THERAPEUTIC GENE THERAPY FOR CANCER WITH INTERLEUKIN-23

by

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Th1-polarizing cytokine IL-12 exhibits potent anti-tumor activity in multiple cancer models; however, therapeutic use of this cytokine is limited due to severe IFN-γ-mediated toxicity. To reduce the amount of IL-12 needed to elicit a therapeutic response, and thereby decrease associated toxicity, it is necessary to characterize novel cytokines to use in conjunction with IL-12. Newly described IL-12 family member IL-23 shares the IL-12 p40 subunit and promotes Th1 immunity by inducing IFN-γ expression and specifically stimulating proliferation of memory CD4+ T-cells. I have demonstrated that injection of an adenovirus expressing IL-23 (Ad.IL-23) into the tumor microenvironment results in significantly enhanced survival and tumor rejection in 40 percent of animals, with concomitant induction of protective anti-tumor immunity. Furthermore, the anti-tumor activity of IL-23 is dependent on IL-12, IFN-γ and CD4+ and CD8+ T-cells, indicating generation of a Th1 response. Delivery of adenovirus expressing IL-12 (Ad.IL-12) into the tumor microenvironment also results in enhanced survival and tumor rejection in up to 90 percent of animals. In contrast to Ad.IL-23, Ad.IL-12 anti-tumor activity requires only IFN-γ and induces protective immunity only 50 percent of the time, suggesting activation primarily of innate immunity. Due to the similar, yet divergent, effector mechanisms used in Ad.IL-12 and Ad.IL-23 anti-tumor effects, I hypothesized that use of these two viruses together would result in synergistic enhancement of tumor eradication. Surprisingly, Ad.IL-12 and Ad.IL-23 do not synergistically enhance anti-tumor effects over use of either
cytokine alone, possibly due to IL-23 p19 sequestration of p40. To circumvent this possibility, adenovirus expressing single chain IL-23 (Ad.scIL-23) was constructed and characterized. Ad.scIL-23 expresses greater levels of cytokine than Ad.IL-23 and treatment of tumor bearing mice results in tumor rejection in 90 percent of animals. However, Ad.scIL-23 does not synergize with Ad.IL-12. Overall, I have shown that IL-23 does possess therapeutic anti-tumor effects, but does not synergize with IL-12 to enhance tumor eradication. Future studies could include characterizing CD4+ T-cell infiltrate of tumors treated with both Ad.IL-12 and Ad.IL-23 to elucidate the mechanism behind the lack of synergy between these two cytokines.
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PREFACE

This work is dedicated to my mother, Maureen, who taught me strength and grace in the face of adversity and who endowed me with the resolve necessary to get to this point in my life and especially to my amazing husband, Dan and wonderful son, Lennon, whose love and support carried me through the adventure of graduate school and gave me the strength to persevere to the end. I love you all much more than you will ever know.

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1.0 INTRODUCTION

1.1 CANCER

In spite of the declaration of the “War on Cancer” by Richard Nixon in 1971 and subsequent billions of federal dollars funneled into research, cancer remains a top public health concern in the United States and abroad. According to the Centers for Disease Control and Prevention, cancer is second only to cardiovascular disease as the leading cause of death amongst men and women in the United States.\(^1\) In fact, 1 in every 2 men and women born in the United States today will be diagnosed with some form of cancer in their lifetimes.\(^2\) Currently, 11,384,892 men and women alive have a history of cancer, with a median age of diagnosis at 66 years of age.\(^2\) In 2009, approximately 1,479,350 individuals were diagnosed with cancer in the United States, with 562,340 succumbing to the disease. Currently, the leading causes of death from cancer amongst men include lung, prostate and colorectal. For women, the leading causes of cancer deaths are lung, breast and colorectal cancer.\(^3\) Overall, cancer incidence is higher for men than women.\(^3\) At most, only 10 percent of cancers arise as a result of germ line mutations; thus the remaining 90 percent are a result of lifestyle or environmental factors. Diet is the highest contributing factor to cancer development at 35%. Exposure to tobacco smoke is the second leading cause (30%), followed by obesity (20%), infections (18%) and radiation and pollutants (7%).\(^4\)

Although cancer remains a leading cause of death in the United States, some progress has been made towards its eradication. 1998 marked the first year since 1930 that a sustained decline in cancer deaths was reported.\(^5\) This trend continued from 1999 to 2006, during which
time diagnoses of new cancers and deaths due to cancer significantly declined amongst all racial and ethnic groups in the United States. This was due largely to declines both in rates of new cancers and deaths from three of the most common cancers in men (lung, prostate and colorectal) and two of the three most common cancers in women (breast and colorectal). Overall, diagnosis of new cancer cases in the United States has declined almost 1 percent per year from 1999 to 2006, while deaths due to cancer have declined 1.6 percent per year from 2001 to 2006.

However, while cancer rates overall have declined, incidence of various cancers in both men and women continue to rise. In men, rates of renal, esophageal and liver cancers, as well as melanoma, myeloma and leukemia, continue to climb. Likewise, in women, incidence of lung, bladder, thyroid and pancreatic cancers continue to mount, in addition to non-Hodgkin lymphoma, melanoma and leukemia. Thus, while some progress is being made to conquer cancer, the development of more effective therapies is still needed.

1.1.1.1 Current Therapies

Traditional treatments for cancers are broadly divided into categories that include radiation, chemotherapy, surgery or biological therapies. Chemotherapy specifically targets rapidly dividing cells, while radiation therapy destroys all cells within the targeted area. Surgery simply involves removal of the tumor mass itself. Due to the ability of the immune system to recognize and eradicate tumor cells, biological therapies that aim to activate the host immune response to tumors have gained much interest and many are currently on the market. Approved biological therapies for cancer include monoclonal antibodies, immune adjuvants and cytokines. Nine monoclonal antibodies against six cancer-specific proteins are currently in use and function by rapidly activating both innate and adaptive immune responses to tumors. In a similar vein, immune adjuvants activate the immune response to tumor antigens and agents such as TLR7
agonist imiquimod and bacilli Calmette-Guérin (BCG) have been used to effectively treat epithelial tumors and bladder cancer, respectively.\textsuperscript{6} Cytokines clinically utilized in cancer therapy include IFN-α, IL-2 and TNF-α. IFN-α has been approved for the treatment of renal cell carcinoma and melanoma.\textsuperscript{7-8} IL-2 has been used to effectively treat metastatic melanoma, as well as renal cell carcinoma.\textsuperscript{9-10} TNF-α has been efficacious in treatment of soft tissue sarcomas.\textsuperscript{11} However, administration of recombinant cytokines is often associated with severe toxicity.\textsuperscript{9} Thus, activation of the host immune response to various tumor antigens using cytokines has proven an effective therapeutic strategy in cancer therapy. However, much work is still needed to identify cytokines possessing both immune stimulating and anti-tumor efficacy with minimal off-target toxicities.

1.1.2 MCA205 mouse fibrosarcoma model

Preclinical animal models of cancer are an efficient means by which to test and characterize a variety of anti-cancer therapies. MCA205 is a mouse model of fibrosarcoma derived by intramuscular injection of carcinogen 3-methylcholanthrene in C57BL/6 mice, is H-2\textsuperscript{b} restricted and considered weakly immunogenic.\textsuperscript{12} A variety of immunotherapies have proven highly effective in activating the host immune response to MCA205 tumors. Stable expression of IFN-α, either alone or in combination with CD80, led to regression of MCA205 tumors and was associated with accumulation of CD4+ and CD8+ T-cells, as well as dendritic cells.\textsuperscript{13} Dendritic cells pulsed with tumor peptides significantly inhibited established MCA205 tumor growth.\textsuperscript{14} Additionally, dendritic cells expressing IL-12 resulted in regression of established MCA205 tumors by induction of a Th1-type anti-tumor response.\textsuperscript{15} Treatment of established MCA205 tumors with IL-18, either alone or in combination with IL-2, resulted in suppressed tumor
Adenoviral-mediated expression of IL-12, IL-18 and IL-1H4 resulted in significant anti-tumor effects in established MCA205 tumors. Treatment of established tumors with IL-21 cDNA also resulted in suppression of tumor growth by enhancing cytotoxic activity of NK cells. Overall, a variety of biological therapies have been proven to effectively activate both innate and adaptive immune responses against the weakly immunogenic MCA205 tumors. Thus, these agents could possibly be broadly applied clinically to combat other weakly immunogenic cancers.

1.1.3 The immune system and cancer

The role the immune system is maintenance of tissue homeostasis, defense against foreign pathogens and destruction of damaged cells. Conceptually, immunity is divided into two arms, innate and adaptive, which differ with respect to timing of activation and recognition of antigen. While cells of innate immune system are rapidly activated, their repertoire for recognizing antigens is limited by germline encoded receptors. Cells involved in innate immunity include monocytes, macrophages, granulocytes (neutrophils, eosinophils, basophils), mast cells, dendritic cells (DCs) and natural killer (NK) cells. NK cells destroy virus infected normal cells and cancer cells. Macrophages and DCs act as sentinels by continuously monitoring tissues for infection or “danger signals.” Upon encountering antigen, macrophages present antigens on their cell surface and produce various cytokines, while DCs migrate to the lymph nodes to present antigens to adaptive immune cells and initiate an adaptive immune response. Adaptive immune cells, in contrast, are activated over days and recognize antigen via diverse antigen receptors that are specific for each antigen encountered. Adaptive immune cells include B-cells, CD4+ T-helper cells and CD8+ cytotoxic T-lymphocytes. CD4+ T-cells drive various types of adaptive immune
responses, B-cells are responsible for antibody production, while CTLs are responsible for mediating the lysis of virus-infected or tumor cells.\textsuperscript{22-23}

1.1.3.1 CD4+ T-cells

CD4+ T-cells are also referred to T-helper cells, due to their ability to activate B-cells, CTLs and macrophages, as well as to direct different types of adaptive immune responses via production of distinct patterns of cytokines and chemokines.\textsuperscript{24} Th1 cells recognize antigens within the context of major histocompatibility complex class II (MHC II) molecules, which are expressed on the cell surface of antigen presenting cells (APCs) such as DCs.\textsuperscript{25} Two types of CD4+ T-cells emerge from the thymus fully differentiated: natural killer T (NKT) cells and natural T regulatory cells.\textsuperscript{24} Other CD4+ T-cells leave the thymus in a “naive” state, with subsequent activation and functional polarization occurring in the periphery. T-cell exposure to antigen in the context of the T-cell receptor via APCs (Signal 1), followed by co-stimulation of the CD28 co-receptor with APC-expressed CD80/86 (Signal 2) leads to activation of the CD4+ T-cell, which is followed by proliferation and differentiation into effector and memory subsets.\textsuperscript{25} The type of T-helper cell generated depends upon cytokines present within the extracellular milieu during activation (Signal 3).\textsuperscript{25} To date, four different subsets of CD4+ T-cells have been identified, each of which appears responsible for driving a distinct type of adaptive immune response. These include Th1, Th2, Th17 and induced T-regulatory cells (Tregs) (Figure 1). In 1986, Tim Mossman and Bob Coffman identified the seminal Th1 and Th2 T-helper cell subsets and showed they could be distinguished based on their cytokine secretion profiles.\textsuperscript{26} Th1 T-cells were ultimately found to drive cell mediated immune responses capable of eradicating intracellular pathogens by activation of cytotoxic T-lymphocytes (CTLs). In addition, Th1 cells have also been implicated in a variety of autoimmune diseases such as experimental autoimmune
encephalitis (EAE) and multiple sclerosis (MS), although the recent discoveries of IL-23 and Th17 T-cells has called their dominance in this role into question.\textsuperscript{27} Th1 cells generate an effective immune response to tumors by generating and enhancing tumor-specific CTL responses.\textsuperscript{25} Th1 cells are characterized by production of IFN-\(\gamma\), IL-2 and lymphotoxin alpha and by expression of transcription factor T-bet.\textsuperscript{26,28} Th1 cell differentiation is driven by production of IL-12 by APCs, which stimulates NK cells to produce IFN-\(\gamma\), which in turn enhances T-bet expression in CD4\(^+\) T-cells via activation of STAT1.\textsuperscript{24} T-bet drives IFN-\(\gamma\) production, which upregulates IL-12R\(\beta\)2 expression (IL-12R\(\beta\)1 is constitutively expressed on naïve CD4\(^+\) T-cells) and sensitizes CD4\(^+\) T-cells to IL-12, which results in additional production of IFN-\(\gamma\) and commitment to the Th1 phenotype.\textsuperscript{24}

Th2 cells support humoral or antibody-mediated adaptive immune responses that are effective in eradicating extracellular pathogens, but have also been implicated in asthma. Th2 cells are characterized by production of IL-4, IL-5, IL-10 and IL-13 and by expression of transcription factor GATA-3. IL-2 and IL-4 are necessary for driving a humoral immunity; IL-4 activates STAT6, which upregulates GATA-3 expression.\textsuperscript{24} While overall Th2 cells are regarded as pro-tumorigenic due their ability to induce angiogenesis and inhibit cell-mediated immune responses to tumors, there have also been reports that Th2 cells may promote anti-tumor immunity under certain circumstances.\textsuperscript{29}

Th17 cells provide immunity to extracellular bacteria and fungi, but are also implicated in a variety of autoimmune diseases. Th17 cells are characterized by expression of IL-17A and IL-17F, as well as IL-6 and TNF.\textsuperscript{30} Th17 cells are generated under priming conditions containing TGF-\(\beta\) and IL-6, which induces T-cell expression of the transcription factor ROR\(\gamma\)t.\textsuperscript{31-33} IL-23 is
necessary for maintenance of the Th17 lineage.\textsuperscript{34} Th17 cells have been shown to possess both pro- and anti-tumor effects.\textsuperscript{35}

Regulatory T-cells are responsible for curtailing immune responses, as well as for maintaining self tolerance. Tregs are characterized by secretion of immunosuppressive cytokines such as IL-10 and TGF-β and by expression of transcription factor Foxp3. IL-2 and TGF-β are necessary for polarization of responder T-cells towards a Treg phenotype, due to STAT5 and Smad3-mediated induction of Foxp3 expression, respectively.\textsuperscript{24} Treg-mediated suppression of anti-tumor immune cells have been implicated in immune tolerance to tumors.\textsuperscript{36}

![Figure 1: CD4+ T-cell subsets](image)

**Immune Response**
- **Th1**
  - T-bet
  - IL-12
  - IL-4
  - IL-23
  - TGF-β
  - IFN-γ
  - Cell-mediated
- **Th2**
  - GATA-3
  - IL-4
  - IL-17
  - GATA-3
  - IL-10
  - Tolerance
- **Th17**
  - RORγt
  - IL-17
  - Extracellular
  - Autoimmune
1.1.3.2 Role of the immune system in carcinogenesis

It has been well established that a link exists between the host immune response and cancer development; however, the precise role of the immune system in cancer pathogenesis remains unclear. In general, there are three possible scenarios for the role of the host immune response to tumors (1) the host immune system recognizes the tumor as foreign and subsequently limits tumor growth or mediates its regression (2) chronic inflammation creates an environment that is conducive to tumor growth, neoangiogenesis and immune suppression or (3) the host immune system is rendered tolerant or ignorant of the cancer, leading to unchecked regression.

Indeed several lines of evidence implicate host immunity and inflammation in the early pathogenesis of cancer. Various types of chronic inflammation are associated with cancer development, such as infections (including *H. pylori* and hepatitis B and C viruses) and presence of inflammatory bowel disease. Likewise, proven risk factors for cancer, such as tobacco smoke and obesity, are also linked to inflammation. Activation of innate immune cells contributes to carcinogenesis using such means as induction of DNA damage by free radicals, promotion of angiogenesis and tissue remodeling by various cytokines, chemokines and matrix metalloproteinases and suppression of anti-tumor immune responses. However, adaptive immune cells can also support tumor progression. Regulatory T-cells (Treg) suppress effector T-cell responses against tumors and generation of humoral immune responses within the tumor increases chronic inflammation.

However, there are reports that the host immune system plays only a minor role in the development and progression of some types of cancers. Some tumors do not exhibit any immune cell infiltrates, which may be a result of physical separation from secondary lymphoid organs,
and thus, T-cells. In this scenario, the tumor remains completely unrecognized by T-cells, but does not actively suppress the host immune response, either.\textsuperscript{38}

In the final scenario, the tumor itself is antigenic due to expression of altered or overexpressed “self” antigens, which is recognized and destroyed by the host immune system utilizing both innate and adaptive components. Indeed, the importance of the host immune response in attenuating cancer pathogenesis has been well established in a variety of clinical cancer studies. In advanced ovarian carcinoma, presence of T-cells within the tumor microenvironment correlated with significantly enhanced 5-year survival compared to patients with tumors lacking T-cell infiltrate (38 versus 4.5 percent survival) and was associated with increased IFN-\(\gamma\).\textsuperscript{39} Likewise, enhanced 5-year survival rates were also associated with enhanced lymphocyte infiltration in rectal cancer patients.\textsuperscript{40} Infiltration of tumors by CD8\(^{+}\) T-cells was associated with better overall survival in colorectal patients.\textsuperscript{41} The association of immune cell infiltrate of tumors with a favorable prognosis suggests that at least some attempt is made by the host immune system to eliminate tumors. Other lines of evidence in a variety of mouse models of cancer highlight the role of the immune system in preventing tumor growth. Mice deficient in B, T and NKT cells are more susceptible to chemical induced carcinogenesis,\textsuperscript{42-43} as are mice deficient in various cytokines associated with a cytotoxic lymphocyte response, such as IFN-\(\gamma\) and IL-12/23 p40.\textsuperscript{42,44-45}

Overall, it is apparent that the immune system can indeed control tumor growth; however, it appears that the timing and type of immunity activated dictates whether the immune system will play friend or foe in carcinogenesis. Generation of a full anti-tumor adaptive immune response should result in eradication of malignancies, while chronic activation of innate cells in premalignant tissues may promote tumorigenesis.\textsuperscript{23} My project is based upon the
hypothesis that the immune system can recognize and eradicate tumors, but that cancer develops and progresses as a result of tumor-induced suppression of an otherwise effective anti-tumor immune response. Specifically, I believe that initially the majority of CD4+ T-helper cells within the tumor are Th1-polarized and can effectively generate an anti-tumor immune response. However, over time, T-cell polarization progresses to a Th2 and eventually a Treg phenotype characterized by immunosuppression and inhibition of an effective anti-tumor response. I propose that local, adenoviral-mediated expression of Th1 polarizing cytokines, such as IL-12 and IL-23, would repolarize the T-helper immune response to tumors from a Treg to a Th1 and mount an anti-tumor immune response capable of eradicating tumors.

1.2 INTERLEUKIN 12

1.2.1 Discovery

Interleukin 12 (IL-12) was first described in the late 1980’s as a cytotoxic T-lymphocyte (CTL) maturation factor upon discovery that supernatants from human leukocytes, PBMCs and B-lymphoblast cells could stimulate CTLs in synergy with IL-2.\textsuperscript{46-48} Simultaneously, T-cell stimulating factor, a secreted product possessing the ability to reactive resting T-cells, was described.\textsuperscript{49} Yet another factor, termed natural killer cell (NK) stimulatory factor, was purified from B-lymphoblast cells and, like its name suggests, was found to induce production of IFN-\(\gamma\) from and enhance mitogen-induced proliferation of PBMCs, as well as stimulate NK cytotoxicity.\textsuperscript{50} Cloning of the IL-12 p35 and p40 genes from B-lymphoblasts led to the
realization that CTL maturation factor, T-cell simulating factor and natural killer cell stimulatory factor were indeed identical and this cytokine was subsequently renamed interleukin 12.\textsuperscript{51}

1.2.2 Expression

IL-12 is heterodimeric cytokine comprised of disulfide-bonded p35 and p40 subunits.\textsuperscript{48,50} The p40 subunit is similar to cytokine receptors, while p35 is related to IL-6 and G-CSF.\textsuperscript{52} Both the p40 and p35 subunits must be co-expressed within the same cell to be secreted as biologically active IL-12.\textsuperscript{51} While p35 is constitutively expressed in many cell types, p40 expression is limited primarily to phagocytic cells. Interestingly, however, within p40-expressing cells, p35 expression appears to be the limiting factor in IL-12 production.\textsuperscript{53}

IL-12 is expressed by a variety of cells, including monocytes, macrophages, dendritic cells, neutrophils and some B-cells.\textsuperscript{54} IL-12 expression can be induced by a variety of pathogens, including bacteria, fungi, parasites and viruses. Additionally, CD40-CD40 ligand engagement between antigen presenting cells (APCs) and T-helper cells induces IL-12 production.\textsuperscript{55} IL-12 elicits IFN-γ expression from resting and activated NK and T-cells.\textsuperscript{50,56-57} T-cell-derived IFN-γ, in turn, primes monocytes for LPS-induced production of IL-12 by upregulating p40 and p35 transcription, thus generating a positive feedback loop for IL-12 production.\textsuperscript{58}

1.2.3 IL-12 Receptor

The IL-12 receptor is likewise comprised of two subunits, IL-12Rβ1 and IL-12Rβ2. Both IL-12Rβ1 and IL-12Rβ2 are Type 1 transmembrane proteins most closely related to the cytokine
receptor gp130. Both subunits must be present to form a high affinity, functional IL-12 receptor. IL-12 binds to its receptor primarily via interaction of the p40 subunit with IL-12Rβ1. IL-12Rβ2 binds both the p35 subunit, which is required for signaling, and the IL-12p70 heterodimer. The cytoplasmic portion of the IL-12Rβ2 subunit contains numerous tyrosine residues and is responsible for signal transduction. Binding of IL-12 to its receptor activates JAK2 and TYK2, leading to the phosphorylation of multiple STAT transcription factors, including STAT1,3, 4, and 5. STAT4 appears to be the most prominent STAT activated, as IL-12 induced activation of NK and T-cells, as well as generation of an effective Th1 immune response, is effectively abrogated in STAT4-deficient mice.

The IL-12 receptor is expressed by activated, but not resting, NK and CD4 and CD8-positive T-cells, as well as some B-cells. IL-12 receptor is also expressed on DCs; however, in these cells, signaling is mediated via NF-κB activation. Activation of T-cells via the T-cell receptor upregulates expression of both chains of the IL-12 receptor. Furthermore, IFN-γ induces transcription factor T-bet expression in T-cells, which in turn upregulates IL-12Rβ2 expression, thereby driving and maintaining a Th1 response.

1.2.4 IL-12 Regulation

IL-12 expression in APCs can be inhibited by a number of cytokines. IL-10 inhibits IL-12 expression by inhibition of p40 transcription, thereby leading to less IL-12 production. TGF-β inhibits production of IL-12 and interferes with IL-12 receptor-mediated signaling, including inhibition of STAT4. Finally, p40 homodimers inhibit IL-12 mediated proliferation and production of IFN-γ from mouse splenocytes and activation of NK cells by binding to the IL-12 receptor.
**1.2.5 Biological Activity**

IL-12 induces activation and proliferation of, as well as IFN-γ production from, NK and T-cells.\(^{50,75}\) IL-12-mediated induction of IFN-γ requires the presence of TNF-α and IL-1β.\(^{72}\) Additionally, IL-12-mediated proliferation of and IFN-γ production from T-cells is synergistically enhanced by IL-2 and B7/CD28 costimulation.\(^{57,76}\) IL-12 also synergizes with IL-18 to enhance IFN-γ production from T\(^{77}\) and NK cells,\(^{78}\) possibly by inducing IL-18 expression and/or upregulating the IL-18Rβ chain.\(^{79}\) Furthermore, in response to microbial infection, IL-12 is produced by activated macrophages, which in turns drives generation of a Th1-type adaptive immune response; therefore, IL-12 acts as a bridge between both innate and adaptive arms of the immune system (Figure 2).\(^{80}\) IL-12 plays a key role in the generation and activation of cytotoxic T-lymphocytes (CTLs) and its absence or presence decides between tolerance or generation of cytotoxic function.\(^{81-82}\) Finally, in the context of cancer, IL-12 may repolarize dysfunctional T-helper cells to a Th1 phenotype.\(^{83}\)

IL-12 is known mainly for its role in driving Th1, or cell-mediated, immune responses. Early studies suggested that IL-12 was required for commitment to the Th1 lineage: IL-12 was shown to induce IFN-γ expression and drive CD4 positive T-cells towards a Th1 phenotype, while blocking expression of IL-4 and a generation of a Th2 response.\(^{84}\) However, further studies have indicated that expression of transcription factor T-bet actually determines lineage fate by upregulating IL-12Rβ2 expression and allowing for expression of IFN-γ independent of IL-12, suggesting that IL-12 is required mainly for proliferation and maintenance, and hence survival, of the Th1 lineage.\(^{85}\) Regardless of the sequence of events, IL-12 is obviously intimately involved with the development of Th1 T-helper cells.
Additionally, the importance of IL-12 in inhibition of carcinogenesis has been well documented. Mice deficient in p40 are more susceptible to UV$^{86-87}$ and methylcholanthrene$^{42}$ induced tumors. Additionally, IL-12 neutralization decreases rejection of transplantable tumors.$^{88}$

**Figure 2: Biological effects of IL-12**

### 1.2.6 IL-12 therapy in preclinical cancer models

Due to the ability of IL-12 to enhance NK and CTL cytotoxic activity, induce IFN-γ expression and drive a Th1 response, while blocking Th2 immunity, IL-12 was expected to be an effective anti-cancer agent. Indeed, not long after its discovery, studies highlighting the ability of IL-12 to enhance cytotoxicity of NK cells against a variety of cancer cell lines *in vitro* were published.$^{89-91}$ Not long thereafter, recombinant mouse IL-12, when injected either systemically or
peritumorally, was shown to effectively eradicate various metastatic and subcutaneous tumors. Further studies supported the anti-tumor activity of IL-12 in murine adenocarcinoma and sarcoma models. However, use of recombinant IL-12 was associated with profound toxicity.

While investigations into the anti-cancer properties of recombinant IL-12 continued, therapies involving restriction of IL-12 expression to the tumor microenvironment were devised. This included either treatment with cells transduced to express IL-12 in vitro or the direct transduction of tumors in vivo with IL-12 cDNA. Such strategies circumvent the toxicity associated with systemic delivery of recombinant IL-12 by restricting IL-12 expression predominantly to the tumor compartment. Additionally, high, local concentrations of IL-12 could possibly enhance induction of potent systemic anti-tumor immunity. Treatment of tumors with cells stably expressing IL-12 was highly successful. Expression of IL-12 in various murine tumor models, including renal cell, pancreatic and colon carcinoma inhibited tumor growth. Likewise, IL-12-expressing syngenic fibroblasts have been shown to successfully hinder tumor growth in various murine models of cancer, including melanoma, ovarian, sarcoma, hepatocellular carcinoma, and pancreatic peritoneal carcinomatosis, as well as inhibit liver metastases in a breast cancer model. Additionally, treatment with syngenic DCs expressing IL-12 have shown therapeutic benefit in a variety of preclinical models including melanoma, sarcoma, adenocarcinoma, as well as inhibit liver metastases in a colon carcinoma model.

Similarly, IL-12-based therapies involving direct transduction of tumor cells with genes encoding this cytokine have also proved highly successful. Transfer of naked IL-12 DNA successfully inhibited tumor growth in various cancer models including melanoma, colon carcinoma, and renal carcinoma. Other nonviral delivery methods including electroporation, gene gun and liposome-mediated transfer of IL-12 have
similarly yielded clinically relevant inhibition of tumor growth in a variety of cancer models. IL-12 cDNA has also been delivered to tumor cells using a variety of viruses, including retrovirus, vaccinia virus, alphaviruses, herpes simplex viruses, with therapeutic benefit demonstrated in a number of cancer models including melanoma, colon adenocarcinoma, neuroblastoma, colorectal, squamous cell carcinoma, and glioma.

The anti-tumor effects of adenovirally delivered IL-12 have been exhaustively investigated. Direct injection of adenovirus expressing IL-12 into tumors results in enhanced survival and induction of protective immunity. Adenoviral-mediated expression of IL-12 yielded therapeutic benefit in a variety of preclinical cancer models including sarcoma, colorectal, hepatocellular carcinoma, adenocarcinoma, glioma, prostate, thyroid, and breast. Additionally, in animals bearing two tumors, treatment of one tumor was associated with regression of the distal tumor. Thus, adenovirus has proven to be an effective tool for delivery of IL-12 into the tumor microenvironment to yield clinical anti-tumor benefit.

1.2.6.1 Mechanism of IL-12-mediated anti-tumor effects

The dual ability of IL-12 to stimulate both innate and adaptive arms of immunity is evident in its mechanisms of tumor eradication. IL-12 induction of IFN-γ production from NK and T-cells is important in tumor eradication, especially early on in the immune response. IFN-γ inhibits angiogenesis, at least in part by induction of chemokines IP-10 and Mig. IFN-γ also induces iNOS expression in tumor cells, leading to NO production and subsequent inhibition of tumor growth. During generation of adaptive immunity, IL-12 activates CD4+ and CD8+ T-cells for effective tumor eradication and generation of long-lasting protective immunity. NKT cells also appear to play an important role in the anti-tumor activity
of IL-12. Additionally, IL-12 may inhibit formation of metastases by inhibiting migration and invasion of tumor cells by upregulation of E-cadherin cell surface molecules.

1.2.7 IL-12 in Clinical Trials

1.2.7.1 Recombinant IL-12

Due to the initial success of IL-12 treatment in preclinical models of cancer, IL-12-based therapies quickly progressed to use in human clinical trials. While IL-12 therapy in preclinical animal models was relatively well tolerated, recombinant IL-12 treatment of advanced malignancies in humans was associated with toxicity in the form of fever, chills, nausea, vomiting and headache. In fact, recombinant IL-12 treatment led to the death of two kidney cancer patients enrolled in a Phase II clinical trial, using a dose previously found to be safe during Phase I studies. It was subsequently discovered that predosing of IL-12 is necessary to ameliorate toxicity associated with subsequent, higher doses. To date, there have been numerous human clinical trials investigating the usefulness of IL-12 as an anti-cancer agent. Recombinant IL-12 has been utilized in the treatment of numerous cancers, including melanoma, renal and bladder. However, clinical efficacy was minimal, with only 0 to 7 percent response based on RECIST grading criteria initially established for chemotherapy agents. IL-12 has also been investigated within the context of cancer vaccines, other cytokines such as IL-2 and IFN-α2b, or antitumor monoclonal antibodies (Trastuzumab), again with only modest clinical success and at most an 11 percent response rate.
1.2.7.2 IL-12 gene therapy

As was indicated before, use of recombinant IL-12 is limited due to severe toxicity in humans. Thus, to simultaneously increase the dose of IL-12 used and decrease associated toxicity, local production of IL-12 within the tumor using various gene therapy approaches has been investigated. IL-12 has been delivered using autologous fibroblasts in advanced cancer patients, with transient anti-tumor effects reported in five of nine treated patients.\textsuperscript{145} Treatment of melanoma patients with autologous tumor cells expressing IL-12 resulted in a moderate clinical response in one patient.\textsuperscript{146} Intratumoral injection of dendritic cells transduced with adenovirus expressing IL-12 for treatment of gastrointestinal tumors yielded similar results, with a partial response reported in only one patient.\textsuperscript{147} In contrast, direct injection of plasmid DNA encoding IL-12 into melanoma lesions yielded more promising results, with two instances of stable disease and one complete remission reported in one study,\textsuperscript{148} and significantly decreased lesion size in more than 30 percent of patients in another.\textsuperscript{149} Canarypox virus has also been used to deliver IL-12 cDNA intratumorally, in combination with B7.1 cDNA, in treatment of melanoma; however, no tumor regressions occurred.\textsuperscript{150} In all trials, little to no toxicity was observed. However, overall clinical efficacy of IL-12 cancer gene therapy has been limited; thus new cytokines are continuously sought out for use in combination with IL-12, to enhance therapeutic efficacy and decrease the effective dose needed.

1.2.8 Augmentation of IL-12 anti-tumor effects

Numerous cytokines have been previously shown to synergistically enhance the anti-tumor activity of IL-12. When administered either locally or systemically, recombinant IL-2 enhances the anti-tumor activity of IL-12 cDNA transduced fibroblasts\textsuperscript{151} and the vaccine effect of TS/A
tumor cells stably expressing IL-2 was synergistically enhanced upon co-treatment with systemic recombinant IL-12. Mice bearing B16 melanoma tumors expressing IL-12 family member IL-27 exhibited synergistically decreased tumor volume when co-treated with an intraperitoneal injection of recombinant IL-12.

Anti-tumor synergism between IL-12 and IL-18 has also been extensively studied due to the ability of IL-18 to synergistically enhance IL-12-mediated IFN-γ production from T and NK cells. Co-delivery of IL-12 and IL-18 using electro-gene therapy synergistically enhanced reduction of tumor volume and was associated with an increase in IFN-γ and CD8+ T-cells in tumors. Likewise, administration of recombinant IL-18 synergistically enhanced vaccine effects of Renca cells stably expressing IL-12 and was dependent upon involvement of CD4+ and CD8+ T-cells and NK cells. Finally, use of systemically delivered recombinant IL-18 and MBT2 mouse bladder cancer cells stably expressing IL-12 synergistically enhanced anti-tumor effects in an IFN-γ dependent fashion.

TNF-α has also been studied at length for its ability to synergize with IL-12, due to its shared ability to induce inflammation and inhibit tumorigenesis. When co-delivered with TNF-α using poly-lactic acid microspheres, IL-12 synergistically enhanced anti-tumor effects in both primary and contralateral B16 and MCA205 tumors, an effect that was dependent upon CD8+ T- and NK cells. IL-12 pretreatment of HT144 melanoma cells followed by treatment with TNF-α also synergistically enhanced survival over use of either cytokine alone. Co-treatment of CT26 colon and Renca renal carcinoma with IL-12 and TNF-α led to synergistically enhanced tumor rejection, due likely to involvement of CD8+ T and NK cells.

The combination of IL-12 and IL-15 has also yielded therapeutic anti-tumor benefit. Like IL-12, IL-15 activates NK cells and aids in maintenance of memory T-cells, as well as
activates tumor-specific CD8+ T-cells. Stable co-expression of IL-12 and IL-15 cDNA in TS/A breast adenocarcinoma cells also resulted in synergistically enhanced anti-tumor effects, which was independent of IFN-γ, but dependent upon CD8+ T-cells. Similarly, stable co-expression of IL-12 and IL-15 cDNA in N592 small-cell lung cancer cells synergistically enhanced tumor rejection, possibly due to activation of macrophages. When injected intratumorally, recombinant IL-12 and IL-15 synergistically enhanced anti-tumor effects in a B16 melanoma model of cancer, most likely due to enhanced IFN-γ production.

Overall, IL-12 has been previously shown to synergize with other cytokines to synergistically enhance anti-tumor effects, commonly using mechanisms characteristic of a Th1 response, including involvement of CD8+ T-cells and enhancement of IFN-γ production. Thus, any potentially Th1 polarizing cytokine has the ability to synergize with IL-12 to enhance its anti-tumor effects.

1.3 INTERLEUKIN 23

1.3.1 Discovery

IL-23 is a relatively newly described member of the IL-6/IL-12 family of heterodimeric cytokines. It shares the IL-12 p40 subunit and is also composed of a unique p19 subunit. IL-23 was identified during a computational screen for IL-6 helical cytokine family members, in which p19 was first identified. Although its discovery came some 11 years after the first description of IL-12, its existence was somewhat anticipated. Mice deficient in p40 were shown to be more susceptible to bacterial infection, died earlier and exhibited higher bacterial burdens than p35
deficient mice, suggesting that p40 may interact with another subunit to form a novel cytokine important in driving Th1 immunity.

1.3.2 Expression

IL-23 is composed of disulfide-bonded p40 and p19 subunits. The p40 subunit is similar to cytokine receptors and is shared with IL-12. The p19 subunit is comprised of four α-helices and is most closely related to IL-12p35. Like p35, p19 is only secreted from cells in the context of p40. Similar to IL-12, IL-23 is expressed by activated antigen presenting cells, including dendritic cells and macrophages, activated monocytes, endothelial cells and T- and B cells. IL-23 expression is induced by activation of Toll-like receptors by microbial products, as well as by Fas and CD40 ligand engagement by dendritic cells.

1.3.3 Receptor

The IL-23 receptor likewise shares the IL-12Rβ1 subunit and is also composed of a unique subunit designated IL-23R. Both subunits must be present to confer IL-23 responsiveness. IL-12Rβ1 is a Type 1 transmembrane protein most closely related to the cytokine receptor gp130. IL-23R is also a Type 1 transmembrane protein and a member of the hemopoietin receptor superfamily related to IL-12Rβ2 and gp130. The extracellular region of IL-23R is comprised of an IgG-like and two cytokine receptor domains and possesses signaling capabilities. The p40 subunit binds to IL-12Rβ1, while p19 binds to the IL-23R. The IL-23 receptor is found on T and NK cells, as well as macrophages and dendritic cells.
IL-23 activates similar JAK/STAT signaling molecules as IL-12, including JAK2, TYK2, and STATS 1, 3, 4 and 5. IL-12Rβ1 binds Tyk2, while IL-23R associates with Jak2. Binding of IL-23 to its receptor leads to JAK activation, which in turn leads to STAT recruitment. Unlike IL-12, however, STAT4 is induced by IL-23 to a lesser extent and STAT3 appears to be the most prominent STAT activated.

1.3.4 Biological activity

Due to its structural similarities to IL-12, IL-23 was expected to share many of its immunostimulatory and Th1-polarizing properties. Like IL-12, IL-23 was found to stimulate proliferation and IFN-γ production from human activated T-cells; however, IL-23 treatment resulted in lower levels of IFN-γ production versus IL-12. Unlike IL-12, IL-23 appeared to preferentially activate both mouse and human memory, but not naïve, T-cells. Overall, it appears as though IL-12 acts early on in the development of a Th1 response, by activating and committing T-cells to this phenotype. In contrast, IL-23, appears to promote maintenance of the Th1 response by enhancing proliferation and activation of the memory T-cell subset. Additionally, IL-23 has also been reported to inhibit Treg-induced immunosuppression in a UVR-induced DNA damage model.

IL-12 had been previously implicated in a variety of inflammatory and autoimmune conditions due to induction of a Th1 response and subsequent IFN-γ production. Indeed, in the absence of p40, conditions such as collagen-induced arthritis (CIA), inflammatory bowel disease (IBD), and experimental autoimmune encephalomyelitis (EAE) exhibit reduced pathogenesis, affirming this assertion. However, studies of CIA, EAE and IBD in mice lacking IFN-γ did not mirror these findings. In fact, the absence of IFN-γ appeared to exacerbate CIA
and EAE disease. These findings suggested (1) a role of IFN-γ in attenuating inflammatory disease and (2) the role of an as of yet undiscovered cytokine involved in autoimmune inflammation. Lack of EAE development in IL-23 (p19) deficient mice confirmed its role as a mediator of autoimmune inflammation. The discovery that IL-23 induces IL-17 production from a unique subset of CD4+ T-cells, followed by the implication of these cells in autoimmune pathogenesis, solidified the role of IL-23 in autoimmune inflammation.

These studies ushered in new paradigms in both the physiologic role of IL-23 and the types of T-helper cells in existence. The primary role of IL-23 appeared not to be in driving a Th1 response, but rather induction of a unique subset of T-cells characterized by expression of IL-17A and F, IL-6 and TNF. These IL-17 producing T-cells, termed Th17, are distinct from traditional Th1 and Th2 helper cell lineages, being generated upon exposure to IL-23 and inhibited by exposure to IFN-γ. Further studies have shown that commitment to the Th17 subset also requires TGF-β and IL-6, which induce expression of Th17-specific transcription factor RORγt. IL-6 also induces expression of IL-21, which enhances RORγt and IL-17 expression. Upregulation of RORγt requires STAT3, while STAT4 is needed for maximal IL-17 production in response to IL-23. RORγt in turn induces transcription of IL-17A and F in naïve T-cells and is responsible for differentiation into the Th17 effector cells. Overall, it appears as though TGF-β and IL-6 are necessary to drive cells towards a Th17 lineage, while IL-23 is required for the maintenance of this subset. However, the precise role of TGF-β is unclear and as it is a strong inhibitor of both Th1 and Th2 cells, TGF-β may indirectly favor polarization to the Th17 subset. Interestingly, Th1/Th17 cells have been discovered in Crohn’s disease patients, which are characterized by expression of both T-bet and RORγt transcription factors, as well as both IL-17 and IFN-γ. Th1/Th17 cells have been since
identified in mice and have also been associated with inflammatory conditions such as arthritis.\textsuperscript{191}

Since its discovery, the IL-23/Th17 axis has been implicated in a variety of autoimmune and inflammatory conditions. The use of IL-23 p19-deficient mice has implicated IL-23 as the major mediator of inflammation in a variety of models, including EAE,\textsuperscript{183} Crohn's disease and ulcerative colitis.\textsuperscript{192} In humans, the IL23 receptor gene is associated with Crohn’s disease.\textsuperscript{193} Additionally, IL-23 promotes expression of IL-22 in Th17 cells, a cytokine implicated in psoriasis, and indeed, increased IL-23 has been observed in psoriatic lesions of patients with psoriasis vulgaris.\textsuperscript{169,194} Increased systemic IL-23 levels are associated with disease activity in rheumatoid arthritis.\textsuperscript{195} IL-23 and Th17 cells have also been implicated in asthma.\textsuperscript{196}

Additionally, IL-23 appears to play a role in the control of infectious disease. In studies investigating susceptibility to a variety of microorganisms, including \textit{Salmonella enteritidis}, \textit{Cryptococcus neoformans}, \textit{T. gondii} and mouse cytomegalovirus, IL-12 p35-deficient mice are more resistant to a variety of microorganisms than their p40-deficient counterparts.\textsuperscript{167,197-199} Furthermore, IL-23 p19 deficient mice are highly susceptible to \textit{Klebsiella pneumoniae} infection, likely due to lack of induction of IL-17 production.\textsuperscript{200} Additionally, administration of IL-23 to p40 deficient mice enhanced resistance to \textit{T. gondii} infection.\textsuperscript{199} Thus, IL-23 appears to play a role in limiting the severity of infectious disease.

\subsection*{1.3.5 Regulation}

Due to their association with highly pathologic inflammatory conditions, it is not surprising that the development of Th17 cells is highly regulated. IL-12, IFN-\(\gamma\) and IL-4\textsuperscript{185,190} all inhibit Th17 development. IL-12/IL-23 family member IL-27 also blocks differentiation of Th17 cells.\textsuperscript{201}
However, regulatory T-cells appear to be somewhat ineffectual against Th17 cells.\textsuperscript{190,202} Additionally, LPS induced IL-23 expression in macrophages can also be inhibited by IFN-\(\gamma\)\textsuperscript{203}. IL-10 can also inhibit p40 transcription, presumably resulting in reduced IL-23 production.\textsuperscript{204} Finally, like IL-12, IL-23-mediated IFN-\(\gamma\) production is inhibited by p40 homodimers.\textsuperscript{205}

1.3.6 IL-23 and cancer

Due to its similarity to IL-12 and ability to induce IFN-\(\gamma\) expression, IL-23 was expected to act as a potent anti-cancer agent. Indeed, initial studies in various establishment models of cancer exemplified its ability to inhibit tumor growth and induce anti-tumor immunity. However, recent studies highlighting the pro-cancer effects of IL-23 call this paradigm into question. Overall, it appears as though pro- or anti-tumor effects of IL-23 depends upon expression levels of this cytokine. At physiologic levels, IL-23 may possess pro-tumor effects, but when expressed at artificially high levels, IL-23 can act as an anti-cancer agent.

1.3.6.1 Anti-tumor activity

Shortly after its discovery, the first study investigating the anti-tumor effects of IL-23 in an establishment model of colon carcinoma was published. Colon carcinoma cells stably expressing a single-chain version of IL-23 experienced inhibited growth and were subsequently rejected, with concomitant induction of tumor specific immunity. Tumor rejection was associated with CD8+ T-cell production of IFN-\(\gamma\) and was dependent upon T-, but not NK, cells.\textsuperscript{206} When stably expressed in B16F1 melanoma cells, single chain IL-23 also inhibited tumor growth and formation of metastases in a CD8+ T-cell dependent, but CD4+ T-cell and NK cell independent, manner.\textsuperscript{207} Studies investigating the anti-tumor effects of single-chain IL-23 in
a variety of establishment models of cancer followed, including pancreatic, human esophageal, colon carcinoma, melanoma, hepatocellular carcinoma, and mouse mammary carcinoma. Mechanisms appeared to exclude the involvement of NK cells, and some discrepancies exist over the importance of T-cells. However, overall, IL-23-mediated anti-tumor immunity appears to involve generation of a Th1 response, with dependence upon CD8+ T-cells and IFN-γ, CD40 expression, and production of Th1 polarizing cytokines, such as IFN-γ, IL-12 and TNF-α.

IL-23 has also been shown to possess anti-cancer effects in therapeutic cancer models as well. Bone marrow-derived neural stem-like cells stably transduced with adenovirus expressing single chain IL-23 significantly enhanced survival of mice bearing intracranial gliomas and established protective immunity against tumor challenge. Induction of anti-tumor immunity in this model was dependent on CD4+ and CD8+ T-cells, as well as NK cells, and was associated with production of IFN-γ, not IL-17. Similarly, treatment of intracranial gliomas with dendritic cells transduced by an adenviral vector to express single chain IL-23 also resulted in tumor rejection and generation of protective immunity mediated primarily by CD8+ T-cells, but also by CD4+ and NK cells as well. Systemic expression of IL-23 by electroporation of muscle cells with IL-23 plasmid DNA decreased tumor burden and enhanced survival of mice using CD4+, CD8+ and NK cells. Interestingly, a study using adenviral-mediated delivery of IL-23 into the tumor microenvironment showed prophylactic, but not therapeutic, anti-tumor activity in a variety of cancer models, including melanoma and colon carcinoma. However, this was in stark contrast to our studies, which indicate that adenviral-mediated delivery of IL-23 into established fibrosarcoma tumors results in potent anti-tumor effects and induction of systemic immunity using mechanisms including IFN-γ and CD4+ and CD8+ T-cells.
IL-23 has also been investigated as a vaccine adjuvant. When used in conjunction with a gp100 peptide-based vaccine, IL-23 suppressed B16 tumor growth and enhanced the cytotoxic activity of vaccine induced CD8+ T-cells.219 While IL-23 therapy was also associated with enhanced IFN-γ production, the anti-tumor effects appeared to be primarily mediated by TNF-α.219 Additionally, B16 cells expressing single chain IL-23 generated protective immunity against parent tumors.153

Investigation of IL-23 synergy with a variety of cytokines has also been conducted. Interestingly, stable co-expression of IL-12 and IL-23 in osteosarcoma cells could only moderately enhance induction of IFN-γ expression from mononuclear cells compared to IL-12 alone.220 However, inoculation with mixed populations of colon carcinoma cells expressing single chain IL-12 and IL-23 did not synergistically enhance anti-tumor effects on a distal parent tumor over use of either cytokine alone.210 Additionally, co-transduction of tumor cells with IL-18 and IL-23 cDNA by gene gun synergistically enhanced anti-tumor effectiveness.221 Similarly, the combination of IL-23-expressing B16 cells and recombinant IL-18 treatment synergistically enhanced anti-tumor immunity.153 Unsurprisingly, co-expression of free p40 and IL-23 inhibited the anti-tumor effects of IL-23 in colon carcinoma cells.205

In summary, IL-23 appears to be a potent anti-cancer agent. Its therapeutic capabilities have been demonstrated in a variety of establishment and therapeutic models of cancer, using a number of methods to achieve local levels of expression within the tumor microenvironment. Moreover, the ability of IL-23 to act as a vaccine adjuvant and to synergize with other Th1-polarizing cytokines has been established as well. Mechanisms of IL-23 mediated tumor eradication and establishment of protective immunity appear to involve activation of innate
immune cells as well as generation of a Th1 immune response, characterized by involvement of CD4+ and CD8+ T-cells, as well as Th1 polarizing cytokines such as IFN-γ, IL-12, TNF-α

1.3.6.2 Pro-tumor effects

While numerous studies have hailed the anti-tumor activity of IL-23, a few recent studies have laid doubt on this claim by declaring that IL-23 is indeed a tumor-promoting cytokine. In a seminal paper, Langowski, et al. showed that IL-23, but not IL-12, expression is enhanced in human tumors and is associated with a decrease in CD8+ T-cell infiltrate and enhanced angiogenesis. Additionally, they demonstrated that inhibition of IL-23 expression leads to increased resistance to both chemically induced and transplanted tumors. The authors explained the discrepancies between their study and numerous others asserting the anti-tumor effects of IL-23 as result of biologically relevant versus artificially inflated levels of IL-23 expression. In their model, at physiologic levels, IL-23 acts as a proinflammatory mediator, inducing expression of factors such as matrix metalloproteinases and IL-17, which act in wound healing and repair responses and create an environment dominated by innate and inflammatory cells. In contrast, when expressed at artificially high levels within the tumor microenvironment, IL-23 may result in enhanced DC, macrophage and granulocyte infiltration, which would lead to tumor destruction. Other studies have supported the pro-tumor assertion. Production of lactic acid by tumors promotes IL-23 production by macrophages, thus creating a proinflammatory, pro-tumor environment. In colorectal carcinoma patients, serum IL-23, as well as p19 mRNA in tumor tissue, was found to be upregulated. Additionally, a correlation was found between IL-23 receptor gene polymorphisms and ovarian cancer risk and prognosis. Thus, much evidence suggests that IL-23, at physiologic levels, may possess pro-tumor activity by promoting inflammation.
Recently, a mechanism has been proposed to explain the role of IL-23 in carcinogenesis. IL-23 signaling results in STAT3 activation.\(^{173}\) Interestingly, STAT3 is also constitutively expressed in a variety of cancers and results in cell cycle progression, inhibition of apoptosis, immune evasion and angiogenesis.\(^{227}\) STAT3 activation also inhibits production of pro-inflammatory cytokines as well as increasing expression of immunosuppressive factors such as IL-10.\(^ {228}\) In tumors, STAT3 activation prompts IL-23 production from tumor associated macrophages. IL-23 then binds to the IL-23 receptor on tumor associated Tregs, activating STAT3 and leading to increased Foxp3 and IL-10 expression.\(^ {229}\) Thus IL-23 may create a pro-tumor environment, at least in part, by enhancing Treg induced immunosuppression.

### 1.3.7 Th17 cells and cancer

As IL-23 has been shown to be a potent anti-cancer agent, yet drives IL-17 expression and is imperative for commitment of CD4+ T-cells to a Th17 phenotype, it begs the question whether either IL-17 or Th17 T-cells also possess anti-cancer activity. Indeed, several studies have implicated IL-17 in anti-cancer immunity. IL-17 deficient mice are more susceptible to lung melanoma\(^ {230}\) and colon carcinoma development.\(^ {231}\) Additionally, expression of IL-17 in hematopoietic tumors was found to significantly inhibit tumor growth in a T-cell dependent fashion.\(^ {232}\) However, IL-17 may also possess pro-cancer effects. Cervical carcinoma cells stably expressing IL-17 exhibit enhanced growth compared to parental cells in nude mice.\(^ {233}\) Additionally, a recent study found that IL-17 receptor-deficient mice are refractory to the development of lymphoma, melanoma, and prostate tumor development and that antibody-mediated depletion of IL-17 inhibits, while systemic administration of recombinant IL-17 enhances, tumor growth.\(^ {234}\)
Numerous studies highlighting the anti-cancer effects of Th17 cells have also been published. CD4+ T-cells specifically recognizing a B16 melanoma antigen polarized to a Th17 phenotype in vitro effectively inhibited melanoma development in an IFN-γ, but not IL-17 or IL-23, dependent manner. Additionally, adoptive T-cell therapy utilizing Th17 cells inhibited tumorigenesis by activation of tumor specific CD8+ T-cells and recruitment of DCs into the tumor microenvironment. Interestingly, in this study, adoptively transferred Th17 cells exhibited more potent anti-tumor effects than their Th1 counterparts.

However, Th17 cells have also been strongly implicated in carcinogenesis. Colonic carcinogenesis associated with human colonic bacterium Bacteroides fragilis expressing B. fragilis toxin is linked with a Th17 response and inhibition of IL-17 or the IL-23 receptor inhibits tumorigenesis. Additionally, melanoma, breast and colon cancers exhibit higher numbers of Th17 tumor infiltrating lymphocytes. Likewise, gastric cancer patients possess greater levels of Th17 cells in peripheral blood and tumor-draining lymph nodes.

Largely, the role of IL-17 and Th17 cells in cancer remains controversial. Numerous studies have correlated IL-17 and Th17 cells with cancer incidence and mice deficient in IL-17 appear to be refractory to development various types of cancers. However, additional studies have shown that IL-17 and Th17 cells exert therapeutic effects utilizing mechanisms such as activation of CD8+ T-cells and induction of IFN-γ. It may be that IL-17 and Th17 cells, like IL-23, possess both pro- and anti-tumor activities, depending upon tumor model, expression level and means of administration. Due to the role of IL-23 in IL-17 production and the purported anti-cancer properties of IL-17, we propose to investigate both the role of IL-17 in IL-23-mediated anti-tumor immunity, as well as investigate any possible anti-tumor synergism between IL-23 and IL-17.
Adenovirus was first identified in the 1950s upon isolation of the virus from adenoid tissue and currently includes 51 different serotypes, which are divided into 6 subtypes based on haemagglutination properties, oncogenic potential and DNA homology.\textsuperscript{239-240} Adenoviruses infect a variety of hosts, including mammals, birds and reptiles.\textsuperscript{241} Adenoviral infection is common in humans, can occur via respiratory, fecal-oral or ocular conjunctival routes and has an incubation period of 2 to 14 days.\textsuperscript{240} Adenovirus types 1, 2 and 5 account for 5 to 10\% of all childhood respiratory diseases and can cause other mild gastrointestinal, urogenital and ocular diseases as well.\textsuperscript{240}

1.4.1 Structure

Adenovirus is a non-enveloped, icosahedral virus approximately 90nm in diameter. A fiber rod protrudes from each of the vertices from a penton base, which is composed of 5 penton polypeptides. The fibers are composed of an N-terminal tail which binds to the penton base, a slender shaft and a C-terminal globular domain that interacts with the cocksackie and adenovirus receptor (CAR) on host cells. The remainder of the virus is comprised of at least 7 additional minor capsid proteins that are believed to play important structural roles. Protein II, also called hexon, is also part of the capsid shell. Proteins IIIa, VI, XIII and IV are involved in capsid stabilization. The core is comprised of the terminal protein, which is linked to the genome, and proteins V, VII and $\mu$, which are involved in DNA packaging. The genome is a linear, approximately 35 kb double stranded DNA molecule which contains 103 nucleotide long inverted terminal repeats in which the origin of replication is embedded.\textsuperscript{242-243}
1.4.2 Replication

The classical pathway of adenovirus entry into cells first involves binding of the fiber knob to the coxsackie and adenovirus receptor (CAR) on host cells. Alternatively, binding of the fiber knob to heparin sulfate glycosaminoglycans is sufficient to initiate entry of adenovirus serotypes 2 and 5. Internalization is mediated by interaction of the RGD motif on the adenovirus penton base with integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$. While still on the surface, adenovirus particles shed the fiber proteins and internalization then occurs via receptor-mediated endocytosis into clathrin-coated pits. Grouping of integrins triggers endocytosis and signaling pathways such as protein kinase A (PKA) and p38/MAP kinase, which direct microtubule-mediated trafficking of adenovirus to the nucleus and others, such as phosphatidylinositol-3-OH (PI3) kinase, which is responsible for cytoskeletal remodeling. Within the endosome, decreased pH is believed to cause a conformational change in the penton base, leading to release of the nucleocapsid into the cytosol. The nucleocapsid then traffics along microtubules to the nucleus, where the remaining capsid is disassembled and DNA released into the nucleus via the nuclear pore complex. Shortly after infection, transcription of the early region 1 (E1) takes place, which includes $E1A$ and $E1B$ genes that activate other early transcription units. Altogether, there are six early transcription units, $E1A$, $E1B$, $E2A$, $E2B$, $E3$ and $E4$. Transcription of $E2A$ results in expression of DNA-binding protein (DBP), while E2B encodes the terminal protein (TP) and DNA polymerase (Pol). TP and Pol dimers, together with DBP and a core origin of replication, maintain basal replication. The E3 gene products inhibit transport of MHC class I molecules to the cell surface, but are nonessential for viral replication in culture. E4 is involved in a variety of functions including shifting translation to viral mRNA. Assembly of adenoviruses takes place within the nucleus of the infected cell and all structural proteins are transported to this site.
following synthesis in the cytoplasm via nuclear localization signals. Newly synthesized particles are then released by lysis of the host cell, enabled, in part, by E1B and E3 encoded proteins, which disrupt the host cell filament networks and the nucleus.²⁴²-²⁴³

1.4.3 Immune response

Shortly after delivery, adenovirus elicits a rapid innate immune response that involves expression of various cytokines, chemokines and neutrophils resulting in necrosis and apoptosis of infected tissues.²⁵⁰-²⁵¹ Adenoviral-mediated activation of innate immunity involves toll-like receptor 9 (TLR9) and cytosolic sensing of adenoviral DNA, leading to production of type-1 interferons.²⁵² Moreover, this response is independent of transgene expression.²⁵⁰ The innate immune response subsequently initiates generation of adaptive immunity. Antigen presenting cells such as DCs and macrophages present adenoviral and transgene products to direct a Th1 and, to a lesser extent, Th2-type immune responses against these antigens.²⁵³ Thus, eliciting of innate and adaptive immune responses against adenovirus can be seen as a barrier to gene therapy efforts focusing on replacement of missing or defective genes. However, in the context of cancer gene therapy, where the ultimate goal is activation of the host immune response to tumors, use of a highly immunogenic vector could be an asset.

1.4.4 Gene Therapy

As a gene therapy vector, adenovirus in particular has received much attention, due to its large insert capacity, ability to infect a wide range of cells, both replicating and quiescent, relative ease of propagation and low toxicity associated with use. Furthermore, DNA encoded by adenovirus
is not germline transmitted; however, transgene expression is limited to a few weeks to months due to activation of both innate and adaptive immune responses.

First generation adenoviral vectors, also referred to as E1-deleted, are the most widely used. The deletion of \( E1A \) and \( E1B \) transcription units disrupts the replication cascade and frees up some 5kb of space for transgene inserts. Some vectors combine E1 and E3 deletions to create even more space for foreign DNA insertions (up to 8kb), as E3 is expendable in viral production.\(^{242}\) First generation adenoviruses are constructed by co-transfection of E1 complementing cell lines, such as 293, with (1) a shuttle plasmid encoding the left ITR, packaging domain and transgene of interest and (2) a plasmid encoding the viral backbone that contains homologous sequences to the shuttle plasmid. Following homologous recombination, E1-deleted viral particles are rescued. First generation adenoviruses are propagated by infection of E1 complementing cell lines, including 293s, which provide viral proteins in \( \text{trans} \).\(^{242}\) However, passage on 293 cells is associated with emergence of \( E1 \) containing, replication competent viruses due to recombination of adenovirus DNA within 293 cells with vector DNA.\(^{254}\) Additionally, deletion of \( E1 \) does not completely abolish remaining viral gene expression, as some cellular proteins can mimic E1 activity in \( \text{trans} \) and activate viral gene expression.

To reduce viral gene expression, reduce immunogenicity and increase carrying capacity, adenoviral vectors deficient in other early genes in addition to \( E1 \) and \( E3 \), such as \( E2A \), \( E2B \) or \( E4 \) were generated, with moderate success.\(^{242}\) Also to this end, high capacity (HC) adenoviral vectors devoid of all viral genes and boasting a 37kb insert capacity were created.\(^{255}\) Production of HC adenoviruses require transfection of vector DNA into \( E1 \) complementing cell lines,
followed by infection with E1-deficient “helper” adenovirus to supply in trans all necessary viral amplification and packaging functions.242

Adenoviruses have been used to replace genes in variety of disease models involving mutations of a particular gene, such as muscular dystrophy256-257 and cystic fibrosis.258 However, due to their high immunogenicity, first generation adenoviruses are uniquely suited to deliver immunostimulatory molecules in a variety of gene therapy of cancer protocols. Indeed, adenoviruses have been used to deliver a variety of Th1 polarizing cytokines intratumorally, including IL-12,18 IL-18,259 and IL-2260 in the treatment of murine fibrosarcoma, melanoma and breast cancer models to name a few. Likewise, adenoviruses have been used in a variety of human clinical trials of cancer and the first approved gene therapy product was an E1-deleted adenovirus aimed at restoring p53 expression in head and neck cancer.242 Thus, adenoviruses are attractive agents for the delivery of immunostimulatory genes in the treatment of cancer.

1.5 SUMMARY

Cancer often arises and progresses as a result of a dysregulated immune response to oncogenic cells. I hypothesize that the problem lies specifically within the CD4+ T-cell response to tumors. Initially, the host immune system recognizes the growing tumor as foreign, due to expression of proteins not typically found on normal cells, and thus mounts a cell mediated immune response aided by Th1-polarized CD4+ T-cells. However, as tumor growth progresses, the CD4+ T-cell response becomes skewed to a Th2 and ultimately a Treg phenotype, which actively inhibits activation of the host immune response. I propose that expression of Th1-polarizing cytokines will reactivate the cell-mediated immune response, leading to tumor destruction and induction of
tumor-specific immunity. Previous studies have shown that expression of IL-12 within the tumor microenvironment leads to tumor regression and generation of tumor-specific immunity utilizing Th1-type mechanisms, but its use is associated with severe toxicity and its efficacy in clinical trials has been unimpressive. Thus, novel cytokines, to use either alone or in combination with IL-12, are continuously sought out. IL-23, a newly described member of the IL-12 family of heterodimeric cytokines, has been reported to posses Th1-polarizing functions similar to IL-12, but also may specifically activate memory T-cells. First generation adenoviruses are an attractive means to attain local expression of immunostimulatory cytokines, due to their large carrying capacity, ease of propagation and ability to activate both innate and adaptive immune responses. Thus, I propose that adenoviral-mediated, intratumoral expression of IL-23 will mount an effective anti-tumor response using mechanisms similar to, yet divergent from, IL-12. Additionally, due to the Th1-polarizing nature of these two cytokines, I propose that adenoviral-mediated co-expression of both IL-12 and IL-23 within the tumor microenvironment will result in synergistic enhancement of anti-tumor effects over use of either cytokine alone.
2.0 THERAPEUTIC, ANTITUMOR ACTIVITY OF ADENOVIRALLY DELIVERED IL-23

2.1 INTRODUCTION

IL-12 is a heterodimeric proinflammatory cytokine composed of p40 and p35 subunits. Its receptor is also a heterodimer, composed of the IL-12Rβ1 and IL-12Rβ2 subunits. IL-12 stimulates IFN-γ production, promotes Th1 differentiation of naïve CD4+ T-cells and links the innate and adaptive immune responses. IFN-γ is responsible for most of the inflammatory actions of IL-12, drives Th1 differentiation and induces IL-12 secretion from DCs, thereby forming a positive feedback loop for Th1 differentiation. IL-12 enhances proliferation of, cytolytic activity of, and IFN-γ production from activated NK and T-cells. The proinflammatory and immunostimulatory activities of IL-12 are responsible for its potent anti-tumor effects in various preclinical animal models of cancer. IL-12 inhibits tumor establishment, causes regression of established tumors and induces tumor-specific immunity. However, systemically delivered IL-12 is associated with severe toxicity. Delivery of IL-12 directly into the tumor microenvironment using adenoviral vectors promotes tumor regression and generation of tumor-specific immunity, while alleviating the toxicity associated with systemic delivery. However, new cytokines are continuously sought out for use in cancer gene therapy either alone and or in combination with IL-12.
IL-23 is a member of the IL-6/IL-12 family of heterodimeric cytokines and is composed of two subunits: p40, which is shared with IL-12, and p19, which is unique to IL-23. p19 is related to the p35 subunit of IL-12 and is active only when co-expressed with IL-12 p40. The IL-23 receptor is composed of IL-12Rβ1 and the IL-23 receptor and is found on human Th0 and Th1 clones and several NK cell lines. IL-23 is expressed by activated dendritic cells and stimulates proliferation of and IFN-γ production from memory Th1 CD4+ T-cells, suggesting an important role for this cytokine in the maintenance of Th1 immunity. IL-23 also acts on dendritic cells to increase IL-12 production and the combination of IL-12 and IL-23 causes dendritic cells to secrete levels of IFN-γ greater than either cytokine alone. Furthermore, IL-23-treated dendritic cells pulsed with an otherwise poorly immunogenic peptide induced a CD4+/CD8+ T-cell response in vivo. Thus, IL-23 is capable of promoting a protective immune response to tumors and, similar to IL-12, may act at the interface of innate and adaptive immunities. Additionally, IL-23 plays an important role in chronic inflammation, as IL-23 exposure can induce IL-17 production from activated CD4+ T-cells. Multiple studies previously performed on various murine carcinoma establishment models have shown that stable expression of IL-23 in tumor lines results in decreased tumor growth, increased survival and ultimately tumor rejection with generation of anti-tumor immunity. When engineered to stably express single chain IL-23, murine colon adenocarcinoma, melanoma, and esophageal tumors exhibited regression and eventual rejection in mice. Tumor rejection due to expression of IL-23 coincided with the generation of a specific adaptive immune response and appears to be dependent on natural killer (NK) and T-cells. Additionally, treatment with dendritic cells expressing IL-23, as well as systemic expression of this cytokine, resulted in inhibition of growth of pre-established tumors utilizing mechanisms that appear to be T and NK
cell dependent.\textsuperscript{215-216} However, the effects of local, intra-tumoral expression of IL-23 in a therapeutic cancer model have yet to be investigated.

Here we have analyzed the anti-tumor activity of adenovirally-delivered IL-23 (Ad.IL-23) in comparison to adenovirally delivered IL-12 (Ad.IL-12) in a day 7 MCA205 murine fibrosarcoma tumor model. Multiple intratumoral injections of Ad.IL-23 resulted in a significant increase in survival time and complete rejection in 40 percent of tumors with subsequent generation of protective immunity and MCA205-specific cytotoxic T-lymphocytes (CTLs). Ad.IL-12 treatment exhibited greater efficiency in tumor eradication compared to Ad.IL-23, but did not generate protective immunity as well as IL-23 treatment. Additionally, we have demonstrated that the anti-tumor activities of IL-12 and IL-23 are similar in their need for IFN-$\gamma$ and lack of requirement for IL-17, perforin and Fas ligand. The anti-tumor mechanisms of the two cytokines diverge in IL-23’s requirement of CD4+ and CD8+ T-cells, which IL-12 is independent of. Taken together, these results demonstrate that direct intratumoral injection of adenovirus expressing IL-23 results in enhanced survival, tumor eradication and generation of protective anti-tumor immunity by generating a Th1 immune response. In contrast, our data suggests that much of the anti-tumor activity of Ad.IL-12 utilizes innate immune mechanisms. Thus, use of similar, yet divergent, mechanisms of action by IL-12 and IL-23 suggest that therapy utilizing both cytokines will result in synergistic anti-tumor effects.
2.2 METHODS

2.2.1 Adenoviruses

Adenoviruses expressing IL-12 (Ad.IL-12) and IL-23 (Ad.IL-23) have been described previously. Ad.IL-12, Ad.IL-23 and Ad.Psi5 (empty vector) were prepared as follows: Viruses were propagated on HEK-293 cells and purified by CsCl banding, followed by dialysis in 3% sucrose solution. Particle titer of purified viruses was determined by spectroscopy using the following equation: (OD_{260})(50)/9.09x10^{-13}. Infectious titers were determined using quantitative real-time PCR as previously described and were approximately 100-fold less than particle titers. Viruses were aliquoted and stored at -80°C until use. Relative cytokine expression of each adenoviral preparation was analyzed by infecting 4x10^4 MCA205 cells with increasing MOIs of Ad.IL-12 or Ad.IL-23 for 1 hour at 37°C/5% CO_2 in serum free media. Complete media was added and cells were then incubated for 72 hours, after which the supernatants were harvested. IL-12 and IL-23 content was analyzed using the mouse IL-12 p70 and mouse IL-23 p19/p40 Ready-Set-Go IL-23 ELISA kits (eBioscience, San Diego, CA), respectively, following manufacturer’s instructions.

2.2.2 Animals

Female C57BL/6 and perforin, Fas ligand, IFN-γ, CD4+ and CD8+ T-cell deficient mice on a C57BL/6 background were obtained from The Jackson Laboratory (Bar Harbor, ME). The IL-17 receptor knockout mice are also on a C57BL/6 background, have been described previously and...
were a kind gift of Dr. Jay Kolls. Mice were used at 6 to 10 weeks of age. Animals were maintained under pathogen free conditions at the Biotechnology Center Animal facility at the University of Pittsburgh. All procedures performed were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

2.2.3 Cell Lines

MCA205 fibrosarcoma, YAC-1 (American Type Culture Collection, Manassas, VA) and B16 melanoma cells were maintained in RPMI supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and 1% penicillin/streptomycin (Gibco, Carlsbad, CA) and L-glutamine (Gibco, Carlsbad, CA). Cells were kept in a humidified chamber at 37°C/5% CO₂ and passaged every 2-3 days.

2.2.4 Cytotoxic T-Lymphocyte Assay

Two to three weeks after tumor resolution, cured mice were sacrificed and spleens harvested. Spleens were mechanically dissociated and treated with Red Cell Lysis buffer (Invitrogen, Carlsbad, CA) to remove all red blood cells. Remaining lymphocytes were cultured with recombinant human IL-2 (R&D Systems, Minneapolis, MN) for 7 days prior to use in CTL assay. CTL assays were performed using the CytoTox 96® Non-Radioactive Cytotoxicity Assay kit (Promega, Madison, WI) following manufacturer’s instructions.
2.2.5 *In Vivo* Tumor Studies

For use in *in vivo* tumor experiments, confluent layers of MCA205 or B16 cells were dissociated by trypsin, washed 3 times with Hanks Balanced Salt Solution (HBSS) (Gibco, Carlsbad, CA) and counted using trypan blue exclusion. Mice were inoculated with $1 \times 10^5$ MCA205 cells in 100uL HBSS subcutaneously in the abdomen. On days 7, 9 and 11 after tumor inoculation, mice were injected intratumorally with $5 \times 10^{10}$ particles (approximately $5 \times 10^8$ PFUs) of either Ad.IL-12, Ad.IL-23 or Ad.Psi5. Tumor volume was monitored using a metric caliper until mice were sacrificed due to excessive tumor size or tumor ulceration. Tumor-free or “cured” mice were subject to tumor challenge 1-2 months after initial tumor resolution with $1 \times 10^5$ MCA205 cells subcutaneously in the abdomen.

2.2.6 Statistics

Kaplan-Meier survival curves were plotted using SPSS version 16.0. Mice were monitored until excessive tumor volume or tumor ulceration, at which time they were sacrificed and recorded as occurrence of an event (death). Cured mice or those with tumors that did not warrant sacrifice by the end of the experiment were censored. Log-rank tests of the survival curves provided p-values. Statistical analyses were 2-tailed, with a p value less than 0.05 considered statistically significant.
2.3 RESULTS

2.3.1 Confirmation of IL-12 and IL-23 expression from adenovirally transduced MCA205 tumor cells

To confirm cytokine expression following Ad.IL-12 or Ad.IL-23-mediated transduction of MCA205 fibrosarcoma tumor cells, $4 \times 10^4$ MCA205 cells were infected with increasing MOIs of adenovirus. Supernatants were harvested 72 hours post-adenoviral infection and analyzed for cytokine expression by ELISA. As shown in Figure 3, transduction of MCA205 cells with either Ad.IL-12 or Ad.IL-23 resulted in release of respective cytokine from the infected cells, but not from Ad.Psi5 or mock infected cells. While both Ad.IL-12 and Ad.IL-23 produce detectible amounts of cytokine, Ad.IL-12 expresses almost 100-fold more cytokine than Ad.IL-23. The biological activity of both Ad.IL-12 and Ad.IL-23 has been previously investigated and published.\textsuperscript{18,269}

![Figure 3: Ad.IL-12 and Ad.IL-23 express detectable levels of cytokine](image-url)
2.3.2 Adenoviral delivery of IL-12 or IL-23 into the tumor microenvironment result in eradication of subcutaneous MCA205 tumors

2.3.2.1 Three injections of Ad.IL-12 or Ad.IL-23 result in enhanced survival and tumor rejection

To examine the anti-tumor activity of Ad.IL-23 in comparison to Ad.IL-12, 6 to 8-week old C57BL/6 mice were inoculated with 1 x 10^5 MCA205 cells subcutaneously. On days 7, 9 and 11 post-tumor inoculation, mice were injected intratumorally with 5 x 10^{10} particles of Ad.IL-12, Ad.IL-23 or Ad.Psi5 in 100 uL of saline. Mice treated with Ad.IL-23 exhibited a statistically significant increase in survival (p=0.000) versus empty vector controls (Figure 4a). In six experiments involving 40 mice treated with three injections of Ad.IL-23, treated mice showed decreased tumor volumes compared to empty vector controls and exhibited an overall tumor rejection rate of 40 percent (Figure 4b), with no evidence of cytokine-mediated toxicity.

As has been reported previously^{18}, Ad.IL-12 exhibited potent anti-tumor activity, significantly increasing survival (p = 0.00) compared to empty vector treated controls and leading to tumor eradication in 70 percent of treated animals (Figures 4a and b). However, there was evidence of IFN-γ mediated toxicity, as some Ad.IL-12 treated animals lost weight, exhibited a hunched appearance and were lethargic; however, none died as a result of Ad.IL-12 treatment.
2.3.2.2 Ad.IL-12 and Ad.IL-23 mediated generation of protective immunity and tumor-specific cytotoxic T-lymphocytes

To determine if Ad.IL-12 and Ad.IL-23 mediated tumor rejection led to generation of protective immunity, cured mice were challenged with 1x10^5 MCA205s in the contralateral flank. Overall, Ad.IL-23 treated, cured mice remained tumor-free for up to three months and all animals challenged with a second dose of MCA205 cells were resistant to tumor development. In contrast, two Ad.IL-12 treated mice that initially experienced tumor rejection later had the tumors recur. In addition, only approximately 50 percent of tumor-free Ad.IL-12 treated mice were resistant to subsequent tumor challenge.

To determine if treatment of MCA205 tumors with Ad.IL-12 or Ad.IL-23 resulted in the generation of tumor-specific CTLs, spleens were harvested from treated, tumor free mice within 2-3 weeks of tumor resolution. Purified lymphocytes were cultured with rhIL-2 for 7 days and re-stimulated with MCA205 cells. CTL activity was measured by LDH release from target cells. Cytotoxic activity was detected against MCA205 tumor cells in splenocytes harvested from both Ad.IL-12 and Ad.IL-23 treated animals (Figures 5a and b). Splenocytes from Ad.IL-23 treated animals exhibited no activity against YAC-1 (NK cell sensitive) cells (Figure 5b), suggesting
that treatment of subcutaneous MCA205 tumors with Ad.IL-23 resulted in generation of anti-
tumor immunity by production of MCA205-specific CTLs. This result is consistent with the
ability of Ad.IL-23 treated, cured mice to resist MCA205 tumor challenge. In contrast,
splenocytes from Ad.IL-12 treated animals showed strong cytotoxicity against YAC-1 cells,
suggesting involvement of an innate immune response in the anti-tumor properties of IL-12.

Figure 5: Ad.IL-23, but not Ad.IL-12, treatment results in generation of tumor-specific CTLs

### 2.3.2.3 Determining the minimal effective dose of Ad.IL-12 and Ad.IL-23

Given that three injections of Ad.IL-23 were highly effective in inducing anti-tumor immunity, we subsequently examined whether two or even one injection of Ad.IL-12 or Ad.IL-23 would be sufficient to mount an anti-tumor response. Established MCA205 tumors were injected intra-
tumorally twice on days 7 and 11. Treatment with two injections of Ad.IL-23 resulted in a 40 percent tumor eradication rate (2 of 5 mice), but did not significantly increase survival compared to empty vector controls (Figures 6a and b). Treatment with two injections of Ad.IL-12 resulted in significant increase in survival compared to Ad.Psi5 and Ad.IL-23 treated animals and resulted in a 100 percent (5 mice total) tumor rejection rate (Figures 6a and b). As before, there was some evidence of IL-12-mediated toxicity, but no animals died as a result of Ad.IL-12
therapy. Interestingly, Ad.IL-23 treated animals that experienced tumor rejection were completely refractory to MCA205 tumor challenge. In contrast, all cured, Ad.IL-12 treated mice developed tumors upon challenge, two of which were not ultimately rejected (data not shown).

Figure 6: Two treatments with Ad.IL-12 or Ad.IL-23 mount an anti-tumor response

In animals treated once on day 7 with 5 x 10^{10} particles of Ad.IL-12 or Ad.IL-23, Ad.IL-12 treatment significantly increased survival compared to Ad.Psi5 treated controls and led to tumor rejection in 60 percent of animals (3 of 5) (Figures 7a and b). Although one treatment with Ad.IL-23 did not lead to tumor rejection in any animals, survival was significantly enhanced compared to empty vector controls (Figures 7a and b). Again, Ad.IL-12 treatment significantly increased survival of tumor bearing mice compared to those treated with Ad.IL-23 (Figure 7a). Similar to previous results, cured, Ad.IL-12 treated animals developed tumors upon MCA205 challenge. Overall, these results suggest that Ad.IL-12 is the more potent anti-tumor agent, exhibiting strong anti-tumor activity even at low doses, but generates only moderate, if any, protective immunity. Furthermore, although Ad.IL-23 treatment is beneficial to survival even at low doses, multiple injections are necessary for maximal anti-tumor effect.
2.3.2.4 Systemic Anti-tumor effects of Ad.IL-12 and Ad.IL-23

To determine if Ad.IL-12 or Ad.IL-23 treatment resulted in induction of systemic immunity, mice bearing two MCA205 tumors were treated on days 7, 9 and 11 in only one tumor with 5x10^10 particles of either Ad.IL-12, Ad.IL-23 or Ad.Psi5. While Ad.IL-23 treatment only led to modest tumor rejection in this model, overall inhibition of tumor growth was observed in both injected and contralateral tumors (Figures 8a-d). In contrast, Ad.IL-12 demonstrated potency both in reducing tumor volume and enhancing tumor rejection compared to empty vector treated controls (Figure 8a-d). These results demonstrate that direct intratumoral injection of adenovirus expressing either IL-12 or IL-23 results in induction of a systemic anti-tumor response, with IL-12 treatment yielding maximal anti-tumor benefits.

Figure 7: 1 treatment with Ad.IL-12 or Ad.IL-23 mounts an anti-tumor response
2.3.3 Ad.IL-23 anti-tumor activity is not model-specific

While the anti-tumor activity of adenovirus expressing IL-12 has been established in other preclinical animal models of cancer,\textsuperscript{18,127,130,270-278} we sought to show that the anti-tumor activity of Ad.IL-23 was not model specific. To this end, C57BL/6 mice were inoculated with $1\times10^5$ B16 melanoma cells in the flank and treated on days 7, 9 and 11 with $5\times10^{10}$ particles of Ad.IL-23. While overall tumor rejection was modest at 20 percent (1 of 5), Ad.IL-23 treated animals survived significantly longer than their untreated counterparts (Figures 9a and b). These data show that the anti-tumor activity of adenovirally delivered IL-23 is not limited to the MCA205 fibrosarcoma model and may potentially have broad applicability.
2.3.4 IL-12 and IL-23 anti-tumor activity is independent of endogenous IL-17 production

The results above clearly demonstrate that adenoviral mediated intra-tumoral delivery of IL-23 results in a significant anti-tumor response. To examine the nature of this immune response in comparison to adenovirally delivered IL-12, the abilities of both Ad.IL-12 and Ad.IL-23 to induce regression of MCA205 tumors was examined in mice deficient for various immunoregulatory molecules or cell types. In vivo, IL-23 strongly induces production of IL-17,30 which has been previously shown to possess anti-tumor activity.232 In contrast, IL-12 induces IFN-γ production, which has been shown to limit IL-17 producing T-cells.279 To determine if IL-23-mediated, endogenous IL-17 production may be responsible for the observed anti-tumor effects or if lack of IL-17 signaling may affect the anti-tumor activity of Ad.IL-12, IL-17 receptor-deficient mice were inoculated with subcutaneous MCA205 tumors and treated with either Ad.IL-12 or Ad.IL-23 on days 7, 9 and 11 post-tumor inoculation. IL-23 treatment significantly increased animal survival (p=0.047) compared to empty vector treated mice (Figure 10a). Additionally, 60 percent (3 of 5) of tumor-bearing mice experienced tumor resolution with
kinetics similar to wild-type mice (Figure 10b) and were resistant to tumor challenge (data not shown).

In marked contrast to IL-23 treatment, Ad.IL-12 therapy resulted in severe, most likely IFN-γ, or possibly TNF-α, mediated toxicity in IL-17 receptor deficient mice. By day 15 post-tumor inoculation, all Ad.IL-12 treated mice had minimal size tumors, but appeared lethargic, hunched over and exhibited matted coats and wasting. All but one mouse, which had experienced tumor rejection, had perished as a result of treatment by day 28. Although statistics on survival could not be generated due to the toxicity of treatment (Figure 10a), it was apparent that Ad.IL-12 therapy did inhibit tumor growth and most likely all treated animals would have rejected the tumors if death due to toxicity would not have occurred prior.

Taken together, these data suggest that adenovirally-delivered IL-23 anti-tumor activity is not mediated by endogenous IL-17 production. Furthermore, while lack of IL-17 signaling does not appear to affect the anti-tumor activity of adenovirally delivered IL-12, it does appear to affect toxicity associated with IL-12 treatment.

![Figure 10: Ad.IL-12 and Ad.IL-23 anti-tumor activity in IL-17R-deficient mice](image)
2.3.5 IL-12 and IL-23 anti-tumor activity is independent of perforin

Perforin is produced by CD8+ T-cells and NK cells and is released upon contact with the target cell to induce cell death. To determine if the anti-tumor activity of adenovirally delivered IL-12 or IL-23 is dependent on perforin-mediated lysis of tumor cells, perforin-deficient mice with established MCA205 tumors were treated with Ad.IL-12 or Ad.IL-23 as described above. Treatment with Ad.IL-23 resulted in a significant increase in survival (p=0.013) (Figure 11a), and led to tumor rejection in 30 percent of mice (3 of 10) (Figure 11b). Cured, perforin-deficient mice experienced tumor rejection in a similar time frame as cured, Ad.IL-23 treated wild-type mice and were resistant to tumor challenge (data not shown).

Similar to Ad.IL-23, Ad.IL-12 treatment in perforin deficient animals led to a significant increase in survival compared to empty vector treated controls (p = 0.005). Likewise, 100 percent (5 mice total) of IL-12 treated animals experienced tumor rejection in a timeframe comparable to wild type mice (Figure 11b). Similar to what was seen in IL-17 receptor deficient mice, there was some evidence of toxicity due to treatment, but none of the perforin deficient animals perished as a result of Ad.IL-12 therapy. Upon challenge, 2 of 3 IL-12 treated, cured, perforin deficient mice developed tumors that did not regress, mirroring the lack of establishment of strong protective immunity seen in wild-type mice.

Overall, these data suggest that adenovirally delivered IL-12 and IL-23 do not utilize perforin-mediated killing as an anti-tumor effector mechanism. Furthermore, lack of perforin does not affect establishment of protective immunity in animals treated with either Ad.IL-12 or Ad.IL-23.
2.3.6 IL-12 and IL-23 anti-tumor activity is independent of Fas ligand

Interactions between Fas and Fas ligand (FasL) are used by activated CD8+ T-cells and some CD4+ effector cells to kill target cells via apoptosis. To determine if the anti-tumor activity of adenovirally delivered IL-12 or IL-23 requires Fas-Fas ligand interactions to eradicate tumor cells and generate anti-tumor immunity, FasL-deficient mice were inoculated with tumors and treated as described above. Interestingly, while Ad.II-23 treatment did not lead to tumor rejection in any treated animals (10 mice total) (Figure 12b), it did significantly increase survival compared to empty vector controls (p=0.000) (Figure 12a). Similar to Ad.II-23, treatment with Ad.II-12 led to enhanced survival compared to empty vector controls (p=0.002) (Figure 12a). Furthermore, treatment led to complete tumor rejection in 60 percent of animals (3 of 5). Ad.II-12 therapy was again associated with some toxicity (in the form of weight loss and lethargy), but did not lead to death in any treated animals. Interestingly, one IL-12 treated, cured animal had a recurrence of the tumor, similar to what was seen in wild-type mice.
The above data leads us to conclude that both adenovirally delivered IL-12 and IL-23 are able to function through a Fas-FasL-independent mechanism to exert their anti-tumor effects. However, in the case of IL-23, given that treatment did not result in eradication of tumors in any of the mice, it is also possible that at least part of the anti-tumor effect of IL-23 involves a Fas-FasL-dependent pathway.

Figure 12: Ad.IL-12 and Ad.IL-23 anti-tumor activity in Fas ligand-deficient mice

2.3.7 Interferon gamma is necessary for IL-12 and IL-23 anti-tumor activity

Cytotoxic T-lymphocytes and a subset of Th1 T-cells secrete IFN-γ upon engagement of the T cell receptor in order to exert their cytotoxic effects on target cells. To determine if IFN-γ is necessary for the anti-tumor activity of adenovirally delivered IL-12 and IL-23, IFN-γ deficient mice with established tumors were treated as described above. Tumor-bearing mice treated with either Ad.IL-12 or Ad.IL-23 showed no significant increase in survival when compared to empty adenoviral vector controls (Figure 13a). Additionally, neither Ad.IL-12 nor Ad.IL-23 treatment led to tumor rejection (6 mice total treated with Ad.IL-12 and 11 mice total treated with Ad.IL-
23) (Figure 13b), suggesting that adenovirally delivered IL-12 and IL-23 both utilize IFN-γ as an effector mechanism to exert their anti-tumor effect.

Figure 13: Ad.IL-12 and Ad.IL-23 anti-tumor activity in IFN-γ-deficient mice

2.3.8 Anti-tumor activity of IL-23, but not IL-12, is dependent on CD4+ T-cells

CD4+ T-cells have a variety of functions, including “helping” the activation of CTLs and directly killing target cells, utilizing mechanisms including Fas-FasL interactions and release of IFN-γ. To determine if the anti-tumor activity of adenovirally delivered IL-12 or IL-23 is dependent on the presence of CD4+ T-cells, CD4 deficient mice were inoculated with MCA205 tumors and treated with either vector as described above. Ad.IL-23 treated mice showed no significant increase in survival when compared to empty adenoviral vector controls (Figure 14a) and none rejected their tumor (10 animals total) (Figure 14b).

In contrast, Ad.IL-12 treated, CD4-deficient mice survived significantly longer than their Ad.Psi5 treated counterparts (p = 0.003) and exhibited an 80 percent (4 of 5) tumor rejection rate (Figures 14a and b). Again, IL-12 treatment resulted in some toxicity, presenting as lethargy and
weight loss in mice, but no animals died as a result of treatment. Additionally, upon MCA205 challenge, all cured, CD4-deficient mice were resistant to tumor challenge.

These data suggest that IL-12 mediated establishment of protective immunity is independent of the presence of CD4+ T-cells, whereas IL-23 requires the activity of CD4+ T-cells to exert optimal anti-tumor effects.

Figure 14: Ad.IL-12 and Ad.IL-23 anti-tumor activity in CD4-deficient mice

2.3.9 IL-23, but not IL-12, utilizes CD8+ T-cells to exert its anti-tumor effects

Since IL-23 anti-tumor activity is dependent on IFN-γ, a mechanism utilized by CD8+ T-cells to exert cytotoxic effects, it is possible that CD8+ T-cells are responsible for the anti-tumor effects of IL-23. Thus CD8+ T-cell deficient mice were inoculated with MCA205 tumors and treated as described above with either Ad.IL-12 or Ad.IL-23. Interestingly, while treatment with Ad.IL-23 did lead to tumor rejection in 20 percent of animals (2 of 10) (Figure 15b), IL-23 therapy did not significantly increase animal survival (Figure 15a).

In contrast, treatment with Ad.IL-12 did significantly enhance survival (p = 0.021) and likewise led to tumor rejection in 40 percent of animals (2 of 5) (Figure 15b). Again, however,
IL-12 treatment was associated with lethal toxicity, with three animals perishing as a result of therapy by day 17 post-tumor inoculation. It must be noted that tumors in these mice were quite small and would likely have regressed entirely had the animals survived.

These results suggest that at least part of the anti-tumor effect of adenovirally delivered IL-23 is mediated through CD8+ T-cell activity, in addition to other effector mechanisms that may be important in tumor elimination. In contrast, CD8+ T-cells are not necessary for IL-12 to exert its anti-tumor effects, as adenovirally delivered IL-12 maintains anti-tumor activity in their absence.

2.4 DISCUSSION

2.4.1 The case for IL-23-mediated gene therapy of cancer

The use of gene therapy for the treatment of cancer has been proven effective in many mouse cancer models and comprises the vast majority of human gene therapy clinical trials performed to date. Here we have investigated the potential anti-tumor activity of the IL-12 family
member IL-23. IL-23 is a relatively new member of the IL-12 family of heterodimeric cytokines and shares many immunostimulatory\textsuperscript{166,173-174} and anti-tumor activities with IL-12.\textsuperscript{92,263-265} IL-23 has been shown to possess anti-tumor activity in the context of various establishment models of cancer.\textsuperscript{206-209,211} and to inhibit established tumor growth when overexpressed systemically or locally via dendritic cells.\textsuperscript{215-216} We chose to investigate the ability of adenovirally delivered IL-23 (Ad.IL-23) to inhibit tumor growth in a therapeutic mouse model of cancer for a number of reasons. First, systemic overexpression of immunostimulatory cytokines may be associated with toxicity, as is the case with systemically delivered IL-12.\textsuperscript{265} Additionally, as IL-23 is associated with a variety of autoimmune inflammatory diseases which are believed to be mediated at least in part by the generation of a specific subset of T-cells that produce IL-17,\textsuperscript{281} we hypothesized that local, rather than systemic, expression would reduce the risk of the unintentional development of any autoimmune pathology. Finally, expression of immunostimulatory cytokines within tumor cells greatly increases the chances of generating an effective immune response against the tumor itself and is technically much simpler than transduction of any intermediate cell type.

2.4.2 \textit{In vitro} expression of IL-12 and IL-23

To ensure adenoviruses expressing IL-12 and IL-23 secrete active cytokine from tumor cells, MCA205 cells were infected with either Ad.IL-12 or Ad.IL-23. Both viruses indeed secreted cytokine, which has been previously shown possess biological activity.\textsuperscript{18,269} Interestingly, Ad.IL-12 secreted approximately 100-fold greater amounts of cytokine compared to Ad.IL-23. This is most likely due to design of the adenoviral vectors. Both viruses express the individual subunits separately using an internal ribosomal entry site (IRES). However, Ad.IL-12 expresses
p40 before the IRES, while Ad.IL-23 expresses the subunit after, which most likely leads to decreased p40 expression from Ad.IL-23. As p19 can only be released from cells in the context of p40, it is possible that less IL-23 is secreted from cells due to lack of sufficient p40. However, though less IL-23 is produced, Ad.IL-23 exhibited potent anti-tumor activity and generated better protective immunity than Ad.IL-12 treatment; thus, production of a vector that expresses greater amounts of IL-23 may result in a more effective anti-tumor agent.

2.4.3 Anti-tumor activity of IL-23

Our results clearly demonstrate that treatment of day 7 MCA205 tumors with repeated intra-tumoral injections of Ad.IL-23 results in a significant increase in animal survival when compared to empty vector treated controls. In a total of 6 experiments involving 40 mice, Ad.IL-23 treated mice overall exhibited decreased tumor volume, with 40 percent of mice rejecting their tumors in about 25 days (Figures 4a and b).

To determine the minimal effective dose of Ad.IL-23, mice with established tumors were treated with two or one injections of Ad.IL-23. Tumor bearing mice treated with two doses of Ad.IL-23 on days 7 and 11 exhibited a 40 percent tumor rejection rate, but treatment did not significantly increase survival compared to empty vector controls (Figures 4a and b). In contrast, mice treated with one dose of Ad.IL-23 on day 7 showed a significant increase in survival compared to controls, but did not exhibit any tumor rejection (Figures 5a and b). We chose three separate injections of Ad.IL-23 as our treatment plan as it yielded both enhanced survival compared to controls and maximum tumor rejection rates. However, increased survival even at the lowest dose has therapeutic implications, as this suggests that use of low dose Ad.IL-23 in
combination with other Th1-polarizing cytokines, such as IL-12, will provide even greater therapeutic response with minimal adenoviral or cytokine mediated toxicity.

Additionally, we have shown that treatment with Ad.IL-23 results in induction of systemic immunity, as treatment of one tumor leads to inhibition distal tumor growth (Figure 8a and b). Complete tumor rejection due to IL-23 treatment was not attained in this model, most likely due to the large tumor burden (Figure 8c and d). Furthermore, we have shown that the anti-tumor activity of Ad.IL-23 is not model-specific, as IL-23 treated mice bearing B16 tumors survived significantly longer than controls (Figure 9a and b). The potency of IL-23 treatment was somewhat less than in the MCA205 model, but this is probably due to differences in immunogenicity of the tumor cell lines.

The anti-tumor activity of IL-23 is consistent with previously published reports showing that stable expression of IL-23 in tumor cell lines leads to tumor rejection in tumor establishment models.\textsuperscript{206-209,211} Although the overall cure rate in our experiments was slightly lower than was obtained in previous studies using establishment models (70 percent versus our 40), this disparity is likely due to differences in the model. An established tumor is more difficult to eradicate than a tumor expressing IL-23 from the onset. Additionally, differences in IL-23 expression levels in therapeutic versus establishment models may also ultimately affect the outcome, as it has been suggested that IL-23 expression levels may dictate whether IL-23 exhibits pro- or anti-tumor effects.\textsuperscript{223} It is also necessary to note that while this work was undergoing, a paper demonstrating a prophylactic, but not therapeutic, effect of adenovirally delivered IL-23 in variety of murine tumors was published.\textsuperscript{217} This disparity in results may be due to the use of different murine tumor models, use of a mutant form of p40 or initiating treatment at a different stage of the tumor model. In our studies, tumor bearing mice were treated on days 7, 9 and 11.
starting with an average tumor size of 4mm compared to the studies performed in the previously mentioned paper, in which treatment was initiated when the average tumor volume reached 7mm in diameter.

2.4.4 Anti-tumor activity of IL-12

As has been previously demonstrated, adenovirus expressing IL-12 possesses potent anti-tumor activity in our hands. Treatment with three injections of $5 \times 10^{10}$ particles of Ad.IL-12 led to enhanced survival compared to empty vector controls and an overall 70 percent tumor rejection rate (Figures 4a and b). IL-12 therapy was, however, associated with some degree of toxicity, presenting as lethargy, weight loss and disheveled coat appearance in treated animals, suggesting a high level of intratumoral IL-12 production. Such toxicity has been reported previously mice treated with recombinant murine IL-12. Ad.IL-12-mediated toxicity observed in treated animals is in accordance with in vitro MCA205 Ad.IL-12 transduction data (Figure 3a), which shows 100-fold greater expression of cytokine compared to Ad.IL-23 (Figure 1b). While toxicity was seen, it must be noted that it was not severe enough be lethal in any wild-type treated animals.

In contrast to Ad.IL-23, Ad.IL-12 maintained its potency as an anti-tumor agent even at low doses and distal sites. Treatment of tumor bearing mice with two doses of Ad.IL-12 led to a significant increase in survival and rejection of 100 percent of tumors (Figures 6a and b). Even at one dose of Ad.IL-12, treated animals survived significantly longer than their Ad.Psi5 treated counterparts and rejected 60 percent of tumors (Figures 7a and b). This is not surprising, as Ad.IL-12 expresses high amounts of cytokine (Figure 3b), which most likely correlates with its
anti-tumor activity. Likewise, mice treated with Ad.IL-12 in one tumor exhibited tumor eradication at both injected and distal sites, exhibiting induction of potent systemic immunity.

2.4.5 Comparison of Ad.IL-12 and Ad.IL-23 anti-tumor activities

Interestingly, at all treatment doses of Ad.IL-12, treated mice survived significantly longer than their IL-23 treated counterparts. This fact, combined with enhanced survival and high tumor rejection rates even at low doses of Ad.IL-12, strongly suggests that Ad.IL-12 is a much stronger anti-tumor agent than Ad.IL-23. However, all (8 mice total) cured, Ad.IL-23 treated mice (regardless of dose) exhibited protective immunity upon tumor challenge up to three months after rejection of the initial tumor. In contrast, only 56 percent (5 of 9) of mice treated with adenovirus-expressing IL-12 were resistant to tumor challenge. IL-12 is a potent activator of innate immunity\textsuperscript{50,262} and it is possible that tumor eradication was due largely to activation of the innate immune response and inhibition of angiogenesis. Thus, upon tumor challenge, cured animals would have no immunologic memory, as the adaptive response was not needed to eradicate the primary tumor. Indeed, CTL assay data from cured, IL-12 and IL-23 treated mice support this assertion. Splenocytes from cured, Ad.IL-23 treated animals show strong MCA205-specific cytotoxicity, with little to no non-specific, YAC-1 (NK cell-mediated) lysis (Figure 5b). In contrast, splenocytes from cured, Ad.IL-12 treated animals exhibited lysis of MCA205 cells, but at levels comparable to those observed in YAC-1 cells (Figure 5a), suggesting involvement of NK cells rather than CTLs.

Overall, these data suggest that, while IL-12 is highly efficient in tumor eradication, IL-23 may activate a robust memory T cell response and generate superior long-term protective immunity. Such results are promising in the context of using IL-23 in conjunction with IL-12.
Presumably using IL-23 in combination with IL-12 will result in efficient tumor eradication using a lower dose of IL-12, thereby reducing toxicity, and enhancement of the duration of protective anti-tumor immunity.

2.4.6 Evaluation of the mechanisms of IL-12 and IL-23-mediated anti-tumor activities

2.4.6.1 Role of CD4+ T cells

CD4+ T-cells perform a variety of functions in the context of anti-tumor immunity, including aiding the generation of specific adaptive immune responses or acting directly on target cells to induce apoptosis. IL-23 utilizes CD4+ T-cells to exert its anti-tumor effects, as IL-23 treatment in tumor-bearing mice deficient in CD4+ T-cells does not result in enhanced survival or lead to tumor rejection (Figures 14a and b). This is in agreement with other published studies detailing the role of CD4+ T-cells in IL-23 anti-tumor effects.212,216

In contrast to IL-23, IL-12 anti-tumor activity does not require CD4+ T-cells in our model. CD4+ T-cell deficient mice treated with adenovirus expressing IL-12 boasted significantly enhanced survival and rejection of 80 of tumors (Figures 14a and b). This is in accordance with earlier reports, as lack of requirement for CD4+ T-cells in the anti-tumor activity of IL-12 has been previously established.92 However, other reports have also implicated a role for CD4+ T-cells in IL-12-mediated tumor eradication.93 Such discrepancies are likely due to IL-12 expression levels; at high doses, IL-12 eradicates tumors using solely innate immune mechanisms, while lower doses require involvement of adaptive immune responses.
2.4.6.2 Role of CD8+ T cells

CD8+ T-cells comprise cytotoxic T-lymphocytes, which are responsible for destruction of tumor cells and somatic cells that are infected with intracellular pathogens. CD8+ T-cell-mediated apoptosis of target cells is induced by release of IFN-γ, perforin or granzymes or via surface expression of Fas ligand. To determine if activation of CD8+ T-cells is responsible for IL-23-mediated tumor eradication, tumor bearing mice deficient in CD8+ T-cells were treated with adenovirus expressing IL-23. While tumor rejection was observed, survival of IL-23 treated animals was not significantly enhanced compared to empty vector controls (Figures 15a and b), leading us to conclude that the anti-tumor activity of IL-23 is dependent on CD8+ T-cells. This is in accordance with previously published reports that confirm the dependence of IL-23 mediated inhibition of tumor growth on CD8+ T-cells.153,212,214-216

In contrast, the anti-tumor activity of adenovirally delivered IL-12 does not require CD8+ T-cells in our model, as tumor bearing, CD8+ deficient mice survived significantly longer than Ad.Psi5 treated controls and experienced tumor regression and rejection (Figure 15a and b). This is in contrast to previous studies indicating a role of CD8+ T-cells.92 Again, this discrepancy is likely due to expression levels of IL-12 and activation of an innate versus an adaptive immune response.

Interestingly, CD8+ T-cell deficient mice were subject to severe toxicity, with 60 percent of mice perishing as a result of IL-12 treatment. These results suggest that CD8+ T-cells may play a role in inhibiting IFN-γ production or attenuating a Th1 response. CD8+ regulatory T-cells with immunosuppressive effects have been described283 and have been shown to produce IL-10,284 which inhibits IL-12 activity285 and thereby may explain observed toxicity in mice deficient in all CD8+ T-cells.
2.4.6.3 Role of IL-17

IL-23 has been shown to induce IL-17 production by aiding the development of Th17 T-cells.\textsuperscript{30} Since IL-17 itself may possess anti-tumor activity\textsuperscript{232} we investigated whether the anti-tumor activity of IL-23 is mediated by endogenous IL-17 production. Using IL-17 receptor deficient mice, we demonstrated that the anti-tumor activity of IL-23 appears to be independent of endogenous IL-17 expression. IL-17 receptor-deficient mice treated with Ad.IL-23 exhibited increased survival and enhanced tumor rejection compared to animals treated with empty adenoviral vector (Figures 10a and b). Thus, although IL-23 has been shown to be a potent activator of IL-17 producing cells,\textsuperscript{184} in the context of adenovirally transduced tumor cells, either Th17 cells do not play a role in the anti-tumor activity or the development of Th1 T-cells is favored.

Anti-tumor activity of adenovirally delivered IL-12 also appeared to be independent of endogenous IL-17 signaling, as MCA205 tumors in IL-17 receptor deficient mice regressed rapidly upon treatment. However, exact statistics were not generated, as IL-12 treated animals all succumbed to presumed IFN-\(\gamma\) mediated toxicity as a result of treatment. While some toxicity was observed in tumor-bearing, Ad.IL-12 treated wild-type mice, the severe toxicity observed in the absence of IL-17 signaling suggests a role of IL-17 in inhibiting either IL-12 or IFN-\(\gamma\) production or attenuating development of Th1 response. Indeed, \textit{in vitro} studies have shown that IL-17 inhibits IL-12 mediated IFN-\(\gamma\) production in PBMCs and Th1 cells by downregulating IL-12 receptor expression.\textsuperscript{286} Thus, toxicity was likely a result of high levels of IL-12 induced IFN-\(\gamma\) production that was unable to be downregulated by IL-17 signaling.
2.4.6.4 Role of perforin

Perforin is released by NK and CD8+ T-cells upon engagement of target cells and forms holes through which granzymes can enter, thereby inducing apoptosis in the target cell. As we have shown that the anti-tumor activity of IL-23 is dependent on CD8+ T-cells, we sought to determine if CD8+ T-cells in IL-23 treated mice utilize perforin as to eradicate tumor cells. The anti-tumor activity of IL-23 does not appear to be dependent on perforin, as treatment of tumor bearing, perforin deficient mice led to significantly enhanced survival and tumor rejection in 30 percent of mice (Figures 11a and b).

Likewise, IL-12 anti-tumor effects are independent of perforin, as IL-12 treated, perforin deficient mice survived significantly longer and rejected 100 percent of tumors (Figures 11a and b). This is in agreement with previous studies investigating the mechanism of adenovirally delivered IL-12 anti-tumor activity. Additionally, cured mice showed similar resistance to MCA205 challenge compared to wild-type mice, suggesting that lack of perforin does not affect adaptive anti-tumor immunity generated by IL-12 treatment.

2.4.6.5 Role of Fas ligand

Fas ligand is used by activated CD8+ T-cells and some CD4+ effector cells to kill target cells via apoptosis. As we have previously shown that the anti-tumor activity of IL-23 is dependent on involvement of CD4+ and CD8+ T-cells, we sought to determine if Fas/Fas ligand interactions were an effector mechanism utilized by T-cells to induce tumor cell death. Upon treatment of tumor bearing, Fas ligand deficient mice with adenovirally delivered IL-23, it was apparent that IL-23 mediated eradication of tumors does not utilize Fas-FasL interactions. Tumor bearing mice deficient in Fas ligand survived significantly longer than their empty vector treated counterparts (Figure 12a). Interestingly, however, IL-23 treatment did not lead to tumor
rejection in any FasL deficient mice (Figure 12b), indicating possibly some role of FasL in complete tumor eradication.

Likewise, the anti-tumor effects of adenovirally delivered IL-12 are independent of FasL, as IL-12 treated, FasL deficient mice survived significantly longer than empty vector treated controls and rejected 60 percent of tumors (Figures 12a and b). This is consistent with the independence of IL-12 anti-tumor activity from CD4+ and CD8+ T-cells. While previous studies have shown a role of Fas/Fas ligand interactions in the anti-tumor effects of adenovirally delivered IL-12, anti-tumor effects are not completely abrogated as a result of inhibition of this pathway,\textsuperscript{288} and thus our results do not entirely contradict previous studies.

2.4.6.6 Role of IFN-γ

Interferon gamma is produced by a variety of innate and adaptive immune cells, including NK cells and T-cells and induces apoptosis in target cells, including tumors. IL-23 has been shown to induce IFN-γ production from CD4+ T-cells.\textsuperscript{166} We thus sought to determine if release of IFN-γ was responsible for the anti-tumor effects of IL-23. Indeed, IFN-γ is indispensable for IL-23 anti-tumor effects, as treated mice deficient in this cytokine experience neither enhanced survival nor tumor rejection (Figure 13a and b).

IL-12 is also a potent activator of IFN-γ production from both T and NK cells\textsuperscript{289} and has been shown to play a role in its anti-tumor activity.\textsuperscript{158,290-291} Likewise, in our model, the anti-tumor effects of IL-12 were dependent upon endogenous IFN-γ expression. Mice deficient in this cytokine were completely refractory to Ad.IL-12 treatment, exhibiting no enhancement of survival or tumor rejection (Figures 13a and b).
2.4.7 Mechanism of anti-tumor activities of IL-12 and IL-23

As IL-12 and IL-23 share the p40 subunit and possess similar immunostimulatory properties,\textsuperscript{166} it was expected that these cytokines would use similar, yet divergent, cytokines and cell types to enact their anti-tumor effects. Indeed, IL-12 and IL-23 anti-tumor effects are similar in their independence of endogenous IL-17, perforin and Fas ligand and their requirement for IFN-\(\gamma\). The only divergence in effector mechanisms lies in the requirement of CD4\(^+\) and CD8\(^+\) T-cells, which IL-23 requires, but IL-12 does not.

With regards to the mechanism of anti-tumor activity of adenovirally delivered IL-23, the requirement for CD4\(^+\) and CD8\(^+\) T-cells along with IFN-\(\gamma\) suggests induction of a Th1 type immune response. Supporting this assertion, cytotoxicity of splenocytes harvested from cured, wild-type animals demonstrate that tumor eradication was mediated, at least in part, by MCA205-specific CTLs (Figure 5b) and is consistent with previous reports.\textsuperscript{215-216} Collectively, these data suggest that the primary mechanism of IL-23 anti-tumor activity is CD4\(^+\) T-cell-mediated activation of CD8\(^+\) T-cells to produce IFN-\(\gamma\), which then induces apoptosis in tumor cells. Our proposed model IL-23 anti-tumor activity is consistent with other reports detailing the mechanism of IL-23 anti-tumor activity.\textsuperscript{216}

The anti-tumor activity of IL-12, in contrast, is dependent strictly on the involvement of IFN-\(\gamma\). This fact combined with the lack of involvement of CD4\(^+\) and CD8\(^+\) T-cells, implies that in our model, IL-12 anti-tumor activity may require not an adaptive immune response for efficient tumor eradication. Indeed, our studies show that IL-12 treatment generates protective anti-tumor immunity in only 50 percent of cases and that tumor-specific CTLs are not generated (Figure 5a). Therefore, as adenovirally delivered IL-12 has been previously shown to eradicate tumors by activation of NK cells and inhibition of angiogenesis,\textsuperscript{128,287} and that IL-12-mediated
inhibition of angiogenesis is dependent upon induction of IFN-γ,\textsuperscript{292} it is likely that the anti-tumor effects of Ad.IL-12 are moderated primarily by utilization of the innate immune response.

2.4.8 Summary

Overall, our results demonstrate that adenoviral delivery of IL-23 into the tumor microenvironment of early stage tumors is therapeutic and able to generate strong protective immunity. Furthermore, our results show that the anti-tumor activity of IL-23 is independent of endogenous IL-17, perforin and, in part, FasL, but dependent on CD4 and CD8 positive T-cells and IFN-γ. Together, these data imply that tumor cell expression of IL-23 drives a Th1-type immune response, which is responsible for its anti-tumor activity.

Adenovirally delivered IL-12 appears to be the more potent anti-tumor agent, causing greater and faster tumor rejection; however, IL-12 treatment generates only limited protective immunity. IL-12 treatment requires only endogenous IFN-γ to exert optimal anti-tumor benefits, suggesting, in our model, generation of a strong innate immune response, with only limited adaptive immune involvement.

In conclusion, the combination of the strong protective immune response generated by IL-23 treatment combined with the rapid and potent eradication of tumors by IL-12 and the utilization of similar, yet divergent effector mechanisms by these two cytokines, suggest that co-treatment of tumors with Ad.IL-12 and Ad.IL-23 together may result in synergistic enhancement of tumor eradication and induction of anti-tumor immunity.
3.0 INVESTIGATION INTO ANTI-TUMOR SYNERGY BETWEEN ADENOVIRALLY DELIVERED IL-12, IL-23 AND IL-17

3.1 INTRODUCTION

IL-12 is a heterodimeric, proinflammatory cytokine comprised of p40 and p35 subunits. IL-12 enhances proliferation of cytolytic activity of, and IFN-γ production from activated NK and T-cells. Furthermore, in response to microbial infection, IL-12 is produced by macrophages, which in turn generates a Th1-type adaptive immune response; therefore, IL-12 acts as a bridge between both innate and adaptive arms of the immune system. IFN-γ is responsible for the majority of the inflammatory activities of IL-12, drives Th1 differentiation, and induces IL-12 secretion from DCs, thereby forming a positive feedback loop for Th1 differentiation. IL-12 activates the JAK/STAT pathway, with STAT4 being preferentially activated. In accordance with the proinflammatory and immunostimulatory activities of this cytokine, IL-12 possesses potent anti-tumor effects. IL-12 has been shown to inhibit tumor establishment, cause regression of established tumors and induce tumor-specific immunity. However, systemically delivered IL-12 is associated with severe toxicity. Delivery of IL-12 directly into the tumor microenvironment using adenoviral vectors promotes tumor regression and generation of tumor-specific immunity, while alleviating the toxicity associated with systemic delivery. Yet new cytokines are continuously sought out for use in cancer gene
therapy, either alone and or in combination with IL-12, to decrease the effective dose needed and enhance long-term protective immunity.

IL-23 is a relatively newly described member of the IL-6/IL-12 family of heterodimeric cytokines and is composed of two subunits: p40, which is shared with IL-12, and p19, which is unique to IL-23. Like IL-12, IL-23 can act on both innate and adaptive arms of immunity. IL-23 stimulates production of IFN-γ from NK cells. Furthermore, IL-23 stimulates proliferation of and IFN-γ production from CD4+ memory T-cells, suggesting an important role in maintenance of Th1 immunity. IL-23 activates the same panel of signaling molecules as IL-12, differing only in that STAT3, as opposed to STAT4, appears to be the most prominent STAT activated. Due to the structural and functional similarities between IL-12 and IL-23, it is not surprising that IL-23 also acts as a potent anti-cancer agent in various establishment and therapeutic models of cancer. We have previously shown that treatment of established MCA205 fibrosarcoma tumors with adenovirus expressing IL-23 leads to significant enhancement of survival, tumor rejection and establishment of protective immunity using mechanisms similar to IL-12.

IL-12 and IL-23 both activate the JAK/STAT pathway, induce IFN-γ production from NK and T-cells, and utilize common mechanisms of tumor eradication. Furthermore, IL-23 acts on DCs to induce IL-12 production and the combination of IL-12 and IL-23 causes DCs to secrete greater levels of IFN-γ than either cytokine alone. We thus proposed that co-delivery of adenoviruses expressing IL-12 and IL-23 in the tumor microenvironment would result in synergistic enhancement of anti-tumor effects due to enhanced IL-12 and IFN-γ production.
In addition to inducing IFN-γ production, IL-23 also strongly drives production of IL-17 from a newly described subset of T-helper cells designated as Th17. In fact, the primary role of IL-23 appears to be expanding and committing T-cells to the Th17 phenotype. Th17 cells are characterized by expression of IL-17A, but can also produce IL-17F. Th17 cells appear to play an important role in activating innate immunity to extracellular pathogens. In addition, IL-23 and Th17 cell-driven inflammation have been implicated in a variety of autoimmune conditions including psoriasis, rheumatoid arthritis, multiple sclerosis and inflammatory bowel disease. Due to their pro-angiogenic and pro-inflammatory activities, Th17 cells and IL-17 have been implicated in tumor promotion. However, IL-17 may also possess therapeutic anti-tumor effects. Th17 T-cells have been shown to exact potent anti-tumor benefit, primarily via activation of tumor-specific CD8+ T-cells. Likewise, treatment with IL-17 leads to tumor rejection in a variety of cancer models.

IL-23 strongly promotes expression of IL-17 from Th17 T-helper cells and stabilizes the Th17 cell population. As IL-17 and Th17 cells have been shown to possess anti-tumor activities, we hypothesized that adenoviral-mediated co-delivery of IL-17 and IL-23 would result in synergistic enhancement of anti-tumor benefits as a result of increased Th17 T-cell production.
3.2 METHODS

3.2.1 Adenoviruses

Adenoviruses expressing IL-12 (Ad.IL-12) and IL-23 (Ad.IL-23) have been described previously\textsuperscript{18,266}. Ad.IL-12, Ad.IL-23 and Ad.Psi5 (empty vector) were prepared as follows: Viruses were propagated on HEK-293 cells and purified by CsCl banding, followed by dialysis in 3\% sucrose solution. Particle titer of purified viruses was determined by spectroscopy using the following equation: \((\text{OD}_{260})(50)/9.09\times10^{-13}\). Infectious titers were determined using quantitative real-time PCR as previously described\textsuperscript{267} and were approximately 100-fold less than particle titers. Viruses were aliquoted and stored at -80\°C until use.

Adenoviruses expressing single-chain (sc) versions of IL-12 and IL-23 were designed as follows: To construct Ad.scIL-12, the IL-12p40 precursor (Met1 to Ser335) was linked to the mature p35 subunit (Arg23 to Ala215) using the previously described 15 amino acid linker (Gly\textsubscript{4}Ser\textsubscript{3}).\textsuperscript{153} To construct Ad.scIL-23, the IL-12 p40 precursor (Met1 to Ser335) was linked to the mature p19 subunit (Leu20 – Ala196) using (Gly\textsubscript{4}Ser\textsubscript{3}). The viruses were constructed and propagated at the University of Pittsburgh’s Vector Core Facility.

3.2.1.1 Cytokine production and biological activity of adenoviruses

Relative cytokine expression of each adenoviral preparation was analyzed by infecting 4\times10\textsuperscript{4} MCA205 cells with increasing MOIs of Ad.IL-12 or Ad.IL-23 for 1 hour at 37\°C/5\% CO\textsubscript{2} in serum free media. Complete media was added and cells were incubated for 72 hours, after which supernatants were harvested. IL-12 and IL-23 content was analyzed using the mouse IL-12 p70,
mouse IL-23 p19/p40 and mouse IL-12/IL-23 p40 Ready-Set-Go IL-23 ELISA kits (eBioscience, San Diego, CA), respectively, following manufacturers’ instructions.

Biological activity of Ad.scIL-23 was assayed as follows: 4x10⁴ MCA205 cells were infected with a MOI 1000 of Ad.scIL-23 or Ad.Psi5 and supernatants harvested 72 hours post-infection. Splenocytes were then harvested from C57BL/6 mice, mechanically dissociated and treated with Red Cell Lysis buffer (Invitrogen, Carlsbad, CA) to remove all red blood cells. Splenocytes were plated at a concentration of 2x10⁶ cells per well in a 24 well plate and 24 hours later treated with supernatants from adenovirus infected MCA205s. Forty-eight hours after treatment, splenocyte supernatants were collected and analyzed for induction of IL-17 expression using the Mouse IL-17 Immunoassay (R&D Systems, Minneapolis, MN).

3.2.2 Animals

Female C57BL/6 and p40-deficient mice on a C57BL/6 background were obtained from The Jackson Laboratory (Bar Harbor, ME). IL-23 (p19) deficient mice have been previously described and were a kind gift of Dr. Jay Kolls. Mice were used at 6 to 7 weeks of age. Animals were maintained under pathogen free conditions at the Biotechnology Center Animal facility at the University of Pittsburgh. All procedures preformed were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

3.2.3 Cell Lines

MCA205 fibrosarcoma cells were maintained in RPMI supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and 1% penicillin/streptomycin (Gibco, Carlsbad, CA) and L-
glutamine (Gibco, Carlsbad, CA). Cells were kept in a humidified chamber at 37°C/5% CO₂ and passaged every 2-3 days.

3.2.4 In Vivo Tumor Studies

For use in in vivo tumor experiments, confluent layers of MCA205 were dissociated by trypsin, washed 3 times with Hanks Balanced Salt Solution (HBSS) (Gibco, Carlsbad, CA) and counted using trypan blue exclusion. Mice were inoculated with 1x10⁵ MCA205 cells in 100μL HBSS subcutaneously in the abdomen. For studies investigating induction of systemic immunity, mice were inoculated with 1x10⁵ MCA205 cells subcutaneously at two distal sites on the abdomen. To analyze Ad.IL-12 and Ad.IL-23 anti-tumor activity in the context of p40 and p19 deficiency, tumor bearing mice were injected on days 7, 9 and 11 post-tumor inoculation with 5x10¹⁰ particles (approximately 5x10⁸ PFUs) of either Ad.IL-12, Ad.IL-23 or Ad.Psi5.

To investigate synergy by delivering Ad.IL-12 and Ad.IL-23 at separate time points, mice were treated with 5x10⁸ particles of Ad.IL-12 or 2.5x10¹⁰ particles of Ad.IL-23 as follows: Ad.IL-12 on day 7, followed by Ad.IL-23 on day 11; Ad.IL-23 on day 7, followed by Ad.IL-12 on day 11; Ad.IL-12 on days 7 and 11; Ad.IL-23 on days 7 and 11; or Ad.Psi5 on days 7 and 11. In studies utilizing mice with two tumors, only one tumor was treated.

To assess synergy by co-delivery of Ad.IL-12 and Ad.IL-23, day 7 tumor-bearing mice were treated once with either Ad.IL-12 and Ad.IL-23 simultaneously or either virus alone. The quantity of virus particles injected were as follows: 1x10⁹ Ad.IL-12 and 5x10¹⁰ Ad.IL-23; 5x10⁸ Ad.IL-12 and 2.5x10¹⁰ Ad.IL-23; and 1x10⁸ Ad.IL-12 and 1x10¹⁰ Ad.IL-23. The total amount of viral particles injected was kept constant between groups by co-delivery of Ad.Psi5. In mice bearing two tumors, only one tumor was treated.
To characterize the anti-tumor activities of Ad.scIL-12 and Ad.scIL-23, tumor-bearing mice were injected intratumorally on days 7, 9 and 11 post-tumor inoculation with $5 \times 10^{10}$ particles of Ad.scIL-12, Ad.scIL-23 or Ad.Psi5. To investigate possible synergy between Ad.scIL-12 and Ad.scIL-23, mice bearing day 7 MCA205 tumors were treated once intratumorally with $1 \times 10^8$ particles of Ad.IL-12 and $1 \times 10^9$ particles of Ad.scIL-23, either virus alone or Ad.Psi5. Total quantity of virus injected was kept constant by use of Ad.Psi5.

In all experiments, tumor volume was monitored using a metric caliper until mice were sacrificed due to excessive tumor size or tumor ulceration. Tumor-free or “cured” mice were subject to tumor challenge 1-2 months after initial tumor resolution with $1 \times 10^5$ MCA205 cells subcutaneously in the abdomen.

### 3.2.5 Immunohistochemistry

Tumors of treated mice were harvested on day 15 post-tumor inoculation (day 8 post-treatment), snap frozen in 2-methylbutane and stored at -80°C. Tumors were then cut by cryostat, ten micron sections placed onto charged slides and stained for CD4, CD8, Foxp3, CD11c and CD31 as follows.

#### 3.2.5.1 CD4 and CD8

For analysis of tumors from mice treated with Ad.IL-12 and Ad.IL-23, sections were fixed in -20°C acetone and slides blocked in Exogenous Peroxidase Block (DAKO, Carpinteria, CA), followed by block in 10% goat serum. CD4 or CD8 antibody (1:250 and 1:150 dilution, respectively)(BD Biosciences, San Jose, CA) was then added, followed by incubation with biotinylated goat anti-rat secondary antibody (1:250)(BD Biosciences, San Jose, CA) in DAKO
Antibody Diluent (DAKO, Carpinteria, CA). Slides were treated with ABC Vectastain kit (Vector Laboratories, Burlingame, CA) and developed using DAB Peroxidase Substrate Kit (Vector Laboratories, Burlingame, CA) following manufacturers’ instructions. Slides were then counterstained with eosin, dehydrated in increasing concentrations of ethanol, followed by xylene, and coverslipped using Permount Media (Fisher, Pittsburgh PA).

For analysis of tumors from mice treated with Ad.scIL-12 and Ad.scIL-23, sections were permeabilized using 10% Triton-X (Sigma, St. Louis, MO), blocked in 2% BSA (Sigma, St. Louis, MO) and incubated with rat anti-mouse CD4 or CD8 antibody (1:50 and 1:100 dilution, respectively)(BD Biosciences, San Jose, CA) overnight at room temperature. Slides were then incubated with Alexa-Flour goat anti-rat secondary (1:500 dilution)(Invitrogen, Eugene, OR) and coverslipped using Flourmount media (SouthernBiotech, Birmingham, AL).

3.2.5.2 Dendritic cells
Sections were stained for DCs using an anti-CD11c primary antibody (1:50 dilution) (BD Biosciences, San Jose, CA) and the Anti-Hamster Ig HRP Detection Kit (BD Pharmingen, San Jose, CA) following manufacturers’ instructions.

3.2.5.3 Foxp3
Sections were permeabilized with 10% Triton-X (Sigma, St. Louis, MO) followed by block in 2% BSA (Sigma, St. Louis, MO). Sections were then incubated with Alexa-Flour 488 conjugated rat anti-mouse Foxp3 (1:100 dilution)(eBioscience, San Diego, CA) for two hours followed by Alexa-Flour goat anti-rat secondary (1:500 dilution)(Invitrogen, Eugene, OR).
3.2.5.4 Angiogenesis

Sections were permeabilized with 10% Triton-X (Sigma, St. Louis, MO) followed by block in 2% BSA (Sigma, St. Louis, MO). Sections were then incubated in rat anti-mouse CD31 primary antibody (1:100) (BD Biosciences, San Jose, CA), followed by Alexa-Flour goat anti-rat secondary (1:500 dilution)(Invitrogen, Eugene, OR).

3.2.6 Statistics

Kaplan-Meier survival curves were plotted using SPSS version 16.0. Mice were monitored until excessive tumor volume or tumor ulceration, at which time they were sacrificed and recorded as occurrence of an event (death). Cured mice or those with tumors that did not warrant sacrifice by the end of the experiment were censored. Log-rank tests of the survival curves provided p-values.

An unpaired T-test was used to analyze immunohistochemistry results if variances between data were equal; if variances were unequal, the Mann-Whitney U test was performed. Statistical analyses were 2-tailed, with a p value less than 0.05 considered statistically significant.
3.3 RESULTS

3.3.1 Anti-tumor activity of Ad.IL-23 requires endogenous IL-12, while Ad.IL-12 does not

Previously, we have shown that adenoviral delivery of IL-23 into the tumor microenvironment results in efficient and long lasting tumor eradication by generating a Th1 immune response that is dependent upon the presence of CD4+ and CD8+ T-cells and endogenous IFN-γ.\textsuperscript{218} Our studies have also indicated a role major role for IFN-γ in the anti-tumor activity of adenovirally delivered IL-12. To further elucidate the potential for IL-12 and IL-23 to synergize in tumor eradication, we sought to determine the role of endogenous IL-12 and IL-23 in Ad.IL-12 and Ad.IL-23 anti-tumor activity. To this end, IL-12/23 p40 deficient mice bearing MCA205 tumors were treated on days 7, 9 and 11 post-tumor inoculation with $5 \times 10^{10}$ particles of Ad.IL-12, Ad.IL-23 or saline. Lack of endogenous p40 abolished the anti-tumor activity of Ad.IL-23, with no enhancement of survival or rejection of tumors in treated mice (8 total) (Figures 16a and b). Thus, IL-23 requires either endogenous IL-12 or IL-23, or both, to exert maximal anti-tumor effects.

In contrast, Ad.IL-12 retained its anti-tumor activity in the absence of endogenous IL-12 and IL-23. When treated with Ad.IL-12, tumor bearing, p40 deficient mice survived significantly longer than saline treated controls ($p = 0.007$) and exhibited tumor rejection in 60 percent of mice (5 total) (Figures 16a and b). Interestingly, there was little evidence of Ad.IL-12 mediated toxicity seen in p40-deficient mice.
3.3.2 Anti-tumor activities of neither Ad.IL-12 nor Ad.IL-23 require endogenous IL-23 expression

To determine the requirement for endogenous IL-23 expression in the anti-tumor effects of Ad.IL-12 and Ad.IL-23, mice deficient in IL-23 p19 were inoculated with MCA205 tumors and treated as described above. Ad.IL-23 retained anti-tumor effectiveness in the absence of endogenous IL-23, with treated mice surviving significantly longer than controls ($p = 0.027$) and attaining a 60 percent (3 of 5) rejection rate (Figures 17a and b). Furthermore, the lack of requirement for IL-23 indicates that Ad.IL-23 anti-tumor activity is dependent upon endogenous IL-12, as p40 knockout studies suggested a role of either IL-12 or IL-23.

Likewise, treatment of tumor bearing, p19 deficient mice with Ad.IL-12 significantly enhanced survival compared to saline treated controls ($p = 0.007$) and initially led to tumor rejection in 100 percent of mice (5 total) (Figures 17a and b). Interestingly, two tumors recurred at day 35 post-tumor inoculation, similar to what was seen previously in wild type mice and again suggesting lack of induction of strong protective immunity.
3.3.3 Ad.IL-12 and Ad.IL-23 co-infection of MCA205 cells in vitro results in enhanced IL-12 and IL-23 expression

We next sought to determine if co-infection of MCA205 cells in vitro with Ad.IL-12 and Ad.IL-23 results in altered cytokine production due to sharing of the common p40 subunit. To this end, 4x10^4 MCA205 cells were transduced with Ad.IL-12 and Ad.IL-23 or either vector alone and supernatants analyzed for IL-12/23 p40, IL-12 and IL-23 expression 72 hours post-infection. Co-infection of MCA205s with Ad.IL-12 and Ad.IL-23 resulted in enhanced IL-12/23 p40 production compared to infection with either virus alone (Figure 18a). Similarly, co-infection with both viruses led to enhanced IL-12 expression when compared to MCA205s treated with Ad.IL-12 alone (Figure 18b). Finally, co-transduction with both Ad.IL-12 and Ad.IL-23 resulted in enhanced IL-23 expression compared to Ad.IL-23 infection alone. (Figure 18c). The above results indicate that co-transduction of MCA205 cells in vitro leads to enhanced IL-12 and IL-23 expression. As we have previously shown that both Ad.IL-12 and Ad.IL-23 possess potent anti-tumor activity, these results suggest that use of both viruses together may result in additive or
Figure 18: Ad.IL-12 and Ad.IL-23 co-infection results in enhanced cytokine expression
even synergistic enhancement of anti-tumor effects due to increased production of both IL-12 and IL-23.

3.3.4 **Investigation of synergism between Ad.IL-12 and Ad.IL-23**

The preceding studies in IL-12/23 p40 and IL-23 p19 deficient mice suggest that Ad.IL-23 exacts at least part of its anti-tumor effects via induction of endogenous IL-12. Furthermore, we have shown that co-infection of MCA205 cells with both Ad.IL-12 and Ad.IL-23 enhances production of both IL-12 and IL-23. Additionally, our previous studies have suggested that IL-12 rapidly eradicates tumors, while IL-23 generates better long-lasting protective immunity. Therefore, we hypothesized that co-treatment of tumors with both Ad.IL-12 and Ad.IL-23 would result in enhanced anti-tumor benefit due to greater cytokine production, which would lead to rapid tumor rejection and subsequent generation of long-lasting protective immunity. To investigate possible synergism between the two viruses, tumor bearing, C57BL/6 mice were treated with Ad.IL-12 and Ad.IL-23 at various doses and time points and animal survival and tumor rejection monitored for enhancement of anti-tumor activity.

3.3.4.1 **Treatment with Ad.IL-12 and Ad.IL-23 at separate time points does not lead to enhanced anti-tumor benefit**

Our previous results have shown that Ad.IL-12 treatment induces rapid tumor rejection, while Ad.IL-23 generates superior protective immunity. We first sought to determine if treatment with Ad.IL-12 followed by Ad.IL-23 would result in rapid tumor rejection followed by induction of
strong protective immunity. To this end, tumor bearing, C57BL/6 mice were treated with two injections of Ad.IL-12 and Ad.IL-23 at suboptimal doses (5x10^8 and 2.5x10^10 particles, respectively) as follows: Ad.IL-12 on day 7, followed by Ad.IL-23 on day 11 (Ad.IL-12,23); Ad.IL-23 on day 7, followed by Ad.IL-12 on day 11(Ad.IL-23,12); Ad.IL-12 on both days 7 and 11 (Ad.IL-12), Ad.IL-23 on both days 7 and 11 (Ad.IL-23) or Ad.Psi5 on days 7 and 11 (Ad.Psi5). Treatment with Ad.IL-12 on day 7 followed by Ad.IL-23 on day 11 did not result in significant enhancement of survival versus treatment with either Ad.IL-12 or Ad.IL-23 alone (Figure 19a). Treatment with Ad.IL-12 followed by Ad.IL-23 resulted in 40 percent tumor rejection (15 mice total) and was not significantly greater than rejection rates in animals treated with either Ad.IL-12 or Ad.IL-23 alone (Figure 19b). A regimen consisting of Ad.IL-23 treatment on day 7 followed by Ad.IL-12 on day 11 yielded similar results, with no significant enhancement of survival or tumor rejection compared to use of Ad.IL-12 or Ad.IL-23 alone (Figures 19a and b).

Figure 19: Ad.IL-12 and Ad.IL-23 do not synergize when delivered at separate time points

To determine if induction of systemic anti-tumor immunity is enhanced by co-treatment with Ad.IL-12 and Ad.IL-23 at separate time points, mice bearing two tumors were treated in
one as described above. In injected tumors, treatment with Ad.IL-12 on day 7 followed by Ad.IL-23 on day 11 resulted in a 29 percent tumor rejection rate, similar to the 43 percent rejection rate achieved with Ad.IL-12 treatment alone (7 mice total per group) (Figure 20a). Likewise, Ad.IL-23 treatment on day 7 followed by Ad.IL-12 on day 11 resulted in rejection of only 11 percent of tumors (9 mice total).

A similar trend was observed in contralateral tumors. Only 14 percent of contralateral tumors in mice treated with Ad.IL-12 on day 7 followed by Ad.IL-23 on day 11 were rejected, less than the 42 percent rejection rate in mice treated with Ad.IL-12 alone (7 mice total per group) (Figure 20b). Taken together, our results suggest that intratumoral delivery of Ad.IL-12 and Ad.IL-23 at separate time points does not enhance local or systemic induction of anti-tumor immunity over use of either virus alone.

3.3.4.2 Co-treatment with Ad.IL-12 and Ad.IL-23 does not enhance anti-tumor benefit

As delivery of Ad.IL-12 and Ad.IL-23 at separate time points did not yield additive or synergistic anti-tumor benefits, we next sought to determine if co-delivery of the viruses would result in enhanced anti-tumor activity. To this end, MCA205 tumor bearing C57BL/6 mice were
treated once on day 7 with suboptimal doses of Ad.IL-12 and Ad.IL-23 (5x10^{10} and 1x10^{9} particles, respectively) or either virus alone. Our previous studies indicated that Ad.IL-23 had only minimal anti-tumor activity at one dose of 5x10^{10} particles, while Ad.IL-12 retained much of its anti-tumor activity at this dose. Thus we chose to use 1x10^{9} particles of Ad.IL-12 in conjunction with 5x10^{10} particles of Ad.IL-23 in our initial investigation into possible synergism between Ad.IL-12 and Ad.IL-23. While co-treatment with Ad.IL-12 and Ad.IL-23 doubled tumor rejection (4 of 5) versus use of either virus alone (2 of 5), survival was not significantly enhanced (Figures 21 a and b). Thus, no additive or synergistic benefit was seen using Ad.IL-12 and Ad.IL-23 at this dose. Three cured, Ad.IL-12 and Ad.IL-23 co-treated mice were resistant to tumor challenge, as was the cured mouse treated with Ad.IL-23 alone. The cured, Ad.IL-12 treated mouse did, however, develop a tumor that did not regress, similar to our previous challenge data in wild-type mice.

Figure 21: Co-treatment with Ad.IL-12 (1x10^{9} particles) and Ad.IL-23 (5x10^{10} particles) does not synergistically enhance tumor eradication

Since some anti-tumor activity was retained at 1x10^{9} and 5x10^{10} particles of Ad.IL-12 and Ad.IL-23, respectively, we decided to use a lower viral titer to possibly unmask any additive
or synergistic anti-tumor benefit between the two viruses. To this end tumor bearing mice were treated once on day 7 with $2.5 \times 10^{10}$ particles of Ad.IL-23 and $5 \times 10^8$ particles of Ad.IL-12 or either virus alone. Mice treated with Ad.IL-12 and Ad.IL-23 survived significantly longer than mice treated with Ad.IL-23 alone ($p = 0.006$), but not longer than Ad.IL-12 alone (Figure 22a). This suggests the enhancement of anti-tumor benefit was due to the contribution of Ad.IL-12 alone and was not synergistic in nature. Treatment with Ad.IL-12 and Ad.IL-23 yielded identical tumor rejection rates at 80 percent, while treatment with Ad.IL-23 alone resulted in a 40 percent overall tumor rejection rate (15 mice total per group) (Figure 22b). Again, tumor rejection data supports the assertion that the anti-tumor benefits of Ad.IL-12 and Ad.IL-23 treatment was due solely to contribution of Ad.IL-12 and was not synergistic. Additionally, all cured mice challenged were resistant to tumor development, suggesting that treatment with the combination of Ad.IL-12 and Ad.IL-23 does not enhance protective anti-tumor immunity.

Figure 22: Co-treatment with Ad.IL-12 ($5 \times 10^8$ particles) and Ad.IL-23 ($2.5 \times 10^{10}$ particles) does not synergistically enhance anti-tumor effects

Again, significant anti-tumor benefit was still observed at a dose of $5 \times 10^8$ particles of Ad.IL-12 and $2.5 \times 10^{10}$ particles of Ad.IL-23. We thus attempted to further titrate down the
doses of Ad.IL-12 and Ad.IL-23 to possibly reveal any additive or synergistic benefits associated with co-treatment. Tumor bearing, C57BL/6 mice were treated once on day 7 with $1 \times 10^8$ particles of Ad.IL-12 and $1 \times 10^{10}$ particles of Ad.IL-23, or either virus alone. Mice treated with both Ad.IL-12 and Ad.IL-23 did not survive significantly longer than animals treated with either Ad.IL-12 or Ad.IL-23 alone (Figure 23a). Obviously a minimum effective dose was reached, as all treatment groups experienced an overall 20 percent tumor rejection rate (5 mice total per group) (Figure 23b); however, no synergistic or even additive benefit was observed in mice treated with both Ad.IL-12 and Ad.IL-23.

**Figure 23:** Co-treatment with Ad.IL-12 ($1 \times 10^8$ particles) and Ad.IL-23 ($1 \times 10^{10}$ particles) does not synergistically enhance anti-tumor activity

Finally, we wished to determine if Ad.IL-12 and Ad.IL-23 co-treatment leads to enhancement of systemic anti-tumor immunity compared to use of either virus alone. To this end, C57BL/6 mice were inoculated with two tumors on the flank and treated once on day 7 with $1 \times 10^9$ particles of Ad.IL-12 and $5 \times 10^{10}$ particles of Ad.IL-23. This treatment regimen yielded maximum tumor eradication as a result of Ad.IL-12 and Ad.IL-23 co-treatment in our previous experiments compared to use of either virus alone (Figure 21b) and thus was the most likely treatment plan to result in additive or synergistic tumor eradication in the systemic model.
Animals co-treated with Ad.IL-12 and Ad.IL-23 rejected 67 percent of injected and 45 percent of contralateral tumors (18 mice total). Similarly, mice treated with Ad.IL-12 rejected 78 percent of injected and 36 percent of contralateral tumors (14 mice total). Ad.IL-23 treatment yielded no tumor rejection in any treated animals (15 total)(Figures 24a and b). Thus, it is evident that co-treatment with Ad.IL-12 and Ad.IL-23 does not result in enhanced generation of local or systemic anti-tumor immunity compared to treatment with Ad.IL-12 alone.

Additionally, all Ad.IL-12 and Ad.IL-23 co-treated, cured mice, regardless of dose, were resistant to MCA205 tumor challenge (7 animals total). All cured Ad.IL-23 treated mice (2 total), but only 75 percent of Ad.IL-12 treated animals (4 total) were resistant to tumor challenge. Thus, co-treatment with Ad.IL-12 and Ad.IL-23 does not appear to generate enhanced MCA205-protective immunity.

Figure 24: Ad.IL-12 and Ad.IL-23 co-treatment does not result in synergistic systemic anti-tumor effects
3.3.5 Determining the mechanism of lack of synergism between Ad.IL-12 and Ad.IL-23

We hypothesized that differences in tumoral immune cell infiltrate or angiogenesis may be behind the lack of additive or synergistic anti-tumor effects between Ad.IL-12 and Ad.IL-23. To investigate, mice inoculated with two tumors in the flank were treated in one on day 7 with $1 \times 10^9$ particles of Ad.IL-12 and $5 \times 10^{10}$ particles of Ad.IL-23 or either virus alone. Tumors were removed at day 15 post-tumor inoculation, snap frozen, cryosectioned and stained for various immune cell infiltrates.

3.3.5.1 Ad.IL-12 and Ad.IL-23 co-treatment does not alter CD4+ T-cell infiltrate

Analysis of CD4+ T-cell infiltrate showed no significant difference between tumors of mice treated with Ad.IL-12 and Ad.IL-23 and those treated with either Ad.IL-12 or Ad.IL-23 alone (Figures 25a and b). In injected tumors, there was no significant difference in CD4+ T-cell infiltrate between Ad.IL-12 and Ad.IL-23, Ad.IL-23 or Ad.IL-12 and control groups (Figure 25a). However, in contralateral tumors, co-treatment with Ad.IL-12 and Ad.IL-23 significantly increased CD4+ T-cell infiltrate compared to control treated mice ($p = 0.00$, Mann-Whitney U test), but was not greater than Ad.IL-12 treatment alone. With the exception of those treated with Ad.IL-12, similar amounts of CD4+ T-cells were present in both injected and contralateral tumors.

3.3.5.2 Ad.IL-12 and Ad.IL-23 co-treatment significantly reduces tumor-infiltrating CD8+ T-cells

Analysis of tumor-infiltrating CD8+ T-cells yielded interesting results. In injected tumors, co-treatment with Ad.IL-12 and Ad.IL-23 resulted in a significant inhibition of infiltrating CD8+ T-
Figure 25: Effects of Ad.IL-12 and Ad.IL-23 co-treatment on CD4+ and CD8+ T-cell infiltrate
cells compared to control treated animals (p = 0.00, Mann-Whitney U test) (Figure 25c). Additionally, co-treatment significantly reduced CD8+ T-cell infiltrate compared to use of Ad.IL-12 alone (p = 0.00, T-test) (Figure 25c). Between groups, tumors of Ad.IL-12 treated animals contained significantly more tumor infiltrating CD8+ T-cells than those treated with either Ad.IL-23 (p = 0.011, T-test) or controls (p = 0.024, T-test) (Figure 25c). Similar to injected tumors, contralateral tumors co-treated with Ad.IL-12 and Ad.IL-23 contained significantly less CD8+ T-cells than tumors treated with either Ad.IL-12 alone (p = 0.004, T-test) or controls (p = 0.035, T-test) (Figure 25d). Overall, with the exception of animals treated with Ad.IL-12, similar amounts of CD8+ T-cells were present in both injected and contralateral tumors.

3.3.5.3 Ad.IL-12 and Ad.IL-23 treatment does not affect dendritic cell infiltrate in tumors
In both injected and contralateral tumors, co-treatment with Ad.IL-12 and Ad.IL-23 did not affect dendritic cell (DC) infiltrate in tumors versus Ad.IL-12, Ad.IL-23 or control treated groups (Figures 26a and b). Likewise, there was no difference in DC infiltrate between tumors treated with Ad.IL-12 alone or Ad.IL-23 alone (Figures 26a and b). Additionally, DC cell infiltrate was roughly equivalent in both injected and contralateral tumors in all treatment groups.

3.3.5.4 Co-treatment with Ad.IL-12 and Ad.IL-23 does not alter Foxp3 infiltrate
Co-treatment with Ad.IL-12 and Ad.IL-23 did not significantly alter Foxp3-positive cells in injected or contralateral tumors compared to controls (Figure 26c and d). Likewise, numbers of tumor-infiltrating Foxp3 positive cells did not significantly differ between animals treated with Ad.IL-12 or Ad.IL-23 alone (Figures 26c and d). Co-treatment with Ad.IL-12 and Ad.IL-23 did
Figure 26: Effects of Ad.IL-12 and Ad.IL-23 co-treatment on dendritic cell and regulatory T-cell infiltrate
result in a trend towards decreased Foxp3 positive cells compared to therapy with Ad.IL-23 alone (p = 0.074, T-test).

In contralateral tumors, Ad.IL-12 and Ad.IL-23 co-treatment did result in significantly less Foxp3 positive cells than treatment with Ad.IL-23 alone (p = 0.013, T-test) (Figure 26d). Likewise, co-treatment resulted in a trend towards decreased Foxp3 positive cells compared to Ad.IL-12 treatment alone (p = 0.064, T-test).

### 3.3.5.5 Ad.IL-12 and Ad.IL-23 co-treatment significantly enhances angiogenesis

To elucidate the effects of Ad.IL-12 and Ad.IL-23 co-treatment on tumor angiogenesis, sections were analyzed for CD31. Interestingly, in injected tumors, co-treatment with Ad.IL-12 and Ad.IL-23 resulted in significantly enhanced CD31 expression versus controls (p = 0.044, T-test) (Figure 27a). Additionally, co-treatment resulted in enhanced angiogenesis compared to treatment with Ad.IL-23 alone (p = 0.001, T-test), but not Ad.IL-12 alone (Figure 27a). Finally, treatment with Ad.IL-12 alone resulted in enhanced angiogenesis compared to Ad.IL-23 alone (p = 0.006, T-test).

In contralateral tumors, Ad.IL-12 and Ad.IL-23 co-treatment did not alter tumor angiogenesis versus controls (Figure 27b). Furthermore, no significant difference in CD31 expression was observed between Ad.IL-12 alone and Ad.IL-23 alone treatment groups (Figure 27b).
3.3.6 Generation and characterization of adenoviruses expressing single chain IL-12 and IL-23

Co-treatment with Ad.IL-12 and Ad.IL-23 did not result in any additive or synergistic anti-tumor effects over use of either cytokine alone and resulted in curious patterns of tumor angiogenesis and CD8+ T-cell infiltrate. Since IL-12 and IL-23 share the common p40 subunit, it is possible that in vivo co-infection of tumors with Ad.IL-12 and Ad.IL-23 leads to preferential expression of one cytokine over another. Therefore, the lack of therapeutic benefit of co-treatment with Ad.IL-12 and Ad.IL-23 may be a result of suboptimal expression of one cytokine combined with over expression of the other. Use of adenoviruses expressing single chain (sc) IL-12 and IL-23 would circumvent this possibility, as subunits could not be shared and the amount of cytokine produced in tumors should correlate linearly with treatment dose. We therefore hypothesized that co-treatment with Ad.scIL-12 and Ad.scIL-23 would result in enhanced therapeutic benefit over use of either virus alone.
3.3.6.1 Adenovirus expressing single-chain IL-12 possesses potent anti-tumor effects, but is characterized by severe toxicity

Adenovirus expressing single chain IL-12 (Ad.scIL-12) was designed to express the complete p40 precursor linked to the mature p35 subunit using a 15 amino acid (Gly4Ser)3 spacer. To ensure cytokine expression, MCA205 cells were infected \textit{in vitro} with Ad.scIL-12. Cells were also infected with Ad.IL-12 for comparison. Ad.scIL-12 infection led to high levels of scIL-12 expression, which was detectible by ELISA in spite of the fact that the p35 and p40 subunits were linked. Expression of IL-12 from Ad.scIL-12 was greater than its double chain counterpart (Figure 28b).

Having shown that Ad.scIL-12 expresses detectable amounts of cytokine from infected MCA205 cells, we next sought to examine the anti-tumor effects of this virus. To this end, tumor bearing, C57BL/6 mice were treated with Ad.scIL-12 or Ad.Psi5 on days 7, 9 and 11 post-tumor inoculation. While Ad.scIL-12 treatment did result in rapid and efficient tumor eradication in all animals (10 total) (Figure 28c), treatment was associated with severe toxicity. Treated mice exhibited classic signs of IFN-\(\gamma\) mediated toxicity, including weight loss, lethargy and ragged appearance. In fact, toxicity associated with Ad.scIL-12 treatment was so severe that all animals perished as a result of treatment by day 35 (Figure 28d). Thus, while Ad.scIL-12 effectively eradicated tumors, survival was not enhanced compared to Ad.Psi5 treated controls due to treatment-associated toxicity. We thus decided against pursuing further investigations into the anti-tumor activity of Ad.scIL-12 due to its association with severe toxicity.

3.3.6.2 Adenovirus expressing single-chain IL-23 possesses potent anti-tumor activity

An adenovirus expressing single chain IL-23 (Ad.scIL-23) was designed to express the complete p40 subunit linked to the mature p19 subunit using a 15-amino acid (Gly4Ser)3 spacer. To
ensure expression of scIL-23 from Ad.scIL-23 transduced cells, MCA205s were infected with Ad.scIL-23 and supernatants analyzed by ELISA. Cells were also infected with Ad.IL-23 for comparison. Ad.scIL-23 expressed very high levels of cytokine, which was detectable by ELISA regardless of its single chain form (Figure 29b). While Ad.IL-23 did express detectible levels of cytokine (approximately 270 pg/mL), its expression was eclipsed by that of Ad.scIL-23.

To ensure that scIL-23 expressed by Ad.scIL-23 was biologically active, splenocytes were treated with supernatants from Ad.scIL-23 infected cells for 48 hours and splenocyte supernatants analyzed for induction of IL-17 expression. Treatment of splenocytes with supernatants from Ad.scIL-23 infected cells resulted in induction of IL-17 expression, while splenocytes treated with supernatants from cells infected with Ad.Psi5 or mock treated did not (Figure 29c). Additionally, splenocytes did not spontaneously release IL-17 (Figure 29c).

We next sought to determine what, if any, anti-tumor activity Ad.scIL-23 possessed. To this end, MCA205-tumor bearing, C57BL/6 mice were treated on days 7, 9 and 11 post-tumor inoculation with 5x10\(^{10}\) particles of Ad.scIL-23 or Ad.Psi5. Treatment with Ad.scIL-23 significantly increased survival compared to Ad.Psi5 treated controls (\(p = 0.00\)), decreased tumor volume and led to an overall 90 percent tumor rejection rate (10 mice total) (Figures 29d and e). Very moderate signs of toxicity were observed in a few Ad.scIL-23 treated animals, mostly in the form of weight loss, but did not result in death. Upon MCA205 tumor challenge, all mice (8 total) remained tumor free up to 45 days after challenge. This indicates induction of strong MCA205 protective immunity, similar to anti-tumor immunity generated by treated with Ad.IL-23.
Figure 28: Characterization of adenovirus expressing single-chain IL-12
3.3.7 Co-treatment with Ad.scIL-23 and Ad.IL-12 does not result in enhanced anti-tumor activity

We next sought to determine if co-treatment with adenovirally delivered IL-12 and IL-23 enhanced local or systemic anti-tumor effects when there is no possibility of subunit switching. To this end, mice bearing two tumors were co-treated in one with $1 \times 10^9$ particles of Ad.scIL-23 and $1 \times 10^8$ particles of Ad.IL-12 or either cytokine alone. We chose against using Ad.scIL-12 in this study due to the toxicity associated with its use. Co-treatment with Ad.IL-12 and Ad.scIL-23 did not enhance tumor rejection in injected or contralateral tumors compared to use of Ad.scIL-23 alone (Figures 30a and b). In both injected and contralateral tumors, 40 percent of co-treated animals experienced tumor rejection, identical to mice treated with Ad.scIL-23 alone (5 mice total per group) (Figures 30a and b). Ad.IL-12-treated mice experienced only a 20 percent rejection rate in injected tumors and did not reject any contralateral tumors (Figures 30a and b). Overall, it is obvious that co-treatment with Ad.IL-12 and Ad.scIL-23 does not result in any additive or synergistic benefits.

Taken together, the absence of any additive or synergistic anti-tumor effects in all of the aforementioned studies strongly suggests that IL-12 and IL-23 do not synergize to enhance anti-tumor immunity.
Figure 29: Characterization of adenovirus expressing single-chain IL-23
3.3.8 Determining the mechanism behind the lack of synergy between Ad.IL-12 and Ad.scIL-23

Overall, these data show no indication of additive or synergistic anti-tumor benefits to use of Ad.IL-12 and Ad.scIL-23 together. To elucidate the precise mechanism underlying the lack of therapeutic benefit, tumor bearing C57BL/6 mice were treated with suboptimal doses of Ad.IL-12 and Ad.scIL-23 once on day 7 as described above. On day 15 post-tumor inoculation, tumors were harvested and snap frozen. Cryosections of tumors were then analyzed for various immune cell infiltrates and angiogenesis.

3.3.8.1 Ad.IL-12 and Ad.scIL-23 co-treatment does not alter CD4+ T-cell infiltrate

Co-treatment with Ad.IL-12 and Ad.scIL-23 did not significantly alter CD4+ T-cell infiltrate compared to treatment with either virus alone or control treated animals (Figure 31a). Likewise, no significant difference was observed in CD4+ T-cell infiltrate between Ad.IL-12 and Ad.scIL-23 treated animals and controls (Figure 31a).
3.3.8.2 Co-treatment with Ad.IL-12 and Ad.scIL-23 does not alter CD8+ T-cell infiltrate versus controls

Ad.IL-12 and Ad.scIL-23 co-treatment significantly enhanced CD8+ T-cell infiltrate of tumors compared to animals treated with Ad.scIL-23 alone (Figure 31b). However, co-treatment did not significantly alter CD8+ T-cell infiltrate compared to use of Ad.IL-12 alone or empty vector (Figure 31b).

3.3.8.3 Co-treatment with Ad.IL-12 and Ad.scIL-23 does not alter Foxp3 positive cell infiltrate

To determine if co-treatment with Ad.IL-12 and Ad.scIL-23 altered regulatory T-cell infiltrate, tumor sections were analyzed for presence of Foxp3 positive cells. No difference in Foxp3 positive cell infiltrate was observed between Ad.IL-12 and Ad.scIL-23 co-treated tumors versus use of either virus alone or Ad.Psi5 (Figure 31c). Additionally, there was no difference in Foxp3 positive cells in tumors treated with Ad.IL-12 or Ad.scIL-23 alone and controls (Figure 31c).

3.3.8.4 DC infiltrate

To determine if co-treatment with Ad.IL-12 and Ad.scIL-23 altered dendritic cell infiltrate, tumor sections were stained for CD11c. Ad.IL-12 and Ad.scIL-23 co-treatment did not enhance tumor infiltrating DCs compared to use of Ad.IL-12 or Ad.scIL-23 alone. Furthermore, neither treatment with both Ad.IL-12 and Ad.scIL-23 nor either virus alone significantly enhanced tumor-infiltrating DCs versus empty vector treated controls (Figure 31d).
3.3.8.5 Angiogenesis

To determine if tumor angiogenesis is altered as a result of co-treatment with Ad.IL-12 and Ad.scIL-23, tumor sections were analyzed for CD31 expression. Co-treatment did not significantly alter CD31 expression compared to use of either Ad.IL-12 or Ad.scIL-23 alone or empty vector controls (Figure 31e). Likewise, no significant difference was observed between Ad.IL-12 alone and Ad.scIL-23 alone treatment groups versus controls (Figure 31e).

3.3.9 Investigation into synergism between Ad.IL-17 and Ad.IL-23

3.3.9.1 Ad.IL-17 co-treatment inhibits Ad.IL-23 anti-tumor effects

In vivo, IL-23 strongly induces production of IL-17,\textsuperscript{30} which has been previously shown to possess anti-tumor activity.\textsuperscript{232} We next sought to determine whether intratumoral co-expression of IL-17 and IL-23 enhances anti-tumor effects. To this end, tumor bearing C57BL/6 mice were co-treated with 2.5\times10^{10} particles of Ad.IL-23 and adenovirus expressing IL-17 (Ad.IL-17) once on day 7 or with either virus alone. Treatment with Ad.IL-17 alone had no anti-tumor effect, as treated mice did not survive significantly longer than Ad.Psi5 treated controls and did not experience tumor rejection (Figures 32a and b). Similar to previous results, Ad.IL-23 treated
Figure 31: Effects of Ad.scIL-23 and Ad.IL-12 co-treatment on immune cell infiltrate and angiogenesis
mice survived significantly longer than controls (p = 0.010) and rejected 60 percent of tumors (10 mice total) (Figures 32a and b). Interestingly, co-treatment with Ad.IL-23 and Ad.IL-17 significantly decreased survival compared to use of Ad.IL-23 alone (p = 0.045) (Figure 32a). Furthermore, use of Ad.IL-17 in conjunction with Ad.IL-23 decreased the tumor rejection rate of Ad.IL-23 by half (30 versus 60 percent, 10 mice total) (Figure 32b). This is suggestive of a pro-tumor effect of IL-17 in our model.

3.3.9.2 Inhibition of IL-17 signaling does not enhance IL-23 anti-tumor activity

As IL-23 is a strong inducer of IL-17 expression, we next sought to determine if inhibition of IL-17 signaling in the tumor microenvironment enhances Ad.IL-23 anti-tumor activity. To block IL-17 signaling, we utilized an adenovirus expressing IL-17 receptor Fc (Ad.IL-17RFc), a fusion of the IL-17 receptor and IgG. To this end, C57BL/6, tumor bearing mice were co-treated with 2.5x10^{10} particles of Ad.IL-23 and 5x10^{10} particles of Ad.IL-17RFc or either virus alone. Ad.IL-23 treatment resulted in an overall 60 percent rejection rate (5 mice total), similar to previous results. Inhibition of IL-17 signaling did not appear to have any discernable anti-tumor effect, as treatment with Ad.IL-17RFc alone did not lead to tumor rejection in any animals.
Furthermore, inhibition of IL-17 signaling did not enhance IL-23 anti-tumor activity, as co-treatment with Ad.IL-23 and Ad.IL-17RFc did not significantly enhance survival or increase tumor rejection compared to animals treated with Ad.IL-23 alone (Figures 33a and b). This is in agreement with our previous results which suggest that Ad.IL-23 treatment most likely results in IFN-γ production and a Th1, not IL-17 and Th17, response to eradicate tumors; therefore inhibition of IL-17 should have no effect anti-tumor activity.

**3.4 DISCUSSION**

**3.4.1 Rationale for IL-12 and IL-23 anti-tumor synergism**

IL-12 is a heterodimeric, proinflammatory cytokine comprised of p40 and p35 subunits. IL-12 induces IFN-γ production from both NK and T-cells, is imperative in driving a Th1 immune response\textsuperscript{50,57-58,76} and strongly activates the JAK/STAT pathway, particularly STAT4.\textsuperscript{289} In accordance with its pro-inflammatory properties, IL-12 possesses potent anti-tumor activities in multiple cancer models, but is associated with severe toxicity.\textsuperscript{92,263-265} Additionally we have
shown that the anti-tumor activity is rapid, but transient. Thus, cytokines are continuously sought out for use in conjunction with IL-12 to decrease the effective dose needed and enhance long-term protective immunity. Indeed, many cytokines have been previously shown to synergistically enhance the anti-tumor activity of IL-12. IL-12 has been shown to synergize with IL-2, IL-18, IL-27, TNF-α and IL-15 commonly using mechanisms involving CD8+ T-cells and enhancement of IFN-γ production.

IL-23 is a relatively newly described member of the IL-6/IL-12 family of heterodimeric cytokines and is comprised of the IL-12 p40 subunit and a unique p19 subunit. IL-23 likewise induces IFN-γ production from NK and T-cells and activates STAT3 of the JAK/STAT pathway. Due to common structural and functional similarities, it is not surprising that IL-23, like IL-12, possesses potent anti-tumor activity in various establishment and therapeutic models of cancer. Additionally, we have previously shown that IL-12 and IL-23 have similar, yet divergent, mechanisms of tumor eradication. Specifically, the anti-tumor activities of adenovirally delivered IL-12 and IL-23 are dependent on induction of IFN-γ.

IL-12 and IL-23 both activate JAK/STAT signaling, induce IFN-γ production from NK and T-cells, and utilize IFN-γ for tumor eradication. Furthermore, co-treatment of DCs with IL-12 and IL-23 results in greater levels of IFN-γ production than use of either cytokine alone. We thus proposed that co-treatment with adenoviruses expressing IL-12 and IL-23 would result in additive or synergistic anti-tumor effects as a result of enhanced intratumoral IFN-γ expression.

To further analyze the potential of adenovirally delivered IL-12 and IL-23 to synergistically enhance anti-tumor function, the roles of endogenous IL-12 and IL-23 in the anti-tumor activities of Ad.IL-12 and Ad.IL-23 were investigated. Tumor-bearing mice deficient in
IL-12/IL-23 p40 were completely refractory to treatment with Ad.IL-23 (Figures 16a and b), indicating that Ad.IL-23 induces production of IL-12, IL-23 or both to exert its anti-tumor effects. To determine whether endogenous IL-12 or IL-23 was induced by Ad.IL-23 treatment, the anti-tumor activity of Ad.IL-23 was evaluated in the context of IL-23 p19-deficient mice. Ad.IL-23 retained its anti-tumor activity in p19-deficient mice, with treatment significantly enhancing survival and resulting in a 60 percent tumor rejection rate (Figures 17a and b). This indicates that Ad.IL-23 treatment induces IL-12 production to exert its anti-tumor effects. Furthermore, Ad.IL-23 treatment either does not induce endogenous production of additional IL-23 or that endogenous IL-23 does not play a role in its anti-tumor activity. As IL-12 eradicates tumors using mechanisms including perforin and Fas ligand, it is certainly intriguing that the anti-tumor activity of Ad.IL-23 is independent of these means. However, the anti-tumor activity of Ad.IL-12, at least in our model, was independent of both perforin and Fas ligand, but completely dependent upon IFN-γ. Thus, it is not surprising that in our model, IL-12-mediated induction of IFN-γ is the primary mechanism utilized by Ad.IL-23 to eradicate tumors. Overall, these results are in accordance with our previous data that suggest the anti-tumor effect of Ad.IL-23 is due to induction of a Th1 immune response, characterized by dependence on CD4+ and CD8+ T-cells and IFN-γ.

Conversely, Ad.IL-12 retained its anti-tumor activity in tumor-bearing mice deficient in IL-12/IL-23 p40, significantly enhancing survival and leading to an overall 60 percent tumor rejection rate (Figures 16a and b). This indicates that Ad.IL-12 either does not induce endogenous IL-12 expression or that Ad.IL-12 can eradicate tumors independently of endogenous IL-12. Interestingly, p40-deficient mice showed no signs of Ad.IL-12-mediated toxicity, as was commonly seen in both wild-type and various knockout mice. This suggests that
Ad.IL-12 induction of endogenous IL-12 expression is at least in part responsible for the toxicity observed. Indeed, IL-12 induces IFN-γ expression, which in turn induces further IL-12 expression, thereby forming a positive feedback loop.58

Like Ad.IL-23, Ad.IL-12 retained potent anti-tumor effectiveness in tumor-bearing IL-23 p19 deficient mice. Treated mice survived significantly longer and initially rejected 100 percent of tumors (Figures 17a and b). This indicates that Ad.IL-12 treatment does not result in IL-23 induction. Similar to previous results in Ad.IL-12 treated animals, two mice had tumors recur, again suggesting lack of induction of strong protective immunity.

Overall, the above data indicates that Ad.IL-23 induces endogenous IL-12 production to eradicate tumors; therefore, use of Ad.IL-12 and Ad.IL-23 together would likely result in synergistic enhancement of anti-tumor benefit due to increased production of IL-12. Likewise, data from co-infection of tumor cells suggest an overall enhancement of cytokine expression (Figure 18a-c). Furthermore, our previous data have indicated that while Ad.IL-12 treatment results in rapid tumor eradication, treatment with Ad.IL-23 results in induction of superior protective immunity. Thus, we decided that Ad.IL-12 and Ad.IL-23 would likely synergize to eradicate tumors as a result of overall enhancement of cytokine expression, leading to enhanced IFN-γ production, rapid tumor regression and IL-23-mediated induction of strong protective immunity.

3.4.2 Ad.IL-12 and Ad.scIL-23 co-treatment does not synergistically enhance anti-tumor effects

We first sought to determine if treatment with Ad.IL-12, followed by Ad.IL-23, would result in synergistically enhanced therapeutic benefit over use of either cytokine alone. As synergism is
defined as “the interaction of two or more agents or forces so that their combined effect is greater than the sum of their individual parts,” we expected that synergy between Ad.IL-12 and Ad.IL-23 would result in tumor eradication rates greater than the sum of treatment with either cytokine alone. For example, if treatment with Ad.IL-12 or Ad.IL-23 alone both resulted in 20 percent tumor rejection rates, then tumor rejection rates of greater than 40 percent in animals treated with both Ad.IL-12 and Ad.IL-23 would be suggestive of synergism. To statistically confirm synergistic activity, tumor rejection rates of animals treated with both Ad.IL-12 and Ad.IL-23, or either virus alone, would then be subjected to least squares regression analysis, which has been used previously to define synergy in cancer therapies.302

Tumor-bearing mice treated on day 7 with Ad.IL-12 followed by Ad.IL-23 on day 11 did not survive significantly longer or experience greater overall tumor rejection than animals treated with Ad.IL-12 or Ad.IL-23 alone (Figures 19a and b). Treatment with Ad.IL-23 on day 7 followed by Ad.IL-12 on day 11 likewise did not enhance anti-tumor benefits over use of either virus alone (Figures 19a and b). Similarly, no enhancement in systemic anti-tumor immunity was observed in mice bearing two tumors treated with Ad.IL-12 and Ad.IL-23, regardless of timing of delivery (Figures 20a and b). These results suggest that delivery of Ad.IL-12 and Ad.IL-23 at separate time points does not result in synergistic or even additive enhancement of anti-tumor effects.

We next sought to determine if co-delivery of Ad.IL-12 and Ad.IL-23 resulted in enhanced anti-tumor benefit over use of either virus alone. We attempted to determine the minimal effective doses of Ad.IL-12 and Ad.IL-23 that would generate maximal synergistic benefit. To this end, various suboptimal doses of Ad.IL-12 and Ad.IL-23 were used to treat tumor bearing mice once on day 7. Doses of $1 \times 10^9$ and $5 \times 10^{10}$; $5 \times 10^8$ and $2.5 \times 10^{10}$; and $1 \times 10^8$
and 1x10^{10} particles of Ad.IL-12 and Ad.IL-23, respectively, did not result in synergistic or even additive enhancement of anti-tumor benefits versus use of either virus alone (Figures 21-23). While co-treatment at 1x10^9 and 5x10^{10} particles of Ad.IL-12 and Ad.IL-23, respectively, did double the overall tumor rejection rate over use of either virus alone (40 versus 20 percent), survival was not significantly enhanced (Figures 21a and b). Additionally, cured, co-treated mice at any dose were no more resistant to tumor challenge than their Ad.IL-23 counterparts. Ad.IL-12 treated, cured mice were again susceptible to tumor challenge, suggesting lack of induction of strong protective immunity by Ad.IL-12.

Likewise, co-treatment with Ad.IL-12 and Ad.IL-23 did not synergistically enhance induction of systemic immunity. Mice bearing two tumors treated in one on day 7 with 1x10^9 particles of Ad.IL-12 and 5x10^{10} particles of Ad.IL-23 did not survive significantly longer than and experienced similar tumor rejection rates to animals treated with Ad.IL-12 alone (Figures 24a and b). Mice treated with Ad.IL-23 at this dose did not reject any tumors, in contrast to the 40 percent tumor rejection rate obtained using this dose in mice bearing only one tumor (Figure 21b). This is most likely due to the enhanced tumor burden.

3.4.3 Determining the mechanism behind the lack of synergism between Ad.IL-12 and Ad.IL-23

We hypothesized that differences in immune cell infiltrate or angiogenesis may be responsible for the lack of additive or synergistic benefit to use of Ad.IL-12 and Ad.IL-23 together. To investigate this possibility, day 15 tumors from mice co-treated once on day 7 with 1x10^9 particles of Ad.IL-12 and 5x10^{10} particles of Ad.IL-23, or either virus alone, were analyzed for various immune cell infiltrates and angiogenesis.
3.4.3.1 CD4 T-cell infiltrate

Similar amounts of CD4+ T-cells were present in tumors of mice co-treated with Ad.IL-12 and Ad.IL-23, or either virus alone. Co-treatment did not enhance CD4+ T-cell infiltrate of tumors over use of either virus alone in either injected or contralateral tumors (Figures 25a and b). In contralateral, but not injected, tumors, co-treatment with Ad.IL-12 and Ad.IL-23 significantly enhanced CD4+ T-cell infiltrate over controls (Figure 25b). This was the only treatment group to significantly enhance CD4+ T-cell infiltrate versus controls; however, this did not translate to therapeutic benefit, as co-treated animals did not exhibit significantly enhanced survival or tumor rejection compared to those treated with Ad.IL-12 or Ad.IL-23 alone (Figures 21a and b). It is rather intriguing that no other treatment enhanced CD4+ T-cell infiltrate versus controls, as there was prolongation of survival and tumor rejection in all groups (Figures 21a and b). This implies that CD4+ T-cell infiltrate at day 15 does not correlate with therapeutic outcome. It is tempting to suggest that CD4+ T-cells are not important in direct MCA205 tumor eradication, as MCA205 cells do not express MHC class II molecules. However, we have previously shown that Ad.IL-23-mediated rejection of MCA205 tumors is dependent upon the presence of CD4+ T-cells.218 Furthermore, it has been established that T-cell-mediated eradication of MCA205 tumors is dependent upon intratumoral CD4+ T-cell infiltration.303 Thus we would expect, at the very least, to see an enhancement of CD4+ T-cells in the Ad.IL-23 treatment groups versus controls. However, as the synergism studies utilized suboptimal doses of Ad.IL-23, it is possible that any therapeutic benefit obtained may have been due to utilization of other mechanisms of action. It is also likely that the activation state and type of CD4+ T-cell present, rather than quantity, is the most important determinant in therapeutic outcome in our model. CD4+ T-cells include Th1, Th2, Th17 and T regulatory-type T-cells, as well as natural killer T-cells.304 Thus, further
characterization of the types of CD4+ T-cells present may be necessary to correlate tumor infiltrate with therapeutic outcome. Additionally, it is possible that day 15 post-tumor inoculation (8 days after treatment) may be too late to distinguish differences in CD4+ T-cell infiltrate. Shortly after day 15, animals responding to treatment begin experiencing tumor rejection; therefore, we chose to analyze tumors at this time point. However, it is possible that involvement of CD4+ T-cells occurred shortly after treatment and thus would have been overlooked in day 15 tumors. Finally, as all tumors are equally analyzed at day 15 and it is impossible to discern whether any given tumor will ultimately respond to treatment, the variable results are likely a result of analysis of both responding and non-responding tumors.

3.4.3.2 CD8+ T-cell infiltrate

CD8+ T-cell infiltrate of tumors likewise yielded unexpected results. Most notably, in both injected and contralateral tumors, Ad.IL-12 and Ad.IL-23 co-treatment resulted in a significant decrease in infiltrating CD8+ T-cells versus controls (Figures 25c and d). However, this did not correlate with observed therapeutic benefit, as co-treated mice experienced tumor rejection and survived significantly longer than controls (Figures 21a and b). Furthermore, co-treatment with Ad.IL-12 and Ad.IL-23 significantly inhibited CD8+ T-cell infiltration of tumors versus treatment with Ad.IL-12 alone (Figures 25c and d). Finally, only treatment with Ad.IL-12 in the injected tumor led to a significant increase in CD8+ T-cells over controls (Figures 25c and d).

CD8+ T-cells recognize and engage antigens in the context of MHC class I molecules on target cells to induce apoptosis. Being “self” in origin, most tumor antigens are displayed in the context of MHC class I molecules, thereby making tumors susceptible to destruction by CD8+ T-cells. In the context of cancer, tumor-specific CD8+ T-cells may be deleted, become unresponsive or are not present in large enough quantities to elicit a therapeutic response.
Thus, conventional wisdom suggests that enhancement of CD8+ T-cells within the tumor microenvironment would correlate with a positive therapeutic outcome. Likewise, others have shown that various successful treatment strategies correlate with CD8+ T-cell infiltration of MCA205 tumors.\textsuperscript{18,303} Thus the lack of correlation between CD8+ T-cell infiltrate and therapeutic anti-tumor benefit was somewhat surprising. Overall, these data suggest that quantity of tumor infiltrating CD8+ T-cells at day 15 is not reflective of therapeutic benefit. This is not to say that CD8+ T-cells do not play a role in tumor eradication, as we, and others, have indicated a role of CD8+ T-cells in IL-12 and IL-23 anti-tumor activity.\textsuperscript{18,218} As was discussed above, these results may be due to timing of tumor analysis, treatment dose, and analyzing both responders and non-responders blindly. Again, the activation state, specificity and cytotoxicity of tumor infiltrating CD8+ T-cells likely plays a greater role in therapeutic outcome than sheer quantities. It is possible that the remaining CD8+ T-cells in co-treated tumors were highly efficacious in eradication and thus a relatively small amount could efficiently eliminate tumors.

The fact that co-treatment with Ad.IL-12 and Ad.IL-23 resulted in significantly less tumor-infiltrating CD8+ T-cells than treatment with Ad.IL-12 alone suggests that either IL-23 is blocking IL-12 mediated recruitment of CD8+ T-cells or that co-infection of tumor cells with Ad.IL-12 and Ad.IL-23 results in expression of a particular cytokine profile that inhibits CD8+ T-cell infiltration of tumors. IL-23 itself may exhibit both pro- or anti-tumor effects, depending upon expression levels.\textsuperscript{223} Furthermore, IL-23 expression in tumors has been shown to decrease CD8+ T-cell infiltration and inhibit CTL activity.\textsuperscript{222} We have shown that co-infection of MCA205 cells \textit{in vitro} enhances IL-23 expression over 30-fold versus infection with Ad.IL-23 alone (Figure 18c). Thus it is possible that enhanced expression of IL-23 in tumors of mice co-
treated with Ad.IL-12 and Ad.IL-23 results in inhibition of CD8+ T-cell recruitment, which may ultimately inhibit any synergistic anti-tumor benefit.

### 3.4.3.3 Dendritic cell infiltrate

Dendritic cells (DCs) play a pivotal role in anti-tumor immunity by enhancing cross presentation of tumor antigens and link innate and adaptive immune responses.\textsuperscript{306} Thus enhancement in tumor-infiltrating DCs would likely yield therapeutic benefit due to increased presentation of tumor antigens and concurrent generation of an anti-tumor immune response. Co-treatment with Ad.IL-12 and Ad.IL-23 did not significantly alter DC infiltrate versus use of either virus alone in either injected or contralateral tumors. Additionally, no difference was observed in DC infiltrate between tumor-bearing mice co-treated with Ad.IL-12 and Ad.IL-23, either virus alone and controls in either injected or contralateral tumors (Figures 26a and b). Again the lack of correlation between therapeutic benefit and DC infiltrate suggests that, at day 15, tumor infiltrating DCs are not indicative of therapeutic outcome. Indeed, this does not suggest that DCs do not play a role in induction of Ad.IL-12 and Ad.IL-23-mediated anti-tumor immunity. This simply suggests that other factors, such as timing of tumor analysis, treatment dose and analysis of both responders and non-responders, may affect immunohistochemical results in our model. Again, it is likely that activation state, not quantity, of DCs most affects therapeutic outcome. In control treated tumors, the population of cells staining positive for CD11c may include immature or anergic DCs, as well as possibly T-cells, and thus not all positive cells would prove useful in mounting an anti-tumor immune response.
3.4.3.4 Regulatory T-cell infiltrate

Regulatory T-cells actively inhibit immune responses and are believed to play a key role in tumor immune evasion. Previous studies have shown that CD4+CD25+ regulatory cells play a role in MCA205 immune evasion.\textsuperscript{307} Thus, we expected that uninhibited MCA205 tumor growth would be associated with greater number of tumor-infiltrating regulatory T-cells and that treatment with Ad.IL-12 and Ad.IL-23 would decrease these numbers. However, in both injected and contralateral tumors, treatment with Ad.IL-12, Ad.IL-23 or both did not alter Foxp3-positive cell infiltrate versus controls (Figures 26c and d). Interestingly, in contralateral tumors, co-treatment with Ad.IL-12 and Ad.IL-23 significantly decreased Foxp3+ cell infiltrate in tumors versus use of Ad.IL-23 alone (Figure 26d). There was also a trend towards decreased Foxp3-positive cells in co-treated tumors versus use of Ad.IL-12 alone, but it was not significant. Overall these data suggest that the decrease of Tregs in co-treated tumors was due solely to the contribution of Ad.IL-12 alone and that co-treatment with Ad.IL-12 and Ad.IL-23 did not synergistically decrease Foxp3 positive cell infiltration. Additionally, similar to results seen in contralateral tumors, co-treatment with Ad.IL-12 and Ad.IL-23 decreased Treg infiltrate versus Ad.IL-23 treatment alone in injected tumors, but the decrease was not statistically significant.

Our results suggest that day 15 decrease in regulatory T-cell infiltration of tumors is not indicative of a positive prognosis. However, as was discussed above, the lack of correlation between therapeutic benefit and Foxp3 positive cell infiltrate can likely be attributed to timing in tumor analysis, dose of treatment and blind analysis of both responders and non-responders. Furthermore, it is possible that treatment with Th1 polarizing cytokines directly enhances the activity of CD4+ and CD8+ T-cells, thereby overcoming Treg induced suppression without directly affecting Tregs themselves. Finally, it must be noted that, while Foxp3 is widely
accepted as a marker for regulatory T-cells, it is also found on a significant, albeit small, population of CD8+ effector T-cells. However, as these cells also have a suppressive function, including them in our analysis would probably not skew results.

### 3.4.3.5 Angiogenesis

Inhibition of tumor angiogenesis is a primary goal in a variety of cancer therapies. The anti-tumor activity of IL-12 is mediated, at least in part, by IFN-γ mediated inhibition of angiogenesis. In contrast, it has been reported that IL-23 may possess pro-angiogenic properties. Thus, we sought to determine the effects of Ad.IL-12 and Ad.IL-23 co-treatment on angiogenesis, as analyzed by CD31 expression. In contralateral tumors, no difference in angiogenesis was observed between animals treated with Ad.IL-12 and Ad.IL-23, Ad.IL-12 alone or Ad.IL-23 alone and controls (Figure 27b). In injected tumors, however, Ad.IL-12 and Ad.IL-23 co-treatment significantly enhanced CD31 expression versus control treated animals (Figure 27a). Furthermore, co-treatment significantly increased angiogenesis compared to treatment with Ad.IL-23 alone in injected tumors (Figure 27a). In contrast to previously published results, Ad.IL-23 alone treatment did not result in enhanced angiogenesis compared to controls (Figure 27a). This may be due to expression levels of IL-23, as the enhanced angiogenesis observed in Ad.IL-12 and Ad.IL-23 co-treated animals may be due to increased IL-23 expression.

### 3.4.4 Summary of investigation into anti-tumor synergy between Ad.IL-12 and Ad.IL-23

In Chapter 2, we demonstrated that adenoviral-mediated delivery of IL-23 into the tumor microenvironment, like IL-12, results in enhancement of survival and tumor eradication.
However, while IL-23 treatment resulted in strong protective immunity, IL-12 treatment was associated with tumor recurrence. Furthermore, we have shown that Ad.IL-12 and Ad.IL-23 uses similar, yet divergent, mechanisms of action to generate anti-tumor immunity. We have subsequently shown that Ad.IL-23 utilizes endogenous IL-12 to exert its anti-tumor effects and that co-infection of MCA205 tumor cells with Ad.IL-12 and Ad.IL-23 results in enhanced production of both cytokines. Thus, we proposed that co-treatment with Ad.IL-12 and Ad.IL-23 would result in enhanced anti-tumor activity, due to use of similar, yet divergent, effector mechanisms and enhanced cytokine expression. However, neither sequential nor concurrent delivery of Ad.IL-12 and Ad.IL-23 resulted in enhanced anti-tumor effects over use of either cytokine alone. To explain the lack of synergy, day 15 tumors of treated and control mice were analyzed for various immune cell infiltrates. Co-treatment inhibited tumor infiltration of CD8+ T-cells and enhanced angiogenesis. IL-23 expression has been previously associated with inhibition of CD8+ T-cell infiltration and enhancement of angiogenesis. Thus, we hypothesized that Ad.IL-12 and Ad.IL-23 co-infection of MCA205 tumors in vivo leads to enhanced IL-23 production, thereby inhibiting any synergism with IL-12.

### 3.4.5 Investigation of synergism between Ad.IL-12 and Ad.scIL-23

To eliminate the possibility of subunit switching, and subsequent enhancement of IL-23 production within the tumor microenvironment in vivo, adenoviruses expressing single chain IL-12 and IL-23 were produced and assayed for expression and anti-tumor activity. Ad.scIL-12 expressed greater quantities of cytokine than Ad.IL-12 and efficiently eradicated MCA205 tumors in vivo (Figure 28c). However, Ad.scIL-12 treatment was associated with severe, likely IFN-γ, or possibly TNF-α, mediated toxicity and all animals perished as a result of treatment by
day 35 (Figure 28d). This toxicity was possibly due to the lack of p40 homodimers, which cannot be formed due to linkage of p40 to p35 and normally attenuate IL-12 activity by binding to the IL-12 receptor. Regardless, due to the severe toxicity associated with use of this virus, further investigation into its therapeutic potential was discontinued.

Ad.scIL-23 expressed almost 1000-fold greater amounts of cytokine that its double-chain counterpart and was biologically active, as indicated by induction of IL-17 expression from splenocytes in vitro (Figures 29b and c). Ad.IL-23 also possessed potent anti-tumor activity, with treatment resulting in significant enhancement of survival, complete tumor rejection in 90 percent of animals and establishment of strong protective immunity (Figures 29d and e). Furthermore, in spite of high expression levels, Ad.scIL-23 treatment showed little to no evidence of toxicity.

We next sought to determine if co-treatment with Ad.IL-12 and Ad.IL-23 would synergistically enhance therapeutic anti-tumor immunity. We chose to use Ad.IL-12, as the toxicity associated with its use was less severe than that associated with Ad.scIL-12. Furthermore, as the IL-23 utilized is in a single chain form, it was unlikely that subunit switching and enhancement of IL-23 production would occur. Mice bearing two tumors were treated in one with suboptimal doses of either both Ad.IL-12 and Ad.scIL-23, either virus alone or Ad.Psi5. Co-treatment resulted in identical tumor eradication rates to treatment with Ad.scIL-23 alone in both injected and contralateral tumors (Figures 30a and b). Thus, co-treatment with Ad.IL-12 and Ad.scIL-23 does not synergistically enhance anti-tumor effects over use of Ad.scIL-23 alone either locally or systemically. Therefore, subunit switching and overproduction of IL-23 is not solely responsible for the lack of synergism between IL-12 and IL-23.
3.4.5.1 Investigating the lack of synergy between Ad.IL-12 and Ad.scIL-23

To determine if co-treatment with Ad.IL-12 and Ad.scIL-23 resulted in altered immune cell infiltrate compared to use of either virus alone and to further elucidate the mechanism underlying the lack of synergy between these two cytokines, day 15 tumors were analyzed for CD4, CD8, CD11c and Foxp3 positive cell infiltrate, as well as angiogenesis.

Overall, co-treatment with Ad.IL-12 and Ad.IL-23 did not augment CD4, CD8, or CD11c positive cell infiltrate compared to control treatment (Figures 31a-d). Similarly, treatment with Ad.IL-12 or Ad.scIL-23 alone did not enhance overall immune cell infiltrate versus controls (Figures 31a-d). Thus, no correlation was observed between enhanced immune cell infiltrate and positive therapeutic outcome. This was similar to our previous immunohistochemical results and, as discussed above, may be due to timing of analysis, treatment dose and activation state of the analyzed cells.

Co-treatment with Ad.IL-12 and Ad.scIL-23 did, however, significantly enhance CD8+ T-cell infiltrate compared to use of Ad.scIL-23 alone (Figure 31b). This is in stark contrast to previous results, in which co-treatment with Ad.IL-12 and Ad.scIL-23 resulted in a significant decrease in tumor-infiltrating CD8+ T-cells compared to treatment with either Ad.IL-12 or Ad.IL-23 alone (Figures 25c and d). This suggests that use of a single chain IL-23 in combination with a double chain IL-12 does indeed alter the cytokine profile expressed from \textit{in vivo} MCA205 tumors. Specifically, less IL-23 may be being produced and therefore CD8+ T-cell infiltration of tumors is not inhibited. However, increase in CD8+ T-cell infiltrate in co-treated tumors compared to use of Ad.IL-23 alone did not translate to enhanced therapeutic benefit (Figures 30a and b).
Similar to immunohistochemical results from treatment with double chain IL-12 and IL-23, treatment with both Ad.IL-12 and Ad.scIL-23, or either cytokine alone, did not result in decreased Foxp3 expression versus controls (Figure 31c). Likewise, co-treatment did not decrease Foxp3 positive cell infiltrate compared to use of either virus alone (Figure 31c).

Finally, angiogenesis was not altered in tumors treated with both Ad.IL-12 and Ad.scIL-23, or either virus alone, compared to controls (Figure 31e). This is in contrast to previous immunohistochemical results utilizing double-chain cytokines, in which co-treatment resulted in significantly enhanced angiogenesis compared to controls. Again, this suggests that use of single-chain IL-23 in combination with IL-12 prevents enhanced expression of IL-23 due to subunit switching, which, in turn, does not lead to enhanced angiogenesis.

3.4.6 Explaining the lack of anti-tumor synergism between IL-12 and IL-23

Overall, co-treatment with Ad.IL-12 and Ad.scIL-23 enhanced tumor infiltrating CD8+ T-cells and did not enhance angiogenesis, yet no anti-tumor synergy was observed. These results support our assertion that increased intratumoral production of IL-23 likely led to decreased CD8+ T-cell infiltrate and enhanced angiogenesis in Ad.IL-12 and Ad.scIL-23 co-treated animals. However, as co-treatment with Ad.IL-12 and Ad.scIL-23 did not result in enhanced anti-tumor activity, it is obvious that IL-23-mediated enhancement of angiogenesis and decrease in CD8+ T-cell infiltration are not solely responsible for the absence of synergy between these two cytokines.
3.4.7 Neither IL-17, nor inhibition of IL-17 signaling, enhances Ad.IL-23 anti-tumor activity

IL-23 is responsible for commitment to a Th17 lineage and induces IL-17 production. IL-17 has been shown to possess anti-tumor activity and Th17 cells have been shown to enhance activity of tumor-specific CTLs. We therefore sought to determine if IL-23 would synergize with IL-17 to enhance anti-tumor effects. Treatment with Ad.IL-23 led to tumor rejection and enhancement of survival, while treatment with Ad.IL-17 did not (Figures 32a and b). Interestingly, use of Ad.IL-17 in conjunction with Ad.IL-23 decreased the tumor rejection rate of Ad.IL-23 by half (Figure 32b). Furthermore, inclusion of Ad.IL-17 with Ad.IL-23 significantly decreased animal survival compared to treatment with Ad.IL-23 alone (Figure 32a). Thus, in our model, Ad.IL-17 at the very least does not possess anti-tumor activity. Furthermore, these results indicate that co-expression of IL-17 with IL-23 decreases the anti-tumor effects of IL-23, possibly by IL-17-mediated inhibition of tumor-specific CTLs or inhibition of an IL-23 driven Th1 response.

IL-23 is a strong inducer of IL-17 expression and IL-17 appears to inhibit the anti-tumor effects of IL-23 (Figures 32a and b). Thus, we decided to investigate whether inhibition of IL-17 signaling in the tumor microenvironment would enhance the anti-tumor effects of IL-23. To block IL-17 signaling, an adenovirus expressing the IL-17 receptor fused to IgG was utilized. Treatment with Ad.IL-17RFc did not result in tumor rejection and survival was comparable to treatment with empty vector. Furthermore, mice co-treated with Ad.IL-23 and Ad.IL-17RFc did not experience enhanced tumor rejection or survival compared to treatment with Ad.IL-23 alone (Figures 33a and b). This is consistent with our previous results, which show that the anti-tumor activity of Ad.IL-23 in IL-17R-deficient mice is retained, not enhanced or diminished, compared
to wild-type mice (Figure 10). These data indicate that inhibition of IL-17 signaling does not affect the anti-tumor activity of IL-23. This suggests two things: (1) Ad.IL-23 treatment either does not result in IL-17 expression in the tumor microenvironment or (2) IL-23 treatment does result in some degree of IL-17 expression, but it is eclipsed by IL-23-mediated IFN-γ production. Overall, these data indicate that IL-23 does not utilize IL-17 and a Th17, but rather IFN-γ and a Th1, immune response to exert its anti-tumor effects.

3.4.8 A step closer to explaining the lack of synergy between IL-12 and IL-23

We expected that co-treatment with adenoviruses expressing IL-12 and IL-23 would synergistically enhance anti-tumor immunity, due to their shared ability to eliminate tumors by activating a Th1 immune response. However, we found no evidence of synergy between these two cytokines. Indeed, previous studies support our assertion: C57BL/6 mice bearing scIL-23-secreting B16 cells do not show enhanced anti-tumor benefit when co-treated with recombinant IL-12 intraperitoneally. Thus, while it was widely accepted and anticipated that IL-12 and IL-23 would synergize in the context of tumor eradication, our studies firmly contradict this notion. Furthermore, the lack of synergy is not entirely due to enhanced IL-23 production, which could result in decreased CD8+ T-cell infiltrate and enhanced angiogenesis. Additionally, we have shown that IL-17 can actively inhibit an IL-23-mediated, Th1-driven anti-tumor response. It is therefore likely that these two cytokines simply do not synergize due to their contrasting and mutually exclusive functions: IL-12 strongly drives IFN-γ expression and a Th1 response, while IL-23 is capable of driving IL-17 expression and a Th17 response. While in our model it did not appear that IL-23-mediated eradication of tumors was mediated by a Th17 response, it is possible that some Th17 T-cells were produced. Furthermore, it has been shown that Th17 cells
inhibit CD8+ T-cell cytotoxicity.\textsuperscript{222,309} Therefore, co-expression of IL-23 with IL-12 may skew the resulting immune response away from a Th1 phenotype and result in lack of enhancement of anti-tumor effects.
4.0 DISCUSSION

We have conclusively shown that adenovirally delivered IL-23 possesses therapeutic antitumor activity in day 7 MCA205 fibrosarcoma and B16 melanoma models. Ad.IL-23 treatment resulted in an overall 40 percent tumor rejection rate and significantly enhanced survival of tumor bearing mice compared to empty vector treated controls. Additionally, IL-23 therapy results in generation of tumor specific CTLs and generation of potent, long-term protective immunity. The anti-tumor activity of IL-23 is independent of endogenous IL-23, IL-17, perforin and Fas ligand, but dependent upon IL-12, IFN-γ and CD4+ and CD8+ T-cells, suggesting generation and utilization of a Th1-type immune response in tumor eradication.

In general, adenovirally delivered IL-12 proved to be the more effective anti-tumor agent with regards to both animal survival and tumor rejection. Ad.IL-12 treatment resulted in a 90 percent rejection rate and significantly enhanced survival compared to both empty vector treated controls and Ad.IL-23 treated animals. Like Ad.IL-23, Ad.IL-12 treatment resulted in generation of tumor-specific CTLs; however, there was indication of involvement of innate immunity, as evidenced by lysis of YAC-1 cells in CTL assays. In our model, IL-12 anti-tumor activity is dependent only on IFN-γ and independent of endogenous IL-12, IL-23, IL-17, perforin, FasL and CD4+ and CD8+ T-cells. Ad.IL-12 treatment generated protective immunity only 50 percent of the time and some tumors that were rejected initially later recurred. Overall,
this suggests that Ad.IL-12 treatment, at least part of the time, activates only an innate response to eradicate tumors.

Due to the similar, yet divergent, mechanisms utilized by Ad.IL-12 and Ad.IL-23 to eradicate tumors, we proposed that use of both cytokines together would synergistically enhance tumor eradication and survival compared to use of either virus alone. However, we have exhaustively shown that neither delivery at separate time points nor co-delivery of Ad.IL-12 and Ad.IL-23 led to synergistic or even additive anti-tumor effects compared to use of either cytokine alone. In elucidating the underlying mechanism, we found that co-treatment with Ad.IL-12 and Ad.IL-23 resulted in significantly decreased tumor infiltrating CD8+ T-cells and significantly enhanced angiogenesis. Our in vitro studies suggested that co-infection with Ad.IL-12 and Ad.IL-23 results in enhanced IL-23 expression. Additionally, previous studies have indicated that IL-23 can inhibit CD8+ T-cell infiltration and enhance angiogenesis. Thus we proposed that IL-23 expression was increased as a result of p19 sequestration of p40, which inhibited synergism with IL-12 due to decreased CD8+ T-cell infiltration of tumors, increased angiogenesis and reduction of IL-12 expression.

To prevent subunit switching between IL-12 and IL-23, adenoviruses expressing single chain IL-12 and IL-23 were constructed. Ad.scIL-23 expressed much greater levels of cytokine and possessed greater anti-tumor activity than Ad.IL-23 (90 versus 40 percent rejection rates). Ad.scIL-12 also expressed greater levels of cytokine and treatment resulted in rapid tumor rejection, but was associated with severe, lethal mortality in mice and therefore use was discontinued. Co-delivery of Ad.scIL-23 and Ad.IL-12 did not result in synergistic enhancement of anti-tumor effects compared to use of either virus alone. Analysis of tumors revealed no inhibition of CD8+ T-cell infiltration and no enhancement of angiogenesis in Ad.scIL-23 and
Ad.IL-12 treated animals. This suggests that subunit switching and enhancement of angiogenesis may have contributed to these observations, but that other factors are definitely involved in the lack of synergism between IL-12 and IL-23.

We also investigated anti-tumor synergism between adenovirally delivered IL-23 and IL-17 due to their shared roles in Th17 development. We found that delivery of IL-17 with IL-23 significantly inhibited the anti-tumor effects of IL-23. However, blocking IL-17 signaling did not enhance the anti-tumor activity of IL-23. Thus we propose that co-expression of IL-17 and IL-23 led to generation of a Th17, rather than a Th1, response. Thus it is possible that under some circumstances IL-23 can drive a Th17 response and therefore the lack of synergism between IL-12 and IL-23 can be explained by IL-23-mediated generation of some Th17 cells which inhibit, not enhance, anti-tumor activity.

Future studies could include characterization of tumor infiltrating CD4+ T-cells in animals co-treated with IL-12 and IL-23 to further elucidate the types of immunity generated. This could be accomplished by analyzing tumor infiltration lymphocytes for cytokine, as well as transcription factor, expression by flow cytometry. To determine if generation of Th17 immunity is indeed responsible for lack of synergism between IL-12 and IL-23, the anti-tumor effects of co-treatment in tumor-bearing mice deficient in either IL-17 or Th17 transcription factor roryt could be investigated.

Overall, we have advanced the knowledge in the cancer gene therapy field in a number of ways. We were the first to show that adenovirally delivered IL-23 exhibited therapeutic anti-tumor benefit by generation of a Th1-type immune response. In doing so, we have reaffirmed the utility of adenovirus as a means to deliver therapeutic immunostimulatory cytokines, as well as add an additional cytokine with potential clinical use in the treatment of cancer. We have also
added yet another line of evidence to suggest that IL-23 possesses anti- tumor effects and can drive Th1 immunity.

Additionally, we have exhaustively shown that IL-12 and IL-23 do not synergize in tumor eradication, possibly by due to IL-23-driven Th17 immunity. This is in stark contrast to the expectation that IL-23 and IL-12 would synergize to enhance anti-tumor effects due to shared IFN-γ inducing and Th1 polarizing abilities. Due to these revelations, further investigations into use of IL-23 as an IL-12 adjuvant should be weighed wisely, especially in the clinic.

Finally, we have shown that adenovirally-delivered single-chain IL-23 exhibits more potent anti-tumor activity than its double-chain counterpart. Thus we have shown that augmentation of IL-23 expression enhances its anti-tumor activity and therefore, if use of IL-23 were to proceed to clinical trials, use of a single chain version may be preferred.

Whether IL-23 proceeds to clinical trials for the treatment of cancer remains to be seen. We have shown that it does possess potent anti-tumor effects with low associated toxicity. However, its association with a variety of inflammatory diseases and its implication in some cancers may inhibit its use therapeutically. The role of IL-23 in cancer certainly needs to be further clarified prior to proceeding to clinical trials.


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