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CYTOKINE THERAPY OF CANCER BY GENE TRANSFER

BY

PETER CAMPBELL REYNOLD EMTAGE. B.Sc., M.Sc.

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Doctor of Philosophy

McMaster University

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CYTOKINE THERAPY OF CANCER BY GENE TRANSFER

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(Medical Sciences)

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AUTHOR: Peter C. R. Emtage, B.Sc., M.Sc., (University of Guelph)

SUPERVISOR: Dr. Jack Gauldie

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ABSTRACT

Many tumors express antigens that are capable of being recognized by effector cells of the immune system. However, these tumors persist in immunocompetent hosts. The mechanisms by which immune effector cells are rendered unresponsive to these antigens is unknown. Previous data from our laboratory demonstrated the use of adenoviral vectors constructed to express single cytokines (IL-2 and IL-12) in mediating regression of tumors in a mouse mammary adenocarcinoma model. The ability of these molecules to enhance the immune response to effectively regress these tumors was hampered by toxicities associated with these factors. To overcome this toxicity and enhance the efficacy of IL-2, double recombinant adenoviral vectors expressing the co-stimulatory molecules B7-1 or B7-2 with IL-2 were constructed to allow for the expression of both immuno-modulatory molecules from the same cell. An alternative approach to enhancing the efficacy of these factors involved the use of double recombinant adenoviral vectors expressing combinations of the chemokine lymphotactin with IL-2 and IL-12.

Mammary tumor cells from transgenic mice expressing polyoma middle T (PyMT) or an oncogenic form of the proto-oncogene Neu (Neu 8142) were transplanted into syngeneic FVB/N recipients to establish the subcutaneous tumor models. Intratumoral injection of adenoviral vectors constructed to express murine B7-1 or B7-2 and IL-2 resulted in regression of PyMT or Neu (8142) tumor bearing mice, superior to that

observed for the single cytokine or co-stimulatory molecule expressing vectors. Cured mice were shown to have generated systemic immunity to a subsequent challenge with fresh tumor cells and tumor specific cytotoxic T lymphocyte activity could be detected.

Intra-tumoral injection of adenoviral vectors constructed to express lymphotactin IL-2 or IL-12 demonstrated complete regression in a small number of PyMT tumor bearing mice. None of these single cytokine or chemokine expressing vectors was capable of inducing regression of Neu (8142) tumors. In contrast, administration of vectors expressing either a combination of lymphotactin and IL-2 or lymphotactin and IL-12 resulted in an increased incidence of complete regression in both tumor models. All regressed mice demonstrated systemic protection and anti-PyMT CTL. The activity of these vectors in the Neu tumor model is different and suggests some variance in the immunogenicity of the two tumor models.

These results demonstrate the ability of double recombinant vectors expressing the co-stimulatory molecules (B7-1 or B7-2) or lymphotactin in combination with IL-2 or IL-12 to enhance the anti-tumor effects of either cytokine alone. These vectors demonstrated no associated toxicities, due to the reduced levels of cytokine that are needed to maximally co-operate with the co-stimulatory or chemokine molecules. Development of this type of vector system will lead to much improved clinical protocols for cancer therapy.

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LIST OF ABBREVIATIONS

| | |
|-----------|--|
| Ad5 | human adenovirus type 5 |
| Ad2 | human adenovirus type 2 |
| APC | antigen presenting cell |
| B7-1 | T cell and NK cell co-stimulatory molecule 1 |
| B7-2 | T cell and NK cell co-stimulatory molecule 2 |
| BAL | bronchoalveolar lavage |
| C | cysteine |
| C-C | cysteine cysteine |
| C-X-C | cysteine-any amino acid-cysteine |
| C-X-X-X-C | cysteine-3x(any amino acid)-cysteine |
| cDNA | complementary deoxyribonucleic acid |
| CTL | cytotoxic T lymphocyte |
| CTLA-4 | cytotoxic T lymphocyte antigen-4 |
| CD28 | T cell receptor for B7-1 and B7-2 |
| DNA | deoxyribonucleic acid |
| E1 | early region 1 |
| E2 | early region 2 |
| E3 | early region 3 |
| E4 | early region 4 |

| | |
|--------------|---|
| FAS | T cell TNF receptor involved in apoptosis |
| GM-CSF | granulocyte macrophage colony stimulating factor |
| IFN γ | interferon gamma |
| IgG | immunoglobulin G |
| IL-1 | interleukin 1 |
| IL-2 | interleukin 2 |
| IL-4 | interleukin 4 |
| IL-5 | interleukin 5 |
| IL-6 | interleukin 6 |
| IL-7 | interleukin 7 |
| IL-8 | interleukin 8 |
| IL-10 | interleukin 10 |
| IL-12 | interleukin 12 |
| IL-18 | interleukin 18 |
| kb | kilobase |
| kDa | kilodalton |
| mRNA | messenger ribonucleic acid |
| Lym | lymphotactin |
| PBS | phosphate buffered saline |
| RANTES | regulated on activation, normal T cell expressed and secreted |

| | |
|--------------|--|
| TGF β | transforming growth factor beta |
| Th1 | Type 1 helper lymphocyte (CD4 ⁺) |
| Th2 | Type 2 helper lymphocyte (CD4 ⁺) |
| TNF α | Tumor necrosis factor alpha |

Chapter 1

INTRODUCTION

The Immune Response

The immune system functions to protect the organism against infection with pathogenic organisms. The diversity of potential pathogenic insults is large and as a result no single immune response can deal with all aspects of immunological function. The regulation of the resulting diversity of immune responses is crucial for proper development of effector mechanisms and survival of the host. The anti-pathogen responses are mediated initially by the activity of the innate immune system, which communicates with the adaptive immune system to provide critical effector activity and memory. The identification of two functionally different CD4⁺ T helper populations of the adaptive immune system (Th1 and Th2) capable of secreting distinct cytokine profiles has provided valuable understanding of the mechanisms by which immune responses are generated *in vivo* to various pathogenic insults (Figure 1) (Mosmann et al., 1986; Abbas et al., 1996; Reiner et al., 1995; Mosmann et al., 1996; O'Garra, 1998). The T helper cells of the type 1 phenotype produce many soluble factors of which IFN γ (Interferon γ) and lymphotoxin are the hallmarks (reviewed in O'Garra, 1998). These soluble factors from Th1 cells induce and regulate cell mediated immune responses which serve to eliminate viruses and other intracellular pathogens through induction of cytotoxic T cells (CTL) (Mosmann et al., 1986; Muraille and Leo, 1998; Abbas et al., 1996; Reiner et al., 1995; Mosmann et al., 1996). Th2 T cells are characterized by a specific cytokine profile (Interleukin 4, 5, 6, 10 and 13) which has implicated this T cell

subset in humoral immunity. Th2 T cells are thought to be involved in antibody production, mediate allergic inflammation and promote eosinophil and mast cell differentiation, activation and function (Muraille and Leo, 1998; Abbas et al., 1996; Reiner et al., 1995; Mosmann et al., 1996). The Th2 subset will not be extensively discussed here since most anti-tumor responses appear to be mediated by Th1 T cells. However, it is important to mention that cytokines from each T helper subset appear to negatively regulate the development of the other subset (reviewed in Muraille and Leo, 1998; O'Garra, 1998). For example, IL-4 and IL-10 produced by Th2 cells inhibits the differentiation of Th1 cells and the antigen presentation and effector function of antigen presenting cells (APCs). Importantly, IL-10 blocks the production of interleukin 12 (IL-12) from APCs and, hence, down regulates the IL-12:IFN γ positive feed back loop which disrupts cell mediated response (Sher and Coffman, 1992; Moore et al., 1993; Muraille and Leo, 1998). Alternatively, IFN γ , a major Th1 and natural killer (NK) cell derived cytokine, inhibits the function and activity of Th2 cells, which in turn results in the inhibition of humoral and allergic responses (Fitch et al., 1992; Muraille and Leo, 1998). The development of a Th1 response is reliant on the innate immune system for developmental signals (IL-12 and IFN γ). Delineation of the T helper subsets (Th1 and Th2) with respect to cytokine and surface antigen expression has revealed fundamental pathways of communication between these two responses (Muraille and Leo, 1998; Abbas et al., 1996; Reiner et al., 1995; Mosmann et al., 1996).

Sentinel cells, like APCs or natural killer cells (NK), encounter pathogens in the non-lymphoid tissues of the body. They immediately respond to these pathogens by releasing toxic mediators (eg lymphotoxin and tumor necrosis factor α (TNF α)) in the case of APCs, or by the direct cytotoxic activity of NK cells on infected cells. This type of effector activity is called the innate immune response since the effector cells involved are non-major histocompatibility complex (MHC) restricted and lack immunological memory (Gazzinelli et al., 1993; Tripps et al., 1993). Cytokines released by these effector cells interact with the same or newly recruited cells (attracted by chemokines) in an autocrine or paracrine fashion to amplify the response (Seder and Paul, 1994; Hsieh et al., 1993). Cytokines, like NK cell derived IFN γ and APC produced IL-12, also act strongly on antigen presenting cells to induce maturation and facilitate the presentation of antigens to CD4⁺ T precursor helper cells. This enables the generation of T helper phenotypes (Muraille and Leo, 1998; Abbas et al., 1996; Reiner et al., 1995; Mosmann et al., 1996). Therefore, the secretion of these molecules links the effector arms of the innate and adaptive immune responses.

The predominant initiator of Th1 mediated responses is IFN γ . IFN γ potently enhances the ability of APCs to produce IL-12 probably within the microenvironment of the inflammatory response (Cassatella et al., 1995; Kubin et al., 1994). IL-12 is the primary cytokine implicated in the differentiation of T helper cell precursors into Th1 T helper cells (Maruo et al., 1996; Murphy et al., 1994; Kennedy et al., 1994; Manetti et

al., 1993; Trinchieri, 1993; Hsieh et al., 1993). Interestingly, the ability of IFN γ to enhance IL-12 production is important since IL-12 strongly increases IFN γ production by NK and T cells (Chan et al., 1991; Trinchieri et al., 1996) providing a positive feed back loop in the microenvironment of the immune response. This feed back mechanism may illustrate a route by which Th1 cells are maintained *in vivo*. The activity of IL-12 and IFN γ on the generation of a Th1 phenotype has been studied extensively in humans and mice (reviewed in Trinchieri, 1998). IL-12 has been shown to exert biological effects on NK cells, T cells and lymphokine activated killer (LAK) cells by inducing the generation of cytotoxic T cells and mediating cytotoxicity in LAK and NK cells (Gately et al., 1992; Chehimi et al., 1992; Bloom and Horvath, 1994). This cytotoxic activity may be regulated in part by the activity of IL-12 in up-regulating cell surface molecules, such as the IL-2 receptor, on T and NK cells, receptors for IL-4, IL-12, TNF α as well as molecules like CD2, CD11a and CD54 on NK cells (reviewed in Brunda, 1994; Trinchieri, 1998). Another important cytokine for the development of Th1 and associated CD8⁺ CTL for cell mediated immunity is IL-2. IL-2 has been shown to be a growth factor for T cells (Morgan et al., 1976), specifically precursor T helper cells (Trinchieri, 1998; Muraille and Leo, 1998; Smith, 1988). In the absence of IL-2, the activating activity of IL-12 is not observed on naïve T cells and, hence, no Th1 immune response can be induced (Chambers and Allison, 1997; Guinan et al., 1994; Gause et al., 1997; Bluestone, 1995).

T helper Dependent and Independent Responses

T helper 1 T cell responses are not only important for the production of cytokines. They also "help" to generate cytotoxic CD8⁺ T cells capable of recognizing specific antigens in the context of MHC class I antigens (Muraille and Leo, 1998; Abbas et al., 1996; Reiner et al., 1995; Mosmann et al., 1996). Factors produced by Th1 CD4⁺ T cells (IL-2, TNF α and IFN γ) augment the cytotoxic activity of CD8⁺ T cells by inducing the maturation of effector functions within these cells (Hart et al., 1997; Boussiotis et al., 1996; Schultze et al., 1996; Guinan et al., 1994). The priming of CD8⁺ T cells to some pathogens via the TCR/Ag:MHC I interaction is not enough to activate CTL function. In most cases, the presence of IL-2 producing CD4⁺ Th1 cells is required to cause maturation and induce proliferation in these cells (Chambers and Allison, 1997; Guinan et al., 1994; Gause et al., 1997; Bluestone, 1995). This is thought to occur in situations where there is inadequate co-stimulation i.e., lack of expression of B7 family members on target cells (Chambers and Allison, 1997; Guinan et al., 1994; Gause et al., 1997; Bluestone, 1995). Interestingly, recent work has demonstrated the presence of B7-1 and B7-2 molecules on both activated CD4⁺ and CD8⁺ T cells (Verwilghen et al., 1994; Prabhu Das et al., 1995; Hollsberg et al., 1997). Although the role of B7-1 and B7-2 on activated T cells is unknown, it has been postulated that these molecules exist to perpetuate the immune response by eliminating the requirement for APC derived co-stimulation.

The mechanism of CTL generation outlined above is dependent on CD4⁺ Th1 T cells. However, this is not the only mechanism by which CD8⁺ CTL can be generated. CD8⁺ T cells can assume a state of "readiness" by interacting with APCs that have a high level of co-stimulatory and accessory molecule expression (Lanier et al., 1995; Gajewski, 1996; Gross et al., 1992; Deeths and Mescher, 1997; Bluestone, 1995; Hart et al., 1997). This "readiness" in association with the formation of the T cell receptor-antigen/MHC I complex has been shown to potently stimulate CD8⁺ T cells to produce IL-2 (Hart et al., 1997). IL-2 synergises with IFN γ derived from NK or CD4⁺ Th1 cells to drive CD8⁺ T cell proliferation and maturation to CTL (Hart et al., 1997; Boussiotis et al., 1996; Schultze et al., 1996; Guinan et al., 1994). The other mechanism of CTL activation is CD4⁺ Th1 cell dependent. The priming of CD8⁺ T cell responses to some pathogens requires the presence of IL-2 producing CD4⁺ Th1 cells to mature and proliferate (Chambers and Allison, 1997; Guinan et al., 1994; Gause et al., 1997; Bluestone, 1995). This is thought to occur in situations where there is inadequate co-stimulation (Chambers and Allison, 1997; Guinan et al., 1994; Gause et al., 1997; Bluestone, 1995). The generation of Th1 cells secreting cytokines which favor the generation of Th1 responses and the production of CD8⁺ CTL are characteristic of infection by viruses and other intracellular pathogens. In addition to inducing a cellular immune response, Th1 cells also augment an antibody mediated response. The antibody isotypes produced during Th1 responses in the mouse are immunoglobulin G2a and G3

(IgG2a and IgG3), which have been shown to play a role in neutralizing pathogens, activating complement and inducing antibody dependent cytotoxicity (ADCC) which is mediated by NK cells (Janeway and Travers, 1997).

The immune response covered here reveals only a fraction of the complexity of the immune system. Enhancement of the Th1 phenotype in cancer immunotherapy is favored by tumor immunologists since the cellular immunity generated by this adaptive response can directly result in tumor cell lysis and in the generation of memory against specific tumor antigens. The key cytokines produced during the development of this phenotype (IL-2, IL-12 and IFN γ) have been shown to exhibit anti-tumor properties. Recently, there have also been reports that demonstrate the activity of some Th2 cytokines in generating anti-tumor responses (IL-4 and IL-10) (Berman et al., 1996; Giovarelli et al., 1995; Bosco et al., 1990; Addison et al., 1995b). However, the literature suggests that these instances are minor compared to the activity elicited by Th1 cytokines.

Tumor Immunology

Malignant transformation results from a multi-step carcinogenic process defined and characterized by DNA mutations or the ectopic expression of normal homeostatic genes. Mutations in proto-oncogenes, mutations in some cellular genes not directly involved in tumor pathogenesis and mutations in genes which regulate cell cycle progression all act to promote malignant neoplastic growth. These mutated self proteins

can potentially provide epitopes for immune recognition allowing responses by the host immune system against these potential rejection antigens (reviewed in Shu et al., 1977). Defining the role of the immune response against tumor rejection antigens has been a complex and problematic undertaking for tumor immunologists during the last 25 years. Presented in this thesis is a detailed examination of the current literature as it pertains to this work. Potential mechanisms utilized by tumors to evade immune-surveillance will be the focus of this section.

Tumor Escape From Immuno-surveillance

The phenomenon of neoplastic growth and progression to cancer is facilitated by the tumor cell's ability to evade the host immune system. A number of mechanisms have been proposed to help identify "escape" parameters. However, mechanisms identified for a specific tumor might not be utilized by a tumor of similar or different origin. A greater understanding of how tumors grow in immuno-competent hosts is starting to emerge with the understanding of the mechanisms behind the induction and function of immune effector cells, predominantly T cells.

Down Regulation or Loss of MHC I Expression

In the case of immunogenic tumors, loss of MHC I expression results in an increase in tumorigenesis (Vora et al., 1997; Hall and Blair, 1997; Kono et al., 1997; Korkolopoulou et al., 1996). This observation suggests that the expression of MHC I molecules is important for tumor recognition and elimination. This is demonstrated by

transfection experiments in which introduction of MHC I genes into murine tumor cell lines results in an increase in immunogenicity and reduces the ability of these tumors to grow in immuno-competent hosts (Wallich et al., 1985; Mandelboim et al., 1995; VandenDriessche et al., 1994). Hence, the ability of some tumors to evade the immune response may in part be due to the ineffective presentation of antigen. In contrast, many murine and human cancers appear to express normal levels of MHC I and still "escape" the immune system. In addition, non-hemopoietic tumors do not express MHC II, therefore, anti-tumor responses are limited to CD8⁺ T cells rather than CD4⁺/CD8⁺ T cell responses. In many cases CD8⁺ T cells need CD4⁺ T helper cells to initiate a proper immune response; in non-hemopoietic tumors this aspect of the immune response is lacking and may also lead to "escape" of the tumor (Callahan and Leach, 1996; Baskar et al., 1994).

Lack of Co-stimulation

The generation of T cell responses relies on both T cell receptor signaling induced by Ag:MHC I or II ligation as well as B7:CD28 ligation on the T cell. As outlined previously, modified immunogenic tumor cells expressing B7-1 or B7-2 fail to grow in immuno-competent hosts (see section Cytokines and B7-1/B7-2 Immunotherapy). This observation suggests an amplification of tumor antigen specific T cell responses mediated by the co-stimulatory molecules. Therefore, the apparent immunogenicity of some tumors appears to be related to their co-stimulatory capacity.

Tumor Cell Selection of Decreased Immunogenicity

It has been demonstrated that tumor cell variants with decreased immunogenicity can grow out of an immunogenic tumor under the selective pressure of the anti-tumor response (North, 1985). These cells usually express different, less dominant epitopes and therefore are resistant to T cell effector mechanisms and easily escape the immune response (North, 1985). However, not all tumor antigens are lost and, hence, it should still be possible to generate immune responses against these antigens using immune modulatory molecules.

Immune Tolerance to Tumor Antigens

Tumor bearing organisms may exhibit immunological tolerance to non-mutated tumor associated self antigens (TAAs). This tolerance is due to central deletion (or peripheral anergy) of self antigens via selection of potential auto-reactive TCR or BCR harboring T or B cells. Alternatively, the host may be rendered tolerant due to the inappropriate presentation of TAAs. Tolerance is an important aspect of tumor immunology since most tumor infiltrating lymphocytes are found to be anergic while they exhibit TCR specificity to some TAAs (Enk et al., 1997; Chaux et al., 1997; Melero et al., 1997; Sotomayor et al., 1996; Kawakami et al., 1996).

Immune Suppression

Many tumors have been shown to produce factors that prevent immune responses from attaining full activity. Soluble factors like IL-10, TGF β , nitric oxide

and PGE₂ all have immuno-suppressive effects and have been shown to be secreted by various tumors (Kundu and Fulton, 1997; Salazar-Onfray et al., 1997; Yue et al., 1997; Young et al., 1996; Alleva et al., 1994). Interestingly, soluble tumor antigens shed from the tumor mass have also been shown to inhibit innate and adaptive immune responses (Ziegler-Heitbrock et al., 1992; Hakim et al., 1983). Recently, a large number of tumors have been shown to express the apoptosis inducing molecules Fas or Fas ligand (Hug, 1997; Zeytun et al., 1997). The ability of these molecules to induce apoptosis of potential anti-tumor T cells is a phenomenon that has been demonstrated for some tumors. The immune-suppressive factors mentioned here not only act at the level of the effector cell but also at the point of antigen presentation to naïve T cells. These effects could block the anti-tumor activity of tumor infiltrating lymphocytes (TILs) or render apparently immunogenic tumors non-immunogenic. The ability of tumors to modulate the immune response adds more complexity to the design of potential anti-tumor immunotherapy protocols.

Cytokines in Tumor Immunotherapy

Cytokines are autocrine and paracrine intercellular messengers capable of inducing proliferation, activation and differentiation of haematopoietic cells. These immune hormones are usually small peptides produced in response to stress in the form of infection (Oppenheim et al., 1994). Current molecular biology techniques have allowed for the identification and expression of many cytokines *in vitro* and *in vivo*.

The activity of these molecules has been shown to be involved in the inflammatory process, in immunity to pathogens, in wound healing and in autoimmunity (Hirano, 1992; Tominaga et al., 1992; Williams et al., 1994; Brennan and Feldmann, 1996). The aberrant regulation of cytokine expression can lead to tissue damage. Hence, there is a need to maintain a homeostatic balance between cytokine function and tissue damage. The mechanism behind this regulation is complex. It is thought that the activities of many cytokines are regulated by the suppressive effects of other cytokines in the local environment of the immune response (Abbas et al., 1991; Oswald et al., 1992; Muraille and Leo, 1998; O'Garra, 1998).

Distinct cytokine profiles are associated with specific CD4⁺ T helper cell populations (Th1 and Th2) (Mosmann et al., 1986; Muraille and Leo, 1998; O'Garra, 1998). The production of IFN γ and IL-2 from Th1 T cells are involved in monocyte/macrophage and cell mediated immune responses. Similarly, Th2 derived IL-4, IL-5 and IL-10 results in antibody production and eosinophil and mast cell function and proliferation (Mosmann and Sad, 1996; Reiner and Seder, 1995; Abbas et al., 1996). The varied and striking effects that cytokines exhibit has led to the incorporation of many cytokines into pre-clinical and clinical models of cancer immunotherapy. The cytokines most relevant to this work are IL-2 and IL-12 for their ability to potentiate Th1 and cell mediated immune responses, and therefore, will be the focus of this thesis.

Interleukin 2 Anti-tumor Activity

IL-2 was first identified in 1978 by Gillis et al, as a factor that could induce the proliferation of T cells. It is a 14-17 kDa protein that is post-translationally modified by glycosylation, which is necessary for its activity. Production of IL-2 is limited to CD4⁺ precursor T cells, differentiated CD4⁺ Th1 cells and CD8⁺ T cells. The proliferative activity acts in an autocrine or paracrine manner depending on the expression of the IL-2 receptor. Cells responding to IL-2 not only proliferate but also produce other cytokines such as TNF α , IFN γ and lymphotoxin. These observations revolutionized immunology and initiated the field of tumor immuno-therapy (Baker et al., 1978; Larsson et al., 1980; Smith et al., 1980; Robb et al., 1981; Robb and Smith, 1981; Leonard et al., 1982; Smith et al., 1983). The ability of IL-2 to mediate anti-tumor effects is based on its ability to activate tumor infiltrating lymphocytes (TILs) (Cameron et al., 1990; Lindgren et al., 1993), and potentiation of cytotoxic activity shown by cytotoxic T, NK and LAK cells (Yron et al., 1980; Fearon et al., 1990; Lotze et al., 1981; Grimm et al., 1982; Vujanovic et al., 1988). TILs are composed of CD4⁺ and CD8⁺ lymphocytes that exhibit anti-tumor cytotoxic activity *in vitro* (Rosenberg et al., 1986; Belldegram et al., 1989). Kradin and co-workers (1989) used TILs activated in the presence of systemic IL-2 to immunize cancer patients. The authors observed a 10-30% response (reduction in tumor size), which at that time was a significant advancement in tumor immunotherapy. IL-2 has also been used systemically in both

pre-clinical and clinical trials (reviewed in Eberlein et al., 1991). Rosenberg and colleagues (1989) demonstrated the efficacy of intravenous recombinant IL-2 protein administration showing that some patients could generate enough of an anti-tumor immune response to reject the tumor. However, this approach was not very efficacious and varying the dosage and administration times could not enhance the patient's response beyond that observed with the high dose IL-2 used in the first clinical trial. The important observation from this and other similar IL-2 trials is the toxicity associated with systemically administering IL-2. Since the protein half life of IL-2 is very short, therapies require the administration of very high doses of IL-2 which result in vascular leak syndrome, anemia, hypotension, fevers, chills, nausea and edema (reviewed in Siegel and Puri 1991). The toxicity associated with the administration of systemic IL-2 stimulated investigators to develop protocols for the local delivery of IL-2 to the tumor site.

Direct injection of recombinant IL-2 to the tumor site resulted in a decrease in IL-2 associated toxicity, longer survival times and, in some cases, much delayed tumor growth in murine tumor models (Yeung et al., 1992; Tohmatsu et al., 1993). Although this therapeutic protocol proved to be more promising, the short half-life of IL-2 requires it to be continually administered to the tumor site. Alternative approaches turned to gene modified tumor cells expressing IL-2 in order to maintain effective levels in the tumor mass for a more prolonged period of time. Interestingly, this approach

resulted in an enhanced anti-tumor activity over the previous approach with the recombinant protein. Tumor cells transduced with retroviruses expressing IL-2 significantly enhanced tumor immunogenicity compared to untransduced tumor cells (Conner et al., 1993; Bannerji et al., 1994; Karp et al., 1993; Gansbacher et al., 1990). *Ex vivo* approaches such as this, are cumbersome and unrealistic for development of human clinical protocols. Haddada and colleagues (1993) took the local administration of IL-2 one step further and constructed an adenoviral vector to express IL-2 and directly administered this vector intra-tumorally in a P815 murine mastocytoma model. Adenoviral vectors are ideal for this approach since they do not require replicating cells to produce the transgene product. In addition, the wide tropism of adenoviruses make them the best candidate for such protocols. The authors report that IL-2 vector delivery to the tumor site resulted in high levels of cytokine production and enhanced immunogenicity. Since this initial observation other investigators have demonstrated the effectiveness of Ad IL-2 vectors at inducing regression and long lasting protection (Haddada et al., 1993; Cordier et al., 1995; Addison et al., 1995; Toloza et al., 1996). The ability of cytokines expressed locally via intra-tumoral injection of Ad vectors to induce anti-tumor effects clearly demonstrates the benefit of using these type of vectors in tumor immunotherapy.

Interleukin 12 Anti-tumor Activity

IL-12 is considered a key regulatory cytokine for its fundamental role in regulating cytokine production and cellular immunity. IL-12 is a heterodimer (with 35 kDa and 40 kDa subunits) produced by antigen presenting cells which include B cells, dendritic cells, monocyte and macrophages (Hall, 1995; Trinchieri et al., 1996; Wong et al., 1998; Stren et al., 1990). Shortly after the identification of IL-12 (Gubler et al., 1991; Wolf et al., 1991), a strong interest was generated in the potential immunomodulatory capacity of this cytokine (Trinchieri, 1994; Brunda and Gately, 1995; Hendrzak and Brunda, 1995). IL-12 has been shown to induce proliferation and activation of T and NK cells, to induce CD8⁺ CTL, NK cells and LAKs, to stimulate cytokine secretion (predominately IFN γ) and to regulate cell mediated immune responses (Schoenhaut et al., 1992; Gately et al., 1992; Naume et al., 1992; Maruo et al., 1996; Murphy et al., 1994; Kennedy et al., 1994; Lederer et al., 1996; Bucy et al., 1994; Chan et al., 1991; Andrews et al., 1993; Muraille and Leo, 1998; O'Garra, 1998). These observations made IL-12 an attractive candidate for cancer immunotherapy protocols. The anti-tumor activity of IL-12 has been shown in many models of murine cancer including lymphomas (O'Toole et al., 1993; Verbik et al., 1995), melanomas (Brunda et al., 1993; Nastala et al., 1994) and carcinomas (Brunda et al., 1993; Nastala et al., 1994; Stern et al., 1993; Voest et al., 1995; Mu et al., 1995). IL-12 has also shown anti-metastatic properties in spontaneous and experimental models of metastasis (Mu et al.,

1995; Gately et al., 1994; Brunda et al., 1993; Nastala et al., 1994). It is evident that pre-clinical trials have demonstrated the potent efficacy of rejection or regression obtained with IL-12 therapy. The precise role of IL-12 in inducing regression is not fully understood. However, it is thought to exert its effect through cytokines such as IFN γ and IFN α and chemokines such as Mig and IP-10 (reviewed in Jenks, 1996; Nastala et al., 1994). IL-12 has also been shown to mediate anti-tumor effects through its activity on T cells. In nude mice and in situations where CD8⁺ T cells have been eliminated (in vivo) by antibody depletion, the anti-tumor effect of IL-12 is significantly reduced (Brunda et al., 1993). This suggests that some of the anti-tumor effects elicited by IL-12 involve CD8⁺ T cells, likely CD8⁺ CTL.

Many groups have initiated clinical trials to evaluate the use of IL-12 as an anti-cancer agent based on the exciting results from animal models. The first trial was initiated by Genetics Institute in association with other research groups (Lotze et al., 1996) as a Phase I dose escalation study. This work showed few side effects which were only evident at the 1,000 ng/kg high dose. In comparison, the Phase II trial which started at 500ng/kg demonstrated serious side effects which eventually led to the temporary termination of this study (Lotze et al., 1996). Side effects associated with IL-12 systemic delivery are anemia, splenomegaly, leucopenia and thrombocytopenia (Tare et al., 1995; Gately et al., 1994; Sarmiento et al., 1994). The toxicity of IL-12 has also been observed by other investigators (Cohen, 1995; Marshall, 1995; Del Vecchio et al.,

1996). These observations forced researchers to evaluate other approaches of IL-12 delivery.

The toxicity induced by high levels of IL-12 prompted the development of similar approaches to those described for IL-2 in order to obtain high local levels of IL-12. Genetic modification of tumor cells is an effective way to obtain high levels of IL-12 within the tumor environment while reducing the toxicity associated with systemic delivery of this cytokine (Colombo et al., 1996; Martinotti et al., 1995; Rodolfo et al., 1996). These reports demonstrate that locally produced IL-12 enhanced the anti-tumor activity significantly above that observed for the systemic administration of recombinant IL-12. In a similar manner to that for IL-2, a number of viral vectors expressing IL-12 were constructed and tested for their effectiveness at inducing anti-tumor responses (Zitvogel et al., 1994; Meko et al., 1995; Caruso et al., 1996; Bramson et al., 1996; Chen et al., 1997). In all cases, investigators observed greatly enhanced anti-tumor responses compared to tumor cells modified to express IL-12. Bramson and co-workers (1996) demonstrated that intra-tumoral injection of an adenoviral vector modified to express both the p35 and p40 subunits of IL-12 mediated regression and delayed tumor growth in 75 % of tumor bearing animals. In this adenocarcinoma model, a high proportion of totally regressed animals were resistant to tumor cell rechallenge. Similarly, other groups have demonstrated the efficacy of adenoviral delivered IL-12 (Chen et al., 1997; Caruso et al., 1996).

The use of recombinant adenoviral vectors in tumor immuno-therapy protocols to deliver immunomodulatory molecules is important. However, depending on the transgene used in the immuno-therapy protocol toxicity can be an issue which could hinder the use of these vector or reduce efficacy (Ad5 IL-2 and Ad5 IL-12). This thesis describes the development of vectors capable of increasing efficacy and reducing toxicity of IL-2 and IL-12.

Co-stimulation and T cell Activation

It is widely accepted that for T cells to become activated they require two signals (Bretscher and Cohn, 1970; Lafferty and Gill, 1993). Signal one is provided by the interaction of the T cell receptor (TCR) and its co-receptors (CD4 and CD8) with either MHC I or MHC II molecules presenting antigen (Hart et al., 1997; Boussiotis et al., 1996; Schultze et al., 1996; Guinan et al., 1994). Signal two is reliant on either exogenous IL-2 or the ligation of T cell surface molecules to provide co-stimulatory signals which augment the signal delivered by the TCR mediated antigen recognition event (Liu and Linsley, 1992; June et al., 1994). Interaction of the TCR complex with antigen:MHC complexes initiates a state of readiness in the T cell but fails to induce proliferation and T cell effector function (Schwartz, 1990; Jenkins, 1992), and without the second signal results in anergy.

Co-stimulatory Molecules The B7:CD28/CTLA-4 Family

Lymphocytes express an 80-90 kDa molecule called CD28, which can be localized to 95% of resting CD4⁺ and 50% of CD8⁺ human peripheral blood human T cells (Turka et al., 1990). Most murine T cells appear to express CD28 (Gross et al., 1992). CD28 exhibits a relatively low affinity for binding its ligands (B7-1 and B7-2), nonetheless, is the most important receptor for T cell co-stimulation (Hart et al., 1997; Boussiotis et al., 1996; Schultze et al., 1996; Guinan et al., 1994). A second, high affinity, receptor called CTLA-4 is expressed on human and murine T cells (Brunet et al., 1987; Linsley et al., 1991b). CTLA-4 shares 31% homology with CD28 (at the amino acid level) and is expressed at high levels on activated but not resting T cells (Harper et al., 1991; Linsley et al., 1991b; Freeman et al., 1992; Lindsten et al., 1993). CTLA-4 is the inhibitory and high affinity receptor for T cells. The T cell receptors CD28 and CTLA-4 both interact with the B7 family of ligands (Brunet et al., 1987; Linsley et al., 1991b). B7-1 and B7-2 interactions with CD28 potentiates IL-2 production and proliferation while interaction with CTLA-4 inhibits proliferation.

The first identified ligand for CD28, B7-1 (CD80), was initially discovered on activated B cells (Freedman et al., 1987; Yokochi et al., 1982). Subsequent investigation has demonstrated the expression of B7-1 on monocytes (Freedman et al., 1991), activated T cells (Azuma et al., 1993; Friccius et al., 1993; Sansom et al., 1993; Yssel et al., 1993), dendritic cells (Larsen et al., 1994) and all other antigen presenting cells.

Anti-B7-1 monoclonal antibodies fail to have an effect in MLR reactions and B7-1 deficient mice respond normally to antigens (Lenschow et al., 1993; Freeman et al., 1993). These observations lead to the subsequent identification of a second CD28 ligand, B7-2. B7-2 (CD86) shares approximately 25% homology with B7-1 and also binds to CTLA-4 on T cells (reviewed in Boussiotis et al., 1996). The difference in the biological consequences resulting from co-stimulation by either B7-1 or B7-2 have not been clearly defined, although the literature is replete with observations that suggest differences between B7-1 and B7-2 (Hart et al., 1997; Boussiotis et al., 1996; Schultze et al., 1996; Guinan et al., 1994).

The biological activity of CD28:B7-1/B7-2 ligation is to induce proliferation and prevent anergy. Interaction of TCR with antigen in the context of MHC molecules results in the generation of intracellular signal by secondary messengers. This signal cascade is not enough to initiate proliferation. On a molecular level, the ligation of CD28 with either B7-1 or B7-2 results in the up-regulation the IL-2 receptor α , β and γ chains (Cerdan et al., 1992; Reiser et al., 1992; Cerdan et al., 1995; Freeman et al., 1995), increased IL-2 gene transcription (Lindsten et al., 1989; Thompson et al., 1989; Fraser et al., 1991; Fraser et al., 1992), up-regulation of CTLA-4 mRNA (Freeman et al., 1992; Lindsten et al., 1993), cytokine secretion and T cell proliferation (Gimmi et al., 1991; Linsley et al., 1991a; Freeman et al., 1993a, b).

Blockade of the CD28:B7 pathway inhibits humoral immunity (Linsley et al., 1992b), graft versus host disease (Blazar et al., 1994), graft rejection (Lenschow et al., 1992; Lin et al., 1993) and prevents autoimmune disease (Milich et al., 1994; Finck et al., 1994). It is therefore evident that since this pathway is extremely important for the generation of immune responses, it provides a potential point of manipulation of the immune response in clinical settings.

Co-stimulation in Cancer Therapy

A number of recent reports have demonstrated that supplying B7-1 or B7-2 to tumors can mediate rejection in immuno-competent hosts. Most human and murine tumors studied so far do not express detectable levels of B7-1 or B7-2 (Hershey et al., 1994; Chen et al., 1994), which may in part explain why potentially antigenic tumors survive in an immuno-competent organism. It follows that the delivery of B7-1 to these antigenic tumor cells might enhance the activation of tumor antigen specific T cells and mediate regression.

The murine B7-1 cDNA was transfected into K1735M2 murine melanoma cells which expresses the tumor rejection antigen human papilloma virus (HPV) E7 (Chen et al., 1991, 1992). The authors demonstrated that B7-1 transfected cells grew in nude mice but failed to grow in immuno-competent syngeneic recipient mice. The authors further demonstrated that the rejection of K1735M2 cells could be blocked by either CTLA-4Ig (soluble CTLA-4 chimeric molecule fused to the human C γ 1 constant region

of the IgG immunoglobulin molecule) or an anti-CD8 depleting antibody, suggesting that B7-1 transfected tumor cells co-stimulated CD8⁺ effector T cells to eliminate the B7-1⁺ tumor cells (Chen et al., 1992). B7-1 transfected tumor cells not only initiated a CD8⁺ response but were shown to be also capable at eliciting a CD4⁺ T cells response. The CD4⁺ response in this study is probably due to the processing and presentation of tumor associated antigens by APCs at the site of tumor cell injection. Baskar and co-workers (1993) co-transfected an MHC II gene along with B7-1 into a mouse sarcoma cell line and demonstrated that these cells induced a strong CD4⁺ T cell response that was capable of rejecting unmodified tumor cells upon re-challenge. Similar results were observed by Marti et al., (1997) using a recombinant vaccinia virus expressing B7-1 and MHC II molecules. These experiments were among the first to suggest the potential for using co-stimulatory molecules to augment an anti-tumor response. However, Ramarathinam and co-workers (1994) demonstrated that the introduction of B7-1 into the murine plasmacytoma cell line J588 induced drastic tumor cell rejection in syngeneic recipients but offered no systemic protection against the untransduced J588 tumor cells. One explanation for this finding is that NK cells are present which are capable of killing only B7-1 transfected tumor cells. The observations presented thus far deal with the consequences of transfecting B7-1 into tumors which are immunogenic.

Chen and colleagues (1994) looked at the consequences of transfecting B7-1 into murine tumor cell lines of varying immunogenicity. Highly immunogenic tumors K1735 (expressing the HPV E6 gene) and RMA lymphoma were compared to tumors of low immunogenicity (the P815 mastocytoma and the EL4 lymphoma) and four non-immunogenic tumor lines (three sarcomas MCA 101, MCA 102, Ag 104 and the melanoma B16). Interestingly, the four immunogenic or low immunogenic tumor cells transfected with B7-1 were rejected, which would not have been rejected without B7-1. In contrast, the B7-1 transfected non-immunogenic tumor cells were not rejected in immuno-competent or nude mice. These results are important since they clearly demonstrate that the effectiveness of B7-1 transfection is limited to immunogenic tumors. Townsend et al. (1994) found similar results with B7-1 expressing tumors of varying degrees of immunogenicity.

The co-stimulatory activity B7-2 was also shown to induce rejection of tumors. B7-2 transduction of MC38 colon carcinoma cells (via a B7-2 vaccinia vector) increased the immunogenicity and rejection of these tumor cells and protected against uninfected MC38 tumor cells (Hodge et al.,1994). Similar to the work of Chen et al. (1994) Yang and co-workers (1995) demonstrated that the transfection of B7-2 into a number of tumor cell lines of varying immunogenicity resulted in rejection of only immunogenic tumors. These findings are the first reports to demonstrate the efficacy and limitation of co-stimulated tumor therapy. Interest has now focused on combinations of co-

stimulatory molecules with immuno-modulatory cytokines in hopes of boosting the anti-tumor immune response.

Cytokine and B7-1/B7-2 Immunotherapy

The ability of the B7 family of co-stimulatory molecules to augment the activity of cytokines of known anti-tumor effects has been extensively studied. There is controversy with respect to the relative functional biological activities of B7-1 and B7-2 (Boussiotis et al., 1996; Schultze et al., 1996). In some reports these molecules have been shown to induce the production of different cytokine profiles (Freeman, et al., 1995; Lenchow et al., 1995; Kuchroo et al., 1995), however, a number of studies have not shown such differences (Lanier et al., 1995; Levine et al., 1995; Schweitzer et al., 1997). Chong et al., (1996) demonstrated that the expression of B7-2 in CMT93 colorectal tumor cells resulted in an increase in the immunogenicity of these cells similar to the effects observed with B7-1 (Chong et al., 1998). Interestingly, with matched expression levels, the B7-2 expressing tumor cells were much more efficient at inducing a protective immunity than the B7-1 expressing tumor cells (Chong et al., 1996). However, when B7-1 and B7-2 have been directly compared their anti-tumor effects have been equal in some cases (Yang et al., 1995), B7-1 dominant in some cases (Gajewski et al., 1996; Matulonis et al., 1996) or B7-2 dominant in other cases (Martin-

Fontecha et al., 1996). The functional differences between B7-1 and B7-2 have not been determined. However, it is clear that these molecules can augment the activity of some cytokines to facilitate anti-tumor responses.

To date the most potent anti-tumor effects of expressing B7 in combination with a cytokine has been observed for B7-1 and IL-12 expressing systems. This combination is by far the most potent combination in a large and varied number of pre-clinical trials. The initial observations on the potency of this combination was made by Kubin et al. (1994) and Murphy et al. (1994). These authors demonstrated the synergy between B7 and IL-12 at inducing proliferation and cytokine production in human and murine T cells. B7-1 transfected tumor cells co-injected with IL-12 secreting tumor or fibroblast cells elicited potent anti-tumor responses (Zitvogel et al., 1996). The administration of rIL-12 systemically to syngeneic tumor bearing mice resulted in the generation of a potent anti-tumor response (Coughlin et al., 1995) and systemic immunity. Chong et al., (1998) demonstrated the anti-tumor effects of combining B7-1 and B7-2 with IL-12 or GM-CSF in the CMT93 and K1735 tumor models. Similarly, Chen et al. (1996) and Parney et al. (1997) also demonstrated the ability of B7-1 and B7-2 to augment the anti-tumor effects of IL-12 and GM-CSF. Rao et al. (1996) used a vaccinia virus expressing B7-1 to augment the anti-tumor response of rIL-12. Cayeux and colleagues (1997) demonstrated the ability of B7-1 and IL-2 co-expressing tumor cells to be rapidly rejected in immuno-competent syngeneic recipients. Salvadori et al., (1995) also

demonstrated similar findings with tumor cells expressing B7-1 and IL-2. Gaken et al. (1997) used NC adenocarcinoma cells transduced with a retrovirus constructed to express B7-1 and IL-2 to demonstrate the potent anti-tumor effects of combining these molecules. Mice that rejected the modified tumor cells were protected from NC tumor cell challenge. B7-1 has also been shown to augment the anti-tumor effects of IL-4 and IL-7 (Cayeux et al., 1995, 1996). Gajewski et al. (1995) combined B7-1, IL-12 and IL-6 and demonstrated the drastic enhancement of anti-tumor responses in the P815 mastocytoma model.

The work presented above deals with the *ex vivo* modification of tumor or fibroblast cells and the subsequent administration of these transduced cells back into the animals. Recently, two publications dealing with the direct intratumoral injection of adenoviral vectors constructed to express B7-1 and either IL-12 or IL-2 into immunocompetent mice have demonstrated the potential of these combinations to induce regression in tumor bearing animals. The combination of B7-1 and IL-12 or IL-2 both resulted in the generation of potent anti-tumor responses in a model of polyoma middle T induced adenocarcinoma (Putzer et al., 1997; Emtage et al., 1998). Both models resulted in the induction of systemic immunity against re-challenge with tumor cells. The findings presented here suggest that the B7 family of co-stimulatory molecules can be used in therapeutic approaches to augment anti-tumor responses either alone or in combination with immuno-modulatory cytokines.

Chemokines

Chemokines are chemotactic cytokines that mediate inflammation. To date over 40 chemokines have been identified, many in the last 7 years. These molecules were first characterized for their ability to attract leukocytes (Schall, 1994; Baggiolini et al., 1994; Schall and Bacon, 1994; Haelens et al., 1996; Horuk and Peiper, 1997; Rollins, 1997; Luster, 1998). Chemokines range in size from about 8 to 20 kd and are approximately 20-70 % homologous at the amino acid level. They are categorized into four main families, distinguished by the spacing of critical cysteine residues near the amino-terminus of the molecule (Baggiolini et al., 1994; Baggiolini et al., 1997). The CXC (X refers to an intervening residue in the amino acid sequence adjacent to the first cysteine) chemokines and CC chemokines comprise the largest families, with the C and CXXXC families having only one member each. The chemokine most relevant to this work is lymphotactin (lym) and will be discussed in detail.

Lymphotactin

The CXC and CC chemokines have been shown to predominantly attract neutrophils and monocytes respectively (Schall, 1994; Baggiolini et al., 1994; Schall and Bacon, 1994; Haelens et al., 1996; Horuk and Peiper, 1997; Rollins, 1997; Luster, 1998). The identification of the chemokine lymphotactin prompted the addition of the γ or C subfamily (Kelner et al., 1994; Yoshida et al., 1995). Lymphotactin is characterized as having only two of the four cysteines (cysteines 2 and 4) found in the

CXC, CC or CXXXC subfamilies (Kelner et al., 1994). Another distinguishing feature of this novel chemokine is its location on chromosome 1 in both mice and humans (Kelner et al., 1994; Yoshida et al., 1995; Muller et al., 1995; Kennedy et al., 1995). For comparison, the CC chemokines are clustered on chromosome 11 in mice (chromosome 17 humans) and the CXC chemokines on chromosome 5 in mice and chromosome 4 in humans (reviewed in Rollins, 1997; Baggiolini et al., 1997; Luster, 1998). Current *in vitro* data shows that lymphotactin is a potent chemotactic factor for human peripheral blood lymphocytes (PBL) (Kennedy et al., 1995) CD4⁺ and CD8⁺ cells, murine CD4⁺ and CD8⁺ thymocytes and splenocytes (Kelner et al., 1994), and on murine and human NK cells (Hedrick et al., 1997; Giancarlo et al., 1996).

Expression of Lymphotactin

Lymphotactin expression has been demonstrated by Northern blot analysis in the spleen and thymus of mice at very low levels (Kelner et al., 1994). In contrast, it appears that lymphotactin expression in human tissues is more widespread since it can be localized to thymus, spleen, small intestine, lung, colon, ovary, testis and PBL (Yoshida et al., 1994; Muller et al., 1995; Kennedy et al., 1995). The expression of lymphotactin has also been demonstrated in specific activated immune cells, predominantly in CD8⁺ cells, CD4⁺ NK.1.1 T cells, $\alpha\beta^+$ CD4⁻ CD8⁻ T cells, $\gamma\delta^+$ intraepithelial T cells, dendritic epidermal T cells, natural killer cells and mast cells (Kelner et al., 1994; Kennedy et al., 1995; Muller et al., 1995; Boismenu et al., 1996; Dorner et al., 1997; Hedrick et al., 1997;

Rumsaeng et al, 1997; Maghazachi et al, 1997). Interestingly, sequence analysis of full length human lymphotactin mRNA demonstrates the presence of many polyadenylation sites suggesting that multiple mRNAs might exist, the consequence of which is unknown (Yoshida et al., 1995; Muller et al., 1995; Kennedy et al., 1995). In contrast, there appears to be only one transcript in mice (Kelner and Zlotnik, 1995).

Two observations make lymphotactin an interesting molecule for further study in the cancer therapy arena. First, the identification of lymphotactin as a T and NK cell chemotactic factor suggests that it might be possible to attract potential anti-tumor effector cells to the tumor site. Secondly, the demonstration that the expression of lymphotactin is specific to activated MHC I restricted CD8⁺ T cells, TCR $\alpha\beta$ ⁺ CD4⁻ CD8⁻ T cells and CD4⁺ NK 1.1⁺ T cells as well as NK cells, intraepithelial $\gamma\delta$ T cells and pro-T cells suggests that lymphotactin plays a very important role in the early stages of the inflammatory response. In an attempt to examine the anti-tumor effects of lymphotactin, Dilloo et al. (1996) used recombinant retroviral vectors expressing lymphotactin or IL-2 to transduce fibroblasts. These transduced fibroblasts were then mixed with A20 tumor cells and transplanted into syngeneic recipients either alone or in combination. The authors were able to demonstrate the ability of lymphotactin in combination with IL-2 to slow tumors growth in mice, but were unable to demonstrate any significant potential for complete tumor regression. It therefore appears that lymphotactin might exert anti-tumor activity. The rationale for using lymphotactin in the

studies presented herein are based solely on this factor's ability to be chemotactic for potential anti-tumor effector populations.

Chemokines in Tumor Immunotherapy

Laning and co-workers (1994) were the first to describe the use of a chemotactic molecule in a pre-clinical tumor immunotherapy protocol. Murine myeloma tumor cell lines were generated to express the chemokine TCA3 (a neutrophil and monocyte chemotactic factor) and then transplanted and monitored for growth in syngeneic recipients. The authors demonstrate that TCA3 expression led to tumor rejection in some animals which was accompanied with long lasting protection. Mule and colleagues (1996) transfected the cDNA for RANTES into a murine fibrosarcoma cell line. Mice were then inoculated with these tumor cells to observe the kinetics of RANTES anti-tumor activity. Modification of the tumor cells with this chemokine resulted in a CD8⁺ mediated rejection of tumor formation. In a similar protocol Huang et al. (1994) looked for immune relevant activity of MCP-1 on metastatic murine colon carcinoma CT-26 cells. The authors demonstrate that MCP-1 transfected tumor cells were rejected by macrophages. Dilloo and co-workers (1996) looked at the ability of lymphotactin to induce rejection of A20 tumors in mice. The authors demonstrate that lymphotactin alone or in combination with IL-2 or GM-CSF was able to reduce (but not regress) the growth of A20 tumors. These data clearly show that chemokines can be

used to modify the growth of tumors in pre-clinical models. Clinical studies of chemokines for tumor immunotherapy have not yet been reported.

Gene Therapy

The introduction of new genes or the replacement of mutated genes *in vivo* has been seen as an attainable goal since the early days of recombinant DNA technology. This concept was first proposed by Friedmann and Roblin in 1972, and resulted in the development of a new scientific discipline termed "Gene Therapy". Recent advances in the field of genetic engineering and cloning have led to the development of systems for the over-expression of therapeutic genes for the treatment of genetic diseases. The rapid progress of molecular biology towards gene identification and function, as well as the use of vectors of both a viral and non-viral nature, have resulted in the design of human cancer gene therapy protocols.

Gene Delivery Vectors

A necessary aspect in the development of *in vivo* gene delivery systems is the safe and efficient transduction of appropriate target cells. To realize this goal research has focused primarily on viral delivery systems which possess the ability to infect human cells. An important function of some viral vectors is their capability to exhibit tropism for particular cells affording the investigator the ability to target molecules to precise cellular compartments. Viral vectors currently under development or in use for *ex vivo* or *in vivo* gene transfer are derived from adenoviruses, murine leukemia

retroviruses, vaccinia viruses and adeno-associated viruses (Rosenfeld et al., 1991; Mastrangeli et al., 1993; Engelhardt et al., 1993; Uckert and Walther, 1994; Jolly, 1994; Moss and Flexner, 1987). Of these systems, adenoviral vectors are the most promising for *in vivo* cancer therapy for many reasons, most important of which are the non-integrating and infectious nature of these vectors (reviewed in Bramson et al., 1996; Hitt et al., 1997). Another advantage of adenoviral vectors in cancer therapy is the transient nature of expression observed both *in vivo* and *in vitro*. This is important since the expression of many immunomodulatory molecules exhibit severe side effects when expressed for prolonged periods. Vector clearance is mediated by CD8⁺ cytotoxic T lymphocyte (CTL) that eliminate virus-infected cells. The anti-virus CTL activity might provide an adjuvant effect which can augment the anti-tumor response by inducing other immune-regulatory molecules.

Adenovirus Structure and Life Cycle

Adenoviruses (Ad) are non-enveloped linear double stranded DNA viruses that have been shown to infect many mammalian species. The first isolation and identification of an adenovirus was made by Rowe et al. (1953). There are currently over 100 serotypes and more than half of these can be found in humans (Hierholzer et al., 1988). The Ad genome is approximately 40 kilobase pairs (kb) with inverted repeats at the ends of the double stranded genome. The viral genome is packaged into

an icosahedral capsid which is composed mainly of virally derived fiber, penton base and hexon proteins (Fields, 1996).

Adenoviruses infect cells via the interactions of the fiber protein with specific cell surface receptors on the surface of target cells (Svensson, 1985; Bergelson et al., 1997). After the viral DNA enters the nucleus it remains episomal where viral transcription is carried out by both host and viral factors. The first genes to be expressed are the early region genes: E1A, E1B, E2, E3 and E4 (reviewed in Nevins, 1987). These early region proteins are essential for viral persistence (dampening of the immune response against virus infected cells) and viral DNA replication (Fields, 1996). Shortly after viral DNA replication, the late region genes (L1-L5) are transcribed from the major late promoter (MLP) and encode virtually all structural capsid proteins (reviewed in Ginsberg, 1984; Field, 1996; Hitt et al., 1997).

Recombinant Adenovirus Vector Construction

There are currently two predominant adenoviral serotypes that are being used to construct recombinant vectors: Ad2 and Ad5 (Roberts et al., 1984; Chroboczek et al., 1992). The type 5 serotype is the more commonly used for gene expression. The E1 and E3 (E3 region is not required for virus propagation in vitro) regions of the Ad genome can be deleted for cloning of foreign DNA in first generation Ad vectors. Deletion of both the E1 and E3 regions allows for a cloning capacity of 8.3 kb (Bett et al., 1994). The construction of such Ad vectors generally requires the cloning of inserts

into shuttle plasmids to produce expression cassettes flanked by Ad sequences which are homologous to the region of the viral genome targeted for DNA insertion (Hitt et al., 1995). The recombinant shuttle plasmid is then co-transfected with the rescue plasmid which contains the bulk of the adenovirus genome and, hence, homologous sequences to those Ad sequences downstream of the expression cassette in the shuttle plasmid. These plasmids are co-transfected into 293 cells which express the E1 region in trans to provide the missing E1 proteins which are necessary for virus replication (Graham et al., 1977; Graham and Prevec, 1991; Bett et al., 1994; Hitt et al., 1995). Homologous recombination between the shuttle and rescue plasmids allows for rescue of the expression cassette into a recombinant adenoviral vector. E1 and E3 deleted adenoviral vectors must be propagated in 293 cells (since E1 must be provided in trans), a process which can result in viral titers up to 10^{12} plaque forming units/ml.

Uses of Recombinant Adenovirus Vectors

Some of the first adenoviral vectors expressed heterologous viral genes for use in anti-viral vaccines. cDNAs for viral antigens were placed in the E3 region of these vectors and used to demonstrate the generation of protective immune responses in various animal models (McDermott et al., 1989; Prevec et al., 1989; Dewar et al., 1989). Since the early Ad vectors retained the E1 region, they were able to replicate in some systems. This is unacceptable for potential human therapeutic protocols since it allows the release of recombinant vectors into the environment. The first generation of Ad

vectors mentioned above allow for replacement of the E1 region of the virus resulting in replication deficient vectors (Bett et al., 1994). This generation of vectors has resulted in the development of new gene delivery protocols for various *in vivo* therapies.

Transduction of various tissues has been demonstrated by numerous groups both in model systems and in clinical settings. Adenoviral vector transduction and vector derived expression have been demonstrated in hepatocytes, myoblasts, endothelial cells, airway epithelial cells, neurons, dendritic cells and in many other cells and tissues (Xing et al., 1994; Rosenfeld et al., 1991; Lemarchand et al., 1992; Morsy et al., 1993; Davidson et al., 1993; Stratford-Perricaudet et al., 1992; Bajocchi et al., 1993; Bramson et al., 1995; Wan et al., 1997). A large number of gene therapies involving adenoviral vectors have been undertaken. Since adenoviruses exhibit a natural tropism for the airway epithelium (and gut), Bajocchi and co-workers (1993) attempted to deliver the cystic fibrosis cDNA to the lungs of individuals with mutations in this membrane conductance protein in an attempt to repair the mutation by gene replacement. The authors were able to demonstrate expression of the transgene in the lungs of individuals receiving treatment. It is now well established that reduced expression of the human clotting factor VIII gene is caused by repression at the level of transcription which results in hemophilia A. Previous attempts to improve factor VIII gene expression by gene therapy have been unsuccessful. However, improved adenovirus-based vectors have been constructed that increase factor VIII expression in animal models. These

vectors have been shown to result in clinically relevant levels of human factor VIII in mice and hemophilic dogs and, therefore, hold promise for human clinical trials (reviewed by Fallaux and Hoeben, 1996). Within the last three years the number of pre-clinical or clinical trials using adenoviral vectors have increased significantly. This thesis will focus on the involvement of adenoviral vector in immunotherapeutic pre-clinical and clinical protocols of cancer therapy.

Adenoviral Vectors In Cancer Immunotherapy

The high transduction efficiency and wide variety of cellular targets for adenoviral infection along with the episomal properties of adenoviral DNA have made adenoviral vectors very attractive for cancer gene therapy. Haddada et al., (1993) assessed the tumorigenic capacity of the murine mastocytoma cell line P815 following IL-2 adenoviral mediated gene transfer. The authors demonstrate that 80% of syngeneic DBA/2 mice receiving Ad IL-2 modified P815 cells failed to develop tumors. Furthermore, animals that did not succumb to lethal dose administration of Ad IL-2 treated P815 cells were shown to exhibit long lasting immunity which was transferable to syngeneic naive recipients.

Nakamura and co-workers (1994) developed an adenoviral mediated adoptive immunotherapy model. The authors constructed and transduced murine T cells with an adenoviral vector expressing IL-2, and were able to achieve 100% gene transduction using a very high multiplicity of infection (MOI). Using cytotoxic T cells transduced

with the Ad IL-2 construct, the authors were able to protect and reduce the metastatic burden in tumor bearing mice.

The previous publications demonstrate the application of adenoviral vectors in the *ex vivo* modification of tumor cells. In an alternative protocol, Cordier and co-workers (1995), used direct administration of an adenovirus expressing IL-2 to P815 tumors. This direct intratumoral delivery of Ad IL-2 resulted in complete regression of P815 tumors in 75% of treated animals. Regressed animals were shown to be protected from challenge with tumor cells. In a similar type of experiments, Addison et al (1995) constructed a human IL-2 adenoviral vector which they injected directly (intra-tumorally) into solid tumors in a murine adenocarcinoma model of breast cancer. This construct was found to induce regression of established tumors and lead to protection from a secondary challenge with wild type tumor cells. In a very similar study using different tumor models (murine fibrosarcoma and mammary carcinomas), Toloza and co-workers (1996), also demonstrated the ability of an IL-2 expressing adenoviral vector to induce regression of established tumors by direct intra-tumoral injection. Addison and co-workers (1995) also demonstrated that an adenoviral vector expressing IL-4 had similar tumor regressing effects.

Bramson et al (1996a and 1996b) used the immuno-modulatory cytokine IL-12 in a mammary adenocarcinoma model and demonstrated the capability of this construct to mediate regression. The majority of mice regressed with this construct remained

tumor free and resistant to challenge with fresh tumor cells. Interestingly, 1 tumor in 11 were not protected from challenge. Caruso et al (1996), in a similar set of experiments, demonstrated the ability of an Ad IL-12 vector to regress MCA-26 colon carcinoma tumors.

The initial reports on adenoviral delivery of cytokine genes for tumor therapy fueled the explosion of reports on the use of many other cytokines. Abe et al (1995) compared the tumor regression capacity of an adenoviral vector expressing GM-CSF to that of a retroviral construct expressing GM-CSF. The authors used a vaccination protocol to compare the efficacies of tumor cells modified to secrete GM-CSF by either of the vectors mentioned above. The Ad GM-CSF vector was not as effective as the retroviral construct at inducing protection from wild type tumor cell challenge. A similar finding was noted for an Ad GM-CSF vector used to modify PyMT tumor cells in a murine adenocarcinoma model (Wan personal communication). Zhang and co-workers (1996) demonstrated the ability of an Ad IFN γ vector to induce complete regression in a nude mouse model of human breast cancer. Abe et al (1996) also demonstrated the ability of Ad IFN γ to augment anti-tumor activity when CD8⁺ CTL were transduced with this vector and delivered to tumor bearing animals.

Other immunomodulatory molecules that can augment the immune response against tumors have also been used in the adenoviral system. La Motte et al (1996) used direct injection of an adenoviral vector expressing the co-stimulatory molecule B7-2 to

demonstrate the efficacy of regression of established P815 tumors in the peritoneal cavity of DBA/2 mice. The most important molecules that give the adaptive immune system its specificity are MHC I and II antigens. The ability of immune effector cells to recognize and respond to target cells is dependent on the ability of target cells (tumor cells in this case) to express MHC I or II associated tumor antigens. Recently, adenoviral vectors which express tumor associated antigens (TAAs) have improved the efficacy of adenoviral vector tumor therapy. Zhai and co-workers (1996) demonstrated the ability of an adenoviral vector constructed to express the melanoma associated antigen gp100 (Ad gp100) to induce rejection of B16 tumor growth in C57b/6 mice. Chen et al (1996) also demonstrated the potential of using TAAs in adenoviral vectors for tumor therapy. Using EL-4 cells stably transfected to express β -galactosidase (β -gal) this group used an Ad β -gal expressing vector to facilitate regression of EL-4 β -gal expressing tumors.

Immune molecules that aid in the effector function of CTLs have been used in adenoviral vectors to directly mediate cytotoxicity in the tumor mass of solid tumors. Arai et al., (1997) constructed an adenoviral vector expressing Fas ligand and demonstrated the ability of this construct to induce apoptosis in tumor cells. Although this finding is important this vector can not be used to induce immunological memory in most cases since effector cells of the immune system express high levels of the Fas receptor and would be killed by the transduced tumor. Marr and co-workers (1997)

utilized the potent pro-inflammatory cytokine TNF α (which is also capable of inducing apoptosis) to regress solid mammary adenocarcinoma tumors in FVB/N mice. Mice that exhibited complete regression were shown to be protected from subsequent challenge with fresh tumor cells

Another route that has been taken to treat tumors is the modification of antigen presenting cells (APC) to secrete cytokines or express TAAs. The rationale for this therapeutic protocol is based on the function of APCs in the presentation of antigens to T helper and cytotoxic T cells for the initiation of the immune response. Feldman et al (1997) used an Ad IFN γ to infect CD34⁺ myeloid stem cells, and showed the highly production of IFN γ from these cells after exposure to 120 pfu of Ad IFN γ . Wan and co-workers (1997), modified APCs by tumor antigen gene transfer in order to load the MHC I or II antigens on the APC population with a tumor specific antigen and, hence, augment antigen presentation and effector cell generation for the initiation of anti-tumor immunity. Bone marrow derived dendritic cells were infected with an Ad encoding the polyoma middle T antigen (PyMT) and then used to vaccinate mice. The group found that all animals treated with TAA modified dendritic cells were protected from challenge with PyMT tumor cells.

Although various protocols for adenoviral mediated delivery of immunomodulatory molecules have proved beneficial to our understanding of immune-regulation and function, the anti-tumor activity of single cytokine vectors in many cases

has been disappointingly low. Recently, in an effort to augment the efficacy of single cytokine adenoviral vectors, a series of recombinant adenoviral vectors were constructed to express the co-stimulatory molecule B7-1 with IL-12 (Putzer et al., 1997). The authors demonstrated the significant augmentation of tumorigenicity using these double recombinant Ad vectors in a murine mammary adenocarcinoma model.

It is therefore clear that adenoviral vectors are capable of delivering the coding information for a diverse group of molecules for the immuno-therapy of cancer. The transient nature of the adenoviral system makes this vector an important tool in cancer therapy protocols. The ability of this system to express multiple molecules will undoubtedly play an important role in gene therapies in general.

Project Outline and Objectives

Current protocols for the treatment of cancer rely on surgical methods, Xray/radiation techniques and chemotherapy. However, the efficacy observed with these various protocols is limited, and in many cases serve only to prolong the life of a tumor bearing host rather than provide a cure. This aspect of current anti-cancer protocols has led to the development of new strategies for the treatment of cancer. The area of cancer immuno-therapy is based on observations which suggest that some immune regulating molecules can be used to boost an immune response and generate immunological memory. Early immuno-therapeutic protocols in our group utilized adenoviral vectors expressing key immune modulatory cytokines like IL-2 and IL-12 to

potentiate anti-tumor responses by delivering high levels of cytokine to the local tumor environment. These cytokines however, were found to exhibit some toxicity at levels which were required for significant therapeutic efficacy. In this thesis our goals were to reduce systemic toxicities associated with high levels of IL-2 and IL-12 and increase efficacy by combining either of these cytokines with other immuno-modulatory molecules in single adenoviral vectors.

In pursuit of this goal we, postulated that combinations of IL-2 with either the co-stimulatory molecule B7-1 or B7-2 would co-operate to increase efficacy and reduce toxicity and used double recombinant adenoviral vectors expressing the co-stimulatory molecules B7-1 or B7-2 with IL-2, to determine the ability of these molecules at generating additive or synergistic effects and reducing IL-2 associated toxicity. Co-stimulatory molecules are required for the proper biological activation of T cells. Specifically we investigated the *in vivo* effects of over-expressing either B7-1 or B7-2 with IL-2 on tumor regression in our two transgenic adenocarcinoma models after intratumoral injection. In this aspect of my thesis we addressed the expression characteristics of B7-1, B7-2 and IL-2 from single and double recombinant adenoviral vectors. We also determined the mechanism of anti-tumor activity in completely regressed animals by performing cytotoxic T lymphocyte assays. For a more stringent assessment of the ability of these constructs to mediate regression in our models we

studied the activity of the various vectors at inducing regression of metastatic pulmonary tumor nodules.

We also attempted to increase the efficacy of IL-2 and IL-12 while reducing their associated toxicities, using combinations of IL-2 and IL-12 with the T and NK cell chemotactic factor lymphotactin. Our postulate was that enhanced accumulation of lymphocytes to the local tumor environment in the presence of IL-2 or IL-12 would enhance anti-tumor immunity. Prior to this study we used a recombinant adenoviral vector expressing lymphotactin (Ad5 mLym) to determine the *in vivo* effects of this chemokine on cell infiltration in the lungs of mice and rats. Specifically, we administered Ad5 mLym to the lungs of mice and rats and examined lung cells over a period of several days. Cell numbers and phenotype were assessed by differential cell counting from the bronchial lavage fluid of Ad5 Lym infected animals. We focused on the ability of lymphotactin to recruit T and NK cells to the lungs and on the phenotype of the infiltrating lymphocytes. This information allowed us to initiate experimental tumor models.

We used recombinant adenoviral vectors expressing a combination of lymphotactin and IL-2 or lymphotactin and IL-12 to determine the ability of these molecules to generate additive or synergistic anti-tumor effects and reduce IL-2 or IL-12 associated toxicity. The levels of cytokine production were assessed *in vitro* on infected cells. Intra-tumoral administration of recombinant vectors was performed in order to

observe augmented IL-2 or IL-12 activity as a result of the addition of immunomodulatory molecules. All completely regressed animals were challenged with fresh tumor cells to determine the induction of immunological memory. Regressed and rechallenged animals were sacrificed and their spleens removed to determine the incidence of antigen specific cytotoxic T lymphocytes in Ad5 mB7-1/hIL-2 treated animals. All other CTL data in this thesis was collected from animals after the initial regression rather than after rechallenge.

Chapter 2

**The Use of Recombinant Adenoviruses Expressing the Co-stimulatory B7-1 in
Combination with IL-2 to Augment
IL-2 Anti-tumor Activity.**

The following article, entitled "A double recombinant adenovirus expressing the co-stimulatory molecule B7-1 (murine) and human interleukin-2 induces complete tumor regression in a murine breast adenocarcinoma model", is published in the Journal of Immunology. 160: 2531-2538, 1998. This publication describes the first use of a double recombinant adenoviral vectors constructed to express B7-1 and IL-2 in a transgenic murine adenocarcinoma model.

The work presented in this study was performed by the author of the thesis. Dr. Wan demonstrated the process of establishing tumors in mice and Dr. Bramson provided assistance in performing the CTL assays. This research paper was written by the author of the thesis. The vector system originated in the lab of Dr. Graham and supervision was provided by Dr. Jack Gauldie resulting in the multiple authorship of this paper.

Running Title:

Complete regression using an Ad expressing mB7-1 and hIL-2

Full Title:

A double recombinant adenovirus expressing the co-stimulatory molecule B7-1 (murine) and human interleukin-2 induces complete tumor regression in a murine breast adenocarcinoma model¹

Authors:

Peter C. R. Emtage^{*§}, Yonghong Wan^{*}, Jonathan L. Bramson^{**#}, Frank L. Graham^{**¶}
and Jack Gauldie^{**‡}

^{*}Department of Pathology, McMaster University, Hamilton, Ontario

[†]Department of Biology, McMaster University, Hamilton, Ontario

[‡]Address correspondence and reprint requests to Dr. Jack Gauldie, Department of Pathology, 1200 Main St. W., Hamilton, Ontario Canada L8N-3Z5.

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[¶]F. L. G. is a Terry Fox Research Scientist of the NCIC (Canada).

[#] J. L. B. was supported by a MRC fellowship.

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Abstract:

Tumors that express tumor specific antigens can maintain growth in an immunocompetent organism. Current hypotheses tend toward T cell anergy as a key component for the inhibition of immuno-reactivity against such tumors. Anergy is thought to occur from hyperactive stimulation of the T cell receptor (TCR) in the absence of co-stimulation (co-stimulation leads to proliferation via interleukin 2 production). Subcutaneous injection of transgenic Polyoma Middle T transformed breast adenocarcinoma tumor cells (PyMT) in the hind flank of FVB/n mice results in the formation of tumor nodules at this site. We determined the MHC class I, II, B7-1 and B7-2 expression in the tumor cells by flow cytometry and showed positive staining for only MHC class I. We show that a single E1 deleted adenovirus constructed to express both the co-stimulatory molecule B7-1 (murine) and human IL-2 genes (Ad5E1 mB7-1/hIL-2) elicits a very potent anti tumor response when administered intratumorally. Ad5E1 mB7-1/hIL-2 induced rapid and complete regression (100%) of all tumors compared to Ad5 E1 mB7-1 (38%), Ad CAIL-2 (42%) and Ad5E1 dl70-3 (control vector) (0%). All mice that exhibited complete tumor regression were fully protected in tumor cell challenge experiments. The systemic immunity generated by intratumoral administration of the Ad vectors was found to be associated with a strong anti PyMT CTL response. These observations indicate that augmenting the immunogenicity of the

tumor with coincident expression of B7-1 in combination with IL-2 may prove beneficial in direct tumor immunotherapy.

Introduction

Current understanding as to how tumors escape immunesurveillance involves T cell anergy as a possible mechanism in models of tumorigenesis. Many tumors express antigenic determinants that are tumor specific or are at least more prevalent in neoplastic tissue (1). Rejection of these tumors is mediated predominately by T lymphocytes (2,3) and the primary T cell activation signal is delivered via the interaction with the T cell receptor (TCR)² of the specific antigenic peptide in association with major histocompatibility molecules (MHC) I or II (4). Normally, activation involves members of the B7 family of co-stimulatory molecules, as the primary antigen signal is not sufficient to elicit the activation response. Anergic T cells, while capable of recognizing the antigenic epitope(s) on tumor cells, fail to eliminate these cells and consequently do not prevent tumor growth.

The first identified member of the B7 co-stimulatory family of molecules was B7-1 (CD80). Molecular characterization of B7-1 has shown that the molecule is a 44-54 kDa member of the immunoglobulin (Ig) superfamily and is the ligand for CD28 and CTLA-4 counter receptors on T cells (5, 6, 7, 8). B7-1 was first described as an activated B cell marker (9, 10), however, B7-1 expression has since been localized to other antigen presenting cells (APC), including dendritic cells, monocytes and macrophages. The interaction of B7-1 with CD28 is vital for the amplification and generation of signals necessary for antigen specific T cell responses and effector

functions (11, 12, 13, 14). B7-1 and CD28 interactions have been shown to contribute to T helper (Th) cell activation and function. In addition, B7-1 has been implicated as a necessary requirement for the generation of CD8⁺ cytotoxic T lymphocytes (CTL) in the absence of help from CD4⁺ T cells (15, 16). Inhibiting the interaction of B7-1 with CD28 while allowing antigen specific interaction can result in T cell anergy, likely by the lack of suitable autocrine growth factor, interleukin 2 (IL-2) production (as reviewed in 17). Anergic tumor infiltrating lymphocytes (TILs) have been demonstrated in some tumors. The use of rIL-2 *in vitro* to overcome anergy, has shown that TILs can recognize tumor specific antigens, and be activated by appropriate signals to develop cytotoxicity (18).

Recently, a number of studies have demonstrated potential for the use of B7 family of co-stimulators in tumor immunotherapy. The expression of B7 family members in murine tumor models, has been shown to activate CD8⁺ T cells and or CD4⁺ T cells against the respective tumor cells (6, 19, 20, 21, 22, 16, 23, 24, 25, 26, 27). Moreover, administration of IL-2 has been shown to promote antitumor immunity presumably by alleviating the anergic block seen in T cells in some tumor models and thereby preventing the onset of anergy (28, 29). We have previously shown (30) that an adenovirus vector expressing human IL-2 administered intratumorally results in approximately 45% regression in a murine adenocarcinoma model. The potential for additive or synergistic effects from expression of both B7-1 and IL-2 prompted us to

investigate the antitumor effects of intratumoral injection of a single adenoviral vector constructed to express both B7-1 and IL-2 from the same cell.

In this study we demonstrate that intratumoral injection of an adenovirus vector (Ad) constructed to express murine B7-1 (Ad5 mB7-1) results in 38% complete regression of tumors while an Ad vector expressing IL-2 (Ad CAIL-2) (30) demonstrated complete regression in 42% of tumor bearing mice. In contrast, intratumoral administration of a dicistronic vector expressing both B7-1 and IL-2 (Ad5 mB7-1/hIL-2) resulted in complete regression in 100% of tumor bearing mice. Cured mice were shown to have generated systemic immunity to a subsequent challenge with fresh tumor cells and tumor specific cytotoxic T lymphocyte activity could be detected.

Materials and Methods

Animals

Six to eight week old FVB/n mice were purchased from Taconic Laboratories and housed in a pathogen free facility until use.

Cell Lines

Cell lines used include the following: A549,(ATCC# CCL-185); WM35, radial phase human melanoma (31); MRC5, human fibroblast cell line (ATCC# CCL-171); PyMT, primary polyoma middle T antigen transformed murine cells obtained from explanted tumors: PTO516, FVB/n kidney derived cells, 516MT3 cells are derived from PTO516 and are stably transformed to express polyoma middle T antigen; 293, human embryonic kidney cells transformed with adenoviral E1 sequences (32); 293N3S, contact independent 293 derivative (33). All cell culture reagents were purchased from GIBCO.

Construction of Ad5 mB7-1

Total RNA was isolated from FVB/n splenocytes using the reagent Trizol (GIBCO). RT PCR was performed using the First Strand cDNA synthesis Kit (GIBCO). Briefly, cDNA was synthesized using oligo(dT) as the primer. PCR was performed using Vent DNA polymerase (New England Biolabs) and the following parameters: denaturation was accomplished at 94⁰C for 1 minute; annealing 55⁰C for 30 seconds; extension at 72⁰C for 1 minute. This was done using the following sense and antisense primers designed to anneal to the 5' and 3' ends of the B7 cDNA sequence as published in

Genbank. The sense primer 5'- AAGATCTCTCCATTGGCTCTAGATTCCTGGC-3' and the antisense primer 5'-GAAAGATCTGATTGTACCTCATGAGCCACATAATA-3', were designed to include Bgl II restriction sites (underlined). The amplified fragment of 1023 base pairs was directly ligated into the Eco RV digested pDK6 shuttle plasmid (Kunskan D. S., M.Sc Thesis, McMaster University) to create pDK6-mB7-1 (Figure 1). The shuttle plasmid pDK6-mB7-1 was amplified and then purified by alkaline lysis and cesium chloride gradient centrifugation. Purified plasmid was then combined with the rescue plasmid pBHG10 (34) and cotransfected into 293 cell to produce Ad5 mB7-1.

Construction of Ad5 mB7-1/hIL-2

The shuttle plasmid containing mB7-1 (pDK6 mB7-1) was sequenced, amplified and then subjected to the following manipulation to generate a dicistronic construct expressing mB7-1 and hIL-2. The human IL-2 open reading frame was amplified using the following 5'-phosphorylated primers, 5'-TACAGGATGCAACTCCTGTCTTGC-3' (sense) and 5'-CTAATTATCAAGTCAGTGTGA-3' (antisense). It should be noted that the sense primer is constructed such that the codon underlined (TAC) is the first amino acid immediately after the ATG start codon. The PCR product of 469 bp was then blunt end ligated into the pCITE 2a plasmid (Novagen) which carries the Encephalomyocarditis virus internal ribosomal entry site (IRES). This plasmid was digested with Nco I (recognition site CCATGG) and subsequently blunt ended (blunting

was performed with Klenow polymerase large fragment from NEB) to produce an ATG start codon, prior to ligation with the IL-2 PCR product. Ligation of the IL-2 PCR product with the pCITE 2a plasmid resulted in pCITE_hIL2 which carries the hIL-2 open reading frame driven by the IRES sequence.

The pCITE_hIL-2 plasmid was used as a template for a second round of PCR. Using the following phosphorylated primer, 5'-TTCCGGTATTTCACCATATTG-3' (IRES sense) and the original hIL-2 antisense primer, a PCR product of 979 bp was amplified corresponding to the IRES/hIL-2 hybrid molecule. This PCR product was blunt end ligated into pDK6 mB7-1 digested with Sal I (3' to the stop codon of mB7-1) to produce pDK6 mB7-1/IRES/hIL-2 (Figure 1). This plasmid was then processed in an identical manner to pDK6 mB7-1 (see Ad5 mB7-1) and cotransfected with pBHG10 to generate Ad5 mB7-1/hIL-2. Both Ad5 mB7-1 and Ad5 mB7-1/hIL-2 were screened using Southern and Northern techniques to characterize the presence of inserted DNA or the production of monocistronic (Ad5 mB7-1) or dicistronic mRNAs (Ad5 mB7-1/hIL-2). In the case of Ad5 mB7-1/hIL-2 Western analysis was performed to detect hIL-2 production. All cloning was confirmed by sequencing.

Ad CAIL-2 and Ad5 dl70-3

A description of these viruses can be found in Addison, 1995 (30).

Flow Cytometry

Flow cytometric analysis was performed on MRC5, A549, WM35 and PyMT cells to characterize mB7-1 expression from both mB7-1 expressing vectors. Cells were infected for 48 hr (MRC5, A549, WM35) prior to analysis and PyMT cells were infected and analyzed over 1-3 days. All groups were harvested at the specific times, washed in PBS and incubated with anti mB7-1 antibody. The PyMT group was also incubated with anti MHC I or II and anti B7-2 antibodies before flow cytometric analysis. Analysis was performed using a flow cytometer (Becton Dickinson).

hIL-2 Assay

MRC5, A549 and WM35 cells were infected at a multiplicity of infection (MOI) of 10 plaque forming units (pfu) per cell of either Ad5 CAIL-2 or Ad5 mB7-1/hIL-2. Infected cultures were incubated for 4 days in the case of MRC5 and WM35, and 5 days for A549 cells. At 24 hr intervals, 200 μ l aliquots were removed and stored at minus 70^oC for quantification. Secreted hIL-2 was quantitated using the hIL-2 DuoSet Kit (Genzyme Diagnostics Inc).

Tumor Cell Preparation and Vector Administration

A transgenic mouse strain (FVB/n) expressing the polyoma middle T (PyMT) antigen under the control of the mouse mammary tumor virus long terminal repeat was the source of the tumor cells used in this study (35). Expression of PyMT antigen results in the spontaneous transformation of the mammary epithelium by 8-10 weeks of age.

Tumors were excised from transgenic mice and subjected to enzymatic digestion to generate a single cell suspension (30). The single cell suspension was washed with PBS, and aliquots of 10^6 tumor cells were injected subcutaneously (SC) into the right hind flank of normal syngeneic FVB/n mice. Palpable tumors (normally 50-75 mm^3) arise in these recipients 21 days after initial at which time appropriate amounts of virus were injected in a volume of 40 μl . After injection of adenoviral vectors, tumors were monitored weekly using calipers. The volume of the tumor was calculated from the longest diameter and average width assuming a prolate spheroid. Mice with tumors not responding to vector treatment were sacrificed when the longest diameter exceeded 20 mm. Regressed mice were left for approximately 3 months and then challenged with 1×10^6 freshly isolated PyMT tumor cells on the left hind flank.

CTL assays

Splenocytes (effectors) were obtained from mice whose tumors had regressed as a result of Ad5 mB7-1, Ad CAIL-2 or Ad5 mB7-1/hIL-2 treatment and co-cultured with 516MT3 cells (stimulators) at a concentration of 1.2×10^5 516MT3 to 1.2×10^7 splenocytes for 5 days in 12 well dishes. Serial dilutions of the effector cells were incubated in a v-bottom 96-well plate with 5×10^3 516MT3 or PTO516 target cells. Target cells (10^6) were labeled with 100 μCi of ^{51}Cr sodium salt for 2 hr prior to co-culture with the effector cells. Cells were co-cultured for 5-6 hr at which time 80 μl of supernatant was removed for counting. The percent specific lysis was calculated as

follows: $100 \times (\text{experimental cpm} - \text{spontaneous cpm}) / (\text{maximal cpm} - \text{spontaneous cpm})$.

Results

Construction of Recombinant Adenoviral Vectors

Recombinant adenoviral vectors which express murine B7-1 (Ad5 mB7-1) or both mB7-1 and human IL-2 (Ad5 mB7-1/hIL-2) were constructed as outlined in figure 1. Expression cassettes for both mB7-1 or mB7-1/hIL-2 were inserted into the E1 deleted region of the human adenovirus type 5 genome. For both constructs, transgenes were flanked by the murine cytomegalovirus immediate-early promoter (mCMV) and a simian virus 40 polyadenylation signal (SV40). In the expression cassette for Ad5 mB7-1/hIL-2, the encephalomyocarditis virus internal ribosome entry site (IRES) (36) was placed between the mB7-1 and the hIL-2 coding sequences resulting in a dicistronic DNA fragment. The IRES functions as an internal ribosome initiation site for the translation of hIL-2 in the resulting dicistronic mRNA.

Expression of the mB7-1 gene and hIL-2 gene (Ad5 mB7-1 and Ad5 mB7-1/hIL-2) was confirmed in human and murine cell lines infected with each recombinant adenoviral vector. We initially decided to characterize freshly isolated PyMT cells for the endogenous expression of crucial immunological molecules. Single cell suspensions of PyMT tumor cells were checked by flow cytometry for surface expression of mB7-1, mB7-2, MHC I and II (Figure 2 A,B,C and D). No mB7-1 expression could be detected on uninfected PyMT tumor cells (Figure 2A). Since no detectable mB7-1 protein could be demonstrated on PyMT cells we proceeded to

determine if the Ad5 mB7-1 and Ad5 mB7-1/hIL-2 vectors could change the PyMT B7 negative phenotype to a mB7-1 positive phenotype. Figure 2E and 2F clearly demonstrate that mB7-1 protein was produced and integrated into the cell membranes of PyMT tumor cells infected with either Ad5 mB7-1 or Ad5 mB7-1/hIL-2. This result confirms that the expression of mB7-1 on PyMT tumor cells infected with both Ad5 mB7-1 or Ad5 mB7-1/hIL-2 vectors is due to the presence of the mB7-1 transgene (Figure 2E and 2F). WM35, A549 and MRC5 cells could also be converted from a mB7-1 negative phenotype to a positive phenotype after infection with the mB7-1 expressing vectors (data not shown).

hIL-2 Expression in Cells Infected with Ad5 mB7-1/hIL-2

The expression of hIL-2 from Ad5 mB7-1/hIL-2 was characterized by Western blot analysis. Supernatants from 293 cell cultures infected with Ad5 mB7-1/hIL-2 and for comparison Ad CAIL-2 were size fractionated by SDS PAGE and Western blotted with an anti-hIL-2 antibody, demonstrating the presence of two polypeptides of 15 and 17 kDa (data not shown). IL-2 from the dicistronic Ad5 mB7-1/hIL-2 and the monocistronic IL-2 control vector Ad CAIL-2 was quantified by ELISA (Figure 3A, B and C). IL-2 levels derived from the Ad5 mB7-1/hIL-2 vector were between 9 to 50 ng/ml compared to hIL-2 levels derived from Ad CAIL-2 which were between 50 to 625 ng/ml for infections with the same MOI. The expression observed per 1×10^6 cells with

Ad5 mB7-1/hIL-2 was approximately 13 fold lower than Ad CAIL-2 (Figure 3A, B and C).

Intratumoral Administration of Adenoviral Vectors

We have previously shown that intratumoral injection of adenoviral vectors expressing IL-2 or IL-12 to polyoma middle T tumor bearing mice cause regression at a dose of 5×10^8 pfu (30, 37). We used this same dose for Ad5 mB7-1, Ad5 mB7-1/hIL-2 and Ad CAIL-2 and compared the effects to those seen with the E1 deleted control vector, Ad5 dl70-3. Administration of control virus did not modify the progression of the polyoma middle T tumor growths in any of the control groups described here or in (30, 37). Conversely, treatment with Ad5 mB7-1 resulted in 38% (9/24 tumors) total regression of established tumors (Table I) and all of the remaining 62% of Ad5 mB7-1 treated tumors showed a pronounced growth retardation as seen in Table I. Ad CAIL-2 treated tumors demonstrated different growth or regression kinetics when compared to Ad5 mB7-1. While, 42% (10/24 tumors) of the Ad CAIL-2 treated mice demonstrated complete regression (Table I), 37% (9/24 tumors) demonstrated a partial growth delay (19/24 tumors or 79% overall responded) and 21% (5/24 tumors) showed no response to treatment. In contrast, administration of Ad5 mB7-1/hIL-2 caused complete regression of all (100%: 38/38) tumors treated suggesting at least additive if not synergistic effects between mB7-1 and hIL-2 expressed from the same cell (Table I). Ad5 dl70-3 treated tumors demonstrated no growth alteration to the vector, as a result these mice were

usually sacrificed between days 30 – 35 post tumor cell injection. All mice exhibiting complete regression, regardless of which vector was used, were tumor free for at least 110 days post injection.

Generation of Systemic Immunity to Polyoma Middle T Tumor Cells

Mice that had undergone complete regression were challenged with 1×10^6 freshly isolated polyoma middle T cells at day 110 post initial vector administration, on the left hind flank (see Materials and Methods). None of the challenged mice developed tumors and all remained tumor free for a further 120 days after challenge (Table II). To ensure tumor forming capacity of these cells, untreated syngeneic mice were included to observe the kinetics of tumor growth (Table II).

CTL Activity in Cured Mice

Two cured mice from each of the following treatment were sacrificed and their spleens removed 120 days after challenge. Splenocytes prepared from Ad5 mB7-1 treated mice (120 days after challenge) demonstrated a 13-23% specific lysis of 516MT3 cells at an effector to target ratio of 3.3:1 (Figure 4). Lysis of PT0516 (no polyoma middle T antigen) was also observed however at a much lower percentage. In contrast, splenocytes from Ad CAIL-2 treated mice (120 days after challenge) demonstrated 57 to 68% specific lysis of 516MT3 cells and undetectable levels of lysis on PT0516 cells (at effector to target ratios of 3.3:1) (Figure 4). Similarly, splenocytes from Ad5 mB7-1/hIL-2 treated mice (120 days after challenge) demonstrated 65 to 73% lysis of

516MT3 targets and undetectable levels of lysis on PT0516 cells (at effector to target ratios of 3.3:1) (Figure 4). When splenocytes from control mice were used, target cell killing was similar to or less than that seen for PyMT negative targets at all effector cell ratios. This suggest the presence of significant numbers of effector cells capable of killing PyMT transformed cells.

Discussion

Immunosurveillance by potential effector cells of the immune system is, in large part, responsible for the rejection of neoplastic cells. However, in individuals with seemingly intact immune systems neoplastic growths arise and are not rejected. This ability of some neoplastic growths to evade the immune system suggests that potentially antigenic tumors elicit specific effects which act to suppress the immune system. Conversely, tumors which do not express associated antigens are cryptic and, hence, offer no target(s) for immune rejection. Thus, understanding the mechanisms of tumor mediated immune-suppression or the parameters encompassing the efficient activation of immune-effector functions will allow for the development of new strategies in tumor immunotherapy.

Recent evidence has demonstrated the ability of certain cytokines to promote tumor rejection and, since the initial IL-2 study by Rosenberg et al. (38), cytokines such as IL-2, IL-4, IL-7 and IL-12 have been studied to assess their potential for mediating tumor rejection in various immunotherapy protocols. Most of these therapies have been limited by the appearance of toxic effects elicited by certain cytokines or the inability of the cytokines under investigation to induce efficient anti-tumor effector activity (39, 40, 41, 42). Tumor infiltrating lymphocytes (TILs) and lymphokine activated killer cells have also been used with only limited success (41, 40, 43). Evidence suggests that genetically modified tumor cells expressing IL-2 or IL-4 either by transfection or

retroviral integration can abrogate the ability of tumors to grow (44, 45, 46, 47). We have recently used adenoviral vectors to deliver cytokines such as IL-2, IL-4 and IL-12 intratumorally (30, 37, 48), and have shown that the transient expression of IL-2, IL-4 and IL-12 could augment the recognition of the tumor by the immune system and result in complete regression in a significant fraction of the tumors treated.

In contrast to the use of cytokine mediators, other tumor immunotherapy regimes have focused on the B7 family of costimulatory molecules. B7 family members are required for the transduction of signals which promote T cell activation via B7 ligation with T cell derived CD28 in the presence of MHC I or II Ag/TCR interactions (50-53). Transfection of tumor cells with B7-1 has been extensively used and demonstrated to result in only moderate effects on tumor regression (21, 24, 26, 54, 55). More pertinent to this work are the observations of a number of groups which show that B7-1 can be used to augment the activity of IL-12, IL-2 and IL-7 *in vivo* (56-60) by positively effecting tumor regression, possibly through interaction with adhesion molecules such as intercellular adhesion molecule-1 (61). Taken together these studies demonstrate that B7 modified tumor cells are capable of delivering, in conjunction with MHC I or II, Ag-specific activation signals to T cells. By far one of the more beneficial effects of B7 enhancement of tumorigenicity is the ability of B7 to directly activate naïve CD8⁺ cytotoxic T lymphocytes in the absence of CD4⁺ help (62).

It is evident from the current literature that the use of cytokines or co-stimulatory molecules in isolation to modulate the immune response against established tumors is of limited efficacy. The biological evidence for the activity of IL-2 and B7-1 on tumor rejection prompted us to examine augmentation of the immune response by supplying both B7-1 and IL-2 to the same cell *in vivo*, reasoning that this might enhance the ability of the immune system to recognize and react against established tumors.

We have demonstrated the efficiency of Ad vectors (Ad5 mB7-1 and Ad5 mB7-1/hIL-2) constructed to express the co-stimulatory molecule B7-1 (murine) at converting B7-1 negative PyMT tumor cells to a B7-1 positive phenotype (Figure 2A, 2E and 2F). We have also demonstrated the ability of the double recombinant vector (Ad5 mB7-1/hIL-2) to produce hIL-2 (Figure 3), however, hIL-2 production was at a level one log lower than that observed for the single vector expressing hIL-2 (Ad CAIL-2) (Figure 3) which we have previously used in this model (30).

Comparison of the effects of intratumoral injection of Ad5 mB7-1 or Ad CAIL-2 versus Ad5 mB7-1/hIL-2 demonstrated that the combination vector was much more effective at inducing complete regression in the PyMT model than either of the vectors expressing mB7-1 or IL-2 alone. Intratumoral administration of Ad5 mB7-1 demonstrated 37.5% total regression (9/24 mice) (Table I), with the other 62.5% (15/24 mice) showing a partial response characterized by a drastic reduction in tumor volume but subsequent relapse into a rapid growth phase (24/24 mice responded). Similar to our previous data

(30). Ad CAIL-2 injection resulted in 10/24 (or 42%) of animals demonstrating complete regression and 37% exhibiting partial reduction in tumor volume or a growth delay (19/24 mice responded), with an overall response of 79%. In contrast, Ad5 mB7-1/hIL-2 treated tumors resulted in complete regression of all tumors treated (100%). Another important observation demonstrated by Ad5 mB7-1/hIL-2 is the shorter time taken for PyMT tumors to regress completely. Consistently, all tumors regressed within 14 days of vector administration. On the other hand tumors undergoing complete regression by treatment with either Ad5 mB7-1 or Ad CAIL-2 took 19 to 27 days to completely regress.

To determine the ability of all vectors to induce protection from freshly isolated PyMT tumor cells, we challenged all completely regressed mice (from experiments 1, 2 and 3, Table I) with freshly isolated PyMT tumor cells 110 days after primary tumor injection. Tumor cell challenge was always administered on the opposite hind flank to the site of the regressed tumor. All mice which exhibited complete regression were found to be protected against challenge, demonstrating that all vectors were capable of generating long lasting systemic immunity (Table II).

To determine a possible mechanism and the specificity of the effector function generated during treatment with the vectors, we examined the cytotoxic T lymphocyte (CTL) activity in the spleen of each completely regressed animal (Figure 4 A, B and C). Ad5 mB7-1 treated animals demonstrated 13-23% specific lysis on 516MT3 polyoma

middle T expressing cell line at a ratio of 3.3:1 (effector:target ratio). This is low but, nevertheless, significant since the same spleen cells resulted in only 1% lysis of the parental line PTO516 that does not express polyoma middle T (Figure 4A). Conversion of the PyMT tumor from a B7-1⁻ to a B7-1⁺ phenotype could enhance the ability of NK cells to target the tumor cells via a mechanism similar to that observed by Yeh et al. (63) and Chambers et al. (64). Therefore, B7-1⁺ PyMT tumor cells could enhance not only MHC restricted effector cells but also non-MHC restricted NK cells. The conversion of PyMT cells from a B7-1⁻ to a B7-1⁺ population potentially provides an environment for the activation of at least two effector cell populations (NK and CD4⁺/CD8⁺) resulting in the 100% response observed for tumors treated with Ad5 mB7-1. Ad5 mB7-1/hIL-2 treatment results in high PyMT-specific CTL activity, 65-73% lysis on 516MT3, as opposed to undetectable lysis on non-specific targets at 3.3:1 effector to target ratio (Figure 4B) similar to that seen in Ad CAIL-2 treated mice that underwent total regression (Figure 4C). In contrast to the single vectors, Ad5 mB7-1/hIL-2 encompasses both effects mentioned above for Ad5 mB7-1 and Ad CAIL-2 into a single system capable of up-regulating IL-2 receptor levels and providing the autocrine activity of IL-2 directly at the site of CD28 (T-cell): mB7-1 (PyMT tumor cells) ligation. mB7-1 ligation with CD28 may act to provide the missing signal(s) necessary to reverse anergy and allow for a greater proliferative response of effector cells. We know that the hIL-2 levels produced by Ad5 mB7-1/hIL-2 are 13 fold lower than that observed for Ad

CAIL-2. Hence, we propose that hIL-2 and mB7-1 act synergistically to overcome anergy by providing a micro-environment maximally conducive to effector function and proliferation.

The findings of this study demonstrate the effectiveness of augmenting the immune response against tumors with adenoviral vectors expressing mB7-1 in combination with hIL-2 (Ad5 mB7-1/hIL-2). Future analyses will determine the effector population activated by the double construct and the effectiveness of this construct in the treatment of other established murine tumors.

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Legend

Figure 1. A schematic diagram of the construction of Ad5 mB7-1 and Ad5 mB7-1/hIL-2. For a more detailed vector construction description see Materials and Methods.

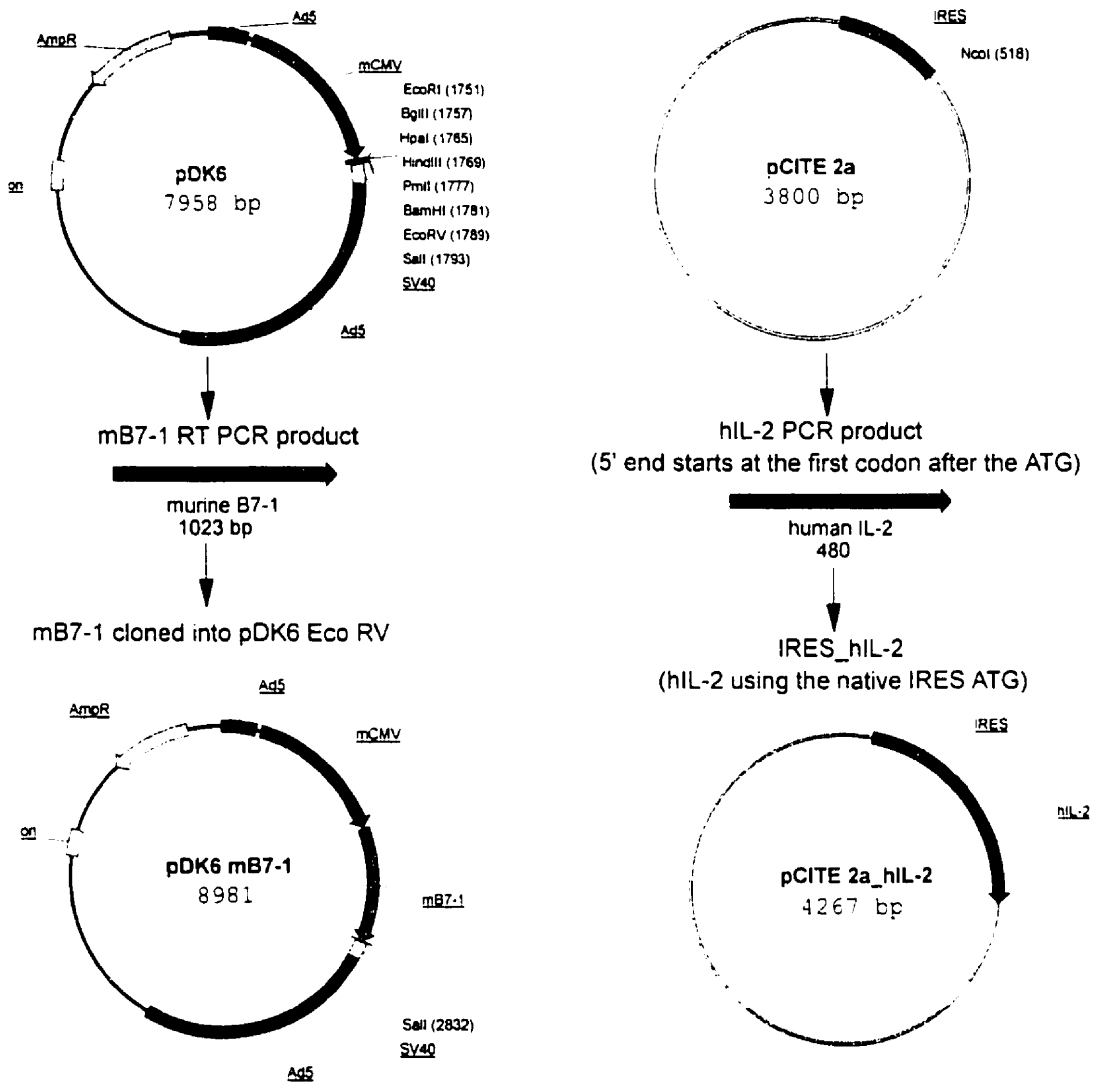
Figure 2. Flow cytometric data demonstrating the expression of important immunoregulatory molecules on PyMT tumor cells (B7-1, B7-2, MHC I and MHC II) (A, B, C and D). Flow cytometry analysis demonstrating vector derived mB7-1 transgene expression from both Ad5 mB7-1/hIL-2 (E) and Ad5 mB7-1 (F) on PyMT tumor cells. PyMT cells were infected with both Ad5 mB7-1 and Ad5 mB7-1/hIL-2 *in vitro* at an MOI of 10 for three days. Single cell suspensions were then subjected to flow cytometry using an anti-mB7-1 antibody.

Figure 3. The production of hIL-2 from Ad5 mB7-1/hIL-2 and Ad CAIL-2 infected A549 (A), MRC5 (B) and WM35 (C). Cells were infected with adenoviral vectors at an MOI of 10 for both Ad CAIL-2 and Ad5 mB7-1/hIL-2. Aliquots were removed and supernatants analyzed by ELISA for human IL-2.

Figure 4. Cytotoxic T lymphocyte activity in totally regressed mice induced by Ad5 mB7-1 (A), Ad5 mB7-1/hIL-2 (B) and Ad CAIL-2 (C). Solid squares represent 516MT3 targets (516MT3 cells express polyoma middle T antigen), open squares

demonstrate PTO516 targets (PTO516 cells do not express polyoma middle T antigen).

Splenocytes were co-cultured with 516MT3 cells for 5-7 days and the checked for CTL activity as outlined in Materials and Methods.



IRES_hIL-2 was amplified and ligated into pDK6 mB7-1 Sal I to construct pDK6 mB7-1/hIL-2. Both constructs were then cotransfected with pBHG10 to make the Ad vectors.

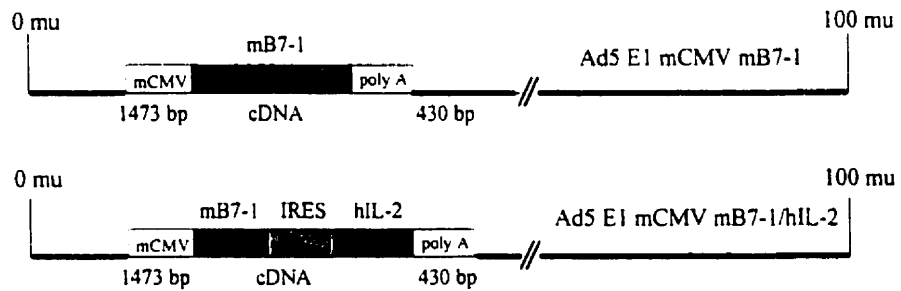


Figure 1

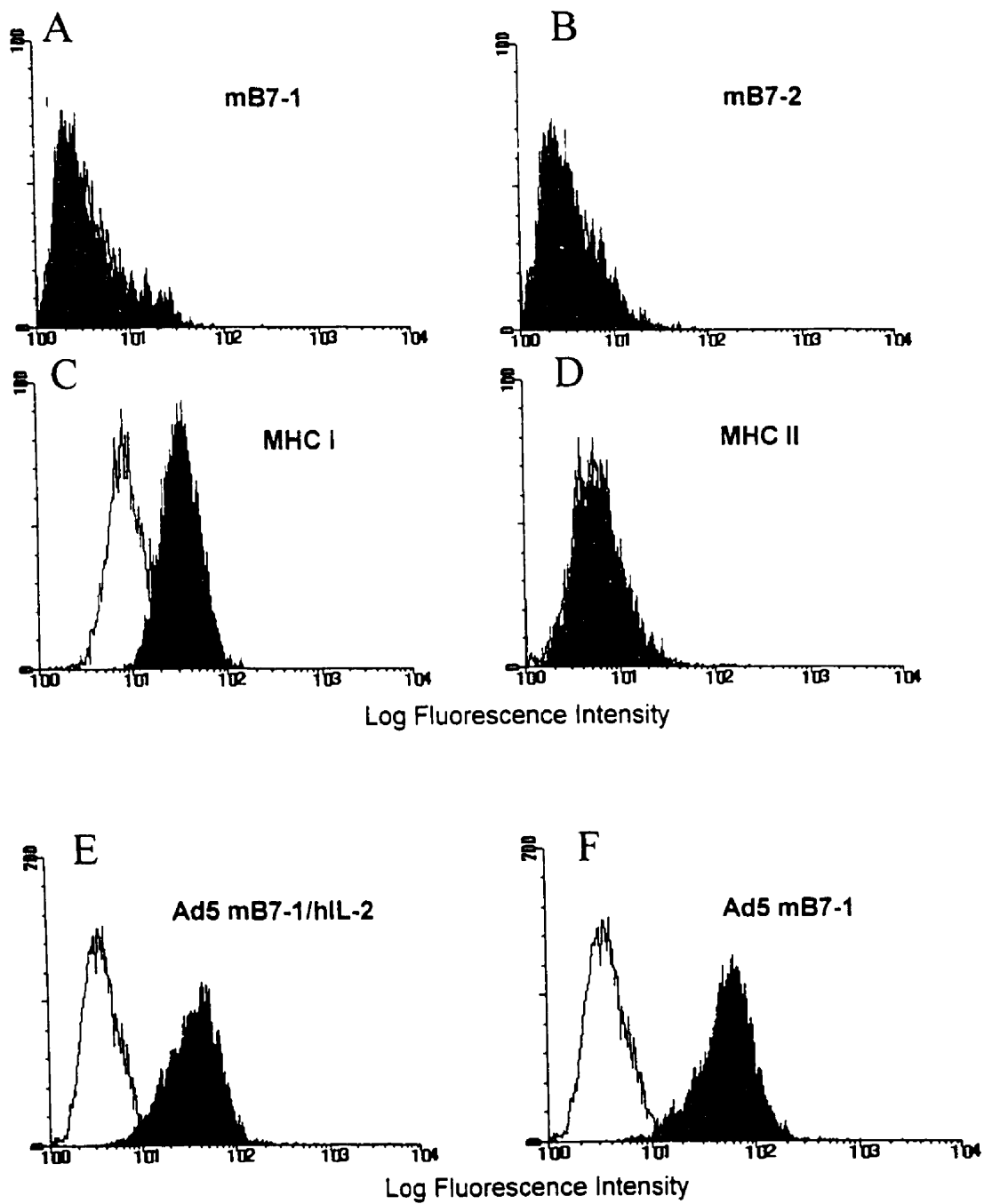


Figure 2

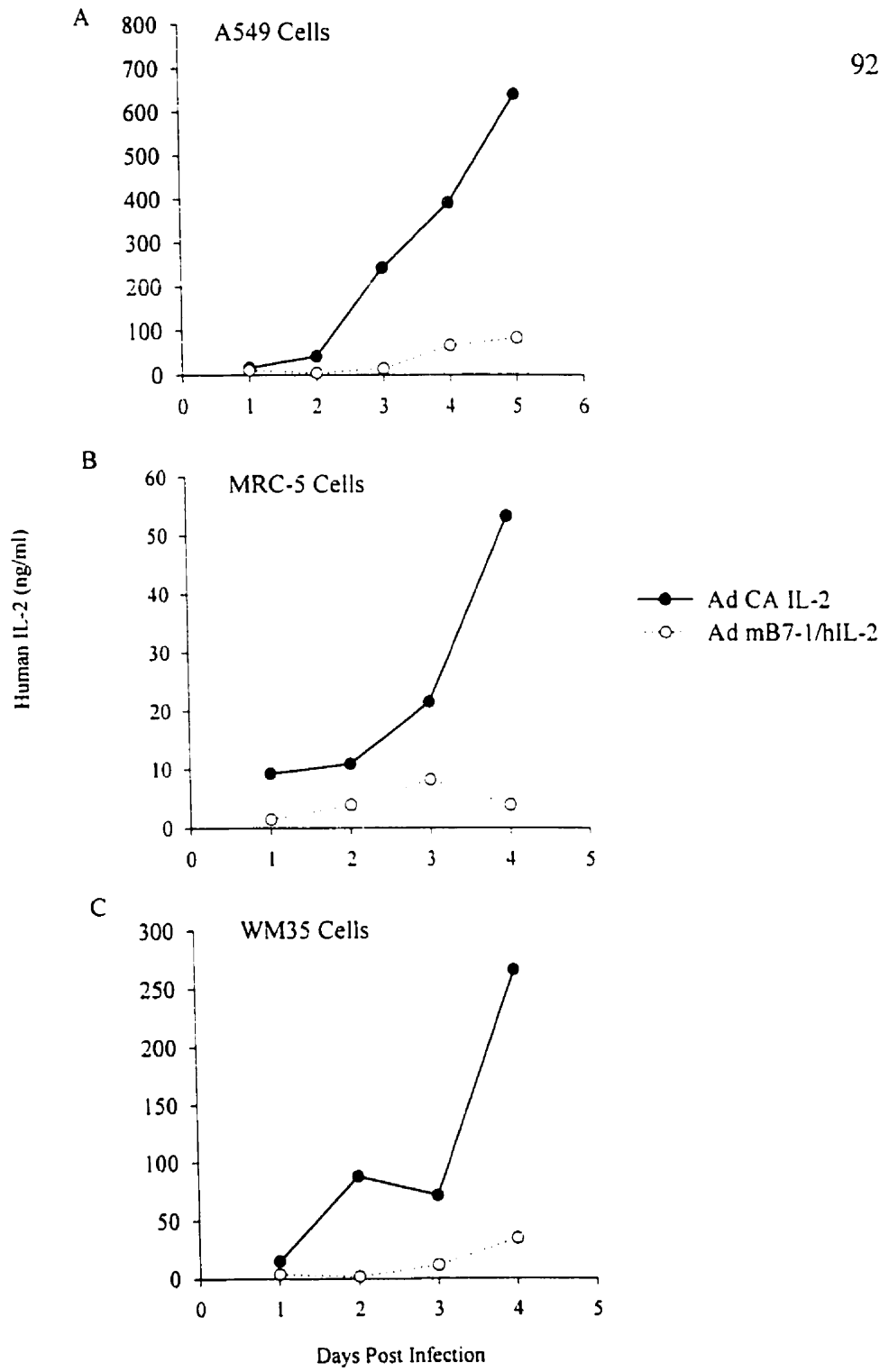


Figure 3

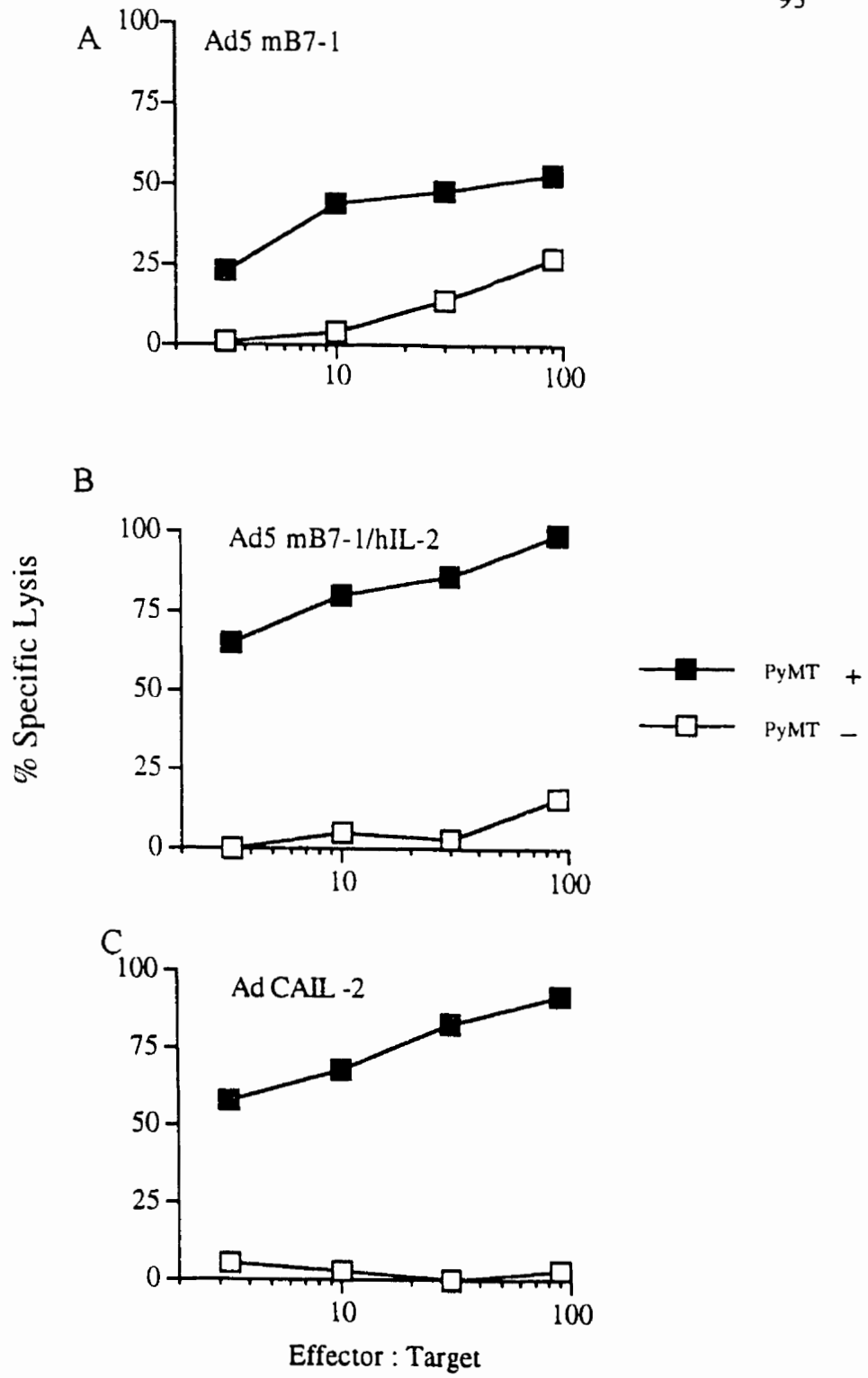


Figure 4

Table 1. Growth response of PyMT tumors in response to intratumoral injection of Ad5 mB7-1, Ad5 CAIL-2 and Ad5 mB7-1/hIL-2.

| Virus | Response | | | | | | Total |
|------------------------|----------|--------|--------|--------|--------|--------|---------------|
| | Exp. 1 | Exp. 2 | Exp. 3 | Exp. 4 | Exp. 5 | Exp. 6 | |
| Ad5 mB7-1 | | | | | | | |
| None | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/4 | 0/24 (0%) |
| Partial | 3/4 | 2/4 | 3/4 | 1/4 | 3/4 | 3/4 | 15/24 (62.5%) |
| Complete | 1/4 | 2/4 | 1/4 | 3/4 | 1/4 | 1/4 | 9/24 (37.5%) |
| Ad5 CAIL-2 | | | | | | | |
| None | 0/4 | 2/4 | 0/4 | 1/4 | 1/4 | 1/4 | 5/24 (21%) |
| Partial | 2/4 | 1/4 | 3/4 | 0/4 | 1/4 | 2/4 | 9/24 (37%) |
| Complete | 2/4 | 1/4 | 1/4 | 3/4 | 2/4 | 1/4 | 10/24 (42%) |
| Ad5 mB7-1/hIL-2 | | | | | | | |
| None | 0/4 | 0/4 | 0/5 | 0/4 | 0/3 | 0/18 | 0/38 (0%) |
| Partial | 0/4 | 0/4 | 0/5 | 0/4 | 0/3 | 0/18 | 0/38 (0%) |
| Complete | 4/4 | 4/4 | 5/5 | 4/4 | 3/3 | 18/18 | 38/38 (100%) |

None = no response to vector treatment

Partial = growth retardation to treatment

Complete = complete regression

Table 2. Protective immunity in mice completely regressed with Ad5 mB7-1, Ad5 CAIL-2 and Ad5 mB7-1/hIL-2. All mice were challenged with freshly isolated PyMT tumor cells 110 days post initial tumor cell administration.

| Virus | Response | | | Total | |
|------------------------|----------|--------|--------|-------|--------|
| | Exp. 1 | Exp. 2 | Exp. 3 | | |
| Ad5 mB7-1 | | | | | |
| Protection | 1/1 | 2/2 | 1/1 | 4/4 | (100%) |
| No Protection | 0/1 | 0/2 | 0/1 | 0/4 | |
| Ad5 CAIL-2 | | | | | |
| Protection | 2/2 | 1/1 | 1/1 | 4/4 | (100%) |
| No Protection | 0/2 | 0/1 | 0/1 | 0/4 | |
| Ad5 mB7-1/hIL-2 | | | | | |
| Protection | 4/4 | 4/4 | 5/5 | 13/13 | (100%) |
| No Protection | 0/4 | 0/4 | 0/5 | 0/13 | |

Protection = no tumor growth for 120 days after challenge

No Protection = tumor growth after challenge

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UMI

Running Title:

B7-1 and B7-2 Augment IL-2 Anti-tumor Activity

Full Title:

Enhanced IL-2 gene transfer immunotherapy of breast cancer by co-expression of B7-1 and B7-2.

Authors:

Peter C. R. Emtage¹, Yonghong Wan¹, William Muller^{1,2}, Frank L. Graham^{1,2} and Jack Gauldie^{1,3}

¹Department of Pathology, McMaster University, Hamilton, Ontario

²Department of Biology, McMaster University, Hamilton, Ontario

³Address correspondence and reprint requests to Dr. Jack Gauldie, Department of Pathology, 1200 Main St. W., Hamilton, Ontario Canada L8N-3Z5.

Tel # 905-525-9140 Ext. 22474

Fax # 905-522-6750

Email gauldie@McMaster.ca

ABSTRACT

The capability of B7-1 to augment the anti-tumor activity of some cytokines has been shown primarily for cytokines like IL-12, IL-7 and to a lesser extent IL-2. In this study we investigate the ability of B7-1 and B7-2 to augment the anti-tumor activity of IL-2. Considering the affinity of both molecules for CD28 (T cell receptor for B7-1 and B7-2) we postulated that their potential to augment IL-2 anti-tumor activity would be similar. Two murine transgenic adenocarcinoma models were chosen to investigate the activity of adenoviral vectors constructed to express either B7-1 and IL-2 or B7-2 and IL-2. Before administering the vector intra-tumorally to tumor bearing mice we determined the expression of B7-1, B7-2, MHC I and MHC II on these tumor cells and demonstrated positive expression of only MHC I. Intra-tumoral injection of the vector expressing B7-1 and IL-2 resulted in the complete regression of all tumors treated (Emtage et al., 1998). In contrast the vector expressing B7-2 and IL-2 was significantly less effective at regressing PyMT tumors. In comparison both double recombinant vectors demonstrated similar levels of complete regression in the Neu (NDL 8142) model. Regressed mice were all protected for rechallenge in both models and demonstrated antigen specific CTL in the PyMT model. These findings indicate that the combination of IL-2 with B7-1 or B7-2 significantly enhances the anti-tumor activity of IL-2.

INTRODUCTION

It is widely appreciated that T cell activation requires two signals ^(1,2). Signal one is provided by the interaction of the T cell receptor (TCR) and its co-receptors (CD4 and CD8) with either MHC I or MHC II molecules presenting antigen ⁽³⁻⁵⁾. Signal two is reliant on either exogenous IL-2 or the ligation of T cell surface molecules to provide co-stimulatory signals which augment the signal delivered by the TCR mediated antigen recognition event ^(6,7). Interaction of the TCR complex with antigen:MHC complexes initiates a state of readiness in the T cell but fails to induce proliferation and T cell effector function ^(8,9). This unresponsiveness is called anergy. Anergic T cells, while capable of recognizing the antigenic epitope(s) on tumor cells, fail to eliminate tumor cells and allow neoplastic progression.

The B7 co-stimulatory family of molecules, B7-1 (CD80) and B7-2 (CD86) are antigen presenting cell accessory molecules required for full activation of T cells. Molecular characterization of the B7 family members has shown that these molecules are members of the immunoglobulin (Ig) superfamily and are ligands for CD28 and CTLA-4 counter receptors on T cells ⁽¹⁰⁻¹³⁾. B7-1 was first described as an activated B cell marker ^(14,15). However, B7-1 and B7-2 expression has since been localized to other antigen presenting cells (APCs), including dendritic cells, monocytes and macrophages. Ligation of CD28 with either B7-1 or B7-2 results in the up-regulation of the IL-2 receptor α , β and γ chains ⁽¹⁶⁻¹⁸⁾, increased IL-2 gene transcription ⁽¹⁹⁻²²⁾, up-regulation

100

of CTLA-4 mRNA levels ^(23,24), cytokine secretion and T cell proliferation ⁽²⁵⁻²⁸⁾. Blockade of the CD28:B7 pathway inhibits humoral immunity ⁽²⁹⁾, graft versus host disease ⁽³⁰⁾, graft rejection ^(31,32) and prevents autoimmune disease ^(33,34). It is therefore evident that since this pathway is extremely important for the generation of immune responses, it provides a potential point of manipulation of the immune response.

Recently, a number of studies have provided evidence for the use of B7-1 or B7-2 co-stimulatory molecules in tumor immunotherapy. Expression of either B7-1 or B7-2 in murine tumor models has been shown to activate CD8⁺ T cells and/or CD4⁺ T cells against the respective tumor cells ⁽³⁵⁻⁴³⁾. There is controversy with respect to the relative functional immune activating biological properties of B7-1 and B7-2 ^(3,4). In some instances these molecules have been shown to induce the production of Th1 or Th2 cytokine profiles in various animal models ⁽⁴⁴⁻⁴⁶⁾. Alternatively, a number of studies have not been able to demonstrate such differences between these two molecules ⁽⁴⁷⁻⁴⁹⁾. In the tumor immunotherapy arena, when B7-1 and B7-2 have been directly compared, their anti-tumor effects have been equal in some cases ⁽³⁷⁾, B7-1 dominant in some cases ^(50,51) and B7-2 dominant in other cases ⁽⁵²⁾. We speculated that B7-1 and B7-2 having the same affinity for CD28 would exhibit similar activities in our tumor models.

We have previously shown that an adenovirus vector expressing human IL-2 administered intra-tumorally results in approximately 42% complete regression in a murine adenocarcinoma model, while the simultaneous expression of B7-1 and IL-2

from a single vector resulted in complete regression of all tumors treated⁽⁵³⁾. The additive or synergistic effects observed from expressing both B7-1 and IL-2 in the same adenoviral vector prompted us to compare the anti-tumor effects of B7-2 by intra-tumoral injection of a single adenoviral vector constructed to express both mB7-2 and hIL-2. In this study we demonstrate that intra-tumoral injection of adenoviral vectors (Ad) constructed to express combinations of B7-1 or B7-2 and the immuno-modulatory cytokine IL-2 results in different outcomes in two separate transgenic murine mammary adenocarcinoma models (PyMT and Neu). The vectors constructed to express B7-2 and IL-2 were considerably less potent than the B7-1/IL-2 combination in the PyMT model. However, they were equipotent when compared in the Neu (NDL 8142) model. Completely regressed mice from both models were shown to have generated systemic immunity to subsequent challenge with fresh tumor cells and potent CTL activity was demonstrated in the PyMT model. Both combinations were able to reduce the incidence of pulmonary metastatic lesions.

MATERIALS AND METHODS

Animals

Six to eight week old FVB/n mice were purchased from Taconic Laboratories and housed in a pathogen free facility until use.

Cell Lines

Cell lines used include the following: PyMT, primary polyoma middle T antigen transformed murine cells obtained from explanted tumors; PTO516, FVB/n kidney derived cells, 516MT3 cells are derived from PTO516 and are stably transformed to express polyoma middle T antigen (Addison et al., 1998), ; 293, human embryonic kidney cells transformed with adenoviral E1 sequences ⁽⁵⁴⁾; 293N3S, contact independent 293 derivative ⁽⁵⁵⁾; the Neu (NDL 8142) tumor cell line was isolated from a tumor bearing transgenic mouse harboring the Neu 8142 deletion outlined in ⁽⁵⁶⁻⁵⁸⁾. All cell culture reagents were purchased from GIBCO.

Construction of Ad5 mB7-2/hIL-2

Total RNA was isolated from 293 cells infected with Ad2 mB7-2 ⁽⁴¹⁾ using the reagent Trizol (the Ad2 mB7-2 construct was kindly provided by Dr. Jeff Bluestone, Chicago, IL). RT-PCR was performed using the First Strand cDNA synthesis Kit (GIBCO). Briefly, cDNA was synthesized using oligo(dT) as the primer. PCR was performed using Vent DNA polymerase (New England Biolabs) and the following parameters: denaturation was accomplished at 94⁰C for 1 minute; annealing 55⁰C for 30

seconds; extension at 72⁰C for 1 minute. This was done using the following sense and antisense primers designed to anneal to the 5' and 3' ends of the B7-2 cDNA sequence as published in Genbank. The sense primer 5'-GGAGCAAGCAGACGCGTAAGAGTG-3' and the antisense primer 5'-CTTTCCTCAGGCTCTCACTGC-3', were designed to amplify the mB7-2 open reading frame. The amplified fragment of 1051 base pairs was directly ligated into the Hpa I digested pDK6 shuttle plasmid⁽⁵³⁾ to create pDK6 mB7-2 (Figure 1). The shuttle plasmid pDK6 mB7-2 was sequenced amplified and then purified by alkaline lysis and cesium chloride gradient centrifugation and used in subsequent cloning steps.

To generate a dicistronic construct expressing mB7-2 and hIL-2. The human IL-2 open reading frame was amplified using the following 5'-phosphorylated primers, 5'-TACAGGATGCAACTCCTGTCTTGC-3' (sense) and 5'-CTAATTATCAAGTCAGTGTTGA-3' (antisense). The sense primer is constructed such that the codon underlined (TAC) is the first amino acid immediately after the ATG start codon. The PCR product of 469 bp was then blunt end ligated into the pCITE 2a plasmid (Novagen) which carries the Encephalomyocarditis virus internal ribosomal entry site (IRES). This plasmid was digested with Nco I (recognition site CCATGG) and subsequently blunt ended (blunting was performed with Klenow polymerase large fragment from NEB) to produce an ATG start codon, prior to ligation with the IL-2 PCR

product. Ligation of the IL-2 PCR product with the pCITE 2a plasmid resulted in pCITE_hIL2 which carries the hIL-2 open reading frame driven by the IRES sequence.

The pCITE_hIL-2 plasmid was used as a template for a second round of PCR. Using the following phosphorylated primer, 5'-TTCCGGTATTTTCCACCATATTG-3' (IRES sense) and the original hIL-2 antisense primer, a PCR product of 979 bp was amplified corresponding to the IRES/hIL-2 hybrid molecule. This PCR product was blunt end ligated into pDK6 mB7-2 digested with Sal I (3' to the stop codon of mB7-2) to produce pDK6 mB7-2/IRES/hIL-2 (Figure 1). This plasmid was then processed in an identical manner to pDK6 mB7-2 and cotransfected with pBHG10⁽⁵⁹⁾ to generate Ad5 mB7-2/hIL-2. Ad5 mB7-2/hIL-2 was screened using Southern and Northern techniques to characterize the presence of inserted DNA or the production of dicistronic mRNAs (Ad5 mB7-2/hIL-2). In the case of Ad5 mB7-2/hIL-2 Western analysis was performed to detect hIL-2 production. All cloning was confirmed by sequencing.

Ad2 mB7-2, Ad5 mB7-1, Ad5 mB7-1/hIL-2, Ad CAIL-2 and Ad5 dl70-3

The Ad2 mB7-2 vector is as described in La Motte et al.⁽⁴¹⁾. The viruses Ad5 mB7-1 and Ad5 mB7-2/hIL-2 are as described in Emtage et al.⁽⁵³⁾. The vector Ad5 IL-2 is described as Ad5 CAIL-2 by Addison et al.⁽⁶⁰⁾. The control vector is described in Addison et al.⁽⁶⁰⁾.

Flow Cytometry

Flow cytometric analysis was performed on PyMT and NDL 8142 cells to characterize mB7-1 and mB7-2 expression from all B7 expressing vectors.. Cells were infected for 48 hr prior to analysis. All groups were harvested at the same time, washed in PBS and incubated with either mB7-1 or mB7-2 monoclonal antibodies (Pharmingen). To determine the expression of immuno-regulatory molecules Neu (NDL 8142) cells were incubated with anti MHC I, MHC II, mB7-1 and mB7-2 antibodies and analyzed by flow cytometry. The expression of mB7-1, mB7-2 and MHC I and II antigens on PyMT tumor cells was previously determined⁽⁵³⁾ Analysis was performed using a flow cytometer (Becton Dickinson).

Tumor Cell Preparation and Tumor Induction

A transgenic mouse strain (FVB/n) expressing the polyoma middle T (PyMT) antigen under the control of the mouse mammary tumor virus long terminal repeat (MMTV) promoter was the source of the tumor cells used in the PyMT portion of this study⁽⁶¹⁾. Expression of PyMT antigen results in the spontaneous transformation of the mammary epithelium in transgenic animals by 8-10 weeks of age. These tumors were excised from transgenic mice and subjected to enzymatic digestion to generate a single cell suspension. The single cell suspension of PyMT tumor cells was washed with PBS, and aliquots of 10^6 tumor cells were injected subcutaneously (sc) into the right hind flank of normal syngeneic FVB/n mice.

The Neu (NDL 8142) model was developed from tumors which arise in transgenic mice harboring a deletion in the extracellular domain of the Neu proto-oncogene. The expression of the NDL 8142 mutation is restricted primarily to the mammary epithelium by the tissue specificity of MMTV long terminal repeat promoter. The Neu (NDL 8142) mutation has been described previously^(56,57). A cell line generated from NDL (8142) transgenic tumors was used to establish tumors (7×10^6 cells/tumor) in syngeneic recipient mice.

Human IL-2 Assay

PyMT and NDL 8142 cells were infected at a multiplicity of infection (MOI) of 10 plaque forming units (pfu) per cell with Ad5 IL-2, Ad5 mB7-1/hIL-2 or Ad5 mB7-2/hIL-2 for 5 days. At 24 hr intervals, 200 μ l aliquots were removed and stored at minus 70^oC for quantification. Secreted hIL-2 was quantitated using the hIL-2 DuoSet Kit (Genzyme Diagnostics Inc).

Vector Administration

Palpable tumors (normally 75-150 mm³) arise in tumor cell recipient mice within 16 to 21 days after which time appropriate amounts of virus were injected intratumorally in a volume of 40 μ l. After injection of adenoviral vectors, tumors were monitored weekly using calipers. The volume of the tumor was calculated from the longest diameter and average width assuming a prolate spheroid. Mice with tumors not responding to vector treatment were sacrificed when the longest diameter exceeded 20

mm. Regressed mice were left for approximately 3 months and then challenged with 1×10^6 freshly isolated PyMT or 7×10^6 Neu (NDL 8142) tumor cells on the left hind flank.

CTL assays

Splenocytes (effectors) were obtained from mice whose tumors had regressed as a result of Ad5 mB7-2 or Ad5 mB7-2/hIL-2 treatment and co-cultured with 516MT3 cells (stimulators) at a concentration of 1.2×10^5 516MT3 to 1.2×10^7 splenocytes for 4 days in 6 well dishes. Serial dilutions of the effector cells were incubated in a v-bottom 96-well plate with 5×10^3 516MT3 or PTO516 target cells. Target cells (10^6) were labeled with 100 μ Ci of ^{51}Cr sodium salt for 2 hr prior to co-culture with the effector cells. Cells were co-cultured for 4-5 hr at which time 80 μ l of supernatant was removed for counting. The percent specific lysis was calculated as follows: $100 \times (\text{experimental cpm} - \text{spontaneous cpm}) / (\text{maximal cpm} - \text{spontaneous cpm})$.

PyMT Pulmonary Metastatic Model

Subcutaneous (sc) PyMT tumors are initiated on the right hind flank of syngeneic recipient mice. Eighteen days later 2.5×10^5 PyMT tumor cells are injected via the tail vein to establish pulmonary metastases. Four to five days later the 75-150 mm^3 sc tumors are injected with the various adenoviral vectors. Fourteen days post intratumoral injection the mice are sacrificed and the lungs removed to assess the number of pulmonary metastases.

RESULTS

Construction of Recombinant Adenoviral Vectors

The recombinant adenoviral vector which expresses both murine mB7-2 and human IL-2 (Ad5 mB7-2/hIL-2) was constructed as outlined in figure 1. The expression cassette for mB7-2/hIL-2 was inserted into the E1 deleted region of the human adenovirus type 5 genome. The dicistronic mB7-2/hIL-2 construct was flanked by the murine cytomegalovirus immediate-early promoter (mCMV) and a simian virus 40 polyadenylation signal (SV40). Separating the mB7-2 open reading frame from the hIL-2 open reading frame is the encephalomyocarditis virus internal ribosome entry site (IRES)⁽⁶²⁾ which results in a dicistronic DNA fragment.

Expression of the mB7-1, mB7-2 and hIL-2 genes (Ad2 mB7-2 and Ad5 mB7-2/hIL-2) was confirmed *in vitro* in the PyMT and Neu (NDL 8142) tumor cell lines infected with each recombinant adenoviral vector. We analyzed early passage Neu (NDL 8142) cells for the endogenous expression of MHC I, MHC II, mB7-1 and mB7-2 (Figure 2A). No MHC II, mB7-1 or mB7-2 expression could be detected on uninfected Neu (NDL 8142) tumor cells (Figure 2A; PyMT tumor cells were previously characterized for the expression of these molecules and were found to only express MHC I⁽⁵³⁾). However, infection of Neu (NDL 8142) tumor cells with vectors expressing mB7-1 or mB7-2 resulted in a positive (mB7-1 or mB7-2) phenotypic conversion (Figure 2B and C). Infection of PyMT cells with Ad2 mB7-2 and Ad5 mB7-

2/hIL-2 vectors converted the mB7-2 negative phenotype to a mB7-2 positive phenotype (Figure 2D). Expression of mB7-1 from Ad5 mB7-1 and Ad5 mB7-1/hIL-2 on PyMT cells was reported previously in Emtage et al. ⁽⁵³⁾. These data demonstrate that the expression of mB7-1 and mB7-2 on PyMT or Neu (NDL 8142) tumor cells is due to the presence of the mB7-1 and mB7-2 transgenes from all vectors. Infection of both PyMT and Neu (NDL 8142) with the control vector Ad5 dl70-3 did not alter or induce the expression of B7-1, B7-2, MHC I or MHC II molecules.

hIL-2 Expression in Cells Infected with Ad5 mB7-2/hIL-2

IL-2 expression from the dicistronic Ad5 mB7-1/hIL-2, Ad5 mB7-2/hIL-2 and the monocistronic IL-2 control vector was quantified by ELISA (Figure 3A and B) from both PyMT and Neu (NDL 8142) tumor cells. IL-2 levels derived from the Ad5 mB7-1/hIL-2 and Ad5 mB7-2/hIL-2 vectors were very similar. Both constructs expressed levels up to 192 ng/ml for Ad5 mB7-1/hIL-2 and up to 157 ng/ml for Ad5 mB7-2/hIL-2, while, the levels derived from Ad5 IL-2 were up to 1129 ng/ml. The expression of IL-2 observed (per 1×10^6 cells) with both double vectors was approximately 10 fold lower than that observed with the single IL-2 vector (Ad5 IL-2).

Intra-tumoral Administration of Adenoviral Vectors

We have previously shown that intra-tumoral injection of adenoviral vectors expressing either IL-2, IL-12 or a combination of B7-1 and IL-2 to PyMT tumor bearing mice caused regression at a dose of 5×10^8 pfu ^(53,60,63). We, therefore, used this same

dose for Ad2 mB7-2 and Ad5 mB7-2/hIL-2 and compared the effects with those seen with the E1 deleted control vector, Ad5 dl70-3. Administration of control virus did not modify the progression of the PyMT tumor growths in any of the control groups described here or in ^(53,60,63). Conversely, treatment of PyMT tumors with Ad2 mB7-2 resulted in 27% (4/15 tumors) total regression of established tumors (Table I). Administration of Ad5 mB7-2/hIL-2 resulted in an increase in regression to 47% of tumors treated suggesting a minor additive or synergistic effect between mB7-2 and hIL-2 expressed from the same cell (Table I). All mice exhibiting complete regression were tumor free up to 120 days post injection.

The activity of the B7-1 or B7-2 expressing vectors were very different in Neu (NDL 8142) tumors compared to that observed for PyMT tumors. In this model the only single vector that could induce regression of tumors was Ad2 mB7-2, which resulted in 33% regression (5/15 tumors) (Table II). No regression was observed with Ad5 mB7-1 or Ad5 IL-2. The double recombinant vectors both mediated regression in this tumor model. Ad5 mB7-1/hIL-2 resulted in 65% regression (13/20 tumors) and Ad5 mB7-2/hIL-2 induced 60% regression (9/15 tumors) (Table II). As with the PyMT tumor model, treatment with the control vector showed no inhibition of tumor growth.

Generation of Systemic Immunity to PyMT and Neu (NDL 8142) Tumor Cells

PyMT or Neu (NDL 8142) mice that had undergone complete regression were challenged with 1×10^6 freshly isolated PyMT or 7×10^6 Neu (NDL 8142) tumor cells

respectively at day 115 post initial vector administration, on the left hind flank (see Materials and Methods). None of the challenged mice developed tumors and all remained tumor free for a further 100 days after challenge (data not shown).

CTL Activity in Cured Mice

Splenocytes prepared from Ad2 mB7-2 treated PyMT mice demonstrated an average of 45% specific lysis of 516MT3 cells (middle T expressing target cells) at an effector to target ratio of 3.3:1. Lysis of PT0516 (control cells) was also observed however at a much lower percentage. Similarly, splenocytes from Ad5 mB7-2/hIL-2 treated PyMT mice demonstrated an average 61% lysis of 516MT3 targets and undetectable levels of lysis on PT0516 cells (at effector to target ratios of 3.3:1) (Figure 4). No target cell killing was seen with control animals (splenocytes from Ad5 dl70-3 treated tumors) We are in the process of characterizing potential Neu target cells to test vector generated CTL activity.

Anti-Metastatic Activity

The ability of the vectors used in this work to reduce or eliminate metastatic growth was assessed in the PyMT model. The Ad5 dl70-3 treated control animals demonstrated significant numbers of pulmonary metastatic nodules in the range of 96-146 (Figure 5). The mB7-1 and hIL-2 showed similar numbers of nodules in the range of 24-65 for Ad5 mB7-1 and 29-67 for Ad5 IL-2. The most potent vector in this model was Ad5 mB7-1/hIL-2 which reduced levels to 0-7 nodules, with 3 out of 5 mice having

no detectable metastatic lesions. The anti-metastatic activity of the mB7-2 vectors varied greatly, with, Ad2 mB7-2 demonstrating nodule levels in the range of 11-113 and Ad5 mB7-2/hIL-2 showing pulmonary metastatic nodules in the range of 3-128 (Figure 5).

DISCUSSION

There are many potential mechanisms by which tumors can evade the immune system. Tumors which express low levels of MHC I do not initiate effective effector T cell recognition. Some tumors have been shown to produce suppressive molecules like TGF β and IL-10, which shut down Th1 responses and, therefore, allow tumor progression. Other tumors have demonstrated the expression of the pro-apoptotic molecule Fas ligand, which could induce the apoptosis of Fas bearing immune effector cells. Important to this work is the finding that most tumors do not express co-stimulatory molecules. The inability of tumor cells to induce efficient effector T cell responses due to the lack of co-stimulatory molecule expression would render T cells unresponsive or anergic. There have been many studies which show the importance of the B7:CD28 pathway in the generation of tumor specific immune responses ^(40,64-66). These observations have led to numerous pre-clinical and clinical trials which utilize the co-stimulatory molecules B7-1 or B7-2 as modulators of anti-tumor immune responses.

B7 family members are required for the transduction of signals which promote T cell activation in the presence of MHC I or II antigen/TCR interactions ^(31,67-69). Transfection of tumor cells with B7-1 or B7-2 has been shown to result in moderate effects on tumor regression or rejection ^(35,36,38,70,71). More pertinent to this work are the observations from a number of groups which show that B7-1 can be used to augment the activity of cytokines, including IL-12, IL-2 and IL-7 *in vivo* ^{(72) (73-75)} by enhancing the

anti-tumor activity of these cytokines. This study demonstrates that B7-1 or B7-2 modified tumor cells are capable of delivering, in conjunction with MHC I or II, antigen specific activation signals to T cells. By far one of the most beneficial effects of B7 enhancement of tumorigenicity is the ability of B7-1 or B7-2 to directly activate naïve CD8⁺ cytotoxic T lymphocytes in the absence of CD4⁺ help⁽⁷⁶⁾.

Cytokines such as IL-2, IL-4, IL-7 and IL-12 have been studied in pre clinical animal models to assess their potential for mediating tumor rejection in various immunotherapy protocols. Most of these therapies have been limited by the associated toxicities of some of the more effective cytokines (IL2 and IL-12) or by the inability of the cytokines under investigation to induce efficient anti-tumor effector activity^(60,77,78). We have recently used adenoviral vectors to deliver cytokines such as IL-2, IL-4 and IL-12 intratumorally^(60,60,63), and have shown that the transient expression of IL-2, IL-4 and IL-12 could augment the recognition of the tumor by the immune system and result in complete regression in a significant fraction of the tumors treated. We have also combined some of these cytokines with the co-stimulatory molecule B7-1. B7-1 in combination with IL-2 or IL-12 resulted in greatly enhanced activity in the regression of PyMT tumors^(53,73). This synergistic or additive activity of B7-1 with either IL-2 or IL-12 prompted us to characterize the activity of B7-2 and it's ability to enhance IL-2 anti-tumor activity.

The ability of B7-1 or B7-2 to modulate anti-tumor activity is a very controversial issue. Yang et al.⁽³⁷⁾, showed that B7-2 transfection into cells from various murine tumor models resulted in rejection levels similar to those observed with B7-1 transfected tumor cells. In contrast, Matulonis and co-workers⁽⁵¹⁾ demonstrated that B7-1 elicited a potent anti-tumor activity in a murine leukemia model whereas B7-2 had no activity. Similarly, Gajewski⁽⁵⁰⁾ showed that P815 tumor cells transfected with B7-1 was more potent at inducing rejection compared to B7-2 transfected tumor cells. Leong and colleagues⁽⁷⁹⁾ illustrated that the expression of B7-1 in a murine malignant mesothelioma model induced rejection. However, the expression of B7-2 in the same cells could not induce the same level of rejection. Alternatively, Martin-Fontecha et al.⁽⁵²⁾ demonstrated the potency of B7-2 in mediating rejection and generating protection from adenocarcinoma or melanoma cells compared to that observed with B7-1.

Here we show the efficiency of Ad vectors constructed to express the co-stimulatory molecules B7-1 or B7-2 (murine) to convert B7-1 and B7-2 negative PyMT or Neu (NDL 8142) tumor cells to a B7-1 or B7-2 positive phenotype (Figure 2C and 2D). We have also demonstrated the ability of the double recombinant vectors (Ad5 mB7-1/hIL-2 and Ad5 mB7-2/hIL-2) to produce significant levels of hIL-2 (Figure 3). The hIL-2 production was 10 fold lower than that observed with the single vector expressing hIL-2 (Ad5 IL-2).

Intra-tumoral injection of Ad2 mB7-2 and Ad5 mB7-2/hIL-2 demonstrated that the combination vector was only slightly more effective at inducing complete regression (27% regression for B7-2 and 47% regression for B7-2 and IL-2) (Table 1) in the PyMT model than the vector expressing mB7-2. We have previously demonstrated the potent synergistic ability of mB-71 and hIL-2 (Ad5 mB7-1/hIL-2) at completely regressing PyMT tumors⁽⁵³⁾. Compared to the activity of Ad5 mB7-1 or Ad5 IL-2 in PyMT tumors (37% and 42%) the ability of mB7-1 to enhance the anti-tumor activity of IL-2 is quite dramatic. In contrast, the ability of mB7-2 to synergise with IL-2 in the PyMT model is much lower suggesting that B7-1 is more potent than B7-2 at augmenting IL-2 activity in this model. This finding is also supported by the observation that both Ad5 mB7-1 and Ad2 mB7-2 result in similar levels of regression in this model.

The activity of these vectors is different in the Neu (NDL 8142) tumor model. In this system Ad5 mB7-1 or Ad5 IL-2 demonstrated no regression in Neu (NDL 8142) tumor bearing animals. In contrast, Ad2 mB7-2 treatment resulted in 33% regression of Neu (NDL 8142) tumors. This is interesting and clearly demonstrates a difference between B7-1 and B7-2 in this system. A synergistic or additive anti-tumor effect is demonstrated by both co-stimulatory molecules in combination with IL-2. The Ad5 mB7-1/hIL-2 and Ad5 mB7-2/hIL-2 both mediated regression of 65% and 60% of Neu (NDL 8142) tumors respectively. The difference between the anti-tumor activity of B7-1 and B7-2 in this model appears to be minimal when both molecules are combined

with IL-2. This suggests a difference in the co-stimulatory activity of these molecules when expressed in this tumor model.

Previously, all PyMT tumors regressed with B7-1 containing adenoviral vectors were shown to be protected from rechallenge with fresh tumor cells⁽⁵³⁾. We therefore, rechallenged mice demonstrating complete regression with B7-2 containing vectors to assess if the ability to generate systemic protection was similar both tumor systems. Freshly isolated PyMT tumor (1×10^6 /mouse) or early passage Neu (NDL 8142) (7×10^6 /mouse) cells were administered to regressed mice from each model 115 days after initial intra-tumoral injection. Tumor cell challenge was always administered on the opposite hind flank to the site of the regressed tumor. Mice which exhibited complete regression were found to be protected against challenge, demonstrating that all vectors were capable of generating long lasting systemic immunity (Table II).

To determine a possible mechanism and the specificity of the effector function generated during treatment with these vectors, we measured antigen specific cytotoxic T lymphocyte (CTL) activity in the spleen of completely regressed animals from the PyMT model. Ad2 mB7-2 treated animals demonstrated an average of 45% specific lysis on 516MT3 PyMT expressing cell line at a ratio of 3.3:1 (effector:target ratio). This is low but, nevertheless, significant since the same spleen cells resulted in approximately 1% lysis of the parental line PTO516 that does not express polyoma middle T (Figure 4). Ad5 mB7-2/hIL-2 treatment resulted in an average PyMT-specific

CTL activity of 61% lysis on 516MT3, as opposed to minimal lysis on non-specific targets at 3.3:1 effector to target ratio. Treatment of tumors with Ad5 dl70-3 resulted in no associated CTL activity on 516MT3 or PTO516 cells. The activity of Ad5 mB7-1 and Ad5 mB7-1/hIL-2 at inducing CTL activity was previously reported in Emtage et al.⁽⁵³⁾, demonstrating that these molecules mediate regression by inducing CTL activity.

We examined the activity of these vectors in a more stringent model. For this we assessed the anti-metastatic ability of all vectors in a PyMT model of pulmonary metastases. The only vector capable of inducing almost complete reduction in metastases was Ad5 mB7-1/hIL-2. In comparison the Ad5 mB7-2/hIL-2 vector showed varied reduction of pulmonary metastatic growth from 3-128 nodules. This observation suggests that the activity of B7-1 and B7-2 are different in this model. The ability of the single vectors to reduce the number of nodules is varied and not as potent as the combination B7-1 and IL-2 expressing vector.

It is clear that the activity of over-expressing B7-1 or B7-2 in these vectors facilitates regression and induces CTL activity that is associated with a long lasting systemic immunity. The activity of B7-2 expressing vectors in both primary and metastatic tumor models (PyMT model) demonstrates that B7-2 is not as potent at inducing regression in the PyMT model in combination with IL-2 when compared to B7-1 expressing vectors. Both molecules exhibit similar activity in the Neu (NDL 8142) model when combined with IL-2. However, when Ad5 mB7-1 and Ad2 mB7-2

vectors are compared in the Neu (NDL 8142) model, B7-2 appears to be more potent at inducing regression. This suggests that the differences observed between these molecules might be due to their ability to co-stimulate effector cells in the Neu (NDL 8142) model, such that in the presence of IL-2 both molecules augment the activity of IL-2 equally. The mechanism associated with differences between B7-1 and B7-2 in this work are unknown. To further complicate this issue, the signal transduction cascades initiated by B7-1 or B7-2 ligation to CD28 appear at present to be identical^(80,81). This, together with the findings of Linsley et al. (1994) that both B7-1 and B7-2 bind CD28 with the same affinity, suggests that these molecules act in an almost identical fashion to co-stimulate T cells via ligation to CD28. A possible explanation for the differences observed in efficacy between B7-1 and B7-2 containing vectors in our tumor models could be related to the signal transduction cascades initiated by B7-1 or B7-2 cytoplasmic domains. According to Borriello et al. (1994) the cytoplasmic domain of murine B7-2 has putative protein kinase C, casein kinase II and cAMP and cGMP-dependent protein kinase phosphorylation sites. Alternatively, the murine B7-1 (cytoplasmic tail I) isoform used in this study does not contain such amino acid sequences. Although this is speculative it is possible that cytoplasmic signals generated within the tumor cells (B7-1 or B7-2 transgene expression) by the ligation of B7-1 or B7-2 with CD28 on the T cell result in negative or positive signals. These signals could alter gene expression from the vector or endogenous gene expression which might either

help or hinder the generation of effective anti-tumor responses. The factors mentioned above could all act to induce the difference observed between B7-1 and B7-2 in this study. Why there appear to be differences in the anti-tumor activity of B7-1 or B7-2 in some tumor models and not others is unknown. It is clear however, that these molecules augment the activity of IL-2 via synergistic or additive effects. This effect is above that observed for either molecule alone (IL-2, B7-1 or B7-2).

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Legends

FIG. 1. Schematic diagram of the construction of Ad5 mB7-2/hIL-2. For a more detailed vector construction description see Materials and Methods.

FIG. 2. FACs analysis of low passage Neu (NDL 8142) tumor cells (A). Phenotypic conversion of Neu (NDL 8142) tumor cells after infection with Ad vectors expressing mB7-1 (B) and mB7-2 (C). Phenotypic conversion of PyMT tumor cells after infection with the Ad vector expressing mB7-2 and hIL-2 (D).

FIG. 3. The production of hIL-2 from Ad5 mB7-1/hIL-2 and Ad5 mB7-2/hIL-2 infected Neu (NDL 8142) (A) and PyMT (B) cells (MOI =10). Aliquots were removed and supernatants analyzed by ELISA for human IL-2 at days 1 to 5.

FIG. 4. Cytotoxic T lymphocyte activity in mice after total tumor regression on treatment with various vectors. The CTL activity generated by the control vector Ad5 dl70-3 is outlined in (A). PyMT specific CTL activity is outlined for the two B7-2 expressing vectors; Ad5 mB7-2 (B) and Ad5 mB7-2/hIL-2 (C). Solid circles represent 516MT3 cells (PyMT targets), open circles represent PTO516 cells (parental non-PyMT expressing cells). Splenocytes were co-cultured with irradiated 516MT3 cells for 5-7 days prior to determination of CTL activity.

FIG. 5. The number of pulmonary metastatic nodules in tumor bearing mice treated with the various mB7-1 and mB7-2 expressing vectors. Mice were sacrificed 14 days after vectors were intra-tumorally administered.

Construction of Ad5 mB7-2/hIL-2

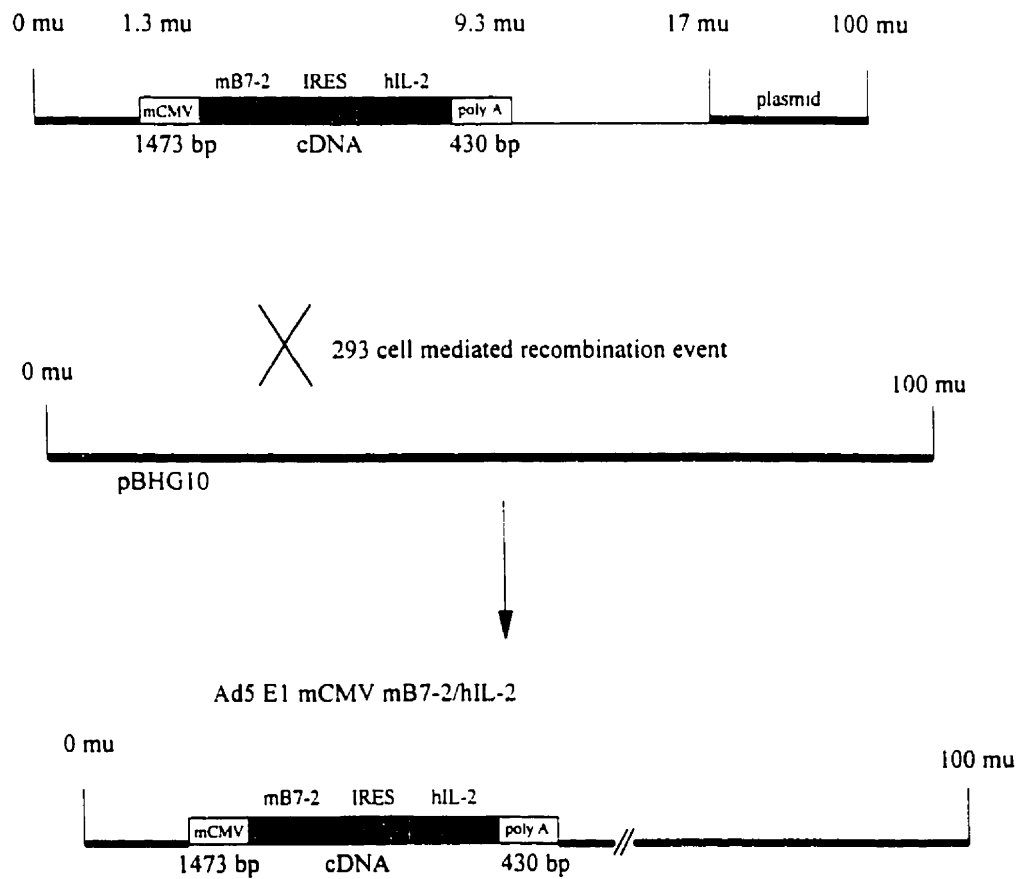


Figure 1

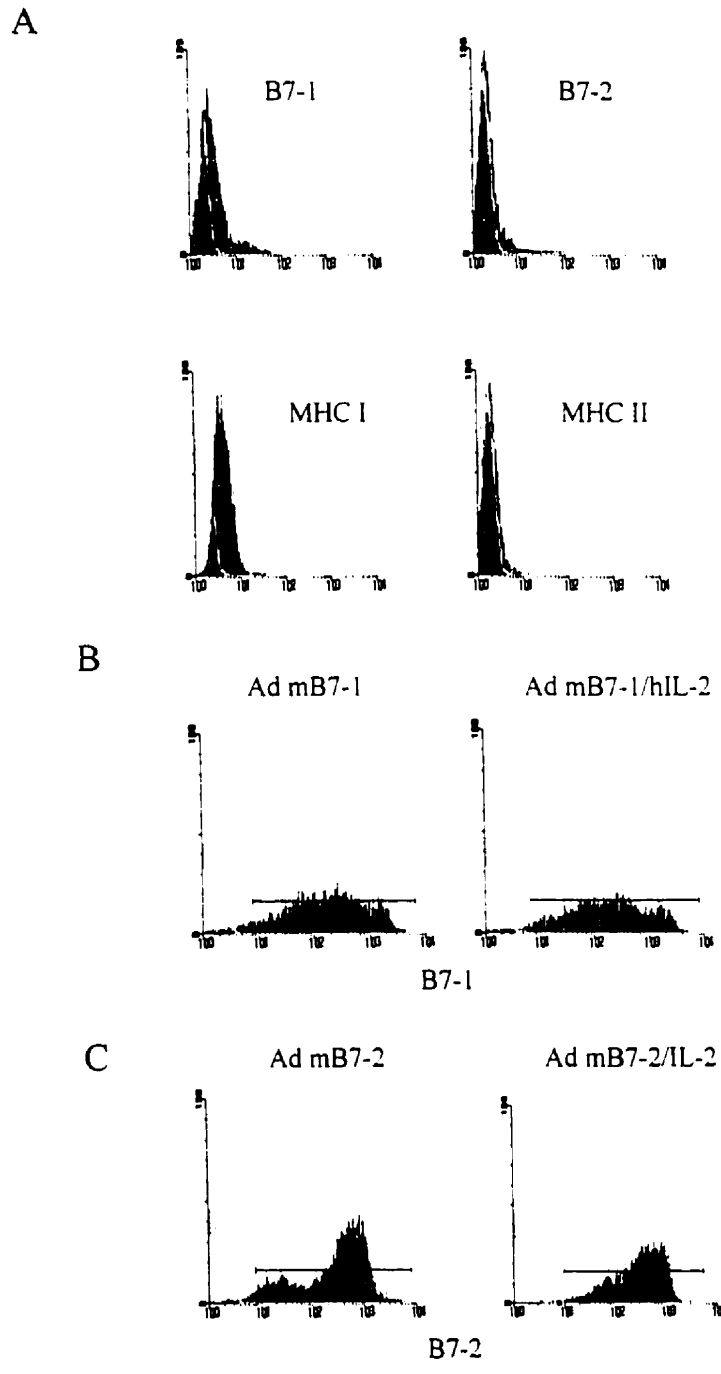


Figure 2

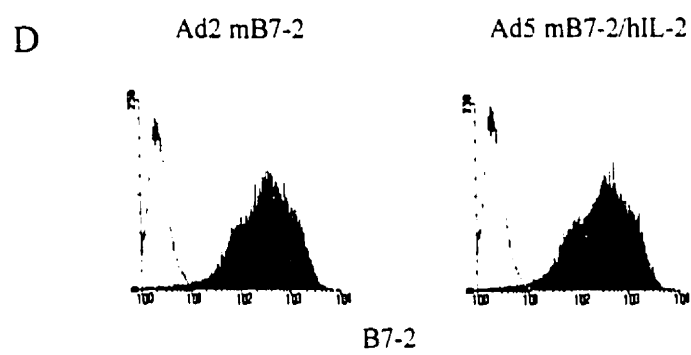
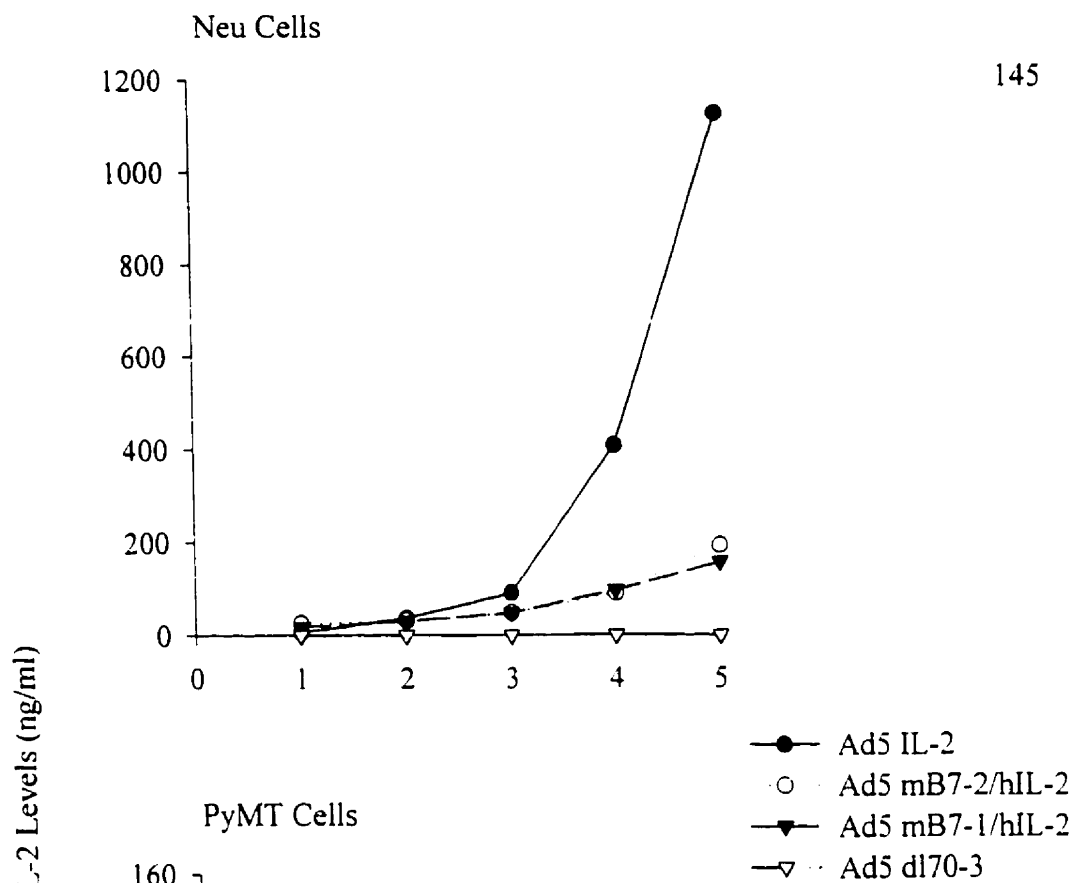


Figure 2

A



B

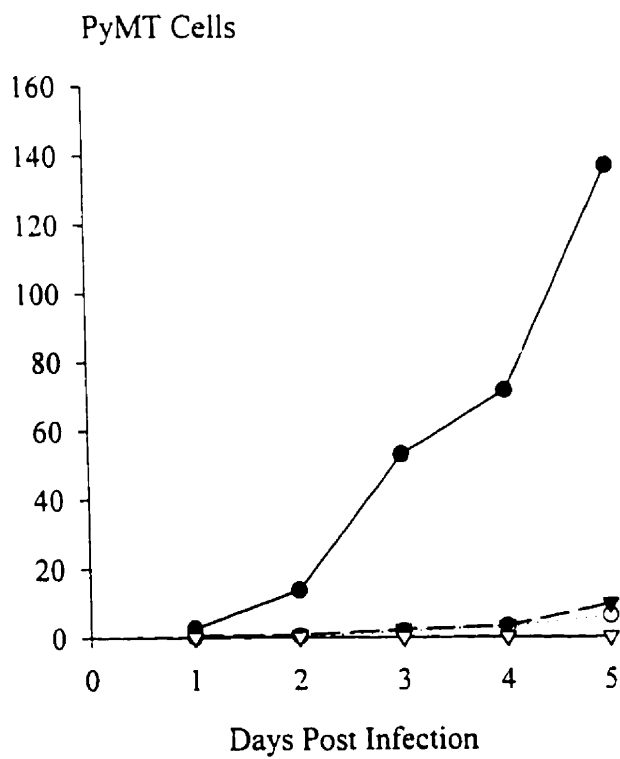


Figure 3

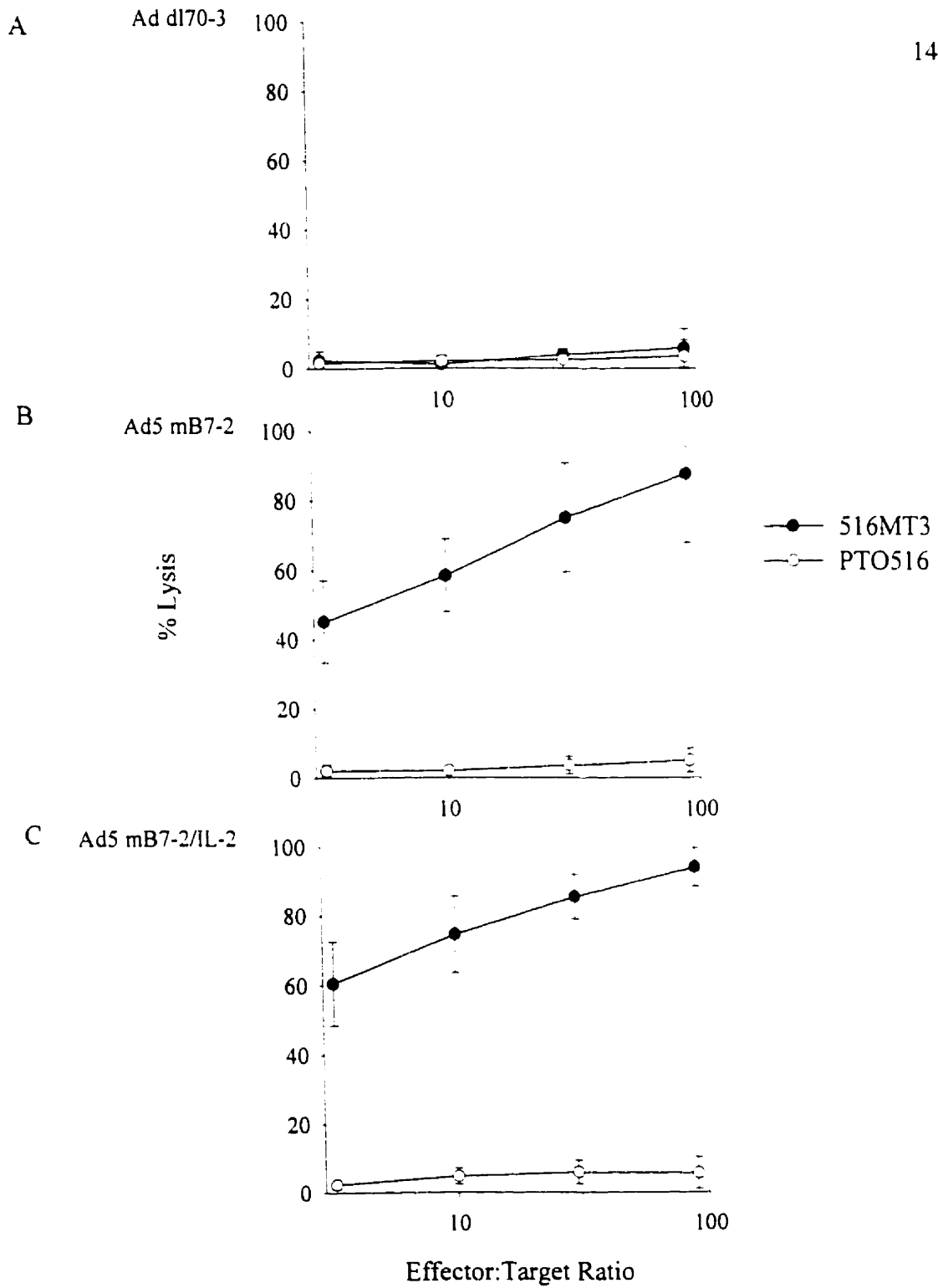


Figure 4

Pulmonary PyMT Metastatic Nodules

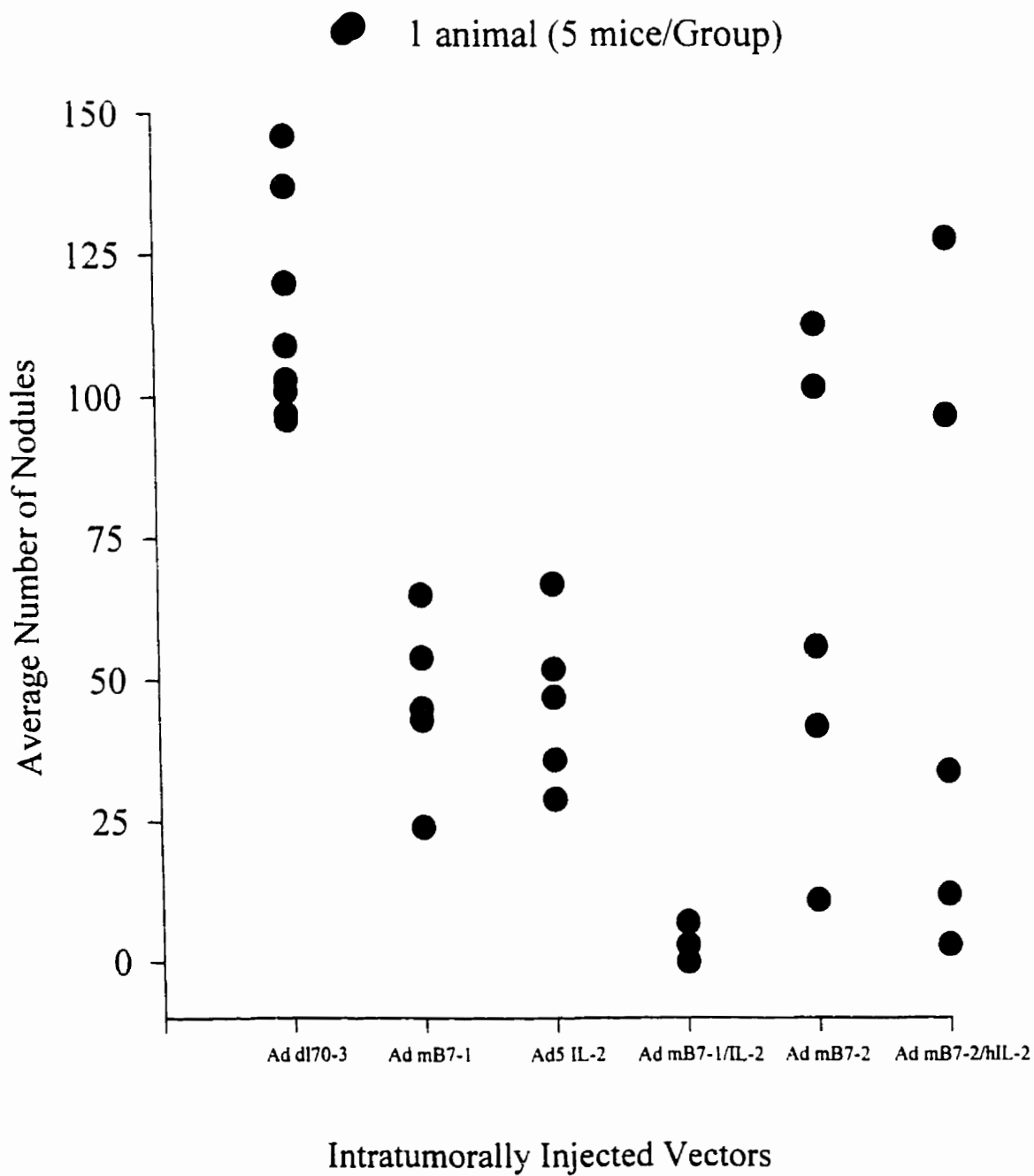


Figure 5

Table 1. Complete regression of PyMT tumors in response to intratumoral injection of Ad vectors

| Treatment | Experiment | | | Percentage Regressed |
|-----------------|----------------------------|-----|-----|----------------------|
| | Survival data (cumulative) | | | |
| Vector | 1 | 2 | 3 | |
| Ad dl70-3 | 0/5 | 0/5 | 0/5 | 0/15 |
| Ad mB7-2 | 2/5 | 1/5 | 1/5 | 4/15 (26%) |
| Ad mB7-2 /hIL-2 | 3/5 | 2/5 | 2/5 | 7/15 (47%) |

Table 2. Complete regression of Neu (NDL 8142) tumors in response to intratumoral injection of Ad vectors.

| Treatment | Experiment | | | | Percentage Regressed |
|-----------------|----------------------------|-----|-----|-----|----------------------|
| | Survival data (cumulative) | | | | |
| Vector | 1 | 2 | 3 | 4 | |
| Ad dl70-3 | 0/5 | 0/5 | 0/5 | 0/5 | 0/20 |
| Ad IL-2 | 0/5 | 0/5 | 0/5 | 0/5 | 0/20 |
| Ad mB7-1 | 0/5 | 0/5 | 0/5 | 0/5 | 0/20 |
| Ad mB7-1/hIL-2 | 2/5 | 3/5 | 4/5 | 3/5 | 13/20 (65%) |
| Ad mB7-2 | 2/5 | 2/5 | 1/5 | | 5/15 (33%) |
| Ad mB7-2 /hIL-2 | 4/5 | 2/5 | 3/5 | | 9/15 (60%) |

Chapter 4

The Use of a Recombinant Adenoviral Vector Expressing the Chemokine Lymphotactin to Investigate the Effects of Chemokine Function In Vivo.

The following article, entitled "Adenoviral mediated gene transfer of lymphotactin to the lungs of mice and rats results in the infiltration and direct accumulation of CD4⁺, CD8⁺ and NK cells", is submitted for publication in the Journal of Immunology, 1998. This publication describes the first use of a recombinant adenoviral vector constructed to express lymphotactin. This study provides data on the effects of over-expressing lymphotactin in the lungs of mice and rats.

The work presented in this study and the manuscript preparation was performed by the author of the thesis. Drs. Y. Wan and Z. Xing provided useful discussion on the results of this work. Dr. A. Zlotnik provided a plasmid containing the lymphotactin cDNA used to construct the Ad5 E1 murine lymphotactin vector. The vector system originated in the lab of Dr. Graham and supervision was provided by Dr. Jack Gauldie resulting in the multiple authorship of this paper.

Running Title:

Chemotaxis of CD4⁺, CD8⁺ and NK cells by lymphotactin

Full Title:

Adenoviral mediated gene transfer of lymphotactin to the lungs of mice and rats results in the infiltration and direct accumulation of CD4⁺, CD8⁺ and NK cells¹.

Authors:

Peter C. R. Emtage^{*§}, Zhou Xing^{*}, Yonghong Wan^{*}, Albert Zlotnik[#], Frank L. Graham^{**†} and Jack Gauldie^{**}.

^{*}Department of Pathology, McMaster University, Hamilton, Ontario

[†]Department of Biology, McMaster University, Hamilton, Ontario

[#]DNAX, Palo Alto, San Francisco California, USA.

[‡]Address correspondence and reprint requests to Dr. Jack Gauldie, Department of Pathology, 1200 Main St. W., Hamilton, Ontario Canada L8N-3Z5.

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[¶]F. L. G. is a Terry Fox Research Scientist of the NCIC (Canada).

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Keywords: Chemokines
Chemotaxis
T Lymphocytes
NK Cells

Abstract

Chemokines are small 8 to 12 kDa chemotactic cytokines that were initially characterized for their ability to control leukocyte trafficking and to a lesser extent leukocyte function. Lymphotactin was first described as a T lymphocyte specific chemotactic factor. However, it has since been shown to also be a potent attractant for natural killer (NK) cells. The chemotactic properties of lymphotactin suggested from *in vitro* data prompted us to study the *in vivo* activity of this chemokine. We constructed an adenovirus vector expressing murine lymphotactin (Ad mLym) and used this construct to over express lymphotactin in the lungs of both mice and rats with similar outcomes. In brief, the accumulation of CD4⁺, CD8⁺ T cells and NK cells surprisingly demonstrated slow kinetics, uncharacteristic of the chemo-attractant potential seen with other chemokines. Lymphocyte accumulation in the lung was not evident prior to 24 hours after gene transfer and reached a peak by day 7 in mice and day 14 in rats. Interestingly, the cellular infiltrate recruited to the lung by lymphotactin was a heterogeneous mixture of lymphocytes, monocytes and neutrophils. Administration of Ad mLym to Balb/c SCID mice demonstrated that the presence of monocytes and neutrophils in the BAL of wild type Balb/c mice was likely due to the action of lymphotactin on lymphocytes. These findings extend the previous *in vitro* findings on the activity of lymphotactin and provide a model for studying the local effects of over-expressing chemokines in various tissues *in vivo*.

Introduction

Chemokines, encompass a family of chemotactic cytokines, small 8 to 12 kDa proteins initially characterized for their ability to modulate leukocyte trafficking and to a lesser extent leukocyte function (1). To date, more than 50 human and rodent chemokines have been identified and placed into 4 groups. Classification depends largely on the structural aspect of the chemokine rather than the genetic relationship (1,2). Chemokines are grouped depending on the placement and presence of cysteine residues within highly conserved regions of the molecule. Of these classes, the CXC and CC families are best characterized for their attractant activity on various cell types of the hematopoietic system. The C family, has only one cysteine residue at the N-terminal and C-terminal domains with lymphotactin as the only known member of this family (3).

Lymphotactin (Lym) was first described as a T lymphocyte specific chemotactic factor (3). However, it has since been shown to also be a potent attractant for natural killer (NK) cells (4,5) and is expressed by a variety of immune effector cells (3-12).

The chemotactic properties of lymphotactin suggested from *in vitro* data prompted us to study the *in vivo* activity of this chemokine in a setting similar to that expected to occur during an ongoing lymphocyte mediated reaction. We constructed a replication deficient adenovirus vector expressing murine lymphotactin (Ad mLym) and used this vector to over-express lymphotactin in the lungs of both mice and rats eliciting

similar effects in both species. The accumulation of CD4⁺, CD8⁺ and NK cells demonstrated slow kinetics, as compared to the chemo-attractant potential seen with CXC and CC chemokines in our laboratory (13,14). Lymphocyte accumulation became evident only after 24 hours from vector instillation and reached a peak by day 7 in mice and day 14 in rats. Control vector demonstrated none or minimal cellular infiltration. The cells accumulating in the lung consisted of a heterogeneous mixture of lymphocytes, monocytes and neutrophils. Administration of Ad mLym to Balb/c SCID mice demonstrated that the presence of monocytes and neutrophils in the lung lavage of wildtype Balb/c mice was most likely due to the action of lymphotactin on the recruited lymphocytes. These findings extend the previous *in vitro* findings on the activity of lymphotactin and provide a model for studying the local effects of over-expressing lymphocyte attracting chemokines in adult animals.

Animals

Six to eight week old Balb/c mice, Balb/c SCIDs and Sprague-Dawley rats weighing 280 g were purchased from Charles River and Taconic Laboratories respectively and housed in a pathogen free facility until use.

Construction of Ad5 mLym

The cDNA for murine lymphotactin was (kindly provided by Dr. Zlotnik, DNAX Corp. Palo Alto, California) excised from the plasmid pJFE14 (3) using Bst XI-Not I restriction enzymes. The lymphotactin fragment was blunt ended with Klenow Large Fragment (NEB) and subcloned into the Eco RV digested shuttle plasmid pDK6 (15) to create pDK6 mLym (Figure 1). The shuttle plasmid pDK6 mLym was amplified and then purified by alkaline lysis and cesium chloride gradient centrifugation. Purified plasmid was then combined with the rescue plasmid pBHG10 (16) and cotransfected into 293 cell to produce Ad5 mLym (Figure 1). A description of the construction of the control vector Ad5 dl70-3 can be found in Addison et al. (17).

Animal Models

Adenoviral vectors were administered to Balb/c mice and Spargue-Dawley rats as follows. Ad5 mLym and Ad5 dl70-3 (E1 deleted control virus) vectors were diluted with sterile PBS pH7.4 to give a total of 1×10^7 pfu per mouse and 1×10^8 pfu per rat.

Vectors were intra-nasally administered to mouse lungs at a dose 1×10^7 pfu of either Ad5 mLym or Ad5 dl70-3 in a total of 20 μ l per mouse. Rats received 1×10^8 pfu in a total of 300 μ l for both vectors, however, the route of delivery was different. In rats, viral infections of lungs were established by intra-tracheal administration of adenoviral vectors as previously described (13, 14, 19). The doses of virus used in these experiments were determined from dose response studies in which 1×10^7 pfu (mice) and 1×10^8 pfu (rats) were observed to result in minimal adenovirus mediated inflammation and allowed for a better assessment of biological activities of lymphotactin transgene protein. In these animals, Ad vector administration leads to gene expression in the bronchial and bronchiolar epithelial cells (19, 20).

Lymphotactin mRNA expression assessed by Northern Blot Analysis and RT-PCR

The production of adenoviral derived lymphotactin was assessed at the level of mRNA by infecting A549 cells (with both control Ad5 dl70-3 and Ad5 mLym vectors; A549 American Type Culture Collection, Rockville, MD: CCL-185). Total RNA was prepared from A549 monolayers infected with the vectors as per the GIBCO BRL protocol for TRIZOL.

The expression of lymphotactin mRNA in the lung tissues of rats and mice infected by Ad5 mLym and Ad5 dl70-3 was assayed by Northern blot analysis. Lung tissue from rats and mice was snap frozen in liquid nitrogen at the time of recovery and stored at -75° C. The frozen tissue was homogenized in 10ml of TRIZOL (GIBCO BRL) and total

RNA was prepared as outlined in the accompanying protocol (GIBCO BRL). Fractionation of the RNA samples from each time point was performed using MOPS/Formaldehyde agarose gel electrophoresis. Fractionated samples were transferred to nylon membranes using capillary transfer and probed with the full length murine lymphotactin cDNA (3).

In order to assay Ad5 mLym specific lymphotactin transcripts, total RNA extracted as outlined above was reverse transcribed using the enzyme Superscript (GIBCO BRL) and the accompanying protocol. PCR primers were constructed to the 5' most nucleotide sequence of murine lymphotactin (5' AGACTTCTCCTCCTGACTTTCCTG 3') and to a region pertaining to the SV40 polyadenylation signal (5' GCTTTATTTGTAACCATTATAAGCTG 3') in the expression cassette. This primer design allowed us to specifically identify the lymphotactin transcripts derived from the adenoviral construct and ignore endogenous lymphotactin transcripts since the endogenous lymphotactin mRNA would not have the SV40 poly A signal. PCR reactions to demonstrate Ad5 mLym specific transcripts were performed as follows; denaturation at 94°C for 4 minutes (initial denaturing step), 55°C annealing for 30 seconds and extension at 72°C for 1 minute, denaturation at 94°C for 1 minute. All PCR reaction were performed on a Perkin Elymer Cetus DNA Thermal Cycler. To semi-quantitatively assess the level of vector derived lymphotactin PCR product, we amplified the highly conserved house keeping gene G6PDH from the same RNA

samples (the sense primer is 5' GGAGGCCATGTAGGCCATGAGGTC 3' and the anti-sense primer is 5' AATGCATCCTGCACCACCAACTGC 3'). Using G6PDH afforded us an internal control to estimate the relative differences in cDNA concentrations added to each reaction. The sizes of the PCR products for lymphotactin and G6PDH are 579 and 555 base pairs respectively.

Bronchoalveolar Lavage (BAL)

Rats were sacrificed at 1, 2, 3, 4, 7, 14 and 21 days after infection, while mice were sacrificed at days 1, 4 and 7. Bronchoalveolar lavage was performed as described previously (19). The cellular pellet obtained from BAL collection was resuspended in 5ml of PBS and counted to determine the total cell number and differential cell counts. Differential cell counting was performed using LeukostatTM (Fisher Diagnostics) stained cytopins (1x10⁵ cells per slide) from the above mentioned BAL samples. The different proportions of the various cell types were obtained by counting a minimum of 500-600 cells randomly distributed throughout the cytopsin.

Lymphocyte Phenotyping

Ad5 mLym and Ad5 dl70-3 were administered to Balb/c mice and BAL fluids were collected at 4 and 7 day intervals. The cellular component was pelleted at 1000 rpm for 5 min and resuspended in 90 μ l of PBS supplemented with 0.2% BSA. Phenotypic identification of lymphocyte subsets was performed by FACs. In short, 10⁶ cells from Ad5 mLym and Ad5 dl70-3 treated mice (to obtain the same number of cells, BAL

fluids from 2 Ad5 dl70-3 treated mice had to be pooled) were incubated with anti-murine CD4 (L3T4, Cy-Chrome labeled), CD8 (Ly-2, FITC labeled) and an anti-murine natural killer cell antibody (DX5, PE labeled) (antibodies were obtained from Pharmingen, San Diego, CA) and prepared for three color FACs analysis using a FACScan flow cytometer (Becton Dickinson, San Jose, CA). The lymphocyte population was gated based on forward scatter and side scatter characteristics and the fluorescent characteristics determined for all samples at the same time with all samples treated identically for each repetition. All antibodies were titered using serial dilutions of each antibody and normal mouse splenocytes, except for the DX5 (NK marker) which was titered on the BAL cells from Balb/c SCID mice infected with Ad5 mLym.

Determination of cell type responsible for monocyte and neutrophil infiltration

Balb/c and Balb/c SCID mice were treated with 1×10^7 pfu of Ad5 mLym and Ad5 dl70-3. BAL fluids were taken at 4 days after vector administration and subjected to total and differential cell counting (using LeukostatTM, Fisher Diagnostics) and FACs analysis (with DX5 anti-NK cell antibody labeled with PE) to determine the phenotype of infiltrating cells.

Lung Histology

Lungs were harvested from rats at days 1, 2, 3, 4, 7, 14 and 21 (mice at days 1, 4 and 7) and fixed by perfusing the lungs with 4% paraformaldehyde and subsequent incubation in 4% paraformaldehyde for 24 hours. After 24 hours the lungs were then serially

passed through ethanol baths to a final ethanol percentage of 70%. Lung tissues were then embedded in paraffin, processed and sectioned. The general morphology of Ad5 mLym and Ad5 dl70-3 treated lungs from both rats and mice was examined by Hematoxylin and Eosin staining.

Immunostaining of Lung Tissue Sections

Immunoperoxidase staining of recombinant Ad5 vector treated lungs was performed using either the mouse anti-monocyte/macrophage antibody (ED1, biotin labeled, Serotec), rabbit anti-CD3 polyclonal antibody (T cell marker, Dako Diagnostic, Canada) or an anti-myeloperoxidase antibody (recognizes neutrophils, Dako Diagnostic, Canada). Paraffin sections were dewaxed in xylene and passed through an ethanol series. Incubating all samples in H₂O₂ methanol-HCL for 30 minutes blocked endogenous peroxidase activity. All slides were subsequently washed in 70% ethanol, distilled water and finally Tris-buffered saline, pH 7.6 (TBS buffer). Specimens were then blocked with non-immune serum and incubated with primary antibodies for 1 hour. A second biotinylated antibody was then applied, incubated and thereafter treated with streptavidin-peroxidase conjugate. All tissues were then incubated with substrate-chromagen mixture for color development and counterstained with hematoxylin for tissue staining.

Results

Construction of Recombinant Adenoviral Vector Ad5 mLym

The recombinant replication deficient adenoviral vector, which expresses murine lymphotactin, was constructed as outlined in figure 1. Using a co-transfection strategy, the cassette expressing murine lymphotactin was inserted into the E1 deleted region of the human adenovirus type 5 genome. The lymphotactin open reading frame was flanked by the murine cytomegalovirus immediate-early promoter (mCMV) and a simian virus 40 polyadenylation signal (SV40). Due to a lack of suitable reagents for detecting murine lymphotactin protein we characterized the viral vector by its ability to produce lymphotactin transcripts in various cell lines (Figure 2A).

Vector derived lymphotactin expression in the lung after Ad5 Lym instillation

The duration and steady state level of adenoviral vector derived lymphotactin mRNA expression was assessed in the lungs of Sprague-Dawley rats and Balb/c mice using RT-PCR. We decided to use RT-PCR since the mRNA sizes for both murine and rat lymphotactin are very similar. The primers were designed to detect mRNA derived from the vector and thus discriminate between the transferred gene and the endogenous gene. Figures 2B and C demonstrates the abundance of cDNA amplified by primers designed to specifically detect Ad5 mLym derived murine lymphotactin transcripts from total RNA extracted and reverse transcribed from lung tissues of rats and mice sacrificed at varying time points. Included in figures 2B and C is the resultant PCR

product for the internal control G6PDH to allow for the quantification of lymphotactin PCR products.

Local effects of lymphotactin over-expression on cellular responses in the lung

To determine if over-expressing lymphotactin has effects in the lungs of mice and rats, BAL fluid samples were collected from animals at various time points after lung administration of Ad5 mLym (1×10^7 for mice and 1×10^8 for rats) and cell analysis carried out. As shown in figure 3 (A and B) both mice and rats responded to lymphotactin over-expression in a qualitatively similar fashion. Cellular accumulation starts after 24 hrs, peaks at day 4 for mice or day 7 for rats and then starts to decline. Compared to the Ad5 mLym treated animals, the E1 deleted control virus (Ad5 dl70-3) does not induce significant levels of cellular accumulation. In mice at 4 days post-infection with the lymphotactin vector the cells of the BAL fluids included monocytes, neutrophils and lymphocytes (Figure 4A and B). Since we were interested in the activity of lymphotactin as a chemotactic factor for lymphocytes we assessed the lymphocyte population in the BAL of Ad5 mLym treated mice at day 4. These day 4 BAL fluids had an average of 9×10^5 lymphocytes compared to the control group, which had an average of 2×10^3 cells. A significant infiltration of lymphocytes was also observed at day 7 such that Ad5 mLym treated mice had an average of 1×10^6 lymphocytes compared to an average of 370 lymphocytes in control vector treated animals.

The activity of lymphotactin over-expression in the lungs of rats followed similar kinetics to that observed for mice. Infiltration of cells in response to lymphotactin into the BAL of these animals begins at day 2 post-infection and is characterized by an average lymphocyte count of 2×10^5 (Figure 5A and 5B). Lymphocyte accumulation peaked in rats at day 14 with an average of 9×10^6 lymphocytes and by day 21 the lymphocyte population declined significantly to 6×10^5 . In contrast, the control treated mice showed no significant change in lymphocyte accumulation over the time course of the experiment (21 days).

Phenotypic characterization of infiltrating lymphocytes

To further characterize the chemotactic activity of lymphotactin *in vivo*, we determined the lymphocyte phenotypes in the BAL infiltrate at days 4 and 7 in mice by FACS analysis. In mice infected with Ad5 mLym, BAL lymphocytes at the 4 day time point showed CD4⁺ cells 45%, CD8⁺ cells 34% and NK cells 21%. These proportions translate to 4×10^5 , 3×10^5 and 2×10^5 cells per each BAL respectively. Interestingly, the relative proportions of CD4⁺, CD8⁺ and NK lymphocytes observed at day 4 changed by day 7 such that CD8⁺ lymphocytes became the predominant lymphocyte subset in the BAL of Ad5 mLym infected animals at day 7 compared to the predominant CD4⁺ population at day 4 (Table 1). At day 7 the lymphocyte population of the BAL consisted of 24% CD4⁺ (4×10^5 CD4⁺ cells), 62% CD8⁺ cells (8×10^5 CD8⁺ cells) and 14% NK cells (2×10^5 NK cells) (Table 1).

Histopathological effects of lymphotactin over-expression in the lung

Prior to day 2, there was no significant inflammatory response in either Ad5 mLym or the control vector treated rats (data not shown). However, by day 3 and remaining until day 14 significant cell infiltration was observed for Ad5 mLym treated animals (Figure 6A and B, both frames represent the gross morphology of day 7 rat lungs treated with the control vector (A) and Ad5 mLym (B) as assessed by H and E staining). The tissue associated infiltration correlated positively with the BAL cellular accumulation (Figure 3). The time points 3, 4, 7 and 14 days are characterized by peribronchial, perivascular and interstitial lymphocytic infiltration revealed by anti-CD3 immuno-histochemical staining at day 7 (Figure 6C). These data are typical of the cell accumulation seen at other time points. Monocytic and neutrophilic infiltration could also be demonstrated at these sites for the day 7 time point (Figure 6D and E). The 21 day time point is similar to the control treated lungs except for minor areas of lymphocyte infiltration characterized by only a few dispersed regions of inflammation similar to that described for the earlier time points (data not shown).

Activity of over-expressing lymphotactin in the lungs of Balb/c SCIDs

Lymphotactin is not known to be chemotactic for neutrophils or monocytes *in vitro* (5). The presence of these cell types in BAL led us to postulate that the infiltrating lymphocytes in Ad5 mLym treated animals expressed a factor(s) capable of inducing the migration and, hence, accumulation of these cell types in the lungs. We examined the

activity of lymphotactin in Balb/c (control group) and Balb/c SCID mice. The Balb/c control group demonstrated similar results to those outlined in figure 4B (for the same time point). However, in the Balb/c SCIDs treated with Ad5 mLym, no lymphocyte, monocyte or neutrophil accumulation could be detected in the BAL fluids. This finding suggests that the accumulation of monocytes and neutrophils in the BAL of mice infected with Ad5 mLym was not a direct effect of lymphotactin but a consequence of lymphocyte accumulation and presumed activation by lymphotactin (Figure 7). However, we observed a significant infiltration of natural killer cells (4×10^5) in the BAL of these SCID animals (as determined by FACs analysis with DX5 antibody). This observation is interesting since it would appear that the activity of lymphotactin on $CD4^+$ and $CD8^+$ T cells results in the accumulation of monocytes and neutrophils. Therefore, it suggests that the activity of lymphotactin on T cells results in different responses than the activity on NK cells.

Discussion

To date there are an estimated 40 to 50 human chemokines, many homologues and unique chemokines have also been described in other animals. Of the four families of chemokines, the gamma or C family, of which lymphotactin is the only known member, is the first chemokine family apparently specific for attracting lymphocytes (3-5). Lymphotactin expression has been demonstrated in very low levels by Northern blot analysis in the spleen and thymus of mice (3). In contrast, it appears that lymphotactin expression in human tissues is more widespread since it has been localized to thymus, spleen, small intestine, lung, colon, ovary, testis and peripheral blood lymphocytes (PBL) (6-8). The expression of lymphotactin has also been demonstrated in specific immune cells. Expression has been localized to CD8⁺ cells, CD4⁺ NK.1.1 T cells, $\alpha\beta$ ⁺ CD4⁺ CD8⁻ T cells, $\gamma\delta$ ⁺ intraepithelial T cells, natural killer cells and mast cells (3-12). Current *in vitro* data shows that lymphotactin is a potent chemotactic factor for human PBL (6) (CD4⁺ and CD8⁺ cells) and murine CD4⁺ and CD8⁺ thymocytes and splenocytes (3). In addition, chemoattractant activity has also been demonstrated on murine and human NK cells (3,4). Considering the specificity of lymphotactin chemotaxis for T lymphocytes *in vitro*, we constructed an adenovirus expressing lymphotactin in order to examine the *in vivo* effects of over-expressing this molecule.

We and others have previously shown that adenoviral delivery to the lung tissue results in expression predominately from bronchial epithelial cells of the respiratory

tract (14,19,20). This approach allows for highly localized expression of lymphotactin for a duration, which lasts over 10-14 days post-infection. Lymphotactin over-expression resulted in a dramatic lymphocytic infiltration into the alveolar and interstitial spaces of the lung. Consistent with lymphocytic accumulation in the parenchyma as determined by anti-CD3 staining of the lung tissues, there were significantly increased numbers of lymphocytes recovered in the BAL in both rats and mice. In mice this infiltration was characterized by the presence of CD8⁺, CD4⁺ and NK cells at day 4, with CD4⁺ cells being most predominant. However, by day 7 this proportion had changed and flow cytometry demonstrated that CD8⁺ cells were predominant. The mechanism for the population switch or expansion of one population over the other is unknown although the presence of adenoviral infection cannot be ruled out as contributing to this switch. The lymphocyte infiltration remains high until day 14 in rats and then returns to basal levels by day 21. The infiltrate is not solely lymphocytes but a mixture of other cell types of which monocytes and neutrophils are a large component. Lymphotactin was previously shown to be lymphocyte specific with no apparent chemotactic effect on monocytes or neutrophils *in vitro* (3,6). Hence, we postulate that these cells were probably attracted to the lung by factors released from the infiltrating lymphocytes. Recently, molecular analysis of T helper type 1 and 2 cells have demonstrated the specific expression of certain chemokine receptors on these cells depending on their helper phenotype (21). It is therefore conceivable that T cells

attracted to the lungs by lymphotactin might express specific chemokine receptors and produce chemokines which result in the accumulation of other mononuclear cells. This was supported by the over-expression of lymphotactin in the lungs of Balb/c SCIDs which resulted in the infiltration of NK cells but demonstrated no monocytic or neutrophilic infiltration. This observation suggests that the monocyte and neutrophil recruitment to the lungs was directed by the activity of CD4⁺ and/or CD8⁺ cells having been acted on by lymphotactin. The recruitment of monocytes and neutrophils to the lung in response to lymphotactin over-expression is interesting since a single *in vivo* administration of recombinant lymphotactin to the peritoneum or foot pads of mice resulted in no inflammatory response and demonstrated only a lymphocytic infiltration (5). It is possible that prolonged exposure of lymphocytes to lymphotactin can cause activation, which would not be present in tissue treated with recombinant lymphotactin (24-48 hours). The changes observed in the Ad5 mLym treated animals are transient and by day 21 in rats the inflammation has resolved, such that only a few minor areas of inflammation remain. There was no evidence of prolonged inflammation or fibrosis as a result of expression of lymphotactin.

It is important to note that the activity of vector derived lymphotactin appears different between rats and mice with respect to the delay of lymphocyte infiltration seen in rats (days 14 compared to day 7 mice). This difference might result from the affinity of murine lymphotactin for the rat lymphotactin receptor, such that there is a lower

affinity when compared to the native receptor in mice allowing for suboptimal chemoattractant potential.

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Legends

FIGURE 1. A schematic diagram of the construction of Ad5 mLym. For a more detailed vector construction description, see Materials and Methods.

FIGURE 2. RT PCR and Northern blot analysis of Ad5 mLym expression *in vitro* and *in vivo*. A549 cells were infected with Ad5 mLym, total RNA was fractionated by gel electrophoresis and subjected to Northern blot analysis with the murine lymphotactin probe (A). RT-PCR amplification of vector derived lymphotactin from total RNA extracted from the lungs of mice and rats treated for various times (B and C).

FIGURE 3. Total cell numbers from the lungs of mice and rats infected with Ad5 dl70-3 and Ad5 mLym. Mice were administered 1×10^7 pfu Ad5 mLym intra-nasally and the lungs removed on Days 1, 4 and 7 post instillation (A). Rats were infected with 1×10^8 pfu of Ad5 mLym intra-tracheally and the lungs removed on Days 1, 2, 3, 4, 7, 14 and 21 (B).

FIGURE 4. Differential cell counts in lungs of Ad5 mLym treated mice. Lung lavage fluids were recovered at Days 1, 4 and 7 from mice infected with Ad5 dl70-3 (A) and Ad5 mLym (B) and total cell differentials carried out on cytopsin preparations.

FIGURE 5. Differential cell counts in lungs of Ad5 mLym treated rats. Lung lavage fluids were recovered at Days 1, 4 and 7 from rats infected with Ad5 dl70-3 (A) and Ad5 mLym (B) and total cell differentials carried out on cytopsin preparations.

FIGURE 6. Representative photomicrographs of the lung histology from rats infected with Ad5 dl70-3 and Ad5 mLym. Rats were infected with 1×10^8 pfu of either Ad5 mLym or Ad5 dl70-3 and the lungs were removed and subjected to H and E or immunohistochemical stains. H and E stains of the lungs of Ad5 dl70-3 and Ad5 mLym treated rats (A and B). Anti-CD3 stain to identify lymphocytes (C). Anti-ED1 stain to localize monocytes and macrophages and an anti-myeloperoxidase stain to demonstrate neutrophils (D and E).

FIGURE 7. Differential cell counts in lungs of Ad5 mLym treated Balb/c and Balb/c SCID mice. Lung lavage fluids were recovered at Day 4 from Balb/c or Balb/c SCID mice infected with 1×10^8 pfu of Ad5 mLym and cell differentials carried out on cytopsin preparations.

Construction of the Ad5 E1 mLym vector

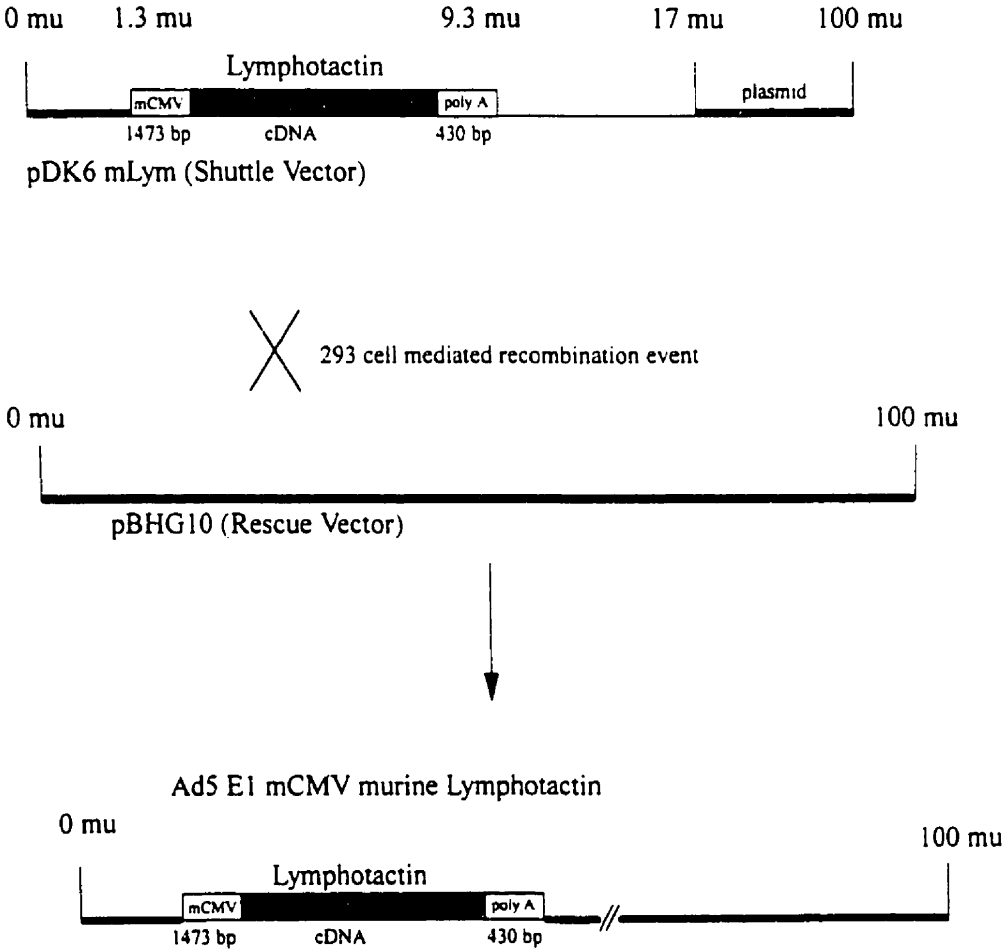
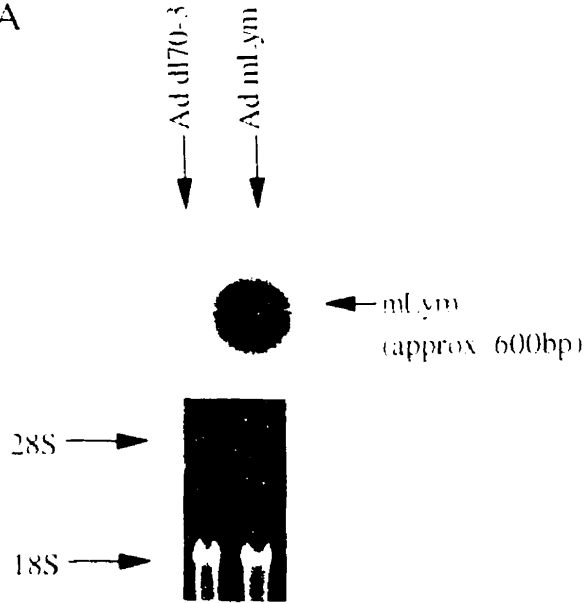
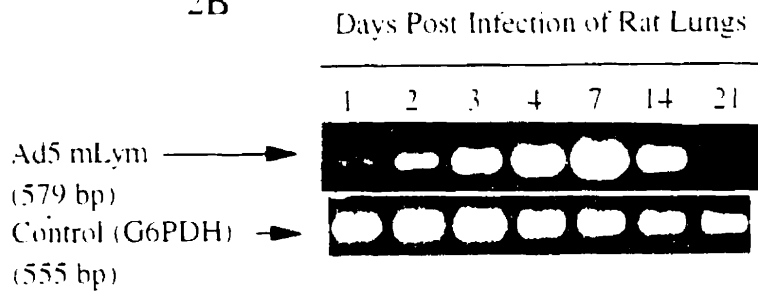


Figure 1

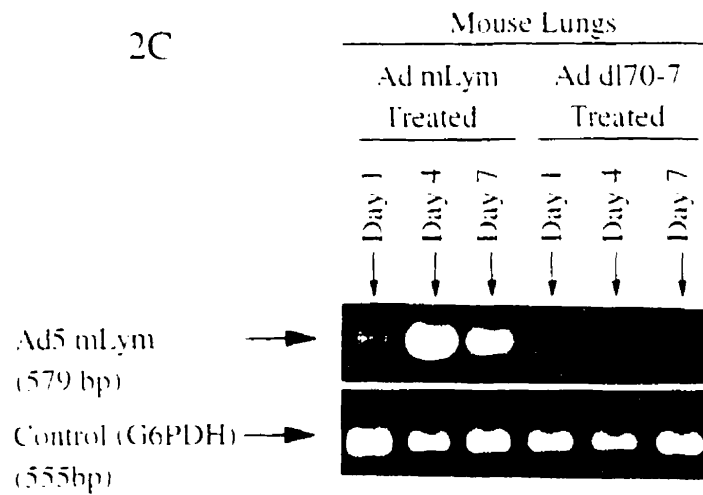
2A



2B



2C



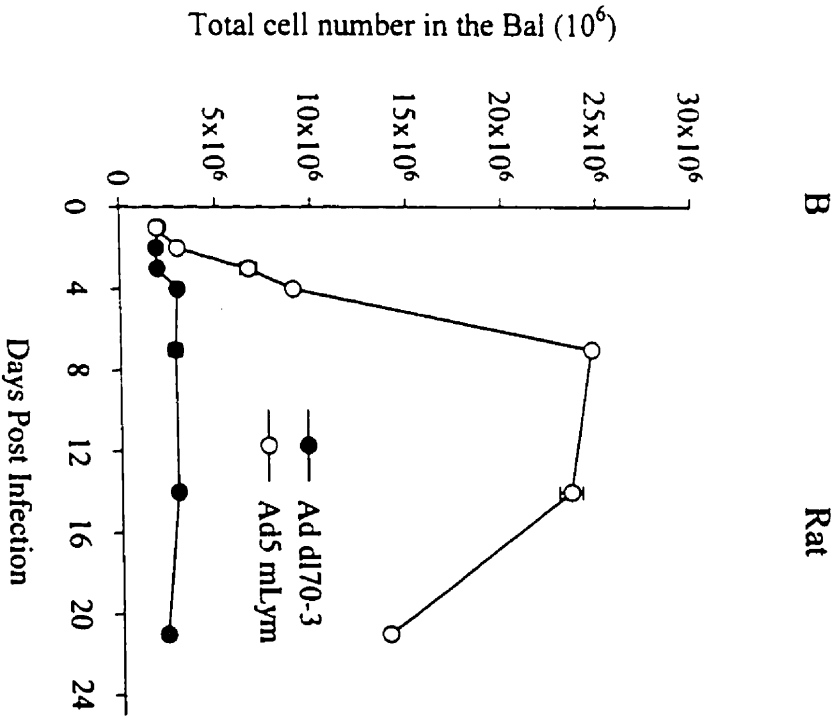
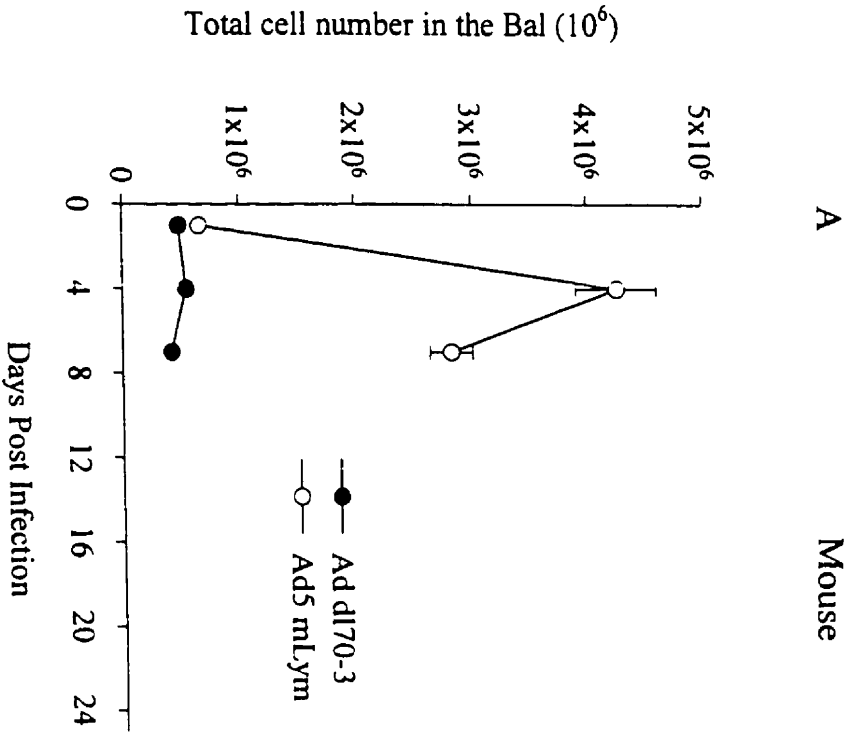
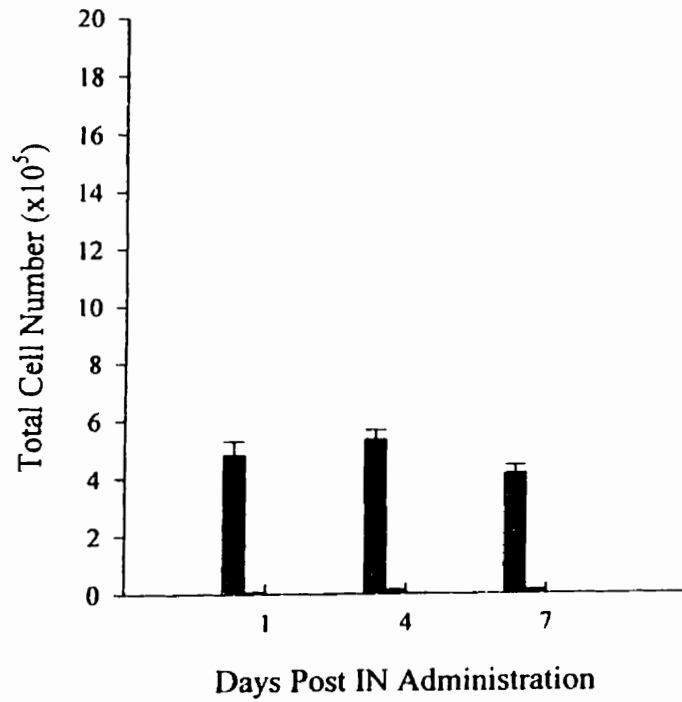


Figure 3

A Cell Type Response to Ad dl70-3



B Cell Type Response to Ad5 mLym

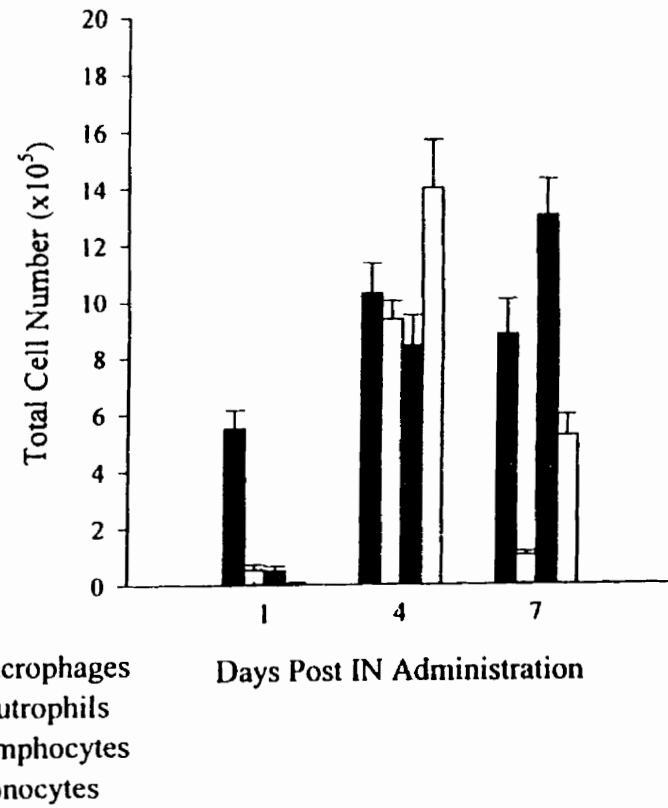
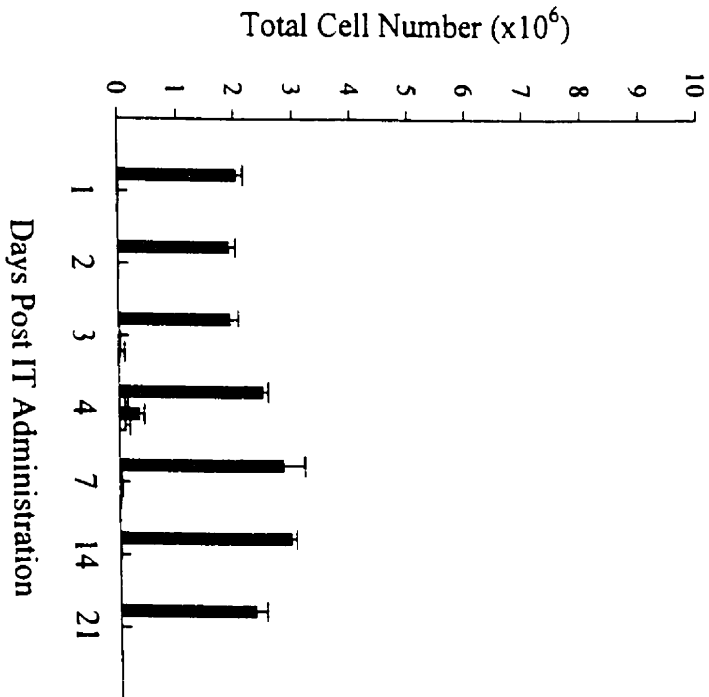


Figure 4

Cell Type Response to Ad5 dl70-3



B

Cell Type Response to Ad5 mLym

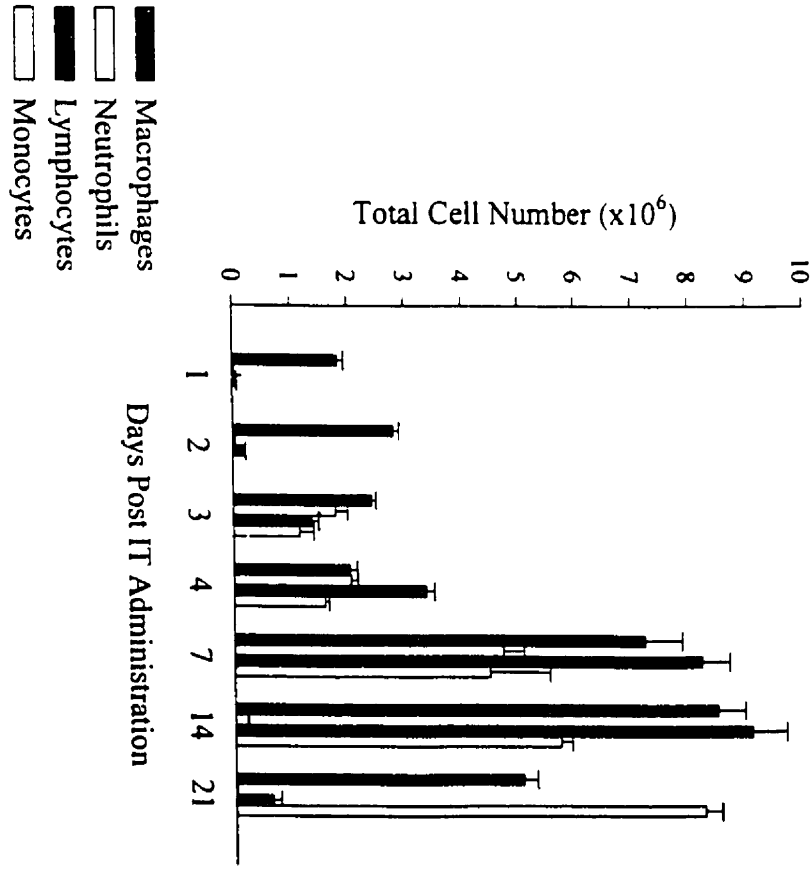
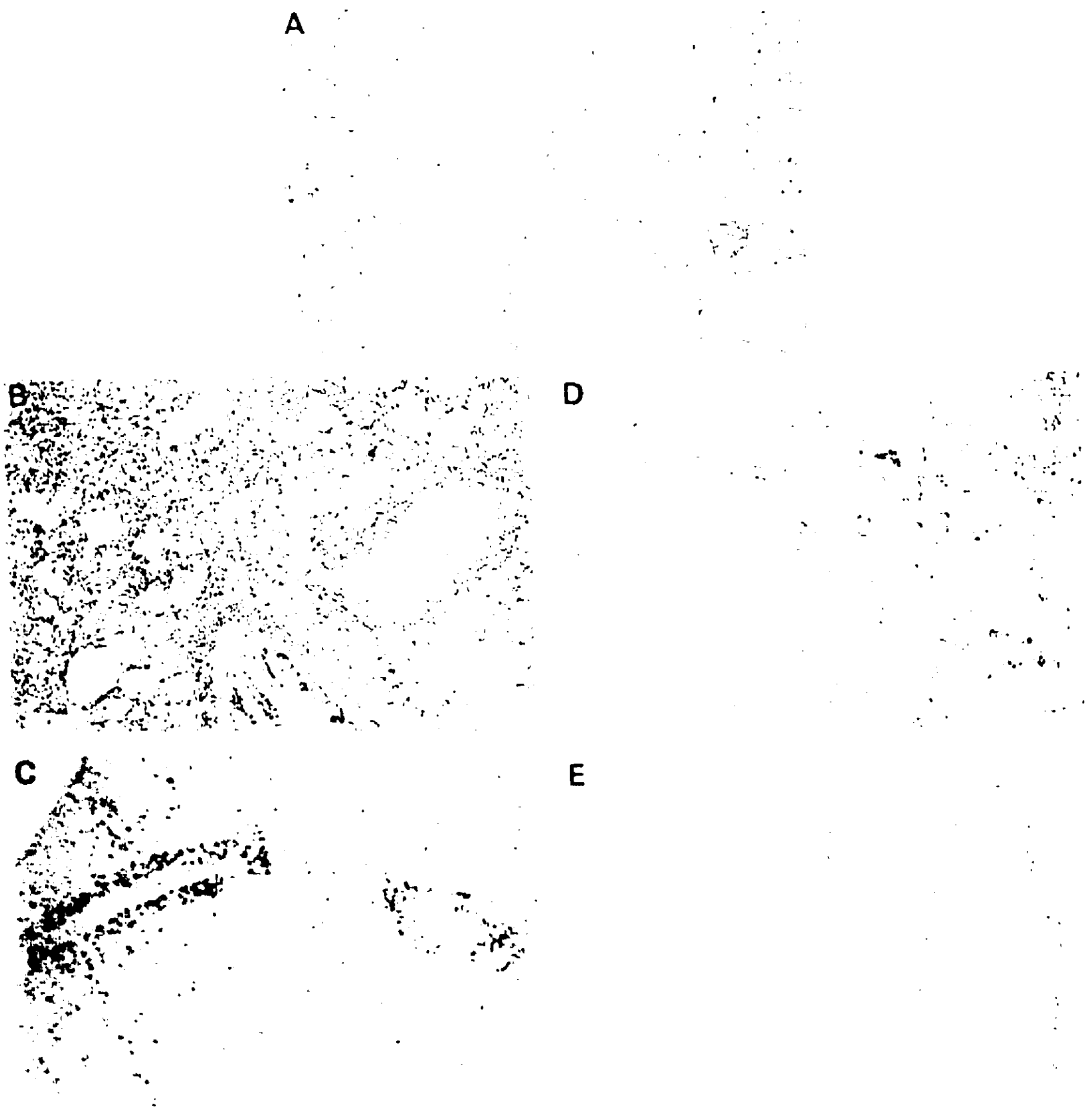


Figure 5



Response to Ad5 mLym in Balb/c SCIDs

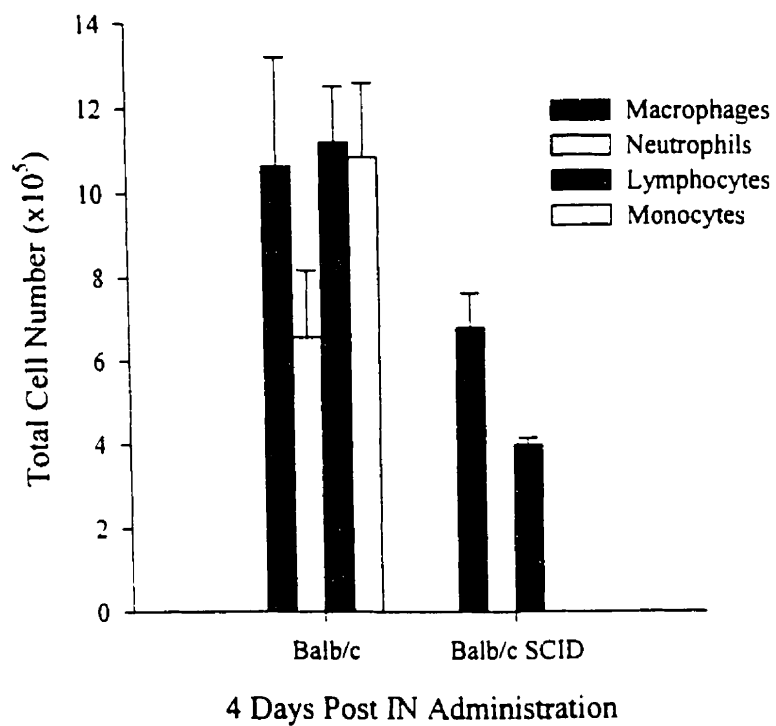


Figure 7

Table I. FACS analysis for lymphocyte phenotypes at day 4 and day 7 in BAL fluid of Balb/c mice treated with Ad5 mLym.

| | Cell Type | Percentage of Lymphocyte Population | Total Cell Number of Individual Phenotypes | Standard Error ^a |
|-------|-----------|-------------------------------------|--|-----------------------------|
| Day 4 | CD4 | 45% | 442050 | ± 34042.18 |
| | CD8 | 34% | 329549 | ± 20272.52 |
| | NK | 21% | 200630 | ± 47162.27 |
| Day 7 | CD4 | 24% | 360120 | ± 30121.00 |
| | CD8 | 62% | 800238 | ± 67814.12 |
| | NK | 14% | 210428 | ± 18921.43 |

^a represents the standard error from three independent experiments

Chapter 5

Lymphotactin in combination with IL-2 or IL-12 greatly enhances the anti-tumors activity of IL-2 or IL-12 alone.

The following article, entitled "Adenoviral vectors expressing lymphotactin and IL-2 or lymphotactin and IL-12 synergise to facilitate tumor regression in murine breast cancer models", is submitted for publication in the Human Gene Therapy, 1998. This publication describes the first use of double recombinant adenoviral vectors constructed to express lymphotactin in combination with IL-2 or IL-12. Data is provide on the effects of these vectors in inducing complete tumor regression.

The work presented in this study and the manuscript preparation was performed by the author of the thesis. Dr. Y. Wan provided useful discussion on the results of this work. Dr. A. Zlotnik provided a plasmid containing the lymphotactin cDNA used to construct the Ad5 E1 murine lymphotactin vector. Dr. M Hitt constructed the Ad5 mIL-12 vector that is used as a control in this study. Dr. W. Muller generated the NDL 8142 tumor cell line used in this paper. The vector system originated in the lab of Dr. Graham and supervision was provided by Dr. Jack Gauldie resulting in the multiple authorship of this paper.

Title:

Adenoviral vectors expressing lymphotactin and IL-2 or lymphotactin and IL-12 synergise to facilitate tumor regression in murine breast cancer models.

Running Title:

Tumor immuno-therapy with combination Ad vectors

Names:

P.C.R. Emtage¹, Y. Wan¹, M. Hitt², F. L. Graham^{1,2}, W. J. Muller², A. Zlotnik³
and J. Gauldie¹.

Departments of ¹Pathology and ²Biology, McMaster University, Hamilton, Ontario L8S4K1, Canada and ³DNAX Corp. Palo Alto, California, USA.

Address reprint requests to:

Dr. J. Gauldie

Department of Pathology

Faculty of Health Sciences

McMaster University

1200 Main Street West

Hamilton, Ontario L8S-4K1

Canada

ABSTRACT

We have previously demonstrated that intratumoral injection with Ad vectors expressing IL-2 or IL-12 can induce regression in a murine breast cancer model. These IL-2 or IL-12 induced anti-tumor responses were mainly mediated by Ag specific T cells. Lymphotactin is a novel lymphocyte chemokine that can cause local accumulation of CD4, CD8 and NK cells. We hypothesized that addition of lymphotactin may enhance the anti-tumor immune responses induced by locally produced IL-2 and IL-12 as we have previously shown. To this end we constructed two double recombinant adenoviral vectors expressing lymphotactin along with either IL-2 (Ad5 Lym/IL-2) or IL-12 (Ad5 Lym/IL-12). Subcutaneous injection of Polyoma Middle T (PyMT) or Neu (8142) transgenic derived breast adenocarcinoma cells, in the hind flank of FVB/n mice results in the formation of tumor nodules in 14-21 days. We show that these constructs elicit potent anti-tumor responses when administered intratumorally. The anti-tumor responses are long lasting as determined by re-challenge experiments and hence demonstrate a protective immunity. These observations indicate that by augmenting the anti-tumor response with adenoviral vectors expressing lymphotactin in combination with IL-2 or IL-12 is a novel way to enhance immunotherapeutic approaches.

OVERVIEW SUMMARY

Intra-tumoral administration of single cytokine gene expressing adenoviral vectors to induce anti-tumor responses is of limited efficacy. The construction of vectors for the delivery of combinations of immuno-modulatory molecules to the tumor site could enhance anti-tumor effects. We examined the anti-tumor activity of Ad-vectors constructed to express combinations of lymphotactin (chemokine) with IL-2 or IL-12 in two murine breast adenocarcinoma models. The combination Ads were compared to single cytokine and chemokine expressing vectors to assess the efficacy of the combination. Combining both lymphotactin and IL-2 or lymphotactin and IL-12 in the same vector significantly enhanced the anti-tumor activity of IL-2 or IL-12.

INTRODUCTION

Tumors can evade elimination in immune competent organisms suggesting that mechanisms exist by which tumors can escape normal immuno-surveillance, persist and grow. In addition to the use of recombinant interleukins and/or growth factors, attempts to augment immune responsiveness against tumors have led to the recent development of many immune cytokine gene delivery protocols and the introduction of genes encoding various immuno-modulatory and stimulatory molecules (Nabel *et al.*, 1993; Foreman *et al.*, 1993; Dranoff *et al.*, 1993; Tahara *et al.*, 1994; Baskar *et al.*, 1995; Matulonis *et al.*, 1995; Addison *et al.*, 1995a,b, Bramson *et al.*, 1996; Putzer *et al.*, 1997; Emtage *et al.*, 1998). Most recently, a great number of cancer immuno-therapy studies have focused on the use of cytokines and co-stimulatory molecules (Freeman *et al.*, 1991; Azuma *et al.*, 1993; Freeman *et al.*, 1993a and 1993b; Chen *et al.*, 1992; Townsend *et al.*, 1993; Yang *et al.*, 1995; Li *et al.*, 1994; Ramarathinam *et al.*, 1994; Townsend *et al.*, 1994; La Motte *et al.*, 1996; Putzer *et al.*, 1997; Emtage *et al.*, 1998). However, it is evident that the use of any one of these molecules alone exhibits limited efficacy in generating an anti-tumor response. Recent data suggests that combinations of these molecules induce tumor regression more efficiently than single molecules (Putzer *et al.*, 1997; Emtage *et al.*, 1998).

The ability to induce an anti-tumor response should be enhanced, in principle, by attracting, to the site of antigen presentation, large numbers of cells capable of eliciting

an effector function upon presentation and activation by tumor antigen. Recently, a new class of chemokine was isolated from human T cell and mouse pro-T cell cDNA libraries (Kelner *et al.*, 1994; Kennedy *et al.*, 1995). Lymphotactin was found to have only one cysteine residue and was placed into a chemokine subclass of its own, the C chemokine. Current *in vitro* data implies that lymphotactin is a potent chemotactic factor for human PBL (Kennedy *et al.*, 1995) (CD4⁺ and CD8⁺ cells) and murine CD4⁺ and CD8⁺ thymocytes and splenocytes (Kelner *et al.*, 1994). In addition, chemoattractant activity has also been demonstrated on murine and human NK cells (Hedrick *et al.*, 1997; Bianchi *et al.*, 1996). We have previously shown (Addison *et al.*, 1995a; Bramson *et al.*, 1996) that recombinant replication deficient adenoviral vectors expressing human IL-2 or murine IL-12 administered intratumorally were partially effective at causing tumor regression in an experimental adenocarcinoma model. The potential for additive or synergistic effects from the expression of a combination of lymphotactin and IL-2 or lymphotactin and IL-12 prompted us to investigate the antitumor effects of intratumoral injection of vectors constructed to express both the chemokine and immune regulating cytokines.

In this study we demonstrate that intratumoral injection of an adenovirus vector (Ad) constructed to express murine lymphotactin (Ad5 mLym) results in 17% complete regression of tumors while an Ad vector expressing IL-2 (Addison *et al.*, 1995a) resulted in complete regression in 25% of tumor bearing mice. Similarly, intratumoral

administration of an Ad vector expressing murine IL-12 resulted in 35% regression. In contrast, administration of dicistronic vectors expressing either a combination of lymphotactin and IL-2 (Ad5 mLym/hIL-2) or lymphotactin and IL-12 (Ad5 mLym/IL-12) resulted in 65% or 75% complete regression in tumor bearing mice respectively. These data demonstrate the potential for incorporating chemokines into current strategies for *in vivo* cancer therapy and show the therapeutic benefits of combining a lymphocyte chemotactic factor along with immune stimulating cytokines into the same adenoviral vector.

MATERIALS AND METHODS

Animals

Six to eight week old FVB/n mice were purchased from Taconic Laboratories and housed in a pathogen free facility until use.

Cell Lines

Cell lines used include the following: PyMT, primary polyoma middle T antigen transformed murine cells obtained from explanted tumors; PTO516, FVB/n kidney derived cells; 516MT3 cells are derived from PTO516 and are stably transformed to express polyoma middle T antigen (Addison *et al.*, 1998); 293, human embryonic kidney cells transformed with adenoviral E1 sequences (Graham *et al.*, 1977); 293N3S, contact independent 293 derivative (Graham *et al.*, 1987); the Neu (NDL 8142) tumor cell line was isolated from a tumor bearing transgenic mouse harboring the NDL 8142 deletion outlined in (Siegel *et al.*, 1994; Siegel and Muller, 1996; Putzer *et al.*, 1998). All cell culture reagents were purchased from GIBCO.

Construction double recombinant Ad vectors

The cDNA for murine lymphotactin was (kindly provided by Dr. Zlotnik, DNAX Corp. Palo Alto, California) excised from the plasmid pJFE14 (Kelner *et al.*, 1994) using Bst XI-Not I restriction enzymes. The lymphotactin fragment was then subjected to PCR to remove the polyadenylation site. PCR was performed using Vent DNA polymerase (New England Biolabs) and the following parameters: denaturation

was accomplished at 94°C for 1 minute; annealing 57°C for 30 seconds; extension at 72°C for 1 minute. This was done using the following sense and antisense primers designed to anneal to the 5' and 3' ends of the lymphotactin open reading frame sequence as published in Genbank; the sense primer 5'-AGACTTCTCCTCCTGACTTTCCTG-3' and the antisense primer 5'-AGGCTGTTACCCAGTCAGGGTTAC-3'. The amplified fragment of 389 base pairs was directly ligated into the Eco RV digested pDK6 shuttle plasmid (Kunskan et al., 1997) to create pDK6 mLym (Figure 1). The shuttle plasmid pDK6 mLym was amplified and then purified by alkaline lysis and cesium chloride gradient centrifugation to obtain DNA for further cloning.

The shuttle plasmid containing mLym (pDK6 mLym) was sequenced, amplified and then subjected to the following manipulation to generate a dicistronic construct expressing mLym and hIL-2. The human IL-2 open reading frame was amplified using the following 5'-phosphorylated primers, 5'-TACAGGATGCAACTCCTGTCYTGC-3' (sense) and 5'-CTAATTATCAAGTCAGTGTGA-3' (antisense). It should be noted that the sense primer is constructed such that the codon underlined (TAC) is the first amino acid immediately after the ATG start codon. The PCR product of 469 bp was then blunt end ligated into the pCITE 2a plasmid (Novagen) which carries the Encephalomyocarditis virus internal ribosomal entry site (IRES). This plasmid was digested with Nco I (recognition site CCATGG) and subsequently blunt ended (blunting

was performed with Klenow polymerase large fragment from NEB) to produce an ATG start codon, prior to ligation with the IL-2 PCR product. Ligation of the IL-2 PCR product with the pCITE 2a plasmid resulted in pCITE hIL2 which carries the hIL-2 open reading frame driven by the IRES sequence.

The pCITE hIL-2 plasmid was used as a template for a second round of PCR. Using the following phosphorylated primer, 5'-TTCCGGTATTTTCCACCATATTG-3' (IRES sense) and the original hIL-2 antisense primer, a PCR product of 979 bp was amplified corresponding to the IRES/hIL-2 hybrid molecule. This PCR product was blunt end ligated into pDK6 mLym digested with *Sai* I (3' to the stop codon of mLym) to produce pDK6 mLym/IRES/hIL-2 (Figure 1). This plasmid was then processed in an identical manner to pDK6 mLym and cotransfected with pBHG10 (Bett *et al.*, 1994) to generate Ad5 mLym/hIL-2. Both Ad5 mLym and Ad5 mLym/hIL-2 were screened using Southern and Northern techniques to characterize the presence of inserted DNA or the production of monocistronic (Ad5 mLym) or dicistronic mRNAs (Ad5 mLym/hIL-2). In the case of Ad5 mLym/hIL-2 Western analysis was performed to detect hIL-2 production. All cloning was confirmed by sequencing.

Construction of Ad5 mLym/mIL-12 was done by using the E1 and E3 regions of the adenovirus genome. The shuttle vector pDK6 mLym was used as the template for PCR and the expression cassette mCMV-lymphotactin-SV40 poly A was amplified using primers designed to the 5' end of the mCMV promoter

(5'ACTCCGCCCGTTTTATGACTAGAACC 3') and the 3' end of the SV40 poly A (5' GATCTTCGATGCTAGACGATCCAGAC 3'). The rescue plasmid pBHG10 was digested with the unique restriction enzyme Pac I and blunt ended with Klenow Large fragment (NEB). For the construction of the pBHG10 E3 mLym rescue vector, we ligated the amplified lymphotactin expression cassette into the blunt ended pBHG10 using T4 DNA ligase (NEB). To construct Ad5 mLym/IL-12, the shuttle vector pMEM12R (contains both the p35 and p40 subunit of IL-12 in an E1 cloning vector Putzer *et al.*, 1997) was co-transfected with the rescue vector pBHG10 E3 mLym into 293 cells to produce Ad5 mLym/IL-12.

Ad5 IL-2, Ad5 dl70-3 and Ad5 mLIL-12

The vector expressing human IL-2 (Ad5 hIL-2) was Ad5 CAIL-2 as described in Addison *et al.*, 1995a; the expressing murine IL-12 vector (Ad5 mLIL-12) was Ad memIL-12R as describe in Putzer *et al.*, 1997 and the control vector Ad5 dl70-3 was outlined in Addison *et al.*, 1995a.

Tumor Cell Preparation and Tumor Induction

A transgenic mouse strain (FVB/n) expressing the polyoma middle T (PyMT) antigen under the control of the mouse mammary tumor virus long terminal repeat (MMTV) promoter was the source of the tumor cells used in the PyMT portion of this study (Guy *et al.*, 1992). Expression of PyMT antigen results in the spontaneous transformation of the mammary epithelium in transgenic animals by 8-10 weeks of age.

These tumors were excised from transgenic mice and subjected to enzymatic digestion to generate a single cell suspension. The single cell suspension of PyMT tumor cells was washed with PBS, and aliquots of 10^6 tumor cells were injected subcutaneously (sc) into the right hind flank of normal syngeneic FVB/n mice.

The Neu (NDL 8142) model was developed from tumors which arise in transgenic mice harboring a deletion in the extracellular domain of the Neu proto-oncogene. The expression of the NDL 8142 protein is restricted primarily to the mammary epithelium by the tissue specificity of MMTV long terminal repeat promoter. The Neu (NDL 8142) mutation has been described previously (Siegel *et al.*, 1994; Siegel and Muller, 1996). A cell line generated from NDL (8142) transgenic tumors was used to establish tumors (7×10^6 cells/tumor) in syngeneic recipient mice.

Quantification of hIL-2 production

PyMT and Neu tumor cells were infected at a multiplicity of infection (MOI) of 10 plaque forming units (pfu) per cell of either Ad5 hIL-2, Ad5 mIL-12, Ad5 mLym/hIL-2 or Ad5 mLym/IL-12. Infected cultures were incubated for 4-7 days and at 24 hr intervals, 200 μ l aliquots were removed and stored at minus 70 $^{\circ}$ C for quantification. Secreted hIL-2 was quantitated using the hIL-2 DuoSet Kit (Genzyme Diagnostics Inc) and IL-12 levels were analysed using the murine IL-12 ELISA Kit from Amersham.

Vector Administration

Palpable tumors (normally 75-150 mm³) arise in tumor cell recipient mice within 16 to 21 days after which time appropriate amounts of virus were injected intratumorally in a volume of 40μl. After injection of adenoviral vectors, tumors were monitored weekly using calipers. The volume of the tumor was calculated from the longest diameter and average width assuming a prolate spheroid. Mice with tumors not responding to vector treatment were sacrificed when the longest diameter exceeded 20 mm. Regressed mice were left for approximately 3 months and then challenged with 1x10⁶ freshly isolated PyMT or 7x10⁶ Neu (NDL 8142) tumor cells on the left hind flank.

Cytotoxic T cell assays

Splenocytes (effectors) were obtained from mice whose tumors had regressed as a result of Ad5 mLym, Ad5 IL-2, Ad5 mIL-12, Ad5 mLym/hIL-2 or Ad5 mLym/IL-12 treatment and co-cultured with 516MT3 cells (stimulators) at a concentration of 1.2 x10⁵ 516MT3 to 1.2 x10⁷ splenocytes for 5 days in 6 well dishes. Serial dilutions of the effector cells were incubated in a v-bottom 96-well plate with 5 x 10³ 516MT3 or PTO516 target cells. Target cells (10⁶) were labeled with 500 μCi of ⁵¹Cr sodium salt in 1 ml of media for 2 hr prior to co-culture with the effector cells. Cells were co-cultured for 5-6 hr at which time 80μl of supernatant was removed for gamma counting. The

percent specific lysis was calculated as follows: $100 \times (\text{experimental cpm} - \text{spontaneous cpm}) / (\text{maximal cpm} - \text{spontaneous cpm})$.

RESULTS

Construction of Recombinant Adenoviral Vectors

Recombinant adenoviral vectors which express murine lymphotactin (Ad5 mLym) alone or in combination with either human IL-2 (Ad5 mLym/hIL-2) or murine IL-12 (Ad5 mLym/IL-12) were constructed as outlined in figure 1A and B. Expression cassettes for lymphotactin or lymphotactin/IL-2 were inserted into the E1 deleted region of the human adenovirus type 5 genome. The lymphotactin expression cassette was cloned into the E3 deleted region and the IL-12 expression cassette was recombined via co-transfection to generate Ad5 mLym/IL-12. In all instances expression cassettes were flanked by the murine cytomegalovirus immediate-early promoter (mCMV) and a simian virus 40 polyadenylation signal (SV40). In the expression cassette for Ad5 mLym/hIL-2, the encephalomyocarditis virus internal ribosome entry site (IRES) (Jang *et al.*, 1988) was placed between the lymphotactin and the hIL-2 coding sequences resulting in a dicistronic DNA fragment. Similarly, the IRES sequence was placed between the p35 and p40 subunits of IL-12 (Putzer *et al.*, 1997) in the Ad5 mLym/IL-12 vector. The IRES functions as an internal ribosome initiation site for the translation of hIL-2 and p40 in the resulting dicistronic mRNAs.

hIL-2 and mLIL-12 Quantification

The expression of hIL-2 from Ad5 mLym/hIL-2 was characterized by Western blot analysis and ELISA quantification. Supernatants from 293 cell cultures infected

with Ad5 mLym/hIL-2 and, for comparison, Ad5 hIL-2 were size fractionated by SDS PAGE and Western blotted with an anti-hIL-2 antibody, demonstrating the presence of two polypeptides of 15 and 17 kDa (data not shown). Human IL-2 from the dicistronic Ad5 mLym/hIL-2 and the monocistronic IL-2 control vector was quantified by ELISA (Figure 2A and B). IL-2 levels derived from the Ad5 mLym/hIL-2 vector were between 11 to 69 ng/ml compared to hIL-2 levels derived from Ad5 hIL-2 which were between 32 to 75 ng/ml for infections with the same MOI in PyMT or Neu tumor cells. The expression observed per 1×10^6 cells with Ad5 mLym/hIL-2 was approximately 3 fold lower than Ad5 hIL-2 (Figure 2A and B) on PyMT tumor cells but equal on Neu tumor cells.

Quantification of murine IL-12 levels was performed by ELISA (Figure 3A and B). Murine IL-12 levels derived from the Ad5 mLym/IL-12 vector were between 610 to 900 ng/ml compared to IL-12 levels derived from Ad5 mIL-12 which were between 700 to 1100 ng/ml for infections with the same MOI assayed on PyMT or Neu tumor cells. The expression of IL-12 from both constructs was very similar as assayed per 1×10^6 cells.

Intratumoral Administration of Adenoviral Vectors

We have previously shown that intratumoral injection of adenoviral vectors expressing IL-2 or IL-12 to polyoma middle T tumor bearing mice cause significant regression at a dose of 5×10^8 pfu (Addison *et al.*, 1995a; Bramson *et al.*, 1996). We

used this same dose for Ad5 mLym, Ad5 mLym/hIL-2 and Ad5 hIL-2 and compared the effects to those seen with the E1 deleted control vector, Ad5 dl70-3 in both PyMT and Neu tumor models. Administration of control virus did not modify the progression of the polyoma middle T or Neu tumor growths in any of the groups described here or in (Addison *et al.*, 1995a; Bramson *et al.*, 1996). Treatment of PyMT or Neu tumors with Ad5 mLym resulted in 20% (4/20 PyMT tumors) and 0% (0/20 Neu tumors) total regression of established tumors (Table 1A and B). Ad5 hIL-2 treated tumors showed 25% (5/20 PyMT tumors) and 0% (0/20 Neu tumors) regression (Table 1 A and B). In contrast, administration of Ad5 mLym/hIL-2 caused regression of 60% (12/20 PyMT tumors) and 45% (9/20 Neu tumors) tumors treated suggesting at least an additive if not synergistic effect between mLym and hIL-2 expressed from the same cell or at the same time (Table 1A and B).

We could not use the same dose of virus (5×10^8 pfu) for the lymphotactin/IL-12 series of experiments as we had in the lymphotactin/IL-2 series, since this dose resulted in toxicity from high IL-12 levels. We determined the least toxic titer of Ad5 mL-12 and Ad5 mLym/IL-12 to be 1×10^8 pfu and used this viral titer for all vectors in both the PyMT and Neu tumor models. Treatment of PyMT tumors with Ad5 mL-12 resulted in a slightly higher regression when compared to Ad5 mLym such that 35% (7/20 PyMT tumors) demonstrated complete regression. However, this treatment was ineffective in the Neu tumor model with 0% (0/25 Neu tumors) of all Ad5 mL-12 treated mice

responding (Table 2 A and B). In contrast, administration of Ad5 mLym/IL-12 caused complete regression of 75% (15/20 PyMT tumors) and 70% (17/25 Neu tumors) of tumors treated. Similar to the findings observed for the lymphotactin/IL-2 construct, the activity of combining lymphotactin with IL-12 results in an additive if not synergistic effect between mLym and IL-12 (Table 2 A and B). All mice exhibiting complete regression, regardless of which vector was used, remained tumor free for at least 100 days post injection.

Generation of Systemic Immunity to PyMT or Neu Tumor Cell Re-challenge

Mice that had undergone complete regression were challenged with 1×10^6 freshly isolated polyoma middle T cells or 7×10^6 Neu tumor cells at day 103 post initial vector administration, on the left hind flank (see Materials and Methods). None of the challenged mice developed tumors and all remained tumor free for a further 120 days after challenge (Table 3 A and B). To ensure tumor forming capacity of these cells, untreated syngeneic mice were included to observe the kinetics of tumor growth for both models.

CTL Activity in Cured PyMT Mice

Three cured mice from each of the treatment groups were sacrificed and their spleens removed 120 days after challenge. Splenocytes prepared from these Ad5 mLym treated mice demonstrated an average of 29% specific lysis of 516MT3 cells at an effector to target ratio of 3.3:1 (Figure 4). Minimal background lysis (3%) of PT0516

(no polyoma middle T antigen) was observed. In contrast, splenocytes from Ad5 hIL-2 treated mice (120 days after challenge) demonstrated 40% specific lysis of 516MT3 cells and 6% lysis on PTO516 cells (Figure 4). The other single cytokine vector Ad5 mIL-12 demonstrated comparable levels of cytotoxic activity (50% on 516MT3 and 5% on PTO516) at an effector to target ratio of 3.3:1 (Figure 4). Similarly, splenocytes from Ad5 mLym/hIL-2 treated mice showed 61% lysis of 516MT3 targets and 3% lysis on control PTO516 cells (Figure 4). A high percentage of lysis was observed in Ad5 mLym/IL-12 splenocytes (85%) at 3.3:1 on 516MT3 cells and low levels on PTO516 cells (2%) (Figure 4). When splenocytes from control mice were used as effectors, target cell killing was similar for both MT3 and PTO516 target populations. These observations suggest a significant presence of effector cells capable of killing PyMT transformed cells. At present we are in the process of generating an appropriate CTL target cell population to test for cytotoxicity in the Neu model.

DISCUSSION

Immunosurveillance by potential effector cells of the immune system is, in large part, responsible for the rejection of neoplastic cells. However, in individuals with seemingly intact immune systems, neoplastic growths arise and are not rejected. The ability of some neoplastic growths to evade the immune system suggests that potentially antigenic tumors elicit specific effects which act to suppress the immune system. Conversely, tumors which do not express associated antigens offer no target(s) for immune rejection. Mechanisms that eliminate immune-effector cell populations by apoptosis such as FAS-FAS ligand interactions, or molecules like TGF β which suppress the activity of potential tumor reactive effector populations or the interruption of crucial steps required to activate a possible anti-tumor response (anergy or ignorance), all aid in the persistence of neoplastic growth in otherwise healthy organisms.

Thus, understanding the mechanisms of tumor mediated immune-suppression or the parameters encompassing the efficient activation of immune-effector functions will allow for the development of new strategies in tumor immunotherapy.

Recent evidence has demonstrated the ability of certain cytokines to promote tumor rejection and, since the initial IL-2 study by Rosenberg *et al.* (1989), recombinant cytokines such as IL-2, IL-4, IL-7 and IL-12 have been studied to assess their potential for mediating tumor rejection in various immunotherapy protocols. Many of these therapies have been limited by the appearance of toxic systemic effects elicited by

certain cytokines or the inability of the cytokines under investigation to induce efficient anti-tumor effector activity (Rosenberg *et al.*, 1989; Addison *et al.*, 1995a,b; Rosenberg *et al.*, 1988; Rosenberg *et al.*, 1985). Tumor infiltrating lymphocytes (TILs) and lymphokine activated killer cells have also been used with only limited success (Rosenberg *et al.*, 1988; Rosenberg *et al.*, 1985; Friedmann *et al.*, 1989). Evidence suggests that genetically modified tumor cells expressing IL-2 or IL-4 either by transfection or retroviral integration can abrogate the ability of tumors to grow (Mattijessen *et al.*, 1991; Rosenberg *et al.*, 1990; Fearon *et al.*, 1990; Ley *et al.*, 1990). We have recently used adenoviral vectors to deliver cytokines such as IL-2, IL-4 and IL-12 intratumorally (Addison *et al.*, 1995a,b; Bramson *et al.*, 1996), and have shown that the transient local expression of IL-2, IL-4 and IL-12 can augment the recognition of the tumor by the immune system and result in complete regression in a significant fraction of the tumors treated.

As a complement to the use of cytokine mediators, other tumor immunotherapy regimes have focused on the B7 family of costimulatory molecules. B7 family members are required for the transduction of signals which promote T cell activation via B7 ligation with T cell derived CD28 in the presence of MHC I or II antigen/TCR interactions (Restifo *et al.*, 1992; Harding *et al.*, 1992; Gimmi *et al.*, 1993; Lenschow *et al.*, 1992). Transfection of tumor cells with B7-1 has been extensively used and shown to result in only moderate effects on tumor regression (Boussiotis *et al.*, 1993; Chen *et*

al., 1992; Chen *et al.*, 1994; Li *et al.*, 1994; Townsend *et al.*, 1994). Recent observations by us and a number of groups show that B7-1 can be used to augment the activity of IL-12, IL-2 and IL-7 *in vivo* (Matulonis *et al.*, 1995; Zitvogel *et al.*, 1996; Putzer *et al.*, 1997; Salvadori *et al.*, 1995; Cayeux *et al.*, 1995; Emtage *et al.*, 1998) by interacting with CD28 and therefore activating tumor specific CD4⁺ and CD8⁺ T cells. Nevertheless, this work demonstrates that B7 modified tumor cells are capable of delivering, in conjunction with MHC I or II, antigen specific activation signals to T cells. Another approach to tumor immunotherapy would be the use of chemotactic cytokines to increase the prevalence of potential populations of immune effector cells.

Chemokines play a crucial role in inflammatory and immune responses (reviewed in Rollins *et al.*, 1997; Baggiolini *et al.*, 1997). These molecules are small 7-20 kDa proteins that act to chemoattract and activate leukocytes to or at specific sites of immune-reactivity (Schall *et al.*, 1993; Rollins *et al.*, 1997; Baggiolini *et al.*, 1997). Chemokines are subdivided into four groups classified as CXC, CC, C and CX₃C. These groups are so termed for the location of cysteine residues in key regions of the molecule. The CXC chemokines, such as GRO α , GRO β , GRO γ and IL-8, chemoattract primarily neutrophils, while the CC chemokines RANTES, MIP-1 α and MIP-1 β attract lymphocytes and monocytes (Reviewed in Rollins *et al.*, 1997; Baggiolini *et al.*, 1997).

Based on these characteristics, many investigators have tested the ability of chemokines to induce tumor rejection by exploiting the chemotactic properties of these molecules. Transfection experiments with tumor cells modified to express TCA3, IP10, MCP1, MIP-1 α , IL-8, MIP-2, RANTES and lymphotactin have been shown to elicit some anti-tumor responses when the modified tumor cells are placed in vivo (Lanig *et al.*, 1994; Mule *et al.*, 1996; Huang *et al.*, 1996; Dilloo *et al.*, 1996; Strieter *et al.*, 1995; Hirose *et al.*, 1995). However, these molecules exhibit less efficacy than the anti-tumor activity of immuno-modulatory cytokines like IL-2, IL-4 and IL-12. With this knowledge, we attempted to increase the efficacy of IL-2 and IL-12 by combining expression of these cytokines with the C chemokine lymphotactin. Lymphotactin has been shown to attract T and NK cells (Kelner *et al.*, 1994; Hedrick *et al.*, 1997; Bianchi *et al.*, 1996). The expression has been localized to CD8⁺ cells, CD4⁺ NK.1.1 T cells, $\alpha\beta$ ⁺ CD4⁻ CD8⁻ T cells, $\gamma\delta$ ⁺ intraepithelial T cells, natural killer cells and mast cells. (Kelner *et al.*, 1994; Kennedy *et al.*, 1995; Muller *et al.*, 1995; Boismenu *et al.*, 1996; Dornier *et al.*, 1996; Hedrick *et al.*, 1997; Rumsaeng *et al.*, 1997; Maghazachi *et al.*, 1997; Bianchi *et al.*, 1996).

The combination of lymphotactin with IL-2 for tumor therapy was previously reported by Dilloo *et al.* (1996). This group used fibroblast transduced with retroviral vectors expressing lymphotactin or IL-2 to supply these molecules at the tumor site. The authors were able to demonstrate the ability of lymphotactin in combination with

IL-2 to reduce the size of tumors in mice, but were unable to demonstrate any significant potential for tumor regression. In our system we observe frequent regression with lymphotactin double vectors. The difference between our findings and the observations of Dilloo *et al.* (1996) may be attributed to two aspects. First, the models might be significantly different in their requirements for regression. This is supported by our observation that PyMT and Neu models respond differently to vector treatment and yet are both breast mammary epithelial derived tumors. Second, intratumorally injecting adenoviral vectors expressing lymphotactin in combination with either IL-2 or IL-12 provides a highly localized micro environment for the generation of a T_H1 type response against the vectors and in the process might facilitate a more potent anti-tumor response. A third possible explanation is the provision of an adjuvant in our system. Adenoviral vectors elicit immune responses which makes the use of these vectors transient. In contrast, the retrovirally infected fibroblast used by Dilloo *et al.* (1996) will not elicit an adjuvant effect and hence, provide no other immuno-modulatory molecules capable of augmenting the anti-tumor response.

It is evident from current literature that use of cytokines, chemokines or co-stimulatory molecules in isolation to modulate the immune response against established tumors is of limited efficacy. The biological evidence for the beneficial simultaneous delivery of adenoviral vectors constructed to express either IL-2 or IL-12 with B7-1 on tumor rejection (Ertage *et al.*, 1998; Putzer *et al.*, 1997) prompted us to examine the

effects of supplying both lymphotactin and IL-2 or IL-12 to the same cell *in vivo*. This approach might enhance the ability of the immune system to recognize and react against established tumors by attracting a larger T cell repertoire to the site of immune response.

We have demonstrated the ability of the double recombinant vectors (Ad5 mLym/hIL-2 and Ad5 mLym/IL-12) to produce IL-2 and IL-12 (Figure 3 and 4). IL-2 production was 50% lower than that observed for the single vector expressing hIL-2 (Ad5 IL-2) in PyMT tumors cell but equal in Neu tumors cells. The IL-12 levels for both Ad5 mL-12 and Ad5 mLym/IL-12 are similar in both PyMT and Neu tumors cells.

Comparison of the effects of intratumoral injection of Ad5 mLym or Ad5 IL-2 versus Ad5 mLym/hIL-2 in both the PyMT or Neu tumor models demonstrated that the combination vector was much more effective at inducing complete regression in the PyMT or Neu models than either of the vectors expressing lymphotactin or IL-2 alone. This additive or synergistic effect can also be seen with the Ad5 mLym/IL-12 experiments.

To determine the ability of all vectors to induce long term protection we challenged all completely regressed mice with freshly isolated PyMT or early passage Neu tumor cells 103-120 days after primary tumor injection. Tumor cell challenge was always administered on the opposite hind flank to the site of the regressed tumor. All mice which exhibited complete regression were found to be protected against challenge,

demonstrating that all vectors were capable of generating long lasting systemic immunity (Table 3).

To determine a possible mechanism and the specificity of the effector function generated during treatment with the vectors, we examined the cytotoxic T lymphocyte (CTL) activity in the spleen of completely regressed animals (Figure 4). Ad5 mLym treated animals demonstrated 29% specific lysis on a 516MT3 polyoma middle T expressing cell line at a ratio of 3.3:1 (effector:target ratio). The lytic activity of splenocytes from Ad5 mLym treated mice on the non-polyoma middle T expressing (PTO516) control cell line was undetectable at all effector:target ratios. Mice regressed with all of the other vectors demonstrated potent anti-PyMT CTL activity regardless of the cytokine used. The ability of Ad5 hIL-2 or Ad5 mLIL-12 to generate a strong CTL response in regressed mice (PyMT tumor model) and yet, have only a 25 and 35% regression activity is intriguing. This observation may be explained as follows. If tumor infiltrating lymphocytes exist in various ratios such that from tumor to tumor the number of CD4⁺, CD8⁺ or NK cells varies in proportion, then one might expect various regression potentials depending on which population is the predominant effector capable of targeting PyMT or Neu tumor cells in response to IL-2 or IL-12 activation. Since total regressions observed for IL-2 and IL-12 are low, it is possible that the local production of these molecules is only sufficient to expand or activate effector populations depending on the relative proportions of CD4⁺, CD8⁺ or NK cells in the

tumor. In contrast, the chemotactic activity of lymphotactin for lymphocytes overcomes the inherent variance in TIL proportions by recruiting CD4⁺, CD8⁺ and NK cells to the tumor site. Another benefit of the activity of lymphotactin is its ability to directly increase the potential tumor reactive TCR repertoire by attracting naïve T cells.

We have previously demonstrated the ability of other double recombinant adenoviral vectors to regress PyMT tumors (Emtage *et al.*, 1998; Putzer *et al.*, 1997). The level of tumor regression observed with the B7-1 and IL-2 expressing double (Emtage *et al.*, 1998) vector demonstrates that the combination of IL-2 with the co-stimulatory molecule B7-1 is a more potent treatment for PyMT tumors than the lymphotactin expressing vectors. However, it is important to note that combining all three molecules (lymphotactin, IL-2, IL-12 and B7-1) might result in a vector more potent than both double vectors. Another interesting aspect of this work is the difference we observe between the two models. Since both models involve potent transforming molecules, whose expression is driven by the MMTV promoter, we should expect the same expression profiles (with respect to tissue distribution) and levels. It is possible that any differences seen between the PyMT and Neu models results from dominance of the epitopes presented by MHC I antigens, such that Neu derived epitopes are less immuno-dominant than PyMT and hence these tumors are less immunogenic. Alternatively, it is possible that Neu mediated transformation results in the production of suppressive factors not observed in PyMT derived tumors and therefore reduces the

immunogenicity of Neu tumors. One can also postulate that genes turned on by PyMT transformation are potential target for effector cells (neonatal or differentiation antigens) more so than by Neu transformation. Regardless, of the mechanism it is clear that tumors are different in their capacity to undergo regression by treatment with the various vectors outlined herein. This observations illustrates an important point, which is that the immunogenicity of many tumors from the same tissue and cell type can and will have different requirements for immuno-therapy. These requirements are most likely dependent on the multi-step events leading to transformation.

The findings of this study demonstrate the effectiveness of augmenting the immune response against tumors with adenoviral vectors expressing lymphotactin in combination with IL-2 or IL-12. These observations demonstrate the potential use of double recombinant vectors expressing chemokines and immunomodulatory cytokines in cancer immnuotherapy.

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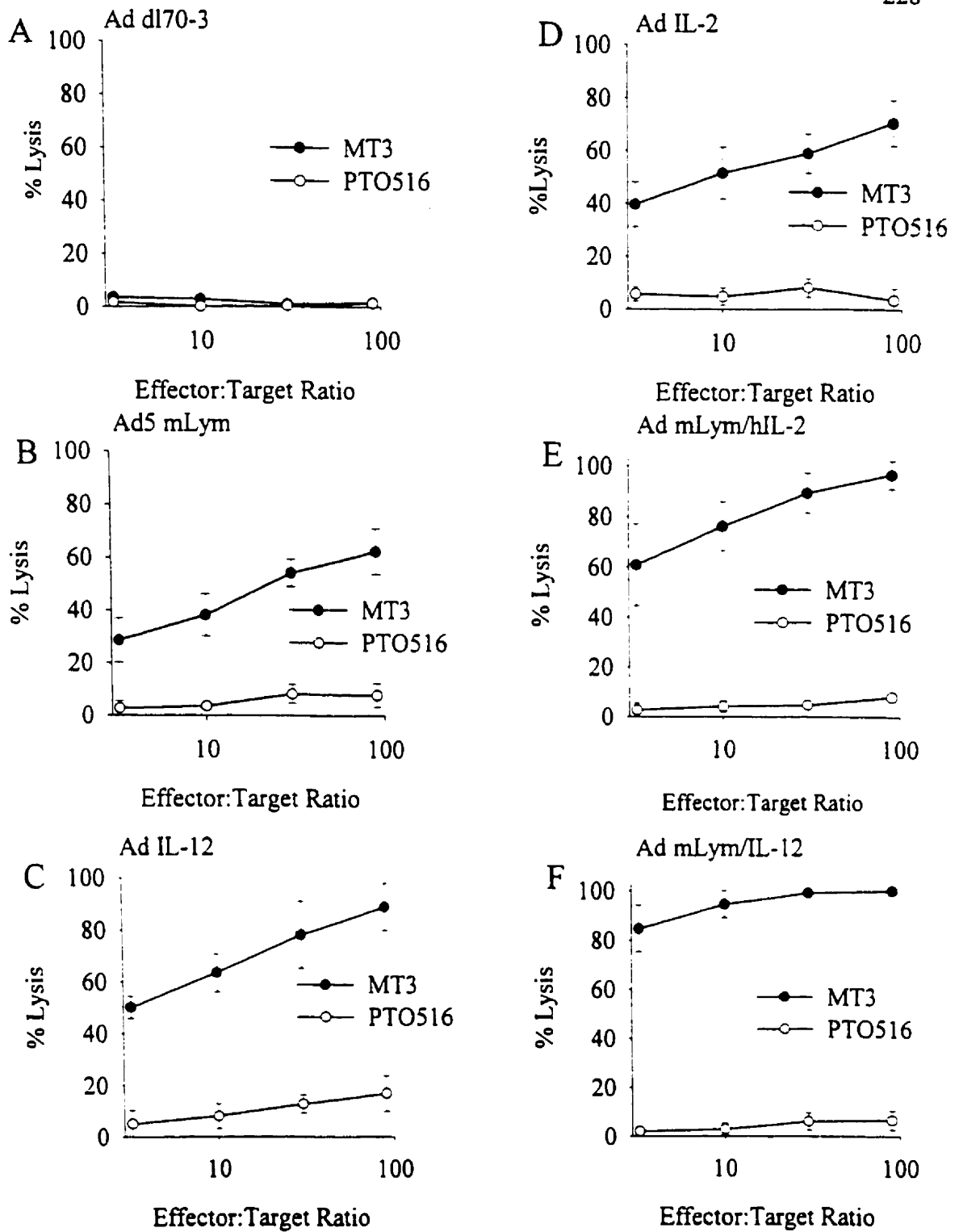


Figure 4

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Legends

FIG 1. Schematic diagram of the construction of Ad5 mLym/hIL-2 (A) and Ad5 mLym/mIL-12 (B). For a more detailed vector construction description see Materials and Methods.

FIG 2. The production of hIL-2 from Ad5 mLym/hIL-2 and Ad5 IL-2 infected Neu (A) and PyMT (B) cells (MOI =10). Aliquots were removed and supernatants analyzed by ELISA for human IL-2 at days 1 to 7.

FIG 3. The production of mIL-12 from Ad5 mLym/mIL-12 and Ad5 mIL-12 infected Neu (A) and PyMT (B) cells (MOI =10). Aliquots were removed and supernatants analyzed by ELISA for murine IL-12 at days 1 to 4.

FIG 4. Cytotoxic T lymphocyte activity in mice after total tumor regression on treatment with various vectors. The CTL activity generated by the control vector Ad5 dl70-3 is outlined in (A). PyMT specific CTL activity is outlined for the various vectors; mLym (B), Ad5 IL-12 (C), Ad5 IL-2 (D), Ad5 mLym/hIL-2 (E) and Ad5 mLym/IL-12 (F). Solid circles represent 516MT3 cells (PyMT targets), open circles represent PTO516 cells (parental non-PyMT expressing cells). Splenocytes were co-

cultured with irradiated 516MT3 cells for 5-7 days prior to determination of CTL activity.

Construction of Ad5 mLym/hIL-2

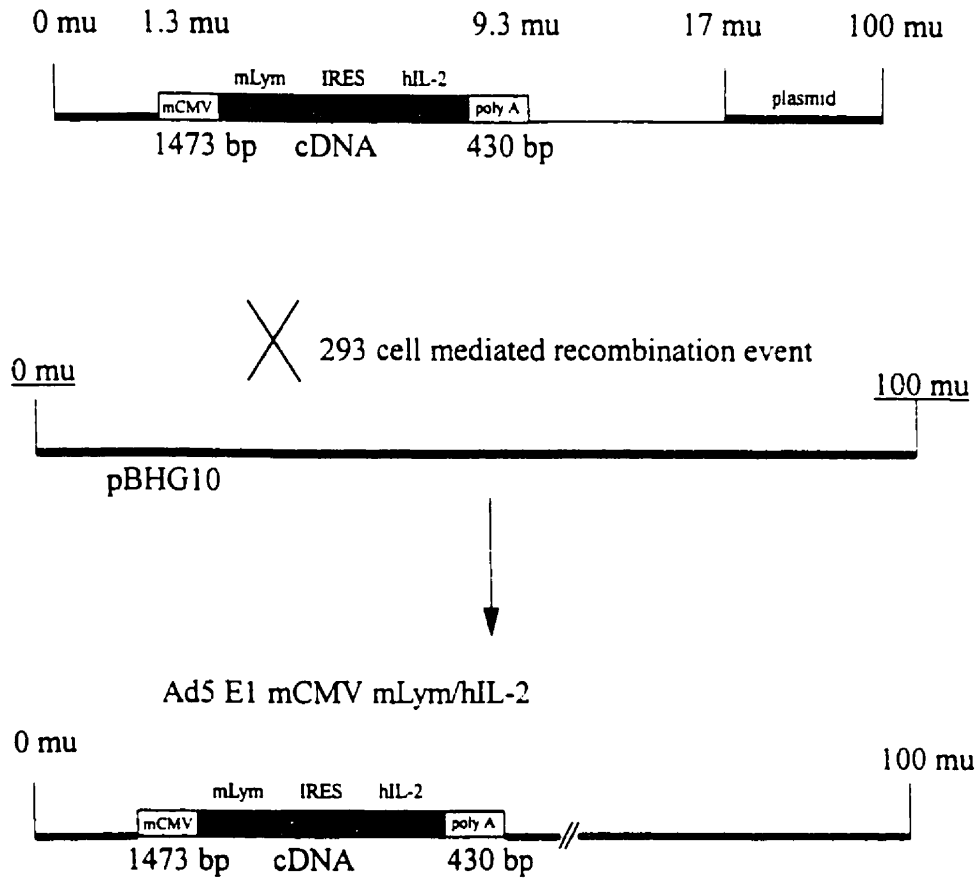


Figure 1A

Construction of the Ad E3 mLym and E1 mIL-12 double recombinant vector

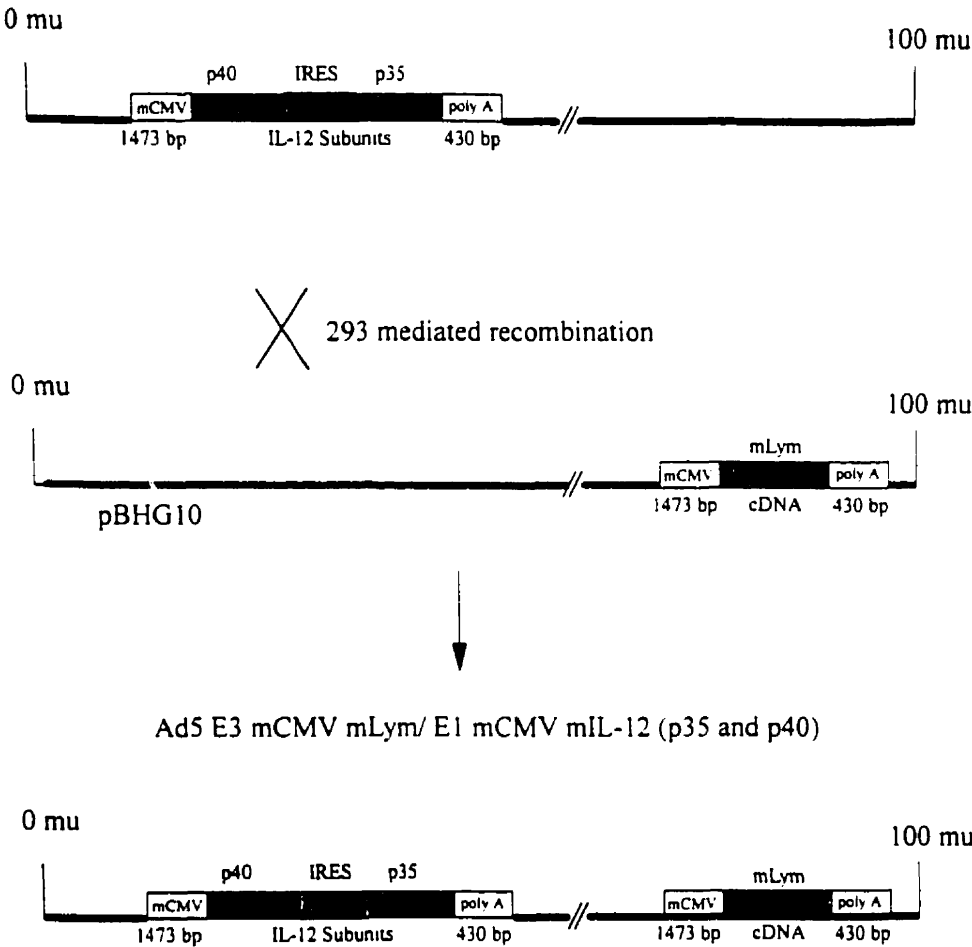


Figure 1B

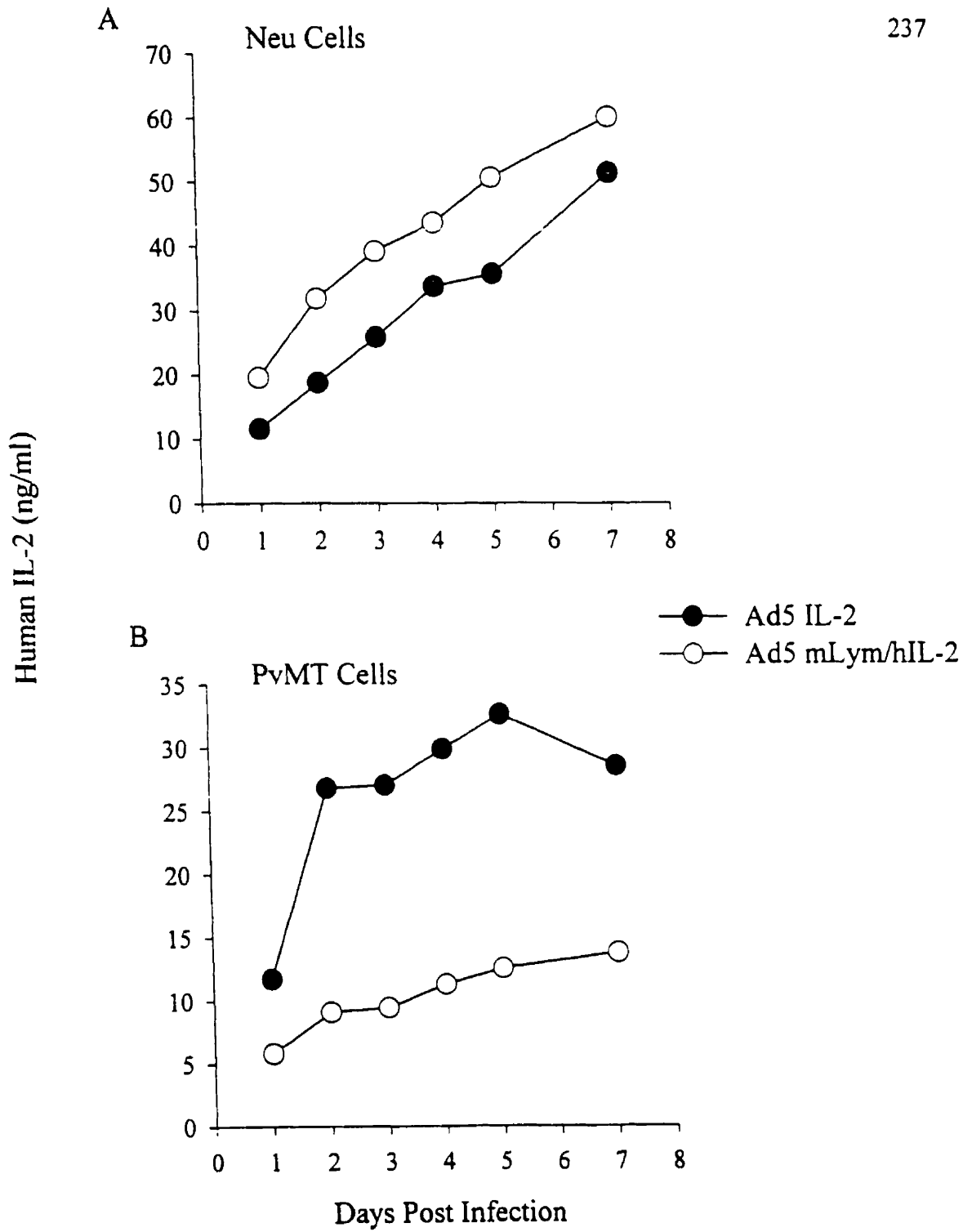


Figure 2

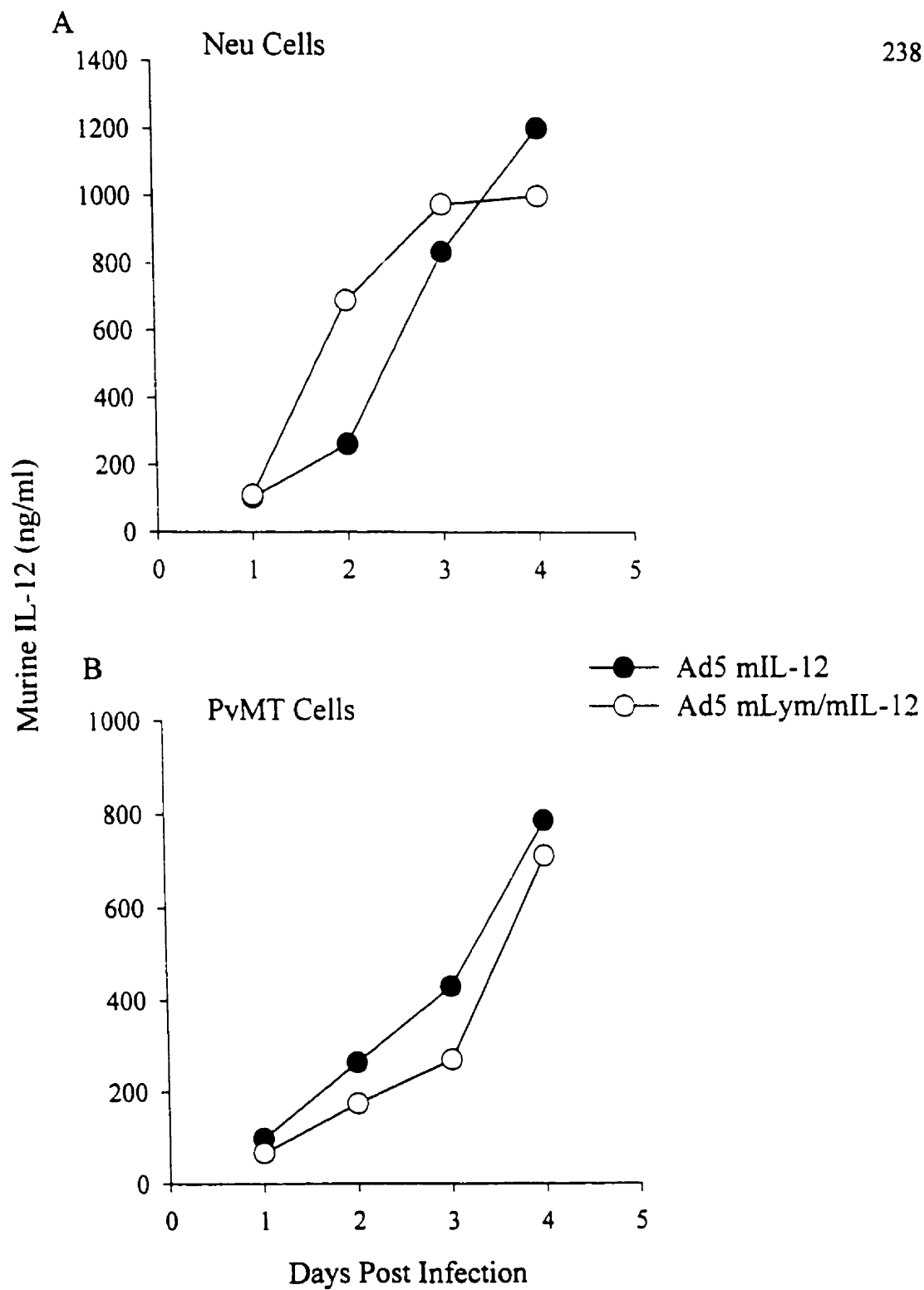


Figure 3

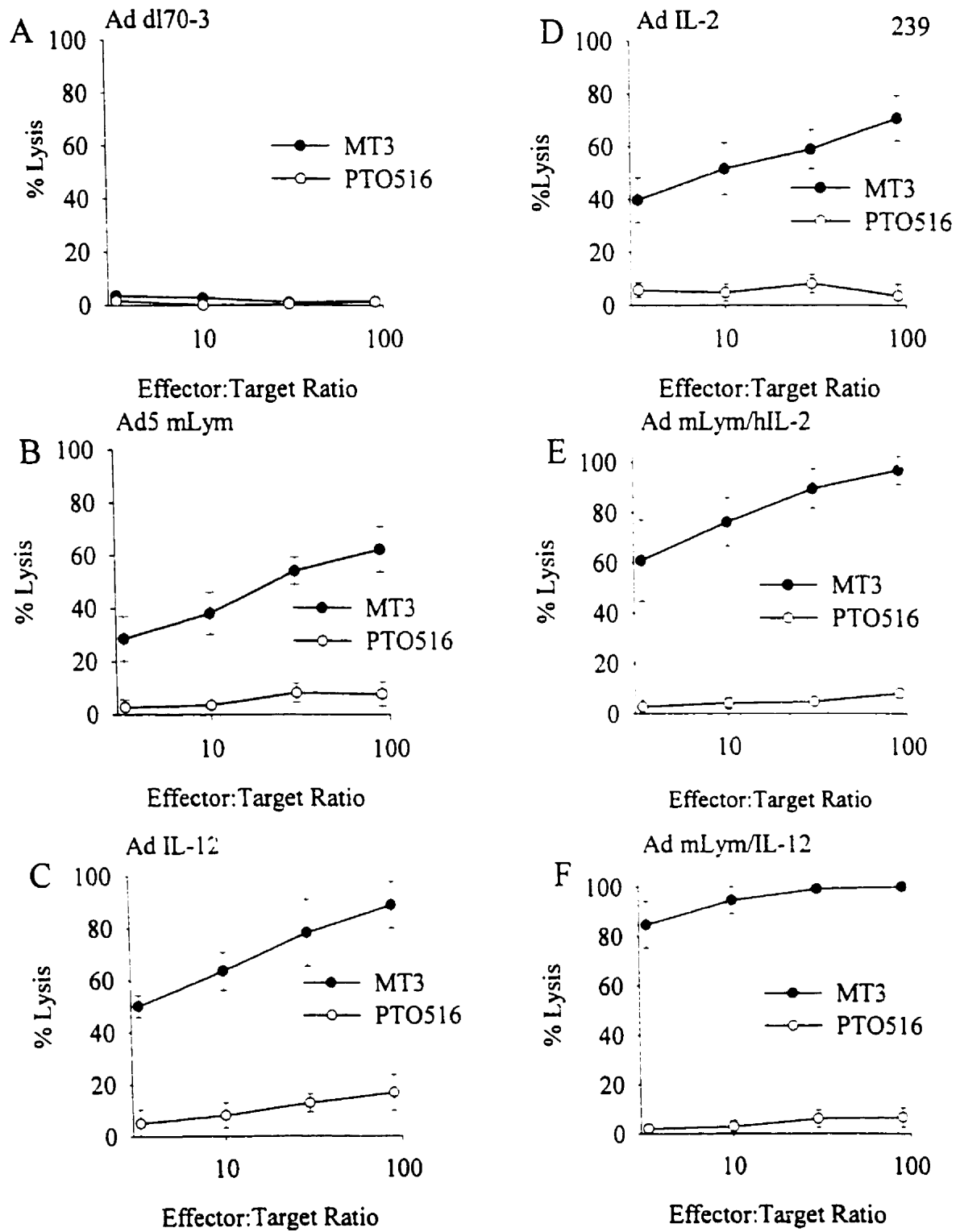


Figure 4

Table 1. Complete regression of PyMT and Neu tumors in response to intratumoral injection of Ad dl70-3, Ad CAIL-2, Ad5 mLym and Ad5 mLym/hIL-2

| PyMT Tumors | Percentage Regressed |
|----------------|----------------------|
| Ad dl70-3 | 0/20 |
| Ad CAIL-2 | 5/20 (25%) |
| Ad mLym | 4/20 (20%) |
| Ad mLym/hIL-2 | 13/20 (65%) |
| Neu Tumors | |
| Ad dl70-3 | 0/20 |
| Ad CAIL-2 | 0/20 |
| Ad mLym | 0/20 |
| Ad mLym /hIL-2 | 9/20 (45%) |

Table 2. Complete regression of PyMT and Neu tumors in response to intratumoral injection of Ad dl70-3, Ad mL-12, Ad5 mLym and Ad5 mLym/IL-12

| PyMT Tumors | Percentage Regressed |
|----------------|----------------------|
| Ad dl70-3 | 0/20 |
| Ad mL-12 | 7/20 (35%) |
| Ad mLym | 3/20 (15%) |
| Ad mLym/IL-12 | 15/20 (75%) |
| <hr/> | |
| Neu Tumors | |
| Ad dl70-3 | 0/25 |
| Ad mL-12 | 0/25 |
| Ad mLym | 0/25 |
| Ad mLym /IL-12 | 17/25 (70%) |

Table 3. Protective immunity in mice completely regressed with Ad mL-12, Ad CAIL-2, Ad5 mLym, Ad5 mLym/IL-12 and Ad5 mLym/hIL-2

| PyMT Regressed | Percentage Regressed |
|----------------------|----------------------|
| Ad CAIL-2 | 5/5 (100%) |
| Ad mLym | 7/7 (100%) |
| Ad mLym/hIL-2 | 13/13 (100%) |
| Ad mL-12 | 7/7 (100%) |
| Ad mLym/IL-12 | 15/15 (100%) |
| Neu Regressed | |
| Ad mLym /hIL-2 | 9/9 (100%) |
| Ad mLym /IL-12 | 17/17 (100%) |

Chapter 6

DISCUSSION

Cancer therapies have focused on protocols which utilize the toxic nature of some chemicals, surgery and the DNA disrupting activity of radiation. The problem with these approaches is two fold. Usually these treatments only extend the life of the patient and offer no protection from reoccurrence should the patient relapse or demonstrate metastatic disease. In the last 15 years there has been an emergence of potential therapies which offer both specificity and protection from metastatic disease. In its infancy the field of tumor immunotherapy focused on the systemic delivery of cytokines capable of boosting an individual's response to a tumor. More recently, gene therapy approaches have arisen from the necessity to increase local tumor concentrations of cytokines and other immuno-regulatory molecules which have been shown to increase efficacy and reduce systemic toxicity. The use of viral and non-viral systems to deliver these molecules to the tumor site has led to over 100 clinical trials world wide. Most of these protocols, however, have not led to any significant enhancement of tumor regression compared to the other non-immunotherapeutic clinical protocols. Many immune-regulating molecules elicit potent responses in pre-clinical tumor models. To this end, we have focused on augmenting the immune-regulating capacity of some of these molecules (IL-2 and IL-12) by combining them with other well-characterized immuno-modulating molecules (B7-1, B7-2 and lymphotactin). This

approach should allow for an enhancement of the efficacy of IL-2 and IL-12 by providing molecules which activate or induce the accumulation of anti-tumor effector cells.

PyMT and Neu (NDL) Tumor Adenocarcinoma Models

Considering the large amount of data collected here at McMaster University using the PyMT tumor model for testing the anti-tumor effects of adenoviral vectors constructed to express IL2 and IL-12 (Addison et al., 1995a; Bramson et al., 1996), we decided to continue using this model for this thesis. In addition, we adopted another mammary adenocarcinoma model: the Neu model (Siegel et al., 1994). Both the PyMT and Neu models make use of tumors that arise in transgenic mice which express either PyMT or Neu (NDL8142) under the control of the mouse mammary tumor virus long-terminal repeat (LTR). Expression of these oncogenes results in transformation of the otherwise normal mammary epithelium (Guy et al., 1992; Siegel et al., 1994; Siegel and Muller, 1996).

For the PyMT model, breast epithelial tumors were removed from PyMT transgenic FVB/n mice, digested with enzymes to obtain a single cell suspension, cultured for 4-5 days and injected sc into syngeneic FVB/n recipient mice. Tumors in the range of 75-150 mm³ formed in these mice within 15-21 days, at which time they were injected with the vectors described in the thesis.

In transgenic Neu (NDL8142) mice, mammary tumors result from the transforming activity of the NDL8142 mutation (deletion of nucleotides 1904-1995) which causes constitutive dimerization of the Neu receptor (Siegel et al., 1994; Siegel and Muller, 1996). Neu transgenic mice bearing sc Neu (8142) tumors for use in pre-clinical experiments can be generated from primary Neu mammary tissue exactly as described for the PyMT model. Alternatively, an epithelial cell line derived from one of these sc tumors is as effective as primary Neu (NDL8142) cancer cells at establishing sc tumors. Use of the Neu cell line obviates the need for maintaining a Neu (8142) transgenic mouse colony which is both costly and time consuming.

The ability of these tumor cells to generate tumors (in 100% of mice treated) upon transplantation in syngeneic recipient mice is interesting since both PyMT and Neu (NDL8142) are foreign/mutated proteins and, therefore, potentially antigenic. The immunogenicity of these tumors is low considering that irradiated tumor cells administered to recipient mice offers no protection from non-irradiated tumor cell challenge (Emtage and Gauldie, unpublished observations). It is therefore likely that other mechanisms exist which allow these transplanted tumors to evade immune-surveillance, grow and eventually kill the host. Suppressive factors produced by tumors are likely to have an effect on the host's immune response. PyMT and Neu (8142) tumors have been shown to produce significant levels of IL-10, TNF α and GM-CSF (Emtage and Gauldie, unpublished observations). It is not known if the levels of IL-10

are immuno-suppressive and inhibit host anti-tumor responses. IL-10 (a cytokine associated with Th2 responses) has, however, been shown to inhibit Th1 responses (reviewed in Muraille and Leo, 1998). It is therefore plausible that both tumor models can deviate or suppress a Th1 response and evade immune mediated rejection. The production of TNF α and GM-CSF by PyMT and Neu (8142) tumors is interesting and at present it is not known how any of these molecules interfere with the generation of anti-tumor responses. Other mechanisms which might allow PyMT and Neu (NDL8142) tumors to grow in immuno-competent host are likely to exist. For example, if the expression of MHC class I antigens on the surface of PyMT and Neu (NDL8142) tumors is too low, then inappropriate presentation of antigens to CD8⁺ T cells can induce anergy (FACs analysis demonstrates that both PyMT and Neu (8142) tumors express equivalent levels of MHC I molecules). This would result in inefficient generation of anti-tumor effector responses and allow tumor growth. Observations made by a number of groups have shown that it is possible for tumors to express MHC I levels that are too low to allow for tumor cell recognition by CD8⁺ T cells (Nabel et al., 1996; Mandelboim et al., 1995). In a similar fashion, tumors which lack the co-stimulatory molecules B7-1 or B7-2 cannot provide the crucial signals necessary for the activation of T cell effector functions (Chambers and Allison, 1997; Guinan et al., 1994; Gause et al., 1997; Bluestone, 1995). Flow cytometric analysis of PyMT and Neu tumors has demonstrated the lack of B7-1 or B7-2 surface expression. This observation

provides another plausible mechanism by which these tumors can grow in an immunocompetent host.

Immunotherapy of PyMT and Neu (NDL8142) Tumors

The generation of Th1 anti-tumor responses has been shown to be required for most tumor rejection or regression responses. However, as mentioned previously, the local tumor microenvironment might be immuno-suppressive as a result of cytokine secretion (IL-10 or TGF β) or the lack of co-stimulatory molecules (B7-1 or B7-2). It is therefore conceivable that for potential therapies to exhibit any efficacy, they must overcome the suppressive effects of the tumor environment. The efficacy of IL-12 is attributed to many functions of which its ability to induce IFN γ , generate Th1 responses and upregulate chemokine expression are most important (Tannenbaum et al., 1996; Bramson et al., 1996; Trinchieri et al., 1996; Zitvogel et al., 1994; Zou et al., 1995; Nastala et al., 1994; Meko et al., 1995). The ability of IL-12 to induce IFN γ from NK and T cells is important since IFN γ has been shown to inhibit Th2 responses which are mediated by IL-4 and IL-10 predominantly (Trinchieri et al., 1996; Mossman and Sad, 1996). This can potentially help to overcome the suppressive effects of tumors that produce IL-10. IFN γ has also been shown to induce MHC I and II upregulation on tumor and antigen presenting cells, facilitating a more potent effector response against the tumor (Trinchieri et al., 1996; Mossman and Sad, 1996; Maruo et al., 1996; Murphy et al., 1994; Kennedy et al., 1994; Manetti et al., 1993; Trinchieri, 1993; Hsieh et al.,

1993). The ability of IFN γ to upregulate the production of the chemokines Mig and IP-10 is also very important. These chemokines have been shown to exhibit angiostatic properties and may serve to limit tumor growth or “debulk” the tumor (Strieter et al., 1995; Angiolillo et al., 1995; Luster et al., 1995).

Many investigators have demonstrated that most tumors induce a state of cytokine unresponsiveness in tumor infiltrating lymphocytes (TILs) populations in various tumor models. TILs have been shown to be rendered anergic. In an attempt to overcome this anergy, our group has postulated the anti-tumor effects of supplying high levels of IL-2 and IL-12 to the local environment of the tumor. As mentioned in previous sections, IL-2 is a crucial growth factor for T cells, especially precursor T helper cell, Th1 T cells and CD8⁺ CTL. By supplying IL-2 to the region of the tumor, we proposed that high local levels of the cytokine would break tolerance induced by the lack of co-stimulatory molecules and overcome the immuno-suppressive environment within the tumor, without the attendant systemic toxicity seen with systemic administration of the cytokine.

Addison and co-workers (1995) demonstrated that intratumoral injection of an adenoviral vector expressing IL-2 could mediate regression in 54% of PyMT tumor bearing mice. The regression was found to be associated with the generation of antigen specific systemic immunity. The authors postulated that high levels of local IL-2 activated T cells by binding to the constitutively expressed low affinity IL-2 receptor

(Abbas et al., 1991). This would initiate proliferation which would eventually lead to the production of the high affinity IL-2 receptor and, hence, bypass the need for co-stimulation.

Bramson and colleagues (1996) constructed an adenoviral vector expressing the p35 and p40 subunits of IL-12. The authors demonstrated that this construct was capable of inducing regression in 30% of all PyMT tumor bearing animals. In a similar manner to that observed with IL-2 therapy, IL-12 also induced PyMT antigen specific systemic immunity. As mentioned previously, IL-12 is a potent inducer of Th1 responses through a positive feed back mechanism with IFN γ . In PyMT tumors IL-12 might counteract the activity of IL-10, if produced at high enough levels, and induce a Th1 response. The activity of IL-12 induced IFN γ would upregulate MHC I on the tumor cells and facilitate tumor cell recognition. The activity of both IL-2 and IL-12 on mediating tumor regression when expressed from adenoviral vectors is limited by cytokine toxicity as the dose of vector is increased in an attempt to enhance efficacy. To augment the efficacy of these cytokines, combination vectors were constructed with either IL-2 and IL-12 and other immuno-modulatory molecules in order to reduce the need of high local levels of cytokine while activating potential effector cells more actively.

B7-1/B7-2 Anti-tumor Activity

Putzer and co-workers (1997) demonstrated that a single adenovirus constructed to express the co-stimulatory molecule B7-1 in combination with IL-12 enhances the anti-tumor activity of IL-12. The authors demonstrate that a single dose of the combination vector expressing IL12 and B7-1 induced regression in 70% of treated animals whereas a single injection of either B7-1 or IL-12 vector alone induced only a delay in tumor progression. All tumors regressed by the combination vector were protected from rechallenge by freshly isolated PyMT tumor cells. The level of efficacy observed with this vector was significantly enhanced above that observed with either molecule alone, while maintaining low levels of toxicity. This work demonstrates that single vector combinations of B7-1 and IL-12 are more potent at inducing regression of established tumors.

In data presented in this thesis we constructed a single adenoviral vector expressing B7-1 and IL-2 and used this vector in the PyMT model to assess its toxicity and regression potential. This work demonstrated that a single vector expressing a combination of B7-1 and IL-2 acted in a synergistic or additive fashion to mediate complete regression of PyMT tumors in tumor bearing animals. The dose of the combination vector expressing mB7-1 and IL-2 used in this study significantly augmented the anti-tumor activity of either molecule alone, and demonstrated no toxicity associated with IL-2. The published work outlined above clearly demonstrates

the benefits of using single adenoviral vectors constructed to express B7-1 in combination with either IL-2 or IL-12.

The efficacy demonstrated by the B7-1 and IL-2 combination prompted us to examine the usefulness of the other B7 family member, B7-2. B7-1 and B7-2 have both been shown to have similar co-stimulatory activity *in vivo* and *in vitro*. To compare the ability of B7-2 to augment the activity of IL-2, we constructed an adenoviral vector to express both B7-2 and IL-2. This construct is similar to the combination vector expressing B7-1 and IL-2 in that the levels of IL-2 are closely matched. In the PyMT tumor bearing mice the vector expressing B7-2 and IL-2 resulted in 47% regression compared to the vector expressing B7-2 alone which demonstrated 26% regression. In the PyMT model, we were therefore unable to augment the activity of IL-2 by combining it with B7-2. However, when we used these vectors in the Neu (NDL8142) tumor model similar levels of regression were observed. The combination vector expressing B7-1 or B7-2 with IL-2 resulted in 65% and 60% regression of Neu (NDL8142) tumor bearing mice respectively. Interestingly, the only single recombinant vector to mediate regression of these tumors was the vector expressing B7-2, suggesting that the activity of B7-1 and B7-2 at inducing anti-tumor activity also differ in this tumor model. Tumor regressed mice demonstrated protection from subsequent tumor cell challenge. The difference observed between B7-1 and B7-2 in both the PyMT and Neu (NDL8142) models are interesting since the studies by Nunes et al. (1996a and b)

show that these molecules are identical at inducing CD28 mediated signal transduction cascades in T cells. This taken together with the observations of Linsley and co-workers (1994), that both B7-1 and B7-2 bind CD28 on the T cell with the same affinity suggested that these molecules should act similarly. The mechanisms by which B7-1 and B7-2 differ with respect to their roles in co-stimulating T cells is unknown. A speculative explanation follows: if the B7-1 and B7-2 transgenes are capable of transducing signals to the nucleus then it is possible that signal transduction cascades initiated by B7-1 or B7-2 ligation to CD28 results in differences in gene expression. This differential regulation could contribute to the variances observed between B7-1 and B7-2 with respect to the two models in this study. The potential for B7-1 and B7-2 mediated signal transduction is demonstrated by different potential phosphorylation sites in the cytoplasmic domains of these molecules (Borriello et al., 1994). Since B7-1 and B7-2 can potentially induce signal transduction cascades within the tumor cell it is therefore plausible that these same signals could interfere with the signals generated by the PyMT and Neu (NDL8142) transforming factors in a negative or positive fashion to hinder or augment anti-tumor responses. Another possible mechanism might involve molecules secreted by the tumor cells. Since PyMT and Neu (NDL8142) are potent and distinct transforming factors it is likely that they affect gene transcription differently such that these molecules might act to positively or negatively modulate anti-tumor activity.

Lymphotactin Anti-tumor Activity

Lymphotactin, as mentioned earlier, is a C chemokine specific for CD4⁺ and CD8⁺ T cells as well as NK cells (Kelner et al., 1994; Hedrick et al., 1997). To augment the anti-tumor activity of IL-2 and IL-12, we postulated that by increasing the number of potential effector cells at the tumor site we would not need very high levels of IL-2 or IL-12 to get maximal regression. In the PyMT models we observed that the double constructs expressing lymphotactin and IL-2 or lymphotactin and IL-12 were more potent at inducing regression than either molecule alone; with 60% and 75% regression respectively. Lymphotactin, IL-2 and IL-12 vectors alone resulted in 17%, 25% and 35% regression respectively. All mice demonstrated protection and antigen specific CTL activity. Considering the activity of the single vectors it is clear that the double vector combinations are more effective at augmenting the activity of IL-2 and IL-12. This could be due to the T cell activating properties of these cytokines.

We turned our attention to the Neu (NDL8142) model to determine how these constructs performed in a less immunogenic system. Similar to the B7-1 and B7-2 series of experiments, none of the single vectors were able to induce regression in this tumor model. The vectors combining lymphotactin with either IL-2 or IL-12 mediated regression in 45% and 70% of Neu (NDL8142) tumor bearing mice respectively. The ability of these vectors to cause regression of such tumors shows an additive or synergistic effect between lymphotactin and IL-2 or IL-12. Regressed mice

demonstrated protection against rechallenge suggesting systemic immunity was enhanced. In both the PyMT and Neu (NDL8142) models both double recombinant vectors resulted in a significantly enhanced efficacy and no associated toxicity. We also looked at the ability of both lymphotactin double vectors to mediate regression of pulmonary metastatic nodules in the PyMT model. In all cases, tumors that had regressed or were regressing had no or significantly reduced metastatic pulmonary nodules. A comparison of the anti-metastatic activity of the vectors expressing combinations of mB7-1 or mB7-2 with IL-2 was performed. The observed kinetics of the mB7-1 and hIL-2 combination vector induced regression is rapid (14-20 days for complete regression). However, the vector combining mB7-2 and IL-2 mediates regression much more slowly (21-30 days). When compared at day 14 post intratumoral injection both vectors induced a significant reduction in the total number of pulmonary metastatic nodules compared to the control or single treated vectors.

Taken together the data presented here demonstrates clearly that it is possible to augment the anti-tumor activity of IL-2 and IL-12 while reducing the toxicity associated with these cytokines. Other approaches to further increase the regression potential of these constructs would include mixtures of the lymphotactin double constructs with the B7 family of constructs. Alternatively, the addition of other cytokines and chemokines like GM-CSF, IL-18, Mig or IP-10 to therapies using any of the previously mentioned double vectors might enhance the immune response and provide greater numbers of

complete regressions by attracting APCs, inducing IFN γ production or inducing an angiostatic environment. In conclusion, the use of the double recombinant vectors in tumor immuno-therapy has been clearly demonstrated in this thesis. Furthermore, the data presented in Chapters 2-5, indicate that at least two different type of immuno-modulatory molecules can be used to enhance anti-tumor activity when combined with IL-2 or IL-12. New approaches to further augment the efficacy of some of these combinations are still required, however the suggestions outlined above will hopefully lead to the development of more therapeutically efficacious protocols for the treatment of cancer.

Chapter 7 APPENDICES**APPENDIX 1.**

This appendix references all plasmids which pertain to this thesis.

Basic "shuttle" plasmids with single inserts

pDK6 mB7-1 -insertion of the open reading frame for murine mB7-1 into the unique Eco RV site in the multiple cloning site of pDK6. Murine B7-1 was cloned by RT-PCR using splenocyte cDNA as the template and the following primers:

mB7-1 Forward 5'- AAGATCTCTCCATTGGCTCTAGATTCCTGGC-3'

Nucleotide 190-214

mB7-1 Reverse 5'- GAAGATCTGATTGTACCTCATGAGCCACATAATA-3'

Nucleotide 1198-1223

PCR amplification was performed using Vent DNA Polymerase which generates 95% blunt ends for easy cloning into Eco RV digested pDK6.

pDK6 mB7-2 -insertion of the open reading frame for murine mB7-2 into the unique Eco RV site in the multiple cloning site of pDK6. Murine B7-2 was cloned by RT-PCR using Ad2 mB7-2 (Obtained for Dr J Bluestone, University of Chicago) vector infected 293 cDNA as the template and the following primers:

mB7-2 Forward 5'-GGAGCAAGCAGACGCGTAAGAGTG-3'

Nucleotide 34-60

mB7-2 Reverse 5'-CTTTCCTCAGGCTCTCACTGC-3'

Nucleotide 1067-1094

PCR amplification was performed using Vent DNA Polymerase which generates 95% blunt ends for easy cloning into Eco RV digested pDK6.

pDK6 mLym (+ Poly A) - insertion of the open reading frame for murine lymphotactin into the unique Eco RV site in the multiple cloning site of pDK6. Murine lymphotactin was subcloned from the plasmid pJFE14 (Kelner et al., 1994) using Bst XI-Not I restriction enzymes. The resulting fragment was blunt ended using Klenow Large Fragment enzyme (NEB) with 1X Klenow buffer in a total of 20 μ l at room temperature to fill in the ends. This fragment was then ligated into the Eco RV digested pDK6 plasmid using T4 DNA ligase (NEB) in 1X T4 DNA ligase buffer in 20 μ l volume at 16^oC overnight.

pDK6 mLym (- PolyA) -insertion of the open reading frame for murine lymphotactin into the unique Hpa I site in the multiple cloning site of pDK6. Murine lymphotactin was cloned by PCR using the plasmid pJFE14 (Kelner et al., 1994) as the template and the following primers:

mLym Forward 5'- AGACTTCTCCTCCTGACTTTCCTG-3'

Nucleotide 9-45

mLym Reverse 5'- AGGCTGTTACCCAGTCAGGGTTAC-3'

Nucleotide 346-369

PCR amplification was performed using Vent DNA Polymerase which generates 95% blunt ends for easy cloning into Hpa I digested pDK6.

pCITE2a IL-2 -the open reading frame for human IL-2 was juxtaposed with the 11th ATG of the IRES (in pCITE2a). The IL-2 fragment lacking the start codon for ribosome initiation was directly amplified from LSXN IL-2 using the following primers:

IL-2 Forward 5'- TACAGGATGCAACTCCTGTCTTGC-3'

Nucleotide 14-38

IL-2 Reverse 5'- CTTAATTATCAAGTCAGTGTTGA-3'

Nucleotide 455-480

The pCITE2a plasmid was digested with Nco I and blunt ended to generate an initiation codon for the translation of hIL-2 (this strategy was used to avoid incorporating extra amino acids to the hIL-2 protein. PCR amplification was performed using Vent DNA Polymerase which generates 95% blunt ends for easy cloning into Nco I blunt ended pCITE2a plasmid.

Basic "rescue" plasmid with single expression cassette inserts

pBHG10 mLym -insertion of the expression cassette mCMV mLym SV40 (Poly A) into the adenoviral rescue vector pBHG10 (Pac I digested). To create this plasmid we first digested pBHG10 with the unique restriction enzyme Pac I which cuts the plasmid

within the E3 region. We then amplified the mLym expression cassette from pDK6 mLym using the following primers:

Expression Cassette Forward Primer

5'-ACTCCGCCCGTTTTATGACTAGAACC-3'

Nucleotide 375-400

Expression Cassette Reverse Primer

5'-GATCTTCGATGCTAGACGATCCAGAC-3'

Nucleotide 1928-1956

The expression cassette was then ligated into the blunted Pac I site of pBHG10. The Pac I digested pBHG10 plasmid was blunt ended using Klenow Large Fragment enzyme (NEB) with 1X Klenow buffer in a total of 20 μ l at room temperature to fill in the ends. This PCR fragment encoding the lymphotactin expression cassette was then ligated into the blunted ended pBHG10 plasmid using T4 DNA ligase (NEB) in 1X T4 DNA ligase buffer in 20 μ l volume at 16⁰C overnight.

Advanced "shuttle" plasmids with two inserts

pDK6 mB7-1 IRES hIL-2 -to construct this plasmid we amplified the IRES hIL-2 segment from pCITE 2a hIL-2 using the following primers and Vent DNA Polymerase:

IRES Forward 5'- TTCCGGTTATTTTCCACCATATTG-3'

Nucleotide 12-36

hIL-2 Reverse 5'- CTTAATTATCAAGTCAGTGTTGA-3'

Nucleotide 455-480

The resulting PCR product was then ligated into the Klenow Large Fragment (NEB) blunted ended Sal I site from pDK6 mB7-1 using T4 DNA Ligase.

pDK6 mLym (- Poly A) IRES hIL-2 -to construct this plasmid we amplified the IRES hIL-2 segment from pCITE 2a hIL-2 using the following primers and Vent DNA Polymerase:

IRES Forward 5'- TTCCGGTATTTTCCACCATATTG-3'

Nucleotide 12-36

hIL-2 Reverse 5'- CTTAATTATCAAGTCAGTGTTGA-3'

Nucleotide 455-480

The resulting PCR product was then ligated into Eco RV site from pDK6 mLym (- Poly A).

APPENDIX II.

Recombinant Adenoviral vectors

This appendix lists the first generation adenoviral vectors constructed and used in this thesis.

Table 1. Recombinant Adenoviral Vectors

| Virus Name | Description | Rescue |
|-----------------|--------------------------|--------|
| Plasmid | | |
| Ad5 mB7-1 | MCMV-mB7-1 | pBHG10 |
| Ad5 mLym | MCMV-Lymphotactin | pBHG10 |
| Ad5 mB7-1/hIL-2 | MCMV-mB7-1 IRES hIL-2 | pBHG10 |
| Ad5 mB7-2/hIL-2 | MCMV-mB7-2 IRES hIL-2 | pBHG10 |
| Ad5 mLym/hIL-2 | MCMV-mLym IRES hIL-2 | pBHG10 |
| Ad5 mLym/mL-12 | MCMV- mLym & MCMV- IL-12 | pBHG10 |

Appendix III.

Effects of Mixtures of Vectors on Tumor Regression

This section describes the results obtained from intra-tumoral injection experiments with mixtures of Ad5 mB7-1/hIL-2 and Ad5 mLym/hIL-2 in Neu (8142) tumors. Tumors were established as outlined in the Materials and Methods sections of chapters 3 and 5.

Complete Regression of Neu (8142) Tumors Using Mixtures of Ad5 mLym/hIL-2 and Ad5 mB7-1/hIL-2 at 2.5×10^8 Respectively.

| Vector | Experiment | Percentage |
|----------------------------------|------------|------------|
| Regressed | | |
| Ad5 dl70-3 | 0/5 | 0% |
| Ad5 mLym/hIL-2 & Ad5 mB7-1/hIL-2 | 6/10 | 60% |

There appears to be no enhanced complete tumor regression associated with the intra-tumoral administration of both double recombinant adenoviral vectors expressing either B7-1 and IL-2 or lymphotactin and IL-2.

APPENDIX IV.**Lytic Activity of NK Cells Collected From the BALs of Ad5 mLym Infected Mice.**

This section describes results obtained from chromium release assays using cells obtained from the lungs of Ad5 mLym (1×10^8 pfu) infected mice. The total cellular infiltrate was collected from the bronchiolar lavage fluids of 6 mice (at each time point: days 4 and 7). We used 6 mice in order to obtain approximately 1.2×10^6 NK cells for the assays. The effector cells were directly incubated with Yac-1 labeled target cells (Yac cells are NK sensitive) in RPMI 1640 for 4 hours prior to the measurement of chromium in the supernatants. Target cells were labeled as follows: Target cells were collected by centrifugation, washed in PBS, centrifuged for 5 min and resuspended in 500 μ l of RPMI 1640. To the target cells we added 50 μ l of 51 Cr and then placed the labeling reaction at 37 $^{\circ}$ C for 1.5 hours. After the incubation target cells were washed with 45 mls of PBS three time and placed in the chromium release assay with the effector cells.

NK activity in the lungs of Ad5 mLym infected mice at days 4 and 7

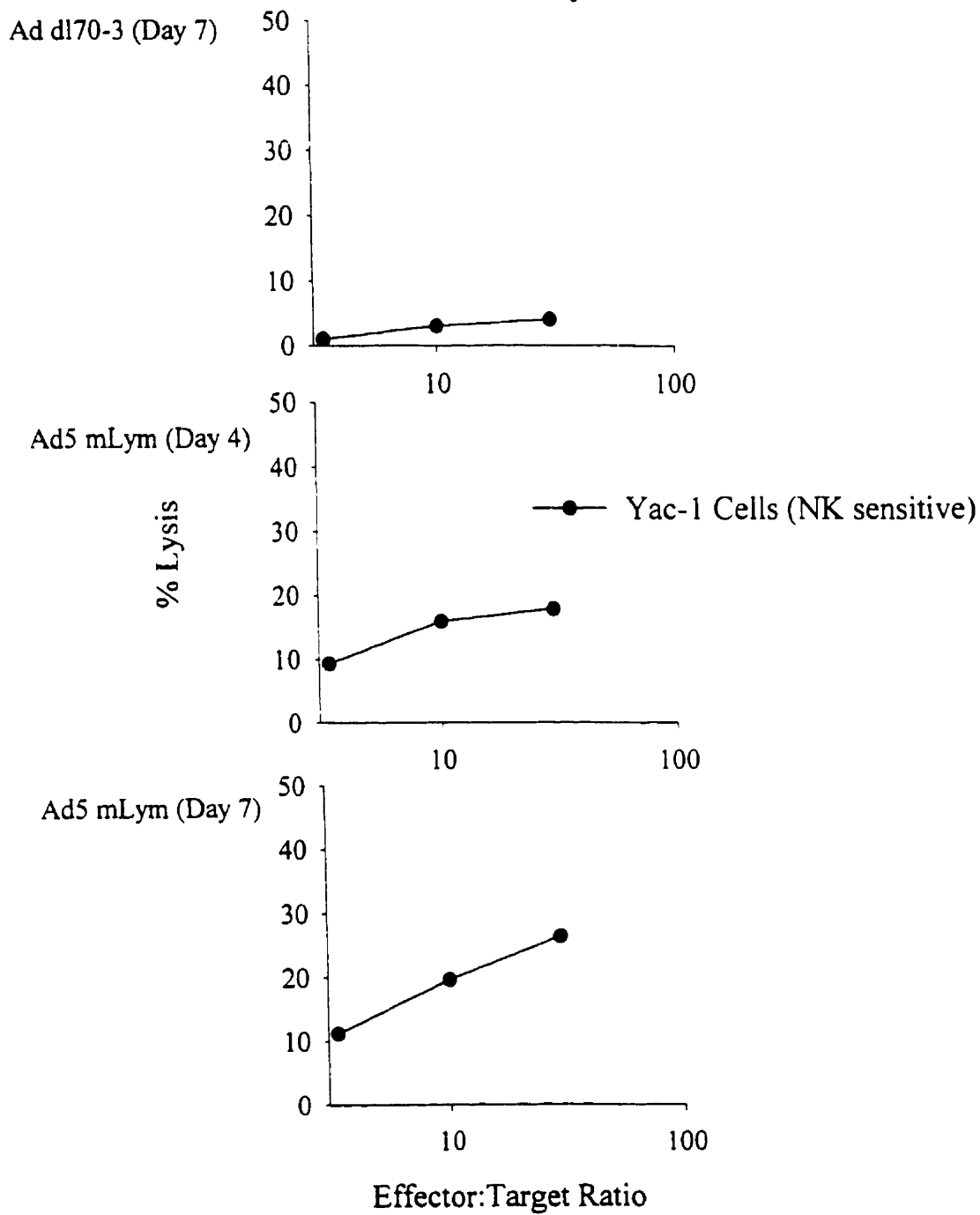


Figure 1

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