Polarization of a type 1 anti-tumor immune response in the context of a strong tumor antigen results in the regression of the mammary adenocarcinoma, 4T1

by
Shawn M. Jensen

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CERTIFICATE OF APPROVAL

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Shawn M. Jensen

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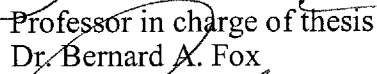
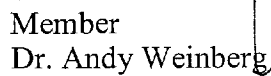


Professor in charge of thesis
Dr. Bernard A. Fox

Member
Dr. David Parker



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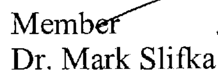

Member
Dr. Mark Slifka

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Abstract

When a tumor vaccine fails to protect an animal from subsequent tumor challenge, that tumor is characterized as nonimmunogenic. However, studies in our lab using a murine melanoma tumor model showed that nonimmunogenic tumors can sensitize tumor-specific T cells, but they display a polarized type 2 immune response and are non-therapeutic (Hu et al., 1998). Conversely, T cells from lymph nodes draining immunogenic tumors, where vaccination confers protection, exhibited a tumor-specific type 1 polarization. These findings have also been observed with four methyl-cholanthrene induced murine sarcomas as well as two oncogene-induced murine prostate tumors (Winter et al., 2003). Here in this study we investigated the hypothesis that augmenting a type 1 anti-tumor immune response would result in a therapeutic response against the poorly-immunogenic murine mammary adenocarinoma, 4T1. Data obtained in three different systems using 4T1 support this claim. First, a subclone of 4T1, 4T1-10, showed enhanced immunogenicity that correlated with a tumor-specific type 1 cytokine response. Second, T cells primed by a GM-CSF-secreting clone of 4T1 displayed a significant increase in tumor-specific IFN-γ which correlated with their enhanced therapeutic efficacy against 4T1 experimental pulmonary metastases. Third, $STAT6^{-/-}$ mice are deficient in the ability to generate a type 2 response and respond with an enhanced type 1 response. $STAT6^{-/-}$ mice rejected 4T1 tumor challenge, and T cells from tumor draining lymph nodes of $STAT6^{-/-}$ mice secreted significantly elevated amounts of IFN-γ in response to 4T1.

In addition to the importance of a type 1 polarized immune response, the lack of a sufficient frequency of tumor-specific T cells in *wt* BALB/c mice challenged with 4T1 was implicated as a reason for the inability to cause tumor regression. T cells from tumor-vaccine draining lymph nodes expanded *in vitro* obtained therapeutic efficacy against 4T1 experimental pulmonary metastases *in vivo*. Also, the regression of the $STAT6^{+}$-expressing 4T1 tumor cell line in $STAT6^{-/-}$ mice was dependent on the presence of STAT6-specific IFN-γ secreting T cells. In the absence of these STAT6-reactive T cells 4T1 grew progressively in $STAT6^{-/-}$ mice. This demonstrated that the ability to mount a type 1 polarized response was not sufficient if a strong tumor antigen was not present to prime a sufficient frequency of tumor-reactive T cells. Together the data presented here show that regression of the mammary adenocarcinoma, 4T1, requires tumor-antigen priming that elicits a sufficient frequency of type 1 polarized tumor-specific T cells.

Chapter 1: Introduction

Breast cancer is the leading cause of cancer among women and only follows lung cancer as the most fatal malignancy. Much progress has been made in the early detection of localized curable disease; however, despite this progress approximately 40,000 women die each year in the United States as a result of metastatic breast cancer (Jemal et al., 2003). For this reason it is imperative that scientists continue to add to the pool of knowledge used for designing future therapies aimed at treating breast cancer. In addition, cancer research benefits a wider scope of science since it includes the investigation of cellular processes and interactions between cells.

Cell growth and cell division are carefully regulated processes that are important for the growth of multicellular organisms. These processes must remain tightly regulated to prevent the dangerous effects of unrestrained cell proliferation. It is not surprising therefore that many regulatory proteins exist that determine whether the cell will remain quiescent or proceed into the cell cycle. However, on rare occasions these regulatory controls are disrupted resulting in unrestrained cell growth. If the descendents of this proliferating cell inherit the propensity to grow without cell regulation then they will expand indefinitely and form a tumor mass. Benign tumors remain localized, often encapsulated in a fibrous capsule. These tumors generally only become a risk if their size impinges on other cells or if they secrete excess amounts of biologically active molecules, such as hormones. However, if the tumor mass invades surrounding tissues and malignant cells enter the bloodstream or lymphatics then the prognosis is much worse for the host. The ability of malignant cells to spread or metastasize to different sites in the host makes metastatic tumors generally impossible to cure surgically. Other

modalites such as chemotherapy are used to treat metastatic cancer, however the impact of toxicity to normal tissues is an important limitation. The diffuse spread of metastatic disease makes it difficult to treat with radiation unless total body irradiation is used which can result in severe deficiencies of hemtopoietic tissue. Therefore treatments that specifically target disseminated metastases must be investigated to provide more successful options of treating cancer.

Immune Surveillance

At the end of the 19th century Robert Koch's research demonstrated that infectious diseases were caused by microorganisms. Koch's work stimulated other researchers including Louis Pasteur, Emil von Behring, and Shibasaburo Kitasato to investigate the ability of the body to defend against potential pathogens. Their work led to the birth of the science of immunology. This understanding of the immune system led scientists to devise strategies to treat cancer using the immune system. In the 1890s Coley treated cancer patients with bacterial extracts that were theorized to boost the immune system (Coley, 1991). Paul Ehrlich proposed in 1908 that the frequency of cancer would be much higher if the immune system did not exist. Burnet coined the term 'immunological surveillance' in 1970 to describe the immune system's ability to prevent tumor formation by detection and destruction of newly developed malignant cells (Burnet, 1970). However, the concept of immunological surveillance was questioned when the absence of a principal component of the immune system, namely T cells, did not significantly alter the incidence of tumor formation. This was shown using athymic 'nude' mice, which despite the apparent absence of T cells did not have an increased frequency of tumors compared to normal mice (Stutman, 1974; Stutman, 1979). But

further research showed that nude mice do not completely lack functional T cells (Maleckar and Sherman, 1987) and other components of the immune system, namely the natural killer cells, could mediate antitumor activity (Smyth et al., 2000a). However, if both these populations of cells were rendered non-functional then a higher incidence of tumor formation was observed (van den Broek et al., 1996). These experiments in conjunction with observations that IFN-γ (Dighe et al., 1994; Kaplan et al., 1998; Shankaran et al., 2001) and perforin (Smyth et al., 2000b) help prevent tumor formation support the theory of immune surveillance and strengthen the argument that the development of an appropriate immune response can destroy malignant cells wherever they may exist in the body (Dunn et al., 2002).

Tumor Antigens

Essential to the concept of immune surveillance is the ability of the immune system to differentiate malignant cells from normal cells. For adaptive immunity to be mounted against a tumor, tumor-associated antigens are needed to trigger an anti-tumor immune response. The fact that most tumor cell proteins are self-proteins also found in the normal cell poses a problem for the immune system, which is programmed to be "non-reactive against self". This "non-reactivity against self" is maintained either by central deletion of self-reactive T cells or host mechanisms that induce tolerance and protect against the induction of T cells that can react against self-proteins. Central deletion of developing T cells that are self-reactive occurs in the thymus when these T cells recognize cognate self-antigen presented by either the thymic epithelium or bone marrow-derived antigen presenting cells (Schwartz, 1990). However, detection of immune responses to self-proteins in patients with cancer has supported the view that not

Table1. Examples of tumor-associated antigens

Category of Antigen	Antigen	Tumors	Normal tissue
Differentiation antigens (overexpressed)	Tyrosinase	Melanoma	Melanocytes
	MART1/Melan-A	Melanoma	Melanocytes
	GP100	Melanoma	Melanocytes
	TRP-2	Melonoma	Melanocytes
Differentiation antigens (normally expressed)	Prostate-specific antigen	Prostate cancer	Prostate
	CD20	B-cell malignancies	B cells
Cancer-testis antigens	MAGE1, MAGE3	Melanoma, others	Testis
	GAGE	Melanoma, others	Testis
	NY-ESO-1	Melanoma, others	Testis
Mutated protein antigens	Beta-catenin	Melanoma, lung, others	ubiquitous
	Ras	Lung, pancreatic, others	ubiquitous
	p53	Breast, colon, others	ubiquitous
	CASP8	Head and neck cancer	ubiquitous
Oncofetal antigens	CEA	Colon cancer, others	Liver
	AFP	Liver cancer	-

all potentially self-reactive T cells are centrally deleted. These T-cell defined tumor antigens (Table 1) can be grouped into four general categories. The first group contains tumor antigens specific to the tissue from which the tumor originated. These tumor antigens might be expressed at levels similar to normal tissue or they might be overexpressed in the tumor compared to the normal tissue. Observations made in clinical trials and mouse experiments with melanocyte differentiation antigens has shown that melanoma rejection is occasionally associated with vitiligo, a local depigmentation of the skin resulting from melanocyte destruction showing that these antigens are not solely specific to the tumor (Rosenberg and White, 1996). The second group of tumor antigens,

cancer testis antigens, consists of transcriptionally reactivated genes. These genes are completely silent in most normal tissues except testis and placenta but are reactivated by the malignant process. The activation of the tumor antigen, MAGE, usually results from a demethylation of its promoter that correlates with a nonspecific genome-wide demethylation (De Smet et al., 1996). Unique antigens that are derived from mutated proteins make up the third group of tumor antigens. These mutations might be a result of chemical carcinogens or radiation. However, antigens from this group also occur in spontaneous tumors suggesting that these mutations might also be a result of mistakes made by the cellular machinery. These mutations might actively contribute to oncogenesis as well as evasion of the immune system. The CASP8 gene encodes the protease caspase-8 which is required for the induction of apoptosis through Fas and tumor necrosis factor receptor 1 (Boldin et al., 1996) (Muzio et al., 1996). The ability of mutated caspase-8 to trigger apoptosis appears to be reduced versus the wild type caspase-8 (Mandruzzato et al., 1997). Mutated proteins can serve as potent immunostimulatory tumor antigens if they are sufficiently different from normal proteins. The oncofetal group of tumor antigens contains proteins expressed during fetal development that become reactived in some tumors soon after transformation. In contrast to the tumor antigens from the groups listed in Table 1 that are self-proteins or are derived from self-proteins, antigens derived from oncogenic viruses constitute another category of potentially useful tumor antigens. The E7 protein of human papilloma virus (HPV)-16 which is present in most cervical carcinomas is capable of eliciting tumor-specific CTLs (Ressing et al., 1995). A long term study has also shown that

administration of a HPV-16 vaccine also reduced the incidence of HPV-16-cervical cancer compared to a placebo group (Koutsky et al., 2002).

Since there is plenty of evidence that potentially self-reactive T cells exist in the periphery of the host and yet the host does not suffer autoimmune disease it argues that peripheral mechanisms of tolerance must regulate the immune system (Linsley and Ledbetter, 1993; Rocken and Shevach, 1996; Schwartz, 1990). This functional tolerance of T cells to self-antigens may be passive, where self-reactive T cells simply ignore the antigen due to the lack of appropriate costimulation (Antonia et al., 1998). Alternatively, tolerance to self-antigens might be exerted by active mechanisms that either eliminate the self-reactive T cells or redirect the function of these T cells so that it is non-destructive to the host.

Peripheral Tolerance mechanisms

Mechanisms of peripheral tolerance can be divided either into deletion, anergy, or regulation. Although most T cell deletion occurs in the thymus, peripheral deletion of mature T cells has also been described (Ferber et al., 1994; Webb et al., 1994). Initially it was suggested that T-cell tolerance could be induced when B cells presented self-antigens to T cells (Epstein et al., 1995). However, more recently it has been shown that self-antigen presentation by peripheral dendritic cells efficiently deletes the majority of antigen-specific T cells and induces anergy in the remaining T cells (Lambolez et al., 2002). This apparent contradiction in the function of dendritic cells as either antigen-presenting cells that stimulate T cells or agents that tolerize self-reactive T cells appears to depend on the maturation status of the dendritic cell (Steinman and Nussenzweig, 2002). In the absence of inflammatory stimuli, dendritic cells remain in an immature

state where they actively acquire antigen but lack costimulatory molecules and do not secrete proinflammatory cytokines such as IL-12 and TNF-α (Lutz and Schuler, 2002) (Fig 1.1). The normal process of cellular turnover provides these immature dendritic cells with self-antigens that they could use to tolerize self-reactive T cells in the periphery (Lutz and Schuler, 2002). Additionally, these immature dendritic cells might induce regulatory T cells that maintain peripheral tolerance (Jonuleit et al., 2000). The mechanisms by which this population of T cells could regulate potentially destructive self-reactive T cells are unclear but could include immunosuppressive cytokines, such as IL-5, IL-10, and TGF-β, which have been reported to suppress naïve T-cell proliferation (Groux et al., 1999).

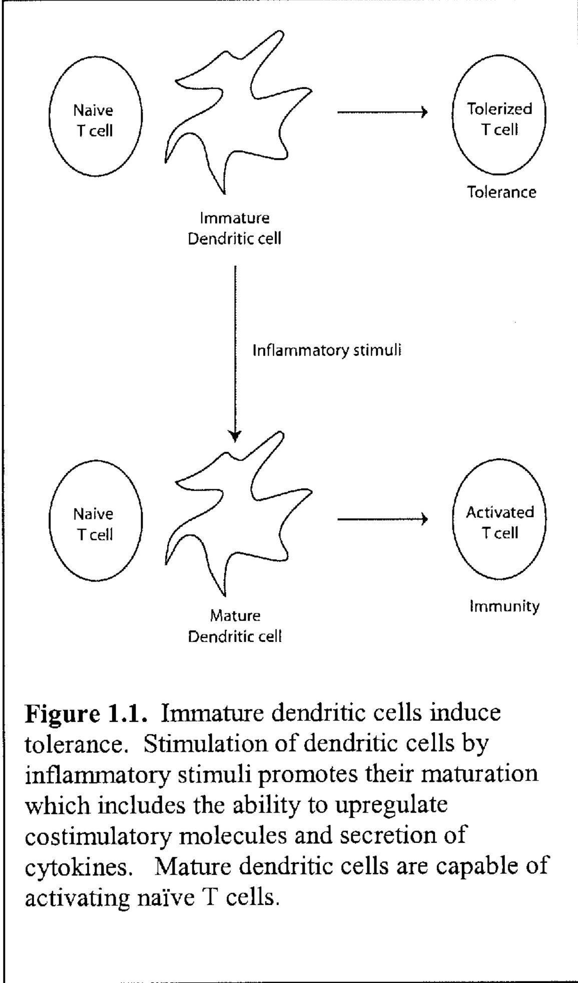

Figure 1.1. Immature dendritic cells induce tolerance. Stimulation of dendritic cells by inflammatory stimuli promotes their maturation which includes the ability to upregulate costimulatory molecules and secretion of cytokines. Mature dendritic cells are capable of activating naïve T cells.

Therefore, it is also possible that the cytokine environment in which T cells become primed might also regulate the immune response. The cytokine environment can polarize the immune response away from a destructive response toward a non-destructive response, a mechanism referred to as immune deviation (Rocken and Shevach, 1996). The original definition of immune deviation was coined by Asherson and Stone who

observed that the induction of a T-cell-dependent antibody response prevented antigen-specific delayed-type hypersensitivity (Asherson and Stone, 1965). Further observations by other groups suggested that an immune response was a composite of two antagonistic T-cell populations (Bretscher, 1974; Parish and Liew, 1972).

It is now generally accepted that both $CD4^+$ T helper (Th) cells and $CD8^+$ T cytotoxic (Tc) cells can be segregated based on their cytokine release patterns (Croft et al., 1994; Li et al., 1997; Mosmann and Sad, 1996; Salgame et al., 1991). A type 1 T cell selectively secretes, IFN-γ, TNF-β /LT and/or IL-2, whereas type 2 T cells secrete IL-4, IL-5, IL-6, IL-10, and/or IL-13. Besides the cytokine milieu in which they are activated, the differentiation of T cells toward one of these two phenotypes is determined by factors including route of antigen entry, the type of adjuvant, costimulation, and strength of T cell signal (Constant and Bottomly, 1997; Seder and Paul, 1994). Although all of these

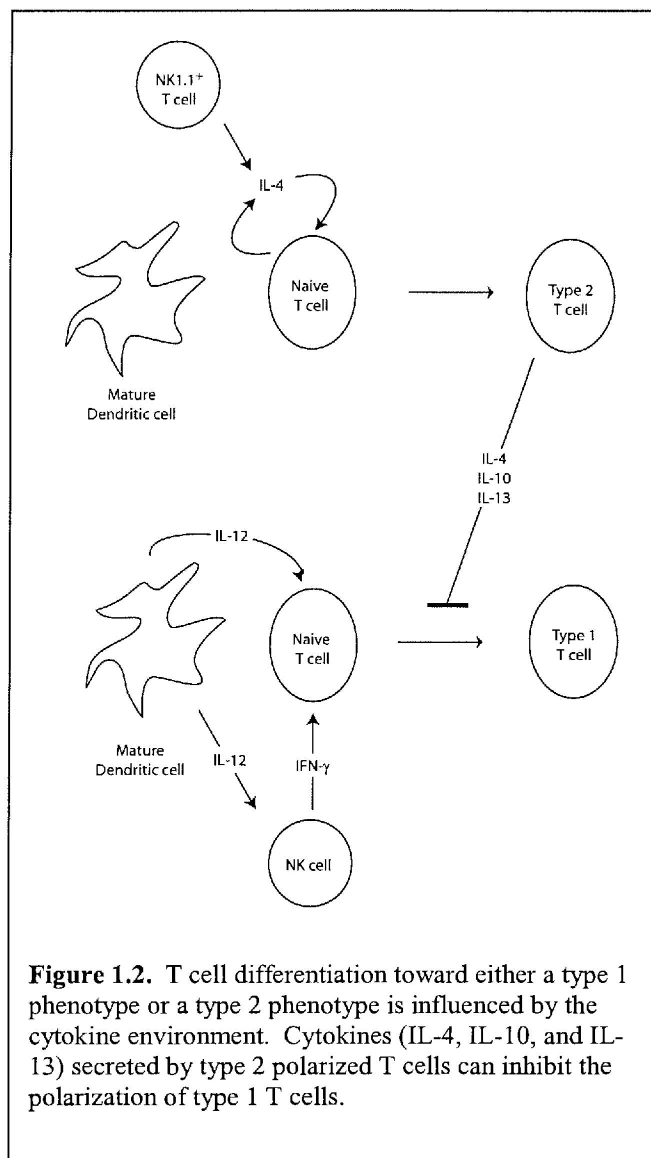

Figure 1.2. T cell differentiation toward either a type 1 phenotype or a type 2 phenotype is influenced by the cytokine environment. Cytokines (IL-4, IL-10, and IL-13) secreted by type 2 polarized T cells can inhibit the polarization of type 1 T cells.

factors have been reported to influence T-cell polarization, the role of the cytokine environment has been studied most intensively. IL-4 has been demonstrated to strongly influence type 2 polarization (Le Gros et al., 1990; Swain et al., 1990) (Fig. 1.2). Moreover, IFN-γ-producing type 1 cells are suppressed at higher doses of IL-4 (Hsieh et al., 1992; Seder et al., 1992). IFN-γ and IL-12 are the major cytokines that promote type 1 polarization. It appears that IL-12 is primarily responsible for driving type 1 polarization (Hsieh et al., 1993; Magram et al., 1996) and IFN-γ prevents the outgrowth of type 2 cells (Gajewski and Fitch, 1988). The relevance of type 1 or type 2 polarized responses to infectious disease states has been studied extensively, especially using the *Leishmania major* (*L. major*) infection model (Rogers et al., 2002). Protection against *L. major* is associated with a type 1 polarized immune response. In contrast, mouse strains that are susceptible to *L. major* infection mount predominantly a type 2 immune response. Converting the type 2 immune response toward a type 1 response in these susceptible mice protected them against *L. major* infection (Sadick et al., 1990). These data demonstrate that a polarized immune response can be inefficient and detrimental to the host. The production of inefficient immune responses that are not capable of reacting destructively against self may be responsible for the poor results associated with many immunotherapies for cancer.

The relevance of immune deviation in tumor models has only recently been investigated. Our own studies and those of others have implicated the type 1/ type2 paradigm in the regulation of the host's immune response to cancer (Aruga et al., 1997; Hu et al., 1998; Tsung et al., 1997). Results from a case study and a clinical trial in patients with melanoma, suggest that type 1 polarized cells have an important role in

mediating tumor regression (Kawakami et al., 1994; Lowes et al., 1997), whereas other groups have suggested that type 2 cells can mediate tumor regression (Dobrzanski et al., 1999; Hung et al., 1998; Rodolfo et al., 1999).

Murine Tumor Models

The past century has witnessed the development of animal models for cancer research. An important experimental tool for cancer research was the production of inbred strains of mice. Although inbred strains of mice were initially produced for commercial purposes tumor biologists noted that tumors could be transplanted successfully from mice of one strain to mice of the same strain (Little, 1924). Inbred mice provided tumor immunologists the system to study if immunological responses could protect mice against tumors. Initial studies demonstrated that immunity could be induced against sarcomas induced by the chemical carcinogen methylcholanthrene (MCA), and it was functionally tumor-specific since normal tissue grafts were not rejected (Foley, 1953; Gross, 1943; Phrehn and Main, 1957). Additionally, methods of selective breeding have made it possible to produce mice that develop 'spontaneous' tumors that can be investigated for mechanisms of tumor formation. Recently, transgenic and knockout mice have enabled investigators to examine specific genetic alterations or deficiencies that cause tumors.

During the early 1980s some studies with transplantable murine lymphoma FBL-3, which is transformed by the Friend Leukemia Virus, demonstrated that nonimmunized mice could reject this strongly antigenic tumor (Greenberg et al., 1988). Adoptive transfer of splenocytes from these immune mice could also protect irradiated tumor-bearing mice. Further examination of this tumor model indicated that tumor rejection

was mediated by a variety of cells including $CD4^+$ T cells, $CD8^+$ T cells, and macrophages (Greenberg, 1986; Mule et al., 1985). These studies corroborated previous work supporting the role of T cells in tumor immune surveillance, but it was apparent that studies using less immunogenic tumors that more closely resembled human cancer were needed. The development of the 100 series of methylcholanthrene (MCA)-induced sarcomas addressed this need as these tumors grew progressively in nonirradiated mice, and only a fraction of mice could be protected from tumor challenge if they were immunized against the tumors using *C. parvum* as an adjuvant (Shu and Rosenberg, 1985). Tumor cell lines from other tissues including lung (Lewis lung), colon (C26), melanocyte (B16), lymphocyte (EL4, YAC-1), liver (RENCA), and breast (SP1) have also been isolated and are currently being used extensively as tumor models.

Numerous tumor models have been developed to investigate the biology of breast cancer. The most useful murine breast tumor model will reflect many of the natural characteristics of human breast cancer. Although no one model can fully replicate the natural history of human breast cancer, each can provide information about an area in which it most closely resembles human breast cancer. The poorly immunogenic tumor, 4T1, shares many immunological characteristics with human breast tumors. It was isolated from a single, spontaneously arising BALB/cfC3H mammary tumor (Dexter et al., 1978; Miller et al., 1983). Subcutaneous injection with the tenth *in vivo* passage of the original, spontaneous tumor led to the creation of the 410LM tumor subline which was derived from a single nodule growing on the right medial lobe of the lung of a BALB/cfC3H mouse. Tumor 410 LM was serially propagated *in vitro* and serially transplanted through four passages *in vivo*, the tumor cells were reestablished in tissue

culture and designated 410.4. The 4T1 tumor cell line is a thioguanine-resistant variant of the 410.4 tumor subline. The metastatic nature of 4T1 was demonstrated by the appearance of metastases in the lymph nodes, lungs, liver, blood, brain, and bone marrow after subcutaneous injection (Pulaski and Ostrand-Rosenberg, 1998). The highly metastatic nature of 4T1 has been shown to be TGF-β dependent, as separate groups have shown that blocking the TGF-β type II receptor significantly restricted the ability of 4T1 cells to establish metastases (McEarchern et al., 2001; Muraoka et al., 2002). Additionally, inhibition of TGF-β secretion by 4T1 reduced the number of metastases to the lungs and liver if it was combined with IFN-γ (Wu et al., 2001). The importance of type 1 cytokines have also been suggested in the 4T1 tumor model. Gene gun-mediated delivery of IL-12 to intradermal 4T1 did not affect primary tumor growth, but it substantially reduced lung metastases (Rakhmilevich et al., 2000). This result appeared to be mediated through NK cells and IFN-γ secretion and not dependent on CD4 or CD8 T cells. A separate group demonstrated substantiating results that IL-12 reduced metastatic disease possibly through a NK cell mediated event (Pulaski et al., 2000). Further work suggested that IFN-γ, possibly produced by NK cells, was critical for reducing metastatic disease through the activation of host macrophages, immature DCs, or neutrophils (Pulaski et al., 2002). T-cell mediated anti-tumor immune responses have also been implicated for enhanced survival against 4T1 tumor. Injection of OX-40L:Ig increased the survival of 4T1-challenged mice implying that engagement of the OX-40 receptor on activated $CD4^+$ T cells with OX-40L:Ig provided enhanced tumor immunity (Morris et al., 2001). The importance of $CD4^+$ T cells was also indicated in experiments

where transfection of 4T1 with MHC class II (I-A^d) resulted in the reduction of metastatic 4T1 tumor cells (Pulaski and Ostrand-Rosenberg, 1998).

Adoptive immunotherapy

Adoptive immunotherapy of cancer is a therapy in which cells with antitumor activity are transferred to a tumor-bearing host. These transferred cells may kill the tumor directly or they may mediate anti-tumor effects indirectly. These indirect effects could be either the secretion of cytokines that recruit other cells that destroy the tumor or by making the tumor environment inhospitable to tumor growth. This type of therapy allows the cells to be activated and expanded *in vitro* and then returned to the tumor-bearing host. The *in vitro* culture period allows for the manipulation of the anti-tumor immune response as well as circumvents immunosuppressive conditions that might exist in the tumor-bearing host.

The description of T-cell growth factor (Morgan et al., 1976) and the subsequent discovery of its active component, IL-2 (Rosenberg et al., 1984), led to the first studies of adoptive immunotherapy in cancer. Lymphokine-activated killer (LAK) cells were first described in 1980 by Yron et al., who demonstrated that normal murine splenocytes could kill a cultured syngeneic sarcoma if they were cultured in T-cell growth factor (IL-2) (Yron et al., 1980). Further studies have defined the LAK phenomenon as cytolytic activity of IL-2-cultured lymphocytes against fresh and cultured tumors, but not against fresh normal tissue. Subsequently, LAK cells have been shown to be non-MHC-restricted (Grimm and Owen-Schaub, 1991; Rayner et al., 1985). Initial murine studies demonstrated that LAK cells could cause the regression of established pulmonary metastases (Mule et al., 1984). Clinical adoptive immunotherapy of cancer patients, with

whole body equivalents of infused LAK cells, was first performed in 1984 when LAK cells were transferred into patients with melanoma or renal cell cancer (Rosenberg et al., 1985).

Subsequent animal studies identified that T cells expanded from lymphocytes infiltrating progressively growing tumors were 50-100 times more effective than LAK cells at mediating regression of established tumors (Rosenberg et al., 1986). These studies led to a second generation of clinical trials using tumor-infiltrating lymphocytes (TIL), and identified characteristics of TIL cultures that had therapeutic activity (Rosenberg et al., 1988). A significant difference of therapeutic TIL cells compared to LAK cells was the tumor-specific cytolytic activity observed by the TIL cells (Aebersold et al., 1991).

The third generation of adoptive immunotherapy trials used an autologous tumor vaccine to prime TVDLN that were subsequently expanded by *in vitro* sensitization (IVS) with autologous tumor cells or purified tumor antigens (Shu et al., 1986). This period of *in vitro* sensitization created or boosted an antitumor immune response under controlled conditions that could be manipulated. Since tumor-reactive T cells might fail to arrest tumor growth *in vivo* due to insufficient numbers or immunosuppressive conditions, *ex vivo* sensitization circumvented possible immunosuppressive mechanisms and enabled expansion of tumor-specific T cells. Subsequent preclinical studies identified that anti-CD3 could replace the requirement for autologous tumor during IVS (Yoshizawa et al., 1991b). This observation led to the fourth generation of clinical adoptive immunotherapy trials, where substantial objective responses in 33% of patients with renal cell carcinoma (2/12 complete response; 2/12 partial response) were observed (Chang et al., 1997). In

addition, primed lymph node cells from 6 of 8 renal cell carcinoma patients developed tumor-specific immune responses (Chang et al., 1997). These results demonstrate that strategies combining autologous tumor vaccination and TVDLN harvest are an effective approach to sensitize or identify tumor-specific T cells.

Outline of Thesis

A thorough understanding of the mechanisms underlying the development of a therapeutic immune response against tumor antigens is critical for the improvement of immune-mediated treatment of cancer. To understand the requirements of an effective response, it is necessary to investigate the mechanisms that impede a therapeutic response against the tumor. Early efforts in our lab suggested that tumors previously classified as nonimmunogenic actually were capable of stimulating an immune response. However, the resulting immune response was inefficient and characterized by secretion tumor-specific type 2 cytokines. These data focused our efforts on the role of immune deviation as a mechanism of tumor evasion. Based on these observations we formulated the following global hypothesis:

> **Augmenting the tumor-specific type 1 immune response will enhance the therapeutic anti-4T1 tumor response**

The work presented herein addresses this hypothesis by answering the following questions:

1. Does the heterogenous population of cells in the 4T1 tumor cell line contain tumor clones of varying immunogenicities; and do more immunogenic tumor clones prime tumor-draining lymph node cells that exhibit augmented tumor-specific type 1 cytokine secretion. (Chapter 2)
2. Does secretion of granulocyte macrophage colony stimulating factor (GM-CSF) by a poorly immunogenic tumor-vaccine augment the type 1 response and improve the therapeutic effectiveness of the T cells primed by the tumor-vaccine? (Chapter 3)

3. Are type 1 polarized T cells from STAT6$^{-/-}$ mice more therapeutic than T cells from *wt* mice? Does the presence of a strong tumor antigen influence the ability of 4T1 to be rejected by STAT6$^{-/-}$ mice? (Chapter 3 & 4)
4. Do type 2 polarized T cells from STAT4$^{-/-}$ mice vaccinated with 4T1 inhibit the anti-tumor efficacy of type 1 polarized T cells from STAT6$^{-/-}$ mice? (Chapter 3)

Chapter 2: Phenotypic and immunogenic diversity of 4T1

Abstract

The 4T1 tumor cell line was isolated from a single, spontaneously arising BALB/c mammary tumor. The poorly immunogenic nature of 4T1 is demonstrated by the inability of prior tumor vaccination to protect mice against 4T1 tumor challenge. Interestingly, 4T1 is a heterogenous tumor cell population that contains tumor subpopulations with distinct phenotypic and immunogenic differences. Two 4T1 subclones, 4T1-9 and 4T1-10, are described here that differ in the expression of cell surface molecules as well as immunogenic potential. Similar to parental 4T1, prior vaccination with 4T1-9 is unable to protect mice against 4T1-9 tumor challenge. In contrast, 4T1-10 is moderately immunogenic protecting approximately 40% of tumor-challenged mice. The expression of the costimulatory molecules, ICAM-1, CD40, and CD86 by 4T1-10 provides a possible explanation for the moderate immunogenicity of this tumor cell clone. Additionally, T cells from 4T1-10 tumor-vaccine draining lymph nodes secreted more tumor-specific IFN-γ than T cells from 4T1-9 tumor-vaccine draining lymph nodes. These data suggest that the more immunogenic subclones of 4T1 can prime a heightened type 1 polarized immune response compared to less immunogenic subclones of 4T1.

Introduction

It has been postulated that tumors arise in a host because they are unable to stimulate an immune response. However, the detection of tumor-reactive T cells in hosts with progressive tumor growth suggests that many cancers can stimulate an immune response (Finke et al., 1992; Ioannides et al., 1991; Miyatake et al., 1986; Muul et al., 1987; Topalian et al., 1989), but that the immune response is insufficient to cause tumor regression. Transplantable murine tumors can be classified based on their tumorgenicity, which can be defined as the lowest dose of tumor cells that results in tumor outgrowth in 100% of the challenged mice (TD_{100}). In regards to immunological responses to tumors, if it requires high doses of tumor cells to be transplanted into immunocompetent, syngeneic recipients for tumors to form then it suggests that these tumors can sufficiently prime the the immune system to prevent tumor outgrowth. Cancers that have been induced by viruses or exposure to carcinogens tend to require quite high doses of cells to cause tumors since these methods of transformation generally result in the acquisition of strong tumor antigens (Klein and Klein, 1977). In contrast, if tumors form when small doses of tumor cells are injected into recipients, it suggests that these tumors lack potent tumor antigens. Spontaneous tumors tend to exhibit this level of tumorgenicity when they are transplanted to syngeneic recipients probably because they arose under the selective pressure of remaining undetected by the immune response. However, tumors may express potentially immunogenic proteins but the selective pressure to remain undetected by the immune system selects for cells with alterations in the antigen-presenting pathway (ie. TAP, proteosome, or MHC class I), which enable the tumor to escape immune recognition (Chen et al., 1996; Vitale et al., 1998). Biological processes

other than the interaction of the tumor cell with the immune system can also dictate whether a tumor will grow progressively. For example, multiple interactions of the stromal microenvironment with the tumor cells will regulate tumor growth (Mueller and Fusenig, 2002). The growth rate of the tumor and its ability to form appropriate blood supply through angiogenesis also contribute to the ability of the tumor to form in its host. Therefore, caution should be taken in drawing a correlation between tumorgenicity and the immunogenic potential of a tumor.

A more direct method of estimating the immunogenicity of a tumor is to vaccinate with possible tumor antigens prior to tumor challenge. In this way the immunogenic potential of the tumor can be directly determined without the complexity of all other factors necessary for tumor formation. In our work we have defined tumor immunogenicity as the ability of prior tumor vaccination with lethally-irradiated tumor to protect mice against twice the TD_{100}. Tumors classified as strongly immunogenic provide protection against a tumorigenic dose of tumor in 100% of tumor-challenged mice. However, if tumor vaccination does not protect any of the mice against tumor challenge then the tumor is classified as poorly immunogenic. Weakly and moderately immunogenic tumors fall between these two extremes.

Prior work by other investigators has demonstrated that 4T1 is a poorly immunogenic mammary adenocarcinoma tumor cell line (Pulaski and Ostrand-Rosenberg, 1998). Our initial observations showed that 4T1 consisted of a hetergenous population of tumor cells with subpopulations demonstrating morphological and phenotypical differences. We attempted to determine whether these separate tumor clones would display varying immunogenicites, and if they did, were the more

immunogenic clones of 4T1 able to prime an enhanced type 1 cytokine response as characterized by IFN-γ secretion?

Materials and Methods

Mice

Female BALB/cJ were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained in a specific pathogen-free environment. Recognized principles of laboratory animal care were followed (Guide for the Care and Use of Laboratory Animals, National Research Council, 1996), and all animal protocols were approved by the Earle A. Chiles Research Institute Animal Care and Use Committee.

Tumor cell lines

4T1 is a 6-thioguanine-resistant cell line that was selected from a tumor cell line derived from a single spontaneously arising mammary tumor in a BALB/c3H mouse (provided by Dr. Suzanne Ostrand-Rosenberg, University of Maryland Baltimore County, MD). All cell lines were maintained in complete media (CM) comprising the following: RPMI 1640 (Bio Whittaker, Walkersville, MD) containing 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, and 50 μg/ml gentamicin sulfate. This was further supplemented with 50 μM 2-mercaptoethanol (Aldrich, Milwaukee, WI) and 10% (vol/vol) fetal calf serum (GIBCO BRL, Grand Island, NY).

4T1 tumor cell clones

4T1-9 and 4T1-10 were cloned from the parental 4T1 tumor cell line by limiting dilution. Briefly, the parental 4T1 tumor cell line was resuspended in CM at 1.5 cells/ml and seeded in a 96-well plate at 200μl/well (0.3 cells/well). Poisson statistics indicate that if <22% of the wells have growing cells (the proportion expected if 0.3 cells/well are plated), then 88% of these wells have only one clone (Taswell, 1981; Yokoyama, 1991). Only 96-well plates with <10% of the wells with growing cells were used for obtaining

tumor subclones. Wells were inspected for monoclonality by looking for tight single clusters using an inverted microscope. Positive wells were selected, tumor cells were expanded *in vitro* and cloned again by limiting dilution.

Tumor phenotyping

4T1, 4T1-9, and 4T1-10 were analyzed by flow cytometric analysis for expression of various cell surface markers. Tumor cells were labeled at 4° C with one or more of the following antibodies, all purchased from BD-PharMingen (San Diego, CA): FITC-labeled H-$2K^d$ (cloneSF1-1.1), FITC-labeled I-A^d/I-E^d (clone 2G9), FITC-labeled CD80 (clone 1G10), PE-labeled CD86 (clone GL-1), PE-labeled CD40 (clone HM40-3), and FITC-labeled ICAM-1 (clone 3E2). Cells were washed and analyzed on a Beckman Coulter EPICS XL-MCL FACS machine (Miami, FL).

Tumorgenicity

BALB/c were injected subcutaneously in the hind flank with 10^3, 5 X 10^3, 10^4 or 10^5 4T1, 4T1-9, or 4T1-10 tumor cells. Tumor growth was determined by multiplying the measured perpendicular diameters of the tumor. Mice were sacrificed when multiplied diameters equaled 150 mm^2.

Tumor immunogenicity

10^7 gamma-irradiated (10000 rad) 4T1, 4T1-9, or 4T1-10 tumor cells were subcutaneously injected in the right hind flank of BALB/c mice. This dose of tumor cells was used based on previous work in our lab determining immunogenicity (Winter et al., 2003). Control mice were subcutaneously injected in the right hind flank with PBS. Fourteen days following tumor vaccination the mice were injected with 2 X TD_{100} (10^4) viable (trypan blue exclusion) 4T1, 4T1-9, or 4T1-10 tumor cells in the contralateral

flank. Tumor growth was determined by multiplying the measured perpendicular diameters of the tumor. Mice were sacrificed when multiplied diameters equaled 150 mm^2.

Tumor vaccination, separation, and activation of vaccine-draining lymph node cells

Tumor cell cultures were trypsinized (Bio Whittaker, Walkersville, MD) and washed twice in Hanks' Balanced Salt Solution (Bio Whittaker, Walkersville, MD). 7.5 X 10^5 4T1-9 or 4T1-10 non-irradiated, live tumor cells were injected subcutaneously into both axillary regions and hind flanks of BALB/c mice. Eight days following vaccination, the superficial inguinal lymph nodes and axillary lymph nodes draining the four vaccination sites were harvested and single cell suspensions were resuspended at 2 X 10^6 cells per ml in CM and cultured in 24 well plates with 50 μl of a 1:40 dilution of 2c11 ascites (anti-CD3). After two days of activation, the cells were harvested and expanded in CM containing 60 IU per ml of rhIL-2 (Chiron, Emeryville, CA) at a starting cell density of 1.25 X 10^5 cells per ml in 300 ml gas permeable tissue culture bags (Nexell Therapeutics Inc., Irvine, CA). After three days, 'effector' T cells were harvested and used in cytokine release assays.

Cytokine release assay

After *in vitro* activation and expansion, 'effector' T cells were washed and incubated alone or were stimulated with 4T1, Renca, CT26, or plate-bound anti-CD3. 'Effector' T cells (2 X 10^6/well) were cultured with tumor cells (2 X 10^5/well) in 2 ml in 24-well plates. Supernatants were recovered 20 hours after stimulation and IFN-γ concentration was measured in duplicate by ELISA using commercially available reagents (BD-

PharMingen, San Diego, CA). The concentration of cytokine in the supernatant was determined by regression analysis.

Statistical Analyses

Cytokine data were analyzed using Student's t test for unequal variances (Microsoft Excel, Redmond, WA). Tumor free survival curves analyzed using Kaplan Meier test (GraphPad Prism, San Diego, CA).

Results

Tumorigenicity and immunogeniciy of 4T1

It was imperative to determine the tumorigenicity of 4T1 before other studies were conducted to establish confidence that the dose of tumor that was injected subcutaneously would reliably cause tumor in 100% of mice. BALB/c mice were injected with tumor cell doses spanning from 10^3 to 10^5 tumor cells. Greater than 30% of BALB/c mice remained tumor free when challenged with the low dose of 10^3 4T1 tumor cells (Fig. 2.1). However, injection of $\geq 5 \times 10^3$ 4T1 tumor cells caused progressive tumor growth and eventual death of 100% of

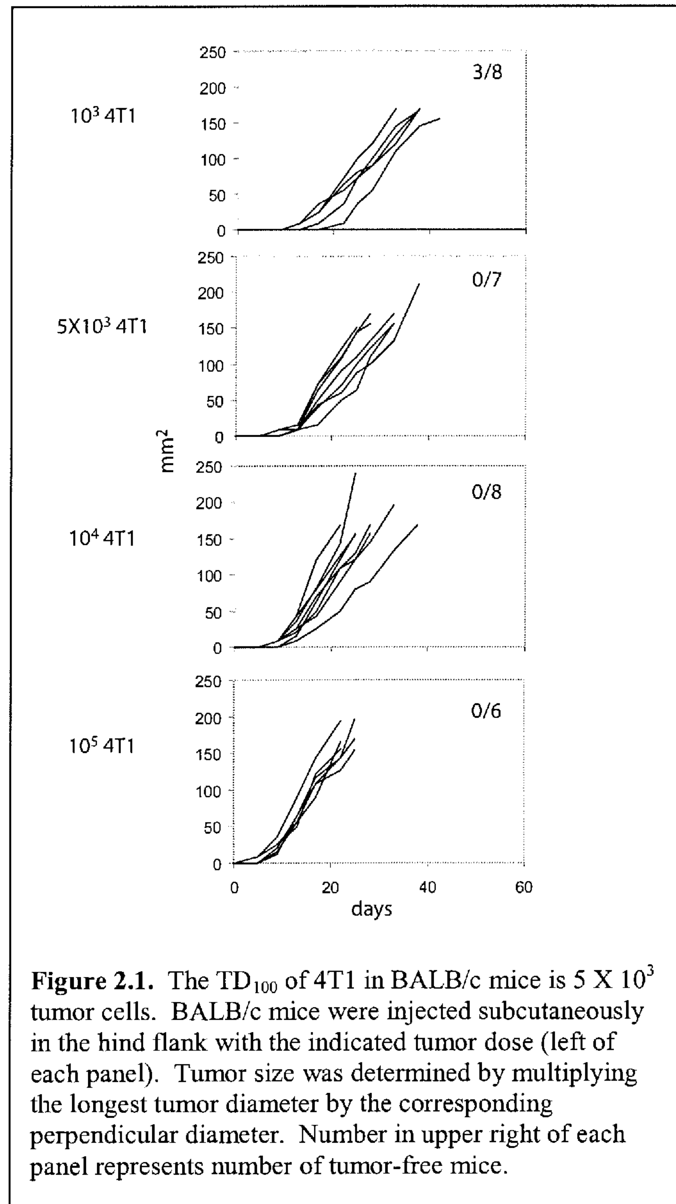

Figure 2.1. The TD_{100} of 4T1 in BALB/c mice is 5×10^3 tumor cells. BALB/c mice were injected subcutaneously in the hind flank with the indicated tumor dose (left of each panel). Tumor size was determined by multiplying the longest tumor diameter by the corresponding perpendicular diameter. Number in upper right of each panel represents number of tumor-free mice.

BALB/c mice. These data demonstrate that the TD_{100} of 4T1 is 5×10^3 tumor cells in BALB/c mice. An additional characteristic of 4T1 that makes it attractive as a tumor

model is its metastatic potential. Metastases from the primary tumor site can be detected in the lung, liver, and brain after subcutaneous challenge (data not shown).

Since a relatively low dose of 4T1 could cause progressively-growing 4T1 tumor in BALB/c mice it was of interest to determine if prior immunization with lethally-irradiated 4T1 cells could protect mice against tumor challenge with twice the TD_{100} of 4T1. Prior vaccination of BALB/c mice with 10^7 irradiated 4T1 tumor cells failed to protect any of the mice against 4T1 tumor challenge (Fig. 2.2), demonstrating that 4T1

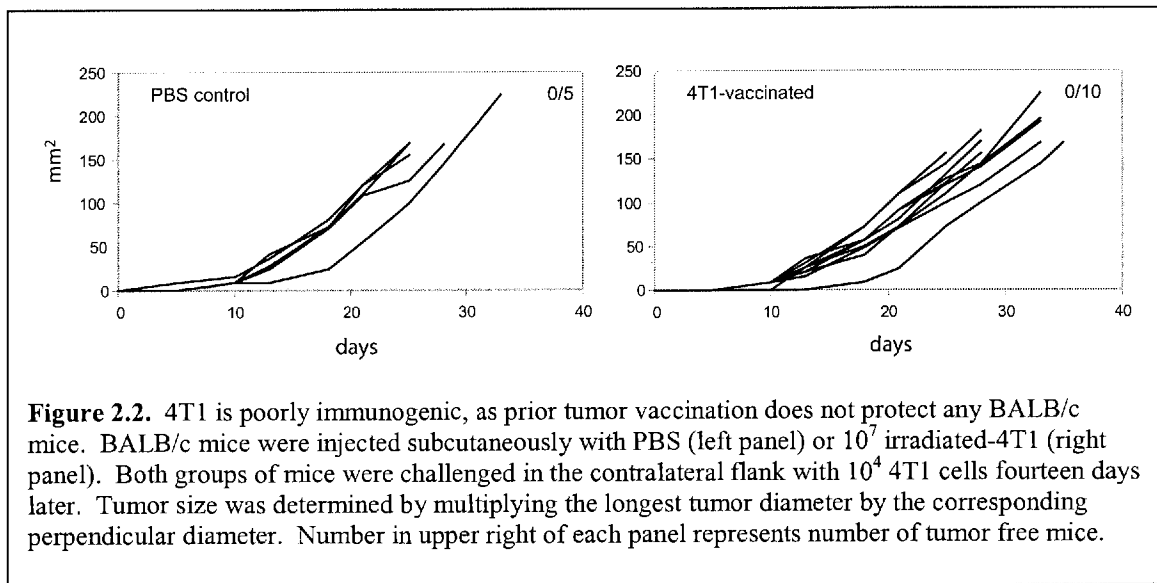

Figure 2.2. 4T1 is poorly immunogenic, as prior tumor vaccination does not protect any BALB/c mice. BALB/c mice were injected subcutaneously with PBS (left panel) or 10^7 irradiated-4T1 (right panel). Both groups of mice were challenged in the contralateral flank with 10^4 4T1 cells fourteen days later. Tumor size was determined by multiplying the longest tumor diameter by the corresponding perpendicular diameter. Number in upper right of each panel represents number of tumor free mice.

was a poorly immunogenic tumor. This characteristic of 4T1 makes it attractive as a tumor model since it mirrors the clinical setting in that most human cancer fail to prime a strong immune response.

Heterogeneity of 4T1

Microscopic examination of the parental 4T1 tumor cell line showed a morphologically heterogeneous population of cells (Fig. 3). Tumor cells that appeared to be loosely adherent and had a ball-like morphology contrasted with tumor cells that

appeared to be adherent to the flask and grew in a cobblestone pattern. It was possible that these different morphologies represented tumor cells at various points in cell cycle growth or alternatively they represented distinct tumor cell clones existing within the parental 4T1 tumor cell line. Subcloning 4T1 by limiting dilution led to the isolation of

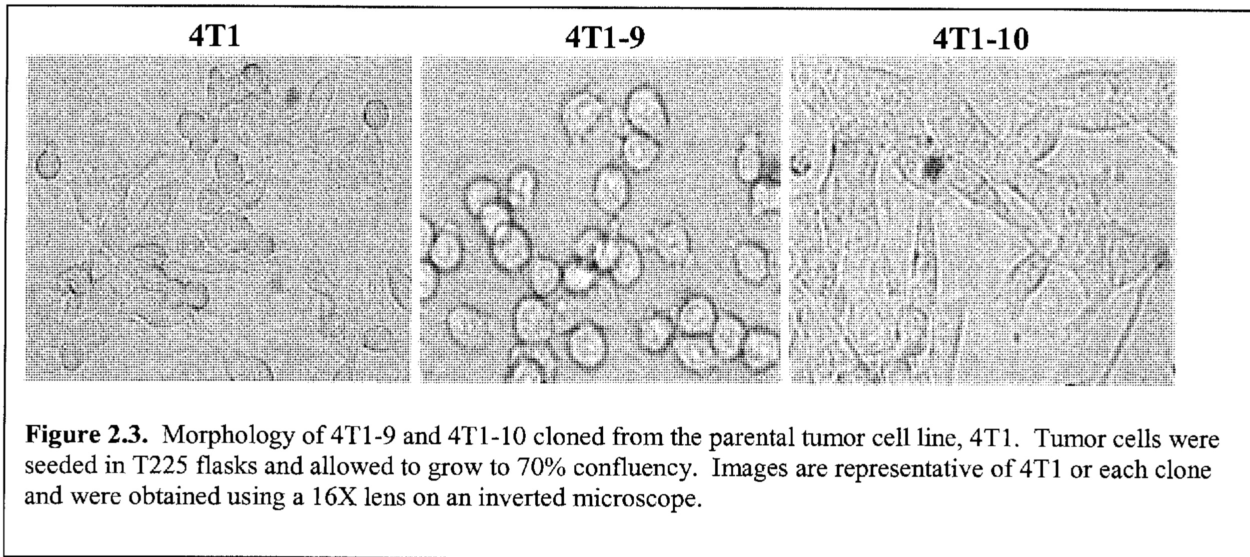

Figure 2.3. Morphology of 4T1-9 and 4T1-10 cloned from the parental tumor cell line, 4T1. Tumor cells were seeded in T225 flasks and allowed to grow to 70% confluency. Images are representative of 4T1 or each clone and were obtained using a 16X lens on an inverted microscope.

multiple subclones from the parental 4T1 tumor cell line. The cells were passed twice through limiting dilution to ensure that monoclonality was achieved. The appearance of each clone remained morphologically stable in cell culture for periods in excess of 3 months suggesting that they were distinct clones and not simply cells at different points in cell cycle growth. Two of twelve subclones that appeared to represent two distinct cell types observed in the parental 4T1 tumor cell line were chosen for further observation. 4T1-9 was loosely adherent to the flask, appeared ball-like and grew as clusters of cells (Fig. 2.3), whereas 4T1-10 was much more adherent and grew as a monolayer in a cobblestone pattern.

These two clones were compared to the parental 4T1 tumor cell line for expression of cell surface markers. 4T1, 4T1-10 and 4T1-9, expressed MHC class I (H-2K^d) on greater than 90% of the cells (Fig. 2.4). The expression of H-2K^d on 4T1-10 was slightly higher compared to either 4T1 or 4T1-9. MHC class II (I-A^d/I-E^d) expression was not detected in either clone or in the parental 4T1 cells. There was no expression of CD80 by any of these tumors, however parental 4T1 and 4T1-10 had both a negative population and a positive population expressing another B7 family member, CD86.

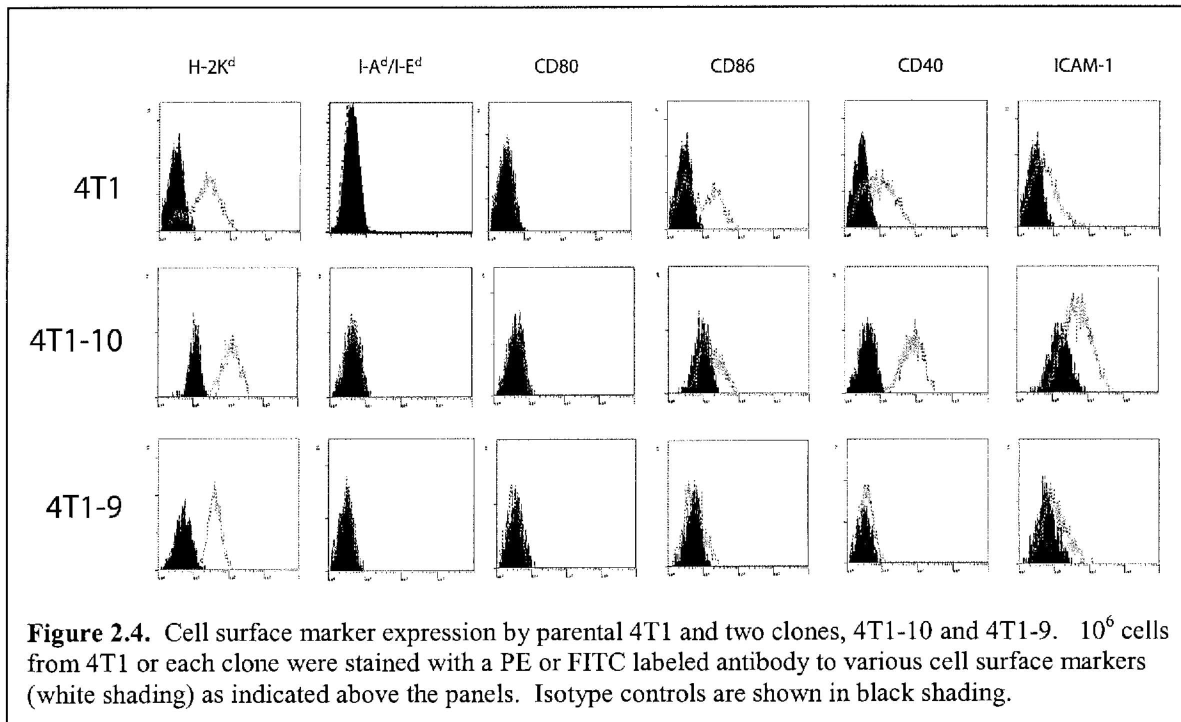

Figure 2.4. Cell surface marker expression by parental 4T1 and two clones, 4T1-10 and 4T1-9. 10^6 cells from 4T1 or each clone were stained with a PE or FITC labeled antibody to various cell surface markers (white shading) as indicated above the panels. Isotype controls are shown in black shading.

CD86 expression was not detected on 4T1-9 cells. The expression of CD40 by 4T1 ranges from strongly positive levels to non-detectable levels. Interestingly, the two clones exhibited quite different expression of this costimulatory molecule. 4T1-10 expressed CD40 on greater than 97% of the tumor cells. In contrast, no detectable levels of CD40 were found on 4T1-9. Expression of the adhesion molecule, ICAM-1, was

observed in a minority of cells from the parental 4T1 tumor cell line. ICAM-1 expression appeared to be higher on 4T1-10 compared to 4T1-9.

Tumorigenicity and immunogenicity of 4T1 clones

The differential expression of various cell surface markers on the 4T1 clones raised the possibility that they could differ in tumorigenicity as well as immunogenicity. The presence of the costimulatory molecule, CD40, on 4T1-10 suggested that it might enhance the activation of T cells (Blotta et al., 1996). There is also evidence that CD40 ligation on tumor cell lines increased their susceptibility to specific lysis by T cells (von Leoprechting et al., 1999). Since 5 X 10^3 parental 4T1 cells was the tumorigenic dose for BALB/c mice we subcutaneously injected either 10^3 or 5 X 10^3 tumor cells from each clone into BALB/c mice. Both 4T1-9 and 4T1-10 displayed similar tumor growth curves when 5 X 10^3 tumor cells were injected into the mice (data not shown). All mice that received this dose of tumor cells developed tumors and eventually succumbed to its progressive growth (Fig.

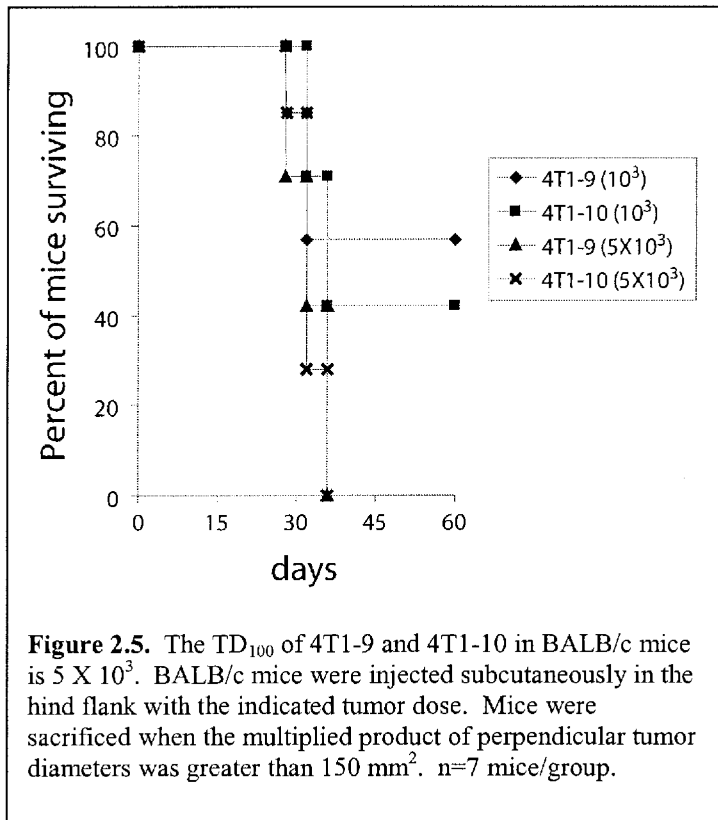

Figure 2.5. The TD_{100} of 4T1-9 and 4T1-10 in BALB/c mice is 5 X 10^3. BALB/c mice were injected subcutaneously in the hind flank with the indicated tumor dose. Mice were sacrificed when the multiplied product of perpendicular tumor diameters was greater than 150 mm^2. n=7 mice/group.

2.5) similar to what was observed with parental 4T1 cells. Forty to sixty percent of the mice developed tumors following injection of 10^3 4T1-9 or 4T1-10 tumor cells.

Although both 4T1-9 and 4T1-10 exhibited the same tumorigenicity as 4T1, we wanted to determine if prior vaccination with either of these clones could protect mice

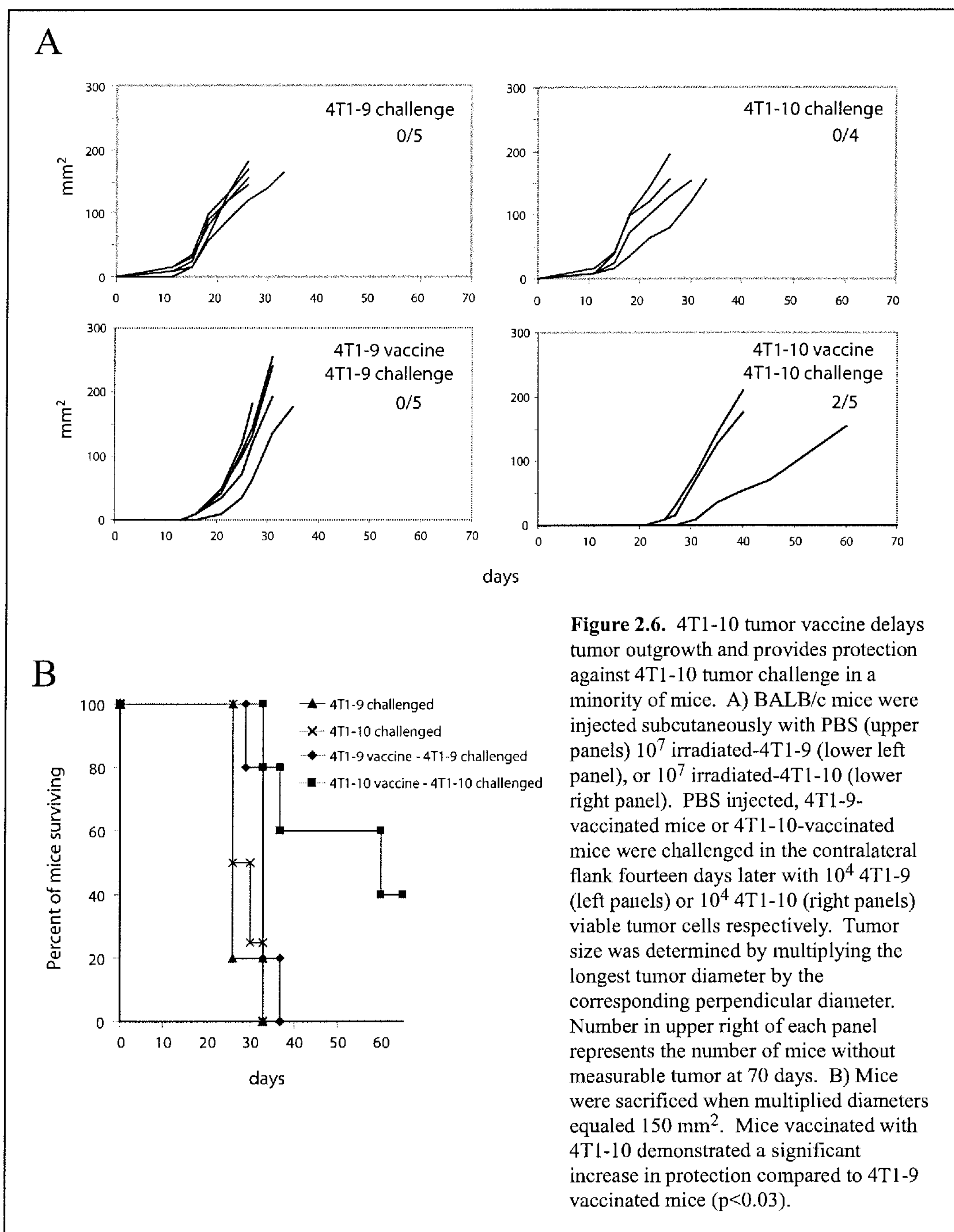

Figure 2.6. 4T1-10 tumor vaccine delays tumor outgrowth and provides protection against 4T1-10 tumor challenge in a minority of mice. A) BALB/c mice were injected subcutaneously with PBS (upper panels) 10^7 irradiated-4T1-9 (lower left panel), or 10^7 irradiated-4T1-10 (lower right panel). PBS injected, 4T1-9-vaccinated mice or 4T1-10-vaccinated mice were challenged in the contralateral flank fourteen days later with 10^4 4T1-9 (left panels) or 10^4 4T1-10 (right panels) viable tumor cells respectively. Tumor size was determined by multiplying the longest tumor diameter by the corresponding perpendicular diameter. Number in upper right of each panel represents the number of mice without measurable tumor at 70 days. B) Mice were sacrificed when multiplied diameters equaled 150 mm^2. Mice vaccinated with 4T1-10 demonstrated a significant increase in protection compared to 4T1-9 vaccinated mice ($p<0.03$).

against a tumor challenge. Mice were vaccinated subcutaneously in the flank with 10^7 lethally-irradiated 4T1-9 or 4T1-10 tumor cells. Fourteen days later the mice were challenged with twice the tumorgenic dose (10^4) of the respective tumor cells in the opposite flank. 4T1-9 tumor challenge grew in all five mice vaccinated with 4T1-9 (Fig. 2.6). Suprisingly, two of the five mice vaccinated with 4T1-10 were able to reject 4T1-10 tumor challenge completely and remained tumor free (Fig. 2.6B). Among the three mice that developed tumors, there seemed to be a significant delay in tumor growth that was particularly evident in one mouse (Fig. 2.6A). These data suggest that in contrast to the poorly immunogenic 4T1 and 4T1-9 tumors, the 4T1-10 clone appears to be moderately immunogenic protecting 40% of mice challenged with 4T1-10 ($p<0.03$).

Tumor-specific IFN-γ secretion by 4T1-10 vaccinated draining lymph node cells

BALB/c mice were injected subcutaneously with either 4T1-10 or 4T1-9 to investigate if the increased immunogenicity of 4T1-10 correlated with enhanced production of type 1 cytokines by tumor-vaccine draining lymph node cells. Tumor-draining lymph node cells were recovered 8 days after tumor injection and were polyclonally-stimulated *in vitro* with soluble anti-CD3 for 2 days and expanded in low-dose IL-2 for three days. This method of polyclonal activation has been shown to support the acquisition of antigen-specific 'effector' function by recently primed T cells (Yoshizawa et al., 1991a; Yoshizawa et al., 1992; Yoshizawa et al., 1991b). 'Effector' T cells from mice injected with 4T1-10 secreted more IFN-γ than 'effector' T cells generated from 4T1-9 injected mice, however this was a modest increase in the secretion of IFN-γ (Fig. 2.7A). There was no significant difference in the amount of IL-4 secreted by these two groups of T cells (Fig. 2.7B). The difference of IFN-γ secretion between

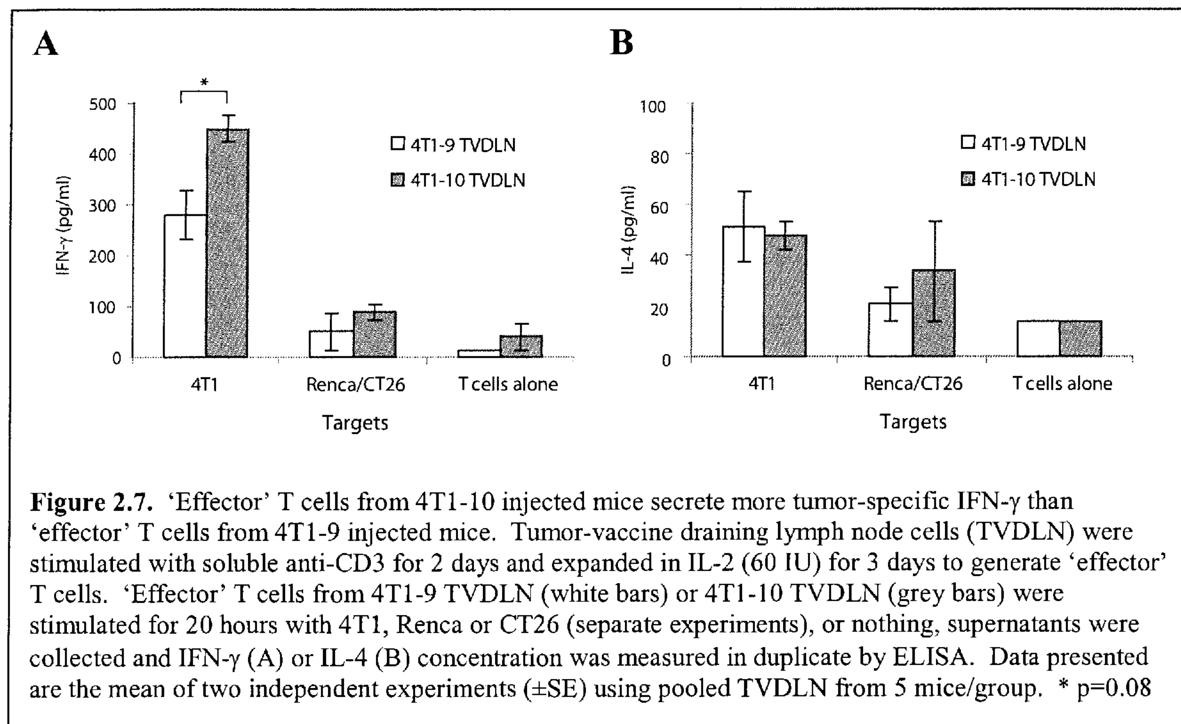

Figure 2.7. 'Effector' T cells from 4T1-10 injected mice secrete more tumor-specific IFN-γ than 'effector' T cells from 4T1-9 injected mice. Tumor-vaccine draining lymph node cells (TVDLN) were stimulated with soluble anti-CD3 for 2 days and expanded in IL-2 (60 IU) for 3 days to generate 'effector' T cells. 'Effector' T cells from 4T1-9 TVDLN (white bars) or 4T1-10 TVDLN (grey bars) were stimulated for 20 hours with 4T1, Renca or CT26 (separate experiments), or nothing, supernatants were collected and IFN-γ (A) or IL-4 (B) concentration was measured in duplicate by ELISA. Data presented are the mean of two independent experiments (±SE) using pooled TVDLN from 5 mice/group. * p=0.08

these two groups did not reach statistical significance (p=0.08), however increasing the number of experiments could decrease the p value to a statistically significant level. The response of 'effector' T cells from 4T1-9 TVDLN as well as 4T1-10 TVDLN was tumor-specific as neither group secreted significant amounts of IFN-γ following stimulation with either the renal cell carcinoma cell line (Renca) or the colon carcinoma cell line (CT26). Although these data do not prove that the type 1 response was responsible for the increased immunogenicity of 4T1-10, they suggest a correlation between IFN-γ secretion by tumor-specific T cells and enhanced immunogenicity.

Discussion

The 4T1 tumor cell line is a heterogenous tumor cell population consisting of cells that are morphologically distinct. It is unclear if the original isolation of the 4T1 tumor cell line resulted in a heterogenous tumor cell population. It should be noted, however, that tumor cells develop spontaneous variants *in vitro* that can have a different genotype, phenotype, or *in vivo* growth behavior than the parent tumor cell population. For this reason, tumor cell cultures described here are never passaged greater than 5 times *in vitro*. This reduces variability between experiments that could result due to tumor heterogeneity. It is this risk of variability that was the motive to initially subclone 4T1. We were successful in isolating multiple subclones, two of which represented two major tumor morphologies microscopically observed in the parental 4T1. Although these clones, 4T1-9 and 4T1-10, had similar doubling times *in vitro* and had similar growth curves *in vivo*, they differed in respect to immunogenicity. It is not clear from the experiments performed here what mechanism is responsible for the protection observed in mice vaccinated with 4T1-10, but other data included here provide some possible explanations. T cells generated from lymph nodes draining the 4T1-10 tumor-vaccine secreted more tumor-specific IFN-γ than T cells generated from mice vaccinated with 4T1-9. Although this difference in secretion of IFN-γ did not quite reach statistical significance ($p=0.08$), it did suggest a correlation between the immunogenicity of 4T1-10 and the secretion of the type 1 cytokine, IFN-γ. This is in accordance to other data generated in our lab that indicates a significant correlation between immunogenicity and the ratio of tumor-specific IFN-γ:IL-4 secreted by tumor vaccine-draining lymph node cells (Winter et al., 2003).

Besides the morphological differences of 4T1-9 and 4T1-10, these clones also displayed differential expression of various cell surface molecules. 4T1-10 expressed slightly higher levels of H-2K^d than either 4T1 or 4T1-9 (Fig. 2.4). This increased expression of MHC class I could be responsible for the enhanced immunogenicity of this clone compared to 4T1-9 or 4T1. It is also possible that the increased expression of costimulatory molecules could be responsible for the increased immunogenicity of 4T1-10. ICAM-1 was clearly expressed at greater levels on 4T1-10 than 4T1-9. T-cell integrin binding to ICAM-1 could promote interactions between T cells and 4T1-10, and lead to activation of tumor-specific T cells (van Seventer et al., 1991). This interaction between T-cell intergrins and ICAM-1 has also been shown to induce T cell responsiveness to CD28:B7 costimulation (Damle et al., 1993), which is interesting since 4T1-10 expresses CD86 (B7.2). It is also possible that CD40 expressed on 4T1-10 could provide costimulation to T cells through CD40L. Most of the biological effects of CD40:CD40L interactions are accounted for by APC activation through CD40, leading to the upregulation of B7.1 and IL-12 secretion (Grewal and Flavell, 1998; Schoenberger et al., 1998). However, it has also been shown that CD40 transfected tumor cells and agonistic anti-CD40L mAbs can trigger proliferation and cytokine production by T cells (Blotta et al., 1996; Cayabyab et al., 1994; Peng et al., 1996). Activation of intracellular signaling molecules JNK/p38-K and p56^{lck} and the resulting tyrosine phosphorylation of intracellular substrates has been observed in T cells stimulated via CD40L (Brenner et al., 1997). Alternatively, activation of CD40 on keratinocytes has led to secretion of TNF-α and augmentation of PHA-driven T cell proliferation (Fuller et al., 2002; Gaspari et al., 1996) suggesting that 4T1-10 might secrete proinflammatory cytokines when CD40 is

engaged by CD40L. Therefore, the expression of these costimulatory molecules on 4T1-10 could possibly provide significant costimulation to naïve tumor-specific T cells to activate them. It should be noted that if costimulation of naïve T cells occurred on 4T1-10 then this would imply that activation of naïve T cells was occurring through direct presentation. Clearly this is unknown and it is also possible naïve T cells might become activated by cross presentation of tumor antigens on APCs. In this case it could be hypothesized that 4T1-10 was more susceptible to lysis by activated T cells or NK cells due to the increased expression of these costimulatory molecules. Of course it is also possible that these molecules have no benefit to immunogenicity and that possibly the tumor-antigen repertoires of 4T1-10 and 4T1-9 account for the difference in immunogenicity of these two subclones.

Clearly studies that investigate these costimulatory molecules on 4T1-10 will need to be performed to conclusively show if there is a role for these molecules in the immunogenicity of 4T1-10. Comparing the immunogenicity of 4T1-10 in CD40L$^{-/-}$ mice to *wt* BALB/c mice; or using methods of blocking protein translation of these costimulatory molecules in 4T1-10 will enable direct assessment of their contribution to the immunogenicity of 4T1-10. It is also of interest to determine if vaccination with 4T1-10 could protect mice against 4T1-9 tumor challenge. These experiments would show whether 4T1-10 could prime a response that is cross-protective against 4T1-9 demonstrating that they share common tumor antigens. In addition, these experiments would provide insight to whether 4T1-10 is more effective at priming an immune response than 4T1-9, possibly due to the expression of the expressed costimulatory molecules. The reverse experiment, using 4T1-9 to vaccinate mice followed by 4T1-10

tumor challenge, would address the susceptibility of 4T1-10 to killing by immune system compared to 4T1-9. Protection against 4T1-10 tumor challenge in 4T1-9 vaccinated mice would suggest that 4T1-10 is more susceptible to lysis than 4T1-9 since this vaccination does not protect 4T1-9 tumor challenged mice.

Chapter 3: GM-CSF secretion by 4T1-9 enhances type 1 polarization which correlates with increased therapeutic efficacy of adoptively-transferred T cells

Abstract

Our recent work suggests that a tumor-specific type 1 immune response is critical for T cell-mediated tumor regression. To further examine the role of the type 1 response we vaccinated mice with a poorly immunogenic subclone of 4T1 that was transduced to express GM-CSF. Tumor-vaccine draining lymph node cells were isolated, activated with soluble anti-CD3 and expanded in low dose IL-2 to generate 'effector' T cells. These 'effector' T cells secreted significantly more tumor-specific IFN-γ ($p<0.05$) and significantly reduced experimental lung metastases in recipient mice ($p<0.05$) compared to 'effector' T cells generated from mice vaccinated with a non-GM-CSF secreting tumor vaccine. These observations led us to question if T cells from tumor-vaccine draining lymph nodes of $STAT6^{-/-}$ mice would be more therapeutic since they exhibit a polarized type 1 response. $STAT6^{-/-}$ 'effector' T cells recognized 4T1 *in vitro*, secreting significantly more IFN-γ compared to 'effector' T cells from *wt* BALB/c mice ($p<0.05$). Interestingly, the adoptive transfer of $STAT6^{-/-}$ 'effector' T cells caused acute loss of body weight in *wt* BALB/c recipients. To avoid this complication $STAT6^{-/-}$ 'effector' T cells were adoptively transferred to tumor-bearing $STAT6^{-/-}$ recipients where they significantly reduced the number of experimental pulmonary metastases compared to the transfer of *wt* BALB/c 'effector' T cells ($p<0.05$). The efficacy of $STAT6^{-/-}$ 'effector' T cells could be partially inhibited by the co-transfer of $STAT4^{-/-}$ 'effector' T cells, which

exhibit a type 2 polarization. These data emphasize the importance of a type 1 polarized immune response for the efficacy of 'effector' T cells against 4T1 lung metastases.

Introduction

Cellular immune responses require the cognate interaction between an antigen-presenting cell (APC) and a T cell. The result of this interaction is either T cell activation or T cell unresponsiveness. One factor that determines whether this interaction will result in T cell activation is the type of costimulation provided by APCs (Abken et al., 2002). Dendritic cells (DC) are recognized as very effective APCs with the ability to prime naïve T cells. They exist in the tissues of the body in an immature state during which they uptake and process antigen. After appropriate stimulation (i.e. LPS or inflammatory cytokines) DCs undergo a maturation process. During maturation they migrate to the lymph nodes where they come in contact with naïve T cells. DCs upregulate MHC class II, CD86, and CD40; thereby increasing their ability to provide the costimulation needed to prime naïve T cells (Steinman et al., 1997). Another critical consequence of DC maturation is the production of IL-12, which plays a pivotal role in the induction of type 1 polarized responses (Macatonia et al., 1995; Mountford et al., 1999).

Since DCs are recognized as the most potent stimulators of primary immune responses they have been used in a variety of therapeutic strategies. These studies have been hampered by the diverse morphological characteristics of DCs and lack of knowledge in regard to which differentiation state of DC is best for *in vivo* immunotherapy. Nonetheless, various groups have demonstrated that DCs can be used to induce immunity to tumors (Celluzzi et al., 1996; Mayordomo et al., 1995; Paglia et al., 1996). Most studies that generate DCs *ex vivo* do so by culturing DC precursors in granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4. Murine GM-CSF induces the maturation and proliferation of bone marrow progenitor cells resulting

the formation of colonies of neutrophils, monocyte/macrophages, as well as DCs (Hamilton, 2002). The role for GM-CSF as a growth factor for DCs and differentiation of DC precursors into mature DCs has been studied extensively *in vitro* (Sallusto and Lanzavecchia, 1994; Schuler and Steinman, 1985). However, the role of GM-CSF *in vivo* is more obscure especially in regards to tumor immunity. In addition to its effects at increasing the population of mature DCs in the host it might also increase inflammation at the site of administration. GM-CSF can cause the degranulation of primary and secondary granules of neutrophils (Richter et al., 1989) that result in the secretion of inflammatory cytokines such as IL-1, IL-6, and TNF (Lindemann et al., 1988; Lindemann et al., 1989). TNFα secretion by monocyte/macrophages is also elicited by GM-CSF (Cannistra et al., 1987). Although it is unclear specifically how *in vivo* GM-CSF influences tumor immunity, GM-CSF was determined to be the most potent cytokine for inducing tumor immunity from a group of 10 molecules that were retrovirally-transduced into tumor cells (Dranoff et al., 1993).

The cytokine environment profoundly biases the polarization of newly-activated T cells (O'Garra, 1998). IL-4 drives the development of type 2 cells and inhibits type 1 cells. On the other hand, IL-12 promotes the development of type 1 cells (Murphy and Reiner, 2002). Two intracellular molecules that belong to the signal transducers and activators of transcription (STAT) family are key to the influence of IL-4 or IL-12 on the cell. The mechanism by which IL-4 drives the development of a type 2 response is dependent on STAT6 (Hou et al., 1994; Quelle et al., 1995). Activation of STAT6 by IL-4 leads to dimerization and translocation to the nucleus where it enhances the transcription of IL-4 inducible genes, including Gata3 and c-maf (Kurata et al., 1999;

Mikita et al., 1998a; Mikita et al., 1998b). The essential role of STAT6 in the polarization of type 2 T cells is supported by data that demonstrates that T cells from $STAT6^{-/-}$ mice fail to develop a type 2 phenotype in conditions that favor type 2 differentiation (Kaplan et al., 1996a; Shimoda et al., 1996; Takeda et al., 1996; Zhu et al., 2001). STAT4 is specifically phosphorylated in response to IL-12 (Jacobson et al., 1995). Mice deficient in STAT4 lack IL-12-induced IFN-γ production and type 1 differentiation and display a type 2 polarization (Kaplan et al., 1996b; Thierfelder et al., 1996).

We examined whether tumor secretion of GM-CSF would improve the therapeutic efficacy of T cells isolated from mice vaccinated with a poorly immunogenic subclone of 4T1. Would T cells from tumor-vaccinated $STAT6^{-/-}$ mice be more therapeutic since they exhibited type 1 polarization? And could therapeutic T cells be inhibited by type 2 polarized T cells from tumor-vaccinated $STAT4^{-/-}$ mice?

Materials and Methods

Mice

Female BALB/cJ, C.129S2-*Stat6*^{tm1Gru} (Kaplan et al., 1996a) (STAT6$^{-/-}$), or C.129S2-*Stat4*^{tm1Gru} (Thierfelder et al., 1996) (STAT4$^{-/-}$), on a BALB/c background, were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained in a specific pathogen-free environment. Recognized principles of laboratory animal care were followed (Guide for the Care and Use of Laboratory Animals, National Research Council, 1996), and all animal protocols were approved by the Earle A. Chiles Research Institute Animal Care and Use Committee.

Tumor cell lines

4T1 is a 6-thioguanine-resistant cell line that was selected from a tumor cell line derived from a single spontaneously arising mammary tumor in a BALB/c3H mouse (provided by Dr. Suzanne Ostrand-Rosenberg, University of Maryland Baltimore County, MD). The colon carcinoma cell line, CT26, was provided by Dr. Gregory Plautz (University of Michigan, MI), the renal cell carcinoma, Renca, was provided by Dr. Bob Wiltrout (Frederick Cancer Research and Development Center, Frederick, MD) and the mammary adenocarcinoma cell line, EMT6, was provided by Dr. Emmanuel T. Akporiaye (University of Arizona, AZ). Each of these tumors was derived from BALB/c mice. 4T1-9 was cloned by limiting dilution from the 4T1 tumor cell line. All cell lines were maintained in complete media (CM) comprising the following: RPMI 1640 (Bio Whittaker, Walkersville, MD) containing 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, and 50 μg/ml gentamicin sulfate. This was further

supplemented with 50 μM 2-mercaptoethanol (Aldrich, Milwaukee, WI) and 10% (vol/vol) fetal calf serum (GIBCO BRL, Grand Island, NY).

GM-CSF transduction of 4T1-9

4T1-9 was seeded into 75 cm^2 flasks with 2 X 10^5 cells and cultured in CM for 48 hours at 37° C. CM was removed and replaced with CM containing polybrene (5μg/ml). 1 ml of GM-CSF retroviral supernatant from retroviral producing cell line (provided by Cell Genesys, Foster City, CA) was added and the flask was incubated at 37° C for 24 hours. 24 hours after infection media was replaced with fresh CM. 72 hours after infection cells were plated by limiting dilution into 96-well plates, supernatants from wells were collected and assayed for GM-CSF secretion by ELISA using commercially available reagents (BD-PharMingen, San Diego, CA). Positive clones were seeded into 6 well plates at 10^6 cells/well for 24 hours. Supernatants were collected and assayed for GM-CSF secretion by ELISA.

Tumor vaccination, separation, and activation of vaccine-draining lymph node cells

Tumor cell cultures were trypsinized (Bio Whittaker, Walkersville, MD) and washed twice in Hanks' Balanced Salt Solution (Bio Whittaker, Walkersville, MD). 7.5 X 10^5 4T1-9 or E10-9 tumor cells were injected subcutaneously into both axillary regions and hind flanks of BALB/c, STAT6$^{-/-}$, or STAT4$^{-/-}$ mice. Eight days following vaccination, the superficial inguinal lymph nodes and axillary lymph nodes draining the four vaccination sites were harvested and single cell suspensions were left unfractionated or separated based on L-selectin expression. L-selectin separation was accomplished using CD62L-MicroBeads (Miltenyi Biotec, CA). For magnetic bead separation, TVDLN were resuspended at 10^8 cells/ml in CM, mixed with 10μl/ml of CD62L-MicroBeads, and

incubated for 20 min at 4°C. Cells were washed with CM, resuspended at 10^8 cells/ml in CM, and passaged over a VarioMACS magnetic depletion column (VarioMACS separator, Miltenyi Biotec). The column was washed extensively with CM, and non-adherent cells (L-selectin$^{low/-}$ cells) were collected. Samples of unseparated, non-adherent, and bound cells were stained with FITC-labeled anti-CD62L, purchased from BD-PharMingen (San Diego, CA), and analyzed on a Beckman Coulter EPICS XL-MCL flow cytometry machine (Miami, FL) for efficiency of separation. Unfractionated and L-selectin^{low} cells were resuspended at 2 X 10^6 cells per ml in CM and cultured in 24 well plates with 50 μl of a 1:40 dilution of 2c11 ascites (anti-CD3). After two days of activation, the cells were harvested and expanded in CM containing 60 IU per ml of rhIL-2 (Chiron, Emeryville, CA) at a starting cell density of 1.25 X 10^5 cells per ml in 300 ml gas permeable tissue culture bags (Nexell Therapeutics Inc., Irvine, CA). After three days, 'effector' T cells were harvested and used in cytokine release assays or adoptive immunotherapy.

CD4 and CD8 depletion of 'effector' T cell populations

'Effector' T cell populations were separated based on CD4 and CD8 expression using CD4-Microbeads (clone GK1.5) or CD8 Microbeads (clone 53-6.7) (Miltenyi Biotec, CA). For magnetic bead separation, 'effector' T cells were resuspended at 10^8 cells/ml in CM, mixed with 10μl/ml of CD8-MicroBeads or CD4-Microbeads, and incubated for 20 min at 4°C. Cells were washed with CM, resuspended at 10^8 cells/ml in CM, and passaged over a VarioMACS magnetic depletion column (VarioMACS separator, Miltenyi Biotec). The column was washed extensively with CM, and non-adherent cells ($CD8^-$) or ($CD4^-$) were collected. Samples of unseparated, non-adherent, and bound cells

were stained with FITC-labeled anti-CD8 (clone 5H10-1) or anti-CD4 (clone RM4-4), purchased from BD-PharMingen (San Diego, CA), and analyzed on a Beckman Coulter EPICS XL-MCL flow cytometry machine (Miami, FL)for efficiency of separation.

Adoptive immunotherapy

Experimental pulmonary metastases were established by i.v. inoculation of BALB/cJ or STAT6$^{-/-}$ mice with 2 X 10^5 4T1 tumor cells. Three days after metastases were established, 'effector' T cells were adoptively transferred intravenously (iv). Starting on the day of T-cell infusion, mice received 90,000 IU of IL-2 i.p. daily for 3 days. Animals were sacrificed 12 days following tumor inoculation, the lungs were resected and fixed in Fekete's solution, and the number of pulmonary metastases were enumerated by visual inspection.

STAT6$^{-/-}$ 'effector' T cell toxicity

As a measure of STAT6$^{-/-}$ 'effector' T cell toxicity weight loss of recipient animals was followed. 'Effector' T cells were adoptively transferred iv into non-tumor bearing BALB/cJ or STAT6$^{-/-}$ mice. Mice were weighed immediately after adoptive transfer (time 0) and at 22, 46, 71, 92, 118, 144, and 190 hours post adoptive transfer.

Cytokine release assay

After *in vitro* activation and expansion, 'effector' T cells were washed and incubated alone or were stimulated with 4T1, EMT6, or plate-bound anti-CD3. 'Effector' T cells (2 X 10^6/well) were cultured with tumor cells (2 X 10^5/well) in 2 ml in 24-well plates. Supernatants were recovered 20 hours after stimulation and IFN-γ or IL-4 concentration was measured in duplicate by ELISA using commercially available reagents (BD-

PharMingen, San Diego, CA). The concentration of cytokine in the supernatant was determined by regression analysis.

Statistical Analyses

Cytokine analysis data and and weight loss data were analyzed using Student's t test for unequal variances (Microsoft Excel, Redmond, WA). Statistical analysis of the number of pulmonary metastases was performed by Wilcoxon rank sum test using S-plus 2000 software (Data Analysis Product Division; Mathsoft, Seattle, WA).

Results

GM-CSF transduction of 4T1-9

We have isolated a poorly immunogenic tumor cell subclone, 4T1-9, from the 4T1 tumor cell line. We transduced 4T1-9 with a retroviral vector encoding the murine GM-CSF gene to examine if the secretion of GM-CSF by a 4T1-9 tumor-vaccine would improve the therapeutic efficacy of tumor-vaccine draining lymph node cells. The tumor cells were cloned by limiting dilution following transduction and individual clones were assayed for GM-CSF production (Fig. 3.1). Neither 4T1-9 nor 4T1 secreted GM-CSF. A number of retrovirally-transduced clones of 4T1-9 expressed low levels of GM-CSF, approximately 1 ng/10^6 cells/24 hours. One clone, E10-9, exhibited significantly more GM-CSF secretion than the other clones, secreting 15 ng/10^6 cells/24 hours. Cell surface marker expression was examined to determine if retroviral-transduction altered the phenotype of E10-9 compared to 4T1-9. E10-9 and 4T1-9 displayed similar morphology and were phenotypically similar in regards to the cell surface markers examined (Fig. 3.2).

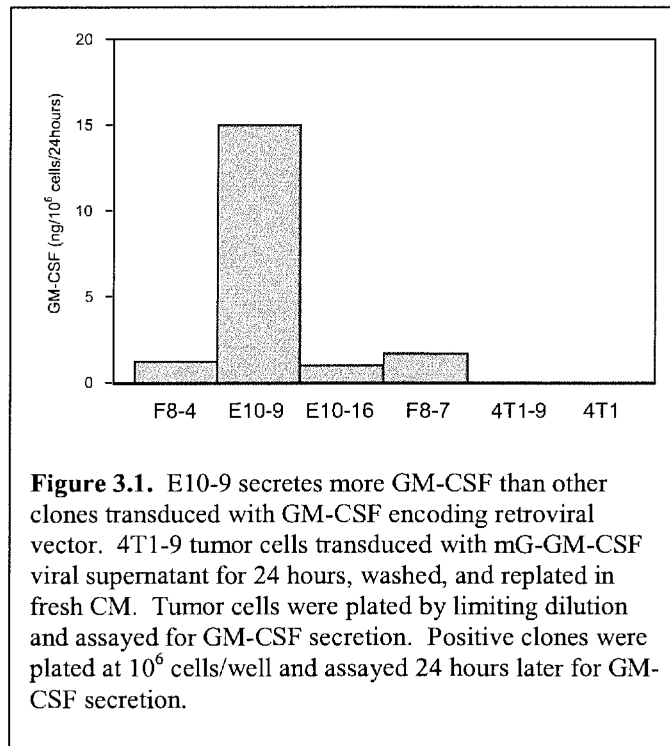

Figure 3.1. E10-9 secretes more GM-CSF than other clones transduced with GM-CSF encoding retroviral vector. 4T1-9 tumor cells transduced with mG-GM-CSF viral supernatant for 24 hours, washed, and replated in fresh CM. Tumor cells were plated by limiting dilution and assayed for GM-CSF secretion. Positive clones were plated at 10^6 cells/well and assayed 24 hours later for GM-CSF secretion.

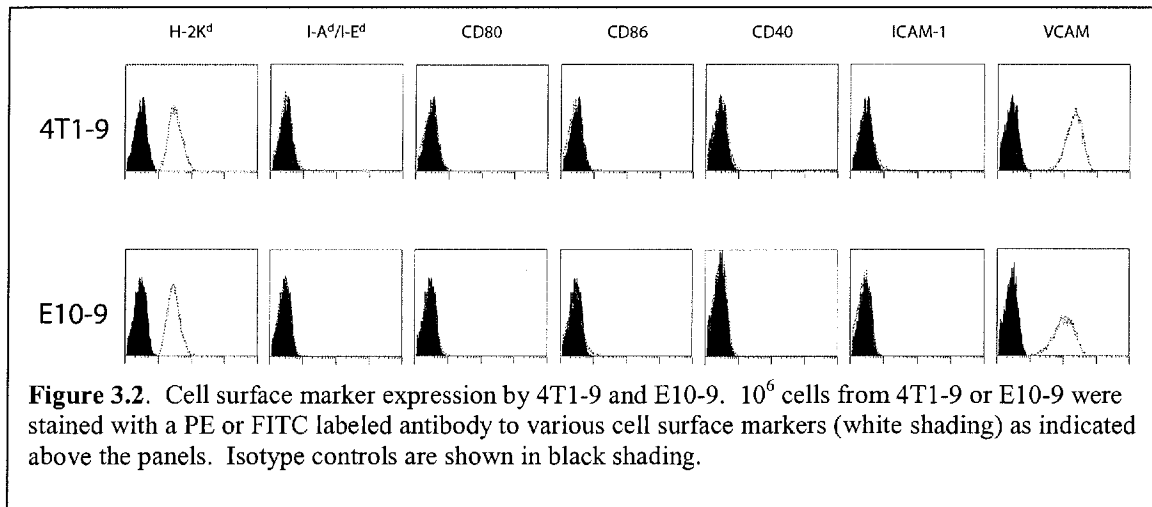

Figure 3.2. Cell surface marker expression by 4T1-9 and E10-9. 10^6 cells from 4T1-9 or E10-9 were stained with a PE or FITC labeled antibody to various cell surface markers (white shading) as indicated above the panels. Isotype controls are shown in black shading.

'Effector' T cells from E10-9 vaccinated mice secrete tumor-specific IFN-γ

Significant secretion of GM-CSF by E10-9 enabled us to investigate if this tumor vaccine would prime type 1 polarized T cells. BALB/c mice were subcutaneously injected with live E10-9 or 4T1-9. Eight days later the tumor-vaccine draining lymph nodes (TVDLN) were removed and TVDLN cells were separated based on L-selectin expression. L-selectin, which is expressed on naïve T cells targets those cells to the

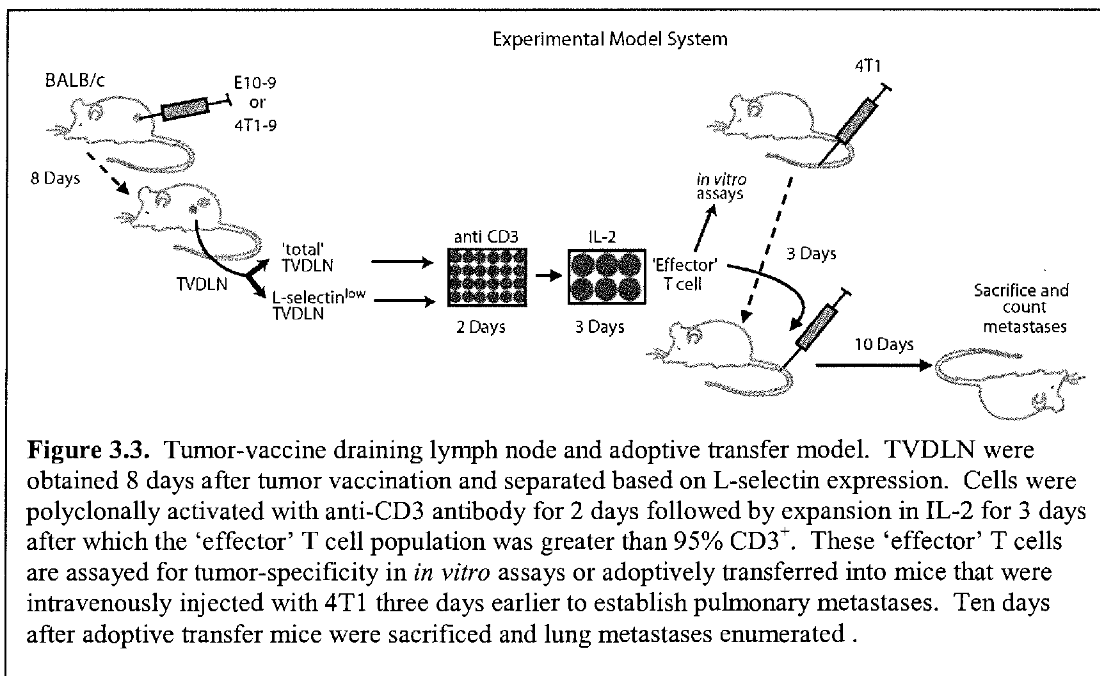

Figure 3.3. Tumor-vaccine draining lymph node and adoptive transfer model. TVDLN were obtained 8 days after tumor vaccination and separated based on L-selectin expression. Cells were polyclonally activated with anti-CD3 antibody for 2 days followed by expansion in IL-2 for 3 days after which the 'effector' T cell population was greater than 95% $CD3^+$. These 'effector' T cells are assayed for tumor-specificity in *in vitro* assays or adoptively transferred into mice that were intravenously injected with 4T1 three days earlier to establish pulmonary metastases. Ten days after adoptive transfer mice were sacrificed and lung metastases enumerated .

lymph nodes (Giblin et al., 1997). Down-regulation of L-selectin is a well established marker for recently activated T cells and memory T cells (Rigby and Dailey, 2000). Other groups have demonstrated that L-selectin^{low} TVDLN are enriched for therapeutic T cells (Cohen et al., 2001; Hu et al., 1998; Kagamu et al., 1996). TVDLN separated according to L-selectin expression, were stimulated *in vitro* with soluble anti-CD3 antibody for 48 hours followed by expansion in low-dose IL-2 (60 IU) for 3 days (Fig. 3.3). After polyclonal activation these 'effector' T cells were tested for their ability to respond to tumor cells *in vitro* (Fig. 3.4). L-selectin^{low} 'effector' T cells generated from mice vaccinated with E10-9 secreted significantly more IFN-γ in response to 4T1 than

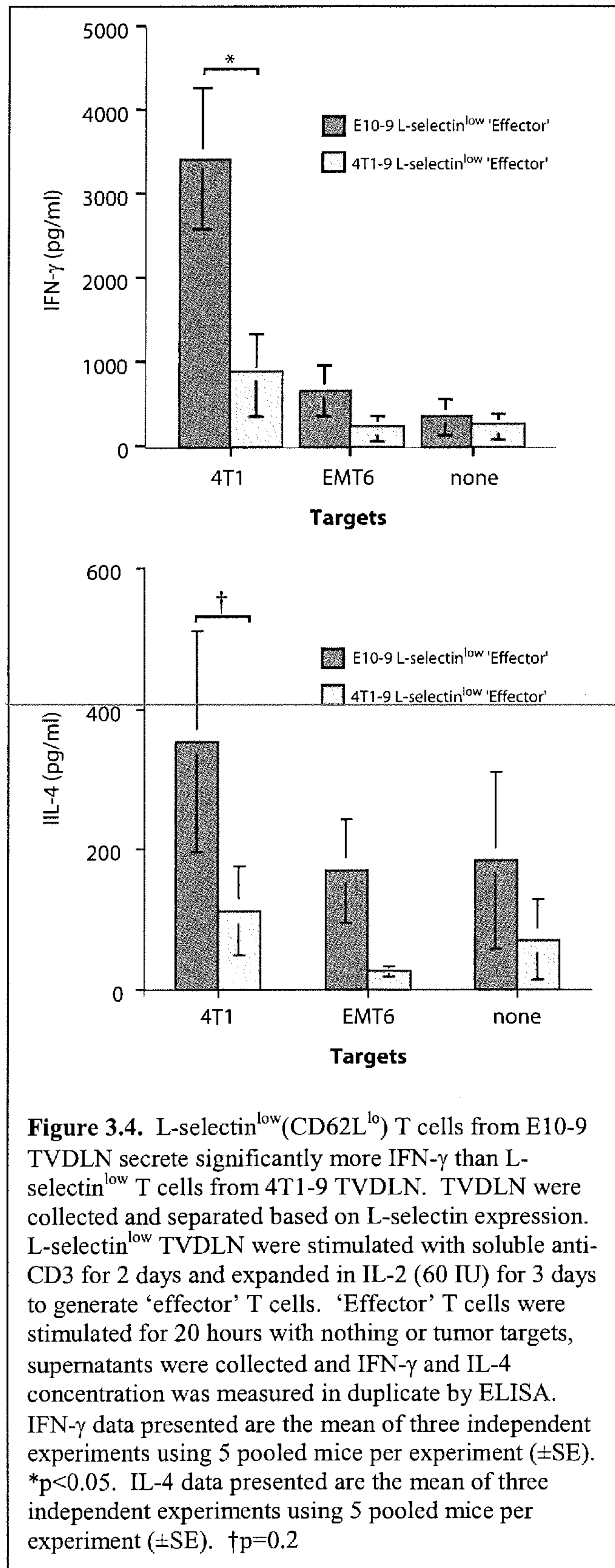

Figure 3.4. L-selectin^{low}(CD62L^{lo}) T cells from E10-9 TVDLN secrete significantly more IFN-γ than L-selectin^{low} T cells from 4T1-9 TVDLN. TVDLN were collected and separated based on L-selectin expression. L-selectin^{low} TVDLN were stimulated with soluble anti-CD3 for 2 days and expanded in IL-2 (60 IU) for 3 days to generate 'effector' T cells. 'Effector' T cells were stimulated for 20 hours with nothing or tumor targets, supernatants were collected and IFN-γ and IL-4 concentration was measured in duplicate by ELISA. IFN-γ data presented are the mean of three independent experiments using 5 pooled mice per experiment (±SE). *$p<0.05$. IL-4 data presented are the mean of three independent experiments using 5 pooled mice per experiment (±SE). †p=0.2

L-selectin^{low} 'effector' T cells from 4T1-9 vaccinated mice ($p<0.05$). This response was tumor-specific as both groups of 'effector' T cells responded only weakly to the mammary adenocarcinoma cell line, EMT6. 'Effector' T cells from E10-9 vaccinated mice also secreted IL-4 but it was not tumor-specific and it was not significantly greater than 'effector' T cells from 4T1-9 vaccinated mice ($p=0.2$). These data demonstrate that the GM-CSF secreting tumor, E10-9, is capable of enhancing the type 1 polarized anti-

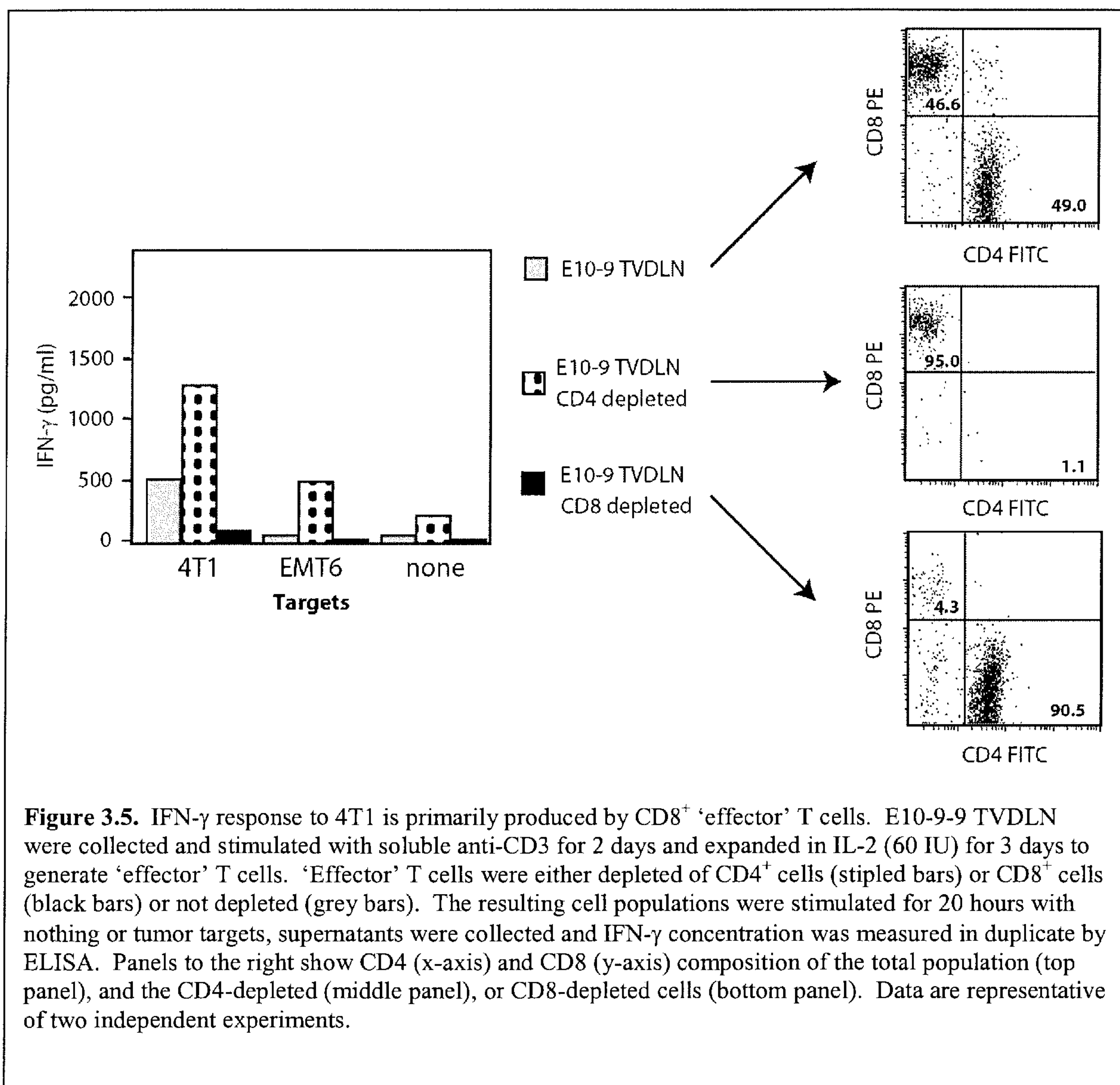

Figure 3.5. IFN-γ response to 4T1 is primarily produced by CD8$^+$ 'effector' T cells. E10-9-9 TVDLN were collected and stimulated with soluble anti-CD3 for 2 days and expanded in IL-2 (60 IU) for 3 days to generate 'effector' T cells. 'Effector' T cells were either depleted of CD4$^+$ cells (stipled bars) or CD8$^+$ cells (black bars) or not depleted (grey bars). The resulting cell populations were stimulated for 20 hours with nothing or tumor targets, supernatants were collected and IFN-γ concentration was measured in duplicate by ELISA. Panels to the right show CD4 (x-axis) and CD8 (y-axis) composition of the total population (top panel), and the CD4-depleted (middle panel), or CD8-depleted cells (bottom panel). Data are representative of two independent experiments.

tumor immune response.

The 'effector' T cell population generated from both E10-9 and 4T1-9 TVDLN was >95% $CD3^+$ T cells and generally comprised equal numbers of $CD4^+$ and $CD8^+$ T cells. Therefore, it was possible that either $CD4^+$ or $CD8^+$ T cells, or both populations, were secreting IFN-γ in response to 4T1. To determine which population was responsible for IFN-γ production, $CD4^+$ or $CD8^+$ 'effector' T cells from mice vaccinated with E10-9 were depleted either of $CD4^+$ or $CD8^+$ T cells. The depleted T cell populations were then stimulated with tumor cells. The $CD8^+$-enriched 'effector' T cell population (CD4-depleted) secreted more IFN-γ than the $CD4^+$ T cell enriched 'effector' T cell population (CD8-depleted) when stimulated with 4T1 (Fig 3.5). These data are not suprising since 4T1 expresses MHC class I but is negative for MHC class II expression. Future experiments that use CTIIA-transduced 4T1 will examine if CD4 responses can secrete IFN-γ if 4T1 expresses MHC class II. These data demonstrate that the 4T1-induced IFN-γ secretion by 'effector' T cells secretion was produced primarily by T_c1 T cells.

Improved Efficacy of E10-9 TVDLN

Since 'effector' T cells from E10-9 vaccinated mice secreted IFN-γ in response to 4T1 *in vitro* it was of interest to determine if these cells would be more therapeutic 'effector' T cells from 4T1-9 vaccinated mice *in vivo.* To test their *in vivo* therapeutic potential BALB/c mice were injected intravenously with 4T1 to establish experimental pulmonary metastases (Fig. 3.2). Three days later 'effector' T cells from either E10-9 or 4T1-9 TVDLN were adoptively transferred into the BALB/c recipient mice that bore 3-day established 4T1 metastases. The adoptive transfer of 4×10^7 'effector' T cells

generated from 4T1-9 TVDLN failed to reduce the number of 4T1 pulmonary metastases, whereas the transfer of 4 X 10^7 'effector' T cells from E10-9 TVDLN reduced the

Table 3.1. T cells from E10-9 vaccinated mice are more therapeutic than T cells from 4T1-9 vaccinated mice

			Mean Number of Pulmonary Metastases (SEM)[d]	
Tumor Vaccine[a]	T cells[b]	IL-2[c]	Exp. 1	Exp. 2
None	None	+	240 (20)	
4T1-9	4 X 10^7 Total	+	211 (41)	
E10-9	4 X 10^7 Total	+	41 (40)[e]	
None	None	+		205 (45)
4T1-9	3 X 10^7 L-selectin^{low}	+		236 (14)
E10-9	3 X 10^7 L-selectin^{low}	+		7 (2)[e]
4T1-9	3 X 10^7 L-selectin^{high}	+		223 (17)
E10-9	3 X 10^7 L-selectin^{high}	+		197 (27)

[a] Mice were vaccinated subcutaneously with 7.5 X 10^5 4T1-9 or E10-9 tumor cells
[b] Single cell suspensions from day 8 tumor-vaccine draining lymph nodes were left unseparated or separated by magnetic separation based on L-selectin expression. The total population, L-selectin^{low} population, and L-selectin^{high} population were stimulated with soluble anti-CD3 for 2 days in CM and then expanded in CM supplemented with 60 IU/ml IL-2 for 3 days. Cells were harvested and adoptively transferred into animals with established 3-day 4T1 pulmonary metastases.
[c] IL-2 (15,000 IU) was administered intraperitoneal daily for 4 consecutive days following adoptive transfer.
[d] Mice were sacrificed 14 days following intravenous injection of 4T1 and the number of pulmonary metastases enumerated.
[e] significantly less than other groups in same experiment ($p<0.05$)

number of 4T1 pulmonary metastases significantly compared to untreated mice or mice that received 4X10^7 'effector' T cells from 4T1-9 TVDLN ($p<0.05$). 'Effector' T cells generated from recently activated T cells (L-selectin^{low} cells) were also significantly more therapeutic if they were from E10-9 TVDLN compared to 4T1-9 TVDLN (Table 3.1).

'Effector' T cells generated from the L-selectin^{low} population of E10-9 TVDLN were also more therapeutic than the L-selectin^{high} population of E10-9 TVDLN cells. These data suggested a correlation between the 4T1-specific secretion of IFN-γ by 'effector' T cells and their therapeutic potential *in vivo* against 4T1 experimental pulmonary metastases.

'Effector' T cells generated from E10-9 vaccinated STAT6$^{-/-}$ mice

The deletion of the STAT6 gene in STAT6$^{-/-}$ mice results in diminished responsiveness to IL-4 and IL-13, and the preferential differentiaton of T cells toward a type 1 phenotype (Kaplan et al., 1996a; Stamm et al., 1998). Since increased tumor-specific IFN-γ secretion resulted in enhanced therapeutic efficacy in our E10-9 tumor model, it was of interest to determine if T cells from E10-9-vaccinated STAT6$^{-/-}$ mice would be more therapeutic than T cells from E10-9-vaccinated *wt* BALB/c mice. TVDLN cells from either E10-9 vaccinated STAT6$^{-/-}$ mice or E10-9 vaccinated *wt* BALB/c mice were used to generate

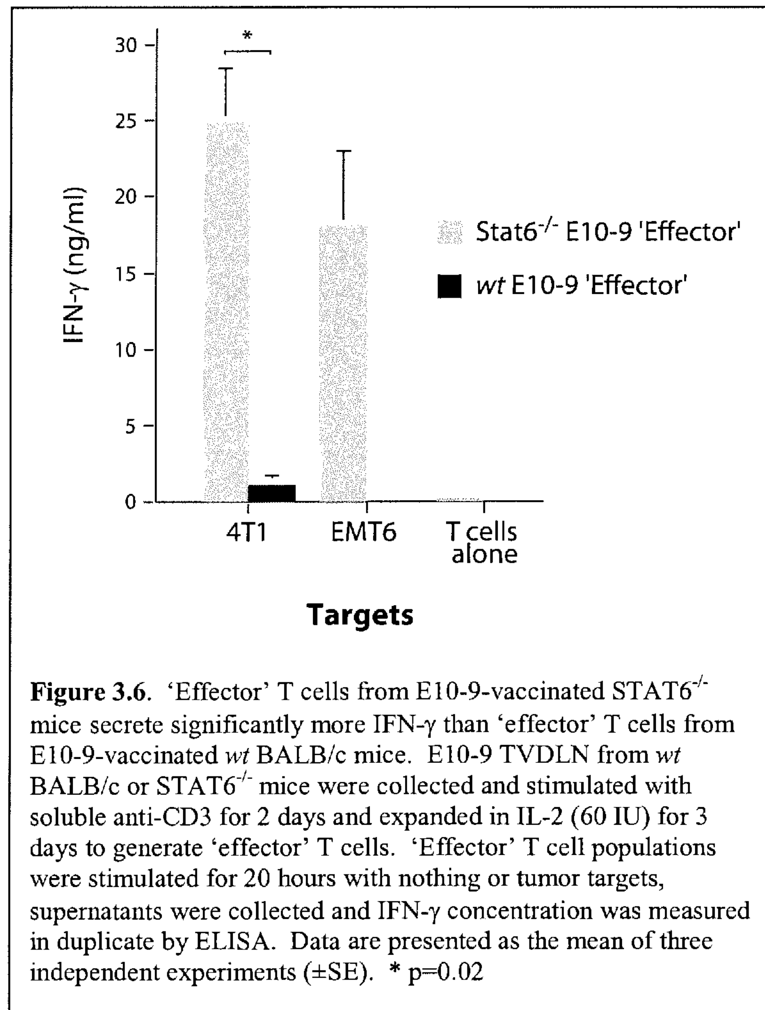

Figure 3.6. 'Effector' T cells from E10-9-vaccinated STAT6$^{-/-}$ mice secrete significantly more IFN-γ than 'effector' T cells from E10-9-vaccinated *wt* BALB/c mice. E10-9 TVDLN from *wt* BALB/c or STAT6$^{-/-}$ mice were collected and stimulated with soluble anti-CD3 for 2 days and expanded in IL-2 (60 IU) for 3 days to generate 'effector' T cells. 'Effector' T cell populations were stimulated for 20 hours with nothing or tumor targets, supernatants were collected and IFN-γ concentration was measured in duplicate by ELISA. Data are presented as the mean of three independent experiments (±SE). * $p=0.02$

'effector' T cells as described above (Fig 3.2). 'Effector' T cells from $STAT6^{-/-}$ mice secreted significantly more IFN-γ in response to 4T1 than *wt* BALB/c 'effector' T cells (Fig. 3.6). The quantity of IFN-γ secreted by $STAT6^{-/-}$ 'effector' T cells was extremely high, the most we have observed from tumor stimulation of 'effector' T cells. Interestingly, 'effector' T cells from E10-9 vaccinated $STAT6^{-/-}$ mice also responded strongly to EMT6, a BALB/c mammary adenocarcinoma, suggesting that the response might be mediated by natural-killer receptors or these two tumors might share a common tumor antigen. Data in the following chapter support the latter conclusion that these tumors share a common antigen. The 'effector' T cells from E10-9 vaccinated $STAT6^{-/-}$ mice did not secrete IFN-γ non-specifically since T cells did not secrete cytokine in the absence of tumor cells.

Table 3.2. 'Effector' T cells from E10-9 vaccinated $STAT6^{-/-}$ mice cause the death of recipient *wt* mice upon adoptive transfer

Mouse strain[a]	T cells[b]	IL-2[c]	# of pulmonary metastases[d]	Mean Number of Pulmonary Metastases (SEM)
	None	+	250, 250, 250, 250, 250, 250, 250 250	250 (0)
$STAT6^{-/-}$	5 X 10^7 L-selectin^{low}	+	Death of 5/5 mice unrelated to pulmonary metastases	
wt BALB/c	5 X 10^7 L-selectin^{low}	+	2, 2, 3, 6, 9	4 (1)[e]

[a] Mice were vaccinated subcutaneously with 7.5 X 10^5 E10-9 tumor cells
[b] Single cell suspensions from day 8 tumor-vaccine draining lymph nodes were separated by magnetic bead separation based on L-selectin expression. The L-selectin^{low} populations were stimulated with soluble anti-CD3 for 2 days in CM and then expanded in CM supplemented with 60 IU/ml IL-2 for 3 days. Cells were harvested and adoptively transferred into BALB/c mice with established 3-day 4T1 pulmonary metastases.
[c] IL-2 (15,000 IU) was administered intraperitoneal daily for 4 consecutive days following adoptive transfer.
[d] Mice were sacrificed 14 days following intravenous injection of 4T1 and the number of pulmonary metastases enumerated. Lungs with >250 metastases were assigned as 250 metastases.
[e] significantly less than the group that received no 'effector' T cells ($p<0.05$)

Effector' T cells generated from either E10-9-vaccinated *wt* BALB/c mice or E10-9-vaccinated STAT6$^{-/-}$ mice were adoptively transferred into *wt* BALB/c mice bearing 3-day 4T1 experimental pulmonary metastases. As was observed before, 'effector' T cells from E10-9-vaccinated *wt* BALB/c mice significantly reduced the number of experimental pulmonary metastases compared to control mice that did not receive T cells. All *wt* BALB/c mice that received 'effector' T cells generated from E10-9-vaccinated STAT6$^{-/-}$ mice died unexpectedly within 72 hours of the adoptive transfer. These deaths were not a result of the experimental pulmonary metastases since upon necropsy there were no macroscopic metastases present in the lungs, liver, spleen, or brain. Within 24 hours of adoptive transfer all mice that received 'effector' T cells from E10-9-vaccinated STAT6$^{-/-}$ mice were moribund showing symptoms including ruffled fur, decreased activity, and weight loss.

This unexpected response could have been the result of a graft-versus-host response from activated T cells from STAT6$^{-/-}$ mice that were transferred into *wt* BALB/c mice. To investigate this possibility, STAT6$^{-/-}$ mice or *wt* BALB/c mice were vaccinated with E10-9 and 'effector' T cells were generated from TVDLN. 'Effector' T cells were adoptively transferred into non-tumor bearing STAT6$^{-/-}$ mice or *wt* BALB/c mice, which were followed for weight loss (Fig. 3.7). Since the adoptive transfer of $5X10^7$ L-selectin^{low} 'effector' T cells resulted in the death of recipient mice (Table 3.2) we transferred $5X10^7$ 'effector' T cells that were generated from the total TVDLN population. Typically, variation in body weight can range from 2-5% in untreated mice. *wt* BALB/c mice that received 'effector' T cells from *wt* BALB/c mice averaged 18.8 grams at adoptive transfer and gained weight to an average of 19.9 grams at 50 hours post

transfer. STAT6$^{-/-}$ recipient mice also gained weight after adoptive transfer of 'effector' T cells from *wt* BALB/c mice, an average of 22.1 grams at adoptive transfer to an average of 22.6 grams at 50 hours post transfer. In contrast, the transfer of 'effector' T cells generated from E10-9-vaccinated STAT6$^{-/-}$ mice to wt BALB/ recipients caused a 14% weight loss by 50 hours post transfer (from an average body weight of 20.3 grams at adoptive transfer to 17.4 grams at 50 hours post transfer). This large decrease in body weight only occurred when 'effector' T cells from STAT6$^{-/-}$ mice where transferred into wt BALB/c mice. The adoptive transfer of 'effector' T cells from STAT6$^{-/-}$ mice into STAT6$^{-/-}$ mice resulted in only a minor weight loss of 6% (average body weight of 19.8 grams at adoptive transfer to 18.5 grams at 50 hours post transfer). Increasing the dose of transferred 'effector' T cells or using L-selectin^{low} separated cells led to an exacerbation of the effect and would ultimately lead to death of the recipient *wt* BALB/c mice.

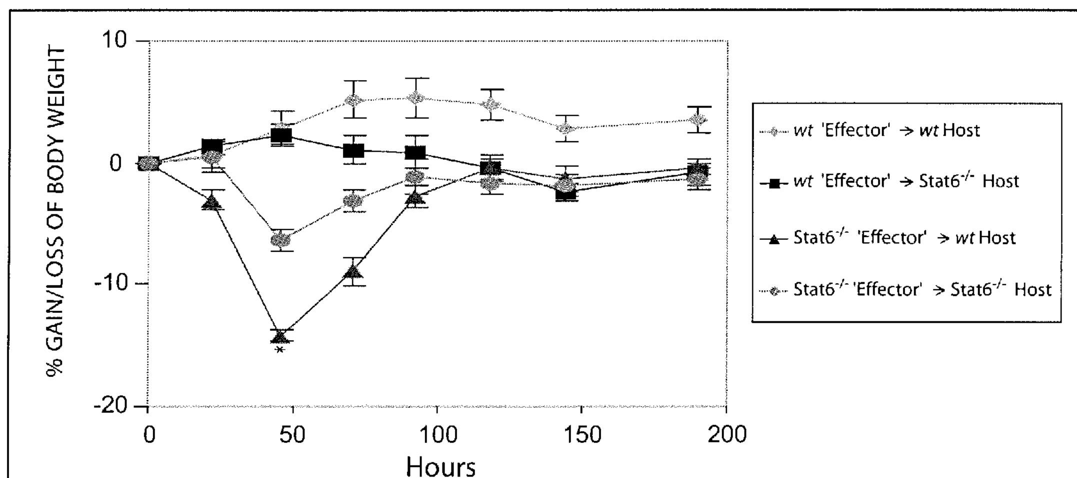

Figure 3.7. 'Effector' T cells from E10-9-vaccinated STAT6$^{-/-}$ mice caused significant weight loss when transferred into *wt* BALB/c mice. Day 8 TVDLN were harvested from E10-9-vaccinated STAT6$^{-/-}$ mice or *wt* BALB/c mice. TVDLN cells were then activated with anti-CD3 for 2 days and expanded in CM supplemented with 60 IU/ml IL-2. After activation and expansion 5 X 10^7 'effector' T cells were adoptively transferred into either STAT6$^{-/-}$ mice or *wt* BALB/c mice. Data are presented as the mean of five mice (±SE). * $p<0.001$ STAT6$^{-/-}$ mice 'effector' T cells → *wt* BALB/c mice compared to all other groups at 50 hours post transfer.

Therapeutic 'effector' T cells from STAT6$^{-/-}$ are inhibited by STAT4$^{-/-}$ 'effector' T cells

Since the adoptive transfer of STAT6$^{-/-}$ 'effector' T cells caused toxicity to *wt* BALB/c recipients, we transferred these 'effector' T cells into STAT6$^{-/-}$ recipients that had 3-day established pulmonary metastases. 25 X 10^6 'effector' T cells from E10-9-vaccinated *wt* BALB/c mice were unable to significantly reduce the number of 4T1 pulmonary metastases in STAT6$^{-/-}$ recipient mice (Table 3.3). In contrast, lungs from STAT6$^{-/-}$ mice treated with 'effector' T cells from E10-9-vaccinated STAT6$^{-/-}$ mice showed no macroscopic tumor metastases. These data demonstrate that the T cells from E10-9-vaccinated STAT6$^{-/-}$ mice are significantly more therapeutic than those from *wt* BALB/c mice ($p<0.05$).

Table 3.3. 'Effector' T cells from E10-9 vaccinated STAT6$^{-/-}$ mice are more therapeutic than 'effector' T cells from E10-9 vaccinated *wt* mice

Mouse strain[a]	T cells[b]	IL-2[c]	# of pulmonary metastases[d]	Mean Number of Pulmonary Metastases (SEM)
	None	+	46, 56, 61, 71, 81	63 (6)
wt BALB/c	25 X 10^6	+	30, 42, 59, 64, 90	57 (10)
STAT6$^{-/-}$	25 X 10^6	+	0, 0, 0, 0, 0	0 (0)[e]

[a] Mice were vaccinated subcutaneously with 7.5 X 10^5 E10-9 tumor cells
[b] Single cell suspensions from day 8 tumor-vaccine draining lymph nodes were stimulated with soluble anti-CD3 for 2 days in CM and then expanded in CM supplemented with 60 IU/ml IL-2 for 3 days. Cells were harvested and adoptively transferred into STAT6$^{-/-}$ mice with established 3-day 4T1 pulmonary metastases.
[c] IL-2 (15,000 IU) was administered intraperitoneal daily for 4 consecutive days following adoptive transfer.
[d] Mice were sacrificed 14 days following intravenous injection of 4T1 and the number of pulmonary metastases enumerated.
[e] significantly less than group that received no 'effector' T cells ($p<0.05$)

'Effector' T cells from E10-9-vaccinated STAT4$^{-/-}$ mice were mixed with 'effector' T cells from E10-9-vaccinated STAT6$^{-/-}$ mice to test if a type 2 polarized response could inhibit the therapeutic response. The adoptive transfer of 'effector' T cells from E10-9-vaccinated STAT4$^{-/-}$ mice are not therapeutic and express significant amounts of the type 2 cytokines IL-4 and IL-5 in response to 4T1 (Fig. 3.8). The addition of 'effector' T cells from E10-9-vaccinated STAT4$^{-/-}$ mice significantly inhibited the therapeutic response of 'effector' T cells from E10-9-vaccinated STAT6$^{-/-}$ mice (Table 3.4). This inhibition was not complete since mice that received the mixed transfer had significantly less pulmonary metastases than mice that did not receive any T cells. The ability STAT4$^{-/-}$

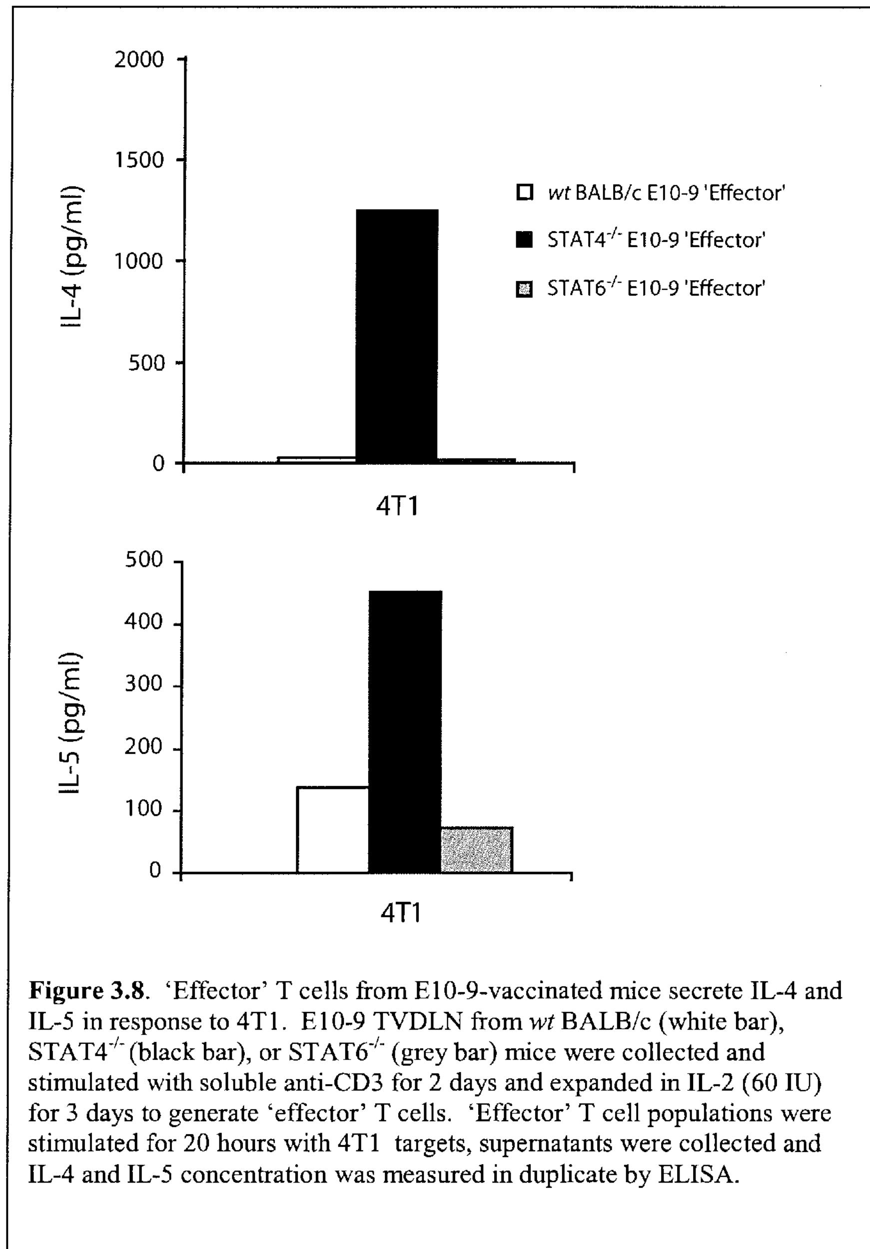

Figure 3.8. 'Effector' T cells from E10-9-vaccinated mice secrete IL-4 and IL-5 in response to 4T1. E10-9 TVDLN from *wt* BALB/c (white bar), STAT4$^{-/-}$ (black bar), or STAT6$^{-/-}$ (grey bar) mice were collected and stimulated with soluble anti-CD3 for 2 days and expanded in IL-2 (60 IU) for 3 days to generate 'effector' T cells. 'Effector' T cell populations were stimulated for 20 hours with 4T1 targets, supernatants were collected and IL-4 and IL-5 concentration was measured in duplicate by ELISA.

'effector' T cells to inhibit was tumor-specific since 'effector' T cells from the colon carcinoma CT26-vaccinated $STAT4^{-/-}$ mice were unable to inhibit the 'effector' T cells from E10-9-vaccinated $STAT6^{-/-}$ mice.

Table 3.4. 'Effector' T cells from E10-9 vaccinated $STAT4^{-/-}$ mice inhibit the therapeutic efficacy of 'effector' T cells from E10-9 vaccinated $STAT6^{-/-}$ mice

			Mean Number of Pulmonary Metastases (SEM)[d]	
$STAT6^{-/-}$ T cells[a]	$STAT4^{-/-}$ T cells[b]	IL-2[c]	Exp. 1	Exp. 2
None	None	+	245 (3)	
15 X 10^6 E10-9	None	+	0 (0)[e]	
15 X 10^6 E10-9	15 X 10^6 E10-9	+	67 (12)[f]	
None	None	+		210 (10)
15 X 10^6 E10-9	None	+		0 (0)[g]
15 X 10^6 E10-9	20 X 10^6 E10-9	+		20 (3)[f]
15 X 10^6 E10-9	20 X 10^6 CT26	+		3 (1)[g]

[a] $STAT6^{-/-}$ mice were vaccinated subcutaneously with 7.5 X 10^5 E10-9 tumor cells. Single cell suspensions from day 8 tumor-vaccine draining lymph nodes were stimulated with soluble anti-CD3 for 2 days in CM and then expanded in CM supplemented with 60 IU/ml IL-2 for 3 days. Cells were harvested and adoptively transferred with or without $STAT4^{-/-}$ T cells into $STAT6^{-/-}$ mice with established 3-day 4T1 pulmonary metastases (5 mice/group).

[b] $STAT4^{-/-}$ mice were vaccinated subcutaneously with 7.5 X 10^5 E10-9 or CT26 tumor cells. Single cell suspensions from day 8 tumor-vaccine draining lymph nodes were stimulated with soluble anti-CD3 for 2 days in CM and then expanded in CM supplemented with 60 IU/ml IL-2 for 3 days. Cells were harvested and adoptively transferred with $STAT6^{-/-}$ T cells into $STAT6^{-/-}$ mice with established 3-day 4T1 pulmonary metastases.

[c] IL-2 (15,000 IU) was administered intraperitoneal daily for 4 consecutive days following adoptive transfer.

[d] Mice were sacrificed 14 days following intravenous injection of 4T1 and the number of pulmonary metastases enumerated.

[e] significantly less than other groups in same experiment ($p<0.05$)

[f] significantly less than no T cell transfer group in the same experiment ($p<0.05$)

[g] significantly less than no T cell transfer group and group containing 'effector' T cells from E10-9 vaccinated $STAT4^{-/-}$ mice ($p<0.05$)

Discussion

In multiple tumor models GM-CSF has proven to be a potent molecule to stimulate tumor immunity (Abe et al., 1995; Armstrong et al., 1996; Dunussi-Joannopoulos et al., 1998; Hu et al., 2000; Krosl et al., 1996; Lee et al., 1997; Levitsky et al., 1996; Mach et al., 2000; Qin and Chatterjee, 1996; Wakimoto et al., 1996). Retroviral transduction of 4T1-9 with a vector encoding GM-CSF has increased the ability of this poorly immunogenic tumor to prime therapeutic T cells. Interestingly, the amount of GM-CSF secreted by E10-9, the GM-CSF clone of 4T1-9, is relatively low (15ng/10^6 tumor cells/24 hours) compared to the effective range (20-200ng/10^6 tumor cells/24 hours) determined by other investigators (Jaffee et al., 1996). Even though E10-9 secreted low amounts of GM-CSF, it still primed 'effector' T cells that were therapeutic against 4T1 pulmonary metastases. The therapeutic potential of the transferred cells resided in the L-selectin^{low} population of the tumor-vaccine draining lymph node cells. This population of cells also exhibited a significant increase in IFN-γ secretion compared to the same population of cells from 4T1-9 vaccinated mice. Taken together these data demonstrate a strong correlation between tumor-specific IFN-γ secretion and therapeutic efficacy of 'effector' T cells from mice vaccinated with a GM-CSF secreting tumor. This is probably a result of recruitment of macrophages, DCs, and granulocytes to the tumor site elicited by the secreted GM-CSF (Dranoff et al., 1993; Kielian et al., 1999). The presence of these cell populations at the tumor site could lead to a proinflammatory cytokine mileu (Cannistra et al., 1987; Lindemann et al., 1989). The expression of the type 1 polarizing cytokine, IL-12, by mature DCs might also explain the augmented IFN-γ response from E10-9 vaccinated mice (Macatonia et al., 1995).

It is unclear if the ability of therapeutic T cells to cause regression of pulmonary metastases is dependent on IFN-γ or if IFN-γ is simply a marker of therapeutic efficacy. It is possible that type 1 polarized cells, as characterized by their IFN-γ secretion, might use other effector molecules to cause tumor regression. Concurrent work in our lab has shown that perforin, IFN-γ, and TNF are the critical effector molecules that characterize therapeutic 'effector' T cells in a melanoma tumor model (Poehlein et al., 2003). A loss of any two of the three molecules reduced the effectiveness of the transferred 'effector' T cells on a per cell basis but they were still able to mediated tumor regression if higher doses of 'effector' T cells were transferred. Loss of all three molecules eliminated therapeutic efficacy entirely.

The polarized type 1 response of $STAT6^{-/-}$ mice provided a good model to examine if a strongly polarized response would be more therapeutic. As expected 'effector' T cells from E10-9-vaccinated $STAT6^{-/-}$ mice secreted the largest amounts of IFN-γ that we have observed by 'effector' T cells. However, we were surprised that the adoptive transfer of these cells into *wt* BALB/c recipients caused severe toxicity and death of recipient mice. Necropsy of these mice showed inflammation of the GI tract, namely the small and large intestines. Interestingly, CFSE-labeled T cells from E10-9-vaccinated $STAT6^{-/-}$ mice could be recovered from the mesenteric lymph nodes of mice that had been adoptively transferred with these T cells. This suggested that these T cells could traffic to locations where the pathology was observed. Toxicity was dependent on the E10-9 tumor-vaccine since naïve $STAT6^{-/-}$ lymph node cells that were activated *in vitro* did not cause toxicity when transferred to *wt* BALB/c recipients. These data led us to believe that E10-9 primes $STAT6^{-/-}$ T cells to an antigen that is only expressed by

normal cells of *wt* BALB/c recipients and not STAT6$^{-/-}$ recipients since no toxicity is observed in STAT6$^{-/-}$ mice. This possibility is investigated further in the next chapter.

The adoptive transfer of STAT6$^{-/-}$ 'effector' T cells did not cause toxicity in STAT6$^{-/-}$ recipient mice, therefore we used these mice as recipients to test the therapeutic efficacy of STAT6$^{-/-}$ 'effector' T cells. The transfer of STAT6$^{-/-}$ 'effector' T cells was significantly more therapeutic than 'effector' T cells from *wt* BALB/c mice ($p<0.05$). These data are encouraging but must be tempered by the possibility that the STAT6$^{-/-}$ 'effector' T cells were primed by an antigen that is not a self-antigen in STAT6$^{-/-}$ mice.

The ability of 'effector' T cells from E10-9 vaccinated STAT4$^{-/-}$ mice to partially inhibit the therapeutic efficacy of 'effector' T cells from E10-9 vaccinated STAT6$^{-/-}$ mice was a bit surprising since these T cells are unaffected by the polarizing effects of IL-4 or IL-13 (Kaplan et al., 1996a; Shimoda et al., 1996; Takeda et al., 1996; Zhu et al., 2001). This suggests that this inhibition must be occurring through another mechanism, possibly the influence other type 2 cytokines on the transferred 'effector' T cells or on the cells of the recipient's immune system. If other type 2 cytokines, such as IL-5 or IL-10, were responsible for this inhibition it remains controversial whether these cytokines would repolarize the transferred T cells. It has been shown that T cells that have undergone a few rounds of division are unable to reexpress cytokines of the opposing phenotype (Grogan et al., 2001). The inhibition was tumor-specific as T cells from CT-26 vaccinated STAT4$^{-/-}$ mice did not reduce the therapeutic efficacy of T cells from E10-9 vaccinated STAT6$^{-/-}$ mice.

Chapter 4: Regression of a mammary adenocarcinoma in $STAT6^{-/-}$ mice is dependent on the presence of STAT6-reactive T cells

Abstract

Polarization of the immune response towards a type 1 cytokine profile has been posited to be associated with a therapeutic anti-tumor immune response. $STAT6^{-/-}$ mice are unable to generate a type 2 immune response and instead mount an enhanced type 1 response. $STAT6^{-/-}$ mice are significantly more resistant to 4T1, a mammary adenocarinoma cell line, resisting a 10-fold higher tumor dose compared to *wt* BALB/c mice. An analysis of the T cells from tumor-bearing $STAT6^{-/-}$ mice revealed that they contained a population primed by a peptide ($STAT6_{531-539}$) of the STAT6 protein expressed in 4T1. The adoptive transfer of T cells from $STAT6_{531-539}$-vaccinated $STAT6^{-/-}$ mice significantly reduced the number of 4T1 pulmonary metastases in recipient mice. Additionally, the role of these $STAT6_{531-539}$-reactive T cells against subcutaneous 4T1 tumor challenge was determined by tumor-challenging *wt* BALB/c mice reconstituted with $STAT6^{-/-}$ bone marrow, thereby assessing if a polarized type 1 immune response in the absence of STAT6-reactive T cells was sufficient to reject a 4T1 tumor challenge. T cells from the $STAT6^{-/-}$ bone marrow chimeras failed to recognize the $STAT6_{531-539}$ and these mice proved to be as susceptible as *wt* BALB/c mice to 4T1 challenge. This demonstrated that the absence of $STAT6_{531-539}$-reactive T cells correlated with the inability to reject 4T1 challenge. Additionally, these data emphasize that the enhanced ability to mount a type 1 polarized immune response is inconsequential if a sufficient anti-tumor immune response is not primed by the tumor.

Introduction

The theory of 'immune surveillance' proposes that the immune system is responsible for the detection and elimination of malignant cells at their inception (Burnet, 1970). Thus, the occurrence of tumors within individuals argues that occasionally a malignant cell escapes 'immune surveillance' and progresses to form a tumor. Many mechanisms have been proposed to explain why this might occur including: the lack of tumor antigens or their presentation (Gilboa, 1999), insufficient costimulation (Abken et al., 2002), or the secretion of immunosuppressive factors (Antonia et al., 1998). It is also possible that the tumor environment might skew the immune response away from a therapeutic response toward a non-therapeutic response, a process known as 'immune deviation' (Rocken and Shevach, 1996). Previous work has demonstrated that type 1 polarized immune responses correlated with a therapeutic anti-tumor immune response whereas type 2 polarized immune responses were markedly less therapeutic or non-therapeutic (Dobrzanski et al., 1999; Hu et al., 1998). Additionally, it was shown that the therapeutic type 2 response was non-therapeutic if type 2 polarized T cells were transferred into IFN-γ knockout mice (Dobrzanski et al., 2001). This demonstrated that the type 1 cytokine, IFN-γ, derived from the host must play a role in type 2-mediated anti-tumor responses.

The commitment of T cells to either a type 1 or type 2 pathway is dependent on many factors including the strength of the antigen (Rogers and Croft, 2000), type of costimulation (Rulifson et al., 1997; Salomon and Bluestone, 1998), and the cytokine environment in which the T cells undergo activation and differentiation (Allen and Maizels, 1997; Murphy and Reiner, 2002; Nakamura et al., 1997). IL-4 drives the

development of type 2 cells and inhibits type 1 cells. The mechanism by which IL-4 drives the development of a type 2 response is dependent on STAT6 (Hou et al., 1994; Quelle et al., 1995). Activation of STAT6 by IL-4 leads to dimerization and translocation to the nucleus where it enhances the transcription of IL-4 inducible genes, including Gata3 and c-maf (Kurata et al., 1999; Mikita et al., 1998a; Mikita et al., 1998b). The essential role of STAT6 in the polarization of type 2 T cells is supported by data that demonstrates that T cells from $STAT6^{-/-}$ mice fail to develop a type 2 phenotype in conditions that favor type 2 differentiation (Kaplan et al., 1996a; Shimoda et al., 1996; Takeda et al., 1996). Furthermore, $STAT6^{-/-}$ mice bred on the *Leishmania*-susceptible BALB/c background are resistant to *Leishmania* infection, which is consistent with differentiation of T cells toward a type 1 phenotype (Stamm et al., 1998).

Various groups have used $STAT6^{-/-}$ mice in tumor models to evaluate 'immune deviation' as a possible mechanism of tumor immune suppression. It was shown that $STAT6^{-/-}$ mice rejected a variant of the mastocytoma P815 at a dose that normally grows progressively in *wt* DBA/2 mice (Kacha et al., 2000). Another group has shown that subcutaneous growth of the mammary adenocarcinoma, 4T1, was delayed in $STAT6^{-/-}$ mice as compared to *wt* BALB/c mice (Ostrand-Rosenberg et al., 2000). This group also showed that the number of spontaneous lung metastases from the primary site of this highly metastatic tumor was reduced in $STAT6^{-/-}$ mice. These results supported the hypothesis that polarization toward a type 1 response led to an enhanced anti-tumor immune response. However, an alternative explanation for the rejection of 4T1 in $STAT6^{-/-}$ mice is that the T-cell repertoire of $STAT6^{-/-}$ mice contains STAT6-reactive T cells that could recognize STAT6 peptide epitopes presented by 4T1. Herein, we report

that 4T1 primes T cells in $STAT6^{-/-}$ mice that recognize 4T1 as well as a STAT6 peptide epitope. Using bone marrow from $STAT6^{-/-}$ mice to reconstitute irradiated *wt* BALB/c mice we produced mice whose immune system was predisposed to a strong type 1 response, but that lacked STAT6-reactive T cells in their T-cell repertoire. This model enabled us to determine whether the rejection of 4T1 by $STAT6^{-/-}$ mice was due to either a strong type 1 response or the combination of the strong type 1 response against a strong foreign antigen (STAT6).

Materials and Methods

Mice

Female BALB/cJ and C.129S2-*Stat6*^{tm1Gru} (Kaplan et al., 1996a), STAT6$^{-/-}$ mice on a BALB/c background, were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained in a specific pathogen-free environment. Recognized principles of laboratory animal care were followed (Guide for the Care and Use of Laboratory Animals, National Research Council, 1996), and all animal protocols were approved by the Earle A. Chiles Research Institute Animal Care and Use Committee.

Tumor cell lines

4T1 is a 6-thioguanine-resistant cell line (provided by Dr. Suzanne Ostrand-Rosenberg, University of Maryland Baltimore County, MD) that was selected from a tumor cell line derived from a single spontaneously arising mammary tumor in a BALB/c3H mouse. The colon carcinoma cell line, CT26, was provided by Dr. Gregory Plautz (University of Michigan, MI), the renal cell carcinoma, Renca, was provided by Dr. Bob Wiltrout (Frederick Cancer Research and Development Center, Frederick, MD) and the mammary adenocarcinoma cell line, EMT6, was provided by Dr. Emmanuel T. Akporiaye (University of Arizona, AZ). Each of these tumors was derived from BALB/c mice. The C57BL/6 melanoma cell line, B16BL6-D5 (D5), was provided by Dr. Suyu Shu (Cleveland Clinic, OH). All cell lines were maintained in complete media (CM) comprising the following: RPMI 1640 (Bio Whittaker, Walkersville, MD) containing 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, and 50 μg/ml gentamicin sulfate. This was further supplemented with 50 μM 2-mercaptoethanol

(Aldrich, Milwaukee, WI) and 10% (vol/vol) fetal calf serum (GIBCO BRL, Grand Island, NY).

Immunoprecipitation and Western blot of STAT6

Tumor cell lines or splenocytes were lysed in cell lysis buffer (250mM NaCl, 25 mM Tris-HCl, 5mM EDTA, 1% NP-40, freshly added protease inhibitors). STAT6 antibody (M-20, Santa Cruz Biotechnology, Santa Cruz, CA) and Protein A-Sepharose (Pharmacia Biotech, Uppsala, Sweden) were added to the total cell lysate and incubated overnight at 4° C. Samples were run on SDS gel and western blotted using STAT6 antibody. Western blots were developed using chemiluminscent Pierce SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, IL).

Peptide loading of STAT6$^{-/-}$ splenocytes

Potential H-2^d-binding STAT6 peptide sequences were determined using a computer program (www-bimas.cit.nih.gov/molbio/hla_bind/) that ranks 9-mer peptides based on a predicted half-time of dissociation from H-2^d class I molecules. The analysis was based on coefficient tables deduced from the published literature by Dr. Kenneth Parker (Parker et al., 1994). Based on their predicted binding scores to H-2K^d three different STAT6 peptides, STAT6$_{18-26}$ (LYVDFPQRL), STAT6$_{129-137}$ (KFTTPLGRL), and STAT6$_{531-539}$ (SYWSDRLII) were synthesized. Predicted binding scores for STAT6 peptides to H-2L^d and H-2D^d were very low, therefore no peptide epitopes were chosen from these groups. The SV40 Large T antigen peptide containing residues 499-507 (DYLDGSVKV), LTA$_{499-507}$, was also synthesized and is a known H-2K^d binding peptide (Newmaster et al., 1998). All peptides were synthesized and purchased from Research Genetics, Huntsville, AL.

Spleens from $STAT6^{-/-}$ mice were collected and mechanically disrupted to create single cell suspensions. Red blood cells were lysed using ACK lysing buffer (Bio Whittaker, Walkersville, MD) and splenocytes were resuspended in 2 ml of CM (10^6 cells/ml) with 20 ng/ml of peptide and incubated at 37° C for 1 hr. Peptide-pulsed splenocytes were washed in CM and used as stimulators in cytokine release assays.

Tumor or peptide vaccination and activation of vaccine-draining lymph node cells

Tumor cell cultures were trypsinized (Bio Whittaker, Walkersville, MD) and washed twice in Hanks' Balanced Salt Solution (Bio Whittaker, Walkersville, MD). 7.5 X 10^5 4T1 tumor cells were injected subcutaneously into both axillary regions and hind flanks of BALB/c and $STAT6^{-/-}$ mice. Eight days following vaccination, the superficial inguinal lymph nodes and axillary lymph nodes draining the four vaccination sites were harvested and single cell suspensions were resuspended at 2 X 10^6 cells per ml in CM and cultured in 24 well plates with 50 μl of a 1:40 dilution of 2c11 ascites (anti-CD3). After two days of activation, the cells were harvested and expanded in CM containing 60 IU per ml of rhIL-2 (Chiron, Emeryville, CA) at a starting cell density of 1.25 X 10^5 cells per ml in 300 ml gas permeable tissue culture bags (Nexell Therapeutics Inc., Irvine, CA). After three days, 'effector' T cells were harvested and used in cytokine release assays, intracellular staining assays, or adoptive immunotherapy

BALB/c and $STAT6^{-/-}$ mice were injected subcutaneously with 25μg of either $STAT6_{531-539}$ or $LTA_{499-508}$ emulsified in CFA. The mice received a second and third subcutaneous injection of peptide in IFA in the opposite flank at 14 days intervals. Ten days following the last peptide injection lymph nodes draining the injection sites were obtained and activated and expanded as described for tumor-vaccine draining lymph node cells.

Adoptive immunotherapy

Experimental pulmonary metastases were established by i.v. inoculation of BALB/cJ or STAT6$^{-/-}$ mice with 2 X 10^5 4T1 tumor cells. Three days after metastases were established, 'effector' T cells were adoptively transferred intravenously (iv). Starting on the day of T-cell infusion, mice received 90,000 IU of IL-2 i.p. daily for 3 days. Animals were sacrificed 12 days following tumor inoculation, the lungs were resected and fixed in Fekete's solution, and the number of pulmonary metastases were enumerated by visual inspection.

Cytokine release assay

After *in vitro* activation and expansion, 'effector' T cells were washed and incubated alone or were stimulated with 4T1, CT26, Renca, D5, peptide-pulsed STAT6$^{-/-}$ splenocytes, or plate-bound anti-CD3. 'Effector' T cells (2 X 10^6/well) were cultured with tumor cells (2 X 10^5/well) or peptide-pulsed STAT6$^{-/-}$ splenocytes (2 X 10^6/well) in 2 ml in 24-well plates. Supernatants were recovered 14 hours after stimulation and IFN-γ or IL-4 concentration was measured in duplicate by ELISA using commercially available reagents (BD-PharMingen, San Diego, CA). The concentration of cytokine in the supernatant was determined by regression analysis.

Intracellular cytokine staining

After *in vitro* activation and expansion, 'effector' T cells (2 X 10^6) were washed and incubated alone or stimulated with 4T1 (2 X 10^5) or peptide-pulsed STAT6$^{-/-}$ splenocytes (2 X 10^6) in 2 ml in 24-well plates. After 1 hour 1 μl GolgiPlug (BD-PharMingen, San Diego, CA) was added to each well. After 16 hours of *in vitro* stimulation cells were harvested and stained with FITC-labeled anti-CD8 antibody (BD-PharMingen, San

Diego, CA), Cy-chrome labeled anti-CD3 antibody (BD-PharMingen, San Diego, CA), and isotype controls (BD-PharMingen, San Diego, CA) for 30 minutes at 4° C. The cells were washed and permeabilized by incubation with Cytofix/Cytoperm solution (BD-PharMingen, San Diego, CA), for 20 minutes at 4° C. After washing and resuspension in Perm/wash solution (BD-PharMingen, San Diego, CA), intracellular cytokine staining with PE-labeled anti-IFN-γ antibody (BD-PharMingen, San Diego, CA) was performed 20 minutes at 4° C. Cells were washed twice with Perm/wash solution, resuspended in FACS-buffer and analyzed on a BD Bioscience FACSCalibur (San Jose, CA). 150,000 gated events based on CD3 expression were collected and analyzed for expression of CD8 and IFN-γ.

Bone marrow reconstitution

BALB/c or $STAT6^{-/-}$ bone marrow was collected by flushing both femurs and tibias with a 27 gauge needle containing CM. $CD4^+$ and $CD8^+$ T cells were depleted by incubating the bone marrow cells with mouse anti-CD4 (L3T4) Microbeads (20μl/10^7 cells) and mouse anti-CD8a (Ly-2) Microbeads (20μl/10^7 cells) (Miltenyi Biotec, Auburn, CA) for 15 minutes at 4° C. Cells were washed in CM and passed over magnetic MACS column. The flow through ($CD4^-$ $CD8^-$ cells) was collected and washed in HBSS twice. 10^7 $CD4^-$ $CD8^-$ bone marrow cells were transferred intravenously to BALB/c or $STAT6^{-/-}$ mice that had been irradiated (700 cGy). Mice were allowed 6-8 weeks to reconstitute their immune system with the bone marrow graft before experiments were performed.

Tumor Challenge

BALB/c, $STAT6^{-/-}$, or bone marrow reconstituted mice were injected subcutaneously in the hind flank with 10^4 or 10^5 4T1 cells ($TD_{100}=10^4$). Tumor growth was determined by

multiplying the measured perpendicular diameters of the tumor. Mice were sacrificed when multiplied diameters equaled 150 mm^2.

Statistical Analyses

Data were analyzed using Student's t test for unequal variances (Microsoft Excel, Redmond, WA).

Results

STAT6$^{-/-}$ mice reject 4T1 tumor challenge

It has been reported previously that growth of the mammary adenocarcinoma, 4T1, is slowed in STAT6$^{-/-}$ mice as compared to *wt* BALB/c mice (Ostrand-Rosenberg et al., 2000). This observation was confirmed by our lab; subcutaneous challenge with 10^4 or 10^5 4T1 cells led to progressive tumor growth in all *wt* BALB/c mice, but after initial tumor formation 4T1 was subsequently rejected by nearly all STAT6$^{-/-}$ mice (Fig. 4.1). Titration of the dose of 4T1 tumor cells showed that 80% of STAT6$^{-/-}$ mice were capable of rejecting a tumor dose that was one log higher than the tumor dose that resulted in tumor growth in 100% of *wt* BALB/c mice.

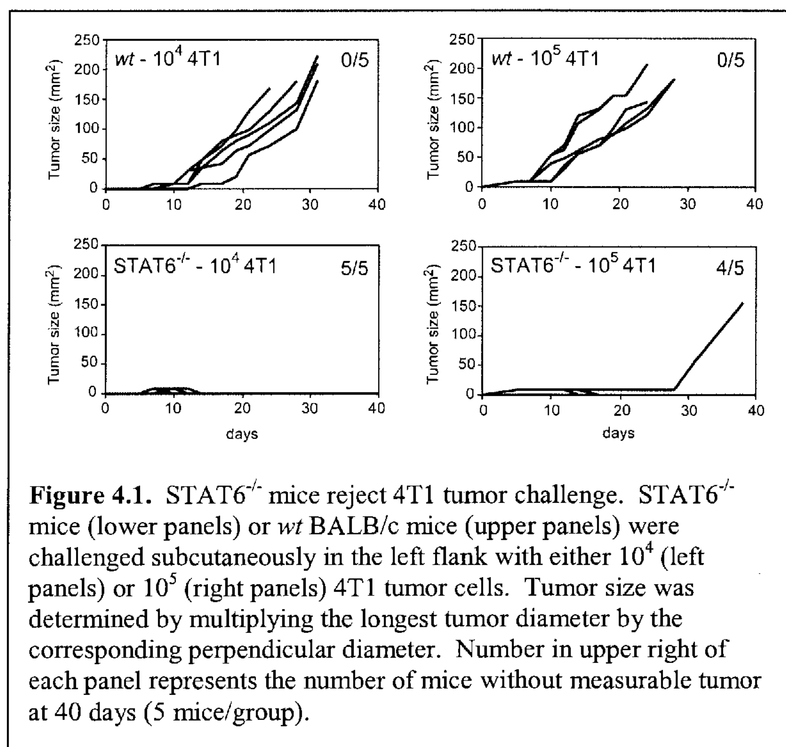

Figure 4.1. STAT6$^{-/-}$ mice reject 4T1 tumor challenge. STAT6$^{-/-}$ mice (lower panels) or *wt* BALB/c mice (upper panels) were challenged subcutaneously in the left flank with either 10^4 (left panels) or 10^5 (right panels) 4T1 tumor cells. Tumor size was determined by multiplying the longest tumor diameter by the corresponding perpendicular diameter. Number in upper right of each panel represents the number of mice without measurable tumor at 40 days (5 mice/group).

STAT6 expression in 4T1

STAT6 was reported to be expressed in mammary tissue (Watson, 2001). To determine whether STAT6 was expressed by 4T1, detergent soluble cellular lysates were immunoprecipitated with anti-STAT6 antibody (Fig. 4.2A). Splenocytes from *wt*

BALB/c mice, STAT4$^{-/-}$ mice, as well as 4T1 and Renca tumor cells expressed the 102 kDa protein; whereas STAT6 was undetectable in STAT6$^{-/-}$ splenocytes. The BALB/c colon and mammary carcinoma cell lines, CT26 and EMT6, as well as D5, a C57BL/6 melanoma, produced STAT6 protein (Fig. 4.2B).

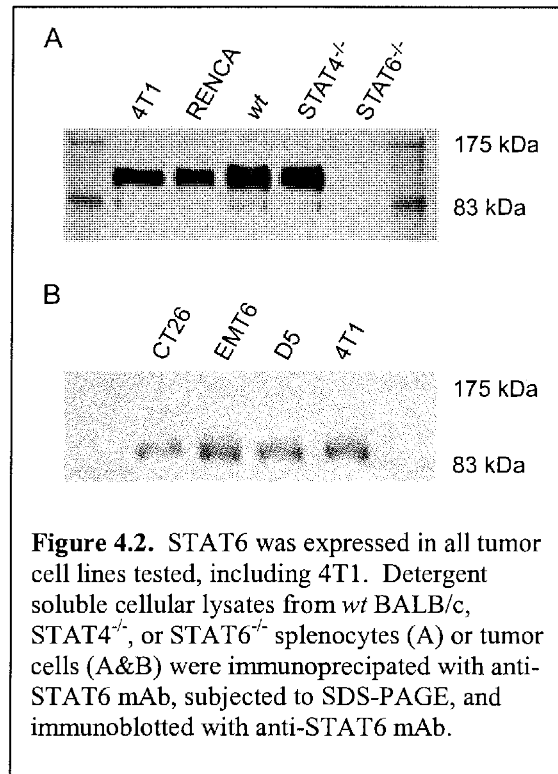

Figure 4.2. STAT6 was expressed in all tumor cell lines tested, including 4T1. Detergent soluble cellular lysates from *wt* BALB/c, STAT4$^{-/-}$, or STAT6$^{-/-}$ splenocytes (A) or tumor cells (A&B) were immunoprecipated with anti-STAT6 mAb, subjected to SDS-PAGE, and immunoblotted with anti-STAT6 mAb.

4T1 vaccination primes STAT6$_{531-539}$ peptide-specific T cells

The identification of STAT6 protein in 4T1 tumor cells raised the possibility that tumor rejection in STAT6$^{-/-}$ mice could be due to endogenous processing of STAT6 protein resulting in the presentation of peptides that stimulate STAT6-specific T cells in STAT6$^{-/-}$ mice. It has been shown that the immune response responsible for the rejection of 4T1 in STAT6$^{-/-}$ mice was dependent on the presence of CD8^{+} cells (Ostrand-Rosenberg et al., 2000). Therefore, we used a computer algorithm to determine theoretical binding affinities of STAT6 peptides for the following MHC class I alleles: H-2K^{d}, H-2L^{d}, and H-2D^{d}. Three peptides predicted to have high or medium binding affinities to H-2K^{d} were selected from a list of possible STAT6 peptides using a computer program that ranks peptides based on a predicted half-time of dissociation from class I molecules. To determine whether 4T1 could prime STAT6-specific T cells in STAT6$^{-/-}$ mice, both *wt*

BALB/c and STAT6$^{-/-}$ mice were vaccinated with 4T1 and the tumor-vaccine draining lymph nodes (TVDLN) were removed 8 days later. TVDLN cells were stimulated *in vitro* with soluble anti-CD3 antibody for 48 hours followed by expansion in low-dose IL-

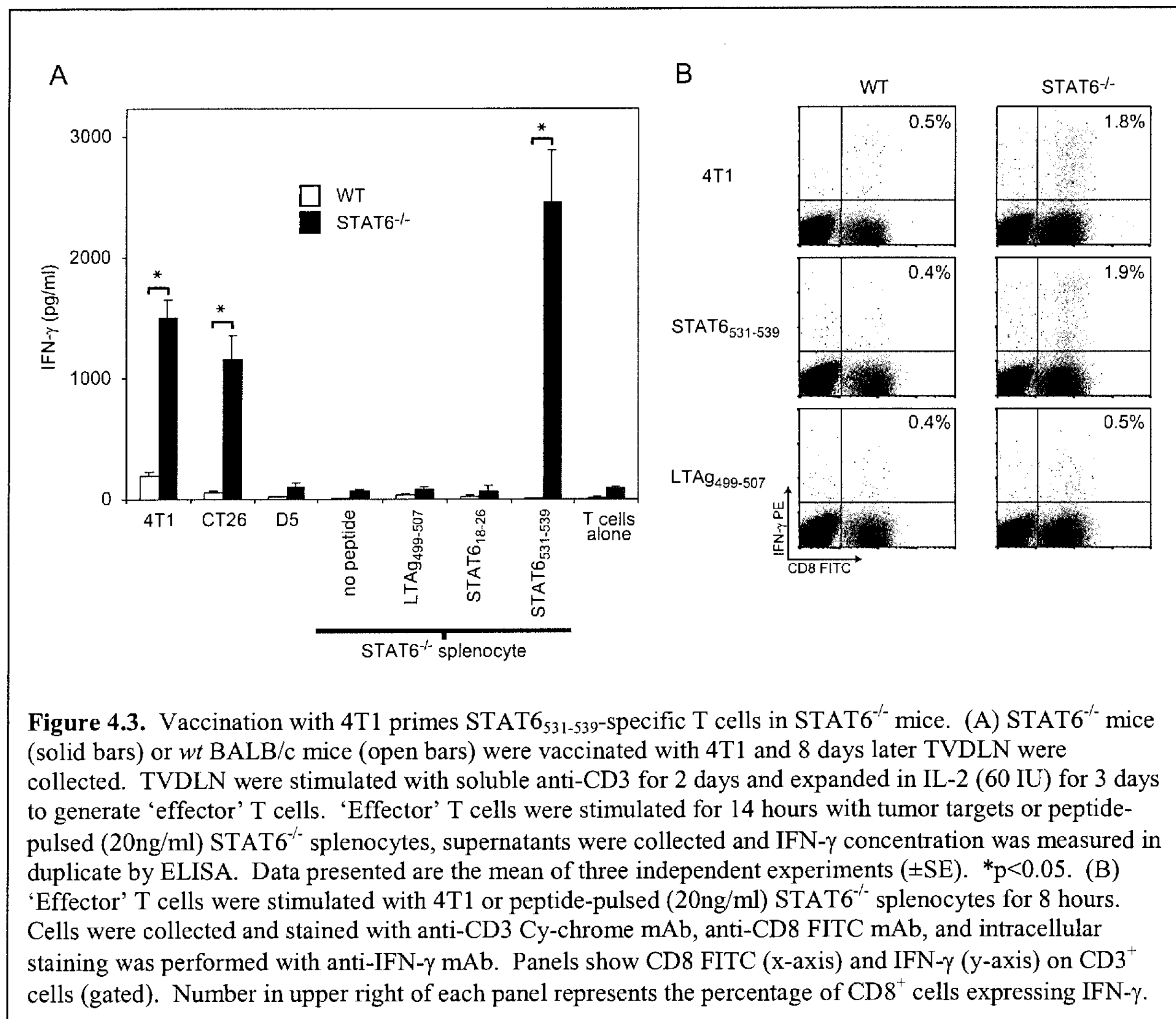

Figure 4.3. Vaccination with 4T1 primes STAT6$_{531-539}$-specific T cells in STAT6$^{-/-}$ mice. (A) STAT6$^{-/-}$ mice (solid bars) or *wt* BALB/c mice (open bars) were vaccinated with 4T1 and 8 days later TVDLN were collected. TVDLN were stimulated with soluble anti-CD3 for 2 days and expanded in IL-2 (60 IU) for 3 days to generate 'effector' T cells. 'Effector' T cells were stimulated for 14 hours with tumor targets or peptide-pulsed (20ng/ml) STAT6$^{-/-}$ splenocytes, supernatants were collected and IFN-γ concentration was measured in duplicate by ELISA. Data presented are the mean of three independent experiments (±SE). *$p<0.05$. (B) 'Effector' T cells were stimulated with 4T1 or peptide-pulsed (20ng/ml) STAT6$^{-/-}$ splenocytes for 8 hours. Cells were collected and stained with anti-CD3 Cy-chrome mAb, anti-CD8 FITC mAb, and intracellular staining was performed with anti-IFN-γ mAb. Panels show CD8 FITC (x-axis) and IFN-γ (y-axis) on CD3$^+$ cells (gated). Number in upper right of each panel represents the percentage of CD8$^+$ cells expressing IFN-γ.

2 (60 IU) for 3 days. This method of polyclonal activation has been shown to support the acquisition of antigen-specific 'effector' function by recently primed T cells (Yoshizawa et al., 1991a; Yoshizawa et al., 1992; Yoshizawa et al., 1991b). After polyclonal activation these 'effector' T cells were assayed for their ability to respond to tumor cells

or peptide-pulsed STAT6$^{-/-}$ splenocytes. T cells generated from 4T1-vaccinated STAT6$^{-/-}$ mice secreted significantly ($p<0.05$) more IFN-γ than T cells from 4T1-vaccinated *wt* BALB/c mice when stimulated with either 4T1 or CT26 tumor cells (Fig. 4.3A). This cross-reactivity was also observed against RENCA and EMT6 (data not shown) suggesting that all of these tumors share an antigen, possibly a peptide from STAT6 since they all express STAT6. Induction of IFN-γ was restricted to H-2^d tumor cells since 'effector' T cells generated from 4T1-vaccinated STAT6$^{-/-}$ mice did not respond to D5, a STAT6$^+$, H-2^b tumor. We determined whether T cells from 4T1-vaccinated STAT6$^{-/-}$ mice could respond to any of the panel of H-2K^d – binding STAT6 peptides and observed very strong responses to the STAT6 peptide comprising residues 531-539 (STAT6$_{531-539}$). T cells from 4T1-vaccinated *wt* BALB/c mice were unable to recognize this peptide. Three other H-2K^d – binding peptides, STAT6$_{18-26}$, STAT6$_{129-137}$, and a SV40 Large T Antigen peptide (LTA$_{499-508}$) (Newmaster et al., 1998) were unable to stimulate IFN-γ secretion from these T cells documenting the specificity of this response. The frequency of CD8$^+$ T cells that secreted IFN-γ in response to 4T1 and STAT6$_{531-539}$ was also much greater in 4T1-vaccinated STAT6$^{-/-}$ mice than in 4T1-vaccinated *wt* BALB/c mice (Fig. 4.3B). These data show that vaccination with 4T1 can prime STAT6$_{531-539}$ peptide-specific T cells and that these T cells are present only in the T-cell repertoire of STAT6$^{-/-}$ mice and not *wt* BALB/c mice.

STAT6$_{531-539}$ peptide vaccination primes STAT6$^{-/-}$ T cells that recognize 4T1

To demonstrate that STAT6$_{531-539}$ was an epitope presented by 4T1 we determined whether STAT6$_{531-539}$-specific T cells from *wt* BALB/c or STAT6$^{-/-}$ mice could recognize 4T1 following vaccination with STAT6$_{531-539}$. Lymph node cells

draining the peptide vaccination site were activated with soluble anti-CD3 and expanded in low dose IL-2 as described for TVDLN. Expanded 'effector' T cells were assayed for tumor-specific IFN-γ secretion. $STAT6_{531-539}$ vaccination primed T cells in $STAT6^{-/-}$ mice that responded to $STAT6_{531-539}$ pulsed $STAT6^{-/-}$ splenocytes (Fig. 4.4). $STAT6_{531-539}$ vaccination of *wt* BALB/c mice also primed T cells that responded to $STAT6_{531-539}$

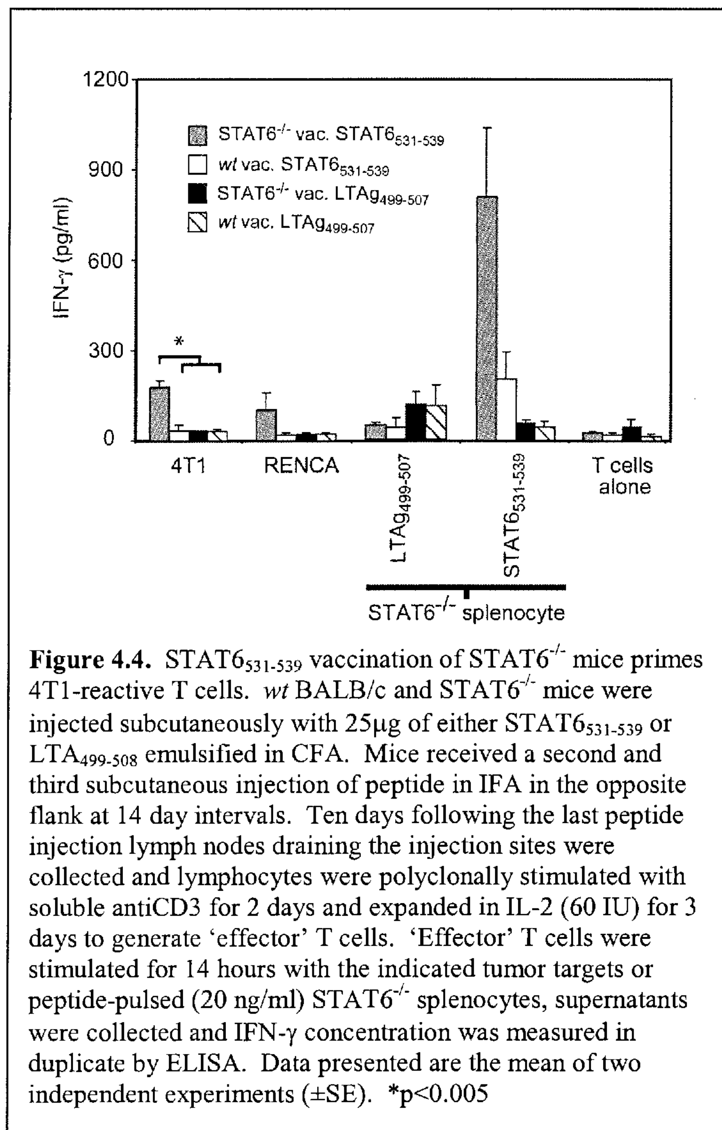

Figure 4.4. $STAT6_{531-539}$ vaccination of $STAT6^{-/-}$ mice primes 4T1-reactive T cells. *wt* BALB/c and $STAT6^{-/-}$ mice were injected subcutaneously with 25μg of either $STAT6_{531-539}$ or $LTA_{499-508}$ emulsified in CFA. Mice received a second and third subcutaneous injection of peptide in IFA in the opposite flank at 14 day intervals. Ten days following the last peptide injection lymph nodes draining the injection sites were collected and lymphocytes were polyclonally stimulated with soluble antiCD3 for 2 days and expanded in IL-2 (60 IU) for 3 days to generate 'effector' T cells. 'Effector' T cells were stimulated for 14 hours with the indicated tumor targets or peptide-pulsed (20 ng/ml) $STAT6^{-/-}$ splenocytes, supernatants were collected and IFN-γ concentration was measured in duplicate by ELISA. Data presented are the mean of two independent experiments (±SE). $^*p<0.005$

but these T cells secreted significantly lower levels of IFN-γ than T cells from $STAT6^{-/-}$ mice. Only T cells from $STAT6^{-/-}$ mice vaccinated with $STAT6_{531-539}$ responded to 4T1 ($p<0.005$). These T cells also responded to the $STAT6^{+}$, H-2^{d} renal cell tumor, Renca. T cells from $STAT6^{-/-}$ mice vaccinated with $LTA_{499-508}$ secreted background levels of IFN-γ in response to 4T1 demonstrating that the ability to respond to 4T1 was dependent on vaccination with $STAT6_{531-539}$. Although *wt* BALB/c mice vaccinated with $STAT6_{531-539}$

mounted a low level response to $STAT6_{531\text{-}539}$-pulsed $STAT6^{-/-}$ splenocytes, they did not respond to 4T1 any better than T cells from $LTA_{499\text{-}508}$ vaccinated *wt* BALB/c mice. These data show that 4T1 presents an epitope that can be recognized by $STAT6_{531\text{-}539}$ peptide-specific T cells generated in $STAT6^{-/-}$ mice.

To determine their therapeutic potential, 'effector' T cells from peptide-vaccinated mice were adoptively transferred into $STAT6^{-/-}$ recipient mice that had 3-day 4T1 pulmonary metastases. $STAT6^{-/-}$ recipient mice that received 'effector' T cells from $STAT6_{531\text{-}539}$-vaccinated $STAT6^{-/-}$ mice had a significant reduction ($p<0.002$) in the number of 4T1 metastases compared to mice that received 'effector' T cells from $STAT6_{531\text{-}539}$-vaccinated *wt* BALB/c mice (Table 4.1). These data demonstrate that

Table 4.1. $STAT6_{531\text{-}539}$ vaccination primed T cells that were therapeutic against experimental 4T1 pulmonary metastases.

Vaccinated mouse (Donor T cells)	Peptide vaccine[a]	Number of cells transferred[b]	IL-2[c]	Mean number of experimental pulmonary metastases (±SE)
$STAT6^{-/-}$	Large T $Ag_{499\text{-}507}$	40×10^6	+	95 (22)
wt BALB/c	$STAT6_{531\text{-}539}$	40×10^6	+	149 (8)
$STAT6^{-/-}$	$STAT6_{531\text{-}539}$	40×10^6	+	0 (0)[d]
None	None	None	+	168 (6)

[a] *wt* BABL/c and $STAT6^{-/-}$ mice were injected subcutaneously with 25 µg of either $STAT6_{531\text{-}539}$ or $LTA_{499\text{-}508}$ emulsified in CFA. Mice received a second and third subcutaneous injection of peptide in IFA in the opposite flank at 14 day intervals.

[b] Ten days following the last peptide injection lymph nodes draining the injection sites were collected and lymphocytes were stimulated with soluble anti-CD3 for 2 days and expanded in IL-2 (60 IU) for 3 days to generated 'effector' T cells. $STAT6^{-/-}$ mice with 3-days experimental 4T1 pulmonary metastases were recipients of transferred 'effector' T cells from either peptide-vaccinated group, or received no T cells.

[c] Mice were injected i.p. with 15,000 IU of IL-2 daily for 3 days starting on the day of adoptive transfer and sacrificed on day 12 when pulmonary metastases were counted.

[d] $p<0.002$ compared to all other groups.

$STAT6_{531-539}$ peptide-specific T cells generated in $STAT6^{-/-}$ mice not only respond to 4T1 *in vitro* but are also sufficient to cause the regression of 4T1 *in vivo.*

Peptide-specific T cells from bone marrow chimeras fail to reject 4T1 tumor challenge

Since $STAT6_{531-539}$ peptide-specific T cells primed in $STAT6^{-/-}$ mice responded to 4T1 *in vitro* and eliminated experimental pulmonary metastases *in vivo* it raised the question whether the rejection of subcutaneous 4T1 challenge in $STAT6^{-/-}$ mice was due to the type 1 polarized immune response of $STAT6^{-/-}$ mice or the presence of $STAT6_{531-539}$-reactive T cells.

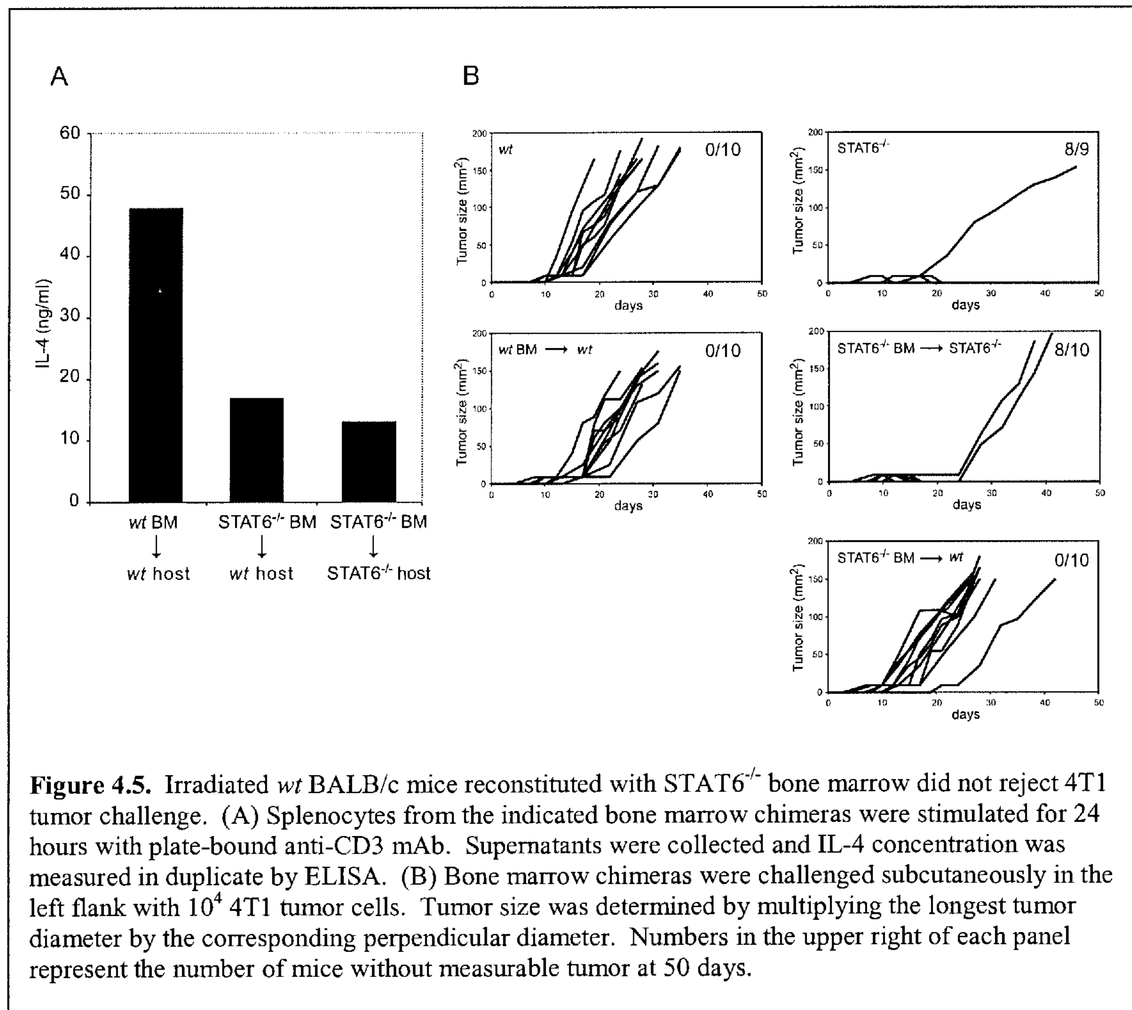

Figure 4.5. Irradiated *wt* BALB/c mice reconstituted with $STAT6^{-/-}$ bone marrow did not reject 4T1 tumor challenge. (A) Splenocytes from the indicated bone marrow chimeras were stimulated for 24 hours with plate-bound anti-CD3 mAb. Supernatants were collected and IL-4 concentration was measured in duplicate by ELISA. (B) Bone marrow chimeras were challenged subcutaneously in the left flank with 10^4 4T1 tumor cells. Tumor size was determined by multiplying the longest tumor diameter by the corresponding perpendicular diameter. Numbers in the upper right of each panel represent the number of mice without measurable tumor at 50 days.

To investigate this question STAT6$^{-/-}$ bone marrow chimeras were generated by injecting STAT6$^{-/-}$ bone marrow into irradiated *wt* BALB/c hosts (STAT6$^{-/-}$BM→ *wt*). These mice would still generate predominantly a polarized type 1 response, but could no longer develop STAT6-reactive T cells because they were either deleted within the *wt* thymus or peripherally tolerized to avoid autoimmunity. *wt* BALB/c bone marrow into *wt* BALB/c chimeras (*wt* BM→ *wt*) and STAT6$^{-/-}$ bone marrow into STAT6$^{-/-}$ chimeras (STAT6$^{-/-}$BM→ STAT6$^{-/-}$) were also produced as controls for the reconstitution. To verify that the bone marrow compartment was reconstituted with donor bone marrow, splenocytes were stimulated with anti-CD3 and the supernatants assayed for IL-4 production by ELISA. Previous work has demonstrated that STAT6$^{-/-}$ lymphocytes secrete significantly less IL-4 than *wt* lymphocytes when stimulated with anti-CD3 (Kaplan et al., 1996a; Zhang et al., 2000). Both (STAT6$^{-/-}$BM→ STAT6$^{-/-}$) mice and (STAT6$^{-/-}$BM→ *wt*) mice made significantly less IL-4 than (*wt* BM→ *wt*) mice demonstrating that the bone marrow grafts were successful (Fig. 4.5A). Subcutaneous tumor challenges with as few as 10^4 4T1 cells caused progressively growing tumors in ten out of ten *wt* BALB/c mice as well as ten out of ten of (*wt* BM→ *wt*) mice (Fig. 4.5B). As expected eight out of nine STAT6$^{-/-}$ mice rejected the 4T1 tumor challenge at 10^4 tumor cells. The (STAT6$^{-/-}$BM→ STAT6$^{-/-}$) mice behaved similar to STAT6$^{-/-}$ mice and eight out of ten were able to reject a 4T1 tumor challenge. On the other hand, (STAT6$^{-/-}$BM→ *wt*) mice were unable to reject a 4T1 tumor challenge; all ten mice developed progressively growing tumors.

Considering our data and previous reports that the regression of 4T1 in STAT6$^{-/-}$ mice is dependent on CD8$^+$ cells (Ostrand-Rosenberg et al., 2000), it appeared that it was

the loss of STAT6-reactive T cells in (STAT6$^{-/-}$BM→ *wt*) mice that explained the growth of 4T1 in these mice. To address whether T cells from (STAT6$^{-/-}$BM→ *wt*) mice responded to 4T1 we collected tumor-draining lymph node cells from the reconstituted mice, and activated and expanded the cells to generate 'effector' T cells. 'Effector' T cells from *wt* BALB/c mice and (*wt* BM→ *wt*) mice did not respond to stimulation by

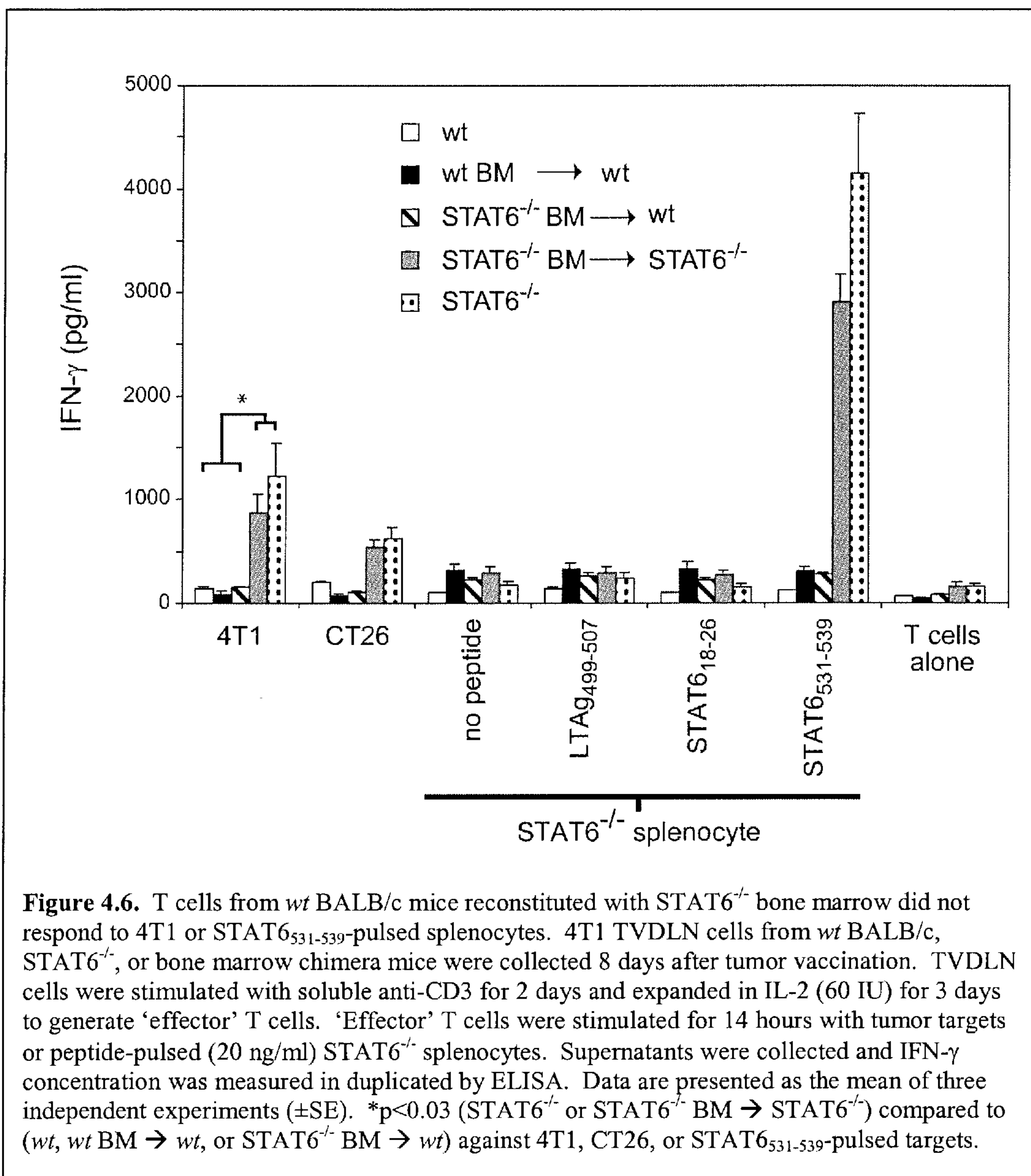

Figure 4.6. T cells from *wt* BALB/c mice reconstituted with STAT6$^{-/-}$ bone marrow did not respond to 4T1 or STAT6$_{531-539}$-pulsed splenocytes. 4T1 TVDLN cells from *wt* BALB/c, STAT6$^{-/-}$, or bone marrow chimera mice were collected 8 days after tumor vaccination. TVDLN cells were stimulated with soluble anti-CD3 for 2 days and expanded in IL-2 (60 IU) for 3 days to generate 'effector' T cells. 'Effector' T cells were stimulated for 14 hours with tumor targets or peptide-pulsed (20 ng/ml) STAT6$^{-/-}$ splenocytes. Supernatants were collected and IFN-γ concentration was measured in duplicated by ELISA. Data are presented as the mean of three independent experiments (±SE). *$p<0.03$ (STAT6$^{-/-}$ or STAT6$^{-/-}$ BM → STAT6$^{-/-}$) compared to (*wt*, *wt* BM → *wt*, or STAT6$^{-/-}$ BM → *wt*) against 4T1, CT26, or STAT6$_{531-539}$-pulsed targets.

4T1 (Fig. 4.6). 'Effector' T cells from STAT6$^{-/-}$ mice as well as 'effector' T cells from (STAT6$^{-/-}$BM→ STAT6$^{-/-}$) mice responded to 4T1 by secreting significantly higher amounts of IFN-γ than the other groups ($p<0.03$). 'Effector' T cells from (STAT6$^{-/-}$BM→ STAT6$^{-/-}$) mice responded less to the 4T1 stimulation than 'effector' T cells from STAT6$^{-/-}$ mice, however the difference was not statistically significant. In contrast, 'effector' T cells from (STAT6$^{-/-}$BM→ *wt*) mice did not respond to stimulation by 4T1 and exhibited levels of IFN-γ secretion similar to *wt* BALB/c mice and (*wt* BM→ *wt*) mice. This demonstrated that the 4T1-specific T cells that are present in (STAT6$^{-/-}$BM→ STAT6$^{-/-}$) mice have been either centrally deleted from the T-cell repertoire, or are nonresponsive in the periphery of the *wt* mouse. As expected, 'effector' T cells from (STAT6$^{-/-}$BM→ *wt*) mice also failed to respond to the $STAT6_{531-539}$ peptide unlike 'effector' T cells from (STAT6$^{-/-}$BM→ STAT6$^{-/-}$) mice. These data demonstrate that the loss of $STAT6_{531-539}$-reactive T cells correlated with the loss of reactivity to 4T1 and the consequence of which is the progressive growth of 4T1 *in vivo*. This is consistent with the conclusion that STAT6$^{-/-}$ mice reject 4T1 because T cells recognize STAT6 as a foreign antigen rather than because of the preference toward developing a type 1 response.

Discussion

STAT6$^{-/-}$ mice may be an important model system to examine the effect of a polarized type 1 immune response on tumor growth. Previous investigators using STAT6$^{-/-}$ mice have concluded that the deletion of the STAT6 gene, which facilitated the development of potent anti-tumor immunity, did so via enhancement of type 1 immune responses (Kacha et al., 2000; Ostrand-Rosenberg et al., 2000). We observed the same findings; however, when T cells from 4T1-vaccinated STAT6$^{-/-}$ mice were adoptively transfered into recipient *wt* BALB/c mice we noticed signs reminiscent of graft-versus-host disease (data not shown). Necropsy of these mice showed inflammation of the small and large intestines suggesting that the transferred cells were responding to a tissue antigen located in the GI tract of *wt* BALB/c mice. These observations led us to test the hypothesis that STAT6 was not only the antigen recognized by T cells mediating the 'graft-versus-host' response, but more importantly, the antigen responsible for the regression of 4T1 observed in STAT6$^{-/-}$ mice. The work presented herein documents the presence of T cells that recognize the STAT6$_{531\text{-}539}$ peptide in 4T1-vaccinated STAT6$^{-/-}$ mice. When the response against other H-2K^{d}-binding STAT6 peptides was examined no reactivity was observed. In bone marrow chimeras (*wt* mice reconstituted with STAT6$^{-/-}$ bone marrow), the absence of STAT6$_{531\text{-}539}$-reactive T cells resulted in mice that were unable to reject the 4T1 challenge, even though the reconstituting STAT6$^{-/-}$ T cells should be predisposed toward a type 1 phenotype. From these experiments it is clear that the rejection of 4T1 tumor cells by STAT6$^{-/-}$ mice is dependent on STAT6$_{531\text{-}539}$-reactive T cells. However, it is not clear if prevention of tumor growth in STAT6$^{-/-}$ mice is solely due to the presence of STAT6$_{531\text{-}539}$-reactive T cells, or if it is the combination of

$STAT6_{531-539}$-reactive T cells and the strong polarization toward a type 1 phenotype that is necessary for tumor rejection. Previous data have shown that the onset of tumor formation of naturally-progressing mouse mammary tumor virus (MMTV)-induced tumors is delayed in $STAT6^{-/-}$ mice compared to *wt* mice (Czarneski et al., 2001). Since these tumors arose endogenously within the mice the complicating issue of STAT6 priming STAT6-reactive T cells in $STAT6^{-/-}$ mice is avoided. Nonetheless, tumors in both *wt* and $STAT6^{-/-}$ mice should express foreign MMTV antigens suggesting that the type 1 polarized response of $STAT6^{-/-}$ mice is beneficial compared to *wt* mice when both immune systems are primed with a strong foreign antigen.

4T1 appears to prime the *wt* immune system insufficiently, as evidenced by the low levels of IFN-γ secreted by tumor-vaccine draining lymph node cells from 4T1-vaccinated *wt* mice when stimulated with 4T1. Since the question of 'immune deviation' is relevant only after T-cells have been primed, the significance of 'immune deviation' to regression of 4T1 is questionable. However, previous studies suggest that 4T1 does posses tumor antigens that can prime tumor-reactive T cell since the adoptive transfer of 'effector' T cells generated from E10-9-vaccinated *wt* mice reduced the number of experimental pulmonary metastases in recipient mice (Table 3.1). These data suggest that either the frequency of *wt* T cells primed by 4T1 is not sufficient to cause regression of subcutaneous 4T1 challenge or the *wt* T cells do not differentiate *in vivo* into 'effector' T cells in the 4T1-challenged animal. Therefore, additional mechanisms apart from 'immune deviation' were responsible for the inability of *wt* BALB/c mice reconstituted with $STAT6^{-/-}$ bone marrow to reject 4T1.

It is also possible that 4T1 might actively suppress the immune response through immunosuppressive factors. Other groups have observed that 4T1 secretes significant amounts of TGF-β, and that blocking TGF-β secretion by 4T1 enhanced the immune response primed by 4T1 (Muraoka et al., 2002; Wu et al., 2001). Increased IFN-γ production by tumor draining lymph node cells was observed when mice were vaccinated with 4T1 transduced with an antisense TGF-β transgene (Wu et al., 2001). TGF-β's role as an antiproliferative factor for T cells appears to work primarily through inhibition of IL-2 production (Gorelik and Flavell, 2002) and inhibition of intracellular activators of T cell differentiation pathways (Gorelik et al., 2002; Gorelik et al., 2000; Heath et al., 2000). If TGF-β secreted by 4T1 was responsible for the inhibition of proliferation of 4T1-specific T cells in *wt* BALB/c mice our data suggest it was unable to exert the same inhibition in $STAT6^{-/-}$ mice. High avidity interactions between $STAT6_{531-539}$-specific T cells and $STAT6_{531-539}$:H-$2K^d$ complexes could induce strong proliferative responses characterized by IL-2 secretion and expression of high affinity IL-2 receptor (Girgis et al., 1999; Heath et al., 1993) that override the inhibition exerted by TGF-β. Further studies using ($STAT6^{-/-}$BM→ *wt*) mice that include strategies that block TGF-β will attempt to examine the role of TGF-β in the initial 4T1-primed proliferation of T cells. More importantly, if the initial priming to tumor antigens can be augmented either by blocking immunosuppressive tumor factors or through enhancing the priming event then the benefits of a polarized type 1 immune response can be investigated in greater detail.

Conclusions

- The 4T1 tumor cell line consists of a heterogenous population of tumor cells that can be separated by subcloning. Two subclones described here, 4T1-9 and 4T1-10, displayed distinct phenotypical and immunological differences. Specifically, 4T1-9 lacked the expression of various costimulatory molecules (CD86, CD40, and ICAM-1) that were expressed by 4T1-10. 4T1-9 was poorly immunogenic similar to the parental 4T1 tumor cell line, whereas 4T1-10 was moderately immunogenic. 'Effector' T cells generated from 4T1-10 TVDLN secreted more IFN-γ in response to 4T1 than did 'effector' T cells from 4T1-9 TVDLN, however this difference did not reach statistical significance (p=0.08). These data concur with previous data in our lab that showed a correlation between enhanced immunogenicity and type 1 polarization (Winter et al., 2003), but it is also possible that the increased expression of costimulatory molecules was responsible for the increased immunogenicity.
- Our results suggest that using a GM-CSF transduced breast cancer vaccine increased the priming of a type 1 immune response, as determined by an increase in tumor-specific IFN-γ secretion *in vitro*. This enhanced type 1 polarized response correlated with increased therapeutic efficacy *in vivo*. Furthermore, we have identified that downregulation of L-selectin expression can be used as a marker for T cells with therapeutic potential in this tumor model of breast cancer.
- Using this GM-CSF secreting tumor vaccine model we have generated effector T cells from *wt* BALB/c mice and STAT6$^{-/-}$ mice. Vaccination of STAT6$^{-/-}$ mice has

consistently produced the most highly type 1 polarized T cells. The observation that infusion of large numbers of highly polarized type 1 'effector' T cells from STAT6$^{-/-}$ mice resulted in substantial toxicity and death of some animals suggests that we have reached a dose limiting toxicity with these highly type 1 polarized T cells. This appears to be the result of STAT6-reactive T cells present in STAT6$^{-/-}$ mice being stimulated by normal cells that express STAT6 in *wt* BALB/c recipients. These findings will need to be considered as we begin to translate novel strategies to develop highly polarized tumor-specific type 1 'effector' T cells for patients with breast cancer since most tumor antigens are self antigens and thus a highly polarized type 1 response against a self antigen could lead to substantial toxicity.

- Interestingly, the highly type 1 polarized T cells from E10-9 vaccinated STAT6$^{-/-}$ mice could be partially inhibited by the cotransfer of T cells from E10-9 vaccinated STAT4$^{-/-}$ mice. The mechanism by which this inhibition is exerted is unknown, but this inhibition is STAT6-independent since the transferred T cells are from STAT6$^{-/-}$ mice that are adoptively transferred into STAT6$^{-/-}$ recipients.
- Our results have shown that the ability of STAT6$^{-/-}$ mice to reject high doses of the poorly immunogenic tumor, 4T1, correlated with the presence of STAT6-reactive T cells that recognize STAT6 epitopes on 4T1. This demonstrates the necessity of a tumor antigen that can be recognized sufficiently by the T cell repertoire to provide primed T cells. Although deletion or peripheral tolerance of STAT6-reactive T cells in *wt* mice reconstituted with STAT6$^{-/-}$ bone marrow correlated with the inability to reject 4T1 tumor challenge, it is also of interest

that $STAT6^{-/-}$/IFN-$\gamma^{-/-}$ double knock-out mice were also unable to reject 4T1 (Ostrand-Rosenberg et al., 2002). This emphasizes the importance of the type 1 cytokine, IFN-γ, since its absence results in the inability of the immune response to reject 4T1 even though STAT6-reactive T cells would be present in $STAT6^{-/-}$/IFN-$\gamma^{-/-}$ double knock-out mice.

- Taken together the data presented here demonstrates that both a tumor antigen that elicits a high frequency of tumor-reactive T cells (*in vitro* expansion of TVDLN in the adoptive transfer tumor model; or STAT6-reactive T cells in the $STAT6^{-/-}$ tumor model) and a strongly polarized type 1 response provide an effective protection against 4T1 tumor challenge.

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Abbreviations

IVS – *in vitro* sensitization
LAK – lymphokine-activated killer
CM – complete media
TVDLN – tumor vaccine-draining lymph node cells
GM-CSF – granulocyte-macrophage colony-stimulating factor
DC – dendritic cell
STAT – signal transducers and activators of transcription
($STAT6^{-/-}$BM→ $STAT6^{-/-}$) - $STAT6^{-/-}$ mice reconstituted with $STAT6^{-/-}$ bone marrow
($STAT6^{-/-}$BM→ *wt*) - *wt* mice reconstituted with $STAT6^{-/-}$ bone marrow
(*wt* BM→ *wt*) - *wt* mice reconstituted with *wt* bone marrow