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MARRYING IMMUNOTHERAPY AND CHEMOTHERAPY: A CANCER THERAPY BASED ON T LYMPHOCYTE EXPANSION AUGMENTED BY ALTERNATE GAMMA CHAIN CYTOKINES AND GEMCITABINE-MEDIATED INHIBITION OF MYELOID DERIVED SUPPRESSOR CELLS

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A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

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Successful adoptive immunotherapy (AIT) for cancer relies on the infusion of in vitro expanded, tumor-reactive lymphocytes with a goal of generating productive tumor immunity. Previously, our lab has developed a protocol to activate selectively tumor-reactive T lymphocytes in vitro using two pharmacologic agents, bryostatin-1 and ionomycin. Following the pharmacological stimulation, conventionally, IL-2 is added to stimulate in vitro proliferation. In this report, alternate cytokines from the common cytokine receptor γ-chain family, namely IL-7 and IL-15, were explored as the alternative

cytokine supplements. We found that tumor DLN cells activated in vitro with B/I and cultured in IL-7/15 alternate common γ-chain cytokines expanded better than IL-2 cultured cells. Furthermore, immunosuppressive myeloid-derived suppressor cells from the tumor microenvironment were targeted with a chemotherapeutic agent, gemcitabine. Despite combining gemcitabine and the T lymphocytes expanded in IL-7/15, AIT failed to induce regression of large established 4T1 mammary flank tumors.

CHAPTER 1 Introduction

1.1 Cancer: Challenges

Despite the advances in cancer research, according to a 2009 American Cancer Society report, cancer accounts for nearly one-quarter of deaths next to only heart diseases in the United States alone. Worldwide, one in eight deaths is due to cancer and it exceeds the number of deaths caused by AIDS, tuberculosis, and malaria combined. The corresponding estimates for total cancer deaths in 2007 were 7.6 million, or about 20,000 cancer deaths a day, with 2.9 million in economically developed countries and 4.7million in economically developing countries. By 2050, the global burden is expected to grow to 27 million new cancer cases and 17.5 million cancer deaths. Moreover, both the direct and indirect financial cost of cancer is also extensive (1).

Cancer cells are defined by the lack of regulation in cell proliferation and homeostasis. Several research findings indicate that tumorigenesis in humans is a multistep process and these genetic altering steps drive the transformation of normal human cells into highly malignant derivatives. Hanahan *et al*. categorizes the six main physical alterations common in cancer cells as: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death (apoptosis), limitless

replicative potential, sustained angiogenesis, and tissue invasion and metastasis (2). Cancer cells escape the terminal differentiation and quiescence steps important in normal cell homeostasis. In order to obtain such properties, tumor cells produce their own growth factors or transform the normal growth factors from the hosts. Furthermore, the signaling pathways of mitogenic stimuli are constitutively activated by overexpression or by mutation of signal transduction signals in tumor cells. Tumor cells can also directly de-regulate cell cycle regulation. Cancer cells obtain these new characteristics to avoid the antitumor defense mechanisms of the normal cells. Deregulations in these multiple regulatory circuits act as rate-limiting steps in tumor progression and add to the complexity of cancer cells (3).

There are several methods to treat cancer including surgery, radiation, chemotherapy, hormones, and immunotherapy. While standard treatments, like chemotherapy and radiation therapy, are used on cancer patients, the toxicity of adverse reactions can be a serious problem (4). On the other hand, immunotherapy is a relatively nontoxic cancer therapy that can be used alone or in conjunction with other therapies (5). The goal of immunotherapy is to use the host immune system to augment protective antitumor immunity and to disrupt the regulatory circuits that maintain tumor progression and tolerance (6).

1.2 Overview of Innate and Adaptive Antitumor Immunity

The idea that the immune system can recognize and respond to carcinomas, which are after all derived from the host, was first proposed by Paul Ehrlich in 1909. However, it was not until the 1950s, as immunologists gained a better understanding of transplantation and tumor immunobiology, that the Ehrlich theory could be validated. With the availability of inbred strains of mice, the animal models demonstrated that the syngeneic transplants of tumors were immunologically distinguishable from normal cells. Macfarlane Burnet and Lewis Thomas formally introduced the term "immunosurveillance" to describe the endogenous immune system components recognizing and eliminating the nascent transformed cells (7, 8).

In addition to identifying the cellular effectors of cancer immunosurveillance, two additional key studies revealed the effector functions of these cellular immune components. First, Dighe *et al*. showed that endogenously produced interferon γ (IFN- γ) protected the host against the growth of transplanted tumor and the formation of primary chemically induced and spontaneous tumors (10). The second important observation was that C57Bl/6 mice lacking perforin were more vulnerable to MCA-induced tumor formation compared to the naïve wild-type mice. Perforin is a part of the cytolytic granules of cytotoxic T cells and natural killer (NK) cells that is involved in lymphocyte-dependent killing of many different target cells, including tumor cells (7, 8).

The host immune system can be categorized into two general responses: innate and adaptive immune response (see appendix A). The innate immune response refers to the first line of barriers to combat a wide range of pathogens but lacks pathogen specificity and

longevity. In addition to the physical and chemical barriers of the host, the innate immune system has cellular component including granulocytes and natural killer cells. In contrast, an adaptive immune response takes time to develop and is highly specific to the pathogen. The adaptive immunity involves the selection and amplification of clones of lymphocytes (B and T cells) bearing receptors that recognize the foreign antigen (a term derived from a substance that can stimulate *anti*body *gen*eration) (11, 12). The adaptive immune system can be further divided into humoral and cellular adaptive immunity. The former refers to the B cell receptors recognizing antigens from the extracellular environment and differentiating into effector plasma cells that secrete antibodies and into memory B cells. However, it is the function of the T lymphocytes, also known as the cell-mediated immune response, which control intracellular pathogens, to activate B cell responses to most pathogens, and play a key role in maintaining antitumor immunity.

T lymphocytes develop in the thymus and are composed of two main classes: one of which has a surface marker CD4 (CD4 T cells) and the other with CD8 (CD8 or cytotoxic T cells). The CD4 T cells are activated by the antigen epitopes bound to MHC II which are only located on the antigen-presenting cells (B cells, macrophages and dendritic cells). The CD4 effector subsets currently characterized are: T helper (T_H) cells (which can be divided into T_H1 and T_H2 cells), T_H17, and several regulatory T cell subsets. T_H1 cells have a dual function of controlling intracellular pathogens and activating macrophages via various cytokines such as interleukin-2 (IL2), tumor necrosis factor-β (TNF-β), and interferon-γ (IFN- γ). T_H2 cells are entirely dedicated to the activation of the naïve B cells to produce antibody. T_H17 cells help to recruit neutrophils to sites of infection early in the

adaptive immune response. Regulatory T cells (Tregs) tend to inhibit immune activities and are important in preventing uncontrolled immune responses and autoimmunity (11, 12).

There are several key findings that demonstrate the cellular basis of cancer immunosurveillance and involvement of both the adoptive and innate immune responses. One of the studies involved the gene-target mice lacking the recombinase activating gene (RAG-2). Mice lacking either RAG-2 or its obligate partner, RAG-1, cannot rearrange lymphocyte antigen receptors, which inhibits production of peripheral αβ T cells, B cells, natural killer cells, or γδ T cells. The importance of RAG-2 study is that RAG-2 expression is limited to cells of the lymphoid system, thus allowing researchers to exclusively study the effects of host lymphocyte deficiency. After subcutaneous injection of the chemical carcinogen 3'-methycholanthrene (MCA), RAG-2 knockout mice (RAG-$2^{-/-}$) developed sarcomas at the injection site faster and with greater frequency when compared with the control group. RAG-$2^{-/-}$ mice were also more prone to spontaneous epithelial tumors. These findings reveal that lymphocytes are important protectors against both chemically-induced and spontaneous tumor formation. Furthermore, Smyth et al. demonstrated that natural killer (components of innate immune system) and natural killer T cells also participate in cancer immunosurveillance. C57BL/6 mice, depleted of both natural killer and natural killer T cells using the anti-NK1.1 mAb, were two or three times more susceptible to MCA-induced tumorigenesis (7, 8, 9).

Cytotoxic T cells carry CD8 surface marker and are stimulated by the antigenic peptide bound to major histocompatibility complex class I (MHC I) which are found on all nucleated cells (12). CD8 T cells have been identified as potent effectors of the adaptive antitumor immune response. The antigenic peptides recognized by these CD8 T cells are mostly non-mutated self-antigens that are also expressed by tumor cells. Although tumor-specific CD4 T cells have also been identified, CD4 T cells can either help or hinder antitumor immune responses due to maintenance of Tregs (13). Since the first human tumor antigen was identified in 1991, many tumor antigens have been cloned. Different types of tumor antigens can be broadly classified into five categories: 1) cancer testis antigen, 2) tissue differentiation antigens, 3) overexpressed antigens, 4) unique tumor antigens and 5) viral antigens. In addition to tumor antigens presented on MHC molecules, transformed tumor cells also overexpress other molecules that can be recognized by the adaptive immune system as neoplastic (8). For example, among the most studied overexpressed oncogenic proteins is HER-2/neu. HER-2/neu is a growth factor receptor that is overexpressed in 30% of breast and ovarian cancers and other adenocarcionmas. Numerous studies have shown that antibody, helper T cell and cytotoxic CD8 T cell respond against HER-2/neu-positive cancer cells (14). Thus due to the immunogenicity of tumor cells, T lymphocytes can be used in immunotherapy to fight cancer.

1.3 Adoptive Immunotherapy: Opportunities

Immunotherapy can be categorized into two types: active and passive. The active immunotherapy stimulates the patient's own immune system by direct administration of therapeutic cancer vaccines *in vivo* (12). Cancer vaccines include genetically modified tumor cells, antigen-loaded dendritic cells, and purified tumor antigens all aim to stimulate the adoptive immune system (17). However, in spite of large number of patients treated with vaccines, exceedingly low objective response rates are reported and cancer vaccines used alone cannot induce regression of invasive, vascularized tumors. Moreover, although immune T cells sensitive to tumor antigens can be generated by direct immunization (active immunotherapy), there are no cancer vaccine models that demonstrate that vacularized tumors can be rejected by this method. On the other hand, the passive immunotherapy has been more encouraging and shown consistent antitumor effects in rejecting large vascularized tumors in mice under appropriate conditions (16). Passive or adoptive immunotherapy (AIT) refers to the reintroduction of the cancer-reactive T cells, originally harvested from the cancer patients, which are then expanded and manipulated in vitro in order to generate productive antitumor responses (12, 6).

In adoptive immunotherapy, various mature T lymphocytes with antitumor activity are transferred into a tumor-bearing host with the goal of eliminating a tumor and preventing its recurrence (15). Three criteria are required for such immunologic destruction of established tumors: 1) sufficient numbers of immune cells must be generated in vivo that are highly sensitive to tumor antigens, 2) these immune components must traffic to and infiltrate the tumor stroma, and 3) once reaching the tumor site, these immune

effectors must manifest appropriate actions such as direct lysis or cytokine secretion than can cause tumor destruction (16).

When planning for successful AIT, the following factors must be taken into consideration: tumor type, route of AIT of immune effector cells, stage or size of tumor to be treated, location of tumor, immune effector cells to be transferred, source of effector cells, and the mechanism for ex vivo stimulation of effector cells including additional drugs or cytokines. When selecting a tumor model, since human tumors are weakly immunogenic, using too immunogenic tumor strain can result in deceptively easy tumor rejection. Also, generation of highly active T lymphocytes with specific reactivity against tumor antigen is critical for effective AIT (18). Although lymphokine-activated killer cells (LAK) and monocytes/macrophages can be used as AIT effectors cells, T cells have been identified as the most potent effectors of the adaptive antitumor immune response (12). Specifically, CD8 cytotoxic T lymphocytes are the primary effector cells used in adoptive T cell therapy.

There are various potential sources of T cells from the tumor-bearing host, including peripheral blood, tumor biopsies, lymph nodes and spleen. Obtaining T cells from the peripheral blood may be easier but higher frequency of tumor reactive T cells can be found in tumor biopsies (tumor-infiltrating lymphocytes) (6). However, harvesting sufficient amount of tumor-infiltrating lymphocytes can be difficult, though, this approach has been used successfully in patients with melanoma (18). Furthermore, tumor-infiltrating lymphocytes may require co-administration of high-dose interleukin-2 in generating antitumor responses in vivo, which may be harmful to the patients. The spleen can also be

a source of lymphocytes though the immunizing tumor cells need to be strongly immunogenic in order to activate the spleen cells. Based on our lab's previous findings, the lymph nodes draining the primary tumor site have been a good source of tumor reactive T lymphocytes. We have found that the popliteal lymph nodes contain T lymphocytes once they are activated by the tumor cells injected into a footpad. Adoptive transfer of these effector cells after further activation and expansion in vitro into the tumor-bearing host can be delivered by intravenous (IV) (12).

For adoptively transferred T cells to proliferate and maintain effector functions in vivo, the host environment must be also prepared to optimize the AIT. One of the ways to precondition a favorable host environment is by lymphodepletion. According to Gattitoni et al., lymphopenic environment enables tumor-reactive T cells to destroy large tumors more effectively (13). Their finding is based on the long observation in mouse models that showed depletion of immune cells before AIT markedly improved the antitumor efficacy of transferred CD8 T cells. Although removing the host immune system for the efficacy of AIT seems counter-intuitive, there are several mechanisms that may explain the augmented efficacy. These factors include the elimination of host immunosuppressive cells such as CD4+ CD25+ regulatory T cells (Tregs), the depletion of endogenous cells competing for activating cytokines, and availability of antigen-presenting cells (APCs) (13).

Host immunosuppression in tumor-bearing hosts can be caused by several factors. Tregs are important for the maintenance of peripheral self-tolerance but suppress antitumor responses. Tregs represent about 5 to 10% of the CD4 T cell population and constitutively express CD25 (also known as IL2-Rα). They are also characterized by expression of the

transcription factor forkhead box P3 (Foxp3). Although Tregs control the key aspect of tolerance to self-antigens, tumor-associated antigens also derived from the self-antigen are masked by the Tregs and avoid the host immune system leading to detrimental immunosuppression (19). Other immune cells, including natural killer (NK) cells, natural killer T (NKT) cells, and most importantly myeloid-derived suppressor cells (MDSCs) have been demonstrated to suppress T cell functions. Although less is revealed about the immunosuppressive effects of NK and NKT cells, more is known about MDSCs. This heterogeneous cell population is composed of myeloid-derived lineage cells at various stages of differentiation, including monocytes, granulocytes, and dendritic cells. Increased frequencies of MDSCs have been reported with tumor progression and T cell dysfunction (13).

Furthermore, adoptively transferred antigen-specific T cells represent a small portion of the total T cell population residing in the host. Competition exists between transferred and irrelevant host T cells for a limited amount of resources such as cytokines and antigens required to support transferred CD8 T cell homeostasis for AIT. Such competition is known as the “cytokine sink” effect. Lymphodepletion can overcome cytokine sink effects and also decreases the competition at the surface of antigen-bearing antigen-presenting cells (APCs). Although lymphodepletion can reduce the number of APCs in vivo, it has been also linked to increasing the number of mature activated APCs which can facilitate the activation of transferred tumor-reactive T cells in vivo (13).

Host preconditioning by lymphodepletion can be achieved by several mechanisms including total body irradiation, genetic alteration, and systematic chemotherapy. Our lab

has administered the cytotoxic chemotherapy agent, cyclophosphamide (CYP), to precondition the host environment for AIT. Cyclophosphamide is a synthetic antineoplastic drug and an alkylating agent that interferes with the growth of rapidly proliferating cells. One possible mechanism of action is by cross-linking of tumor cell DNA (20). Cyclophosphamide has also been shown to remove tumor-induced suppressor T cells. Lutisak et al. demonstrated the mechanisms by which CYP can both decrease the number of Tregs and inhibit the suppressive capability of Tregs. Their results showed that low-dose CYP administration decreases expressions of GITR and Foxp3 which are important in Treg function while enhancing apoptotic events and suppressing homeostatic proliferation of Tregs (19).

1.4 Cytokines: Supplement to AIT

Successful adoptive immunotherapy requires not only a transfer of tumor-reactive T cells into the tumor bearing host, but also in vitro expansion of cancer-reactive T cells (6). Administration of cytokines has been known to amplify the AIT effect (12). Especially, the cytokines that use the receptors of the common cytokine-receptor γ-chain family (γc cytokines) are important for regulating T cell responses and stimulating T cell growth (21). Among the γc cytokines, interleukin-2 (IL-2), IL-7, and IL-15 are the critical cytokines for regulating lymphoid homeostasis. These cytokines can stimulate T cell activation, survival and proliferation in vitro. The common γc component in the receptors for these cytokines stimulates intracellular MAP kinase and PI3 kinase pathways that result in mitogenic and antiapoptotic signals (22).

Activated CD4 and CD8 T cells produce IL-2 in response to T cell receptor ligation which binds to the heterotrimeric receptors that include IL-2Rα, IL-2/15Rβ, and γc (12, 22). IL-2Rα recognizes IL-2 specifically with low affinity and has a short cytoplasmic domain. IL-2/15Rβ, as the name indicates, is a receptor chain that is shared both by the IL-2 and IL-15 receptor. Once activated, it stimulates JAK3-, STAT5-, and AKT-dependent signaling pathways which are involved in cellular survival and proliferation. Studies with gene-targeted knockout phenotypes revealed IL-2 is critical in supporting T cell activation, and to a lesser degree, macrophages, natural killer cells, and B cells (12).

IL-15 is a both T cell and natural killer cell stimulatory factor (23). IL-15 and IL-15Rα are expressed in thymus and support the intrathymic differentiation of T cells. The IL-15 receptor resembles the IL-2 receptor in that it is also a heterotrimeric receptor with IL-15Rα chain instead of the IL-2Rα chain and it binds to IL-15 with a high affinity (22). Although both IL-2 and IL-15 are similar in structure and function, they do have some contrasting functions. IL-2 can promote activation-induced T cell death while IL-15 inhibits cellular death (23). Moreover, IL-15 enhances the proliferative capacity of memory T cell subsets (24). Memory T lymphocytes are a dynamic repository of antigen-sensitized T lymphocytes that accumulate overtime. They provide immediate protection in peripheral tissues and increase recall responses to antigens in secondary lymphoid organs (25). Schluns et al. demonstrated that IL-15 and IL-15Rα are required to generate memory phenotype CD8 T cells in unimmunized mice and tumor-antigen specific memory CD8 T cells (21).

While IL-15 signals are important in supporting memory CD8 T cell proliferation, the survival of the memory T cell population is supported by another common γc chain cytokine, IL-7. IL-7 contributes to the survival and proliferation of thymocytes during early stages of T cell development (23). The IL-7 receptor is a heterodimer with an IL-7α component which binds specifically to IL-7. Animals with deficient IL-7 or IL-7Rα demonstrated severe defects in T cell differentiation and deficiencies of both B and T lymphocytes indicating the importance of IL-7 in T cell development. IL-7 may also promote thymocyte survival by upregulating antiapoptotic thymocyte Bcl-2 expression (22). Furthermore, in vitro administration of IL-7 has been shown to enhance proliferation and survival of mature T cells, while exogenous IL-7 in vivo administration increased the numbers of CD4 and CD8 T cells (23).

CHAPTER 2 Phenotyping different stages of T cell differentiation stimulated by both pharmacological agents and alternate gamma-chain cytokine family

2.1 Introduction

Successful adoptive immunotherapy for cancer relies on the infusion of in vitro expanded, tumor-reactive lymphocytes with the goal of generating protective tumor immunity (6). Among the lymphocytes, CD8 cytotoxic T cells have been demonstrated as potent mediators of antitumor immunity (13). In order to activate and grow tumor-specific cytotoxic T lymphocytes, these lymphocytes must be periodically stimulated with autologous tumor antigen. Tumor cells can be used as stimulators but they may secrete immunosuppressive factors and are not clinically practical, since large amount of tumor cells are required. Thus, our lab has developed a protocol to activate selectively the tumor-reactive T lymphocytes in vitro using two pharmacologic agents, bryostatin-1 and ionomycin. When T cells from the tumor-sensitized murine draining lymph nodes were activated with bryostatin-1 and ionomycin along with low-dose IL-2, not only did the lymphocytes expand better in vitro but also demonstrated antitumor efficacy in vivo (26, 27).

Exposure to both bryostatin-1 and ionomycin mimics an important T cell activation pathway (28). Following the ligation of tumor antigen with the T cell receptor, various proximal signaling complexes lead to activation of phospholipase C (PLC) which includes Ca^{2+} and DAG-induced responses. Phospholipase C is phosphorylated and activated by SLP-76, Vav 1, and LAT after T cell receptor ligation. Activated PLC then hydrolyzes the membrane lipid PI (4,5)P_2 producing the second messengers inositol triphosphate (IP_3) and diacylglycerol (DAG). The second messenger IP_3 stimulates Ca^{2+} permeable ion channel receptors (IP_3R) on the endoplasmic reticulum (ER) membrane, leading to the release of ER Ca^{2+} stores into the cytoplasm. T cell receptor induced production of DAG results in the activation of two pathways involving Ras and protein kinase C (PKC). PKC then phosphorylates numerous protein substrates downstream which activate genes involved in the function, survival, activation and homeostasis of T cells (29).

Brostatin-1 belongs to a class of macrocyclic lactones and is derived from marine bryozoans, *Bugula neritina*. It binds to the regulatory domains of protein kinase C (PKC) and regulates the pathway by both activation and inhibition. Short-term treatment of bryostatin-1 activates PKC while a long-term exposure results in PKC inhibition (30). It also has many immunomodulating properties including stimulation of IL-2 production and interferon-gamma in human peripheral blood lymphocytes, induction of IL-2 receptor on human T lymphocytes and inhibition of natural killer and lymphokine-activated killer (LAK) cells (31). However, it does not have tumor promoting properties that some other PKC activators (phorbol esters) demonstrate. Ionomycin is a calcium ionophore which

increases the intracellular Ca^{2+} concentration which are universal second messengers in eukaryotic cells (29, 33).

In addition to the pharmacologic agents, cytokines are also known to regulate T lymphocyte differentiation, survival and proliferation (23). Cytokines also play an important role in the differentiation of memory T cells. Memory is the hallmark of the adaptive immune system and is the ultimate goal of a protective immune response. There are four main events linked to the generation of memory T cells. These events include: initiation, clonal expansion, contraction and memory generation (21). Once the naïve T cells complete their maturation in the thymus, mature T cells circulate through the bloodstream and peripheral (secondary) lymphoid organs. Before activation, these naïve T cells maintain a low metabolic rate and divide only occasionally (homeostatic proliferation). T cells are activated when their receptors recognize a foreign antigen bound to MHC molecules and by interacting with the costimulatory receptor, which initiates a cascade of transcriptional and translational signals that allows for clonal expansion and acquisition of effector functions. After a vigorous clonal expansion phase, the great majority of effectors cells are eliminated by apoptosis during the contraction phase. A subpopulation of T cells remaining persists as memory T cells (33).

While IL-2 is critical in promoting effector T cell survival, T cell activation, and to a lesser degree, macrophages, natural killer cells, and B cells, IL-2 also enhances activation-induced cell death (AICD) of lymphocytes as a mechanism of self-tolerance (12, 34). Although death of lymphocytes is an important homeostatic mechanism in the immune system and maintenance of peripheral tolerance to self antigens, AICD of

lymphocytes is disadvantageous in adoptive immunotherapy. Moreover, IL-2 is essential for regulatory T cells (Tregs) differentiation which may also hinder the effectiveness of adoptive immunotherapy. Naturally occurring Tregs (CD4+ CD25+ T cell population with constitutively expressed transcription factor forkhead box P3 or Foxp3) are important in controlling immunological tolerance to self-antigens but tumor-associated antigens can also be expressed as self which can enhance tumor protection (35).

Thus, other cytokines from the common cytokine receptor γ-chain family, namely IL-7 and IL-15, were explored as the alternative cytokine supplements. Both IL-7 and IL-15 are essential regulators of T cell survival (33). Also, clear roles for IL-7 and IL-15 have been described in the maintenance of memory CD8 T cells. The memory CD8 T cell pool consists of effector memory cells that reside in peripheral tissues and central memory T cells located in lymphoid organs. Effector *memory* CD8 cells resemble phenotypic and functional similarities to effector cytotoxic CD8 T cells but persist even after antigen clearance. Central memory CD8 T cells are distinct from naïve, effector, and effector memory CD8 T cells. Characteristic phenotype properties of central memory cells include the expressions of $CD44^{high}$, $CD62L^{high}$, IL-2Rβ+, $Ly\text{-}6C^{high}$, and CCR7+. These markers are downregulated on effector memory T cells (36).

Physiologically, IL-7 is produced by a variety of stromal cells, keratinocytes, dendritic cells, neurons, and endothelial cells. IL-7 is critical for lymphopoiesis, T cell homeostasis, maintenance, and it also promotes T cell cytolytic responses. IL-7 is also an attractive cytokine agent for restoration of T cell activities in tumor-bearing hosts due to high level of the IL-7 receptor (CD127) expression on naïve T cells (37). While IL-7

receptors are found on naïve T cells, they are first downregulated after activation but then re-expressed on a subset of activated CD8 T cells that progress to become memory T cells (33). Memory T cells have an increased ability to survive in vivo when compared with naïve T cells. As IL-7 is a survival factor and memory CD8 T cells express high levels of IL-7Rα, so IL-7 may transmit the same survival signals to memory CD8 T cells as it does to naïve CD8 T cells (22).

Although IL-15 was originally identified as a cytokine with IL-2-like activity due to its common β- and γ-chain receptors, there are numerous data that support the idea that, unlike IL-2, IL-15 signals are important in maintenance of memory CD8 T cells (38). In fact IL-15 is a more potent proliferative agent for memory CD8 T cells than IL-2. Furthermore, IL-15 and IL-15Rα are required to maintain normal numbers of antigen-specific memory CD8 T cells. Tan et al., using a cytokine knockout animal model, showed that in the absence of both IL-7 and IL-15, homeostatic proliferation of memory CD8 T cells completely failed.

Our lab has previously shown that bryostatin-1 and ionomycin (B/I) selectively activate tumor-reactive T cells with low expression of the lymph node homing receptor, L-selectin ($CD62L^{low}$), considered a marker for active effector cells. These $CD62L^{low}$ lymphocytes demonstrated higher anti-tumor activity both in vitro and in vivo. However, these lymphocytes were cultured only in IL-2 (28). In a more recent report, we combined the effects of IL-7 and IL-15 on tumor DLN lymphocytes also activated in B/I. Compared to the cells grown in IL-2 alone, these IL-7/15 cells demonstrated greater proliferation and survival in the B16 melanoma model. Also IL-7/15 cells yielded higher in vitro interferon-

gamma release and higher number of CD8 T cells with a central memory phenotype. However, in vivo adoptively transferred IL-7/15 cells were only slightly more effective against B16 lung metastases when compared to IL-2 cultured cells (39). However, in our unpublished report, we have used the 4T1 mammary carcinoma model to show significantly greater anti-tumor responses of IL-7/15 grown cells when used for AIT even at lower doses (20 and 40 million cells) (see appendix B). 4T1 is a poorly immunogenic, highly aggressive, metastasizing cancer. Unlike treating i.v. infusion-induced pulmonary metastases of B16 melanoma model, the 4T1 model aims to treat subcutaneous solid tumors and spontaneous metastases. Based on these findings, using the less immunogenic 4T1 mammary carcinoma model, we aimed to investigate the proliferation and phenotypes of the tumor DLN lymphocytes activated in B/I and expanded in IL-7/15 compared to culture in IL-2.

2.2 Materials and Methods

Mice

Virus-free BALB/c mice (National Cancer Institute) were used between 8 and 12 weeks of age. Animals were caged in groups of 6 or fewer and provided food and water *ad libitum*. All guidelines at Virginia Commonwealth University, which conform to the American Association for Accreditation of Laboratory Animal Care and the U.S. Department of Agriculture recommendations for the care and humane experimental use of animals, were followed.

Tumor Cell Line

4T1 mammary tumor cells were kindly provided by Dr. Jane Tsai at the Michigan Cancer Foundation, Detroit, Michigan. Cells were maintained in complete Dulbecco's Modified Essential Medium (DMEM) with 10% heat-inactivated fetal calf serum (Hyclone, Logan,UT), 1 mM sodium pyruvate (Mediatech, Inc, Herndon, VA), 100 U/ml penicillin (Invitrogen, Grand Island, NY), 100 mg/ml streptomycin, 0.075% sodium bicarbonate, and 10mM HEPES buffer. All cells were incubated in 250 ml T-flasks (PGC, Gaithersburg, MD) at 37 °C in humidified air with 5% CO_2. Tumor cells were harvested for inoculation of mice with 0.05% trypsin–EDTA (Invitrogen), washed twice with 1× PBS and resuspended in 1× PBS.

Draining Lymph Node (DLN) Sensitization and Harvesting DLN Cells

BALB/c mice were inoculated in one hind footpad with 1 x 10^6 viable 4T1 mammary tumor cells. Ten days after sensitization, mice were euthanized by CO_2 inhalation and ipsilateral popliteal lymph nodes were harvested into complete RPMI under sterile conditions. Then the lymph node cells were crushed through a metal screen and single cell suspensions were prepared in complete RPMI.

In Vitro Pharmacologic (Bryostatin and Ionomycin) Activation of Lymphocytes

The single cell suspensions of DLN were washed with RPMI and resuspended in RPMI at 1 x 10^6 cells/ml. DLN cells were incubated for 18 h with 5nM bryostatin 1 (kindly provided by the National Cancer Institute, Bethesda, MD), 1mM ionomycin (Calbiochem, San Diego, CA), and 80U/ml of rIL-2 (Chiron, Emeryville, CA) at 37 °C in humidified air with 5% CO_2.

Expansion of Lymphocytes in Culture

Bryostatin-1/ionomycin-activated DLN cells were washed three times with complete warm RPMI and resuspended at 1 x 10^6 cells/ml. DLN cells were then expanded in complete RPMI supplemented with an additional 5% heat-inactivated fetal calf serum with either 40U/ml of rIL-2 or 10ng/ml each of IL-7 and IL-15 (Peprotech Inc, Rocky Hill, NJ). The cells were allowed to proliferate in culture for an additional 7-9 days and were split every 2-3 days with fresh cytokines in order to maintain 1x10^6 cells/ml concentration.

CD8 T Cell Phenotype Assay and Treg Staining by Flow Cytometry

DLN cells were washed with flow buffer (2% fetal bovine serum and phosphate buffered saline of pH7.4) and brought up to 1 x 10^6 cells/ml in 100µl. Cells were first treated with anti-mouse CD16/CD32 (Mouse BD Fc Block) (BD Pharmingen, San Diego, CA). Cells were stained for 30 minutes for surface markers: anti-mouse PE-conjugated anti-CD62L-mAb (Biolegend, San Diego, CA); anti-mouse FITC-conjugated anti-CD44-mAb and anti-CD25-mAb (Biolegend, San Diego, CA); and anti-mouse PE/CY5-conjugated anti-CD8-mAb and anti-CD4-mAb (Biolegend, San Diego, CA). Unstained cells were used as the negative control. PE-conjugated rat IgG_{2a},λ was used as the isotype control for CD62L and Foxp3 (Biolegend, San Diego, CA). FITC-conjugated rat IgM was used as the isotype control for CD44 and CD25. Secondary intracellular staining for Foxp3 was carried out after fixation and permeabilization using a PE-conjugated anti-mouse FoxP3 (FJK-16s) staining set (eBioscience, SanDiego, USA). PE/CY5-conjugated rat IgG_{2b},κ was used the isotype control for CD8 and CD4 (Biolegend, San Diego, CA). The freshly stained cells were analyzed on an ELITE Beckman Coulter flow cytometer. 50,000 viable cells per sample were analyzed.

Cytokine Release Assay

DLN cells that have been activated with B/I cultured in either IL-2 or IL-7/15 were assayed for IFN-γ secretion in response to specific antigen. The lymphocytes were cultured in 24-well plates at $2x10^6$ cells/ml with either no stimulants (negative control) or with a stimulator (irradiated 4T1 mammary tumor cells). Lymphocyte to stimulator ratio was 10:1. After 24 hours of culture, supernatant was harvested and stored at -20°C until assayed using a mouse IFN-γ ELISA kit (BD Biosciences Pharmingen, San Diego, CA). Results reported are the mean values of duplicate ELISA wells ± SD.

2.3 Results

DLN cells grown in IL-7/15 expand better in vitro than in IL-2

Wildtype BALB/c mice were inoculated in one hind footpad with viable 4T1 mammary tumor cells to sensitize the lymph nodes. Ten days after inoculation, mice were euthanized by CO_2 inhalation and ipsilateral popliteal lymph nodes were harvested. Then the lymph node cells were activated with B/I regimen and were grown in vitro either in IL-2 or IL-7/15 cytokines for 10 days. As indicated in Figure 1, IL-7/15 cells expand to 12 fold increase by day 3 and IL-2 cells have expanded to 11.5 fold. By day 6, the fold increase difference between IL-7/15 and IL-2 was striking (40 fold for IL-7/15 and 11.4 fold for IL-2). IL-7/15 cells continued to have higher number of viable cells extending to day 10 (68 fold for IL-7/15 and 18.9 fold for IL-2).

Phenotypic polarization of cells cultured in IL-7/15 vs. IL2

We have previously found that B/I regimen preferentially activate $CD62L^{low}$ cells, which lead to a T cell population that is functionally enriched for tumor antigen-specific clones with antitumor activity. Such adoptively transferred cells seemed to reside predominantly in the CD8 T cell subset. However, these cells had previously only been expanded in IL-2 in vitro. Thus, we investigated whether the phenotypes of IL-2 expansion were different from the IL-7/15 expanded cells. On days 2, 6, and 9 of cell expansion, samples were taken from both IL-2 and IL-7/15 groups and were stained for CD8, CD44 and CD62L. CD8 is a marker used for cytotoxic T cells. CD44 is a cell surface glycoprotein that is expressed by many cells but among CD8 T cells, its expression is upregulated after activation and remains high on memory T cells. CD62L, also known as L-selectin, acts as a homing receptor for T cells to lymph nodes. CD62L marker can also be used to distinguish subpopulations of the CD8 T memory cell as central memory CD8 T cells ($CD62L^{high}$) and effector memory CD8 T cells ($CD62L^{low}$). Figure 2 shows percentages of DLN cell by triple staining for (CD8, CD44, and CD62L) and histogram (for CD8) on days 2, 6, and 9. On day 2, when both IL-2 and IL-7/15 cells were expanding on the upward trend of the expansion curve, there is not much difference in the proportion of CD8+ cells (42.63% for IL-2 and 42.84% for IL-7/15). Percentages representing central memory (triple positive) for IL-2 and IL-7/15 were also similar (71.51% for IL-2 and 77.11% for IL-7/15). By day 9, when the expansion difference between IL-2 and IL-7/15 cultured cells is the greatest, IL-2 cells have significantly reduced central memory phenotype ($CD44^{high}/CD62L^{high}$) but the IL-7/15 group maintained the memory phenotype (24.44% for IL-2 vs. 52.61% for IL-7/15). Also, IL-7/15 cultured cells maintained a high

proportion of CD8+ T cells while IL-2 cultured CD8+ cells began to decline (43.9% for IL-2 and 59.8% for IL-7/15).

Treg staining higher in IL2 group than IL7/15

DLN cells expanded in either IL-2 or IL-7/15 were also analyzed for the presence of T regulatory cells (Tregs) on the same days. As shown in Figure 3, triple positive staining for CD4, CD25 and Foxp3 markers indicates Tregs. The histograms for each day also show viable CD4+ cells for each cytokine groups. On day 2, when the expansion difference is minimal, both the CD4+ cell population and percentages of Tregs are similar between IL-2 and IL-7/15 groups. However, as IL-7/15 cultured cells proliferate and expand more effectively, both the CD4 subset and Tregs decreased for IL-7/15 group compared to IL-2 group. Treg population declined from 15% to 2.6% of CD4+ cells on day 9. Of note, since the CD4+ population is declining as well (from 41.7% to 35.9%), this triple stained Treg population is even more reduced in IL-7/15 cultured cells compared to cells from IL-2. However, IL-2 maintained both the CD4+ subset (42.1%, 43.1% and 52.1% on days 2, 6, and 9 respectively) and the Treg population (14.2%, 7.3%, and 7.6% on days 2, 6, and 9 respectively).

Tumor specificity by IL-7/15 Group vs.IL-2 Group

DLN cells that have been sensitized by B/I regimen and cultured in either IL-2 or IL-7/15 were assayed for IFN-γ secretion in response to specific antigen. The lymphocytes were cultured in 24-well plates at $2x10^6$ cells/ml with either no stimulant (negative control) or with a stimulator (irradiated 4T1 mammary tumor cells). Lymphocyte to stimulator ratio was 10:1. Results are shown in Figure 4 and are reported as the mean values of duplicate

ELISA wells ± SD. Data shown are representative of three independent experiments. Contrary to our previous findings in B16 melanoma model, IL-2 cultured cells secreted more IFN-γ than IL-7/15 cultured cells when exposed to irradiated 4T1 mammary tumor cells. This high in vitro IFN- γ response of IL-2 group to 4T1 cells increased further by day 10 of expansion. Previous studies have shown that this IFN-γ response is tumor antigen-specific (60).

2.4 Discussion

Adoptive cancer immunotherapy relies on the infusion of tumor reactive T cells into tumor-bearing hosts. This therapy utilizes ex vivo expansion of tumor reactive T cells where stimulatory pharmacological agents and cytokines can be applied to generate large numbers of such immune effector cells (40). In the experiments described in this chapter, we found that tumor DLN cells activated in vitro with B/I and cultured in IL-7/15 alternate common γ-chain cytokines expanded better than IL-2 cultured cells. This in vitro expansion finding is consistent with our previous report on the beneficial effects of combining IL-7 and IL-15 comparative to IL-2 alone in B16 melanoma model. B16 melanoma-sensitized lymph node cells from C57B1/6 mice cultured in IL-7/15 consistently resulted in much greater expansion of viable cells than in IL-2 in more than ten separate experiments. Not only did IL-7/15 induce greater rapid proliferative activity, but it also induced prolonged growth of T cells (39).

In addition to generating a large number of ex vivo antigen specific T lymphocytes to transfer, T cells must also preserve their effector functions and homing abilities of

trafficking to lymph nodes to induce tumor regression and elimination and protection from recurrence (41). The major function of CD8 effector T cells is to kill infected cells or malignant cells expressing viral or tumor antigen. Since cytotoxicity requires direct contact with the target cell, these cytotoxic cells must be able to migrate to different tissues, especially to sites of inflammation. This capacity of lymphocytes to enter tissues from blood depends upon the ability to adhere to endothelial cells while withstanding hydrodynamic shear stresses exerted by flowing blood. Tethering and rolling mechanisms are initiated by members of the selectin family (42).

Numerous research findings have indicated that antigen-activated CD8 T cells differentiate differently in trafficking phenotypes and functions when exposed to various common receptor γ-chain family cytokines. According to Klebanoff et al., in the presence of IL-15 cytokine, the CD8 T cells differentiated into central memory cells (a subset of memory phenotype) while IL-2 stimulated cells assumed a highly differentiated, effector and effector memory CD8 T cells (43). Our CD8 phenotype staining comparison for both IL-2 and IL-7/15 showed marked differences between the two groups on day 9 when their expansion differences are most pronounced. As expected, IL-7 and IL-15 maintained $CD62L^{high}$ memory CD8 T cell population while IL-2 cultured cells lost much of CD62L expansion.

Central memory T cells ($CD62L^{high}$) are located in secondary lymphoid issue with limited migratory potential and have been proposed to show little cytolytic activity. On the other hand, effector memory cells ($CD62L^{low}$) reside in non-lymphoid tissues with promiscuous circulatory potential and exhibit cytolytic activity (21). Thus, T cells used for

current adoptive immunotherapy trials are commonly targeted for effector and effector memory CD8 T cell phenotypes and functions due to their strong lytic capacity and release of high IFN-γ (13). However, long-term immunological protection and ability to undergo self-renewal that can be manifested by memory CD8 T cell population has also attracted researchers to examine the therapeutic effects of memory T cell transfer therapy (43, 44, 45). Klebanoff et al. evaluated the same IL-2 and IL-15 cultured CD8 T cell populations as mediators of immune response to an established B16 melanoma. They found that IL-15 cultured antigen-specific cells with central memory T cell phenotype showed greater in vivo recall response compared to effector memory T cell population. This enhanced response was associated with the complete eradication of large, established B16 melanoma tumors (43, 44). We have also found significant anti-tumor responses of IL-7/15 grown cells when used for AIT in 4T1 mammary carcinoma model. Although AIT with effector phenotype IL-2 group was successful in inducing tumor regression at a maximum AIT dose of 75 million cells, it was only memory-centered IL-7/15 group that consistently showed complete elimination of 4T1 tumor when lower doses (20 and 40 million cells) of AIT was utilized (unpublished Bear lab data).

Although previously we have observed a correlation between antitumor activity in vivo and IFN-γ release in vitro, this 4T1 model study showed that tumor regression may not be completely dependent on this cytokine (28). Klebanoff et al. showed that either IL-2 or IL-15 group produced comparable, dose-dependent amounts of IFN-γ secretion (43, 44). Contrary to our previous findings in B16 melanoma model (39), IL-2 cultured cells in this report secreted more IFN-γ in vitro than IL-7/15 cultured cells when exposed to irradiated

4T1 mammary tumor cells. This finding corresponds to findings of Gattinoni et al. in which they found that CD8 T cells that acquire effector properties and exhibit increased antitumor reactivity in vitro (e.g. IFN--γ release) are actually less effective at triggering tumor regression and cures in vivo. Furthermore, unlike IL-7/15, IL-2 enhances activation-induced cell death (AICD) of lymphocytes as a mechanism of self-tolerance and is essential for regulatory T cells (Tregs) differentiation which may also hinder the effectiveness of adoptive immunotherapy (12, 34). We have found that IL-2 cultured cells maintained higher Treg populations compared to IL-7/15 group.

The result reported here, which demonstrates a different responsiveness of tumor DLN cells to IL-2 and IL-7/15 for in vitro expansion and memory phenotypes may contribute to achieving successful AIT. Our findings of IL-7/15 cultured cells effectively expanding and proliferating ex vivo after B/I treatment correlated with inducing tumor regression and complete elimination even with smaller doses of T cells needed for AIT transfer. This phenomenon may be further explored in terms of treating larger established tumors with fewer transferred cells; perhaps T memory cells grown in IL-7/15 can demonstrate faster and longer in vivo recall responses than cells grown in IL-2.

Figure 1: IL-7/15 induces greater growth of tumor DLN cells than IL-2 in vitro
BALB/c mice were inoculated in one hind footpad with 1 x 10^6 viable 4T1 mammary tumor cells. Ten days after sensitization, mice were euthanized by CO_2 inhalation and ipsilateral popliteal lymph nodes were harvested. DLN cells were incubated for 18 h with 5nM bryostatin 1, 1mM ionomycin, and 80U/ml of rIL-2 at 37 °C in humidified air with 5% CO_2. DLN cells were then expanded in complete RPMI supplemented with an additional 5% heat-inactivated fetal calf serum with either 40U/ml of rIL-2 or 10ng/ml of IL-7 and IL-15. IL-7/15 cells expand to 12 fold increase by day 3 while IL-2 cells have only expanded to 11.5 fold. By day 6, the fold increase difference between IL-7/15 and IL-2 is significant (40 fold for IL-7/15 and 11.4 fold for IL-2). IL-7/15 cells continue to have higher number of cells even to day 10 (68 fold for IL-7/15 and 18.9 fold for IL-2).

Figure 1

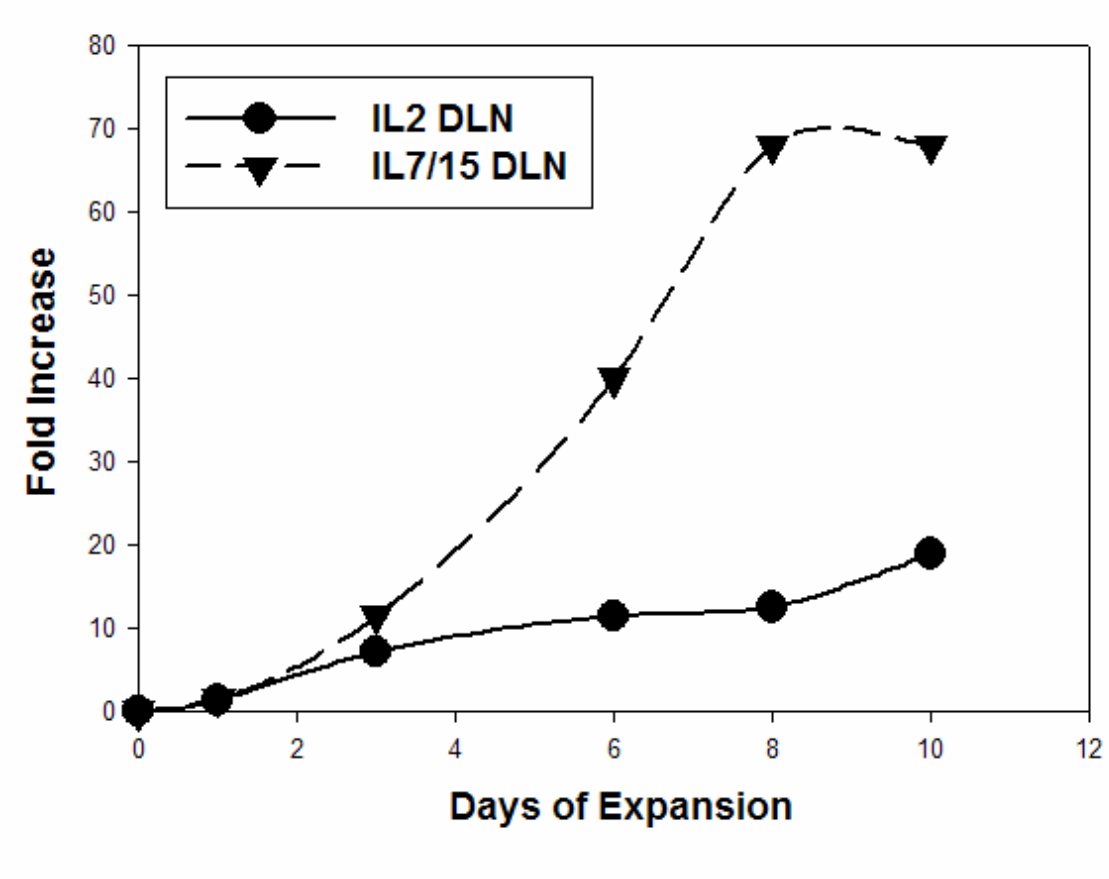
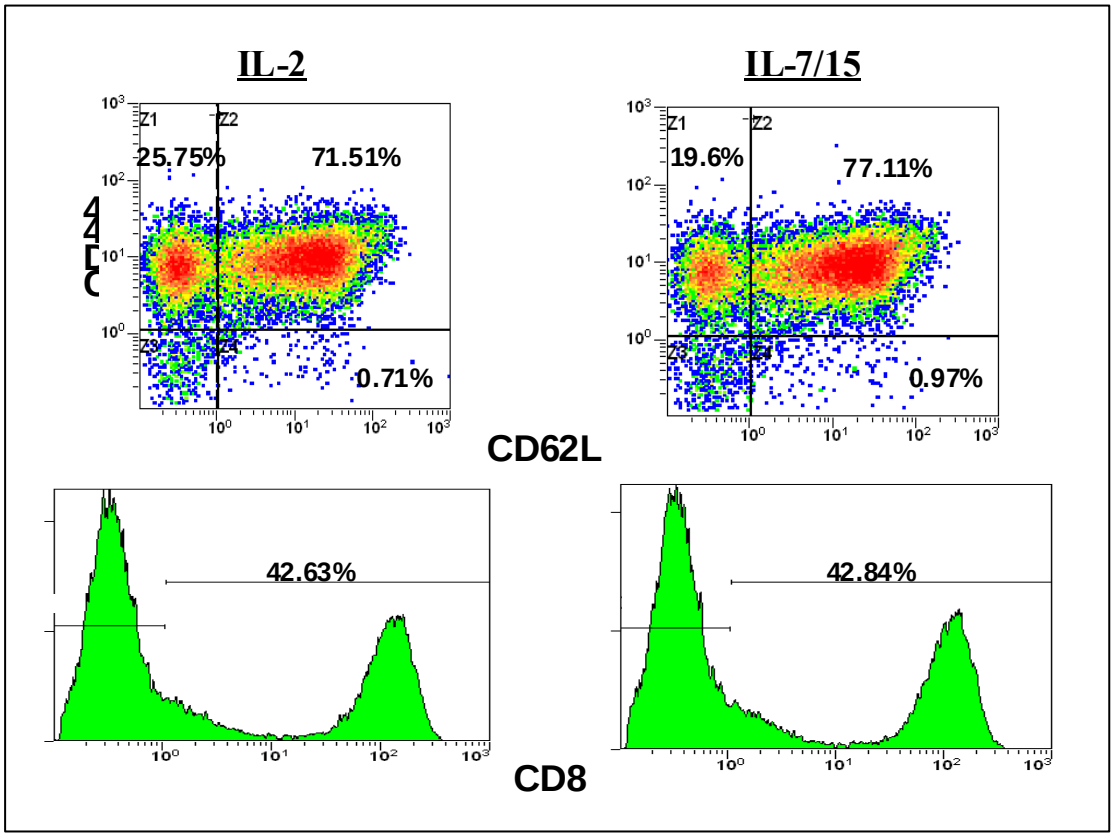

IL2 DLN
IL7/15 DLN
Fold Increase
Days of Expansion
0
10
20
30
40
50
60
70
80
0
2
4
6
8
10
12

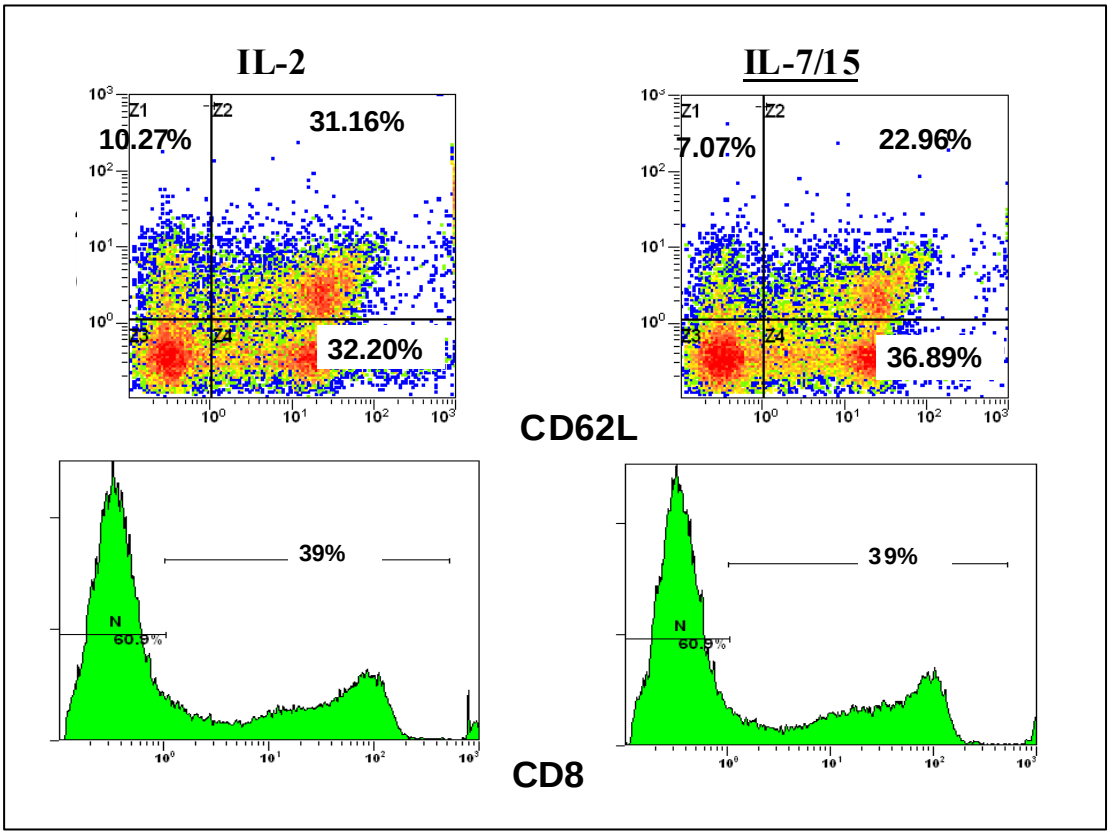
Figure 2: IL-7/15 expanded DLN cells maintain central memory CD8 T cell subsets and show higher CD8 T cells.
4T1 mammary tumor cells sensitized DLN cells were incubated for 18 h with 5nM bryostatin 1, 1mM ionomycin, and 80U/ml of rIL-2. DLN cells were then expanded in either 40U/ml of rIL-2 or 10ng/ml of IL-7 and IL-15. On days 2 (2A), 6 (2B), and 9 (2C) of cell expansion, DLN cell samples were taken for CD8 memory phenotype surface staining. Percentages of triple positive staining gated on viable CD8 memory population is indicated by CD8+ CD62L+CD44+ staining in the upper right quadrant. The lower histogram indicates the percentages for CD8+ T cell population. Fluorescence of 50,000 viable cells per sample was analyzed by flow cytometry. Data shown are representative of three independent experiments.

Figure 2A



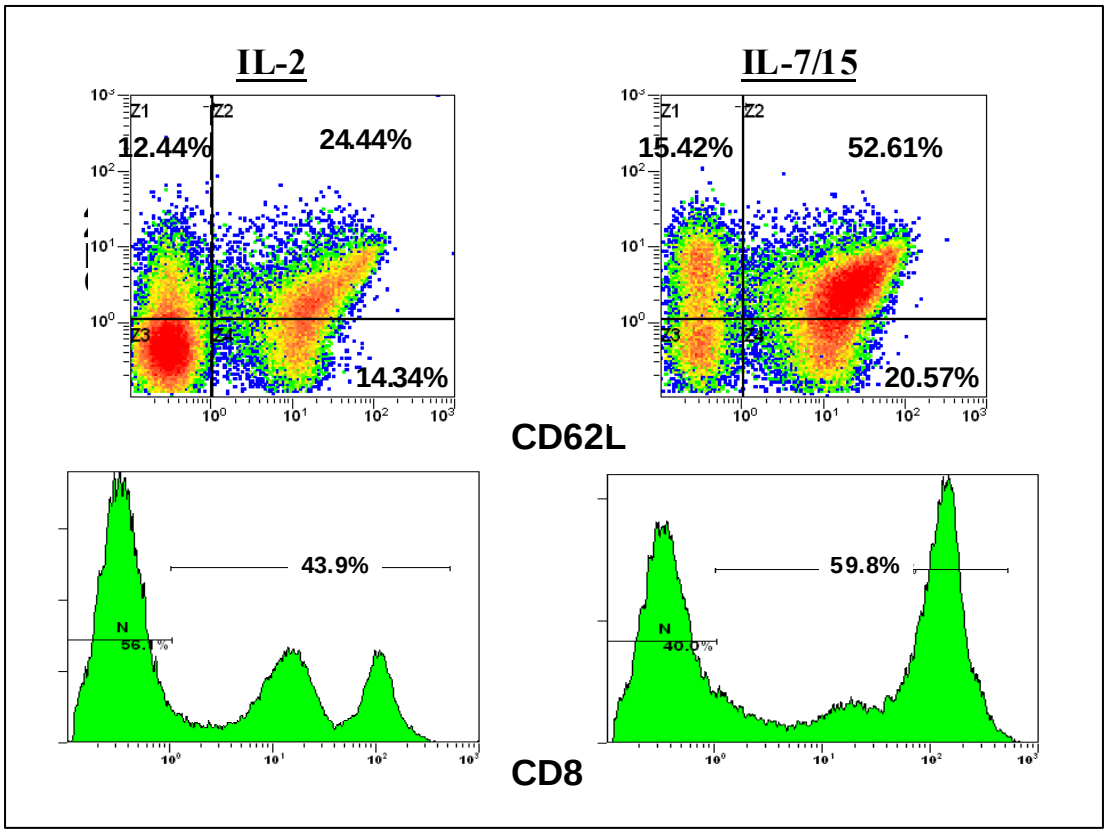
Day 2 CD8+ CD62L+ CD44+ memory phenotype staining

Figure 2B



Day 6 CD8+ CD62L+ CD44+ memory phenotype staining

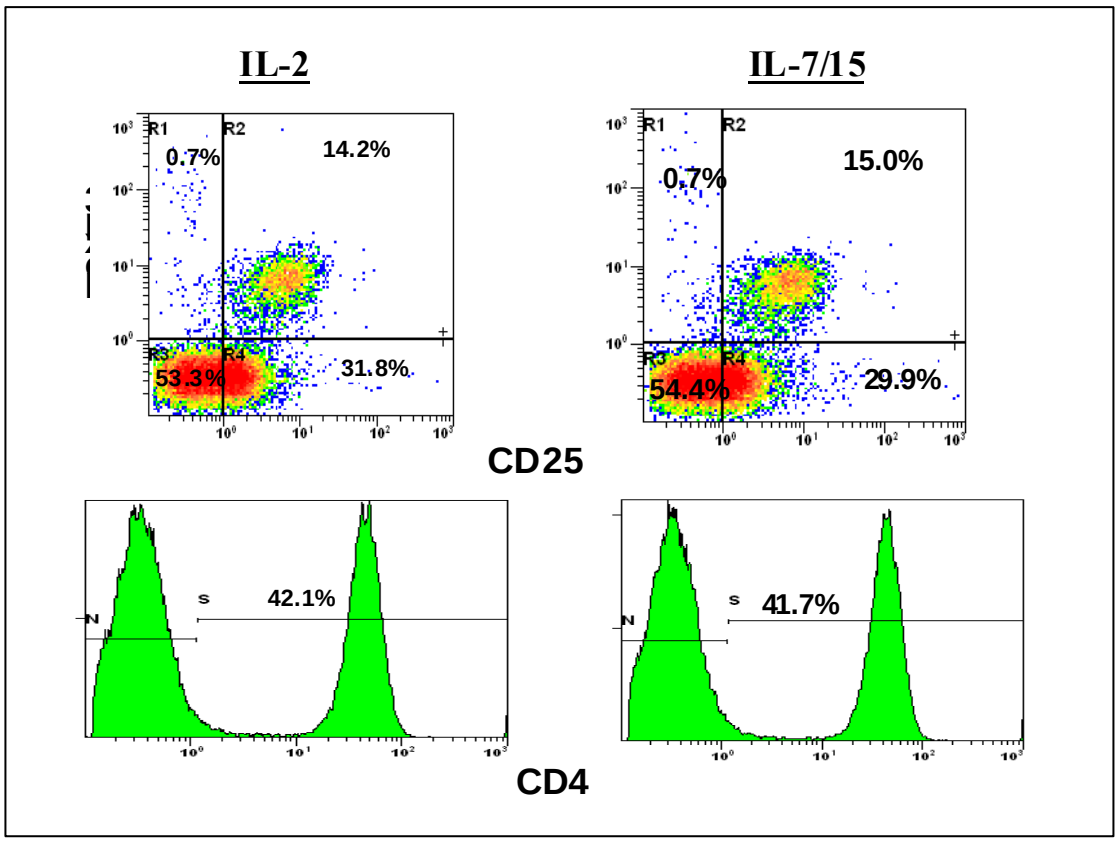
Figure 2C



Day 9 CD8+ CD62L+ CD44+ memory phenotype staining

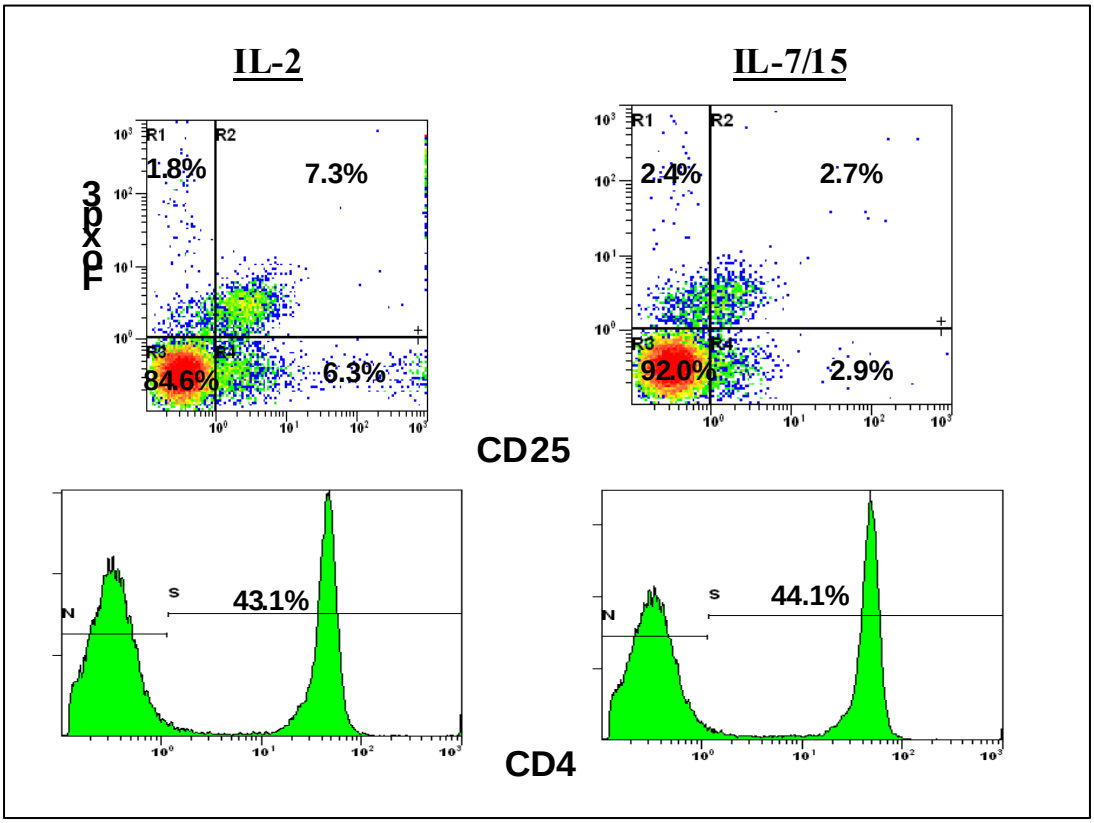
Figure 3: IL-2 maintains higher number of immunosuppressive T regulatory cells (Tregs) when compared to IL-7/15 cell group.
DLN cells expanded in either 40U/ml of rIL-2 or 10ng/ml of IL-7 and IL-15 were also analyzed for Treg (CD4+ CD25+ Foxp3+) population. On days 2 (3A), 6 (3B), and 9 (3C) of cell expansion, DLN cell samples were stained for Tregs following using a PE-conjugated anti-mouse FoxP3 (FJK-16s) staining set. Percentages of triple positive staining gated on viable CD4 cell population are shown in the upper right quadrant. The lower histogram indicates the percentages of viable CD4+ T cell population. Fluorescence of 50,000 viable cells per sample was analyzed by flow cytometry. Data shown are representative of three independent experiments.

Figure 3A



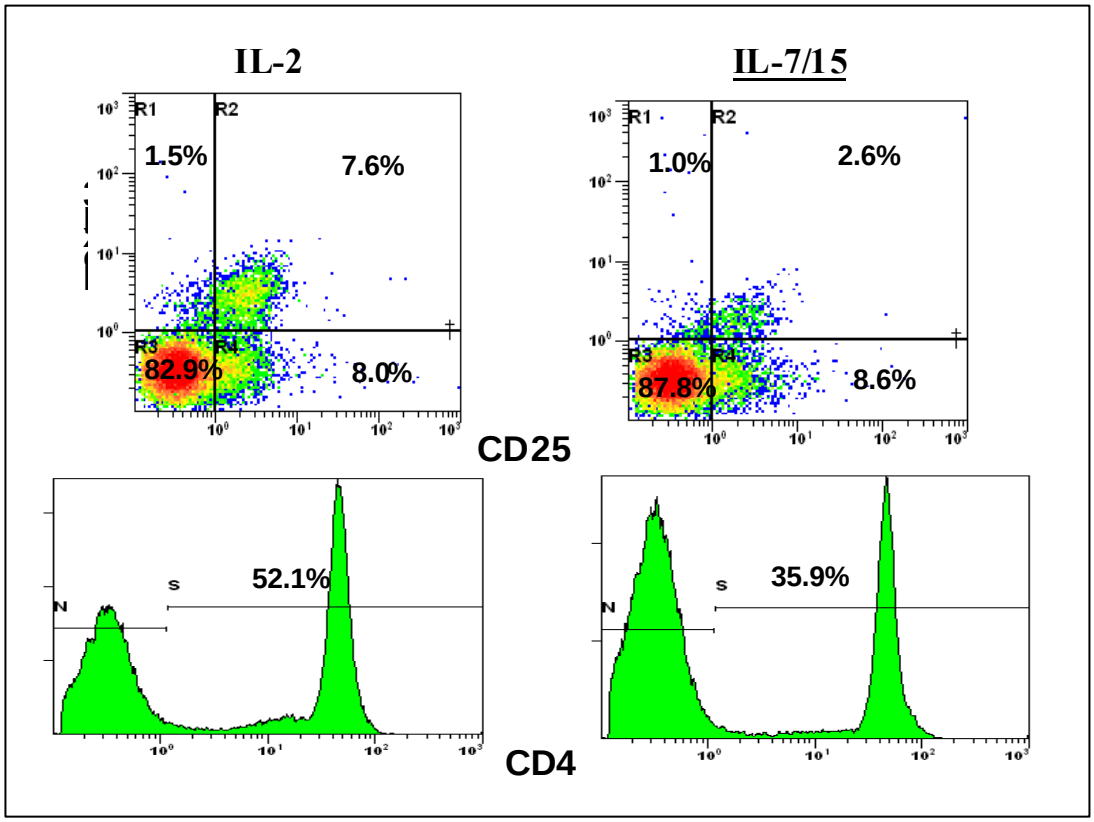
Day 2 Treg (CD4+ CD25+ Foxp3+) Triple Staining

Figure 3B



Day 6 Treg (CD4+ CD25+ Foxp3+) Triple Staining

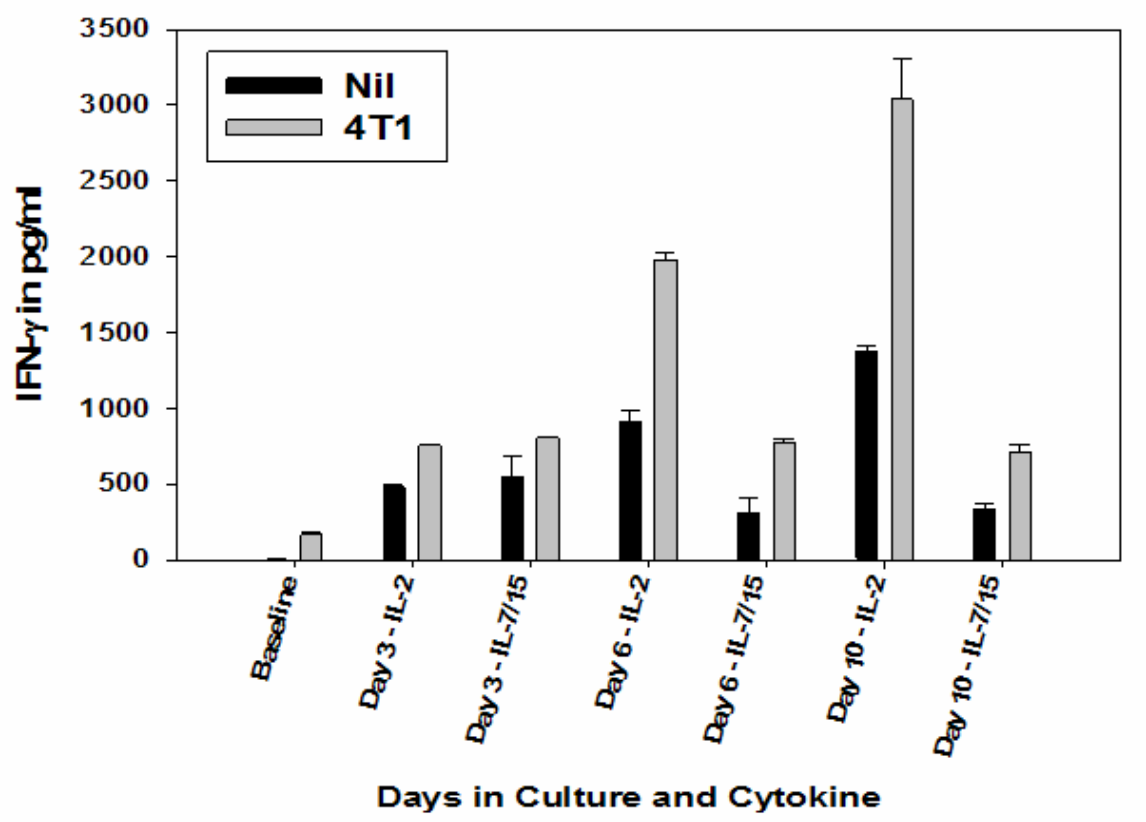
Figure 3C



Day 9 Treg (CD4+ CD25+ Foxp3+) Triple Staining

Figure 4: IL-2 expanded DLN cells show an increase in IFN-γ secretion in response to specific 4T1 antigen when compared to that of IL-7/15 group over the course of ex vivo culture.
DLN cells that have been sensitized by bryostatin 1 and ionomycin cultured in either IL-2 or IL-7/15 were assayed for IFN-γ secretion in response to specific and nonspecific antigen. The lymphocytes were cultured in 24-well plates at $2x10^6$ cells/ml with either no stimulants (negative control) or with a stimulator (irradiated 4T1 mammary tumor cells). Lymphocyte to stimulator ratio was 10:1. After 24 hours of culture, supernatant was harvested and stored at -20°C until assayed using a mouse IFN-γ ELISA kit. Results reported as the mean values of duplicate ELISA wells ± SD. Data shown are representative of three independent experiments.

Figure 4


3500
3000
2500
2000
1500
1000
500
0
IFN-γ in pg/ml
Nil
4T1
Baseline
Day 3 - IL-2
Day 3 - IL-7/15
Day 6 - IL-2
Day 6 - IL-7/15
Day 10 - IL-2
Day 10 - IL-7/15
Days in Culture and Cytokine

CHAPTER 3 Gemcitabine-Mediated Suppression of Myeloid-Derived Suppressor Cells Combined with Adoptive Immunotherapy to Treat Established Tumor

3.1 Introduction

Adoptive immunotherapy focuses on inducing and expanding cytotoxic T cells and improving the immune recognition of weak antigens expressed by tumors. However, tumor-induced immune suppression found in human patients and experimental animals with malignant tumors significantly impedes the effectiveness of immunotherapy and cancer immunosurveillance (46). There are several proposed mechanisms that may modulate tumor-induced immune suppression. Cancer-associated immune suppression can be facilitated by factors secreted by the tumor, the tumor microenvironment or by suppressive immune cells such as regulatory T cells. Among these factors, myeloid-derived suppressor cells (MDSC) are identified as a major contributor to tumor-associated immunosuppression (47).

MDSCs are a heterogeneous population of undifferentiated cells that are derived from the myeloid, macrophage, and dendritic cell lineage. This population includes granulocyte, macrophage, dendritic cell, and early myeloid progenitors. Increased levels of MDSCs are reported with numerous pathologic conditions. In animal models, frequency of

MDSCs can be found in the spleens than in the tumor microenvironment. Human MDSCs have been reported in the bone marrow and peripheral blood of cancer patients with lung, breast, and head and neck cancer (46). MDSCs are also known as “immature myeloid cells,” “inhibitory macrophages,” and “early myeloid cells.” However, immunosuppressive MDSCs have a key phenotype that can be identified by two major cell surface markers, CD11b and Ly6G (previously known as Gr-1). CD11b is a specific marker for myeloid cells of the macrophage lineage and Gr-1 is a marker for granulocytes. MDSCs express low levels of MHC class II molecules and costimulatory molecules; thus, they do not induce effective antitumor responses due to the inability to process and present antigens (48). Furthermore, Yang et al. demonstrated that these CD11b+ Gr-1+ cells can acquire endothelial cell properties in the tumor microenvironment that can contribute to tumor angiogenesis in promoting tumor growth. MDSCs also downregulate expressions of the CD3ζ chains of T cell receptors, inhibiting antigen-specific responses from activated CD4 and CD8 T lymphocytes (49). MDSCs have also been linked to triggering apoptotic death of CD8 T cells (50).

The immunosuppressive effects of MDSCs can be mediated by multiple mechanisms. MDSCs stimulate immunosuppressive cytokines, nitric oxide synthase type 2 (iNOS or NOS2) and arginase-I (ARG1) metabolism of L-arginine into reactive oxygen species (ROS) and nitric oxide (NO). The expansion of MDSCs is associated with increased levels of growth factors including vescular endothelial growth factor (VEGFs) which inhibit dendritic cell differentiation. Also, MDSC reduction of CD3ζ chains of T

cell receptors has been accompanied by an increase in ARG1 activity. Activation of ARG1 limits L-arginine availability in the tumor microenvironment and the formation of urea which can alter translation of mRNAs, further suppressing T cell functions. Additionally, high levels of iNOS and NO accompanied by MDSCs suppress IL-2 receptor signaling and increase reactive oxygen species and reactive nitrogen oxide species. These highly reactive oxidizing agents damage biological targets and contribute to immunosupression by T cell apoptosis, increased IL-2 instability, depressed T cell proliferation, and depression of CD3ζ chain expression (46). By direct contact with macrophages, MDSCs can also decrease macrophage IL-12 production while increasing IL-10 secretion (47).

Based on these immunosuppressive effects of MDSCs, elimination of MDSCs from the tumor bearing hosts has been proposed to enhance the adoptive immunotherapy. Kusmartsev et al. described a strategy of in vivo administration of all-trans-retinoic acid (ATRA) to tumor bearing animals. ATRA is a naturally occurring isomer of retinoic acid capable of inducing differentiation of a human leukemia cell line and acute promyelocytic leukemia cells. In vitro experiments with ATRA significantly reduced the presence of MDSCs in humans and mice. Combining the administration of ATRA and cancer vaccine demonstrated reduced immunosuprression and enhanced efficacy of the tumor vaccine in the animal model (52). Suzuki et al. examined the potential use of a chemotherapeutic agent, gemcitabine, in combination with immunotherapy to address the immunosuppressive effects of MDSCs. Gemcitabine HCl (GEM) is 2'-deoxy-2', 2'-difluorocytidine monohydrochloride (β-isomer) is a nucleoside analogue of cytidine that halts the DNA synthesis phase (S-phase) and blocks the progression of cells through the

G1/S-phase boundary (53). Their rationale for using GEM treatment were based on the observations of Nowak et al. that antiproliferative gemcitabine selectively preserves tumor-specific CD4 and CD8 T cells while reducing the number of B cells. This study showed that administration of GEM at a physiological dose can significantly reduce the number of MDSCs in the spleens of tumor bearing animals while maintaining CD4 T cells, CD8 T cells, natural killer cells, and macrophages. Furthermore, the antitumor activity of CD8 T cells and activated natural killer cells were enhanced (54).

In the weakly immunogenic 4T1 mammary carcinoma model, we have recently reported that GEM can directly inhibit MDSCs and indirectly reduce the number of MDSCs by its cytotoxicity to the tumor cells. Based on these findings, AIT was performed to cure 4-day old 4T1 flank tumors. The *donor* AIT spleen cells were prepared from tumor-bearing mice after in vivo GEM treatment which augmented the in vitro expansion of the splenic T cells and restored T cell function. However, the AIT treatment with these splenic T cells was ineffective against the established tumors (55). In this study, we investigate whether in vivo administration of GEM to the AIT *recipient* tumor-bearing mice has a therapeutic effect in eliminating established 4T1 mammary carcinoma. AIT T lymphocytes from the tumor-draining lymph nodes, rather than spleen cells, were prepared using the more effective IL-7/15 cytokine expansion protocol mentioned in the previous chapter.

3.2 Materials and Methods

Mice

Virus-free BALB/c mice (National Cancer Institute) were used between 8 and 12 weeks of age. Animals were caged in groups of 6 or fewer and provided food and water *ad libitum*. All guidelines at Virginia Commonwealth University, which conform to the American Association for Accreditation of Laboratory Animal Care and the U.S. Department of Agriculture recommendations for the care and humane experimental use of animals, were followed.

Tumor Cell Line

4T1 mammary tumor cells were kindly provided by Dr. Jane Tsai at the Michigan Cancer Foundation, Detroit, Michigan. Cells were maintained in complete Dulbecco's Modified Essential Medium (DMEM) with 10% heat-inactivated fetal calf serum (Hyclone, Logan,UT), 1 mM sodium pyruvate (Mediatech, Inc, Herndon, VA), 100 U/ml penicillin (Invitrogen, Grand Island, NY), 100 mg/ml streptomycin, 0.075% sodium bicarbonate, and 10mM HEPES buffer. B16F10 melanoma tumor cells were kindly donated by Dr. Rodney Prell at Cell Genesys, Inc., South San Francisco, California. All cells were incubated in 250 ml T-flasks (PGC, Gaithersburg, MD) at 37 °C in humidified air with 5% CO_2. Tumor cells were harvested for inoculation of mice with 0.05% trypsin–EDTA (Invitrogen), washed twice with 1× PBS and resuspended in 1× PBS.

Draining Lymph Node (DLN) Sensitization and Harvesting DLN Cells

BALB/c mice were inoculated in one hind footpad with 1 x 10^6 viable 4T1 mammary tumor cells. Ten days after inoculation, mice were euthanized by CO_2 inhalation and ipsilateral popliteal lymph nodes were harvested into complete RPMI under sterile

conditions. Then the lymph node cells were crushed through a metal screen and single cell suspensions were prepared in complete RPMI.

In Vitro Bryostatin and Ionomycin Activation

The single cell suspensions of DLN were washed with RPMI and resuspended in RPMI at 1 x 10^6 cells/ml. DLN cells were incubated for 18 h with 5nM bryostatin 1 (kindly provided by the National Cancer Institute, Bethesda, MD), 1mM ionomycin (Calbiochem, San Diego, CA), and 80U/ml of rIL-2 (Chiron, Emeryville, CA) at 37 °C in humidified air with 5% CO_2.

Expansion of Lymphocytes in Culture

Bryostatin-1/ionomycin-activated DLN cells were washed three times with complete warm RPMI and resuspended at 1 x 10^6 cells/ml. DLN cells were then expanded in complete RPMI supplemented with an additional 5% heat-inactivated fetal calf serum with 10ng/ml each of IL-7 and IL-15 (Peprotech Inc, Rocky Hill, NJ). The cells were allowed to proliferate in culture until peak growth and were split every 2-3 days in order to maintain 1x10^6 cells/ml concentration.

Treatment of Established Tumors (Adoptive Immunotherapy-AIT)

Recipient BALB/c mice were inoculated subcutaneously into their shaven left flanks with 1 x 10^6 4T1 mammary tumor cells in 0.05ml phosphate buffered saline of pH7.4 (PBS). These tumor bearing mice were then randomly divided into five experimental groups: 1) untreated control, 2) CYP-only treated control, 3) CYP and GEM treated, 4) CYP and AIT and 5) CYP, GEM and AIT treated group. Nine days after 4T1 flank injection, cyclophosphamide (CYP) groups received 100mg/kg of CYP

intraperitoneally (ip) on the left flank. The first administration of in vivo gemcitabine (GEM) was also given on this day at 60mg/kg intraperitoneally (ip) on the right flank. A subsequent GEM administration was given on weekly basis from this day on. Twenty-four hours later after first day administration (day 10), 75 million of IL7/15-expanded DLN cells for AIT were infused iv in 0.5ml of PBS, 10 days after flank inoculation.

Tumor Measurements

Flank tumor growth was monitored by biweekly measurements of perpendicular diameters using calipers. When the tumor area (calculated as a product of the two perpendicular measurements) was greater than $100mm^2$ or if the mouse appeared sick, the animal was euthanized by CO_2 inhalation.

Sensitization of Spleen Cells and *In Vitro* Expansion of Splenocytes

BALB/c mice were inoculated subcutaneously into their shaven left flank with 50,000 4T1 mammary cells. On the 20^{th} day, mice were treated with 60mg/kg of GEM intraperitoneally. Six to 24 h after GEM treatment, spleens were harvested in complete RPMI, weighed, and crushed through a cell strainer. Splenocytes were resuspended in 1× ammonium chloride solution to lyse red blood cells and were stained with 0.04% trypan blue to exclude dead cells. The viable spleen cell numbers were counted under a light microscope using a Neubauer type hemacytometer. Splenocytes that were expanded were first subjected to Ficoll density gradient centrifugation to isolate splenic mononuclear cells. These cells were then washed and brought to a concentration of 1×10^6 cells/ml and activated by incubation with bryostatin, ionomycin and IL2 (as above) in 50 ml conical polystyrene tubes at 37 °C, 5% CO2, for 18 h. Cells were washed three times with warm

complete RPMI and resuspended at 1×10^6 cells/ml. Splenocytes were expanded in complete RPMI supplemented with an additional 5% heat-inactivated fetal calf serum with IL-7+IL-15 (10 ng/ml each, Peprotech Inc, Rocky Hill, NJ). The cells were allowed to proliferate in culture until peak growth was reached and were split every 2–3 days to 1×10^6 cells/ml.

Staining for Myeloid Derived Suppressor Cells (MDSC)

Spleen cells were washed with flow buffer (2% fetal bovine serum and phosphate buffered saline of pH7.4) and brought up to 1 x 10^6 cells/ml in 100µl. Cells were first treated with anti-mouse CD16/CD32 (Mouse BD Fc Block) (BD Pharmingen, San Diego, CA). Then the cells were stained for 30 minutes with: anti-mouse (FITC-conjugated) CD11b and anti-mouse (PE-conjugated) Ly-6G/Ly-6C(Gr-1) (Biolegend, San Diego, CA). Unstained cells were used as a negative control. FITC-conjugated rat IgM was used as the isotype control or CD11b and PE-conjugated rat IgG_{2a},λ was used as the isotype control for Ly-6G/Ly-6C(Gr-1) (Biolegend, San Diego, CA). Staining with anti-CD11b alone or anti-Gr-1 alone was used as single color positive controls. Stained cells were analyzed (50,000 viable cells per sample) on an ELITE Beckman Coulter flow cytometer.

Separation of MDSC from Splenocytes

BALB/c mice were injected in the flank with 50,000 4T1 carcinoma cells. Twenty-one days later, splenocytes were harvested and prepared as described above. Some splenocytes were set aside as a control group while the remaining cells were sorted to positively select out the myeloid derived suppressor cells using an Easy Sep PE Selection Kit (StemCell Technologies, Vancouver, BC, Canada). Splenocytes were treated with FcR

blocking antibody (BD Biosciences Pharmingen, San Diego, CA) and PE-conjugated anti-mouse Ly-6G/Ly- 6C (Gr-1) (Biolegend, San Diego, CA). The positive MDSC cells adhered to the magnetic beads while the MDSC-depleted splenic lymphocytes were collected separately. Both the MDSC positive and depleted cell groups were counted using a Neubauer type hemacytometer, and the percentages of MDSC originally present were calculated. Using the total number of cells for the control groups as the starting point, the number of cells in the MDSC positive group and MDSC-depleted groups were adjusted by the calculated MDSC percentage to ensure that the same number of lymphocytes was plated in each well. In some groups, MDSCs removed with the magnetic beads were added back to the MDSC-depleted cells at a two-to-one splenic lymphocytesto- MDSC ratio. Then all groups of splenocytes (control group, MDSCdepleted group and MDSC-added-back group)were activated with B/I plus IL-2 (80 U/ml), and expanded with a combination of IL-7 and IL-15 (10 ng/ml).

Proliferation Assay

Splenic lymphocytes (unseparated, MDSC-depleted or with MDSC added back, as described above) were activated with B/I plus IL-2, and cultured in IL-7 and IL-15. On day 4, the activated cells were added to 96-well plates at 5×10^4 cells/well. After 2 days of culture, the cells were labeled with 1 μCi/ml of [3H] thymidine (PerkinElmer Life and Analytical Sciences, Boston, MA) and were harvested 24 h later with a semi-automated harvester (PHD: Cambridge Technology, Inc., Cambridge, MA). Thymidine uptake was determined by liquid scintillation counting and expressed as the mean cpm±SD of triplicate wells.

Statistical Analysis

Differences between two groups were analyzed with the Student's t-test. Differences among more than two groups were examined by analysis of variance (ANOVA) and Tukey–Kramer honestly significant difference test (Tukey's HSD) using JMPIN software (SAS Institute Inc., Cary, N.C.). Results of tumor growth are presented as the means±standard errors (SE) of tumor area in each treatment group. In vivo experiments included three to six mice per group. In vitro assays were repeated at least twice. A $p < 0.05$ was used throughout to determine significant differences.

3.3 Results

In vitro proliferation of splenic T lymphocytes from 4T1 tumor-bearing mice is suppressed by MDSCs

BALB/c mice were inoculated subcutaneously in the flank with 50,000 4T1 mammary carcinoma cells. Twenty-one days later, spleens were harvested and MDSCs were depleted using the magnetic beads. All experimental groups, the unseparated tumor bearing splenocytes (TB control), MDSC-depleted splenocytes (TB MDSC depleted), and splenic lymphocytes with MDSC added back in a similar proportion to what was removed (TB MDSC first depleted then MDSC added back) were pulsed for 18h with bryostatin-1 (5nM), ionomycin (1mM), and IL-2 (80U/ml) then expanded for 8 days in IL-7/15 (10ng/ml). Figure 5A shows the expansion curves for all three groups. The MDSC-depleted group viable cell number expanded 23.4 fold, and both the control groups with MDSCs showed suppression of growth. Figure 5B shows similar results of proliferation of

lymphocytes measured by thymidine incorporation. Each cell group was pulsed with ^{3}H-thymidine on day 5 and cells were harvested for beta-scintillation counting the following day.

In vivo administration of Gemcitabine reduces MDSCs in the tumor bearing host spleen

Using two-color flow cytometry, the number of CD11b+ and Gr-1+ (MDSCs) population was analyzed in mice bearing large 4T1 flank tumors. As shown in Figure 6, CD11b+ Gr-1+ cells made up 29.5% of the spleen cells in an animal bearing a large tumor (100 mm^2 in size). Gemcitabine was able to reduce this CD11b+ Gr-1+ cell population with a single dose of 60mg/kg administered shortly before spleen harvest. Even after a short period of 6h of GEM treatment, the percentage of CD11b+ Gr-1+ cells in the tumor spleens begins to decrease to 22.7%. By 12h and 24h, the MDSC population was reduced to 18.3% and 17.7 % respectively.

Gemcitabine-treated 4T1 tumor bearing host failed to reduce the late stage 4T1 flank tumor

Recipient BALB/c mice were inoculated subcutaneously with 50,000 4T1 mammary tumor cells in the flank. After 9 days, cyclophosphamide (CYP) groups received 100mg/kg of CYP intraperitoneally (ip) on the left flank. The first administration of in vivo gemcitabine (GEM) was also given on this day at a concentration of 60mg/kg. A subsequent GEM administration was given on a weekly basis from this day on. On day 10, IL7/15-expanded DLN cells were infused iv. As shown in Figure 7B, despite our previous findings of GEM's cytotoxicity in vitro to 4T1 cells and its inhibiting effects on MDSCs in vivo, rapid 4T1 tumor kinetics inhibited a complete tumor regression and therapeutic

effects of this combination chemo-immunotherapy. AIT alone carried out under the same conditions did not inhibit tumor growth.

3.4 Discussion

Tumor-induced immunosuppression is a major obstacle to successful immunotherapy and a possible mechanism of tumor escape from immunosurveillance (52, 56). Several studies have identified myeloid-derived suppressor cells (MDSC) with phenotype of CD11b+ Gr-1+ as an important contributor to tumor immune evasion (50, 54, 56, 57). A significant increase in the level of MDSC in tumor-bearing spleens and bone marrow is associated with impaired immune reactivity (48). Accumulation of MDSC in both preclinical models and in human samples has been shown to be associated with defective dendritic cell function and inhibition of antigen specific T cell responses (57). Our findings here show that the presence of MDSC suppresses in vitro proliferation of splenic T cells harvested from tumor-bearing hosts. On the other hand, depletion of MDSC restored in vitro proliferative capacity.

The mechanisms of MDSC-mediates immunosuppression are diverse. One pathway involves nitric oxide (NO) as an intercellular multivalent signaling molecule. NO is synthesized by the oxidation of L-arginine to L-citrulline and NO, a reaction catalyzed by a family of enzymes called nitric oxide synthases (NOS) and other cofactors including flavones, tetrahydrobiopterin, and Ca^{2+}. Three types of NOS are known: types 1 and 3 are constitutively expressed in neuronal tissue and endothelium. An inducible form of NOS (iNOS or type 2) is expressed in a variety of tissue and cell types, including vascular

endothelium and macrophages. NO released by MDSC works downstream of the IL-2 receptor, inhibiting the phosphorylation of STAT5, Erk, and Akt, three components of the IL-2 receptor signaling pathway. NO can also interfere with signaling of many other cytokines such as IL-1, IL-6, IL-8, IL-10, IL-12, IFN-γ, TNF-α, and TGF-β. Animal studies with NOS inhibitors and depletion of iNOS have shown NO to be as an important mediator of MDSC-mediated immunosuppression. In vitro and in vivo studies performed in $iNOS^{-/-}$ knockout mice indicated that NO was required to inhibit T cell proliferation in the presence of MDSC. Also, in the presence of superoxide, NO gives rise to peroxynitrite ($ONOO^-$). Peroxynitrite induces antigen-primed T lymphocyte apoptosis by inhibiting protein tyrosine phosphorylation via nitration of critical tyrosine residues. However, the inhibition of T cell proliferation in the presence of either splenic or bone marrow MDSC from tumor bearing mice was completely reversed when an iNOS inhibitor and a superoxide dismutase mimetic were added. Suppressive mechanisms that allow MDSC to inhibit CD8 function require cell-cell contact. This contact is not to induce T lymphocyte apoptosis but rather to trigger NO secretion by MDSC by IFN-γ (58). Furthermore, tumor-infiltrating MDSC seem to be the primary source of ARG1 activity in tumors, and treatment with ARG1 inhibitors impairs tumor formation in immunologically intact mice. Increased ARG1 activity by MDSC has been linked to reduced CD3ζ chains in T cells and diminished production of IL-2 and IFN-γ. ARG1 activity also leads to depletion of L-arginine in the local tumor environment, which induces iNOS to produce superoxide (46).

Other suppressive mechanisms that allow MDSC to inhibit T cell immunity involve IL-12 and IL-10 production. MDSC can downregulate IL-12 production by macrophages

and increase their own production of IL-10 in response to signals from macrophages. IL-12 produced by macrophages promotes tumoricidal natural killer cell activity. MDSC may minimize this NK activity by downregulating IL-12 production. MDSC release of IL-10 interferes with DC maturation and high levels of IL-10 may indirectly block DC function (47).

Thus, eliminating these myeloid immunosuppressive cells in vivo has been a goal with the potential of enhancing the antitumor activities of transferred T cells in adoptive immunotherapy. Surgical removal of the cancer has shown a short-term reduction of MDSC and restoration of antitumor immunity, though, surgical removal of most metastatic tumors is not possible. Depletion of MDSC by treating tumor-bearing mice with Gr-1 antibodies has also been linked to reduction of MDSC and reduced tumor growth. However, because of poor specificity of the Gr-1 monoclonal antibodies, mature granulocytes were also eliminated, jeopardizing the host's immune system (58). Kusmartsev et al. demonstrated both in vitro and in vivo that administration of all-trans-retinoic acid can dramatically reduce the number of MDSC in tumor-bearing animals and increased antitumor CD4 and CD8 T cell responses.

More recently, the cytotoxic chemotherapy agent, gemcitabine, has been combined with AIT to reduce MDSC and enhance antitumor activity. GEM is a commonly used agent in combination chemotherapy for the treatment of several types of cancers (mammary, bladder, lung, and pancreatic cancers). Gemcitabine HCl (GEM), 2'-deoxy-2', 2'-difluorocytidine monohydrochloride (β-isomer), is a nucleoside analogue of cytidine that halts the DNA synthesis phase (S-phase) and blocks the progression of cells through

the G1/S-phase boundary, which targets rapidly diving tumor cells (53). GEM has been reported not only to reduce MDSC but also to lower IL-10 levels, thus, restoring macrophage production of IL-12 (47). Also, GEM can induce apoptosis of tumor cells, which can generate more antigen cross presentation. In this report, we showed that even a short 6 hour GEM in vivo treatment reduces MDSC in the spleens of tumor-bearing mice. By 24 hour, MDSC were significantly reduced. Accordingly, Suzuki et al. administered a single dose of GEM (120 mg/kg) in vivo, similar to the equivalent dose used in patients and observed reduction in MDSC accompanied by increased antitumor activity of CD8 T cells and activated natural killer cells. Ko et al. also used GEM (60 mg/kg) combined with immunotherapy to treat Her2/neu induced mammary tumors and showed that this combined chemotherapy and immunotherapy induced therapeutic antitumor immunity (54, 59).

In our previous report, we found that GEM can reduce the number of CD11b+ Gr-1+ population by both its direct inhibitory effect on MDSC and indirectly as a result of its cytotoxicity to the tumor cells (55). By eliminating the tumors directly, the number of MDSC maintained by factors secreted from the tumor mass declined. Although GEM reduced MDSC and restored T cell in vitro functions of splenocytes prepared for AIT, these splenic T cells still failed to induce tumor regression when transferred to treat 4-day established 4T1 flank tumors. In this report, we aimed to treat a large 10 day established 4T1 flank tumor (>60mm^2) by treating the AIT *recipients* with gemcitabine. Moreover, we have prepared the T lymphocytes for AIT from tumor DLN cells which were stimulated in vitro with B/I and cultured in IL-7/15 for 10 days before AIT. Unlike our previous AIT

attempts with a limited number of splenic T cells, we have transferred a maximum cell dose (75 million cells) of T lymphocytes to treat large 4T1 tumors. Despite the successful AIT preparation, combined therapy of GEM and AIT failed to induce significant tumor regression of such large established tumors. Also, unlike Suzuki et al. who administered 120 mg/kg of GEM, we used a lower GEM dose (60 mg/kg) to lessen the adverse effects of the antineoplastics on the activated tumor-specific cytotoxic T cells. Thus, although GEM treatment can attenuate the MDSC-mediated tumor-suppressive environment, when treating large tumors in the future, it should be accompanied by other methods such as surgery or additional cytotoxic agents.

Figure 5. In vitro proliferation of splenic T lymphocytes from 4T1 tumor-bearing mice is suppressed by MDSCs.

BALB/c mice were inoculated subcutaneously in the flank with 50,000 4T1 mammary carcinoma cells. Twenty-one days later, spleens were harvested and MDSCs were depleted using the magnetic beads. All experimental groups, the unseparated tumor bearing splenocytes (TB control), MDSC-depleted splenocytes (TB MDSC depleted), and splenic lymphocytes with MDSC added back in a similar portion to what was removed (TB MDSC first depleted then MDSC added back) were pulsed for 18h with bryostatin-1 (5nM), ionomycin (1mM), and IL-2 (80U/ml) then expanded for 8 days in IL-7/15 (10ng/ml). Figure 5 **(A)** shows the expansion curves for all three groups. The MDSC-depleted group expanded to 23.4 fold increase in cell number while both the control groups with MDSCs showed suppression in growth. Figure 5 **(B)** shows similar results of proliferation of lymphocytes measured by thymidine incorporation. Each cell groups were pulsed with ^{3}H-thymidine on day 5 and cells were harvested for beta-scintillation counting the following day. Data shown are representative of three independent experiments.

Figure 5A

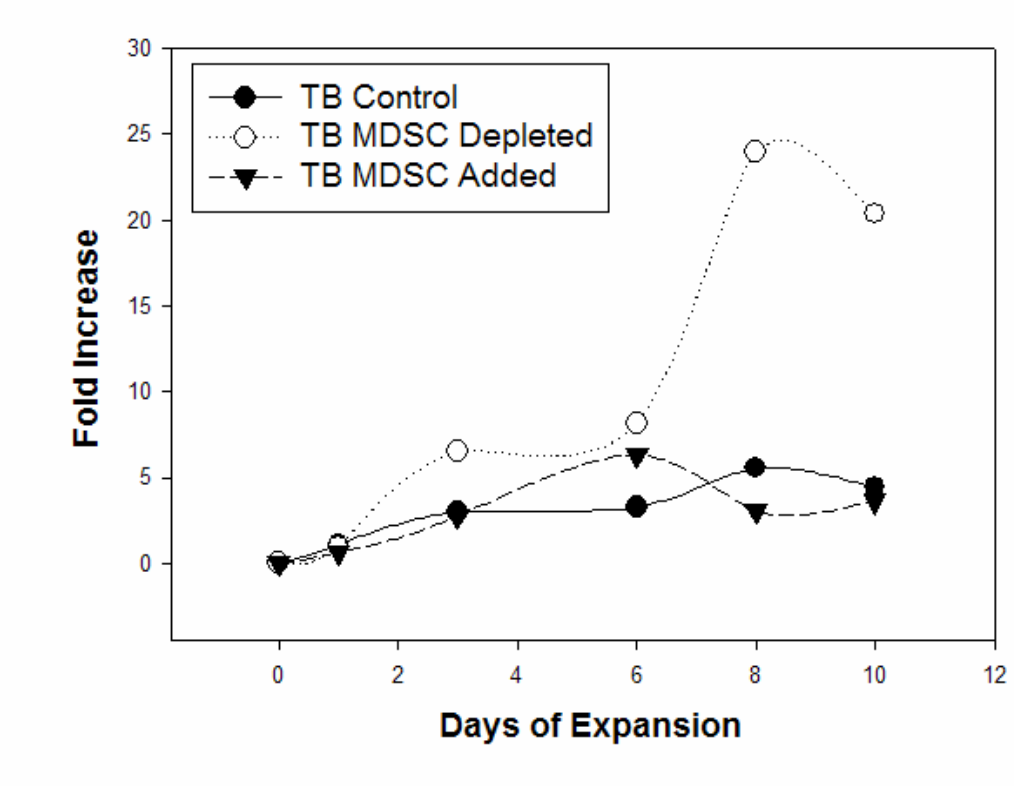

TB Control
TB MDSC Depleted
TB MDSC Added
Fold Increase
Days of Expansion
0
5
10
15
20
25
30
0
2
4
6
8
10
12

Figure 5B

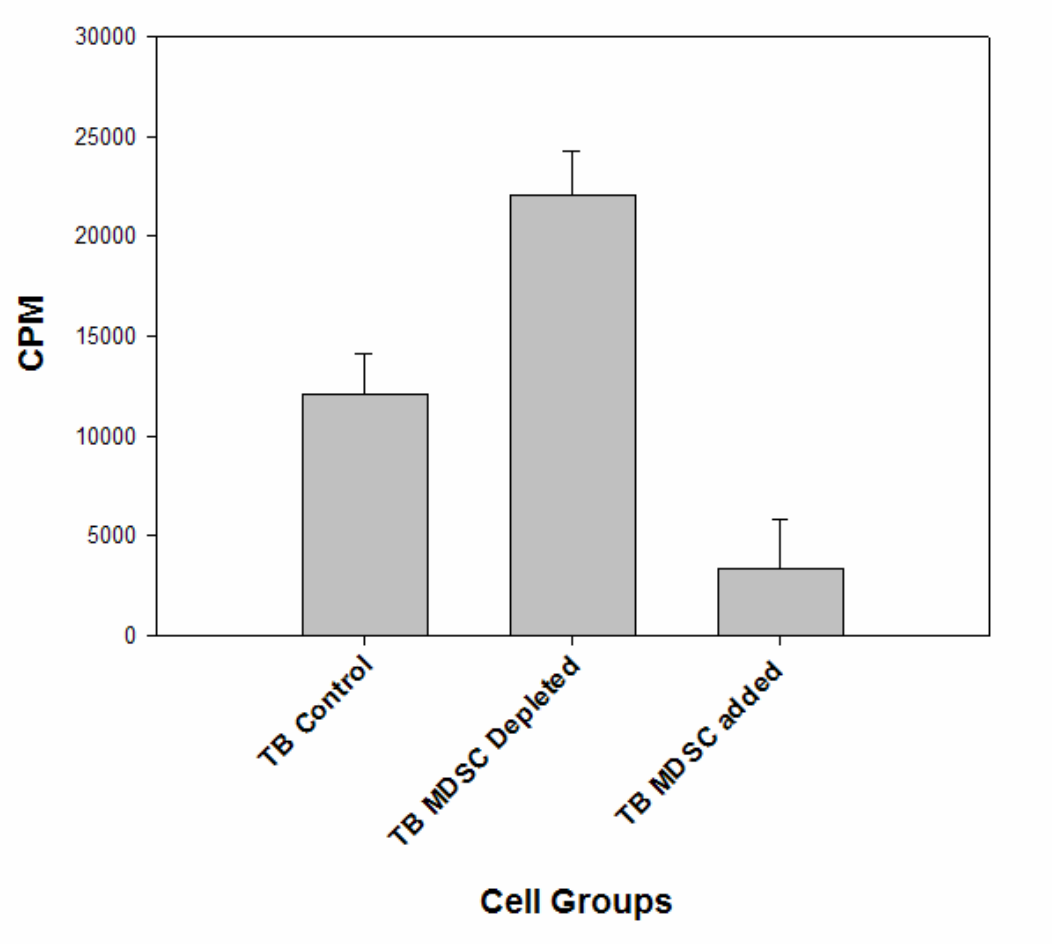

30000
25000
20000
15000
10000
5000
0
CPM
TB Control
TB MDSC Depleted
TB MDSC added
Cell Groups

Figure 6: In vivo administration of Gemcitabine reduces MDSCs in the tumor bearing host spleens

Using two-color flow cytometry, the number of CD11b+ and Gr-1+ (MDSCs) population was analyzed in mice bearing large 4T1 flank tumors. As shown in Figure 6, CD11b+ Gr-1+ cells made up 29.5% of the spleen cells in an animal bearing a large tumor (100 mm^2 in size). Gemcitabine was able to reduce this CD11b+ Gr-1+ cell population with a single dose of 60mg/kg in vivo administration. Even after a short period of 6h of GEM treatment, the percentage of CD11b+ Gr-1+ cells in the tumor spleens begins to decrease to 22.7%. By 12h and 24h, the MDSC population has been reduced to 18.3% and 17.7 % respectively. Data shown are representative of three independent experiments.

Figure 6

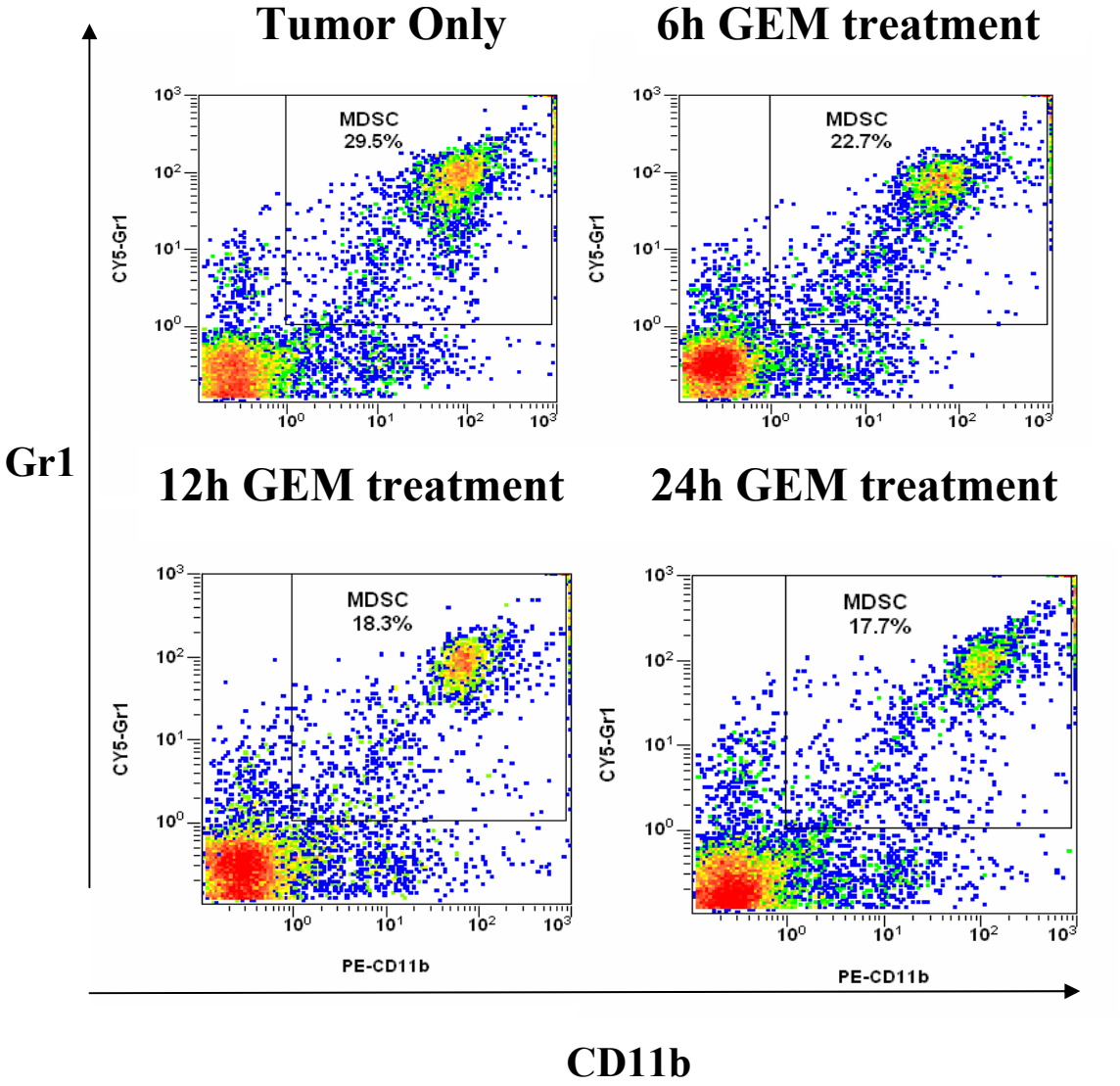

Tumor Only
6h GEM treatment
12h GEM treatment
24h GEM treatment
MDSC
29.5%
MDSC
22.7%
MDSC
18.3%
MDSC
17.7%
CY5-Gr1
PE-CD11b
Gr1
CD11b

Figure 7: Adoptive immunotherapy of 75 million IL-7/15 cultured DLN cells in combination with in vivo administration of GEM were ineffective against 10 day old subcutaneous 4T1 flank tumors.

Recipient BALB/c mice were inoculated subcutaneously into their shaven left flank with 1 x 10^6 4T1 mammary tumor cells in 0.05ml phosphate buffered saline of pH7.4 (PBS). These tumor bearing mice were then randomly divided into five experimental groups: 1) untreated control, 2) Cyclophosphamide-only treated control (CYP only), 3) Cyclophosphamide and gemcitabine treated (CYP & GEM), 4) Cyclophopshamide and AIT (CYP & AIT) and 5) CYP, GEM and AIT treated group (CYP/GEM & AIT). After 9 days of 4T1 flank injection, cyclophosphamide (CYP) groups received 100mg/kg of CYP intraperitoneally (ip) on the left flank. The first administration of in vivo gemcitabine (GEM) was also given on this day concentration of 60mg/kg on the right flank. A subsequent GEM administration was given on weekly basis from this day on. Twenty-four hours later, 75 million (max dose) of IL7/15-expanded DLN cells for AIT were infused iv in 0.5ml of PBS, 10 days after flank inoculation. **(A)** IL-7/15 cultured DLNs expanded to 65 fold by day 10 (AIT day) (B) Data shown is mean tumor area ± SE (6 mice per group). A significant difference in mean tumor area was found using ANOVA and Tukey-Kramer honestly significant difference test (Tukey's HSD) using JMPin software.

Figure 7A

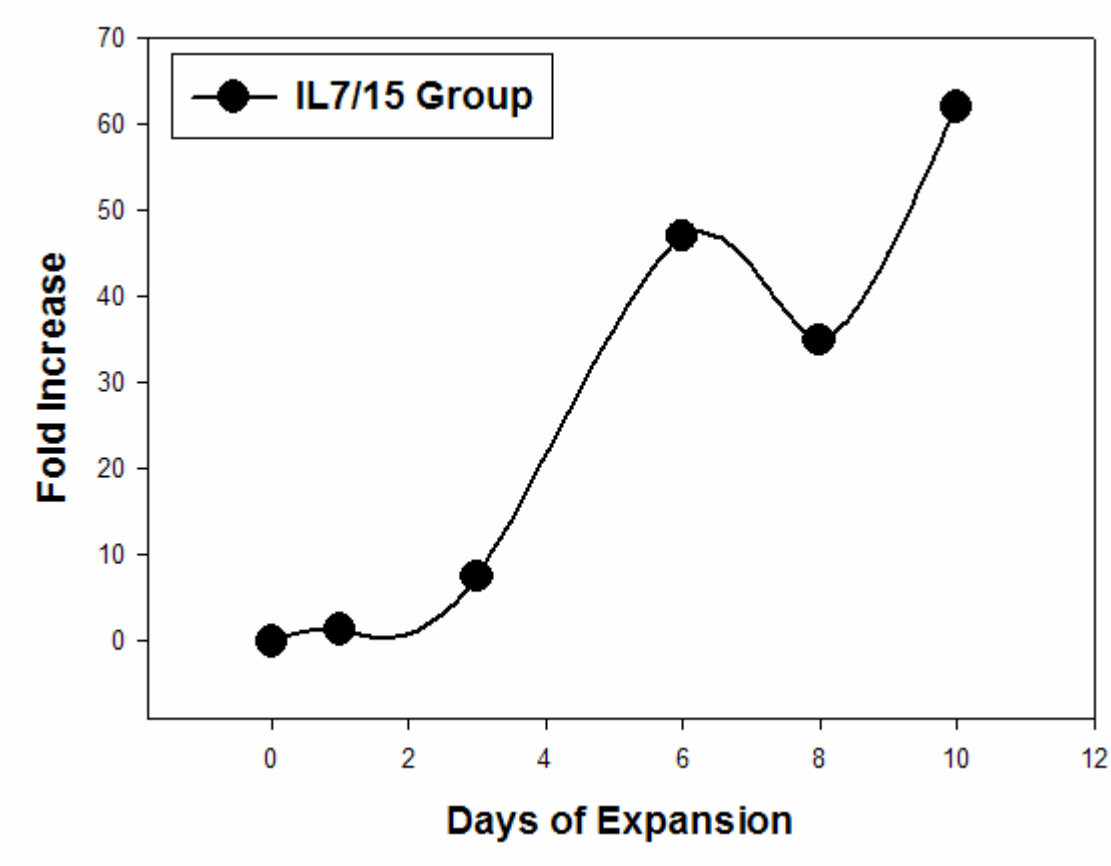
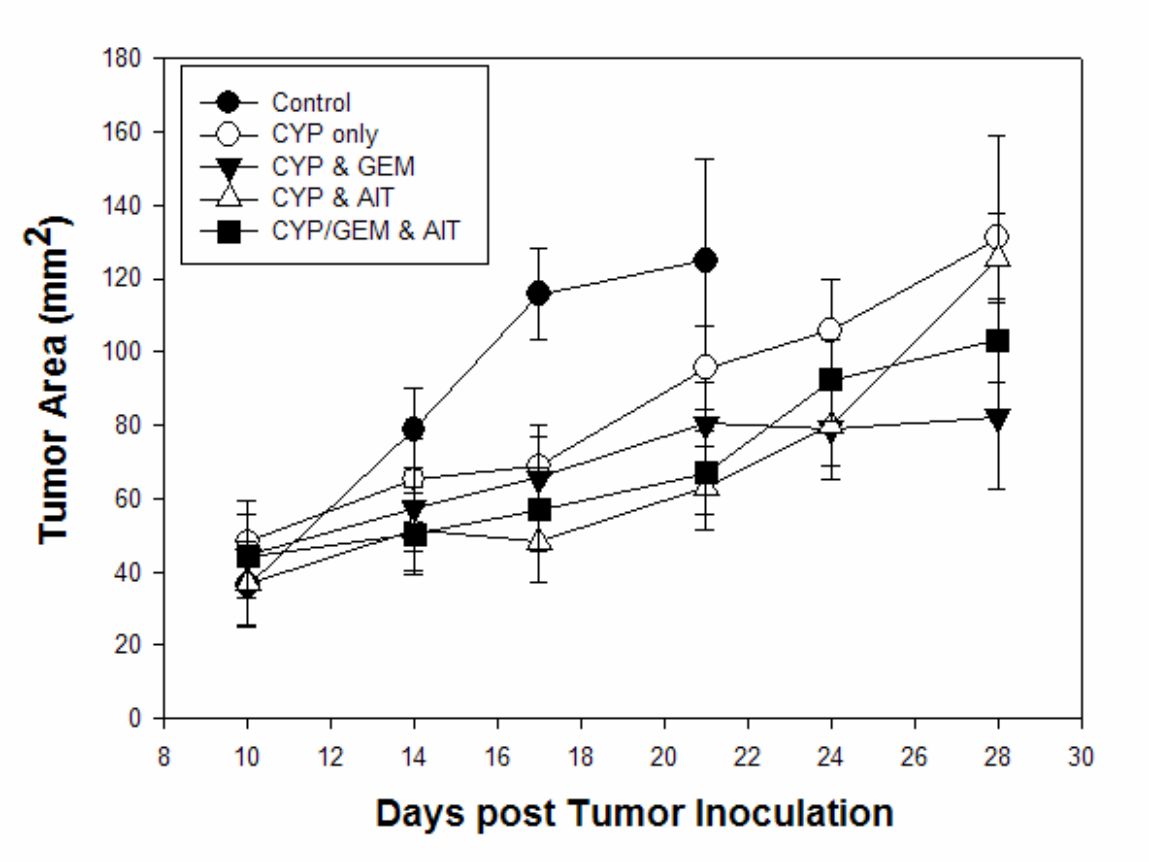

IL7/15 Group
Fold Increase
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
Days of Expansion

Figure 7B


Control
CYP only
CYP & GEM
CYP & AIT
CYP/GEM & AIT
Tumor Area (mm2)
Days post Tumor Inoculation
0
20
40
60
80
100
120
140
160
180
8
10
12
14
16
18
20
22
24
26
28
30

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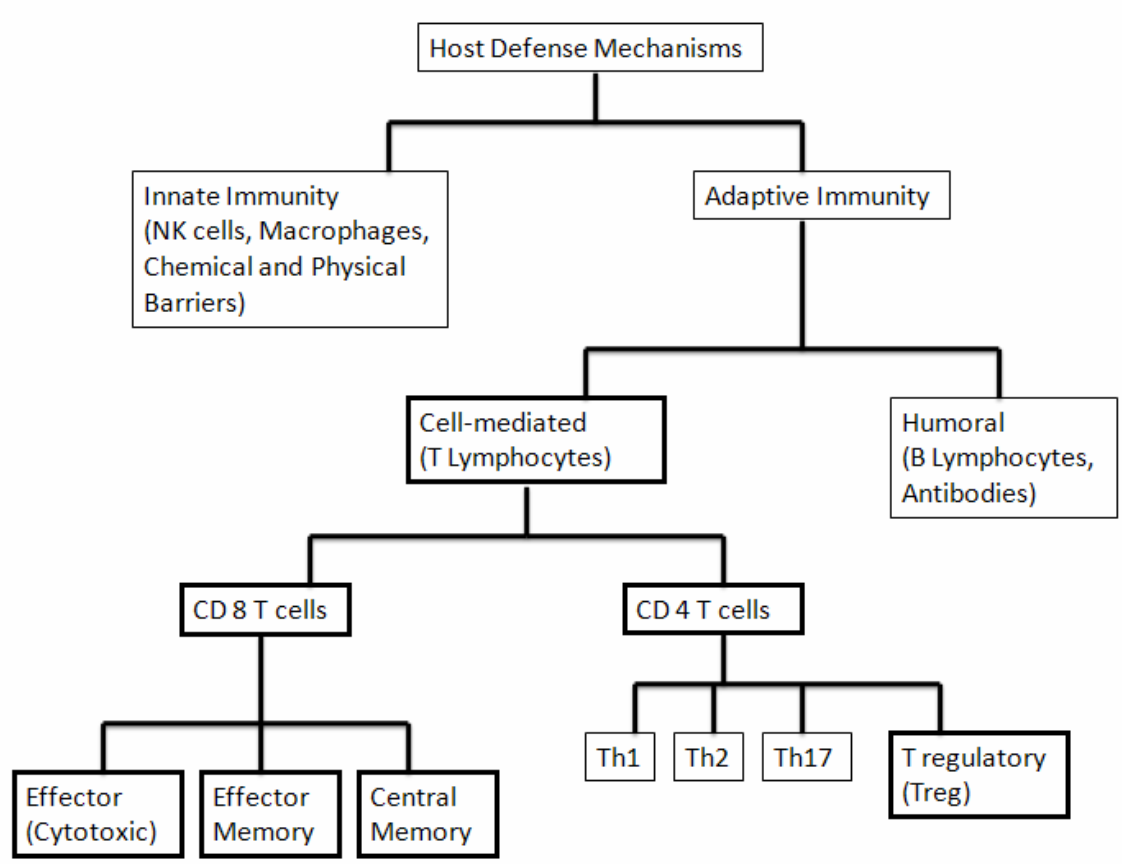
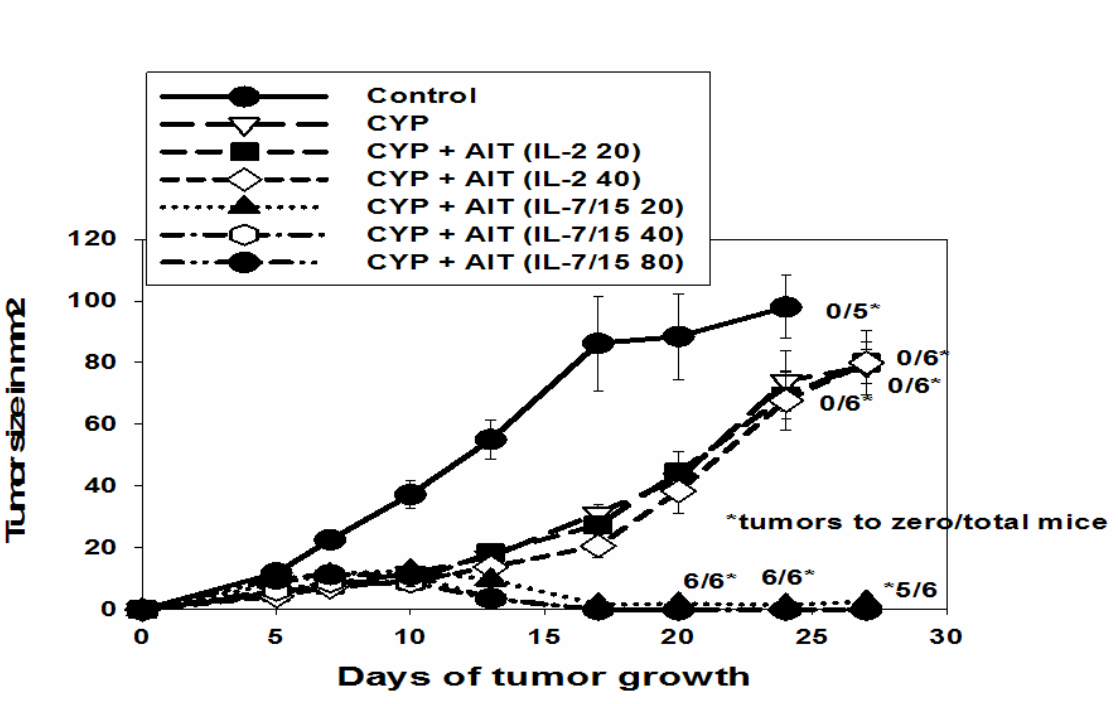
APPENDIX A



The Host Defense Mechanisms.

APPENDIX B



Recipient BALB/c mice were inoculated subcutaneously 50,000 4T1 mammary carcinoma. These tumor bearing mice were then randomly divided into five experimental groups:

1) Untreated control
2) Cyclophosphamide-only treated control (CYP)
3) Cyclophosphamide and AIT with IL-2 expanded 20 million DLN cells (CYP + AIT (IL-2 20))
4) AIT with IL-2 expanded 40 million DLN cells (CYP + AIT (IL-2 40))
5) AIT with IL-7/15 expanded 20 million DLN cells (CYP + AIT (IL-7/15 20))
6) AIT with IL-7/15 expanded 40 million DLN cells (CYP + AIT (IL-7/15 40))
7) AIT with IL-7/15 expanded 80 million DLN cells (CYP + AIT (IL-7/15 80))

After 3 days of 4T1 flank injection, cyclophosphamide (CYP) groups received 100mg/kg of CYP intraperitoneally (ip) on the left flank. AIT was performed the following day to cure 4 day tumor. All IL-7/15 AIT group induced complete tumor regression while IL-2 AIT group had similar kinetics as the CYP only control group. Data shown is mean tumor area ± SE (6 mice per group). A significant difference in mean tumor area was found using ANOVA and Tukey-Kramer honestly significant difference test (Tukey's HSD) using JMPin software

VITA

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