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AUGMENTATION OF T CELL EXPANSION FOR ADOPTIVE IMMUNOTHERAPY BY ALTERNATE GAMMA CHAIN CYTOKINES AND BY 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.

by

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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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Major Director: George Ford
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Successful treatment of cancer with adoptive immunotherapy (AIT) is dependent on the ability to produce large numbers of tumor-specific, functional T cells. The purpose of this thesis is to explore ways in which T cell expansion could be augmented. We have focused on exploring alternate gamma chain cytokines as stimulators of T cell proliferation and differentiation in addition to investigating the potential usefulness of gemcitabine (GEM) in abrogating the immunosuppressive effects of myeloid derived suppressor cells (MDSCs). B16 melanoma sensitized draining lymph node cells that have been activated *in vitro* with bryostatin-1 and ionomycin (B/I) were expanded in either IL-7/15 or in IL-2.

We found that IL-7/15 was superior to IL-2 in expanding T cells for AIT of pulmonary metastases. Expansion of antitumor T cells was also improved by suppressing accumulation of MDSCs in mice bearing 4T1 mammary carcinoma using GEM. GEM directly inhibits both 4T1 mammary carcinoma cells and MDSCs. Its inhibition of MDSCs rescued tolerant T cells, augmenting both expansion and response to tumor antigen.

{CHAPTER 1: Introduction}

Current Trends in Cancer:

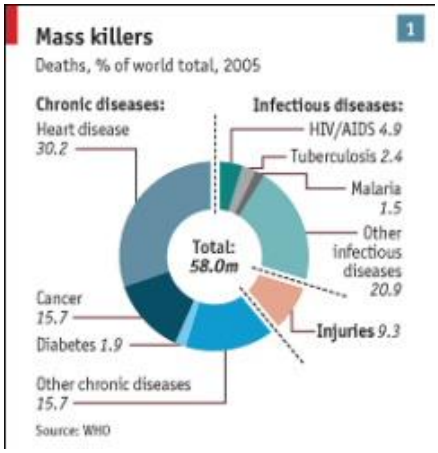
In the United States, cancer is the second most common cause of death, accounting for 1 in every four. According to the American Cancer Society, 2,437,180 new cases of cancer are expected to be diagnosed in the United States in 2008, not including noninvasive cancer of sites outside the urinary bladder [1]. This is in addition to approximately 10.8 million Americans surveyed in 2004 living with a history of cancer. Over 1500 people are expected to die per day because of cancer this year, with an expected death toll of 564,650[1]. With so much of the population affected, either as a patient or living with someone with malignancy, developing better, more effective treatments is of the utmost importance.

Cancer is a disease that afflicts all nations of the world, not just the United States. It accounts for 15.7% of total global deaths and has a greater mortality than HIV/AIDS, tuberculosis and malaria combined (Fig. 1). However, there has been a shift in the world

population that is affected by cancer. Success in preventing and treating infectious diseases and in improving sanitation across the globe has helped increase life expectancy (from 50 in 1965 to 65 in 2005), allowing chronic diseases like cancer to become a growing concern internationally [2]. Globalization has led to the spread of Western living standards, like smoking, unhealthy diets, lack of exercise and obesity. This influence has fostered a rise in the incidence of cancer in poor and middle-income countries. Cancer is a relatively new concern in these countries, so while death rates for chronic illnesses have decreased in affluent countries due to early detection, preventative measures, public education, and better diagnosis and treatment options, there is an increase in death rates caused by chronic illnesses in developing nations. In 2005, one-quarter of the deaths observed in the industrialized nations were due to chronic illnesses compared to three-quarters in developing countries[2]. Much of the developing world is armed against acute infectious diseases but lack the support systems necessary to tackle the cost of public education and long term medical care. In the United States, $8.1 billion is spent to diagnose and treat breast cancer each year with clinics and specialists available across the country. In contrast, Pune, India has a single facility that provides comprehensive breast cancer services for about 3.5 million women[3]. Half of all Indian women with breast cancer receive nothing in the way of treatment. 50% of cancers are caught early, in stage 0 or 1, in the U.S., but only 5% in South Africa. In the face of these challenges, it becomes all the more imperative that effective treatments for cancer are developed. Not just effective, but inexpensive and practical. AIT as it is right now, is neither of these. Therefore, much work is still needed to make this treatment option optimal for combating cancer. In

addition to building up the cancer support infrastructure, increasing awareness, bettering prophylactic measures, improving diagnoses, and funding more cancer-related research, developing manageable and accessible treatment options will help ensure equal access to care for cancer patients world-wide.

Figure 1. Causes of Death Worldwide in 2005 [2]



Cellular Immunotherapy as a Therapeutic Approach:

The foundations of cellular therapy was established in 1957, when Barnes and Loutit provided the first evidence of the ability of the immune system to target and

eradicate cancer[4]. Using a murine model of leukemia, they hypothesized that irradiation alone is not enough to cure leukemia due to radiation resistant leukemic cells that can cause relapse in treated mice. However, irradiation followed by homologous bone marrow transplantation may eliminate surviving leukemic cells by stimulating an immune response against the host. Mice with leukemia that have been irradiated and grafted with homologous bone marrow were completely cured while mice grafted with isologous bone marrow were not. The graft versus host effect induced by the homologous marrow was able to eliminate residual leukemic cells that survived irradiation. These mice showed no signs of leukemia upon histological examination and suspensions made from their spleens did not cause leukemia in normal mice when injected intraperitoneally, which stands in contrast to mice grafted with isologous marrow. The results of these experiments showed that cellular immunity can be manipulated against cancer and launched a new direction for research in the treatment of cancer.

Following this, Southam *et al.* demonstrated that the growth of cancer is amenable to control by immune mechanisms present in the host through autologous and homologous transplant experiments admixed with leukocytes[5]. When autologous leukocytes were present, there was inhibition of tumor growth in 21 of 42 patients implanted subcutaneously with their own tumor cells. In groups implanted with autologous cancer cells in addition to homologous leukocytes or autologous plasma, there was a 33% inhibition of cancer growth (3 of 9 patients and 7 of 21 patients, respectively). In patients receiving homografts, 7 of 9 patients showed tumor inhibition when leukocytes from the

cancer donor were admixed and 2 of 8 patients showed inhibition when control leukocytes from healthy donors were used. Although the sample size of the study is too small to give statistically significant results, this study suggests the presence of specific inhibitory leukocytes in cancer patients that perhaps exert some degree of control on the growth of cancer cells of that individual.

The studies by Barnes and Loutit and Southam *et al.* suggest that a defense mechanism against cancer growth exists in patients and involves cellular immunity. This is an important concept because cancer cells are not foreign to the host body. Thus, without support of hard science, it would be inconceivable that the host immune system would recognize and annihilate good cells that have gone bad. Since then, there has been much evidence of endogenous immune responses against cancer that warrant developing immunotherapy as a treatment for cancer. For example, Her-2/neu is an oncogenic protein that is overexpressed in about 20% of human adenocarcinomas and is also overexpressed in cancers of the breast, ovary, uterus, stomach, and lung[6]. Using quanititative ELISA for the detection of human HER-2/neu specific antibody response or Western Blotting, humoral immunity (IgG and IgA) has been detected in patients bearing cancers overexpressing Her-2/neu, like colorectal, ovarian, and breast cancer[7-9]. Tumor specifc CD-4 T cells have also been detected in the peripheral blood of patients bearing breast or ovarian cancer and these cells proliferated in response to HER-2/neu protein and peptides, demonstrating the existence of cell mediated immunity against Her-2/neu cancers[8, 9]. Measuring interferon-gamma (IFN-γ) release and tumor cytolysis, endogenous antitumor

CD-8 T cells have been observed in melanoma patients[10, 11]. Immunocytochemical analyses of human tumors show the presence of tumor infiltrating lymphocytes (TILs), including machrophages, neutrophils, natural killer cells, eosinophils, T cells, and B cells[12]. The accumulation of antigen specific T and B cells at the tumor site supports the notion that cancer cells are immunogenic. The presence of tumor reactive T cells and the circulation of tumor targeting antibodies show that priming of the immune system against malignant cells is functional during neoplastic transformation. Further evidence that the immune system is capable of fighting cancer *in situ* comes from experiments that show the ability of TILs isolated from tumor sites to expand *in vitro* and cause tumor regression in autologous patients upon adoptive transfer[13]. The observation that patients develop spontaneous responses to antigens being expressed by autologous tumor suggests that it may be possible to boost and optimize this immunity to therapeutic levels through immunotherapy. If the host immune system is inhibited by cancer then its restoration should increase responsiveness to tumor tissue and aid in the inhibition of malignancy.

Although spontaneous immune mechanisms exist against cancer, they are unable to control the growth of even antigenic tumors. Tumors evade host immune system through masquerading strategies and through suppression of immune cells. Many mechanisms have been proposed to account for this immune dysfunction, including the secretion of inhibitory factors (e.g., transforming growth factor (TGF-ß), interleukin-10 (IL-10), phosphatidyl serine, gangliosides, nitric oxide, prostaglandin E2, and reactive oxygen intermediates), downregulation of major histocompatability complexes and costimulatory

signals on tumor cell surface, loss of tumor antigen expression, and induction of regulatory immune cells[12, 14, 15]. These changes prevent tumor recognition and inhibit immune effector functions. TILs harvested at tumor sites are often deficient in proliferative response and cytotoxic function *in vitro*, but are able to recover these biologic processes after purification from tumor cells. A hypothesis proposed by Radoja and Frey suggests that T cells receive an abortive apoptotic signal from tumor cells that inactivates T cell receptor (TCR)-mediated signal transduction and causes T cell anergy[12]. This mechanism involves the incomplete activation of the apoptotic pathway, which leads to proteolysis of TCR-ζ and inhibits transduction of TCR signaling when the T cell recognizes a tumor antigen. Inactivation of TCR- ζ prevents the activation of the MAP kinase pathway, which results in down-regulated cytokine transcription, failure to transit the cell cycle, loss of proliferation, and loss of effector function. Thus, TILs remain in G0/G1 and are functionally anergic. Upon removal of the pro-apoptotic signal, TILs can again express TCR-ζ and reenter the cell cycle. This hypothesis is based on the observation that TILs express both Fas and FasL and are unable to respond to stimulation via the TCR [16]. Lymphocytes recovered from patients with advanced head and neck cancer have abnormalities in signaling via the TCR. Compared with normal lymphocytes, those isolated from cancer patients have defects in expression and function of signaling molecules involved in the TCR pathway, including decreased expression of the ζ and ε chains, decreased Ca2+ flux, and impared kinase activity. Defects in TCR are posttranscriptional and inhibitors of apoptosis prevent ζ chain loss induced by TIL interaction with tumor cells [16]. These observations suggest that signaling defects found in

lymphocytes from cancer patients might be caused by apoptosis induced by the tumor. Through this immunosuppressive mechanism and others, the tumor microenvironment effectively puts the host cells in a state of tumor tolerance.

Failure of immune surveillance is caused by both immunosuppression as well as immune ignorance. Peripheral tumors may successfully grow because they remain outside the immune system and are ignored long enough to become well established and no longer amenable to rejection by the endogenous immune responses. Ochsenbein *et al.* demonstrated a correlation between lack of CTL priming and absence of tumor antigens in the lymph nodes and spleens of mice with growing fibrosarcoma tumors that were initially implanted as solid tumor pieces[17]. Tumor escape in these mice was not by T cell anergy or deletion as revealed by following CFSE labeled T cells after adoptive transfer and was not caused by immune escape or MHC I modulation. Isolated tumor cells were all susceptible to effector CTL specific for the tumor antigen, glycoprotein of lymphocytic choriomeningitis (LCMV). Thus, it was hypothesized that tumor cell processing in secondary lymphoid organs may be crucial in the induction of an immune response against peripheral tumors. Antigens that do not enter lymph tissues at sufficient levels do not induce an efficient CTL response, thus, allowing peripheral tumors that are highly antigenic to be ignored by the immune system. Subcutaneous inoculation of ALY x ALY mice lacking all secondary lymphoid organs with 10^7 MC-GP fibrosarcoma cells in suspension always caused tumors but never in C57BL/6 control mice. Control mice were positive for both LCMV-GP-Specific PCR of DNA extracted from the lymph nodes and

primed CTL activity. No CTL response was induced in ALY x ALY mice. However, inoculation directly into the spleen induced a CTL response. This verifies the importance of antigen presentation. Growing tumors in C57BL/6 mice implanted with MC-GP solid tumor pieces were susceptible to rejection if a strong and long-lasting antigen-driven CTL response was induced and maintained *in vivo*. Thus, restoration of immune surveillance by immunization against the established tumors was key in the success of treatment.

Despite barriers to immune function against cancer, a very important observation is made: TIL dysfunction is reversible. Although CD8+ TILS cannot lyse tumors expressing immunogenic antigens in situ, they are able to *in vitro*. This implies that the T cells have intact effector function, which is inhibited at the site of tumor growth. Removal of the T cells from the tumor environment and culture with IL-2 reversed the dysfunction [14]. In addition, excision of the tumors from patients or animals restored ζ chain expression and the TCR signal transduction pathway.

The discovery of endogenous immune responses against cancer has validated immunotherapy of cancer by confirming three important points: 1) cancer cells express immunogenic proteins, 2) the body has cells that can recognize and mount a response against these proteins, and 3) this response is often immunosuppressed by the cancer microenvironment. Subsequent unveiling of tumor associated antigens further boosts immunotherapeutic efforts. Many tumor associated antigens have been identified, allowing for a very controlled and highly selective targeting of cancer cells. Because of the

predominant role of cell-mediated immunity in cancer eradication, efforts to identify tumor antigens have focused on those recognized by T cells. Antigens specifically involved in cancer rejection have been identified by isolating tumor infiltrating lymphocytes from patients with metastatic melanoma that can induce tumor rejection when adoptively transferred to the autologous cancer patient[18, 19]. Recognition of tumors by TILs can be assayed by *in vitro* tumor cell lysis or by cytokine release. TILs associated with tumor regression were used to screen cDNA libraries derived from tumors. Genes that encode TIL-reactive antigens were identified by transfecting cDNA libraries from melanoma into breast cancer cells. Stable transfectants were isolated and tested for the ability to induce cytokine release from TILs. An alternative method that was used employed a transient expression system that screens pools of cDNA for TIL-reactivity. The genes were then cloned and tested for immunogenicity. Six genes recognized by TILs were thus found, including MART-1, gp100, tyrosinase, tyrosinase-related protein 1, p15, and ß-catenin[18, 19]. Of these, all but ß-catenin are non-mutated forms of genes found in normal cells. MART-1, gp100, and tyrosinase are differentiation antigens present on melanomas and normal melanocytes. Tyrosinase-related protein 1 is a normal melanoma differentiation antigen that is the product of translation from an open reading frame different from the normal protein. Likewise, the p15 antigen is derived from a normal gene but expression of this antigen is limited to melanoma and absent at the cell surface of normal cells. The discovery of these antigens exemplify a biological process that further supports the use of immunotherapy against tumors. That is, tumors cells, due to genetic instability, often express proteins that are usually limited in expression on normal cells and therefore, below

threshold level for T cell recognition. Immunotherapy can be used to utilize this difference in expression to break T cell tolerance and target malignant cells with little or no harm to healthy cells. The ß-catenin antigen represents another class of antigens that can be exploited: unique tumor associated antigens that arise from mutations of normal genes.

New cancer antigens have also been identified by the serologic analysis of recombinant cDNA expression libraries (SEREX) and by eluting peptide antigens from antigen presenting cells (APCs) and screening autologous tumor-specific T cells for reactivity[20]. At least 90 human tumor antigens recognized by T cells have so far been identified, lending promise to clinical application [18-23]. Identification of these immunogenic epitopes allows host T lymphocytes to be sensitized *in vitro* by host APCs, expanded to therapeutic numbers, and eventually reintroduced into cancer patients to lyse antigen bearing tumor cells and secrete cytokines that inhibit tumor progression[21, 23, 24]. Clinically, TILs sensitized to gp100 epitopes have been shown to cause >50% tumor regression when adoptively transferred to autologous melanoma patients along with IL-2 and similar tumor specific immune responses have been observed *in vivo* with transfer of T cells generated by *in vitro* stimulation with autologous APCs pulsed with specific tumor associated antigens[22, 25, 26]. Infusion of TILs recognizing tyrosinase with IL-2 into a patient with multiple established lung, mucosal, and subcutaneous metastases caused a complete remission and retreatment upon relapse led to complete regression [19].

Because tumor antigens tend to be poorly immunogenic, modifications can be made to immunodominant peptides to increase binding to major histocompatability

complexes[6]. Immunotherapy can be performed with immunodominant peptides alone, combined with adjuvants, pulsed onto APCs, or in combination with cytokines and costimulatory molecules to enhance the immune response[6, 18, 22, 24-27]. With known antigens, T cell receptors can be engineered to bind with high affinity to tumor cells, allowing for more potent and more selective immune responses. Hanson *et al.* created a murine TCR transgenic model for the methylcholanthrene-induced fibrosarcoma CMS5 called DUC18[28]. Transfer of DUC18 splenocytes containing transgenic T cells with TCRs that are H-2K^{d} restricted and specific for syngeneic CMS5 fibrosarcoma rejection antigen, mutated ERK2, to normal BALB/c mice conferred a resistance to CMS5 challenge. Using DUC18 splenocytes, adoptive immunotherapy of established CMS5 tumors caused rejection of the tumor without the aid of other forms of therapy. Rejection of tumors by AIT is preceded by lymphocyte infiltration of the tumor site as revealed by histological examination. By defining specific targets, the identification of tumor associated antigens has been demonstrated to augment immunotherapeutic efforts against cancer.

Evidence of spontaneous immune responses against tumors, of immunosuppression induced by the cancer milieu, of immunological ignorance, and of tumor associated antigens all suggest that the host immune system can be manipulated and optimized to target cancer. Traditional therapies, such as irradiation, chemotherapy, and surgery, do not prevent metastatic spread of disseminated tumor cells. Immunotherapy attempts to prevent metastatic spread, eliminate the tumor, and prevent recurrence through the development of memory lymphocytes.

{CHAPTER 2: Principles of Adoptive Immunotherapy}

Immunotherapy uses the body's natural defenses against pathogens to target harmful cancer cells. Adoptive immunotherapy (AIT) relies on manipulation of a host's lymphocytes to produce tumor-specific cells that are optimized in effector functions. T cells are harvested from a patient and cancer specific T cells are expanded *in vitro* then reintroduced into the host. These optimized T cells will hopefully mount a response against the malignant cells and eradicate the tumor mass. AIT circumvents the *in vivo* constraints that influence the magnitude and avidity of T cell responses against tumor, allowing the generation and activation of lymphocytes away from the suppressive tumor environment. In addition, it allows for the treatment of the host before reintroduction of the selected cells; thereby, providing the optimal environment for antitumor responses. Successful adoptive immunotherapy depends on several factors. The type of cells chosen for transfer mediates the potency of the immune response induced against the tumor. The effectiveness of the treatment is contingent upon the ability to generate and expand, *in vitro*, tumor specific and reactive lymphocytes from tumor bearing hosts. The *in vitro* avidity of these lymphocytes must extend *in vivo* to the cancer site. Thus, creating a lymphodepleted host environment and reversal of immunosuppression in the host become essential in achieving therapeutic results.

Using T cells for Adoptive Transfer:

Cytotoxic CD8+ T cells have many advantages over other cells as the focus for adoptive immunotherapy. They can specifically target tumor cells through recognition of differentially expressed tumor proteins, have long clonal lifespans, are amenable to genetic manipulation, have multiple, potent tumor-killing mechanisms, and can provide long-term protection by developing memory responses. CD8+ T cells that have become activated can kill tumor directly through cytolysis or secrete cytotoxic cytokines (e.g., tumor necrosis factor), cytokines that recruit or activate other immune cells that are tumoricidal (e.g., granulocyte-monocyte colony stimulating factor and interferon-γ), and cytokines that affect tumor vasculature (e.g., IL-12).

Common sources of CD8+ T cells are the tumor tissue (tumor infiltrating lymphocytes – TILs), spleen, blood, and draining lymph nodes (DLNs). TILs represent a heterogeneous population of lymphocytes that are found within the tumor tissue. Their migration to the tumor site is thought to have been the result of a tumor antigen (Ag)-specific immune response. Hence, TILs are already exposed to tumor cells and recognize them as immunogenic. Schiltz *et al.* characterized TILs from melanoma, colorectal cancer, renal cell carcinoma, breast, and sarcoma and found that they are dominated by T cells, either CD4+ or CD8+, depending on culture conditions[29]. TILs expanded for low-dose therapy (TIL reinfusion numbers of $5x10^8$-10^9) were predominantly CD4+ in 76% of 42 cultures while high-dose TILs (infusion numbers of > $5x10^9$-10^{10}) were predominantly CD8+ in 84% of 44 cultures.

TILs are isolated from the tumor mechanically or through enzymatic digestion and made into a single cell suspension. The suspension is then incubated in IL-2, which enriches for T lymphocytes and allows *ex vivo* expansion to sufficient numbers [29, 30]. Successful therapy using TILs has been noted in several *in vivo* studies[30, 31]. Adoptive transfer of TILs with systemic administration of IL-2 in a lymphodepleted host has resulted in the elimination of established micrometastases in several tumor models, including MCA-105 sarcoma, MC-38 colon adenocarcinoma, the B16 melanoma, MCA-106 sarcoma, and 1660 bladder carcinoma [30]. The use of TILs has translated into human therapy causing significant regression of tumor masses in the clinic[13, 19, 22]. However, despite promising results, harvesting TILs with specific reactivity *in vivo* has met with limited success and the overall process is time and labor intensive. The mean number of days to reach successful initiation of culture is 35±24 days and expansion from culture to treatment ranged from an average of 59 days for low-dose TIL therapy to an average of 80 days for high-dose therapy.

While TILS provide an attractive source of Ag sensitized T cells, this source is quite limited. Not all cancer patients have solid tumors that are accessible, making TILs available only to a minority of patients. TILs are a heterogeneous population of lymphocytes and may only contain a few cells that have antitumor activity. Thus, much effort has been made to generate tumor specific T cells derived from other sources. Our lab uses the draining lymph nodes as the source of T cells after vaccination with whole

tumor cells. The use of DLNs is based on observations that tumor antigen presentation after vaccination of the host occurs in the lymph nodes that drain the tumor site[32]. The works by Maass and colleagues revealed that after subcutaneous inoculation of mice with IL-2-secreting M-3 melanoma cells, there was no indication of T cell priming at the site of injection. Rather, mRNA recovered at the vaccination site showed an accumulation of natural killer cells, macrophages, and granulocytes. Here, macrophages ingest tumor cells and are hypothesized to migrate to DLNs, where they act as antigen presenting cells to prime T cells. This hypothesis is supported by several observations: latex beads are transferred from tumor cells to macrophages at the vaccination site, draining lymph nodes become enlarged in vaccinated animals, and mRNA markers for activated T cells (IL-2, IL-4, CTLA-4, and CD69) with cytotoxic activity (granzyme B and IFN-γ) are found in the DLN only after vaccination. Identifying and isolating DLNs in the clinic has become practical with the development of sentinel node mapping using isosulfan blue (IB) dye and radioactive tracers[33]. IB injection has allowed for the consistent identification of immunologically active DLNs within a regional lymphatic basin that stems from the primary tumor. Injection of the dye into the footpad 10 days after vaccination with tumor cells using the same inoculation site led to blue stained popliteal lymph nodes and injection into the flank stained axillary and inguinal lymph nodes. T cells harvested from DLNs stained with IB show anti-tumor activity upon adoptive transfer, causing complete regression of 4T07 mammary carcinoma in all treated mice. Sentinel node mapping is important in translating work in mice to human clinical trials, allowing for the reduction of morbidity associated with extensive and unnecessary removal of irrelevant lymph nodes.

Unlike TILs that have been exposed to tumor cells and may contain tumor reactive lymphocytes, CD8+ T cells from sites outside the tumor must be sensitized. Subjects can be primarily sensitized to the tumor *in vivo* by antigen immunization and secondarily sensitized *in vitro* with the same antigen to select for T cells with better tumor reactivity. Numerous tumor antigens have been identified and can be exploited towards this end. Many protocols also employ whole tumor cells or tumor cell lysates as the source of tumor antigens for vaccination.

Generation and Activation of Tumor Reactive T cells:

Effective adoptive therapy depends on lymphocytes that can mount a specific immune response against tumor cells while leaving normal cells unharmed. Tumor antigen specific T cells can be generated using a variety of methods. Classical protocols have used whole tumor cells as the source of tumor antigens to sensitize and stimulate T cells. Advances in technology have allowed for the identification of specific tumor antigens that are conducive to a more selective immune response. Pharmacologic agents have also been developed that activate T cells without the need for tumor cells or antigens.

There are many advantages to using whole tumor cells to sensitize and stimulate T cells. Whole cells expose T cells to diverse antigens that are present on the tumor without knowing the exact peptides responsible for tumor rejection. A polyclonal repertoire of T cells can be generated that mitigate the impact of tumor escape mechanisms, like mutation of specific antigen(Ag) or downregulation of antigen expression. In addition, Dalyot-

Herman *et al.* found that whole tumor cells expressing an antigen recognized by T cells are more immunogenic than soluble Ag peptide[34]. This is perhaps due to better Ag presentation or stronger costimulation provided by the tumor cells. To generate tumor specific T cells in our lab, we inoculate naive mice with whole tumor cells and harvest draining lymph nodes. This protocol has induced tumor reactive T cells to several tumor models, including fibrosarcomas, 4T07-IL-2 and 4T1 mammary tumor cells, P815 mastocytoma cells, and B16 melanoma cells[33, 35, 36].

Shu *et al.* performed *in vitro* sensitization with viable tumor cells and IL-2 using the weakly immunogenic murine tumor, MCA105 [37]. Their work showed that nontherapeutic cytotoxic T cells can acquire antitumor reactivity capable of mediating regression of established pulmonary metastases. Therapeutic lymphocytes harvested from the draining lymph nodes can be generated by *in vitro* sensitization with whole tumor cells for a broad range of host tumor burdens as well as durations of tumor growth.

The usefulness of tumor cells is limited by availability. Not all patients have cells that can readily be isolated. Another disadvantage of using tumor cells is that they are generally not immunogenic. However, scientific advances have arisen to circumvent this problem. For example, tumor cells can be transfected with genes for adjuvants that are highly immunogenic. For example, one of the tumor models used to generate tumor-specific lymphocytes in our lab is B16–GMCSF. GM-CSF is a cytokine that acts as an adjuvant by stimulating dendritic cells, macrophages, and other granulocytes, activating and attracting these effector cells to the tumor. Another method to enhance T cell

responsiveness to tumor cells is to genetically engineer tumor cells to secrete factors like IL-2 that enhance the cellular immune response.

In addition to tumor cells, T cells can be sensitized and activated by proper Ag presentation using autologous antigen presenting cells (APCs) pulsed with a specific tumor peptide. However, this requires precise knowledge of the relevant tumor antigen epitope. Synthetic peptides are readily available; and thus, bypass the quantitative limitation that thwarts the use of whole tumor cells. APCs pulsed with synthetic peptides have been used to induce the formation of effector T cells that prevent the growth of solid tumor in C57BL/6 mice when adoptively transferred 5 days after subcutaneous injection of EG7 OVA transfected thymoma cells[34]. Similarly, Valmori *et al.* exploited a known melanocyte associated antigen, Melan-A, to synthesize an analogue that demonstrates improved *in vitro* T cell expansion and cytotoxicity when pulsed onto peripheral blood mononuclear cells derived from melanoma patients[38]. Yee *et al.* has transitioned this protocol to clinical trials with successful regression of metastases using MART-1 and gp100-specific CD8+ T cells generated from the peripheral blood of patients with metastatic melanoma[39]. These T cells were sensitized to melanoma by incubation with autologous dendritic cells pulsed with the specific peptide epitopes.

As demonstrated by Valmori *et al.*, better recognition of known tumor epitopes has been achieved by genetically modifying the antigen to create analogues with high affinity binding. T cell receptors can also be enhanced to foster antigen recognition. In their

experiments, Dalyot-Herman *et al.* used an OT-1 TCR transgenic T cell model that is specific for OVA peptide bound to H-2Kb and demonstrated both the strengths and weaknesses of using T cells with transgenic TCR recognizing a single peptide [34]. The use of a transgenic TCR that recognizes an immunogenic tumor peptide ensures that once activated, all the T cells are Ag responsive; thereby, ensuring quality with limited quantity. Dalyot-Herman *et al.* were able to delay the establishment of solid tumor for 45 days in 40% of mice with adoptive transfer of antigen specific effector CD8+ T cells and this tumor free condition lasted up to 60 days. However, the tumor eventually grew, escaping the immune response by loss of the antigen that was recognized by OT-1 T cells. Tumor cells harvested from these mice no longer triggered cytolytic activity by OVA specific CD8+ T cells. Tumor antigen loss variants generated by mutation of the recognized antigen or loss of expression have also been observed in human therapy [39]. Observations such as these argue against becoming reliant on a single antigen for therapy, despite the ability to specifically target cancer cells over healthy cells.

Whether using whole tumor cells or specific antigens to activate T cells, the optimal protocol for generating T cells with strong antitumor specificity and reactivity combines *in vivo* immunization or priming with *ex vivo* sensitization. Although tumor bearing hosts already have a milieu of tumor antigens, these antigens may be concealed from the immune system, whether by physical barriers or tumor induced defects in antigen presentation. Vaccination of the host exposes the immune system to tumor antigens that may otherwise be inaccessible *in vivo*. Without *in vivo* priming to the tumor, *in vitro*

sensitization is not therapeutic. Luking and colleagues demonstrated this concept using the A20 BALB/c derived B cell lymphoma and found that among the *in vitro* stimulated T cell lines, only those from vaccinated mice provided protective immunity[40]. T cells from naive mice showed A20 reactivity *in vitro* but were not protective *in vivo*. They establish that T cells derived from vaccinated mice accumulate rapidly during *in vitro* stimulation compared to those from naive mice and have better *in vivo* survival after withdrawal of the antigenic stimulus. The need for *in vivo* priming is also substantiated by the works of Shu *et al.*, which verify that activation by *in vitro* sensitization is a secondary response dependent on primary *in vivo* activation[37]. Luking *et al.* argued that along with *in vivo* priming, *in vitro* sensitization of T cells is also necessary for optimal adoptive therapy. When T cells were enriched *in vitro* without restimulation, a nonprotective population with a broad TCR Vβ repertoire resulted. This indicates that clonal expansion was not induced because the TCR repertoire expressed by the T cell population would have been restricted as a consequence. Because of the heterogeneity of lymphocytes obtained from a host, *in vitro* resensitization allows for the enrichment of lymphocytes with tumor-reactivity. It also prevents death by neglect, a process by which T cells that are not properly stimulated through the TCR undergo senescence and apoptosis. Proper *in vitro* sensitization improves T cell fitness and allows optimal response to homeostatic cytokines.

Ex vivo activation of T cells can be achieved without the need for tumor antigens. T cells can be stimulated pharmacologically through the use of small molecules that activate pathways involved in T cell activation via the TCR. Clonal expansion of naive T

cells requires a primary signal from the recognition of a peptide presented by a major histocompatibility complex (MHC) as well as a secondary co-stimulatory signal provided by the antigen presenting cell. The recognition of the MHC:peptide complex is performed by the T cell receptor, which is a heterodimer composed of two transmembrane glycoprotein chains called αand β that each contain a variable domain and a constant domain. The complete TCR is a complex of this antigen recognition component and invariant signaling proteins, CD3γ, CD3δ, CD3ε, and the ζ chain. These invariant accessory chains have immunoreceptor tyrosine-based activation motifs (ITAMs) sequences. The ITAMs contain tyrosine residues that are phosphorylated by receptor-associated Src-family tyrosine kinases, Lck and Fyn. In addition, antigen receptor signaling is enhanced by co-receptors, CD8 in cytotoxic T cells. Upon recognition of an MHC:peptide complex by the TCR and CD8 binding to the α_3 domain of MHC class I, clustering of the receptors is induced to bring together players necessary for signal transduction. This allows the CD45 tyrosine phosphatase to remove inhibitory phosphates at the carboxy terminus of the Src-family kinases and thereby, allow the activation of Lck and Fyn. Lck is constitutively associated with the CD8 co-receptor and phosphorylates the CD3ε chains. Fyn phosphorylates the ζ chains which recruits the ζ –chain associated protein (ZAP-70). ZAP-70 is phosphorylated and activated by Lck and subsequently phosphorylates the adaptor proteins LAT and SLP-76, which binds and activates Tec kinases that phosphorylate phospholipase C-γ (PLC-γ) and guanine exchange factors (GEFs) that activate Ras G protein. PLC-γ cleaves phosphatidylinositol biphosphate (PIP_2) into diacylglycerol (DAG) and inositol triphosphate (IP_3). IP_3 increases intracellular

Ca^{2+} levels activating calcineurin, a phosphatase that activates the nuclear factor of activated T cells (NFAT). DAG and Ca^{2+} activate protein kinase C (PKC), which activates NFκB. Ras activates the MAP kinase cascade which activates Fos, a part of the AP-1 complex. NFAT, NFκB, and AP-1 are transcription factors that induce specific gene expression, resulting in IL-2 production, upregulation of high affinity IL-2 receptor, T cell proliferation, and T cell differentiation.

The need for antigen and co-receptor stimulation are circumvented by the use of bryostatin-1 and ionomycin, a protocol that was first established in our lab. Bryostatin-1 is a macrocyclic lactone that activates PKC during short exposure, but induces PKC inhibition by causing depletion of the kinase during prolonged exposure[41]. It has also been shown to have *in vitro* and *in vivo* antitumor activities, inhibiting growth, activating apoptosis, inducing differentiation, and enhancing the effects of chemotherapy. Bryostatin-1 as a single antitumor agent has gone through phase I and phase II clinical trials against various cancers with little efficacy[41]. However, the ability of short duration stimulation with B/I to activate PKC serves as a promising drug to activate T cells used for AIT.

Bryostatin-1 in conjunction with ionomycin, a calcium ionophore stimulate the TCR by activating PKC and increasing intracellular calcium concentrations. Stimulation with bryostatin-1 and ionomycin (B/I) alone do not expand lymphocytes in culture but together induced 269-28,206-fold expansion of human breast cancer DLN T cells cultured

in IL-2 [35, 42]. Breast DLN cells after a single dose of B/I at 50nM pulsed for 18hr caused expansion in IL-2 for up to 10 days, after which cell number declined. However, a restimulation with B/I +IL-2 extended proliferation for at least another week[42].

Because the DLN are composed of a mixed population of lymphocytes, we questioned whether B/I is specific in its mode of action. Precursor frequency analysis of antigen sensitized cytotoxic T cells from tumor vaccinated mice show 100-fold increase in the incidence of sensitized CD8+ T cells after pulsing with B/I [42-44]. Unsenstitized T cells show no significant cytolytic activity after expansion with B/I + IL-2. Thus, it was concluded that B/I+ IL-2 treatment of DLN lymphocytes selectively activates T cells already sensitized to the tumor. Indirect evidence was provided for this selective expansion of pre-effector lymphocytes sensitized to tumor cells. No cytotoxic T cell activity was observed *in vitro* against autologous tumor despite recognition of the tumor resulting in IFN-γ secretion and *in vivo* tumor regression upon AIT [36, 42]. Direct evidence comes from experiments performed on $CD62L^{low}$ cells enriched from DLNs of tumor bearing mice. CD62L is a lymph node homing receptor that is down-regulated in antigen sensitized T cells, allowing the lymphocytes to traffic to tumor sites. Thus, L-selectin is a marker for effector ($CD62L^{low}$) versus naive ($CD62L^{high}$) T cells. B/I stimulation and 11 days of culture in IL-2 caused a 12-fold greater expansion of $CD62L^{low}$ cells than $CD62L^{high}$ populations and 33-fold more IFN-γ secretion in response to autologous tumor cells[44]. AIT of 4 day old and 10 day old 4T07 flank tumors showed complete regression when cells transferred were $CD62L^{low}$ or unsorted compared to no tumor regression when

$CD62L^{high}$ lymphocytes were used. In addition, it was demonstrated that cured animals were protected from tumor rechallenge a month later. Thus, B/I preferentially expands primed lymphocytes that can eliminate tumor as well as provide memory and resistance against tumor relapse. This activation is probably specific to sensitized T cells because of the need for costimulation to activate naive, but not primed T cells. The potency of B/I expanded DLN cells against tumors was demonstrated even when transferred without exogenous IL-2[36]**.** B/I expanded DLN cells are able to cause complete regression of lung, metastases, liver metastases, and intradermal tumors and protects cured hosts from tumor challenge[35, 36]. The antitumor effect is specific and demonstrates the ability of DLN cells to traffic to the tumor site.

A well supported model of T cell activation suggests that proliferation, differentiation and death are progressive processes. This progression is regulated by signal strength which is determined by a multitude of factors: antigen concentration and antigen affinity determines the rate of TCR triggering, the presence of costimulation regulates the extent of signal amplification, and the duration of the interactions between T cells and APCs determines the duration of signaling. Gett *et al*. has shown that T cells that receive strong stimulation (anti-CD3 antibody + IL-2 + anti-CD28 antibody) survive in the absence of cytokines and accumulate in the presence of IL-7 and IL-15 while those that received weak stimulation (anti-CD3 + IL-2) did not accumulate in response to the cytokines and died by neglect[45]. The effect of signal strength also carries on *in vivo*. T cells receiving a strong signal proliferate extensively compared to those receiving a weak

signal. We have demonstrated that B/I is able to provide strong TCR stimulation that allows T cell survival and function *in vivo* without the need for concomitant application of IL-2, which is needed with other protocols[22, 25, 26]. This not only reduces the toxicities associated with infusion of exogenous cytokines, but also cuts back cost of the treatment. Thus, it is our preferred method for T cell activation.

In vitro Expansion of Tumor Reactive Lymphocytes

Expansion of T cells *in vitro* is dependent on cytokines of the interleukin family, especially the subset that signals via the common γ chain. Interleukins are soluble signaling peptides produced by leukocytes that regulate the activity of cells of the immune system in an autocrine or paracrine manner. They function in controlling the growth, development, and differentiation of lymphocytes and act as effector molecules of activated T cells. Interleukin-2 (IL-2) has been the standard cytokine for T cell expansion both in animal models and in clinical settings[13, 14, 22, 24, 29, 35, 36, 43]. Its receptor consists of three subunits: α, β, and γ. The β and γ chains are found on resting T cells and together form a receptor for IL-2 with intermediate affinity, allowing resting T cells to respond to high levels of IL-2. In activated T cells, the high affinity receptor is expressed and consists of all three chains, allowing activated T cells to respond to low levels of IL-2. When IL-2 binds its receptor, Janus kinases (Jak) are recruited and phosphorylate the IL-2 receptor, creating phosphorylated tyrosines that serve as docking sites for adaptor proteins such as Shc protein and STAT5[46, 47]. Shc activates the Ras-Raf-MAP kinase and P13 kinase/Akt

pathways leading to cytokine transcription, survival, and cell-cycle entry and growth. STAT5 translocates into the nucleus and acts as a transcription factor to upregulate expression of mitogenic and survival genes, like the anti-apoptotic Bcl-2 family members and the Pim family.

The γ chain is shared by receptors for other cytokines, including IL-4, IL-7, IL-9, and IL-15, and activates the Jak/Stat and PI-3 kinase/Akt pathway mentioned above. This redundancy suggests that other cytokines might also be effective for expanding T cells. Indeed, the results of several research groups reveal that other cytokines, notably IL-7 and IL-15, might be more effective for culturing tumor reactive T cells than the traditional IL-2[48-50]. Like IL-2, both IL-7 and IL-15 have important roles in the homeostasis of T cells [47, 50-52]. Homeostatic proliferation of naive T cells requires TCR contact with self ligand:MHC and stimulation from IL-7. IL-7 promotes survival by inhibiting the mitochondrial pathway of apoptosis through the induction of Bcl-2 expression and by inhibiting nutrient withdrawal-induced apoptosis through the control of glucose uptake[47]. In excess, IL-2 and IL-15 can induce strong proliferation of naive T cells with a preference for CD8+ T cells. Naive T cells that survive positive and negative selection and are maintained within the immune system become mature T cells and ultimately, memory and memory phenotype T cells. Mature T cells are long-lived and maintained through continuous contact with self ligand:MHC and γ-chain cytokines, like IL-7 and IL-15. Resting memory phenotype cells are independent of MHC stimulation and have a heightened sensitivity to γ-chain cytokines. Both IL-7 and IL-15 are important in

regulating background survival and turnover of memory phenotype CD8+ T cells and IL-7 is important for CD4+ cells [50].

IL-7 and IL-15 are important for the homeostasis of immunologically naive CD8+ T cells ($CD44^{low}$) and memory phenotype T cells ($CD44^{high}$). Several primary sources have also noted the roles of these cytokines in the proliferation and survival of primed effector T cells ($CD44^{high}$ $CD62^{low}$)[49, 50, 53, 54]. A review by Boyman and colleagues noted that IL-2 is needed by CD8+ T cells for optimal *in vitro* expansion, survival at low cell densities, IFN-γ production, and cytotoxic function. Because of the shared γ-chain motif, it is possible that IL-15 and IL-7 can supplement for IL-2 in stimulating primed CD8+ T cells. The *in vivo* effect of IL-2, IL-7 and IL-15 on Ag-specific T cell proliferation were observed by injecting immunized mice with the cytokines and measuring T cell number and IFN-γ secretion in response to antigen [50]. Compared to IL-2, IL-7 and IL-15 were more potent in increasing the number of CD8+ effector and especially memory T cells specific for the tumor antigen. IL-7 had a significant effect in prolonging the survival of tumor challenged mice when given in conjunction with immunization[50]. At day 21, IL-7 and IL-15 treated mice had a smaller tumor volume than IL-2 treated mice and survived longer than IL-2 treated mice, with IL-7 treated mice having the most prolonged lifespan. IL-15 at a dose of 50ng/ml is able to stimulate proliferation of tolerant and memory CD8+ T cells *in vitro* but not naive cells and selectively expands CD8+ T cells over CD4+ T cells in peripheral lymph nodes by augmenting cellular proliferation *in vivo* [49, 55]. The proliferation of tolerant CD8+ T cells in response to IL-15 is caused by a restoration of Ag

responsiveness, which allowed the lymphocytes to proliferate in response to tumor *in vivo* and prolong survival in all tumor bearing mice, leading to complete tumor regression in 50% of AIT recipients[49]. What is also interesting is that IL-15 seemed to be a better stimulator than IL-2 in their model. IL-2 at physiologic or low doses (<100U/ml) did not stimulate proliferation of tolerant T cells and a dose of ≥1000U/ml was needed to mimic the response observed with 50ng/ml IL-15. IL-2 also restores Ag responsiveness but in a smaller proportion of T cells and with blunted proliferation. Klebanoff *et al.* compared T cells cultured in IL-2 versus IL-15 and found that the two cytokines induce phenotypically and functionally different populations of CD8+ T cells. IL-2 produces T cells with an effector memory phenotype ($CD44^{high}$, $CD62L^{low}$, CCR7-, CD69+) while IL-15 produces central memory cells ($CD44^{high}$, $CD62L^{high}$, CCR7+, CD69-)[54, 56, 57]. Effector memory CD8+ T cells share similar phenotypic and functional properties as effector T cells and eventually become central memory cells that have enhanced sensitivity to antigen and the greatest potential for long term persistence *in vivo*[57]. IL-2 and IL-15 cultured T cells secrete comparable levels of IFN-γ and TNF-α, but expressed different levels of IL-2 and IL-10 [54]. Only IL-2 cultured T cells secrete IL-10, an immunosuppressive cytokine, and only IL-15 cultured cells secrete their own IL-2. IL-15 cultured T cells also show much better *in vivo* expansion than IL-2. IL-15 cultured T cells, when adoptively transferred were significantly better at improving survival of tumor bearing mice. When given in conjunction with AIT, IL-15 helps prolong the resulting tumor regression and delays relapse longer than IL-2[56]. In lymphodepleted hosts, IL-7 and IL-15 are critical for the maintenance of CD8+ memory T cells[53]. When anti-IL-7 antibody was used to deplete IL-

7 in lymphodepleted hosts, a significant reduction in Ag-specific T cells was observed at the peak and contraction phases of T-cell expansion in response to antigen stimulation *in vivo*. Although IL-15 knockout mice showed little difference in T cell numbers compared to controls, elimination of IL-15 in IL-7 depleted mice caused a greater reduction in T cell numbers. These results demonstrate the need for IL-7 in the expansion and persistence of primed T cells in lymphodepleted mice after vaccination.

Although IL-2 can promote T cell activation and proliferation, it also plays a pivotal role in the development of T regulatory cells and activation-induced cell death (AICD) of T cells. These two effector functions of IL-2 may preclude it from being the optimal cytokine for T cell activation and expansion. Activation-induced apoptosis is a FasL and IL-2 mediated pathway triggered by repeated antigen stimulation of primed T cells and is proposed to be a means of eliminating autoreactive T cells. AICD is induced in WT T cells that are activated by antigen but not in naive T cells [58]. Apoptosis, increased FasL expression, increased FasL transcripts, and decreased FLIP transcripts are observed in activated IL-2-/- T cells when IL-2 is added but not in untreated cells. These results definitively demonstrate the role of IL-2 in AICD and suggest that IL-2 functions as a transcriptional activator of FasL and an inhibitor of suppressors of FasL signaling (e.g. FLIP). Research by Van Parijs *et al.* further elucidates the components of IL-2 signaling that lead to AICD[59]. IL-2Rβ knockout cells were not sensitive to AICD and sensitivity was restored when cells were rescued with WT IL-2Rβ or a mutated form that prevents Shc binding and Akt activation (Y338F). However, supplementing IL-2Rβ-/- cells with

forms of the receptor that cannot activate STAT5 (Δ355) failed to restore sensitivity. These cells also had lower FasL expression than IL-2Rβ-/- cells expressing WT or Y338F mutants, suggesting that STAT5 may be involved in AICD by decreasing FasL expression. The role of IL-2 in inducing AICD seems to rest on specific IL-2Rβ signaling motifs that involve STAT5 and eventually leads to the upregulation of FasL. While other cytokines, namely those that signal through the common γ, share signaling pathways with IL-2, AICD and regulation of autoreactive T cells seems to be unique to IL-2 in potency. For example, IL-4 induction of proliferation does not seem to involve STAT5 and IL-4 does not inhibit FLIP[58, 59]. Compared to IL-2, IL-15 given exogenously in addition to AIT has been shown to increase anti-apoptotic bcl-2, which is upregulated in memory lymphocytes and aids in their long term survival[56]. IL-15 transgenic T cells expressing human IL-15 are resistant to AICD and this resistance is abolished when anti-IL-15 antibody is added[55].

Regulatory T cells (Treg) are suppressive cells that are important in the maintenance of peripheral tolerance by inhibiting T cells, B cells, dendritic cells, natural killer cells, and natural killer T cells. They can be identified by the phenotype, CD4+CD25+(IL-2Rα)FOXP3+(forkhead box P3)[46]. Increased Treg cells were observed in patients with various cancers, including non-small-cell lung cancer, ovarian, breast, colorectal, oesophageal, gastric, and lung. A comprehensive review by Weiping Zou noted the correlation between reduced Treg numbers and reduced tumor volume when various Treg depleting techniques are employed[60]. These regulatory cells differentiate in the thymus and traffic to tumour sites under the influence of CC-chemokine ligand 22

(CCL22) secreted by cancer cells. Treg cells can also be converted from CD4+CD25- T cells by the tumor microenvironment. There are multiple proposed immunosuppressive mechanisms employed by these cells. Treg cells can induce the production of immunosuppressive molecules, like IL-10 from other lymphocytes. IL-10 inhibits differentiation of T cells, secretion of cytokines, presentation of antigen, expression of costimulatory molecules, and dysregulation of cytolyic effector functions. Competition for IL-2 and direct lysis of immune cells are other strategies used by Tregs to regulate the immune response.

IL-2 is required for the induction of FOXP3, a definitive intracellular marker for Tregs that is also important for their development and function[61, 62]. CD4+CD25+FOXP3+ T cells can be derived from human CD4+CD25+ and CD4+CD25- naive T cells after activation with anti-CD3 and anti-CD28 antibodies and exposure to high dose IL-2. These cells suppress the proliferation of CD4+CD25- T cells and this inhibitory function correlates with the expression of FOXP3[61]. IL-2 also protects Tregs from apoptosis[62]. Mice that lack IL-2 develop lethal autoimmune disease that has been linked to a failure in Treg development, which can be restored by transfer of Tregs from WT mice or by treatment with IL-2[62]. Similarly, the development of Treg cells in patients with autoimmune hepatitis is impaired and can be restored by exposure to IL-2[61]. Although, the IL-2Rα chain has been implicated as the IL-2 receptor component that mediates IL-2 induced Treg development, recent research suggest that the IL-2Rγ and its activation of STAT5 may be more important in regulating Treg cells[62]. IL-7 and IL-15 have both been

shown to upregulate the expression of FOXP3 in CD4+CD25+ T cells and can maintain the suppressive potency of Tregs *in vitro*. However, the concentration of IL-7 and IL-15 needed to induce these effects were 20 to 50-fold higher than IL-2, respectively. Thus, despite redundancy, IL-2 seems to be the primary cytokine involved in the ontogeny of Treg cells. Although other cytokines can substitute for IL-2 in maintaining immunosuppression by Tregs, their potency is much less in comparison.

Based on these findings, part of this thesis project investigates the combined effectiveness of IL-7 and IL-15 in culturing antigen specific T cells compared to the classical protocol using IL-2.

Creating a Host Environment Conducive to AIT: Lymphodepletion

To optimize conditions for adoptively transferred T cells to persist and maintain effector functions *in vivo*, it is necessary to create a host environment more conducive to the success of AIT. Two factors that hinder the success of AIT are competition from irrelevant lymphocytes for scarce resources and tumor-induced immunosuppression.

Transferred T cells with tumor specificity make up only a small portion of the total lymphocyte population that resides in the host. These irrelevant lymphocytes compete with tumor specific T cells for lymphoid space, antigens, and cytokines important in the promoting proliferation and persistence. Lymphodepletion of the host before adoptive transfer is a technique that promotes the survival and function of effector T cells by

eliminating competition for limited resources. A lymphopenic environment can be achieved through genetic manipulations, irradiation, and chemotherapy (e.g., cyclophosphamide). Lymphodepletion of fibrosarcoma MCA tumor bearing hosts augments adoptive immunotherapy of pulmonary metastases, subcutaneous tumors, and intracranial tumors by reducing the T cell dose needed to completely eliminate metastases by 3-fold, 12-fold, and 4-fold, respectively[48]. This better antitumor response is attributable to greater *in vivo* proliferation of effector cells at the tumor site in irradiated hosts compared to nonirradiated[48, 53]. Enhanced proliferation of effector T cells may be caused by lymphodepletion-mediated increase in the availability of cytokines and other homeostatic factors, increase in lymphoid space to which lymphocytes can traffick, decrease immunosuppression by Tregs, and/or induction of cytotoxicity to cancer cells. Unlike the fibrosarcoma MCA model, Gattinoni *et al.* found that in the BI6 melanoma model there was more of a qualitative than a quantitative difference in effector T cells adoptively transferred to irradiated and nonirradiated hosts[63]. Pmel-1 T cells from lymphodepleted mice produced higher levels of IFN-γ, IL-2, granulocyte-macrophage colony stimulating factor, and TNF-α, and macrophage inflammatory protein-1α. Through a set of subtle experiments, Gattinoni *et al.* establish cellular sink eradication as one of the key mechanisms underlying better success of AIT after lymphodepletion.

Cyclophosphamide, an alkylating agent that mediates DNA crosslinking, may cause lymphodepletion in treated mice. Experiments using cyclophosphamide (CYP) and AIT to treat Friend leukemia cells and RBL-5 melanoma cells resulted in complete tumor

regression in 100% of mice with no relapse for up to 100 days after treatment[64]. The success with CYP and AIT mirror that of treatment with irradiation and AIT. In these two models, CYP enhance AIT by inducing nonspecific proliferation of both naive and antigen specific T cells, upregulating a cytokine storm of GMCSF, IL-7 and IL-2 in the bone marrow and/or spleen of treated mice, and promoting antigen driven homing of transferred lymphocytes to secondary lymphoid organs. Pretreatment with cyclophosphamide and fludarabine before AIT with autologous T cells and high dose IL-2 of patients with metastatic melanoma resulted in objective clinical responses in 6 out of 13 patients that manifested in the rapid growth of MART-1 specific T cells *in vivo* and tumor regression [65].

Lymphodepletion may also abrogate the immunosuppressive effects of Tregs, which are recruited and stimulated by tumor cells. In addition to the elimination of cellular sinks, the lymphopenic effect of CYP also helps reduce the number of Treg cells in tumor bearing animals and has been used in conjunction with AIT for that purpose[66]. The mechanism by which CYP acts on Treg cells was elucidated by Lutsiak *et al.*[67]. When exposed to Tregs in culture, T cells from CYP treated mice showed 50%-70% greater proliferation than T cells from untreated mice. Besides decreasing the immunosuppressive activity of Tregs, CYP also decreases Treg cell number *in vivo* by increasing sensitivity to apoptosis, decreasing proliferation, and downregulating expression of surface molecules like GITR and FoxP3 that are important in Treg function.

Creating a Host Environment Conducive to AIT: Targeting Myeloid Derived Suppressor Cells

Although recognition of self antigens is desired in the adoptive immunotherapy of cancer, autoreactivity in normal circumstances results in severe autoimmune diseases, like Graves disease, Lupus, and Multiple Sclerosis. The immune system has evolved special cell populations, like Treg cells, to prevent autoimmunity by lymphocytes that react strongly to self peptides. In addition to Treg cells, autoimmunity is dampened by myeloid derived suppressor cells (MDSCs), a heterogenous population of immature cells of the myeloid/macrophage/dendritic cell lineage that has recently gained much attention in tumor immunology[68-71]. MDSCs accumulate in tumor bearing hosts in bone marrow, blood, spleen, lymph nodes, and at the tumor site and are important in murine as well as human cancer progression. For example, in patients with renal cell carcinoma(RCC), there is an upregulation of CD11b+CD14- MDSCs compared to healthy individuals (from ~10% in peripheral blood mononuclear cells to ~40% in RCC patients) [72]. In mice, MDSCs are identified by the phenotype CD11b+Gr1+ and also express CD31, CD124, IL-4Rα, CD115, and MCSF-R. MDSCs that have been identified in humans have the phenotype: Lin-, CD14-, HLA-DR-, CD15+, CD34+, CD11b+, CD33+, and CD13+[69, 72-74]. Accumulation of MDSCs is caused by the tumor microenvironment through secreted factors that stimulate myelopoiesis (e.g., granulocyte/macrophage colony stimulating factor, Flt3, monocyte CSF, and IL-3) and prevent differentiation of immature myeloid suppressor cells (e.g., vascular endothelial growth factor)[70]. *In vitro* studies have shown

that MDSCs also secrete autocrine factors that drive their maturation and differentiation. The maturation/differentiation of MDSCs is correlated with increased suppressive function so that MDSCs found circulating in the blood or in the spleen are less potent than tumor infiltrating MDSCs, which are more mature and have constitutively activated arginase-1 (ARG-1) and nitric oxide synthease (NOS-2)[70]. CD11b+ splenocytes from tumor bearing mice but not naive mice produce IFN-γ, a Th1 cytokine, and IL-13, a Th2 cytokine. IFN-γ, from activated CD8+ T cells and MDSCs, as well as IL-13 are needed for the full activation of MDSC effector activity. MDSC production of reactive oxygen species also maintains their undifferentiated status and promotes proliferation[75].

MDSCs reduce antigen specific CD8+ T cell proliferation, increase T cell death by apoptosis, foster T cell tolerance, and change the profile of cytokines secreted by activated T lymphocytes[70, 72, 74, 76]. They exert their immunosuppressive effects primarily through the secretion of arginase-1 (ARG-1) and nitric oxide synthase (NOS2). ARG-1 causes the depletion of L-arginine in the local environment and leads to the production of urea and ornithine [70]. L-arginine starvation and increased urea concentrations impair translation and is correlated with the downregulation of CD3ζ expression and IL-2 and IFN-γ production. NOS2 catalyzes the reaction between O_2 and L-arginine to generate L-citrulline and nitric oxide (NO), which inhibit intracellular signaling pathways needed for T cell proliferation and survival and favors apoptotic signaling. In the absence of L-arginine, superoxide anion (O_2-) is generated and reacts with other molecules to create reactive nitrogen oxide and reactive oxygen species that cause biological damage. Of note, O2- and NO react to

form peroxynitrite (ONOO-), which modifies proteins through oxidation and nitration of amino acids.

ARG-1 and NOS2 have a myriad of inhibitory effects on T cells[72, 73, 75, 77]. Arginase activity is upregulated in renal cell carcinoma patients along with MDSC accumulation. This is accompanied by a decrease in CD3ξ expression on T cells, a decrease in L-arginine levels, a decrease in activated T cell production of IL-2, IFN-γ, IL-4, and IL-10, and an increase in L-ornithine levels[72]. This decrease in IFN-γ production in humans contrast with results in mice, where MDSCs inhibit proliferation but not IFN-γ secretion[70, 75]. The role of IFN-γ in promoting or inhibiting MDSC activity also varies[70, 77, 78]. Suppression of T cells by MDSCs seems to be both specific and nonspecific and can occur during both the early and late stages of T cell activation [76, 79, 80]. MDSCs inhibit the early stage of T cell activation, MHC:Ag binding, by inducing Ag-specific MHC class I-restricted tolerance[79]. When MDSCs are present *in vivo*, CD8+ T cells do not bind MHC and do not secrete IFN-γ in response to specific peptide. It is hypothesized that this nonresponsiveness is the result of MDSC generation of reactive oxygen species (ROS) and peroxynitrite (ONOO-), which modifies TCR and CD8 molecules. MDSCs from mice that cannot produce ROS ($gp91^{phox-/-}$) did not induce T cell tolerance, and treatment with uric acid, which specifically neutralizes ONOO-, rescues tolerant T cells. Through the nitration of tyrosines in the MHC-TCR complex, ONOO- induces a rigidity that interferes with TCR-MHC interaction. Culture with functional MDSCs increases nitrotyrosine in CD8+ T cells, which is not seen when T cells are cultured with MDSCs from $gp91^{phox-/-}$ or iNOS-/-

mice. Kusmartsev *et al.* argues that MDSC suppression of T cells is Ag specific and MHC class I dependent in a different manner [78]. They found that inhibition of CD8+ T cell responsiveness is mediated by MDSC production of ROS. Activation of ROS production by MDSCs is caused by stimulation of adhesion molecules that are upregulated in MDSCs from tumor-bearing mice and requires stable interaction fostered by Ag specific T cells but not naive T cells.

MDSCs also suppresses T cells during the late stage of activation at the level of IL-2R signaling[76]. T cell proliferation stimulated by both mitogenic factors and Ag specific activation is blocked by MDSCs that prevent the lymphocytes from entering the cell cycle. This inhibition is reversible and is caused by impaired IL-2R signaling pathways (JAK/STAT, RAS/MAPk, and P13k/Akt). Even in the presence of excess IL-2 and mitogenic factors, phosphorylation of STAT5, ERK1/2 and Akt is absent. NO is believed to mediate this late suppression. Addition of NO to T cell cultures inhibited mitogen stimulated proliferation and phosphorylation of JAKs and STATs and inhibition of NOS reversed MDSC mediated inhibition of proliferation.

The expression of ARG-1 and NOS2 is stimulated by cyclooxygenase-2 (cox2), which is overexpressed in most human cancers. In addition, IFN-γ has been shown to increase NOS2 mRNA and protein levels in a dose dependent manner and IL-13 upregulates ARG-1 mRNA and protein concentrations[70, 76]. Both cytokines are believed to work in an additive manner to activate fully the suppressive arms of MDSCs. Inhibition of

either cytokine suppresses MDSC function. Kusmartzev *et al.* demonstrated that STAT1, but not STAT3 or STAT6, is involved in the signaling pathway that mediates MDSC suppression of T cells[73]. MDSCs from STAT1 knockout mice do not suppress T cell responses and lack ARG-1 activity and NOS-2 expression and NO production. These results implicate the Jak/Stat signaling pathway in the regulation of tumor immunity through modulation of MDSC function.

In addition to direct inhibition of T cells through ARG-1 and NOS2 production, MDSCs also mediate indirect mechanisms of inhibition that are independent of these enzymes. Sinha *et al.* demonstrate cell contact dependent cross talk between MDSCs and macrophages that resulted in increased IL-12 production by macrophages and increased IL-10 production by MDSCs[81]. This skews T cell immunity from one that promotes tumor rejection (Th1) to one that favors tumor progression (Th2) by driving the development of M2 macrophages (IL-12^{low}, IL-10^{high}) over M1 macrophages (IL-12^{high}, IL-10^{low}) that promote CD8+ T cell differentiation and decrease MDSC numbers [77, 82]. In addition, IL-10 inhibits dendritic cell maturation. Both dendritic cells and macrophages play important roles in antigen presentation and secrete factors that promote the development of cellular immunity.

The lack of consensus on the specific mechanisms by which MDSCs exert their immunosuppressive effects on T cells suggests the existence of different subsets of MDSCs with diverse inhibitory functions. These MDSCs present a barrier to cellular immunity and abrogation of their influence may increase the likelihood of successful AIT.

Depletion of MDSCs in mice augments AIT and depletion of CD11b+ MDSCs in RCC patients increases proliferation, IFN-γ secretion, and CD3ζ expression in peripheral blood mononuclear cells[70, 72] . Several methods have been developed to inhibit MDSCs. Anti-Gr-1/Ly6G antibody has been used to deplete MDSCS, but because this marker is also expressed on neutrophils, use of this antibody may lead to severe immunosuppression[71, 83]. Blocking ONOO- with uric acid has been shown to prevent T cell tolerance in tumor bearing mice and to delay tumor growth[79]. Blocking the products of MDSCs, through uric acid or ARG-1 and NOS2 inhibitors, however, does not inhibit the known and unknown immunosuppressive mechanisms that rely on cell-cell contact. Thus, it may be more effective to directly eliminate MDSCs in tumor bearing hosts. One way to do this is to stimulate differentiation of MDSCs into dendritic cells using IL-4 and *all-trans* retinoic acid[80]. Another method that has attracted attention because of its ease of application is a chemotherapy drug called gemcitabine (GEM), which was first shown to deplete MDSCs by Suzuki *et al.* in 2005[85]. GEM given to animals bearing large TC-1 tumors (1000mm^3) reduced splenic MDSCs from 28.5% in control mice to 9.3% in treated mice while leaving important CD4+ T cell, CD8+ T cell, and B cell populations unperturbed. MDSC immunosuppression of T cells is also reduced by GEM. CD8+ T cells given in conjunction with splenocytes from GEM treated animals and TC-1 tumor cells significantly inhibited tumor development compared to combination with splenocytes from untreated mice. GEM has also been shown to slow the growth of primary tumors and decrease MDSC proportion in the blood of tumor bearing mice[81]. These results prompted

the question: does GEM act directly on MDSCs or does direct anti-tumor effect decrease tumor-load and thereby decrease MDSCs indirectly?

Gemcitabine or 2',2'-difluorodeoxycytidine is a pyrimidine antimetabolite that has been used clinically to treat patients with non-small-cell lung cancer, ovarian cancer, and breast cancer[86]. This antimetabolite works during the S phase of the cell cycle and requires phosphorylation to become active; it is phosphorylated by deoxycytidine kinase, deoxycytidine monophosphate kinase, and nucleoside diphosphate kinase to difluorodeoxycytidine monophosphate, difluorodeoxycytidine diphosphate, and difluorodeoxycytidine triphosphate. The triphosphate form inhibits DNA synthesis by competitive inhibition of DNA polymerase and by incorporation into DNA and is believed to have a tumoricidal effect by inducing apoptosis caused by early termination of DNA synthesis. The diphosphate form inhibits ribonucleotide reductase and thereby reduces the pools of deoxyribonucleotides available for DNA synthesis. The advantage of using GEM is that it is already approved by the Food and Drug Administration to treat metastatic cancer and is well tolerated in patients. Although GEM has been associated with myelosuppression, it has not been applied clinically as an agent to abrogate the immunosuppression caused by MDSCs. The second part of this thesis explores the application of GEM to determine how MDSCs mediated escape from antitumor immunity can be reversed. We believe that combating MDSCs using gemcitabine as a therapeutic intervention will greatly aid AIT.

{CHAPTER 3 B/I Activated DLN Cells Cultured with IL-7/15 Show Greater Expansion *in vitro* than Cells Cultured with IL-2 and Demonstrate *in vivo* Antitumor Reactivity }

Introduction

Adoptive immunotherapy (AIT) relies, at least in part, on the ability to produce large numbers of tumor specific lymphocytes. Our lab has developed a protocol for effective activation and expansion of draining lymph node (DLN) cells harvested from tumor bearing animals, using bryostatin-1 (B) and ionomycin (I). Bryostatin-1 activates protein kinase C and has been shown to induce the expression of IL-2Rα on human CD4+ and CD8+ lymphocytes[87]. Ionomycin is a calcium ionophore that in conjunction with bryostatin-1 induces IL-2 expression and secretion and augments proliferation of T cells[41, 87]. Together the two mimic signaling pathways activated by antigen:MHC stimulation of the T cell receptor. Stimulation with B/I caused 2-3x greater proliferation than *in vitro* stimulation with autologous tumor cells and caused the number of T cells to increase from 40% to 100% after B/I + IL-2 stimulation[35, 43, 44]. B/I was selective in its mode of action, preferentially activating antigen sensitized T cells, but not naive lymphocytes[36, 42-44]. The effectiveness of these agents was apparent in both murine and human DLN cells. DLNs from breast cancer patients expanded 269-28,206-fold in IL-2 after exposure to B/I[42]. B/I

expanded DLN cells trafficked to tumor sites and exerted tumor specific cytotoxicity[35, 36]. In combination with partial lymphodepletion induced by cyclophosphamide treatment, B/I expanded T cells were able to cause complete regression of lung metastases, liver metastases, and intradermal tumors and protected cured hosts from tumor challenge[36, 88].

Despite the efficacy of this B/I regimen, bryostatin-1 was toxic to DLN cells, inducing apoptosis in 84% of the cells by inhibiting interleukin-2 (IL-2) secretion[89]. Administration of IL-2 protected against B/I induced cell death, but about half the DLN cells still died. The upregulation of cell death in the absence of IL-2 demonstrates the important role cytokines played in the persistence and function of T cells. IL-2 is a key cytokine in the expansion and survival of T cells and promotes cytokine production in activated lymphocytes. For these reasons, IL-2 has been the standard cytokine used for T cell expansion in murine models and in the clinic[13, 14, 22, 24, 29, 35, 36, 43]. However, other γ chain cytokines may be more efficient for this purpose. While IL-2 promotes growth and development of predominantly effector T cells, IL-7 and IL-15 generate central memory T cells, which may be more effective in adoptive immunotherapy, since memory lymphocytes can persist long-term and require lower antigen doses and less costimulation to mount a rapid and potent immune response. This was demonstrated when OT-1 T cells were rechallenged with OVA peptides after culture with IL-2, IL-7 or IL-15[57]. IL-7 and IL-15 cultured T cells, which expressed a central memory phenotype, responded with strong proliferation and IFN-γ secretion when rechallenged with OVA peptide[57, 90]. However, T cells cultured with IL-2 did not proliferate when re-exposed to Ag but

underwent activation induced cell death(AICD) instead. AICD is mediated by IL-2 upon restimulation of the TCR of previously activated T cells, which is undesirable for the purposes of AIT. In addition, IL-2 is crucial in the development of T regulatory (Treg) cells, which maintain peripheral tolerance by inhibiting other lymphocytes and can inhibit anti-tumor immunity. While other STAT5 activating cytokines can substitute for the absence of IL-2, they are much less important in this process[46, 61, 62].

The induction of AICD and T regulatory cells by IL-2 prompted us to explore other cytokines for use in T cell expansion. Because IL-2 belongs to a subset of interleukins that share signaling pathways mediated by the common γ chain, our investigation began with cytokines that share that receptor, namely IL-7 and IL-15. Both IL-7 and IL-15 regulate homeostatic proliferation of T cells and protect against apoptosis[47, 50-52]. However, the two cytokines have slightly different functions. IL-7 maintains both naive and memory T cells and protects against apoptosis by 1) increasing bcl-2 expression and 2) controling glucose uptake to prevent apoptosis caused by nutrient withdrawal[47]. IL-15 acts primarily on memory T cells and protects against IL-2 induced AICD[55, 56]. Both IL-7 and IL-15 have demonstrated the ability to reverse tolerance in T cells from tumor bearing mice and drive the expansion of effector and memory lymphocytes [49, 53, 54]. Mechionda *et al.* showed that IL-7 and IL-15 were comparable or better than IL-2 at expanding Ag sensitized T cells when given as an adjuvant *in vivo* along with immunization [50]. However, it seems that *in vitro*, IL-2 induced more tumor responsive T-cells with a greater capacity to proliferate in comparison to either IL-7 or IL-15 alone [57]. This contrasted our preliminary data, in which IL-15 induced >2-fold higher expansion of activated T cells than IL-2 and IL-7 induced 2-

3-fold greater expansion. The difference in cytokine activity might be due to our use of B/I to activate sensitized T cells. Observation of IL-15 and IL-7 knockout mice revealed that IL-15 is required to maintain the complete functionality of CD8+ T cells but not the number and conversely, IL-7 maintains proliferation and survival, but not the function of adoptively transferred cells[63]. Knockout of both cytokines impaired both quantity and quality of CD8+ T cells. Because IL-7 and IL-15 given individually was not drastically more effective than IL-2 and because the two complement each other in qualitative and quantitative function, we aimed to investigate the combined effects of IL-7 and IL-15 on T cell expansion to drive the development of potent antitumor T lymphocytes.

Materials and Methods

Mice

Virus-free C57Bl/6 (National Cancer Institute) and female pmel-1 TCR transgenic mice (bred in-house) 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 of the Virginia Commonwealth University Institutional Animal Care and Use Committee, 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 Lines

B16-GMCSF and B16-F10 melanoma tumor cell lines and were kindly provided by Dr. Richard Dutton at the Trudeau Institute, Saranac Lake, NY. and Dr. Rodney Prell at Cell Genesys, Inc., South San Francisco, California. After thawing from storage in liquid nitrogen, B16-GMCSF cells were cultured in complete Dulbecco's Modified Essential Medium (DMEM) with 10% heat-inactivated fetal calf serum (Hyclone, Logan, UT), 1mM sodium pyruvate (Mediatech, Inc, Herndon, VA) , 100 U/ml penicillin (Invitrogen, Grand Island, NY), 100 ug/ml streptomycin, 0.075% sodium bicarbonate, and 10mM HEPES buffer. B16F10 melanoma cells were cultured in complete RPMI 1640 with 10% heat-inactivated fetal calf serum, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 0.075% sodium bicarbonate, 2mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM Hepes buffer, and 5×10^{-5}M 2-mercaptoethanol (Sigma, St. Louis, MO). Tumor cells were harvested for inoculation of mice with 0.05% trypsin-EDTA (Invitrogen), washed twice with 1xPBS and resuspended in 1xPBS. All cells were incubated in 250 ml T-flasks (PGC, Gaithersburg, MD) at 37ºC in humidified air with 5% CO_2.

Draining Lymph Node (DLN) Sensitization and Harvest

Donor pmel-1 mice were injected with $1x10^6$ B16-GMCSF cells in the footpad. Ten days after inoculation of footpads, mice were sacrificed and ipsilateral popliteal draining lymph nodes were harvested into complete RPMI using sterile technique. DLNs were crushed through a metal screen and single cell suspensions were prepared in complete RPMI.

In vitro Activation of DLN Cells and Expansion

DLN cells were brought to a concentration of $1x10^6$ cells/ml activated by incubation with 5 nM bryostatin-1 (provided by the National Cancer Institute, Bethesda, MD), 1 uM ionomycin (Calbiochem, San Diego, CA) (B/I), and 80U/ml of rIL-2 (Chiron, Emeryville, CA) or 10ng/ml each of IL-7/15 (Peprotech Inc, Rocky Hill, NJ) in 50ml conical polystyrene tubes at 37°C, 5% CO_2, for 18 hours. Cells were washed three times with warm complete RPMI and resuspended at $1x10^6$ cells/ml. DLN cells were 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. The cells were allowed to proliferate in culture for an additional 7-9 days and were split every 2-3 days in order to maintain $1x10^6$ cells/ml concentration.

Adoptive Immunotherapy

Host C57Bl/6 mice were inoculated intravenously (IV) with 250,000 B16-F10 melanoma cells and randomized into different treatment groups: a) untreated control b) cyclophosphamide (CYP) only c) CYP + AIT with various doses of lymphocytes. On day 3, mice were pretreated with intraperitoneal (IP) injection of 100mg/kg CYP (Bristol-Myers Squibb Company, Princeton, NJ). On day 4, the B/I activated and expanded DLN lymphocytes were washed twice in serum free medium (RPMI 1640) and injected intravenously into host mice at a dose of $2x10^6$, $7.5x10^6$, or $15x10^6$. After 24 days, mice were sacrificed and lungs were harvested and placed in Fekete's Solution (85% ethanol,

10% formaldehyde, and 5% acetic acid). Metastases form black nodules and were counted using a dissecting light microscope. Mice with metastases too numerous to count were assigned an arbitrary value of 250 because this is the largest number of nodules that could be reliably enumerated per mouse. Data are shown as mean number of metastases ± standard error (SE) with 6 mice/group.

Phenotype Assay

Cells were brought to $1x10^6$ cells/ml in 100ul and stained for 30 minutes with anti-mouse, PE-conjugated anti-CD4-mAb, anti-CD8-mAb, anti-CD62L-mAb, anti-CD44-mAb, and anti-CD69-mAb (BD Pharmingen, San Diego, CA). Unstained cells were used as the negative control. Rat IgG_{2b},κ was used as the isotype control for CD4 and CD44, rat IgG_{2a},κ for CD8 and CD62L, and Hamster IgG1, κ for CD69. Stained cells were fixed with 2% paraformaldehyde and analyzed within 7 days of staining on an ELITE Beckman Coulter flow cytometer. 15,000 cells per sample were analyzed.

Interferon γ release assay

Antigen sensitized, B/I activated T-cells that had been cultured in 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, irradiated (10,000 rads) spleen cells (negative control), or irradiated spleen cells pulsed with GP100 peptide (KVPRNQDWL). Lymphocyte to stimulator ratio was 10:1. After 24 hours of culture, supernatant was harvested and stored at -20°C until assayed using a

murine IFN-γ ELISA kit (BD Biosciences Pharmingen, San Diego, CA). Results reported are the mean values of duplicate ELISA wells ± SD.

Apoptosis Assay

Apoptosis was determined using the standard Annexin V-FITC Apoptosis Detection kit (BD Biosciences Pharmingen, San Diego, CA). B/I activated and expanded DLN cells were washed twice with 1xPBS and resuspended in 1x binding buffer at a 200,000 cells/100ul and double stained for Annexin V and propidium iodide (PI). Unstained cells were used as negative controls and staining with Annexin V alone or PI alone was used as single color positive controls. 20,000 cells per sample were analyzed.

Statistical analysis

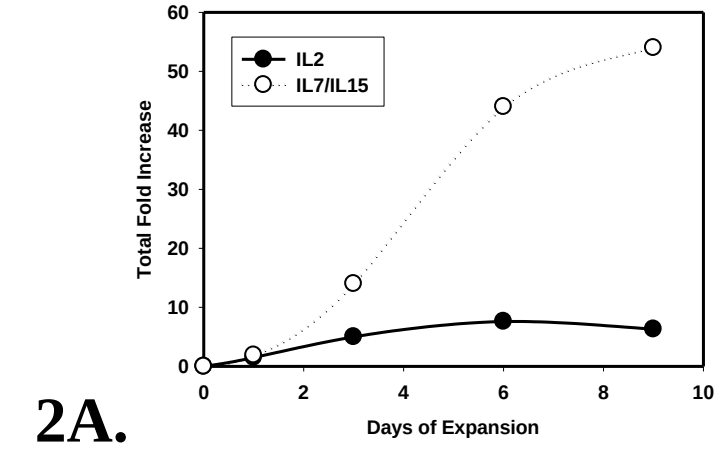
Differences in pulmonary metastases were assessed by analysis of variance (Wilcoxon-Rank Sum Test) using JMPIN software (SAS Institute Inc., Cary, N.C.). Results are presented as the means ± standard error (SE) in each treatment group. *In vivo* experiments included six mice per group. *In vitro* assays were repeated as indicated. An $\alpha < 0.05$ was used throughout to determine significant differences.

Results

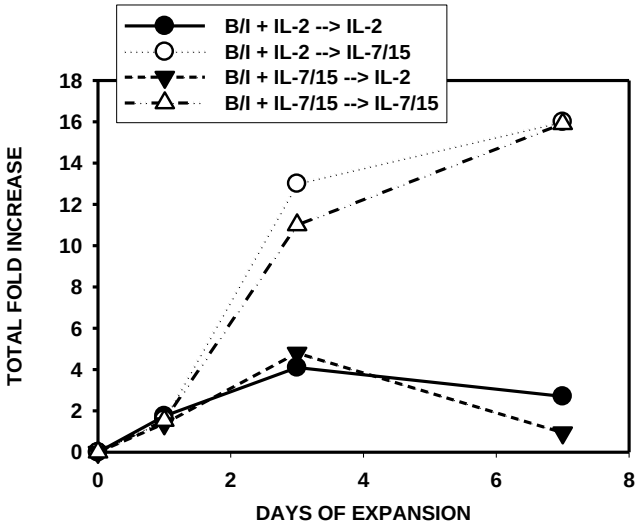
IL-7/15 Supports Greater Expansion of B/I Activated DLN Cells than IL-2

Preliminary data showed that IL-7 and IL-15 expanded B/I activated DLN cells 2-3-fold and 5-fold better than IL-2, respectively. Thus, we were curious to see whether or

not there would be an additive or synergistic effect when the two cytokines were combined. Pmel-1 mice were injected with $1x10^6$ B16-GMCSF cells in the footpad. Ten days later ipsilateral popliteal DLNs were harvested and pulsed for 18hr at $1x10^6$ DLN cells/ml in bryostatin-1(5nM)/ionomycin(1uM) (B/I) + IL-2 (80U/ml) or B/I + IL-7/15 (10ng/ml each) then expanded in either IL-2 (40U/ml) or IL-7/15 (10ng/ml each). After six days of expansion, B/I activated DLN cells cultured in IL-7/15 showed greater expansion than IL-2 expanded cells (Fig. 2). IL-7/15 cultures showed a total of 44-fold increase in cell number compared to a 7.6-fold increase in IL-2 cultures (Fig. 2A). This was a 479% increase. At the end of nine days, there was a 757% difference in fold expansion between IL-7/15 and IL-2 cultures. In addition, while IL-2 cultured DLN cells reached a peak at around day 6 and began to decline in numbers, IL-7/15 DLN cells continued to proliferate until day 9 of culture and beyond. A repeat of the experiment verify a >400% difference in expansion between IL-7/15 and IL-2 cultures by day 6 and this difference in expansion did not depend on the cytokine regimen used in the B/I pulse step (Fig. 2B).



2A.



2B.

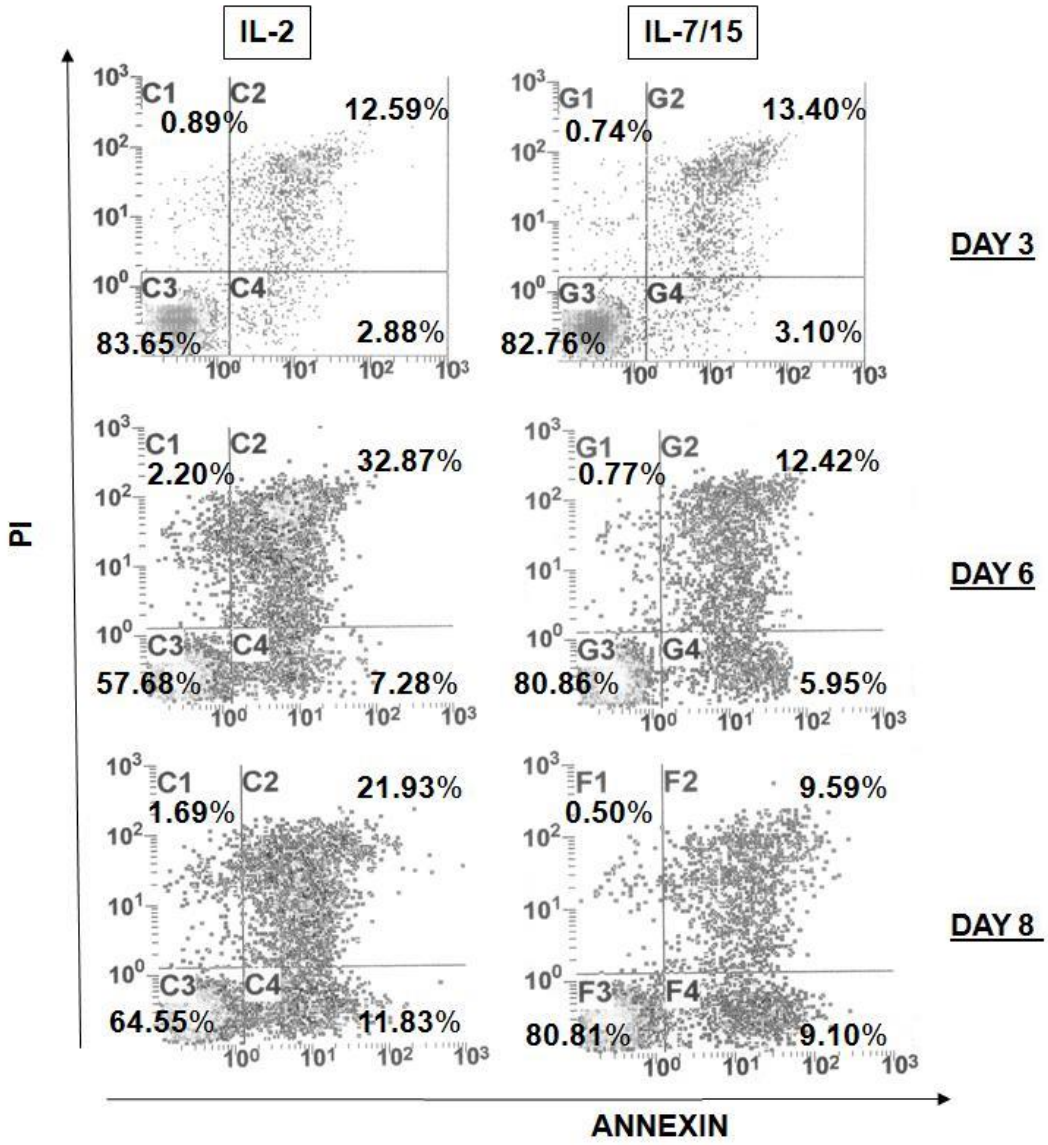
{Figure 2: IL-7/15 induces 8-9 fold greater growth of DLN cells than IL-2.}

(A) Pmel-1 mice were injected with $1x10^6$ B16-GMCSF cells in the footpad. Ten days later ipsilateral popliteal DLNs were harvested and pulsed for 18hr at $1x10^6$ cells/ml in B(5nM)/I(1uM) + IL-2 (80U/ml) or B/I + IL-7/15 (10ng/ml each) then expanded in IL-2 (40U/ml) or IL-7/15 (10ng/ml each), respectively, for 9 days.

(B) DLN cellss were harvested and brought to $1x10^6$ DLN cells/ml. The cells were divided into two pulse conditions. Cells were either pulsed with B(5nM)/I(1uM) + IL-2 (80U/ml) or B/I + IL-7/15 (10ng/ml each) for 18hr. Each pulse group was then divided into two cultures so that one received IL-2 (40U/ml) and the other IL-7/15 (10ng/ml each).

Greater Expansion by IL-7/15 was Partly due to Protection against Apoptosis

IL-2 has been associated with activation induced cell death (AICD) of T lymphocytes while IL-7 and IL-15 are known promoters of survival, protecting against apoptosis induced by nutrient starvation and AICD. Because there was such a significant difference in expansion when B/I activated DLN cells were cultured in IL-7/15 compared to IL-2, we wondered whether or not there was a difference in the rate of apoptosis between the two culture conditions. Samples of B/I activated DLN cells were taken at various time points during culture and stained with annexin V and propidium iodide (PI). Cells that only stained for PI were necrotic or dead. Those that only stained for annexin V were in early apoptosis and cells that stained for both were in late apoptosis (Fig. 3). On day 3 of expansion, B/I activated DLN cells in IL-2 and IL-7/15 cultures showed similar rates of apoptosis (15.5% and 16.5%, respectively). However, by day 6, there was a significant difference in proportion of cells undergoing apoptosis. The IL-7/15 cultured cells were 80.9% viable cells and 18.4% apoptotic (early + late) cells, compared to 57.7% viable cells and 40.2% apoptotic cells in the IL-2 culture. This was a 40% increase in viability and a 54.2% decrease in the apoptotic rate. The difference in the rate of apoptosis remained at day 8 of expansion and contributed to the differences in the observed cell numbers.



{Figure 3: IL-7/15 expanded B/I activated DLN cells have a smaller proportion of apoptotic cells than IL-2 expanded cultures.}

B16-GMCSF sensitized DLNs from pmel-1 mice were harvested and pulsed for 18hr at $1x10^6$ DLN cells/ml in B(5nM)/I(1uM) + IL-2 (80U/ml) or B/I + IL-7/15 (10ng/ml each) then expanded in IL-2 (40U/ml) or IL-7/15 (10ng/ml each), respectively. Samples were taken on days 3, 6 and 8 of expansion and stained for Annexin V-FITC and propidium iodide (PI). Fluorescence of at least 20,000 cells per sample was analyzed by flow cytometry. The differences in apoptosis between IL-2 and IL-7/15 cultures are representative of three independent experiments.

IL-7/15 Induces Similar Subsets of T cells as IL-2 and Maintains Central Memory CD8+ T Cells Better than IL-2

An earlier experiment in the Bear lab showed that induction of tumor regression by adoptive transfer of B/I activated cells is dependent on CD8+ T cells[88]. We next questioned whether or not IL-7/15 maintained similar CD8+ T cells subsets as IL-2. B/I activated DLN cell cultures were sampled and cell surface phenotypes were determined by immunofluorescence staining using monoclonal antibodies against CD4, CD8, CD44, CD62L and CD69 (Table 1). CD4 is a marker for helper T cells, CD8 is a marker for cytotoxic T cells, CD44 is a marker for T cells with the memory phenotype and CD69 is a marker for activated T cells. CD62L (or L-selectin) is a lymph node homing molecule that is downregulated upon T cell activation in association with an increase in T cell effector function. As T cells transition to the central memory phenotype CD62L expression is increased. On days 1 and 3, when DLN cells were on the upward part of their growth curves, IL-7/15 and IL-2 expanded DLN cells showed similar proportions of activated CD4+ and CD8+ T cells. Compared to fresh DLN cells, expanded DLN cells increased the proportion of activated T cells by day 1, indicated by a decrease in CD62L expression and an increase in CD69 expression. CD69 expression is decreased as expected after day 1. By day 3 of expansion, there was an increase in the CD8+ population in both cultures and an increase in T cells with the memory phenotype. By day 7, when IL-2 cultured cells have begun to decline in numbers, there was a shift in the CD4+ and CD8+ populations compared to IL-7/15 cultured cells. Specifically, there was an increase in the CD4+ subset

in IL-2 cultures and a decline in the CD8+ and CD44+ subsets. In contrast, IL-7/15 cultures maintained high proportions of CD8+ and CD44+ cells with no change in the CD4+ subset. There also seemed to be a shift in IL-7/15 cultures from an effector memory ($CD44^{high}CD62L^{low}$) to a higher proportion of central memory phenotype ($CD44^{high}CD62L^{high}$) while IL-2 cultures maintained an effector phenotype. Although the results were not definitive, they indicate that IL-7/15 was able to maintain equal or greater proportions of activated CD4+ and CD8+ T cells when compared to IL-2 and favored the induction of central memory CD8+ T cells over time. Both expansion regimens enriched for T cells so that the DLN pool, which was about 50% or less T cells when freshly harvested, was composed of 100% T cells by day 7 of culture. In addition, Figure 2B + 4 and Table 1 show that the proliferation, phenotype, and antitumor response of DLN cells depended on the cytokines used for expansion and did not depend on what cytokine regimen was used during pulsing with B/I.

Table 1: IL-7/15 expands similar T cell subsets as IL-2 and maintains central memory CD8+ T cells better than IL-2.

B16-GMCSF sensitized DLNs were harvested from pmel-1 mice and pulsed for 18hr at $1x10^6$ DLN cells/ml in B(5nM)/I(1uM) + IL-2 (80U/ml) or B/I + IL-7/15 (10ng/ml each) then expanded in either IL-2 (40U/ml) or IL-7/15 (10ng/ml each). Samples were taken on days 1, 3 and 7 of expansion and incubated with mAb against CD4, CD8, CD44, CD62L, and CD69. Fluorescence of 15,000 cells per sample was analyzed by flow cytometry. Numbers represent percentage of cells with the indicated phenotype.

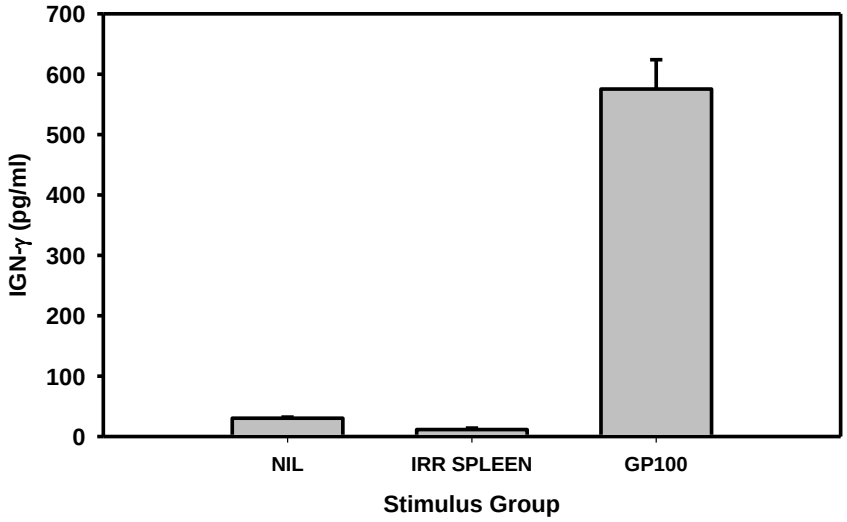
		CD4	CD8	CD44	CD62L	CD69
Pre-pulse		7.30	34.6	58.7	72.5	13.1
DAY 1	**B/I + IL-2 → IL-2**	3.79	42.0	72.6	46.0	50.4
	B/I + IL-2 → IL-7/15	4.96	35.1	74.5	52.1	60.0
	B/I + IL-7/15 → IL-2	5.13	44.4	79.1	51.1	65.7
	B/I + IL-7/15 → IL-7/15	6.05	36.2	76.8	53.3	67.2
DAY 3	**B/I + IL-2 → IL-2**	9.93	75.6	86.2	32.7	14.1
	B/I + IL-2 → IL-7/15	6.29	80.0	91.3	**57.1**	13.1
	B/I + IL-7/15 → IL-2	11.6	74.0	90.2	43.0	27.7
	B/I + IL-7/15 → IL-7/15	6.94	87.3	95.2	**69.7**	43.8
DAY 7	**B/I + IL-2 → IL-2**	**27.0**	52.8	--	33.8	15.1
	B/I + IL-2 → IL-7/15	6.06	**94.3**	**87.7**	**71.7**	43.4
	B/I + IL-7/15 → IL-2	**24.9**	56.9	59.8	27.0	6.14
	B/I + IL-7/15 → IL-7/15	6.61	**93.4**	**93.7**	**60.5**	35.0

IL-7/15 Expanded B/I activated DLN Cells Respond Specifically to Tumor Antigen

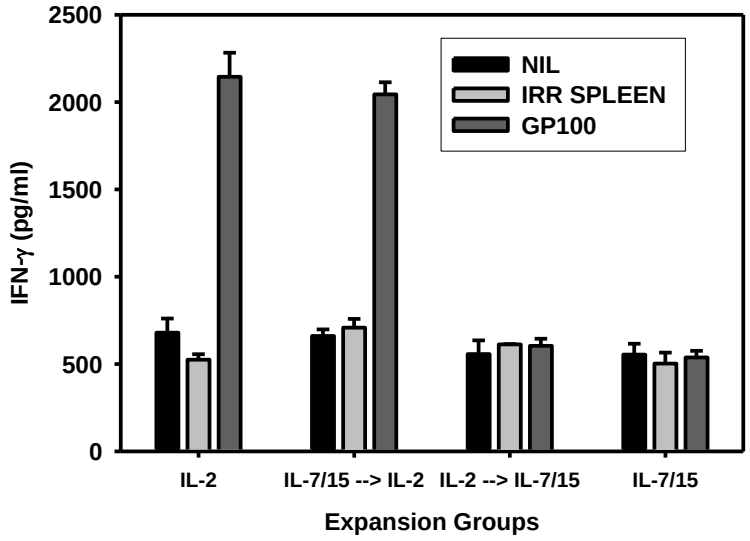
Our lab has shown that IFN-γ secretion plays a key role in tumor regression mediated by CD8+ DLN cells and is therefore used as a marker for antitumor reactivity [91]. To compare the tumor response of IL-2 and IL-7/15 expanded DLN cells, we stimulated expanded cells with plain media (negative control), irradiated splenocytes (negative control), or the gp100 peptide. The gp100 peptide, KVPRNQDWL, is a differentiation antigen upregulated on melanoma cells and is the cognate epitope for the transgenic TCR of pmel-1 mice. This peptide was used to assay the antigen specific reactivity of DLN cells that were sensitized to B16-GMCSF melanoma. After 24 hours, supernatant was collected and IFN-γ levels were measured (Fig. 4). Pre-expansion cultures that have not yet been expanded showed reactivity to gp100 as expected, but not to media alone or to the negative control (Fig. 4A). Specificity to gp100 but not to irradiated spleen was maintained throughout expansion for all cultures (Fig. 4A-D). Interestingly, DLN cells expanded in IL-2 started with potent reactivity to gp100 on day 1, with high levels of IFN-γ secretion but showed a decrease in antitumor response over time. In contrast, IL-7/15 cultured cells had minimal antitumor response on Day 1 of expansion but increased IFN-γ secretion in response to gp100 over time so that by day 3, the concentration of INF-γ secreted by IL-7/15 expanded cells was similar to that of IL-2 expanded cells on Day 1 and surpassed the declining response of IL-2 cultured cells. This high response to gp100 by IL-7/15 expanded cells increased further by day 7 of expansion, when IL-2 expanded cells showed a flat or even declining IFN-γ response. Because IFN-γ is a product of activated T

cells, the change in the production of this cytokine in IL-2 cultures in response to specific peptide may reflect the decline in proportion of CD8+ T cells over time (Table 1). Similarly, the maintenance of high antitumor responses by IL-7/15 expanded T cells on Day 3 and 7 may reflect the maintenance of a large, viable and functional CD8+ T cell population in IL-7/15 cultures (Table 1).

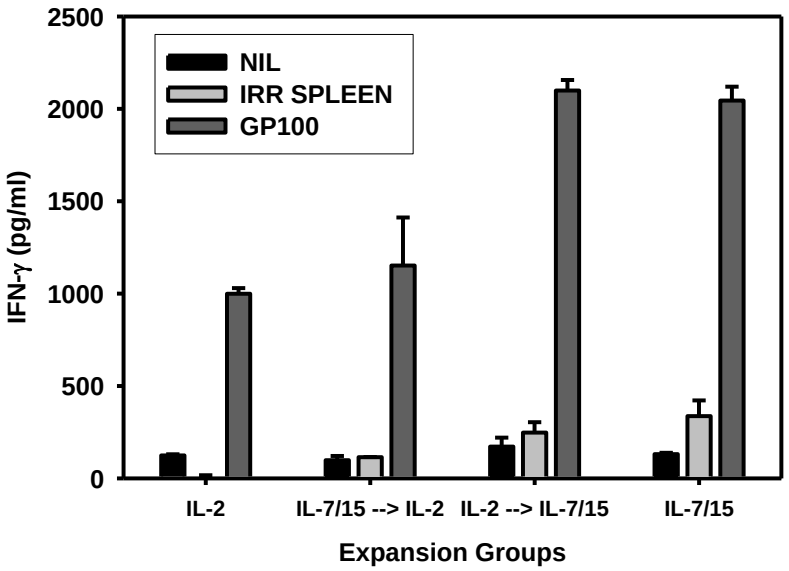
4A: Day 0



4B: Day 1



4C: Day 3



4D: Day 7

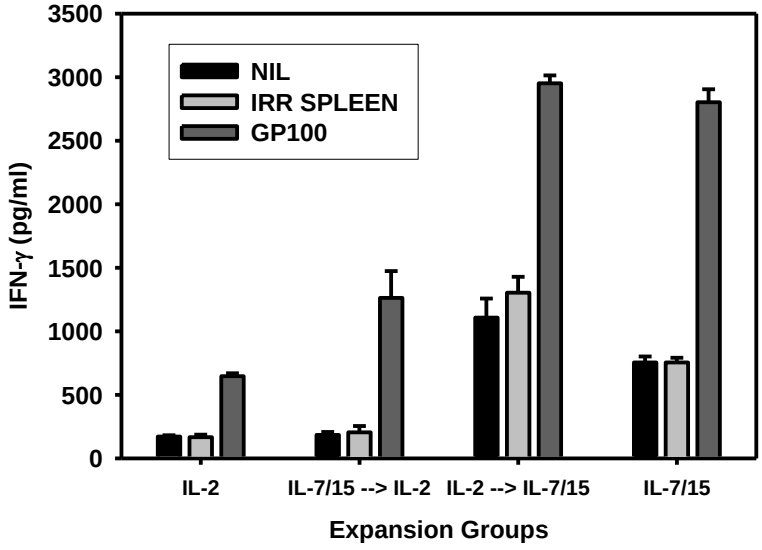


Figure 4. IL-7/15 expanded DLN cells show an increase in IFN-γ secretion in response to specific antigen while IL-2 expanded DLN cells decline in reactivity over the course of *ex vivo* culture.

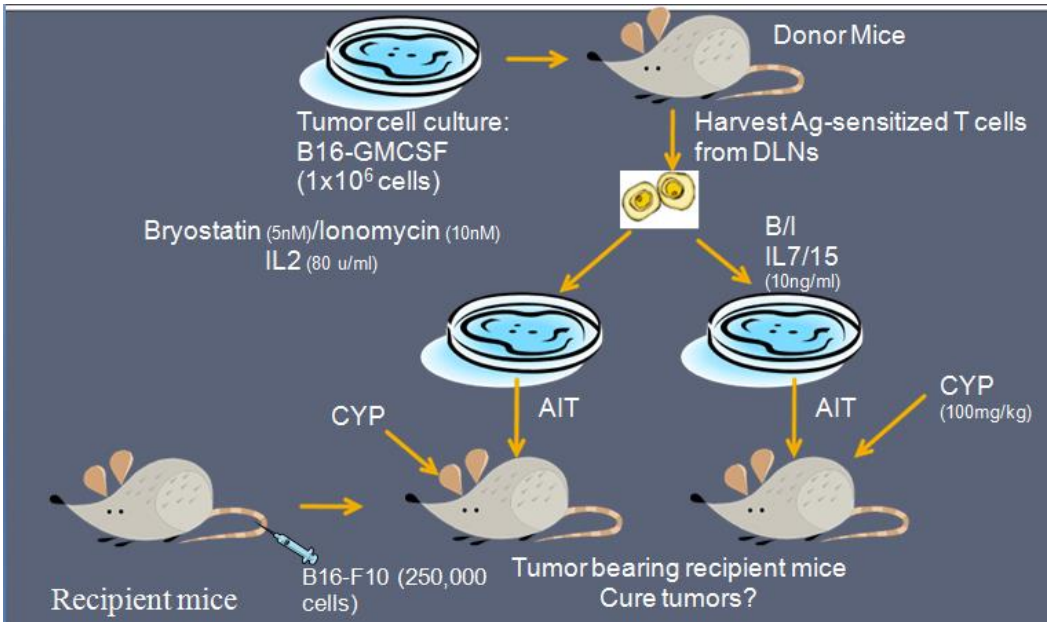
B16-GMCSF sensitized DLNs from pmel-1 mice were harvested and pulsed for 18hr at $1x10^6$ DLN cells/ml in B(5nM)/I(1uM) + IL-2 (80U/ml) or B/I + IL-7/15 (10ng/ml each) then expanded in either IL-2 (40U/ml) or IL-7/15 (10ng/ml each). Samples were taken on days **(A)** 0, **(B)** 1, **(C)** 3, and **(D)** 7 of expansion and stimulated with media alone (NIL), with irradiated splenocytes (IRR SPLEEN), or irradiated splenocytes pulsed with the specific peptide, gp100 (GP100). Cells were incubated with stimulants with a ratio of 10:1 in 24 well plates at a concentration of $2x10^6$/ml. Supernatant was collected 24hrs after exposure to stimulants and assayed for IFN-γ using the murine IFN-γ ELISA kit. Data is shown as the mean of duplicates ± SD.

Adoptive Transfer of IL-7/15 Expanded B/I Activated DLN Cells Eliminates Melanoma Lung Metastases

Lymphocytes grown in IL-7/15 showed a potent IFN-γ response to specific peptide *ex vivo.* Therefore, we hypothesized that this activity would extend *in vivo* and mediate tumor regression. In an adoptive immunotherapy experiment, C57Bl/6 mice inoculated with B16F10 melanoma cells intravenously were treated with B/I activated DLN cells expanded in different cytokines. Mice were given different doses of DLN cells to determine the optimal T cell dose needed to cure B16F10 pulmonary metastases generated by an initial tumor cell dose of 250,000 cells per mouse. Summary statistics are shown in Table 2. All treated groups were significantly different from the untreated control group (CON), p-value < 0.003 (Fig. 5). Groups treated with AIT at T cell doses of $7.5x10^6$ and $15x10^6$ were significantly different from CYP only group (p-value < 0.004). Cyclophosphamide is an alkylating agent that may have direct antitumor effect and causes lymphodepletion in mice, eliminating cytokine sinks and regulatory T cells that compete with and inhibit the adoptively transferred T cells. Thus, mice treated with AIT are generally given cyclophosphamide in our lab. When given as a single drug agent, cyclophosphamide slowed tumor progression (Fig. 5, CYP). Both IL-2 and IL-7/15 expanded DLN cells were ineffective at a dose of $2x10^6$/mouse, compared to CYP alone. IL-2 and IL-7/15 expanded cells were equally potent against the melanoma cells at a dose of $7.5 x10^6$. Complete tumor regression was observed in most mice when DLN cells were given at $15x10^6$ cells per mouse. At this concentration, IL-7/15 expanded DLN cells were

significantly more effective than IL-2 expanded cells, causing complete tumor regression in all mice while IL-2 only caused complete remission in one out of six mice. Thus, IL-7/15 expanded B/I activated DLN cells were as potent if not more potent against B16F10 melanoma as IL-2 expanded cells.

5A.


Donor Mice
Tumor cell culture:
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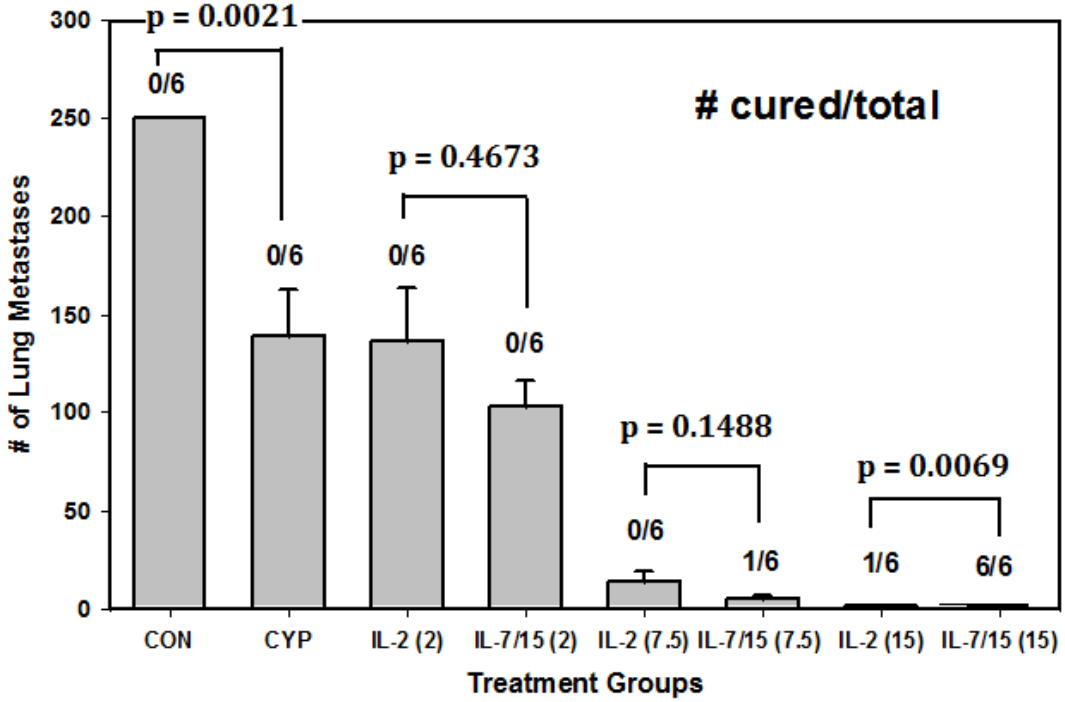
(1x10^6 cells)
Harvest Ag-sensitized T cells
from DLNs
Bryostatin (5nM)/Ionomycin (10nM)
IL2 (80 u/ml)
B/I
IL7/15
(10ng/ml)
CYP
AIT
AIT
CYP
(100mg/kg)
Recipient mice
B16-F10 (250,000
cells)
Tumor bearing recipient mice
Cure tumors?

5B.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# cured/total
p = 0.4673
0/6
0/6
0/6
p = 0.1488
p = 0.0069
0/6
1/6
1/6
6/6
300
250
200
150
100
50
0
# of Lung Metastases
CON
CYP
IL-2 (2)
IL-7/15 (2)
IL-2 (7.5)
IL-7/15 (7.5)
IL-2 (15)
IL-7/15 (15)
Treatment Groups

Figure 5: IL-7/15 expanded DLN cells were as effective as IL-2 expanded cells at curing B16F10 Lung Metastasis.

Recipient C57BL/6 mice were injected intravenously with 250,000 B16F10 melanoma cells and randomized to 8 treatment groups: untreated control (CON), cyclophosphamide (CYP) treated (100mg/kg), and CYP + AIT with IL-2 or IL-7/15 expanded DLN cells at doses of 2, 7.5, and $15x10^6$ cells/mouse (doses shown in parenthesis). CYP was given 3 days after tumor inoculation and AIT was performed the following day. After 24 days, lungs were harvested and lung metastasis were counted. The data shown is the mean number of lung metastasis ± SE of 6 mice per group. All treated groups were significantly different from CON and groups treated with AIT at T cell doses of $7.5x10^6$ and $15x10^6$ were significantly different from CYP only group (Wilcoxon rank sum test, p-value < 0.05). **(A)** AIT Schemata **(B)** Results

Table 2: Summary Statistics of Figure 5B.

GROUPS	N	Mean	SE	Lower 95%	Upper 95%
CON	6	250.0	0.00	250.0	250.0
CYP	6	139.3	22.94	80.36	198.3
IL-2 (2)	6	136.7	27.27	66.55	206.8
IL-2 (7.5)	6	13.83	4.915	1.198	26.47
IL-2 (15)	6	1.33	0.422	0.249	2.417
IL-7/15 (2)	6	102.7	13.88	66.98	138.4
IL-7/15 (7.5)	6	5.00	1.897	0.123	9.877
IL-7/15 (15)	6	0.00	0.00	0.00	0.00

Conclusions

Our preliminary data showed that IL-7 and IL-15 supported expansion of B/I activated DLN cells 2-3 and 5-fold greater than IL-2. When we combined IL-7 and IL-15, there was an additive effect so that IL-7/15 expanded DLN cells 8.6-fold greater than IL-2 (Fig. 2). As we've discovered, this difference in proliferation was partly attributed to the pro-survival role of these alternate cytokines. IL-7/15 expanded B/I activated DLN cells have a smaller proportion of apoptotic cells than IL-2 expanded cultures (Fig. 3). This protection from apoptosis also allowed IL-7/15 expanded cells to expand for a longer period than IL-2 expanded cells. B/I activation and expansion with IL-2 and IL-7/15 enriched for T cells resulting in a population composed almost entirely of CD4+ and CD8+ T cells (Table 1). During the growth phase of expansion, IL-7/15 induced similar T cell subsets as IL-2, but maintained central memory CD8+ T cells better than the prototypical cytokine on Day 7 (Table 1). IL-7/15 expanded DLN cells showed an increase in IFN-γ secretion in response to specific antigen while IL-2 expanded DLN cells declined in reactivity over the course of *ex vivo* culture (Fig. 4). This response to tumor antigen by IL-7/15 expanded cells extended *in vivo,* causing 100% regression of pulmonary metastases at a dose of $15x10^6$ cells per mouse, which is significantly better than the 17% cure rate achieved with IL-2 expanded cells (Fig. 5, Table 2).

{Chapter 4: Treating Tumor Bearing Mice with Gemcitabine Eliminates Myeloid Derived Suppressor Cells and Improves Expansion of T cells }

Introduction

Myeloid derived suppressor cells (MDSCs) are a heterogeneous population of immature monocytes and granulocytes that cause T cell dysfunction in tumor bearing mice and humans[68-73, 75]. These CD11b+/Gr-1+ cells accumulate in the bone marrow, blood, spleen, lymph nodes, and at the tumor site in tumor bearing hosts. Accumulation of MDSCs is caused by the tumor microenvironment through secreted factors that stimulate myelopoiesis and maintain the immature state of myeloid cells[70].

MDSCs present a barrier to successful AIT and therefore, are potential targets for augmenting treatment of cancer. Several methods have been developed to deplete MDSCs directly, to decrease MDSC numbers indirectly by stimulating differentiation into nonsuppressive mature immune cells, and to inhibit products secreted by MDSCs [71, 79, 80, 83]. One of the most promising of these methods is administration of 2',2'-difluorodeoxycytidine or gemcitabine (GEM). GEM is a cytidine analogue that has been used as an antimetabolite against non-small-cell lung cancer, ovarian cancer, and breast cancer[86]. GEM works during the S phase of the cell cycle, acting as a competitive inhibitor of DNA polymerase and incorporating into DNA. In addition, the drug inhibits ribonucleotide reductase and thereby reduces the pools of deoxyribonucleotides available

for DNA synthesis. GEM is believed to be tumoricidal, inducing apoptosis caused by early termination of DNA synthesis. In mice, GEM has a plasma half life of 0.28 hr after a dose of 20mg/kg given intravenously and 86.3% is excreted in urine 24 hrs after administration [92]. The drug is quickly distributed in the spleen, thymus, testicles, kidney, femur, small intestines, and lymph nodes.

Although GEM has been associated with myelosuppression, it has not been applied clinically as an agent to abrogate the immunosuppression caused by MDSCs. The purpose of the following studies is to elucidate the mechanism by which GEM exerts its inhibitory effects. Our goal is to determine whether the observed suppression of MDSCs is a direct effect of the drug or an indirect result of cytotoxicity to tumor cells. Doing so will help clarify whether gemcitabine is a good treatment option for abrogating the immunosuppression caused by MDSCs.

Materials and Methods

Mice

Virus-free BALB/c and athymic nude 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 of the Virginia Commonwealth University Institutional Animal Care and Use Committee, 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 Lines

4T1 mammary tumor cells were kindly provided by Dr. Jane Tsai at the Michigan Cancer Foundation, Detroit, Michigan. Cells were passaged in complete Dulbecco's Modified Essential Medium (DMEM) with 10% heat-inactivated fetal calf serum (Hyclone, Logan, UT), 1mM sodium pyruvate (Mediatech, Inc, Herndon, VA) , 100 U/ml penicillin (Invitrogen, Grand Island, NY), 100 ug/ml streptomycin, 0.075% sodium bicarbonate, and 10mM HEPES buffer. Meth A sarcoma, an unrelated tumor cell line (ATCC, Rockville, MD) was maintained in complete RPMI 1640 with 10% heat-inactivated fetal calf serum, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 0.075% sodium bicarbonate, 2mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM Hepes buffer, and 5×10^{-5}M 2-mercaptoethanol (Sigma, St. Louis, MO). All cells were incubated in 250 ml T-flasks (PGC, Gaithersburg, MD) at 37ºC in humidified air with 5% CO_2. Tumor cells were harvested for inoculation of mice with 0.05% trypsin-EDTA (Invitrogen), washed twice with 1xPBS and resuspended in 1xPBS.

Tumor Inoculation

BALB/c and nude mice were inoculated subcutaneously (S.C.) in the flank with 50,000 4T1 mammary carcinoma cells. Tumor growth was monitored with bidirectional tumor measurements every 2-3 days. The product of the two perpendicular measurements

measured with a caliper was recorded as the tumor area in mm^2. Results are reported as the mean tumor area ± standard error (SE) with 3-6 mice per group.

Cyclophosphamide and Gemcitabine Treatments

Cyclophosphamide (CYP) was given at a dose of 100mg/kg via intraperitoneal (I.P.) injection on day 5 after tumor inoculation (Bristol-Myers Squibb Company, Princeton, NJ). Gemcitabine-HC1 (GEM) was given at 60mg/kg via I.P. injection (Eli Lilly and Company, Indianapolis, IN). Two GEM treatment regimens were employed: 1) EARLY GEM: GEM was given on day 5 after 4T1 tumor inoculation and repeated once weekly 2) LATE GEM: was given in a single dose on day 20-25.

Spleen Harvest and Expansion of Splenocytes

Spleens were harvested 24-48hr after GEM treatment in complete RPMI, weighed, and crushed through a cell strainer. Splenocytes were resuspended in 1x ammonium chloride solution to lyse red blood cells. Splenocytes were stained with 0.04% tryphan blue to exclude dead cells and 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 lymphocytes. Splenic lymphocytes were brought to a concentration of $1x10^6$ cells/ml and activated by incubation with 5 nM bryostatin-1 (provided by the National Cancer Institute, Bethesda, MD), 1 uM ionomycin (Calbiochem, San Diego, CA) (B/I), and 80U/ml of rIL-2 (Chiron, Emeryville, CA) in 50ml conical polystyrene tubes at 37°C, 5% CO_2, for 18 hours. Cells were washed three times with

warm complete RPMI and resuspended at $1x10^6$ cells/ml. Splenocytes were expanded in complete RPMI supplemented with an additional 5% heat-inactivated fetal calf serum with either 40U/ml of rIL-2 or an alternating regimen whereby B/I activated splenocytes were incubated for 24hrs in IL-7/15 (10ng/ml each, Peprotech Inc, Rocky Hill, NJ) then exposed to IL-2 for 24hrs and recultured in IL-7/15 for the remainder of the expansion period. The cells were allowed to proliferate in culture until peak growth was reached and were split every 2-3 days in order to maintain $1x10^6$ cells/ml concentration.

Bone Marrow Harvest and Collection of Peripheral Blood

Tibia and femur from euthanized mice were collected and flushed with complete RPMI to collect bone marrow. Red blood cells were lysed with a 1x ammonium chloride solution, washed twice with 1xPBS, brought to $1x10^6$/100uL and stained for MDSCs. Blood from the hearts of euthanized mice was collected into an anti-clotting solution (5uM EDTA in PBS), washed with FACS Buffer (2% FBS + 0.1% sodium azide in PBS), resuspended in 100uLs FACS buffer and stained for MDSCs. After staining, red blood cells were lysed with 1x ammonium chloride solution. Cells were fixed in 2% paraformaldehyde for later analysis.

Staining for Myeloid Derived Suppressor Cells

Cells were brought to $1x10^6$ in 100ul and stained for 30 minutes with anti-mouse CD11b and anti-mouse Ly-6G/Ly-6C(Gr-1) (Biolegend, San Diego, CA). Unstained cells were used as a negative control and rat IgG_{2b},κ was used as the isotype control. Staining

with Cd11b alone or Gr-1 alone were used as single color positive controls. Stained cells were fixed with 2% paraformaldehyde and analyzed within 7 days of staining on an ELITE Beckman Coulter flow cytometer. 25,000 cells per sample were analyzed.

Interferon-γ Release Assay

Interferon γ (IFN-γ) release from tumor sensitized, B/I activated and expanded splenic lymphocytes in response to various stimulants was assayed using ELISA. The splenic lymphocytes were cultured in 24-well plates at $2x10^6$ cells/ml with either no stimulants, irradiated (10,000 rads) 4T1 mammary carcinoma cells, or irradiated MethA sarcoma (negative control). Lymphocyte to stimulator ratio was 10:1. After 24 hours of culture, supernatants were harvested and stored at -20°C until assayed using a murine IFN-γ ELISA kit (BD Biosciences Pharmingen, San Diego, CA). Results reported are the mean values of duplicate ELISA wells ± SD.

In vitro Assay of Gemcitabine Effect on 4T1 Mammary Carcinoma Cells

Two methods were used: 1) 4T1 mammary cells were cultured in 24 well plates as duplicates at a concentration of 200,000 cells/ml in media, 60ng/ml GEM, 300ng/ml GEM, or 1500 ng/ml GEM. Cells were stained 0.04% tryphan blue and counted at 0hr, 24hr and 48hr using a light microscope and a Neubauer type hemacytometer. Data shown are means of duplicate wells ± SEM. 2) MTT assay: 4T1 mammary carcinoma cells were treated with 0, 3.6ng/ml, 32.4ng/ml, or 97.2ng/ml GEM and incubated in 96 well plates for 48hrs and 72hrs at 2,000, 5,000, 10,000, and 20,000 cells per well. After incubation, 10ul

of MTT yellow tetrazolium salt was added and standard MTT assay protocol was followed, with absorption at 550nm (MTT Proliferation Kit I, Roche Applied Science, Indianapolis, IN). Data generated are means of duplicate wells ± SE.

Adoptive Immunotherapy Using GEM to Treat Recipients

Recipient BALB/c mice were inoculated with 50,000 4T1 cells in the flank and randomly divided into four treatment groups with 6 mice per group: untreated controls (CON), GEM treated (GEM ONLY), AIT treated (AIT ONLY), and GEM combined with AIT (GEM + AIT). GEM treatment was given on days 6 and 11 at a dose of 60mg/kg. AIT was given on day 7 via I.V. using 4T1 sensitized DLN cells at a dose of $50x10^6$ cells per mouse.

Adoptive Immunotherapy Using GEM to Treat Donors

BALB/c mice were injected in the flank with 50,000 4T1 cells. Mice were either treated with GEM (60mg/kg) or untreated on Day 20 and spleens were harvested 48hrs later. Mononuclear lymphocytes were isolated via ficoll density gradient centrifugation. Splenic lymphocytes were activated and expanded as above. Recipient BALB/c mice were inoculated in the flank with 10,000 4T1 cells. Three days later, mice were randomly divided into treatment groups with 6 mice per group: untreated controls (CON), CYP ONLY, CYP + AIT with untreated donor lymphocytes (CYP + AIT CON), and CYP + AIT with GEM treated donor lymphocytes (CYP + AIT GEM). CYP (100mg/kg) was given three days after tumor inoculation and AIT was given 24hrs after CYP treatment at a dose of $18x10^6$ T cells/mouse.

Statistics

Differences between two groups were analyzed with the student's t-test. Differences among more than two groups were assessed 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. Tumor growth experiments were repeated at least 2 times. *In vivo* experiments included three to six mice per group. *In vitro* assays were repeated at least twice. An $\alpha < 0.05$ was used throughout to determine significant differences.

Results

Early Gemcitabine Treatment inhibits 4T1 flank tumor growth in BALB/c Mice, reverses splenomegaly, and suppresses MDSC accumulation in the spleen.

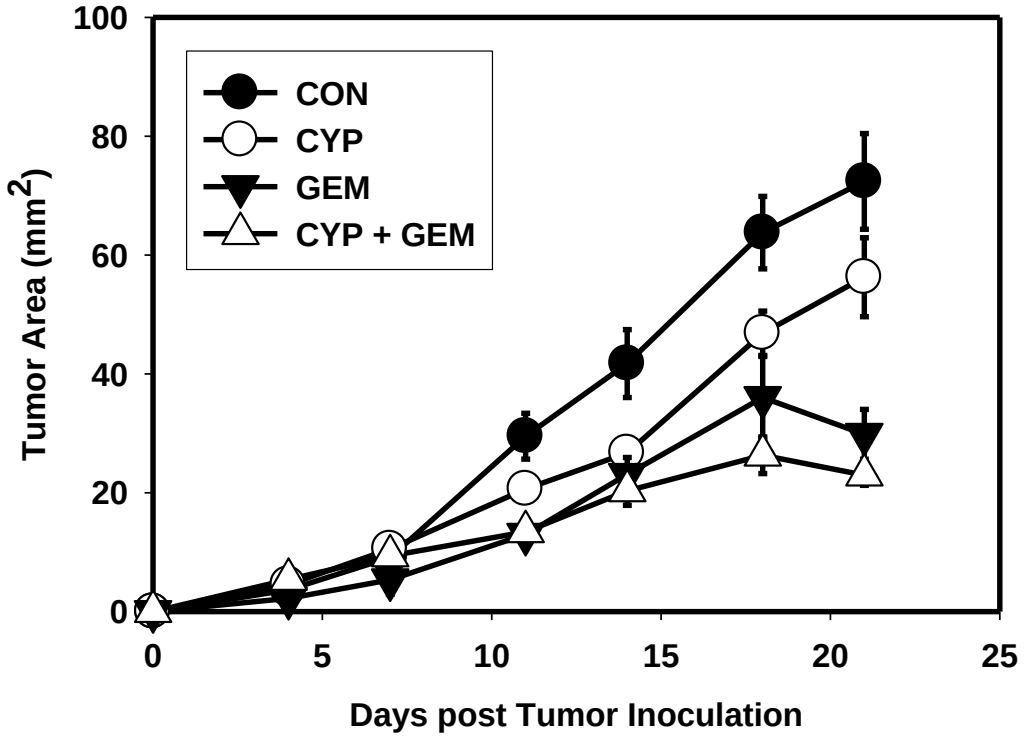
Sinha *et al.* reported that gemcitabine (GEM) treatment given one day after injection of 7000 4T1 cells into BALB/c mice resulted in delayed primary tumor growth and lower MDSC numbers in the blood of tumor bearing mice[77]. They argued that the decrease in MDSCs is a direct effect of GEM treatment, which then slowed tumor growth. Tumor cells secrete factors that recruit and induce the accumulation of MDSCs. Therefore, we questioned the validity of the conclusion drawn by Sinha *et al.* because GEM may directly inhibit tumor growth and thereby, indirectly inhibit MDSC accumulation as a secondary effect. With a small tumor dose and treatment 24hr after injection, the tumor cells have not had the time to adjust to the host environment and may,

therefore, be highly susceptible to GEM toxicity. We wanted to repeat the experiment to verify that the effects of GEM on tumor growth is measurable even after the tumor has been established, typically 5 days after injection, and at a higher dose of tumor inoculum. If GEM has no effect on established tumor growth, then its inhibitory influence on MDSCs must be direct. In addition, we wanted to investigate the combined effect of GEM and cyclophosphamide (CYP), a lymphodepleting drug used in our adoptive immunotherapy protocols. Therefore, BALB/c mice were injected subcutaneously with 50,000 4T1 cells in the flank and either treated with GEM (60mg/kg) on day 5, with CYP (single dose, 100mg/kg), with CYP and GEM, or left untreated. GEM was given once a week after the first dose and spleens were harvested at various times for analysis.

Like Sinha *et al.*, we observed delayed tumor progression in GEM treated animals, even at a higher dose of tumor inoculation and at a later time of GEM treatment. After 21 days of tumor growth, untreated controls had tumors that were 72.4±8.06mm^2 compared to 29.8±4.2 for the GEM treated group and 23.0±1.7 for CYP + GEM group (Fig. 6A). The difference in tumor area between the untreated group and groups treated with GEM was significant (p-value < 0.05). In tumor bearing mice, splenomegaly was observed with increasing tumor burden (Fig. 6B+C). In naive mice, spleen weight averaged 138.5±4.65mg with an absolute splenocyte count of 103.79±7.14 x10^6 and 2.81±0.28% MDSCs (average of 13 mice). In untreated tumor bearing mice, an increase of 200-300% can be observed in spleen weight and an increase of 300-700% in splenocyte numbers, depending on the size of the tumor. Accumulation of MDSCs was observed with increasing tumor burden so that by day 18, MDSCs made up 30% of the spleen cells in

untreated controls (Fig. 6D). The two groups treated with GEM showed a drop to 10% and 13% MDSCs in the spleen. The difference in MDSCs between control and GEM treated groups is even more dramatic for the absolute number of MDSCs (Fig. 6E). The combined treatment with GEM and CYP did not have an additive or synergistic effect on tumor progression. CYP alone had only a weak effect on tumor growth, and did not inhibit MDSCs and when combined with GEM, the results on tumor growth and MDSCs mirrored those of the GEM group.

Because gemcitabine inhibited both tumor growth and accumulation of MDSCs, it is difficult to make conclusions about the pharmacologic mechanism. If this drug is to be used to combat MDSCs then it is important to confirm that it in fact acts on these cells. We were left questioning whether or not the reduction of MDSC accumulation in tumor bearing BALB/c mice is caused by a direct effect of gemcitabine on MDSCs or by an indirect cytotoxic effect of the drug on 4T1 mammary carcinoma cells or both.


100
80
60
40
20
0
Tumor Area (mm2)
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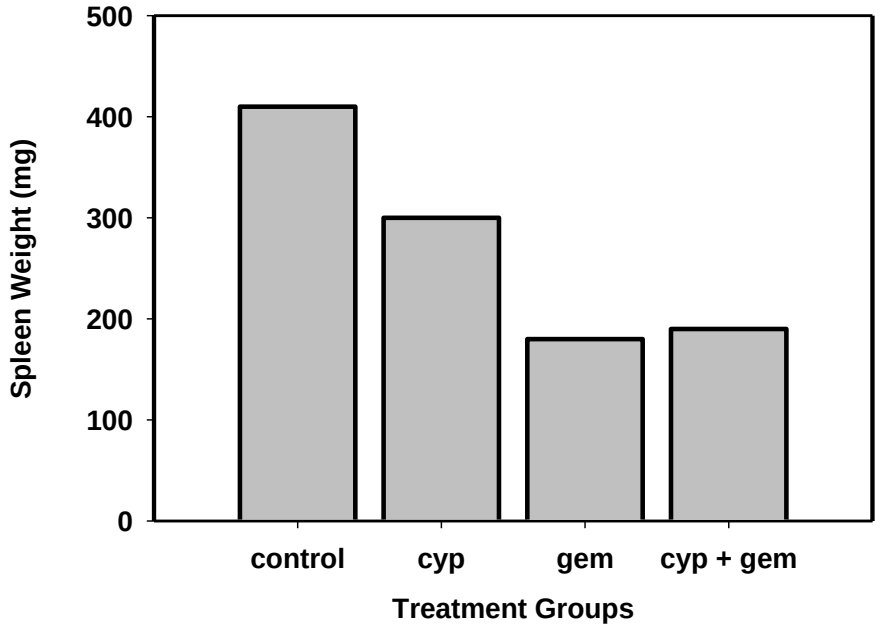
CYP
GEM
CYP + GEM
0
5
10
15
20
25
Days post Tumor Inoculation

6A.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100
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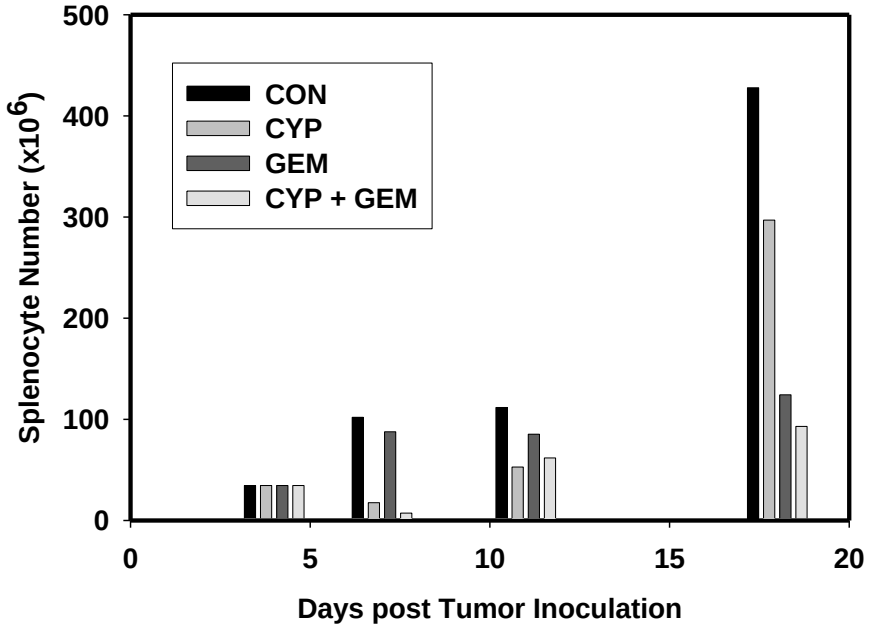
Spleen Weight (mg)
control
cyp
gem
cyp + gem
Treatment Groups

6B.


500
400
300
200
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Splenocyte Number (x10^6)
CON
CYP
GEM
CYP + GEM
0
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15
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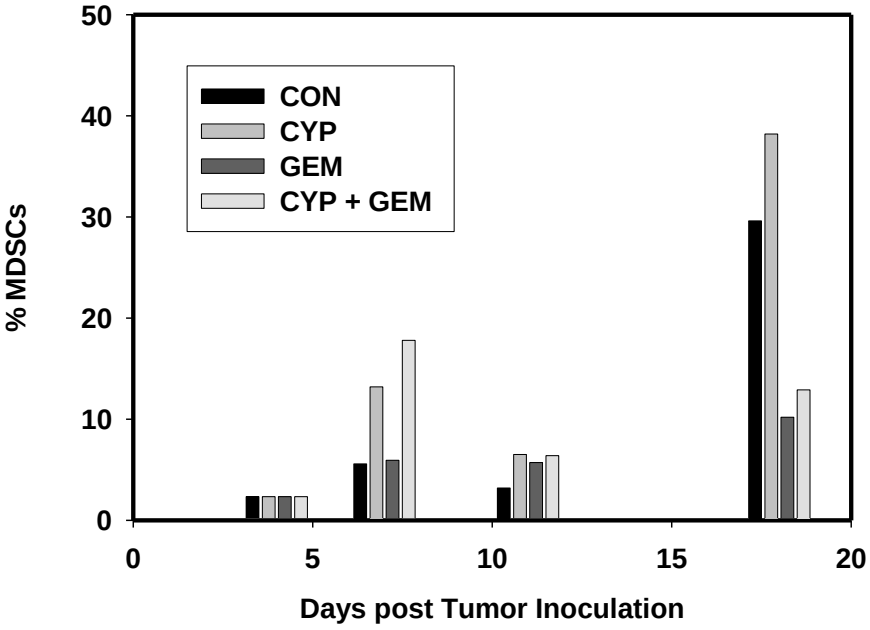
Days post Tumor Inoculation

6C.


50
40
30
20
10
0
% MDSCs
CON
CYP
GEM
CYP + GEM
0
5
10
15
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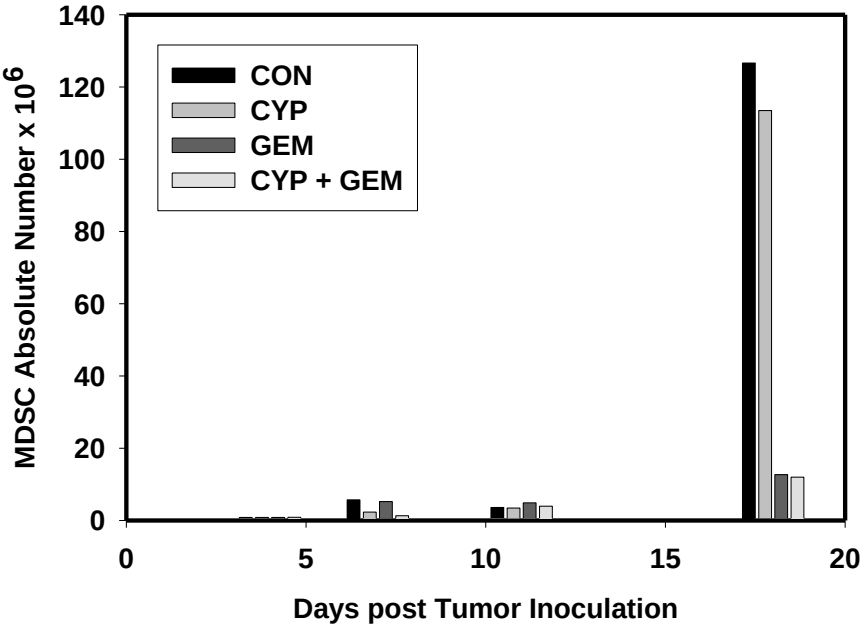
Days post Tumor Inoculation

6D.



6E.

Figure 6: Early gemcitabine treatment of BALB/c mice bearing established 4T1 flank tumors delays tumor growth, reverses splenomegaly, and inhibits MDSC accumulation in the spleen.
BALB/c mice were injected with 50,000 4T1 cells in the flank and were randomly assigned to one of five treatment groups: untreated controls (CON), cyclophosphamide (CYP) treated (2mg/dose), gemcitabine (GEM) treated (60mg/kg), and CYP+GEM. GEM treatments were given on days 5, 15, and 21. Spleens from representative animals were harvested on days 4, 7, 11, and 18. The spleens were weighed and stained for MDSCs using anti-CD11b antibody (FITC) and anti-GR-1 antibody (PE). Gemcitabine's effect on **(A)** tumor growth represented by area in mm^2±SE (5 mice per group)**, (B)** spleen weight on day 18, **(C)** absolute number of splenocytes, **(D)** percentage MDSCs in the spleen, and **(E)** absolute number of MDSCs are shown. Statistical analysis was performed on tumor areas measured on Day 18. The groups' mean tumor area were compared using ANOVA and found to be significantly different ($F(3,16) = 9.5$, p-value = 0.0008). Using Tukey's HSD, it was determined that the untreated control had significantly larger tumor than the GEM and GEM+CYP treated groups ($p < 0.05$). Within the three treated groups: CYP, GEM, CYP + GEM, there was no significant difference. Differences shown between control and GEM treated groups are typical of three independent experiments.

Gemcitabine Directly Inhibits 4T1 Mammary Carcinoma Cell Growth

To begin answering the question we posed, we tested the direct effects of gemcitabine on 4T1 mammary carcinoma cells *in vitro*. Duplicate wells of 4T1 cells were incubated for 24 and 48hr with a physiologically relevant concentration of GEM (300ng/ml) and doses 5-fold lower and higher. Viable cell numbers were counted via trypan blue exclusion (Fig. 7A). Untreated controls showed a 3-fold increase in numbers after 24 hours and after reaching confluence, cell numbers declined sharply. GEM treated groups showed a dose dependent inhibition of cell growth. Table 3 represents data from an MTT assay that verifies the results shown in figure 7. Again, untreated cells showed an increase in cell number over time, which is indicated by an increase in the optical density. GEM treated groups showed a dose dependent inhibition of proliferation for all starting concentrations of 4T1 cells. A concentration as little as 3.6ng/ml of GEM is enough to effectively prevent progression through the cell cycle. Because GEM is an antimetabolite, its inhibitory effect on cell division is expected, especially on rapidly dividing cancer cells. The results of these *in vitro* assays suggest that GEM acts directly on 4T1 cells and this inhibition on tumor growth may contribute to the decrease in MDSCs observed in treated mice.

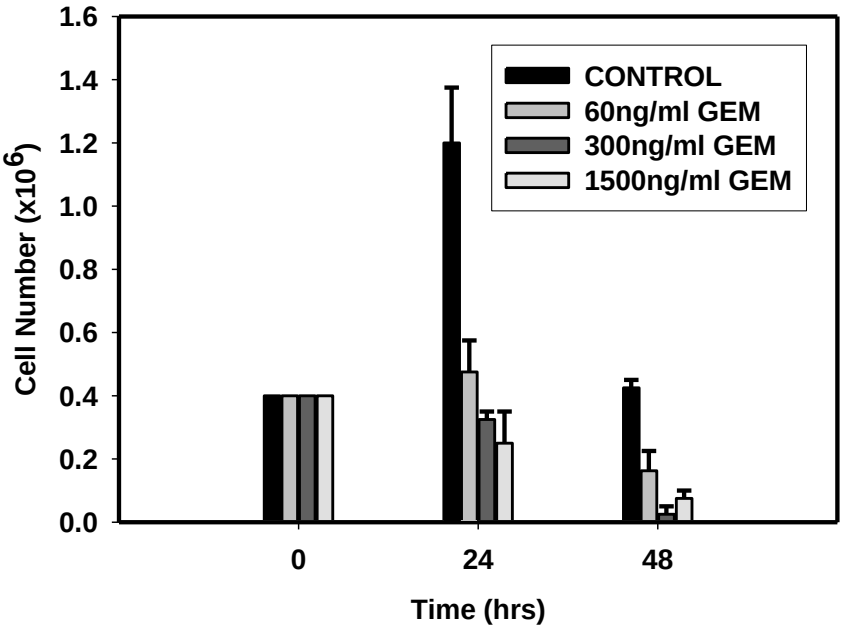


Figure 7: Gemcitabine Has a Direct Effect on 4T1 Mammary Carcinoma Cell Growth

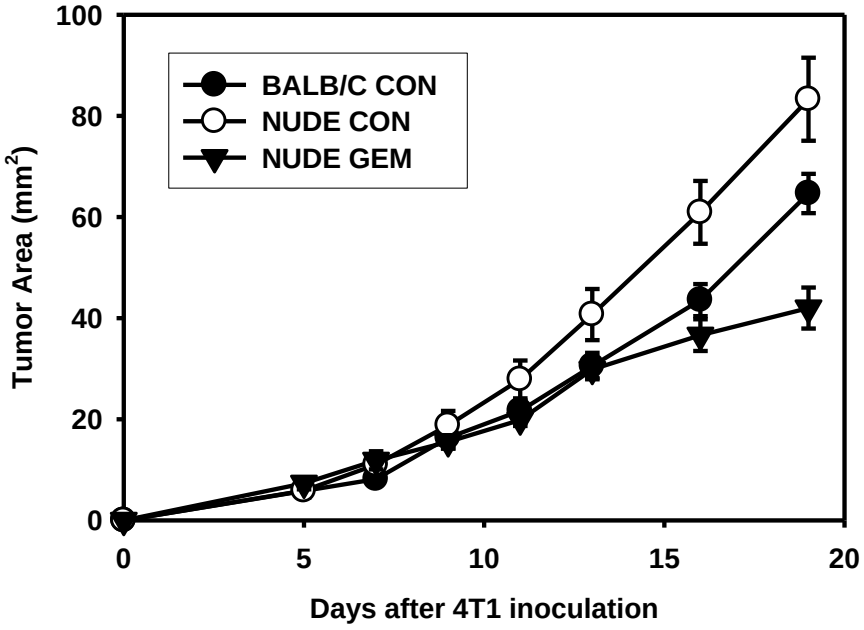
4T1 mammary carcinoma cells were cultured in 24 well plates in duplicates at a concentration of 200,000 cells/ml in media, 60ng/ml GEM, 300ng/ml GEM, or 1500 ng/ml GEM. Cells were counted at 0hr, 24hr and 48hr and data shown is mean cell number ± SE. Differences are typical of two independent experiments.

Table 3: MTT Assay of Gemcitabine Effect on 4T1 Mammary Carcinoma Cell Proliferation.
4T1 cells treated with 0, 3.6ng/ml, 32.4ng/ml, or 97.2ng/ml of GEM and incubated in 96 well plates for 48hrs at 2,000, 5,000, 10,000, and 20,000 cells per well. After incubation, 10ul of MTT yellow tetrazolium salt was added and standard MTT assay protocol was followed. Data shown is mean optical density±SE of duplicates.

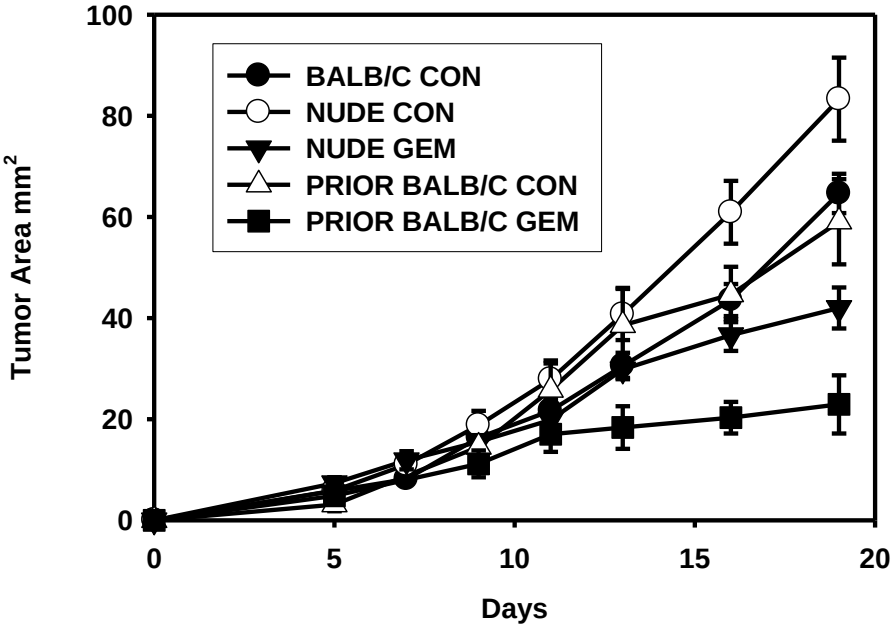
	2,000 4T1 Cells		**5,000 4T1 Cells**		**10,000 4T1 Cells**		**20,000 4T1 Cells**	
GEM	**48 hrs**	**72 hrs**	**48 hrs**	**72 hrs**	**48 hrs**	**72 hrs**	**48 hrs**	**72 hrs**
0 ng/ml	0.436±0.0130	0.914±0.0300	0.593±0.0500	1.201±0.0130	0.828±0.0540	1.488±0.0800	0.971±0.0600	1.567±0.0175
3.6 ng/ml	0.338±0.0075	0.278±0.0705	0.461±0.0060	0.517±0.0365	0.616±0.0095	0.629±0.0250	0.721±0.0300	0.735±0.0790
32.4 ng/ml	0.177±0.0160	0.124±0.0040	0.247±0.0070	0.161±0.0040	0.334±0.0225	0.210±0.0050	0.507±0.0125	0.324±0.0010
97.2 ng/ml	0.166±0.0015	0.116±0.0050	0.217±0.0025	0.163±0.0120	0.306±0.0190	0.172±0.0125	0.487±0.0300	0.296±0.0260

The Inhibitory Activity of Gemcitabine on 4T1 Growth, Splenomegaly, and MDSC Accumulation in the Spleen of BALB/c Mice is Mediated by the Drug's Direct Effect on MDSCs.

Although GEM has a direct effect on 4T1 mammary carcinoma cells, it is possible that the chemotherapy drug also directly inhibits MDSCs. We hypothesized that if MDSCs were directly inhibited by GEM then immunosuppression of T cells would be lifted and the delayed tumor growth might result from an antitumor immune response. Therefore, we tested the effects of GEM on tumor growth in nude mice. Nude mice were inoculated with 50,000 4T1 cells and were either untreated or treated with GEM once a week starting on day 5. Untreated BALB/c mice injected with the same tumor inoculum were used for comparison of tumor kinetics. While there were no significant differences in tumor kinetics between BALB/c mice and nude mice, tumor growth was significantly delayed in nude mice that were treated with GEM (Fig. 8, Tukey's HSD, $p\text{-value}<0.05$). We superimposed the tumor growth curves in this experiment with a prior experiment performed in BALB/c mice following the same protocol. The tumor growth curves in BALB/c mice were the same in both experiments, allowing us to compare the extent of growth inhibition by GEM in nude mice versus BALB/c mice. GEM had similar potency against tumor growth in both nude and BALB/c mice, inhibiting growth by 2-fold and 2.6-fold, respectively. Thus, it appears as though the inhibitory effect of GEM on tumor progression when administered at Day 5 and then weekly is independent of T cells.


100
80
60
40
20
0
Tumor Area (mm2)
BALB/C CON
NUDE CON
NUDE GEM
0
5
10
15
20
Days after 4T1 inoculation

8A.

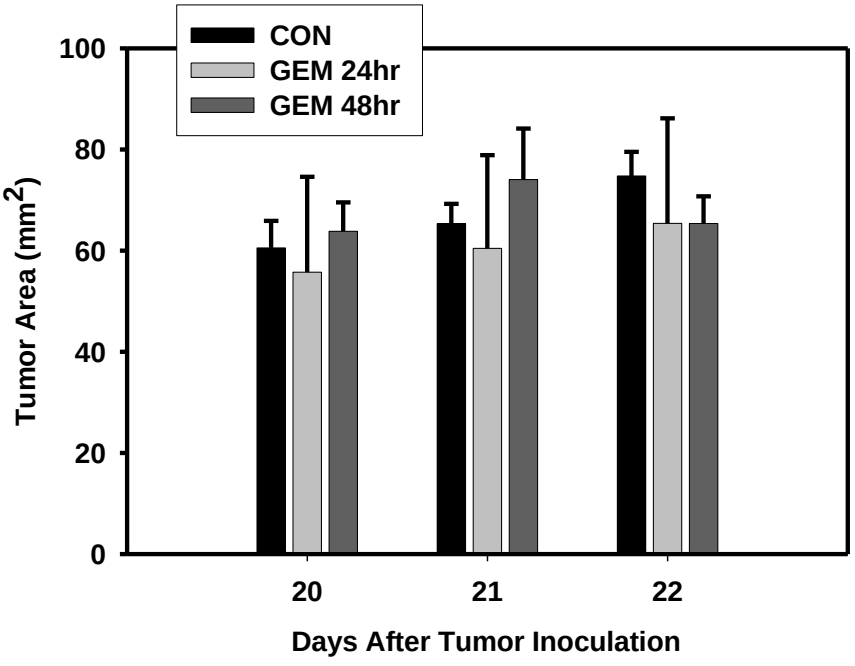

100
80
60
40
20
0
Tumor Area mm2
BALB/C CON
NUDE CON
NUDE GEM
PRIOR BALB/C CON
PRIOR BALB/C GEM
0
5
10
15
20
Days

8B.

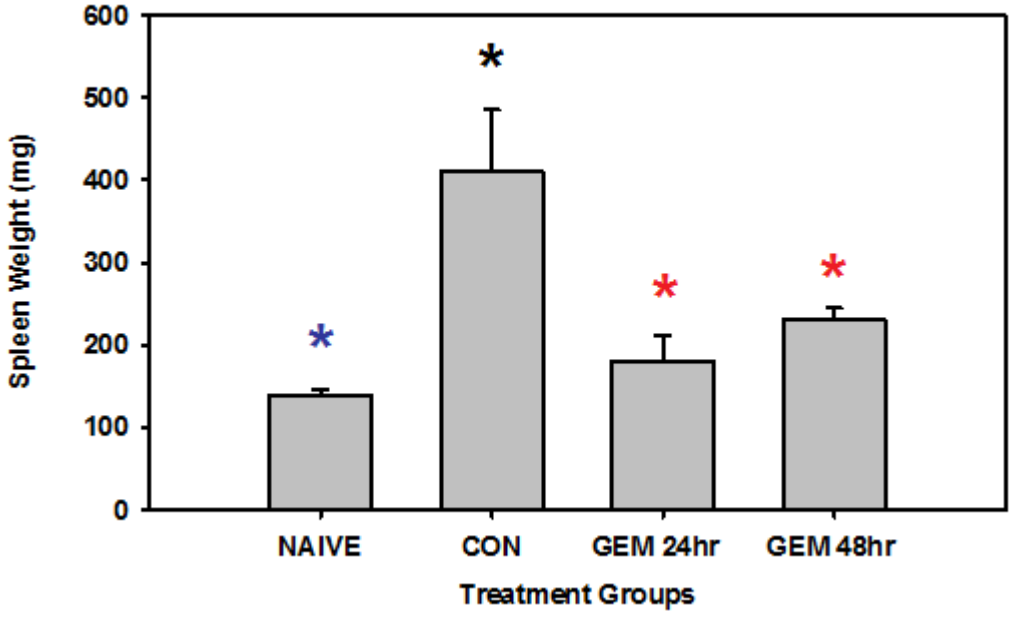
Figure 8: Gemcitabine inhibits 4T1 flank tumor growth in nude mice. Nude mice were injected with 50,000 4T1 cells in the flank and were randomly assigned to one of two treatment groups: untreated controls (CON) and gemcitabine (GEM) treated (160mg/kg). GEM treatments were given on days 5, 12, and 17. BALB/c mice given the same tumor inoculation without GEM treatment were used for comparison of tumor growth. **(A)** Gemcitabine's effect on tumor growth represented by area in $mm^2 \pm SE$ (6 mice/group). **(B)** Data from the same experiment performed in BALB/c mice (PRIOR BALB/C) were superimposed on data from nude mice experiment for comparison. Statistical analysis was performed on tumor areas measured on Day 19. ANOVA: $F(2,15) = 13.8$, p-value = 0.0004.

To continue investigating whether or not GEM directly inhibits MDSCs, we allowed 4T1 flank tumors to grow to a large size, ≥60mm^2, before treating with a single dose of GEM (60mg/kg). Spleens were harvested 24 hr or 48 hr after treatment. We hypothesized that within this short period of time tumor size would not change significantly. Therefore, if gemcitabine administered only once at 3 weeks of tumor growth decreased MDSC proportion in the spleen, it is likely to be through a direct effect on these cells and not an indirect effect caused by inhibition of tumor growth. GEM treatment for as little as 24hr was enough to reverse the splenomegaly observed in untreated controls and decreased the proportion and absolute number of MDSCs by 172% and 664%, respectively (Fig. 9). This inhibition on MDSCs occurred even though there was no significant difference in tumor size between untreated and treated groups 24 hr and 48hr after treatment (Fig. 9 + 10).

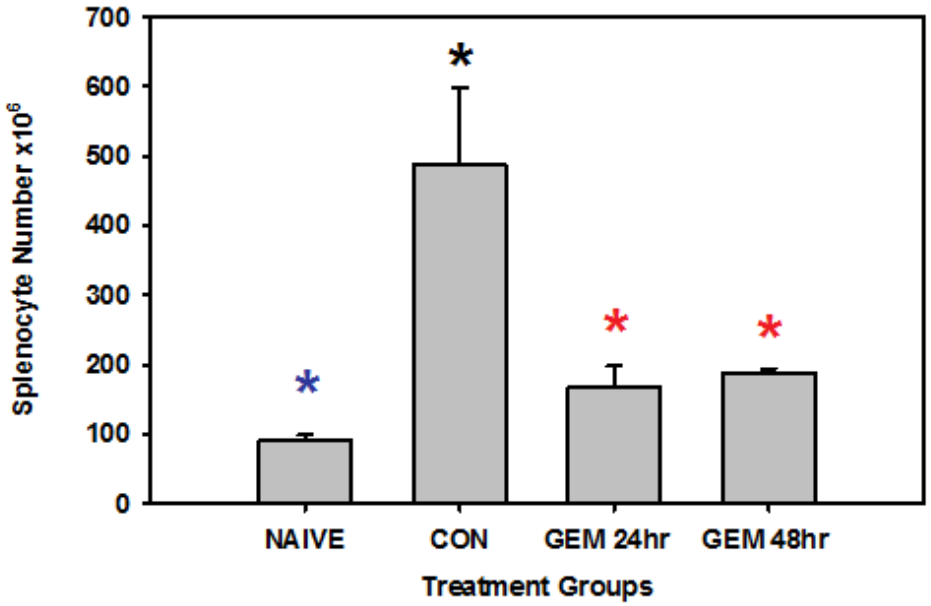
In addition to the spleen, MDSCs accumulate in the bone marrow and peripheral blood of tumor bearing mice. We examined at the bone marrow and peripheral blood to determine whether or not the influence of GEM on MDSCs is widespread. In the bone marrow and peripheral blood, MDSCs make up 32.6±2.19% and 23.2±3.79%, respectively (Fig. 10). MDSCs accumulate in the bone marrow to 78.8±3.29% and peripheral blood to 76.4±2.03% in tumor bearing mice, an increase of greater than 100%. Although less effective in the bone marrow, GEM decreased MDSC development here as well as the peripheral blood.


CON
GEM 24hr
GEM 48hr
100
80
60
40
20
0
Tumor Area (mm2)
20
21
22
Days After Tumor Inoculation

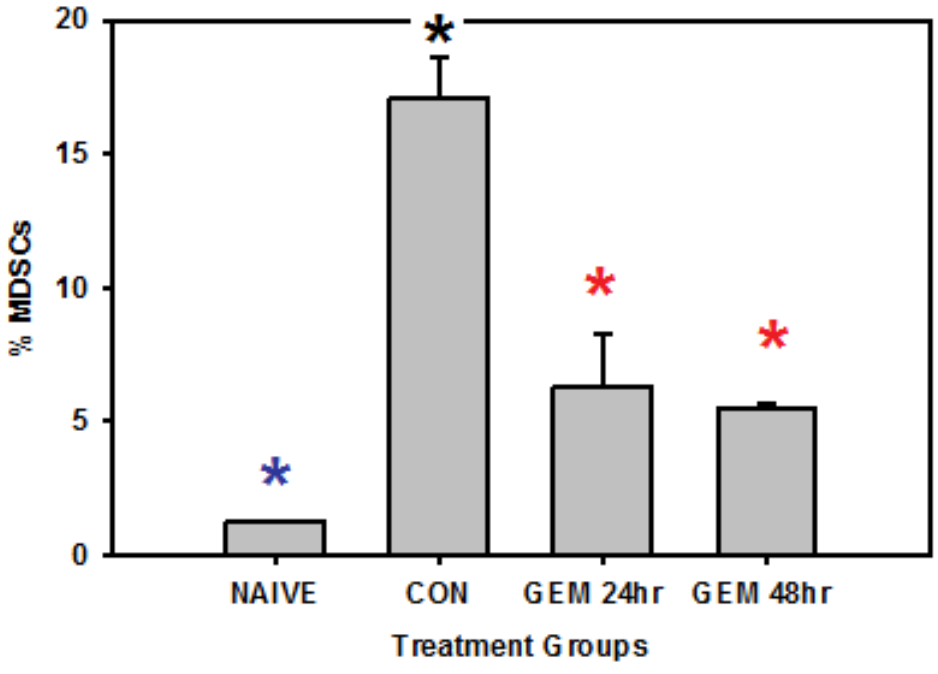
9A.


600
500
400
300
200
100
0
Spleen Welght (mg)
*
*
*
*
NAIVE
CON
GEM 24hr
GEM 48hr
Treatment Groups

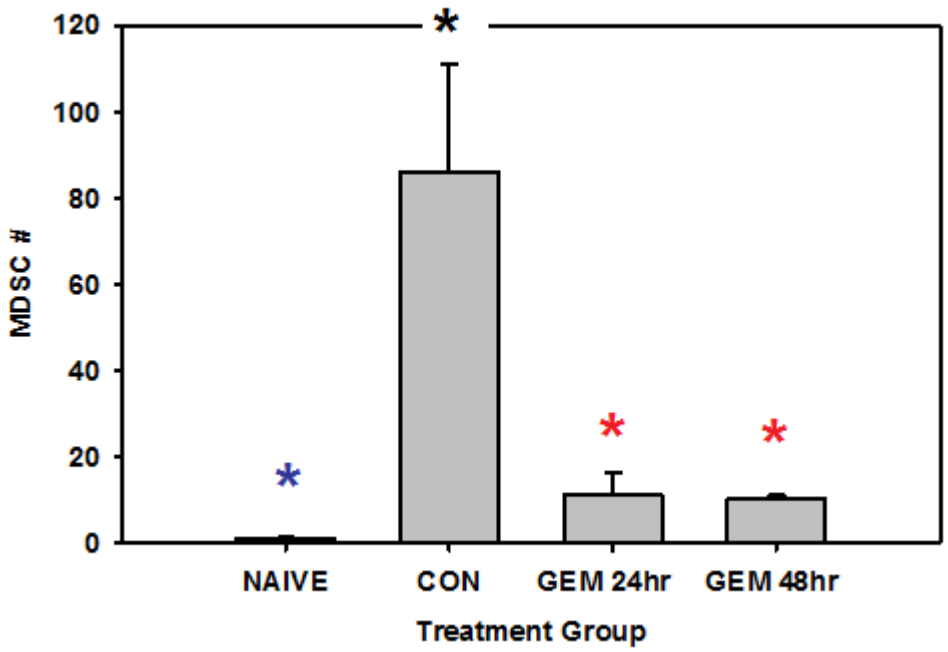
9B.


700
600
500
400
300
200
100
0
Splenocyte Number x10^6
NAIVE
CON
GEM 24hr
GEM 48hr
Treatment Groups

9C.

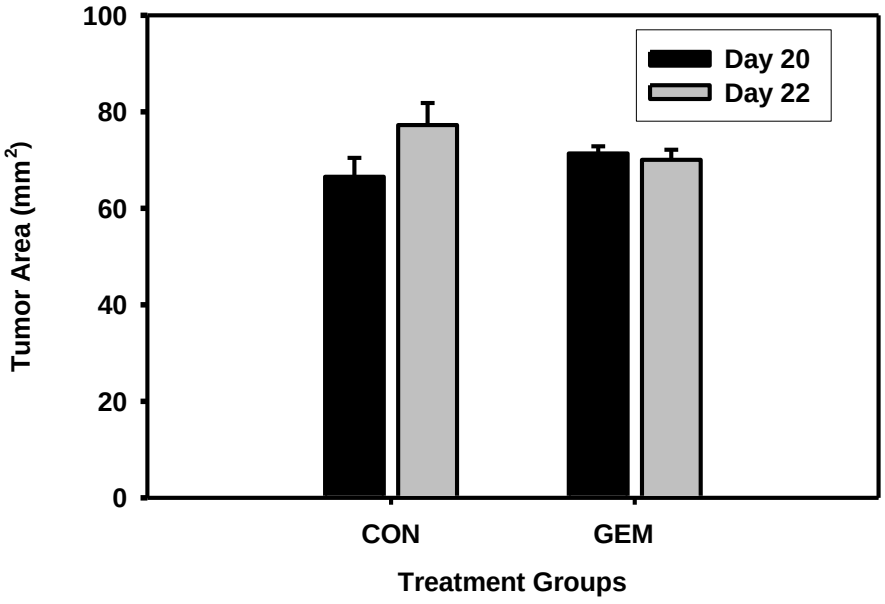

20
15
10
5
0
% MDSCs
NAIVE
CON
GEM 24hr
GEM 48hr
Treatment Groups

9D.

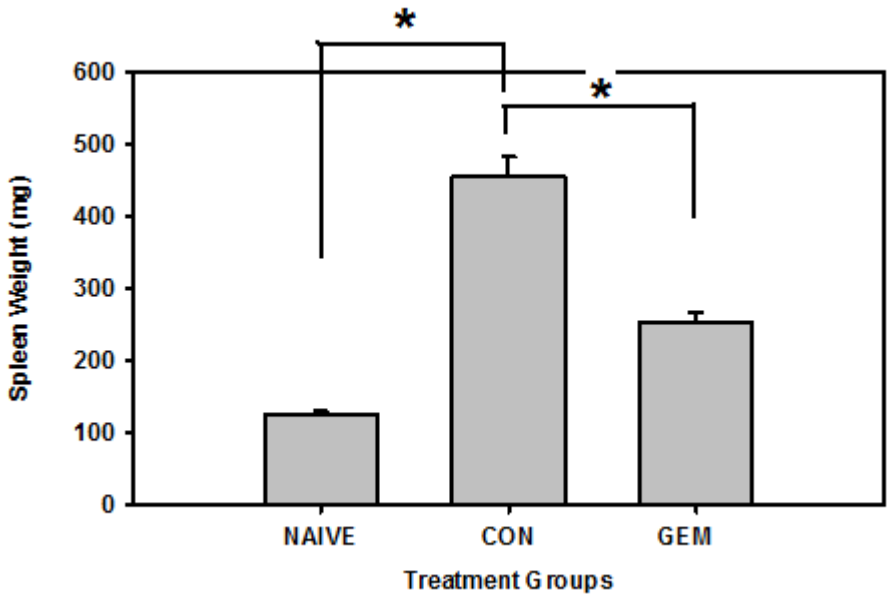


9E.

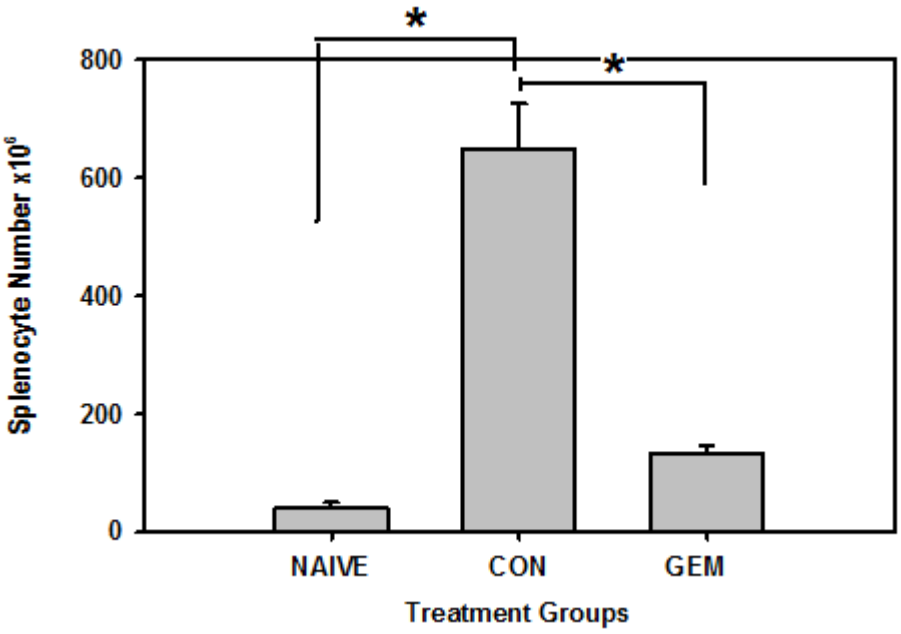
Figure 9: Late Treatment of Gemcitabine Reverses Splenomegaly and Suppresses MDSC Accumulation in the Spleen, Despite No Effect on Tumor Size.
BALB/c mice were injected with 50,000 4T1 cells in the flank and were randomly assigned to one of three treatment groups: untreated controls (CON), GEM treatment for 24hrs(GEM 24hr, 60mg/kg), and GEM treatment for 48hr (GEM 48hr). 3 tumor-free mice were used as naive controls (NAIVE). GEM was given once on day 20 to the 48hr group and on Day 21 to the 24hr group. Spleens were harvested on day 22. Tumor size was measured on days 20, 21, and day 22. The spleens were weighed and stained for MDSCs using anti-CD11b antibody (PE, 5uL) and anti-GR-1 antibody (FITC, 1.25uL). Gemcitabine's effect on **(A)** tumor growth, **(B)** spleen weight, **(C)** absolute number of splenocytes, **(D)** percentage MDSCs in the spleen, and **(E)** absolute number of MDSCs calculated as the product of C and D are shown. Statistical analysis was performed on day 22 measurements. Data are shown as mean±SE (3 mice/group). Differences between untreated control and mice treated with late GEM regimen are representative of three independent experiments. **(A)** $F(2, 3.6) = 0.74$, p-value = 0.5327, **(B)** $F(3,3.7) = 11.0$, p-value = 0.0258, **(C)** $F(3,4.0) = 25.4$, p-value = 0.0048, **(D)** $F(3,8) = 28.3$, p-value = 0.0001. **(B-D)** Mean values of NAIVE, GEM 24hr, and GEM 48hr were significantly different from CON as determined by Tukey-Kramer HSD. Asterisks of different colors represent significant differences between the means as determined by Tukey-Kramer HSD (p-value < 0.05).


100
80
60
40
20
0
Tumor Area (mm2)
Day 20
Day 22
CON
GEM
Treatment Groups

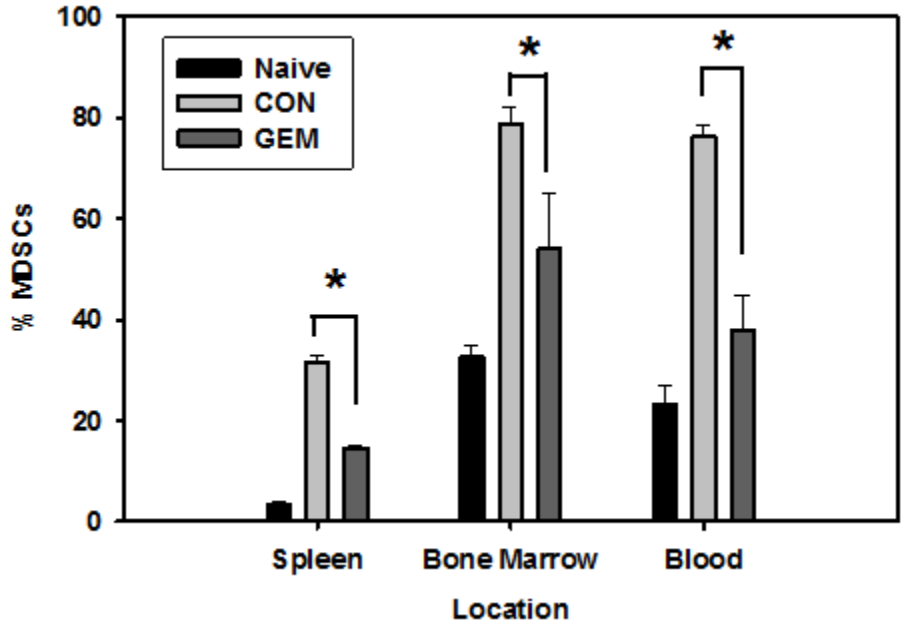
10A.


*
*
600
500
400
300
200
100
0
Spleen Weight (mg)
NAIVE
CON
GEM
Treatment Groups

10B.


*
*
800
600
400
200
0
Splenocyte Number x10^6
NAIVE
CON
GEM
Treatment Groups

10C.


100
80
60
40
20
0
% MDSCs
Naive
CON
GEM
*
*
*
Spleen
Bone Marrow
Blood
Location

10D.

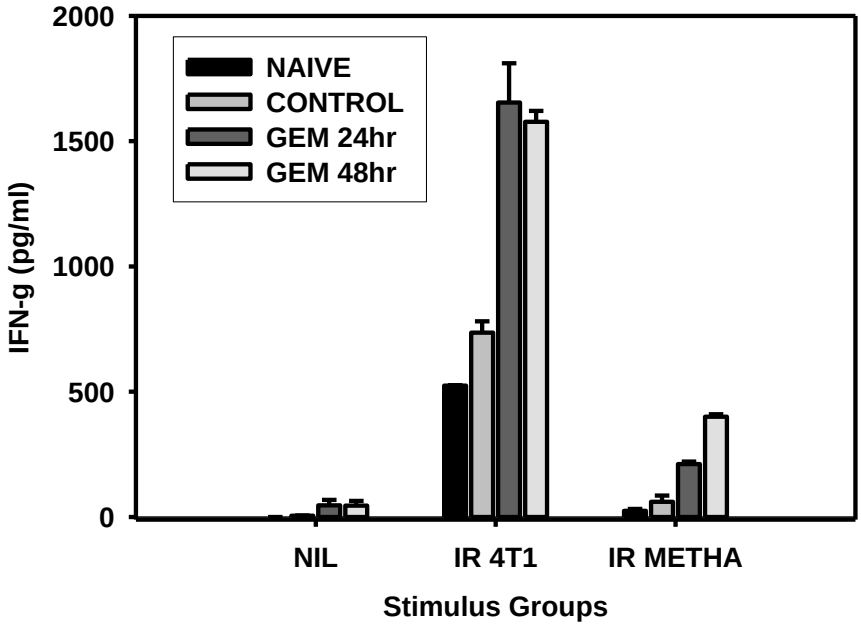
Figure 10: Late Treatment of Gemcitabine inhibits MDSC accumulation in the spleen, bone marrow, and blood.
BALB/c mice were inoculated in the flank with 4T1 mammary carcinoma cells. 20 days later, mice were randomly assigned to untreated control group (CON) or GEM treated group (GEM). GEM was given I.P. at 60mg/kg. Three mice were used as tumor free controls (NAIVE). Spleens, bone marrow and blood was harvested 48hrs after GEM treatment. GEM effect on **(A)** tumor area, **(B)** spleen weight, **(C)** splenocyte number, and **(D)** MDSC proportion in spleen, bone marrow, and blood. **(A+B)** Mean±SE (6 mice/group). **(C+D)** Mean±SE (3 mice/group). **(A)** two-tailed student's t-test. Day 20: $t = 1.16$, $df = 6.41$. p-value = 0.2890, Day 22: $t=1.43$, $df = 7.02$, p-value = 0.1950 **(B)** ANOVA: $F(2,12) = 54.0$, p-value < 0.0001 **(C)** ANOVA: $F(2,6) = 54.2$, p-value = 0.0001 **(D)** spleen: $F(2, 3.4) = 293.5$, p-value = 0.0002, bone marrow: $F(2,3.5) = 29.1$, p-value = 0.0008, blood: $F(2, 3.4) = 70.0$, p-value = 0.0018. Asterisks indicate significant differences.

Gemcitabine Treatment of Tumor Bearing Mice Helps Restore CD8+ T cell Immune Function

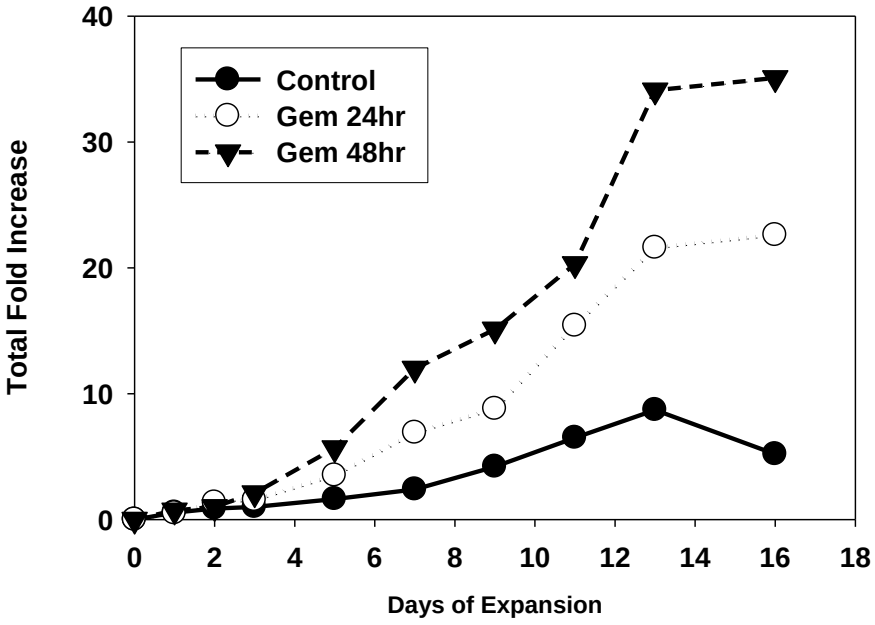
MDSCs inhibit T cells through various direct and indirect mechanisms [70, 72-76, 79-81]. If GEM inhibits MDSCs, then immunosuppression of T cells should be abrogated by GEM. We decided to investigate MDSC suppression of activated T cell proliferation and response to tumor antigen. Splenocytes harvested from untreated and GEM treated mice bearing large tumors were enriched for mononuclear lymphocytes through ficoll density gradient centrifugation. Splenic lymphocytes were cultured for 24hrs with media, irradiated 4T1 mammary carcinoma cells, or irradiated Meth-A sarcoma cells. Meth-A sarcoma cells were used as a negative control for these 4T1 primed T cells, which should respond to 4T1 cells and secrete interferon- γ (IFN-γ). Naive splenic lymphocytes were also used as a negative control. These unsensitized T cells should have little to no specificity for 4T1 cells. Splenic T cells from untreated, tumor bearing mice had dampened responses to antigen stimulation so that IFN-γ concentrations were as low as those of unsensitized, naive T cells (Fig. 11A). This tolerance was lifted when GEM was given to tumor bearing mice.

Splenic lymphocytes from mice in this experiment were also activated with bryostatin-1/ionomycin plus IL-2, and expanded with an alternating regimen of IL-7/15 and IL-2 cytokines. Our preliminary data showed that this alternating regimen resulted in the greatest expansion of T cells. Even at its peak of growth, on day 13, splenic lymphocytes from untreated tumor bearing mice had 150% less expansion than lymphocytes from mice treated with GEM 24hrs before harvest and 300% less than

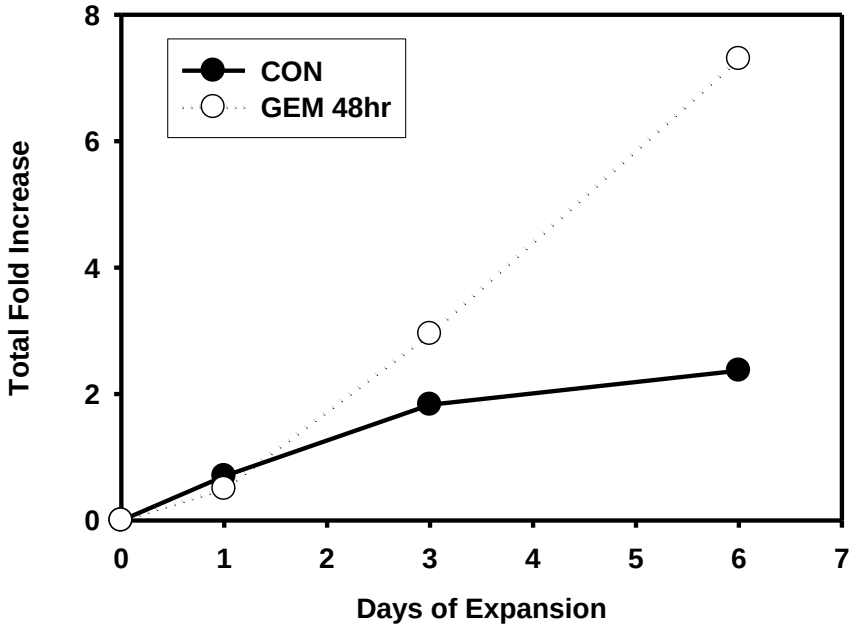
lymphocytes from mice treated with GEM 48hrs before harvest (Fig. 11B). After 16 days of expansion the difference in growth of T cells from mice that were untreated and those treated for 48hr with GEM reached >500%. To confirm this difference, splenic lymphocytes from the CON and GEM 48hr groups were expanded in IL-2 or IL-7/15 (Fig. 11C+D). A similar difference in proliferation was observed between the two groups. In addition, after 11 days of expansion away from the immunosuppressive influence of MDSCs, T cells from untreated tumor bearing mice were rescued from tolerance, secreting IFN-γ in response to 4T1 cells, but still at lower levels comparable than T cells from mice treated with GEM for 24hrs (Fig. 11E).


2000
1500
1000
500
0
IFN-g (pg/ml)
NAIVE
CONTROL
GEM 24hr
GEM 48hr
NIL
IR 4T1
IR METHA
Stimulus Groups

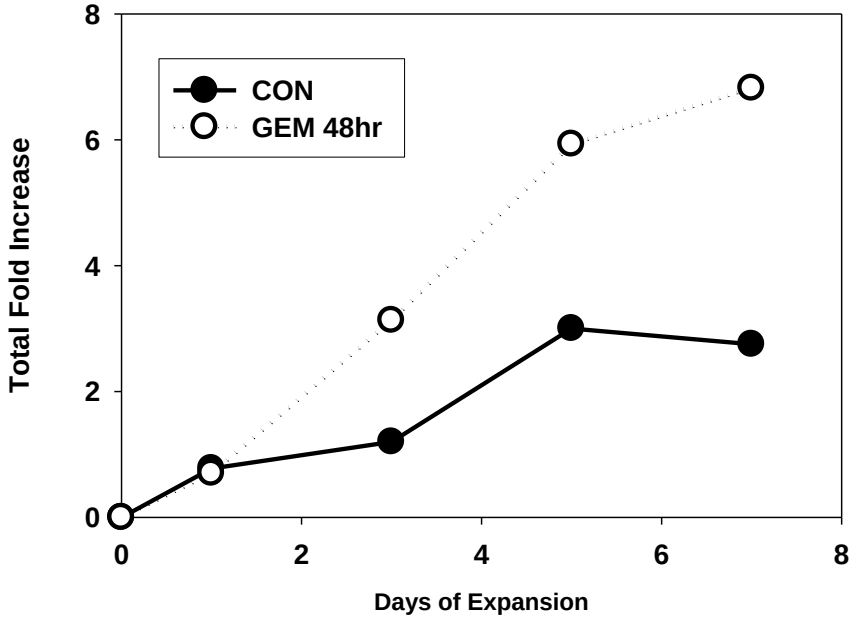
11A.


40
30
20
10
0
Total Fold Increase
Control
Gem 24hr
Gem 48hr
0
2
4
6
8
10
12
14
16
18
Days of Expansion

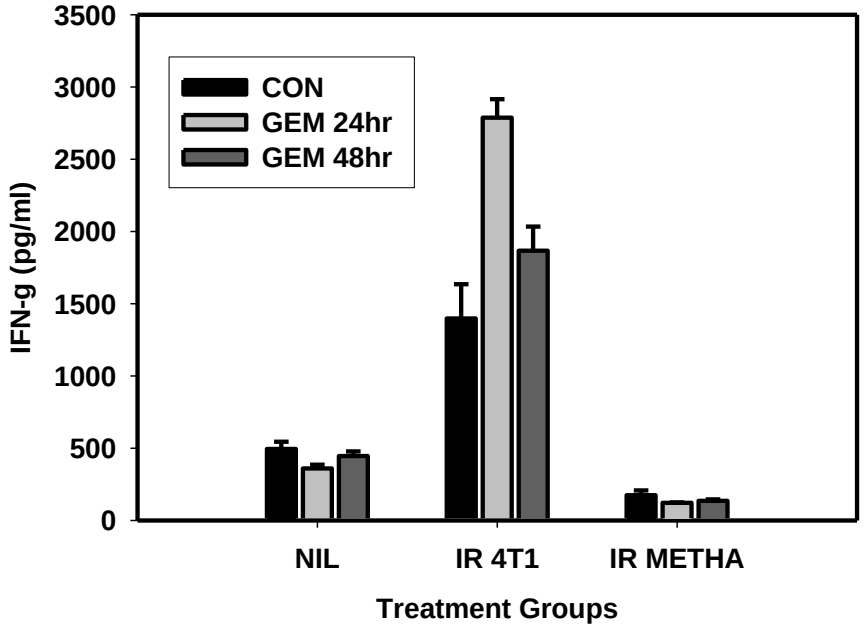
11B.


CON
GEM 48hr
Total Fold Increase
Days of Expansion
0
2
4
6
8
0
1
2
3
4
5
6
7

11C.


CON
GEM 48hr
Total Fold Increase
Days of Expansion
0
2
4
6
8
0
2
4
6
8

11D.



11E.

Figure 11: **Gemcitabine treatment augments splenic T cell expansion *in vitro* and restores T cell ability to respond to antigenic stimulus.**
BALB/c mice were injected with 50,000 4T1 cells in the flank and were randomly assigned to one of three treatment groups: untreated controls (CON), GEM treatment for 24hrs (GEM 24hr, 60mg/kg), and GEM treatment for 48hr (GEM 48hr). GEM was given once on day 20 for the 48hr group and on Day 21 for the 24hr group. Spleens were harvested on day 22. Three tumor-free mice were used as naive controls (NAIVE). Splenocytes were enriched for lymphocytes by ficoll density gradient centrifugation or by passage through a nylon wool column. **(A)** Splenic lymphocytes were incubated with (irradiated 4T1 cells or irradiated MethA cells) or without stimulus for 24 hours and IFN-γ secretion was measured pre-expansion. **(B)** Splenic lymphocytes were pulsed for 18hr in B(5nM)/I(1uM) + IL-2 (80U/ml) then expanded using the alternate cytokine regimen, where cells were expanded on day 1 in IL-7/15 (10ng/ml) then exposed to IL-2 (40U/ml) on day 2, and placed back in IL-7/15 on day 3. Differences between untreated and treated mice are typical of two independent experiments. **(C)** Splenic lymphocytes from CON and GEM 48hr group expanded in IL-2 (40U/ml). **(D)** Splenic lymphocytes from CON and GEM 48hr group expanded in IL-7/15 **(E)** Splenic lymphocytes were incubated with (irradiated 4T1 cells or irradiated MethA cells) or without stimulus for 24 hours and IFN-γ secretion was measured on day 11 of expansion. Data is shown as mean ± SD **(A+E)**. Results typical of two independent experiments.

Successful Adoptive Immunotherapy Partly Depends on Administration of a Large Dose of Tumor Specific T cells

We have shown that splenic T cells from tumor bearing mice that have been treated with GEM expand to a greater extent in response to mitogenic cytokines than untreated tumor bearing mice and demonstrate greater antitumor function *in vitro*. We next questioned whether or not this GEM rescued T cell function can inhibit tumor progression *in vivo*. Thus, an AIT experiment was performed using GEM treated and untreated BALB/c mice bearing large 4T1 tumors as donors (Fig. 12). Tumor growth in mice treated with AIT + CYP showed similar kinetics as tumor growth in mice treated with CYP alone ($p>0.05$). Thus, AIT with $18x10^6$ T cells harvested from GEM treated and untreated BALB/c mice bearing 22 day old tumors was ineffective. The results of this experiment suggest that with all other variables remaining constant, a larger dose of T cells may be necessary to exert a significant inhibition on tumor progression. This will have to be tried in future experiments.

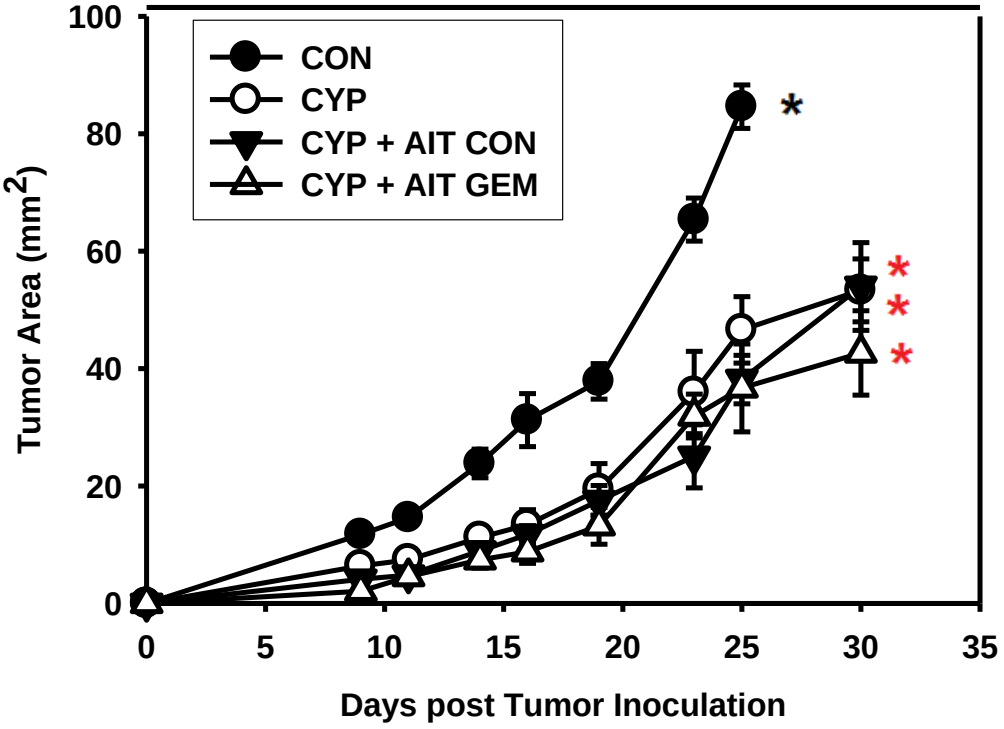


Figure 12: AIT with T cells from mice with 22 day old 4T1 tumors at a dose of $18x10^6$ T cells does not inhibit 4 day old 4T1 tumors.
BALB/c mice were injected in the flank with 50,000 4T1 cells. Mice were either treated with GEM (60mg/kg) or untreated on Day 20 and spleens were harvested 48hrs later. Mononuclear lymphocytes were isolated via ficoll density gradient centrifugation. Splenic lymphocytes were activated and expanded as in Fig. 11B. Recipient BALB/c mice were inoculated in the flank with 10,000 4T1 cells. Three days later, mice were randomly divided into treatment groups: untreated controls (CON), CYP ONLY, CYP + AIT with untreated donor lymphocytes (CYP + AIT CON), and CYP + AIT with GEM treated donor lymphocytes (CYP + AIT GEM). CYP (100mg/kg) was given three days after tumor inoculation and AIT was given 24hrs after CYP treatment at a dose of $18x10^6$ T cells/mouse. Data shown is mean tumor area ± SE (6-12 mice/group). A significant difference in mean tumor area was found using ANOVA ($F(3,26) = 11.7$, p-value < 0.0001). Asterisks of different colors represent significant differences (Tukey-Kramer HSD, p-value <0.05).

Rapid Tumor Kinetics Inhibit Therapeutic Effects of Combination Chemoimmunotherapy

We have attempted to use GEM to augment the immune function of donor T cells. Similarly, GEM might also be used to improve immune function in the recipient. By inhibiting MDSC accumulation in the recipient, GEM treatment might prevent MDSC mediated suppression of T cell immunity and thereby, increase antitumor immunity after AIT. Therefore, we performed an AIT experiment in combination with GEM treatment (Fig. 13). AIT, under the conditions we have set, did not inhibit tumor growth without GEM. AIT with GEM inhibited tumor growth, but this inhibition was caused by the direct tumoricidal effects of GEM and not by antitumor activities of transferred T cells.

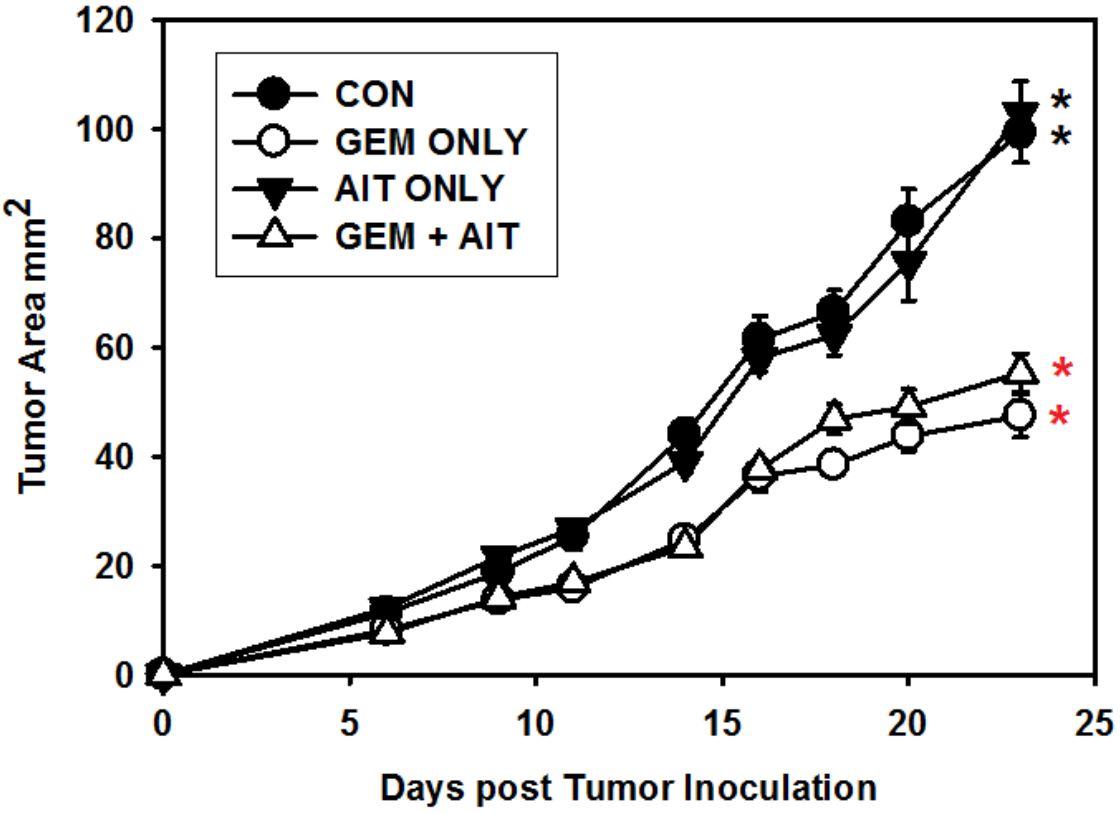


Figure 13: AIT in Combination with early treatment of GEM is Ineffective against 7 Day Old 4T1 Flank Tumors Given at an Initial Dose of 50,000 4T1 cells/mouse. Recipient Balb/c mice were inoculated with 50,000 4T1 cells in the flank and randomly divided into four treatment groups: untreated controls (CON), GEM treated (GEM ONLY), AIT treated (AIT ONLY), and GEM combined with AIT (GEM + AIT). GEM treatment was given on days 6 and 11 at a dose of 60mg/kg. AIT was given on day 7 using 4T1 sensitized DLN cells at a dose of $50x10^6$ cells per mouse. Data shown is mean tumor area ± SE (6 mice/group). A significant difference in mean tumor area was found using ANOVA ($F(3,20) = 36.8$, p-value<0.0001). Asterisks of different colors represent significant differences (Tukey-Kramer HSD, p-value <0.05).

Conclusions

Our results demonstrate that GEM caused delayed tumor growth and suppression of MDSCs, in a T cell independent manner (Fig. 6-8). We performed follow up experiments to determine whether or not the elimination of MDSCs was a direct effect of the drug or an indirect effect that resulted from an inhibition of tumor growth. We found that GEM acts on both cell populations. GEM inhibited 4T1 mammary carcinoma cell growth in a dose dependent manner and directly inhibited MDSCs, in a manner independent of its effect on tumor growth (Fig. 7, Table 3). The latter conclusion is based on the observation that late gemcitabine treatment of 20+ day tumor bearing BALB/c mice abrogated splenomegaly, reduced MDSC proportion in the spleen, bone marrow and blood, and restored T cell proliferation and effector function, despite little change in tumor size (tumor size was not greatly affected with just 24hr-48hr gemcitabine treatment) (Fig. 9, 10, 11). AIT performed in conjunction with GEM treatment of donors or recipients was not successful as expected and reveal technical problems that need to be addressed in the future.

{CHAPTER 5: Discussion}

Employing Alternate Common Gamma Chain Cytokines for T cell Expansion

IL-2 is the primary cytokine that drives the induction of cellular immunity. Therefore, it is the prototypical cytokine used for the expansion of CD8+ T cells. Supporting this status quo, Carrio *et al.* showed that IL-2 always induced greater proliferation and IFN-γ secretion than IL-7 or IL-15 alone [57]. This difference is partly due to the preferential selection of effector T cells by IL-2 and selection of memory T cells by IL-7 and IL-15[50, 57]. Mice treated with IL-7 or IL-15 in conjunction with tumor antigen showed exaggerated contraction of the effector pool which led to improved survival of the much smaller memory pool. On the other hand, mice treated with IL-2 showed inferior contraction of the effector pool which may account for the much greater number of T cells observed with IL-2 stimulation. Despite this, our preliminary data contrast data presented by Carrio *et al.*, showing that IL-7 or IL-15 expands T cells to a greater than IL-2. The contradictory results may be caused by a difference in the protocol used to produce activated T cells. Carrio *et al.* activated T cells by pulsing splenocytes with specific peptide while our protocol employs pharmacological activation with bryostatin-1 and ionomycin. The different activation protocol used may result in a difference in T cell fitness. T cell fitness is defined by resistance to cell death and responsiveness to homeostatic cytokines. Withdrawal of antigen signaling causes death by neglect and continued T cell persistence requires survival signals through survival promoting receptors.

IL-7 and IL-15 are homeostatic cytokines that promote survival. The signal strength received through the TCR also plays a role in prolonging T cell survival both *in vitro* and *in vivo* by regulating the capacity of primed T cells to respond to homeostatic cytokines, to survive cytokine withdrawal, and to accumulate *in vivo*[45]. It is possible that T cell activation with bryostatin-1 and ionomycin produces stronger TCR signaling which increases the capacity of sensitized T cells to respond to homeostatic cytokines, like IL-7 and IL-15.

Melchionda *et al.* showed that IL-7 and IL-15 were comparable or better than IL-2 at expanding Ag sensitized CD8+ T cells when given as an adjuvant *in vivo* along with immunization[50]. There was no additive or synergistic effect when IL-7 and IL-15 were combined. In our *in vitro* B/I activation model, we have shown that the combination of IL-7 and IL-15 has an additive effect on expansion, increasing T cell numbers 8 to 9-fold over IL-2 (Fig.2). We found that this difference in expansion was partly due to the protective roles of IL-7 and IL-15 against apoptosis[47, 51, 52, 55, 56]. By day 6 of expansion, 40% of IL-2 expanded T cells were in apoptosis (Fig. 3). In contrast, only about 18% of IL-7/15 expanded T cells were apoptotic. The greater rate of apoptosis in IL-2 expanded cells may be the result of IL-2 mediated activation induced cell death[58, 59]. To strengthen this argument, future experiments should look for differences in expression levels of FasL and anti-apoptotic molecules, like Bcl-2 and Bcl-X_L, and pro-apoptotic molecules, like Bax.

Similar to B/I activation + IL-2 and culture in IL-2, expansion with IL-7/15 also selects for T cells (Table1). After activation with B/I + IL-2 or IL-7/15, we saw an increase in CD44, a decrease in CD62L and an increase in CD69. CD44 is a marker for the memory cells. CD62L is a marker for effector cells and in combination with CD44 differentiate between effector memory and central memory T cells. CD69 is a marker for activated cells that is upregulated after T cell activation and is downregulated after 24 hours. As expected, we saw a decline in CD69 after Day 1. Several publications have noted that IL-2, IL-7, and IL-15 induce different subsets of T cells[48, 50, 54]. Both IL-7 and IL-15 are important in regulating background survival and turnover of central memory T cells ($CD44^{high}CD62L^{high}CD69^{-}$) while IL-2 maintains the effector phenotype ($CD44^{high}CD62L^{low}CD69^{+}$). Mechionda *et al.* found that, *in vivo*, IL-7 and IL-15 initially expanded effector T cell pools similar to IL-2, but this expansion resulted in long-term development of antigen specific memory T cells[50]. Similar to their results, we found that IL-7/IL-15 initially induced similar T cell subsets as IL-2 (Table 1). These cytokines selected for effector memory CD8+ T cells ($CD44^{high}CD62L^{low}$), which displayed similar phenotypic and functional properties as effector cells but persist longer after Ag clearance[57]. However, by day 3 we began to see a divergence and by day 6 there was a substantial difference in T cell subsets. IL-7/15 expanded cells maintained a central memory CD8+ T cells ($CD44^{high}CD62L^{high}$), which are functionally and phenotypically different from effector memory cells (T_{EM}). This induction of the central memory phenotype (T_{CM}) is desirable because less differentiated, central memory like T cells have

been dubbed the optimal population for AIT because of their greater fitness compared to effector cells[63]. On the other hand, IL-2 favored expansion of CD4+ T cells and T cells with an effector phenotype with a downregulation of the memory phenotype, which may help explain the cells' lack of persistence. Because IL-2 plays a pivotal role in the development of Treg cells (CD4+CD25+FOXP3+), it may be possible that the shift to CD4+ cells is indicative of an increase in Treg cells[46, 61, 62]. This would contribute to the impaired expansion of IL-2 stimulated cells compared to IL-7/15 stimulated cells. To confirm this, future experiments should look for FOXP3+ cells in addition to the other T cell subsets.

In addition, despite the increase in T_{CM} cells, we also found that IL-7/15 expanded cells increased their IFN-γ response to specific antigen stimulation over time while IL-2 cultured cells showed a decline in IFN-γ response (Fig. 4). Because IFN-γ is a product of activated CD8+ T cells, the change in the production of this cytokine in response to specific peptide may reflect the decline in the proportion of CD8+ T cells over time in IL-2 cultures (Table 1). Similarly, the maintenance of high antitumor responses by IL-7/15 expanded T cells on Day 3 and 7 reflect the persistence of a large, viable CD8+ T cell population in IL-7/15 cultures. In addition, it may reflect a transition from an effector memory to a central memory phenotype. Central memory cells have enhanced sensitivity to Ag that leads to a rapid activation of effector functions, which may explain our observed increase in response to Ag over time [57].

One of the concerns of *in vitro* expansion is that it shortens the lifespan of antigen-sensitized T cells *in vivo* after adoptive transfer. Repeated *in vitro* stimulation of human T cells resulted in progressive decrease in telomerase activity and shortening of telomeres [93]. This eventually led to replicative senescence and an impaired ability of the adoptively transferred T cells to mediate antitumor responses. In addition, *in vitro* conditions for generating large numbers of antigen specific T cells may alter the function of CD8+ T cells *in vivo*. In fact, a review by Gattinoni *et al.* suggested an inverse relationship of *in vitro* and *in vivo* antitumor function of adoptively transferred T cells [63]. The ability of IL-7/15 cells to persist in culture for a longer period of time might be indicative of their ability to persist *in vivo* (Fig. 2). Our results showed that IL-7/15 expanded cells were able to persist *in vivo* and mediated regression of pulmonary metastases in 100% of treated mice compared to 17% with the same number of IL-2 expanded cells. Although our treatment was therapeutic, it is difficult to tell whether or not it correlates with the differences observed in IL-2 and IL-7/15 cultures *in vitro*. Therefore, *in vivo* assays should be conducted to show that IL- 7/15 cells expand and persist better *in vivo* and maintain their potent central memory phenotype and functions.

We have shown that with alternate gamma chain cytokines, like the combination of IL-7 and IL-15, greater numbers of T cells with the same or greater antitumor efficacy as IL-2 expanded cells can be produced. This is invaluable for the success of AIT, which is dependent on both the quantity and quality of effector cells. The ratio of antigen specific T cells to tumor cells determines the kinetic balance between the rate of tumor growth and the strength and duration of the immune response. The ability to get large numbers of T

cells that can persist and respond to tumor *in vivo* in less time will help drive the development of more efficient and less costly treatment plans for cancer patients.

Abrogating Immunosuppression of Myeloid Derived Suppressor Cells with Gemcitabine

Even if large numbers of functional T cells are administered, adoptive T cell therapy may still be unsuccessful in controlling infection or malignancy, due to multiple mechanisms of tumor evasion. Conversely, tumor-induced immunosuppression may inhibit the production of T cells with therapeutic activity from the tumor bearing host. One such mechanism of immunosuppression is caused by myeloid derived suppressor cells (MDSCs), which accumulate in association with increasing tumor burden, leading to splenomegaly (Fig. 6). MDSCs reduce antigen specific CD8+ T cell proliferation, increase death by apoptosis, foster T cell tolerance, and change the profile of cytokines secreted by activated T lymphocytes[70, 72, 74, 76]. They exert their immunosuppressive effects primarily through the secretion of arginase I (ARG-1) and nitric oxide synthase, leading to L-arginine starvation, production of urea, generation of NO, and the creation of reactive nitrogen oxide and reactive oxide species (NOS-2) [70, 72, 75, 77-80]. In addition to direct inhibition of T cells through ARG-1 and NOS-2, MDSCs also indirectly inhibit development of cytotoxic T cells by driving the formation of alternate macrophages that favor a Th2 response and thereby, promote tumor progression[81]. MDSCs also inhibit T cell function by inducing the development of CD4+CD25+FoxP3+ T regulatory cells *in vitro* and *in vivo*[95]. This inhibition was dependent on IFN-γ stimulated secretion of IL-10

and transforming growth factor (TGF-β) from MDSCs. Thus, the induction of MDSCs represents a mechanism of tumor induced immune suppression that may be responsible for the inability of T cells from tumor bearing hosts to recognize tumor antigen, expand in vitro and/or eliminate tumor cells.

Depletion of MDSCs may help to create a better host environment that is more conducive to the activation of antitumor immunity after AIT and may rescue antitumor immunity in tumor bearing hosts. Gemcitabine has recently been reported to be an effective treatment against MDSCs without severely harming activated immune cells[81, 85, 96-97]. An investigation of the effect of GEM on immune cells in patients with pancreatic adenocarcinoma revealed that GEM decreased memory T cell function, but enhanced naive cell activation[97]. Treatment with GEM did not interfere with Th1-cell activation and production of Th1 cytokines but may inhibit Th2 immunity. Thus, while GEM does have some negative side effects, it seems to produce more positive than negative responses.

Before application against MDSCs, GEM was used against metastatic cancers as an FDA approved antimetabolite with tumoricidal effects[86]. This cytidine analogue acts during the S phase of the cell cycle. Thus, rapidly dividing cells, like cancer cells, are highly susceptible to the drug's impairment of DNA synthesis. If the tumor microenvironment induces myelopoeisis, it is possible that GEM would also act on MDSCs to inhibit their accumulation by inhibiting the formation of immature myeloid cells. In addition, GEM distributes to areas in which MDSCs have been known to collect, like the spleen, femur, and lymph nodes, which gives the drug the opportunity to operate

on this cell population. We have observed that in mice with established 4T1 flank tumors, GEM, administered soon after tumor inoculation and repeated once a week at a dose of 60mg/kg, delayed tumor progression, prevented splenomegaly, and suppressed MDSC accumulation in the spleens (Fig. 6). Our results verified the work by Sinha *et al*. who had similar data from the same 4T1 tumor model and work by Ko *et al*, who used a tolerogenic Her-2/neu induced tumor model[85, 96]. Both groups claimed that the inhibition of tumor progression was a result of direct inhibition of MDSCs by GEM. However, their experiments lacked rigorous evidence to support a direct effect of GEM on MDSCs. Because GEM has an influence on both tumor growth and MDSC numbers, it is difficult to determine which effect comes first. The purpose of our studies was to elucidate the mechanisms by which GEM exerted its pharmacologic effects on MDSCs. Our goal was to determine whether the observed suppression of MDSCs is a direct effect of the drug or an indirect result of cytotoxicity to tumor cells. Doing so may help determine whether GEM is a good treatment option for abrogating the immunosuppression caused by MDSCs, especially for tumors that are not susceptible to GEM's direct cytotoxic effect.

Figure 14 represents the ways in which GEM may act. In tumor bearing mice, the tumor cells secrete factors that lead to the development of MDSCs, which in turn, inhibit T cell function by the secretion of arginase-1 and nitric oxide synthase and indirectly by the induction of Treg cells and M2 macrophages (Fig. 14A). If GEM directly inhibits MDSC accumulation, we would see an abrogation of immunosuppression, which would allow the immune response to recover and impair tumor progression. Thus, any decrease in tumor

size after GEM administration would be a result of an immune response rescued by GEM treatment, acting directly on MDSCs. (Fig. 14B). On the other hand, if the drug directly inhibits tumor growth, it would prevent MDSC accumulation (Fig. 14C). Therefore, we would see a decrease in both tumor size and MDSC accumulation in treated animals. However, the inhibition of tumor progression would be predominantly a direct cytotoxic effect of GEM on tumor cells, not an immune mediated one. The last possible scenario is one in which GEM acts on both cell populations (Fig. 14D).

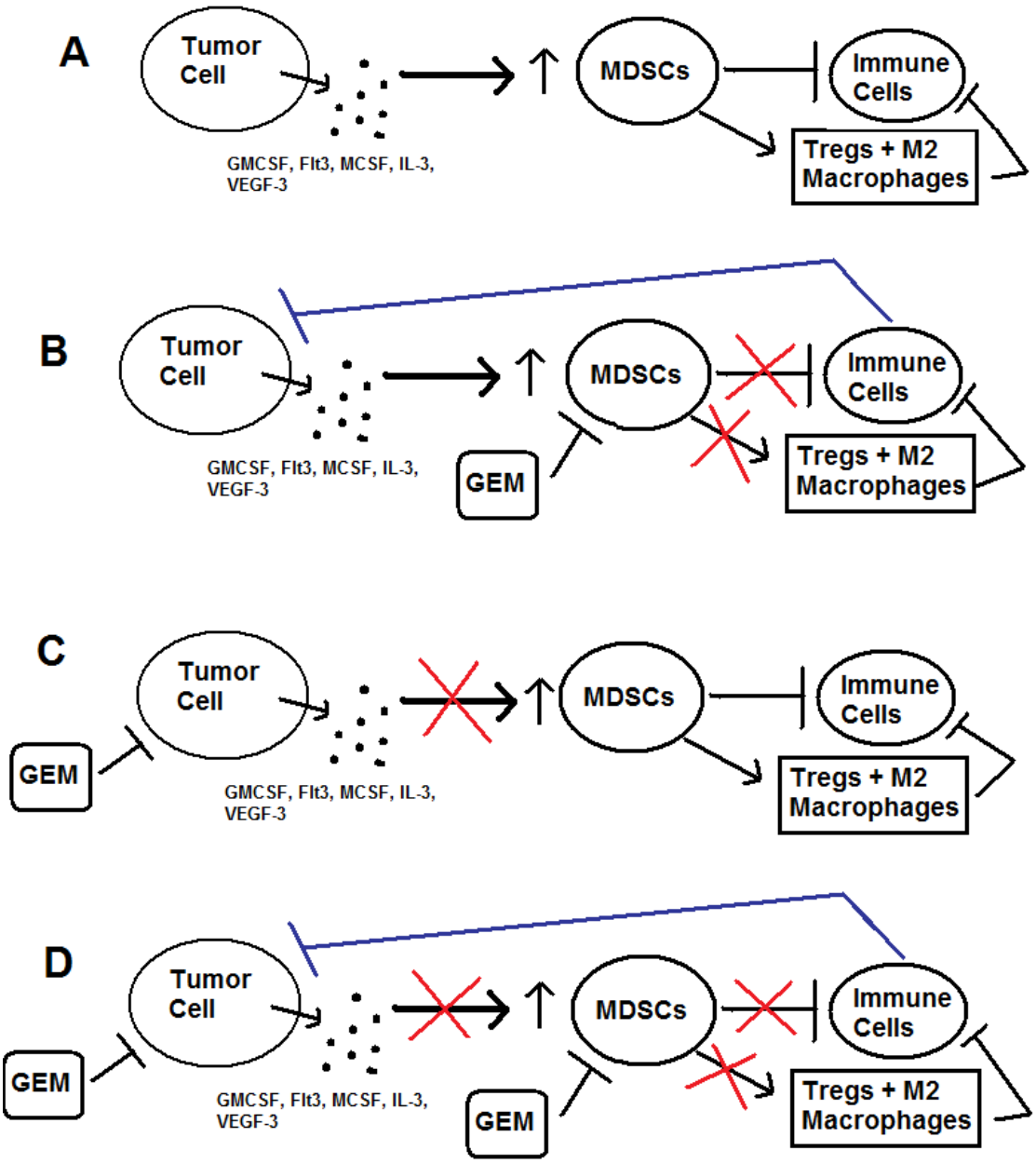


Figure 14: Potential Mechanisms of GEM Pharmacologic Effects.

(A) In untreated mice, tumor cells secrete factors that induce MDSC development and cause T cell dysfunction. **(B)** GEM acts directly on MDSCs and by releasing T cells from inhibition by MDSCs, mediates immune dependent inhibition of tumor progression **(C)** GEM inhibits tumor cells directly, inhibiting tumor growth and indirectly preventing the accumulation of MDSCs and impair tumor growth pharmacologically. **(D)** GEM inhibits both MDSCs and tumor cells directly.

Through *in vitro* assays, we have shown that GEM does indeed directly inhibit 4T1 tumor cell growth (Fig. 7, Table 3), corroborating the dose dependent cytotoxicity observed by Smith *et al.* on endometrial cancer cells[94]. We also hypothesized that despite this direct effect on tumor cells, GEM might still directly inhibit MDSCs. Since MDSCs suppress T cell immunity, we reasoned that part of the impaired tumor growth seen in GEM treated mice might be caused by abrogation of T cell inhibition. Therefore, if GEM were given to athymic nude mice that lacked T cells, the inhibition of tumor growth in GEM treated mice would be dampened. This would provide evidence that at least part of GEM's effect on tumor growth *in vivo* results from direct inhibition of MDSCs. However, contrary to this hypothesis, GEM treatment caused a similar delay in tumor growth in nude mice as in BALB/c mice (Fig. 8). Thus, it would seem that even if GEM does act directly on MDSCs, the inhibition of tumor progression *in vivo* is independent of T cells. The results here substantiated previous findings that elimination of CD8+ T cells had no effect on the antitumor effect of this chemotherapy drug[96]. Although GEM mediated effects on tumor growth *in vivo* were independent of T cells, the amplified immune response generated by AIT in combination with GEM was dependent on C8+ T cell activity[96, 98].

Even though GEM has a potent direct anti-tumor effect in the 4T1 model, it may also inhibit MDSC directly and have potential for augmenting immunotherapy (Fig. 14D). Although the results of our experiment in nude mice did not demonstrate whether or not GEM acts directly on MDSCs, our next experiment did help answer the question. When we treated mice bearing large established tumors (>60mm^2) with GEM, we saw little

change in the tumor size compared to untreated mice (Figs. 9 and 10). Nevertheless, splenomegaly was reversed within 24hr after treatment and MDSC numbers were decreased in the blood, bone marrow, and spleen. All three tissues are known sites where MDSCs accumulate and where GEM is distributed after administration[92]. These results support the hypothesis that GEM inhibits MDSCs directly and verify in the 4T1 tumor model experiments presented by Suzuki *et al* in the TC-1 lung epithelial tumor model and by Ko *et al*. in the Her-2/neu breast cancer model[85, 96]. Although their results, which were obtained 48hrs after GEM treatment, were convincing, our analysis after only 24hrs and with half the dose of GEM further substantiates the direct effect of GEM on MDSCs. In just 24hr or 48hr after GEM treatment, when our analysis was performed, there was not enough time for tumor size to change, so the GEM effects on MDSCs are unlikely to be attributable to the drug's cytotoxicity against the tumor. Therefore, it seems likely that GEM works on both tumor cells and MDSCs. To verify further that GEM acts directly on MDSCs, future experiments could assay for differences in MDSC products, like ARG-1 and NOS-2 in the spleen and bone marrow of treated and untreated mice. Apoptotic cells at the tumor site and at MDSC locations should be measured to confirm inhibition of these cells by GEM.

The inhibitory effects of GEM on MDSCs suggest that GEM treated tumor bearing mice should have less immunosuppression than untreated mice with upregulated suppressor cells. Indeed, we showed a novel association between GEM treatment , decreased MDSCs, and improved T cell proliferation. Splenic lymphocytes from treated mice were

better able to respond to activation signals and to proliferate in response to cytokines than lymphocytes from untreated controls (Fig. 11). This improvement was correlated to reduction in MDSCs in tumor bearing mice after GEM treatment. In addition, lymphocytes from untreated 4T1 bearing mice were unresponsive to tumor, but were rescued by removal from the immunosuppressive host environment and B/I activation, so that after expansion (albeit markedly reduced compared to GEM-treated mice), splenic lymphocytes secreted IGN-γ in response to specific tumor antigen at levels similar to cells from treated mice (Fig. 11).

The ability of GEM to rescue tolerant T cells from hosts with established tumors may prove useful for augmenting AIT in the clinic. At the time of diagnosis, many patients have advanced tumor progression, with T cells that are already immunosuppressed and dysfunctional. Treatment with GEM mitigated this suppression and functional T cells were harvested. To help combat the kinetics of disease progression, it is important to be able to harvest functional Ag-specific T cells and quickly expand these cells to therapeutic numbers for adoptive transfer as soon as possible. Thus, GEM has the potential to augment AIT in two ways. Donors can be treated to increase the responsiveness of tumor primed T cells at the time of harvest to *in vitro* manipulation. In addition, treatment of tumor-bearing recipients (identical to the donor in most human AIT paradigms) before infusion of T cells might create a more conducive environment for T cell proliferation and persistence.

Other researchers have demonstrated the benefits of inhibiting MDSCs in cancer therapy[71, 79, 83, 84]. Using anti-Gr-1 antibody, Seung *et al* showed that depletion of MDSCs alone was able to cause complete rejection of a progressive tumor induced by UV light[83]. Sinha *et al*. suggested that induction of M1 macrophages and reduction of MDSCs by surgical removal of established 4T1 mammary carcinoma and treatment with all-*trans*-retinoic acid (ATRA) could result in rejection of established tumors[71]. Treatment of mice bearing C3 fibrosarcoma or DA3-HA mammary adenocarcinoma with ATRA decreased MDSC number in the spleen, lymph nodes, and bone marrow by inducing differentiation into mature dendritic cells[84]. Kusmartsev *et al.* demonstrated that ATRA given in combination with vaccination to mice with C3 fibrosarcoma broke T cell tolerance and increased expansion of Ag-specific, IL-2 producing CD4+ T cells as well as expansion of Ag-specific IFN-γ producing CD8+ T cells, and led to impaired tumor growth. Similar to our data, their study found that only T cells from MDSC depleted tumor bearing mice showed Ag-specific IFN-γ responses. All of these findings suggest that mitigating the immunosuppressive effects of MDSCs can augment cancer therapy. Therefore, GEM might also display similar successes when combined with AIT by inhibiting MDSCs.

As of today, GEM has not been used in the clinic for the purpose of inhibiting immunosuppression by MDSCs. Its success in the clinic has been attributed to its direct tumoricidal effects[86, 94]. Therefore, it has been used as a single agent or in combination with other chemotherapy drugs and in conjunction with AIT. When GEM has been combined with AIT, positive responses were attributed to the drug's direct antitumor

effects[98, 101]. For example, Nowak *et al*. used GEM in conjunction with AIT and activating anti-CD40 ligand, which led to complete regression of AB1 murine malignant mesothelioma and induced protective immune memory[98]. This response could not be achieved without GEM treatment. The researchers asserted that GEM augmented combination therapy by inducing apoptosis of tumor cells and by increasing CD4+ and CD8+ T cell infiltration of the tumor[98, 99]. The authors attributed the latter to GEM's direct anti-tumor effect and abrogation of humoral immunity. Specifically, the researchers found that GEM treatment of tumor bearing mice increased CD8+ T cell proliferation and cytolytic function. They reasoned that this was caused by an increase in tumor antigen cross-presentation induced by GEM mediated tumor cell apoptosis[99]. In addition, they found that GEM treatment of mice with established AB1 murine mesothelioma resulted in depleted serum antibody titer[100]. However, their results could also have been explained by GEM mediated suppression of MDSCs. The tumor site is one location where MDSC accumulation is observed in mice with large tumor burdens. If GEM causes the MDSC proportion to decrease at the tumor site, an increase in the proportion of other cell populations, like CD4+ and CD8+ T cells, would result. Because MDSCs inhibit T cells and drive the development of a Th2 immune response, suppression of MDSCs by GEM would also lead to increased T cell responsiveness and decreased humoral responses, as observed by Nowak et *al*[70, 72, 74, 76, 81]. As demonstrated, positive results can be obtained by using GEM as a traditional tumoricidal antimetabolite in conjunction with AIT. However, better results and more optimal conditions for AIT might be obtained by focusing on the pharmacologic targets of GEM (i.e. by targeting both tumor cells and MDSCs).

Ko *et al.* have begun utilizing GEM in combined chemoimmunotherapy in a tolerogenic Her-2/neu murine tumor model. Similar to our strategy, they targeted Treg cells and attempted to abrogate immunosuppression by MDSCs. When we examined the combined effects of cyclophosphamide, which can inhibit Treg cells, and GEM we saw antitumor effects that mirrored the groups treated with GEM alone, with no additive effects. Using anti-GITR antibody to deplete Treg cells in combination with GEM, Ko *et al.* made the same observations[96]. Targeting both Treg cells and MDSCs may not have eradicated established tumors on their own, but in combination with AIT, this chemoimmunotherapy led to better outcomes than either AIT with Treg cell depletion or AIT with MDSCs depletion. In fact, AIT combined with inhibition of Treg cells and MDSCs led to a cure rate of 100% and protected cured mice against tumor rechallenge 90 days after the first tumor inoculation.

Following the success of this group and other groups that have used GEM in AIT, our next set of experiments aimed to investigate the effects of GEM in our model of AIT. However, our initial combination of AIT with GEM treatment of recipients did not yield significant improvement in outcomes of 4T1 tumor bearing BALB/c mice (Fig. 14). We attempted to treat 7 day old 4T1 tumors given at an initial concentration of 50,000 4T1 cells/mouse with $50x10^6$ T cells/dose. The recipient mice were treated with GEM 24hrs before AIT and again a week later. Failure of this combination therapy to inhibit tumor progression might be attributed to several factors. The first is the failure of GEM treatment at day 7 and day 11 to inhibit MDSCs that only accumulate in our model when

mice have developed large tumor burdens (Fig. 6). It is therefore difficult to use this model to show that GEM inhibition of MDSCs augment AIT. It is not practical to use GEM to treat MDSCs at their time of accumulation, when tumor area has reached $\geq 60mm^2$, because the tumor burden would too great to be treated by AIT before mice have to be euthanized. Giving GEM early and repeatedly would make it difficult to distinguish direct tumoricidal effects from MDSC inhibition. Therefore, to investigate benefits of GEM induced inhibition of MDSCs in AIT, a different tumor model should be employed, one that induces the accumulation of MDSCs at the early stages of tumor development. It is also likely that mice given an inoculum of 50,000 4T1 cells/mouse have tumors that are already too advanced at day 7 to be treated by $50x10^6$ T cells. The T cells may have antitumor function and proliferative capacity, but are unable to match the rapid growth of the tumor cells. Future experiments can be optimized by carrying out a cell dose assay to determine what dose of T cells yield positive outcomes for a specific tumor inoculum and for a specific treatment timeline. Furthermore, it should be noted that other immunosuppressive mechanisms exist besides MDSCs that thwart T cell immunity, like Treg cells and cytokine sinks. Our lab typically combines AIT with CYP to create a host environment more conducive to AIT. CYP may be important in helping maintain T cell fitness *in vivo* and should be used in future AIT experiments.

While other groups have used GEM to treat recipient mice, we additionally questioned whether or not GEM augments AIT when used to treat donors. Because T cells from GEM treated mice with large tumors showed a significant qualitative and quantitative difference compared to T cells from untreated, tumor bearing mice, we wondered whether or not GEM rescued T cell function would extend *in vivo* and eradicate 4T1 flank tumors. 4T1 flank tumors are difficult to cure with T cells from Ag vaccinated mice. Thus, if T cells from mice with such advanced tumors can mediate an immune response against established tumor, a strong argument can be made for the use of GEM to treat donors before T cell harvest.

To test this hypothesis we performed a preliminary AIT experiment using T cells harvested from mice bearing 22 day old tumors (Fig. 12). The mice were either untreated or treated with GEM 48hrs before T cell harvest. The failure of AIT using lymphocytes from donors with advanced tumors to inhibit tumor progression demonstrate difficulties faced in the clinic. T cells from hosts with well established tumors are dysfunctional or absent due to immunosuppression from a variety of tumor induced mechanisms making it difficult to harvest sufficient numbers of tumor-specific, functional T cells. We used $18x10^6$ T cells/mouse, which was not therapeutic. It is likely that even if the T cells were able to respond to tumor antigens, as shown in figure 11, this dose was too small to compete with the rapid growth of tumor cells at the given dose of 10,000 4T1 cells/mouse. Following AIT experiments should employ a larger dose of T cells. In addition, because the T cells were harvested from mice with advanced tumors, they were likely to be less fit

and therefore, less able to persist and carry out effector functions *in vivo*. Future studies would have to follow T cell lifespan and proliferation *in vivo* to confirm this. Tumor escape mechanisms, such as mutation of specific antigen or downregulation of key MHC molecules, could also account for the lack of T cell effectiveness.

AIT has had such limited success in the clinic against most cancers because it is dependent on so many factors and overcoming multiple obstacles. Successful AIT depends on the type of cells used for adoptive transfer, the reversal of tolerance and immune dysfunction, the generation and expansion Ag-specific cells, and the *in vivo* persistence and avidity of transferred cells. In addition, immune suppression in the adoptive host must be abrogated to ensure that fit Ag-specific cells can traffick to the tumor site and target tumor cells. We have found that by exploring new ways to augment T cell expansion, we may make adoptive immunotherapy more effective as a treatment against cancer. Not only would we increase the likelihood that AIT is successful against tumor development, we would be able to make the treatment less costly in terms of labor, time and expenses. This will prove prudent in a world where cancer is quickly becoming a problem for both resource rich and resource poor countries.

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