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EX VIVO EXPANSION OF TUMOR-SPECIFIC T CELLS WITH SEQUENTIAL COMMON GAMMA CHAIN CYTOKINES RENDER THEM REFRACTORY TO MDSC UPON ADOPTIVE IMMUNOTHERAPY.

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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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List of Abbreviations

ADH……..Atypical ductal hyperplasia

AICD…….Activation Induced Cell Death

AIT……Adoptive Immunotherapy

ANV……..Antigen Negative Variant

APC……...Antigen Presenting Cells

B/I……..Bryostatin1/Ionomycin

CD…….....Cluster of Differentiation

cDNA……Complementary DNA

CDDO-Me…….C-28 methyl ester of 2-cyano-3, 12-dioxooleana-1,9,-dien-28-oic acid

CFSE…… 5,6 Carboxyfluorescein diacetate succinidimyl ester

Cy5……... Cyanine 5

CYP……..Cyclophosphamide

DC…… Dendritic Cells

DNA……. Deoxyribonucleic Acid

DCIS…… Ductal Carcinoma in situ

E:T…….Effectors:Target

ECD……. Extracellular domain of neu

EGFR…… Epidermal growth factor receptor

EGF……... Epidermal Growth Factor

ELISA……Enzyme-linked Immunosorbent Assay

FBS…….. Fetal Bovine Serum

FDA……. Food and drug administration

Gem……. Gemcitabine

GM-CSF…….Granulocyte macrophage colony stimulating factor

GPI…… Glycosyl Phosphatidylinositol

GrB…….Granzyme-B

Her-2……..... Human epidermal growth factor receptor 2

HLA……Human Leukocyte Antigen

HRP……Horse radish peroxidase

H_2SO_4…….Sulphuric acid

IACUC……Institutional Animal Care and Use committee

i.d…… Intradermal

i.p…….Intraperitoneal

i.v…….Intravenous

IDO……..Indoleamine 2,3-dioxygenase

IFN……...Interferon

Ig……..Immunoglobulin

IL…….....Interleukin

IL-2R……Interleukin-2 receptors

Ly6……...Lymphocyte antigen 6 complex

MAPK……..Mitogen activated protein kinase

MCA/Meth A……...3'-methycholanthrene

MDSC……..Myeloid-derived suppressor cells

MFI…….Mean Fluorescence Intensity

MHC…….. Major Histocompatibility Complex

MMC…….. Mouse Mammary Carcinoma

MMTV……Mouse Mammary Tumor Virus

NK……..Natural killer

NKT……Natural killer T cells

NO……..Nitric oxide

O.D…….Optical density

PBS…….Phosphate buffered saline

PE……Phycoerythrin

PI ……Propidium iodide

PI3 kinase……Phosphatidylinositol kinase

PMN……...Polymorphonuclear cells

Prf……...Perforin

RAG…….. Recombinase Activating Gene

RPM……Rotation per minute

RPMI……..Roswell Park Memorial Institute

ROS……Reactive Oxygen Species

s.c ……..Subcutaneous

SD…… ..Standard Deviation

SEM……Standard Error of Mean

SH2 domain……Src homology 2 domain

STAT……..Signal transducers and activators of transcription

SV40…….. Simian virus 40

T_{CM}……..Central memory of T cells

TCR…….T cell receptor

T_E…….Effector of T cells

T_{EM}…….. Effectors memory of T cells

TGFβ…….. Transforming Growth factor beta

TIL……..Tumor Infiltrating lymph node

T_N……Naive T cell population

VEGF……..Vascular endothelial growth factor

Abstract

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Myeloid derived suppressor cells (MDSCs) are heterogeneous population of immature cells at various stages of differentiation, characterized by the presence of CD11b and Gr1 in mice. They are major contributors of the tumor-induced immune suppression against the tumors. So far, various strategies have been introduced to

overcome the endogenous MDSCs. Most of these approaches rely on the elimination of MDSCs and it is not clear whether tumor-reactive T cells may be differentiated towards phenotypes that are refractory to MDSCc. Our laboratory has previously shown that high affinity T cells derived from tumor-sensitized wild-type FVB mice and expanded *ex vivo* with the alternating common gamma chain cytokine formulation (initiation of culture with IL-7 + IL-15 followed by one day pulse with IL-2 and continuation of culture with IL-7 + IL-15) can successfully induce tumor regression in FVBN202 transgenic mouse model of breast carcinoma upon adoptive immunotherapy (AIT), only when combined with the depletion of endogenous MDSCs. In this study we have introduced a novel formulation of the sequential common gamma chain cytokines (initiation of culture with IL-7 + IL-15 followed by the expansion with IL-2 until 6 days) for the *ex vivo* expansion of the autologous and tumor-sensitized low affinity T cells derived from FVBN202 mice and further used for AIT. This novel formulation induced differentiation of tumor-reactive CD8+ T cells mainly towards effector and effector/memory phenotypes that were refractory to MDSCs *in vitro* and *in vivo*. AIT by using these T cells induced rejection of primary neu positive tumors and generated long-term memory responses against the recall tumor challenge. Importantly, these T cells also resulted in the inhibition of neu antigen negative relapsed tumor cells. Our findings in the present study provide a platform for AIT of breast cancer patients.

CHAPTER 1 Introduction

1.1. Cancer-Statistics and Challenges

Despite extensive ongoing cancer research and newly emerging therapeutic strategies, cancer remains to be a devastating disease in the world. According to the American Cancer Society report in 2009, cancer accounts for nearly one-quarter of deaths in the United States, exceeded only by heart diseases. Worldwide, one in eight deaths is due to cancer, exceeding the mortality rate of AIDS, malaria and tuberculosis combined. The corresponding estimates for total cancer deaths in 2007 were 7.6 million, or about 20,000 cancer deaths a day, with 2.9 million in economically developed countries and 4.7 million in economically developing countries. By 2050, the global burden is expected to grow to 27 million new cancer cases and 17.5 million cancer deaths. Moreover, the direct and indirect costs of cancer are extensive (1) and are a financial burden for the society. In women, breast cancer is the second most prevalent fatal cancer exceeded by lung cancer and followed by colon and rectum cancer.

1.2. Breast Cancer and Importance of ErbB Family of Receptor Tyrosine Kinase-Structure and Function in Cancer Initiation-

Breast cancer is the most frequently occurring cancer in women in the United States, with an overall risk of developing invasive breast cancer 1 in 8 (2, 3). Her-2/neu

(human epidermal growth factor receptor 2), is an onco-protein that upon activation triggers intracellular signaling events, crucial for cell growth, proliferation, differentiation and survival (4). It has been reported that the Her-2/neu proto-oncogene is amplified in 25-30% of human primary breast cancers (5), which has been associated with tumor aggressiveness and unfavorable clinical prognosis (6). Overexpression of Her-2/neu is evident by the gene amplification as well as protein overexpression. A variety of carcinomas including ovarian, prostate, lung, liver, kidney, bladder, and gastric cancer also overexpress Her-2/neu (4). Therefore, effective therapeutic formulation that targets Her-2/neu may be applicable to all types of carcinomas with the overexpression of Her-2/neu. A correlation was established between Her-2/neu gene amplification/Her-2/neu receptor overexpression and other positive indications of tumor progression such as tumor size, absence of hormone receptor expression, aneuploidy and proliferation index, all of which are associated with poor prognosis (7-9).

Her-2/neu or rat neu with 89% homology to human Her-2/neu (10) is a member of the epidermal growth factor receptor (EGFR) family, also known as ErbB family of tyrosine kinase receptors. The Epidermal Growth Factor (EGF) is known to stimulate epidermal proliferation and eventual keratinization. The EGFR was discovered in 1975 as a specific target for epidermal growth factor (11, 12), and it is the first cell surface receptor known to be linked directly to cancer. The EGFR family encompasses 4 members, ErbB1 (EGFR itself), ErbB2 (also known as Her-2/neu), ErbB3 and ErbB4 (13). All members of this family are structurally related and composed of closely related

single chain modular glycoproteins with a heavily glycosylated extracellular ligand binding region (~620 residues), a single transmembrane domain (~23 residues) and an internal tyrosine kinase domain (~260 residues) that is flanked by juxtamembrane (~40 residues) and a C terminal (~232 residues) regulatory regions. The extracellular domain (ECD) is composed of 2 homologous large domains and 2 cysteine rich domains (13). Sub-domains I (ECDI), located most distally from the cell membrane, together with sub-domain III (ECDIII), are the homologous members of the leucine rich repeat family and participate in binding to extracellular ligands (13-17). Sub-domains II (ECDII) and IV (ECDIV) are cysteine rich and ECDII acts as a dimerization arm. In the presence of ligands ECDII is exposed and facilitate heterodimerization with the ECDII of other ErbB molecules (13-15). In the absence of the ligands the ErbB molecules assume a "tethered" structure, and during this orientation ECDIV interacts with ECDII thereby blocking its dimerization interface (13-15). Her-2/neu is unique among the family with its ECDII dimerization arm being constitutively exposed. This structural peculiarity makes it accessible to other ErbB family members for heterodimer formation (18). In fact, it is the most highly preferred heterodimerization partner of the other ligand bound family members. Her-2/neu has no known direct activating ligand, which provides a satisfying explanation for the unique biological properties of this orphan receptor, since ligand binding to ECDI and ECDIII facilitates a conformational change that exposes ECDII in other ErbB molecules, ultimately resulting in a unique dimerization that is purely receptor mediated (13, 14, 16, 17). The receptor molecules of EGFR family in general, in the ligand-induced dimer conformation become tyrosine autophosphorylated

in *trans*. The resulting phosphotyrosine recruits the SH2 domain of multiple downstream signaling molecules thus initiating an array of intracellular signaling pathways (13). In the case of Her-2/neu, overexpression of the receptor alone favors increased dimerization with other ErbB family members or its homodimerization, thereby triggering signaling cascades that ultimately result in uncontrolled proliferation and cell survival (14, 19). The intracellular signaling pathway of Her-2/neu is thought to involve ras-MAPK, MAPK independent S-6 kinase and phospholipase c-gamma signaling pathways (7). The above mentioned observations explain the reasons why overexpression of Her-2/neu can cause cell transformation. Several cancer therapeutic strategies targeting Her-2/neu have been introduced in the past few years namely monoclonal antibody therapy, tyrosine kinase inhibitors, and vaccines. A monoclonal antibody Trastuzumab (Herceptin®) that was approved by the Food and Drug Administration (FDA) recognizes ECDIV of Her-2/neu and is effective against 30% of the Her-2/neu positive breast cancers, though majority of patients develop resistance within 9 months of treatment. Trastuzumab is not effective against Her-2/neu overexpressing ovarian cancers, therefore, other monocolonal antibodies that target different sub-domains of the Her-2/neu ECD are being developed. Among these, Pertuzumab recognizes ECDII and has been shown to be effective against Her-2/neu overexpression breast and ovarian cancer in clinical trials (20).

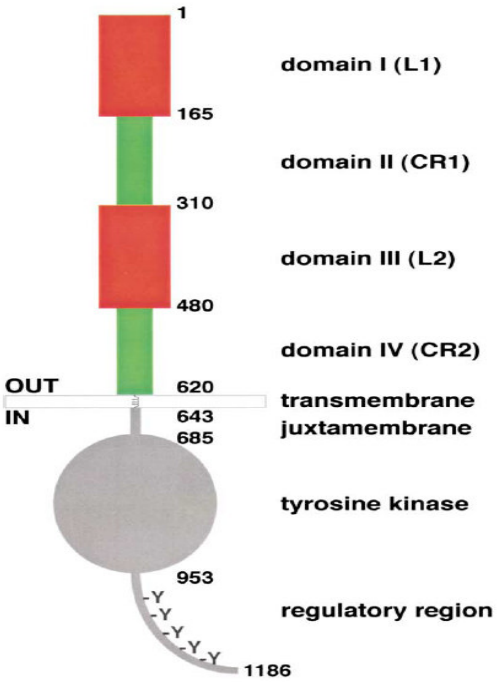
Figure 1A: Domain organization of a typical member of the Epidermal Growth Factor Receptor Family.

The receptor is composed of an extracellular region, a transmembrane domain and a tyrosine kinase domain. The tyrosine kinase domain is flanked by a juxtamembrane region and a regulatory C-terminal region. The extracellular domain (ECD) is composed of four sub domains, namely Domain I (ECDI), Domain II (ECDII), Domain III (ECDIII) and Domain IV (ECDIV).

Figure 1B: The structure and dimerization pattern of ErbB molecule

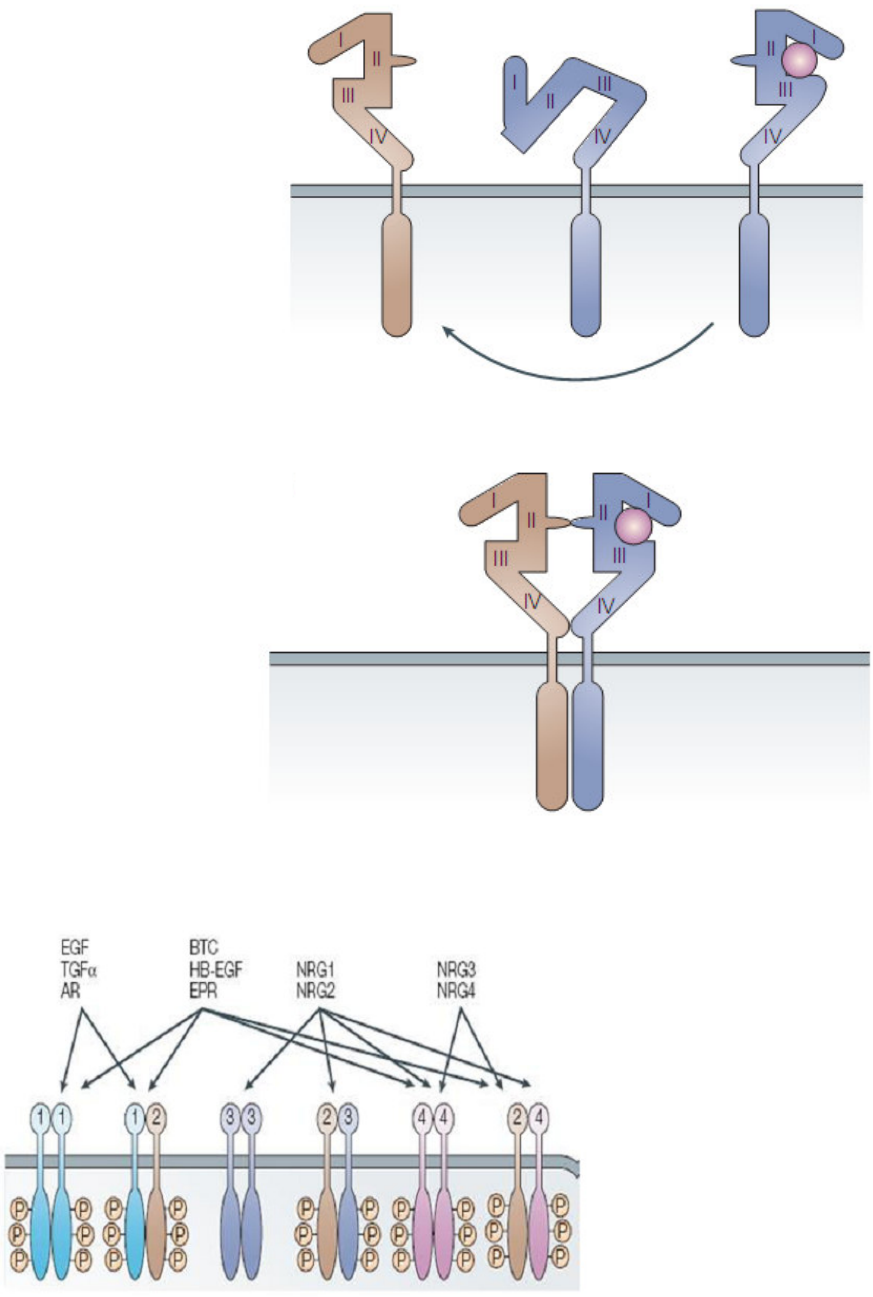
The top panel shows ErbB2 (left) with a constitutively exposed dimerization arm (ECDII) even in absence of ligand whereas ErbB1, 3 and 4 adopt the "tethered" conformation in absence of the ligand (centre). Upon ligand binding (right) ErbB1, 3 and 4 acquire a conformational change that exposes the dimerization arm for binding. The middle panel shows that ErbB2 molecule exhibiting a fixed conformation resembling a ligand activated state of EGFR and ErbB3 molecules. The bottom panel shows ligand binding to ErbB receptors which induces the homo- and hetero-dimerization and subsequent activation of the intrinsic kinase domain which results in phosphorylation of specific tyrosine residues within the cytoplasmic tail. These phosphorylated residues serve as docking sites for a range of proteins, the recruitment of which leads to the activation of intracellular signal pathways.

Figure 1A



Burgess, A. W., H. S. Cho, C. Eigenbrot, K. M. Ferguson, T. P. Garrett, D. J. Leahy, M. A. Lemmon, M. X. Sliwkowski, C. W. Ward, and S. Yokoyama. *An open-and- shut case? Recent insights into the activation of EGF/ErbB receptors. Mol. Cell* 12(3):541-552, 2003.

Figure 1B



Hynes,N.E. and H.A.Lane. *ERBB receptors and cancer. The complexity of targeted inhibitors. Nat.Rev.Cancer* 5(5):341-354, 2005.

1.3. Mouse Model

The FVBN202 mouse model of neu overexpressing spontaneous mammary carcinoma provides a clinically relevant model for investigating the immunotherapy of Her-2/neu positive breast cancer. These mice develop spontaneous mammary tumors within 4-12 months of age as a result of overexpression of the unactivated form of rat neu oncogene in their mammary glands under the control of the mouse mammary tumor virus (MMTV) promoter (21). The MMTV/unactivated neu is isogenic to the MMTV/activated neu except for the absence of the activating mutation in the transmembrane domain and the presence of a SalI restriction endonuclaese site between the neu cDNA and SV40 polyadenylation splicing signals.

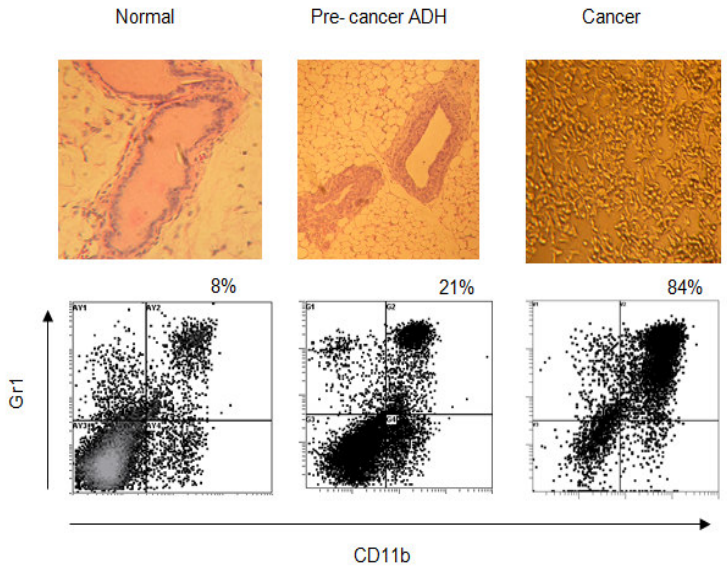
FVBN202 mice develop premalignant mammary hyperplasia similar to ductal carcinoma in situ (DCIS) prior to the development of spontaneous carcinoma within 1.5-2 months of age. Spontaneously arising mammary tumors are harvested from these mice and processed in the laboratory to establish neu + mouse mammary carcinoma cell lines, referred to as MMC. The endogenous overexpression of neu oncogene in these transgenic mice makes them immunologically tolerant to neu oncoproteins. This makes the animal model clinically relevant, mimicking the normal condition of human breast cancer, where malignancy develops from “self” tissues thereby escaping the body’s own immune system. The T cells harvested from these mice are of low affinity for the neu tumor antigen. On the contrary, wild type FVB mice do not express the rat neu oncogene, so the immune system of these mice can exhibit robust responses, when the animals are inoculated with neu+ mouse mammary carcinoma (MMC) tumors derived

from spontaneous mammary tumor of FVBN202 mice. The animals can subsequently reject MMC, because of harboring the neu specific T cells that express high affinity receptors for the neu antigen. It has been reported previously, that FVBN202 mice show an increased level of myeloid-derived suppressor cells (MDSCs) during the initiation and establishment of spontaneous breast carcinoma (22). **Figure 1C** shows the correlation of the increased abundance of CD11b+Gr1+ MDSCs along with the progression and establishment of spontaneous breast carcinoma. Slides show the section of the normal mammary duct followed by the gradual proliferation of the epithelial layer during premalignant atypical ductal hyperplasia (ADH) and finally with the establishment of malignant breast tumor. Uncontrolled proliferation of the epithelial layer coincided with the increased level of MDSCs (22).

Figure 1C: Increased level of MDSCs along with the progression and establishment of breast carcinoma

The figure shows an increased CD11b+Gr1+ MDSCs along with the gradual progression and establishment of mammary carcinoma. Starting from the normal level in FVBN202 mice with healthy mammary epithelial ducts, the MDSCs show consistent increase during the atypical ductal hyperplasia (ADH) and invasive breast cancer.

Figure 1C



Kmieciak et al. **"***Danger signals and nonself entity of tumor antigen are both required for eliciting effective immune responses against HER-2/neu positive mammary carcinoma: implications for vaccine design.".* l *Cancer Immunol Immunother.* 57: 1391-1398, 2008.

1.4. Myeloid -Derived Suppressor Cells (MDSCs) – How do they abrogate anti-tumor immune responses *in vivo*

Tumor development is often accompanied by a peculiar alteration of hematopoesis that leads to a progressive accumulation of myeloid cells in bone marrow, blood, spleen and at the tumor site. These cells share the markers CD11b and Gr1 and their accumulation correlates with the inhibition of T cell responses to antigenic stimulation both *in vivo* and *in vitro*. CD11b+ Gr1+ cells are heterogenous and are commonly known as myeloid derived suppressor cells (MDSCs). The abundance of this cell population is correlated with many types of cancer namely murine colon carcinoma MCA-26, Lewis lung carcinoma, highly metastatic breast carcinoma, 4T1, neu+ breast carcinoma and melanoma (B16) (23, 24-27). Increased abundance of circulating MDSCs correlates with clinical cancer stage and metastatic burden of breast cancer. Stage IV patients with extensive metastatic tumor burden had the highest percentage and absolute number of MDSCs (28). Increased abundance of MDSCs during tumor progression is associated with enhanced myelopoesis, because soluble factors secreted from tumors including GM-CSF and VEGF are known to stimulate myelopoesis or block the maturation of hematopoetic progenitor cells, respectively (23, 29). It is still not clear which signaling pathway is specifically involved in the induction of MDSCs. A number of pathways including STAT1, STAT3, c-kit ligand/ receptor have been suggested to be responsible for induction of MDSCs. The heterogenous population of MDSCs characterized by CD11b+Gr1+ actually represents a macropopulation. Different epitopes of the Gr1 molecules have been identified, namely Ly6G and Ly6C in different subsets of MDSCs

characterized by different functional attributes (25, 30, 31). The Ly6 family members are cysteine rich molecules that are glycosyl phosphatidylinositol (GPI)-anchored to the cell surface; it has recently been reported that expression of Ly6G represents a granulocytic phenotype whereas Ly6C correlates with a monocytic phenotype. The mononuclear cell MDSCs are akin to inflammatory monocytes whereas polymorpho nuclear (PMN) MDSCs are closer to immature neutrophils. Blocking IFN-γ or disrupting STAT1 impaired suppression by the former while IFN-γ was required for suppression by PMN MDSCs (25, 30). Recent reports reveal that the monocytic phenotype contributes to the suppressive function of MDSCs (32, 33).

The exact mechanism of T cell suppression by MDSCs remains elusive. It has been reported that MDSCs can acquire endothelial cell property in tumor microenvironment and supports angiogenesis, thereby favoring tumor growth while ignoring the immune sentinels. They are also known to cause apoptotic death of CD8+ T cells (34). Gabrilovich *et al.* have shown antigen specific contact-dependant suppression of T cells by MDSCs. They showed that Gr1+ cells express myeloid cell marker and MHC class I but lack MHC class II and do not affect T cell proliferation or IL-2 production induced by Concanavalin A or by MHC class II but they do exert a detrimental effect on the number of IFN-γ producing CD8+ T cells in response to specific peptides presented by MHC class I (35). Other reports reveal that MDSCs may work through downregulation of T cell receptor (TCR) zeta chain and can cause dysfunction of T cells described in cancer, infectious diseases and autoimmune disorders (36). Morales *et al.* also reported that MDSCs inhibit T cell activation in FVBN202 mice in a contact-dependent manner

(37). Another probable mode of suppression by MDSCs has been argued to be dependent on the production of nitric oxide (NO) as well as other soluble factors. It has been shown that suppression is due to the impairment of IL-2 receptor signaling pathways mediated by and requires NO, which is secreted by MDSCs in response to signals from activated T cells, including IFN-γ and a contact-dependent stimulus (38). Gabrilovich *et al.* proposed that antigen specific CD8+ T cell tolerance is one of the major mechanisms of tumor escape. They have shown that the tolerance is induced by MDSCs. They have found that MDSCs directly disrupt the binding of the specific peptide-MHC complex dimers via nitration of tyrosines within the TCR/CD8 complex, which results in the inability of CD8+ T cells to respond to the specific peptides. Nitration of the TCR by MDSCs is thought to be induced by hyperproduction of reactive oxygen species (ROS) and peroxinitrite during cell-cell contact (39). Some reports showed inhibitory effect of arginase I produced by MDSCs. Arginase I metabolizes L-arginine to urea and ornithine whereas nitric oxide synthase 2 converts it to citrulline and NO. Depletion of L-arginin by any one of these pathways results in T cell dysfunction. High arginase activity is reported in lung and colon cancer (23, 27). The same group confirms that MDSC-mediated down regulation of L-selectin to be a major cause of T cell suppression as T cells must express L-selectin (high) in order to home to lymph nodes and sites of inflammation where they encounter antigen and are activated. This downregulation perturbs T cell trafficking patterns thereby inhibiting T cell activation (40, 41). Although several groups have proposed various mechanisms of

suppression by MDSCs, the exact mode of action remains unclear. It is likely that different mechanisms of suppression exist in different cancer models.

1.5. Cancer Therapeutics- An overview of adoptive immunotherapy

Paul Ehrlich was one of the first to conceive the idea that the immune system could repress a potentially "overwhelming frequency" of carcinomas arising from 'self' tissues. To a large extent, the revisiting of Ehrlich's proposal had to await the maturation of the developing field of Immunology. In 1950, the work of Medawar and colleagues clarified the critical role of cellular components of immunity in mediating allograft rejection. This work cast doubt on data that were used to argue for the existence of tumor antigen. Although it was generally accepted that the immune response was capable of recognizing and destroying transplanted tumors derived from outbred strain of mice, it soon became clear that the underlying mechanism was that of allograft rejection rather than tumor specific rejections. With the introduction of inbred strains of mice, it became clear that the syngeneic tumor transplant can be immunologically distinguishable from the normal "self" entities. The endogenous immune system components recognizing and abrogating the nascent transformed cells were described as "immunosurveillance" by Sir Macfarlane Burnet and Lewis Thomas. They speculated that lymphocytes acted as sentinels in recognizing and eliminating continuously arising nascent transformed cells and that tumor cell specific neoantigens could provoke an effective immunologic reaction that would eliminate cancer development (42, 43).

Along this line of thought Dighe *et al.* reported that endogenous production of IFN-γ can protect the host against growth of transplanted tumors along with the formation of the primary chemically induced and spontaneous tumors (43). It was shown that administration of the neutralizing mAbs against IFN-γ into mice transplanted with Meth A tumors blocked LPS-induced tumor rejection. It was also proved that transplanted fibrosarcomas grew faster in mice treated with IFN-γ blocking antibodies (43, 44). Early studies identified perforin (Prf) as a critical cytolytic molecule in the primary host's anti-tumor responses. After challenge with Meth A tumors, Prf-/- mice formed 2-3 times more tumors than wild type mice. In addition 50% of aging Prf-/- C57BL/6 mice developed spontaneous disseminated lymphomas as compared to 1/16 wild type mice (42, 43).

Availability of immune deficient mice allowed testing of tumor immunosurveillance hypothesis. Genetically manipulated mice lacking recombinase activating gene, RAG-2 or its obligate partner RAG-1 cannot somatically rearrange lymphocyte antigen receptors, and thus cannot produce peripheral αβ T cells, B cells, NKT cells or γδ T cells. RAG-2 expression is limited to the cells of lymphoid system; therefore RAG-2-/- mice provide an appropriate model to study the effect of host cell lymphocyte deficiency on tumor development. It has been shown that following subcutaneous injection of Meth A tumor cells, 129/ SvEv RAG-2-/- mice developed tumor at the injection site faster than the wild type control group. NK and NKT cells also participate in cancer immunoserveillance. C57BL/6 mice, depleted of both natural killer and

natural killer T cells using the anti-NK1.1 mAb, were two to three times more susceptible to Meth A-induced tumorigenesis (43).

Presence of tumor immunosurveillance supported by the above-mentioned evidences provides a platform for immunotherapy of cancers. This suggests that cancer patients or tumor-bearing animals harbor tumor-reactive T cells that, if expanded *ex vivo*, can result in tumor regression upon adoptive T cell immunotherapy (AIT) (45). This method has gained some success in melanoma and in the treatment of patients with cancers expressing viral antigen such as Epstein Barr Virus (46).

T cells for AIT can be harvested from various sites from the tumor-bearing hosts, including peripheral blood, tumor biopsies, resected lymph nodes, malignant effusions and spleens. Obtaining T cells from the peripheral blood may be easier, but higher frequency of tumor reactive T cells can be found in tumor biopsies (tumor-infiltrating lymphocytes, TIL) (47). However, harvesting sufficient number of TIL can be difficult, though this approach has been used successfully in patients with melanoma (47). Successful AIT depends on the type of T cells transferred, their effector functions, ability to reach tumor location and the ability of the cells to overcome any tolerance or immune suppression in the host. The immunogenic potential of the tumor antigen, size and location of tumor, and the regimen used for of the *ex vivo* expansion of autologous T cells determine efficacy of the therapy. Three major criteria for successful AIT are (48, 49):

- Sufficient numbers of T cells with high reactivity against the tumor *in vivo*
- Trafficking of T cells to and infiltrating in the tumor stroma

- Activation at the tumor site to manifest appropriate effector mechanisms such as direct lysis or cytokine secretion capable of tumor destruction

It has been shown that efficacy of AIT depends on T cell phenotype. Terminally differentiated CD8+ T cells were nearly 100- fold less effective *in vivo* on a per cell basis than T cells at an early stage of differentiation. Less differentiated T cells have high proliferation potential and are less prone to apoptosis than more differentiated cells and have a higher ability to respond to homeostatic cytokines as they express receptors IL-7 Rα chain (45). For an effective and successful AIT, T cells should possess effector functions and the ability to generate memory responses (50). The major cytotoxic function of the CD8+ T cells demands direct cellular contact with the target cells. To induce the tumor specific cell death, the effector CD8+ T cells should reach the tumor site. This potential extravasations' capacity depends on their ability to adhere to endothelial walls while withstanding shear stresses exerted by flowing blood. The tethering and rolling mechanism is governed by the selectin family (51). It has been reported that loss of expression of the lymphoid homing molecules such as CD62L or CCR7 and acquisition of CD44 expression can be associated with increased anti-tumor effects of adoptively transferred T cells (45). Moreover, expression of CD44 has been shown to be the most potent stimulator of Fas ligand expression on human T cells (52) which in turn may induce apoptosis in Fas positive target cells.

Previous preclinical studies indicated that immune ablation is an effective preconditioning regimen that can increase T cell responses after adoptive

immunotherapy, as lymphodepletion increases the efficacy of transferred T cells. Transfer of small numbers of antigen-specific T cells into a lymphopenic host results in the expansion and activation of the transferred T cell population, known as homeostatic proliferation. Adoptively transferred antigen-specific T cells represent a small portion of the total T cell population residing in the host. Host mediated inhibitions of the proliferation of adoptively transferred T cells might involve direct cellular contact or competition between transferred and endogenous host T cells for a limited amount of resources such as cytokines that are required to support transferred T cells homeostasis. This is known as the "cytokine sink" effect. Lymphodepletion can overcome cytokine sink effects and also decrease the competition at the surface of antigen-bearing antigen presenting cells (APC). Gattitoni *et al.* reported that a lymphopenic environment enables tumor-reactive T cells to destroy large tumors more effectively (45). The same group has also reported that though lymphodepletion reduces the number of APC *in vivo*, it is also linked to the increasing the number of mature activated APCs which can facilitate the activation of transferred cells (45). To create a lymphopenic host environment, several methods including total body irradiation, genetic alteration and chemotherapy can be applied. In this study we have used cyclophosphamide (CYP) for lymphodepletion. CYP is a synthetic antineoplastic drug and an alkylating agent that interferes with the growth of rapidly proliferating cells (53). CYP is also known to remove tumor- induced suppressor T cells. The above discussion clearly depicts that the elimination of the host immune suppressive environment is a primary pre-requisite for effective AIT. The MDSCs are known to be a major contributor to the tumor associated

immune suppression in a number of carcinomas. Tumor cells secrete immune suppressive cytokines such as IL-10, TGFβ and VEGF. Tumors express immunosuppressive enzyme indoleamine 2,3-dioxygenase which is involved in tryptophan metabolism. This enzyme appears to exert its suppressive effect through depletion of tryptophan and production of anti- inflammatory tryptophan metabolites (47). All these suppressive elements which can abrogate T cell functions should be eliminated prior to AIT.

Though several ways have been introduced to circumvent these endogenous MDSCs, none of them has achieved complete success. The abundance of these suppressive cells in the tumor microenvironment remains a critical challenge for successful AIT.

1.6. Bryosatin-1/Ionomycin treatment and application of novel cytokine formulation for the *ex vivo* expansion of tumor-reactive T cells prior to AIT

Bryostatin-1 belongs to a class of macrocyclic lactones derived from the marine bryozoans, *Bugula neritina*. It binds to the regulatory domain of Protein Kinase C (PKC) and governs its activation as well as inhibition. Short-term treatment with Bryostatin-1 activates PKC while long exposure may have a detrimental effect (37). Ionomycin is a calcium ionophore that increases the intracellular Ca^{2+} concentration, which acts as a second messenger in various signaling cascades (54). The treatment with Bryostatin-1 and Ionomycin (B/I) induces selective activation of tumor-specific T cells without the need for identification of tumor antigen (55). Exposure to both bryostatin-1 and ionomycin (B/I) mimics signaling through the CD3/TCR complex and leads to activation and proliferation of effector T cells (55).

Adoptive immunotherapy of cancer patients utilizes the *ex vivo* expansion of tumor-reactive T cells by using cytokines or other pharmacological agents to generate a large number of such immune effector cells (56). Among the common gamma chain (γc) cytokines (IL-2, IL-4, IL-7, IL-9, IL-15, IL-21) mainly IL-2, IL-7, IL-15, and/or IL-21 have been used to improve the efficacy of AIT. The common γ chain component of the receptors of these cytokines stimulates MAP kinase and PI3 kinase pathways that result in anti-apoptotic and mitogenic signals (57).

The endogenous naïve T cells characterized by the presence of CD45RA can differentiate into quiescent memory cells bearing the characteristic of CD45RO surface protein and effector cells when first encounter the antigen. The quiescent memory cells may also arise from the differentiation of effector cells following activation. There are two types of memory T cells. Central/memory T cells are characterized by the expression of CD44 and $CD62L^{high}$ whereas effector/memory T cells are characterized by the expression of CD44 and $CD62L^{low}$. The latter mature rapidly into effector cells after re-stimulation and secrete large amounts of IFN-γ or IL-4 (58). The *ex vivo* manipulations of T cells during the expansion can preferentially support the survival and proliferation of these various effector and memory phenotypes of T cells.

IL-2 is the major T cell growth factor cytokine that is produced by activated T cells. The IL-2 receptor is expressed by activated T cells, activated B cells and NK cells. The receptor comprises three polypeptide subunits: IL-2 receptor α chain (CD25), IL-2Rβ chain (CD122) and IL-2Rγ chain (CD132). IL-2Rβ and γ chains participate in signal transduction whereas the α chain augments binding affinity for IL-2 (55). IL-2 is known

to cause activation-induced cell death (AICD), and it preferentially supports differentiation of CD8+ T cells towards effector and effector/memory phenotypes by down regulation of the lymph node homing receptors CD62L (59, 55). Culture of B/I activated human T cells from healthy donors with IL-2 *ex vivo* resulted in the *de novo* generation of the CD4+CD25+ FoxP3+ T cells without any detectable suppressive function (60).

IL-7 is the hematopoietic growth factor cytokines which is secreted by the stromal cells, epithelial cells and fibroblasts but is not produced by lymphocytes. The receptor for this cytokine is expressed by T cells, pre-B cells and dendritic cells (DCs). IL-7 receptor consists of an α chain (CD127) and shares common γ chain (CD132) with IL-2 receptor. IL-7 is crucial for the survival and homeostatic expansion of naïve and memory CD8+ T cells. Injection of IL-7 results in the expansion of both CD4+ and CD8+ T cells as well as the relative reduction of CD4+ Tregs (55, 61).

IL-15 is produced by monocytes, dendritic cells (DCs) and epithelial cells. IL-15 receptor consists of an α subunit and shares common β and γ subunits with the IL-2 receptor. IL-15 receptor is expressed by T cells and NK cells. IL-15 induces expression of anti-apoptotic Bcl-2 as well as inhibition of pro -apoptotic Bim in CD8+ T cells expressing the receptors for IL-7, thereby decreasing AICD in those specific subsets of CD8+ T cells (62). IL-15 is known to favor the differentiation and proliferation of the CD8+ central memory subset of T cells. While IL-7 and IL-15 are responsible for supporting the primary memory CD8+ T cells, only IL-15 is known to support the secondary memory phenotypes (63). This cytokine is also reported to render T

effector/memory cells refractory to the suppressive function of Tregs via activation of the PI3 kinase cascades but does not involve the rescue of those T cells from apoptosis (64). IL-15-expanded CD8+ T cells display only effector functions *in vitro* but are highly effective *in vivo*. This is likely because the IL-15-induced differentiation of T central/memory subsets are responsible for generating prolonged memory responses *in vivo* whereas the effector T cells undergo apoptosis after several cycles of proliferation or cytolytic function against tumor *in vitro* (55).

In this study we have aimed to introduce a novel cytokine regimen for the *ex vivo* expansion of T cells that can render the T cells refractory to the inhibitory effects of MDSCs and generate long-term memory responses following AIT.

CHAPTER 2 Materials and Methods

Mouse model

FVBN202 transgenic female mice (Charles River Laboratories) were used between 6 to 8 weeks of age throughout these studies. These mice overexpress an unactivated rat neu transgene under the regulation of MMTV promoter (21). These mice develop premalignant mammary hyperplasia similar to ductal carcinoma in situ (DCIS) prior to the development of spontaneous carcinoma (22). The studies have been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Commonwealth University. The mice were sacrificed before they attained a tumor volume of 2000mm³.

Tumor cell lines

The MMC cell line was established from a spontaneous tumor harvested from an FVBN202 transgenic mouse as described previously (65). The antigen negative variant (ANV) cell line, characterized by a loss of neu expression, was derived from a relapsed MMC tumor in the FVB strain as previously described (65). Both cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). Mice were challenged with 3-5x10^6 tumor cells by subcutaneous (s.c.) inoculation wherever indicated in this study.

Expansion of effector T cells derived from FVBN202 mice

Female FVBN202 mice were inoculated with $4X10^6$ MMC cells and the splenocytes were harvested after 10-21 days. Splenocytes (10^6cells/ml) were stimulated in a warmed complete medium containing 15% FBS with Bryostatin-1 (5nM) and Ionomycin (10nM) along with 80U/ml of IL-2 (Peprotech) for 16 hours. Cells were washed three times and cultured at 10^6 cells/ml in complete medium (RPMI-1640 supplemented with 100U/ml of penicillin, 100µg/ml streptomycin, 10% FBS, glutamine and β-mercaptoethanol) with 10-20ng/ml of IL-7 and IL-15 (Peprotech). After 24 hours 20U/ml of IL-2 was added to the medium. On the next day cells were washed three times and cultured (10^6cells/ml) in complete medium with 40U/ml of IL-2. Cells were counted and cultured at 10^6cells/ml in complete medium with 40U/ml of IL-2 every other day for a total of 6 days.

Adoptive immunotherapy

24 hours prior to adoptive immunotherapy (AIT), FVBN202 mice were treated with Cyclophosphamide (CYP, 100mg/Kg) by intraperitoneal (i.p) injection in order to induce lymphopenia. Mice were challenged with $3X10^6$ MMC cells (s.c.) and then received $70X10^6$ *ex vivo* expanded T cells by tail vein (i.v) injection later on the same day. Tumor growth was monitored by digital calipers and tumor volumes were calculated by: Volume (v) = [L (length) x W (width) ²]/2. Blood was collected from the orbital sinus to determine the level of circulating CD11b+Gr1+ cells by flow cytometry.

At the termination of the experiments, splenocytes were harvested and stained using the appropriate antibodies for several surface markers and internal molecules.

Cytotoxicity assay

Freshly harvested lymphocytes or *ex vivo* expanded T cells were cultured with MMC or with CFSE labeled ANV at 10:1 ratio, in 3 ml complete medium (RPMI-1640 supplemented with 100U/ml of penicillin, 100µg/ml streptomycin, 10% FBS, glutamine and β- mercaptoethanol) and 20U/ml of IL-2 (Peprotech) in 6 well culture dishes. Prior to co-culture, ANV cells were stained with CFSE, to gate out the CFSE+neu- ANV population for estimating viability of the tumor cells. After 48 hours, MMC cells were harvested and stained for neu (anti-c-Erb2/c–Neu, Clone-4, Calbiochem) along with FITC anti mouse IgG, Annexin V-PE and Propidium Iodide (PI) according to manufacturer's protocol (BD Pharmingen). In case of ANV, neu staining was omitted. Flow cytometry was used to analyze the viability of neu positive cells.

CFSE staining

$5X10^6$ ANV cells were resuspended in 5 ml of HBSS and spun for 10 minutes at 1200 rpm at 4°C. The HBSS was discarded and cells were stained with 1.5µM of CFSE in 1 ml HBSS for 5 minutes at room temperature in the dark. After incubation cells were washed with 1 ml of FBS, followed by three more washes with HBSS+10% FBS. Finally cells were resuspended in complete media and desired numbers of cells were used for cytotoxicity assay.

Flow cytometry

Flow cytometry analyses were performed as standardized by our laboratory. The freshly isolated spleens were homogenized into a single cell suspension and red blood cells were lysed using 1X ACK (NH_4Cl, $KHCO_3$, $EDTA.Na_2.2H_2O$) lysis buffer for 6 minutes at room temperature. Cells were washed with 2 volumes of RPMI1640 supplemented with 10% FBS, and then resuspended in Flow Cytometry cell staining buffer (1X PBS with 1 % FBS and 0.1% of sodium azide). Cells were counted and aliquoted into each sample tube (10^6 cells/tube). The splenocytes were used either freshly isolated from the mice or after co-culturing for 24 hours with and without MMC; or after a 6-day expansion with a special cytokines regimen. Non-specific binding to Fc receptors was blocked with anti-CD16/32 antibody (Biolegend, San Diego, CA) for 20 minutes on ice. Cells were stained with surface antibodies namely against CD4 (GK1.5), CD8 (53-6.7), CD3 (145-2C11), CD69 (H1.2F3), CD11B (M1/70), Gr1 (RB6-8C5), Ly6G (1A8), CD62L (MEL-4) and CD44 (IM-7) and incubated on ice in the dark for 20 min. All antibodies were used at the manufacture's recommended concentration. Cells were washed twice and fixed with 1% paraformaldehyde. For internal staining with anti-perforin (eBioOMAK-D) antibody (eBioscience), FoxP3 Fix/ Perm Buffer system was used according to the manufacturer's protocol (Biolegend, San Diego, CA). For Granzyme-B and IFN-γ (XMG1.2) (eBioscience) staining, cells already stained for extracellular molecules were fixed with 2.5% paraformaldehyde for 10 min on ice, permeabilized by washing with

1ml and then with 2ml of 0.1% saponin buffer, and stained with internal antibodies for 30 minute on ice in the dark. Especially for IFN-γ staining, transport blocker (Monensin) was added to the growing cell culture medium 4 hours prior to staining. Lastly, cells were washed twice with normal cell staining buffer and resuspended in 500 µl of the same. For Annexin V staining, after cells were stained for respective surface markers followed by a wash with Flow Cytometry cell staining buffer, they were washed again with 1X Annexin V buffer (BD Pharmingen) and the Annexin V staining protocol was followed according to manufacturer's recommended protocol. Samples were run on a Beckman Coulter FC 500 and analyzed using Summit version 4.3 software.

IFN-γ ELISA

Tumor-sensitized freshly isolated effector lymphocytes or *ex vivo* expanded T cells were cultured in complete medium at a 10:1 ratio with irradiated MMC cells for 24 hours. Supernatants were collected and stored at-80°C until used. IFN-γ was detected using a Mouse IFN-γ ELISA set (BD Pharmingen) according to the manufacture's protocol.

Protein ELISA

ELISA was performed as previously described by our group (37). Blood was collected from experimental animals from the retro-orbital sinus, allowed to sit at room temperature for 10 minutes, and then spun for 10 minutes at 10,000 rpm. Serum was harvested and stored at -80°C until used. For measuring the antibody response against neu, 96 well plates were coated with 10 µg/ml of the ECDII and incubated overnight at 4° C. Plates were washed with PBS+0.05% Tween-20 and blocked with 2% skim milk for one hour. After washing, 5 and 50-fold serial dilutions of the sera were added (100µl/well) and incubated for 2 hours at room temperature. Horse-radish-peroxidase (HRP)-conjugated anti-mouse IgG1 was added at a 1:2000 dilution for 1 hour. Plates were washed and reactions developed by adding 100µl/well of the TMB Microwell peroxidase substrate (Kierkegaard & Perry). The reaction was stopped with 2M H_2S0_4, and the O.D. read at 450 nm. Mean antibody titers were calculated.

Isolation of Myeloid-Derived Suppressor Cells (MDSCs) *in vitro*

MDSCs were isolated from tumor-bearing FVBN202 animals using an EasySep PE selection kit from StemCell Technologies following manufacturer's protocol. For the isolation purpose, cells were stained with Gr1-PE antibody (Biolegends, San Diego, CA).

Suppression assay

In suppression assay T cells were cultured in the presence of isolated Gr1-PE labeled MDSCs in a ratio of 2:1. Such mixed cell population was co-cultured with MMC at a ratio of 10:1:5 of Effector:Target:MDSC cells. Flow cytometry analysis was performed to examine various surface markers and internal molecules expressed by T cells upon stimulation/suppression. Supernatants from these co-cultures were collected for IFN-γ ELISA. For cytotoxicity assay, the cell population (T+MMC+MDSC) was stained with purified mouse monoclonal antibody against rat neu (anti-c-Erb2/c–Neu, Clone-4, Calbiochem) along with FITC- anti mouse IgG, Annexin V-PE and Propidium Iodide (PI) according to manufacturer's protocol (BD Pharmingen).

Tumor challenge studies

FVBN202 mice received MMC inoculations earlier the same day prior to receiving AIT. After successful rejection of primary tumors, they were re-challenged for second (n=3) and third (n=1) time on day 23 and day 61, respectively, with 5 $X10^6$ MMC by s.c. inoculation. The recipients were also challenged with $4X10^6$ ANV (n=2) on day 50. Subsequent tumor inoculation was done on the contralateral side. Tumor growth was monitored at regular intervals.

Statistical analysis

Graphical data are presented as averages with standard errors. Statistical comparisons between groups were made using Student's T test with $P < 0.05$ being statistically significant.

CHAPTER 3 Study Rationale and Results

Study Rationale

Our laboratory has had a longstanding interest in developing an effective means of treating neu positive mammary tumors by using tumor-specific T cells. The FVBN202 mouse model is clinically relevant for investigating adoptive immunotherapy (AIT) because of endogenous expression of rat neu oncoprotein. These transgenic mice develop atypical ductal hyperplasia (ADH) preceding the initiation and establishment of spontaneous breast carcinoma (21). Our laboratory has already shown ADH coincided with elevated levels of MDSCs in FVBN202 mice, which in turn resulted in the failure of AIT against mammary carcinoma (22, 37). **Figure 1C** shows the correlation of the increased abundance of CD11b+Gr1+ MDSCs along with the progression and establishment of spontaneous breast carcinoma. Uncontrolled proliferation of the epithelial layer was associated with the increased level of MDSCs (22). Previous attempts in our laboratory to use AIT to cure neu+ tumors in FVBN202 mice have proven unsuccessful, despite the fact that highly active effector T cells were obtained from wild type FVB mice that could successfully reject the neu+ tumor challenge. Efforts to identify the reasons lead to the understanding of the key inhibitory role of CD11b+Gr1+ MDSCs in FVBN202 mice. Adoptive transfer of high affinity T cells

derived from wild type FVB mice, activated with Bryostatin-1/Ionomycin (B/I) and expanded with the alternating common gamma chain cytokines (culture of T cells with IL-7 + IL-15 for 24 hours after the B/I activation followed by the overnight pulse with IL-2 and continuation of the culture with IL-7 + IL-15) *ex vivo* resulted in tumor rejection, only when combined with the depletion of MDSCs *in vivo* (37). However, non-self source of T cells (from wild type FVB into FVBN202 mice) reduced clinical application of the treatment. Therefore, in the present research we used autologous low affinity T cells for the AIT of FVBN202 mice and developed an *ex vivo* protocol for the expansion of these T cells which render them refractory to MDSCs. We first tested an alternating common gamma chain cytokine protocol and then improved it into a sequential cytokine formulation (IL-7+IL-15 followed by IL-2). We hypothesized that initiation of culture with IL-7 and IL-15 would support the differentiation of tumor-reactive memory T cells and continuation of the culture with IL-2 would result in the induction of activation-induced cell death (AICD) in fully differentiated T cells, rescuing early differentiated T cells from apoptosis. AIT by means of these T cells at their early stage of differentiation would induce robust tumor rejection and protect animals against recall tumor challenge by generating memory responses.

Alternating common gamma-chain cytokines expanded tumor-reactive T cells that failed to overcome MDSCs *in vivo* following AIT

Splenic T cells were isolated from tumor-bearing female FVBN202 mice and expanded *ex vivo* with an alternating gamma chain cytokine formulation

(IL7/IL15→IL2→IL7/IL15) following B/I activation (37). This protocol resulted in a 2.6 and 4-fold expansion of splenocytes on day 7 compared to day 0 and post-B/I activation respectively (**Figure 2**). The B/I activation resulted in a significantly decreased splenocytes from 72 million to 46.94 million followed by a consistent expansion attaining 188.3 million cells on day-7. **Figure 3** shows the proportions of CD8+ and CD4+ T cells from freshly isolated and *ex vivo* expanded lymphocytes.

Figure 2: *Ex vivo* expansion of splenocytes with alternating (IL-7/IL-15→IL-2→IL-7/IL-15) gamma chain cytokines

Spleen harvested from MMC-sensitized large tumor-bearing (tumor volume≤2000mm³) female FVBN202 mouse was crushed and processed to yield single cell suspensions which were subjected to a 7-day expansion with the alternating gamma chain cytokines. Splenocytes were incubated with 5nM Bryostatin-1 and 10nM Ionomycin along with 80 U/ml of IL-2 in complete medium for 16 hours followed by expansion with IL-7/IL-15 (10ng/ml), alternating with IL-2 (40U/ml) and again with IL-7/IL-15. The figure shows the fold increase of T lymphocytes at different time intervals during the expansion protocol. Cell counts were determined by trypan blue exclusion.

Figure 2

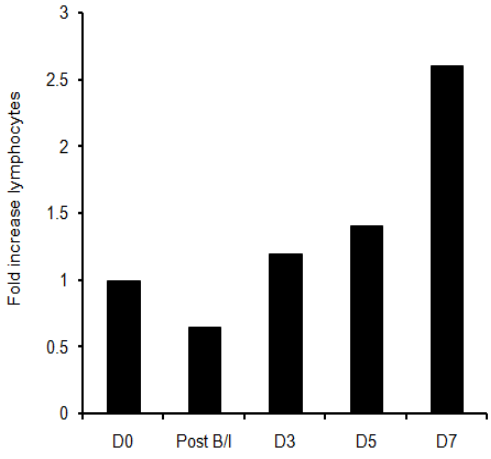
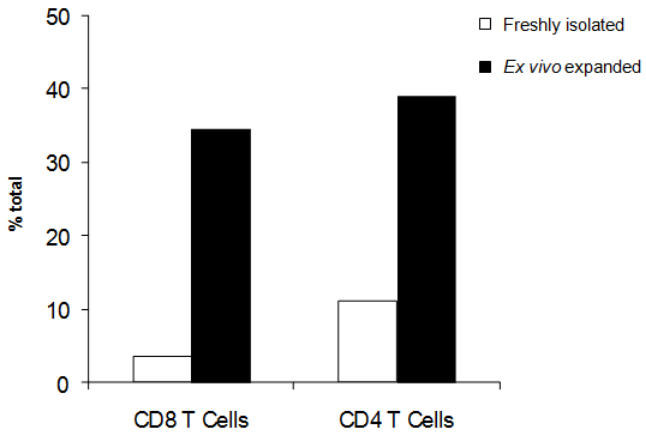


Figure 3: Expansion of splenocytes with the alternating gamma chain cytokines results in the expansion of CD8+ and CD4+ T cells

Spleen harvested from MMC sensitized large tumor-bearing (tumor volume≤2000mm³) female FVBN202 mouse was crushed and processed to yield single cell suspensions which were subjected to a 7-day expansion with alternating gamma chain cytokines. The figure shows the percent total population of the CD8+ and CD4+ T cells before and after the expansion with the alternating gamma chain cytokine formulation.

Figure 3



Freshly isolated splenocytes contained 3.72% and 11.22% CD8+ and CD4+ T cells, respectively. Culture with the alternating gamma chain cytokines resulted in increase in the T cell subsets. CD8+ and CD4+ T cells expanded 9.27 and 3.48 fold, reaching 34.51% and 39.05%, respectively.

In order to determine viability (AnnexinV-/PI-) of T cells during the *ex vivo* expansion flow cytometry analysis was performed. High viabilities for CD8+ and CD4+ T cells were detected prior to expansion (**Figure 4, upper panel**) or following a 7-day expansion (**Figure 4. lower panel**).

In order to determine phenotype distribution of T cells and the effects that *ex vivo* expansion may have on the differentiation of T cells, we performed flow cytometry analyses. Freshly isolated CD8+ T cells contained mainly central/memory and naïve phenotypes accounting for 51.43% of central/memory (T_{CM}: CD44+CD62L^{high}) and 30.71% of naïve (T_N: CD44-CD62L+) T cells as well as 7.52% of effector phenotype (T_E: CD44+CD62L-) and 9.52% of effector/memory phenotypes (T_{EM}: CD44+CD62L^{low}). Freshly isolated CD4+ T cells contained mainly central/memory and effector phenotypes accounting for 57.85% of T_{CM} and 29.03% of T_E cells as well as 8.08% of T_{EM} and 5.61% of T_N cells (**Figure 5, upper panel**). *Ex vivo* expansion with the alternating gamma chain cytokines generated increased T_E phenotypes for CD8+ (16.76%) and CD4+ (36.86%) T cells (**Figure 5**, lower panel). However, T_{CM} remained the most abundant phenotypes after the *ex vivo* expansion of CD8+ and CD4+ T cells

(**Figure 5**, lower panel). The MFI for CD44 expression in the expanded CD8+ and CD4+ T cells were 5.61 and 7.28, respectively.

In order to determine whether the *ex vivo* expansion of T cells may enrich for the tumor-reactive T cells, we determined production of IFN-γ and cytotoxic function of T cells in the presence (+) and absence (-) of MMC tumor cells at an effector:target (E:T) ratio of 10:1. Supernatants were collected after a 24-hour culture and assayed for IFN-γ by ELISA. **Figure 6** shows that freshly isolated splenocytes produced IFN-γ when stimulated with irradiated MMC *in vitro* (3580.5 pg/ml; p= 0.0000005). Importantly, the *ex vivo* expanded T cells showed significantly higher production of tumor-induced IFN-γ than freshly isolated T cells (8.2 fold; 29400 pg/ml p = 0.036).

Figure 4: *Ex vivo* expansion of T cells with the alternating gamma chain cytokine formulation supports maintenance of highly viable lymphocyte population

Spleen harvested from MMC sensitized large tumor-bearing (tumor volume≤2000mm³) female FVBN202 mouse was crushed and processed to yield single cell suspensions which were subjected to a 7-day expansion with alternating gamma chain cytokines. The cells were gated on CD8+ or CD4+ cells and analyzed for AnnexinV and PI.

Figure 4

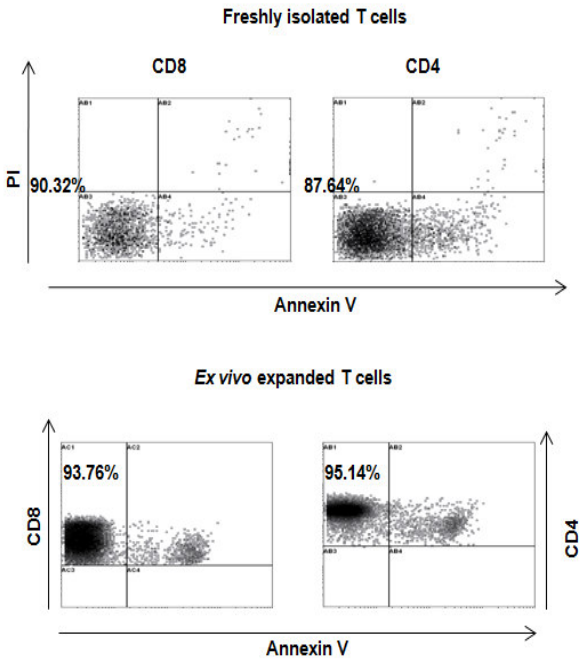


Figure 5: *Ex vivo* expansion of T cells with the alternating gamma chain cytokines supports effector (T_E) and central/memory (T_{CM}) T cell phenotypes

Spleen harvested from MMC sensitized large tumor-bearing (tumor volume≤2000mm³) female FVBN202 mouse was crushed and processed to yield single cell suspensions which were subjected to a 7-day expansion with alternating gamma chain cytokines. The *ex vivo* expanded and freshly isolated T cells were stained with CD62L, CD44 and CD8/CD4 antibodies. Gated CD8+ and CD4+ T cells were analyzed within 50,000 viable cells for the expression of CD44 and CD62L. The CD44+CD62L- (T_E), CD44+ $CD62L^{low}$ (T_{EM}), CD44+$CD62L^{high}$ (T_{CM}) and CD44-CD62L+ (T_N) phenotypes are shown.

Figure 5

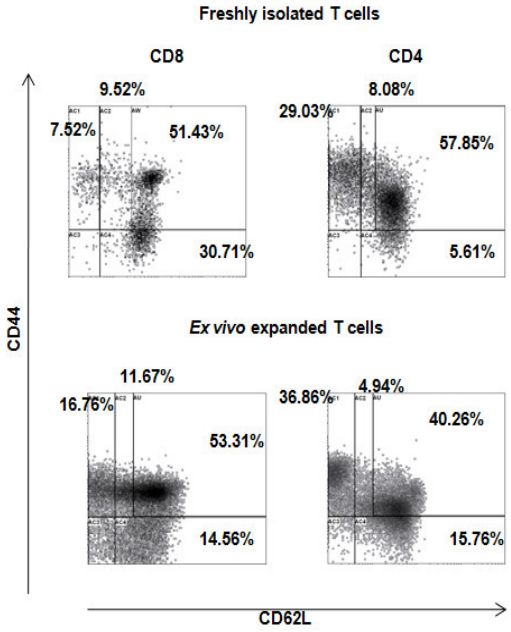
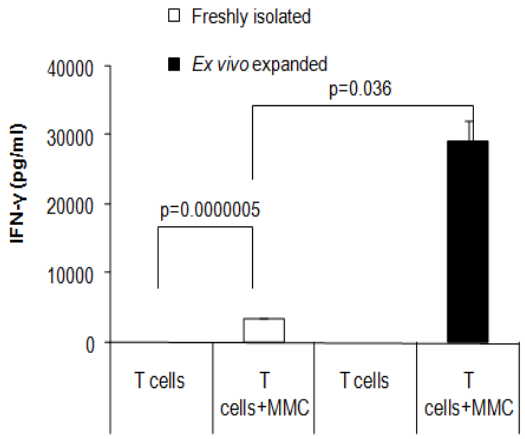


Figure 6: *Ex vivo* expanded T cells with the alternating gamma chain cytokines produce more IFN-γ than freshly isolated T cells upon *in vitro* stimulation with neu positive MMC

The *ex vivo* expanded and freshly isolated T cells were cultured either alone or with irradiated neu+ MMC (15000 rad) in the effector:target ratio of 10:1 for 24 hours and supernatants were tested for IFN-γ secretion by using ELISA.

Figure 6



To confirm the anti-tumor efficacy *in vitro,* T cells were co-cultured with viable MMC target cells (E:T ratio of 10:1) for 48 hours followed by staining with antibodies directed towards neu, Annexin V and PI. Control wells were seeded with MMC in the absence of T cells. Gated neu positive MMC cells were analyzed for Annexin V and PI to determine apoptosis of tumor cells by freshly isolated as well as the *ex vivo* expanded T cells. **Figure 7** shows that viability (Annexin V-/PI-) of MMC decreased from 92.26% in the absence of T cells to 68.57% and to 61.69% in the presence of freshly isolated and the *ex vivo* expanded T cells, respectively.

To test the anti-tumor response potential *in vivo*, the *ex vivo* expanded T cells were used for AIT in FVBN202 mice challenged with neu positive MMC. Prior to transfer of the *ex vivo* expanded T cells, animals were treated with Cyclophosphamide (CYP) for lymphodepletion. Control mouse did not receive AIT. **Figure 8** shows the failure of T cells expanded with the alternating gamma chain cytokines to induce tumor rejection.

Since the alternating common gamma chain cytokine formulation could not support the expansion of T cells that could cause tumor regression in FVBN202 mice, we came up with the sequential common gamma chain cytokine regimen for the *ex vivo* expansion of T cells. We hypothesized that continuation of T cell culture with IL-2 following initial culture with IL-7 + IL-15 could support the expansion of CD8+ T_E cells that, compared to naïve or central/memory T cells, may be refractory to MDSCs and result in tumor rejection upon AIT even in the presence of endogenous MDSCs.

Figure 7: *Ex vivo* expanded T cells with the alternating gamma chain cytokines did not show an enhanced anti-tumor efficacy compared to the freshly isolated T cells

The freshly isolated and *ex vivo* expanded T cells were cultured with neu positive MMC in an effector:target ratio of 10:1, the control wells were seeded with MMC alone and cultured for 48 hours. The cells were stained with antibodies against neu, AnnexinV and PI. Gated neu+ population were analyzed for Annexin V and PI.

Figure 7

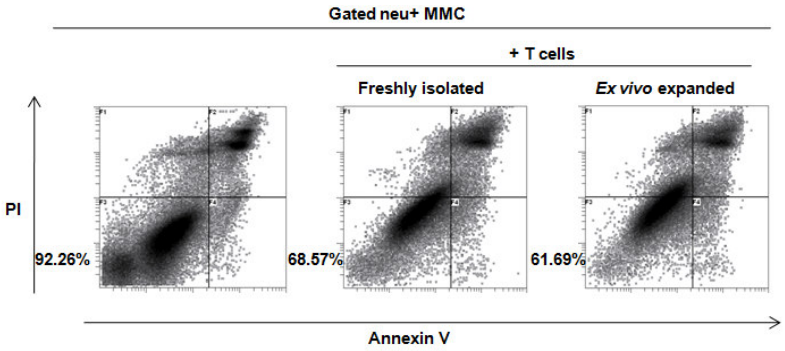
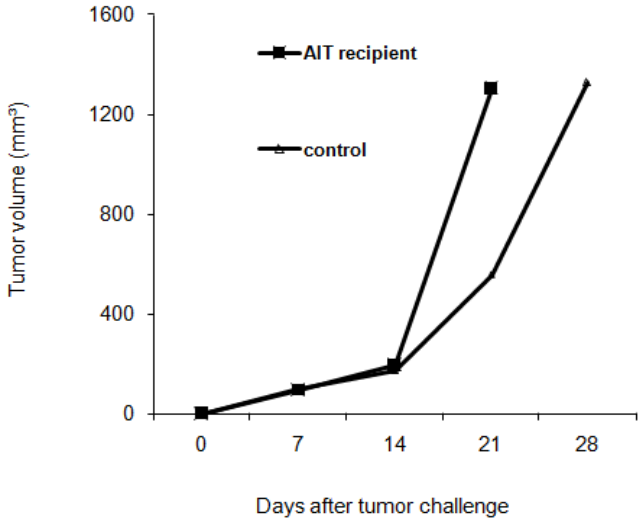


Figure 8: AIT by using T cells expanded with the alternating gamma chain cytokines fail to cause tumor regression *in vivo*

FVBN202 mice were inoculated with MMC (3 million per mouse) and then injected i.v. with 70 million of the *ex vivo* expanded T cells. Prior to AIT, mice received CYP treatment to cause lymphodepletion. Control mice received CYP treatment and MMC challenge but no AIT. The tumor growth was presented as volume.

Figure8



Results

Experimental designs

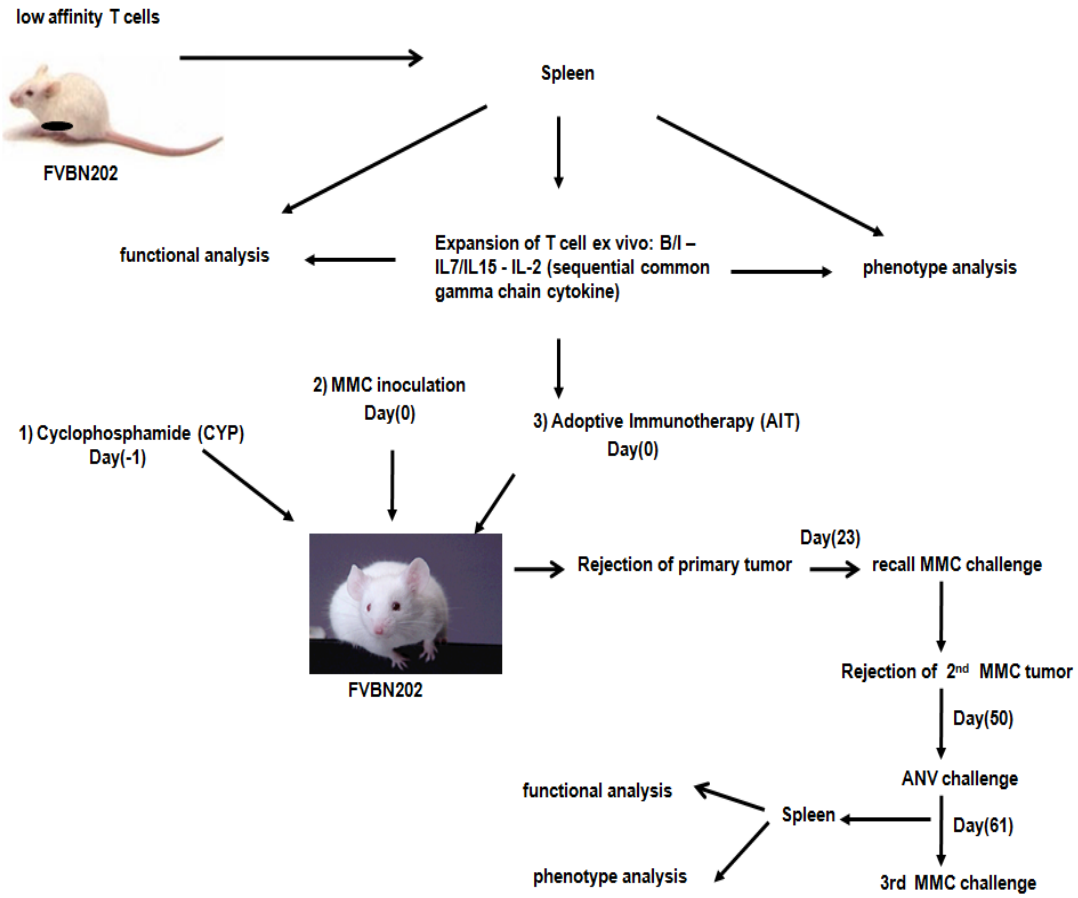
In order to improve anti-tumor efficacy of AIT we made two modifications in our protocol. First, we decided to collect T cells from FVBN202 donors early during tumor development when tumor volume was 200-400 mm^3 rather than late during tumor burden when tumor volume reached 2000 mm^3. Secondly, we changed the alternating gamma chain cytokine formulation to a sequential cytokine formulation (IL-7+ IL-15 followed by IL-2). Since IL-2 can cause activation-induced cell death (AICD), T cells at late stage of differentiation would die and those at early stage of differentiation would survive. This could enrich for effector and effector/memory phenotypes that may last longer *in vivo*. **Figure 9** shows the schematic representation of the experimental procedures. Spleens were harvested from MMC-sensitized tumor-bearing female FVBN202 mice, crushed and processed to obtain single cell suspension. Freshly isolated splenocytes were used for functional assay, *in vitro* anti-tumor responses, and phenotype analysis as well as the *ex vivo* expansion. Splenocytes were stimulated with Bryostatin-1/Ionomycin (B/I) + IL-2 (80 U/10^6 cells/ml) for 16 hours followed by an initial culture with IL-7+IL-15 (10-20 ng/10^6 cells/ml) for 24 hours then pulsed with IL-2 (20U/10^6 cells/ml). One day after, cells were split and cultured with IL-2 (40U/10^6 cells/ml) for the next 3-4 days. The *ex vivo* expanded T cells were used for *in vitro* assays and AIT by i.v. route (7 x 10^7 cells/mouse). The recipients of AIT received cyclophosphamide (100mg/Kg) 24 hours prior to AIT and were subjected to MMC

challenge a few hours before AIT. Twenty three days after, when animals had rejected primary tumors, they were further challenged with MMC without any cyclophosphamide in order to determine memory responses. On days 50 and 61 following primary tumor challenge, animals were challenged with ANV and MMC, respectively. While one group received ANV on the contralateral side and MMC on the same side, another group was challenged with ANV on the contralateral side only. Naïve mice challenged with the tumor served as control. Animals were then sacrificed and their T cells were analyzed *in vitro*.

Figure 9: Schematic representation of the experimental design

Spleens were harvested from MMC-sensitized tumor-bearing (either bearing small or large tumors) female FVBN202 mice, crushed and processed to obtain single cell suspension. Freshly isolated splenocytes were used for functional assay, *in vitro* anti-tumor responses, and phenotype analysis as well as *ex vivo* expansion with sequential common gamma chain cytokine formulation for AIT. Twenty three days after AIT, when animals had rejected MMC tumors, they were further challenged with MMC without any cyclophosphamide in order to determine memory responses. On days 50 and 61 following primary tumor challenge, animals were challenged with ANV on the contralateral side and/or with MMC, respectively. While one group received ANV on the contralateral side and MMC on the same side, another group was challenged with ANV on the contralateral side only. Naïve mice challenged with the tumor served as control. 71- 80 days after the primary tumor challenge animals were sacrificed and their splenic T cells were subjected to *in vitro* analyses.

Figure 9



Sequential common gamma-chain cytokines expand T cells, preferentially CD4+ T cells, from FVBN202 donor mice bearing small tumors

Spleens were harvested from FVBN202 donors 10-14 days after MMC challenge, when tumor volume was between 150mm³-300mm³. These tumor-sensitized T cells were nurtured and proliferated in the presence of sequential gamma chain cytokines and exhibited high anti-tumor efficacy. We first determined the level of MDSCs in the spleen of donor mice. **Figure 10A** shows that MDSCs accounted for 17.91% gated and 6.25% total splenocytes. **Figure 10B** shows two major subsets of MDSCs. Ly6C+Ly6G- subsets accounted for 18.44% on gated CD11b+ and 0.68% of total splenic cells while Ly6C+Ly6G+ subsets accounted for 69.51% gated CD11b+ and 2.1% of total splenic cells. Prior to, during and after the *ex vivo* expansion of splenocytes, trypan blue exclusion was performed to determine the fold expansion. **Figure 11** shows the fold expansion of splenocytes that was comparable to that of the alternating gamma chain cytokines (**Figure 2**). There was about 2.9 fold reduction after B/I stimulation compared to day 0 baseline. On day three, five and six, number of cells increased consistently, ultimately expanding up to 1.9 and 6.5 fold on day 6 compared to day 0 and post-B/I activation. We then performed flow cytometry analysis of T cell subsets to determine their relative proportions. All gated CD4+ or CD8+ cells were found to be CD3+ which proves they were *bona fide* T cells (**Figure 12, lower panel**). Both CD8+ and CD4+ T cells were expanded in the presence of the sequential gamma chain cytokines (**Figure 13**). Rate of expansion was relatively lower than when the alternative gamma chain cytokines was used **(Figure 3**). Unlike the alternating common

gamma chain cytokines, the sequential cytokines preferentially expanded CD4+ T cells (**Figures 3 and 13**) such that the proportion of CD4+ T cells was higher than CD8+ T cells after the expansion.

Figure10: FVBN202 donor mice bearing small tumors have a moderate level of endogenous splenic MDSCs

Spleens harvested from MMC-sensitized tumor bearing (tumor volume=150-300mm³) female FVBN202 mice were stained with antibodies against CD11b, Ly6G, and Gr1 (Ly6C). A total of 100,000 cells were analyzed by flow cytometry to examine the level of endogenous MDSCs and its monocytic and granulocytic subpopulation. (A) percent total and percent gated population of CD11b+Gr1+ (upper right quadrant) MDSCs. (B) percent gated and percent total population of CD11b+Gr1+ subsets. The Ly6C+Ly6G- (upper left quadrant) shows the suppressive monocytic subpopulation whereas the Ly6C+Ly6G+ (upper right quadrant) depicts non-suppressive subpopulation. The bar graphs with error bars show the average values from three different mice±SD.

Figure 10

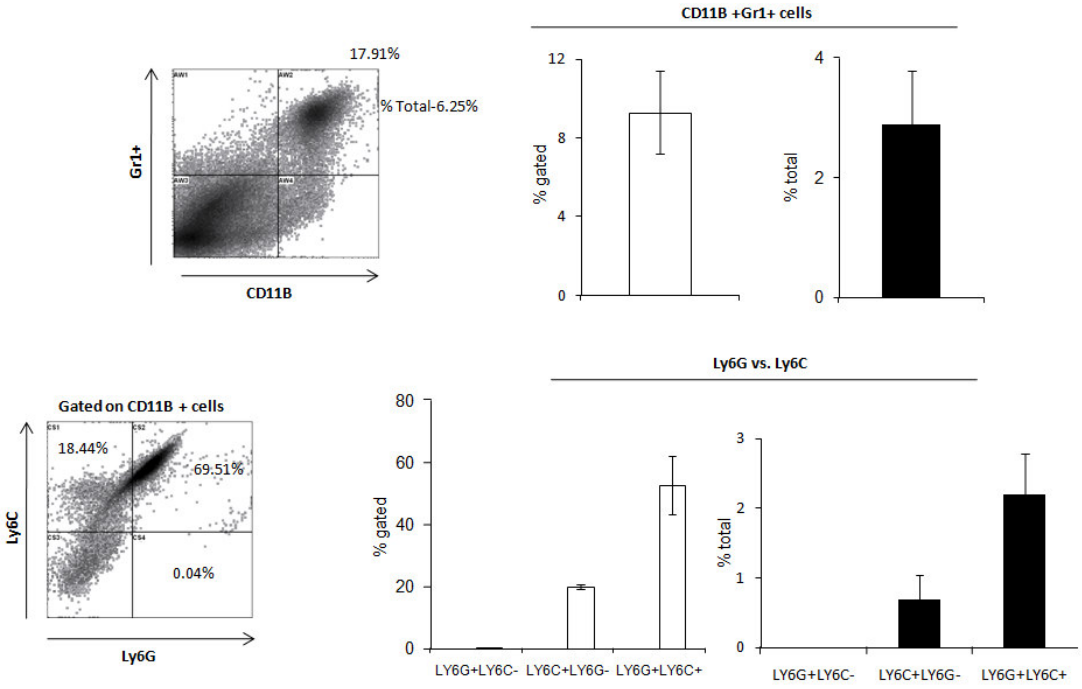


Figure 11: *Ex vivo* expansion of splenocytes with sequential (IL-7/IL-15→IL-2) gamma chain cytokines

Spleens harvested from MMC-sensitized tumor bearing (tumor volume=150-300mm³) female FVBN202 mice (n=2) were crushed and processed to yield single cell suspensions which were subjected to 6 day expansion with sequential common gamma chain cytokines. T cells were incubated with 5nM Bryostatin-1 and 10nM Ionomycin along with 80 U/ml of IL-2 in complete medium for 16 hours followed by expansion with IL-7/IL-15 (10ng/ml), and then with IL-2 (40U/ml). Cell counts were performed using trypan blue exclusion.

Figure 11

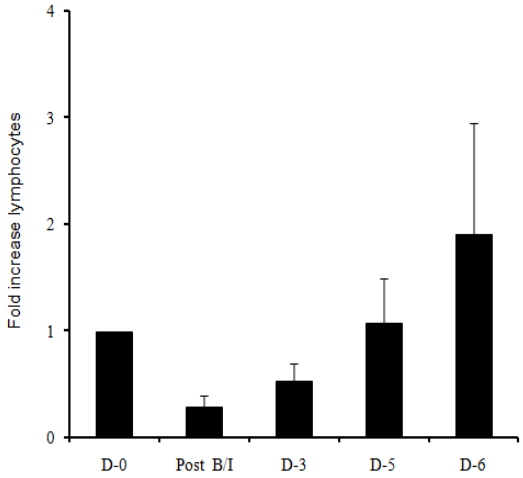


Figure 12: The sequential common gamma chain cytokine formulation preferentially expands CD3+ T cells

The *ex vivo* expanded T cells were stained with CD8, CD3 and CD4 antibodies and 100,000 viable cells from each sample were analyzed by flow cytometry to examine the percentage of CD3+ cell among the expanded population. Gated CD8+ or CD4+ cells were then analyzed for the expression of CD3.

Figure 12

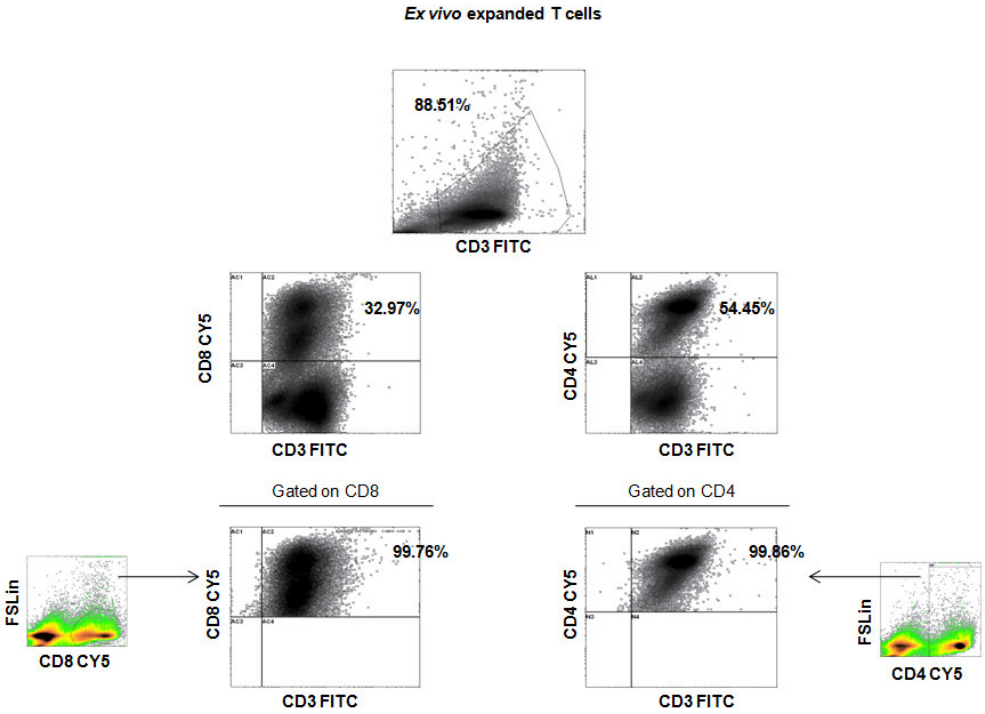
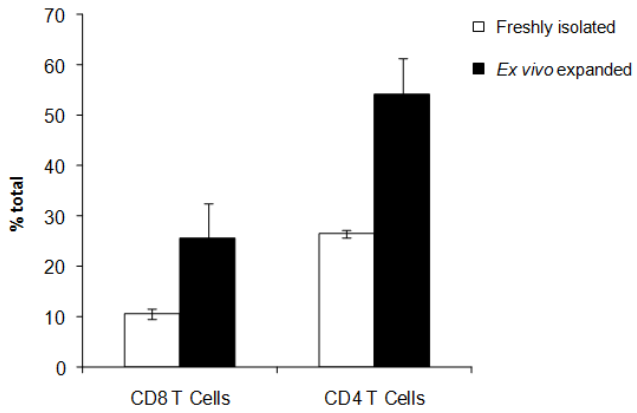


Figure 13: Expansion of splenocytes with the sequential common gamma chain cytokines results in the expansion of CD8+ and CD4+ T cells

Splenocytes were stained with antibodies towards CD8 and CD4 prior to and after a 6-day expansion. Percent total of gated CD8+ and CD4+ T cells were determined.

Figure 13



The *ex vivo* expanded T cells show reduced viability

The success of adoptive immunotherapy depends on viability of the *ex vivo* expanded T cells. First, in order to determine whether MMC stimulation may affect viability of T cells, freshly isolated splenocytes were cultured in the presence or absence of MMC for 24 hours. As shown in **Figure 14**, Annexin V- viable T cells did not change dramatically after the stimulation with MMC ($p > 0.2$). Viability of freshly isolated CD8+ T cells was 61.67% of which $CD8^{high}$ T cells had higher viability than $CD8^{low}$ T cells (**Figure 14A**; 72.05% vs. 14.13%, $p = 0.0000031$). Because of a 24 hour culture of the freshly isolated T cells there was a reduction in viability which made it impossible to compare it with the viability of T cells after the *ex vivo* expansion. However, we know that viability of the freshly isolated T cells is above 87% (**Figure 4**). Considering that expansion of T cells with the sequential common gamma chain cytokines resulted in a reduced viability of CD8+ and CD4+ T cells (**Figure 14B)** compared to T cells expanded with the alternating common gamma chain cytokines (**Figure 4**).

Ex vivo* expanded T cells with the sequential common gamma chain cytokines show an increased tumor-specific IFN-γ production *in vitro

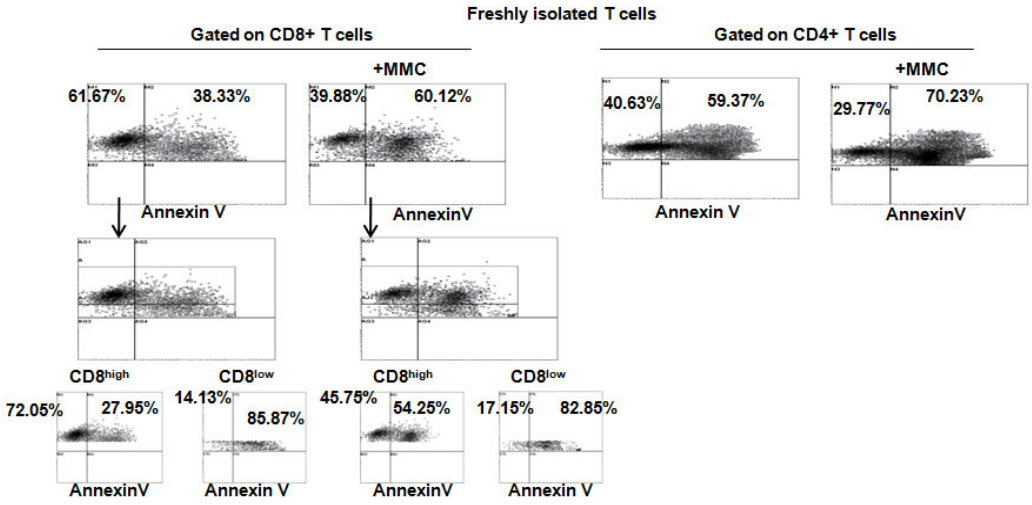
In order to determine whether T cells expanded with the sequential common gamma chain cytokines were enriched for tumor-reactive phenotypes, we performed *in vitro* assays. Freshly isolated T cells or *ex vivo* expanded T cells were cultured either with (+) or without irradiated MMC (-) for 24 hours and then analyzed for their activation. **Figure 15A** shows negligible production of MMC-induced IFN-γ by freshly isolated T

cells whereas the *ex vivo* expanded T cells produced significant amounts of IFN-γ upon stimulation with MMC (p=0.002), as determined by IFN-γ ELISA. In order to determine the cellular source of the IFN-γ production, flow cytometry analyses were performed. **Figure 15B** shows a 5.3 fold higher proportion of CD8+ IFN-γ+ T cells upon MMC stimulation (from 1.23% to 6.53%, p=0.04) whereas CD4+ T cells did not show any significant increase in IFN-γ production (p=0.18). Increased IFN-γ production were noticeable in both $CD8^{high}$ (p=0.042) and $CD8^{low}$ (p=0.037) T cells.

Figure 14: Viability of freshly isolated and *ex vivo* expanded T cells

The lymphocytes were harvested from MMC-sensitized small tumor-bearing (tumor size=150-300 mm^3) female FVBN202 mice and subjected to 6 day expansion with the sequential common gamma chain cytokines. Freshly harvested lymphocytes were cultured alone or with irradiated MMC in the effector:target ratio of 10:1 for 24 hours and stained with antibodies towards CD8, CD4 and AnnexinV. The *ex vivo* expanded T cells were cultured alone and stained similarly. The upper left quadrants representing CD8+AnnexinV-/CD4+AnnexinV- are considered to be viable. The middle panel shows the gating pattern, how CD8 high and low population was separated. Representative dot plots (A) as well as average of the means for two separate experiments (B) are shown.

Figure 14A



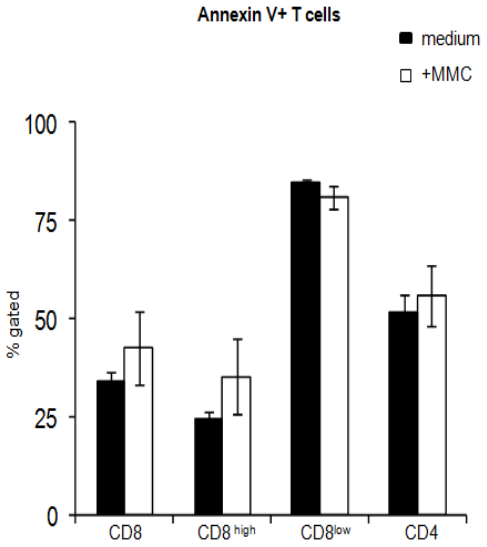


Figure 14B

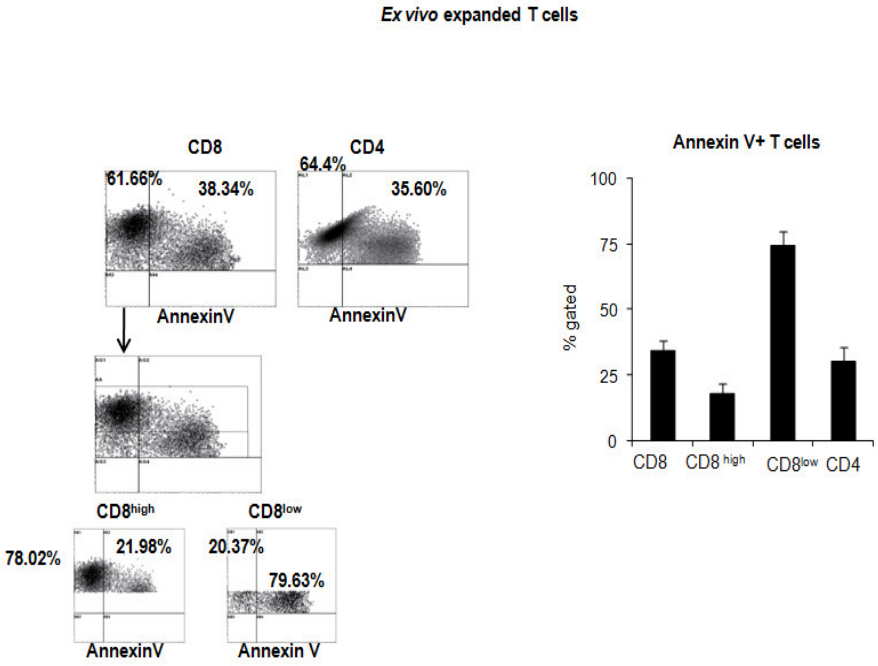


Figure 15: T cells expanded with the sequential common gamma chain cytokine formulation can produce more IFN-γ than freshly isolated T cells upon *in vitro* stimulation with MMC tumor cells

Freshly isolated and *ex vivo* expanded T cells were either cultured alone or with irradiated MMC in an effector:target ratio of 10:1 for 24 hours and supernatants were tested for IFN-γ secretion. (A) data from IFN-γ ELISA, representing IFN-γ secretion by freshly isolated (left panel) and *ex vivo* expanded (right panel) T cells with and without MMC stimulation. (B) represents dot plots from flow cytometric analyses of IFN-γ staining for CD8+ and CD4+ T cells. A total of 100,000 cells of each sample was analyzed. The bar graph with error bars represents the average of percent gated population of IFN-γ+CD8+, IFN-γ+CD8^{high}+, IFN-γ+CD8^{low}+, IFN-γ+CD4+ populations from two different experiments.

Figure 15A

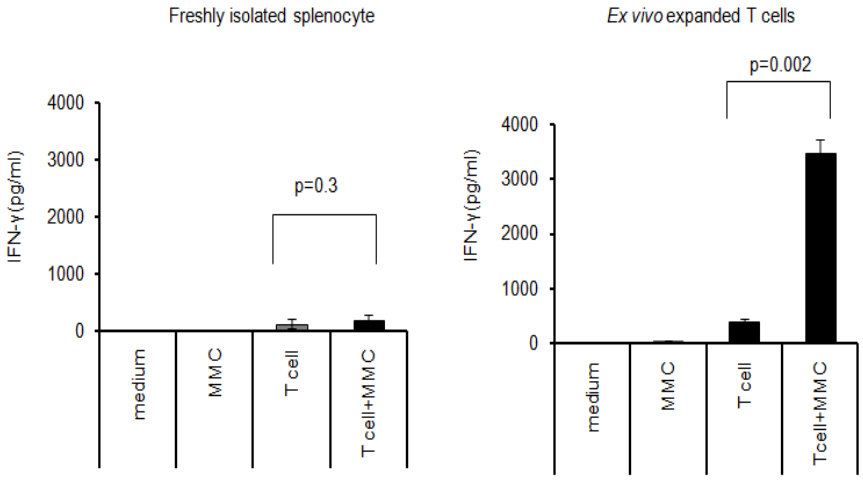
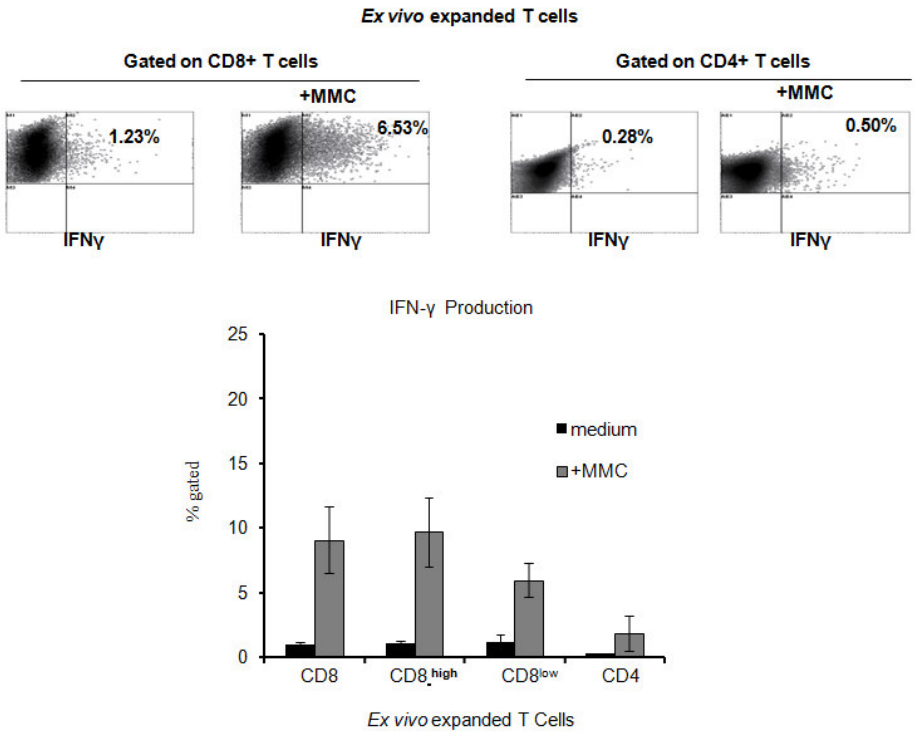


Figure 15B



Ex vivo* expanded T cells with the sequential common gamma chain cytokines show an increased tumor-specific perforin (Prf) production as well as granzyme B (GrB) and CD69 expression in *vitro

Perforin (Prf) is a cytotoxic protein aids in delivering contents of granules into the cytoplasm of target cells. These granules can cause apoptosis of the target cells. The increased production of Prf by T lymphocytes can be considered as "marker" of cytotoxic potential of T cells. Therefore, we looked at the ability of T cells to produce Prf in tumor-specific fashion. Freshly isolated CD4+ T cells were found to have marked increase in the number of cells producing perforin (Prf) upon MMC stimulation (0.63% vs. 2.98%, p=0.02) whereas CD8+ T cells did not show any appreciable changes (1.49% vs. 2.94, p=0.3) (**Figure 16**). The *ex vivo* expanded T cells did not show any marked increase in Prf production upon MMC stimulation (**Figure 17**). However, compared to freshly isolated T cells, only marginal increase in Prf production was detected in the *ex vivo* expanded CD8+ T cells (**Figures 16 and 17**; 1.49% vs. 2.74%, p=0.17).

Granzyme-B is a serine protease which activates apoptosis once within the target cells. Both CD4+ and CD8+ T cells, either freshly isolated (**Figure 18**) or *ex vivo* expanded (**Figure 19**), showed high expression of GrB (> 90%) and it did not increase upon MMC stimulation *in vitro*.

The early activation marker, CD69, showed a 2.82 fold increase in freshly isolated CD8+ T cells (10.23% vs. 28.86%, p=0.002) and a 1.99 fold increase on CD4+ T cells

(19.13% vs. 38.19%, p=0.001) when stimulated with MMC (**Figure 20**). Importantly, the *ex vivo* expanded CD8+ T cells showed significantly higher expression of CD69 compared to freshly isolated ones (**Figures 20 and 21**; 10.23% vs. 81.06%, p=0.04) whereas no increases were detected on CD4+ T cells following the *ex vivo* expansion (**Figures 20 and 21**; 19.13% vs. 14.85%, p=0.16). These *ex vivo* expanded CD8+ T cells and CD4+ T cells showed no significant increase in CD69 expression upon MMC stimulation *in vitro* (**Figure 21**, CD8+ T cells: 81.06 vs. 84.41%, CD4+ T cells: 14.85% vs. 18.87%, p=0.2).

Figure 16: Freshly isolated CD4+ T cells show increased production of Prf upon *in vitro* stimulation with MMC

Splenocytes of FVBN202 donors bearing small tumors were stained with CD8, CD4 and Prf antibodies before and after a 24-hour stimulation with irradiated MMC. A total of 100,000 cells per sample were analyzed to detect the production of Prf. Gated CD8+ and CD4+ T cells were analyzed for the detection of Prf. The bar graphs with error bars represent the average values from three different animals.

Figure 16

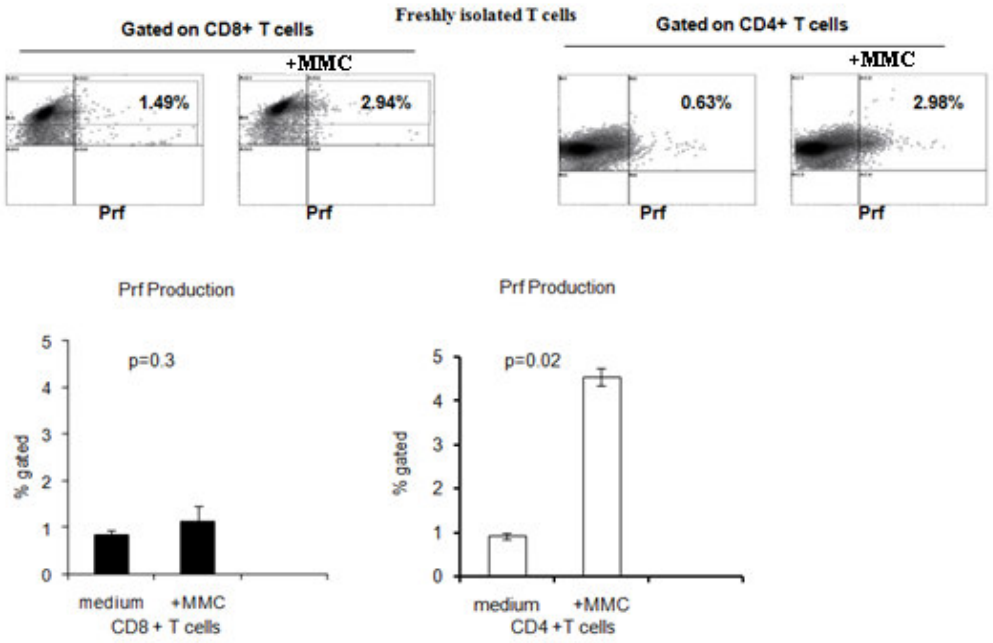


Figure 17: T cells expanded with the sequential common gamma chain cytokine formulation show higher basal level of endogenous perforin (Prf) than freshly isolated T cells but fail to increase Prf upon MMC stimulation

Splenocytes harvested from MMC-sensitized small tumor-bearing female FVBN202 mice were subjected to 6 day expansion with the sequential common gamma chain cytokines and were stained with CD8, CD4 and Prf antibodies before and after stimulation with MMC. A total of 100,000 cells per sample were analyzed by flow cytometry. Gated CD8+ and CD4+ cells were analyzed for the expression of Prf. The bar graph with error bars represents the average values of two different experiments.

Figure 17

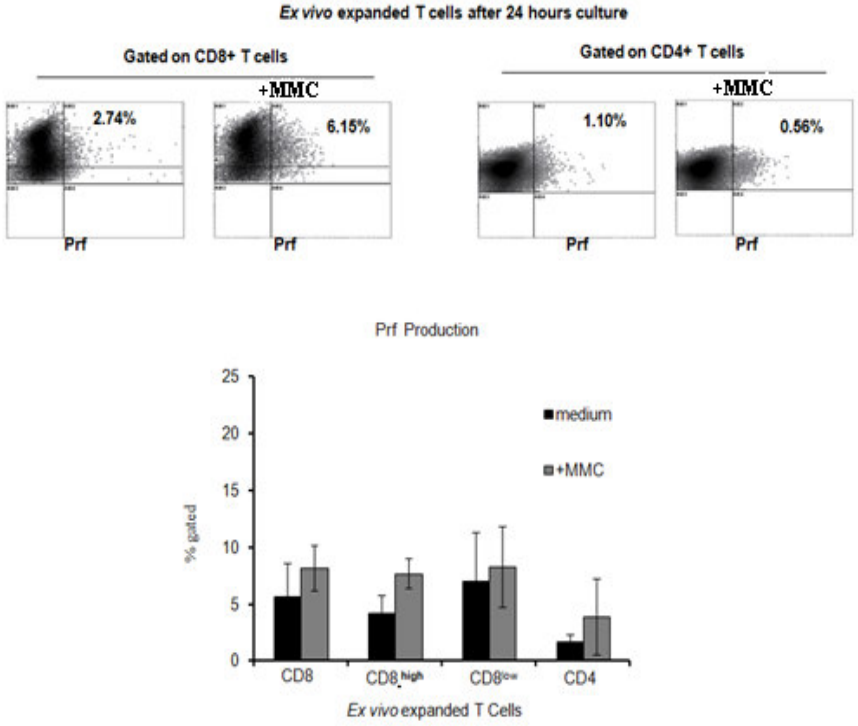


Figure 18: Freshly isolated T cells show high level of granzyme-B (GrB) production

A total of 100,000 cells per sample were analyzed to detect the production of GrB on gated CD8+ and CD4+ cells before and after MMC stimulation. The bar graphs with error bars represent the average values from three different animals examined separately in a single experiment.

Figure 18

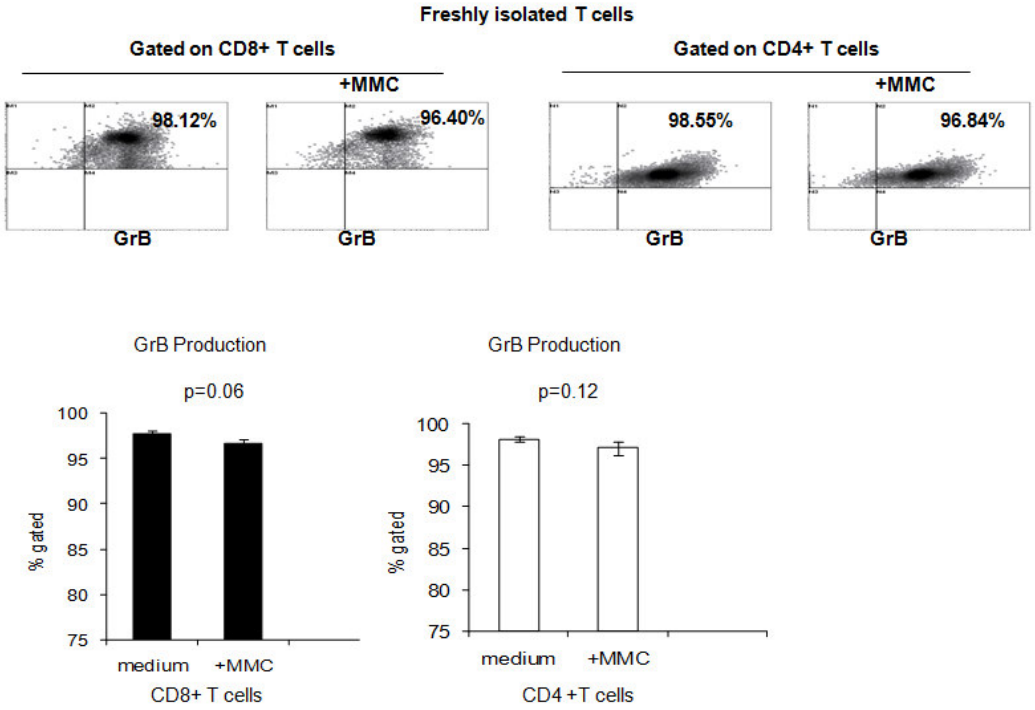


Figure 19: T cells expanded with the sequential common gamma chain cytokine formulation show high level of granzyme B (GrB)

A total of 100,000 cells per sample were analyzed to detect the production of GrB on gated CD8+ and CD4+ cells before and after MMC stimulation. The bar graphs with error bars represent the average values from three different animals examined separately in a single experiment.

Figure 19

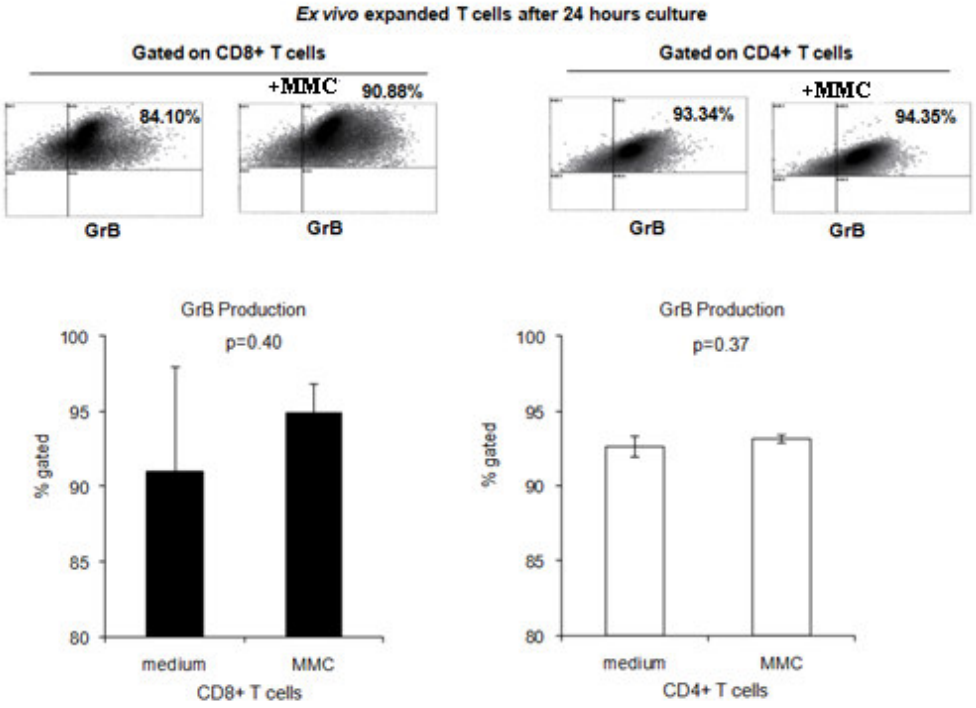


Figure 20: The CD8+ and CD4+ T lymphocytes harvested freshly from MMC-sensitized small tumor-bearing (tumor volume=150-300mm^3) female FVBN202 mice show significant expression of CD69 on MMC stimulation

A total of 100,000 cells per sample were analyzed to detect the expression of CD69 on gated CD8+ and CD4+ cells before and after MMC stimulation. The bar graphs with error bars represent the average values from three different animals examined separately in a single experiment.

Figure 20

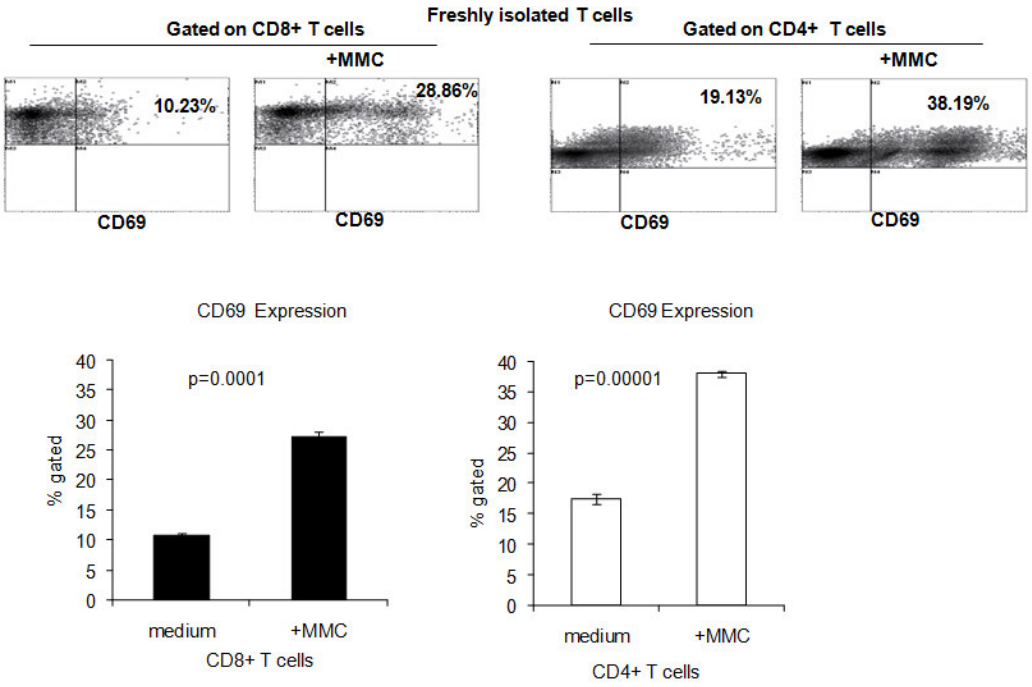
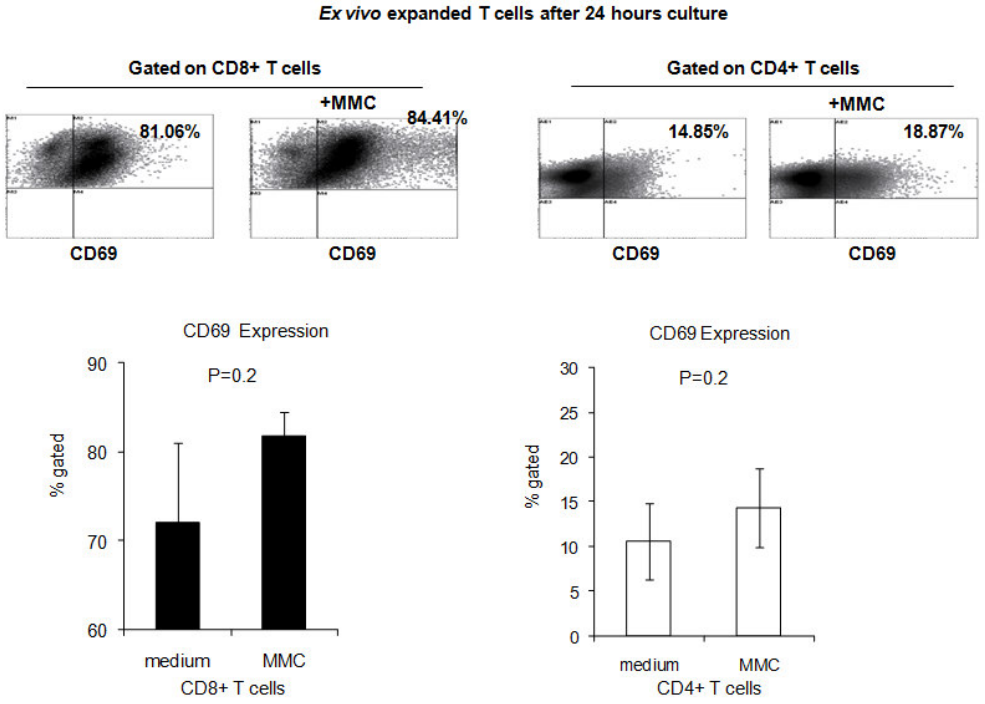


Figure 21: The *ex vivo* expanded T cells with the sequential common gamma chain cytokines show an increased basal level of CD69 expression even in the absence of MMC stimulation *in vitro*

A total of 100,000 cells per sample were analyzed to detect the expression of CD69 on gated CD8+ and CD4+ cells before and after MMC stimulation. The bar graphs with error bars represent the average values from three different animals examined separately in a single experiment.

Figure 21



T cells expanded with the sequential common gamma chain cytokines are highly effective against the tumor and are refractory to MDSCs *in vivo*

In order to determine whether the *ex vivo*-expanded tumor-reactive T cells may also protect FVBN202 mice against tumor challenge even in the presence of endogenous MDSCs, AIT was performed. FVBN202 mice were challenged with MMC 24 hours after cyclophosphamide injection. Animals were split into two groups; the control group remained untreated whereas the test group was subjected to AIT in the absence of MDSC depletion *in vivo*. **Figure 22** shows complete rejection of the tumor following AIT. The control mice (without AIT) developed tumors, as expected.

In order to determine the phenotype distribution of T cells, flow cytometry analyses were performed. **Figure 23** shows the phenotypic distribution of CD8+ and CD4+ T cells in the freshly isolated splenocytes. T_{CM} and T_N phenotypes were the most abundant cells for both CD8+ and CD4+ T cells. CD8+ T cells consisted of 5.62% effector (T_E), 9.91% of effector memory (T_{EM}), 57.23% central memory (T_{CM}) and 26.28% of naïve (T_N) phenotypes. The CD4+ T cells were of 7.97% effector, 7.77% effectors memory, 59.98% central memory and 22.61% of naïve phenotypes. Phenotypic distribution of the *ex vivo* expanded T cells is shown in **Figure 24**. The *ex vivo* expanded CD8+ T cells showed marked increases in T_E and T_{EM} phenotypes whereas CD4+ T cells showed an increased T_E and T_{CM} phenotypes compared to the freshly isolated T cells (**Figures 23 and 24**). The *ex vivo* expanded CD8+ T cells consisted of 43.90% effector, 16.40% effectors memory, 36.43% central memory, and 1.75% naïve phenotypes. The CD4+ T

cells were of 26.33% effector, 9.58% effector memory, 61.27% central memory, and 1.95% naïve phenotypes.

Figure 22: T cells expanded with the sequential common gamma chain cytokines are highly effective against the tumor and are refractory to MDSCs *in vivo*

The *ex vivo* expanded T cells were used for AIT in FVBN202 mice (n=3) challenged with 3 million MMC. Mice were treated with CYP for lymphodepletion. The control mice (n=2) received CYP treatment and MMC challenge but no AIT. The figure shows the tumor growth pattern of the control (upper panel) and the recipient of AIT (lower panel).

Figure 22

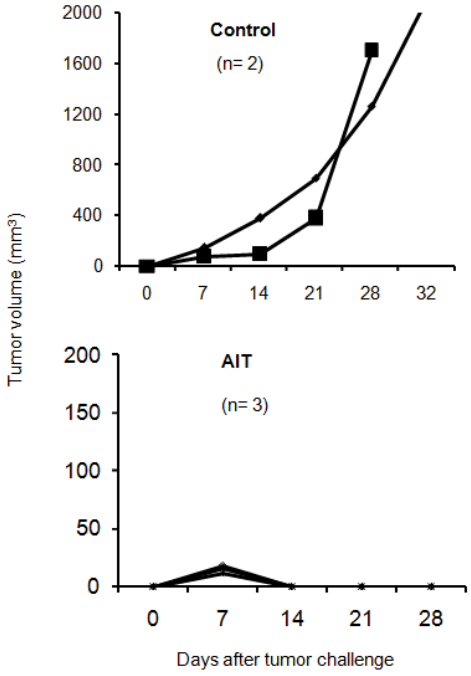
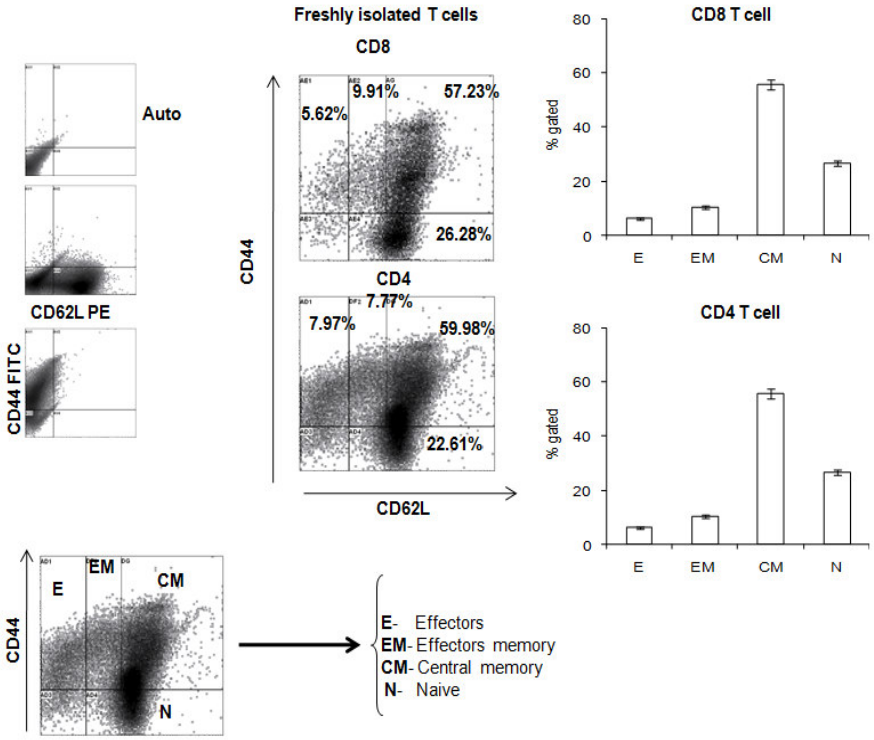


Figure 23: Phenotypic distribution of freshly isolated T cells harvested from MMC-sensitized small tumor-bearing FVBN202 mice

The freshly isolated T cells were stained with CD44, CD62L and CD8/CD4 antibodies and fluorescence of 100,000 viable cells were analyzed by flow cytometry. The lowest panel is a representative dot plot with the quadrants marked for the distribution of various phenotypic subsets of T cells. The upper left panel represents the auto fluorescence of the sample and the fluorescence for single colored antibodies. The upper middle panel shows the phenotypic distribution of the CD8+ and CD4+ T cells. The bar graphs with error bars (upper right panel) represent the average of 3 different animals examined in a single experiment.

Figure 23



Compared to freshly isolated CD8+ T cells, the *ex vivo* expanded cells showed a marked shift toward effector cells (**Figures 23 and 24**; 5.62% vs. 43.9%, p=0.02) and effector/memory cells (9.91% vs.16.4%, p=0.004) whereas central/memory cells decreased from 57.23% to 36.4% (p=0.006). A marked decrease was also observed in naïve CD8+ T cells (26.28% vs. 1.7%, p=0.0001). The MFI for CD44 expression in CD8+ T cells was 85.2 which were over 14 fold higher than that expressed in T cells expanded with the alternating gamma chain cytokines (**Figure 5**). Compared to freshly isolated CD4+ T cells, the *ex vivo* expanded CD4+ T cells showed an increase in effector phenotypes (7.97% vs. 26.33%, p=0.1) while no changes were observed in effector/memory (7.77% vs. 9.58%, p=0.28) and central/memory (59.98% vs. 61.27%, p=0.42) phenotypes. Naïve CD8+ T cells showed a marked decrease following the *ex vivo* expansion (22.61% vs. 1.95%, p=0.001). The MFI for CD44 expression in CD4+ T cells was 51.5 which was 8-10 fold higher than that expressed in T cells expanded with the alternating gamma chain cytokines (**Figure 5**).

Sequential common gamma chain cytokines support the proliferation and differentiation of T cells that are highly effective against the tumor and are refractory to MDSCs *in vitro*

Since the *ex vivo*-expanded T cells with the sequential common gamma chain cytokines showed an increased tumor-reactivity *in vitro* evidenced by the production of IFN-γ and CD69 expression as well as an anti-tumor efficacy *in vivo*, we sought

to determine whether the expanded T cells would show an enhanced cytotoxicity against the tumor cells *in vitro*. Freshly isolated or *ex vivo* expanded T cells were co-cultured with MMC target cells (E:T ratio of 10:1) for 48 hours followed by staining with antibodies directed towards neu, Annexin V and PI. Control wells were seeded with MMC in the absence of T cells. Gated neu positive cells were then analyzed to detect apoptosis (AnnexinV+ and PI+ cells). **Figure 25** shows the result of cytotoxicity assays *in vitro*. The control MMC cells in the absence of T cells showed 87.50% viability (AnnexinV-/PI-). Viability of MMC dropped to 50.79% in the presence of freshly isolated T cells. When co-cultured with the *ex vivo* expanded T cells the viability of MMC dropped from 68.8% to 17.6% (p=0.000027). By normalizing the viability of control MMC to 100%, the *ex vivo* expanded T cells were found to be more efficient in inducing apoptosis of tumor cells than freshly isolated T cells.

Since AIT with the *ex vivo* expanded T cells using sequential gamma chain cytokines but not alternating gamma chain cytokines induced tumor rejection, even in the absence of MDSC depletion *in vivo*, we hypothesized that the sequential gamma chain cytokine regimen supported proliferation and differentiation of T cells that are refractory to the inhibitory effects of MDSCs. In order to test this, T cells were co-cultured with MMC in the presence or absence of MDSCs *in vitro*. **Figure 26A** shows that the *ex vivo* expanded T cells produced a significantly more IFN-γ in the presence of irradiated MMC when compared with T cells alone (T vs. T+MMC p=0.035). In addition, presence of MDSCs at 2:1 ratio (T: MDSCs) did not inhibit MMC-specific IFN-γ production, with a marginal increase in the level of IFN-γ (p= 0.09). No significant

secretion of IFN-γ was detected in MMC, MDSCs or T cells alone. To determine cellular source of IFN-γ production we performed flow cytometry analyses. The gating patterns used for the analyses are shown in **Figure 26B**; the lymphocyte region was first gated and CD8+ T cells and CD4+ T cells were re-gated out in order to determine IFN-γ production by the T cell subsets. **Figure 26C** shows that the *ex vivo* expanded CD8+ T cells produced IFN-γ when stimulated with MMC in the absence or presence of MDSCs (0.75% vs. 11.67% or vs. 30.04%). The CD4+ T cells also showed increased IFN-γ production upon MMC stimulation in the absence or presence of MDSCs (0.29% vs.3.24% or vs.11.23%). There were significant increases in IFN-γ production by CD8+ (p=0.04), $CD8^{high}$ (p=0.04) and $CD8^{low}$ (p=0.037) T cells upon MMC stimulation whereas CD4+ T cells showed a negligible increase (p=0.18) in IFN-γ production.

Figure 24: Phenotypic distribution of the T cells harvested from MMC-sensitized small tumor-bearing FVBN202 mice and expanded with the sequential common gamma chain cytokines

T cells were stained with CD44, CD62L and CD8/CD4 antibodies and fluorescence of 100,000 viable cells were analyzed by flow cytometry. The far left panel represents the auto fluorescence of the sample and the fluorescence for single colored antibodies. The middle panel shows the phenotypic distribution of the CD8+ and CD4+ T cells. The bar graphs with error bars (right panel) represent the average values of two different experiments.

Figure 24

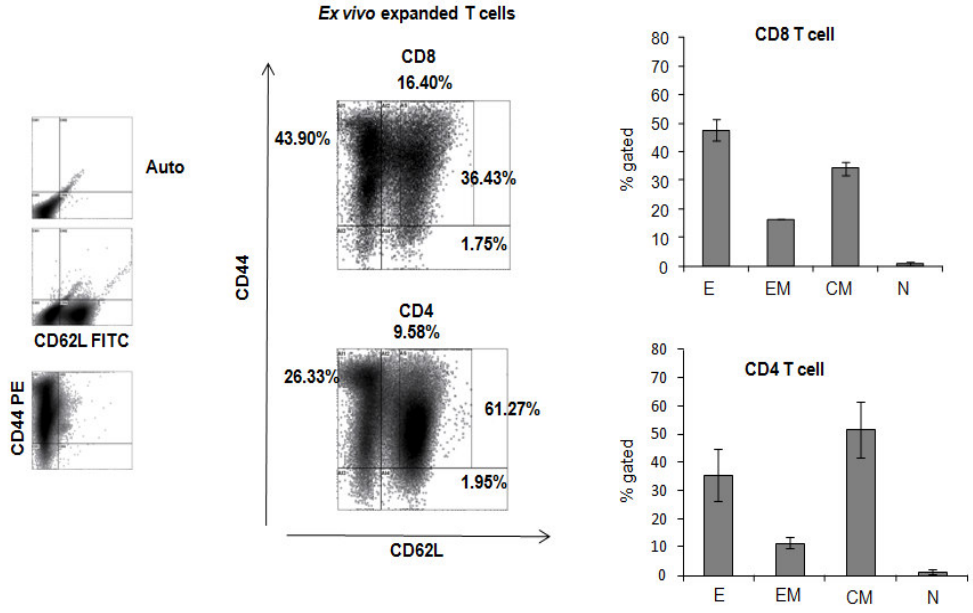
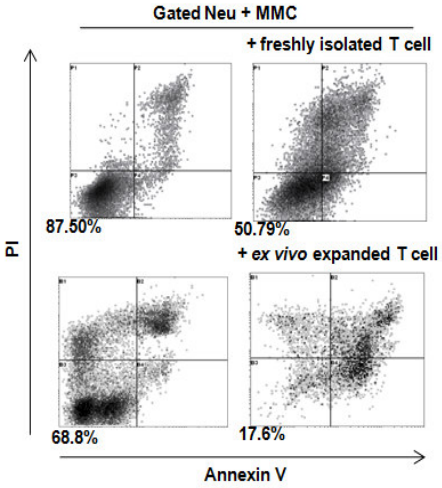


Figure 25: T cells expanded with the sequential gamma chain cytokines show more anti-tumor efficacy than freshly isolated lymphocytes

The cells were stained with antibodies against neu, AnnexinV and PI. The neu+ cells were gated and viability was determined. The lower left panel represents the viable population (AnnexinV- PI-). The figure shows the viability of MMC control and MMC cultured with freshly isolated lymphocytes(upper panel) and MMC control and MMC cultured with *ex vivo* expanded T cells (lower panel). In the bar graph the viability of control MMC has been normalized to 100%. The bar graph (above) represents the average of 3 animals and the bar graph (below) shows the average values from 2 different experiments.

Figure25



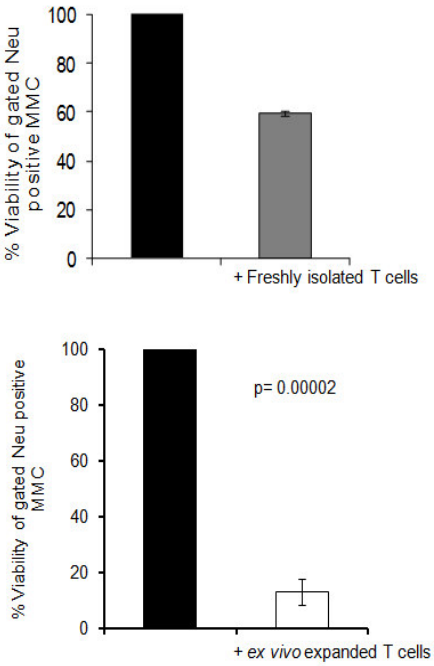


Figure 26: T cells expanded with sequential common gamma chain cytokines produce tumor-specific IFN-γ even in the presence of MDSCs

The T cells expanded *ex vivo* with sequential common gamma chain cytokines were cultured alone, with irradiated MMC and together with irradiated MMC and MDSC in the Effector:Target:MDSC ratio of 10:1:5 for 24 hours and the supernatants were tested for the level of secreted IFN-γ. (A) represents data of IFN-γ ELISA and shows that T cells can produce more IFN-γ on stimulation with MMC even in presence of MDSC. The error bars show the average values from two different experiments. (B) represents the gating pattern followed to gate out the CD8+ and CD4+ T cells during flow cytometry analyses, in order to confirm that the potential source of IFN-γ is the T lymphocytes but not the MDSC. (C) *ex vivo* expanded T cells were stained with the CD8, CD4 and IFN-γ antibodies and flow cytometry analyses were done. The upper panel shows the amount of IFN-γ produced by T cells alone and by T cells on MMC stimulation. The middle panel represents the histogram showing that the contribution of MMC in IFN-γ production was negligible, and the lower panel shows the level of IFN-γ secreted by cells when cocultured with MDSCs. The bar graph with error bars represents the average values from two different experiments.

Figure 26A

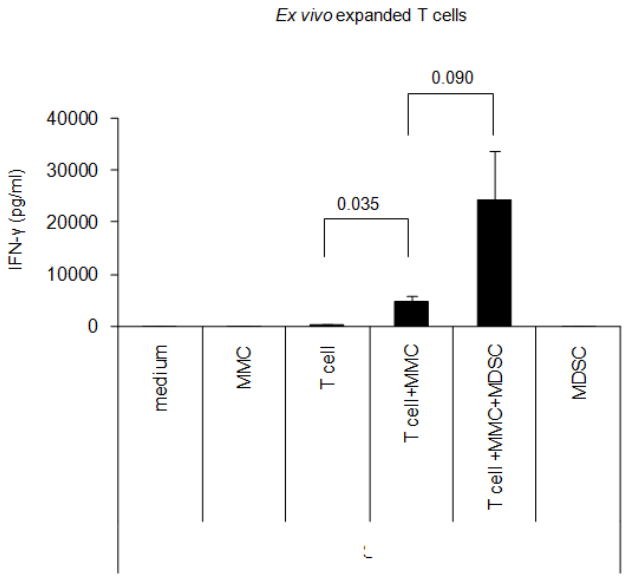


Figure 26 B

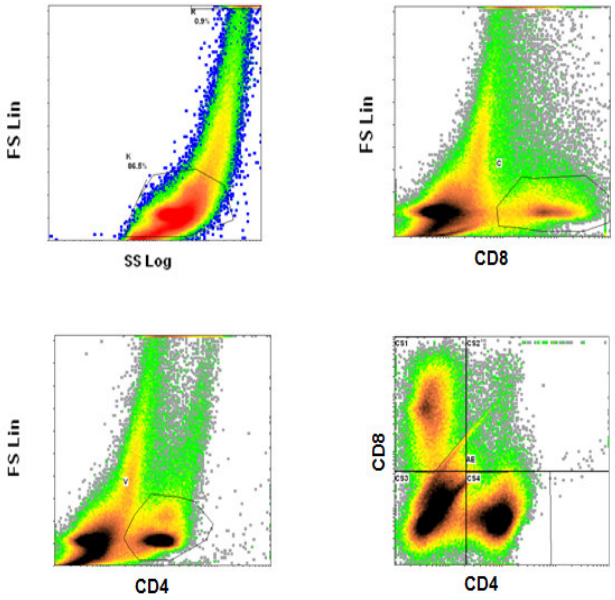
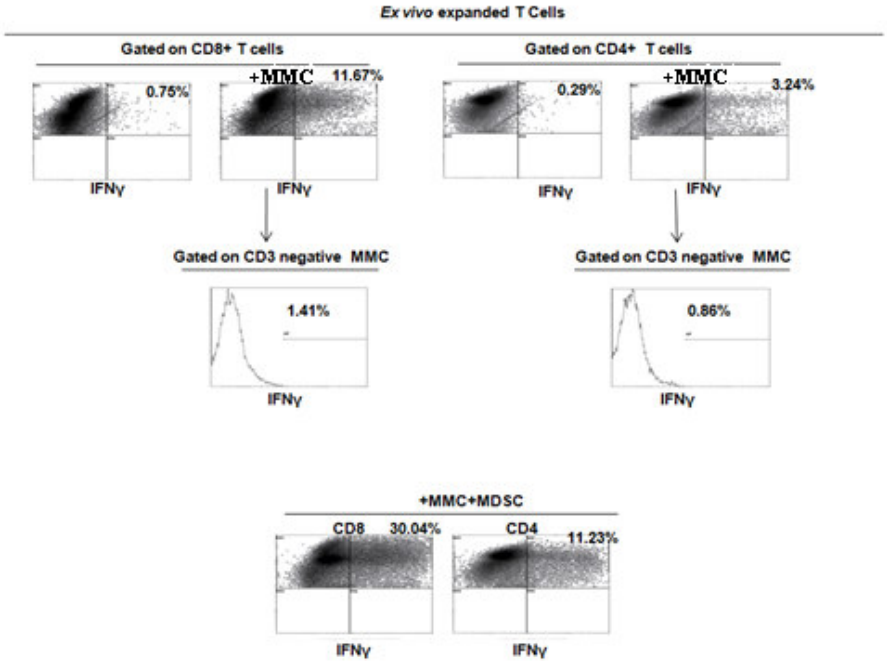


Figure26C



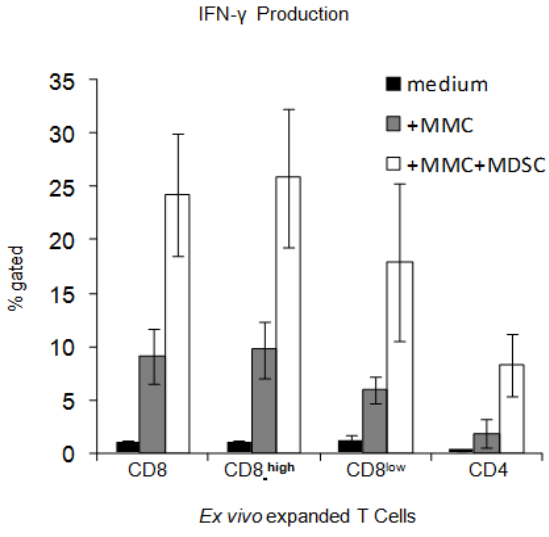
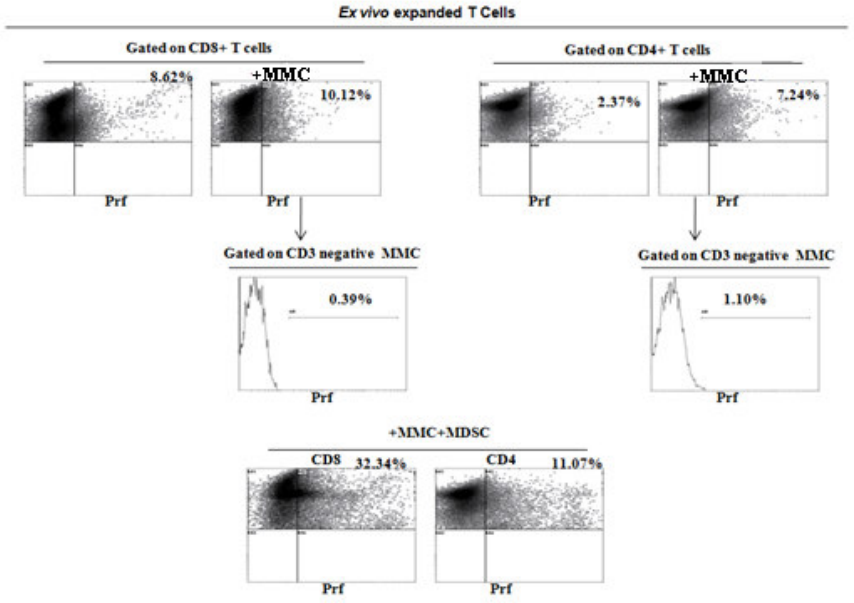
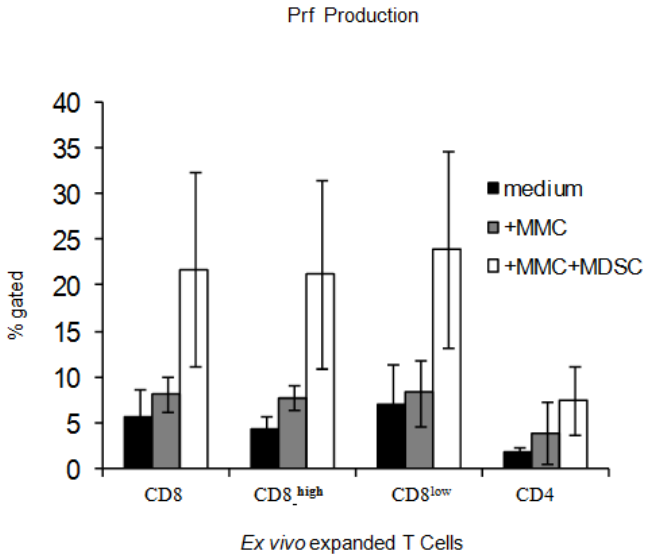


Figure 27: T cells expanded with the sequential common gamma chain cytokines are refractory to the inhibitory effects of MDSCs and can produce perforin (Prf) upon MMC stimulation even in presence of MDSCs

The T cells expanded *ex vivo* with sequential common gamma chain cytokines were cultured alone, with irradiated MMC and together with irradiated MMC and MDSC in the Effector:Target:MDSC ratio of 10:1:5 for 24 hours and the supernatants were tested for the level of Prf. The *ex vivo* expanded T cells were stained with CD8, CD4 and Prf antibodies and flow cytometry analyses were done. The upper panel shows the amount of Prf produced by T cells alone and by T cells on MMC stimulation. The middle panel represents the histogram showing the contribution of MMC in Prf production was negligible, and the lower panel shows the level of IFN-γ secreted by cells when cocultured with MDSCs. The bar graph with error bars represents the average values from two different experiments.

Figure27





Presence of MDSCs slightly increased IFN-γ production by CD8+ T cells upon MMC stimulation, though it was not found to be significant (p=0.09). A similar trend as to the effects of MDSCs on T cell responses was detected for perforin production by the *ex vivo* expanded T cells (**Figure 27**). Initially 8.62% of CD8+T cells were found to be positive for perforin, the percentage increased slightly to 10.12% and further to 32.34% when co-cultured with MMC alone and MMC+MDSC, respectively. The percentage of CD4+ Prf+ T cells increased from 2.37% to 7.24% with MMC alone and further to 11.07% with MMC + MDSCs. Although there was increased perforin production by T cells stimulated with MMC in presence or absence of MDSCs, the statistical analyses of the repeat experiments revealed no significant differences (for CD8+ cells; T vs. T+MMC p=0.28; T vs. T+MMC+MDSC p= 0.14).

Figures 28 and **29** show that addition of MDSCs did not alter the Granzyme-B production nor did alter the expression of CD69.

Finally, *in vitro* cytotoxicity assays revealed that the *ex vivo* expanded T cells can kill MMC even in the presence of MDSCs (**Figure 30**). Neu positive MMC target cells were co-cultured with the *ex vivo* expanded T cells in the presence or absence of MDSCs (E:T:MDSC ratios of 10:1:5) for 48 hours followed by staining with antibodies directed towards neu, Annexin V and PI. Control wells were seeded with MMC in the absence of T cells. Gated neu positive cells showed apoptosis in the presence of T cells regardless of the presence or absence of MDSCs. Viability of MMC dropped from 74.87% to 3.66% (p= 0.004) when co-cultured with T cells and was further reduced to

1.22% (p =0.003) in the presence of MDSCs. There were no significant differences in the apoptosis of MMC induced by T cells in the presence or absence of MDSCs (p= 0.3).

Figure 28: T cells expanded with the sequential common gamma chain cytokines are refractory to the inhibitory effects of MDSCs and can maintain unaltered Granzyme-B (GrB) upon MMC stimulation and in the presence of MDSC

The *ex vivo* expanded T cells were stained with the CD8, CD4 and GrB antibodies and flow cytometry analyses were done. The upper panel shows the amount of GrB produced by T cells alone and by T cells upon MMC stimulation and the lower panel shows the level of GrB secreted by cells when co-cultured with MDSCs. There is no increase in GrB production upon MMC stimulation as the endogenous level has reached saturation.

Figure 28

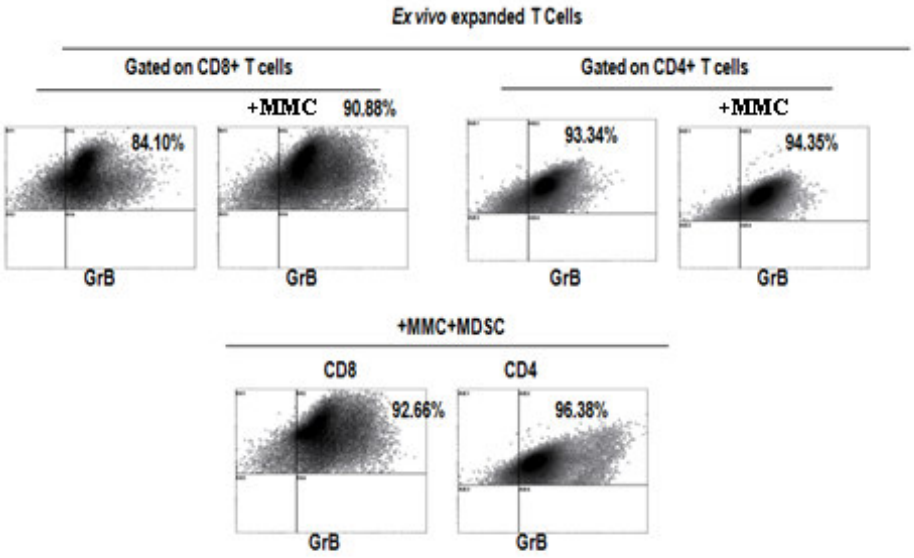


Figure 29: T cells expanded with the sequential common gamma chain cytokines are refractory to the inhibitory effects of MDSCs and can maintain unaltered expression of CD69 upon MMC stimulation even in presence of MDSCs

The *ex vivo* expanded T cells were stained with the CD8, CD4 and CD69 antibodies and flow cytometry analyses were done. The upper panel shows the amount of CD69 expressed by T cells alone and by T cells upon MMC stimulation and the lower panel shows the level of CD69 expression by cells when co-cultured with MDSCs. There is no marked increase in CD69 expression as the endogenous level has reached saturation.

Figure 29

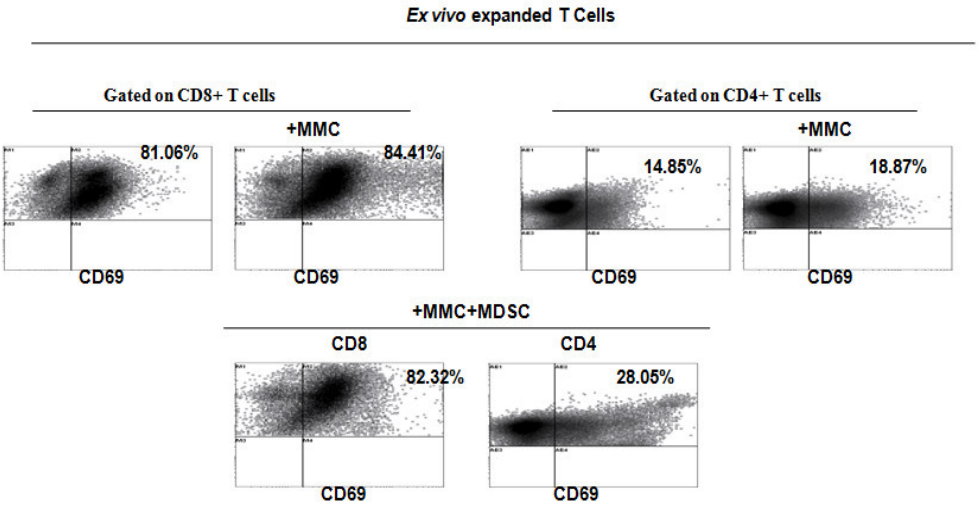
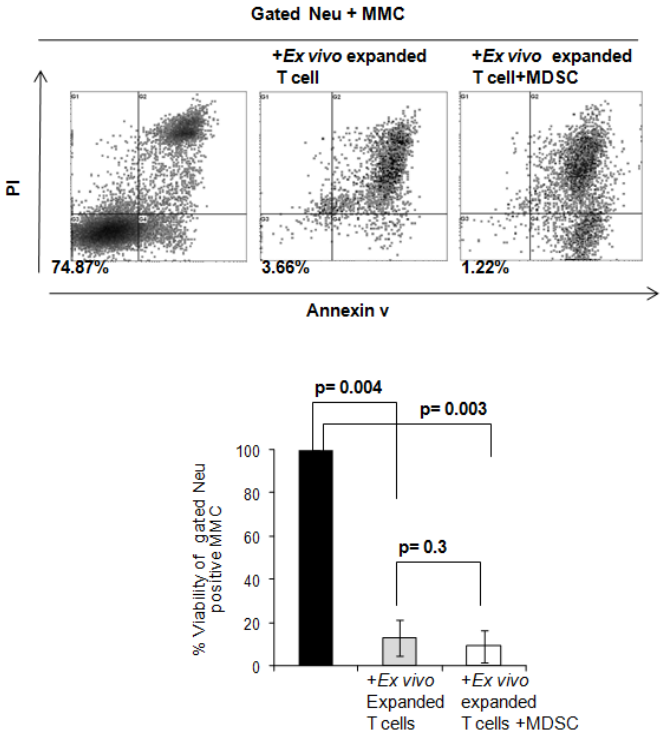


Figure 30: T cells expanded with the sequential common gamma chain cytokines show an enhanced cytotoxicity against MMC compared to freshly isolated T cells even in the presence of MDSCs

The freshly isolated and the *ex vivo* expanded T cells were cultured with MMC or with MMC and MDSC in the Effector: Target:MDSC ratio of 10:1:5. The control wells were seeded with MMC alone and cultured for 48 hours. The cells were stained with neu, AnnexinV and PI antibodies. The neu+ cells were gated and viability was determined. In the bar graph the viability of control MMC has been normalized to 100%. The dot plots represent the viability of the control MMC and MMC co-cultured with T cells and MDSCs. The bar graph shows the average values from 2 different experiments.

Figure 30



Sequential common gamma chain cytokines expand tumor-reactive T cells derived from the FVBN202 donors bearing large tumors

Since donors of T cells that were expanded with the alternating gamma chain cytokines were harboring large tumors whereas those expanded with the sequential gamma chain cytokines had small tumors, we sought to determine whether the source of T cells during early versus late tumorigenesis may be responsible for the differential effects that we observed. In order to test this possibility, we collected T cells from FVBN202 donors carrying large tumors (>1000 mm^3) and expanded them with sequential gamma chain cytokines followed by *in vitro* and *in vivo* analyses. **Figure 31** shows the level of MDSCs in the spleen of donors, accounting for 73.4% of the granulocyte gated and 9.3% of the total splenocytes. Spleens from two donors were harvested 21 days after MMC challenge, pooled together, and subjected to a 6-day expansion with the sequential gamma chain cytokines. **Figure 32** shows a drop from 150 million cells to 77.7 million cells after the B/I activation followed by the expansion to 805.98 million cells on day six. There was 5.37 and 10.36 fold increase of T lymphocytes compared to day 0 and post B/I activation respectively. T cells expanded with the sequential common gamma chain cytokines showed better expansion potential than with those expanded with the alternating gamma chain cytokine regimen. **Figure 33** shows that the *ex vivo* expanded CD8+ T cells increased from 4.06% to 25.88% (6.37 fold) and CD4+ T cells increased from 12.15% to 46.78% (3.85 fold). The expanded T cells were used for further *in vitro* experiments to assess the anti-tumor responses. **Figure 34A** shows a marked increase in IFN-γ production by the *ex vivo* expanded T cells stimulated with

MMC (CD8+ T cells: 5% vs. 30%; CD4+ T cells: 0.1% vs. 1%). A similar trend was detected when T cells were analyzed for the expression of perforin (**Figure 34B**). CD8+Prf+ T cells were increased from 58% to 84% and CD4+Prf+ T cells were increased from 7% to 16.4%, when stimulated with MMC *in vitro*. Compared to the expanded T cells derived from donors bearing small tumors (**Figure 17**), those from donors with large tumors (**Figure 34B**) showed increase in CD8+Prf+ T cells in the absence (2.74% vs. 58%) or presence of MMC (6.15% vs. 84%). Similar trends were detected for CD4+Prf+ T cells in the absence (1.1% vs. 7%) or presence (0.56% vs. 16.4%) of MMC. Similar to the T cells derived from mice with small tumors (**Figure 28**); T cells derived from mice with large tumors (**Figure 34C**) expressed high levels of GrB before or after MMC stimulation. Similar trends were observed for the expression of CD69 (**Figure 34D**). CD8+CD69+ T cells were increased from 63% to 79%, when stimulated with irradiated MMC. CD4+CD69+ T cells were also increased from 6.4% to 10%, when stimulated with MMC. There were no significant differences in the expression of CD69 comparing the T cells derived from mice with small tumors (**Figure 29**) and those from mice with large tumors (**Figure 34D**).

Figure 31: The level of endogenous MDSCs is very high in the FVBN202 donors bearing large tumors

The splenocytes were harvested from the MMC tumor-bearing female FVBN202 mice and were stained with CD11b and Gr1 antibodies to determine the level of endogenous splenic MDSCs. The figure represents a very high CD11b+Gr1+ MDSC level (upper right quadrant). Both the percent total and percent gated values are being shown in the data.

Figure31

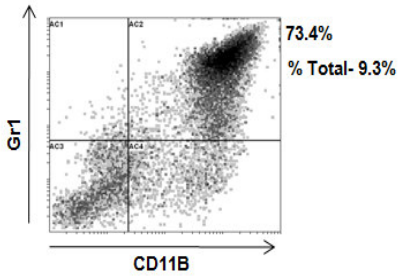


Figure 32: *Ex vivo* expansion of splenocytes of FVBN202 donors bearing large tumors using the sequential (IL-7/IL-15→IL-2) gamma chain cytokines

Spleens harvested from MMC-tumor bearing (tumor size> 1000mm³) female FVBN202 mice were crushed and processed to yield single cell suspension which were subjected to a 6-day expansion with the sequential common gamma chain cytokines. Cell counts were determined by trypan blue exclusion.

Figure 32

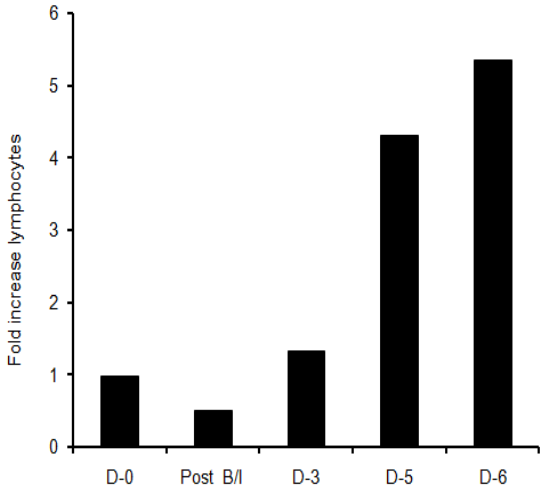


Figure 33: *Ex vivo* expansion of T cells of FVBN202 donors bearing large tumors using the sequential (IL-7/IL-15→IL-2) gamma chain cytokines

Flow cytometry analyses of the percent total population of CD8+ and CD4+ T cells before and after the expansion with the sequential common gamma chain cytokine formulation.

Figure 33

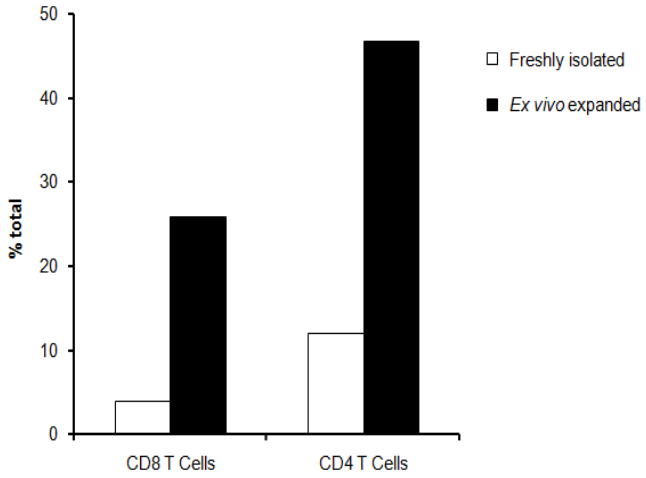
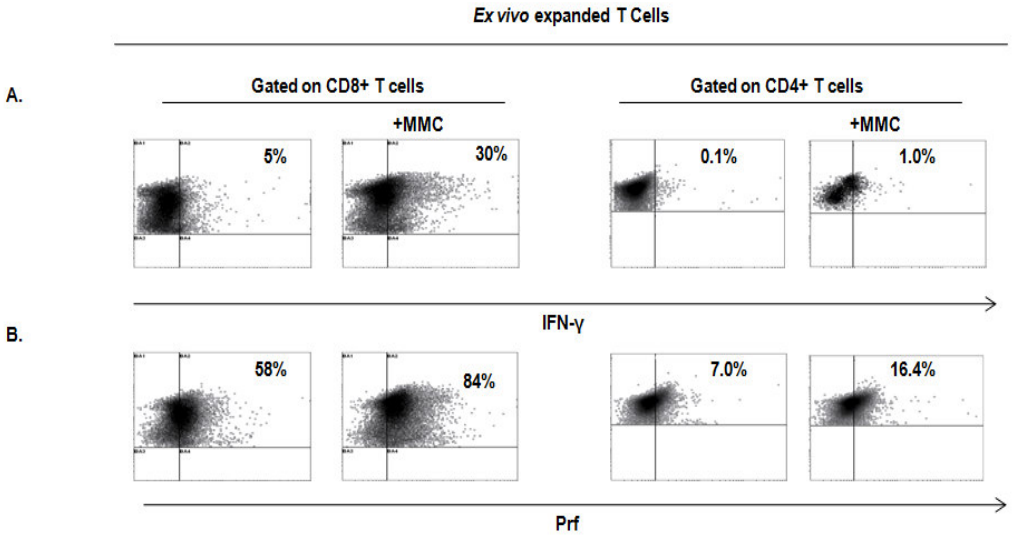
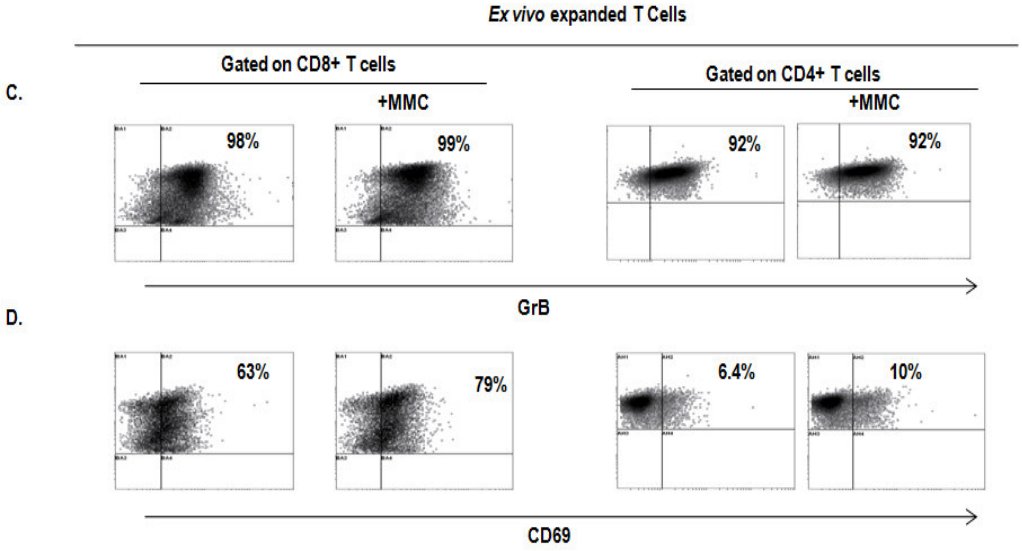


Figure 34: Production of IFN-γ, perforin and granzyme B as well as the expression of CD69 by the *ex vivo* expanded T cells harvested from FVBN202 donors bearing large tumors

The *ex vivo* expanded T cells were cultured alone or with irradiated MMC in the ratio of 10:1 for 24 hours and stained with CD8, CD4 and specific antibodies against various surface and internal molecules which are considered to be the marker of anti-tumor responses. Fluorescence for 100,000 viable cells was analyzed by flow cytometry. (A) represents MMC-induced IFN-γ production by CD8+ and CD4+ T cells. (B) perforin production by both CD8+ and CD4+ T cells. (C) Granzyme-B production by both CD8+ and CD4+ T cells. (D) expression of CD69 by both CD8+ and CD4+ T cells upon MMC stimulation.

Figure 34





We also tried to compare the phenotypic distribution of freshly isolated and the *ex vivo* expanded T cells. It is clear from the data that the *ex vivo* expanded CD8+ T cells were of effector and effector/memory while CD4+ T cells were of effector and central/memory phenotypes (**Figure 35**) similar to what was detected when T cells were isolated from FVBN202 donors bearing small tumors and expanded with the sequential gamma chain cytokines (**Figure 24**). The *ex vivo* expanded T cells showed 56% effector, 16% effectors/memory, 22% central/memory, and 4% naïve phenotypes (**Figure 35**). The MFI for CD44 was 30.9 which was over 6 fold higher than that expressed in CD8+ T cells expanded with the alternating common gamma chain cytokines (**Figure 5**). The CD4+ T cells consisted of 26% effector, 10% effector/memory, 60% central/memory, and 3.5% naïve phenotypes. The MFI for CD44 was 33.1 which was 4-5 fold higher than that expressed in CD4+ T cells expanded with the alternating common gamma chain cytokines (**Figure 5**). Stimulation of these T cells with irradiated MMC, *in vitro*, did not change the proportion of T cell phenotypes (**Figure 35**). These phenotypic distributions remained the same after MMC stimulation.

***Ex vivo* expansion of T cells with the sequential common gamma chain cytokines render them refractory to MDSCs *in vivo* and generate long term memory responses against mammary tumors**

Since CD8+ T cells harvested from donors with large or small tumors showed predominant effector and effector/memory phenotypes upon expansion with the sequential gamma chain cytokines, we hypothesized that presence of effector/memory T

cells might protect the mice against recall tumor challenge and might also provide protection against relapsed tumors not expressing neu (ANV). To test our hypotheses, FVBN202 recipients were inoculated with 5 million MMC for the second time on day 23 after the first inoculation without receiving cyclophosphamide or AIT. Control naïve mice were inoculated with an equal number of MMC cells. **Figure 36A** shows that all animals (n=3) rejected the recall tumor challenge within 14 days. Control mice developed tumors, as expected. To determine whether memory T cell responses might also protect the mice against neu negative relapsed ANV tumor cells, animals were split into two groups. Group 1 was inoculated with 4 million ANV on the contralateral side, 13 days after rejecting the recall MMC, and with 5 million MMC (3^{rd} challenge) 11 days after ANV challenge. Group 2 was only challenged with ANV on the contralateral side. The control group was inoculated with an equal number of ANV cells only. **Figure 36B** shows complete rejection of MMC and significant inhibition of ANV compared to the control group. Group 2 also showed inhibition of ANV compared ANV control mouse, though to a lesser degree compared to those inoculated with MMC, simultaneously. In order to determine phenotypic distribution of T cells in these recipient mice as well as the anti-tumor efficacy against MMC and ANV *in vitro*, animals were sacrificed and their splenocytes were analyzed. **Figure 37** represents the phenotypic distribution of the T lymphocytes derived from the spleen of the animal which received AIT and were challenged with MMC (2^{nd} inoculation) and ANV. When sacrificed the ANV tumor was about 600mm³. The data show predominant effector phenotypes for both CD8+ and CD4+ T cells.

In order to confirm reactivity of the T cells with both MMC and ANV, animals that were challenged with MMC and ANV (group 1) were sacrificed when the ANV tumor was about 74 mm^3. **Figure 38A** shows the level of CD11B+Gr1+ MDSCs in the spleen of the recipient mouse, accounting for 4.24% of the gated granulocyte region and 1.12% of the total population. Gated CD11B+ population showed 5.67% Ly6G+Ly6C- and 17.93% of Ly6G-Ly6C+ as well as 12.87% of Ly6G+Ly6C+ population. As shown in **Figure 38B** T cells showed an increased cytotoxicity against MMC (viability of MMC reduced from 72.91% in the absence of T cells to 62.01% in the presence of T cells). T cells also showed an increased cytotoxicity against CFSE-labeled ANV (**Figure 38C;** from 84.19% in the absence of T cells to 51.88% in the presence of T cells).

Protein ELISA was conducted on the serum collected from the animals which received tumor challenge followed by AIT and successfully rejected the tumor and showed memory responses. 5 and 50 fold dilution series of the serum was prepared for the ELISA; no antibody mediated response towards the tumor antigen was detected in these animals (data not shown).

Figure 35: Phenotypic distribution of the *ex vivo* expanded T cells harvested from FVBN202 donors bearing large tumors and expanded with the sequential common gamma chain cytokines

The *ex vivo* expanded T cells were stained with CD44, CD62L and CD8/CD4 antibodies and fluorescence of 100,000 viable cells was analyzed by flow cytometry.

Figure 35

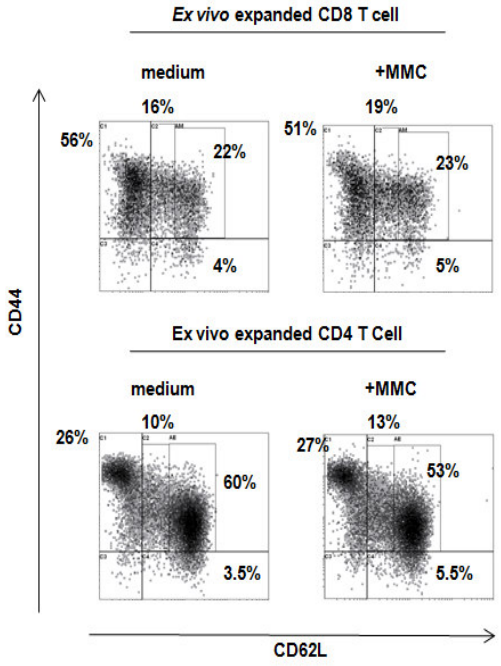


Figure 36: *Ex vivo* expansion of T cells with the sequential common gamma chain cytokines generate long term memory responses against mammary tumors

The animals represented in Figure 22, when successfully rejected the primary tumor after receiving AIT with the *ex vivo* expanded T cells were further re-challenged with 5 million of MMC (A). This time no CYP treatment or AIT was given to the animals. On day 50 they received 4 million of ANV on the contralateral side and lastly on day 61, one of them received a 3rd MMC re-challenge on the contralateral side of the previous inoculation (B).

Figure 36

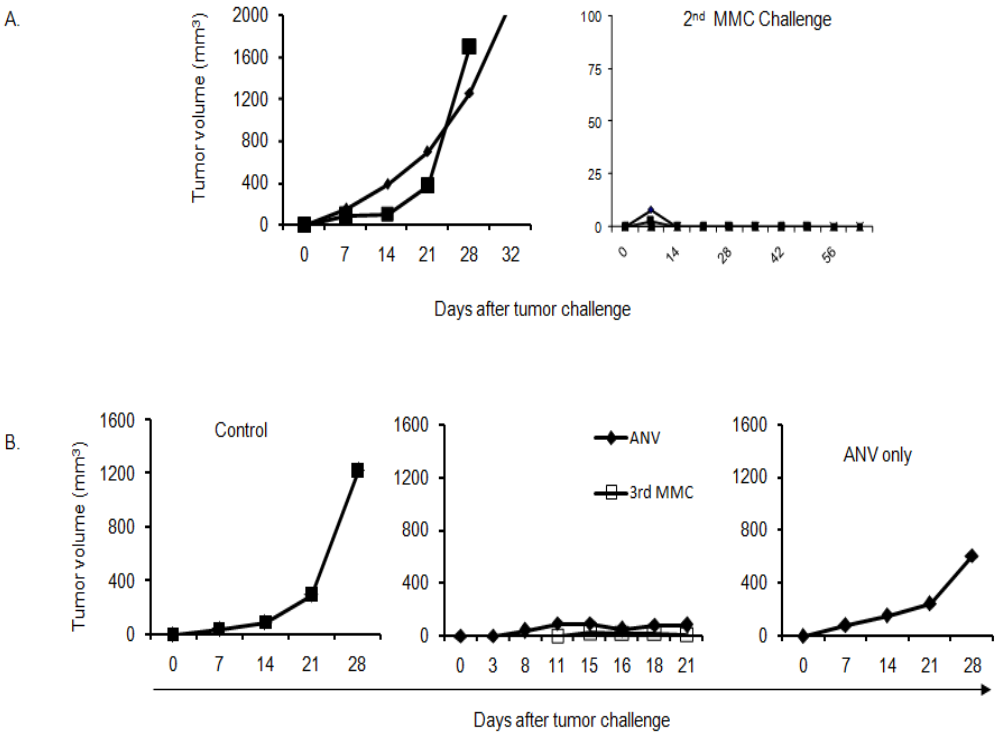


Figure 37: Phenotypic distribution of T cells derived from the AIT recipients

The animal which received the second MMC challenge and ANV challenge was sacrificed when the ANV tumor reached 600 mm^3 volume and splenocytes were harvested. The freshly isolated splenocytes were stained with CD44, CD62L and CD8/CD4 antibodies. The phenotypic distribution was analyzed by flow cytometry. It showed marked resemblance with the phenotypic distribution of the freshly isolated donor's lymphocytes.

Figure 37

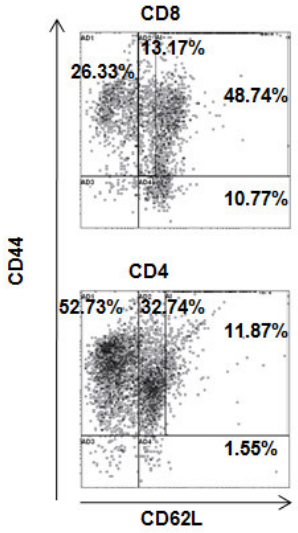
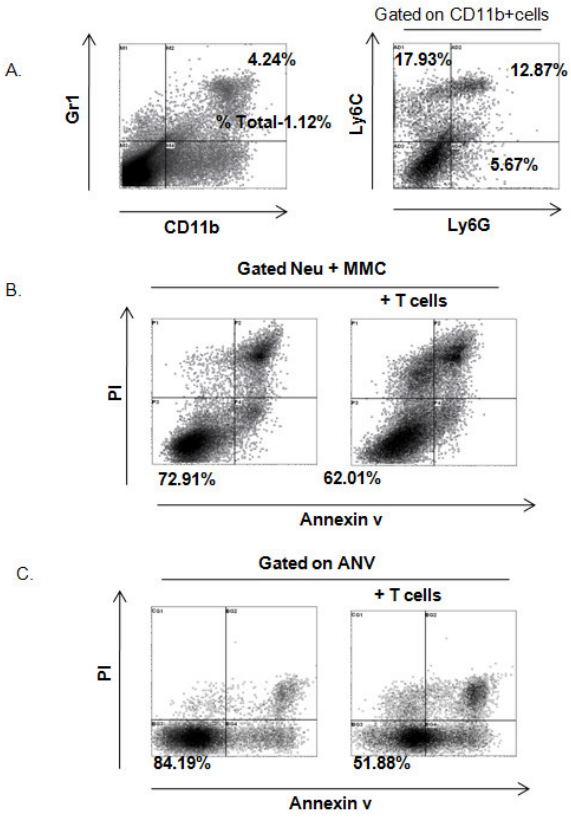


Figure 38: Presence of MDSCs in the spleen of the AIT recipients and anti-tumor efficacy of their T cells against MMC and ANV

The recipient of AIT which received the ANV and the third MMC challenge was sacrificed when the ANV tumor reached 76mm³ and the MMC tumor was about to disappear (10mm³), the splenocytes were stained for detection of endogenous MDSCs (A) and to test their cytotoxic potential against MMC (B) or ANV (C).

Figure 38



CHAPTER 4 Discussions

Adoptive immunotherapy (AIT) includes isolation of lymphocytes from cancer patients, expansion of tumor-reactive T cells *ex vivo* and infusion of these tumor-reactive T cells back into the patient (45). Current protocols for AIT of cancers utilize the *ex vivo* expansion of tumor-reactive T cells in the presence of T cell growth factors such as IL-2 or other pharmacological agents to generate a large number of such immune effector cells (66). Recently, common gamma chain cytokines including IL-7, IL-15, and IL-21 have been used for the expansion of T cells (66-68). Previous pre-clinical reports have suggested that the antigen-sensitized CD8+ T cells exhibit different trafficking and phenotypic distribution as well as functional attributes when exposed to various common receptor gamma chain cytokines. In the present study, we conducted comparative analyses of two different cytokine regimens used in our laboratory for the expansion of tumor-reactive T cells for AIT of breast carcinoma. We have recently used the alternating (IL-7/IL-15→IL-2→IL-7/IL-15) and sequential (IL-7/IL-15→IL-2) common gamma chain cytokine formulations for the *ex vivo* expansion of the autologous T cells derived from the FVBN202 transgenic mouse model of Her-2/neu positive breast carcinoma. In addition to determining the MDSC sensitivity of T cells expanded with these two different cytokine regimens, we also determined whether the

state of tumor burden during early or late tumorigenesis may affect the anti-tumor efficacy of donor T cells after reintroduction into the tumor bearing animals.

Alternating and sequential common gamma chain cytokines expand tumor-reactive T cells *ex vivo*

We have previously reported that B/I activation of the neu-specific high affinity T cells derived from wild type FVB mice, and expansion in the presence of the alternating common gamma chain cytokines enhanced anti-tumor efficacy of the T cells *in vitro*. However, AIT using these T cells protected FVBN202 mice against challenge with the neu positive MMC, only when combined with the depletion of MDSCs *in vivo* (37). In the present studies we used FVBN202 transgenic mice as donors of low affinity T cells against neu positive MMC. We found that the alternating and sequential common gamma chain regimens *ex vivo* expanded tumor-reactive T cells, as evidenced by an enhanced tumor-induced IFN-γ production as well as cytotoxicity against MMC *in vitro*. These data argue against the notion that tumor-bearing FVBN202 animals are tolerant to their tumor antigens because they harbored pre-existing tumor-specific T cells that were expanded *ex vivo*. A recent report on pre-existing Her-2/neu-specific immune responses in breast cancer patients (69) is consistent with our observation and both argue against the existence of immunological tolerance in cancer patients.

T cells expanded with the sequential common gamma chain cytokines showed comparable expansion and enhanced *in vitro* cytotoxicity but had a lower ratio of CD8+ to CD4+ T cells, lower production of the tumor-induced IFN-γ and a higher

proportion of apoptotic T cells compared to those expanded with the alternating common gamma chain cytokines regimen

The alternating and sequential gamma chain cytokine formulations were equally effective in fold expansion of T cells, regardless of the status of tumor burden of the donors. However, these two regimens had differential effects on the expansion of CD4+ and CD8+T cells. While the alternating formulation results comparable yield of CD8+ and CD4+ T cells, the sequential formulation preferentially expands CD4+ T cells. The expansion pattern revealed that the alternating gamma chain cytokine formulation supported 9.2 fold and 3.4 fold expansion of the CD8+ and CD4+ T cells such that at the end of the expansion the proportion of both subsets were similar. With sequential cytokine regimen abundance of CD4+ T cells were maintained, regardless of the status of tumor burden in the donors. However, fold expansion of CD8+ and CD4+ T cells with the sequential gamma chain cytokines was lower when donors had small tumor burdens.

Despite the lower ratio of CD8+ T cells over CD4+ T cells and greater numbers of apoptotic T cells following the expansion with sequential gamma chain cytokines, these T cells showed greater cytotoxic effects on MMC tumor cells *in vitro*. This greater cytotoxicity was associated with lower titer of tumor-induced IFN-γ production by these T cells compared to those expanded with the alternating gamma chain cytokines (3500 pg/ml vs. 30,000 pg/ml). Flow cytometry analysis revealed that tumor-induced IFN-γ production was evident in CD8+ T cells but not in CD4+ T cells. These data suggest the

importance of IFN-γ-independent mechanisms of tumor killing such as perforin/granzyme B production by the tumor-reactive T cells. While freshly isolated CD8+ and CD4+ T cells from FVBN202 mice showed minimal perforin production (less than 3%) upon stimulation with MMC *in vitro*, majority of the *ex vivo*-expanded T cells produced perforin in response to MMC stimulation (6-84% of CD8+ T cells and 0.56%-16% of CD4+ T cells). T cells isolated from animals with small tumor burdens and expanded with the sequential gamma chain cytokines also showed a higher percentage of CD8+Prf+ T cells compared to freshly isolated T cells (2-6% vs. 1.5%), though less than T cells derived from the donors with large tumor burden. All T cells showed high levels of granzyme B production regardless of cytokine formulation or tumor burden in the donors. We have previously reported that high affinity T cells can reject neu positive MMC tumor cells that lack expression of the receptor for IFN-γ, suggesting the existence of IFN-γ-independent mechanisms of tumor rejection by T cells (65). Significant *in vitro* tumoricidial activity of T cells expanded with sequential gamma chain cytokines was evident regardless of the status of tumor burden in the T cell donors. Others also reported that the presence of tumor-specific IFN-γ has been producing T cells was not correlated with objective clinical responses in patients with melanoma (70). Importantly, IFN-γ found to render some tumors, including uveal melanoma cells, resistant to granule-mediated lysis by cytotoxic lymphocytes (71). It is evident from these studies that sequential common gamma chain cytokine formulation supports expansion of tumor reactive T cells that can preferentially induce IFN-γ-independent mechanism of tumor regression.

Sequential common gamma chain cytokine regimen was more efficient than the alternating common gamma chain cytokine formulation for the expansion of effector and effector/memory CD8+ T cells as well as effector and central/memory CD4+ T cells

For effective and successful AIT, T cells should possess the effector function and homing abilities to lymph nodes to induce tumor regression and protection from future recurrence (50). The major cytotoxic function of the CD8+ T cells demands direct cellular contact with the target cells. To induce tumor specific cell death, the effector CD8+ T cells should reach the tumor site from blood. This potential extravasation capacity depends on their ability to adhere to endothelial cells while withstanding hydrodynamic shear stresses exerted by flowing blood. The tethering and rolling mechanism is governed by the selectin family (51). According to Klebanoff *et al*, in the presence of IL-15, CD8+ T cells differentiated to the central/memory phenotype while IL-2 preferentially supported effector and effector/memory CD8+ T cells (72). As expected, the alternating gamma chain cytokine formulation maintained CD8+ and CD4+ central memory phenotypes after a 6-day expansion (CD8+ T cells: from 51.4% to 53.3% and CD4+T cells: from 58% to 40%) with small increases in effector phenotypes (CD8+ T cells: 2.2. fold increase and CD4+T cells: 1.3 fold increase). Overall, central memory and effector phenotypes were abundant after the expansion with alternating gamma chain cytokines. This regimen caused a marked reduction of naïve T cell population for the CD8+ subsets. On the other hand, the sequential common gamma chain cytokine formulation supported the expansion of CD8+ effector

and effectors/memory phenotypes, regardless of tumor burden of the donors. However, this formulation provided a different protection for CD4+ T cells such that central memory phenotypes were the most abundantly expanded cells. Given the expression of MHC class I but not MHC class II by the tumor cells, CD8+ T cells are expected to play a more important role against MMC breast tumors than CD4+ T cells. This prediction is supported by our observation that tumor-specific IFN-γ and perforin productions were markedly increased in CD8+ rather than in CD4+ T cells upon stimulation with MMC tumor cells *in vitro*. In addition, a higher frequency of CD8+ effector T cells with the sequential gamma chain cytokines compared to those with the alternating gamma chain cytokines (44-56% vs. 16.8%) was associated with greater tumoricidial activity of T cells *in vitro*. Importantly, the number of the CD44 receptors per CD8+ T cells was greater with sequential gamma chain cytokines compared to those with alternating gamma chain cytokines (MFI: 30.9 vs. 5.6). Expression of CD44 has been shown to be the most potent stimulator of Fas ligand expression on human T cells (52) which in turn may induce apoptosis in Fas positive target cells. We have already shown the expression of Fas in MMC tumor cells (65). These data suggest that CD8+ effector T cells may work better against the tumor cells compared to CD8+ central memory T cells. In fact *in vivo* tumor challenge studies further confirmed higher efficiency of CD8+ effector T cells in tumor rejection. While AIT by means of T cells expanded with the alternating gamma chain cytokines provided no protection against tumor challenge, those expanded with the sequential common gamma chain cytokines provided complete protection against tumor challenge even without depletion of MDSCs *in vivo*. These

differences in the efficiency of T cells were related to different gamma chain cytokine formulation rather than the status of tumor burden in the donors, because in both cases T cells were derived from animals bearing large tumors. Importantly, T cells grown with the sequential common gamma chain cytokines generated long-term memory responses not only against MMC tumor cells but also to some extent against challenge with the neu antigen negative ANV relapsed tumors without need for cyclophosphamide treatment because of the reconstitution of tumor-reactive T cells following AIT. Such T cell reconstitution was confirmed by phenotype analysis of T cells derived from the recipient animals after the rejection of recall tumor challenge. All the animals showed high levels of CD8+ effector T cells (26.3%) compared to those derived from the donors prior to the expansion *ex vivo* (6-7%). These T cells also showed anti-tumor efficacy against MMC and ANV *in vitro* without further expansion *ex vivo*. These findings are consistent with previous reports showing that loss of expression of the lymphoid homing molecules and acquisition of CD44 expression can be associated with increased anti-tumor effects of adoptively transferred T cells (45).

Status of tumor burden in FVBN202 donors was found to have significant impact on the efficacy of tumor-specific T cells *in vitro*. Compared to CD8+ T cells derived from animals with small tumor burden, those derived from animals with large tumor burden and expanded with the sequential gamma chain cytokines showed higher levels of tumor-induced IFN-γ (30% vs. 6.5%) and perforin (84% vs. 6.15%) production as well as a higher proportion of CD44+ effector phenotypes (56% vs. 44%). Higher efficacy of these T cells was also confirmed by showing that T cells derived from donors with large

tumor burden and expanded with the sequential gamma chain cytokines protected animals against primary and recall tumor challenge whereas those derived from donors with small tumor burden and expanded with the same sequential gamma chain cytokine protocol protected animals against primary tumor challenge but whether or not they may protect animals against recall tumor challenge remains to be determined.

Higher anti-tumor efficacy of T cells expanded with the sequential gamma chain cytokines compared to alternating gamma chain cytokines was observed despite the higher levels of apoptotic cells and lower proportion of CD8+ T cells over CD4+ T cells. This may be related to the fact that continuous presence of IL-7 during the expansion (with the alternating gamma chain cytokine regimen) could protect T cells from apoptosis and as a result increase the frequency of T cells that are at late stage of differentiation and may not last long *in vivo* after AIT. Higher frequency of central memory T cells also supports this possibility. On the other hand, continuation of culture with IL-2 (with the sequential common gamma chain cytokines) results in apoptosis of T cells that are at late stages of differentiation and rescues those at early stages of differentiation that could undergo further differentiation *in vivo* after AIT. Higher frequency of apoptotic T cells as well as lower proportion of CD8+ central memory T cells supports this possibility. However this was only the case for CD8+ T cells and could not explain high proportion of CD4+ central memory T cells. Therefore, a key element would be whether T cells grown with the sequential gamma chain cytokines may acquire resistance to inhibitory function of MDSCs, as suggested by *in vivo* efficacy of AIT by means of these T cells in the absence of MDSC depletion.

Sequential common gamma chain cytokine protocol facilitates differentiation and expansion of tumor-reactive T cells that are refractory to the inhibitory effects of MDSCs

MDSCs are known to be a major contributor in tumor-induced immune suppression. Gabrilovich *et al.* have shown antigen specific contact-dependency of these cells. MDSCs did exert a detrimental effect on the number of IFN-γ producing CD8+ T cells in response to specific peptides presented by MHC class I (35). Other reports reveal that MDSCs may work through downregulation of TCR zeta chain and can cause dysfunction of T cells described in cancer, infectious diseases and autoimmune disorders. Alternatively, their suppressive function may be dependent on their derivation from a chronically inflamed environment (36). Our previous work in FVBN202 transgeic mouse model of breast carcinoma showed that MDSCs inhibit T cell activation in a contact-dependent fashion (37).

In vitro co-culture assays showed that T cells expanded with the sequential gamma chain cytokines were refractory to MDSCs, as evidenced by unaltered production of the tumor induced IFN-γ, perforin, granzyme B, or expression of the early activation marker CD69. Importantly, the presence of MDSCs did not inhibit the tumor killing function of T cells *in vitro*. Given that the T cell inhibitory function of MDSCs have been reported by using CD3 activation or tumor-induced activation *in vitro*, it is likely that MDSCs could inhibit activation rather than effector function of T cells. Therefore, they would be expected to inhibit naïve and central memory T cells rather than already

activated effector T cells. A higher proportion of CD8+ effector T cells with the sequential gamma chain cytokines compared to those with the alternating gamma chain cytokines support this possibility. In order to test this possibility we should sort effector and central memory T cells and perform *in vitro* suppression assays in the presence or absence of MDSCs. These experiments will determine whether only CD44+ effector T cells are refractory to MDSCs and higher proportion of these phenotypes within the expanded T cells cause them to be refractory to MDSCs. In line with our observation, other groups reported that MDSC-mediated down regulation of L-selectin to be the major cause of T cell suppression as T cells must have L-selectin (high) phenotype to home to lymph nodes and sites of inflammation where they encounter antigen and are activated. This down regulation perturbs T cell trafficking patterns, thereby inhibiting T cell activation (41). Again, this report supports the hypothesis that CD62L+ memory T cells are more sensitive to MDSCs than CD62L- effector T cells.

Recently, there have been several reports on strategies to overcome MDSCs. A previous pre- clinical report suggests the *in vivo* administration of all-trans-retinoic acid can eliminate MDSCs (73). All-trans-retinoic acid is a naturally occurring isomer of retinoic acid capable of causing differentiation of a human leukemia cell line and acute promyelopoetic leukemia cells. *In vitro* experiments with all-trans-retinoic acid show marked success in removing MDSCs in humans and mice. Combined application of this compound and cancer vaccines showed reduced immune suppression and better efficacy of the vaccines (73). Anti-inflammatory triterpenoids such as the synthetic C-28 methyl ester of 2-cyano-3, 12-dioxooleana-1,9,-dien-28-oic acid (CDDO-Me) have been

proven to abrogate the immune suppressive functions of MDSCs (74). Other therapeutic strategies involve application of cytokines, drugs and antibodies for elimination of MDSCs. Nowak *et al.* showed that anti- mitotic Gemcitabine can maintain tumor specific CD8+ and CD4+ T cells, preferentially reducing the B cells (75). Based on Nowak's observation, Suzuki *et al.* tested the application of Gemcitabine, a common chemotherapeutic agent, along with immunotherapy for neutralizing the immunosuppressive effects of MDSCs. Gemcitabine HCL (GEM 2'-deoxy-2',2'-difluorocytidine monohydrochloride) is a base analogue of cytidine that can block DNA synthesis in the S-Phase thereby stopping cell cycle progression (76). Though all these strategies achieved partial success, none of them could observe complete reduction of MDSCs. The novel cytokine regimen that we have established can successfully make T cells refractory to the inhibitory effects of MDSCs. The *ex vivo* expanded T cells can exhibit marked anti-tumor efficacy *in vitr*o as well as *in vivo* even in presence of MDSCs. They can even show memory responses without elimination of endogenous MDSCs. The exact mechanism by which the T cells can overcome the effects of MDSCs is not known, but one report suggests that Ox-40 ligation can break T cell anergy and can restore the proliferative capacity of tumor reactive T cells (77).

CONCLUSIONS

1) T cell expansion with the sequential common gamma chain cytokines results in comparable expansion, enhanced *in vitro* cytotoxicity, a lower ratio of CD8+ over CD4+ T cells, lower levels of tumor-induced IFN-γ production and poorer viability of the expanded T cells in comparison to the T cells expanded by alternating gamma chain cytokines.
2) The alternating common gamma chain cytokine formulation supports preferential differentiation of central/memory phenotypes and effector phenotype of T cells but the sequential gamma chain cytokine regimen supports differentiation of effector and effector/memory phenotypes of CD8+ T cells as well as effector and central/memory phenotype of CD4+T cells. The latter increases the number of CD44 receptors on CD8+ and CD4+ T cells.
3) T cells expanded with the sequential common gamma chain cytokines can protect animals from primary neu positive tumor challenge and can exhibit strong memory responses against neu positive and neu negative tumor challenge.
4) The anti-tumor efficacy of the isolated T cells after the expansion depends on the status of tumor burden of the donors in addition to the cytokine regimen. T cells harvested from donors bearing larger tumor burden show greater anti-tumor efficacy in terms of increased production of IFN-γ and perforin and exhibiting a higher percentage of CD44+ effector T cell phenotypes.

5) T cells expanded with the sequential common gamma chain cytokines are refractory to MDSCs whereas those expanded with the alternating gamma chain cytokines are susceptible to MDSCs.

The major observations of this study are reproducible and have been repeated 3-4 times. The underlying mechanisms of some of our observations need further investigation. Thus the future directions of this investigation include:

1) To determine which phenotype of the *ex vivo* expanded T cell is most effective in overcoming tumor escape and is refractory to MDSCs.
2) To determine whether T cells harvested from donors with low tumor burden can exhibit memory responses after the expansion with sequential gamma chain cytokines.
3) To examine the cytokine-induced expression of receptors (Ox-40/Ox-40L) on T cells that may render them refractory to MDSCs.
4) To determine the therapeutic efficacy of sequential common gamma chain cytokines-expanded T cells against spontaneous and transplanted mammary tumors.

Literature Cited

1. American Cancer Society. (n.d.). Retrieved from American Cancer Society: Global facts and figures of cancer http://www.cancer.org/docroot/home/index.asp

2. Jemal, A., R. Siegel, E. Ward, Y. Hao, J. Xu, T. Murray, and M. J. Thun. 2008. Cancer statistics. *CA Cancer. J. Clin. 58: 71-96.*

3. seer.cancer.gov/csr/1975_2000/results_merged/topic_lifetime_risk.pdf

4. Potti A., Forseen S., Koka V.et al. 2004. Determination of HER-2/neu overexpression and clinical predictors of survival in a cohort of 347 patients with primary malignant brain tumors. *Cancer Investigation. 22(4): 537-544.*

5. Slamon, D. J., W. Godolphin, L. A. Jones, J. A. Holt, S. G. Wong, D. E. Keith, W. J.Levin, S. G. Stuart, J. Udove, and A. Ullrich. 1989. Studies of the HER-2/neu protooncogene in human breast and ovarian cancer. *Science 244: 707-712.*

6. Slamon, D. J., G. M. Clark, S. G. Wong, W. J. Levin, A. Ullrich, and W. L. McGuire. 1987. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu *oncogene. Science 235: 177-182.*

7. Kurebayashi, J. 2001. Biological and clinical significance of HER2 overexpression in breast cancer. *Breast Cancer 8: 45-51.*

8. Maguire, H. C.,Jr and M. I. Greene. 1989. The neu (c-erbB-2) oncogene. *Semin. Oncol.16:148-155*

9. Eladdadi, A. and D. Isaacson. 2008. A mathematical model for the effects of HER2 overexpression on cell proliferation in breast cancer. *Bull. Math. Biol. 70: 1707-1729.*

10. Bhattacharya, R., R. Bukkapatnam, I. Prawoko, J. Soto, M. Morgan, and R. R.Salup.2002. Efficacy of vaccination with plasmid DNA encoding for HER2/neu or HER2/neu- eGFP fusion protein against prostate cancer in rats. Int. *Immunopharmacol. 2: 783-796.*

11. Cohen, S. 1965. The stimulation of epidermal proliferation by a specific protein (EGF). *Dev. Biol. 12: 394-407.*

12. Carpenter, G., K. J. Lembach, M. M. Morrison, and S. Cohen. 1975. Characterization of the binding of 125-I-labeled epidermal growth factor to human fibroblasts. *J. Biol. Chem. 250: 4297-4304.*

13. Burgess, A. W., H. S. Cho, C. Eigenbrot, K. M. Ferguson, T. P. Garrett, D. J. Leahy, M. A. Lemmon, M. X. Sliwkowski, C. W. Ward, and S. Yokoyama. 2003. An open-and- shut case? Recent insights into the activation of EGF/ErbB receptors. *Mol. Cell 12: 541-552.*

14. Hynes, N. E. and H. A. Lane. 2005. ERBB receptors and cancer: the complexity of targeted inhibitors. *Nat. Rev. Cancer. 5: 341-354.*

15. Schlessinger, J. 2002. Ligand-induced, receptor-mediated dimerization and activation of EGF receptor. *Cell 110: 669-672.*

16. Garrett, T. P., N. M. McKern, M. Lou, T. C. Elleman, T. E. Adams, G. O. Lovrecz, H. J. Zhu, F. Walker, M. J. Frenkel, P. A. Hoyne, R. N. Jorissen, E. C. Nice, A. W. Burgess and C. W. Ward. 2002. Crystal structure of a truncated epidermal growth factor receptor extracellular domain bound to transforming growth factor alpha. *Cell 110: 763-773.*

17. Ogiso, H., R. Ishitani, O. Nureki, S. Fukai, M. Yamanaka, J. H. Kim, K. Saito, A. Sakamoto, M. Inoue, M. Shirouzu, and S. Yokoyama. 2002. Crystal structure of the complex of human epidermal growth factor and receptor extracellular domains. *Cell 110:775-787.*

18. Garrett, T. P., N. M. McKern, M. Lou, T. C. Elleman, T. E. Adams, G. O. Lovrecz, M. Kofler, R. N. Jorissen, E. C. Nice, A. W. Burgess, and C. W. Ward. 2003. The crystal structure of a truncated ErbB2 ectodomain reveals an active conformation, poised to interact with other ErbB receptors. *Mol. Cell 11: 495-505.*

19. Graus-Porta, D., R. R. Beerli, J. M. Daly, and N. E. Hynes. 1997. ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. *EMBO J. 16: 1647-1655.*

20. Kristjansdottir K, Dizon D. 2010. HER-dimerization inhibitors: evaluating pertuzumab in women's cancers. *Expert Opin Biol Ther. Feb;10(2):243-50.*

21. Guy, C. T., M. A. Webster, M. Schaller, T. J. Parsons, R. D. Cardiff, and W. J. Muller. 1992. Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. *Proc. Natl. Acad. Sci. U. S. A. 89: 10578-10582.*

22. Kmieciak, M., J. K. Morales, J. Morales, E. Bolesta, M. Grimes, and M. H. Manjili.2008. Danger signals and nonself entity of tumor antigen are both required for eliciting effective immune responses against HER-2/neu positive mammary carcinoma: implications for vaccine design. *Cancer Immunol. Immunother.Sep;57(9):1391-1398*

23. Gallina, G., L. Dolcetti, P. Serafini, C. De Santo, I. Marigo, M. P. Colombo, G. Basso, F. Brombacher, I. Borrello, P. Zanovello, S. Bicciato, and V. Bronte. 2006. Tumors induce a subset of inflammatory monocytes with immunosuppressive activity on CD8+ T cells. *J. Clin. Invest. 116: 2777-2790.*

24. Kusmartsev, S. A., Y. Li, and S. H. Chen. 2000. Gr-1+ myeloid cells derived from tumor-bearing mice inhibit primary T cell activation induced through CD3/CD28 costimulation. *J. Immunol. 165: 779-785.*

25. Youn, J. I., S. Nagaraj, M. Collazo, and D. I. Gabrilovich. 2008. Subsets of myeloid-derived suppressor cells in tumor-bearing mice. *J. Immunol. 181: 5791-5802*.

26. Habibi, M., M. Kmieciak, L. Graham, J. K. Morales, H. D. Bear, and M. H. Manjili. 2008. Radiofrequency thermal ablation of breast tumors combined with intralesional administration of IL-7 and IL-15 augments anti-tumor immune responses and inhibits tumor development and metastasis. *Breast Cancer Res. Treat*.

27. Rodriguez, P. C., C. P. Hernandez, D. Quiceno, S. M. Dubinett, J. Zabaleta, J. B. Ochoa, J. Gilbert, and A. C. Ochoa. 2005. Arginase I in myeloid suppressor cells is induced by COX-2 in lung carcinoma. *J. Exp. Med. 202: 931-939.*

28. Diaz-Montero, C. M., M. L. Salem, M. I. Nishimura, E. Garrett-Mayer, D. J. Cole, and A. J. Montero. 2008. Increased circulating myeloid-derived suppressor cells correlate 286 with clinical cancer stage, metastatic tumor burden, and doxorubicin-cyclophosphamide chemotherapy. *Cancer Immunol. Immunother.*

29. Kusmartsev, S. and D. I. Gabrilovich. 2002. Immature myeloid cells and cancer-associated immune suppression. *Cancer Immunol. Immunother. 51: 293-298.*

30. Movahedi, K., M. Guilliams, J. Van den Bossche, R. Van den Bergh, C. Gysemans, A. Beschin, P. De Baetselier, and J. A. Van Ginderachter. 2008. Identification of discrete tumor-induced myeloid-derived suppressor cell subpopulations with distinct T cellsuppressive activity. *Blood 111: 4233-4244*.

31. Sawanobori, Y., S. Ueha, M. Kurachi, T. Shimaoka, J. E. Talmadge, J. Abe, Y. Shono, M. Kitabatake, K. Kakimi, N. Mukaida, and K. Matsushima. 2008. Chemokine-mediated rapid turnover of myeloid-derived suppressor cells in tumor-bearing mice. *Blood 111: 5457-5466.*

32. Morales JK, Kmieciak M, Knutson KL, Bear HD, Manjili MH.2009. GM-CSF is one of the main breast tumor-derived soluble factors involved in the differentiation of CD11b-Gr1- bone marrow progenitor cells into myeloid-derived suppressor cells. Nov 8. *Breast Cancer Res Treat.*

33. Peranzoni E, Zilio S, Marigo I, Dolcetti L, Zanovello P, Mandruzzato S, Bronte V. 2010. Myeloid-derived suppressor cell heterogeneity and subset definition.*Curr Opin Immunol.Apr; 22(2):238-44.*

34. Apollini, E., Bronte, V., Mazzoni, A., Serafini, P., Cabrelle, A., Segal, D., et al. (2000). Immortalized myeloid suppressor cells trigger apoptosis in antigen-activated T lymphocytes. *Journal of Immunology. 165: 6723 - 6730.*

35. Gabrilovich, D. I., M. P. Velders, E. M. Sotomayor, and W. M. Kast. 2001. Mechanism of immune dysfunction in cancer mediated by immature Gr-1+ myeloid cells. *J. Immunol. 166: 5398-5406.*

36. Ezernitchi, A. V., I. Vaknin, L. Cohen-Daniel, O. Levy, E. Manaster, A. Halabi, E. Pikarsky, L. Shapira, and M. Baniyash. 2006. TCR zeta down-regulation under chronic inflammation is mediated by myeloid suppressor cells differentially distributed between various lymphatic organs. *J. Immunol. 177: 4763-4772.*

37. Morales JK, Kmieciak M, Graham L, Feldmesser M, Bear HD, Manjili MH.2009. Adoptive transfer of HER2/neu-specific T cells expanded with alternating gamma chain cytokines mediate tumor regression when combined with the depletion of myeloid-derived suppressor cells. *Cancer Immunol Immunother.Jun;58(6):941-53.*

38. Mazzoni, A., V. Bronte, A. Visintin, J. H. Spitzer, E. Apolloni, P. Serafini, P. Zanovello, and D. M. Segal. 2002. Myeloid suppressor lines inhibit T cell responses by an NO-dependent mechanism. *J. Immunol. 168: 689-695.*

39. Nagaraj S, Schrum AG, Cho HI, Celis E, Gabrilovich DI.2010. Mechanism of T cell tolerance induced by myeloid-derived suppressor cells. *J Immunol.Mar 15;184(6):3106-16*

40. Srivastava MK, Sinha P, Clements VK, Rodriguez P, Ostrand-Rosenberg S.2010. Myeloid-derived suppressor cells inhibit T-cell activation by depleting cystine and cysteine. *Cancer Res.Jan 1;70(1):68-77*

41. Ostrand-Rosenberg S.2010. Myeloid-derived suppressor cells: more mechanisms for inhibiting antitumor immunity. *Cancer Immunol Immunother.Apr 23*

42. Dunn, G., Bruce, A., Ikeda, H., Old, L., & Schreiber, R. 2002. Cancer Immunoediting: From immunosurveillance to tumor escape. *Nature Immunology. 3 (11): 991 - 998.*

43. Dunn, G., Old, L., & Schreiber, R. 2004. The immunobiology of cancer immunsurveillance and immunoediting. *Immunity. 21: 137 - 148.*

44. Dighe, A., Richards, E., Old, L., Schreiber, R. 1994. Enhanced in vivo growth and resistance to rejection of tumor cells expressing dominant negative IFN-gamma receptors. *Immunity. 1: 447 – 456.*

45. Gattinoni, L., Powell, J., Resenberg, S., & Restifo, N. 2006. Adoptive Immunotherapy for Cancer: Builing on Success. *Nature Reviews: Immunology.* 6: 383 - 393.

46. Rosenberg, S. A., N. P. Restifo, J. C. Yang, R. A. Morgan, and M. E. Dudley. 2008. Adoptive cell transfer: a clinical path to effective cancer immunotherapy. *Nat. Rev. Cancer. 8: 299-308.*

47. Dougan, M., & Dranoff, G. (2009). Immune Therapy for Cancer. *Annual Review of Immunology* . 27: 83 - 117.

48. Michalek, J., Buchler, T., & Hajek, R. (2004). T lymphocyte therapy of cancer. *Physiological Research*. 53: 463 - 469.

49. Rosenberg, S., Yang, J., & Restifo, N. (2004). Cancer immunotherapy: moving beyond current vaccines. *Nature Medicine*. 10: 909 - 915.

50. June, C. (2007). Principles of adoptive T cell cancer therapy. *Journal of Clinical Investigation*. 117: 1204 - 1212.

51. Weninger, W., Crowley, M., Manjunath, N., Andrian, & U. (2002). Migratory properties of naive, effector, and memory CD8 T cells. *Journal of Experimental Medicine*. 194: 953 - 966.

52. Nakano K, Saito K, Mine S, Matsushita S, Tanaka Y. Engagement of CD44 up-regulates Fas ligand expression on T cells leading to activation-induced cell death. *Apoptosis. 2007 Jan;12(1):45-54*

53. Proietti, E., Greco, G., Garrone, B., Baccarini, S., Mauri, C., Venditti, M., et al. (1998). Importance of cyclophosphamide-induced bystander effect on T cells for a successful tumor eradication in repsonse to adoptive immunotherpay in mice. *Journal of Clinical Investigation. 101: 429 - 441.*

54. Keller, A., & Borst, J. (2006). Control of peripheral T cell survival: a delicate divison of labor between cytokines and costimulatory molecules. *Human Immunology. 67: 469 - 477.*

55. Manjili MH and Kmieciak M. (2010). Cancer immunotherapy and cytokines. In V.R. Preedy (Ed.), Modern insights into disease from molecules to man: cytokines, 1st edition, Science publishers (in press)

56. Klebanoff, C., Khong, H., Antony, P., Palmer, D., & Restifo, N. (2005). Sinks, suppressors and antigen presenters: how lymphodepletion enhances T cell-mediated tumor immunotherapy. *Trends in Immunology. 26: 111 - 117.*

57. Ma, A., Koka, R., & Burkett, P. (2006). Diverse functions of IL2, IL15 and IL7 in Lymphoid Homeostasis. *Annual Review of Immunology. 24: 657 - 679.*

58. Goldrath, A., Sivakumar, P., Glaccum, M., Kennedy, M., Bevan, M., Benoist, C., et al. (2002). Cytokine requirements for acute and basal homeostatic

proliferation of naive and memory CD8+ T cells. *Journal of Experimental Medicine. 195: 1515 - 1522.*

59. Murphy, K., Travers, P., Walport, M. Janeway's Immunobiology. Seventh edition. New York: Garland Science, 2008.

60. Klebanoff CA, Finkelstein SE, Surman DR, Lichtman MK, Gattinoni L, Theoret MR, Grewal N, Spiess PJ, Antony PA, Palmer DC, Tagaya Y, Rosenberg SA, Waldmann TA, Restifo NP. 2004. IL-15 enhances the in vivo antitumor activity of tumor-reactive CD8+ T cells. *Proc Natl Acad Sci U S A.Feb 17;101(7):1969-74.*

61. Kmieciak M, Gowda M, Graham L, Godder K, Bear HD, Marincola FM, Manjili MH. 2009. Human T cells express CD25 and Foxp3 upon activation and exhibit effector/memory phenotypes without any regulatory/suppressor function. *J Transl Med.Oct 22;7:89.*

62. Rosenberg SA, Sportès C, Ahmadzadeh M, Fry TJ, Ngo LT, Schwarz SL, Stetler-Stevenson M, Morton KE, Mavroukakis SA, Morre M, Buffet R, Mackall CL, Gress RE.2006. IL-7 administration to humans leads to expansion of CD8+ and CD4+ cells but a relative decrease of CD4+ T-regulatory cells. *J Immunother. May-Jun;29(3):313-9.*

63. Inoue S, Unsinger J, Davis CG, Muenzer JT, Ferguson TA, Chang K, Osborne DF, Clark AT, Coopersmith CM, McDunn JE, Hotchkiss RS.2010. L-15 prevents apoptosis, reverses innate and adaptive immune dysfunction, and improves survival in sepsis. *J Immunol. Feb 1;184(3):1401-9.*

64. Sandau MM, Kohlmeier JE, Woodland DL, Jameson SC.2010. IL-15 regulates both quantitative and qualitative features of the memory CD8 T cell pool. *J Immunol.Jan 1;184(1):35-44.*

65. Kmieciak, M., K. L. Knutson, C. I. Dumur, and M. H. Manjili. 2007. HER-2/neu antigen loss and relapse of mammary carcinoma are actively induced by T cell-mediated anti-tumor immune responses. *Eur. J. Immunol. 37: 675-685*

66. Bear, H. D., & Chin, C. S. (2001). B/I activated T cellsmediated regression of established tumors. *Journal of Surgical Research. 415 - 434.*

67. Klebanoff, C., Khong, H., Antony, P., Palmer, D., & Restifo, N. (2005). Sinks, suppressors and antigen presenters: how lymphodepletion enhances T cell-mediated tumor immunotherapy. *Trends in Immunology. 26: 111 - 117.*

68. Schluns, K., & Lefrancois, L. (2003). Cytokine control of memory T-cell development and survival. *Nature Reviews Immunology. 3: 269 - 279.*

69. Goodell V, Waisman J, Salazar LG, de la Rosa C, Link J, Coveler AL, Childs JS, Fintak PA, Higgins DM, Disis ML. Level of HER-2/neu protein expression in breast cancer may affect the development of endogenous HER-2/neu-specific immunity. *Mol Cancer Ther. 2008 Mar;7(3):449-54.*

70. Rosenberg SA, Sherry RM, Morton KE, Scharfman WJ, Yang JC, Topalian SL, Royal RE, Kammula U, Restifo NP, Hughes MS, Schwartzentruber D, Berman DM, Schwarz SL, Ngo LT, Mavroukakis SA, White DE, Steinberg SM. 2005. Tumor progression can occur despite the induction of very high levels of self/tumor antigen-specific CD8+ T cells in patients with melanoma. *J Immunol. 175(9):6169-76.*

71. Hallermalm K, Seki K, De Geer A, Motyka B, Bleackley RC, Jager MJ, Froelich CJ, Kiessling R, Levitsky V, Levitskaya J. 2008.Modulation of the tumor cell phenotype by IFN-gamma results in resistance of uveal melanoma cells to granule-mediated lysis by cytotoxic lymphocytes. *J Immunol. Mar 15;180(6):3766-74.*

72. Klebanoff, C., Gattinoni, L., Torabi-Parizi, P., Kerstann, K., Cardones, A., Finklestein, S., et al. 2005. Central memory self/tumor reactive CD8+ T cells confer superior antitumor immunity compared with effector memory T cells. *PNAS. 102: 9571 - 9576.*

73. Kusmartsev, S., Cheng, F., Yu, B., Nefedova, Y., Sotomayor, E., Lush, R., et al. 2003. All-trans-retinoic acid eliminates immature myeloid cells from tumor-bearing mice and improves the effect of vaccination. *Cancer Research. 63: 4441 - 4449.*

74. Nagaraj S, Youn JI, Weber H, Iclozan C, Lu L, Cotter MJ, Meyer C, Becerra CR, Fishman M, Antonia S, Sporn MB, Liby KT, Rawal B, Lee JH, Gabrilovich DI.2010. Anti-inflammatory triterpenoid blocks immune suppressive function of MDSCs and improves immune response in cancer. *Clin Cancer Res. 16(6):1812-23*

75. Ugel S, Delpozzo F, Desantis G, Papalini F, Simonato F, Sonda N, Zilio S, Bronte V.2009. Therapeutic targeting of myeloid-derived suppressor cells. *Curr Opin Pharmacol. 9(4):470-81.*

76. Nowak AK, Robinson BW, Lake RA.2002. Gemcitabine exerts a selective effect on the humoral immune response: implications for combination chemo-immunotherapy. *Cancer Res. 2002 Apr 15;62(8):2353-8.*

77. Redmond WL, Gough MJ, Weinberg AD.2009. Ligation of the OX40 co-stimulatory receptor reverses self-Ag and tumor-induced CD8 T-cell anergy in vivo. *Eur J Immunol. 39(8):2184-94.*

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