OREGON HEALTH & SCIENCE UNIVERSITY
SCHOOL OF MEDICINE – GRADUATE STUDIES

Identification of a Novel Human $CD4^+$ T Cell Population and Overcoming the Immunosuppressive Environment In Large Established Tumors

A DISSERTATION
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Todd Ashley Triplett

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Advisor: Andrew Weinberg, PhD

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CERTIFICATE OF APPROVAL

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This is to certify that the PhD dissertation thesis of

Todd Ashley Triplett

has been approved

_____
Andrew Weinberg/Advisor

_____
David Parker/Chair

_____
Nancy Haigwood

_____
Mark Slifka

_____
Philip Stork

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Dedication

This thesis is dedicated to my mother, Donna Triplett. You have always been a source of inspiration and unquestionable support. Your diagnosis of Lupus is what piqued my interest in immunology and your continuing struggle is a constant reminder of why I do research, which is to better understand the immune system in the hopes that new discoveries will one day be used to improve lives.

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List of Abbreviations

Ag: antigen

AICD: activation induced cell death

APC: antigen-presenting cell

bp: base pair

CMV: cytomegalovirus

CTL: control

CTLA-4: cytotoxic T-lymphocyte antigen 4

DC: dendritic cell

FACS: fluorescence activated cell sorting

Flu: influenza

FWB: flow wash buffer

GrzB: granzyme B

IFNγ: interferon-γ

i.p.: intraperitoneal

LAK: lymphokine-activated killer

MHC: major histocompatibility complex

NK: natural killer

NKT: natural killer T-cell

PAMP: pathogen associated molecular pattern

PBMC: peripheral blood mononuclear cell

PCR: polymerase chain reaction

PMA: phorbol 12-myristate 13-acetate

p:MHC: peptide-MHC complex

PRR: pattern recognition receptor

RAG: recombinase activating gene

s.c.: subcutaneous

SEB: staphylococcal enterotoxin B

SLE: systemic lupus erythematosus

STAT: signal transducer and activator of transcription

TCR: T-cell receptor

TGF-β: transforming growth factor-β

Th: T helper

TIL: tumor infiltrating lymphocyte

TLR: toll-like receptor

TME: tumor microenvironment

TNFRSF: tumor necrosis factor receptor superfamily

TRAF: TNF receptor associated factor

Treg: regulatory T cell

VEGF: vascular endothelial growth factor

WT: wild type

YFP: yellow fluorescence protein

Thesis Abstract

T cell proliferation, survival and effector function are tightly regulated by the presence of cytokines and expression of their receptors. Interleukin-2 (IL-2) was the first cytokine discovered and studies have shown that IL-2 availability and IL-2 receptor (CD25) expression promotes T cell activation, proliferation and survival. CD25 expression by murine T cells is primarily restricted to $CD4^{+}Foxp3^{POS}$ Tregs. In contrast, it has been reported that a large proportion of $CD4^{+}FOXP3^{NEG}$ non-Tregs express low-intermediate levels of CD25 in humans. This $CD25^{LO/INT}FOXP3^{NEG}$ population of human T cells was originally thought to represent recently activated cells, however this T cell population had not been studied in depth. Given the current practice of targeting the IL-2 signaling pathway in the clinic, we sought to fully characterize this population. In chapter 2, we show that contrary to what has been reported in the literature, these cells are not recently activated but represent a quiescent memory subpopulation. Our analyses found that this $CD25^{INT}$ population was phenotypically and functionally distinct from the $CD25^{NEG}$ memory $CD4^{+}$ T cells *in vitro* and was differentially affected *in vivo* by IL-2 treatment in cancer patients. We and others have not been able to find a mouse equivalent for this population, therefore studies performed in mice regarding the role of IL-2/CD25 may not necessarily translate to humans. Hence, these findings improve our understanding of the role of CD25 in human immunology and may also have clinical implications by helping to illuminate the mechanisms and potentially improve the efficacy of therapies that target IL-2 and CD25.

In contrast to IL-2, transforming growth factor-β (TGF-β) has been shown to negatively influence T cell proliferation and effector function and is known to inhibit

anti-tumor T cell responses, especially in large established tumors. Therefore, we sought to determine whether TGF-β signaling inhibits αOX40 mediated anti-tumor therapy in a pre-clinical mouse cancer model. In chapter 3, we found that while αOX40 therapy alone was ineffective at treating large established tumors, it was able to elicit complete regression in ~85% of the mice when given in conjunction with a TGF-β receptor inhibitor (SM16). Evaluation of tumor infiltrating T cells showed that αOX40/SM16 dual therapy resulted in an increase in proliferating granzyme B expressing $CD8^+$ T cells which were better able to produce IFNγ. These results show that combining αOX40 with TGF-β inhibitors is potentially a promising cancer immunotherapy that hopefully will be tested in future clinical trials.

Chapter 1

Introduction

Immune System

The following sections describe the fundamentals of the mammalian immune system, which will provide background information for this thesis.

Innate Immune System

The immune system is a collection of tissue structures, cell types and processes that has evolved to protect organisms from foreign pathogens such as harmful viruses, bacteria, fungi and parasites, as well as control to the outgrowth of neoplastic cells. The mammalian immune system is broken down into two parts: the innate and the adaptive. The innate immune system is an ancient part of the immune system that evolved to recognize a limited set of molecular motifs found in microbes. Cells of the innate immune system include neutrophils, dendritic cells (DCs) and macrophages. These innate cells express germ-line encoded pattern recognition receptors (PRRs), which act as sensors of pathogens by detecting certain pathogen associated molecular patterns (PAMPs), such as cell wall components and nucleic acid structures unique to pathogens. Activation of PRRs, such as toll-like receptors (TLRs), leads to increased inflammation, immune cell recruitment and up-regulation of immune stimulatory proteins [1]. These PAMPs are not normally produced by host cells (self) and are usually conserved by microbes (non-self). Thus, receptors of the innate immune system constitute a rudimentary system for detecting foreign invaders.

T Cells

The mammalian adaptive immune system are made up of bone marrow derived lymphocytes that either mature in the bone marrow to become B cells, or migrate to the

thymus to mature into T cells. This section will focus on T cells, specifically αβ T cells. Unlike the innate immune system, which has a myriad of fixed receptors encoded in the germline, T cells express T cell receptors (TCR) that are assembled by somatic DNA recombination. Proteins expressed by pathogens that activate the adaptive immune system are termed antigens (Ags). However, TCRs don't recognize whole antigens, but rather protein segments (8-17 amino acids) of antigens termed epitopes that bind to and are presented by major histocompatibility complexes (MHC) on cells (two forms: MHCI and MHCII) [2]. Typically each T cell expresses thousands of identical TCRs that are made up of a heterodimer protein consisting of an α and a β-chain, which together recognize specific peptide:MHC complexes (pMHC) [3].

Thymic Selection of T Cells

Since TCR formation is stochastic, T cell development in the thymus is tightly regulated to ensure that (1) TCRs are capable of binding to MHC complexes during a process termed positive selection and that (2) T cells expressing high affinity TCRs for MHC presenting peptides derived from self proteins are deleted during a process termed negative selection [4]. Negative selection is essential for maintaining a healthy immune balance since tissues are constantly presenting self-pMHC, and therefore this deletion limits the efflux of self-reactive high affinity T cells into the periphery, which can cause autoimmunity.

T cells with TCRs that recognize pMHCI in the thymus develop to express CD8 (co-receptor) ($CD8^+$ T cells) whereas T cells that recognize pMHCII develop to express CD4 (co-receptor) ($CD4^+$ Tcells) [5]. These two T cell subsets differ in their role during

immune responses. $CD8^+$ cells can directly *kill* infected or tumor cells whereas $CD4^+$ T cells *help* orchestrate other immune cells by providing signals such as immune-modulating soluble proteins (cytokines) and membrane bound proteins during immune responses. Hence $CD8^+$ T cells are termed cytotoxic T cells and $CD4^+$ T cells are termed helper T cells.

Activation of Antigen-Presenting Cells

During an immune response, activation of T cells starts within lymphoid tissues where pathogens and their products are taken up by a group of innate immune cells termed antigen presenting cells (APCs), such as DCs and macrophages [6]. During an infection, APCs engulf pathogens and infected cells through a process termed phagocytosis. Activation of APCs through their PRRs results in the release of cytokines and chemokines and increased expression of T cell co-stimulatory molecules and pMHC complexes on their cell surface [1]. These signals help recruit and activate other immune cells to alert them to an infection. Naïve $CD4^+$ T cells become activated (effector) cells when their TCRs recognize specific peptides in the context of MHCII on APCs (signal 1) in conjunction with co-stimulatory (signal 2) and cytokine signals (signal 3) provided by the activated APCs [7, 8]. Signals provided by activated APCs direct the differentiation of $CD4^+$ T cells into distinct helper subsets (e.g. Th1, Th2, Th17 or Treg) that ultimately secrete certain cytokines tailored for the appropriate immune response. This discussion will focus on the T helper type I (Th1) response, which is the appropriate response to enhance $CD8^+$ T cell mediated killing of infected cells and tumor cells [9].

$CD4^+$ Th1 Helper Response

A Th1 immune response is characterized by APCs secreting IL-12 which helps differentiate $CD4^+$ T cells into cells that express the master transcription factor, Tbet, and subsequently secrete IFNγ [10]. In addition, Th1 $CD4^+$ T cells also up-regulate expression of co-stimulatory molecules on their surface, such as CD40 ligand (CD40L), which interacts with the CD40 receptor (CD40R) expressed on APCs. Both IFNγ and CD40L increase expression of pMHC I/II, cytokines and co-stimulatory molecules such as the B7 ligands by APCs. B7 ligands (CD80 and CD86) on APCs bind to the T cell co-receptor CD28 to enhance T cell activation, IL-2 production and proliferation [11]. This positive crosstalk between APCs and $CD4^+$ cells enables APCs to activate (license) $CD8^+$ cytotoxic cells by presenting cognate pMHC I (signal 1) along with co-stimulatory (signal 2) and pro-inflammatory signals (signal 3). These licensed effector $CD8^+$ cells then become less dependent on co-stimulation for cytolytic function when encountering their target and express functional effector proteins such as perforin, granzymes and CD95L [12].

Cytotoxic Mechanisms of $CD8^+$ T cells

Licensed $CD8^+$ effector cells circulate and traffic to inflamed tissues to form conjugates with infected or tumor cells expressing the cognate pMHC I on their cell surface [12]. These conjugates form stable interactions, releasing perforin monomer from granules within $CD8^+$ T cells that then polymerize on the membrane of the targeted cell to form pores. These pores allow water and salts to cross the membrane, thereby disrupting cellular homeostasis and causing cell death. Granzymes are also able to pass

through these pores to activate a cascade involving caspases that results in degradation of DNA and apoptosis of targeted cells [13]. $CD8^+$ T cells can also kill cells by activating death receptors on targeted cells. For instance, CD95L (Fas ligand) on $CD8^+$ T cells can bind to the death receptor CD95R (Fas) on target cells and cause caspase activation and apoptosis [14].

Expansion and Contraction of T cells

During the initial exposure to antigens, activated antigen-specific T cell populations undergo rapid proliferation (expansion phase), followed by rapid cell death of approximately 90-95% of the expanded population from the peripheral blood (contraction phase). After contraction, the 5-10% of the T cells that survive are termed memory T cells and can be identified by their expression of certain unique proteins [12]. In C57BL/6 mice, memory and activated cells transition from being $CD44^{LO}$ to $CD44^{HI}$ [15], while in humans memory T cells transition from being $CD45RA^{POS}CD45RO^{NEG}CD95^{NEG}$ to $CD45RA^{NEG}CD45RO^{POS}CD95^{POS}$ [16, 17]. These clonally expanded memory cells are found at a higher frequency than their naïve progenitors and are epigenetically imprinted to be less dependent on co-stimulation and can undergo rapid proliferation during subsequent encounters with antigen [12]. These enhanced secondary immune responses are the basis of vaccination leading to protective immunity.

T cell responses are highly regulated by the presence of certain cytokines and other activating signals during all phases of an immune response. In the absence of these signals (signals 2 and 3), T cells that react to self-pMHC complexes can undergo

apoptosis (clonal deletion) or become unresponsive (anergic). These mechanisms of peripheral tolerance are important because normal tissues are constantly presenting self-pMHC complexes and not all self-reactive T cells are deleted during negative selection in the thymus [18]. Thus T cell activation and homeostasis are tightly controlled by immune signals, such as expression of IL-2 and its receptor (CD25), which are described in detail below and are the main subject of Chapter 2 of this thesis.

T cell Differentiation

During an ongoing immune response, T cells up-regulate expression of certain proteins termed activation markers (CD69, CD25 and KLRG1). Furthermore, memory T cell lineages can be identified by a particular expression pattern of certain proteins. For instance, during an immune response, $CD8^+$ T cells that are $KLRG1^{Hi}CD127^{Lo}$ become terminally differentiated effector cells while $KLRG1^{Lo}CD127^{Hi}$cells become long-lived memory cells [19]. Such cell differentiation is determined by immune signals delivered during activation. For example, studies have shown that cells which express and maintain CD25 on their surface and receive IL-2 signals during activation are more likely to become terminally differentiated $KLRG1^{Hi}CD127^{Lo}$ cells compared to cells that do not receive IL-2 signals during activation, which become $KLRG1^{Lo}CD127^{Hi}$ long-lived memory cells [20, 21].

Surface markers are also used to identify late-differentiated cells involved in chronic immune responses. Under ideal circumstances, the pathogen, inflammation and foreign antigens are cleared, leaving resting memory T cells in humans that are $CD95^+CD28^+$. However during autoimmune diseases and chronic viral infections,

human $CD4^+$ and $CD8^+$ T cells are chronically stimulated and can be driven towards a late differentiated phenotype. These late differentiated cells in humans are identified by their loss of CD28 and have unique characteristics such as poor proliferative capacity, increased ability to produce inflammatory cytokines and more resistance to apoptosis [22-24]. Cytomegalovirus (CMV) reactive T cells are mainly found within the $CD28^{NEG}$ subset and this subset is increased in persons with autoimmune diseases (e.g. rheumatoid arthritis and systemic lupus erythematosus) [22, 25-27].

IL-2 and CD25

The following sections will provide background information for chapter 2, which will discuss the phenotypic and functional characterization of a poorly characterized $CD4^+CD25^{INT}FOXP3^{NEG}$ T cell population found in humans.

IL-2 and CD25 Background

Interleukin-2 (IL-2) is a 15.5 kDa α-helical protein first identified by its ability to culture T cells long-term *in vitro* [28, 29]. IL-2 is primarily produced by $CD4^+$ and $CD8^+$ T cells shortly after activation [30-32], but is also produced to a lesser extent by NK cells, NKT cells, DCs and mast cells [33-36]. IL-2 gene expression is induced by TCR activation and stabilized by co-stimulation through CD28 [37]. The IL-2 receptor is composed of three subunits: CD25 (α-chain), CD122 (β-chain) and CD132 (common gamma chain, γc). CD122 and CD132 are also subunits for other cytokine receptors, whereas CD25 is specific for the IL-2 receptor [38]. Of the three components, CD25 has the highest affinity for IL-2 by itself, but does not directly participate in IL-2 signaling

[39-41]. Therefore, at high concentrations of IL-2, signaling can occur in the absence of CD25 through the CD122/CD132 intermediate affinity IL-2 receptor. However, cells require CD25 to respond to lower concentrations of IL-2 by forming the CD25/CD122/CD132 high affinity IL-2 receptor.

CD25 Expression by Activated and Regulatory T Cells

CD25 was originally discovered in 1981 using a monoclonal antibody that recognized an antigen transiently expressed on activated, but not resting human T cells. This protein was originally called Tac (short for T cell activation) [42, 43] and subsequent studies went on to molecularly characterize CD25, demonstrating its role in IL-2 signaling [41, 44-47]. CD25 expression and IL-2 signaling have been shown to increase T cell activation, expansion, and survival, thus promoting T cell responses [20, 21, 46, 48, 49]. However, extensive studies have been published using mouse models that show CD25/IL-2 signaling also promotes immune tolerance.

In 1995, Sakaguchi showed in elegant experiments that depleting $CD4^+CD25^+$ T cells during adoptive cell transfer experiments resulted in fatal systemic autoimmune disease, which could be rescued if the mice were reconstituted with a small number of $CD4^+CD25^+$ T cells [50]. Though it was originally thought that $CD4^+CD25^+$ cells were recently activated T cells, it later became known that these regulatory T cells (Tregs) constitutively express CD25, can develop in the thymus and suppress immune responses [51, 52]. Subsequent genetic studies found that Tregs are controlled by the gene *FOXP3*, which encodes the protein FOXP3 (FOXP3 in humans, Foxp3 in mice) [53, 54]. Since then, Sakaguchi and others have extensively shown that $Foxp3^+$ Tregs require IL-2

signaling for their survival through CD25 [55-60]. Based on these studies, some immunologists believe that IL-2 is more important for immune suppression rather than enhancing T cell activation and proliferation [61]. However, the precise role of CD25 in immunology may be species-specific.

Differences in CD25 Expression Between Mice and Humans

In 2001 Hafler's, group attempted to translate the $CD4^+CD25^+$ Treg findings from mice to humans. Hafler found that in contrast to mice, CD25 was not a dependable marker for Tregs in humans since a much larger proportion of $CD4^+$ T cells from PBMCs expressed CD25, and only the $CD4^+CD25^{HI}$ cells had suppressor function indicative of Tregs, while $CD4^+CD25^{LO/INT}$ cells did not [62] (Figure 1.1, left). These findings were later confirmed after FOXP3 was discovered and analyzed in humans [63] (Figure 1.1, right). While this $CD25^{LO/INT}FOXP3^{NEG}$ non-Treg population has been mentioned in several studies as being recently activated $CD4^+$ T cells that have transiently up-regulated CD25, the primary focus of these studies were Tregs [62-66]. The absence of the $CD4^+CD25^{INT}$ population from laboratory mice has been postulated to be due to the relatively germ-free

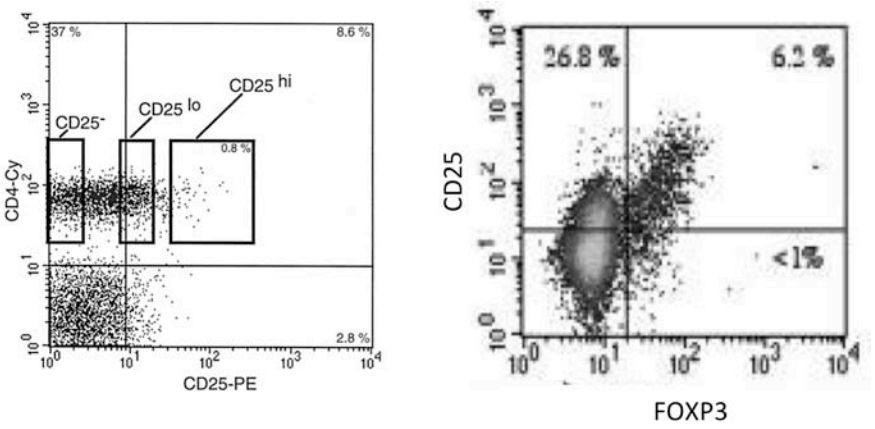


Figure 1.1: Reports of $CD4^+CD25^{INT}$ non-Tregs in humans. Evaluation of CD25 expression on $CD4^+$ T cells isolated from PBMCs of healthy individuals. (Left) Adapted from Baecher-Allan et al. (2001) J Immunol. 167(3): 1245-53, with permission from the Journal of Immunology. (Right) Adapted from Roncador et al. (2005) Eur. J. Immunology. 35: 1681-1691, with permission from John Wiley and Sons provided by Copyright Clearance Center

housing of mice compared to humans [62, 64, 66]. However, to our knowledge, no group has characterized the human $CD4^{+}CD25^{INT}FOXP3^{NEG}$ population to determine if they are truly activated T cells.

Given the importance of IL-2 in immune responses and homeostasis, and the current practice of targeting the IL-2 signaling pathway in the clinic by administering IL-2 to cancer patients or anti-CD25 blocking antibodies to transplant patients, it is important to understand the function of the $CD25^{LO/INT}FOXP3^{NEG}$ population. Therefore, we sought to thoroughly characterize this population. In Chapter 2, we show for the first time that these T cells are not recently activated cells, but rather represent a novel quiescent memory population that is uniquely affected by IL-2 treatment in cancer patients. We propose that the absence of this $CD25^{INT}Foxp3^{NEG}$ population in mice is not due to housing conditions and that the existence of this population in humans may explain differences that have been reported in the literature on the role of IL-2/CD25 between mice and humans [67-69]. These findings not only improve our understanding of the role of CD25 in human immunology, but may also have clinical implications by helping to illuminate the mechanisms and potentially improve the efficacy of therapies that target IL-2 and CD25.

Tumor Immunology

The following sections will provide background information for Chapter 3, which will discuss the therapeutic benefit of αOX40 + SM16 combination immunotherapy for treating large established tumors in a pre-clinical mouse cancer model.

History of Tumor Immunology

The concept that the immune system is capable of recognizing and killing tumor cells has been around since the seminal observations made by the bone surgeon and cancer researcher William Coley in the 1890's. Coley's idea of immune responses against tumors started with a patient with an unresectable sarcoma. After unsuccessful surgeries to remove the sarcoma, Coley's patient contracted severe cases of erysipelas (acute *streptococcus* infection of the upper dermis) during which the sarcoma completely regressed [70]. This observation led Coley to the literature where he found several other cases of complete tumor regression during erysipelas [70]. Though the field of immunology was young and little was understood about the mechanisms during Coley's time, he hypothesized that the *streptococcus* infections were somehow stimulating a productive anti-tumor immune response.

Due to the lack of understanding of how the immune system works, the field of tumor immunology was largely unexplored until the field of immunology started to mature in the 1950's. Using inbred mice, researchers were able to show that mice could be prophylactically vaccinated using irradiated tumor cells against transplants of syngeneic tumors. These vaccination strategies led to tumor specific immunity, thus suggesting the existence of tumor antigens in mouse cancer models [71, 72].

Immunosurveillance

As the understanding of cellular immunity advanced, Macfarlane Burnet developed the concept of "immunosurveillance" and Lewis Thomas hypothesized that the cellular immune system evolved not only to fight infections, but also to prevent the

outgrowth of neoplastic cells in order to maintain tissue homeostasis [73]. To test the hypothesis of immunosurveillance, researchers used athymic nude mice, which were thought to be completely immunodeficient at the time [74]. These experiments added doubt to the idea of tumor immunosurveilance since tumors occurred at the same rate in athymic nude mice as immune-intact mice [75, 76]. However, it was later shown that nude mice contained some functional T cells and were thus not completely immunodeficient [77]. It wasn't until later that a better immune deficient mouse model was developed, the $RAG^{-/-}$ (Recombinase Activation Gene) mice, which completely lack natural killer T (NKT) cells, T cells and B cells, and this model showed that the adaptive immune system plays a role in controlling tumor growth [78]. Subsequent studies have since shown the contribution of αβ T cells, γδ T cells, NK cells and NKT cells in controlling tumor outgrowth in transgenic mouse models [79, 80].

Mechanisms of Tumor Immunology

Our understanding of the specific mechanisms by which the immune system is able to control and shape tumor growth has been improved using a variety of experimental techniques. Though there are a myriad of cell types and pathways that mediate anti-tumor immune responses, the Th1/Cytotoxic immune response has been shown to be important by many research groups for controlling tumor growth in cancer models [81-83]. The canonical cytokine of Th1 cells is IFN-γ, which further promotes Th1 differentiation, increases MHC I up-regulation on tumor cells and activates APCs to increase expression of co-stimulatory and MHC molecules that further promote T cell

recruitment and activation. This ultimately provides help leading to a potent cytotoxic $CD8^+$ T cell response.

It was found that neutralizing IFN-γ or over-expression of a dominant negative IFN-γ receptor by tumor cells resulted in faster tumor growth, suggesting that IFN-γ enhances the immunogenecity of tumor cells [84]. It was subsequently shown in multiple models that tumors in $IFN\gamma^{-/-}$ mice grew faster, more frequently and metastasized more often compared to wild type mice [85, 86]. In addition, IFN-γ and STAT1 deficient mice develop tumors at a greater frequency and more rapidly than wild type controls when crossed to $p53^{-/-}$ mice, which spontaneously develop tumors. Perforin and granzymes (e.g. granzyme B) are packaged in cytolytic granules found in NK and cytotoxic T cells and are also important in Th1/cytotoxic responses [87]. The perforin cytolytic pathway has also been shown to be important in anti-tumor immune responses [85, 88].

Human Tumor Immunology

With the use of transgenic mice and molecular techniques, it is now unequivocal that anti-tumor immune responses occur in a variety of mouse tumor models. However, proving the existence of anti-tumor immune responses to spontaneous tumors in humans is more difficult due to experimental limitations. Nevertheless, the accumulating evidence cited below has shown that tumor specific immunity does exist in humans and that the immune system can be manipulated to cause regression of established tumors.

Since the 1970's it has been known that people who are immunocompromised have a higher incidence of cancers, both of viral origin as well as *de novo* tumors [89-95]. It has also been shown that lymphocytes infiltrate tumors, and importantly, an increased

accumulation of lymphocytes in the tumor is positively correlated with clinical outcome in multiple tumor types [96-102]. Though these findings suggest a role for the immune system in controlling tumor growth, they are merely correlative.

More definitive evidence for the existence of anti-tumor immunity in humans was published in the 1980's showing the existence of cytotoxic T cells that could be cloned from cancer patients which specifically recognized autologous tumor cell lines [103-105]. Subsequent studies showed that these cytolytic T cells recognized tumor cells in an MHC I dependent manner and have been utilized to identify tumor antigens [106-108]. Importantly, Rosenberg and others have shown the clinical effectiveness of using these T cells to elicit long-term responses in patients with late-stage metastatic melanoma [109].

Lack of Endogenous Immune Response Against Established Tumors

Except for occasional cases of spontaneous tumor regression in melanoma and basal cell carcinoma patients, the immune system seems incapable of meditating regression of large established tumors on its own [110]. This is most evident in studies that show an accumulation of lymphocytes within progressing tumors. Thus, it is clear that lymphocytes are able to access the tumor but the endogenous immune response ultimately fails to control tumor growth. The inability of the immune system to cause tumor regression in most cancers is thought to be due to the presence of immune suppressing signals, T cell anergy and clonal deletion of tumor reactive T cells due to chronic TCR activation in the absence of co-stimulation (signal 2) and activating cytokines (signal 3). T cell anergy and clonal deletion are most common when the antigens are not from mutated genes but rather from overexpressed or ectopically

expressed normal genes [109]. Also, tumors are notoriously difficult targets for T cells since they often downregulate MHC I expression on their surface and do not express co-stimulatory signals [111, 112].

Therefore studies are currently underway to determine how to boost the immune system to successfully overcome immunosupression and anergy and to spur a strong anti-tumor immune response that results in long term regression of established tumors. Below is a discussion of two broad categories of cancer immunotherapy, those that add agonists of immune activation or antagonists of immune suppression.

Cancer Immunotherapies

The following sections will provide background information for chapter 2, which will discuss the response of the $CD4^{+}CD25^{INT}FOXP3^{NEG}$ population to IL-2 treatment in cancer patients, as well as provide background information for chapter 3, which will discuss the ability of αOX40 + SM16 combination therapy to cure mice of large-established tumors.

Approaches to Increase a Productive Anti-Tumor Immune Response

Various groups have experimented with different immunotherapies to treat cancer over the years with limited success. One approach is vaccination with irradiated tumor cells or tumor antigens. This approach has often proven ineffective [113], possibly because tumor antigens are low affinity and differ between patients as well as between tumors within the same individual. In addition, a recent study suggests that different regions within a single primary tumor may express different antigens [114]. Therefore,

strategies that systemically activate the immune system, with the intent of stimulating T cell responses against multiple endogenous tumor antigens within an individual, are theoretically more promising. This section will focus primarily on cancer immunotherapies that target the cytokine signaling pathways of IL-2, transforming growth factor-β (TGF-β), as well as the T cell co-stimulatory receptor OX40.

IL-2 Immunotherapy

There are two approaches to targeting cytokine signaling in cancer patients, either infusing patients with immune-activating cytokines or blocking immune-suppressing cytokines (e.g. blocking antibodies, small molecule inhibitors). An example of the former is IL-2, which was originally discovered for its ability to increase T cell survival and proliferation in culture. In the early 1980's, Steve Rosenberg, an early proponent of cancer immunotherapy, immediately saw the therapeutic potential for using IL-2 in treating cancer. Rosenberg went on to show that tumor infiltrating lymphocytes from mice could be isolated and expanded in culture with IL-2, and that IL-2 enabled these cells to lyse tumor cells *in vitro* [115]. These IL-2 stimulated cells were later termed lymphokine-activated killer (LAK) cells which could be derived from spleens and could lyse tumor cell lines that were resistant to natural killer cells [116].

Rosenberg originally envisioned using IL-2 to culture and activate T cells derived from patients in order to adoptively transfer these LAK cells back into the same patients. He demonstrated the promise of this approach in mice by isolating tumor infiltrating T cells, expanding them in culture using IL-2 and adoptively transferring them back into the mice [115, 117-119]. However, this type of therapy was technically challenging for

treating cancer patients at the time. Instead, he was able to show that direct infusion of mice with recombinant human IL-2 (rhIL-2) resulted in the production of LAK cells and caused regression of established tumors [120, 121]. These encouraging results led to human trials using rhIL-2, which was subsequently approved by the FDA and is now used to treat patients with metastatic melanoma and renal cell carcinoma, eliciting partial responses in 15% and complete durable responses in 6% of these patients [122, 123].

TGF-β

Another approach to targeting cytokines in tumor-bearing hosts is to block cytokines that suppress immune activation. An example of an immune suppressing cytokine in tumor-bearing hosts is transforming growth factor-β (TGF-β). TGF-β is produced by a variety of tumor types [124-128] as well as tumor associated immune cells [129-131], and elevated levels of TGF-β in cancer patients have been reported to be associated with tumor progression in a variety of tumor types [126-128, 132-134]. TGF-β has many pro-tumor functions that act directly on tumor cells [131] as well as inhibit the function of immune cells, including monocytes, dendritic cells, NK cells and T cells [130, 131]. These actions include inhibition of antigen presentation, T cell differentiation, cytotoxic activity of CD8 T cells and CD4 Th1 cytokine production [130, 131, 135, 136]. The impact of TGF-β on immune regulation is evident in $tgfb1^{-/-}$ mice, which ultimately succumb to fatal autoimmunity [137]. Direct experimental evidence showing the suppressive effects of TGF-β on T cells was demonstrated in mouse studies using a dominant-negative form of the TGF-βRII transgene under the control of a T-cell-specific promoter [138]. Like $tgfb1^{-/-}$ mice, these mice also develop systemic

autoimmune disease. Lastly, TGF-β signaling has been shown to be important for the expansion, survival and suppressive function of Tregs *in vivo* [139-141].

Collectively, these studies provide rationale for blocking TGF-β as a strategy to enhance anti-tumor immune responses. This has been achieved using neutralizing antibodies [142], fusion proteins [143] and small molecule TGF-β signaling inhibitors, which have resulted in regression of primary tumor growth and metastasis in several mouse tumor models [144-146]. Though TGF-β is known to have direct effects on tumor cells, tumor regression during TGF-β signaling blockade is greatly diminished in SCID and athymic nude mice, suggesting that the mechanism is primarily immune mediated [145, 147, 148]. In addition, expression of the dominant-negative TGF-βRII on T cells resulted in the enhancement of tumor reactive T cells and tumor regression [149, 150]. Based on these findings, antibody and small molecule antagonists of TGF-β signaling have already been developed by pharmaceutical companies and are currently undergoing testing in clinical trials [151, 152].

OX40

The recent FDA approval and therapeutic benefit of ipilimumab (αCTLA-4) has added excitement for other cancer therapies that target T cell co-receptors, such as OX40. OX40 (CD134, TNFRSF4) is a member of the tumor necrosis factor receptor superfamily (TNFRSF) expressed primarily on activated $CD4^+$ and $CD8^+$ T cells [153-155]. OX40 was originally discovered in the 1980's by a group at Oxford who found that a monoclonal antibody they developed, termed OX-40, recognized T cells and enhanced their proliferation [156]. It has subsequently been shown that OX40 expression on T

cells is up-regulated by TCR ligation and IL-2 signaling [157], whereas the ligand is expressed primarily by activated APCs [158]. Both the OX40L and OX40R form trimers on their respective cells [159]. Once engaged, OX40 signaling is mediated by the recruitment of TNF receptor associated factors (TRAFs) that initiate various signaling pathways, including JNK and NF-κB [160, 161].

Many groups have shown in mouse models that engagement of OX40 with cognate ligand or agonist antibodies increases T cell proliferation, survival, cytokine production and the generation of memory T cells [162, 163]. In addition to recently activated T cells, regulatory T cells constitutively express OX40 in mice, and under certain conditions, treatment with OX40 agonists mitigates Treg development and function while endowing $CD8^+$ effector cells with the ability to resist Treg-mediated suppression [164-167].

These findings led to testing OX40 agonists in mice to treat cancer. Using an OX40L:Ig fusion protein and agonist antibodies, it has been shown that stimulating OX40 on T cells can cause tumor regression in a variety of tumor models [154, 168, 169]. These anti-OX40 mediated anti-tumor responses were dose dependent, required $CD4^+$ and $CD8^+$ T cells and resulted in long-term tumor immunity [170, 171]. Though the mode of action was originally thought to be through the activation of $CD4^+$ T cells, which then activate $CD8^+$ T cells, it was later shown that direct engagement of OX40 on $CD8^+$ T cells is also important for the anti-tumor immune responses [172, 173].

These finding were also tested in non-human primates, and it was shown that agonist OX40 antibodies caused activation of circulating lymphocytes with no overt signs of toxicity [174]. Collectively, these studies provided rationale for developing and

testing an OX40 agonist murine antibody in cancer patients. Treatment with the OX40 agonist antibody has recently completed a phase I clinical trial and resulted in activation of $CD4^+$ and $CD8^+$ circulating lymphocytes and tumor regression in some of these late-stage cancer patients (unpublished data). Therefore, humanized antibodies and ligands specific for OX40 are currently being developed for multiple dosing in future clinical trials.

Combination Therapies

In mouse tumor models, single agent immunotherapies are often successful at treating cancer if given shortly after tumor inoculation, but often fail when tumors are allowed to establish. For example, αOX40 has been shown to be efficient at treating mice if given shortly after tumor challenge when tumors are small or not yet palpable [154, 168, 169, 171], but less effective when given later when tumors are larger [170, 172, 175]. Larger tumors most likely better resemble tumors observed in the clinic in which patients have had tumors present within their bodies for months or years [176, 177].

A potential explanation for the inability of αOX40 to cause tumor regression of large tumors is that the microenvironments of larger tumors contain immune suppressive cytokines such as TGFβ, IL-10, vascular endothelial growth factor (VEGF) and adenosine [178]. Therefore, a successful tumor immunotherapy may require not just an agonist to boost effector T cell function, but also an inhibitor(s) of T cell suppression in order to eradicate large established tumors. Chapter 3 will discuss the ability of combining OX40 with a TGF-β receptor inhibitor (SM16) to cure mice of large established tumors.

Chapter 2

Defining a functionally distinct subset of human memory $CD4^+$ T cells that are $CD25^{POS}$ and $FOXP3^{NEG}$

Abstract

Surface expression of the IL-2 receptor α-chain (CD25) has been used to discriminate between $CD4^{+}CD25^{HI}FOXP3^{+}$ regulatory T cells (Tregs) and $CD4^{+}CD25^{NEG}FOXP3^{-}$ non-Tregs. However, this study reports that the majority of resting human memory $CD4^{+}FOXP3^{-}$ T cells express intermediate levels of CD25 and that CD25 expression can be used to delineate a functionally distinct memory subpopulation. The $CD25^{NEG}$ memory T cell population contains the vast majority of late differentiated cells that respond to antigens associated with chronic immune responses and are increased in patients with systemic lupus erythematosus (SLE). In contrast, the $CD25^{INT}$ memory T cells respond to antigens associated with recall responses, produce a greater array of cytokines and are less dependent on co-stimulation for effector responses due to their expression of CD25. Lastly, compared to the $CD25^{NEG}$ and Treg populations, the $CD25^{INT}$ memory population is lost to a greater degree from the blood of cancer patients treated with IL-2. Collectively, these results show that in humans, a large proportion of $CD4^{+}$ memory T cells express intermediate levels of CD25, and this $CD25^{INT}FOXP3^{-}$ subset is a functionally distinct memory population that is uniquely affected by IL-2.

Introduction

T cell survival and effector function are sensitive to the availability of essential cytokines during development, homeostasis and activation. Interleukin-2 (IL-2) is a 15.5 kDa α-helical protein discovered for its ability to culture T cells long-term *in vitro* [28]. IL-2 has broad effects on T lymphocytes, including survival, proliferation, activation-induced cell death (AICD), T cell differentiation, cytokine production and immune tolerance [31, 179, 180]. The high-affinity receptor for IL-2 (IL-2R) is composed of three subunits, the α-subunit (CD25), β-subunit (CD122) and the common γ-chain (CD132). CD122 and CD132 are also subunits for other cytokine receptors, whereas CD25 is specific to the IL-2 receptor. IL-2 signaling occurs exclusively through the cytoplasmic tails of CD122 and CD132; CD25 has a short cytoplasmic tail and is not involved in IL-2 signaling. Instead, CD25 has the highest affinity for IL-2 among the individual subunits and acts as an affinity converter [31]. At high concentrations, IL-2 can signal in the absence of CD25 through CD122 and CD132, which form the intermediate affinity IL-2R. However, CD25 in addition to CD122 and CD132 is required to respond to low concentrations of IL-2 by forming the high affinity IL-2 receptor [31]. Once formed, the IL-2/CD25/CD122/CD132 quaternary complex is short-lived ($t_{1/2}$ 10-20 minutes) on the cell surface [181]. Upon internalization, IL-2, CD122 and CD132 are targeted for lysosomal degradation, whereas CD25 is recycled to the cell surface [182, 183].

Though CD25 has been shown to influence effector function of lymphocytes, CD25 is thought to play a greater role in immune tolerance in mice [31, 61]. Initially it was found that depletion of $CD4^+CD25^+$ T cells from adoptive cell transfer experiments

into nude mice resulted in systemic autoimmune disease [50]. These $CD4^+CD25^+$ cells were later shown to express the transcription factor Foxp3 (FOXP3 in humans) and are now termed regulatory T cells (Tregs), which comprise 5-15% of $CD4^+$ T cells in humans [184]. Tregs depend on IL-2 signaling for their survival *in vitro* and *in vivo* [55, 56, 185]. Therefore, constitutive expression of CD25 on Tregs is thought to be crucial to their survival and maintenance of immune homeostasis. This idea is supported by studies of mice deficient in CD25 or IL-2, which have low numbers of Tregs and develop severe systemic autoimmune disease as they age [59, 68]. Despite the positive effects of IL-2 on effector and memory T cells, CD25/IL-2 deficiency in mice does not appear to greatly hinder T cell immunity, reviewed elsewhere [61]. Therefore, it is thought that in mice, CD25/IL-2 plays a dominant role in immune tolerance and less for adaptive immunity, perhaps because CD25 is expressed only transiently on activated effector cells and constitutively on Tregs. However, expression of CD25 and its role in immunology may be species dependent, since CD25 appears to play a larger role in T cell effector responses in humans compared to mice, and may be dispensable for the maintenance of Tregs as seen in patients treated with CD25 blocking antibodies [69, 186, 187]. This notion has been discussed elsewhere in the literature [188, 189] and is supported by the phenotype of CD25 deficiency in humans, who in contrast to mice, are severely immunocompromised and have a normal frequency of Tregs [67, 190-192].

This difference between mice and humans may be related to the presence of a large population of $CD4^+FOXP3^-$ T cells in humans that express intermediate levels of CD25, a population that has not been found in mice [193]. Given the importance of IL-2 in the immune system and in the clinic, we sought to determine if resting $CD4^+FOXP3^-$ T

cells that expressed CD25 represent a functionally distinct human T cell population that responds to IL-2 immunotherapy in cancer patients. We report that $CD4^{+}CD25^{INT}FOXP3^{-}$ cells comprised up to 65% of resting human $CD4^{+}$ T cells and constituted the majority of the $CD4^{+}$ memory compartment in healthy individuals. Further evaluation revealed that $CD4^{+}CD25^{NEG}$ memory and $CD4^{+}CD25^{INT}$ memory populations are composed of functionally distinct memory subsets. Also, $CD25^{INT}$ T cells exhibit enhanced effector function when activated in the absence of co-stimulation that is in large part due to IL-2 signaling. Lastly, we found that compared to the $CD25^{NEG}$ and Treg populations, the $CD25^{INT}$ population proliferated more vigorously to rhIL-2 *in vitro* and decreased in the peripheral blood of cancer patients undergoing IL-2 immunotherapy. Together, these data show that in humans there exist a larger proportion of resting $CD4^{+}FOXP3^{-}$ T cells that express CD25 than previously thought, and that $CD25^{INT}$ cells are a functionally distinct memory population that may play a role in IL-2 immunotherapy in cancer patients. Ultimately, further studies of this population may help us understand and improve the efficacy of immunotherapies that influence IL-2 signaling.

Materials and methods

Lymphocyte isolation and phenotypic analysis

Human PBMCs were isolated by centrifugation of heparinized blood over Ficoll-Plaque™ PLUS (GE Healthcare). Isolated PBMCs were either analyzed fresh or were frozen in 45% RPMI/45% FBS/10% DMSO and then thawed for analysis. Staining for flow cytometry was done at either 4°C or room temperature for 30 minutes with antibodies to: CD3 (UCHT1), CD4 (SK3), CD8 (SK2), CD25 (Miltenyi, 4E3), CD25 (BD, M-A251), CD95 (DX2), CD45RA (HI100), CD45RO (UCHL1), CD127 (eBioRDR5), CD28 (CD28.2), CD134 (ACT35), CCR5 (2D7/CCR5) or CD319 (162.1). For intracellular staining, cells were prepared with Foxp3 Staining Buffer Set (eBioscience) according to the manufacturer's instructions and incubated at either 4°C or room temperature for 30 minutes with antibodies to: EOMES APC (WD1928), FOXP3 (236A/E7), Ki67 (B56), pSTAT5 (47), IL-17A (BL168), Granzyme B (GB11), BCL-2 (100), IL-2 (MQ1-17H12) or IFN-γ (B27). Antibodies were acquired from Miltenyi, eBioscience, BD Biosciences, BioLegend, Invitrogen and Beckman Coulter. All samples were run on an LSR II flow cytometer or FACSAria II and analyzed by FlowJo or Winlist. Sorting experiments were done using $CD4^+$ cells enriched by Miltenyi LS columns from fresh PBMCs that were stained and sorted using a BD FACSAria II Cell Sorter.

Healthy donors and patients

PBMCs from individuals (10 females, 5 males; mean age, 36; age range, 27-61) without known autoimmune disease or cancer were used as healthy donors in this study.

Patients with SLE (10 females; mean age, 40; age range, 20-49) that took part in the study fulfilled the American College of Rheumatology revised classification criteria for lupus [194]. Patients had active (n=7) or inactive (n=3) renal nephritis and were being treated with a variety of drugs (hydroxychloroquine n=9, mycophenolate n=4, prednisone=7). Cancer patients undergoing IL-2 immunotherapy (3 females, 7 males; mean age, 53; age range, 24-68) had either metastatic melanoma or renal metastatic carcinoma. Analysis of PBMCs from healthy donors and SLE patients was done on fresh samples. Samples from IL-2 treated patients were frozen PBMCs that had been collected immediately before treatment and 18 hours, 1 week and 2 weeks after the first infusion. All IL-2 patients received 600,000 IU/kg of rhIL-2 (Proleukin) every 8 hours by intravenous bolus for up to 14 doses. Two cycles of IL-2 immunotherapy were given at 2 week intervals following which clinical response was determined and further IL-2 was administered at the discretion of their physician for patients with stable or responding disease.

IL-2 in vitro stimulations

Enriched $CD4^+$ or sorted cells from fresh PBMCs were cultured in 10% complete RPMI and incubated at a concentration of 100,000 cells/100 μl in 96 well plates. For pSTAT5 analysis, cells were incubated for 1 hour at 37°C with or without 2μg/ml of anti-CD25 blocking antibody (R&D Systems, clone #22722) and stimulated with rhIL-2 (Proleukin) for 15 minutes. The cells were then fixed and permeabilized with the Fix & Perm Cell Permeabilization Reagents from Invitrogen following the methanol modified protocol and stained for pSTAT5. For survival and proliferation assay, sorted cells were

cultured for 7 days with or without rhIL-2 and evaluated for survival by Annexin V/7AAD staining (BD Biosciences) and proliferation by intracellular Ki67.

Antigen stimulation and intracellular cytokine staining

Frozen PBMCs from healthy individuals were thawed and cultured at 37°C in 10% complete RPMI at a concentration of 1 x 10^6 cells/100 μl in 96 well plates. Cells were cultured with 5 μg/ml of anti-CD28/49d alone or with Flu Vaccine (afluria®, 3 μg/ml), SEB (Toxin Techonology Inc., 1μg/ml) or CMV lysate (Advanced Biotechnologies Inc.,10 μg/ml) for 1 hour, after which brefeldin A (5 μg/ml) was added. After 18 hours, cells were stained for extracellular CD3, CD4, CD95 and CD25 and then stained for the intracellular cytokines IFN-γ and IL-2 after permeabilization. CD25 MFI background was determined by staining for all markers except CD25 in each assay.

Anti-CD3 stimulation assays

Fresh PBMCs were sorted, suspended in 10% RPMI at a concentration of 50,000 cells/100 μl in 96 well plates that were uncoated or pre-coated with 5 μg/ml anti-CD3 (OKT3). All samples were done in triplicate with and without 2μg/ml of anti-CD25 blocking antibody (R&D Systems, clone #22722). Cells were cultured for 3 days, after which 100 μl of supernatant was collected and the cells were transferred to uncoated 96 well plates and given 100 μl of fresh media with and without anti-CD25 (2 μg/ml). Two days after re-plating, proliferation was analyzed by counting cells with a hemocytometer and survival was determined by Annexin V/7AAD staining (Invitrogen) analyzed by flow cytometry.

Statistical Analysis

Statistical significance was determined by unpaired or paired student t-test using Prism software (GraphPad, San Diego, CA); a p-value of <0.05 was considered significant.

Results

CD25 expression on human $CD4^+FOXP3^-$ memory T cells

The IL-2 receptor alpha chain (CD25) has been used as a marker for Treg cells ($CD4^+CD25^{HI}FOXP3^+$) as well as activated T cells [31]. However, analysis of $CD4^+$ cells using two different monoclonal antibodies to CD25 clearly revealed a population of resting $FOXP3^-$ human $CD4^+$ T cells that expressed intermediate levels of CD25 [193]. We found that these two commercially available anti-human CD25 antibodies revealed a significant proportion of $CD4^+FOXP3^-$ T cells expressed intermediate levels of CD25 (Figure 2.1). We subsequently used clone 4E3 for the remainder of this study and found that $CD25^{INT}$ $CD4^+$ T cells were found in all individuals studied, comprising 35-65% of all $CD4^+$ T cells in normal donors. Representative FACS plots from four individuals are shown in figure 2.2A.

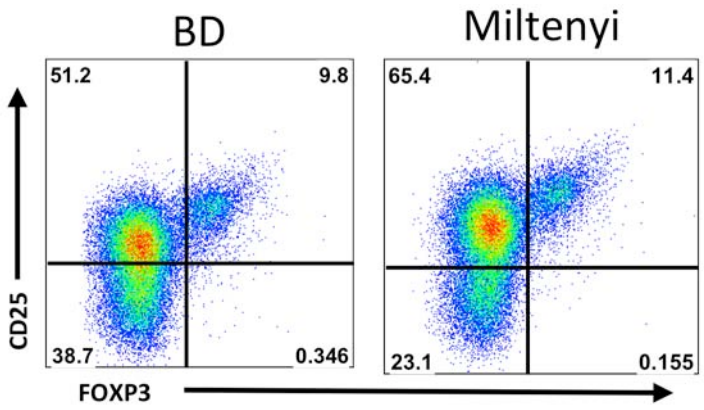


Figure 2.1: CD25 detection using two monoclonal antibodies. $CD3^+CD4^+$ PBMCs from the same individual evaluated for CD25 expression using an anti-human CD25 antibody from BD (clone M-A251) or Miltenyi (clone 4E3). Plots are representative of 3 individuals

The specificity of this cell population was verified by blocking with a CD25:Ig fusion protein at various concentrations prior to staining cells. As shown in figure 2.2B, there was a dose-dependent decrease in both the $CD25^{INT}$ and $CD25^{HI}$ population with increasing concentrations of CD25:Ig. To show that the 4E3 clone recognized functional CD25, $CD4^+$ T cells from fresh PBMCs were stimulated with various concentrations of

rhIL-2 and then evaluated for up-regulation of intracellular pSTAT5, as pSTAT5 is downstream of IL-2 signaling (Figure 2.2C). Cells expressing higher levels of CD25 responded to lower concentrations of IL-2, while cells expressing little or no CD25 required higher concentrations of rhIL-2. When pre-incubated with an anti-CD25 blocking antibody that does not interfere with binding of the 4E3 anti-CD25 antibody, the cells expressing intermediate and high levels of CD25 were unable to respond to the lower concentrations of rhIL-2 but did respond to a higher dose of rhIL-2, presumably through the β and γ chains of the IL-2 receptor (Figure 2.2C).

Although we found the $CD25^{INT}FOXP3^{-}$ cells mainly among $CD4^{+}$ T cells, a small proportion of resting $CD8^{+}$ T cells also expressed CD25 (Figure 2.2D). $CD25^{INT}$ $CD4^{+}$ T cells were interrogated by flow cytometry for expression of markers of naïve and memory cells. The majority of $CD25^{INT}$ cells expressed the memory marker CD95 (Figure 2.3) [16]. This observation was reaffirmed by the expression of the naïve and memory markers CD45RA and CD45RO (Figure 2.3) [17]. In the normal individuals studied, $CD25^{INT}$ T cells comprised the majority (as much as 80%) of memory cells in the $CD4^{+}$ T-cell compartment (data not shown). We were unable to find a significant relationship between the percent of $CD4^{+}$ that were $CD25^{INT}$ as a function of age within the cohort of healthy individuals used in this study (data not shown).

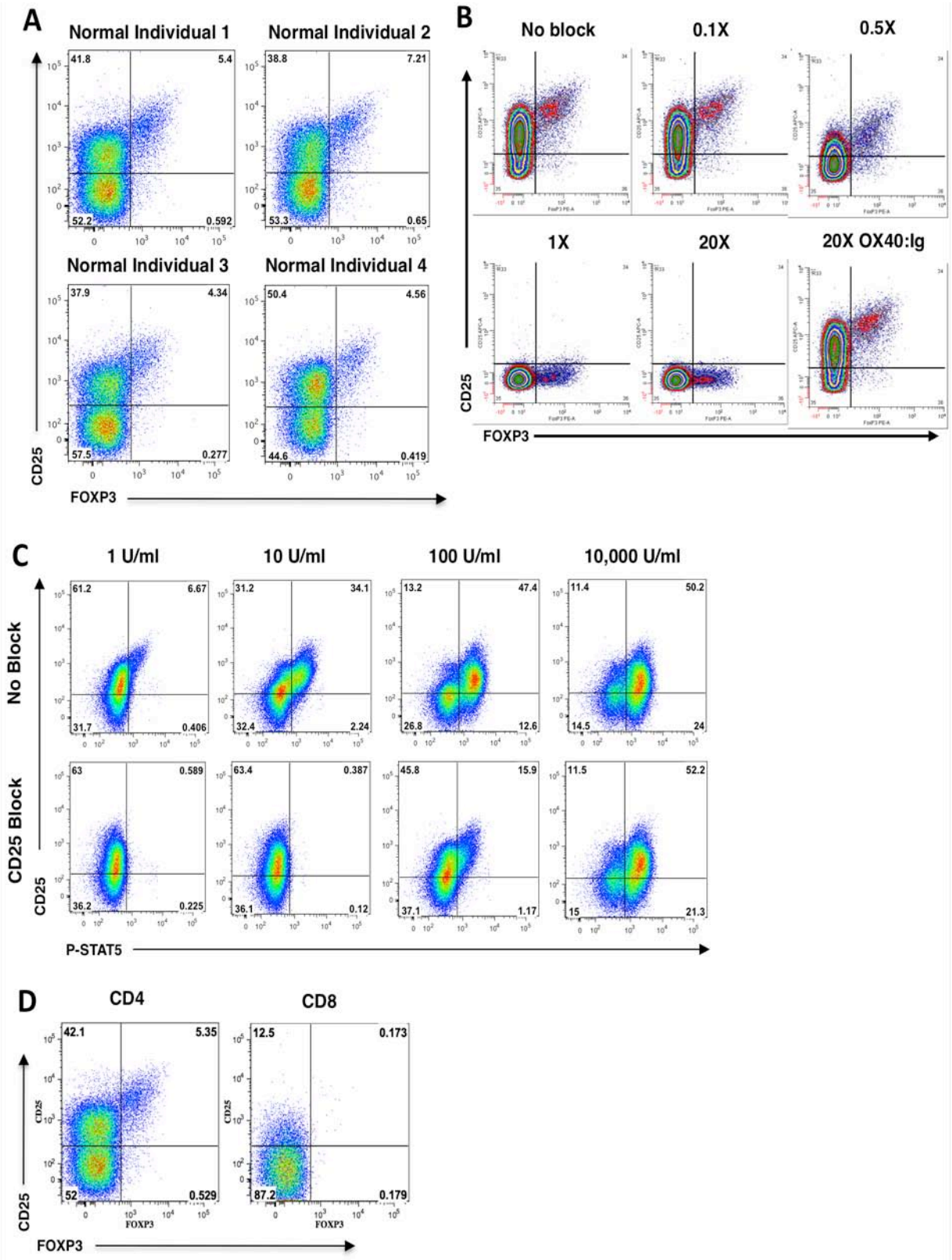
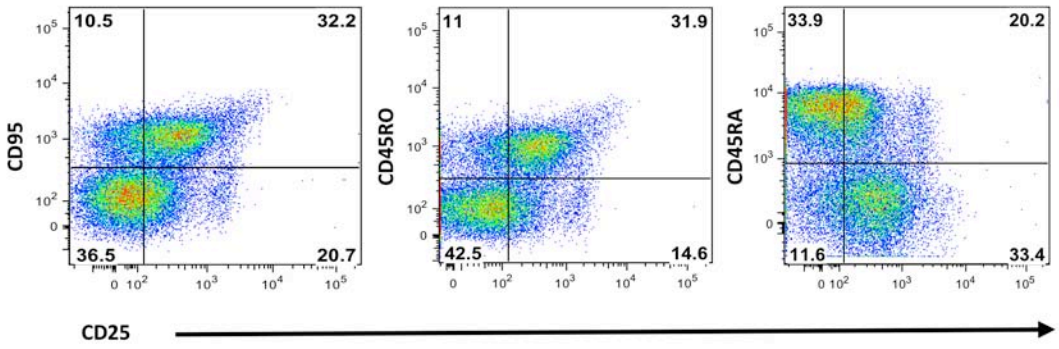


Figure 2.2: A large proportion of CD4$^+$ T cells are CD25^{INT}FOXP3^{NEG}. (A) CD25 and FOXP3 staining of CD3$^+$CD4$^+$ T cells from 4 of 10 individuals. **B)** Anti-CD25 antibody was incubated with different concentrations of CD25:Ig or OX40:Ig fusion protein for 30 minutes at room temperature. PBMCs were then added and evaluated for binding of anti-CD25 antibodies. **(C)** Enriched CD4$^+$ cells from fresh PBMCs were stained for CD25 prior to incubation at 37° C with various concentrations of rhIL-2 (U/ml) in the presence or absence of anti-CD25 blocking antibodies for 15 minutes and evaluated for intracellular pSTAT5. Plots are representative of two independent experiments using PBMCs from different health individuals **(D)** CD25 and FOXP3 staining of CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ T cells from the same individual. Plots are representative of 10 different healthy individuals.



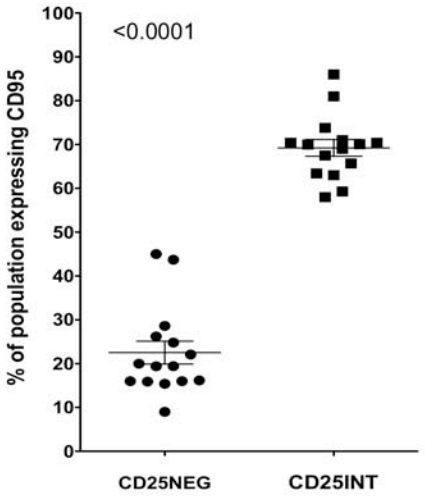


Figure 2.3: CD25^{INT}FOXP3^{NEG} population are mostly memory cells.
(Above) CD3^{+}CD4^{+}PBMCs from the same individual evaluated for CD95, CD45RA and CD45RO. Plots are representative of 5 healthy individuals. **(Right)** The percentage of CD3^{+}CD4^{+}CD25^{NEG} and CD3^{+}CD4^{+}CD25^{INT} cells that express CD95 was determined in PBMCs from healthy donors (n=15). The graphs show the data from all 15 individuals; each symbol represents an individual, the wide horizontal bar represents the mean +/- SD. Statistical significance was determined by paired student's t-test analysis.

We next evaluated whether CD95^{+}CD25^{NEG}FOXP3^{-} and CD95^{+}CD25^{INT}FOXP3^{-} CD4^{+} T cells maintain their respective CD25 phenotype over time. CD4^{+} T cells were enriched from fresh blood samples and then sorted into four groups: naïve (CD95^{-}), memory CD25^{NEG}, memory CD25^{INT} and Treg cells (Figure 2.4A,B) [64]. The sorted cells were cultured without stimulation and re-evaluated for expression of CD25 two and five days later. These sorted populations maintain their relative levels of CD25, suggesting the CD25^{INT} memory cells were not recently activated cells with transient CD25 expression (Figure 2.4C). These data imply that CD25^{INT} and CD25^{NEG} memory populations represent two distinct resting memory populations.

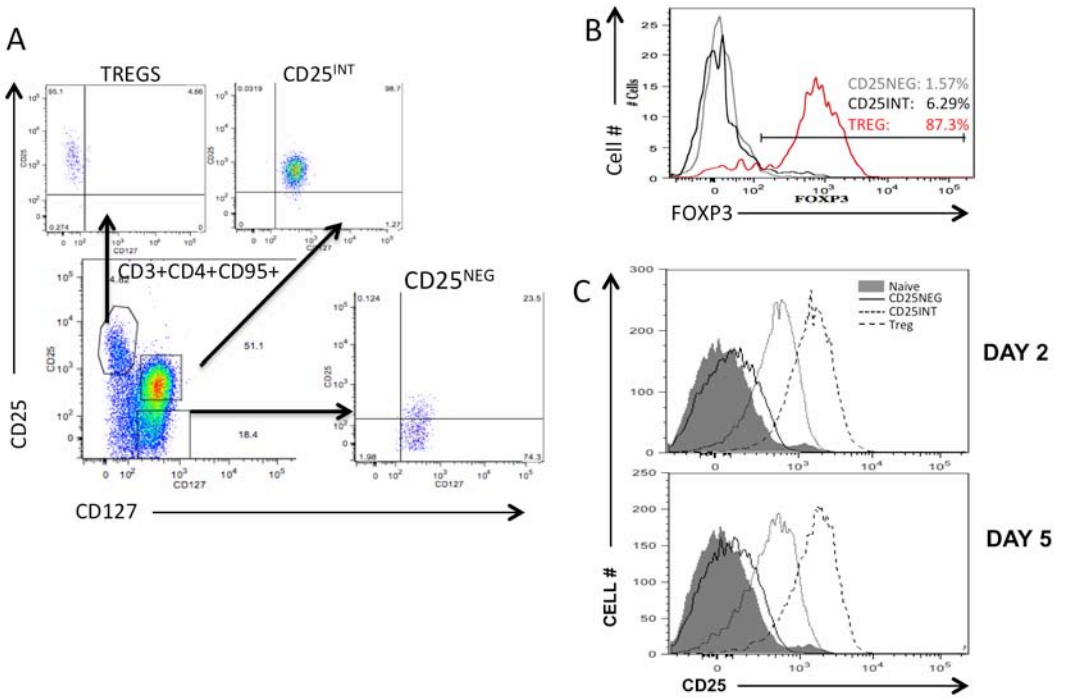


Figure 2.4 $CD25^{INT}FOXP3^{NEG}$ memory cells are not recently activated cells. (A) Gating scheme and purities for sorting naïve ($CD95^{NEG}$), memory $CD25^{NEG}$ ($CD95^{POS}CD25^{NEG}CD127^{POS}$), memory $CD25^{INT}$ ($CD95^{POS}CD25^{INT}CD127^{POS}$) and Treg cells ($CD95^{POS}CD25^{HI}CD127^{NEG}$). **(B)** FOXP3 expression by sorted populations. **(C)** Sorted populations evaluated for CD25 expression 2 and 5 days after being sorted. Plots are representative of three healthy individuals.

Phenotypic evaluation of $CD25^{INT}$ and $CD25^{NEG}$ memory T cells

Next, we tested the hypothesis that $CD25^{INT}$ memory cells were distinct from their memory $CD25^{NEG}$ counterparts by examining differences in differentiation/activation markers which are expressed by memory cells. The majority of $CD4^{+}$ naïve and memory cells from normal donors express CD28. However, others have shown that individuals with ongoing chronic immune responses, such as autoimmune disease, have a higher proportion of late-differentiated memory $CD4^{+}$ T cells that do not express CD28 [25, 26]. We found the majority of these memory $CD4^{+}CD28^{NEG}$ cells were within the $CD25^{NEG}$ population (Figure 2.5A). The memory $CD4^{+}CD28^{NEG}$ population has been reported to produce cytolytic proteins such as granzyme B [27], which are typically expressed by $CD8^{+}$ T-cell subsets. We found that memory $CD4^{+}$ T

cells that produce granzyme B were within the $CD25^{NEG}$ population and not found in the $CD25^{INT}$ population (Figure 2.5A).

To further assess the differences between the $CD25^{NEG}$ and $CD25^{INT}$ memory populations, we performed a microarray analysis with RNA from sorted $CD95^{+}$ memory populations. Two genes whose expression levels were lower in the $CD25^{INT}$ cells were CD319, a member of the signaling lymphocyte activation molecule family (SLAMF) family receptors, and the T-box transcription factor, Eomesodermin (EOMES), both of which are up-regulated in activated $CD8^{+}$ and NKT cells. Previous studies have shown that granzyme B is regulated in part by EOMES, while CD319 has activating properties on NKT cells, but little information regarding these two proteins is available for human $CD4^{+}$ T cells [195-197]. Therefore, we evaluated intracellular and surface expression levels of EOMES and CD319 protein in $CD4^{+}$ T cells from normal individuals. We found EOMES and CD319 were preferentially expressed within the $CD4^{+}CD25^{NEG}$ population, confirming our microarray data (Figure 2.5B). In contrast, the co-stimulatory TNF-receptor family member OX40 (CD134) was preferentially expressed on the surface of $CD25^{INT}FOXP3^{-}$ population within normal individuals (Figure 2.5C).

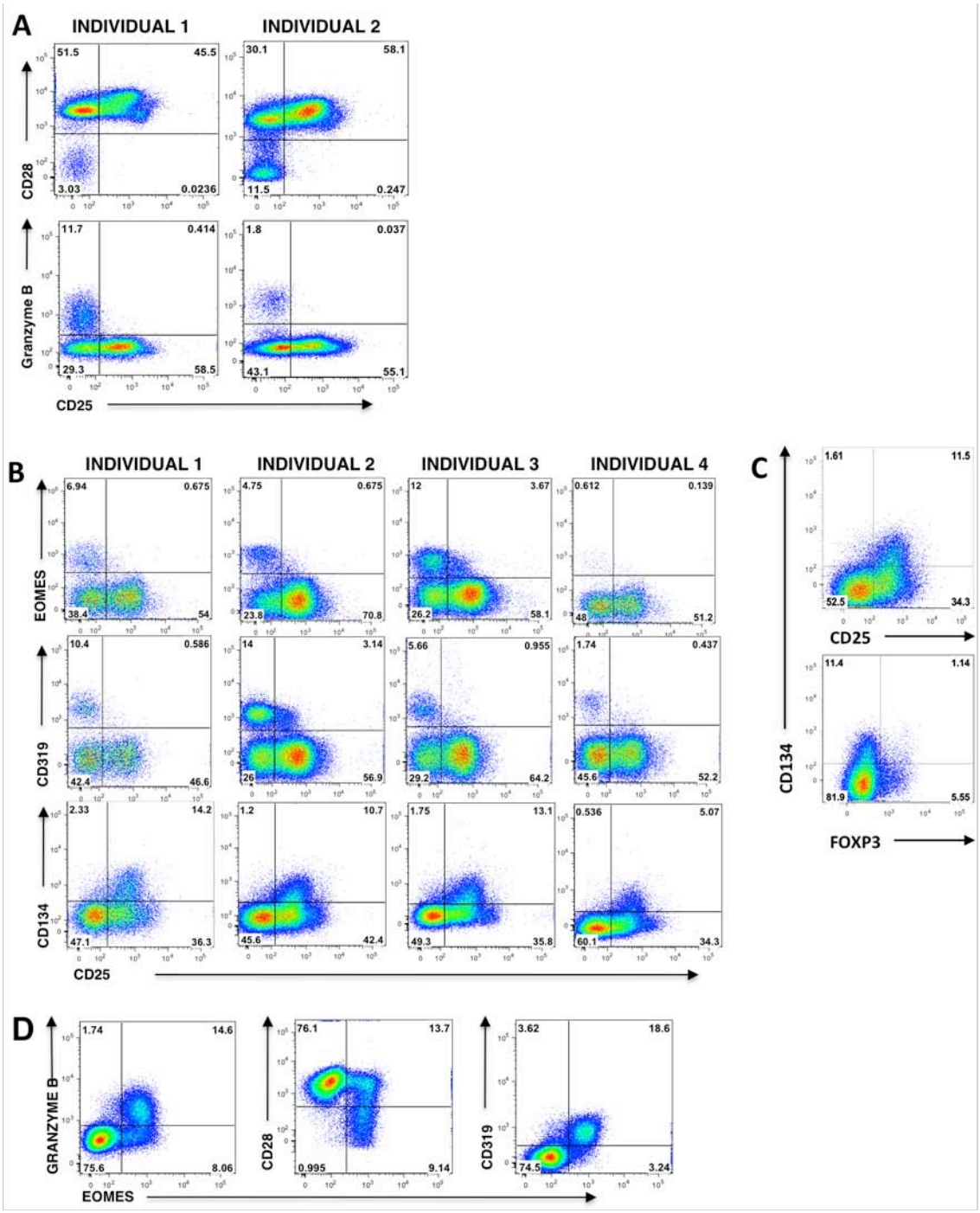


Figure 2.5: CD25^{NEG} and CD25^{INT} populations contain distinct memory subsets. CD3^{+}CD4^{+} T cells from fresh PBMCs obtained from healthy donors evaluated for **(A)** CD25, CD28 and Granzyme B expression, **(B)** CD25, EOMES, CD319 and CD134, and **(C)** Evaluation of CD3^{+}CD4+ PBMCs from the same individual for expression of CD134 versus CD25 and FOXP3 **(D)** Evaluation of PBMCs from the same individual for expression of EOMES, Granzyme B, CD28 and CD319. Plots A, B and C are representative of 10 individuals. Plot D is representative of 3 individuals.

EOMES partly controls granzyme B expression, therefore we addressed whether EOMES was expressed in granzyme B^+ and/or $CD28^{NEG}$ cells. We observed that the majority of both the $CD28^{NEG}$ and the granzyme B^+ cells co-expressed EOMES, but not all of the $EOMES^+$ cells were $CD28^{NEG}$ or granzyme B^+ (Figure 2.5D). Lastly, since granzyme B, EOMES and CD319 are expressed by cytolytic $CD8^+$ T cells, we wanted to determine if a similar trend was found in $CD8^+$ T cells. As mentioned, most of the human $CD8^+$ T cell populations are $CD25^{NEG}$. However, we observed a high proportion of $CD8^+$ T cells that express intermediate levels of CD25 in some cancer patients. The majority of the $CD8^+$ T cells that express granzyme B, EOMES, CD319 and lack CD28 are within the $CD8^+CD25^{NEG}$ subpopulation (Figure 2.6). We did not find clear differences in expression of the differentiation markers CCR7, CD62L or CCR5 between $CD95^+CD25^{NEG}$ and $CD95^+CD25^{INT}$ $CD4^+$ memory T cells (Figure 2.7) [198-200]. However, CCR7 for the most part was co-expressed on the $CD25^{INT}$ subpopulation. Collectively, these results show that the $CD25^{NEG}$ and $CD25^{INT}$ memory cells are stable populations that contain distinct markers associated with known memory subsets.

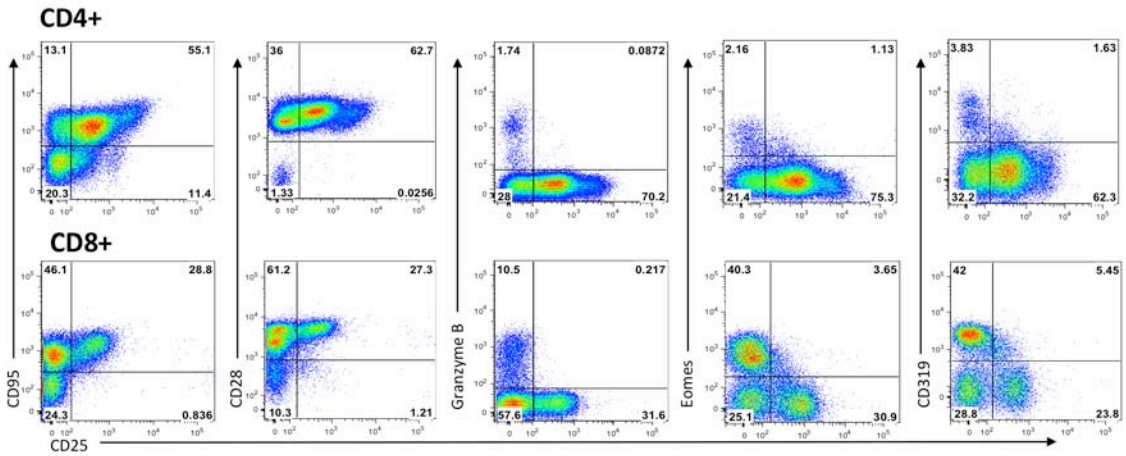


Figure 2.6: CD4^{+} and CD8^{+} cells have similar expression pattern of CD25 in relation to differentiation markers. Expression of CD25 versus CD95, CD28, granzyme B, EOMES and CD319 on CD3^{+}CD4^{+} and CD3^{+}CD8^{+} PBMCs from the same cancer patient. Plots are representative of two cancer patients.

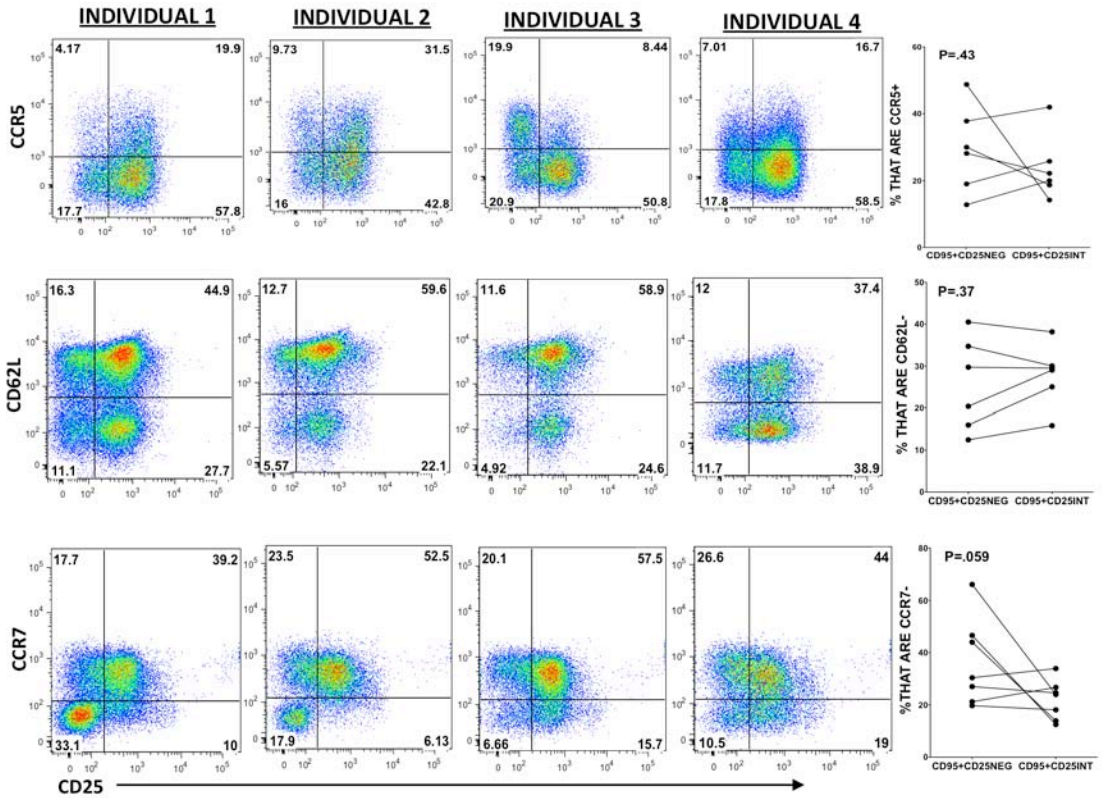


Figure 2.7: No distinct differences in expression of CCR5, CD62L and CCR7. between CD25^{NEG} and CD25^{INT} memory cells. Flow cytometry plots of CD3^{+}CD4^{+}CD95^{+} cell from healthy individuals evaluated for CD25 versus CCR5, CCR7 and CD62L (left) and the percentage of CD95^{POS}CD25^{NEG} and CD95^{POS}CD25^{INT} that are CCR5^{POS}, CCR7^{NEG} and CD62L^{NEG} (right).

CD25^{INT} memory cells are not associated with chronic immune responses

Since late-differentiated memory cells were associated with the CD25^{NEG} but not the CD25^{INT} memory population (Figures 2.5), we hypothesized that CD25^{NEG} memory cells would preferentially respond to antigens associated with chronic infections in humans. To test this hypothesis, we evaluated cytokine responses of memory CD4^{+} T cells after activation with antigens associated with a typical recall memory response (Influenza) and antigens associated with chronic immune responses (HCMV). CD4^{+} T cells stimulated with the superantigen Staphylococcal Enterotoxin B (SEB) served as a positive control for cytokine stimulation. CMV-specific T cells were skewed towards the CD25^{NEG} population when compared to SEB, whereas responses to Influenza were skewed towards the CD25^{INT} population (Figure 2.8A,B).

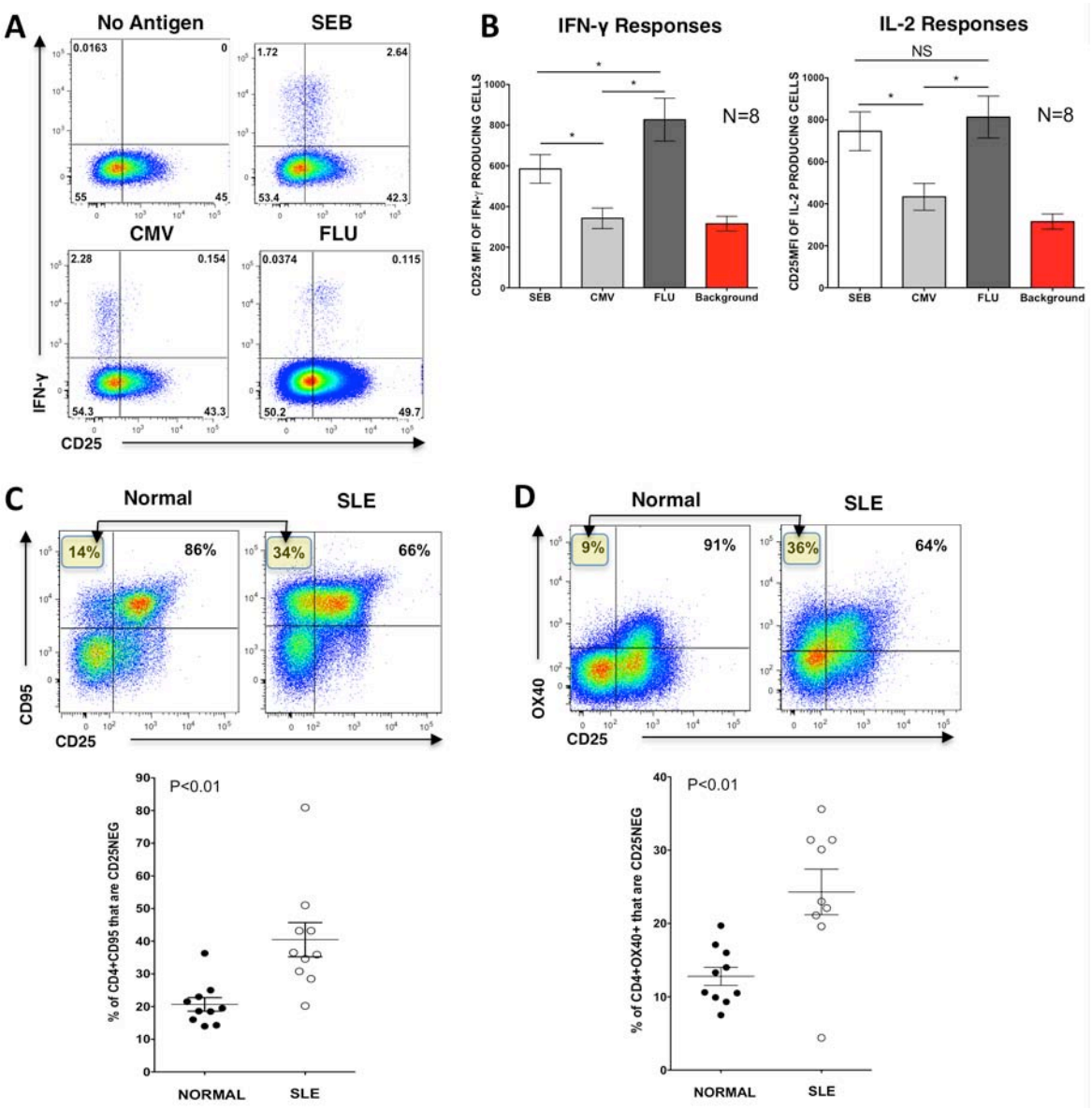


Figure 2.8: CD25^{NEG} memory cells are associated with chronic immune responses. (A) CD3^{+}CD4^{+} PBMCs from a healthy donor were cultured with either SEB (1μg/ml), flu vaccine (3 μg/ml) or CMV lysate (10 μg/ml) in the presence of anti-CD28/49d (5 μg/ml) and Brefeldin A (5 μg/ml) for 18 hours and stained for IFN-γ. Flow cytometry plots are representative of one individual's responses **(B)** CD25 MFI intensity of CD3^{+}CD4^{+} IFN-γ and IL-2 producing cells after stimulation. The bar graphs depict the cumulative mean +/- SD from 8 independent experiments using PBMCs from different healthy individuals. *=P<.05 **(C,D)** Fresh CD3^{+}CD4^{+} PBMCs from sex matched healthy donors and SLE patients were evaluated for the proportion of CD95^{+} **(C)** and OX40^{+} **(D)** that are CD25^{NEG} (N=10). The flow cytometry plots show the data from one healthy individual and one SLE patient. The graphs show the data from all 10 individuals per group; each symbol represents an individual, the wide horizontal bar represent the mean and the error bars represent mean +/-SD. Statistical significance was determined by one-way ANOVA analysis (B) or by unpaired student's t-test (C,D).

The production of cytokines by the $CD25^{NEG}$ memory cells in response to HCMV suggests that they are involved in chronic immune responses. Therefore, we hypothesized that patients with systemic lupus erythematosus (SLE), who suffer from chronic inflammation, would have a greater proportion of $CD4^+$ memory T cells skewed toward the $CD25^{NEG}$ population. We compared $CD4^+$ T cells from SLE patients and gender-matched healthy volunteers using CD95 and CD134 as markers of memory and activation, respectively. As reported by others, we observed a higher percentage of memory ($CD4^+CD95^+$) and activated memory cells ($CD4^+CD134^+$) in SLE patients compared to healthy donors (data not shown) [201, 202]. We also found that the memory/activated cells were skewed toward the $CD25^{NEG}$ compartment in SLE patients compared to normal donors (Figure 2.8C, D). These data suggest that the late-differentiated $CD4^+$ memory T cells are primarily within the $CD25^{NEG}$ memory population, which are expanded in SLE patients.

Functional characterization of $CD25^{INT}$ and $CD25^{NEG}$ memory $CD4^+$ T cells

Next, we wanted to determine whether there were functional differences between $CD95^+CD25^{NEG}$ and $CD95^+CD25^{INT}$ memory cells upon activation with anti-CD3. We observed that sorted $CD95^+CD25^{INT}$ memory cells (Figure 2.4) stimulated with anti-CD3 alone (in the absence of co-stimulation) formed larger clusters of cells compared to sorted $CD95^+CD25^{NEG}$ cells (Figure 2.9A). Expression of CD25 prior to activation may provide the $CD95^+CD25^{INT}$ memory population with an advantage in the absence of added co-stimulation by allowing them to respond to lower levels of IL-2. CD25 is known to be greatly up-regulated on T cells after activation and which would negate any benefit of

CD25 expression prior to activation [20, 42]. However, we found that only the $CD95^{+}CD25^{INT}$ population up-regulated CD25 in response to anti-CD3 alone (Figure 2.9B). Since CD25 up-regulation on activated cells is enhanced by IL-2 signaling [203], we evaluated IL-2 responses by intracellular pSTAT5 levels and found that only the $CD95^{+}CD25^{INT}$ memory population increased pSTAT5 levels (Figure 2.9C). Stimulation in the presence of high concentrations of exogenous IL-2 demonstrated that both populations are capable of up-regulating both CD25 and pSTAT5 levels (Figure 2.9BC).

Next, we tested the function of CD25 expression on the $CD95^{+}CD25^{INT}$ population during activation in the absence of co-stimulation. We found that anti-CD25 blocking antibodies interfered with the ability of $CD25^{INT}$ cells to form aggregates, up-regulate CD25 and phosphorylate STAT5 (Figure 2.9A,B,C). The decrease in CD25 staining was not due to blocking of the anti-CD25 detection antibodies, since the anti-CD25 blocking antibodies do not interfere with the anti-CD25 detection antibody (Figure 2.2C and Figure 2.9C). To further compare differences between $CD95^{+}CD25^{NEG}$ and $CD95^{+}CD25^{INT}$ memory cells and the role of CD25 during activation in the absence of co-stimulation, proliferative responses were determined. When stimulated with anti-CD3 alone, the $CD95^{+}CD25^{INT}$ but not the $CD95^{+}CD25^{NEG}$ cells proliferated robustly (Figure 2.10A). However, blocking CD25 on the $CD95^{+}CD25^{INT}$ cells interfered with their ability to proliferate (Figure 2.10A). Conversely, when stimulated in the presence of anti-CD28 or exogenous rhIL-2, both the $CD95^{+}CD25^{INT}$ and $CD95^{+}CD25^{NEG}$ populations proliferated robustly, demonstrating that the $CD95^{+}CD25^{NEG}$ cells are capable of proliferation (data not shown).

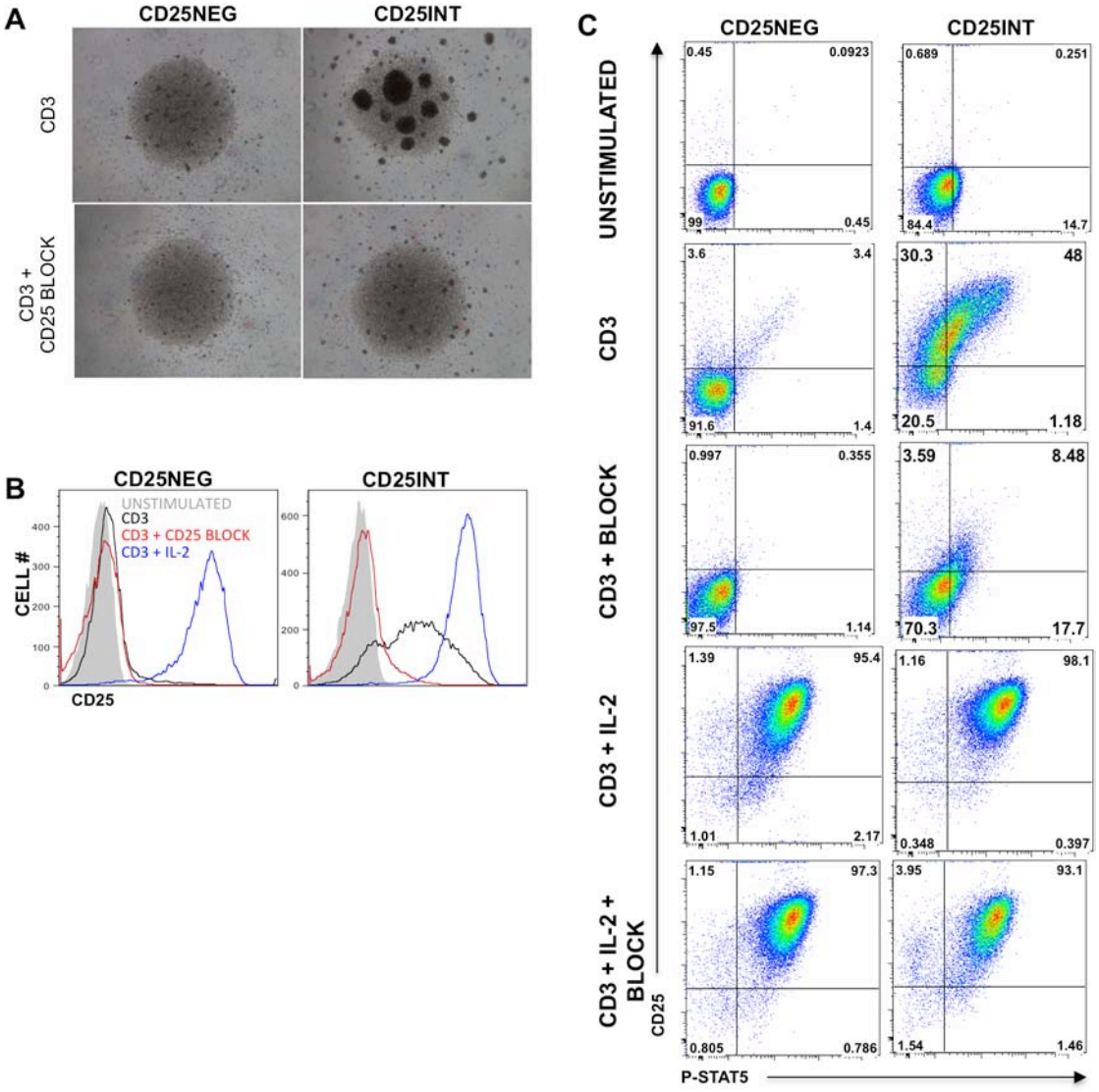


Figure 2.9: CD25^{INT} memory cells have increased expression of CD25 and pSTAT5 in response to anti-CD3 alone compared to CD25^{NEG} memory cells. (A-C) CD95^{+}CD25^{NEG} and CD95^{+}CD25^{INT} cells were sorted from fresh PBMCs s and plated at 50,000 cells/well in triplicate **(A)** Image of sorted cells stimulated with plate bound anti-CD3 in the presence or absence of soluble anti-CD25 for 48 hours. **(B)** CD25 expression on sorted cells 72 hours after culture with or with out anti-CD3 +/- either anti-CD25 blocking antibody or rhIL-2 (5,000 U/ml). **(C)** 72 hours after stimulation as in (B), the cells were stained for CD25 in culture for 20 minutes and then fixed and evaluated for intracellular pSTAT5 by flow cytometry. All images and flow cytometry plots are representative of 3 individual experiments using different healthy individuals performed in triplicate.

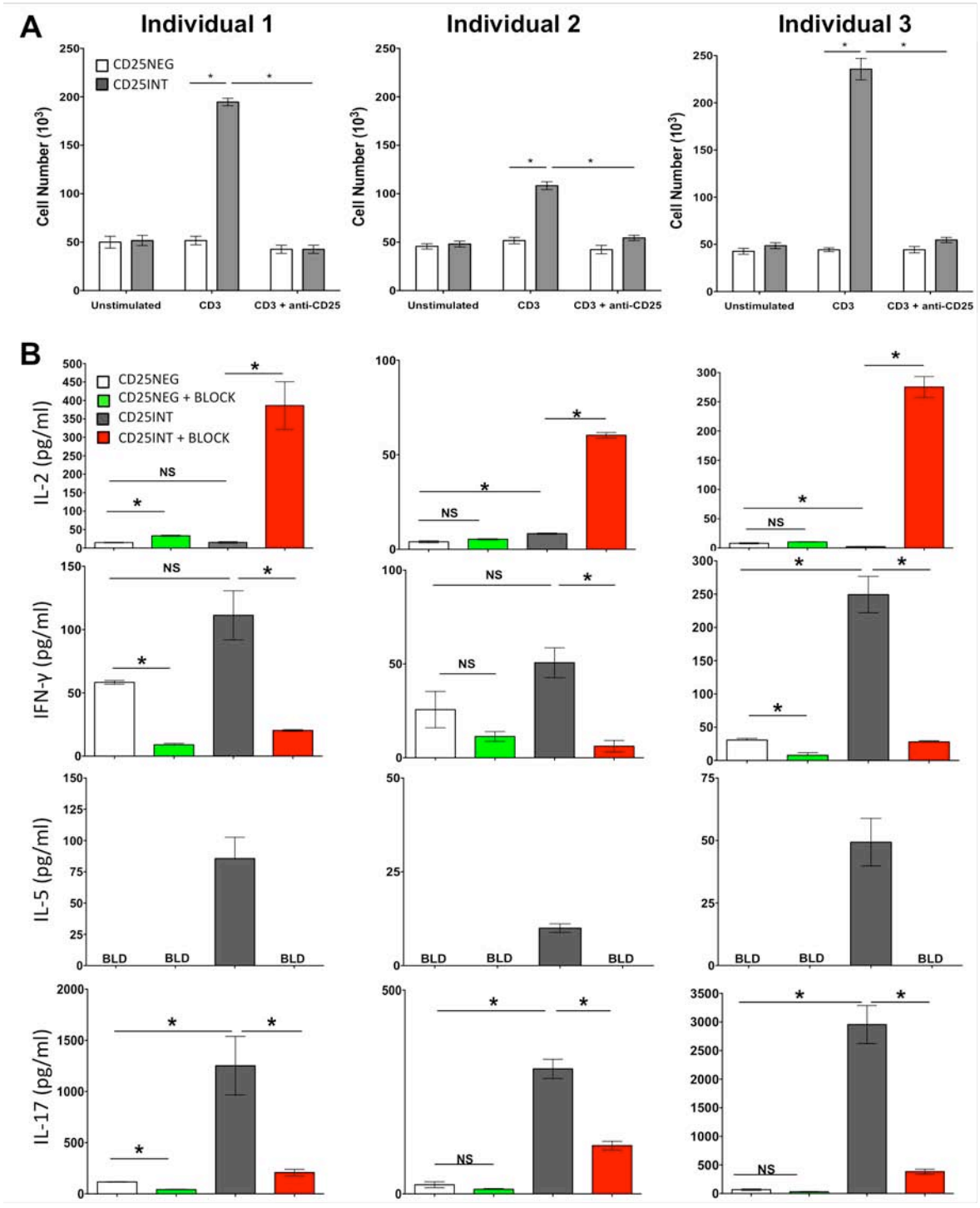


Figure 2.10: CD25^{INT} cells proliferate and produce more cytokines in response to anti-CD3 alone due to their expression of CD25 . (A) Total cell numbers after culture of the sorted cells with or without anti-CD3+/- anti-CD25 blocking antibody for 72 hours and resting for 48 hours without anti-CD3+/-. **(B)** Cytokine concentration in the supernatant of the cultures described in (A) taken 72 hours after stimulation. BLD: below level of detection. NS= not statistically significant. Bar graphs depict the mean of triplicate wells_/ SD for three independent experiments using sorted cells from healthy individuals. Statistical significance was determined by one-way ANOVA analysis. *p<.05.

Lastly, cytokine concentrations determined from supernatant showed that $CD95^{+}CD25^{INT}$ cells produced more cytokines than the $CD95^{+}CD25^{NEG}$ population and that blocking CD25 had a negative impact on these cytokine levels (Figure 2.10B). Interestingly, blocking CD25 on the $CD95^{+}CD25^{INT}$ population increased levels of detectable IL-2. This observation may be explained by a lack of IL-2 internalization and also a lack of negative feedback on IL-2 production. Collectively, this data suggests that $CD95^{+}CD25^{INT}$ cells stimulated in the absence of co-stimulation are able to respond to lower concentrations of IL-2 due to their expression of CD25 prior to activation.

$CD25^{INT}$ memory cells differ in their sensitivity and functional responses to rhIL-2 *in vitro*

Since CD25 expression increased the ability of $CD95^{+}CD25^{INT}$ memory cells to proliferate and produce cytokines (Figure 2.10), we investigated their functional responses to rhIL-2 alone. Cells were sorted from fresh PBMCs (Figure 2.4) and stimulated with various concentrations of rhIL-2 (no anti-CD3). To determine their sensitivity to rhIL-2, cells were analyzed for intracellular pSTAT5 (Figure 2.11A). The majority of cells in the Treg and $CD95^{+}$ memory populations up-regulated pSTAT5 following stimulation with high concentrations of rhIL-2 (1,000 U/ml). However, each population differed in their response to lower concentrations of rhIL-2, showing an expected gradient of decreasing sensitivity to low concentrations of rhIL-2 from Treg cells to $CD95^{+}CD25^{INT}$ to $CD95^{+}CD25^{NEG}$ to naïve cells.

The effect of rhIL-2 on survival was evaluated in sorted populations cultured for 7 days with or without rhIL-2 (Figure 2.11B). We found that the majority of the Treg

populations were dead/dying when cultured alone and that exogenous rhIL-2 rescued the Treg cells from cell death (Figure 2.11B). The $CD95^{+}CD25^{NEG}$ cells were dependent on the addition of exogenous rhIL-2 for cell survival to a lesser extent than the Tregs. In contrast, the $CD95^{+}CD25^{INT}$ cells survived well without exogenous rhIL-2. We also found that compared to the $CD95^{+}CD25^{NEG}$ population, the $CD95^{+}CD25^{INT}$ population was better able to survive when stimulated with anti-CD3 in the absence of co-stimulation and had higher levels of the pro-survival protein BCL-2 *ex vivo* (Data not shown).

Proliferative responses induced by rhIL-2 in the absence of TCR stimulation were evaluated by expression of intracellular Ki67. Co-incubation with increasing concentrations of rhIL-2 induced proliferation by $CD25^{INT}$ cells and to a lesser extent $CD25^{NEG}$ cells (Figure 2.11C). The Treg population did not proliferate in response to increasing concentrations of rhIL-2 alone, which has been reported by others [204]. Since IL-2 is known to regulate CD25 and FOXP3, we examined expression of these proteins in response to rhIL-2 (Figure 2.11D) [203, 205]. Surprisingly, the $CD95^{+}CD25^{NEG}$ population showed no change in CD25 expression, while the Treg population greatly increased CD25 levels. In contrast, the $CD95^{+}CD25^{INT}$ population displayed a bimodal expression of CD25 in response to rhIL-2, with some of the cells increasing and some decreasing expression of CD25. In addition, the Treg cells up-regulated FOXP3 to a greater degree compared to the $CD95^{+}CD25^{NEG}$ and $CD95^{+}CD25^{INT}$ cells. These results were consistent among the three individuals tested. Together, these results show that these distinct populations differ in their sensitivity and functional responses to rhIL-2 *in vitro*.

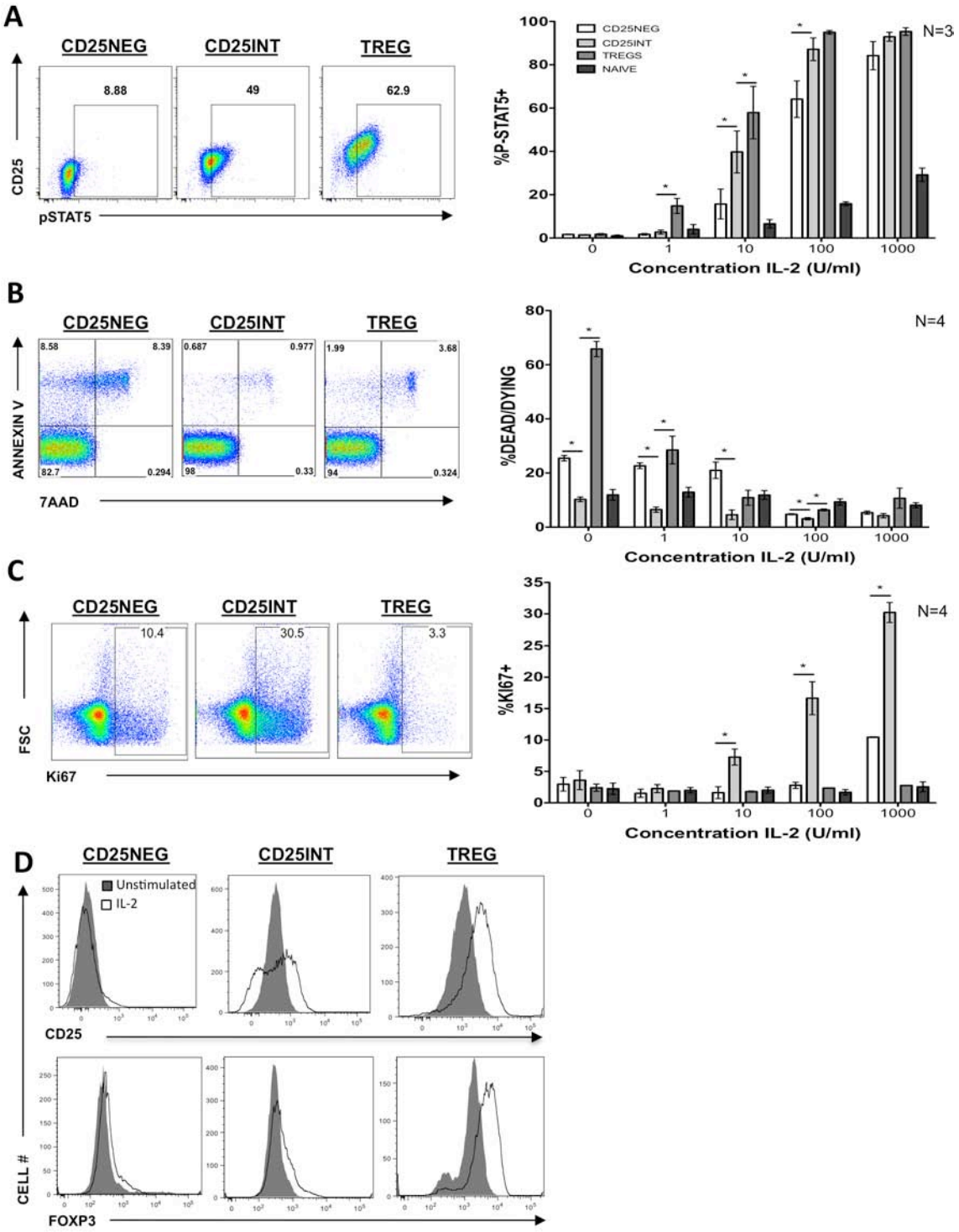


Figure 2.11: Tregs, CD25^{NEG} and CD25^{INT} memory cells differ in sensitivity and functional responses to IL-2. Sorted populations from fresh healthy donor PBMCs were cultured with the indicated **c**oncentrations of rhIL-2 for **(A)** 15 minutes and evaluated for intracellular pSTAT5. (Left) Flow cytometry plots of one of three healthy individuals at 10 U/ml and (Right) bar graphs of the cumulative mean +/- SD from 3 independent experiments using PBMCs from different individuals **(B)** 7 days and evaluated for 7AAD and Annexin V staining. (Left) Flow cytometry plots of one of three healthy individuals at 10 U/ml and (Right) bar graphs of the cumulative mean +/- SD from 4 independent experiments using PBMCs from different individuals or **(C)** intracellular Ki67 levels (Left) Flow cytometry plots of one of three healthy individuals at 1,000 U/ml and (Right) bar graphs of the cumulative mean +/- SD from 4 independent experiments using PBMCs from different individuals. **(D)** Sorted populations cultured for 48 hours with or without rhIL-2 (5,000 U/ml) and evaluated for expression levels of CD25 and FOXP3. The flow cytometry plots show the data are representative of 3 independent experiments using PBMCs from different healthy individuals. The wide horizontal bar represents the mean +/- SD Statistical significance was determined by one-way ANOVA analysis.*=P<.05

IL-2 immunotherapy differentially affects $CD25^{INT}$ $CD4^{+}$ T cells

Based on the differential responses by the $CD25^{INT}$ subset to rhIL-2 *in vitro*, we evaluated CD25 expression on $CD4^{+}$ T cells isolated from cancer patients receiving immunotherapy with high-dose IL-2. Analysis of $CD4^{+}$ T cells before and after the first infusions revealed that the $CD25^{INT}$ population as a percentage of total $CD4^{+}$ T cells, was significantly reduced, while the percentage that were $CD25^{NEG}$ increased (Figure 2.12A,B). The decrease in proportion of $CD25^{INT}$ cells with a concomitant increase of $CD25^{NEG}$ cells was a trend observed in 10 patients (Figure 2.12A,B). In contrast, no significant change was found in the proportion of $FOXP3^{+}$ Treg cells (Figure 2.12B). These changes began within 30 minutes of IL-2 infusion,

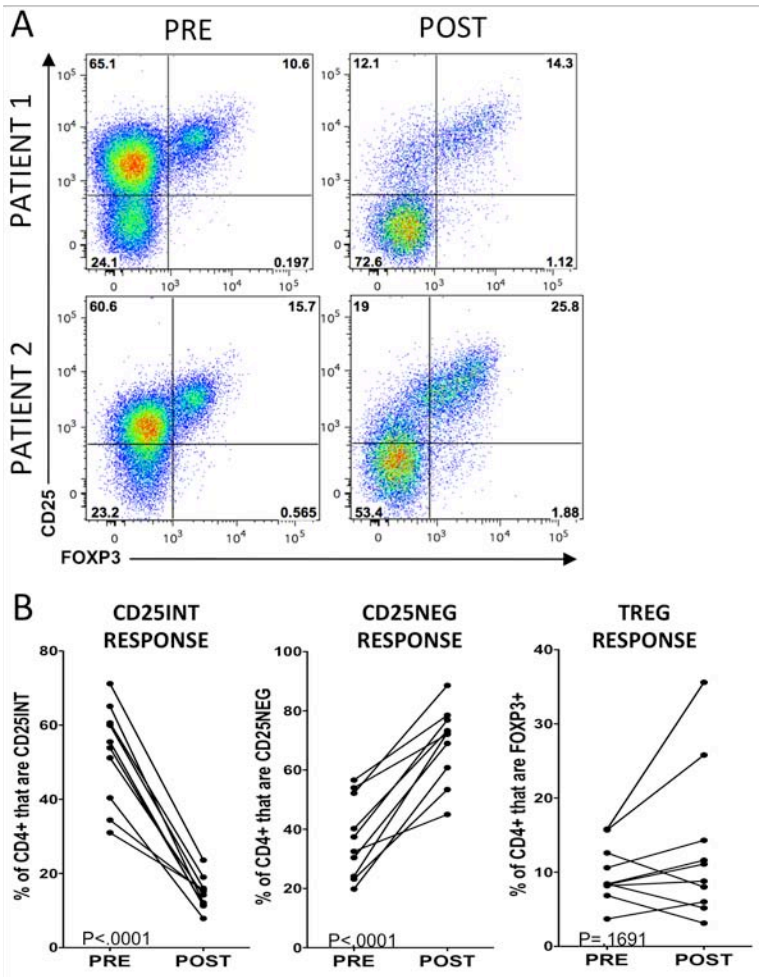


Figure 2.12: $CD25^{INT}$ population is differentially affected by IL-2 treatment in cancer patients. $CD3^{+}CD4^{+}$ T cells from PBMCs collected from cancer patients with metastatic melanoma or renal cell carcinoma over the course of IL-2 immunotherapy and subsequently frozen were analysed. **(A)** Representative flow cytometry plots of $CD3^{+}CD4^{+}$ cells collected from two patients (n=10 in total) immediately before and 18 hours after the start of IL-2 immunotherapy and evaluated for CD25 and FOXP3 expression. **(B)** Cumulative results from all patients (n=10) following analysis as detailed in (A). Each dot are results from an individual before and after the start of treatment. Statisical analysis was determined by student's paired t-test.

suggesting that the effect is due to direct rhIL-2 stimulation and not downstream effects (Figure 2.13).

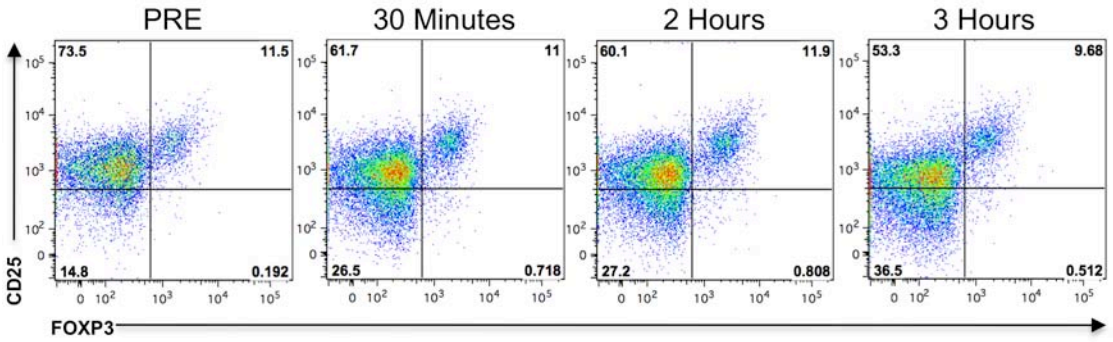


Figure 2.13: Kinetics of CD25^{INT} population's response to IL-2 treatment. CD25 and FOXP3 expression on CD3^{+}CD4^{+} T cells from one patient during the first 3 hours of IL-2 immunotherapy. Flow cytometry plots are representative of one of three experiments using different patients.

Since rhIL-2 binds to CD25, we wanted to confirm that the disappearance of the CD25^{INT} cells was not due to blocking of the anti-CD25 detection antibody by rhIL-2. We noted that pre-incubation with rhIL-2 does not interfere with binding of the CD25 antibody used in these studies (2.14A). Moreover, if rhIL-2 did block the CD25 detection antibody, we would not expect to observe CD25 staining on the Treg cells after

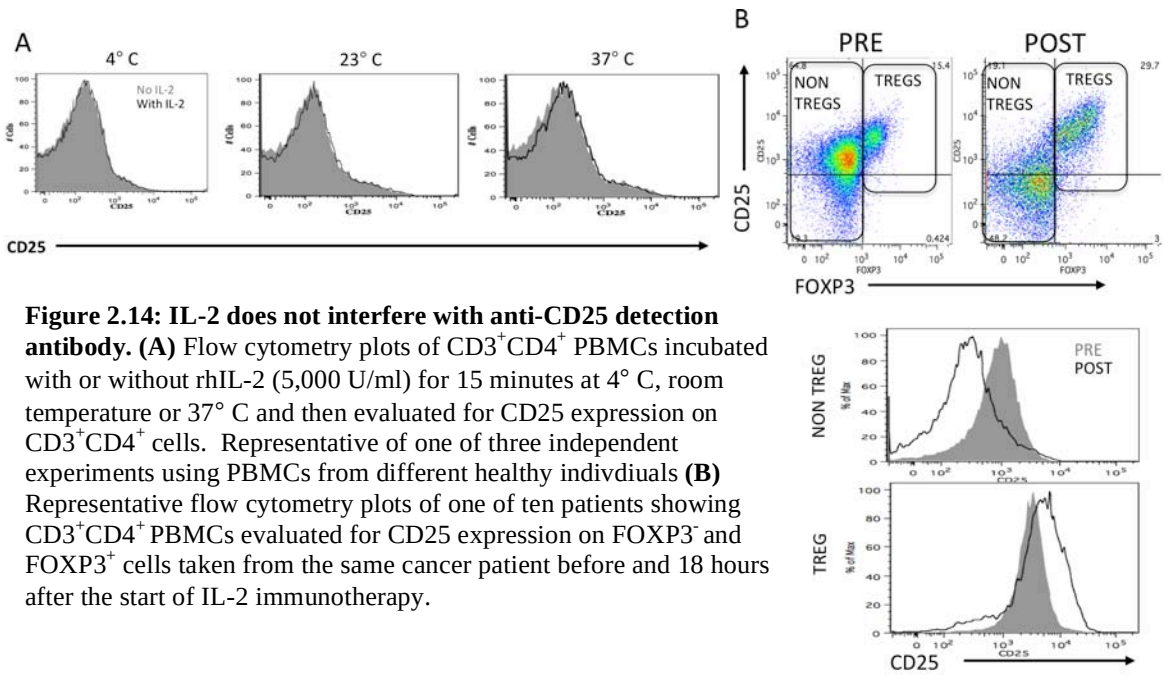


Figure 2.14: IL-2 does not interfere with anti-CD25 detection antibody. (A) Flow cytometry plots of CD3^{+}CD4^{+} PBMCs incubated with or without rhIL-2 (5,000 U/ml) for 15 minutes at 4° C, room temperature or 37° C and then evaluated for CD25 expression on CD3^{+}CD4^{+} cells. Representative of one of three independent experiments using PBMCs from different healthy indivduals **(B)** Representative flow cytometry plots of one of ten patients showing CD3^{+}CD4^{+} PBMCs evaluated for CD25 expression on FOXP3^{-} and FOXP3^{+} cells taken from the same cancer patient before and 18 hours after the start of IL-2 immunotherapy.

IL-2 treatment. Instead, we observed an overall increase in CD25 expression on the Treg cells (2.14B). This is consistent with our previous *in vitro* finding (Figure 2.11D) and was further confirmed with sorted cells (2.15).

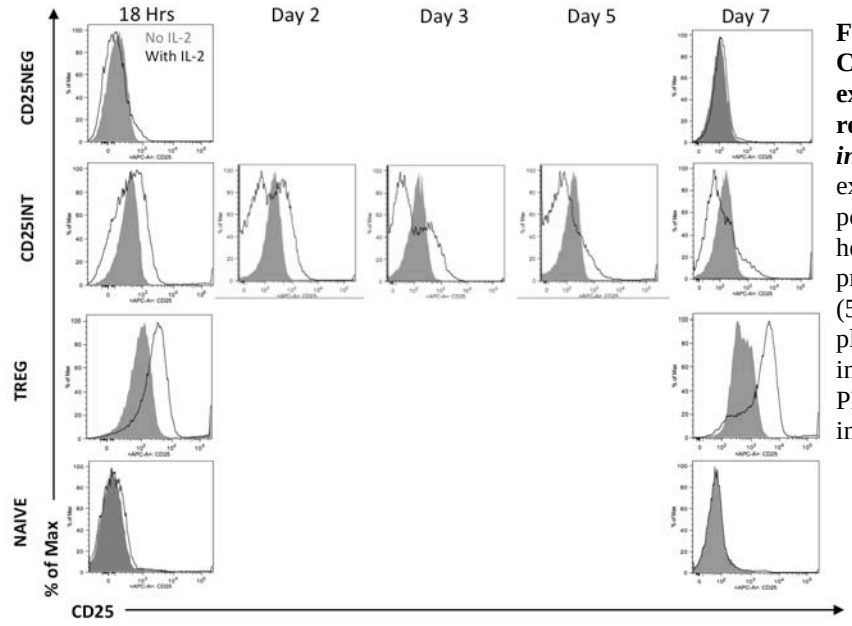


Figure 2.15: Tregs and CD25^{INT} cells differ in their expression of CD25 in response to IL-2 *in vitro* and *in vivo*. Evaluation of CD25 expression over time on sorted populations taken from a healthy donor incubated in the presence or absence of rhIL-2 (5,000 U/ml). Flow cytometry plots are representative of 2 independent experiments using PBMCs from different healthy individuals.

Lastly, we wanted to determine whether IL-2 immunotherapy modulated the CD4^{+} T-cell compartment in a transient or lasting fashion. Therefore, patients were evaluated over time after the start of IL-2 therapy, which was between 4 to 11 days after the final infusion. We observed that within a few days after the last IL-2 infusion, the CD25^{INT} population returned and remained at near pre-treatment levels in 4 individual patients (Figure 2.16). In contrast, the Treg data was not consistent between patients. Taken together, it is apparent that the CD25^{INT} population is differentially affected by IL-2 and could potentially be playing an integral role in anti-tumor immunity in cancer patients undergoing IL-2 immunotherapy.

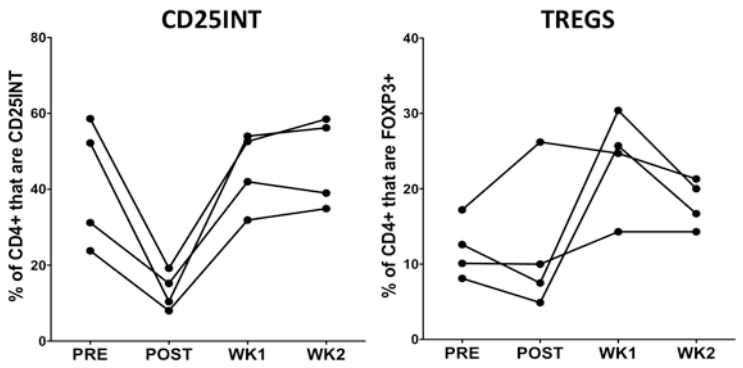


Figure 2.16: CD25^{INT} response to IL-2 treatment is transient. Evaluation of $CD3^{+}CD4^{+}$ T cell subsets from 4 patients' PBMCs before, 18 hours, 1 week and 2 weeks after the start of IL-2 immunotherapy. Each line represents data from one of 4 independent experiments using different patients.

Discussion

Previous studies in mice and humans have shown that CD25 is expressed primarily on resting $FOXP3^+$ Treg cells and transiently on activated T cells. Here we have shown that a large proportion of resting $CD4^+$ T cells in humans express intermediate levels of CD25 and are $FOXP3^-$. We have found no mouse equivalent for this population when staining $CD4^+$ T cells for CD25 and Foxp3 in our mouse colony in either young, old or tumor bearing C57BL/6 male and female mice. In addition, when enriched resting $CD4^+$ cells from mice are stimulated *ex vivo* with low concentrations of IL-2, much fewer cells from mice up-regulated pStat5 compared to human cells (7% vs 40%) (data not shown). Though there have been some reports of variable levels of $CD4^+CD25^+Foxp3^-$ cells in mice under certain inflammatory conditions, it is unclear if these are activated cells that have transiently up-regulated CD25 or represent a resting memory population similar to what we have found in humans [206-209]. Therefore, there may be differences in the expression and role of IL-2/CD25 in cellular immunology between laboratory mice and humans. This notion has been discussed by other groups [67, 188, 189] and is supported by studies of humans who lack functional CD25, and appear to have functional and phenotypic differences when compared to $CD25^{-/-}$ mice. In contrast to mice, CD25 deficiency in humans is accompanied by severe immunodeficiency that is characterized by susceptibility to opportunistic pathogens and a normal Treg frequency [50, 59, 67, 68, 190-192]. In addition, IL-2 deficient mice are fully capable of rejecting allografts, whereas CD25 deficient humans are not [192, 210, 211]. Thus CD25 may play a larger role for effector responses and less of a role for homeostasis of Tregs in humans compared to mice. This may explain why blocking

CD25 during tumor immunotherapies has not translated well from mice to humans [212]. Discrepancies between mouse and human immunology have been described elsewhere and is not unexpected since the species diverged 65-75 million years ago [213]. Therefore, studies conducted in mice on the role of IL-2 in T cell function may not exactly translate to humans, and this study may offer one possible explanation for these differences.

We believe that the discovery of this $CD4^{+}CD25^{INT}$ population is particularly important for therapies that target CD25/IL-2, and that hopefully by studying the response of this population we can better understand the mechanism of these therapies and improve their clinical efficacy. We evaluated the response of the $CD4^{+}CD25^{INT}FOXP3^{NEG}$ population to IL-2 immunotherapy. Over the course of IL-2 immunotherapy in cancer patients, the percentage of $CD4^{+}$ T cells that were $CD25^{INT}$ population decreased, while the $CD25^{NEG}$ increased and Treg populations stayed relatively stable, suggesting these populations were differentially affected by the therapy (Figure 2.12). From these studies, it was clear that the $CD25^{INT}$ population was affected by the IL-2 therapy, however it is currently not known exactly how the $CD25^{INT}$ population responded to the therapy. One possibility is that the $CD25^{INT}$ cells may have down-regulated or shed CD25 [214]. However we did not see diminution of CD25 on the Treg cells, and we demonstrated that not all of the $CD25^{INT}$ population down-regulated expression of CD25 in response to rhIL-2 *in vitro* and that some even increased CD25 expression (Figures 2.13 and 2.14). In addition, *in vitro* stimulation with rhIL-2 also suggested that the $CD25^{INT}$ cells are differentially responsive to rhIL-2, as shown by Ki67 staining, and could therefore be activated to a greater degree than the $CD25^{NEG}$ and

Treg populations (Figure 2.11). Therefore, we believe that the disappearance of the $CD25^{INT}$ population observed in IL-2 cancer patients is most likely a combination of events, including decreased surface expression of CD25 and increased activation, which might have led to AICD and/or egress from the blood to tissue. Nevertheless, it is clear that the $CD25^{INT}$ population is greatly affected by IL-2 immunotherapy and may be integral to the anti-tumor immune response.

In addition to studying the $CD25^{INT}$ population in the context of IL-2/CD25 therapies, these different memory populations also contained phenotypically and functionally distinct subsets. For instance, a higher percentage of the $CD25^{INT}$ population co-expressed OX40, while the $CD25^{NEG}$ population contained the majority of $EOMES^{POS}$, $SLAMF7^{POS}$, $CD28^{NEG}$ and granzyme B^{POS} memory cells (Figure 2.5). $CD28^{NEG}$ and granzyme B^{POS} cells have been shown to be late differentiated memory cells associated with chronic immune responses. Interestingly, all late differentiated $CD28^{NEG}$ and granzyme B^{POS} cells also co-expressed EOMES, which suggests that EOMES could be a master transcription factor for $CD28^{NEG}$/Granzyme B^{+} late-differentiated $CD4^{+}$ T cells. In addition, we found that the $CD25^{NEG}$ memory population contained the majority of CMV reactive cells and were expanded in SLE patients. Collectively, this data suggested that the $CD4^{+}$ memory T cells associated with chronic immune responses are $CD25^{NEG}$.

In summary, we report in this study that a large percentage of memory $CD4^{+}$ T cells in humans express intermediate levels of CD25. CD25 expression on the $CD25^{INT}$ memory population appears to be important biologically and that the $CD25^{INT}$ population is greatly affected by IL-2 immunotherapy in cancer patients. These findings not only

improve our understanding of the role of CD25 in human immunology, but may also have clinical implications by helping to illuminate the mechanisms and potentially improve the efficacy of therapies that target IL-2 and CD25.

Chapter 3

Combining anti-OX40 with an inhibitor of TGF-β overcomes the immunosuppressive environment in established tumors

Abstract

Single agent cancer immunotherapies are often successful in mouse tumor models at treating small tumors but are often ineffective once the tumors become larger. Therefore, tumor immunotherapy may require more than one strategy to successfully treat large tumors. In this study, we show that combining αOX40 antibodies with an inhibitor of TGF-β (SM16) synergizes to elicit complete regression of large established tumors, resulting in long-term survival in ~85% of the αOX40/SM16 treated mice compared to ~15% with SM16 alone and 0% with αOX40 alone. Evaluation of tumor infiltrating T cells showed that SM16/αOX40 dual therapy resulted in an increase in proliferating granzyme B expressing $CD8^+$ T cells which were better able to produce IFNγ. Using OX40-YFP reporter mice, we also found an increase in $CD8^+YFP^+$ T cells in the tumors of SM16 and SM16/αOX40 treated mice. Collectively, these results show that combining αOX40 with TGF-β inhibitors is a promising therapy for future treatment of cancer patients.

Introduction

OX40 (CD134, TNFRSF4) is a member of the tumor necrosis factor receptor (TNFR) superfamily expressed on activated $CD4^+$ and $CD8^+$ T cells [153-155]. Engagement of OX40 with cognate ligand or soluble agonist enhances T cell proliferation, survival and cytokine production by memory T cells [155]. In addition to OX40 being an activation antigen on T cells, regulatory T cells (Tregs) constitutively express OX40 in mice. Under certain conditions, treatment with OX40 agonist antibodies (αOX40) inhibits Treg development and function while enhancing effector cell function when co-cultured with Tregs [164-167, 215]. Importantly, a murine anti-human OX40 antibody has recently completed a phase I clinical trial for the treatment of cancer patients with encouraging results. Currently, humanized OX40 agonists are being developed to conduct future trials in cancer patients.

Although αOX40 therapy has been effective at controlling small tumors (0-50 mm^2) in a variety of mouse tumor models [154], it has been less effective at eradicating large established tumors (>50 mm^2) [170-172, 175]. A potential explanation for this deficiency is that the microenvironments of these larger tumors may become immunesuppressive, which may more accurately reflect tumors observed in late-stage cancer patients [176, 177]. Therefore, it is conceivable that a strategy that blocks immunosuppression will improve the efficacy of αOX40 immunotherapy especially against large established tumors.

TGF-β is an immune suppressing cytokine that is produced by a variety of tumor types [124-128] as well as by immune cells found within the tumor microenvironment [129-131] and it has been shown that elevated levels of TGF-β are associated with tumor

progression [126-128, 132-134]. TGF-β has many pro-tumor functions that act directly on tumor cells [131] as well as by inhibiting the anti-tumor immune responses via its direct actions on immune cells, including monocytes, dendritic cells, NK cells and T cells [130, 131]. These actions include inhibition of antigen presentation, T cell differentiation, cytotoxic activity of CD8 T cells, and Th1 cytokine production by CD4 T cells [130, 131, 135, 136]. In addition to suppression of effector T cells, TGF-β signaling in peripheral Tregs has also been shown to be important for their expansion, survival and suppressive function *in vivo* [139-141].

Collectively, the previously discussed studies provide a rationale for blocking TGF-β to enhance an anti-tumor immune response. This can been achieved by using neutralizing antibodies [142], fusion proteins [143] and small molecule TGF-β signaling inhibitors [144] all of which have resulted in suppression of primary tumor growth and metastasis in several mouse tumor models. However, unlike antibodies and fusion proteins, whose ability to effectively restore immune function depends on complete neutralization of circulating TGF-β, small molecule TGF-β inhibitors, which interfere with TGF-β receptor signaling, allow immune cells to regain function in TGF-β-rich environments. Of the small molecule inhibitors, SM16 is orally bioavailable and has demonstrated anti-tumor efficacy when administered in the diet [148]. SM16 binds to the kinase active site of TGF-β type I receptor (ALK5), thus inhibiting phosphorylation of SMAD proteins and downstream signal transduction [145]. We hypothesized that using an agonist to boost effector T cell function (αOX40) in conjunction with an inhibitor of T cell suppression (SM16) would provide the immune system with ability to eradicate large established tumors. Indeed this combination has recently been shown to be effective in

eradicating tumors via an immune-dependent mechanism in the 4T1 breast carcinoma model [216]. Therefore, we wanted to know if this combination would work to cure large-established tumors in other tumor models.

Our data demonstrate that SM16 plus αOX40 administration stimulates a vigorous anti-tumor response that results in complete and durable tumor regression in ~85% of mice bearing large (50-120 mm^2) well-established MCA205 tumors that resulted in resistance to tumor re-challenge. Examination of the tumors revealed an increase in tumor infiltrating T cells (TIL) in mice receiving αOX40 + SM16 therapy that were phenotypically and functionally distinct from T cells found in tumors of the other treatment groups. The combination therapy increased the frequency of TIL expressing OX40, IFNγ producing $CD8^+$ T cells, and proliferating $CD8^+$granzyme B^+ T cells. Collectively, these results demonstrate that combining OX40 agonists with an orally available inhibitor of TGF-β signaling can overcome the immunosuppressive environment in large established tumors resulting in tumor regression and long-lived anti-tumor immune response. Therefore, combining SM16 and αOX40 could be a potentially promising approach for future treatment of cancer patients.

Materials and Methods

Mice

Six to eight week old C57BL/6 female mice were purchased from Jackson Laboratory (Bar Harbor, ME). C57BL/6 OX40-Cre mice were kindly provided by Dr. Nigel Killeen (UCSF, San Francisco, CA) and were crossed with mice from Jackson labs carrying the Rosa26-loxP-STOP-loxP-YFP allele [217]. In the double transgenic OX40-Cre/Rosa-YFP mice, cells that express OX40 driven cre protein will knock out a floxed stop cassette that is juxtaposed between a promoter that is constitutively active in lymphocytes and a gene encoding YFP [218]. All mice were maintained under specific pathogen-free conditions in the Providence Portland Medical Center animal facility in accordance with the Principles of Animal Care (NIH publication no.85-23, revised 1985). All studies were reviewed and approved by the institutional animal care and use committee (IACUC) of the Earle A. Chiles Research Institute.

Tumor Cell Line

MCA205 sarcoma cells were propagated *in vitro* using (complete media) RPMI (Lonza, Walkersville, MD) containing 0.292 ng/ml glutamine (Hyclone Laboratories, Logan, UT), 100 U/ml streptomycin (Hyclone), 100 U/ml penicillin (Hyclone), 1X non-essential amino acids (Lonza BioWhittaker), 1mM sodium pyruvate (Lonza BioWhittaker) and 10mM HEPES (Sigma). The MCA205 cell line was routinely tested and shown to be free of mycoplasma contamination.

Reagents

The following antibodies were used for analysis by flow cytometry: CD4 Pacific Blue or PerCP-Cy5.5 (ebioscience, GK1.5), CD8 PerCP-Cy5.5 (ebioscience, 53-6.7), OX40 PE (Biolegend, OX-86), CD25 APC (ebioscience, PC61.5), Ki67 FITC (BD, 35), Foxp3 Pacific Blue (ebioscience, FJK-16s), Granzyme B PE (Invitrogen, GB12) and IFNγ APC (ebioscience, 4S.B3). Live cells were identified using the eFluor® 780 viability dye (ebioscience). The OX40 agonists are rat anti-mouse OX40 antibody (clone 86). Rat IgG1 (sigma) antibodies were used as isotype controls. SM16 was synthesized by Biogen Idec (Cambridge, MA) and was incorporated into standard Purina rodent chow (#5001) by Research Diets (New Brunsick, NJ) at a concentration of 0.3 g SM16 per kg of chow (0.03%). A calorie and nutrient-matched diet without SM16 (Purina) was used as the control diet. Delivery of SM16 in chow avoids the impracticality of oral delivery in capsule or pill form in mouse models yet has translational potential as the drug can be made orally bioavailable to humans in pill form.

Flow cytometry

Cells (1-5 million) in 100 μl of flow wash buffer (FWB, PBS with 0.5% FBS) were incubated with the appropriate antibodies at 4°C for 30 minutes. For intracellular staining, cells were fixed and permeabilized with the Foxp3 Staining Buffer Set (eBioscience) according to the manufacturer's protocol. Intracellular staining was done at 4°C for 30 minutes with the appropriate antibodies to detect specific intracellular proteins. Cells were analyzed using an LSR II flow cytometer (BD) and FlowJo4 software.

In vivo tumor growth and survival experiments

For primary tumor challenge, mice were injected subcutaneously (s.c.) in the right flank with 4 x 10^5 MCA205 tumor cells. Eleven days (~50-120 mm^2) post-tumor inoculation, mice were randomized into the following treatment groups: control diet + rat IgG1 (isotype control for αOX40), control diet + αOX40 (OX86), SM16 diet (0.03% SM16) + rat IgG1 and SM16 + αOX40. Mice were then given intraperitoneal (i.p.) injections of 250 μg of either αOX40 or rat IgG1 on days 2 and 6 after starting diet. Mice were taken off the control and SM16 diets after 2 weeks and monitored for tumor growth 2-3 times a week by measuring tumor length (L) and width (W) to determine tumor size (LxW, mm^2). All mice were sacrificed when the tumors either ulcerated or reached >150 mm^2. For re-challenge experiments, mice were injected s.c. with 4 x 10^5 MCA205 tumor cells in the right flank and 4 x 10^5 3LL cells into the left flank and were examined for tumor growth in the absence of any additional therapy.

Phenotyping of TIL and splenocytes

Mice were injected with 4 x 10^5 MCA205 cells s.c.. Once tumors were established for 11 days (~50-120 mm^2), mice were put on SM16 or control diet and administered 250 μg of either αOX40 or Rat IgG1 on days 2 and 6 after starting diet. Seven days after starting feed, tumor and spleens were harvested. Resected tumors were minced with scissors and disaggregated using triple enzyme digest cocktail containing 10 mg/ml of collagenase type IV (Worthington Biochemical Corp. Lakewood, NJ), 1 mg/ml hyaluronidase (Sigma-Aldrich, St. Louis, MO), 200 μg/ml DNAse I (Roche Applied Sciences, Indianapolis, IN) in complete media for 30 minutes with agitation at 37° C

using a shaker. The tumor digest was then passed through 70-µm nylon filter, subjected to red blood cell lysis using ACK lysing buffer (Lonza) and then washed and used for analysis. Spleen cells were minced between slides, subjected to red blood cell lysis and passed through 70-µm nylon filter, washed and used for analysis. Cells were then incubated with fluorescently labeled antibodies and analyzed by flow cytometry.

In vitro stimulation of isolated immune cells

Half of tumor digest or 1 x 10^7 spleen cells were re-suspended in 1 ml of complete media and simulated for 1 hour at 37° C with 1X PMA/ionomycin cocktail (eBioscience) in 48 well plates. Brefeldin A (Biolegend) was added for a final concentration of 5 µg/ml and incubated with the cells for an additional 4 hours at 37° C. Cells were then stained for surface markers, then fixed and permeabilized and evaluated for intracellular IFNγ as described above.

Sorting Experiments

OX40-CRE/ROSA-YFP mice were injected with 4 x 10^5 MCA205 into left and right flanks. After tumors established for 11 days (50-120 mm^2), mice were put on control or SM16 diet and given injections of αOX40 or Rat IgG antibodies on days 2 and 6 after starting diet. Tumors were resected after 7 days of SM16 or control diet. Tumor infiltrating cells were isolated as described above and stained for surface CD4 and CD8. Cells from tumor were sorted using a FACSAria II (BD)bv into the following populations: $CD4^+YFP^-$, $CD4^+YFP^+$, $CD8^+YFP^-$ and $CD8^+YFP^+$.

Results

αOX40/SM16 dual therapy cures mice with large established tumors

αOX40 therapy has been shown to cure mice in several different mouse tumor models if administered shortly after tumor challenge when tumors are either small or not yet palpable (Figure 3.1A) (0-50 mm^2). However, αOX40 has been less effective at curing large established tumors (>50 mm^2), either having no impact or only delaying tumor growth with no increase in long-term survival (Figure 3.1B) [170-172, 175]. Examination of the tumor microenvironment after treatment revealed that the αOX40 antibody was able to bind to T cells isolated from large tumors (data not shown). Yet, despite being able to penetrate large tumors and bind to infiltrating lymphocytes, it was unable to initiate a strong anti-tumor immune response. This inability of αOX40 to activate an effective anti-tumor T cell response may be due to immune suppressing signals present in large tumors [219].

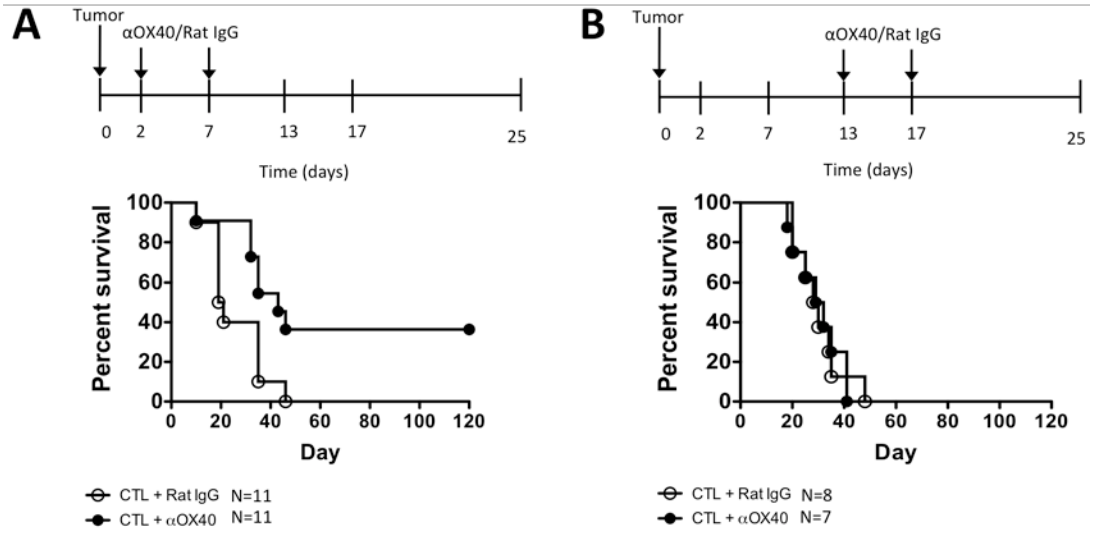


Figure 3.1: αOX40 treatment is ineffective at curing mice of large established tumors. Mice were inoculated with 4 x 10^5 MCA205 cells into the right flank and given 250μg of either αOX40 or isotype control antibodies on **(A)** days 2 and 7 or **(B)** days 13 and 17. Survival data is representative of 2 independent experiments with 7 mice per group.

Therefore, we hypothesized that using OX40 agonist antibodies in conjunction with a small molecule inhibitor of the TGF-β receptor (SM16) would initiate a potent anti-tumor immune response with the potential to cure mice harboring large tumors. To test this, MCA-205 sarcoma cells were implanted into mice and allowed to grow until the tumors were ~50-120 mm^2 (11 days). The mice were then given SM16 or control feed for 14 days, during which they were treated with either rat IgG control or αOX40 agonist antibodies as depicted in Figure 3.2A. When evaluating tumor growth, αOX40 alone had no noticeable effect on these established tumors (Figure 3.2A, B). However, when given in conjunction with SM16, αOX40 was able to cause complete regression of large tumors leading to long-term tumor-free status (Figure 3.2A, B). These cures led to complete and durable responses in ~80% of the mice treated with the SM16/αOX40 dual therapy compared to ~15% of mice treated with SM16 alone, and 0% of the mice treated with αOX40 alone or rat IgG (Figure 3.2A). Therefore, an αOX40 agonist in conjunction with a TGF-β signaling inhibitor (SM16) provided a therapeutic immune boosting combination that caused regression of large established tumors.

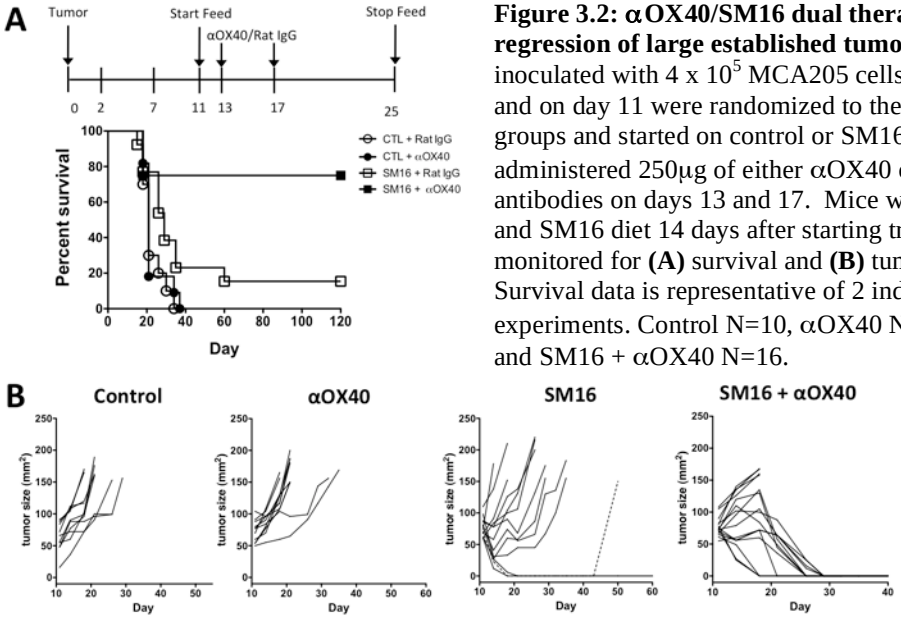


Figure 3.2: αOX40/SM16 dual therapy causes regression of large established tumors. Mice were inoculated with 4 x 10^5 MCA205 cells into the right flank and on day 11 were randomized to the various treatment groups and started on control or SM16 diet and administered 250μg of either αOX40 or isotype control antibodies on days 13 and 17. Mice were taken off control and SM16 diet 14 days after starting treatment and monitored for **(A)** survival and **(B)** tumor growth. Survival data is representative of 2 independent experiments. Control N=10, αOX40 N=11, SM16 N=13 and SM16 + αOX40 N=16.

SM16/αOX40 therapy increases the frequency of $CD4^+$ and $CD8^+$ T cells in the tumor

To understand how SM16/αOX40 mediates tumor regression, tumors were evaluated for tumor infiltrating lymphocytes (TIL) in the tumor microenvironment (TME). For all phenotyping of the TME, mice with large tumors were put on diet, administered 2 injections of control or αOX40 antibodies and sacrificed 7 days after starting diet (Figure 3.3A). Tumors were then removed and subjected to triple-enzyme digest without density gradient centrifugation so as not to enrich for immune cells and thus evaluate the proportion of total cells within the tumor digest that were $CD4^+$ or $CD8^+$ T cells. Using the gating strategy shown in figure 3.3B, we found an increase in the proportion of cells that were $CD4^+$ and $CD8^+$ in the SM16 and SM16/αOX40 treated mice compared to the control and αOX40 treated mice (Figure 3.3C). Interestingly, no increase in proportion of $CD4^+$ and $CD8^+$ T cells was found in the spleens, therefore our future phenotyping focused on T cells within the tumor. The increase of T cells within the tumors suggested that the mechanism of tumor regression in the SM16 and SM16/αOX40 treated mice was immune-mediated. However, TGFβ is known to have pro survival effects directly on tumors, which are independent of immune suppression [131]. Therefore, to confirm the role of lymphocytes in SM16 mediated anti-tumor responses, we performed therapy experiments in $RAG^{-/-}$ mice. As shown in figure 3.3D, SM16 was unable to control the outgrowth of large tumors in the absence of lymphocytes.

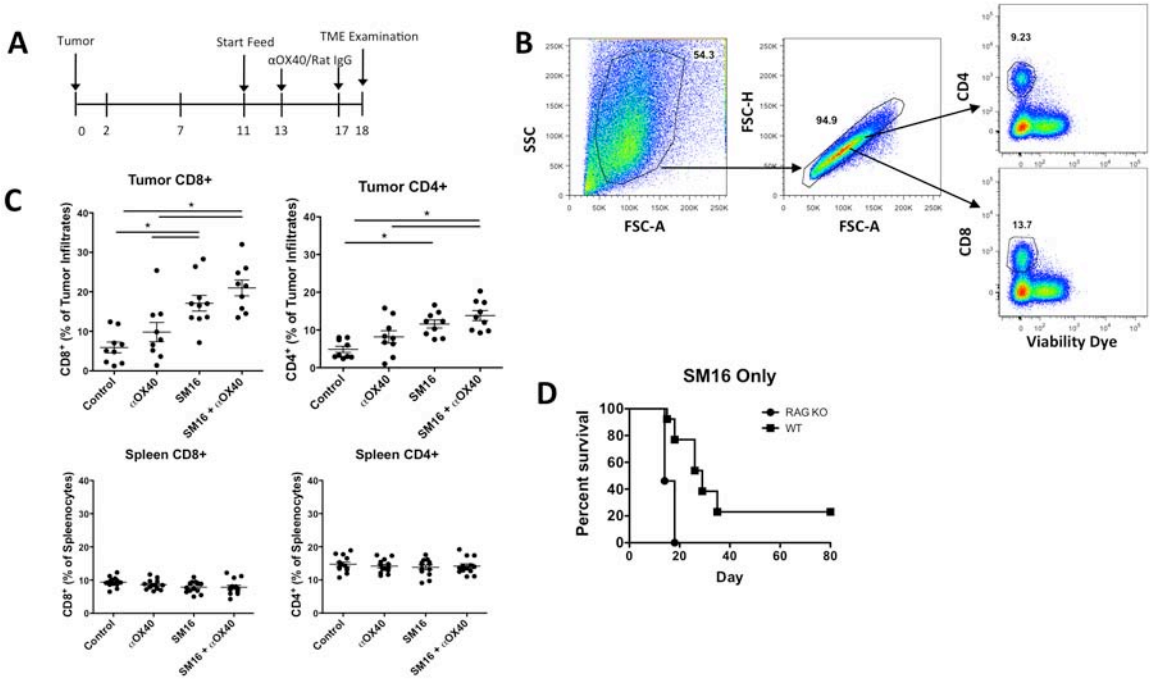


Figure 3.3: αOX40/SM16 dual therapy increases the frequency of CD4$^+$ and CD8$^+$ TIL. Mice were inoculated with 4 x 10^5 MCA205 cells into the right flank and on day 11 were randomized to the various treatment groups and started on control or SM16 diet and administered 250μg of either αOX40 or isotype control antibodies on days 13 and 17. 7 days after starting SM16/control diet evaluation of the tumor for infiltrating lymphocytes was determined. **(A)** Schematic representation of treatment protocol for evaluation of the tumor microenvironment. **(B)** Gating strategy for evaluating tumor infiltrating CD4$^+$ and CD8$^+$ T cells. **(C)** Proportion of tumor digest and splenocytes that were CD4$^+$ or CD8$^+$ from 3 independent experiments with each dot representing data from an individual mouse, the wide horizontal bar represents the mean +/- SD. Statistical significance was determined by one-way ANOVA analysis. *=P<.05. **(D)** WT and RAG$^{-/-}$ mice were treated as in figure 3.2 and evaluated for tumor growth. (N=13/group).

SM16/αOX40 combination therapy changes the phenotype and effector function of TIL

We further evaluated the tumor infiltrating T cells to determine if there was simply an increase of T cells in the tumors or if these T cells were phenotypically and functionally different between the treatment groups. Since IFNγ is a known beneficial anti-tumor cytokine [78, 85, 86], we evaluated the ability of the tumor infiltrating T cells from the treated mice to produce IFNγ *ex vivo*. When stimulated with PMA/Ionomycin, we found an increase in the frequency of CD4$^+$ and CD8$^+$ TIL capable of producing IFNγ from mice treated with SM16/αOX40 (Figure 3.4A).

In addition to IFNγ, the perforin/granzyme B cytolytic pathway has been shown to be important for effective anti-tumor immune responses [47, 88, 220, 221]. Therefore,

we tested the hypothesis that SM16/αOX40 would increase the proportion of tumor-isolated $CD8^+$ T cells that expressed granzyme B. When evaluating the TIL for granzyme B expression, we found an increase in the proportion of $CD8^+$ that were granzyme B^+ in the tumors of SM16/αOX40 treated mice compared to tumors from mice

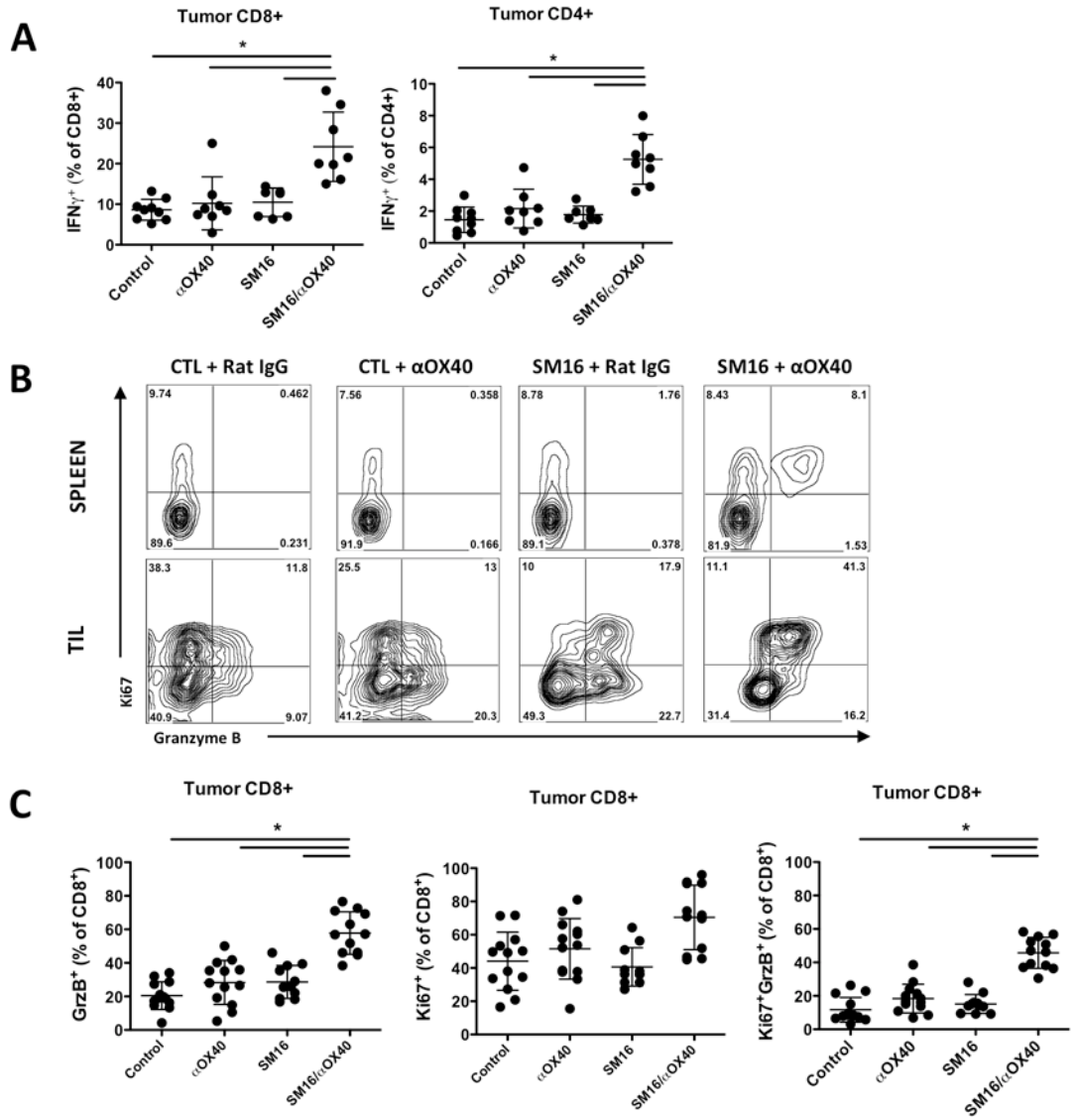


Figure 3.4: Tumor regression is associated with an increase in proliferating $CD8^+GrzB^+$ T cells. Mice were treated as in figure 3.3. **(A)** Tumor digests were stimulated for 1 hour with PMA/Ionomycin (1x), after which brefeldin A (5 μg/ml) was added for an additional 4 hours. $CD4^+$ and $CD8^+$ cells were then evaluated for intracellular IFNγ. Graphs show the proportion of $CD4^+$ and $CD8^+$ cells that were $IFN\gamma^+$ from 3 independent experiments. **(B)** Representative FACs plots of $CD8^+$ cells from the spleen and tumor for expression of Ki67 and granzyme B. **(C)** Proportion of $CD8^+$ cells from the tumor that were granzyme B^+, $Ki67^+$ or granzyme B^+Ki67^+. Results are from 3 independent experiments, the wide horizontal bar represents the mean +/- SD. Each dot on all graphs represents results from individual mice. Statistical significance for all graphs was determined by one-way ANOVA analysis. *=P<.05

in the other treatment groups (Figure 3.4B,C). To further characterize these cells, we determined the proportion of $CD8^+$ cells that were actively undergoing proliferation by Ki67 expression. Though we found no difference in the proportion of $CD8^+$ cells proliferating in the tumors of SM16/αOX40 treated mice, there was a significant increase in proliferating $CD8^+$granzyme B^+ cells (Figure 3.4C). Together, this data shows that SM16/αOX40 treatment led to an increase in $CD8^+$ cells that functionally and phenotypically resemble effector cytolytic cells compared to the $CD8^+$ T cells found in the tumors of mice from the other treatment groups.

SM16/αOX40 combination therapy does not affect the proportion of TIL that are $Foxp3^+$

Previous studies have shown that TGF-β signaling can promote Treg proliferation and survival [139-141]. Therefore, one would expect to see a decrease in the frequency of Tregs when TGFβ signaling is blocked. However, we found that in the mice from the SM16 treated groups there was an increase in the proportion of $CD4^+$ cells that were $Foxp3^+$ in the spleen and no statistical difference in the tumor (Figure 3.5). Also, we found there was an increase in the proportion of Tregs undergoing proliferation in the spleens of SM16/∝OX40 treated mice (data not shown). This expansion of Tregs in the absence of TGFβ signaling has also been observed in other tumor models [129, 216].

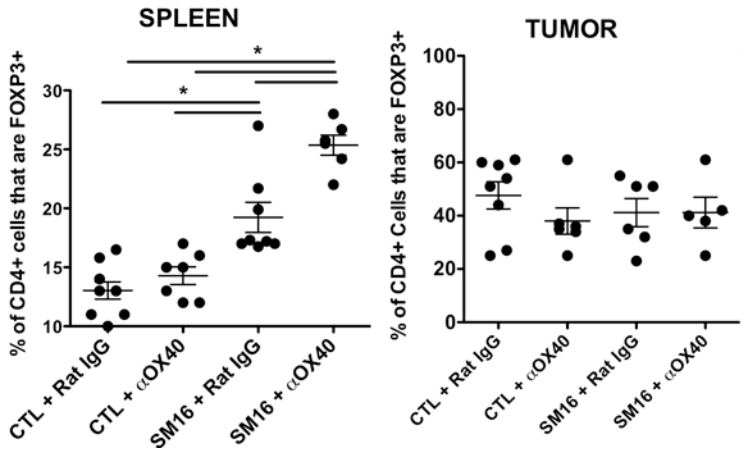


Figure 3.5: No change in frequency of Tregs in the tumors of αOX40/SM16 treated mice. Mice were treated as in Figure 3.3 and the proportion of $CD4^+$ cells that were $FOXP3^+$ from the spleen and tumors were evaluated. Graphs show the results from 2 independent experiments each symbol represents results from an individual mouse, the wide horizontal bar represents the mean +/- SD. Statistical significance for all graphs were determined by one-way ANOVA analysis.

SM16/αOX40 combination therapy increases the proportion of OX40 expressing TIL

OX40 expressing tumor infiltrating T cells are most likely the targets for αOX40 therapy. Therefore, we wanted to determine if there was an increase in the proportion of T cells that express OX40 in the SM16 and SM16/αOX40 treated mice. However, since our anti-OX40 detection antibody is the same clone as the therapeutic antibody, our alternative strategy was to use $OX40^{Cre}/ROSA^{YFP}$ reporter mice to detect OX40 expression by T cells [217]. These reporter mice have several advantages over using detection antibodies. As opposed to using detection antibodies, which gives you only a snapshot of OX40 expression by TIL, these mice allow one to detect cells that currently express OX40 or had previously expressed OX40. In addition, OX40 expression on $CD8^+$ TIL is low and therefore difficult to detect using antibodies, especially after enzymatic digestion. Using these mice, we found an increase in the proportion of $CD8^+$ TIL that were YFP^+ in the SM16 and SM16/αOX40 group (Figure 3.6A,B). There was no difference in the proportion of tumor infiltrating $CD4^+$ that were YFP^+ since the

majority of them were YFP^+ in all groups (data not shown). This increase in OX40 expressing $CD8^+$ T cells in the tumor is most likely relevant to the mechanism of tumor destruction since previous work has shown that direct engagement of OX40 by $CD8^+$ cells is necessary to overcome anergy and initiate an αOX40 driven anti-tumor immune response [170, 172, 173].

Since there was an increase in the proportion of $CD8^+$ cells that were YFP^+ in the SM16/ αOX40 treated mice, we wanted to address whether the $CD8^+YFP^+$ cells were functionally distinct compared to the $CD8^+YFP^-$ cells. Therefore, cells were sorted from pooled tumors from multiple mice in order to evaluate expression of intracellular proteins. When evaluating the sorted cells from tumors, a greater proportion of the $CD8^+YFP^+$ cells expressed granzyme B compared to the $CD8^+YFP^-$ cells in all of the treatment groups (Figure 3.6C). Next, we evaluated the ability of the sorted cells to produce IFNγ from the different treatment groups. In all of the treatment groups, a greater percentage of $CD8^+YFP^+$ cells isolated from the tumor were able to produce IFNγ compared to the $CD8^+YFP^-$ cells (Figure 3.6D). Taken together, the SM16 treated groups had an increased percentage of YFP^+ (OX40 expressing) cells within the tumor, and these $CD8^+YFP^+$ cells expressed higher levels of granzyme B compared to the $CD8^+YFP^-$ cells.

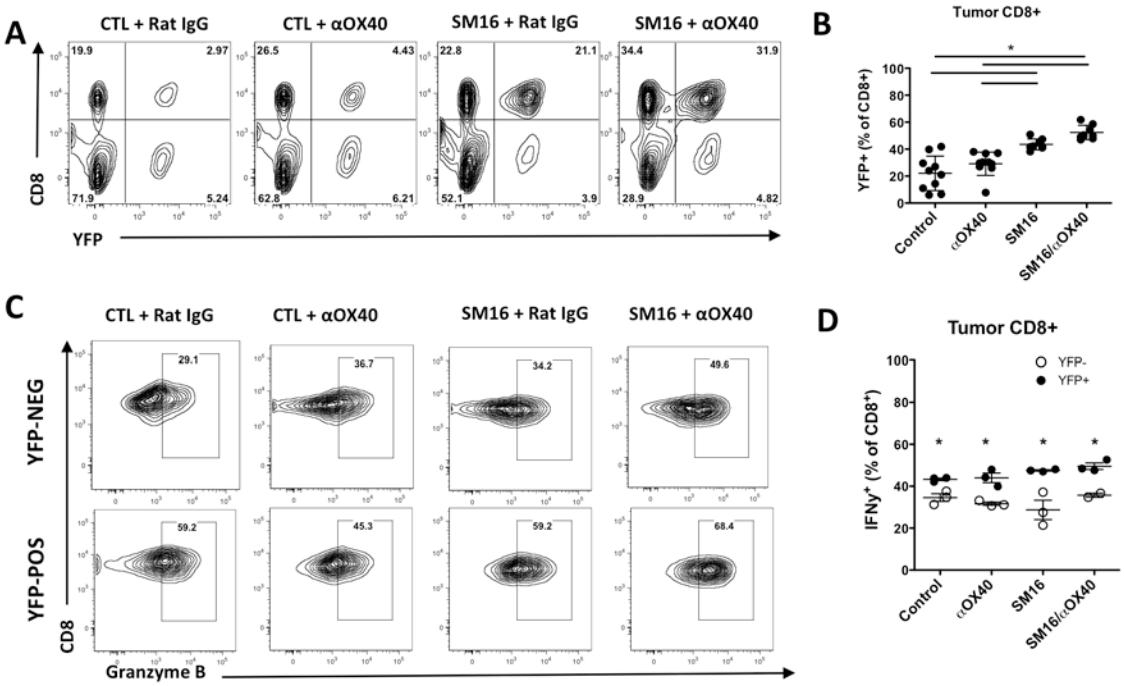


Figure 3.6: Increase in OX40 expressing CD8$^+$ T cells in the tumors of αOX40/SM16 treated mice. YFP reporter mice were treated as in Figure 3.3. **(A)** Representative flow cytometry plots of tumor digest for expression of YFP versus CD8. **(B)**Proportion of CD8$^+$ and CD4$^+$ cells from the tumor that were YFP$^+$. Results are from 3 independent experiments with each individual dot representing results from an individual mouse, the wide horizontal bar represents the mean +/- SD. Statistical significance for graphs was determined by one-way ANOVA analysis. *=P<.05 **(C,D)** Tumor digest pooled from tumor-bearing mice (N=4/group) were used to sort CD8$^+$YFP$^-$ and CD8$^+$YFP$^+$ cells. **(C)** Sorted cells were evaluated for expression of granyme B. **(D)** Sorted cells were stimulated in triplicate as in Figure 3.4A with PMA/Ionomycin and evaluated for proportion of CD8$^+$ that were IFNγ$^+$. Results from C and D are from one experiment. Statistical significance for D was determined by paired student's t-test between YFP$^-$ and YFP$^+$ cells in each group. *=P<.05

SM16/αOX40 cured mice develop long-term anti-tumor immunity

Lastly, we wanted to determine if mice cured by SM16 and SM16/αOX40 develop long-term immunity to the MCA-205 tumor cell line. Therefore we re-challenged mice with the MCA205 tumor cells that had a complete response to treatment with SM16 or SM16/αOX40. As shown in figure 3.7, 0/7 of the mice from the SM16/αOX40 treated group developed tumors, whereas a fraction of the SM16 treated mice developed tumors (2/7) and all the naïve mice developed tumors (7/7). In contrast, neither the SM16 cured or SM16/αOX40 cured mice were protected from primary challenge with 3LL tumor cells (data not shown). These results indicate that SM16/αOX40 mediated tumor regression resulted in long-term tumor-specific immunological memory that protects against future tumor growth.

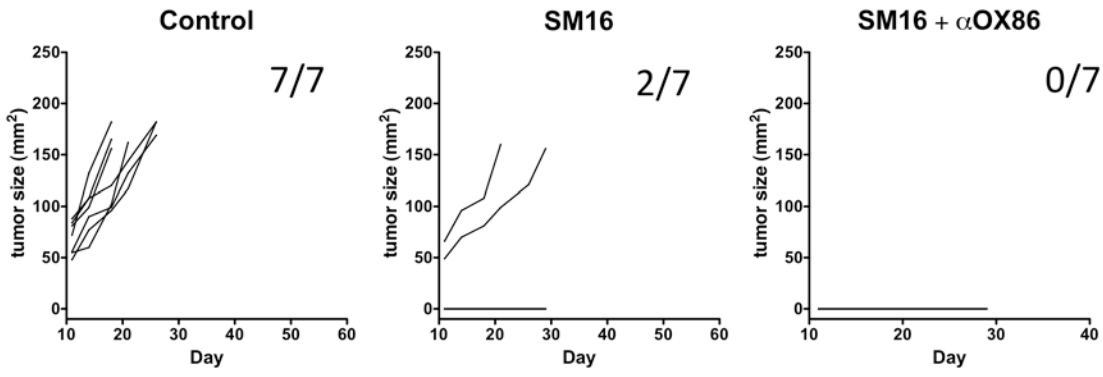


Figure 3.7: αOX40/SM16 cured mice are able to resist tumor-specific re-challenge. Mice were treated as in Figure 3.2. Naïve mice and mice that had complete tumor regression and were tumor free for at least two weeks after treatment with SM16 or SM16 + αOX40 were inoculated with 4 x 10^5 MCA205 cells into the left flank and were monitored for tumor growth and survival. Results are from one experiment (N=7/group).

Discussion

It has been reported that the microenvironment of large tumors is immunosuppressive due to the presence of inhibitory cytokines. Therefore, a successful tumor immunotherapy may require both an agonist to boost effector T cell function and an inhibitor(s) of T cell suppression [170-172, 175]. In this study, we found that while αOX40 therapy alone was ineffective at treating large established MCA205 tumors, αOX40 was capable of causing complete regression of large tumors if given in conjunction with a TGF-β inhibitor (SM16). The dual treatment resulted in long-term survival in ~80% of the αOX40/SM16 treated mice compared to ~15% of mice treated with SM16 alone (Figure 3.2). Furthermore, complete tumor regression was accompanied by the host's ability to resist tumor re-challenge in all of the mice cured by αOX40 + SM16 therapy to MCA-205, but were unprotected from an unrelated tumor cell line (Figure 3.7).

Examination of the tumor microenvironment showed that the immune composition was considerably altered in the tumors of αOX40/SM16 treated mice. These changes included an increase in tumor infiltrating $CD4^+$ and $CD8^+$ T cells associated with enhanced T cell differentiation toward Th1/cytotoxic effector phenotypes. Functional analysis showed that a greater proportion of $CD4^+$ and $CD8^+$ cells from αOX40/SM16 treated mice were capable of producing IFN-γ *ex vivo* (Figure 3.4A). Phenotypic analysis revealed a significant increase in the proportion of $CD8^+$ T cells expressing granzyme B in the tumors of the αOX40/SM16 (~57%) treated mice when

compared to the SM16 (~25%) and αOX40 (~27%) treated mice. Interestingly, a large proportion of these $CD8^+GrzB^+$ cells co-expressed the proliferation marker Ki67 (~77%) in the αOX40/SM16 treated mice. These proliferating $CD8^+GrzB^+$ cells represent a newly defined subpopulation of terminally differentiated T cells that have been previously described as senescent end-stage cells [222].

Previous studies have shown that OX40 signaling increases $CD8^+$ T cell proliferation, IFN-γ production and granzyme B expression [223]. Conversely, TGF-β signaling by $CD8^+$ T cells inhibits all these functions [136]. However, it is clear that SM16 and αOX40 mono-therapies are unable to significantly affect the phenotype of the T cells in this tumor model. These data show that promoting OX40 signaling while simultaneously inhibiting TGF-β signaling has a synergistic effect on tumor infiltrating $CD8^+$ T cell differentiation and proliferation, which most likely led to tumor destruction. This synergy may partly be explained by the ability of SM16 to increase the proportion of $CD8^+$ T cells that can express OX40 in the tumor (Figure 3.6).

Since OX40 and TGF-β signaling have been shown to modulate Treg proliferation, survival and function [139-141, 164-167, 215], we expected to find differences in the frequency of tumor infiltrating Tregs. Surprisingly, we found no statistical change in the proportion of $CD4^+$ T cells that expressed $Foxp3^+$ in the tumor between the different treatment groups (Figure 3.5). This finding suggests that abrogation of Treg frequency in the tumors is probably not involved with the efficacy of αOX40/SM16 treatment. It is noteworthy that despite the high frequency of $CD4^+$ cells that are $Foxp3^+$ (~40%) in the tumors of αOX40/SM16 treated mice, $CD8^+$ cells appear capable of proliferating and differentiating into effector $CD8^+GrzB^+$ cells. It currently

remains unknown if αOX40/SM16 treatment affects Treg function and/or alters $CD8^+$ T cell susceptibility to Treg suppression.

In summary, we found that combining αOX40 antibodies with an inhibitor of TGF-β (SM16) resulted in complete and durable regression of large established tumors. These responses were accompanied by changes in the T cell frequencies and phenotype within these tumors and the ability to resist tumor re-challenge. Collectively these findings suggest that αOX40 synergizes with SM16 to initiate a powerful anti-tumor immune response that can cure mice of large established tumors. Based on these findings, and the clinical development of both OX40 agonists and TGF-β antagonists, this combination therapy could be an effective future strategy for treating cancer patients.

Chapter 4

A Novel Transgenic Mouse to Study the Role of Stat3 signaling in OX40 Driven Anti-Tumor Immune Responses

Abstract

The role of Stat3 signaling in T cells during anti-tumor immune responses has not been extensively studied, especially in response to immunotherapies (e.g. αOX40). Therefore, we developed a novel transgenic mouse that conditionally deletes the *Stat3* gene specifically in OX40 expressing T cells. In this chapter, we show that activated T cells from these transgenic mice expressed decreased levels of Stat3, had a decreased propensity to become IL-17 producing cells, and tumor infiltrating T cells isolated from these mice contained less pStat3. In summary, these transgenic mice provide a unique tool for studying the role of Stat3 signaling in T cell responses.

Introduction

As discussed in chapter 3, αOX40 agonist antibodies are less effective at causing regression of large tumors, which is most likely due to immune suppressive signals within the tumor microenvironment. Recent studies have identified signal transducer and activator of transcription 3 (Stat3) as a potential negative regulator of anti-tumor T cell responses. Activated pStat3 has been found to be persistently present in tumor cells and tumor associated immune cells [224]. Since cytokines and growth factors that are regulated by Stat3 in turn activate Stat3, it has been hypothesized that a positive feedback loop for Stat3 activation can be established within the tumor microenvironment (TME) [225]. This constitutive Stat3 activation within the TME is propagated by crosstalk between tumor and immune cells and promotes tumor growth by three mechanisms [225]. First, studies have shown that pStat3 can act as an oncogene within the tumor cells [226]. Second, pStat3 in tumor cells promotes the secretion of immune suppressing factors, including vascular endothelial growth factor (VEGF), IL-6 and IL-10 [227-229]. Third, studies have shown that activated Stat3 in haematopoietic cells can promote tolerance and suppress anti-tumor immune responses [224, 225, 228, 230, 231].

However, the effects of activated Stat3 in tumor infiltrating T cells have not been thoroughly addressed. In support of the notion that Stat3 signaling inhibits anti-tumor T cell responses, a recent study showed that deletion of the *Stat3* gene in adoptively transferred OT-I $CD8^+$ T cells enhanced T cell responses against ova-expressing tumors in $RAG^{-/-}$ recipients [232]. However, other studies have shown that Stat3 is important for T cell proliferation, survival and memory formation [233-235], all of which may play a crucial role in anti-tumor T cell responses. Therefore we sought to determine the role of

Stat3 in OX40 mediated anti-tumor T cell responses by creating a novel transgenic mouse that specifically deletes the *Stat3* gene upon OX40 expression.

Materials and Methods

Mice and genotyping

Mice which have an IRES-*cre* element inserted into the third exon of the *OX40* gene (OX40-Cre) [217] were crossed with mice carrying *Stat3* alleles with exons 16-21 flanked by *loxP* sites (STAT3flx/flx) [236]. All experiments used male and female progeny that were $OX40^{Cre/Wt}/Stat3^{Flx/Flx}$. For controls, age-matched male and female $OX40^{Cre/Wt}/Stat3^{Wt/Wt}$ mice were used. Genotyping was performed by PCR amplification of tail DNA. The following primers were used for genotyping of the *Stat3* floxed allele: primer 1 (5'- GAA GGC AGG TCT CTC TGG TG- 3') and primer 2 (5'- AGG CTG CCA ACA GCC ACT GCC -3'). The PCR for the *Stat3* floxed allele was performed at 94°C for 2 min, followed by 30-35 cycles of 94°C for 30s, 62°C for 30s, and 72°C for 30 s. This primer pair amplifies a 140 bp product for wildtype *stat3* alleles and a 260 bp product for floxed *stat3* alleles [237]. The following primers were used for genotyping of the *OX40-cre* allele: primer 1 (5'- GCC-ATT-GAG-AAG-GAG-CAA-GT -3') and primer 2 (5'- TCG-TCA-AGA-AGA-CAG-GAC-CA -3'). The PCR for *Cre* allele was performed at 94°C for 2 min, followed by 30 cycles of 94°C for 15s, 62°C for 15s, and 72°C for 45s. This primer pair amplifies a ~350 bp product for an *OX40-cre* allele [217].

Tumor Cell Line

MCA205 sarcoma cells were propagated *in vitro* using (complete media) RPMI (Lonza, Walkersville, MD) containing 0.292 ng/ml glutamine (Hyclone Laboratories, Logan, UT), 100 U/ml streptomycin (Hyclone), 100 U/ml penicillin (Hyclone), 1X non-essential amino acids (Lonza BioWhittaker), 1mM sodium pyruvate (Lonza

BioWhittaker) and 10mM HEPES (Sigma). The MCA205 cell line was routinely tested and shown to be free of mycoplasma contamination.

Flow cyotmetry

Cells (1-5 million) in 100 μl of flow wash buffer (FWB, PBS with 0.5% FBS) were incubated with the appropriate surface antibodies and a viability dye (ebioscience) at 4°C for 30 minutes. For intracellular staining of RORγt (ebioscience; AFKJS-9), Foxp3 (ebioscience; FJK-16s), Gata3 (ebioscience; TWAJ), Tbet (ebioscience; 4B10) and IL-17 (ebioscience; eBio17B7), cells were fixed and permeabilized with the Foxp3 Staining Buffer Set (eBioscience) according to the manufacturer's protocol. For analysis of intracellular Stat3 (BD Biosciences; M59-50) and pStat3 (BD Biosciences; 4/P-Stat3), cells were fixed and permeabilized with the Fix & Perm Cell Permeabilization Reagents from Invitrogen following the methanol modified protocol. All intracellular staining was done at 4°C for 30 minutes with the appropriate antibodies to detect specific intracellular proteins. Cells were analyzed using an LSR II flow cytometer (BD) and FlowJo4 software.

Phenotyping of TIL and splenocytes

Mice were injected with 4 x 10^5 MCA205 cells s.c.. At different times, tumors were resected, minced with scissors and disaggregated using triple enzyme digest cocktail containing 10 mg/ml of collagenase type IV (Worthington Biochemical Corp. Lakewood, NJ), 1 mg/ml hyaluronidase (Sigma-Aldrich, St. Louis, MO), 200 μg/ml DNAse I (Roche Applied Sciences, Indianapolis, IN) in complete media for 30 minutes with agitation at

37° C using a shaker. The tumor digest was then passed through 70-μm nylon filter, subjected to red blood cell lysis using ACK lysing buffer (Lonza) and then washed and used for analysis. Spleen and lymph node cells were minced between slides, subjected to red blood cell lysis and passed through 70-μm nylon filter, washed and used for analysis. Cells were then incubated with fluorescently labeled antibodies and analyzed by flow cytometry.

T helper differentiation

Spleens from $OX40^{Cre/Wt}$/Stat3 $^{Wt/Wt}$ and $OX40^{Cre/Wt}$/Stat3 $^{Flx/Flx}$ mice were processed as described above. $CD4^{+}CD62L^{+}$ were then isolated by negative enrichment of $CD4^{+}$ T cells followed by positive enrichment of $CD62L^{+}$ cells (Miltenyi). Cells were then washed and resuspended in complete RPMI at a concentration of 1 x 10^{6} cells/ml, from which 200μl were added to a 96 well plate pre-coated with anti-CD3 (2.5 μg/ml; Biolegend) and anti-CD28 (5 μg/ml; Biolegend). For Th17 conditions, cells were cultured with TGF-β (5 ng/ml; R&D Systems), IL-6 (20 ng/ml; R&D Systems), anti-IFNγ (10 μg/ml), anti-IL-12 (10 μg/ml), anti-IL-4 (10 μg/ml) and anti-IL-2 (10 μg/ml). For Treg conditions, cells were cultured with TGF-β (5 ng/ml; R&D Systems), rhIL-2 (1,000 U/ml; Novartis), anti-IFNγ (10 μg/ml), anti-IL-12 (10 μg/ml) and anti-IL-4 (10 μg/ml). After 72 hours, all cells were washed and transferred to a new 96 well plate with fresh media and cultured for an additional 48 hours with rhIL-2 (1,000 U/ml). Cells were then simulated for 1 hour at 37° C with 1X PMA/ionomycin cocktail (eBioscience). Brefeldin A (Biolegend) was added for a final concentration of 5 μg/ml and incubated with the cells for an additional 4 hours at 37° C. Cells were then stained for surface

markers, then fixed and permeabilized and evaluated for intracellular IL-17 and Foxp3 as described above.

Results

pStat3 levels in tumor infiltrating T cells

As shown in chapter 3, αOX40 therapy is less effective at curing mice of large established tumors due to immune suppressing signals. Some studies have suggested that up-regulation of pStat3 suppresses T cell responses in the tumor microenvironment. To determine if pStat3 is up-regulated in T cells in the MCA-205 tumor model, we evaluated $CD3^+$ T cells from tumors of different sizes for pStat3. We found that tumor infiltrating T cells from larger tumors had increased levels of pStat3 compared to T cells isolated from smaller tumors (Figure 4.1). However, it was not known if pStat3 signaling by T cells was in part suppressing the anti-OX40 mediated anti-tumor immune responses in larger tumors.

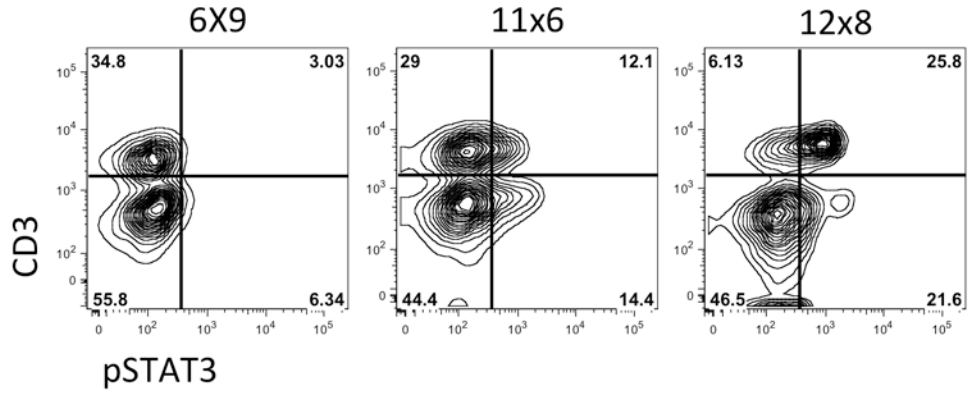


Figure 4.1: pSTAT3 levels are increased in T cells from larger tumors. Mice were inoculated with 4 x 10^5 MCA205 cells into the right flank. After 12 days, tumors of different sizes were digested, and $CD3^+$ TIL were evaluated for intracellular pSTAT3 *ex vivo*. Results are from one experiment.

Generation of $OX40^{Cre/Wt}/Stat3^{flx/flx}$ mice

To address the role of Stat3 in suppressing OX40 mediated tumor regression, we generated mice that conditionally knock out the *Stat3* gene upon OX40 expression. This

was accomplished by crossing mice that have an IRES-*cre* element in one of their OX40 exons with mice carrying *Stat3* alleles flanked by *loxP* sites. To determine if Stat3 expression was down-regulated in OX40 expressing T cells within these mice, splenocytes were stimulated anti-CD3 + anti-CD28 + IL-2, which is known to induce OX40 expression [238]. After resting the T cells for an additional 48 hours, $OX40^{Cre/Wt}/Stat3^{flx/flx}$ cells were incubated with or without IL-6 and evaluated for intracellular pStat3. As Figure 4.2 shows, pStat3 levels were lower in T cells from the $Stat3^{Tg}$ mice ($OX40^{Cre/Wt}/Stat3^{flx/flx}$) compared to control mice ($OX40^{Cre/Wt}/Stat3^{Wt/Wt}$) and were less responsive to IL-6 up-regulation of pSTAT3.

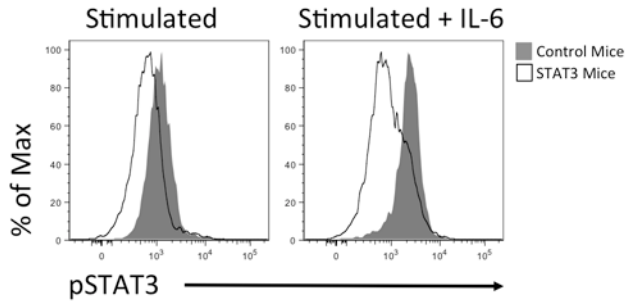


Figure 4.2: STAT3 is down-regulated by T cells from $OX40^{Cre/Wt}/STAT3^{flx/flx}$ mice after activation. Enriched $CD4^+$ cells from the spleens of $OX40^{Cre}/STAT3^{wt/wt}$ and $OX40^{Cre}/STAT3^{flx/flx}$ mice were stimulated with plate bound anti-CD3/CD28 (both 5 μg/ml) in the presence of rhIL-2 (1,000/ml). After 48 hours, cells were washed, re-plated and rested for an additional 48 hours. Cells were then cultured with or without IL-6 (50 ng/ml) for 20 minutes and evaluated for intracellular pSTAT3. Data is representative of 5 independent experiments.

Further verification of Stat3 deletion

Since Stat3 is involved in Th17 differentiation [233], we evaluated the ability of $CD4^+$ T cells from $Stat3^{Tg}$ mice to differentiate into Th17 cells *in vitro*. As expected, fewer $CD4^+$ T cells from $Stat3^{Tg}$ mice differentiated into IL-17 producing cells (Figure 4.3). Interestingly, during Th17 differentiation conditions (IL-6 + TGF-β) a larger proportion of the conditional knock out T cells differentiated into $Foxp3^+$ cells that were unable to make IL-17 (Figure 4.3). This enhanced Treg differentiation under Th17

conditions is presumably due to TGF-β signaling in the absence of IL-6 signaling (via pStat3). These findings were further confirmed *in vivo* which showed that $CD4^+$ T cells from the $Stat3^{Tg}$ mice expressed lower levels of RORγt, the master transcription for Th17 cells (Figure 4.4). In contrast, there were no major changes in expression of Tbet, Gata3 or Foxp3, the master transcription factors for Th1, Th2 and Treg cells. Lastly, we determined that in the $Stat3^{Tg}$ mice, pStat3 levels were significantly decreased in both $CD4^+$ and $CD8^+$ tumor infiltrating T cells, but not non-T cells, when analyzed directly *ex vivo* (Figure 4.5).

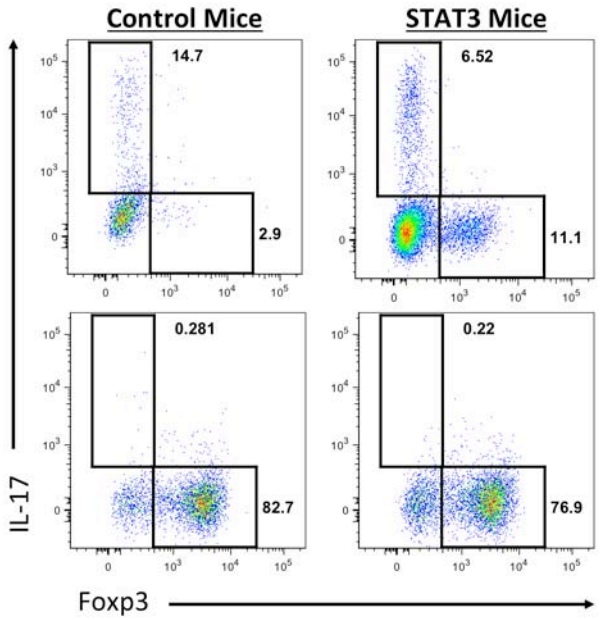


Figure 4.3: $CD4^+$ cells from $OX40^{Cre/Wt}/STAT3^{flx/flx}$ mice have abnormal responses to Th17 differentiation conditions *in vitro*. Enriched $CD4^+CD62L^+$ and cells from the spleens of $OX40^{Cre}/STAT3^{wt/wt}$ and $OX40^{Cre}/STAT3^{flx/flx}$ mice were stimulated with plate bound anti-CD3/CD28(both 5 μg/ml) in **(top)** Th17 conditions or **(bottom)**Treg conditions for 72 hours. Cells were then washed and re-plated in the presence of rhIL-2 (1,000 U/ml). After 48 hours cells were stimulated with PMA/Ionomycin (1x), after which brefeldin A (5 μg/ml) was added for an additional 4 hours. Cells were then evaluated for intracellular IL-17 and Foxp3. Results are representative of 2 independent experiments done in duplicate.

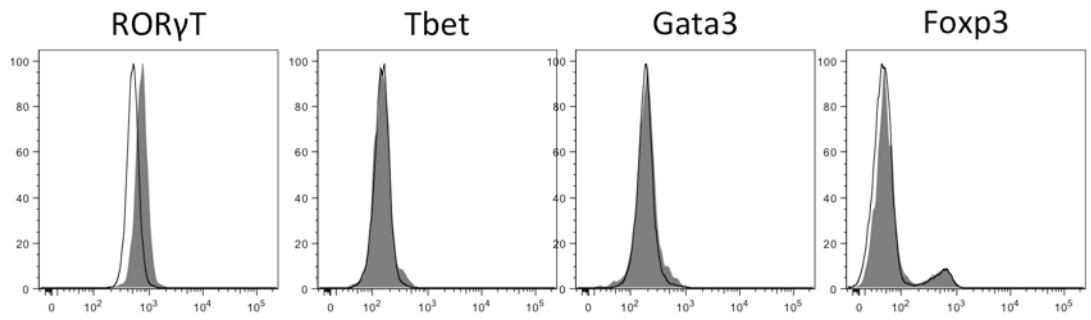


Figure 4.4: CD4^{+} cells from OX40$^{Cre/Wt}$/STAT3$^{flx/flx}$ mice have decreased expression of RORγt *ex vivo*. CD4^{+} cells from spleens from OX40^{Cre}/STAT3$^{wt/wt}$ (grey) and OX40^{Cre}/STAT3$^{flx/flx}$ mice (black) were evaluated for RORγt, Tbet, Gata3 and Foxp3 *ex vivo*. Results are representative of 2 independent experiments.

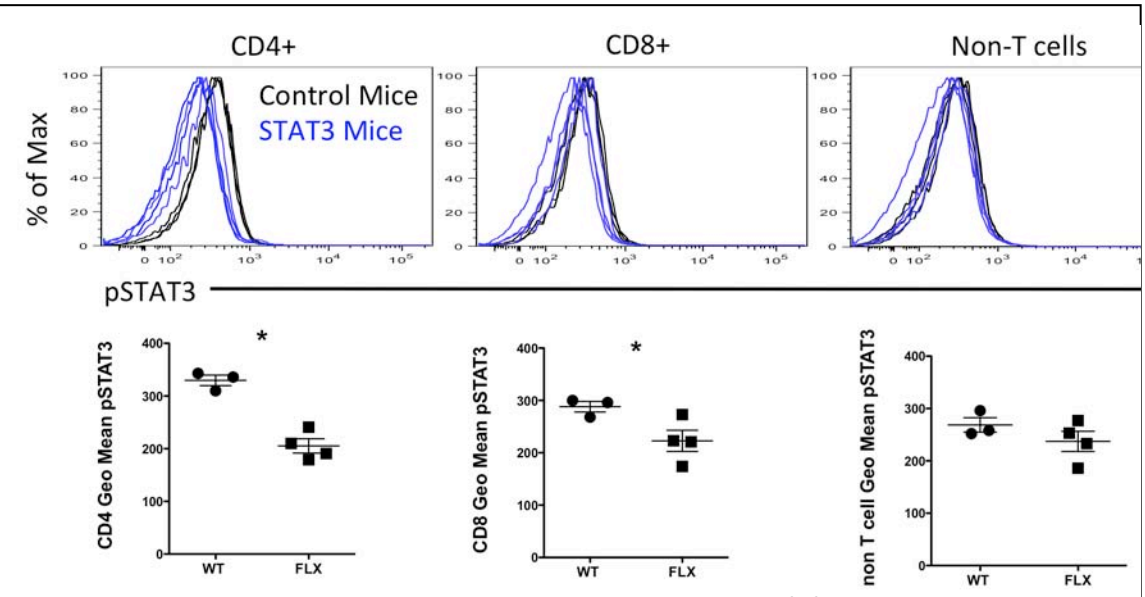


Figure 4.5: Tumor infiltrating lymphocytes from OX40$^{Cre/Wt}$/STAT3$^{flx/flx}$ mice have down-regulated levels of pSTAT3. OX40^{Cre}/STAT3$^{wt/wt}$ and OX40^{Cre}/STAT3$^{flx/flx}$ mice were inoculated with 4 x 10^{5}MCA205 cells into the right flank. After 11 days CD4^{+}, CD8^{+} and non-T cells (CD4^{-}CD8^{-}) from the tumors were evaluated for intracellular pSTAT3 *ex vivo*.

Discussion

The inability of αOX40 to cure mice of large established tumors may be due to the presence of immune suppressive signals. In this chapter, we show that as tumors progressed in size, higher levels of pStat3 were detected in tumor infiltrating T cells. However, it is unclear whether Stat3 signals were involved with suppressing anti-tumor T cell responses in these larger tumors, specifically in response to αOX40 therapy. To address this, we created a novel transgenic mouse that specifically deletes the *Stat3* gene upon OX40 expression. In this chapter, we show that activated T cells from these transgenic mice expressed lower levels of pStat3 and were less responsive to IL-6 compared to littermate control mice. When stimulated under Th17 differentiation conditions *in vitro*, a smaller percentage of the transgenic $CD4^+$ T cells produced IL-17 and expressed less RORγt when analyzed *ex vivo*. Lastly, we determined that TIL from these experimental mice contained less pStat3 compared to TIL from control mice. In summary, we created a novel transgenic mouse that conditionally deletes *Stat3* upon OX40 expression which will be used in future studies to examine the role of Stat3 signaling in T cells during αOX40 treatment of mice with established tumors.

Chapter 5

Conclusions and Future Directions

$CD4^+CD25^{INT}FOXP3^{NEG}$ Human Memory Population

CD25 expression is restricted primarily to $CD4^+Foxp3^{POS}$ Tregs in mice, however a large proportion of $CD4^+FOXP3^{NEG}$ non-Tregs express low-intermediate levels of CD25 in humans. While some studies have suggested that this $CD25^{LO/INT}FOXP3^{NEG}$ population are recently activated T cells, the same publications were primarily focused on the $CD25^{HI}FOXP3^{POS}$ Tregs [62-66]. To our knowledge, no previous study has thoroughly characterized this $CD25^{LO/INT}FOXP3^{NEG}$ human T cell population. Therefore, given the broad effects of IL-2 on T cells, and the current practice of targeting the IL-2 signaling pathways in the clinic by administering IL-2 to cancer patients or anti-CD25 blocking antibodies to transplant patients, it is important to define the role that $CD25^{LO/INT}FOXP3^{NEG}$ cells play in immune function. Studying this population may have clinical ramifications by helping to illuminate the mechanism of IL-2 action and potentially improve upon the therapies that target IL-2 and CD25.

Using a relatively new anti-CD25 antibody (clone 4E3; Miltenyi), we were able to reveal that a larger proportion of $CD4^+$ cells from healthy individuals were $CD25^{INT}FOXP3^{NEG}$ than previously reported (Figure 1.1 vs 2.2A). For the first time we show that contrary to the literature, the $CD25^{INT}FOXP3^{NEG}$ cells from human PBMC are not recently activated, but actually represent a stable quiescent memory population that makes up the majority of memory $CD4^+$ T cells (~75%). However, not all memory cells expressed intermediate levels of CD25, as a significant proportion of memory cells (~20%) were $CD25^{NEG}FOXP3^{NEG}$. Therefore, a primary focus of this thesis was to determine whether the resting memory cells that express CD25 were phenotypically and functionally distinct from $CD25^{NEG}$ memory T cells.

Examination of phenotypic markers, responses to antigens and evaluation of memory cells in SLE patients suggested that the compared to the $CD25^{INT}$ memory population, the $CD25^{NEG}$ memory population was associated with chronic immune responses (Figures 2.5 and 2.8). Based on this set of data, we propose a novel differentiation scheme for $CD4^+$ T cells in humans. The model predicts that as memory cells become chronically activated, they loose expression of CD25, then gain expression of EOMES/SLAMF7, followed by the loss of CD28 and gain expression of granzyme B (Figure 5.1). This loss of CD25 and gain of SLAM7 follows other trends reported for human T cells in the literature of the loss of traditional T cell co-receptors (e.g. CD28, CD27) and the concomitant acquisition of non-traditional NK cell co-receptors (e.g. NKG2D, KIR) as $CD4^+$ T cells become chronically stimulated [23, 24].

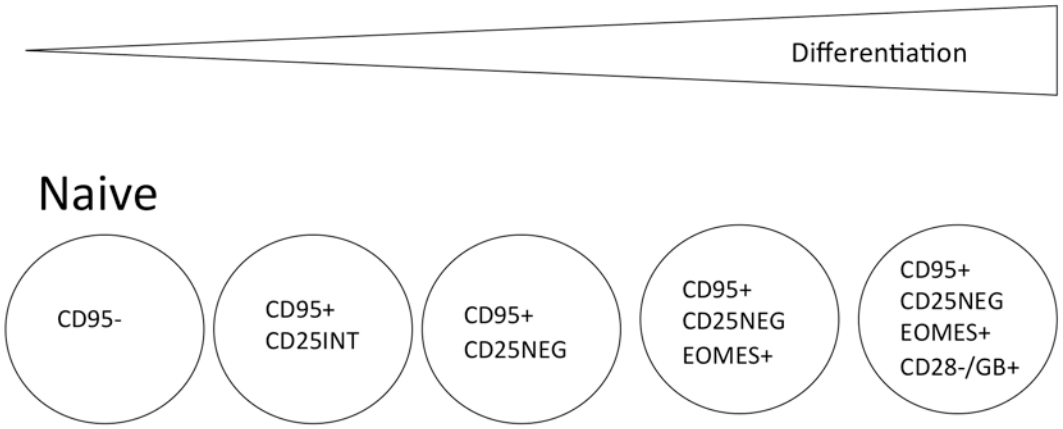


Figure 5.1: Proposed differentiation scheme for human $CD4^+$ T cells

Though studies in humans are often difficult, this differentiation model could be further validated experimentally. First, sorted naïve cells could be stimulated repeatedly *in vitro* to determine if the phenotype of the cells differentiate as predicted. Second, studies have shown that as individuals age they accumulate late-differentiated effector T

cells, therefore a large cohort of healthy individuals of different ages could be studied [23, 239]. If the hypothesis is correct, memory cells in young individuals should be primarily $CD25^{INT}EOMES^{NEG}CD28^{POS}$. Then, as individuals age, there would be an accumulation of $CD25^{NEG}EOMES^{NEG}CD28^{POS}$ memory cells followed by the accumulation of $CD25^{NEG}EOMES^{POS}CD28^{NEG}$ memory cells. Lastly, one could follow CMV reactive cells in recently CMV infected individuals to determine if they too change phenotype as described above. Future studies of these different memory subsets may provide further insight into their role in chronic immune diseases and responses to treatments.

Since this $CD4^{+}$ memory population does express the high affinity IL-2 receptor, another focus within this thesis was to determine if the $CD25^{INT}$ population was differentially affected by IL-2 when compared to the $CD25^{NEG}$ memory and Treg populations. Interestingly, we found differences between these three populations that included IL-2 mediated regulation of CD25 and FOXP3 protein expression, sensitivity to rhIL-2, proliferation, and their dependence on exogenous IL-2 for survival (Figures 2.11, 2.13). These differences also occurred at higher concentrations of rhIL-2 where all three populations were capable of responding to rhIL-2 through the lower affinity IL-2 receptor, suggesting there are differences independent of IL-2 sensitivity. For instance, in addition to STAT5 activation, some studies have implicated pSTAT3, and the PI3K and MAPK pathways as being involved in IL-2 signaling [240]. Therefore, these populations may differ in their IL-2 signaling patterns [241], which could be evaluated by quantifying phosphorylation of signaling molecules by ELISAs and using specific signaling inhibitors during IL-2 stimulation. There also may be differences in the pattern

of phosphorylation of STAT5 and other signaling proteins when comparing these subsets, which could be tested by analyzing for phosphorylated isoforms [242].

These findings may have important clinical implications for improving IL-2 immunotherapy by selectively blocking responses of particular T cell subsets during IL-2 treatment. For instance, past studies have shown that $FOXP3^{POS}$ Tregs are sometimes increased following IL-2 treatment and these increases negatively correlated with clinical outcome [243]. Therefore, if Tregs are uniquely dependent on particular IL-2 signaling pathways, Treg expansion could potentially be blocked without affecting effector T cell subsets during IL-2 treatment. Conversely, selective inhibitors could be used to specifically inhibit IL-2 effector T cell signaling in transplant patients.

The $CD25^{INT}FOXP3^{NEG}$ T cell population also differed from the other populations in their dependence on exogenous IL-2 for survival *in vitro* (Figure 2.11). While the Treg population was dependent on exogenous rhIL-2 for survival, the $CD25^{INT}$ population was not. We tried to determine if the $CD25^{INT}$ memory cells were making their own IL-2 *in vitro* by intracellular cytokine staining and ELISA, but we were unable to detect IL-2 production by these methods. However, it is possible that we were unable to detect IL-2 production by $CD25^{INT}$ cells because IL-2 was constantly being bound to the IL-2 receptor, internalized, and then degraded [182, 183]. Therefore, future studies could be performed to test the role of endogenous IL-2 for survival *in vitro* by co-incubating the different subsets with anti-IL-2 neutralizing and anti-CD25 blocking antibodies. If the $CD25^{INT}$ population is dependent on endogenous IL-2 production for survival, we would expect an increase in cell death by administering the blocking antibodies. In addition, future studies could be performed to test whether CD25 is

involved with homeostatic survival and/or proliferation of the $CD25^{INT}FOXP3^{NEG}$ population *in vivo*. This could be accomplished by following this population in transplant patients receiving anti-CD25 blocking antibodies and looking for changes in expression of Ki67 and annexin V, as well as whether the proportion of $CD4^{+}CD25^{INT}FOXP3^{NEG}$ change over time.

Another surprising finding within this thesis was the $CD25^{INT}FOXP3^{NEG}$ population's response to IL-2 treatment in cancer patients (Figure 2.12). This unique population is distinctly affected by IL-2 treatment in cancer patients and may be involved with regression of tumors in these patients. Therefore approaches involving manipulation of Tregs in clinical situations by inhibiting the function of CD25 [244] or depleting CD25 expressing cells [245, 246], may also affect the $CD25^{INT}FOXP3^{NEG}$ population and could have a negative effect upon the desired outcome. As mentioned prior, a more promising strategy may be to use inhibitors that selectively inhibit different $CD4^{+}$ T cell populations during IL-2/CD25 specific immunotherapies.

However, it still remains unclear exactly how the $CD25^{INT}$ population responded to the *in vivo* IL-2 immunotherapy. Further studies to understand the response of $CD25^{INT}FOXP3^{NEG}$ cells over the course of IL-2 infusions could include 1) evaluating changes in Ki67 expression to indicate proliferation; 2) evaluation of changes in chemokine receptor expression or conformational changes of integrins which may indicate increased trafficking from the blood to tissues; 3) evaluation of 7AAD/Annexin V to indicate increased cell death; and 4) evaluation of patient serum for soluble CD25 protein levels, which would indicate increased shedding of CD25.

Lastly, we were unable to find a mouse equivalent for the $CD25^{INT}FOXP3^{NEG}$ $CD4^+$ T cell population in either young, old or tumor bearing C57BL/6 male and female mice. Therefore, we believe that there are differences in the expression and role of IL-2/CD25 for $CD4^+$ T cells between mice and humans. This notion has been discussed by other groups [67, 188, 189] and is supported by phenotypic differences observed between CD25 deficient mice and humans [31, 67, 69, 190-192]. For instance, in contrast to mice, CD25 doesn't not appear to be essential in humans for maintaining normal Treg frequencies *in vivo* [67, 69, 186, 187]. Also, CD25 deficiency in humans is accompanied by a severe immunodeficiency characterized by susceptibility to opportunistic infections and an inability to reject allografts [67, 190-192], whereas CD25 and IL-2 deficient mice do not have these traits [58, 68, 210, 211]. The existence of the $CD25^{INT}FOXP3^{NEG}$ population in humans may explain some of these differences, especially given the role of CD25 on the $CD25^{INT}FOXP3^{NEG}$ population during activation in the absence of co-stimulation (Figure 2.10). Thus CD25 may play a larger role for effector responses and less of a role for homeostasis of Tregs in humans compared to mice. These differences need to be taken into account when trying to translate CD25 and IL-2 studies from mice to humans. Ultimately, future studies in humans with the $CD4^+CD25^{INT}FOXP3^{NEG}$ population may help us understand the role of IL-2 in human immunology and improve the efficacy of immunotherapies that influence IL-2 signaling.

Overcoming the Immunosuppressive Environment In Large Established Tumors

Despite the existence of tumor reactive T cells and the accumulation of lymphocytes within progressing tumors, the immune system present within these tumors

seems incapable of meditating regression spontaneously [110]. Therefore the goal of cancer immunotherapy is to stimulate a potent anti-tumor immune response in order to successfully control or cause regression of large tumors. Though single agent cancer immunotherapies are usually successful in mouse tumor models if administered shortly after tumor challenge, they are often ineffective once the tumors become established [177, 247, 248].

A potential explanation for the inability of the immune system to cause tumor regression is that the malignant microenvironment contains immune suppressive cytokines which can dampen T cell function [170, 177, 247, 249-252]. Anti-OX40 in pre-clinical models has been effective at treating small but not large tumors in mouse cancer models, therefor it seemed logical to combine OX40 agonists with agents that can inhibit immune suppressive pathways. In Chapter 3, we showed that while αOX40 therapy was ineffective at treating large MCA205 tumors (Figure 3.1), it was able to cause complete regression of large established tumors in ~85% of the mice if combined with the TGF-β receptor inhibitor SM16 (Figure 3.2). Examination of the tumor microenvironment showed that the tumor immune composition was considerably altered in mice treated with αOX40/SM16. One of the striking results was an increase in tumor infiltrating $CD4^+$ and $CD8^+$ T cells (Figure 3.3). However, it was not clear if this accumulation of T cells in the tumor was due to increased proliferation within the tumor or enhanced recruitment. Analysis of Ki67 expression revealed that there was no overall increase in proliferating T cells within the tumors of αOX40/SM16 treated mice (Figure 3.4), suggesting that improved T cell recruitment may be a primary factor. Future studies could determine the role of trafficking by analyzing T cells for expression of chemokine

receptors and evaluating the tumor microenvironment for the presence of increased chemokine expression. Using blocking antibodies and other means, one could further evaluate the role of these chemokines in the accumulation of T cells within the tumors.

In addition to an overall increase in T cells within the tumors of αOX40/SM16 treated mice, phenotypic analysis revealed a significant increase in the proportion of $CD8^+$ T cells expressing granzyme B in the tumors of the αOX40/SM16 treated mice. Interestingly, a large proportion of these $CD8^+GrzB^+$ cells co-expressed the proliferation marker Ki67 (Figure 3.4). These $CD8^+GrzB^+Ki67^+$ T cells represent a newly defined subpopulation of terminally differentiated T cells [222] and suggest that there is an active immune response occurring within these tumors.

Previous studies have shown that signaling through OX40 increases $CD8^+$ T cell proliferation, IFN-γ production and granzyme B expression. Conversely, TGF-β signaling by $CD8^+$ T cells inhibits all these functions. However, it was clear that SM16 and αOX40 therapies alone were unable to change the phenotype of the T cells in this tumor model and therefore suggests there was a synergistic effect on tumor infiltrating $CD8^+$ T cell differentiation and proliferation, which most likely led to tumor destruction. Though αOX40 therapy is known to target OX40 expressing T cells, TGF-β can have direct and indirect inhibitory effects on T cells [130, 131, 135, 136]. For example, TGF-β has been shown to inhibit antigen presentation and activation by APCs, which could indirectly affect the phenotype and function of $CD8^+$ T cells [130]. Ultimately, TGF-β receptor (Alk5) conditional knock out mice could be used to better understand the mechanism of how SM16 synergizes with αOX40.

Since OX40 and TGF-β signaling have been shown to modulate Treg proliferation and survival [139-141, 164-167, 215], we expected to find differences in the frequencies of tumor infiltrating Tregs. Surprisingly, we found no statistical change in the proportion of $CD4^+$ T cells that expressed $Foxp3^+$ in the tumor between the different treatment groups (Figure 3.5), which suggests that alteration of Treg frequencies in the tumors may not be involved with the αOX40/SM16 treatment mechanism. It is striking that despite the high frequency of $CD4^+$ that are $Foxp3^+$ (~40%) in the tumors of αOX40/SM16 treated mice, $CD8^+$ cells appear capable of proliferating and differentiating into effector $CD8^+GrzB^+$ cells. However, it currently remains unknown whether αOX40/SM16 treatment affects Treg function and/or alters $CD8^+$ T cell susceptibility to Treg suppression. Therefore future studies could be performed to test these two possibilities using Treg suppression assays. Furthermore, $Alk5^{flx/flx}$ mice could be crossed to FOXP3-Cre mice to determine if blocking TGF-β signaling specifically in Tregs affects the therapeutic outcome in response to αOX40 treatment. If it is determined that SM16 affects Treg suppressive function, further phenotypic and functional studies could be performed to fully understand the effects on Tregs.

In addition to TGF-β signaling, activation of Stat3 in immune cells has also been shown to inhibit anti-tumor immune responses [225]. In the MCA205 tumor model, we found that as tumors progressed, T cells contained higher levels of pStat3 (Figure 4.1). However, the role of Stat3 signaling in anti-tumor T cell responses remains unknown, particularly in response to immunotherapies (e.g. αOX40). Therefore, we developed a novel transgenic mouse that conditionally deletes the *Stat3* gene upon OX40 expression by T cells. In chapter 4, we showed that *in vitro* activation of T cells from these

transgenic mice resulted in down-regulation of pStat3 (Figure 4.2) and that tumor infiltrating T cells had lower levels of pStat3 (Figure 4.5). Future experiments are currently underway to determine if deleting the *Stat3* gene in OX40 expressing T cells alters the ability of immunotherapies to cause regression of large established tumors. In addition to these transgenic mice, studies are planned to determine the efficacy of combining αOX40 with systemic Stat3 signaling inhibitors, which could potentially be used to treat cancer patients.

In summary, we found that while αOX40 was able to cure mice of tumors if administered shortly after tumor challenge, it was less effective if the tumors were larger. Given that these larger tumors may more accurately reflect tumors seen in cancer patients, and that OX40 therapy has recently completed phase I clinical trials, there is a need to improve the αOX40 mediated anti-tumor immune responses. In chapter 3, we showed that combining an αOX40 agonist with an inhibitor of TGF-β (SM16) resulted in complete and durable regression of large established tumors and could be an effective future strategy for treating cancer patients. Therefore studies are underway to understand the mechanism(s) of αOX40/SM16 dual therapy and to find other agents that will synergize with the αOX40 therapy.

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