

**IL-17 SECRETING CD8+ T CELLS:
INDUCTION, PLASTICITY AND ROLE IN
ANTI-TUMOR IMMUNITY**

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ABSTRACT

CD8⁺ T cells activated in the presence of IL-6 and TGF- β secrete IL-17, and are known as Tc17 cells. In mice, adoptive T cell immunotherapy for cancer results in tumor regression and long term survival. In previous work, it was shown that efficacy of Tc17 cell adoptive immunotherapy correlates with Tc17 conversion from IL-17 to IFN- γ production (plasticity). In these studies, we sought to understand the factors that determine Tc17 plasticity, the requirements for Tc17 plasticity, and the role of Tc17 plasticity in an effective anti-tumor response. To investigate Tc17 plasticity *in vitro*, we first sorted Tc17 cells to obtain pure IL-17⁺IFN- γ ⁻ cells. Purified Tc17 cultured with IL-2 or IL-12 resulted in conversion to an IFN- γ secreting phenotype. Correspondingly, IL-2 and IL-12 also increased expression of the T cell transcription factor T-bet. To understand the factors important for Tc17 plasticity *in vivo*, we adoptively transferred antigen-specific Tc17 cells, then specifically activated them using a modified vaccinia virus that expresses their cognate antigen. Tc17 activation in a pro-inflammatory environment resulted in conversion to IFN- γ secretion. In addition, vaccinia infection increased the expression of T-bet in Tc17 cells. Based on our *in vitro* data, we tested whether IL-2 or IL-12 were required for Tc17 plasticity *in vivo*, and found that neither were absolutely required. However, Tc17 conversion required the presence of T-bet. Consistent with those results, we found expression of T-bet was absolutely required for Tc17 adoptive immunotherapy in cancer models. Taken together, these data provide new insight into the plasticity of Tc17, which are becoming more prominent in the fields of autoimmunity and tumor immunology.

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CHAPTER 1

INTRODUCTION

Immune Activation and CD8+ T cells

When the body encounters a pathogen such as influenza, the immune system fights the pathogen. The innate immune system recognizes the pathogen and alerts the adaptive immune system. The adaptive immune system consists of two arms, cellular and humoral immunity. B cells are the main components of humoral immunity, and their main function is to secrete proteins known as antibodies that help eliminate the pathogen. The second arm is cellular immunity, which consists of T cells. T cells are a critical component of an effective immune response, more specifically CD8+ T cells. CD8+ T cells play an important role during the elimination of intracellular pathogens, such as viruses and some bacteria¹. The activation and differentiation of naïve CD8+ T cells is a complex process and can be divided into different phases.

During the acute phase of an infection, a naïve CD8+ T cell encounters its cognate antigen in the context of co-stimulatory molecules and an inflammatory milieu. After the initial encounter, a CD8+ T cell enters the expansion phase of the infection, undergoing rapid proliferation and differentiation into a cytotoxic T lymphocyte (CTL)². The primary function of a CTL is to secrete cytotoxic factors in order to kill and eliminate a target cell. When a cell in the body becomes infected with a foreign pathogen, a CTL can find and remove the cell. This process helps to stop the spread of the pathogen to the rest of the body. The granular cytotoxic molecule perforin is critical during a CD8+ T cell response against lymphocytic choriomeningitis virus (LCMV) and *Listeria monocytogenes* (LM)^{3, 4}. Another important CTL cytotoxic molecule used against pathogens is granzyme B⁵. This cytotoxic function peaks around day 7 of an infection⁶. In addition to cytotoxicity, a CTL secretes the pro-inflammatory cytokines

interferon-gamma (IFN- γ) and tumor necrosis factor alpha (TNF- α). After the expansion phase of the infection, CD8+ T cells enter the contraction phase, during which only a small percentage (~5-10%) of the activated CD8+ T cells will survive^{7, 8}. The cells that survive constitute the pool of functional memory CD8+ T cells.

CD8+ T cell Memory

Immunologic memory is an important aspect of the immune system, because it allows for a quicker response upon secondary encounter to the same foreign antigen. CD8+ T cells persist for several months after an infection, and maintain cytotoxic function. These memory CD8+ T cells protect a mouse against a secondary infection^{8, 9}. A memory CD8+ T cell can expand and produce cytotoxic molecules more rapidly than a naïve CD8+ T cell¹⁰. There are specific characteristics to distinguish CD8+ T cells being activated during the acute and expansion phases of an infection from those that survive and become memory cells. Interleukin-7 receptor alpha (IL-7R α or CD127) is an important receptor on naïve and memory CD8+ T cells. While IL-7 and its receptor CD127 are not required for CD8+ T cells expansion, they are very important for memory formation¹¹. During the acute phase of an infection, CD127 can be down-regulated on naïve CD8+ T cells. When this happens, cells up-regulate the effector molecule killer cell lectin-like receptor G-1 (KLRG-1)¹². These CD127^{low}KLRG-1^{high} cells are also known as short-lived effector cells (SLECs). SLECs are a subset of CD8+ T cells shown to be terminally differentiated and critical for killing infected cells during an infection. On the other hand, memory precursor effector cells (MPECs) do not lose CD127 expression or

up-regulate KLRG-1. Cells that maintain high CD127 expression will become part of the memory pool of CD8+ T cells during the contraction phase^{10, 13}.

Common Gamma Chain Cytokines and Receptors

CD127 belongs to the common gamma chain receptor family. There are several other important CD8+ T cell common gamma chain receptors including IL-2 receptor alpha (CD25), IL-2 receptor beta (CD122), and IL-2 receptor gamma (CD132). These receptors interact with the T cell cytokine IL-2, which is an important cytokine during all phases of an infection. IL-2 receptor signaling promotes both CD8+ T cell growth and differentiation. The receptors CD122 and CD132 are constitutively expressed on naïve CD8+ T cells and necessary for IL-2 signal transduction. Conversely, CD25 is induced on naïve CD8+ T cells upon activation. The trimeric high affinity IL-2 receptor (IL-2R $\alpha\beta\gamma$) is formed after CD25 up-regulation. This trimeric receptor increases affinity for IL-2 by 10³ to 10⁴ fold, which is required for physiologic IL-2 signaling in mice¹⁴. IL-2 can be secreted by CD8+ T cells or other lymphocytes. The ability of a CD8+ T cell to secrete IL-2 is dependent on both the initial TCR stimulation and co-stimulation. More specifically, either increasing TCR stimulation or CD28 co-stimulation or both will increase IL-2 secretion^{15, 16}. IL-2 helps CD8+ T cells after the initial antigen encounter, by promoting antigen-independent proliferation and expansion⁸. Intriguingly, the level of IL-2 secreted in the environmental milieu affects CD8+ T cell effector functions. *In vitro* activated CD8+ T cells in the presence of low levels of IL-2 have decreased cytotoxic activity. In addition, when CD8+ T cells are activated during LCMV infection without

IL-2 receptor signaling, they express less KLRG-1 and maintain higher CD127 expression¹⁷. IL-2 plays an important role to promote CD8+ T cell memory formation.

During an LCMV infection, CD8+ T cells separate into two different types of cells. Cells expressing low levels of CD25 are less sensitive to IL-2 and maintain higher CD127 expression. Conversely, cells that maintain high CD25 expression proliferate more rapidly, exhibit better effector function and are more terminally differentiated¹⁸. There is contradictory evidence about the requirement of IL-2 during CD8+ T cell expansion and memory formation *in vivo*. During the acute and expansion phases of either an LCMV or vesicular stomatitis virus (VSV) infection, CD8+ T cells proliferate in the absence of IL-2. While it is not critical for expansion, IL-2 is still important during the priming of a CD8+ T cell. During priming, IL-2 will program a CD8+ T cell to become a memory cell. In other words, IL-2 signaling during priming is required for a robust secondary response to infection^{16, 19}. Another group showed IL-2 is important for proliferation and short-lived (SLEC) formation, whereas memory cell (MPEC) formation was completely intact¹³. As mentioned previously, the availability of IL-2 in the microenvironment is important. When there are high levels of IL-2, cells will up-regulate CD25 and down-regulate CD127, which leads to an increase in effector cells rather than memory cells. In addition, CD127 down-regulation is determined by the amount of IL-2 present during activation¹⁷. The receptor CD122 is also important during activation and memory formation.

During the course of an infection, a CD8+ T cell will lose CD25 expression and express more CD122²⁰. CD122 expression promotes functional memory formation, because with CD215, it forms the IL-15 receptor complex²¹. IL-15 is a cytokine that

plays a critical role during CD8⁺ T cell memory development. The CD8⁺ T cell memory compartment is greatly reduced in IL-15R knockout (KO) mice. In addition, memory CD8⁺ T cells with high CD122 expression are dependent on IL-15 for both proliferation and survival^{7, 22-24}. IL-15 is an important cytokine for effector memory survival, because of the loss of CD127 expression²⁵. Many of the mechanisms described above are controlled by two CD8⁺ T cell transcription factors, T-bet and eomesodermin.

CD8⁺ T cell Transcription Factors

The T-box transcription factor T-bet, encoded by *tbx21*, is referred to as the “master regulator” of CD8⁺ T cell differentiation. The transcription factor eomesodermin (eomes) is another CD8⁺ T cell transcription factor, more important during memory formation. Both eomes and T-bet control the transcription of numerous CTL effector molecules such as IFN- γ , granzyme B and the homing receptor CXCR3^{26, 27}. CD8⁺ T cells that do not express T-bet secrete fewer effector cytokines and have diminished cytotoxicity against LCMV infection²⁸. Conversely, in the absence of T-bet, CD8⁺ T cells can protect against LM infection²⁹. The pro-inflammatory cytokine IL-12 is an important driver of T-bet up-regulation during the initial CD8⁺ T cell antigen encounter. IL-12 actually represses eomes expression while increasing T-bet expression³⁰. T-bet is associated with CD8⁺ T cells responding during the acute phase of an infection, whereas eomes is expressed after T-bet and increases over time in CD8⁺ T cells^{2, 31}. T-bet will be discussed further in Chapter 7. Even though eomes is associated with memory formation, mutations present in both T-bet and eomes of these leads to a

void in the CD8⁺ T cell memory response against LCMV²⁶. The characteristics described above are important for the basic function of a CD8⁺ T cell, but recent literature has shed light on other potential roles/functions of CD8⁺ T cells.

IL-17 Secreting T cells and Tumor Immunity

Helper T cells are also known as CD4⁺ T cells, and consist of different subsets including Th1, Th2, Th17 and Th22. Similarly, CD8⁺ T cells can be divided into different subsets. CD8⁺ T cells can be activated and skewed to secrete IL-4 (Tc2) or IL-22 (Tc22)³²⁻³⁴. More interestingly, like CD4⁺ T cells that secrete IL-17 (Th17), CD8⁺ T cells can secrete IL-17 (Tc17). One study showed CD8⁺ T cells co-cultured with bacterial exposed dendritic cells secrete IL-17³⁵. Another study showed CD8⁺ T cells without functional T-bet expression have an unusual inclination to secrete IL-17 during viral infection³⁶. Unlike traditional killer CD8⁺ T cells, there is contradicting data on Tc17 cell cytotoxicity^{33, 37-39}. If a Tc17 cell cannot kill, IL-17 secretion must be important for an effective anti-tumor response.

The role of IL-17 in the context of the tumor microenvironment is controversial. There is evidence to show IL-17 is pro-tumorigenic and increases tumor growth. One study showed IL-23, which is an important cytokine in the activation of IL-17 secreting T cells, was increased in the tumor microenvironment and promote tumor growth⁴⁰. IL-17 secreting T cells are increased in the tumor microenvironment of numerous mouse and human cancers, perhaps contributing to tumor pathogenesis⁴¹. Alternatively, studies have

shown adoptively transferred Th17 cells to slow or inhibit the growth of melanoma tumors^{42, 43}.

Similar to Th17 cells, the purpose and role of Tc17 cells in the tumor microenvironment is controversial. In a mouse model of chemical carcinogenesis, a subset of CD8+ T cells, so called “T-pro”, emerged. These T-pro cells secreted IL-17, expressed high levels of the transcription factor high retinoic acid-related orphan receptor (ROR γ T) and were non-cytotoxic. T-pro cells were associated with malignant progression⁴⁴. There is an association between increased percentages of Tc17 cells in tumor tissue and tumor progression in patients with hepatocellular carcinoma⁴⁵. In gastric cancer patients, higher percentages of Tc17 cells not only correlate with tumor progression but also promote myeloid derived suppressor cell formation⁴⁶. Conversely, Tc17 adoptive immunotherapy in mouse tumor models has shown promising results. In these studies, Tc17 adoptive T cell therapy led to tumor regression in both flank and lung melanoma models⁴⁷⁻⁴⁹. Tc17 cells can be found in other diseases and disease models, where the role is not completely understood.

Tc17 Cells

The role of Tc17 cells has yet to be determined, but there is evidence to support a similar role to Th17 cells. In the absence of CD4+ T cells, CD8+ T cells secrete IL-17 in response to fungal infection in mice, as a compensatory response^{50, 51}. Tc17 cells are depleted in both monkeys and humans infected with the immunodeficiency virus (SIV/HIV), suggesting an important role for these during infection⁵²⁻⁵⁴. In a model of

atopic dermatitis, when mice were depleted of CD4⁺ T cells, CD8⁺ T cells secreted IL-17 in the skin, again suggesting a compensatory role⁵⁵. Interestingly, this phenomenon was also shown in a mouse model of colitis. In the absence of major histocompatibility complex (MHC) II, mice infected with bacteria had increased percentages of endogenous Tc17 cells in the colon⁵⁶. In a mouse model of brain inflammation known as experimental autoimmune encephalomyelitis (EAE), Tc17 cells were found in both the draining lymph node and central nervous system (CNS)⁵⁷. In several human autoimmune diseases including psoriasis, rheumatoid arthritis, multiple sclerosis and lupus, Tc17 cells are located in both the peripheral blood mononuclear cells (PBMC) and tissue^{34, 58-62}. Infectious viral mouse models also show a small percentage of endogenous Tc17 cells^{63, 64}. One issue with studying Tc17 cells is they are usually a very small percentage of the total CD8⁺ T cell compartment, which limits the characterization of these cells.

Tc17 Induction

CD8⁺ T cells can be skewed *ex vivo* to produce IL-17 under specific conditions in large numbers. Several groups have shown *in vitro* generated Tc17 cells can mimic natural Tc17 cells by secreting large percentages of IL-17 and very little IFN- γ . They also express ROR γ T and low levels of the T-bet and eomes^{33, 37, 48, 65}. ROR γ T is the key transcription factor that influences the production of IL-17 in Th17 cells⁶⁶. The details of CD8⁺ T cell skewing to an IL-17 secreting phenotype will be discussed further in Chapter 3. The influence of various cytokines and transcription factors during activation of Tc17 cells has been studied. When IFN- γ KO CD8⁺ T cells are activated, they secrete more IL-17 than IFN- γ wild type (WT) CD8⁺ T cells^{37, 64, 67}. Suppressor of cytokine

signaling (SOCS) 3 inhibits Tc17 differentiation by inhibiting the critical modulator of IL-17 secretion, signal transducer and activator of transcription (STAT) 3⁶⁸. Similarly, activated interferon regulatory factor 3 (IRF3) KO CD8+ T cells secreted more IL-17 than the WT counterpart, showing a role of IRF3 in modulating Tc17 differentiation⁶⁹. In Th17 cells, IL-2 restricts Th17 development through the STAT5 signaling pathway⁷⁰. Some studies examined molecules required for Tc17 development, including IRF4. IRF4 KO CD8+ T cells are unable to skew to Tc17, showing the requirement for IRF4 in Tc17 differentiation⁷¹. These data address the requirements and role of specific factors during Tc17 induction, but not the function of Tc17 cells *in vivo*.

Tc17 Cell Adoptive Transfer Models

The function of Tc17 cells has been investigated through the use of animal models. One question is whether Tc17 cells could be important during an infection, other than compensation in the absence of CD4+ T cells. In a model of viral infection, *in vitro* generated Tc17 cells were adoptively transferred into mice infected with influenza. The adoptively transferred cells showed a protective advantage against lethal influenza infection. There was a reduction in this protective effect if IFN- γ KO Tc17 cells were adoptively transferred⁶³. During a vaccinia infection, adoptively transferred Tc17 cells are able to promote viral clearance similarly to Tc1 cells⁶⁴. As previously mentioned, natural Tc17 cells are present in multiple human autoimmune diseases, and they are often associated with inflammation and pathogenicity. In a self-antigen model where hemagglutinin (HA) is expressed in the lung, adoptively transferred antigen specific Tc17 cells caused lung pathology. This is in contrast to adoptively transferred IL-12 activated CD8+ T cells (Tc1), which were significantly less pathogenic³³. In a murine model of

diabetes, using the ectopic expression of OVA in the pancreatic islet cells, adoptively transferring antigen-specific Tc17 cells previously activated in the presence of IL-6 and TGF- β did not result in pancreatic pathogenicity. This was in contrast to IL-23-activated Tc17 cells, which were diabetogenic upon adoptive transfer³⁷. In a different diabetic model, where HA antigen is expressed in the pancreatic islet cells, adoptively transferred antigen specific Tc17 cells were non-pathogenic. Interestingly, upon simultaneous transfer with Th1 cells, Tc17 became pathogenic, causing hyperglycemia and death in the mice⁶⁵.

As previously mentioned, Tc17 adoptive T cell therapy in tumor models has shown promising results. In two separate studies, antigen-specific Tc17 cells were adoptively transferred into mice bearing B16 tumor in the flank. In one of the studies, adoptive transfer with Tc17 cells led to a superior anti-tumor response when compared to transfer with non-polarized CD8+ T cells⁴⁸. In the other study, even though Tc17 cells caused tumor regression, adoptive transfer with Tc1 cells showed a superior anti-tumor response compared to Tc17 cells⁴⁷. Tc17 cells have an anti-tumor response when adoptively transferred into mice bearing metastatic melanoma, where B16 is administered intravenously and forms lung metastases. Similar to other studies, adoptive transfer with Tc1 cells led to a superior response compared to Tc17 cells. Surprisingly, this was not seen when the tumor cells did not express the IFN- γ receptor. In the absence of the IFN- γ R on the tumor, Tc17 cells provided a better anti-tumor response than adoptively transferring Tc1 cells⁴⁹. In one flank tumor study, adoptively transferred IFN- γ KO or TNF- α KO Tc17 cells had a weaker anti-tumor response compared to IFN- γ WT or TNF- α WT Tc17 cells⁴⁷. This was also seen in an infection model, where adoptively

transferred IFN- γ KO Tc17 cells were unable to protect mice against lethal influenza infection⁶³. IFN- γ secretion seems to be important for an effective Tc17 anti-tumor response.

Adoptively transferred Tc17 cells expressing both IL-17 and IFN- γ had a superior anti-tumor response compared to Tc17 cells expressing IL-17 alone in mice bearing lymphoma in the flank³⁸. There is contradicting evidence on the importance of Tc17 cells switching from IL-17 to an IFN- γ production in the context of an effective anti-tumor response⁴⁷⁻⁴⁹. Tc17 cells can produce IFN- γ in the context of both infection and autoimmunity. These topics will be discussed further in chapter 5. This conversion also occurs in Th17 cells, and is also known as cellular plasticity.

T Cell Plasticity

Th17 plasticity has been shown in B16 tumor models. When Th17 cells were adoptively transferred into B16 tumor bearing mice, they switch to produce IFN- γ . Interestingly, Th17 cells that could not produce IFN- γ did not promote an effective anti-tumor response⁷². Th17 plasticity has also been shown in specific autoimmune mouse models. The role of conversion is still controversial in autoimmune pathogenicity. In one study, Th17 cells that were unable to produce IFN- γ did not affect EAE pathogenesis. A different study showed Th17 cells that could not produce IFN- γ actually ameliorated EAE pathogenesis⁷³⁻⁷⁵. In an autoimmune diabetes model, only IFN- γ producing Th17 cells conferred diabetic pathogenesis⁷⁶. Previously activated Th17 cells cultured *in vitro* in the presence of pro-inflammatory cytokines can be re-programmed to secrete IFN- γ ⁷⁷. Similarly to Th17 cells, Tc17 cells cultured in the presence of pro-inflammatory

cytokines have been shown to re-program and secrete IFN- γ *in vitro*. This was also dependent on specific transcription factors^{38, 64, 68}. *In vitro* plasticity of Tc17 cells will be discussed further in Chapter 4.

The factors that affect Tc17 cell plasticity remain unclear. IL-12 has been shown to be an important player in Tc17 plasticity, but perhaps there are other cytokines or factors that can influence Tc17 plasticity. Gamma chain cytokines and receptors play an important role during CD8⁺ T cell immunity to infection, but the role of gamma chain cytokines in Tc17 plasticity has yet to be elucidated. Transcription factors are required for Th17 plasticity, but the requirements for Tc17 plasticity are still unknown. In addition, Th17 plasticity is a critical component in both autoimmune pathogenesis and anti-tumor response. The role of Tc17 plasticity during an anti-tumor response is still unknown. In the following study, we sought to characterize factors that drive Tc17 plasticity, define the requirements for Tc17 plasticity and determine the role of Tc17 plasticity in effective anti-tumor immunity.

CHAPTER 2

MATERIALS & METHODS

Mice

C57BL/6 (WT) and C57BL/6-Tg (CAG-OVA) 916Jen/J mice were purchased from Jackson Laboratories. B6-LY5.2/Cr mice were purchased from NCI Fredrick Laboratories. OT-1/RAG KO transgenic mice on a C57BL/6 (H-2^b) background were originally obtained from Dr. H. Levitsky (Johns Hopkins University). These mice express a transgenic TCR V β 2 and V β 5 specific for the SIINFEKL peptide of OVA in the context of MHC Class I. Homozygous IL-2 KO and T-bet KO were purchased from Jackson Laboratories and bred onto the OT-1/RAG KO background. OT-1, cytokine/transcription factor-deficient mice were selected from the F2 generation. OT-1/IL-12R β 1 transgenic mice were kindly provided by J. Harty (U Iowa).

Flow Cytometry

Monoclonal antibodies with the following specificities were used in this study: CD8 (5H10 & 53-6.7; Life Technologies), CD25 (7D4 & PC61.5; eBioscience), CD44 (IM7; eBioscience), CD45.2 (104; Biolegend), CD62L (MEL-14; Biolegend), CD122 (TM-b1; Biolegend), CD127 (A7R34; Biolegend), eomesodermin (Dan11mag; eBioscience), IL-17a (TC11-18H10.1; Biolegend), IFN- γ (XMG1.2; Biolegend), KLRG-1 (2F1; eBioscience), Live/Dead Fixable (Invitrogen), ROR γ T (B2D; eBioscience), T-bet (eBio4B10), Thy1.1 (OX-7; BD Pharmingen). To analyze the cytokine production and transcription factors of cells for *in vitro* studies, cells were stimulated with PMA (phorbol 12-myristate 13-acetate; 50 ng/mL; Sigma) and ionomycin (0.5 ug/mL; Sigma) for 4 hours. For analysis of the cytokine production and transcription factors of adoptively transferred cells, cells were stimulated with pOVA (10 ug/mL) for 4 hours. Protein

transport inhibitor cocktail was added during all stimulations (eBioscience). Cells were not stimulated for extracellular staining analysis. Samples were acquired on a BD LSR flow cytometer (BD Biosciences) and FlowJo software (Tree Star) was used for data analysis.

Differentiation of CD8⁺ T cells

OT-1+/RAG KO spleen and lymph node cells were collected, pooled and activated with pOVA (1 ug/mL) and cultured for 5 days in the presence of mIL-1 β (20 ng/mL; Shenandoah Biotechnology, Inc), mIL-6 (20 ng/mL; Shenandoah Biotechnology, Inc), mIL-23 (20 ng/mL, eBioscience), hTGF- β 1 (5 ng/mL; Shenandoah Biotechnology, Inc), anti-mouse IL-4 antibody (10 ug/mL; 11B11; NCI), anti-mouse IFN- γ antibody (10 ug/mL; XMG1.2; BioXCell) and anti-mouse CD28 antibody (1 ug/mL; 37.51) for the generation of Tc17 cells. OT-1+/RAG KO spleen and lymph node cells were pooled and activated with pOVA (1 ng/mL) and cultured for 4 days in the presence of mIL-2 (1 ng/mL; Peprotech), hIL-12 (10 ng/mL; Peprotech), anti-mouse IL-4 antibody (10 ug/mL; 11B11; NCI) and anti-mouse CD28 antibody (1 ug/mL; 37.51). In reactivation cultures, differentiated Tc17 cells were sorted on day 5 and then cultured in the presence of mIL-2 (20 ng/mL), mIL-7 (20 ng/mL), mIL-12 (10 ng/mL), or mIL-15 (20 ng/mL). The sorted cells were also cultured with soluble anti-CD28 (1 ug/mL) and the previous mentioned cytokines on anti-CD3 (2 ug/mL) coated plates. Sorted Tc17 cells were cultured for 4 days and then analyzed for cytokine production, transcription factor expression and cell surface receptor expression.

Tc17 Sorting

After *in vitro* polarization and re-stimulation with PMA (100 ng/mL) and ionomycin (1 ug/mL) for 2 h, IL-17 or IFN- γ -secreting CD8⁺ T cells were stained with bi-specific CD45/cytokine mAbs according to the manufacturer's protocol (Miltenyi Biotec) and then sorted using a BD FACSAria instrument. 7-AAD was utilized to exclude dead cells and CD8⁺ T cells were sorted to >97% pure IL-17+IFN- γ - cells.

Quantitative Real-Time Polymerase Chain Reaction (qPCR)

mRNA was extracted from T cells with TRIzol Reagent (Life Technologies) and the TRIzol RNA Isolation protocol. cDNA was synthesized with a RNA to cDNA EcoDry Premix kit (Clontech). All primers were purchased from Life Technologies-Applied Biosystems; reactions were performed in triplicate using an Applied Biosystems StepOnePlus Instrument.

Recombinant Vaccinia Virus

A recombinant vaccinia virus (VV) encoding full-length ovalbumin (OVA) was provided by Dr. D. Pardoll. VV-OVA was propagated in TK- cells, purified from cell lysates by sucrose banding, titered by plaque assay and stored at a concentration of 1×10^8 PFU/mL in a -80°C freezer. VV-OVA was thawed on ice and diluted in PBS to 1×10^6 PFU per mouse in a volume of 200 uL. This was co-administered with Tc cells by retro-orbital injection.

Adoptive Cell Transfers

For adoptive transfer experiments, 1×10^6 - 2×10^6 *in vitro* generated Tc17 cells were adoptively transferred into CAG-OVA, RAG KO or CD45.1+ mice in 200 uL PBS via retro-orbital injection. Cells were harvested 3 days later from CAG-OVA mice or 7 days later from RAG KO or CD45.1+ mice. For infection models, 1×10^6 PFU recombinant VV-OVA was adoptively transferred with $1-2 \times 10^6$ *in vitro* generated Tc17 cells into CD45.1+, C57B/6 or IL-2 KO/RAG KO mice. These injections were in 200 uL PBS via retro-orbital injection. Cells were harvested 7 days later.

Isolation of Tissue Lymphocytes

Spleen, lymph node, lung and liver lymphocytes were isolated as followed: all tissues were harvested and single cells suspensions were obtained by mechanical disruption. Liver lymphocytes were re-suspended in a Percoll Plus (GE Healthcare) gradient. The lymphocyte buffy coat was removed and washed in PBS. For cytokine and transcription factor intra cellular staining (ICS), cells were stimulated with pOVA (10 ug/mL) for 4 hours. Protein transport inhibitor cocktail was added during all stimulations (eBioscience). Cells were not stimulated for extracellular staining analysis.

***In Vivo* Killing Assay**

5×10^5 Tc1 and Tc17 cells were injected into CD45.1+ recipients without VV-OVA or 1×10^4 - 2×10^5 cells were injected into CD45.1+ recipients infected with VV-OVA. Five days after Tc cell injection, recipient mice were injected with 5.5×10^6 SIINFEKL-pulsed splenic cells stained with 5 uM CFSE, and 4.5×10^6 non-pulsed splenic cells stained with

0.5 μ M CFSE. Eighteen hours later, spleen cells were harvested and the ratio of live CFSE^{hi} to CFSE^{lo} cells was determined using flow cytometry.

Adoptive T cell Immunotherapy Model

For tumor challenge, female CD45.1 mice received a subcutaneous injection of 1.6×10^5 B16F10-OVA melanoma cells. On day 14, mice with palpable, established tumors (100-250 mm³ respectively) received a retro-orbital injection of 5×10^6 *in vitro* generated Tbet WT or Tbet KO Tc17 cells with or without VV-OVA. Control groups received VV-OVA alone or no treatment. Tumor volume was calculated by using the following formula: tumor volume (mm³) = (length) x (width)² x 0.5. Mice with tumor volumes that equaled or exceeded 1500 mm³ were humanely sacrificed.

Isolation of Tumor-Infiltrating Lymphocytes (TIL)

Individual subcutaneous tumors were harvested from mice and wet weights measured. Single cells suspensions were obtained by mechanical disruption. Tumor cells were washed 3-4 times in PBS followed by re-suspension in a Percoll Plus (GE Healthcare) gradient. The lymphocyte buffy coat was removed and washed in PBS. For cytokine and transcription factor intra cellular staining (ICS), cells were stimulated with pOVA (10 μ g/mL) for 4 hours. Protein transport inhibitor cocktail was added during all stimulations (eBioscience). Cells were not stimulated for extracellular staining analysis.

Statistical Analysis

Statistical significance was calculated by unpaired Student's t test with Prism software. All p values <0.05 were considered significant.

CHAPTER 3

PHENOTYPE OF *IN VITRO* GENERATED TC17 CELLS

INTRODUCTION

As mentioned in Chapter 1, similar to the Th17 subset, CD8⁺ T cells secrete IL-17 in different diseases. However, their usual small numbers preclude extensive phenotyping and functional analyses. In order to circumvent this issue, the skewing conditions to promote CD8⁺ T cell secretion of IL-17 have been studied^{33, 37, 63}. Similar to Th17 cell skewing, TGF- β and IL-6 are critical for Tc17 differentiation. IL-1 β and IL-23 are dispensable, but do increase IL-17 secretion. It is critical to block IFN- γ during initial activation to shift the production from IFN- γ towards IL-17 secretion. Normally during CD8⁺ T cell activation, IL-2 is added to promote proliferation and growth, but it has been shown that IL-2 actually inhibits IL-17 production^{33, 37, 63}. If CD8⁺ T cells are activated under optimal conditions, they can secrete upwards of 90% IL-17 with very little IFN- γ production. In this chapter we examined characteristics of *in vitro* generated Tc17 cells. In order to define these characteristics, we skewed CD8⁺ T cells under IL-17 promoting conditions *in vitro*. We hypothesized we would obtain high percentages of Tc17 cells.

The expression profile of Tc17 cell transcription factors has been studied by using qPCR, Tc17 cells express much higher levels of ROR γ T compared to a Tc1 cell. Interestingly, T-bet is expressed slightly less or equivocally between Tc17 cells and Tc1 cells^{33, 48, 68}. We wanted to investigate the protein expression of transcription factors in Tc17 cells by flow cytometry. We hypothesized, similar to the expression of mRNA transcripts, Tc17 cells analyzed by flow cytometry would express high percentages of ROR γ T protein. Additionally, we hypothesized Tc17 cells would express very little T-

bet or eomes protein. In order to do this we skewed CD8⁺ T cell and stained for ROR γ T, T-bet and eomes.

The expression of cell surface receptors on Tc17 cells has not been studied extensively, but similarly to Th17 cells, Tc17 cells up-regulate the IL-23 receptor and CCR6 during activation. Additionally, the IL-12 receptor is expressed on Tc17 cells but less than Tc1 cells^{33, 37}. We wanted to determine the expression of the common gamma chain receptors on Tc17 cells. We hypothesized they would express CD127, but not the high affinity IL-2 receptor. In order to do this we skewed CD8⁺ T cells towards IL-17 secretion and stained CD25, CD122 and CD127.

As mentioned in Chapter 1, one of the important characteristics of a Tc17 cell is their decreased cytotoxic activity compared to Tc1 cell or CTL. The ability of Tc17 cells to kill *in vitro* and *in vivo* remains controversial^{63, 64, 78}. We sought to determine the cytotoxicity of the Tc17 cells we generated *in vitro*. In order to do this we used an *in vivo* killing assay to compare the specific lysis of *in vitro* generated Tc17 cells compared with *in vitro* generated Tc1 cells.

RESULTS

Cytokine Profile of Skewed Tc17 Cells

To investigate the characteristics of *in vitro* generated Tc17 cells, we activated OT-1 CD8⁺ T cells under IL-17 polarizing conditions. We verified the cytokine production by flow cytometry and found the activation to yield >90% IL-17+IFN- γ - Tc17 cells (Figure 3-1).

***In vitro* Generated Tc17 Cells have an Activated Phenotype**

To determine the expression of the common gamma chain receptors on the Tc17 cells we stained CD25, CD122 and CD127. We found after activation Tc17 cells maintained CD127 expression. Tc17 cells had increased CD25 expression similarly to Tc1 generated OT-1 cells. Tc17 cells expressed significantly less CD122 than Tc1 cells (Figure 3-2). We next studied whether this receptor expression mirrored the transcriptional level by qPCR. We found CD25 was similar to protein expression on the RNA transcript level, but there was no difference between the CD122 RNA levels of Tc1 and Tc17 cells. Tc17 cells had significantly more CD127 and CCR7 transcript than Tc1 cells, again suggesting a more memory phenotype. The RNA transcript levels of the IL-12 receptor was not different between Tc1 and Tc17 cells, but Tc17 cells expressed more CCR6, a common receptor found on Th17 cells. In addition, Tc17 cells express less CXCR3 than Tc1 cells, a common receptor found on Th1 cells (Figure 3-3).

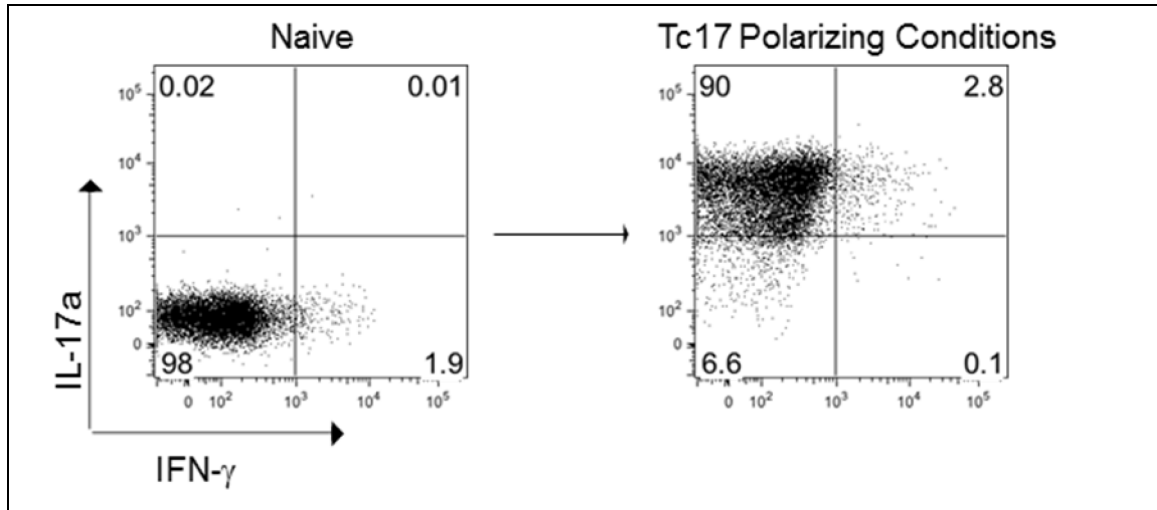
***In Vitro* Generated Tc17 Cells are ROR γ T^{hi}, Tbet^{lo} and Minimally Cytotoxic**

In order to further characterize the Tc17 cells, we examined the expression of transcription factors profile in activated T cells. We performed qPCR analysis and found that Tc17 cells had significantly more ROR γ T transcript and less T-bet transcript compared to Tc1 cells, which was confirmed on the protein level by flow cytometry. Tc17 cells down-regulated comes on the transcriptional level significantly more than Tc1 cells, again shown on the protein level (Figures 3-4). To determine Tc17 cytotoxicity, we performed an *in vivo* killing assay and showed Tc17 cells can kill, but significantly less than Tc1 cells (Figure 3-5).

SUMMARY

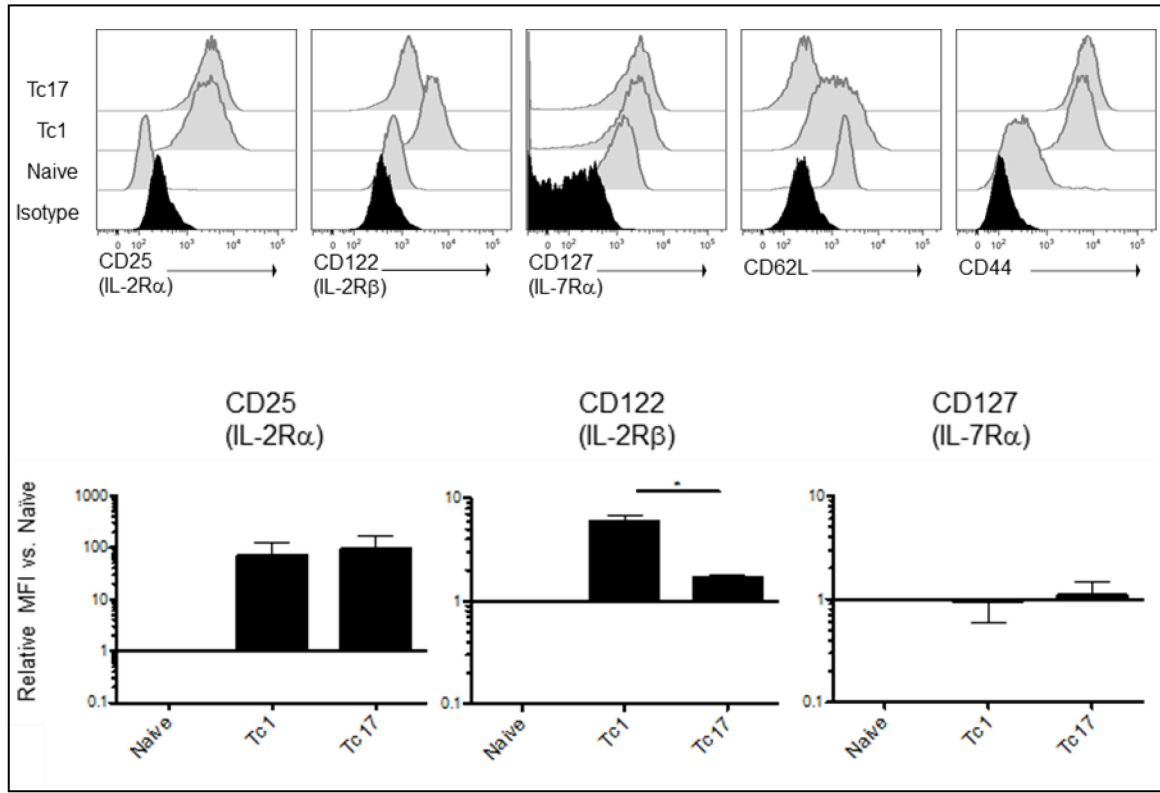
We polarized naïve CD8⁺ T cells *in vitro* to secrete >90% IL-17 with very little IFN- γ secretion. In addition, Tc17 cells expressed the traditional Th17 transcription factor ROR γ T, and had very low expression of the Th1 transcription factors T-bet and Eomes. This suggests Tc17 cells could have a long-term memory phenotype²⁶. Both Tc1 and Tc17 cells expressed CD44 and CD25 upon activation, but did not lose CD127 expression. This suggests a predilection toward a memory phenotype¹⁰. High CD25 expression suggests Tc17 cells have an activated phenotype, which may play an important role in their plasticity. Interestingly, Tc17 cells expressed significantly less CD122 as compared to Tc1 cells, suggesting a diminished high affinity IL-2 receptor signaling complex¹⁵. This was not reflected on the RNA level, suggesting post-translational control of CD122 expression. Both Tc1 and Tc17 cells expressed the IL-12 receptor transcript, suggesting the Tc17 skewing conditions did not alter CD8⁺ T cell affinity for IL-12. We saw Tc17 cells kill significantly less than Tc1 cells, suggesting they are not as effective at normal CTL functions.

Figure 3 - 1: Flow Cytometry, Cytokine Profile of Tc17 Cells



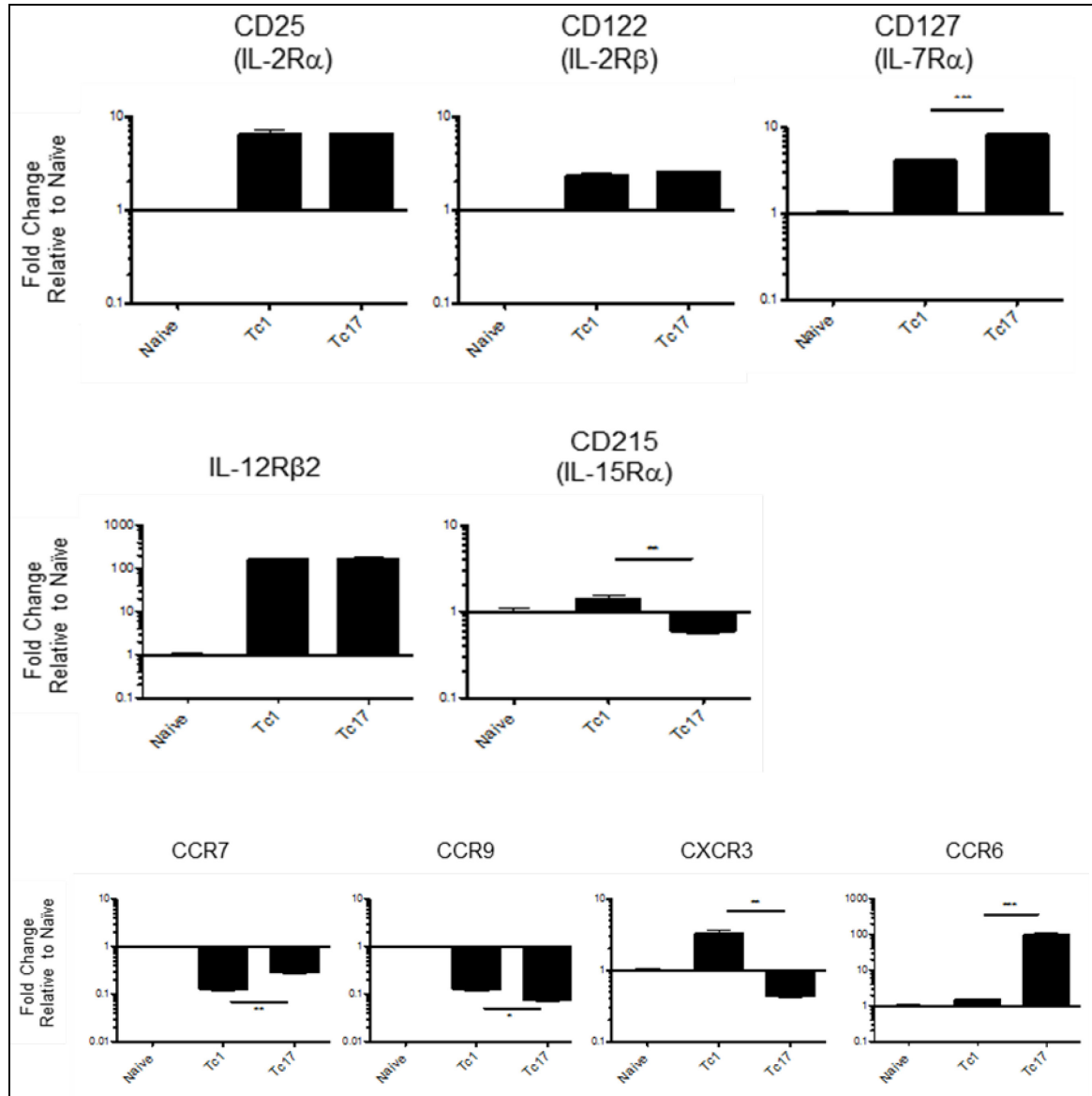
OT-1+/RAG KO spleen and lymph node cells were collected, pooled and activated with OVA peptide and cultured for 5 days in the presence of mIL-1 β , mIL-6, mIL-23, hTGF- β 1, anti-mouse IL-4 antibody, anti-mouse IFN- γ antibody and anti-mouse CD28 antibody. For analysis of cytokine production, cells were stimulated with PMA and ionomycin. Protein transport inhibitor cocktail was added during stimulation. Cytokine staining was analyzed using intracellular staining and flow cytometry.

Figure 3 – 2: Flow Cytometry, Receptor Profile of *In Vitro* Activated CD8+ T cells



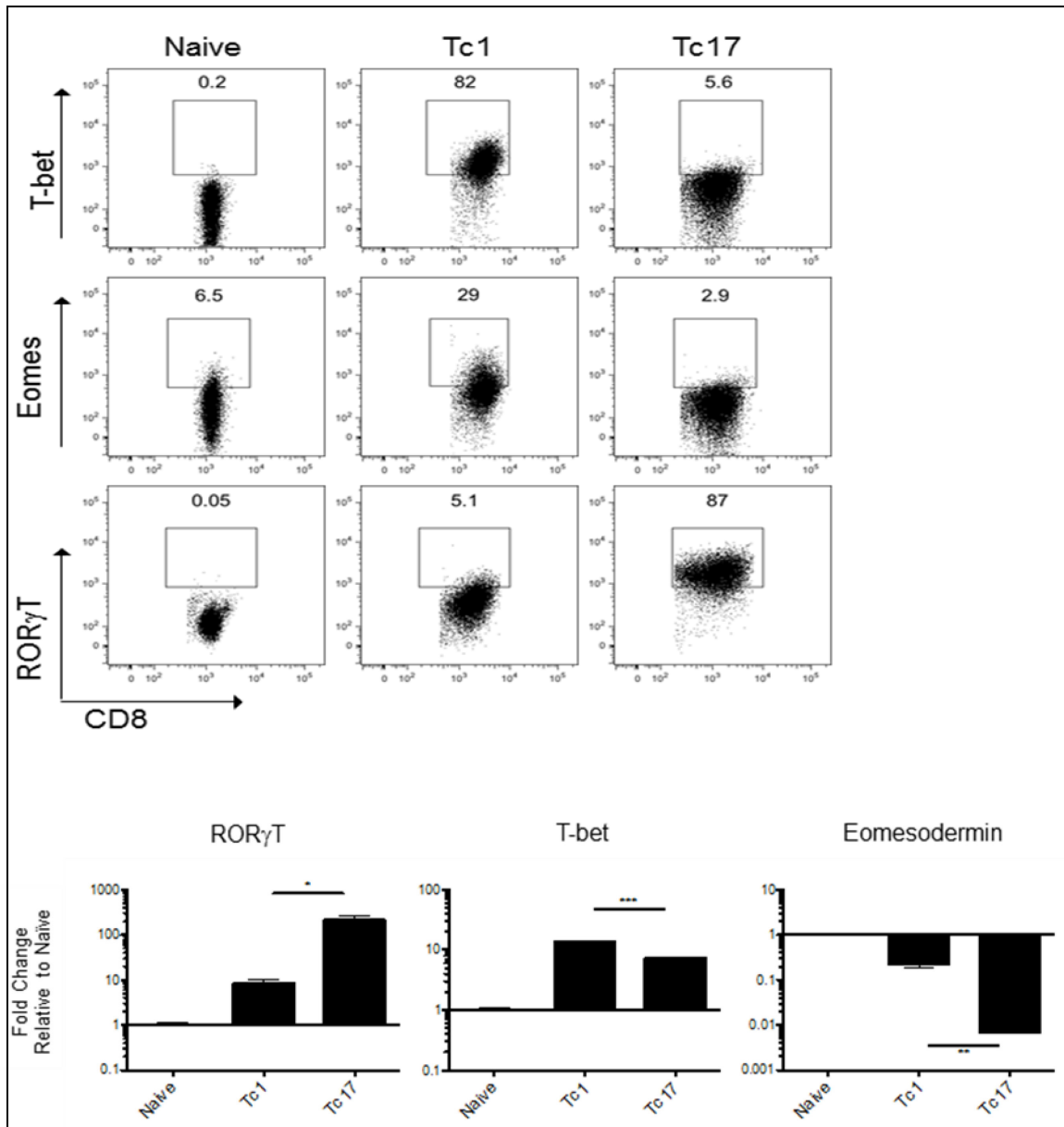
OT-1+/RAG KO spleen and lymph node cells were collected, pooled and activated with OVA peptide and cultured for 5 days in the presence of mIL-1 β , mIL-6, mIL-23, hTGF- β 1, anti-mouse IL-4 antibody, anti-mouse IFN- γ antibody and anti-mouse CD28 antibody. Receptors were analyzed using extracellular staining and flow cytometry. The values represent the means \pm SEM (n=2). *p<0.05

Figure 3 – 3: qPCR, Receptor Profile of *in vitro* Activated CD8+ T cells



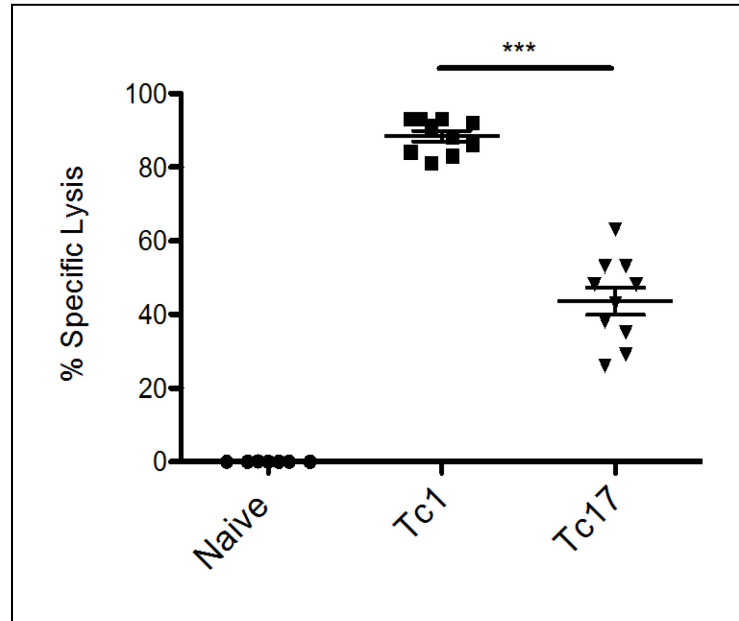
OT-1+/RAG KO spleen and lymph node cells were collected, pooled and activated with OVA peptide and cultured for 5 days in the presence of mIL-1 β , mIL-6, mIL-23, hTGF- β 1, anti-mouse IL-4 antibody, anti-mouse IFN- γ antibody and anti-mouse CD28 antibody. mRNA was extracted from T cells and cDNA synthesized. qPCR was performed in triplicate. The values represent the means \pm SEM (n=3). *p<0.05, **p<.01, ***p<.001

Figure 3 – 4: Transcription Factor Profile of *In Vitro* Activated CD8+ T cells



OT-1+/RAG KO spleen and lymph node cells were collected, pooled and activated with OVA peptide and cultured for 5 days in the presence of mIL-1 β , mIL-6, mIL-23, hTGF- β 1, anti-mouse IL-4 antibody, anti-mouse IFN- γ antibody and anti-mouse CD28 antibody. For analysis of transcription factors, cells were stimulated with PMA and ionomycin. Protein transport inhibitor cocktail was added during stimulation. Transcription factor staining was analyzed using intracellular staining and flow cytometry. mRNA was extracted from T cells and cDNA synthesized. qPCR was performed in triplicate. The values represent the means \pm SEM (n=2). *p<0.05, **p<.01, ***p<.001

Figure 3 – 5: *In vivo* Killing Assay Comparing *In Vitro* Activated CD8+ T cells



In vitro generated Tc1 and Tc17 cells were prepared and 5×10^5 cells were injected into CD45.1+ recipients without VV-OVA. Five days after Tc cell injection, recipient mice were injected with 5.5×10^6 SIINFEKL-pulsed splenic cells stained with 5 uM CFSE, and 4.5×10^6 non-pulsed splenic cells stained with 0.5 uM CFSE. Eighteen hours later, spleen cells were harvested and ratio of live CFSE^{hi} to CFSE^{lo} cells was determined using flow cytometry. ***p<.001

CHAPTER 4

TC17 PLASTICITY *IN VITRO*

INTRODUCTION

One of the important characteristics of both Th17 and Tc17 cells is the capacity to switch from IL-17 to IFN- γ secretion. This is in stark contrast to Th1 and Tc1 cells, which do not switch from IFN- γ to IL-17 production. Previously activated Th17 cells cultured in the presence of the pro-inflammatory cytokines IL-12 or IL-23 can be re-programmed to secrete IFN- γ . This plasticity is dependent on STAT4, which is an important transcription factor for IFN- γ secretion⁷⁷. Similarly to Th17 cells, Tc17 cells cultured in the presence of IL-12 secrete IFN- γ *in vitro*^{64, 68}. This was dependent on STAT4, as well, as T-bet. IFN- γ secretion by Tc17 cells was concomitant to increased expression of T-bet but not ROR γ T which remained unchanged. Interestingly, previously skewed Tc17 cells re-cultured in the presence of IL-2 did not switch their phenotype³⁸.

In this chapter we addressed the factors that affect Tc17 plasticity *in vitro*. We sought to investigate Tc17 plasticity using a homogenous population of Tc17 cells. It was important to ensure Tc17 cells that switched from IL-17 to IFN- γ secretion came from a population of only IL-17 secreting cells. In order to do this we sorted Tc17 cells to obtain a pure IL-17+IFN- γ - population.

Although IL-12 affects plasticity, we investigated other factors that could affect conversion^{64, 68}. In Chapter 2, we showed Tc17 cells express CD25, CD127 but down-regulated CD122 and CD215. We hypothesized IL-2 and IL-7 would cause Tc17 cells to secrete IFN- γ , but IL-15 would not. In Chapter 2 we also demonstrated Tc17 cells expressed the IL-12 receptor. We hypothesized IL-12 would affect Tc17 plasticity. In order to test this we cultured sorted Tc17 cells in IL-2, IL-7, IL-12 and IL-15 and stained

for cytokines. When Tc17 cells are adoptively transferred into mice bearing their cognate antigen, they convert to an IFN- γ secretion phenotype^{48, 64}. We examined the role of the T cell Receptor (TCR) stimulation in Tc17 plasticity. We hypothesized TCR stimulation would affect Tc17 plasticity. In order to do this we cultured sorted Tc17 cells with CD3 and CD28 and stained for cytokines.

The gamma chain receptor expression of Tc17 upon conversion has not been shown. We hypothesized Tc17 cells would maintain CD127 expression upon conversion with cytokines, but lose CD127 expression in the presence of TCR stimulation. We also hypothesized Tc17 cells would express the high affinity IL-2 receptor upon conversion *in vitro*. In order to test these hypotheses we cultured sorted Tc17 cells with specific cytokines and TCR stimulation and stained for CD25, CD122 and CD127.

We then sought to investigate the transcription factor expression of Tc17 cells upon conversion. We hypothesized Tc17 cells would lose ROR γ T expression and have increased T-bet and eomes expression upon conversion. In order to test this hypothesis we cultured sorted Tc17 cells with specific cytokines and TCR stimulation and stained for T-bet, ROR γ T and eomes.

RESULTS

Tc17 Post-Sort Purity

In order to perform these studies, we chose to start with an absolutely pure IL-17+IFN- γ - population of cells. In order to test this we sorted the *in vitro* generated Tc17 cells using a cytokine capture assay, where the cells are not permeabilized and can be used for further studies (Figure 4-1). We determined Tc17 cells can be sorted to a >98% pure IL-17+IFN- γ - population.

IL-2, IL-7 and IL-12 Determine Tc17 Conversion *in vitro*

Based on the data in Figures 3-2 and 3-3, we hypothesized common gamma chain cytokines would play a critical role in Tc17 plasticity. To test this hypothesis, sorted Tc17 cells were cultured in specific cytokines in the absence of TCR stimulation. We found IL-2 and IL-7 but not IL-15 drove pure Tc17 cells to produce IFN- γ . This result was obtained in the absence of any further TCR stimulation. In addition, IL-12 by itself drove Tc17 plasticity (Figure 4-2).

Conversion does not Dictate Receptor Expression

We sought to determine the expression of gamma chain receptors of cultured Tc17 cells. In order to do this, we cultured sorted Tc17 cells with specific cytokines without TCR stimulation. Tc17 cells cultured with IL-15 were in a more quiescent state, and lost the expression of the high affinity IL-2 receptor. In the presence of IL-2, Tc17 cells maintained CD25 expression and had a significant increase in CD127 expression as compared to sorted Tc17 cells. In contrast, IL-7, IL-12 and IL-15 all led to a significant

decrease in CD25 expression. Tc17 cells cultured in the presence of IL-7 caused a significant decrease of CD127 as compared to sorted Tc17 cells, suggesting a more terminal effector state (Figure 4-3)

Conversion is Associated with T-bet Expression

Next we determined the transcription factor expression of the cultured Tc17 cells. In order to do this we cultured sorted Tc17 cells without TCR stimulation in the presence of specific cytokines. In the presence of IL-2 or IL-7, even though the cells secreted IFN- γ , ROR γ T expression did not change. Oppositely, Tc17 cells cultured with IL-12 had decreased ROR γ T expression. Eomes was not affected by conversion. Most interestingly, conversion was associated with increased T-bet expression, which is a major transcription factor for IFN- γ (Figure 4-4).

TCR Stimulation Determines Tc17 Cell Conversion *in vitro*

In order to further understand the requirements for plasticity *in vitro*, we sought to combine TCR stimulation with specific cytokines. In order to do this, we again chose to start with an absolutely pure IL-17+IFN- γ - population of cells (>98% pure). Sorted Tc17 cells were cultured in the presence of plate bound CD3 and soluble CD28 in combination with or without only those cytokines we know to drive conversion. In the presence of TCR stimulation alone, Tc17 cells secreted very little IFN- γ . However, the addition of IL-2, IL-7 or IL-12 drove Tc17 cells to produce IFN- γ . The effect was only minimally synergistic and, similar to results we showed in Figure 4-2, IL-12 had the biggest effect on Tc17 conversion (Figure 4-5).

Conversion with TCR Stimulation does not Dictate Receptor Expression

Next we examined the role of TCR stimulation and cytokine addition on the expression of common gamma chain receptors. In order to do this we cultured sorted Tc17 cells with CD3 and CD28 in combination with specific cytokines. Compared to sorted Tc17 cells, Tc17 cells cultured with TCR stimulation alone or in combination with IL-12 had decreased CD25 and CD122, but increased CD127 expression. Contrarily, addition of IL-2 with TCR stimulation did not significantly change any of the receptors. Similar to results seen in Figure 4-3, TCR stimulation in the presence of IL-7 led to decreased CD127 expression (Figure 4-6).

Conversion with TCR Stimulation is Associated with T-bet Expression

We next investigated the transcription factor expression in these TCR stimulated cells. In order to do this we cultured sorted Tc17 cells with CD3 and CD28 in combination with specific cytokines. TCR stimulation alone increased T-bet expression of Tc17 cells, as well as, TCR stimulation in combination with IL-2, IL-7 and IL-12. Tc17 cells cultured in presence of IL-12 and TCR stimulation had decreased ROR γ T expression similar to results shown in Figure 4-4. Additionally, there was no effect on eomes during conversion (Figure 4-7).

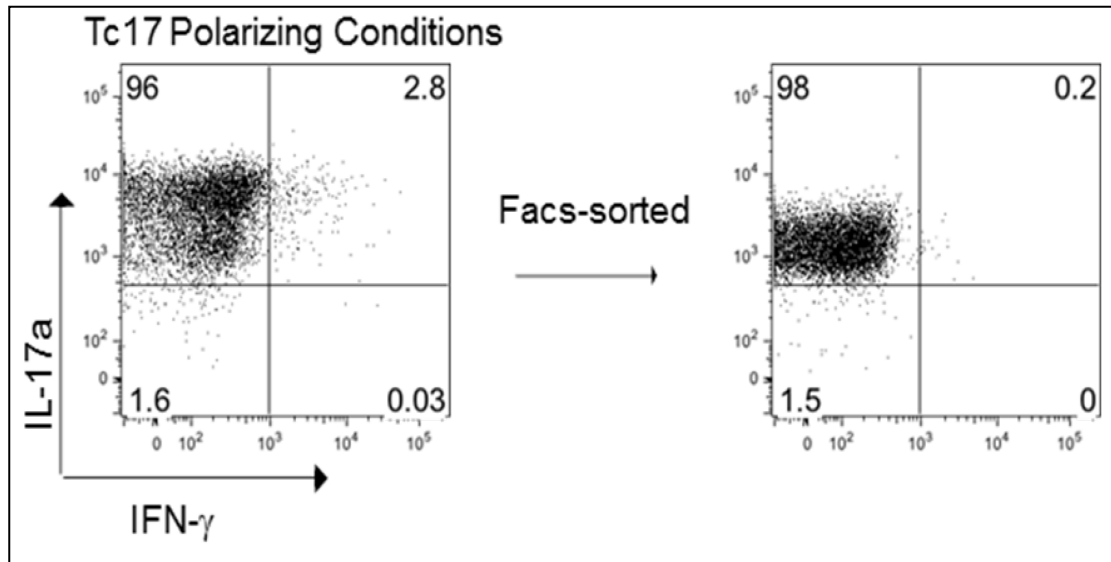
SUMMARY

The cytokine capture assay is a valuable tool to purify IL-17+IFN- γ - Tc17 cells for further investigation *in vitro*. Sorted Tc17 cells cultured in the presence of IL-2, IL-7 or IL-12 produced IFN- γ . IL-12 being the most potent driver of conversion, with the greatest percentage of an IFN- γ single positive population. From Chapter 3, we know Tc17 cells expressed CD25, CD127 and the IL-12 receptor. This expression was important for Tc17 cell conversion *in vitro*. In addition, skewed Tc17 cells had decreased expression of the IL-15 receptor, predicting IL-15 would not be important for Tc17 conversion. Finally, TCR stimulation was minimally important for Tc17 conversion. This suggests the functional plasticity of Tc17 was driven by cytokines and the equivalent cytokine receptor expression of these cells.

Sorted Tc17 cells stimulated with TCR alone or TCR with IL-12 had decreased CD25 and CD122 expression compared to sorted Tc17, but increased CD127 expression. This suggests a Tc17 cells stimulated with TCR have a long lived memory phenotype. In the presence of IL-2, Tc17 cells maintained CD25 expression but had increased CD127 expression. This was in comparison to sorted Tc17 cells, suggesting a maintained activated state, with potential for long lived memory. Contrarily, addition of IL-2 with TCR stimulation did not significantly change any of the receptors, suggesting a continued activated state or terminal effector differentiation. We found IL-7 with or without TCR stimulation decreased CD127 expression, suggesting a more terminal effector phenotype^{10, 18}.

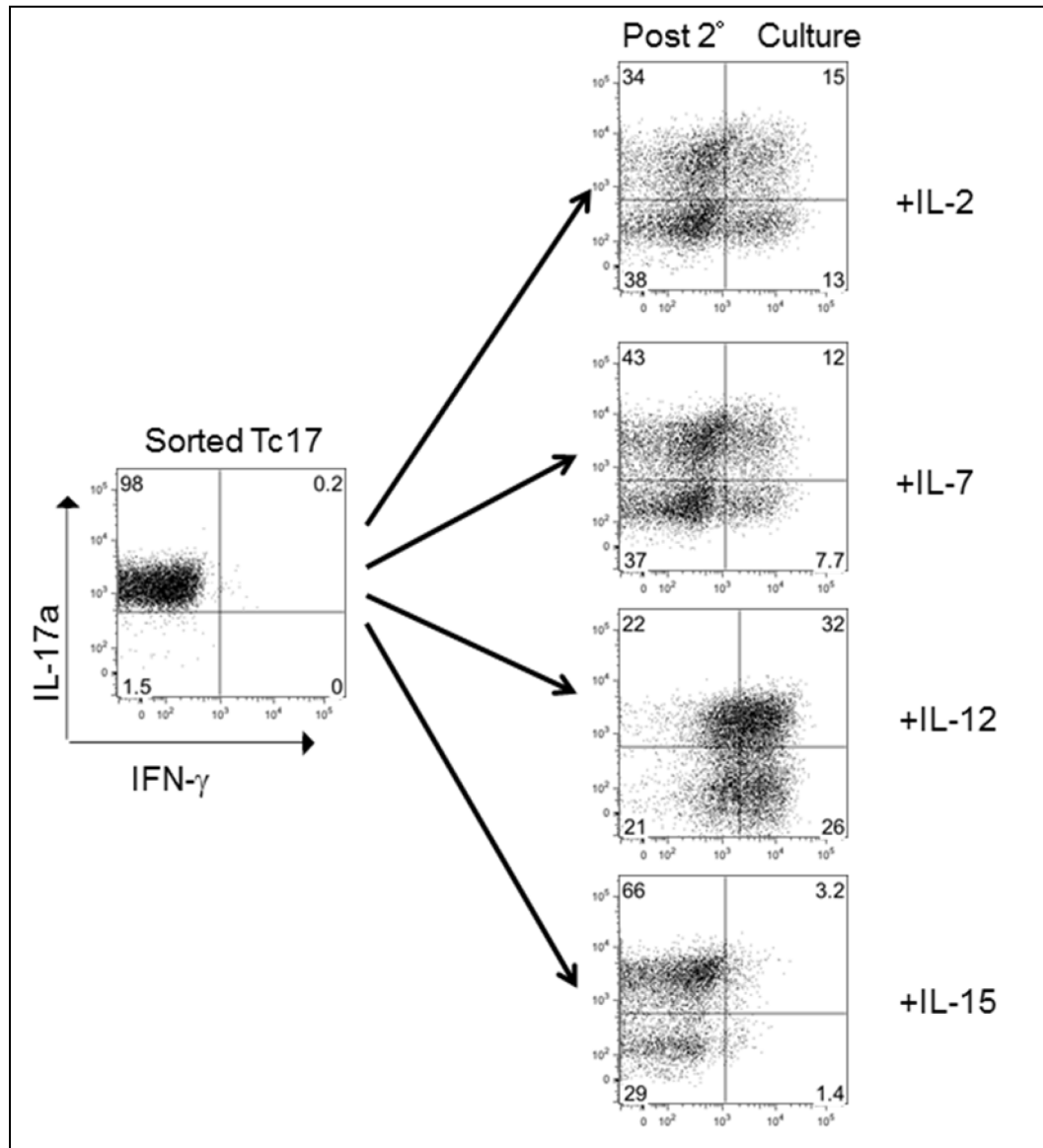
Tc17 cells expressed T-bet during conversion, which was seen most prominently in the presence of IL-12. Interestingly, Tc17 cells lost ROR γ T expression when cultured with IL-12. Conversely, Tc17 cells cultured with IL-2 and IL-7 did not lose ROR γ T expression. This suggests IL-12 is the only cytokine able to affect transcription factor plasticity of Tc17 cells. All of these data suggest the mechanism of conversion could be potentiated through T-bet expression.

Figure 4-1: Tc17 Sorting



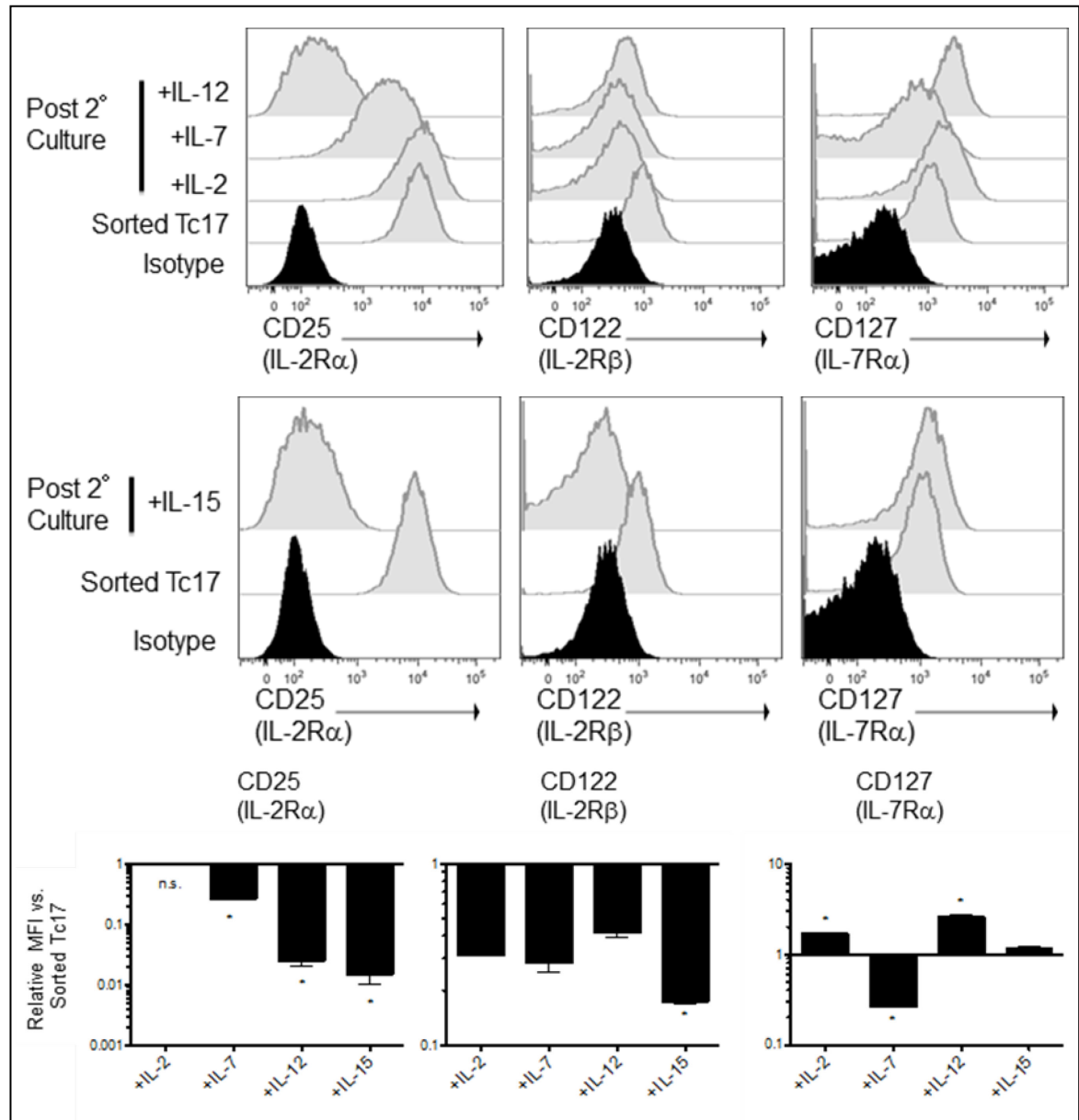
After *in vitro* polarization and re-stimulation with PMA and ionomycin for 2 hours, IL-17 or IFN- γ -secreting CD8⁺ T cells were stained with bi-specific CD45/cytokine mAbs and then sorted to >97% purity. 7-AAD was utilized to exclude dead cells and CD8⁺ T cells were sorted on IL-17⁺IFN- γ ⁻ cells.

Figure 4-2: *In vitro* Conversion with Specific Cytokines



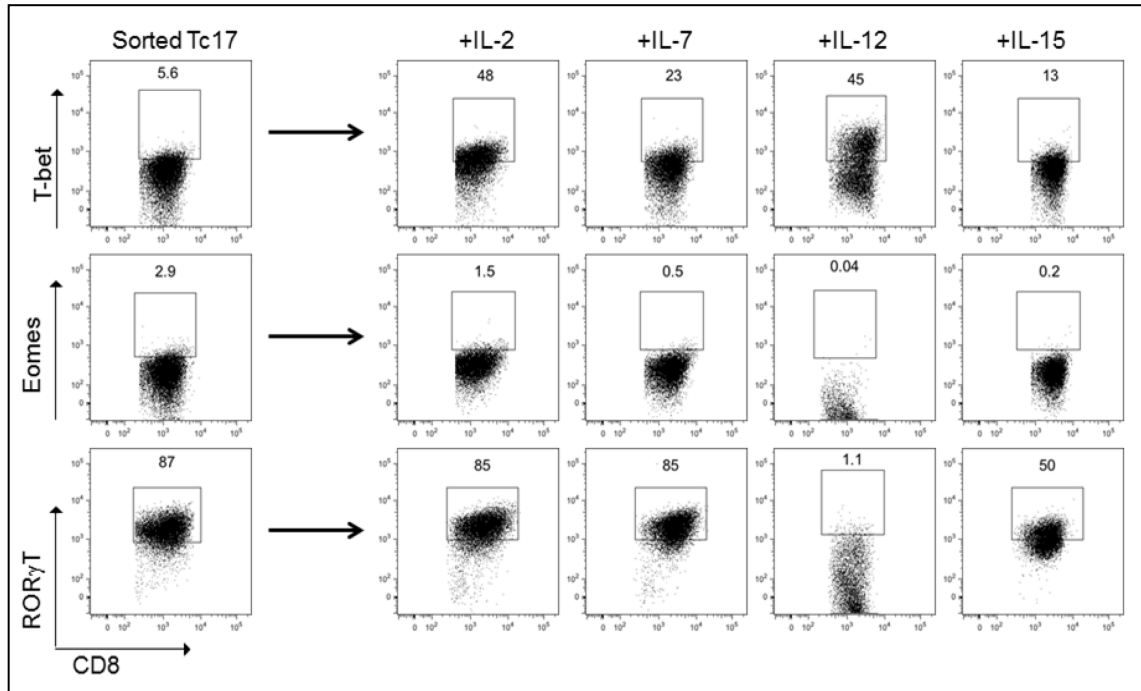
Tc17 cells were sorted on day 5 and then cultured with mIL-2, mIL-7, mIL-12, or mIL-15 for 4 days. For analysis of cytokine production, cells were stimulated with PMA and ionomycin. Protein transport inhibitor cocktail was added during stimulation. Cytokine staining was analyzed using intracellular staining and flow cytometry

Figure 4-3: Receptor Profile of Cultured Tc17 Cells



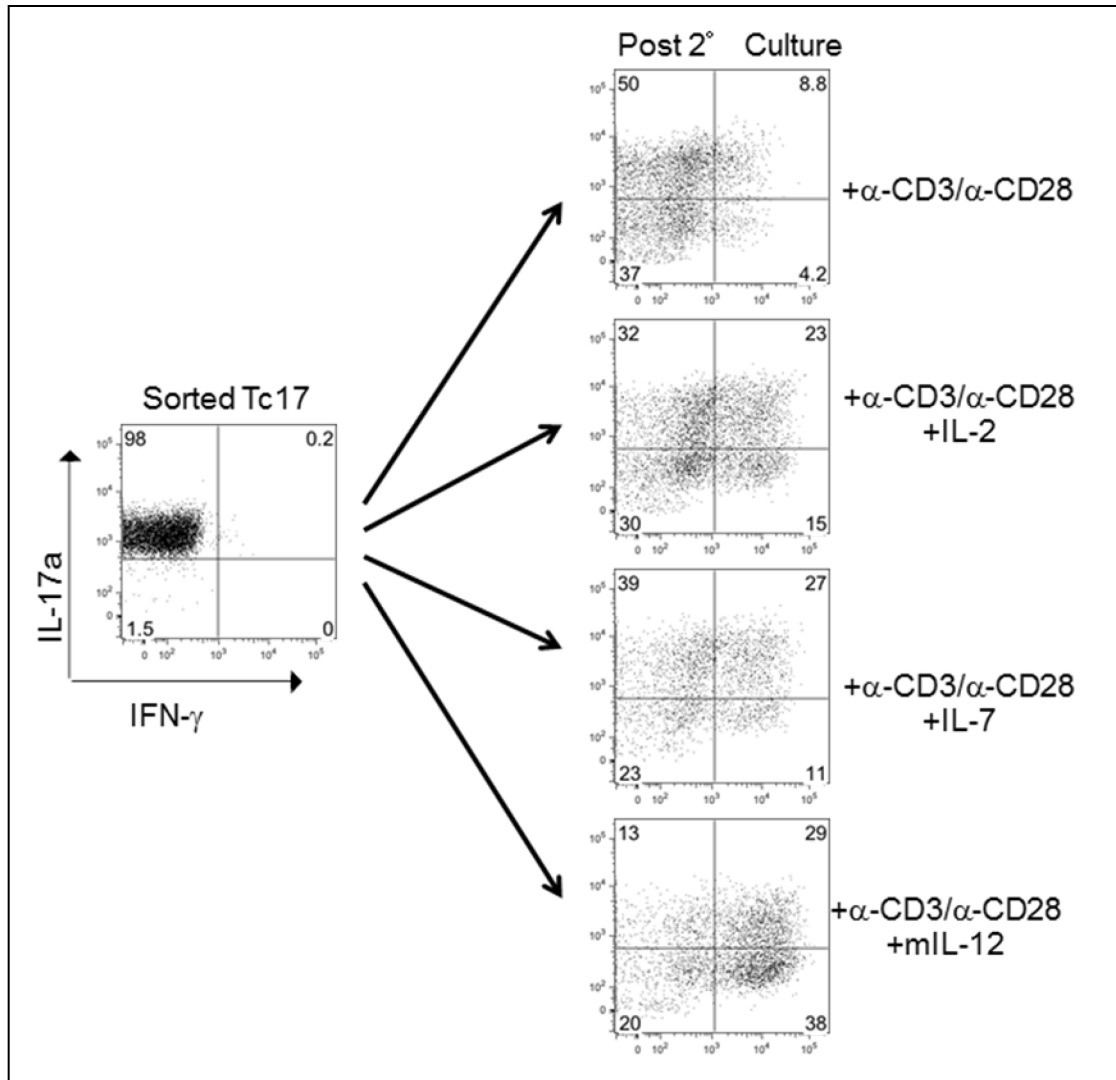
Tc17 cells were sorted on day 5 and then cultured with mIL-2, mIL-7, mIL-12, or mIL-15 for 4 days. Receptors were analyzed using extracellular staining and flow cytometry. The values represent the means \pm SEM (n=2). *p<0.05

Figure 4-4: Transcription Factor Profile of Cultured Tc17 cells



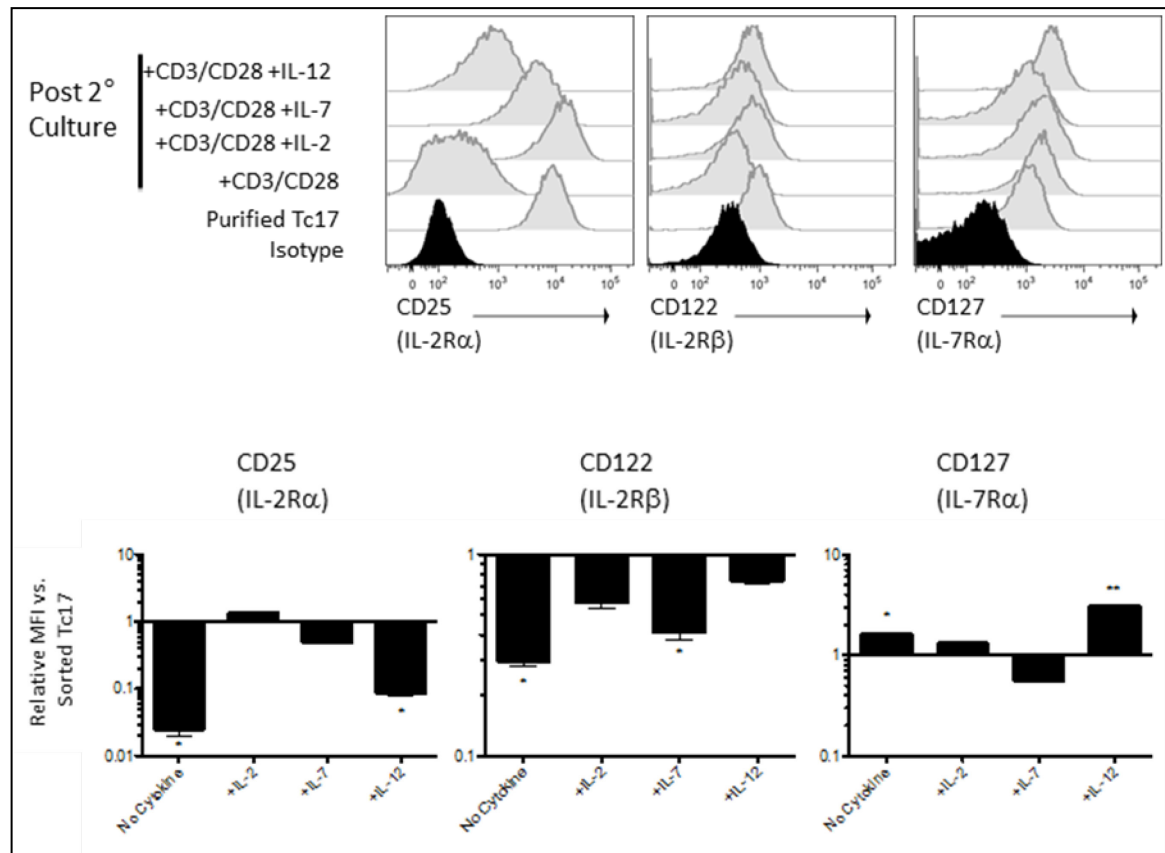
Tc17 cells were sorted on day 5 and then cultured with mIL-2, mIL-7, mIL-12, or mIL-15 for 4 days. For analysis of transcription factors, cells were stimulated with PMA and ionomycin. Protein transport inhibitor cocktail was added during stimulation. Transcription factor staining was analyzed using intracellular staining and flow cytometry.

Figure 4-5: *In Vitro* Conversion with TCR Stimulation



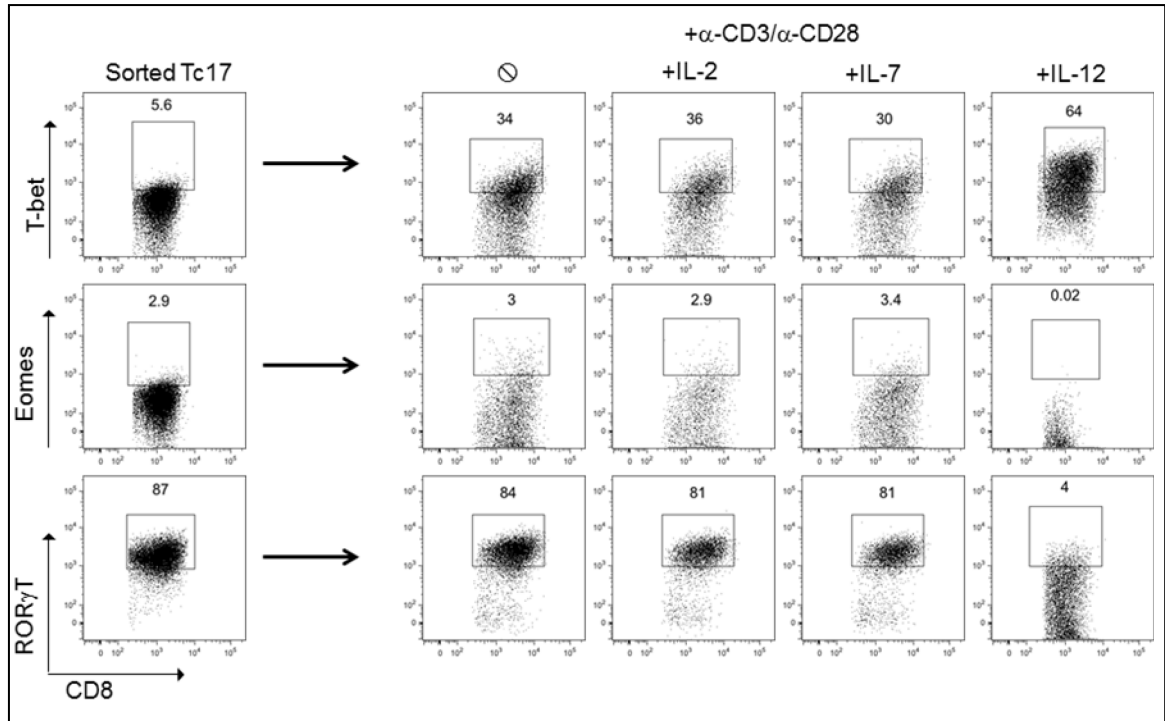
Tc17 cells were sorted on day 5 and then cultured on anti-CD3 coated plates with soluble anti-CD28 in the presence of mIL-2, mIL-7, mIL-12, or mIL-15 for 4 days. For analysis of cytokine production, cells were stimulated with PMA and ionomycin. Protein transport inhibitor cocktail was added during stimulation. Cytokine staining was analyzed using intracellular staining and flow cytometry.

Figure 4-6: Receptor Profile of TCR Stimulated Tc17 Cells



Tc17 cells were sorted on day 5 and then cultured on anti-CD3 coated plates with soluble anti-CD28 in the presence of mIL-2, mIL-7, mIL-12, or mIL-15 for 4 days. Receptors were analyzed using extracellular staining and flow cytometry. The values represent the means \pm SEM (n=2). *p<0.05, **p<.01

Figure 4-7: Transcription Factor Profile of TCR Stimulated Tc17 Cells



Tc17 cells were sorted on day 5 and then cultured on plate bound anti-CD3 with soluble anti-CD28 in the presence of mIL-2, mIL-7, mIL-12, or mIL-15 for 4 days. For analysis of transcription factors, cells were stimulated with PMA and ionomycin. Protein transport inhibitor cocktail was added during stimulation. Transcription factor staining was analyzed using intracellular staining and flow cytometry.

CHAPTER 5

TC17 PLASTICITY *IN VIVO*

INTRODUCTION

In this chapter we examined Tc17 plasticity *in vivo*. In Chapter 4, we demonstrated sorted Tc17 cells cultured with IL-15 did not convert *in vitro*. We sought to look at the stability of these cells *in vivo*. We hypothesized Tc17 cells would retain IL-17 production and not produce IFN- γ in the absence of any stimulation. To test this hypothesis we adoptively transferred Tc17 cells into mice in the absence of cytokine or antigen stimulation. Adoptively transferred Tc17 cells switch from IL-17 to IFN- γ secretion in the presence of VV-OVA⁶⁴. We next sought to verify whether inflammation triggers the conversion of Tc17 cells⁶⁴. In Chapter 4, we showed sorted Tc17 cells cultured with the pro-inflammatory cytokines IL-2 or IL-12 converted *in vitro*. We hypothesized inflammation would drive conversion to IFN- γ secretion *in vivo*.

Tc17 plasticity also occurs in mouse self-antigen models. Normally when a CD8⁺ T cell encounters a self antigen without inflammation it is tolerized and loses its ability to produce cytokines⁷⁹. In a diabetes model where self antigen is expressed on pancreatic islet cells, one group showed Tc17 cells co-transferred with Th1 cells did not lose cytokine production. Conversely, the Tc17 cells started to produce IFN- γ ⁶⁵. We showed antigen-specific Tc17 cells adoptively transferred into mice expressing self-antigen in the lung switched to an IFN- γ secreting phenotype³³. The role of self antigen recognition on Tc17 conversion remains unclear^{33, 37, 65}. We hypothesized Tc17 cells exposed to self antigen in the absence of inflammation would convert to IFN- γ secretion. To test this hypothesis we adoptively transferred Tc17 cells into OVA-transgenic mice

that express OVA antigen throughout the entire body. After adoptive transfer into these specific microenvironments, we stained for cytokine expression.

Based on the data from Chapter 4, we found IL-7 drove Tc17 cells to secrete IFN- γ *in vitro*. Therefore, we hypothesized Tc17 cells would convert in a V(D)J recombination activation gene (RAG-1) KO mouse. RAG KO mice lack any adaptive immune system, and have an abundance of IL-7 and IL-15. As mentioned in Chapter 1, adoptively transferred cells undergo homeostatic proliferation in this type of microenvironment⁸⁰. To test this hypothesis we adoptively transferred Tc17 cells into RAG KO mice, and looked at the cytokine expression after adoptive transfer.

The nature of the receptors expressed by Tc17 cells after adoptive transfer is expected to influence their *in vivo* conversion to IFN- γ secreting cells. Tc17 cells can exhibit a memory precursor phenotype, and maintain high percentages of CD127^{hi}KLRG-1^{low} populations after anti-tumor responses^{48, 49}. Many of the common gamma chain cytokine receptors have never been studied on adoptively transferred Tc17 cells. In Chapter 4 we showed Tc17 cells that did not convert lost CD25 expression and had increased CD127 expression. We hypothesized Tc17 cells adoptively transferred into mice without stimulation would also lose CD25 expression. In Chapter 4, we demonstrated Tc17 cells expressed CD25 in the presence of both IL-2 and IL-2 with TCR stimulation. We hypothesized the high affinity IL-2 receptor complex would be associated with conversion in the presence of inflammation (VV-OVA). We found Tc17 cells lost CD127 expression in the presence of IL-7 in Chapter 4. We hypothesized Tc17 cells would lose CD127 expression in RAG KO mice. To investigate these questions we adoptively transferred Tc17 cells into mice without stimulation, mice infected with VV-

OVA, RAG KO mice and CAG-OVA mice. We stained CD25, CD122 and CD127 after adoptive transfer.

The transcription factor expression of Tc17 cells is well established, but not the expression after adoptive transfer^{33, 63}. In Chapter 4, we showed Tc17 cells express T-bet upon conversion *in vitro*. In addition, Tc17 cells lost ROR γ T in the presence of IL-12 but not under any other conditions. We hypothesized Tc17 cells adoptively transferred in the absence of stimulation would maintain the same transcription factor expression as before transfer. We also hypothesized Tc17 cells adoptively transferred in the presence of inflammation or in self-antigen would express T-bet and lose ROR γ T expression. Additionally, we predicted Tc17 cells adoptively transferred into RAG KO mice would express T-bet but not lose ROR γ T expression. In order to test these hypotheses we adoptively transferred Tc17 cells into mice without stimulation, mice infected with VV-OVA, RAG KO mice and CAG-OVA mice. We stained T-bet, eomes and ROR γ T after adoptive transfer.

Cytotoxicity is an important characteristic of a CTL. Tc17 cells converted in the presence of vaccinia acquire cytotoxic potential. This was shown by sorting Tc17 cells directly *ex vivo* and performing an *in vitro* killing assay⁶⁴. Another group showed Tc17 cells converted *in vitro* with IL-12 were more cytotoxic than the non-converted counterparts³⁸. In Chapter 3, we showed Tc17 cells killed significantly less than Tc1 cells *in vivo*. We hypothesized Tc17 cells adoptively transferred into VV-OVA mice would be cytotoxic in an *in vivo* killing assay, but less so than Tc1 cells. To test this

hypothesis we used an *in vivo* killing assay to compare Tc17 cells to not only Tc1 cells but also naïve cells adoptively transferred into mice infected with VV-OVA⁶⁴.

RESULTS

Inflammation Determines Tc17 Conversion *in vivo*

In Chapters 3 and 4 we showed IL-2 and IL-12 drove conversion of Tc17 cells *in vitro*, so decided to look at conversion in an inflammatory microenvironment. We used the pro-inflammatory pathogen VV-OVA. We found after adoptive transfer into mice infected with VV-OVA, Tc17 cells switched from IL-17 to IFN- γ secretion. We observed this conversion in all organs of the mouse, the strongest conversion being in the liver. In contrast, we also adoptively transferred Tc17 cells into mice without cytokine or TCR stimulation. We found Tc17 cells maintain IL-17 secretion in this microenvironment (Figure 5-1). The conversion in mice infected with VV-OVA was observed whether or not the cells were sorted to >98% purity. This would suggest the IFN- γ population does not expand from the existing population but from the IL-17 population. We used unsorted cells in all further *in vivo* experiments (Figure 5-2).

Neither Homeostatic Proliferation Conditions nor Self-Antigen Determine Conversion *in vivo*

We next sought to understand the role of other *in vivo* microenvironments on Tc17 plasticity. The data in Chapter 3 suggested a role for IL-7 in conversion, so we examined the conversion of Tc17 cells in homeostatic proliferation conditions. When adoptively transferred into RAG KO mice, homeostatic proliferation did not drive Tc17 cells toward IFN- γ secretion. Interestingly, the spleen and liver appeared to be the best microenvironments to maintain IL-17 secretion in a RAG KO host. We also examined conversion of Tc17 cells adoptively transferred into mACT-OVA (CAG-OVA), which

express OVA ubiquitously throughout the mouse. We found Tc17 cells do not convert, but begin to lose IL-17 secretion in all organs in the presence of self-antigen (Figure 5-3).

Host Microenvironment Affects Receptor Expression *in vivo*

In order to understand the mechanism of conversion, we examined the expression of common gamma chain cytokine receptors on adoptively transferred Tc17 cells *in vivo*. In order to do this we adoptively transferred Tc17 cells into mice without antigen, mice infected with VV-OVA, RAG KO mice and CAG-OVA mice. We stained receptors on adoptively transferred Tc17 cells from the spleen. Compared to Tc17 cells from VV-OVA infected mice, cells without any antigen stimulation have decreased CD122 expression and increased CD127 expression. Conversely, even though homeostatic proliferation did not impact Tc17 plasticity, it did affect receptor expression. Tc17 cells transferred into Rag KO mice had an increase in CD25 and CD122 expression, but lower CD127 expression compared to converted Tc17 cells. In a tolerogenic environment, Tc17 cells had decreased CD127 and CD122 expression (Figure 5-4 and 5-5). In addition, we sought to understand the memory phenotype of these adoptively transferred cells. We found Tc17 cells adoptively transferred into RAG KO mice were CD127^{high}KLRG-1^{high}, whereas self-antigen led to an early effector phenotype or a CD127^{low}KLRG-1^{low} population. With or without antigen stimulation, Tc17 cells maintained a memory precursor phenotype (Figure 5-5).

Converted Tc17 Cells Express T-bet and lose ROR γ T *in vivo*

In order to further understand the mechanism of conversion, we examined the expression of transcription factors of Tc17 cells after adoptive transfer. In order to do this we

adoptively transferred Tc17 cells into mice without antigen, mice infected with VV-OVA, RAG KO mice and CAG-OVA mice. We determined Tc17 cells adoptively transferred into mice without any stimulation did not express T-bet or eomes, and maintained expression of ROR γ T. Similar to the cytokine profile, Tc17 cells lost expression of all transcription factors in the presence of self-antigen. In a homeostatic proliferation environment, Tc17 cells had increased T-bet expression, which was not mirrored by IFN- γ secretion. Interestingly, converted Tc17 cells had increased T-bet expression and lost ROR γ T expression, a result corroborated by our previous *in vitro* data (Figure 5-6).

Converted Tc17 Cells can Lyse Targets as Effectively as Tc1 Cells

We next sought to understand the Tc17 function upon conversion. In order to do this, we performed an *in vivo* killing assay. We adoptively transferred naïve CD8⁺ T cells into mice without VV-OVA as a negative control and mice infected with VV-OVA as a positive control. We also adoptively transferred Tc1 and Tc17 cells into mice infected with VV-OVA. We found smaller numbers of Tc17 cells (1×10^4) had significantly lower specific lysis than transferring larger numbers of Tc17 cells (2×10^5). This was compared to naïve CD8⁺ T cells activated in mice infected with VV-OVA. Tc17 cells, even upon conversion, were significantly less efficient at killing as naïve CD8⁺ T cells. Intriguingly, Tc1 cells are not significantly better at killing than converted Tc17 cells (Figure 5-7).

SUMMARY

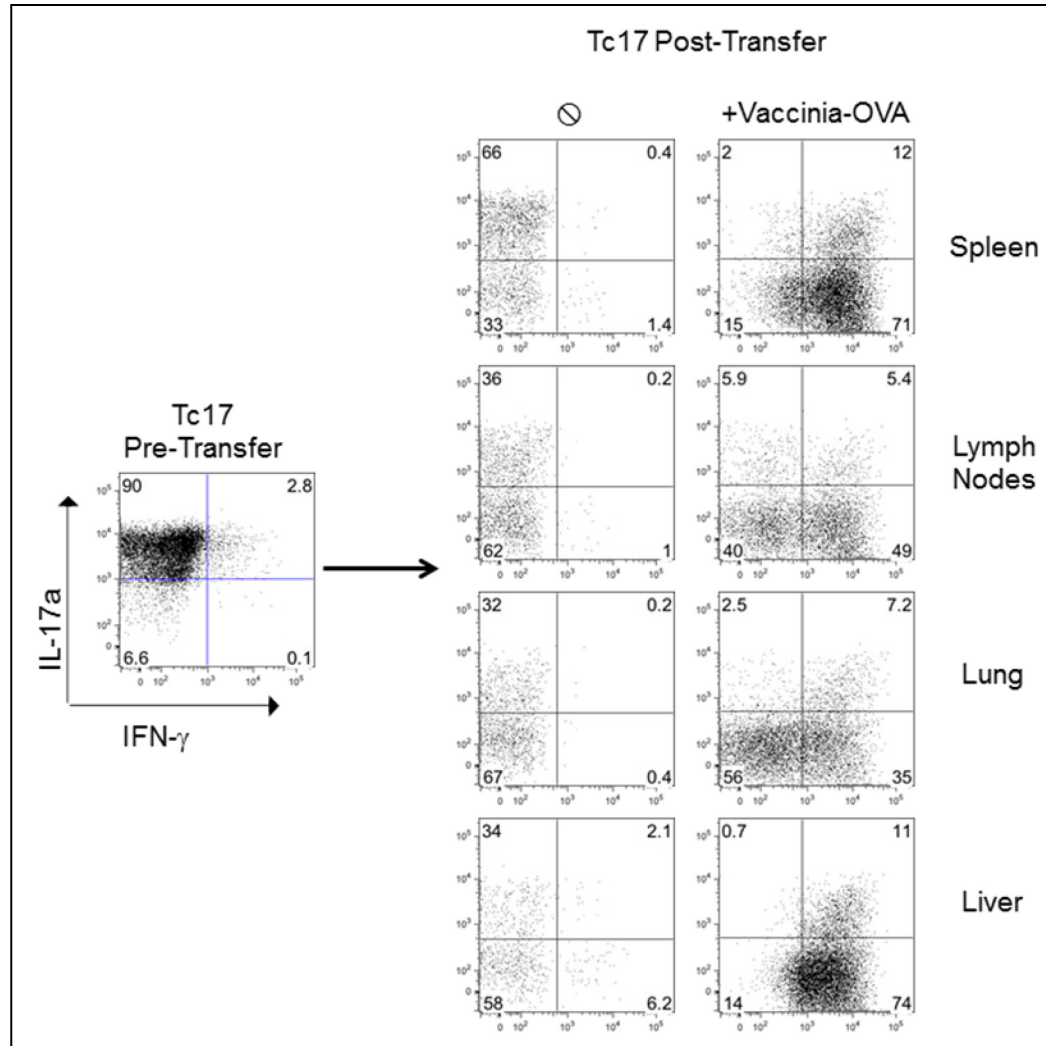
Tc17 plasticity occurred in mice infected with VV-OVA *in vivo*. Unexpectedly, Tc17 cells did not convert under homeostatic proliferation. This suggests the role of antigen stimulation in combination with a specific cytokine milieu is important to promote conversion *in vivo*. Tc17 cells did not convert, but began to lose IL-17 secretion in all organs in the presence of self-antigen, suggesting Tc17 cells can be tolerized.

Compared to converted Tc17 cells, cells without any antigen stimulation have decreased CD122 expression and increased CD127 expression, suggesting a long-term memory phenotype. Tc17 cells transferred into Rag KO mice have increased expression of CD25 and CD122, but decreased expression of CD127, suggesting they were terminally differentiated state. In a tolerogenic environment, Tc17 cells lost both CD127 and CD122 expression, suggesting a terminal effector state. Tc17 cells maintained CD127 expression and did not readily express KLRG-1, suggesting a more memory precursor phenotype even during a VV-OVA infection.

We found Tc17 cells adoptively transferred into mice infected with VV-OVA expressed T-bet and lost ROR γ T. This was similar to observations made in Chapter 4, again suggesting the mechanism of conversion could be potentiated through T-bet expression. In a homeostatic proliferation environment, Tc17 cells had a slight increase in T-bet expression, suggesting a minor acquisition of an effector phenotype. Tc17 cells adoptively transferred into self antigen bearing mice did not express any of the transcription factors, suggesting a tolerized state.

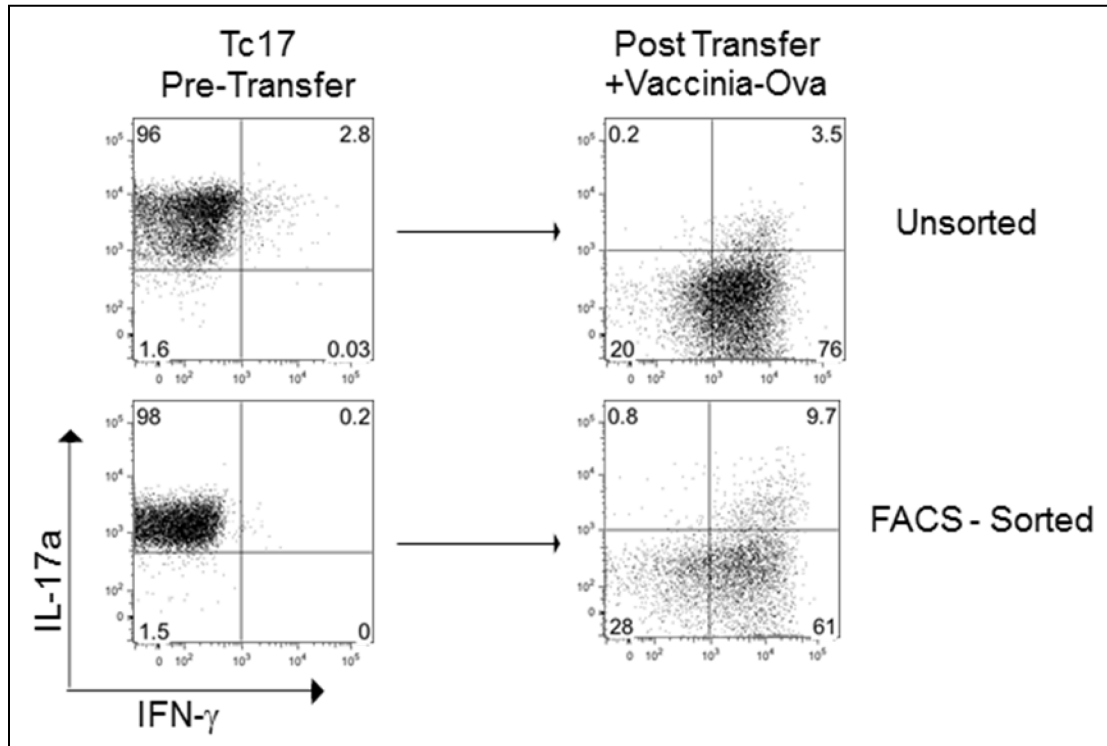
Tc17 cells acquired killing abilities upon conversion, suggesting conversion controls Tc17 effector function. Although there was a significant difference between Tc17 cells and naive CD8⁺ T cells adoptively transferred into mice infected with VV-OVA. This suggests Tc17 cells are not efficient killers even upon conversion compared to newly activated CD8⁺ T cell. Converted Tc17 cells were not significantly different than Tc1 cells.

Figure 5-1: Adoptive Transfer with or without VV-OVA



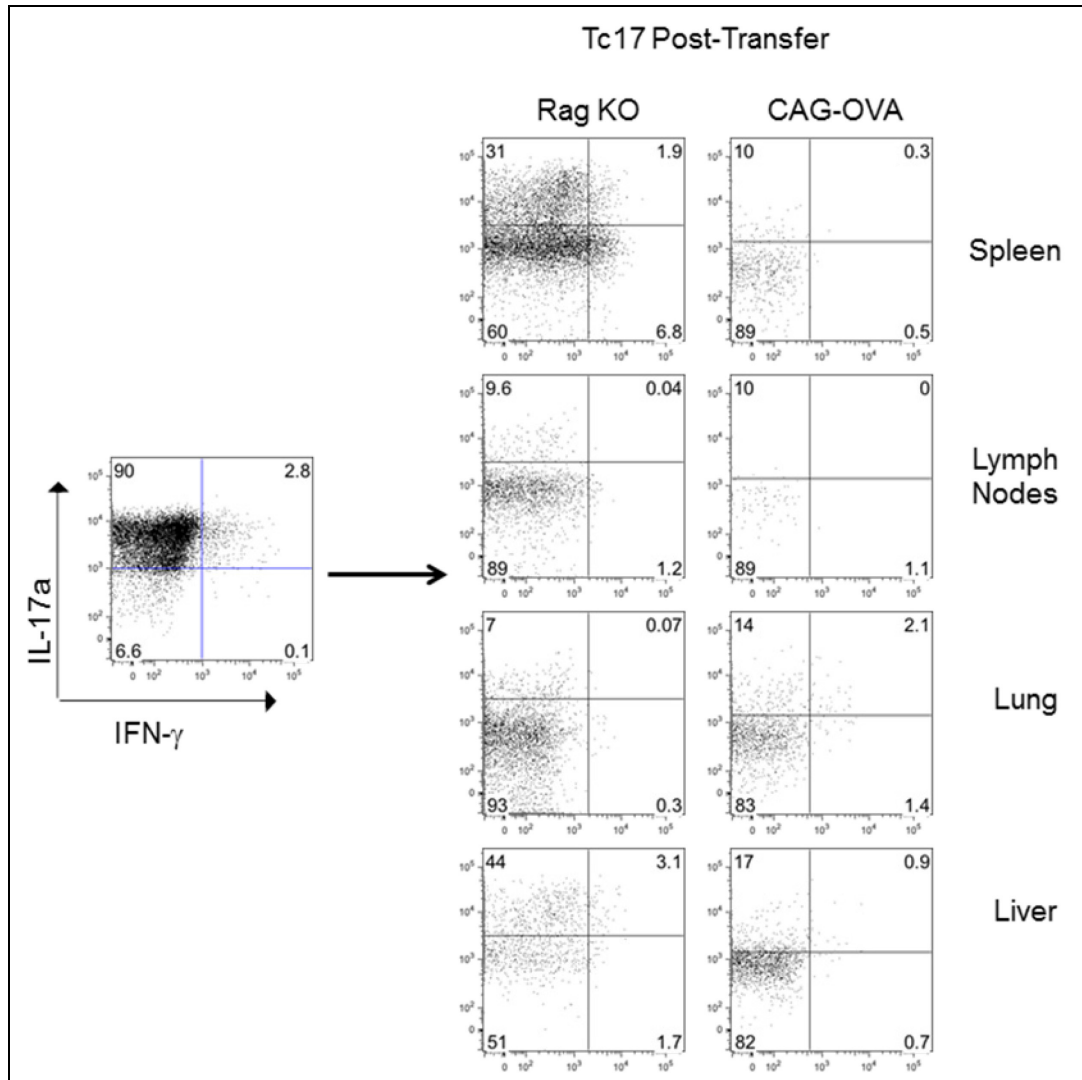
1-2x10⁶ *in vitro* generated Tc17 cells were adoptively transferred into CD45.1+ recipients with or without VV-OVA. 5 days later, respectively, organs were harvested. For analysis of cytokine production, cells were stimulated with OVA peptide. Protein transport inhibitor cocktail was added during stimulation. Cytokine staining was analyzed using intracellular staining and flow cytometry.

Figure 5-2: Adoptive Transfer of Sorted Cells with VV-OVA



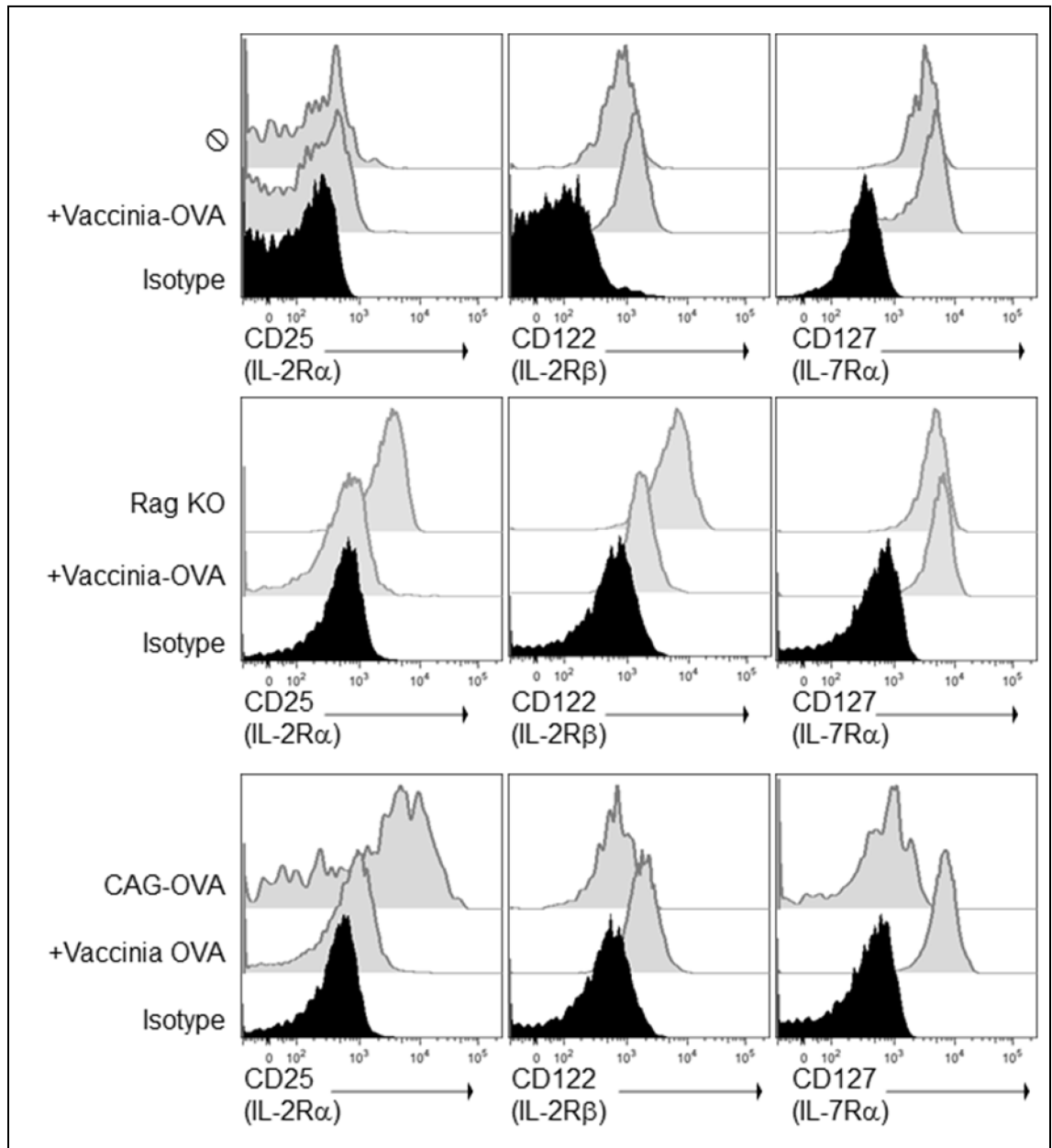
In vitro generated Tc17 cells were sorted and 2×10^5 cells were adoptively transferred into CD45.1+ recipients with VV-OVA. 7 days later organs were harvested. For analysis of cytokine production, cells were stimulated with OVA peptide. Protein transport inhibitor cocktail was added during stimulation. Cytokine staining was analyzed using intracellular staining and flow cytometry.

Figure 5-3: Adoptive Transfer into RAG KO and CAG-OVA Mice



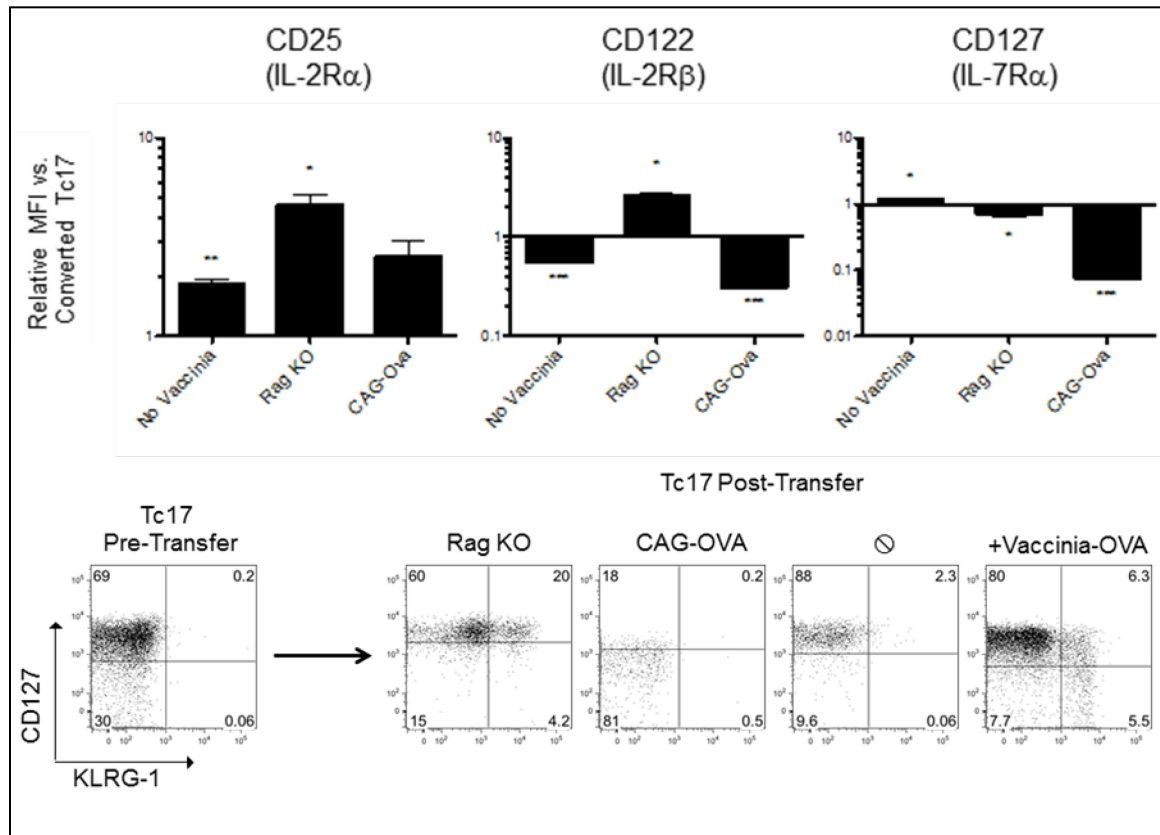
1-2x10⁶ *in vitro* generated Tc17 cells were adoptively transferred into CAG-OVA or RAG-KO recipients. 3 or 7 days later, respectively, organs were harvested. For analysis of cytokine production, cells were stimulated with OVA peptide. Protein transport inhibitor cocktail was added during stimulation. Cytokine staining was analyzed using intracellular staining and flow cytometry.

Figure 5-4: Receptor Profile of Adoptively Transferred Tc17 Cells



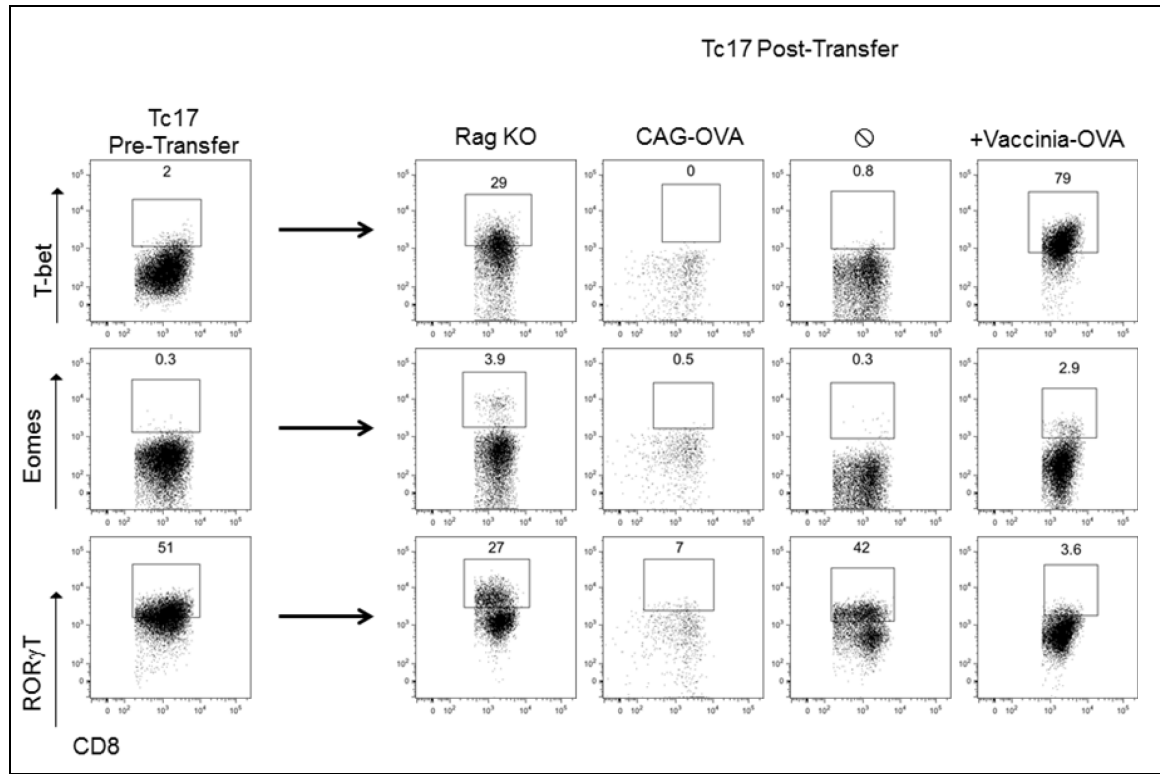
1-2x10⁶ *in vitro* generated Tc17 cells were adoptively transferred into CAG-OVA, RAG KO or CD45.1+ recipients. 3 or 7 days later, respectively, organs were harvested. Receptors were analyzed using extracellular staining and flow cytometry.

Figure 5-5: Relative MFI Differences of Receptors and Memory Phenotype of Adoptively Transferred Cells



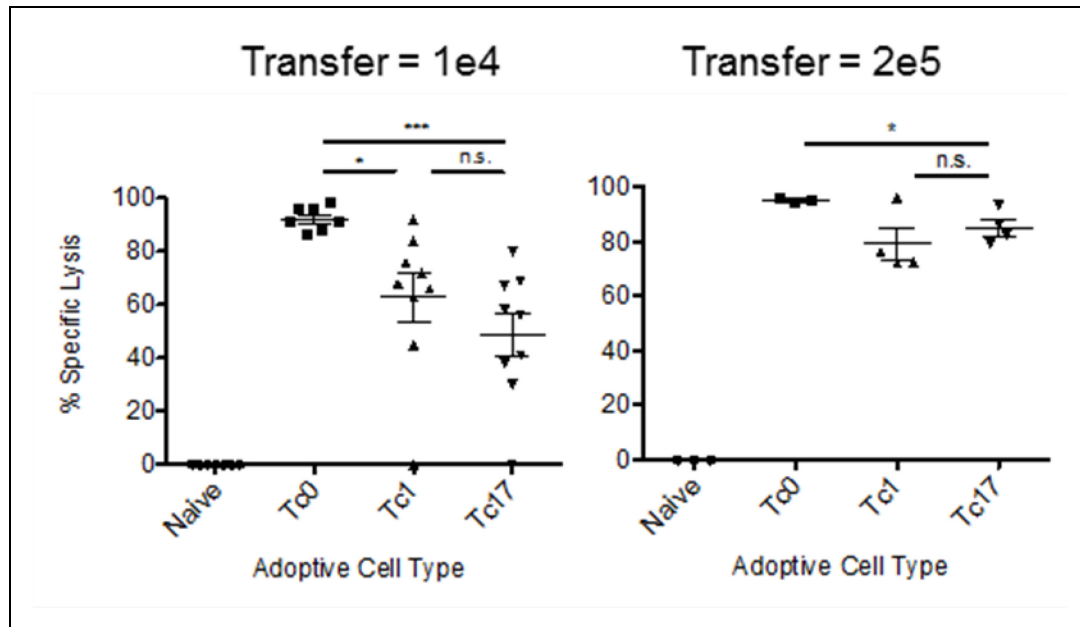
1-2x10⁶ *in vitro* generated Tc17 cells were adoptively transferred into CAG-OVA, RAG KO or CD45.1+ recipients. 3 or 7 days later, respectively, organs were harvested. Receptors were analyzed using extracellular staining and flow cytometry. The values represent the means +/- SEM (n=4). *p<0.05, **p<.01, ***p<.001

Figure 5-6: Transcription Factor Profile of Adoptively Transferred Tc17 Cells



1-2x10⁶ *in vitro* generated Tc17 cells were adoptively transferred into CAG-OVA, RAG-KO or CD45.1+ recipients. 3 or 7 days later, respectively, organs were harvested. For analysis of transcription factors, cells were stimulated with OVA peptide. Protein transport inhibitor cocktail was added during stimulation. Transcription factor staining was analyzed using intracellular staining and flow cytometry.

Figure 5-7: *In Vivo* CTL Assay of Converted Tc17 Cells



1×10^4 or 2×10^5 naïve CD8⁺ T cells were injected into CD45.1⁺ recipients with or without VV-OVA. In addition, 1×10^4 or 2×10^5 *in vitro* generated Tc1 and Tc17 cells were prepared and injected into CD45.1⁺ recipients with VV-OVA. Five days after Tc cell injection, recipient mice were injected with 5.5×10^6 OVA-pulsed splenic cells stained with 5 μ M CFSE, and 4.5×10^6 non-pulsed splenic cells stained with 0.5 μ M CFSE. Eighteen hours later, spleen cells were harvested and ratio of live CFSE^{hi} to CFSE^{lo} cells was determined using flow cytometry. The values represent the means \pm SEM (n=4-8). *p<0.05, ***p<.001

CHAPTER 6
REQUIREMENTS FOR TC17 CONVERSION *IN VIVO*

INTRODUCTION

The requirements for T cell conversion *in vivo* have not been studied extensively. As mentioned in Chapter 4, IL-12 can be a potent driver of both Th17 and Tc17 conversion *in vitro*^{38, 64, 77}. IL-12 is an important pro-inflammatory cytokine produced mainly by phagocytic cells such as macrophages and neutrophils. The IL-12 receptor is composed of two different subunits, IL-12R β 1 and IL-12R β 2. The IL-23 receptor shares the IL-12R β 1 subunit with the IL-12 receptor. IL-12 is important during an infection, because it causes natural killer cells and T cells to secrete IFN- γ . IL-12 is required for resistance to some bacteria and parasitic infections⁸¹. Conversely, IL-12 is not required during some Th1 responses⁸². In Chapter 4, we demonstrated IL-2 drove conversion of Tc17 cells *in vitro*. We discussed in Chapter 1 the importance of IL-2 during CD8+ T cell activation. Autocrine IL-2 is required for an effective CD8+ memory response to infection⁸³.

In this chapter, we sought to understand the requirements for Tc17 conversion from IL-17 to IFN- γ secretion *in vivo*. In Chapter 4, we demonstrated IL-2 drove Tc17 conversion *in vitro*. We hypothesized autocrine or exocrine IL-2 would be required for conversion to an IFN- γ secreting phenotype *in vivo*. In order to test the requirement for exocrine IL-2 during conversion, Tc17 cells were adoptively transferred into IL-2 KO mice infected with VV-OVA. In order to test the requirement for autocrine IL-2 during conversion, we adoptively transferred IL-2 KO Tc17 cells into mice infected with VV-OVA. Finally, we adoptively transferred IL-2 KO Tc17 cells into IL-2 KO mice to understand conversion in the complete absence of IL-2. We looked at cytokine expression after adoptive transfer.

In Chapter 4, we demonstrated IL-12 drove Tc17 conversion *in vitro*. We hypothesized that similar to IL-2, Tc17 cells would not convert in the absence of IL-12. In order to test this hypothesis we adoptively transferred IL-12R β 1 KO CD8⁺ T cells into mice infected with VV-OVA, and looked at cytokine expression after adoptive transfer.

We also wanted to look at the transcription factor expression in the absence of IL-2 or IL-12. In Chapter 3, we showed sorted Tc17 cells cultured with IL-2 or IL-12 expressed T-bet. In Chapter 4, we showed adoptively transferred Tc17 cells expressed T-bet upon conversion *in vivo*. We hypothesized if IL-2 or IL-12 increases T-bet expression, Tc17 cells adoptively transferred into mice infected with VV-OVA in the absence of IL-2 or IL-12 would not be able to express T-bet. We also hypothesized Tc17 cells would maintain ROR γ T expression in the absence of IL-2 or IL-12. Essentially, without IL-2 or the IL-12 receptor, Tc17 cells transferred into VV-OVA infected mice would act similarly to transfer into mice without stimulation. In order to test these hypotheses, we adoptively transferred IL-2 KO Tc17 cells into IL-2 competent or IL-2 KO mice infected with VV-OVA, and stained for T-bet, ROR γ T and eomes. We also adoptively transferred IL-12R β 1 KO CD8⁺ T cells into mice infected with VV-OVA and stained for T-bet, ROR γ T and eomes.

Finally, we were interested to know if the absence of either IL-2 or IL-12 would change the expression of common gamma chain receptors. In Chapter 4, we showed sorted Tc17 cells cultured with IL-2 expressed similar levels of CD25 and CD127 compared to sorted Tc17 cells. Conversely, we showed sorted Tc17 cells cultured with IL-12 expressed less CD25 and more CD127 compared to sorted Tc17 cells. We

hypothesized in the absence of either IL-2 or IL-12, adoptively transferred Tc17 cells in mice with VV-OVA infection would act similarly to transfer into mice without infection. Without IL-2 or the IL-12 receptor, they would not convert and therefore would express more CD127 and less CD122 than IL-2 or IL-12 competent Tc17 cells. In order to test this hypothesis, we adoptively transferred IL-2 KO Tc17 cells into IL-2 competent or IL-2 KO mice infected with VV-OVA. We also adoptively transferred IL-12R β 1 KO CD8⁺ T cells into mice infected with VV-OVA. We stained CD25, CD122 and CD127 after adoptive transfer.

RESULTS

Exocrine IL-2 is not required for Tc17 Conversion *in vivo*

To understand the requirement of exocrine IL-2 in Tc17 conversion, we crossed IL-2 KO mice onto the RAG KO background. Next, we adoptively transferred IL-2 WT Tc17 cells into IL-2 KO/RAG KO recipients into mice infected with VV-OVA. We harvested splenocytes from the IL-2KO/RAG KO mice and found Tc17 cells adoptively transferred in the absence of IL-2 lost IL-17 production and secreted IFN- γ , although not as strongly as seen in IL-2 competent hosts. This showed exocrine IL-2 is not required for conversion *in vivo* (Figure 6-1).

Role of Exocrine IL-2 on Tc17 Cell Transcription Factor Expression *in vivo*

We next hypothesized if Tc17 cells converted without exogenous IL-2, the transcription factor profile would be similar to Tc17 cells converted with exogenous IL-2. To test this hypothesis we adoptively transferred IL-2 WT Tc17 cells into IL-2 KO/RAG KO recipients infected with VV-OVA. We found IL-2 WT Tc17 cells adoptively transferred into IL-2 KO recipients have increased T-bet expression and decreased ROR γ T expression. This was similar to adoptive transfer of Tc17 cells into IL-2 competent recipients. Tc17 cells adoptively transferred into mice without exogenous IL-2 express more eomes compared to adoptive transfer into mice with exogenous IL-2. The difference in eomes expression did not impact IL-17 or IFN- γ production, suggesting eomes is not as important for Tc17 plasticity (Figure 6-2).

Autocrine IL-2 is not required for Tc17 Conversion *in vivo*

Next we sought to examine the requirement of autocrine IL-2 for Tc17 conversion *in vivo*. To test this we crossed IL-2 KO mice onto the OT-1/RAG KO background. We tested the ability of IL-2 KO OT-1 CD8⁺ T cells to be skewed under IL-17 polarizing conditions. We found IL-2 KO CD8⁺ T cells robustly skewed to an IL-17 secreting phenotype. IL-2 KO Tc17 cells actually express less IFN- γ than the IL-2 competent counterpart (Figure 5C). We then adoptively transferred IL-2 KO Tc17 cells into mice infected with VV-OVA. IL-2 KO Tc17 cells converted, losing IL-17 expression and secreting IFN- γ . This was similar to adoptive transfer of IL-2 competent Tc17 cells. We then adoptively transferred IL-2 KO Tc17 cells into IL-2 KO recipients infected with VV-OVA. We found Tc17 cells adoptively transferred in the absence of both autocrine and exocrine IL-2 did not convert as well as Tc17 cells in the presence of IL-2. These cells had decreased IL-17, but lacked equivalent IFN- γ production compared to adoptive transfer into IL-2 competent recipients (Figure 6-3).

Role of Autocrine IL-2 on Tc17 Cell Transcription Factor Expression

We then investigated the role of autocrine IL-2 on the expression of transcription factors and hypothesized if Tc17 cells converted without autocrine IL-2, the transcription factor profile would be similar to Tc17 cells converted with autocrine IL-2. To assess this hypothesis we adoptively transferred IL-2 KO Tc17 cells into mice with VV-OVA infection. Similar to IL-2 competent Tc17 cells, IL-2 KO Tc17 cells adoptively transferred into an IL-2 competent host had increased T-bet expression and decreased ROR γ T expression. IL-2 KO Tc17 cells expressed more *omes* than IL-2 WT cells, but

that did not impact IL-17 or IFN- γ expression. We sought to determine the transcription factor expression in the absolute absence of IL-2. To do this we adoptively transferred IL-2 KO Tc17 cells into IL-2 KO recipients. IL-2 KO Tc17 cells adoptively transferred into an IL-2 KO host expressed less T-bet and more ROR γ T than IL-2 KO Tc17 cells transferred into an IL-2 competent host. IL-2 KO Tc17 cells transferred into an IL-2 KO host expressed much higher percentages of eomes than those transferred in the presence of IL-2, suggesting a role for IL-2 signaling in eomes expression (Figure 6-2 and 6-4).

Role of IL-2 on Tc17 Cell Receptor Expression

We next wanted to understand the expression of common gamma chain receptors in the absence of IL-2. We found all of the adoptively transferred Tc17 groups had significantly less CD122 and CD127. WT or IL-2 KO Tc17 cells adoptively transferred into IL-2 KO hosts had significantly more CD25 expression. IL-2 KO Tc17 cells adoptively transferred into IL-2 competent hosts had a higher percentage of SLECs (Figure 6-5).

IL-12 Receptor Signaling is not required for Tc17 Conversion *in vivo*

In Chapter 4 we demonstrated IL-12 drove Tc17 conversion *in vitro*. Therefore, we hypothesized IL-12 signaling would be a requirement for conversion *in vivo*. In order to test this hypothesis we skewed OT-1/IL-12R β 1 KO cells under IL-17 polarizing conditions. We found IL-12R β 1 KO cells skewed to a >80% IL-17+IFN- γ - cell population. We next adoptively transferred IL-12R β 1 KO Tc17 cells into mice infected with VV-OVA. We found the IL-12R β 1 was not required for Tc17 conversion *in vivo*.

IL-12R β 1 KO Tc17 cells lost IL-17 secretion and started secreting IFN- γ as robustly as cells that could signal through the IL-12 receptor (Figure 6-6).

Role of the IL-12 Receptor on Tc17 Cell Transcription Factor Expression *in vivo*

To determine if there were any differences between the transcription factor profile of WT Tc17 cells and IL-12 receptor KO Tc17 cells adoptively transferred into virally infected mice, we adoptively transferred IL-12R β 1 KO Tc17 cells into mice infected with VV-OVA. Similar to IL-12R β 1 competent Tc17 cells, IL-12R β 1 KO Tc17 cells had increased T-bet expression and decreased ROR γ T expression. Similar to IL-2 KO Tc17 cells, IL-12R β 1 KO Tc17 cells expressed more cytokines than the WT counterpart (Figure 6-2 and 6-7).

Role of the IL-12 Receptor on Tc17 Cell Receptor Expression

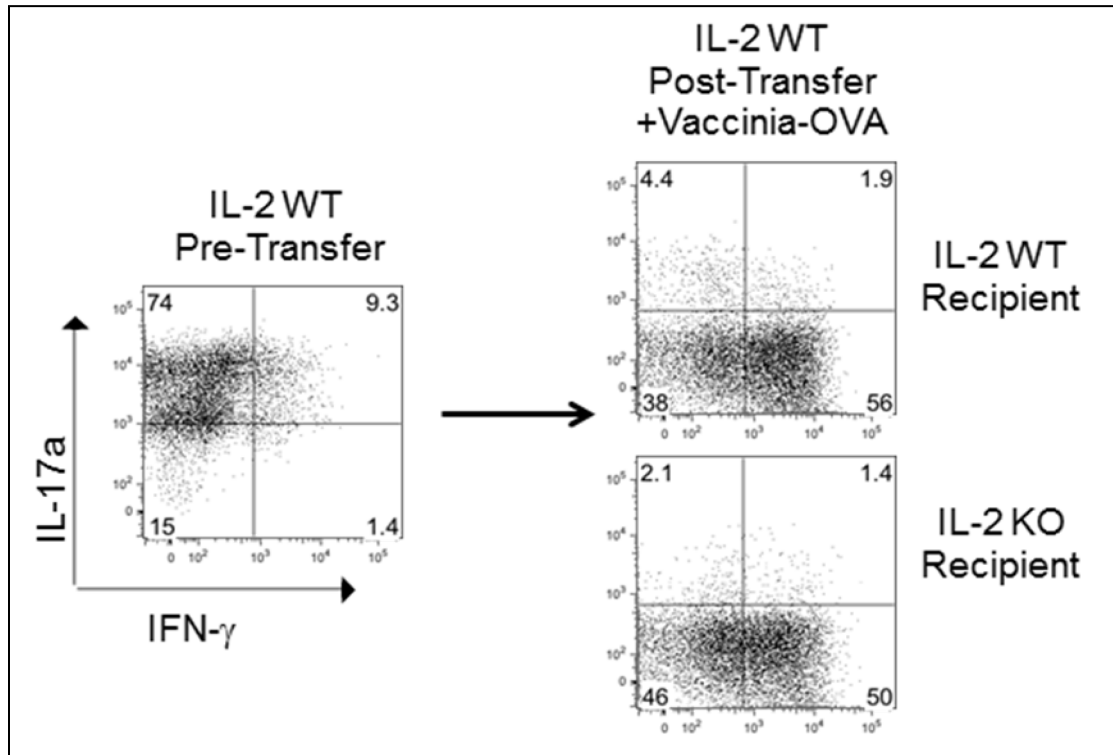
To investigate the gamma chain receptor expression of IL-12 receptor KO Tc17 cells upon conversion, we adoptively transferred IL-12R β 1 KO cells into VV-OVA infected mice. In the absence of IL-12R β 1, Tc17 cells adoptively transferred into infected mice had significantly less CD25 and CD127. CD122 expression was not significantly different between WT and KO IL-12R β 1 Tc17 cells. IL-12R β 1 KO Tc17 cells had a higher percentage of SLEC's, suggesting a more effector phenotype (Figure 6-8)

SUMMARY

Neither IL-2 or IL-12 were required to switch Tc17 cells from IL-17 to IFN- γ secretion *in vivo*. In the absence of IL-2, Tc17 cells had significantly less CD122 and CD127, suggesting a more short-term effector phenotype. Furthermore, in the absence of IL-12, Tc17 cells expressed higher CD25 and lower CD127, suggesting a more short-term effector phenotype. This was not reflected by a dramatic increase in SLECs, so the role of gamma chain receptors during conversion is still unclear.

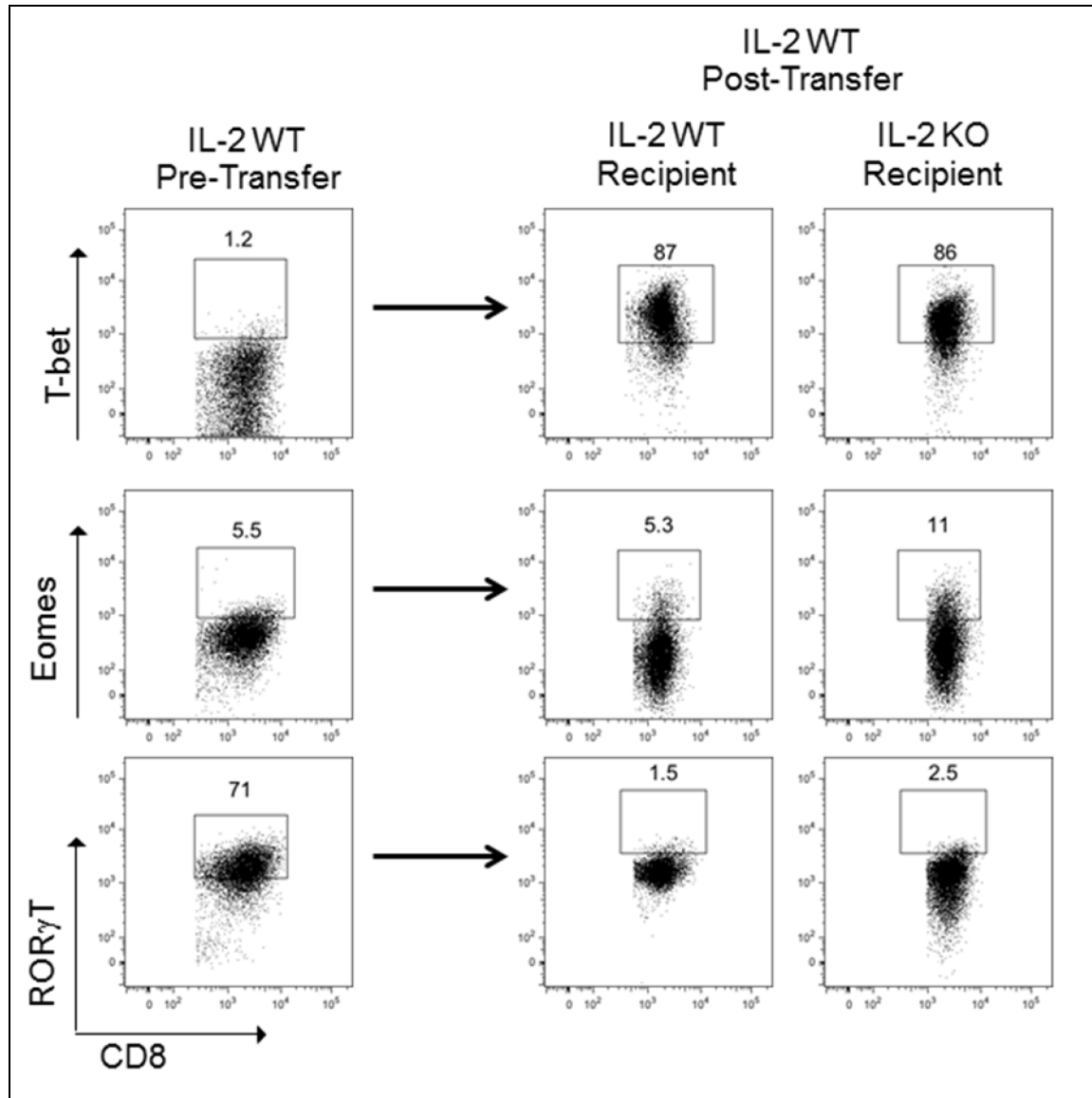
In the absence of autocrine or exocrine IL-2, Tc17 cells expressed T-bet and lost ROR γ T expression. Conversely, in the complete absence of IL-2, Tc17 cells expressed less IFN- γ and T-bet. This suggests IL-2 is sufficient and important for Tc17 plasticity, but not absolutely required. In the absence of the IL-12 receptor, Tc17 cells expressed T-bet and lost ROR γ T expression. Similar to IL-2, while IL-12 was sufficient to drive conversion *in vitro*, it is not required *in vivo*.

Figure 6-1: Role of Exocrine IL-2 in Tc17 Conversion (Cytokine Profile)



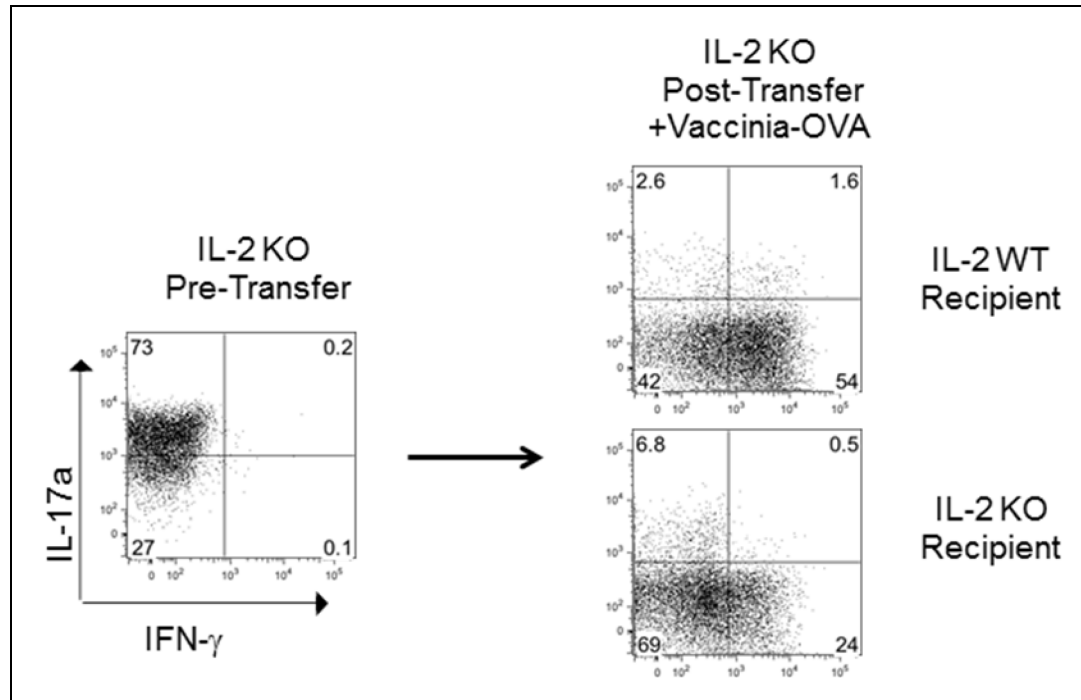
1x10⁶ *in vitro* generated IL-2 WT Tc17 cells were adoptively transferred into CD45.1+ or IL-2 KO/RAG KO recipients with VV-OVA. 7 days later organs were harvested. For analysis of cytokine production, cells were stimulated with OVA peptide. Protein transport inhibitor cocktail was added during stimulation. Cytokine staining was analyzed using intracellular staining and flow cytometry.

Figure 6-2: Role of Exocrine IL-2 in Tc17 Conversion (Transcription Factor Profile)



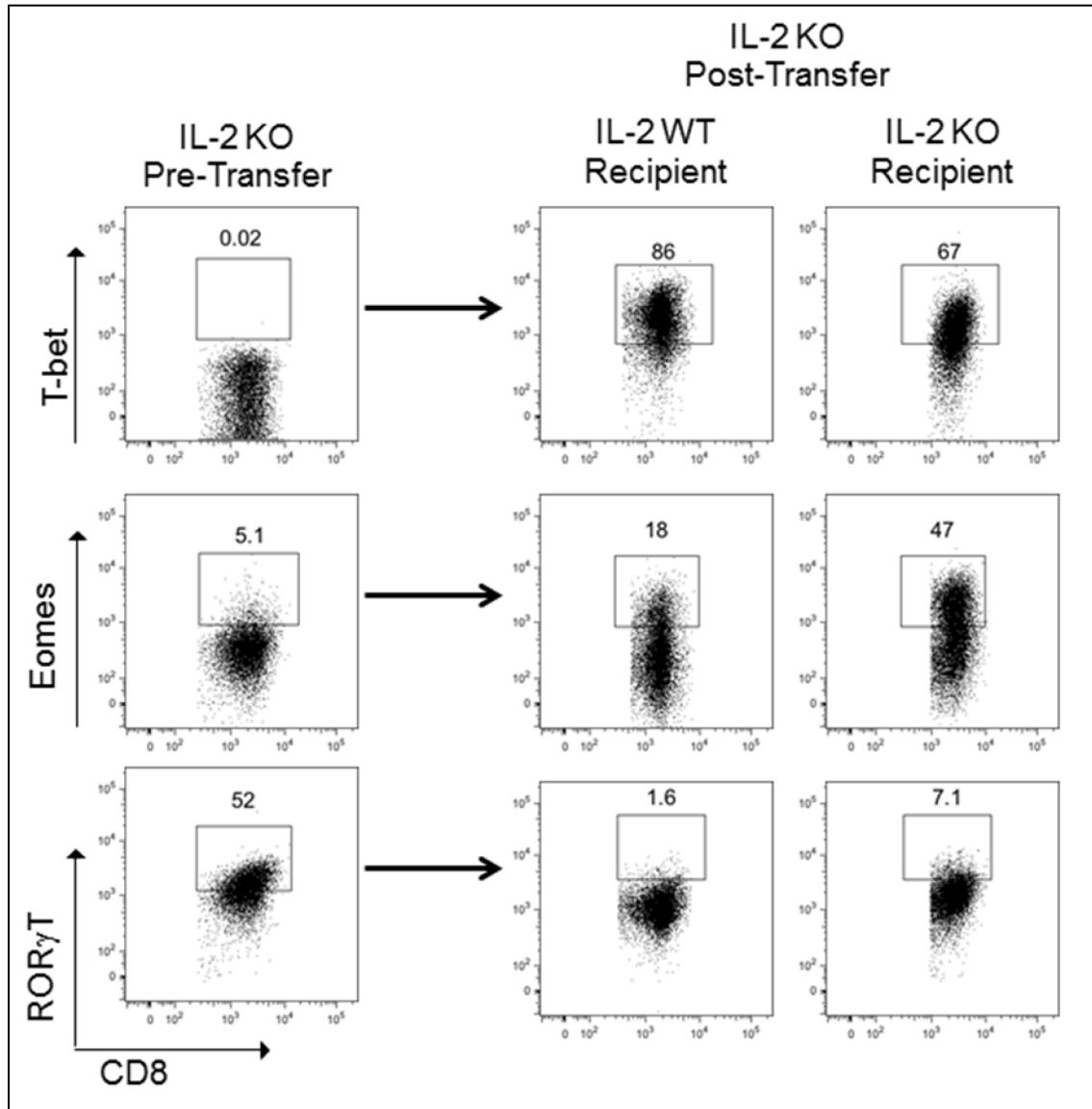
1×10^6 *in vitro* generated IL-2 WT Tc17 cells were adoptively transferred into CD45.1+ or IL-2 KO/RAG KO recipients with VV-OVA. 7 days later organs were harvested. For analysis of transcription factors, cells were stimulated with OVA peptide. Protein transport inhibitor cocktail was added during stimulation. Transcription factor staining was analyzed using intracellular staining and flow cytometry.

Figure 6-3: Role of Autocrine IL-2 in Tc17 Conversion (Cytokine Profile)



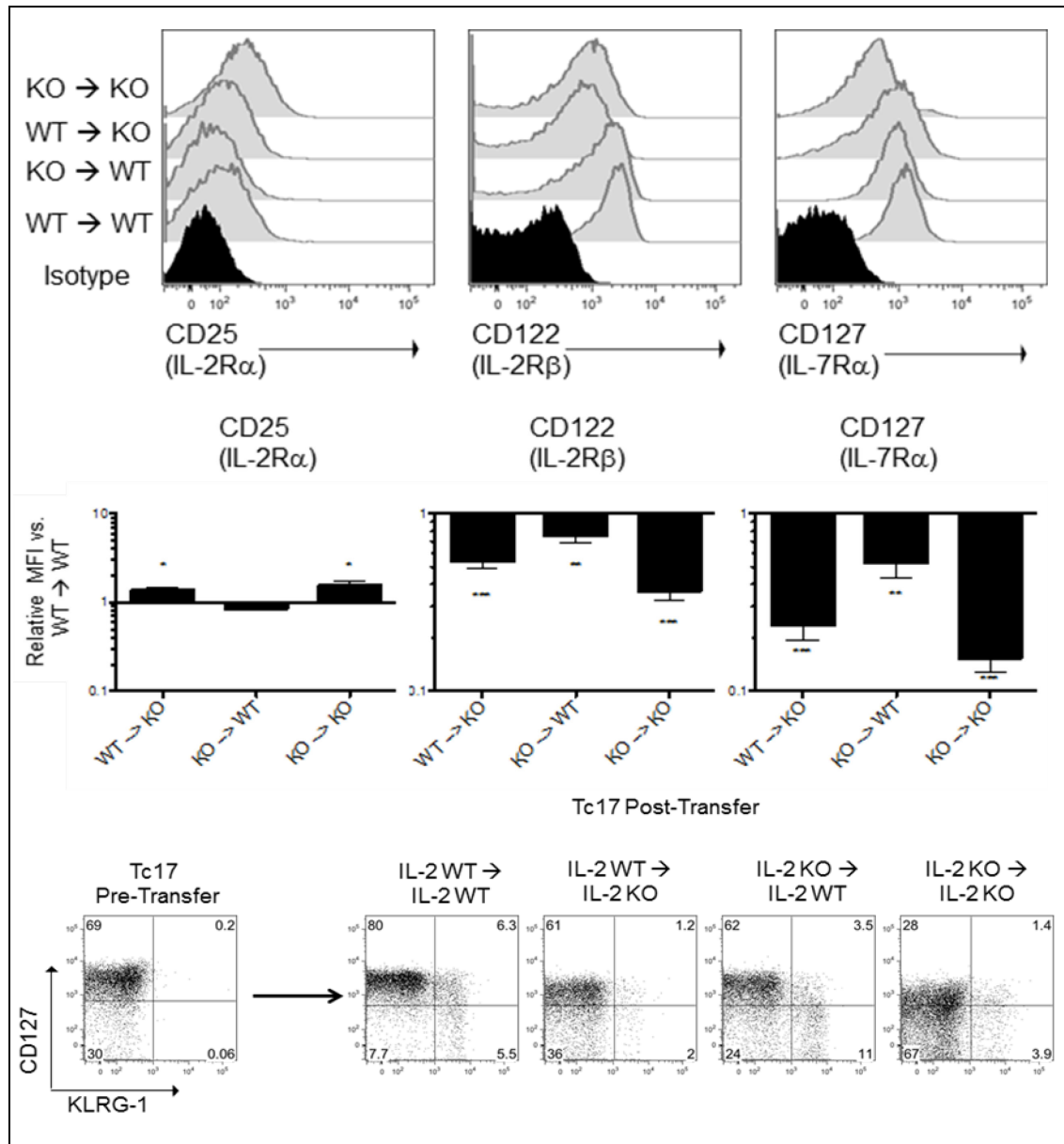
1×10^6 *in vitro* generated IL-2 KO Tc17 cells were adoptively transferred into CD45.1+ or IL-2 KO/RAG KO recipients with VV-OVA. 7 days later organs were harvested. For analysis of cytokines, cells were stimulated with OVA peptide. Protein transport inhibitor cocktail was added during stimulation. Cytokine staining was analyzed using intracellular staining and flow cytometry.

Figure 6-4: Role of Autocrine IL-2 in Tc17 Conversion (Transcription Factor Profile)



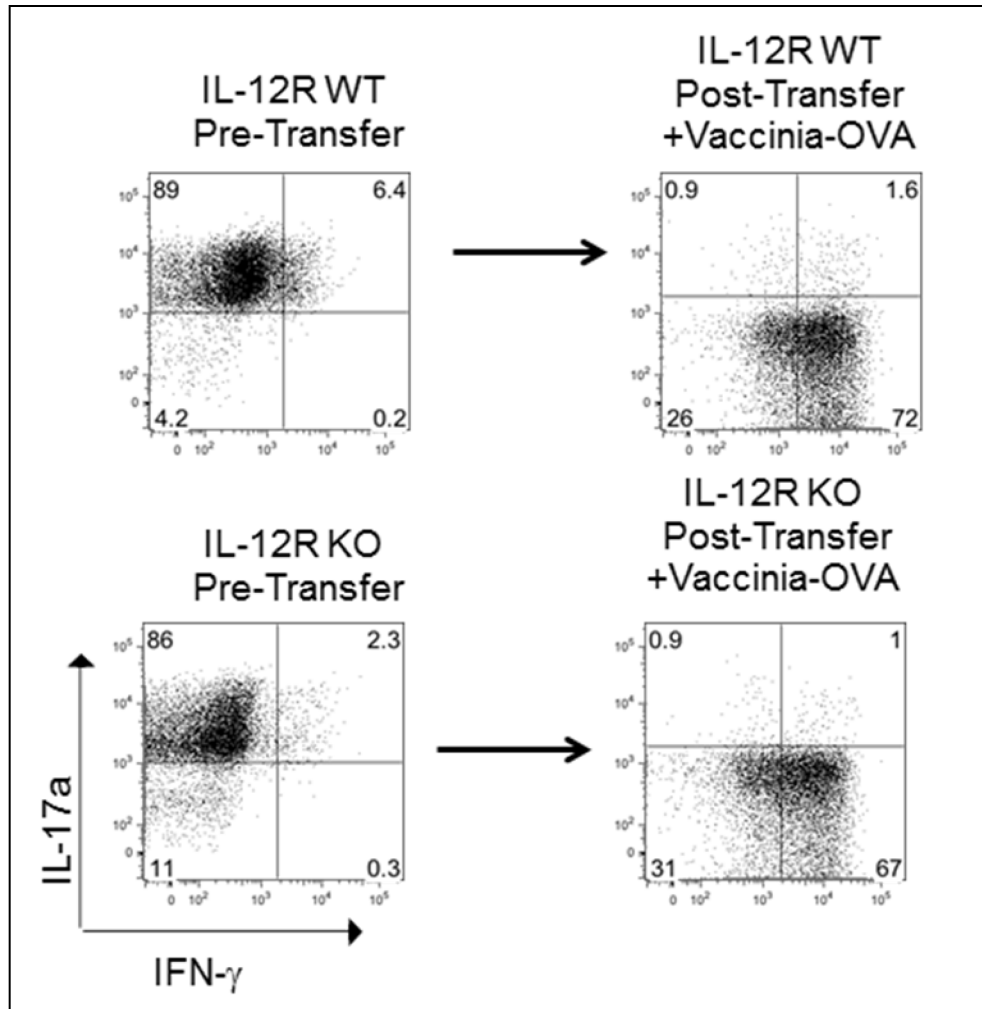
1×10^6 *in vitro* generated IL-2 KO Tc17 cells were adoptively transferred into CD45.1+ or IL-2 KO/RAG KO recipients with VV-OVA. 7 days later organs were harvested. For analysis of transcription factors, cells were stimulated with OVA peptide. Protein transport inhibitor cocktail was added during stimulation. Transcription factor staining was analyzed using intracellular staining and flow cytometry.

Figure 6-5: Role of Exocrine and Autocrine IL-2 in Tc17 Conversion (Receptor Profile)



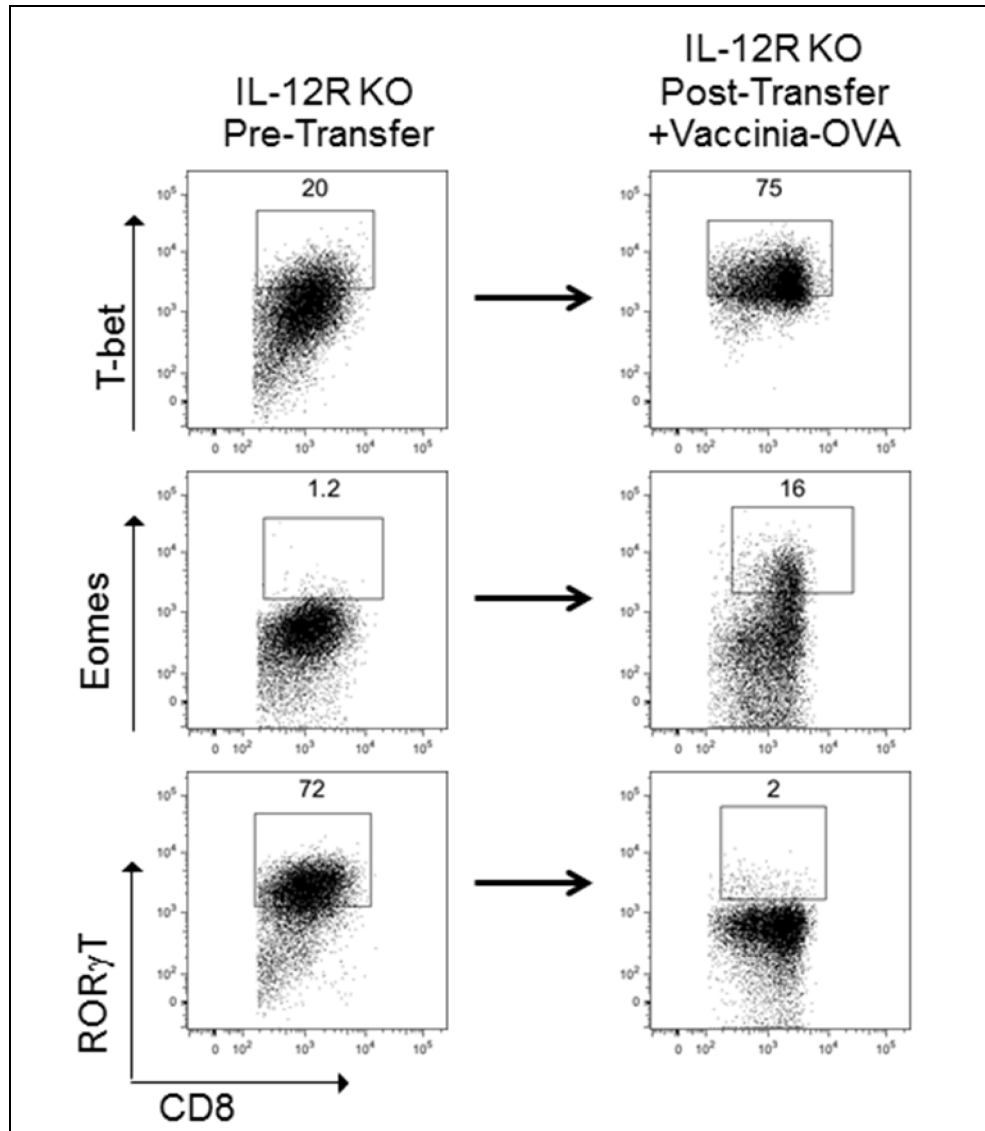
1x10⁶ *in vitro* generated WT or IL-2 KO Tc17 cells were adoptively transferred into CD45.1+ or IL-2 KO/RAG KO Recipients. 7 days later, respectively, organs were harvested. Receptors were analyzed using extracellular staining and flow cytometry. The values represent the means +/- SEM (n=4). *p<0.05, **p<.01, ***p<.001

Figure 6-6: Role of IL-12 in Tc17 Conversion (Cytokine Profile)



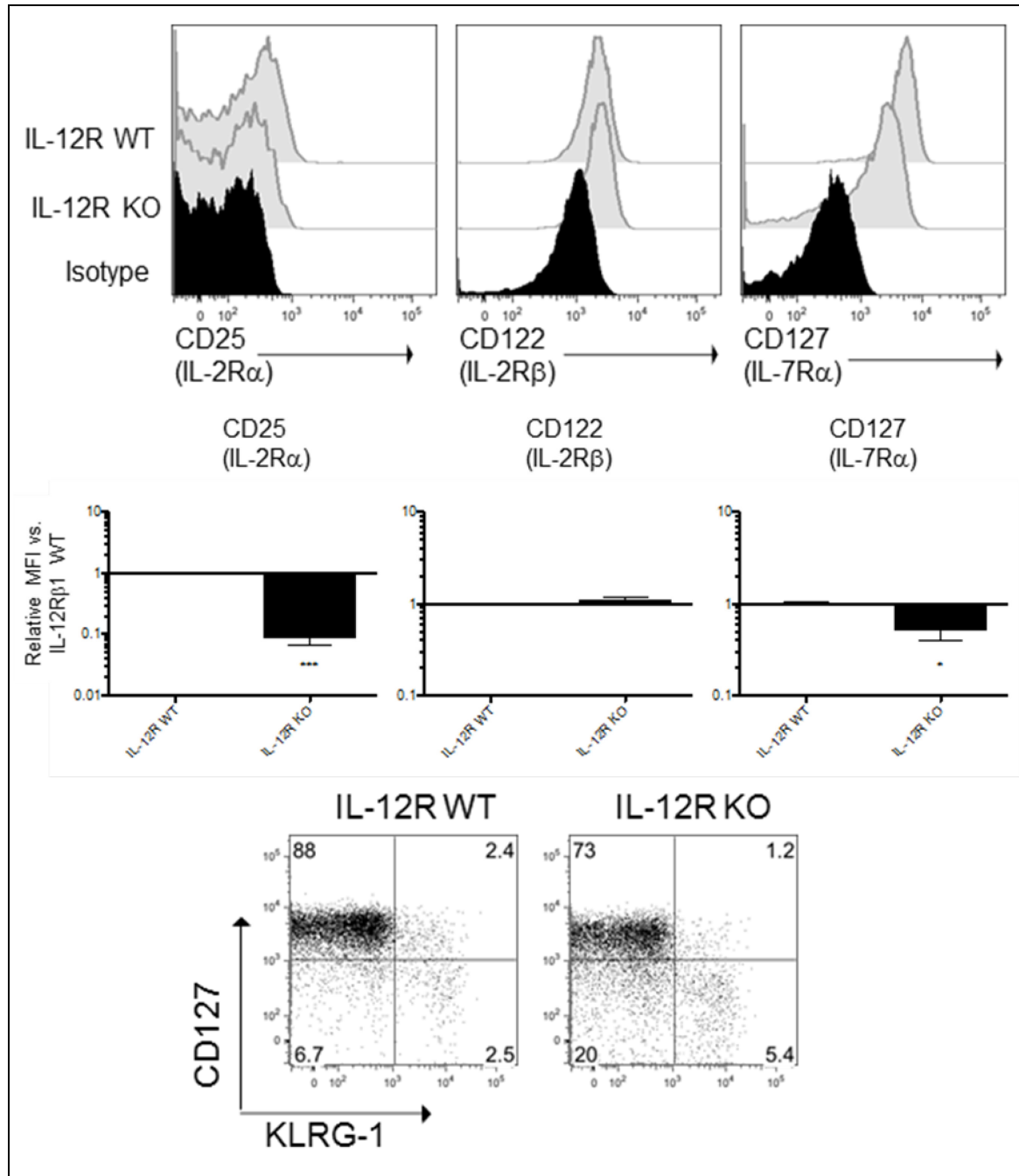
1x10⁶ *in vitro* generated WT or IL-12Rβ1 KO Tc17 cells were adoptively transferred into CD45.1+ or C57B/6 recipients with VV-OVA. 7 days later organs were harvested. For analysis of cytokine production, cells were stimulated with OVA peptide. Protein transport inhibitor cocktail was added during stimulation. Cytokine staining was analyzed using intracellular staining and flow cytometry.

Figure 6-7: Role of IL-12 in Tc17 Conversion (Transcription Factor Profile)



1×10^6 *in vitro* generated IL-12R β 1 KO Tc17 cells were adoptively transferred into C57B/6 recipients with VV-OVA. 7 days later organs were harvested. For analysis of transcription factor expression, cells were stimulated with OVA peptide. Protein transport inhibitor cocktail was added during stimulation. Transcription factor staining was analyzed using intracellular staining and flow cytometry.

Figure 6-8: Role of IL-12 in Tc17 Conversion (Receptors Profile)



1x10⁶ *in vitro* generated WT or IL-12R β 1 KO Tc17 cells were adoptively transferred into CD45.1+ or C57B/6 Recipients. 7 days later, respectively, organs were harvested. Receptors were analyzed using extracellular staining and flow cytometry. The values represent the means \pm SEM (n=4). *p<0.05, ***p<.001

CHAPTER 7

DEFINING THE MOLECULE REQUIRED FOR

TC17 CONVERSION *IN VIVO*

INTRODUCTION

As mentioned in Chapter 1, the T-box transcription factor T-bet, encoded by *tbx21*, is referred to as the “master regulator” of CD8⁺ T cell differentiation. T-bet controls the transcription of numerous CTL effector molecules such as IFN- γ and granzyme B^{26, 27}. T-bet plays an important role during T cell induction. T-bet KO CD8⁺ T cells activated under IL-17 promoting conditions secrete more IL-17 than T-bet competent CD8⁺ T cells^{37, 64, 84}. During LCMV infection, T-bet KO CD8⁺ T cells secrete IL-17 as opposed to the normal IFN- γ secretion³⁶. As discussed in Chapter 1, T-bet KO Th17 cells do not promote an effective anti-tumor response. T-bet KO Th17 cells are unable to convert from IL-17 to IFN- γ secretion. T-bet controls the plasticity of the Th17 cells and subsequently the anti-tumor response⁷². This phenomenon also occurs in autoimmune models. In one study, adoptively transferred T-bet KO Th17 cells did not affect EAE pathogenesis. Conversely, a different study showed adoptively transferred T-bet KO Th17 cells actually improved EAE pathogenesis^{73, 75}.

In this chapter, we sought to find the absolute requirement for Tc17 conversion *in vivo*. In Chapters 4-6, we showed an association between T-bet expression and IFN- γ production *in vitro* and *in vivo*. While T-bet is required for the functional plasticity of Th17 cells, we sought to understand the role of T-bet in Tc17 plasticity⁷²⁻⁷⁵. We hypothesized T-bet would be required for conversion *in vivo*. In order to investigate this hypothesis, we adoptively transferred T-bet KO CD8⁺ T cells into mice infected with VV-OVA and looked at IL-17 and IFN- γ secretion.

We also sought to determine the transcription factor expression in the absence of T-bet. In Chapters 4-6, we demonstrated robust conversion both *in vitro* and *in vivo* was associated with loss of ROR γ T. Therefore, we hypothesized Tc17 cells unable to convert in the absence of T-bet would maintain ROR γ T expression. In addition, the only time we saw eomes expression was during conversion in VV-OVA infected mice. If T-bet is required for conversion and may influence eomes expression, we predicted T-bet KO Tc17 cells would express less eomes than T-bet competent Tc17 cells. In order to test these hypotheses, we adoptively transferred T-bet KO CD8⁺ T cells into mice infected with VV-OVA. We stained T-bet, ROR γ T and eomes after adoptive transfer.

Finally, we wanted to investigate the gamma chain receptor expression of T-bet KO Tc17 cells after adoptive transfer into VV-OVA infected mice. In Chapter 5, we showed the gamma chain cytokine receptor expression of Tc17 cells adoptively transferred into mice without stimulation. We hypothesized T-bet KO Tc17 cells adoptively transferred into mice infected with VV-OVA would act similarly to WT Tc17 cells adoptively transferred into mice without stimulation; T-bet KO Tc17 cells would express less CD122, more CD25 and similar CD127. In order to test this hypothesis we adoptively transferred T-bet KO CD8⁺ T cells into mice infected with VV-OVA. We stained CD25, CD122 and CD127 after adoptive transfer.

RESULTS

T-bet is required for Tc17 Conversion *In Vivo*

To test the requirement for T-bet, we bred T-bet KO mice onto the OT-1/RAG KO background. We first cultured T-bet KO Tc17 cells under Tc17 polarizing conditions *in vitro*. We obtained a >98% IL-17+IFN- γ - population without sorting, which demonstrated the negative role of T-bet during Tc17 induction. Next we adoptively transferred T-bet KO Tc17 cells into mice infected with VV-OVA. We harvested splenocytes and found T-bet KO Tc17 cells could not convert *in vivo*. T-bet KO Tc17 cells did not secrete any IFN- γ , and maintained expression of IL-17 in the liver and spleen. Interestingly, T-bet KO Tc17 cells produced less IL-17 in the lymph nodes and lung. This did not correlate with increased IFN- γ secretion, just loss of IL-17. T-bet may affect IL-17 secretion in certain tissues (Figure 7-1). These data show the important role of T-bet during Tc17 conversion *in vivo*.

T-bet KO Tc17 Cells do not lose ROR γ T Expression in an Inflammatory Milieu

We next wanted to understand the expression of ROR γ T and eomes in T-bet KO Tc17 cells in an inflammatory milieu. In order to do this we adoptively transferred T-bet KO Tc17 cells into mice infected with VV-OVA. We harvested splenocytes and found T-bet KO Tc17 cells do not lose ROR γ T expression in an inflammatory milieu. This could contribute to the inability of these cells to expression IFN- γ *in vivo*. As confirmation, we showed T-bet KO Tc17 cells do not express T-bet. We found T-bet KO Tc17 cells express very little eomes in VV-OVA infected mice, suggesting the importance of T-bet for eomes up-regulation (Figure 7-2).

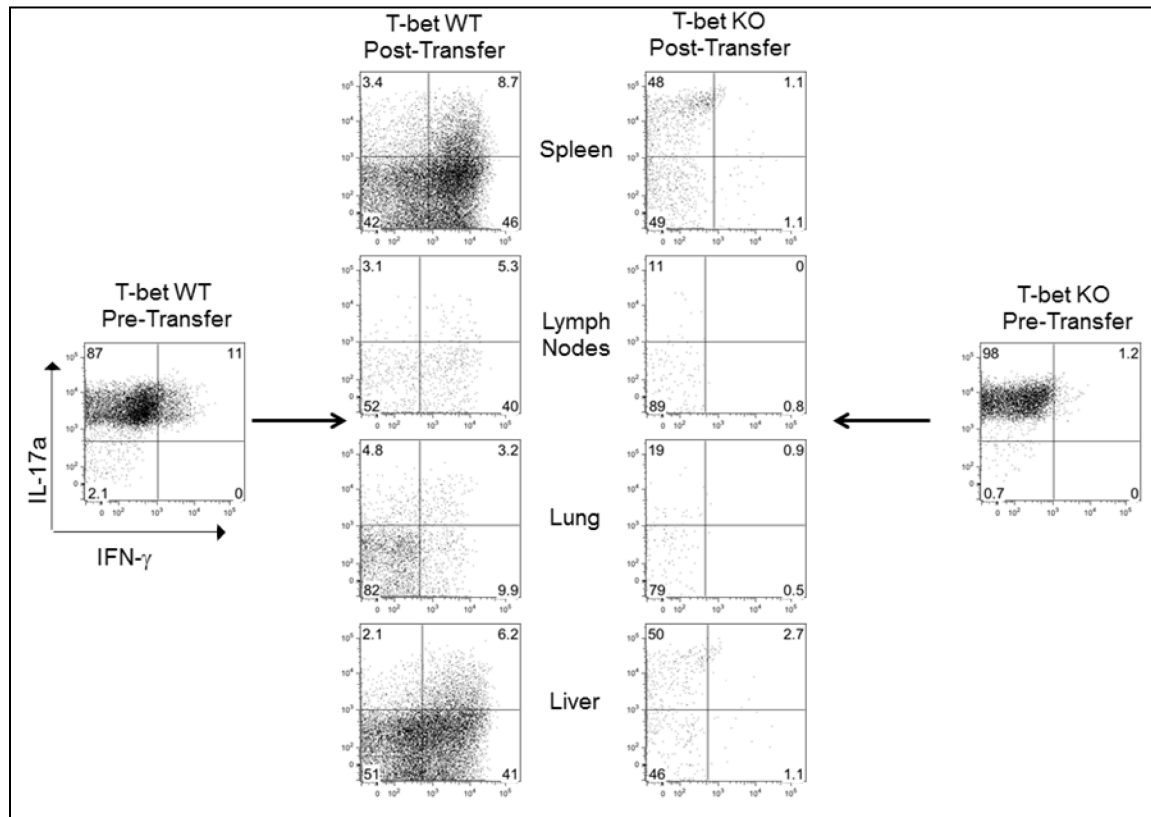
Role of T-bet on Receptor Expression of Tc17 Cells *in vivo*

We next sought to determine the expression of common gamma chain cytokine receptors on T-bet KO Tc17 cells in an inflammatory milieu, so we adoptively transferred T-bet KO Tc17 cells into mice infected with VV-OVA. We found they express significantly less CD122 than T-bet competent Tc17 cells. T-bet KO Tc17 cells also express significantly more CD25 than T-bet competent Tc17 cells. Furthermore, we found T-bet KO Tc17 cells have a lower percentage of SLEC's as compared to T-bet competent Tc17 cells in an inflammatory milieu, suggesting the important of T-bet for an effective effector response (Figure 7-3).

SUMMARY

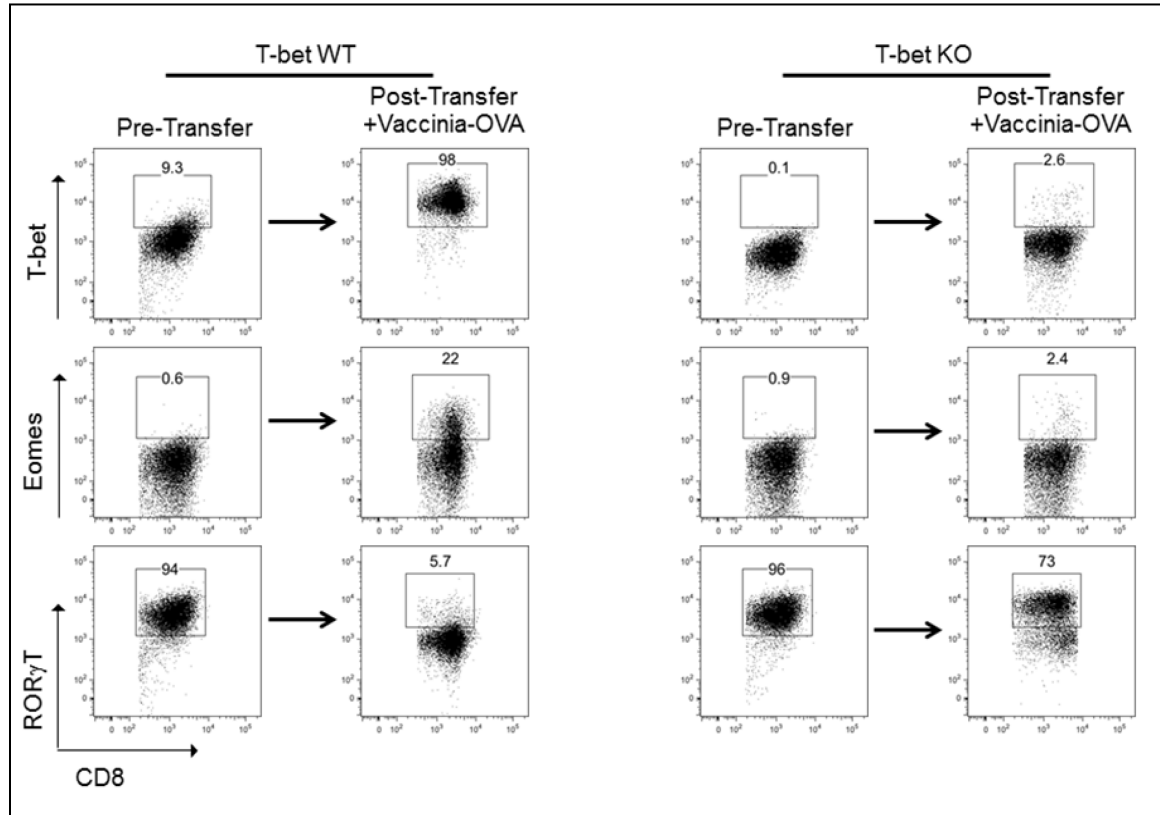
These data show T-bet is required for conversion of Tc17 cells *in vivo*. We found T-bet KO Tc17 cells do not lose ROR γ T expression even with a strong inflammatory milieu provided by VV-OVA. This suggests in the absence of T-bet, the Tc17 transcriptional program is very stable. In addition, after adoptive transfer, T-bet KO Tc17 cells express less eomes as compared to T-bet WT Tc17 cells. This suggests T-bet controls expression of eomes in Tc17 cells. We found T-bet KO Tc17 cells have significantly less CD122 expression than T-bet WT Tc17 cells, suggesting a potential role for the high affinity IL-2 receptor during conversion. We also found T-bet KO Tc17 cells do not express KLRG-1 in the spleen as compared to T-bet WT Tc17 cells. This suggests T-bet drives the expression of KLRG-1 in Tc17 cells and acquisition of an effector phenotype.

Figure 7-1: Role of T-bet in Tc17 Conversion (Cytokine Profile)



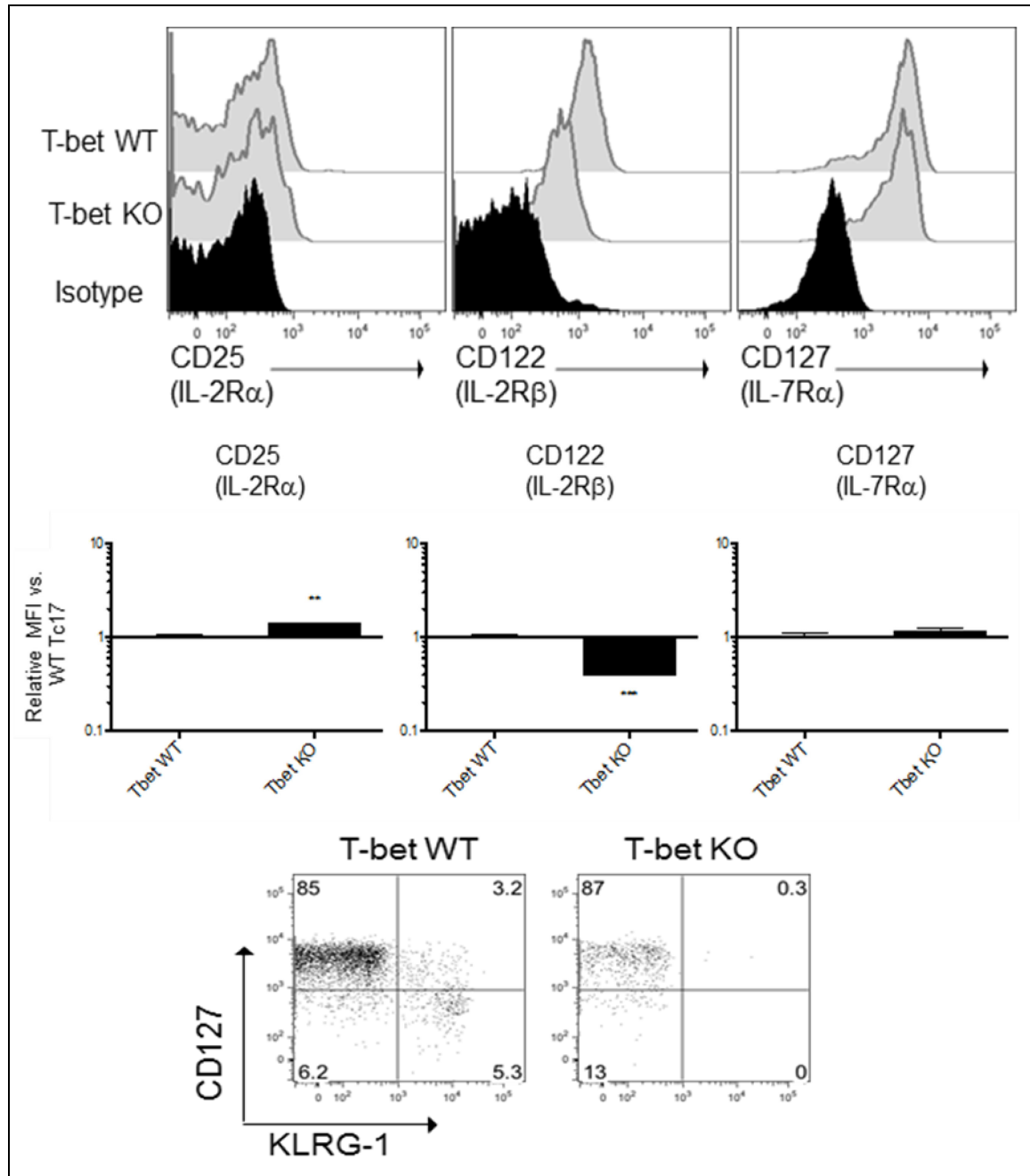
1×10^6 *in vitro* generated WT or T-bet KO Tc17 cells were adoptively transferred into CD45.1+ recipients with VV-OVA. 7 days later organs were harvested. For analysis of cytokine production, cells were stimulated with OVA peptide. Protein transport inhibitor cocktail was added during stimulation. Cytokine staining was analyzed using intracellular staining and flow cytometry

Figure 7-2: Role of T-bet in Tc17 Conversion (Transcription Factor Profile)



1×10^6 *in vitro* generated WT or T-bet KO Tc17 cells were adoptively transferred into CD45.1⁺ recipients with VV-OVA. 7 days later organs were harvested. For analysis of transcription factor expression, cells were stimulated with OVA peptide. Protein transport inhibitor cocktail was added during stimulation. Transcription factor staining was analyzed using intracellular staining and flow cytometry

Figure 7-3: Role of T-bet in Tc17 Conversion (Receptor Profile)



1x10⁶ *in vitro* generated WT or T-bet KO Tc17 cells were adoptively transferred into CD45.1⁺ recipients with VV-OVA. 7 days later, organs were harvested. Receptors were analyzed using extracellular staining and flow cytometry. The values represent the means \pm SEM (n=4). **p<.01, ***p<.001

CHAPTER 8

ROLE OF TC17 PLASTICITY IN ANTI-TUMOR IMMUNITY

INTRODUCTION

As referenced in Chapter 1, adoptively transferred antigen-specific Tc17 cells promote tumor regression in both flank and lung tumors^{38, 47, 48}. This anti-tumor response was associated with Tc17 plasticity in flank tumor bearing mice, but not lung bearing mice tumor⁴⁷⁻⁴⁹. Tc17 cells converted with IL-12 prior to adoptive transfer have a better anti-tumor effect than un-converted Tc17 cells³⁸. As discussed in previous chapters, T-bet KO Th17 cells could not promote an effective anti-tumor response. These T-bet KO Th17 cells were unable to convert from IL-17 to IFN- γ secretion. T-bet controlled the plasticity of the Th17 cells and subsequently the anti-tumor response⁷².

In this chapter, we sought to understand if T-bet is required for an effective Tc17 cell anti-tumor response. Tc17 cells can promote an anti-tumor response, but the requirement for conversion during this response has never been investigated⁴⁷⁻⁴⁹. In Chapter 7, we showed T-bet was required for conversion *in vivo*. We hypothesized T-bet competent Tc17 cells would promote tumor regression, but T-bet KO Tc17 cells would be equivalent to untreated mice. In order to test this hypothesis we adoptively transferred T-bet WT and T-bet KO Tc17 cells into tumor-bearing mice and monitored tumor growth.

There is contradicting evidence on the role of vaccination in conjunction with Tc17 cell adoptive immunotherapy⁴⁷⁻⁴⁹. In Chapters 5-7, we showed VV-OVA drove conversion of adoptively transferred Tc17 cells. We hypothesized VV-OVA in combination with adoptively transferred Tc17 cells, would positively affect the anti-tumor response. In Chapter 7, we demonstrated T-bet was required for Tc17 plasticity in

mice infected with VV-OVA. We hypothesized VV-OVA would not affect the anti-tumor response of adoptively transferred T-bet KO Tc17 cells. In order to test these hypotheses we adoptively transferred T-bet KO and T-bet WT Tc17 cells with or without VV-OVA and monitored tumor growth.

The transcription factor expression of adoptively transferred cells after an anti-tumor response has never been investigated. In Chapter 5-7, we showed adoptively transferred Tc17 cells express T-bet and convert in mice infected with VV-OVA, but Tc17 cells do not express T-bet or convert without VV-OVA infection. We hypothesized adoptively transferred Tc17 cells would express T-bet in tumor-bearing mice infected with VV-OVA. Conversely, we hypothesize Tc17 cells adoptively transferred into tumor-bearing mice without vaccine will not express T-bet or convert from IL-17 to IFN- γ secretion. Additionally, T-bet expression would be associated with an effective anti-tumor response, as well as, loss of ROR γ T. In order to test these hypotheses we adoptively transferred Tc17 cells into tumor-bearing mice with or without VV-OVA infection and looked for transcription factor expression after transfer.

In Chapter 7, we showed T-bet KO Tc17 cells did not lose ROR γ T expression. We hypothesized T-bet KO Tc17 cells adoptively transferred into tumor bearing mice would also not lose ROR γ T expression. In Chapter 7, adoptively transferred T-bet KO Tc17 cells expressed less eomes than T-bet competent Tc17 cells. We hypothesized this would also occur in tumor-bearing mice. In order to test these hypotheses we adoptively transferred T-bet KO and T-bet WT Tc17 cells with or without VV-OVA and looked for transcription factor expression.

Expression of gamma chain receptors on adoptively transferred Tc17 cells in tumor bearing mice has not been thoroughly investigated⁴⁸. In Chapter 5, we showed Tc17 cells adoptively transferred into mice without stimulation expressed more CD127 and less CD122 than Tc17 cells adoptively transferred into mice infected with VV-OVA. We hypothesized Tc17 cells adoptively transferred into tumor-bearing mice without VV-OVA infection would behave similarly expressing more CD127 and less CD122 than transfer with VV-OVA. Additionally, Tc17 cells adoptively transferred into tumor-bearing mice with VV-OVA infection would express more CD122 and have a larger pool of SLECs compared to cells adoptively transferred without VV-OVA infection. In order to test these hypotheses we adoptively transferred Tc17 cells into tumor-bearing mice with or without VV-OVA infection and looked at the expression of gamma chain receptors.

In Chapter 7, we showed T-bet KO Tc17 cells expressed more CD127 and less CD122 than T-bet competent Tc17 cells in mice infected with VV-OVA. T-bet KO Tc17 cells also did not express KLRG-1 or become SLECs as compared to T-bet competent Tc17 cells. We hypothesized T-bet KO Tc17 cells adoptively transferred into tumor-bearing mice would not become SLECs and express more CD127 than T-bet competent cells. We also hypothesized adoptive transfer with or without VV-OVA infection would not change the phenotype. In order to test these hypotheses we adoptively transferred Tc17 cells into tumor-bearing mice with or without VV-OVA infection, and looked at CD25, CD122 and CD127 expression on adoptively transferred cells.

RESULTS

T-bet is required for Effective Tc17 Anti-Tumor Immunity

To test the requirement for T-bet expression during Tc17 cell adoptive immunotherapy, we implanted wild type mice with B16F10-OVA melanoma. When the mice had established, vascularized tumors, we adoptively transferred WT or T-bet KO Tc17 cells with or without VV-OVA infection. Additionally, we administered VV-OVA alone without T cells as a negative control. We found adoptive transfer of WT Tc17 cells but not T-bet KO Tc17 cells mice showed improved survival. The most profound anti-tumor response was shown with WT Tc17 cell in combination with VV-OVA treatment. Established melanoma tumors, some as large as 1200 mm³, regressed upon adoptive T cell therapy. A couple mice that received T-bet KO Tc17 cells in combination with VV-OVA infection had tumor regression, but relapsed after several days. VV-OVA treatment alone was similar to untreated tumors (Figures 8-1 and 8-2).

Tumors Treated with Tc17 Cells have Significantly Smaller Tumors than Untreated Tumors

We next sought to examine the effect of treatment on the size of tumors *ex vivo*. In order to do this, we harvested tumors 7 days after treatment. We measured tumor wet weights and found tumors treated with WT Tc17 cells and VV-OVA were significantly smaller than tumors treated with T-bet KO Tc17 cells and VV-OVA. Untreated tumors were significantly greater than tumors treated with Tc17 cells and VV-OVA (Figure 8-3).

Homing of Tc17 Cells to Tissue of Tumor-Bearing Mice

We next determined if the deficiency in an effective anti-tumor response was because T-bet KO Tc17 cells could not infiltrate the DLN or tumor. In order to do this we harvested cells from the spleen, draining lymph node and tumors and looked for the adoptively transferred population. We found there was not a significant difference between the percentages of adoptively transferred WT or T-bet KO Tc17 cells with VV-OVA infection in the DLN. Interestingly, there were a significantly higher percentage of T-bet KO Tc17 cells in the tumor (Figure 8-4).

Anti-Tumor Immunity Correlates with Tc17 Cell Conversion

To test the cytokine phenotype of the adoptively transferred cells we stimulated cells from all organs and analyzed the cytokine profile. WT Tc17 cells adoptively transferred with VV-OVA converted to an IFN- γ secreting phenotype more efficiently in both the spleen and DLN than Tc17 cells without VV-OVA. WT Tc17 cells that migrated to the tumor secreted very little IFN- γ , even with VV-OVA. Conversely, as was seen in Chapter 7, T-bet KO Tc17 cells did not convert in the spleen, DLN or tumor. This was with or without VV-OVA treatment. T-bet KO Tc17 cells also did not lose IL-17 expression (Figure 8-5).

Anti-Tumor Immunity is Associated with T-bet Expression of Adoptively Transferred Tc17 cells

We next hypothesized T-bet expression in adoptively transferred Tc17 cells would be important in conversion and an effective anti-tumor response. We found WT Tc17 cells adoptively transferred with VV-OVA expressed T-bet and had lower ROR γ T expression

than WT Tc17 cells without VV-OVA in the DLN and TIL. Interestingly, the difference in transcription factor expression is less in the TIL between WT Tc17 cells with or without VV-OVA. As expected, T-bet KO Tc17 cells maintained ROR γ T expression in all organs (Figures 8-6, 8-7 and 8-8).

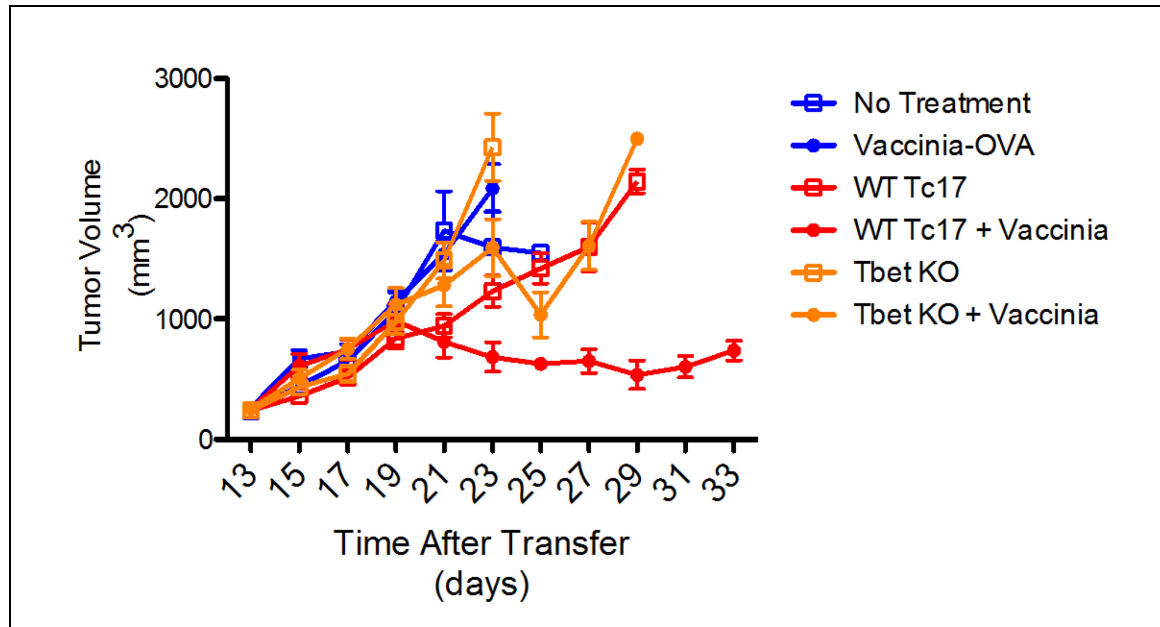
Role of T-bet on Receptor Expression of Tc17 Cells in Tumor-Bearing Mice

Next we characterized the expression of gamma chain receptors on the adoptively transferred cells populations. We hypothesized the receptors would be different between WT versus T-bet KO Tc17 cells during the anti-tumor response. Similar to what we showed in Chapter 7, we found T-bet KO Tc17 cells have significantly less CD122 expression as compared to WT mice, and significantly more CD25 expression in the DLN and spleen. This was independent of combination with VV-OVA infection. However, this difference was lost in the TIL (Figures 8-9 and 8-10). Furthermore, similar to Chapter 7, we found T-bet KO Tc17 cells have a lower percentage of SLEC's as compared to T-bet competent Tc17 cells in an inflammatory milieu (Figure 8-11).

SUMMARY

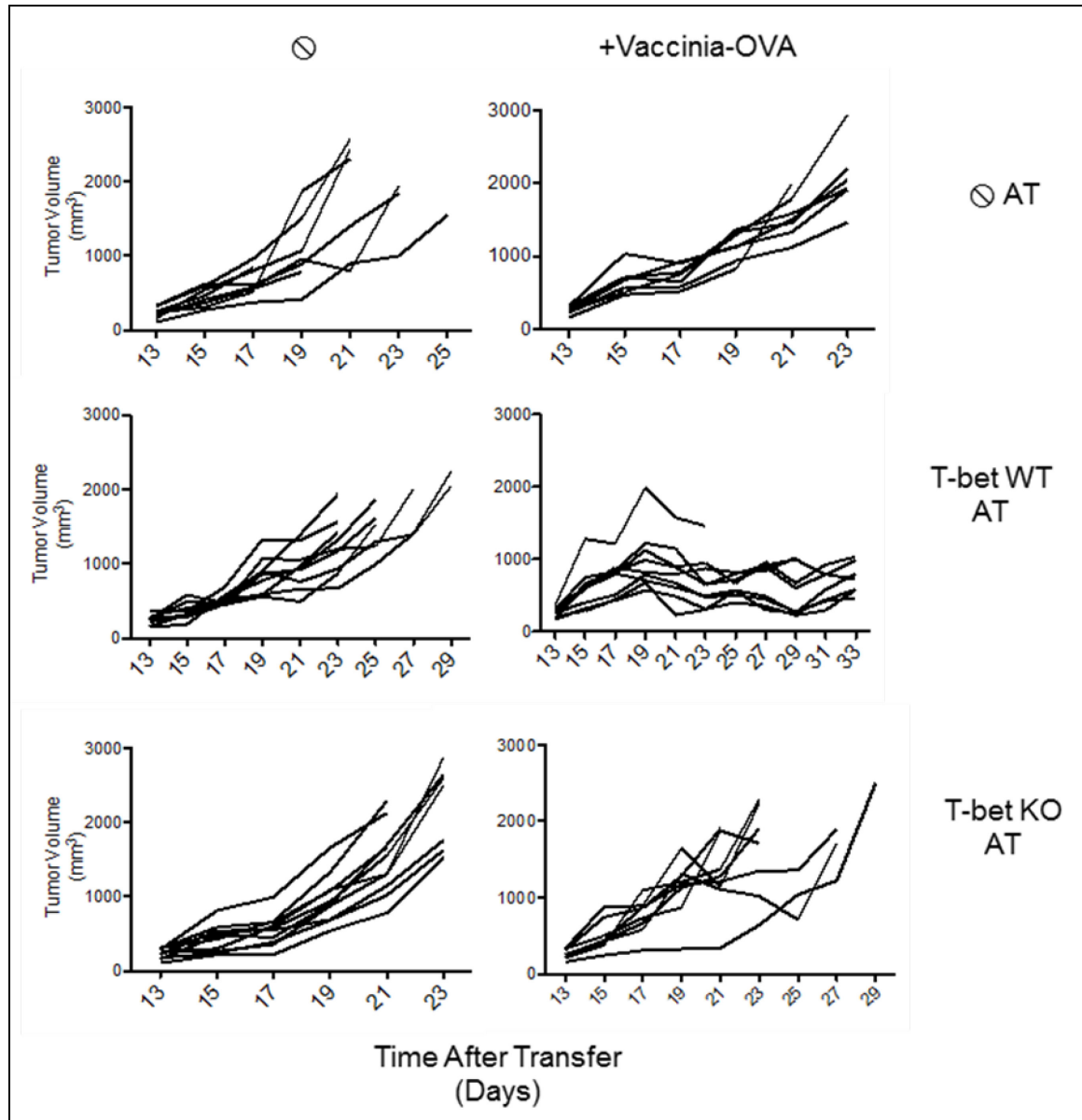
T-bet and IFN- γ expression in Tc17 cells are critical for tumor regression. This suggests the importance of functional plasticity during an anti-tumor response. We found in the absence of VV-OVA there was only a small survival advantage. Conversely, Tc17 cells adoptively transferred with VV-OVA promoted tumor regression and increased survival, thus proving Tc17 conversion was required to mediate anti-tumor immunity. T-bet KO Tc17 cells were detected in the draining lymph node and in the tumor. This finding demonstrated that although T bet KO Tc17 cells trafficked to the tumor, their inability to produce IFN- γ was detrimental to their anti-tumor functions. We found T-bet KO Tc17 cells maintained ROR γ T expression, suggesting this is an important mechanism for functional plasticity. In addition, the efficiency of the anti-tumor function was dependent on the level of T-bet expression because the VV-OVA injection in mice receiving Tc17 led to higher T-bet expression, and enhanced tumor regression. Our data demonstrate T-bet is the mastermind transcriptional factor driving Tc17 plasticity and subsequently anti-tumor immunity.

Figure 8-1: Tumor Growth Curves



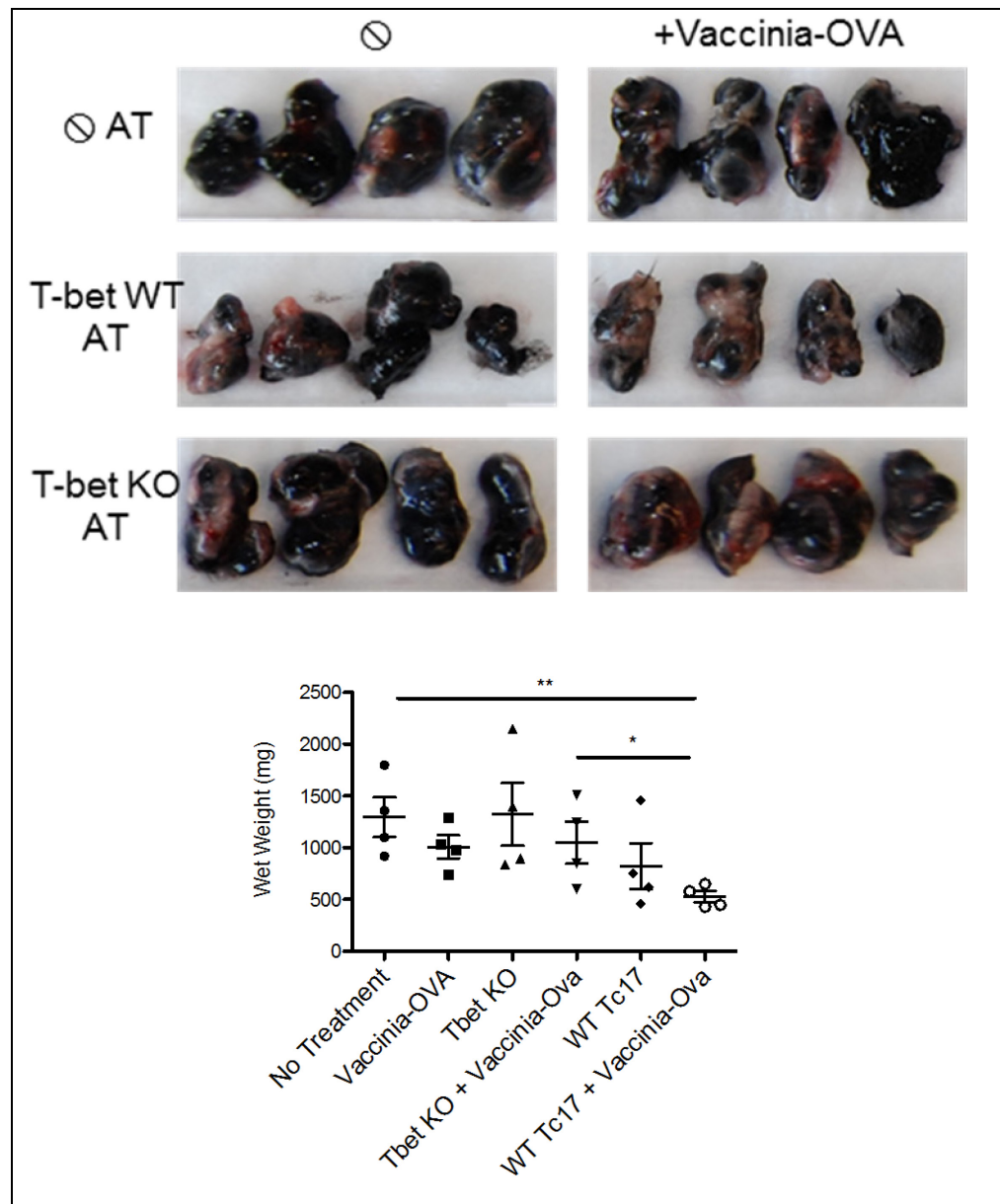
1.6×10^5 B16F10-OVA melanoma cells were injected into CD45.1+ recipients. On day 14, mice with palpable, established tumors received an adoptive transfer of 5×10^6 *in vitro* generated WT or T-bet KO Tc17 cells. This was with or without VV-OVA. Control groups received VV-OVA alone or no treatment. Tumor volume was calculated by using the following formula: tumor volume (mm^3) = (length) x (width)² x 0.5. The values represent the means \pm SEM (n=8-10).

Figure 8-2: Spaghetti Plots



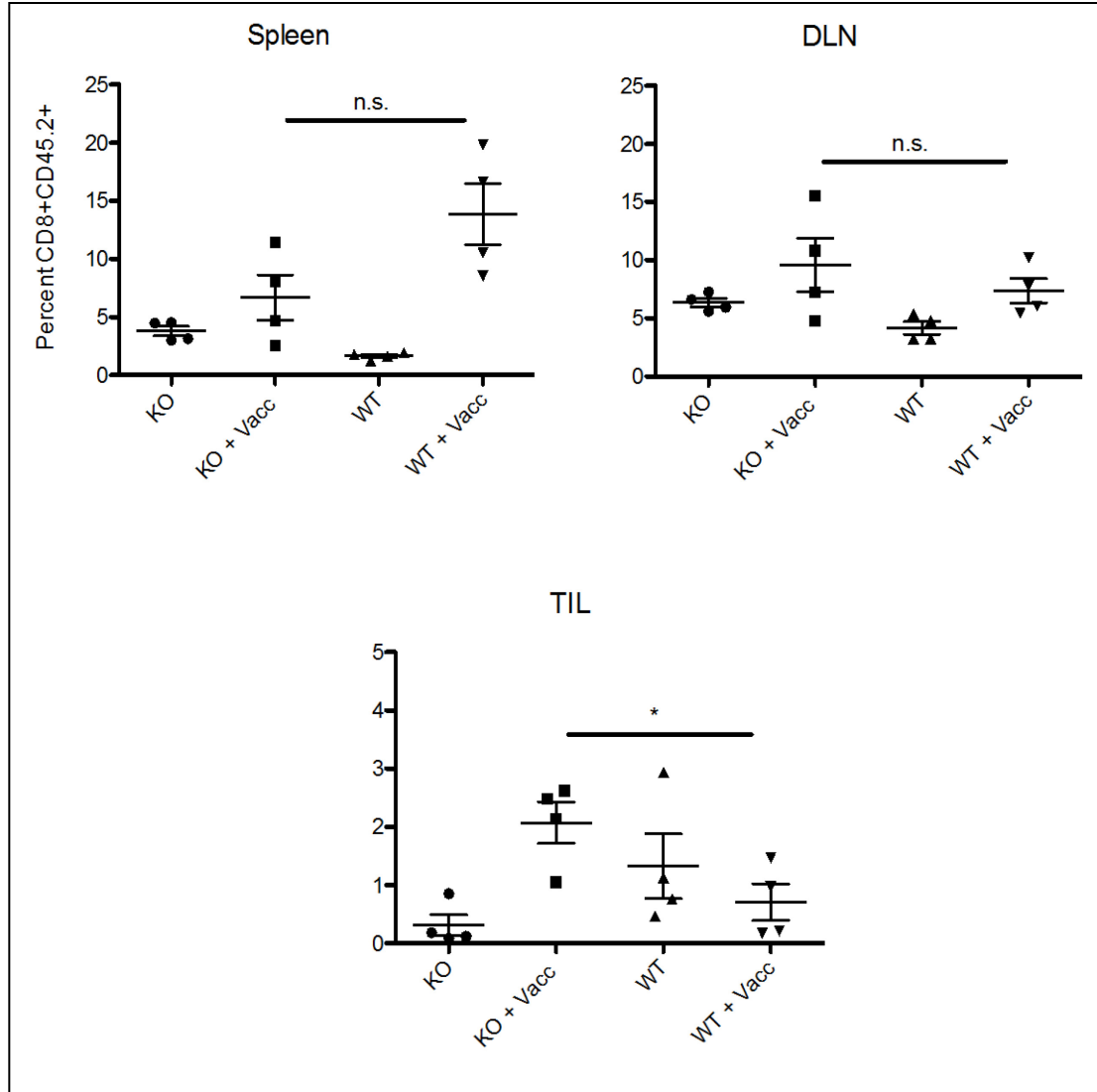
1.6×10^5 B16F10-OVA melanoma cells were injected into CD45.1+ recipients. On day 14, mice with palpable, established tumors received an adoptive transfer of 5×10^6 *in vitro* generated WT or T-bet KO Tc17 cells. This was with or without VV-OVA. Control groups received VV-OVA alone or no treatment. Tumor volume was calculated by using the following formula: tumor volume (mm³) = (length) x (width)² x 0.5.

Figure 8-3: Tumor Sizes and Wet Weights



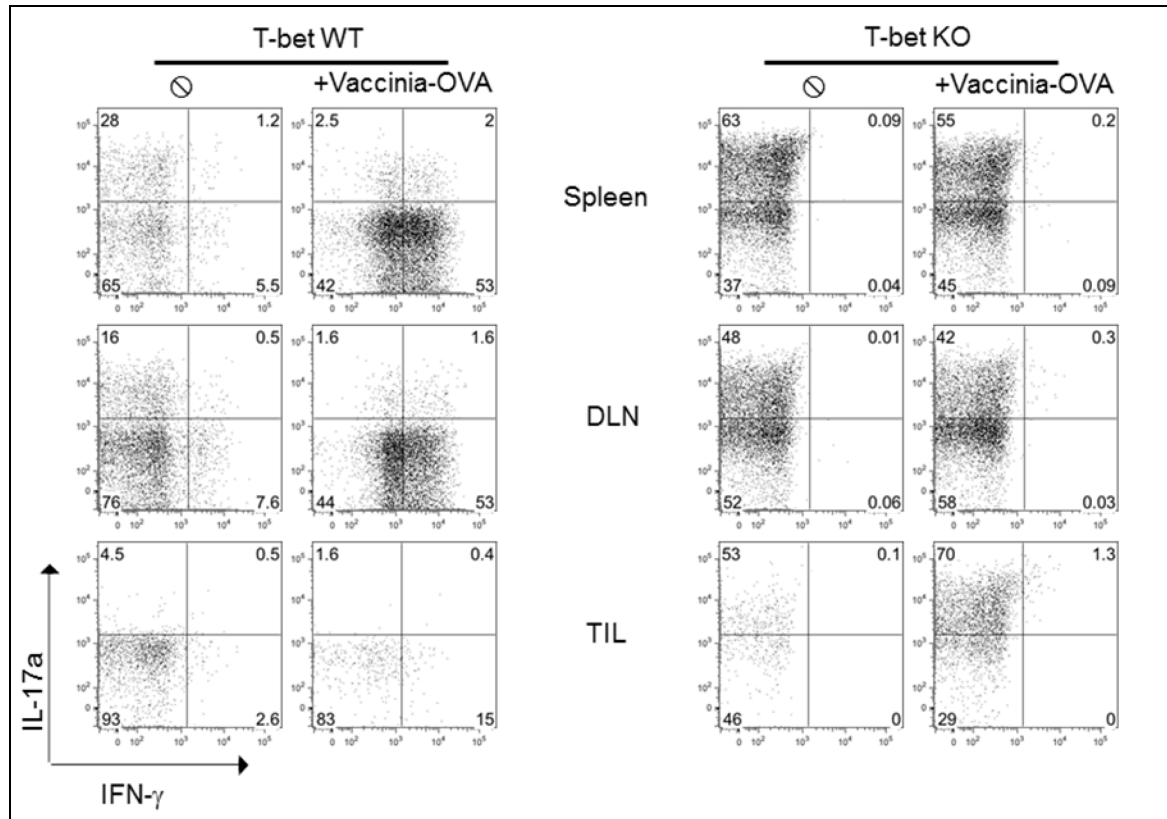
1.6×10^5 B16F10-OVA melanoma cells were injected into CD45.1+ recipients. On day 14, mice with palpable, established tumors received an adoptive transfer of 5×10^6 *in vitro* generated WT or T-bet KO Tc17 cells. This was with or without VV-OVA. Control groups received VV-OVA alone or no treatment. Tumors were harvested and wet weights measured. The values represent the means \pm SEM (n=4). *p<0.05, **p<0.01

Figure 8-4: Cell Percentages of Adoptively Transferred from Tumor Bearing Mice



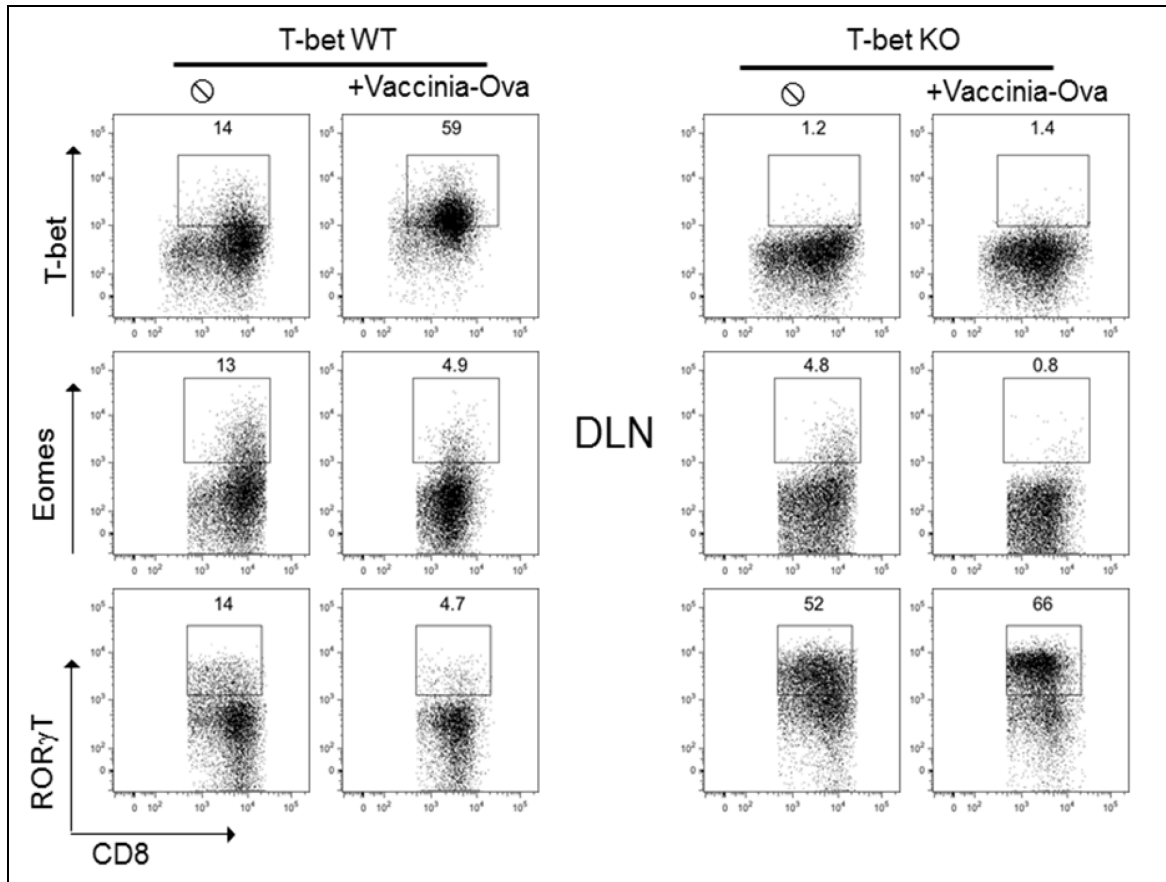
1.6×10^5 B16F10-OVA melanoma cells were injected into CD45.1+ recipients. On day 14, mice with palpable, established tumors received an adoptive transfer of 5×10^6 *in vitro* generated WT or T-bet KO Tc17 cells. This was with or without VV-OVA. Control groups received VV-OVA alone or no treatment. 7 days later, organs were harvested. The values represent the means \pm SEM (n=4). *p<0.05

Figure 8-5: Cytokine Profile of Adoptively Transferred Tc17 Cells from Tumor-Bearing Mice



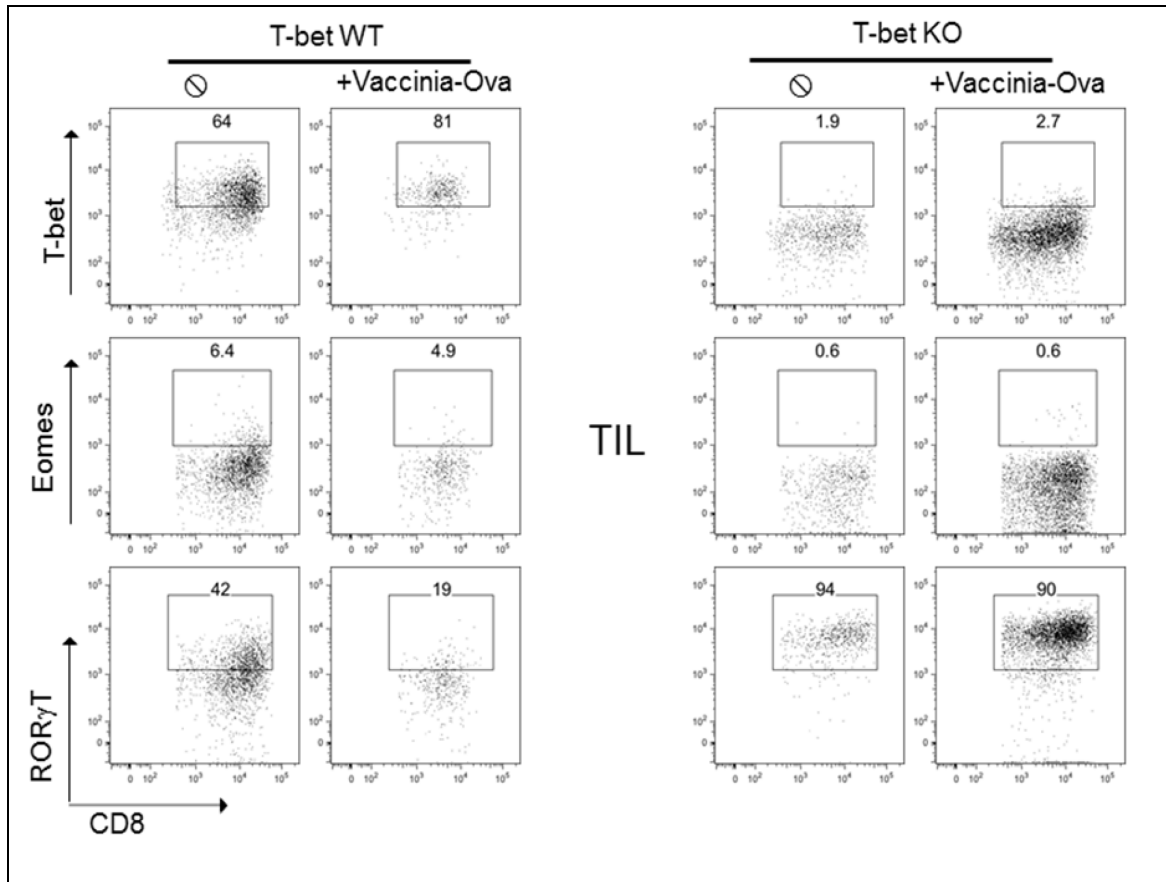
1.6x10⁵ B16F10-OVA melanoma cells were injected into CD45.1+ recipients. On day 14, mice with palpable, established tumors received an adoptive transfer of 5x10⁶ *in vitro* generated WT or T-bet KO Tc17 cells. This was with or without VV-OVA. Control groups received VV-OVA alone or no treatment. 7 days later organs were harvested. For analysis of cytokine production, cells were stimulated with OVA peptide. Protein transport inhibitor cocktail was added during stimulation. Cytokine staining was analyzed using intracellular staining and flow cytometry.

Figure 8-6: Transcription Factor Profile of Adoptively Transferred Tc17 Cells from Tumor-Bearing Mice (DLN)



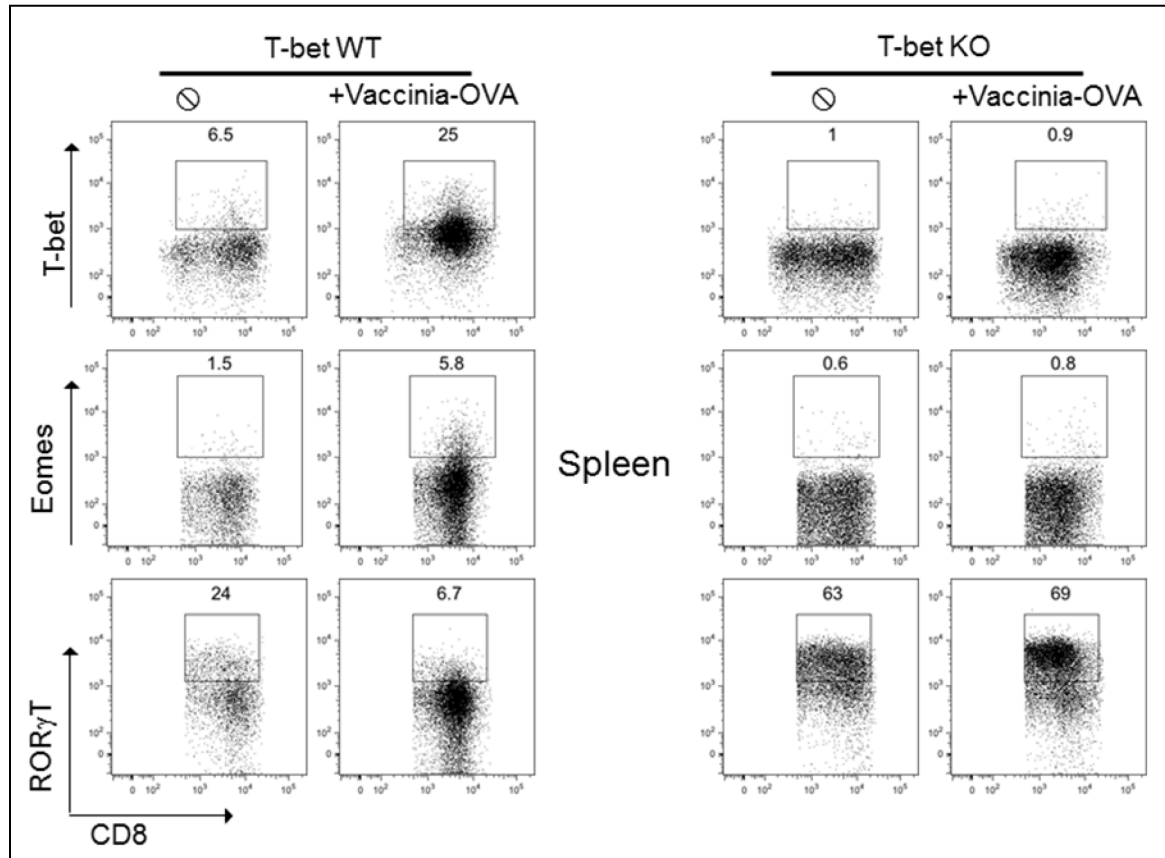
1.6x10⁵ B16F10-OVA melanoma cells were injected into CD45.1+ recipients. On day 14, mice with palpable, established tumors received an adoptive transfer of 5x10⁶ *in vitro* generated WT or T-bet KO Tc17 cells. This was with or without VV-OVA. Control groups received VV-OVA alone or no treatment. 7 days later organs were harvested. For analysis of transcription factors, cells were stimulated with OVA peptide. Protein transport inhibitor cocktail was added during stimulation. Transcription factor staining was analyzed using intracellular staining and flow cytometry.

Figure 8-7: Transcription Factor Profile of Adoptively Transferred Tc17 Cells from Tumor-Bearing Mice (TIL)



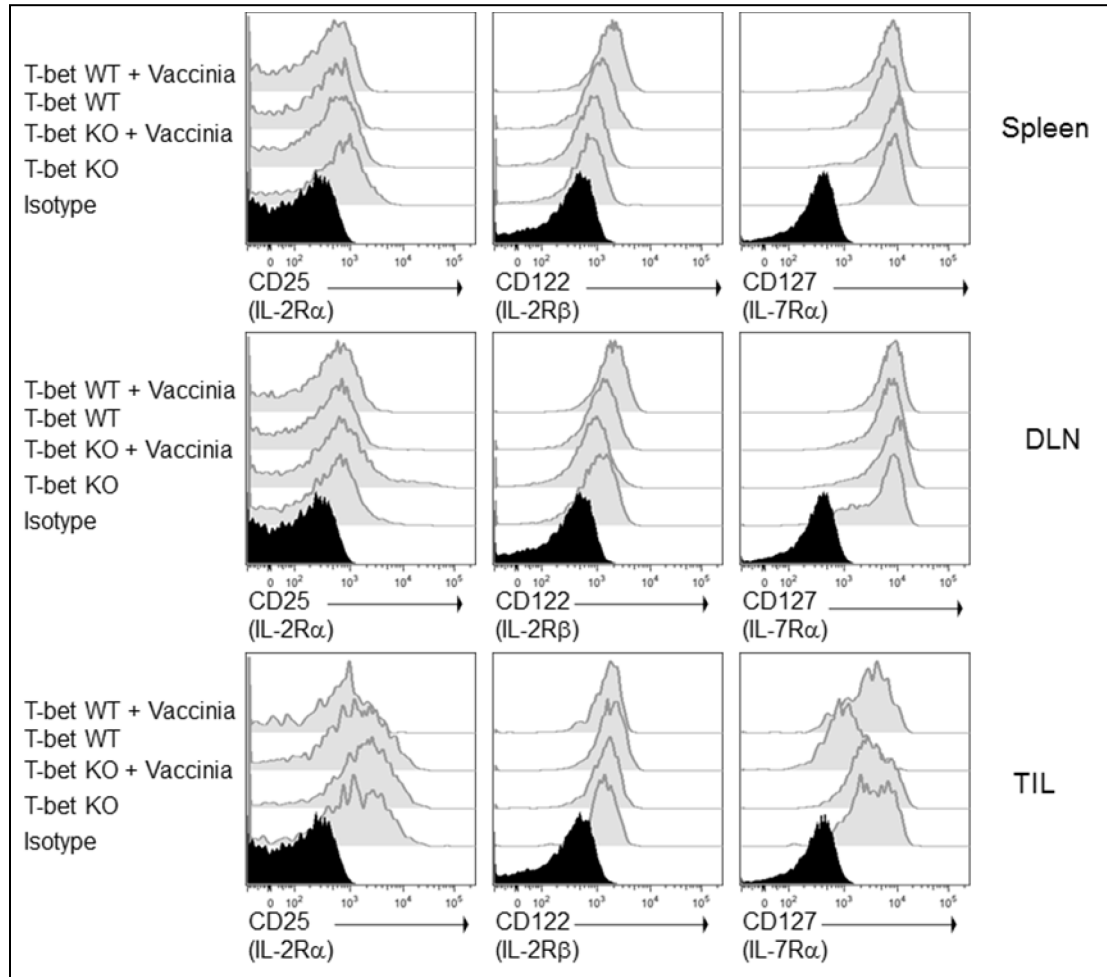
1.6×10^5 B16F10-OVA melanoma cells were injected into CD45.1+ recipients. On day 14, mice with palpable, established tumors received an adoptive transfer of 5×10^6 *in vitro* generated WT or T-bet KO Tc17 cells. This was with or without VV-OVA. Control groups received VV-OVA alone or no treatment. 7 days later organs were harvested. For analysis of transcription factors, cells were stimulated with OVA peptide. Protein transport inhibitor cocktail was added during stimulation. Transcription factor staining was analyzed using intracellular staining and flow cytometry.

Figure 8-8: Transcription Factor Profile of Adoptively Transferred Tc17 Cells from Tumor-Bearing Mice (Spleen)



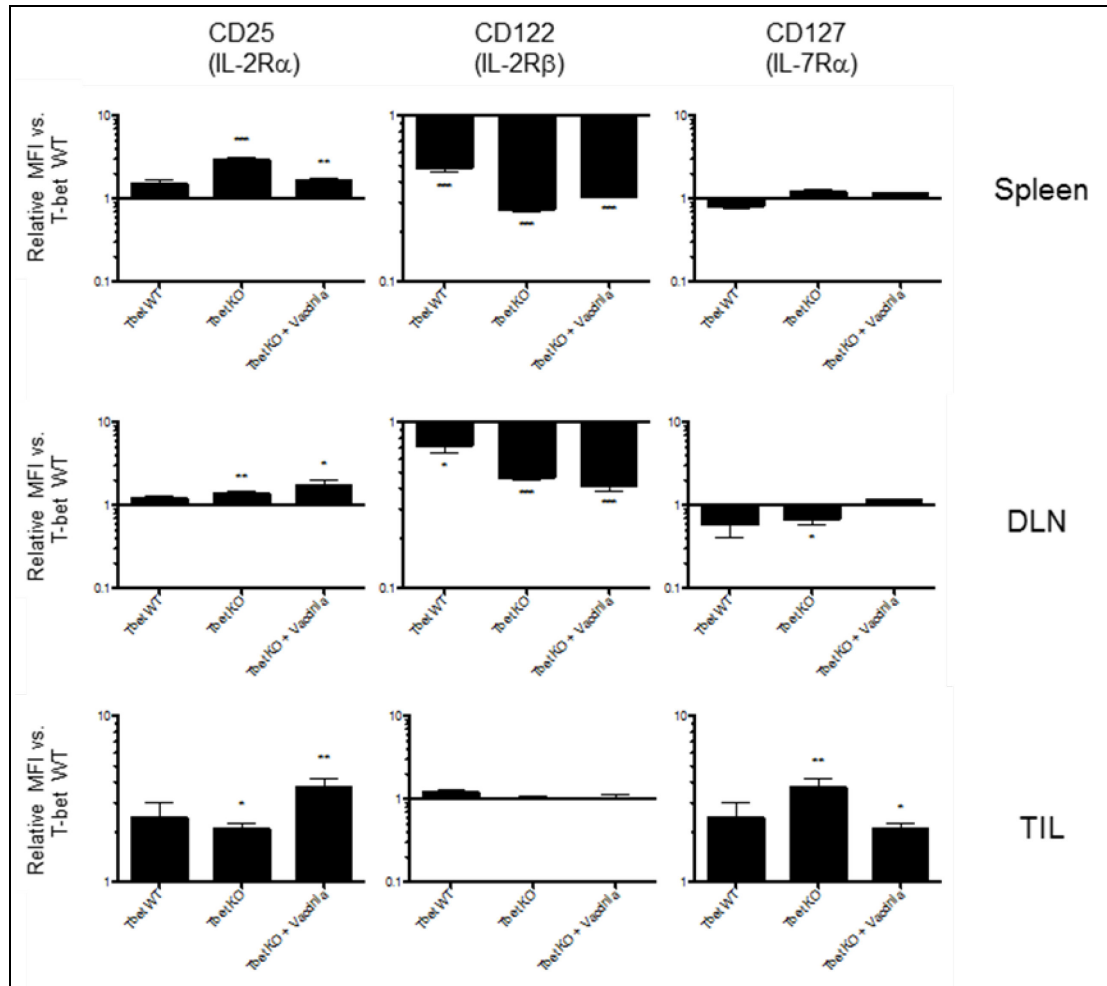
1.6×10^5 B16F10-OVA melanoma cells were injected into CD45.1+ recipients. On day 14, mice with palpable, established tumors received an adoptive transfer of 5×10^6 *in vitro* generated WT or T-bet KO Tc17 cells. This was with or without VV-OVA. Control groups received VV-OVA alone or no treatment. 7 days later organs were harvested. For analysis of transcription factors, cells were stimulated with OVA peptide. Protein transport inhibitor cocktail was added during stimulation. Transcription factor staining was analyzed using intracellular staining and flow cytometry.

Figure 8-9: Receptor Profile of Adoptively Transferred Tc17 Cells from Tumor-Bearing Mice



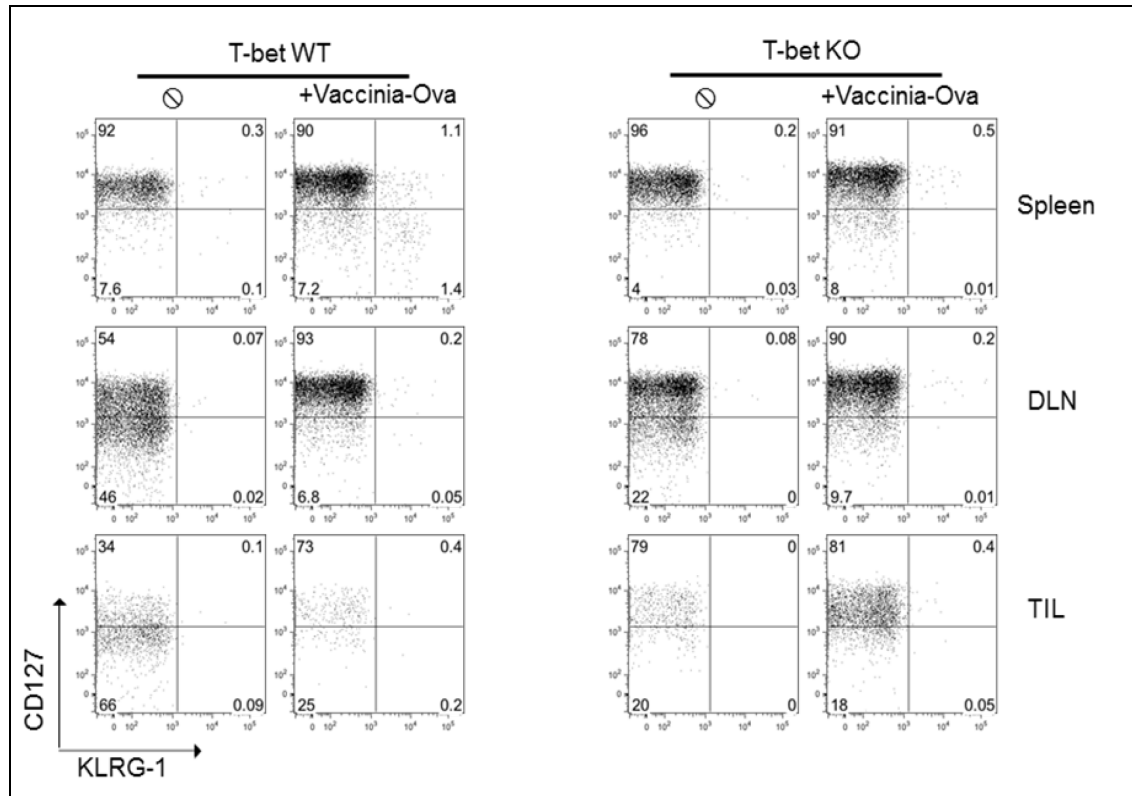
1.6x10⁵ B16F10-OVA melanoma cells were injected into CD45.1+ recipients. On day 14, mice with palpable, established tumors received an adoptive transfer of 5x10⁶ *in vitro* generated WT or T-bet KO Tc17 cells. This was with or without VV-OVA. Control groups received VV-OVA alone or no treatment. 7 days later organs were harvested. Cell surface receptor staining was analyzed using intracellular staining and flow cytometry.

Figure 8-10: Relative Receptor MFIs of Adoptively Transferred Tc17 Cells from Tumor-Bearing Mice



1.6×10^5 B16F10-OVA melanoma cells were injected into CD45.1+ recipients. On day 14, mice with palpable, established tumors received an adoptive transfer of 5×10^6 *in vitro* generated WT or T-bet KO Tc17 cells. This was with or without VV-OVA. Control groups received VV-OVA alone or no treatment. 7 days later organs were harvested. Cell surface receptor staining was analyzed using intracellular staining and flow cytometry. The values represent the means \pm SEM (n=4). *p<0.05, **p<0.01, ***p<0.001

Figure 8-11: Memory Phenotype of Adoptively Transferred Tc17 Cells from Tumor-Bearing Mice



1.6x10⁵ B16F10-OVA melanoma cells were injected into CD45.1+ recipients. On day 14, mice with palpable, established tumors received an adoptive transfer of 5x10⁶ *in vitro* generated WT or T-bet KO Tc17 cells. This was with or without VV-OVA. Control groups received VV-OVA alone or no treatment. 7 days later organs were harvested. Cell surface receptor staining was analyzed using intracellular staining and flow cytometry.

CHAPTER 9

DISCUSSION & CONCLUSIONS

Normally, CD8⁺ T cells are cytotoxic and produce IFN- γ . Under certain conditions CD8⁺ T cells secrete IL-17 and have poor cytotoxicity. Tc17 cells can switch from producing IL-17 to IFN- γ , a termed known as plasticity. IL-12 is a cytokine that affects Tc17 plasticity; VV-OVA and autoimmunity also affect Tc17 plasticity^{33, 38, 64}. Other factors that could affect Tc17 plasticity are not well characterized. In order to characterize potential factors *in vitro*, we utilized a sorting technique to obtain a pure population of Tc17 cells. Similar to published data, we showed sorted Tc17 cells cultured in IL-12 switched from IL-17 to IFN- γ secretion *in vitro*. We also demonstrated IL-2 and IL-7 as factors that affect Tc17 plasticity *in vitro*, which is contrary to previously published data where IL-2 was not sufficient for Tc17 plasticity *in vitro*³⁸. The role of TCR stimulation on Tc17 plasticity *in vitro* is not well characterized. We determined TCR stimulation had a minimal effect on Tc17 plasticity compared to IL-2, IL-7 or IL-12. These data suggest cytokines, but not TCR stimulation, are important factors for Tc17 plasticity *in vitro*.

Tc17 cells express ROR γ T and very low levels of T-bet^{33, 64}. We were interested to know the transcription factor expression of Tc17 cells upon conversion *in vitro*. Tc17 cells cultured in IL-12 expressed T-bet and lost of ROR γ T expression, which is contrary to published literature showing ROR γ T expression did not go down upon conversion³⁸. We also determined the transcription factor expression of Tc17 cells upon conversion *in vivo*. Tc17 cells adoptively transferred into mice infected with VV-OVA up-regulated T-bet and lost ROR γ T expression, which is similar to previously published data⁶⁴.

The requirements for Tc17 plasticity are not well characterized. Based on our *in vitro* data we investigated the role of IL-2 or the IL-12 receptor in Tc17 plasticity. We determined neither IL-2 nor IL-12 receptor signaling to be required for conversion *in vivo*. This suggests that both cytokines are sufficient but not necessary for conversion, and there is a different molecule required for Tc17 plasticity. In the absence of IL-2 or the IL-12 receptor, T-bet was still strongly expressed and ROR γ T expression lost. This suggests T-bet is the master regulator of conversion rather than one single cytokine or signal. We showed in several chapters T-bet was expressed upon conversion both *in vitro* and *in vivo*. We determined T-bet to be the molecule necessary for Tc17 conversion in an inflammatory milieu. We found T-bet KO Tc17 cells did not lose IL-17 secretion in the presence of VV-OVA.

In several studies, Tc17 cells were used as an adoptive immunotherapy^{47, 48}. The requirement for conversion for an anti-tumor response is not well defined. We determined conversion was required for an effective immune response. This suggests in the B16 model, Tc17 that only secrete IL-17 are not sufficient for an anti-tumor response. Conversely, it was Tc17 cells that became IFN- γ secretors which promoted effective anti-tumor responses. In addition, we showed Tc17 cells adoptively transferred without Vaccinia-OVA did not exhibit an effective tumor response when compared to Tc17 cells transferred with Vaccinia-OVA. This is different than what has been published in the literature, where Tc17 cell transfer alone was sufficient for an anti-tumor response⁴⁷.

As mentioned in chapter 1, Tc17 cells are negatively associated with human autoimmune diseases and cancer^{34, 45, 46, 58-62, 85, 86}. These data provide valuable information to define factors that could affect human Tc17 cell conversion. These factors

could lead to treatments that reverse negative outcomes in patients. The use of Tc17 cells in adoptive immunotherapy has tremendous promise. The requirement for conversion for an effective anti-tumor response was not well characterized. We think it is critical to understand this in order to move forward with Tc17 cells as an immunotherapeutic option. Tc17 cells could be detrimental to a patient if they do not convert to IFN- γ secretion, because they could promote greater inflammation and potential tumor progression. These data have important implications for clinical scenarios such as cancer treatment as well as other disease treatments in the future.

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Publications (Peer Reviewed):

1. Jackson CM, **Ceccato CM**, Nirschl CJ, Durham NM, Ruzevick J, Baxi E, Meeker AK, Taube JM, Calabresi PA, Lim M, Drake CG. "CNS tumor mediated immune tolerance is reversible with radiation and immunotherapy". (In preparation)
2. **Ceccato CM**, Nirschl CJ, Durham NM, Jackson CM, Alme A, Drake CG. "Tc17 plasticity is determined by the inflammatory milieu". (In Preparation)
3. Durham NM, Elias J, Jackson CM, Nirschl CJ, **Ceccato CM**, Drake CG. "Lymphocyte Activation Gene 3 (LAG-3) Modulates the Ability of CD4 T-cells to be Suppressed In Vivo". Under review in PLOS One.

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Poster Abstracts:

1. **Ceccato C**, Nirschl C, Durham N, Jackson C, Alme, A, Drake C: "Tc17 Plasticity is Determined by the Inflammatory Milieu". At the 21st Annual International Cancer Immunotherapy Symposium (Dynamics of Host-Tumor Interaction), September 30-October 2, 2013.
2. **Ceccato C**, Nirschl C, Durham N, Alme A, Jackson C, Drake C: "Determining Molecular Mechanisms of Tc17 Plasticity *In Vitro* and *In Vivo*". At Immunology 2013 the 100th Annual American Association of Immunologists Conference, May 3-7, 2013.
3. **Ceccato C**, Jackson C, Nirschl C, Durham N, Alme A, Lim M, Drake C: "Combining Radiotherapy and Immunotherapy in the Treatment of CNS Cancer". At the 20th Annual International Cancer Immunotherapy Symposium (From Milestones to Medicines: Translating Tumor Immunology Research into Immunotherapies), October 1-3, 2012.
4. **Ceccato C**, Durham, N, Nirschl C, Alme A, Pan X, Tam A, Drake C: "Determining Mechanisms that Drive IL-17 Secreting CD8 Cells (Tc17) Toward IFN- γ Production *In Vitro*". At the Keystone Symposium: Th17 Cells in Health and Disease, February 5-10, 2012.
5. **Ceccato C**, Durham, N, Nirschl C, Alme A, Pan X, Tam A, Drake C: "IL-2 and IL-7 Drive IL-17 Secreting CD8 Cells (Tc17) Toward IFN- γ Production *In Vitro*". At the 19th Annual International Cancer Immunotherapy Symposium (Immune Effector Mechanisms in Tumor Immunity), October 2-4, 2011.
6. **Ceccato C**, Durham N, Bruno T, Nirschl C, Tam A, Drake C: "Elucidating the Mechanisms of CD8⁺ T cell Plasticity". At Immunology 2011 the 98th Annual American Association of Immunologists Conference, May 13-17, 2011.

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