CD8 T cell tumor infiltration following Tc1 or Tc2 therapy

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ABSTRACT

Meredith L. Burgents: CD8 T cell tumor infiltration following Tc1 or Tc2 therapy
(Under the direction of Patrick M. Flood)

Type I (Tc1) $CD8^+$ T cells have been shown to be more effective than type II (Tc2) $CD8^+$ T cells for adoptive cell transfer therapy in several tumor models. Migration differences between Tc1 and Tc2 cells were previously proposed to contribute to this difference in therapeutic efficacy. In order to evaluate Tc1 and Tc2 migration *in vivo*, we developed a model using transfected EL-4 thymoma tumor cells expressing the p33 peptide antigen from lymphocytic choriomeningitis virus (p33.EL-4). We used P14 mice, which are transgenic for the T cell receptor specific for p33 peptide that is expressed by these p33.EL-4 tumors. We crossed UBI.GFP mice that ubiquitously express GFP with the P14 mice, in order to generate mice that express both GFP and the p33-specific TCR in the $CD8^+$ T cell population. Splenocytes from these mice were cultured to generate Tc1 and Tc2 cells, which were injected intraveinously into tumor-bearing mice. Donor cells were phenotyped before transfer and on days 3 and 7 after transfer. The $CD8^+$ T cells were examined for GFP and adhesion molecule expression by flow cytometry. We examined gene expression of T-bet and enzymes important for selectin ligand glycosylation in Tc1 and Tc2 cultures, as well as the gene expression of cytokines, chemokines, and chemokine receptors in Tc1 and Tc2 treated mice.

We found significantly more Tc1 than Tc2 cells in TDLNs and tumors on days 3 and 7 after transfer. Both Tc1 and Tc2 donor cells were found in TDLN and tumor sites of p33

positive-positive tumors compared to sites of p33 antigen-negative tumors. More importantly, all $CD8^+$ T cells isolated from these tumors on days 3 and 7 after therapy, regardless of host or donor origin and Tc1 or Tc2 phenotype, expressed high levels of adhesion molecules important for T cell migration. This suggests that antigen does not alter the adhesion molecule expression of tumor infiltrating $CD8^+$ T cells. These cells expressed high levels of CD44, leukocyte function-associated antigen-1 (LFA-1), and P-selectin glycoprotein ligand 1 (PSGL-1, CD162), suggesting a required "tumor infiltrating phenotype". Before transfer and 3 days after transfer, Tc1 cells expressed higher levels of this tumor infiltrating phenotype compared to Tc2 cells. Thus, increased Tc1 cell migration to TDLN and infiltration of tumors may be due to higher expression of a tumor infiltrating phenotype compared to Tc2 cells. We found that T-bet expression is higher in cells from Tc1 vs. Tc2 cultures, which may promote the type I phenotype, including higher adhesion molecule expression. However, we did not find a significant difference between the gene expression of selectin ligand glycosylating enzymes or PSGL-1 gene expression in Tc1 vs. Tc2 cells. In addition, when we examined the gene expression of chemokines in the tumors of Tc1 vs. Tc2 treated mice, we found that type I interferon (IFN)–γ inducible protein (IP)-10 is more highly expressed compared to macrophage derived chemokine (MDC) or macrophage inducing protein (MIP)-1α in either treatment group. Also, donor and host $CD8^+$ cells in Tc1 and Tc2 treated mice express chemokine receptor CXCR3 and cytokine IFN–γ, but no interleukin (IL)-4. This data suggest that in addition to surface expression of adhesion molecules CD44, LFA-1, PSGL-1, expression of type I cytokine IFN-γ and chemokine receptor CXCR3 is also a characteristic of a "tumor infiltrating phentoype."

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ACT	adoptive cell transfer
APC	antigen presenting cell
B4GalT-I	β1, 4-galactosyltransferase-I
C2GlcNAcT-I	β1, 6-glucosaminyltransferase-I
CCR4	C-C chemokine receptor 4
CCR5	C-C chemokine receptor 5
CCR7	C-C chemokine receptor 7
CXCR3	C-X-C chemokine receptor 3
CD62L	L-selectin
DC	dendritic cell
ERM	Ezrin/Radixin/Moesin
FucT-VII	α1, 3-fucosyltransferase-VII
GATA-3	GATA binding transcription factor 3
GFP	green fluorescent protein
HA	hyaluronic acid
ICAM	intercellular adhesion molecule
IFN–γ	interferon-gamma
IL	interleukin
IP-10	IFN–γ inducible protein-10, CXCL10
i.v.	intraveinous
LFA-1	leukocyte function-associated antigen-1, CD11a/CD18
LN	lymph node

MDC	macrophage derived chemokine, CCL22
MHC	major histocompatabilty complex
MIP-1α	macrophage inducing protein –1α, CCL3
NK	natural killer (cell)
PI3K	phosphatidylinositol 3-kinase
PKC	protein kinase-C
PSGL-1	P-selectin glycosylated ligand-1, CD162
RIL	recombinant inerleukin
s.c.	subcutaneous
SMAC	supramolecular activation complex
ST3GalT-IV	sialyl transferase-IV
ST3GalT-VI	sialyltransferase-VI
STAT	signal transactivator of T cells
TAA	tumor associated antigen
T-bet	T-box 21 transcription factor
TCR	T cell receptor
Tc1	type I cytotoxic T cell
Tc2	type II cytotoxic T cell
T_{CM}	central memory T cell
TDLN	tumor draining lymph node
T_{EM}	effector memory T cell
Th1	type I helper T cell
Th2	type II helper T cell

Treg	$CD4^+CD25^+$ regulatory T cell
TSA	tumor specific antigen
UV	ultraviolet light

Chapter I

Introduction

Immune response to tumors

Development of tumors

Tumors develop as a result of uncontrolled growth of cancerous cells (1). Transformation of normal cells into cancerous cells involves several steps collectively termed carcinogenesis (1). Initiation of carcinogenesis occurs as a cell or population of cells undergoes an event leading to DNA damage (1). Internal (intrinsic) or environmental (extrinsic) events such as point mutations or ultraviolet radiation, respectively, can cause damage to DNA (1, 2). Cells have repair mechanisms designed to recognize damage and restore DNA (1). Unfortunately, not all DNA damage is repaired, and if the damage interrupts particular genes, this unrepaired damage leads to uncontrolled growth of the individual cells into an expanded cell population (1). This expansion, or promotion stage, can be a result of mutations in tumor-suppressor or proto-oncogenes, which regulate cell cycle and proliferation (1). The final stage of carcinogenesis, progression, is where these expanded cell populations undergo transformation into malignant cells, forming tumors (1).

Importantly, the immune system is able to respond to tumors and participate in the regulation of tumor growth (1-5). It is believed that mechanisms in the immune system are capable of surveying the body for the generation of pre-cancerous cells and mediating the removal of these cells from tissues (1-5). This process, known as immunosurveillance, has been off-set by the more recent understanding of the process called immunosubversion (1-5). Taken together, these models offer a complex dichotomy for not only immune system contributions to tumor cell rejection but also progression (1-5). Furthermore, it is currently thought that tumors progress uncontrollably without intervention when the balance is shifted away from immunosurveillance towards immunosubversion (1-5).

Immunosurveillance

Our understanding of the immune system and its response to tumors has come a long way since the original proposal by Ehrlich in 1909 that one role of the immune system is to reject an "overwhelming frequency" of carcinomas (3, 4). Later, in the next half-century Burnet would propose the term "immunosurveillance" for the immune system's role in recognizing tumor cells and mounting a response against tumor antigens capable of clearing the tumor cells prior to the presentation of clinical symptoms (6, 7). Thomas also agreed with this theory, suggesting that the body must have mechanism(s) for managing the inevitable development of mutated cells and that immune mechanisms are a plausible candidate (8). Due to the vast knowledge concerning tumor cell recognition and clearance by immune mechanisms, the current model of immunosurveillance is rather complicated compared to these earlier versions, which were quite simplistic but fitting with knowledge in the field at that time (1-8).

The theory of immunosurveillance has two major components: 1) Tumor cells are recognized by immune cells; and 2) Immune responses can eliminate tumor cells (1-4). Natural killer (NK) cells and cytotoxic $CD8^+$ T lymphocytes are key components of anti-tumor responses because they effect cell-mediated killing of targeted tumor cells (1-4). NK cells recognize and kill target cells based on their expression of ubiquitous proteins, such as laminin, coupled with the loss of major histocompatabilty complex (MHC) class-I molecule expression on the surface of target cells, including most cancer cells (1-4). MHC class- I signals through the killing inhibitory receptor on NK cells to prevent target cell killing, so the loss of MHC class-I is detected by the lack of this inhibitory signal (1-4). NK cells can also

be activated by detecting MHC-I-like molecules upregulated during stress responses (3). Indeed, the concept of immune recognition and response to stress also applies to T cell responses primed through contact with antigen presenting cells (APCs) (3). Dendritic cells (DCs) are "professional" APCs thought to play an important role in tumor cell recognition and tumor antigen presentation to cells of the adaptive immune system (1-5, 9-11). The "danger model" of tumor cell recognition proposes that expression of danger signals such as heat shock proteins by cells with damaged DNA may activate DCs, which then present tumor antigens to B and T lymphocytes of the adaptive immune response (3, 11). B cells secrete large amounts of immunoglobulin that can bind epitopes on tumor cells promoting their rejection (1-4, 11). T cell mediated tumor clearance is detailed below.

$CD8^+$ T cells responses to tumors

T cells of the adaptive immune response recognize peptides presented in the context of MHC complexes on the cell surface, and recognition of target cell MHC:peptide complexes by the T cell receptor (TCR) can result in direct killing of antigen expressing cancer cells (1-4, 8-14). Sequences encoding regions of TCRs recognizing the MHC are conserved, while sequences encoding regions that recognize the peptide within the MHC are highly variable and undergo recombination to generate a vast repertoire of TCR specificities (12). Due to allelic exclusion, only one recombined TCR sequence is expressed per T cell (12). Because of the unique specificity of an individual T cell's expression of the TCR and the large number of T cells, the repertoire of peptide antigens that T cells can recognize is estimated to be $1x10^8 – 1x10^{11}$ (12). This vast range of antigen recognition is important for T

cell responses against tumors, because tumor cells can express a wide variety of antigens (1-5, 9-12).

Tumor antigens are presented by APCs in tumor draining lymph nodes (TDLNs), where a single T cell can expand into a clonal population of epitope-specific T cells (1-5, 9-15). This population includes effector cells as well as cells that will continue to develop into central or effector memory cells (10, 15). T cells express accessory molecules with the TCR called CD4 or CD8 (12). Each $CD4^+$ T cell recognizes a specific peptide in the context of a specific MHC class-II complex, and $CD4^+$ T cells include both helper (Th) and regulatory T cells (Tregs) that respond by secreting large amounts of cytokines (15-18). Th cells can be further divided into type I (Th1) and type II (Th2), based on their secretion of type I and type II cytokines, respectively (17). Effector $CD8^+$ T cells also respond by secreting cytokines, but unlike $CD4^+$ T cells, activated $CD8^+$ T cells upregulate molecules related to cytolytic activity (17-19). $CD8^+$ T cells can actually kill tumor cells via cell to cell contact through engaging ligands with death domain/receptor motifs or by releasing perforin followed by granzyme B (1, 2, 5, 9-11, 18, 19). Targeted tumor cells undergo apoptosis, which further promotes the immune response and can ultimately lead to further presentation of tumor antigens and tumor cell rejection (20, 21).

Tumor antigens and antigen-loss variants

Antigens expressed by cancer cells within tumors are generally classified as either tumor-associated antigens (TAAs) or tumor specific antigens (TSAs) (3, 12, 22). While TAAs are normally expressed by some tissues but induced inappropriately in cancer cells, TSAs are only expressed on cancer cells (12, 22). Dominant antigenic epitopes may be those

epitopes that are expressed more abundantly by cancer cells and are highly immunogenic/antigenic, while minor antigenic epitopes may be those that are not expressed as abundantly and/or are minimally immunogenic (11, 12, 22). Within the tumor mass there are a variety of cell types that express tumor antigenic epitopes, but generally they are either cancer cells or stromal cells (11, 12, 21-24). Cancer cells are the cells derived from the original malignantly transformed cell, while stromal cells are cells supporting the growth of the cancerous cells (11, 21, 23, 24). Stromal cells include bone marrow-derived immune cells and non-bone marrow-derived fibroblasts and endothelial cells (11, 21, 23, 24). Tumor antigens can be shed from the cancer cells and their antigenic epitopes are then presented by MHC molecules on APCs in the TDLNs or on the nearby stromal cells (11, 20-24). Killing of stromal cells has been shown to be an important and effective therapy for mediating tumor rejection (21, 23, 24).

Although T cells clearly contribute to the rejection of tumors by directly and indirectly mediating cancer cell killing, T cells can also promote immunoselection (1-5, 9-11, 25). The following description is an overview of how $CD8^+$ T cell responses against TAA or TSA promote the immunoselection process, where immune responses promote survival of cancer cell populations instead of rejecting them (1-5, 9-11, 25). Animal models have shown that tumors can express more than one antigenic epitope recognizable by the T cell repertoire, which can therefore serve as T cell antigens (22). Due to the unique specificity of T cells to respond to only one epitope antigen on one type of class I molecule, the response of T cells is restricted to a small number of dominant epitopes (11, 24, 25). As the T cell response targets these specific dominant epitopes, cancer cells expressing sufficient levels of these epitopes may be killed by the T cells (11, 24, 25) However, those cancer cells not

expressing these epitopes or those cells which did express the epitopes but have down-regulated the expression of these epitopes may escape T cell killing (1, 11, 24, 25). These antigen loss variant cells which lost epitope expression continue to persist and the tumor continues to grow, even though these cells may express other T cell antigenic epitopes, the so-called minor antigens (1, 11, 24, 25). Following the loss of dominant antigenic epitopes, minor antigen-specific T cells may now be activated; leading to another round of antigen-specific responses and cancer cell killing (1, 11, 24, 25). As the cycle of antigen recognition and killing of cancer cells bearing these antigenic epitopes continues, eventually the T cell repertoire is exhausted or suppressed, unable to effectively respond to antigens that may be expressed on these cancer cells (1, 11, 24, 25). The remaining cancer cells no longer express antigenic epitopes that are either recognized by the T cells or which can effectively stimulate a tumor-protective response, and the tumor burden increases (1, 11, 24, 25). Even as new T cells are elicited from the thymus, they cannot compete with the increased tumor burden and this immunoselection favors the survival of cancer cells not expressing T cell antigenic epitopes capable of leading to effective tumor surveillance (1, 11, 24, 25). Again, these cancer cells without T cell-stimulating antigenic epitopes are called antigen-loss variants (1, 11, 24, 25). Selection for these antigen loss variants *in vivo* has been demonstrated, and we are realizing that immunoselection may also be a product of immunosubversion, described below (1, 2, 9, 11, 23, 25).

Immunosubversion

Even though immune responses can clearly promote tumor rejection, immune responses can also contribute to tumor growth (1-5, 9-11, 25). Immunoselection is one

aspect of the negative effects immune responses can have on tumor clearance (1-5, 9-11, 25). The cycle of immunoselection may be perpetuated by suppressive immune responses (1, 2, 4, 9-11, 25). Despite the positive effects of T cell responses towards promoting tumor rejection, some T cells can promote tumor growth by suppressing immune responses against tumors (1, 2, 5, 9-11, 25). For example, Tregs suppress immune responses through the production of interleukin (IL)-10 and transforming growth factor-β (TGF-β) (11, 15, 16, 25). Additionally, other suppressive cells in the tumor microenvironment also promote suppression of effective anti-tumor responses (1, 2, 5, 9-11). These cells include myeloid suppressor cells and even $CD8^+$ T suppressor cells (1, 2, 5, 9-11). IL-10 is considered to be an important immunosuppressive cytokine produced by these cells and has been shown to promote the priming of type II responses by APCs (11). Several studies have shown that therapies to reduce suppressive cell populations responding to tumors will result in increased tumor cell killing (26-29). Therefore, part of the current paradigm of immunotherapies incorporates inhibiting suppressive activity and increasing $CD8^+$ T cell activity (26-29).

Adoptive cell transfer therapy

"Active" and "Passive" immunotherapy

Immunotherapy approaches to treat cancer may be classified as "active" or "passive" therapies (11). Active therapies are those which promote the host immune response against tumor antigens, while passive therapies are those which transfer components of established immune responses to promote tumor cell clearance (11, 27). Vaccines with peptide-pulsed APCs or genetically engineered tumor cells are examples of active therapy (11, 27). These therapies are designed to facilitate priming of host anti-tumor responses, such as T and B lymphocytes (11, 27). Instead, T cell or antibody therapies are passive therapies, where the priming of the T and B lymphocytes occurs under controlled conditions *in vitro* (10, 11, 26, 29). Therefore, active therapies promote a broader spectrum of tumor rejection, while passive therapies offer a specific mechanism of anti-tumor activity. Passive T cell therapies are designed to target tumor cells and aid host responses, resulting in the complete rejection of tumor cells, the prevention of antigen loss variants or metastasis, and the establishment of immunologic memory to respond to any recurrent developments of cancer cells (10, 11, 26, 29).

T cell therapy

Adoptive cell transfer (ACT) therapy with $CD4^+$ or $CD8^+$ T cells has been proven to be successful in promoting rejection of tumors in a number of animal tumor models, as well as in clinical trials (9-11, 26, 29). Donor T cells are harvested from naïve animals or tumor-bearing animals (or patients) and are then cultured under controlled conditions *in vitro* to prime and/or expand antigen-specific T cells (9-11, 26, 29). If possible, T cells specific for

TSA and not TAA are generated in order to reduce any cross-reactivity with non-tumor cells that would result in autoimmune responses deleterious to the patient (9-11, 22, 26, 29). In addition, the donor cells must be stimulated to obtain optimal effector function and migration properties (11, 26, 29, 30). This is critical for the therapy to be effective, because not all T cells have the same ability to target tumor cells, migrate to tumor draining lymph nodes, or infiltrate into the tumors (11, 26, 29, 30). It is important to note that, if possible, monoclonal T cells against TSA can be generated, which limits the generation of contaminating suppressive T cells, including Treg cells, which must be removed from the donor cell population before transfer in order for the therapy to ultimately be effective (11, 26, 29, 31). Therefore, lymphodepletion is sometimes used to deplete the host lymphocytes, including activated suppressive cell populations, but this does not necessarily eliminate the development of effector cells subsequent to depletion (11, 26, 29). These therapies also require that these epitope-specific effector T cells used in adoptive therapy exhibit the ideal phenotype, stage of differentiation, and migration properties to effectively seek out and destroy the tumor at the site of growth (11, 26, 29, 31-35). Given the diversity of cancer cell origins and tumor locations coupled with the complex nature primary and metastatic tumor growth, studies discerning the critical properties needed by adoptively transferred cells to be most effective in therapy are vital to the ultimate success of ACT therapy.

Phenotype of T cells for therapy

One crucial factor which ultimately determines the effectiveness of ACT is the ability of adoptively transferred epitope-specific T cells to home to the TDLN as well as the site of the tumor, and to effectively infiltrate the tumor mass (10, 11, 26, 31). While it was

originally believed that T cells with the highest cytolytic activity *in vitro* would be the most effective population for ACT therapy, it is now known that these cells actually confer limited tumor rejection activity *in vivo* (26, 32-35). This is because while these cells were most effective at killing the tumor cells *in vitro*, they were significantly less effective in migrating to the site of the tumor and infiltrating the tumor mass (33-35). This is due to the fact that activated T cells progress through various stages of activation before becoming memory cells (given a linear development of memory cells), and transition into these different activation stages are accompanied by changes in proliferation capacity, cytokine expression, and in the cell surface expression of molecules needed for optimal migration *in vivo* (26, 33-35). Immediately after stimulation, the T cells have high proliferative capacity, produce large amounts of IL-2, but low amounts of IFN-γ, and how low cytolytic activity *in vitro* (33-35). These early effector cells begin to down-regulate CD62L and up-regulate CD44 (33, 35). After subsequent stimulations, their proliferative capacity and IL-2 production decreases, while IFN-γ and cytotlytic activity increases (33, 35). These intermediate and late effector stage T cells progressively down-regulate CD62L and up-regulate CD44 (33, 35). Interestingly, it is the early effector cells which have the highest anti-tumor activity after transfer *in vivo* (26, 33, 35). It is believed that the expression of CD62L is important for the donor cells to home to the TDLNs for restimulation and subsequent tumor infiltration (11, 26, 31, 33-35).

Type I (Tc1) and type II (Tc2) $CD8^+$ T cell therapy

Tc1 and Tc2 cells

$CD8^+$ T cell therapy has been applied to treatment of many tumors including melanoma (10, 11, 26, 27, 29, 31). Numerous animal studies have investigated the use of type I Tc1 vs. type II Tc2 $CD8^+$ T cells for therapy (36-48). Tc1 and Tc2 cells, like Th1 and Th2 cells, are generated in different cytokine conditions and/or by conditions or stimuli which generate different signaling patterns through the TCR (17, 49-52). Th1 and Tc1 cells are generated by inducing a stronger calcium flux signal, while Th2 and Tc2 cells are generated by activating a stronger protein kinase C (PKC) signal (50). In addition, like Th1 vs. Th2 polarization, we know that altered peptide ligands can generate Tc1 vs. Tc2 cells (50). *In vitro* the Tc1 cells are normally generated by adding IL-12 /IFN–γ and anti-IL-4 antibody to cultures during antigen-specific activation, while Tc2 cells are generated by adding IL-4 and anti-IFN–γ and anti-IL-12 antibodies, to stimulating culture conditions (49, 52). T cell cultures can be stimulated *in vitro* by adding antigenic peptides to whole splenocytes cultures, adding peptide-pulsed APCs to purified T cells, or by simply adding anti-CD3 and anti-CD28 antibodies to T cells (52). It has also been reported that *in vivo* generation of Tc1 vs. Tc2 cells can occur depending on the T cell priming environment (51, 53). In two recent examples, exposure of animals to ultraviolet radiation (UV) prior to T cell priming through the exposed skin promotes the development of Tc2 cells, while animals vaccinated with 48 hour peptide-pulsed DCs, instead of 8 hour pulsed DCs, generated more Tc2 than Tc1 cells against the peptide antigen (51,53). Therefore, it appears that the generation of polarized Tc1 and Tc2 cells can occur under natural conditions *in vivo*, and

therefore understanding the differential effectiveness of these cells in anti-cancer responses represents an important step toward developing effective anti-cancer therapies.

Although there are numerous methods for generating Tc1 and Tc2 cells, the function of these cell populations is consistent regardless of the methods used for generating these cells (52). Tc1 cells secrete large amounts of IFN–γ, but Tc2 cells secrete limited amounts of IFN–γ and significant amounts of IL-4, IL-5, and IL-10 (17, 49-52). Like Th1 and Th2 cells, Tc1 and Tc2 cells express type I and type II chemokine receptors (17, 54). Tc1 cells express chemokine receptors CCR5 and CXCR3, but Tc2 cells express chemokine receptors CCR4 and CXCR3 (17, 54). In addition, these two polarized $CD8^+$ T cell populations reportedly have similar proliferation and cytolytic responses *in vitro* and *in vivo* (49, 52). This similar proliferative capacity and cytolytic activity by Tc1 and Tc2 cells *in vitro* suggested that antigen-specific Tc1 and Tc2 cells would be equally responsive *in vivo* and led to numerous investigations of Tc1 and Tc2 ACT tumor therapies.

Efficacy of Tc1 and Tc2 therapy

Over the past 15 years, a number of investigations have evaluated Tc1 and Tc2 therapies, in both the B16 metastatic and EL-4 non-metastatic tumor models (36-48). In contrast to the results expected from *in vitro* data (described above), several studies have shown evidence that cultured antigen-specific donor Tc1 cells are more effective than Tc2 donor cells when transferred into tumor-bearing host animals (37, 39-41, 45, 46, 48). For all of these studies, antigen-specific T cells were generated *in vitro* and then transferred i.v. into recipient mice either on the same day as tumor injections or on empirically-determined days following tumor injections (36-48). In some cases both Tc1 and Tc2 therapies were effective

at reducing tumor growth or promoting tumor rejection, but in other cases Tc2 treated animals showed no improvement compared to controls (36, 38, 42-44, 47). Studies that did report effective therapy with Tc2 cells demonstrated that Tc2 therapy, while effective, does require significantly more donor cells than Tc1 therapy (37, 39). As a result of these findings, the properties of Tc1 and Tc2 cell function during tumor therapy were investigated.

Functions of $CD8^+$ T cells mentioned above include proliferation, cytolytic killing, and cytokine production (17, 18). Proliferation and cytolytic killing efficiency appears to be the same for Tc1 and Tc2 cells both *in vitro* and *in vivo* (17, 37-40, 45, 49, 52). The only clear difference between these Tc1 and Tc2 cells to date is their cytokine profile listed above: Tc1 cells secrete large amounts of IFN–γ, and Tc2 cells secrete little IFN–γ but large amount of type II cytokines such as IL-4, IL-5, and IL-10 (17, 49-52). Whether type–specific cytokine production by donor cells is required for effective Tc1 or Tc2 therapy is not clear, because studies evaluating the importance of effector-cell cytokines have shown conflicting results (37-40, 43, 44, 47, 48). However, donor or host derived IFN–γ appears to be important for therapy (37-41, 43, 44, 46-48, 55, 56). This is supported by evidence that IFN–γ promotes upregulation of chemokines shown to increase the recruitment of tumor infiltrating T cells (11, 47, 48, 57-59). This suggests that therapy with Tc1 cells, which produce more IFN–γ compared to Tc2 cells, would result in increased infiltration of tumors by T cells. Indeed, one study found higher numbers of host T cells in TDLNs of Tc1 vs. Tc2 treated animals, and another study concluded that IFN–γ signaling to host cells was important for increased host tumor infiltration during Tc1 therapy (Tc2 therapy was not examined) (39, 47, 48). What was still unclear was whether donor Tc1 and Tc2 cells infiltrate tumors with equal efficiency. It is important to determine whether Tc1 or Tc2

therapy promotes not only donor, but also host cell infiltration into the tumors and migration to TDLNs, because trafficking of T cells to TDLNs and tumors has been shown to indicate a positive prognosis in patients who have been given immunotherapy (9, 11). Thus, we evaluated donor and host cell migration and tumor infiltration in Tc1 and Tc2 treated animals in our study.

Tc1 vs. Tc2 trafficking

Although it was not yet understood or clearly demonstrated, a few observations from two key studies suggested that Tc2 cells may have different migration properties than Tc1 cells: 1) Tc1 cells were found in higher number in TDLNs than Tc2 cells, 2) Tc2 cells arrived later in the TDLNs than Tc1 cells, and 3) Tc2 cells appeared to preferentially migrate to the spleen (39, 40). One of these two studies has evidence to suggest that Tc1 cells also infiltrate tumors in higher numbers than Tc2 cells, however, due to limitations of the study, statistical significance could not be evaluated (40). Importantly, both studies which support migration differences between Tc1 and Tc2 cells were done in s.c. tumor models (39, 40). Other studies have suggested that Tc1 and Tc2 cell tumor infiltration is similar in a pulmonary metastasis tumor model (37, 43).

The phenotype of Tc1 and Tc2 cells has been evaluated to a limited extent, particularly in regard to migration properties (52). As stated earlier, it has already been shown that expression of adhesion molecules CD44 and CD62L are important for effective tumor therapy (26, 32-35). One study examined the expression of activation-associated surface markers and function of Tc1 and Tc2 cells derived under various culturing methods (whole splenocyte cultures with peptide, T cell cultures with generated APCs and peptide,

and T cells with anti-CD3 with anti-CD28 antibodies) (52). This study found different levels of CD44 and CD62L expression, based on culture methods (52). In general, CD44 was slightly higher and CD62L was higher overall on cells in the Tc1 culture compared to cells in the Tc2 culture (52). Since CD62L is important for homing to LNs, the higher expression of this molecule by Tc1 cells *in vitro* could lead to higher migration to TDLNs by Tc1 vs. Tc2 cells (60). If Tc1 cells do actually migrate and infiltrate tumors more effectively than Tc2 cells, it is reasonable to expect a higher number of Tc2 cells would be required for therapy, and it appears that this is the case (37, 39). Tc2 therapy has been shown to require up to 100-fold more cells than Tc1 therapy to demonstrate equivalent efficacy (39). (Also, because Tc1 and Tc2 cells express different levels of chemokine receptors, it is presumed that these cells would respond with different efficiencies to various chemokines (17).) Taken together, these data suggest that Tc1 cells migrate more effectively than Tc2 cells to TDLN, thus requiring a smaller population of donor cells for effective therapy. Because T cell migration and tumor infiltration are important for effective tumor clearance and tumor therapy, if Tc1 and Tc2 cells have different migration and tumor infiltration properties *in vivo*, these differences could contribute to the differnences in therapeutic efficacy for Tc1 vs. Tc2 therapy.

T cell trafficking and adhesion molecules

Migration

Although little is known about Tc1 vs. Tc2 cell trafficking specifically, we do know that T cell trafficking is regulated by a variety of molecules, including chemokines, which bind to chemokine receptors on T cells, and numerous molecules expressed by the T cells (ligands and receptors), which are important for T cell adhesion (60). (These molecules are collectively referred to as adhesion molecules in this document as an abbreviated form of a list of molecules.) Chemokines play an important role in attracting T cells to specific sites of inflammation or to specific tissues (60). Adhesion molecules control T cell retention at certain sites as well as migration across endothelium (60-64). Different chemokines and adhesion molecules are important for naïve, effector, and memory T cell migration (60, 61). Naïve T cells circulate through the blood, lymphatics, and secondary lymphoid organs including lymph nodes (LNs) (60, 61). Naïve T cells express both CD62L and chemokine receptor CCR7 that binds CCL19 and CCL21, which are critical for T cell homing to LNs (60, 61, 65). T cells enter into LNs from the blood by crossing high endothelial venules (61, 63, 65). After activation in the LNs following contact with APCs, effector T cells down-regulate CCR7 and CD62L but up-regulate other adhesion molecules and chemokine receptors for migration to the site of inflammation (61, 64, 65). Memory cells are subdivided into two distinct subsets- central and effector memory cells (61, 64). Central memory T cells circulate similarly to naïve T cells and express molecules important for LN homing (60, 61, 64). Effector memory T cells do not circulate through LNs, but instead migrate primarily through non-lymphoid tissues (60, 61, 64). As mentioned briefly above, the T cells used in

ACT therapy must have an appropriate phenotype for migrating to TDLNs and tumors (26, 33-35).

In addition to the expression of the appropriate chemokine receptors and homing molecules such as CCR7 and CD62L, the expression of cell surface receptors that facilitate loose or tight adhesion and transendothelial migration is an important component needed for appropriate T cell trafficking (60-64). These adhesion and migration processes are needed for T cells to migrate from one site to another when crossing vasculature (60-64). As stated above, T cells cross high endothelial venules to enter into LNs (including TDLNs), and in order for T cells to infiltrate inflamed tissues or tumors they must cross the activated endothelium at the site of inflammation (60-64). Specific chemokines recruit activated T cells to the inflammation site, such as macrophage inducing protein (MIP)-1α, macrophage derived chemokine (MDC), or IFN-inducible protein (IP)-10, which bind chemokine receptors CCR5, CCR4, and CXCR3, respectively (9, 11, 17, 30, 46, 54, 57-61). The binding of these or other chemokines expressed by activated endothelium signals through G-coupled protein chemokine receptors to promote changes in adhesion molecules on T cells (60, 66-70). These changes include switching from inactive to active conformation for some adhesion molecules (66-70). The T cells then undergo loose and tight adhesion to the endothelium, followed by transendothelial migration through the endothelium (62, 63). Below is a brief review of several of these adhesion molecules important in $CD8^+$ T cell adhesion, diapedesis, or infiltration, and what is known about their differential expression on Tc1 vs. Tc2 cells.

L- selectin (CD62L)

Adhesion molecules regulate each step of this migration into an inflamed site (60-70). These adhesion molecules include integrins, selectins, and their ligands (60-70). Selectin CD62L binding is critical for effective T cell homing to LNs (65). CD62L has been reported to be expressed higher on Tc1 cultures compared to Tc2 cultures (52). There are two other known selectins, E- and P- selectin (65). Selectins are expressed on bone-marrow derived cells (including T cells) as well as endothelial cells (65). Members of the selectin family have similar structure: C-type lectin, with N-terminal lectin domain, an epidermal growth factor-like domain, repeat sequences, followed by C-terminal trans-membrane and cytoplasmic domains (65). While L-selectin is important for homing to the LNs, it can also play a role in adhesion to peripheral node addressin on inflamed endothelium (65). Expression of CD62L is regulated in part by proteolytic cleavage of surface bound CD62L, which in turn has been shown to upregulate CD62L gene expression (71). In addition, the soluble CD62L generated by cleavage of the surface bound CD62L can inhibit T cell migration *in vivo*, particularly the homing to LNs (72). Therefore, we examined the expression of CD62L by donor cells. Examining CD62L expression by donor cells not only aids in determining the activation/memory phenotype of the cells but also may suggest whether CD62L expression is important for regulating donor cell migration into TDLNs, where the donor cells encounter APCs presenting tumor antigens.

P- selectin glycosylated ligand-1 (CD162, PSGL-1)

P-selectin glycoprotein ligand-1 (CD162, PSGL-1) is the primary T cell ligand for P-selectin on activated endothelium (63, 65). PSGL-1 functions as a homodimer, which

requires glycosylation of the sialic acid backbone structure for optimal binding to selectins (65). It is expressed on all T cells, although not necessarily in the glycosylated form expressed on activated T cells (65). PSGL-1 mediates loose adhesion or rolling of T cells along activated endothelium (63, 65). There is evidence that PSGL-1 has other functions, as well (73-75). Cross-linking of PSGL-1 can induce apoptosis through the mitochondrial pathway, Syk signaling through ezrin/radixin/moesin (ERM) proteins, as well as upregulation of colony stimulating factor-1 transcription through Syk (73-75). PSGL-1 has also been implicated in chemotactic T cell responses not dependent on the adhesion function of PSGL-1 (76). PSGL-1 expression and function may be different between type I and type II T cells (65, 77). In Th1 cells, it has been shown that cross-linking of PSGL-1 promotes the binding of integrin leukocyte function-associated antigen-1 (CD11a/CD18, LFA-1) to its ligand, intercellular adhesion molecule (ICAM-1), but the expression of optimally glycosylated PSGL-1 in Th2 cells is significantly impaired compared to Th1 cells (77, 78). Differences in the glycosylation of PSGL-1 in Th1 and Th2 cells has been linked to differences in the expression of T-box 21 transcription factor (T-bet) and its regulation of glycosylating enzymes (77-78). PSGL-1 expression and role for migration of Tc1 vs. Tc2 cells is still unknown and differences in PSGL-1 exprsesion may contribute to the observed differences in Tc1 vs Tc2 migration *in vivo*. Differences in the level and regulation of PSGL-1 expression by Tc1 and Tc2 cells may affect their transendothelial migration in a manner similar to that observed for Th1 and Th2 cells. Therefore, we examined the expression of PSGL-1 on Tc1 and Tc2 donor cells *in vitro* and *in vivo*.

Leukocyte function-associated antigen-1 (CD11a/CD18, LFA-1)

Before-mentioned LFA-1 is an integrin adhesion molecule (66-70). Integrins are heterodimers of alpha and beta subunits and have inactive and active conformations (62). LFA-1 consists of subunits αL and β2, both containing several domains including cytoplasmic domains for signaling (69, 70). Chemokine signaling through small GTPases promotes the change in LFA-1 conformation from inactive to active, changing from low affinity to the highest affinity (69, 70). LFA-1 is then able to readily bind ICAM-1 (as well as ICAM-2 or ICAM-3) mediating tight adhesion of T cells to the activated endothelium (66-70). LFA-1 is also able to bind molecules at the tight junctions between endothelial cells, such as junctional adhesion molecules, and mediate migration across the endothelium (67, 68). Like PSGL-1, LFA-1 can also mediate signaling (69, 70). Cross-linking of LFA-1 leads to signaling through cytohesin-1 to MAP kinases (69). Due to the adhesion and signaling functions of LFA-1, it is interesting that LFA-1 localizes to lipid rafts and redistributes on the cell surface (69, 70, 79-81). LFA-1 localizes initially to the middle of SMACs and then moves to the peripheral SMAC after TCR signaling, as the TCRs centralize (70). In activated T cells, the TCRs and LFA-1 localize similarly, forming the immunological synapse, a ring of molecules at the junction between T cells and target cells (70). Thus, conformation, signaling, and distribution of LFA-1 are important for T cell functions (66-70, 79-81ref). LFA-1 has been shown *in vitro* to promote Th1 cell migration in conjunction with CD44, another adhesion molecule that localizes within the SMAC and immunological synapses (82). Thus, LFA-1 is an important molecule in mediating T cell migration and signaling functions. Whether Tc1 and Tc2 cells express LFA-1 similarly and

whether it functions efficiently in both cell populations is unknown. We examined the expression of LFA-1 by Tc1 and Tc2 donor cells *in vitro* and *in vivo*.

CD44

CD44 molecules are class I transmembrane glycoproteins encoded by one gene (83-85). At least 10 isoforms are generated through alternative splicing and post-translational modifications (83-85). CD44 is upregulated after T cell activation and is also expressed on memory cells, as noted previously (61, 64). As could be expected due to the variety of CD44 isoforms, CD44 has a variety of functions (83, 84f). CD44 can function as a co-receptor to mediate signaling, although whether CD44 can directly mediate signal transduction has not yet been determined (83, 84). A few of the signaling proteins that associate with the cytoplasmic tail of CD44 are Rho kinase, PKC, and the TCR signaling molecules LCK and FYN (83, 84). We are only beginning to understand the complex nature of CD44 signaling. Importantly, CD44 interacts with the extracellular matrix and binds hyaluronic acid (HA) (83, 84). As stated above, the adhesion function of CD44 is important for Th1 cell transendothelial migration, but not for Th2 transendothelial migration (82). CD44 expression and function are not known for Tc1 and Tc2 cells. Again, because Th1 and Th2 cells show a different dependence on CD44 for transendothelial migration *in vitro* and CD44 is involved in T cell signaling, we investigated the expression of CD44 by Tc1 and Tc2 cells.

Tumor infiltration by T cells

Adhesion molecules

All of the above adhesion molecules (CD62L CD44, LFA-1, and PSGL-1) have been implicated in T cell tumor infiltration (26, 33-35, 85-87). The ability of T cells to infiltrate tumors is important for tumor clearance, especially for T cells used in tumor therapy (9, 11, 26, 31). Host responses to tumors requires CD62L and ICAM-1 (LFA-1 ligand) expression by T cells or other cells, as shown by knockout models (88). It is proposed that this is due to the need for T cell activation in the TDLNs and for effective transendothelial migration, where CD62L and ICAM-1 play a role (88). As shown in previous ACT therapy studies, CD62L and CD44 expression by donor cells is important for effective tumor therapy (26, 33-35). Again, CD62L expression is believed to contribute to homing of the donor cells to TDLNs for further antigen presentation and expansion of the donor cell population (26, 33-35, 88). Why CD44 is important is not yet known, however it may be to direct T cell migration in response the to extracellular matrix (83, 84). In addition, cancer cells have been shown to express higher levels of HA compared to normal tissue (89). Thus, the high expression of the CD44 ligand, HA, by cancerous tissue may help recruit T cells expressing higher levels of CD44 into the tumor (89). In addition, PSGL-1 has also been implicated in tumor infiltration of $CD8^+$ T cells because, $CD8^+$ T cells with a high avidity for P-selectin have been shown to secrete more IFN–γ in response to tumors than cells with lower avidity for P-selectin (85). Therefore, expression of these adhesion molecules may be important for tumor infiltration by donor Tc1 and Tc2 cells during ACT therapy (ref). So, we examined the expression level of these molecules implicated in tumor infiltration by T cells in order to determine if, as expected, the cells would express high levels of these adhesion molecules.

The role of antigen in T cell migration

Expression of antigen has been shown to affect T cell interactions with endothelial cells, particularly *in vitro* (90). T cell recognition of antigen presented by the endothelial cells can lead to arresting of the T cells, killing of the endothelial cells, or transmigration (91-93). Several *in vivo* studies have shown that antigen presentation on inflamed endothelium promotes the transmigration of antigen-specific T cells (90, 93). As we were conducting the following study, several labs independently reported that antigen-specific T cells infiltrate antigen-positive tumors more than antigen-negative tumors (94-97). How the expression of antigen promotes T cell migration is unknown, and whether antigen presentation by endothelial cells promotes the migration of both type I and type II T cells similarly has not been reported.

Summary

T cell responses are a critical component of the immune response against tumors, and both $CD4^+$ and $CD8^+$ T cells are being used effectively in ACT tumor therapies (9-11, 26-29). Several studies have recently examined the efficacy of Tc1 and Tc2 cell therapies in animals (36-48). Although we and others have found that Tc1 therapy is more effective than Tc2 therapy, why Tc1 therapy is more effective is not yet clear (37, 39-41, 45, 46, 48, 98). After Tc1 or Tc2 cells are transferred i.v. the cells must migrate to tumor draining lymph nodes and infiltrate the tumor (10, 11, 26, 31). Adhesion molecules and chemokines are important for effective T cell migration and tumor infiltration (9. 11, 60-68, 83, 84). Differences in the trafficking of Tc1 and Tc2 cells have been proposed as a reason that Tc1 therapy is more effective than Tc2 therapy (39, 40). We propose that the differences in Tc1 vs. Tc2 cell migration as well as the recruitment of host $CD8^+$ T cells contributes to the differences in Tc1 vs. Tc2 therapy. Therefore, in the following study, we investigated host $CD8^+$ T cell and donor Tc1 vs. Tc2 cell migration and tumor infiltration *in vivo* (99).

References

1. Jakobisiak M, Lasek W, Golab J. Natural mechanisms protecting against cancer. Immunol Lett. 2003 Dec 15;90(2-3):103-22. Review.

2. Zitvogel L, Tesniere A, Kroemer G. Cancer despite immunosurveillance: immunoselection and immunosubversion. Nat Rev Immunol. 2006 Oct;6(10):715-27.

3. Smyth MJ, Godfrey DI, Trapani JA. A fresh look at tumor immunosurveillance and immunotherapy. Nat Immunol. 2001 Apr;2(4):293-9. Review.

4. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. Nat Immunol. 2002 Nov;3(11):991-8. Review.

5. Swann JB, Smyth MJ. Immune surveillance of tumors. J Clin Invest. 2007 May;117(5):1137-46.

6. Burnet M. Cancer; a biological approach. I. The processes of control. Br Med J. 1957 Apr 6;1(5022):779-86.

7. Burnet M. Implications of immunological surveillance for cancer therapy. Isr J Med Sci. 1971 Jan;7(1):9-16.

8. Thomas L. On immunosurveillance in human cancer. Yale J Biol Med. 1982 May-Aug;55(3-4):329-33.

9. Gajewski TF, Meng Y, Blank C, Brown I, Kacha A, Kline J, Harlin H. Immune resistance orchestrated by the tumor microenvironment. Immunol Rev. 2006 Oct;213:131-45. Review.

10. Klebanoff CA, Gattinoni L, Restifo NP. $CD8^+$ T-cell memory in tumor immunology and immunotherapy. Immunol Rev. 2006 Jun;211:214-24. Review.

11. Neeson P, Paterson Y. Effects of the tumor microenvironment on the efficacy of tumor immunotherapy. Immunol Invest. 2006;35(3-4):359-94. Review.

12. Abbas AK, comp. Cellular and Molecular Immunology. Philadelphia: Saunders. 2003.

13. Tseng SY, Dustin ML. T-cell activation: a multidimensional signaling network. Curr Opin Cell Biol. 2002 Oct;14(5):575-80. Review.

14. Mempel TR, Henrickson SE, Von Andrian UH. T-cell priming by dendritic cells in lymph nodes occurs in three distinct phases. Nature. 2004 Jan 8;427(6970):154-9.

15. Hiura T, Kagamu H, Miura S, Ishida A, Tanaka H, Tanaka J, Gejyo F, Yoshizawa H. Both regulatory T cells and antitumor effector T cells are primed in the same draining lymph nodes during tumor progression. J Immunol. 2005 Oct 15;175(8):5058-66.

16. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science. 2003 Feb 14;299(5609):1057-61.

17. Woodland DL, Dutton RW. Heterogeneity of CD4($^{+}$) and CD8($^{+}$) T cells. Curr Opin Immunol. 2003 Jun;15(3):336-42. Review.

18. Glimcher LH, Townsend MJ, Sullivan BM, Lord GM. Recent developments in the transcriptional regulation of cytolytic effector cells. Nat Rev Immunol. 2004 Nov;4(11):900-11. Review.

19. Wowk ME, Trapani JA. Cytotoxic activity of the lymphocyte toxin granzyme B. Microbes Infect. 2004 Jul;6(8):752-8. Review.

20. Guermonprez P, Amigorena S. Pathways for antigen cross presentation. Springer Semin Immunopathol. 2005 Jan;26(3):257-71. Review.

21. Kammertoens T, Schuler T, Blankenstein T. Immunotherapy: target the stroma to hit the tumor. Trends Mol Med. 2005 May;11(5):225-31. Review.

22. Stauss H, Kawakami Y, Parmiani G, comps and eds. Tumor antigens recognized by T cells and antibodies. New York: Taylor & Francis. 2003.

23. Blankenstein T. The role of tumor stroma in the interaction between tumor and immune system. Curr Opin Immunol. 2005 Apr;17(2):180-6. Review.

24. Spiotto MT, Schreiber H. Rapid destruction of the tumor microenvironment by CTLs recognizing cancer-specific antigens cross-presented by stromal cells. Cancer Immun. 2005 Jun 6;5:8.

25. Pawelec G. Tumour escape: antitumour effectors too much of a good thing? Cancer Immunol Immunother. 2004 Mar;53(3):262-74. Review.

26. Gattinoni L, Powell DJ Jr, Rosenberg SA, Restifo NP. Adoptive immunotherapy for cancer: building on success. Nat Rev Immunol. 2006 May;6(5):383-93. Review.

27. Bhardwaj N. Harnessing the immune system to treat cancer. J Clin Invest. 2007 May;117(5):1130-6. Review.

28. Curiel TJ. Tregs and rethinking cancer immunotherapy. J Clin Invest. 2007 May;117(5):1167-74. Review.

29. June CH. Principles of adoptive T cell cancer therapy. J Clin Invest. 2007 May;117(5):1204-12. Review.

30. Fisher DT, Chen Q, Appenheimer MM, Skitzki J, Wang WC, Odunsi K, Evans SS. Hurdles to lymphocyte trafficking in the tumor microenvironment: implications for effective immunotherapy. Immunol Invest. 2006;35(3-4):251-77. Review.

31. Yee C, Thompson JA, Byrd D, Riddell SR, Roche P, Celis E, Greenberg PD. Adoptive T cell therapy using antigen-specific $CD8^+$ T cell clones for the treatment of patients with metastatic melanoma: *in vivo* persistence, migration, and antitumor effect of transferred T cells. Proc Natl Acad Sci U S A. 2002 Dec 10;99(25):16168-73.

32. Ikeda H, Chamoto K, Tsuji T, Suzuki Y, Wakita D, Takeshima T, Nishimura T. The critical role of type-1 innate and acquired immunity in tumor immunotherapy. Cancer Sci. 2004 Sep;95(9):697-703. Review.

33. Gattinoni L, Klebanoff CA, Palmer DC, Wrzesinski C, Kerstann K, Yu Z, Finkelstein SE, Theoret MR, Rosenberg SA, Restifo NP.. Acquisition of full effector function *in vitro* paradoxically impairs the *in vivo* antitumor efficacy of adoptively transferred $CD8^+$ T cells. J Clin Invest. 2005 Jun;115(6):1616-26.

34. Klebanoff CA, Gattinoni L, Torabi-Parizi P, Kerstann K, Cardones AR, Finkelstein SE, Palmer DC, Antony PA, Hwang ST, Rosenberg SA, Waldmann TA, Restifo NP. Central memory self/tumorreactive $CD8^+$ T cells confer superior antitumor immunity compared with effector memory T cells. Proc Natl Acad Sci. U S A 2005 Jul 5;102(27):9571-6.

35. Hinrichs CS, Gattinoni L, Restifo NP. Programming $CD8^+$ T cells for effective immunotherapy. Curr Opin Immunol. 2006 Jun;18(3):363-70. Review.

36. Cerwenka A, Carter LL, Reome JB, Swain SL, Dutton RW. *In vivo* persistence of CD8 polarized T cell subsets producing type 1 or type 2 cytokines. J Immunol. 1998 Jul 1;161(1):97-105.

37. Dobrzanski MJ, Reome JB, Dutton RW. Therapeutic effects of tumor-reactive type 1 and type 2 $CD8^+$ T cell subpopulations in established pulmonary metastases. J Immunol. 1999 Jun 1;162(11):6671-80.

38. Dobrzanski MJ, Reome JB, Dutton RW. Type 1 and type 2 $CD8^+$ effector T cell subpopulations promote long-term tumor immunity and protection to progressively growing tumor. J Immunol. 2000 Jan 15;164(2):916-25.

39. Helmich BK, Dutton RW. The role of adoptively transferred CD8 T cells and host cells in the control of the growth of the EG7 thymoma: factors that determine the relative effectiveness and homing properties of Tc1 and Tc2 effectors. J Immunol. 2001 Jun 1;166(11):6500-8.

40. Kemp RA, Ronchese F. Tumor-specific Tc1, but not Tc2, cells deliver protective antitumor immunity. J Immunol. 2001 Dec 1;167(11):6497-502.

41. Chamoto K, Kosaka A, Tsuji T, Matsuzaki J, Sato T, Takeshima T, Iwakabe K, Togashi Y, Koda T, Nishimura T. Critical role of the Th1/Tc1 circuit for the generation of tumor-specific CTL during tumor eradication *in vivo* by Th1-cell therapy. Cancer Sci. 2003 Oct;94(10):924-8.

42. Chang ML, Chen YT, Su YC, Kung JT. Cytotoxic T lymphocytes generated by short-term *in vitro* TCR stimulation in the presence of IL-4 are therapeutically effective against B16 melanoma. J Biomed Sci. 2003;10(6 Pt 1):644-50.

43. Dobrzanski MJ, Reome JB, Hollenbaugh JA, Hylind JC, Dutton RW. Effector cellderived lymphotoxin alpha and Fas ligand, but not perforin, promote Tc1 and Tc2 effector cell-mediated tumor therapy in established pulmonary metastases. Cancer Res. 2004 Jan 1;64(1):406-14.

44. Dobrzanski MJ, Reome JB, Hollenbaugh JA, Dutton RW. Tc1 and Tc2 effector cell therapy elicit long-term tumor immunity by contrasting mechanisms that result in complementary endogenous type 1 antitumor responses. J Immunol. 2004 Feb 1;172(3):1380-90.

45. Do JS, Choi YH, Shin SH, Yi HK, Hwang PH, Nam SY. Committed memory effector type 2 cytotoxic T (Tc2) cells are ineffective in protective anti-tumor immunity. Immunol Lett. 2004 Aug 15;95(1):77-84.

46. Nishimura F, Dusak JE, Eguchi J, Zhu X, Gambotto A, Storkus WJ, Okada H. Adoptive transfer of type 1 CTL mediates effective anti-central nervous system tumor response: critical roles of IFN-inducible protein-10. Cancer Res. 2006 Apr 15;66(8):4478-87.

47. Hollenbaugh JA, Dutton RW. IFN-gamma regulates donor CD8 T cell expansion, migration, and leads to apoptosis of cells of a solid tumor. J Immunol. 2006 Sep 1;177(5):3004-11.

48. Dobrzanski MJ, Reome JB, Hylind JC, Rewers-Felkins KA. CD8-mediated type 1 antitumor responses selectively modulate endogenous differentiated and nondifferentiated T cell localization, activation, and function in progressive breast cancer. J Immunol. 2006 Dec 1;177 (11):8191-201.

49. Sad S, Marcotte R, Mosmann TR. Cytokine-induced differentiation of precursor mouse $CD8^+$ T cells into cytotoxic $CD8^+$ T cells secreting Th1 or Th2 cytokines. Immunity. 1995 Mar;2(3):271-9.

50. Noble A, Truman JP, Vyas B, Vukmanovic-Stejic M, Hirst WJ, Kemeny DM. The balance of protein kinase C and calcium signaling directs T cell subset development. J Immunol. 2000 Feb 15;164(4):1807-13.3.

51. Boni A, Iezzi G, Degl'Innocenti E, et al. Prolonged exposure of DCs to maturation stimuli favors the induction of type-2 cytotoxic T lymphocytes. Eur J Immunol. 2006 Dec;36(12):3157-66.

52. Kemp RA, Backstrom BT, Ronchese F. The phenotype of type 1 and type 2 $CD8^+$ T cells activated *in vitro* is affected by culture conditions and correlates with effector activity. Immunology. 2005 Jul;115(3):315-24.

53. Haque, S., Burgents M. L., and Flood P. M. Loss of immunologic protection against highly immunogenic cancer cells and outgrowth of antigen loss variants correlates with a shift in CD8 T cell activity from a Tc1 to a Tc2 phenotype. Submitted.

54. Siveke JT, Hamann A. T helper 1 and T helper 2 cells respond differentially to chemokines. J Immunol. 1998 Jan 15;160(2):550-4.

55. Nakajima C, Uekusa Y, Iwasaki M, Yamaguchi N, Mukai T, Gao P, Tomura M, Ono S, Tsujimura T, Fujiwara H, Hamaoka T. A role of interferon-gamma (IFN-gamma) in tumor immunity: T cells with the capacity to reject tumor cells are generated but fail to migrate to tumor sites in IFN-gamma-deficient mice. Cancer Res. 2001 Apr 15;61(8):3399-405.

56. Qin Z, Schwartzkopff J, Pradera F, Kammertoens T, Seliger B, Pircher H, Blankenstein T. A critical requirement of interferon gamma-mediated angiostasis for tumor rejection by $CD8^+$ T cells. Cancer Res. 2003 Jul 15;63(14):4095-100.

57. Huang H, Liu Y, Xiang J. Synergistic effect of adoptive T-cell therapy and intratumoral interferon gamma-inducible protein-10 transgene expression in treatment of established tumors. Cell Immunol. 2002 May-Jun;217(1-2):12-22.

58. Yang X, Chu Y, Wang Y, Zhang R, Xiong S. Targeted *in vivo* expression of IFN-gamma-inducible protein 10 induces specific antitumor activity. J Leukoc Biol. 2006 Dec;80(6):1434-44.

59. Gorbachev AV, Kobayashi H, Kudo D, Tannenbaum CS, Finke JH, Shu S, Farber JM, Fairchild RL. CXC chemokine ligand 9/monokine induced by IFN-gamma production by tumor cells is critical for T cell-mediated suppression of cutaneous tumors. J Immunol. 2007 Feb 15;178(4):2278-86.

60. Mora JR, von Andrian UH. T-cell homing specificity and plasticity: new concepts and future challenges. Trends Immunol. 2006 May;27(5):235-43. Review.

61. Weninger W, Manjunath N, von Andrian UH. Migration and differentiation of $CD8^+$ T cells. Immunol Rev. 2002 Aug;186:221-33. Review.

62. Hogg N, Laschinger M, Giles K, McDowall A. T-cell integrins: more than just sticking points. J Cell Sci. 2003 Dec 1;116(Pt 23):4695-705. Review.

63. Steeber DA, Venturi GM, Tedder TF. A new twist to the leukocyte adhesion cascade: intimate cooperation is key. Trends Immunol. 2005 Jan;26(1):9-12. Review.

64. Lefrancois L. Development, trafficking, and function of memory T-cell subsets. Immunol Rev. 2006 Jun;211:93-103. Review.

65. Ley K, Kansas GS. Selectins in T-cell recruitment to non-lymphoid tissues and sites of inflammation. Nat Rev Immunol. 2004 May;4(5):325-35. Review.

66. Hogg N, Henderson R, Leitinger B, McDowall A, Porter J, Stanley P. Mechanisms contributing to the activity of integrins on leukocytes. Immunol Rev. 2002 Aug;186:164-71. Review.

67. Hogg N, Smith A, McDowall A, Giles K, Stanley P, Laschinger M, Henderson R. How T cells use LFA-1 to attach and migrate. Immunol Lett. 2004 Mar 29;92(1-2):51-4. Review.

68. Pribila JT, Quale AC, Mueller KL, Shimizu Y. Integrins and T cell-mediated immunity. Annu Rev Immunol. 2004;22:157-80. Review.

69. Dustin ML, Bivona TG, Philips MR. Membranes as messengers in T cell adhesion signaling. Nat Immunol. 2004 Apr;5(4):363-72. Review.

70. Kinashi T. Intracellular signalling controlling integrin activation in lymphocytes. Nat Rev Immunol. 2005 Jul;5(7):546-59. Review.

71. Venturi GM, Conway RM, Steeber DA, Tedder TF. $CD25^{+}CD4^{+}$ regulatory T cell migration requires L-selectin expression: L-selectin transcriptional regulation balances constitutive receptor turnover. J Immunol. 2007 Jan 1;178(1):291-300.

72. Tu L, Poe JC, Kadono T, Venturi GM, Bullard DC, Tedder TF, Steeber DA. A functional role for circulating mouse L-selectin in regulating leukocyte/endothelial cell interactions *in vivo*. J Immunol. 2002 Aug 15;169(4):2034-43.

73. Urzainqui A, Serrador JM, Viedma F, Yanez-Mo M, Rodriguez A, Corbi AL, Alonso-Lebrero JL, Luque A, Deckert M, Vazquez J, Sanchez-Madrid F. ITAM-based interaction of ERM proteins with Syk mediates signaling by the leukocyte adhesion receptor PSGL-1. Immunity. 2002 Oct;17(4):401-12.

74. Chen SC, Huang CC, Chien CL, Jeng CJ, Su HT, Chiang E, Liu MR, Wu CH, Chang CN, Lin RH. Cross-linking of P-selectin glycoprotein ligand-1 induces death of activated T cells. Blood. 2004 Nov 15;104(10):3233-42.

75. Ba XQ, Chen CX, Xu T, Cui LL, Gao YG, Zeng XL. Engagement of PSGL-1 upregulates CSF-1 transcription via a mechanism that may involve Syk. Cell Immunol. 2005 Sep;237(1):1-6.

76. Veerman KM, Williams MJ, Uchimura K, Singer MS, Merzaban JS, Naus S, Carlow DA, Owen P, Rivera-Nieves J, Rosen SD, Ziltener HJ. Interaction of the selectin ligand PSGL-1 with chemokines CCL21 and CCL19 facilitates efficient homing of T cells to secondary lymphoid organs. Nat Immunol. 2007 May;8(5):532-9.

77. van Wely CA, Blanchard AD, Britten CJ. Differential expression of alpha3 fucosyltransferases in Th1 and Th2 cells correlates with their ability to bind P-selectin. Biochem Biophys Res Commun. 1998 Jun 18;247(2):307-11.

78. Atarashi K, Hirata T, Matsumoto M, Kanemitsu N, Miyasaka M. Rolling of Th1 cells via P-selectin glycoprotein ligand-1 stimulates LFA-1-mediated cell binding to ICAM-1. J Immunol. 2005 Feb 1;174(3):1424-32.

79. Lin J, Miller MJ, Shaw AS. The c-SMAC: sorting it all out (or in). J Cell Biol. 2005 Jul 18;170(2):177-82. Review.

80. Cairo CW, Mirchev R, Golan DE. Cytoskeletal regulation couples LFA-1 conformational changes to receptor lateral mobility and clustering. Immunity. 2006 Aug;25(2):297-308.

81. Gonzalez PA, Carreno LJ, Figueroa CA, Kalergis AM. Modulation of immunological synapse by membrane-bound and soluble ligands. Cytokine Growth Factor Rev. 2007 Feb-Apr;18(1-2):19-31.

82. Katakai T, Hara T, Sugai M, Gonda H, Nambu Y, Matsuda E, Agata Y, Shimizu A. Chemokine-independent preference for T-helper-1 cells in transendothelial migration. J Biol Chem. 2002 Dec 27;277(52):50948-58.

83. Martin TA, Harrison G, Mansel RE, Jiang WG. The role of the CD44/ezrin complex in cancer metastasis. Crit Rev Oncol Hematol. 2003 May;46(2):165-86. Review.

84. Ponta H, Sherman L, Herrlich PA. CD44: from adhesion molecules to signalling regulators. Nat Rev Mol Cell Biol. 2003 Jan;4(1):33-45. Review.

85. Tanigawa K, Takeshita N, Craig RA, Phillips K, Knibbs RN, Chang AE, Stoolman LM. Tumor-specific responses in lymph nodes draining murine sarcomas are concentrated in cells expressing P-selectin binding sites. J Immunol. 2001 Sep 15;167(6):3089-98.

86. Blank C, Brown I, Kacha AK, Markiewicz MA, Gajewski TF. ICAM-1 contributes to but is not essential for tumor antigen cross-priming and $CD8^+$ T cell-mediated tumor rejection *in vivo*. J Immunol. 2005 Mar 15;174(6):3416-20.

87. Jenkinson SR, Williams NA, Morgan DJ. The role of intercellular adhesion molecule-1/LFA-1 interactions in the generation of tumor-specific $CD8^+$ T cell responses. J Immunol. 2005 Mar 15;174(6):3401-7.

88. Yamada M, Yanaba K, Hasegawa M, Matsushita Y, Horikawa M, Komura K, Matsushita T, Kawasuji A, Fujita T, Takehara K, Steeber DA, Tedder TF, Sato S. Regulation of local and metastatic hostmediated anti-tumour mechanisms by L-selectin and intercellular adhesion molecule-1. Clin Exp Immunol. 2006 Feb;143(2):216-27.

89. Toole BP. Hyaluronan: from extracellular glue to pericellular cue. Nat Rev Cancer. 2004 Jul;4(7):528-39. Review.

90. Marelli-Berg FM, Jarmin SJ. Antigen presentation by the endothelium: a green light for antigen-specific T cell trafficking? Immunol Lett. 2004 May 15;93(2-3):109-13. Review.

91. Marelli-Berg FM, Frasca L, Weng L, Lombardi G, Lechler RI. Antigen recognition influences transendothelial migration of $CD4^+$ T cells. J Immunol. 1999 Jan 15;162(2):696-703.

92. Marelli-Berg FM, Scott D, Bartok I, Peek E, Dyson J, Lechler RI. Activated murine endothelial cells have reduced immunogenicity for $CD8^+$ T cells: a mechanism of immunoregulation? J Immunol. 2000 Oct 15;165(8):4182-9.

93. Marelli-Berg FM, James MJ, Dangerfield J, Dyson J, Millrain M, Scott D, Simpson E, Nourshargh S, Lechler RI. Cognate recognition of the endothelium induces HY-specific $CD8^+$ T-lymphocyte transendothelial migration (diapedesis) *in vivo*. Blood. 2004 Apr 15;103(8):3111-6.

94. Boissonnas A, Combadiere C, Lavergne E, Maho M, Blanc C, Debre P, Combadiere B. Antigen distribution drives programmed antitumor CD8 cell migration and determines its efficiency. J Immunol. 2004 Jul 1;173(1):222-9.

95. Joncker NT, Marloie MA, Chernysheva A, Lonchay C, Cuff S, Klijanienko J, Sigal-Zafrani B, Vincent-Salomon A, Sastre X, Lantz O. Antigen-independent accumulation of activated effector/memory T lymphocytes into human and murine tumors. Int J Cancer. 2006 Mar 1;118(5):1205-14.

96. Mrass P, Takano H, Ng LG, Daxini S, Lasaro MO, Iparraguirre A, Cavanagh LL, von Andrian UH, Ertl HC, Haydon PG, Weninger W. Random migration precedes stable target cell interactions of tumor-infiltrating T cells. J Exp Med. 2006 Nov 27;203(12):2749-61.

97. Boissonnas A, Fetler L, Zeelenberg IS, Hugues S, Amigorena S. *In vivo* imaging of cytotoxic T cell infiltration and elimination of a solid tumor. J Exp Med. 2007 Feb 19; 204(2):345-56.

98. Qian, L, Lorena, S, Burgents, M, and Flood, PM. Increased $CD8^+$ T cell Antigenicity of Murine Tumors Increases Tumor Resistance. Manuscript in preparation.

99. Burgents ML, Flood PM. Tc1 Cells Infiltrate Tumor Sites More Efficiently than Tc2 Cells Due to Enhanced Expression of a Tumor Infiltrating Phenotype. Submitted.

Chapter II

A Model for Evaluating Migration and Tumor Infiltration of Antigen-Specific T Cells

Abstract

ACT therapy is an effective treatment for tumors in animals and patients. Tc1 cells have been shown to be more effective for tumor therapy than Tc2 cells. In addition, studies have suggested that Tc1 cells migrate to tumor draining lymph nodes and tumors more effectively than Tc2 cells, however, differences in Tc1 and Tc2 cell migration are not yet understood. In order to develop a model for evaluating Tc1 and Tc2 migration, we utilized both *in vitro* and *in vivo* approaches for studying T cell migration. We found that the *in vitro* approach for evaluating T cell chemotaxis and transendothelial migration was difficult to duplicate for Tc1 and Tc2 cells. The MS1-VEGF endothelial cell line we investigated for use in transendothelial migration studies expressed an activated phenotype after overnight stimulation, suggesting that these cells may be used for transendothelial migration studies. However, because optimal conditions for evaluating Tc1 and Tc2 cell chemotaxis were not obtained, our transendothelial migration model is not complete and requires additional development before it may be appropriate for further investigations. Importantly, the *in vivo* model we developed, however, was highly reproducible and offers many advantages for examining Tc1 and Tc2 cell migration and tumor infiltration. In this model, we established EL-4 thymoma tumors expressing the p33 antigen from lymphocytic choriomeningitis virus in host mice and injected these mice with polarized Tc1 or Tc2 cells generated *in vitro* from splenocytes of GFPxP14 mice. These Tc1 and Tc2 cells are specific for p33, expressing the Vb8 T cell receptor specific for p33, and express green fluorescent protein (GFP). In addition, these GFPxP14 Tc1 and Tc2 cells express type I and type II cytokine and chemokine receptor profiles, respectively. Tc1 and Tc2 therapy using these cells yielded reduced tumor growth compared to untreated control animals, as observed in other Tc1 and

Tc2 tumor models, with Tc1 therapy providing higher reduction in tumor growth compared to Tc2 therapy. Also, when we used this *in vivo* model to examine Tc1 and Tc2 migration, we were able to identify the donor Tc1 and Tc2 cells following transfer. Tc1 cells could be found in TDLNs and tumors as early as day 1 after transfer. Both Tc1 and Tc2 cells could be found in TDLNs and tumors on day 3, 7, and 10 following transfer. While more Tc1 cells were found in TDLNs and tumors compared to Tc2 cells, the Tc2 cells appeared to be more abundant in the spleen compared to Tc1 cells. Also, there appeared to be no significant difference between donor cell migration to contralateral and ipsilateral lymph nodes. Therefore, our GFPxP14 model is appropriate for investigating the migration and tumor infiltration of Tc1 and Tc2 cells *in vivo*, and these studies suggest that, indeed, Tc1 and Tc2 cells may have different migration properties.

Introduction

CD8+ Tc1 and Tc2 cells have been used for tumor therapy in animals (1-13). Studies have shown that Tc1 therapy is more effective at reducing tumor growth or rejecting tumors compared to Tc2 therapy (2, 4-6, 10, 11, 13). A few studies have suggested that differences in Tc1 vs. Tc2 migration may contribute to this difference in therapeutic efficacy between Tc1and Tc2 cells (4, 5, 11). In addition, *in vitro* studies have suggested that CD4+ Th1 and Th2 cells have different migration properties (14, 15). Therefore, we proposed to evaluate Tc1 and Tc2 cell transendothelial migration *in vitro* and migration to TDLNs and tumors *in vivo*. In order to do this, we first needed to develop a model to track the Tc1 and Tc2 cells.

Transendothelial migration assays are a method to evaluate T cell migration *in vitro* (14-17). Transwell plates (Corning) with transwell filters separating lower and upper wells of the plate are used for this assay. Chemokines are added to the lower well, and the migrating cells are added to the upper well. After a desired time period, the cells within the lower well are counted. In order to evaluate transendothelial migration, endothelial cells are cultured on the transwell filters (coated with extracellular matrix components). This approach has been used to evaluate migration of CD4+ T cells, including polarized Th1 and Th2 cells (14, 15). However, studies examining CD8+ T cell migration have focused on *in vivo* migration, and there is limited knowledge of CD8+ T cell migration *in vitro* (18). In fact, to our knowledge, there are no reports of Tc1 or Tc2 migration *in vitro*.

Studies examining T cell migration *in vivo* have been limited by previous methods used to track the donor cells. There have been three general approaches to tracking donor cells *in vivo*: intracellular labeling of amines, antibody detection of cell surface proteins, and GFP expression by transferred cells (4, 5, 7-9, 11-13, 18). Labeling amines with

carboxyfluoroscein succinimidyl ester and other methods for labeling amines in cells are limited by the dilution of the labeling due to proliferation of the donor cells. Using antibodies to detect donor cells based on expression of a unique cell surface protein, such as Thy1.1, has limited applications. Because of the numerous applications for analyzing GFP expressing cells, including *in vivo* imaging, fluorescence microscopy, and flow cytometry, GFP-based methods are well suited for tracking cell migration *in vivo* (18). Therefore, in order to distinguish the donor Tc1 and Tc2 cells from host CD8+ T cells, we proceeded to develop a model using GFP expression as a method to identify the donor cells.

In this study we worked to develop an *in vitro* transendothelial migration assay and an *in vivo* migration assay to evaluate Tc1 and Tc2 cell migration. After finding less than optimal results with the transendothelial migration *in vitro* approach, we focused on developing an *in vivo* model. We developed a GFP-based murine model for investigating donor T cell migration and tumor infiltration *in vivo*. GFPxP14 mice were generated by crossing UBI.GFP mice with P14 mice (19, 20). T cells from GFPxP14 mice were characterized by flow cytometry *ex vivo* and after culture *in vitro*. Tc1 and Tc2 donor cells were generated *in vitro* from GFPxP14 splenocytes and used for tumor therapy. We then examined the spleens, TDLNs, and tumors of animals given Tc1 or Tc2 therapy to evaluate whether these Tc1 and Tc2 cells generated from GFPxP14 splenocytes could be identified after transfer into tumor bearing animals. Our GFPxP14 murine model will allow for further investigation of T cell migration and tumor infiltration *in vivo*.

Materials & Methods

Mice

C57BL/6J (B6) and UBI.GFP mice were obtained from The Jackson Laboratory (Bar Harbor, Maine) (19). CD8 T cell vβ8 TCR transgenic P14 mice specific for LCMV peptide p33-41 (KAVYNFATC) in context of H-2D^b were originally obtained from Dr. Pam Ohashi (20). GFPxP14 mice were generated in our lab by crossing the UBI.GFP strain with the P14 strain and using the F1 generation. Spleens of GFPxP14 mice were used as the source for our donor cells, as detailed below. B6 mice were used as recipient animals. Female mice aged 8-12 weeks of age were used throughout these experiments. All mice were maintained in specific pathogen free conditions by University of North Carolina's Department of Laboratory Animal Medicine and all animal procedures were approved by the university's Institutional Animal Care and Use Committee.

Comparison of B6, P14, and GFPxP14 T cells

The phenotype of T cell subsets in B6, P14, and GFPxP14 mice was determined by harvesting splenocytes, thymocytes, and lymph node cells of these mice, then staining the cells as indicated. Cultures of splenocytes of these mice were stimulated with 2 μg/mL of ConcanavalinA and analyzed by flow cytometry.

MS1-VEGF cells

The MS1-VEGF cell line (ATCC) was maintained in DMEM (UNC-CH Tissue Culture Facility) (21). When cells were 80-90% confluent, the cultures were split 1:3. This cell line was originally developed from pancreatic endothelial cells, which were transfected with

vascular endothelial growth factor to promote the endothelial cell phenotype. Cells were stimulated overnight with 1 ng/mL of IL-1β or TNF-α (R&D Systems) before analyzing the cell phenotype by flow cytometry.

Tumor cell lines and construction of p33 encoding plasmid

As a source of antigen-bearing tumor cells, our lab has previously generated LCMV peptide p33 -expressing EL4 thymoma tumor cells (EL-4.p33 cells, 22). Briefly, EL4 cells obtained from ATCC were transfected with a PcDNA3.0 plasmid (Invitrogen) containing an insert encoding the LCMV peptide p33 linked to human beta-2 Microglobulin (22). Linking peptides to human β2 microglobulin has been shown to enhance the peptide presentation in transfected cells, evidenced by increased specific lysis compared to target cells transfected with peptide alone (23-26). P33.EL4 tumor cells were selected and maintained using RPMI-1640 medium containing G418 (Sigma-Aldrich). Transfection was confirmed by PCR and expression of the p33 antigen was confirmed by using the transfected cells as targets of P14 T cells in cytolytic assays (22). Both parental EL4 (p33 antigen-negative) cells and transfected p33.EL4 (p33 antigen-positive) cells were used in this study, as detailed below.

Tc1/Tc2 cultures

Splenocytes from GFPxP14 mice were harvested and then stimulated at $5x10^6$ cells/mL for 3 days with 2μM p33 peptide (KAVYNFATC) along with 2 units/mL rIL-2 in RPMI medium. For Tc1 cells, we also added rIL-12 at 12.5ng/mL and anti-IL-4 antibody at 2.5ng/mL, but for Tc2 cells we added rIL-4 at 27.5ng/mL, anti-IL-12 antibody at 5.5ng/mL, and anti-IFN-γ antibody at 5.5ng/mL. Recombinant murine cytokines and anti-murine cytokine antibodies

were all obtained from R&D Systems. Tc1 and Tc2 cultures were both over 90% GFP+CD8+vβ8+ after three days of culture, as determined by flow cytometry.

Flow cytometry analysis of surface molecule expression

For cell surface expression analysis, cultured cells or cells from spleen, thymus, lymph node and tumor tissues were harvested. Then, single-cell suspensions were made and the cells were stained for various cell surface markers, as indicated, using antibodies against the following: for endothelial cell staining-- CD11b (Mac-1), CD31 (PECAM-1), CD54 (ICAM-1), CD62P (P-selectin), CD102 (ICAM-2), and CD106 (VCAM-1); or for T cell staining-- CD4, CD8, Vβ8, and CD25 (Pharmingen). Endothelial cells or lymphocytes were gated based on forward and side scatter. Where noted, donor and host cells in TDLNs and tumors were identified by gating for GFP. All flow cytometry samples were run on a FACSCalibur (BD Bioscience) and analyzed using FlowJo software (Treestar).

Tc1 and Tc2 therapy

$1x10^6$ EL4.p33 tumor cells were injected s.c. into the right ventral flank of B6 mice. On the same day either $1x10^6$ Tc1 or $1x10^6$ Tc2 in 200uL of PBS were injected i.v. into the tail vein of these mice. Control mice were injected with PBS alone. Tumor growth was assessed every 2-3 days using calipers to measure perpendicular widths of the palpable tumors through day 18. Tumor areas of animals within each treatment group were determined. Four animals were used per treatment group.

Real Time RT- PCR

Tc1 and Tc2 cultures were analyzed for IFN-γ, IL-4, CXCR3, CCR4, and CCR5 mRNA expression using TaqMan Gene Expression Assays from Applied Biosystems.. Total RNA was isolated from both cultures using RNEasy Minikits from Qiagen followed by DNase I treatment (Promega). mRNA was converted to cDNA using Superscript III (Invitrogen). This cDNA was then used in Real Time PCR reactions using an ABI 7700 thermocyclyer. Samples were run in triplicate. Data was analyzed according to the methods of Livak and Schmittgen (27). Ct values for the cytokine and chemokine receptor genes were normalized to GAPDH expression for the same mRNA source. The lowest level of gene expression was found for IL-4 expression in Tc1 culture. This expression level was set as a fold expression value of 1.00. Then, the other gene expression values were determined relative to this fold expression value.

Chemotaxis assay

5μm pore transwell plate filters were coated with matrigel (BD Bioscience). Medium containing MIP-1α chemokine (0ng/mL-100ng/mL as noted), was added to the lower well of the plated. The matrigel coated filters were then inserted and $1x10^5$ Tc1 or Tc2 cells were added to the upper well. Loaded transwell plates were incubated at 37 for the duration of the assay. After a certain period of time (4-28 hours as noted), the number of cells in the lower well was determined by counting the number of cells in a sample from the lower well using a hemocytometer. Assay samples were run in triplicate.

Migration and Tumor Infiltration of Tc1 and Tc2

5 or 7 days before donor cell transfer, B6 mice were injected s.c. in the right ventral flank with $1x10^6$-$1.5x10^6$ p33.EL4 tumor cells. For experiments examining the role of antigen, $1x10^6$-$1.5x10^6$ EL4 tumor cells were also injected into the mice but into the left ventral flank on the same day. Then on day 0, $1x10^6$ Tc1 or $1x10^6$ Tc2 donor cells in 200uL of PBS were injected i.v. into the tail vein of these mice. On days 3 and 7 after donor cell transfer, TDLNs (axillary and superficial inguinal lymph nodes) and tumors were collected then analyzed by flow cytometry. Two animals per group were used for each time point in each experiment.

Statistical Analysis

Experiments were repeated at least twice and data from one representative experiment is shown for each figure. Where shown, a two-tailed Student's T-test was utilized to test for significance with $p \leq .05$ considered significant.

Results

In vitro and *in vivo* models have been used to evaluate T cell migration (14-18). In order to develop a model to examine Tc1 vs. Tc2 migration, we applied both approaches. *In vitro* models offer more control over variables that affect T cell migration, such as the amount and type of chemokines attracting the T cells. *In vitro* models allow us to address the role of individual molecules and cells, while *in vivo* models are more difficult to control and interpret because of the complexity of *in vivo* systems. GFP expression is an effective method for identifying and tracking T cells *in vitro* and *in vivo* (18, 28). Therefore, we generated GFP expressing TCR transgenic mice specific for p33 antigen. We then identified an endothelial cell line appropriate for *in vitro* migration assays and examined Tc1 and Tc2 chemotaxis *in vitro*. Finally, we developed an *in vivo* model for evaluating donor cell tumor infiltration.

Generation and characterization of GFPxP14 mice

We chose to utilize GFP technology for tracking the Tc1 and Tc2 cells, because GFP expression can be detected in a number of assays/applications, allowing more options for evaluating Tc1 and Tc2 cell migration and tumor infiltration (18). UBI.GFP mice (Jackson) express GFP under the human ubiquitin promoter (19). We crossed these mice with P14 T cell receptor Vβ8 transgenic mice to generate GFPxP14 mice. Importantly, the P14 mice are specific for the p33 antigen from lymphocytic choriomeningitis virus (20). Previously in the lab, an EL-4 thymoma tumor cell line was transfected to express this p33 antigen (EL-4.p33, 22). This model would allow us to evaluate the migration and tumor infiltration by the p33 antigen-specific T cells *in vitro* and *in vivo*.

We phenotyped the T cell populations in the GFPxP14 mice (F1 generation of the GFPxP14 cross) by flow cytometry. We examined the T cell populations of thymus, spleen, and lymph nodes from GFPxP14 mice and compare these populations with T cells from B6 and P14 mice (Figure 2.1). Cells were stained with monoclonal antibodies against CD4, CD8, and Vβ8. Lymphocytes were gated and analyzed for expression of these molecules and GFP. Over 97% of cells in GFPxP14 mice express GFP, whereas cells from B6 and P14 mice do not. As expected, CD4+ cells are higher in tissues of B6 mice compared to either GFPxP14 or P14 mice, which have less than half as many CD4+ cells in the spleen and LNs. CD8+ cells are higher in the spleen and LNs of GFPxP14 and P14 mice compared to B6 mice. Importantly, over 80% of the CD8+ cells in the GFPxP14 and P14 mice are Vβ8+, while only 20% or fewer of the CD8+ cells in B6 mice express Vβ8. Therefore, the thymocytes, splenocytes, and lymph node cells of GFPxP14 mice are similar to those cells of P14 mice but express GFP. Additionally, because previous studies using GFP to track T cells have reported that the T cells lost GFP expression after stimulation, we stimulated GFPxP14 splenocytes with Concanavalin A for 3 days and evaluated the phenotype of the cultured T cells (Figure 2.2) (28). The culture maintained GFP expression and over 90% of the CD8+ cells were CD25+Vβ8+. Therefore, the T cells of GFPxP14 mice are similar to the P14 mice T cells but express GFP, even after stimulation.

GFPxP14 Tc1 and Tc2 cells

Once we had developed the GFPxP14 mice and confirmed that T cells from these mice maintain GFP and Vβ8 expression after culture, we then confirmed that these cultured GFPxP14 splenocytes could be polarized to Tc1 and Tc2 cells. Tc1 and Tc2 cultures were

generated by stimulating GFPxP14 splenocytes for 3 days in polarizing conditions as described and the cultures were phenotyped for GFP, CD8, CD25, and Vβ8 expression using flow cytometry (Figure 2.3). These Tc1 and Tc2 cells derived from GFPxP14 splenocytes were GFP+CD8+ and over 90% of the cells are CD25+Vβ8+. Real time RT-PCR was used to confirm the expression of IFN-γ, IL-4 and chemokine receptors indicative of type I and type II cultures (Figure 2.6) (29, 30). Tc1 cells expressed higher levels of IFN-γ and CCR5 than Tc2 cells, which expressed higher levels of IL-4 and CCR4. Both cultures expressed similar levels of CXCR3. Therefore, not only do GFPxP14 derived Tc1 and Tc2 cells express GFP and an activated T cell phenotype after stimulation, but the cells also express phenotypes indicative type I and type II polarization, respectively.

In order to evaluate whether the Tc1 and Tc2 cells behaved similarly to Tc1 and Tc2 in other tumor therapy models, we transferred GFPxP14 Tc1 and Tc2 cells into mice receiving EL-4.p33 tumor cells (Figure 2.7) (1-13). B6 mice were injected with 1x10^6 tumor cells s.c. and injected with 1x10^6 Tc1 or Tc2 cells i.v. on the same day, while control animals received tumor cells s.c. and only PBS i.v. (This dose of Tc1 and Tc2 donor cells falls within the range of donor cell doses reported by others to provide tumor protection (13).) Every 2-3 days after the injections, animals were evaluated for palpable tumors. Once tumors were palpable, tumor growth was evaluated every 3 days through day 18. While both Tc1 and Tc2 treatments reduced tumor growth compared to control animals, the animals receiving Tc1 treatment had significantly reduced tumor growth compared to Tc2 treated animals, as well. Interestingly, while animals given Tc1 therapy exhibit effective retardation of tumor growth starting on day 10, animals given Tc2 therapy do not exhibit impeded tumor growth until day 12. After this initial delay in the reduction of tumor growth for Tc2 vs. Tc1

therapy, both therapies continue to be effective at reducing tumor growth through day 18 compared to control animals. Importantly, we continued to use this effective dose of Tc1 and Tc2 cells to develop our model to examine tumor infiltration. Overall, these observations of Tc1 and Tc2 cell treatments show that Tc1 and Tc2 cells generated from GFPxP14 splenocytes exhibit therapeutic efficacy similar to previous reports of Tc1 and Tc2 therapies --Both Tc1 and Tc2 therapies reduce tumor growth, but Tc1 therapy is more effective than Tc2 therapy (1-13).

In vitro T cell migration assays

We sought to develop a transendothelial migration assay to be used to evaluate Tc1 and Tc2 migration *in vitro*. Two important components of transendothelial migration assays are the endothelial cells and the chemokine that is used to attract T cells (14-17). For transendothelial migration assays, the endothelial cells are cultured until confluency on the transwell filters. These filters are coated with extracellular matrix to aid in the adherence of the endothelial cells. Endothelial cells used in transendothelial migration assays can either be endothelial cell lines or endothelial cells isolated mice and then cultured *in vitro* (15-17). We chose to use a cell line for convenience and because cells isolated from mice have a risk of being contaminated with other cells, making their purity less reliable. The MS1-VEGF cell line (ATCC) has been used previously to examine dendritic cell transendothelial migration (21). These MS1-VEGF cells are pancreatic cells transfected to express vascular endothelial growth factor (VEGF). Unstimulated endothelial cells do not express the necessary chemokines and adhesion receptors or ligands needed for T cell migration, so cultured

endothelial cells must be stimulated/activated with inflammatory cytokines before they can be used in transendothelial migration assays (14-17, 21).

In order to determine whether the MS1-VEGF cell line was appropriate for use in the T cell transendothelial migration assay, we evaluated the phenotype of the MS1-VEGF cells after overnight stimulation with either IL-1β or TNF-α. We stained the activated cells and control unstimulated cells for the expression of CD11b, CD31, CD54, CD62P, CD102, and CD106 (Figure 2.6). After overnight stimulation, the cells treated with either IL-1β orTNF-α had higher expression of these molecules than the untreated cells. Also, it appears that stimulation with TNF-α induced higher expression of these molecules compared to IL-1β stimulation. Therefore, MS1-VEGF cells, particularly those stimulated with TNF-α, express an activated phenotype appropriate for use in a transendothelial migration assay.

We then wanted to determine which chemokine would be optimal for establishing a transendothelial assay. To do this, we initially set up chemotaxis assays for polarized Tc1 and Tc2 cells. Tc1 and Tc2 cells express different chemokine receptors, including CCR5 and CCR4, respectively (29, 30). Studies have shown that Th1 cells migrate more efficiently in the presence of MIP-1α compared to Th2 cells (14). Additionally, MIP-1α has been shown to play a role in recruiting CD8+ T cells to tumors (22, 31). Our lab has previously found that transfection of tumor cells with MIP-1α increased tumor infiltration by CD8+ cells and that clearance of these tumors was impaired in CD8 knockout mice (22). Therefore, in order to determine the appropriate kinetics for CD8+ T cell migration in our system, we chose to examine migration of Tc1 and Tc2 cells in response to the biologically relevant MIP-1α (Figure 2.7). We expected that MIP-1α would recruit Tc1 cells more efficiently than Tc2 cells, and the two cells types could then serve as positive and negative controls, respectively.

We titrated the doses of chemokine in the lower well (0ng/mL-100ng/mL) and varied the time allowed for migration (4-28h, only 4-12h are shown). We were able to find migrated cells in the lower wells at the earliest time point of 4 hours and many cells had migrated to the lower wells at time points 12 hours or later (Figure 2.7 A-C). However, despite the increase in the number of migrated cells over time, we did not observe a chemokine dose-dependent change in the number of migrated cells. Thus, it appears that despite numerous modifications of kinetics and chemokine concentrations, we were only able to observe spontaneous migration of the T cells into lower wells and found no differences between Tc1 and Tc2 cells in these conditions. Furthermore, because this chemotaxis assay with polarized Tc1 and Tc2 cells needs further modifications before the assay is appropriate for examining either chemotaxis or chemotaxis-driven transendothelial migration by these polarized T cells, we decided to concentrate our efforts on developing an *in vivo* system.

GFPxP14 Tc1 and Tc2 cell migration and tumor infiltration in vivo

An *in vivo* model would be ideal, because the models in previous studies suggesting that Tc1 and Tc2 cells have different migration properties were tumor therapy models (4, 5, 11). We needed a model to examine not only donor cell migration but also tumor infiltration. So, we confirmed that the Tc1 and Tc2 cells could be tracked *in vivo* based on GFP expression after transfer into tumor bearing mice. B6 mice were injected with $1x10^6$ EL-4.p33 tumor cells s.c. in order to establish tumors. Then 7 days later, the mice were injected with either $1x10^6$ Tc1 or Tc2 cells i.v. We examined spleen, lymph node, and tumor tissues for GFP+CD8+ cells by flow cytomertry on various days after transfer. We found GFP+CD8+ cells in both Tc1 and Tc2 treated mice by day 3 after transfer and continued to

find the donor cells on days 6 and 8 after transfer (Figure 2.8-2.11). Interestingly, we found a limited number of donor cells, if any, in the tissues on day 1 and day 10 after transfer, suggesting that days 3-8 were the optimal time period for examining Tc1 and Tc2 cells *in vivo*. Since we established that Tc1 and Tc2 cells derived from GFPxP14 splenocytes were tracable *in vivo*, we proceeded to examine migration and tumor infiltration *in vivo* (32).

Discussion

Tc1 and Tc2 therapy studies have suggested that Tc1 therapy is more effective than Tc2 therapy due to differences in Tc1 and Tc2 migration (4, 5, 11). Previous studies tracking T cells *in vivo* have utilized a variety of techniques which have limited applications (4, 5, 7-9, 11-13, 18). In this study we developed a model utilizing GFP expression of antigen-specific donor Tc1 and Tc2 cells to examine their migration and tumor infiltration *in vivo* after transfer into tumor bearing mice. We generated GFPxP14 mice that express both GFP and the p33 specific TCR. T cell populations from these mice maintain GFP expression even after stimulation and express the Vβ8 TCR specific for p33 peptide. Cultured Tc1 and Tc2 cells derived from GFPxP14 splenocytes express type I and type II cytokine and chemokine receptor phenotypes, respectively(29, 30). When these cultured cells are transferred into EL-4.p33 tumor bearing mice, we can identify tumor infiltrating donor CD8+ cells 3 and 7 days after transfer. Thus, our GFPxP14 murine model for generating donor cells is a useful model for evaluating T cell migration *in vivo*.

This GFPxP14 model has numerous applications. *In vivo* imaging is of particular interest. As a new and evolving technique, *in vivo* imaging is being used by others to investigate T cell migration *in vivo* (18). Since this technique requires the labeling of the T cells, our model is applicable to this technique and is not restricted by the potential loss of T cell labeling. In addition, applying two photon microscropy to the GFPxP14 model will allow us to evaluate T cell contact with the other cells *in vivo*, providing even more details of the interaction of the T cells in the tumor mass, as well as transendothelial migration of the infiltrating T cells. These are just two of the additional approaches that can utilize the

GFPxP14 model to evaluate T cell migration and tumor infiltration *in vivo*, including polarized donor Tc1 and Tc2 cells.

As stated previously and described above, an *in vivo* model is an important tool for examining Tc1 and Tc2 cell migration and tumor infiltration, allowing for the complexity of an *in vivo* system (18). We used our GFPxP14 model to investigate the migration and tumor infiltration of Tc1 and Tc2 cells in a subsequent study (32). However, despite the advantages for using an *in vivo* model, having an *in vitro* model would allow for future studies to contribute to our understanding of the mechanisms of Tc1 and Tc2 cell migration. Currently, our *in vitro* model still requires additional optimizing. We still need to determine the optimal chemokines (type and concentration) to attract Tc1 and Tc2 cells to the lower well, as well as the time duration for the assay. The data do suggest that four hours is too short of a time period and that overnight, approximately 18 hours, is possibly too long. Also, we used MIP-1α to attract the Tc1 vs. Tc2 cells to the lower well, based on previous reports of Th1 and Th2 chemotaxis and the chemokine receptor expression by the Tc1 and Tc2 cells (14, 29, 30). Because we did not find a dose-dependent migration of neither Tc1 nor Tc2 cells over time, we believe that we observed only chemokine-independent, spontaneous migration. This may be because, unlike Th1 cells, neither Tc1 nor Tc2 cells respond to MIP-1α (14). (Perhaps naïve CD8+ T cells would exhibit a chemotactic response.) It is possible that MIP-1α is not the optimal chemokine for attracting polarized CD8+ T cells. Because CXCR3 is expressed by both Tc1 and Tc2 cells, we could use chemokine CXCL11, a ligand for CXCR3 that has been implicated in tumor therapy (33).

Because stimulation of the MS1-VEGF cell line induces an activated phenotype appropriate for use in a transendothelial migration assay, we believe that developing a

trasnendothelial migration assay with Th1 or Th2 cells would be an important next step towards an assay for Tc1 and Tc2 cells using this MS1-VEGF cell line. Other studies have already evaluated Th1 and Th2 cell chemotaxis and transendothelial migration *in vitro*, so the kinetics and optimal chemokines for theTh1 or Th2 cell assay have been determined by others (14, 15). While we were not able to apply these findings directly to Tc1 and Tc2 cells, adapting current protocols and using the MS1-VEGF cell line as the source for the endothelial cells will allow us to demonstrate whether the MS1-VEGF cell line can be used for T cell transendothelial migration assays. Therefore, we will need to continue to optimize this assay before it can be used to compare the migration of Tc1 and Tc2 cells.

Whether the GFPxP14 model is used for *in vitro* or *in vivo* T cell migration, there are two key components of our model: antigen specificity and lasting GFP expression of the CD8+ T cells. Antigen-specific T cell receptor transgenic mice are an invaluable tool for examining T cell immune responses, because they allow us to investigate the response of a monoclonal pool of T cells. In our model, the T CD8+ T cells are specific for the p33 antigen, which is expressed by the EL-4.p33 tumor cells (22). Therefore, we are able to examine the response of tumor antigen-specific T cells. The GFP expression by these T cells is critical to our model. Other methods for tracking donor cells are limited because they require antibody staining or cannot track donor cells long-term (18). Although at least one other group has developed a similar GFP model, where the T cells lost GFP expression after stimulation, our model is unique in that we have the ability to track our donor cells long-term by GFP expression which does not decrease after stimulation (28). Therefore, our GFPxP14 model for generating Tc1 and Tc2 cells is an invaluable tool for examining the migration and tumor infiltration of T cells, particularly *in vivo* (32).

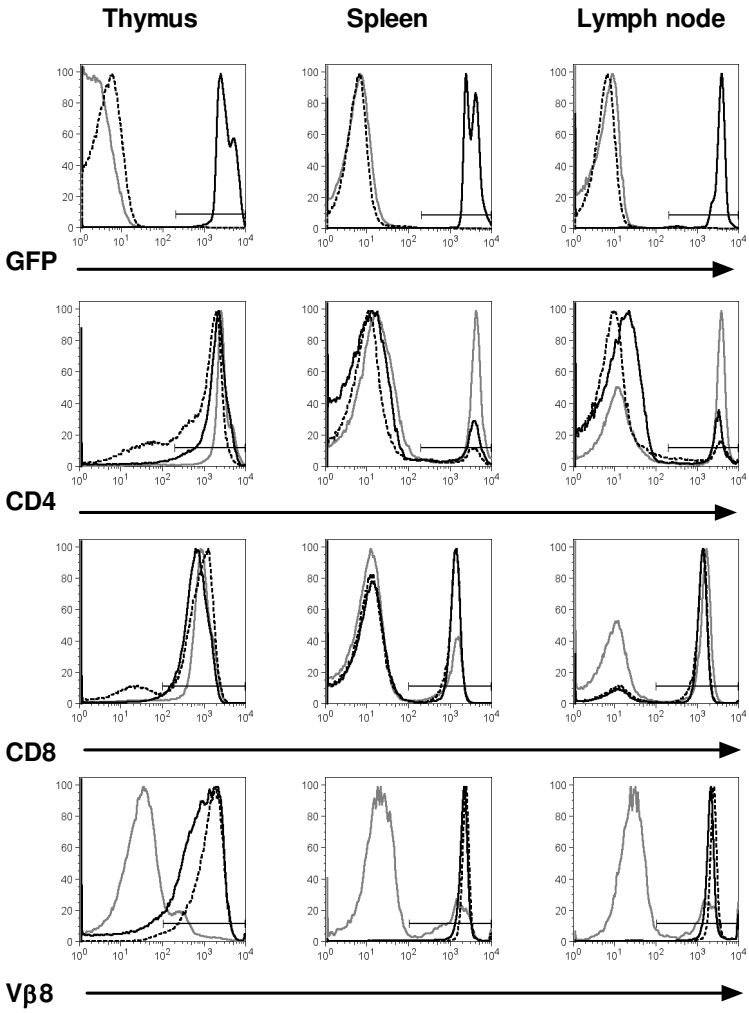

Figure 2.1: Comparison of B6, P14, and GFPxP14 phenotype. Thymocytes, splenocytes, and lymph node cells from these mice were harvested and stained for CD4, CD8, and Vβ8. Expression of these molecules and GFP on lymphocytes in these itssues was determined by flow cytometry and are shown in the histograms for B6 (gray line), P14 (dotted black line), and GFPxP14 mice (solid black line).

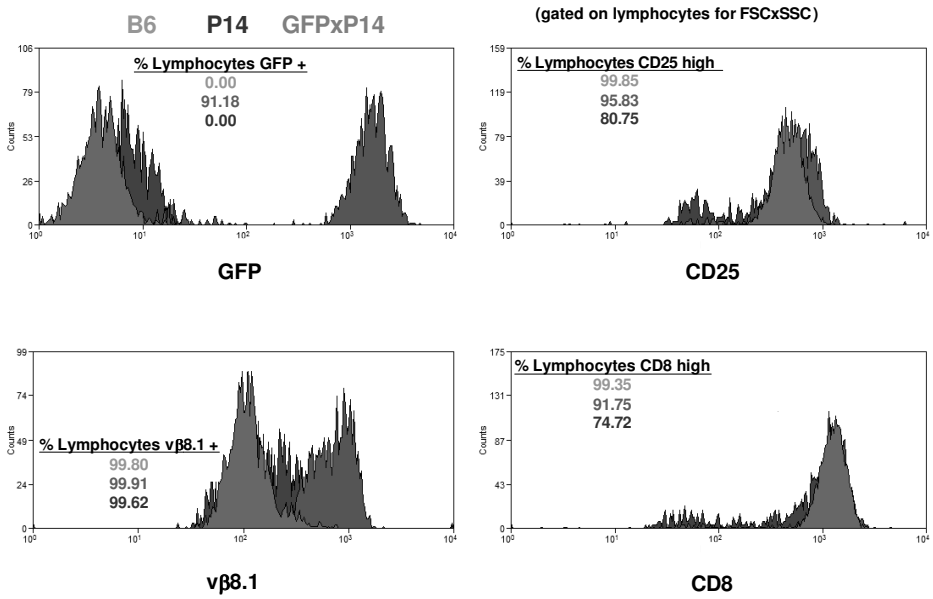

Figure 2.2: Comparison of B6, P14, and GFPxP14 splenocyte cultures. Splenocytes from B6 (light gray histogram), P14 (black histogram), and GFPxP14 (medium gray histogram) mice were stimulated with Concanavalin A for 3 days. The expression of GFP, CD25, Vβ8, and CD8 was determined by flow cytometry.

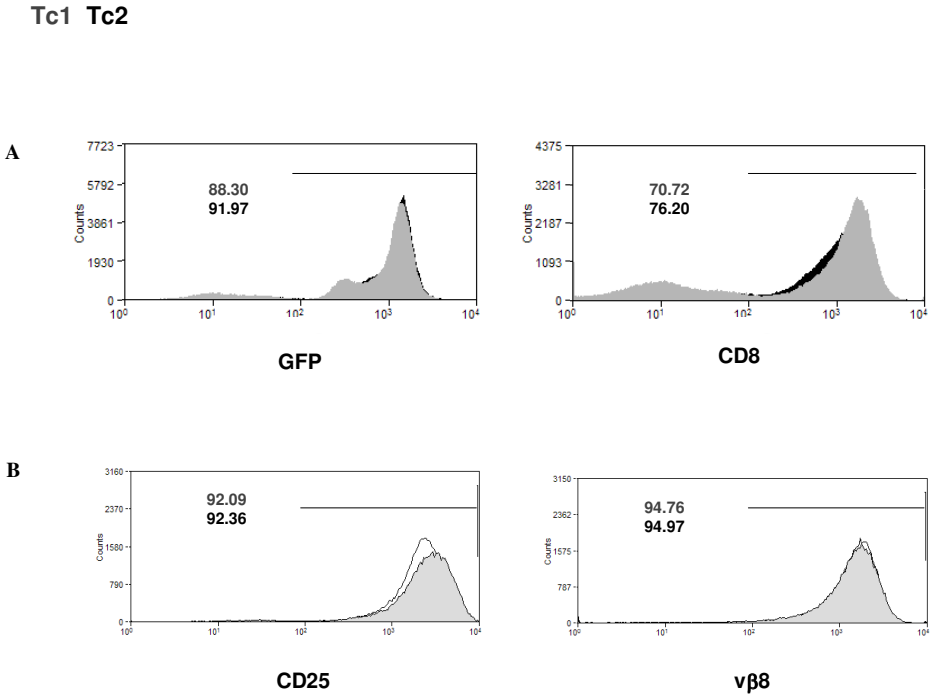

Figure 2.3: Phenotype of GFPxP14 Tc1 and Tc2 cultures. GFPxP14 mice were generated by crossing P14 and UBI.GFP mice. Splenocytes from the GFPxP14 mice were stimulated with the addition of p33 peptide and cultured in polarizing conditions for 3 days. Histograms in A and B show the expression of GFP and CD8 by lymphocytes in the culture and expression of Vβ8 and CD25 by the CD8+ cells, respectively. Cells from the Tc1 culture are shown in the filled gray histograms (A-B), while cells from the Tc2 culture are shown in the filled black (A) or open black lined (B) histograms.

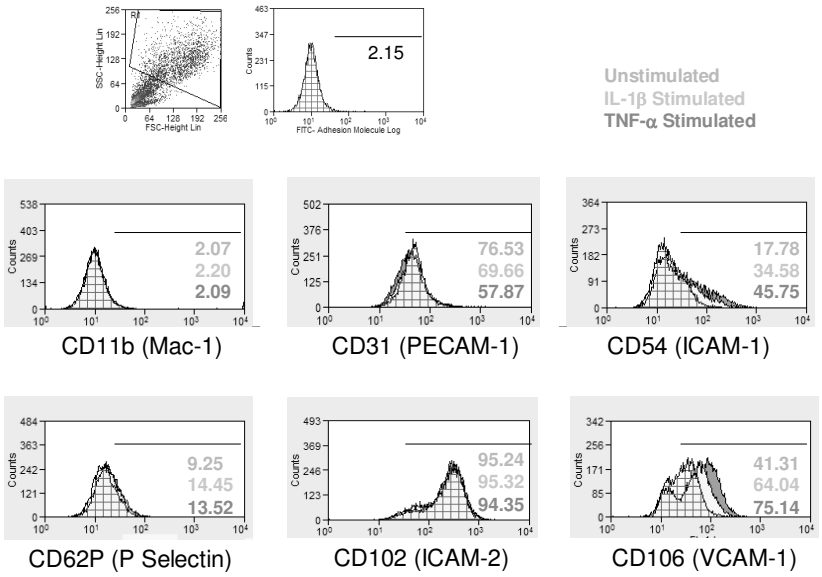

Figure 2.4: Phenotype of cytokine stimulated MS1-VEGF cells. Cultured MS1-VEGF cells were either unstimulated (cross pattern histogram, top number) or stimulated overnight with IL-1β (open histogram, middle number) or TNF-α (dark histogram, bottom number). The cells were then stained for the following before being analyzed by flow cytometry: CD11b, CD31, CD54, CD62P, CD102, and CD106. FSCxSSC and isotype control staining are shown in the first two panels.

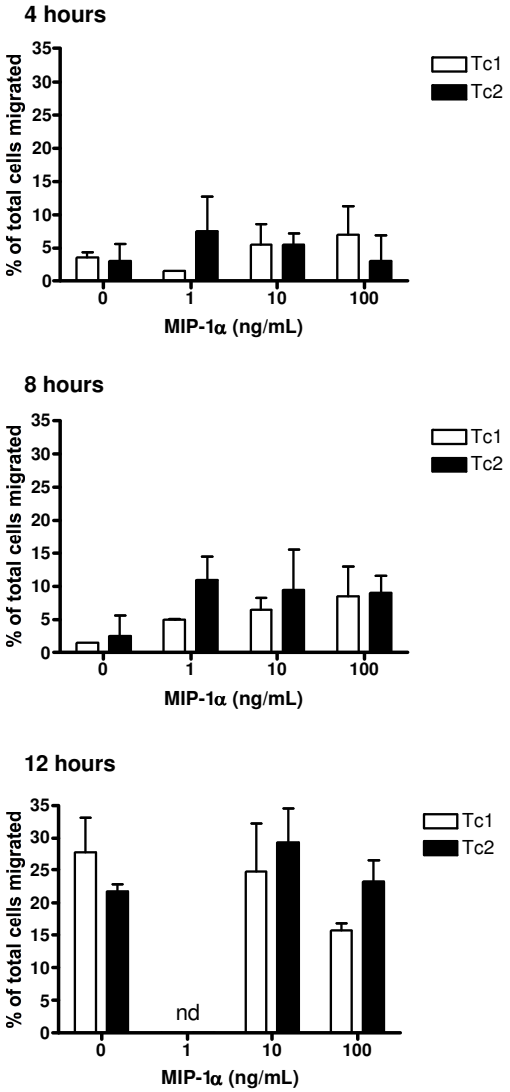

Figure 2.5: Chemotaxis of Tc1 and Tc2 cells *in vitro*. $1x10^5$ polarized GFPxP14 Tc1 or Tc2 cells were added to the upper well of transwell plates with matrigel coated filters (no endothelial cells). MIP-1α (0-100ng/mL) was added to the lower well. After 4, 8, or 12 hours, migrated cells in the lower wells was determined. *nd,* not determined.

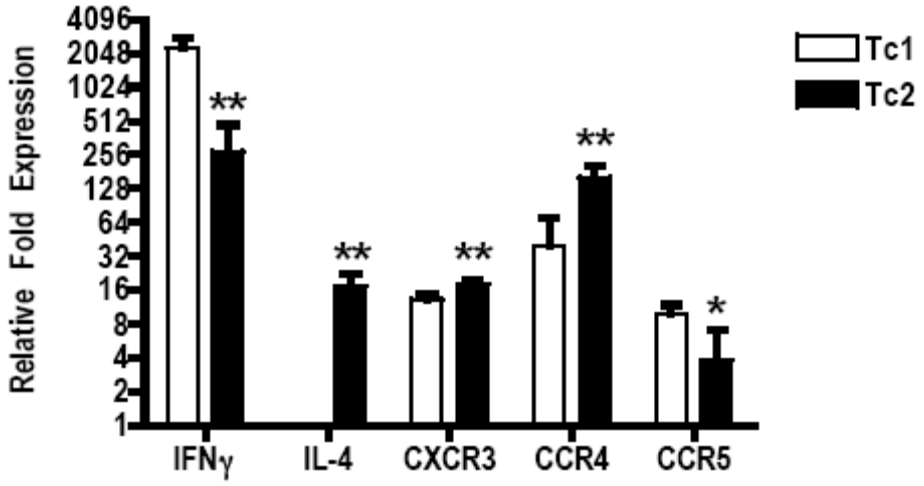

Figure 2.6- Real time RT-PCR analysis of IFNγ, IL-4, CXCR3, CCR4, and CCR5 gene expression by Tc1 and Tc2 cell cultures. mRNA from cells in GFPxP14 Tc1 and Tc2 cell cultures were harvested and gene expression was analyzed. Relative fold expression of cytokine and chemokine receptors is expressed relative to Tc1 expression of IL-4, set to a value of 1. Data shown is from one experiment, representing two experiments. $p \leq .05$, * or $p \leq .01$, ** gene expression inTc1 vs. Tc2 cells.

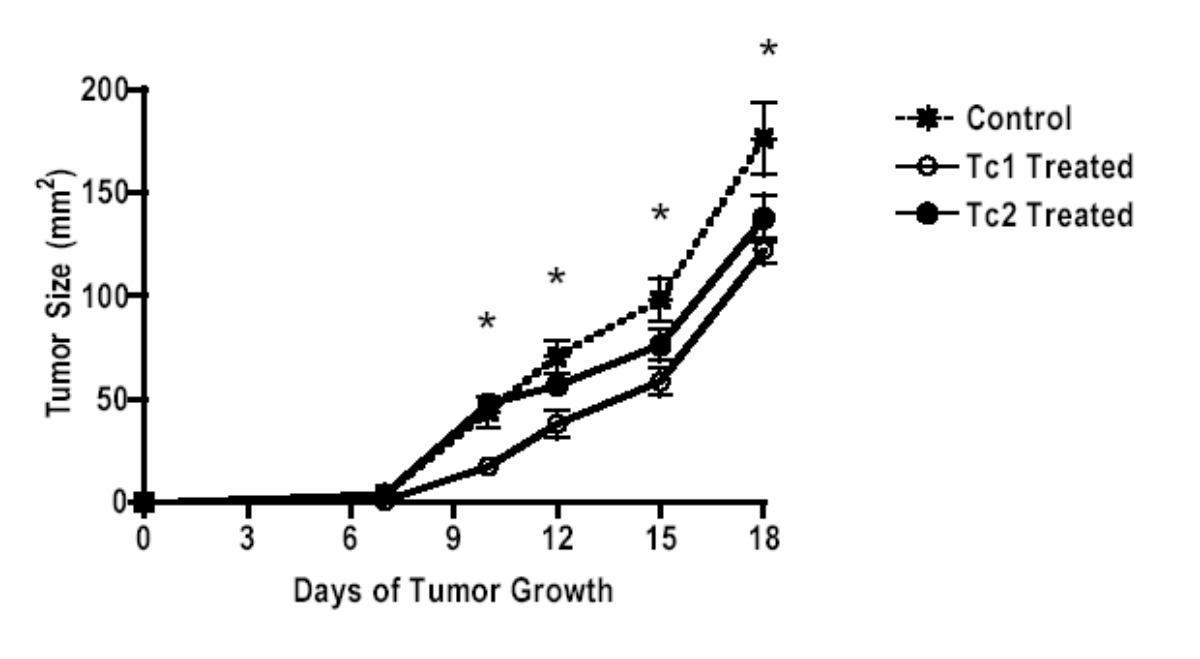

Figure 2.7: Tumor growth following transfer of Tc1 or Tc2 cells. $1x10^6$ EL4.p33 tumor cells were injected s.c. into B6 mice, followed by an injection of PBS alone or either $1x10^6$ polarized Tc1 or Tc2 cells. Tumor growth was assessed every 2-3 days. Data shown is from one experiment, representing at least two experiments. $p \leq .05$, * Tc1 vs. PBS control (days 10, 12, 15, and 18), Tc2 vs. PBS control (days 12, 15, and 18), and Tc1 vs. Tc2 (days 10, 12, 15, and 18).

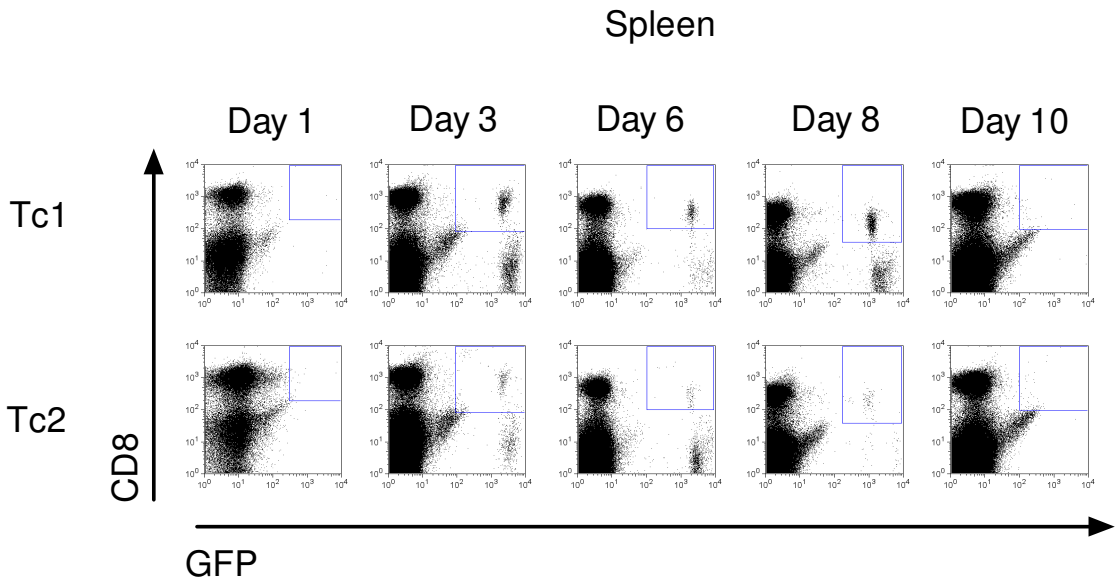

Figure 2.8: Donor cells in spleens of Tc1 or Tc2 treated tumor bearing mice.
On day 0, $1x10^6$ Tc1 (A and B) or Tc2 (C and D) cells generated from GFPxP14 splenocytes were transferred i.v. into B6 mice bearing p33.EL-4 s.c. tumors. On days 1, 3, 6, 8, and 10 after transfer, splenocytes were harvested, and the cells were stained for CD8. Lymphocytes were analyzed for GFP and CD8 expression to identify GFP^+CD8^+ donor cells using FlowJo.

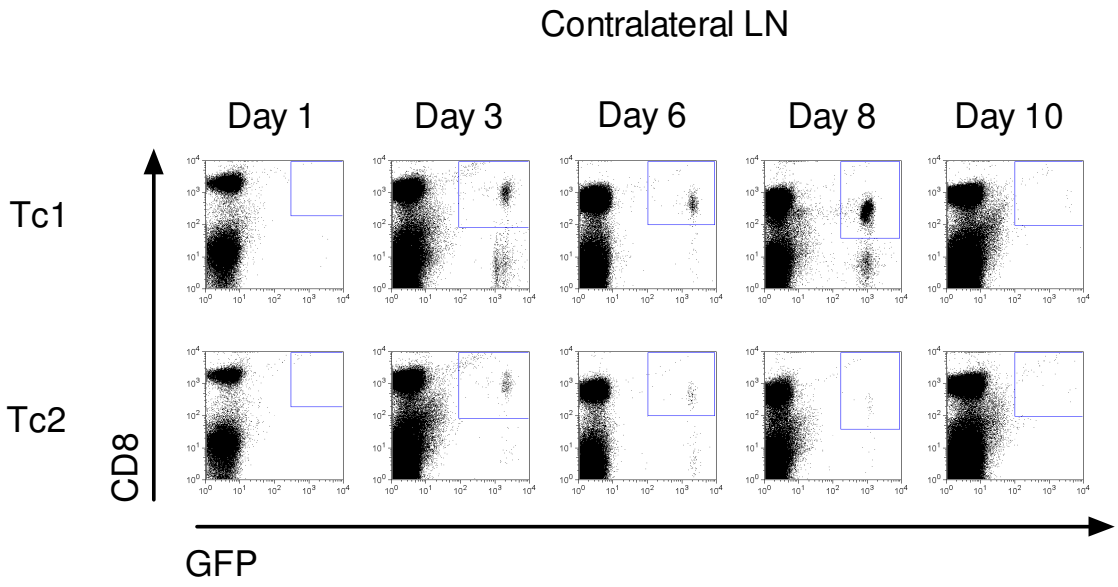

Figure 2.9: Donor cells in contralateral LNs of Tc1 or Tc2 treated tumor bearing mice. On day 0, $1x10^6$ Tc1 (A and B) or Tc2 (C and D) cells generated from GFPxP14 splenocytes were transferred i.v. into B6 mice bearing p33.EL-4 s.c. tumors. On days 1, 3, 6, 8, and 10 after transfer, the contralateral LNs were harvested, and the cells were stained for CD8. Lymphocytes were analyzed for GFP and CD8 expression to identify GFP^+CD8^+ donor cells using FlowJo.

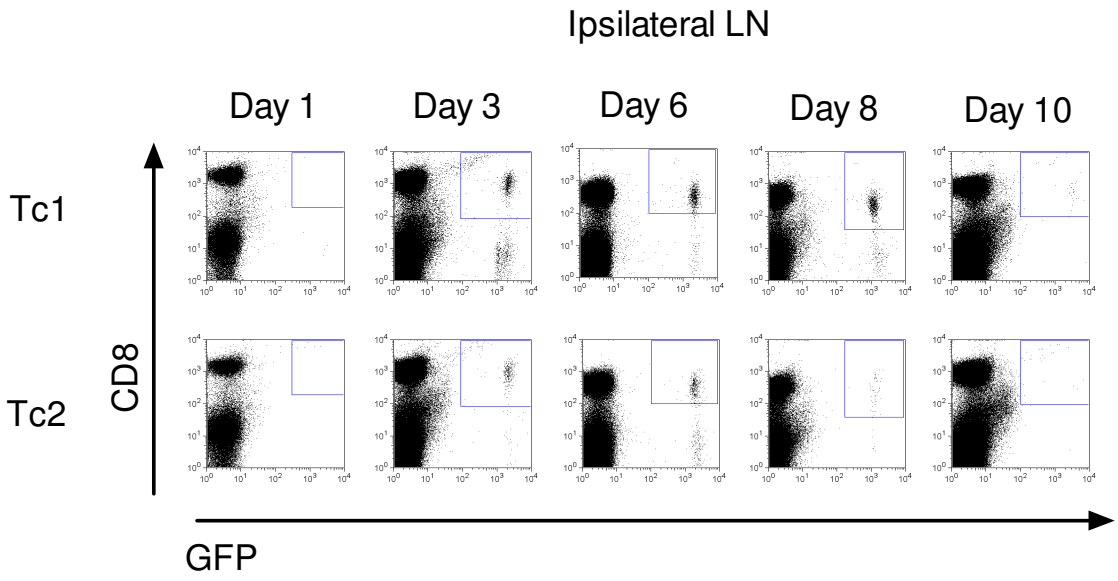

Figure 2.10: Donor cells in ipsilateral LNs of Tc1 or Tc2 treated tumor bearing mice. On day 0, $1x10^6$ Tc1 (A and B) or Tc2 (C and D) cells generated from GFPxP14 splenocytes were transferred i.v. into B6 mice bearing p33.EL-4 s.c. tumors. On days 1, 3, 6, 8, and 10 after transfer, ipsilateral LNs were harvested, and the cells were stained for CD8. Lymphocytes were analyzed for GFP and CD8 expression to identify GFP^+CD8^+ donor cells using FlowJo.

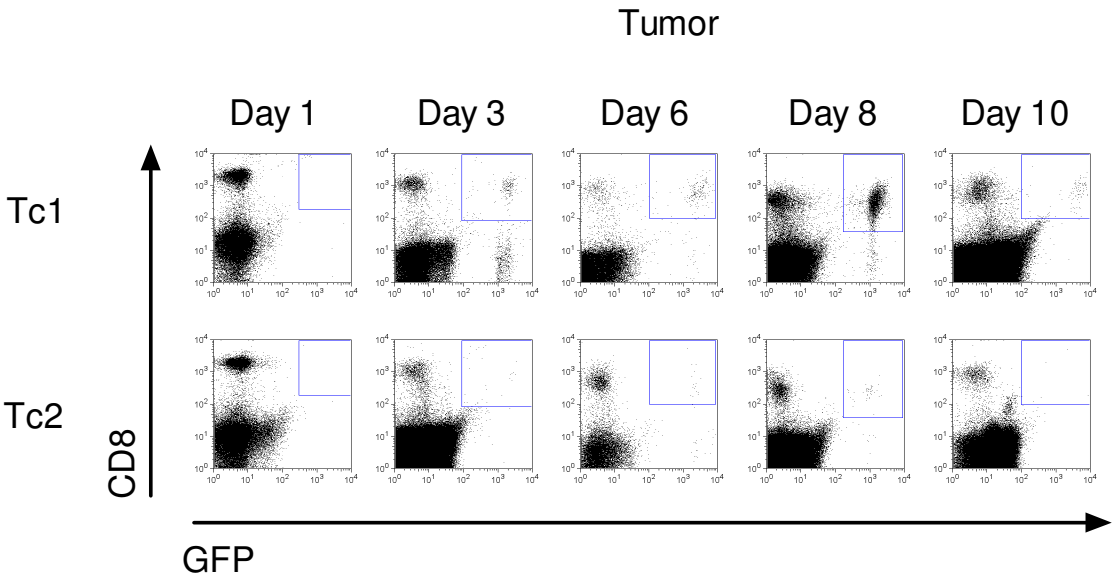

Figure 2.11: Donor cells in tumors of Tc1 or Tc2 treated tumor bearing mice.
On day 0, $1x10^6$ Tc1 (A and B) or Tc2 (C and D) cells generated from GFPxP14 splenocytes were transferred i.v. into B6 mice bearing p33.EL-4 s.c. tumors. On days 1, 3, 6, 8, and 10 after transfer, tumors were harvested, and the cells were stained for CD8. Lymphocytes were analyzed for GFP and CD8 expression to identify GFP^+CD8^+ donor cells using FlowJo.

References

1. Cerwenka A, Carter LL, Reome JB, Swain SL, Dutton RW. *In vivo* persistence of CD8 polarized T cell subsets producing type 1 or type 2 cytokines. J Immunol. 1998 Jul 1;161(1):97-105.

2. Dobrzanski MJ, Reome JB, Dutton RW. Therapeutic effects of tumor-reactive type 1 and type 2 $CD8^+$ T cell subpopulations in established pulmonary metastases. J Immunol. 1999 Jun 1;162(11):6671-80.

3. Dobrzanski MJ, Reome JB, Dutton RW. Type 1 and type 2 $CD8^+$ effector T cell subpopulations promote long-term tumor immunity and protection to progressively growing tumor. J Immunol. 2000 Jan 15;164(2):916-25.

4. Helmich BK, Dutton RW. The role of adoptively transferred CD8 T cells and host cells in the control of the growth of the EG7 thymoma: factors that determine the relative effectiveness and homing properties of Tc1 and Tc2 effectors. J Immunol. 2001 Jun 1;166(11):6500-8.

5. Kemp RA, Ronchese F. Tumor-specific Tc1, but not Tc2, cells deliver protective antitumor immunity. J Immunol. 2001 Dec 1;167(11):6497-502.

6. Chamoto K, Kosaka A, Tsuji T, Matsuzaki J, Sato T, Takeshima T, Iwakabe K, Togashi Y, Koda T, Nishimura T. Critical role of the Th1/Tc1 circuit for the generation of tumor-specific CTL during tumor eradication *in vivo* by Th1-cell therapy. Cancer Sci. 2003 Oct;94(10):924-8.

7. Chang ML, Chen YT, Su YC, Kung JT. Cytotoxic T lymphocytes generated by short-term *in vitro* TCR stimulation in the presence of IL-4 are therapeutically effective against B16 melanoma. J Biomed Sci. 2003;10(6 Pt 1):644-50.

8. Dobrzanski MJ, Reome JB, Hollenbaugh JA, Hylind JC, Dutton RW. Effector cellderived lymphotoxin alpha and Fas ligand, but not perforin, promote Tc1 and Tc2 effector cell-mediated tumor therapy in established pulmonary metastases. Cancer Res. 2004 Jan 1;64(1):406-14.

9. Dobrzanski MJ, Reome JB, Hollenbaugh JA, Dutton RW. Tc1 and Tc2 effector cell therapy elicit long-term tumor immunity by contrasting mechanisms that result in complementary endogenous type 1 antitumor responses. J Immunol. 2004 Feb 1;172(3):1380-90.

10. Do JS, Choi YH, Shin SH, Yi HK, Hwang PH, Nam SY. Committed memory effector type 2 cytotoxic T (Tc2) cells are ineffective in protective anti-tumor immunity. Immunol Lett. 2004 Aug 15;95(1):77-84.

11. Nishimura F, Dusak JE, Eguchi J, Zhu X, Gambotto A, Storkus WJ, Okada H. Adoptive transfer of type 1 CTL mediates effective anti-central nervous system tumor response: critical roles of IFN-inducible protein-10. Cancer Res. 2006 Apr 15;66(8):4478-87.

12. Hollenbaugh JA, Dutton RW. IFN-gamma regulates donor CD8 T cell expansion, migration, and leads to apoptosis of cells of a solid tumor. J Immunol. 2006 Sep 1;177(5):3004-11.

13. Dobrzanski MJ, Reome JB, Hylind JC, Rewers-Felkins KA. CD8-mediated type 1 antitumor responses selectively modulate endogenous differentiated and nondifferentiated T cell localization, activation, and function in progressive breast cancer. J Immunol. 2006 Dec 1;177 (11):8191-201.

14. Siveke JT, Hamann A. T helper 1 and T helper 2 cells respond differentially to chemokines. J Immunol. 1998 Jan 15;160(2):550-4.

15. Katakai T, Hara T, Sugai M, Gonda H, Nambu Y, Matsuda E, Agata Y, Shimizu A. Chemokine-independent preference for T-helper-1 cells in transendothelial migration. J Biol Chem. 2002 Dec 27;277(52):50948-58.

16. Marelli-Berg FM, Frasca L, Weng L, Lombardi G, Lechler RI. Antigen recognition influences transendothelial migration of $CD4^+$ T cells. J Immunol. 1999 Jan 15;162(2):696-703.

17. Marelli-Berg FM, Scott D, Bartok I, Peek E, Dyson J, Lechler RI. Activated murine endothelial cells have reduced immunogenicity for $CD8^+$ T cells: a mechanism of immunoregulation? J Immunol. 2000 Oct 15;165(8):4182-9.

18. Weninger W, Manjunath N, von Andrian UH. Migration and differentiation of $CD8^+$ T cells. Immunol Rev. 2002 Aug;186:221-33. Review.

19. Schaefer BC, Schaefer ML, Kappler JW, Marrack P, Kedl RM. Observation of antigen-dependent CD8+ T-cell/ dendritic cell interactions *in vivo*. Cell Immunol 2001 Dec 15;214(2):110-22.

20. Pircher H, Michalopoulos EE, Iwamoto A, et al. Molecular analysis of the antigen receptor of virus-specific cytotoxic T cells and identification of a new V alpha family. Eur J Immunol 1987 Dec;17(12):1843-6.

21. Colvin BL, Lau AH, Schell AM, Thomson AW. Disparate ability of murine CD8alpha- and CD8alpha+ dendritic cell subsets to traverse endothelium is not determined by differential CD11b expression. Immunology. 2004 Nov;113(3):328-3722.

22. Qian, L, Lorena, S, Burgents, M, and Flood, PM. Increased CD8+ T cell Antigenicity of Murine Tumors Increases Tumor Resistance. Manuscript in preparation.

23. Mottez E, Langlade-Demoyen P, Gournier H, et al. Cells expressing a major histocompatibility complex class I molecule with a single covalently bound peptide are highly immunogenic. J Exp Med 1995 Feb 1;181(2):493-502.

24. Uger RA, Barber BH. Creating CTL targets with epitope-linked beta 2-microglobulin constructs. J Immunol. 1998 Feb 15;160(4):1598-605.

25. White J, Crawford F, Fremont D, Marrack P, Kappler J. Soluble class I MHC with beta2-microglobulin covalently linked peptides: specific binding to a T cell hybridoma. J Immunol 1999 Mar 1;162(5):2671-6.

26. Uger RA, Chan SM, Barber BH. Covalent linkage to beta2-microglobulin enhances the MHC stability and antigenicity of suboptimal CTL epitopes. J Immunol 1999 May 15;162(10):6024-8.

27. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}{}_{T}$ method. Methods 2001 Dec; 25: 402-408.

28. Manjunath N, Shankar P, Stockton B, Dubey PD, Lieberman J, von Andrian UH. A transgenic mouse model to analyze CD8(+) effector T cell differentiation *in vivo*. Proc Natl Acad Sci U S A. 1999 Nov 23;96(24):13932-7.

29. Woodland DL, Dutton RW. Heterogeneity of CD4($^{+}$) and CD8($^{+}$) T cells. Curr Opin Immunol. 2003 Jun;15(3):336-42. Review.

30. Cerwenka A, Morgan TM, Harmsen AG, Dutton RW. Migration kinetics and final destination of type 1 and type 2 CD8 effector cells predict protection against pulmonary virus infection. J Exp Med. 1999 Jan 18;189(2):423-34.

31. Gough M, Crittenden M, Thanarajasingam U, Sanchez-Perez L, Thompson J, Jevremovic D, Vile R. Gene therapy to manipulate effector T cell trafficking to tumors for immunotherapy. J Immunol. 2005 May 1;174(9):5766-73.

32. Burgents ML, Flood PM. Tc1 Cells Infiltrate Tumor Sites More Efficiently than Tc2 Cells Due to Enhanced Expression of a Tumor Infiltrating Phenotype. Submitted.

33. Hensbergen PJ, Wijnands PG, Schreurs MW, Scheper RJ, Willemze R, Tensen CP. The CXCR3 targeting chemokine CXCL11 has potent antitumor activity *in vivo* involving attraction of CD8+ T lymphocytes but not inhibition of angiogenesis. J Immunother (1997). 2005 Jul-Aug;28(4):343-51.

Chapter III
Tc1 Cells Infiltrate Tumor Sites More Efficiently than Tc2 Cells Due to Enhanced Expression of a Tumor Infiltrating Phenotype*

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Abstract

Tc1 cells have been shown to be more effective than Tc2 T cells for adoptive cell therapy against tumors. Differences in migration and tumor infiltration between Tc1 and Tc2 cells may contribute to this difference in therapeutic efficacy. In this study we investigated migration and tumor infiltration of Tc1 and Tc2 *in vivo*. We generated donor cells from green fluorescent protein expressing TCR transgenic mice (specific for lymphocytic choriomeningitis virus p33 antigen) and investigated the migration and tumor infiltration of these cells in mice bearing p33 antigen-negative and p33 antigen-positive tumors. We report that Tc1 cells migrate to TDLNs and infiltrate tumor sites faster and in higher numbers than Tc2 cells following injection, regardless of tumor p33 antigen expression. *In vitro* and in TDLNs, Tc1 cells express significantly higher levels of several adhesion molecules important for T cell migration compared to Tc2 cells, specifically CD62L, LFA-1, CD44, and PSGL-1. However, we found that all host and donor $CD8^+$ cells infiltrating tumors in Tc1 or Tc2 treated mice uniformly expressed high levels of LFA-1, CD44, and PSGL-1, regardless of tumor expression of p33 antigen. These results suggest that there is a tumor infiltrating phenotype required for $CD8^+$ T cells to migrate to and infiltrate tumor sites; and the higher expression of this phenotype by Tc1 cells compared to Tc2 cells contributes to enhanced efficiency of Tc1 vs. Tc2 cell migration to TDLNs and infiltration of tumor sites.

Introduction

$CD8^+$ T cells are classified as either type I or type II based on their cytokine secretion profile (1). While Tc1 cells secrete large amounts of IFN-γ, Tc2 cells, in addition to a limited amount of IFN-γ, secrete predominantly IL-4, IL-5, and IL-10 (1-6). Studies have evaluated the effectiveness of using *in vitro* generated Tc1 and Tc2 cells for tumor therapy in order to determine if transferred Tc1 or Tc2 cells provide protection against tumor growth or promote tumor rejection in tumor-bearing mice (7-18). In a number of these studies, both Tc1 and Tc2 transferred cells were effective in protection against tumor growth, although several studies showed that Tc1 therapy was more effective than Tc2 therapy (7-18). Several studies suggested that a possible reason for this difference in therapeutic efficacy was differential migration of Tc1 vs. Tc2 cells *in vivo*, since these studies found more Tc1 cells in TDLNs or tumors compared to Tc2 cells after transfer (9, 16). Conversely, results by others showed that there is no difference between the number of Tc1 and Tc2 cells that migrate to TDLNs or tumors following injection (10). Therefore, the efficacy of Tc1 vs. Tc2 cell tumor therapy and the efficiency of Tc1 vs. Tc2 cell migration to TDLN and tumor sites are still unclear.

Adhesion molecules such as selectins, integrins, and their ligands play an important role in $CD8^+$ T cell migration to lymphoid tissues and infiltration of tumor sites (19-21). Molecules such as CD62L, PSGL-1, LFA-1, and CD44 have previously been identified as adhesion molecules which play a critical role in T cells crossing activated endothelium (22-25), and several reports suggest they are important for strong anti-tumor responses by transferred T cells (26-31).

In addition to adhesion molecules, antigen expression by tumor cells has also been shown to regulate $CD8^+$ T cell migration and tumor infiltration *in vivo* (32-35). A recent imaging study showed that more antigen-specific $CD8^+$ T cells were found in the antigen-positive tumor compared to the antigen-negative tumor within the same animal (35). Furthermore, these $CD8^+$ T cells infiltrated further into the tumor mass and were more evenly distributed throughout the antigen-positive tumor (35). Although studies have suggested an important role for antigen in $CD8^+$ T cell migration and tumor infiltration, the effect of antigen on the migration and tumor infiltration of polarized $CD8^+$ T cells is still unknown.

In this study, we utilized GFP expression to track donor Tc1 and Tc2 cells *in vivo* during tumor therapy. We evaluated differences in migration to TDLNs, infiltration of established antigen-positive vs. antigen-negative tumors, and expression of adhesion molecules by Tc1 and Tc2 cells *in vitro* and *in vivo*. In this study, we report that Tc1 cells express a higher level of adhesion molecules than Tc2 cells *in vitro* following activation and in the TDLNs 3 days following transfer *in vivo*. Importantly, the adhesion molecule phenotype of the tumor infiltrating Tc1 and Tc2 cells is similar, namely PSGL-1 high, LFA-1 high, and CD44 high, The adhesion molecule phenotype of Tc1 and Tc2 cells was identical in antigen-positive vs. antigen-negative tumor sites, despite that antigen does enhance the number of Tc1 and Tc2 cells found within the tumor sites. Overall, this study suggests that enhanced expression of adhesion molecules defines a tumor infiltrating phenotype for both Tc1 and Tc2 cells, but different expression levels of these adhesion molecules following *in vitro* activation contributes to more abundant migration and tumor infiltration by Tc1 vs. Tc2 cells *in vivo*.

Materials & Methods

Mice

C57BL/6J (B6) and UBI.GFP mice were obtained from The Jackson Laboratory (Bar Harbor, Maine) (36). $CD8^+$ T cell Vβ8 TCR transgenic P14 mice specific for LCMV peptide p33-41 (KAVYNFATC) in context of $H\text{-}2D^b$ were originally obtained from Dr. Pam Ohashi (37). GFPxP14 mice were generated in our lab by crossing the UBI.GFP strain with the P14 strain and using the F1 generation (See Chapter 2). Spleens of GFPxP14 mice were used as the source for our donor cells, as detailed below. B6 mice were used as recipient animals. Female mice aged 8-12 weeks of age were used throughout these experiments. All mice were maintained in specific pathogen free conditions by University of North Carolina's Department of Laboratory Animal Medicine and all animal procedures were approved by the university's Institutional Animal Care and Use Committee.

Tc1/Tc2 cultures

Tc1 and Tc2 cells were generated *in vitro* from splenocytes harvested from GFPxP14 mice and stimulated at $5x10^6$ cells/mL for 3 days with p33 peptide (KAVYNFATC) along with 2 units/mL rIL-2 in RPMI medium. For generating Tc1 cells, we also added rIL-12 at 12.5ng/mL and anti-IL-4 antibody at 2.5ng/mL, and for generating Tc2 cells we added rIL-4 at 27.5ng/mL, anti-IL-12 antibody at 5.5ng/mL, and anti-IFN-γ antibody at 5.5ng/mL. Recombinant murine cytokines and anti-murine cytokine antibodies were all obtained from R&D Systems. Tc1 and Tc2 cultures were both over 90% $GFP^+CD8^+V\beta8^+$ after three days of culture, as determined by flow cytometry. Where noted polarized Tc1 or Tc2 cells were

restimulated in culture with peptide in identical polarizing conditions for an additional 3 days before analysis. Functional studies confirmed that Tc1 and Tc2 cells generated using this protocol proliferate at the same rate and specifically kill p33-loaded targets with equal efficiency (38). Also, we confirmed type-I and type-II cytokine cand chemokine receptor gene expression in Tc1 and Tc2 cells generated using this protocol (Figure 2.6).

Tumor cell lines and construction of p33 encoding plasmid

As a source of antigen-bearing tumor cells, we generated LCMV peptide p33 -expressing EL-4 thymoma tumor cells (EL-4.p33β2M cells) (38). For simplicity, EL-4.p33β2M cells are referred to as "p33.EL-4" cells in this publication. Briefly, EL-4 cells obtained from ATCC were transfected with a PcDNA3.0 plasmid (Invitrogen) containing an insert encoding the LCMV peptide p33 linked to human beta-2 Microglobulin (38). Linking peptides to human β2 microglobulin has been shown to enhance the peptide presentation in transfected cells, evidenced by increased specific lysis compared to target cells transfected with peptide alone (39-42). P33.EL-4 tumor cells were selected and maintained using medium containing G418 (Sigma-Aldrich). Transfection was confirmed by PCR and expression of the p33 antigen was confirmed by using the transfected cells as targets of P14 T cells in cytolytic assays. Both parental EL-4 (p33 antigen-negative) cells and transfected p33.EL-4 (p33 antigen-positive) cells were used in this study, as detailed below.

Migration and Tumor Infiltration of Tc1 and Tc2

5 or 7 days prior to donor cell transfer, B6 mice were injected s.c. in the right ventral flank with $1x10^6$-$1.5x10^6$ p33.EL-4 tumor cells. For experiments examining the role of antigen, $1x10^6$-$1.5x10^6$ EL-4 tumor cells were also injected into the mice but into the left ventral flank on the same day. On day 0, $1x10^6$ Tc1 or $1x10^6$ Tc2 donor cells in 200uL of PBS were injected i.v. into the tail vein of these mice. On days 3 and 7 after donor cell transfer, TDLNs (axillary and superficial inguinal lymph nodes) and tumors were collected then analyzed by flow cytometry. Four animals were used per treatment group for each time point in each experiment.

Flow cytometry analysis of surface molecule expression

For cell surface expression analysis, cultured cells or lymph node and tumor tissues were harvested. Tc1 and Tc2 cell cultures were harvested after one or two stimulations. TDLNs and tumors were harvested on days indicated and single-cell suspensions were made. Cells were then stained using monoclonal antibodies (Pharmingen) against the following cell surface markers and adhesion molecules important for $CD8^+$ T cell trafficking: CD8, Vβ8, CD25, LFA-1, CD44, CD49d, CD62L, PSGL-1, and $\alpha_4\beta_7$ integrin (19-31). $1x10^5$ or $5x10^5$ cells were analyzed for TDLN and tumor samples, respectively, to allow for comparing lymphocyte and total $CD8^+$ cell numbers between samples. Lymphocytes were gated based on forward and side scatter. Donor and host cells in TDLNs and tumors were identified by gating for GFP. The mean fluorescence intensity (MFI) of surface molecule expression was determined in addition to the percent of cells positive for the surface marker. All flow

cytometry samples were run on a FACSCalibur (BD Bioscience) and analyzed using FlowJo software (Treestar).

Statistical Analysis

Experiments were repeated at least twice and data from one representative experiment is shown for each figure. Two-tailed Student's T-test was utilized to test for significance with $p \leq .05$ considered significant.

Results

Differential Tc1 vs. Tc2 migration to draining lymph nodes and tumor infiltration in vivo

We sought to examine Tc1 and Tc2 cell migration and tumor infiltration into established p33.EL-4 and EL-4 tumors using GFP expression to track the donor cells. UBI.GFP mice (36) were crossed with P14 mice (37) to generate a mouse strain, GFPxP14, whose $CD8^+$ T cells express both GFP and the p33-specific TCR from P14 transgenic mice. The GFP^+ cells from these splenocyte cultures are over 90% $CD8^+$, $CD25^+$, and $V\beta8^+$ (Figure 2.3). Tc1 and Tc2 cells were generated from GFPxP14 splenocytes in 3 day cultures using polarizing conditions, and then the expression of IFN-γ and IL-4 cytokines as well as CXCR3, CCR4, and CCR5 chemokine receptors was examined using real time RT-PCR (Figure 2.6). CXCR3 was expressed similarly by both cultures. Tc1 cells expressed high levels of IFN-γ and CCR5 mRNA expression but low levels of CCR4 with minimal expression of IL-4 mRNA, indicative of type I polarization (Figure 2.6). Tc2 cultures exhibited high levels of IL-4 and CCR4 mRNA expression, but relatively low expression of IFN-γ and CCR5 mRNA, indicative of type II polarization (Figure 2.6). We also confirmed that these *in vitro* generated Tc1 cells showed significantly higher therapeutic efficacy in reducing p33.EL-4 tumor growth than an identical number of *in vitro* generated Tc2 cells injected i.v., while showing no effect on the growth of parental EL-4 cells (Figure 2.7).

Tc1 and Tc2 donor cell migration to TDLNs and infiltration of tumor sites was evaluated using our p33.EL-4 tumor model. P33.EL-4 tumors were established in B6 mice by injection of $1x10^6$ tumor cells s.c. in the right ventral flank of animals 7 days before the injection of *in vitro* generated Tc1 or Tc2 cells. This dose established palpable tumors within 7 days. On day 0, the animals were injected i.v. with either $1x10^6$ Tc1 or Tc2 donor cells.

Then, on days 1, 3, and 7 after therapy, lymph node and tumor tissues were harvested, and GFP^+ donor and GFP- host cells were examined by flow cytometry. A limited number of GFP^+CD8^+ cells were found in the TDLNs and at the tumor site in some of the Tc1 treated animals, but not in Tc2 treated animals, on day 1 following transfer (Figures 2.10 and 2.11). By day 3, a measurable number of GFP^+CD8^+ cells were found in both Tc1 and Tc2 treated mice (Figure 3.1). More importantly, there were significantly more donor cells in the TDLNs and tumors of Tc1 treated mice compared to Tc2 treated mice on both day 3 and day 7 after transfer (Figure 3.1). The numbers of lymphocytes and $CD8^+$ cells in Tc1 treated mice found in the TDLN or the tumor site were identical to the number found in the same sites of Tc2 treated mice on either day 3 or day 7, demonstrating that animals treated with Tc1 or Tc2 therapy had similar numbers of total $CD8^+$ T cell migration to TDLN and tumor infiltration (Figure 3.1).By day 7, there were more total lymphocytes and total $CD8^+$ cells in both TDLNs and tumor sites than on day 3, and the percentage of GFP^+ Tc1 and Tc2 donor cells increased in both the TDLNs and the tumors sites when compared to the total lymphocyte population found in the TDLNs and tumor sites (Figure 3.1). The number of both Tc1 and Tc2 donor cells began to significantly decline after day 7 following transfer (Figure 2.11). These results demonstrate that injection of donor Tc1 or Tc2 cells results in their migration to both the TDLN and the tumor site; and the increase in the percentages of GFP^+ Tc1 and Tc2 donor cells from day 3 to day 7 were not due to a decrease in overall lymphocyte or $CD8^+$ cells found at that site. These results also suggest that the higher percentage of Tc1 vs. Tc2 donor cells on both day 3 and day 7 is due to a higher number of Tc1 cells within the $CD8^+$ population, rather than increased lymphocyte or total $CD8^+$ cells in Tc1 treated animals. Therefore, it appears that Tc1 cells may migrate more rapidly and in

greater numbers to both TDLN and to tumor sites than Tc2 cells following transfer into tumor-bearing mice.

Tc1 and Tc2 exhibit a different adhesion phenotype following in vitro activation and in TDLN but not in tumor infiltrates

We examined whether differences between Tc1 and Tc2 cell migration and tumor infiltration above were due to differences in adhesion molecule expression by Tc1 and Tc2 cells. Monoclonal antibodies to adhesion molecules LFA-1, CD44, CD49d, CD62L, PSGL-1, and $\alpha_4\beta_7$ integrin were used to phenotype *in vitro* generated Tc1 and Tc2 cells before transfer and at various times after transfer in the TDLN and tumor mass. GFPxP14 splenocytes were stimulated with peptide and cultured for 3 days in polarizing conditions, then analyzed or restimulated for 3 additional days before analysis by flow cytometry. Tc1 and Tc2 cells exhibit different profiles of adhesion molecules following one (Figure 3.2A) and two (Figure 3.2B) stimulations *in vitro*. In particular, it was found that after primary *in vitro* polarization, the level of CD62L and PSGL-1 expression is higher on Tc1 cells than on Tc2 cells, while both Tc1 and Tc2 cells expressed comparably high levels of CD44 and LFA-1 (Figure 3.2A). More significant differences in Tc1 and Tc2 cell phenotypes were found following a second round of *in vitro* polarization, where Tc1 cells expressed higher levels of LFA-1, CD44, CD62L and PSGL-1 (Figure 3.2B), as well as even stronger polarization to the Tc1 or Tc2 phenotype (data not shown). In contrast, CD49d and $\alpha_4\beta_7$ integrin were only minimally expressed by both Tc1 and Tc2 cells, even after the second stimulation (Figure 3.2B). Since Tc1 and Tc2 cells express similar levels of CD25 after both primary and secondary stimulation, the level of activation does not appear to contribute to the observed differences in migration and tumor infiltration (Figure 3.2).

In order to evaluate whether the difference in adhesion phenotype between Tc1 and Tc2 donor cells was maintained *in vivo* and results in differential migration of these cells to lymphoid and tumor sites, we examined Tc1 and Tc2 cells from the TDLNs and tumors of treated mice on days 3 and 7 after injection. P33.EL-4 tumor bearing mice were injected with $1x10^6$ Tc1 or Tc2 donor cells generated *in vitro*. TDLNs and tumors were harvested from the mice and the cells were examined for their expression of Vβ8, CD25, LFA-1, CD44, CD62L, and PSGL-1 by flow cytometry. When we examined donor cell phenotypes in the TDLN (Figure 3.3), we found that on day 3 Tc1 cells had increased expression of CD62L, CD44, PSGL-1, and LFA-1. However on day 7, only the difference in CD62L remained significant (Figure 3.3). Our results demonstrate that the MFI of CD44, LFA-1, and PSGL-1 adhesion molecules was significantly higher on donor Tc1 cells than Tc2 cells on day 3 (Figure 3.3A), while on day 7 these differences in expression levels between Tc1 and Tc2 cells had disappeared (Figure 3.3B). However, the MFI of CD62L expression was significantly higher on Tc1 cells on both day 3 and day 7 (Figures 3.3A and 3.3B) when compared to Tc2 cells, suggesting that the higher level of CD62L expression by Tc1 cells could contribute to the higher number of Tc1 cells migrating to TDLNs compared to Tc2 cells. When activation status for these Tc1 and Tc2 cells was examined, CD25 expression on donor cells was low/moderate and Vβ8 expression high (data not shown). Therefore, the overall adhesion phenotype profile of Tc1 vs. Tc2 cells suggests that only a subset of Tc2 cells have the appropriate expression of adhesion molecules to effectively home to the TDLNs following injection.

We also examined the phenotype of donor cells which had infiltrated into the tumor mass on days 3 and 7 following injection. In contrast to their phenotype in TDLN, the

phenotype of both Tc1 and Tc2 cells which had infiltrated the tumors were identical on day 3 as well as day 7 after injection: They expressed very high levels of CD44, LFA-1, and PSGL-1 (Figure 3.4) and moderate levels of CD25 and high Vβ8 (data not shown). When we analyzed the phenotype of the host $CD8^+$ cells in the tumors of Tc1 and Tc2 treated mice, we found that likewise, these cells expressed the same "tumor infiltrating phenotype": LFA-1 high, CD44 high, and PSGL-1 high (data not shown). This data supports that in order for cells to infiltrate a tumor, they must express a high level of certain adhesion molecules necessary for transendothelial migration, and that a much smaller number of Tc2 cells express the appropriate phenotype needed to infiltrate into tumors when compared to Tc1 cells. Therefore, it appears that the ability of Tc1 cells to migrate to tumor sites more efficiently than Tc2 cells may be due to their inherent ability to express higher levels of adhesion molecules critical for tumor infiltration.

The role of antigen in Tc1 and Tc2 migration and tumor infiltration

Presentation of antigen by tumor cells and/or APC and the recognition of this antigen by antigen-specific T cells plays an important role in regulating $CD8^+$ T cell trafficking (32-35), so we evaluated the migration to and infiltration of antigen-positive and antigen negative tumors by p33-specific Tc1 vs. Tc2 cells. B6 mice were injected s.c. with EL-4 (left flank) and p33.EL-4 (right flank) tumor cells and after 7 days, Tc1 or Tc2 donor cells were injected i.v. and TDLNs and tumor infiltration were analyzed by flow cytometry. As expected, there were a larger number of both Tc1 and Tc2 cells in the p33.EL-4 tumor compared to the EL-4 tumor on day 3, while we found that by day 7, the number of Tc1 cells infiltrating the EL-4 tumor was equivalent to the number found in the p33.EL-4 tumors (Figure 3.5). We also

found that there continued to be more Tc1 cells migrating to both the p33.EL-4 and the EL-4 tumor sites than Tc2 on both days 3 and 7, suggesting that antigen expression at the site of the tumor mass does not play a role in the preferential migration of antigen-specific Tc1 cells to tumor sites, or on the kinetics of their migration (Figure 3.5).

We next evaluated the tumor-infiltrating phenotype of Tc1 and Tc2 cells infiltrating both p33.EL-4 and EL-4 tumors by looking at the cell surface expression of LFA-1, CD44, and PSGL-1. Surprisingly, we found no significant differences in the MFI of these adhesion molecules for both Tc1 and Tc2 cells in antigen-positive p33.EL-4 vs. antigen-negative EL-4 tumors (Figure 3.6), namely they were LFA-1, CD44, and PSGL-1 high. Likewise, host cells that migrate into both p33.EL-4 and EL-4 tumors following injection of either Tc1 or Tc2 cells expressed this identical phenotype (3.7), indicating again that there is a tumor-infiltrating phenotype that dictates whether cells can infiltrate tumors. These data suggest that while antigen may play a role in increasing the amount of cellular infiltrate for both Tc1 and Tc2 donor cells, it does not play a role in the kinetics of infiltration, the tumor-infiltrating phenotype of $CD8^+$ T cells, or the preferential migration of Tc1 cells to tumor cells following injection.

Discussion

In this study, we examined migration and tumor infiltration of Tc1 and Tc2 by transferring GFP-expressing p33-specific donor cells into tumor bearing mice. When we examined the migration to TDLNs and infiltration into tumors, we found more Tc1 cells than Tc2 cells at both sites on days 3 and 7 after transfer. We also found that while Tc1 and Tc2 cells have different adhesion molecule profiles *in vitro* following antigenic stimulation and in TDLNs following injection, the donor Tc1 and Tc2 cells that infiltrate the tumor have identical expression of adhesion molecules regardless of the time we examined them or whether or not these tumors express p33 antigen. Moreover, host cells infiltrating tumors also express this identical phenotype-- high levels of LFA-1, CD44, and PSGL-1 expression. Therefore, it appears that the selective advantage of Tc1 cells to migrate to and infiltrate tumor sites appears to be due to their intrinsically higher expression of several adhesion molecules critical for tumor infiltration, and this expression does not appear to be altered by the presence of antigen at the tumor site. These results suggest that tumor infiltration by activated $CD8^+$ T cells requires the expression of a "tumor infiltrating phenotype", and that Tc1 cells generated *in vitro* naturally express this phenotype (which is characterized by a high expression level of LFA-1, CD44, and PSGL-1) more abundantly than Tc2 cells.

Our extensive analysis of adhesion molecule phenotypes suggests a critical role for LFA-1, CD44, and PSGL-1 in the differences in tumor infiltration we see between Tc1 and Tc2 cells. Our results demonstrate that Tc1 and Tc2 cells infiltrating the tumors on days 3 and 7 have similar adhesion profiles, a "tumor infiltrating phenotype" that is LFA-1 high, CD44 high, and PSGL-1 high. This phenotype is expressed by a greater number of Tc1 vs. Tc2 cells following *in vitro* activation and at TDLNs on day 3, which may promote earlier

and more effective tumor infiltration by Tc1 cells compared to Tc2 cells. The existence of a "tumor infiltrating phenotype" is supported by previous findings suggesting a role for CD44, LFA-1, and PSGL-1 in $CD8^+$ T cell-mediated tumor rejection (26-31). Although requirement of these specific adhesion molecules for tumor infiltration has not yet been demonstrated, our results indicate that high expression of CD44, LFA-1 and PSGL-1 may play a critical role in tumor infiltration.

Another important consideration for differences in T cell migration is the effector/memory phenotype of the cells, which is indicated in part by CD44 as well as CD62L expression (19-21, 30, 43-46). Importantly, $CD62L^{high}CD44^{high}$ cells have been shown to be more effective in tumor therapy than $CD62L^{low}CD44^{high}$ cells, possibly due to the cells trafficking through TDLNs (30, 43-46). CD44 is upregulated on effector T cells as well as both effector and central memory T cells (T_{EM} and T_{CM}, respectively), while CD62L is expressed on naïve T cells and only T_{CM} (19-21, 43-46). We and others find CD62L expression is also higher for Tc1 cells than Tc2 cells following activation (10), indicating that Tc1 cultures may contain more cells with the T_{CM} phenotype ($CD62L^{high}CD44^{high}$) compared to Tc2 cultures that have more cells with the T_{EM} phenotype ($CD62L^{low}CD44^{high}$). Since CD62L is important for homing to lymph nodes (19-21, 25), the higher level of CD62L expression by Tc1 cells vs. Tc2 cells *in vitro* and *in vivo* could contribute to the increased migration of Tc1 cells to TDLNs compared to Tc2 cells *in vivo*. Lower expression of CD62L by Tc2 cells may also contribute to the limited anti-tumor function of Tc2 cells observed *in vivo*.

While previous studies have reported higher numbers of $CD8^+$ T cell infiltrating further into antigen-positive vs. antigen-negative tumors (34, 35), the role of antigen

expression by tumor endothelium in promoting $CD8^+$ T cell infiltration remains unclear. In particular, whether antigen expression on tumors impacts Tc1 and Tc2 cell tumor infiltration is undefined. Interestingly, we found that while the number of both donor Tc1 and Tc2 cells was higher in antigen-positive tumors compared to antigen-negative tumors, the expression of antigen did not appear to affect any of the following: the kinetics of donor cell infiltration; the enhanced infiltration of tumors by Tc1 cells compared to Tc2 cells; or the adhesion phenotype profiles of the infiltrating Tc1 and Tc2 donor cells. The Tc1 and Tc2 cells, as well as host $CD8^+$ T cells, in both the antigen-positive and antigen-negative tumors all expressed the same LFA-1^{high}CD44^{high}PSGL-1^{high} "tumor infiltrating phenotype". Since Tc1, Tc2, and host $CD8^+$ T cells within the p33.EL-4 and EL-4 tumors express this phenotype, the role of antigen expression by tumors may be to simply expand the population of cells responding to the tumor. However, because antigen presentation does alter the expression of these adhesion molecules on $CD8^+$ T cells (19-25), the enhanced infiltration by Tc1 and Tc2 cells to antigen-positive tumors may also be due to differences in either adhesion molecule distribution or altered conformation (22-25).

In summary, this study shows evidence of migration and tumor infiltration differences between Tc1 and Tc2 cells. Tc1 cells migrate to TDLNs and infiltrate tumors more efficiently than Tc2 cells. Adhesion molecule profiles of Tc1 and Tc2 cells *in vitro* prior to transfer suggest that the enhanced efficiency of Tc1 cell migration and infiltration compared to Tc2 cells is due to increased expression of CD62L and an LFA-1^{high}CD44^{high}PSGL-1^{high} tumor infiltrating phenotype. Tc1 and Tc2 cells in the antigen-negative tumors express this phenotype, and Tc1 cells infiltrate these tumors in higher number than Tc2 cells. This suggests that the intrinsic differences in adhesion molecule expression between Tc1 and Tc2

cells before transfer contributes to antigen-independent trafficking of the antigen-specific Tc1 and Tc2 cells into tumors. Therefore, Tc1 cells are recommended for adoptive cell therapy, due to their high expression of a tumor infiltrating phenotype and superior migration and tumor infiltration properties *in vivo*.

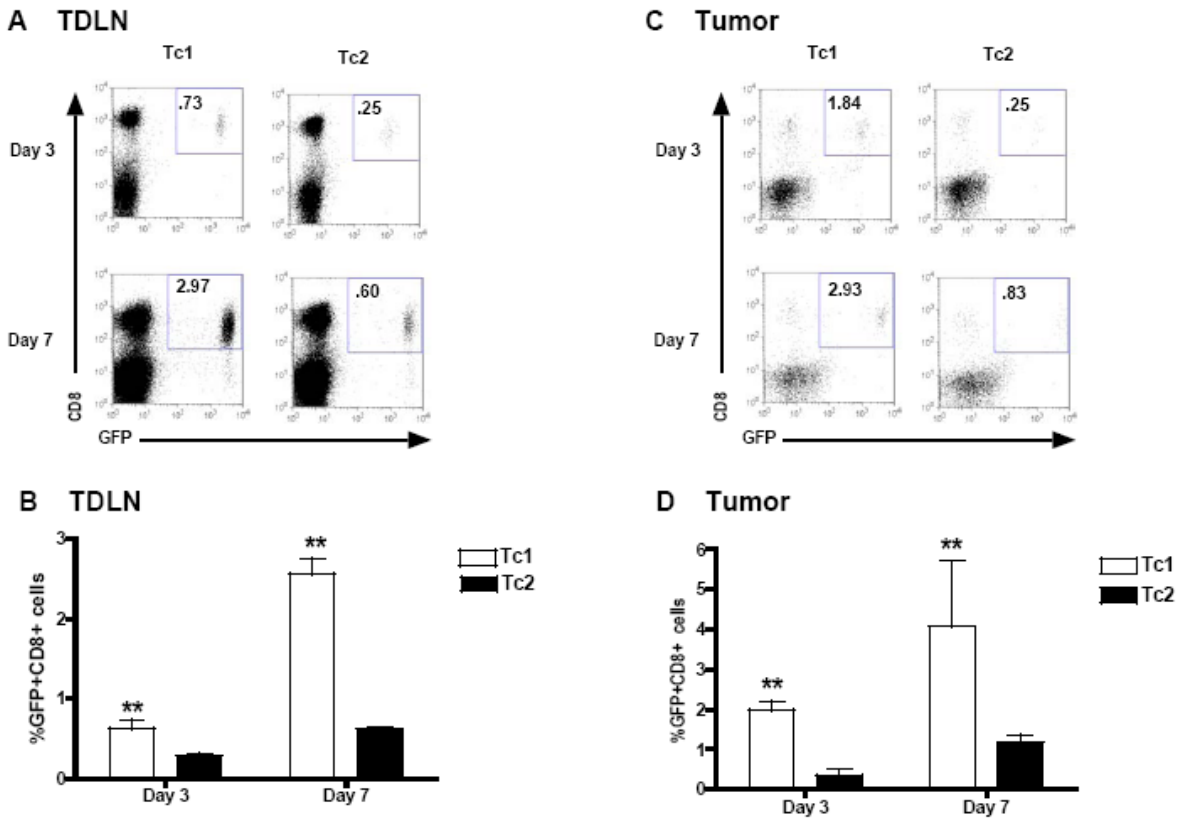

Figure 3.1: Tc1 and Tc2 donor cell migration to TDLNs and infiltration of tumors *in vivo*. On day 0, 1x10^6 Tc1 (A and B) or Tc2 (C and D) cells generated from GFPxP14 splenocytes were transferred i.v. into B6 mice bearing p33.EL-4 s.c. tumors. On days 3 and 7 after transfer, TDLNs and tumors were harvested, and the cells were stained for CD8. Lymphocytes were analyzed for GFP and CD8 expression to identify GFP$^+$CD8$^+$ donor cells using FlowJo. A and C show dot plots of GFP and CD8 staining from one of four animals in each group. B and D show collective data from all four animals in each group. Data shown represent two experiments. ** $p \leq .01$ Tc1 vs. Tc2 cells on day 3 and day 7 in TDLNs. **$p \leq .01$ Tc1 vs. Tc2 cells on day 3 and day 7 in tumors.

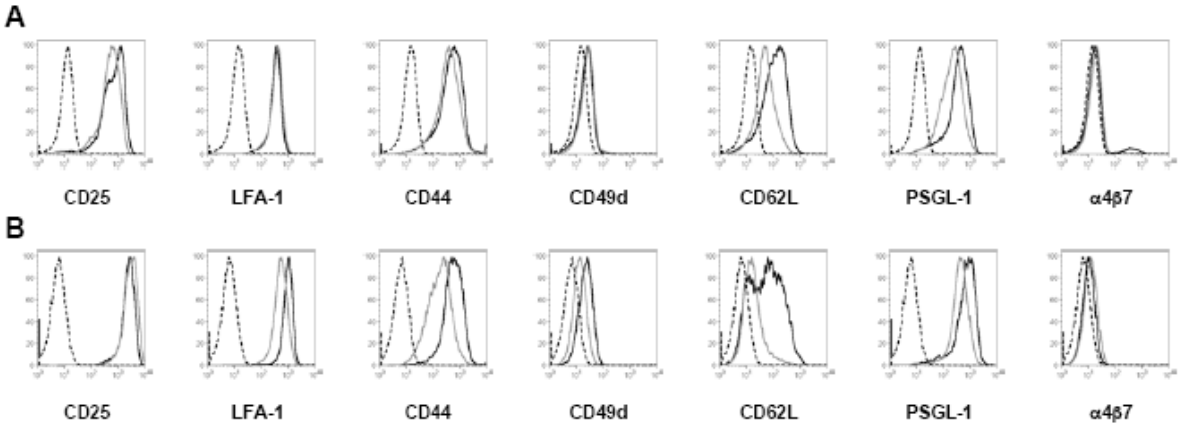

Figure 3.2: Phenotype of Tc1 and Tc2 cells *in vitro*. Tc1 (black line) and Tc2 (grey line) cells were stained for the expression of CD25, LFA-1, CD44, CD49d, CD62L, PSGL-1, and $\alpha_4\beta_7$ integrin on day three of culture (A) and after three days of restimulation *in vitro* (B). Staining for expression was analyzed using FlowJo.

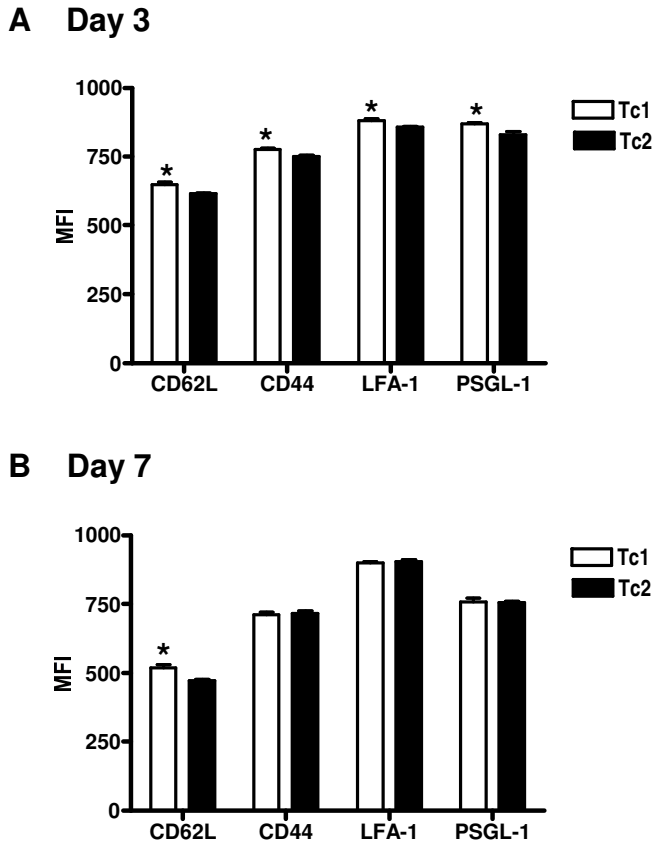

Figure 3.3: Phenotype of Tc1 and Tc2 cells in TDLNs 3 and 7 days after transfer into mice with established tumors. On day 0, 1x10^6 Tc1 *or* Tc2 cells generated *in vitro* from GFPxP14 splenocytes were transferred i.v. into B6 mice bearing p33.EL-4 tumors. On days 3 (A) and 7 (B) after transfer, TDLNs were harvested and stained for CD8, CD62L, CD44, LFA-1, and PSGL-1 expression. GFP^+CD8^+ lymphocyte Tc1 (open bars) and Tc2 (shaded bars) cells were gated and analyzed for MFI of adhesion molecule expression using FlowJo. Data shown is from four animals per group and is representative of two experiments. * $p \leq .05$ Tc1 vs. Tc2 expression of CD25, CD62L, CD44, LFA-1, and PSGL-1 on day 3. *$p \leq .05$ Tc1 vs. Tc2 expression of CD62L on day 7.

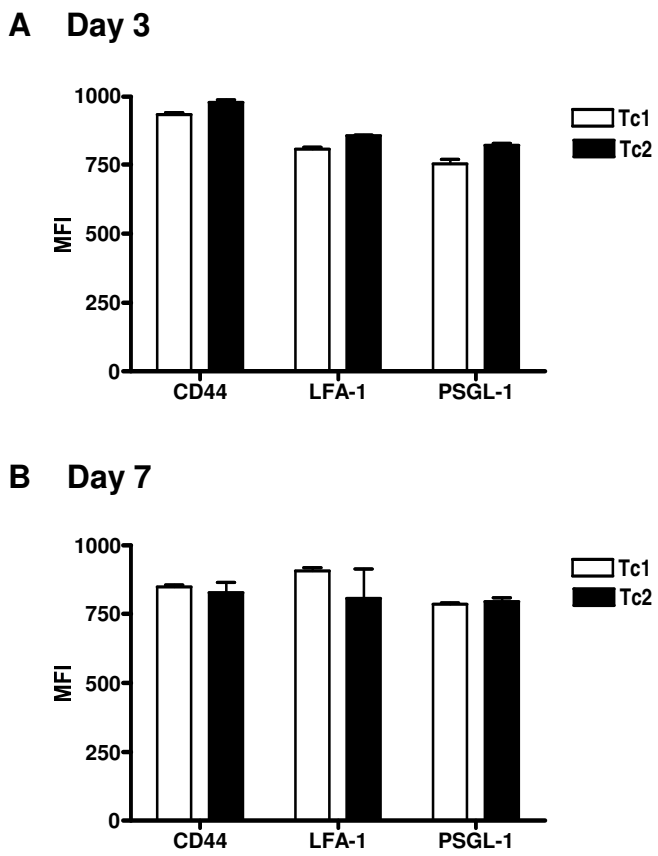

Figure 3.4: Phenotype of Tc1 and Tc2 in tumors 3 and 7 days after transfer into mice with established tumors. $1x10^6$ Tc1 *or* $1x10^6$ Tc2 cells generated *in vitro* from GFPxP14 splenocytes were transferred i.v. into B6 mice bearing p33.EL-4 tumors. On days 3 (A) and 7 (B) after transfer, tumors were harvested and stained for the following markers: CD8, CD44, LFA-1, and PSGL-1. Tc1 (open bars) and Tc2 (shaded bars) cells were gated based on GFP^+CD8^+ expression and the MFI of staining for both populations was analyzed using FlowJo. Data shown is from four animals per group and is representative of two experiments.

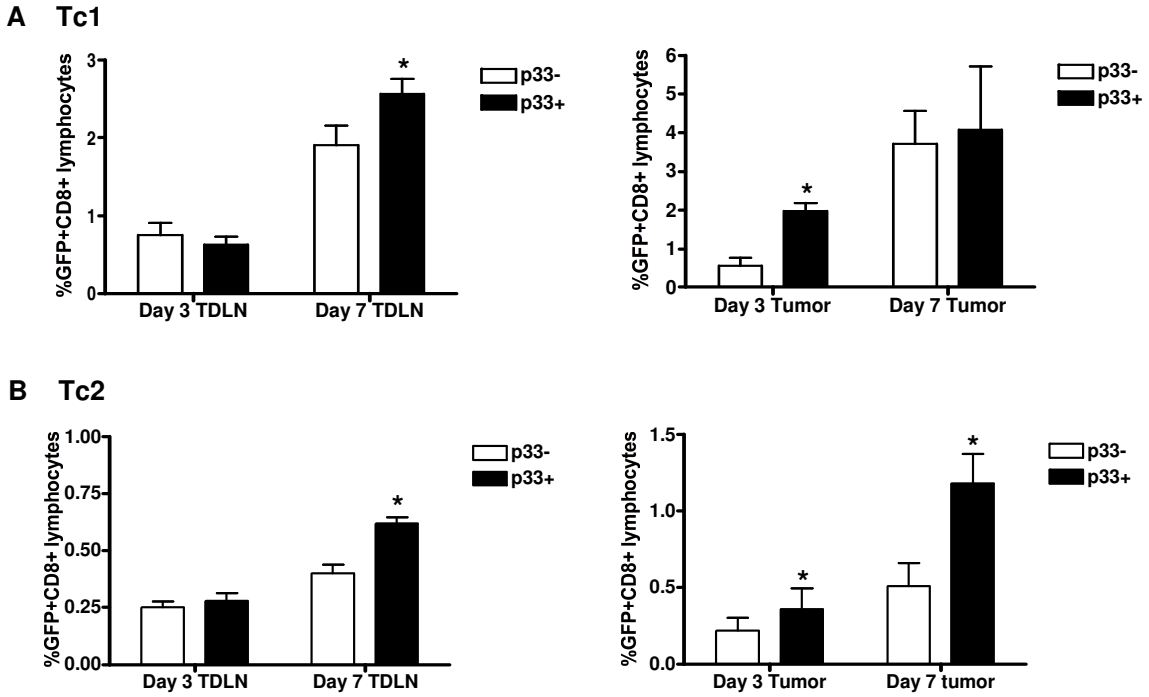

Figure 3.5: Tc1 and Tc2 cell migration to TDLNs and tumor sites of antigen-positive and antigen-negative tumors. On day 0, $1x10^6$ Tc1 (A) or Tc2 (B) cells generated from GFPxP14 splenocytes were transferred i.v. into B6 mice bearing EL-4 (left flank) and p33.EL-4 (right flank) s.c. tumors. On days 3 and 7 after transfer, TDLNs and tumors were harvested, and the cells were stained for CD8. Lymphocytes were analyzed for GFP and CD8 expression to identify GFP^+CD8^+ donor cells using FlowJo. Data shown is from four animals per group and is representative of two experiments. * $p \leq .05$ Tc1 cells in EL-4 vs. p33.EL-4 TDLNs on day 7 and tumors on day 3 after transfer. *$p \leq .05$ Tc2 cells in EL-4 vs. p33.EL-4 TDLNs on day 7 and tumors on days 3 and 7 after transfer.

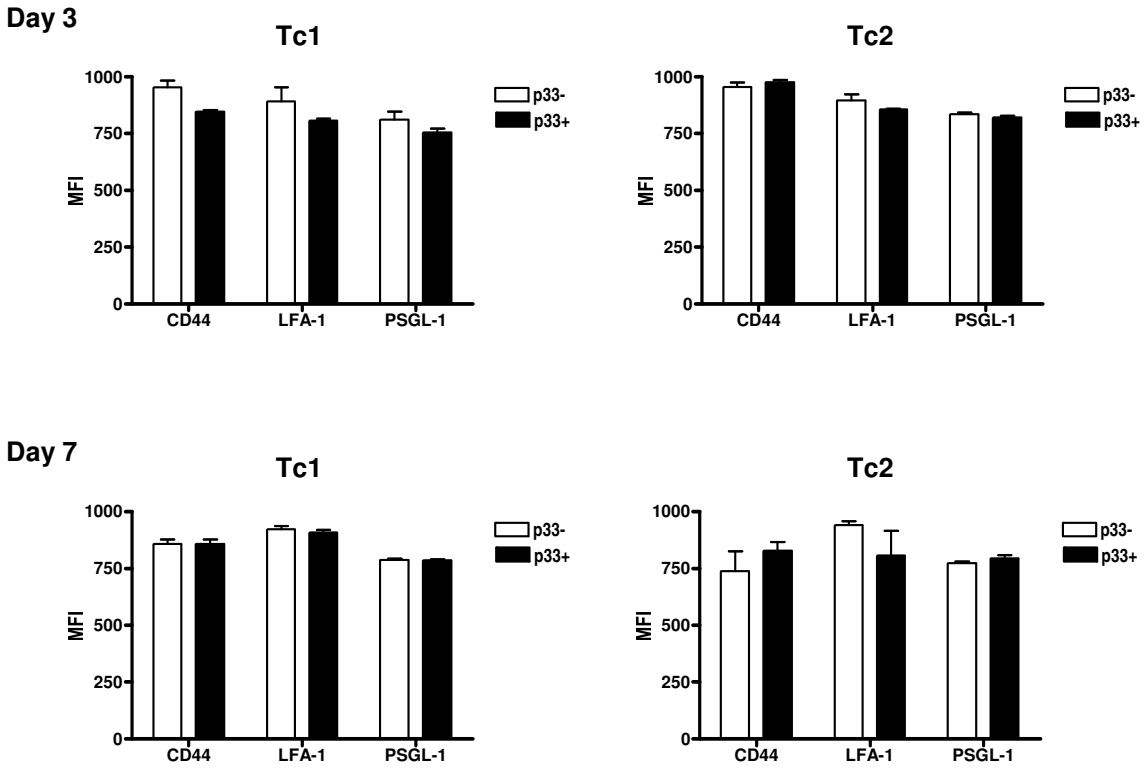

Figure 3.6: Phenotype of Tc1 and Tc2 cells in antigen-positive vs. antigen-negative tumors. On day 0, $1x10^6$ Tc1 or Tc2 cells generated from GFPxP14 splenocytes were transferred i.v. into B6 mice bearing EL-4 (left flank) and p33.EL-4 (right flank) s.c. tumors. On days 3 (A) and 7 (B) after transfer, tumors were harvested, and the cells were stained for CD8, CD44, LFA-1, and PSGL-1. GFP^+CD8^+ donor lymphocytes were gated and analyzed for adhesion molecule expression using FlowJo. Data shown is from four animals per group and is representative of two experiments.

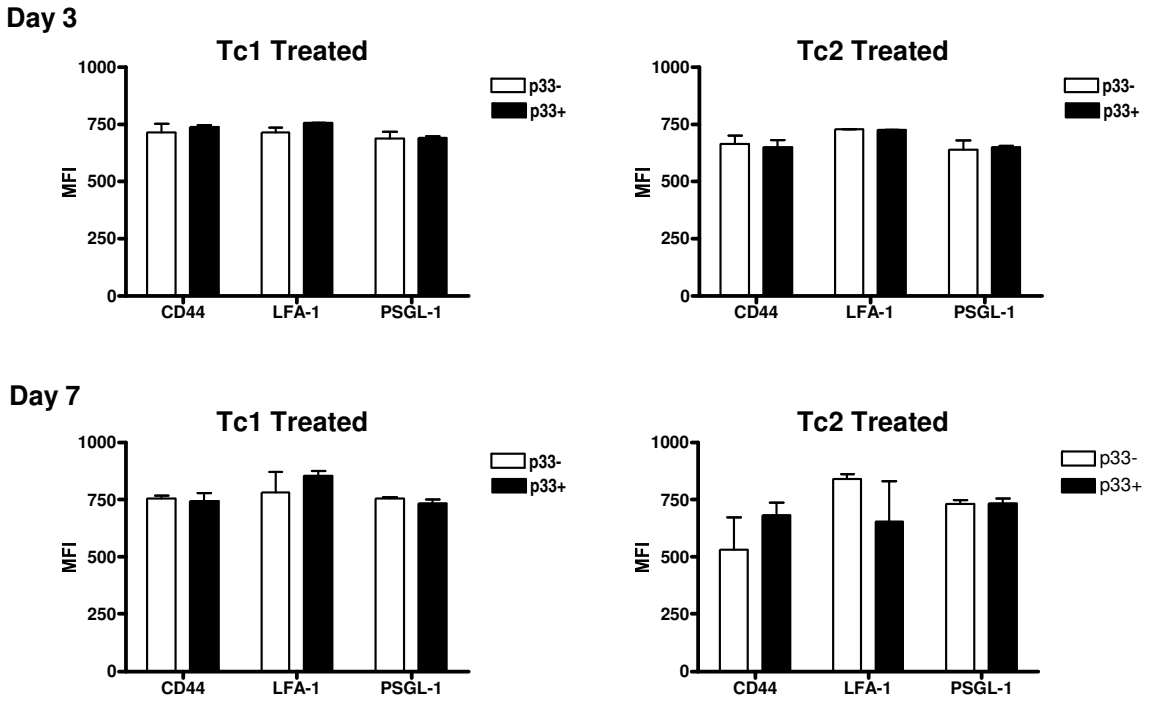

Figure 3.7: Phenotype of host CD8$^+$ cells in antigen-positive vs. antigen-negative tumors in Tc1 or Tc2 treated animals. On day 0, 1x10^6 Tc1 or Tc2 cells generated from GFPxP14 splenocytes were transferred i.v. into B6 mice bearing EL-4 (left flank) and p33.EL-4 (right flank) s.c. tumors. On days 3 (A) and 7 (B) after transfer, tumors were harvested, and the cells were stained for CD8, CD44, LFA-1, and PSGL-1. GFP$^-$CD8$^+$ host lymphocytes were gated and analyzed for adhesion molecule expression using FlowJo. Data shown is from four animals per group and is representative of two experiments.

References

1. Woodland DL, Dutton RW. Heterogeneity of CD4(+) and CD8(+) T cells. Curr Opin Immunol 2003; Jun;15(3):336-42.

2. Sad S, Marcotte R, Mosmann TR. Cytokine-induced differentiation of precursor mouse CD8+ T cells into cytotoxic CD8+ T cells secreting Th1 or Th2 cytokines. Immunity 1995 Mar;2(3):271-9.

3. Cerwenka A, Carter LL, Reome JB, Swain SL, Dutton RW. *In vivo* persistence of CD8 polarized T cell subsets producing type 1 or type 2 cytokines. J Immunol 1998 Jul 1;161(1):97-105.

4. Noble A, Truman JP, Vyas B, Vukmanovic-Stejic M, Hirst WJ, Kemeny DM. The balance of protein kinase C and calcium signaling directs T cell subset development. J Immunol 2000 Feb 15;164(4):1807-13.3.

5. Kemp RA, Backstrom BT, Ronchese F. The phenotype of type 1 and type 2 CD8+ T cells activated *in vitro* is affected by culture conditions and correlates with effector activity. Immunology 2005 Jul;115(3):315-24.

6. Boni A, Iezzi G, Degl'Innocenti E, et al. Prolonged exposure of dendritic cells to maturation stimuli favors the induction of type-2 cytotoxic T lymphocytes. Eur J Immunol 2006 Dec;36(12):3157-66.

7. Dobrzanski MJ, Reome JB, Dutton RW. Therapeutic effects of tumor-reactive type 1 and type 2 CD8+ T cell subpopulations in established pulmonary metastases. J Immunol 1999 Jun 1;162(11):6671-80.

8. Dobrzanski MJ, Reome JB, Dutton RW. Type 1 and type 2 CD8+ effector T cell subpopulations promote long-term tumor immunity and protection to progressively growing tumor. J Immunol 2000 Jan 15;164(2):916-25.

9. Helmich BK, Dutton RW. The role of adoptively transferred CD8 T cells and host cells in the control of the growth of the EG7 thymoma: factors that determine the relative effectiveness and homing properties of Tc1 and Tc2 effectors. J Immunol 2001 Jun 1;166(11):6500-8.

10. Kemp RA, Ronchese F. Tumor-specific Tc1, but not Tc2, cells deliver protective antitumor immunity. J Immunol 2001 Dec 1;167(11):6497-502.

11. Chamoto K, Kosaka A, Tsuji T, et al. Critical role of the Th1/Tc1 circuit for the generation of tumor-specific CTL during tumor eradication *in vivo* by Th1-cell therapy. Cancer Sci 2003 Oct;94(10):924-8.

12. Chang ML, Chen YT, Su YC, Kung JT. Cytotoxic T lymphocytes generated by short-term *in vitro* TCR stimulation in the presence of IL-4 are therapeutically effective against B16 melanoma. J Biomed Sci 2003;10(6 Pt 1):644-50.

13. Dobrzanski MJ, Reome JB, Hollenbaugh JA, Hylind JC, Dutton RW. Effector cellderived lymphotoxin alpha and Fas ligand, but not perforin, promote Tc1 and Tc2 effector cell-mediated tumor therapy in established pulmonary metastases. Cancer Res 2004 Jan 1;64(1):406-14.

14. Dobrzanski MJ, Reome JB, Hollenbaugh JA, Dutton RW. Tc1 and Tc2 effector cell therapy elicit long-term tumor immunity by contrasting mechanisms that result in complementary endogenous type 1 antitumor responses. J Immunol 2004 Feb 1;172(3):1380-90.

15. Do JS, Choi YH, Shin SH, Yi HK, Hwang PH, Nam SY. Committed memory effector type 2 cytotoxic T (Tc2) cells are ineffective in protective anti-tumor immunity. Immunol Lett 2004 Aug 15;95(1):77-84.

16. Nishimura F, Dusak JE, Eguchi J, et al. Adoptive transfer of type 1 CTL mediates effective anti-central nervous system tumor response: critical roles of IFN-inducible protein-10. Cancer Res 2006 Apr 15;66(8):4478-87.

17. Hollenbaugh JA, Dutton RW. IFN-gamma regulates donor CD8 T cell expansion, migration, and leads to apoptosis of cells of a solid tumor. J Immunol 2006 Sep 1;177(5):3004-11.

18. Dobrzanski MJ, Reome JB, Hylind JC, Rewers-Felkins KA. CD8-mediated type 1 antitumor responses selectively modulate endogenous differentiated and nondifferentiated T cell localization, activation, and function in progressive breast cancer. J Immunol 2006 Dec 1;177 (11):8191-201.

19. Weninger W, Manjunath N, von Andrian UH. Migration and differentiation of CD8+ T cells. Immunol Rev 2002 Aug;186:221-33.

20. Mora JR, von Andrian UH. T-cell homing specificity and plasticity: new concepts and future challenges. Trends Immunol 2006 May;27(5):235-43.

21. Lefrancois L. Development, trafficking, and function of memory T-cell subsets. Immunol Rev 2006 Jun;211:93-103.

22. Hogg N, Laschinger M, Giles K, McDowall A. T-cell integrins: more than just sticking points. J Cell Sci 2003 Dec 1;116(Pt 23):4695-705.

23. Hogg N, Smith A, McDowall A, et al. How T cells use LFA-1 to attach and migrate. Immunol Lett 2004 Mar 29;92(1-2):51-4.

24. Steeber DA, Venturi GM, Tedder TF. A new twist to the leukocyte adhesion cascade: intimate cooperation is key. Trends Immunol 2005 Jan;26(1):9-12.

25. Ley K, Kansas GS. Selectins in T-cell recruitment to non-lymphoid tissues and sites of inflammation. Nat Rev Immunol 2004 May;4(5):325-35.

26. Tanigawa K, Takeshita N, Craig RA, et al. Tumor-specific responses in lymph nodes draining murine sarcomas are concentrated in cells expressing P-selectin binding sites. J Immunol 2001 Sep 15;167(6):3089-98.

27. Blank C, Brown I, Kacha AK, Markiewicz MA, Gajewski TF. ICAM-1 contributes to but is not essential for tumor antigen cross-priming and CD8+ T cell-mediated tumor rejection *in vivo*. J Immunol 2005 Mar 15;174(6):3416-20.

28. Jenkinson SR, Williams NA, Morgan DJ. The role of intercellular adhesion molecule-1/LFA-1 interactions in the generation of tumor-specific CD8+ T cell responses. J Immunol 2005 Mar 15;174(6):3401-7.

29. Yamada M, Yanaba K, Hasegawa M, et al. Regulation of local and metastatic hostmediated anti-tumour mechanisms by L-selectin and intercellular adhesion molecule-1. Clin Exp Immunol 2006 Feb;143(2):216-27.

30. Gattinoni L, Powell DJ Jr, Rosenberg SA, Restifo NP. Adoptive immunotherapy for cancer: building on success. Nat Rev Immunol 2006 May;6(5):383-93.

31. Fisher DT, Chen Q, Appenheimer MM, et al. Hurdles to lymphocyte trafficking in the tumor microenvironment: implications for effective immunotherapy. Immunol Invest 2006; 35(3-4):251-77.

32. Marelli-Berg FM, Jarmin SJ. Antigen presentation by the endothelium: a green light for antigen-specific T cell trafficking? Immunol Lett 2004 May 15;93(2-3):109-13.

33. Boissonnas A, Combadiere C, Lavergne E, et al. Antigen distribution drives programmed antitumor CD8 cell migration and determines its efficiency. J Immunol 2004 Jul 1;173(1):222-9.

34. Joncker NT, Marloie MA, Chernysheva A, et al. Antigen-independent accumulation of activated effector/memory T lymphocytes into human and murine tumors. Int J Cancer 2006 Mar 1;118(5):1205-14.

35. Boissonnas A, Fetler L, Zeelenberg IS, Hugues S, Amigorena S. *In vivo* imaging of cytotoxic T cell infiltration and elimination of a solid tumor. J Exp Med 2007 Feb 19;204(2):345-56.

36. Schaefer BC, Schaefer ML, Kappler JW, Marrack P, Kedl RM. Observation of antigen-dependent CD8+ T-cell/ dendritic cell interactions *in vivo*. Cell Immunol 2001

Dec 15;214(2):110-22.

37. Pircher H, Michalopoulos EE, Iwamoto A, et al. Molecular analysis of the antigen receptor of virus-specific cytotoxic T cells and identification of a new V alpha family. Eur J Immunol 1987 Dec;17(12):1843-6.

38. Qian, L, Lorena, S, Burgents, M, and Flood, PM. Increased CD8+ T cell Antigenicity of Murine Tumors Increases Tumor Resistance. Manuscript in preparation.

39. Mottez E, Langlade-Demoyen P, Gournier H, et al. Cells expressing a major histocompatibility complex class I molecule with a single covalently bound peptide are highly immunogenic. J Exp Med 1995 Feb 1;181(2):493-502.

40. Uger RA, Barber BH. Creating CTL targets with epitope-linked beta 2-microglobulin constructs. J Immunol. 1998 Feb 15;160(4):1598-605.

41. White J, Crawford F, Fremont D, Marrack P, Kappler J. Soluble class I MHC with beta2-microglobulin covalently linked peptides: specific binding to a T cell hybridoma. J Immunol 1999 Mar 1;162(5):2671-6.

42. Uger RA, Chan SM, Barber BH. Covalent linkage to beta2-microglobulin enhances the MHC stability and antigenicity of suboptimal CTL epitopes. J Immunol 1999 May 15;162(10):6024-8.

43. Gattinoni L, Klebanoff CA, Palmer DC, et al. Acquisition of full effector function *in vitro* paradoxically impairs the *in vivo* antitumor efficacy of adoptively transferred CD8+ T cells. J Clin Invest 2005 Jun;115(6):1616-26.

44. Klebanoff CA, Gattinoni L, Torabi-Parizi P, et al. Central memory self/tumorreactive CD8+ T cells confer superior antitumor immunity compared with effector memory T cells. Proc Natl Acad Sci U S A 2005 Jul 5;102(27):9571-6.

45. Hinrichs CS, Gattinoni L, Restifo NP. Programming CD8+ T cells for effective immunotherapy. Curr Opin Immunol 2006 Jun;18(3):363-70.

46. Wherry EJ, Teichgraber V, Becker TC, et al. Lineage relationship and protective immunity of memory CD8 T cell subsets. Nat Immunol 2003 Mar;4(3):225-34.

Chapter IV

Gene Expression in Donor and Host Cell in Tc1 and Tc2 Treated Animals Which May Contribute to Differences in Tc1 vs. Tc2 Migration

Abstract

Previously, we demonstrated that Tc1 and Tc2 cells have different migration and tumor infiltration properties *in vivo*. In addition, we found that CD8+ cells in tumors of Tc1 and Tc2 treated animals express a "tumor infiltrating phenotype" characterized by expression of specific adhesion molecules such as PSGL-1. It is unknown how PSGL-1 protein levels are regulated in CD8+ T cells. In the following preliminary study, we used real time RT-PCR to evaluate whether molecules implicated in the regulation of PSGL-1 in CD4+ T cells (T-bet, selectin glycosylating enzymes, and PSGL-1) are also involved in the regulation of PSGL-1 in CD8+ T cells. We examined the gene expression of CD43, another selectin ligand believed to be regulated by T-bet and glycosylating enzymes. We found that T-bet and CD43 genes are significantly higher in Tc1 cells compared to Tc2 cells, which suggest that T-bet may have a role in the regulation of a Tc1 vs. Tc2 phentoype. mRNA levels of PSGL-1 and the glycosylating enzymes were similar in both Tc1 and Tc2 cells, suggesting that differences in cell surface levels of PSGL-1 protein by Tc1 vs. Tc2 cells is not due to differences in the transcription of genes for these molecules. Migration is also regulated by chemokines such as IP-10, MDC, and MIP-1α, whose receptors, CXCR3, CCR4, and CCR5, are differentially expressed by Tc1 and Tc2 cells. We evaluated gene expression of these chemokines and chemokine receptors in Tc1 and Tc2 treated animals by real time RT-PCR. We found that IP-10 was highly expressed in tumors of both Tc1 and Tc2 treated animals compared to MDC and MIP-1α. We also found that tumor infiltrating host and donor CD8+ cells expressed high levels of CXCR3 and IFN-γ, indicating that tumor infiltrating cells express a type I phenotype.

Introduction

As described earlier, T cell migration is regulated by molecules important for adhesion and chemotaxis (See Chapter 1) (1-4). We and others have found that Tc1 and Tc2 cells have different expression profiles of these molecules, and studies have also suggested that Tc1 and Tc2 cells have different migration properties (Figures 2.6 and 3.2-3.4) (5-8). We found that Tc1 cells migrate to TDLNs and infiltrate tumors more efficiently than Tc2 cells (Figure 3.1). We also found that Tc1 cells had higher cell surface levels of adhesion molecules *in vitro* compared to Tc2 cells (Figure 3.2). The differences in adhesion molecule cell surface expression likely play a role in the migration differences observed between Tc1 and Tc2 cells *in vivo*. However, it is not clear how these adhesion molecules are regulated differently in Tc1 and Tc2 cells.

One of the adhesion molecules Tc1 cells express more than Tc2 cells on the cell surface is PSGL-1 (Figure 3.2) (9). Expression of PSGL-1 is regulated both at the level of gene expression and post-translational modification of the core protein by glycosylating enzymes (9). PSGL-1 gene expression is believed to be under the control of transcription factor T-bet and its associated signaling molecules (10-12). Interestingly, T-bet is expressed in Th1 but not Th2 cells, suggesting that T-bet may be a type I specific transcription factor (11-13). Despite confirmed expression of T-bet by CD8+ T cells, expression of T-bet has not yet been confirmed in polarized Tc1 or Tc2 cells (10). T cells from T-bet knockout mice exhibit impaired transendothelial migration *in vitro* that is restored by forced expression of T-bet (11, 12). This is thought to be due to the decreased level of selectin ligand synthesis in T-bet knockout cells, because T-bet has been shown to regulate expression of glycosylating enzymes critical for selectin ligand synthesis (11, 12). These enzymes include α1,3-

fucosyltransferase-VII (FucT-VII), β1,4-galactosyltransferase-I (B4GalT-I), sialyl transferase-IV (ST3GalT-IV), sialyltransferase-VI (ST3GalT-VI), and β1,6-glucosaminyltransferase-I (C2GlcNAcT-I) (8, 11, and 12). In order to examine the mechanism of PSGL-1 regulation in Tc1 and Tc2 cells, we evaluated the gene expression of PSGL-1 itself, as well as T-bet and the glycosylating enzymes, in both Tc1 and Tc2 cells polarized *in vitro*.

While adhesion molecules are important for infiltration of tumors, chemokines and the expression of the appropriate chemokine receptors by T cells are important for migration of T cells to the tumor site (1-4). Tc1 and Tc2 cells express different levels of chemokine receptors, and therefore are likely to migrate in response to different chemokines (5). Tc1 cells express CCR5 and CXCR3, while Tc2 cells express CCR4 and CXCR3 (Figure 2.6) (5). The different expression of chemokine receptors may play a role in the different migration efficencies of Tc1 and Tc2 cells that we observed *in vivo* (Figure 3.1). Chemokines such as IP-10, MDC, and MIP-1α bind to CXCR3, CCR4, and CCR5, respectively (14-19). Tc1 therapy has been shown to promote upregulation of chemokines in treated animals compared to untreated animals (20). Differences in chemokine expression in Tc1 vs. Tc2 treated animals may also play a role in the different migration efficencies of Tc1 and Tc2 cells, as well as the different therapeutic effectiveness of Tc1 and Tc2 therapies. For these reasons, we examined the expression of these chemokines in tumors of Tc1 vs. Tc2 treated mice and the expression of these chemokine receptors by Tc1 and Tc2 cells in TDLNs and tumors after transfer.

In following preliminary studies, we evaluated gene expression by donor Tc1 and Tc2 cells *in vitro* and by host and donor cells *in vivo* after ACT therapy using real time RT-PCR .

We found that T-bet is expressed higher in Tc1 vs. Tc2 cells, and that PSGL-1 mRNA levels are similar between Tc1 and Tc2 cells. Glycosylating enzymes were also expressed similarly between Tc1 and Tc2 cells. CD43, another selectin ligand which may be under control of T-bet, was also expressed higher in Tc1 compared to Tc2 cells (9-12). The chemokine IP-10, that binds CXCR3, was expressed higher than either MIP-1α or MDC in tumors of Tc1 and Tc2 treated animals. IP-10 expression was similar between Tc1 and Tc2 treated animals on day 3 but significantly higher in Tc1 treated animals on day 7. CXCR3 was also the highest expressed chemokine receptor by host cells in tumors of Tc1 and Tc2 treated animals. CXCR3 was expressed by both Tc1 and Tc2 cells in the tumors on day 3 and 7 after transfer, but Tc1 cells expressed significantly higher levels of CXCR3 gene on day 7. We examined whether Tc1 and Tc2 donor cells are still polarized in TDLNs and tumors after transfer by evaluating IFN-γ and IL-4 expression in addition to the chemokine receptors CXCR3, CCR4, and CCR5. Donor cells in the tumors expressed IFN–γ on day 3 after transfer, and only Tc1 cells expressed IFN–γ on day 7 after transfer. Neither Tc1 nor Tc2 cells in the tumors on days 3 or 7 expressed IL-4. In addition we evaluated gene expression in host cells, and we found that like donor cells, host cells in tumors expressed IFN-γ but not IL-4 after donor cell transfer.

Methods

Mice

C57BL/6J (B6) and UBI.GFP mice were obtained from The Jackson Laboratory (Bar Harbor, Maine) (21). CD8 T cell vβ8 TCR transgenic P14 mice specific for LCMV peptide p33-41 (KAVYNFATC) in context of H-2D^b were originally obtained from Dr. Pam Ohashi (22). GFPxP14 mice were generated in our lab by crossing the UBI.GFP strain with the P14 strain and using the F1 generation (See Chapter 2). Splenocytes of these GFPxP14 mice were used as the source for our donor cells, as detailed below. B6 mice were used as recipient animals. Female mice aged 8-12 weeks of age were used throughout these experiments. All mice were maintained in specific pathogen free conditions by University of North Carolina's Department of Laboratory Animal Medicine and all animal procedures were approved by the university's Institutional Animal Care and Use Committee.

Tc1/Tc2 cultures

Splenocytes from GFPxP14 mice were harvested and then stimulated at 5x10^6 cells/ml for 3 days with p33 peptide (KAVYNFATC) along with 2 units/mL rIL-2 in RPMI medium. For Tc1 cells, we also added rIL-12 at 12.5ng/mL and anti-IL-4 antibody at 2.5ng/mL, but for Tc2 cells we added rIL-4 at 27.5ng/mL, anti-IL-12 antibody at 5.5ng/mL, and anti-IFN-γ antibody at 5.5ng/mL. Recombinant murine cytokines and anti-murine cytokine antibodies were all obtained from R&D Systems. After 3 days in culture, Tc1 and Tc2 cells were either used for real time RT-PCR analysis of gene expression or for transfer into tumor bearing mice.

Tumor cell lines and construction of p33 encoding plasmid

As a source of antigen-bearing tumor cells, our lab has previously generated LCMV peptide p33 -expressing EL4 thymoma tumor cells (EL-4.p33 cells, 23) Briefly, EL4 cells obtained from ATCC were transfected with a PcDNA3.0 plasmid (Invitrogen) containing an insert encoding the LCMV peptide p33 linked to human beta 2 microglobulin (23). P33.EL4 tumor cells were selected and maintained using RPMI-1640 medium containing G418 (Sigma-Aldrich). Transfection was confirmed by PCR and expression of the p33 antigen was confirmed by using the transfected cells as targets of P14 T cells in cytolytic assays (23). Both parental EL4 (p33 antigen-negative) cells and transfected p33.EL4 (p33 antigen-positive) cells were used in this study, as detailed below.

Real Time RT- PCR

Cells from Tc1 and Tc2 cultures were analyzed for expression of numerous genes: IFN-γ, IL-4, CXCR3, CCR4, CCR5, T-bet, PSGL-1, CD43, FucT-VII, B4GalT-I, ST3GalT-IV, ST3GalT-VI, and C2GlcNAcT-I. Sorted donor GFP+CD8+ and host GFP-CD8+ cells from Tc1 and Tc2 treated mice were analyzed on days 3 and 7 after transfer for expression of IFN-γ, IL-4, CXCR3, CCR4, and CCR5. These cells were sorted using a MoFlo flowcytometer from Cytomation. Tumors from Tc1 and Tc2 treated mice on days 3 and 7 after transfer of donor cells were analyzed for expression of MIP-1α, MDC, and IP-10. mRNA expression various genes was examined using TaqMan Gene Expression Assays from Applied Biosystems. Total RNA was isolated from *in vitro* and *in vivo* samples using RNEasy Minikits from Qiagen followed by DNase I treatment (Promega). mRNA was converted to cDNA using Superscript III (Invitrogen). This cDNA was then used in Real Time PCR

reactions using an ABI 7700 thermocycler. Samples were run in triplicate. Data was analyzed according to the methods of Livak and Schmittgen (24). Ct values for the cytokine and chemokine receptor genes were normalized to GAPDH expression for the same mRNA source. The lowest level of specific gene expression by a sample was set to a value of 1.00, as noted. Then, all other gene expression values were determined relative to this fold gene expression value.

Statistical Analysis

Experiments have not yet been repeated and therefore data from one experiment is shown for each figure. Four animals were used per group for each time point in each experiment and cells from these animals were pooled to make one representative mRNA source. Two-tailed Student's T-test was utilized to test for significance with $p \leq .05$ considered significant.

Results

Gene expression of T-bet and genes regulating selectin ligand synthesis

Previously we found that PSGL-1 is more highly expressed on the cell surface of Tc1 than Tc2 cells, as determined by flow cytometry (Figure 3.2). Because molecules important for surface expression of PSGL-1 expression have been identified in Th1 cells, we evaluated the expression of these genes in Tc1 and Tc2 cells (9, 11, 12). We harvested RNA from cultured GFPxP14 splenocyte derived Tc1 and Tc2 cells on day 3 of culture. We then used real time RT-PCR to evaluate the mRNA expression of the following genes: PSGL-1 itself, T-bet (transcription factor known to modulate PSGL-1 in Th1 cells), CD43 (another selectin ligand thought to be modulated by T-bet), and the glycosylating enzymes FucT-VII, B4GalT-I, ST3GalT-IV, ST3GalT-VI, and C2GlcNAcT-I (9-12). Expression of these genes was compared to IFN-γ and IL-4 expression by the Tc1 and Tc2 cells.

We first confirmed that the cells from the Tc1 culture were polarized, expressing a significantly higher level of IFN-γ and significantly lower level of IL-4 gene expression compared to Tc2 cells (Figure 4.1). As expected from previous studies in Th1 vs. Th2 cells, Tc1 cells express significantly higher levels of T-bet compared to Tc2 cells (Figure 4.1) (10-13). Despite the higher level of PSGL-1 on the surface of Tc1 vs. Tc2 cells we observed previously, we found no significant difference between the gene expression of PSGL-1 or the glysosylating enzymes between Tc1 and Tc2 cells (Figure 4.1). In contrast, we did find a significant difference in the gene expression of CD43, which is thought to be regulated similarly to PSGL-1 in Th1 cells (Figure 4.1) (11, 12). This suggests that the higher PSGL-1 expression in Tc1 vs. Tc2 cells is not due to differences in the mRNA level of PSGL-1 or the

glycosylating enzymes we examined. Our data also suggest that indeed, T-bet may be an important transcription factor contributing to the Tc1 vs. Tc2 phenotype.

Chemokine gene expression by tumors in Tc1 and Tc2 treated animals

Once we had briefly examined gene expression, which may contribute to the different level of PSGL-1 on Tc1 and Tc2 cells, we then began to evaluate whether expression of chemokines and their receptors may have contributed to the differences in Tc1 and Tc2 migration. Because Tc1 therapy has been shown to increase IP-10 chemokine production compared to no treatement, we evaluated chemokine expression by cells in tumors of Tc1 and Tc2 treated animals (Figure 4.2) (20). We chose to examine the gene expression of IP-10, MDC, and MIP-1α, which can bind chemokine receptors CXCR3, CCR4, and CCR5, respectively (1-5, 14-19). These chemokine receptors are differentially expressed on Tc1 and Tc2 cells (Figure 2.6) (5). We found no difference in MIP-1α expression between Tc1 and Tc2 treated animals on either day 3 or 7 after donor cell transfer (Figure 4.2). We also found no difference the expression of MDC or IP-10 in Tc1 vs. Tc2 treated animals on day 3 after transfer (Figure 4.2). However, we did find significantly higher expression of both MDC and IP-10 by cells in the tumors of Tc1 vs. Tc2 treated animals on day 7 after donor cell transfer (Figure 4.2). Importantly, IP-10 was expressed significantly higher than either MIP-1α or MDC at each time point in both Tc1 and Tc2 treated animals (Figure 4.2). Therefore, these data suggest that MDC expression may be induced following Tc1 treatement, but both Tc1 and Tc2 treatment may induce significant expression of IP-10, particularly on day 7 in Tc1 treated animals.

Cytokine gene expression of donor cells and host cells in Tc1 and Tc2 treated animals

Cytokine signaling can upregulate the expression of chemokines (*e.g.* IFN-γ induces IP-10) (4, 14-19). Therefore, although we had previously shown that the Tc1 and Tc2 cells were distinctly polarized populations *in vitro* (Figure 2.6), we examined whether the transferred donor cells maintained the Tc1 and Tc2 cytokine profile *in vivo*. We compared IFN–γ and IL-4 gene expression of donor cells in TDLNs and tumors in order to evaluate the maintenance of donor cell polarization after transfer (Figures 4.3). This also allowed us to evaluate whether Tc1 donor cells may continue to express more IFN-γ *in vivo* after transfer compared to Tc2 cells, which could promote the increased level of IP-10 production in the tumors of Tc1 vs. Tc2 treated animals on day 7, as seen above (Figure 4.2). We found that on days 3 in TDLNs, both Tc1 and Tc2 cells had the appropriate levels of IFN-γ to indicate type I (high IFN-γ) and type II (low IFN-γ) polarization, respectively (Figure 4.3). IL-4 was expressed minimally by both Tc1 and Tc2 donor cells on day 3. In contrast on day 7, not only did Tc2 donor cells express higher amounts of IFN-γ that were comparable to Tc1 cell IFN-γ gene exprsesion, but also, the Tc2 cell expression of IL-4 was not detected (Figure 4.3). (IL-4 was also not detected in Tc1 cells on day 7.(Figure 4.3)) When we examined the donor cells in tumors on day 3, we found that Tc1 cells have higher expression of IFN-γ compared to Tc2 cells, indicative of type I and type II polarization (Figure 4.3). On day 7 in the tumors, Tc1 donor cells had less IFN-γ expression than on day 3 after transfer, but Tc2 donor cells had no detectable expression of IFN-γ (Figure 4.3). Neither donor cell population expressed IL-4 on day 3 or day 7 after transfer (Figure 4.3). Therefore, these data suggest that while the Tc2 cells may convert to a type I phenotype in the TDLNS, still expressing

IFN–γ on day 7 and no IL-4, Tc2 cells may ultimately down-regulate cytokine gene expression at the tumor sites, not expressing IFN–γ or IL-4.

Cytokine production by Tc1 and Tc2 donor cells has been shown to be important for host responses, which are predominantly type I responses (20, 25-28). Therefore, because cytokine signaling promotes T cell polarization, we compared gene expression of IFN-γ and IL-4 by host CD8+ cells in Tc1 and Tc2 treated mice (Figure 4.4). The profile of higher IFN-γ vs. low/undetectable IL-4 expression in the host cells from both Tc1 and Tc2 treated animals suggests that host cells exhibit a type I phenotype in the TDLNs and tumors (Figure 4.4). In TDLNs IFN-γ was more highly expressed than IL-4 in host cells from Tc1 and Tc2 treated animlas on days 3 and 7 after donor cell transfer (Figure 4.4). Expression of IFN–γ in host cells of Tc1 treated animals was significantly higher on day 3 compared to host cells of Tc2 treated animals (Figure 4.4). IL-4 expression was barely detectable in host cells of both Tc1 and Tc2 treated animals in the TDLNs (Figure 4.4). In tumors IFN-γ was more highly expressed by host cells from Tc1 vs. Tc2 treated animals on day 3, but was expressed similarly by host cells from both treatement groups on day 7 (Figure 4.4). IL-4 was undetected at both time points in both treatment groups (Figure 4.4). Therefore, it appears that host CD8+ cells in both Tc1 and Tc2 treated animals exhibit a predominant type I phenotype, based on their cytokine gene expression in TDLNs and tumors.

Chemokine Receptor gene expression of donor cells and host cells in Tc1 and Tc2 treated animals

Simialry to cytokine expression, chemokine receptor expression is also an indicator of type I or type II polarization (5). Since we had observed differences in the level of

chemokine receptor expression by Tc1 and Tc2 cells (Figure 2.6) and differences in the expression of chemokines by tumors in Tc1 vs. Tc2 treated animals (Figure 4.2), we evaluated the expression of CXCR3, CCR4, and CCR5 chemokine receptors by donor and host CD8+ cells in TDLNs and tumors on days 3 and 7 after donor cell transfer (Figures 4.5 and 4.6). In TDLNs CXCR3 expression by Tc1 and Tc2 cells was higher than expression of CCR4 or CCR5 (Figure 4.5). Tc1 and Tc2 cells expressed similar levels of CXCR3 on day 3 after transfer, but Tc1 cells expressed significantly higher levels of CXCR3 expression on day 7 (Figure 4.5). CCR4 was minimally expressed by both Tc1 and Tc2 cells on day 3 after transfer, but only minimally detectable in Tc1 cells on day 7 (not detectable in Tc2 cells on day 7) (Figure 4.5). CCR5 expression was higher in Tc1 than Tc2 cells on days 3 and 7 after transfer (Figure 4.5). Tc2 cell expression of CCR5 was undetected on day 7 (Figure 4.5). In the tumors, we found that CXCR3 was expressed similarly by Tc1 and Tc2 cells on day 3 but was higher for Tc1 cells on day 7 compared to Tc2 cells, where CXCR3 expression was undetected (Figure 4.5). CCR4 gene expression was detected only at minimal levels in Tc1 cells on day 7 (Figure 4.5). CCR5 was also detectable only in Tc1 cells, but was found on days 3 and 7 (Figure 4.5).

We then examined the chemokine receptor gene expression in host cells from TDLNs and tumors in Tc1 and Tc2 treated animals. As we observed in donor cells, CXCR3 was more highly expressed than CCR4 or CCR5 in TDLNs and tumors of Tc1 and Tc2 treated animals (Figure 4.6). In TDLNs, while on day 3 the CXCR3 expression was higher in Tc1 treated animals, on day 7 it was higher in Tc2 treated animals (Figure 4.6). CCR4, although only expressed at low or minimal levels in host cells of TDLNs, was also expressed higher in Tc1 treated animals on day 3 but higher in Tc2 treated animals on day 7 (Figure 4.6). CCR5,

likewise, being expressed at low or minimal levels in host cells of TDLNs, was also expressed higher on day 3 in Tc1 treated animals and on day 7 in Tc2 treated animals (Figure 4.6). In the tumors of the Tc1 and Tc2 treated animals, CXCR3 was expressed higher by host cells than CCR4 or CCR5 Figure 4.6). CXCR3 expression was higher in host cells of Tc1 treated animals on day 3 but was higher in host cells of Tc2 treated animals on day 7 (Figure 4.6). CCR4 expression was only detected in host cells of Tc2 treated animals on day 7 after donor cell transfer (Figure 4.6). CCR5 expression was found in host cells from Tc1 and Tc2 treated mice on days 3 and 7 in the tumors (Figure 4.6). The expression of CCR5 was higher on the host cells from Tc2 vs. Tc1 treated animals on day 7 (Figure 4.6). Overall, we found that CXCR3 is more highly expressed by donor and host cells compared to expression levels of CCR4 and CCR5 in TDLNs and tumors on days 3 and 7 after donor cell transfer.

Discussion

We have shown previously that cultured Tc1 and Tc2 cells are polarized into type I and type II populations, respectively, and that these populations of cells not only have different levels of PSGL-1 cell surface protein and gene expression of chemokine receptors but also appear to migrate to TDLNs and infiltrate tumors with different efficiencies (Figures 2.6, 3.1, and 3.2). Here we examined donor, host, and total tumor cells by real time RT-PCR for gene expression to evaluate genes which may contribute to the increased cell surface expression of PSGL-1 on Tc1 vs. Tc2 cells and to evaluate the role of chemokines in the recruitment of Tc1 vs. Tc2 cells to TDLNs and tumors. We also evaluated cytokine gene expression to indicate whether the donor cells remained polarized after transfer. We found that T-bet and the selectin ligand CD43 are expressed significantly higher in Tc1 than Tc2 cells. PSGL-1 and the selectin glycosylating enzymes we examined were not differentially expressed (mRNA) in Tc1 vs. Tc2 cells. We also found that chemokine IP-10 was more highly expressed than either MIP-1a or MDC, particularly in tumors of Tc1 treated animals on day 7, and MDC gene expression was also higher in tumors of Tc1 treated animals on day 7 compared to tumors of Tc2 treated animals. Overall, CXCR3 was expressed higher than either CCR4 or CCR5 by host and donor CD8+ cells in TDLNs and tumors of Tc1 and Tc2 treated animals. Finally, both host and donor CD8+ cells in Tc1 and Tc2 treated animals expressed higher levels of IFN–γ gene expression, overall, compared to IL-4.

Although we previously found different levels of PSGL-1 selectin ligand on the cell surface of Tc1 and Tc2 cells by flow cytometry, we did not find differences in the mRNA levels of PSGL-1 or the glycosylating enzymes we examined (Figures 3.2 and 4.1). Therefore, it is possible that regulation of PSGL-1 expression in Tc1 vs. Tc2 cells is not

regulated at the transcription level. Instead, mRNA of PSGL-1 and the enzymes we examined may be degraded more quickly in Tc2 cells or the glycosylating enzymes may not be as active in Tc2 cells compared to Tc1 cells. Interestingly, we did find higher expression of T-bet and CD43 in Tc1 cells compared to Tc2 cells. Even though PSGL-1 and CD43 are both P-selectin ligands, it appears that Tc1 and Tc2 cells express and possibly regulate the expression of these selectin ligands differently. Our observation of T-bet expression being higher in Tc1 cells compared to Tc2 cells is an important finding. Despite growing knowledge of type I and type II regulation in CD4+ T cells, including T-bet expression, very little is known about the transcriptional control of type I and type II phenotypes in CD8+ T cells. T-bet is important for Th1 cell functions, and we suggest from our observations that T-bet is also a key transcription factor for Tc1 cells (10-13). It may be that T-bet is moderatlely expressed in Tc2 cells but is off-set by the expression of another, as yet unknown, transcription factor. For example, T-bet type I activity is off-set by transcription factor GATA binding transcription factor-3 (GATA-3) type II activity in CD4+ T cells (10, 13). Thus, our data, together with studies on CD4+ T cell polarization, suggest that T-bet may be a key transcription factor for type-I signaling in both CD4+ and CD8+ T cells.

When we examined type I polarization of donor and host cells in the Tc1 and Tc2 treated animals, we found that gene expression of IFN–γ was higher than IL-4, which was undetected in many samples. This dominating IFN–γ expression indicates that the host cells in tumors of the Tc1 and Tc2 treated animals are type I cells, overall, and is supported by previous findings suggesting that type I host responses are required for both Tc1 and Tc2 therapy (25-28). We propose that IFN–γ expression may be another indicator of "tumor infiltrating phenotype" for CD8+ T cells, in addition to adhesion molecule expression (See

Chapter 3). Importantly, IFN–γ gene expression by Tc2 cells in the tumors on day 7 after transfer is undetectable in addition to IL-4 expression and all three chemokine receptors we examined. This suggests that Tc2 cells may be down-regulating gene expression after infiltrating into tumors. It is unknown whether Tc1 cells are less susceptible to Treg activity in tumors, but we do know that giving recombinant IL-12, a type I cytokine used to polarize Tc1 cells *in vitro*, can reverse Treg activity in at least one tumor therapy study (29). Treg cells are known to suppress anti-tumor activity and are a critical consideration for ACT therapy (30, 31). Therefore, evaluating Treg activity in Tc1 vs. Tc2 treated animals, as well as the importance of type I cytokines, will be important components of our understanding of Tc1 and Tc2 therapy.

Another important consideration in evaluating the efficacy of Tc1 vs. Tc2 therapy is the role of type-specific or promiscuous chemokines in attracting both donor and host cells to reject the tumor (4, 14-20). Notably, we found that the only type-specific chemokine we investigated that was upregulated in Tc1 vs. Tc2 treated animals was IP-10. Type I chemokine IP-10 is induced through IFN-γ signaling and is expected to recruit predominantly type I T cells (4, 16, 18-20). Because type I T cells are thought to be more effective in clearing tumor cells than type II T cells, several studies have examined the role of IP-10 in the recruitment of tumor infiltrating T cells (18-20). Tumors cells transfected to express IP-10 had increased infiltration by CD8+ T cells (19). We found that IP-10 is highly expressed overall in the tumors of both Tc1 and Tc2 treated animals on days 3 and 7 after donor cell transfer. Because we found that IP-10 is expressed significantly higher in tumors of Tc1 treated animals on day 7, we suggest that Tc1 therapy may enhance the recruitment of type I CD8+ T cells to the tumor site through upregulation of IP-10. However, we did not see an

increase in the number of total lymphocytes or CD8+ cells in tumors of Tc1 vs. Tc2 treated mice on days 3 and 7 (Figure 3.1). We believe that an increase in the number of CD8+ cells recruited to tumors in Tc1 vs. Tc2 treated animals may occur after day 7, especially because IP-10 production in Tc1 treated animals has been shown to be bimodal in nature, declining after approximately 7 days following therapy and then peaking again at approximately 21 days after therapy (20). The observed high gene expression of IP-10 may not affect the number of CD8+ cells recruited to the tumors in Tc1 vs. Tc2 animals but may preferentially recruit type I CD8+ T cells to the tumors. We need to evaluate IP-10 production and the recruitment of type I vs. type II CD8+ cells to the tumors in Tc1 and Tc2 treated animals to address these questions. Expression of IP-10 receptor CXCR3 by tumor infiltrating donor and host cells suggests further that IP-10 may be critical for the tumor infiltration in the Tc1 and Tc2 treated animals.

Our results further suggest that differential expression of chemokine receptors by donor and host cells may also play a role in ACT therapy. When we examined CXCR3 expression we found that it was expressed higher overall by donor and host CD8+ cells in TDLNs and tumors of Tc1 and Tc2 treated animals compared to CCR4 or CCR5 on days 3 and 7 after donor cell transfer. Type I specific receptor CCR5 expression by Tc1 cells was higher than Tc2 cells in TDLNs and tumors on both days 3 and 7, but type II specific receptor CCR4 expression was limited or undetected in both Tc1 and Tc2 cells. These results together with the cytokine expression data suggest that the Tc1 donor cells maintain the Tc1 phenotype *in vivo* through day 7 after transfer but Tc2 cells may convert to a type I phenotype *in vivo* as early as day 3 after transfer. A conversion of Tc2 to a type I phenotype *in vivo* may contribute to the delay or decreased efficacy of Tc2 vs. Tc1 therapy we and

others have observed. Furthermore, Tc2 cells may need to alter their phenotype *in vivo* to express the "tumor infiltrating phenotype" we described previously as being expressed higher on Tc1 compared Tc2 cells.

Overall, these preliminary studies suggest roles for several molecules in the regulation of Tc1 and Tc2 phenotype, as well as the recruitment of CD8+ cells to tumors in Tc1 and Tc2 treated animals. T-bet is a strong candidate for further investigation in the regulation of the Tc1 vs. Tc2 phenotype (See also Chapter 5) (10, 13). PSGL-1 cell surface expression is higher in Tc1 than Tc2 cells, but it does not appear to be due to differential gene expression of either the PSGL-1 molecule itself of the glycosylating enzymes which post-translationally modify PSGL-1 for optimal selectin binding (9, 11, 12). On the other hand, differential expression of the CD43 does appear to be due at least in part to differences in gene expression. In addition, IFN–γ and its expression by donor cells, as expected from work by others, may be important for Tc1 and Tc2 therapy, as well as for promoting recruitment of cells to tumors (25-28). IP-10 expression may be important for the recruitment of type I CD8+ cells in Tc1 vs. Tc2 treated animals, in conjunction with the expression of the CXCR3 chemokine receptor. Future studies will need to confirm these real time RT-PCR observations and evaluate the requirement for these molecules, in order to validate the suggested importance of these molecules in Tc1 vs. Tc2 biology and therapy.

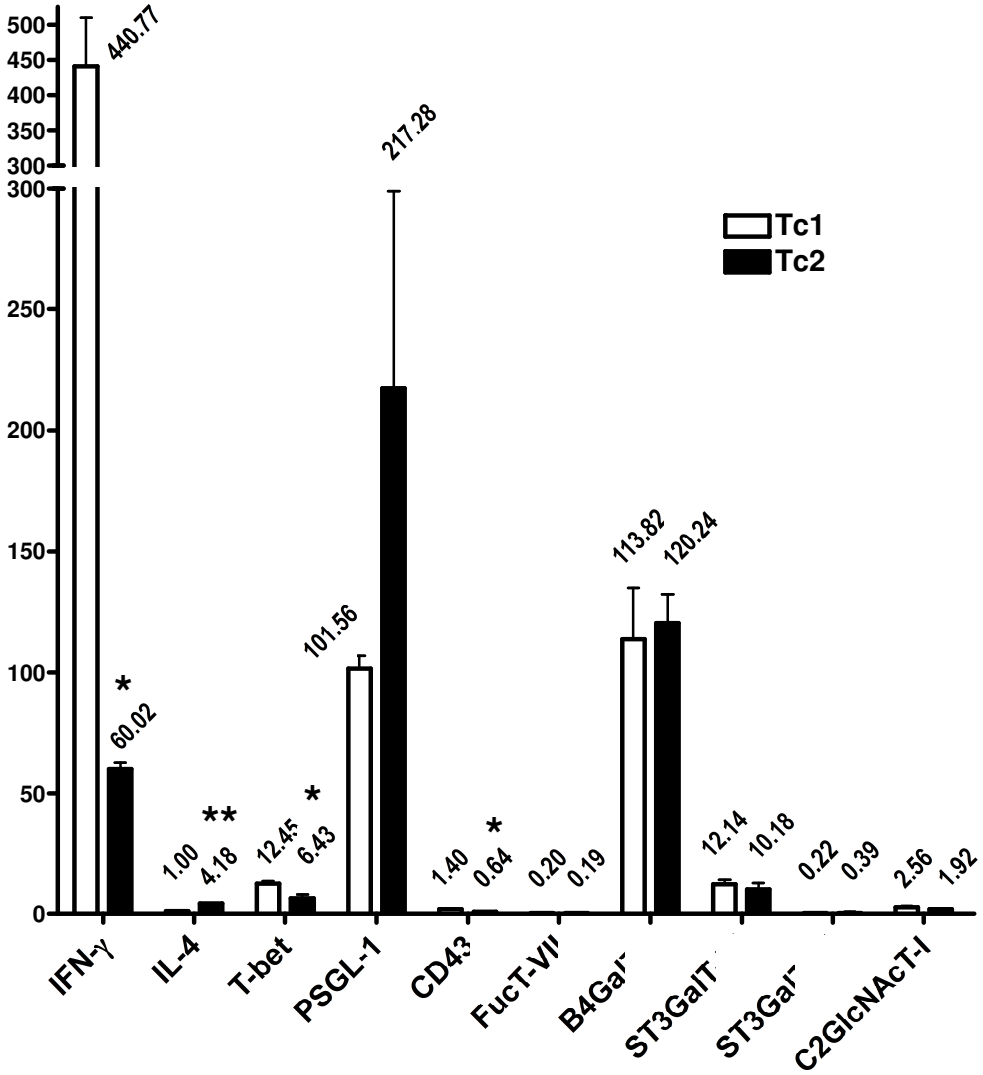

Figure 4.1: Tc1 and Tc2 cell expression of genes important for selectin ligand synthesis. RNA was harvested from cultured Tc1 and Tc2 cells. Expression of the following genes was evaluated using real time RT-PCR: IFN-γ, IL-4, T-bet, PSGL-1, CD43, FucT-VII, B4GalT-I, ST3GalT-IV, ST3GalT-VI, and C2GlcNAcT-I. All gene expression values are shown relative to the IL-4 gene expression by Tc1 cells (set to a value of 1.00). Tc1 vs. Tc2, $p \leq .05$*, $p \leq .01$**.

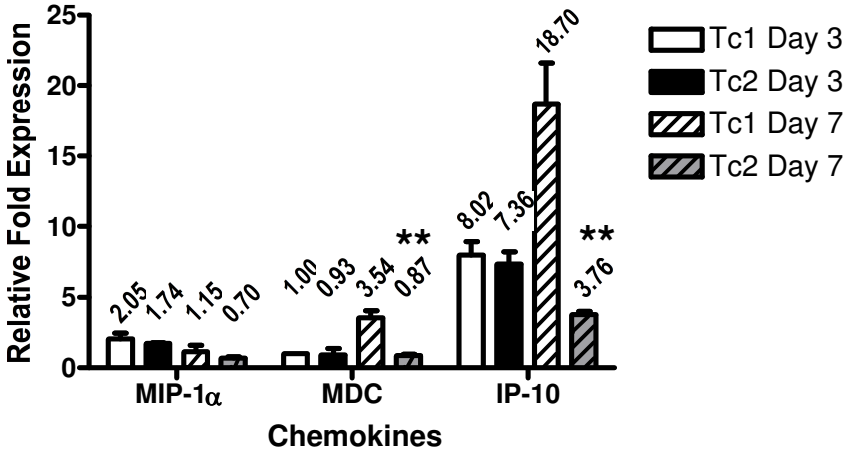

Figure 4.2: Chemokine expression in tumors of Tc1 and Tc2 treated animals. Tumors from Tc1 and Tc2 treated animals were harvested 3 and 7 days after Tc1 and Tc2 treatment. RNA was harvested from the cells in the tumors and the expression of MIP-1α, MDC, and IP-10 was evaluated using real time RT-PCR. All values are shown relative to the MDC gene expression by Tc1 cells on day 3 (set to a value of 1.00). Tc1 vs. Tc2, $p \leq .01$**.

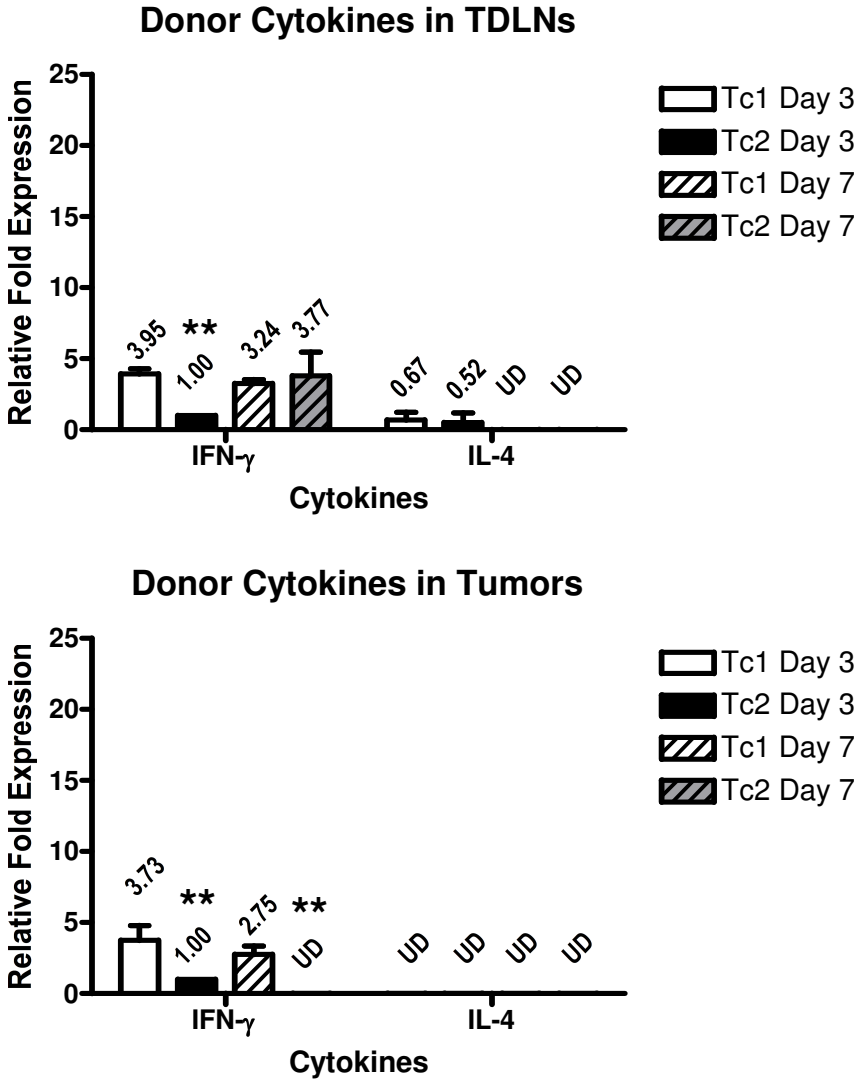

Figure 4.3: Cytokine expression by donor Tc1 and Tc2 cells in Tc1 and Tc2 treated animals. Cells in TDLNs and tumors from Tc1 and Tc2 treated animals were harvested 3 and 7 days after treatment. Cells were sorted for GFP^+CD8^+ cells. RNA was harvested from these cells and expression of IFN-γ and IL-4 was evaluated by real time RT-PCR. All values are shown relative to the IFN-γ gene expression by Tc2 cells on day 3 (set to a value of 1.00). Tc1 vs. Tc2, $p \leq .01$**.

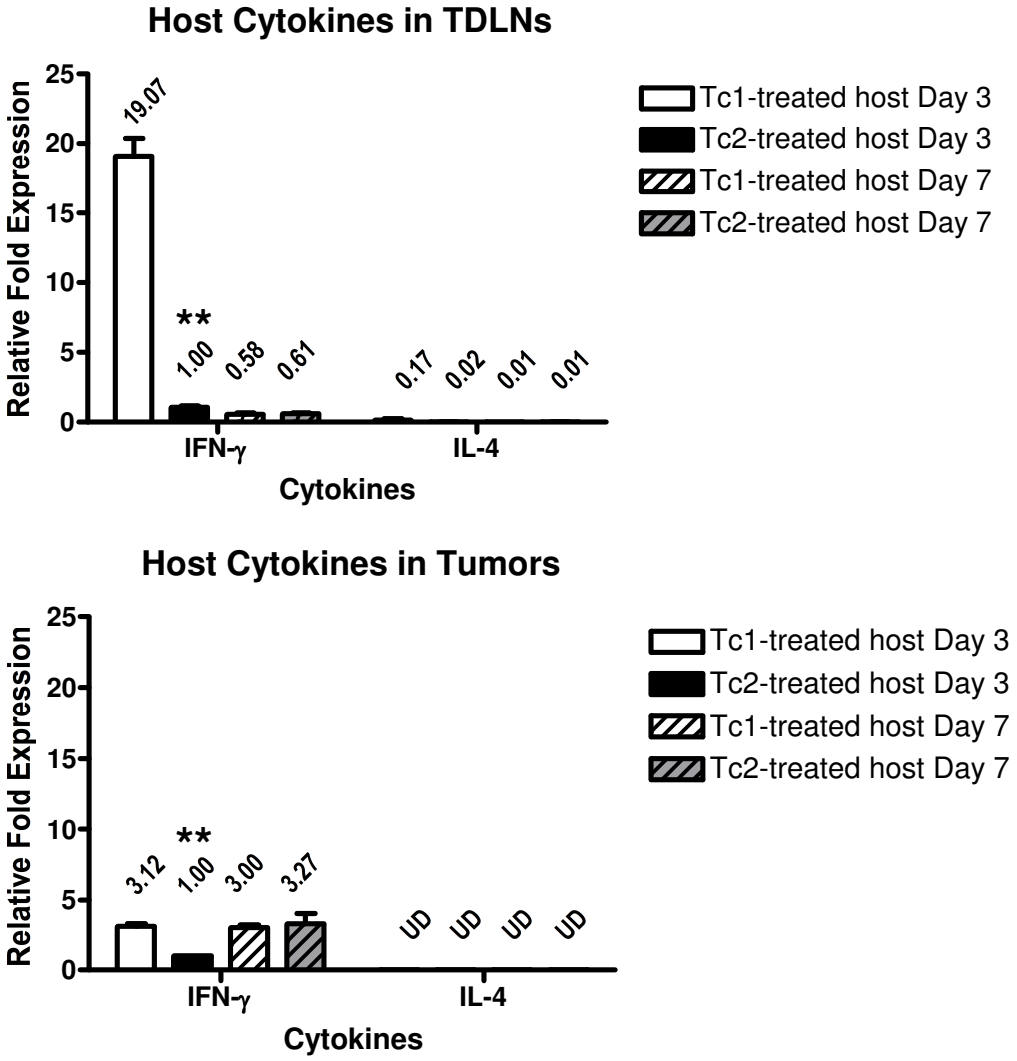

Figure 4.4: Cytokine expression by host CD8+ cells in Tc1 and Tc2 treated animals. Cells in TDLNs and tumors from Tc1 and Tc2 treated animals were harvested 3 and 7 days after treatment. Cells were sorted for $GFP^{-}CD8^{+}$ cells. RNA was harvested from these cells and expression of IFN-γ and IL-4 was evaluated by real time RT-PCR. All values are shown relative to the IFN-γ gene expression by host $CD8^{+}$ cells in Tc2 treated animals on day 3 (set to a value of 1.00). Tc1 vs. Tc2, $p \leq .01$**.

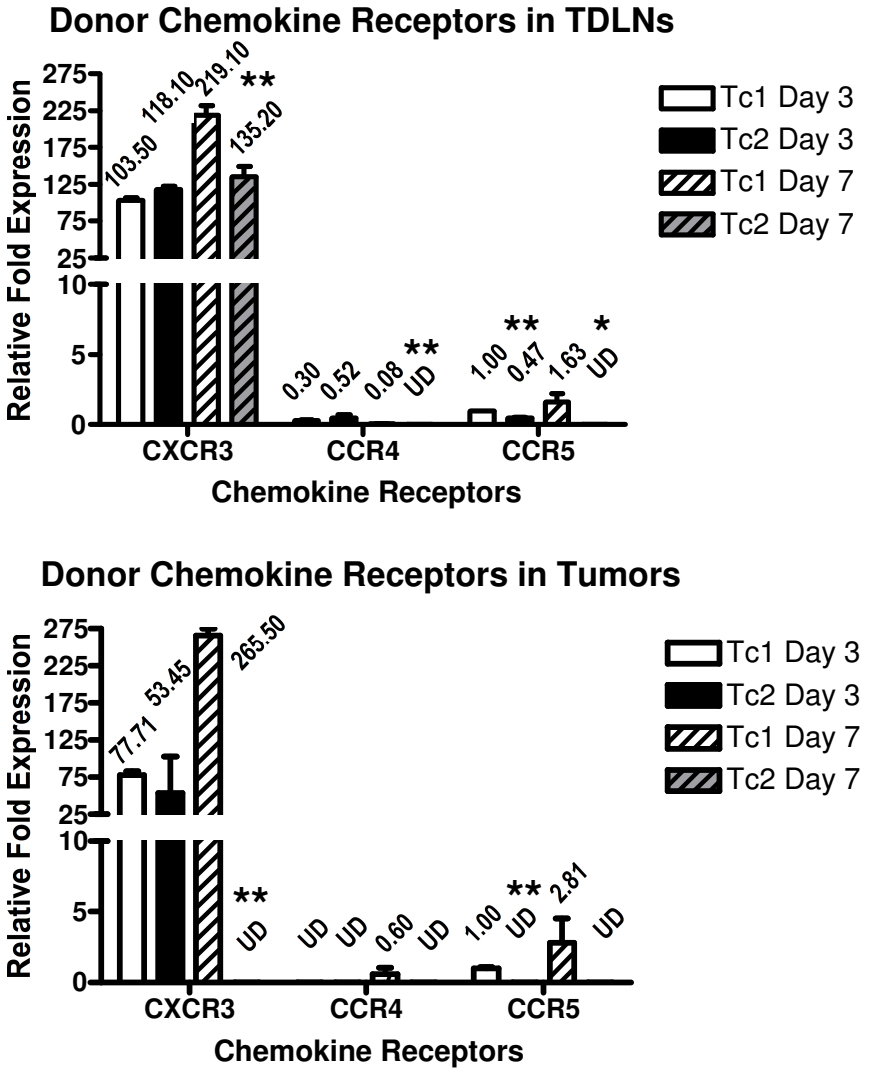

Figure 4.5: Chemokine receptor expression by donor Tc1 and Tc2 cells in Tc1 and Tc2 treated animals. Cells in TDLNs and tumors from Tc1 and Tc2 treated animals were harvested 3 and 7 days after treatment. Cells were sorted for $GFP^{+}CD8^{+}$ cells. RNA was harvested from these cells and expression of CXCR3, CCR4, and CCR5 was evaluated by real time RT-PCR. All values are shown relative to the CCR5 gene expression by Tc1 cells on day 3 (set to a value of 1.00). Tc1 vs. Tc2, $p \leq .05$* $.p \leq .01$**.

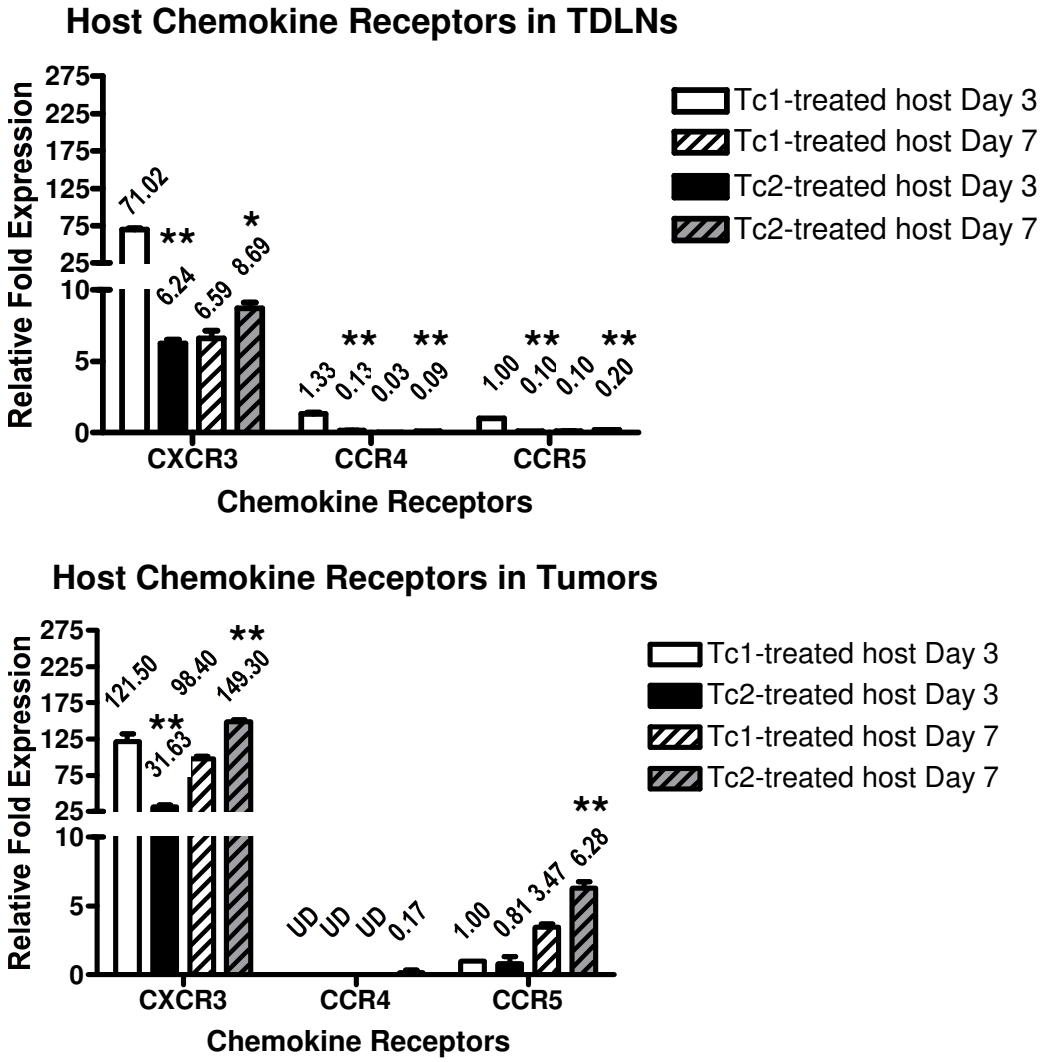

Figure 4.6: Chemokine receptor expression by host CD8+ cells in Tc1 and Tc2 treated animals. Cells in TDLNs and tumors from Tc1 and Tc2 treated animals were harvested 3 and 7 days after treatment. Cells were sorted for $GFP^{-}CD8^{+}$ cells. RNA was harvested from these cells and expression of CXCR3, CCR4, and CCR5 was evaluated by real time RT-PCR. All values are shown relative to the CCR5 gene expression by Tc1 cells on day 3 (set to a value of 1.00). Tc1 vs. Tc2, $p \leq .05$* $.p \leq .01$**.

References

1. Weninger W, Manjunath N, von Andrian UH. Migration and differentiation of $CD8^+$ T cells. Immunol Rev. 2002 Aug;186:221-33. Review.

2. Mora JR, von Andrian UH. T-cell homing specificity and plasticity: new concepts and future challenges. Trends Immunol. 2006 May;27(5):235-43. Review.

3. Lefrancois L. Development, trafficking, and function of memory T-cell subsets. Immunol Rev. 2006 Jun;211:93-103. Review.

4. Viola A, Contento RL, Molon B. T cells and their partners: The chemokine dating agency. Trends Immunol. 2006 Sep;27(9):421-7. Review.

5. Cerwenka A, Morgan TM, Harmsen AG, Dutton RW. Migration kinetics and final destination of type 1 and type 2 CD8 effector cells predict protection against pulmonary virus infection. J Exp Med. 1999 Jan 18;189(2):423-34.

6. Helmich BK, Dutton RW. The role of adoptively transferred CD8 T cells and host cells in the control of the growth of the EG7 thymoma: factors that determine the relative effectiveness and homing properties of Tc1 and Tc2 effectors. J Immunol. 2001 Jun 1;166(11):6500-8.

7. Kemp RA, Ronchese F. Tumor-specific Tc1, but not Tc2, cells deliver protective antitumor immunity. J Immunol. 2001 Dec 1;167(11):6497-502.

8. Nishimura F, Dusak JE, Eguchi J, Zhu X, Gambotto A, Storkus WJ, Okada H. Adoptive transfer of type 1 CTL mediates effective anti-central nervous system tumor response: critical roles of IFN-inducible protein-10. Cancer Res. 2006 Apr 15;66(8):4478-87.

9. Ley K, Kansas GS. Selectins in T-cell recruitment to non-lymphoid tissues and sites of inflammation. Nat Rev Immunol. 2004 May;4(5):325-35. Review.

10. Glimcher LH, Townsend MJ, Sullivan BM, Lord GM. Recent developments in the transcriptional regulation of cytolytic effector cells. Nat Rev Immunol. 2004 Nov;4(11):900-11. Review.

11. Lord GM, Rao RM, Choe H, Sullivan BM, Lichtman AH, Luscinskas FW, Glimcher LH. T-bet is required for optimal proinflammatory CD4+ T-cell trafficking. Blood. 2005 Nov 15;106(10):3432-9.

12. Underhill GH, Zisoulis DG, Kolli KP, Ellies LG, Marth JD, Kansas GS. A crucial role for T-bet in selectin ligand expression in T helper 1 (Th1) cells. Blood. 2005 Dec 1;106(12):3867-73.

13. Agnello D, Lankford CS, Bream J, Morinobu A, Gadina M, O'Shea JJ, Frucht DM. Cytokines and transcription factors that regulate T helper cell differentiation: new players and new insights. J Clin Immunol. 2003 May;23(3):147-61. Review.

14. Andrew DP, Chang MS, McNinch J, Wathen ST, Rihanek M, Tseng J, Spellberg JP, Elias CG 3rd. STCP-1 (MDC) CC chemokine acts specifically on chronically activated Th2 lymphocytes and is produced by monocytes on stimulation with Th2 cytokines IL-4 and IL-13. J Immunol. 1998 Nov 1;161(9):5027-38.

15. Tang HL, Cyster JG. Chemokine Up-regulation and activated T cell attraction by maturing dendritic cells. Science. 1999 Apr 30;284(5415):819-22.

16. Dufour JH, Dziejman M, Liu MT, Leung JH, Lane TE, Luster AD. IFN-gamma-inducible protein 10 (IP-10; CXCL10)-deficient mice reveal a role for IP-10 in effector T cell generation and trafficking. J Immunol. 2002 Apr 1;168(7):3195-204.

17. Maurer M, von Stebut E. Macrophage inflammatory protein-1. Int J Biochem Cell Biol. 2004 Oct;36(10):1882-6. Review.

18. Mohan K, Cordeiro E, Vaci M, McMaster C, Issekutz TB. CXCR3 is required for migration to dermal inflammation by normal and *in vivo* activated T cells: differential requirements by CD4 and CD8 memory subsets. Eur J Immunol. 2005 Jun;35(6):1702-11.

19. Yang X, Chu Y, Wang Y, Zhang R, Xiong S. Targeted *in vivo* expression of IFN-gamma-inducible protein 10 induces specific antitumor activity. J Leukoc Biol. 2006 Dec;80(6):1434-44.

20. Dobrzanski MJ, Reome JB, Hylind JC, Rewers-Felkins KA. CD8-mediated type 1 antitumor responses selectively modulate endogenous differentiated and nondifferentiated T cell localization, activation, and function in progressive breast cancer. J Immunol. 2006 Dec 1;177 (11):8191-201.

21. Schaefer BC, Schaefer ML, Kappler JW, Marrack P, Kedl RM. Observation of antigen-dependent CD8+ T-cell/ dendritic cell interactions *in vivo*. Cell Immunol 2001 Dec 15;214(2):110-22.

22. Pircher H, Michalopoulos EE, Iwamoto A, et al. Molecular analysis of the antigen receptor of virus-specific cytotoxic T cells and identification of a new V alpha family. Eur J Immunol 1987 Dec;17(12):1843-6.

23. Qian, L, Lorena, S, Burgents, M, and Flood, PM. Increased CD8+ T cell Antigenicity of Murine Tumors Increases Tumor Resistance. Manuscript in preparation.

24. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}{}_{T}$ method. Methods 2001 Dec; 25: 402-408.

25. Dobrzanski MJ, Reome JB, Dutton RW. Therapeutic effects of tumor-reactive type 1 and type 2 $CD8^+$ T cell subpopulations in established pulmonary metastases. J Immunol. 1999 Jun 1;162(11):6671-80.

26. Dobrzanski MJ, Reome JB, Dutton RW. Type 1 and type 2 $CD8^+$ effector T cell subpopulations promote long-term tumor immunity and protection to progressively growing tumor. J Immunol. 2000 Jan 15;164(2):916-25.

27. Dobrzanski MJ, Reome JB, Hollenbaugh JA, Hylind JC, Dutton RW. Effector cellderived lymphotoxin alpha and Fas ligand, but not perforin, promote Tc1 and Tc2 effector cell-mediated tumor therapy in established pulmonary metastases. Cancer Res. 2004 Jan 1;64(1):406-14.

28. Dobrzanski MJ, Reome JB, Hollenbaugh JA, Dutton RW. Tc1 and Tc2 effector cell therapy elicit long-term tumor immunity by contrasting mechanisms that result in complementary endogenous type 1 antitumor responses. J Immunol. 2004 Feb 1;172(3):1380-90.

29. Kilinc MO, Aulakh KS, Nair RE, Jones SA, Alard P, Kosiewicz MM, Egilmez NK. Reversing tumor immune suppression with intratumoral IL-12: activation of tumor-associated T effector/memory cells, induction of T suppressor apoptosis, and infiltration of CD8+ T effectors. J Immunol. 2006 Nov 15;177(10):6962-73.

30. Curiel TJ. Tregs and rethinking cancer immunotherapy. J Clin Invest. 2007 May;117(5):1167-74. Review.

31. June CH. Principles of adoptive T cell cancer therapy. J Clin Invest. 2007 May;117(5):1204-12. Review.

Chapter V
Discussion

Study Summary

ACT therapy has been used to treat animals and patients with tumors (1-4). Tc1 cells have been shown to be more effective than Tc2 cells for therapy in several tumor models (5-11). Migration differences between Tc1 and Tc2 cells were previously proposed to contribute to this difference in therapeutic efficacy (6, 7, 10). We developed a model to examine Tc1 vs. Tc2 trafficking *in vivo* (See Chapter 2) (12). Previously in the lab, we transfected EL-4 thymoma tumor cells to express the p33 peptide antigen from lymphocytic choriomeningitis virus (p33.EL-4) (13). In order to evaluate monoclonal $CD8^+$ T cell responses specific for these tumors, we used P14 mice, which are transgenic for the TCR specific for p33 peptide in the context of MHC-I molecule $H\text{-}2D^b$ that is expressed by these p33.EL-4 tumors (14). Other models have used intracellular labeling of amines or staining of cell surface proteins with antibodies to identify antigen-specific donor cells (6, 7, 10). These methods have limitations, so we utilized green fluorescent protein (GFP) expression to track our donor cells (12, 15-17). We crossed UBI.GFP mice that ubiquitously express GFP with the P14 mice, in order to generate a F1 generation of mice that express both GFP and the p33-specific TCR in the $CD8^+$ T cell population (12). We stimulated splenocytes from these mice with peptide under polarizing conditions to generate Tc1 and Tc2 cells for transfer (12). Donor cells were phenotyped before transfer using flow cytometry and real time RT-PCR. Tc1 or Tc2 donor cells were injected i.v. into tumor bearing mice. On days 3 and 7 after transfer, TDLNs and tumors were harvested. The $CD8^+$ T cells were examined for GFP and adhesion molecule expression by flow cytometry.

We found significantly more Tc1 than Tc2 cells in TDLNs and tumors on days 3 and 7 after transfer (Figure 3.1) (12). Both Tc1 and Tc2 donor cells were found in TDLN and

tumor sites of p33 antigen-positive tumors and sites of p33 antigen-negative tumors (Figure 3.5) (12). More importantly, all $CD8^+$ T cells isolated from the tumors on days 3 and 7 after therapy, regardless of host or donor origin and Tc1 or Tc2 phenotype, expressed high levels of adhesion molecules important for T cell migration (Figures 3.3, 3.4, and 3.7) (12). This was the case in p33 antigen-positive and antigen-negative tumors, which suggested that antigen does not alter the adhesion molecule expression of tumor infiltrating $CD8^+$ T cells (Figures 3.6 and 3.7) (12). These cells expressed high levels of CD44, LFA-1, and PSGL-1, suggesting a "tumor infiltrating phenotype" that is required for tumor infiltration by $CD8^+$ T cells (12). Before transfer and 3 days after transfer, Tc1 cells expressed higher levels of the tumor infiltrating phenotype compared to Tc2 cells *in vitro* and in TDLNs (Figure 3.2) (12). Thus, increased Tc1 cell migration to TDLN and infiltration of tumors may be due to the higher expression of the tumor infiltrating phenotype compared to Tc2 cells.

We then used real time RT-PCR to examine the gene expression of molecules which may contribute to the increased adhesion molecule expression on Tc1 cells we observed. Cultured Tc1 and Tc2 cells expressed similar levels of the PSGL-1 gene, as well as similar levels of genes for a variety of selectin glycosylating enzymes (Figure 4.1). The gene expression of CD43, a ligand for P-selectin other than PSGL-1, was higher in Tc1 than Tc2 cells (Figure 4.1). More importantly, the gene expression of transcription factor T-bet was significantly higher in Tc1 than Tc2 cells (Figure 4.1). Therefore, gene transcription of PSGL-1 and the glycosylating enzymes does not appear to contribute to the regulation of the surface expression of PSGL-1 by Tc1 and Tc2 cells. Additionally, our findings suggest that T-bet is a strong candidate as a regulator of Tc1 vs. Tc2 phenotype.

When we used real time RT-PCR to examine the gene expression of cytokines, chemokines, and chemokine receptors by the cells in Tc1 or Tc2 treated animals, we found evidence of a predominant type-I response in both the Tc1 and Tc2 treated groups: IFN-γ, IP-10, and CXCR3 expression. Both donor and host CD8+ cells in tumors of Tc1 and Tc2 treated animals expressed IFN-γ but not IL-4 (Figure 4.3 and 4.4). Chemokine IP-10 was more highly expressed than either MIP-1α or MDC in the tumors of both Tc1 and Tc2 treated animals (Figure 4.2). Chemokine receptor CXCR3, which binds IP-10, was highly expressed by both donor and host CD8+ cells in the tumors (Figure 4.5 and 4.6). Together these data suggest that IFN-γ, IP-10, and CXCR3 may be important molecules in anti-tumor responses, particularly for CD8+ T cell mediated responses.

Therefore, our studies suggest that Tc1 and Tc2 therapies may be differentially effective due to differences in Tc1 and Tc2 donor cell migration and tumor infiltration *in vivo*. These differences may result from differences in the cell surface expression of adhesion molecules. Antigen does not appear to affect the phenotype of the donor cells after transfer, but does increase the number of infiltrating CD8+ cells. Both Tc1 and Tc2 treatment of tumor bearing animals results in type I responses, including the gene expression of IFN-γ, IP-10, and CXCR3, suggesting that differences in Tc1 vs. Tc2 therapy is not due to a qualtitative change in the phenotype of cells in the tumors, but perhaps due to differences in the quantitative expression of a type I phenotype in Tc1 vs. Tc2 treated animals.

Tumor infiltrating phenotype and Tc1 vs. Tc2 therapy

Tc1 therapy is more effective than Tc2 therapy in several tumor models (5-11). We investigated whether differences in Tc1 and Tc2 cell migration and tumor infiltration may contribute to the difference in therapeutic efficacy based on evidence by previous studies that Tc1 and Tc2 cells may migrate differently (12). We showed that in both Tc1 and Tc2 treated tumor bearing animals, the donor and host tumor infiltrating $CD8^+$ T cells were all CD44 high, LFA-1 high, and PSGL-1 high (Figures 3.4 and 3.7) (12). This tumor infiltrating phenotype was more highly expressed by Tc1 and Tc2 cells *in vitro* and on day 3 in TDLNs after transfer (Figures 3.2 and 3.3) (12). We also examined the expression of CD49d and $\alpha 4\beta 7$ integrins but found little or no expression of these molecules by Tc1 and Tc2 cells *in vitro* (Figure 3.2) (12). Since we concentrated our *in vivo* studies on those molecules that showed a significant difference in expression between Tc1 and Tc2 cells prior to transfer, we do not have conclusive data that the expression of these integrin molecules does not change after transfer, particularly for Tc1 cells, which may contribute to the differences in initial migration by Tc1 and Tc2 cells. (This initial migration may be affected by Tc1 or Tc2 cells being held within the lung instead of continuing into circulation.) However, since Tc1 cells migrate and infiltrate tumor more effectively than Tc2 cells, we believe that the difference in Tc1 and Tc2 cell phenotype before transfer contributes to these differences in migration and tumor infiltration. Migration to TDLNs and tumor infiltration by transferred cells has been shown to be important for ACT therapy (1, 18-20). Taken together this suggests that the increased migration and tumor infiltration by Tc1 vs. Tc2 cells *in vivo* contributes to the increased efficacy of Tc1 vs. Tc2 cell therapy.

How the trafficking of Tc1 vs. Tc2 cells may affect therapy is not yet clear. However we do know that host responses are important for both therapies, so the increased number of Tc1 vs. Tc2 cells at TDLNs and tumors may affect host cell responses differently (21-23). One proposed theory is that Tc1 donor cells promote the recruitment of more type I host $CD8^+$ T cells to the tumor sites compared to Tc2 donor cells (23). We found that the number of tumor infiltrating total lymphocytes and $CD8^+$ T cells was the same in both Tc1 and Tc2 treated animals, so Tc1 and Tc2 therapy do not promote different numbers of host cells in our model (Figure 3.1) (12). We do use only $1x10^6$ donor cells, which is sufficient to observe differences in Tc1 vs. Tc2 therapy and trafficking *in vivo*, but this therapeutic dose may not be sufficient to significantly impact the number of tumor infiltrating host $CD8^+$ T cells that has been reported by others (23).

Type I effector T cell responses, particularly because of interferon (IFN)–γ production, and tumor infiltration by T cells have been shown to be important for tumor clearance (3, 21-32). Tc1 cells do produce more IFN-γ than Tc2 cells, and our preliminary studies suggest that CD8+ cells in tumors of both Tc1 and Tc2 treated mice produce IFN–γ, but no IL-4 (Figure 4.3). This suggests that while Tc1 and Tc2 donor cells express different type I vs. type II cytokine genes before transfer, tumor infiltrating CD8+ cells express only type I cytokine gene production (Figure 4.1 and 4.3). A possible role for IFN–γ in T cell tumor infiltration is that IFN–γ promotes the production of chemokine IP-10. Expression of IP-10 by tumor cells has beeen shown to increase tumor infiltration by CD8+ T cells (31). Because IP-10 binds to receptor CXCR3, which is expressed by Tc1 and Tc2 cells before and after transfer, IP-10 may be the key chemokine for recruiting donor cells to the tumor site. More Tc1 cells may infiltrate tumors in response to IP-10 because IP-10 may promote the

recruitment of Type-I cells (31). We did find increased production of IP-10 in the tumors of Tc1 treated animals on day 7 compared to Tc2 treated animals, which supports the proposal that higher IFN-γ production by Tc1 cells in the tumors may promote increased chemokine production, which may in turn preferentially recruit type-I cells (Figure 4.2). Even though we did find a difference in donor cell tumor infiltration, we did not find a difference in the number of tumor infiltrating host $CD8^+$ T cells in Tc1 and Tc2 therapy (Figure 3.1). However, it is important to note that we did not evaluate activity of the infiltrating host cells other than cytokine and chemokine receptor gene expression that suggested type-I host responses in both Tc1 and Tc2 treated animals (Figures 4.4 and 4.6). Other functions of host CD8+ T cells, such as cytolytic activity, may be impacted significantly by the cytokine production of Tc1 vs. Tc2 cells, altering the efficacy of Tc1 and Tc2 therapies.

Type I and type II cytokines have been shown to have different effects on responding anti-tumor effector $CD8^+$ T cells (3, 30). Type I cytokines IFN–γ and interleukin (IL)-12 have been shown to promote tumor rejection by $CD8^+$ T cells, while type II cytokine IL-10 has been shown to suppress the activity of $CD8^+$ T cells responding to tumors (3, 30). Therefore, because IFN–γ along with IL-12 are produced by Tc1 cells and IL-10 is produced by Tc2 cells, the transferred donor cells may promote anti-tumor responses or suppress anti-tumor responses by host cells, respectively. While we and others have found that both Tc1 and Tc2 cells produce IFN–γ, it is likely that the low level of IFN–γ production by Tc2 cells is not sufficient to promote the same effects as the high level of IFN–γ produced by Tc1 cells (45).

IFN–γ promotes several aspects of $CD8^+$ T cell activity (33). IFN–γ signals through signal transactivator of T cells (STAT)-1 and promotes IL-12 production, which in turn

promotes further IFN–γ production through STAT-4 signaling (33). Together, IFN–γ and IL-12 can promote expression of adhesion molecules by T cells (34). Therefore, the IFN–γ production in animals with Tc1 or Tc2 therapy may promote the upregulation of adhesion molecules on host and donor $CD8^+$ T cells to the tumor infiltrating phenotype. This type I STAT signaling could contribute to the observed change in Tc2 adhesion molecule expression from *in vitro* to *in vivo* phenotype. *In vitro* and on day 3 in TDLNs after transfer, Tc2 cells expressed adhesion molecules but not at high levels (12). On day 7 in TDLNs after transfer, Tc2 cells expressed high levels of adhesion molecules (12). Since Tc2 cells produce low levels of IFN–γ, it is likely that IFN–γ production by host cells rather than donor Tc2 cells leads to the enhanced adhesion molecule expression on donor Tc2 cells.

The cytolytic activity of $CD8^+$ T cells is also affected by IFN–γ and IL-12 signaling (33). During T cell priming, APCs provide the necessary primary (MHC:peptide complex) and secondary (co-stimulatory molecules) signaling needed for T cell activation, but recently it has been reported that a third signal is needed for optimal effector cell activity, which may explain previous reports of incomplete priming or non-responsiveness of T cells in animals injected with tumor cells (34-44). Both IFN–γ and IL-12 can provide this important third signal *in vitro* and *in vivo* (37-40, 42, 44). Therefore, while donor cells are activated before transfer, the cytokine production by Tc1 cells may contribute to the needed third signal for both host and donor cell activity, leading to increased tumor clearance early after Tc1 cell therapy. Host $CD8^+$ T cells may also contribute IFN–γ and IL-12 to the third signal for effector cell activity. In contrast, Tc2 production of IL-10 may suppress this signaling, thereby suppressing the activity of host $CD8^+$ T cells within tumors, leading to diminished efficacy in Tc2 vs. Tc1 therapy. This IL-10 production by Tc2 cells may work in synergy

with IL-10 production by regulatory T (Treg) cells to suppress host anti-tumor activity in situ rather than tumor infiltration (3, 30). Additionally, it is not known whether Tregs affect Tc1 vs. Tc2 cell activity equally. Because Tregs are known to accumulate in tumors, the sensitivity and response of donor Tc1 vs. Tc2 cells to Treg activity may be an important factor determining the efficacy of Tc1 vs. Tc2 therapy (3, 30).

Why more Tc1 than Tc2 cells in TDLNs and tumors?

Our study, along with data from other studies, clearly demonstrates that more Tc1 than Tc2 cells are found in TDLNs and tumors. There are several possible explanations as to why more Tc1 than Tc2 cells are found at these sites: 1) Tc1 cells undergo expansion and/or Tc2 cells undergo contraction after transfer; 2) More cells in Tc1 cultures express the appropriate activation/adhesion phenotype for trafficking *in vivo* than cells in Tc2 cultures; and 3) Tc2 cells express a phenotype sufficient for migration and tumor infiltration but exhibit less than optimal adhesion molecule function *in vivo*. We address these explanations and what role antigen may play in $CD8^+$ T cell tumor infiltration in the following discussion.

Proliferation, Apoptosis, and Activation (or Memory) Phenotype

During T cell responses, there is an expansion phase dominated by proliferation and a contraction phase dominated by apoptosis regulating the number of responding T cells (increasing and decreasing cell numbers, respectively). Donor cells have proliferation potential at the time that they are transferred into the tumor-bearing recipient mice, and since the donor cells can come in contact with antigen after transfer *in vivo*, it is expected that the donor cells would proliferate. As anticipated, studies have shown evidence suggesting that both tumor-specific Tc1 and Tc2 cells proliferate after transfer into tumor-bearing mice (13, 23). It is therefore unlikely that differences in expansion of Tc1 vs. Tc2 cells can explain our differences in the number of donor cells in TDLNs and tumors, since we and others have evidence demonstrating that Tc1 and Tc2 cell populations proliferate with similar efficiencies *in vitro* and *ex vivo* (Flood PM, unpublished observations, 45). Therefore, differences in proliferation rates are not likely to contribute to the difference in the number of

Tc1 vs. Tc2 cells in TDLNs and tumors after donor cell transfer. However, studies have not reported on Tc1 vs. Tc2 cell apoptosis. It is possible that the Tc2 cells undergo a contraction phase earlier after transfer than the Tc1 cells.

Signals that promote T cell survival can also contribute to the development of memory cell subsets (46-48). We have evidence to suggest that Tc1 and Tc2 cell cultures have different activation phenotypes, potentially reflecting the development of different memory cell populations (12). Following activation, T cells upregulate CD44 and downregulate CD62L (46-48). However, as cells develop into memory cell subsets, these cells remain $CD44^+$ but can be distinguished by CD62L expression (46-48). Effector memory (T_{EM}) cells are CD62L- and central memory (T_{CM}) cells are $CD62L^+$ (46-48). Presumably, this applies to "classical" $CD8^+$ T cells. Whether this applies similarly to polarized $CD8^+$ T cells is not clear. We found that Tc1 cell cultures are $CD44^+$ and are $CD62L^+$/high (Figure 3.2) (12). Tc2 cell cultures are predominantly $CD44^+$ but CD62L- (Figure 3.2) (12). Therefore, Tc1 cultures may contain more cells with the T_{CM} phenotype, and Tc2 cultures may have more cells with the T_{EM} phenotype. Studies have shown that $CD44^+$ T cells and $CD62L^+$ T cells are more effective in adoptive cell transfer therapy (1, 19, 20). Therefore, this CD44 and CD62L phenotype of Tc1 vs. Tc2 cultures likely contributes to differences in Tc1 vs. Tc2 therapy. Importantly, since both CD44 and CD62L are adhesion molecules, this further suggests that trafficking of cells may contribute to whether T cells are effective for tumor therapy and differences in Tc1 vs. Tc2 therapy may be due in part to the difference in phenotype.

Regulation of adhesion molecule expression by Tc1 and Tc2 cells

Adhesion molecules including integrins, selectins, and their ligands regulate T cell trafficking (17, 34, 47, 49-53). As stated above, we examined the expression of adhesion molecules by Tc1 and Tc2 cells *in vitro* before transfer and also after transfer. In addition to CD62L and CD44 expression, we also determined the expression of LFA-1 and PSGL-1. Tumor infiltrating $CD8^+$ T cells all expressed high levels of CD44, LFA-1, and PSGL-1 (Figure 3.4 and 3.7) (12). It is not clear whether each of these molecules is individually important for tumor infiltration or whether ligand binding for one adhesion molecule promotes the expression of the other adhesion molecules. Although we now know that Tc1 cells express higher levels of LFA-1, CD44, CD62L, and PSGL-1 compared to Tc2 cells *in vitro*, we have not determined why Tc1 cells express higher levels of these molecules (Figure 3.2). Expression of adhesion molecules can be regulated at several levels, including gene transcription and signaling or post-translational modification (34, 51, 54). Below we discuss examples transcription factors and signaling that can promote the upregulation of adhesion molecules and examples only of modifications made to PSGL-1 and CD44.

Gene Transcription and Signaling

Key molecules and transcription factors which regulate the expression of adhesion molecules have been identified, however we have limited knowledge of type I vs. type II response regulation of these molecules (33). This limited knowledge is further complicated by the current lack of understanding of type I vs. type II signaling in $CD8^+$ T cells, because most studies examining signaling and gene expression in T cells have focused on the $CD4^+$ populations. Methods to generate Tc1 and Tc2 cells *in vitro* provide some insight into what

transcription factors and signaling pathways are important for type I and type II polarization in $CD8^+$ T cells. In a study examining calcium vs. protein kinase C (PKC) signaling to polarize $CD8^+$ T cells, Noble and Kemeny found that Tc1 and Tc2 cells are generated by increased calcium and PKC signaling following TCR engagement, respectively (55). Additionally, when generating Tc1 and Tc2 cells *in vitro*, recombinant type-specific cytokines and antibodies against type-specific cytokines are used (5-13, 21-24, 56). Type I polarization includes adding rIL-12 and blocking IL-4, while type II polarization includes adding IL-4 and blocking IL-12 and IFN–γ (5-13, 21-24, 56). Cytokine signaling through cytokine receptors leads to STAT signaling (33, 57). Type I signaling goes through STAT1 and STAT4, but type II signaling goes through STAT6 (33, 57). Downstream of STAT1 and STAT4 is T-bet (33, 57). T-bet is perhaps the key transcription factor regulating type I $CD4^+$ T (Th1) cell activity (57). T-bet has also been identified in $CD8^+$ T cells and shown to regulate genes involved in cytolytic activity (33). In type II $CD4^+$ (Th2) cells, GATA-binding transcription factor 3 (GATA-3) is a key transcription factor (57). Unlike T-bet, GATA-3 is not expressed in $CD8^+$ T cells and actually promotes the development of $CD4^+$ T cells (58). Thus, T-bet is of particular interest as a candidate for differences in Tc1 vs. Tc2 signaling and cellular activity.

T-bet is possibly the key transcription factor responsible for the increased level of adhesion molecule expression on Tc1 vs. Tc2 cells. T-bet has been shown to be critical for migration and expression of enzymes important for adhesion molecule expression by Th1 cells (34, 59-61). IL-12 and IFN–γ expressed by type I cells bind their respective receptors and signal through STAT-4 and STAT-1, respectively (33, 57). This promotes T-bet activity, which in turn promotes IFN–γ and IL-12 productions (33, 57). This cyclic signaling is

believed to occur in $CD8^+$ T cells as well (33). Therefore, by adding IL-12 in Tc1 cultures and blocking IL-12 and IFN–γ in Tc2 cultures, T-bet signaling is likely promoted in Tc1 and blocked in Tc2 cells. Our preliminary studies do suggest that Tc1 cells express higher levels of T-bet gene expression (Figure 4.1). Furthermore, if T-bet is indeed upregulated in Tc1 cells, then adhesion molecule expression regulated by Tc1 cells may be upregulated through T-bet.

Post-Translational Modification

Adhesion molecule expression is not only regulated at the transcriptional level but also at post-translational modification (34, 51, 54, 62). For example, both CD44 and PSGL-1 undergo post-translational modification (34, 51, 54, 62). In order for PSGL-1 to be active, it must be glycosylated at numerous sites (34). Several enzymes are known to be important for PSGL-1 expression in type I T cells, including core 2 β1,6 N-acetylglucosaminyl-transferase I, fucosyltransferase VII, and α 2,3-sialyltransferase IV (34, 62, 63). Considering the possible role for T-bet signaling in Tc1 cell function, it is interesting that T-bet knockout studies have shown that these enzymes are regulated by T-bet (59, 60). Consequently, our observation of higher expression of PSGL-1 on Tc1 cells *in vitro* and on day 3 after transfer, could be due to upregulation of T-bet as well as the enzymes responsible for PSGL-1 expression in Tc1 cells compared to Tc2 cells. Although our preliminary studies do suggest a higher level of T-bet gene expression in Tc1 vs. Tc2 cells, the gene expression of these enzymes does not appear to be different between Tc1 and Tc2 cells (Figure 4.1). Therefore, through T-bet signaling, Tc1 cells may upregulate translatation or activity of these enzymes

more than Tc2 cells, leading to higher expression of PSGL-1. The role T-bet plays in the post-translational modification of CD62L, CD44, and LFA-1 is not known.

CD44 molecules are class I transmembrane glycoproteins encoded by one gene (54). The isoforms are generated through alternative splicing and post-translational modifications (54, 64). As could be expected due to the variety of CD44 isoforms, CD44 has a variety of functions (54, 64, 65). CD44 expression is also regulated at the cell surface, through proteolytic cleavage (54, 64). The enzyme mediating the cleavage has not yet been identified (54). CD44 is upregulated after T cell activation and is also expressed on memory cells (46-48). We found that both Tc1 and Tc2 donor cells express CD44 *in vitro* after stimulation and also at TDLNs and tumors after transfer into tumor-bearing mice (Figures 3.2-3.4) (12). Tc1 cells expressed higher levels of CD44 *in vitro* and 3 days after transfer in the TDLNs (Figures 3.2 and 3.3) (12). Because of the multidisciplinary functions and isoforms of CD44, it is difficult to determine the impact of higher CD44 expression on Tc1 vs. Tc2 cells. We believe that higher CD44 expression does play a role in increased migration and tumor infiltration by Tc1 vs. Tc2 cells *in vivo*. Importantly, we do not know whether Tc1 and Tc2 cells express similar levels of the various CD44 isoforms, only that CD44 expression overall is higher in Tc1 cells *in vitro* and in TDLNs on day 3 after transfer.

Adhesion molecule function in Tc1 and Tc2 cells

We have limited knowledge concerning the regulation of adhesion molecule function in $CD8^+$ T cells (17, 34, 47, 49-53). There is some evidence of how adhesion molecules function in $CD4^+$ T cells, although not necessarily in polarized Th1 or Th2 cells (17, 34, 47, 49-53). Additionally, most studies have focused on the change in adhesion molecule

function in naïve vs. activated T cells, not subsequent regulation of function after T cells have been activated. In general, upon activation, T cell adhesion molecules convert to the active conformation, redistribute on the cell surface, and associate with intracellular signaling molecules (49-52, 54). While we have shown that Tc1 and Tc2 cells express different levels of adhesion molecules CD62L, CD44, LFA-1, and PSGL-1 *in vitro* but similar levels of adhesion molecules within tumors, we do not know whether these adhesion molecules function equally in both Tc1 and Tc2 cells (Figures 3.2-3.4) (12). Therefore, in addition to the difference in expression, Tc1 and Tc2 cells may migrate differently due to differences in adhesion molecule functions. Since expressed adhesion molecules function not only to mediate adhesion but also to convey signaling, the function of adhesion molecules is not purely based on the expression level of the adhesion molecule, but also on several other factors (34, 47, 49-53, 66). Many adhesion molecules have inactive and active conformations, as well as the distribution of the adhesion molecule in active conformation, particularly within the immunological synapse, is critical for adhesion and signaling (66-69). At each of these levels, the function of adhesion molecules can be controlled. Below, we've focused on the functions of LFA-1.

Adhesion

Most adhesion molecules are known for their adhesive properties. Integrins, including LFA-1 have active and inactive conformations, which result in the alteration of the affinity of the LFA-1 α_L and β_2 subunits (49-51, 66- 68). LFA-1 changes from inactive to active conformation as a result of chemokines binding to their receptors (66). This inside-out signaling stimulated by the binding of chemokines involves the small GTPase RAP1,

phosphatidylinositol 3-kinase (PI3K), and mitogen-activated protein kinase pathways (66). LFA-1 is believed to have three affinity states termed low, intermediate, and high affinities (68). It has been proposed that each of these affinity states may have different functions (68). The low affinity state may mediate loose or rolling adhesion, permitting transient interaction between LFA-1 and its ligands, such as ICAM-I (68). Intermediate affinity is proposed more variety in interactions between LFA-1 and its ligands (68). Importantly, the high affinity state is proposed to be the optimal state for LFA-1 mediated signaling, which is described below (68).

Studies have shown that Th1 and Th2 cells respond differently to chemokines, which is not surprising because the cells express different chemokine receptors (70, 71). However, what is surprising is that Th1 cells have been shown to migrate across endothelial cells independently of chemokines (72). This chemokine-independent transmigration was blocked by antibodies against either CD44 or LFA-1 and was blocked by inhibitors of PI3K, suggesting that although LFA-1 affinity is enhanced after chemokine binding, the affinity of LFA-1 may be sufficient for function without increasing the affinity through chemokine signaling (72). Notably, this did not occur in Th2 cells, suggesting that Th1 cells require different signals for migration than Th2 cells (72). Perhaps Tc1 and Tc2 cells also differ in chemokine-independent regulation of transendothelial migration through LFA-1 and CD44. It is possible that Tc1 and Tc2 cells may express LFA-1 at similar levels (valency) but not necessarily in the same affinity state or even require the same chemokine signaling to change the affinity of LFA-1. We have shown evidence that more cells in Tc1 cultures express high levels of LFA-1 than cells in Tc2 cultures, but we do not know whether Tc1 and Tc2 cells

with high LFA-1 expression have similar LFA-1 affinity states or respond similarly to signaling dependent or independent of chemokines (Figure 3.2) (12).

Signaling

Many adhesion molecules also have signaling functions and may act as co-receptors for signaling molecules. Clustering of molecules is important for signaling transduction. Both LFA-1 and CD44 undergo clustering (54, 68). As mentioned above, it is proposed that the high affinity of LFA-1 is the conformation that mediates signaling (68). Lateral mobility of LFA-1 in lipid rafts permits the clustering of LFA-1 molecules (68, 73). Clustering of LFA-1 has been examined primarily during the priming of naïve T cells. During T cell activation, LFA-1 localizes within the central supramolecular activation complex (SMAC) and then moves to the peripheral SMAC, where Talin is localized (66, 68). When LFA-1 is clustered and binding to intercellular adhesion molecule-1 (ICAM-I), it is proposed that Talin anchors the clusters to the cytoskeleton but molecules can still interact with LFA-1 to transduce signaling (66, 68, 73). LFA-1 binding to ICAM-I results in calcium signaling and promotes further signaling and transcription activation (66, 68). The dynamic function of LFA-1 to mediate adhesion and signaling, particularly in the SMAC, and the different affinities of LFA-1 conformation states demonstrates the complexity of adhesion molecules function and regulation in T cells. Again, while we demonstrated that Tc1 cells express higher LFA-1 *in vitro* compared to Tc2 cells, we don't know whether the localization of LFA-1 in lipid rafts or mobility in and out of SMACs is different between Tc1 and Tc2 cells (Figure 3.2) (12).

What is the role of antigen in Tc1 and Tc2 cell trafficking?

Antigen presentation to T cells not only activates and restimulates the T cells, which leads to changes in gene expression and phenotype, but also regulates T cell function (45, 74). Specifically, target cell antigen recognition by antigen-specific T cells promotes target cell killing and antigen recognition of antigen expression on endothelial cells promotes transendothelial migration (74). *In vitro* studies have shown that antigen expression by endothelial cells affects whether T cells initially kill the endothelium, arrest along the endothelium during migration, or continue to proceed in transendothelial migration into inflamed tissue (74). Tumor infiltration by $CD8^+$ T cells has been shown to be enhanced by antigen expression by tumor cells (75-78). Previously, it was unclear whether Tc1 or Tc2 cells migrated or infiltrated tumors differently, including whether they respond differently to antigen expression by tumors.

In order to examine the role of antigen expression by tumors in Tc1 vs. Tc2 trafficking *in vivo*, we injected mice with p33-expressing EL-4 tumor (p33.EL-4) cells in the right flank and parental (p33 negative) EL-4 tumor cells in the left flank. Tc1 and Tc2 cells generated from GFPxP14 splenocytes were then transferred i.v. into the tumor-bearing mice 5-7 days later. We then compared the number and phenotype of donor Tc1 and Tc2 cells in TDLNs and tumors 3 and 7 days after transfer. We found donor cells in both EL-4 and p33.EL-4 TDLNs and tumors on both days (Figure 3.5) (12). Tc1 cell numbers were higher compared to Tc2 cell numbers at the same site (Figure 3.5) (12). Again, we conclude that Tc1 cells have more efficient trafficking than Tc2 cells. Interestingly, we found that the number of both Tc1 and Tc2 cells was higher in the p33.EL-4 vs. EL-4 TDLNs and tumors, suggesting that while the expression of antigen does not appear to determine the differences

in Tc1 vs. Tc2 cell migration, it does play a role in the migration and tumor infiltration of antigen-specific Tc1 and Tc2 cells (Figure 3.5) (12). Expression of antigen may promote the expansion of donor cells, inhibit the contraction of donor cells, or mediate the migration and tumor infiltration process, thus leading to the observed increase in Tc1 and Tc2 donor cells in TDLNs and tumor sites of p33.EL-4 tumors. It is also important to note that host $CD8^+$ T cell expression of Vβ8 TCR, which is the TCR expressed by donor cells specific for p33 antigen, was higher in the host $CD8^+$ T cells isolated from p33.EL-4 tumors, also supporting a role for antigen expression in the recruitment of endogenous $CD8^+$ T cells (data not shown) (12). Whether endothelial cells sufficiently present antigen to stimulate proliferation is still in question. Although antigen exposure can promote changes in T cell phenotype, we found no significant differences in the phenotypes of donor cells in the p33 antigen-positive vs. antigen-negative sites (Figure 3.6) (12). It is not clear whether antigen expression by tumors affected the Tc1 and Tc2 cells similarly other than the increased number of cells in the TDLNs and tumors.

Antigen expression by endothelial cells and transendothelial migration

As stated above, the expression of antigen by endothelial cells impacts T cell interaction with the endothelial cells (74). Using several antigen-specific T cell models, $CD4^+$ and $CD8^+$ T cells have been shown to transmigrate across activated endothelium presenting cognate antigen more efficiently than activated endothelium not expressing cognate antigen (79-81). Antigen-specific $CD4^+$ T cells migrated across IFN–γ activated endothelial cell layers *in vitro* and did not proliferate, and those T cells that proliferated in response to antigen expression on endothelial cells did not migrate (79). Two studies

examining the response of antigen-specific $CD8^+$ T cells have suggested that antigen expression by endothelial cells may regulate the responsiveness of $CD8^+$ T cells (80-81). Effector $CD8^+$ T cells killed cytokine-activated peptide pulsed endothelial cells but did not kill resting peptide pulsed endothelial cells (80). Also, $CD8^+$ T cells rested for two weeks after restimulation were examined for migration properties (80). These $CD8^+$ T cells migrated readily across cytokine-activated endothelial cell layers but not across resting endothelial cells, resting peptide-pulsed endothelial cells, or even activated peptide-pulsed endothelial cells (80). This suggested that antigen presentation by endothelial cells may inhibit T cell migration, but this study was limited to only those $CD8^+$ T cells which had been resting and likely progressed into memory cells, not effector cells. Additionally, while this study was conducted *in vitro*, another study conducted by the same group to examine the role of antigen in T cell transendothelial migration *in vivo*, they found that antigen presentation by endothelial cells promoted $CD8^+$ T cell diapedesis (81). Whether antigen definitively promotes transendothelial migration by activated $CD8^+$ T cell is still being investigated. Our study and others demonstrate that antigen does affect tumor infiltration, although the exact mechanism is unknown (Figure 3.5) (12, 75-78). It is certainly unknown whether polarized Tc1 and Tc2 cells migrate differently across endothelium presenting antigen.

Antigen expression and tumor infiltration

Previous studies have shown that antigen expression by tumors can regulate the infiltration and distribution of antigen-specific T cells in a tumor (75-78). An early study by Boissonas et al. that examined the role of antigen in $CD8^+$ tumor responses found that activated antigen-specific $CD8^+$ T cells when transferred into mice bearing antigen-negative

tumors still infiltrated into the tumors despite the lack of cognate antigen expression (75). Another study using a different model showed that indeed activated T cells, both $CD4^+$ and $CD8^+$ cells, are able to infiltrate cognate antigen-negative tumors, provided that the cells are primed elsewhere, i.e. *in vitro* or at another site *in vivo* (76).

More recent studies have examined the actual movement of adoptively transferred $CD8^+$ T cells within antigen-positive and antigen-negative tumors using two-photon microscopy (77, 78). Antigen presentation by tumors was shown to promote continued migration of antigen-specific $CD8^+$ T cells (77). Boissonas et al conducted a similar study where antigen-specific $CD8^+$ T cells were shown to arrest when in contact with antigen-positive cells, presumably to mediate cell killing, and then migration was restored and upon contact with another tumor cell, the $CD8^+$ T cells arrested, again (78). As a result, Boissonas et al. proposed a model for the role of antigen in the $CD8^+$ T cell tumor infiltration and tumor cell killing where the cycle of migration, arrest, and killing continues until the T cells are exhausted or suppressed (78). The movement of the antigen-specific $CD8^+$ T cells in this study was very interesting. Early after transfer, the donor cells in the antigen-positive tumor had very specific-somewhat limited movement, while the donor cells in antigen-negative tumors had more random movement (78). Later after transfer and after significant tumor cell killing, the donor cells in the antigen-positive tumors had increased movement, comparable to the movement of the donor cells in antigen-negative tumors (78). Importantly, this study and others have documented increased tumor infiltration by donor cells in antigen-positive vs. antigen-negative tumors (75-78).

We found the same results in our study: donor Tc1 and Tc2 cells infiltrate the antigen-positive tumors in higher numbers compared to the antigen-negative tumors (Figure

3.5) (12). The before-mentioned study also found that the donor cells appear to infiltrate tumors form the peripheral edges and then infiltrate further into the center of the tumor (78). Donor cells infiltrated only the peripheral edges of the antigen-negative tumors, but infiltrated further into the center of the antigen-positive tumor (78). It would be interesting to determine whether Tc1 and Tc2 cells penetrate into the center of the antigen-positive tumors in a similar pattern. Because we considered tumor infiltrating cells to include those cells which we could collect by simply harvesting the tumor, it is possible that many of the donor cells we identified were located within the tumor vasculature. More Tc2 cells may arrest in the tumor vasculature compared to Tc1 cells, since we know that the mechanisms of transendothelial migration for Th1 and Th2 cells appear to differ and Tc1 cells appear to have higher migration efficiency overall *in vivo*. Also, it would be interesting to find whether Tc1 and Tc2 cells only penetrate into the peripheral edges of the antigen-negative tumors.

How does this apply to ACT therapy?

Our study suggests that indeed differences between Tc1 and Tc2 therapies may be due to the difference in migration properties of Tc1 and Tc2 cells, including their adhesion molecule expression (Figures 3.1-3.4) (12). Because $CD8^+$ T cells are being used for tumor therapy in patients, Tc1 and Tc2 tumor therapy models offer a unique approach for understanding $CD8^+$ T cell migration and tumor infiltration *in vivo*, particularly how $CD8^+$ T cell trafficking affects the efficacy of ACT therapy (1-12, 21-24). For example, all the tumor infiltrating $CD8^+$ T cells express a tumor infiltrating phenotype: CD44 high, LFA-1 high, and PSGL-1 high (Figures 3.4 and 3.7) (12). Expression of CD44, LFA-1, and PSGL-1 may be important for tumor infiltration by mediating loose and tight adhesion followed by transendothelial migration into the tumors. Several studies have suggested that these adhesion molecules work together to mediate adhesion (82-84). For example, crosslinking of PSGL-1 has been shown to stimulate clustering of LFA-1 and subsequent binding to ICAM-1 in Th1 cells *in vitro* (84). We do not yet know whether these adhesion molecules work in concert, in sequence, or individually. We did find that both donor and host $CD8^+$ T cells in the tumor express the tumor infiltrating phenotype, this suggests that cells cultured *in vitro* for tumor therapy need to express this tumor infiltrating phenotype, either before transfer or by acquiring the phenotype *in vivo*, in order to effectively migrate to and infiltrate the tumor (Figures 3.4 and 3.7) (12).

We propose that adhesion molecule expression and antigen specificity of T cells used in ACT therapy is an important consideration when preparing the cells for transfer. Our study and those by others have clearly demonstrated that donor cell expression of CD62L, CD44, LFA-1, and PSGL-1 is important for promoting migration of donor cells and tumor

rejection (Figures 3.2-3.4) (1, 2, 12, 18- 20, 85-89). Transferred cells must have the appropriate phenotype and state of activation, neither naïve nor late effector cells (1-4, 30). The donor cells should also be specific for tumor antigens that are expressed by tumor cells (1-4, 30). Development of antigen loss variants will complicate the antigen-specific response by transferred donor cells (1-4, 30). However, we have corroborating evidence that activated T cells will infiltrate tumor not expressing the cognate antigen (12, 75-78). The lack of antigen expression may limit the efficacy of therapy by permitting premature exhaustion or contraction of the donor cell populations (1, 2).

While ACT therapy certainly is feasible in patients, it is important to remember that treatment protocols for individual patients will need to be tailored to their needs (4, 90). The current paradigm for treatment considered by tumor immunologists is that immunotherapies, including ACT therapy, will probably have to be combined with more traditional treatments and other immunotherapies (4, 90). For example, patients will likely still require surgery, when possible, to remove as much of a tumor as possible, followed by conventional methods of radiation or chemotherapy to control the growth of residual tumor cells (90). Immunotherapies may require a complex approach of promoting effector immune responses while diminishing suppressive immune responses (1-4, 30, 90, 91). Dendritic cell vaccines, ACT therapies, and even doses of antibodies may need to be coupled with lymphodepletion or cytokine therapy to inhibit suppressors of the response, such as Tregs (1-4, 30, 90-92). Additionally, patients may be genetically prone to suppressive immune responses (for example IL-10 production), which may require more aggressive approaches to inhibiting immunosuppressive activity (93).

Conclusions

Tc1 cells had previously been shown to deliver more effective tumor therapy compared to Tc2 cells (5-11). Data suggested that Tc1 cells may migrate more efficiently than Tc2 cells after transfer into tumor-bearing mice (6, 7,10). We developed a model to examine the migration and tumor infiltration of these donor cells *in vivo*, as well as examine whether antigen expression increased tumor infiltration (See Chapters 2 and 3) (12). We found that Tc1 cells were more abundant in TDLNs and tumors than Tc2 cells, even in sites of antigen-negative tumors (Figures 3.1 and 3.5) (12). We phenotyped the adhesion molecule expression of the donor cells before and after transfer (Figures 3.2-3.4) (12). Tc1 cells expressed higher levels of CD62L, CD44, LFA-1, and PSGL-1 *in vitro* compared to Tc2 cells (Figure 3.2) (12). After three days *in vivo*, the Tc1 cells still expressed higher levels of these molecules compared to Tc2 cells (Figures 3.3 and 3.4) (12). On day 7 after transfer, only CD62L expression was higher on Tc1 cells than Tc2 cells (Figures 3.3 and 3.4) (12). All of the $CD8^+$ T cells in the tumors expressed high levels of CD44, LFA-1, and PSGL-1 (Figures 3.4 and 3.7) (12). We define this adhesion molecule expression as a tumor infiltrating phenotype, because this phenotype was expressed by both donor and host $CD8^+$ T cells in the antigen-positive and antigen-negative tumors of Tc1 and Tc2 treated animals (Figures 3.4 and 3.7) (12). The expression of cognate antigen by the tumors did not appear to affect the expression of this tumor infiltrating phenotype but did affect the number of infiltrating Tc1 and Tc2 cells (Figures 3.5 and 3.6) (12). These findings are important for advancing our understanding of adoptive cell therapies in the treatment of tumors, particularly the importance of generating donor cells with the appropriate phenotype to facilitate the necessary migration and tumor infiltration after transfer.

We believe that Tc1 cells migrate and infiltrate tumors more efficiently than Tc2 cells and that the differences in the expression of a tumor infiltrating phenotype before transfer contributes to the observed differences in Tc1 vs. Tc2 cell trafficking. However, our work suggests many future studies: 1) We still need to demonstrate the requirement for the tumor infiltration phenotype by donor cells, perhaps by sorting for Tc1 and Tc2 cells that express this phenotype *in vitro* before transfer and comparing Tc1 vs. Tc2 cell trafficking. 2) How Tc1 cells regulate higher expression of these adhesion molecules or whether the efficiency of adhesion molecule function is different for Tc1 and Tc2 cells is unknown. Regulation of gene expression by T-bet, resulting in expression of different isoforms and modifications of the adhesion molecules, is a strong candidate for transcriptional differences between Tc1 and Tc2 cells. This is supported by the increased expression of T-bet we observed in Tc1 cells compared to Tc2 cells, but T-bet activity still needs to be confirmed. 3) Differences in mobility of adhesion molecules in lipid rafts and in SMAC formation is also another possible explanation for differences in Tc1 and Tc2 cell trafficking. 4) Furthermore, antigen expression and chemokine expression, particularly CXCR3 ligand IP-10, by tumors may affect Tc1 and Tc2 cell trafficking differently. In general, it is still unclear how antigen promotes tumor infiltration by $CD8^+$ T cells or which chemokines are responsible for tumor infiltration by CD8+ T cells, especially donor cells.

Although we have contributed significantly to our knowledge of Tc1 vs. Tc2 biology and the use of polarized cells for ACT therapy, many questions remain. Future studies addressing these questions will begin to provide invaluable information for improving ACT therapies for use in clinical trials as well as the contrasting role of Tc1 vs. Tc2 cells. Perhaps Tc1 and Tc2 cells may be appropriate for different therapies, migrating preferentially to

different tissues or responding more effectively against different diseases. Undoubtedly, this is a solid beginning to broad future for investigating Tc1 vs. Tc2 trafficking and the use of Tc1 and Tc2 cells for therapies.

References

1. Gattinoni L, Powell DJ Jr, Rosenberg SA, Restifo NP. Adoptive immunotherapy for cancer: building on success. Nat Rev Immunol. 2006 May;6(5):383-93. Review.

2. Klebanoff CA, Gattinoni L, Restifo NP. CD8+ T-cell memory in tumor immunology and immunotherapy. Immunol Rev. 2006 Jun;211:214-24. Review.

3. Neeson P, Paterson Y. Effects of the tumor microenvironment on the efficacy of tumor immunotherapy. Immunol Invest. 2006;35(3-4):359-94. Review.

4. June CH. Principles of adoptive T cell cancer therapy. J Clin Invest. 2007 May;117(5):1204-12. Review.

5. Dobrzanski MJ, Reome JB, Dutton RW. Therapeutic effects of tumor-reactive type 1 and type 2 CD8+ T cell subpopulations in established pulmonary metastases. J Immunol. 1999 Jun 1;162(11):6671-80.

6. Helmich BK, Dutton RW. The role of adoptively transferred CD8 T cells and host cells in the control of the growth of the EG7 thymoma: factors that determine the relative effectiveness and homing properties of Tc1 and Tc2 effectors. J Immunol. 2001 Jun 1;166(11):6500-8.

7. Kemp RA, Ronchese F. Tumor-specific Tc1, but not Tc2, cells deliver protective antitumor immunity. J Immunol. 2001 Dec 1;167(11):6497-502.

8. Chamoto K, Kosaka A, Tsuji T, Matsuzaki J, Sato T, Takeshima T, Iwakabe K, Togashi Y, Koda T, Nishimura T. Critical role of the Th1/Tc1 circuit for the generation of tumor-specific CTL during tumor eradication *in vivo* by Th1-cell therapy. Cancer Sci. 2003 Oct;94(10):924-8.

9. Do JS, Choi YH, Shin SH, Yi HK, Hwang PH, Nam SY. Committed memory effector type 2 cytotoxic T (Tc2) cells are ineffective in protective anti-tumor immunity. Immunol Lett. 2004 Aug 15;95(1):77-84.

10. Nishimura F, Dusak JE, Eguchi J, Zhu X, Gambotto A, Storkus WJ, Okada H. Adoptive transfer of type 1 CTL mediates effective anti-central nervous system tumor response: critical roles of IFN-inducible protein-10. Cancer Res. 2006 Apr 15;66(8):4478-87.

11. Dobrzanski MJ, Reome JB, Hylind JC, Rewers-Felkins KA. CD8-mediated type 1 antitumor responses selectively modulate endogenous differentiated and nondifferentiated T cell localization, activation, and function in progressive breast cancer. J Immunol. 2006 Dec 1;177 (11):8191-201.

12. Burgents ML, Flood PM. Tc1 Cells Infiltrate Tumor Sites More Efficiently than Tc2 Cells Due to Enhanced Expression of a Tumor Infiltrating Phenotype. Submitted.

13. Qian L, Lorena S, Burgents M, and Flood PM. Increased CD8+ T cell Antigenicity of Murine Tumors Increases Tumor Resistance. Manuscript in preparation.

14. Pircher H, Michalopoulos EE, Iwamoto A, Ohashi PS, Baenziger J, Hengartner H, Zinkernagel RM, Mak TW. Molecular analysis of the antigen receptor of virus-specific cytotoxic T cells and identification of a new V alpha family. Eur J Immunol. 1987 Dec;17(12):1843-6.

15. Manjunath N, Shankar P, Stockton B, Dubey PD, Lieberman J, von Andrian UH. A transgenic mouse model to analyze CD8(+) effector T cell differentiation *in vivo*. Proc Natl Acad Sci U S A. 1999 Nov 23;96(24):13932-7.

16. Schaefer BC, Schaefer ML, Kappler JW, Marrack P, Kedl RM. Observation of antigen-dependent CD8+ T-cell/ dendritic cell interactions *in vivo*. Cell Immunol. 2001 Dec 15;214(2):110-22.

17. Weninger W, Manjunath N, von Andrian UH. Migration and differentiation of CD8+ T cells. Immunol Rev. 2002 Aug;186:221-33. Review.

18. Gattinoni L, Klebanoff CA, Palmer DC, Wrzesinski C, Kerstann K, Yu Z, Finkelstein SE, Theoret MR, Rosenberg SA, Restifo NP.. Acquisition of full effector function *in vitro* paradoxically impairs the *in vivo* antitumor efficacy of adoptively transferred CD8+ T cells. J Clin Invest. 2005 Jun;115(6):1616-26.

19. Klebanoff CA, Gattinoni L, Torabi-Parizi P, Kerstann K, Cardones AR, Finkelstein SE, Palmer DC, Antony PA, Hwang ST, Rosenberg SA, Waldmann TA, Restifo NP. Central memory self/tumorreactive CD8+ T cells confer superior antitumor immunity compared with effector memory T cells. Proc Natl Acad Sci. U S A 2005 Jul 5;102(27):9571-6.

20. Hinrichs CS, Gattinoni L, Restifo NP. Programming CD8+ T cells for effective immunotherapy. Curr Opin Immunol. 2006 Jun;18(3):363-70. Review.

21. Dobrzanski MJ, Reome JB, Hollenbaugh JA, Hylind JC, Dutton RW. Effector cellderived lymphotoxin alpha and Fas ligand, but not perforin, promote Tc1 and Tc2 effector cell-mediated tumor therapy in established pulmonary metastases. Cancer Res. 2004 Jan 1;64(1):406-14.

22. Dobrzanski MJ, Reome JB, Hollenbaugh JA, Dutton RW. Tc1 and Tc2 effector cell therapy elicit long-term tumor immunity by contrasting mechanisms that result in complementary endogenous type 1 antitumor responses. J Immunol. 2004 Feb 1;172(3):1380-90.

23. Hollenbaugh JA, Dutton RW. IFN-gamma regulates donor CD8 T cell expansion, migration, and leads to apoptosis of cells of a solid tumor. J Immunol. 2006 Sep 1;177(5):3004-11.

24. Dobrzanski MJ, Reome JB, Dutton RW. Immunopotentiating role of IFN-gamma in early and late stages of type 1 CD8 effector cell-mediated tumor rejection. Clin Immunol. 2001 Jan;98(1):70-84.

25. Nakajima C, Uekusa Y, Iwasaki M, Yamaguchi N, Mukai T, Gao P, Tomura M, Ono S, Tsujimura T, Fujiwara H, Hamaoka T. A role of interferon-gamma (IFN-gamma) in tumor immunity: T cells with the capacity to reject tumor cells are generated but fail to migrate to tumor sites in IFN-gamma-deficient mice. Cancer Res. 2001 Apr 15;61(8):3399-405.

26. Huang H, Liu Y, Xiang J. Synergistic effect of adoptive T-cell therapy and intratumoral interferon gamma-inducible protein-10 transgene expression in treatment of established tumors. Cell Immunol. 2002 May-Jun;217(1-2):12-22.

27. Mitola S, Strasly M, Prato M, Ghia P, Bussolino F. IL-12 regulates an endothelial cell-lymphocyte network: effect on metalloproteinase-9 production. J Immunol. 2003 Oct 1;171(7):3725-33.

28. Huang H, Xiang J. Synergistic effect of lymphotactin and interferon gamma-inducible protein-10 transgene expression in T-cell localization and adoptive T-cell therapy of tumors. Int J Cancer. 2004 May 10;109(6):817-25.

29. Ikeda H, Chamoto K, Tsuji T, Suzuki Y, Wakita D, Takeshima T, Nishimura T. The critical role of type-1 innate and acquired immunity in tumor immunotherapy. Cancer Sci. 2004 Sep;95(9):697-703. Review.

30. Gajewski TF, Meng Y, Blank C, Brown I, Kacha A, Kline J, Harlin H. Immune resistance orchestrated by the tumor microenvironment. Immunol Rev. 2006 Oct;213:131-45. Review.

31. Yang X, Chu Y, Wang Y, Zhang R, Xiong S. Targeted *in vivo* expression of IFN-gamma-inducible protein 10 induces specific antitumor activity. J Leukoc Biol. 2006 Dec;80(6):1434-44.

32. Gorbachev AV, Kobayashi H, Kudo D, Tannenbaum CS, Finke JH, Shu S, Farber JM, Fairchild RL. CXC chemokine ligand 9/monokine induced by IFN-gamma production by tumor cells is critical for T cell-mediated suppression of cutaneous tumors. J Immunol. 2007 Feb 15;178(4):2278-86.

33. Glimcher LH, Townsend MJ, Sullivan BM, Lord GM. Recent developments in the transcriptional regulation of cytolytic effector cells. Nat Rev Immunol. 2004 Nov;4(11):900-11. Review.

34. Ley K, Kansas GS. Selectins in T-cell recruitment to non-lymphoid tissues and sites of inflammation. Nat Rev Immunol. 2004 May;4(5):325-35. Review.

35. Prevost-Blondel A, Zimmermann C, Stemmer C, Kulmburg P, Rosenthal FM, Pircher H. Tumor-infiltrating lymphocytes exhibiting high *ex vivo* cytolytic activity fail to prevent murine melanoma tumor growth *in vivo*. J Immunol. 1998 Sep 1;161(5):2187-94.

36. Shrikant P, Mescher MF. Control of syngeneic tumor growth by activation of CD8+ T cells: efficacy is limited by migration away from the site and induction of nonresponsiveness. J Immunol. 1999 Mar 1;162(5):2858-66.

37. Schmidt CS, Mescher MF. Peptide antigen priming of naive, but not memory, CD8 T cells requires a third signal that can be provided by IL-12. J Immunol. 2002 Jun 1;168(11):5521-9.

38. Valenzuela J, Schmidt C, Mescher M. The roles of IL-12 in providing a third signal for clonal expansion of naive CD8 T cells. J Immunol. 2002 Dec 15;169(12):6842-9.

39. Curtsinger JM, Johnson CM, Mescher MF. CD8 T cell clonal expansion and development of effector function require prolonged exposure to antigen, costimulation, and signal 3 cytokine. J Immunol. 2003 Nov 15;171(10):5165-71.

40. Curtsinger JM, Valenzuela JO, Agarwal P, Lins D, Mescher MF. Type I IFNs provide a third signal to CD8 T cells to stimulate clonal expansion and differentiation. J Immunol. 2005 Apr 15;174(8):4465-9.

41. Curtsinger JM, Lins DC, Johnson CM, Mescher MF. Signal 3 tolerant CD8 T cells degranulate in response to antigen but lack granzyme B to mediate cytolysis. J Immunol. 2005 Oct 1;175(7):4392-9.

42. Mescher MF, Curtsinger JM, Agarwal P, Casey KA, Gerner M, Hammerbeck CD, Popescu F, Xiao Z. Signals required for programming effector and memory development by CD8+ T cells. Immunol Rev. 2006 Jun;211:81-92. Review.

43. Hargadon KM, Brinkman CC, Sheasley-O'neill SL, Nichols LA, Bullock TN, Engelhard VH. Incomplete differentiation of antigen-specific CD8 T cells in tumor-draining lymph nodes. J Immunol. 2006 Nov 1;177(9):6081-90.

44. Curtsinger JM, Gerner MY, Lins DC, Mescher MF. Signal 3 availability limits the CD8 T cell response to a solid tumor. J Immunol. 2007 Jun 1;178(11):6752-60.

45. Kemp RA, Backstrom BT, Ronchese F. The phenotype of type 1 and type 2 CD8+ T cells activated *in vitro* is affected by culture conditions and correlates with effector activity. Immunology. 2005 Jul;115(3):315-24.

46. Wherry EJ, Teichgraber V, Becker TC, Masopust D, Kaech SM, Antia R, von Andrian UH, Ahmed R. Lineage relationship and protective immunity of memory CD8 T cell subsets. Nat Immunol. 2003 Mar;4(3):225-34. Review.

47. Lefrancois L. Development, trafficking, and function of memory T-cell subsets. Immunol Rev. 2006 Jun;211:93-103. Review.

48. Haring JS, Badovinac VP, Harty JT. Inflaming the CD8+ T cell response. Immunity. 2006 Jul;25(1):19-29. Review.

49. Hogg N, Laschinger M, Giles K, McDowall A. T-cell integrins: more than just sticking points. J Cell Sci. 2003 Dec 1;116(Pt 23):4695-705. Review.

50. Hogg N, Smith A, McDowall A, Giles K, Stanley P, Laschinger M, Henderson R. How T cells use LFA-1 to attach and migrate. Immunol Lett. 2004 Mar 29;92(1-2):51-4. Review.

51. Pribila JT, Quale AC, Mueller KL, Shimizu Y. Integrins and T cell-mediated immunity. Annu Rev Immunol. 2004;22:157-80. Review.

52. Steeber DA, Venturi GM, Tedder TF. A new twist to the leukocyte adhesion cascade: intimate cooperation is key. Trends Immunol. 2005 Jan;26(1):9-12. Review.

53. Mora JR, von Andrian UH. T-cell homing specificity and plasticity: new concepts and future challenges. Trends Immunol. 2006 May;27(5):235-43. Review.

54. Ponta H, Sherman L, Herrlich PA. CD44: from adhesion molecules to signalling regulators. Nat Rev Mol Cell Biol. 2003 Jan;4(1):33-45. Review.

55. Noble A, Truman JP, Vyas B, Vukmanovic-Stejic M, Hirst WJ, Kemeny DM. The balance of protein kinase C and calcium signaling directs T cell subset development. J Immunol. 2000 Feb 15;164(4):1807-13.3.

56. Sad S, Marcotte R, Mosmann TR. Cytokine-induced differentiation of precursor mouse CD8+ T cells into cytotoxic CD8+ T cells secreting Th1 or Th2 cytokines. Immunity. 1995 Mar;2(3):271-9.

57. Agnello D, Lankford CS, Bream J, Morinobu A, Gadina M, O'Shea JJ, Frucht DM. Cytokines and transcription factors that regulate T helper cell differentiation: new players and new insights. J Clin Immunol. 2003 May;23(3):147-61. Review.

58. Hernandez-Hoyos G, Anderson MK, Wang C, Rothenberg EV, Alberola-Ila J. GATA-3 expression is controlled by TCR signals and regulates CD4/CD8 differentiation. Immunity. 2003 Jul;19(1):83-94.

59. Lord GM, Rao RM, Choe H, Sullivan BM, Lichtman AH, Luscinskas FW, Glimcher LH. T-bet is required for optimal proinflammatory CD4+ T-cell trafficking. Blood. 2005 Nov 15;106(10):3432-9.

60. Underhill GH, Zisoulis DG, Kolli KP, Ellies LG, Marth JD, Kansas GS. A crucial role for T-bet in selectin ligand expression in T helper 1 (Th1) cells. Blood. 2005 Dec 1;106(12):3867-73.

61. Chen GY, Osada H, Santamaria-Babi LF, Kannagi R. Interaction of GATA-3/T-bet transcription factors regulates expression of sialyl Lewis X homing receptors on Th1/Th2 lymphocytes. Proc Natl Acad Sci U S A. 2006 Nov 7;103(45):16894-9.

62. Daniels MA, Hogquist KA, Jameson SC. Sweet 'n' sour: the impact of differential glycosylation on T cell responses. Nat Immunol. 2002 Oct;3(10):903-10. Review.

63. Smithson G, Rogers CE, Smith PL, Scheidegger EP, Petryniak B, Myers JT, Kim DS, Homeister JW, Lowe JB. Fuc-TVII is required for T helper 1 and T cytotoxic 1 lymphocyte selectin ligand expression and recruitment in inflammation, and together with Fuc-TIV regulates naive T cell trafficking to lymph nodes. J Exp Med. 2001 Sep 3;194(5):601-14.

64. Martin TA, Harrison G, Mansel RE, Jiang WG. The role of the CD44/ezrin complex in cancer metastasis. Crit Rev Oncol Hematol. 2003 May;46(2):165-86. Review.

65. Toole BP. Hyaluronan: from extracellular glue to pericellular cue. Nat Rev Cancer. 2004 Jul;4(7):528-39. Review.

66. Kinashi T. Intracellular signalling controlling integrin activation in lymphocytes. Nat Rev Immunol. 2005 Jul;5(7):546-59. Review.

67. Hogg N, Henderson R, Leitinger B, McDowall A, Porter J, Stanley P. Mechanisms contributing to the activity of integrins on leukocytes. Immunol Rev. 2002 Aug;186:164-71. Review.

68. Dustin ML, Bivona TG, Philips MR. Membranes as messengers in T cell adhesion signaling. Nat Immunol. 2004 Apr;5(4):363-72. Review.

69. Lin J, Miller MJ, Shaw AS. The c-SMAC: sorting it all out (or in). J Cell Biol. 2005 Jul 18;170(2):177-82. Review.

70. Siveke JT, Hamann A. T helper 1 and T helper 2 cells respond differentially to chemokines. J Immunol. 1998 Jan 15;160(2):550-4.

71. Syrbe U, Siveke J, Hamann A. Th1/Th2 subsets: distinct differences in homing and chemokine receptor expression? Springer Semin Immunopathol. 1999;21(3):263-85. Review.

72. Katakai T, Hara T, Sugai M, Gonda H, Nambu Y, Matsuda E, Agata Y, Shimizu A. Chemokine-independent preference for T-helper-1 cells in transendothelial migration. J Biol Chem. 2002 Dec 27;277(52):50948-58.

73. Cairo CW, Mirchev R, Golan DE. Cytoskeletal regulation couples LFA-1 conformational changes to receptor lateral mobility and clustering. Immunity. 2006 Aug;25(2):297-308.

74. Marelli-Berg FM, Jarmin SJ. Antigen presentation by the endothelium: a green light for antigen-specific T cell trafficking? Immunol Lett. 2004 May 15;93(2-3):109-13. Review.

75. Boissonnas A, Combadiere C, Lavergne E, Maho M, Blanc C, Debre P, Combadiere B. Antigen distribution drives programmed antitumor CD8 cell migration and determines its efficiency. J Immunol. 2004 Jul 1;173(1):222-9.

76. Joncker NT, Marloie MA, Chernysheva A, Lonchay C, Cuff S, Klijanienko J, Sigal-Zafrani B, Vincent-Salomon A, Sastre X, Lantz O. Antigen-independent accumulation of activated effector/memory T lymphocytes into human and murine tumors. Int J Cancer. 2006 Mar 1;118(5):1205-14.

77. Mrass P, Takano H, Ng LG, Daxini S, Lasaro MO, Iparraguirre A, Cavanagh LL, von Andrian UH, Ertl HC, Haydon PG, Weninger W. Random migration precedes stable target cell interactions of tumor-infiltrating T cells. J Exp Med. 2006 Nov 27;203(12):2749-61.

78. Boissonnas A, Fetler L, Zeelenberg IS, Hugues S, Amigorena S. *In vivo* imaging of cytotoxic T cell infiltration and elimination of a solid tumor. J Exp Med. 2007 Feb 19;204(2):345-56.

79. Marelli-Berg FM, Frasca L, Weng L, Lombardi G, Lechler RI. Antigen recognition influences transendothelial migration of CD4+ T cells. J Immunol. 1999 Jan 15;162(2):696-703.

80. Marelli-Berg FM, Scott D, Bartok I, Peek E, Dyson J, Lechler RI. Activated murine endothelial cells have reduced immunogenicity for CD8+ T cells: a mechanism of immunoregulation? J Immunol. 2000 Oct 15;165(8):4182-9.

81. Marelli-Berg FM, James MJ, Dangerfield J, Dyson J, Millrain M, Scott D, Simpson E, Nourshargh S, Lechler RI. Cognate recognition of the endothelium induces HY-specific CD8+ T-lymphocyte transendothelial migration (diapedesis) *in vivo*. Blood. 2004 Apr 15;103(8):3111-6.

82. Steeber DA, Tang ML, Green NE, Zhang XQ, Sloane JE, Tedder TF. Leukocyte entry into sites of inflammation requires overlapping interactions between the L-selectin and ICAM-1 pathways. J Immunol. 1999 Aug 15;163(4):2176-86.

83. Kadono T, Venturi GM, Steeber DA, Tedder TF. Leukocyte rolling velocities and migration are optimized by cooperative L-selectin and intercellular adhesion molecule-1 functions. J Immunol. 2002 Oct 15;169(8):4542-50.

84. Atarashi K, Hirata T, Matsumoto M, Kanemitsu N, Miyasaka M. Rolling of Th1 cells via P-selectin glycoprotein ligand-1 stimulates LFA-1-mediated cell binding to ICAM-1. J Immunol. 2005 Feb 1;174(3):1424-32.

85. Kagamu H, Touhalisky JE, Plautz GE, Krauss JC, Shu S. Isolation based on L-selectin expression of immune effector T cells derived from tumor-draining lymph nodes. Cancer Res. 1996 Oct 1;56(19):4338-42.

86. Tanigawa K, Takeshita N, Craig RA, Phillips K, Knibbs RN, Chang AE, Stoolman LM. Tumor-specific responses in lymph nodes draining murine sarcomas are concentrated in cells expressing P-selectin binding sites. J Immunol. 2001 Sep 15;167(6):3089-98.

87. Blank C, Brown I, Kacha AK, Markiewicz MA, Gajewski TF. ICAM-1 contributes to but is not essential for tumor antigen cross-priming and CD8+ T cell-mediated tumor rejection *in vivo*. J Immunol. 2005 Mar 15;174(6):3416-20.

88. Jenkinson SR, Williams NA, Morgan DJ. The role of intercellular adhesion molecule-1/LFA-1 interactions in the generation of tumor-specific CD8+ T cell responses. J Immunol. 2005 Mar 15;174(6):3401-7.

89. Yamada M, Yanaba K, Hasegawa M, Matsushita Y, Horikawa M, Komura K, Matsushita T, Kawasuji A, Fujita T, Takehara K, Steeber DA, Tedder TF, Sato S. Regulation of local and metastatic hostmediated anti-tumour mechanisms by L-selectin and intercellular adhesion molecule-1. Clin Exp Immunol. 2006 Feb;143(2):216-27.

90. Bhardwaj N. Harnessing the immune system to treat cancer. J Clin Invest. 2007 May;117(5):1130-6. Review.

91. Curiel TJ. Tregs and rethinking cancer immunotherapy. J Clin Invest. 2007 May;117(5):1167-74. Review.

92. Gilboa E. DC-based cancer vaccines. J Clin Invest. 2007 May;117(5):1195-203. Review.

93. Jin P, Panelli MC, Marincola FM, Wang E. Cytokine polymorphism and its possible impact on cancer. Immunol Res. 2004;30(2):181-90. Review.

APPENDICES

APPENDIX 1:
CD43 Expression on Tc1 and Tc2 cells in TDLNs and tumors of Tc1 and Tc2 treated animals

Earlier we demonstrated that Tc1 and Tc2 cells express different levels of adhesion molecules *in vitro*, including PSGL-1, particularly after restimulation. After transfer into tumor bearing animals, Tc1 cells expressed higher levels of PSGL-1 on day 3 in TDLNs compared to Tc2 cells, but on day 7, both Tc1 and Tc2 cells expressed high levels of PSGL-1. In both antigen-positive and antigen-negative tumors, all donor and host CD8+ cells expressed high levels of PSGL-1. Therefore, we concluded that high PSGL-1 expression is a phenotype of tumor infiltrating CD8+ cells, one characteristic of a "tumor infiltrating phenotype" (Chapter 3). In order to evaluate why Tc1 cells have higher cell surface levels of PSGL-1, we examined the gene expression of PSGL-1 as well as the gene expression of molecules known to regulate PSGL-1 in CD4 T cells, using real time RT-PCR (1-4). We also examined the gene expression of another selectin ligand, CD43 (1). While we did not find a difference between the gene expression of PSGL-1 by Tc1 and Tc2 cells, we did find a significant difference between the gene expression of CD43 in Tc1 vs. Tc2 cells.

CD43 is one of the ligands for P-selectin (1). Due to the increased gene expression of CD43 in Tc1 vs. Tc2 cells *in vitro*, we examined the cell surface levels of CD43 on Tc1 and Tc2 cells *in vivo* after transfer. According to the methods described in Chapter 3, we transferred GFPxP14 Tc1 or Tc2 cells into tumor bearing animals and subsequently evaluated CD43 expression by flow cytometry. We found that on day 3 after donor cell transfer, Tc1 cells expressed significantly more CD43 in TDLNs of antigen-negative and

antigen-positive tumors (Figure A1). In tumors on day 7, Tc1 cells expressed higher levels of CD43 only in antigen-postive tumors (Figure A1). Although the majority of donor cells in the tumors are positive for CD43 expression on day 3 and day 7, there are donor cells that are negative for CD43 expression. Therefore, it does not appear that CD43 expression is a component of the "tumor infiltrating phenotype" described earlier (Chapter 3). Interestingly, the expression of CD43 in Tc1 vs. Tc2 cells in TDLNs is different on day 7 (but not day 3) for TDLNs of antigen-negative vs. antigen-positive tumors. In TDLNs of antigen-negative tumors, Tc1 and Tc2 cell staining profiles are broad, with Tc1 cells having a population peak at high CD43 expression and Tc2 cells having a population peak at minimal CD43 expression. As stated above, this CD43 expression is significantly different for Tc1 and Tc2 cells. However, in the TDLNs of antigen-positive tumors, both Tc1 and Tc2 cells have narrow expression profile, with a population peak at a high level of expression. These data suggest that antigen may promote up-regulation of CD43 expression by antigen-specific T cells. How this may occur is unclear. To our knowledge, there are no reports on CD43 expression changing after antigen recognition by T cells.

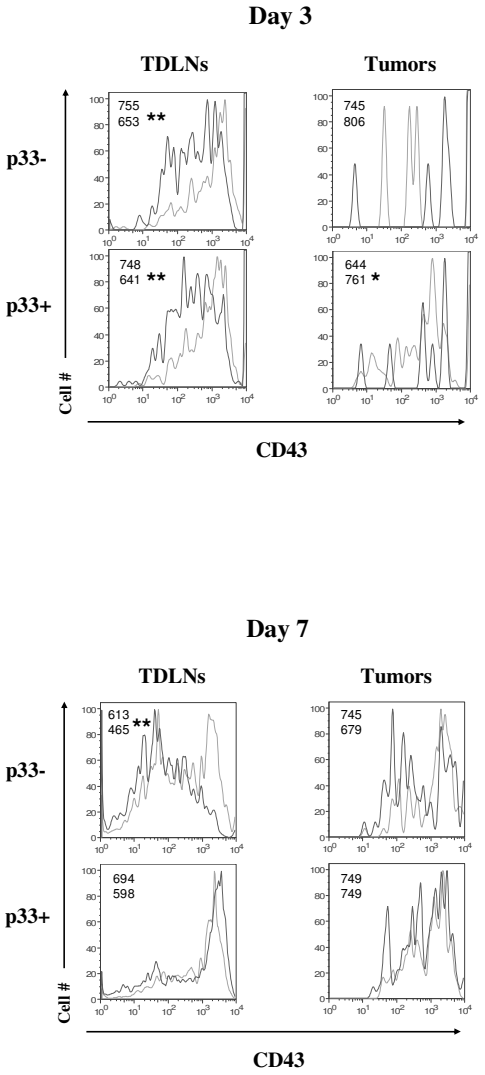

Figure A1: CD43 expression by Tc1 and Tc2 cells in TDLNs and tumor sites of antigen-negative and antigen-positive tumors. Following the methods in Chapter 3, we stained cells in TDLNs and tumors for CD43. Gated GFP+CD8+ cells were examined, and the MFI of CD43 expression on Tc1 (top #) and Tc2 (bottom #) cells was determined. Histograms show the profile for Tc1 (gray line) and Tc2 (black line) cells.
$p \leq .05$ * or $p \leq .01$ ** for Tc1 vs. Tc2 MFI.

APPENDIX 2:
A Brief Study on Acute Ultraviolet Light Exposure in Aged Animals

In addition to investigating Tc1 and Tc2 cell migration and tumor infiltration *in vivo*, we briefly examined how acute exposure to ultraviolet light (UV) affects T cell responses in aged animals. Aging and UV exposure alter T cell responses (5-8). Knowledge of how either aging or UV exposure promotes immunosuppression is not clear. However, we do know that both aging and UV exposure affect CD4+ T cells (5-8). Therefore, we chose to evaluate CD4+ T cells in aged animals either untreated or exposed to UV compared to untreated or UV exposed young animals.

The CD4+ T cell population includes Treg cells known for their suppressive activity of both effector CD4+ and CD8+ T cells (9). Because both UV exposure and aging alter T cell responses, we wanted to examine whether Treg cells are more abundant in aged or UV exposed animals. Treg cells are identified as CD4+CD25+ and also have been reported to express intermediate levels of CD45RB (9). Therefore, we chose to evaluate the percentage of cells expressing this phenotype in the spleens of young vs. aged animals that were untreated or exposed to UV. Splenocytes were stained with fluorochrome-conjugated monoclonal antibodies specific for CD4, CD25, and CD45RB and the numbers of CD4+CD25+CD45RB^{int} determined by flow cytometry.

When we compared splenocytes from control vs. UV exposed young animals, we found that UV exposed young animals had a significantly higher percentage of cells with the Treg phenotype (Figure A2.1). We also found this to be the case for control vs. UV exposed old animals (Figure A2.1). In addition, when we compared the percentage of cells with the

Treg phenotype in control young vs. old animals, we found that old animals had a significantly higher percentage of Treg cells (Figure A2.1). Therefore, it appears that both age and UV exposure increase the numbers of Treg cells. It is important to note that identification of Treg cells based on the cell surface phenotype is not the most accurate method of identifying Treg cells. The most definitive marker for Treg cells is expression of the forkhead-winged-helix transcription factor Foxp3 (9). Therefore, we cannot definitively say that Treg cells are higher in UV treated vs. control and old vs. young animals. We can only conclude that cells with the Treg phenotype of CD4+CD25+CD45RB^{int} are increased.

Because we observed a higher percentage of cells with the Treg phenotype in aged animals or animals exposed to UV and Treg cells suppress proliferation responses of effector T cells, we then evaluated T cell proliferation in UV exposed aged animals vs. untreated aged animals. We stimulated splenocytes from these animals with either polyclonal stimulator concanavalin A or the LCMV p33 peptide that stimulates CD8+ T cells. After 48 hours of culture, we assessed cell proliferation using alamar blue. Basal level of proliferation was determined from unstimulated cultures. We found that splenocytes from the old animals proliferated in response to both peptide and concanavalin A (Figure A2.2). Importantly, proliferation of the splenocytes from the UV exposed old animals was significantly lower than controls (Figure A2.2). This suggests that UV exposure does impair proliferative responses in old animals. Whether this impaired proliferation is due to Treg cells is not clear. Future studies will need to confirm the presence of Treg cells by Foxp3 staining and confirm the proliferation data by a suppression assay.

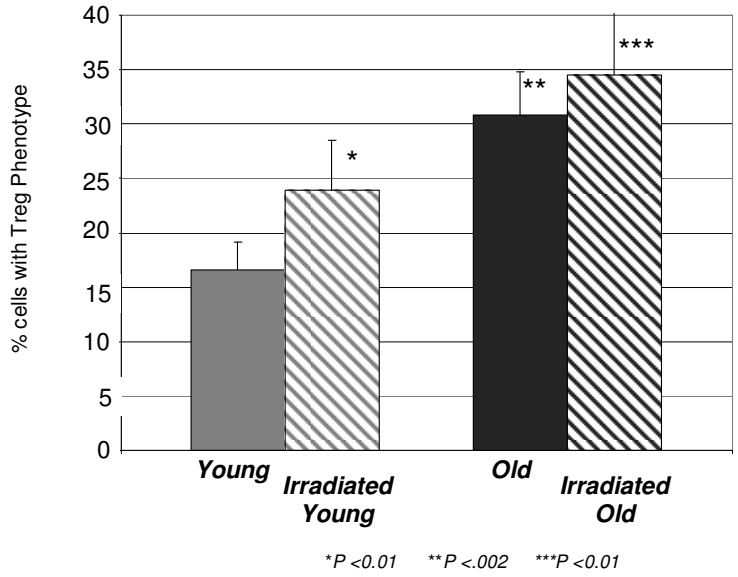

Figure A2.1: $CD4^+CD25^+CD45RB^{int}$ cells in young and old animals following acute exposure to UV. Young (8-12 weeks old) or old (12 months old) P14 transgenic mice specific for the p33 peptide were shaved on their dorsal side and either not exposed (control) or exposed to UV. Four animals were used per group. Animals were exposed to UV for 30 minutes per treatment. A total of 6 treatments were given over a 2 week period, with only 3 treatements per week. Each athree times a week for 2 weeks for a total of 6 doses. The total acute UV dose was 3 hours, or approximately $4.1J/m^2$. 2 days following the last exposure to UV, splenocytes of the treated mice and control mice were harvested. Single cell suspensions were stained for CD4, CD25, and CD45RB. Cells samples were run on a FACSCalibur (BD Bioscience). Data for gated lymphocytes were analyzed using Summit Software. Student's t-test was used to determine statistical significance.

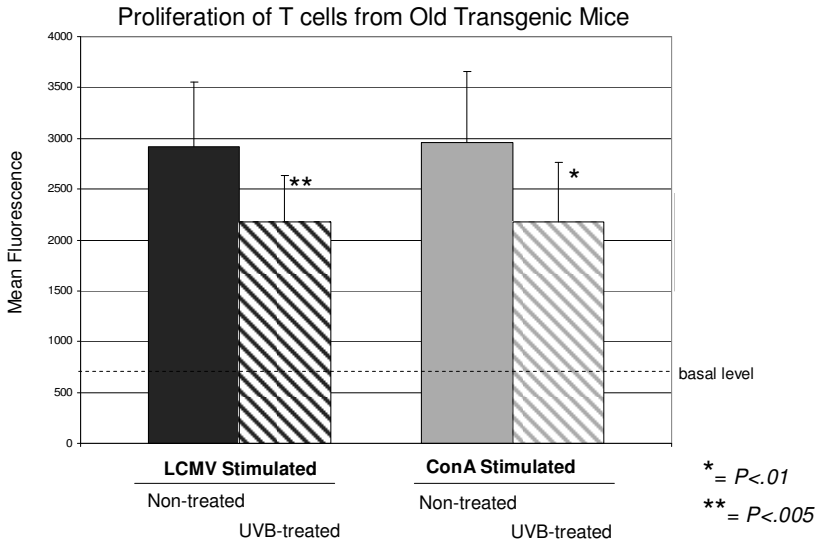

Figure A2.2: Proliferation of T cells in control vs. UV exposed old animals. Old P14 mice (12 months old) mice were shaved on their dorsal side and either not exposed (control) or exposed to UV. Four animals were used per group. Animals were exposed to UV according to methods in Figure A2.1. 2 days following the last exposure to UV, splenocytes of the treated mice and control mice were harvested and stimulated *in vitro* using LCMV p33 peptide or concanavalinA. Unstimulated cells were cultured as a control for basal proliferation. After 48 hours, alamar blue was added to the culture and after 4 additional hours the plate was read using a fluorimeter. Student's t-test was used to determine statistical significance.

References

1. Ley K, Kansas GS. Selectins in T-cell recruitment to non-lymphoid tissues and sites of inflammation. Nat Rev Immunol. 2004 May;4(5):325-35. Review.

2. Glimcher LH, Townsend MJ, Sullivan BM, Lord GM. Recent developments in the transcriptional regulation of cytolytic effector cells. Nat Rev Immunol. 2004 Nov;4(11):900-11. Review.

3. Lord GM, Rao RM, Choe H, Sullivan BM, Lichtman AH, Luscinskas FW, Glimcher LH. T-bet is required for optimal proinflammatory CD4+ T-cell trafficking. Blood. 2005 Nov 15;106(10):3432-9.

4. Underhill GH, Zisoulis DG, Kolli KP, Ellies LG, Marth JD, Kansas GS. A crucial role for T-bet in selectin ligand expression in T helper 1 (Th1) cells. Blood. 2005 Dec 1;106(12):3867-73.

5. Linton PJ, Haynes L, Tsui L, Zhang X, Swain S. Age-related changes in CD4 T cells of T cell receptor transgenic mice. Mech Ageing Dev. 1997 Feb;93(1-3):95-105. Review.

6. Miller RA. Effect of aging on T lymphocyte activation. Vaccine. 2000 Feb 25;18(16):1654-60. Review.

7. Schwarz T. Regulatory T cells induced by ultraviolet radiation.
Int Arch Allergy Immunol. 2005 Jul;137(3):187-93. Review.

8. Norval M. The mechanisms and consequences of ultraviolet-induced immunosuppression. Prog Biophys Mol Biol. 2006 Sep;92(1):108-18. Review.

9. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science. 2003 Feb 14;299(5609):1057-61.