Intracellular expression of Fas Ligand and Granzyme B by tumor infiltrating or *in vitro* activated CD8+ T cell populations

by

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Abstract

CD8+ T cells can recognize infected or cancerous cells and eliminate them by exocytosing cytolytic molecules or presenting death ligands on their surface, both of which can initiate apoptosis in target cells. Granzyme B (GzmB) and Fas Ligand (FasL) are two of the effector proteins that CD8+ T cells can use to kill infected or cancerous cells. GzmB is a soluble protein that shares a vesicle with other cytolytic enzymes that enter the target cell upon degranulation, and FasL is a transmembrane ligand that can engage with the Fas death receptor on target cells. Past research has shown that these two cytolytic proteins can be stored and trafficked within the cell differently, and can be presented to the target cell in response to different T cell receptor signal strength in the context of target cell recognition. Furthermore, there is debate as to whether FasL is required for, contributes to, or is dispensable for clearance of tumors. Most of these studies have not characterized whether FasL protein is actually present in the cells responding to the tumors, or whether extrinsic factors can influence FasL protein expression in CD8+ T cells. As CD8+ T cells have the potential to be exploited therapeutically for patients with cancer, it is important to better understand expression patterns of effector mechanisms in activated CD8+ T cells and how these can be manipulated.

I used two approaches to examine the relationship between FasL and GzmB expression in CD8+ T cells and the environment these cells are in. First, I activated naïve CD8+ T cells under controlled conditions *in vitro* and characterized FasL and GzmB protein expression dynamics after changes to the cytokine milieu. Second, I injected mice with subcutaneous or intraperitoneal EG.7 lymphoma and analyzed the CD8+ T cell

response in secondary lymphoid tissue and in the tumor sites. I found that overall, multiple CD8+ subsets can intracellularly express FasL, either coexpressed with GzmB or in the absence of GzmB. FasL expression in CD8+ T cells is influenced by the *in vitro* activation and *in vivo* tumor environment in ways unique from GzmB, with some conditions that are unfavorable for GzmB expression eliciting intracellular FasL expression. Furthermore, FasL protein expression in tumor-responding CD8+ T cells is not influenced by T cell reactivity to the immunodominant tumor antigen, unlike GzmB. Both memory and effector CD8+ T cells can express intracellular FasL and/or intracellular GzmB, though FasL/GzmB coexpression is more likely to be associated with an effector or recently activated PD-1hi phenotype. Overall, these findings shed light on the independent expression of FasL and GzmB by CD8+ T cells and the ubiquity of intracellular FasL expression in activated CD8+ T cell subtypes.

Preface

This thesis is an original work by Amanda N. Scott. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name Maturation and activation of T cells, Animal Use Protocol No. 305, 1992.

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List of Abbreviations

ADAM10	A Disintegrin and Metalloprotease10
AICD	Activation-induced cell death
APC	Antigen presenting cell
BSA	Bovine serum albumen
CAR	Chimeric antigen receptor
c-FLIP	Cellular FLICE-like inhibitory protein
CTL	Cytotoxic T Lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen-4
cLN	Contralateral (non-draining) lymph node
DC	Dendritic cell
DICE	Death induced by CD95 elimination
DISC	Death inducing signaling complex
dLN	Draining lymph node
dPBS	Dulbecco's PBS
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
FADD	Fas-associated death domain
FACS	Fluorescence assisted cell sorting
FasL	Fas Ligand
gld	"general lymphoproliferative disease" (FasL mutant mouse)
GzmA	Granzyme A
GzmB	Granzyme B
GzmC	Granzyme C
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HVEM	Herpesvirus entry mediator
IFN-α	Interferon-alpha
IFN-γ	Interferon-gamma
IL-2, -7. -12…	Interleukin-2, Interleukin-7, etc.
IP	Intraperitoneal
KLRG1	KLRG1 killer cell lectin like receptor G1
LAMP-1	Lysosomal membrane associated protein 1
LAMP-2	Lysosomal membrane associated protein 2
LCMV	Lymphocytic ChoriomeningitisVirus
LN	Lymph node
lpr	lymphoproliferation mutation (Fas mutant mouse)
MDSC	Myeloid-derived suppressor cells
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MPEC	Memory precursor effector cells
MTOC	Microtubule organizing complex
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NFAT	Nuclear factor of activated T cells
NK cells	Natural Killer cells
OVA	Ovalbumin
PBS	Phosphate-buffered saline

PD-1	Programmed cell death protein 1
PD-L1	Programmed death-ligand 1
qPCR	Quantitative polymerase chain reaction
PEL	Peritoneal exudate lymphocytes
PMA	Phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear leukocytes
RPMI	Roswell Park Memorial Institute medium
SC	Subcutaenous
SLEC	Short-lived effector cells
SMAC	Supramolecular activation complex
TAM	Tumor-associated macrophage
Tcm	Central memory T cells
TCR	T cell receptor
Teff	Effector T cell
Tem	Effector memory T cell
Th	Helper T cell
TIL	Tumor infiltrating lymphocytes
Tmem	Memory T cell
Tn	Naïve T cell
TNF	Tumor necrosis factor
Treg	Regulatory T cell
Tscm	Stem cell memory T cell

Chapter 1:
Introduction

Introduction to the Immune System

The immune system: innate and adaptive work together

The immune system is comprised of a diverse set of leukocytes that work together to detect foreign threats, signal danger to other cells, and clear infected and dysfunctional cells. All potential routes of entry to the body are surveyed or populated by immune cells specialized in controlling infection. The innate immune system responds immediately and is capable of detecting infections through various receptors. These include pattern recognition receptors, such as Toll-like receptors, intracellular RIG-I like receptors, and Nod-like receptors that recognize pathogen-associated molecular patterns and host-derived danger signals on macrophages and dendritic cells (DC), as well as other cells. These "other" innate cells include granulocytes such as neutrophils, eosinophils, and basophils, which release the contents of granules containing vasodilators, reactive oxygen species, and other mediators to increase inflammation and recruit other cells to the site of infection.

The lineage of innate cells that have the most impact on my areas of research in T cell activation are the classic antigen presenting cells (APC), which include macrophages and DC. These can line barrier tissues, and once they are stimulated by pathogen, they can migrate to secondary lymphoid organs, and present foreign antigens or phagocytosed antigens from apoptotic cells to the adaptive immune system: to B cells as naked antigen or to T cells as peptides on MHCI or MHCII [1]. Multiple lineages of dendritic cells have been found capable of eliciting CD8+ T cells responses to pathogens [2], but for all of

them, the cytokines or costimulatory ligands presented to the T cells along with MHC are affected by the pathogen the DC have encountered [3]. I will later review some of these DC-generated signals and the effect they can have on CD8+ T cells after activation.

While the most common lymphocytes are the adaptive T and B cells, the innate Natural Killer (NK) lymphocytes share some similarities. They can secrete cytokines in response to or kill infected or otherwise "perturbed" cells. Unlike T cells, their effector activity is not triggered in an antigen-specific manner, and it is debated whether NK cells can generate true memory, though antigen-specific memory-like NK cells have been characterized [4, 5].

The adaptive immune response is comprised of B and T lymphocytes; they both rearrange the genes for their cell receptors during initial cell development to generate populations with a massive repertoire of potential recognition. Once B cells are activated by ligation of their B cell receptor to antigen presented by follicular DC in lymphoid tissue, and receive secondary signals from the immune environment and helper cells, they undergo both somatic hypermutation of their receptor genes and affinity maturation. This is selection of the most avid B cell clones for further hypermutation, activation, and differentiation into antibody-secreting plasma cells and long-lived memory cells [6].

T cells are activated in the lymphoid organs upon interaction with antigen-presenting cells, and can then go on to exit into the tissue and influence other immune cells by secreting cytokines such as IFN-γ or IL-10, providing costimulatory "help" to B cells and other lymphocytes in the form of CD40, or killing other cells through cytolytic effector mechanisms. In all cases, some of the T cells with these responsibilities can remain as long-lived memory cells to respond again upon reinfection.

T cells: Helpers, Regulators, Killers

Before being allowed to exit to the periphery, T cells with newly rearranged TCR undergo education in the thymus. T cells are first provided signals for further survival if their TCR has sufficient affinity for self peptide:MHC as part of positive selection. Those that fail, die by atrophy. Positively selected cells also undergo negative selection, where T cells with too strong of a reaction to self peptide:MHC undergo apoptosis [7, 8]. Some T cells that have moderate to high self-affinity become natural Tregs [9, 10] when supplemented by costimulatory signals in the thymus [11, 12]. Those that pass positive selection and are not strongly reactive to self antigen pass negative selection, become mature, exit the thymus, and can seek activation in the secondary lymphoid tissue.

The two main lineages of T cells that contain the alpha and beta chains of the T cell receptor are CD4+ T cells and CD8+ T cells. CD4+ T cells recognize class II MHC, on professional antigen-presenting cells and some other immune cells, like B cells, that can present MHCII. Therefore, a significant amount of their effector activity happens near other immune cells, and their primary role is to exert stimulatory or suppressive effects on the immune response via cytokine secretion. Activated CD4+ cells can also present CD40L to DC, which can prime them to help CD8+ T cells [13, 14]. They can also differentiate into numerous lineages of T helper cells. Th1 CD4+ cells can secrete proinflammatory and proliferative cytokines such as IL-2 and IFN-γ, which can amplify inflammatory responses. Th2 CD4+ T cells can secrete cytokines such as IL-10, IL-4, and IL-5, which can promote humoral responses but also contribute to allergy and asthma.

Other T cell lineages include Th17 and Th22 CD4+ T cells [15-17], as well as Treg cells. Tregs suppress active CD4+ and CD8+ responses [18].

As mentioned earlier, Tregs can emerge as an alternative to death via negative selection in the thymus in populations that have increased affinity for self-antigen [19, 20], but they can also be induced in the periphery when CD4+ T cells are exposed to, among other factors TGF-beta [21, 22]. CD4+ Tregs can suppress conventional T cell responses by a number of methods. Tregs are important for inducing tolerance and preventing autoimmunity [23], though the specifics of these mechanisms are still under active research. However, this tolerogenic activity can be a challenge in antitumor responses [24, 25]. Furthermore, as lymphocytes can often be recruited in high numbers to tumor environments where a high amount of self-antigen is present, it is interesting to note that tumor-infiltrating Tregs and conventional T cells often have different antigen specificity [25, 26].

There is some evidence that there are CD8+ Tregs. One group has found that a CD8+ Treg-like population, identified by CD122+ and a debated range of other antigens [27], can suppress a DC vaccine-boosted antitumor response in mice [28]. Furthermore, recent work suggests that CD8+ Treg-like suppression of allograft rejection is partly dependent on FasL [29]. The possibility that FasL can be used as both a suppressive and cytotoxic molecule (to be discussed later in this chapter) by CD8+ T cells is interesting.

The large proportion of CD8+ T cells are not regulatory, but serve in an effector capacity by secreting some cytokines and by killing target cells. CD8+ T cells recognize peptide in the context of MHCI, which is present on most somatic cells. Because almost

all cells are their potential targets, they can exert their effects anywhere in the body that they can enter.

T cell Activation

Once a CD8+ T cell has survived positive and negative selection in the thymus and entered the secondary lymphoid organs to survey for its antigen, it will require three signals for full activation (Fig 1-1). Most importantly, T cells require interaction of the TCR with cognate antigen in the form of MHCI with peptide, most commonly on an antigen-presenting cell. In addition, these cells will require costimulation, from ligands such as B7.1 or B7.2 that interact with the CD28 receptor, or CD70 that interacts with the pro-memory CD27 receptor, or LIGHT that interacts with the HVEM receptor. Finally, within a few hours to days, cells will require extrinsic cytokines to become fully activated and reach maximum proliferative potential [30].

There is a complex network of signals from both innate and adaptive cells that result in T cell activation. In addition to antigen-presenting cells, helper CD4+ T cells are required for a robust CD8+ T cell response: helper T cells, upon activation, can present CD40L to the CD40 receptor on dendritic cells, which helps "license" them to become effective antigen presenting cells [13, 14]. This is especially critical for long-term memory development [31], particularly if the innate stimulus is suboptimal [32]. Furthermore, IL-2 from activated CD4+ cells can contribute to the expansion and activation of CD8+ populations [33].

Once cells have received all three signals, they proliferate and begin differentiating to memory and effector cells, which I will review later in this section. In addition, activated cells acquire effector ability, or the ability to secrete cytokines and kill

other cells when they encounter target cells. One of the things that they begin to express is CD44, a receptor for hyaluronan, which is expressed in abundance in the periphery; CD44 on the cell surface allows them to patrol the periphery in search of target cells [34, 35].

T cells recognize antigen in the context of peptide on MHC. During protein synthesis, misfolded and recycled cellular as well as viral proteins are cleaved by the proteasome [36], then transported to the endoplasmic reticulum where the peptides are assembled into the peptide-binding groove of class I MHC [37, 38]. Overhanging residues of the bound peptides are trimmed, then the assembled peptide:MHCI complex is transported via the golgi complex to the cell surface. This provides a running display of all the proteins synthesized inside the cell. Almost all somatic and immune cells express MHCI for CD8+ surveillance, though this process can be disrupted by some viruses like poxviruses [39] and MHCI expression has been found downregulated in some cancers [40-42]. In addition to MHCI, MHCII is expressed by cells capable of phagocytosing exogenous protein. This protein is degraded in the phagolysosome to short peptides, which can bind to MHCII when their phagolysosome fuses with the acidified endosome containing MHCII plus chaperone molecules. Peptide:MHCII is then trafficked to the cell surface, where it provides to CD4+ T cells a sampling of peptides from extracellular matrix proteins as well as from nearby apoptosing cells and free pathogens. It has been suggested that tumor-tolerant monocytes have reduced MHCII expression [43].

The antigen-specificity of the interaction between TCR and peptide:MHC has been exploited by immunologists in various manners. TCR transgenic mice have

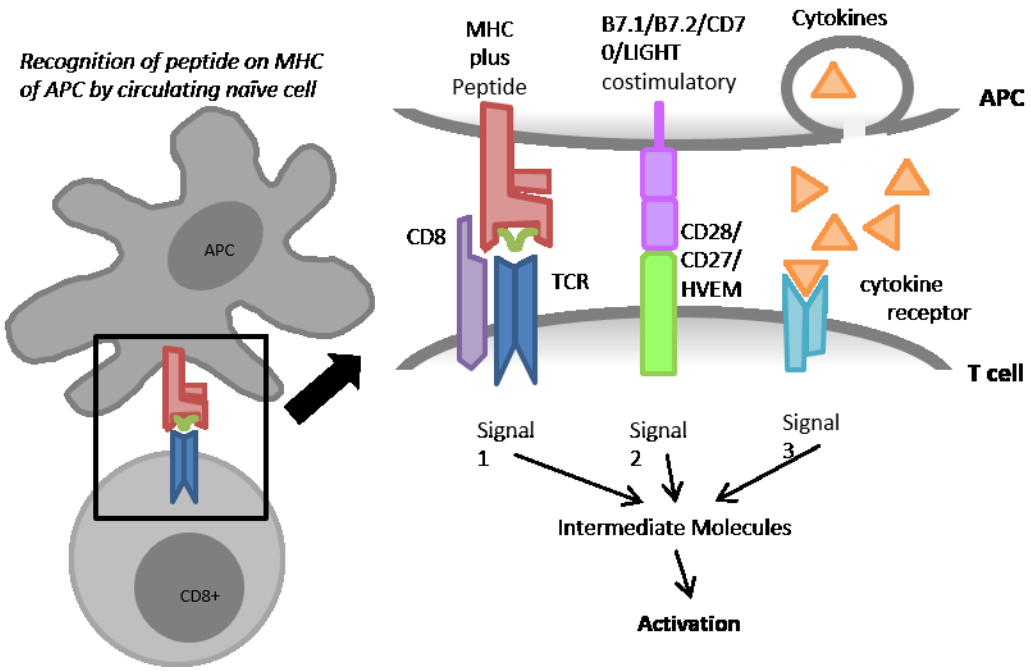


Figure 1-1. Three signals are required for CD8$^+$ T cell activation.
In order to fully give rise to effector and memory populations, and proliferate, CD8+ T cells require three signals. First, the T cell receptor interacts with Peptide/MHC on the antigen-presenting cell (APC) surface (Signal 1). In addition, interaction of costimulatory receptors with their costimulatory ligands is required (Signal 2). Finally, cytokines are required to fully enhance T cell expansion and differentiation (Signal 3).

completely rearranged genes for TCR alpha and TCR beta genes inserted into the genome; the TCR expressed by these mice will have a reproducible antigen specificity and are often used as antigen-specific models. For example, OT-I mice have CD8+ T cells specific for the SIINFEKL peptide of ovalbumin presented on H-2K^{b} MHCI [44]. Alternatively, fluorescent tetramers of peptide-loaded MHC can be used to bind to and identify antigen-specific T cells with unmanipulated or unknown TCR rearrangements. However, in order to get functional antigen-specific cells in a durable response, in the beginning, TCR signaling, costimulation, and cytokine signals must convene to fully activate a T cell.

TCR Signaling

Once an activated T cell recognizes its peptide:MHC ligand on a target cell in the periphery via TCR and CD4 or CD8, clustering of the receptors initiates phosphorylation events that trigger a signaling cascade, resulting in transcription upregulation, calcium influx, and polarization of the microtubules towards the synapse with the target cell.

TCR and CD8 or CD4 clustering in response to peptide:MHC signal results in the SRC-family kinase Lck, which is associated with the intracellular domain of CD4 and CD8, recruited to the TCR signaling complex[45]. While the α and β chains of the TCR confer antigen specificity, the components of the TCR complex that are associated with signal propagation are the CD3ε, -γ, -δ and -ζ chains [46]. CD3ε cross-linking antibodies are often used *in vitro* to mimic target cell engagement of the TCR. When Lck is proximal to the CD3 chains, Immunoreceptor Tyrosine-based Activation Motifs (ITAMs) on CD3 chains are phosphorylated by Lck, allowing the kinase Zap70 to be recruited to the TCR/CD3 complex, where it too is phosphorylated by Lck or a similar kinase Fyn,

and activated [45]. Active, phosphorylated Zap70 is then able to move to other signaling molecules and activate them, such as LAT and SLP76, which result in propagation of cascades to activate other signaling molecules [47]. Principal among these pathways are the release of Ca^{2+} from the endoplasmic reticulum into the cytoplasm. This elevated Ca^{2+} eventually results in signals that allow nuclear transport of transcription factor NFAT [48]. Another pathway that is activated is the cascade downstream of the GTPase Ras. Active Ras eventually results in the activation of transcription factors that can turn on immune signaling genes, and the release of antiapoptotic proteins to promote survival of stimulated cells[49]. In addition, the Mammalian target of rapamycin (mTOR) pathway is activated following TCR signaling. Downstream signaling complexes can phosphorylate targets that resulting in increased protein translation, and also result in actin rearrangement [50], which is critical for T cell extravasation and motility.

Often multiple signaling pathways contribute to the same outcome from TCR signaling. For example, Zap70, SLP-76, LAT, and Erk1/2 all contribute to the regulation of MTOC reorientation [51].

In naïve cells, genes turned on as a result of this TCR signal can turn on proliferation and differentiation when combined with costimulatory signals, and in mature activated CTL, these can trigger degranulation or exocytosis.

Integrin interactions between the target cell and CTL are also reinforced at this point, and a strong adhesion molecule-ringed supramolecular activation complex usually (SMAC) forms, though it is not required for all cytolytic activities in CD8+ T Cells [52, 53]. Most of the effector molecules are directed to this site for exocytosis into the immunological synapse. Vesicles containing effector molecules dock at the cell

membrane, and the vesicle membrane fuses with the T cell membrane at the immunological synapse [54, 55]. Effector molecules diffuse towards the target cell, or transmembrane proteins then migrate along the T cell membrane to interact with proximal receptors on the target cell surface. The activity of these effector mechanisms will be discussed in greater detail later in this introduction.

Costimulatory Receptors

Costimulatory receptors on T cells include molecules such as CD28, 4-1BB, OX40, and HVEM, which can interact with costimulatory ligands on activated phagocytic cells. Signaling through these receptors enhances the signals from the T cell receptor. These signals can enhance proliferation [56], and promote survival by increasing anti-apoptotic proteins [57]. Naïve cells receiving TCR stimulation in the absence of any CD28 (or other costimulatory signal) become anergic [58-61]. Originally it was believed that CD28 ligation was absolutely necessary for T cell activation, but since then it has been found that T cells can be activated as long as there are alternate costimulatory signals in addition to TCR stimulation if CD28 is absent [62]. Other costimulatory receptors, such as 4-1BB, are absent on naïve cells but are upregulated quickly on cells after activation and their ligation can also have effects on the differentiation of T cells [63, 64]. The balance of costimulatory receptor signals can alter the differentiated fate of CD8+ T cells [65]. Anergy in the absence of costimulatory signal is a means of peripheral tolerance, preventing self-reactive T cells that have managed to avoid negative selection from becoming dangerously activated when encountering self antigen in the periphery.

Cytokines

Cytokines are soluble molecules that serve as immune mediators. Their interaction with receptors on the surface of immune and somatic cells results in conformational changes to the intracellular domains of the receptor, and activation of signaling cascades via intracellular mediators, primarily members of the member of the JAK-STAT (Janus activated kinase-signal transducer and activator of transcription) family [66]. Different cytokine receptors elicit tyrosine phosphorylation and dimerization of different combinations of JAK-STAT family members, resulting in both unique and shared downstream gene activation[67]. Activated JAKs can also interact with intracellular signaling pathways. Below, I will review some of the cytokines that act on T cells.

Common Cytokine-Receptor Gamma-Chain Cytokines

These cytokines (IL-2, IL-4, IL-7, IL-9, 1L-15, and IL-21) share a common γ-chain subunit (CD132) of their receptors [68]. They all have effects on the differentiation and homeostasis of CD8+ T cells, but I will briefly review only a few of them below.

IL-2 was originally named T Cell Growth Factor for its observed ability to regulate the growth of T cells [69]. In addition to the CD132 γ-chain receptor subunit, the IL-2 receptor is also comprised of a CD122 β-chain plus the CD25 α-chain subunit to make a high-affinity receptor for soluble IL-2 [70]. IL-2 is a characteristic T cell cytokine involved in T cell and NK cell proliferation and expansion [71]. IL-2 does not seem to be essential for expansion of CTL in secondary lymphoid organs [72], but in the periphery it is more important: first for expansion of the activated CTL in the periphery, and then eventually for the upregulation of apoptosis, although it is disputed that this is required for contraction [72]. CD122 (IL-2 and IL-15 receptor) signaling can be dispensable for CD8+ T cell activation and initial proliferation, but it amplifies post-infection expansion

[73, 74]. IL-2 also increases following activation and differentiation [75], and it can induce activated cells to be more susceptible to FasL-mediated fratricide [76, 77]. Furthermore, Esser *et al* found that IL-2 added directly to pre-activated Th1 CD4+ and CD8+ T cell clones can induce FasL-mediated cytotoxicity in the absence of TCR stimulation [78].

IL-7 is important for the homeostasis of naïve T cells and persistence of memory populations [79]. IL-7Rα (CD127) is initially high on naïve cells, then downregulated in effector cells and maintained or upregulated on memory populations [80-82]. IL-7 is important for long-term memory population development *in vivo* [83, 84].

IL-15, which shares the receptor β-chain subunit CD122 with IL-2 in addition to the common γ-chain receptor subunit, has its unique CD215 α-chain subunit [85]. IL-15 is presented in *trans*, as it is presented on the cell surface bound to the α-chain subunit, and signaling occurs when the complexed β and γ receptor subunits on another cell detects the α-chain presented IL-15, allowing IL-15 communication between cells [86]. IL-15 positively affects homeostatic proliferation, effector cell expansion, and CD8+ T cell memory development and maintenance [68, 87-89].

Inflammatory Cytokines

Innate immune cells can produce inflammatory cytokines upon recognition of infection via ligation of toll-like receptors or other pattern recognition receptors that sense pathogen-associated molecular patterns [1, 90-93], or danger-associated patterns, such as cytosolic DNA from tumor cells [94, 95]. Cytokines such as IL-12 and IFN-α can be secreted by several cells in the activation milieu including antigen-presenting cells during initial activation [96, 97].

IFN-α is well known to impact antigen presenting cells as well as lymphocytes [98], and its antiviral effects have been well-characterized [99]. IFN-α can impact the subsequent expression of a wide range of cytokines , such as IL-8, TNF-α, and IFN-γ [100]. IFN-α and IL-12 have some redundant activities and also some divergent effects on lymphocytes [101]. IL-12 and IFN-α are also known to boost the TH1 cytolytic response [102], and CD8+ T cells from mice lacking receptors for IFN-α have dysfunctions in GzmB-mediated killing [103]. IL-12 delivered to tumor sites resulted in the destruction of the tumor stroma in a Fas-dependent manner [104]

CD8+ T cell Differentiation

CD8+ T cell differentiated types

CD8+ T cells are not a single homogeneous population, but instead are made up of several subpopulations of differentiated cells, with a growing number of subcategories.

Naïve T cells (Tn)

The never previously activated state of CD8+ T cell is known as naïve. Naïve CTL have no effector molecules, high in cytokine receptors for survival such as CD127 (IL-7Rα), and have high levels of CD62L to allow them to transit through secondary lymphoid tissue to survey for cognate antigen [105, 106].

Effector T cells (Teff)

Effector CTL are essential for many immune responses, expanding in large numbers following activation (Fig 1-2) and contracting following pathogen clearance. In mice, effector cells bear the hallmark of activation, high levels of CD44 expression, allowing them to enter peripheral tissues. CD62L gene expression in these cells is halted, and remaining CD62L protein present on the cell is cleaved off by metalloproteases

[107], prohibiting them from patrolling the lymphoid nodes. These cells are therefore low in CD62L and have also been found to be high in KLRG1[108], and have high levels of effector mechanisms, particularly degranulation-based effectors GzmA, B, and Perforin. Some groups also use the term short-lived effector cells (SLEC) to specify effector cells that are terminally differentiated and unable to survive long following infection [109]. In addition to short-lived effector cells, some KLRG1lo-int, CD62Llo, CD127hi effector cell populations have been identified as precursors to memory differentiation [82, 110, 111]. Their phenotype between effector cells is indicative of current effector ability as well as the potential to persist as long-term memory [82].

Memory Cells

Memory cells are considered as such because they are capable of persisting long after infection (Fig 1-2) and give rise to another CD8+ T cell expansion following reinfection. While the prime characteristic of memory is long-term persistence, there are subsets of memory cells, some more consistently used in the literature than others, to describe certain functional and phenotypic variations:

Central Memory Cells (Tcm)

These cells are the "poster children" of memory cells. They have elevated CD45RO (in humans), the chemokine receptor CCR7, CD127 and CD62L expression. This CD62L is one of the receptors that allow them to once again patrol the spleen and lymph nodes, while retaining their ability to enter peripheral tissue via CD44. They can be found months after primary infection, and have reduced perforin and GzmB expression in general, as well as elevated IL-2 production [109]. Some have argued that

Tcm are not effectively cytotoxic [112]. They can have elevated longevity post-adoptive transfer in tumor models [113, 114].

Effector Memory Cells (Tem)

Effector Memory cells have similar transcriptional and phenotypic profile ($CD44^{hi}$ $CD62L^{lo}$ $CD127^{lo}$) to effector cells, but persist long after infection[115]. They are often found residing in peripheral tissues [115]. Reactivated Tem can be used in adoptive transfer for tumor clearance [116, 117], though less effectively than can central memory or stem cell memory [114, 117, 118]. They have more effective immediate cytotoxicity than central memory cells [80, 112].

Tissue-Resident Memory Cells

These are memory CTL present in tissues following infection. They are resident in peripheral tissues, do not recirculate, and capable of high levels of cytotoxicity [115]. It is not always clear if they can be distinguished from recent immigrants or if effector memory and tissue-resident memory cells are distinct phenotypes [119], but CD103 expression has been used by many as an indicator of terminal tissue residency [120].

Stem Cell Memory Cells (Tscm)

These cells are $CD44^{lo}$, $CD62L^{hi}$, and are often characterized as expressing CD122, CXCR3, and Fas receptor [114, 121]. These have the greatest proliferative potential and can give rise to other memory lineages of CD8+ T cells[122, 123]. They are also associated with positive outcomes upon adoptive transfer against tumors [114].

Virtual Memory Cells

There are also cells in humans and mice that are have an activated, $CD44^{hi}$ phenotype in the absence of antigenic stimulation [124]. They have been found with

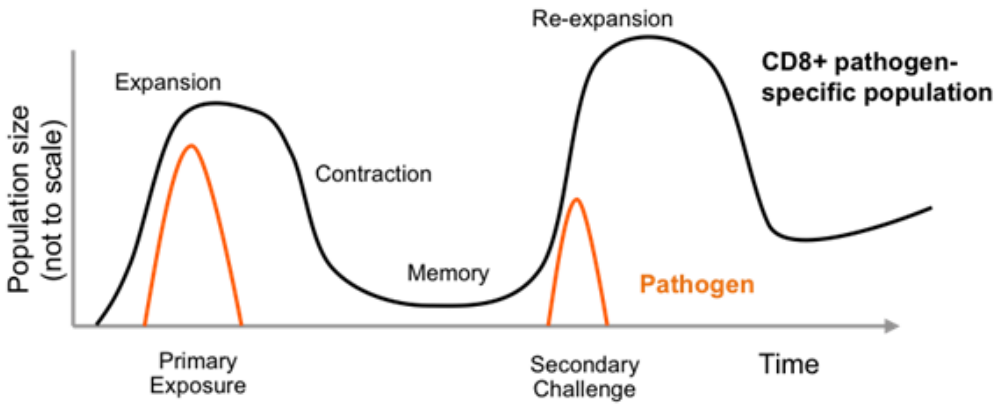


Figure 1-2. General CD8+ T cell population expansion, contraction, expansion in response to pathogen.
In an acute primary immune response, cells expand upon exposure to a pathogen, then contract after clearance, leaving a small population of memory cells. Upon secondary infection, there is rapid re-expansion of the antigen-specific cells and a more effective clearance of pathogen.

reactivity to antigens that experimental mice were never exposed to [125]. However, these cells can proliferate rapidly and produce IFN-γ upon restimulation [126, 127]. They have also been found to express FasL transcripts at higher levels than for GzmB, but neither as high as for IFN-γ [116, 128].

Keep in mind that many studies do not finely differentiate between the subsets within general memory-like of effector-like populations, so where possible I have used the same nomenclature as the researchers used in their primary literature.

Effect of Extrinsic Factors on Memory/Effector development

Regardless of which model of CD8+ T cell differentiation is happening, extrinsic factors from antigen presenting cells, CD4+ T cells, or from within the CD8+ population can have effects on what proportions of Tmem and Teff emerge during expansion. As I mentioned briefly before, T cells require three signals for activation: T cell receptor engagement, costimulatory receptor engagement, and cytokines. The combination of the strength and type of signals that the differentiating T cells are exposed to will influence its fate.

While the length of time that naïve CD8+ T cells are exposed to peptide-pulsed DC *in vivo* affects the overall proliferation of the activated cells and subsequent expanded population size, the length of antigen exposure does not affect the rate of differentiation of CTL into memory, nor does it affect the effector ability of that memory population upon immune challenge [129]. There is some evidence that TCR avidity affects the quality of the resultant response [130] and possible memory recall [131] but the general consensus is that once past a basic threshold, TCR avidity does not significantly alter the quality of the immune response [132]. In addition, level of TCR engagement or antigen

density does not alter the quality of memory generation, but when fewer TCR are engaged, fewer memory cells emerge [133, 134], and at the opposite end of the spectrum, too much antigen has deleterious effects on the T cell response [135]. This is linked to the Uneven Stimulus model described above, but other ascribe it to strength of the stimulus affecting the rate of conversion from Tem to Tcm [136].

Cytokine exposure also has an effect on differentiation. IL-2 signaling is associated with enhancing the effector or Tem populations [76, 137, 138]and high expression of degranulation effector mechanisms, while low IL-2 is associated with a central memory phenotype [137, 139, 140]. Signaling through the shared CD122 β-subunit of IL-2 and IL-15 receptors seems to operate on a continuum: low signals favor central memory differentiation, intermediate favor effector memory, and high CD122 signals favor effector memory differentiation [137]. IL-2 and IL-15 often have divergent roles in differentiation. Conversely, IL-15 positively affects homeostatic proliferation and CD8+ memory development and maintenance [68, 87-89]. IL-15 has also been found to contribute to development of virtual memory T cells, but that necessity of IL-15 is inversely related to affinity for self-antigen [128].

IL-12 increases the proportion of effector cells shortly after activation, *in vitro* [82, 141] or *in vivo* [142]. IL-12 can make some effector memory cells transition to effector cells [143] and can prime Tmem cells for secondary expansion to effector populations upon re-exposure to antigen [143]. IL-12 may influence whether cells differentiate to a memory precursor phenotype (low IL-12) or short-lived effector phenotype (high IL-12) [82]. However, IL-12 has also been shown by some to be essential for generation of Memory cells, both *in vitro* [144] [140] and *in vivo* [141, 145,

146], and has been found to enhance survival and cytolytic activity of memory-phenotype (CD62Lhi) cells [147]. In fact, CD62Lhi CD8+ T cells from cultures treated with IL-12 have similar tumor in mice clearance to their CD62Llo counterparts [147]. Like IL-12, IFN-α enhances expansion [148-150] and initial activation of cells [102, 148]. IL-12 and IFN-α may have a reciprocal relationship for CD8+ T cell Memory and effector development, with IL-12 favoring effector populations and IFN-α favoring memory; when both cytokines are added to cells together, two populations arise, bearing distinct memory or effector characteristics [151]. IFN-α is essential for memory generation in the absence of IL-12, and aids in the persistence of memory cells [141, 146] [148]. IFN-α can also sustain expansion of virtual memory populations [152]. However, IFN-α has been found to drive differentiation of SLEC cells, as well [110].

As seen in a more holistic fashion, infections with the same pathogen that received modulated antibiotic timing, or where inflammation is artificially induced during the same infection have varied balances of memory and effector cells [153].

CD8+ T Cell Effector Functions

Upon ligation of the T cell receptor to MHC on a target cell, a cytoplasmic signaling cascade is initiated by clustering of the transmembrane domains of the TCR complex and other signaling molecules. As a downstream result of this, Ca^{2+} enters the cytoplasm from intracellular stores as well as from the extracellular environment, the MTOC reorients towards the immune synapse, and vesicles transit to the immune synapse [55]. In human cells, it is debated whether FasL, granzymes, and perforin are stored in the same cytolytic granules or FasL is stored separately [154, 155].

Release of Cytokines

CD8+ T cells can secrete cytokines such as IFN-γ and TNF-α, which can have direct effects on other immune cells, or on tumor cells. These can have direct effects on infected cells as well, by upregulating MHCI presentation, both via upregulated MHCI expression and by enhancing peptide processing for MHC presentation [156]. IFN-γ can also enhance proliferation of the CD8+ T cell pool [157].

Degranulation: Granzymes and Perforin

Degranulation is the best-characterized cytolytic effector mechanism of T cells. Cytolytic granules are lysosomally derived [158], and contain perforin and granzymes (Fig 1-3), as well as transmembrane molecules such as lysosomal-associated membrane protein (LAMP)-1 and LAMP-2. Cell surface display of LAMP1 (CD107a), found in cytolytic granules, is used as an indicator of past cytolytic activity [112, 159]. Upon ligation of the T cell receptor and CD8 coreceptor with their ligand, peptide:MHCI, the calcium influx and protein phosphorylation that occur following the TCR signaling cascade are necessary for trafficking cytolytic vesicles to the immune synapse and their subsequent exocytosis. Perforin enters the target cell via endocytosis [160, 161], though in some conditions perforin can facilitate Gzm entry into target cells [162-164].Once inside the cells, granzymes, which are serine proteases, initiate apoptosis by cleaving procaspase 3, Bid, and DNA, among other targets.

Not all cells express all granzymes constitutively. For example, NK cells, which as mentioned before can have similar cytolytic pathways to CD8+ T cells, can express Granzyme B abundantly and GzmC only when required [165]. Furthermore, although

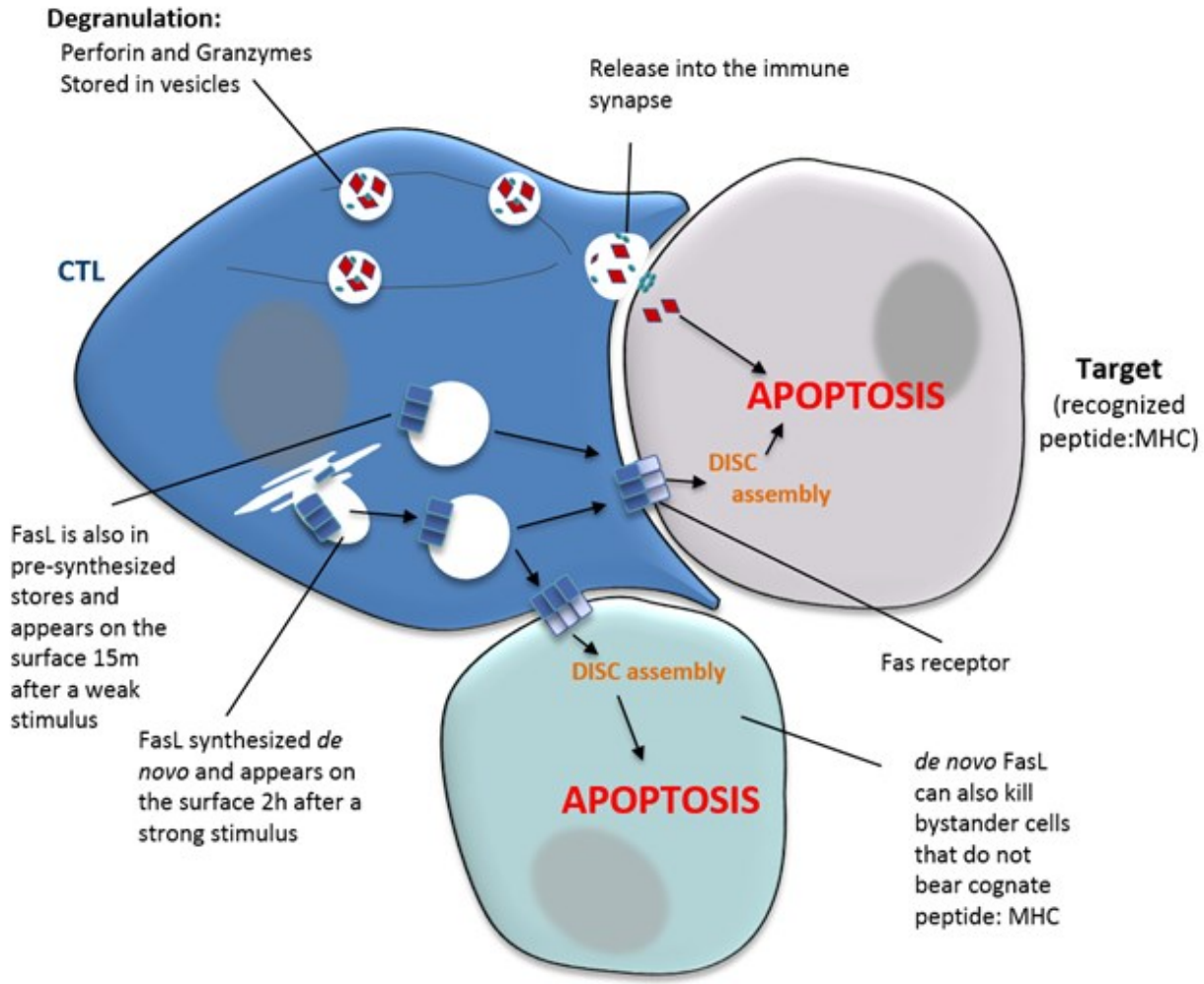


Figure 1-3. Cytolytic Effector Mechanisms from CD8+ T cells.
When an activated CD8+ T cell engages with a target cell bearing the cognate antigen of peptide/ MHCI (left out of the figure for clarity, but top-right cell is the one bearing this antigen), it degranulates granzymes and perforin towards the target cell. Facilitated by perforin, granzymes enter the cell and initiate apoptosis. In addition, CD8+ T cells also initiate *de novo* synthesis and surface presentation of FasL to target cells as well as bystander cells. In the presence of a weaker stimulus, CD8+ T cells can rapidly translocate FasL from pre-synthesized stores and present to recognized target cells. In either case, FasL engages Fas receptor on the surface of target cells, where it initiates formation of the DISC (Death-inducing signaling complex) around the cytoplasmic domain of Fas, which eventually leads to apoptosis.

perforin and granzymes A, B, and C are all induced upon activation of naïve CD8+ T cells, individual cells within a single population have differing levels of transcripts of these effector mechanisms [166].

Fas Ligand

FasL is a TNF-superfamily transmembrane protein. This death ligand family also includes molecules such as TNF and TRAIL, all of which can be presented on a CTL surface, [167]. All of these can interact with their respective receptors on the target cell to initiate the extrinsic (receptor mediated) apoptosis pathway. The Fas receptor is constitutively expressed on many cells. FasL-mediated apoptosis can kill infected or cancerous cells, and there is some debate on whether it may be involved in T cell population contraction following a cellular immune response. FasL can be expressed by CTL and NK cells, and upregulated on some other immune cells such as B cells, CD4+ T cells, and macrophages. FasL can also be expressed constitutively by many cells in "immune privileged" sites, such as testes and neural tissue [168, 169]. It can also be upregulated on the tumor endothelium as a barrier to invasion by lymphocytes [170].

Regulation of FasL expression

FasL expression at the transcriptional level is controlled by multiple transcription factors. Among others, there are transcription factor binding sites upstream of the *fasl* promoter for NFAT and AP-1, which also influence GzmB expression, and NF-κB can bind distally upstream of the FasL gene [171]. NFAT is critical for FasL transcription, both by directly binding upstream, as well as for eliciting the recruitment of other transcription factors from the early growth factor family [172]. CD28 signaling has been found to directly affect FasL transcription, with a CD28 responsive element upstream of

fasl as well [173]. When integrating signals from cytokines on FasL gene expression, we have some information: The FasL promoter contains interferon-responsive elements bound by IFN regulatory factor transcription factor family members [174]. IL-2 has been shown to activate the promoter of the *fasl* gene [175]. Addition of IL-12 and IFN-α to cultured CD8+ T cells can enhance FasL mRNA in the first 3 days after activation [176]. Higher dose IL-2 also enhances GzmA, GzmB and perforin expression in CD8+ T cells [140, 177]. IL-12 and IFN-α have been found to increase GzmB expression and chromatin accessibility [176, 178]. While it is tempting to draw parallels between the regulation of FasL and the better characterized Granzymes and perforin, factors upregulating Perforin protein do not consistently upregulate FasL [179, 180]. In addition, in some animal infection models, mixed populations have been observed containing cells of the same differentiated phenotype that may only degranulate, or only secrete IFN-gamma [181], but characterization for FasL is lacking.

There is also continuing investigation as to epigenetic control of FasL and GzmB in activated CD8+ T cells. GzmB epigenetic modulation has been characterized in human [182] and mouse [183, 184] CD8+ T cells. While two studies have examined histone modifications required to permit FasL gene expression in human T cells [185, 186], no information is known regarding the transcriptional control of FasL in various memory or effector lineage cells. Beyond epigenetic control, there is some evidence that FasL mRNA levels are higher in memory cells than in effector cells [187], but this relationship to FasL synthesis or surface expression is completely missing. Most expectations of what happens to FasL as cells differentiate are based on the assumption that FasL is similarly regulated *in vivo* compared to other effector mechanisms like GzmB and Perforin.

FasL storage and translocation

In mouse CD8+ T cells, FasL and degranulation are differentially regulated. While it has long been known that perforin-competent mice can still kill target cells without Granzyme release [188], the mechanism was unclear, though FasL was later identified as the culprit [162, 189]. FasL is stored in a vesicle absent of perforin or granzymes [190, 191]. These vesicles traffic to the cell surface upon restimulation in a manner regulated by intracellular Ca^{2+} release [190] and upon exocytosis can kill cells in a FasL-dependent manner. In unstimulated CTL FasL traffics through the surface of CTL en route after synthesis from the *trans* golgi network to the FasL storage vesicle [192]. This pre-synthesized FasL can appear on the CTL surface within 15 minutes after target cell engagement (Fig 1-3). In addition to translocation of pre-synthesized FasL, there is a second temporally distinct wave of FasL surface presentation. *de novo* synthesized FasL appears on the cell surface within 2 hours of target cell engagement or mitogen stimulation (Fig 1-3). Furthermore, they are executed under different biological stimuli: a strong target signal is required for later, *de novo* synthesized FasL, while a weak signal can still stimulate early FasL translocation[190]. These distinct waves of FasL on the cell surface also have different biological outcomes: early FasL only kills specific target cells, while late FasL can also kill bystander cells (Fig 1-3) [190]. CD4 cells have also been found *in vivo* to kill bystander cells in a Fas-dependent manner that also partially depended on bystander cell MHC expression[193]. The FasL wave with the greatest potential for destruction has a stronger requirement, while the "quieter" early FasL is less selective with regards to stimulus strength. These could have different outcomes in the tumor environment where there is a mix of strong tumor antigen responses and weak self-antigen or weak neoantigen responses. However, at this point we are still not sure

whether FasL even has the potential to be deployed consistently by CTL in the tumor environment.

Signaling through the Fas receptor

Fas (CD95) is the receptor for Fas Ligand. Fas-dependent cell death initiates the signaling for the extrinsic apoptotic pathway through the death domain in its cytoplasmic tail. Although Fas is already in trimers on the cell surface [194]. FasL binding Fas results in conformationtional changes that allow initiation of apoptosis through the recruitment of FADD (Fas-associating protein with Death Domain) to is cytoplasmic tail. The recruited FADD then recruits procaspase 8 to this complex, also known as the DISC (Death-inducing signaling complex), where procaspase 8 dimerizes and proteolytically cleaves itself to active Caspase 8 [167, 195]. Caspase 8 is released into the cytoplasm, where it then goes on to initiate apoptosis. In Type I (Fas-sensitive) cells, this is via cleavage of effector caspase procaspase 3. In type II (Fas-resistant) cells, Caspase 8 cleavage of Caspase 3 is insufficient for apoptosis activation, and so the later cleavage of antiapoptotic protein Bid by Caspase 8 is required, to activate the intrinsic apoptotic pathway[196].

Fas-mediated cell death can be blocked by engagement of cellular FLICE-like inhibitory protein (c-FLIP) proteins, which have structural homology to procaspase 8 but no catalytic ability. c-FLIP can form dimers or multimers with procaspase 8 at the DISC, and after such interaction, Caspase 8 gets incorrectly cleaved or not cleaved at all, and therefore remains stuck at the DISC, so that it cannot initiate apoptosis [197]. The ratio of procaspase 8 to cFLIP is important for cell death fate signaling [198, 199]. In addition, CD8+ T cell susceptibility to FasL-mediated fratricide is partly modulated by c-FLIP

levels[200], and different cytokines can affect the level of c-FLIP protein in cells [201]. Furthermore, Tregs are less susceptible to Fas-mediated cell death due to high c-FLIP levels [202], which would help them to execute suppressor activity in immune-privileged sites. In addition, exposure to stimuli such as TNF, or CD40L can increase c-FLIPS in cancer cells, reducing their susceptibility to Fas-mediated death [203]. Therefore, there can be resistance in tumors to FasL-mediated death in spite of abundant cell-surface Fas.

Another consequence of FasL signaling is the upregulation of NF-κB-regulated genes in cells with elevated c-FLIP$_L$ levels [199, 204], allowing survival and proliferation pathways [205]. This could have undesirable outcomes in cancer in c-FLIP high cancers when FasL "killing" is initiated by killer cells, only to be turned into a survival signal in the target cells.

There is some evidence that loss of Fas in cancer cells can itself cause cell death, though not through the apoptosis pathway. The Peter group proposed that tumor cells can have Death Induced by CD95R/L Elimination (DICE), a necroptosis-like pathway, when Fas-FasL signaling is abrogated, primarily by reduction of Fas expression [206-208]. It seems as though this is a backup mechanism the evolved to eliminate cells that lose/mutate both copies of the Fas gene.

Impact of Fas/FasL mutations

While the above scenario sounds like too risky of an outcome to desire FasL-mediated killing at all in the tumor environment, one of its major biological roles is to curtail autoimmunity. Mouse models with defects in the FasL and Fas genes have lymphoproliferation and autoimmune disease. The *lpr* mouse, which has an insertion causing defective Fas receptor expression [209, 210], *lpr-like*, which has a point mutation

blocking apoptotic signaling from the Fas receptor [211], and the *gld* mouse, which has defects in the FasL gene [209, 212]. All of these mice have Lupus-like autoimmune disease that worsens with the age of mice, autoantibodies, and high levels of a an aberrant T cell population, with a B220+ CD4- CD8- phenotype [213, 214]. Humans can suffer from Autoimmune Lymphoproliferative Syndrome (ALPS), which present similar autoimmune symptoms and overabundance of CD4-CD8- T cells, as a result of mutations in the Fas gene [215].

FasL in the control of infection

With respect to pathogen clearance, there is definitely mixed evidence on the necessity of FasL in the control of the immune response. The research is conflicted on the necessity of FasL for clearance of infection [162, 216-219] and tumors [220-224], Potential biological roles of FasL during an infection include clearing infected cells. The requirement for FasL varies depending on the pathogen. FasL is not required to clear LCMV infections in mice, though its contribution has not been rules out [216, 219], and contributes to clearance of West Nile virus [225]. While one effector mechanism may be sufficient, often the ability to use multiple effector mechanisms will result in an optimal cellular response. For example, FasL can simply enhance the ability of individual perforin, GzmA, and GzmB to control acute Friend virus infection in mice, but also FasL is absolutely essential for control of chronic Friend virus infection [226]. Price and colleagues found that in influenza-infected mice, perforin-deficient or FasL-deficient mice can both independently control infection, but eventually escape mutants of the influenza virus emerge. Mice with intact FasL and perforin can control influenza and restrict viral epitope mutations [227]. Overall, it seems that virus strain and pathology

affect the utility of FasL or degranulation in viral clearance, but it is also likely that the expression of multiple effector mechanisms can result in mutational escape of viral infections. Might this be the case in cancer as well?

Other non-apoptotic FasL signals

FasL can also be cleaved by matrix metalloproteases such as ADAM10, resulting in soluble FasL [228]. Soluble FasL does not have proapoptotic function, but its cleavage serves to reduce FasL-mediated apoptosis both by removing FasL from the cell surface and by freeing soluble FasL to bind to Fas and block it from interacting with membrane-bound FasL in a proapoptotic manner. Soluble FasL in serum or ascites has been correlated with tumor progression in some human and murine models [229]. There is also the possibility that soluble FasL bound to Fas can initiate a mobility-inducing signaling complex (MISC), a pro-survival pathway mediated through PI3 Kinase signaling [230, 231].

The FasL-Fas interaction is not a one-way signal. Reverse signaling from FasL can affect sensitivity to TCR signaling[232, 233], and CD8+ T cells from *gld* mice have reduced proliferative capacity [234].

Klebanoff and colleagues also showed that when mixed CD8 and CD4 populations containing effector and control memory populations plus naïve populations are used for adoptive cell therapy against tumors, the memory and effector cells can have negative impact on the naïve cells, causing them to differentiate without generating antitumor memory. This is driven by Fas on the naïve cells receiving FasL signaling from their activated counterparts, but instead of initiating cell death, results in Akt-mediated

signaling [114]. They have termed this "precocious differentiation" and it appears to be another way to maintain homeostasis between differentiated populations of T cells.

Activated T cells can eventually become exhausted

Coinhibitory Receptors contribute to peripheral tolerance by dampening activation of T cells, either by directly interfering with the TCR-mediated intracellular signaling cascade, competing with costimulatory receptors for their ligands, or by generating more signals that lead to alternate differentiation. PD-1 is one of the best known of these inhibitory receptors. It is expressed on T cells after TCR stimulation [235]. The ligands for PD-1 are PD-L1[236] and PD-L2. PD-L1 in particular can be expressed on many tumor cells lines and *in vivo* [237]. Upon its ligation, PD-1 recruits intracellular signaling molecules SHP-1 and SHP-2 that dephosphorylate TCR-mediated signaling molecules [238-240]. PD-1 inhibits cells in a manner independent of another coinhibitory receptor, CTLA-4 [241]. PD-1 is upregulated on cells following antigen-specific stimulation and then downregulated in the absence of further stimulation [242]. It not only halts proliferation [243], it regulates autocrine IL-2 production and increases the TCR signaling threshold required for CTL restimulation [243]. IL-2 can rescue cells from a dysfunctional state, in spite of PD-1 [242]. PD-1 has also been found to decrease the "stop rolling" signal in CTL, preventing PD-1+ cells from easily entering inflamed tissue [244].

CTLA4 is another coinhibitory molecule, from the same family as costimulatory receptor CD28. It does not have an inhibitory signaling motif in its tail, but it can outcompete CD28 for binding to B7.1 and B7.2[245], and may directly disrupt signaling cascades. Other inhibitory receptors that can be elevated in CD8+ T cells by the tumor

and by chronic infection include B and T cell lymphocyte attenuator (BTLA) [246] and Tim-3 [247, 248]. Multiple coinhibitory receptors together on T cells are associated with an increased state of exhaustion [249-252].

T cell exhaustion is the state in which T cells progressively lose effector ability, as a result of chronic antigen exposure, and some have argued that programming for exhaustion versus eventual exhaustion is determined at the MPEC stage [253, 254]. Chronic neoantigen exposure from tumors is a major driver of CD8+ T cell exhaustion [255], resulting in diminished expression of transcription factors critical in expression of many effector-associated and memory-associated genes[120, 255]. This is often seen in chronic viral infection and in cancer [249]. Schietinger and colleagues have shown evidence suggesting that CD8+ T cell exhaustion is initiated early on in the tumor response, at first in a reversible form (loss of CD127 expression, loss of TNF-α and IFN gamma expression) [256]. Cells can initially be rescued by IL-2 [242] or PD-1 or CTLA4 blockade [257], but if allowed to continue unchecked, can eventually become irreversibly dysfunctional [255, 258]. This is a total loss of cytokine secretion, loss of cytolytic mechanisms, blockage of TCR signal transduction, and increased susceptibility to apoptosis [255, 258]. Schietinger and colleagues found that *fasl* gene expression is upregulated in exhausted cells, while *gzmb* gene expression is downregulated in exhausted cells from melanoma lesions [255]. I have not been able to find evidence on how FasL protein expression is modulated in exhausted CD8+ T cells.

Exhaustion is, to some degree, similar from a T cell's perspective whether its antigen is from self in the context of autoimmunity, or from tumor in the context of cancer. The T cells are present in an environment with sufficient target antigen, and

stimulation at sufficiently high levels to then turn on coinhibitory receptors. As it encounters more antigen over time, the suppressive signals turned on accumulate, and eventually the suppressive signals override stimulatory environment enough that the cell is no longer able to exert effects on other cells. How this can be manipulated or prevented to the advantage of cancer clearance while avoiding autoimmunity is a challenge for cancer immunotherapy.

Cancer and the Immune System

A brief overview of the tumor microenvironment

Tumors are heterogeneous environments comprised of transformed cells, vasculature, and stromal cells. The tumor cells themselves are transformed and accumulate mutations to evade growth suppression, enable immortal replication, and increase secretion of and sensitivity to proliferative signals and growth factors [259]. Some transformed cells can also turn on genes that allow for metastasis and invasion. This is often facilitated by undergoing endothelial-mesenchymal transition (EMT), often via loss of E-cadherin-mediated adhesion to other cells [260] or upregulation of chemokines like CCL5 [261].To supply resources to the growing mass of solid tumors, oncogene signaling can upregulate expression of factors like Vascular endothelial growth factor (VEGF) to induce angiogenesis [262].

Within tumors are a multitude of somatic and immune cells that are themselves not transformed, which make up the tumor stroma. These help make tumors into complex tissues that are in many ways distinct from the tissues/organ surrounding it [263]. Stromal cells can include fibroblasts, pericytes that surround and support the vascular endothelium [264], cancer stem cells [265], and a variety of myeloid and lymphoid

immune cells. The tumor cells themselves can recruit inflammatory myeloid cells, which can assist in remodeling the extracellular environment [266]. Inflammatory immune cells can also activate epithelial-mesenchymal transition (EMT), and release reactive oxygen species that can contribute to further mutation of the tumor cells [267]. In addition to these proinflammatory myeloid cells, lymphocytes are recruited to tumors, where they can have both antitumor and immune-suppressive effects. Hanahan and Weinberg added four new hallmarks of cancer. Among these, the most important to this thesis is the hallmark of avoiding immune destruction [268].

Barriers to an effective T cell response in cancer

While traditional cancer chemotherapy can induce tumor cells to be directly more immunogenic [269], and can initiate immunogenic cell death [270, 271], it is a constant battle between immune evasion by the tumors, avoidance of autoimmunity/damage to self, and effective clearance of tumor cells. The numerous ways in which tumors can persist in spite of an active immune response makes obvious the need to better understand which aspect of the cellular immune response are actually effective. Thompson and colleagues found that naïve cells can transit directly to tumors in the absence of lymph nodes and become activated and develop effector ability [186, 272], but of course there are significant challenges from the tumor environment prohibiting their entry.

Suppressor cells in the immune environment

Tregs and Myeloid-derived suppressor cells can contribute significantly to the dampening of the immune response to tumors by inhibiting proliferation and activation of tumor-reactive lymphocytes [273]. Tregs can be recruited to the tumor site by chemokines produced by the tumor itself or by tumor-associated macrophages and

MDSC [274], which are discussed in more detail below. Tumor cells themselves can also directly interact with T cells in a suppressive manner. For example, one group found evidence suggesting that PD-L1+ tumor cells can induce Fas-FasL mediated CD8+ T cell death in the tumor, but did not clarify whether this death was from FasL on the CTL, in a fratricidal manner, or FasL upregulated on the carcinoma cells [237].

Loss of antigen on tumor cells/lack of CTL specific for tumor antigen

Downregulation of MHC by tumor cells [42], variable levels of tumor antigen, and the ability of tumors to respond to selective pressure from the immune system by losing immunodominant peptide antigens is a well-known problem. [275, 276]. Conversely, chronic exposure to immunodominant antigens can lead to exhaustion and dysfunction of the T cell population [277]. In addition, methods of tolerance to avoid autoimmunity can inhibit the expansion or even presence of T cells that recognize the tumor. Negative selection in the thymus prevents self-reactive T cells from reaching maturation [8], and many tumor antigens are slightly modified self antigens. The tumor may not produce significantly "foreign" antigen to be recognized by circulating T cells. Furthermore, T cells that are activated by tumor antigens can often become anergic [278]. Unless antigen is presented along with costimulatory ligands, T cells do not become properly activated [279] by an antigen presenting cell. If the tumor is the first site on which a T cell sees its cognate antigen, it will become anergic rather than active [278].

Physical and Metabolic/Hypoxic barriers

Changes to the vasculature in tumors can result in the loss of ligands for T cell homing or physical reorganization that mechanically prevents CD8+ T cell infiltration [280, 281]. In addition to restricted trafficking into the tissue, the tumor

microenvironment, particularly in solid tumors, can be hostile to T cells through limited oxygen availability. Typically, active T cells use aerobic glycolysis to produce ATP to fuel rapid proliferation and biosynthesis of effector proteins [282], but hypoxic tumor environments can limit the availability of oxygen. While CTL can survive and lyse target cells in hypoxic environments [283], their proliferation can be negatively impacted [283]. In addition, the hypoxic environment can induce tumors to upregulate PD-L1 [284], enhancing the suppressive effect on any nearby CD8+ T cells that have upregulated PD-1.

Myeloid-Derived Suppressor Cells and Tumor-associated Macrophages

An emerging player in the suppression of the antitumor immune response is a family of cells called Myeloid-Derived Suppressor Cells (MDSC). They are of myeloid origin and can resemble monocytes as M-MDSC, or resemble neutrophils as PMN-MDSC [285]. M-MDSC are more plentiful than PMN-MDSC in tumors, and can differentiate into suppressive tumor-associated macrophages (TAM) [286]. Both M-MDSC and TAM can inhibit CD8+ T cell activity: M-MDSC and TAM can deplete nutrients needed for CTL metabolism and secrete reactive oxygen and nitrogen species to promote T cell anergy [287, 288]. When the presence of TAM is upregulated, TAM can outcompete DC for antigen presentation to CD8+ T cells and inefficiently activate them, preventing CD8+ T cells from encountering highly stimulatory DC and being efficiently activated [289]. MDSC can also enhance activation of Treg cells [290].

There is mixed evidence about the relationship between CTL and MDSC with respect to Fas/FasL. MDSC can express the Fas receptor and undergo apoptosis in a Fas-dependent manner [291], but *in* vivo studies in FasL and Fas-deficient mice suggest that

FasL-FasL interactions between CTL and tumor and indirectly recruit MDSC to the tumor site [292], or may polarize the proportion of MDSC populations [293]. Furthermore, some PMN-MDSC can express FasL themselves to kill tumor-infiltrating CTL [294].

Tumor-derived exosomes

Tumor cells can also be a source of immunomodulatory exosomes. These are small (20-100nm), membrane-bound spheroids that are formed by inward budding of a vesicular membrane, and are exocytosed when the exosome-containing vesicle fuses with the outer cell membrane. Tumor-derived exosomes (TDE) can contain transmembrane proteins as well as soluble proteins and nucleic acids to act on secondary cells that they fuse with [295]. They can act on innate and adaptive immune cells, and can arise from solid tumors as well as lymphomas and ascetic tumors [295]. With respect to human T cells, tumor conditioned medium and enriched microparticles can induce suppressive activity in CD8+ T cells [296], which are capable of suppressing proliferation of conventional CD8+ T cells. Prior to this, TDE from cancers had been shown to kill CTL in a Fas-dependent manner [297, 298] [299], and promote conversion of CD4+ T cells into GzmB Tregs [298]. In addition, TDE can promote tumor growth in a mouse model by reducing CD8+ numbers, presumably via their demonstrated cytotoxic effects on CTL [300].

Immunotherapy of Cancer

Immunotherapy has been a theoretical possibility for decades, and currently there are numerous clinically applied as well as promising immune based therapies against cancer. Antigen non-specific approaches involve changing the immune environment to enhance

the antitumor response or reduce suppression. Cytokine therapy has shown some spectacular successes and failures in the clinic [301, 302]. IL-2 was the first cytokine immunotherapy for cancer with real, broad benefits for patients. It is given to patients as an immunomodulatory drug, as an adjuvant to adoptive cell therapy, and used to expand patient TIL populations for autologous immunotherapy [303]. Modulating the IL-2 effects on donor cells as well as post-ACT secretion of IL-2 is still a field of intense study to improve efficacy while reducing side-effects [304]. As it can expand any CD25+ T cell population, therapeutic IL-2 can expand both desirable effector T cell populations, as well as CD4+ Treg populations, which as discussed in the previous section may suppress the antitumor response. IL-12 is still intriguing to researchers as an immune therapy, though it has many adverse effects on patients [305]. It is promising as an adjuvant to adoptive cell therapy in preclinical studies [306] or to condition adoptively transferred cells prior to cell therapy [307, 308]. Some suggest that combination of cytokine therapy with other immunotherapy would bring the best benefits [309, 310].

Checkpoint blockade therapy operates on the principle of blocking the "stop" signals that coinhibitory receptors and their ligands provide to T cells. The purpose of all of these are to prevent T cells that are expressing coinhibitory receptors from receiving inhibitory signals and therefore become less exhausted and retain or possibly regain function in response to target cells [249, 257]. Ipilimumab, which binds to and blocks CTLA-4, is the first such antibody approved by the FDA for use in humans. It has shown success in treatment against melanoma, though with severe side effects in some patients [311]. Blockade against PD-1 and its ligand PD-L1 are also under active investigation. The presence of PD-1 has also been suggested to be associated with recently activated T

cells in tertiary lymphoid structures in tumors [312], so maintaining cells active at these sites in spite of elevated checkpoint inhibitor expression should be beneficial to the antitumor response.

In some cases, checkpoint blockade therapy against PD-1 and CTLA-4 on activated cells approaching exhaustion seems to be most successful when initiated against targets that have a high mutational burden [313], but there is extremely wide variation in the success of checkpoint blockade therapy, likely due to variation between intrinsic immunogenicity of tumors, as well as the other factors mentioned in this section [314]. Furthermore, strongly exhausted cells seem to be resistant to any effects from checkpoint blockade [315], so therapeutic effects are limited by the proportion of PD-1+ cells that are not exhausted, or are at least less dysfuntional.

Of course, if CD8+ T cells are unable to penetrate a tumor at all, checkpoint inhibitors will not enhance direct cytotoxic activity against the tumor cells. Furthermore, blocking the activity of checkpoint inhibitors also increases the chances of autoimmunity [301].

A targeted approach that integrates a larger repertoire of immune cells is cancer vaccination, commonly through injection of a tumor antigen directly [316], or through injection of peptide loaded DC or injection of antigen coupled to DC-targeting antibodies [317]. Over the years these approaches have become increasingly personalized for patients [318].

Adoptive cell therapy is the transfer of patient-derived, tumor-reactive T cells into the patient, where they can recognize target cells and then execute therapeutic cytolytic, immunostimulatory (or, if desired, suppressive) effects. While a distinct advantage of this

approach is its specificity for tumor cell antigen, it only has specificity for as long as the tumor continues to express the antigens that the transferred T cells recognize [319]. One version of this is autologous cell therapy, whereby tumor-infiltrating lymphocytes (TIL) are isolated from the patient. The CTL can be expanded by culture with cytokines such as IL-2, stimulated by tumor-associated antigens, or transduced with genes for TCR against a defined tumor epitope [320]. A more dramatic manipulation of the T cells for therapy involves transfecting the cells with chimeric antigen receptors (CAR), which are a fusion protein. This fusion protein typically has the extracellular domain of an antibody against a tumor antigen, and the intracellular domain of TCR or costimulatory receptors, to elicit an intracellular signal similar to a TCR being bound [321]. This has shown exceptional promise for patients with B-cell acute lymphoblastic leukemia (B-ALL), a leukemia often seen in children. The chimeric antigen receptor is targeted against CD19 on the surface of B cells[322]. There is hope that this success in leukemia can be translated to therapy for solid tumors, but the antigens recognized by the CAR need to be designed against solid tumor antigens in a manner that that will not result in autoimmunity [323]. In addition to transferred cells being susceptible to suppression and dysfunction like native T cells, another significant challenge with all adoptive cell therapies against cancer is that tumor cells can adapt to lose expression of the target protein [324].

Another concern when choosing which cells to manipulate for adoptive cell therapy is their activity and proliferative potential *in vivo*, with stem cell memory possibly having the most potential for lasting therapeutic effects in patients [325]. However, if other differentiated CD8+ T cells are available or emerge during

manipulation, it is important to thoroughly characterize their therapeutic potential, especially if they express diverse effector mechanisms.

CD8+ T cell response to tumors

Many groups have examined the CTL response to tumors in mice particularly in B16 melanoma [272, 326-328], and EG.7 lymphoma cells [329-337]. Most have used these models to ask questions about the efficacy of differently activated CTL as therapy for solid tumors, since often the endogenous response of CD8+ T cells to solid tumors is not sufficient for tumor elimination [220, 308, 327, 328, 331, 338, 339]. However, for CTL to effectively contribute to an antitumor response, they need to directly contact the tumor cells [340], so CD8+ T cells can use contact-dependent cytolytic mechanisms to kill cancer cells. Some have used the endogenous response as experimental controls [341, 342] with limited information about the characteristics of the resultant CTL, aside from a brief mention that CD8+ T cells in subcutaneous EG.7 are high in Granzyme B [341].

What do we know in general about the cytolytic ability of CD8+ T cells in tumors? Some tumors increase Fas receptor expression when cultured *in vivo* [343, 344], which may suggest that cytolytic activity via FasL is not a strong selective force against tumors. However, in a metastatic renal carcinoma model, FasL-deficient *gld* mice have increased mortality [345]. A substantial amount of work has compared tumor clearance in mice receiving adoptive cell therapy of activated CD8+T cells with manipulated effector protein expression. Conflictingly, FasL has been shown to contribute significantly to clearance [338, 345, 346], either while degranulation is dispensable [345, 347], or in combination with degranulation [336]. Others have found that therapeutic CD8+ T cells need neither FasL nor degranulation to clear tumors, and it is dependent upon the release

of effector cytokines such as IFN-γ by CD8+ T cells [326, 337]. In addition, one group has shown that cytolytic effector mechanism use changes over time, as CD8+ T cells in an allograft tumor response increasingly use FasL to kill other cells as time progresses following tumor engraftment [180].

With all of these positive and negative influences on the survival and entry into tumors, FasL and degranulation must have differing biological regulation *in vivo* in order to fulfill distinct biological activities ascribed to them. Would they also be regulated during the activation of naïve cells prior to adoptive cell therapy, and have an impact on the adoptive transfer experiments described above? Furthermore, what does "normal" look like for FasL and Gzm B expression in mice, and how does that change in cells responding exclusively to the tumor or its antigens? What does that expression look like as the populations emerge against the tumor, both effector and memory, and potentially exhaustion? To better exploit immune therapy against cancers, we need to understand what effector mechanisms are employed by CD8+ T cells in tumors and how we man manipulate the expression of these mechanisms.

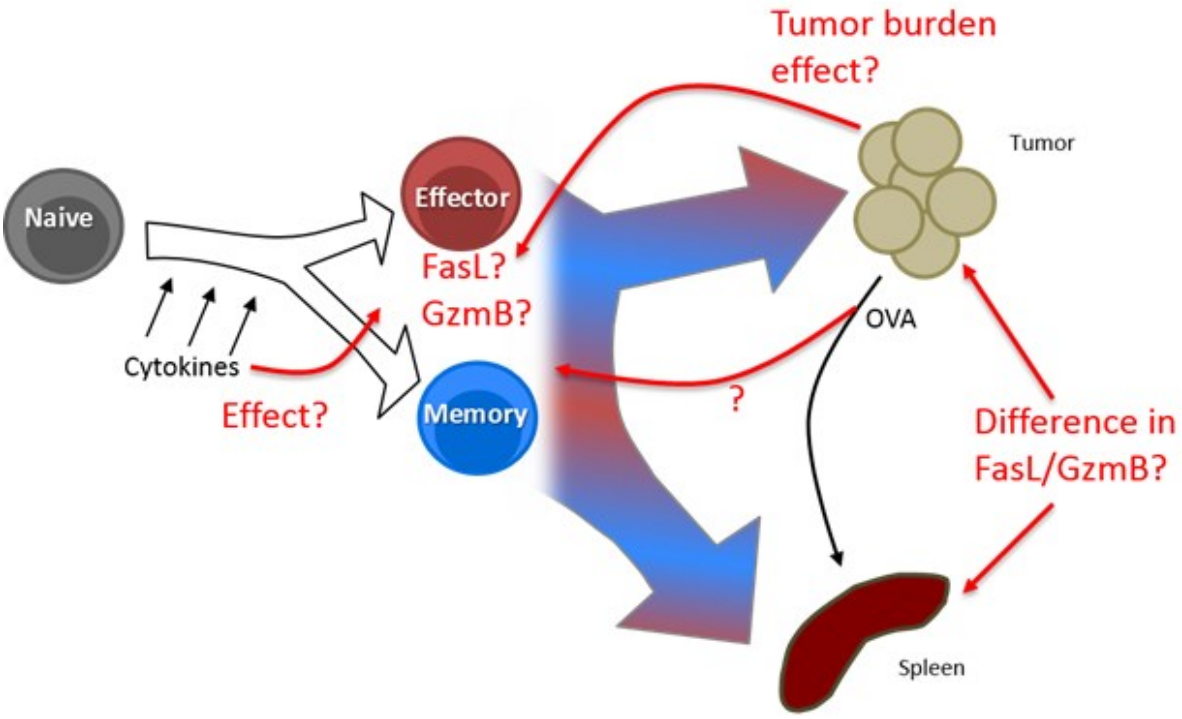


Figure 1-4. Questions addressed by this thesis:
Following the activation of naïve cells to activated cells, and differentiation to memory and effector lineages, what effect do cytokines have on FasL or GzmB expression? Does tumor burden, or presence in the tumor compared to the spleen, affect FasL or GzmB dynamics by activated CD8+ T cells? Is this different between tumor antigen specific CTL and the general CTL population? Is this the same for both memory and effector cells?

Hypothesis

As other researchers have shown that FasL and degranulation can have distinct biological roles in viral clearance and tumor models, I hypothesize that FasL is regulated by cytokine signals in the *in vitro* activation milieu in a manner distinct from GzmB, and that FasL protein is expressed by CD8+ T cells in a biologically distinct manner from GzmB in cells responding to tumors *in vivo*.

Objectives

1. Determine whether FasL protein expression by CD8+ T cells *in vitro* is affected by cytokine activation conditions known to increase GzmB stores.
2. Determine the dynamics of FasL and GzmB protein expression in tumor-infiltrating CD8+ T cells.
3. Determine whether FasL intracellular storage and surface expression by CTL *in vivo* is preferentially expressed by memory or effector cells, and whether this is affected by antigen specificity to the tumors they infiltrate.

In this thesis I will present data demonstrating that FasL stores in CD8+ T cells are not strongly affected by cytokines following activation *in vitro*. Furthermore, under naïve cell stimulation conditions that result in a majority of activated cells expressing GzmB, FasL is not always expressed by a majority of CD8+ T cells. I will also show that converse to this, *in vivo* CD8+ T cells in mice bearing EG.7 tumors are more likely to have intracellular stores of FasL than GzmB. I will show that unlike GzmB, FasL protein expression is not associated with specificity for specific tumor-associated antigens. I will also demonstrate that intracellular FasL expression is not restricted by tumor volume in mice, and can be found in cells of all examined phenotypes.

Chapter 2:
Materials and Methods

Mice

Female C57/BL6 mice from Charles River Laboratories were used as recipient mice and sources for cells for all experiments unless noted otherwise. Female OT-I mice [348] on a C57/B6 background as well as female CD45.1/CD45.2 heterozygous mice were a generous gift from the Baldwin lab at the University of Alberta. Ly5.1 (CD45.1) mice on a C57/BL6 background were from Charles River Canada. All mice were caged in conventional facilities and handled according to protocols approved by the University of Alberta animal care and use committee (Protocol AUP#305).

T cell enrichment

Mice not receiving any injections, and used as a source of lymphocytes for naïve CD8+ T cell enrichment, had the following organs removed and pooled: spleen; axillary, brachial, cervical, inguinal, and popliteal lymph nodes. All were transferred to Dulbecco's PBS (dPBS) (Invitrogen). All pooled tissues were homogenized in a glass Dounce homogenizer in dPBS.

Cells were strained through a 70uM nylon filter and washed with dPBS three times and counted by haemocytometer, without including erythrocytes in the counts. Cells were resuspended such that one mouse spleen plus LN cells were combined with 1x volume of reagents for the EasySep mouse CD8+ T cell negative enrichment kit (STEMCELL technologies # 19853). Reagents were scaled accordingly for experiments using cells from multiple pooled mice. Incubation times and reagents were used according to the protocol in the kit.

To enrich for naïve $CD8^+$ T cells, biotinylated anti-CD44 clone IM7 (BD Biosciences) was added to the cell mixture at the same time as the CD8 enrichment cocktail in the STEMCELL protocol mentioned above. 0.03μg of antibody was added per $1x10^6$ of counted cells. After enrichment, cells were washed, counted, and resuspended in PBS for adoptive transfer, or in culture media for *in vitro* stimulation.

In vitro activation of naïve T cells

16-20 hours prior to stimulation, 24-well TC-treated plates were coated with anti-CD3ε (clone 145-2C11, HO lab stock) and anti-CD28 (clone 37.51, eBioscience): Concentration of coating antibodies are noted in results sections. Antibodies were added to dPBS and mixed gently before aliquoting 180 μL per well. Plates were gently tapped to ensure that entire well bottom was coated, then plates were wrapped in plastic wrap and incubated overnight at 4ºC. Wells without a complete meniscus the next morning were not used. Prior to plate-bound stimulation of cells, coated wells were washed twice with dPBS, blocked for 30 minutes at 37ºC with sterile 2% BSA in PBS, then washed three times and kept filled with dPBS at room temperature until needed.

Naïve $CD8^+$ T cells prepared as described above were resuspended at $3.0x10^5$ cells/ml in Culture Medium: RPMI (Invitrogen) plus 10% FCS (Fisher), 2mM L-glutamine (Gibco), 100ug/ml Penicillin (Invitrogen), 100ug/ml Streptomycin (Invitrogen), 0.1mM Non-essential Amino Acids (Gibco), 1mM Sodium Pyruvate (Gibco), and 53 nM 2-Mercaptoethanol (Sigma-Aldrich). If any inflammatory cytokines were used (see section below), they were added to master mixes of cells at the proper final concentration. All dPBS was removed from coated wells, and then the mixtures were aliquoted at 1mL/well. Cells were incubated for 48 hours at 37ºC with 5% CO_2.

After 48-50 hours (Day 2), cells were harvested by resuspending T cells with a transfer pipette . Cells with the same treatment were pooled into a tube, spun down and resuspended in the same volume of culture medium. After counting with a haemocytometer, cells concentration was adjusted again to $3.0x10^5$ cells/ml and divided into cytokine treatment groups, if required. IL-2 (and other cytokines, as indicated) was added to the cells to the desired final concentration, then cells were aliquoted at 1mL/well into fresh 24-well TC plates.

Cells were typically split on days 3, 6, and 9 post-start. Wells of cells that were the same treatment group were harvested with a transfer pipette and pooled. For each one harvested well, 1.3 mL of fresh media (containing the appropriate amount of cytokine for 1.3 mL) was added. Cells were gently mixed and aliquoted in a fresh TC-treated 24-well plate at 1ml/well.

PMA/Ionomicin Stimulation of Activated T cells

Cells were harvested from culture by transfer pipette and pooled into a tube, spun down and resuspended in the same volume of culture medium. Cells were counted, then spun down again and resuspended at $1x10^7$ cells/ml in RPMI+ 2% FCS. In a v-bottom plate, 94 µL ($0.94x10^6$ cells) of cells was aliquoted. To each well being stimulated, I added 6 µL of a master mix containing 8.33µM Ionomicin (Sigma-Aldritch) and 1.66 µg/ml Phorbol Myristate Acetate (PMA) (Sigma-Aldritch) diluted in RPMI+2% FCS. Final stimulus conditions were 100 ng/ml PMA, plus 0.5µM Ionomicin. Wells were gently mixed with a pipette, then covered and incubated at 37^{o}C for 15 minutes or two hours. After incubation, cells were immediately washed twice with cold PBS and pelleted in a 4^{o}C centrifuge prior to staining for surface FasL.

Cytokines

Recombinant IL-2 was prepared in-house from Z7 bacterial stock, and units/ml of stock was assayed using Ostergaard lab AB.1 clone assays in addition to CTLL-2 proliferation assays. Stock IL-2 was stored at 2000U/ml and stored at -20ºC when not in use. Aliquots were thawed a maximum of 5 times before being discarded. A range of 5U/ml to 100U/ml was used in experiments; the actual concentration is listed in each experiment description.

Recombinant universal interferon-alpha (IFN-α) was purchased from PBL Interferonsource (#11200-2) and stock solution was prepared at $1x10^6$U/ml in 0.1%BSA in PBS. Stock solution was stored at -70ºC. Aliquots were thawed once and stored at 4ºC to add to cells for one experiment and then discarded. Stock of IFN-α was added to cultures to achieve a final concentration of 1000U/ml, as used previously to differentiate naïve CD8+ T cells [176].

Recombinant mouse IL-12 was purchased from Biolegend (#577002) and stock solution was prepared at $1x10^5$ U/ml in 1% BSA in PBS. Stock solution was stored at -70ºC. Aliquots were thawed a maximum of three times to prepare a 1/100 diluted refrigerator stock in Culture Media, this refrigerator stock was used to add to cells for one experiment and then discarded. Working stock of IL-12 was added to cultures to achieve a final concentration of 2U/ml, as used previously [141, 176].

RNA extraction

$1x10^6$ cells were pelleted, then RNA was extracted from cell pellets using an RNeasy Mini Kit (Qiagen, # 74104) following the kit instructions. As part of the procedure, I followed the instructions for including use of QiaShredder columns (Qiagen #79654),

with on-column DNA digestion using the RNase-Free DNase kit (Qiagen). Following extraction, RNA was stored at -80ºC. Prior to reverse transcription, RNA was quantified using a Nanodrop 2000 spectophotometer (Thermo Scientific) and analyzed using Nanodrop 2000 software (Thermo Scientific).

Reverse transcription to cDNA template

50 ng of RNA template was added to the reagents for reverse transcription reaction from the Superscript III Reverse Transcription kit (Invitrogen) according to the product instructions. cDNA was synthesized according to the reverse transcription kit instructions, in an Eppendorf Mastercycler gradient model thermocycler. cDNA template was stored at -80C until PCR was carried out.

Quantitative PCR (qPCR)

Oligonucleotide primers were purchased from Integrated DNA Technologies and stored as 20 μM stocks in water. The internal reference gene hypoxanthine guanine phosphoribosyl transferase (HPRT) was used, detected by a primer pair designed by Dr. Deanna Hockley. The most efficient primer pair for FasL was a pair published previously[349]. All primer sequences for intron-spanning primers are summarized in Table 2-1. cDNA templates were diluted 1/4 in RNAse/DNAse-free water and 2 μL was added to an 18 μL volume of reaction master mix, with each single reaction containing 10 μL of Power SYBR Green PCR kit (Life Technologies), plus 0.2 μL of each forward and reverse primer stock (table 2-1) and 7.6 μL of water; the final reaction volume was 20uL. Samples were pipetted into twin-tec PCR plates (Eppendorf), sealed with film, gently vortexed, and centrifuged to pool liquid. Samples were immediately run on an Eppendorf Realplex2 Mastercycler epigradient PCR machine using a temperature cycle as follows:

50ºC – 2 minutes
95ºC – 10 minutes
40x of: {95ºC – 15 seconds
{50ºC – 60 seconds
Melt curve of 60ºC → 95ºC – 20 minutes
95ºC – 15 seconds
4ºC – hold

Data was analyzed using Ep Realplex Software (Eppendorf). Increases in FasL or GzmB transcripts were calculated relative to the HPRT control gene and then within experiments fold-change in expression was calculated compared to the values for cells cultured at 5U/ml IL-2.

Table 2-1 Primers used in qPCR.

Primer	**Sequence**
HPRT forward	5'- CAGTCCCAGCGTCGTGATTAGCG -3'
HPRT reverse	5'- CCTTGAGCACACAGAGGGCCAC -3'
GzmB forward	5'- ACCCTACATGGCCTTACTTTC-3'
GzmB reverse	5'-TGACATTTATTATACTTCCTTCACAG -3'
FasL forward	5'- CGTGAGTTCACCAACCAAAGC -3'
FasL reverse	5'- CCCAGTTTCGTTGATCACAAG -3'

Tumor cells

EG.7 cells [350], which are EL4 lymphoma cells transfected with ovalbumin DNA, were a generous gift from the Kane lab at the University of Alberta. Cells were cultured in RPMI (Invitrogen) + 8% FCS (Thermo Fisher). Cells possessing the OVA gene were selected by supplementing the culture with 0.4mg/ml Geneticin (G418) (Invitrogen) at 37ºC with 5% CO_2. Cell cultures were periodically checked to ensure expression that the dominant SIINFEKL peptide of albumin was presented on the cell surface in MHC. This was checked by surface antibody staining against H-2K^b-SIINFEKL (Table 2-2). Cultures were discarded if not all live cells were found to be

expressing the peptide-antigen complex and replaced with fresh culture from frozen stock. 16-26 hours prior to injection, cells were resuspended in media lacking G418. Cells were washed three times in serum-free, Ca-Mg-free Dulbecco's PBS (dPBS) (Invitrogen) and resuspended in dPBS at a concentration to result in the desired number of cells in 200 μL of injectate.

Adoptive transfers and tumor implantation

1 mL syringes were filled with >200 μL cells at the desired concentration and stored horizontally at room temperature until used. Less than one hour elapsed between syringe filling and final injection of mice. Syringes were fitted with sterile 27G needles. Immediately prior to injection, cells were resuspended by gently moving syringe plunger until mixture was homogenous, without creating bubbles. Any air was removed, volume was adjusted to 200ul, and liquid was injected into mice. Note that 200 μL is the total injected volume, and does not include volume remaining in the needle. Different injections required different concentrations of cells to deliver the desired number of cells in 200ul, as described below.

Subcutaneous (SC), Intraperitoneal (IP), and Intravenous (IV) injections were carried out according to standard animal use protocols and approved by University of Alberta Ethics Committee. Subcutaenous injections of EG.7 tumors contained $2.0x10^6$ cells in 200 μL and were always injected into the right flank following inhalation anesthesia by Isoflurane. Intraperitoneal injections of EG.7 tumors contained $2.5x10^6$ cells in 200 μL and were always injected into peritoneal cavity following inhalation anesthesia by Isoflurane. Intravenous injections of naïve CD8+ T cells contained $1.0x10^6$ cells in 200ul. Mice were allowed to recover before being returned to animal housing.

Tumor monitoring and harvesting

Injected mice were assessed for health status every 2-3 days and conditions were recorded on a log sheet. External tumor volume for subcutaneous tumors was measured by the same researcher every time by the same pair of calipers. Volume was calculated as (H x W x L) ÷ 2 in mm^3, which is a slight modification of the methods previously published for ellipsoid tumors[351, 352], to allow for more irregularly-shaped tumors. Mice were euthanized when tumors were a range of volumes, from first palpable (27 mm^3) to maximum volume allowed. Once external volume reached 2000 mm^3, mice had to be euthanized. Some individual mice had a final volume over 2000 mm^3 due to a rapid volume increase after a previous smaller volume, or overnight growth after a mouse was measured as at less than 2000 mm^3 and was placed aside for sacrifice the next morning. Mice were euthanized by Isoflurane inhalation followed by cervical dislocation. As dictated by the experiment, the individual tissues were harvested.

For mice with SC tumors, draining (right side, dLN) and contralateral (left side, cLN) inguinal and popliteal lymph nodes removed. Spleen was removed. Tumor was removed by gently peeling back skin and connective tissue. Tumors were measured with calipers immediately after dissection. In animals with multi-lobed tumors, the measurement was taken of the entire mass together. All tissues were placed in wells of chilled 6-well plates containing RPMI. Larger tumors were cut in half if needed to remain submerged in media. All samples were pressed through a metal mesh screen (152um) with a syringe plunger and rinsed with RPMI + 2.5% FCS. No collagenase was added. Samples were kept cold (4-8^{o}C) up to 1 hour when not being actively processed. Spleen and tumor samples were treated with ammonium chloride to lyse red blood cells. All samples were subsequently washed 3 times with RPMI + 2.5% FCS.

For mice injected with tumors IP, peritoneal cells were harvested by the following lavage protocol: Abdominal skin was cut and peeled back, leaving abdominal membrane intact. A 5 mL syringe was filled with 3 mL of dPBS and 2 mL of air, and fitted with a 27G needle. The liquid was injected into the abdominal cavity, aiming the stream in multiple directions. Air was also injected to inflate the abdominal cavity. The mouse was held in one hand and gently shaken in an anterior-posterior motion 5-8 times. The mouse was then placed onto an orbital shaking device on medium speed for 8-12 minutes. After shaking, a 3mL syringe fitted with a 21G needle, bevel-up, was used to remove cells from the abdomen and added to a chilled tube filled with approximately 2 ml of PBS + 5% serum. The peritoneum was immediately rinsed with 1mL additional dPBS that was removed and added to sample. Rinsing was repeated again if possible. On average 4 mL was recovered per mouse. Any draw of peritoneal fluid that contained blood was discarded and collection was stopped.

Sample staining

All cell suspensions to be stained were kept on ice prior to staining and during cell aliquoting. Approximately 1-2x10^6 cells were used in each 100 µL staining reaction. Tumor samples were scaled up when possible; twice the number of cells were resuspended in twice the "1x mastermix" volume of media (Table 2-2) plus antibodies.

Cells were aliquoted into v-bottom 96-well plates (Corning) and centrifuged at 2100xg, then flicked to remove supernatant. Cells were resuspended in prepared Fc Block mastermix (1x = 98 µL Surface Stain Buffer (dPBS + 2% FCS + 5mM EDTA) +2 µL mouse Fc block). and incubated at 4ºC for 30 minutes. Cells were centrifuged and

supernatant flicked off. Pellets were resuspended in 100 µL (or 200ul, for tumor samples) of surface antibody mastermix.

Antibody mastermixes were prepared no more than 30 minutes before staining and stored on ice before use. Mixes were prepared by aliquoting half the final volume of required buffer, adding all required antibodies, and then adding remaining half of buffer and mixing gently to combine. For example, for a 5x mastermix with 2 antibodies each used at 0.5/100, the final volume would be 500ul: 247 µL buffer + 2.5 µL antibody A + 2.5 µL Antibody B + 247.5 µL buffer; 100 µL would be added to each stained sample. For surface staining, antibodies were added to Surface Stain Buffer (dPBS + 2% FCS + 5mM EDTA). For surface staining with multiple dyes off of the violet laser, I used a mixture of one part Surface Stain Buffer and 1 part Brilliant Stain Buffer (BD Biosciences). See Table 2-2 for antibody clones and final concentrations used. Surface stains were incubated for 30 minutes at 4^{o}C, and then cells were washed three times with PBS.

After washing, cells were resuspended in freshly prepared 2% formalin in PBS. Cells were fixed at room temperature in the dark for 12 minutes. Cells were washed twice with PBS. For samples not requiring permeabilization (i.e. stains involving surface FasL), cells were resuspended in PBS for remaining steps. For all other samples, cells were washed once in Perm Stain Buffer, centrifuged, and then resuspended in 100 µL of prepared intracellular antibody mastermix (using same methods described above). Note that "intracellular FasL" described in results chapters detected by this method could include cell-surface FasL stained during this method as well, as staining for surface FasL was not blocked. For intracellular staining, antibodies were added to Permeabilization

Stain Buffer (dPBS + 0.2% Saponin +4% FCS). Cells were incubated at 4ºC for 30 minutes, then washed three times with PBS. All samples regardless of permeabilization were resuspended in PBS + 0.5% formalin and stored at 4ºC for 1-24 hours before analysis.

Samples were analyzed on a BD LSRII, BD LSRForetessa, or BDFACSAriaII cytometer and data was analyzed using FlowJo Software (TreeStar). Gates for intracellular staining and expression were determined by comparison against a "fluorescence minus one" (FMO) control if necessary.

Table 2-2 Antibodies used in flow cytometry staining.

Antigen	Clone	"1x"	Brand	Note
Fas Ligand	MFL3	1/100	BD Biosciences	Always PE. Surface OR intracellular
Granzyme B	GB12	2/100	Life Technologies	Always APC. Always intracellular
CD8α	53-6.7	0.5/100	BD Biosciences	Changed colour depending on panel
CD44	IM7	0.5/100	BD Biosciences	Changed colour depending on panel
CD62L	MEL-14	0.5/100	BD Biosciences	Changed colour depending on panel
CD127	AR734	0.5/100	BioLegend for BV711, eBioscience for PerCP-Cy5.5 in earlier experiments	
Vα2 TCR	B20.1	0.5/100	eBioscience	APC-ef780
Vβ5.5,5.2 TCR	MR9-4	0.5/100	BD Biosciences	FITC
CD45.1	A20	0.5/100	BD Biosciences	Changed colour depending on panel
CD45.2	104	0.5/100	BD Biosciences	Changed colour depending on panel
PD-1	J43	1/100	eBioscience	PerCP-ef710
H-2K^{b}-SIINFEKL	25-D1.16	1/100	eBioscience	APC
Isotype for above	P3.6.2.8.1	1/100	eBioscience	APC
Mouse Fc block	2.4G2	2/100	BD Biosciences	N/A

Data Analysis

Statistical analyses were calculated in Microsoft Excel and GraphPad Prism. For normalized MFI values presented in many data chapters, Mean Fluorescent Intensity (MFI) of stained samples was divided by MFI of a matched control sample of the same cell type but lacking the antibody of interest.

Chapter 3: The effect of inflammatory cytokines on FasL expression by newly activated CD8+ T cells

Introduction

FasL and GzmB represent distinct cytolytic activities, and past work from our lab has shown that expression of FasL, and degranulation, in response to antigenic and mitogenic stimuli is differentially regulated [353, 354]. Expression of Granzyme B has been more thoroughly characterized. We know its expression dynamics *in vivo* in some viral [217, 355, 356] and tumor [220-224], models. However, the research is conflicted on the necessity of FasL for clearance of infection [162, 216-219] and tumors [220-224]. Previous work from our lab has also shown that FasL can be presented by CTL under lower antigen stimulus conditions than are optimal for degranulation [354], which further underlines the possibility that these two effector mechanisms have distinct biological roles. This shows the need to better understand the effect of the environment in which the T cell finds itself on FasL protein expression. One approach is to examine this in CD8+ T cells activated under defined conditions, which is the focus of this chapter.

In addition, as autologous and chimeric antigen receptor therapies emerge [357-359], understanding the relationship between *in vitro* activation environment and effector mechanisms is increasingly important. For example, does the environment favor particular effector mechanisms? Do all CD8+ T cells synthesize FasL and GzmB. Are they expressed by only some subpopulations? Are they influenced in a similar manner by the same activating signals?

The parameter I have focused on examining in the context of activation is the cytokine signal present during or after activation. The cytokines released by APC *in vivo* are modulated by pathogen danger signals [360], and the dose received by CTL can be

affected by proximity to innate cells and other lymphocytes. Furthermore, cytokines are used therapeutically to modulate cell therapy products for human treatment [116, 303, 305]. While cells can proliferate in the absence of extrinsic cytokine under many conditions, this third signal increases the cytotoxic response of CD8+ T cells [30, 78]. Furthermore, inflammatory cytokines can drive differentiation of activated CD8+ T cells into populations of effector and memory T cells [141, 176]. In this chapter, I quantified FasL protein levels in cells in response to varied cytokine environments and in response to post-activation CTL differentiation. I found that cytokines that can elevate GzmB protein levels have less of an effect on the FasL protein levels. I also found, however, that the cytokines that are present during activation of the cells can differently affect FasL and GzmB coexpression in different CD8+ T cell subsets.

Results

A model to examine naïve CD8+ T cell activation *in vitro*

To first examine the effect of the cytokine environment on FasL and GzmB protein expression, I developed a model that would allow me to stimulate naïve CTL *in vitro*, based on similar studies by other groups [140, 178, 361]. I chose plate-bound anti-CD3ε and anti-CD28, as these were used by other researchers who have examined the impact of inflammatory cytokines on CD8+ T cell differentiation [140, 176, 178]. Furthermore, this allowed me to keep activation signals 1 (TCR) and 2 (costimulation, via CD28 signaling) consistent across experiments and easily removed from the cells after a defined period. Much of the IL-2 dependent programming occurs after 48 hours following signals 1 and 2 (TCR and costimulation)[77, 362], so I opted to add IL-2 on

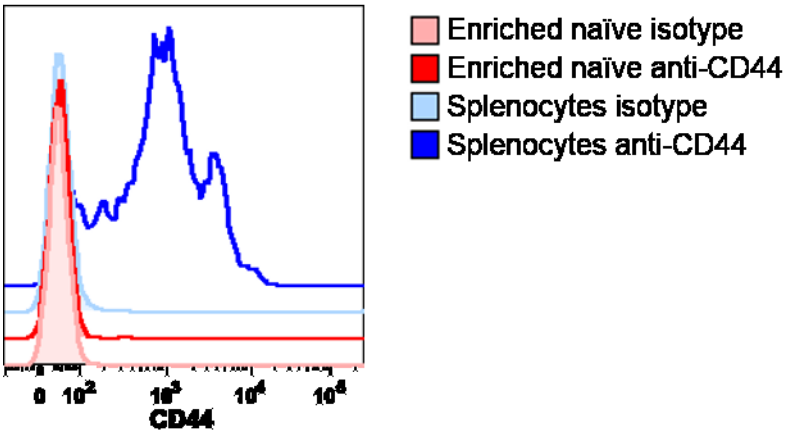
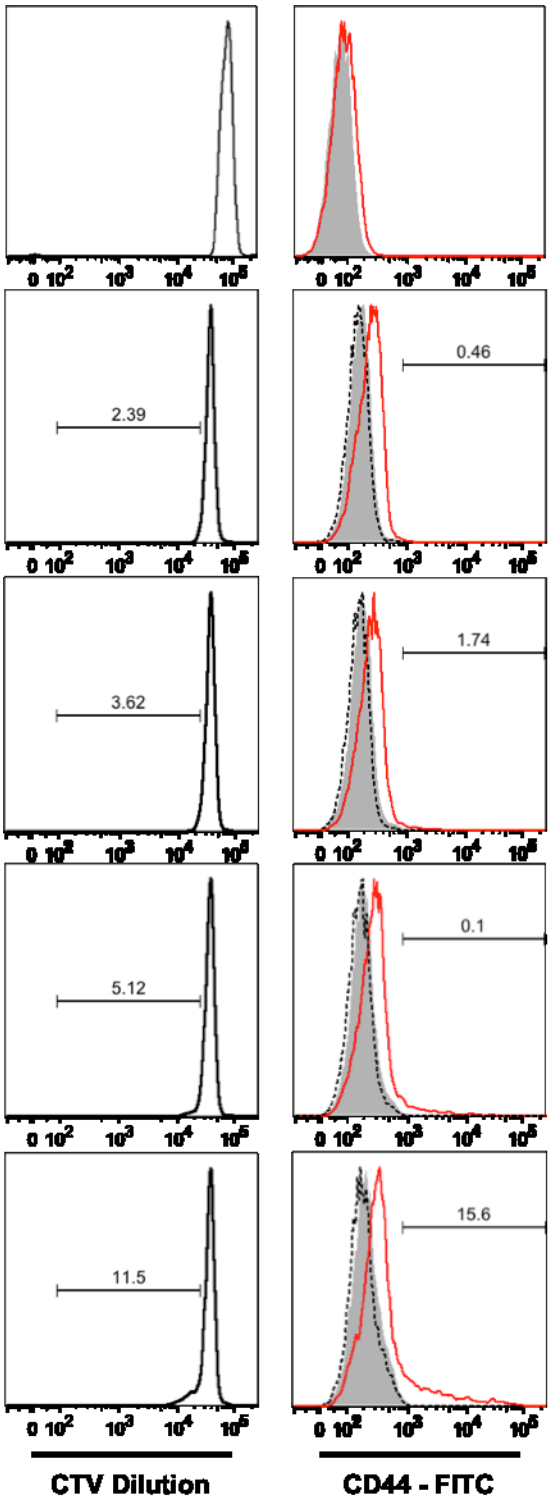


Figure 3-1. Expression of CD44 on enriched naïve and splenic CD8$^+$ T cells.
Cells from pooled spleen and lymph nodes were enriched, or not, by negative selection with a CD8+ T cell negative enrichment kit supplemented with Streptavidin anti-CD44. Cells were stained for CD8α and either CD44 or its matched isotype control. Displayed data is of cells gated on the CD8+ population.

day 2 onwards. Furthermore, the highest proportion of CD8+ cell differentiation occurs after 48 hours *in vitro* [363] and *in vivo* [129].

The CD8+ T cells used from this study were from cells isolated from pooled spleen and LN of healthy, conventionally housed female C57/BL6 mice. I enriched them for naïve CD8+ T cells by using a StemCell Technologies CD8+ T cell negative enrichment kit, supplemented by biotinylated anti-CD44, to also remove any activated, CD44+ cells. All desired cells are not bound by enrichment antibodies prior to experimental stimulation or staining. I had on average 98% purity for CD8+ enrichment. The cells resulting from the negative enrichment were confirmed to be naïve as they were $CD44^{lo}$ (Figure 3-1).

As I wanted to focus primarily on the effects of cytokines after activation, I initially chose a combination of anti-CD3ε and anti-CD28 that would elicit a large proportion of activated cells after 48h. I initially looked for a concentration of anti-CD3ε and anti-CD28 antibodies that would result in a fully activated population by day 2, as indicated by as many cells as possible having undergone proliferation and CD44 surface expression. I titrated the concentration of anti anti-CD3ε and anti-CD28 used to coat 24-well plates for activation of enriched naïve CD8+ T cells. Cell Trace Violet (CTV) dilution was used to indicate proliferation. Tissue culture treated plates coated in the highest tested concentration of antibodies, at 10 ug/ml of each, result in the greatest proportion of CD44+, proliferating cells at 48h (Figure 3-2), so this concentration was used for future studies. Activation of naïve cells with this condition results in a positive shift in intracellular FasL for the population of cells at 48h compared to 0h (Fig 3-3).



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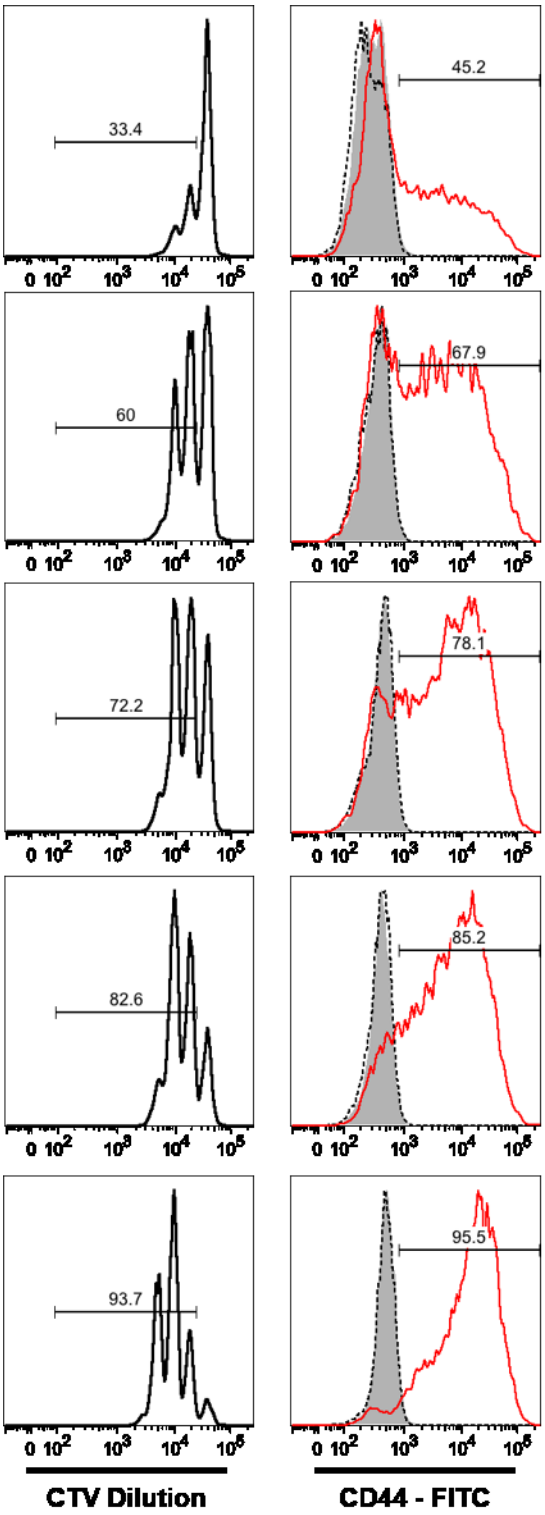

33.4
45.2
48 hours
1.25 µg/mL each
anti-CD3ε, anti-CD28
60
67.9
48 hours
2.5 µg/mL each
anti-CD3ε, anti-CD28
72.2
78.1
48 hours
3.33 µg/mL each
anti-CD3ε, anti-CD28
82.6
85.2
48 hours
5.0 µg/mL each
anti-CD3ε, anti-CD28
93.7
95.5
48 hours
10.0 µg/mL each
anti-CD3ε, anti-CD28
0 10^2 10^3 10^4 10^5
CTV Dilution
CD44 - FITC

Figure 3-2. CD44 expression and proliferation corresponds with anti-CD3 plus anti-CD28. Naïve enriched $CD8^+$ cells were labeled with Cell Trace Violet (CTV) and plated at $0.3x10^6$ cells per well of a 24-well plate coated with the indicated concentrations of antibodies against CD3ε and CD28. For example, 0.15 μg/mL indicates that a solution of 0.15μg/mL of anti-CD3ε plus 0.15 μg of anti-CD28 in PBS was used to coat wells of plate. After 48 hours, cells were harvested, examined for CTV dilution, and stained for CD44 (red line). Dotted line is unstained; grey histogram is isotype control.

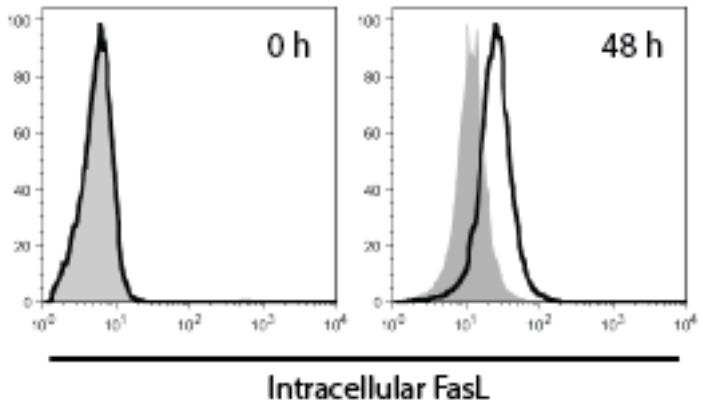


Figure 3-3. 48h of plate-bound Signal 1 and Signal 2 activation in the absence of exogenous cytokines is sufficient to stimulate FasL expression by $CD8^+$ T cells.
Naïve CD8+ T cells were plated at $3x10^6$ per well of 24-well plates previously coated with 10ug/mL anti-CD3and anti-CD28. They were incubated for 48 hours and then stained for intracellular FasL via fixation and permeabilization. Any small amount of FasL that was on the cell surface on these cells would also be captured during this staining. Grey histogram is isotype control.

Therefore, I have a model that results in optimal activation of naïve CD8+ T cells, and also results in the production of intracellular FasL.

In the presence of high anti-CD3 and high anti-CD28, increased IL-2 does not increase intracellular FasL protein

Knowing that FasL protein is present following *in vitro* activation by the conditions I selected, I wanted to modulate the cytokine environment to see if FasL levels could be manipulated. IL-2 is one of the cytokines reaching T cells from both autocrine and paracrine sources [364]. IL-2 increases following T cell activation [75], and it supports T cell proliferation [71] and GzmB expression [177]. One group has shown earlier that IL-2 added to splenic T cells increased FasL expression compared to the mitogen PMA alone [175]. Furthermore, IL-2 added directly to previously activated Th1 CD4+ and CD8+ T cell clones can induce FasL-mediated cytotoxicity in the absence of TCR stimulation [78]. I added IL-2 at varying concentration to the cells from 48h onwards, when the cells were removed from the anti-CD3ε and anti-CD28-coated plate and transferred to fresh media. I chose concentrations ranging from 5U/ml to 100U/ml since 10-100 U/ml is the concentration range most commonly used by others to examine various effects of IL-2 on T cells [140]. I also included 5U/ml IL-2, which is generally insufficient to support growth but may reveal a dose-dependent effect. Furthermore, I found that cultures not supplemented with IL-2 did not survive long enough to thoroughly compare with IL-2 treated cells.

At all concentrations of IL-2, most CTL remain GzmBhi after 48h of cytokine exposure, but higher concentrations of IL-2 result in elevated GzmB protein expression (Fig 3-4A). Furthermore, increasing IL-2 concentration increases relative GzmB expression (Fig 3-4B). This is in alignment with previous research [76, 140, 177]. This

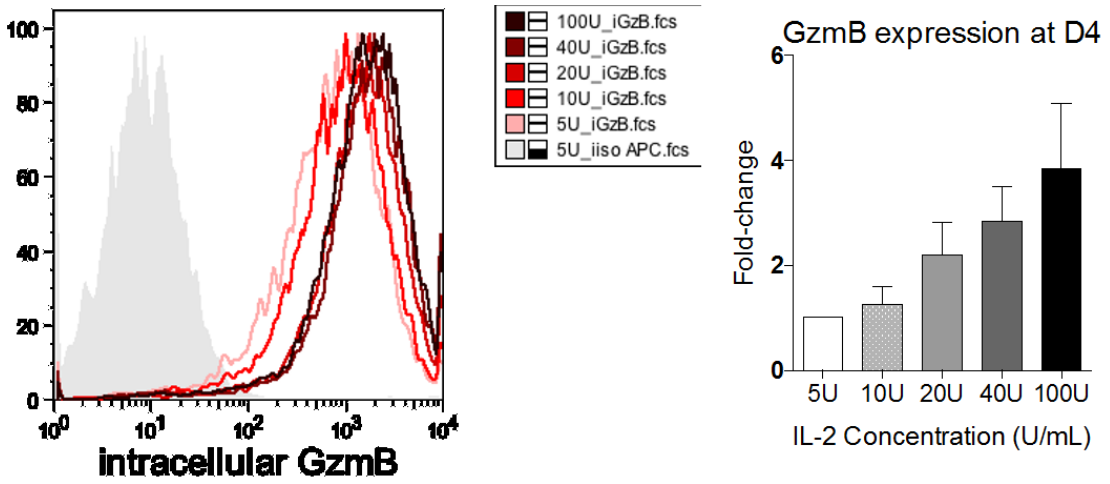


Figure 3-4. Cells cultured in a range of IL-2 concentrations are all GzmB positive, but higher IL-2 concentrations result in higher intracellular GzmB protein stores and increased GzmB gene transcription.
Naïve CD8+ T cells were plated at $3x10^6$ per well of 24-well plates previously coated with 10ug/mL anti-CD3ε and anti-CD28. They were incubated for 48 hours and then transferred to cultured with varying concentrations of IL-2. Cells were harvested after 48h of IL-2 exposure. A) Cells were fixed, permeabilized, and stained from GzmB. Representative histogram (of 3 experiments) shown. B) Cells were assessed for *GzmB* gene expression by quantitative PCR, using the internal control gene *HPRT*. Fold-change is the relative expression compared to cells cultured in 5U/mL IL-2. Bar graph is an average of three experiments, with error bars indicating the SEM.

also confirms that the concentrations of IL-2 that I am using are sufficient to elicit effects on a known target of IL-2 induced gene regulation.

I next set out to examine the effects of IL-2 on FasL expression. I found that there is not a strong increase in intracellular FasL protein in response to increased concentrations of IL-2 when examined at days 4, 7, and 10 (Fig 3-5A-C). Examining the overall trends in FasL levels over time, there is a divergence of intracellular FasL protein that corresponds with IL-2 dose (Fig 3-5D): Cells treated with higher IL-2 concentrations (40, 100 U/ml) increase intracellular FasL and those differences are maintained over the ten days studied. The expression differences are sufficiently subtle that I conclude that concentration of extrinsic IL-2 has negligible effect on intracellular FasL protein, at least when signals 1 and 2 are optimal. When analyzed by a two-way ANOVA, there is no significant effect of IL-2 concentration, time, or an interaction of time and IL-2 concentration on FasL staining.

I also stained for surface FasL in the absence of restimulation or permeabilization to determine if surface FasL levels change with IL-2 concentration. Very low levels of surface FasL are present at any of the time points I examined (Fig 3-5E-F), with a small increase only occurring in cells treated with 100U/ml IL-2 at day 10 following activation. At no other time point is there a discernible IL-2 mediated effect on cell-surface expression of FasL protein.

Overall, I found that following optimal CD3 and CD28 stimulation, a range of IL-2 concentrations that have a dose-dependent effect on GzmB expression and protein storage do not have a significant effect on FasL protein inside or on the surface of cells.

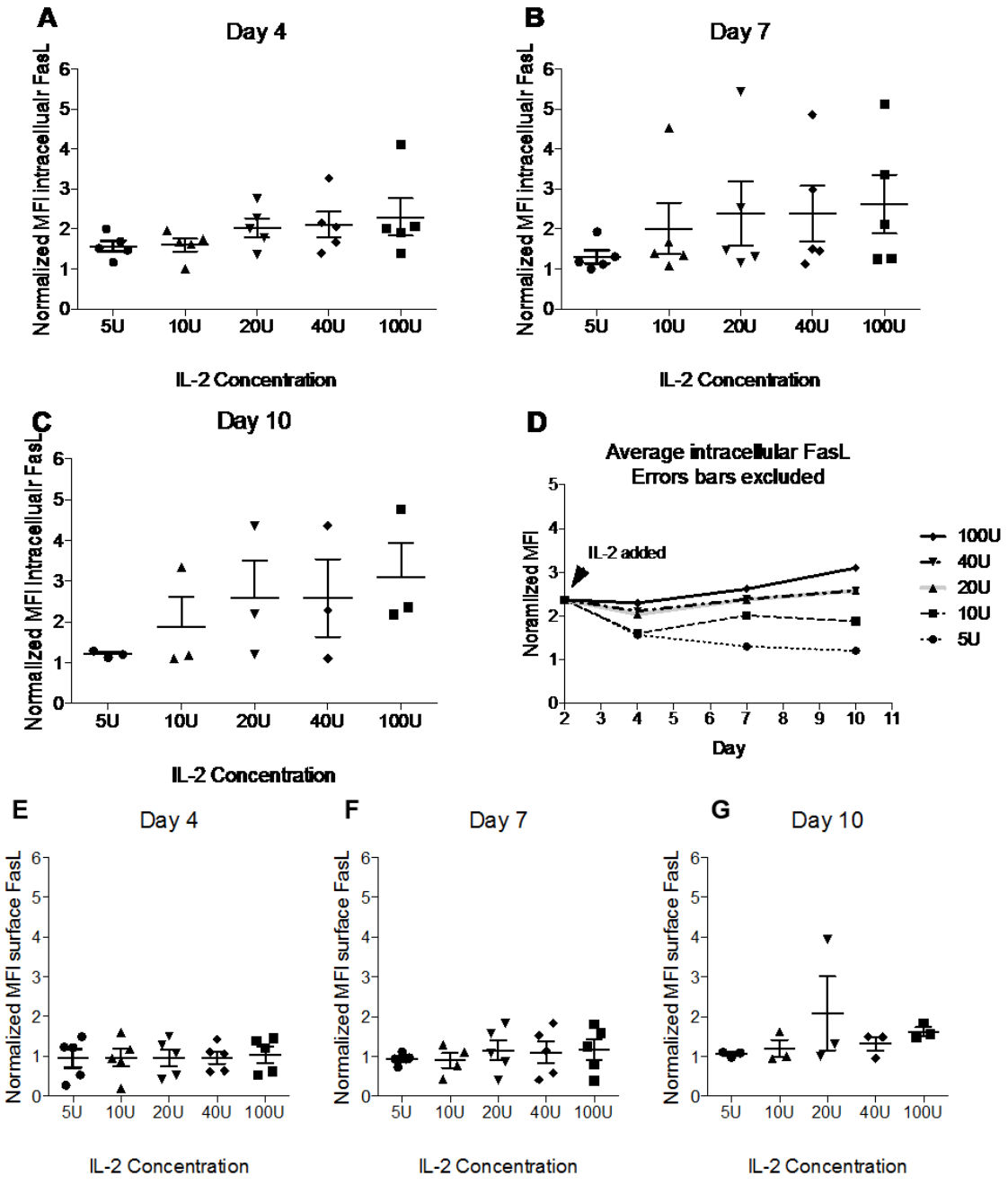


Figure 3-5. Cells cultured in a range of IL-2 concentrations trend towards increased intracellular stores of FasL, while cell-surface FasL remains minimal.
Naïve CD8+ T cells were plated at $3x10^6$ per well of 24-well plates previously coated with 10ug/mL anti-CD3ε and anti-CD28. They were incubated for 48 hours and then transferred to cultures with varying concentrations of IL-2. Data from at least three experiments per time point are shown. Error bars are SEM. A, B, C,. Cells were harvested after 4d, 7d, or 10d of IL-2 exposure and fixed, permeabilized, and stained for intracellular FasL. D. Averaged values for each time point and sample group. Error bars excluded for clarity. Two-way ANOVA = n.s. E, F, G. . Cells were harvested after 4d, 7d, or 10d of IL-2 exposure and stained for cell-surface FasL in the absence of permeabilization or restimulation.

IL-2 does not increase FasL mRNA in dose-dependent manner in activated cells

Although I saw very small increases in FasL inside and on the surface of cells in response to IL-2 concentration, I wanted to know whether increasing IL-2 concentration at least gives CD8+ T cells the potential to induce increased message levels of FasL. Previous research has shown that the addition of IL-2 to CD4+ T cell culture can increase FasL mRNA[365] in the absence of extrinsic TCR stimulation. I examined FasL expression by qPCR on Day 4 as this was when cytokine-dependent effects on GzmB had been detected by other researchers [140, 176]. All FasL levels were standardized against the internal control gene HPRT, and then scaled relative to the expression level in cells treated with 5U/ml IL-2. All concentrations of IL-2 from 10U/ml to 100 U/ml increase the relative transcription of FasL (Fig 3-6), but not significantly. When examining the raw data for my qPCR analyses, the CT values for HPRT at 100U/ml are slightly decreased, so the highest IL-2 concentration has a slight effect on control gene transcription, which could explain the decrease in fold-change at this concentration. This could be due to a global increase in gene expression. My results still suggest that unlike GzmB, FasL transcription is not regulated in a dose-dependent manner by IL-2 at the highest concentration of 100U/ml in CD3/CD28 activated CD8+ T cells.

Surface FasL in response to restimulation is affected by IL-2 concentration

FasL on the cell surface in response to target cell or mitogenic stimuli can come from two pools: intracellular pre-synthesized, as well as *de novo* synthesized [353]. Not all intracellular or stored FasL in the cell is translocated to the surface upon target cell encounter [190]. I wanted to determine if the conditions in which I saw a small but non-significant increase in intracellular FasL from added IL-2 result in more surface FasL after restimulation.

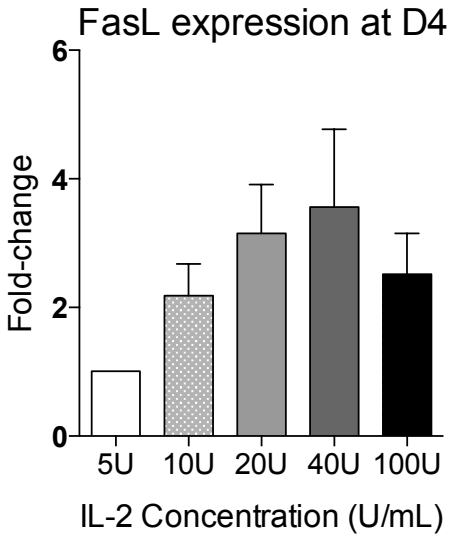


Figure 3-6. The concentration-dependent effects of IL-2 on relative FasL gene expression peaks at 40U/mL on day 4 post-activation.
Naïve CD8+ T cells were plated at $3x10^6$ per well of 24-well plates previously coated with 10ug/mL anti-CD3ε and anti-CD28. They were incubated for 48 hours and then transferred to cultures with varying concentrations of IL-2. After another 48 hours of culture, cells were harvested and assessed for *FasL* gene expression by quantitative PCR, using the internal control gene *Hprt*. Fold-change is the relative expression compared to cells cultured in 5U/mL IL-2. Bar graph is an average of three experiments, with error bars indicating the SEM. Summary data from 3 experiments.

I tested cells at D7, as this is when I started to detect a slight difference in FasL (Fig 3-5D). For restimulation I used the pharmacological activators PMA and Ionomicin (PMA/I) at 37^{o}C to mimic a TCR-mediated target cell signal. We know that the FasL from intracellular stores appears on the surface within 15 minutes of PMA/I restimulation [190]. I found that the fold-change in translocated surface FasL by this time point is significantly higher on cells cultured in higher amounts of IL-2 (20U, 40U, 100U) compared to cells cultured in the lowest 5U/ml IL-2 (Fig 3-7A). As FasL presented on the cell surface is the ligand target cells would see in a killing situation, it makes sense that the cytokine environment may modulate this response. I also quantified the increase in surface FasL after 2h of PMA/I, at which point surface FasL is known to have been synthesized *de novo* in response to the stimulus [353]. I saw a broader range of surface FasL accumulation, with cells cultured with 20U/ml and 40U/ml IL-2 having a significantly greater increase than those cultured at 5U/ml (Fig 3-7B). Although cells from 100U/ml are all higher for surface FasL than 5U/ml, the spread in data for 100U/ml was too broad to allow for statistical significance. My data show that surface FasL on CD8+ T cells 2h post-restimulation is increased with 20U/ml and 40U/ml IL-2, which is consistent with previous data that IL-2 increases FasL mRNA transcripts when added to CD4+ T cells cultures [365] Furthermore, other researchers have found that IL-2 can enhance Fas-mediated cytolytic activity of mouse $CD4^{+}$ cells, though, but which FasL pathway was affected was not examined [366]. Overall, it is interesting that increasing IL-2 in culture shows very little difference in intracellular or surface FasL in “resting” CD8+ T cells, but still result in differences at the effector level, in increased cell-surface FasL on these same cells upon restimulation.

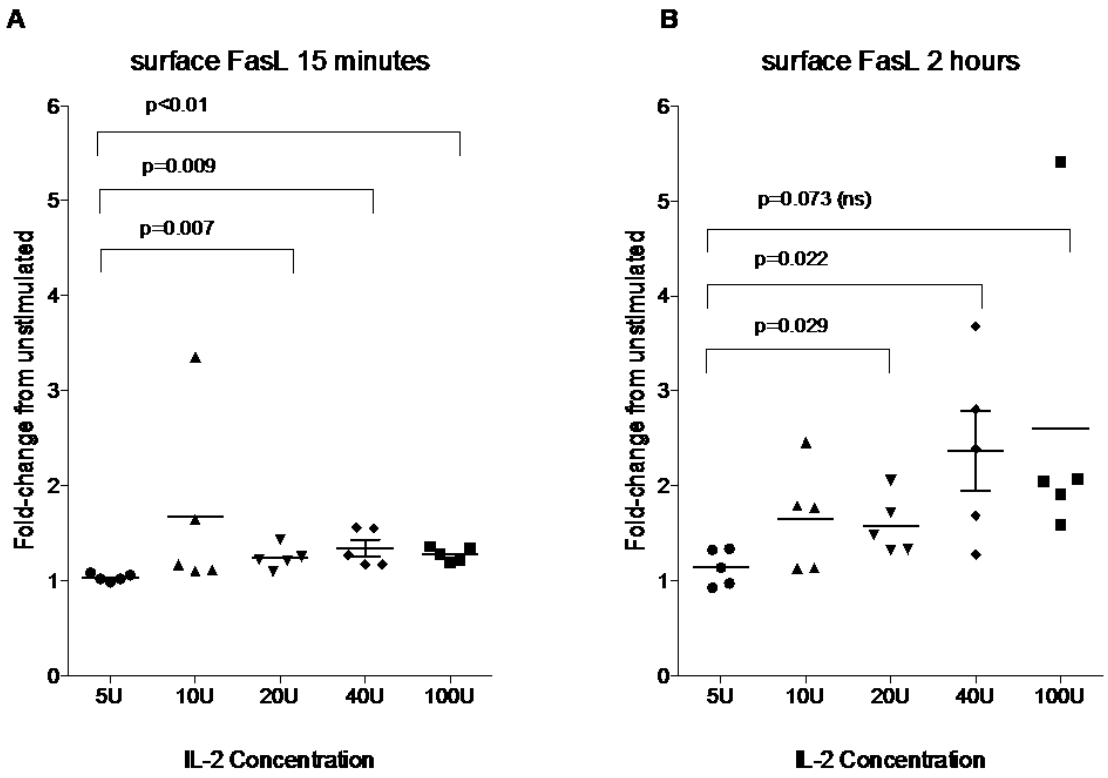


Figure 3-7. Cells cultured with increased IL-2 concentrations have increased surface expression of FasL in response to PMA/Ionomicin stimulation.
Naïve CD8+ T cells were plated at $3x10^6$ per well of 24-well plates previously coated with 10ug/mL anti-CD3ε and anti-CD28. They were incubated for 48 hours and then transferred to cultures with varying concentrations of IL-2. Cells were harvested after 7d and stimulated with PMA/Ionomicin for 15 minutes or 2 hours, then immediately stained for surface FasL. Data from five experiments are shown. Error bars are SEM. P values obtained by 2-way, paired Student's t-test.

Activated CD8+ T cells are more likely to have intracellular GzmB than FasL irrespective of IL-2 concentration

The previous sections showed that although GzmB expression is regulated by IL-2, the same is not true of intracellular FasL. Next, I wanted to see whether the IL-2 concentration affected the fraction of cells expressing intracellular FasL or GzmB and if the fraction of cells expressing these proteins is modulated by IL-2.

I analyzed the same cells assessed before for levels of FasL and GzmB, but scored them as being Gzm+ FasL+, GzmB+ FasL-, GzmB- FasL+, or GzmB- FasL-, based on gating strategy shown in the representative FACS plot in Fig 3-8. At day 4 post-activation, almost all (over 90%) are GzmB+, aligning with what was shown in figure 3-4. The majority are GzmB+ FasL- (Fig 3-8A), and the relative proportion of GzmB+ FasL+ to GzmB+ FasL- does not change in response to IL-2 concentration. At D7, there is more variability between IL-2 treated cultures with respect to proportions of GzmB+ FasL+ and GzmB+ FasL-, and an elevated proportion of cells express neither FasL nor GzmB (Fig 3-8B). However, there is no significant increase or decrease that can be attributed to IL-2 concentration. Under the conditions I have used, the IL-2 concentration in culture does not affect the proportion of cells expressing GzmB and/or FasL. It is also important to note that this data clearly shows that not all *in vitro* activated CD8+ T cells coexpress FasL and GzmB protein in the same cell.

In cells treated with sub-optimal levels of anti-CD3 and anti-CD28, increased IL-2 helps sustain intracellular FasL over time

The previous three sections showed that when TCR stimulus and costimulation are optimal, IL-2 alters neither the overall levels of intracellular FasL nor its coexpression with GzmB. However, restimulated CD8+ T can express it on the cell surface in an IL-2

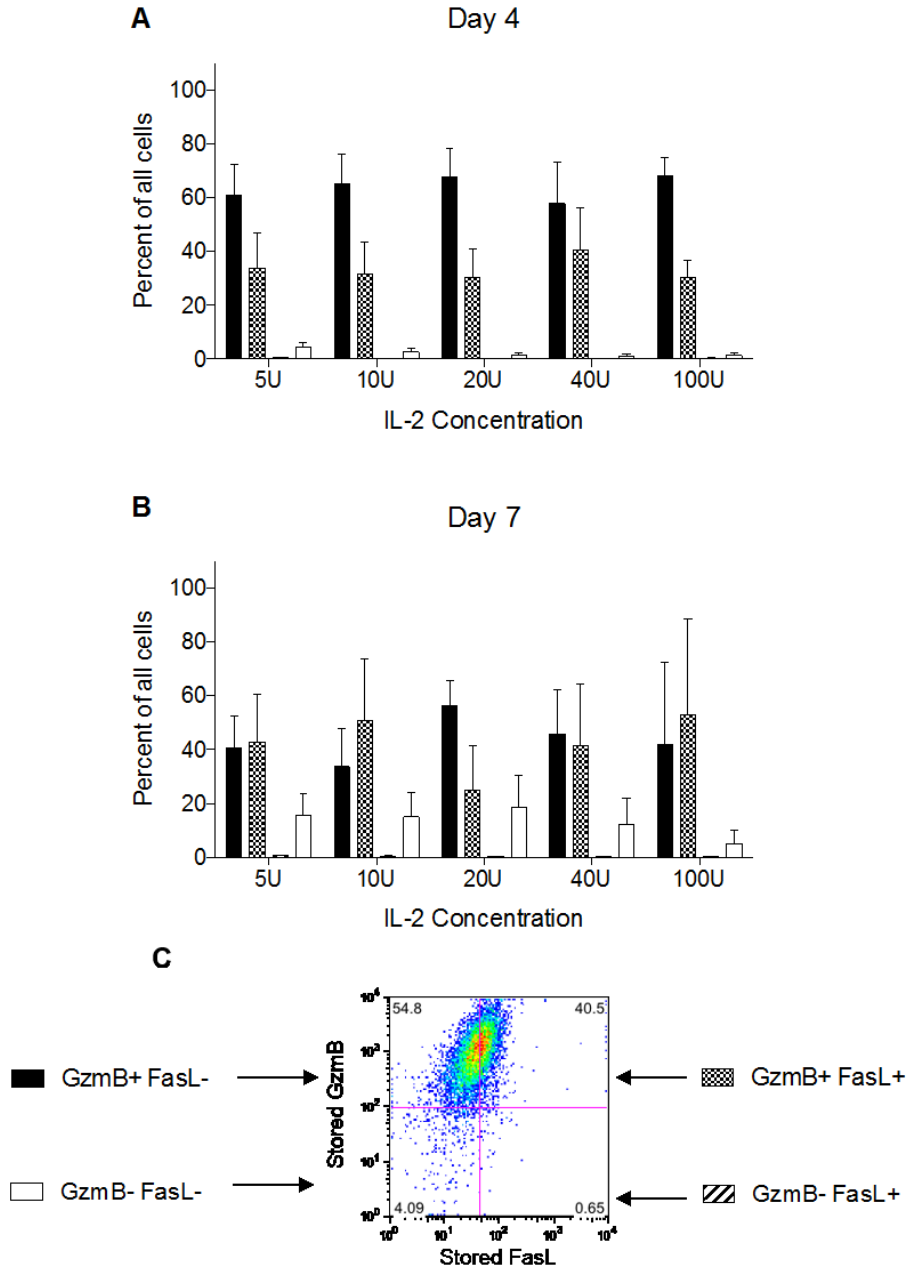


Figure 3-8. In cells activated with optimal anti-CD3 and high anti-CD28, IL-2 does not change the distribution of cells expressing intracellular FasL and GzmB.
Naïve CD8+ T cells were plated at $3x10^6$ per well of 24-well plates previously coated with 10ug/mL anti-CD3ε and anti-CD28. They were incubated for 48 hours and then transferred to cultures with varying concentrations of IL-2. Cells were harvested on (A) D4 or (B) D7 post-stimulation, then fixed and permeabilized and stained for intracellular FasL and GzmB. Data from thee experiments are shown. Error bars are SEM. No significant P values from 2-way paired student's t-test. C, representative data showing gating for intracellular FasL and GzmB. FasL+ and GzmB+ boundaries determined using cells stained for FasL but not GzmB, cells stained for GzmB but not FasL, and neither FasL nor GzmB.

influenced manner. It may be that high CD3 and CD28 signals are just overriding the programming sensitive to cytokines, so weaker CD3 and CD28 signals are needed to better dissect the cytokine response. Furthermore, my colleague Wayne Juang simultaneously found in cells activated by CD3epsilon and plate-bound B7 ligand *in vitro* in a similar model, there appears to be an inverse relationship between the strength of B7::CD28 signal and FasL protein expression [367]. To determine if IL-2 regulates FasL expression following suboptimal stimulation, I used plates coated with 3ug/ml of each antibody to activate the cells, as a very similar tested concentration resulted in the majority of cells becoming activated and proliferating, but had only gone through 2 or fewer cells division (Fig 3-2, 3.33ug/ml). I kept the protocol for stimulations and cell culture the same, and compared low (5U/ml) and high (40U/ml) concentrations of IL-2.

I found that the proportion of cells high for intracellular FasL generally lowers over the course of 12 days in these experiments, while the proportion of GzmB+ cells remains high (Fig 3-9A). While the proportion of FasL high cells is higher in the 40U/ml treated cells at the latest time point (Fig 3-9A), this is not a statistically significant difference. Similarly, the higher IL-2 results in more cells with high GzmB throughout the experiment, but the difference is not statistically significant at any day except D7. This is also meaningful because prior work with *in vitro* activation followed by flow cytometry of PCR did not follow to such a late time point [140]. It is possible to see longer-term effects on FasL and GzmB expression *in vitro*.

I continued to also examine the normalized MFI of FasL in these cells, as cells treated differently may be both "positive" for FasL, but may store different amounts of this effector molecule. This is also meaningful for GzB, where cells are mostly positive

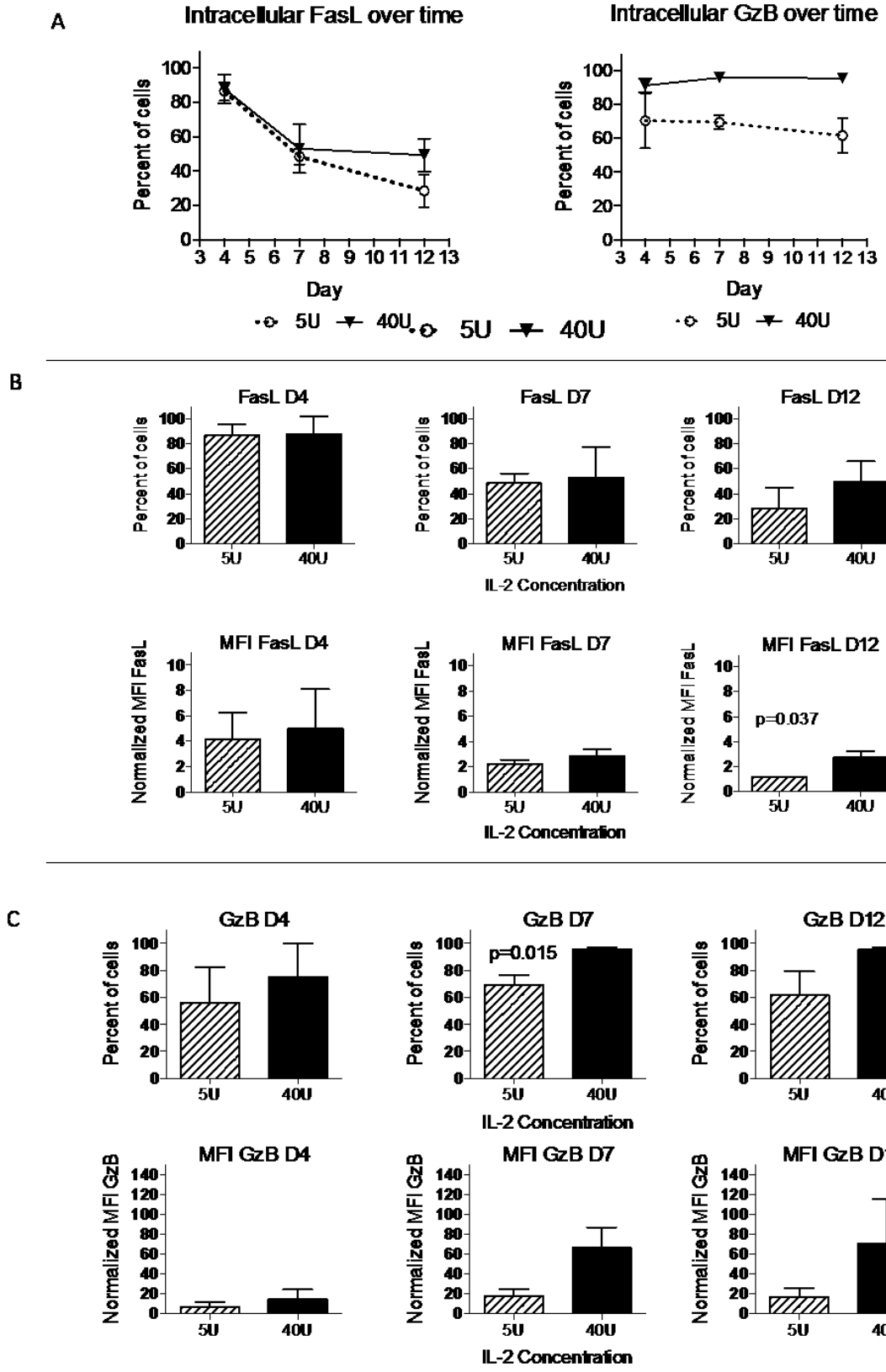

A
Intracellular FasL over time
Intracellular GzB over time
Percent of cells
Day
5U
40U
B
FasL D4
FasL D7
FasL D12
IL-2 Concentration
MFI FasL D4
MFI FasL D7
MFI FasL D12
Normalized MFI FasL
p=0.037
C
GzB D4
GzB D7
GzB D12
p=0.015
MFI GzB D4
MFI GzB D7
MFI GzB D12
Normalized MFI GzB

Figure 3-9. In the presence of reduced anti-CD3 and anti-CD28, increasing IL-2 concentration slightly sustains GzmB and FasL expression.
Naïve CD8+ T cells were plated at $3x10^6$ per well of 24-well plates previously coated with 3ug/mL anti-CD3ε and anti-CD28. They were incubated for 48 hours and then transferred to cultures with 5U/mL or 40U/mL IL-2. Cells were harvested on D4, D7, or D12 post-stimulation, then fixed and permeabilized and stained for intracellular FasL and GzmB. A, Summary of percent of cells positive for intracellular FasL or GzmB over time. B, Percent Positive (top row) and normalized MFI (bottom row) for intracellular FasL. C, Percent Positive (top row) and normalized MFI (bottom row) for intracellular GzmB.
Data from three experiments are shown. Error bars are SEM. P values are determined by student's 2-way paired t test. Only statistically significant values shown. For A and B, no significant difference between 5U/mL and 40U/mL.

throughout. As time progresses, cells express less intracellular FasL (Fig 3-9B), but this decrease over time is less dramatic in cells treated with 40U/ml IL-2. By D12, the difference in FasL level of protein expression between low and high IL-2 is indeed significant. I would have liked to sustain the experiment for a longer period of time, but the proliferation of cells treated with 5U/ml IL-2 and originally activated with 3ug/ml coated plates is so low at later time points that it was challenging to sustain a culture of cells for 2 weeks and beyond and retain sufficient numbers of for analysis. However, from the time span seen here, it seems that IL-2 helps cells sustain intracellular FasL levels over time.

It appears that cells activated in these conditions are slow to ramp up their GzB protein expression, though most cells are still positive. There is no significant difference in intracellular GzmB between cells treated with 5U/ml and 40U/ml IL-2, except at D7. However, cells treated with 5U/ml IL-2 had staining intensities greater than 20x the staining control, and cells treated with 40U/ml IL-2 had to 120x the intensity of the isotype control on D12 (Fig 3-9C). There is still no significant difference between these groups, though.

Although I had expected lower signals 1 and 2 to have a diminished impact on subsequent FasL protein expression, leaving more "room" for the influence of IL-2 levels, it appears that at early time points, cells follow a programmed dynamic for FasL expression and are only affected by extrinsic IL-2 levels at later times.

FasL- GzmB- cells are present in cultures that were suboptimally activated and then exposed to low IL-2

As previously discussed, in CD8+ T cells activated with maximal anti-CD3 and anti-CD28 stimulation conditions, the dominant "phenotype" is GzmB+ FasL- at D4,

with a population of GzmB+ FasL+ cells emerging by Day 8, with some at D8 GzmB- FasL- (Fig 3-8). As this progression of populations is independent of IL-2 concentration, I wanted to examine whether this changes with reduced anti-CD3/CD28 stimulation, but variable IL-2. In cells activated with 3ug/ml anti-CD3 and 3ug/ml anti-CD28, even though most cells are still GzmB+, the difference from earlier experiments is that GzmB is co-expressed with FasL in suboptimally stimulated cells (Fig 3-10). FasL+ GzmB+ cells are the dominant phenotype at D4, and over time there is a migration to dominance of GzmB+ FasL- by D12, while still maintaining a GzmB+ FasL+ population. For all days, most populations are similar between 5U/ml and 40U/ml IL-2, with the exception of a GzmB- FasL- population present in the 5U/ml IL-2 treated culture, which slightly increases over time. This GzmB- FasL- population is always significantly higher in low-IL-2 treated cells. Other frequent populations (GzmB+ FasL-, GzmB+ FasL+) do not differ between 5U and 40U treated cells, but do change over time. It seems like the GzmB+ FasL- population emerges from the GzmB+ FasL+ population over time, as if the expression of FasL along with GzmB is transient in cells stimulated with low anti-CD3/CD28, but intracellular FasL can be expressed at time points by cells receiving insufficient stimulation to express intracellular GzmB.

Inflammatory cytokines present during and after activation have a late effect on GzmB protein expression and minimal effect on FasL protein expression

Previously, it has been found that IL-12 and IFN-α enhance short-term increases in GzmB and FasL transcripts [176, 368], and IFN-α increases soluble FasL[368] in newly-activated CD8+ T cells, but no studies have examined in depth the protein expression within these cells to see if FasL and GzmB are coexpressed, or whether FasL is intracellular or on the cell surface.

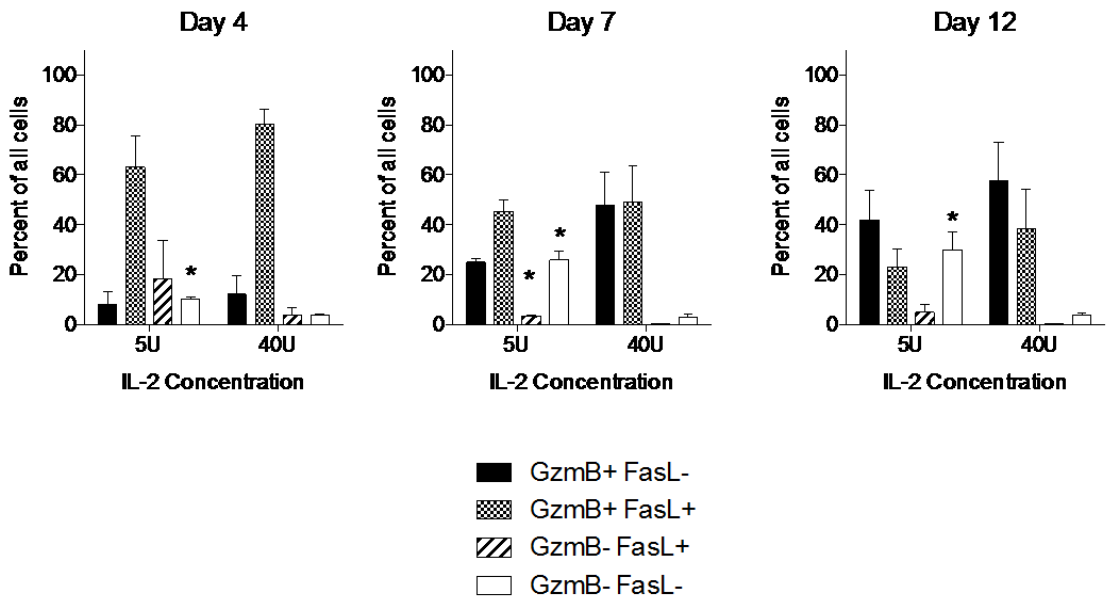


Figure 3-10. In cells activated with suboptimal anti-CD3/CD28 and 5U/ml IL-2, but not 40U/mL IL-2, a small GzmB$^-$ FasL$^+$ population is present.
Naïve CD8+ T cells were plated at 3x10^6 per well of 24-well plates previously coated with 3ug/mL anti-CD3ε and anti-CD28. They were incubated for 48 hours and then transferred to cultures with varying concentrations of IL-2. Cells were harvested on D4, D7, or D12 post-stimulation, then fixed and permeabilized and stained for intracellular FasL and GzmB. Data from thee experiments are shown. Error bars are SEM. * indicates significant ($P<0.05$) difference between 5U/mL and 40U/mL IL-2 as determined by a 2-way student's t-test.

I activated the cells with plates coated with 10ug/ml anti-CD3ε plus 10ug/ml anti-CD28 conditions as described in the first section, with IL-12, IFN-α, or both cytokines together added to cells from 0h onwards. Minimal (5U/ml) IL-2 was added to cells to sustain proliferation from 48h onwards. The inflammatory cytokines were added earlier than IL-2, because *in vivo* they are more likely to be present prior to T cell activation, as other, non-T cells are secreting them. I examined cells at D4 and D7 after activation. I found that cells cultured in the presence of IL-12 started to die in increasing numbers after approximately 10 days of culture and so only earlier time points were considered.

I found that most cells are positive for GzmB at D4, but the addition of IL-12 and/or IFN-α sustains GzmB expression by D7 (Fig 3-11A). The lack of an additive effect in cells treated with both IFN-α and IL-12 for the proportion of cells positive for GzmB is not surprising, mostly because so many cells are already positive. However, when the amount of GzmB per cell was examined, by D7 there is still not an additive effect on GzmB. There is no significant increase in intracellular GzmB protein at D4, but by D7 there is a significant increase in intracellular GzmB in cells treated with inflammatory cytokines compared to cells receiving just IL-2 (Fig 3-11C). However there is no additive effect on GzmB in cells that receive both IL-12 and IFN-α compared to their counterparts with only IL-12 or IFN-α (Fig 3-11B, C).

Adding IL-12 and/or IFN-α increases the proportion of cells that express intracellular FasL at the early time point of D4, but this increase is no longer statistically significant for all added cytokine conditions by D7 (Fig 3-12A). However, at no point in that I examined is the amount of intracellular FasL significantly increased by the addition of IL-12 and/or IFN-α (Fig 3-12B, C).

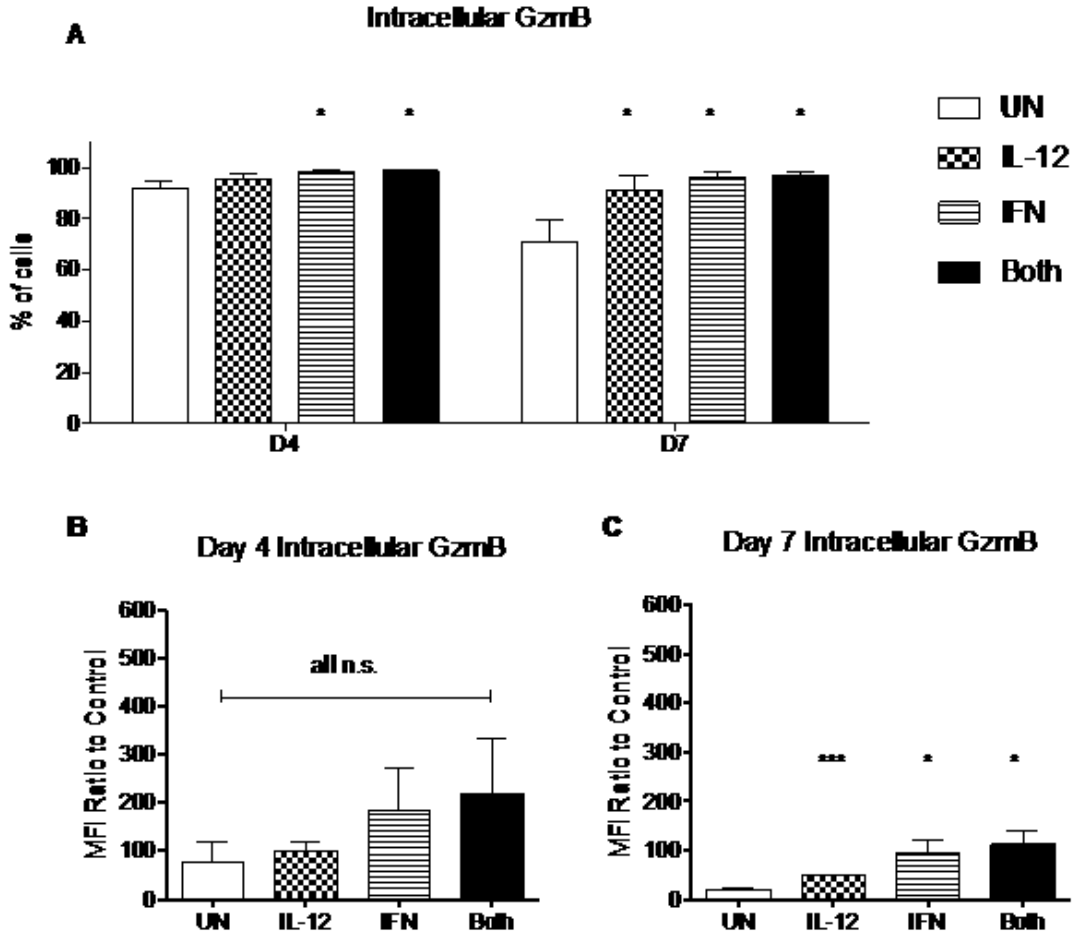


Figure 3-11. Inflammatory cytokines only increase the proportion of cells positive for GzmB or the intracellular expression of GzmB at D7.
Naïve CD8+ T cells were plated at $3x10^6$ per well of 24-well plates previously coated with 10ug/mL anti-CD3ε and anti-CD28. Cells treated with IFN-α, IL-2, or both together, had cytokines added at D0. "UN" cells did not have inflammatory cytokines added. All were incubated for 48 hours and then transferred to cultures 5U/mL of IL-2. Cells were harvested on D4 or D7 post-stimulation, then fixed and permeabilized and stained for intracellular GzmB. Data from at least four experiments are shown. Error bars are SEM. P values from a paired, two-way student's t-test against "UN" data. *=P<0.05, ***=P<0.001.

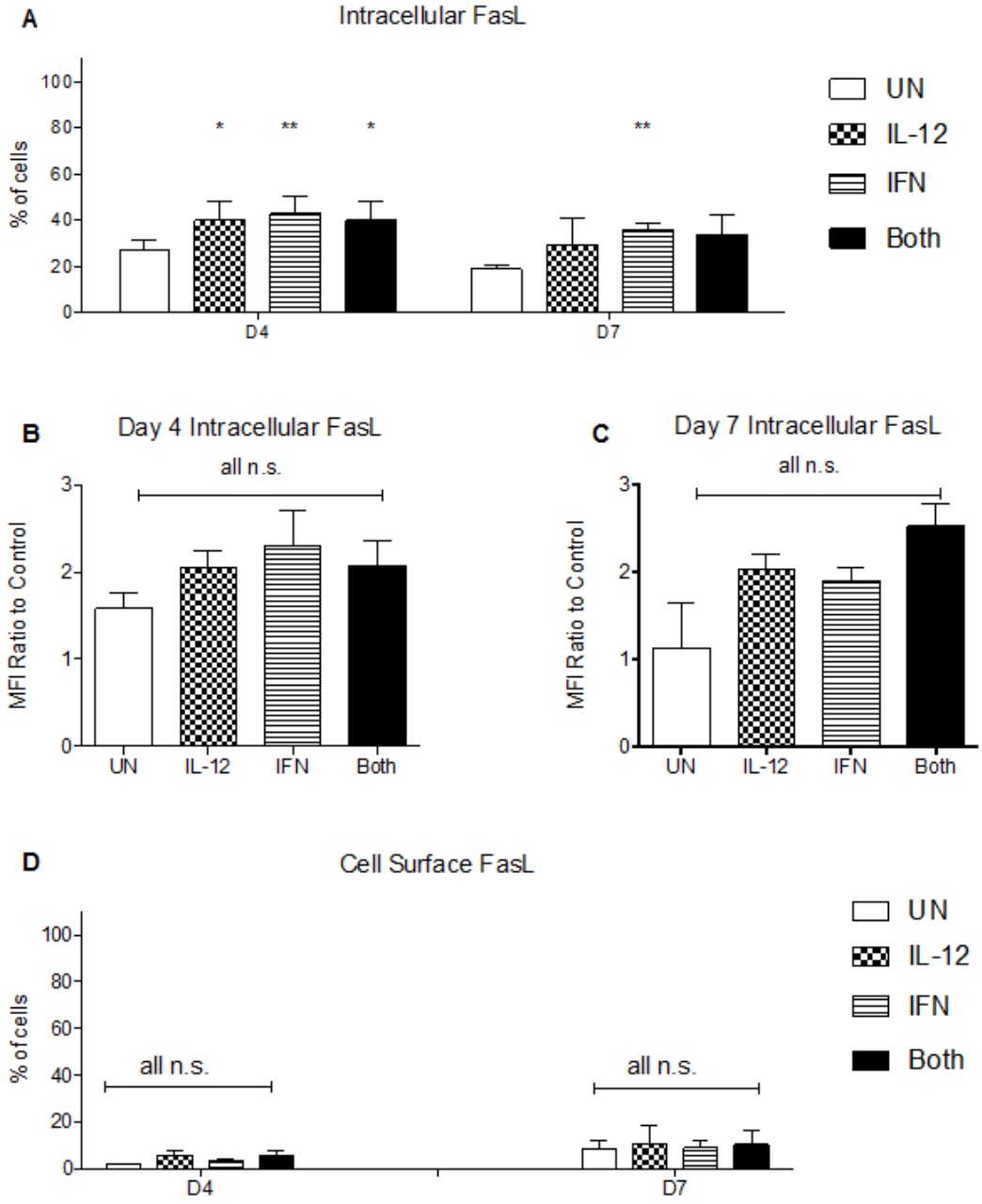


Figure 3-12. Inflammatory cytokines increase the proportion of cells high for intracellular FasL at D4.

Naïve CD8+ T cells were plated at $3x10^6$ per well of 24-well plates previously coated with 10ug/mL anti-CD3ε and anti-CD28. Cells treated with IFN-α, IL-2, or both together, had cytokines added at D0. "UN" cells did not have inflammatory cytokines added. All were incubated for 48 hours and then transferred to cultures 5U/mL of IL-2. Cells were harvested on D4 or D7 post-stimulation, then fixed and permeabilized and stained for intracellular FasL, or not permeabilized and stained for cell surface FasL without restimulation. Data from at least four experiments are shown. Error bars are SEM. P values from a paired, two-way student's t-test against "UN" data. *=P<0.05, **=P<0.01

I have included surface FasL measurement by flow cytometry in this section, as I cited work earlier by Kaser and colleagues where they claimed FasL cell-surface expression after 48h of CD8+ T cells exposure to IFN-α. I found that very few cells in my model are positive for surface FasL in the absence of restimulation regardless of cytokine treatment (Fig 3-12D).

Overall, I have not found evidence for the addition of IL-12 or IFN-α to CD8+ T cells during activation to have a significant effect on the intracellular amount of FasL following activation, but they do have an effect on the proportion of FasL+ cells early following activation. Conversely, these same cytokines have an effect on proportion of GzmB+ cells and intracellular GzmB amount, but at a later time post-activation.

Intracellular FasL and GzmB vary between effector and memory CD8+ T cells

The differences in populations treated with cytokines, even though they appear to have similar proportions of FasL-positive or GzmB positive cells, become more apparent when examining the differentiation of cell cultured with these cytokines. When cells are stimulated in culture, they can obtain effector (CD62Llo, CD127lo) or central memory (CD62Lhi, CD127hi) phenotypes. Could these populations have different expression of FasL and GzmB?

I analyzed differentiated cells obtained from culture experiments as described in the previous section, in the presence of different inflammatory cytokines. I used CD62L and CD127 staining to gate for central memory (CD62Lhi, CD127hi) and effector (CD62Llo, CD127lo) populations. Note that henceforth I am referring to these populations as effector and memory. Overall, there is a shift from cells of a central memory phenotype on D4 in all conditions to cells of a more effector-like phenotype by

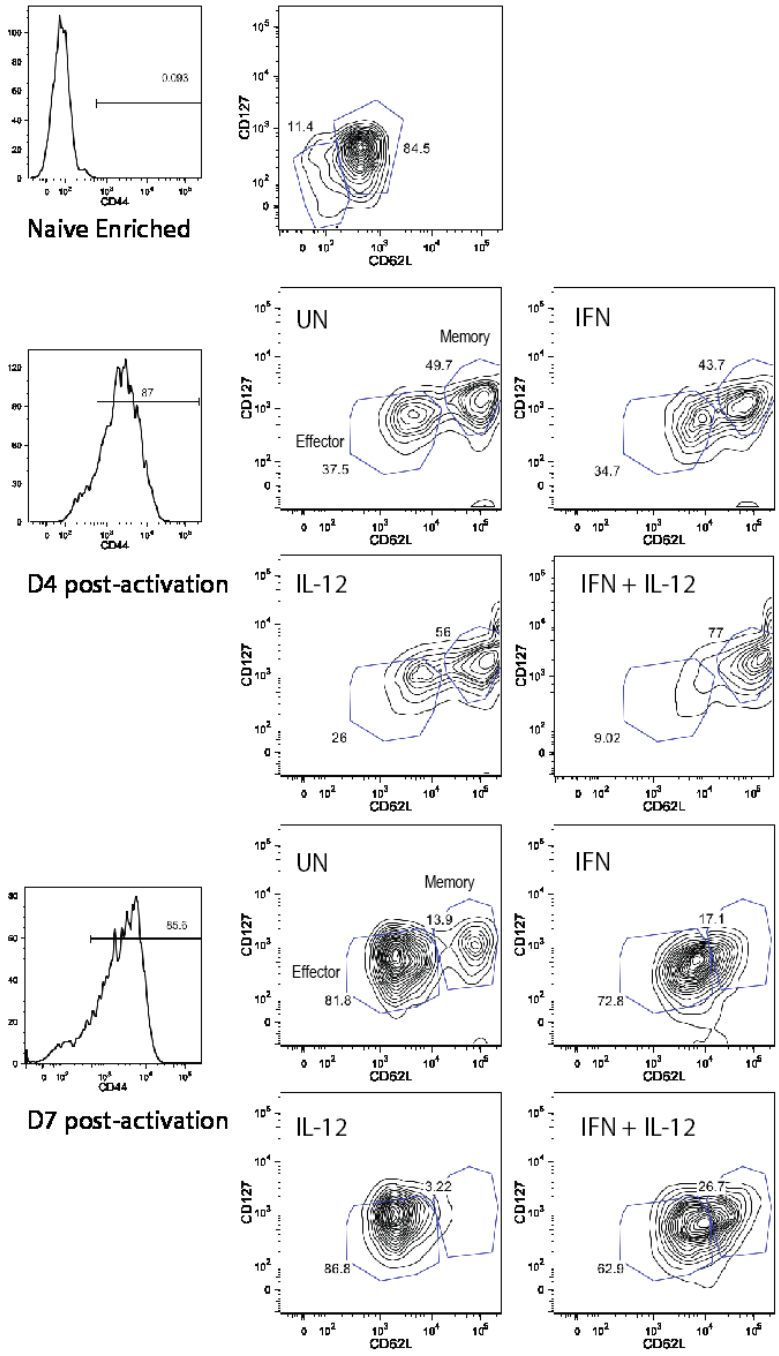


Figure 3-13. Both effector and memory phenotypes emerge in cells activated and cultured with inflammatory cytokines *in vitro*.
Naïve CD8+ T cells at $3x10^6$ per well of 24-well plates previously coated with 10ug/mL anti-CD3ε and anti-CD28 for 48h. Cells were cultured from 0h on with added 2U/mL IL-12, 1000U/mL IFN-α (IFN), or both cytokines. All cultures, including negative control (UN) received 5U/mL IL-2 from 48h onwards. Cells were harvested at D4 or D7 and stained for CD44, CD62L and CD127. In each plot, gated left are effector, right are memory. Representative data from one of three experiments shown.

D7 (Fig 3-13). These populations can be analyzed for intracellular FasL and GzmB expression (Fig 3-14).

The protein expression of FasL and GzmB differs with respect to cytokines and effector versus central memory phenotype (Fig 3-14). Considering that GzmB remains high in almost all of my experiments described in previous sections, it is not surprising that both differentiated effector and memory populations have predominantly GzmB-positive populations (figure 3-14). However, FasL coexpression is notably variable, both depending on cytokine and on differentiation. This would explain some of the issues discussed in the previous section with respect to FasL expression in response to IL-12 and CD8+ T cells addition.

Not all subpopulations of cells from all cytokine environments have unique patterns of these two effector mechanisms, which makes sense recalling that there is little difference between the proportions of gross FasL+ or between the gross GzmB+ populations in Fig 3-11. However, there are some trends that stand out:

All cytokines conditions can give rise to effector and memory phenotype populations. Both memory and effector cells are capable of expressing FasL and GzmB , and have FasL- GzmB+ and FasL+ GzmB+ populations as early as D4. In spite of differentiation being a large part of cellular destiny, not all effector cells are in lock-step with respect to effector mechanism expression, and can vary their effector molecule protein stores. This is seen at D7 in cells treated with IL-12, in which more cells coexpress FasL and GzmB. (Fig 3-15). The addition of IL-12 or IFN-α reduces the transition to FasL- GzmB- by effector phenotype cells.

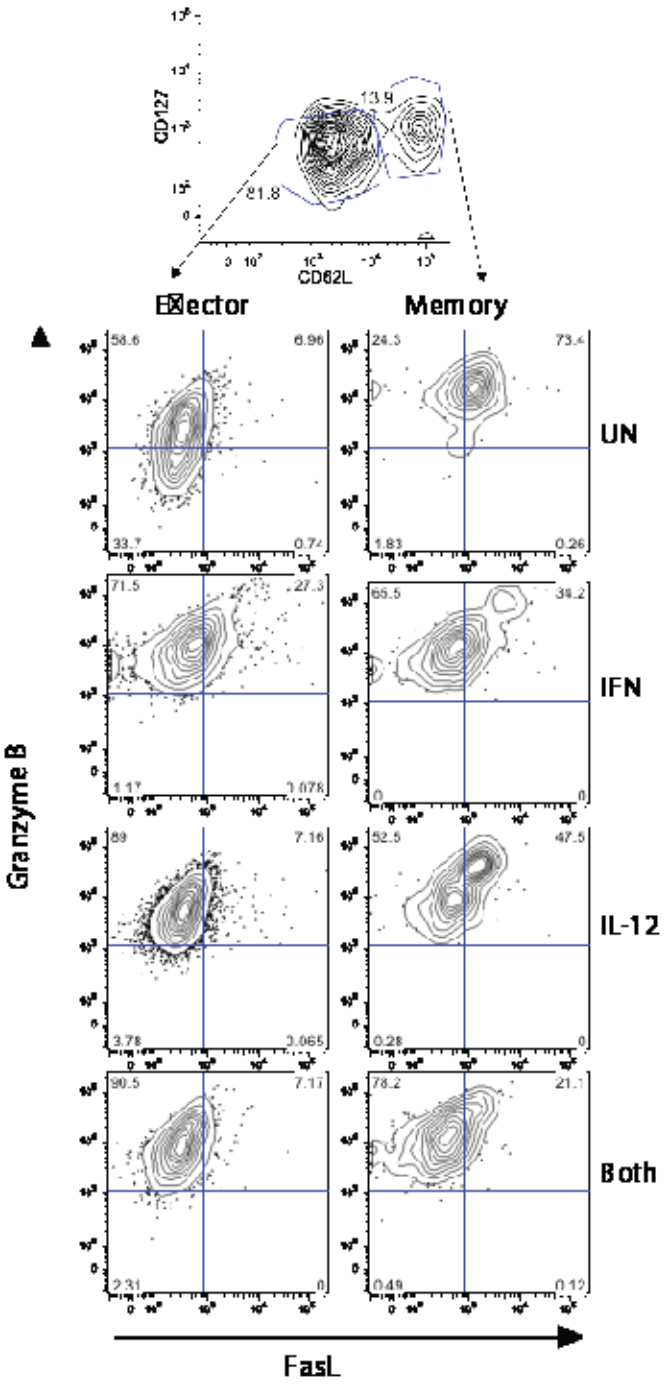


Figure 3-14. Representative memory and effector cells differ in intracellular FasL and GzmB coexpression.
Cells were cultured from 0h onwards with added 2U/mL IL-12, 1000U/mL IFN-α (IFN), or both cytokines (Both). All cultures, including the negative control (UN) received 5U/mL IL-2 from 48h onwards. Cells were harvested at 7 days post activation and stained for CD44, CD62L and CD127, then intracellularly for FasL and GzmB. Representative D7 data shown from one of three experiments. Summary data shown in figure 3-15.

Surprisingly, FasL is less common in most effector populations than in memory phenotype populations by D7. (Fig 3-10, Fig 3-12). In fact, D7 shows a shifting of most effector cells from being split between GzmB single positive or double positive for both, to either GzmB alone or neither effector mechanism (Fig 3-14). It appears that these effector cells are losing intracellular FasL, except perhaps in cells treated with IL-12.

In addition, it seems as though memory cells activated without IFN-α or IL-12 transition to a greater proportion having FasL+ GzmB+ coexpression from D4 to D7, suggesting that there they are "catching up" with the memory cells from cultures receiving IFN-α or IL-12, all of which do not change much between D4 and D7 (Fig 3-15). The only notable exception to this is memory cells from cultures treated with IFN-α, which at D4 had significantly fewer GzmB+ FasL- cells and significantly more GzmB+ FasL+ cells than their counterparts that had not received IFN-α. This IFN-α treated memory population seems to be ahead of the trend for FasL and GzmB coexpression over time. Cytokines present in the activation environment can have a very slight effect on FasL protein expression, but it is more strongly modulated by time and differentiation.

Discussion

In this chapter, I showed that intracellular FasL and GzmB are not always coexpressed together in *in vitro* activated CD8+ T cells. In general, GzmB and FasL are influenced independently by cytokines: while IL-2 affects GzmB protein, IL-2 does not appear to modulate FasL expression but may help sustain FasL expression over time. IL-2 does not affect surface levels of FasL in activated but resting CD8+ T cells, but does seem to enhance its surface expression upon restimulation. Inflammatory cytokines can increase intracellular FasL expression early after activation, but sustain GzmB expression

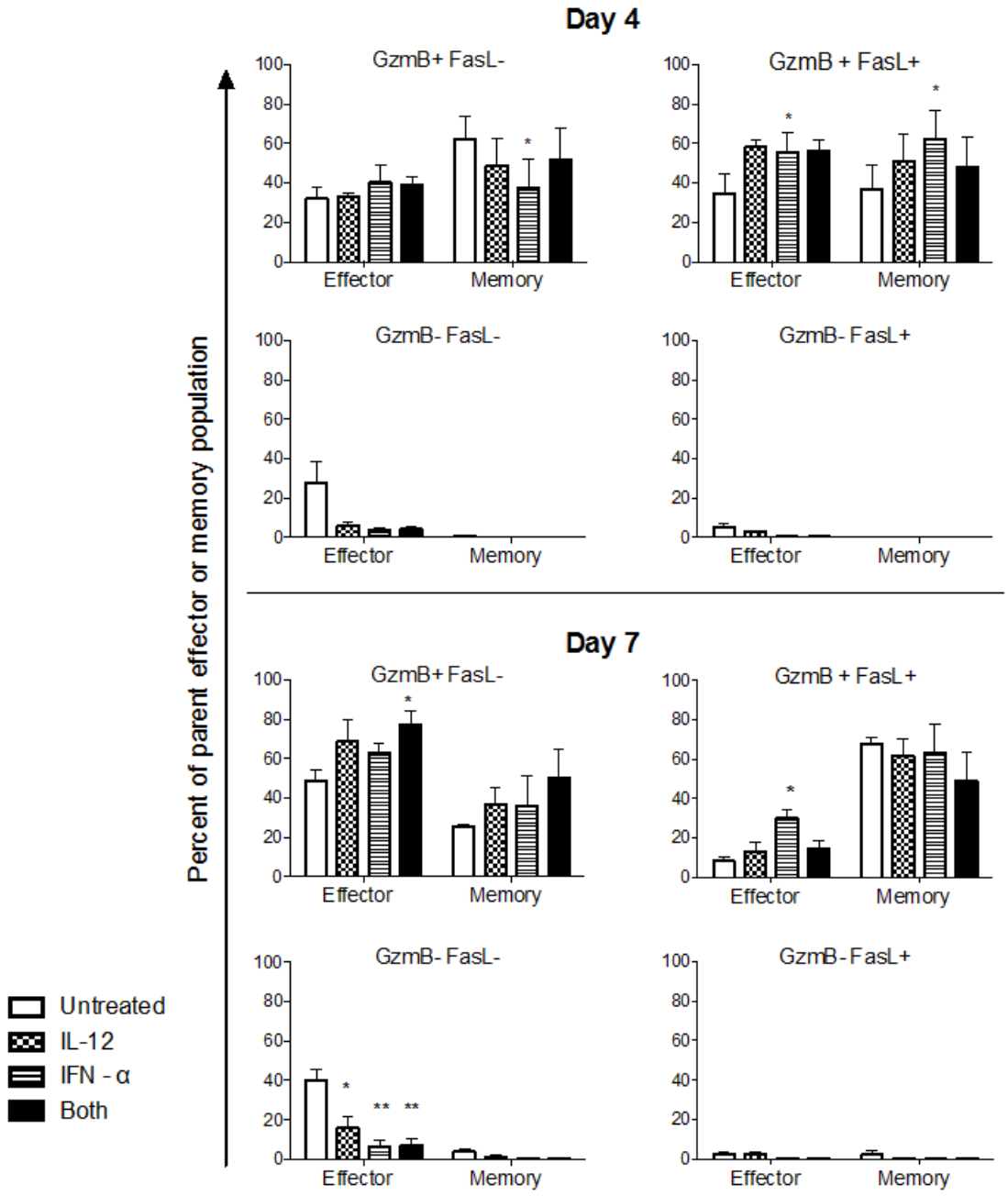


Figure 3-15. Memory and Effector cells differ in intracellular FasL and GzmB coexpression.

Cells were cultured from 0h onwards with added 2U/mL IL-12, 1000U/mL IFN-α, or both cytokines (Both). All cultures, including the negative control (Untreated) received 5U/mL IL-2 from 48h onwards. Cells were harvested at 4 or 7 days post activation and stained for CD44, CD62L and CD127, then intracellularly for FasL and GzmB. Populations were gated as effector or memory cells, then divided into quadrants for FasL and GzmB expression as represented in Figure 3-12. Cytokine-treated samples were compared against "UN" sample by paired student's t-test. *=P<0.05, **=P<0.01

later after activation. I also showed that in differentiated CD8+ T cell populations generated *in vitro*, effector phenotype cells seems to lose FasL expression over time but central memory phenotype cells may retain FasL and GzmB coexpression.

I found that populations that express FasL and GzmB independently or together do not do so consistently over time. Depending on the strength of TCR and CD28 stimulus the cells received, and the cytokines they were exposed to, the FasL+ GzmB+ population appears early or late in my analyses, and the FasL+ GzmB- population is rare and transient. I wondered whether there is a consistent pathway for progressing to or from the FasL+ GzmB- phenotype. My colleague Wayne Juang suggested in his thesis that FasL+ GzmB- is the default state after activation, with GzmB expression added in transiently and an eventual return to a FasL+ GzmB- state [367](Fig 3-16A). However, this model does not agree with most of the data in this chapter, where a FasL- GzmB+ population is present at some point in all experiments. In fact, the first data I show, wherein FasL expression is not nearly as dominant as GzmB at the early day and FasL+ GzmB- cells are virtually nonexistent, with a slightly increasing FasL- GzmB- population (Fig 3-8), seems to fit better with a model where cells start out as FasL- GzmB+ and progress to FasL+ GzmB+ before eventually losing expression of both, irrespective of IL-2 concentration (Fig 3-16B). Memory cells exposed to inflammatory cytokines seem to follow this pattern as well (Fig 3-15). In contrast, effector cells in the same experiment seem to follow the inverse pattern: Cells are first FasL+ GzmB+ coexpressors, losing FasL over time to become FasL- GzmB+ and eventually FasL- GzmB- (Fig 3-16C). Cells activated with suboptimal anti-CD3/CD28 and high IL-2 follow this pattern (Fig 3-10) as

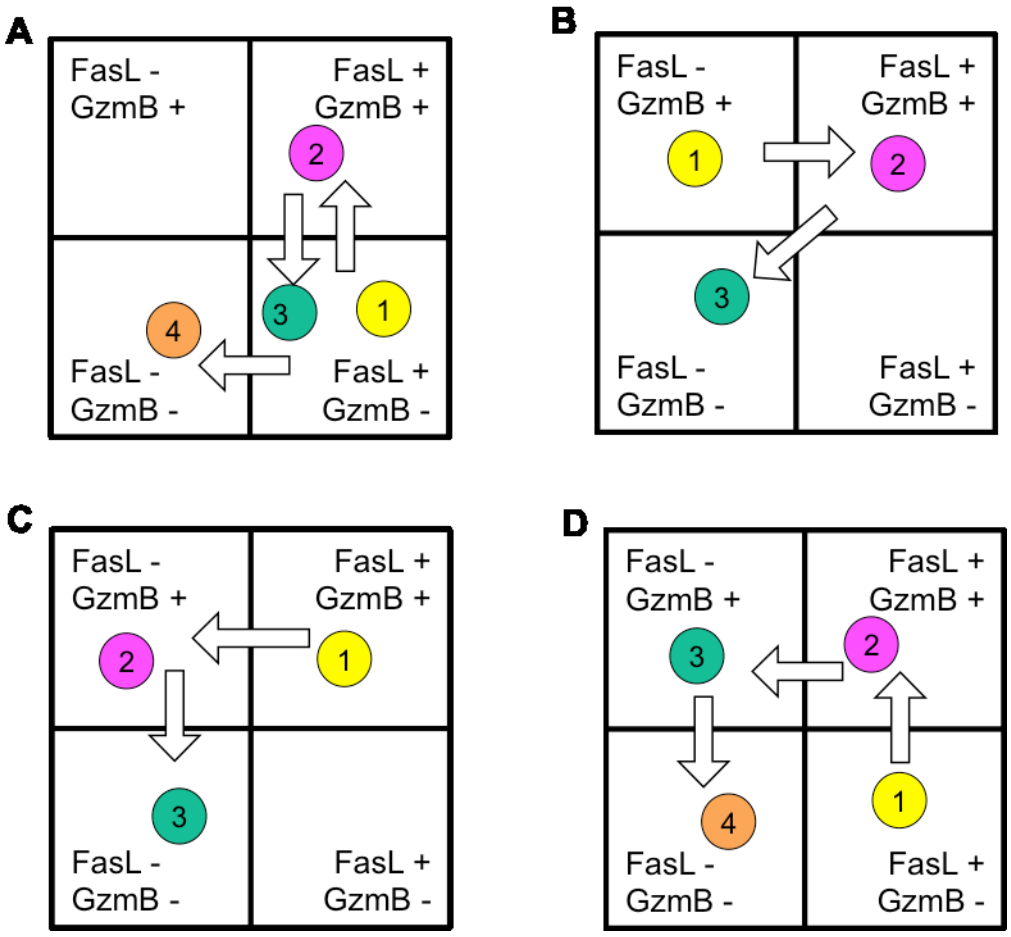


Figure 3-16. Potential models for intracellular FasL and GzmB expression by CD8+ T cells after naive cell activation by plate-bound anti-CD3/CD28.
In all models, naïve CD8+ T cells are FasL- GzmB-. (1) indicates the first expression pattern following activation, and the arrows indicate changes in expression over time. A. Cells first become FasL+ GzmB-, then become FasL+ GzmB+, and then return to FasL+ GzmB-, but both eventually become FasL- GzmB-. B. Cells first express only intracellular GzmB as FasL- GzmB+, then add intracellular FasL expression to be FasL+ GzmB+, then lose both and are FasL- GzmB-. C. Cells first coexpress both as FasL+ GzmB+, then lose FasL to become FasL- GzmB+, then lose GzmB to become FasL- GzmB-. D. Cells first express only FasL as FasL+ GzmB-, then become FasL+ GzmB+, then transition to FasL- GzmB+, then neither as FasL- GzmB-.

well. However, there is a small population of FasL+ GzmB- cells when CD8+ T cells are activated with suboptimal anti-CD3/CD28 and receive only low IL-2. As this small population is lost over time, it is possible that the first state for these cells after activation is actually FasL+ GzmB-, before transitioning to FasL+ GzmB+ and then FasL- GzmB+ before eventually losing both (Fig 3-16D). Experiments that I mention fitting into model C may also fit into model D, but it may just be that the higher stimulus (for effector cells) or the elevated IL-2 is able to push them past the FasL+ GzmB- state more quickly and I did not assess these cells early enough. While from the data in this chapter I cannot resolve whether there is one programmed course among activated cells for FasL and GzmB expression, they all suggest that FasL+ GzmB+ is a transient state in *in vitro* activated CD8+ T cells.

I had wondered whether the cytokine environment would similarly affect both FasL and GzmB. As discussed above, the major intracellular FasL/GzmB coexpression patterns do not change much between cytokine treated groups with the sameCD3/CD28 activation strength. However, the presence of FasL or GzmB and their quantity may be controlled independently. The actual quantity of GzmB is increased with higher concentrations of extrinsic IL-2, though optimal stimulation results in the majority of cells being GzmB+ following *in vitro* activation. This is not the case for Fas Ligand. First, my experiments have shown FasL expression, is not significantly increased by high concentrations of IL-2 in culture, following activation with high anti-CD3/CD28.

While some of the low impact of IL-2 concentration on FasL stores might be explained by high stimulation via CD3 and CD28 overriding the effect on FasL by IL-2, this does not agree with my data post-PMA/I stimulation where the cells treated with

higher IL-2 do have increased FasL on the surface after restimulation. Perhaps there is a maximum limit of FasL maintained at any point intracellularly in activated cells, though as I discuss below, that maximum amount may change depending on original TCR plus CD28 activation strength. It could also be that FasL is still synthesized at increased levels in accordance with mRNA levels as IL-2 concentration increases, but that FasL gets cleaved at the cell surface. My colleague Ana Clementin found that FasL is trafficked to the cell surface after synthesis prior to endocytosis and storage [192], so excess synthesized FasL may end up cleaved from the cell surface. Since I saw very little surface FasL at any day except a small increase at D10 in 100U/ml treated cells, if this trafficking is occurring it would have to be immediately cleaved [369]. Future work could assess soluble FasL by ELISA to check this. Alternatively, IL-2 may not affect basal FasL synthesis, but may permit FasL gene to be more accessible for rapid transcription and then synthesis following PMA/I or target cell encounter.

Some studies have focused on FasL-dependent killing in response to IL-2: In one study where CD4+ T cells were stimulated *in vitro* with peptide followed by culture with or without supplemented 40U/ml of IL-2, GzmB-mediated killing is enhanced by culture in IL-2, while FasL-dependent killing by CD4+ T cells is not enhanced by IL-2 [370]. Another study found that in already-activated CD8+ T cell clones, the addition of IL-2 is sufficient to trigger FasL-mediated cytotoxicity in the absence of TCR stimulation[78]. However, neither of these studies confirmed that this cytotoxicity actually is associated with an increase in FasL on the CTL surface. My research suggests that increasing IL-2 concentration in cell culture enhances cell-surface FasL in response to restimulation. Both of the studies cited above merely examined whether the presence of a single

concentration of IL-2 can enhance FasL killing, but not whether more IL-2 further enhances FasL expression in an IL-2 dependent manner. It is also possible that the ranges of IL-2 which caused increases in post-stimulation surface FasL that I saw in my experiments do not translate to increased cytotoxicity in target cells; this is a possible topic for future investigation.

Work by Pipkin and colleagues [140] in CD8+ T cells activated *in vitro* and followed by culture with high (100U/ml) and low (10U/ml) IL-2 found that, as detected by Western Blot, GzmB is high by D4 and stays high, slightly increasing, in high-IL-2 treated cells, while decreasing in low-IL-2 treated cells, continuing in both directions through D6. I found that when assessed by flow cytometry my cells follow a similar trend, with a peak difference in GzmB at D7. My assessment via flow cytometry takes this further, however, by examining not just average intracellular GzmB within a population, but also the percentage of cells positive for GzmB. While it is important to know how much GzmB is in a cell, what is also meaningful is how many CTL have the potential to kill, and are armed with FasL, GzmB, or both. Since I have found that not all cells express both proteins at once, qPCR or Western blots from pooled populations lacks the nuance to distinguish these coexpression patterns.

GzmB is not significantly higher in cells that were exposed to both IL-12 and IFN-α than their counterparts with only IL-12 or IFN-α (Fig 3-11B,C). Furthermore, the addition of IL-12 or IFN-α had the most significant effect on GzmB expression a week after activation, and not at the earlier D4. While this at first seems to contradict findings that IL-12 or IFN-α can increase GzmB expression, Agarwal and colleagues found that in a 3-day activation study similar to mine, GzmB mRNA peaks at 48 hours and starts to

decrease by 72h [176]. It could be that by the point I examined the cells, general transcription is low enough that additive effects on protein levels are minimal, and the best effect that IL-12 and IFN-α have on GzmB expression (or survival of GzmB+ cells) is to sustain it, rather than to further increase it. Inflammatory cytokines also only significantly increase the proportion of CD8+ T cells positive for FasL in cells at D4. The Agarwal study I mentioned saw cytokine-dependent increases in FasL mRNA transcripts up to 72h[176], but did not go out to the 4 days that I started measuring at, and transcript-level changes do not always translate into protein. Although the proportion of cells high for intracellular FasL is increased under most conditions at D4 and under all conditions at D7, the actual amount in these positive cells is sufficiently variable to not render the differences in intracellular FasL statistically significant (Fig 3-12B, C).

What surprised me the most in differentiated-phenotype cells s that there is a CD127hi CD62Lhi phenotype population at both D4 and D7 that express GzmB. While groups have found cytotoxic memory T cells [371, 372], but it is interesting to see that "memory" cells are strongly armed without restimulation first. However, we must take this with a grain of salt as I am looking at one week following activation, not 4+ weeks following an *in* vivo response. As I was unable to get consistently satisfactory KLRG1 staining in my samples (data not shown), it may be that in this model, I'm seeing Tcm as the original, first phenotype to develop, and as the CD8+ population expand *in vitro*, they are gaining GzmB and FasL before the memory surface markers are switched off, so my memory-like, FasL+ GzmB+ cells are a transitional cell type. Regardless of which model this supports, I will investigate in later chapters of my thesis whether memory-phenotype cells can express GzmB *in vivo* as well.

Overall, in this chapter I have shown that FasL and GzmB have separate protein expression regulation in *in vitro* activated cytotoxic T cells. Not only do activated CD8+ T cells heterogeneously express intracellular GzmB and/or FasL, but also FasL and GzmB are modulated differently by extrinsic cytokines over time. *In vitro* derived memory and effector phenotype cells can both express FasL or GzmB protein, and have different time-based transitions between FasL+ GzmB+, FasL-GzmB+ and FasL-GzmB- phenotypes. Future rwork should examine whether *in vitro* derived cells with different FasL and GzmB expression patterns have distinct cytolytic or therapeutic activity when transferred *in vivo*.

Chapter 4:
FasL is expressed by CD8+ T cells in the EG.7 tumor model

Introduction

I have shown in the previous chapter that cytokines present in the activation environment, combined with time following activation and differentiation of $CD8^+$T cells, can influence the balance of FasL and Granzyme B protein expression by these cells. Since the cytokine environment is modulated following an *in vivo* immune response, I wanted to see what the effects of an immune response were on FasL and GzmB expression in mice. Studying this response in tumors is scientifically interesting as it deviates from the textbook activation-expansion-clearance-contraction-memory cycle associated with an acute infection. As tumors that are unsuccessfully or ineffectively cleared by the immune system present persist, there are also the factors of time and tumor burden that could affect effector mechanism expression in CD8+ T cells found in these tumors, similar to what can be seen in chronic infections [249, 250, 255]. There is still debate as to whether FasL and/or Granzyme B are essential to clearance of tumors, but very little work has been done to determine whether the proteins themselves are expressed in tumor infiltrating cells. Cellular immunotherapy is increasingly acknowledged as a viable clinical intervention for patients with a number of cancers [358, 373]. To fully understand how the immune system responds to cancer we need to understand more about CD8+ T cell function and regulation in cancer. In particular, are FasL and GzmB both expressed by CD8+ T cells in response to tumors? Does presence in the tumor, or the size of the tumor itself, have an effect on FasL or GzmB expression by CD8+ T cells? In this chapter, I demonstrate that CD8+ T cells responding to

intraperitoneal and subcutaneous tumors in mice express FasL protein, and GzmB is only expressed by a subset of CD8+ T cells.

I examined the immune response to the same tumor injected in two different sites: intraperitoneally as ascites, and subcutaneously as a solid tumor. I injected the EG.7 lymphoma in mice, which is transfected with cDNA for ovalbumin (OVA) [350]. One well-defined OVA antigen is the SIINFEKL peptide of OVA on MHC on the cell surface (K^b-SIINFEKL). This is recognized as foreign by the immune system. The parental line of tumor cells is of T-cell origin from female C57/B6 mice treated with carcinogens [374], and can grow as ascites or as solid tumors, depending on their site of injection. Any T cell populations that recognize OVA peptides could therefore be considered as tumor antigen-specific. This model antigen was also chosen as it is recognized by a defined rearranged TCR on CD8+ T cells, which have been permanently introduced into OT-I transgenic mice [348]. These can be recognized by anti-Vα2 and anti-Vβ5 antibodies.

Results

Injection of EG.7 cells intraperitoneally elicits a small but detectable Vα2+/Vβ5+ CTL population in the peritoneum

The first model I employed was the intraperitoneal injection of EG.7 cells into mice that had been previously adoptively transferred with naïve CD8+ T cells from OT-I transgenic mice (Fig 4-1). The OT-I cells strongly recognize the K^b-SIINFEKL [348] on EG.7 cells and can be activated in response to EG.7. Following an intraperitoneal injection of EG.7 cells, CD8+ T cells have been shown to infiltrate the peritoneum within 48 hours [375], but I wished to see the effects of established tumor cells on FasL expression, potentially as an antigen-specific response expands, which can take 7-14

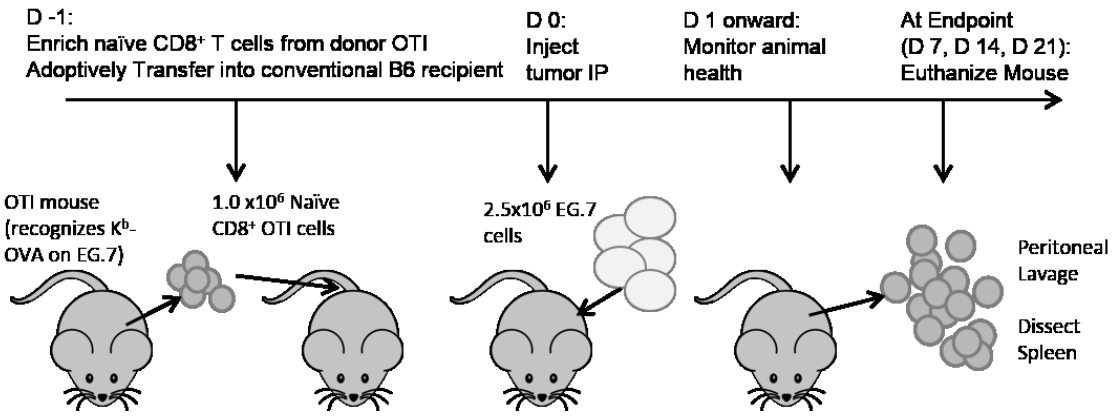


Figure 4-1. Schematic of injection timeline for mice bearing intraperitoneal tumors following adoptive transfer of naïve OTI cells.

days. I tested this model without prior adoptive transfer, but a small proportion of these mice develop morbid abdominal swelling in the second and third weeks, preventing me from ethically using mice responding to EG.7 with only the native immune response. The addition of a naïve OT-I population enhances the frequency of circulating naïve cells potentially specific for the tumor that can expand and respond to the EG.7 cells, giving the mouse a slight advantage for survival while the EG.7 cells expand. However, this is not as directly therapeutic as transferring in activated OT-I cells, as the naïve OT-I cells still need to be activated within the host.

In order to identify OT-I T cells, or other endogenous cells bearing the Vα2/Vβ5 domains of the TCR rearrangement that can be be specific for K^b-SIINFEKL [44], I used antibodies against the rearranged TCR chains (Fig 4-2). These are capable of detecting the majority of cells from an OT-I mouse, and have a frequency of detection below 2% in conventional B6 CD8+ T cells, as these mice likely have some rearranged TCR that are detected by these antibodies. Not all or even most Vα2+/Vβ5+ cells will be OVA-specific, as they may have different D and J regions of the TCR alpha and beta chains that differ from the rearranges OT-I ones, but OVA-specific T cells will be contained in the Vα2+/Vβ5+ population.

I first examined the number of cells recovered from the peritoneum of mice without tumors, and at D7, D14, and D21 post EG.7 injection. Increasingly large numbers of cells are recovered over time by peritoneal lavage from mice bearing tumors, though there is enough variability in sampling that these increases are not statistically significant compared to peritoneal samples from mice without tumors (Fig 4-3A). This represents a mix of tumor cells, CD8+ T cells, and other leukocytes.

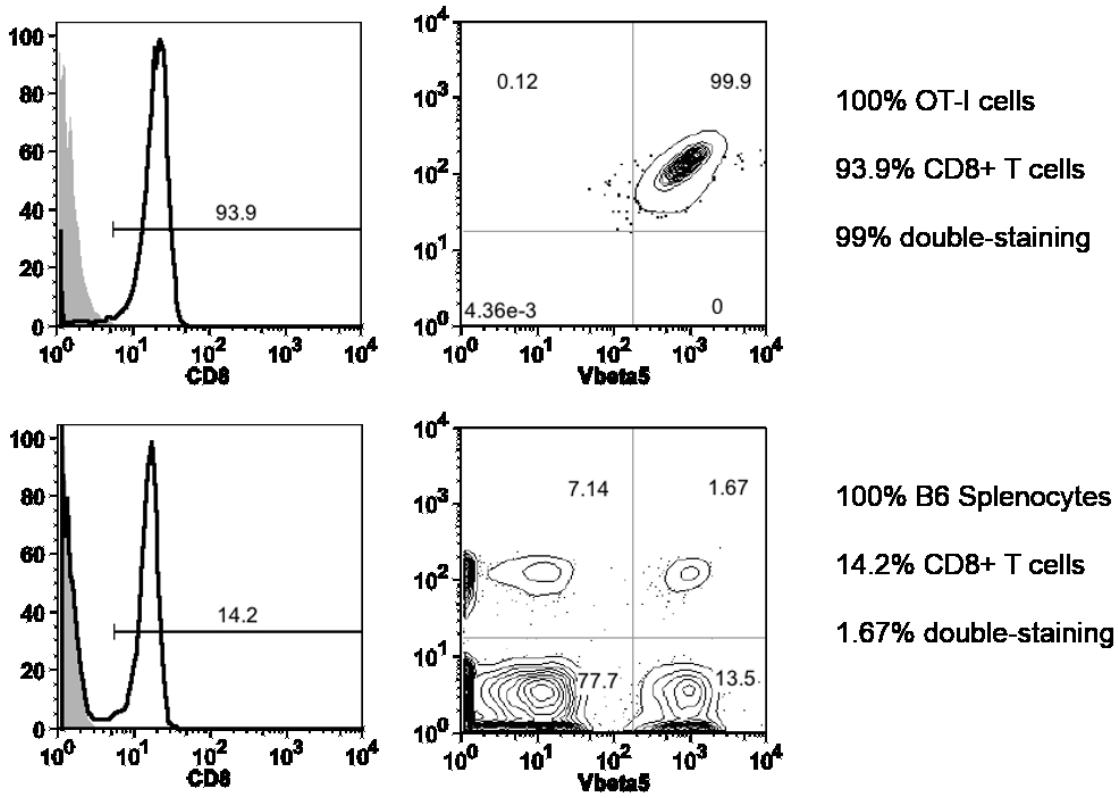


Figure 4-2. Combined staining for Valpha2 and Vbeta5 rearrangements of TCR detects OT-I T cells.

A pure culture of OT-I T cells, or bulk fresh splenocytes from a conventional B6 mouse, were stained for CD8 and Vα2 and Vβ5 TCR. Cells were gated on the $CD8^+$ population prior to examining TCR coexpression Grey region of histogram indicates unstained control.

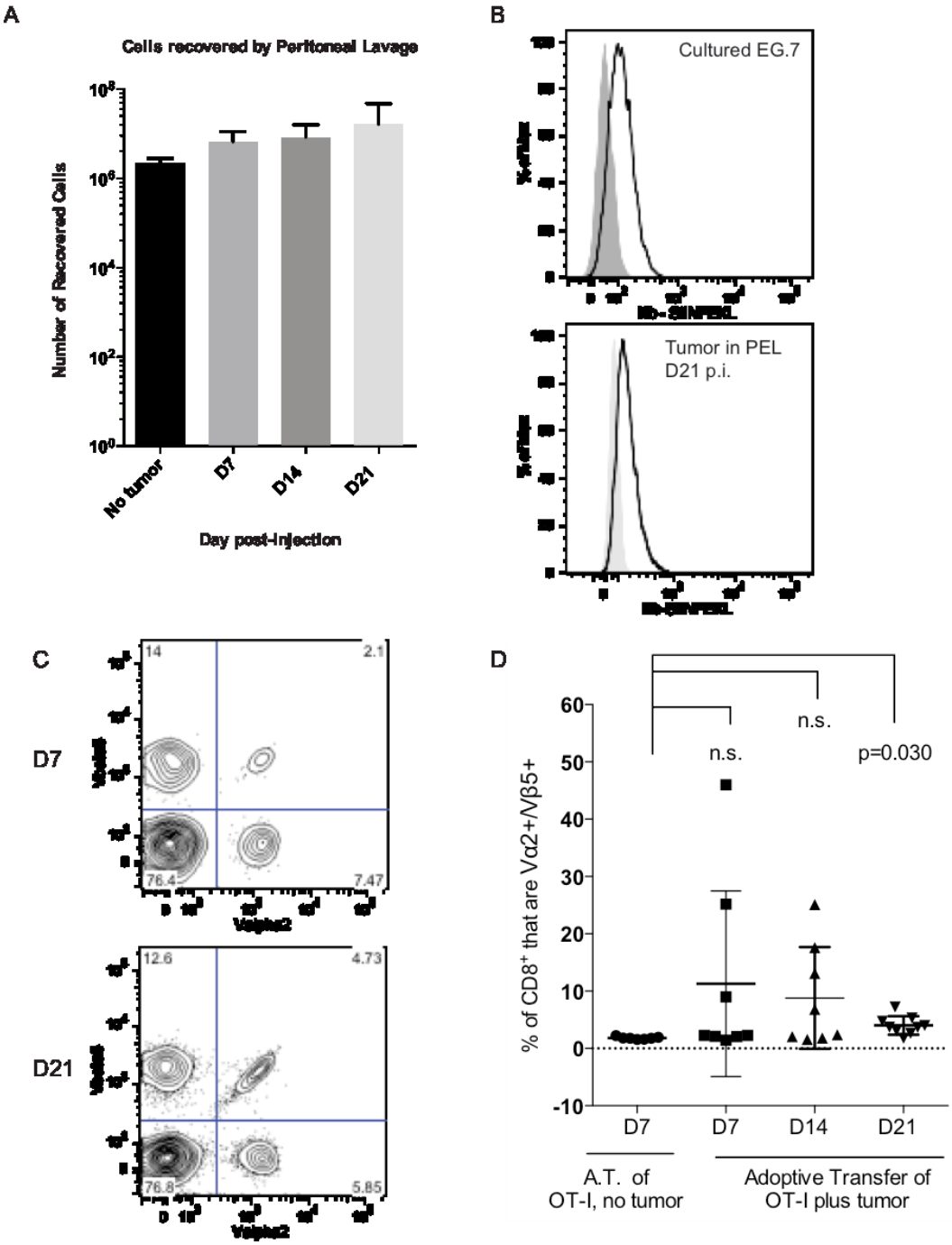


Figure 4-3. Though K^b-SIINFEKL-positive cells remain in the peritoneum after 21 days, there is only a limited Vα2+/Vβ5+.

Mice received adoptive transfers of Naïve OTI T cells on day -1, then were injected with PBS (no tumor), or $2.5x10^6$ EG.7 cells. A. Absolute counts of cells recovered by peritoneal lavage. B. Expression of Kb-SIINFEKL prior to and after growth in mouse peritoneum, C. Representative Vα2/Vβ5 TCR staining of CD44+ CD8+ T cells from peritoneal exudate of tumor-injected mice, D. Overall proportion of CD44+ $CD8^+$ T cells bearing Vα2+/Vβ5+ TCR in mice. P values from unpaired 2-way student's t-test.

It is important to characterize the expression of antigen on the tumor cells to determine if there is a selection of non-antigen bearing tumor cells, as this could affect the antigen specificity of the subsequent T cell response. The tumor cells from peritoneal exudate retain expression of K^b-SIINFEKL on their surface for up to 21 days (Fig 4-3B), suggesting that there is not a detectable selective pressure against KB-SIINFEKL expression. The proportion of Vα2+/Vβ5+ CD8+ T cells remains small through all days examined following tumor injection (Fig 4-3C,D). There is only a significant increase in activated Vα2+/Vβ5+ CD8+ T cells compared to the non-tumor control at day 21 (Fig 4-3C and D). This conflicts with work showing that the influx of antigen-specific OT-I cells into tumor-bearing peritoneum is transient, with antigen-specific cells leaving for secondary lymphoid tissue after this time [375]. The later increase I saw in Vα2+/Vβ5+ cells at D21 may be due to a an increased infiltration into or reduced emigration from the tumor at later time points than Shirkant and colleagues examined. Of course, not all tumor-specific T cells are going to be bearing the OT-I TCR, some may be against minor undefined antigens, so there may be a population of antigen-specific T cells present, but these are invisible in the current model.

Injection of EG.7 cells subcutaneously elicits a local polyclonal response

I also injected EG.7 cells subcutaneously. Unlike a peritoneal tumor, a solid tumor has challenges to $CD8^+$ T cell infiltration, changes in vasculature, or change in nutrient/oxygen availability in the tumor [280]. However, I was able to track health status and tumor burden of mice by external palpation. This also does not require pre-tumor adoptive transfer of naïve OT-I cells (Fig 4-4).

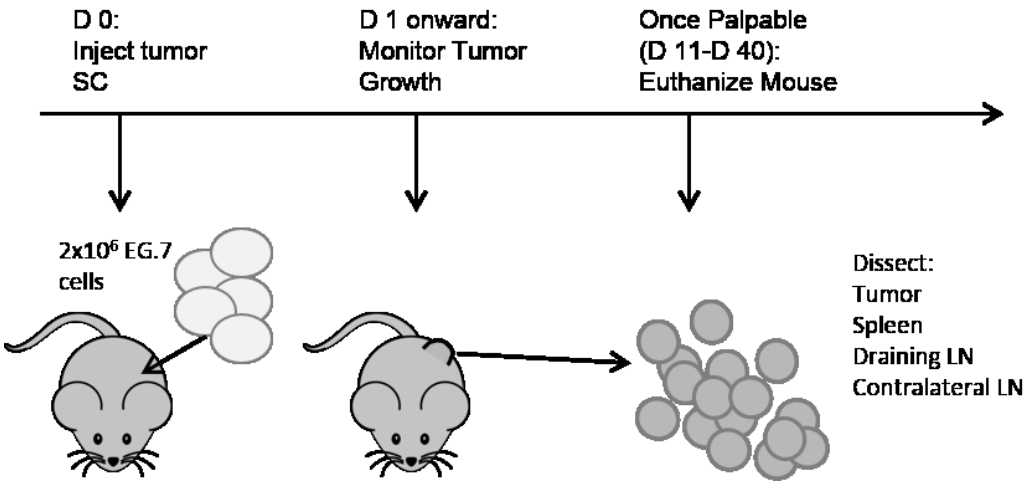


Figure 4-4. Schematic of injection timeline for mice subcutaneous tumors in previously unmanipulated mice

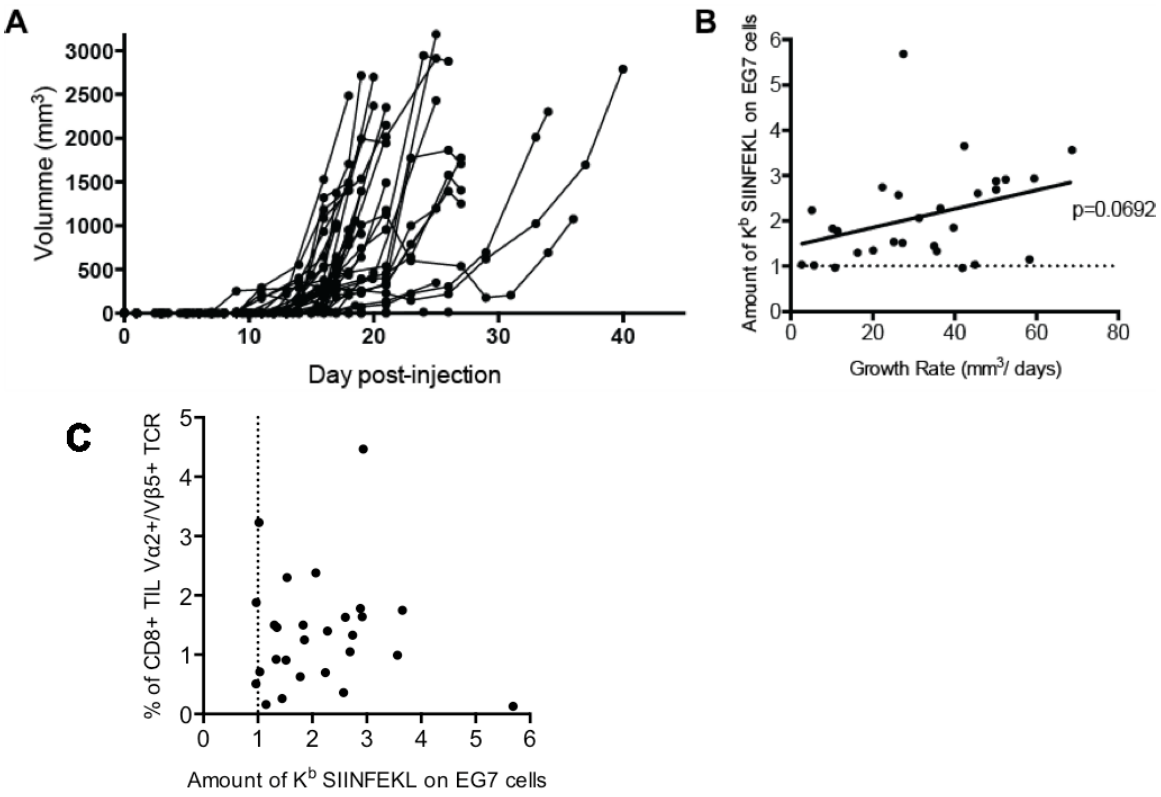


Figure 4-5 Solid subcutaneous tumors progress in mice independently of K^b-SIINFEKL on EG.7 cell surface.

Mice were injected SC in right flank with $2.0x10^6$ EG.7 cells on Day 0. A. External volume of SC tumors over time, B. Growth rate of tumors (final dissected volume over number of days to dissection) versus K^b-SIINFEKL staining of dissected tumor cells (MFI of K^b-SIINFEKL relative to isotype control) after dissection. P value from linear regression between surface K^b-SIINFEKL and growth rate. P value is not significant. C. Proportion of Vα2+/Vβ5+ cells in the activated CD44+ CD8+ TIL population compared to K^b-SIINFEKL staining on tumor cells from the same mouse. No significant correlation is present.

Mice developing tumors were first palpable typically at between D12 and D19. (Fig 4-5A). Most tumors grew consistently over time, but a few tapered off for growth over time. Only one mouse had a reduction in tumor volume and then regained tumor volume, but data from this mouse was not exceptional in any of the characteristics covered in this section. There is a wide range of Kb-SIINFEKL staining on tumor cells after dissection, but there is no significant correlation between growth rate of the tumor and post-dissection K^b-SIINFEKL staining (Fig 4-5B) on tumor cells or frequency of Vα2+/Vβ5+ T cells in the tumor (Fig 4-5C). I can assume that any Vα2+/Vβ5+ CD8+T cell population that arose was elicited by the tumor, but not sufficient for its clearance.

An average of $2x10^7$ cells were recovered from subcutaneous tumors (Fig 4-6A). As already mentioned for the IP tumor, this includes both tumor and immune cell populations. There are significantly more cells in the draining versus contralateral lymph node, indicating a local immune response. I stained cells for CD44, as this is upregulated on T cells once they have become activated. Unlike in my *in vitro* experiments, I found three CD44 staining populations in the spleen: CD44hi, CD44int, and CD44lo (Fig 4-6B). After gating on all three, the CD44hi population is elevated for CD8+ T cells in the tumor, significantly more than in the spleen (Fig 4-6C). CD44hi cells are the population that are most consistently detectible in the TIL population. Even though some CD8+ cells fall into the CD44int gate, they appear to be a mostly continuous population with the CD44hi cells. Therefore, I will focus on CD44hi cells. CD44hi and CD44+ may be used interchangeably in later sections of this thesis, always referring to the CD44hi gated population when discussing activated cells. In spite of the elevated numbers of cells recovered from the dLN compared to the cLN, there is no difference in the proportion of

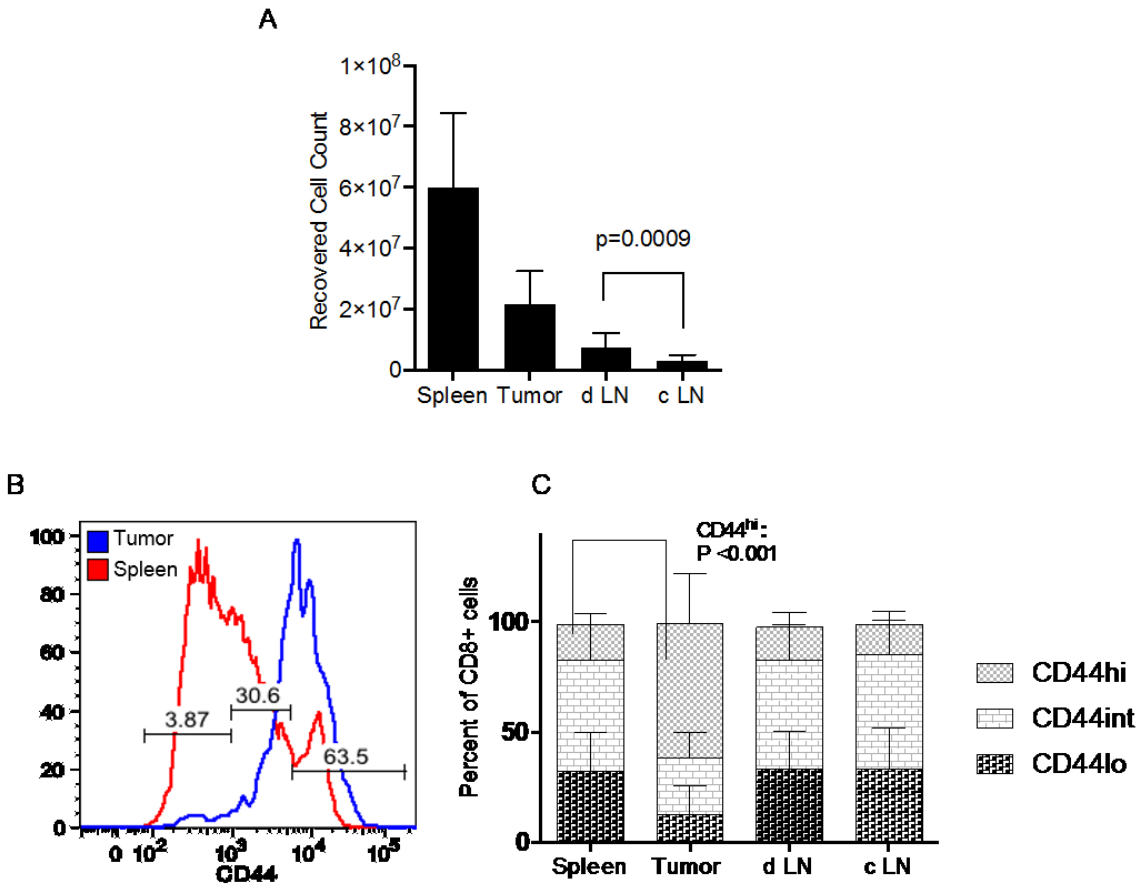


Figure 4-6 While the draining LN has in increased number of cells compared to its contralateral counterpart, only the tumor has an increased proportion of activated CTL. Mice were injected SC in right flank with 2.0x10^6 EG.7 cells. A. Counts of recovered, live cells post-dissection from tumor-bearing mice. B. Representative CD44 stain for CD8+ cells from spleen (red) or tumor (blue). Displayed frequencies are for tumor CD8+ T cell sample. C. Proportion of $CD8^+$ T cells with high, intermediate, and low levels of CD44. Mice euthanized at different stages of tumor progression were pooled. Data from at least 18 mice over 4 independent experiments. P values from two-way, unpaired students' t-test.

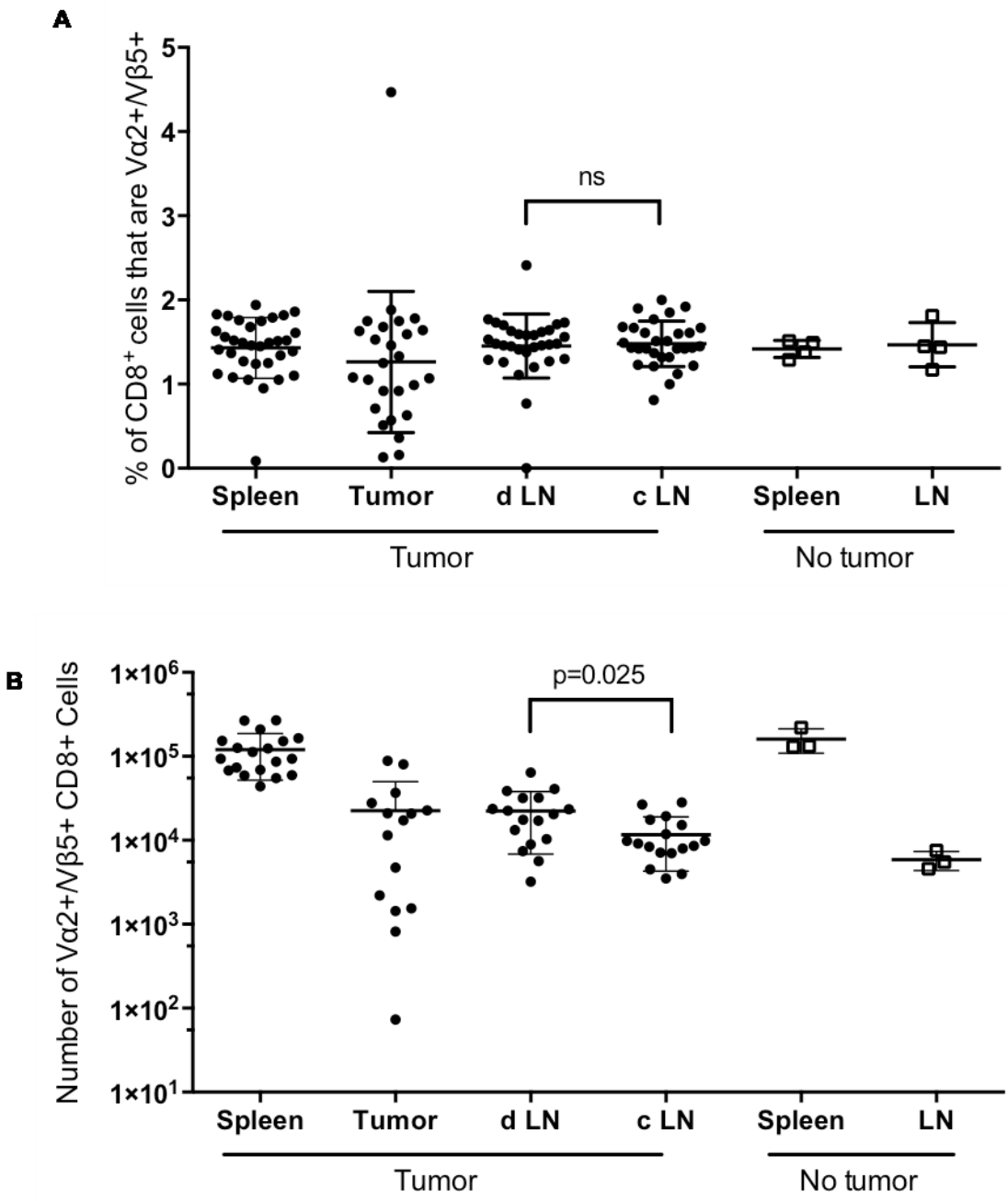


Figure 4-7 No relative increase in the fraction of percentage of Vα2+/Vβ5+ CD8+ T cells is detected in response to tumors.

Mice were injected SC in right flank with $2.0x10^6$ EG.7 cells on Day 0. A. Proportion of Vα2+/Vβ5+ $CD8^+$T cells from tissues in SC tumor-bearing mice and non-tumor control mice B. Absolute numbers of Vα2+/Vβ5+ $CD8^+$T cells from tissues in SC tumor-bearing mice and non-tumor control mice. P value from paired two-way student's t-test. Data from at least 14 mice over at least four independent experiments (tumor mice) or a least 3 mice over three experiments (no tumor).

CD8+ cells that are CD44hi in the dLN or cLN (Fig 4-6C). There does not appear to be preferential activation of CD8+ T cells in the draining LN, just increased numbers of cells recruited to or staying in the dLN.

The proportion of Vα2+/Vβ5+ T cells is not elevated in the tumor or in the draining lymph node compared to the spleen or contralateral lymph node, respectively (Fig 4-7A). The absolute number of these cells is higher in the draining lymph node compared to its nondraining counterpart (Fig 4-7B), but this is likely simply due to increased cells numbers in the draining lymph node, and not an antigen-specific expansion. However, it has been shown that in a B16-OVA model, CD8+ T cells can be activated directly in the tumor itself [272], so it may be that the naïve cells are recruited directly to the tumor for activation. All of this together indicates that there is a population of activated $CD8^+$ T cells with unknown specificity in the subcutaneous tumor.

Most activated CD8+ T cells from conventionally housed tumor-free mice express intracellular FasL

Before looking at data from tumor-bearing mice, I wanted to establish the normal effector molecule expression by activated CD8+ T cells in healthy, conventionally housed, unmanipulated mice. In $CD8^+$ splenocytes stained for CD44, there are high, intermediate, and low CD44 expressing populations (Fig 4-8A). $CD44^{lo}$ are naïve cells, and comprise a small proportion of the total splenic $CD8^+$ T cells. These naïve cells are almost exclusively negative for FasL and GzmB, as expected (Fig 4-8C). $CD44^{hi}$ cells, the typical activated population, from the same sample and the same gating strategy, are mostly single positive for intracellular FasL, with a distinct but small population expressing neither FasL nor GzmB intracellularly (Fig 4-8C, D). Therefore, this is the *status quo* of the $CD8^+$ T cells in my negative control splenic CD8+ T cells: mostly FasL

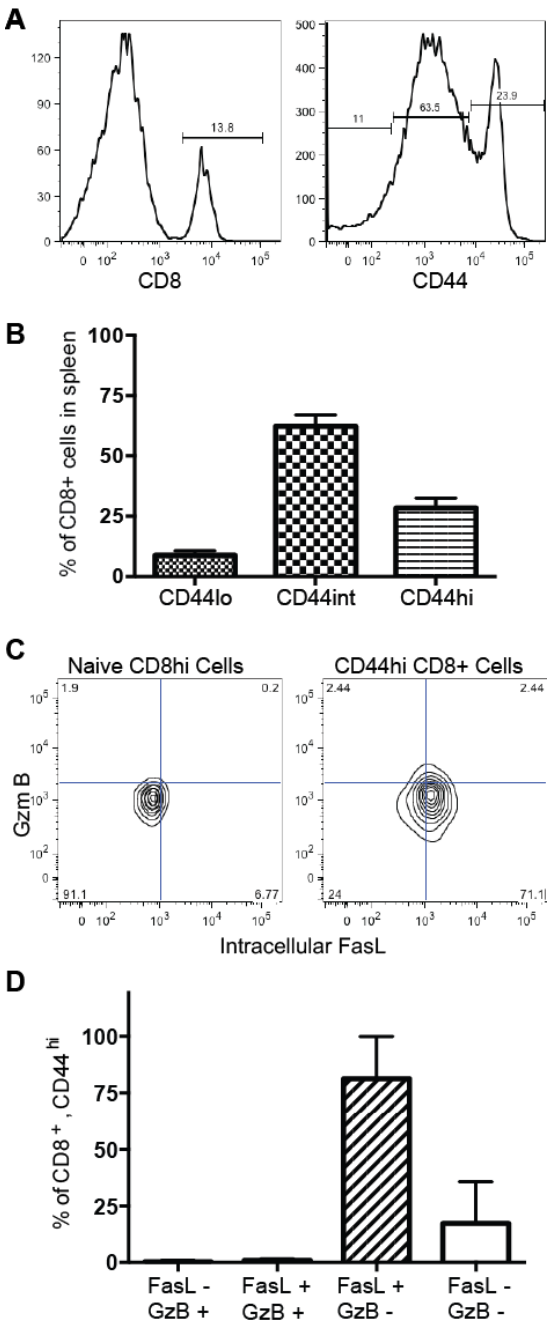


Figure 4-8 Splenocytes of mice without tumors predominantly express FasL alone. Splenocytes from mice housed in conventional conditions, with no tumors, were stained for CD8, CD44, and intracellular FasL and GzmB. A. Gating for activated $CD8^+$ cells, first by CD8 expression and then by CD44 expression. B. Frequencies of $CD44^{lo}$, $CD44^{int}$, and $CD44^{hi}$ cells. C. Representative staining for intracellular FasL and GzmB in Naïve cells ($CD44^{lo}$) and $CD44^{hi}$ $CD8^+$ cells. D. Summary data showing distribution of intracellular FasL/Granzyme expression in $CD44^{hi}$ $CD8^+$ cells. Data from at least seven mice over three independent experiments.

expressing, very few cells expressing GzmB, either alone or in combination with FasL. I see a very similar population distribution when $CD44^{hi}$ $CD8^{+}$ T cells from the peritoneum of mice lacking tumors are examined (Fig 4-9A) Again, most of the cells are FasL positive, with very little GzmB positive, and approximately 20% expressing neither effector protein. In summary, intracellular FasL expressed by activated CD8+ T cells sin the spleen, while in contrast GzmB+ cells are very rare this same T cell population..

GzmB is expressed by some CTL when EG.7 tumor cells are present

Given that the majority of activated CD8+ T cells form the spleen and peritoneum of normal mice express FasL, I wanted to determine whether FasL or GzmB expression changes upon introduction of a tumor. When mice were injected with Eg.7 cells intraperitoneally, I found that in the activated CD8+ T cell population, many cells continue to primarily express FasL alone (Fig 4-9B, C). However, an increased proportion of activated CD8+ T cells express neither FasL nor GzmB compared to the healthy control. Furthermore, there is an increase in the proportion of cells that express intracellular GzmB and FasL, and by Day 21 there are significantly more cells expressing GzmB plus FasL relative to the proportion of cells expressing GzmB alone (Fig 4-9B). As the average proportion of $CD8^{+}$ T cells storing FasL decreases in tumor-bearing mice compared to healthy mice, it may be that FasL protein expression is turned off or decreased in tumor-infiltrating cells, or that FasL-positive cells leave the peritoneum, as suggested by other with respect to highly cytolytic cells [375] , or die off, in spite of some GzmB storage increasing.

Considering that in Chapter 3 I showed that intracellular GzmB is nearly universally highly expressed in recently *in vitro* activated T cells, it is surprising that

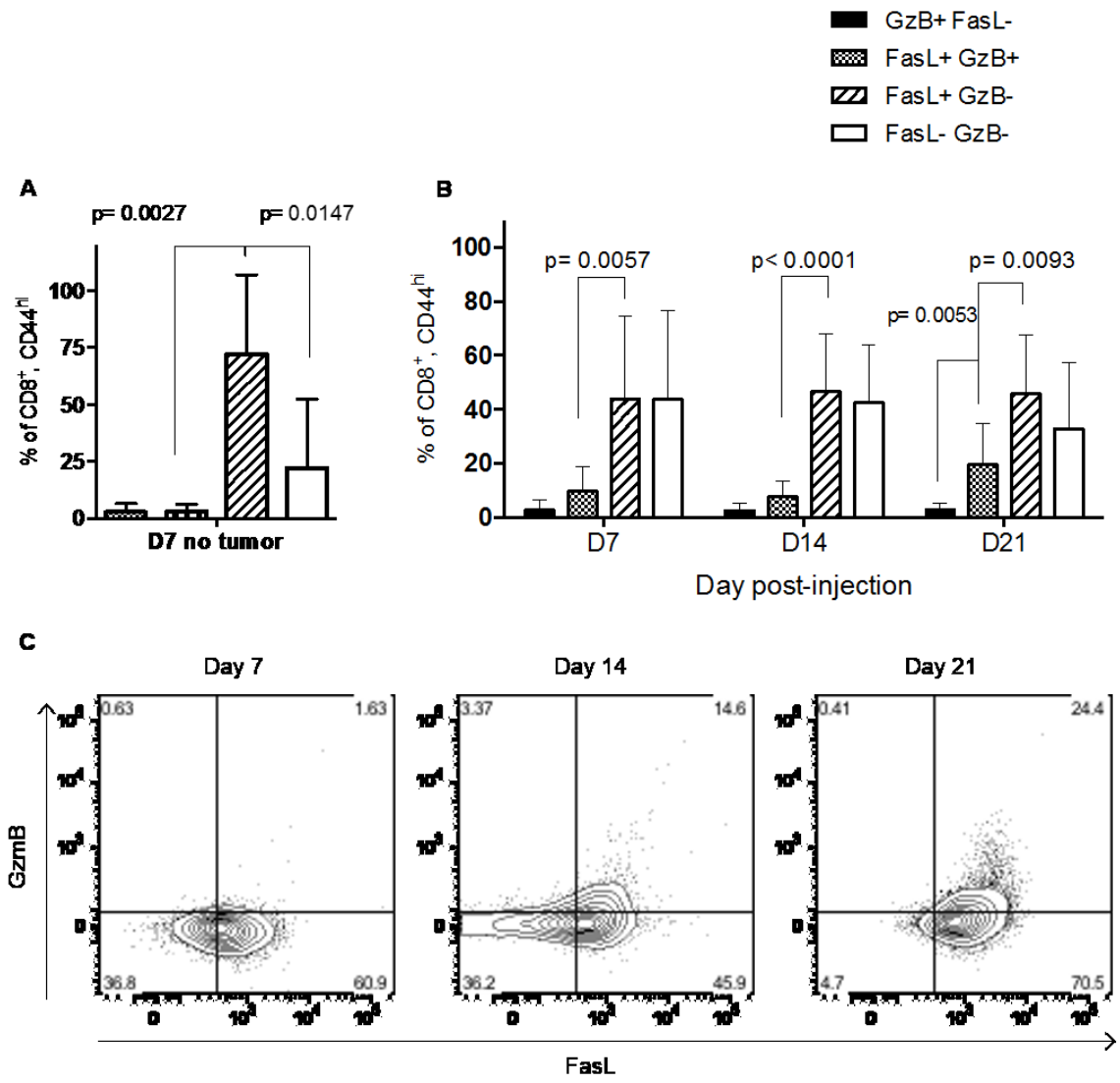


Figure 4-9 In the presence of IP EG.7 tumor cells, most infiltrating peritoneal CD8$^+$ T cells express either FasL alone or neither FasL nor GzmB.
A. Distribution of intracellular FasL/Granzyme B expressing cells in CD44^{hi} CD8$^+$ PEL from mice adoptively transferred with naïve OTI cells but not injected with tumor. B. Distribution of intracellular FasL/Granzyme expressing cells in CD44^{hi} CD8$^+$ PEL from mice adoptively transferred with naïve OTI cells but not injected with tumor. All p values from paired student's t-test. Data from at least seven mice over three independent experiments. C. Representative staining for intracellular FasL and GzmB in naïve and CD44^{hi} CD8$^+$ cells in mice adoptively transferred with naïve OTI cells and then injected with EG.7 tumor cells.

there is not a higher level of GzmB+ cells in the tumor bearing peritoneum. Possibly circulating T cells that are not newly activated are recruited to the peritoneum, or GzmB expression peaks at a time point earlier than I have examined, or activation stimuli are not strong enough from the ascites tumor to elicit widespread expression of GzmB by CD8+ T cells. The fact that very little difference was seen among the three time points examined supports both of these possibilities. As there is a small but significant increase in OT-I TCR+ T cells in the PEL of tumor-bearing mice by Day 21, I had expected that this might be indicative of a change in the quality of the response to the tumor, but this is not the case when looking at the general activated CD8+ T cell population.

CD8$^+$ solid tumor TIL have an increased frequency of GzmB+ cells

As there are more CD44hi CD8+ T cells in the draining lymph nodes of mice bearing subcutaneous tumors (Fig 4-6B), I suspected that this was indicative of a strong CD8+ response to the tumors. I expected to detect the majority of CD8+ TIL expressing GzmB. However, if the tumor environment has a suppressive effect on effector mechanism expression, I might see a local effect on the CD8+ effector mechanism expression, relative to distal lymphoid tissue. I found that the majority of activated cells from both draining and contralateral lymph nodes express FasL alone (Fig 4-10). The lack of any relative change in the draining lymph node suggests no significant local effect of the presence of the tumor on FasL or GzmB expression by the draining CD8+ T cell population. A greater proportion of cells coexpress FasL and GzmB in the TIL population compared with lymphoid tissues, but there are still significantly fewer of these FasL+ GzmB+ cells than FasL+ GzmB- cells in the TIL population. Like in the IP tumor model, very few cells express GzmB alone (Fig 4-10). Therefore, some cells are stimulated to

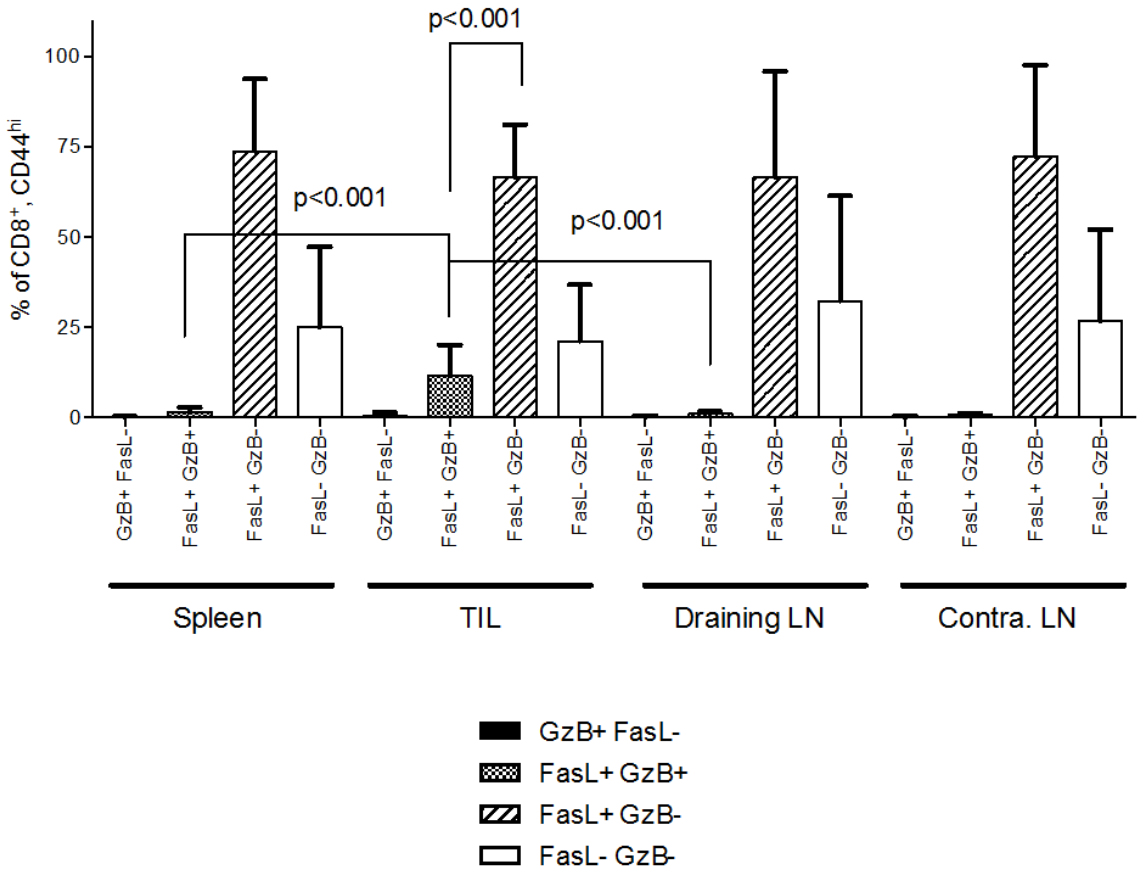


Figure 4-10 In mice bearing subcutaneous EG.7 tumors, the TIL population has a significant increase in GzB+ FasL+ coexpressing cells.
Distribution of intracellular FasL/Granzyme expressing cells in $CD44^{hi}$ $CD8^{+}$ T cells isolated from mice with subcutaneous with EG.7 tumors. All p values from paired student's t-test. Data from 31 mice over five independent experiments.

produce GzmB in, or prior to, entering the tumor environment, but most retain the same phenotype of FasL-SP that is seen in activated CD8+ T cells from the of both tumor-bearing and control mice. There is a very small decrease in the TIL FasL- GzmB- population compared to the spleen, apparently due to the increase of GzB+ cells.

I speculated that there might be changes in the relative proportions of FasL+ and GzmB+ cells as the tumor size changes. Surprisingly, there is no significant correlation between tumor burden (volume) and proportion of CD8+ TIL possessing intracellular FasL+ GzmB-, FasL+ GzmB+, or FasL- GzmB+, in any tissue examined, even in the tumor (4-11). In addition, I did not see any correlation between intracellular FasL or GzmB MFI and tumor volume or growth rate (data not shown). I had expected to see loss of effector mechanism expression in CD8+ T cells in large tumors, but I did not find see a correlation between tumor volume and FasL- GzmB- cells in the tumor (4-11). In addition, in the range of tumor volumes examined, tumor volume does not play as much of a role in the change in effector molecule coexpression by CD8+ T cells (e.g. From FasL+ GzmB- to FasL+ GzmB+) as does just being in the tumor itself. However, the overall takeaway is that while their presence in tumors increases GzmB in CD8+ T cells, their expression does not relate to tumor size, and GzmB+ FasL+ coexpression is clearly not emerging in CD8+ T cells until they are in the tumor.

Adoptively transferred naïve cells show antigen-specific recruitment to solid tumor

I described earlier that I detected a very low number of Vα2+/Vβ5+ cells in the TIL population of mice with SC tumors. Furthermore, these numbers are not significantly increased compared to the numbers found in the spleen in naïve mice, suggesting that mice with subcutaneous tumors do not have a detectable OVA-specific immune response

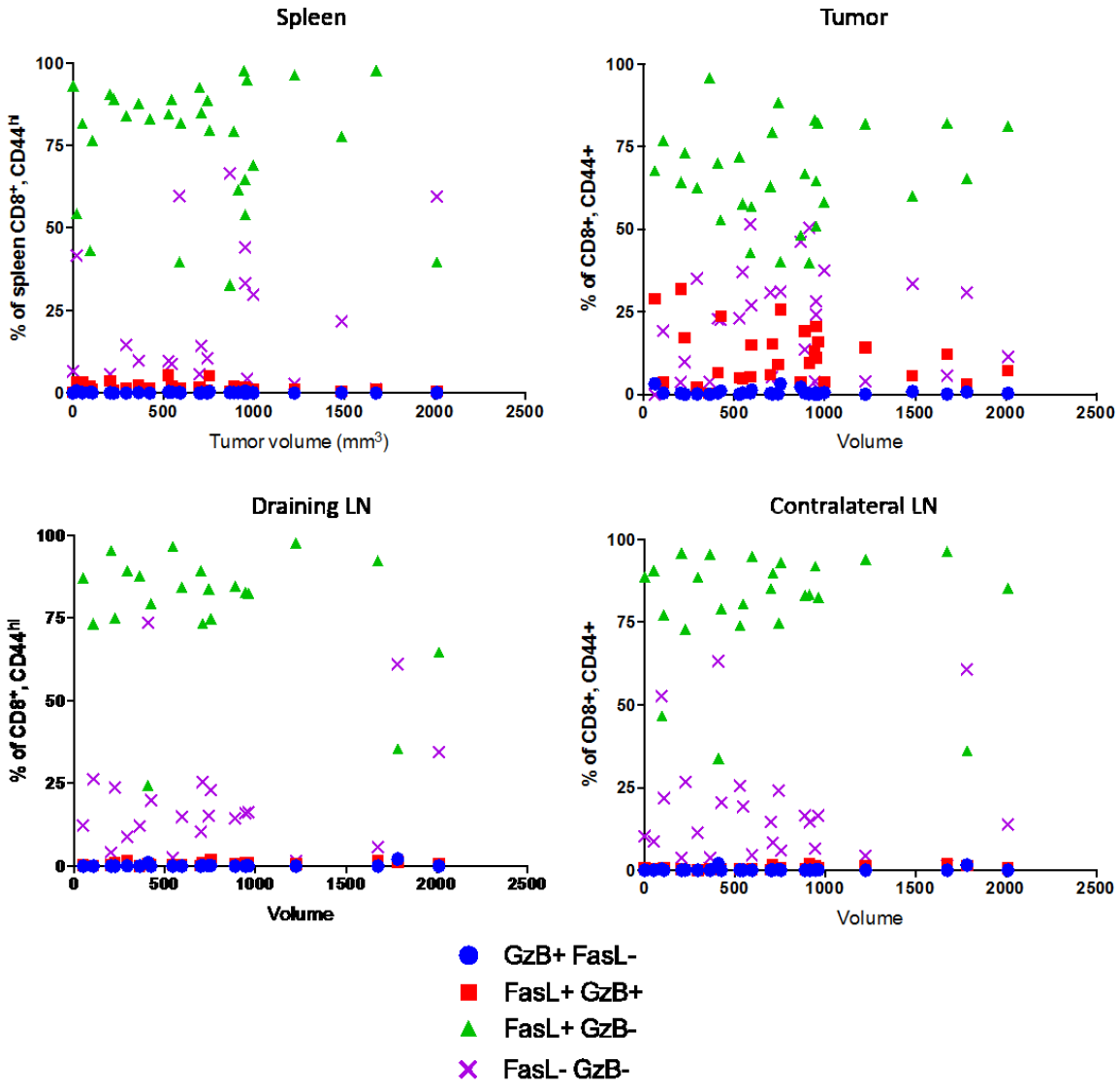


Figure 4-11 There is no correlation between subcutaneous tumor burden and proportion of activated CD8$^+$ cells expressing intracellular FasL/Granzyme.
Distribution of intracellular FasL/Granzyme expressing cells in CD44^{hi} CD8$^+$ T cells from mice injected subcutaneously with EG.7 tumor cells. Linear regressions of volume vs. proportion of activated CD8$^+$ T cells for all tissues and combinations of effector proteins yielded no significant results. Data from 31 mice over five independent experiments.

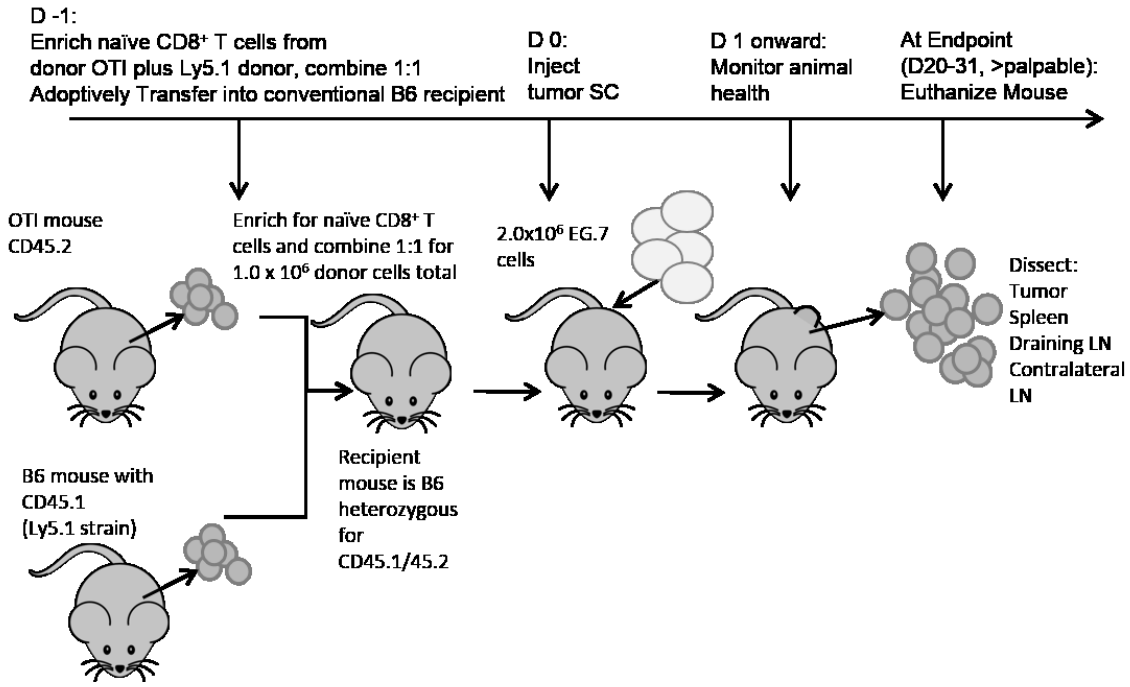


Figure 4-12. Schematic of injection timeline for mice bearing subcutaneous tumors following adoptive transfer of naïve OTI plus naïve B6 cells.

(Fig 4-7). It is not clear if the activated CD8+ T cells within the tumor are antigen-specific or if they are just previously activated cells that migrate to the inflamed tumor like they would into an infected lung or gut, with no reactivity to the tumor. I also cannot determine whether FasL and GzmB coexpression by activated CD8+ TIL is in response to a specific stimulus. Building on my subcutaneous tumor injection method, I added in a pre-tumor adoptive transfer that contains equal numbers of naïve, CD8+ T cells from OT-I (OVA-specific) and conventional C57/B6 mice (polyclonal) (referred to henceforth as B6 donor). Donor cells were injected intravenously 24 hours prior to tumor injection (Fig 4-12). I used different combinations of CD45.1/CD45.2 alleles to distinguish the B6 donor, OT-I donor, and recipient cells. Due to the short time between adoptive transfer and tumor injection, activation of naïve donor cells will occur after adoptive transfer and would most likely be tumor specific.

After tumor establishment, donor cells of both types can be found in the tissues examined (Fig 4-13A). There is no significant difference in the proportion of CD8+ cells in the spleen that are of OT-I or B6 donor origin (Fig 4-13Bi), but a greater proportion of the OT-I donor cells in the spleen are CD44+ than their B6 donor counterparts (Fig 4-13Bii). Even though equal proportions of OT-I and B6 donor cells can end up in the spleen, activation of the splenic donor cells is tumor antigen (OVA)-specific. There are proportionately more activated OT-I donor cells in all sites relative to activated B6 donor cells, with an average of over 50% of donor cells being of OT-I origin in all tissues (Fig 4-13C). However, the data is most consistent, and dramatic, in the tumor. A significantly higher proportion of donor-origin cells in the tumor are OT-I, compared to ratios found in the spleen (Fig 4-13C). Furthermore, there are significantly more OT-I donor cells per

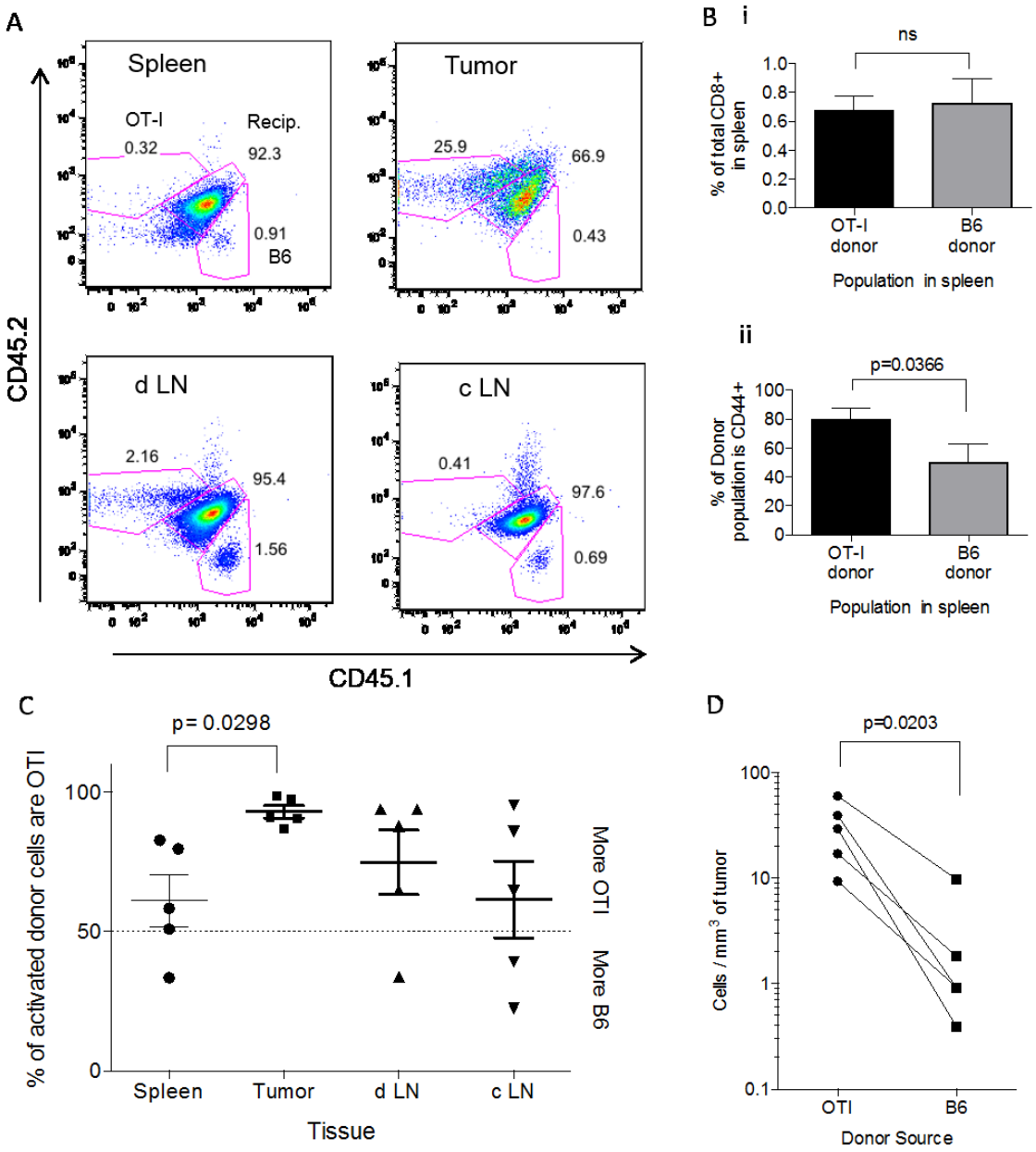


Figure 4-13. Significantly more OT-I donor cells than B6 donor cells are present in the tumor.

Mice were adoptively transferred with a mix of naive OT-I and B6 donor cells, then injected 24 hours later with EG.7 tumor cells subcutaneously. Tumor and other tissues were dissected after 20-30 days post-injection and stained for CD45.1, CD45.2, CD8, CD44. A. Representative plots showing donor/recipient populations. B. Recruitment to and activation of donor cells in spleen: In spleen samples, CD8+ cells were gated as OT-I donor or B6 donor and then assessed (i) proportion of entire CD8+ splenocytes, and (ii) proportion of that donor population that is CD44+. C. Total CD8+ CD44+ OT-I donor cells, divided by number of B6 donor plus OT-I donor cells. D. Cells/mm^3 in SC tumor. All error bars are SEM. All P values from paired student's t-test.

mm^3 of tumor mass than there are of B6 donor cells (Fig 4-13D).

If recently activated cells were recruited to or proliferating in tumors independent of tumor specificity (either OVA or polyclonal tumor antigens), I would have expected to see similar proportions of activated CD8+ cells from both donor types in the tumor, which I did not observe. There is an OVA-specific response in the tumor, and OVA tumor antigen-specific activated CD8+ T cells are able to recruit to or persist in that environment, but they are still dwarfed by the polyclonal cells of the native response. Regardless, these data show that there is an antigen-specific population among newly activated T cells present in the subcutaneous EG.7 subcutaneous tumor.

Vα2+/Vβ5+ CD8+ T cells in solid tumor TIL are only weakly associated with GzmB expression

I next wanted to examine the impact of this antigen specificity and recent tumor-specific activation on intracellular FasL/GzmB expression by the CTL. Are the GzmB+ FasL+ coexpressors or the FasL- GzmB- CD8+ T cells in SC TIL due to recent antigen-specific activation? I compared not only donor OT-I cells and donor polyclonal B6 cells, but also recipient CD8+ T cell populations. In addition to the recipient cells of unknown specificity, there is a very small population of CD8+ cells detected by Vα2+/Vβ5+ TCR staining. While the proportion of Vα2+/Vβ5+native cells stayed the same as in earlier SC experiments, I acquired larger flow cytometry samples to provide sufficient numbers of these cells for comparative analysis. I compared activated populations of OT-I donor, B6 donor, Vα2+/Vβ5+ CD8+ cells of recipient origin, and all other CD8+ T cells (polyclonal recipient).

In the spleen, where there was closest to an equal proportions of activated OT-I donor and B6 donor cells (Fig 4-13C), all four donor and recipient subpopulations have

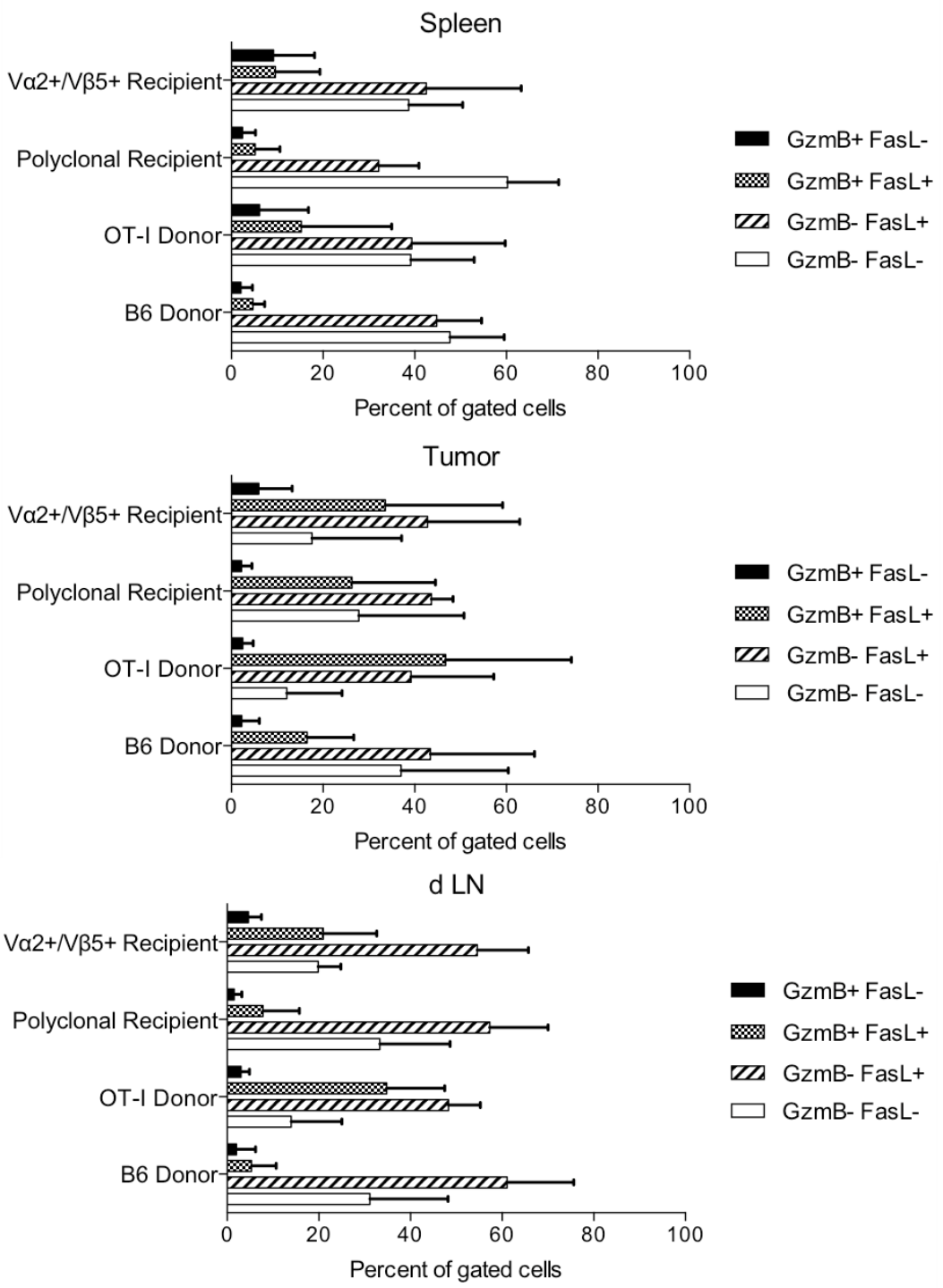


Figure 4-14. OT-I TCR+ donor and Vα2+/Vβ5+ recipient cells have elevated numbers of GzmB+ FasL+ cells in all sites compared to polyclonal donor and recipient cells.
Mice were adoptively transferred with a mix of naive OT-I and B6 donor cells, then injected 24 hours later with EG.7 tumor cells subcutaneously. Tumor and other tissues were dissected after 20-30 days. OT-I and B6 donor cells, and Vα2+/Vβ5+ and polylconal recipient cells were gated and then assessed for intracellular storage of FasL and GzmB.

similar division of FasL and GzmB expressing populations (Fig 4-14), with a few exceptions of note. First, the polyclonal recipient population has a higher proportion of GzmB- FasL- cells in these experiments compared to the earlier experiments (Fig 4-10, Fig 4-11). It is also worth noting that this GzmB- FasL- population is highest in splenic polyclonal recipients than in Vα2+/Vβ5+ recipients in the spleen or either donor group in the spleen (Fig 4-14). In addition, slightly more, but not significantly so, of the OT-I donor and the Vα2+/Vβ5+ recipient populations are GzmB+ in the spleen than their polyclonal counterparts.

In the tumor itself, OT-I or OT-I like Vα2+/Vβ5+ (donor or recipient) populations have increased GzmB+ FasL+ coexpressing cells compared to polyclonal populations, but not significantly so (Fig 4-14). In addition, there is not much difference in general between OT-I donor and Vα2+/Vβ5+ recipient cells. There was very little difference between the polyclonal B6 donor cells and the polyclonal recipient cells with respect to FasL and GzmB expression (Fig 4-14). In the tumor, it seems like there is more of a difference between populations containing OVA-specific cells and populations with unknown specificity, although not statistically significant, than between recently activated donors and native recipient responders.

All four donor/recipient groups in the tumor have FasL- GzmB- populations. Coming back to the question at the beginning of the description of this model, are CTL lacking both FasL and GzmB recently activated? In regards to antigen-specific populations, I would say no. In addition, the increase in FasL+ GzmB+ CD8+ T cells seen in tumors cannot be solely attributed to influx of a population recently activated cells, or to only tumor antigen-specific cells. a

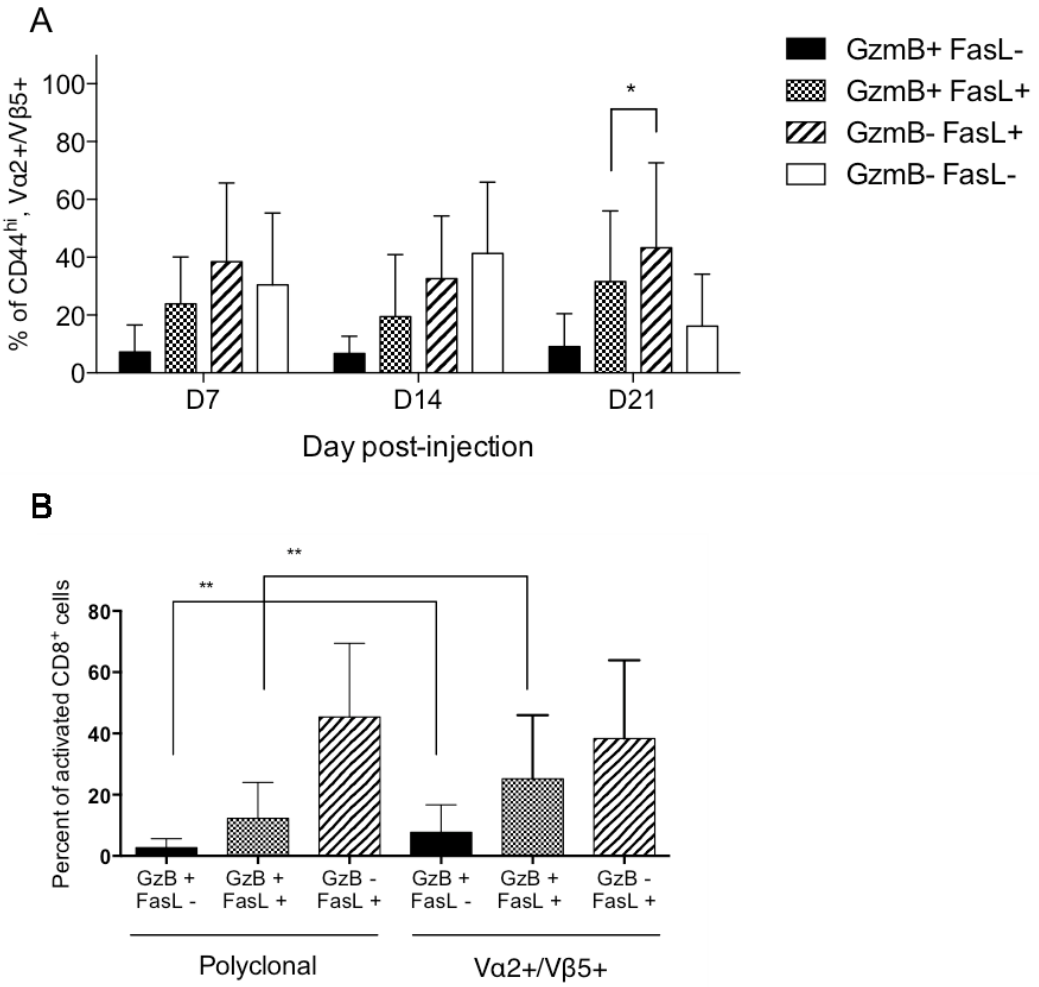


Figure 4-15. Cells with Vα2+/Vβ5+ TCR in mice bearing intraperitoneal tumors have a greater proportion of cells expressing GzmB compared to the entire CD44+ CD8+ peritoneal population.

A. Mice received adoptive transfers of naïve OT-I T cells prior to IP tumor injection, as described in figure 4-1. Cells were stained for Vα2+/Vβ5+ TCR as well as for other surface and intracellular antigens, then characterized for intracellular FasL and GzmB expression. A. Expression of FasL and GzmB in OT-I-TCR+ T cells in mice bearing IP tumors. B. Difference between Vα2+/Vβ5+ and polyclonal responders in FasL and GzmB expression in mice bearing IP tumors, pooled across all days. P values from paired two-way student's t-test. Error bars are SEM. Data from 10 mice over 4 independent experiments.

Vα2+/Vβ5+ TCR expression in peritoneal CD8+ T cells is associated with GzmB

In mice responding to intraperitoneal tumors, recipient and donor mice (Fig 4-1) were not of different CD45 alleles, so no distinctions of that kind could be made to distinguish activated OT-I+ donors and other Vα2+/Vβ5+ cells. However, there are comparisons to make between potentially OVA-specific and polyclonal activated CD8+ T cells in the peritoneal exudate. Vα2+/Vβ5+ cells have similar trends in FasL and GzmB expression (Fig 4-15A) to their polyclonal counterparts (Fig 4-9B). Activated CTL express FasL, and a minority of cells expresses GzmB, mostly in combination with FasL. However, if the Vα2+/Vβ5+ and polyclonal cells are pooled across all three time points, there is a significant increase in the proportion of Vα2+/Vβ5+ cells expressing GzmB alone compared to polyclonal cells. There is a similarly significant increase in FasL+ GzmB+ cells in Vα2+/Vβ5+ cells compared to the polyclonal cells. However, there is no significant difference between the populations of cells expressing FasL alone. These results suggest that potentially tumor-specific CD8+ T cells in the peritoneum are responding to the EG.7 cells and expressing more GzmB compared to their polyclonal counterparts.

Activated CD8$^+$ T cells in both sites express surface FasL without restimulation

Intracellular FasL indicates the potential of a cell to kill by FasL, but not its history. As my colleague Ana Clementin demonstrated with *in vitro* stimulated CTL clones, FasL remains on the cell surface temporarily after TCR-mediated stimulation, and then is either cleaved or endocytosed. [192] I found in Chapter 3 that there is a modest IL-2 induced increase in the amount of FasL that can transit to the cell surface following restimulation. This means that CTL may be flexible with respect to quantity of FasL

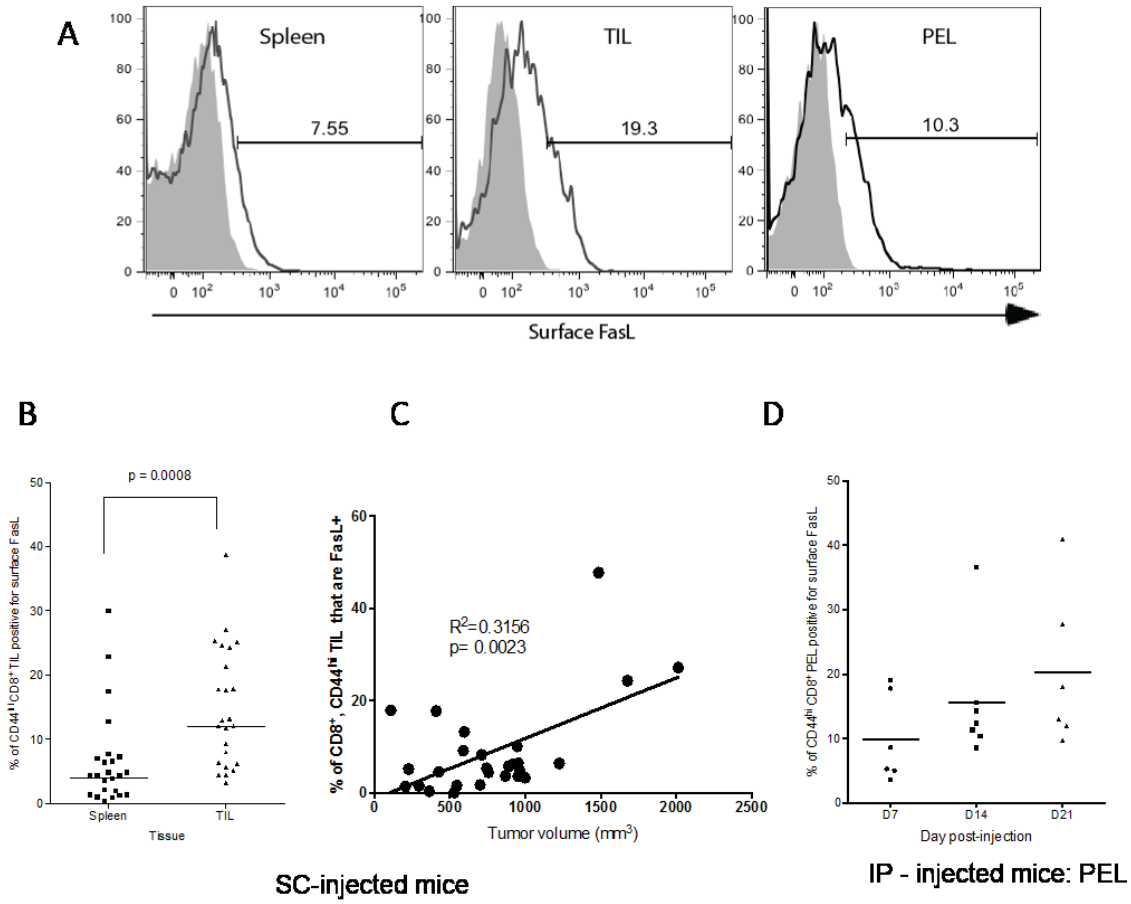


Figure 4-16. Activated CD8$^+$ T cells from mice bearing EG.7 tumors or cells retain surface expression of FasL in absence of *in vitro* restimulation.

A. Representative staining of CD8$^+$ CD44$^+$ T cells from mice bearing tumors. Cells were neither stimulated nor permeabilized prior to FasL staining. Solid histogram: FMO control, Line: Stained sample. B. Comparison of cells positive for surface FasL from spleen and tumor in SC-tumor bearing mice. p values obtained by paired student's t-test. C. Linear regression of tumor volume versus percent of activated CD8+ T cells positive for cell surface FasL. D. Activated CD8+ T cells positive for surface FasL over three weeks from peritoneum of mice bearing intraperitoneal tumors. No significant differences detected between groups in data from panel D. Data from at least six mice over three independent experiments.

translocation in face of the same stimulus, and I wanted to see whether the tumor environment could increase the amount of FasL on the surface of activated CD8+ T cells. To examine this, I stained for cell surface FasL on CD44+ CD8+ cells from the peritoneum, or tumor of mice injected with EG.7 cells.

Cells from tumor-bearing mice clearly have detectable surface FasL (Fig 4-16A). In mice with subcutaneous tumors, a significantly greater proportion of activated CTL from the TIL are positive for surface FasL, compared to their counterparts in the spleen (Fig 4-16B). There is a positive correlation between tumor volume and the proportion of $CD8^+$ CD44+ that are positive for surface FasL (Figure 4-16C). Even though the expression of intracellular FasL of CTL does not change in relation to tumor burden, FasL on the cell surface does. I saw parallels in mice with IP tumors: there is a possible trend of more surface FasL-positive cells at later time points following tumor injection (Figure 4-9D), but the change over time is not statistically significant. However, TIL in SC and peritoneal tumors are clearly stimulated as a result of their environment to express FasL protein on their surface.

The question remains of whether the surface FasL is triggered by tumor antigen. This cannot be directly tested in a tumor environment, however I did assess whether surface FasL is associated with Vα2+/Vβ5+ cells. When I examined CD44+ CD8+ T cells from the tumor stained for surface FasL, surface FasL positive cells in the tumor have no significant difference in frequency of cells that are Vα2+/Vβ5+ than the general CD44+ CD8+ cell population from the tumor (Fig 4-17). There is a significant increase in the proportion of cell-surface-FasL positive CD44+ CD8+ T cells from the spleen that are Vα2+/Vβ5+. Recall that there are significantly more CTL bearing cell-surface FasL in

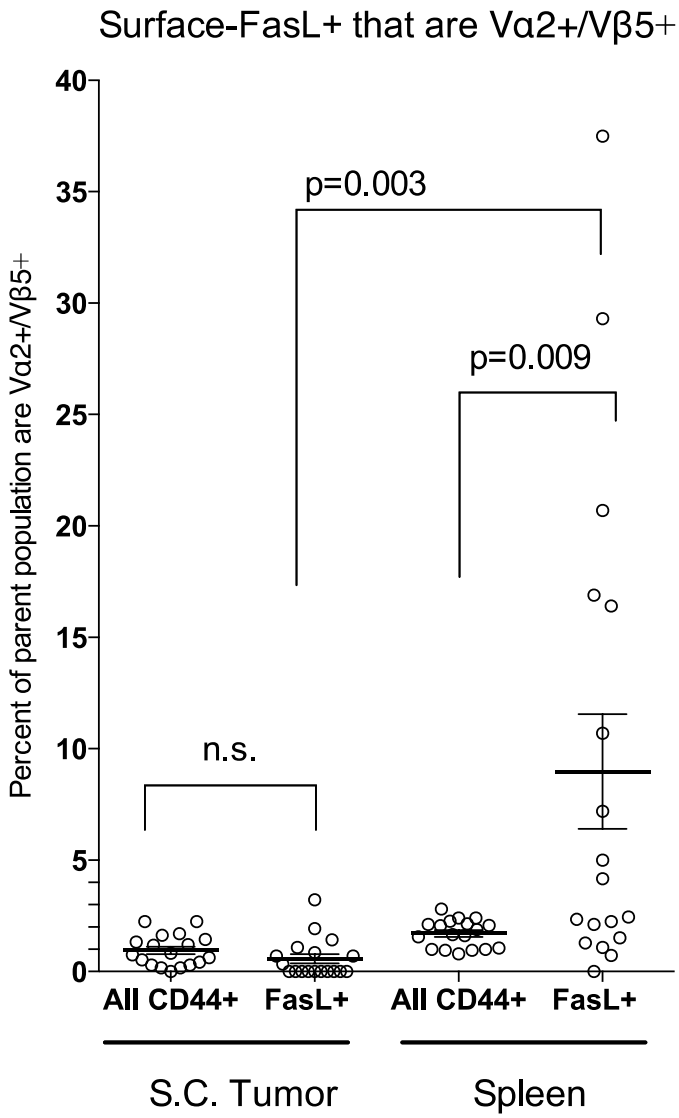


Figure 4-17. For CD8$^+$ T cells from mice bearing SC EG.7 tumors, surface FasL$^+$ population is polyclonal, while in spleen, surface FasL$^+$ population bears more Vα2+/Vβ5+ CD8+ T cells.

CD8$^+$ CD44$^+$ T cells from mice bearing tumors were neither stimulated nor permeabilized prior to FasL staining, and also assessed for being Vα2+/Vβ5+. Data from 18 mice over five independent experiments. P values obtained by paired student's t-test. Error bars represent SEM. "Parent population" is indicated below column ("All CD44+", etc).

the tumor compared to the spleen, but the Vα2+/Vβ5+ CD8+ T cells with cell-surface FasL are not expanding, or are dying off.

Discussion

In this chapter, I have shown that in contrast to *in vitro* newly activated CD8+ T cells, which mostly are GzmB+, I found that *in vivo* CD8+ T cells from mice injected with EG.7 tumor cells almost exclusively express intracellular FasL, either alone or in combination with GzmB. The tumor antigen specific CD8+T cell population in the peritoneum has elevated frequency of GzmB expression, suggesting that antigen-specific stimulation can increase GzmB expression. Furthermore, cell-surface FasL is elevated in on activated CD8+ T cells in the tumor, independent of antigen specificity.

Before even characterizing the tumor-infiltrating cells, I showed that activatedCD8+ T cells in healthy mice can express intracellular FasL protein. In both the spleen and tumor of mice not injected with tumors, a large proportion of CD44+ CD8+ cells express FasL, but almost none of the cells express GzmB. Although these mice are not fighting tumors, they may be responding to other pathogens that they encounter in conventional, non germ-free, housing. It may also be possible that most activated CD8+ T cells express FasL protein while they patrol the spleen and periphery.

It is was surprising to find such dramatic differences between my *in vitro* activated cells, which are GzmB dominant, and my CD8+ T cells isolated from the tumor, which are FasL dominant. Clearly CD8+ T cells have a wide potential range of FasL and GzmB intracellular expression, as both experiments use cells of C57/B6 mouse origin. However, activation duration and signal strength are very different between the cells in a tumor and the cells in culture. As the data from cultured cells activated *in vitro*

with the lowest stimulatory conditions (3ug/ml anti-CD3/CD28, 5U/ml IL-2) in Chapter 3 suggest that at lower stimulus thresholds FasL is still retained in cells while GzmB is not, this would fit in with the generally low-stimulus environment usually seen *in vivo*. FasL protein expression is retained by CD8+ T cells *in vivo* even under conditions that are not favorable for GzmB expression. It would be interesting to see how little TCR stimulation is required for maintenance of FasL expression in the CD8+ population. Is tonic, nonspecific stimulation sufficient, or is at least low antigen specific stimulus in the context of an infection required?

Since emerging neoantigens and selection against tumors presenting T cell antigens is a challenge to an effective T cell response to tumors [120, 376], I wanted to examine whether CD8+ T cells that are tumor-specific are at all different with respect to FasL or GzmB expression. While the OVA antigen-specific response was quite small, models that others have used which did generate a large, antigen-specific response were different than mine. CD8+ T cells were injected after tumor establishment [74, 377], or co-injected with antigen-specific CD4+ T cells [74], or at a higher number [74]. One group has elicited an endogenous response to IP injected EG.7 cells[378], but no one has used the EG.7 tumor subcutaneously with endogenous T cell repertoire as I used in most of my SC experiments. However, my mixed-adoptive transfer experiment showed that there can be tumor antigen-specific activation of CD8+ T cells in response to a SC EG.7 tumor. When these tumor-specific activated CD8+ T cells are examined for FasL/GzmB expression, there are only minor, not statistically significant increases in GzmB expression compared to the entire polyclonal CD8+ T cell populations in the same tissue.

I did, however, show that Vα2+/Vβ5+ CD8+ T cells, which may include some OT-I like OVA-specific cells, in the peritoneal tumor environment have elevated proportions of GzmB+ FasL- and GzmB+ FasL+ compared to the general polyclonal population. Elevation of GzmB expression may be part of the antigen-specific response. If we presume that these intracellular stores are indicative of exactly what the CD8+ TIL are about to degranulate or translocate to the cell surface, this aligns with what Shanker and colleagues found in a model where transgenic CTL responded to renal carcinoma cells: high density tumor antigen elicited degranulation-mediated killing, while low-density antigen elicited FasL-mediated killing [345].

I found that at D21 there are elevated Vα2+/Vβ5+ CD8+ T cells in the IP model, but there is not a significant difference in the quality of the FasL/GzmB response of antigen-specific or polyclonal responses between D21 and D7 or D14 (Figs 4-15 and 4-9, respectively). In other words, the detectable tumor antigen-specific response in the peritoneum is either constant over the first three weeks post-injection, or below the limit of detection. It may also be that the three-week period I examined is too early. Mowat and colleagues examined EG.7-reactive cytotoxicity of the PEL from mice with IP tumors, and found that mice reach peak killing capacity at 4 weeks post-injection, with killing at 1 and 2 weeks post-injection being no better than controls [378]. However, while they did not do any tests to quantify OVA-specific TCR-bearing cells (by tetramer or TCR staining), the observed cytolytic response was not to non-OVA tumor antigens, as injection with parental (non-OVA) EL4 cells does not elicit the same responses as EG.7 4 weeks post-injection [378]. Perhaps the OVA-specific CTL population in my mice would be different with respect to FasL and GzmB expression at times beyond D21.

I showed that CD44+ CD8+ T cells in the solid tumor and in the peritoneum of EG.7-injected mice have FasL on their cell surface without needing *in vitro* restimulation. Experiments carried out Jinshu He show that there is minimal FasL on the surface of CD8+ T cells in the spleen or peritoneum of mice responding to an allogeneic priming, without *ex vivo* restimulation [190]. However, I believe that these findings can be reconciled by the different nature of the response: In past experiments, there were not detectable tumor cells in the PEL at time of sacrifice 10-12 d post-injection [190]. However, there was still an active response to the still-present tumor in my mice at the time of sacrifice, potentially providing constant stimulus for CD8+ TIL. Furthermore, these two experiments may actually be part of the same system wherein FasL is transiently elevated, but upon successful tumor cell clearance the constant FasL on the cell surface is reduced.

GzmB+ cells are increased in a manner that could be OVA-specific in CD8+ PEL, and marginally so in SC TIL, but there is no such relationship between antigen specificity of SC TIL and surface FasL presentation that I was able to detect. The positive correlation between tumor volume and CD8+ cell-surface FasL expression is not driven in an OVA-specific manner. This reinforces that FasL and GzmB may not have redundant biological roles or be turned on by the same stimuli *in vivo*.

In a colorectal cancer study in humans, gene expression of granzymes and perforin is significantly higher in tissues that have high memory T cell infiltration, and correlated with better outcomes [379]. I did not find a correlation, whether positive or negative, between GzmB protein in CD8+ T cells and subcutaneous tumor volume. It may be that there are elevated transcripts seen in the above study, but constant protein

storage as seen over increasing tumor volume as shown in this thesis. In this case, the CTL would be poised to synthesize GzmB quickly after target cell encounter. Another possible reason why I did not find any correlation between tumor volume and %GzmB+ FasL+ or % GzmB+ FasL- cells is that I only examined mice that had palpable tumors that grew and had sufficient cells for FACS analysis afterwards. It is unknown whether the few mice that I injected with EG.7 cells but did not present palpable tumors had different FasL/GzmB compared to mice bearing dissectible tumors, as there was no "TIL" population do examine at that point.

There is some correlation in the field of research between poor outcomes and soluble FasL levels, though that varies between serum soluble FasL and FasL in the tumor itself [380]. How much of the intracellular FasL or cell surface FasL that I detected is destined to be cleaved off and floating in the ECM? It would be interesting to see if the surface FasL+ cells are adjacent to apoptosing cells, knowing that surface FasL+ increases with tumor size. Furthermore, I showed that there is no increase in Vα2+/Vβ5+ staining on CD8+ cells with elevated surface FasL, and likely no OVA-specificity. What is the function and specificity of the CD8+ T cells with elevated cell surface FasL? From the data reviewed in this chapter, however, the elevation of surface FasL in correspondence with tumor size has potential as an indicator of tumor progression.

In general, the conditions in the tumor environment, whether in the peritoneum or in an SC tumor, are sufficient to retain FasL expression on most cells and elicit GzmB expression in some cells. The relative expression of these mechanisms is not influenced by tumor burden or over the period of time I examined, suggesting that the presence in the tumor is sufficient to allow enhancement of GzmB expression, and further enhance it

if cells are specific for a defined tumor antigen. Conversely, the lack of change with volume or time also suggests that presence in the tumor is not sufficient to change intracellular FasL stores by CD8+ TIL compared to CD8+ T cells in secondary lymphoid tissue, but that FasL on CD8+ TIL is elevated on the cell surface in tumors. FasL and GzmB protein expression by CD8+T are differentially affected by CD8+ infiltration into tumor-bearing sites, and even differ depending on the tissue in which the tumor is.

Chapter 5: Examination of FasL and GzmB expression in differentiated populations of tumor-infiltrating CD8+ T cells

Introduction

At any time in a healthy individual, there is a mix of effector and memory cells responding to immune threats. While most indications of effector ability *in vivo* are associated with GzmB expression, many have suggested that the best outcomes for adoptive cell therapy against tumors are actually with Tscm and Tcm [114, 116], which have been shown by others that they can have a low level of FasL expression [116, 128]. Also, it has been shown in a melanoma model that previously established memory CD8+ T cells can be resistant to tumor-mediated suppression [381], so they may have an active cytolytic role in the response to tumors, in spite of work showing that antiviral memory cells can degranulate but not kill target cells. I have previously shown in this thesis that memory phenotype cells *in vitro* can express FasL and GzmB. Can both memory and effector cells express FasL and GzmB in response to EG.7 tumors?

PD-1 expression is upregulated on activated cells and its ligation can dampen CTL proliferation, TCR sensitivity, and IL-2 production [238, 382]. This is a way to counter autoimmunity, but in overstimulated CD8+ populations, can lead to exhaustion [256, 383]. While one group found that PD-1 on CD8+ T cells can identify tumor-specific cells in a polyclonal population [384], these T cells were not confirmed to be effectively cytolytic against tumor cells. In addition, the Dai group identified a set of PD-1 positive "memory" cells that were regulatory CD8+ T cells [385]. As Chen and colleagues showed that FasL may be present in regulatory CD8+ T cells [386], do FasL-GzmB- CD44+ CD8+ T cells from tumors express PD-1?

The overarching purpose of this chapter is to determine whether there is a differentiated CD8+ phenotype associated with FasL or GzmB expression in tumor-infiltrating lymphocytes. In this chapter, I show that both memory and effector cells are capable of expressing FasL and GzmB, but that effector phenotype cells not only express more intracellular FasL and GzmB, but also are more likely to express both effector proteins at the same time. Furthermore, I show that FasL and GzmB coexpression is associated with elevated expression of coinhibitory receptor PD-1.

Results

CD8+ Memory and Effector phenotype cells are present in mice with tumors

Using the in vitro tumor models described in Chapter 4, I examined the cells responding to IP or SC tumors for effector and memory phenotypes. Memory phenotype cells in this thesis are considered CD44hi CD127hi CD62Lhi, and effector phenotype cells being CD44hi CD127lo CD62Llo, although this second population could be a mix of effector memory cells and short-lived effector cells. In general, memory phenotype cells are more common than effector phenotype cells in both PEL from mice with IP tumors (Fig 5-1A) and in the spleen of mice bearing SC tumors (Fig 5-1B). However, an effector phenotype is more abundant in the tumor-infiltrating CD8+ population of mice bearing SC tumors (Fig 5-1B). In addition, the proportion of effector cells in the TIL population is not correlated with tumor volume (Fig 5-1C), so data from mice with varying tumor volume is pooled into one group for SC tumor data.

Effector phenotype cells express more FasL and GzmB than memory cells

I wanted to examine whether there is a difference between effector and memory phenotype cells with respect to FasL and GzmB expression, so cells were gated as

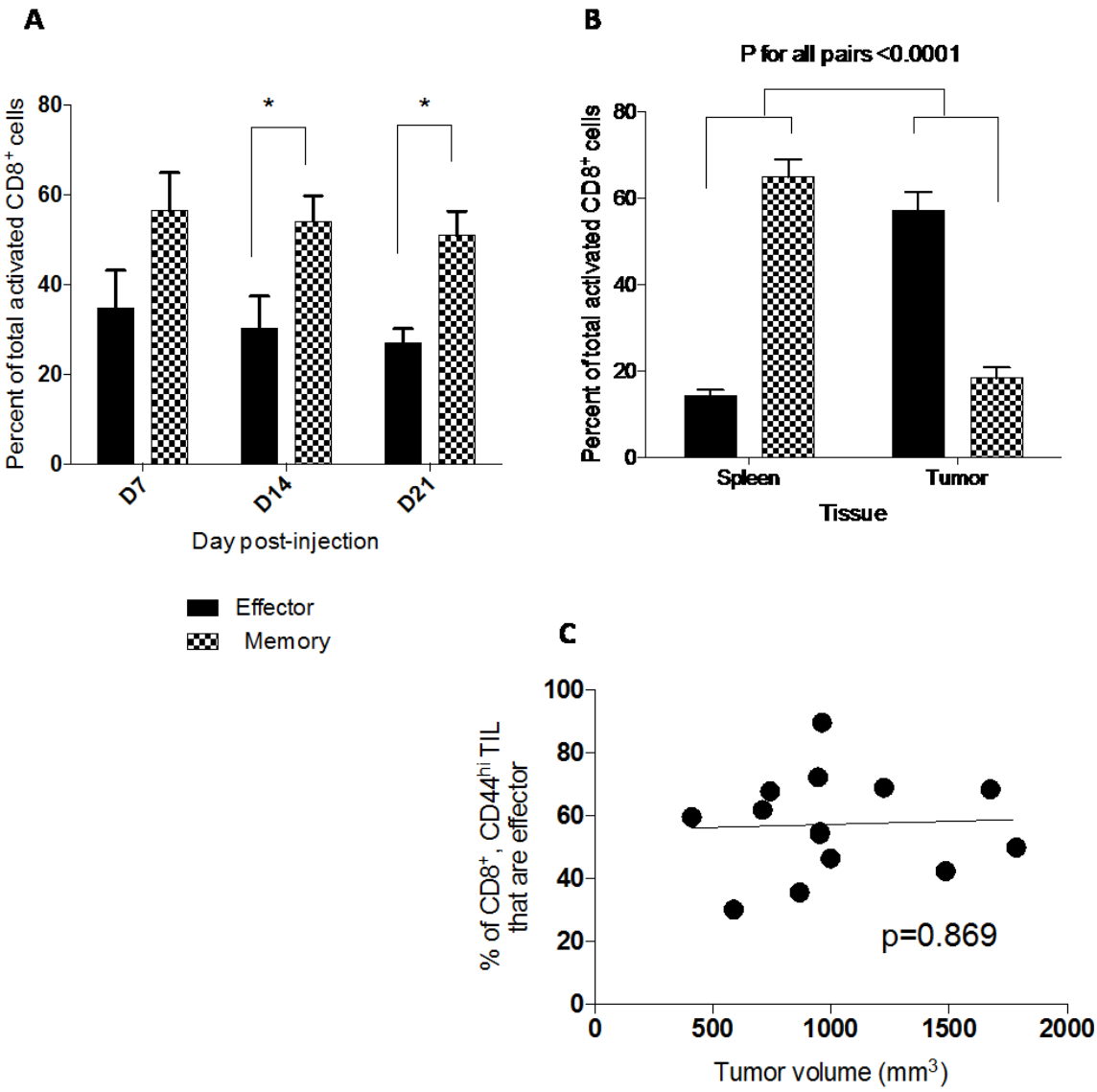


Figure 5-1. Expression of Effector and Memory phenotypes in activated CD8$^+$ T cells in tumor.

Cells recovered by peritoneal lavage from mice bearing EG.7 intraperitoneal tumors, or from spleen and tumor of mice bearing subcutaneous EG.7 tumors, were stained for CD8, CD44, CD62L, CD127, and intracellular FasL and GzmB. Effector phenotype cells were gated as CD8+ CD44^{hi} CD62L^{lo} CD127$^{lo;}$ Memory phenotype cells were gated as CD8+ CD44^{hi} CD62L^{hi} CD127^{hi}

A. Peritoneal activated CD8+ T cells. * = P<0.05 B. PEL and Spleen from mice bearing SC tumors, all days pooled. P values from two-way, paired student's t-test.. C. Distribution of percent of activated CD8+ T cells that are effector phenotype. Line is linear regression. No significant relationship. Each point represents one mouse, with six mice or more per time point from at least five experiments. Error bars are SEM.

effector or memory then the ratio of MFI to the staining control was quantified for the two populations, as a measure of the level of protein expression. When examined over time, the only significant difference between effector and memory cells is that at D14 and D21, effector cells have significantly higher surface FasL than memory phenotype cells (Fig 5-2A). Since the proportions of effector and memory do not significantly change over time in PEL (Fig 5-1A), I have grouped them together for most remaining analyses in this chapter. When pooled over all time points, effector cells have significantly more intracellular FasL and GzmB than memory phenotype cells (Fig 5-1B). Not surprisingly, bulk effector cells also have significantly more FasL on the cell surface than memory phenotype cells. Effector cells in the peritoneum appear to be more "armed" than their memory counterparts with respect to FasL and GzmB expression.

When I examined TIL from mice bearing subcutaneous tumors, I saw a similar trend. Effector phenotype cells have significantly more intracellular FasL and GzmB than their memory counterparts. Significantly more FasL is on the surface of effector phenotype tumor infiltrating CD8+ T cells than memory phenotype cells (Fig 5-3). In spite of the solid subcutaneous and fluid intraperitoneal tumor environments harboring different proportions of effector and memory phenotype cells (Fig 5-1), it seems in both tumor sites the effector cells are both stocked up with more cytolytic effector proteins and are transporting FasL to the cell surface.

Memory CD8+ T cells can coexpress FasL and GzmB *in vivo*

While Tmem have less intracellular FasL and GzmB than Teff, I sought to confirm whether these CD8+ differentiated populations express both effector proteins. Are the cells coexpressing intracellular FasL plus GzmB versus intracellular FasL alone,

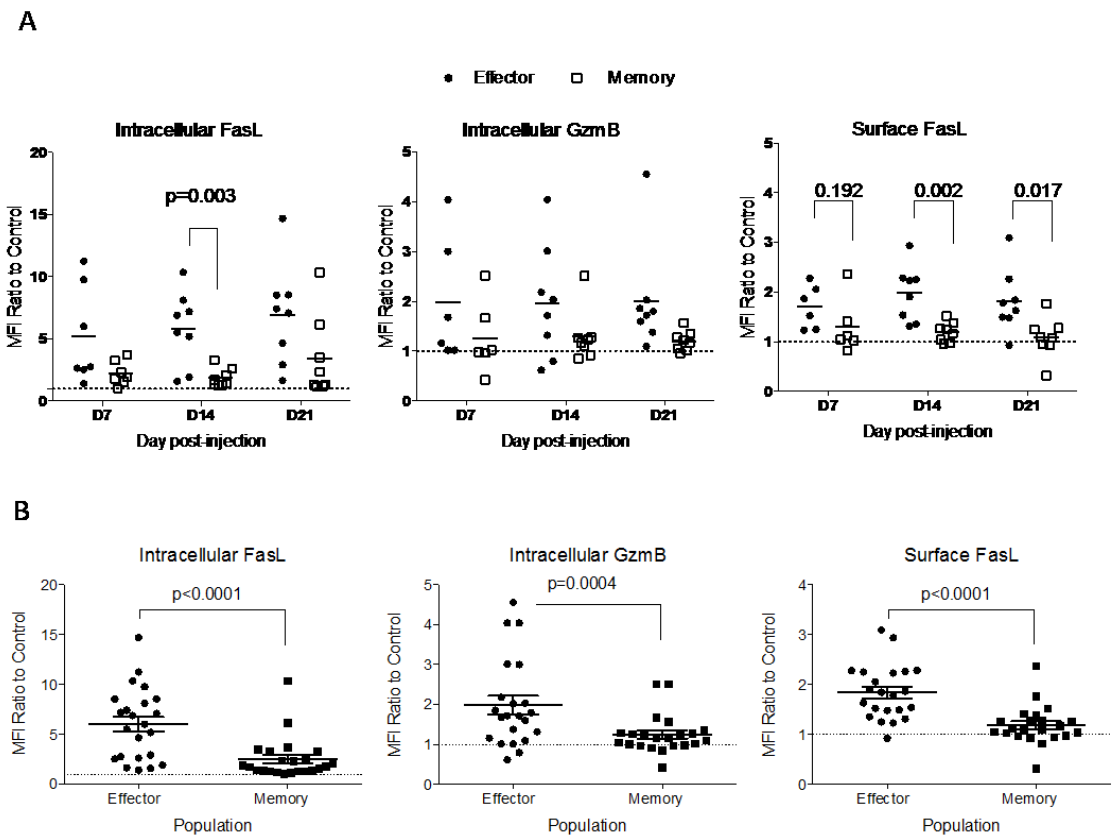


Figure 5-2. Peritoneal effector phenotype cells from mice bearing IP EG.7 tumors have overall significantly more FasL, GzmB, and surface FasL expression than peritoneal memory phenotype populations.

Cells at days 7, 14, and 21 post-injection IP with EG.7 were harvested by peritoneal lavage and stained for CD8, CD44, CD62L, CD127, FasL, and GzmB. A. Effector molecule population staining in populations, divided by day post-injection. B. Effector mechanisms in pooled effector and memory phenotype cells. P values from paired, 2-way student's t-test. Each point represents one mouse, with six mice or more per time point from at least five experiments. Error bars are SEM. Dotted line indicates ratio of 1.0, at which point mean fluorescent intensity for effector mechanism is the same as for staining control.

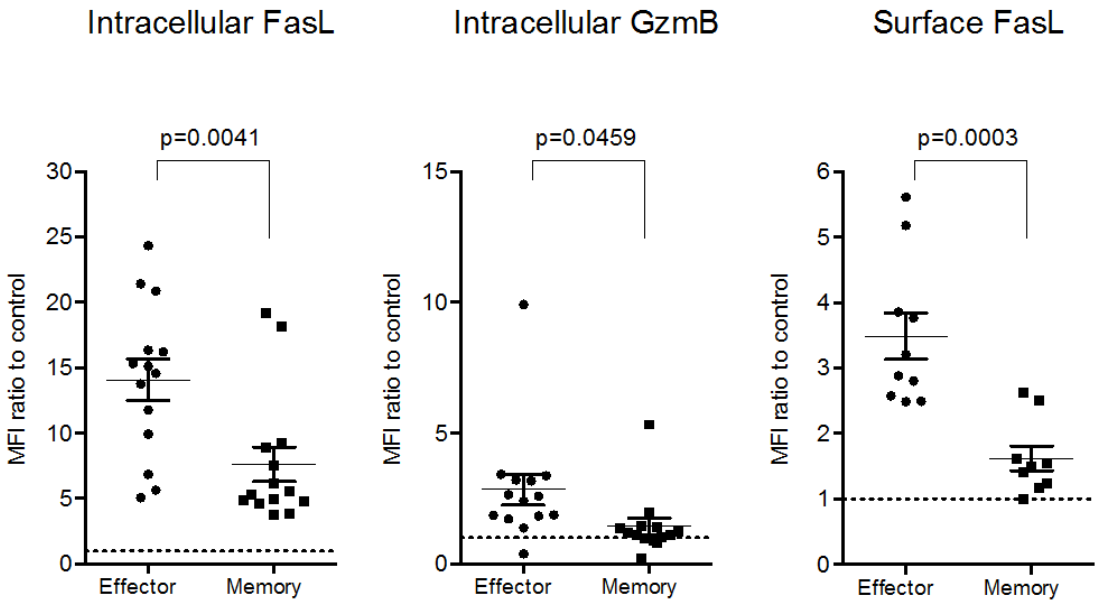


Figure 5-3. Solid tumor infiltrating effector phenotype population has significantly more FasL, GzmB, and surface FasL expression than the TIL memory phenotype population. Cells from tumor and spleen of mice bearing SC tumors from all days were stained for CD8, CD44, CD62L, CD127, FasL, and GzmB. MFI ratio control indicates that MFI of fully-stained cells was normalized by division my the MFI of FMO control cells with the same gating strategy. P values from paired two-way T test. Error bars are SEM.

or even neither protein, are they more likely to be of an effector or memory phenotype? Past researchers have found that expression of GzmB to be an indicator of good tumor clearance/prognosis in cancer patients [387, 388], however it is not known whether this is due to expression by effector or memory cells.

Unsurprisingly, I found that in activated CD8+ TIL from mice bearing subcutaneous tumors, effector is always the dominant phenotype (Fig 5-4A, left columns), When cells are grouped as to whether they express FasL plus GzmB, FasL alone, or neither, the effector cells are always more abundant than memory cells (Fig 5-4A). There were too few GzmB+ FasL- cells from the subcutaneous TIL population to further sub-analyze for effector or memory phenotype in a statistically reliable manner. Cells coexpressing intracellular GzmB and FasL have the largest increase in the proportion of effector cells, and significantly reduced memory cells, compared to all activated CD8+ TIL (Fig 5-4). Combining this knowledge with the information from Figure 5-3, it appears that while overall both memory and effector CD8+ TIL are capable of expressing FasL protein, effector phenotype cells express more of it, and are more likely to also be coexpressing GzmB. In addition, in splenic CD8+ T cells of the same mice, FasL+ GzmB- and FasL- GzmB- cells preserve the same balance of Effector and memory phenotypes as the general splenic CD44+ population, but GzmB+ FasL+ cells have a significantly increased proportion of effector cells (Fig 5-3B). That said, splenic CD8+ T cells in tumor-bearing mice have a memory, GzmB+ FasL+ population as well. Memory cells do not have to be in the tumor to have intracellular GzmB expression.

In the peritoneal population, I found that CD8+ T cells in the PEL coexpressing FasL and GzmB are effector dominant (Fig 5-5). A significantly higher proportion of the

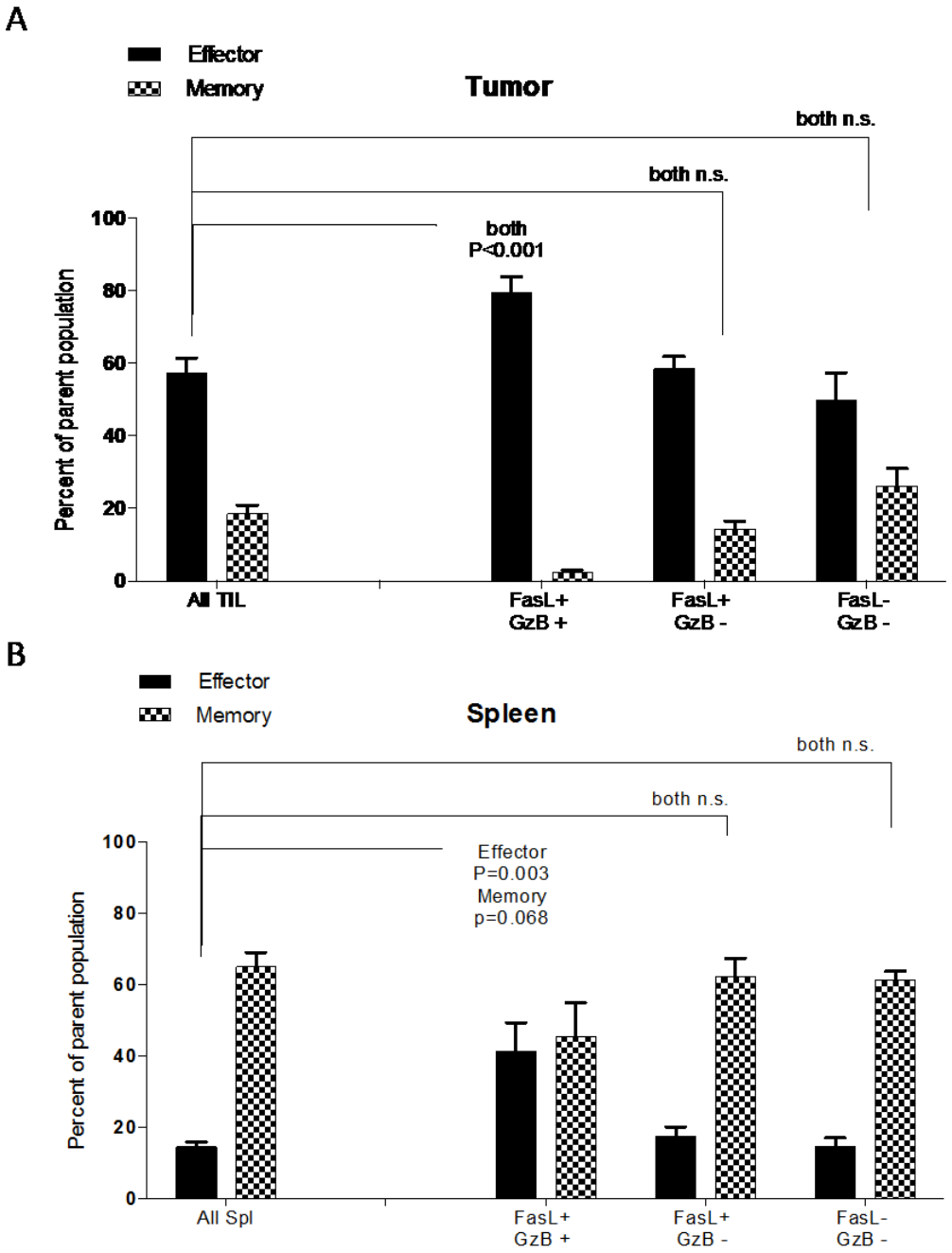


Figure 5-4. In mice with SC tumors, FasL+ GzmB+ CD8+ TIL cells have elevated proportions of effector phenotype and lower proportion of memory phenotype than in the general TIL or splenic population.

Cells from tumor (A) and spleen (B) of mice bearing SC tumors were stained for CD8, CD44, CD62L, CD127, FasL, and GzmB. CD44+ CD8+ cells were gated according to intracellular FasL and GzmB staining then gated as memory or effector phenotype. The percent of parent population summarizes data from 14 mice over three independent experiments. P values from paired two-way T test. Error bars are SEM.

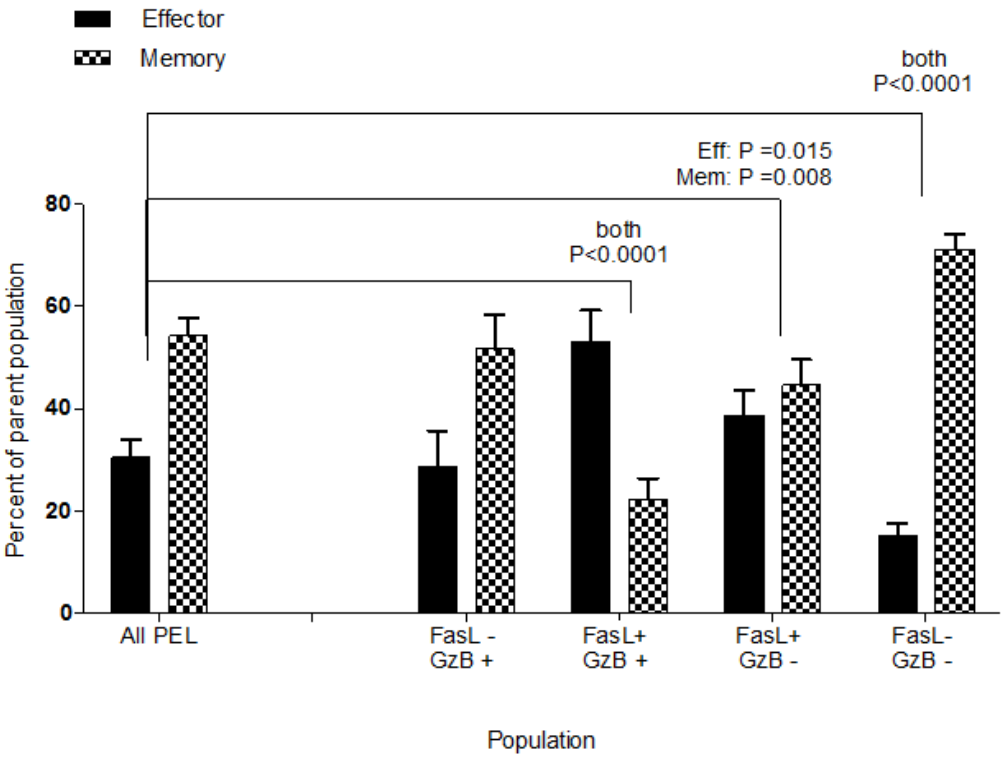


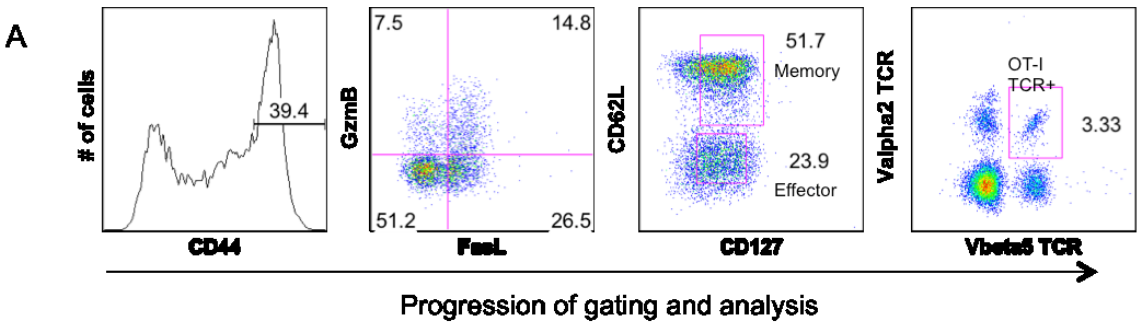
Figure 5-5. Compared to the total PEL population, Effector phenotype cells dominate the FasL+ GzmB+ population, and are increased in the FasL+ GzmB- population.
Cells at days 7, 14, and 21 post-injection with EG.7 tumors were harvested from peritoneal lavage and stained for CD8, CD44, CD62L, CD127, FasL, and GzmB. All days were pooled in this figure. Cells were gated on intracellular FasL and GzmB staining, and then proportion of each population that had effector or memory phenotype was quantified. P values from paired, 2-way student's t-test. Each point represents one mouse, with 14 or more per group over four independent experiments. Error bars are SEM.

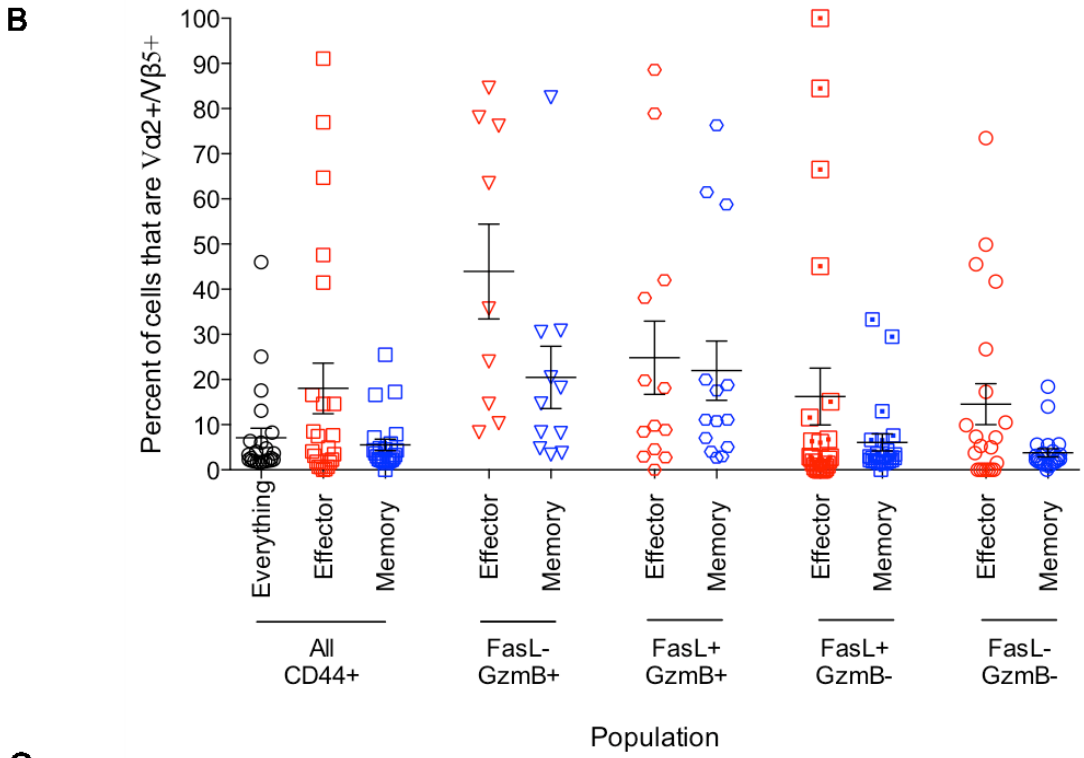
FasL+ GzmB+ cells are effector cells, compared to the frequency of effector cells in the entire activated CD8+ population. This agrees with what I found in the subcutaneous tumor. There is not a significant increase in effector proportion in FasL- GzmB+ cells, surprisingly, as I have not found reports of FasL- GzmB+ memory CD8+ T cells in the literature. In addition, FasL-only cells are almost equally likely to be either effector or memory phenotype cells, which is a statistically significant departure compared to the distribution in the overall activated CD8+ population. The novel finding from this section is that there are memory phenotype cells present in all FasL/GzmB expression groups (Fig 5-5) even though they express less FasL and GzmB overall (Fig 5-3B), and are the dominant phenotype for CD8+ PEL cells lacking either protein in the (Fig 5-5). Some memory phenotype cells in the peritoneum have retained FasL, and to a lesser extent GzmB expression.

GzmB+ FasL- Effector cells are more likely to be Vα2+/Vβ5+ than GzmB+ Memory cells

In the previous chapter, I demonstrated that cells belonging to a Vα2+/Vβ5+ population, which may be OVA-specific, in the tumor-bearing peritoneum are more likely to express GzmB than their polyclonal counterparts. I did not find a similarly significant difference in the FasL- GzmB- populations. However, I showed earlier in this chapter that both memory and effector cells can be FasL- GzmB-. Do the effector and memory cells that lack FasL and GzmB have the same frequency of potentially OVA antigen-specific Vα2+/Vβ5+ TCR?

When the GzmB+ FasL- population is further dissected and the memory and effector cells are assessed for Vα2+/Vβ5+ TCR expression (Fig 5-6A), the effector phenotype cells in the GzmB+ FasL- group are significantly more likely to be





C

Statistics for graph above:			FasL- GzmB+		FasL+ GzmB+		FasL+ GzmB-		FasL- GzmB-	
All CD8+	All Effector	All Memory	Effector	Memory	Effector	Memory	Effector	Memory	Effector	Memory
Vs all:	0.0749	0.5389	0.0000	0.0234	0.0120	0.0143	0.1680	0.7221	0.1356	0.1597
	Vs all eff:	0.0351	0.0264		0.4848		0.8330		0.6326	
		Vs all mem:		0.0056		0.0040		0.8141		0.2591
		Pairs:	0.0289		0.8287		0.2255		0.0181	

Figure 5-6. In activated CD8$^+$ T cell population from peritoneum of mice bearing I.P. EG.7 cells, significantly more cells expressing GzmB are Vα2+/Vβ5+ compared to all CD8+ cells. Cells were stained for CD8, CD44, intracellular FasL and GzmB, CD62L, CD127, and OT-I TCR chains. A. Gating strategy to examine Vα2+/Vβ5+ memory and effector cells in FasL/GzmB populations. B. Summary of data. C. Table of statistics. P values obtained by paired student's t-test. Grey cells: comparison not applicable. Green cells: statistically significant. Values are from 15 mice over three experiments.

Vα2+/Vβ5+ than the GzmB+ FasL- memory cells (Fig 5-6B). Even though we see more memory phenotype cells than effector cells that are only expressing GzmB in Fig 5-4, more of those effector cells are Vα2+/Vβ5+.

For the GzmB- FasL- cells, FasL- GzmB- effector cells have a significantly higher proportion of Vα2+/Vβ5+ cells than FasL- GzmB- memory cells (Fig 5-6), again in spite of the fact that memory GzmB- FasL- cells are far more frequent than effector GzmB-FasL- cells. This is surprising, as I expected that tumor antigen-specific effector cells would retain some form of FasL or GzmB expression due to the strong OT-I TCR stimulus some of these cells may receive from antigen.

Cells coexpressing FasL and GzmB express high PD-1 on the cell surface

As cells exposed to chronic antigen exposure can become exhausted [120], I thought that any tumor antigen-specific cells (of any specificity, OVA or otherwise) would be prime candidates for exhaustion. Are these exhausted? The marker I chose as a preliminary indicator of potential exhaustion was PD-1, as it can also provide clues about recent activation. It is a marker of both recent activation and exhaustion, as it dampens TCR signaling[240].

Cells from mice with subcutaneous tumors were also stained for PD-1 and assessed for relative PD-1 expression in cells with different FasL and GzmB expression patterns. Note that FasL- GzB+ cells were not included in this analysis, as too few samples from SC tumors had enough cells in this group for further analysis. I found that cells coexpressing FasL and GzmB have significantly more PD-1 on their surface than cells expressing FasL alone or expressing neither effector molecule (Fig 5-7A, Ci). This is true for cells found both in the spleen and in the tumor, although overall samples from

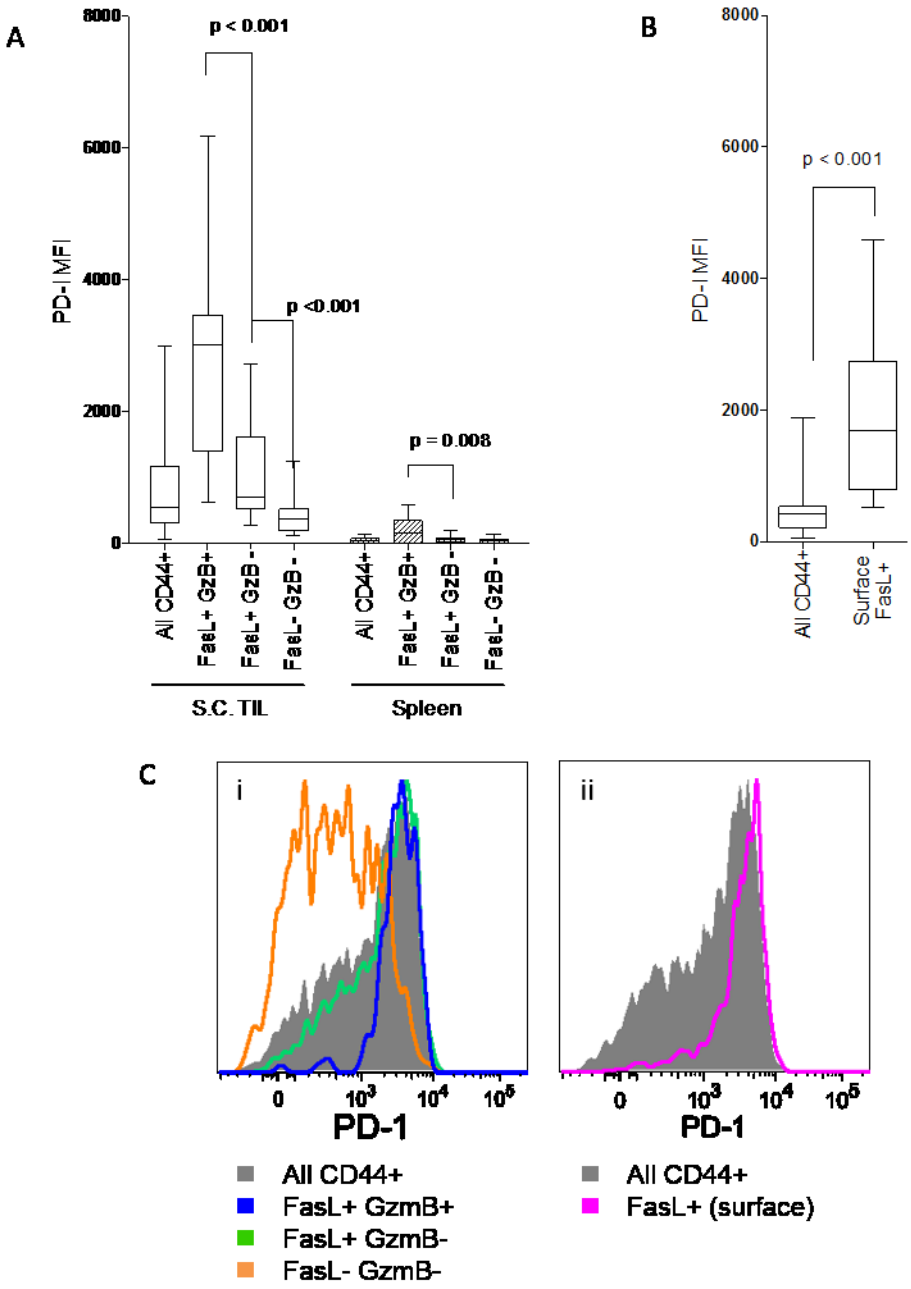


Figure 5-7. In activated CD8$^+$ T cells from mice bearing S.C. EG.7 tumors, PD-1 is significantly higher in populations positive for both intracellular FasL and GzmB or for cells positive for cell-surface FasL.

A. Cells were stained for surface phenotype markers as well as for intracellular FasL and GzmB, and gated on FasL/GzmB expression for subsequent analysis. B. Cells were stained for surface phenotype markers as well as for surface FasL without restimulation, and gated on FasL/GzmB expression for subsequent analysis. Summary of plot elements in A and B: whiskers indicate minimum to maximum values, box is 25th to 75th percentile, and line indicates median. p values obtained by paired student's t-test. Values are from at least 13 mice over three experiments. FasL-GzB+ were not included as too few cells were present to be statistically reliable. C. Representative histograms of cells stained for intracellular (i) or surface (ii) FasL.

the tumor have more PD-1 than their corresponding populations in the spleen. However, in TIL populations, cells expressing FasL alone also have significantly more PD-1 than cells expressing neither FasL nor GzmB. I can conclude that cells present in the tumor have elevated PD-1 in general, but PD-1 expression is elevated along with increasing FasL and then GzmB expression.

Since I have shown previously that FasL on the surface of non-restimulated cells increases as tumor volume increases (Fig 4-16), I wondered whether it was possible that FasL on the cell surface is indicative of a dysfunctional or exhausted cell state. I compared PD-1 expression on subcutaneous CD8+ TIL positive for cell-surface FasL to PD-1 on the entire CD44+ population, and found that PD-1 is higher on CD8+ T cells that have cell surface FasL (Fig 5-7B, Cii).

Contrary to my expectations, tumor infiltrating CD8+ T cells lacking FasL and GzmB have the lowest PD-1 expression compared to cells expressing any combination of FasL and GzmB. FasL+ GzmB+ CD8+ T cells and CD8+ T cells with cell-surface FasL have the highest PD-1 surface expression. Whether high PD-1 expression is due to recent activation that elicited intracellular GzmB and FasL coexpression needs to be examined.

Recent activation is associated with PD-1 elevation on FasL+ TIL CD8+ T cells

The section above shows that PD-1 is elevated on FasL+ GzmB+ cells, which may be due to recent activation. Repeated antigen exposure can also lead to PD-1 upregulation [256]. I showed that Vα2+/Vβ5+ CD8+ T cells have more FasL and GzmB coexpression. Would OVA-specific cells, or the Vα2+/Vβ5+ that may contain OVA-specific cells, be especially high for PD-1 in the FasL+ GzmB+ population, or be PD-1

high regardless of FasL/GzmB expression due to an antigen-specific effect on PD-1 expression?

As a reminder, I have detected for potentially OVA-specific cells by staining for the Vα2/Vβ5 TCR chains, rather than by MHC:K^bSIINFEKL tetramer. Therefore, junctional diversity in Vα2/Vβ5+ TCR expressing T cells may result in some endogenous "OT-I TCR+" CD8+ T cells that could recognize, and become activated by, antigens other than OVA peptide. If I adoptively transfer naïve OT-I T cells shortly before tumor injection, this donor population is likely to have been activated by antigens arising from the tumor. If FasL+ GzmB+ cells are high for PD-1 due to recent activation, would adoptively transferred naïve OT-I cells give rise to different FasL/GzmB/PD-1 expression patterns than endogenous Vα2+/Vβ5+ cells of unknown activation history? This is not a perfect comparison, as the all donor OT-I cells are OVA-specific, while Vα2+/Vβ5+ may be OVA-specific and may be specific to other antigens.

When examining the TIL population in mice that have received mixed OVA-specific and polyclonal naïve cells before injection (model reviewed in Fig 4-12), PD-1 is significantly elevated for the total CD44+ CD8+ population, on OVA-specific OT-I donor origin cells (Fig 5-8), compared to OT-I TCR+ cells originating from the recipient. While the overall dynamics of PD-1 expression on cells of different FasL/GzmB expression groups is quite similar between the donor OT-I and recipient Vα2+/Vβ5+ cells, PD-1 is significantly higher on donor cells in both FasL+ GzmB+ and FasL+ GzmB- populations (Fig 5-8). This suggests that recent activation, as well as antigen-specificity, contribute to PD-1 elevation on FasL-expressing cells. There is higher PD-1 on polyclonal FasL+ GzmB+ recipient cells in these experiments, but PD-1 levels were

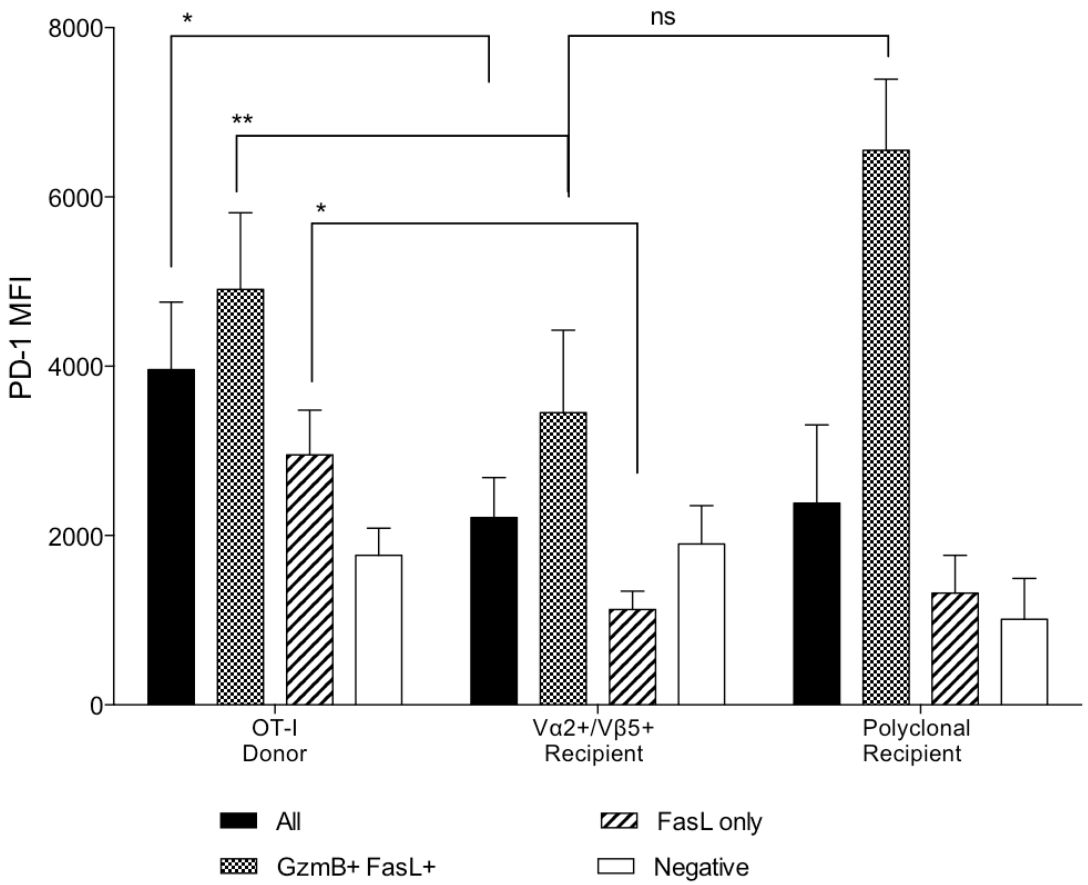


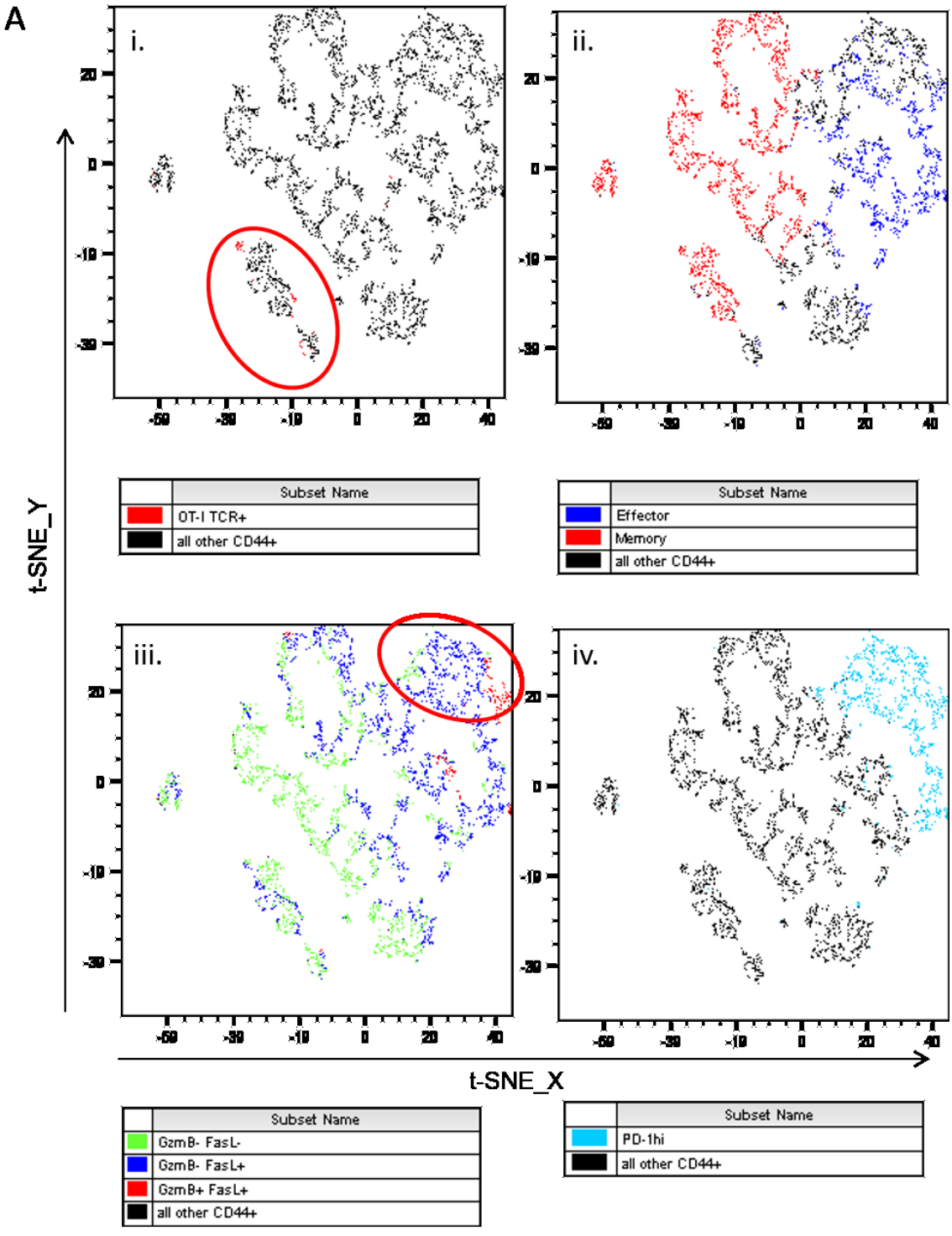
Figure 5-8. PD-1 surface expression on CD8+ S.C. TIL differs between populations depending on FasL/GzmB coexpression, as well as cell origin.
Mice received adoptive transfer of naïve OT-I and naïve polyclonal CD8+ cells prior to subcutaneous tumor injection. After tumor dissection, cells were stained for CD45.1 and CD45.2 to distinguish donors and recipients, and surface PD-1 and OT-I TCR, as well as stored FasL and GzmB. Numbers of polyclonal donor cells was too low to further analyze for PD-1 expression. Columns represent mean, error bars are SEM. P values obtained by two-way, paired t-test. *=P<0.05 **=P<0.01.

variable enough that this difference compared to OT-I TCR+ donor or Vα2+/Vβ5+ recipient cells is not significant.

As Figure 5-7 shows that PD-1 expression is higher on FasL- GzmB- activated CD8+ cells in the tumor than their counterparts in the spleen, there is still the question of whether some exhaustion is occurring to cause a loss of FasL and GzmB. FasL- GzmB- OT-I TCR+ activated CD8+ T cells, either of donor or Vα2+/Vβ5+ recipient origin, have more surface PD-1 than their polyclonal counterparts (Fig 5-8), which suggests that this FasL- GzmB- population may be partly exhausted in an antigen-dependent manner. It is also possible that these populations are actually heterogeneous and some are activated while some are starting to become exhausted. However, without additional surface markers, we cannot be certain.

Heterogeneous population groupings are present in TIL and PEL CD8+ T cells

A limitation to these phenotypic results is that true biological differentiation is not the linear, categorical progression implied by the rigid effector and memory gates shown at the start of this chapter. Polyclonal memory cells may be of varying age and specificity, and even Vα2/Vβ5+ cells in the OT-I TCR rearranged native population will have TCR rearrangement variability and therefore variable antigen affinity and activation signal strength. Furthermore, I observed cell events outside of $CD127^{hi}$ $CD62L^{hi}$ memory phenotype and $CD127^{lo}$ $CD62L^{lo}$ effector phenotype in my activated CD8+ T cells, and these populations were not included in analysis. It is possible that some of them are part of the PD-1hi, CD62Lhi, CD127lo CD8+ Treg population Dai *et* al identified [385]. I took a few representative samples from experiments discussed earlier in this chapter and analyzed the entire CD44+ population from a TIL or PEL sample by



Subcutaneous tumor CD8+ T cells

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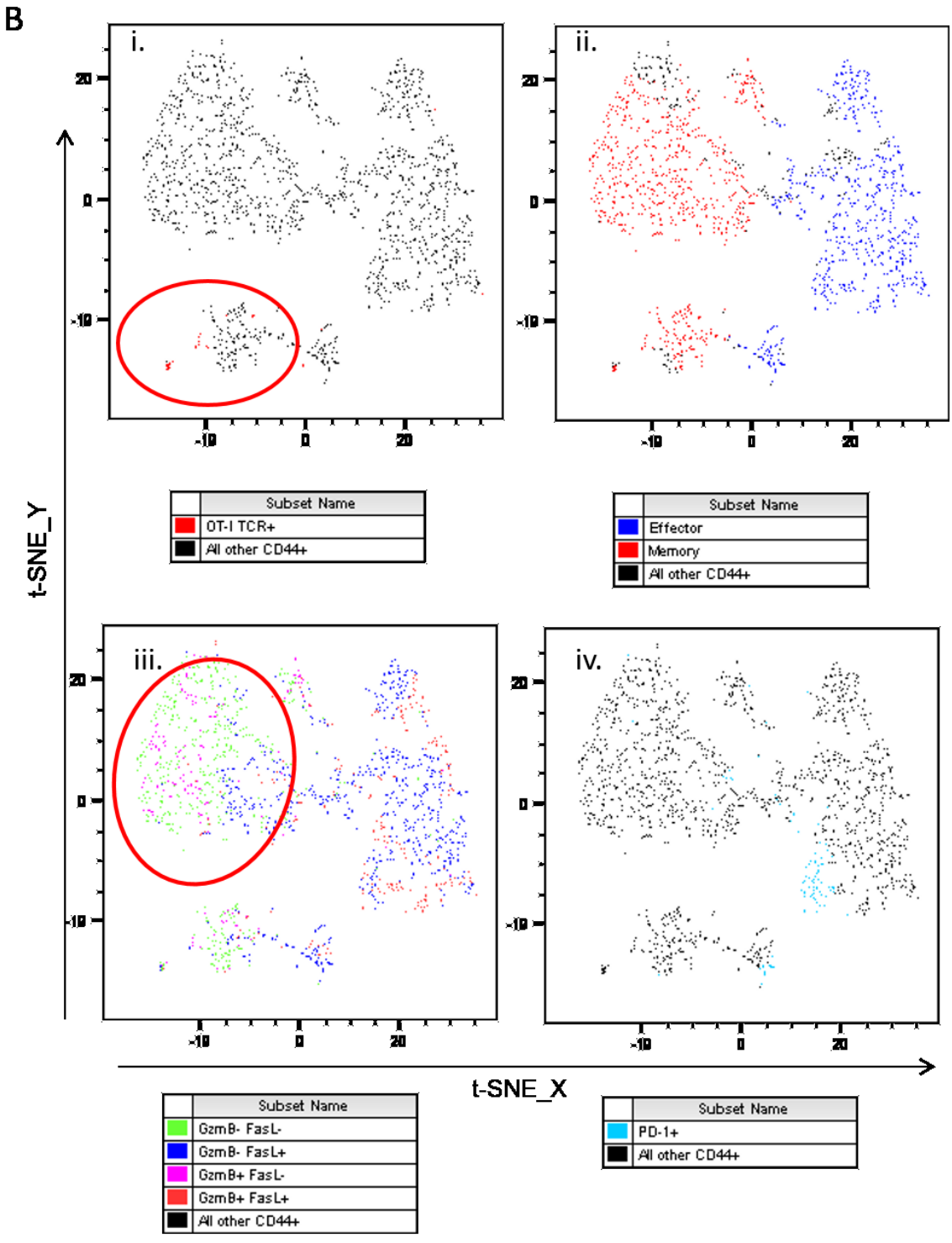

Intraperitoneal tumor CD8+ T cells

Figure 5-9. Additional phenotypes emerge when analyzing CD44+ CD8+ cells from mice bearing tumors via t-SNE.
A single mouse data file from a samples stained for CD8, CD44, CD127, CD62L, PD-1, TCR Vα2, TCR Vβ5, and intracellular FasL and GzmB was analyzed via t-SNE (t-Distributed Stochastic Neighbor Embedding) algorithm in FlowJo software. First cells were gated as CD8+ CD44+, then algorithm was run using all compensated fluorescent parameters. Note that "OT-I TCR+" indicates Vα2+/Vβ5+ TCR.
A. CD8+ CD44+ TIL from SC-tumor bearing mouse. Events:3770 Iterations: 1000, Perplexity: 25, Learning/Eta: 200. B, CD8+ CD44+ PEL from SC-tumor bearing mouse. Events:1594 Iterations: 1000, Perplexity: 20, Learning/Eta: 200. In both: i. Distribution of population clusters from t-SNE with OT-I TCR+ cells overlaid in red. ii. Traditionally gated effector and memory phenotype events overlaid on same data from plot iii. Populations gated on intracellular FasL and GzmB protein expression, iv. PD-1-hi cells overlaid in blue.
Areas of particular interest for discussion in the text are circled in red.

t-SNE (t-Distributed Stochastic Neighbor Embedding) [389], which is an automated algorithm to cluster populations without gating, similar to a principal component analysis. Some typically gated populations fall within a small number of nearby clusters, like the "OT-I TCR+" Vα2+/Vβ5+ cells circled in 5-10Ai and 5-10Bi. However, there are clusters present that defy classical effector/memory phenotypes (Fig 5-9 Aii, Bii).

Cells that I had identified as memory or effector based on classic gating fall into multiple clusters, as can be seen by the memory and effector "islands" separated from the bulk of the population clusters in both 5-9Aii and 5-9Bii. Furthermore, the bulk clusters of memory cells are quite heterogeneous. Examining the circled region in 5-9Biii, memory phenotype cells in this region have a mix of GzmB+ FasL-, GzmB- FasL+, GzmB- FasL-, and a few GzmB+ FasL+ cells. All of them are sufficiently similar in other phenotypic aspects to be clustered in the same region.

I noticed that in my subcutaneous TIL sample, there is a non-memory, non-effector population that is high for PD-1 and seems to express intracellular FasL without GzmB. These might be regulatory CD8+ T cells as PD-1hi CD8+ Tregs have been identified[385], but functional or further phenotypic analysis is needed to confirm this. It would benefit future researchers to examine additional memory and exhaustion markers such as CD27, CD103, CTLA4, and CD122, as well as indicators of ongoing proliferation like Ki-67. Unfortunately, the sample size to give this analysis true power is greater than the size of most of my thesis samples, and the application of this analysis method to conventional flow cytometry data was not commonly used by immunologists before most of my samples were acquired. Therefore, the data in figure 5-9 is for interest rather than for drawing hard conclusions about the population composition of tumor

infiltrating cells, but it certainly suggests that the tumor-infiltrating CD8+ population is complex and has heterogeneous expression of FasL and GzmB across multiple differentiated populations.

Discussion

In this chapter I have shown that intracellular FasL protein is present in a wide range of cell types, including memory or effector, and cells that are polyclonal or antigen-specific. Effector cells express significantly more FasL and GzmB protein, but memory cells can be found in SC tumor, tumor-bearing peritoneum, and spleen that coexpress FasL and GzmB. Tumor-infiltrating T cells positive for FasL protein expression, either intracellularly or on the cell surface, are associated with elevated PD-1 expression levels.

By using CD62L and CD127 staining, I examined CTL from mice via the same phenotyping as was done for my *in vitro* studies in the previous chapter. While I have been using the terms Effector and Memory, these are rather broad categories, as CD127lo CD62Llo could be both Teff and Tem; CD127hi CD62Lhi memory cells are most likely Tcm, but other intermediate memory phenotypes may exist, as seen by others [390]. Not all the cells fell into neat CD62Lhi CD127hi or CD62Llo CD127lo categories, but most clustered in the predicted phenotypes. I was able to find effector and memory cells at all times and in all mice that I examined, and did not notice any time-dependent effects on distribution of effector versus memory, or any significant correlation between tumor volume and percent effector/memory.

In spite of this, there is a difference in the effector to memory ratio of the CD44+CD8+ from intraperitoneal tumor response versus the subcutaneous response. At the times I examined, the peritoneal responders are more memory-heavy, while SC TIL

responders are more effector-heavy. Others have found that in mice bearing B16 melanoma lesions adoptively transferred with *in vitro* activated antigen-specific CTL plus CD4+ T cells, at all times in the lung metastases the majority of CTL have an effector phenotype rather than Tcm, Tem, or naïve phenotype [391]. It is interesting that in spite of the difference in memory-bias versus effector bias in the two sites, in both cases FasL+ GzmB+ coexpressors are elevated for effector cells compared to the total CD44+ effector distribution in that tissue's CD8+ population.

While it is not surprising that I found effector cells in both tumor sites to express more intracellular FasL, more intracellular GzmB, and more cell-surface FasL than is in memory cells, it is striking that Memory cells can express GzmB protein. It is highly likely that the tumor environment is sufficiently stimulatory to allow GzmB expression by memory cells. Furthermore, a very recent paper showed that when memory cells emerge as daughter cells from effector precursors, they can retain high levels of cytotoxicity in an influenza model [371]. Memory cells persisting long-term after influenza infection can retain GzmB gene expression, though protein was not characterized[372]. Murine splenic antigen-specific cells in an LCMV model are capable of producing GzmB and IFN-γ upon restimulation, regardless of whether they are memory or effector cells, but very few of those memory cells retain GzmB expression without restimulation [112]. These results seem to align with my findings, but in a viral model. I think that my results represent a legitimate expression of FasL and GzmB by tumor-reactive memory cells.

Other researchers found that in a tumor allograft model, cells that were outside of the traditional Tcm or Tem phenotypes had the highest levels of FasL [180]. The

heterogeneous populations I found that that defy traditional memory/effector gating are increasingly in line with our understanding of intermediate phenotypes in differentiated CD8+ T cells. For example, one group found that CD62L- CCR7+ cells, which do not have a classic "memory" phenotype, behave in a memory-like manner [392], and another found that memory establishment can occur regardless of precursor CD62L phenotype [393]. This can likely be interpreted partly as transient, intermediate phenotypes emerging the differentiation to effector lineages from memory. However, as they are found in the periphery where the tumor response is happening, more investigation is needed into the biological roles played by these intermediate-phenotype cells.

In the context of a DNA vaccination, Brentville and colleagues found that higher avidity CTL mediate tumor clearance and can become part of the memory response. They can develop into functionally stronger memory cells, but at supra high levels of antigen stimulation, the memory cells appear to become exhausted [131]. This is interesting given that I found, in the peritoneal tumor CD44+ CD8+ population, significantly more Vα2/Vβ5 TCR+ cells in the memory population that are likely to be cytotoxic (Tmem FasL-GzmB+, Tmem FasL+ GzmB+) compared to the proportion of memory cells that are antigen-specific in the general population (Fig 5-6). These may be, but are not limited to being, OT-I like and OVA-specific. Some of these OT-I TCR+ Tmem could be the high-affinity memory cells and therefore be stimulated to produce or retain expression of more GzmB. Furthermore, the elevated antigen-specificity in FasL-GzmB- Teff compared to FasL-GzmB- Tmem could be indicative of antigen-specific CTL that have been so highly stimulated that they lost or are in the process of losing effector mechanism expression, and are on the way to exhaustion. It is disappointing that I carried out these

experiments before I added PD-1 to my panels, as examining these antigen-specific, FasL-GzmB- effector cells for PD-1 and, in addition, for other markers of exhaustion like CTLA4 and Tim-3 would be very useful. However, comparing this population to the general SC TIL FasL- GzmB- population in mice with SC tumors, cells without FasL or GzmB appear to have the lowest expression of PD-1 (Fig 5-7). Erkes examined CD8+ TIL that are specific to non-tumor antigen in a tumor model, and found that they are PD-1+ but functionally impaired [394], although the PD-1 these cells express is nonfunctional, truncated PD-1.

The data I show in figure 5-7 does not support my original expectation that GzmB-FasL- cells would be PD-1 hi, due to exhaustion. I suspect in the GzmB+ FasL+ population, high PD-1 is due to recent activation, rather than exhaustion. However, others have shown that PD-1hi TIL had more functional avidity than PD-1lo TIL [395], and one group examining the tumor response in humans used PD-1 to identify clonally-expanded tumor-specific cells [384]. In this same experiment, PD-1 hi cells have increased tumor-specific lysis although specific antigens are undefined. Another group looked at PD-1 antigen-specific CD8+ T cells in human melanoma and found that they were functionally impaired: They had reduced ability to produce cytokines *in vitro,* and found a positive association between dysfunction and antigen specificity for tumor[396] They also found that PD-1+ cells were more often CD127lo compared to PD-1-. At first this seems to conflict with my own findings, where FasL+ GzmB+ cells are highest in PD-1. However, these cells that I see as FasL+ GzmB+ PD-1hi may have lost function only for things that I did not examine.

Wu and colleagues examined PD-1+ CD8+ T cells, in tumor and dLN of human colorectal cancer patients, and found that PD-1+ and PD-1- cells have similar GzmB intracellular staining by FACS in tumor, but PD-1+ cells have more GzmB than their PD-1- counterparts [397] in the lymph node, which agrees with my finding that among splenic CD8+ cells, GzmB+ cells have highest PD-1 expression. In addition, while the PD-1+ tumoral CTL are dysfunctional for cytokine secretion, the lymph node PD-1+ CTL are not dysfunctional [397]. These studies together suggest that in human models PD-1 expression may sometimes go along with dysfunction in CTL, even if they are "armed" like functional T cells would be. In addition, location adds contextual clues to whether or not PD-1+ T cells are exhausted. It has been shown that presence in the tumor environment is not sufficient to induce exhaustion, as cells in the tumor becoming exhausted must also be specific for tumor antigens [255]. Therefore, the high PD-1 levels I saw on FasL- GzmB- OT-I TCR+ TIL in mice with adoptively transferred CD8+ cells were either very recently activated and not yet expressing FasL and GzmB, or have a high degree of tumor specificity for undefined tumor antigens and are becoming exhausted. Future experiments that integrate memory and effector phenotyping along with PD-1 staining and proliferation tracking in the same cells will help us better integrate my findings of FasL and GzmB expression and its elevated presence on memory and effector cells with the life history of those CD8+ T cells.

Chapter 6: Discussion

Overall, I have presented evidence in this thesis that Fas Ligand and Granzyme B proteins are expressed independently in CD8+ T cells in response to extrinsic stimuli, resulting in heterogeneous expression in a CD8+ T cell population. Expression of FasL and GzmB protein is not homogeneous in cells, and there is a wide range of coexpression patterns, with cells activated *in vitro* with high CD3 and CD28 stimulation at one end, and activated CD8+ cells in normal, tumor free mice at the other extreme. Activated CD8+ T cells in SC and IP tumors display a range of FasL and GzmB phenotypes overlapping these two.

Summary of Results

While naïve CD8+ T cells express neither FasL nor GzmB protein (Fig 6-1, left), this is less common in activated CD8+ T cells in healthy, conventionally housed mice. In activated CD8+ T cells from the spleen or peritoneum of tumor-free mice, most of the population expresses intracellular FasL in the absence of GzmB (Fig 6-1, left). However, if the naïve cells are activated *in vitro* instead, they almost all express GzmB protein, either alone, or coexpressed with FasL (Fig 6-1 centre). Only following sub-optimal stimulation of cells, by low TCR and costimulatory receptor ligation and low levels of added cytokine, do *in vitro* activated cells take on a FasL+ GzmB- or FasL- GzmB- phenotype (Fig 6-1, centre). Furthermore, I found that cytokine conditions that are known to elevate GzmB expression do not significantly enhance FasL protein expression in resting CD8+ T cells. Cells that were activated *in vitro*, do not have high levels of cell-surface FasL without restimulation of the T cells.

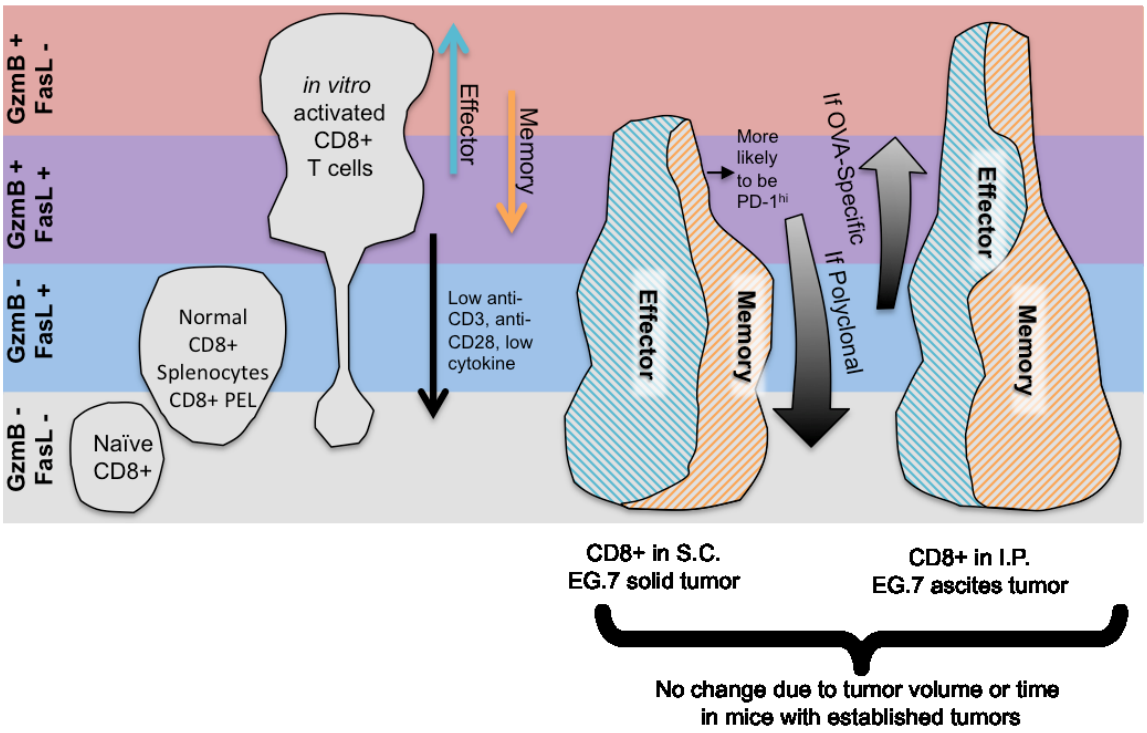

Figure 6-1. Model of influences on FasL and GzmB protein expression in vivo and in vitro in activated CD8+ T cell.
Four bands represent different expression patterns of stored FasL and GzmB. Grey blob shapes represent different populations studied in this thesis. Shape of populations represents relative frequency of that cell type having FasL and/or GzmB. Population blobs are not to scale between sample types. Arrows indicate influences on effector mechanism expression: for example, OVA-specific CD8+ cells are more likely to be FasL+ GzmB+ or FasL- GzmB+.

FasL is expressed by activated CD8+ T cells in the spleen and peritoneum of healthy mice without tumors (Fig 6-1, left). The vast majority of these activated CD8+ T cells in both tissues express intracellular FasL in the absence of GzmB. This suggests that FasL is kept as a baseline by most circulating or peritoneal-patrolling CD8+ T cells for a currently unknown purpose. It is possible that some of these cells are recently activated in response to minor infections that the mice are fighting, or they could be engaged in regulatory activity, or some undefined combination of both.

When examining activated CD8+ T cells in tumor bearing mice *in vivo*, most cells express FasL alone or express neither FasL not GzmB. CD8+ TIL in subcutaneous tumors are similar to the CD8+ T cells in spleen, draining LN, and contralateral LN when it comes to FasL+ GzmB- dominance, followed by FasL- GzmB-. However, presence in the tumor environment significantly increases the population coexpressing FasL and GzmB together, so presence in the tumor does have a positive effect on GzmB protein stores (Fig 6-1, right).

The question of whether tumor burden or time can change the quality of the response with respect to FasL/GzmB expression arose once I found that presence in the tumor itself elevates intracellular GzmB. While there may be an effect in mice that were injected with EG.7 cells but did not develop tumors, the lack of a tumor to examine precluded my ability to make any association between TIL GzmB or FasL and effective clearance. Over the time periods I used, there is no relationship between time or tumor burden and the dynamics of FasL/GzmB expression, with one exception: surface expression of FasL on activated T cells in the tumor. While there is very low surface FasL on resting CD8+ T cells *in vitro*, CD8+ T cells from mice with tumors have FasL

on the cell surface, and more on CTL from the tumor than CTL from the spleen. This is a novel finding, and furthermore the proportion of surface FasL+ cells increases slightly with time in IP populations, and correlate with tumor volume in SC tumors. However, at least in the SC tumors, this is independent of antigen specificity.

The notable difference between CD8+ T cells in the peritoneum and in solid tumors is that activated CD8+ T cells in the peritoneum of IP EG.7 tumor bearing mice can express GzmB in the absence of FasL, which is very rare in subcutaneous tumor CD8+ TIL (Fig 6-1, right). The expression of GzmB may also be associated with OVA-specificity, as seen in an OT-I adoptive transfer model in SC tumors, or is associated with expression of the Vα2+/Vβ5+ T cell receptor in the IP tumor response. While cell-surface FasL is, as discussed above, elevated on tumor-infiltrating CD8+ T cells in both tumor sites, there is no relationship, between this FasL surface expression and known TCR specificity or TCR rearrangement.

I found that subcutaneous tumor-infiltrating CD8+ T cells that are the most "weaponized", either with high cell-surface FasL, or with FasL and GzmB coexpression, have the highest levels of PD-1 (Fig 6-1), though this is possibly associated with recent activation, rather than exhaustion. Finally, while the two tumor sites had differing balances of memory and effector phenotype populations, with SC being effector-dominant and IP being memory-dominant, every combination of FasL and GzmB expression was found in both memory and effector cells *in vivo* (Fig 6-1). The *in vivo* finding of FasL+ and GzmB+ memory and effector phenotype cells echoes that seen following *in vitro* activation. If we were to imagine a continuum of FasL/GzmB phenotypes extending from FasL- GzmB-, through FasL+ single expressors, to FasL+

GzmB+ coexpressors, to GzmB+ single expressors, effector cells are closer to the GzmB-heavy end, while memory cells are closer to the FasL or negative end (Fig 6-1). Depending on method of activation, location *in vivo* and TCR rearrangement, CD8+ T cells can be found across this spectrum. Since the phenotypes that I saw *in vitro* are very different with respect to FasL and GzmB protein expression than those seen *in vivo*, it is another reminder that not all *in vitro* work can be extrapolated to biological relevance in patients. The expression of FasL and GzmB protein is very heterogeneous within the tumor-responding CD8+ T cell population *in vivo.*

FasL and GzmB expression heterogeneity in activated CD8+ T cells

Even within cells of the same differentiated phenotype, there can be variable storage of FasL and GzmB (Fig 6-1). *In vitro*, this varies with time and a modulated cytokine environment, and the t-SNE data *in vivo* data in chapter 5 shows that there can be variation in other factors, such as coexpressed surface phenotype markers, including TCR alpha and beta chains. I suspect that there is a wide range of antigen specificities in the polyclonal FasL+ GzmB+ population that actually represent diverse recruitment – some tumor specific, some nonspecific to tumor the tumor but recruited via inflammation, and some specific for self antigens and suppressive. As the data in chapter 4 regarding the mixed pre-tumor adoptive transfer experiments shows, there is definitely antigen-specific recruitment to the tumor occurring. However, the difference in overall PD-1 expression on OT-I TCR+ TIL from the donor population compared to Vα2+/Vβ5+ TIL from the recipient population suggests that there is also a contribution from the activation history of those CD8+T cells. This is possibly also due to the OT-I donor population being a pure OVA-specific population rather than only expressing the

Vα2+/Vβ5+ TCR that may include OT-I like cells. Progression to exhaustion in CD8+ T cells is progressive [250, 255, 383], so it may just be that what I have gated as single populations, for example, the PD-1hi, FasL+, GzmB+ may comprise a heterogeneous population with some cells not exhausted and some others progressing to exhaustion. Additional markers of exhaustion, such as CTLA4 and LAG3, as well as assessment for loss of IFN-γ production upon restimulation, should be examined in future studies of this nature.

FasL or GzmB expression in memory cells

It surprised me to find in both *in vitro* activated CD8+ T cells as well as in CD8+ T cells from mice bearing EG.7 tumors that a memory population can express GzmB and FasL. While groups have found cytotoxic memory T cells [371, 372], it is interesting to see that memory cells are strongly armed without ex-vivo restimulation. I could be seeing either former effector cells that have retained GzmB expression, or memory cells in transition to effector cells. Future researchers could use adoptive transfer of labeled GzmB+ memory phenotype cells [164] into tumor-bearing mice and as a tool to track whether these GzmB+ memory cells can give rise to effector cells, or even eventually lose FasL expression.

While the phenotype of CD127hi CD62Lhi captured the Tcm phenotype for gating purposes in my analysis, there may be other memory cells that do not share this phenotype that could be present. There were some CD44+ CD8+ cells falling outside of the bounds of my effector and memory gates that were not included in the analysis of my thesis. These could be intermediate cells in the process of transitioning between Teff and Tcm.

Stem cell memory cells confer adoptive cell therapy immunity [114, 122, 123], but I have not assessed whether they are elevated in spleen or tumor of tumor-bearing mice. They are CD44lo, so I did not examine them under the scope of this work. Zhang and colleagues recently published data suggesting that adoptively transferred human CD8+ stem cell memory (Tscm) use FasL and possibly GzmB to contribute to tumor cell cytotoxicity. These mechanisms are not for survival of the adoptively transferred Tscm, but they use FasL for effective lysis of target cells resulting in tumor regression [398]. CCR7 staining, in addition to examination of CD44lo populations in peripheral tissue, will help clarify whether Tscm populations are present at all during the native response to tumors, and whether they are storing or translocating FasL.

It is possible that the GzmB+ FasL+ memory population that I did gate on *in vivo* is quite heterogeneous for more than just FasL and GzmB. It is possible that some resident memory (Trm) may be included in the "effector" population, as they are CD62Llo. Why should we care whether the effector-like cells are resident memory or transient effector populations? Even though FasL+ GzmB+ cells are elevated in OVA-specific populations, there is still an increased presence of FasL+ GzmB+ in polyclonal TIL and PEL compared to polyclonal healthy spleen or peritoneum. As Trm can be nonspecifically recruited in response to other antigens and still display effective cytolytic ability after bystander activation in an acute infection [399, 400], these may be valuable targets for cancer vaccination. CD103 is an integrin involved in retention of T cells in tissue [401] and is often used as an identifier for Trm cells [402]. At a minimum, CD103 should be added to future panels[119] to clarify whether Trm have the same FasL and GzmB dynamics as the effector or memory cells. However, data from this thesis adds to

the papers that state that a subset of Tcm-like memory cells can express GzmB. Evidence in this thesis supports that memory-phenotype CD8+ T cells in mice bearing tumors can definitely store FasL.

FasL: possibly optimal mechanism for sub-optimal stimulatory conditions

In CD8+ T cells activated *in vitro,* A GzmB- but FasL+ positive population is present following suboptimal anti-CD3/CD28 activation. Furthermore, intracellular FasL protein is also present in most CD44+ CD8+ T cells *ex vivo*, which suggests that FasL is preferentially stored in CD8+ T cells in low-antigen conditions. The greatest difference between OT-I TCR+ T cells and polyclonal populations with respect to effector mechanisms is the presence or absence of GzmB, but not FasL. It would seem that FasL without GzmB is a more common state.

While it is rare to detect FasL- GzmB+ cells *in vivo*, they are the most common *in vitro*. This is probably partly due to the strong stimulus given to cells *in vitro* that boosts GzmB expression until it is dominant, without similarly affecting FasL. In addition, OT-I and Vα2+/Vβ5+ TCR populations have higher prevalence of GzmB+ cells (Fig 6-1). The signal mediated through the OT-I TCR:K^b-SIINFEKL interaction is known to be strong [403]. It would be interesting to examine a similar model to my naïve adoptively transferred mice bearing tumors, but with naïve CD8+ T cells from OT-3 mice instead of OT-I mice being transferred prior to tumor injection. OT-3 mice also recognize K^b-SIINFEKL, but with a significantly weaker affinity than OT-I cells [403]. In this case, the actual antigen itself would be the same frequency from the tumor, but recognized by a receptor with weaker affinity. Segal and colleagues asked a similar question when assessing the success of adoptive cell therapy in mice, and found that *in vitro* activated

OT-I and OT-3 cells can similarly clear small μ-myc-OVA tumors, but neither can control large established tumors. However, they only assessed IFN-γ production in these cells, and used it as a therapeutic model, not as a way to assess development of the antigen-specific response with respect to cytolytic effector mechanisms. Would the resultant antigen-specific TIL still have a significant increase in GzmB+ cells compared to OT-I cells? I would predict that the OT-3 cells would have reduced GzmB expression compared to the OT-I cells, but expression of FasL would be retained.

The suppressive environment found in the tumor relative to in *in vitro* cultures could contribute to the differences I see in GzmB/FasL expression patterns between *in vitro* and *in viv*o. In the tumor, T cells not only encounter other T cells, tumor cells, and proinflammatory innate immune cells, but they also can encounter myeloid-derived suppressor cells, which can have negative effects on the T cell population by competition for metabolic resources, secretion of suppressive cytokines like IL-10, and direct suppression through ROS secretion to T cells [286]. It is remarkable that even in the suppressive environment of a tumor, FasL expression can be retained in a high proportion of CD8+ T cells.

Surface FasL, PD-1, and regulatory activity

The Dai group has provided some interesting data suggesting that CD8+ CD122+ Tregs may use FasL for survival while it suppresses effector CD8+ T cells responses. They found that these CD8+ Tregs can kill CTL *in vitro* in a FasL-dependent manner, but *in vivo* blockade of Fas or FasL is insufficient to dampen their suppressive ability [29]. However, Fas receptor-deficient CD8+ Tregs are not capable of having as significant of an effect on CTL activity as Fas-competent CD8+ Tregs [29]. CD8+ Tregs can kill CD4+

T cells *in vitro* in a Fas-dependent manner, and contribute to depletion of CD4+ T cell populations *in vivo* [29, 404]. It seems like CD8+ Tregs might be using FasL as a personal defense mechanism, killing off sufficient numbers of nearby CTL that they are capable of surviving to dampen the effector CTL response via IL-10. If future researchers could examine the cytokine profile of FasL+, particularly surface FasL+, cells, I expect that FasL could be found in different cells expressing multiple cytokine types such as IFN-γ and IL-10. Not all at the same time of course, but FasL would be present in cytokine-heterogeneous CD8+ T cell populations.

The Berke group also performed some basic experiments to show that in an alloreactive tumor model, CD8+ T cells are capable of killing bystander cells, as a means of contraction, via FasL[180]. However, they did not characterize the CD8+ T cell lineage they are. They arise within days of allogeneic tumor injection, but these could still be either effectors or reactivated memory. It is possible that a portion of the cells killing via FasL are Tregs, especially as bystander killing via FasL can be TCR specificity-independent [354]. Since I saw no increase in Vα2+/Vβ5+ expression in CD8+ TIL positive for surface FasL, the polyclonal, surface FasL+ TIL may be Tregs in my experiments. I did not examine markers such as CD122 or CD25 to determine whether some of these CD8+ T cells could potentially be Tregs, but that is something that future researchers should examine.

The high PD-1 that I observed on surface FasL+ CD8+ T cells would agree with my suggestion above that some of the CD8+ TIL may be Tregs, as PD-1+ CD122+ CD8+ T cells have been shown to be suppressive [385, 405, 406]. PD-1 is also expressed on CD8+T cells immediately after activation [235], and is upregulated on functionally

exhausted cells. However, it is less likely that active presentation of surface FasL would be considered functional exhaustion, unless FasL presentation is retained during the early stages of exhaustion, and only upon accumulation of multiple exhaustion markers is FasL no longer expressed. Schietingen found in human melanoma tumors, exhausted cells had elevated FasL transcripts compared to naïve cells, but did not show whether this is progressively modulated over time, like IFN-γ is[255]. Furthermore, Erkes and colleagues found functional heterogeneity in the PD-1+ CD8+ TIL population in a mouse melanoma model, demonstrating that PD-1+ tumor-antigen specific cells are dysfunctional, while PD-1+ T cells specific to CMV infected cells are functional [394]. They further found that the PD-1 expressed by the virus-specific cells is not full-length and cannot bind PD-L1 and PD-L2 ligands. I did not use the same antibody clones they used, and did not assess PD-1 binding by its ligands, but the clone J43 that I used can be used to block binding of PDL-1 and PDL-2 to its targets [407], so it may be that I have detected functional PD-1. Overall, the combined surface FasL, PD-1 expression, and lack of antigen specificity suggests that some of the surface FasL+ cells may be CD8+ Treg cells, but significant additional phenotypic and functional characterization is needed. Furthermore, future researchers should sort out CD8+ Tregs from tumors and assess whether they can suppress CTL proliferation or even kill them in a FasL-dependent manner.

One may wonder why cells would be armed with FasL for both antitumor and suppressive function. Why does the homeostatic state of the body let this happen? One could argue that cancer may not have been as great of an evolutionary selective pressure for shaping the immune system as, say, acute viral, parasitic, or bacterial infections.

However, humans and complex organisms in general have consistently evolved in the presence of chronic viral infections starting in childhood, such as cytomegaloviruses. In the presence of these chronic antigenic challenges, usage of the weapons primarily in a suppressive capacity would have long-term benefits for a host… or human. It is up to us in the labs and clinics to figure out how to exploit these responses for the benefit of people with cancer.

Surface FasL expression: Indicative of poor prognosis?

Oncologists have historically tried to find immunological indicators of tumor progression or prognosis. While histological analysis searching for correlation between TIL and patient outcomes has been researched for decades, several years ago the Immunoscore consortium gained significant attention for well-validated data suggesting that the best predictor of clinical outcomes in colorectal and gastric cancer, as well as brain metastases, is the high level of infiltration of CD3+ CD8+ T cells into the tumor as assessed by histology [387, 388, 408-410]. Similar results have also been found in some breast cancers [411], but CD8+ infiltration was found to be associated with aggressive progression in other breast cancers [412].

Researchers have also tried to extend these immune associated outcomes to other markers in tumor tissues, like PD-L1, with inconsistent success across solid tumor types [411-414]. The histological presence of GzmB in some tumor tissues is associated with overall survival in squamous cell carcinoma [415], transitional carcinoma [416], and gastric cancer [410], and increases in GzmB+ CD8+ T cells in rectal cancer post-therapy is associated with positive outcomes [417]. However, older research showed that a high proportion of cells staining for GzmB in Hodgkin's Lymphoma was found to have a

negative correlation with overall survival in Hodgkin's Lymphoma [418]. The difference between GzmB-sensitive and GzmB-insensitive tumors may also be due to their susceptibility to apoptosis by GzmB+ cells, either by down regulating surface MHC or by inducing dysfunction or exhaustion in the GzmB+ TIL population. However, overall there is some promising data that at least in some carcinomas, infiltration of CD8+ T cells and expression of GzmB protein is associated with positive outcomes. Whether this would also be seen in my EG.7 model, itself a T cell-origin lymphoma, remained to be seen. When I began my research, I had hoped that FasL may be some kind of prognostic indicator, but of course in a mouse tumor model where the "patients" are euthanized at the time of tumor sampling, I cannot get the same "prognostic" power. However, I wanted to know whether I could find evidence that FasL expression alone or in combination with GzmB would be associated with some stage of solid tumor progression. As FasL can kill cells under conditions that do not elicit degranulation [354], it seemed plausible that FasL may be associated with tumor progression or regression in a manner independent of degranulation/GzmB staining. In one small cell lung cancer experiment, FasL was found expressed on TIL but not on tumor cells, but that this FasL expression score was not associated with long-term survival in patients [419]. However, as evidenced by the inconsistencies in Immunoscore findings summarized above, this may be an exception, rather than the rule.

When analyzing subcutaneous TIL by flow cytometry, I did not find any significant correlation between tumor volume of subcutaneous EG.7 tumors and the proportion of CD8+ T cells positive for intracellular FasL alone, FasL in combination with GzmB, GzmB alone, or neither mechanism, I also did not find a relationship

between tumor volume and GzmB or FasL MFI of TIL (data not shown). In my model, static storage of these effector mechanisms is not associated with tumor progression. However, location of FasL+ and/or GzmB+ CD8+ cells within the tumor may play a major role. How close are FasL+ CD8+ cells to the tumor margins, or to the vascular endothelium? While flow cytometry has the advantage of high throughput, there is no spatial context within the tumors for assessing infiltration of these GzmB+ FasL+ into the tumor, so future examination by histology would add value to these findings.

Cell-surface FasL is significantly correlated with tumor volume (Fig 4-16C). I have already discussed above the possibility that these may be presented by CD8+ Treg as a survival mechanism, but in addition it may be that tumor cells are subverting CTL FasL expression for their own survival. It has recently been shown that Fas signaling to colorectal cells induces stemness (epithelial to mesenchymal transition), though it appears that the Fas Ligand is presented by the cancerous epithelial cells themselves [420-422]. I did not examine my tumor cells for morphological changes or differentiation, but it is an interesting possibility that FasL on the CTL contribute to the expansion of the tumor itself. It may also be that high surface FasL presentation by CD8+ T cells in the tumor is harming the antitumor response and promoting tumor survival by indirectly recruiting Myeloid-derived suppressor cells (MDSC). This has been suggested by a mouse lung tumor model to be possibly mediated by T cell presentation of FasL to Fas on tumor cells, which turns on Prostaglandin E2 (PGE2) secretion by tumor cells, which can recruit MDSC to the tumor site [292]. Movahedi and colleagues have shown that MDSC can emerge in subcutaneous EG.7 tumors in mice, but that the M-MDSC population (which would differentiate to suppressive monocytes) is inefficient at T cell

suppression *in vitro* compared to M-MDSC derived from another murine tumor, BW-Sp3 [285]. Taken together, it may be that there could be increased MDSC recruitment associated with the elevated FasL expression by CTL in the tumor, but whether these are actively suppressive MDSC is yet to be determined. It would be interesting for future researchers to see in this model whether MDSC are present in high numbers, and whether Fas-deficient tumors have different MDSC recruitment. It is possible that FasL and GzmB are damaging tissue in addition to being part of the immune response. Regardless of the activity of FasL within the tumor itself, its positive correlation to tumor volume may at least be used as an indicator of CD8+ T cell activity, even if not productive for anti-tumor clearance.

Future Directions

Is the presence of FasL on CD8+ T cells associated with any apoptosis *in situ* in the tumor? Is apoptosis in tumors, or in lymphocytes? It would be appropriate to compare this in implanted tumors of known Fas-resistant and Fas-sensitive cell tumor lines along with samples from human patients whose disease progression can be tracked. Note that for human samples, Toomey *et al* have described testing of certain clones that work better than others for immunohistochemistry of FasL [419].

I hope that future researchers investigate whether the cells with high cell surface FasL are the same as those high for GzmB and/or intracellular FasL. This would help clarify whether cytokines or *in vivo* factors that affect surface FasL translocation are simultaneously affecting GzmB production or storage. However, proof of concept experiments that I conducted never resulted in a satisfactory protocol to distinguish surface and intracellular FasL by flow cytometry. Were a BV421 or similarly bright

conjugate of the anti-FasL MFL3 clone to become available for costaining with the PE conjugate of the same clone (surface versus intracellular), it would be a good idea for future researchers to revisit this question. Of course, this could also be visualized through confocal microscopy.

One natural progression for my research would be to examine FasL and GzmB in the context of cancer immunotherapy. I had aspirations to combine cytokine culture conditions and the phenotyping data I saw *in vivo* with adoptive cell therapy experiments in mice, similar to those published by Diaz-Montero and colleagues [147, 308] where CTL are conditioned by cytokines in culture, but a good model was not developed fully in time and at the appropriate scale to be included in this thesis. However, I can suggest some directions to take for future researchers who wish to continue this. In cells that do get transferred into mice, does the FasL expression change over time in the memory population as the tumor gets cleared? Since I have ascribed FasL and GzmB in TIL numerous potential roles in the above sections, it would be useful to see whether any of them are affected by cellular immunotherapy or checkpoint blockade. For example, if FasL were used by CD8+ Treg to dampen antitumor responses, would one lose or reduce surface FasL population in TIL following checkpoint blockade? Furthermore, comparative functional studies of TIL extracted from tumors to determine whether they can kill tumor cells in degranulation and/or FasL dependent or independent manner, or use FasL to suppress or differentiate other CD8+ T cell populations would illuminate the functional roles I have suggested may be associated with the FasL/GzmB expression phenotypes. Does checkpoint blockade have deleterious effects on some of these functions from the TIL?

I have presented data in this thesis that shows the heterogeneous nature of FasL and GzmB storage in tumor-infiltrating and activated cells. I found vastly different FasL and GzmB coexpression phenotypes between CD8+ T cells isolated directly from tumors and cells activated from naïve cells *in vitro* and cultured with extrinsic cytokines. Activation conditions and presence near tumors can sway whether a CD8+ cell will express FasL or GzmB, and this is also variable within memory or effector differentiated cells. Next, we will need to examine what the biological utility of these diverse populations is, and whether they harm or help the antitumor response. From there, we can better adjust our immunotherapies to exploit or suppress these effector proteins.

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