Development and Function of Tissue-Resident Memory CD8$^+$ T Cells

By

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List of abbreviations

Ag	Antigen
AKT	Protein Kinase B
AMP	Adenosine Monophosphate
APC	Antigen Presenting Cell
APL	Altered Peptide Ligand
ATAC	Assay for Transposase-Accessible Chromatin
BSA	Bovine Serum Albumin
BCR	B cell receptor
CD	Cluster of Differentiation
CCL	Chemokine (C-C motif) Ligand
CCR	C-C motif Chemokine Receptor
CFSE	Carboxyfluorescein Succinimidyl Ester
cGAMP	Cyclic GMP-AMP
cGAS	Cyclic GMP-AMP Synthase
CoA	Coenzyme A
CXCL	Chemokine (C-X-C motif) Ligand
CXCR	C-X-C motif Chemokine Receptor
DNA-PK	DNA-dependent Protein Kinase
DTH	Delayed Type Hypersensitivity
EFC	Entry Fusion Complex
EV	Extracellular Virion
FAO	Fatty Acid Oxidation
FBS	Fetal Bovine Serum
GFP	Green Fluorescent Protein
gMFI	Geometric Mean Fluorescence Intensity
GMP	Guanosine Monophosphate
GPCR	G-protein Coupled Receptor
GSEA	Gene Set Enrichment Analysis
HIV	Human Immunodeficiency Virus
HSV	Herpes Simplex Virus
IBD	Inflammatory Bowel Disease
IFN	Interferon
IFNAR	Interferon alpha/beta Receptor
IL	Interleukin
IRF	Interferon Regulatory Factor
iSALT	Inducible Skin Associated Lymphoid Tissue
ISG	Interferon Stimulated Gene
JAK	Janus Activating Kinase
KLRG1	Killer-cell Lectin like Receptor G1
LC	Langerhans Cell

LCMV	Lymphocytic Choriomeningitis Virus
LEC	Lymphatic Endothelial Cell
LN	Lymph Node
MHC	Major Histocompatibility Complex
MV	Mature Virion
NF-kB	Nuclear Factor Kappa-light-chain-enhancer of Activated B Cellls
NKT	Natural Killer T cell
OAS	Oligoadenylate Synthase
PBS	Phosphate Buffered Saline
PFU	Plaque Forming Unit
PI3K	Phosphoinositide 3-kinase
PKR	Protein Kinase R
PRR	Pattern Recognition Receptor
qPCR	Polymerase Chain Reaction
RGS	Regulator of G-protein Signaling
S1P	Sphingosine-1-phosphate
SD	Standard Deviation
SEM	Standard Error of the Mean
STAT	Signal Transducer and Activator of Transcription
TCA	Tricarboxylic Acid Cycle
TCM	Central Memory T cell
TCR	T Cell Receptor
TEM	Effector Memory T Cell
TGF-β	Transforming Growth Factor Beta
TK	Thymidine Kinase
TLR	Toll-like Receptor
TNF	Tumor Necrosis Factor
TRAIL	TNF-related Apoptosis-inducing Ligand
TRM	Tissue-resident Memory T cell
VACV	Vaccinia Virus
YFP	Yellow Fluorescent Protein

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Abstract

Tissue-resident memory (T_{RM}) $CD8^+$ T cells permanently reside within non-lymphoid tissues where they provide a first-line of defense against invading pathogens. However, the mechanisms regulating their development and the long-term functional consequences following their activation in situ are poorly defined. Here, I use a model of epicutaneous Vaccinia virus infection to investigate two main research questions regarding the development and function of T_{RM} $CD8^+$ T cells. I begin by describing the role that antigen recognition within the skin microenvironment plays in the development of T_{RM} $CD8^+$ T cells. Next, I determine how repeated antigen encounters by mature T_{RM} $CD8^+$ T cells impacts the composition and function of the T_{RM} population.

To determine mechanistically how T cell receptor (TCR) engagement regulates T_{RM} formation, I developed an interferon-gamma (IFNγ)-YFP reporter system and found that only a fraction of antigen-specific $CD8^+$ T cells transiently produced IFNγ in the skin during viral infection in an antigen-dependent manner. Transcriptomic profiling revealed that TCR-signaling promoted T_{RM} differentiation and elevated metabolic activity, while suppressing gene networks that control tissue egress and the development of circulating memory T cells. Notably, I identified Blimp1 as a critical target downstream of TCR-dependent signaling within the skin microenvironment to enforce tissue-retention and T_{RM} differentiation. Thus, these findings show that access to antigen and strength of TCR signaling within non-lymphoid tissues are key factors regulating the acquisition of the tissue-residency transcriptional program.

Following their formation, mature T_{RM} $CD8^+$ T cells are able to accelerate local clearance of secondary infections, but whether these specialized T cell popula-

tions can be readily boosted to increase protective immunity is poorly understood. Here, I demonstrate that repeated activation of T_{RM} $CD8^+$ T cells using only topical application of antigenic peptide caused a delayed-type hypersensitivity reaction and increased the number of antigen-specific T_{RM} $CD8^+$ T cells specifically in the challenged skin by approximately 15 fold. Expanded T_{RM} $CD8^+$ T cells in the skin were derived from memory T cells recruited out of the circulation that became $CD69^+$ tissue-residents following a local antigen encounter. Notably, recruited circulating memory $CD8^+$ T cells of a different antigen-specificity could be coerced to become tissue-resident using a dual peptide challenge strategy. Expanded T_{RM} $CD8^+$ T cells significantly increased anti-viral protection, suggesting this could be a strategy to rapidly boost tissue-specific cellular immunity. Taken together, the data presented in this thesis support a model where antigen-dependent formation of T_{RM} $CD8^+$ T cells is an iterative process that boosts T_{RM}-mediated immunity specifically at sites of previous pathogen encounter.

Chapter 1

Introduction

1.1 General T cell background

Naive T cell activation and generation of memory T cells

Immunological memory is mediated by the adaptive immune system, which is comprised of B- and T-cells. Both types of lymphocytes encode an antigen receptor that undergoes recombination during their development (B cell receptor (BCR) and T cell receptor (TCR), respectively), thereby allowing them to respond to a nearly unlimited number of antigens rather than the relatively small number of conserved features of pathogens that are detected by innate immune cells[1]. B-lymphocytes develop from hematopoetic precursors in the bone marrow and are able to recognize extracellular antigens and contribute to protective immunity through the secretion of antibodies. In contrast, T cells develop in the thymus and recognize short peptides that are presented on the surface of cells by major histocompatibility complexes (MHC). T cells are generally divided into two main categories based on which class of MHC molecule they are able to recognize; $CD4^+$ T cells are MHC-II restricted, while $CD8^+$ T cells are MHC-I restricted[2]. Following their development in the thymus, naïve $CD4^+$ and $CD8^+$ T cells enter the circulation and actively survey secondary lymphoid organs for antigen-presenting cells (APCs) displaying cognate peptide-bound major histocompability complex(pMHC)[3]. T cells are carried throughout the body in the circulatory system, and extravasate into either lymph

nodes or non-lymphoid tissues in a three step process called extravasation. Initially, relatively low affinity interactions between glycosylated ligands and selectin receptors cause T cells to slow down and 'roll' along the endothelial cells. Integrin activation then mediates the firm adhesion and arrest of T cells. Finally, T cells rearrange their cytoskeleton and 'squeeze' between endothelial cells and into the tissue parenchyma in a process known as transendothelial migration. Thus, T cells use the blood vasculature to survey the entire body, and extravasation into either lymph nodes or non-lymphoid tissues is regulated by the coordinated expression of selectins, integrins, and chemokine receptors by both T cells and endothelial cells. Naive T cells are quiescent and their function is essentially limited to surveillance of lymph nodes for APCs displaying cognate pMHC complexes[3]. Antigens are continuously delivered to lymph nodes in several ways, including active transport by migratory APCs and passive flow in lymph. Antigens that passively drain in lymph are then phagocytosed by APCs resident within lymph nodes. In both cases, this system allows the population of rare, antigen-specific naïve T cells to respond to infections that occur within non-lymphoid tissues despite their limited range of immunosurveillance[4]. For example, the skin contains a specialized population of APCs called Langerhans cells that are able to detect pathogens and migrate from the skin into the skin-draining lymph node, where they are able to activate naive T cells. Because T cells are highly potent in their capacity to cause inflammation and kill target cells, there is a high threshold of activation that prevents inadvertent tissue damage. Thus, activation of naive T cells requires three signals - recognition of cognate peptide-MHC through the TCR, co-stimulatory interactions such as stimulation through CD28, and the presence of inflammatory cytokines, such as interleukin-12 or type I interferon.

Following activation, naïve T cells undergo proliferative clonal expansion and differentiation to generate a large population of effector cells. During this process of ex-

pansion, widespread epigenenetic remodeling allows effector cells to execute specific effector functions that contribute to the ongoing immune response[5, 6]. The effector functions of $CD4^+$ T cells are quite varied and lineage-specific, ranging from providing isotype switching signals to antibody-producing B cells (folicular helper T cells) to dampening immune responses through secretion of anti-inflammatory cytokines (regulatory T cells)[7]. In contrast, $CD8^+$ T cells have relatively limited effector functionality that is largely limited to direct cytolysis of target cells and expression of inflammatory cytokines[8]. There are multiple mechanisms of cytotoxicity used by $CD8^+$ T cells, including Perforin-mediated delivery of cytotoxic granules to target cells and activation of death receptors through the expression of Fas ligand, tumor-necrosis factor α (TNFα), or TNF-related apopotosis-inducing ligand (TRAIL)[9, 10]. All of these mechanisms converge on the activation of caspases that ultimately cause the target cell to undergo apoptosis. In addition to direct killing, $CD8^+$ T cells secrete inflammatory cytokines such as interferon gamma (IFNγ), which causes nearby cells to express anti-viral genes and serves to prevent pathogen spread[11]. Because pMHC-TCR interactions require direct cell-to-cell contact, effector T cells must be able to infiltrate infected non-lymphoid tissues to perform these effector functions. Thus, unlike naïve T cells, effector cells express adhesion molecules and chemokine receptors that allow their trafficking into inflamed tissues[3]. Although potent in their capacity to fight infections, effector cells are very short-lived and the majority of effector T cells will undergo apoptosis approximately 1-2 weeks after activation in a process known as contraction[12]. The long-lived T cells that survive the contraction phase are called memory T cells, and these populations are able to execute effector functions and undergo proliferation in response to TCR stimulation alone. Critically, memory T cells are present at higher frequencies than the pool of antigen-specific naive T cells, allowing them to detect and respond to secondary infections more efficiently. These features of memory T cells allow them

to provide protective immunity against a wide variety of viral, bacterial, and parasitic infections.

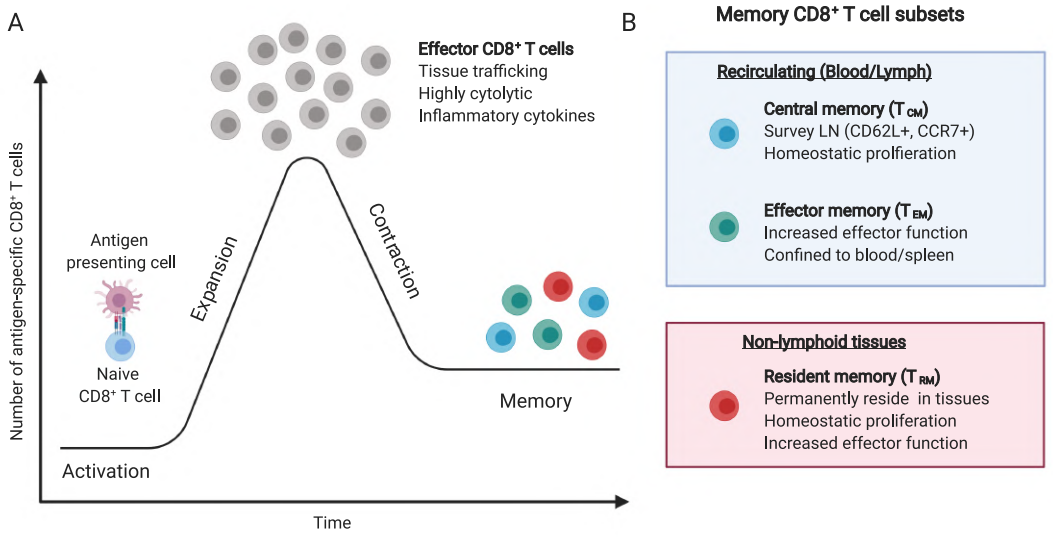

Figure 1.1: Activation of naïve CD8⁺ T cells and generation of memory
(A) Activation of naïve CD8⁺ T cells occurs in the lymph node and initiates a program of proliferative expansion, generating a clonal population of effector CD8⁺ T cells that are able to execute specific effector functions that contribute to pathogen clearance. Following the short-lived effector phase (1-2 weeks), the vast majority of effector CD8⁺ T cells undergo apoptosis in a process called contraction. Cells that survive contraction comprise the memory population. (B) The total memory CD8⁺ T cell population is comprised of distinct subsets that can be broadly categorized based on migration patterns. T_{CM} and T_{EM} are found in the circulation, while T_{RM} cells premanently reside within tissues. Figure is adapted from **Nolz, Butler, and Harty, *Cell Microbiol*., 2011**.

Memory CD8⁺ T cell subsets

In early studies, T cells were typically isolated from the blood, primarily due to sample availability and convenience. In a landmark paper, Sallusto et al found that memory T cells in human blood were heterogenous and could be divided into functionally distinct subsets based on expression of lymph-node homing C-C chemokine receptor (CCR7)[13]. Cells that lacked CCR7 displayed higher levels of Perforin and secreted high levels of the pro-inflammatory cytokine IFNγ upon ex vivo stimulation, resulting in the label of "effector memory" T (T_{EM}) cells. In contrast, CCR7⁺ cells expressed low levels of Perforin and IFNγ but had higher expression

of another lymph-node homing molecule, L-selectin (CD62L), and proliferated extensively following restimulation, leading to the label of “central memory” (T_{CM}) T cells. Although T_{EM} cells did not express lymph node homing molecules, they did express higher levels of chemokine receptors and adhesion molecules involved in the migration of T cells into non-lymphoid tissues. These observations led to a model in which T_{EM} cells were proposed to actively survey non-lymphoid tissues and T_{CM} cells were thought to limit immunosurveillance to lymphoid organs.

Based on this model, T cells isolated from non-lymphoid tissues were assumed to be T_{EM} cells that were surveying the tissue and would return to the circulation through the lymphatic system. However, certain features of T cells isolated form tissues were inconsistent this model. For example, CCR7 plays an important role in tissue egress, raising the question of how T_{EM} cells (defined by their lack of CCR7 expression) would re-enter the circulation following their recruitment into non-lymphoid tissues[14]. Additionally, T_{EM} and T_{CM} cells were equally efficient at homing to the lung following adoptive transfer into naïve hosts, suggesting that non-lymphoid tissue surveillance is not restricted to either subset alone[15]. Further, T cells isolated from non-lymphoid tissues expressed a different suite of surface markers than any individual cell type found in the circulation, suggesting the possible existence of a tissue-resident (T_{RM}) subset of memory T cells[16–18]. The molecules CD69 and CD103 were very rarely expressed by circulating cells but were nearly always expressed by T cells isolated from non-lymphoid tissues, suggesting that T_{RM} cells could be identified by these markers. Transplantation studies confirmed the resident nature of this T cell subset by showing that $CD69^+$ $CD103^+$ T cells remained within the grafted tissue and could not be recovered from the circulation[18]. Since their discovery, T_{RM} cells have been shown to provide enhanced protective immunity against a wide variety of pathogens, as well as having a pathogenic role in several autoimmune diseases[19–21]. These important

functions have generated a strong interest in understanding the mechanisms that regulate their development and protective capacity, with the ultimate goal of enhancing T_{RM} formation in vaccine design and inhibiting T_{RM} function in autoimmune diseases. In this thesis, I examine the development and function of T_{RM} $CD8^+$ T cells, using a model system of Vaccinia virus skin infection.

1.2 Vaccinia Virus

Poxvirus life cycle

Poxviruses are a large family of dsDNA viruses that are responsible for several important human diseases, including smallpox, monkeypox, and molluscum contagium[22]. In addition to their relevance to human health, poxviruses are powerful laboratory tools due to their use as an expression vector and their ability to induce robust immune responses in laboratory animals[23]. Poxviruses are characterized by the large size of their genome and their unique life cycle, which involves replication within the cytoplasm and the production of two distinct forms of infectious virus – mature virions (MVs) and extracellular virions (EVs) [24]. The production of two distinct virions is a feature shared by all poxviruses, but they are best-studied in the laboratory model poxvirus VACV. MVs consist of a unique biconcave core structure that contains the viral genome and is flanked by two protein-rich 'lateral bodies' that are immediately deployed into the cytosol following entry[25]. EVs consist of an MV that is enclosed in an additional lipid bilayer membrane that contains a distinct set of proteins from the MV envelope. During infection, EVs either remain attached to the infected cell or are exported into the extracellular environment, while MVs are only released upon cell lysis[26]. One intriguing hypothesis to explain this dimorphic nature of the poxvirus life cycle is that MVs are responsible for transfer between animals, while EVs are responsible for viral spread within an infected animal. Consistent with this idea, EVs contain fewer viral proteins on

its surface and are the dominant form in the circulation of infected animals, suggesting that the production of EVs may be an evolutionary strategy to 'cover' the antigens on the surface of MVs[27, 28]. Studies in rabbits have shown that antibodies directed against EV are more protective than antibodies against MV-specific targets, and the neutralizing antibody response in humans immunized with VACV is strongly biased towards proteins present only on MVs[29, 30]. Although the role of these distinct forms of VACV are largely speculative, it is clear that both forms are produced during infection in a process that is exclusively found in poxviruses.

Due to their large size, cellular entry of both MVs and EVs is dependent on macropinocytosis[24, 31]. In vitro studies have identified several virus-host protein interactions that mediate attachment of MVs, but the contribution of these interactions in vivo is not well understood, and the factors that lead to EV attachment and shedding of its extra membrane are almost entirely unknown[25]. Regardless, both forms of VACV induce membrane 'blebbing' and actin rearrangements characteristic of macropinocytosis, and ultimately end up within endosomes[32, 33]. Entry into the cytoplasm occurs following acidification of the endosome, and is dependent on a group of 12 proteins within the MV envelope, termed the entry fusion complex (EFC)[34]. Activation of the EFC delivers the viral genome and lateral bodies into the cytoplasm, which includes all of the machinery required for RNA synthesis, as well as a capping enzyme, poly(A) polymerase, and 2'-O-methyltransferase, such that mRNA molecules can be produced within the viral cores immediately following cell entry[35].

Similar to many other virus families, poxvirus genes can be categorized based on their expression kinetics. The temporal regulation of early, intermediate, and late genes is accomplished through a cascade mechanism, where early genes contribute to expression of intermediate genes, and intermediate genes contribute to

the subsequent expression of late genes, and finally, late gene products are packaged into virions and allow immediate expression of early genes upon the next round of infection. All VACV genes are transcribed by the virally-encoded RNA polymerase, which can pair with a variety of transcription factors that recruit the RNA-polymerase complex to specific promoter sequences that are associated with early, intermediate, and late expression[36]. Expression of early genes is regulated by a single transcription factor, named 'early transcription factor', while several transcription factors can initiate intermediate and late gene expression. Functionally, early genes generally serve to initiate genome replication and evade host immune defenses, while intermediate and late genes are generally involved in virion assembly[36].

Following the deployment of the viral core into the cytoplasm, VACV genome replication occurs in cytoplasmic 'viral factories' that are adjacent to the nucleus. The precise mechanism by which VACV DNA replication occurs is not clear, but DNA hairpins at the end of the genome support a self-priming model of replication where hairpins provide a free 3' end for the VACV-encoded DNA polymerase, E9[35]. DNA replication is actually a requirement for the expression of intermediate and late genes, and the newly replicated viral genomes are thought to act as the template for transcription of these genes[36]. As intermediate and late genes are expressed, crescent-shaped structures begin to form within the viral factories. These spherical structures contain at least one lipid bilayer, and continue to grow in length until they form a complete sphere[24]. Following the formation of these immature virions, the VACV protease I7 cleaves several viral core proteins, resulting in the formation of mature virions[37]. A small number of MVs are then wrapped in two layers of host-derived membranes and delivered to the cell surface as an EV, while assembled MVs are released upon cell lysis.

Immunomodulatory functions of VACV

The large number of virally encoded proteins and the cytoplasmic site of replication provides ample opportunities for immune detection of poxvirus infection. To evade this host response, poxviruses devote approximately 1/3-1/2 of their genomes to genes with immunomodulatory functions[38]. The functions of poxvirus immunomodulatory genes are best studied in VACV, but this species is largely limited to the laboratory and is rarely found in natural outbreaks[39]. It is likely that more virulent poxviruses found in nature have retained more genes with immunomodulatory function[40]. Regardless, this astounding allocation of genetic material highlights the important role of immunomodulation during poxvirus infection, and enables poxviruses to interfere with nearly every level of host defense.

Viruses are often detected by the innate immune system through the activation of pattern recognition receptors (PRRs) that detect conserved structural features or replication intermediates. PRRs are located on the cell surface, within endosomes, and in the cytoplasm, which provides opportunities for detection of pathogens before, during, and after cell entry. Activation of PRRs initiates signaling cascades that ultimately converge at the activation of NF-kB, IRF3, and/or IRF7, which then can drive expression and secretion of pro-inflammatory type I interferons[41]. Upon ligand binding, the type I IFN receptor (IFNAR) dimerizes and activates the JAK1/TYK2 kinases, which then phosphorylate STAT1 and STAT2, ultimately leading to transcription of a large class of IFN-stimulated genes (ISGs)[42]. Many of these ISGs are involved in anti-viral defenses, and nearly every cell expresses IFNAR, such that pathogen detection (and subsequent secretion of type I IFN) by one cell creates an anti-viral state in the surrounding cells, thereby preventing pathogen spread.

VACV has evolved to combat these innate immune defenses at nearly every step,

beginning with pathogen detection. As VACV is a dsDNA virus that resides in the cytoplasm, it has the potential to activate cytoplasmic nucleic acid sensors such as cyclic GMP-AMP synthase (cGAS) and DNA-dependent protein kinase (DNA-PK) (DNA sensors) or oligoadenylate synthase (OAS) and protein kinase R (PKR) (RNA sensors). To counteract this detection pathway, the VACV protein E3 contains both a dsDNA binding domain and a dsRNA binding domain that sequester these ligands away from cytoplasmic PRRs[43]. Other VACV proteins, such as C4 and C16, bind Ku, the DNA-sensing subunit of DNA-PK, thereby preventing the formation of a functional DNA sensor[44]. Beyond inhibiting the initial detection of nucleic acids, VACV also encodes proteins that interfere with the signaling pathways downstream of PRR activation. For example, the VACV protein B3 degrades 2'-3' Cyclic guanosine monophosphate–adenosine monophosphate (cGAMP), which is a small molecule second messenger that is synthesized by the dsDNA sensor cGAS and drives type I IFN expression[45]. In addition to these cytosolic nucleic acid sensors, VACV also encodes a large number of genes that inhibit signaling pathways downstream of toll-like receptor (TLR) activation. Interestingly, nucleic acid sensing TLRs do not appear to be activated by VACV, but TLR2 and TLR4 have been demonstrated to play protective roles against VACV infection[46, 47]. These TLRs typically are activated by structural components of microbial cell walls (e.g. lipopolysaccharide is a strong activator of TLR4)[48], suggesting that components of the VACV virion other than nucleic acids are also sensed by innate immune cells. However, TLR2 and TLR4 can be activated by a wide variety of bacterial and fungal structural components[48], and the molecular components of the VACV virion that interact with TLR2 and TLR4 are not known. Regardless of which part of the virion is detected by TLRs, the large number of VACV-encoded genes that interfere with TLR signaling suggests that TLR-dependent detection of VACV infection plays an important role in host defense[49]. Lastly, many of these PRR

signaling pathways converge at the activation of transcription factors that drive type I IFN expression (such as nuclear factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and interferon regulatory factor 3 (IRF3)), which generally are mediated by phosphorylation events that allow the transcription factors to translocate to the nucleus. VACV prevents these final steps through a variety of different mechanisms that mainly involve sequestration of the active forms in the cytoplasm or stabilization of inhibitory proteins that prevent nuclear translocation[38]. Thus, there are many layers of VACV-mediated immunomodulation that the immune system must overcome in order to successfully express type I IFNs and initiate an anti-viral response.

However, if these interference pathways are not effective and type I IFNs are produced (or are produced by an uninfected cell), VACV has additional strategies to prevent the induction of ISGs. First, during late stages of infection, VACV expresses decoy type I IFN receptors that are secreted from infected cells and prevent type I IFNs from reaching other nearby host cells[50]. Second, VACV encodes a phosphatase (VH1) that dephosphorylates STAT1 and STAT2 heterodimers, thereby preventing gene expression downstream of IFNAR signaling[51] (as well as downstream of the type III IFN receptor, which is not thought to play a significant role in VACV infection). VH1 also dephosphorylates STAT1 homodimers, which are generated downstream of type II IFN receptor ligation (IFNγ receptor), thus extending VACV immunomodulatory activity to the adaptive immune system[51]. Apart from IFNγ receptor signaling, VACV inhibits adaptive immunity by secreting a decoy IFNγ receptor and several chemokine-binding proteins that prevent the recruitment of B and T cells to the site of infection[52, 53]. Lastly, VACV also inhibits MHC-II antigen-presentation, thereby potentially inhibiting both $CD4^+$ T cell activation and their ability to identify target cells[54, 55]. However, despite the impressive number of immunomodulatory genes devoted to inhibition of the innate and adaptive

immune response, VACV remains a highly immunogenic virus that has potential to be used as a powerful vaccine vector[56].

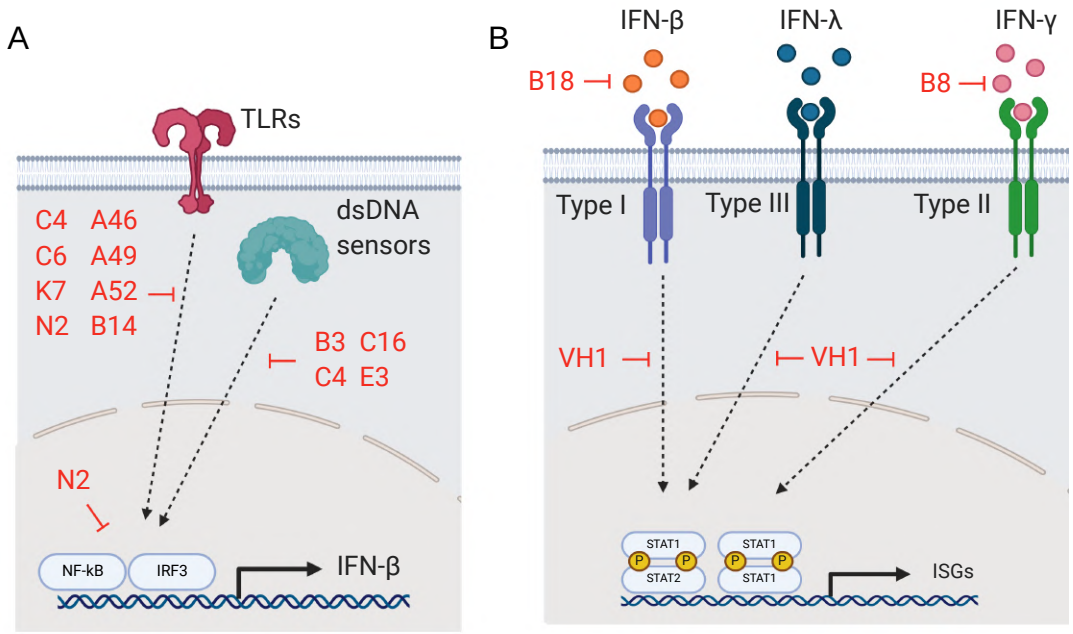

Figure 1.2: Immunomodulatory functions of poxviruses
(A) Viral recognition by either TLRs or intracellular nucleic acid sensors initiates signaling cascades that culminate with the activation of transcription factors such as NF-kB and/or IRF3 to drive expression of IFN-β. VACV encoded proteins that interfere with this detection pathway are indicated in red. (B) VACV also encodes proteins that prevent the action of IFNs on nearby cells by secreting decoy cytokine receptors or by dephosphorylating STAT1/2 dimers downstream of IFN receptor activation.

Use of VACV as a vaccine vector

VACV is the virus that was used to immunize against smallpox, and it is the only human vaccine to date that has resulted in the elimination of its target pathogen[57]. Given the success of VACV in eradicating smallpox, it was reasoned that recombinant VACV strains could be used to immunize against other pathogens of interest by expressing a given antigen in a VACV vector. Because of its large genome size, up to 25kb can be inserted into the viral genome, meaning that multiple recombinant antigens can be expressed simultaneously. The characterization of early, intermediate, and late promoters also allows for fine tuning of the expression of

recombinant proteins in VACV vectors. For most immunology studies, an early promoter (such as the very commonly used, naturally occuring p7.5 promoter) are used in order to avoid effects of virally-induced cytotoxicity and to ensure that the recombinant protein is expressed, even in cells that are not permissive to VACV replication[58]. Further, promoters have been designed that incorporate both early and late promoter elements such that a recombinant gene is expressed throughout infection[59]. Similarly, the strength of expression can be manipulated by small changes in the promoter sequences that either enhance or inhibit expression. Together, all of these options make VACV vectors flexible in terms of level and timing of recombinant gene expression. Additionally, due to its replication within the cytoplasm, integration of VACV into the genome and inadvertent activation of an oncogene is extremely unlikely. Logistically, VACV is very temperature stable, easing the practical concerns of vaccine delivery and storage[60]. Lastly, despite the large number of immunomodulators in its genome, VACV still generates a robust humoral and cellular immune response against foreign antigens[57]. These features make VACV a very attractive potential vaccine vector, and there are clinical trials using VACV-based vectors against HIV-1, Malaria, Tuberculosis, and influenza, and VACV expressing rabies virus glycoprotein has been widely used in controlling rabies infection of wild animals[61, 62]. However, there have been several significant adverse events associated with VACV-immunization, which has led to development of attenuated VACV strains that are safe to use in humans. To this end, one extremely safe alternative to VACV is Modified Vaccinia Ankara (MVA), which was derived from VACV following over 500 serial passages in chicken fibroblasts[63]. MVA has lost approximately 30kb of the VACV genome and is replication-incompetent in human cells, making MVA an extremely safe alternative to more virulent VACV strains. Regardless, no VACV or MVA vector-based vaccine has been approved for human use, underscoring the need to continue to uncover the function of immune

evasion genes with the goal of developing vaccine vectors with increased safety and immunogenicity.

1.3 Skin as an immunological organ

Barrier function

The skin provides a physical barrier between the body and the outside environment and therefore plays a critical role in protection against chemical and pathogenic insults. This barrier function is mediated by keratinocytes, which comprise the vast majority of cells found in the epidermis. Keratinocytes are derived from basal stem cells that are physically attached to the basement membrane, and over the course of approximately 8-10 days in mice (40-55 days in humans), keratinocytes will progress through three distinct differentiation states as they migrate outward towards the interface with the environment – resulting in the formation of the stratum spinosum, stratum granulosum, and the outermost layer, stratum corneum[64, 65]. Each stage of differentiation serves a specific purpose that contributes to the barrier function of the epidermis as a whole. The stratum spinosum supports the resident immune cells that aid in pathogen detection, and as recently divided keratinocytes first move through this layer, they begin to express the proteins required for desmosome formation and express filamentous keratin proteins. Above that, in the stratum granulosum, desmosome-mediated tight junctions between keratinocytes create a barrier that prevents diffusion of molecules larger than 70 kDa into the body[66]. The stratum corneum is the outermost layer and consists of terminally differentiated keratinocytes called corneocytes. These cells are enucleated and are embedded in a dense matrix of lipids and filamentous keratin proteins that prevents water loss and represents a formidable barrier to incoming pathogens[67]. Thus, the epidermis represents a continually replenished front-line defense that efficiently protects the body from chemical insults and pathogen invasion.

Pathogen detection

Apart from providing a physical barrier, keratinocytes within the epidermis participate in detection of pathogens through expression of PRRs and the subsequent expression of inflammatory cytokines such as type-I IFN, TNFα, interleukin-33 (IL-33), and members of the IL-1 family[68, 69]. In addition to pathogen detection and production of inflammatory cytokines, the epidermis contributes to immunosurveillance by supporting the residence of both innate (Langerhans cells and $\gamma\delta$ T cells) and adaptive (mostly $CD8^+$ T_{RM} cells). Skin-resident $\gamma\delta$ T cells play an important role in wound healing in mice, but are very rare in humans[70]. However, both human and mouse skin contain Langerhans cells (LCs), which are a specialized subset of dendritic cells that are seeded in the skin during development. These cells are able to capture antigens that permeate into the stratum spinosum (the keratinocyte layer directly above the basal stem cell layer), cross the basement membrane, and ultimately migrate to the lymph node where they are able to present antigens to naive T cells[71]. In contrast to LCs, adaptive immune cells are not seeded during development but instead differentiate following the resolution of infection or inflammation within non-lymphoid tissues. Within the skin, T_{RM} $CD8^+$ T cells generally localize to the epidermal layer adjacent to the basement membrane and exhibit a dendritic morphology that allows them to make frequent contacts with LCs, despite being densely embedded in a layer of keratinocytes[72]. In fact, recruitment of recently activated $CD8^+$ T cells to the epidermis is sufficient to generate a T_{RM} population, demonstrating the inherent support of T_{RM} $CD8^+$ T cell residence by keratinocytes in the stratum spinosum[73]. Thus, epidermal keratinocytes play a critical role in skin immunity by both actively detecting pathogens and supporting the residence of T_{RM} $CD8^+$ T cells.

Keratinocytes are the predominant cell type infected by VACV following skin scar-

ification, and replication occurs in discrete foci of infection throughout the epidermis[74]. VACV also infects a relatively small number of inflammatory monocytes that are recruited to keratinocyte lesions. Although cultured keratinocytes detect VACV infection through cytosolic nucleic acid sensing pathways, the pathways that are used by keratinocytes or inflammatory monocytes to elicit inflammation in vivo are not well understood[75, 76]. Interestingly, effector $CD8^+$ T cells are recruited to the borders of VACV infection foci in vivo, but are largely excluded from entering the lesion of infected keratinocytes. Rather, effector $CD8^+$ T cells directly kill inflammatory monocytes outside of the keratinocyte lesion, potentially to prevent viral dissemination throughout the host[74]. Combined with other studies, it is clear that $CD8^+$ T cells contribute to protective immunity, and the combination of $CD4^+$ and $CD8^+$ T cells are required for host survival following VACV infection by scarification[77].

In addition to epidermal defenses, the dermal layer of skin has also developed to enable effective pathogen detection and subsequent immune responses. Similar to Langerhans cells in the epidermis, professional antigen presenting cells (such as dendritic cells or macrophages) reside within the dermis and migrate to lymph nodes to present antigens to T cells[69, 71]. Apart from transporting antigens to the lymphatic system, the biology of the dermis is also uniquely set up to enable the recruitment of leukocytes into the skin through postcapillary venules, which exhibit increased permeability in response to inflammatory signals. This increased permeability allows high molecular-weight proteins (such as immunoglobulins) to permeate the tissue and also promotes the recruitment of circulating T and B cells[69, 78]. Within the skin, perivascular macrophages surround these postcapillary venules and secrete chemokines that attract dermal dendritic cells, thereby promoting the localization of professional APCs to the site of T cell extravasation[79]. These cellular clusters surrounding postcapillary venules are called inducible skin associated

lymphoid tissues (iSALT), and are critical for the induction of contact hypersensitivity responses against allergens[80]. However, as nearly every cell in the dermis and epidermis is able to express MHC-I and many skin cells can even express MHC-II in response to particular cytokines[81, 82], the role of iSALT in antiviral or antibacterial responses is not clear. Regardless, these physiological features of the dermis and epidermis make the skin well-suited for immune responses and an important layer of protection against environmental and pathogenic insults.

1.4 Tissue-resident memory T cell differentiation

Epithelial localization of T_{RM} $CD8^+$ T cells

In mouse models of viral infections, T_{RM} $CD8^+$ T cells are predominantly found in epithelial layers, particularly in barrier tissues such as the gut, skin, and lung[83–86]. Because the epithelium may allow access to specific signals (such as transforming growth factor beta (TGF-β)), it was proposed that migration into the epithelium may be a critical step in the T_{RM} differentiation process. This was confirmed using a herpes simplex virus (HSV-1) skin infection model, where expression of the chemokine receptor CXCR3 is required for both epithelial entry and subsequent upregulation of the T_{RM} markers CD69 and CD103[83]. This role of CXCR3-mediated epithelial migration also applies to the female reproductive tract, where topical application of the CXCR3 ligands CXCL9 and CXCL10 is sufficient to recruit effector $CD8^+$ T cells into the epithelium and generate a long-lived T_{RM} population[73]. Interestingly, effector $CD8^+$ T cells that do not express the terminal differentiation marker killer cell lectin-like receptor subfamily G member 1 (KLRG1) express higher levels of CXCR3 and are better able to migrate towards CXCL9 and CXCL10[83]. Adoptive transfer of purified $KLRG1^-$ or $KLRG1^+$ effector populations formally demonstrated that T_{RM} $CD8^+$ T cells are derived from $KLRG1^-$ effector cells. Thus, early studies in the skin and female reproductive tract demonstrated that CXCR3-mediated mi-

gration into the epithelial layer was required for the differentiation of $CD103^+$ T_{RM} $CD8^+$ T cells, and that similar to circulating memory T cells, T_{RM} $CD8^+$ T cell precursors are contained within the $KLRG1^-$ population. However, other models of viral skin infection such as VACV scarification have shown that T_{RM} $CD8^+$ T cells can also persist in the dermis, although these cells are less abundant and typically lack CD103 expression[19]. Human skin is rich with both $CD69^+/CD103^-$ dermal and $CD69^+/CD103^+$ epidermal T cells[87], suggesting that the epidermal localization may promote CD103 expression but is not strictly required for T_{RM} differentiation.

Transcriptional regulation of T_{RM} differentiation

In order to persist within non-lymphoid tissues, T_{RM} cells must adapt to their tissue of residence and upregulate pathways of tissue retention. These unique biological features suggest that T_{RM} $CD8^+$ T cells engage a different gene expression profile from memory T cells in the circulation. Indeed, transcriptional analyses of T_{RM} $CD8^+$ T cells isolated from a variety of both barrier and non-barrier tissues has revealed a core set of approximately 40 differentially expressed genes that distinguish circulating memory $CD8^+$ T cells and T_{RM} $CD8^+$ T cells[83]. Although this set of genes was defined in mice, human biopsy samples have identified T_{RM} $CD8^+$ T cells in lung, intestine, and skin that share many of these features of the core T_{RM} transcriptional program, suggesting this small set of T_{RM}-defining genes is largely conserved across species[88].

Within this small group of T_{RM}-defining genes, two main pathways stand out: repression of tissue egress and increased expression of adhesion molecules that enforce retention. Lymphocyte migration is largely mediated by G-protein coupled receptors (GPCRs) that sense chemotactic gradients[89]. While many studies have identified signals and receptors that mediate naïve T cell migration within lymphoid organs have been studied extensively, to date there are only two GPCRs that have

a known role in regulating $CD8^+$ T cell tissue egress: CCR7 and sphingosine-1-phosphate receptor 1 (S1PR1))[90]. The ligands for these receptors, CCL19 and CCL21 for CCR7, and S1P for S1PR1, are maintained at low levels in non-lymphoid tissues and at high levels in lymph, providing directional cues that guide T cell motility out of non-lymphoid tissues and thereby allowing them to recirculate[91, 92]. The gradients of these individual chemokines are maintained by two separate mechanisms. CCL21 is uniquely expressed and secreted by lymphatic endothelial cells (LECs), and binds to sulfated sugars in the extracellular environment, leading to high concentrations surrounding lymphatic vessels[93]. This gradient is sensed by both T cells and dendritic cells (DC) as they enter into afferent lymphatic vessels that drain non-lymphoid tissues such as the skin. CCL19 is also capable of binding CCR7, but is dispensable for both DC and T cell egress from the skin[94]. Similar to CCL21, S1P is actively secreted by LECs such that local concentrations of S1P are highest near the afferent lymphatic vasculature. However, in addition to being highly expressed by LECs, S1P concentrations are high in plasma due to high expression and secretion of S1P by red blood cells[95]. These gradients are further reinforced through the expression of the S1P-degrading enzymes, S1P lyase and lipid phosphate phosphatase 3 (LPP3). S1P lyase expression by parenchymal cells is required for establishing the gradient of S1P between tissues and blood or lymph, while LPP3 does not affect bulk S1P concentrations within tissues, but is instead uniquely expressed on basolateral surfaces of endothelial cells such that S1P gradients are maintained across endothelial cell layers[91, 95]. Similar to CCL21, both DCs and T cells use this S1P gradient to guide their motility to lymph nodes, and these chemokines also appear to play important roles in cellular positioning within lymph nodes[96–98]. Thus, the selective secretion of S1P and CCL21 by LECs, together with the selective degradation of S1P by parenchymal cells, establishes a gradient between lymph and tissues, thereby allowing T cells to re-enter

the circulation following tissue immunosurveillance.

Interestingly, CCR7 repression is not included in the core transcriptional profile despite experimental evidence that CCR7$^{-/-}$ effector T cells become T_{RM} cells in the skin more efficiently than their WT counterparts[83]. In contrast, S1PR1 is one of the most down-regulated genes in many T_{RM} populations and is included in the core T_{RM} CD8^{+} T cell transcriptional profile. Forced expression of S1PR1 prevents the formation of T_{RM} populations, demonstrating that repression of this particular S1PR family member is a critical step during T_{RM} differentiation[99]. Decreased S1PR5 expression is also included in the core transcriptional profile, but a functional role for this receptor has yet to be demonstrated[83]. Thus, repression of S1PR1 is a critical step in T_{RM} differentiation in all tissues, and the roles for other S1PR family members or other chemokine receptors such as CCR7 is less clear and may be dependent on the tissue being studied.

In addition to repression of tissue egress receptors themselves, T_{RM} CD8^{+} T cells also express genes that inhibit downstream signaling by these receptors. Cells have many different mechanisms of attenuating GPCR signaling, including 'regulator of G protein signaling' (RGS) proteins that increase the rate of GTP hydrolysis by the Gα subunit of heterotrimeric G proteins, thereby rendering the Gα subunit inactive[100]. Increased expression of Rgs1 and Rgs2 are included in the core T_{RM} transcriptional profile, suggesting that tissue egress is repressed at both the receptor and downstream signaling levels. Additionally, CD69 is one of the most widely expressed markers of T_{RM} CD8^{+} T cells and is commonly used in their identification[101]. Functionally, CD69 physically interacts with S1PR1 on the surface of cells and causes the internalization and degradation of both proteins[102, 103]. Therefore, it has been assumed that the near universal expression of CD69 by T_{RM} CD8^{+} T cells is yet another mechanism to repress S1PR1-mediated egress. How-

ever, transcript levels of CD69 are often not different between circulating memory and T_{RM} $CD8^+$ T cells[83, 99]. One possible explanation for this discrepancy is that repression of S1PR1 leads to decreased levels of S1PR1 protein on the cell surface, and therefore T_{RM} $CD8^+$ T cells display increased surface expression of CD69 protein even in the absence of increased transcription[104]. Although this precise role of CD69 has not been formally demonstrated in T_{RM} $CD8^+$ T cells, $CD69^{-/-}$ T cells generate T_{RM} populations that are approximately 10-fold smaller than their wild type counterparts, demonstrating that at least some level of CD69 expression is required for T_{RM} differentiation in the skin[83, 105]. Interestingly, the requirement for CD69 appears to be tissue-dependent, as $CD69^{-/-}$ T cells developed fewer T_{RM} cells in the lung and kidney following influenza infection, while T_{RM} development in the liver was not impacted. This phenomonenon also was dependent on the viral infection model, as $CD69^{-/-}$ T_{RM} cells developed normally in the lung (but not kidney) following acute lymphocytic choriomeningitis virus (LCMV) infection. Thus, prevention of tissue egress is a universal requirement for T_{RM} development, but the precise mechanisms used to inhibit tissue egress appear to vary depending on the infection and tissue being studied.

While inhibition of tissue egress is clearly an important feature of T_{RM} $CD8^+$ T cell differentiation, increased expression of adhesion molecules that promote tissue retention through physical binding of ligands is also widely observed in T_{RM} $CD8^+$ T cells[18, 106, 107]. For example, expression of CD103, which encodes the α_E integrin, is upregulated in the core T_{RM} transcriptional profile and is often used as a marker to identify T_{RM} $CD8^+$ T cells. The $\alpha_E\beta_7$ integrin pair can bind E-cadherin, which is present at adherens junctions within epithelial layers[108, 109]. It is therefore tempting to speculate that $\alpha_E\beta_7$ binding to E-cadherin is a mechanism to retain $CD8^+$ T cells within the epithelial layer, and recent studies suggest this interaction does occur between epithelial cells and T_{RM} $CD8^+$ T cells in the lung. However,

CD103 has also been implicated in directing cytotoxic polarization of T_{RM} $CD8^+$ T cells, and E-cadherin is also expressed by T_{RM} $CD8^+$ T cells themselves, suggesting that the precise role(s) of CD103 in the differentiation, maintenance, and function of T_{RM} $CD8^+$ T cells likely extend beyond binding epithelial cell junctions[110]. Another gene in the core transcriptional profile that is often used to identify T_{RM} $CD8^+$ T cells is α_1 integrin CD49a. Together with the β_1 integrin, this complex is known as very late antigen-1 (VLA-1) and is able to bind type IV collagen, which is highly abundant within the basement membrane[72, 111]. $CD49a^+$ T_{RM} $CD8^+$ T cells in the lung appear to migrate along the basement membrane, and CD49a deficient $CD8^+$ T cells are less motile than their wild type counterparts, suggesting that CD49a mediates T_{RM} motility[112]. However, the precise substrates used for motility and the role of any 'outside-in' signaling mediated by CD49a has not been examined in detail. Regardless of their role, $CD8^+$ T cells deficient in either CD103 or CD49a have a diminished ability to form long-lived T_{RM} populations, demonstrating that these genes are more than simply markers of T_{RM} $CD8^+$ T cells[83, 113].

In addition to these mechanisms of tissue retention that are shared by all T_{RM} $CD8^+$ T cells, there are also tissue-specific changes in gene expression that allow metabolic adaptation to a given tissue microenvironment[114]. This process is best studied in the skin, where T_{RM} $CD8^+$ T cells express fatty acid binding proteins (FABPs) 4 and 5, which facilitate the uptake of fatty acids and promote fatty acid oxidation (FAO)[115]. Genetic ablation of these receptors had no effect on the initial recruitment or early stages of differentiation, but did result in diminished survival of T_{RM} $CD8^+$ T cells, suggesting that the uptake of fatty acids and their subsequent use in FAO is required for T_{RM} maintenance in the skin. This metabolic dependency on FAO has also been observed in other non-lymphoid tissues despite their lack of expression of FABP4 and 5, which prompted researchers to test whether T_{RM} $CD8^+$ T cells use different isoforms of FABPs based on their tissue of resi-

dence[116, 117]. Indeed, following acute LCMV infection, development of liver T_{RM} $CD8^+$ T cells is dependent on FABP1, while intestinal T_{RM} $CD8^+$ T cells use FABP2 and FABP6[118]. Interestingly, the supernatant of different tissues was sufficient to induce changes in FABP isoform expression, offering a clear demonstration of T_{RM} $CD8^+$ T cell adaptation in response tissue-specific factors.

Transcription factors that regulate the T_{RM} transcriptional profile

Distinct functions of memory T cell populations are largely regulated by the expression of lineage-specific transcription factors[119–122]. One such transcription factor is Kruppel-like factor 2 (Klf2), which is highly expressed in naïve and T_{CM} $CD8^+$ T cells where it directly induces S1PR1 and CCR7 expression[123]. Because repression of these tissue-egress receptors is a critical step of T_{RM} differentiation, Klf2 repression was predicted to be required for the formation of T_{RM} $CD8^+$ T cells. Using Klf2-GFP reporter mice, it was found that effector $CD8^+$ T cells downregulate Klf2 expression following their infiltration into non-lymphoid tissues, leading to a subsequent decrease in S1PR1 and CCR7 transcript levels[99]. Similar to S1PR1, forced expression of Klf2 prevents T_{RM} formation, and decreased expression of Klf2 is included in the core T_{RM} transcriptional profile, demonstrating that Klf2 antagonizes T_{RM} formation through induction of tissue egress pathways.

Similar to Klf2, the T-box transcription factors T-bet and Eomesodermin (Eomes) are known to be important regulators of circulating memory $CD8^+$ T cell development[124]. In this context, these transcription factors have an antagonistic relationship, with T_{EM} cells expressing high levels of T-bet, and long-lived T_{CM} cells expressing high levels of Eomes[125]. In the skin and lung, T_{RM} $CD8^+$ T cells downregulate both T-bet and Eomes upon tissue entry, and this downregulation is required for expression of CD103[126]. However, while Eomes expression remains extremely low in mature T_{RM} $CD8^+$ T cells, a small increase in T-bet expression is

required for the maintenance of these cells. Mechanistically, this residual T-bet expression drives expression of CD122 (the IL-2/15Rβ chain), thereby allowing T_{RM} $CD8^+$ T cells to sense IL-15. This cytokine is required for the survival of T_{RM} $CD8^+$ T cells in the skin, but is not required in other organs such as the intestine[122, 127, 128]. Thus, the coordinated downregulation of Eomes and low level of T-bet expression play a critical role in skin-resident memory $CD8^+$ T cell formation and maintenance, but the extent that these transcription factors regulate T_{RM} cells in other tissues is unknown.

The role of Klf2, T-bet, and Eomes in T_{RM} differentiation was predicted based on their known roles in other populations of $CD8^+$ T cells. Using a more global, unbiased approach that involved integration of assay for transposase-accessible chromatin-sequencing (ATAC-seq) data, RNA interference screening, and computational predictions, Milner et al identified the transcription factor Runx3 as a potential central regulator of the T_{RM} transcriptional program[129]. Over-expression of Runx3 in effector $CD8^+$ T cells was sufficient to recapitulate the majority of the T_{RM} transcriptional profile, and Runx3 expression was required for T_{RM} development. Runx3 appears to play a role in both the differentiation and maintenance of T_{RM} $CD8^+$ T cells, as deletion at either early timepoints or at later timepoints, well after viral clearance, both resulted in defective T_{RM} formation. Further, T_{RM} formation following viral infection was impaired in a wide variety of non-lymphoid tissues and in tumor models, suggesting that Runx3 plays a universal role in regulating T_{RM} $CD8^+$ T cell differentiation. Interestingly, Runx3 is not differentially expressed between T_{RM} $CD8^+$ T cells and circulating memory $CD8^+$ T cells, suggesting that Runx3 activity is not regulated transcriptionally. The post-transcriptional mechanisms that govern Runx3 activity are not known, highlighting the knowledge gaps about how precursor cells initiate the T_{RM} differentiation process.

In addition to αβ $CD8^+$ T cells, several other innate lymphocyte cell populations are resident within non-lymphoid tissues, and these populations share many of the transcriptional features of αβ T_{RM} $CD8^+$ T cells[130]. However, the role of Klf2, T-bet, Eomes, and Runx3 were all largely defined in antigen-specific $CD8^+$ T cells using viral infection models. Therefore, investigators continued to search for transcription factors that controlled the transcriptional program shared among all tissue-resident lymphocyte populations. To this end, Mackay et al identified two transcriptional repressors, Blimp1 and homolog of blimp1 (Hobit), that act cooperatively to establish the transcriptional profile of multiple tissue-resident lymphocyte populations[122]. Mechanistically, Blimp1 and Hobit directly repress Klf2, S1PR1, and CCR7 in both liver-resident natural killer T (NKT) cells and in $CD8^+$ T cells, suggesting that repression of this tissue egress pathway is a universal feature of tissue resident lymphocytes. Interestingly, Blimp1 is expressed by effector $CD8^+$ T cells in both non-lymphoid tissues and in the circulation, where it has a known role in regulating the expression of effector genes. In contrast, Hobit expression was only detected in mature T_{RM} populations, suggesting that Hobit may be a master regulator of T_{RM} $CD8^+$ T cells[122, 131, 132]. However, deficiency in either transcription factor alone did not impact T_{RM} $CD8^{++}$ T cell formation, suggesting that Blimp1 and Hobit must act together to function as a master regulator of tissue-resident lymphocytes.

Signals that regulate T_{RM} differentiation

Alongside investigations into the transcriptional regulators of T_{RM} differentiation, significant effort has been directed towards identifying the molecular signals within non-lymphoid tissues that induce the expression and activity of those regulators. The discovery of universal regulators such as Runx3, Blimp1, and Hobit, and the fact that there is a conserved expression pattern of a core set of T_{RM}-defining genes suggests that there might be universal signal that regulates T_{RM} differentiation in

all tissues. To date, the cytokine TGF-β is the only universal regulator of T_{RM} differentiation that has been described, but TGF-β signaling does not directly contribute to the activity of the master T_{RM} regulators Runx3, Blimp1, or Hobit[83, 106, 133]. Critically, the upstream signals that cause expression of the master regulators are poorly defined, and represent one of the major knowledge gaps in T_{RM} biology.

TGF-β is constitutively expressed in nearly every tissue and is secreted in a latent form that requires activation by integrins, proteolytic cleavage, or reactive oxygen species. In the skin and gut, $\alpha_6\beta_6$ integrin can activate TGF-β, and deletion this integrin pair prevented T_{RM} $CD8^+$ T cell formation in both tissues[134]. Active TGF-β can signal through three distinct receptor complexes, TGF-BR1-3, although TGF-BR2 is thought to be the major site of TGF-β binding[135], and TGF-$BR2^{-/-}$ $CD8^+$ T cells fail to form mature T_{RM} populations in the skin, lung, and gut[83, 106]. Canonically, TGF-β binds to a TGF-BR2 dimer and recruits two TGF-BR1 subunits, forming a heterotetrameric complex that phosphorylates Smad2 and Smad3, which then are able to bind Smad4 and this Smad2/3/4 complex enters the nucleus to drive expression of TGF-β target genes[135]. Mechanistically, TGF-β exposure promotes T_{RM} development by driving CD103 expression and also causes repression of Klf2 and S1PR1[99, 136]. Interestingly, CD103 expression is Smad3 dependent, while the repression of Klf2 and S1PR1 appears to act through a non-canonical PI3K/Akt pathway[99]. Pro-inflammatory cytokines such as TNFα, IL-33, and type I IFNs act synergistically with TGF-β to repress the Klf2 and S1PR1 in vitro, but the role of these accessory cytokines in vivo is not clear, as type I IFNs do not impact T_{RM} formation in the skin and actually inhibit T_{RM} differentiation in the gut during Yersinia pestis infection[86, 105]. TGF-β clearly regulates T_{RM} development in nearly all non-lymphoid tissues, but the specific pathways that regulate this process remain unclear and likely depends on the environmental and inflammatory context in which TGF-β signaling occurs.

Apart from TGF-β, the cytokine requirements for T_{RM} differentiation vary by tissue. For example, IL-15 is required for the development of T_{RM} $CD8^+$ T cells in the skin and salivary gland, but is dispensable in the gut[83, 126, 128]. In T_{CM} $CD8^+$ T cells, IL-15 leads to expression of the costimulatory molecule 4-1BB, which in turn promotes expression of the anti-apoptotic molecules Bcl2 and Bcl-X_L[137, 138], thereby promoting survival of circulating memory $CD8^+$ T cells. IL-15 was also found to be critical for both the initial recruitment of effector $CD8^+$ T cells and subsequent maintenance of T_{RM} populations in the skin[83, 139, 140]. The precise consequences of IL-15 signaling in T_{RM} $CD8^+$ T cells is unclear, but is presumed to engage the same survival mechanisms as it does in circulating memory $CD8^+$ T cells. Under steady state conditions in the skin, IL-15 is derived from hair-follicles, supporting the idea that stromal or epithelial cells provide tissue-specific signals that regulate adaptation T_{RM} $CD8^+$ T cells to their environmental niche[127].

While local cytokine exposure clearly regulates T_{RM} $CD8^+$ T cell formation, the role of local antigen recognition is less clear. The observation that intradermal transfer of effector $CD8^+$ T cells into uninfected skin generated a T_{RM} population suggests that local antigen recognition is not a strict requirement for T_{RM} development in the skin, as is the case in the lung and brain[84, 141, 142]. However, we and others recently demonstrated that local antigen promotes T_{RM} formation following VACV infection, suggesting that antigen recognition contributes to but is not required for T_{RM} development in the skin[143–145]. In contrast, persistent antigen in the intestine actually prevents CD103 expression, suggesting that antigen recognition might inhibit the formation of T_{RM} $CD8^+$ T cells in the gut[136]. Clearly, the role of antigen in T_{RM} development varies by tissue, and the consequences of antigen recognition within tissues and the mechanisms by which TCR signaling regulates T_{RM} differentiation remains unclear. Thus, chapter 2 of this thesis aims to fill this knowledge gap by examining transcriptional consequences of antigen recognition

within the skin, and to define how those changes in gene expression promote T_{RM} differentiation.

1.5 Function of tissue-resident memory T cells

Protective capacity of T_{RM} cells

In contrast to naïve laboratory mice, biopsy samples have revealed that human skin contains a high density of T_{RM} cells. In fact, it is estimated that human skin harbors approximately 20 billion T cells, roughly twice as many as in the entire circulatory system[146]. This high density of T_{RM} cells also holds true for mice that were not raised in clean laboratory environments, suggesting that T_{RM} cells play an important role in protection against the constant encounters with environmental microbes and pathogens that occur outside of the laboratory environment[147]. While their protective role in humans is difficult to determine experimentally, mouse studies have definitively proven that T_{RM} $CD8^+$ T cells confer enhanced protective immunity compared to their circulating counterparts. Because most immunization strategies generate both a circulating and tissue-resident memory $CD8^+$ T cell population, a variety of experimental approaches have been used to study the functions of T_{RM} $CD8^+$ T cells in isolation. For example, T_{RM} $CD8^+$ T cells are protected from systemic administration of depleting antibodies, offering a relatively easy method to eliminate circulating memory populations while leaving T_{RM} populations intact[126, 148, 149]. This technique can be combined with B-cell deficient μMT mice such that T_{RM} populations are the only component of adaptive immunity left intact[18, 19]. Alternatively, pharmacological inhibition of S1PR1 with the FDA-approved drug FTY720 results in retention of T cells within lymphoid organs and thereby prevents any contribution of circulating memory T cells to protective immunity within peripheral tissues[19, 150]. Similarly, blockade of receptors required for T cell trafficking into non-lymphoid tissues eliminates the contribution of circulating memory

T cells[151]. Using these techniques, T_{RM} cells have been shown to provide protection against viruses, bacteria, and parasites in a wide variety of both barrier and non-barrier tissues[106, 152–154].

While the protective capacity of T_{RM} $CD8^+$ T cells was initially described in the context of infection models, it has now become clear that these cells also play a critical role in anti-tumor immunity[155]. The first evidence for this came from analysis of tumor infiltrating lymphocytes (TIL), some of which were found to express the T_{RM} markers CD69, CD49a, and CD103[156, 157]. Transcriptomic analysis has confirmed that many of the TILs expressing these markers also exhibit similar gene expression profiles as T_{RM} $CD8^+$ T cells, suggesting tumor-residence[158]. While the presence of TIL has long been used as a prognostic factor for cancers, recent analysis has found that the abundance of $CD103^+$ $CD8^+$ T is a better predictor of patient outcomes, suggesting that T_{RM}-like TILs are the most efficient anti-tumor component of the adaptive immune system[159, 160]. Indeed, using a mouse model of melanoma, it was recently demonstrated that artificially increasing the number of T_{RM} $CD8^+$ T cells through over expression of Runx3 results in better tumor control[129]. Thus, T_{RM} $CD8^+$ T cells central mediators of optimal immune protection against both pathogens and tumors.

T_{RM} $CD8^+$ T cell immunosurveillance within tissues

The ability of T_{RM} $CD8^+$ T cells to provide enhanced, antigen-specific protective immunity suggests that they are able to survey the local microenvironment for pMHC. In vivo imaging studies in mice have demonstrated that T_{RM} $CD8^+$ T cells in the skin extend dendrites that reach between the densely packed keratinocyte layer and make contacts with Langerhans cells embedded within the epidermal layer[72]. This dendritic morphology has also been confirmed in human skin-resident $CD8^+$ T cells and is dependent on the actin cytoskeleton, suggesting active regulation of this

process[161, 162]. In addition to the sampling of the nearby environment through dendrites, T_{RM} $CD8^+$ T cells within the skin are also motile during steady state[162, 163]. The signals that regulate T_{RM} motility during homeostasis are not known, but GPCRs do not appear to be involved as treatment with pertussis toxin has no effect. The migration occurs along the basement membrane, raising a possible role for integrins expressed by T_{RM} cells. Indeed, recent studies in the lung have shown that CD49a expression by influenza-specific T_{RM} $CD8^+$ T cells is required for movement along the basement membrane[112]. In contrast, CD103 localizes to regions of contact with epithelial cells and restricts steady-state motility in both the lung and skin[72]. $CD103^{-/-}$ $CD8^+$ T cells in the skin also exhibited increased motility, suggesting that T_{RM} $CD8^+$ T cells in the skin and lung use similar mechanisms to perform local immunosurveillance.Interestingly, despite their expression of CD49a, T_{RM} $CD8^+$ T cells in the small intestine do not display this motility program but rather are sessile in steady-state and increase motility in response to inflammation[164]. Altogether, the precise molecular interactions that regulate T_{RM}-mediated immunosurveillance are just beginning to be uncovered and will be important considerations in either enhancing T_{RM}-mediated protection or inhibiting T_{RM}-mediated autoinflammatory conditions.

Mechanisms of protection utilized by T_{RM} $CD8^+$ T cells

One of the hallmark features of the core T_{RM} transcriptional profile is elevated expression of both cytolytic machinery and pro-inflammatory cytokines, suggesting these cells are transcriptionally poised to rapidly execute effector functions in response to secondary infections[165]. Indeed, T_{RM} $CD8^+$ T cells are able to directly kill target cells ex vivo and express high levels of IFNγ upon restimulation[154, 166], demonstrating that both mechanisms of canonical $CD8^+$ T cell-mediated protective immunity are intact in T_{RM} cells. However, whether both pathways contribute to

T_{RM}-mediated protective immunity in vivo is difficult to determine. In humans, HSV-specific T_{RM} populations have been shown to produce Perforin at sites of HSV reactivation during asymptomatic viral shedding, suggesting rapid control of reactivated HSV by T_{RM} cells[167]. Further evidence for the cytotoxicity of T_{RM} $CD8^+$ T cells comes from studies in the brain, where T_{RM}-mediated protection against viral re-infection was Perforin dependent[168]. However, complete protection in this model was also dependent on IFNγ, suggesting that both pathways contribute to T_{RM}-mediated protection against viral infections.

In contrast to the lack of direct evidence of cytotoxic function in vivo, it is well documented that T_{RM} $CD8^+$ T cells rapidly express the inflammatory cytokine IFNγ upon reactivation. In fact, topical application of antigenic peptide to skin or mucosal tissues that contain T_{RM} $CD8^+$ T cells is sufficient to cause expression of IFNγ within hours of TCR stimulation[20, 169, 170]. Using this technique, it was found that IFNγ secretion by T_{RM} $CD8^+$ T cells prevents viral infection of the surrounding tissue[20, 171]. In addition to its potent induction of anti-viral defenses, IFNγ also activates vascular endothelial cells to express adhesion molecules that are critical for the recruitment of circulating lymphocytes to the site of T_{RM}-activation[170, 171] (Figure 1.3). Thus, local activation of T_{RM} $CD8^+$ T cells initiates an extremely potent inflammatory response that can prevent pathogen replication at the site of infection.

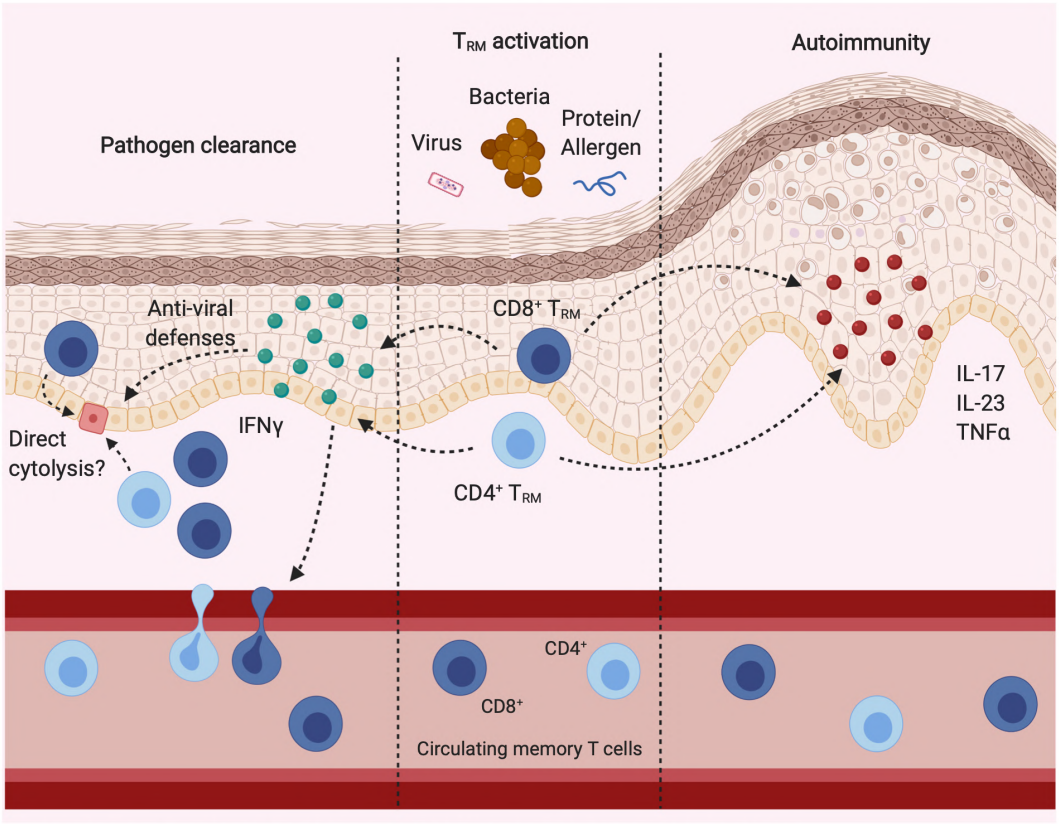

Figure 1.3: The functional consequences of T_{RM} activation within the skin.
$CD4^+$ and $CD8^+$ T_{RM} cells reside within the skin and initiate inflammatory responses upon secondary exposure to either pathogen or environmental antigen (i.e. allergen). In the context of a secondary pathogen infection, T_{RM} cells express IFNγ, which causes nearby cells to express anti-viral genes and also recruits memory T cells from the circulation. It is unclear to what degree T_{RM}-mediated protection is due to IFNγ as compared to direct killing of infected cells. In contrast, in autoimmune diseases such as psoriasis, pathogenic T_{RM} cells express the cytokines IL-17, IL-23, and TNFα, which act on the surrounding keratinocytes causing hyperproliferation and disease. The environmental antigens that cause psoriasis and the role of T_{RM} cells in other autoinflammatory disorders are active areas of research.

$CD4^+$ T_{RM} cells

Optimal protective immunity against many model infections requires $CD4^+$ T_{RM} cells, which have been identified in most tissues but exhibit significant differences from $CD8^+$ T_{RM} cells[172, 173]. In the skin, $CD4^+$ T_{RM} cells are typically found within the dermis, where they express CD69 but not CD103 and exhibit greater motility than their epidermal $CD8^+$ counterparts[79, 174]. Studies in mice have demonstrated that this population of dermal $CD69^+$ $CD4^+$ T cells exist under homeostatic conditions, but increase in number following viral, bacterial, or parasitic infection, similar to $CD8^+$ T_{RM} cells[175, 176]. However, experiments using photoconvertible cells and parabiosis have demonstrated that $CD4^+$ T_{RM} undergo a greater degree of recirculation than $CD8^+$ T_{RM}[79]. Circulating $CD4^+$ T cells are constantly replenishing the skin, and $CD4^+$ T cells lose CD69 expression as they migrate towards the lymph node, suggesting a continual turnover of $CD4^+$ T_{RM} cells in the skin. Despite their recirculation, $CD4^+$ T_{RM} cells play critical roles in the coordination of immune responses within non-lymphoid tissues, and provide enhanced protection against many infections including influenza, VACV, Mtb, HSV, and Leishmania[18, 152, 177, 178]. The mechanisms by which $CD4^+$ T_{RM} cells provide protection are not clear, but appear to involve expression of chemokines that organize immune cell ‘clusters’ that provide opportunities to interact with MHC-II^+ dendritic cells or macrophages[172, 179]. The cellular composition of these clusters depends on the tissue and infection model being studied, but disruption of these clusters through elimination of specific chemokines or cell types diminishes protective immunity, underscoring their critical role in non-lymphoid tissue responses[176].

T_{RM} cells in autoimmunity

As described above, T_{RM} $CD8^+$ T cells are strong inducers of antigen-specific inflammation and play an important role in the control of peripheral infections. How-

ever, this potent inflammatory capacity but can also be the source of immunopathology when T_{RM} cells are specific for environmental or self-antigens. After their discovery, clinicians quickly became suspicious that pathogenic T_{RM} cells might mediate inflammatory diseases that reflect their functional properties; namely the ability to induce rapid and potent inflammatory responses and long-term persistence at specific anatomical locations. T_{RM} cells have been implicated in many autoimmune disorders, but their role in the pathogenesis of autoimmune inflammatory diseases of the skin are best studied[21].

One prototypical T_{RM}-mediated skin disease is psoriasis, which is characterized by finely demarcated lesions of hyperproliferative keratinocytes that result in dry, itchy, and scaly patches of skin. Early studies found that a toxin that selectively depletes T cells, but not keratinocytes, ameliorated disease, demonstrating that psoriasis is a T cell-mediated disease and not a consequence of keratinocyte disfunction[180]. Further evidence came for T cell involvement in psoriasis came when clinicians found that IL-17 was highly expressed in psoriatic lesions, and IL-17 producing $CD4^+$ and $CD8^+$ T cells were enriched in lesional skin[181]. These studies clearly implicated T cells in psoriasis, but did not offer insight into whether those T cells were resident within the lesions or recirculating as a result of local inflammation. Several lines of investigation answered this question definitively. First, blocking E-selectin, which effectively prevents T cell migration into the skin, had no effect on clinical outcome, suggesting that the pathogenic T cells are not being recruited from the blood vasculature[182]. Second, transplantation of non-lesional skin from psoriasis patients onto immunodeficient mice resulted in the development of psoriasis in the transplanted skin, demonstrating that the pathogenic T cells are resident in pre-psoriatic skin and can cause disease independently of any circulating T cells[183]. Lastly, blockade of TNFα, IL-17, and IL-23 temporarily alleviates the symptoms of psoriasis, but do not impact the number or localization of T cells within

psoriatic skin. The disease generally returns in the same anatomical location once treatment is stopped[184, 185], suggesting that pathogenic T cells will become re-activated and continue to cause disease after treatment. These cytokines play protective roles against certain fungal and bacterial infections, but their aberrant expression by T_{RM} cells in repsonse to self antigens result in disease. While the antigens that drive psoriasis are unknown, TCR sequencing of psoriatic lesions found particular $\alpha\beta$ TCRs that were shared among psoriatic patients but not found in healthy individuals, suggesting that psoriasis may be driven by the same antigens in different individuals[186]. The antigenic basis of psoriasis, along with the mechanisms that regulate the maintenance of the pathologic T_{RM} populations will be critical next steps in treating psoriasis and may be extended to a wide variety of autoimmune diseases.

To this end, a recent study of human patients with inflammatory bowel disease (IBD) found increased numbers of $CD4^+$ T cells with increased expression of CD69, CD103, and inflammatory cytokines, consistent with a T_{RM} phenotype[187]. Following this observation, the investigators then moved to a variety of mouse models of IBD to perform mechanistic studies that confirmed the requirement for $CD4^+$ T_{RM} in the development of IBD. Critically, this study also included a strategy to deplete established T_{RM} through the treatment of mice with NAD^+, which binds a recently described purinergic receptor, P2RX7, that is highly expressed by T_{RM} cells and causes cell death[188]. Treatment with NAD^+ prevented IBD, confirming the pathogenic role of $CD4^+$ T_{RM} and also offering a new drug target that was discovered through research on the basic biology of T_{RM} cells.

In summary, T_{RM} cells are now appreciated to be a major subset of memory T cells that are present in nearly every human tissue. Their discovery has led to a newfound appreciation that the majority of human T cells likely do not recircu-

late, challenging a decade-old model of T cell immunosurveillance. The molecular mechanisms regulating their development and function are just beginning to be understood, and the rapidly growing number of studies focused on T_{RM} cells will likely lead to improved knowledge of their development and function, which may ultimately provide opportunities to improve vaccine strategies and disease treatments.

1.6 Aims of thesis

T_{RM} $CD8^+$ T cells are a specialized subset of memory $CD8^+$ T cells that permanently reside within non-lymphoid tissues. Their anatomical positioning enables these cells to rapidly provide protective immunity in the context of pathogen infection, but their dysregulation also contribute to several inflammatory autoimmune diseases. These features of T_{RM} biology have generated a strong interest in understanding the mechanisms that regulate their development and function, with the ultimate goal of improving vaccine design and developing treatments for autoimmune diseases. In this thesis, I use a model of epicutaneous VACV infection to investigate two main research questions regarding both the *development* and *function* of T_{RM} $CD8^+$ T cells.

First, what is the role of antigen-recognition within non-lymphoid tissues during T_{RM} $CD8^+$ T cell differentiation? We have recently demonstrated that antigen recognition in the skin microenvironment enhances T_{RM} formation by approximately 50-100 fold. The aims of chapter 2 are to 1) develop an IFNγ-YFP reporter system to identify effector $CD8^+$ T cells receiving TCR stimulation within the VACV-infected skin microenvironment and 2) perform transcriptional profiling of TCR-stimulated effector $CD8^+$ T cells within VACV-infected skin to determine the molecular mechanisms by which TCR stimulation promotes or inihibits T_{RM} differentiation.

Second, what are the functional consequences of activating established T_{RM} $CD8^+$ T cell populations within the skin? We have previously demonstrated that topical application of antigen peptide activates T_{RM} $CD8^+$ T cells and causes a local inflammatory reaction. The aims of chapter 3 are to 1) determine the inflammatory and protective capacity of VACV-specific T_{RM} $CD8^+$ T cells following repeated antigenic challenges and 2) determine whether this simple method of T_{RM} $CD8^+$ reactivation can boost the T_{RM} population in order to improve local protective immunity.

Chapter 2

Antigen recognition by effector CD8$^+$ T cells in VACV-infected skin initiates a transcriptional program of tissue residency

2.1 Introduction

Following their activation and proliferative expansion within the draining lymph node, effector CD8$^+$ T cells enter the circulation and subsequently infiltrate inflamed non-lymphoid tissues to execute effector functions that contribute to pathogen clearance[3]. Once inflammation has subsided, a subset of these effector T cells permanently remain within non-lymphoid tissues and differentiate into long-lived tissue-resident memory (T_{RM}) CD8$^+$ T cells[18]. Due to their anatomical location, T_{RM} CD8$^+$ T cells are poised to rapidly execute effector function and initiate inflammatory responses in response to pathogen detection[189]. This potent inflammatory capacity can also be detrimental and result in immunopathology, as T_{RM} T cells have been suggested to be the causative agent of several autoinflammatory diseases including psoriasis, allergic contact dermatitis, and inflammatory bowel disease[21]. Thus, understanding the mechanisms that either promote or limit the formation of this important cell type are highly relevant for vaccine design and treatment of autoinflammatory conditions.

The formation of T_{RM} CD8$^+$ T cells is accompanied by the engagement of a unique transcriptional program that enforces their permanent residence within the tissue microenvironment. This retention program is mediated by several recently identified transcription factors including Runx3, Hobit, and Blimp1[122, 129]. These tran-

scription factors govern tissue retention in part by repressing genes that promote tissue egress; namely the G-protein coupled receptor *S1pr1* and its transcriptional activator, *Klf2*. Forced expression of either Klf2 or S1pr1 inhibits T_{RM} formation, suggesting that repression of tissue egress pathways is a necessary element of the T_{RM} differentiation process[190]. In contrast to the repression of tissue-egress, T_{RM} 'master' regulators also drive expression of several genes that promote tissue retention, including α_E (CD103) and α_1 (CD49a) integrins, which are proposed to increase tissue-residence through physical interaction with their ligands within the tissue microenvironment. CD103 pairs with the β7 integrin chain to form a receptor for E-cadherin[109], thereby retaining T_{RM} T cells within epithelial layers, while CD49a is able to bind type I and type IV collagen and is thought to regulate T_{RM} T cell motility along collagen fibers during local immunosurveillance[112]. Thus, multiple signaling pathways are engaged in T_{RM} $CD8^+$ T cells that enforce their residence within tissues, but how diverse signaling pathways collectively control the development of T_{RM} cells in vivo are not completely understood.

Following their recruitment into inflamed non-lymphoid tissues, effector $CD8^+$ T cells are exposed to a range of tissue-derived factors that have been shown to regulate particular aspects of the T_{RM} transcriptional program. For example, inflammatory cytokines including TNFα, IL-33, or type I IFNs contribute to repression of the S1PR1 tissue egress pathway[99, 136]. These inflammatory cytokines act synergistically with TGF-β, which is a well-characterized driver of CD103 expression, suggesting that the local cytokine environment regulates the acquisition of the T_{RM} phenotype. Indeed, TGF-β responsiveness is universally required for T_{RM} development and the tissue microenvironment can be sufficient to promote T_{RM} differentiation[83]. Persistent antigen presentation in the small intestine inhibits CD103 expression, suggesting that sustained antigen recognition can prevent TGF-β-mediated T_{RM} differentiation[191]. In contrast, antigen recognition has

been shown to be necessary for optimal T_{RM} formation following infection of the brain or lung[141, 142]. We have also shown that transient exposure to antigen within the skin strongly enhances T_{RM} $CD8^+$ T cell differentiation following the clearance of acute VACV infection[143]. Thus, whether antigen recognition within non-lymphoid tissues ultimately promotes or inhibits T_{RM} differentiation remains controversial. One major limitation in pursuing studies of antigen-promoting T_{RM} differentiation has been the inability to clearly identify the T cells actively engaging in cognate antigen recognition in vivo, as both the overall antigen load as well as potential restricted access to antigen-presenting cells are additional factors that could also control the ultimate fates of individual T cells that are recruited into tissue microenvironments.

In this study, we used an IFNγ-YFP reporter system to definitively identify effector $CD8^+$ T cells actively receiving TCR stimulation within VACV-infected skin. By directly comparing IFNγ^+ and IFNγ^- T cells from the same infected skin, we were able to identify the transcriptional consequences of TCR stimulation within the context of the local inflammatory microenvironment. On a phenotypic and transcriptional level, recently activated effector T cells receiving antigenic stimulation and executing effector functions already resembled mature T_{RM} cells and expressed high levels of the key T_{RM}-promoting transcription factor Blimp1 and reduced S1P-mediated migration, suggesting that TCR stimulation contributes to engagement of the canonical T_{RM} transcriptional profile. Thus, we find that antigen recognition within the skin is a critical signal that promotes T_{RM} differentiation, findings which could provide insights into therapies designed to either enhance or inhibit the formation of T_{RM} $CD8^+$ T cells within non-lymphoid tissues.

2.2 Results

2.2.1 A subset of tissue-infiltrating effector CD8$^+$ T cells express IFNγ during viral skin infection in an antigen-dependent manner

Epicutaneous infection with VACV generates robust populations of T_{RM} CD8$^+$ T cells in a manner that is highly dependent on transient, local recognition of cognate antigen within the skin microenvironment[143, 144]. To understand the mechanisms by which local antigen promotes T_{RM} CD8$^+$ T cell differentiation, we sought to identify the effector CD8$^+$ T cells that were actively receiving TCR stimulation within VACV-infected skin, as it is unclear whether all antigen-specific T cells engage in cognate antigen recognition or if spatiotemporal factors within the tissue microenvironment limits their access to antigen-presenting cells. To do this, we utilized TCR-transgenic (TCR-tg) P14 CD8$^+$ T cells (specific for the LCMV-derived epitope GP_{33-41} presented by H2-D^b) that expressed a single copy of an IFNγ-YFP reporter gene. Following transfer of naïve IFNγ-YFP P14 CD8$^+$ T cells, mice were then infected with VACV expressing GP_{33-41} (VACV-GP33) on the left ear skin by scarification. To first determine if the IFNγ-YFP reporter system accurately reflected IFNγ protein expression, we stimulated P14 CD8$^+$ T cells from the spleen on day 7 post-infection with increasing concentrations of GP_{33-41} and directly compared YFP expression to intracellular IFNγ staining. The percentage of T cells expressing YFP and IFNγ protein was equivalent at all concentrations (Figure 2.1), demonstrating that expression of YFP faithfully reports IFNγ protein expression. Following VACV-GP33 infection, IFNγ expression was essentially undetectable in T cells isolated from lymphoid organs, but 20-30 percent of P14 CD8$^+$ T cells within VACV-infected skin consistently expressed IFNγ through days 4-7 post-infection (Figure 2.2A,B). VACV replicates robustly in keratinocytes and is cleared from the skin between days

10-15 after infection[74, 143]. IFNγ expression by P14 CD8$^+$ T cells was lost coincident with viral clearance (Figure 2.2B,C) and was also enriched in the epidermis (Figure 2.2D,E), suggesting that infiltration into the antigen-rich, VACV-infected skin microenvironment is required for antigen-specific CD8$^+$ T cells to express IFNγ.

Exposure to particular combinations of inflammatory cytokines can be sufficient to cause effector CD8$^+$ T cells to express IFN[192]. To determine if IFNγ expression in VACV-infected skin was solely due to recognition of cognate antigen, we co-infected mice on the right ear skin with VACV and on the left ear skin with VACV-GP33 (Figure 2.2F). Recruitment of effector CD8$^+$ T cells into the skin is inflammation-dependent but antigen-independent[139, 143, 193], and thus, effector P14 CD8$^+$ T cells were recruited equally to both sites of infection (Figure 2.2G,H), as similar local inflammatory environments were caused by both VACV infections. However, IFNγ expression was highly enriched within the VACV-GP33-infected skin compared to VACV-infected skin lacking expression of the immunogenic peptide (Figure 2.2G,I), demonstrating that IFNγ-YFP expression can be used to identify T cells actively receiving TCR stimulation in vivo. As we have previously reported[143], the presence of cognate antigen within the VACV-infected skin microenvironment also significantly enhanced the subsequent formation of CD69$^+$ T_{RM} CD8$^+$ T cells within previously infected skin (Figure 2.2J), demonstrating that local antigen is required for IFNγ expression by effector CD8$^+$ T cells during infection as well as the subsequent formation of T_{RM} CD8$^+$ T cells following viral clearance.

Because only a subset of CD8$^+$ T cells were expressing IFNγ in the skin on day 7 after infection, we next tested whether all of the effector CD8$^+$ T cells isolated from the skin had the potential to express IFNγ in response to TCR stimulation. To do this, we cultured effector P14 CD8$^+$ T cells from the spleen, VACV-infected skin, and VACV-GP33 infected skin in the presence or absence of saturating concentrations

of GP_{33-41}. Interestingly, a large portion (approximately 70 percent) of P14 $CD8^+$ T cells in VACV-GP33-infected skin cultured as a single cell suspension began to express IFNγ in the absence of any additional peptide, suggesting that spatiotemporal dynamics within the VACV-infected skin microenvironment likely limits the ability of $CD8^+$ T cells to interact with cells presenting peptide (Figure 2.3). However, essentially all P14 $CD8^+$ T cells from either VACV- or VACV-GP33-infected skin became IFNγ^+ after stimulation with GP_{33-41} peptide (Figure 2.3), demonstrating that antigen-specific 'bystander' $CD8^+$ T cells have the full potential to express IFNγ, but are not actively engaging cognate pMHC-I within VACV-infected skin.

To determine whether this finding of limited execution of effector functions was generalizable to other T cell populations, we next examined IFNγ-YFP expression by OT-I TCR-tg $CD8^+$ T cells (specific for the peptide sequence SIINFEKL ($OVA_{257-264}$) presented by H2-K^b) following VACV-SIINFEKL infection. Similar to our previous reports using VACV-GP33, VACV-SIINFEKL infection peaked on day 5 and virus was cleared by day 20 post infection (Figure 2.4A). IFNγ expression was again restricted to only a subset of effector OT-I $CD8^+$ T cells in the infected skin, was lost following viral clearance, and expression was enriched in the epidermis (Figure 2.4B-F). The endogenous VACV-specific $CD8^+$ T cells specific for H2-K^b-$B8R_{20-27}$ also exhibited similar patterns of IFNγ expression (Figure 2.5) during the course of VACV skin infection. Taken together, these results demonstrate that IFNγ expression identifies the subset of effector $CD8^+$ T cells that are actively engaging antigenic peptide within the VACV-infected skin microenvironment.

2.2.2 TCR signal strength within the skin microenvironment regulates expression of IFNγ and subsequent T_{RM} $CD8^+$ T cell formation

The previous data demonstrated that the presence of cognate antigen was required

for both IFNγ expression and optimal T_{RM} $CD8^+$ T cell formation. However, this system tested the complete presence or absence of cognate antigen and did not address whether there is a threshold of TCR signal strength that is required for IFNγ expression and/or T_{RM} differentiation. To test this, we utilized the OT-I TCR-tg $CD8^+$ T cell system in conjunction with a series of well-characterized amino acid variants at the 4^{th} position of SIINFEKL that exhibit decreased affinity for the OT-I TCR[194]. To test the sensitivity of the OT-I TCR to these altered peptide ligands (APLs), we isolated effector OT-I $CD8^+$ T cells from the spleen on day 7 post VACV-SIINFEKL infection and determined the concentration of peptide required for a half-maximal IFNγ-YFP response (EC_{50}). Similar to what has been reported previously, IFNγ-YFP OT-I $CD8^+$ T cells exhibited a wide range of sensitivities to the APLs (Figure 2.6A,B), demonstrating that the same concentration of antigen leads to different levels of OT-I TCR engagement and subsequent IFNγ expression.

We therefore reasoned that infection with VACV strains expressing these SIINFEKL APLs could be used to vary the strength of TCR stimulation for OT-I $CD8^+$ T cells without changing the overall inflammatory milieu or antigen load within the VACV-infected skin microenvironment. To this end, we generated VACV that expressed SIINFEKL or one of the lower affinity variants. Because the strength of TCR stimulation directly impacts the degree of $CD8^+$ T cell proliferation, we first tested the functionality of VACV-APLs by measuring activation of naive OT-I $CD8^+$ T cells within the draining lymph node. Each VACV strain was equally infectious (Figure 2.7A), but cellular proliferation and expression of the activation markers CD25 and CD69 (expressed before cell division begins) correlated with APL affinity (Figure 2.7B-E), demonstrating that the low-affinity variant peptides are expressed and presented. This decreased level of activation resulted in lower frequencies of effector OT-I $CD8^+$ T cells in the circulation (Figure 2.7F), confirming that VACV-APLs differentially activate naïve OT-I $CD8^+$ T cells.

To control for different levels of activation by the VACV-APLs, we used a co-infection system where the skin of the left ear is infected with VACV-SIINFEKL (control skin) and the right ear skin is infected with one of the VACV-APL strains (experimental skin; Figure 2.6C). In contrast to a single infection, the magnitude of the effector response was equivalent in co-infected mice (Figure 2.6D), suggesting that the VACV-SIINFEKL infection was the dominant driver of OT-I $CD8^+$ T cell activation and expansion using this model. OT-I $CD8^+$ T cells were recruited equally to both the control (N4-infected) and experimental (APL-infected) skin on day 7 post infection, but the levels of $IFN\gamma$ expression directly correlated with the strength of TCR signal received within the VACV-infected skin microenvironment (Figure 2.6E-G). On day 40 post infection, the number of $CD69^+$ OT-I T_{RM} $CD8^+$ T cells was reduced in the skin where lower affinity peptides were presented (Figure 2.6H), suggesting that the strength of TCR stimulation required for $IFN\gamma$ expression is largely equivalent to that required for T_{RM} $CD8^+$ T cell differentiation, whereas the number of memory OT-I $CD8^+$ T cells in the circulation was not impacted (Figure 2.6I). Together, these data demonstrate that the strength of TCR stimulation received within the VACV-infected skin microenvironment is a critical regulator for both the execution of effector functions (e.g. production of cytokines) and the subsequent development of T_{RM} $CD8^+$ T cells.

2.2.3 $IFN\gamma^+$ $CD8^+$ T cells in the skin exhibit phenotypic and transcriptional features of T_{RM} differentiation

Given their impressive protective capacity, generation of T_{RM} cells is an attractive goal for vaccine strategies and many studies have identified gene sets that are expressed by developing and/or mature skin-T_{RM} $CD8^+$ T cells[83, 195, 196]. To test whether T cells receiving TCR stimulation within VACV-infected skin were undergoing T_{RM} differentiation, we first analyzed expression of known T_{RM}-associated genes on day 7 post-infection. Nearly all $IFN\gamma^+$ $CD8^+$ T cells were protected from

intravenous labeling, expressed low levels of Ly6C and KLRG1, and high levels of CD69, CD122, PD-1, and ICOS, all of which are phenotypic features of mature T_{RM} $CD8^+$ T cells in the skin (Figure 2.8A,B)[83, 151, 196]. Apart from cell surface phenotypes, T_{RM} $CD8^+$ T cells also exhibit a distinct transcriptional profile compared to memory $CD8^+$ T cells in the circulation. Thus, we next determined whether TCR stimulation within the skin caused changes in gene expression that promote T_{RM} differentiation. To do this, we sort purified $IFN\gamma^+$, $IFN\gamma^-$, and splenic P14 $CD8^+$ T cells on day 7 post-infection (Figure 2.8C) and analyzed the transcriptional profile of these three T cell populations. Several hundred genes were differentially expressed (Figure 2.8D,E) and the three populations were clearly distinct based on both principal component analysis and hierarchical clustering (Figure 2.8F,G). These data also show that there are significant changes in gene expression associated with both entry into the tissue microenvironment (Spleen vs. $IFN\gamma^-$), as well as subsequent TCR stimulation within the skin. Strikingly, genes that are highly expressed in $IFN\gamma^+$ cells are also upregulated in the core T_{RM} transcriptional profile, and genes that are expressed at low levels in $IFN\gamma^+$ cells are repressed in the core T_{RM} transcriptional profile (Figure 2.8G). Together, these data demonstrate that $IFN\gamma^+$, $IFN\gamma^-$, and splenic effector $CD8^+$ T cells are three distinct populations, and of those three populations, $IFN\gamma^+$ cells uniquely exhibit known features of mature T_{RM} $CD8^+$ T cells. In addition to cell-surface phenotypes associated with T_{RM} development, a suite of genes with known roles in T_{RM} differentiation has also been identified[83, 99, 126, 129]. We therefore next analyzed expression of these genes between TCR-stimulated effector $CD8^+$ T cells ($IFN\gamma^+$) cells, antigen-specific bystander T cells from the same skin microenvironment ($IFN\gamma^-$), and effector cells that have not yet been exposed to the skin microenvironment or received a second antigen encounter (spleen). $IFN\gamma^+$ cells had higher transcript levels of *Ifng* as well as other TCR-dependent effector genes including *Prf1*, *Ccl3*, and *Ccl4*

(Figure 2.9A), offering further support that IFNγ-YFP$^+$ T cells in the skin are exhibiting multiple TCR-dependent effector functions. We also found that IFNγ^+ cells had decreased expression of many genes that antagonize T_{RM} formation, including *Klf2*, *Tcf7*, *S1pr1*, *Sell*, and *Klrg1* (Figure 2.9A)[83, 99, 126]. Additionally, the T_{RM}-promoting/signature genes *Ccr8*, *Cxcr6*, *Prdm1*, and *Il2rb* were more highly expressed in IFNγ^+ cells[122, 126, 127, 161]. In contrast, other T_{RM}-antagonizing genes appear to be regulated by exposure to the tissue microenvironment, including *Tbx21*, *Eomes*, and *S1pr5* (Figure 2.9A), suggesting that TCR stimulation and microenvironmental factors both contribute to changes in gene expression and may regulate distinct pathways of T_{RM} differentiation. We next focused on the global differences between IFNγ^+ and IFNγ^- cells in order to understand whether TCR stimulation in the context of the VACV-infected skin microenvironment promoted T_{RM} differentiation. To do this, we performed gene set enrichment analysis (GSEA) comparing the set of genes that were up- or down-regulated in IFNγ^+ cells (identified in Figure 2.8D) to the published expression profile of mature VACV-specific T_{RM} and circulating effector memory (T_{EM}) CD8$^+$ T cell populations[115]. GSEA revealed that the set of genes upregulated in day 7 IFNγ^+ T cells were also more highly expressed in mature T_{RM} populations, and the set of genes downregulated in IFNγ^+ cells were more highly expressed in mature, circulating T_{EM} populations (Figure 2.9B). These data suggest that effector T cells receiving TCR stimulation within the skin microenvironment have already undergone changes in gene expression that resemble mature T_{RM} CD8$^+$ T cells, whereas previous studies found that the T_{RM} transcriptional program is not fully engaged until more than 25 days post infection when analyzing bulk T cell populations within the skin. Thus, TCR engagement within the skin promoted expression of genes necessary for T_{RM} differentiation and retention, while repressing gene networks that promote tissue egress and the development of circulating memory CD8$^+$ T cells, suggesting that CD8$^+$ T

cells encountering cognate antigen are the major T_{RM} precursors.

To further understand the transcriptional changes associated with antigen recognition within virally infected skin, we performed pathway enrichment analysis on the set of differentially expressed genes between IFNγ^+ and IFNγ^- cells. Many metabolic pathways were enriched in IFNγ^+ cells (Figure 2.9C), suggesting that TCR stimulation triggers metabolic adaptation to the tissue microenvironment; a feature of T_{RM} differentiation that is just beginning to be understood. Survival of T_{RM} CD8$^+$ T cells in the skin is dependent on mitochondrial fatty acid oxidation, and these cells express fatty acid binding proteins (FABPs) 4 and 5 that allow the uptake and subsequent oxidation of extracellular lipids[115]. Circulating memory CD8$^+$ T cells also preferentially utilize oxidative phosphorylation and are therefore able to derive energy from a wide variety of nutrients[197]. A recent study demonstrated that circulating memory CD8$^+$ T cells do not directly uptake or store lipids, but rather use the glycolysis pathway to synthesize fatty acids and use lysosomal acid lipase A (*Lipa*) to enable their subsequent use in fatty acid oxidation[198]. Interestingly, IFNγ^+ cells upregulated the glycolysis pathway and had high expression of glucose transporters (*Slc2a1*, *Slc2a3*), *Lipa*, and *Fabp5* (Figure 2.9D), suggesting that both synthesis and direct uptake of fatty acids could be occurring. To test whether increased expression of glucose transporters translated to increased glucose uptake, we cultured cells from the skin in the presence of the fluorescent glucose analog 2-NBDG and measured 2-NBDG uptake by flow cytometry. Because 2-NBDG and YFP are detected in the same channel, we used ICOS expression as a surrogate marker for IFNγ expression (Figure 2.8A). Indeed, TCR-stimulated effector cells from the skin displayed more efficient uptake of the glucose analog 2-NBDG (Figure 2.9E,F). Thus, these analyses demonstrate that TCR stimulation promotes the uptake of diverse nutrient sources and promotes the expression of key enzymes that divert these sources towards fatty acid oxidation, which is required for the long-

term maintenance of T_{RM} $CD8^+$ T cells.

2.2.4 TCR stimulation drives elevated expression of Blimp1 and represses expression of key genes involved in tissue egress

Blimp1 is a transcription factor that is encoded by the gene *Prdm1* and has been shown to promote engagement of a transcriptional profile (together with Hobit) that enforces tissue retention in some lymphocyte populations[122]. In our transcriptional analysis, $IFN\gamma^+$ cells displayed higher expression of *Prdm1* (Figure 2.9A), suggesting a possible mechanism by which TCR stimulation contributes to retention and/or T_{RM} $CD8^+$ T cell differentiation. Notably, we did not identify *Hobit* as being differentially expressed between $IFN\gamma^+$ and $IFN\gamma^-$ T cells from the skin. We first confirmed increased expression of Blimp1 in $IFN\gamma^+$ cells on a protein level by flow cytometry and found high Blimp1 expression in $IFN\gamma^+$ cells (Figure 2.10A,B). We next used Blimp1-YFP reporter P14 $CD8^+$ T cells to determine the antigen-dependence and kinetics of Blimp1 expression during VACV skin infection. Compared to naïve $CD8^+$ T cells, effector P14 $CD8^+$ T cells in the spleen and VACV-infected skin expressed Blimp1 as has been previously reported. However, a subset of P14 $CD8^+$ T cells in VACV-GP33 infected skin expressed even higher levels of Blimp1 that was lost coincident with viral clearance (Figure 2.10C,D). Furthermore, ex vivo TCR stimulation of effector P14 $CD8^+$ T cells (but not naïve $CD8^+$ T cells) was sufficient to promote elevated Blimp1 expression (Figure 2.10E,F), suggesting that the Blimp1-mediated T_{RM} transcriptional program is uniquely engaged following a 'second' antigen encounter and not during the initial activation of naïve $CD8^+$ T cells. To test whether induction of Blimp1 was dependent on the strength of TCR stimulation, we stimulated effector OT-I $CD8^+$ T cells with an equal concentration SIINFEKL APLs, and found that similar to $IFN\gamma$ expression and T_{RM} formation, TCR signal strength is an important factor that promotes elevated Blimp1 expression (Figure 2.10G,H).

Mechanistically, Blimp1 antagonizes T_{CM} development through repression of *Tcf7* and inhibits tissue-egress through direct repression of *Klf2*, *S1pr1*, and *Ccr7*. Of these four Blimp1 targets, *Klf2*, *S1pr1*, and *Tcf7* were transcriptionally repressed in IFNγ^+ cells (Figure 2.9A), suggesting that these components of the 'canonical' Blimp1-dependent transcriptional program are engaged downstream of TCR stimulation. TCR stimulation was also sufficient to repress these key Blimp1 target genes, but did not impact *Ccr7* expression in vivo (Figure 2.9A) or in vitro (Figure 2.10I), suggesting suppression of *Ccr7* is not a feature of antigen-dependent T_{RM} differentiation. Interestingly, *Cd69* transcript levels were also not different between IFNγ^+ and IFNγ^- cells (Figure 2.9A), despite increased surface expression of CD69 protein on IFNγ-YFP$^+$ cells (Figure 2.8A). S1PR1 and CD69 physically interact on the cell surface, causing the internalization and degradation of both proteins[102]. Thus, transcriptional repression of *S1pr1* can result in increased expression of CD69 on the cell surface, independent of changes in *Cd69* transcription[103]. In agreement with that model, ex vivo TCR stimulation of purified effector CD8$^+$ T cells did not cause any changes in *Cd69* transcript abundance, but did result in stable expression of CD69 protein on the cell surface and prevented S1P-dependent migration of effector CD8$^+$ T cells (Figure 2.10I-K), suggesting that TCR-driven repression of *S1pr1* contributes to the sustained expression of CD69 on the surface of mature T_{RM} CD8$^+$ T cells. In contrast, effector CD8$^+$ T cells were unable to efficiently migrate in response to the CCR7 ligand CCL21 and TCR stimulation did not alter their migratory capacity to this chemokine (Figure 2.10M). These findings demonstrate that TCR stimulation of effector CD8$^+$ T cells uniquely prevents S1P-mediated migration, but does not control any potential CCR7-mediated egress from tissues. Together, these data show that TCR stimulation is a major driver of Blimp1 expression and directly contributes to the repression of the S1P-mediated egress pathway.

2.2.5 Blimp1 is required for CD69 expression and tissue retention following TCR stimulation in the skin

To test whether elevated expression of Blimp1 in response to TCR stimulation contributes to antigen-dependent T_{RM} differentiation, we co-transferred congenically distinct, Cre-ERT2^{+} P14 CD8^{+} T cells that were either wild type (WT) (*Prdm1*$^{+/+}$) or contained loxP sites that flanked exons 5-8 within the *Prdm1* gene (*Prdm1*$^{FLOX/FLOX}$; referred to as KO in Figure 2.11). Mice were then infected on the left ear skin with VACV-GP33 and treated with tamoxifen on days 3-7 after infection (Figure 2.11A). On day 15 post infection, *Prdm1*$^{FLOX/FLOX}$ CD8^{+} T cells in the spleen expressed higher levels of CD62L (Figure 2.11B,C), recapitulating a known phenotype of Blimp1$^{-/-}$ CD8^{+} T cells and demonstrating that tamoxifen treatment was effective at deleting expression of Blimp1 in most cells. The ratio of KO/WT cells was significantly lower in the skin compared to the spleen (Figure 2.11D,E), and *Prdm1*$^{FLOX/FLOX}$ T cells had significantly lower expression of CD69 (Figure 2.11F,G), consistent with a failure to repress the *S1pr1* tissue egress pathway. Importantly, CD103 expression was not impacted by Blimp1 deficiency (Figure 2.11H,I), which agrees with our previous work demonstrating that local antigen regulates sustained CD69 expression, but that expression of CD103 occurs via an antigen-independent mechanism[143]. At day 40 post-infection, some *Prdm1*$^{FLOX/FLOX}$ T cells in the skin displayed a unique phenotype (CD69^{-}/CD103^{+}), which is extremely rare for WT skin T_{RM} CD8^{+} T cells following VACV infection (Figure 2.12). One possible explanation for this phenotype is that some *Prdm1*$^{FLOX/FLOX}$ T cells were exposed to TGF-β and/or other CD103-inducing signals in the skin, but were unable to repress S1PR1-mediated egress and express CD69 following TCR stimulation. Consistent with this notion, a significant proportion of *Prdm1*$^{FLOX/FLOX}$ T cells in the circulation expressed CD103, and CD103^{+} T cells were most highly enriched within the skin-draining lymph (Figure 2.12). Collectively, these data demonstrate that Blimp1 is

required for optimal CD69 expression by antigen-specific $CD8^+$ T cells in the skin and are consistent with a role for TCR-stimulated expression of Blimp1 in promoting antigen-dependent retention by limiting S1PR1-mediated tissue egress.

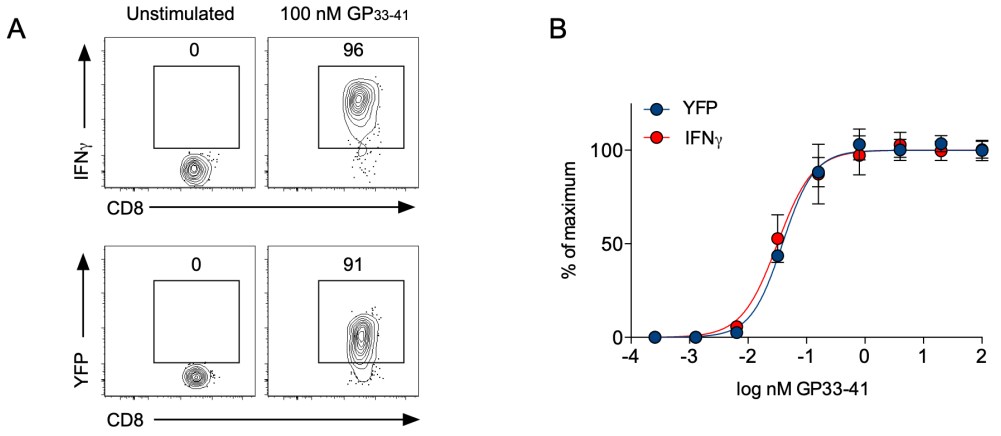

Figure 2.1: IFNγ-YFP reporter mice accurately report IFNγ protein expression.
(A) Mice received naïve IFNγ-YFP P14 CD8$^+$ T cells and were infected on the ear skin with VACV-GP33. Splenocytes were stimulated with the indicated concentration of GP_{33-41} and YFP expression was compared to IFNγ intracellular staining by flow cytometry. **(B)** Quantification of (A).

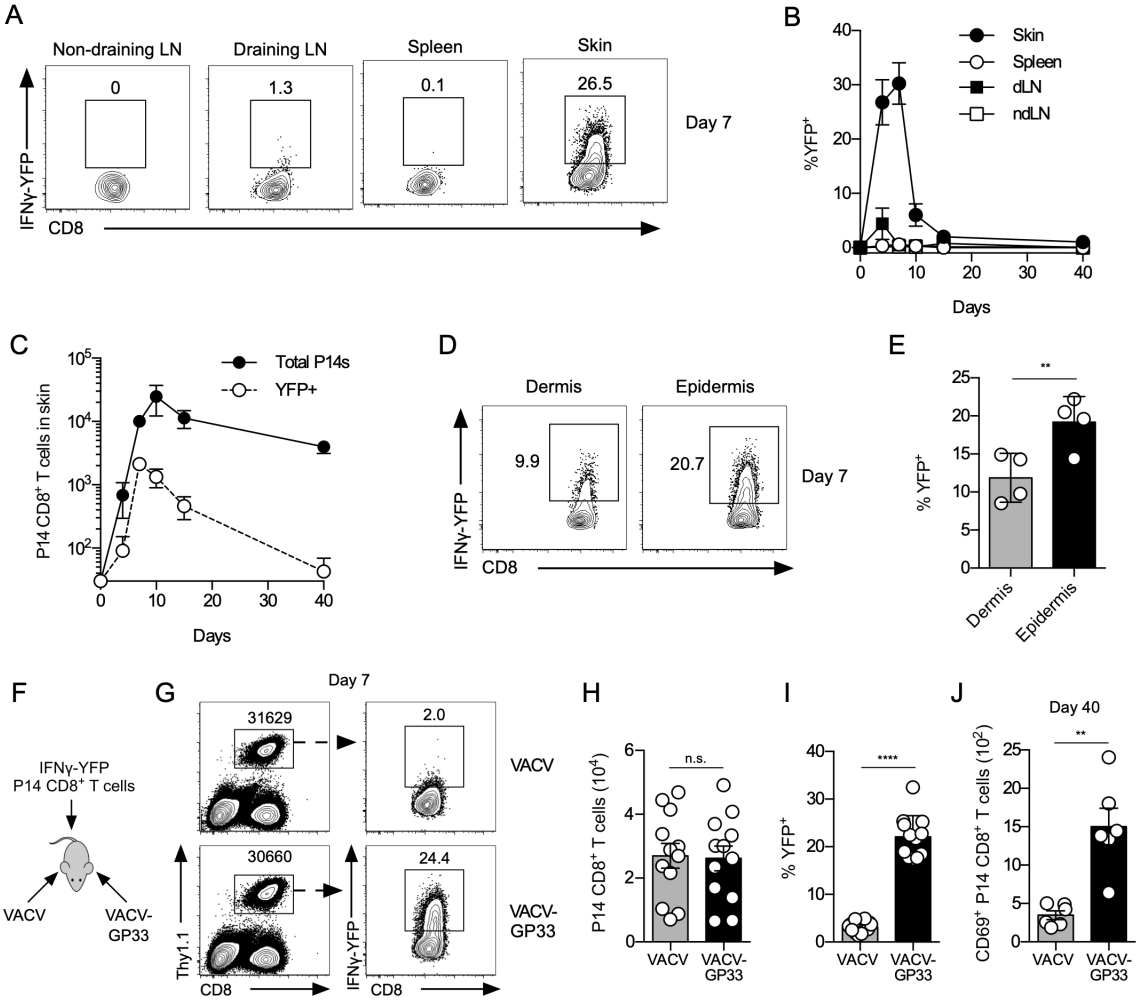

Figure 2.2: Expression of IFNγ in the skin is transient and depends on local cognate antigen. (A) Mice received naïve IFNγ-YFP P14 CD8+ T cells and were infected on the ear skin with VACV-GP33. YFP expression in the indicated organs was determined by flow cytometry on day 7 post infection. **(B)** Quantification of (A) at the indicated timepoints after infection. **(C)** Quantification of the total number and YFP+ P14 CD8+ T cells over time. **(D)** Mice were treated as in (A) and YFP expression was determined by P14 CD8+ T cells in the dermis and epidermis on day 7 post infection. **(E)** Quantification of (D). **(F)** Co-infection experimental design. **(G)** Mice were treated as in (A) except co-infected on the right ear skin with VACV and the number of P14 CD8+ T cells and their expression of YFP was determined by flow cytometry. **(H)** Quantification of the number of P14 CD8+ T cells in (G). **(I)** Quantification of YFP expression in (G). **(J)** Mice were treated as in (F) and the number of CD69+ P14 CD8+ T cells in each ear skin was determined by flow cytometry. Statistical significance was calculated using a paired t-test. Error bars represent SEM.

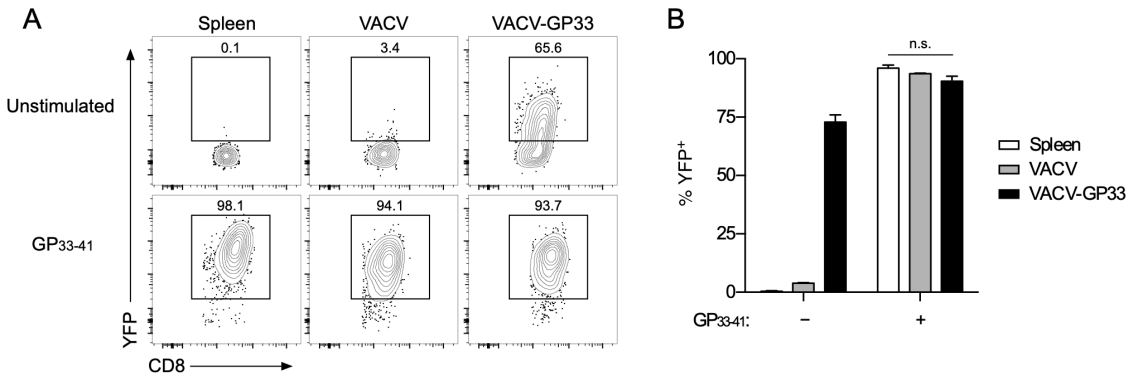

Figure 2.3: All effector CD8⁺ T cells within the skin are capable of expressing IFNγ. (A) Mice received naïve IFNγ-YFP P14 CD8⁺ T cells and were infected on the left ear skin with VACV-GP_{33-41} and on the right ear skin with VACV. On day 7 post infection, cells were isolated from the skin and spleen and stimulated with the indicated concentration GP_{33-41} for 18 hours and YFP expression was measured by flow cytometry. (B) Quantification of A.

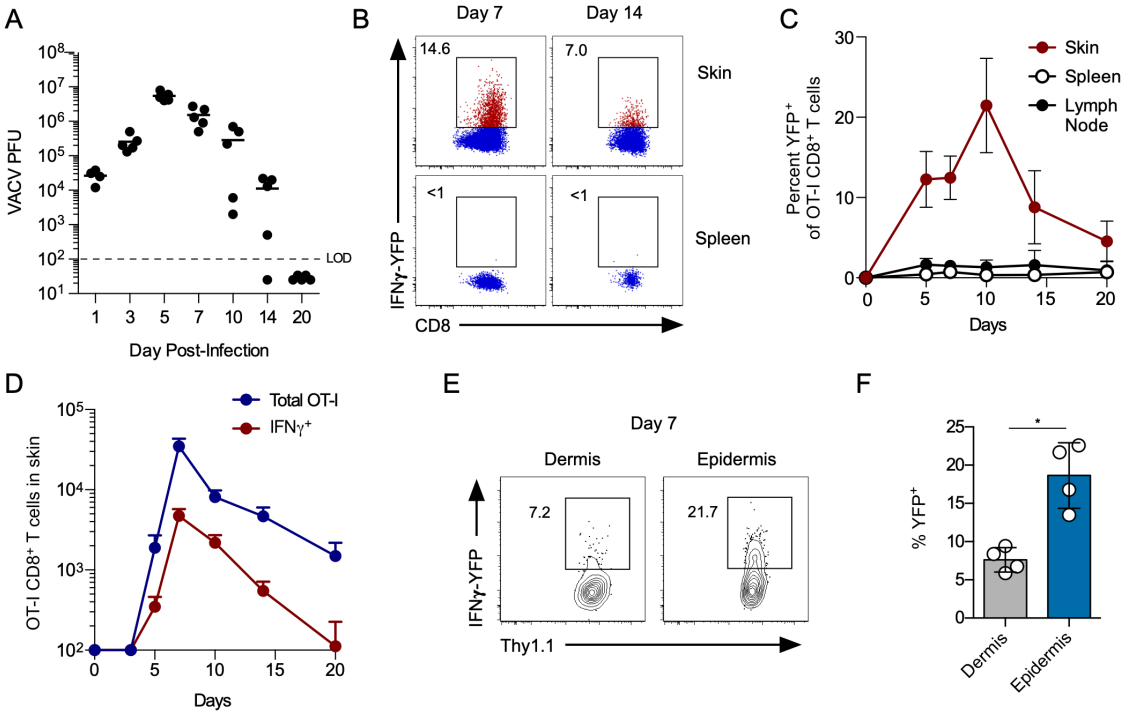

Figure 2.4: Kinetics of IFNγ expression by OT-I CD8⁺ T cells in response to VACV-SIINFEKL infection. (A) Mice were infected on the left ear skin with VACV-SIINFEKL by scarification and viral titers were quantified on the indicated day post infection. **(B)** Mice received naïve IFNγ-YFP OT-I CD8⁺ T cells and were infected on the left ear skin with VACV-SIINFEKL and YFP expression was determined in the spleen and skin by flow cytometry on the indicated day post infection. **(C)** Quantification of (B). **(D)** The total number of OT-I CD8⁺ T cells and the number of IFNγ⁺ OT-I CD8⁺ T cells in the skin was quantified on the indicated day post infection by flow cytometry. **(E)** Expression of IFNγ by OT-I CD8⁺ T cells isolated from the dermis or epidermis on day 7 post VACV-SIINFEKL infection. **(F)** Quantification of (E). Statistical significance was determined using a paired t-test. Error bars represent SEM.

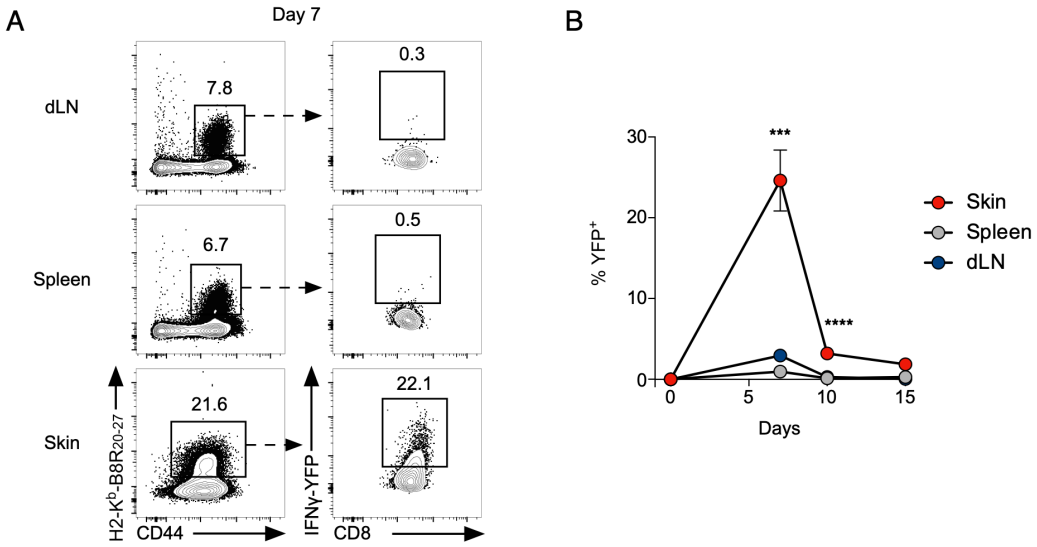

Figure 2.5: Endogenous VACV-specific CD8⁺ T cells transiently express IFNγ during viral skin infection. (A) Mice were infected on the ear skin with VACV and B8R-specific CD8⁺ T cells were identified in the indicated organ by tetramer stain on day 7 post infection. YFP expression by B8R-specific CD8⁺ T cells was determined by flow cytometry. **(B)** Quantification of the data from (A). Statistical significance was determined using a paired t-test. Error bars represent SD.

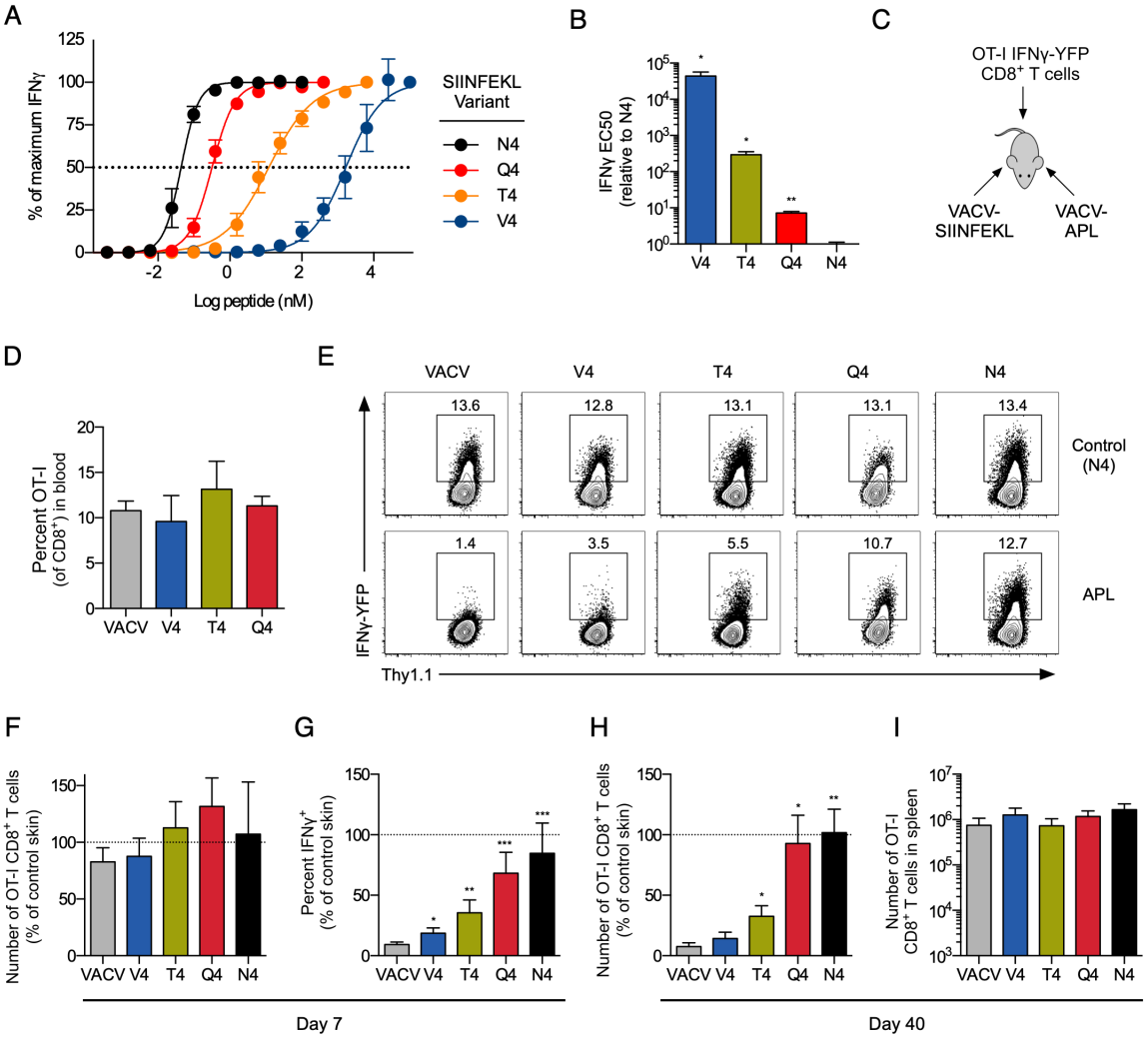

Figure 2.6: TCR signal strength dictates IFNγ expression and T_{RM} $CD8^+$ T cell formation. (A) IFNγ-YFP OT-I $CD8^+$ T cells and were isolated from the spleen on day 7 post VACV-SIINFEKL infection and stimulated with the indicated peptide concentrations and IFNγ-YFP expression was measured by flow cytometry. Data are normalized to frequency of $IFN\gamma^+$ cells at the highest peptide concentration. **(B)** Quantification of the IFNγ EC_{50} values for the indicated APL. **(C)** Co-infection experimental design. **(D)** Mice received naïve IFNγ-YFP OT-I $CD8^+$ T cells and were infected on the left ear skin with VACV-N4 (control skin) and on the right ear skin with VACV expressing one of the altered peptide ligands (experimental skin). The frequency of OT-I $CD8^+$ T cells in the blood was determined on day 7 post co-infection. **(E)** Representative flow cytometry plots depicting IFNγ-YFP expression in the control and experimental skin on day 7 post infection. **(F)** Quantification of the number of OT-I $CD8^+$ T cells in (E). **(G)** Quantification of IFNγ-YFP expression by OT-I $CD8^+$ T cells in (E). **(H)** Mice were treated as in (C) and the number of $CD69^+$ OT-I $CD8^+$ T cells in the skin was quantified by flow cytometry on day 40 post infection. **(I)** Number of OT-I $CD8^+$ T cells in the spleens of mice from (H). Data in (F-H) are normalized to control (N4-infected) skin. Statistical significance was calculated using an unpaired t-test. Error bars represent SEM.

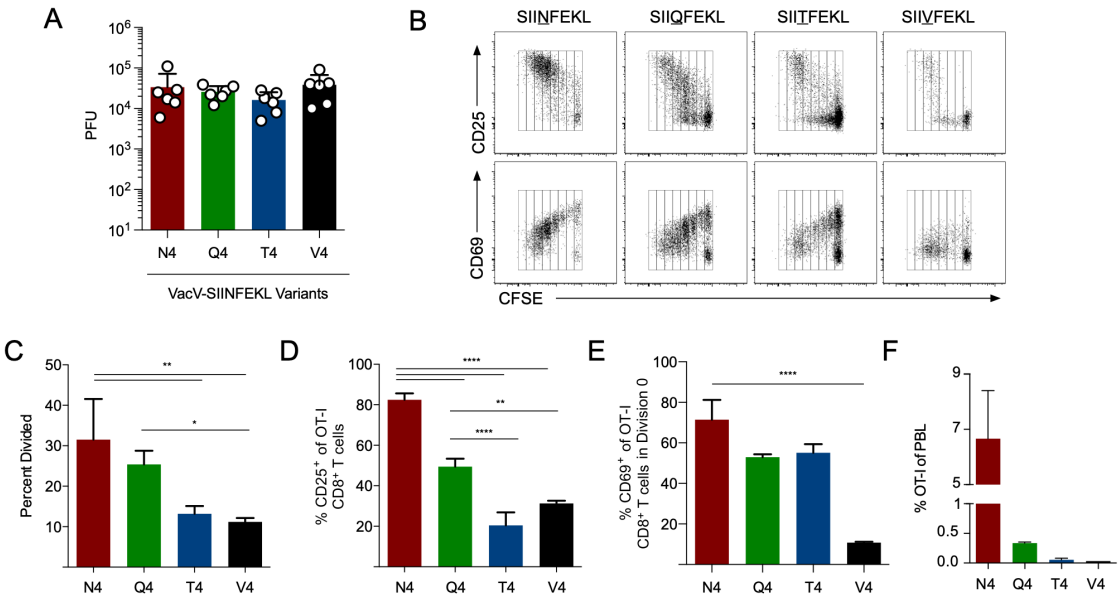

Figure 2.7: Activation of naïve OT-I CD8⁺ T cells in response to VACV-APL infection.
(A-E) Mice received CFSE-labeled naïve OT-I CD8⁺ T cells and were infected on the left ear skin with the indicated VACV-SIINFEKL variant. **(A)** Viral titers were quantified 48 hours post infection. **(B)** CFSE dilution and expression of CD25 and CD69 was determined by flow cytometry at 48 hours post infection. **(C)** Quantification of the percent of OT-I CD8⁺ T cells that underwent division from the data in (C). **(D)** Quantification of the percentage of OT-I CD8⁺ T cells expressing CD25 from the data in (C). **(E)** Quantification of expression of CD69 in undivided OT-I CD8+ T cells from the data in (C). **(F)** Mice received naïve OT-I CD8⁺ T cells and were infected on the left ear skin with the indicated VACV strain. The frequency of OT-I CD8⁺ T cells in the blood was determined on day 7 post infection. PBL = peripheral blood lymphocytes. Statistical significance was determined using an unpaired t-test. Percent divided was calculated using FlowJo 9.9 proliferation platform.

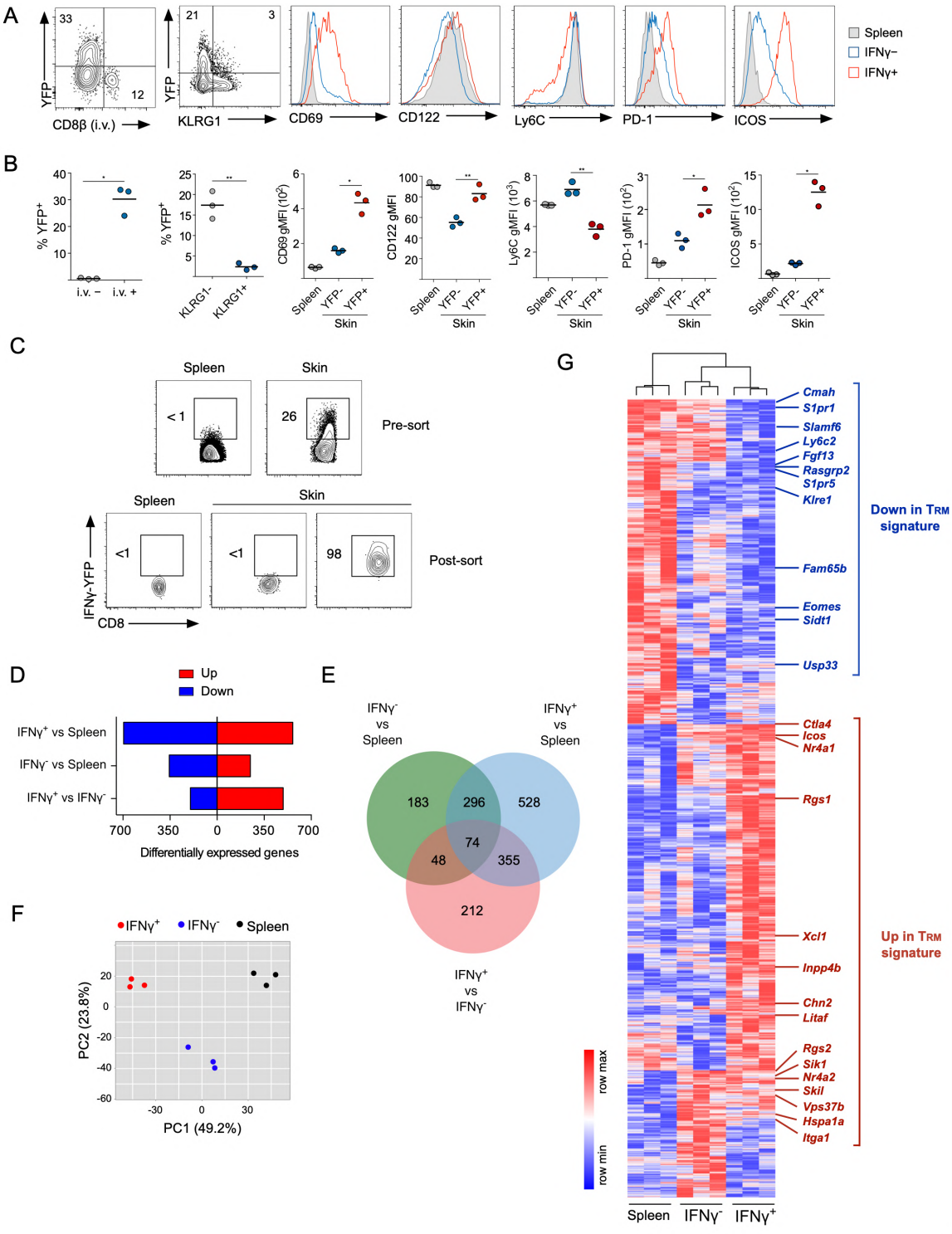
A
33
12
YFP
CD8β (i.v.)
21
3
KLRG1
CD69
CD122
Ly6C
PD-1
ICOS
Spleen
IFNγ−
IFNγ+
B
% YFP+
i.v. −
i.v. +
KLRG1-
KLRG1+
CD69 gMFI (10²)
CD122 gMFI
Ly6C gMFI (10³)
PD-1 gMFI (10²)
ICOS gMFI (10²)
Spleen
YFP-
YFP+
Skin
C
Spleen
Skin
< 1
26
Pre-sort
<1
98
Post-sort
IFNγ-YFP
CD8
D
Up
Down
IFNγ+ vs Spleen
IFNγ- vs Spleen
IFNγ+ vs IFNγ-
700
350
0
Differentially expressed genes
E
IFNγ- vs Spleen
IFNγ+ vs Spleen
183
296
528
74
48
355
212
IFNγ+ vs IFNγ-
F
IFNγ+
IFNγ-
Spleen
PC2 (23.8%)
PC1 (49.2%)
G
Cmah
S1pr1
Slamf6
Ly6c2
Fgf13
Rasgrp2
S1pr5
Klre1
Down in TRM signature
Fam65b
Eomes
Sidt1
Usp33
Ctla4
Icos
Nr4a1
Rgs1
Xcl1
Inpp4b
Chn2
Litaf
Rgs2
Sik1
Nr4a2
Skil
Vps37b
Hspa1a
Itga1
Up in TRM signature
row max
row min
Spleen
IFNγ-
IFNγ+

Figure 2.8: IFNγ$^+$ effector CD8$^+$ T cells within the skin exhibit phenotypic and transcriptional features of mature T_{RM} CD8$^+$ T cells. (A) Expression of phenotypic features associated with mature T_{RM} CD8$^+$ T cells by IFNγ$^+$ P14 CD8$^+$ T cells on day 7 post infection. **(B)** Quantification of the data in (A). **(C)** Effector P14 CD8$^+$ T cells were sorted from the skin and spleen based on YFP expression. Post sort purity was determined by flow cytometry. **(D)** The number of differentially expressed genes between the sorted populations. **(E)** Venn diagram depicting the number of differentially expressed genes that are shared between each pairwise comparison. **(F)** Principal component analysis of the sorted populations. **(G)** Heatmap displaying relative expression levels of differentially expressed genes between the three sorted populations. Genes that are up- or down-regulated in the T_{RM} signature are labeled. Statistical significance was determined using a paired t-test. Error bars represent SD.

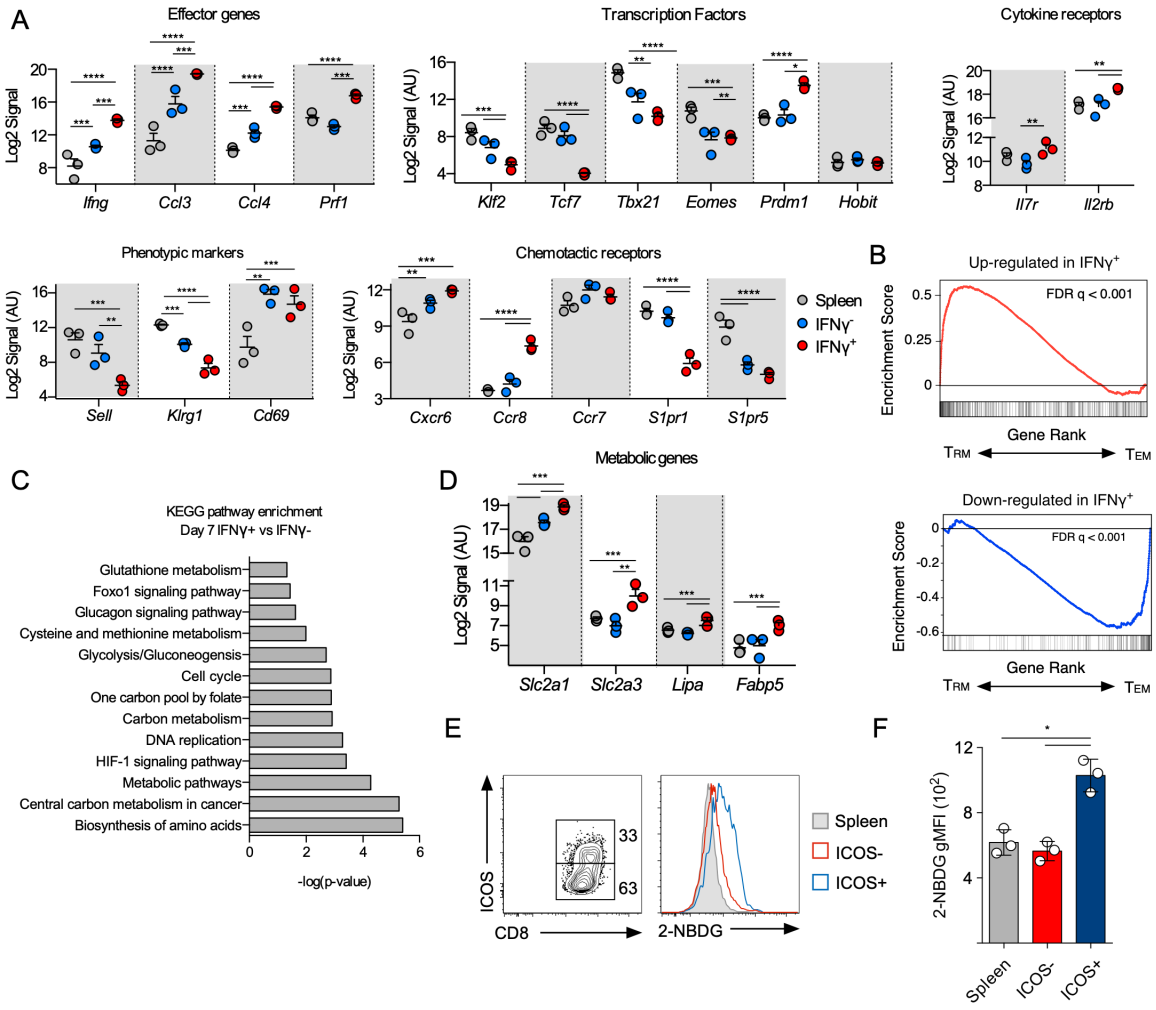

Figure 2.9: Gene expression profile of IFNγ⁺ effector CD8⁺ T cells promotes T_{RM} differentiation. (a) Expression levels of key Individual genes associated with T_{RM} differentiation. **(b)** GSEA analysis comparing genes up- or down-regulated in IFNγ^+ cells to transcriptional profiles of mature VACV-specific T_{RM} or T_{EM} populations. **(c)** KEGG pathway enrichment of differentially expressed genes from Figure 2.9D. **(d)** Expression levels of key metabolic genes derived from the data in Figure 2.9D. **(e)** Mice received naïve P14 CD8⁺ T cells and were infected on the ear skin with VACV-GP33. On day 7 post infection, cells were isolated from the skin and cultured in the presence of 2-NBDG for 30 minutes and uptake was measured by flow cytometry. ICOS was used as a surrogate for IFNγ-YFP expression. **(f)** Quantification of (e). AU = arbitrary units. Statistical significance was determined using a one-way ANOVA test (a,d) or a paired t-test (f). Error bars represent SD.

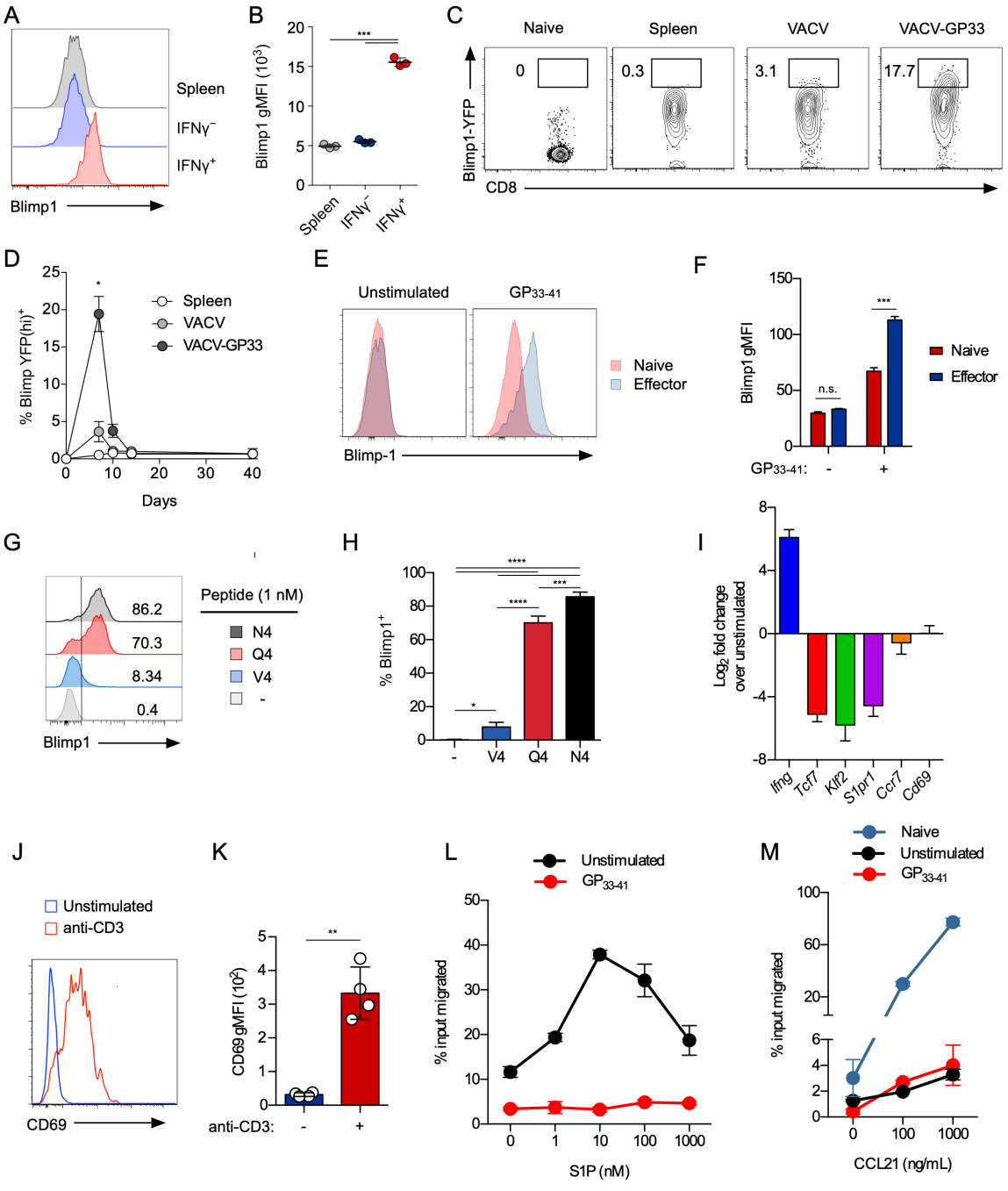
A
Spleen
IFNγ⁻
IFNγ⁺
Blimp1
B
Blimp1 gMFI (10³)
***
Spleen
IFNγ⁻
IFNγ⁺
C
Naive
Spleen
VACV
VACV-GP33
0
0.3
3.1
17.7
Blimp1-YFP
CD8
D
% Blimp YFP(hi)⁺
Spleen
VACV
VACV-GP33
Days
E
Unstimulated
GP33-41
Naive
Effector
Blimp-1
F
Blimp1 gMFI
n.s.
***
Naive
Effector
GP33-41: - +
G
86.2
70.3
8.34
0.4
Peptide (1 nM)
N4
Q4
V4
-
Blimp1
H
% Blimp1⁺
****
****
***
*
- V4 Q4 N4
I
Log2 fold change over unstimulated
Ifng
Tcf7
Klf2
S1pr1
Ccr7
Cd69
J
Unstimulated
anti-CD3
CD69
K
CD69 gMFI (10²)
**
anti-CD3: - +
L
Unstimulated
GP33-41
% input migrated
S1P (nM)
M
Naive
Unstimulated
GP33-41
% input migrated
CCL21 (ng/mL)

Figure 2.10: TCR stimulation induces Blimp1 expression and inhibits tissue egress pathways in effector CD8$^+$ T cells. (A) IFNγ-YFP P14 CD8$^+$ T cells were isolated from VACV-GP33-infected skin on day 7 post infection, and YFP and Blimp1 expression was measured by intracellular staining and flow cytometry. **(B)** Quantification of (A). **(C)** Mice received naïve Prdm1-YFP P14 CD8$^+$ T cells and were infected on the left ear skin with VACV-GP33 and on the right ear skin with VACV. Cells were isolated from the skin and spleen and expression of YFP was determined by flow cytometry. **(D)** Quantification of (A) at the indicated timepoint post infection. **(E)** Naïve or day 7 effector P14 CD8$^+$ T cells were isolated from the spleen and stimulated with the indicated concentration of GP_{33-41} for 24 hours. Blimp1 expression was determined by flow cytometry. **(F)** Quantification of (E). **(G)** OT-I CD8$^+$ T cells were isolated from the spleens on day 7 post VACV-SIINFEKL infection and stimulated with 1 nM of the indicated SIINFEKL variant. Blimp1 expression was determined by flow cytometry ('-' indicates no peptide). **(H)** Quantification of (G). **(I)** On day 7 post VACV-GP33 infection, purified P14 cells from the spleen were cultured in the presence or absence of plate-bound anti-CD3 for 18 hours and changes in gene expression were measured by RT-PCR. **(J)** Mice were treated as in (I), and CD69 surface expression was determined by flow cytometry. **(K)** Quantification of (J). **(L)** On day 7 post VACV-GP33 infection, splenocytes were stimulated with 100 nM GP_{33-41} for 18 hours and the ability of effector P14 CD8$^+$ T cells to migrate towards the indicated concentration of S1P was determined by flow cytometry. **(M)** Cells were stimulated as in (L) and the ability of effector P14 CD8$^+$ T cells to migrate towards the indicated concentration of CCL21 was determined by flow cytometry. Naïve CD8$^+$ T cells are included as positive control. Statistical significance was determined using a paired t-test (B,D,H,K) or unpaired t-test (F). Error bars represent SEM.

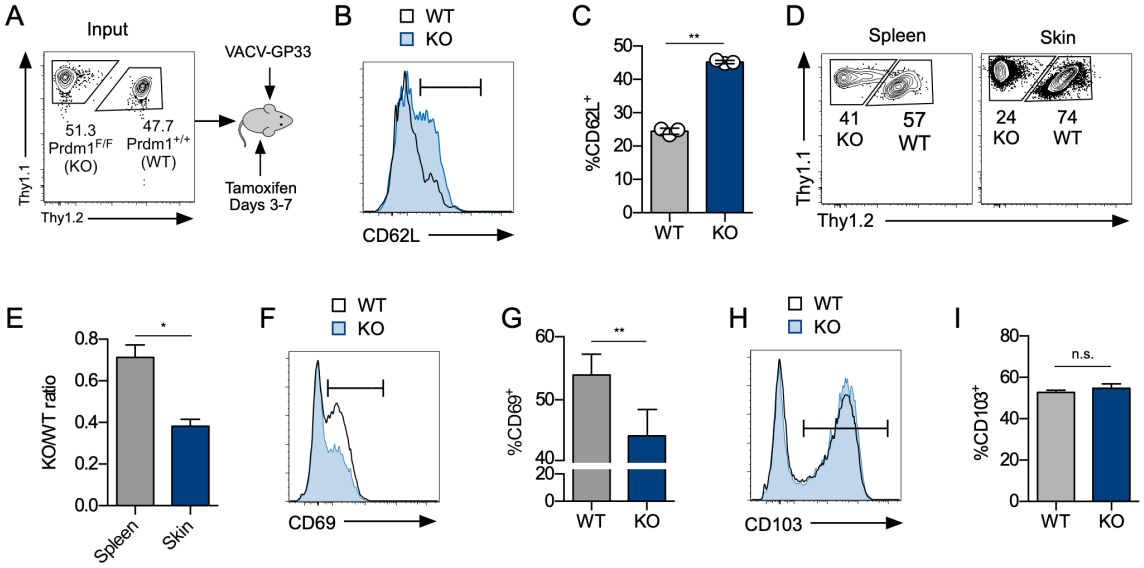

Figure 2.11: Blimp1 is required for optimal tissue retention of CD8⁺ T cells. (A) Experimental design. Mice received a ~1:1 mixture of congenically distinct Prdm1$^{F/F}$ or Prdm1$^{+/+}$ Cre-ERT2⁺ P14 CD8⁺ T cells and were infected on the left ear skin with VACV-GP33. Mice were treated with tamoxifen on days 3-7 post infection and were harvested on day 15 post infection. **(B)** Representative flow cytometry plots of CD62L expression by WT or KO P14 CD8⁺ T cells. **(C)** Quantification of (B). **(D)** Ratio of KO/WT P14 CD8⁺ T cells in the spleen and skin. **(E)** Quantification of (D). (F) CD69 expression by KO or WT P14 CD8⁺ T cells in the skin. **(G)** Quantification of (F). **(H)** CD103 expression by KO or WT P14 CD8⁺ T cells in the skin. **(I)** Quantification of (H). Statistical significance was determined using a paired t-test. Error bars represent SEM.

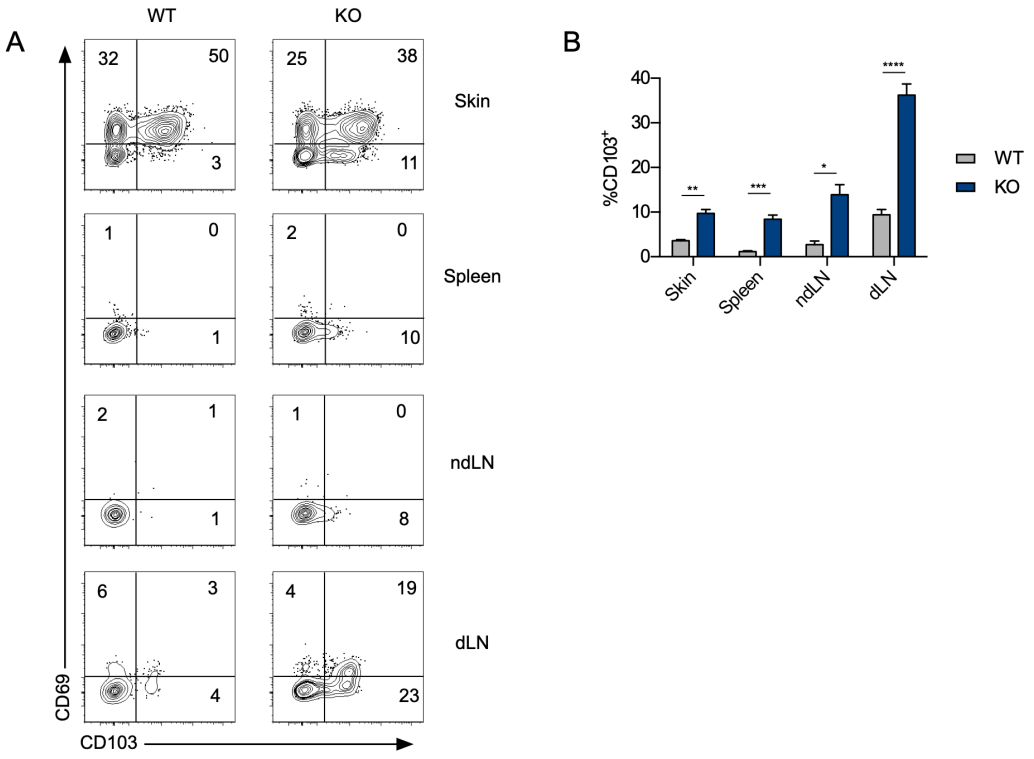

Figure 2.12: Blimp1$^{-/-}$ CD8^{+} T cells express CD103 in the circulation. (A-B) Mice received a 1:1 mixture of congenically distinct Cre-ERT2^{+} P14 CD8^{+} T cells that were either Prdm1$^{F/F}$ (KO) or Prdm1$^{+/+}$ (WT) and were infected on the left ear skin with VACV-GP33, followed by treatment with tamoxifen on days 3-7 post infection (as in Figure 2.11). **(A)** Representative flow cytometry plots depicting expression of CD69 and CD103 in the indicated organ by WT and KO cells on day 41 post infection. **(B)** Quantification of CD103 expression from the data in (B) ndLN = non-draining lymph node, dLN = skin draining lymph node. Statistical significance was determined using a paired t-test.

2.3 Discussion

Skin-T_{RM} CD8+ T cells were first formally described using a model of HSV-1 infection, which was used to demonstrate that T_{RM} formation is highly enriched at sites of previous infection[18]. However, infection with HSV strains lacking specific antigens did not diminish the subsequent formation of T_{RM} cells specific for that given antigen, and exposure to tissue microenvironments is sufficient to generate T_{RM} populations[73, 84]. These results led to a model where the local inflammatory environment instructed T_{RM} development and antigen recognition with non-lymphoid tissues was dispensable. This is in contrast with studies that have now demonstrated that local antigen recognition enhances T_{RM} differentiation following epicutaneous VACV infection[143, 144]. One explanation for this discrepancy is based on the viruses used in the respective studies. HSV rapidly (within 1-2 days) enters the sensory nerve endings at the site of primary infection and migrates towards the dorsal root ganglia, where it establishes latent infection[199]. By the time effector $CD8^+$ T cells are recruited to the skin, virus is largely cleared from the site of primary infection, offering a limited window of potential T cell-pathogen interaction. VACV replicates robustly in keratinocytes for 10-15 days, providing enough time and cellular targets for effector $CD8^+$ T cells to infiltrate infected skin and interact with VACV-infected cells[74].

While antigen-independent pathways of T_{RM} differentiation clearly exist, this work and our previously published studies have demonstrated that the presence of local antigen results in more robust T_{RM} development than exposure to the tissue microenvironment alone. By using the IFNγ-YFP approach described in this study, we were able to compare three populations of effector cells; those within the spleen that have not yet been exposed to the tissue environment, cells in the skin that have not engaged cognate pMHC-I, and cells in the skin that are actively receiving TCR

stimulation. Thus, this approach allowed us to examine the transcriptional consequences of TCR stimulation in addition to exposure to the tissue microenvironment alone. Consistent with this notion, we found that expression of particular T_{RM}-associated genes appear to be regulated by exposure to the VACV-infected skin microenvironment (*Tbx21, Eomes, S1pr5*; Figure 2.9A) and others that are regulated in response to TCR stimulation within VACV-infected skin (*Tcf7, Il2rb, Prdm1, S1pr1*; Figure 2.9A). Thus, these data illuminate which pathways are engaged in antigen-dependent T_{RM} differentiation and those that are engaged in response to exposure to the inflammatory microenvironment.

Using this IFNγ-YFP reporter system, we also show that TCR signaling in effector $CD8^+$ T cells is a major driver of changes in gene expression that promote T_{RM} development, including induction of Blimp1 and inhibition of Klf2-S1PR1-mediated tissue egress (Figure 2.13). In contrast to the temporary repression of *Klf2* and *S1pr1* following TCR stimulation of naive $CD8^+$ T cells, these genes are permanently repressed in effector cells undergoing T_{RM} differentiation[83]. While Blimp1 has been shown to directly bind the *Klf2* and *S1pr1* genes in effector $CD8^+$ T cells, the mechanisms by which it represses these target genes is unclear[122]. Blimp1 is known to recruit epigenetic-silencing enzymes to the *Il2ra* and *Cd27* loci in effector cells[200], which are critical steps in the development of long-lived circulating memory $CD8^+$ T cells. In fact, Blimp1 contains a SET domain[201], suggesting that Blimp1 accomplishes repression of its target genes at least in part through direct epigenetic silencing. Our previous study showed that TCR stimulation of effector cells regulated the sustained expression of CD69 and data from this study suggests that repression of *S1pr1* may contribute to the sustained surface expression of CD69. Thus, expression of Blimp1 in effector, but not naïve, $CD8^+$ T cells supports a model where a second antigen encounter permanently represses *S1pr1* through epigenetic modifications, thereby promoting T_{RM} differentiation and driving

sustained surface expression of CD69.

In summary, we have used an IFNγ-YFP reporter system to identify T cells receiving TCR stimulation within the VACV-infected skin microenvironment. IFNγ^+ T cells exhibit phenotypic, transcriptional, and functional features of mature T_{RM} CD8$^+$ T cells, suggesting that IFNγ^+ cells comprise a significant population of T_{RM} precursors. Mechanistically, TCR stimulation drove expression of the transcription factor Blimp1, and also repressed *Klf2* and *S1PR1*, suggesting that local antigen recognition promotes T_{RM} differentiation, at least in part, through the Blimp1-dependent T_{RM} transcriptional program. Additionally, our data suggest that the transcriptional profile of IFNγ^+ T_{RM} precursor cells may be leveraged as a resource for generating hypotheses about novel genes and pathways involved in antigen-dependent and independent T_{RM} differentiation, such as changes in metabolic programming that enable CD8$^+$ T cells to adapt to the skin microenvironment. Altogether, this study demonstrates that T cells receiving TCR stimulation are T_{RM} precursors and defines the transcriptional pathways engaged therein, which could ultimately be leveraged to optimize T_{RM} formation in the context of vaccine design.

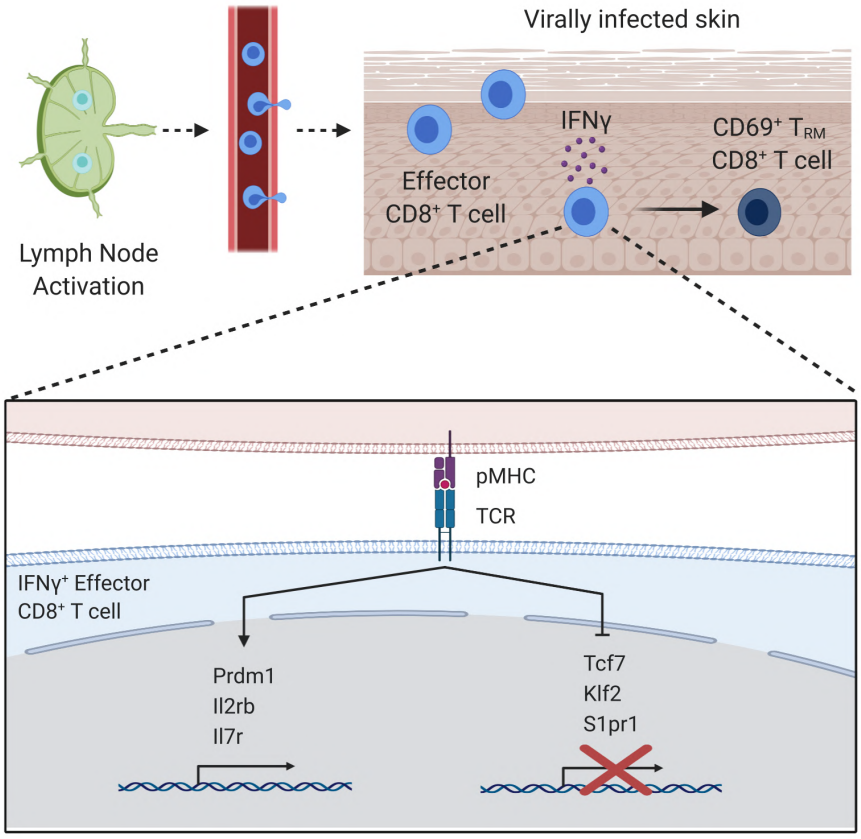

Figure 2.13: Antigen-dependent T_{RM} differentiation during VACV skin infection.
Following activation of naïve CD8⁺ T cells in the lymph node, the resulting effector populations enter the circulation and are recruited to VACV-infected skin in an antigen-independent manner. Antigen recognition within the skin microenvironment triggers changes in gene expression that promote development into a mature CD69⁺ T_{RM} CD8⁺ T cell.

Chapter 3

Targeted expansion of tissue-resident CD8$^+$ T cells to boost cellular immunity in the skin

3.1 Introduction

Cellular immunity is largely mediated by CD4$^+$ and CD8$^+$ T cells, and requires direct recognition of 'non-self' peptides presented on major histocompatibility complexes (MHC). Because many intracellular infections occur within non-lymphoid tissues, memory T cells must either be already positioned at the site of pathogen entry or able to rapidly localize to inflamed tissues following re-infection. Traditionally, the goal of vaccination strategies targeting the formation of cellular immunity has been to generate large populations of circulating antigen (Ag)-specific memory T cells with booster immunizations and strong adjuvants[202, 203]. In theory, expanding the number of memory T cells in the circulation would lead to increased surveillance of peripheral tissues and responsiveness to secondary challenge. However, in human vaccination trials targeting the prevention of AIDS, tuberculosis, and malaria, the numbers of circulating memory T cells does not correlate with protection, even after successful heterologous boosting[204, 205]. This lack of protection by circulating memory T cells has generated a strong interest in developing vaccines that seed tissue-resident memory (T_{RM}) T cells at sites of pathogen entry.

Although the factors governing the differentiation of T_{RM} cells are not completely understood, recruitment of effector T cells into peripheral tissues can be sufficient to generate a T_{RM} population[84, 136]. Thus, one approach to seed T_{RM} cells within a target tissue is to prime a T cell response and recruit effector T cells into the tis-

sue microenvironment by delivering recombinant chemokines or other non-specific inflammatory agents. Recent studies have reported that T_{RM} cells generated using this “prime and pull” approach are highly protective against both infections and tumors[73, 84, 206]. However, the chemokines used in the recruitment phase specifically recruit effector (and not memory) $CD8^+$ T cells into the tissue, and as a result, this technique only allows a short time-frame in which seeding of T_{RM} cells can occur and cannot be used to boost existing T_{RM} populations[73]. Further, the large population of effector and memory cells resulting from the transfer of monoclonal T cell receptor transgenic (TCR-tg) T cells may not accurately reflect the same trafficking and localization patterns of the relatively rare, polyclonal endogenous Ag-specific $CD8^+$ T cell repertoire[207]. Here, we show that topical application of antigenic peptide to skin harboring endogenous T_{RM} $CD8^+$ T cells causes inflammation and locally expands the Ag-specific (but not bystander) tissue-resident population by recruiting new T_{RM} precursors from the circulation. This mechanism of T_{RM} expansion significantly improved protective immunity in the skin, suggesting its potential utility as a tissue- and Ag-specific vaccine boosting strategy.

3.2 Results

3.2.1 Viral skin infection generates protective circulating and tissue-resident memory T cells

Skin infection with poxvirus vectors has become an attractive and widely used vaccine approach[56]. Using a procedure similar to the smallpox immunization strategy[74], we infected the left ear skin of naive B6 mice with attenuated, thymidine kinase deficient (*tk*-) VACV[208] and analyzed the accumulation of $CD8^+$ T cells in the skin that were specific for the immunodominant epitope of VACV ($H2\text{-}K^b\text{-}B8R_{20\text{-}27}$). B8R-specific $CD8^+$ T cells trafficked into the infected skin between days 7 and 15 post-infection and a stable population of 50-150 B8R-specific memory $CD8^+$ T cells formed in the previously infected skin 80 days after infection (Figure 3.1A-C). B8R-specific $CD8^+$ T cells that remained in the skin expressed the canonical T_{RM} markers CD69 and CD103, whereas memory B8R-specific $CD8^+$ T cells in the spleen did not (Figure 3.1D-F). Together, these data demonstrate that VACV skin immunization generates Ag-specific memory $CD8^+$ T cells in both the circulation and in the skin.

VACV infection generates robust humoral and cellular immunity that accelerates clearance of a secondary infection[209]. To quantify the amount of protection provided by these arms of adaptive immunity, we treated mice that were immunized only on the left ear skin with control (IgG) or anti-CD4/CD8 depleting antibodies (αCD4/8), which eliminated nearly all T cells from the spleen (Figure 3.1G) and $CD69^-$ cells from the skin, but did not deplete $CD69^+$ T cells at the vaccination site (Fig. 3.1H,I). Both the left and right ear skin were then infected with the more virulent parent strain of VACV (Western Reserve, VACV-WR). IgG treated animals prevented VACV infection at the site of immunization, as well as in distal unimmunized skin (Figure 3.1J, IgG), demonstrating that the combination of circulating memory

T cells and antibodies can be sufficient to rapidly control viral skin infection. However, when circulating memory T cells were eliminated, viral titers were reduced in immunized skin 50-fold compared to distal skin (Figure 3.1J, αCD4/8). These data demonstrate that VACV skin infection generates highly protective adaptive immunity, but endgogenous T_{RM} cells provide site-specific protection even within the context of functional humoral immunity.

3.2.2 Activation of T_{RM} $CD8^+$ T cells causes delayed type hypersensitivity and local accumulation of antigen-specific T_{RM} $CD8^+$ T cells

Topical application of antigenic peptide to T_{RM}-containing skin causes delayed type hypersensitivity (DTH), characterized by tissue swelling and the recruitment of circulating lymphocytes[20, 143, 170, 171, 210]. Because only 50-150 B8R-specific T_{RM} $CD8^+$ T cells formed in the skin after VACV infection, we next tested whether this small population could also initiate a DTH response. We infected the left and right ear skin with *tk*- VACV and 35 days post-infection topically applied a DMSO/acetone solution containing $B8R_{20-27}$ to the left ear skin and control peptide to the contralateral ear skin. B8R-challenged skin became inflamed (Figure 3.2A) and rapidly accumulated $CD45^+$ leukocytes, $CD8^+$ T cells, and B8R-specific $CD8^+$ T cells (Figure 3.2B-D). DMSO/acetone was required for peptide penetration, as leukocytes did not accumulate in skin challenged with $B8R_{20-27}$ in an olive oil emulsion (Figure 3.3A). T_{RM} $CD8^+$ T cells were necessary for this DTH reaction, because skin of i.v. infected mice, which generated a robust population of circulating B8R-specific memory $CD8^+$ T cells, but limited numbers of T_{RM} $CD8^+$ T cells in the skin (Figure 3.3B-D), did not become inflamed after $B8R_{20-27}$ challenge (Figure 3.2A). To determine the duration and durability of the DTH response, we allowed inflammation to subside (approximately 10 days), and then re-challenged

the skin with $B8R_{20-27}$ two additional times. The inflammatory response was a similar magnitude and duration following each challenge (Figure 3.2E), demonstrating that Ag-specific T_{RM} $CD8^+$ T cells remain highly functional after repeated activation.

We next determined whether repeated activation caused any changes to the B8R-specific T_{RM} $CD8^+$ T cell population in the challenged skin. Following resolution of the final episode of inflammation, the number of B8R-specific T_{RM} $CD8^+$ T cells increased significantly in B8R-stimulated skin compared to control skin (Figure 3.2F,G). B8R-specific T_{RM} $CD8^+$ T cells in skin challenged with control peptide remained mostly $CD69^+/CD103^+$, whereas the expanded B8R-specific T_{RM} population became largely $CD69^+/CD103^-$ (Figure 3.2F,H), which we have recently shown to be the dominant T_{RM} $CD8^+$ T cell population that forms following secondary VACV skin infection[211]. Notably, T_{RM} $CD8^+$ T cells that were not B8R-specific did not expand and remained $CD69^+/CD103^+$ (Figure 3.4A-C). B8R-specific $CD8^+$ T cells in the spleen were not significantly boosted by peptide challenge (Figure 3.2I), demonstrating that expansion was site-specific and restricted to the Ag-specific T_{RM} $CD8^+$ T cell population (Figure 3.2J). Critically, the increased number and $CD69^+/CD103^-$ phenotype of B8R-specific $CD8^+$ T cells were maintained 40 days after peptide challenge (Figure 3.4D-F), demonstrating the stability of the expanded B8R-specific T_{RM} population.

Because B8R-specific T_{RM} $CD8^+$ T cells expanded exclusively in the skin following three rounds of $B8R_{20-27}$ peptide challenge, we next determined if boosting T_{RM} $CD8^+$ T cells of a single Ag specificity would improve protection compared to the primary T_{RM} population. We administered 3 rounds of $B8R_{20-27}$ or control peptide to VACV-immunized skin (as in Figure 3.2E), depleted circulating $CD4^+$ and $CD8^+$ T cells, and infected both distal and immunized skin with VACV-WR (as in Figure 3.1J). In immunized skin, boosting the number of B8R-specific T_{RM} $CD8^+$ T cells

increased protection by 50-fold compared to the primary T_{RM} population (Figure 3.2K), whereas protection in distal skin was not affected. Together, these data demonstrate that activation of endogenous skin T_{RM} $CD8^+$ T cells causes acute DTH, but also generates a larger population of secondary Ag-specific T_{RM} $CD8^+$ T cells that provide site-specific protective immunity.

3.2.3 Circulating memory $CD8^+$ T cells traffic into the skin following local T_{RM} activation and accumulate in an antigen-specific manner

To determine if the rapid accumulation of $CD8^+$ T cells following T_{RM} activation was due to the recruitment of circulating memory $CD8^+$ T cells, we treated mice with CD8 depleting antibody before peptide challenge, which eliminates $CD8^+$ T cells from the circulation, but spares T_{RM} $CD8^+$ T cells in the skin (Figiure 3.1G-I). When circulating $CD8^+$ T cells were depleted, the number of $CD8^+$ T cells and B8R-specific cells did not increase after peptide challenge (Figure 3.5A-C), demonstrating that the increase of $CD8^+$ T cells in the skin within 40 hours of peptide challenge was due to the recruitment of memory $CD8^+$ T cells from the circulation. Trafficking of memory $CD8^+$ T cells into inflamed skin is dependent on their ability to bind P- and E-selectin on vascular endothelium[193]. B8R-specific memory $CD8^+$ T cells in the circulation expressed P- and E-selectin ligands (Figure 3.5D,E) and blocking P- and E-selectin prevented the recruitment of $CD45^+$, $CD8^+$ T cells (Figure 3.6A,B), and B8R-specific $CD8^+$ T cells into the skin (Figure 3.5F). To determine if recruitment of circulating Ag-specific memory $CD8^+$ T cells was required for the subsequent formation of an expanded, stable secondary T_{RM} $CD8^+$ T cell population, we depleted circulating $CD8^+$ T cells and challenged the ear skin with 3 rounds of $B8R_{20\text{-}27}$. As shown previously (Figure 3.2F-H), B8R-specific $CD8^+$ T cells in the skin of IgG-treated mice expanded and formed a $CD69^+/CD103^-$ secondary T_{RM} population (Figure 3.5G-I). In contrast, mice that lacked circulating memory $CD8^+$

T cells failed to accumulate B8R-specific T_{RM} $CD8^+$ T cells and remained mostly $CD69^+/CD103^+$ (Figure 3.5G-I). Together, these data demonstrate that the accumulation of $CD69^+/CD103^-$ Ag-specific T_{RM} $CD8^+$ T cells requires the recruitment of memory $CD8^+$ T cells from the circulation.

3.2.4 'Recruit and capture' strategy to establish antigen-specific T_{RM} $CD8^+$ T cells in the skin

It has been reported that recruitment of effector/memory $CD8^+$ T cells into non-lymphoid tissues can be sufficient to generate T_{RM} $CD8^+$ T cells[73, 83, 136, 148, 151]. To test if this occurs during a T_{RM}-mediated DTH reaction, we transferred naïve P14 $CD8^+$ T cells and infected mice with LCMV, which generates robust circulating memory populations, but limited numbers of P14 $CD8^+$ T cells can be isolated from the skin[211]. LCMV-immune mice were then infected on the ear skin with VACV to generate B8R-specific T_{RM} $CD8^+$ T cells. Mice were then challenged with $B8R_{20-27}$ or control peptide and recruitment of P14 and B8R-specific $CD8^+$ T cells was analyzed (Figure 3.7A). Memory P14 and B8R-specific $CD8^+$ T cells both rapidly trafficked into the skin within 40 hours of $B8R_{20-27}$ challenge (Figure 3.7B,C; 40h), demonstrating that the recruitment of circulating memory $CD8^+$ T cells is antigen-independent. However, in skin that was challenged with B8R peptide, only B8R-specific $CD8^+$ T cells were retained 10 days after the final challenge (Figure 3.7B,C; 10d), demonstrating that there is essentially no bystander T_{RM} differentiation of recruited memory $CD8^+$ T cells. This finding suggested that the $B8R_{20-27}$ peptide was responsible for both initiating recruitment and the subsequent accumulation of B8R-specific memory $CD8^+$ T cells in the skin. Thus, we next tested whether a new T_{RM} population could be established by recruiting circulating memory $CD8^+$ T cells with $B8R_{20-27}$, and then 'capturing' memory $CD8^+$ T cells of a different Ag-specificity by including an additional antigenic peptide (Figure 3.7D). To test this, we infected LCMV-immune mice on the left and right ear skin with

VACV and 30 days later challenged the left ear skin with $B8R_{20-27}/GP_{33-41}$ and the right ear skin with $B8R_{20-27}$/control peptide 3 times (Figure 3.7E). Thirty days after the final peptide challenge, B8R-specific $CD8^+$ T cells accumulated at both sites equally, but significantly more GP_{33-41}-specific $CD8^+$ T cells were retained in the $B8R_{20-27}/GP_{33-41}$ challenged skin compared to $B8R_{20-27}$/control skin (Figure 3.7F-K). B8R-specific $CD8^+$ T cells were largely $CD69^+/CD103^-$ at both sites, while the majority of GP_{33-41}-specific $CD8^+$ T cells were $CD69^-/CD103^-$ in $B8R_{20-27}$/control-challenged skin and mostly $CD69^+/CD103^-$ in $B8R_{20-27}/GP_{33-41}$ skin (Figure 3.7F-K). Similar results were observed when TCR-tg P14 $CD8^+$ T cells were used as the "capture" population (Figure 3.8). Together, these data demonstrate that activation of established T_{RM} $CD8^+$ T cells is sufficient to recruit circulating memory $CD8^+$ T cells, but the capture and subsequent differentiation into $CD69^+$ T_{RM} requires Ag recognition within the skin.

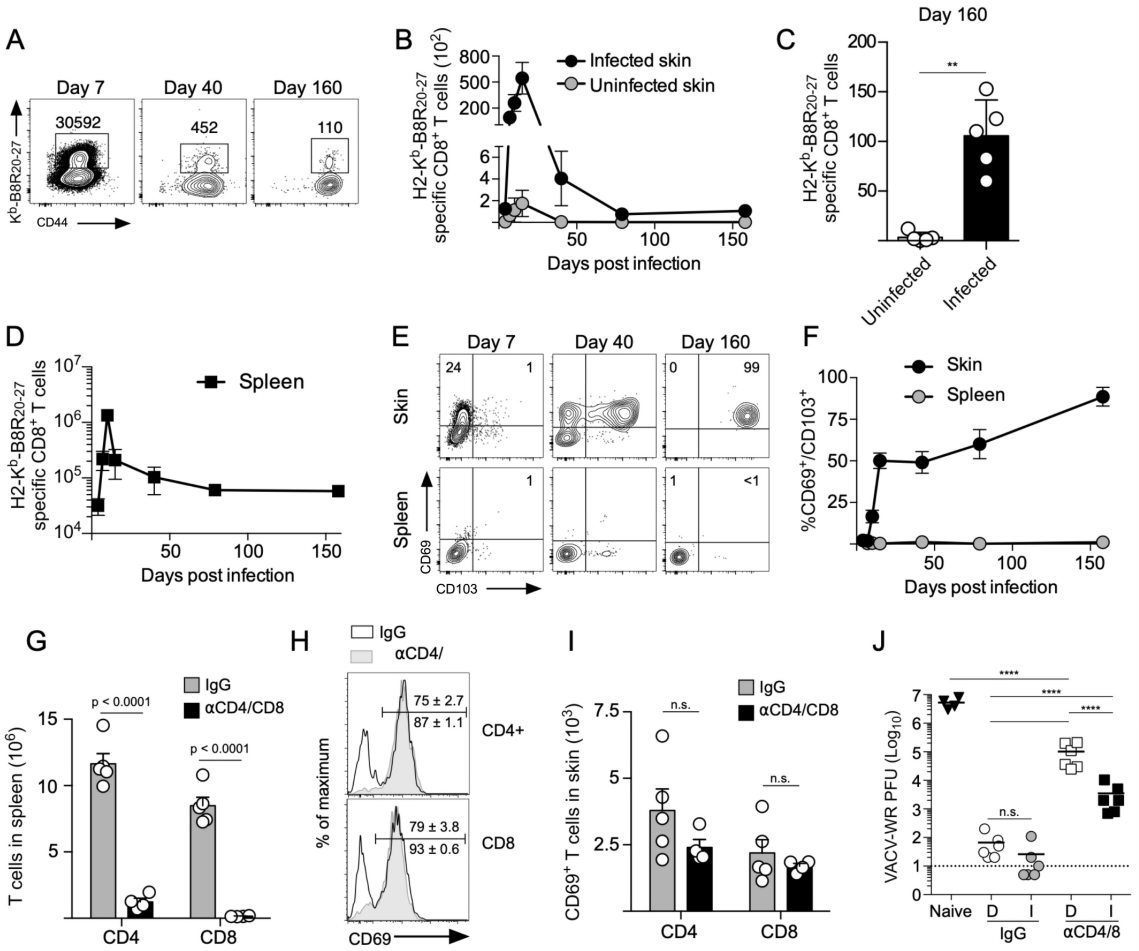

Figure 3.1: VACV skin infection generates protective circulating and tissue-resident memory T cell populations. (A) Mice were infected on the left ear skin with VACV and B8R-specific CD8⁺ T cells from infected skin were identified at the indicated times post-infection by flow cytometry. **(B)** Quantification of (A). **(C)** Quantification of the final timepoint in B. **(D)** Quantification of B8R-specific CD8⁺ T cells in the spleen. **(E)** CD103 and CD69 expression by B8R-specific CD8⁺ T cells. **(F)** Quantification of (E). **(G-I)** Mice were infected as in A and received IgG or CD4/CD8 depleting antibodies on day 35 post-infection. **(G)** Number of CD4⁺ and CD8⁺ T cells in the spleen. **(H)** CD69 expression by CD4⁺ and CD8⁺ T cells in the skin. **(I)** Number of CD69⁺ CD4⁺ and CD8⁺ T cells in the skin. **(J)** Mice were treated as in (G) and challenged on left and right ear skin with VACV-WR. Viral load was quantified day 3 post-infection. Dashed line indicates limit of detection. D: distal skin. I: immunized skin. Statistical significance was determined using a paired t-test (C), unpaired t-test (G,I), or one-way ANOVA with Tukey's multiple comparison test (J). Error bars represent SEM. Data in A-I are representative of at least 2 independent experiments (n = 3-5). Data in I are pooled from 2 independent experiments (n = 3).

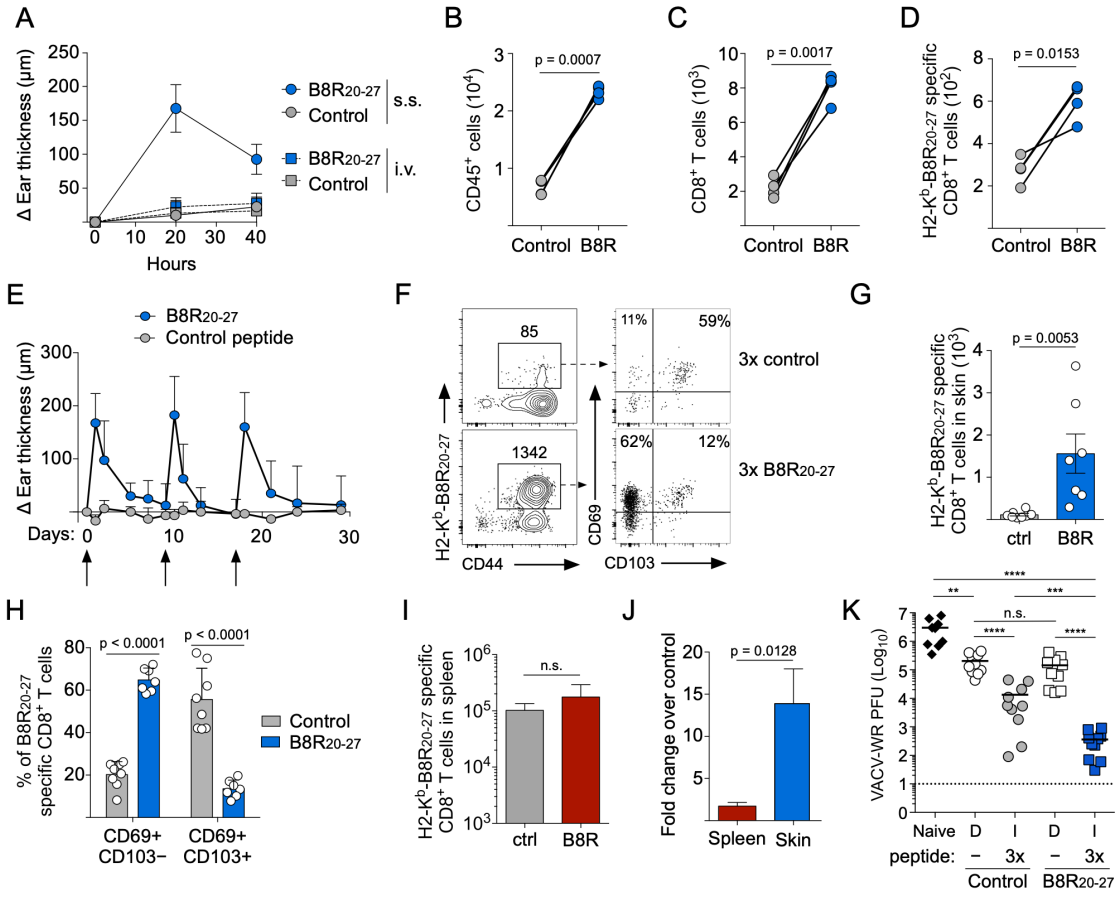

Figure 3.2: Topical application of cognate peptide activates T_{RM} CD8$^+$ T cells and boosts the quantity of antigen-specific T_{RM} CD8$^+$ T cells in the skin. (A) Mice were infected with VACV i.v. or on the left ear skin by scarification (s.s.; see Fig. 3.3). On day 40 post infection, B8R$_{20-27}$ or control peptide (NP$_{396-404}$ of LCMV) were applied to the left ear skin and change in ear thickness was measured. **(B-D)** Mice were infected by scarification as in A, and 40 hours after peptide application the number of CD45$^+$ (B), CD8$^+$ (C), and B8R-specific CD8$^+$ T cells (D) in the skin was determined by flow cytometry. **(E)** Mice were infected on the left and right ear skin with VACV. On day 35 post-infection, skin was challenged with B8R$_{20-27}$ or control peptide and swelling was monitored. Once swelling had subsided, mice were re-challenged two more consecutive times (arrows indicate peptide challenge). **(F)** Representative flow cytometry plots depicting B8R-specific CD8$^+$ T cells and expression of CD69 and CD103 10 days after the final peptide challenge (see Fig. 3.4). **(G,H)** Quantification of (F). **(I)** Number of B8R-specific CD8$^+$ T cells in the spleen of mice challenged with control peptide or B8R$_{20-27}$. **(J)** Fold increase in B8R-specific CD8$^+$ T cells in spleen or skin after B8R$_{20-27}$ challenge. **(K)** Mice were treated as in (E) and were given anti-CD4/CD8 depleting antibodies 10 days after the last peptide challenge. Mice were then infected on both ear skins with VACV-WR and viral load was quantified day 3 post-infection. Dashed line indicates limit of detection. D, distal skin. I, immunized skin. Statistical significance was determined using a paired t-test (B-D), unpaired t-test (G-J), or one-way ANOVA with Tukey's multiple comparison test (K). Error bars represent SEM. Data in A-J are representative of at least 3 independent experiments (n=3-8). Data in (K) are pooled from 2 independent experiments (n=4-5).

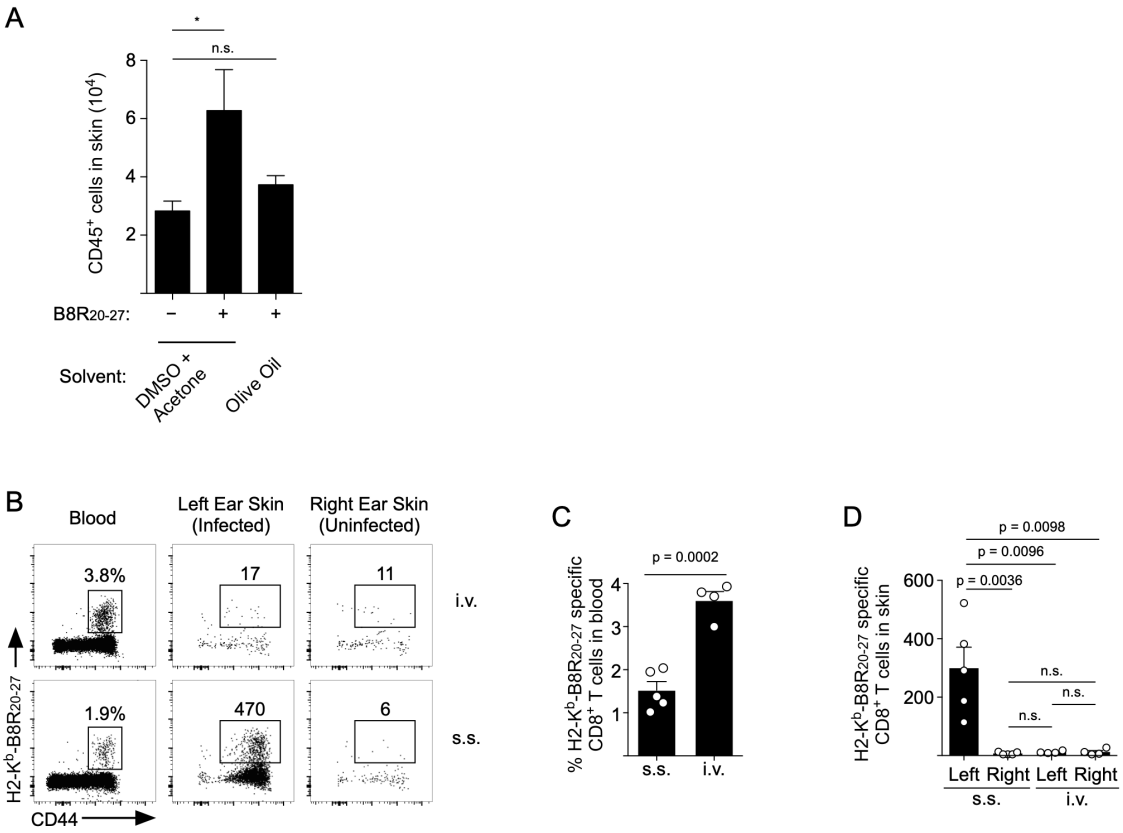

Figure 3.3: DMSO is required to deliver peptide to skin-T_{RM} CD8$^+$ T cells and skin infection is required to generate functional VACV-specific skin-T_{RM} CD8$^+$ T cells (Related to Figure 3.2). (A) Mice were infected on the left ear skin with VACV and 30 days later were challenged with B8R$_{20-27}$ in DMSO + acetone or an olive oil emulsion and the number of CD45$^+$ cells in the challenged skin was quantified 40 hours after challenge. **(B)** Mice were infected with VACV either i.v. or on the left ear skin by scarification (s.s.). On day 35 post infection, B8R-specific CD8$^+$ T cells were identified in the blood and skin by flow cytometry. **(C)** Quantification of the frequency of B8R-specific CD8$^+$ T cells in the blood. **(D)** Quantification of the number of B8R-specific CD8$^+$ T cells in the left (infected) and right (uninfected) ear skin of mice infected i.v. or by scarification. Statistical significance was determined using an unpaired t-test. Data are represented as mean ± SEM. Data are representative of two independent experiments (n = 3-5).

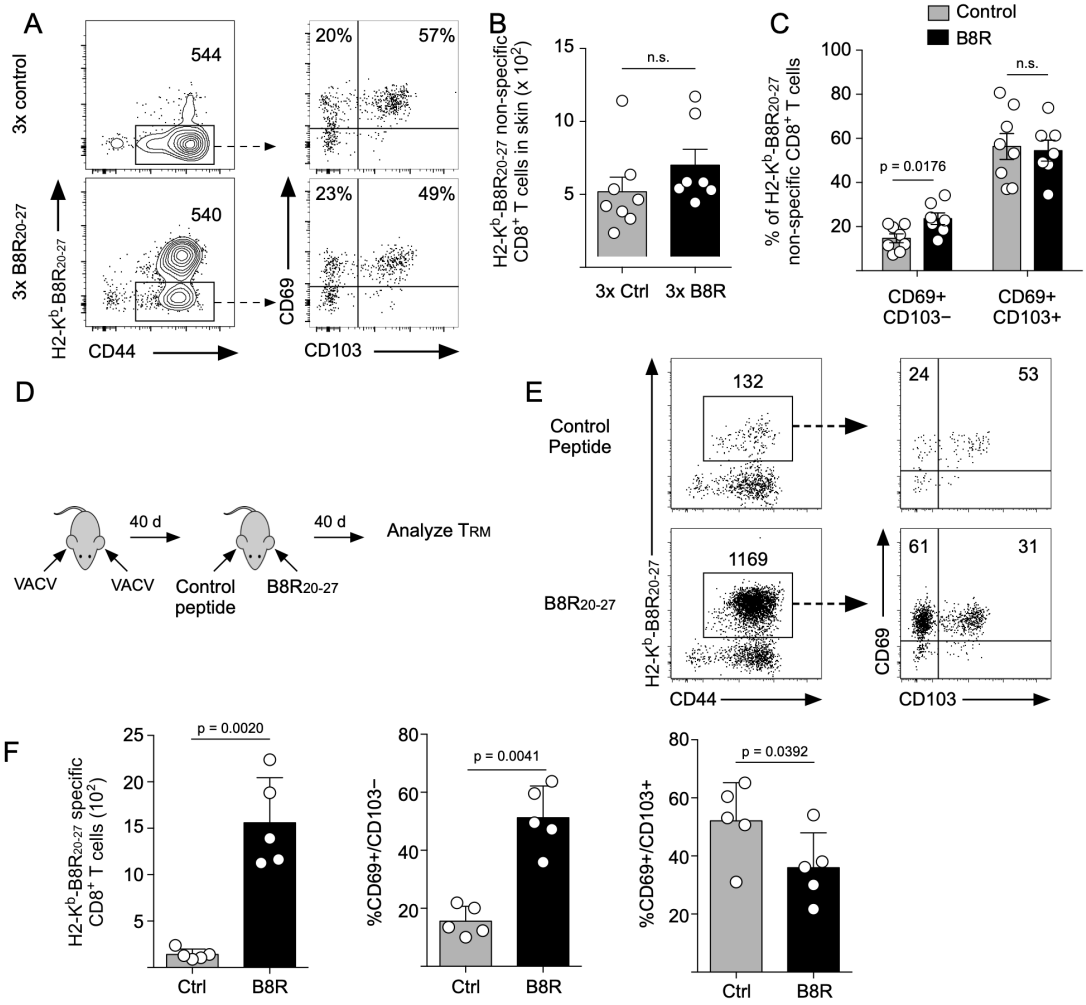

Figure 3.4: Antigen non-specific CD8⁺ T cells do not accumulate in challenged skin and boosted antigen-specific T_{RM} CD8⁺ T cells are stably retained. (A-C) Mice were infected on the left and right ear skin with VACV. On day 35 post-infection, skin was challenged with $B8R_{20-27}$ or control peptide and skin swelling was monitored. Mice were re-challenged twice more when swelling had subsided (as in Fig. 3.2E) and the B8R-non-specific T_{RM} CD8⁺ T cells were analyzed 10 days after the final peptide challenge. **(A)** Representative flow cytometry plots depicting B8R-non-specific CD8⁺ T cells and their expression of CD103 and CD69 (same data as in Figure 3.2F-H). **(B)** Quantification of the number of non-B8R-specific CD8⁺ T cells in (A). **(C)** Quantification of CD103 and CD69 expression by non-B8R-specific CD8⁺ T cells in (A). **(D)** Experimental setup to test whether the boosted B8R-specific T_{RM} population is maintained 40 days after a single round of peptide challenge. Mice were infected on the left and right ear skin with VACV and 40 days later challenged with control peptide ($NP_{396-404}$) on the right ear skin or $B8R_{20-27}$ on the left ear skin. **(E)** Mice were treated as in (D) and the number of B8R-specific T_{RM} CD8⁺ T cells and their expression of CD103 and CD69 40 days after peptide challenge was determined by flow cytometry. **(F)** Quantification of (E). Statistical significance was determined using an unpaired (B,C) or paired (F) t-test. Data are represented as mean ± SEM. Data are representative of at least two independent experiments (n = 3-8).

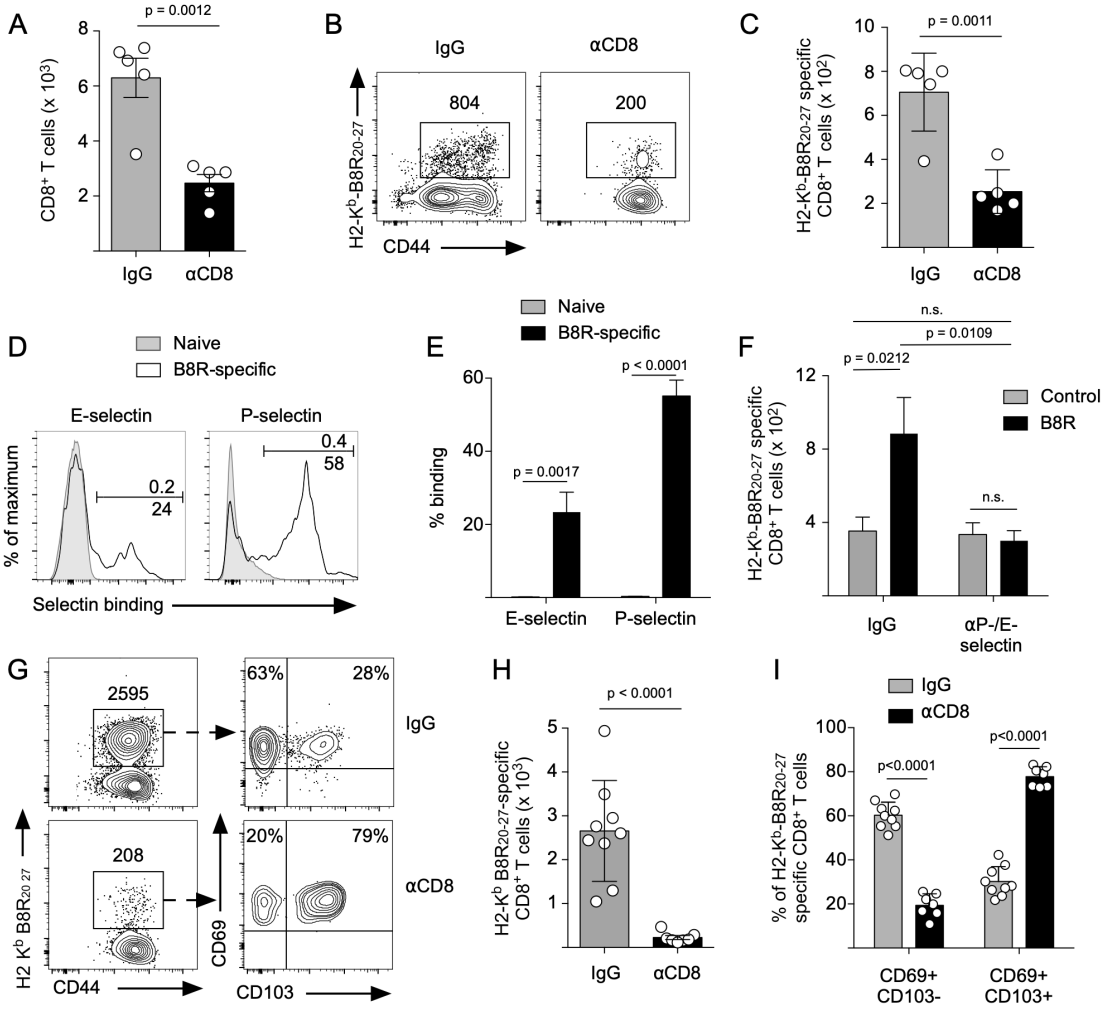

Figure 3.5: Circulating memory CD8⁺ T cells are recruited to the site of T_{RM} activation and are the source of secondary T_{RM} CD8⁺ T cells. (A-C) Naive B6 mice were infected on the left ear skin with *tk*-VACV and on days 30 and 35 after infection mice received control IgG or CD8 depleting antibody. Mice were then challenged on the left ear skin with $B8R_{20-27}$ and the number of CD8⁺ T cells (A) and $B8R_{20-27}$-specific CD8⁺ T cells (B-C) was quantified 40 hours after peptide challenge. **(D-F)** Mice were infected on the left and right ear skin with VACV and on day 35 after infection, $B8R_{20-27}$ specific CD8⁺ T cells in the blood were identified by tetramer and analyzed for expression of P- and E- selectin ligands (D-E). Naive cells were identified as $CD62L^{+}/CD44^{-}$ and used as a negative control for selectin ligand detection. (E) Quantification of data shown in (D). (F) Mice received IgG or P-/E-selectin blocking antibodies on day 35 post infection, were challenged with $B8R_{20-27}$ on the left ear skin and control peptide on the right ear skin, and the number of B8R-specific CD8⁺ T cells in the skin 40 hours after challenge was determined by flow cytometry (see Fig. 3.6). **(G-I)** Mice received IgG or CD8 depleting antibody as in (A) and were then challenged three times with $B8R_{20-27}$ (as in Fig. 3.2E) and analyzed 10 days after the last peptide challenge. (G) Representative flow cytometry plot of B8R-specific CD8⁺ T cells and their expression of CD69 and CD103. (H-I) Quantification of the data shown in (G). Statistical significance was determined using an unpaired t-test. Error bars represent SEM. Data in (A-C) are representative of 2 independent experiments, n=3-5 per group. Data in (D-F) are representative of 3 independent experiments, n = 3-5 per group. Data in (G-I) are pooled from 2 independent experiments, n=4-5 per group.

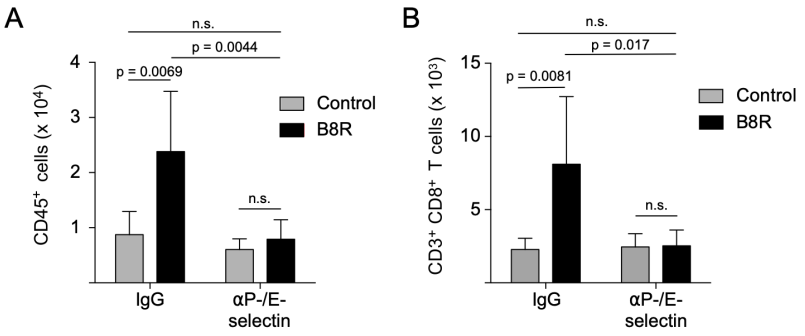

Figure 3.6: Cellular recruitment associated with T_{RM}-mediated DTH is dependent on P- and E-selectin. (A-B) Mice were infected on the left and right ear skin with VACV and 35 days after infection were given control IgG or anti-P- and anti-E-selectin blocking antibodies (as in Fig. 3.5F). Mice were then challenged on the left ear skin with $B8R_{20-27}$ and the right ear skin with control peptide and the number of $CD45^+$ cells (A), and $CD8^+$ T cells (B) in the skin 40 hours after challenge was determined by flow cytometry. Statistical significance was determined using an unpaired t-test. Data are represented as mean ± SEM. Data are representative of 3 independent experiments (n = 3-10).

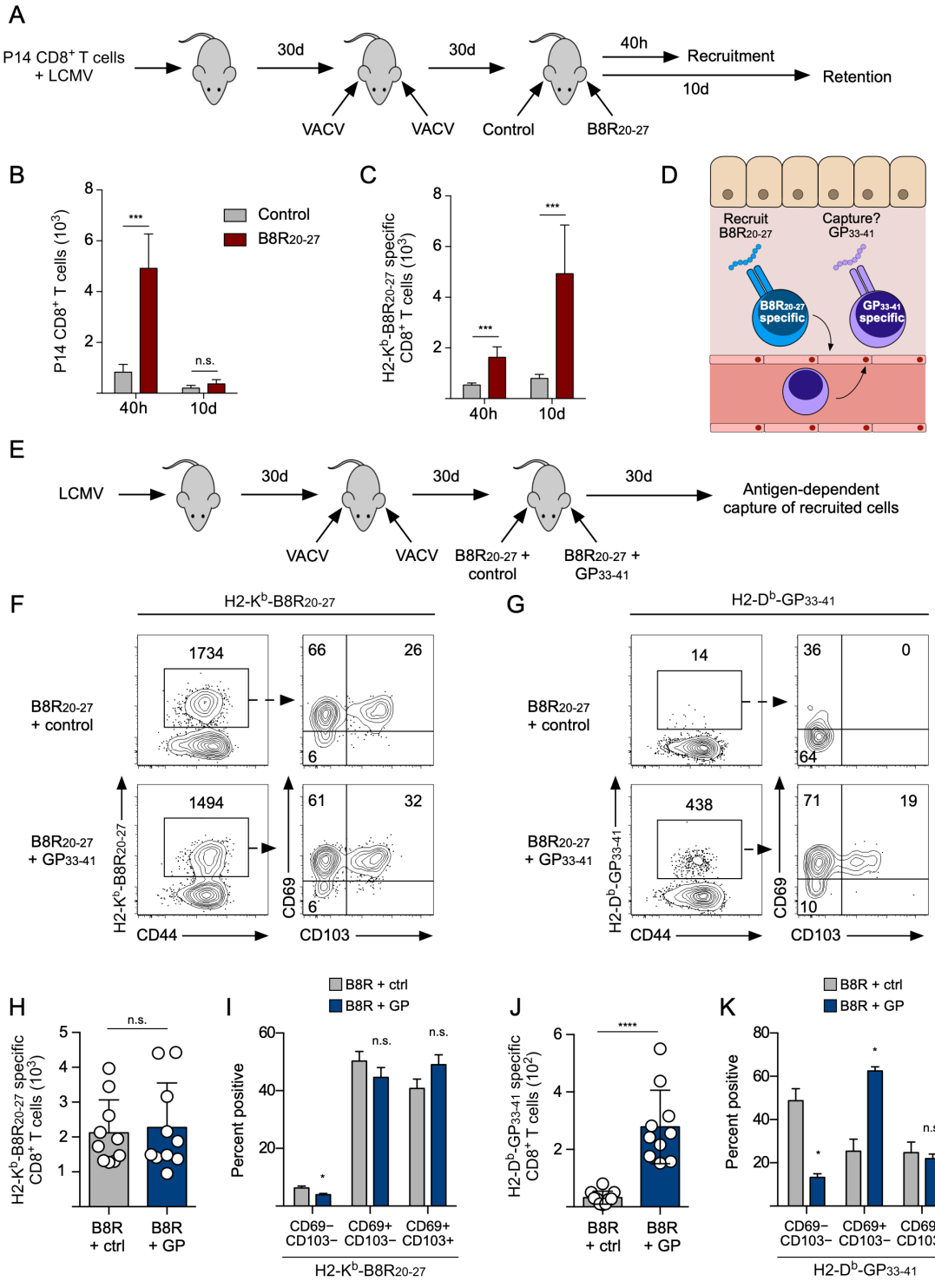
A
P14 CD8+ T cells + LCMV
30d
30d
40h
Recruitment
10d
Retention
VACV
VACV
Control
B8R20-27
B
P14 CD8+ T cells (10^3)
Control
B8R20-27
***
n.s.
40h
10d
C
H2-Kb-B8R20-27 specific CD8+ T cells (10^3)
***
***
40h
10d
D
Recruit B8R20-27
Capture? GP33-41
B8R20-27 specific
GP33-41 specific
E
LCMV
30d
30d
30d
Antigen-dependent capture of recruited cells
VACV
VACV
B8R20-27 + control
B8R20-27 + GP33-41
F
H2-Kb-B8R20-27
B8R20-27 + control
B8R20-27 + GP33-41
1734
66
26
6
1494
61
32
6
H2-Kb-B8R20-27
CD44
CD69
CD103
G
H2-Db-GP33-41
B8R20-27 + control
B8R20-27 + GP33-41
14
36
0
64
438
71
19
10
H2-Db-GP33-41
CD44
CD69
CD103
H
H2-Kb-B8R20-27 specific CD8+ T cells (10^3)
n.s.
B8R + ctrl
B8R + GP
I
B8R + ctrl
B8R + GP
Percent positive
n.s.
n.s.
*
CD69− CD103−
CD69+ CD103−
CD69+ CD103+
H2-Kb-B8R20-27
J
H2-Db-GP33-41 specific CD8+ T cells (10^2)
****
B8R + ctrl
B8R + GP
K
B8R + ctrl
B8R + GP
Percent positive
*
*
n.s.
CD69− CD103−
CD69+ CD103−
CD69+ CD103+
H2-Db-GP33-41

Figure 3.7: Circulating memory CD8$^+$ T cells recruited during DTH form a de novo T_{RM} CD8$^+$ T cell population following local antigen recognition. (A-C) Mice received naïve P14 CD8$^+$ T cells and were infected with LCMV. Mice were then infected with VACV on the left and right ear skin followed by challenge with either control peptide ($NP_{396-404}$) or $B8R_{20-27}$. (A) Experimental design. (B-C) Number of P14 (B) or B8R-specific CD8$^+$ T cells (C) in the skin 40 hours after peptide challenge or 10 days after three rounds of peptide challenge. **(D)** Schematic of the 'recruit and capture' strategy. **(E-K)** Mice were infected with LCMV and 30 days later were infected with VACV. Mice were then challenged three times with a mixture of B8R + control peptide ($OVA_{257-264}$) or B8R + GP_{33-41}, and skin was analyzed 30 days after the final peptide challenge. (E) Experimental design. (F, G) Representative flow cytometry plots depicting the number of B8R-specific (F) or GP_{33-41}-specific (G) CD8$^+$ T cells and their expression of CD69 and CD103. (H,I) Quantification of F. (J,K) Quantification of G (see Fig. 3.8). Statistical significance was determined using a paired t-test. Error bars represent SEM. Data in (B-C) are representative of 2 independent experiments (n = 5). Data in (F-K) are representative of 3 independent experiments (n=4-10).

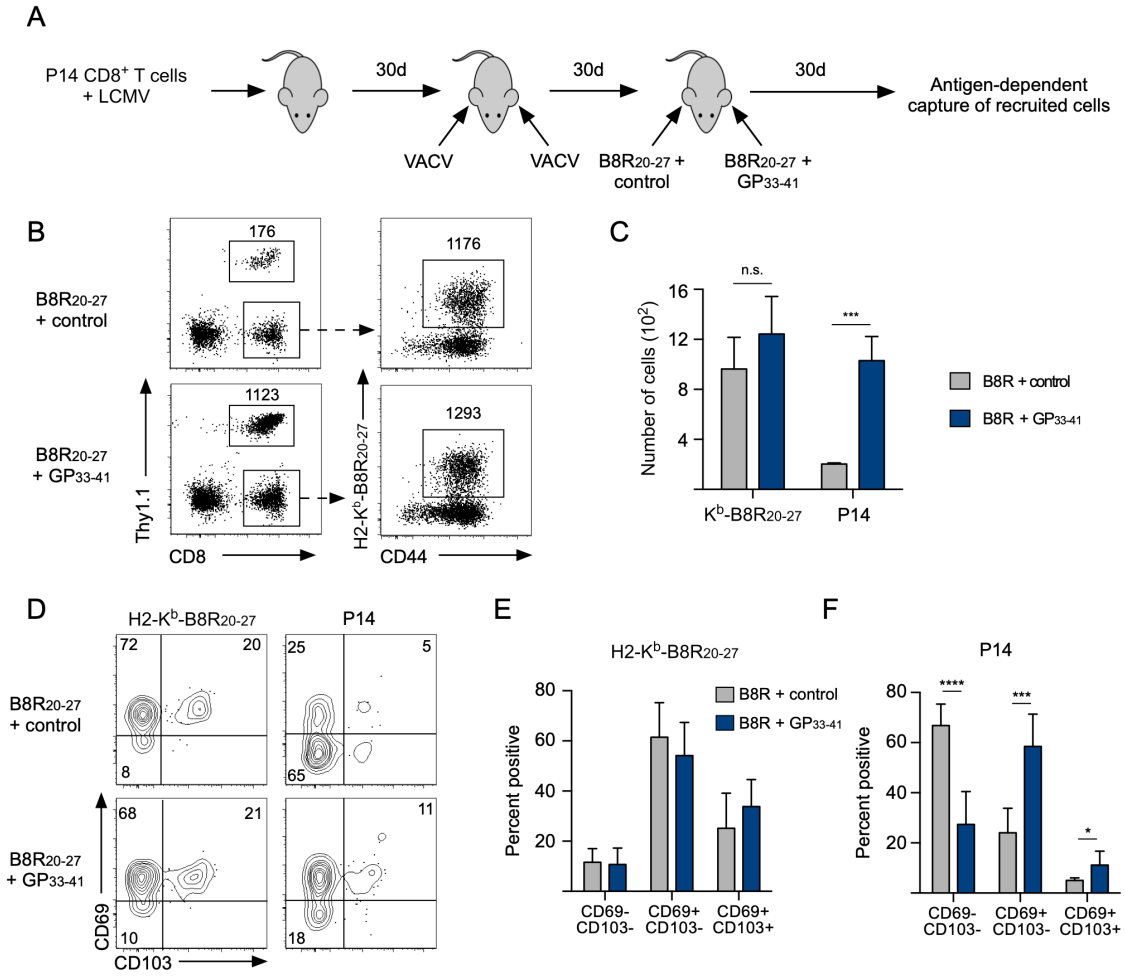

Figure 3.8: Circulating memory TCR-tg CD8⁺ T cells recruited during DTH form a de novo T_{RM} CD8⁺ T cell population following local antigen recognition. Mice received naïve P14 CD8⁺ T cells and were infected with LCMV. Mice were then infected on the left and right ear skin with VACV 30 days after LCMV infection. Ear skin was then challenged with $B8R_{20-27}$+control peptide ($OVA_{257-264}$) or $B8R_{20-27}$ + GP_{33-41} three times and T_{RM} cells were analyzed 30 days after the final peptide challenge (as in Fig. 4E). **(A)** Experimental design. **(B)** Representative flow cytometry plots depicting the number of Thy1.1⁺ P14 CD8⁺ T cells and B8R-specific CD8⁺ T cells. **(C)** Quantification of (B). **(D)** Representative flow cytometry plots of CD103 and CD69 expression by P14 and B8R-specific CD8⁺ T cells identified in (B). **(E)** Quantification of CD103 and CD69 expression by B8R-specific CD8⁺ T cells identified in (D). **(F)** Quantification of CD103 and CD69 expression by P14 CD8⁺ T cells identified in (D). Statistical significance was determined using a paired t-test. Data are represented as mean ± SEM. Data are representative of 2 independent experiments (n = 5).

3.3 Discussion

Recently, we reported that the presence of local antigen enhances the formation of T_{RM} $CD8^+$ T cells during a primary VACV skin infection[143], which agrees with our data presented here demonstrating that antigen recognition by circulating memory $CD8^+$ T cells within the skin microenvironment also controls the formation and retention of secondary T_{RM} cells. This finding is in contrast to a recent study demonstrating bystander T_{RM} differentiation following HSV-1 skin infection[196], suggesting that antigen-dependent T_{RM} differentiation may not occur during herpesvirus infections. Most secondary T_{RM} $CD8^+$ T cells do not express CD103, which is consistent with our recent observation that skin-T_{RM} $CD8^+$ T cells that are derived from circulating memory cells are intrinsically unable to express CD103, but are in fact tissue-resident[211]. Interestingly, human skin contains approximately twice as many T cells as the entire vascular system, and these skin-resident T cells are nearly all $CD69^+$ but heterogeneous in terms of CD103 expression[21]. In contrast, T_{RM} populations generated in laboratory mice following a single infection are generally smaller than circulating T cell populations and nearly all $CD69^+/CD103^+$[18, 83]. Our results suggest that recurring encounters with pathogens or environmental antigens may contribute to the increased size and varied composition of the $CD8^+$ T cell populations observed in human skin.

In summary, we demonstrate that topical application of a single antigenic peptide boosted the number of antigen-specific T_{RM} $CD8^+$ T cells in the skin and was sufficient to increase local protective immunity against a homologous infection. The secondary T_{RM} $CD8^+$ T population was derived from circulating memory $CD8^+$ T cells that were recruited into the skin and differentiated into a $CD69^+/CD103^-$ T_{RM} $CD8^+$ T cell following local antigen encounter (Figure 3.9). Importantly, because this mechanism of expansion only relies on stable circulating memory T cell popu-

lations, boosting T_{RM} populations within the skin could occur at any time after successful immunization. Additionally, because all circulating memory $CD8^+$ T cells are recruited to the site of challenge, de novo T_{RM} populations can be generated by including additional peptides that will capture recruited memory $CD8^+$ T cells. Thus, our study demonstrates that topical application of antigenic peptides may offer a cheap and simple strategy to boost protective T_{RM} $CD8^+$ T cell populations at environmental barrier tissues such as the skin.

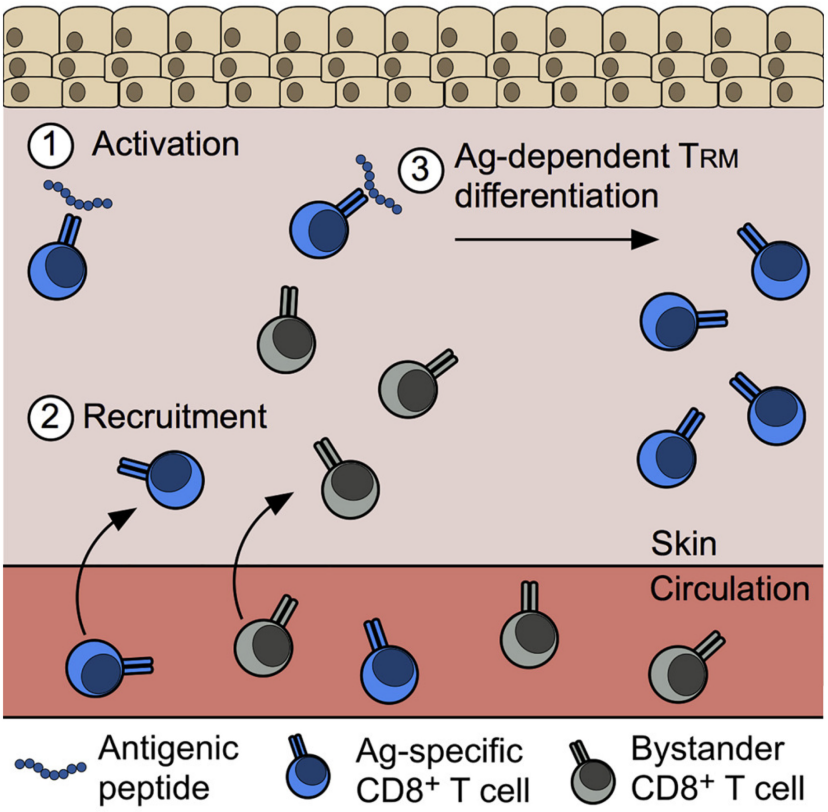

Figure 3.9: Targeted expansion of tissue-resident memory $CD8^+$ T cells following topical application of antigenic peptide. (1) Topical application of antigenic peptide activates T_{RM} $CD8^+$ T cells in the skin and initiates a local contact hypersensitivity response. (2) As a result of the contact hypersensitivity, memory $CD8^+$ T cells from the circulation are recruited to the inflamed skin independent of antigen specificity. (3) Antigen-specific memory $CD8^+$ T cells from the circulation form a secondary T_{RM} population while bystander $CD8^+$ T cells are not retained within the skin. This increase in T_{RM} $CD8^+$ T cells provides increases local protective immunity.

Chapter 4

Summary, conclusions, and avenues of future investigation

The work presented in this thesis focused on both the differentiation and function of T_{RM} $CD8^+$ T cell populations following viral skin infection. Specifically, I examined the transition from effector to T_{RM} $CD8^+$ T cell and determined that TCR engagement by effector $CD8^+$ T cells induces significant transcriptional changes that promote T_{RM} differentiation. I then investigated the role of established T_{RM} $CD8^+$ T cell populations in mediating local hypersensitivity reactions using a simple technique of topical administration of an antigenic peptide. This method of T_{RM} $CD8^+$ T cell reactivation was sufficient to boost the local antigen-specific T_{RM} population and subsequently increase T_{RM}-mediated protective immunity. Together, the data in this thesis support a model in which effector or circulating memory $CD8^+$ T cells are recruited to sites of inflammation and thereby provide systemic immunosurveillance, while encounters with cognate antigen within those tissues induces T_{RM} differentiation, thereby positioning pathogen-specific immunosurveillance specifically at sites of pathogen invasion and/or replication.

T_{RM} development

To understand how TCR stimulation promotes T_{RM} $CD8^+$ T cell differentiation during the primary response to VACV skin infection, I began with a characterization of IFNγ expression by effector $CD8^+$ T cells using an IFNγ-YFP reporter system.

I found that IFNγ was not expressed by cells in the blood or lymphoid organs but was restricted to a subset of effector cells within the skin. IFNγ expression became undetectable coincident with viral clearance and was dependent on the presence of local cognate antigen, demonstrating that IFNγ expression can be used to identify cells that were actively receiving TCR stimulation in vivo. Using this reporter system, I then compared genome-wide expression profiles of IFNγ^+ and IFNγ^- CD8$^+$ T cells and found that IFNγ^+ cells are engaging known pathways that promote T_{RM} differentiation, and that IFNγ^+ cells on day 7 post infection already resemble mature T_{RM} CD8$^+$ T cells on a transcriptional level. This transcriptional analysis agreed with flow cytometric analysis that showed increased expression of key surface markers that are known to be associated with T_{RM} development, providing strong evidence that IFNγ^+ cells are precursors of long-lived T_{RM} CD8$^+$ T cells. Mechanistically, I found that TCR stimulation prevents S1PR1-mediated tissue egress in effector CD8$^+$ T cells, a step that is required for the development of T_{RM} CD8$^+$ T cells. Further, TCR stimulation drove expression of the transcription factor Blimp1, which was recently found to regulate several critical transcriptional changes that promote T_{RM} development, including repression of S1PR1 as well as transcription factors that promote T_{CM} development. TCR stimulation of effector CD8+ T cells was sufficient to cause repression of these critical Blimp1 target genes, suggesting that T_{RM} differentiation following antigen recognition within the skin is at least in part regulated through known Blimp1-dependent pathways.

To begin to address the specific role of Blimp1 expression downstream of TCR stimulation, we have performed preliminary studies using Blimp1$^{FLOX/FLOX}$ mice in conjunction with a tamoxifen-inducible Cre. Intriguingly, Blimp1$^{FLOX/FLOX}$ mice expressed lower levels of CD69 on day 15 post infection than their WT counterparts, suggesting a possible lack of S1PR1 repression. However, there was not a significant defect in T_{RM} formation at later timepoints, suggesting that Blimp1 may

act in cooperation with other transcription factors to regulate T_{RM} development. This result is consistent with the published role of Blimp1 acting in concert with Hobit to regulate T_{RM} development following HSV skin infection[122]. Although it has not been reported whether Blimp1 and Hobit physically interact, Blimp1 often accomplishes changes in gene expression through physical interaction with other transcriptional regulators[212]. One such partner proteins is Zbtb32, which cooperates with Blimp1 in effector $CD8^+$ T cells to regulate the development of T_{EM} and T_{CM} populations following systemic viral infection[213]. Interestingly, our transcriptional analyses in Chapter 2 demonstrated that Zbtb32 is also highly expressed in response to TCR stimulation in the skin, suggesting a possible additional partner protein for Blimp1 may cooperate with multiple proteins to regulate T_{RM} formation. Future studies investigating potential alternative Blimp1 partner proteins would provide insight into the transcriptional networks that regulate T_{RM} development.

In this study, I focused exclusively on T_{RM} differentiation downstream of antigen recognition, which is in contrast to many of the initial studies which found that antigen was dispensable for the development of T_{RM} $CD8^+$ T cells in skin. Rather, these studies focused on the role of tissue-derived cytokines and interactions with tissue-specific components of the extracellular matrix during T_{RM} formation and maintenance. While antigen-independent pathways that promote T_{RM} differentiation clearly exist, this work and our previously published studies have demonstrated that the presence of local antigen results in more robust T_{RM} development than exposure to the tissue microenvironment alone. By using the IFNγ-YFP approach described in this thesis, we were able to compare three populations of effector cells; those within the spleen that have not yet been exposed to the tissue environment, cells in the skin that have not engaged cognate pMHC, and cells in the skin that are actively receiving TCR stimulation. Thus, this approach allowed us to examine the transcriptional consequences of TCR stimulation on top of exposure

to the tissue microenvironment alone. Consistent with this notion, we found that expression of particular T_{RM}-associated genes appear to be regulated by exposure to the VACV-infected skin microenvironment, while others are regulated in response to TCR stimulation within VACV-infected skin. Thus, the transcriptional profiles of T_{RM} precursor cells is a rich data source that could generate hypothesis about novel antigen-dependent and -independent pathways that regulate the formation of T_{RM} $CD8^+$ T cells.

To begin to use this dataset for hypothesis generation, I performed pathway enrichment analysis to determine whether genes involved in specific biological processes were over-represented in the $IFN\gamma^+$ transcriptional profile. I found that many diverse metabolic pathways are engaged by $IFN\gamma^+$ T_{RM} precursor cells, suggesting that TCR stimulation within the skin microenvironment may enable effector $CD8^+$ T cells to utilize the various nutrient sources that are present in non-lymphoid tissues and are not encountered in the circulation. For example, T_{RM} $CD8^+$ T cells in the skin require FABP4 and 5, which allows them to directly uptake extracellular fatty acids that are abundant within the epidermis[115]. This uptake was also coupled to an increase in the ability of T_{RM} $CD8^+$ T cells to oxidize fatty acids, thereby generating acetyl CoA and deriving energy from the TCA cycle and oxidative phosphorylation. Similarly, long-lived T_{CM} cells in the circulation also preferentially utilize oxidative phosphorylation, but do not display increased uptake of extracellular fatty acids[198]. Rather, these cells synthesize lipids from glucose or glycerol precursors, which are then hydrolyzed by lysosomal lipase A (LipA), which enables transport into the mitochondria for FAO by transporters on the mitochondrial membrane. Interestingly, there are many mitochondrial transporters of unknown function, and many of them are predicted to be capable of transporting shorter chain fatty acids, based on homology to other long-chain fatty acid transporters[214]. Interestingly, one such transporter (Slc25a45) is highly expressed in $IFN\gamma^+$ cells

within VACV infected skin. This increased mitochondrial transporter expression, together with increased expression of diverse metabolic pathways, Lipa, and nutrient transporters, suggests that TCR stimulation may enable effector $CD8^+$ T cells to utilize a wide variety of nutrients to perform FAO. These ideas could be tested by inhibiting various metabolic pathways and nutrient transporters through either pharmacological or genetic strategies, or a combination of both, and determining whether specific metabolic pathways are required for T_{RM} $CD8^+$ T cell development. Alternatively, forced expression of particular transporters or enzymatic pathways could potentially lead to increased T_{RM} formation, which would provide insights into which metabolic programs are optimal for T_{RM} cells. Given the differences between various non-lymphoid tissues, it would be interesting to determine whether there is a universal T_{RM} metabolic program or whether tissue-specific adaptations dominate T_{RM} metabolism.

Apart from metabolic shifts, I also identified approximately 20 transcription factors that are differentially expressed following TCR stimulation in the skin, and the vast majority of these have unknown roles in T_{RM} development. Because T cell differentiation programs are generally driven by the coordinated action of lineage-defining transcription factors, systematic inhibition or over-expression of these transcription factors may uncover novel regulators of the T_{RM} $CD8^+$ T cell transcriptional program. T cell lineage decisions are also reinforced through epigenetic inhibition of other differentiation programs. Therefore, comparing epigenetic profiles of $IFN\gamma^+$ and $IFN\gamma^-$ cells would provide insight into epigenetic differences driven by TCR stimulation. Together with the transcriptional data presented in this thesis, this paired dataset would provide a powerful system to uncover epigenetic remodeling that promotes or inhibits T_{RM} differentiation.

T_{RM} function

In the second part of this thesis, I examined the function of T_{RM} $CD8^+$ T cells following reactivation. Specifically, I found that application of antigenic peptide to skin that contained T_{RM} $CD8^+$ T cells was sufficient to cause a localized delayed type hypersensitivity reaction that was characterized by tissue swelling and the rapid accumulation of many different leukocyte populations, including memory $CD8^+$ T cells, to the site of T_{RM} activation. Application of antigenic peptide to T_{RM} $CD8^+$ T cells did not boost circulating memory $CD8^+$ T cell levels, suggesting that circulating memory $CD8^+$ T cells were not re-activated, but instead were recruited to the skin directly from the circulation. This is consistent with other studies from our laboratory demonstrating that 'resting' T_{CM} $CD8^+$ T cells in the circulation express molecules that serve as ligands for P- and E-selectin, which enables this subset of circulating memory $CD8^+$ T cells to traffic directly into inflamed tissues independently of lymph node re-priming[139, 211]. As a result, circulating memory $CD8^+$ T cells are rapidly recruited to the site of T_{RM} activation independent of their antigen specificity. However, following the resolution of this acute inflammatory response, the number of T_{Rm} $CD8^+$ T cells specific for the boosting peptide was increased dramatically, whereas bystander T_{Rm} $CD8^+$ T cells of a different antigen specificity were not affected, suggesting that antigen recognition within the skin was a major driver of 'secondary' T_{RM} differentiation. Indeed, inclusion of an additional peptide was sufficient to 'capture' a subset of recruited circulating memory $CD8^+$ T cells and generate a de novo T_{RM} population. The numerical increase in antigen-specific T_{Rm} $CD8^+$ T cells offered increased protective immunity compared to a primary T_{RM} population, suggesting that this simple method of T_{RM} activation could be used as a tissue and antigen specific boosting strategy.

Interestingly, human skin is densely populated with T_{RM} cells. In fact, it is estimated

that human skin on average contains approximately 20 billion T cells, approximately twice the number of T cells in the blood and lymphoid organs[21]. T_{RM} populations in human skin also share the majority of the transcriptional features that were used to define T_{RM} cells in laboratory mice, suggesting a conserved transcriptional program of tissue residency across species. However, despite the overall similarities, there are significant differences between T_{RM} populations in humans and laboratory-mice. For example, nearly all mouse T_{RM} $CD8^+$ T cells generated in response to a single infection reside in the epidermal layer and express CD103 at late timepoints after infection, whereas human T_{RM} $CD8^+$ T cells are often resident in the dermis and a significant portion of human $CD8^+$ T_{RM} do not express CD103[88]. Additionally, when circulating memory T cells become T_{RM} cells, they do not express CD103, and T_{RM} cells generated in mice after a single infection are usually vastly outnumbered by circulating memory T cells[147, 211]. We observed that repeated encounter with cognate antigen not only boosted the number of T_{RM} $CD8^+$ T cells, but also altered the phenotype of the T_{RM} population to become largely $CD69^+/CD103^-$, suggesting that this mechanism of T_{RM} reactivation might contribute to the disparity in size and composition between T_{RM} populations found in humans and laboratory mice. Intriguingly, T_{RM} populations of 'dirty' mice that were not raised in clean laboratory environments are similar to human T_{RM} populations in size and phenotype, further suggesting that repeated encounter with environmental and microbial antigens can drastically shape the T_{RM} population.

Reactivation of T_{RM} $CD8^+$ T cells by topical peptide application is also an attractive method to study the mechanisms by which T_{RM} cells provide protection and cause inflammation. Although T_{RM} $CD8^+$ T cells express IFNγ following application of antigenic peptide, preliminary experiments with either IFNγ receptor deficient mice or IFNγ and TNFα blocking antibodies had no effect on the local hypersensitivity response (data not shown), suggesting that the canonical pro-inflammatory

cytokines produced by $CD8^+$ T cells may not be involved. However, this is in contrast with reports from the literature that demonstrate IFNγ expression is required for T_{RM}-mediated recruitment of circulating lymphocytes to the female reproductive tract. These previously published experiments were performed using OT-I TCR-transgenic $CD8^+$ T cells, whereas our study examined the polyclonal endogenous repertoire of T_{RM} $CD8^+$ T cells, which encodes a large variety of TCRs of varying affinity for pMHC and therefore may respond differently to restimulation. Additionally, the concentration of antigenic peptide used in our study was approximately 10-fold lower than previously published studies, further suggesting that the strength of TCR stimulation may impact the downstream consequences of T_{RM} $CD8^+$ T cell activation. Regardless of any experimental caveats, the simple model system described in this thesis could be useful in determining the molecular mechanisms by which T_{RM} $CD8^+$ T cells provide protective immunity, initiate inflammatory responses, and cause immunopathology.

The majority of work in the T_{RM} field (and this thesis) has focused on the development and function of $CD8^+$ T_{RM} cells, largely due to technical considerations that make the identification of antigen-specific $CD4^+$ T cells challenging. However, many autoimmune diseases are thought to be mediated at least in part by $CD4^+$ T_{RM} cells, and the majority of T_{RM} cells in human skin are $CD4^+$[88]. These $CD4^+$ T_{RM} cells exhibit several features that distinguish them from $CD8^+$ T_{RM}; namely dermal localization and increased rates of recirculation[79]. However, a recent study of human $CD4^+$ and $CD8^+$ T_{RM} cells found a shared transcriptional program that also closely resembles the T_{RM} transcriptional profile of mouse $CD8^+$ T_{RM} cells[87]. Thus, despite significant phenotypic and functional differences, the transcriptional mechanisms of tissue retention appear to be at least partly conserved between $CD4^+$ and $CD8^+$ T_{RM} cells. Epicutaneous VACV infection clearly generates $CD69^+$ $CD4^+$ T cells in the skin, but whether TCR stimulation of effector $CD4^+$ T cells

enhances or inhibits T_{RM} differentiation is largely unknown. MHC-II restriction of $CD4^+$ T cells suggests that fewer pMHC-expressing targets are available in non-lymphoid tissues, although the extent to which non-hematopoietic expression of MHC-II changes during the course of viral skin infection has not been thoroughly investigated. In a separate part of my graduate work, we generated novel VACV strains that target recombinantly expressed peptides to the MHC-II presentation pathway and enable robust $CD4^+$ T cell responses against MHC-II restricted model peptides[215]. These viruses could be used in similar coinfection experiments with IFNγ-YFP reporter mice to begin to determine whether TCR-stimulation contributes to $CD4^+$ T_{RM} differentiation. Additionally, these viruses could be used to generate a $CD4^+$ T_{RM} population of a known antigen specificity that could then be activated by topical application of antigenic peptide. These experiments would begin to illuminate differences and similarities in the differentiation and function of $CD4^+$ and $CD8^+$ T_{RM} cells, which could ultimately provide insights into diverse autoimmune diseases.

In addition to understanding the basic biology of T_{RM} $CD8^+$ T cell development and function, the ultimate long-term goal of this work is to apply this knowledge to design vaccines that generate T_{RM} populations at specific anatomical locations. Traditionally, the goal of T cell-based vaccines has been to generate a large population of circulating antigen-specific memory T cells and efforts to maximize the number of circulating memory T cells have focused on using booster immunizations[202, 203, 216]. In theory, expanding the number of memory T cells in the circulation would lead to increased surveillance of peripheral tissues and responsiveness to secondary challenge. However, in many vaccination trials the numbers of circulating memory T cells do not correlate with protection, even after successful heterologous boosting[204, 205, 217]. This lack of protection by circulating memory T cells has generated a strong interest in developing vaccines that seed T_{RM} cells at sites of

pathogen entry. Based on the work in this thesis, vaccine designs that incorporate antigen recognition within the desired tissue might be more effective than systemic vaccination strategies that are currently used. Additionally, topical application of antigenic peptides may offer a simple and affordable method to boost protective T_{RM} populations in a desired anatomical location.

Conclusion

Altogether, this thesis demonstrates that antigen recognition within the skin promotes the local formation of T_{RM} $CD8^+$ T cells, and this increase in T_{RM} formation provides improved local protective immunity. Antigen-dependent T_{RM} differentiation held true for both primary effector $CD8^+$ T cells responding to VACV infection and for circulating memory $CD8^+$ T cells recruited to the skin following T_{RM} activation. This mechanism of antigen-dependent T_{RM} differentiation positions T_{RM} cells at sites of previous infection, and therefore increases the chances of detecting future re-infections by the same pathogen. Future studies that extend these observations to $CD4^+$ T_{RM} cells, diverse infectious agents, and other non-lymphoid tissues will offer further insight into T_{RM} biology, and may ultimately be leveraged to improve vaccine efficacy or to develop new treatments for autoimmune conditions.

Chapter 5

Methods

Mice

C57BL/6N mice (6-10 weeks of age) were purchased from either Charles River/NCI or from the Jackson Laboratory. IFNγ-YFP[218], Blimp1-YFP[219], and CD45.1 mice were purchased from the Jackson Laboratory. P14[220] and OT-I[221] mice were described previously and were maintained by sibling to sibling mating. For adoptive transfers, 2.5×10^4 Thy1.1$^+$ P14 CD8$^+$ T cells or 10^4 Thy1.1$^+$ OT-I CD8$^+$ T cells were injected i.v. in 200µl of PBS. Cre-ERT2 mice were purchased from the Jackson Laboratory and were described previously[222]. Prdm1^{Flox} mice have been described previously[223] were obtained from Dr. Noah Butler (University of Iowa). All animal experiments were approved by the OHSU Institutional Animal Care and Use Committee.

Infections

LCMV-Armstrong infections (2×10^5 pfu) were performed by i.p. injection in a volume of 200 µl. All VACV strains were maintained by propogation in BSC-40 cells as previously described[224]. VACV skin infections were performed on anesthetized mice by placing 10^7 pfu of virus (in 10 µl of PBS) on the ventral side of the ear pinna, and then poking the virus-coated skin 25 times with a 27-gauge needle. Intravenous VACV infections were performed by injection of 2×10^7 pfu *tk*- VACV in a volume of 200 µl. VACV-SIINFEKL variants were generated by homologous

recombination as described previously [224] by Dr. James Gibbs in the laboratory of Dr. Jon Yewdell.

Quantification of viral titers

VACV titers from infected skin was determined using standard plaque assays. Infected ears were removed and homogenized in 1 ml of RPMI containing 1% FBS. Homogenates were subjected to three rounds of freeze-thaw and 0.1 ml of serial 10-fold dilutions were used to infect BSC-40 cells in a 12 well plate for 1 hour. Infected BSC-40 cells were then covered with 1% Seakem agarose and plaques were visualized three days later after overnight incubation with neutral red dye. All infectious agents were approved by the OHSU Institutional Biosafety Committee.

Topical peptide challenge

$B8R_{20-27}$, GP_{33-41}, $NP_{396-404}$, and $OVA_{257-264}$ peptides were dissolved in 20-40 µl of 4:1 acetone/DMSO or olive oil/H2O. The DMSO/acetone formulation was chosen to enhance the penetration of the peptides and to aid in covering the entire surface area of the ear skin. The peptide solution was applied to anesthetized mice on the dorsal and ventral side of previously infected ears using a pipette tip. Ear pinna thickness was measured with a dial micrometer (Ames).

Leukocyte isolation from skin

Skin tissue was incubated for 1.5 h at 37°C with 1 ml HBSS (Gibco) containing $CaCl_2$ and $MgCl_2$ supplemented with 125 U/ml collagenase II (Invitrogen) and 60 U/ml DNase-I (Sigma-Aldrich). Leukocytes were purified from whole-tissue suspensions by resuspending the cells in 10 ml of 35% Percoll (GE Healthcare)/HBSS followed by centrifugation at 500g for 10 minutes at room temperature with no brake. The number of T cells within infected skin was quantified by flow cytometry.

Dermis/Epidermis separation

Dermal and epidermal sections of skin were prepared by incubating ear skin in dispase (2.5 mg/ml) for 90 minutes at 37°C in PBS, followed by manual separation of the epidermal sheet from the dermis. Epidermal sheets were then digested in 0.25% Trypsin + 0.1% EDTA and dermal sections were digested in 125U/ml collagenase II and 60 U/ml DNase-I. Digested dermis and epidermis were then forced through a mesh screen to generate a single cell suspension that was then stained for flow cytometry.

In vivo antibodies

Depleting antibodies (100-200 µg) targeting CD4 (GK1.5) or CD8α (2.43) were delivered i.p. and CD8β (YTS156.77) and CD4 (RM4-5) fluorescent antibodies were used to confirm depletion three to five days after antibody administration. P- and E-selectin (RB40/9A9, respectively) blocking antibodies (200 µg) were administered 18 hours before and at the time of peptide challenge.

Cell staining and flow cytometry

Spleens or lymph nodes of infected mice were harvested and single cell suspensions were generated by gently forcing the organ through a mesh screen. Red blood cells were lysed in spleen samples by resuspending cell pellets in 150 mM NH4Cl, 10 mM KHCO3, and 0.1 mM Na-EDTA and staining for surface antigens was performed in PBS/1% fetal bovine serum for 15 minutes at 4° C. Tetramer staining was performed in PBS/1% fetal bovine serum for 45 - 60 minutes at room temperature, followed by surface staining as described below. Staining for surface antigens was performed in PBS containing 1% fetal bovine serum for 15 minutes at 4° C. P/E-selectin binding was determined by incubating cells with P- or E-selectin human IgG Fc chimeric proteins (RD Systems) for 30 minutes in 1% FBS/Dulbecco's PBS containing Ca^{2+} and Mg^{2+} (Gibco) at room temperature. Se-

lectin binding was detected using anti-human IgG Fc phycoerythrin (eBioscience). Cells were then stained with fluorescent antibodies as described above. Blimp1 staining was performed using True-Nuclear fixation and permeabilization buffers as described by the manufacturer (Biolegend). In experiments involving fluorescent reporters (IFNγ-YFP and Prdm1-YFP), cells were not fixed before flow cytometric analysis. Data was acquired using either a BD LSRII Flow Cytometer or a BD Fortessa Flow Cytometer in the OHSU Flow Cytometry Core Facility. Flow cytometry data was analyzed using FlowJo software, version 9.9 or 10.

Cell sorting and microarray analysis

Ear skin and spleens of mice containing IFNγ-YFP P14 CD8$^+$ T cells were harvested and CD45$^+$, CD8$^+$, Thy1.1$^+$ cells were sorted directly into 500 µl of trizol using a BD InFlux sorter. Between 58,000-88,000 IFNγ^+ cells, 100,000 IFNγ^- cells, and 100,000 splenic P14 cells were collected for each replicate. RNA was isolated using a chloroform-ethanol extraction followed by purification using RNEasy columns (Qiagen) according to manufacturer's instructions. Labeled cDNA was synthesized using the GeneChip Pico assay (Applied Biosystems). Amplified and labeled cDNA target samples were each hybridized to an Affymetrix GeneChip Clariom S Mouse microarray and image processing was performed using Affymetrix Command Console (AGCC) v3.1.1. and expression analysis was performed using Affymetrix Expression Console software build 1.4.1.46. Differential gene expression was analyzed using Affymetrix Transcriptome Analysis Console v.4.0.2. Differential expression between conditions was determined by one-way ANOVA using eBayes ccorrection and significance cutoffs were set at $p < 0.01$ and 2-fold or greater fold change. Principal component analysis was performed on the set of differentially expressed genes between all 3 conditions using Clutergrammer[225]. Heatmap was generated by performing hierarchical clustering of all differentially ex-

pressed genes using a one minus pearson correlation within the Morpheus webtool (Broad Institute, https://software.broadinstitute.org/morpheus). Gene set enrichment analysis was performed with expression data from Pan et al[115] (GSE79805) using the java desktop application (Broad Institute[226]). KEGG pathway enrichment was performed using gProfiler[227, 228].

Ex vivo peptide stimulation and intracellular cytokine stain

Spleens or skin of mice were harvested and a single cell suspension was generated as described in 'Cell staining and flow cytometry' and 'Leukocyte isolation from skin'. Bulk splenocytes or skin leukocytes were then cultured in a 96 well plate in the presence of Brefeldin A (Biolegend) and the indicated peptides at the indicated concentrations for 5 hours unless otherwise indicated. Surface staining was then performed, followed by fixation and permeabilization using CytoFast Fix/Perm buffers (Biolegend) according to the manufacturer's instructions. Intracellular cytokine staining was performed at 4° C for 20 minutes.

Effector cell purification and in vitro stimulations

Spleens of VACV-GP33 infected mice containing P14 $CD8^+$ T cells were harvested on day 7 post infection and a single cell suspension was generated as described in 'Cell staining and flow cytometry'. Single cell suspensions were washed with RPMI containing 5 % FBS and then stained with anti-Thy1.1 in PBS containing 1 percent FBS on ice for 15 minutes. Cells were washed with PBS containing 1% FBS and incubated with anti-PE or anti-APC magnetic beads (Miltenyi) on ice for 10 minutes. Cells were washed twice and then purified using the Miltenyi AutoMACS Pro cell separator. Purified effector cells were then stimulated with plate-bound anti-CD3ε (10 µg/ml) for 18 hours.

Inravascular labeling

To label intravascular cells, 3 mg of fluorescently labeled anti-CD8β was injected i.v. in 200 µl of PBS, and tissues were harvested 3 minutes later. Single cell suspensions were kept on ice and processed as described in 'Cell staining and flow cytometry'.

CFSE labeling

Naive OT-I $CD8^+$ T cells were isolated from the spleen and washed twice with PBS before labeling with 1 µM CFSE at 37 °C for 15 minutes. Cells were then washed twice in RPMI + 10% FBS and transferred into naive recipients. The following day, mice were infected on the left ear skin with the indicated VACV strain and cells were harvested from the draining lymph node and stained as described in 'Cell staining and flow cytometry'. Proliferation was analyzed by CFSE dilution using FlowJo software version 9.9.6. The percentage divided and expansion index were calculated using the FlowJo proliferation platform[229].

Quantitative PCR

Purified effector P14 $CD8^+$ T cells were stimulated as described and RNA was isolated using an RNeasy mini kit and cDNA was synthesized using the SuperScript III First Strand Kit (Invitrogen) according to the manufacturer's instructions. qPCR reactions were performed using Power SYBR green PCR Master Mix (ThermoFisher) and analyzed on a Step One Plus Real Time PCR system (Applied Biosystems). Changes in gene expression were quantified using the $\Delta\Delta$Ct method, using TATA binding protein for normalization. The following primers were used: IfngF: AGCAACAGCAAGGCGAAAA IfngR: GAATGCTTGGCGCTGGA S1pr1F: GTGTAGACCCAGAGTCCTGCG S1pr1R: AGCTTTTCCTTGGCTGGAGAG Tcf7F: CAATCTGCTCATGCCCTACC Tcf7R: CTTGCTTCTGGCTGATGTCC Klf2F: CTCAGCGAGCCTATCTTGCC Klf2R: CACGTTGTTTAGGTCCTCATCC TbpF: TGGAATTGTACCGCAGCTTCA TbpR: ACTGCAGCAAATCGCTTGGG

S1P migration assay

Spleens of VACV-GP33 infected mice containing P14 $CD8^+$ T cells were harvested on day 7 post infection and single cell suspensions were generated as described in 'Effector cell purification and in vitro stimulations'. Cells were washed twice in RPMI 1640 containing L-glutamine, Penicillin/Streptomycin, 10 mM HEPES buffer, and 0.5% fatty-acid free BSA. Cells (1-2 x 10^6 cells/ml) were then cultured in media alone or in media containing 100 nM GP_{33-41} for 18 hours before transfer to Transwell inserts with a pore size of 5 µM and a diameter of 6.5mm in 24 well plates (Corning Costar). A gradient was established by plating 100 µl of cells in the upper well and 580 µl of media containing the indicated concentration of S1P (RD Systems) in the lower well. Plates were incubated at 37°C in 5% CO2 for 3 hours and the number of cells in each well were counted by flow cytometry.

2-NBDG uptake

Leukocytes were isolated from the skin on day 7 post VACV-GP33 infection and cultured in RPMI + 10% FBS in the presence of 100 µM 2-NBDG for 30 minutes. Cells were then stained and 2-NBDG uptake was quantified by flow cytometry.

Tamoxifen treatment

Tamoxifen (Sigma) was dissolved in corn oil (Sigma) at a concentration of 10 mg/ml by incubation at 37°C on a rocking plate for 3-5 hours. Mice received 100 µl of 0.45 µM filter-sterilized tamoxifen solution i.p. daily for 5 days. Tamoxifen solutions were freshly made for each experiment.

Figure generation

Figures 1.1, 1.2, 1.3, and 2.13 were created with Biorender. The venn diagram was generated using jvenn[230].

Statistical Analysis

Statistical tests and experimental details for each experiment are stated in the figure legend. Statistical tests were performed using Prism software (version 6.0; GraphPad Software). * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

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