The Role of Gammadelta T Cells and CD8+ Memory T Cells
in Vaccinia Viral Infection

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Dissertation submitted in partial
fulfillment of the requirements for the degree
of Doctor of Philosophy in the Department of
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ABSTRACT

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Abstract

Immune responses against viral infections are mediated through a complex process by diverse populations of cells, that can be harnessed for tumor immunotherapies and vaccinations. Vaccinia virus (VV) is the most studied member of the poxvirus family and is responsible for the successful elimination of smallpox worldwide. It is unique among well-studied viruses in that it replicates solely in the host cytoplasm and can elicit one of the longest lasting immunity in recorded human history. Its success in vaccination has led to the development of adjuvants with VV epitopes and recombinant VV vectors for other infectious diseases and cancer immunotherapy. However, the mechanism behind how VV elicits such a strong immune response from the immune system remains insufficiently understood.

Previous studies have shown that although activation of NK cells is critical for the initial control of VV infection, efficient activation of CD8$^+$ T cell response is required for the eradication of VV infection. It has also been demonstrated that $\gamma\delta$T cells play an important part in the immune response against VV infection. However, both processes remain relatively undefined. What promotes CD8$^+$ T cell activation and subsequent generation of CD8$^+$ memory T cells in response to VV infection is still not very well dissected, and the mechanisms that govern $\gamma\delta$T cells response to

VV are relatively unknown. This thesis examines these questions through three main aims: 1) influence of γδT cells on CD8$^+$ T cell activation, 2) γδT cell direct cytotoxicity against VV infection, and 3) mechanisms that govern CD8$^+$ memory T cell formation. The overall goal of this thesis is to understand the mechanisms behind γδT and CD8$^+$ T cells responses against VV infection.

We found that γδT cells play an important role in promoting CD8$^+$ T cell response to VV infection. We showed that γδT cells serve not only as antigen presenting cells to CD8$^+$ T cell activation, but also as mediators of other signals of CD8$^+$ T cell response *in vivo*. We further demonstrated that cell intrinsic MyD88 signaling in γδT cells is required for activation of CD8$^+$ T cells. Contrary to conventional expectations, we found that NKG2D expression in both NK and CD8$^+$ T cells only have partial effect on the elimination of VV post-infection. Instead, we found that NKG2D is an important activator of γδT cell cytotoxicity for VV clearance. Lastly, we demonstrated that Notch1, but not Notch2, deficiency increases the formation of CD8$^+$ memory T cells, through modulating the expression of TCF1/*Tcf7*. We discovered that cleaved Notch1 intracellular domain binds upstream of *Tcf7* and controls the expression of *Tcf7* for CD8$^+$ memory T cell formation.

These results demonstrated a critical role for γδT cells in viral clearance and the regulation of adaptive T cell response, with insights into the formation of CD8$^+$

memory T cells. Collectively, this dissertation seeks to better understand how $\gamma\delta$T and CD8^{+} T cells respond to VV infection, with the hopes of shedding additional light on the design of more effective vaccine strategies based on the precise manipulation of immune cell populations for infectious diseases and cancer immunotherapy.

Dedication

I would like to dedicate this dissertation to my parents—thank you for your

unwavering love and support.

谁言寸草心，报得三春晖

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List of Abbreviations

Ab   Antibody

Ag   Antigen

AdB8R   Recombinant Adenovirus encoding B8R

ADCC   Antibody-Dependent Cellular Cytotoxicity

APC   Antigen Presenting Cell

B8R   Vaccinia virus epitope

CD   Cluster of Differentiation

cDNA   Complementary DNA

CFSE   Carboxyfluoroscein Succinimidyl Ester

ChIP   Chromatin immunoprecipitation

CML   Chronic Myelogenous Leukemia

CMV   Cytomegalovirus

DC   Dendritic Cell

DT   Diphtheria Toxin

DTR   Diphtheria Toxin Receptor

EAU   Experimental Autoimmune Uveitis

FACS   Fluorescence-Activated Cell Sorting

FITC	Fluorescein Isothiocyanate
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
GSI	Gamma-Secretase Inhibitor
HA	Hemagglutinin
HBSS	Hank's Buffered Salt Solution
ICS	Intracellular Cytokine Staining
IFN-α	Interferon alpha
IFN-β	Interferon beta
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
i.p.	Intraperitoneal
i.v.	Intravenous
Kbp	Kilobase pair
LN	Lymph Node
LPS	Lipopolysaccharide
MACS	Magnetic-Activated Cell Sorting
MHC-I	Major Histocompatibility Complex Class I

MHC-II	Major Histocompatibility Complex Class II
MOI	Multiplicity of infection
MVA	Modified Vaccinia Virus Ankara
MyD88	Myeloid Differentiation Primary Response gene 88
NICD	Notch Intracellular Domain
NK	Natural Killer Cell
NKG2D	NK Group 2 member D
PAMP	Pathogen-Associated Molecular Pattern
PD-L1	Programmed Death Ligand 1
PE	Phycoerythrin
PE-Cy5	Phycoerythrin Cyanine 5
PerCP	Peridinyl Chlorophyllin
PFU	Plaque Forming Units
PMA	Phorbol 12-myristate 13-acetate
PPD	Purified Protein Derivative
PRR	Pattern Recognition Receptor
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcription Polymerase Chain Reaction

SD	Standard Deviation
T$_{CM}$	Central Memory Cells
TCR	T Cell Receptor
T$_{EM}$	Effector Memory Cells
TGF-β	Transforming Growth Factor - beta
T$_{RM}$	Resident Memory Cells
TRAIL	TNF-related apoptosis-inducing ligand
TRIF	TIR-domain-containing adapter-inducing IFNβ
TLR	Toll-Like Receptor
TNF-α	Tumor Necrosis Factor alpha
IFN-I	Type I Interferon
VV	Vaccinia Virus
VV-Ova	Recombinant Vaccinia Virus Expressing Ovalbumin
WR	Western Reserve
WT	Wild Type

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Chapter 1. Introduction

For tumor immunosurveillance and viral infections, CD8^{+} T cells are the ultimate effector cells responsible for controlling subsequent pathology. Published studies have noted that though NK cells are necessary for initial immune response against viral infections, CD8^{+} T cells are required to eliminate the virus [1-5]. Following resolution of the infection, generation of CD8^{+} memory T cells also allows the immune system to prepare rapid and robust responses against future repeat infections. By directly killing virus-infected cells, CD8^{+} T cells eliminate the contagion, and CD8^{+} memory T cells build a strong immunity against similar pathologies, years after clearance of the initial insult [2].

Gammadelta T cells (γδT cells) cells are a small subpopulation of T cells but have a disproportionally large effect on the immune system. They are key components of numerous disease processes, including cancer immunosurveillance, infection control, and autoinflammation [6]. One of the main ways that γδT cells exert this role is through their interaction with other cells in the immune system. Published findings have found that γδT cells not only exert a strong influence on CD8^{+} T cells, but also on other immune cells involved in the activation of CD8^{+} T cells, such as dendritic cells [7-10]. By modulating the effect of other immune cells, γδT cells can control how other cells in the immune system react to various stimuli.

Vaccinia virus is a particularly unique virus in among well-studied viruses in virology. It is responsible for the complete elimination of smallpox worldwide, and despite being one of the first vaccines developed in human history, it remains as one of the few vaccines that can exert a life-long immunity without the need for boosters [11, 12]. This chapter will investigate how γδT cells influence cells in the immune system for innate and adaptive cytotoxicity, especially focusing on the activation and generation of CD8+ memory T cells following VV infections. Furthermore, we will explore strategies for immunization against viral pathogens and potential insights into immunotherapy for cancer.

1.1 γδT cells

γδT cells constitute less than 1% of all circulating cells in healthy adult humans, but their effects extend beyond what the population size would suggest in the immune system [13-15]. In a meta-analysis of expression signatures and overall survival outcomes across 39 human malignancies, intra-tumoral γδT cells are the most significant favorable prognostic population of immune cells [16]. Other studies have also demonstrated γδT cells' importance in cancer immunosurveillance [17-19], autoinflammation [6, 20], infection control [21, 22], and others. In addition to exerting direct cytotoxicity, γδT cells can influence other cells in the immune system

through direct antigen presentation, expression of immune-specific ligands, and secretion of both autoinflammatory and anti-inflammatory cytokines.

γδT cells can have such a varied influence in the immune system because there are several functionally distinct subsets. However, unlike conventional αβT cells, where the different subsets can be identified based on their coreceptors and cytokine production, a definitive system to categorize γδT cells remains elusive [23-26]. Initial proposals to define γδT cells based on their different TCR expression in both mice and humans have had to be revisited [27, 28]. Recently, a more functional definition has been proposed, by segregating γδT cells into either antitumor or protumor populations [24, 29-35]. Because the definition of different subsets of γδT cells remains fluid [27, 36], many studies have focused on the effect of γδT cells as a whole. For this dissertation, we will also analyze γδT cells as a population, with a brief discussion into the two functional populations of antitumor and protumor γδT cells.

1.1.1 γδT cells and activation of CD4⁺ and CD8⁺ T cells

γδT cells bridge the innate and adaptive immune systems, by not only directly inducing cytotoxicity, but also acting as antigen presenting cells (APCs) to activate the adaptive immune system. The role of γδT cells as APCs to αβT cells was

first demonstrated in cattle [37], and subsequently illustrated in humans and mice [38, 39].

Professional APCs, such as dendritic cells, activate CD8^{+} T cells via three main pathways: 1) presentation of antigen peptide on major histocompatibility complex class I (MHC-I), 2) co-stimulation with CD80 and/or CD86 ligands, and 3) cytokine production for paracrine activation [40]. γδT cells exhibit similar findings upon stimulation.

Like professional APCs, stimulated γδT cells also express antigen presenting cell molecules, such as CD80, CD86, CD40, CD54, and HLA-DR at similar rates compared to LPS-matured dendritic cells [39, 41]. *In vitro* co-culture of γδT and αβT cells under specific stimuli results in strong induction of αβT cells proliferation and differentiation [38]. Following IPP-activation, γδT cells' endosomes and lysosomes cluster intracellularly, with corresponding increase in expression of HLA-DR on the cell surface, like immature and mature dendritic cells. Subsequent incubation of purified protein derivative (PPD) treated γδT cells with CD4^{+} αβT cells resulted in antigen-specific activation and proliferation of CD4^{+} T cells with similar magnitudes as dendritic cells. The same findings were also seen with PPD-activated γδT cell incubation with CD8^{+} αβT cells [42].

γδT cells are the only lymphoid-lineage cells that are capable of phagocytosis. Direct visualization of γδT cells has demonstrated that γδT cells can opsonize and process tumor cell fragments for presentation of tumor-associated antigens [43]. Human γδT cells are capable of phagocytosing foreign antigens, including E. coli and synthetic beads. Confocal and transmission electron microscopy have found that γδT cells possess pseudopod-like dendritic processes and express CD16 (FcγRIII), that when activated, opsonize and process antigens for antigen-specific presentation. In contrast, other lymphoid cells that express CD16, such as NK cells, are not capable of these functions [44].

The specific mechanisms that govern how antigen-presentation functions in γδT cells remain an active area of investigation. Several studies have found that γδT cells can be activated independently of T cell receptor engagement. Cytomegalovirus (CMV) infections induce intrinsic antibody dependent cell-mediated cytotoxic (ADCC) via CD16 [45]. Tumor cells are opsonized by γδT cells after recognition by tumor-specific antibodies [43]. Experimental autoimmune uveitis (EAU) model has also demonstrated that γδT cells can be induced to exhibit APC function via cytokines, given sufficient priming [39]. Listeria monocytogenes is also able to induce human γδT cells phagocytosis and antigen presentation following activation

by anti-CD3e antibodies [46]. Similar findings have been replicated in mycobacteria [47] and plasmodium falciparum [48].

After activation, γδT cells also appear to affect αβ memory T cell formation in nonimmunized mice. Interestingly only specific depletion of subpopulations of γδT cells, but not pan-δTCR-knockout mice, exhibit changes in αβ memory T cell populations. Mice with specific knockout of Vγ4/Vγ6 TCR demonstrate significant increase in CD8^{+} memory and CD4^{+} αβT cells [49]. The effect is abrogated in mice with complete knockout of γδT cells.

γδT cells are intricately involved in activation of αβT cells through numerous pathways, and more studies are needed to fully dissect the mechanisms behind the relationship.

1.1.2 γδT cells and dendritic cells

In addition to intrinsic antigen presenting capabilities, γδT cells also influence other antigen presenting cells (APC), such as dendritic cells (DC). γδT cells can induce maturation of DC through secretion of GM-CSF [50, 51]. In turn, DC can also influence the activation and proliferation of γδT cells through CD86 ligand interaction and cell-to-cell contact [52]. However, the effect of that interaction appears to be dependent on the stimulus, and the mechanism behind the interaction is not well understood.

In several infection and *in vitro* models, dendritic cells appear to play a crucial

role in the activation and expansion of γδT cells. In Brucella infection, dendritic cells are

required for induction of IFN-γ in γδT cells [53]. In *in vitro* cultures, DCs are required for

the activation of pamidronate-stimulated γδT cells [52, 54-56]. The effect appears to be

mediated mainly through cytokines, such as IL-12 production from DCs.

Conversely, γδT cells also influence dendritic cell maturation [50]. However, the

relationship seems to be dependent on the stimulus. In mycobacterial infection, γδT cells

inhibit dendritic cells [47]. Under EAU models and *in vitro* culture in contrast, phospho-

antigen-activated γδT cells induce dendritic cell maturation [10, 50]. Similarly in Brucella

infection, γδT cells are required for dendritic cells for full maturation and expression of

CD86 and IL-12 [53]. γδT cells induce dendritic cell maturation when co-cultured *in vitro*

via contact-dependent and cytokines induction in response to phospho-antigen [57].

Studies have found that dendritic cells can also activate or inhibit γδT cells,

depending on the stimulus [8]. After initial potentiation with phospho-antigens,

dendritic cells activate γδT cells [58]. However, in HIV infection, HIV-exposed DCs

inhibit human γδT cell proliferation and IFN-γ production in response to antigens [59].

The impairment of γδT cell proliferation is directly correlated with HIV-induced

suppression of IL-12 secretion in DC/γδT cell cocultures.

The interplay between γδT cells with DC and other immune cell populations can

actively contribute to the immune response by bridging the innate and adaptive

immunity. The conflicting findings in the interaction between γδT cells and dendritic cells may be due to a difference in the effects of different stimuli against different populations of γδT cells. However, there is still a significant area open for investigation in the interaction between DC and γδT cell crosstalk.

1.1.3 γδT cells and NK/NKT cells

Like γδT cell antigen presentation to αβT cells, γδT cells can also present antigens to CD1d-restricted invariant natural killer T cells (iNKT). In this process, γδT cells phagocytose CD1d-containing membrane fragments from phosphoantigen-expressing cells and then activate iNKT [60]. In antitumor models, γδT cells have been demonstrated to induce robust NK cell-mediated antitumor cytotoxicity through CD137 engagement [61]. $\delta TCR^{-/-}$ mice exhibited decreased NK cell antitumor responses [62]. In Listeriosis models, γδT cells were responsible for early IFN-γ secretion by NK cells [63]. Further findings have determined that γδT cells appear to influence NK cell function through an interplay with DC-like cells [64]. However, the interaction between γδT cells and NK cells has not been verified in all models and is still in nascent stages of research.

NK cells' influence on γδT cells is similarly not well established. Following stimulation by Mycobacterium tuberculosis, NK cells induce γδT cell proliferation *in vitro* [65]. However, *in vivo* models suggest that there may be a competitive nature to the interaction between NK and γδT cells [66].

1.2 Functional definitions of γδT cells

Successful immunotherapy requires a delicate balance in maintaining a tightly controlled homeostasis between antitumor cytotoxicity and protumor immune tolerance. Adoptively transferred αβT cell populations provide intense cytotoxicity against specific targets but are limited by their specificity. Dendritic cells lack cytotoxic functions by themselves. And NK cell immunotherapies have had inconsistent results in clinical trials. γδT cells provide a rare combination of cytotoxicity and induction of adaptive immune response. They can potentially allow short-term antitumor effects, while providing long-term protection with antigen-presentation.

γδT cells have two main hallmarks that distinguish them from other immune cells in their potential for cancer immunotherapy. The first is γδT cells' innate ability to infiltrate tumors. Beyond all other immune cells, infiltration of γδT cells into tumors is one of the most beneficial prognostic markers for survival outcomes [16]. Both *in vitro* and *in vivo* experiments show that γδT cells can infiltrate into developing malignancies. After infiltration, γδT cells exhibit cytotoxicity and modulate the tumor microenvironment to either promote protumor or antitumor functions of other immune cells. By understanding how γδT cells diverge between antitumor and protumor phenotypes, there is tremendous potential to control not

only direct cytotoxicity, but also how other immune cells infiltrate and respond to specific tumors.

Secondly, γδT cells' potential to recognize antigens beyond those presented by major histocompatibility complexes provides a powerful way to circumvent the limitations associated with tumor associated antigens. This could allow γδT cells to target antigens that were inaccessible to αβT cell immunotherapies, and it could also broaden the potential to synergize with extrinsic αβT cells through antigen presentation. Future immunotherapies do not have to be limited to one type of cells. γδT cells provide a potential mechanism to fine-tune the immunotherapies to decrease adverse effects, while maintaining potent cytotoxic and tumor-killing effects. However, there is still significant amount of knowledge to be gleaned in understanding how γδT cells mechanistically respond to different stimuli.

Human trials with γδT cells in cancer immunotherapy interestingly have been relatively lackluster. A review of clinical trials conducted in the past decade demonstrates that γδT cell-based immunotherapies are well tolerated, but the benefits are moderate at best [67]. Ongoing studies have also demonstrated unexpected protumor functions of γδT cells in mouse models [29] and human patients [30, 31]. This difference between expectation and reality of γδT cells in cancer immunotherapy indicates that significant more work is needed to dissect the

various functions of γδT cells and to outline specific strategies in how to best harness

the effects of γδT cells in cancer immunotherapy.

1.2.1 Cytotoxic and antitumor γδT cells

Despite the numerous unknowns that still need to be addressed, γδT cells

have tremendous potential in cancer immunotherapy. They are one of the first

responders and bridge the gap between innate and adaptive immune responses, by

producing cytokines [18], exerting cytotoxicity [45] activating CD8$^+$ αβ T cells [42],

and inducing dendritic cell maturation [50].

γδT cell's role in cancer immunosurveillance was demonstrated in 2001 when

δTCR$^{-/-}$ mice exhibited increased rate of methylcholanthrene- or

dimethylbenzanthracene-induced cutaneous tumors and transplanted squamous cell

carcinoma compared to that of wild-type mice [17]. Similar findings corroborated

γδT cells' function in immunosurveillance in other tumor models [68, 69]. Further

investigation revealed that skin exposure to carcinogens in mice lead to increased

expression of stress ligands RAE-1 and H60 by keratinocytes that bind to NKG2D

receptors [70, 71]. Engagement of NKG2D activates cytolytic responses in human γδT

cells, through secretion of pro-apoptotic granzyme B and perforin [72]. *In vitro*

investigations revealed that γδT cells can express TNF-related apoptosis-inducing

ligand (TRAIL) for *in vitro* killing of Chronic Myelogenous Leukemia (CML) cells

[73], as well as expression of Fas ligand to bind to cancer cell-lines that express Fas

receptors. Similarly, human γδT cells induce antibody-dependent cellular

cytotoxicity (ADCC) via recognition of Fc on antibodies bound to target cells [45].

For indirect antitumor functions, human γδT cells can act as professional

antigen presenting cells to αβT cells *in vitro* [42]. Vγ9δ2^{+} T cells can express MHC

class II, costimulatory molecules, and lymph node-homing chemokine receptors,

such as CCL7 [38]. Direct visualization of γδT cells have also demonstrated that γδT

cells can opsonize and process tumor cell fragments for presentation of tumor

associated antigens [43]. The indirect impact of γδT cells on antitumor immunity also

relates to its stimulation of NK cell cytotoxicity via CD137 (4-1BB) [61], induction of

immunoglobulin class switching of peripheral B cell populations [74], secretion of

GM-CSF for maturation of dendritic cells [50, 51], and secretion of IFN-γ for

promotion of MHC-I regulation on cancer cells.

γδT cells are pre-committed in the thymus to either secrete IL-17 or IFN-γ

depending on whether there is strong or weak ERK-dependent TCR signaling [75].

Increased TCR signal strength promotes the development of IFN-γ secreting γδT

cells, while weak antibody-induced TCR signal promotes IL-17^{+} phenotype.

However, in healthy humans, there are few IL-17^{+} γδT cells in the bloodstream [76],

which suggests that different populations of γδT cells are further expanded following activation in response to different stimuli.

A combination of γδT cells' interaction with the tumor microenvironment, surrounding immune cells, and response to cytokines have been demonstrated to play a complex role in pushing γδT cells towards IFN-γ$^+$ antitumor responses. In humans, stimulation with IL-2 and IL-15 of immature γδ thymocytes directs γδT cells towards differentiation into IFN-γ$^+$ cells [77]. IL-2, IL-12, IL-15, IL-18, and IL-21 [78] have all been demonstrated to potentiate γδT cells towards antitumor functions, specifically towards cytotoxicity and IFN-γ production.

There are several immune cell types that directly interact with γδT cells. Dendritic cells secrete IL-15 to induce antitumor effects of γδT cells [79]. Regulatory T cells, in contrast, secret TGF-β and IL-10 to inhibit antitumor functions of γδT cells [80]. Circulating neutrophils secrete arginase-1 and reactive oxygen species (ROS) to inhibit γδT cells [81]. Myeloid cells under hypoxic tumor microenvironments induce γδT cell exhaustion through PD-L1 expression, to downregulate IFN-γ production, cytotoxicity, and ADCC [82].

1.2.2 Protumor γδT cells

Despite the numerous mechanisms that γδT cells can use to target tumor cells, human clinical trials of intrinsically activated or adoptively transferred γδT cells, especially against solid tumors, have been lackluster. Out of 9 human clinical trials with *in vivo* autologous stimulation and 12 with *ex vivo* autologous expansion, less than 10 percent of patients fully responded to the treatment [83, 84]. Interestingly, *ex vivo* expansion of γδT cell populations from donor T cells during bone marrow transplantations for blood malignancies have the highest response rates [85-87]. Response to intrinsic activation of γδT cell therapy in follicular lymphoma is increased with concurrent rituximab-depletion of CD20$^+$ B cells, than similar activation of γδT cells alone [88]. This suggests that γδT cells could be more effectively used as an adjuvant therapy to existing chemotherapies that otherwise would adversely inhibit immune cell proliferation. γδT cells may act in a balancing role that inhibits the immune system for protumor effects under inflammation and promotes cytotoxicity when other immune cells are otherwise impaired.

Infiltrating γδT cells isolated from breast cancer biopsies inhibit the function of naïve and effector T cell responses and block the normal function of dendritic cells. This suggests that γδT cells can promote tumor growth by inhibiting intrinsic immune responses against cancers [9]. Protumor function of γδT cells appears to

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derive primarily from its production of IL-17. IL-17 knockout mice have distinctly

smaller tumors in mouse models of breast cancer, fibrosarcoma [89], hepatocellular

carcinoma [31], lung cancer [90], melanoma [91], and ovarian cancer [29]. IL-17^{+} γδT

cells can promote immunosuppressive phenotypes of neutrophils by producing G-

CSF [92]. Neutralization of IL-17 in mouse model of breast cancer reduce G-CSF

levels and decreased neutrophil infiltration into tumors. Interestingly, IL-17-

producing γδT cells are scarce in healthy individuals, suggesting that diseased states,

such as meningitis or cancer [76], drive the differentiation of γδT cells towards IL-17,

potentially as a way to dampen the immune system against a mounting or

overactive immune reaction.

Separate of IL-17 mediated pro-tumor functions, γδT cells also directly inhibit

T cell responses via secretions of Galectin-1, Galectin-9, and IL-4 [93]. γδT cells

express high levels of exhaustion ligands and comprise ~40% of tumor-infiltrating T

cells in human pancreatic ductal carcinoma with exhausted αβT cells. Mouse models

have found that depletion of γδT cells or PD-L1 blockade restores CD4^{+} and CD8^{+} T

cell infiltration and functionality to inhibit tumor progression [94]. γδT cells can also

kill activated macrophages via Fas-Fas ligand interaction to resolve home immune

response to Listeria monocytogenes infections in mice [95]. Increasing evidence of

γδT cells promoting tumor cell growth suggests that γδT cells serve a nuanced role in the immune system.

For the tumor microenvironment, γδT cells have demonstrated an ability induce tumor cell resistance to anti-VEGF therapy to promote tumor growth [96]. Following activation with IL-2 and Zoledronic acid, γδT cells secrete VEGF *in vitro*, along with αβT cells and NK cells [97]. Similarly, γδT cells can directly impact epithelial cells of tumors to promote tumor cell proliferation via secretion of IL-22 and amphiregulin [98].

Generation of IL-17^{+} γδT cells appears to depend on combination of IL-1β, IL-6, IL-23, and TGF-β to stimulate IL-17 production by human γδT cells [76]. Stimulation of human myeloid cells with microbial products via MyD88-dependent pathway to produce IL-1β and IL-23 is sufficient to differentiate IL-17-producing γδT cells [98]. Human trials of IL-1β antibody similarly have demonstrated decreased lung cancer incidence and associated decreased mortality, potentially suggesting that canakinumab may dampening pro-tumor γδT cell functions [99].

1.3 CD8$^+$ memory T cells

1.3.1 Phases of CD8$^+$ T cell response to an acute viral infection

Following viral infections, there are three main phases of CD8$^+$ T cell response: expansion, contraction, and stabilization. Formation of CD8$^+$ memory T cells occur during these three phases and persist after resolution of the infection [40].

The first phase of CD8$^+$ T cell response, expansion, begins when naïve CD8$^+$ T cells encounter antigen presenting cells (APC) within a secondary lymphoid organ. APC interact and present foreign antigen peptides on major histocompatibility complex class I (MHC-I) to the naïve CD8$^+$ T cells to provide the requisite signals to efficiently activate the T cell via T cell priming.

There are three main signals required for this interaction. Signal one is the recognition of the T cell receptor (TCR) on the naïve T cell for the peptide presented on MHC-I on the surface of the APC. Signal two is the interaction between costimulatory molecules present on the surface of the APC with those on the T cell. The constituent APC costimulatory molecules are classically composed of B7 family of cell surface molecules, CD80 and CD86, which interact with CD28 on the naïve T cells. Combined activation through Signal one and two are necessary for full activation of CD8$^+$ T cells, as isolated signaling through TCR only induces anergy or functional unresponsiveness, and CD28-only activation has no stimulating effect on

naïve T cells. For full differentiation of effector CD8^{+} T cells, additional factors such as cytokines are also required during CD8^{+} T cell priming and are collectively known as Signal three.

Following activation, different cell surface phenotypes are expressed that functionally distinguish between naïve and newly-activate CD8^{+} T cells. Activated CD8^{+} T cells upregulate CD44, CD25, and CD69, and downregulate CD62L or otherwise known as L-selectin [100, 101]. CD25 is the alpha chain of the IL-2 receptor complex, and upon upregulation, the cell becomes more sensitive to IL-2, which is important for CD8^{+} T cell expansion and survival [102]. CD62L expression is required for T cell homing to lymph nodes, and down-regulation of the molecule allows the cell to exit the lymph node and migrate to sites of inflammation and infection [103, 104].

Over the following seven to ten days, CD8^{+} T cells undergo rapid division approximately every 24 hours, increasing antigen-specific CD8^{+} T cells by a factor of ten- to one-hundred-thousand-fold [103, 105]. At the same time, the activated CD8^{+} T cells gain effector functions that are able to eliminate virally-infected cells from the host, leading to resolution of the infection via direct release of cytolytic perforin and granzyme B proteins into attached cells, and indirect production of effector

interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) cytokines [106-108].

The expansion phase peaks approximately seven to ten days following initial antigenic exposure and is then superseded by the contraction phase with apoptosis of 90 to 90% of the antigen specific CD8^{+} effector T cells. As the infection clears, inflammation decreases with associated decline in growth factors that were necessary for prior T cell expansion. Associated decrease in growth factors that were previously necessary for the survival of CD8^{+} T cells leads to the initiation of apoptosis. Members of the interleukin-2 (IL-2) family, e.g., IL-2, IL-7, and IL-15, as well as type I interferons (type I IFNs) have been implicated in this passive role of T cell apoptosis following clearance of infection [109, 110].

Previously published studies have also found that there is also an active process of T cell apoptosis via ligation of death receptors and their respective ligands, both present on CD8^{+} T cells, or through directly signaling of apoptosis-inducing cytokines e.g., IFN-γ and inflammation [111-113].

Following contraction, the remaining 5-10% of CD8^{+} T cells are known as memory cell precursors, which differentiate into CD8^{+} memory T cells via the stabilization phase. Per conventional model, the precursor cells differentiate in a linear fashion to acquire characteristic CD8^{+} memory T cell phenotype in the weeks

following antigen clearance [103]. This process of CD8^{+} memory T cell differentiation

remains an active area of investigation and significant interest in the development of

vaccines and immunotherapy for cancers.

1.3.2 Subsets and hallmarks of CD8^{+} memory T cells

CD8^{+} memory T cells are an integral part of the adaptive immune system to

provide long-term protection from reoccurring infections. This protection stems

from the ability of CD8^{+} memory T cells to elicit a significantly more rapid and

effective response to lower antigen concentration upon repeat encounters, compared

to primary responses by naïve CD8^{+} T cells. CD8^{+} memory T cells require less

dependence on co-stimulatory activation and have better mechanisms to produce

and secrete cytokines and cytotoxic mediators.

Phenotypically, there are several different subsets of CD8^{+} memory T cells,

with three main categories: central memory CD8^{+} T cells (T_{CM}), effector memory

CD8^{+} T cells (T_{EM}), and tissue resident memory CD8^{+} T cells (T_{RM}) (Table 1). The

differences between the three subsets are mainly based on differences of the cells in

function, homing, and phenotypic characteristics. All three subsets, by definition,

have high levels of expression for CD44, a marker for antigen exposure, and CD127,

also known as IL-7Rα.

The most conventional subset of memory CD8^{+} T cells are T_CM, which are found predominantly in secondary lymphoid tissue, such as the spleen or peripheral lymph node with high expression of lymph node-homing molecule CD62L and chemokine receptor CCR7. T_CM proliferate to a greater degree than both T_EM and T_RM and have a greater functional ability to protect the host against subsequent infections, with homeostatic proliferation [114-116].

T_EM are circulating CD8^{+} memory T cells with low expression of CD62L and CCR7, and therefore decreased homing to lymph nodes and lymphoid organs [105, 117]. Following infection, T_EM readily localize to peripheral tissue and non-lymphoid organs. Functionally, T_EM resemble CD8^{+} effector T cells and demonstrate greater expression of granzyme B and greater *ex vivo* lytic ability compared to T_CM [118].

A more recently identified population of CD8^{+} memory T cells, T_RM, are permanent residents within tissues [119, 120]. They are mainly identified based on expression of CD103 and CD49a [121, 122], which aid in tissue entry, and CD69 for tissue retention [123]. Similar to T_EM, T_RM also have decreased expression of CCR7 and CD62L, but each population appears to differ depending on the tissue of residence. T_RM appear to play a role in sensing and alarm, by recognizing antigen and production of IFN-γ, for increasing expression of chemokine ligands and to

promote the recruitment and effector functions of the innate and adaptive immune

system to the site of infection [124-126].

Table 1: Summary of CD8$^+$ memory T cell subsets

Subset	Location	CD44	CD62L	CD127
T$_{CM}$	Circulation, lymphoid organs	High	High	High
T$_{EM}$	Circulation	High	Low	High
T$_{RM}$	Skin and non-lymphoid organs	High	Low	High

1.4 Vaccinia virus

Vaccinia virus (VV) is the most extensively studied member of the Poxviridae family and is responsible for the eradication of smallpox worldwide [12, 127, 128]. Poxviruses are unique among viruses in that they replicate exclusively in the host cell cytoplasm. The enveloped VV genome is composed of approximately 190 kilobase pairs (kbp) of double-stranded DNA that encodes all of the enzymes and proteins required for viral DNA replication and transcription in the host cell cytoplasm [129].

Beginning around 2 hours after infection, the early viral genes encoded after cell entry uncoats the virus, to begin transcription of intermediate and late genes responsible for the formation of new viral particles [130]. Replication of VV relies primarily on the central ~100 kbp portion of the genome. The rest of the genome are involved in the virulence and regulation of the host immune response [131].

There are two main forms of VV following replication: intracellular mature virus (IMV) and extracellular enveloped virus (EEV). IMV is formed first with membrane cisternae from the intermediate compartment between the endoplasmic reticulum and Golgi apparatus surrounding VV DNA. A small portion of IMV then becomes surrounded by endosomal or trans-Golgi network membranes to form intracellular enveloped virion (IEV), which translocates to the cell surface to fuse

with the plasma membrane and become double-encapsulated extracellular

enveloped virus (EEV) [132, 133]. EEV is crucial for viral dissemination, as

antibodies directed against EEV can inhibit viral release, and antibodies against EEV

proteins protect host mammals against VV challenge more effectively than

antibodies to IMV proteins [134, 135].

VV encodes numerous proteins for immune evasion, some of which are

immunodominant and can trigger VV-specific T cell immune responses in mice [136-

139] (Table 2). B8R is an immune-prevalent antigen in VV that contains the only

major dominant epitope in C57BL/6 mice, triggering >5% of total CD8$^+$ T cell

response to VV and can be used to identify VV-specific CD8$^+$ T cells [136]. It

normally serves as an IFN-γR mimic in non-murine species to inhibit host IFN-γ

from binding to IFN-γR for subsequent signaling. However, B8R is not functionally

active in mice, and can serve as a marker for activated VV-specific CD8$^+$ T cells via

tetramer staining [140-142]. In this thesis, we use B8R tetramer staining to identify

VV-specific CD8$^+$ T cells.

The goal of this thesis aims to investigate the role of γδT and CD8$^+$ T cells in

response to VV infection, with hopes of better understanding the immune system for

vaccination and cancer immunotherapy. The success of VV in vaccination has led to

the development of recombinant VV as a vaccine vehicle for other infectious diseases

such as influenza, HIV, malaria, and cancer immunotherapy [143-146]. In most cases, immunization with recombinant VV generates significantly greater immune responses than immunization with the corresponding protein or peptide epitopes with standard adjuvants. The distinctive ability of VV to generate a potent immunity most likely derives from its ability to activate both the innate and adaptive immune systems.

Table 2: VV-derived immunoprevalent antigens

Ag Name	VV-WR Name	Peptide Sequence	Restriction
—	148	SIYQYVRL	H-2Kb
—	148	KYQQDRDTL	H-2Kd
—	148	IPAALIILL	H-2Ld
A3L	122	KSYNYMLL	H-2Kb
A3L	122	YSPSNHHIL	H-2Db
A3L	122	IYSPSNHHI	H-2Kd
A47L	173	AAFEFINSL	H-2Kb
A47L	173	TMMINPFMI	H-2Db
A47L	173	AHINALEY	H-2Db
A47L	173	KIIQKSSSI	H-2Kd
B8R	190	TSYKFESV	H-2Kb
D1R	106	LGYIIRYPV	H-2Kb
D1R	106	SMYCSKTFL	H-2Db
D1R	106	KYEGPFTTT	H-2Kd
D1R	106	KYFYGEIAL	H-2Kd
D1R	106	KFINGASTM	H-2Kd
D5R	110	SKIFINSII	H-2Kd
D5R	110	TYTTMDTLI	H-2Kd
D5R	110	YYFSLQQRL	H-2Kd
E9L	65	RMNSNQVCI	H-2Db
E9L	65	NPLSNPFYM	H-2Ld
J6R	98	INFEFVCL	H-2Kb

J6R	98	KYAANYTKI	H-2Kd
J6R	98	KYFFTVSNI	H-2Kd
J6R	98	RYNVIASSI	H-2Kd
M1L	30	TSNVITDQTV	H-2Db

1.5 Thesis Prospectus

As cellular populations required for the efficient clearance and subsequent generation of effective memory against viral pathogens, $\gamma\delta$T cells and CD8$^+$ T cells play important roles in the formation of immune memory against viral pathogens. VV is a potent stimulator of both $\gamma\delta$T and CD8$^+$ T cells and is a powerful tool in vaccine development and immunotherapy. However, the mechanism behind how VV elicits such a strong immune response from the immune system remains incompletely explored. To investigate these mechanisms, the overall goal of this thesis is to understand how VV stimulates $\gamma\delta$T and CD8$^+$ T cells, and the subsequent effects on viral clearance and memory T cell formation. To do this, we examined the influence of $\gamma\delta$T cells on CD8$^+$ T cell activation, directly cytotoxicity of $\gamma\delta$T cells against VV infection, and the mechanisms that govern CD8$^+$ memory T cell formation following VV infection. We addressed this thesis project with three chapters to answer the following questions:

Chapter 3: How does VV stimulate $\gamma\delta$T cells for CD8$^+$ T cell activation?

Chapter 4: What is the role of NKG2D in $\gamma\delta$T cell clearance of VV infection?

Chapter 5: How is T cell-specific deficiency of Notch1 associated with CD8$^+$ memory

   T cell formation?

Chapter 2. Materials and Methods

2.1 Animals

Eight- to ten-week-old C57BL/6, *δTCR*$^{-/-}$, *OT-1*, *LckCre*$^+$, *RosaCre*$^+$, and *b2m*$^{-/-}$ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). *MyD88*$^{-/-}$ on C57BL/6 background were kindly provided by Shizuo Akira (Osaka University, Osaka, Japan). *NKp46Cre*$^+$ and *NKG2D*$^{F/F}$ mice were created in-house and crossed with *LckCre*$^+$, *NKp46Cre*$^+$, or *RosaCre*$^+$ mice on C57BL/6 background to create *LckCre*$^+$ *NKG2D*$^{F/F}$, *NKp46Cre*$^+$ *NKG2D*$^{F/F}$, *LckCre*$^+$ *NKp46Cre*$^+$ *NKG2D*$^{F/F}$, and *RosaCre*$^+$ *NKG2D*$^{F/F}$ mice. All experiments involving the use of mice were done in accordance with protocols approved by the Animal Care and Use Committee at Duke University and the Ohio State University.

2.2 Vaccinia virus (VV)

2.2.1 VV purification and storage

Western Reserve (WR) strain of VV was purchased from American Type Culture Collection (Manassas, VA). Recombinant VV-OVA was provided by Jonathan Yewdell at NIH. The viruses were grown in TK-143B cells and purified by centrifugation through a 35% sucrose cushion as previously described [147]. The titer was determined by plaque assay on TK-143B cells and subsequently stored at -80°C until use. For *in vivo* studies, $5x10^6$ pfu of live VV in 0.1mL Tris-Cl was injected into mice intraperitoneally, unless otherwise specified.

2.2.2 Plaque assay

Viral load in the peritoneum is measured by plaque-forming assay as described [1]. Mice were euthanized 3 days after infection, and the peritoneum is washed with PBS, and stored at -80°C. Peritoneum washings were macerated with bead homogenizer (MP Biomedical, Irvine, CA), and serial dilutions were performed to determine virus titers by plaque assay on confluent TK-143B cells.

2.2.3 VV quantitative real-time PCR

3 days post-VV inoculation, total DNA was isolated from peritoneal fluid as previously described [148]. Real-time quantitative PCR was used to analyzed VV

E3L gene in duplicates using SYBR Green Real-Time PCR Master Mix (Bio-Rad, Hercules, CA). PCR conditions were 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 50 seconds, followed by a melt curve capture on CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Primer sequences are provided below in Table 4. Relative gene expression levels for each respective gene were calculated using threshold cycle ($2^{-\Delta\Delta CT}$) and normalized to *Gapdh*.

2.3 Adenovirus

Adenovirus encoding B8R (Ad-B8R) was grown in 293 cells (American Type Culture Collection), purified by two rounds of CsCl density centrifugation, and desalted by gel filtration through Sephadex G-25 column (PD-10 column; Amersham Biosciences, Piscataway, NJ). The titer of the virus was determined by plaque-forming assay on 293 cells [149, 150]. In all instances, mice were infected with 2×10^9 plaque-forming units (PFU) of Ad-B8R i.p.

2.4 Generation and culture of DC using bone marrow (BM) culture

Femurs and tibiae of mice were harvested and bone marrow cells were flushed with DC medium (RPMI-1640 with 5% fetal bovine serum [FBS], 2mM L-glutamine, 10mM HEPES, 50μM β-mercaptoethanol, 100 IU/mL penicillin, and 100 IU/mL streptomycin), as previously described[147]. After lysis of red blood cells with ACK lysis buffer (Gibco Life Technologies, Waltham, MA), the bone marrow cells were cultured in 6-well plates at density of $3x10^6$ cells/mL in 3mL DC medium in the presence of mouse granulocyte macrophage-colony stimulating factor (GM-CSF; 1000 U/mL; R&D Systems, Minneapolis, MN) and interleukin 4 (IL-4; 500 U/mL; R&D Systems). GM-CSF and IL-4 were replenished on day 2 and 4. On day 5, DCs were harvested, and $CD11c^+$ DCs were transferred onto a new 24-well plate at a density of $0.85 x 10^6$ cells/mL in 2mL DC media.

2.5 γδT cells

2.5.1 γδT cell *in vivo* VV activation and isolation

Splenocytes were harvested from C57BL/6 mice 2 days after peritoneal inoculation with VV. γδT cells were isolated from harvested splenocytes with pan-T cell microbeads, followed by anti-γδTCR microbeads (Cat #130-092-125, Miltenyi Biotec, Auburn, CA). The isolated γδT cells were assessed via flow cytometry for confirmation.

2.5.2 Adoptive transfer of γδT cells

Naïve γδT cells were isolated from pooled spleens and lymph nodes donor mice on C57BL/6 background, with pan-T cell microbeads, followed by anti-γδTCR microbeads (Miltenyi Biotec). The isolated cells were confirmed via flow cytometry for confirmation and suspended in phosphate buffered saline (PBS). The cells were then injected intravenously via the tail vein into recipient mice on C57BL/6 background at 1×10^6 cells/mouse, unless otherwise specified.

2.6 Splenic T Cell culture

T cells were isolated from splenocytes of C57BL/6 mice using pan-T cell isolation microbeads (Miltenyi Biotec), and cultured at 37°C in RPMI-1640 with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, 100 IU/mL streptomycin, 2mM L-glutamine, and 50μM β-mercaptoethanol, with or without 500μM LY411575 (GSI; Stemgent, Cambridge, MA) to inhibit Notch activation.

2.7 Plate-bound DLL4 stimulation

Plates were coated with 0.5μg/mL of recombinant mouse DLL4 (R&D, Minneapolis, MN) in 1x PBS overnight at 4°C. Afterwards, they were washed with 1x PBS and air-dried prior to use for stimulation of isolated T cells at 37°C for 48 hours.

2.8 CD8⁺ T cell proliferation assay.

CD8$^+$ T cells were isolated from splenocytes of OT-I mice on C57BL/6 background using anti-CD8a microbeads (Miltenyi Biotec), and then fluorescently labeled with carboxyfluorescein succinimidyl ester (CFSE). Labeled CD8$^+$ T cells and OVA-I peptide were then cocultured with matured DCs or VV-activated γδT cells at 1:1 ratio in 96 well plates. The cells were incubated at 37°C for 72 hours, and then assessed via flow cytometry.

2.9 Cell sorting.

Lymphocytes were isolated from spleens and lymph nodes of wild-type, *Notch1^{ΔT}*, or *Notch2^{ΔT}* mice on C57BL/6 background, with anti-CD8a microbeads (Miltenyi Biotec). The isolated cells were then stained with CD8-PerCy5 and B8R-PE, confirmed via flow cytometry, and resuspended in PBS for cell sorting with BC MoFlo Astrios Cell Sorter (Beckman Coulter, Brea, CA).

2.10 Antibodies and Flow Cytometry

2.10.1 Antibodies and flow cytometry analysis

The list of used antibodies is provided below in (Table 3). Cells were suspended in PBS buffer with 2% heat-inactivated FBS and 0.1% sodium azide. After staining, cells were washed twice, and analyzed with FACSCanto flow cytometer (BD Biosciences) using FlowJo software (BD Biosciences)

Table 3: Summary of antibodies and fluorophores

Antibody	Fluorophore	Company	Catalog Number
CD3e	APC	BD	553066
CD4	APC	BD	553051
CD44	APC	BD	559250
IFN-γ	APC	BioLegend	505810
MHC-I H2D	APC	eBioscience	17-5998-30
TCRβ	APC	BioLegend	109211
CD62L	Biotin	BD	553159
CD4	FITC	BD	553047
CD8a	FITC	BD	553031
GranzymeB	FITC	eBioscience	11-8898-82
TCRγδ	FITC	eBioscience	11-9959-42
CD80 (B7.1)	PE	BioLegend	104709
CD86 (B7.2)	PE	BD	553692
H-2k(b) TSYKFESV	PE	NIH	7716
CD4	PE	BD	553730
CD8a	PE	BD	553032
TCRγδ	PE	BioLegend	118107
H2Kb SIINFEKL	PE	BioLegend	141603
CD3e	PE-Cy5	BD	553065
CD4	PE-Cy5	BD	553050
CD8a	PE-Cy5	BD	553034
CD127	PE-Cy5	eBioscience	15-1271-83

TCRγδ	PE-Cy7	eBioscience	25-5711-80
CD4	PE-Cy7	BioLegend	100421
Streptavidin	PE-Cy7	BD	557598

2.10.2 Intracellular cytokine staining

Splenocytes were re-stimulated specifically for CD8+ T cells with 2 μg/mL

B8R peptide (TSYKFESV, BD Pharmingen) and 5 μg/mL Brefeldin A (Invitrogen) for

5 hours at 2 μg/mL at 37°C. Splenocytes were stimulated specifically for γδT cells

with 50 ng/mL Ionomycin, 100 ng/mL PMA, and 5 μg/mL Brefeldin A for 2.5 hours

at 37°C. After staining with cell surface markers, the cells were fixed and

permeabilized with Cytoperm/Cytofix solution (BD Biosciences) for 20 minutes and

incubated with anti-IFN-γ antibodies for 30 minutes. The cells were washed twice

with Permeabilization buffer (BD Biosciences) and analyzed with FACSCanto flow

cytometer using FlowJo software (BD Biosciences)

2.10.3 MHC/peptide tetramer

The VV-specific epitope B8R$_{20-27}$, TSYKFESV, is a synthetic peptide based on

modified vaccinia virus Ankara (MVA) sequence[138]. Peptide MHC I tetramers

consisting of B8R$_{20-27}$/K^b conjugated to allophycocyanin were obtained from the NIH

Tetramer Core Facility (Emory University, Atlanta, GA, USA). Cells were stained

with the tetramer for 30 minutes at room temperature in the dark together with

surface staining and subsequently analyzed by flow cytometry.

2.11 *In vivo* antibody blocking assay

Mice were treated with anti-NKG2D neutralizing antibody (HMG2D; Bio X

Cell, Lebanon, NH) or a control Ig. On day 0, mice were inoculated with VV

intraperitoneally (5×10^6 pfu). 3 days later, peritoneal fluid was harvested and

assayed for viral titers and $\gamma\delta$T cells by flow cytometry

2.12 Chromatin Immunoprecipitation (ChIP)

ChIP assays were performed as previously described. Briefly, 1×10^7 cells were

left untreated, treated with plate-bound DLL4, or treated with both plate-bound

DLL4 and 500µM GSI for 48 hours prior to crosslinking for 10 mins with 1%

formaldehyde. Antibody recognizing mouse cleaved Notch1 (Val1744; NICD) was

purchased from Cell Signaling Technology (Danvers, MA). Normal rabbit IgG (Cell

Signaling Technology) was used as negative control. qRT-PCR for regions proximal

to *Tcf7* was performed with specific primers (sense: 5'-

CTTGAGCTGAGGGGTCCTTG -3'; antisense: 5'- GCCTAAGCGGGTACTCTGAC -

3'). PCR products were further analyzed with agarose gel electrophoresis.

2.13 Real-time PCR

Total RNA was isolated from MACS-isolated γδT cells using RNeasy Plus kit (Qiagen, Hilden, Germany) according to manufacturer's recommendations. Genomic DNA contamination was eliminated with gDNA eliminator or on column DNase digestion (Qiagen). Reverse transcription was performed with Superscript First-Strand Synthesis (Promega, Madison, WI), with random and oligo-(dT)15 primer mixture at 1:1 ratio. Subsequent cDNA was analyzed in duplicates using SYBR Green Real-Time PCR Master Mix (Bio-Rad, Hercules, CA). PCR conditions were 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 50 seconds, followed by a melt curve capture on CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Primer sequences are provided below (Table 4). Relative gene expression levels for each respective gene were calculated using threshold cycle ($2^{-\Delta\Delta CT}$) and normalized to *Gapdh*.

Table 4: List of qRT-PCR primers

Gene	F Sequence	R Sequence
β–Actin	AGCCATGTACGTAGCCATCC	CTCTCAGCTGTGGTGGTGAA
CD8	ACTACCAAGCCAGTGCTGCGAA	ATCACAGGCGAAGTCCAATCCG
c-Myc	CGGACACACAACGTCTTGGAA	AGGATGTAGGCGGTGGCTTTT
Dapl1	GAAAGCTGGAGGGATGCGAATC	TGTGTCCGTCAGAGCATCCAAC
E3L	AAGCTCTGTACGATCTTCAACG	TCAGCCATAGCATCAGCATC
GAPDH	CCTCGTCCCGTAGACAAAATG	TGAAGGGGTCGTTGATGGC
Gzmb	CCTCCAGGACAAAGGCAG	CAGTCAGCACAAAGTCCTCTC
IL-1a	GATTCACAACTGTTCGTGAGC	GATGAGTTTTGGTGTTTCTGGC

IL-1b	ACGGACCCCAAAAGATGAAG	TTCTTCACAGCCACAATGAG
IL-6R	CAAGAATCCTCGTCCATGTCC	TCGTCTTGCTTTCCTTCTCAG
IL-10	CCAAGCCTTATCGGAAATGA	TTTTCACAGGGGAGAAATCG
IL-12a	CTTTGATGATGACCCTGTGC	GCAGAGTCTCGCCATTATGA
IFN-α	AGGATTTTGGATTCCCCTTG	GTCAGAGGAGGTTCCTGCAT
IFN-β	CCCTATGGAGATGACGGAGA	TCCCACGTCAATCTTTCCTC
IFN-γ	ACTGGCAAAAGGATGGTGAC	TGAGCTCATTGAATGCTTGG
IRF1	CACCAGGAACCAGAGGAAAG	GTCCATCAGAGAAAGTGTCCG
IRF3	GACACCAATGGCAAAAGCAG	AGAAGGATCAGAAACAATAGCCAG
IRF4	CTCGTCGTGGTCAGCTCTTT	AATCCCCATTGAGCCAAGCA
IRF7	CCAGTTGATCCGCATAAGGT	GAGGCTCACTTCTTCCCTATTT
IRF8	GTTTACCGAATTGTCCCCGAG	CGGCCACACTCCATCTCAG
MyD88	CTGTCTCCAGGTGTCCAACAG	ATGTAGACAGGACGGCATCAG
Prf1	GATGTGAACCCTAGGCCAGA	GGTTTTTGTACCAGGCGAAA
Runx1	CACCGTCTTTACAAATCCGCCAC	CGCTCGGAAAAGGACAAACTCC
TNF-α	TCAGTAGACAGAAGAGCGTGGT	GAGCACAGAAAGCATGATCC
Tcf7	CCTGCGGATATAGACAGCACTTC	TGTCCAGGTACACCAGATCCCA
EnhTcf7	CTTGAGCTGAGGGGTCCTTG	GCCTAAGCGGGTACTCTGAC
Trib2	CCTGGTGCTGCGTGACCTCAA	GCCATGCTTGTCAGAGAGTGAG

2.14 Statistical analysis

Results are expressed as mean ± SEM. Differences between groups were examined for statistical significance using Kolmogorov-Smirnov test, Mann-Whitney test, or unpaired t-test with Welch's correction. *P*-values less than 0.05 are considered to be significant.

Chapter 3. $\gamma\delta$T cells are required for CD8^{+} T cell response to Vaccinia viral infection

3.1 Introduction

Vaccinia virus (VV), an enveloped double-stranded DNA virus, is a member of the *Orthopoxvirus* genus of the Poxviridae family. It has approximately 190kbp genome that encodes all the proteins required for cytoplasmic viral replication in host cells [128]. It is responsible for the worldwide elimination of smallpox, and as a result has been developed as recombinant vaccine vehicle for infectious diseases and cancer immunotherapy [129]. It is unique among viral agents to be able to elicit both potent and long-lasting immunity [12]. Though its natural route of infection is via the skin, many studies have noted that intraperitoneal, intravenous, and intramuscular modes of VV inoculation provides similar clinical efficacy in both mice and humans [151-153].

We have previously shown that the unique potency of intraperitoneal VV inoculation in the activation of CD8^{+} T cell responses is dependent on efficient activation of the innate immune system through Toll-like receptor (TLR)-dependent and -independent pathways [4, 154]. Specifically, we have demonstrated that intrinsic TLR2-MyD88 (myeloid differentiating factor 88) signaling in CD8^{+} T cells is critical for clonal expansion and long-lived memory formation [2]. In addition, TLR-independent production of type I interferons (IFNs) is also important for efficient CD8^{+} T cell responses [155, 156]. However, despite these advances, the mechanisms by conventional

antigen-presenting cells are unable to fully explain the unique potency of VV in the activation of CD8^{+} T cell responses.

γδT cells are a unique population of lymphocytes that exert a strong influence on the immune system [19]. Previous studies have also shown that there are several subpopulations of γδT cells with distinct functions. However, a definitive system to categorize the different subpopulations has remained elusive. Initial proposals to define γδT cells based on their different TCR expression in both mice and humans have had to be revisited [23-28]. As a result, we assessed the effects of γδT cells as a whole for this study.

γδT cells act as a bridge between the innate and adaptive immune responses, with characteristics of both. They can exert direct cytotoxicity and enhance the adaptive immune responses [6, 38, 39, 42]. Studies have found that γδT cells are important in the immune response against many mycobacterial, parasitic, and viral infections [21, 46, 145, 157-165]. Similarly, previous studies have demonstrated that γδT cells express CD80 and CD86 at similar levels to professional antigen presenting cells and is able to promote CD8^{+} T cells activation [38, 42]. However, it remains largely unknown exactly how γδT cells promote adaptive immune responses.

In this study, we found that γδT cells play a critical role in promoting CD8^{+} T cell response to VV infection. We showed that activation of γδT cells by VV presented viral antigens in the context of MHC class I for CD8^{+} T cell activation *in vivo*. We further

demonstrated that cell-intrinsic MyD88 signaling in γδT cells is required for γδT cell activation and CD8⁺ T cell responses. These results demonstrated a critical role for γδT cells in the regulation of CD8⁺ T cell response to viral infection and may shed light on the design of more effective vaccine strategies based on manipulation of γδT cells.

3.2 Results

3.2.1 γδT cells are required for CD8⁺ T cell response to VV

To address whether γδT cells play a role in regulating CD8⁺ T cell responses, we first examined the activation status of γδT cells in response to VV infection in vivo. C57BL/6 mice were injected with VV intraperitoneally, and at different time points after infection, γδT cells were examined for IFN-γ production.

We found that in both spleen (Figure 1A) and peritoneal cavity (Figure 1B), IFN-γ⁺ γδT cell count reached its peak around day 4 following VV infection, with subsequent decline in the days following. This is in contrast to VV-specific CD8⁺ T cell response in that IFN-γ⁺ CD8⁺ T cell count reached its peak around day 7 (Figure 1). These results indicated that the activation of γδT cells peaked prior to that of CD8⁺ T cells. We found that naïve γδT cells secreted IFN-γ after stimulation, therefore we subsequently always comparatively determined IFN-γ gating within each experiment between inoculated versus naïve mice (Figure 1C, D).

Figure 1. 5 x 10^6 pfu of VV were injected into wild-type C57BL/6 mice

intraperitoneally. 2, 3, 4, 5, 6, 7 and 8 days after infection, CD8$^+$ T cells from spleen

and peritoneal cavity were assayed for IFN-γ production by intracellular staining by

FACS, and IFN-γ$^+$ γδT cells and CD8$^+$ T cells from the spleen (**A**) spleen and

peritoneal cavity (**B**) are shown. IFN-γ$^+$ γδT cells are first gated on CD3e+ and

γδTCR$^+$, then assessed on IFN-γ expression. VV-specific B8R$^+$ IFN-γ$^+$ CD8$^+$ T cells

were first gated on B8R$^+$ CD8$^+$ T cells, and then assessed for IFN-γ expression. The

mean of each time point is plotted. Representative of 2 independent studies, each

with 3 biological replicates. Gating strategy used to generate the data are

represented by (**C**) and (**D**). The gating for IFN-γ$^+$ γδT cells is determined against

IFN-γ$^+$ γδT cells from control naïve mice for each experiment. Mann-Whitney test, P <

0.05.
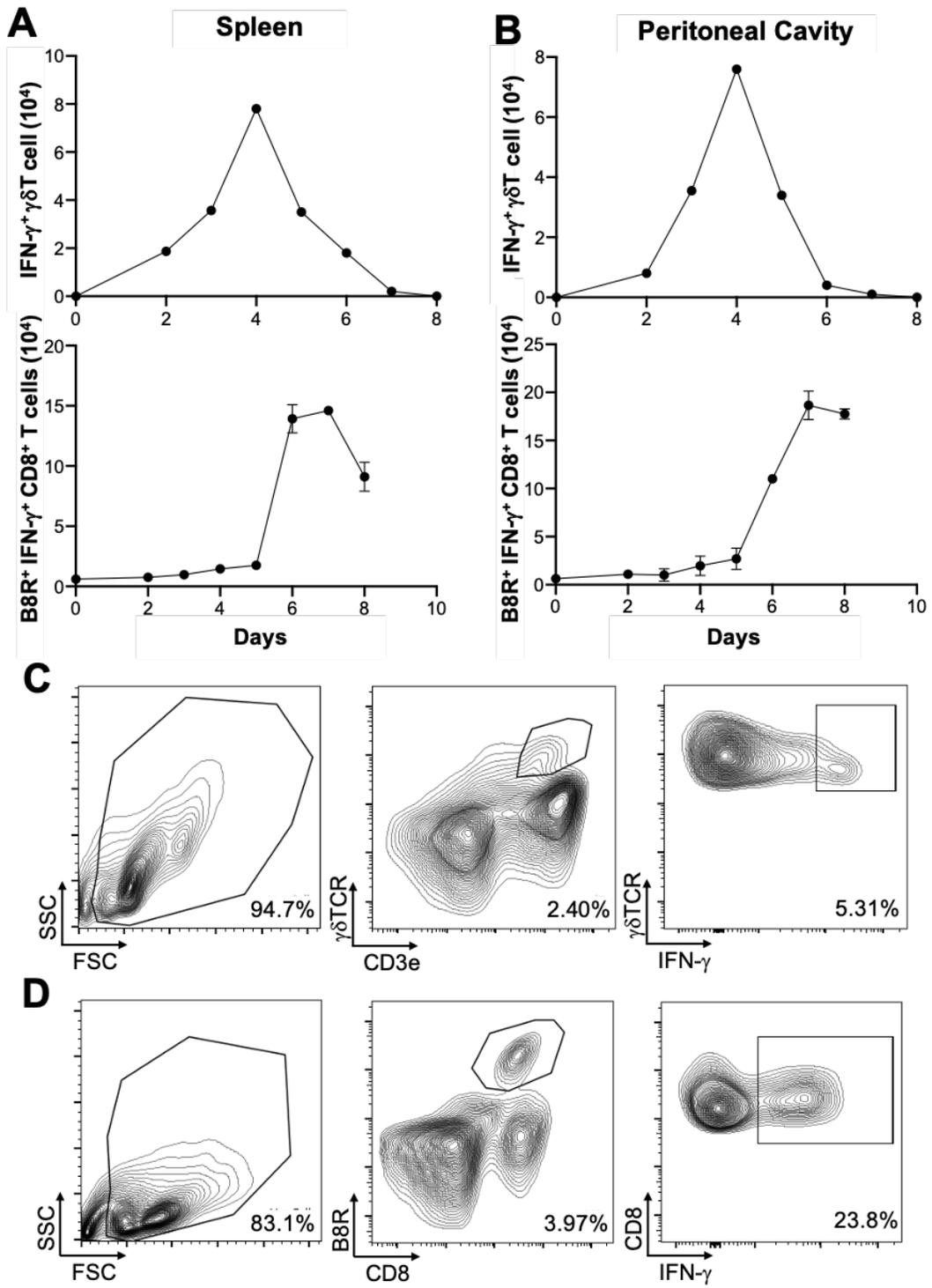
Figure 1: Activation of γδT cells in response to VV infection We next

determined if γδT cells play a role in CD8⁺ T cell response to VV. We inoculated

wild-type (WT) and *δTCR⁻/⁻* C57BL/6 mice intraperitoneally with VV and assessed for

VV-specific B8R⁺ CD8⁺ T cell activation 7 days post-inoculation. B8R is a VV epitope

that is recognized by VV-specific CD8⁺ T cells; B8R⁺ CD8⁺ T cells are specifically

activated by VV. We found that there is a significant decrease in VV-specific B8R⁺

(Figure 2C, E) and functional IFN-γ⁺ (Figure 2D, F) CD8⁺ T cells in *δTCR⁻/⁻* mice that

lack γδT cells, compared to that of WT mice ($P < 0.005$). We subsequently found that

this defect can be rescued with adoptively transferred WT γδT cells. VV inoculation

of *δTCR⁻/⁻* mice with adoptive transfer of WT γδT cells had significantly greater VV-

specific B8R⁺ and IFN-γ⁺ CD8⁺ T cells, compared to that of *δTCR⁻/⁻* mice with VV

inoculation alone ($P < 0.005$). VV-specific B8R⁺ and IFN-γ⁺ CD8⁺ T cell response in

δTCR⁻/⁻ with adoptive transfer of WT γδT cells following VV inoculation also

approximated the same response as WT mice with VV inoculation alone (*P* not

significant). This suggested that γδT cells play a critical role in CD8⁺ T cell activation

following VV infection.

Figure 2. 5 x 10⁶ pfu of VV were injected intraperitoneally into wild-type C57BL/6

(WT) or *δTCR⁻/⁻* mice (γδT⁻/⁻). Concurrently, a different population of *δTCR⁻/⁻* mice

were also adoptively transferred with 1×10^6 WT γδT cells (γδT$^{-/-}$ VV + WT γδT),

followed by VV inoculation. (**A**) 3 days post-adoptive transfer and VV inoculation,

there is a detectable population of γδT cells in the mesenteric lymph nodes, with

γδTCR-PECy7 and CD3e-PerCy5 staining. (**B**) Representative plot of purified γδT

cells used for adoptive transfer. 7 days post-inoculation, the spleens were harvested

and stained for B8R$^+$ CD8$^+$ T cells by tetramer and IFN-γ$^+$ CD8$^+$ T cells by intracellular

staining. (**C**) Representative FACS plots first gated on CD8$^+$ CD4$^-$ T cells and then

plotted against B8R$^+$ and CD8$^+$ T cells for B8R$^+$ CD8$^+$ T cells. (**D**) Representative FACS

plots first gated on CD8$^+$ CD4$^-$ T cells and then assessed for IFN-γ expression. (**E, F**)

Quantification of FACS plots. Values are mean ± SEM, representative of 3

independent studies, each with at least 3 biological replicates. Kolmogorov-Smirnov

nonparametric t-test, *$P < 0.005$.
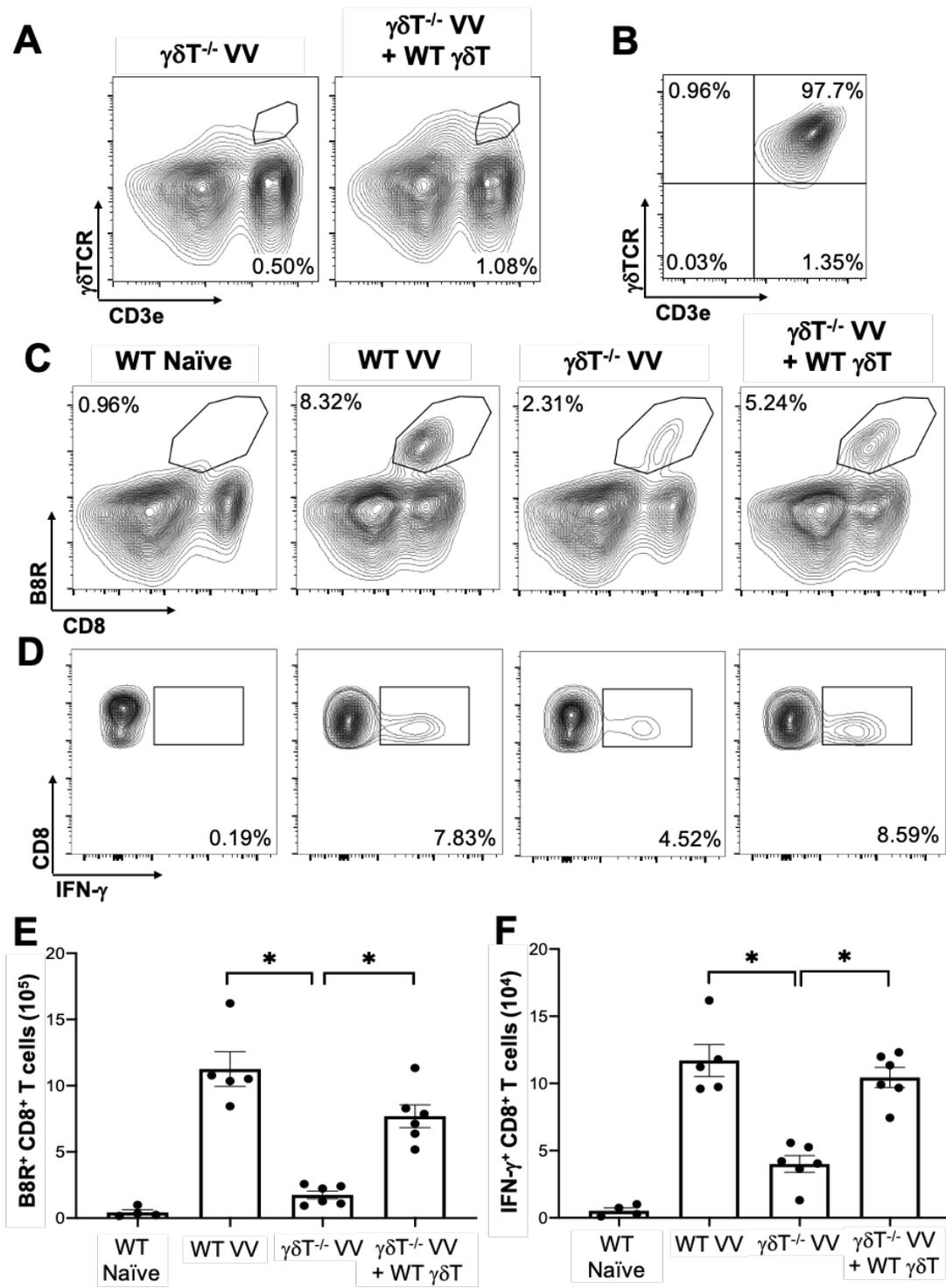
Figure 2 γδT cells is required for CD8+ T cell response to VV

3.2.2 VV activates γδT cells to present MHC-I peptide and upregulate CD80 and CD86

To determine how γδT cells promote the activation CD8$^+$ T cells to VV infection, we explored whether γδT cells contributed to signals that are required to activate CD8$^+$ T cells: 1) direct presentation of VV-specific peptide on MHC-I, 2) co-stimulation with CD80 and CD86 ligands [166-168]. To assess peptide presentation on MHC-I, we inoculated WT mice with VV or VV encoded with OVA (VV-OVA). We then assessed γδT cells for expression of H2K^b specific for SIINFEKL peptide on MHC-I. We found that there is an increase in H2K^b SIINFEKL$^+$ γδT cells in mice inoculated with VV-OVA, compared to that of naïve or mice inoculated with VV (Figure 3A).

We also found that following VV infection, there is an increase in CD80 and CD86 expression on the surface of γδT cells by flow cytometry. CD86 is expressed first as the initial co-stimulatory ligand, and CD80 is expressed after antigen-presenting-cell activation [169]. We found that 4 days post-inoculation, there is a significant increase in CD86 (Figure 3C; $P < 0.01$), and a corresponding increase in CD80 (Figure 3B; $P < 0.05$). These results suggests that γδT cells could provide the necessary signals for CD8$^+$ T cell activation after VV infection.

Figure 3. C57BL/6 mice were inoculated intraperitoneally with 5 x 10^6 pfu of VV or VV encoding OVA (VV-OVA). (**A**) Representative FACS plots first gated on CD3e$^+$ and γδTCR$^+$ T cells, and then assessed for SIINFEKL peptide expression on mouse MHC-I H2K^b via anti-H2K^b SIINFEKL in spleens harvested 2 days post-infection. (**B, C**) 4 days post-inoculation, splenocytes were extracted and stained for CD3e$^+$ and γδTCR$^+$ T cells. The gated cells were then assessed for (**B**) CD80 and (**C**) CD86 expression. Values are mean ± SEM, representative of 3 independent studies, each with at least 3 biological replicates. Kolmogorov-Smirnov nonparametric t-test, *$P <$ 0.05.
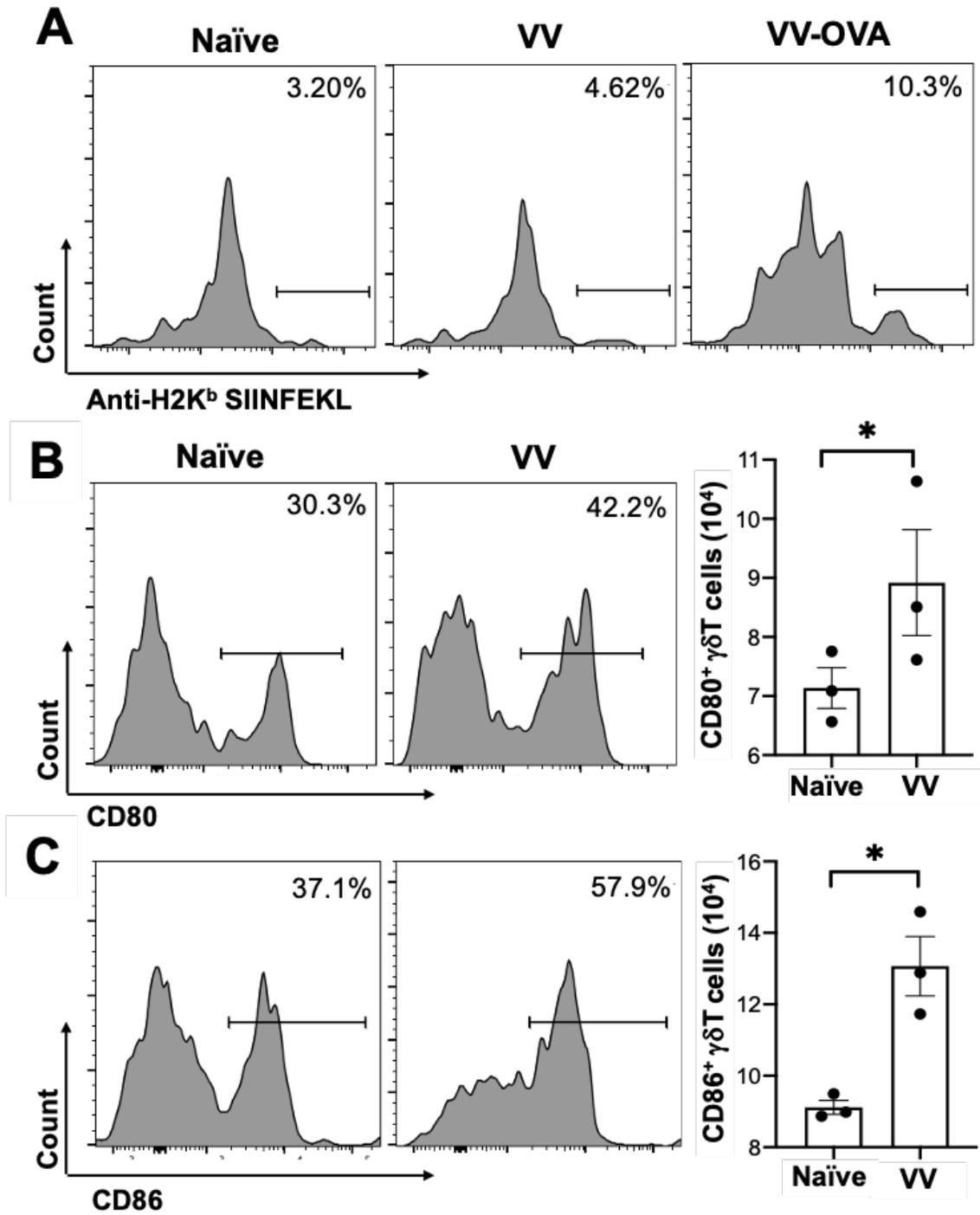
Figure 3 VV activates γδT cells to present peptide on MHC-I and upregulate CD80/CD86 expression.

Signal 3 of effector CD8^{+} T cell activation is mainly associated with type I

interferon, IL-1, and IL-12[166, 170, 171]. 4 days post-VV inoculation, we found that

there is a significant increase in expression of IL-1 and IFN-α in γδT cells compared

to that of naïve γδT cells (Figure 4; $P < 0.001$). γδT cells also secrete a basal level of IL-

12 that does not change following VV inoculation but is significantly decreased

following depletion of MyD88 (Figure 5**B**, $P < 0.001$). This suggests that γδT cells can

provide the necessary signals for CD8^{+} T cell activation after VV infection.

Figure 4. 4 days following VV inoculation, γδT cells were isolated from splenocytes

from naïve and VV-inoculated WT mice with MACS. Total RNA was extracted from

the isolated cells and assessed by qRT-PCR for IFN-α, IL-1, and IL-12 expression.

Values are mean ± SEM, representative of 3 independent studies. Kolmogorov-

Smirnov nonparametric t-test, *$P < 0.05$.
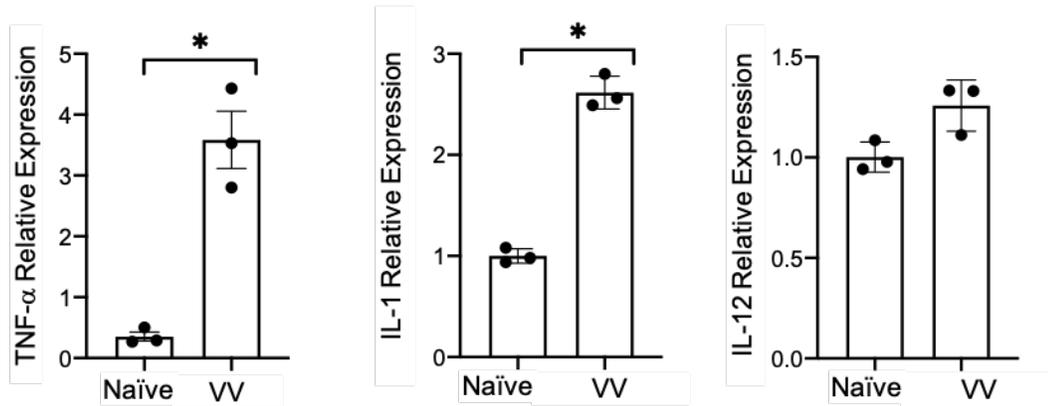
Figure 4 VV activates γδT cells to increase cytokine expression

Figure 5. Wild-type and *MyD88*^{-/-} mice were inoculated with VV intraperitoneally and 4 days post-inoculation, splenocytes were harvested. γδT cells were isolated with MACS. Total RNA was extracted from the isolated cells and assessed by qRT-PCR for expression of **(A)** IRF-1, and **(B)** IL-1, IL-12, and IFN-α
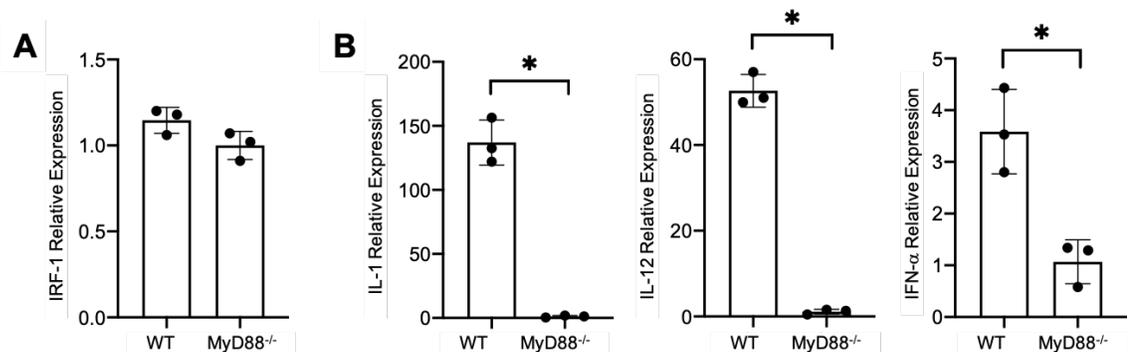
Figure 5 Expression comparison between WT and MyD88^{-/-} mice inoculated with VV

3.2.3 γδT cells also directly activate CD8$^+$ T cells via MHC-I

We next explored whether γδT cells directly activates CD8$^+$ T cell following

VV infection *in vivo*. To determine if VV can activate γδT cells to act as antigen

presenting cells, we isolated γδT cells from WT C57BL/6 mice that had been

inoculated intraperitoneally with VV 48 hours prior. CD8$^+$ T cells were obtained

from OT-I mice on C57BL/6 background and pulsed with CFSE. The CFSE-labeled

CD8$^+$ T cells were then co-cultured with the VV-activated γδT cells and OVA-I

peptide. 72 hours after co-incubation, CD8$^+$ T cell proliferation was assayed by CFSE

dilution. As a control, the CD8$^+$ T cells were also incubated with matured DCs and

OVA-I peptide. We found that CD8$^+$ T cells proliferated at a similar magnitude when

cocultured with VV-activated γδT cells as with matured DCs (Figure 6A). The same

proliferation is not seen if CD8$^+$ T cells are incubated with γδT cells or OT-I peptide

alone. This suggests that VV is able to activate γδT cells to become antigen

presenting cells.

We then determined if γδT cells functionally acts as professional APCs via

MHC-I by assessing if CD8$^+$ T cell activation could be rescued via adoptive transfer

of *β2m$^{-/-}$* γδT cells. β2m is a necessary component of MHC-I. We found that adoptive

transfer of WT γδT cells into *δTCR$^{-/-}$* followed by VV infection significantly increased

percentages and cell count of B8R$^+$ and IFN-γ$^+$ CD8$^+$ T cells compared to *δTCR$^{-/-}$* with

VV infection alone (Figure 6B-E; $P < 0.001$). However, $\beta 2m^{-/-}$ $\gamma\delta$T cells into $\delta TCR^{-/-}$

followed by VV infection resulted in similar percentages and cell count of B8R$^+$ and

IFN-γ^+ CD8$^+$ T cells as $\delta TCR^{-/-}$ with VV infection alone (Figure 6B-E; $P < 0.001$). This

suggests that $\gamma\delta$T cells also activate CD8$^+$ T cell via presentation of epitope on MHC-I

for CD8$^+$ T cell recognition.

Figure 6. CD8$^+$ T cells were obtained from splenocytes of OT-I$^+$ mice via magnetic-

activated cell sorting. (**A**) The CD8$^+$ T cells were labeled with Carboxyfluorescein

diacetate succinimidyl ester (CSFE) and measured for proliferation. The labeled cells

were co-cultured *in vitro* with OVA-I peptide plus LPS-matured dendritic cells or

VV-activated $\gamma\delta$T cells for 3 days, at a ratio of 1:1. Cells were harvested and stained

for CD8 and CD3e. Control CD8$^+$ T cells are incubated with OT-I peptide or $\gamma\delta$T cells

alone. They were subsequently assessed via flow cytometry for CSFE. (**B**, **C**) 5 x 10^6

pfu of VV was inoculated intraperitoneally into WT or $\delta TCR^{-/-}$ mice, with and

without adoptive transfer of 1 x10^6 cells of WT or $\beta 2m^{-/-}$ $\gamma\delta$T cells. 7 days post-

inoculation, splenocytes were stained for CD8$^+$ CD4$^-$ lymphocytes, and assessed for

B8R$^+$ and IFN-γ^+ CD8$^+$ T cells. (**B**) Representative FACS plots first gated on CD8$^+$

CD4$^-$ T cells and then plotted against B8R$^+$ and CD8$^+$ expression for B8R$^+$ CD8$^+$ T

cells. (**C**) Representative FACS plots first gated on CD8$^+$ CD4$^-$ T cells and then

assessed for IFN-γ expression. **(D, E)** Quantification of FACS plots. Values are mean

± SEM, representative of 3 independent studies. Kolmogorov-Smirnov

nonparametric t-test, *$P < 0.005$.
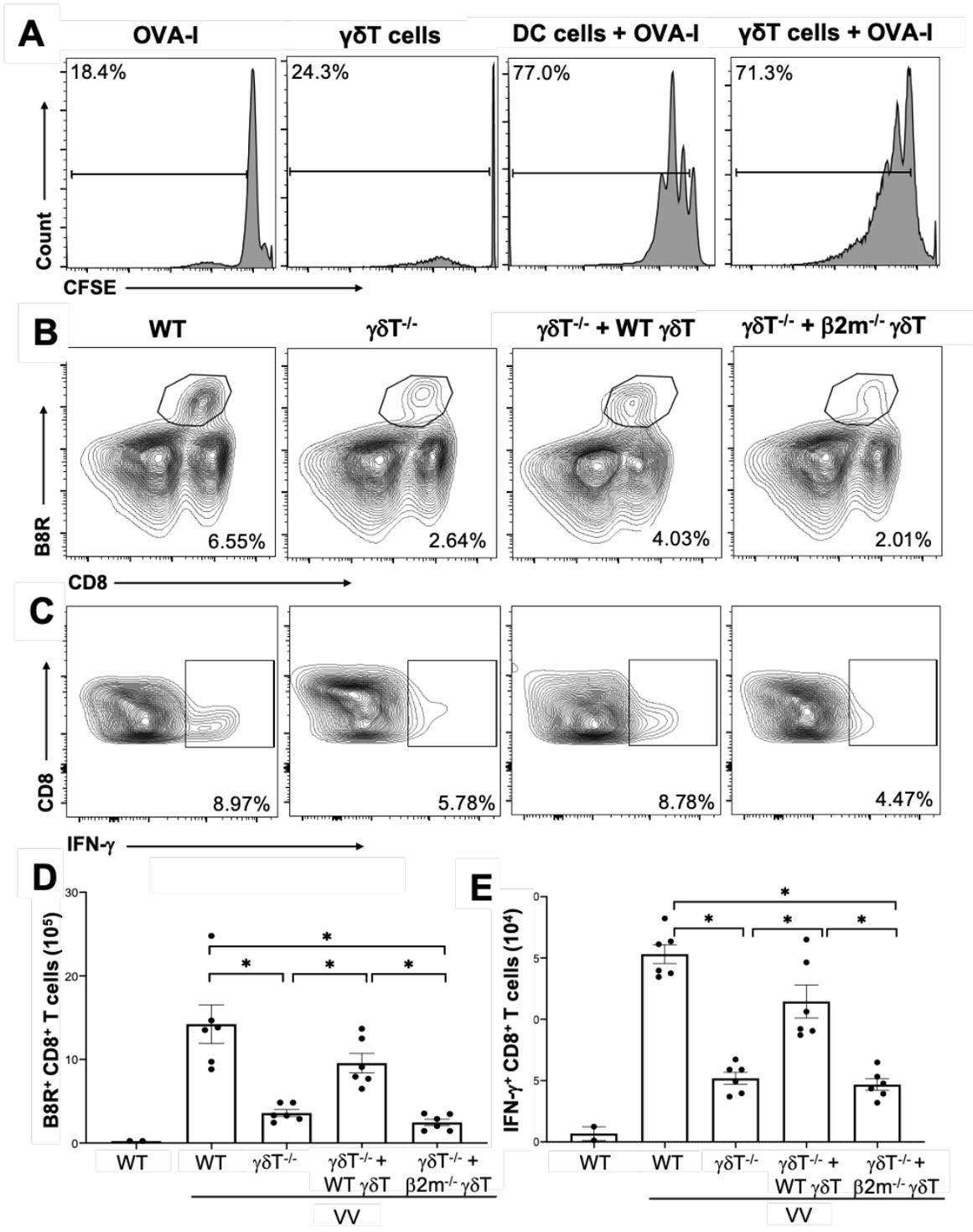
Figure 6 γδT cells directly activate CD8⁺ T cells via MHC-I

3.2.4 MyD88 signaling in γδT cells promotes CD8⁺ T cell response to VV

      To determine how VV activates γδT cells for antigen presentation, we screened

mice defective for innate signaling pathways and assessed for γδT cell proliferation and

IFN-γ⁺ γδT cell functional activation. VV was inoculated in WT, IFN-αβR$^{-/-}$, IFN-γR$^{-/-}$,

TNF-αR$^{-/-}$, and MyD88$^{-/-}$ mice and splenic cells were assessed via flow cytometry 4 days

afterwards. We found that there was a significant deficit in γδT cell proliferation and

IFN-γ secretion in MyD88$^{-/-}$ mice (Figure 7), but not in mice with other deficient signaling

pathways. This suggests that MyD88 signaling is required for VV activation of γδT cells.

Figure 7. Wild-type (WT), IFN-αβ receptor deficient (IFN-αβR$^{-/-}$), IFN-γ receptor

deficient (IFN-γR$^{-/-}$), MyD88 deficient (MyD88$^{-/-}$), and TNF- α receptor deficient

(TNF- αR$^{-/-}$) mice were inoculated with VV intraperitoneally and splenocytes were

harvested 4 days after inoculation. Harvested cells were gated for CD3e⁺ γδTCR⁺ T

cells and subsequently assessed IFN-γ positivity.
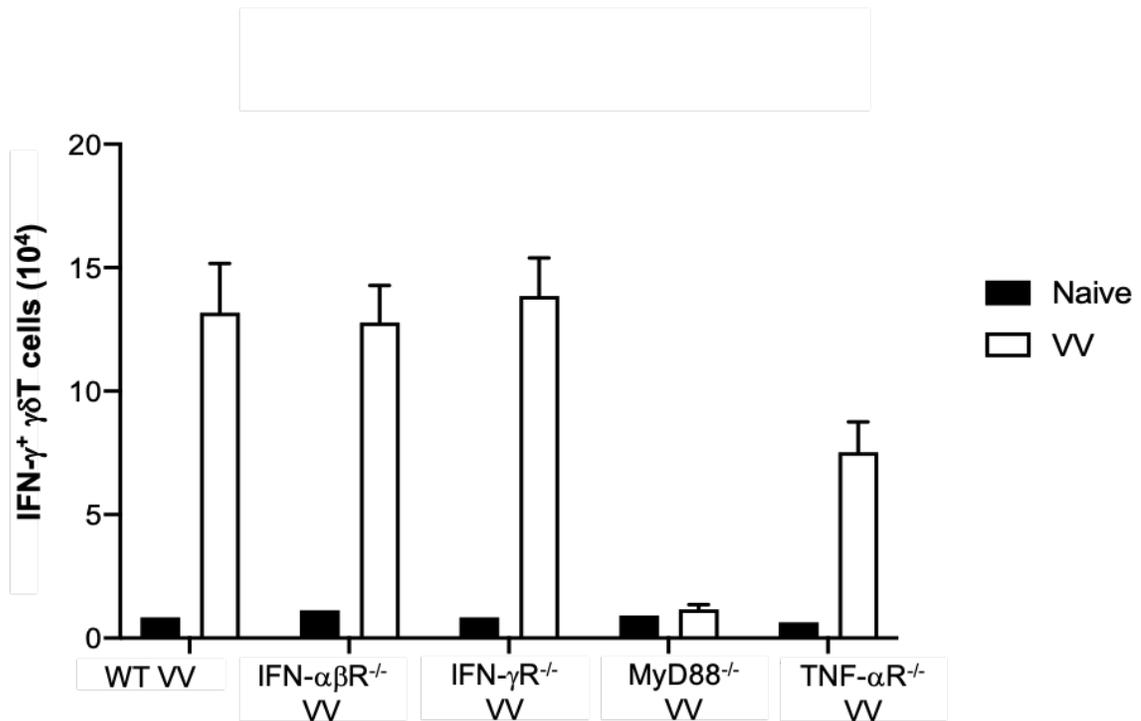
Figure 7 *In vivo* **screen for signaling pathways required for γδT cell activation following VV infection**

We next addressed if intrinsic MyD88 activation in γδT cells is sufficient for VV activation, opposed to signaling from other cells, we adoptively transferred WT γδT cells into MyD88$^{-/-}$ mice and assessed for γδT cell activation. We found that adoptive transfer of WT γδT cells into MyD88$^{-/-}$ mice is sufficient to rescue γδT cell proliferation and IFN-γ$^+$ secretion (Figure 8; P < 0.001), suggesting intrinsic MyD88 signaling is required for activation of γδT cells by VV.

Figure 8. 5×10^6 pfu of VV was inoculated into WT and *MyD88$^{-/-}$* mice with and without adoptive transfer of 1×10^6 cells of WT $\gamma\delta$T cells. Mice splenocytes were harvested 4 days after inoculation and assessed via flow cytometry. (**A**) Representative FACS plots of live cells plotted for CD3e$^+$ $\gamma\delta$TCR$^+$ splenocytes, 4 days after VV inoculation. (**B**) Representative FACS plots first gated on CD3e$^+$ $\gamma\delta$TCR$^+$ splenocytes, and then assessed for IFN-γ positivity. The gating for IFN-γ is determined against $\gamma\delta$T cells from control naïve mice for each experiment. (**C**) Summary graph of a representative experiment for positive CD3e$^+$ $\gamma\delta$TCR$^+$ T cells in mice, 4 days post-VV inoculation. (**D**) Summary graph of a representative experiment for IFN-γ^+ CD3e$^+$ $\gamma\delta$TCR$^+$ T cells in mice, 4 days post-VV inoculation. Representative of 3 independent experiments. Kolmogorov-Smirnov nonparametric t-test, *$P < 0.001$,
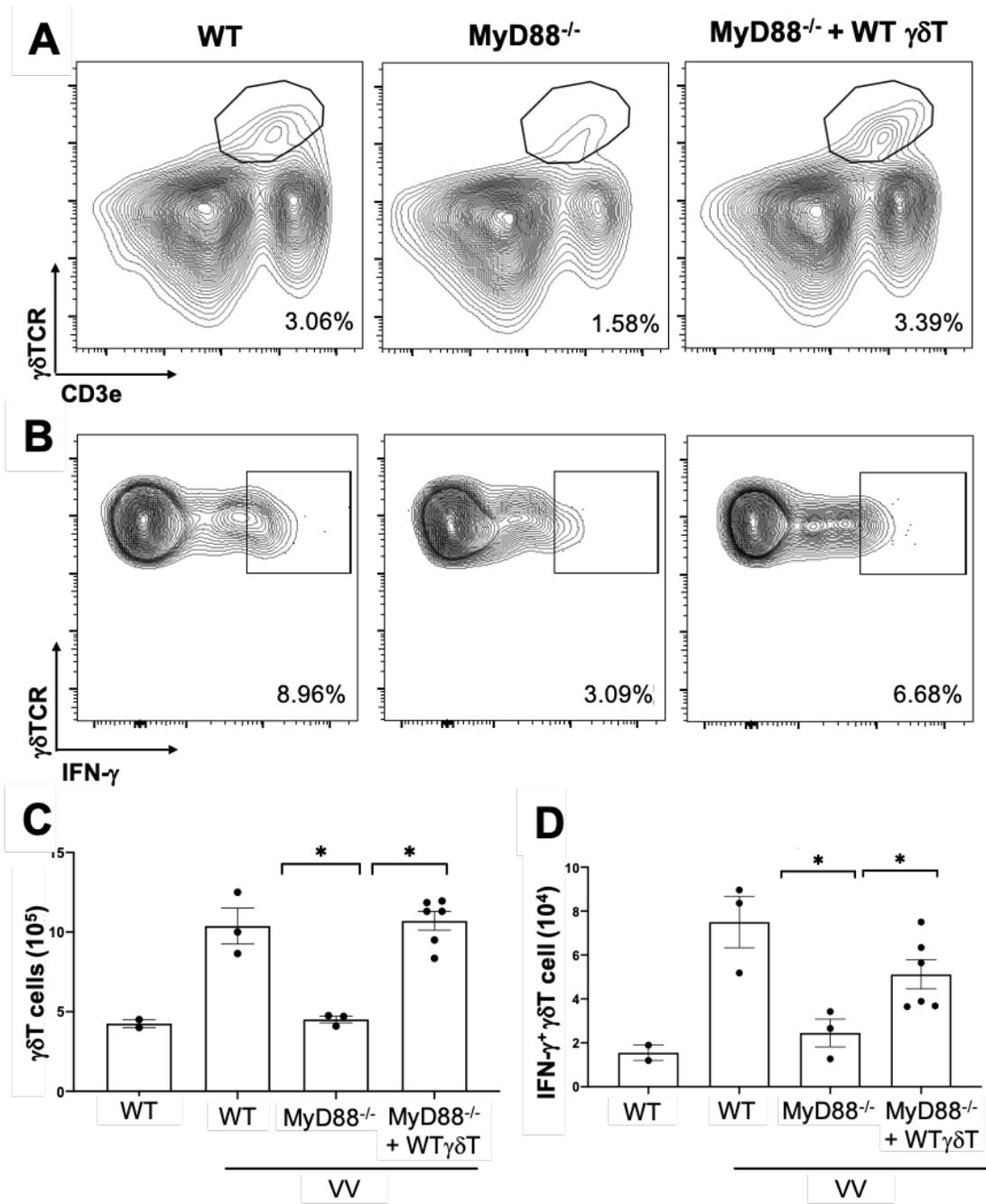
Figure 8 Intrinsic MyD88 signaling is required for activation of γδT cells by VV

We then investigated if MyD88 signaling in γδT cells is needed for subsequent CD8$^+$ T cell activation. We adoptively transferred WT or MyD88$^{-/-}$ γδT cells into $\delta TCR^{-/-}$ mice and found that there was similar γδT cell reconstitution (Figure 9A), but that was a significant decrease in VV-specific B8R$^+$ and IFN-γ$^+$ CD8$^+$ T cell proliferation in $\delta TCR^{-/-}$ adoptively transferred with MyD88$^{-/-}$ γδT cells when compared to that of WT γδT cells (Figure 9B-E; P < 0.005).

Figure 9. $\delta TCR^{-/-}$ mice were adoptively transferred with WT or $MyD88^{-/-}$ γδT cells and inoculated with VV. (**A**) 3 days after inoculation, there is no significant difference between $dTCR^{-/-}$ mice adoptively transferred with WT vs MyD88$^{-/-}$ γδT cells. 7 days after inoculation, splenocytes were obtained and stained for CD8$^+$ CD4$^-$ lymphocytes and plotted via flow cytometry for (**B**) B8R$^+$ CD8$^+$ T cells or (**C**) IFN-γ$^+$ CD8$^+$ T cells. (**D**) Representative FACS plots of splenocytes first gated on CD8$^+$ CD4$^-$ lymphocytes, and then plotted for CD8$^+$ B8R$^+$ T cells. (**E**) Representative FACS plots of splenocytes first gated on CD8$^+$ CD4$^-$ lymphocytes, and then assessed for IFN-γ positivity. Unpaired student T-test, *P < 0.005. Each panel is representative of 3 independent studies.
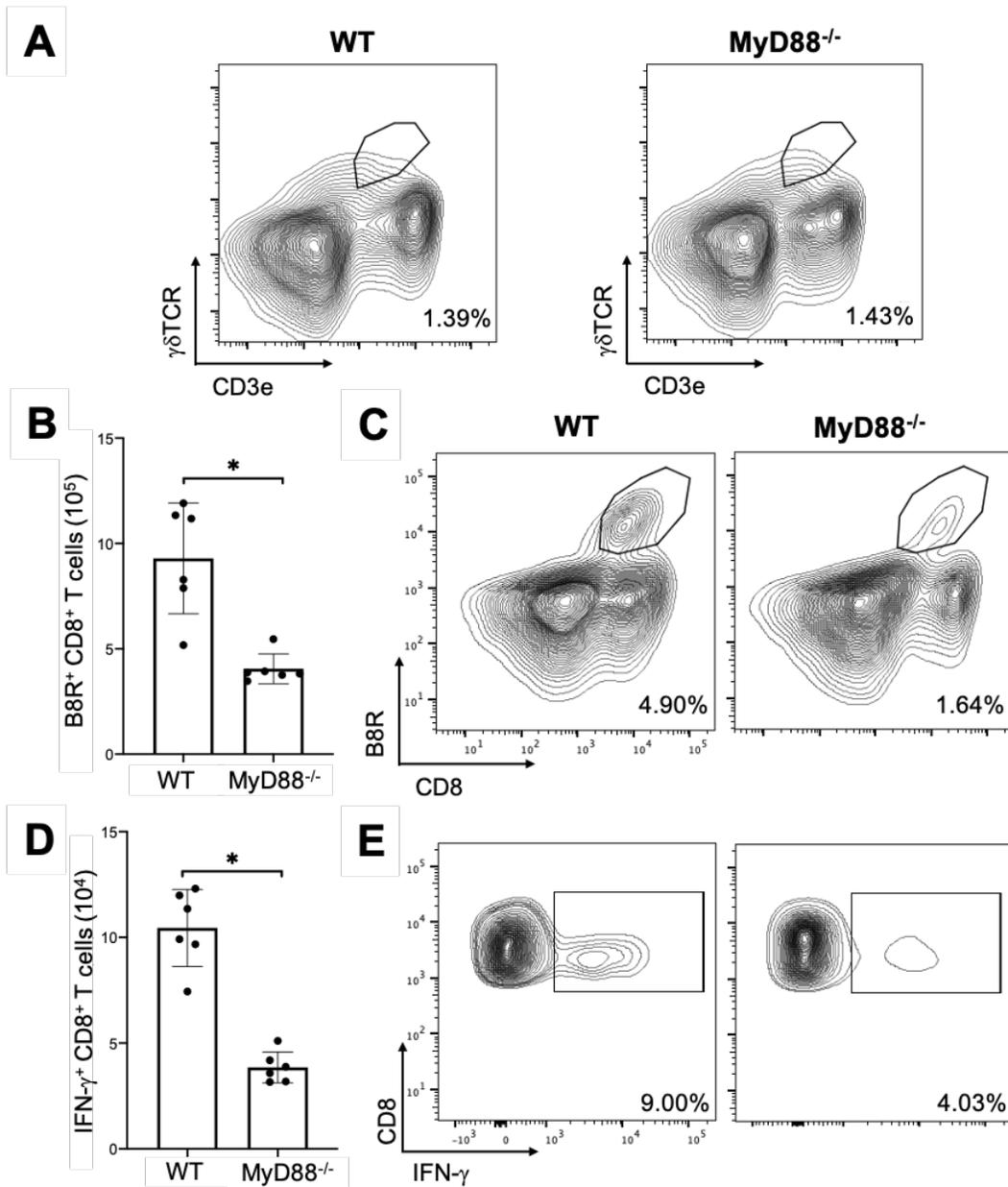
Figure 9 MyD88 is required for CD8+ T cell activation by γδT cells

To determine the downstream signaling responsible for interferon activation

by VV in γδT cells, we assessed qRT-PCR for IRF-1, 3, 4, 7, and IRF-8 in wild-type

and *MyD88^-/-* γδT cells. We found that there is a significant decrease in expression of

IRF-3, 4, and 8 in *MyD88^-/-* γδT cells, compared to that of wild-type γδT cells (Figure

10; $P < 0.001$). This suggests that activation of IRF-3, 4, and 8 transcription factors

require MyD88 signaling following VV infection.

Figure 10. 4 days following VV inoculation, γδT cells were isolated from splenocytes

from naïve and VV-inoculated WT mice with MACS. Total RNA is extracted from

the isolated cells and assessed by qRT-PCR for expression of IRF-3, 4, 7, and 8.

Unpaired student T-test, *$P < 0.005$.
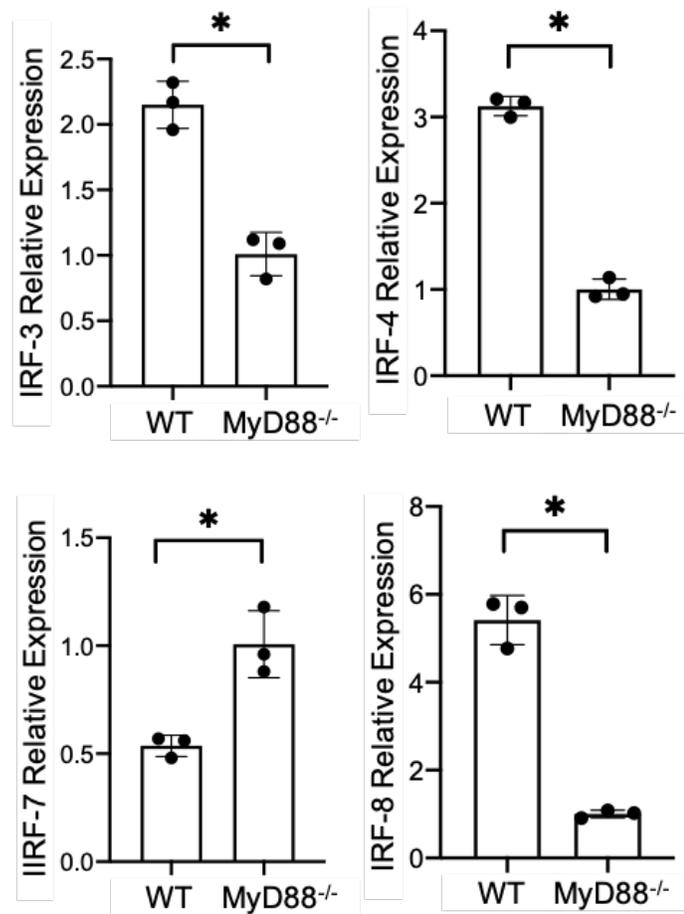
Figure 10 MyD88 deficiency in γδT cells influences downstream signaling

3.2 Discussion

Here we showed that VV can activate γδT cells via the MyD88 signaling

pathway. We further showed that VV-activated γδT cells can present antigens to

activate and induce VV-specific CD8+ T cell response. Our results further

demonstrate MyD88 has a critical role in VV activation of γδT cells to promote

specific CD8+ T cell response.

γδT cells represents approximately 0.7% of the peripheral blood and play an important role in the integration of the innate and adaptive immune system [6]. Previous studies have shown that although activation of NK cells is critical for the initial control of VV infection [4, 154], efficient activation of CD8^{+} T cell response is required for the eradication of VV infection [2-5]. What promotes the activation of CD8^{+} T cell response to VV infection remains incompletely defined. Understanding how γδT cells activate CD8^{+} T cells will better elucidate the mechanisms that govern CD8^{+} T cell activation and how to better employ them for future strategies in vaccination or immunotherapy.

γδT cells play an active role in the control of parasitic, bacterial, and viral infections, such as malaria, Listeria monocytogenes, Salmonella, EBV, and HSV [21, 46, 48, 145, 158, 159, 172, 173]. Unlike other innate immune cells, γδT cells require activation by various antigens prior to exhibiting cytotoxic characteristics [174]. Currently, most strategies that target γδT cells employ phosphoantigens to activate γδT cells. However, recent evidence suggests that phosphoantigen activation is nonspecific, and induce both inflammatory and anti-inflammatory functions in the targeted cells [29, 175-177]. This may be due to the numerous subpopulations of γδT cells that exert balancing functions against each other [36, 93, 178]. The goal is therefore to investigate a method that would preferentially activate one

subpopulation of γδT cells, however categorization of γδT cells remain controversial [24, 27]. Recent studies have shown that even γδT cells with the same γ and δ TCR chains appear to have distinctly different functions [32-35]. Given the long-lasting immunity that VV produce in clinical evaluations, understanding how VV activates γδT cells could provide insights into strategies to shifting γδT cells towards cytotoxicity immunity overall.

In this study, we demonstrate that γδT cells is required for the full activation of CD8+ T cells after VV infection. *δTCR*^{-/-} mice have deficient VV-specific CD8+ T cell proliferation and functional response. We find that deficiency of γδT cells results in over 3-fold decrease in CD8+ T cell response. Given previous studies that demonstrate γδT cells' influence on the immune system, it is possible that γδT cells may be directly responsible for CD8+ T cell activation following VV infection [37, 38, 42]. To investigate this possibility, we investigated whether VV upregulates the 3 conventional signals of CD8+ T cell activation in γδT cells. We found that following infection with VV-OVA, SIINFEKL peptide is present on MHC-I on the surface of γδT cells. We also found that there is a significant decrease in CD8+ T cell activation in *δTCR*^{-/-} mice adoptively transferred with deficient MHC-I γδT cells, compared to that transferred with wild-type γδT cells. Similarly, there is a significant increase in expression of CD86, IFN-γ, and IL-1 in γδT cells following VV infection for signals 2

and 3 that are required for CD8+ T cell activation. This suggests that γδT cells can provide all 3 signals necessary for CD8+ T cell activation, and that γδT cells directly influence CD8+ T cell activation following VV infection.

To determine how VV activates γδT cells for antigen presentation, we determined γδT cell activity in mice with deficiencies in IFN-αβ, IFN-γ, TNF-α, and MyD88 signaling. We found that only mice with deficient MyD88 signaling presented with impaired γδT cell response to VV infection. Subsequently, we investigated whether the γδT cell deficiency see in *MyD88-/-* mice are due to signaling from other cells that require MyD88 signaling or MyD88 in γδT cells alone. We found that *MyD88-/-* mice that were adoptively transferred with WT γδT cells exhibited normalized γδT cells response to VV. This indicates that VV activates γδT cells via MyD88-associated PRRs. Additionally, when *MyD88-/-* γδT cells are adoptively transferred to *δTCR-/-* mice, there is a deficient CD8+ T cell response. This means that MyD88 activation on γδT cells is required for CD8+ T cell response.

In conclusion, our study reveals that VV activates γδT cells via MyD88 signaling pathway, which leads to direct antigen presentation to CD8+ T cells. *In vivo*, this bridge between the innate and adaptive immune pathways plays a critical role in the activation of CD8+ T cell response to VV. Furthermore, we demonstrate that cell-intrinsic MyD88 signaling in γδT cells is required for activation of CD8+ T

cells. These results demonstrate a critical role for γδT cells in the regulation of

adaptive T cell response to viral infection and may shed light on the design of more

effective vaccine strategies based on manipulation of γδT cells.

Chapter 4. NKG2D activation is required for γδT cell activation

4.1 Introduction

NKG2D, encoded by *Klrk1*, is a type II transmembrane activating receptor

expressed on cytotoxic cells, including CD8^{+} T cells, NK cells, macrophages, and γδT

cells [179-181]. Previous studies have found that NKG2D plays an important role in the

recognition and elimination of cells under stress, specifically tumor cells [182], virally

infected cells [183], and organ transplants [184]. NKG2D ligands, MICA/B and Rae1

respectively in humans and mice, are almost exclusively upregulated in abnormally

stressed cells, such as tumor cells [185-188] and virally infected cells [189]. MICA/B and

Rae1 are not expressed in most normal cells of postnatal humans or mice [180].

Upregulation of MICA/B and Rae1 in tumor cells is associated with increased immune

response in humans and mice [186, 187]. Tumor cells with high NKG2D expression are

attacked by NK cells *in vitro* [72], with subsequent rapid development of protective CD8^{+}

T cell immunity [190]. This makes activating NKG2D particularly favorable for targeting

tumors and eliminating viral infections. However, the role of NKG2D in various cell

types in the context of the entire immune system has not been fully elucidated.

Vaccinia virus (VV) is a poxvirus that produces one of the longest cellular

immunity in humans and is responsible for the elimination of smallpox worldwide [12,

127]. Previous findings have demonstrated that NK cells are required for initial immune

response against VV infection [4, 154, 191], however αβT cells is required for the complete elimination of VV [2]. NKG2D is integral in the immunity against VV [192], however the specific activation of NKG2D in different cell types by VV has not been completely delineated.

Activation of NKG2D in CD8^{+} T cells is associated with enhanced TCR activation and function, cytotoxicity, and enhanced T cell survival [72, 187, 193-195], with subsequent CD8^{+} T cell antitumor immune surveillance [196, 197], and viral anti-immunity [189, 198]. Inhibition of NKG2D in NK cells is also associated with immune evasion by virus [183, 199, 200]. However, the contribution of NKG2D activation in CD8^{+} T cells, NK cells, and γδT cells against viral infection is not well understood. The role of NKG2D expression in different cytotoxic cells in viral immunity remains to be elucidated.

γδT cells is a small, but important, population of T cells that differ from conventional CD4^{+} or CD8^{+} αβT cells in that they do not require presentation by conventional antigen-presentation cells for activation. Instead, they are able to act as antigen-presenting cells and present antigens directly to CD8^{+} T cells [38, 42]. At the same time, γδT cells are capable of exerting direct cytotoxicity against virally infected [201-204] and tumor cells [205-207]. Previous studies have found that γδT are an important component of cancer immunosurveillance [208] and immunity against intracellular bacteria [21, 46] and viruses [172, 201-204, 209].

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Targeting NKG2D in specific cell types is important for developing vaccination strategies against viruses and tumors. In this study, we demonstrate that NKG2D expression in αβT cells and NK cells are important for immune response to VV but is not able to explain the full role of NKG2D in viral immunity. We find that NKG2D expression in γδT cells plays a significant role in viral immunity in the absence of NKG2D expression in αβT and NK cells. This provides significant insight into potential strategies for future vaccine development.

4.2 Results

4.1.1 NKG2D on αβT cells and NK cells partially contributes to VV clearance

Studies have shown that activation of NKG2D receptors is important for NK cell activation and VV clearance [191]. To determine the effect of NKG2D activation on NK and αβT cells for VV clearance *in vivo*, we assessed VV viral load 3 days post-inoculation in mice with deficient NKG2D in αβT cells (*LckCre+ NKG2D^{F/F}*; NKG2D^{ΔT}), NK cells (*NKp46Cre+ NKG2D^{F/F}*; NKG2D^{ΔNK}), and both (*LckCre+ NKp46Cre+ NKG2D^{F/F}*; NKG2D^{ΔT/ΔNK}). We found that following VV inoculation, there is no significant difference in viral load change between NKG2D^{ΔT}, NKG2D^{ΔNK}, or NKG2D^{ΔT/ΔNK} mice. Furthermore, we inoculated NKG2D^{ΔT/ΔNK} mice with VV and anti-NKG2D neutralizing antibody. We found that there was a significant difference

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in VV viral load between mice injected with anti-NKG2D neutralizing antibody versus NKG2D$^{\Delta T/\Delta NK}$ mice with deficient NKG2D in only NK and T cells (Figure 11). This suggests that other cells besides NK and T cells contribute to viral clearance following activation of NKG2D receptor.

Figure 11. Mice with deficient NKG2D in αβT cells and/or NK cells were inoculated with 5x10^6 pfu of VV intraperitoneally. 3 days post-VV inoculation, the intraperitoneal cavity was washed with 1xPBS and assessed for viral load via quantitative real-time PCR (qPCR) for VV gene E3L (**A**). Mice with deficient NKG2D in both αβT cells and NK cells (*LckCre$^+$ NKp46Cre$^+$ NKG2D$^{F/F}$*, NKG2D$^{\Delta T/\Delta NK}$), with and without complete neutralization with anti-NKG2D antibody, were inoculated with 5x10^6 pfu of VV intraperitoneally. 3 days post-VV inoculation, the intraperitoneal cavity was washed with 1xPBS and assessed for viral load via (**B**) qPCR for VV gene E3L and (**C**) plaque assay. Values are mean ± SEM, representative of 3 independent studies. Kolmogorov-Smirnov nonparametric t-test, *$P < 0.01$.
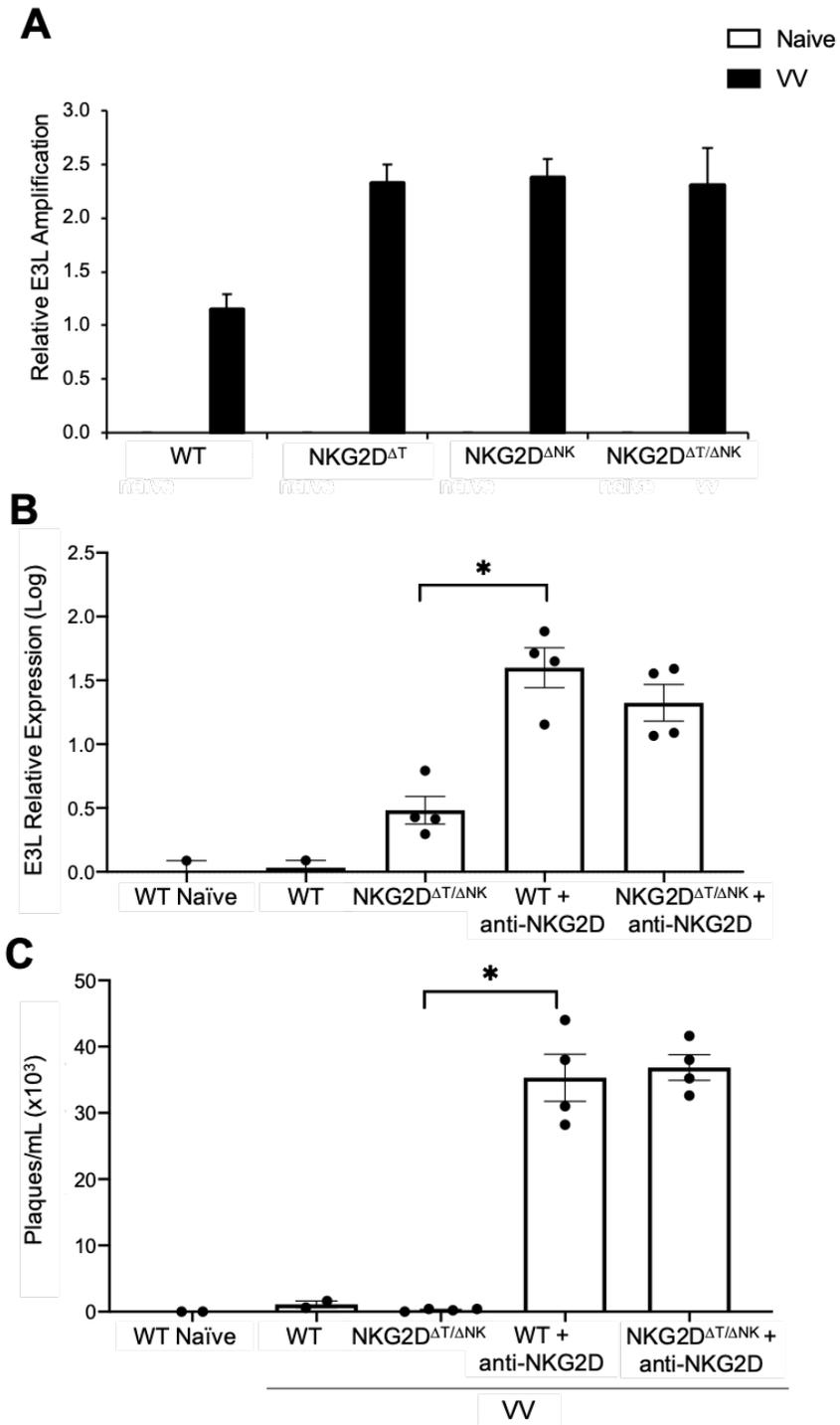
Figure 11 NKG2D on αβT cells and NK cells partially contributes to VV clearance

4.1.2 γδT cells are important for VV clearance

We suspected that γδT cells may contribute to VV viral clearance, and found that kinetically, γδT cells are activated by VV around 4 days post-inoculation, compared to NK cells, which are maximally activated around 2-3 days post-inoculation (Figure 12A, B). We subsequently inoculated γδT cell-deficient mice ($\delta TCR^{-/-}$; γδT$^{-/-}$) and found that there is a significant increase in VV viral load compared to that of wild-type C57BL/6 (WT) mice. Following adoptive transfer of WT γδT cells, we found that the deficiency in VV clearance can be rescued with the adoptively transferred cells (Figure 12C, D). This suggests that γδT cells are important for VV clearance.

Figure 12. Flow cytometry of NK cells and IFN-γ$^+$ γδT cells, of naïve (day 0) and day 2-8 after intraperitoneal (i.p.) injection of 5x10^6 pfu of VV into wild-type C57BL/6 mouse (WT). Splenocytes were extracted from the (**A**) spleen and (**B**) site of inoculation with 1xPBS lavage of the peritoneal cavity and gated on CD3e$^+$ γδTCR$^+$ γδT cells and assessed for IFN-γ positivity via flow cytometry. (**C, D**) WT and γδT cell-deficient mice ($\delta TCR^{-/-}$, γδT$^{-/-}$) with or without adoptive transfer of WT γδT cells were inoculated with VV intraperitoneally. 3 days post-inoculation, the intraperitoneal cavity was wash with 1xPBS, and assessed for viral load via (**C**)

qPCR for E3L VV gene and (**D**) plaque assay. Values are mean ± SEM, representative

of 3 independent studies. Kolmogorov-Smirnov nonparametric t-test, *P < 0.01.

Figure 12 γδT cell is important for VV clearance

4.1.3 NKG2D expression is important for VV activation of γδT cells and VV clearance

To determine if NKG2D is important for activation of γδT cells, we analyzed γδT cells expansion and IFN-γ secretion 4 days following VV inoculation in WT and NKG2D$^{ΔT/ΔNK}$ mice with and without anti-NKG2D neutralizing antibodies. We found that there is an increase in γδT cells proliferation and activation in NKG2D$^{ΔT/ΔNK}$ mice, that is abolished with injection of anti-NKG2D antibodies (Figure 13). This suggests that VV activation of NKG2D is important for γδT cells activation.

Figure 13. Mice with deficient NKG2D in αβT cells and NK cells, with and without complete neutralization with anti-NKG2D antibody, were inoculated with 5x10^{6} pfu of VV intraperitoneally. 4 days post-VV inoculation, splenocytes were isolated and gated for (**A, C**) CD3e^{+} γδTCR^{+} γδT cells and assessed for (**B, D**) IFN-γ positivity. Representative FACS flow plots for (**A**) CD3e^{+} γδTCR^{+} γδT cells and (**B**) IFN-γ secretion. Values are mean ± SEM, representative of 3 independent studies. Kolmogorov-Smirnov nonparametric t-test, *$P < 0.05$.
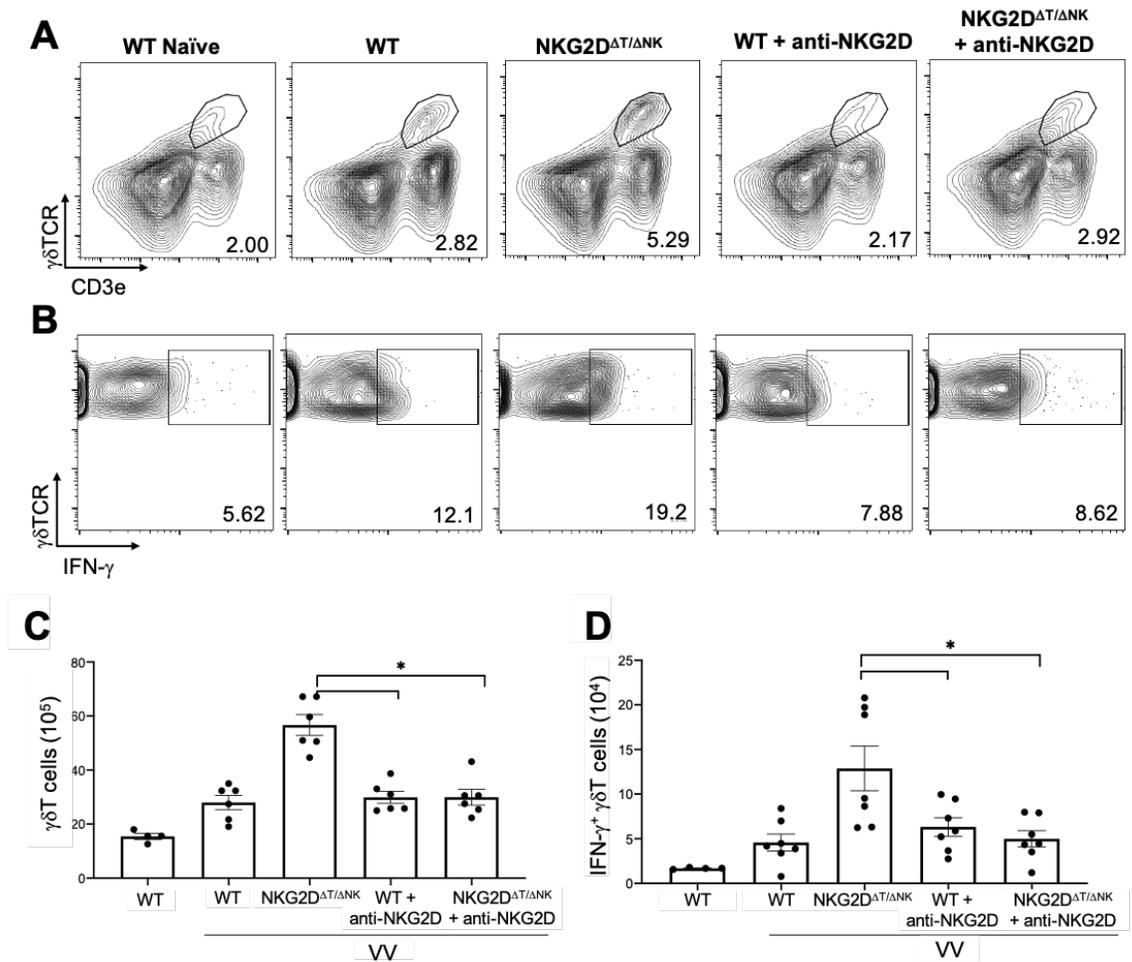
Figure 13 NKG2D expression on γδT cells is important for γδT cells activation by VV

Subsequently, to determine if γδT cell activation is impaired in NKG2D^{-/-} mice. We investigated if there is a deficiency in γδT cell proliferation and activation in NKG2D^{-/-} mice, compared to that of WT mice. We found that NKG2D^{-/-} γδT cells is associated with decreased IFN-γ expression following VV inoculation (Figure 14A-D). Similarly, we find that there is an impairment in VV viral clearance in NKG2D^{-/-}

mice compared to that of WT mice (Figure 14E). This indicates that deficiency of

NKG2D inhibits γδT cells proliferation and activation following VV inoculation.

Figure 14. Mice with deficient NKG2D in all cells (*RosaCre$^+$ NKG2D$^{F/F}$*, NKG2D$^{-/-}$),

and WT mice were inoculated with 5x10^6 pfu of VV intraperitoneally. 4 days post-

inoculation, splenocytes were harvest and gated on CD3e$^+$ γδTCR$^+$ γδT cells and

assessed for IFN-γ$^+$ secretion. (**A, B**) Representative flow cytometry plots of CD3e$^+$

γδTCR$^+$ γδT cells and IFN-γ$^+$ γδT cells in WT and NKG2D$^{-/-}$ mice. (**C, D**)

Quantification of γδT cells and IFN-γ$^+$ γδT cells from WT and NKG2D$^{-/-}$ mice with

and without VV inoculation. (**E, F**) 3 days post-VV inoculation, the peritoneum from

WT and NKG2D$^{-/-}$ mice were washed with 1xPBS and assessed for VV load via

plaque assay (**E**) and E3L qPCR (**F**). Values are mean ± SEM, representative of 3

independent studies. Kolmogorov-Smirnov nonparametric t-test, *$P < 0.005$.
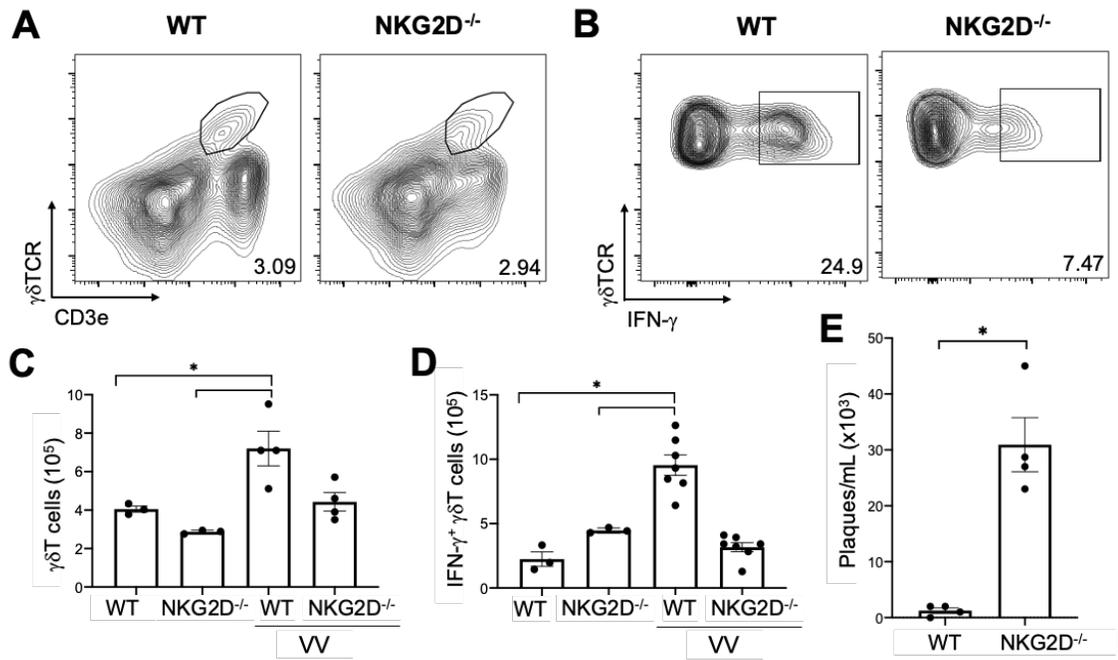
Figure 14 NKG2D is important for VV clearance

To assess if NKG2D expression on γδT cells is responsible for VV clearance,

we then adoptively transferred NKG2D$^{-/-}$ γδT cells into γδT$^{-/-}$ mice, following VV

inoculation, and assessed for viral clearance. We found that adoptive transfer of

NKG2D$^{-/-}$ γδT cells is unable to sufficiently control VV viral load compared to that of

WT γδT cells (Figure 15). This suggests that NKG2D expression in γδT cell is

important for in vivo VV clearance.

Figure 15. WT and γδT$^{-/-}$ mice were inoculated with 5x10^6 pfu of VV

intraperitoneally, with or without adoptive transferred of WT γδT cells or NKG2D$^{-/-}$

γδT cells. 3 days post-VV inoculation, the peritoneum cavity was washed with 1xPBS

and assessed for viral load via plaque assay. Values are mean ± SEM, representative

of 3 independent studies. Kolmogorov-Smirnov nonparametric t-test, *$P < 0.005$.

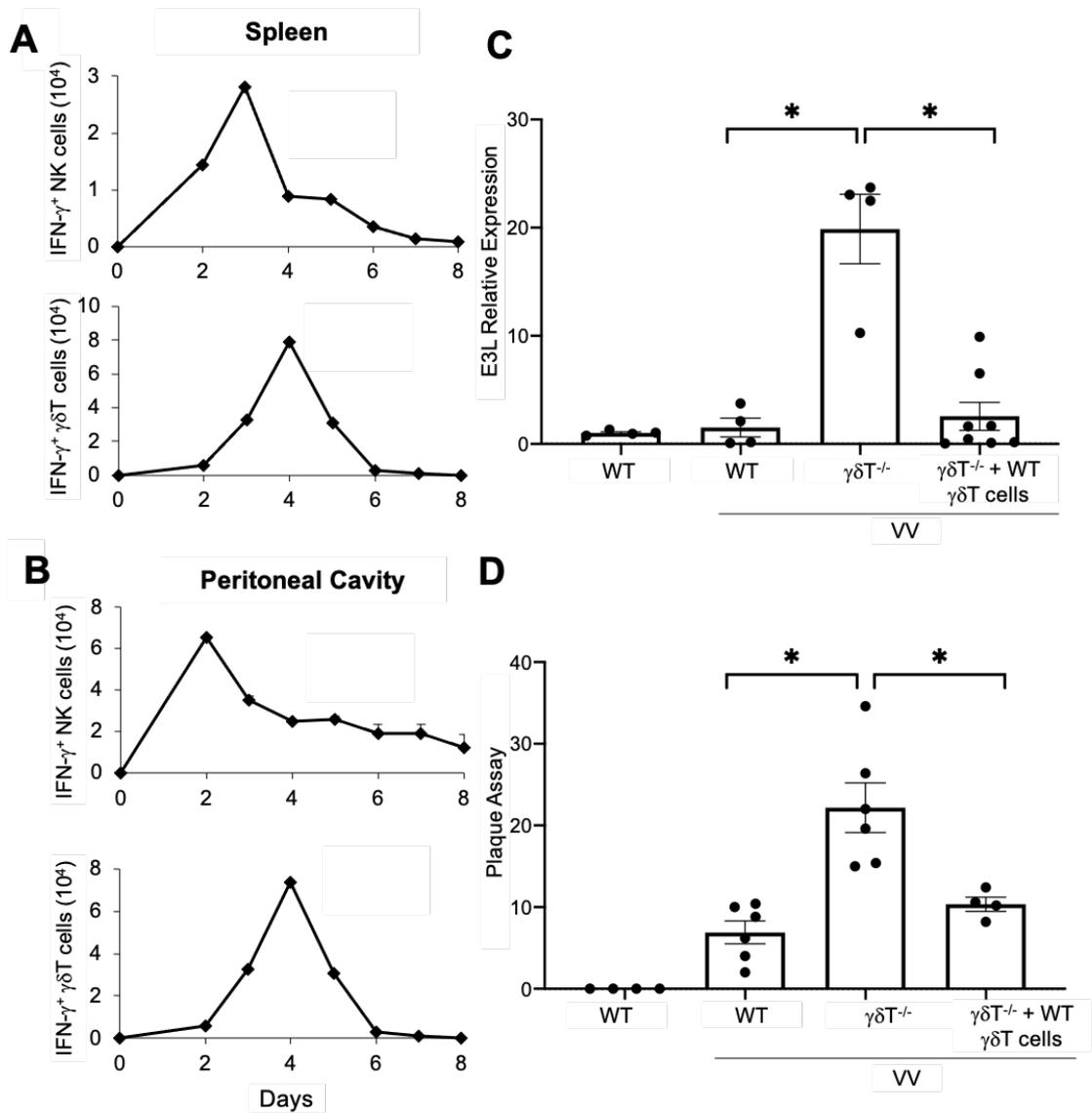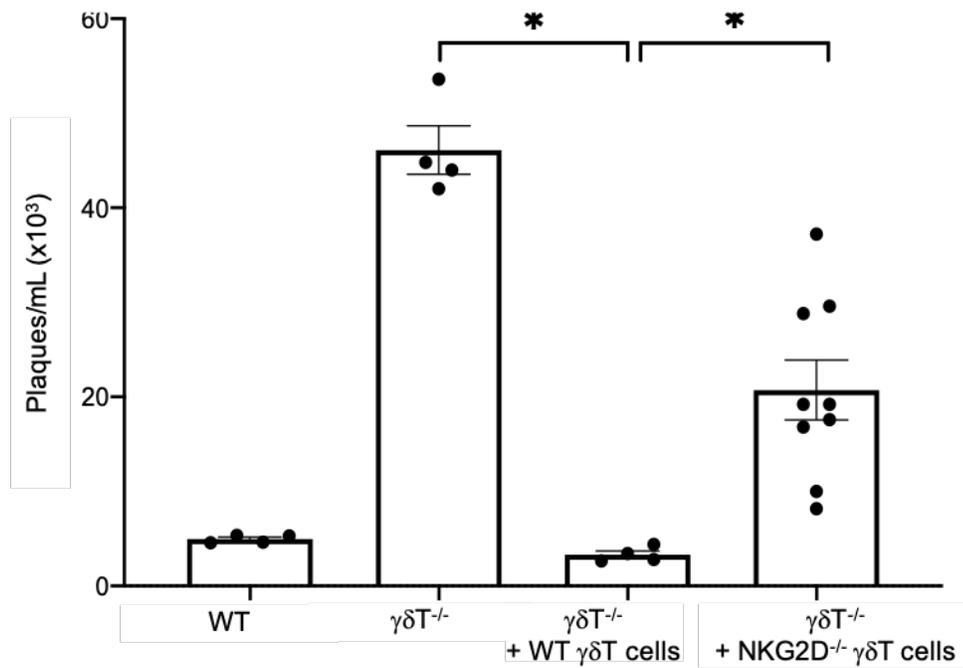**Figure 15 γδT cell activation by NKG2D is important for VV clearance**

4.3 Discussion

Because of the selective expression of NKG2D ligands in stressed cells that

have been infected or are cancerous, NKG2D is a highly favorable target for

development of immunotherapy against viral infections and cancers. Previous studies have illustrated that activation of NKG2D in CD8^{+} T cells and NK cells is responsible for cytotoxicity against virally infected and cancerous cells. However, the contribution of NKG2D in different cell types against viral infections has not been fully elucidated.

Previous studies have found that expression of NKG2D contributes to γδT cells tumor immunosurveillance in both humans [72, 210] and mice [17, 70]. Here, we demonstrated that NKG2D expression in CD8^{+} T cells and NK cells is important for VV clearance but is not able to explain the role of NKG2D in viral immunity. Inhibition of NKG2D by anti-NKG2D antibody or in mice with complete knockout of NKG2D results in greater deficiency of VV clearance than just depletion of NKG2D in CD8^{+} T cells and NK cells alone. We found that adoptive transfer of γδT cells with deficient NKG2D into *δTCR*^{-/-} mice results in significantly decreased VV clearance, compared to that of adoptive transfer with wild-type γδT cells. This suggests that expression of NKG2D in γδT cells also plays an important role in VV clearance.

γδT cells are an important but understudied population of immune cells. They represent approximately 0.7% of the peripheral blood and play an important role in the viral clearance [6]. They exhibit characteristics of both innate and adaptive

immune systems. Like conventional αβT cells, but unlike other innate immune cells, γδT cells require activation for cytotoxicity [174]. However, unlike αβT cells, γδT cells do not require antigen presentation for TCR activation [211]. The rules that govern γδT cell activation remains an area of active investigation [212]. Here we found that activation of NKG2D in γδT cells is crucial for γδT cell activation and subsequent VV clearance. VV activation of NKG2D is important for γδT cells secretion of IFN-γ in response to VV infection.

In conclusion, our study reveals that activation of NKG2D in γδT cells is important for activation and subsequent VV clearance. This suggests that NKG2D expression in γδT cells is able to compensate for NKG2D deficiency in CD8+ T cells and NK cells, and that selective deficiency of NKG2D in γδT cells is sufficient to hinder immune clearance of VV. These results demonstrate a critical role for NKG2D in γδT cells in the immunity against viral infection and may shed light on the design of more effective vaccine strategies based on the manipulation of γδT cells.

Chapter 5. Activation of CD8⁺ T cells

5.1 Introduction

Following primary infections, the achieved immunity against the attacking agent is mediated mainly through memory cells. CD8⁺ memory T cells is responsible for protective memory immunity against many viral infections and tumor antigens. Understanding the molecular mechanisms that underly the process of CD8⁺ memory T cell development remains one of the biggest hurdles in vaccination efforts for infectious diseases and tumor immunotherapy [213, 214].

After initial infection, most activated antigen-specific CD8⁺ T cells undergo apoptosis. Only a small population of activated T cells survive to differentiate into CD8⁺ memory T cells. This process is composed of mainly three phases: expansion, contraction, and stabilization. Naïve CD8⁺ T cells are first activated by antigen presenting cells (APC) that present antigen peptides on major histocompatibility complex class I (MHC-I) with costimulatory activators. In this initial expansion phase, a heterogenous population composed mainly of CD8⁺ effector T cells (T_{EFF}) proliferate to fight against the infection. Afterwards, during the contraction phase, 90-95% of all CD8⁺ T cells that compose the peak of expansion undergo apoptosis, leaving only a small population composed of memory T cell precursors to persist long term [215, 216]. After approximately 40 days, a stable population of memory T

cells form. However how this critical population of T cells is formed remains incompletely understood [217].

Furthermore, there are several subpopulations of memory T cells. Of which, central memory T cells (TCM) are considered to have the longest longevity and strongest response upon secondary stimulation [103, 105]. The signals that are responsible for TCM differentiation are also similarly not fully delineated.

Notch is a highly conserved molecular pathway integral to many binary cell-fate differentiation points during T cell development [218]. Ligand-mediated activation of the receptor leads to a series of proteolytic cleavages to result in the release of Notch intracellular domain (NICD). Once release, NICD enters the nucleus and binds to other transcription factors for the expression of target genes. Previous studies have found that Notch plays a central role in the differentiation of CD8^{+} TEFF [219]. Signals that induce differentiation of CD8^{+} TEFF upregulate Notch signaling, and corresponding activation of Notch signaling increases differentiation of the CD8^{+} TEFF populations. In mice with deficient Notch1 and Notch2 in CD8^{+} T cells, there is also a correlated decrease in IFN-γ response to viral infections. Further analysis of Notch1 and Notch2 deficient CD8^{+} T cells indicate that there may be an equivalent increase in expression of TCM-specific genes [220-222]. This suggests that

inhibition of Notch could preferentially decrease differentiation of T$_{EFF}$ in favor of CD8$^+$ memory T cells, specifically T$_{CM}$.

To further understand the mechanisms behind this process, we found that Notch1-specific deficiency in T cells is associated with increased population of CD8$^+$ T$_{CM}$ following primary infection. Upon rechallenge, we found that mice with Notch1, but not Notch2, deficient T cells have greater antigen-specific secondary response. After examining potential downstream mediators, we discovered that Notch1-specific deficiency is associated with increased expression of Transcription Factor 1 (TCF1 or *Tcf7*), a major mediator of memory T cell differentiation [223]. ChIP analysis of NICD binding indicates that Notch1 could mediate *Tcf7* mRNA expression by occupying a potential proximal enhancer. This modulation could be further abrogated with a small molecule gamma-secretase inhibitor (GSI) that prevents Notch activation. Our findings indicates that Notch1 is targetable mediator of CD8$^+$ memory T cells differentiation, by modulating the expression of memory T cell transcription factor TCF1.

5.2 Results

5.2.1 Deficiency of Notch1, but not Notch2, is associated with increased Vaccinia virus-specific B8R$^+$ CD8$^+$ T cells.

To determine if deficiency of Notch1 or Notch2 is associated with increase in antigen-specific response, we inoculated mice intraperitoneally with 5×10^9 pfu of Vaccinia virus (VV) and assessed CD8$^+$ T cell response at 1, 2, 3, and 7 weeks post-infection. Using MHC tetramer staining for B8R (TSYKFESV), a VV-specific epitope, we were able to evaluate the population of CD8$^+$ T cells that were activated by VV infection. We found that at 1 week post-inoculation, mice with T cell deficiency in Notch1 (Notch1$^{\Delta T}$) had a smaller VV-specific B8R$^+$ CD8$^+$ T cell population than that of wild-type (WT) mice or mice with T cell deficiency in Notch2 (Notch2$^{\Delta T}$; Figure 16, Figure 17A). This difference is abrogated by week 2 and then reversed by week 3. By 7 weeks post-inoculation, Notch1$^{\Delta T}$ mice had significantly more B8R$^+$ CD8$^+$ T cells compared to that of WT or Notch2$^{\Delta T}$ (Figure 16, Figure 17B).

Figure 16. Mice with deficient Notch1 and Notch2 in T cells (*LckCre$^+$ Notch1$^{F/F}$*, Notch1^{DT}; *LckCre$^+$ Notch2$^{F/F}$*, Notch2^{DT}) are inoculated with 5×10^6 pfu of VV, and assessed for **(A)** VV-specific B8R$^+$ CD8$^+$ T cells and **(B)** activated CD8$^+$ T cell

production of IFN-g at 7, 14, 21, and 49 days post-VV inoculation via flow

cytometry.
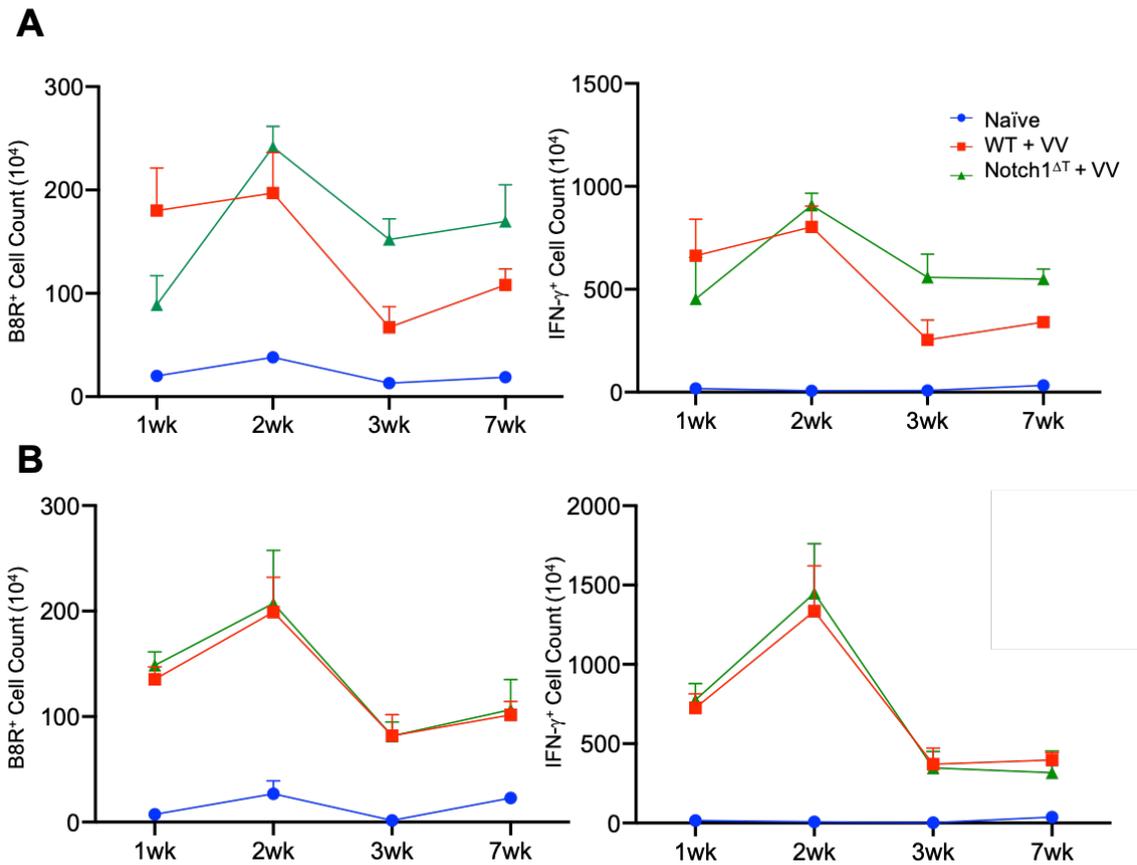
Figure 16 Notch1^{ΔT} mice exhibit prolonged VV-specific CD8+ T cell response

Figure 17. Representative flow cytometry plots of VV-specific B8R⁺ CD8⁺ T cells at

(A) 7 days and **(B)** 49 days post-VV inoculation. Representative flow cytometry plots

of IFN-g⁺ CD8⁺ T cells at **(C)** 7 days and **(D)** 49 days post-VV inoculation Values are

mean ± SEM, representative of 3 independent studies. Kolmogorov-Smirnov

nonparametric t-test, *$P < 0.05$.
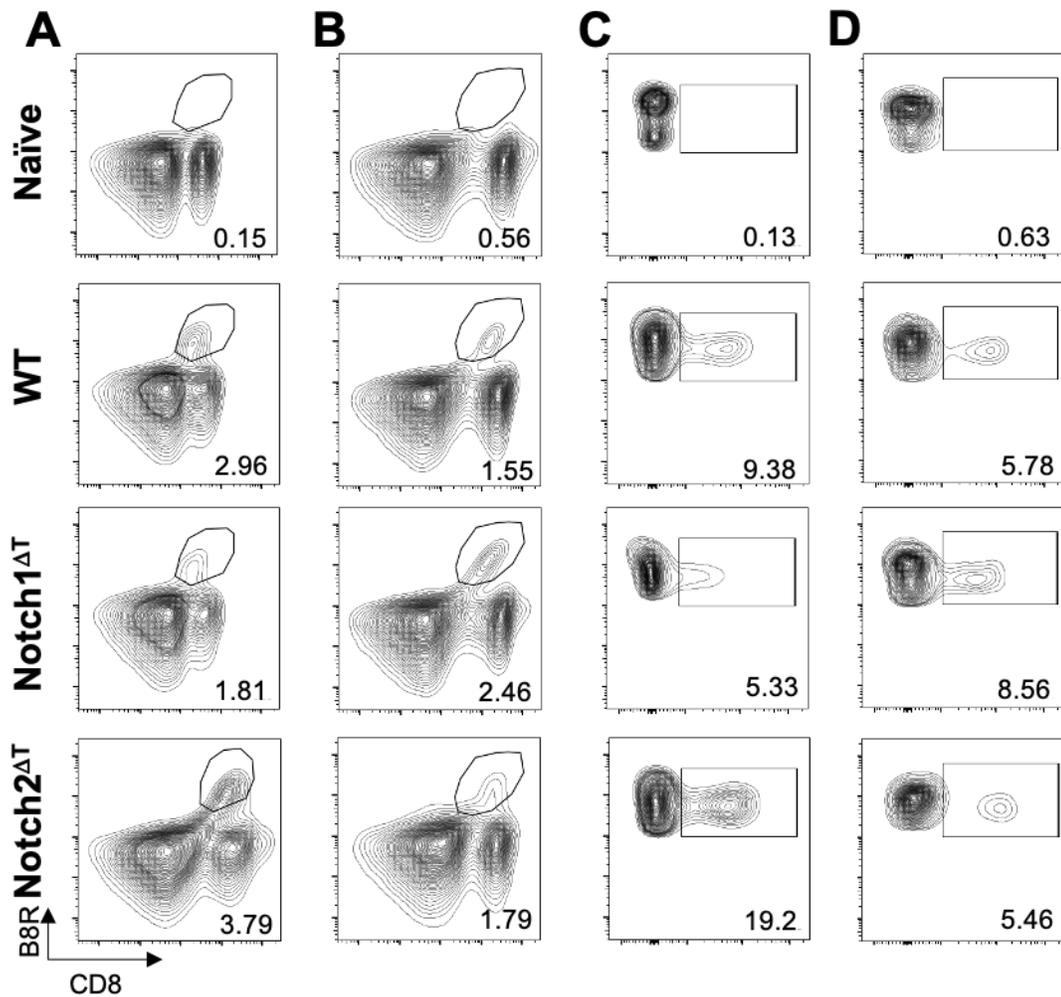
Figure 17 Representative flow cytometry plots

Next, we investigated whether the difference in VV-specific population of B8R$^+$ CD8$^+$ T cells in Notch1$^{\Delta T}$ compared to WT and Notch2$^{\Delta T}$ translated functionally to IFN-γ secretion over time. At 1, 2, 3, and 7 weeks post-inoculation, we isolated cells from WT, Notch1$^{\Delta T}$, and Notch2$^{\Delta T}$ mice and stimulated the cells with B8R (TSYKFESV) peptide for 5 hours with Brefeldin A. We then assessed the cells' functional production of IFN-γ via intracellular staining in response to stimulation. We found that similar to the population number of B8R$^+$ CD8$^+$ T cells, there were fewer IFN-γ^+ CD8$^+$ T cells and less IFN-γ production in Notch1$^{\Delta T}$ mice compared to that of WT or Notch2$^{\Delta T}$ at 1 week post-inoculation (Figure 16, Figure 17C). The difference disappears by week 2 and reverses by week 3. At week 7 (49 days) post-infection, Notch1$^{\Delta T}$ mice demonstrated significantly more IFN-γ^+ CD8$^+$ T cells compared to that of WT or Notch2$^{\Delta T}$ (Figure 16, Figure 17D). This suggests that deficiency of Notch1 in T cells increases not only antigen-specific population of CD8$^+$ T cells after contraction, but also have functionally greater IFN-γ^+ CD8$^+$ T cells.

5.2.2 Deficiency of Notch1, but not Notch2, is associated with greater response to rechallenge with secondary infection

By definition, the hallmark capability of memory T cells is its ability to mount a potent recall response upon secondary exposure to antigen and serves as the gold standard in measuring memory cell formation and functionality. To investigate

whether Notch1 or Notch2 deficiency in T cells impacts this ability, we rechallenged

VV-inoculated mice with 2×10^9 pfu of adenovirus encoding B8R (AdB8R) at 6 weeks

(42 days) post-VV infection. At 7 weeks post-VV, or 1 week (7 days) post-AdB8R

infection, we analyzed the mice for VV-specific B8R$^+$ CD8$^+$ T cells and found that

there is a significantly larger population of B8R$^+$ CD8$^+$ T cells in Notch1$^{\Delta T}$ than WT or

Notch2$^{\Delta T}$ (Figure 18A, C). This indicates that Notch1, but Notch2, deficiency in T

cells is associated with greater recall response to secondary rechallenge.

This conclusion is further solidified with functional IFN-γ production

analysis. After stimulation with B8R peptide, intracellular staining for IFN-γ in CD8$^+$

T cells from AdB8R rechallenged Notch1$^{\Delta T}$ mice is significantly greater at 7 weeks

than that of WT and Notch2$^{\Delta T}$ mice (Figure 18B, D). This demonstrates that Notch1

deficiency in T cells is associated with greater memory T cell response.

Figure 18. Mice with deficient Notch1 or Notch2 in T cells are inoculated with 5×10^6

pfu of VV intraperitoneally. 6 weeks after inoculation, the mice are rechallenged

intraperitoneally with 2×10^9 pfu of AdB8R. 1 week after rechallenge or 7 weeks post-

VV inoculation, the spleen is extracted, and assessed via flow cytometry for **(A)** VV-

specific B8R$^+$ CD8$^+$ T cell response and **(B)** activated CD8$^+$ T cell production of IFN-g.

Graphical representation of **(C)** B8R$^+$ and **(D)** IFN-γ^+ CD8$^+$ T cells. Values are mean ±

SEM, representative of 3 independent studies. Kolmogorov-Smirnov nonparametric

t-test, *$P < 0.05$.
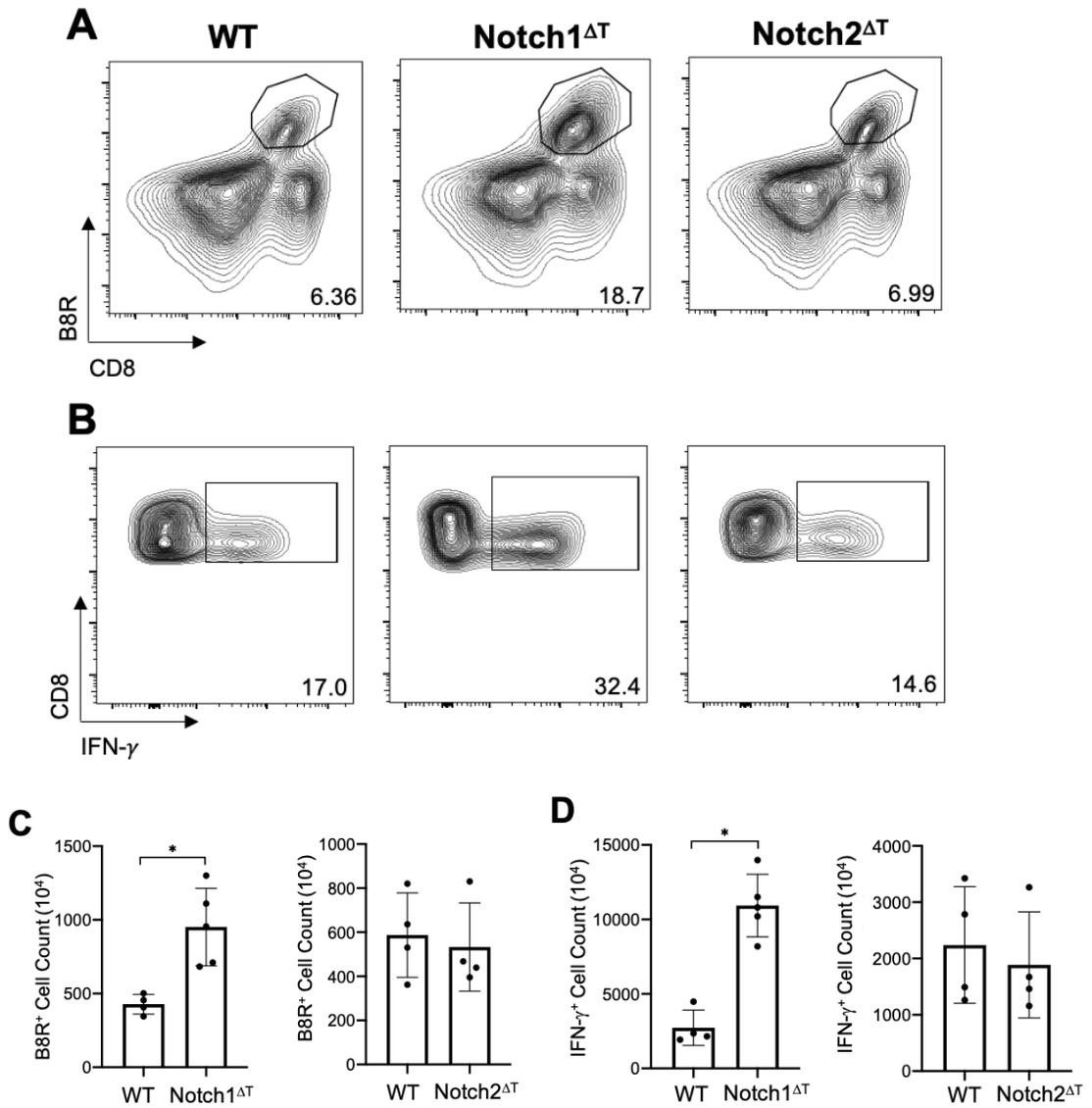
Figure 18 Restimulation in Notch1$^{\Delta T}$ mice results in greater response than in Notch2$^{\Delta T}$ mice at 7 weeks post-VV inoculation

5.2.3 Notch1 deficiency in T cells is associated with increased T_{CM} at 7 weeks post-VV infection 1 week after post-AdB8R rechallenge

To understand the specific subpopulation of CD8$^+$ memory T cells that are influenced by Notch1 deficiency in T cells, we analyzed VV-specific B8R$^+$ CD8$^+$ splenic T cells at 52 days post-infection for cell surface markers of T_{CM} and T_{EM}. CD44 is a marker of antigen exposure and CD62L is a L-selectin ligand that is required for homing to lymph node and lymphoid organs. T_{CM} is primarily located in lymphoid organs and is characterized by high expression of CD44^{hi} CD62L^{hi}. In contrast, CD62L is downregulated in circulating T_{EM}, which is associated with CD44^{hi} CD62L^{lo}. CD127 or IL-7R is a surface protein that is a reliable marker for memory T cells and is highly expressed in both T_{CM} and T_{EM}.

1 week after AdB8R rechallenge, which is also 7 weeks post-VV inoculation, we found that there are larger populations of CD44^{hi} CD62L^{hi} T_{CM} in both the spleen and site of inoculation peritoneal cavity in B8R$^+$ CD8$^+$ T cells from Notch1$^{\Delta T}$ compared to that of WT mice (Figure 19A).

Interestingly, the population of CD44^{hi} CD127^{hi} memory T cells is relatively the same in the spleen of both Notch1$^{\Delta T}$ and WT mice, but there is a significantly greater population of CD44^{hi} CD127^{hi} B8R$^+$ CD8$^+$ memory T cells in Notch1$^{\Delta T}$ at the site of inoculation peritoneal cavity, compared to that of WT mice (Figure 19B). This

indicates that Notch1 deficiency may be preferentially associated with increased

population of T$_{CM}$, opposed to that of T$_{EM}$.

Figure 19. Mice with and without deficient Notch1 in T cells are inoculated with

5x10^6 pfu of VV intraperitoneally. 6 weeks post-VV inoculation, the mice are

rechallenged intraperitoneally with 2x10^9 pfu of AdB8R. 1 week after rechallenge or

7 weeks post-VV inoculation, the spleen is extracted, the peritoneal cavity site of

infection is washed with 1xPBS, and both are assessed via flow cytometry for

expression of **(A)** CD44 and CD62L or **(B)** CD44 and CD127 in VV-specific B8R$^+$

CD8$^+$ T cells. Flow cytometry plots are representative of 3 independent studies.
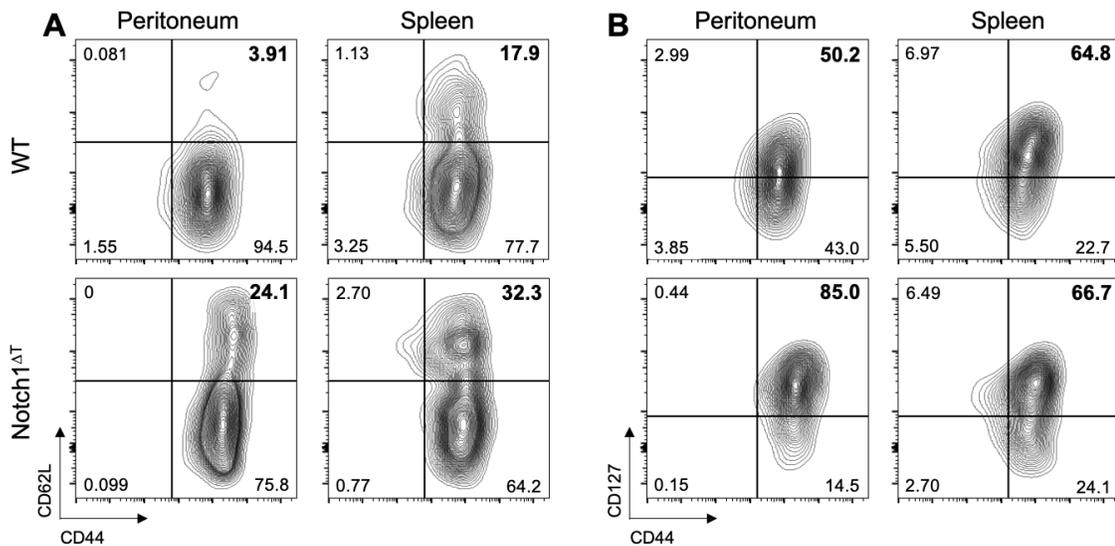
Figure 19 AdB8R restimulated Notch1$^{\Delta T}$ mice exhibit greater CD44^{hi} CD62L^{hi} expressing B8R$^+$ CD8$^+$ T cells than that of WT mice at 7 weeks post-VV inoculation

5.2.4 Deficiency of Notch1, but not Notch2, is associated with relative increased expression of Tcf7

In order to investigate the downstream pathways that Notch1 may govern to influence CD8$^+$ memory T cell formation, we examined published databases for genes that are upregulated in memory T cells and mice with T cell deficiencies in Notch1 versus Notch2 [219, 224-233]. We narrowed down our focus to a list of genes that included *Tcf7*, *Dapl1*, *Trib2*, *Runx1*, and *c-Myc*.

Mice were infected intraperitoneally with 5x10^6 pfu of VV and splenic cells were harvested 3 weeks later to sort for B8R$^+$ CD8$^+$ T cells. The sorted B8R$^+$ CD8$^+$ T cells were then processed for RNA isolation, and assessed for *Tcf7*, *Dapl1*, *Trib2*, *Runx1*, and *c-Myc* expression via qRT-PCR (Figure 20). Our findings indicated that deficiency of Notch1 in T cells is associated with relatively greater expression of *Tcf7*, *Dapl1*, and *Runx1* (Figure 20A-C). This suggests that Notch1 may be a modulator their expression. Most published literature has found that Notch is usually part of a transcriptional activation complex with other conventional transcription activators. Our findings seem indicate that loss of Notch1 increases the expression of some associated genes and may serve as a modulator in gene expression.

Figure 20. Mice with deficient Notch1 or Notch2 in T cells are inoculated with 5×10^6 pfu of VV and harvested 21 days later for cell sorting of splenic VV-specific B8R$^+$ CD8$^+$ T cells. The sorted cells were then assessed via quantitative real-time reverse transcription PCR (qRT-PCR) for relative genetic expression of **(A)** *Tcf7*, **(B)** *Dapl1*, **(C)** *Runx1*, **(D)** *Trib2,* and **(E)** *c-Myc* against *Gapdh*.
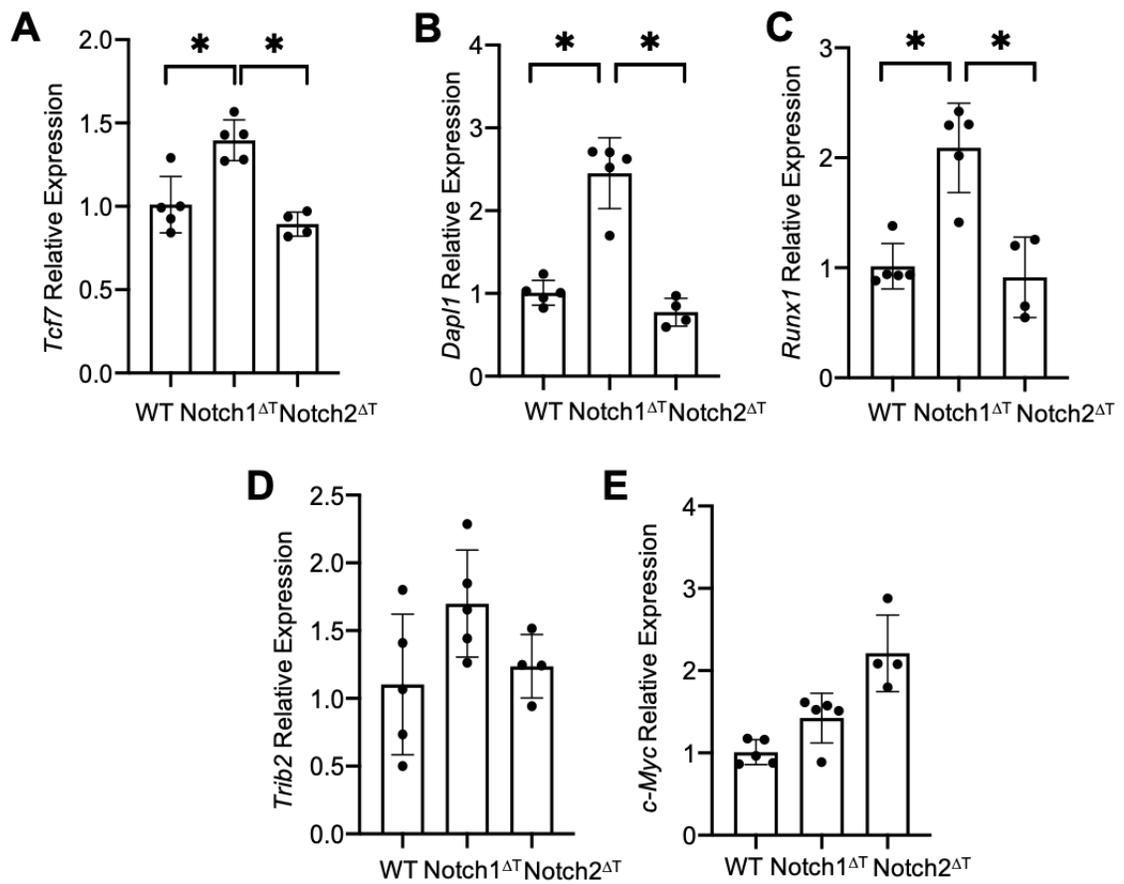
Figure 20 Increase activation of *Tcf7* in Notch1$^{\Delta T}$ mice compared to Notch2$^{\Delta T}$ and WT mice 3 weeks after VV activation

To better understand how Notch1 can modulate the expression of *Tcf7*, *Dapl1*, and *Runx1*, we analyzed publicly available Notch1-specific ChIP-seq data in both mice and humans (GSM1123916; GSM1694090). Our analyses found that Notch1 intracellular domain binds upstream of *Tcf7* in both mice and humans (Figure 21). This suggests that NICD may modulate *Tcf7* expression by occupying proximal enhancers of *Tcf7*.

Figure 21. ChIP-sequencing data were obtained from public database for Notch1 NICD for mouse (GSM1123916; black) [224] and human (GSM1694090; blue) [226]. UCSC Genome Browser views [234] show sequencing coverage for Notch1 ICD-specific ChIP-seq peaks for regions up- and downstream of **(A)** *Tcf7*, **(B)** *Dapl1*, and **(C)** *Runx1*.
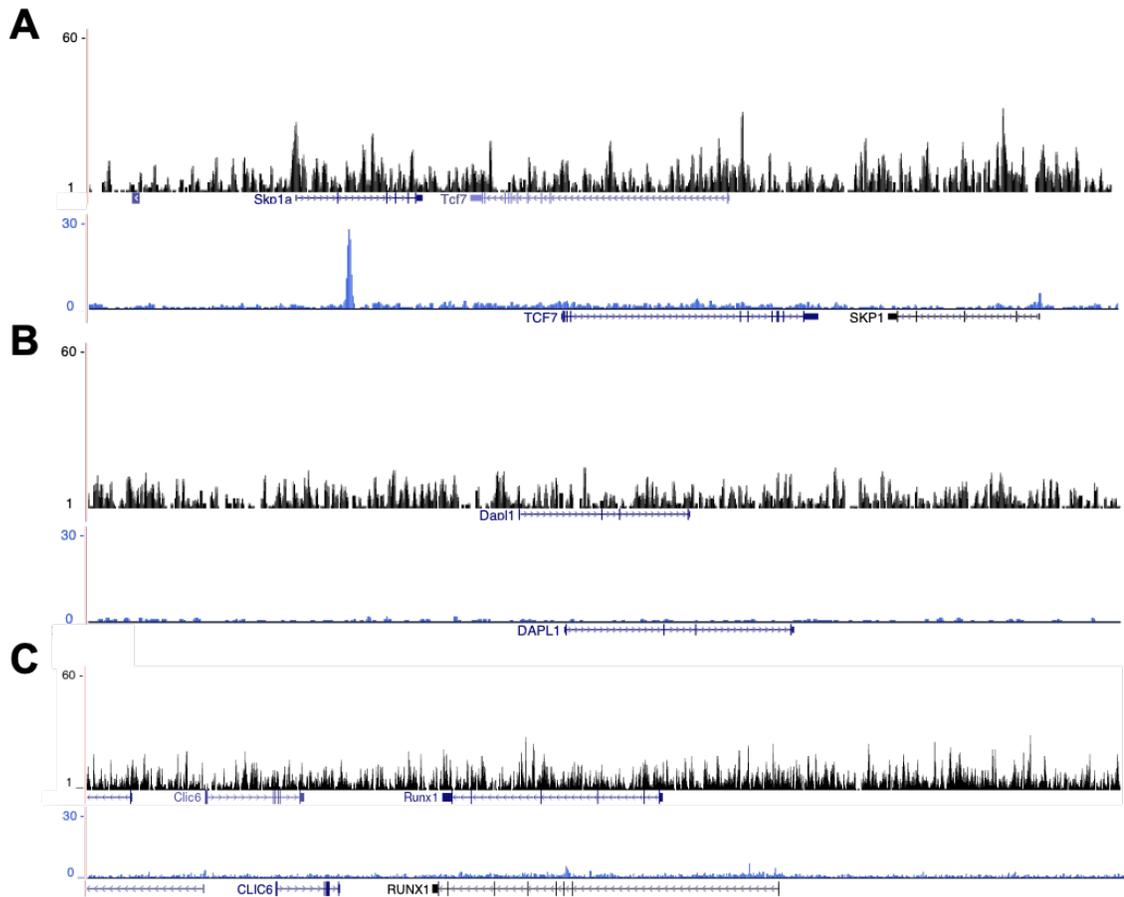
Figure 21 Notch1 ICD binds proximally to target genes

5.2.5 Notch1 NICD binds proximally to Tcf7 and stimulation of Notch with DLL4 increases Tcf7 expression

To further dissect our findings against conventional understanding of

Notch1's role as transcriptional activators, we isolated splenic T cells from wild-type

C57BL/6 mice with MACS selection and stimulated them with plate-bound Delta

Like canonical Notch Ligand 4 (DLL4). DLL4 preferentially activates Notch1 and

Notch4, and it is a crucial component of T cell development and maturation [225].

We found that after incubating splenic T cells with plate-bound DLL4, there is a significant increase in expression of *Tcf7* at 48 hours by qRT-PCR (Figure 22). This further supports our hypothesis that Notch1 is a modulator of *Tcf7* gene expression.

Figure 22. T cells were isolated from wild-type C57BL/6 mice (WT) via magnetic bead selection. Then the cells were stimulated *in vitro* for 24 or 48 hours with plate-bound DLL4 ligand and assessed via qRT-PCR for relative *Tcf7* expression against *Gapdh*.
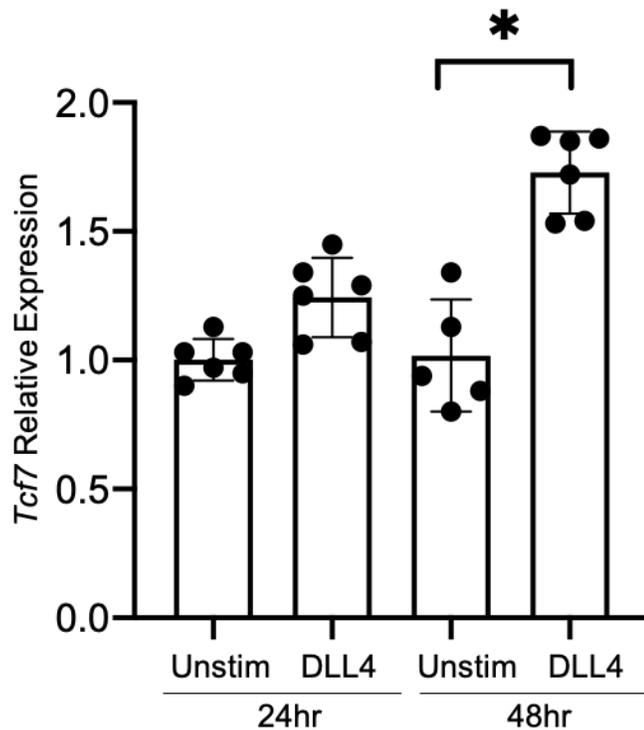
Figure 22 DLL4 stimulation increases *Tcf7* expression

We then examined whether Notch1 NICD binds to purported enhancer regions of *Tcf7* by performing ChIP-qPCR with anti-Notch1 NICD monoclonal antibody in splenic T cells for peaks found in previous ChIP-seq dataset analyses. We found that Notch1 NICD binds to a region upstream of *Tcf7* (Figure 23A). Binding to the enhancer increases after stimulation with plate-bound DLL4 and decreases with inhibition by GSI. In T cells isolated from Notch1$^{\Delta T}$, there is no significant NICD binding to the region as assessed by ChIP-qPCR (Figure 23B). This indicates that Notch1 NICD binds to proximally to a potential enhancer and thereby modulates the expression of *Tcf7*.

Figure 23. T cells from WT and Notch1$^{\Delta T}$ mice were isolated and culture *in vitro* for 48 hours with plate-bound DLL4 with or without LY411575 Notch γ-secretase inhibitor (GSI). After 48 hours, the cells were harvested for ChIP with anti-Notch1 ICD and assessed with quantitative real-time PCR (qPCR) followed by **(A)** gel electrophoresis and **(B)** quantitative analysis for a noncoding DNA region upstream of *Tcf7* with forward 5′- CTTGAGCTGAGGGGTCCTTG - 3′ and reverse 5′ - GCCTAAGCGGGTACTCTGAC - 3′ primers.
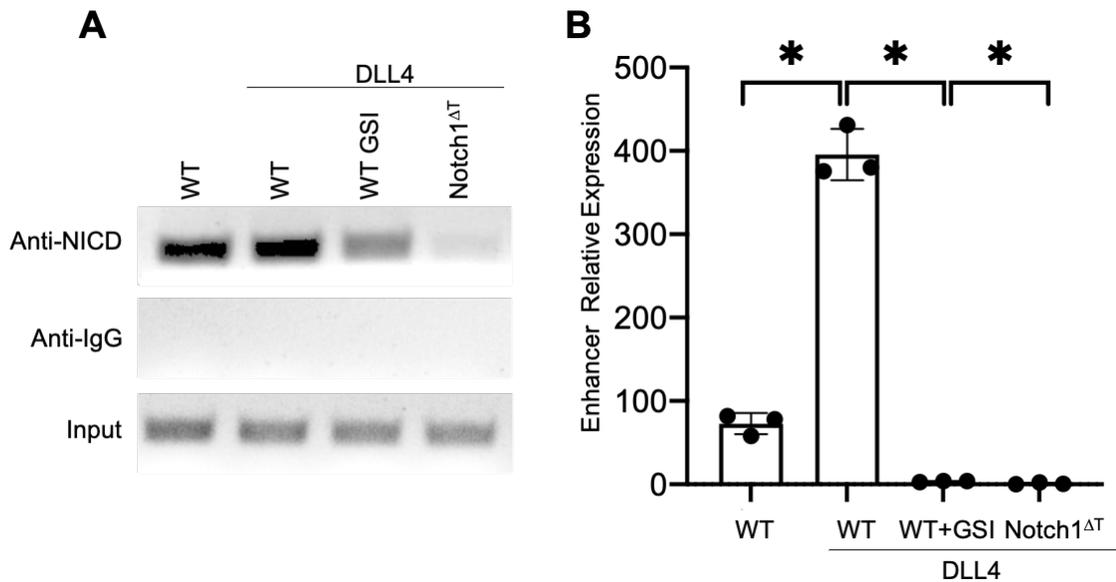
Figure 23 Notch1 NICD binds proximally to *Tcf7*

5.3 Discussion

The ability to pharmacologically target memory T cell formation is crucial for

the development of vaccines and cancer immunotherapy. TCF1/*Tcf7* is a well-

established transcription factor that promotes the differentiation of memory T cells.

However, attempts to target it pharmacologically have not been successful. Notch is

a cell surface receptor targeted by many therapeutic agents widely use in research

and clinical trials. Here we show that Notch1 is a modulator of TCF1, and that

inhibition of Notch increases the expression of *Tcf7* and is associated with

phenotypically greater memory T cell responses to infection.

We found that interestingly though Notch is conventionally considered a transcriptional activator for many genes, it may in fact serve as a modulator for *Tcf7* gene expression. Recently published studies of the mechanisms behind Notch signaling affirm our suspicions. Jarrett et al. found that Notch1 has a self-negative feedback system termed Notch-regulated ankyrin repeat protein (NRARP) [235]. Notch increases the expression of NRARP and recruits it to the transcriptional activator complex [236, 237]. The simultaneous engagement of NRARP to NICD1 and RBPJ complex results in the degradation of the transcriptional activating complex [235].

Notch is part of the transcriptional activator complex, but loss of Notch does not impact the expression of DNA-binding transcription factor RBPJ and co-activator Mastermind-like (MAML) that compose the rest of the transcriptional activation complex [238, 239]. It may be possible that without Notch, the transcriptional complex is minimally active enough to continue transcription of some genes, but it is also not hindered by the negative feedback forces that govern Notch degradation [240-242]. More extensive studies on the mechanisms behind Notch activation of specific genes need to be accomplished, prior to any conclusions about the mechanisms that govern Notch regulation of *Tcf7* for the differentiation of memory T cells.

Nevertheless, our results confirm that deficiency of Notch1, but not Notch2, is associated with greater CD8$^+$ memory T cell in response to viruses. It indicates that Notch1 is potentially a pharmacologically druggable target that is upstream of TCF1 and can be potentially used to manipulate the expression of *Tcf7* for formation of memory CD8$^+$ T cells. This suggests that inhibition of Notch1 is potential target to create more efficacious vaccines.

Chapter 6. Conclusion

As the vaccine used to achieve the only successful eradiation of a pathogenic infection in human history, Vaccinia virus (VV) can provide powerful potential insights into the underpinnings immune memory, and strategies of how to best design future immunotherapies and vaccines against infectious diseases and cancers. Already, insight into the pathogenesis of VV has provided many vaccine vectors through the use of recombinant viruses encoding a specific antigen. Protection against influenza was first achieved with a recombinant VV vector encoding the influenza-derived hemagglutinin gene [143]. However, there are numerous adverse effects and limited efficacies to using VV vectors in order to elicit immune responses so bluntly. Attempts to use VV vectors to vaccinate against cancers have only been met with limited successes. As a result, it is important to understand the mechanisms behind how VV induce immunity in cellular populations that results in long term memory even after elimination of the infection.

6.1 The role of $\gamma\delta$T cells as antigen presenting cells to CD8$^+$ T cells

Previously published studies have demonstrated that VV is a potent stimulator of TLR pathway. Upon VV infection *in vivo*, direct TLR2-MyD88 signaling in CD8$^+$ T cells is necessary for the efficiency expansion, survival, and subsequent differentiation in memory T cell populations, through the activation of the PI3K-Akt

pathway [2]. These findings suggest that activation of TLR signaling is not exclusive

to the innate immune response, but it is also crucial for activation of the adaptive

immune system. Prior studies have also shown that VV activation of TLR-dependent

and -independent pathways are necessary for subsequent adaptive immune

responses. VV activates dendritic cells (DCs) and promote the production of pro-

inflammatory cytokines, specifically IL-6, IL-1, and IL-12 through TLR2-MyD88-

dependent pathways and elicit production of IFN-β and other type I IFN through

TLR-independent mechanisms [4, 150]. The question is therefore if cellular

populations that bridge the innate and adaptive immune system require MyD88-

dependent and/or -independent pathways for activation, and what is the subsequent

effect of that stimulation.

γδT cells are part of an emerging population of cells that exhibit features of

both innate and adaptive immunity. They respond to infections with and without

activation by antigen presenting cells (APCs) [38, 42], with the ability generate a

more diverse and potent population of cells than conventional innate cells in

response to an insult. They are one of the first responders and bridge the gap

between innate and adaptive immune responses. *In vitro* experiments have

demonstrated that they produce cytokines, exert cytotoxicity, and activate other

cells in the immune system. Human γδT cells *in vitro* can directly present and

induce antigenic response in CD8$^+$ αβT cells. However, what controls the activation of γδT cells and their functions *in vivo* remains incompletely defined [173].

In this thesis, we have demonstrated that γδT cells are activated in response to VV infection in wild-type C57BL/6 mice. Furthermore, there is significant increase in viral titers in δT-cell receptor knockout C57BL/6 (*δTCR*$^{-/-}$) mice, which suggests that γδT cells play a critical role in VV control. We have also found that γδT cell activation is defective in *MyD88*$^{-/-}$ mice, suggesting a role for MyD88 pathway in regulating γδT cell activation. We further demonstrated that although the activation of NK cells is not affected, CD8$^+$ T cell response is compromised in *δTCR*$^{-/-}$ mice, suggesting a role of γδT cells in promoting the CD8$^+$ T cell response to VV infection.

To further investigate these findings, we demonstrated that γδT cells present antigens via Major Histocompatibility Complex Class I (MHC-I) directly to CD8$^+$ T cells, and that this process is required for the subsequent clearance of VV *in vivo*. These findings provide direct evidence for the role that γδT cells play in the activation of CD8$^+$ T cells. Previously published work has found that γδT cells can phagocytose microbeads and antigens. Here we illustrate that γδT cells is able to subsequently process the antigens for presentation to CD8$^+$ T cells, and further

strengthens the importance of γδT cells in the role of CD8^{+} T cell activation. This also provides a potential reason for recent findings that inhibition of professional antigen presenting cells does not abrogate the activation of CD8^{+} T cells [243].

More research is needed to understand the mechanisms behind CD8^{+} T cell activation, and the role of γδT cells to determine if γδT cells can also activate CD8^{+} T cells through a path independent of antigen presentation. In this thesis, we have demonstrated that γδT cells can activate CD8^{+} T cells via antigen presentation through MHC-I.

6.2 γδT cell cytotoxicity against viral infections

NKG2D is a C-type lectin-like stress-sensing transmembrane receptor that is expressed on many immune cells including γδT cells, CD8^{+} αβT cells, and NK cells [180, 190]. NKG2D canonically responds to stress-induced ligands that act as generalized danger signal to the immune system [181, 244]. *In vitro* studies have shown that activation of NKG2D alone could directly stimulate γδT cells without TCR engagement [245], and that blockade of the NKG2D pathway in γδT cells could inhibit the cytotoxicity of human Vγ9Vδ2 T cells [246].

Previous *in vitro* studies have indicated that γδT cells can be activated by a variety of ligands and multiple non-T cell receptor (TCR) activating receptor

pathways such as NKG2D [179, 245, 247, 248]. However, the specific role and subsequent effect of NKG2D activation in γδT cells is not well understood, and it has been particularly hard to dissect the role of NKG2D *in vivo* separate from that of other cell types. Prior investigations of NKG2D *in vivo* have only been able to use mice with full-body deficiency of the receptor. Here we present an analysis with mice that have NKG2D alleles flanked by LoxP sequences and can be used to study NKG2D in specifically targeted immune cells.

In this thesis, we have demonstrated that NKG2D expression in γδT is required for the complete elimination of VV infection. NKG2D expression in αβT cells and NK cells does not encompass the entirety of NKG2D's effect on the immune system in response to VV infection. This is particularly important because most investigation into the function of NKG2D has focused on its effect in NK cells and cytotoxic T cells [181, 249]. Our studies demonstrate that γδT cells also play a significant role in the effect of NKG2D cytotoxicity.

This broadens our understanding of the mechanisms behind NKG2D functioning and gives us particular insight into interactions that the receptor has with other pathways. Initial survey of our data reveals that there may be significant dual roles or parallel compensatory pathways to NKG2D function following selective embryonic depletion. Our initial findings reveal that acute inhibition of

NKG2D with inhibitory antibodies has a greater effect in γδT cells than congenital depletion of NKG2D in NKG2D$^{-/-}$ mice. This finding is replicated not only in γδT cells functional assays, but also viral clearance.

We found that mice injected with neutralizing anti-NKG2D antibodies have distinctly greater deficiency in Vaccinia viral clearance than NKG2D$^{-/-}$ mice. Similarly, adoptive transfer of NKG2D$^{-/-}$ γδT cells are sometimes able to partially rescue some of the deficiencies in Vaccinia viral clearance of δTCR$^{-/-}$ mice. This suggests that there may be compensatory or dual mechanistic roles to intrinsic NKG2D signaling in γδT cells. Prior published studies also reveal similar trends [250-252]. However, there is not enough evidence fully conclude the identity of the compensatory pathway and whether these findings reveal a more complex signaling cascade behind NKG2D activation.

Future experiments examining the mechanistic interactions of NKG2D with other parallel or intersection signaling pathways can better reveal the complex role that NKG2D plays in cytotoxicity of not only γδT cells, but also NK and CD8^{+} T cell. NKG2D is a fascinating signaling pathway that has tremendous implications for viral vaccination and cancer immunotherapy, however until we better understand the various intricacies behind its activation in different immune cells under contrasting conditions, we may find ourselves surprised more often than not.

6.3 Formation of antigen-specific CD8$^+$ memory T cells

One of the hallmarks of a strong immunity is the ability to mount a secondary response months and years after the initial insult. CD8$^+$ memory T cells are by definition required for the process. CD8$^+$ Memory T cells are a key component of the acquired immune response that provides a long-lived and rapid protection against various antigens. By understanding the mechanisms behind CD8$^+$ memory T cell formation in Vaccinia viral model, we can better understand how to generate more memory T cells to more precisely manipulate the immune system for cancer immunotherapy and vaccine development.

Vaccinia virus is able to generate a unique long lasting response in the immune system after a short exposure. In this thesis, we find that selective depletion of Notch1 receptor in T cells generates increased CD8$^+$ memory T cell formation than wild-type or Notch2 receptor deficiency alone. We further illustrate that this deficiency is modulated through TCF1/*Tcf7*, a potent memory T cell transcription factor. We found that Notch1 acts not as a pure activator or inhibitor of TCF1/*Tcf7*, but as a modulator whose activation and deficiency are both associated with increased *Tcf7* expression. This is particularly significant because it provides a pharmacologically targetable method to modulate *Tcf7* expression for CD8$^+$ memory T cell formation.

TCF1/*Tcf7* is a Wnt pathway effector that is key to the differentiation and persistence of CD8^{+} memory T cells [223]. The most widely accepted current model of memory T cell differentiation denotes concurrent formation of precursor memory and effector T cells following initial phases of stimulation and expansion. Most of the effectors cells succumb to apoptosis during the contraction phase, and the surviving precursor cells differentiate into functional memory T cells with cell-specific qualities of self-renewal and rapid expansion upon secondary stimulation [105].

Previously published findings have found that Notch1 or Notch2 is a key decision point for the differentiation of effector cells versus memory cells [219]. Our findings investigate this further to show that Notch1, but not Notch2, signaling is crucial to the differentiation of memory T cell pathway by modulating the expression of *Tcf7* through potential proximal enhancers.

For future investigations, it is important to understand how to best pharmacologically target Notch1 for CD8^{+} memory T cell formation. Our findings reveal that Notch1 modulates *Tcf7* expression, and that both inhibition and activation of Notch1 are associated with increased *Tcf7* expression. A more in-depth mechanistic understanding of how Notch1 modulation can impact *Tcf7* expression

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will reveal a more comprehensive strategy for differentiation of the most optimal CD8$^+$ memory T cells.

The epigenetic mechanisms behind how Notch1 modulate other genes are also particularly interesting and could provide explicit insight into how Notch functions and the functional differences between the various versions of the receptor and its ligands. Notch is one of the most significant receptor pathways during and after development. The mechanisms that govern its regulation and effects remain incompletely defined, and our studies provide a glimmer of insight into how it could potentially affect other intersecting and parallel pathways.

Similarly, it is important to note that our findings here only provide initial proof-of-concept in mouse *in vivo* and *in vitro* models. More comprehensive replications in humans and clinical models are required prior to significant translation into clinical therapies.

In conclusion, from its first initial success in eradicating smallpox in humans to its current effects in vaccination and cancer immunotherapy, VV has powerful effects on numerous cell types in the immune system, the mechanisms of which have yet to be completely elucidated. This dissertation has demonstrated only a small proportion of the effects the virus has on $\gamma\delta$T cells and CD8$^+$ T cells in the immune system. These provide significant implications for potential strategies of vaccination

and cancer immunotherapy, but more in-depth investigations of VV for future

immunotherapies are required to create more efficient and powerful therapies

against infectious diseases and cancers.

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Biography

Rui graduated from Beachwood High School in Beachwood, OH in May 2009, and attended Duke University in Durham, NC from August 2009 to May 2013, as an Angier Buchanan Duke Memorial Scholar.

During this time, she worked with Dr. Erich Jarvis, Ph.D. and Dr. Ute Hochgeschwender, M.D. on the differentiation and maintenance of avian stem cells. She also worked over the summer as an undergraduate research intern in the lab of Dr. Michael Bader, Ph.D. at the Max Delbrück Institute for Molecular Medicine on induced pluripotent stem cells, and the lab of Dr. Hilal Lashuel, Ph.D. at the École Polytechnique Fédérale de Lausanne on chemical biosynthesis of modified α-synuclein. In 2013, she graduated with a Bachelor of Science in Neuroscience with distinction. Subsequently, Rui entered the MD-PhD Medical Scientist Training Program at Duke University School of Medicine in August 2013, and joined the lab of her advisor, Dr. Yiping Yang, M.D., Ph.D.

Following her PhD dissertation, Rui will apply to residencies to pursue a career in academic medicine and research., with the hopes of specializing in interventional radiology, specifically interventional oncology.