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**LATE ANTIGEN REGULATES THE DIFFERENTIATION OF
CYTOTOXIC CD4 T CELLS IN INFLUENZA INFECTION.**

A Dissertation Presented

By

ALLEN MINH VONG

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester

In partial fulfillment of the requirements for the degree of

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IMMUNOLOGY AND MICROBIOLOGY

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ABSTRACT

CD4 T cells differentiate into multiple effector subsets that mediate pathogen clearance. ThCTL are anti-viral effectors with MHC-II restricted cytotoxicity. The factors regulating ThCTL generation are unclear, in part due to a lack of a signature marker. I show here that in mice, NKG2C/E identifies ThCTL that develop in the lung during influenza A virus (IAV) infection. ThCTL phenotype indicates they are highly activated effectors with high levels of binding to P-selectin, T-bet, IFN γ production, and degranulation. ThCTL express increased levels of granzymes and perforin and lower levels of genes associated with memory and recirculation compared to non-ThCTL lung effectors. ThCTL are also restricted to the site of infection, the lung in IAV and systemically in LCMV. ThCTL require Blimp-1 for their differentiation, suggesting a unique effector CD4 population. As ThCTL are highly activated, they also require antigen signaling post priming during IAV infection. Late antigen was necessary and sufficient for the differentiation of ThCTL. In the context of late antigen encounter, ThCTL surprisingly do not require CD80 and CD86 costimulation for their differentiation. Additionally ThCTL do not require late IL-2 for their differentiation and instead require late IL-15 signals for their efficient generation. Thus these data suggest ThCTL are marked by the expression of NKG2C/E and represent a unique CD4 effector population specialized for cytotoxicity.

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LIST OF ABBREVIATIONS

7-AAD	7-Aminoactinomycin D
Ag	Antigen
Ab	Antibody
APC	Antigen presenting cell
Ascl-2	Achaete-scute family bHLH transcription factor 2
B6	C57BL/6
BAL	Bronchoalveolar lavage
Bcl-6	B cell lymphoma 6
Blimp-1	B lymphocyte-induced maturation protein
CAR	Chimeric antigen receptor
CMV	Cytomegalovirus
CRTAM	Class I-restricted T cell-associated molecule
CTLA-4	Cytotoxic T-lymphocyte protein 4
DC	Dendritic cell
dLN	Draining lymph nodes
dpi	Days post infection
Eomes	Eomesodermin
FoxP3	Forkhead box P3
GATA-3	GATA binding protein 3
GC-Tfh	Germinal center T follicular helper
GFP	Green fluorescent protein

HA	Hemagglutinin
HIV	Human immunodeficiency virus
IAV	Influenza A virus
ICCS	Intracellular cytokine secretion assay
ICOS	Inducible T cell costimulator
IEL	Intraepithelial lymphocyte
i.n.	Intranasal
i.p.	Intraperitoneal
ITIM	Immunoreceptor tyrosine-based inhibition motif
i.v.	Intravenous
KLR	Killer lectin-like receptor
KLRG1	Killer cell lectin-like receptor subfamily G 1
LCMV	Lymphocytic choriomeningitis virus
LD50	Median lethal dose
Lef-1	Lymphoid enhancer binding factor 1
M1	Matrix 1
M2	Matrix 2
MCMV	Murine cytomegalovirus
MHC-I	Major Histocompatibility complex class I
MHC-II	Major Histocompatibility complex class II
MS	Multiple sclerosis
NA	Neuraminidase

Necl-2	Nectin-like molecule 2
NKG2X	NKG2A/C/E
NLR	NOD like receptors
NP	Nucleocapsid protein
Ova	Ovalbumin
PD-1	Programmed cell death protein 1
p.f.u.	Plaque forming units
PSGL-1	P-selectin glycoprotein ligand-1
qPCR	Quantitative reverse transcription polymerase chain reaction
RLR	RIG I like receptor
ROR γ t	RAR-related orphan receptor gamma
Runx3	Runt-related transcription factor 3
SAP	SLAM Associated Protein
SLAM	Signaling lymphocytic activation molecule 1
TCF-1	T cell factor-1
TCR	T cell receptor
TFH	T follicular helper
Tg	Transgenic
ThCTL	Cytotoxic CD4 T cells
Thpok	T helper-inducing POZ/Krüppel factor
TLR	Toll like receptor
Treg	T regulatory cell

VLS Vascular leak syndrome
VSV Vesicular stomatitis virus

PREFACE

This dissertation is submitted to fulfill the requirement for a Doctor of Philosophy at the University of Massachusetts Medical School at Worcester. This dissertation represents original work that I conducted at the University of Massachusetts Medical School under the supervision of Dr. Susan L Swain. Parts of texts and figures from the methods, results, and discussion sections were taken from the following manuscript:

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CHAPTER I: INTRODUCTION

Influenza infects and hospitalizes thousands of individuals annually leading to morbidity and mortality (1). The immune system employs multiple strategies to combat influenza infections and vaccines are utilized to generate immune memory to confer protection against influenza virus. Since the virus mutates and the annual strains of influenza circulating each year are different (1), vaccines need to be made annually to try to vaccinate individuals against circulating strains. Better understanding how the immune system combats influenza can help design better therapeutics to protect people against this constantly changing threat.

Influenza virus can also be a model system to understand how the immune system combats a viral infection. The influenza system has been utilized to study the mechanisms of how CD4 T cells, CD8 T cells, and B cells respond to a viral infection. In particular, we utilize influenza to study how CD4 T cells respond to viral infection. CD4 T cells are capable of orchestrating the adaptive immune system in part by secreting effector cytokines that influence CD8 T cells and B cells (2). Notably a subset of CD4 effector cells also possess cytotoxic function, an anti-viral mechanisms normally attributed to CD8 T cells and NK cells. These cytotoxic CD4 T cells, termed ThCTL, represent a unique CD4 effector subset.

The following is an introduction to the influenza virus, the immune response to influenza, and cytotoxic CD4 T cells in immunity.

Influenza Virus

Influenza is a single stranded negative sense enveloped RNA virus belonging to the *Orthomyxoviridae* family (3). *Orthomyxoviridae* include four genera, influenza A, B, and C, and Thogovirus. Influenza nomenclature states the antigenic hemagglutinin (HA) and neuraminidase (NA) subtypes, of which there are 15 HA and 9 NA. Influenza virus genome has eight RNA segments encoding viral structural and machinery important for viral replication. The structure of the intact influenza A virus, include the HA and NA spikes on the outside of the enveloped virus. Matrix (M2) protein is also on the surface, although at small numbers. Matrix (M1) surrounds the viral core under the lipid bilayer of the virus. The segmented RNA associates with nucleocapsid protein (NP) and the RNA polymerase machinery (PB1, PB2, PA). The virus also encodes two nonstructural proteins NS1 and NS2. Many of these proteins can be recognized by the immune system. Influenza virus has been found to naturally infect birds, swine, humans, horses, seals, whales, and mink (3). Experimentally, influenza can adapt and infect mice and ferrets allowing for experimental studies. The high mutation rate of influenza contributes to the pathogenicity and immunity towards influenza. The RNA polymerase for virus replication leads to a higher mutation rate compared to DNA polymerases (3–5). The accumulation of mutations in genes leading to protein changes in the antigenic sites of HA or NA contributes to antigenic drift of HA and NA. The gene segments of an influenza virus can also exchange with another of the same genera leading to gene reassortment. The exchange of antigenic HA and NA during reassortment contributes to antigenic shift seen in influenza. For influenza A virus, antigenic shift can lead to the creation of a pandemic strain of influenza (3).

Influenza infection in humans has estimated to cost around \$10 billion dollars annually in direct medical costs in the U.S. based on data collected in 2003, with the total economic burden at over \$80 billion (6). Annual deaths from influenza have been estimated to be around 20,000 individuals annually (7). Current vaccine strategies are not optimal as estimated efficacy of vaccine for the 2016-2017 season has been around 34% for influenza A and 56% for influenza B (8). This issue is compounded by the poor in vivo response of aged CD4 T cells (9) with the aged (>65 years of age) having the highest rate of hospitalization due to influenza (8). Additionally new vaccine strategies has been mixed in their success, with live attenuated influenza vaccine not being recommended for the 2016-2017 flu season by the CDC. Thus there has been great interest in designing a universal influenza vaccine to help protect against multiple circulating strains (10, 11). In search for better vaccines, T cell focused vaccines offer benefits. T cells recognize peptides derived form influenza virus, but they have been shown to recognize some internal proteins that have not mutated as much as the external HA and NA (12). Promoting immune responses and memory toward conserved parts of the virus is one avenue to target multiple strains of influenza.

The immune response to Influenza virus

Influenza virus infects the epithelial cells of the lung and replicates in these cells (13, 14). The innate immune system detects the virus through multiple receptors for viruses. The Toll Like Receptor (TLR) system recognizes influenza through TLR7 that recognize single stranded RNA of the virus in the endosome. TLR7 and TLR3 have been

implicated in the interferon response to influenza (15–17). Additionally cytosolic receptors like RIG-I, belonging to RIG-I like receptors (RLR), can also contribute to recognition by binding to the 5' triphosphate in the RNA (16, 18). NOD-like receptors (NLR) have been also shown to be important for survival against influenza infection (19). The activation of these receptors by influenza infection ultimately leads to downstream signaling events that culminate in the production of anti-viral cytokines, notably type I interferon (IFN)(15). The release of type I IFN leads to viral suppression in cells and can also influence signaling in other cells.

Alveolar macrophages line the respiratory tract and can be infected with influenza virus. These cells constantly take up particles in the lung and thus can also be infected with the virus (13). Dendritic cells also capture and sense influenza virus, most likely through the capture of debris from infected epithelial cells (13). Activation of the dendritic cells leads to migration to the draining lymph node to prime the adaptive immune response. Viral peptides can be presented through MHC-II to prime CD4 T cells or through MHC-I through cross presentation to prime CD8 T cells. Activation of these T cells leads to proliferation and differentiation into effector cells capable of mediating viral clearance.

The adaptive immune response to influenza infection

Influenza infection generates a robust T and B cell response in the mouse (20), making it a useful tool to study immune responses against viral infection. In learning about how the immune system clears the virus, work by various groups have interrogated

the role of the adaptive immune response to influenza virus. Notably the different arms of the immune system are capable of compensating for each other. B cells play an important role in clearing virus through anti-viral antibodies (21), but in the absence of B cells or CD4 help, CD8 T cells can clear the virus (22, 23). The same is true when CD8 T cells are eliminated in the host; the antibody response is enough to clear influenza virus (24). Thus influenza infection in the mouse activates a robust immune response capable of clearing the virus through multiple mechanisms.

CD8 T cells clear the virus mainly through perforin dependent mechanisms but can also contribute through their cytokine production (25, 26). Tc1 and Tc17, subsets of CD8 effectors, are capable of clearing virus leading to protection against lethal challenge of influenza (27). The perforin dependent mechanisms leads to direct lysis of the infected cell where perforin allows the entry of granzymes that activate the apoptosis pathway through cleavage of caspases (28).

B cells contribute to the clearance of influenza by producing anti-viral antibodies that can contribute to viral clearance (21). Much work has been done to examine the antibody response to influenza, as there is evidence for prophylactic potential in humans (29, 30) and mice (31). Antibodies against influenza can prevent and protect against infection through various mechanisms including direct neutralization. The isotype of the antibody can also contribute to clearance where certain IgG subtypes are important for the efficacy of broadly neutralizing antibodies against influenza (32). Because B cells can differentiate into long lived plasma cells producing antibodies for the life of the host (33),

these cells have been targeted in vaccination to generate protective antibodies against influenza infection (11).

CD4 T cells contribute to both pathways by helping the B cell response and through perforin dependent mechanisms (34). CD4 effector cells with the capability to promote and help the B cell response are characterized as T follicular helpers (Tfh) (35). Tfh possess the ability to secrete IL-21 and IL-4, which are important cytokines for the germinal center response allowing for isotype switching and somatic hypermutation (36). Tfh that enter the germinal center are termed GC-Tfh and these cells can be identified by the expression of GL-7 along with canonical Tfh markers CXCR5 and Bcl-6 (37). Tfh are important for the generation of protective and isotype switched antibodies as evidenced when mice lack the SAP (Slam associated protein) and Tfh are reduced leading to a loss of high affinity and isotype switched antibodies against influenza infection (38, 39) which are important for protection against influenza (32, 40).

Cytotoxic CD4 T cells (ThCTL)

Activated CD4 T cells will differentiate into effector subtypes including Th1, Th2, and Th17 cells. Additionally, cells responding to self-antigen can also differentiate into T regulatory cells. Further, CD4 T cells with a defined function Tfh and ThCTL are also found during viral infection. Cytotoxic CD4 T cells, ThCTL, are defined by their function of cytotoxic capability against target cells (41). Akin to CD8 T effector cells, these ThCTL utilize perforin to kill infected or target cells. ThCTL have been described in humans and mice (41). The early reports of human ThCTL describe a phenomenon

where CD4 T cells cultured extensively in vitro can become cytotoxic (42). Later, these cells were found in humans with the help of direct staining of cytotoxic molecules like granzyme and perforin (43). Analysis of cytotoxic CD4 T cells in disease states revealed their association with protective capacity. For example, ThCTL correlated with improved disease outcomes in HIV infected individuals, where CD4 T cells expressing Granzyme A are predictive of controllers of infection (44). In influenza infection, the memory response of CD4 T cells correlates with better disease outcomes after rechallenge in humans and these memory CD4 T cells exhibited cytotoxicity when assayed ex vivo (45). During Dengue vaccination, the responses of individuals with cytotoxic CD4 T cells were predictive of better protection against Dengue infection (46). These human studies and others in sum demonstrate the protective capability of ThCTL.

In mice, further studies extend the ability of ThCTL to protect against viral infections (41). Our lab and others have shown ThCTL to be protective during influenza infection in mice (34, 47). The regulation of ThCTL, however, is not well understood. Further understanding of how ThCTL are generated and regulated will better inform the development of vaccines or therapies to promote protection in human disease. Current challenges of the ThCTL field are defining a marker of ThCTL, defining what factors promote ThCTL, and whether memory ThCTL forms. The following reviews ThCTL regulation and immunity in the field.

Co-stimulation and ThCTL

Multiple groups have studied the role of co-stimulation during the generation of ThCTL. As naïve T cells encounter their cognate antigen on antigen presenting cells, they receive multiple signals that contribute to their activation. Signals through the T cell receptor, co-stimulation, and cytokines in sum lead to the activation, proliferation, and differentiation of T cells. The contribution of the strength of T cell receptor signaling has been less well studied in terms of ThCTL formation, although the strength can influence the differentiation of other CD4 T cell subsets (48, 49). In vitro culture of ThCTL suggests low antigen doses can promote cytotoxic formation (50), whether this is also occurring in vivo remains to be studied. Co-stimulation, however, has been shown to greatly influence the differentiation of ThCTL.

OX-40 and 4-1BB

OX-40 (CD134) and 4-1BB (CD137) both have been shown to promote ThCTL formation. OX-40 can be induced after activation and the triggering of the receptor leads to increased proliferation and expansion of CD4 T cells. 4-1BB is also inducible after activation and the triggering of the receptor leads to similar co-stimulation of proliferation and activation of CD4 T cells. In mouse models of self-reactivity or super-antigen, addition of CD137 and CD134 agonist antibodies leads to the formation of CD4 T cells with cytotoxicity (51). These cytotoxic CD4 T cells generated with activating OX-40 and 4-1BB upregulated granzyme B protein and perforin mRNA. The upregulation of these cytotoxic molecules leads to direct lysis of target cells both ex vivo and in vivo. Notably, OX-40 alone can up-regulate granzyme B expression, while 4-1BB activation

leads to greater expansion of cytotoxic CD4 T cells. Additionally the treatment of activating CD137 and CD134 leads to a greater response to tumor challenge with B16 melanoma with a concomitant increase in the expression of granzyme B and interferon in the CD4 T cells. With great interest in tumor immunotherapies, other groups have focused on utilizing co-stimulation to promote cytotoxic CD4 T cell responses to tumors (52, 53). The addition of agonist 4-1BB to a tumor vaccine model where B16 cells expressing Fl3t-ligand can be therapeutically administered to B16 melanoma bearing mice leads to the accumulation of tumor infiltrating cytotoxic CD4 and CD8 T cells. The cytotoxic CD4 T cells upregulate multiple genes associated with cytotoxicity, including granzymes A, B, K and perforin. The multiple cytotoxic genes correlate with the increased cytotoxic activity of tumor infiltrating CD4 T cells against melanoma cells (52). OX-40 has also been shown to be capable of inducing tumor eradicating cytotoxic CD4 T cells as well (53). Again using the B16 melanoma tumor model, addition of cyclophosphamide and OX-40 agonist leads to the eradication of the established tumor in mice. This treatment induces the CD4 T cells in the mice to become cytotoxic as demonstrated by the upregulation of granzyme B and direct ex vivo cytotoxicity against melanoma cells. Notably the use of OX-40 agonist also induces human CD4 T cells ex vivo to become cytotoxic against tumor target cells. The mechanism for how CD134 and CD137 programs cytotoxic CD4 T cells is less well studied. However, experiments have shown that the activation of these pathways leads to the expression of the transcription factor Eomesodermin (Eomes) (51–53). Eomes plays an important role in development of CD8 T cell effector function including the ability to secrete interferon gamma, express

granzyme B, and cytotoxic killing of target cells (54). In CD8 T cells, expression of Eomes in resting cells can drive the expression of perforin (55), suggesting a possible mechanism of where OX-40 and 4-1BB induces Eomes expression which turns on cytotoxic genes like perforin.

Additional Co-stimulatory molecules

Although multiple co-stimulatory molecules exist on CD4 effector T cells, not all are capable of promoting cytotoxicity. GITR and CD27 were explored and were both unable to induce Eomes expression on tumor infiltrating CD4 T cells (52). CD40 and blocking PD-1 was also investigated showing limited efficacy against tumor rejection by CD4 T cells (53), however whether this was through an indirect mechanism or the inability to promote cytotoxicity in CD4 T cells needs to be studied further. CTLA-4 engagement leads to an inhibitory signal towards effector cells. Blocking CTLA-4 does lead to an increase in cytotoxic CD4 T cells in tumors (56). The tumor infiltrating CD4 effectors exhibit cytotoxicity and blocking CTLA-4 leads to an expansion of these cells, suggesting blocking CTLA-4 is not required for the induction of ThCTL but may be important for the expansion of these cells.

The selectivity of certain co-stimulatory molecules for inducing ThCTL suggest a regulated environment where ThCTL are preferentially generated under the right conditions. Further understanding how these conditions could be modulated would help therapies that could better target ThCTL for diseases where ThCTL serve a protective function.

Cytokines and ThCTL

After antigen recognition and TCR stimulation of naïve CD4 and CD8 T cells, cytokines act as a ‘signal 3’ to modulate the differentiation of effector cells. In vitro cultures polarizing naïve CD4 T cells into different helper subtypes add different polarizing cytokines to promote the differentiation of CD4 T cells (2). Cytokines potentially affecting the differentiation of ThCTL have been investigated and explored below.

Interleukin 2

IL-2, a cytokine produced by activated T cells, promotes the expansion and proliferation of activated T cells (57). The role of IL-2 beyond the proliferative effects has been studied in regards to T cell differentiation. Notably, IL-2 is important for the homeostasis of regulatory T cells (Treg) in vivo (58). In terms of ThCTL development, IL-2 has been shown to promote the development of cytotoxicity in CD4 T cells. In vitro differentiation of naïve CD4 T cells into effector CD4 T cells supplemented with a ‘high’ dose of exogenous IL-2 leads to the increase expression of granzyme B and cytotoxic activity (50). Increasing the dose of IL-2 in culture also leads to an increase in the production of perforin protein in CD4 T cells (59), as well as in CD8 T cells (55). Thus IL-2 can induce the production of cytotoxic molecules and the differentiation of ThCTL from naïve CD4 T cells in vitro. However, work done in vivo has shown less clear results. The use of CD25^{-/-} CD4 T cells *in vivo* is complicated due to reduced expansion

of these cells during influenza challenge (59). Although these cells have reduced granzyme B expression, the CD25^{-/-} CD4 T cells degranulate similarly to wild type CD4 T cells, and have similar *in vivo* cytotoxicity. It is unclear whether the high dose of exogenous IL-2 used *in vitro* that induces the cytotoxic program is also a physiologically relevant dose *in vivo*. Additionally, during viral infections, the induction of multiple inflammatory cytokines can potentially provide compensating signals in the absence of IL-2, leading to similar CD4 and CD8 effector numbers (60).

Type I Interferon

The type I family of interferons are cytokines important for anti-viral immunity. This family consists of multiple subtypes including IFN α , IFN β , and others (61). Type I IFN signal through the interferon receptor, IFNAR, which acts as a signal three to co-stimulate T cells. Thus loss of IFNAR leads to reduced CD8 T cell expansion and function (62), including the reduced expression of granzyme B. Accordingly, the loss of IFN signaling on CD4 T cells also leads to reduced expansion of anti-viral CD4 cells *in vivo* (63). However, the ability of these LCMV specific CD4 T cells to secrete IFN γ was unaffected in the absence of IFNAR (63). As the type I IFN played critical roles in anti-viral immunity, whether these cytokines played a role for ThCTL was explored in detail. *In vitro* culture of naïve CD4 T cells supplemented with high IL-2 leads to increased granzyme B expression, and the further addition of IFN α leads to more granzyme expression (64). Importantly, the combination of IL-2 and IFN α lead the cells to transcribe perforin and exhibit peptide specific cytotoxicity. Influenza infection of

IFNAR deficient mice revealed CD4 T cells in the lung express less granzyme B protein compared to infected wild type mice. Addition of neutralizing IL-2 to these mice lead to a further decrease in granzyme B expression (64). Thus the type I IFN could play an important role in vivo in programming ThCTL formation.

Interferon γ

Type II interferon, interferon γ , plays an important role in protection against many viruses (65). During anti-viral immunity, CD4 T cells routinely produce IFN γ as a potent anti-viral effector cytokine (2). Naïve CD4 T cells differentiated into Th1 cells produce IFN γ and IFN γ helps promote the Th1 subset (66). Because, Th1, or IFN γ producing cells are found during viral infections, it is of interest to see whether IFN γ is important for ThCTLs as well. When naïve CD4 T cells are polarized in vitro under Th1 conditions, CD4 T cells displaying cytotoxic activity can be found (50), although high IL-2 conditions generate a higher cytotoxic activity. Naïve CD4 T cells lacking the IFN γ gene and polarized under these conditions also lead to no change in cytotoxic activity (34). Loss of IFN γ in either polyclonal CD4 T cells or TCR transgenic CD4 T cells activated by viral infection does not impact ex vivo cytotoxicity against peptide pulsed target cells (47). Thus IFN γ does not play an appreciable role in promoting the differentiation of ThCTL.

ThCTL exhibit peptide specific cytotoxicity, which demonstrates the need for MHC-II presentation on target cells. MHC-II is normally expressed only on professional antigen presenting cells, however MHC-II can be modulated by IFN γ . IFN γ leads to the

activation of the MHC II transactivator gene, CIITA, which can upregulate genes for MHC II antigen presentation (67, 68). Indeed the lung epithelial cells of influenza infected mice express MHC-II, while uninfected mice display little MHC-II (47) on lung epithelium. Although, IFN γ may not be needed for cytotoxic CD4 T cell induction, IFN γ may be an important cytokine to help mediate the cytotoxicity by inducing target cells to upregulate MHC-II.

Other Cytokines

While other cytokines have not been thoroughly investigated for ThCTL induction during viral infection, IL-27, IL-10, and IL-15 have been shown to be important for ThCTL induction in tumor models. Mice lacking IL-27, IL-10, or IL-15 vaccinated and re-challenged with tumor cells have tumor infiltrating CD4 T cells that express less granzyme B compared to wild type host mice (52).

When naïve CD4 T cells are polarized under Th2 conditions, these effector CD4 T cells exhibit low cytotoxic activity, suggesting a suppressive environment during Th2 polarization. Addition of exogenous IL-4 to the IL-2 alone cultures also leads to suppression of cytotoxicity, suggesting IL-4 of the Th2 polarizing conditions to be suppressive of ThCTL differentiation (50).

Further studies are warranted to understand the cytokine environment that promotes or inhibits ThCTL induction in the context of therapies that could potentially target cytotoxic CD4 T cell generation.

ThCTL transcription factors

CD4 T cell differentiation into effector cells involves transcription factors that enable the expression of a multitude of effector genes that endow the cells to promote pathogen clearance. Canonical CD4 subsets involve ‘master’ transcription factors that are essential to program the cells to become T helper subsets: T-bet for Th1, GATA-3 for Th2, ROR γ t for Th17, and FoxP3 for Treg (2). However, for certain specialized subsets, it is becoming clear that multiple transcription factors are required. Tfh cells are programmed by Bcl-6 expression (69); however CD4 cells also utilize Ascl-2 (70), Maf (71, 72), LEF-1, and TCF-1 (73) to promote the entire Tfh program. Thus, multiple aspects of the Tfh program are regulated by different transcription factors. ThCTL is also a specialized subset of CD4 effector cells and depending on the model system, multiple transcription factors have been proposed to be important for programming cytotoxic function in CD4 effectors.

Eomesodermin (*Eomes*)

Eomesodermin (*Eomes*) is a T-box family transcription factors involved in development. T cells can express *Eomes*, and CD8 T cell expression of *Eomes* is important for effector generation (54) as well as memory CD8 T cell formation (74, 75). The expression of *Eomes* on CD4 T cells is less well studied, however multiple model systems have shown *Eomes* to be required for cytotoxicity. In a self-reactive model of CD4 T cell activation, the induction of ThCTL through co-stimulating CD134 and CD137 leads to the upregulation of *Eomes* expression (51). In a superantigen model, the

addition of CD134 and CD137 co-stimulation also leads to the upregulation of Eomes on CD4 T cells as well as the upregulation of granzyme B. The loss of Eomes on CD4 T cells results in a loss of granzyme B expression as well (51). These data suggest Eomes may be important in programming cytotoxic CD4 T cells by regulating granzyme expression. In the melanoma model of ThCTL induction, where 4-1BB agonist can induce ThCTL responses, Eomes is highly upregulated. Again, the loss of Eomes in this model leads to a loss of granzyme B expression on CD4 T cells (52). In a similar model, downregulation of Eomes through retroviral transduction of ThCTLs leads to a reduction in ex vivo cytotoxic function, suggesting Eomes is required for programming cytotoxicity (53).

T-bet (*Tbx21*)

T-bet is another T-box family transcription factor that plays an important role in programming Th1 CD4 T cells (2, 66) as well as CD8 T cell effector cells (75). Because in vitro cultures of Th1 cells displayed cytotoxicity (50), the Th1 program perhaps is compatible with the ThCTL program. Although T-bet is not required for granzyme B expression in a self-reactive model of CD4 activation (51), T-bet could play a role during viral infection where a large majority of the CD4 response produces IFN γ . Indeed, loss of T-bet leads to a reduction in CD4 T cell expression of granzyme B during influenza infection (64). In vitro culture of CD4 T cells under ThCTL promoting conditions, reveals T-bet can be upregulated and bound to cytotoxic genes *Gzmb* and *Prfl* (64). Although IFN γ is not required for the cytotoxic phenotype to develop in vivo (47), T-bet

could play a role in promoting cytotoxic genes when it is induced by other cytokines, like IL-12 (76).

Blimp-1 (*Prdm1*)

Blimp-1 is a transcriptional repressor important in programming B cell plasma cell differentiation (33) and CD8 T cell effector differentiation (77). In CD8 T cells, Blimp-1 regulates the cytotoxicity, where CD8 T cells lacking Blimp-1 isolated from infected mice are unable to effectively kill target cells (77). Notably Blimp-1 also affects the expression of other transcription factors, as loss of Blimp-1 leads to a reduction of T-bet and an increase of Eomes expression in CD8 T cells. Because Blimp-1 may play a role in programming cytotoxic function, it has been looked at for ThCTL programming (64). CD4 T cells lacking Blimp-1 during influenza infection does lead to a reduction in granzyme B expression and a loss of cytotoxicity (64). The interplay between Blimp-1 and T-bet was explored in vitro, where T-bet binding to cytotoxic genes was lost when Blimp-1 was genetically deleted from CD4 T cells (64). Together, these data suggest Blimp-1 could be regulating cytotoxic CD4 T cell differentiation in a manner similar to CD8 T cells.

Thpok (*Zbtb7b*) and Runx3

Thpok and Runx3 are transcription factors that regulate the CD4 and CD8 single positive development in the thymus. During T cell development, Thpok is important for inducing the CD4 T cell lineage while suppressing the CD8 T cell lineage (78, 79).

Runx3 helps stop the CD4 lineage and induce the CD8 T cell lineage (80). Although Thpok is important for CD4 T cell lineage, not all mature CD4 T cells express Thpok. Using a Thpok GFP reporter mouse system, CD4 T cells found in the intestine, including the intraepithelial lymphocytes express CD4 but lose Thpok (81). The loss of Thpok also corresponds to the increase expression of CD8a. These CD8a positive, Thpok negative intraepithelial lymphocytes (IEL) displayed MHC-II restricted cytotoxicity in terms of functionally killing target cells as well as degranulation as evidenced by CD107a⁺ staining (81). Thus the cytotoxic program for CD4 T cells is allowed by ‘reprogramming’ the cells due to a loss of Thpok.

ThCTL immunity

ThCTL are generated in response to a pathogen insult. Various groups have looked at immune responses against viruses and have explored whether or not cytotoxic CD4 T cells are generated. Additionally, cytotoxic CD4 T cells could potentially be important for the viral clearance or protection against lethal challenges of virus or pathogen. Viruses are continually evolving and are under immune selection pressure and thus have various ways to avoid the immune system. Disrupting MHC-I presentation is a typical feature of viral infection (82), and thus additional mechanisms of viral clearance are warranted including NK cell mediated clearance or MHC-II restricted clearance. The role of ThCTL in multiple infections is reviewed below.

Influenza Virus

The immune response to influenza generates multiple defense mechanisms to combat influenza infection. CD8 T cells can contribute to influenza clearance in mice (25), although mice lacking CD8 T cells can also clear virus (24), suggesting redundant mechanisms for viral clearance including the humoral immune response. Antibody mediated clearance is also important for protection, as antibody alone can rescue mice infected with influenza (21). CD4 T cells can contribute to protection through two mechanisms: antibody helper function and perforin mediated cytotoxicity (34). In mice infected with influenza, CD4 T cells with cytotoxic function can be found in the infected lungs (47). These ThCTL are capable of mediating protection against a lethal challenge of influenza infection, even in the absence of antibody help (34). Further, ThCTL can drive perforin dependent immune evasion by influenza, suggesting direct control of virus in vivo (83). In humans, memory CD4 T cells specific for influenza internal proteins can be a marker of protective responses (45). These memory CD4 T cells exhibit cytotoxic function ex vivo, where they are CD107a⁺ and lyse autologous peptide pulsed target cells. Although primary immune responses were not analyzed, the memory response suggests that cytotoxic CD4 T cells can be protective against influenza infection in humans. The T cell specificity for internal proteins can also lead to heterosubtypic protection against other strains of influenza (12).

Lymphocytic Choriomeningitis Virus

LCMV has been utilized extensively to understand the T cell response against viral infections. Although CD8 T cells are required for clearance of LCMV, the removal

of CD8 T cells in $\beta 2m$ deficient mice leads to the generation of CD4 T cells with cytotoxic activity (84), suggesting a compensatory mechanism. These data also suggest that cytotoxic CD4 T cells only arise when CD8 T cells are defective. However, in wild-type mice infected with LCMV, cytotoxic CD4 T cells can be found (85). The cytotoxic CD4 T cells can kill target cells in a peptide dependent manner in vivo. Importantly, the response was specific to LCMV derived peptides, suggesting the endogenous polyclonal CD4 response can be cytotoxic.

West Nile Virus

West Nile Virus is a member of the *flaviviridae* family and is found throughout the United States where transmission occurs through mosquitoes. Infection with West Nile Virus is one of the most common causes of neuroinvasive diseases and currently lacks a vaccine for preventative treatment (86). In mice infected with West Nile Virus, both CD8 T cells (87) and CD4 T cells (88) contribute to immunity. When the CD4 T cells were characterized, they displayed both IFN γ producing capability as well as cytotoxicity. The effector cytotoxic CD4 T cells could lyse West Nile Virus derived peptide pulsed target cells both in vivo and ex vivo (89). Whether the protective ability of CD4 T cells is derived from their cytotoxic potential or their antibody helper ability remains to be studied.

Ectromelia Virus

Ectromelia virus is a mousepox virus that is similar to the human smallpox virus. When mice are infected with Ectromelia virus, T cell cytotoxicity and antibody responses are important for clearance of virus (90). When CD4 T cell responses are analyzed after Ectromelia virus infection, the majority of activated CD4 T cells in the draining lymph nodes express granzyme B. These cytotoxic CD4 T cells were able to lyse target cells in vivo as well. Importantly, the removal of perforin from CD4 T cells in mice, led to an increase of viral titers after infection suggesting cytotoxic CD4 T cells contribute to viral clearance (91).

Dengue Virus

Dengue virus is a Flavivirus spread by mosquitoes. Over 300 million people are estimated to have been infected by Dengue (92). Although the antibody response and CD8 response to Dengue has been studied, the CD4 response has been less well studied. Using Dengue derived peptides, MHC-II restricted CD4 T cell responses have been studied in individuals infected with Dengue virus. These activated CD4 T cells showed a characteristic cytotoxic phenotype, where they expressed perforin, granzyme B, and degranulated (46). Further, when these cells were isolated and assayed for cytotoxic function, the ThCTL could kill target cells pulsed with Dengue derived peptides. These early results suggest ThCTL could be generated during Dengue virus infection, and that the cells could contribute to viral clearance.

Epstein-Barr Virus and Murine γ -herpes virus

Epstein-Barr Virus is a γ herpes virus infects humans, and murine γ herpes virus is used a model system in mice to study immune responses against γ herpes viruses. Infection of mice with γ herpesvirus 68 leads to typical viral control a week after infection and subsequent viral latency. CD4 T cells contribute to protection of mice to infection, where mice lacking MHC-II succumb to viral infection and virus being detected well into the latency period (93). The reemergence of virus in mice lacking CD4 T cells, suggest that CD4 T cells play a critical role in controlling virus during latency. Analysis of the CD4 T cell response during latent infection reveals many of the CD4 T cells degranulate as measured by CD107a staining (94), and display in vivo and ex vivo cytotoxicity (94). These CD4 T cells can also prevent viral reactivation in vitro. A transgenic CD4 T cell specific against an immunodominant γ herpesvirus 68 epitope activated in vivo also displays cytotoxicity against peptide pulsed target cells both in vivo and ex vivo (95).

Epstein-Barr virus is widespread in people and is usually controlled by the immune system, where primary infection can present as infectious mononucleosis (96). CD8 T cells are expanded and specific for many of the early and lytic genes. However, CD4 T cells are also found specific for many of the latent genes in EBV immune individuals (97). These specific CD4 T cells can be cloned out and the clones demonstrate cytotoxicity as measured by ex vivo lysis of target cells as well as staining for granzyme B and perforin.

Cytomegalovirus

Cytomegalovirus (CMV) is a beta herpes virus that infects humans and mice. In humans, infection is controlled in normal immunocompetent individuals and virus remains latent throughout life. CMV infected individuals have shown to possess circulating CD4 T cells with a cytotoxic phenotype including expression of granzyme A, B, and perforin (98). Further, during a primary CMV infection of an individual, CD4 T cells with a cytotoxic phenotype emerge when virus loads peak suggesting these cells are responding to the infection (99).

Mouse cytomegalovirus (MCMV) is also used to study the immune response against CMV. CD4 effector T cells can promote viral clearance of MCMV, where transfer of effector CD4 T cells leads to lower viral loads in immunocompromised mice (100). The endogenous CD4 T cell response to MCMV has been characterized using tetramers loaded with MCMV derived peptide. The polyclonal CD4 T cell response to MCMV includes cells expressing granzyme B, with enrichment in the liver. Pulsing target cells with MCMV peptides reveals CD4 T cell mediated cytotoxicity in vivo (101).

Human Immunodeficiency Virus

Human Immunodeficiency Virus (HIV), a retrovirus, infects CD4 T cells causing a large decline of CD4 T cells that eventually leads to Acquired Immune Deficiency Syndrome (AIDS). The loss of CD4 T cells points to the importance of this subset in immunity against pathogens. Individuals with HIV infection have circulating CD4 T cells that are cytotoxic (43). Some individuals with HIV infection have normal CD4 T cell levels, and are termed non-progressors. A non-progressor showed CD4 T cells with

cytotoxic phenotype, including granzyme and perforin expression (98). Additionally, granzyme A expression can be used to predict the clinical outcome of HIV infection. The presence of high granzyme A positive CD4 T cells at initial HIV infection correlates with increased time individuals had high CD4 T cell counts, increased time off antiretroviral therapy, and increased time with controlled viremia compared to individuals with low granzyme A CD4 T cells (44). These data suggest cytotoxic CD4 T cells contribute to the immune response against HIV infection.

Cancer

Immune surveillance of cancer cells is an important function of a healthy immune system and is revealed with increased cancer in individuals that have some form of immune suppression. Cancer cells also develop immune evasion mechanisms to avoid being cleared by the immune system. Cytotoxic CD4 T cells have been proposed to also promote clearance of cancer (53, 56) in mouse models of melanoma. Vaccine strategies to promote protective responses against cancer growth also induce cytotoxic CD4 T cells that mediate killing of melanoma cells (52). Further work is required to understand whether these strategies could also translate into treatments for human cancers. CD4 T cells were found to be specific to a neo-antigen generated by mutations in an epithelial cancer. After ex-vivo expansion of these specific CD4 T cells and transfer back into the patient as an immunotherapy, these cells were able to promote tumor regression. Phenotyping of the activated CD4 T cells reveals antigen-specific cytotoxic responses as indicated by CD107a⁺ degranulation (102).

Markers of ThCTL

Phenotyping ThCTL has been hindered by the inability to stain for many cytotoxic proteins in the mouse. The defining feature of ThCTL is cytotoxic function, but conducting cytotoxic assays prevents the use of flow cytometry based phenotyping as well as gene expression analyses to address questions about ThCTL differentiation. Additionally, tracking ThCTL through development isn't feasible using functional cytotoxicity assays. Thus, the field has sought for a marker of cytotoxic CD4 T cells.

KLRG-1

Killer cell lectin-like receptor G1 is a marker of activated CD8 T cells that are short lived effector cells with reduced capacity to become memory cells (103). In a tumor vaccine model, where agonist 4-1BB is used to expand anti-tumor T cells, around half of the tumor infiltrating CD4 T cells upregulate KLRG1 (104). KLRG1 expressing CD4 T cells are enriched in expression of cytotoxic genes compared to KLRG1 negative CD4 T cells, suggesting a potential marker of ThCTL. When KLRG1 positive versus negative CD4 T cells were isolated from tumors, the KLRG1 positive CD4 T cells were enriched in tumor killing ex vivo, indicating KLRG1 marks the ThCTL in the tumor infiltrating CD4 T cells (52). In this setting, KLRG1 also identifies Treg cells, however, when Treg cells were removed, KLRG1 still marked the cytotoxic CD4 T cells. Further expanding the relevance of this marker to other settings, KLRG1 expressing CD4 T cells could be found in mice infected with LCMV or *Listeria monocytogenes* (52). Gene expression

analysis revealed the KLRG1 expressing CD4 T cells have higher expression of cytotoxic genes compared to KLRG1 negative CD4 T cells, including 2 fold higher *prfl* expression. It is unclear, however, whether the KLRG1 negative cells are as activated as the KLRG1 positive cells. Whether or not the KLRG1 had a preferential enrichment of cytotoxic genes compared to other effector genes remains to be studied, although there was no enrichment of T-bet.

The function of KLRG1 on T cells is less well known. KLRG1 can be inhibitory on NK cells due to an ITIM motif in the cytoplasmic region. During MCMV infection, KLRG1 marks NK cells with less IFN γ producing capability, and in vitro cross linking of KLRG1 reduces the amount of IFN γ produced after activation (105), although these NK cells were overexpressing KLRG1. Studies have indicated E-cadherin as the ligand for KLRG1 (106), suggesting a specific effect on T or NK cells when they encounter target cells expressing E-cadherin, like epithelial cells. Whether KLRG1 plays a functional inhibitory role on ThCTL remains to be studied.

CRTAM

The MHC class I restricted T cell associated molecule (CRTAM) is not expressed on naïve NK and CD8 T cells, but upregulated after activation (107). CRTAM binds a Nectin like molecule, Necl-2. Necl-2 is a tumor suppressor gene that mediates cell-cell junctions in different cell types like neurons and epithelial cells (107). Induction of CRTAM on NK cells or CD8 T cells increases cytotoxicity and cytokine production respectively. CD4 T cells can also express CRTAM upon activation (108), albeit not as

high as CD8 T cells in terms of frequency. Further culture of activated cells and re-stimulation results in even higher CRTAM expression. In vitro culture of CD4 T cells under typical ThCTL conditions (50), generates CRTAM⁺ and CRTAM⁻ CD4 T cells. The CRTAM⁺ CD4 T cells shows an enrichment of granzyme B and perforin compared to CRTAM⁻ CD4 T cells (108), as well as display peptide specific cytotoxicity. These results suggest CRTAM as a marker of cytotoxic CD4 T cells. CD4 T cells found in the lungs of influenza-infected mice also express CRTAM, however only after ex vivo re-stimulation. CRTAM can modulate ThCTL function, as CRTAM knockout mice have reduced MHC-II restricted peptide specific cytotoxicity (108). Whether CRTAM is specifically important for the generation of ThCTL or for the modulation of cytotoxicity remains to be studied. Because CD4 T cells needs to be re-stimulated for their expression of CRTAM, it is more difficult to use this marker to track ThCTL.

CD8a

Intraepithelial lymphocytes (IEL) can display cytotoxicity, including CD4 T cells. These ThCTL found in the intestine can express CD8a, normally expressed on CD8 T cells (81). CD4 IEL down-regulate their expression of Thpok allowing for the expression of cytotoxic genes driven by Runx3 expression. A consequence of the Runx3 activation is the expression of CD8a. The ThCTL found in the gut are then marked by CD8a expression (81). When IEL are sorted and assayed for cytotoxic function, the CD4⁺CD8a⁺ and the CD4⁻CD8ab⁺ cells displayed cytotoxicity. Interestingly, the interplay between Thpok and Runx3 may also be occurring elsewhere besides the gut. CD8a gene

expression can be found enriched in CRTAM⁺ CD4 T cells (108), as well as cytotoxic CD4 T cells found in influenza infection (109). Although in both cases, the CD8a protein is not expressed, suggesting perhaps more stringent regulation of the protein in the periphery compared to the gut. Thus, CD8a appears to mark ThCTL in the gut but may not apply to ThCTL found in other tissue sites or in other inflammatory settings.

Thesis Objectives

Because ThCTL have been implicated in the protective function of the immune response against viral infections and cancer, further study of this population is warranted. This thesis will characterize the ThCTL subset of CD4 effector cells and explore the regulation of this subset. In brief, this thesis will put forth a marker of ThCTL, namely the expression of NKG2A/C/E, as a defining feature of ThCTL found in the infected mouse lung. These ThCTL are highly activated and are tissue restricted to the site of infection. ThCTL require Blimp-1 for their differentiation, suggesting additional factors are important for their formation. The encounter with antigen late in the response is also important for ThCTL formation. During this late antigen phase, costimulation through CD80 and CD86 is not required, while non-ThCTL do require costimulation. Furthermore, the role of IL-15 is important during this stage for full ThCTL differentiation. Thus ThCTL, in contrast to other effectors with the exception of Tfh, normally develop only when a pathogen persists and in the tissue site of pathogen replication, providing a novel paradigm of tissue-restricted effector generation.

CHAPTER II: MATERIALS AND METHODS

Mice

BALB/cByJ, C57BL/6 (B6), and B6.PL-*Thy1^a*/CyJ (B6.Thy1.1) mice were obtained from The Jackson Laboratory. *Prdm1^{fl/fl}* mice were originally received from Dr. Alexander Tarakhovsky (The Rockefeller University, New York) and were bred with *Cd4-cre⁺* (Blimp-1 CKO) (110). Blimp-1 CKO OT-II cells were obtained by crossing *Prdm1^{fl/fl} Cd4-cre⁺* mice with OT-II TCR transgenic (Tg) mice. B6.OT-II.Thy1.1.*Hcst^{-/-}/Tyrobp^{-/-}* (OT-II.Thy1.1 DAP10/12 KO) cells were obtained by breeding *Hcst^{-/-}/Tyrobp^{-/-}* mice (111)(kindly provided by Dr. Toshiyuki Takai, Tohoku University and Dr. Lewis Lanier, University of California, San Francisco) with OT-II.Thy1.1 TCR Tg mice. *H2-t23^{-/-}* (Qa-1 KO) (112) cells were kindly provided by Dr. Harvey Cantor (Dana Farber Cancer Institute). *Cd80^{-/-}Cd86^{-/-}* was obtained from Dr. Joonsoo Kang (UMMS). *Tcra^{-/-}Tcrb^{-/-}* mice were obtained from Dr. Raymond Welsh (UMMS). *Il15ra^{-/-}* mice were obtained from The Jackson Laboratory. *Il15ra^{fl/fl}* mice were obtained from The Jackson Laboratory and bred with *Cd4-Cre⁺* mice. The subsequent *Il15ra^{fl/fl}Cd4-Cre⁺* mice were then bred to OT-II TCR transgenic mice. *Nr4a1^{GFP}*(Nur77GFP) mice were obtained originally from The Jackson Laboratory, and bred with the OT-II TCR transgenic to obtain OT-II Nur77 GFP. HNT mice express a TCR recognizing amino acid 126-138 of A/PuertoRico/8/34 (PR8, H1N1) HA and OT-II mice express a TCR recognizing amino acid 323-339 of chicken ovalbumin (OVA). OT-II.Thy1.1 TCR Tg and HNT.Thy1.1 TCR Tg mice were obtained from the animal breeding facility at Trudeau Institute or

University of Massachusetts Medical School (UMMS). SMARTA TCR transgenic mice (kindly provided by Dr. Raymond Welsh, UMMS) express a TCR recognizing LCMV epitope gp₆₁₋₈₀. DO11.10 and *IL2*^{-/-} DO11.10 cells (113) were obtained from Dr. Kai McKinstry and Dr. Tara Strutt from the University of Central Florida. All mice were at least 8 weeks old at time of infection. Naïve CD4⁺ cells were obtained from 5-8 week old mice. Experimental animal procedures were conducted in accordance with the UMMS Animal Care and Use Committee guidelines.

Naïve cell isolation

In adoptive transfer experiments, naïve cells were isolated from naïve mice as previously described (114). Briefly, peripheral lymph nodes and spleens were harvested from mice and processed into single cell suspensions. Cells were then centrifuged through a percoll gradient (GE healthcare) and the layer between 80% and 62% was isolated. Cells were then enriched for CD4 T cells using magnetic enrichment (Miltenyi Biotec). Naïve cells are routinely >90% TCR transgenic⁺ and expressed a naïve phenotype of CD44^{lo} and small size. 0.5 x 10⁵ purified naïve cells were adoptively transferred in 200 ul of PBS intravenously into mice. Mice were then subsequently infected with virus on the same day.

Virus stocks and infections

Mouse-adapted influenza viruses A/Puerto Rico/8/34 (A/PR8), (H1N1) originating from stocks at St. Jude Children's Hospital and A/PR8-OVA_{II} (H1N1) (kindly

provided by Dr. Peter Doherty) were grown in the allantoic cavity of embryonated hen eggs at the Trudeau Institute. Mice were infected intranasally (i.n.) under light isoflurane anesthesia (Piramal Healthcare) with virus in 50 μ l PBS. Mice received a 0.3 LD₅₀ dose of IAV. Mice that received adoptively transferred T cells were infected on the same day as cell transfer. For LCMV experiments, mice were given 5×10^4 p.f.u. of LCMV Armstrong (generously provided by Dr. Raymond Welsh, UMMS) strain with intraperitoneal injection.

Cell preparation from tissues.

At the time points indicated after infection, mice were euthanized and lungs, spleen, and draining mediastinal lymph nodes (dLN) were harvested and single cell suspensions prepared by mechanical disruption of organs and passage through 70 μ m nylon mesh. For some experiments, mice were euthanized and bronchioalveolar lavage (BAL) was performed. Peripheral blood was collected with cardiac puncture and mice were perfused with 10 mL of PBS and the lungs, dLN, spleen, kidney, and liver was taken. The lungs, kidney, and liver were digested with collagenase P (Roche) and passed through a nylon membrane. Liver cell suspensions were layered over percoll (GE Healthcare) and the interface between the 40% and 70% layers was collected.

Flow cytometry and sorting

Cell suspensions were washed, resuspended in PBS for incubation on ice with 1 μ g anti-FcR (2.4G2) followed by staining for viability. Cells were then resuspended in

FACS buffer (PBS + 0.5% bovine serum albumin and 0.01% sodium azide; Sigma-Aldrich) and incubated with combinations of fluorochrome-labeled antibodies (Ab) for surface staining: anti - CD4 (GK1.5, RM4-4, RM4-5), CD25 (3C7), CD27 (LG.7F9), CD44 (IM7), CD69 (H1.2F3), CD90.1 (Thy1.1, OX-7 and HIS51), CD127 (A7R34), CD150 (SLAM, TC15-12F12.2), CD152 (CTLA-4, UC10-4B9), CD183 (CXCR3, CXCR3-173), CD185 (CXCR5, SPRCL5), CD186 (CXCR6, SA051D1), CD195 (CCR5, HM-CCR5), CD215 (IL-15R α , DNT15Ra), CD279 (PD-1, 29F.1A12), CD335 (NKp46, 29A1.4), GL-7 (GL-7), MHC-II (I-A/I-E, M5/114), NKG2A/C/E (20d5), NKG2A (16a11), NK1.1 (PK136). Ab purchased from eBioscience, BioLegend, and BD Biosciences. Dead cells were excluded using Live/Dead Fixable Amine Dye (Invitrogen). For P-selectin binding, cells were incubated with mouse P-selectin-IgG fusion protein (BD Biosciences), washed and detected with fluorescent goat anti-human IgG (Jackson Immunoresearch) secondary Ab. For NKG2A and NKG2A/C/E co-staining, cells were incubated with anti-NKG2A first, washed and then incubated with anti-NKG2A/C/E to avoid steric blocking. For tetramer staining, cells were stained for 1 hr at 37°C with fluoroconjugated I-A^b-NP₃₁₁₋₃₂₅ tetramer obtained from the NIH tetramer facility or obtained from Dr. Lawrence Stern (UMMS) prior to surface marker staining.

For intracellular cytokine staining, cells were stimulated with Phorbol 12,13-dibutyrate (Sigma) and Ionomycin (Sigma) or peptide pulsed B cells (stimulated with LPS and dextran sulfate), brefeldin A (Sigma), monensin (BD Golgi Stop), and anti-CD107a (LAMP-1, 1D4B, Biolegend). Cells were then surface stained and fixed for 20 min in 4% paraformaldehyde followed by permeabilization for 15 min by 0.1% saponin

buffer (PBS plus 1% FBS, 0.1% NaN₃ and 0.1% saponin; Sigma-Aldrich) and stained with anti-IFN γ (XMG1.2, eBioscience) for 30 minutes. IL-21 was stained with anti-IL21R Fc fusion protein (R&D Systems) with subsequent secondary antibody for detection. Granzyme B (GB11, Thermo Fisher) expression was determined by intracellular staining directly *ex vivo*. For transcription factor staining, cells were fixed and permeabilized according to manufacturer's protocol (eBioscience) and then stained with labeled anti-Tbet (4B10), anti-Eomes (Dan11mag), anti-FoxP3 (FJK-16s), and anti-Bcl6 (K112-91) (eBioscience and BD Biosciences).

All flow cytometry results were acquired using LSRII flow cytometers (BD Biosciences) and analyzed with FlowJo (Tree Star) analysis software.

For flow sorting, lungs were pooled and target cells were enriched by magnetic enrichment (MACS, Miltenyi Biotec) for CD90.1 according to manufacturer's protocol. Enriched cells were then stained to isolate NKG2A/C/E⁺ or NKG2A/C/E^{neg} of CD4⁺ CD90.1⁺ CD8a^{neg} NK1.1^{neg} I-A^{b neg} cells using the FACS Aria cell sorter (BD Biosciences). We routinely get 75-90% purity of NKG2A/C/E⁺ effectors and >95% purity for the NKG2A/C/E^{neg} effectors.

Cytotoxic Assays

For *ex vivo* cytotoxic assays, effector cells were isolated either through magnetic enrichment (MACS, Miltenyi Biotec) or by flow sorting from pooled lungs of influenza infected mice. Effectors were pre-incubated with anti-CD178 (Fasl, MFL3, eBioscience). Target cells were generated 2 days prior by stimulating CD19 MACS enriched spleen

cells with 25 $\mu\text{g/ml}$ LPS and 25 $\mu\text{g/ml}$ dextran sulfate (Sigma). Targets were separated into two fractions (targets and bystanders) and labeled with either 1 μM or 0.4 μM of dye (Carboxyfluorescein succinimidyl ester (CFSE) or CellTrace Violet, Thermo Fisher). Targets were pulsed with cognate peptide at 5 μM for 1hr at 37°C, including OVA₃₂₃₋₃₃₉ (ISQAVHAAHAEINEAGR), NP₃₁₁₋₃₂₅ (QVYSLIRPNENPAHK), NP₂₁₆₋₂₃₀ (RIAYERMCNILKGKF) (115), all from New England Peptide. gp₆₁₋₈₀ (GLNGPDIYKGVYQFKSVEFD) was obtained from Dr. Raymond Welsh (UMMS). Targets and bystanders were mixed at 1:1 and co-cultured with effectors at the indicated effector to target ratios for 4 hr at 37°C and 5% CO₂. In some cases, anti-MHC-II Ab (M5/114, BioXcell) was added at 20 $\mu\text{g/ml}$ or anti-NKG2A/C/E (20d5, eBioscience) was added at 10 $\mu\text{g/ml}$. Plates were then harvested, washed, and stained with Annexin V, 7-Aminoactinomycin D (7-AAD) (Sytox), or Live/Dead Amine (all Thermo Fisher). Specific killing was calculated as 100 x (1 - (live targets/live bystanders normalized to no effector control wells)). Peptide pulsing and cytotoxic assays were done in complete RPMI media (cRPMI) (RPMI 1640 containing 7.5% fetal bovine serum, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 100 IU penicillin, 100 $\mu\text{g/ml}$ streptomycin and 10 mM HEPES).

For *in vivo* cytotoxicity assays (85), CD90.2 depleted spleens (MACS) were split into targets and bystanders and labeled and pulsed with peptide like above. Targets and bystanders were mixed at a 1:1 ratio and injected i.v. into host mice. 18 hr later, mice were sacrificed and cells from the spleen were isolated for staining. Specific killing was

calculated as $100 \times (1 - (\text{live MHC-II}^+ \text{ targets}/\text{live MHC-II}^+ \text{ bystanders normalized to the ratio found in uninfected mice}))$).

In vitro cultures

Cells were isolated using magnetic beads (MACS) to enrich for CD90.1 (Miltenyi Biotec). 96 U bottom plates were pre-coated with anti-CD3 antibodies (2C11) at 0.5 $\mu\text{g}/\text{ml}$. Cells were plated at $1-2 \times 10^5$ cells per well and cultured with complete T cell media. CD28 (37.51) was added in solution to some wells at 20 $\mu\text{g}/\text{ml}$. IL-2 generated in house was added to some wells at the indicated concentrations. Anti-CD25 (PC61) and anti-CD122 (TM- β 1) was also added at 10 $\mu\text{g}/\text{ml}$ to some wells (BD Biosciences). Cells were cultured for 2 days at 37°C with 5% CO₂.

Intravenous labeling

2.5 μg of anti-CD4 clone (clone RM4-5) was injected i.v. into infected host mice. Mice were euthanized 3-5 minutes after injection and harvested quickly. Peripheral blood was taken via cardiac puncture and the mouse was perfused with 10 mL of PBS. Organs were taken and single cell suspensions were stained with anti-CD4 (clone RM4-4) as normal. Staining of peripheral blood showed CD4 positive (clone RM4-4) cells were also >95% CD4 (clone RM4-5) positive indicating sufficient i.v. labeling.

Effector isolation and transfer

At 6 dpi, dLN and spleens were isolated from infected mice and pooled. Single cell suspensions were obtained and enriched with magnetic beads (Miltenyi Biotec) for CD90.1. Positively selected cells were then washed and adoptively transferred into hosts in 200 μ L PBS i.v. Isolation and processing was all done at room temperature except incubation with magnetic beads, which was done at 4°C. Care was taken to minimize the time effector cells were outside of the mouse, and experiments routinely averaged ~2 hours from start to finish.

Neutralizing antibody and IL-2 complex treatment

Neutralizing IL-2 antibodies clones S4B6 and JES6-1A12 or isotype IgG2A control (BioXcell) were mixed (250 μ g each) (113) and administered in 200 μ L PBS i.p. into mice. For IL-2 complex: 2 μ g of recombinant mouse IL-2 (ebioscience) was mixed with 20 μ g of anti-IL-2 (S4B6, BD Biosciences) (113) for 20 min at room temperature. IL-2 complexes were then administered i.n. into lightly anesthetized mice.

Real Time-PCR and microarray analysis

Isolated cell populations from flow sorting were immediately placed in RNA cell protect (Qiagen) and frozen at -80° C until extraction. RNA was extracted (Qiagen) and complementary RNAs were labeled and hybridized onto Affymetrix 2.0 ST arrays according to manufacturer's protocols. Data was normalized with the RMA algorithm and log transformed using Affymetrix expression console. P-values were generated using

unpaired ANOVA in Affymetrix Transcriptome Analysis Console 3.0. Heat maps were generated using Gene-E (The Broad Institute). Differential expression of selected genes was validated by reverse transcribing RNA and amplifying using Taqman gene expression assays (Thermo Fisher). The fold increase in expression of NKG2A/C/E⁺ relative to NKG2A/C/E^{neg} was determined with the “ $2^{-\Delta\Delta CT}$ ” method.

Statistical analysis

Unpaired, two-tailed, Students t-tests with an $\alpha = 0.05$, were used to assess whether the means of two normally distributed groups differed significantly. Cell numbers were routinely not normally distributed and were \log_{10} transformed. The Welch-correction was applied when variances were found to differ. Paired analysis was done when comparing populations within the same mouse. When comparing three groups, one-way ANOVA was used to determine significance with post-hoc Tukey test to determine significance between individual groups. Statistical analyses were performed using GraphPad Prism. Significance is indicated as * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$, and **** $P < 0.0001$. All error bars represent the standard error of the mean.

CHAPTER III: ThCTL CHARACTERIZATION AND REGULATION

NKG2A/C/E Identifies Cytotoxic CD4 T Cells.

Although cytotoxic CD4 T cells have been characterized in various infection and tumor models, a search for a definitive marker of these cells has been elusive. One of the challenges of studying a subset of T cells identified by their function is the difficulty with identifying their phenotype and characteristics and studying the regulation of their generation with single-cell level resolution. The use of a surface marker enables the isolation of these cells without damaging the cells with intracellular staining or alteration of the cells with the requirement of stimulation. Previously, candidate markers of cytotoxic CD4 T cells in other settings have been proposed: CD8a marks cytotoxic CD4 T cells in the gut (81) and Eomes marks cytotoxic CD4 T cells in the tumor environment (52). Additionally, CRTAM can also mark cytotoxic CD4 T cells in influenza infection but only when the cells are stimulated *ex vivo* (108). We sought to find a marker of cytotoxic CD4 T cells in influenza infection and after preliminary screens of NK-associated markers, honed in on a family of NK cell receptors. The NKG2A/C/E family of receptors is part of the killer cell lectin-like receptors (KLR) with C-type lectin domains. NKG2A/C/E (KLRC) (NKG2X) exists as a homodimer with CD94 and is found on NK cells (116) and CD8 T Cells (117). The NKG2A receptor has two immunoreceptor tyrosine-based inhibitory motifs (ITIM) that is proposed to deliver an inhibitory signal to the cell when the receptor is engaged (116–118). NKG2C and NKG2E lack the ITIM motif and are proposed to activate the cell through association

with adaptor proteins DAP10 and DAP12(119, 120). It has been shown that one ligand for NKG2A/C/E is the non-classical class I molecule, Qa-1 (116, 121), but there may be additional unidentified ligands. Surface NKG2A/C/E expression has been found on some CD4 T cells in both mice (122) and humans (123). In mice, NKG2A/C/E expression is induced only on in vitro activated CD4 T cells polarized towards the Th1 subset but not on cells polarized towards the Th2 subset (122). Interestingly, NKG2A/C/E begins to be expressed after 3 rounds of in vitro activation and polarization, suggesting high or repeated activation signals could be important for CD4 expression of NKG2A/C/E. Further, activated CD4 T cells in patients with multiple sclerosis (MS) can express NKG2C and have a cytotoxic phenotype (123). These NKG2C⁺ CD4 T cells are detectable in the brain tissue of MS patients. These data suggest that NKG2A/C/E could mark a highly activated subset of effector CD4 T cells, and could potentially identify ThCTL during viral infection.

To determine whether CD4 T cells express the NKG2X family of receptors during influenza, I infected B6 mice with a sub-lethal dose of influenza virus PR8. At day 9 post infection, I isolated cells from the lung tissue of infected mice and stained for CD4 effectors and stained with the antibody clone 20d5 that identifies all three of the family members of NKG2X: NKG2A/C/E. To identify antigen specific CD4 T cells, I also used a tetramer loaded with the NP₃₁₁₋₃₂₅ peptide derived from influenza nucleoprotein. Figure 3.1A shows that indeed antigen specific CD4 T cells express NKG2A/C/E during the peak of influenza infection. I also looked at TCR transgenic systems and found that the

Figure 3.1: Lung effector CD4 T cells express NKG2A/C/E

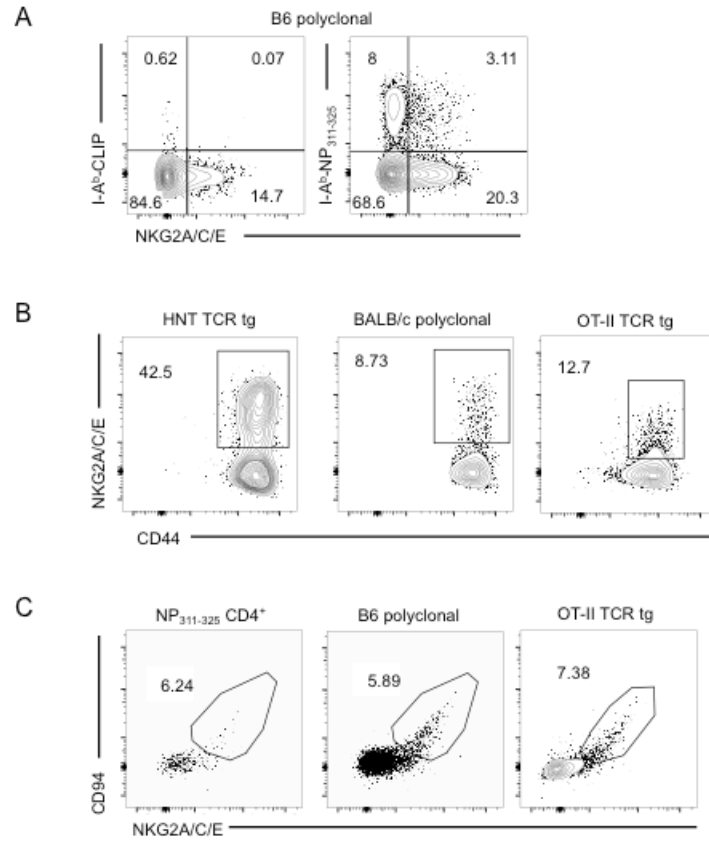


Figure 3.1: Lung effector CD4 T cells express NKG2A/C/E

(A) Naïve B6 mice were infected with PR8-OVA_{II} and lungs were stained at 8 dpi. Representative flow plots of NKG2A/C/E expression and NP₃₁₁₋₃₂₅ tetramer staining is shown. Gated on live CD4 T cells. **(B)** Expression of NKG2A/C/E on different CD4 populations. Naïve HNT TCR transgenic or OT-II TCR transgenic CD4 T cells were transferred into BALB/c or B6 mice, respectively and infected with PR8 or PR8-OVA_{II} respectively. 8 dpi, lungs were stained for NKG2X and representative flow plots are shown for HNT effectors (left), polyclonal Balb/c effectors (middle), and OT-II effectors (right). Cells were gated on live CD4⁺ CD44^{hi} and donor congenic marker. **(C)**. Expression of CD94 with NKG2X. Representative flow plots of NKG2X and CD94 staining gated on NP₃₁₁₋₃₂₅ tetramer⁺ (left), polyclonal CD44^{hi} CD4⁺ in B6 mice (middle), and OT-II effectors (right). Data is representative of at least n = 5 mice per population, and at least two independent experiments.

OT-II TCR transgenic and the HNT TCR transgenic also expressed NKG2X at the peak of the influenza response (Figure 3.1B). Additionally the polyclonal CD4 effectors in the lungs of infected BALB/c mice had NKG2X expression (Figure 3.1B). Because NKG2X is normally expressed as a homodimer with CD94, I co-stained with CD94 and saw that the NP₃₁₁₋₃₂₅ specific CD4 effectors, the polyclonal CD4 effectors in B6 mice, and the OT-II TCR transgenic CD4 effectors also co-express NKG2X with CD94 at the peak of the influenza response (Figure 3.1C). These results also confirm what the lab has shown previously that the HNT TCR transgenic CD4 effectors in the lung co-express NKG2X and CD94 (109) at the peak of the influenza response.

Based on the previous results where NKG2X expression was found on highly polarized Th1 cells (122) and that human NKG2C⁺ CD4 T cells displayed cytotoxicity (123), we hypothesized that NKG2X marks the ThCTL subset of CD4 effectors during influenza infection. To test whether NKG2X marks ThCTL I isolated CD4 effectors from influenza infected mice and assayed the effector's ability to lyse peptide pulsed target cells. I transferred naïve OT-II CD4 T cells into naïve B6 mice and infected the mice with PR8-OVA_{II} strain of influenza, which was engineered to encode the OT-II cognate antigen (OVA₃₂₃₋₃₃₉) in the HA of PR8 (124). At day 8 post infection, I isolated the lungs and flow sorted for NKG2A/C/E expressing donor OT-II cells and non-expressing donor cells. I then assayed both population's ability to lyse peptide pulsed target cells and found that the NKG2A/C/E⁺ CD4 T cells were able to kill target cells in a peptide-specific manner (Figure 3.2). Importantly, blocking MHC-II on the target cells abolished the killing activity, indicating the cytotoxicity is dependent on peptide-MHC-II

Figure 3.2: NKG2A/C/E marks cytotoxic CD4 T cells in influenza infection

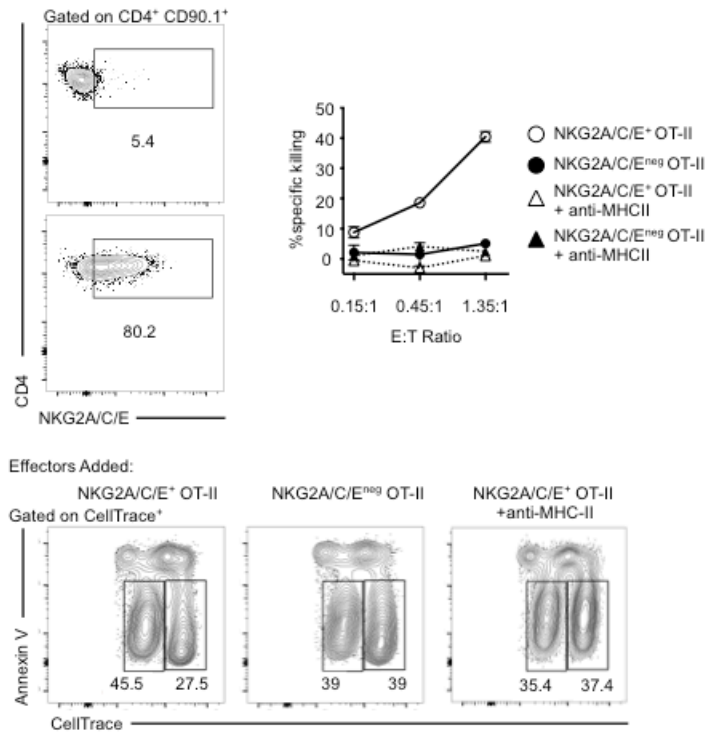


Figure 3.2: NKG2A/C/E marks cytotoxic CD4 T cells in influenza infection

Naïve OT-II CD4 T cells were transferred into B6 mice and infected with PR8-OVA_{II}. 8 dpi, lungs were harvested and pooled. Donor OT-II cells were flow sorted based on the expression of NKG2X. Representative purities are shown of the NKG2X⁺ and NKG2X^{neg} population (Top left, and middle left). Cytotoxicity of each population was assayed ex vivo. MHC-II was blocked with anti-MHC-II antibodies (Top right). Representative staining of the cytotoxicity assays, where target cells identified by CellTrace staining were assayed for staining of Annexin V (Bottom panels). Representative data from at least two independent experiments, where cells were isolated from pooled n=10 infected lungs each time. Mean ± SEM.

Figure 3.3: NKG2A/C/E⁺ CD4 effectors express proteins associated with cytotoxicity

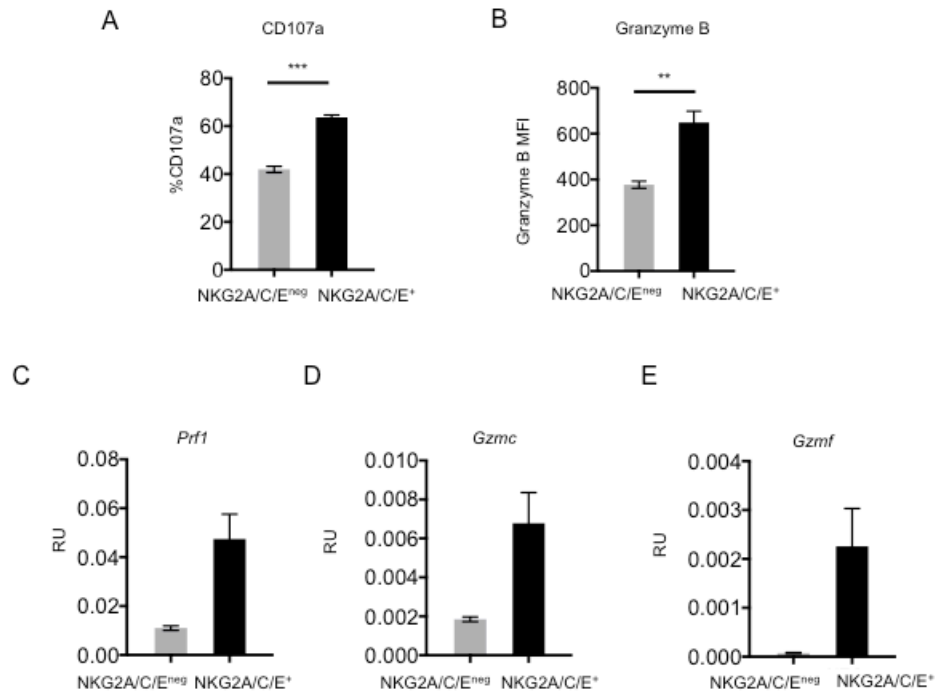


Figure 3.3: NKG2A/C/E⁺ CD4 effectors express proteins associated with cytotoxicity

(A) Naïve OT-II CD4 T cells were adoptively transferred into B6 mice and infected with PR8-OVA_{II}. At 8 dpi, lung donor cells were enriched and assayed for degranulation by stimulation from activated APC plus cognate peptide. CD107a expression was compared between NKG2X⁺ and NKG2X^{neg}. (B) Similarly donor cells were stained for the expression of Granzyme B by flow cytometry. (C-E) Naïve OT-II CD4 T cells were transferred into B6 mice and infected with PR8-OVA_{II}. At 8 dpi, lung donor cells were enriched and sorted based on the expression of NKG2X. RNA was isolated from sorted cells and gene expression was determined using Taqman primers for (C) Perforin (D) Granzyme C (E) Granzyme E. Data is representative of 2 independent experiments with n = 5 each experiment (A, B) or from pooled lungs from n=10 mice each experiment (C-E). Mean ± SEM. ** $P < 0.005$, *** $P < 0.001$.

interactions. These experiments were also performed in the BALB/c system with HNT CD4 TCR transgenic mice, indicating the NKG2A/C/E marks cytotoxic CD4 T cells in both B6 and BALB/c mice (109). To further look at the phenotype of the NKG2A/C/E⁺ CD4 effectors, I assayed various markers of cytotoxicity. CD107a is a marker of degranulation and is a correlate with cytotoxic function (125). I enriched for donor OT-II CD4 effectors and stimulated the cells ex vivo with peptide pulsed APCs to measure CD107a expression. The NKG2A/C/E⁺ donor CD4 T cells are enriched in CD107a expression, suggesting more degranulation and cytotoxicity (Figure 3.3A). Granzyme B is a cytotoxic effector molecule that promotes apoptosis once inside a target cell (28). At the peak of infection, ThCTL marked by NKG2X expression have higher expression of granzyme B protein (Figure 3.3B). To assay gene expression of cytotoxic molecules by ThCTL, I isolated NKG2X⁺ and NKG2X^{neg} OT-II CD4 T cells from the lungs at 8 dpi and purified the RNA for quantitative reverse transcription polymerase chain reaction (qPCR). NKG2X⁺ cells were enriched for expression of perforin (*Prfl*), granzyme C (*Gzmc*), and granzyme F (*Gzmf*) compared to NKG2X^{neg} CD4 T cells, suggesting the NKG2X⁺ CD4 effectors are enriched in cytotoxic molecule expression (Figure 3C-3E). These data together suggest that NKG2A/C/E marks the cytotoxic CD4 T cells in influenza infection.

NKG2X minimally impacts cytotoxicity of ThCTL.

Since NKG2X marks ThCTL, we asked whether NKG2X plays a role in ThCTL function. In NK cells, NKG2X can promote or inhibit the cytotoxicity of NK cells (116,

126). NKG2A is inhibitory, while NKG2C and NKG2E are activating. To understand which isoforms ThCTL express in B6 mice, I utilized the 16A11 clone antibody that specifically binds to NKG2A in B6 mice. First staining with 16A11 and then subsequently staining with 20d5 enables one to see co-expression of NKG2A and NKG2C/E or combinations of them. I transferred naïve OT-II CD4 T cells into B6 mice and infected with PR8-OVA_{II}. On day 8 post infection, I harvested the lungs for flow staining of the NKG2 family of receptors. Staining for NKG2A reveals that few of the donor effector cells express NKG2A compared to NK cells. Most of the NKG2X receptor on ThCTL thus are NKG2C/E, while NK cells express all three and are NKG2A⁺C/E⁺ (Figure 3.4A). Taken together, these data show that ThCTL express activating receptors of the NKG2X complex. Because ThCTL express the activating receptors and these receptors have been shown to co-stimulate cytotoxicity in NK cells (126), I hypothesized that the NKG2C/E are co-stimulating the cytotoxicity of ThCTL. To test whether these receptors played a role in cytotoxicity of ThCTL, I blocked NKG2X function in two ways. First, I isolated effector OT-II CD4 T cells from the lung at 8 dpi and assayed their cytotoxicity against target cells lacking Qa-1 (112). Qa-1 is a non-classical MHC-I protein that is the ligand for the NKG2A/C/E-CD94 heterodimer complex (116). ThCTLs were equally able to kill wild type and Qa-1 deficient target cells (Figure 3.4B) above control splenic donor OT-II, which display minimal cytotoxicity (47). Because the 20d5 antibody is reported to be blocking and can block NK cell killing of target cells (121), I utilized this blocking antibody to block NKG2X. Addition of the 20d5 antibody to the cytotoxic assay with isolated effector CD4 T cells show that

Figure 3.4: NKG2X receptors minimally impact cytotoxicity of ThCTL

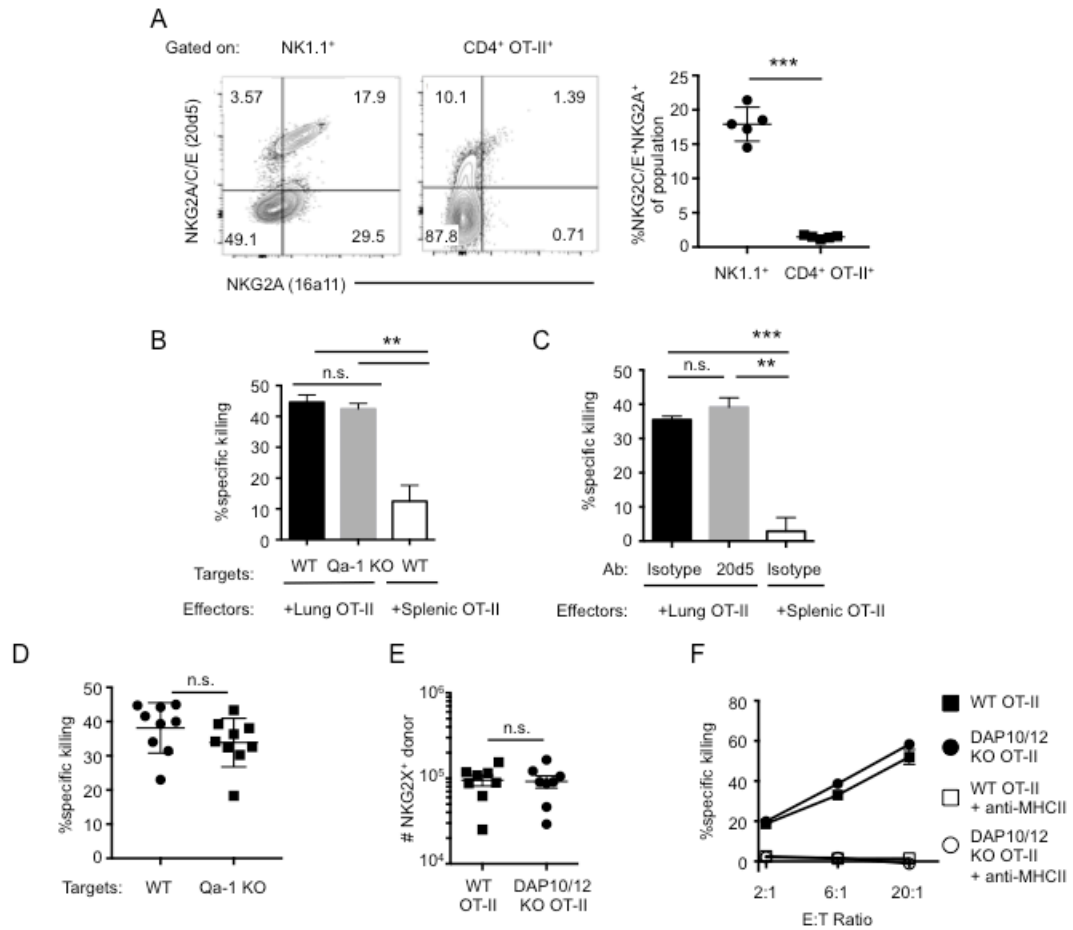


Figure 3.4: NKG2X receptors minimally impact cytotoxicity of ThCTL

(A) Naïve OT-II CD4 T cells were adoptively transferred into B6 mice and infected with PR8-OVA_{II}. At 8 dpi, lungs were isolated and stained with NKG2A and NKG2A/C/E. Representative plots of NKG2A and NKG2X expression on NK cells (left) or donor OT-II effector cells (middle). Quantification of the percent NKG2A⁺NKG2A/C/E⁺ (right). **(B)** Lung donor OT-II CD4 T cells were isolated from infected lungs and assayed for cytotoxicity against peptide pulsed target wild type and Qa-1 KO cells. Spleen donor OT-II CD4 T cells were also isolated and assayed for cytotoxicity against peptide pulsed wild type target cells. **(C)** Lung donor OT-II CD4 T cells were isolated from infected lungs and assayed for cytotoxicity against peptide pulsed target wild type cells. Anti-NKG2A/C/E was added in the assay as well. Spleen donor OT-II CD4 T cells were also isolated and assayed for cytotoxicity against peptide pulsed wild type target cells. **(D)** Naïve OT-II CD4 T cells were adoptively transferred into B6 mice and infected with PR8-OVA_{II}. At 7 dpi, peptide pulsed wild type or Qa-1 KO target cells were transferred i.v. into hosts. At 8 dpi, the in vivo peptide specific cytotoxicity was assayed. **(E)** Naïve wild type or DAP10/12 KO OT-II CD4 T cells were adoptively transferred into B6 mice and infected with PR8-OVA_{II}. At 8 dpi, the number of NKG2X⁺ donor CD4 T cells was enumerated. **(F)** Naïve wild type or DAP10/12 KO OT-II CD4 T cells were adoptively transferred into B6 mice and infected with PR8-OVA_{II}. At 8 dpi, lung donor CD4 T cells were isolated and assayed for peptide specific cytotoxicity. Anti-MHC-II was added in control wells. Data is representative of at least 2 independent experiments (A-C,F) with an n=5 mice each time (A) or pooled from n = 10 mice each time (B-C,F). Data was pooled from 2 independent experiments (D-E) with n = 8-9 each genotype. Target cells were pooled from n = 2 mice each time. Mean ± SEM. ** *P* < 0.005, *** *P* < 0.001.

cytotoxicity was not impacted compared to isotype control treated wells (Figure 3.4C). Both isotype and 20d5 treated lung effectors had increased cytotoxicity above control splenic effector CD4 T cells. These data suggest that NKG2X has minimal impact on the ex vivo cytotoxicity of ThCTL. Although I assayed killing ex-vivo, the role of NKG2X may be more apparent in vivo. To test if NKG2X plays a role in in vivo cytotoxicity by ThCTL, I transferred naïve OT-II CD4 T cells in naïve B6 mice and infected with PR8-OVA_{II} to generate effector CD4 T cells in vivo. On day 7 post infection, I transferred OT-II peptide pulsed either WT or Qa-1 KO target cells into mice. 18 hours after transfer, I harvested the mice and target cells were enumerated for their relative viability compared to unpulsed bystander cells to calculate specific cytotoxicity in vivo. ThCTL in the infected mice killed both wild type and knockout target cells equally in vivo, suggesting that in vivo these cells do not need activation of NKG2X to co-stimulate cytotoxicity (Figure 3.4D). Since Qa-1 is the reported ligand for NKG2X, I utilized Qa-1 KO targets to test my hypothesis. However, there could be multiple uncharacterized ligands for NKG2X. To test the role of NKG2X independent of Qa-1, I utilized mice deficient in the signaling adaptors for NKG2C and NKG2E. DAP10 and DAP12 are signaling adaptors that transduce the activation signal made by NKG2C and NKG2E (119, 120). In order to test the T cell intrinsic requirement for DAP10 and DAP12, I crossed the DAP10/12 KO mice (111) to the OT-II CD4 transgenic mouse and generated OT-II CD4 T cells lacking DAP10 and DAP12. DAP10 and DAP12 are responsible for the expression of Ly49H (127) as well as NKG2D on NK cells (128), but are not needed for NKG2X expression (127). I found that DAP10/12 KO OT-II CD4 T cells are able to generate similar numbers

of ThCTL by expression of NKG2A/C/E indicating these adaptor molecules are not required for ThCTL generation (Figure 3.4E). Although ThCTL was assayed using the expression of NKG2X, whether the DAP10/12 KO OT-II were functionally defective, I tested the cytotoxicity of these cells. Isolating WT and DAP10/12 KO OT-II CD4 effector cells from lungs at 8 dpi and assaying their peptide specific cytotoxicity shows that both WT and DAP10/12 KO OT-II cells are both equally able to kill target cells in a MHC-II dependent manner (Figure 3.4F). Taken together these data suggest that the NKG2X complex, although marking ThCTL, do not play a significant role in modulating the cytotoxicity of ThCTL. These data are in contrast to reported roles of NKG2X in co-stimulating NK cells (126), which suggests NKG2X may be acting differently in CD4 T cells compared to NK cells. Alternatively, NKG2X may play a role other than cytotoxicity, which will require further study. One possibility is that the requirement of NKG2X is only required for killing certain kinds of targets. Thus it will require further study to fully understand the role of NKG2X in ThCTL, but the most obvious role in induction of cytotoxicity is not seen.

ThCTL are highly activated effectors in influenza infection.

One of the major drawbacks in studying a subset of effector cells characterized by function, is the inability to track and phenotype these cells. Further, it is unknown whether ThCTL also transition to a memory cell after clearance of influenza infection. Because NKG2X marks ThCTL during influenza infection, we can further assess the phenotype of ThCTL. I infected wild type B6 mice with PR8-OVA_{II} and harvested cells

Figure 3.5: ThCTL phenotype and kinetics

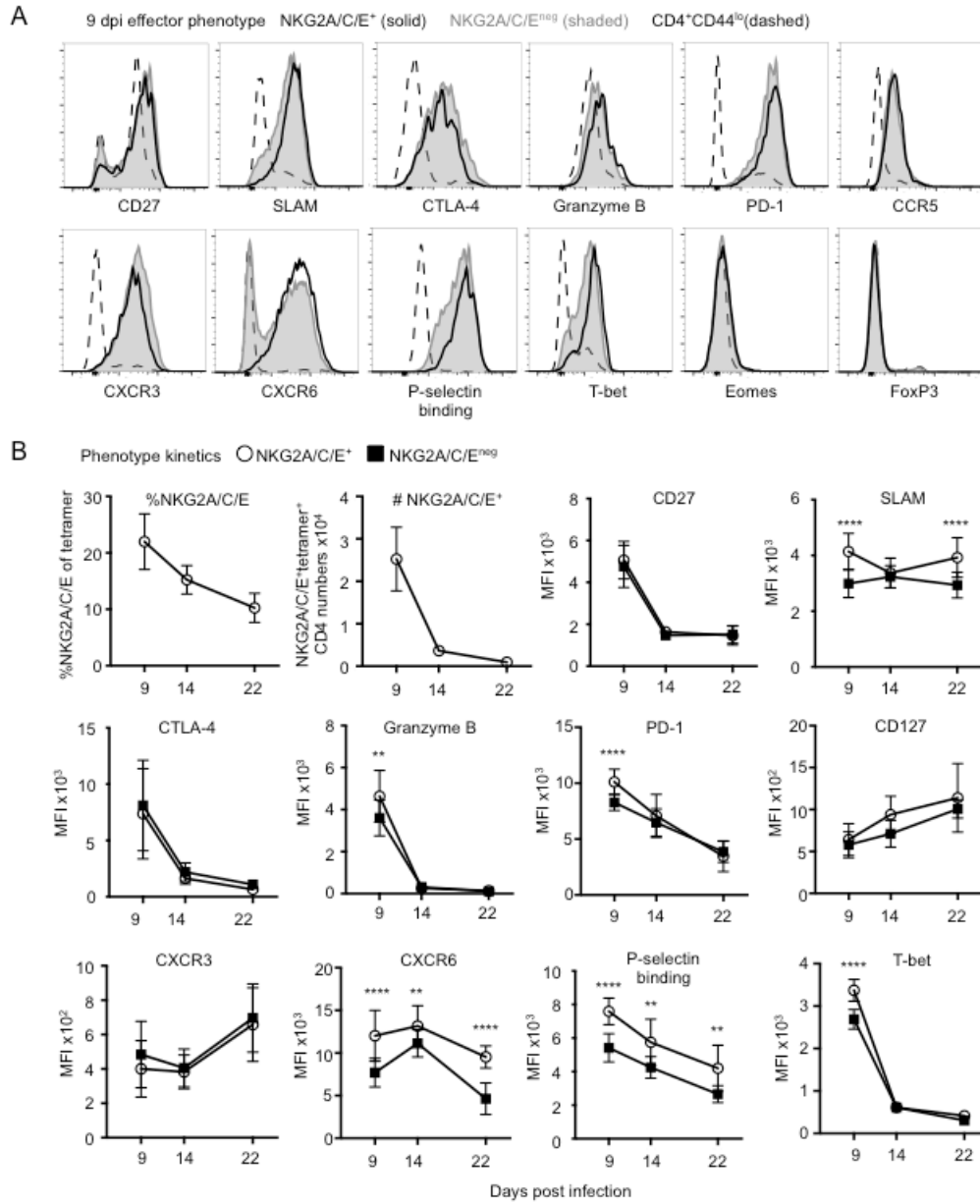


Figure 3.5: ThCTL phenotype and kinetics

(A) Representative histograms of the expression of phenotypic markers on either naïve cells CD4⁺ CD44^{lo} (dashed lines), NKG2A/C/E^{neg} (shaded), or NKG2A/C/E⁺ (solid line). Naïve B6 mice and infected with PR8-OVA_{II} and at 9 dpi lungs were isolated and stained for the markers shown below each histogram overlay. Cells were gated on live CD4⁺ NP₃₁₁₋₃₂₅ tetramer⁺ cells. **(B)** Naïve B6 mice and infected with PR8-OVA_{II} and at 9 dpi, 14 dpi, and 22 dpi, lungs were isolated and stained for the indicated markers above each graph. The numbers and percent of NKG2X⁺ NP₃₁₁₋₃₂₅ tetramer⁺ cells was also enumerated. Data are representative of at least 2-3 independent experiment with n=4-5 each experiment (A). Data are pooled from 2-3 independent experiments with n = 9-10 for each time point. Mean ± SEM. ** $P < 0.005$, **** $P < 0.0001$.

from infected lungs at 9, 14, and 22 dpi. I then compared the effector CD4 T cell phenotype for a number of effector and memory associated cell surface proteins by gating on the NKG2X positive (ThCTL) and negative (non-ThCTL) fractions. At the peak of the response, 9 dpi, ThCTL resemble a highly activated effector subset (Figure 3.5A). As expected, ThCTL have increased granzyme B expression. Overall, ThCTL have higher expression of SLAM, PD-1, CXCR6, T-bet, and binding to P-selectin compared to non-ThCTLs (Figure 3.5B). Signaling lymphocytic activation molecule 1, (SLAM, CD150), is expressed on many T cells. Activated CD4 T cells can increase expression of SLAM, while Tfh cells express lower levels of SLAM (37), and Bcl-6 over-expression leads to reduced SLAM expression (69). The increased SLAM expression on ThCTL suggests at least that they have suppressed Bcl-6 and or the Tfh program. Programmed cell death protein 1, (PD-1, CD279), is an inhibitory receptor of the CD28 family and can be expressed on exhausted and activated T cells (129). The increased PD-1 expression on ThCTL suggests that these cells are highly activated, and although this receptor is associated with exhaustion, ThCTL do not appear to be functionally exhausted (Figure 3.6). CXCR6 (CD186), is a chemokine receptor for the chemokine CXCL16, and is important for the recruitment of T cells in inflamed sites (130). Notably, all ThCTL express CXCR6, while non-ThCTL is a mixture of both CXCR6⁺ and CXCR6^{neg}, which suggests ThCTL are enriched in trafficking into inflamed sites. T-bet is a known Th1 subset transcription factor (2). Most of the lung effector cells express high levels of T-bet (Figure 3.5), consistent with the known Th1 polarizing environment during influenza infection. T-bet could also regulate cytotoxic genes in CD4 T cells (64), suggesting T-bet

may be important for ThCTL as well as Th1 cells. P-selectin and P-selectin glycoprotein Ligand 1 (PSGL-1) are adhesion molecules that regulate the adhesion and recruitment of activated T cells into non-lymphoid sites like the gut (131). Incubating the cells with a P-selectin fusion protein and subsequent detection with secondary antibodies allows for the detection of P-selectin binding ability. ThCTL have higher binding to P-selectin compared to non-ThCTL (Figure 3.5) suggesting ThCTL have a higher capacity to be recruited to the non-lymphoid sites like the lungs, and is consistent with the increased CXCR6 expression to promote infiltration into inflamed sites. Other markers that were not significantly different in expression between ThCTL and non-ThCTL in the lung were, CD27, CTLA-4, and CXCR3. CD27 is a co-stimulatory molecule important for T cell memory (132). Both ThCTL and non-ThCTL have similar CD27 expression, suggesting similar costimulation potential for memory formation. Additionally, activation of CD27 in tumor infiltrating CD4 T cells does not induce cytotoxicity (52). Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4, CD152), is an inhibitory receptor on activated T cells and T regulatory cells as well. Similar expression of CTLA-4 suggests that both ThCTL and non-ThCTL are both equally able to be regulated by CTLA-4. Blocking CTLA-4 can induce cytotoxicity in tumor infiltrating CD4 T cells (56), suggesting blocking CTLA-4 might also be utilized to increase ThCTL in influenza, though we have not yet tested this possibility. CXCR3 is a chemokine receptor for the chemokines CXCL9, CXCL10, and CXCL11 and is important for the trafficking of activated T cells into the lung during infection (133). In CD8 T cells, CXCR3 helps locate infected cells in the skin environment (134) and marks protective lung memory cells (135). ThCTL and

non-ThCTL both express similar CXCR3 (Figure 3.5), which suggests both populations are able to home to the infected lung and perhaps are recruited to areas containing infected cells within the lung.

Looking at transcription factors, Eomes and FoxP3 are both expressed at low levels on CD4 effector cells from the lung, including ThCTL. Eomes appears to be important for ThCTL found in other models like tumors (52, 53) and self-reactive T cells (51). The lack of Eomes expression therefore suggests that ThCTL induced in the lung by influenza infection may utilize other transcription factors for programming cytotoxicity, although whether ThCTL require Eomes during influenza infection remains to be studied. FoxP3 is important for T regulatory cells (58) and the lack of FoxP3 expression suggests that ThCTL are not a part of the conventional Treg population, which can display cytotoxicity (136).

In summary ThCTL at 9 dpi display a unique phenotype that includes high-level expression of effector associated and lung homing receptors that is similar to the non-ThCTL with more enrichment in markers of cytotoxicity and trafficking into inflamed tissues. Since not much is known about ThCTL memory CD4 T cells, or even if ThCTL become memory CD4 T cells, I followed the contraction of the CD4 T cell response after influenza. At 14 and 22 dpi, NKG2X can still be found on CD4 T cells in the lung (Figure 3.5B), with a decline in numbers of NKG2X⁺ CD4 T cells typical of a contracting CD4 T cell response (114). During this contraction, many of the effector molecules highly expressed at 9 dpi are downregulated on CD4 T cells transitioning into memory cells. CD27, CTLA-4, Granzyme B, PD-1, CXCR6, T-bet, and binding to P-selectin were

all downregulated at 14 and 22 dpi (Figure 3.5B). Interestingly, ThCTL enrichment of CXCR6 and binding to P-selectin are retained during the contraction phase, suggesting ThCTL that are transitioning to memory still have this preferential homing capability. Expression of markers of memory cells including the IL-7Ra (CD127) and CXCR3 were subsequently increased as CD4 T cells contracted, with no observable difference in expression between ThCTL and non-ThCTL. These data suggest that ThCTL likely contract into a small resting population of memory cells. Although 22 dpi is an ‘early’ memory time point, multiple effector markers have already been downregulated, which suggests that the cells have already become resting memory cells. Previous studies have shown the transition from effector to memory formation can occur rapidly (137). Although NKG2X has been used to track the ThCTL population, it is unclear if the NKG2X at 22 dpi marked ThCTL found at 8 dpi. Fate mapping experiments to track ThCTL found at the effector stage are needed to address these concerns about memory ThCTL. Nonetheless, the NKG2X⁺ CD4 cells retained trafficking enrichment compared to NKG2X^{neg} throughout contraction.

The phenotypic markers assayed on ThCTL suggest this population of effectors is highly activated. To accompany the phenotypic data, I asked the question whether or not these cells were also functionally highly active as well. Based on previous results, ThCTL are endowed with cytotoxic function, however their cytokine potential was not assayed due to a lack of a defining marker (47). To test the functional capability of ThCTL, I transferred naïve CD4 OT-II cells into naïve B6 mice and infected the mice with PR8-OVA_{II}. On day 8 post infection, I isolated effector donor OT-II cells and

Figure 3.6: ThCTL have increased effector function

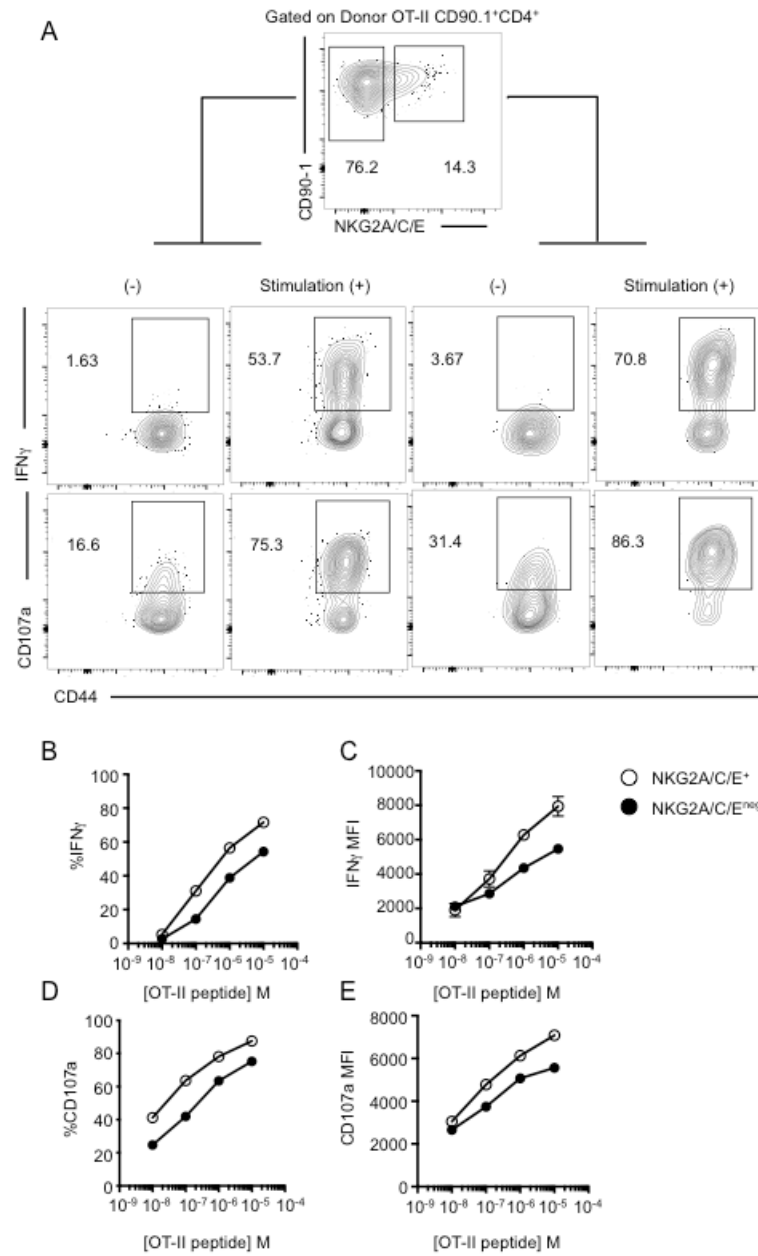


Figure 3.6: ThCTL have increased effector function

(A) Naïve OT-II CD4 T cells were adoptively transferred into B6 mice and infected with PR8-OVA_{II}. At 8 dpi, lungs were isolated and donor CD4 T cells were enriched and assayed for degranulation and cytokine secretion by stimulation with peptide pulsed activated APC. Shown are representative gating and flow plots of degranulation (CD107a) and cytokine production (IFN γ). (B) The percent IFN γ ⁺ of gated NKG2X⁺ (open circle) or NKG2X^{neg} (closed circle) CD4 effectors plotted against increasing peptide concentrations. (C) The median fluorescence intensity of IFN γ ⁺ NKG2X⁺ (open circle) or NKG2X^{neg} (closed circle) CD4 effectors. (D) The percent CD107a⁺ of gated NKG2X⁺ (open circle) or NKG2X^{neg} (closed circle) CD4 effectors plotted against increasing peptide concentrations. (E) The median fluorescent intensity of CD107a⁺ NKG2X⁺ (open circle) or NKG2X^{neg} (closed circle) CD4 effectors plotted against increasing peptide concentrations. Data are representative of 2 independent experiments, where donor cells were isolated from pooled infected lungs n=10 each time. Mean \pm SEM.

assayed the cytokine production and degranulation capability of ThCTL and non-ThCTL *ex vivo*. To rule out any differences in TCR affinity when examining a polyclonal population, I utilized the TCR transgenic system with a single T cell receptor. *Ex vivo* stimulation with varying doses of peptide on antigen presenting cells reveals a typical dose-response curve for both IFN γ secretion by intracellular cytokine staining and degranulation by CD107a staining (Figure 3.6). IFN γ , although not required for ThCTL induction (34, 47), or influenza survival (138), can be important for inducing MHC-II expression on target cells (47, 67). Stimulating ThCTL and non-ThCTL with increasing doses of peptide pulsed APCs reveals higher doses is accompanied by an increased proportion that secrete IFN γ in both populations. In the IFN γ positive fraction of cells, the amount of IFN γ protein increases with increasing peptide dose as shown as median fluorescence intensity of the population (MFI). NKG2X⁺ ThCTL have an increase in the proportion and amount of intracellular IFN γ protein in response to cognate antigen compared to non-ThCTL, suggesting the ThCTL population are more able to secrete this effector cytokine (Figure 3.6A-C). These data are also consistent with the increased T-bet expression on ThCTL in the lung (Figure 3.5). These data also indicate that non-ThCTL also express high levels of IFN γ , indicating ThCTL are not the only IFN γ producing CD4 effector subset in the infected lung.

To look at degranulating cells, I measured CD107a expression during the *ex vivo* stimulation. CD107a marks degranulating cells (125) and is a common functional marker of cytotoxic cells (53, 59, 81). NKG2X⁺ ThCTL have increased proportion of CD107a⁺ compared to the NKG2X^{neg} lung CD4 effectors at every peptide dose examined,

suggesting the ThCTL have a higher degranulating capacity compared to non-ThCTL (Figure 3.6D,E). Of the CD107a⁺ portion of cells, the ThCTL additionally have higher protein staining as well, suggesting more degranulation on a per cell basis. Notably, CD107a expression is not an exclusive marker of cytotoxicity as the non-ThCTL also express CD107a. Cytotoxicity and degranulation can be decoupled in CD8 T cells, notably in memory CD8 T cells (139), suggesting the non-ThCTL cells could have cells degranulating that are not cytotoxic (Figure 3.2). Nonetheless, the ThCTL have higher degranulating capacity compared to the non-ThCTL found in the lungs at the peak of infection.

ThCTL gene expression.

Because ThCTL are marked by NKG2X expression in the infected lung, we can analyze the gene expression of ThCTL compared to non-ThCTL CD4 effectors. Although both populations are found in the same tissue, ThCTL are differentiated by their cytotoxic ability (Figure 3.2), suggesting perhaps a unique program drives their differentiation. The effector phenotype of the population, however, is largely similar to the non-ThCTL lung effectors (Figure 3.5), indicating that they probably overlap in their differentiation. To explore the gene expression of ThCTL as a population of specialized effectors, I compared the global gene expression of ThCTL and non-ThCTL in the lung by microarray. Transfer of naïve HNT CD4 transgenic cells into wild type mice and subsequent infection with PR8 yields a robust effector population in the lung. On day 7 post infection, the peak of the response in this transgenic system, I isolated the lung

Figure 3.7: ThCTL gene expression

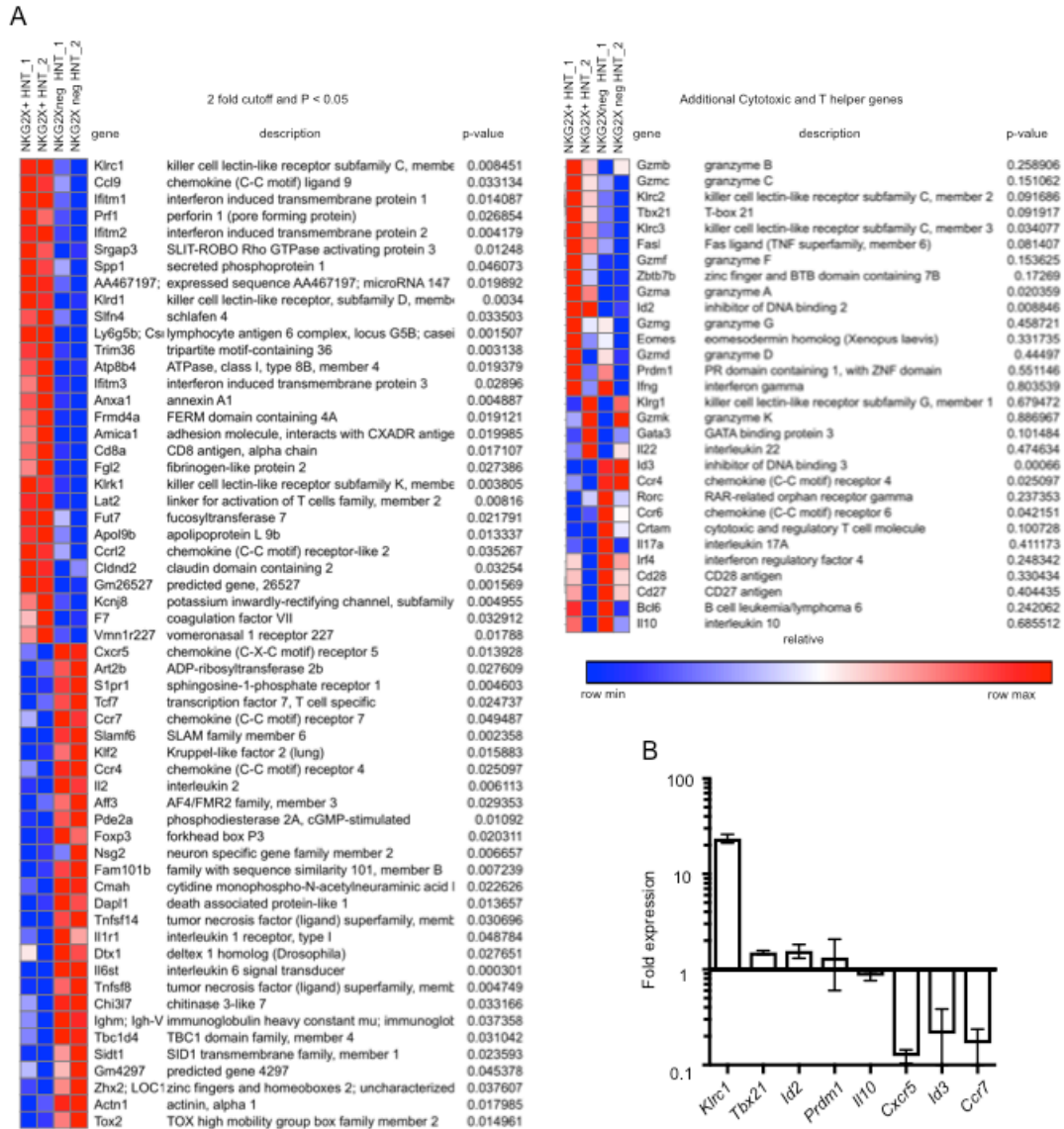


Figure 3.7: ThCTL gene expression

(A) Gene heat maps of the ThCTL and non-ThCTL. Naïve HNT CD4 T cells were adoptively transferred into BALB/c mice and infected with PR8. At 8 dpi, lungs were isolated, pooled, and sorted for donors and NKG2X expression. RNA was isolated and microarray analysis was performed. Shown are the relative expression of the indicated populations and the genes with a 2 fold difference and $P < 0.05$ (left) as well as for selected cytotoxic genes (right). **(B)** Naïve OT-II CD4 T cells were adoptively transferred into B6 mice and infected with PR8-OVA_{II}. At 8 dpi, lungs were isolated and donor CD4 T cells were sorted based on NKG2X expression. RNA was isolated and gene expression measured using Taqman primers for the indicated genes. The fold expression of NKG2X⁺ over NKG2X^{neg} is shown. Data from each microarray run is shown as _1 and _2 and are from cells isolated from pooled infected lungs n=10 each time (A). Data are pooled from at least 2 experiments where cells were isolated from pooled infected lungs n=10 each time (B). Mean \pm SEM.

effector cells and sorted the effectors by their expression of NKG2X. The RNA was isolated and prepared for microarray analysis comparing the whole mouse genome. Indeed only a few genes are differentially expressed in these two populations, as they both have the same TCR and are found in the same anatomical site, the lung. But of the genes that are differentially expressed, many affirm the cytotoxic potential of ThCTL (Figure 3.7). As expected there was an increase in the expression of genes encoding the NKG2X complex, *Klrc1*, *Klrc2*, and *Klrc3* in NKG2X⁺ ThCTL compared to non-ThCTL. Perforin and granzymes are also enriched in the ThCTL population compared to the non-ThCTL population, consistent with the cytotoxic capability of ThCTL. Genes associated with tissue residency are also enriched in ThCTL, notably the downregulation of *Klf2*, a transcription factor that regulates *S1p1r* (140, 141). The downregulation of both these genes indicates a transition to residency (140), and ThCTL have lower expression of both compared to non-ThCTL (Figure 3.7). Factors associated with memory formation were also downregulated including *Il2* and *Tcf7*, encoding TCF-1. IL-2 promotes survival of effector CD4 T cells (114), and can promote memory formation after influenza infection (113). Additionally, IL-2 is regulated by Blimp-1 in effector CD8 T cells (110, 142, 143) and CD4 T cells (144), suggesting Blimp-1 could be acting to reduce IL-2 expression in ThCTL effectors. Blimp-1 can repress expression of *CCR7* (77, 142), again consistent with the downregulation of *Ccr7* in ThCTL compared to non-ThCTL. TCF-1 is important for memory survival in CD8 T cells (145). The downregulation of both *Il2* and *Tcf7*, suggest the preferential lack of canonical survival factors and calls into question whether ThCTL survive long term into memory CD4 T cells. Although kinetics data suggest they

do (Figure 3.5), ThCTL may utilize a different mechanism of memory formation that could be different than circulating memory cells (113, 145). The upregulation of *Cd8a* is interesting in the context of what happens to gut ThCTL (81), and is consistent with the concept of a downregulation of ‘helper CD4’ genes and an upregulation of ‘cytotoxic CD8’ genes in ThCTL. The upregulation of *Cd8a* suggests this may be occurring in ThCTL, but not to the same extent as gut ThCTL. ThCTL from stimulated cells can also upregulate *Cd8a* but do not express the protein CD8a (108). *Cxcr5* was also downregulated in ThCTL compared to non-ThCTL, suggesting differential responsiveness to CXCL13. CXCR5 also marks Tfh cells suggesting ThCTL could be suppressing the Tfh lineage as well. The transcription factors Id2 and Id3 regulate CD8 effector and memory differentiation (146, 147). Id2 promotes effector cell survival (147) while Id3 promotes memory formation in CD8 effector cells (146). Interestingly, ThCTL have higher *Id2* expression and lower *Id3* expression compared to non-ThCTL (Figure 3.7A,B). The pattern of this expression of Id2 and Id3 suggests ThCTL resemble more the more differentiated or terminal effector phenotype seen in CD8 T cells. Additionally, genes regulated by Id2 and Id3 were also seen differentially expressed in the array. *Ccr7*, *Cxcr5*, and *Il2* were upregulated in *Id3*^{hi} CD8 T cells, consistent with the lower expression in non-ThCTL, which have higher *Id3* expression (Figure 3.7). Conversely, *Gzma* and *Cxcr6* are downregulated in *Id3*^{hi} CD8 T cells consistent with the higher expression in ThCTL, which have higher *Id2* expression (Figure 3.7, 3.5). *Prdm1*, encoding Blimp-1, was also downregulated in *Id3*^{hi} CD8 T cells (146) suggesting a possible interplay between *Id3*, *Id2*, and Blimp-1 in promoting ThCTL, although further

studies are required to define such a relationship. Id2 and Id3 can be upregulated by cytokine exposure on CD8 T cells (146), possibly suggesting a role for differential cytokines in promoting ThCTL versus non-ThCTL. The gene expression data taken together suggests that although ThCTL share many features of non-ThCTL, a program similar to terminal effector CD8 T cells appears to be upregulated in ThCTL and is consistent with the cytotoxic effector function of ThCTL.

ThCTL are restricted to the lung during influenza infection.

Given the gene expression of ThCTL indicating tissue residency by downregulation of *Klf2* and *Slp1r*, I hypothesized ThCTL were localized to the lung tissue in infected mice. To test whether ThCTL are localized to the lung, I transferred naïve OT-II transgenic CD4 T cells into mice and infected with PR8-OVA_{II}. On day 8 post infection, I isolated lymphocytes from the bronchoalveolar lavage (BAL), lungs, draining lymph nodes (dLN), spleen, liver, and the blood. CD4 effector cells are known to infiltrate multiple organs (148) in the mice, and I recovered effector cells from all places I isolated cells from (Figure 3.8A,B). The majority of the effector cells I recovered were from the lung and spleen with similar numbers found in the BAL, dLN, and liver. Staining for ThCTL with anti-NKG2X revealed that ThCTL are enriched in the BAL and lungs, while much fewer cells can be found in the dLN, liver, spleen, and blood (Figure 3.8A,C,D). The greatest numbers of ThCTL recovered was from the lungs of infected mice. The lack of ThCTL in the other organs that had effector cells in them, suggest ThCTL are enriched in the infected tissue. During the course of influenza infection, the

Figure 3.8: ThCTL are restricted to the site of infection

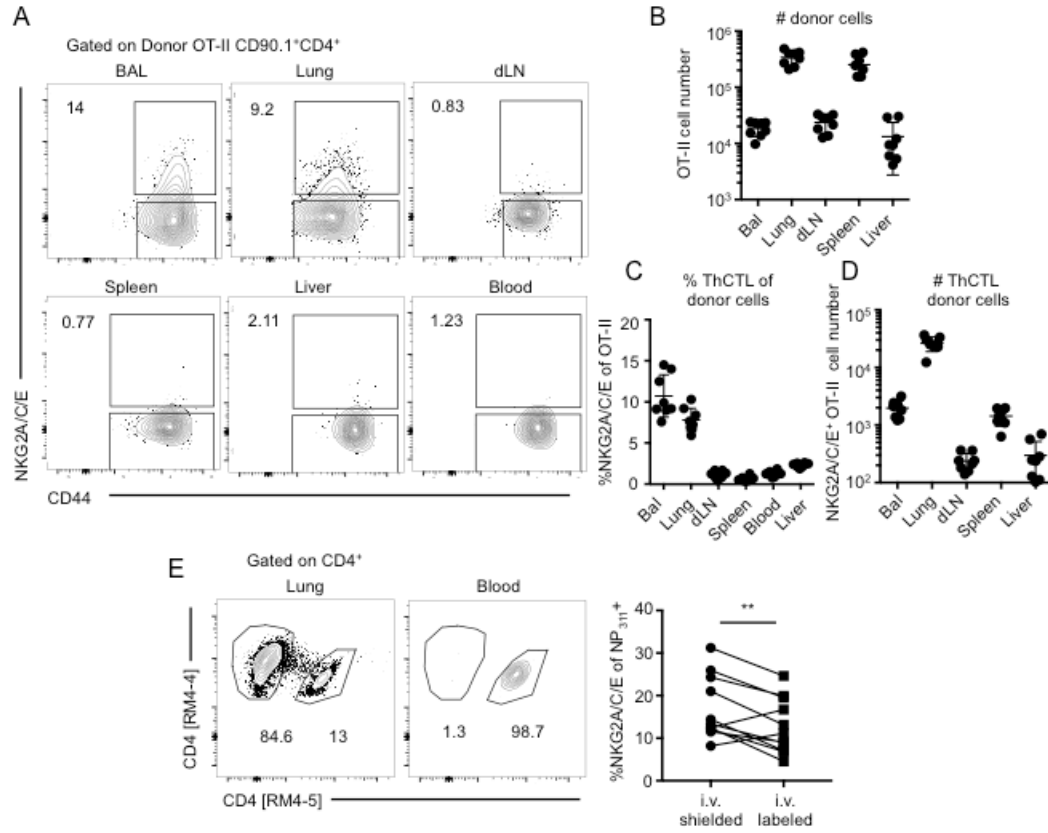


Figure 3.8: ThCTL are restricted to the site of infection

(A) Naïve OT-II CD4 T cells were adoptively transferred into B6 mice and infected with PR8-OVA_{II}. At 8 dpi the indicated tissues were isolated and stained for expression of NKG2X on donor CD4 T cells. Representative flow plots are shown. (B). The number of donor cells recovered from the indicated tissues. (C) The percent NKG2X expression on donor cells recovered from the indicated tissues. (D) the number of NKG2X donor cells recovered from the indicated tissues. (E) Naïve B6 mice were infected with PR8-OVA_{II}. At 8 dpi, CD4 antibody was administered i.v. and lungs harvested and stained. Representative flow plots of lung and blood for i.v. CD4 and ex vivo CD4 staining (left). The quantitation of the percent NKG2X⁺ expression on NP₃₁₁₋₃₂₅ tetramer CD4⁺ cells in the lung (right). Data are representative of at least two independent experiments (A, E). Data are pooled from two independent experiments with n = 8. (B-D) or n = 11 (E). Mean ± SEM. ** $P < 0.005$.

virus replicates only in the lung (13), indicating ThCTL preferentially localize to the site of infection. Whether signals in the infected lung drive pre-ThCTL effectors to become ThCTL or whether ThCTL are generated in the secondary lymphoid organs and then preferentially retained in the infected environment remain to be studied. Within the lung tissue, T cells can be exposed to the vasculature or are in the parenchyma of the lung (149). To ask whether ThCTL are preferentially localized within the lung tissue, I performed intravenous (i.v.) antibody labeling in infected wild type mice. Intravenous CD4 antibody labels CD4 T cells exposed to the vasculature, while cells in the lung parenchyma are shielded from labeling. Subsequent staining with a non-sterically hindered CD4 antibody clone enables discrimination of i.v. labeled and i.v. shielded CD4 effector cells in the lung (150). To examine the polyclonal antigen specific CD4 T cells, NP₃₁₁₋₃₂₅ loaded tetramer staining was also conducted. As expected, the CD4 T cells in the blood were all labeled with i.v. CD4 antibody, while lung CD4 effectors segregated into i.v. labeled and i.v. shielded (Figure 3.8E). The polyclonal NP₃₁₁₋₃₂₅ specific ThCTL are preferentially enriched in the shielded proportion of CD4 T cells, suggesting a unique localization of these cells compared to non-ThCTL. These data together suggest ThCTL are uniquely localized to the site of infection and are enriched in the parenchyma of the lung tissue. The location of T cells within the parenchyma may have implications for their function, as parenchyma associated CD4 effectors are more functional (151), while tissue resident memory can protect better than splenic memory against influenza infection (152).

Figure 3.9: LCMV ThCTL are found in multiple tissues

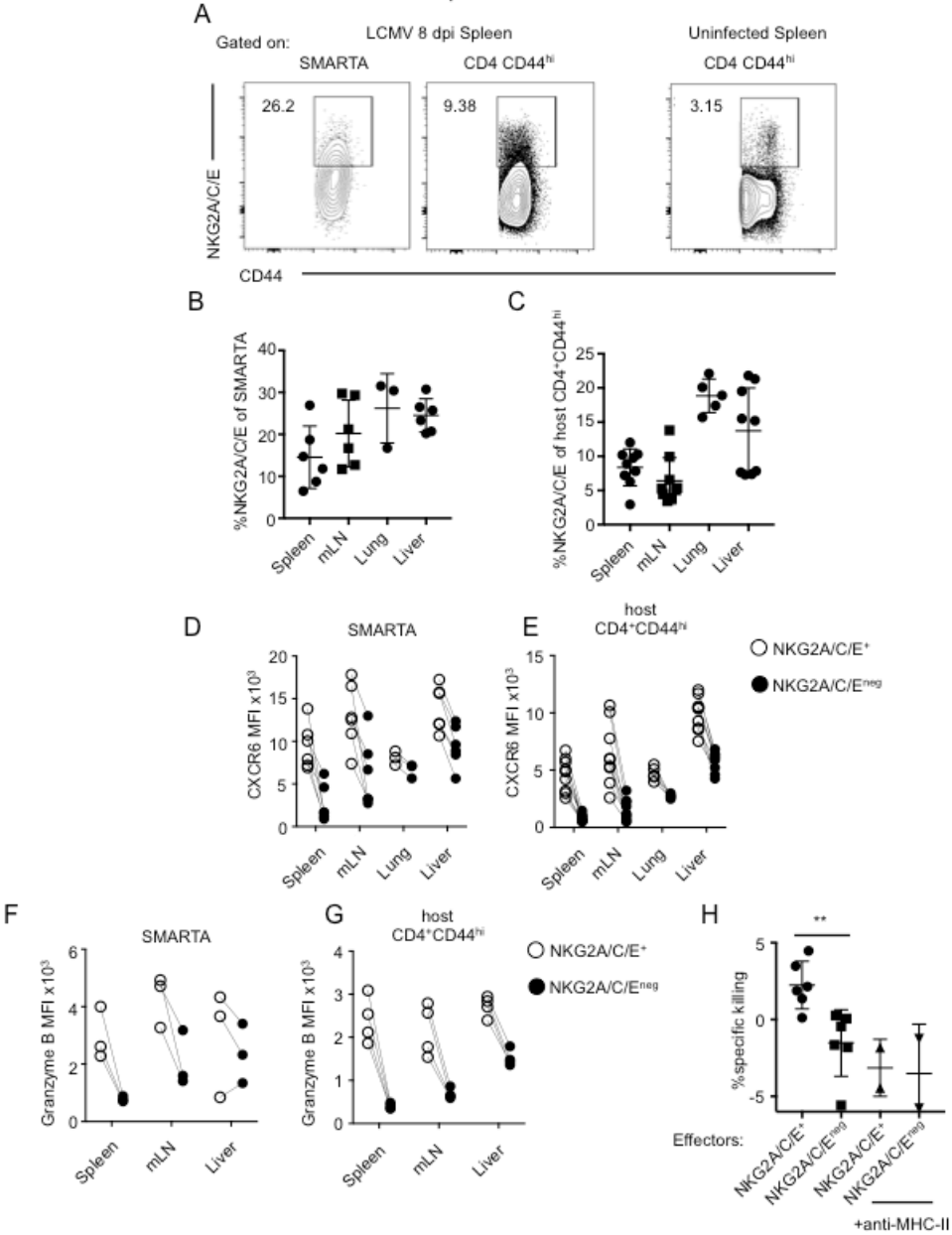


Figure 3.9: LCMV ThCTL are found in multiple tissues

(A) Naïve SMARTA CD4 T cells were adoptively transferred into B6 mice and infected with LCMV Armstrong. At 8 dpi the indicated tissues were harvested and stained for NKG2X expression. Representative staining of NKG2X on gated SMARTA effectors (left), CD44^{hi} host effectors (middle) or uninfected mice (right) in the spleen. (B) The percent of SMARTA donor cells expressing NKG2X (C) The percent of host CD4 CD44^{hi} effectors expressing NKG2X (D) The expression of CXCR6 in the indicated tissues gated on SMARTA NKG2X⁺ (open circle) and NKG2X^{neg} (closed circle). Lines connect NKG2X⁺ and NKG2X^{neg} of each individual mouse. (E) The expression of CXCR6 in the indicated tissues gated on host CD44^{hi}CD4 NKG2X⁺ (open circle) and NKG2X^{neg} (closed circle). Lines connect NKG2X⁺ and NKG2X^{neg} of each individual mouse. (F) The expression of Granzyme B in the indicated tissues gated on SMARTA NKG2X⁺ (open circle) and NKG2X^{neg} (closed circle). Lines connect NKG2X⁺ and NKG2X^{neg} of each individual mouse. (G) The expression of Granzyme B in the indicated tissues gated on host CD44^{hi}CD4 NKG2X⁺ (open circle) and NKG2X^{neg} (closed circle). Lines connect NKG2X⁺ and NKG2X^{neg} of each individual mouse. (H) Naïve SMARTA CD4 T cells were adoptively transferred into B6 mice and infected with LCMV Armstrong. At 8 dpi the spleens were harvested and SMARTA cells were flow sorted based on their expression of NKG2X and assayed for cytotoxicity ex vivo. Anti-MHC-II as added in control wells. Data are representative of at least two independent experiments (A). Data are pooled from two experiments (B-H) or of one experiment (F). Mean ± SEM. ** $P < 0.005$.

NKG2X marks ThCTL in LCMV infection.

ThCTL are enriched in the site of viral replication during influenza infection, where the virus grows in lung epithelial cells (14). However, other viruses display different cell tropisms and replicate in other cells or organs. To ask whether ThCTL are found in other tissue sites, where the virus is present, we also examined mice infected with lymphocytic choriomeningitis virus (LCMV). I transferred naïve SMARTA transgenic CD4 cells into wild type B6 mice and then infected with 5×10^4 p.f.u of LCMV Armstrong. The SMARTA transgenic T cell receptor is specific for LCMV gp₆₁₋₈₀ (153). This acute infection drives the proliferation of CD4 and CD8 T cells in the spleen (153) and subsequently generates a MHC-II restricted cytotoxic response (85), suggesting ThCTL are found in the LCMV response. At 8 dpi, I harvested spleens from infected mice and stained for NKG2X expression on the host and donor antigen specific transgenic CD4 effector cells. Both donor and host effector cells in the spleen expressed NKG2X (Figure 3.9A), a contrast to the spleen in influenza infected mice (Figure 3.8A). Importantly, uninfected mice did not display high NKG2X expression (Figure 3.9A), suggesting the infection drives the differentiation of NKG2X ThCTL. This data suggest that ThCTL are not unique to the lung, as they can be found in the spleen when the spleen is one of the sites of infection (154). Notably, LCMV can spread to the lungs, liver, spleen, and mesenteric lymph nodes, which constitutes a systemic infection (154, 155). In contrast to influenza infection (Figure 3.8), NKG2X is expressed on donor antigen specific CD4 T cells in the spleen, mesenteric lymph nodes (dLN for intranasal influenza), and liver (Figure 3.9B). The host response also shows similar expression in

these organs as well (Figure 3.9C). The data is consistent with the concept of ThCTL being localized to sites of infection, which in the case of influenza is the lung, while LCMV is systemic. To also assay the phenotype of these ThCTL found in LCMV, I stained CXCR6 and Granzyme B, two phenotypic markers that are enriched in ThCTL found during influenza (Figure 3.5). Like in influenza infection, the ThCTL found in the spleen, mLN, lung, and liver following challenge with LCMV had higher expression of CXCR6 and granzyme B compared to non-ThCTL (Figure 3.9D,F). Further, the host response also displays the same phenotypic differences between ThCTL and non-ThCTL for CXCR6 and granzyme B (Figure 3.9E,G). These phenotypic data suggests that ThCTL found in LCMV infection are similar to ThCTL found in influenza, at least for these ThCTL markers assayed. The increased granzyme B expression also suggests that ThCTL in LCMV are enriched in cytotoxic capability, and perhaps marks the cytotoxic MHC-II restricted population found in LCMV (85). To ask whether these NKG2X⁺ CD4 effectors had cytotoxic potential I isolated either the NKG2X⁺ expressing or non-expressing SMARTA TCR transgenic CD4 effector cells from the spleen at 8 dpi and assayed their ability to lyse peptide pulsed target cells. Indeed, the NKG2X⁺ expressing ThCTL had enriched cytotoxicity against peptide pulsed target cells (Figure 3.9H). Importantly, blocking MHC-II via antibody inhibits cytotoxicity suggesting the observed cytotoxicity is MHC-II restricted. Taken together, these data suggest that NKG2X⁺ marks the ThCTL population found in LCMV infection. Importantly, these ThCTL are also found in the spleen and other organs where LCMV replicates in contrast to influenza infection where the virus replicates only in the lung, suggesting ThCTL are enriched in

sites where virus rapidly replicates. Whether this is because the inflammatory environment is conducive to ThCTL differentiation or whether the inflammatory environment retains ThCTL remains to be studied. Nonetheless, these data suggest ThCTL are found at sites of viral replication.

Blimp-1 is necessary for ThCTL differentiation.

Since ThCTL arise in a unique location and possess a specialized effector function, we wanted to further study what factors regulate their generation. The CD4 T cell response to pathogens can be characterized into helper types, mainly Th1, Th2, Th17, Tregs, and Tfh (2). However less is known about that genetic program regulates ThCTL differentiation. From the gene expression data (Figure 3.7), multiple genes repressed by Blimp-1 appear to be downregulated in ThCTL compared to non-ThCTL including, *Ccr7* (77), *Tcf7* (142), and *Il2* (143, 144). Blimp-1 is also important for programming the effector differentiation of CD8 T cells, including cytotoxic function (77). Additionally, Blimp-1 has been shown to regulate granzyme B production and cytotoxic function of CD4 T cells (64). These data led us to hypothesize that Blimp-1 programs the differentiation of the cytotoxic ThCTL population. To test whether Blimp-1 played a role in ThCTL differentiation, I used mice conditionally deficient in Blimp-1 in T cells. *Prdm1^{fl/fl}* were crossed with *Cd4-Cre* mice to generate Blimp-1 conditionally knocked out mice (CKO) (110). Further, I crossed these Blimp-1 CKO mice to the OT-II CD4 transgenic system to generate OT-II Blimp-1 CKO CD4 cells to test the T cell intrinsic loss of Blimp-1. To ask whether Blimp-1 was important for cytotoxic CD4 T cell

Figure 3.10: ThCTL require Blimp-1 for their differentiation

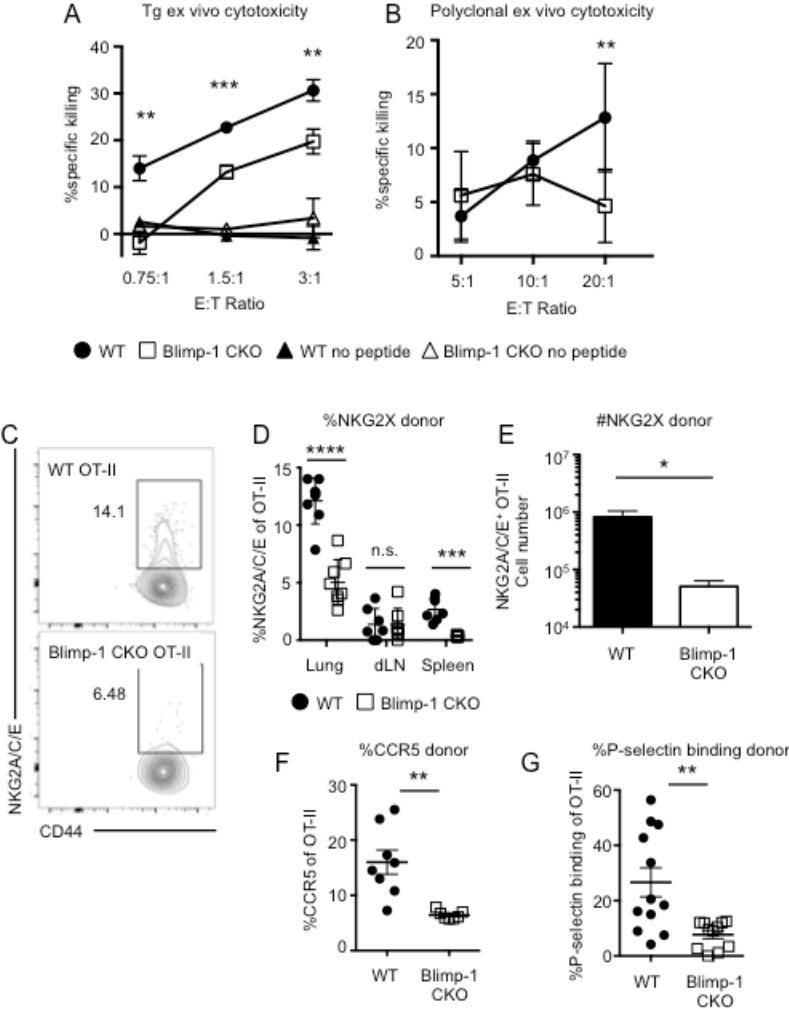


Figure 3.10: ThCTL require Blimp-1 for their differentiation

(A) Naïve wild type or Blimp-1 CKO OT-II CD4 T cells were adoptively transferred into B6 mice and infected with PR8-OVA_{II}. At 8 dpi the lungs were harvested and wild type (closed circle) and Blimp-1 CKO (open square) donor cells enriched and assayed for peptide specific cytotoxicity. Target cells lacking peptide were also assayed with wild type (closed triangle) or Blimp-1 CKO (open triangle) effectors. **(B)** Naïve wild type or Blimp-1 CKO mice and infected with PR8. At 8 dpi CD4 CD44^{hi} lung effector cells flow sorted from either wild type (closed circle) or Blimp-1 CKO (open square) mice were assayed for peptide specific killing. **(C)** Naïve wild type or Blimp-1 CKO OT-II CD4 T cells were adoptively transferred into B6 mice and infected with PR8-OVA_{II}. At 8 dpi the lungs were harvested and stained. Representative flow plots gated on lung donor cells for the expression of NKG2X. **(D)** The percent donor cells expressing NKG2X was measured in the indicated tissues from either wild type (closed circle) or Blimp-1 CKO (open square). **(E)** The numbers of NKG2X⁺ donor OT-II cells recovered from the lungs. **(F)** The percent donor cells expressing CCR5 in the lung. **(G)** The percent donor cells binding to P-selectin in the lung. Data are representative of at least two independent experiments (A, C, E). Lungs were pooled from infected mice of n = 10 per genotype (A) or n = 20 per genotype (B). Data are pooled from two independent experiments (B, D, F, G), with n = 8 each genotype (D), or n = 7-8 each genotype (F), or n = 11-12 each genotype (G). Mean ± SEM. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$, and **** $P < 0.0001$.

function, I transferred either wild type or Blimp-1 CKO naïve OT-II CD4 cells into congenically marked hosts and infected the hosts with PR8-OVA_{II}. At 8 dpi, I isolated the donor cells from the lungs of infected hosts and assayed their cytotoxic potential against OT-II peptide pulsed target cells. The wild type OT-II CD4 effectors were able to kill peptide pulsed target cells, while the Blimp-1 CKO OT-II were less able to do so at the entire range of effector to target ratios assayed (Figure 3.10A). These data suggest a T cell intrinsic loss of Blimp-1, as the host is wild type, leads to a reduced cytotoxic function in CD4 effector cells. To ask whether the polyclonal population of effector cells also require Blimp-1 for cytotoxicity, I infected either wild type or Blimp-1 CKO mice with PR8. At 8 dpi, I isolated the effector cells by sorting on CD4⁺ and CD44^{hi} double positive effector cells from the lungs and assayed their cytotoxic activity against influenza NP peptides (NP₃₁₁₋₃₂₅ and NP₂₆₁₋₂₇₅) (47, 115) pulsed target cells. The cytotoxic response of the polyclonal population is less compared to transgenic CD4 response, as expected since only a fraction of the polyclonal population would be specific for the NP peptides assayed. The Blimp-1 CKO CD4 effector cells showed a statistically significant reduction in specific cytotoxicity compared to wild type CD4 effector cells at the highest effector to target ratio assayed (Figure 3.10B). These data suggest that both the transgenic and polyclonal CD4 effector T cells require Blimp-1 for cytotoxic function at the peak of the influenza T cell response. To understand whether differentiation of ThCTL was impacted, I measured the phenotype of the effector CD4 T cells when Blimp-1 was conditionally deleted. Blimp-1 deficient OT-II CD4 transgenic effector cells at 8 dpi had a decrease in NKG2X expressing cells compared with wild type OT-II

effectors (Figure 3.10C,D), suggesting ThCTL require Blimp-1 for their differentiation. The loss of ThCTL was also reflected in the number of ThCTL recovered from the lungs, where fewer NKG2X⁺ OT-II donor cells were recovered in the absence of Blimp-1. CD8 T cells lacking Blimp-1 have altered homing potential due to a loss of CCR5 expression (77) and subsequent accumulation in the dLN of influenza infected mice. Indeed the proportion of effector OT-II donor cells expressing CCR5 was decreased in the absence of Blimp-1. However, assaying for ThCTL in the dLN and spleen shows that the ThCTL defect could not solely be explained by deficient trafficking, as ThCTL was not found in either dLN or spleen (Figure 3.10D). ThCTL express high levels of binding to P-selectin (Figure 3.5), which is dependent on Blimp-1 as well (Figure 3.10G). These data suggest that Blimp-1 is critical for the differentiation of ThCTL during influenza infection. Not only is the cytotoxic function impaired, but the ThCTL phenotype of NKG2X expression and high binding to P-selectin is also reduced in the absence of Blimp-1. The requirement for Blimp-1 is consistent with the concept that ThCTL are similar to highly differentiated terminal effector cells found in the CD8 T cell compartment (142). The high activation effector phenotypes and function (Figure 3.5,3.6) align with this concept as well and leads us to wonder whether ThCTL survive well into the memory compartment or are short lived. After respiratory infection, populations of CD8 T cells in the lung have different persistence and division properties (156). Further studies are needed to address these questions.

Antigen recognition by CD4 effector T cells correlates with effector phenotypes.

ThCTL have similar characteristics of a highly activated effector CD4 T cell subset (Figure 3.5,3.6,3.7). Early reports of cytotoxic CD4 T cells came from long term culture and activation of CD4 clones that gained cytotoxicity after restimulation (42). Human ThCTL have been characterized to be ‘antigen experienced’ and exhibit a phenotype of high activation and extensive differentiation as well (43). These results suggest that ThCTL may require continued and/or a high level of antigen signaling for their differentiation, although in vitro differentiation of mouse ThCTL suggests lower antigen doses promote cytotoxicity (50). Whether ThCTL require continued antigen stimulation or just restimulation at a particular time point remains understudied. In vivo activation of CD4 T cells benefit from continued antigen presence for proliferation and cytokine production (157). The requirement of 4-1BB and OX-40 costimulation in tumor models also suggests that additional activation is beneficial for ThCTL generation (51–53). Further, NKG2X is expressed on in vitro cultured CD4 T cells only after repeated rounds of activation and polarization (122). These observations led us to hypothesize that CD4 effectors require additional antigen stimulation in vivo for their differentiation to ThCTL.

To study antigen recognition in vivo, I utilized the OT-II Nur77-GFP reporter mouse generated in our lab (114). *Nr4a1* (encoding Nur77) is an immediate early gene downstream of the T cell and B cell receptor (158). Nur77-GFP mice transiently express green fluorescent protein (GFP) after the T cell receptor is activated and the strength of activation correlates with GFP expression (158). These transgenic OT-II CD4 T cells will transiently express GFP, where GFP is expressed after 24 hours post TCR stimulation

Figure 3.11: Antigen recognition at 6 dpi correlates with specialized effector subsets

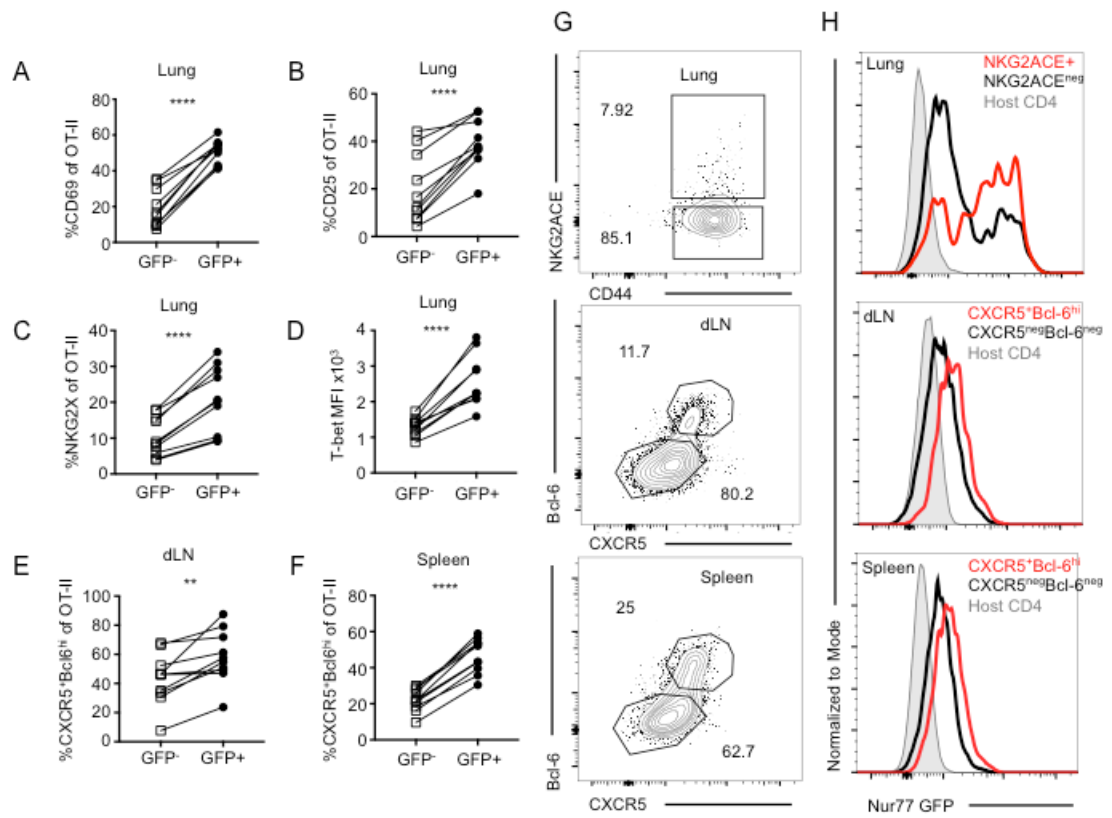


Figure 3.11: Antigen recognition at 6 dpi correlates with specialized effector subsets

Naïve Nur77 GFP OT-II CD4 T cells were adoptively transferred into B6 mice and infected with PR8-OVA_{II}. At 6 dpi the lungs were harvested stained for the indicated markers. Shown is the percent of GFP⁺ (closed square) and GFP^{neg} (open square) donor cells expressing the indicated markers. Lines connect GFP⁺ and GFP^{neg} of each individual mouse. **(A)** CD69 expression in the lungs. **(B)** CD25 expression in the lungs. **(C)** NKG2X expression in the lungs. **(D)** T-bet median fluorescence intensity in the lungs. **(E)** CXCR5⁺Bcl-6^{hi} of donor cells in the dLN. **(F)** CXCR5⁺Bcl-6^{hi} of donor cells in the spleen. **(G)** Representative flow plots of the expression of NKG2X, CXCR5, and Bcl-6 on donor cells from the indicated tissues. **(H)** Representative overlaid histograms of Nur77 GFP expression from the host CD4 T cells (shaded), NKG2X⁺ (red line), and NKG2X^{neg} (black line) donor CD4 T cells in the lungs (top). Representative overlaid histograms of Nur77 GFP expression from the host CD4 T cells (shaded), CXCR5⁺Bcl-6^{hi} (red line), and CXCR5^{neg}Bcl-6^{neg} (black line) donor CD4 T cells in the dLN (middle). Representative overlaid histograms of Nur77 GFP expression from the host CD4 T cells (shaded), CXCR5⁺Bcl-6^{hi} (red line), and CXCR5^{neg}Bcl-6^{neg} (black line) donor CD4 T cells in the spleen (bottom). Data are representative of two independent experiments (G, H). Data are pooled from two independent experiments (A-F) with n = 10. ** $P < 0.005$, **** $P < 0.0001$.

and is substantially reduced 24 hours after removal of stimulation (114). In mice infected with influenza virus expressing the OT-II epitope, OT-II CD4 T cells are primed early in the response around day 3 post infection (114). Antigen recognition peaks about day 3-4, is present from day 5-7 and decline thereafter. This timing is congruent with that seen for antigen recognition and cytokine signaling 5-8 dpi that is required for the generation of memory CD4 T cells during influenza infection (113, 114). To address if antigen recognition during this time window was also important for effector subset generation, I assayed the phenotype of Nur77-GFP⁺ cells. I transferred naïve OT-II Nur77-GFP CD4 T cells into B6 mice and infected the hosts with PR8-OVA_{II}. At 6 dpi, I isolated lungs, draining lymph nodes, and spleens to assay GFP expression. Gating on the donor OT-II cells, I gated on either GFP positive or negative populations and compared the phenotype between the two. As expected, in the lungs of infected mice there is a bimodal expression of GFP, while the dLN and spleens have a more uniform expression of GFP (Figure 3.11H)(114). Since the lung is where the virus replicates and the peak of viral replication is around 4-6 dpi (159), the high GFP expression could be due to the high levels of infection in the lung compared to the dLN and spleen. In the lungs the expression of GFP correlated with antigen recognition as GFP⁺ transgenic donor cells were enriched in CD69 and CD25 expression compared to GFP^{neg} transgenic donor cells (Figure 3.11A,B). NKG2X expression was also enriched in the GFP expressing population of donor cells, suggesting antigen recognition at this time point correlated well with ThCTL formation. Although 6 dpi is an early time point (109), and the levels of NKG2X are low (Figure 3.11G), there is consistent and statistically significant enrichment for NKG2X expression

in the GFP⁺ population. Similarly, the GFP⁺ donor cells consistently express higher levels of T-bet protein compared to GFP^{neg} donor cells (Figure 3.11D). ThCTL also express more T-bet protein at the peak of infection (Figure 3.5B, 3.7). These data suggest that antigen recognition at or right before 6 dpi correlated with ThCTL phenotype. In the dLN and spleen of infected mice, GFP⁺ donor cells were enriched with Tfh phenotype of CXCR5⁺ and B cell lymphoma 6 (Bcl-6^{hi}). Tfh have been shown to continually interact with B cells in the follicle (160) and the continued antigen signaling may be important for their differentiation (161) during LCMV infection. These data taken together suggest that CD4 T cells encounter antigen after priming and that the post-priming encounter with antigen correlates with increased effector phenotypes including ThCTL and Tfh.

Antigen recognition at the effector stage is necessary for continued ThCTL and Tfh differentiation.

Because antigen recognition by CD4 effectors at 6 dpi correlated with ThCTL and Tfh differentiation, we hypothesized that antigen recognition at 6 dpi is necessary for continued differentiation of ThCTL and Tfh. To test whether antigen post priming is necessary for the differentiation of these effector subsets, I removed antigen by transfer of effectors into hosts lacking antigen. I generated effectors CD4 T cells by adoptive transfer of naïve OT-II CD4 transgenic cells into naïve B6 hosts and subsequently infected the hosts with PR8-OVA_{II}. At 6 dpi, where antigen recognition is still occurring and well after priming (114), I isolated effector cells from the dLN and spleens of mice by enrichment based on their donor congenic marker CD90.1. The majority of the

effector response at 6 dpi is located in the dLN and spleen (114, 162) with few in the lungs, making isolating effectors from the lung at 6 dpi difficult. Additionally, if the majority of the response is primed in the dLN and spleen with subsequent infiltration into the lungs after 6 dpi, isolating the effectors before this event allows one to ask whether antigen is necessary to support the differentiation of 'pre-ThCTL' effectors into mature ThCTL in the lung. Indeed, the effectors isolated from the dLN and spleen are NKG2X^{neg} (Figure 3.12A). After isolation of effector CD4 T cells at 6 dpi, I transferred the cells into new hosts that were 6 dpi with PR8-OVA_{II}, PR8, or uninfected, termed the sequential transfer model (114) (Figure 3.12A). Donor OT-II CD4 effectors transferred into PR8 hosts are not exposed to OVA_{II} antigen but would experience the inflammatory environment from the ongoing PR8 infection. Importantly, the extent of inflammation induced by PR8-OVA_{II} and PR8 are similar (114). Effector CD4 T cells transferred into uninfected hosts would be exposed to neither inflammation nor antigen. After transfer of effector cells, I harvested the lungs, dLN, and spleen of the second hosts after 2 days post transfer, or 8 dpi altogether. 2 days was chosen to allow the effectors to continue differentiating while still assessing the effector response at the peak of the infection. At 8 dpi, recovery of donor OT-II cells reveals that antigen contributes to the size of the effector population, presumably as a result of continued expansion of these cells (114), although with varying effect size (Figure 3.12B). In the lungs of the second hosts, lack of antigen (PR8 group) resulted in a small but statistically significant reduction in the recovery of donor cells (Figure 3.12C) compared with the presence of antigen (PR8-OVA_{II} group). Removal of both antigen and inflammation dramatically reduced the

Figure 3.12: Antigen after 6 dpi promotes survival and expansion of effectors in the lung and SLO

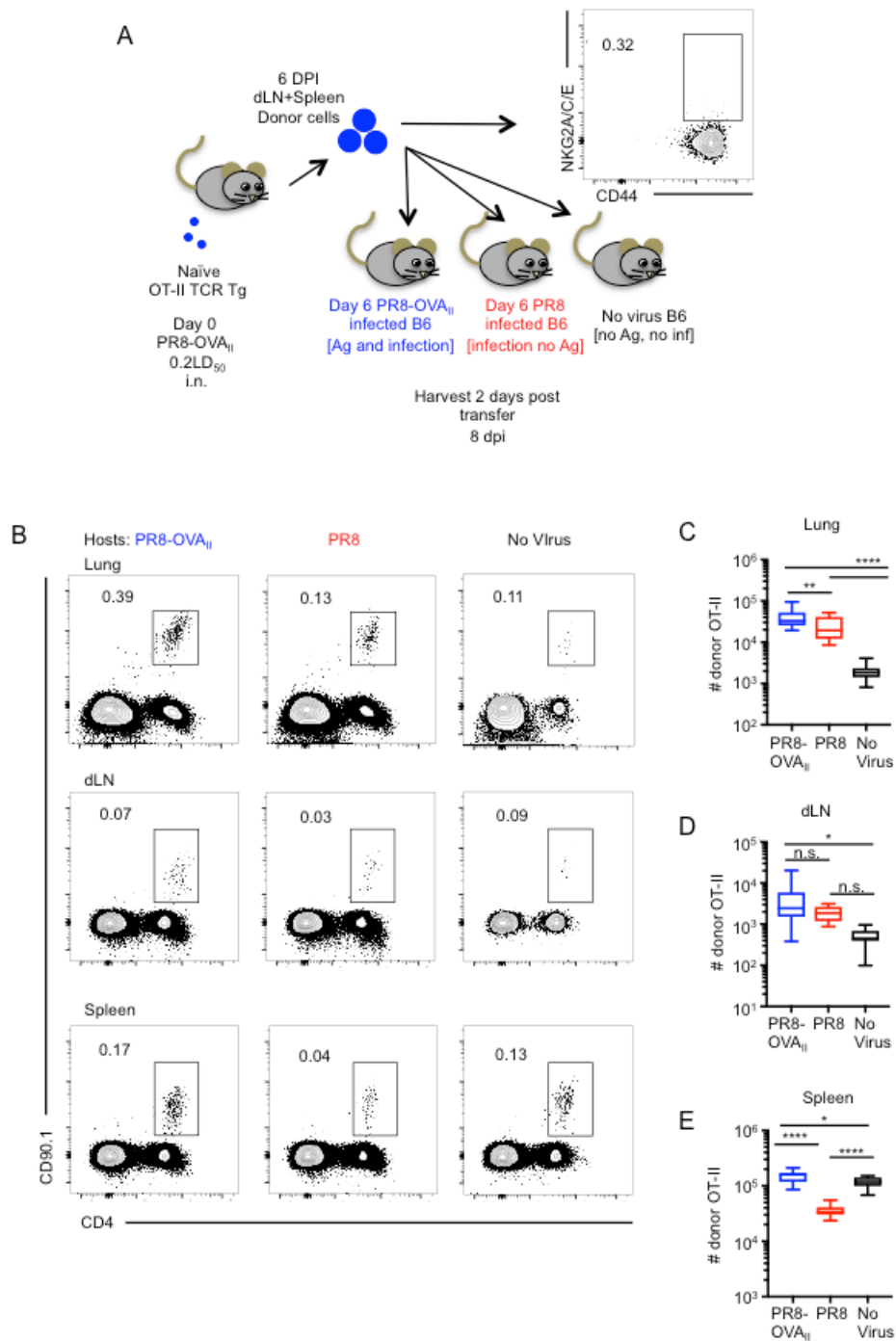


Figure 3.12: Antigen after 6 dpi promotes survival and expansion of effectors

(A) Experimental outline model. Naïve OT-II CD4 T cells were adoptively transferred into B6 mice and infected with PR8-OVA_{II}. At 6 dpi effectors were isolated from the dLN and spleen and transferred into 6 dpi PR8-OVA_{II} B6 mice, 6 dpi PR8 B6 mice, or uninfected B6 mice. 2 days post transfer, donor cells were enumerated from the indicated tissues. **(B)** Representative flow plots of the donor cells recovered from the lungs (top row), dLN (middle row), and spleens (bottom row) of host mice that were infected with PR8-OVA_{II} (left panels), PR8 mice (middle panels), or uninfected mice (right panels). **(C)** Number of donor cells recovered from the lungs. **(D)** Number of donor cells recovered from the dLN. **(E)** Number of donor cells recovered from the spleen. Data are representative of at least two independent experiments (B). Data are pooled from four independent experiments with $n = 19$ each group (C), or from two independent experiments with $n = 10$ each group (D,E). Boxes extend from the 25th to 75th percentile with the median as a line. Whiskers show the minimum and maximum values. * $P < 0.05$, ** $P < 0.005$, and **** $P < 0.0001$.

amount of donor cells recovered compared to just removal of antigen alone. These data suggest that inflammation may play an important role in recruiting effector CD4 T cells into the lungs, as would be expected with the roles of chemokines and adhesion molecules during inflammation (130, 131). Lack of antigen had no statistically significant effect on donor cell recovery in the dLN, suggesting antigen does not play a significant role in effector CD4 T cell survival or proliferation in the dLN (Figure 3.12D). Removal of both antigen and inflammation did lead to a reduction in donor cells recovered from the dLN suggesting both these factors are important for dLN CD4 effector cells. Whether inflammation and antigen promote survival, proliferation, trafficking or a combination thereof requires further study. Although *ex vivo* investigations suggest that antigen alone can promote survival and proliferation of 6 dpi effector cells from influenza infected mice (114), removal of antigen or both antigen and inflammation reduced the number of donor cells recovered from the spleen (Figure 3.12E), suggesting optimal recovery of splenic CD4 effectors requires both antigen and inflammation. It is unclear why the splenic effectors require antigen for their survival or trafficking in these organs while effectors in the dLN do not. It is possible the dLN directly draining the inflamed lungs have high or recent enough inflammatory signals to promote effector CD4 survival or migration into the dLN. Nonetheless, these data suggest that antigen recognition after 6 dpi overall plays only a small role in the survival or migration of effector CD4 T cells into the lungs, dLN, and spleen. As long as there is inflammation, the effector CD4 T cells can survive and migrate appropriately for at least 2 days *in vivo*, while the removal of inflammation leads to a more pronounced reduction in the numbers of donor cells

recovered (Figure 3.12). However, eventually the removal of antigen leads to a dramatic decline in the number of cells recovered, suggesting the window during which inflammation benefits CD4 effectors could be short (114), consistent with the concept of a “checkpoint” for effectors to see antigen to survive and transition to long-lived resting memory (113, 114).

Loss of ThCTL when antigen after 6 dpi is absent.

To address whether generation of ThCTL was affected by their transfer to a host lacking antigen or lacking both antigen and inflammation, I phenotyped the recovered donor cells from the sequential transfer and assayed the expression of NKG2X. NKG2X was expressed by donor cells recovered from mice infected with PR8-OVA_{II} suggesting the sequential transfer model sufficiently reproduces the induction of ThCTL in mice. Transfer to a host without antigen at 6 dpi led to a profound loss of ThCTL as measured by a reduced proportion of NKG2X expressing donor cells (Figure 3.13A,B). The number of ThCTL recovered was also reduced when late antigen was not available (Figure 3.13C). Inflammation alone was not sufficient to rescue ThCTL formation as no difference was seen in the expression of NKG2X between donor cells recovered from PR8 or uninfected mice. The further reduction in the number of ThCTL recovered in uninfected mice is likely due to the reduced T cell infiltration in the lungs of uninfected mice (Figure 3.12B). Further phenotyping of the donor cells reveals a loss of ThCTL function as well. Loss of exposure to antigen after 6 dpi led to reduced expression of granzyme B and reduced ability of donor cells to degranulate (Figure 3.13D,E). To

Figure 3.13: Antigen after 6 dpi promotes ThCTL differentiation and function

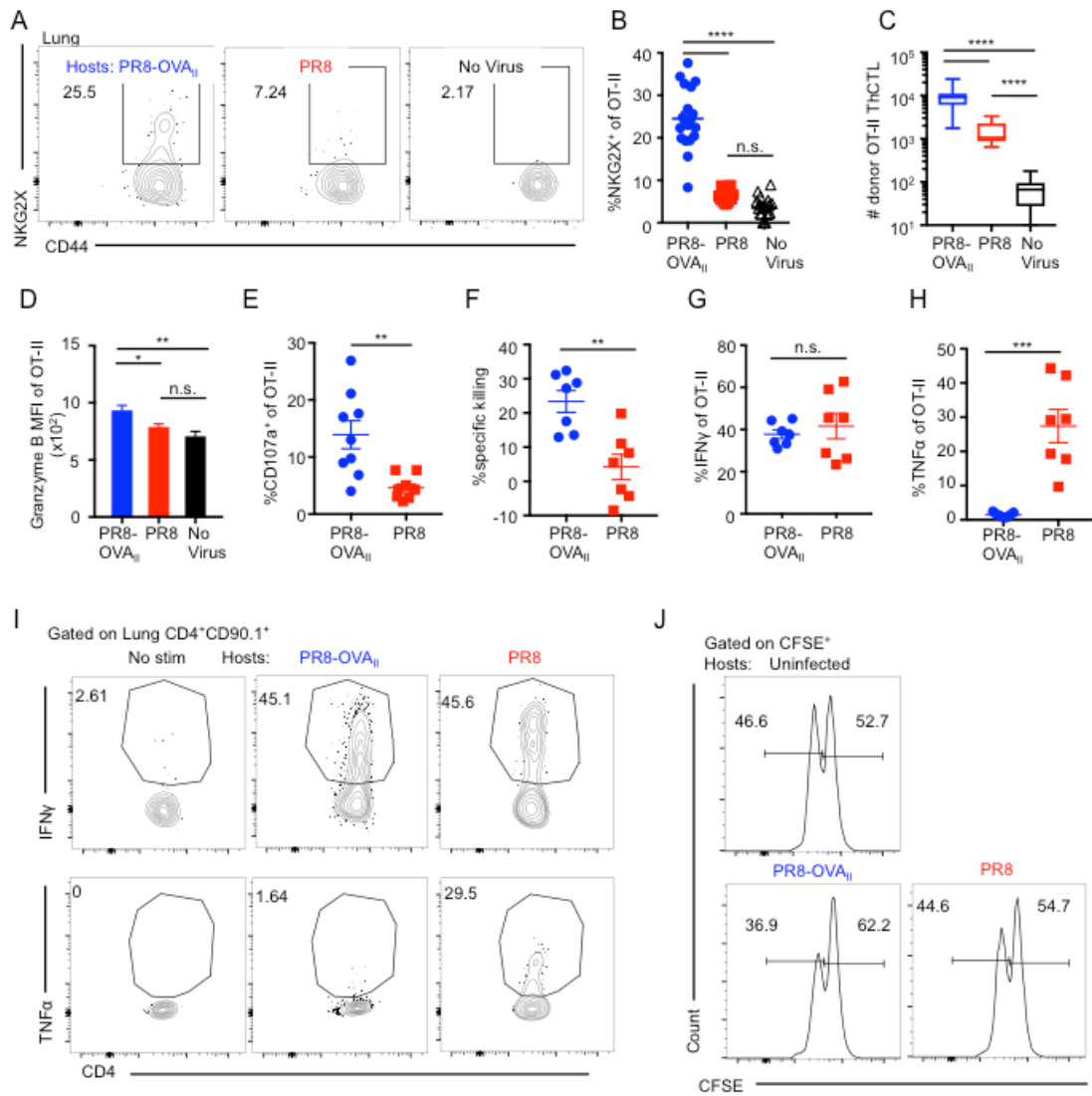


Figure 3.13: Antigen after 6 dpi promotes ThCTL differentiation and function

Naïve OT-II CD4 T cells were adoptively transferred into B6 mice and infected with PR8-OVA_{II}. At 6 dpi effectors were isolated from the dLN and spleen and transferred into 6 dpi PR8-OVA_{II} B6 mice (blue circles), 6 dpi PR8 B6 mice (red squares), or uninfected B6 mice (open triangles). 2 days post transfer, donor cells were analyzed from the lungs. **(A)** Representative staining of NKG2X expression on donor cells from the lungs of the indicated mice. **(B)** Percent NKG2X expression on donor cells isolated from the lungs of indicated mice. **(C)** Numbers of NKG2X⁺ donor CD4 T cells from the lungs of the indicated mice. **(D)** Median fluorescence intensity of donor cells from the lungs of indicated mice. **(E)** Naïve OT-II CD4 T cells were adoptively transferred into B6 mice and infected with PR8-OVA_{II}. At 6 dpi effectors were isolated from the dLN and spleen and transferred into 6 dpi PR8-OVA_{II} B6 mice or 6 dpi PR8 B6 mice. 2 days post transfer, lungs were isolated and stimulated with peptide pulsed APCs and stained for CD107a expression. **(F)** Naïve OT-II CD4 T cells were adoptively transferred into B6 mice and infected with PR8-OVA_{II}. At 6 dpi effectors were isolated from the dLN and spleen and transferred into 6 dpi PR8-OVA_{II} TCR α/β KO mice or 6 dpi TCR α/β KO mice and the in vivo cytotoxicity was assayed. **(G)** Lung cells from the indicated mice were stimulated ex vivo with CD3 and CD28 and assayed for percent IFN γ . **(H)** Lung cells from the indicated mice were stimulated ex vivo with CD3 and CD28 and assayed for percent IFN γ . **(I)** The representative flow plots of the cytokine production of IFN γ (top row) and TNF α (bottom row) from the donor cells in the indicated mice. **(J)** The representative flow plots of the target and bystander cells recovered from the in vivo cytotoxic assay in the indicated mice. Shown are percentages of the targets (CFSE^{lo}) and bystanders (CFSE^{hi}). Data are representative of 4 (A) or 2 (I, J) independent experiments or pooled data from 4 (B, C) n = 19 each group, or two independent experiments (D-H) n = 9-10 each group. Boxes in (C) extend from the 25th to 75th percentile with the median as a line. Whiskers show the minimum and maximum values. The rest are mean \pm SEM. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$, and **** $P < 0.0001$.

directly address whether the cytotoxic ability of donor cells was affected, I assayed the in vivo cytotoxic potential of the donor cells. I repeated the sequential transfer model, but instead of transferring into B6 mice, I transferred the 6 dpi effectors into 6 dpi (with either PR8-OVA_{II} or PR8) TCR α/β KO mice. These hosts lack their own T cells so there is no host cytotoxic response allowing one to assay the cytotoxicity of only the transferred donor cells. On 7 dpi, I transferred differentially labeled peptide pulsed target or bystander cells. At 8 dpi, I harvested the mice to assess the relative survival of target and bystander cells (85)(Figure 3.13J). Transfer into hosts lacking antigen at 6 dpi led to a reduction of in vivo cytotoxic function of the transferred donor cells (Figure 3.13F,J). The loss of cytotoxic function is consistent with the reduction of granzyme and degranulation in the absence of late antigen. These data taken together suggests that ThCTL require late antigen recognition for their differentiation from pre-ThCTL to ThCTL as transfer of 6 dpi effectors into hosts lacking antigen led to a loss of ThCTL phenotype and function. To ask whether other effector functions of lung CD4 effectors would be impacted, I also assessed the cytokine producing ability of the recovered donor cells by intracellular cytokine staining (ICCS). Transfer of donor cells into hosts without antigen did not affect the ability of lung donor cells to make IFN γ (Figure 3.13G,I). Interestingly, the lung donor cells exposed to antigen at 6 dpi do not secrete TNF α but develop the ability to secrete TNF α when transferred to hosts lacking antigen, suggesting the donor cells could be differentiating into a different subset of effector cells in the presence and absence of antigen recognition (Figure 3.13H,I). Overall these data suggest that antigen at 6 dpi promotes effector CD4 T cells to further differentiate into ThCTL

and gain cytotoxic function. Although IFN γ production is a marker of effector CD4 T cells, the requirement of late antigen for cytotoxic function suggests that ThCTL and cytotoxicity is associated with further effector differentiation.

Antigen recognition after 6 dpi upregulates effector phenotypes in the lung associated with ThCTL.

Since late antigen promotes ThCTL differentiation but does not affect cytokine secretion, we asked whether other effector phenotypes are also regulated by antigen at the effector phase. Phenotyping indicated that PD-1 expression that is high on all lung effectors, and higher on ThCTL (Figure 3.5), is reduced when late antigen recognition was absent after transfer (Figure 3.14A). Inflammation was not sufficient to drive PD-1 expression in the absence of antigen recognition by effectors. The high PD-1 expression with prolonged antigen suggests the possibility of additional regulation or inhibition of activated effectors to prevent continued or overt expansion, similar to the role of PD-1 in controlling overly stimulated CD8 T cells in chronic infections (129). CD27 was also dependent on effector recognition of antigen transfer of effectors to hosts lacking antigen led to a reduction in CD27 expression (Figure 3.14B). Again inflammation was not sufficient to rescue CD27 expression in the absence of antigen. Since CD27 promotes CD4 memory formation in influenza infection (113) and in CD8 cells (132), these data are consistent with the role of effector recognition of antigen at 6 dpi in promoting CD4 memory (114). Binding to P-selectin was not dependent on antigen after 6 dpi but did depend on PR8 infection induced inflammation (Figure 3.14C). These

Figure 3.14: Antigen after 6 dpi promotes lung effector phenotypes

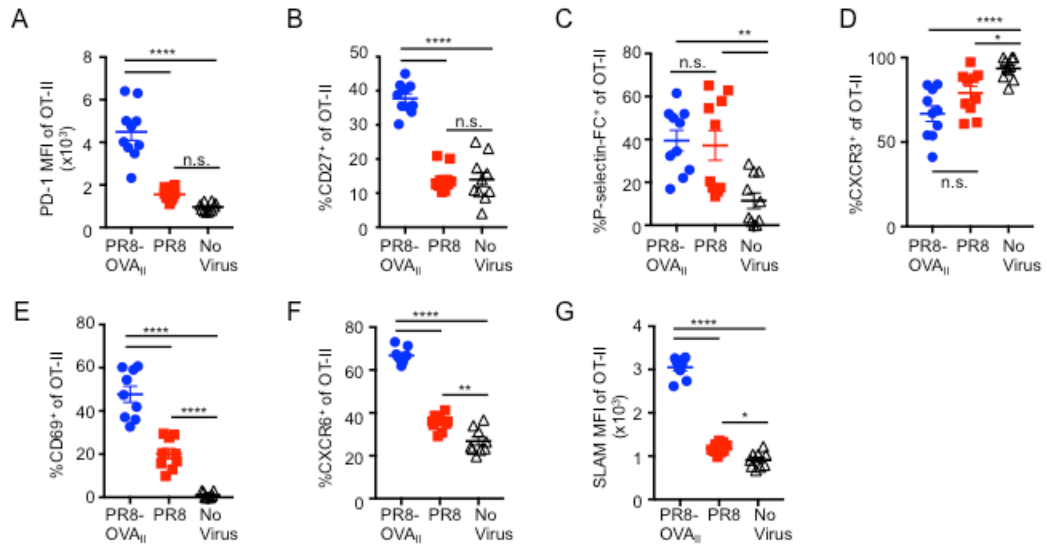


Figure 3.14: Antigen after 6 dpi upregulates effector phenotypes in the lung

Naïve OT-II CD4 T cells were adoptively transferred into B6 mice and infected with PR8-OVA_{II}. At 6 dpi effectors were isolated from the dLN and spleen and transferred into 6 dpi PR8-OVA_{II} B6 mice (blue circles), 6 dpi PR8 B6 mice (red squares), or uninfected B6 mice (open triangles). 2 days post transfer, donor cells were analyzed from the lungs. **(A)** Median fluorescence intensity of PD-1 expression on donor cells from the indicated mice. **(B)** Percent CD27 expression on donor cells from the indicated mice. **(C)** Percent binding to P-selectin on donor cells from the indicated mice. **(D)** Percent CXCR3 expression on donor cells from the indicated mice. **(E)** Percent CD69 expression on donor cells from the indicated mice. **(F)** Percent CXCR6 expression on donor cells from the indicated mice. **(G)** Median fluorescence intensity of SLAM expression on donor cells from the indicated mice. Data are pooled from two independent experiments with $n = 9-10$ for each group. Mean \pm SEM. * $P < 0.05$, ** $P < 0.005$, and **** $P < 0.0001$.

results are consistent with the role of P-selectin and activated PSGL-1 in promoting infiltration into inflamed sites (131). CXCR3 expression also was not dependent on antigen after 6 dpi and surprisingly effector transfer to uninfected hosts led to the highest expression of CXCR3 on donor cells (Figure 3.14D). Although CXCR3 helps recruit effectors to infected cells (134), the expression of CXCR3 increases when effector CD4 T cells become more resting (Figure 3.5B). Alternatively CXCR3 could be downregulated after encounter with chemokines CXCL9 and CXCL10, which are produced in the inflamed lungs. CD69 is an early activation marker expressed after TCR stimulation. As expected, CD69 expression is reduced when effectors are transferred into hosts lacking antigen (Figure 3.14E). Inflammation alone was able to induce some CD69 expression compared to donor cells transferred into uninfected hosts. CD69 can be induced by inflammatory cytokines in CD8 T cells (163), suggesting some of the CD69 expression is due to inflammation. CXCR6 is a chemokine receptor enriched on ThCTL. Transfer of donor CD4 effectors into hosts lacking antigen reduced the expression of CXCR6 (Figure 3.14F). Inflammation alone led to a small but statistically significant increase in CXCR6, but the majority of CXCR6 expression appears to be regulated by late antigen. As binding to P-selectin and CXCR6 are involved in trafficking to inflamed sites, induction of these two pathways appear to be differentially regulated by inflammation and antigen, suggesting redundancy in effector CD4 T cell migration to the inflamed lung and is consistent with the recovery of donor cells in the sequential model transfers (Figure 3.12B). SLAM expression showed a similar pattern as CXCR6, suggesting antigen promotes high SLAM expression, with a small effect of inflammation on SLAM

expression. Since ThCTL also express high levels of SLAM, the role of antigen in promoting SLAM is consistent with antigen recognition after 6 dpi also promoting ThCTL differentiation. These data taken together suggest that antigen recognition at the effector stage promotes the highly differentiated effector phenotypes of lung effectors in general and ThCTL in particular. Notably, not all effector associated cell surface marker changes depend on antigen, with binding to P-selectin and CXCR3, being instead dependent on inflammation. Thus multiple signals during 6-8 dpi promote the differentiation of effector CD4 T cells in the lung into ThCTL.

Antigen recognition after 6 dpi promotes Tfh in the dLN and spleens of influenza infected mice.

Tfh require multiple instructing signals from their interactions with dendritic cells (DC) and B cells (164). Further, in LCMV infection, Tfh require repeated interactions with APC for their continued differentiation as well as their induction (161). Phenotyping of 6 dpi effectors in the dLN and spleen suggests that antigen recognition after 6 dpi could also be important for Tfh formation during influenza infection (Figure 3.11E,F). To ask whether Tfh also depend on antigen after 6 dpi, I assayed the phenotype of the transferred donor CD4 OT-II cells in the sequential transfer model system. Donor Tfh cells were further induced as seen by the high expression of CXCR5 and Bcl-6. Transfer of effector CD4 T cells to hosts lacking antigen led to a reduced Tfh population as a proportion of donor CD4 effector cells and reduced numbers of donor Tfh recovered from the spleen 2 days post transfer (Figure 3.15A-C). Since the 6 dpi CD4 effector cells

Figure 3.15: Antigen recognition after 6 dpi promotes Tfh

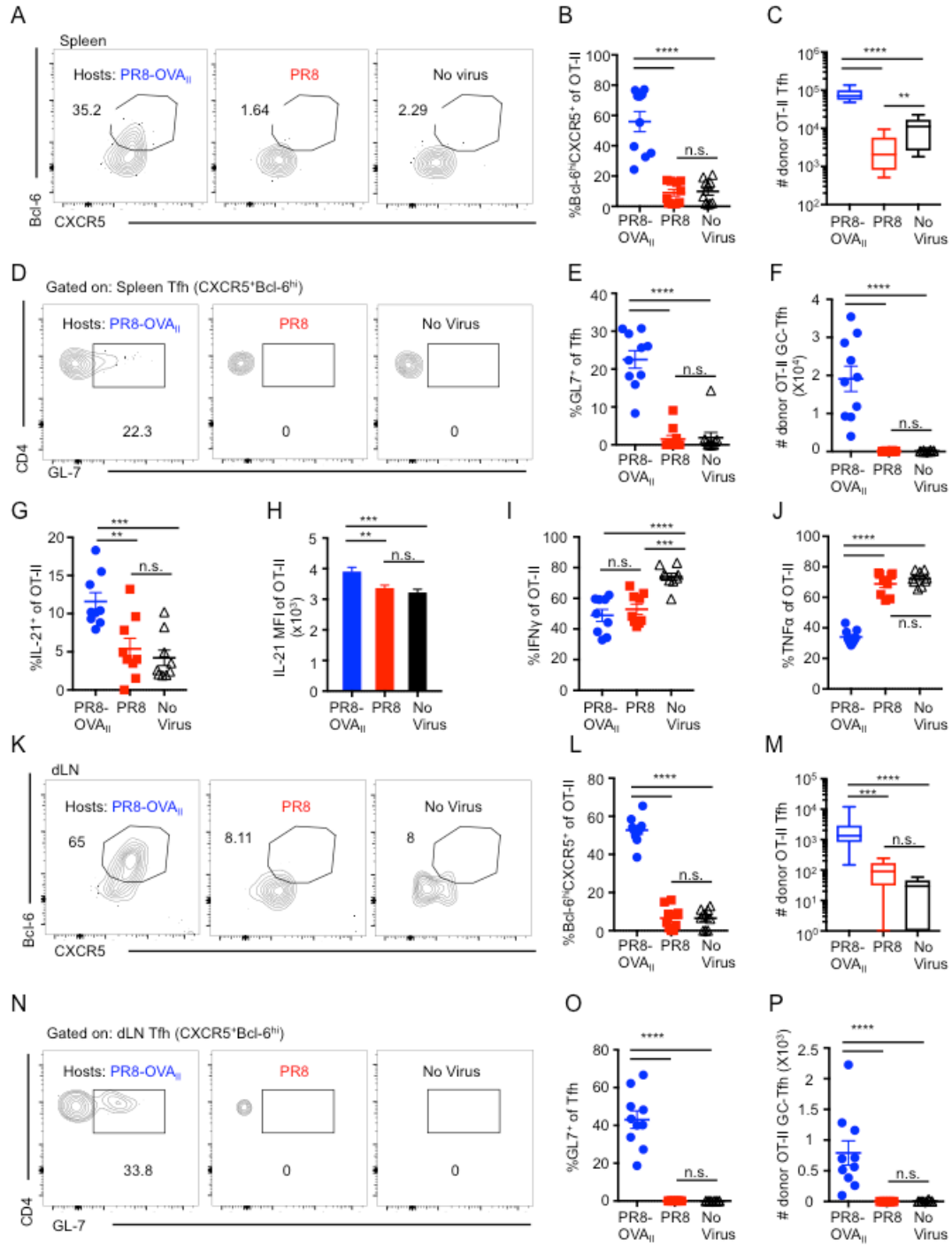


Figure 3.15: Antigen recognition after 6 dpi promotes Tfh

Naïve OT-II CD4 T cells were adoptively transferred into B6 mice and infected with PR8-OVA_{II}. At 6 dpi effectors were isolated from the dLN and spleen and transferred into 6 dpi PR8-OVA_{II} B6 mice (blue circles), 6 dpi PR8 B6 mice (red squares), or uninfected B6 mice (open triangles). 2 days post transfer, donor cells were analyzed from the spleen and dLN. **(A)** Representative flow plots of CXCR5 and Bcl-6 expression on donor cells recovered from the indicated mice. **(B)** Percent of donor cells expressing CXCR5⁺Bcl-6^{hi} from the spleens of indicated mice. **(C)** Number of CXCR5⁺Bcl-6^{hi} donor cells recovered from the spleens of indicated mice. **(D)** Representative flow plots of GL-7 expression on donor CXCR5⁺Bcl-6^{hi} cells from the spleens of the indicated mice. **(E)** Percent of donor CXCR5⁺Bcl-6^{hi} cells expressing GL-7. **(F)** The number of CXCR5⁺Bcl-6^{hi}GL-7⁺ donor cells recovered from the spleens of indicated mice. Spleens from the indicated mice were stimulated in vitro with PdBu and Ionomycin and gated donor cells were analyzed for cytokine expression. **(G)** Percent of donors expressing IL-21. **(H)** Median fluorescence intensity of IL-21 of donor cells. **(I)** Percent IFN γ of donor cells. **(J)** Percent TNF α of donor cells. **(K)** Representative flow plots of CXCR5 and Bcl-6 expression on donor cells recovered from the dLN of indicated mice. **(L)** Percent of donor cells expressing CXCR5⁺Bcl-6^{hi} from the dLN of indicated mice. **(M)** Number of CXCR5⁺Bcl-6^{hi} donor cells recovered from the dLN of indicated mice. **(N)** Representative flow plots of GL-7 expression on donor CXCR5⁺Bcl-6^{hi} cells from the dLN of the indicated mice. **(O)** Percent of donor CXCR5⁺Bcl-6^{hi} cells expressing GL-7 in the dLN. **(P)** The number of CXCR5⁺Bcl-6^{hi}GL-7⁺ donor cells recovered from the dLN of indicated mice. Data are representative of two independent experiments (A, D, K, N). Data are pooled from two independent experiments (B, C, E, F, G-J, L, M, O, P) with n = 9-10 for each group. Boxes in (C, M) extend from the 25th to 75th percentile with the median as a line. Whiskers show the minimum and maximum values. The rest are mean \pm SEM. ** $P < 0.005$, *** $P < 0.001$, and **** $P < 0.0001$.

already have Tfh cells (Figure 3.11E,F), these results are unable to distinguish whether antigen recognition after 6 dpi is important for maintenance or induction of Tfh, although results in the LCMV system suggests it could be both (161). Nonetheless, transfer of effectors to hosts where antigen is absent leads to a severe loss of Tfh after 2 days. Further analysis of germinal center Tfh (GC-Tfh) by expression of GL-7 (37) reveals that both the proportion of Tfh expressing GL-7 and the number of GC-Tfh cells recovered are severely reduced when antigen is absent (Figure 3.15D-F). Inflammation alone was insufficient to rescue the loss of Tfh and GC-Tfh. To test whether the function of these cells was also affected by antigen recognition after 6 dpi, I measured the production of IL-21 ex vivo by ICCS. IL-21 is an important Tfh cytokine that promotes the germinal center response (36) as well as Tfh differentiation (165, 166). Antigen recognition after 6 dpi was important for the production of IL-21 (Figure 3.15G,H), suggesting that the loss of Tfh was accompanied with the loss of germinal center B cell helper function. Assaying the production of IFN γ and TNF α cytokines reveals a similar pattern seen on the lung donor cells in the sequential model transfer. Transfer of effectors to hosts lacking antigen did not impact the ability of the cells to make IFN γ (Figure 3.15I), while also promoting the ability of the cells to make TNF α (Figure 3.15J). These data suggest that not all effector functions in the spleen are promoted by antigen after 6 dpi, however Tfh function requires this prolonged recognition. Tfh in the dLN displayed a similar pattern as the Tfh from the spleen. Tfh were severely reduced in proportion and numbers when effectors were transferred into hosts without antigen after 6 dpi (Figure 3.15K-M). GC-Tfh were similarly reduced when effectors were not exposed to antigen (Figure 3.15N-P). These

data taken in sum suggest that both ThCTL and Tfh require recognition of antigen from 6-8 dpi for their continued differentiation. As in the lung, not all effectors in the secondary lymphoid organs require antigen after 6 dpi and the remaining non-ThCTL and non-Tfh effectors are capable of producing IFN γ and TNF α and by inference promote other anti-viral effector mechanisms. The phenotypic and functional changes observed implies that antigen recognition after 6 dpi promote further differentiation to specialized functions of CD4 effector cells of cytotoxicity and germinal center help. Notably IL-21 production and cytotoxicity was dependent on antigen. These results also imply that the CD4 T cell response is continually specializing when antigen is still available to potentially promote additional mechanisms that enhance viral clearance (34).

Tfh and ThCTL differentially require CD80/CD86 costimulation after 6 dpi.

Since antigen recognition after 6 dpi was critical for the generation of both Tfh and ThCTL, we also explored the context in which antigen is presented. Antigen is normally presented by APC that express various co-stimulatory molecules. Tfh require Inducible T-cell Costimulator (ICOS, CD278) and ICOSL interactions with APC (164). ThCTL have been shown to be dependent on OX-40, 4-1BB or both for their generation (51–53). Notably, these studies with ThCTL have not looked at CD80 and CD86 co-stimulation. Although CD28-CD80/CD86 interactions are important for priming of T cells and for IL-2 induction, there are redundant mechanisms that assist T cell activation during viral infections. Interestingly, CD28 deficient mice mount a normal CD8 cytotoxic response against LCMV infection (167), suggesting cytotoxicity does not require

CD80/CD86 co-stimulation. Nonetheless, CD28 has been reported to be needed for CD8 cytotoxicity during influenza infection (168). These studies did not address the role of CD80/CD86 during initial priming or after priming at the effector stage. To address this, I utilized the sequential transfer model to transfer effector CD4 T cells into mice lacking both CD80 and CD86. I transferred naïve OT-II CD4 T cells into B6 mice and infected with PR8-OVA_{II}. At 6 dpi, I harvested and isolated donor cells by enriching for their donor congenic marker CD90.1. The effector cells were then transferred into either WT or CD80/CD86 KO hosts that were also infected with PR8-OVA_{II} 6 days earlier. The CD80/CD86 KO hosts provide antigen and inflammation, but their APC would not provide CD80/CD86 costimulation. Assaying the donor cells after 2 days post transfer again allowed sufficient time for differentiation into ThCTL and Tfh cells (Figure 3.16A,G). In the absence of CD80 and CD86 costimulation after 6 dpi there was an increase in the proportion of donor cells expressing NKG2X (Figure 3.16A,D) and an increase in the amount of protein expressed as measured by median fluorescence intensity of NKG2X (Figure 3.16B,C). However there was no increase in the numbers of ThCTL recovered from the mice lacking CD80/CD86 costimulation compared to wild type mice (Figure 3.16E). These data suggest CD80/CD86 costimulation after 6 dpi is not necessary for efficient induction of ThCTL. The loss of non-ThCTL could explain the increase in the proportion of NKG2X expression without a change in the total numbers of ThCTL recovered. We measured ex vivo degranulation to address whether the ThCTL generated in the absence of CD80 and CD86 costimulation were functional. ThCTL generated with or without late CD80/CD86 costimulation were equally able to degranulate as indicated

Figure 3.16: CD80/86 interactions at the effector stage differentially regulate ThCTL and Tfh

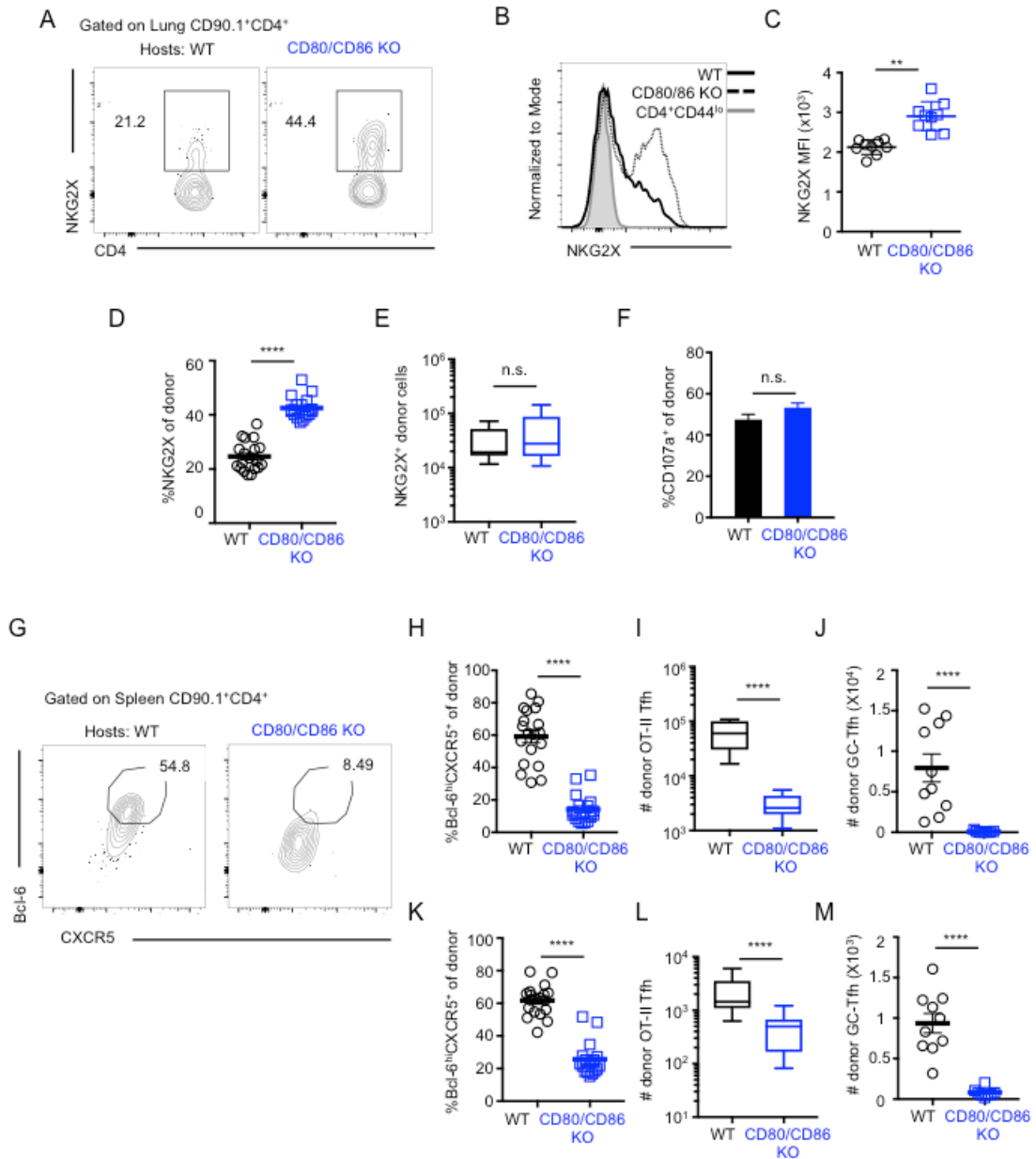


Figure 3.16: CD80/CD86 interactions at the effector stage differentially regulate ThCTL and Tfh

Naïve OT-II CD4 T cells were adoptively transferred into B6 mice and infected with PR8-OVA_{II}. At 6 dpi effectors were isolated from the dLN and spleen and transferred into 6 dpi PR8-OVA_{II} B6 mice (open circle) or 6 dpi PR8-OVA_{II} CD80/CD86 KO mice (open blue squares). 2 days post transfer, donor cells were analyzed from the lungs, dLN, and spleens. **(A)** Representative NKG2X expression of lung donor cells from wild type mice (left) or CD80/CD86 KO mice (right). **(B)** Representative overlaid histograms of NKG2X expression on lung naïve CD4⁺ CD44^{lo} (shaded), lung donor cells from wild type mice (solid) or from CD80/CD86 KO mice (dashed). **(C)** Median fluorescence intensity of NKG2X on donor lung cells from the indicated mice. **(D)** Percent NKG2X expression on donor lung cells from the indicated mice. **(E)** Numbers of NKG2X⁺ donor cells recovered from the lungs of the indicated mice. **(F)** Lung cells from the indicated mice were stimulated with activated peptide pulsed APCs and stained for CD107a expression on donor cells. **(G)** Representative flow plots of CXCR5 and Bcl-6 expression on donor cells recovered from the spleens wild type mice (left) or CD80/CD86 KO mice (right). **(H)** Percent of donor cells expressing CXCR5⁺Bcl-6^{hi} from the spleens. **(I)** Number of CXCR5⁺Bcl-6^{hi} donor cells recovered from the spleens. **(J)** The number of CXCR5⁺Bcl-6^{hi}GL-7⁺ donor cells recovered from the spleen. **(K)** Percent of donor cells expressing CXCR5⁺Bcl-6^{hi} from the dLN. **(L)** Number of CXCR5⁺Bcl-6^{hi} donor cells recovered from the dLN. **(M)** The number of CXCR5⁺Bcl-6^{hi}GL-7⁺ donor cells recovered from the dLN. Data are representative of 4 (A) or 2 (B, F, G) independent experiments. Data are pooled from 4 (D, H, K) n = 16-19 per group, 3 (E, I, L) n = 12-15 per group, or 2 (C, J, M) n = 8-10 per group, independent experiments. Boxes in (E, I, L) extend from the 25th to 75th percentile with the median as a line. Whiskers show the minimum and maximum values. The rest are mean ± SEM. ** *P* < 0.005, **** *P* < 0.0001.

by CD107a expression, suggesting that cytotoxic function of ThCTL is also independent of costimulation by CD80 and CD86 after 6 dpi. These data are consistent with reports of CD80 and CD86 costimulation being important for priming, but not for reactivation of polarized CD4 effector cells (169). Additionally, these data are consistent with human ThCTL not expressing CD28 (43). Taken together, these data indicate that functional ThCTL are efficiently generated in the absence of CD80/CD86 co-stimulation after 6 dpi. As Tfh also require antigen after 6 dpi, I assayed Tfh and GC-Tfh formation in the absence of CD80 and CD86 costimulation. In contrast to ThCTL, Tfh and GC-Tfh were severely reduced when effectors were transferred into hosts lacking CD80/CD86 costimulation (Figure 3.16G). The absence of CD80/CD86 costimulation after 6 dpi led to a reduction of the proportion of Tfh cells and the number of Tfh recovered from both the spleens and dLN of mice (Figure 3.16H,I,K,L). The numbers of GC-Tfh were severely reduced in the absence of CD80 and CD86 costimulation (Figure 3.16J,M). The loss of Tfh and GC-Tfh suggests that this subset of specialized CD4 effectors require additional costimulation for either their induction or maintenance. Although post priming CD80/CD86 is not required for Tfh cells in protein immunization models (170), these data are consistent with reports that CD28 post-priming is important for Tfh cells during infection (171), although the exact timing was not explored. Here we show that at least at 6 dpi, Tfh still require CD80/CD86 costimulation. Additionally, CD80/CD86 costimulation is important for germinal center formation (172), and since germinal centers are important for Tfh cells (161), the role of CD80 and CD86 could be both direct and indirect for the formation of Tfh cells. In summary, late CD80 and CD86

costimulation during influenza infection has different roles for ThCTL and Tfh. ThCTL are independent of late CD80/CD86 costimulation, while Tfh require CD80 and CD86 costimulation. These data suggest that these two specialized effector populations while both depending on additional antigen recognition utilize different signals for their regulation.

TCR signaling on effectors is sufficient to drive ThCTL formation in vitro.

Since antigen recognition at the effector stage is important for ThCTL formation, I asked whether antigen was sufficient to drive ThCTL formation. To test whether antigen was sufficient to drive ThCTL, I activated effectors in vitro with TCR stimulation. As in vivo infected mice would have multiple factors that could influence ThCTL, in vitro stimulation is the simplest method to test whether antigen was sufficient. I generated in vivo 6 dpi effectors as before, and isolated the effectors from infected mice by enrichment with the congenic marker CD90.1. To provide antigen signals, I stimulated the effectors with anti-CD3 coated wells. Additionally, to test CD80 and CD86 costimulation, I also added agonist CD28 antibody. After 2 days of in vitro culture with CD3 stimulation, CD4 effector cells expressed NKG2X (Figure 3.17A-C). In contrast, 2 days of CD3 and CD28 stimulation did not induce NKG2X expression (Figure 3.17A-C). The increase in the proportion of NKG2X expressing donor cells correlated with an increase in the numbers of ThCTL recovered after 2 days of in vitro stimulation. As expected, CD28 costimulation led to an increase in donor cell recovery (Figure 3.17D). However this increase in donor cell recovery was not associated with an increase in the

Figure 3.17: TCR stimulation of 6 dpi effectors sufficiently drives ThCTL in vitro

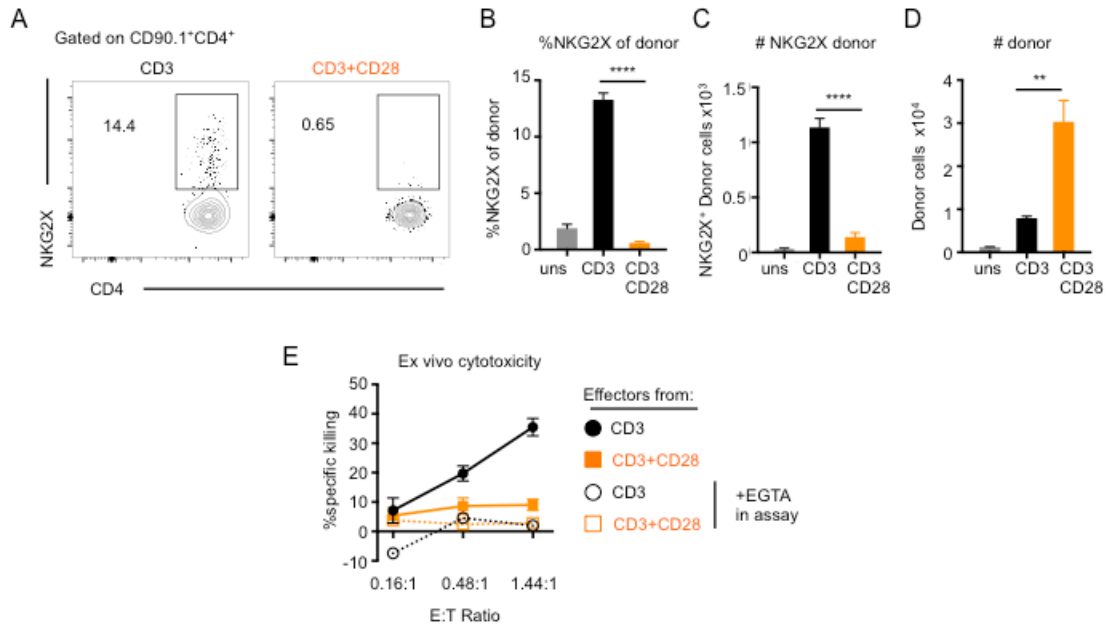


Figure 3.17: TCR stimulation of 6 dpi effectors sufficiently drives ThCTL in vitro

Naïve OT-II CD4 T cells were adoptively transferred into B6 mice and infected with PR8-OVA_{II}. At 6 dpi effectors were isolated from the dLN and spleen and donor cells were isolated and stimulated in culture for 2 days with either anti-CD3 or anti-CD3 and anti-CD28. **(A)** Representative flow plots of NKG2X expression on donor cells stimulated with CD3 (left) or CD3 and CD28 (right). **(B)** Percent of donor cells expressing NKG2X. **(C)** Number of donor NKG2X recovered. **(D)** Number of donor cells recovered. Donor cells were re-isolated after 2 days in cultured and assayed for their peptide specific cytotoxicity **(E)**. EGTA was added in control wells. Data are representative of at least 2 independent experiments (A, E). Data are pooled from 2-3 independent experiments with n = 6-9 for each group. Mean ± SEM. ** $P < 0.005$, **** $P < 0.0001$.

numbers of ThCTL recovered when CD28 was added. These data suggest that TCR stimulation was sufficient to drive ThCTL formation, while CD28 costimulation inhibited ThCTL. To address whether TCR stimulation was sufficient for ThCTL function, I re-isolated donor CD4 effectors after 2 days of in vitro stimulation. Assaying the peptide specific cytotoxicity of these effectors reveals that late TCR stimulation was sufficient to drive cytotoxicity (Figure 3.17E). CD28 costimulation was also inhibitory for the observed cytotoxicity induced by late TCR stimulation. These data are consistent with the in vivo requirement for antigen for the differentiation of ThCTL. These data however are at odds with the in vivo role of CD80/CD86, as there was no observed increase in ThCTL recovered in the absence of CD80/CD86 costimulation after 6 dpi (Figure 3.16E). The in vitro conditions may not fully recapitulate the conditions that occur in vivo, and the mechanism of the suppression of ThCTL will be explored further. Nonetheless, these data suggest that late TCR stimulation in vitro is sufficient for at least a portion of 6 dpi effectors to differentiate into ThCTL. Notably not all the effectors became ThCTL as measured by NKG2X expression, suggesting antigen alone may not be the full signal for ThCTL differentiation.

IL-2 at 6 dpi prevents ThCTL formation in vitro.

To further understand how CD28 stimulation in vitro led to a suppression of ThCTL formation from effectors isolated at 6 dpi (Figure 3.17), I further explored the role of IL-2. CD28 costimulation is an important driver of increased IL-2 production by T cells (169, 173). To test the role of IL-2 on the in vitro cultures of the 6 dpi effectors, I

Figure 3.18: IL-2 in vitro hinders ThCTL development from 6 dpi effectors

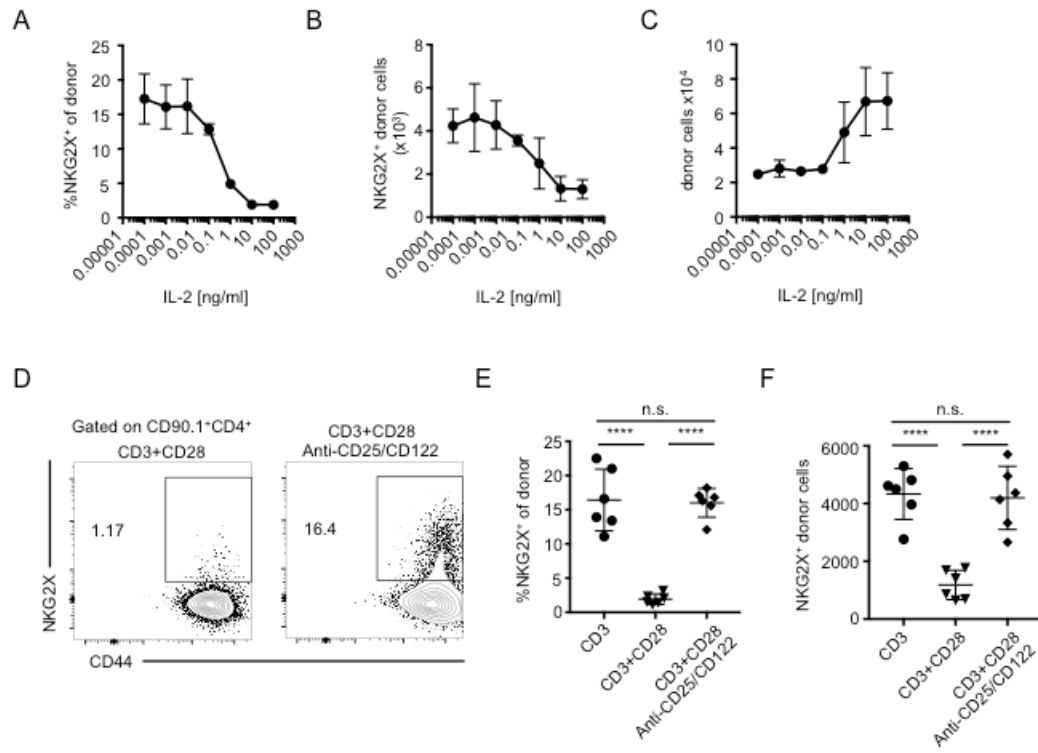


Figure 3.18: IL-2 in vitro hinders ThCTL development from 6 dpi effectors

Naïve OT-II CD4 T cells were adoptively transferred into B6 mice and infected with PR8-OVA_{II}. At 6 dpi effectors were isolated from the dLN and spleen and donor cells were isolated and stimulated in culture for 2 days with anti-CD3. Exogenous IL-2 at the indicated concentrations was added in the culture. **(A)** Percent of donor cells expressing NKG2X. **(B)** The number of NKG2X donor cells recovered. **(C)** The number of donor cells recovered. Naïve OT-II CD4 T cells were adoptively transferred into B6 mice and infected with PR8-OVA_{II}. At 6 dpi effectors were isolated from the dLN and spleen and donor cells were isolated and stimulated in culture for 2 days with anti-CD3 and anti-CD28. **(D)** Representative flow plots of NKG2X expression on donor cells with anti-CD25/CD122 treatment (right) or without (left). **(E)** The percent of NKG2X expression on donor cells in the indicated conditions. **(F)** The numbers of NKG2X donor cells recovered. Data are pooled from 2 independent experiments with $n = 6$. Mean \pm SEM. **** $P < 0.0001$.

stimulated 6 dpi effectors like in Figure 3.17 with anti-CD3. I titrated exogenous recombinant IL-2 into the cultures and after 2 days of stimulation, I assayed the formation of ThCTL. The proportion and number of ThCTL recovered was decreased as the dose of exogenous IL-2 was increased in the in vitro stimulation cultures (Figure 3.18A,B), suggesting IL-2 is suppressive in vitro for the ThCTL phenotype. As expected, the number of donor cells increased as the amount of exogenous recombinant IL-2 was added in the cultures (Figure 3.18C). These data suggest that high levels of IL-2 in vitro lead to a loss of ThCTL in culture. To address whether CD28 costimulation led to IL-2 production which in turn leads to the loss of ThCTL, I blocked IL-2 signaling. Blocking CD25 (IL-2R α) and CD122 (IL-2R β) on cells stimulated with CD3 and CD28 lead to an increase in the proportion of donor cells expressing NKG2X compared to cells stimulated without blocking antibodies (Figure 3.18D,E). Blockade of the IL-2 receptor also led to an increase in the number of ThCTL recovered in the wells after two days of stimulation (Figure 3.18F). These data suggest that CD28 costimulation promotes IL-2 production, which ultimately leads to the loss of ThCTL in culture. Whether IL-2 directly suppresses ThCTL is unclear as the cultured effectors are initially a heterogeneous population of 6 dpi effectors with possibly different potentials for further differentiation. IL-2 could be expanding an IL-2 responsive population and outcompeting the ThCTL in vitro as all the cells are in the same well. These in vitro conditions are in contrast to in vivo conditions where there is spatial regulation and where ThCTL may develop in the lung in a specialized environment. However these data suggest that ThCTL can at least exist in the absence of high levels of IL-2, as long as high levels of TCR stimulation is present.

Differentiation of ThCTL is independent of IL-2 in vivo.

To further explore the role of IL-2 in ThCTL differentiation, I turned to in vivo experiments to interrogate IL-2. To ask whether ThCTL require IL-2 signals at the effector stage, I blocked IL-2 in vivo by adding neutralizing antibodies of known efficacy (113). I infected wild type mice with PR8-OVA_{II} and at day 6 and day 7 post infection, I administered intranasal neutralizing IL-2 antibodies (113) to target the lung infiltrating T cells. At 8 dpi, I harvested the lungs and assessed ThCTL. Neutralizing IL-2 antibody in vivo did not affect the differentiation of ThCTL as similar proportions and numbers of ThCTL were recovered in the lung after neutralization of IL-2 in vivo (Figure 3.19A,B). These data suggest that ThCTL do not require IL-2 at 6-7 dpi for their formation and that IL-2 during this time is not suppressive for ThCTL formation in vivo. IL-2 production however does have a restricted pattern of expression during influenza. IL-2 is mainly produced in the dLN and spleen with lower expression in the lungs of influenza infected mice (47, 159, 162), suggesting IL-2 may not be highly produced in the infected lung environment. Therefore, to test whether addition of IL-2 could affect ThCTL development, I administered exogenous IL-2 to mice. I transferred naïve OT-II CD4 transgenic cells into wild type mice and infected with PR8-OVA_{II}. On day 6 and 7 post infection, I administered exogenous IL-2 in the form of IL-2 complexes. The bioavailability of recombinant IL-2 is short lived and the complex with anti-IL-2 antibodies greatly increases the half life of IL-2 (174). Addition of IL-2 complexes led to an increase in CD25 expression of donor cells in the lung (Figure 3.19C). However, the

Figure 3.19: ThCTL develop in the absence of autocrine IL-2 signaling in vivo.

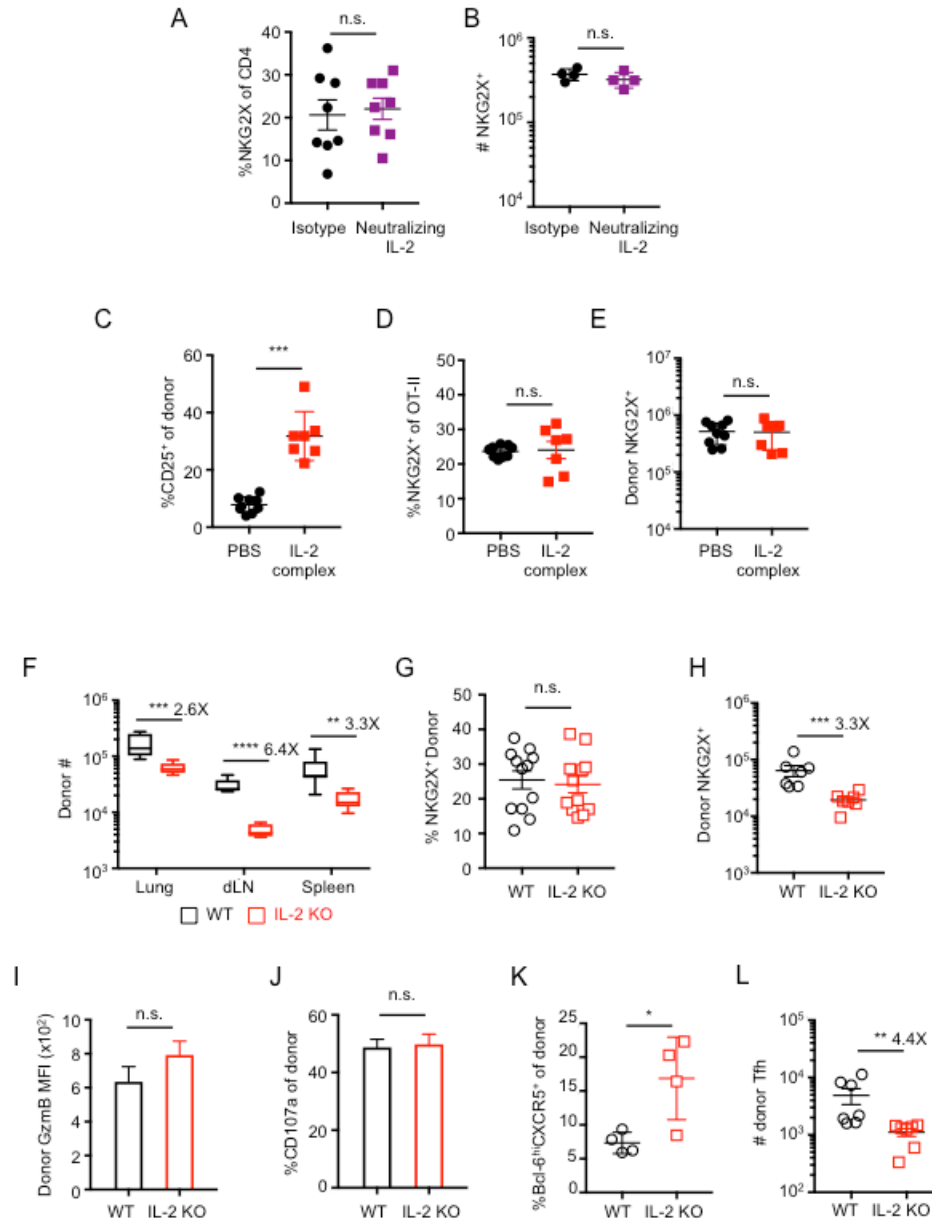


Figure 3.19: ThCTL develop in the absence of autocrine IL-2 signaling in vivo

B6 mice were infected with PR8-OVA_{II}. At 6 dpi and 7 dpi, neutralizing IL-2 antibodies were administered i.n. At 8 dpi, lungs were assayed for **(A)** percent of effector CD4⁺ CD44^{hi} cells expressing NKG2X. **(B)** The number of NKG2X⁺ CD4⁺ CD44^{hi} effector cells recovered. Naïve OT-II CD4 T cells were adoptively transferred into B6 mice and infected with PR8-OVA_{II}. At 6 dpi and 7 dpi, IL-2 complexes were administered i.p. At 8 dpi, lungs were harvested and analyzed for **(C)** CD25 expression on lung donor cells. **(D)** Percent of lung donor cells expressing NKG2X. **(E)** The number of NKG2X donor cells recovered from the lungs. Naïve DO11.10 or IL-2 KO DO11.10 CD4 T cells were adoptively transferred into BALB/C mice and infected with PR8-OVA_{II}. At 8 dpi, lungs, dLN, and spleens were analyzed. **(F)** The number of wild type (open black box) or IL-2 KO (open red box) donor cells recovered from the indicated tissues. **(G)** The percent donor cells expressing NKG2X in the lungs. **(H)** The numbers of NKG2X donor cells recovered from the lungs. **(I)** The median fluorescence intensity of granzyme B expression on donor cells from the lungs. **(J)** Lung cells were stimulated ex vivo with peptide pulsed activated APC and stained for CD107a expression on donor cells. **(K)** Percent of donor cells expressing CXCR5⁺Bcl-6^{hi} from the dLN. **(L)** Number of CXCR5⁺Bcl-6^{hi} donor cells recovered from the dLN. Data are representative of 2 experiments (B, J, K) n = 4-5 each group each time. Data are pooled from 2 (A, C-E, I, L) n = 7-9 each group, or 3 (F-H) n = 12 per group, independent experiments. Boxes in (F) extend from the 25th to 75th percentile with the median as a line. Whiskers show the minimum and maximum values. The rest are mean ± SEM. * *P* < 0.05, ** *P* < 0.005, *** *P* < 0.001.

addition of IL-2 did not lead to a decrease in the proportion or numbers of ThCTL recovered compared to PBS treated control mice (Figure 3.19D,E). These data suggest that addition of a supra-physiological amount of IL-2 at 6-7 dpi does not alter the differentiation of ThCTL. These data are consistent with the idea that the in vitro exogenous IL-2 addition was not affecting ThCTL directly but was acting on other cells that outcompete ThCTL in vitro. Addition of exogenous IL-2 in vivo could also lead to other potential indirect effects.

Many immune cells could respond to IL-2 like CD8 T cells and NK cells as well as endothelial cells that can lead to toxicity (175), further complicating the interpretation of nonspecific IL-2 addition in vivo. To address a T cell intrinsic requirement for IL-2 in ThCTL differentiation, I utilized CD4 TCR transgenic mice lacking IL-2. I transferred either wild type or IL-2 deficient naïve DO11.10 CD4 transgenic cells into wild type mice and infected with PR8-OVA_{II}. On 8 dpi, I harvested the mice and assayed for ThCTL differentiation. In these mice, only the transferred donor cells lack autocrine IL-2 production, while the host is wild type. As IL-2 is important during priming, the numbers of donor cells recovered in the lungs, dLN, and spleen were reduced when donor cells lacked autocrine IL-2 compared to wild type (Figure 3.19F). However the reduction of donor cells wasn't so severe that the phenotype could not be assessed. Similar proportion of donor cells lacking autocrine IL-2 expressed NKG2X compared to wild type donor cells (Figure 3.19G), suggesting autocrine IL-2 is not necessary for ThCTL differentiation. The numbers of ThCTL recovered was reduced in the absence of autocrine IL-2 (Figure 3.19H), echoing the similar overall reduction in donor cells

recovered (Figure 3.19F). Because the donor cells lacked autocrine IL-2 throughout the response, these data suggest that IL-2 was important for proliferation of the donor cells but not for ThCTL differentiation. To address whether the lack of autocrine IL-2 affected ThCTL function, I also measured granzyme B expression and degranulation capacity. Wild type and IL-2 KO donor cells had similar granzyme B protein expression (Figure 3.19I) and had similar capacities to degranulate (Figure 3.19J), suggesting ThCTL function is independent of autocrine IL-2. Although the host can make IL-2 and there could be some paracrine IL-2 to compensate, it isn't enough to compensate to wild type levels in terms of donor cell recovery. Tfh have been reported to be inhibited by IL-2 (176, 177), and there was an increase in the proportion of IL-2 deficient donor cells expressing CXCR5 and Bcl-6 compared to wild type donor cells (Figure 3.19K). The increase in proportion, however could not overcome the deficiency in donor cell recovery leading to a decrease in the numbers of Tfh recovered in the absence of autocrine IL-2 (Figure 3.19L). These data taken together with the IL-2 neutralization and exogenous IL-2 addition, suggest that IL-2 plays a minimal role in ThCTL differentiation. The role of IL-2 in vivo for ThCTL appears to be for maximal proliferation and/or survival, however ThCTL are not completely defective in the absence of IL-2. The lack of a requirement for IL-2 is consistent with the lack of a requirement for CD80 and CD86 costimulation (Figure 3.16), as this costimulatory pathway helps induce IL-2 production (167, 169). These data are also consistent with the lack of IL-2 being produced by CD4 T cells in the lung (47, 159, 162), suggesting ThCTL are not in a high IL-2 environment in the lung. In

sum, ThCTL are independent of IL-2, further making this subset of effectors unique in terms of the pathways that mediate their differentiation.

IL-2 independent CD4 memory T cells in the lung after influenza infection.

The lack of a requirement for IL-2 in ThCTL differentiation calls into question whether these cells could potentially transition into the memory pool. Previous results suggest that ThCTL could be characterized as ‘terminally’ differentiated as this population has downregulated many memory associated genes (Figure 3.7), require Blimp-1 for their differentiation (Figure 3.10), and express Blimp-1 (109). IL-2 plays a critical role in CD4 memory generation (113), and promotes survival of CD4 effector cells (114). To address whether CD4 T cells lacking autocrine IL-2 generate memory CD4 T cells after influenza infection, I transferred naïve wild type or IL-2 deficient DO11.10 CD4 transgenic cells into wild type mice and infected with PR8-OVA_{II}. At 28 dpi, a memory time point well after effectors have become resting (Figure 3.5), I harvested and analyzed cells from the lungs, dLN, and spleen. As expected, the lack of autocrine IL-2 led to a severe reduction in cells recovered compared to wild type cells (Figure 3.20A,B). The loss of donor memory cells is greater than the already reduced numbers of effector cells recovered at the peak of the infection (Figure 3.19F), suggesting the continued requirement of IL-2 for memory formation. Looking at the individual organs reveals that although the IL-2 deficient memory cells in the dLN and spleen are near the limit of detection, there is a population of IL-2 deficient memory cells in the lungs (Figure 3.20A). The fold reduction of numbers of IL-2 deficient lung memory cells

Figure 3.20: A population of lung CD4 memory develops in the absence of autocrine IL-2

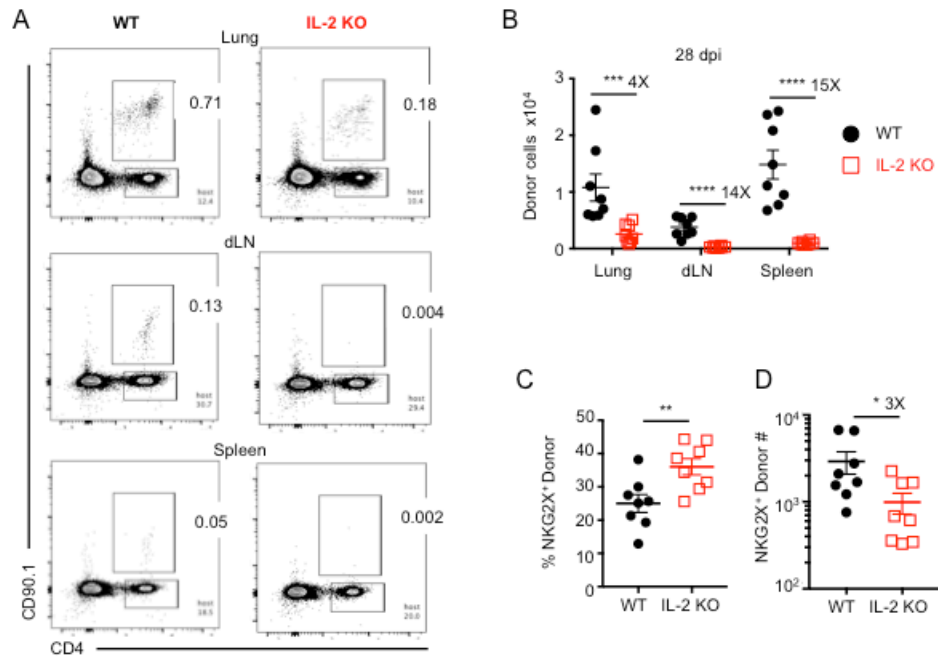


Figure 3.20: A population of lung CD4 memory develops in the absence of autocrine IL-2

Naïve DO11.10 or IL-2 KO DO11.10 CD4 T cells were adoptively transferred into BALB/C mice and infected with PR8-OVA_{II}. At 28 dpi, lungs, dLN, and spleens were analyzed. **(A)** Representative flow plots of donor wild type (left) or IL-2 KO (right) cells from the lungs (top), dLN (middle), and spleen (bottom). **(B)** Numbers of wild type (black circle) or IL-2 KO (red open square) donor cells recovered from the indicated tissues. **(C)** The expression of NKG2X on donor cells recovered from the lungs. **(D)** The number of NKG2X donor cells recovered from the lungs. Data are representative (A) or pooled (B-D) of 2 independent experiments, n = 8 each group. Boxes in (B) extend from the 25th to 75th percentile with the median as a line. Whiskers show the minimum and maximum values. The rest are mean ± SEM. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$, and **** $P < 0.0001$.

was less compared to IL-2 deficient memory cells in the dLN and spleen; 4X to 15X (Figure 3.20B). Because there are cells to analyze in the lung, phenotyping of the IL-2 deficient lung memory CD4 cells was possible. Assessing the expression of NKG2X reveals that IL-2 deficient memory CD4 T cells had a minor increase in the proportion of cells expressing NKG2X (Figure 3.20C). The reduction in the total number of memory cells however leads to a reduction of total numbers of NKG2X⁺ memory CD4 cells in the lung (Figure 3.20D). These data suggest that although autocrine IL-2 was necessary for CD4 memory formation, a population of lung memory CD4 cells is independent of IL-2. In addition, this population also expresses NKG2X, suggesting they represent ThCTL memory, as ThCTL at the effector stage was independent of IL-2 as well. However these data cannot rule out NKG2X expression at the memory time point is the same population of ThCTL that existed on 8 dpi and fate-mapping experiments would be necessary to address that question. Nonetheless, these data suggest that IL-2 is not required for both ThCTL at the effector stage and lung NKG2X cells at the memory stage.

ThCTL partially require IL-15.

Since ThCTL was independent of IL-2 for their differentiation, we wanted to ask whether other cytokines could be important for their differentiation. Although IL-2 and interferons have been previously studied (50, 64), the role of cytokines at the effector stage for ThCTL differentiation has not been studied. Local IL-15 promotes CD8 effector cells in the lungs of influenza infected mice (178). Additionally, local IL-15 promotes resident memory CD8 T cells (179), suggesting IL-15 could affect local cell populations.

As ThCTL appear to be resident in the lung tissue, IL-15 could potentially promote ThCTL during influenza infection. To test whether ThCTL require late IL-15, I generated 6 dpi effectors like before by transfer of naïve OT-II CD4 T cells into wild type mice and infected with PR8-OVA_{II}. I then transferred the donor OT-II cells at 6 dpi into either wild type or IL-15R α deficient hosts that were also infected with PR8-OVA_{II} 6 days prior. Hosts deficient in IL-15R α are unable to provide IL-15 signals to the transferred donor cells as IL-15 is mainly presented in trans to T cells (180–182). 2 days post transfer (8 dpi), I harvested the lungs, dLN, and spleens of mice to analyze ThCTL formation. Staining of IL-15R α showed that the deficient mice were indeed deficient in IL-15R α (Figure 3.21A). Comparing IL-15R α expression in the three organs shows that the lungs had higher expression of IL-15R α compared to the dLN and spleens (Figure 3.21A). These data are consistent with the report of increased IL-15 expression in the lung after influenza infection (178). Recovery of donor cells from the lungs, dLN, and spleen revealed no significant defect in the number of donor cells (Figure 3.21B), suggesting a minimal role of IL-15 in promoting the infiltration and survival of CD4 effector cells. This is in contrast to the defects seen in CD8 effector cells (178) during influenza. As expected, the lack of IL-15R α leads to a severe reduction in NK cells in all three organs analyzed (Figure 3.21C). IL-15 transpresentation is important for NK cell homeostasis (183, 184). To address whether ThCTL were impacted, I phenotyped the lung donor cells. Donor cells transferred into hosts lacking IL-15R α showed a reduced proportion of cells expressing NKG2X compared to donor cells transferred into wild type hosts (Figure 3.21D). The total numbers of ThCTL recovered was also reduced when the effectors

Figure 3.21: IL-15 signals promote 6 dpi effectors to differentiate into ThCTL

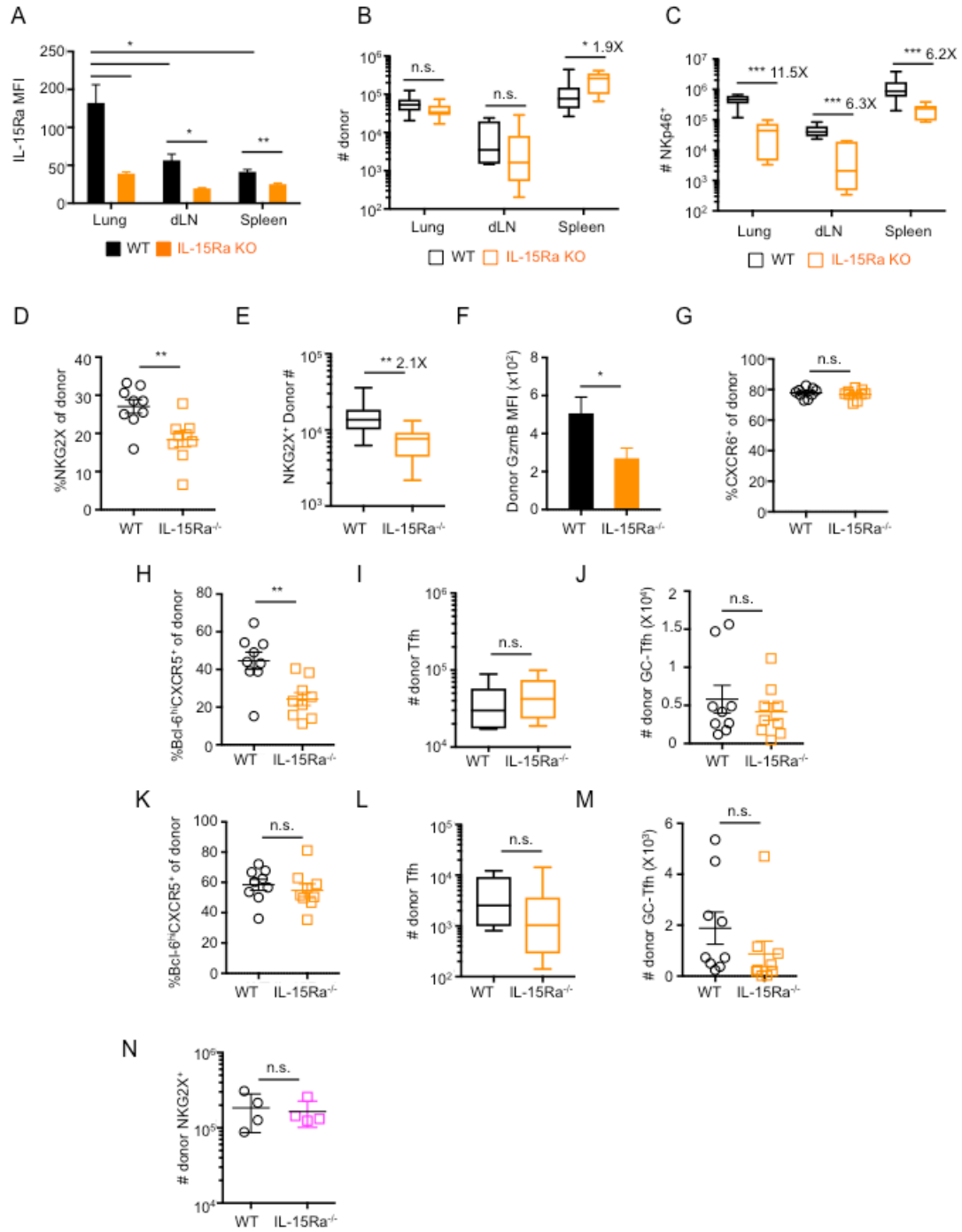


Figure 3.21: IL-15 signals promote 6 dpi effectors to differentiate into ThCTL

Naïve OT-II CD4 T cells were adoptively transferred into B6 mice and infected with PR8-OVA_{II}. At 6 dpi effectors were isolated from the dLN and spleen and transferred into 6 dpi PR8-OVA_{II} B6 mice or 6 dpi PR8-OVA_{II} IL-15Ra KO mice. 2 days post transfer, donor cells were analyzed from the lungs, dLN, and spleens. **(A)** Median fluorescence intensity of live cells from the indicated tissues of either wild type (black bars) or IL-15Ra KO (orange bars) mice. **(B)** The numbers of donor cells recovered from the indicated tissues from wild type (black boxes) or IL-15Ra KO (orange boxes) mice. **(C)** The number of NK cells (NKp46⁺) recovered from the indicated tissues. **(D)** The percent of lung donor cells expressing NKG2X. **(E)** The numbers of NKG2X donor cells recovered from the lungs. **(F)** The median fluorescence intensity of granzyme B expression on donor cells in the lung. **(G)** The percent of lung donor cells expressing CXCR6. **(H)** Percent of donor cells expressing CXCR5⁺Bcl-6^{hi} from the spleens. **(I)** Number of CXCR5⁺Bcl-6^{hi} donor cells recovered from the spleens. **(J)** The number of CXCR5⁺Bcl-6^{hi}GL-7⁺ donor cells recovered from the spleen. **(K)** Percent of donor cells expressing CXCR5⁺Bcl-6^{hi} from the dLN. **(L)** Number of CXCR5⁺Bcl-6^{hi} donor cells recovered from the dLN. **(M)** The number of CXCR5⁺Bcl-6^{hi}GL-7⁺ donor cells recovered from the dLN. **(N)** The number of NKG2X expressing donor cells from wild type or IL-15Ra KO donor OT-II cells in the lungs at 8 dpi. Data are from one experiment (A, N) n = 4 per group or pooled from 2 independent experiments (B-M) with n = 9 per group. Boxes in (B, C, E, I, L) extend from the 25th to 75th percentile with the median as a line. Whiskers show the minimum and maximum values. The rest are mean ± SEM. * *P* < 0.05, ** *P* < 0.005, *** *P* < 0.001.

were transferred into mice deficient in IL-15R α . These data suggest that IL-15 presented in trans is important for effectors to differentiate into ThCTL. Donor cells also express lower amounts of granzyme B when they were transferred into hosts lacking IL-15R α , suggesting IL-15 signals presented in trans promotes ThCTL function. Another lung effector and ThCTL marker, CXCR6, was not impacted by the loss of trans IL-15 signaling on effectors after 6 dpi, suggesting not all lung effector markers are affected. The reduction in ThCTL in the absence of late IL-15 transpresentation however is not as severe that when antigen is absent after 6 dpi (Figure 3.13), suggesting other cytokines or inflammatory signals could compensate for the differentiation of ThCTL. Nonetheless, IL-15 transpresentation after 6 dpi can promote the differentiation of a portion of ThCTL. To address whether Tfh were also similarly affected, I analyzed the Tfh population in the spleens and dLN of mice. The loss of transpresentation of IL-15 after 6 dpi did not lead to a defect in the numbers of Tfh cells recovered in both the spleen and dLN (Figure 3.21I,K,L). There was a reduction in the proportion of donor cells in the spleen expressing CXCR5 and Bcl-6, however the total numbers of Tfh were unchanged as the total numbers of spleen donor cells was increased in the absence of IL-15R α (Figure 3.21B). The loss of NK cells could potentially explain this phenomenon as NK cells can regulate the number of antiviral CD4 T cells (185). Numbers of GC-Tfh were also unaffected in the absence of IL-15 after 6 dpi (Figure 3.21J,M) in the dLN and spleen. To test the T cell intrinsic requirement for IL-15R α , I transferred a 1:1 mix of wild type or Il15ra^{-/-} OT-II CD4 (IL-15R α KO) transgenic cells into wild type host B6 mice and infected with PR8-OVA_{II}. Co-transferring the wild type and knockout T cells allows for

both populations to compete as well as ensuring they are differentiating in the same environment. At 8 dpi, in the lungs of infected mice, I recovered similar numbers of NKG2X expressing wild type and IL-15R α KO donor cells (Figure 3.21 N). These data suggests that the CD4 T cells themselves do not require IL-15R α for their differentiation into ThCTL, and is consistent with reports that the majority of IL-15 signaling is through transpresentation. These data together suggest that IL-15 signals after 6 dpi promote ThCTL while not affecting Tfh. The preference of IL-15 on ThCTL is consistent with IL-15 having roles on local tissue populations (178, 179) and the increased IL-15R α expression in the lung compared to the dLN and spleen (Figure 3.21A). TLR4 and TLR3 stimulation as well as stimulation with type I or II interferons can induce macrophages to make IL-15, suggesting molecules enriched in the sites of infection can help promote IL-15 production (186). Whether IL-15 promotes the survival of ThCTL remain to be studied, as IL-15 is known to promote survival of CD8 and NK cells (178, 183). And since IL-15 can be important for tissue resident memory (179) and CD8 memory (187), whether IL-15 promotes the survival of ThCTL derived memory also remains to be studied.

CHAPTER IV: DISCUSSION

ThCTL differentiation in summary

The results presented above illustrate ThCTL differentiation in the mouse during viral infection (Figure 4.1). Naïve CD4 T cells encounter their cognate antigen on antigen presenting cells and become activated. After activation these cells proliferate and differentiate into effector CD4 T cells. During this differentiation, different signals from the immune system instruct the effector cells to follow programs of differentiation into more specialized effector cells with unique functions. In the mouse and in humans, CD4 T cells can differentiate into cells with cytotoxic function. These ThCTL are unique in their requirements and differentiation compared to other CD4 subsets. ThCTL are regulated by late antigen as well as Blimp-1. These cells also are tissue restricted, in that they are only found in the site of infection. In the case of influenza, where the virus replicates in the lung, ThCTL are found in the lung. In the case of LCMV infection, where the virus is found in multiple sites including the spleen, ThCTL can be found in those sites. The requirement of late antigen suggests that ThCTL are further differentiated compared to non-ThCTLs. Indeed phenotyping ThCTL in the lung reveals higher expression of activation and effector markers compared to non-ThCTL in the lung. The requirement of antigen also is integrated with the requirements of costimulation and cytokines; signals typically associated with T cell – APC interactions. Notably, CD80 and CD86 are not required at this time point for ThCTL formation, while these signals are important for other effector cells like non-ThCTL lung effectors and Tfh cells. ThCTL

Figure 4.1: Model for the regulation of ThCTL, Tfh, and memory

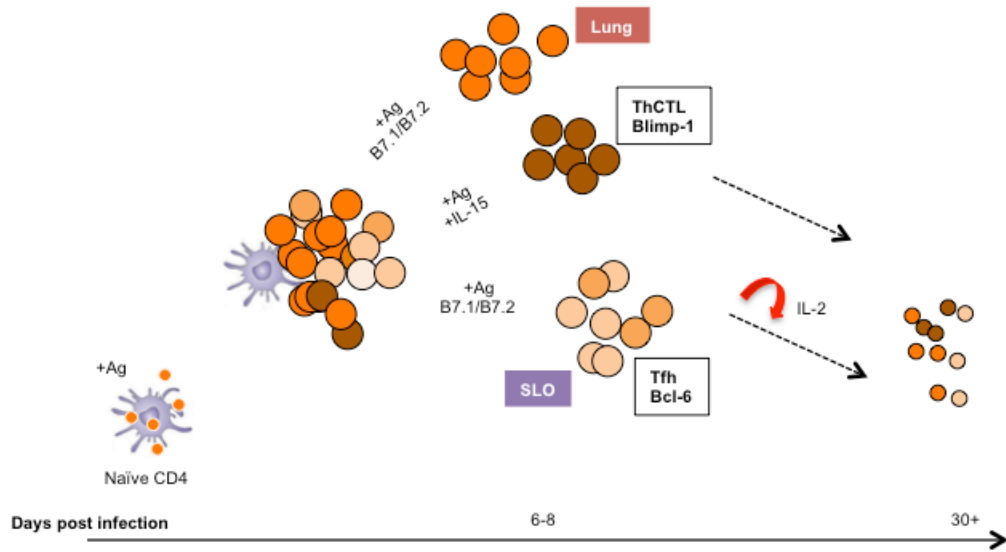


Figure 4.1 A model for the regulation of ThCTL, Tfh, and CD4 memory by antigen exposure at the effector phase.

Antigen recognition at the effector phase promotes the continued differentiation of effectors into ThCTL, Tfh, and memory. CD80/CD86 costimulation and IL-2 at the effector stage is important for Tfh and memory formation. IL-15 promotes ThCTL differentiation without the need for CD80/CD86 or IL-2. The differential regulation of these effectors suggest unique pathways in controlling the formation of these CD4 subsets.

are independent of autocrine IL-2 in vivo, instead some ThCTL require IL-15 signaling in vivo for their differentiation or survival. These results show that the regulation of ThCTL encompass multiple signaling pathways during the late antigen phase suggesting CD4 effector cells and the CD4 immune response is constantly incorporating information from the ongoing viral infection to tailor the response. Better appreciation of the requirements for ThCTL generation would lead to better strategies to elicit CD4 cytotoxic responses where these cells have shown to be therapeutically beneficial in human viral infections.

Early events in ThCTL differentiation

Although much of the work presented here focuses on the roles of signals that occur post priming and mainly on 6 dpi, there are questions about what signals are important for ThCTL formation before 6 dpi. Previous studies have looked at in vitro cultures of CD4 effectors suggesting IL-2 and low dose antigen being important for the acquisition of cytotoxicity (50). In the context of T helper polarizing cultures, Th2 polarizing conditions leads to an absolute reduction in the cytotoxicity of CD4 effectors (50). Th1 polarizing conditions are permissive for cytotoxicity, as Th1 effectors displayed measurable cytotoxicity, while ThCTL conditions had the highest cytotoxicity. ThCTL conditions in these cultures were with high dose IL-2 and blocking IL-4, to prevent the development of Th2 effectors. Thus although Th1 cells are considered to be helper cells that secrete IFN γ , the polarizing conditions during Th1 polarization are permissive for ThCTL development. Interestingly, Th1 conditions are also permissive for Tfh formation in vivo (188). Whether Th1 conditions are necessary for ThCTL

development was addressed in a self-reactive model, where T-bet deficient CD4 T cells could still become ThCTL (51). However during a viral infection, the inflammatory conditions generally prime a highly Th1 polarized environment. Yet, when IFN γ is removed from mice during influenza infection, ThCTL still develop (47), although T-bet deficiency has not been tested in this model. The Th1 polarizing environment is permissive to ThCTL because we observe Th1 associated gene expression on ThCTL. ThCTL have high expression of T-bet and secrete IFN γ at the peak of influenza infection, suggesting ThCTL develop in conjunction with acquisition of canonical Th1 effector functions. An additional layer of ThCTL differentiation is the involvement of Blimp-1. Here we show that Blimp-1 is an important driver of ThCTL differentiation. Although the timing of Blimp-1 has not been looked at, Blimp-1 could be playing a role early. During LCMV infection, a bifurcation of Blimp-1 and Bcl-6 can be seen as early as 3 dpi (164). Blimp-1 and Bcl-6 are transcriptional repressors that are antagonistic regulators of each other, where Bcl-6 is important for Tfh (69, 189, 190) and Blimp-1 for ThCTL (64, 109). Thus for ThCTL to develop, an early suppression of the Tfh differentiation pathway may be needed. How 'early' ThCTL suppress Tfh may involve antigen and cytokines. Indeed the early Blimp-1 expression is associated with high IL-2R α expression (164, 191). These data are consistent with the early in vitro experiments showing high IL-2 in culture being important for ThCTL differentiation (50), as well as IL-2 signaling being suppressive for Tfh in vivo (176, 177). Cytokines can also influence Blimp-1 expression where IL-2, IL-12, or IL-4 can induce Blimp-1 expression (55, 143). Additionally the role of antigen dose and affinity have been shown to regulate CD4 effector differentiation (48, 49),

where high affinity can lead to more IL-2R α expression early. Asymmetric division (192, 193) could also contribute to the early bifurcation of the response, but whether this division can also segregate IL-2R α expression remains to be studied. Costimulation can also alter the Bcl-6 and Blimp-1 balance as exogenous OX-40 stimulation leads to an increase in Blimp-1 expression early during LCMV infection (191). OX-40 can also induce ThCTL induction (51, 53) and whether this is through induction of Blimp-1 remains to be studied. Thus early events during priming may set the landscape for ThCTL development, either through initial suppression of the Tfh pathway and/or the induction of Blimp-1 through antigen, costimulation, and cytokines.

Late events in ThCTL differentiation

After priming CD4 effectors continue to see antigen (114) as revealed through the Nur77 GFP reporter system. Early work has shown that continued antigen is important for the development of CD4 effector cells (157, 161). Here we show that recognition of late antigen was important for the differentiation of both ThCTL and Tfh during influenza infection. The requirement of late antigen suggests that ThCTL and Tfh require additional regulation compared to non-ThCTL or non-Tfh in the response. Notably, IFN γ production was not impaired when late antigen was removed suggesting that the Th1 response by itself does not require late antigen. Where the late antigen is coming from remains to be addressed. During influenza infection, antigen can persist weeks past clearance of virus (159, 194). APC late in the response may also be uniquely able to activate effector cells (195) and promote Tfh differentiation. The germinal center

response is also another source of antigen that can help regulate Tfh differentiation (161). However, which APC induce ThCTL differentiation remains to be studied. Certainly the late antigen presented on APC need not have costimulation through CD80 and CD86 to promote ThCTL differentiation, a contrast to Tfh differentiation. The lack of a requirement of late CD80 and CD86 suggests that ThCTL may have enough activation signals where they do not need further costimulation, unlike the proposed events occurring early during priming (51–53). The lack of the need for CD28 is reminiscent of human CD4 ThCTL that was originally described as CD28 negative (43). As CD28 also promotes survival and proliferation of cells (196) the lack of a requirement for late CD80/CD86 may suggest a regulatory role where increased proliferation and survival is not turned on to prevent overt expansion of cytotoxic ThCTL beyond the effector response. ThCTL being independent of late CD80/CD86 is also consistent with the independence of late IL-2 as well. Although IL-2 may be important early to prime the cells for ThCTL induction, perhaps through induction of Blimp-1, IL-2 becomes unnecessary late for ThCTL. Blimp-1 can regulate IL-2 expression (143, 144). Thus after ‘pre-ThCTL’ induce Blimp-1, they subsequently may suppress IL-2 production and responsiveness to IL-2 (110). Indeed gene expression on ThCTL revealed *Il2* being suppressed. These data are also similar to human ThCTL where perforin⁺ CD4 T cells are also IL-2^{neg} (43). The lack of IL-2 induction or responsiveness also suggests continued regulation of the survival and proliferation of ThCTL, where the cells will not respond to signals promoting their continued expansion or survival. Using APC lacking CD80/CD86 did not alter the expression of IFN γ of effector cells but does limit their IL-2 production

(169). And even if the IL-2 is limited, the presence of Blimp-1 may even further reduce IL-2 production and prevent ThCTL from responding to paracrine or serum IL-2 due to repression of IL-2R α (110). IL-2 can have pleiotropic effects in the lung and can lead to vascular leak syndrome (VLS) (174). Therefore, the expansion of Blimp-1⁺ CD4 effectors including ThCTL may also constrain IL-2 production in order to prevent overt damage in the lung tissue, where disrupting a vital organ can be fatal. Indeed little IL-2 is detected by lung effectors (159, 162). As ThCTL is independent of IL-2 in vivo, how does IL-2 inhibit ThCTL formation in vitro (Figure 3.18)? It is clear from the early phenotyping that the effectors at 6 dpi are a heterogeneous population of effectors (Figure 3.11). Thus multiple cell populations could be responding to IL-2. First the IL-2 responsive population can be driven to proliferate and outcompete the ThCTL, as they would be unresponsive to IL-2. Additionally IL-2 could be inducing factors that are inhibitory towards ThCTL differentiation. IL-2 is an important cytokine to induce Th2 differentiation in CD4 T cells (197, 198). IL-2 can help keep the *Il4* gene open for transcription (198) and is important for IL-4 protein production by stimulated CD4 T cells (199). The source of IL-4 during either in vivo influenza infection or the in vitro stimulation of effectors remains understudied. A possible source of IL-4 can be Tfh cells. Tfh in the germinal center can make IL-4 during LCMV infection (37) and the IL-4 made by Tfh is important for germinal center B cells to transition to plasma cells (36). Since Tfh are found in the germinal center, pre-ThCTL could be segregated from the source of IL-4 and thus are not suppressed in vivo, but are in vitro when these cells are forced together. CD8 T cells that enter the germinal center during LCMV infection

downregulate their expression of cytotoxic genes like perforin and granzyme (200). IL-4 is known to be suppressive towards ThCTL (50) and can reduce perforin mediated cytotoxicity in CD8 T cells (201, 202). Thus the anatomical compartmentalization of different CD4 effector cells could lead to ThCTL avoiding IL-2 and IL-4 mediated suppression. Indeed IL-2 can be found in the draining lymph nodes of influenza infected mice (203), where ThCTL are not found (47). Because the IL-2 pathway could be actively suppressed in ThCTL, other factors may help regulate their continued differentiation or survival. As the high affinity receptor for IL-2 (IL-2R α) may be downregulated, the remaining IL-2 receptor (IL-2R β and common γ chain) can also respond to IL-15. Here we show that late in the response ThCTL can respond to IL-15 presented in trans that promotes the differentiation or survival of ThCTL in the lung. IL-15 can be important for NK cell and CD8 memory homeostasis and can be presented by APCs and radio-resistant cells (178, 179) could be promoting ThCTL responses at non lymphoid sites, like the lung during influenza. Although Blimp-1 can also suppress the responsiveness to IL-15 in CD8 T cells (110), it is unclear to what extent this occurs in ThCTL. Indeed, the defect in removing late IL-15 is not as overt as compared to removing late antigen. These results suggest that ThCTL late in the response have additional regulatory elements in terms of antigen, costimulation, and cytokines in promoting differentiation. It appears that with antigen stimulation ThCTL are allowed to differentiate and expand further, but are constrained in their survival or proliferation by becoming unresponsive to canonical survival/proliferation signals of IL-2 and CD80 and CD86. The ignorance of these signals can at least partially be overcome with alternate

signals like IL-15. Whether IL-15 helps promote the long-term survival and persistence of ThCTL remains to be studied.

ThCTL and terminally differentiated effectors

Since ThCTL depend on Blimp-1 and express high levels of Blimp-1 (109) they may resemble the terminal effector population of cytotoxic CD8 T cells (142). Indeed CD8 terminal effectors depend on IL-15 for survival (103) and require Blimp-1(142), similar to ThCTL requiring both these factors as well. Although the term terminally differentiated effectors imply that these cells are short lived, evidence suggests that a proportion of terminal effectors survive into the memory pool (103) during LCMV infection. But comparing infection with *Listeria monocytogenes* or vesicular stomatitis virus (VSV) revealed different amounts of and long term survival of terminal effector cells (204), suggesting inflammation or antigen could influence the survival of the terminally differentiated effector cells. Thus ThCTL may utilize alternate pathways for survival. Indeed IL-2 largely controls the formation of CD4 memory T cells (113), yet ThCTL may not need IL-2 for long term survival. The presence of an IL-2 independent CD4 memory population in the lungs of influenza infected mice suggests other signals can be used for long term survival. ThCTL also appear to suppress *Tcf7*, an important transcription factor for Tfh differentiation (73) as well as survival of CD8 memory cells in the secondary lymphoid organs (145). Indeed CD4 effectors during influenza that suppress *Tcf7* are enriched in the lungs of infected mice (193), and memory CD8 T cells in the lung have low TCF-1 expression (205). Thus, ThCTL may be downregulating the

conventional factors important for circulating memory formation found in the secondary lymphoid organs. ThCTL may utilize the environment they are resident in to promote survival. The regulation imposed by Blimp-1, IL-2, CD80/CD86, TCF-1 altogether suggests that ThCTL may heavily suppress their capability to survive and expand by conventional methods. The requirement of antigen, which induces some survival signals, may be enough to promote the short term survival of ThCTL. However, the lack of costimulation or IL-2 will lead to the majority of the cells to die when antigen is removed (114). The increased regulation may prevent overt activation or persistence of cytotoxic cells to prevent autoimmunity or immunopathology. Yet, memory CD4 T cells are important for protection against viruses (83, 162), and the presence of memory CD4 cytotoxic cells could be beneficial (45, 83). Therefore mechanisms may be in place to permit a small number of ThCTL to transition to memory CD4 T cells.

ThCTL as a therapeutic target

The results presented here reveal additional regulatory mechanisms in place to control the generation of ThCTL during viral infections. Notably, the requirement for continued antigen to program CD4 effectors into the ThCTL phenotype differs than the requirements for Th1 cells. These results also suggest that conventional methods to promote survival of CD4 effectors are not sufficient to target ThCTL. The requirements for generating ThCTL memory will require further study to better inform vaccine approaches for influenza or other viral infections. Generating ThCTL for tumor immunotherapy however can utilize additional antigen to promote cytotoxic CD4

responses. Indeed CD4 T cells can be specific for endogenous tumor antigens (102). As Blimp-1 and the regulation of IL-2 in ThCTL suggests, the induced ThCTL may be short-lived and additional rounds of antigen could be potentially beneficial. The short-lived nature of ThCTL could also be an underappreciated boon. Over stimulated effectors during checkpoint blockade therapy can lead to toxicities (206) or cytokine release syndrome associated with chimeric antigen receptor (CAR) T cell therapies (207). Thus activating ThCTL can promote cytotoxicity without over stimulating other effectors, via a lack of CD80/CD86 costimulation, in the tumor environment can be an attractive approach to limit toxicity. Further work understanding the regulation of ThCTL in tumors and viral infection will help promote therapeutic approaches in the clinic.

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