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The Role of Late Antigen in CD4 Memory T Cell Formation during Influena [i.e. Influenza] Infection: A Dissertation

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THE ROLE OF LATE ANTIGEN IN CD4 MEMORY T CELL FORMATION DURING INFLUENA INFECTION

A Dissertation Presented

By

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The signature of the Dean of the Graduate School of Biomedical Sciences signifies that the student has met all graduation requirements of the School.

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Abstract

While memory CD4 T cells are critical for effective immunity to pathogens, the mechanisms underlying their generation are poorly defined. Although extensive work has been done to examine the role of antigen (Ag) in shaping memory formation, most studies focus on the requirements during the first few days of the response known as the priming phase. Little is known about whether or not Ag re-encounter by effector T cells (late Ag) alters CD4 memory T cell formation. Since influenza infection produces a large, heterogeneous, protective CD4 memory T cell population, I used this model to examine the role of late Ag in promoting CD4 memory T cell formation.

In the experiments presented in this thesis, I demonstrate that late Ag is required to rescue responding CD4 T cells from default apoptosis and to program the transition to long-lived memory. Responding cells that failed to re-encounter Ag had decreased memory marker expression and failed to produce multiple cytokines upon re-stimulation. Ag recognition is required at a defined stage, as short-term Ag presentation provided 6 days after infection is able to restore canonical memory formation even in the absence of viral infection. Finally, I find that memory CD4 T cell formation following cold-adapted influenza vaccination is boosted when Ag is administered at this stage. These findings imply that persistence of viral Ag presentation into the effector phase is the key factor that determines the efficiency of memory generation. They also suggest that administering Ag during the effector stage may improve vaccine efficacy.

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Figure 4.3	Devarajan, P., B. Bautista, A. M. Vong, K. K. McKinstry, T. M. Strutt, and S. L. Swain. 2016. New Insights into the Generation of CD4 Memory May Shape Future Vaccine Strategies for Influenza. *Front. Immunol.* 7: 136. Distributed under the terms of the Creative Commons Attribution license (http://creativecommons.org/licenses/by/4.0/)

List of Abbreviations

7-AAD	7-Aminoactinomycin D
Ab	Antibody
Ag	Antigen
AICD	Activation induced cell death
APC	Antigen presenting cell(s)
B6	C57BL/6J mice
BAL	Bronchoalveolar lavage
Bcl-2	B-cell lymphoma 2
Bcl-6	B-cell lymphoma 6
Bim	Bcl-2 interacting mediator of cell death
ca	cold-adapted
CDC	Centers for Disease Control and Prevention
CFSE	Carboxyfluorescein succinimidyl ester
CTV	Cell trace violet
CXCR	C-X-C chemokine receptor
DAVID	Database for Annotation, Visualization and Integrated Discovery
DC	Dendritic cell
DLN	Draining lymph node
DNA	Deoxyribonucleic acid
dpi	days post infection / immunization
dpt	days post transfer

DTR	Diphtheria toxin receptor
FBS	Fetal bovine serum
Foxo	Forkhead box protein O
GFP	Green fluorescent protein
HA	Hemagglutinin
HA0	HA precursor
HAI	Hemagglutination-inhibition
i.v.	Intravenous
IAV	Influenza A virus
ICOS	Inducible T cell costimulatory
Id2	Inhibitor of DNA binding 2
IFN	Interferon
IIV	Inactivated influenza vaccine
IL	Interleukin
KLRG1	Killer cell lectin like receptor G1
LAIV	Live attenuated influenza vaccine
LCMV	Lymphocytic Choriomeningitis virus
LN	Lymph node
LPS	Lipopolysaccharide
Ly6C	Lymphocyte antigen 6 complex, locus C1
M2e	Ion channel matrix ectodomain
MDCK	Madin-Darby Canine Kidney

MFI	Median Fluorescence Intensity
MHC-II	Major histocompatibility complex class II
miRNA	Micro ribonucleic acid
MPEC	Memory precursor effector cells
mRNA	Messenger ribonucleic acid
MVA	Modified Vaccinia Ankara
NA	Neuraminidase
NIAID	National Institute of Allergy and Infectious Disease
NIH	National Institutes of Health
NK	Natural killer
NP	Nucleoprotein
OVA	Ovalbumin
PA	Polymerase acidic
PD-1	Programmed cell death protein 1
PMA	Phorbol myristate acetate
PPARγ	Peroxisome proliferator-activated receptor gamma
PR8	A/Puerto Rico/8/34
PRR	Pattern recognition receptors
RBC	Red blood cells
RDC	Respiratory dendritic cells
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute medium

RT	Room temperature
SA	Sialic acid
sciIV	Single-cycle infectious influenza virus
SD	Standard deviation
SEM	Standard error of mean
SLEC	Short lived effector cells
SLO	Secondary lymphoid organs
STAT	Signal transducer and activator of transcription
TCF-1	T cell factor 1
TCR	T cell receptor
Tfh	Follicular helper T cells
Tg	Transgenic
ThCTL	Cytotoxic CD4 T cells
TLR	Toll like receptor
TNF	Tumor necrosis factor
Treg	Regulator T cells
Trm	Tissue-resident memory T cells
UMMS	University of Massachusetts Medical School
US	United States
vRNP	viral ribonucleoprotein
VSV	Vesicular stomatitis virus
WSN	A/WSN/33

Preface

This dissertation is submitted to fulfill the requirement for a degree of Doctor of Philosophy at the University of Massachusetts Medical School. This dissertation represents original work that I conducted under the supervision of Dr. Susan Swain. Parts of the results and discussion sections both text and figures have been taken from the following manuscript:

> **Bautista, B**., Devarajan, P., McKinstry, K., Strutt, T., Vong, A., Jones, M., Kuang, Y., Mott, D., Swain, S. 2016. Short-lived Antigen Recognition but not Viral Infection at a Defined Checkpoint Programs Effector CD4 T Cells to become Protective Memory. In Press. *J. Immunol.*

Data from Figure 3.7 and Figure 3.8 were experiments I conducted and were presented in the following manuscript:

> McKinstry, K. K., T. M. Strutt, **B. Bautista**, W. Zhang, Y. Kuang, A. M. Cooper, and S. L. Swain. 2014. Effector CD4 T-cell transition to memory requires late cognate interactions that induce autocrine IL-2. *Nat. Commun.* 5: 5377.

Figure 3.4D contains a viral titer graph. I performed the infections for these experiments and Yi “Katie” Kuang performed the RNA isolation and qRT-PCR. Finally, Dr. Priyadharshini Devarajan and Dr. Susan Swain have helped in editing this dissertation.

CHAPTER I: Introduction

Overview

The mechanisms that govern the selection of a few of the many Ag-specific effector CD4 T cells present at the peak of the response to form memory are largely unclear. While many studies have suggested that memory fate determination occurs early in the response, others have demonstrated that signals received at later stages can alter memory T cell formation. Recent work from our lab has identified a checkpoint that occurs during the effector phase of the response during which autocrine IL-2 signals are required for CD4 memory T cell formation (1). Since a dominant driver of IL-2 production by CD4 T cells is cognate Ag recognition, I sought to test if Ag recognition at this checkpoint was required to induce IL-2 production and promote memory formation.

The aim of my thesis work was to understand what role Ag presentation during the effector stage of the response plays in shaping CD4 memory T cell formation. To this end, I used a mouse-adapted influenza infection model that is known to induce potent CD4 memory T cell generation (2–4). Not only is a long-lived heterogeneous memory CD4 T cell population formed following influenza infection, it is capable of mediating heterosubtypic protection (3, 5). While studying CD4 memory T cell formation in this model provides valuable insights to a basic mechanism of memory generation, it also has translational relevance. Given that memory CD4 T cells enhance antibody (Ab) responses (6, 7), CD8 T cell responses (8–11), innate responses (12), as well as mediate direct effector activity

(2, 4, 13, 14), understanding the requirements for their generation is critical to rational vaccine design. T cell responses are largely directed against highly conserved epitopes, this suggests that a vaccine that effectively enhances T cell memory might lead to a more broadly protective vaccine (15). In fact, many vaccination efforts are underway that focus on enhancing memory T cell formation (15). This thesis may help guide those efforts by establishing the Ag requirements for effective memory generation.

The following introduction will provide background on the influenza virus and its pathogenesis. Understanding its mode of infection provides insight into Ag presentation and the effectiveness of vaccination strategies. I will discuss T cell responses to influenza infection to orient readers to the specifics of viral and T cell kinetics as well as the phenotype and subset differentiation of T cells during influenza infection. I will then focus on T cell responses in general and introduce a few key molecules that arise in my thesis and discuss their relevance to contraction and memory T cell formation. I will end with a discussion of the role of Ag in shaping both effector and memory T cell responses. Overall this introduction will explain the relevant work in the field and illuminate the contributions presented in this thesis. It will also put the work into the greater context of how it may ultimately aid translational efforts in influenza vaccination.

Influenza viruses

A. Influenza Viruses: An overview

Influenza viruses are part of the family *Orthomyxoviridae*. Influenza B and C viruses predominantly infect humans, while Influenza A viruses (IAV) infect a wide range of mammals and birds (16). Influenza C viruses are endemic and cause mild respiratory disease, while Influenza A and B are responsible for seasonal epidemics (17). Since Influenza A virus (IAV) can infect many different animals, gene segment rearrangement can occur resulting in novel strains which are responsible for pandemic outbreaks (16, 18). IAV are classified based on their hemagglutinin (HA) and neuraminidase (NA) subtypes with HA having 18 subtypes and NA having 11 subtypes (19–21). IAV HA phylogeny can be further classified into group 1 (H1,H2, H5, H6, H8, H9, H11, H12, H13, H16, H17, and H18) or group 2 (H3, H4, H7, H10, H14, and H15) (22). The World Health Organization guidelines for influenza nomenclature are Type (A, B, C) / host (if non-human) / place of isolation / isolation number / year of isolation (17).

B. Influenza Viruses: Structure and Genome

Influenza viruses are enveloped, negative-sense, single-stranded RNA viruses containing seven (Influenza C) or eight (Influenza A and Influenza B) gene segments. The eight gene segments in Influenza A and Influenza B viruses are PB1, PB2, PA, HA, NP, NA, M, and NS (Figure 1.1). Polymerase basic 1 and 2 (PB1 and PB2) and Polymerase acidic (PA) encode proteins that together form a viral RNA-dependent RNA polymerase complex (23). Influenza C viruses have a

Figure 1.1 Influenza Virus Structure and Genome

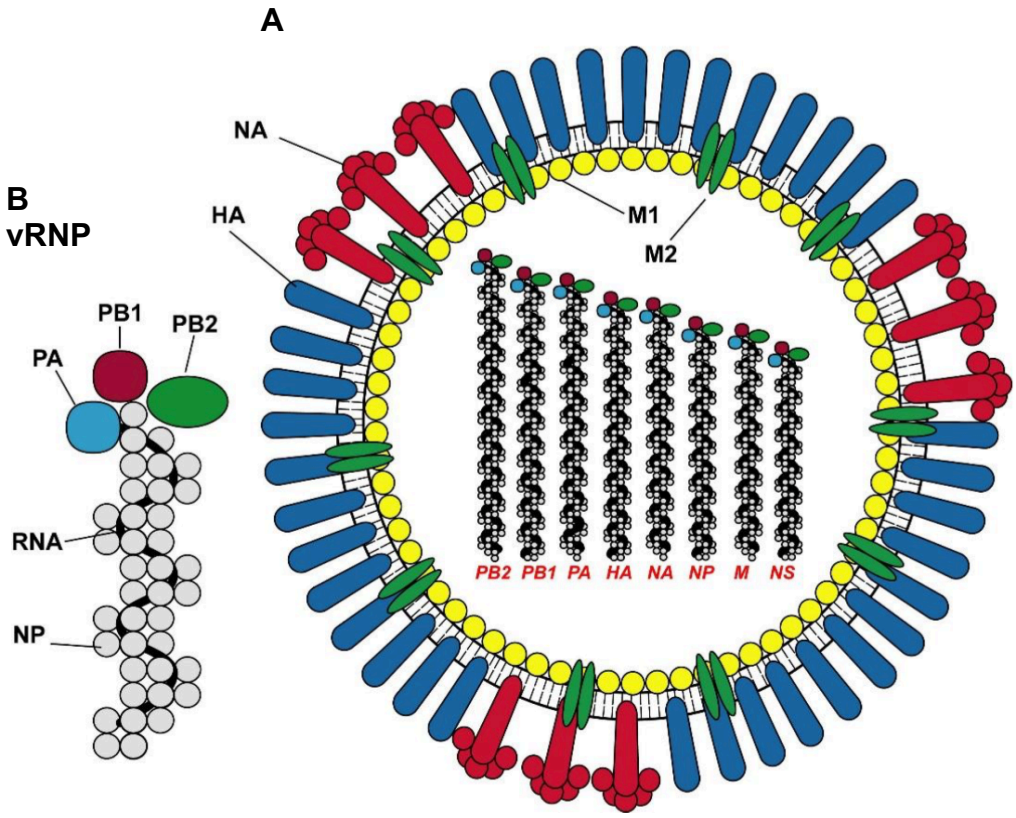


Figure 1.1. Influenza Virus Structure and Genome

(A) Influenza A virus (IAV) structure. IAV is an enveloped, negative-sense, single-stranded RNA virus. The viral envelope consists of a host cell-derived lipid bilayer along with viral hemagglutinin (HA) and neuraminidase (NA). The ion channel matrix (M2) is a transmembrane protein with a small, highly conserved ectodomain exposed at the viral surface. The inner surface envelope matrix 1 (M1) protein forms a protein layer under the lipid bilayer. The genome consists of eight gene segments including Polymerase basic 1 (PB1), Polymerase basic 2 (PB2), Polymerase acidic (PA), HA, nucleoprotein (NP), NA, matrix (M), and non-structural (NS). **(B)** Viral gene segments are coated with many NP proteins and one RNA polymerase complex composed of PB1, PB2, and PA per gene segment. This structure is termed viral ribonucleoprotein (vRNP).

Figure adapted from: Sridhar, S., K. Brokstad, and R. Cox. 2015. Influenza Vaccination Strategies: Comparing Inactivated and Live Attenuated Influenza Vaccines. *Vaccines* 3: 373–389. Creative Commons Attribution license.

subunit called polymerase 3 (P3) instead of PA (23). HA encodes hemagglutinin that forms a trimeric glycoprotein present in the viral envelope. HA binds sialic acid on the surface of host cells to mediate cell entry (24). NP encodes the nucleoprotein that encapsulates all gene segments in the virion and is essential for viral replication and transcription. NA encodes neuraminidase which is a tetrameric glycoprotein in the viral envelope that mediates the exit of newly formed virions from the host cell by cleaving sialic acid linkages (25). M encodes the inner surface envelope matrix 1 (M1) protein which forms a protein layer under the host cell-derived lipid bilayer. M also encodes the ion channel matrix (M2) that is present in the viral membrane. NS encodes the non-structural protein (NS1) which is an antagonist of Type I IFN (26). Through alternative splicing, NS also encodes nuclear export protein (NEP, also known as NS2) which mediates the export of viral ribonucleoprotein (vRNP) complexes from the nucleus to the cytoplasm (17, 20, 27–29).

The viral envelope consists of a host cell-derived lipid bilayer which contains cholesterol rich lipid rafts (28, 30). The envelope contains the viral proteins HA, NA, and M2. HA is by far the most abundant protein, making up about 80%, NA makes up about 17%, and M2 is only present at about 16 to 20 molecules per virion (29). Virions are pleiomorphic but are generally spheroid in shape and are around 100nm in diameter (31). Under this lipid bilayer is a protein layer that consists of M1 and small amounts of NEP. The viral core consists of helical vRNPs which are composed of gene segments encapsulated with many NP molecules

and associated with one viral RNA polymerase complex per gene segment (Figure 1.1).

C. Influenza Viruses: Viral entry

HA binds to sialic acid (SA) bound to the terminal end of glycoproteins found on host cell surfaces. HA molecules have varying affinities for different SA linkages. IAVs that have evolved to infect epithelial cells of the human respiratory tract have HA molecules with a high affinity for α2-6 SA, while those that have evolved to infect birds have a high affinity for α2-3 SA which is the most abundant linkage present in avian gut epithelial cells. α2-3 SA linkages are also present at a low frequency in the human lower respiratory tract which is why humans can be infected with avian-evolved IAV (32). Interestingly, swine tracheal epithelial cells have both SA linkages and are therefore thought to provide an important source of pandemic strain formation as a result of co-infection.

Prior to activation, the HA precursor (HA0) must be cleaved into the HA1 and HA2 subunits. This requires a host cell serine protease. For human IAV, HA0 contains a monobasic cleavage site that can only be cleaved by a few trypsin-like proteases thought to be primarily present in the lung epithelia. Cleavage can be mediated by secreted proteases or transmembrane proteases which may cleave the HA0 prior to viral budding (33–35). It is therefore possible for local cells lacking the required enzyme to become infected by virions coated with pre-cleaved HA but these cells are unlikely to propagate the virus due to their inability to cleave HA.

For highly pathogenic H5 and H7 strains, the HA0 contains a multibasic cleavage site which can be cleaved by the more ubiquitous furin-like proteases allowing productive infection of a broader range of cell types (35).

The HA1 subunit contains the sialic acid binding domain. The HA2 subunit contains the fusion peptide. Cleavage is necessary for viral fusion because it mediates a conformational change in HA that releases the fusion peptide from the C-terminus of HA1, preparing it to function at low pH (35). Once HA binds sialic acid on the host cell surface, receptor-mediated endocytosis occurs and the virus enters an endosome within the cell cytoplasm. As the pH in the endosome becomes more acidic (pH 5-6), HA undergoes a conformational change exposing the fusion peptide. The fusion peptide is then inserted into the host cell membrane and through further conformational changes the viral and host membranes are brought in close proximity to each other and form a stalk that eventually collapses forming a pore. This process likely requires multiple HA interactions with SA on the cell surface. Low pH also allows vRNPs to dissociate from M1 via the M2 ion channel. Once the pore is formed the vRNPs are released into the host cell cytosol (24, 28, 36).

D. Influenza Viruses: Transcription and Replication

Unlike many RNA viruses, influenza viral replication occurs in the nucleus instead of the cytoplasm. Once the vRNPs are in the nucleus both transcription (the generation of mRNA for translation into viral protein products) and replication

(the generation of more negative-sense RNA for packaging into new virions) are carried out. Viral transcription of the negative-sense RNA is executed by the viral RNA polymerase complex associated with each vRNP. The PA and PB2 subunits cleave 5' caps from host mRNA (termed "cap-snatching"), these serve as primers to initiate transcription. The PA C-terminal domain moves single-stranded RNA into the active site located on the "large domain" of the polymerase that consists of PB1 and PB2. Transcription is terminated with polyadenylation (Poly-A). Resulting mRNAs are then transported to the cytosol where they are transcribed into protein by host cell machinery (37, 38). Viral replication requires the generation of intermediate positive-sense complimentary RNPs (cRNP) that lack 5' Caps and Poly-A tails. These cRNP then serve as efficient templates for the synthesis of many negative-sense RNPs that can be packaged into new virions. Translation of mRNA into more viral NP and viral RNA polymerase is required for replication to take place. (23, 37, 39).

E. Influenza Viruses: Viral Budding

Viral budding is a complex process that is initiated by targeting of HA and NA to lipid rafts on the host cell surface. M1 then binds the cytoplasmic tails of HA and NA and forms a docking site for vRNPs. This is followed by recruitment of M2 which is responsible for facilitating the curvature of the cell membrane at the budding site which leads to membrane scission and virion release (29, 40, 41). NA plays a critical role in this process in cleaving SA moieties to prevent HA binding

to the host cell membrane or causing aggregation with other new virions (20, 25).

F. Influenza Viruses: Disease

Although influenza viral infections are generally self-limiting, they cause significant morbidity and mortality in the young, elderly, and immune-compromised (17, 22, 27). Seasonal influenza related deaths vary drastically from year to year, ranging from around 3,000-49,000 deaths in the United States per year (42). Pandemic influenza strains can be much more catastrophic, with the most extreme example being the 1918 Spanish Influenza pandemic which resulted in around 50 million deaths worldwide (16). Fatal complications following influenza infection include pneumonia and secondary bacterial infections (16, 17). In fact, most of the deaths seen in the 1918 Spanish flu pandemic as well as those seen in seasonal epidemics were associated with a secondary bacterial infection (17). Additionally, mutations in viral genes can lead to increased virulence, most notably a mutation found in the HA0 cleavage site of H5 and H7 broadens the host protease requirements enabling the virus to infect cells other than bronchial epithelium (16, 17). Therefore, influenza poses a significant health risk that warrants further examination of how effective vaccination can be achieved.

Influenza vaccination

A. Influenza vaccination: Challenges

IAV is an extremely agile virus, rapidly acquiring mutations to respond to strong selection pressure by neutralizing HA-specific Ab as well as anti-viral drugs including those targeted against NA. These mutations are rapidly acquired due to the lack of proofreading function of the influenza RNA polymerase resulting in a mutation rate of about 1-8 X 10^{3} substitutions per site per year (16). Mutations acquired in the HA or NA glycoproteins is termed “antigenic drift”. Additionally, “antigenic shift” occurs when a host is co-infected with two or more different influenza strains resulting in gene reassortment that includes the HA and NA genes. Wild aquatic birds are thought to be the dominant hosts in which this occurs, as IAV generally results in asymptomatic gastrointestinal infections and mixed co-infections are quite common (43). Since most vaccine-induced immune responses target the HA glycoprotein, both antigenic shift and antigenic drift necessitate new vaccine development every season (22). Not only is this process onerous and expensive, the need to predict which circulating strains will dominate in any given season often leads to reduced vaccine efficacy in seasons where predictions were incorrect and when pandemic strains arise (44, 45).

B. Influenza Vaccination: Inactivated Influenza Vaccines (IIV) and Recombinant Vaccines

IIV have been used since the 1940s and are the most commonly administered influenza vaccines. Traditionally these have been trivalent vaccines including Influenza A H1N1, H3N2 and an Influenza B strain. Recently,

quadrivalent vaccines have been approved that include both circulating Influenza B lineages (18, 46). IIV include whole, split (treated with ether/detergent), and subunit (semi-purified HA and NA) vaccines (47). Production of these vaccines is a lengthy process involving passage of viral stocks in embryonated eggs. Given this onerous development process, the emergence of pandemic strains is extremely problematic in that a novel IAV strain would be able to spread much more rapidly than our ability to respond and generate a specific vaccine.

In 2013, a trivalent (replaced in 2016 by a quadrivalent) cell-based, IIV (ccIIV3 and ccIIV4) was licensed for use in the US. Flucelvax is produced via the passage of virus through Madin-Darby Canine Kidney (MDCK) cells in culture (48). This platform has several benefits including being safe for those with egg allergies, not being limited by embryonated egg supply, and eliminating the potential for egg-adapting mutations that may occur during passage through embryonated eggs (49). Although not an IIV, a recombinant HA vaccine (Flublok) has also been approved for use in the United States. In this system, purified HA is expressed in insect cells using a baculovirus expression system (50). Adaption of new platforms like these will be critical moving forward to increase or ability to rapidly generate a vaccine should an unexpected pandemic strain arise.

C. Influenza vaccination: Live attenuated Influenza Vaccine (LAIV)

LAIV consists of a cold-adapted, attenuated live virus. Like IIV, seasonal predictions are made to select an H1N1, H3N2, and either one (trivalent) or two

(quadrivalent) Influenza B lineages. The HA and NA of each virus is then inserted into a cold-adapted, temperature sensitive backbone derived from A/Ann Arbor/6/60 and B/Ann Arbor/1/66 viruses (51). These viruses replicate in the upper respiratory tract but not in the lower respiratory tract providing a limited amount of virus replication to stimulate the innate immune system and allow for processing of Ag for T cell recognition (52, 53).

LAIV was first licensed for use in the US in 2003 and since then has been very commonly used, particularly in children. However, in June 2016, the CDC's Advisory Committee on Immunization Practices decided to not recommend vaccination with LAIV for the 2016-2017 flu season. Although it has been well established that these vaccines have limited efficacy in adults as pre-existing immunity prevents replication of the attenuated virus, there have been many studies showing increased effectiveness in children compared to IIV (18, 54–56). However, in the 2010-2011, 2013-2014, and 2015-2016 seasons, LAIV underperformed compared to IIV in protecting against circulating H1N1 in children ages 2-17 (48, 57). The reason for this unreliable efficacy is unclear, however disparities between the efficacy of different vaccine shipments within the same flu season suggest that vaccine handling may have been a cause (57).

D. Influenza vaccination: Universal Influenza vaccine

All currently licensed influenza vaccines fail to generate broadly protective responses. Current methods for measuring the efficacy of vaccines rely heavily on

hemagglutination inhibition (HAI) assays that measure the concentration of serum Ab that blocks HA binding to sialic acid on red blood cells (RBC). Therefore, this assay will only detect Ab that interfere with sialic acid binding, presumably via binding the globular head (HA1 subunit) of the HA molecule. While these Ab are likely to neutralize the virus by preventing receptor binding and viral entry, they are also likely to only recognize the immunizing strain, as this region can undergo extensive mutation without affecting viral fitness (22). There are efforts to develop broadly reactive (within a given subtype) Ab against the receptor binding region of the HA. This involves the use of various methods of sequence analysis to develop a synthetic HA molecule with shared or "centralized" sequences among all the known viruses within a given subtype (58–60).

The stalk region, made up mostly of the HA2 subunit, is far less tolerant of mutations than the globular head due to its critical function during viral fusion. The stalk region is fairly conserved and therefore Ab that target this region have broad reactivity, generally among an entire phylogenetic group of HA molecules (61–64). Unfortunately, stalk reactive Ab are not as immunodominant as those generated against the globular head and are not generated by current IIV vaccination (65–67). However, they are generated following infection with live virus (65, 68, 69) as well as following vaccination with pandemic H5, H7, and swine H1 strains (65, 70–72). Presumably, when the immunizing Ag includes a globular head that has never been seen by the immune system (as is the case with pandemic strains) the low levels of pre-existing cross-reactive stalk specific Ab are favored over the primary

anti-globular head response which is likely to be delayed (47). This discovery has led to efforts to generate vaccines with chimeric HA constructs that include an exotic globular head (generally from avian IAV) attached to the H1 stalk region. Following serial immunizations with chimeras using different globular head constructs attached to the same H1 stalk region, stalk specific Ab can be continually boosted. So far, this immunization regimen has only been tested in mice and ferrets (73, 74). One important component of any vaccine aimed at boosting anti-HA stalk Ab is to ensure that the Ab being boosted has a similarly inhibitory function across different influenza subtypes. There have been instances were a stalk binding Ab actually aided viral fusion and exacerbated disease (75).

Another promising target for universal vaccine design is the highly conserved ectodomain of M2 (M2e). Although on its own it is a poor immunogen, several groups have had success generating constructs to increase the immunogenicity of this small 22-23 amino acid domain in rodent models of vaccination (76–80).

Influenza T cell epitopes are much more highly conserved than influenza Ab epitopes (81). This is due to the fact that many T cell epitopes are derived from highly conserved internal viral proteins (81–84). Given this, one would predict that some memory T cells generated following live infection would be cross-reactive against pandemic influenza strains. Indeed, many studies have shown that infection and/or vaccination with seasonal influenza strains generate memory T cell responses that cross-react against previously un-encountered pandemic

strains (82, 85–91). Studies testing PBMC of healthy donors find the highest frequency of cross-reactivity for both CD8 and CD4 T cells in the influenza M1 protein, with significant responses also seen against NP and PB1 epitopes (15, 84, 86).

Current efforts to develop a T cell based universal vaccine have made use of recombinant, replication-deficient Modified Vaccinia virus Ankara (MVA) which is a highly attenuated virus that has been used as the “boost” step in many prime-boost regimens. Since most adults have been previously exposed to influenza, MVA (encoding influenza NP and M1) is administered in an effort to “boost” pre-existing memory T cell populations (92–94). Thus far, this platform has had success in boosting cross-reactive T cell memory in clinical trials in both 18-45 aged cohorts as well as in the elderly (92, 95–97). Additionally, a small, preliminary study has shown that the MVA-NP+M1 vaccine can reduce viral shedding and symptom prevalence after challenge with a virus against which volunteers had no pre-existing neutralizing Ab (as determined by HAI Ab titers) (98). Importantly, one dose of vaccine appears to be enough to boost pre-existing T cell populations therefore obviating the problem of vector-directed immunity (99). For infants lacking pre-existing T cell immunity, a strategy using both adenovirus and MVA vectors to perform a prime/boost regime has been tested in mice (100).

A few other T cell based vaccines have had some success in phase I clinical trials. The Multimeric-100 vaccine comprised of conserved linear epitopes from HA, NP, and M1 has been shown to be safe and immunogenic (101). Another

vaccine comprised of several conserved polypeptides consisting of CD8 T cell epitopes was found to be somewhat immunogenic but did not appear to result in decreased symptoms or viral shedding following influenza challenge (102, 103). While DNA vaccines have had more success in mouse models than in larger animals (104), a phase I clinical trial has demonstrated safety and some immunogenicity in humans (105). Phase I clinical trials have also been completed using a simian adenoviral vector expressing NP + M1 as well as using a recombinant virus-like particle (VLP) including HA, NA, and M1 (106, 107). Finally, single-cycle infectious influenza viruses (sciIV) have demonstrated T cell-dependent heterosubtypic protection in both mice and ferrets (108–110). Peptide vaccination has also had some success in providing heterosubtypic protection in mice. It however remains to be seen how effective they will be in clinical trials.

E. Influenza Vaccination: Mediators of protection: Sterilizing Immunity

Sterilizing immunity is defined as an immune response that completely eliminates infection. This can be achieved through preventing viral receptor binding, fusion, replication, or budding. While HAI titers are used as the main correlate of sterilizing immunity, other Ab specificities can also provide sterilizing immunity. For example, Ab specific for the HA stalk can neutralize virus by preventing the cleavage of HA0 into the HA1 and HA2 subunits. They can also inhibit the conformation change of HA that is required for successful fusion thereby preventing the virus from successfully infecting the host cell. Additionally, stalk-

specific Ab may interfere with viral budding, preventing cell to cell transmission and viral propagation.

Additionally, it should not be overlooked that Tfh are required for the generation of long-lived plasmablast and memory B cell formation. Indeed, studies have found that induction of influenza-specific CD4 T cells with a Tfh signature strongly correlate with neutralizing Ab titer as well as protection (111, 112). Studies suggest that the sterilizing Ab produced by seasonal vaccination may only last for about 6 months (70). While this is acceptable for our current seasonal vaccination system, if the goal is to move toward more broadly neutralizing and long-lasting vaccines, a focus on inducing sufficient Tfh formation should be a top priority.

F. Influenza Vaccination: Mediators of protection: Limiting disease severity

Given that often the morbidity and mortality associated with influenza viruses is correlated with the extent of disease pathology, the potential for inducing immune responses that limit disease severity is an important protection mechanism that deserves serious consideration. Both cell-mediated and non-neutralizing Ab responses can provide protection by limiting viral propagation and overall disease severity.

One main mechanism by which non-neutralizing Ab provides protection is via antibody-dependent cell-mediated cytotoxicity (ADCC) (78, 113–115). This is a process in which viral Ag on the surface of an infected cell is bound by Ab. Natural killer (NK) cells recognize the Fc region of the Ab and kill the infected cell

through the release of lytic enzymes (116). Other mechanisms that are especially important for protection mediated by anti-M2e Ab are antibody-dependent cell-mediated phagocytosis and complement dependent cytotoxicity (78, 80, 115). Although these mechanisms do not block initial infection, they do limit viral propagation and can therefore have a dramatic effect on protection (78, 113–115).

While T cell responses cannot neutralize the virus, there is substantial evidence that T cells can provide heterosubtypic protection by limiting the severity of disease. Many mouse studies have shown that both CD8 and CD4 T cells can mediate heterosubtypic protection (2, 3, 83, 117, 118). Additionally, various human studies have shown that cross-reactive T cell responses correlate with decreased disease severity (119–122). One study in particular found that cross-reactive CD4 T cells with cytotoxic function correlated with decreased disease severity following influenza challenge in humans (120).

Although current seasonal LAIV has fallen short of expectations likely due to manufacturing problems as well as adult pre-existing immunity preventing viral propagation as evidenced by decreased viral shedding and low immunogenicity (44, 56, 123), it's success in boosting broadly reactive protection can be attributed to its superior induction of T cell responses (52, 53, 124–127). The similarity to viral infection of LAIV may be what drives its success in initiating T cell responses. One study demonstrates that viral infection is best at generating multiple cytokine producing CD4 T cells, a characteristic shown to mark functional superiority (128), suggesting that a vaccination scheme that mimics live viral infection may be the

best way to generate potent memory CD4 T cells (129). Although vaccine delivery may need to be re-worked, it is clear that inducing T cell responses is a worthwhile effort in that they can prevent severe disease in the absence of neutralizing Ab.

T cell Response to Influenza

A. T cell Response to Influenza: Antigen Presentation

Activation of the T cell response is initiated largely by migratory $CD11c^{+}CD103^{+}$ and $CD11c^{+}CD11b^{+}$ respiratory dendritic cell (RDC) populations. RDC are resident in the lung and upon uptake of Ag (either by endocytosis of viral particles and/or dead cellular debris, or direct infection) migrate to the SLO and present Ag to naïve T cells. The extent to which direct viral infection of dendritic cells (DC) is required for T cell responses is unclear. CD4 T cells recognize epitopes bound to MHC-II which are loaded in endosomal compartments (130). It is therefore traditionally thought that CD4 T cell epitopes are derived from endocytosis of viral particles or cellular debris from infected cells. However, a recent study found that processing of endogenous Ag rather than exogenous Ag mediated CD4 T cell responses following influenza infection (131). This study suggests that rather than originating from the endosomal compartment, presented Ag likely originates from the cytosol indicating a critical role for direct viral infection of antigen presenting cells (APC).

However, determining if an APC population is infected *in vivo* is somewhat

difficult, many studies rely on IAV-GFP reporters to identify virally infected cells. Unfortunately, these reporters cannot distinguish between infected cells and cells carrying endocytosed viral material (132, 133). A few studies have employed methods to address this, including staining for surface HA (one of the first proteins to be expressed on the surface of virally infected cells) and use of a hematopoietic miRNA targeted against viral NP that specifically limits viral replication in APC. The study using surface HA staining to mark virally infected cells demonstrated that $CD103^+$ and $CD11b^+$ RDCs present in the DLN 48 hours post infection were NS1-GFP^+, indicating a significant amount of intracellular NS1, but were surface HA negative, suggesting that they were not infected (134). They went on to suggest that these DCs were protected from infection via a Type I IFN-dependent mechanism (134). A study using hematopoietic specific miRNA found that CD8 T cell responses were not altered when viral replication was inhibited in APC, suggesting that presentation of exogenous Ag via cross-priming of CD8 T cells was sufficient (135). However, endogenous Ag processes may play a role in Ag encounter of effector T cells in the lung because lung macrophage populations can be infected (134) and MHC-II is upregulated on lung epithelia following infection (14). While the mode of Ag uptake by migratory DC is still up for debate, studies comparing CD4 T cell activation following inactivated versus live virus have clearly demonstrated that infectious virus is required to initiate an effective CD4 T cell response (131).

Once migratory DC enter the DLN, they can transfer Ag to LN resident DC

populations although the extent to which this is required for the generation of the T cell response is unclear (136). While both $CD103^+$ and $CD11b^+$ DC present Ag early to initiate priming in the DLN, $CD11b^+$ DC peak at 5 days post infection (dpi) in the DLN and 7 dpi in the lung suggesting that Ag encounters at the effector stage of the response are likely mediated by this subset (137). It was shown that $CD11b^+$ DC effectively endocytose viral Ag and are able to present to CD8 T cell via cross-priming (137–139), given that the classical MHC-II Ag presentation pathway includes processing of exogenous Ag, it is likely that these cells are able to present Ag to CD4 T cells as well.

B. T cell Response to Influenza: Kinetics of the T cell Response

Migratory DC arrive in the lung about 24 hours after infection and by day 3 of infection virtually all Ag-specific T cells have been stimulated with Ag in the secondary lymphoid organs (SLO) but have not yet begun to proliferate. At 4 dpi, T cells have undergone several rounds of division and a few cells begin to migrate to the lung around 5-6 dpi (140). Both CD4 and CD8 T cells peak in the lung around 8-9 dpi (14, 141). Following the peak of infection, CD4 T cells sharply contract and form a stable memory population by 20 dpi (1) (Figure 1.2). The contraction of CD8 T cells is a bit more delayed particularly in the lung and BAL (141). Viral titer peaks around 4 dpi and is cleared by 10-12 dpi (141) (Figure 1.2).

The primary effector CD4 T cell response is heterogeneous, and is

Figure 1.2. Kinetics of the CD4 T cell response to influenza.

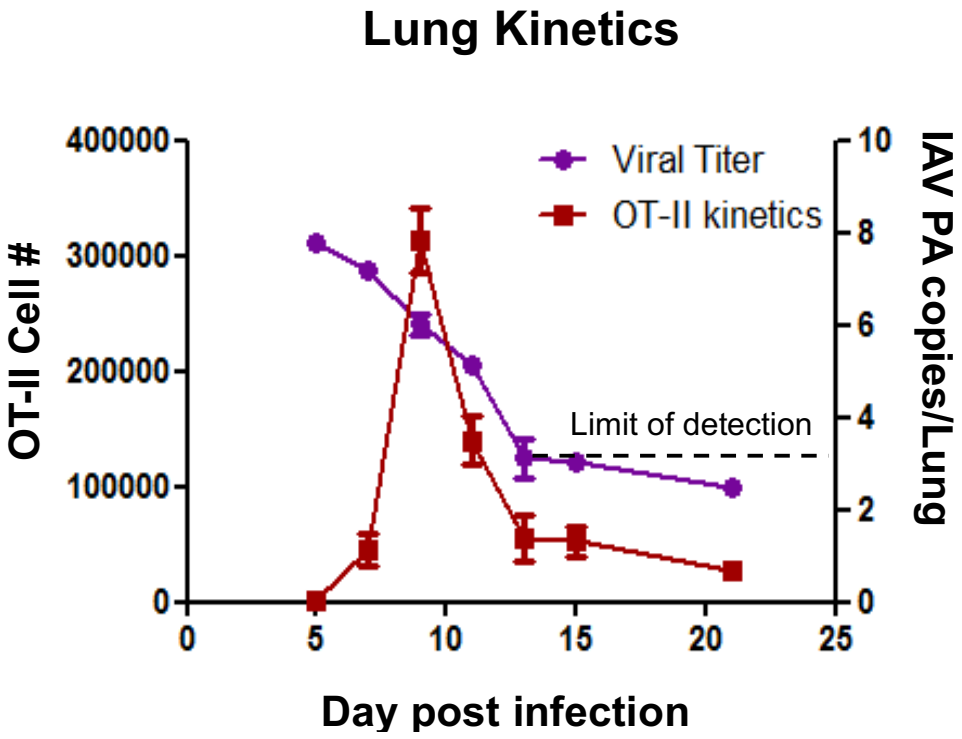


Figure 1.2 Kinetics of the CD4 T cell response to influenza.

$5x10^4$ naïve OT-II.Thy1.1$^{+/}$ were transferred to B6 mice followed by infection with a sublethal dose of PR8-OVA_{II}. OT-II cell numbers are enumerated on the left y-axis over the course of the response. IAV PA copy number per lung is enumerated on the right y-axis over the course of the response. The limit of detection refers to the PA copy number reading of uninfected B6. OT-II cell numbers peak at day 9 of infection. Virus is cleared by day 13 of infection.

composed of Th1, SLO-restricted T follicular helper cells (Tfh), and lung-restricted cytotoxic CD4 T cells (ThCTL). A Th1 phenotype predominates in all organs with T-bet expression and IFNγ production being present in the lung, spleen, and DLN. Lung cells largely produce IFNγ with little IL-2 and TNF, whereas CD4 T cells in the SLO produce IFNγ, IL-2, and TNF (4, 14, 140). The IAV Ab response is highly dependent on CD4 T cells (6), and the generation of long-lived plasma cell formation requires CD4 T cell expression of SAP (7). In addition to the Tfh-mediated germinal center reactions, CD4 T cells promote an early IgA response (6). CD4 T cell "licensing" of DC via CD40-CD40L interactions helps CD8 T cells overcome Treg suppression (8). CD4 T cells also promote CD8 T cell memory formation and recall responses (9, 10). Additionally, CD8 T cell resident memory localization and function requires IFNγ production by CD4 T cells during IAV infection (11). Finally, lung restricted ThCTL can mediate direct killing of infected cells resulting in reduced weight loss and protection in the absence of Ab (4, 13, 14).

C. T cell Response to Influenza: Memory T cells

A heterogeneous population of memory CD4 T cells is generated following influenza infection (4, 142). Memory CD4 T cells enhance innate immune responses in the lung (12). They provide enhanced B cell help compared to naïve CD4 T cells. They provide CD8 T cell help and, via IFNγ, provide direct protection against lethal IAV (3). Upon activation, memory CD4 T cells accumulate in the lung

to a greater extent than do primary CD4 T cells and have greater multi-cytokine producing potential (2). Additionally, whereas Tfh are restricted to the SLO during the primary response, during a secondary response, Tfh can be found in the lung (2).

Recent work has demonstrated the increased protective ability of tissue resident memory T cells (Trm) (5, 53, 143). Teijario et al. found that memory CD4 T cells isolated from the lung provided superior protection when compared to memory CD4 T cells isolated from the spleen (5). CD8 Trm can be identified by CD103 and CD69 expression, CD4 Trm are identified by high CD69 expression and a slight upregulation of CD11a (LFA-1) (5, 143). While it has been shown that CD8 Trm require Ag presentation in the lung (144) as well as CD4 T cell help (11), little is known about what regulates CD4 Trm formation following influenza infection.

Tfh memory has been identified in several infections and circulating influenza-specific Tfh-like memory cells can be found in the human population (111, 112, 145–147). Interestingly, memory $CXCR5^+$ CD4 T cells retain some plasticity upon secondary infection. Instead of being restricted to Tfh differentiation, they are able to differentiate into multiple different subsets (147–149). It is likely that the heterogeneity of memory CD4 T cells is critical to protection and more work is needed to fully elucidate the requirements for the generation of each subset.

Factors that Govern T cell Responses

A. Factors that Govern T cell Responses: Contraction

Overview

The T cell response terminates with the apoptosis of around 90% of responding T cells leaving a small population of memory T cells. Therefore, surviving this stage of the response is critical for memory formation. There are several processes that have been proposed to regulate the cell death that occurs during this stage. The major proposals involve extrinsic or death receptor-mediated cell death and intrinsic or Bcl-2 family member mediated cell death (150, 151). The following subsections will examine the proposed role for both of these pathways in contributing to cell death during the termination of the T cell response. A mechanism involving the death receptor pathway is activation induced cell death (AICD) in which activated T cells undergo apoptosis when re-encountering Ag. Although some reports have shown this can occur in a death receptor-independent fashion (152, 153), the majority of studies demonstrate this is a death receptor (Fas or CD95 in particular)-driven mechanism (154–161). The intrinsic cell death model involves programmed or passive cell death in which activated T cells die at the end of the immune response due to the withdrawal of critical survival factors (162, 163).

Activation-induced cell death (AICD)

AICD describes the phenomenon of activated T cells undergoing cell death when re-stimulated. With the exception of a few studies suggesting a role for granzyme B or hematopoietic progenitor kinase 1 (HPK1) (152, 153), the term AICD is used to describe a Fas (CD95)-mediated mechanism of cell death (154–161). Most of these studies examine *in vitro*-generated effector T cells cultured in the presence of exogenous IL-2. Since IL-2 has been shown to sensitize T cells to AICD (157, 164), these conditions may cause *in vitro*-generated effector T cells to be more susceptible to AICD than *in vivo*-generated effector T cells.

The role of AICD *in vivo* is unclear. Fas-mediated AICD seems to play a role in maintaining peripheral tolerance, limiting autoimmunity, and superantigen-mediated deletion (165–167). Some reports find AICD of human activated T cells during HIV infection (168). Additionally, some have found a role in Fas-mediated cell death in regulating T cell contraction during persistent lymphocytic choriomeningitis virus (LCMV) infection (169). However, Fas-mediated AICD appears to have limited to no role in regulating T cell contraction following acute viral infection (165, 166, 170, 171). Given that the models in which AICD appears to be important are those in which Ag stimulation is very strong (superantigen) and/or continuous (autoimmunity and persistent infections) or in the presence of unphysiological levels of IL-2 (*in vitro* generation of effectors), it is likely that an acute viral infection does not generate effectors that are as susceptible to AICD. It is also unclear how extensive Ag re-encounter is during the effector phase of the response, an issue that is addressed in this thesis.

Programmed or Passive Cell Death

Programmed or passive cell death involves intrinsic cell death mediated by the Bcl-2 family members discussed in the following subsection. It is also often referred to as cytokine or survival factor withdrawal. The rationale for this model is that T cell activation induces many pathways involved in cell death (172), and cytokines are needed to counterbalance these effects by providing survival signals. Studies demonstrating that responding T cells required cytokines in addition to TCR stimulation and co-stimulation for survival (173–176) led to the hypothesis that as pathogen decreases and T cell numbers expand, survival inducing cytokines may become limiting, leading to the default death of effector T cells (150, 162, 163). While one study found that Ag can promote the survival of effectors late in the response (177), it is generally believed that inflammation induced cytokines promote the survival of effector T cells even in the absence of Ag (178, 179). Some of the key factors in this process including Bcl-2-interacting mediator of cell death (Bim), IL-7, and IL-2 are discussed further below.

The role of Bim during contraction.

Bim is a member of the B cell lymphoma 2 (Bcl-2) family of proteins that mediate intrinsic cell death. Intrinsic cell death is initiated by growth factor deprivation, stress, UV, or viral infection. It is a mechanism that leads to pore formation in the outer mitochondrial membrane leading to release of cytochrome c and Second Mitochondria-derived Activator of Caspase (SMAC, also known as

DIABLO) resulting in caspase 9 activation, ultimately leading to caspase 3 activation and cell death.

The Bcl-2 family of proteins can be further differentiated into three classes based on their expression of Bcl-2 homology domains 1-4 (BH1-4). The first includes the anti-apoptotic proteins including Bcl-2, Bcl-XL, Bcl-w, A1, and myeloid leukemia cell differentiation protein 1 (Mcl-1) which include BH1-4. The second class includes pro-apoptotic molecules that contain BH1-3, including Bcl-2-associated X protein (Bax) and Bcl-2 homologous antagonist killer (Bak) which are the two proteins directly involved in pore formation in the outer mitochondrial membrane (180, 181). The final class is the BH3-only proteins, including Bim, Bcl-2 antagonist of cell death (Bad), and Bcl-2-interacting killer (Bik), Bcl-2 interacting domain death agonist (Bid), Bcl-2 modifying factor (Bmf), Bcl-2/adenovirus E1B 19KD protein-interacting protein 3 (pNIP3), Harakiri (Hrk), Noxa, and p53-upregulated modulator of apoptosis (Puma) (182–184). These proteins are thought to act by binding class I anti-apoptotic Bcl-2 proteins and blocking their inhibition of Bax and Bak (184, 185). However, Bim and Bid have also been shown to directly activate Bax and Bak (180, 185, 186).

The pro-apoptotic protein Bim and anti-apoptotic Bcl-2 have been shown to play a dominant role in T cell survival with the ratio of these two determining the level of naïve T cell survival (187, 188). Additionally, the overexpression of Bim promotes effector T cell death and contraction is significantly reduced in Bim knockout T cells (171, 189–191). Importantly, although some studies have

suggested a requirement for Bim in activation of autoreactive T cells (192), Bim knockout cells are able to proliferate and are functional following pathogen challenge (189, 191). The regulation of Bim expression during T cell activation is fairly complex. Studies have demonstrated that it is increased following TCR stimulation (193). Others have shown that Foxo3a promotes the expression of Bim. Since phosphorylated AKT (pAKT) [downstream of, among other things, the TCR (194)] leads to phosphorylation of Foxo3a, resulting in its exit from the nucleus and sequestration in the cytoplasm preventing the transcription of its targets, it is possible that TCR signaling can directly lead to decreased Bim expression as seen in memory CD4 T cells (195). Additionally, Id2 is thought to inhibit Bim expression (196), since Id2 expression is regulated by STAT4 and STAT5 (197), there is a potential mechanism by which cytokines can inhibit Bim expression in addition to promoting Bcl-2 expression (198). Although Bim is an important mediator of cell death during contraction, more work must be done to elucidate what the key signals are that regulate its expression during a T cell response.

IL-7 and its receptor CD127 during contraction and memory formation.

CD127 is the alpha subunit of the IL-7 receptor. IL-7 is critical for the long term survival of memory T cells (189, 199, 200). The role of IL-7 in regulating the generation of memory CD4 T cells is unclear. Studies have shown that blocking IL-7 prior to memory formation had no effect on contraction during Vaccinia or LCMV infection (201, 202), it did however, limit memory formation of secondary

effectors during influenza infection particularly in the SLO (1). Given the near uniform upregulation of CD127 on memory CD4 T cells it is clear that it is an essential marker of memory formation (203).

Although CD127 marks memory precursor effector CD8 T cells (204), it is unclear to what extent it predicts memory formation of CD4 effector T cells. Although a study of secondary effectors found that CD127 expression at the effector stage correlated with cells that would form memory (1), since secondary effectors express more CD127 than do primary cells (2), it is unclear whether or not this is the case in the primary response. In fact, a study in which $CD127^{h}$ and $CD127^{o}$ CD4 T cells were sorted at the effector stage and transferred to infection-matched hosts showed no difference in their ability to become memory (205). This was due to the ability of $CD127^{o}$ cells to upregulate CD127 during the termination of the response. While the role of CD127 expression in selecting which effector CD4 T cells become memory is unclear, it is clear that memory CD4 T cells upregulate CD127 expression (203, 205) and are dependent on IL-7 for their maintenance (189, 199, 200).

<u>The role of IL-2 in memory T cell formation.</u>

IL-2 signaling during priming is critical for CD4 and CD8 memory T cell formation (206, 207). Conversely, IL-2 signaling during priming can also lead to terminal differentiation and increased susceptibility to AICD (157, 164, 208). Similarly, increased CD25 expression on early CD8 effectors is thought to mediate

increased IL-2 signaling leading to terminal differentiation (208). In CD4 T cells, early $CD25^{h}$ effector cells are thought to be driven toward Th1 differentiation as opposed to Tfh differentiation (149). Although there is some similarity in that Tfh-like cells are thought to give rise to central memory cells (148, 149), Th1 cells are also capable of becoming memory (148, 209) and therefore are not terminally differentiated.

Timing is likely critical in determining the outcome of IL-2 signaling. Studies that examine late IL-2 signaling largely conflict those that examine early IL-2 signaling. For example, in CD8 T cells, studies suggest that IL-2 signaling late during the response promotes memory formation of CD8 T cells (210, 211). When $CD25^{h}$ and $CD25^{o}$ CD8 T cells are sorted at day 8 of LCMV infection and transferred to infection-matched hosts, $CD25^{h}$ cells formed memory to a greater extent than did $CD25^{o}$ cells (210). Additionally, addition of exogenous IL-2 late during LCMV infection boosts CD8 memory T cell formation (211). Studies from our lab have shown that effector CD4 T cells could be rescued from programmed apoptosis with the addition of IL-2 and TGFβ *in vitro* (212). Additionally, McKinstry et al. showed that IL-2 was required from day 5-7 of influenza infection for CD4 memory T cell formation (1).

B. Factors that Govern T cell Responses: CXCR3 and memory T cells

CXCR3 is a chemokine receptor that is expressed on effector and memory T cells. It is upregulated on CD4 effector and memory cells following Th1-inducing

inflammation, and is regulated by the Th1 transcription factor T-bet (213, 214). Its ligands include CXCL9, CXCL10, and CXCL11 that can be induced by IFNγ. CXCL10 can also be upregulated by IFNα/β as well as NF-κB induction (215, 216). Therefore, CXCL10 is preferentially expressed following TLR ligation. During priming, CXCR3 is critical for effective Th1 differentiation and for migration of Ag-specific T cells out of the T cell zone into the interfollicular (site of T cell and DC interactions) and medulla (site of drainage of soluble Ag) regions (217). It is also critical for directing activated T cells to peripheral sites of inflammation (218–220).

CXCR3 has been shown in CD8 and human CD4 T cells to mark memory cells that have a heightened ability to participate in recall responses (221, 222). Additionally, CD8 T cell studies have shown that $CXCR3^+$ memory cells are preferentially localized in the cortical ridge area near the interfollicular regions where they rapidly migrate upon activation (223, 224). Memory CD4 T cells also seem to preferentially localize to the cortical ridge (225), although while it is likely, it has not been shown that CXCR3 mediates this localization. CXCR3 expression in memory CD8 T cells is also critical for lung surveillance and protection following influenza infection (226). While this role has not yet been examined in CD4 T cells, given that memory CD4 T cells also express CXCR3, it is likely that they migrate toward its ligands in a similar fashion.

C. Factors that Govern T cell Responses: Bcl-6 and memory T cells

Bcl-6 is a transcriptional repressor most prominently known for its role in promoting Tfh formation (227–229). However, it has been shown to interact with T-bet in Th1 cells to modulate Th1 genetic programs (230, 231). For example, Oestreich et al. found that during Th1 differentiation T-bet bound Bcl-6 at the *Socs1, Socs2, Tcf7,* and *Ifng* loci to inhibit gene expression (230). Interestingly, the inhibition of IFNγ expression by T-bet bound to Bcl-6 was only present late during Th1 differentiation, suggesting that the association of T-bet with Bcl-6 may play a role in limiting effector differentiation. However, this study was done following *in vitro* Th1 polarization so it is unclear to what extent these interactions occur *in vivo*.

Bcl-6 is also important for both CD8 and CD4 memory T cell formation (232–234). Ichii et al. found that although effector expansion was not affected, Bcl-6 deficient CD4 T cells were unable to form a long-lived memory population following immunization with OVA and LPS (232). They found that Bcl-6 was important for cell survival during the late stages of the response. This finding is interesting given that Bcl-6 was recently found to play a role in repressing glycolysis which is a critical step in transitioning to a resting memory cell that relies primarily on oxidative phosphorylation mediated by fatty acid oxidation (235–237). Additionally, Bcl-6 knockouts appear to have a decrease in molecules required for fatty acid oxidation in adipose tissue (238). Although the role of Bcl-6 in inhibiting glycolysis during the transition to memory has not been thoroughly examined *in vivo* these findings are consistent with the fact that Tfh (which express very high levels of Bcl-

6) utilize glycolysis much less than Th1 cells (239). Thus, although it is clear that Bcl-6 plays a critical role in memory T cell formation, more work must be done to elucidate what role it plays in mediating the metabolic transition of memory T cells.

D. Factors that Govern T cell Responses: The role of Ag in effector generation.

In vitro studies have found a very minimal requirement for Ag to program T cells to undergo proliferation and gain effector function. The consensus being around 24-40 hours of strong TCR stimulation for CD8 T cells (240–244) and 48 hours of strong TCR stimulation for CD4 T cells (243, 245–248). One recent study found that 2 days of αCD3 and αCD28 *in vitro* was not sufficient for continued proliferation of CD4 T cells transferred to mice without Ag (249). The potential difference in this study may have been the absence of exogenous IL-2 in the *in vitro* cultures, since IL-2 has been found to be necessary for the survival of cells undergoing Ag-independent proliferation *in vitro* (242, 245, 248, 250). However, although cells may continue to proliferate without Ag, some have found that proliferation is increased if Ag is present for up to 60-96 hours of culture (247, 251, 252).

Studies in which priming occurred *in vivo* had more conflicting results. Studies using the *Listeria monocytogenes* (*L.monocytogenes*) infection model in which Ampicillin (Amp) was used to truncate infection at 24 hours resulted in conflicting reports from two different groups. In studies from the Harty lab and

others, both responding CD8 and CD4 T cells peaked a day earlier than the no Amp control and never reached the peak cell number seen in the control (253–256). However, Bevan and colleagues found that CD8 effector T cell numbers were similar, while CD4 T cell numbers were reduced following Amp treatment after 24 or 48 hours compared to untreated controls (257). Despite these differences, at 7 dpi (the peak when infection was truncated) both studies found similar numbers of CD8 T cells when Amp was used at 24 hours and similar number of CD4 T cells when Amp was used at 48 hours compared to untreated control mice, suggesting that cells did undergo some Ag-independent proliferation *in vivo* (253, 257).

The use of antibiotics to truncate infection results in a decrease in Ag presentation as well as inflammation which has been shown to promote continued proliferation of CD8 T cells during *L.monocytogenes* infection (258). To address this caveat, a few studies have examined the role of Ag while leaving inflammation intact. One such study found that deleting APC via a diphtheria toxin (DT) system at 48 hours had no effect on the numbers of CD8 effector T cells. However, this study used the transfer of peptide-pulsed CD11c-DTR APC to initiate a response which is unlikely to adequately replicate Ag presentation during pathogen infection (259). Using an elegant transgenic mouse strain termed TIM (tet-inducible invariant chain with MCC) that expresses $MCC_{93\ 103}$-bound MHC-II in an inducible manner, Obst et al. found that CD4 T cells do not undergo full autopilot proliferation after 2 days of stimulation (249, 260). A caveat that is not fully addressed in these studies is that the APC are likely not as activated as those present during pathogen

infection.

A couple of studies have made use of the Y-Ae Ab that is specific for the Eα peptide bound to I-A^b (261) to specifically block Ag recognition to transgenic TEa CD4 T cells specific for this epitope. One study used a model with peptide-pulsed APC, while the other used a VSV recombinant that expressed the Eα peptide. The peptide-pulsed APC model showed that Ag was needed beyond 36 hours for full expansion (262). The VSV model showed that full effector CD4 T cell expansion was reached after 4 days of infection (263). It should be noted that although the peak of the CD4 T cell response in the VSV model is not shown, it appears to be no later than 5 dpi (263). This indicates that Ag was required for almost the entire duration of the expansion phase of the response.

A few CD8 T cell studies have been done in influenza with mixed results. A couple of groups have found that CD8 T cells required Ag stimulation beyond 7 dpi for full effector expansion (263, 264). However, although both studies used the influenza model WSN-OVA_I and measured OT-I recovery at 10 dpi, their results differed in the magnitude of the effect. Blair et al. found that blocking OVA-K^b resulted in similarly low numbers of effector cells regardless of whether they blocked on day 0 or day 7 of infection (263). Dolfi et al. found a very modest decrease in CD8 T cells following deletion of CD11c on 6 dpi. They went on to show that OT-I effectors isolated at 8 dpi were present at much higher numbers at 5 days post transfer (dpt) if transferred to WSN-OVA_I-infected hosts compared to uninfected or PR8-infected hosts. However, 8 dpi OT-I effectors were transferred

into 5 dpi infected hosts, this mismatched timing may not accurately replicate what occurs during an endogenous T cell response (264). Others suggest there are epitope specific differences in the length of the Ag requirement for CD8 T cells (138, 139). These studies suggest that CD8 T cells directed against certain epitopes are dependent on prolonged Ag presentation that requires cross-presentation mediated by virus-specific Ab binding FcγR on DC (138, 139). Given the differences in MHC-I and MHC-II peptide loading pathways, it is unclear if CD4 T cells are regulated in a similar way.

The fact that, in some *in vivo* models, T cells require more than 2 days of Ag stimulation for full effector expansion appears to contradict findings *in vitro* that suggest that sustained Ag stimulation for more than 2 days is detrimental to effector survival (246). However, these differences may be explained by the fact that Ag contacts after 24 hours *in vivo* may be more transient than those *in vitro*. Microscopy studies have illuminated the timing of priming of both CD8 and CD4 T cells in the draining lymph node following administration of peptide/APC (265–269). The initial phase is characterized by multiple transient interactions between T cells and DC expressing cognate Ag. During this phase T cells integrate successive signals and if they reach a certain signaling threshold they progress to the next phase of more stable contacts with Ag/DC which are required to induce activation over tolerance (270, 271). After about 24 hours, T cells separate from DC and undergo cytokine-driven proliferation (266–268). At this time, occasional contacts with DC may be made but they are rare with most T cells regaining their

full mobility (267–269). Interestingly, in a study that added a second wave of DC, investigators found that CD4 T cells could form stable contacts during this third phase (269, 272), however these additional contacts did not result in increased proliferation suggesting that proliferation at this time is likely Ag-independent (269). For clarity, most of these studies add labeled Ag bearing DCs and Ag-specific T cells in a controlled manner and block LN trafficking to synchronize the response. It is therefore unclear what the kinetics are during a live viral infection when both T cell and Ag-bearing DC migration and numbers are dynamic.

Overall, it is likely that initial priming is followed by a phase of Ag-independent proliferation as *in vitro* studies found (245) and *in vivo* microscopy studies appear to confirm (266–268). However, this programmed proliferation may be for a limited duration and subsequent Ag stimulation may be required for the sustained proliferation seen during a pathogen infection. Of course given the largely conflicting literature, this is not certain and is likely infection specific. The ability for Ag to continue to have a positive influence on effector T cell generation is likely dependent on the breaks from Ag stimulation that are likely to occur *in vivo* (266, 273).

E. Factors that Govern T cell Responses: The role of Ag in memory T cell formation.

The time frame during which memory fate determination is made has been a controversial topic. Rather than identifying a point at which fate decisions are set

in stone, many studies have revealed steps that inform fate determination, leaving open the possibility that future interactions may affect the ultimate fate of a cell. There has been some debate about the extent to which naïve T cells are predestined for certain effector or memory fates. Studies in both CD4 and CD8 T cells have shown that single naïve clones undergo different differentiation patterns upon activation likely due to, among other things, TCR avidity (274–278). Work from the Allen lab has shown that even cells with similar avidity for cognate Ag may undergo different effector and memory differentiation due to their affinity for self-ligand (279, 280). However, given that a single cell can differentiate into both effector and memory cells (275), and that IFNγ-producing effector cells can give rise to memory (209, 281), fate determination is likely not solidified at the naïve T cell stage.

Many of the studies that examined the Ag requirements for T cell proliferation also tested the Ag requirements for memory formation. A few studies demonstrated that although limiting infection may inhibit effector cell numbers generated at the peak of the response, it either had no effect on or increased memory cell numbers (138, 139, 253–255, 263). These findings along with others led to a model of memory differentiation in which the bifurcation of effector and memory cells occurs very early in a response. This early bifurcation model led to the belief that molecules that promote effector expansion and differentiation are often in opposition to memory cell formation (282).

One prominent finding critical to the formation of this model is the

observation that when T cells form stable interactions with DC during priming, they undergo an asymmetric division. During these stable interactions a supramolecular activation cluster (SMAC) is formed between the T cell and DC in which many signaling molecules are clustered. The result is that when the T cell divides, the daughter closest to the DC will retain more of the signaling molecules that have coalesced at the synapse, in addition to T-bet (283) and CD25 (284), both factors that when present at higher levels in cells early during the response lead to terminal differentiation of effectors (208, 285, 286).

While this phenomenon is well documented in CD8 T cells, less has been done to study the effect of this early bifurcation in CD4 T cells (287). While studies suggest that segregation of LFA-1, CD4, IFNγR, STAT1 and in some cells CRTAM (284, 288–290) concentrate in the immunological synapse, it is suggested this first asymmetric division may mediate Th1 versus Tfh cell fate (291). This is an intriguing hypothesis since others have found an early bifurcation of those effector destinies (149, 292). Since both these subsets can further differentiate into memory cells (149, 293), it is unlikely that this first division is solely responsible for memory fate determination in CD4 T cells.

One prominent feature in CD8 T cell literature is the ability to differentiate between short-lived effector cells (SLECs) and memory precursor effector cells or (MPECs) that can be defined by their expression of KLRG1 and CD127 fairly early on during the T cell response. It was found that when sorted at the effector stage, $CD127^{h}$ CD8 T cells were far more likely to form memory than $CD127^{o}$ cells (204).

Using these markers as a surrogate for memory versus effector differentiation, studies of CXCR3$^{-/-}$ CD8 T cells demonstrated less KLRG1 expression due to limited migration to sites of greater Ag density (294–296). These observations suggested that increased encounters with Ag lead to terminal differentiation of effectors (294–296). Although, these studies don't adequately separate Ag recognition from inflammation which can also cause terminal differentiation (285, 297), so their interpretation is unclear. However, recent findings have underscored the fact that these markers do not cleanly define terminally differentiated effectors and memory cells. For instance, KLRG1^{h} cells are present at memory time points and have been shown to mediate optimal protection in models of Vaccinia and *L.monocytogenes* (298). It is therefore misleading to assume that factors that promote KLRG1 expression are antagonistic to memory formation.

Unfortunately, CD127 expression at the effector stage is not a reliable marker for effector CD4 T cells destined to become memory (205). A study by Marshall et al. suggested that Ly6C marked terminally differentiated effector cells, however their results were not as dramatic as those seen with CD127 expression in CD8 T cells (205). Additionally, Ly6C^{h} cells are also present at significant numbers at the memory stage and seem to identify a Th1-like memory population (148). In fact, many studies have used Ly6C as a memory CD4 T cell marker (299, 300). It would therefore be similarly misleading to assume that factors that result in Ly6C upregulation were antagonistic to memory formation.

Moreover, there is growing evidence in CD8 T cell studies that Ag

recognition during the effector phase, while not affecting memory cell numbers, enhances the function of memory cells (137–139, 144, 301). During influenza infection, if Ag was blocked after 7 dpi, certain CD8 T cell specificities were unable to optimally proliferate, produce cytokines, and protect following secondary infection (138, 139). This is despite forming similar numbers of memory cells with a similar CD127 and KLRG1 phenotype (138, 139), further suggesting that these markers are of limited utility. Others have shown in a vaccination model that adding additional Ag 5 days after immunization results in efficient memory CD8 T cell generation (302, 303).

Although no CD4 T cell studies examine the role of Ag beyond the priming phase, there are studies that suggest that stronger TCR stimulation favors memory CD4 T cell formation (304, 305). Williams et al. found that SMARTA transgenic T cells specific for the gp61 epitope were able to form memory during LCMV infection but not during Listeria Monocytogenes-gp61 (LM-gp61) infection despite substantial effector expansion. They found this correlated with a reduced functional avidity of SMARTA cells compared to endogenous cells in LM-gp61 infected mice (304). While this is a largely correlative study, it does explain the observation that polyclonal memory populations have increased functional avidity compared to effector cells (304). This suggests that there is some selection pressure on TCR avidity in determining which clones survive and become memory, an obvious candidate being a role for late Ag presentation in selecting which cells become memory. Conversely, Blair et al. found that blocking Ag at 4 dpi following VSV

infection resulted in more memory CD4 T cell formation (263). However, there were very few memory CD4 T cells in the control and no phenotype or functional analysis was done. Thus, the role of Ag at the effector phase of the response in promoting or inhibiting CD4 memory T cell generation is a largely unexplored area.

Thesis Objectives

Memory CD4 T cells provide protection during influenza infection via several mechanisms. Through their helper activity they enhance Ab responses (6, 7), CD8 T cell responses (8–11), and innate responses (12), additionally they are able to mediate direct effector function (2, 4, 13, 14). The ability of memory CD4 T cells to orchestrate an effective immune response combined with the fact that many T cell epitopes are derived from highly conserved IAV proteins make them ideal targets for vaccination. However, vaccination strategies are stunted by our lack of understanding of the mechanisms that govern the formation of a large, functional CD4 memory T cell population. Efforts to illuminate the mechanisms involved in this process could have great implications in rational vaccine design.

Current vaccines are generated under the assumption that the Ag recognition that occurs during priming is sufficient for CD4 memory T cell formation. However, recent studies suggest that Ag recognition during the effector phase of the response results in a more functional CD8 memory T cell population (138, 139, 144). While the role of Ag recognition at the effector stage in promoting CD4 memory T cell formation is unclear, work from our lab demonstrate that

autocrine IL-2 signals are required at this time for efficient memory formation (1).

I sought to examine the role of Ag recognition at the effector phase of the response, termed “late Ag” in shaping the ongoing CD4 T cell response and promoting CD4 memory T cell formation. I found that Ag recognition was required for full expansion of CD4 effector T cells. Late Ag promoted the survival of responding CD4 T cells in a Bim-dependent manner. This pro-survival effect of late Ag recognition was significantly decreased in the absence of IL-2 and co-stimulation. Unlike CD8 T cells (138, 139), the number of CD4 memory T cells was significantly decreased in the absence of late Ag. Late Ag promoted the immediate expression of CD25, a marker that when expressed late during a response correlates with increased ability to form memory (210). It also promoted memory associated factors CD27 and Bcl-6. The memory T cells that form in the absence of late Ag have reduced memory cell markers CD127 and CXCR3 and have a reduced ability to secrete multiple cytokines upon re-stimulation.

Importantly, I have established that the signals required late in the response can be fulfilled by a short-term Ag/APC population in the absence of virus-induced inflammation. The late addition of Ag/APC that are only capable of presenting Ag for up to 2 days *in vivo* was sufficient to restore CD4 memory T cell numbers, function, and phenotype in both the lung and SLO. Additionally, when provided at 6 dpi, this short lived Ag/APC was sufficient to generate a protective CD4 memory T cell population. Finally, I demonstrate that adding Ag/APC at 6 dpi can boost CD4 memory T cell formation in a cold-adapted immunization model.

This late Ag dependent model of CD4 memory T cell formation suggests that persisting Ag, indicative of a continuing threat, is required for the commitment of resources to memory T cell formation. Additionally, the work presented in this thesis establishes the importance in developing vaccines that can provide enough Ag to enable Ag re-encounter at later stages of the response.

CHAPTER II: Materials and Methods

Mice

Naïve CD4 T cells were isolated from OT-II.Thy1.1$^{+/}$, OT-II.*Nr4a1*^{eGFP}.Thy1.1$^{+/}$, OT-II.*Bcl2l11*$^{+/}$, or OT-II.Osb.eGFP mice bred at the UMMS breeding facility. CD11c Tg.*H2-Ab1*$^{/}$ and OT-II.*Bcl2l11*$^{+/}$ bred at the UMMS breeding facility were used. Hosts were B6 male mice ordered from Jackson Laboratories (JAX). *Nr4a1*^{eGFP} mice originally obtained from JAX were bred at the UMMS breeding facility were also used. Mice used in experiments were 8-12 weeks of age. The Institutional Animal Care and Use Committee of the University of Massachusetts Medical School approved all animal procedures.

Viral Stocks, Infections, and Immunizations

For all influenza viral infections described, mice were lightly anesthetized with isoflurane (Piramal Healthcare) before intranasal infection with 50μL of virus diluted in PBS. Influenza A PR8-OVA$_{II}$ and PR8 (H1N1) viruses were produced in the allantoic cavity of embryonated hen eggs from stock obtained from Dr. Peter Doherty of St. Jude Children's Hospital. A sublethal dose of 0.3LD$_{50}$ was used. Protection experiments were performed using a lethal dose of 2LD$_{50}$. Cold-adapted, attenuated ca.A/Alaska/72/CR9 (H3N2) was originally supplied by S. Epstein (NIH, Bethesda, MD) then grown at the Trudeau Institute (83). Mice were immunized with 2500 TCID$_{50}$ ca.Alaska, a dose shown to elicit T cell mediated

protection (83). Influenza A/Philippines/2/82/x-79 (H3N2) was supplied by S. Epstein (NIH, Bethesda, MD). Mice infected with 100 PFU.

Naïve CD4 T cell Isolation and Effector Generation in Primary Hosts

Spleens and peripheral LNs were harvested from 6-10 week old TCR transgenic or WT mice. Resting cells were enriched using a percoll gradient. CD4 T cells were then isolated using CD4 MACS beads (Miltenyi). Naive CD4 T cells were washed twice, re-suspended in PBS, and a total of $3x10^5$-$5x10^5$ cells were transferred by i.v. injection into hosts. Hosts were infected with PR8-OVA_{II} on the same day.

Isolation of 6 dpi Effector CD4 T cells

Spleen and DLN (Lung Draining Lymph Nodes) were harvested from B6 mice on 6d after PR8-OVA_{II} infection. Cell suspensions were pooled and donor cells were isolated by either Thy1.1 or CD4 MACS isolation (Miltenyi). Cells were resuspended in PBS and 1-$4x10^6$ effector cells were transferred by i.v. injection to hosts. All steps were conducted at RT (except for one 15 minute incubation at 4ºC) to maintain effector phenotype. This minimal protocol ensures that effector cells are only out of mice for 2.5 hours.

Bone Marrow Dendritic Cell Preparation

Bone marrow was harvested from B6 mice and washed with RPMI including 1% FBS. Cells were plated at 7-$8x10^6$ cells/mL in RPMI with 7.5% FBS including

10ng/ml GMCSF. After 7 days, cells were harvested and $CD11c^+$ cells were isolated by MACS. Purified cells were then re-plated at $2x10^6$ cells/ml and stimulated with Poly I:C at 10µg/ml for 1 day in culture and used as DC. DC were harvested, pulsed with 10µM OVA_{II} or $NP_{311\ 325}$-peptide at 37ºC for 1 hour with shaking. Cells were resuspended in PBS and $3\text{-}5x10^5$ cells per mouse were injected i.v.

PR8-infected Splenic APC Preparation and *In Vitro* Culture

Spleens from PR8-infected B6 mice were harvested 6 dpi. Cell suspensions were pooled and washed with RPMI containing 1% FBS. Cells were depleted of $Thy1.2^+$ cells using MACS beads. Cells were irradiated with 3000 Rads. This APC population was then co-cultured with isolated 6 dpi effectors at a ratio of 5:1 APC : OT-II. OVA_{II} peptide was added to culture at 0.5µM. IL-7 was added to cultures at 0.1ng/ml (a concentration that does not promote proliferation). All blocking antibodies were used at 10µg/ml.

APC for the Protection Experiment

Spleen cells were harvested from uninfected B6 mice. $Thy1.2^+$ cells were depleted using MACS beads. The Thy1.2-depleted fraction was then plated at $3x10^6$ cells/ml in RPMI containing 7.5% FBS and 10ng/ml LPS and 10ng/ml dextran sulfate. After 2 days in culture, these activated APC enriched cells were harvested and pulsed

with 10μM OVA_{II} peptide at 37ºC with shaking for 1 hour. APC were transferred to hosts with 6 dpi effectors at a ratio of 1:1.

Histology

B6 mice were infected with $0.3LD_{50}$ PR8 or PR8-OVA_{II}. Lungs were harvested at 6 dpi and fixed in 10% buffered formalin. 10μm sections were taken and stained with hematoxylin and eosin (H&E) stain. Lungs were scored as follows: (1) Healthy looking bronchioles with consolidation and mononuclear infiltrates comprising under 5% of the lung. (2) Mild bronchiolitis with consolidation and mononuclear infiltrates comprising over 5% of the lung. (3) Moderate bronchiolitis with consolidation and mononuclear infiltrates comprising equal to or greater than 15% of the lung. (4) Moderate bronchiolitis with consolidation and mononuclear infiltrates comprising equal to or greater than 25% of the lung. (5) Severe bronchiolitis with consolidation and mononuclear infiltrates comprising over 50% of the lung. Scoring was done blind and four sections of each lung were scored and the average is presented.

Viral Titers

Viral titers of PR8 or PR8-OVA_{II}-infected lungs were determined by quantification of viral RNA. Whole lungs were homogenized in TRIsol/Chloroform (Sigma-Aldrich) and RNA was extracted using the VWR E.Z.N.A kit and Turbo DNA-free kit (Thermo Fisher Scientific). 2.0 μg of RNA was reverse transcribed into cDNA

using the High capacity cDNA reverse transcription kit (Thermo Fisher Scientific). Quantitative PCR was performed to amplify the acidic polymerase (PA) gene using the Bio-Rad CFX96 Realtime PCR system with 50ng of cDNA per reaction. The following primers and probe were used: forward primer: 5'CGGTCCA AATTCCTGCTGA-3'; reverse primer: 5'-CATTGGGTTCCTTCCATCCA-3'; probe: 5'-6-FAM-CCAAGTCATGAAGGA GAGGGAATACCGCT-3'. Data were analyzed using the CFX Manager Software Version 20 (Bio-Rad). A standard curve generated using a PA-containing plasmid obtained from Dr. Rob Webster at St. Jude's Children's Research Hospital was used to calculate the PA gene copy number per 50 ng of cDNA. This was used to calculate the total PA copy number per lung.

Flow Cytometry: Cytokine and Other Intracellular Staining

For cytokine staining, total splenoyctes were stimulated with PMA and Ionomycin for 4 hours at 37ºC. Brefeldin A (10µg/ml) was added after 2 hours of stimulation. Following a surface stain, cells were fixed in 4% paraformaldehyde and permeabilized in 0.1% saponin for 30 min at 4ºC. Cytokines were then stained for 30 minutes at 4ºC. Bim, Bcl-2, Ki67, Bcl-6, and T-bet were stained using the eBioscience Foxp3 staining buffer kit following manufacturer's recommendations. Bim Ab was stained with a fluorescent Goat α-Rabbit Ab from Invitrogen. Host IA^b-$NP_{311\ 325}$-specific CD4 T cells were stained with the IA^b-$NP_{311\ 325}$-APC tetramer obtained from the NIH Tetramer Core Facility. All antibodies were obtained from

eBioscience except anti-Bim (Cell Signaling) and anti-Bcl-6-PE (BD biosciences). Samples were run on LSRII instruments (BD biosciences) and analysis was done using Flowjo (Tree Star) analysis software.

Flow Cytometry: Phospho-STAT3 Staining

After 4 hours of culture, cells were washed with PBS and stained for 20 minutes on ice with Invitrogen Live/Dead yellow. Cells were then incubated in BD Fix/Perm Buffer for 10 minutes at 37°C. Cells were then incubated in BD Perm Buffer III for 30 minutes on ice. Finally, cells were stained with fluorescently labeled pY705 STAT3 Ab (BD Biosciences) for 1 hour at RT protected from light. Samples were run immediately after staining.

Microarray Analysis

RNA was isolated from sorted 6 dpi effector OT-II cells, 6 dpi OT-II cultured in media for 2 days, and 6 dpi OT-II cultured with αCD3 and αCD28 for 2 days using the same protocol as was used for viral titers. Then a microarray was performed using Affymetrix Mouse Gene 2.0 ST arrays by the UMMS Genomics Core Facility. The Affymetrix Expression Console was then used for RMA normalization and the Affymetrix Transcriptome Console was used to identify genes that were differentially expressed by over 2-fold. The NIAID DAVID platform was then used to classify genes into functional categories.

Statistical Analysis

Groups of at least 3 mice were used for all experiments to ensure sufficient power. MFI in all graphs is median fluorescence intensity. For analysis comparing more than 2 samples, a one-way ANOVA analysis was conducted with GraphPad prism software. To compare 2 samples an unpaired, two-tailed student's t-test was conducted with GraphPad prism software. All data was included unless found to be a significant outlier using the Grubb's test (ESD method) available through GraphPad prism software. Welch's correction was applied when the standard deviations were unequal. Significance is indicated by * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$, **** = $P<0.0001$.

CHAPTER III: Late Ag is Required for CD4 Memory T cell Generation during Influenza Infection

Introduction

While much progress has been made in defining the early activation events required for the generation of effector CD4 T cell subsets, the pathways that drive a cohort of effector T cells to successfully transition to a memory state remain poorly defined. It is unclear to what extent programming during initial cognate interaction of T cells with APC determines the fate of effector T cells and if later signals affect memory generation.

Various models defining the role of Ag in effector and memory differentiation have been proposed. Some suggest that the initial interaction with Ag/APC is sufficient to program a cohort of T cells to become memory and further exposure to Ag and inflammation drive terminal differentiation of effector T cells (242, 245, 251, 253, 282). In contrast, other studies suggest that late Ag enhances the function but not the number of memory CD8 T cells (138, 139). It has been shown that CD4 T cells require more Ag stimulation for effector and memory generation than do CD8 T cells, but most of these analyses have been limited to the priming phase of the response (249, 260, 269, 305). Other studies have concluded that while prolonged Ag stimulation can enhance effector CD4 T cell proliferation, it is deleterious to memory formation (263), and continuous Ag stimulation may drive CD4 T cells to a state of reduced responsiveness (246, 300). *In vivo*, responding T cells disengage from APC 24 hours after initial interaction, engaging in few APC

contacts during the last phase of priming (266, 269). Thus, it remains unclear how often responding CD4 T cells encounter Ag after the initial priming phase of the response and if later Ag exposure impacts memory generation.

During a response to a live pathogen, it would be advantageous for the quality and quantity of the effector and memory response to be determined at the effector stage when the immune system could sense whether there is still a threat. In an *in vivo* model of IAV infection, our lab recently found that autocrine IL-2 production by effector CD4 T cells during a defined checkpoint (5-7 dpi) was essential to promote survival and memory formation (1). Similarly, addition of IL-2 complexes late in the response promoted memory formation during LCMV infection (211). Since IL-2 production is typically induced by cognate Ag recognition, I investigated whether the interaction of effector CD4 T cells with APC during this checkpoint is the key event that drives them to make IL-2, to survive, and to differentiate into long-lasting memory cells. A defined stage of effector CD4 T cell development, where CD4 effector fate is determined by cognate Ag interaction, would suggest a new paradigm in which the formation of memory depends on a cohort of cells being selected by persisting Ag to become memory cells.

In most *in vivo* studies heretofore, it has not been possible to define the necessary timing and duration of the signals needed for the rescue of effectors from apoptosis and exessive contraction. Additionally, as T cells reach the effector stage, the roles that ongoing infection play in promoting memory have not been

definitively examined in an *in vivo* model of infection. Defining these elements is critical for rational vaccine design.

To address these gaps in our understanding, I use a well-defined model of IAV infection to determine the role that Ag presentation and ongoing infection, during the effector phase, play in shaping memory CD4 T cell formation. IAV induces a highly protective memory CD4 T cell population that synergizes with B cells and CD8 T cells to provide protection from challenge with supralethal viral doses (2, 3, 14, 120). The response thus epitomizes successful memory CD4 T cell generation in response to infection and is therefore well-suited to reveal the mechanisms involved in effective memory generation.

I find that effector CD4 T cells, induced by IAV infection, require cognate Ag recognition at 6 dpi for continued expansion, survival, and all but a minor fraction of memory generation. In well-controlled adoptive transfer models, I find that Ag/APC encounter at the effector stage (6 dpi) enhances the recovery of memory cells in SLO and in the lung at least 10 to 100-fold. Notably, other infection-induced effects, such as inflammation, are not required for this increased memory generation. Effector T cells, exposed to Ag/APC for as little as 2 days, expressed higher levels of memory-associated molecules CD25, Bcl-6, CD127, and CXCR3. The memory cells generated by Ag encounter between 6-8 dpi had enhanced ability to make cytokines and provided better protection against a lethal dose of IAV than those that were not exposed to checkpoint Ag. Moreover, in a cold-adapted vaccine model, I found very little Ag presentation during this late

checkpoint, but when additional Ag/APC were introduced at this time, memory CD4 T cell formation was enhanced. This suggests that low levels of Ag presentation from 6-8 dpi may limit efficacy of vaccines that do not provide high levels of persisting Ag. These findings imply that whether pathogen infection persists into the effector stage determines effector fate by supplying late Ag/APC that are needed to program memory formation and that interventions to achieve this need not involve long-lived infection and its potentially deleterious effects.

Antigen Recognition at the Effector Phase of Influenza Infection is Limited.

Previously, it has been difficult to assess exactly when responding CD4 T cells encounter Ag *in vivo.* Transfer of naïve CD4 T cells at various times following IAV infection demonstrates that Ag presentation occurs up to 3 weeks post infection (306). However, naïve and effector CD4 T cells may have different patterns of trafficking. Additionally, *in situ* effectors may localize to specific niches and may not be able to access the same Ag depots that intravenously administered naïve cells can. It is therefore unclear if responding CD4 T cells recognize Ag for 3 weeks post infection. Microscopy studies have suggested that only about ~35% of IAV-specific effector CD4 T cells undergo arrest and produce IFNγ directly ex vivo suggesting recent Ag encounter in the lung at 7 dpi (307). Although this study was conducted by transferring *in vitro* generated Th1 effectors into infected mice and was therefore not necessarily representative of Ag recognition by endogenous CD4 T cells. To more directly test when responding CD4 T cells recognize Ag *in*

situ, I utilized the *Nr4a1*^{eGFP} (Nur77^{GFP}) mice that transiently express GFP following TCR stimulation (243, 308).

To determine when responding CD4 T cells encounter Ag *in vivo* following IAV infection, I crossed OT-II.Thy1.1$^{+/}$ (Ovalbumin$_{323\ 339}$ (OVA_{II})-specific TCR transgenic mice) to Nur77^{GFP} mice. To evaluate the feasibility of using Nur77^{GFP} as an indicator of recent Ag-induced TCR stimulation in effector T cells, I isolated CD4 T cells from Nur77^{GFP} mice and stimulated them *in vitro*. GFP expression was rapidly induced and remained high with continued TCR stimulation (Figure 3.1A), but was significantly reduced within 24 hours following removal of stimulation (Figure 3.1B). Additionally, GFP was rapidly re-expressed following secondary exposure to Ag (Figure 3.1C) and did not decrease with division (Figure 3.1D) (243).

To determine the kinetics of IAV Ag recognition *in vivo,* I transferred naïve OT-II.Nur77^{GFP}.Thy1.1$^{+/}$ cells to C57BL/6J (B6) mice and infected with a sublethal dose of A/Puerto Rico/8/34-Ovalbumin$_{323\ 339}$ (PR8-OVA_{II}) (Figure 3.2A-3.2D). As expected, during priming (3 dpi) most cells were GFP^{+} indicating recent Ag exposure. However, by 5 dpi, only a fraction of effector CD4 T cells had recently encountered Ag and by 9 dpi (the peak of the lung effector T cell response) a very low percentage of cells express GFP (Figure 3.2C). An examination of the kinetics of GFP^{+} cell numbers demonstrates that the Ag recognition occurs in both the lung and SLO and peaks around 7 dpi (Figure 3.2D). The low percentage of GFP expressing cells was not due to the transfer of a non-physiological number of OT-

Figure 3.1. Nur77^{GFP} is a reliable reporter of TCR signaling in mature effector CD4 T cells.

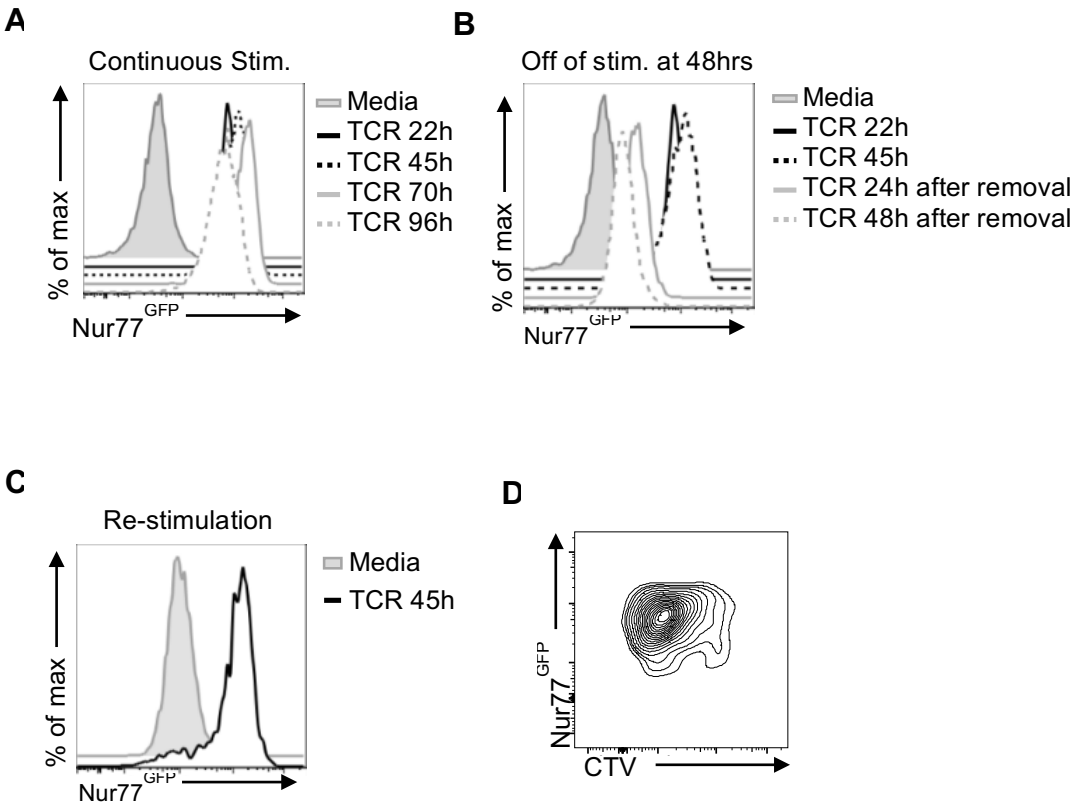


Figure 3.1. Nur77^{GFP} is a reliable reporter of TCR signaling in mature effector CD4 T cells.

(A, B) CD4 T cells were isolated from Nur77^{GFP} mice and cultured with anti-CD3 + anti-CD28 either continuously for 4 days (A) or removed from stimulation at 48 hours and re-plated (B). GFP expression was determined by flow cytometry. **(C)** OT-II.Nur77^{GFP}.Thy1.1$^{+/}$ cells were stimulated *in vitro* with irradiated OVA_{II}-pulsed APCs for 2 days with 5ng/mL of IL-2, then rested for 3 days in culture. Cells were then re-stimulated in culture with OVA_{II}-pulsed APC for 2 days. **(D)** Nur77^{GFP} CD4 T cells were labelled with cell trace violet (CTV) and stimulated with anti-CD3 + anti-CD28 for 3 days in culture. (A-D) Representative data, n=6, 2 experiments.

II cells, as a similar GFP kinetics was seen in polyclonal host responses following IAV infection of Nur77^{GFP} mice (Figure 3.2E). Thus, effector CD4 T cells only intermittently respond to cognate antigen *in vivo* and the Ag recognition that does occur, is mostly limited to just before the peak of the effector T cell response. These findings suggest that Ag recognition at the effector stage could act to select a limited number of effectors to become memory.

Although the GFP median fluorescence intensity (MFI) is lower in the SLO than in the lung, it is higher than at the memory time point (31 dpi) (Figure 3.2B). Additionally, when OT-II.Nur77^{GFP} cells are isolated at 6 dpi and transferred to hosts without Ag (PR8-infected hosts) they do not express GFP (Figure 3.3A). This suggests that although it may not be strong TCR stimulation, the GFP signal in the SLO at later time points may reflect continuing Ag presentation. Since follicular helper CD4 T cells (Tfh) are thought to recognize residual Ag in germinal centers (309–311) it is likely that the population that expresses GFP at these later time points are Tfh. Indeed, when GFP^{+} cells are compared to GFP cells in the spleen and DLN, GFP^{+} cells have higher expression of the Tfh markers CXCR5 and Bcl-6 at 9 dpi (Figure 3.3B, 3.3C).

Late Ag is Required for Memory CD4 T cell Formation.

Since several previous studies have looked at the role of Ag using antibiotics to truncate infection resulting in a reduction in both Ag and general pathogen-induced inflammation, I sought to examine the role of late Ag using a

Figure 3.2. Ag recognition at the effector phase of influenza infection is limited.

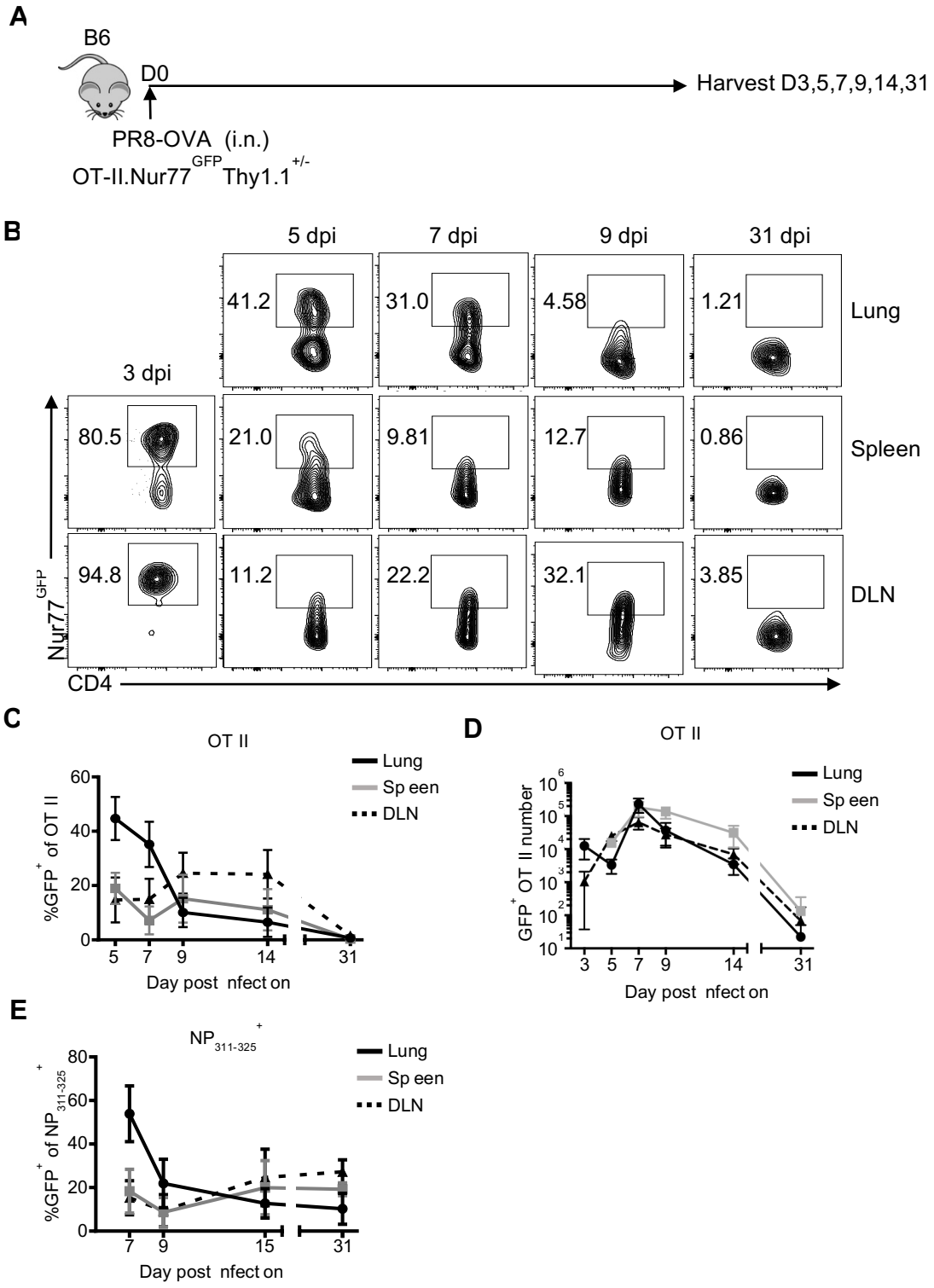


Figure 3.2 Antigen recognition at the effector phase of influenza infection is limited.

(A-D) $5x10^5$ naïve OT-II.Nur77^{GFP} were transferred to B6 mice. Mice were infected with PR8-OVA_{II}. Lung, spleen, and DLN were harvested at various time points and GFP expression of donor cells was analyzed. (A) Experimental schematic. (B) Representative flow cytometry plots of Nur77^{GFP} expression at various time points following infection. (C) Kinetics of GFP expression by OT-II.Nur77^{GFP} cells during PR8-OVA_{II} infection in B6 mice. (D) Kinetics of OT-II GFP$^+$ cell number. (E) GFP expression kinetics of $NP_{311\ 325}$ tetramer$^+$ cells during PR8-OVA_{II} infection of Nur77^{GFP} mice. (B, D) Representative data, 4 experiments, n=3-4 each. (C, E) Pooled data, n=12-16, 3-4 experiments, mean ± SD.

Figure 3.3. Low level Ag presentation late in the SLO marks Tfh.

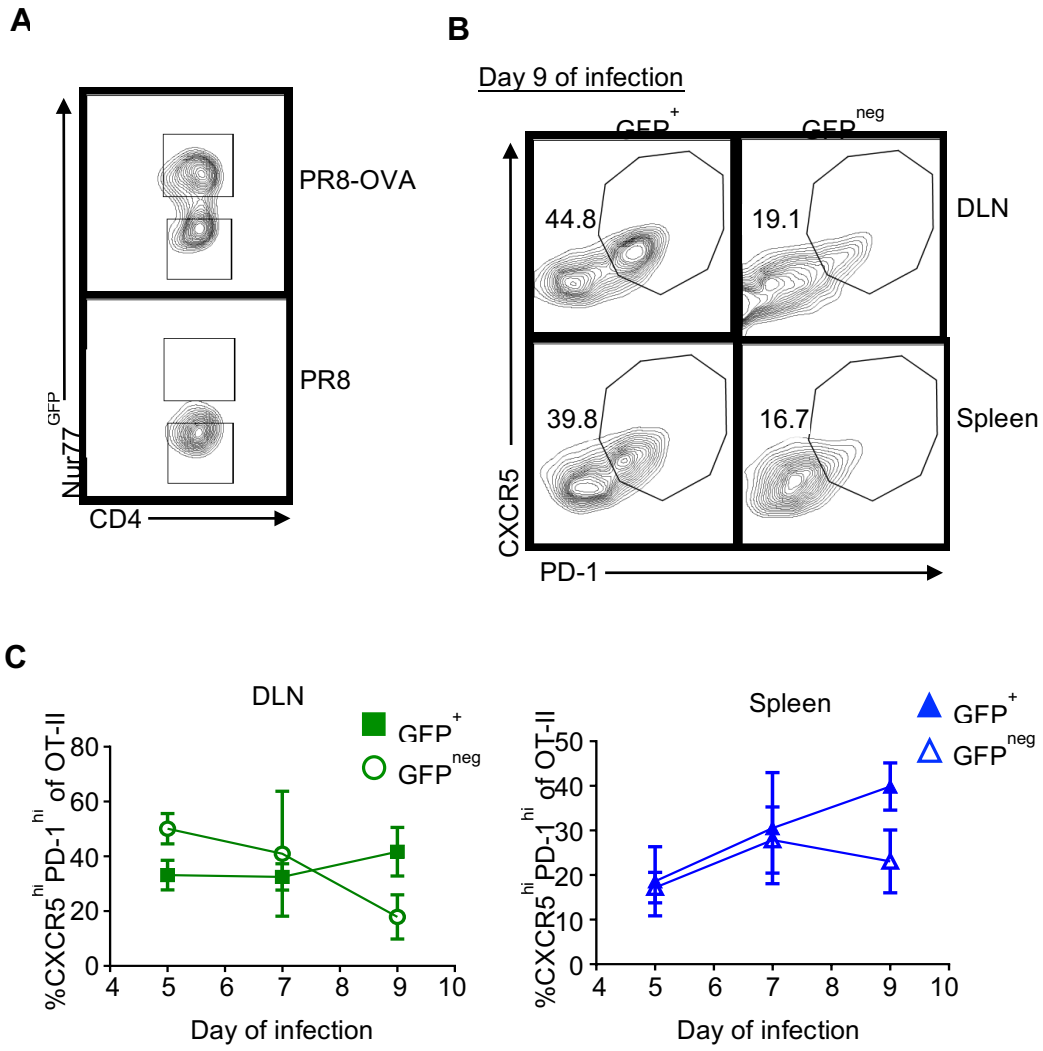


Figure 3.3. Low level Ag presentation late in SLO marks Tfh.

(A) OT-II.Nur77^{GFP}.Thy1.1$^{+/}$ naïve cells were transferred to B6 mice followed by infection with PR8-OVA$_{II}$. Donor cells were isolated at 6 dpi and transferred to either infection matched PR8-OVA$_{II}$-infected or PR8-infected hosts. Lung cells were harvested 18 hours after transfer. GFP expression in the lung is shown. **(B-C)** OT-II.Nur77^{GFP}.Thy1.1$^{+/}$ naïve cells were transferred to B6 mice followed by infection with PR8-OVA$_{II}$. Spleen and DLN were harvested at 5,7, and 9 dpi. (B) Representative flow plots of Tfh in GFP^{+} and GFP populations at 9 dpi in the spleen and DLN. (C) Tfh kinetics between GFP^{+} and GFP cells. Representative data, 3-4 experiments, n=3-4 each.

model that could distinguish between the effects of Ag versus those of the pro-inflammatory cytokine milieu. To this end, I used the PR8 and PR8-OVA_{II} viruses to generate hosts with inflammation alone and inflammation with cognate OT-II Ag. To ensure that these infections were similar in aspects other than OT-II peptide presentation, I used the IA^b-$NP_{311\ 325}$ tetramer to measure the $NP_{311\ 325}$ specific endogenous CD4 T cell response during the course of PR8 and PR8-OVA_{II} infection. I found that the CD4 T cell response followed a similar kinetics in both viruses (Figure 3.4A). This indicates that with respect to factors that govern the CD4 T cell response, these viruses are very similar. Additionally, both PR8 and PR8-OVA_{II} infections generated a similar amount of lung pathology at 6 dpi (Figure 3.4B, 3.4C). Finally, both viruses had similar viral titers at 6 dpi and had cleared infection by 14 dpi (Figure 3.4D).

I next tested if Ag recognition during the effector phase had any effect on memory generation. For this, I performed a sequential adoptive transfer experiment outlined in Figure 3.5A. I first transferred naïve OT-II.Thy1.$1^{+/}$ cells to B6 mice and infected with a sublethal dose of PR8-$OVA_{II.}$ At 6 dpi donor OT-II.Thy1.$1^{+/}$ effector cells were isolated from the SLO of IAV infected hosts. These 6 dpi effectors were fully activated, having undergone extensive division as evidenced by CFSE dilution, upregulation of CD44, CXCR3, and PD-1 and downregulation of CD62L (Figure 3.5B). Donor cells were transferred into 3 groups of recipients, also infected 6 days previously with PR8-OVA_{II} (Ag and virus), PR8

Figure 3.4. PR8 and PR8-OVA$_{II}$ infections are similar.

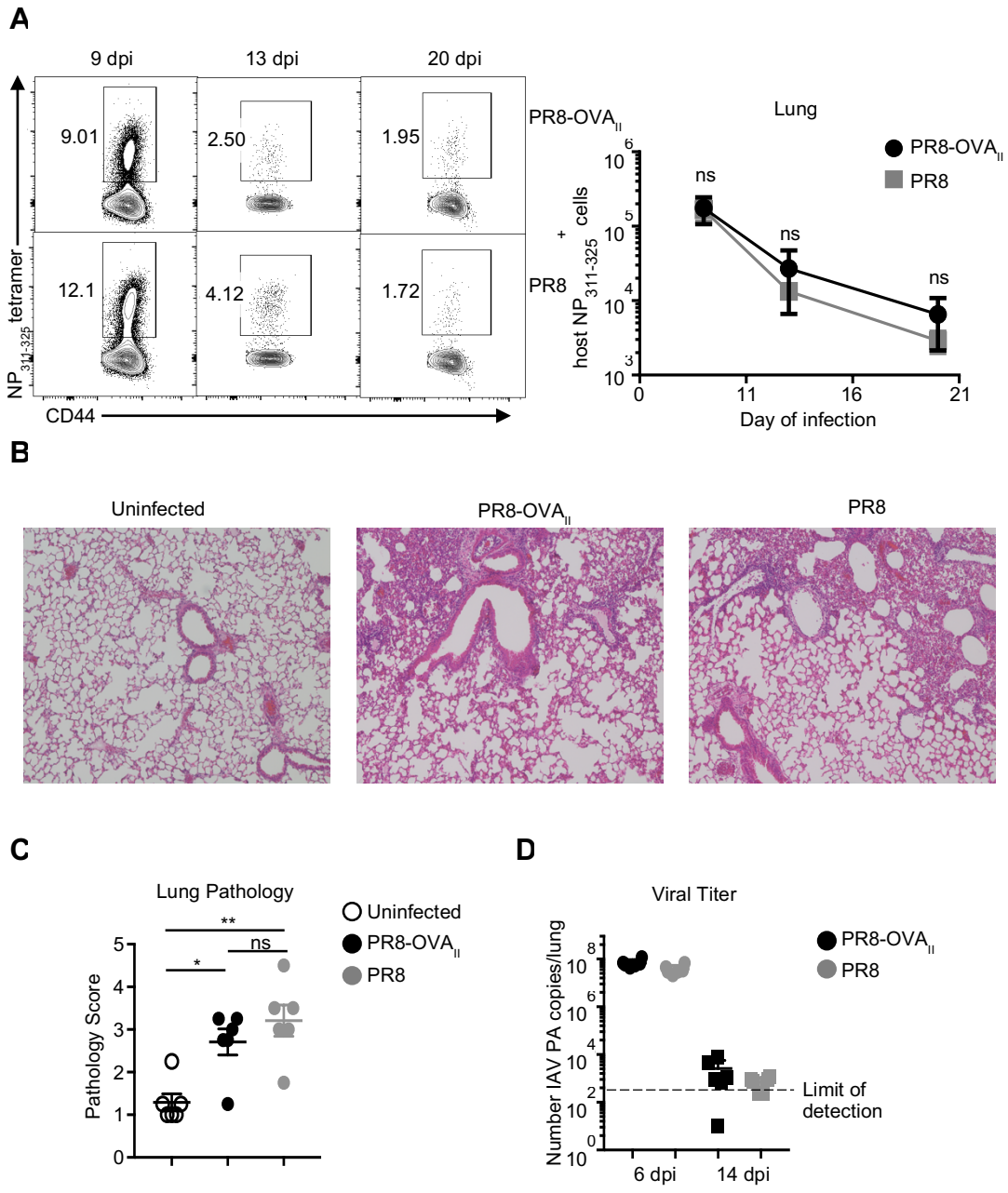


Figure 3.4 PR8 and PR8-OVA_{II} infections are similar.

(A) B6 mice were infected with $0.3LD_{50}$ PR8-OVA_{II} or PR8. The endogenous CD4 T cell response in the lung was measured at 3, 7, and 14 dpt by staining with the IA^b-$NP_{311\ 325}$ tetramer. Data is representative, n=9, 3 experiments, mean ± SD. **(B)** B6 mice were infected with PR8-OVA_{II}, PR8, or not infected as in (A). On 6 dpi, lungs were harvested and fixed in 10% buffered formalin. Pictures are shown of H&E stained lung sections at 10X magnification. **(C)** Pathology scoring of lung sections. **(D)** Viral titer of PR8-OVA_{II} or PR8-infected lungs harvested at 6 dpi and 14 dpi. Limit of detection determined by uninfected lung results. (A, B) Data is representative of 2 experiments, n=3 each, mean ± SD. (C, D) Data is pooled from 2 experiments, n=3 each, mean ± SD.

(virus without Ag), or no virus (Figure 3.5A). I did not include lung effector T cells, since they are more likely to have recently encountered Ag (Figure 3.2).

I enumerated donor cells in the lung, spleen, and draining lymph nodes (DLN) at 3, 7, and 14 days post-transfer (dpt). At 7 dpt, there were 60-200x more donor OT-II cells in the lung, 15-30X more in the spleen, and 80-400X more in the DLN of PR8-OVA_{II}-infected hosts compared to PR8-infected or uninfected hosts which were equally poor in supporting donor cell recovery (Figure 3.5C, 3.5D). In PR8-OVA_{II} hosts, donor OT-II numbers peaked at 3 dpt (9 dpi) and then contracted slowly over the subsequent 12 days (Figure 3.5E) mimicking the endogenous CD4 T cell response (140) (Figure 1.2). However, in PR8-infected and uninfected hosts donor cells underwent a sharp, immediate contraction and by 14 dpt (20 dpi) were reduced to close to the limit of detection (Figure 3.5E). A highly significant difference in memory recovery was still seen at 53 dpt (Figure 3.5F). These results imply that re-exposure to Ag at or after 6 dpi is necessary to maximize the effector CD4 T cell response, prevent excessive contraction, and generate a long-lived memory population, and that infection without Ag has little if any impact on memory formation.

To test if the ability of Ag recognition to promote memory is transient or instead persists to later time-points, I isolated donor OT-II effectors at 14 dpi instead of 6 dpi and transferred them to kinetically-matched PR8-OVA_{II}, PR8, or uninfected hosts (Figure 3.6A). The presence of Ag in the hosts had little or no impact on recovery of these 14 dpi donor cells (Figure 3.6B) indicating that, at 14

Figure 3.5. Late Ag is required for memory CD4 T cell formation.

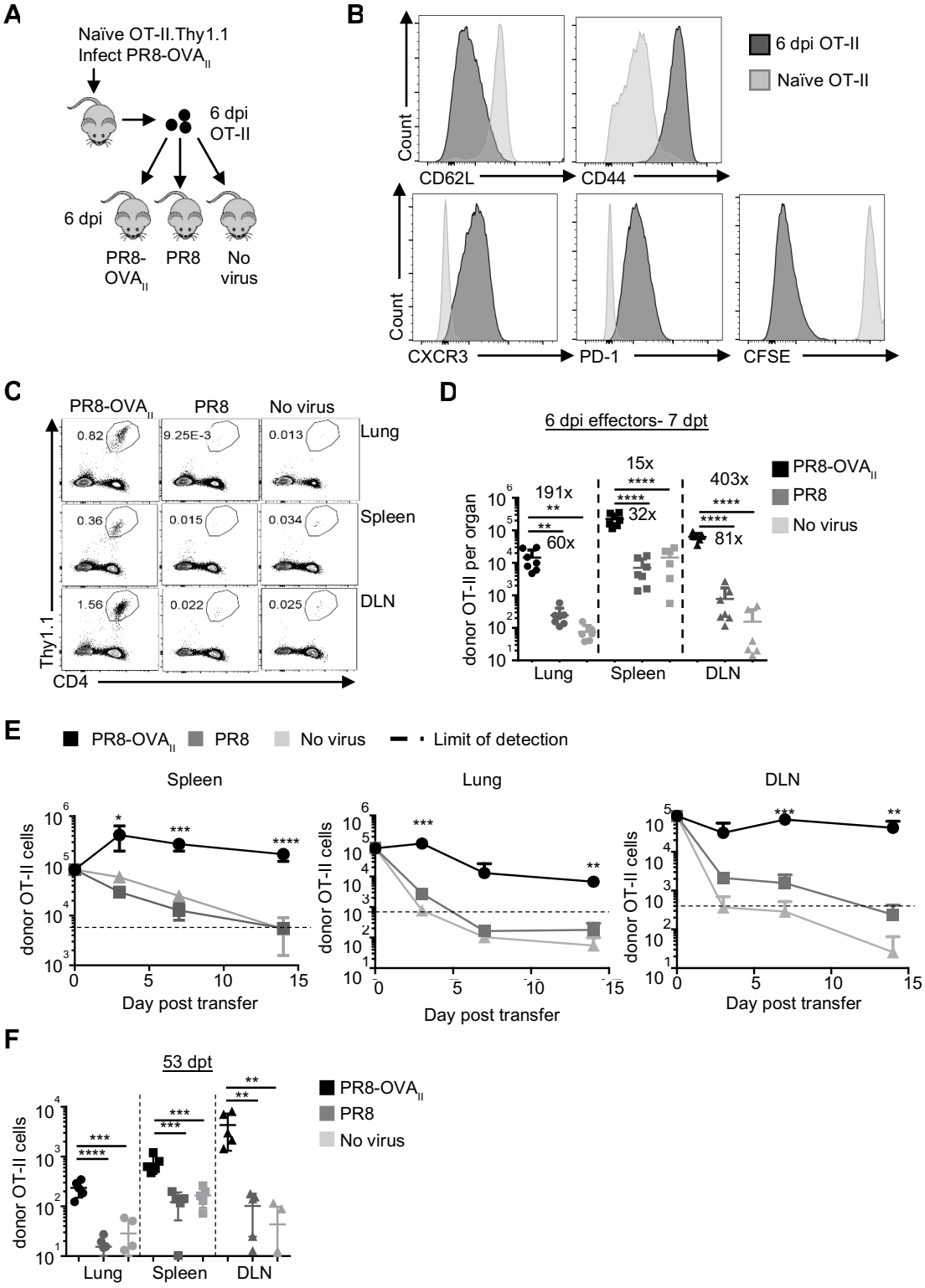


Figure 3.5. Late Ag is required for memory CD4 T cell formation.

(A-F) $5x10^5$ naïve OT-II.Nur77^{GFP}.Thy1.1$^{+/}$ were transferred to B6 hosts. Hosts were infected with PR8-OVA_{II}. On the same day, groups of B6 mice were either infected with PR8-OVA_{II} or PR8. On 6 dpi, donor OT-II cells were isolated from the spleen and DLN of infected mice. $2x10^6$ 6 dpi effectors were transferred to the PR8-OVA_{II}, PR8, or uninfected hosts. (A) Experimental schematic. (B) Representative flow cytometry plots showing the phenotype of isolated 6 dpi OT-II effectors compared to naïve OT-II cells. (C) Representative flow cytometry plot gated on live cells at 7 dpt. (D) Quantification of donor cell recovery at 7 dpt. (E) Kinetics of donor cell recovery in the spleen, lung, and DLN. (F) Quantification of cells harvested 53 dpt. Data is representative, n=3-5 each, 3 experiments, mean ± SD.

dpi, CD4 T cells are no longer require Ag recognition for memory formation. Interestingly, while 6 dpi effectors underwent extensive proliferation when transferred to PR8-OVA_{II} infection matched hosts, 14 dpi effectors failed to proliferate in any host (Figure 3.6C). This is despite the presence of Ag presentation evidenced by the proliferation of naïve cells (306). I therefore postulate that late Ag recognition must occur within a limited time frame in order to promote sustained proliferation and survival into memory.

Late Ag is Required for Effector and Memory Formation *In Situ.*

To determine if this late requirement for Ag was seen during an intact response without the isolation and transfer of effectors, I tested if blocking MHC-II, during the effector phase of the response, with the anti-IA^b Ab (M5114) resulted in reduced effector and memory cell numbers. Initial attempts to block MHC-II in WT hosts were unsuccessful likely due to the abundance of MHC-II expression during IAV infection (data not shown). I therefore sought to establish a model with limited MHC-II expression that still promoted normal memory CD4 T cell formation. To this end, I compared CD4 memory T cell formation in B6 mice to CD11cTg.*H2-Ab1*$^{-/-}$ (CD11cTg) mice. CD11cTg mice are MHC-II knockout mice with a transgene that expresses MHC-II under the CD11c promoter. Therefore, these mice only express MHC-II on $CD11c^+$ cells (Figure 3.7A).

To test if memory formation occurred normally in these mice, I transferred naïve OT-II cells into B6 or CD11cTg mice and infected with PR8-OVA_{II}. Similar

Figure 3.6. 14 dpi effectors no longer require Ag to form memory.

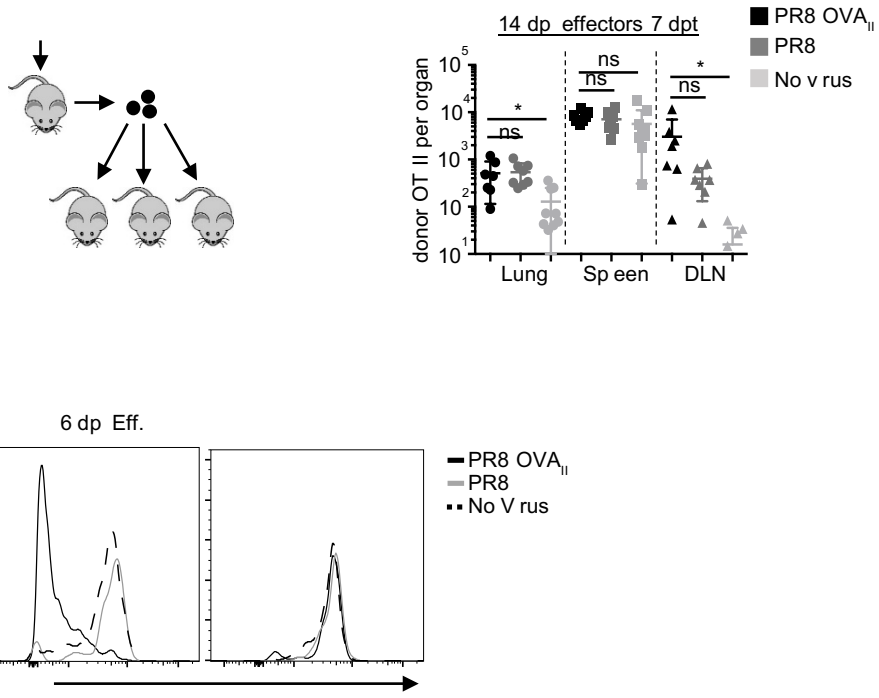


Figure 3.6. 14 dpi effectors no longer require Ag to form memory.

(A-B) $5x10^5$ naïve OT-II.Nur77^{GFP}.Thy1.1$^{+/}$ were transferred to B6 hosts. Hosts were infected with PR8-OVA_{II}. On the same day, groups of B6 mice were either infected with PR8-OVA_{II} or PR8. On 14 dpi, donor OT-II cells were isolated from the spleen and DLN of infected mice. $2x10^6$ 14 dpi effectors were transferred to the PR8-OVA_{II}, PR8, or uninfected hosts. (A) Experimental schematic. (B) Cell recovery at 7 dpt. **(C)** Comparison of CFSE dilution between 6 dpi effectors and 14 dpi effectors 3 dpt into PR8-OVA_{II}-infected, PR8-uninfected, or uninfected second hosts. Data is representative, n=3-4 each, 3 experiments, mean ± SD.

numbers of effectors were generated at 9 dpi and similar numbers of memory cells were generated in all organs at 28 dpi (Figure 3.7B). Memory cells generated in CD11cTg mice appeared to be functional, as a similar percentage produced IFNγ and IL-2 following peptide re-stimulation *ex vivo* (Figure 3.7C). This finding indicates that MHC-II on the $CD11c^+$ compartment is sufficient for memory formation.

Given that memory formation occurred normally in the CD11cTg mice, I used them as hosts for a MHC-II blocking experiment. I transferred naïve OT-II cells into CD11cTg hosts and infected with PR8-OVA_{II}. Then I treated the mice with anti-MHC-II Ab (clone M5114) on day 4-7 of infection (Figure 3.8A). At 7 dpi, most MHC-II expression was significantly blocked especially in the SLO (Figure 3.8B). Only a modest reduction was seen in the lung (Figure 3.8B). At 7 dpi, the number of donor cells was significantly reduced in all organs when MHC-II was blocked during the effector phase (Figure 3.8C). This is consistent with the immediate contraction seen in 6 dpi effectors transferred to hosts without Ag (Figure 3.5E). Importantly, priming did not appear to be affected as the donor cells in both mice produced similar levels of IFNγ upon *ex vivo* re-stimulation (Figure 3.8D). These findings suggest that responding CD4 effector T cells require Ag recognition during the effector phase for their full expansion.

At a memory time point (27-28 dpi), there was a significant reduction in memory cell formation in the lung and the spleen, however no reduction was seen in the DLN (Figure 3.8E). Interestingly, the memory cells formed in the DLN in the

Figure 3.7. MHC-II on CD11c^{+} cells is sufficient for CD4 memory T cell formation.

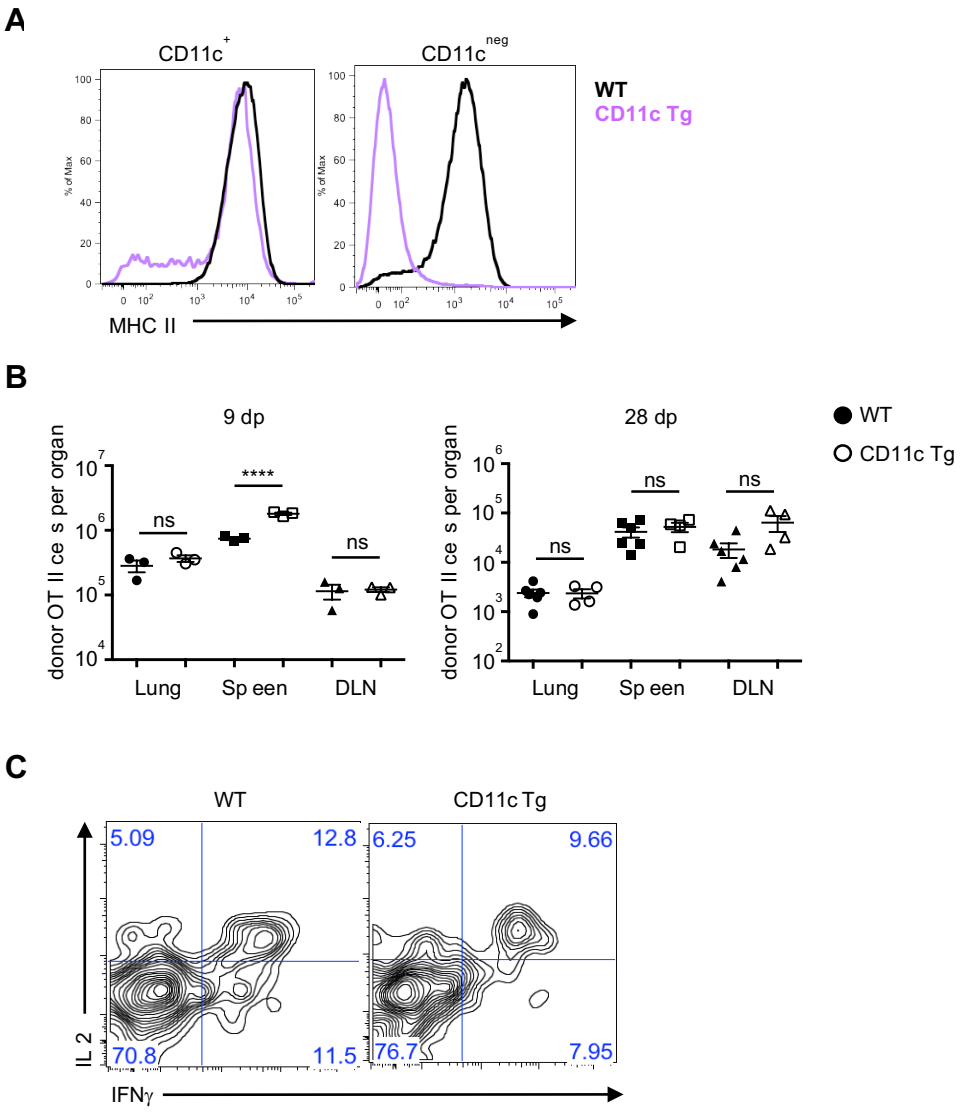


Figure 3.7. MHC-II on CD11c^{+} cells is sufficient for CD4 memory T cell formation.

(A) Representative flow cytometry plot of MHC-II expression on CD11c^{+} and CD11c cells in either WT B6 or CD11cTg mice in the spleen. **(B-C)** OT-II.Thy1.1$^{+/}$ naïve cells were transferred to WT or CD11cTg mice followed by infection with a sublethal dose of PR8-OVA$_{II}$. (B) Donor cell recovery at 9 and 28 dpi in WT or CD11cTg mice in the lung, spleen, and DLN. (C) Representative flow cytometry plots showing IFNγ and IL-2 production of donor cells in the spleen following 4 hours of re-stimulation with αCD3 and αCD28 Ab at 28 dpi. Data is representative of 2 independent experiments, n=3-5 each, mean ± SEM.

Figure 3.8. Late MHC-II is required for CD4 memory T cell formation.

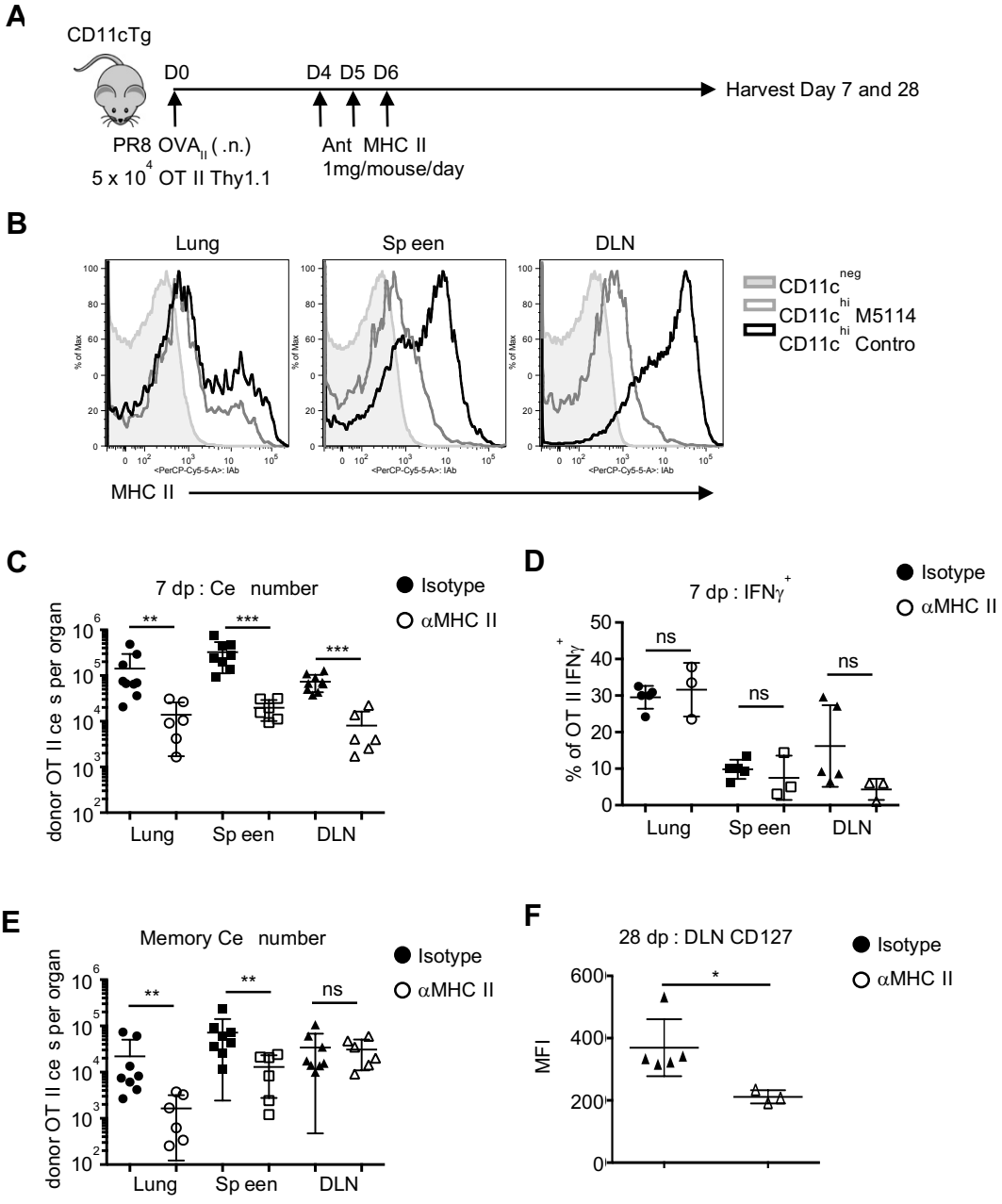


Figure 3.8. Late MHC-II is required for CD4 memory T cell formation.

(A-F) $5x10^4$ naïve OT-II.Thy1.1$^{+/}$ cells were transferred to CD11cTg mice followed by sublethal PR8-OVA_{II} infection. Mice were then treated with αMHC-II Ab (M5114) at 1mg/mouse/day administered via i.v. injection. (A) Experimental schematic. (B) Representative flow cytometry plots showing MHC-II staining in the lung, spleen, and DLN of CD11c$^+$ cells in treated and untreated mice compared to CD11c cells. (C) Donor cell recovery at 7 dpi. (D) IFNγ production of donor cells at 7 dpi following 4 hours of re-stimulation with αCD3 and αCD28 Ab. (E) Donor cell recovery at 27-28 dpi. (F) CD127 expression of donor cells in the DLN of treated and untreated mice at 28 dpi. (B, D, F) Representative data of 2 independent experiments, n=3-5 each, mean ± SD. (C, E) Pooled data, 2 experiments, n=3-5 each, mean ± SD.

absence of late Ag had reduced CD127 expression. This suggests that they may not be as fit to receive the IL-7 signals required for long term survival. The fact that no impact was seen in the DLN is surprising given that large effects were seen in all three organs in the transfer model. It is possible that MHC-II blocking was not complete enough to effect memory formation in the DLN. Alternatively, since CD11cTg mice have a deficiency in endogenous CD4 T cells, survival niches may be more available to donor CD4 T cells resulting in less competition and therefore less stringency in the requirements for memory formation. However, the fact that blocking MHC-II during the effector phase largely mimicked the results of the transfer experiments provides evidence that the transfer model largely replicates what occurs during the course of the endogenous CD4 T cell response to IAV.

Late Ag is Required for Full Expansion.

Some studies suggest that effector CD4 T cell division is programmed by initial Ag encounter (247, 248), while others suggest that CD4 T cells do not undergo such "autopilot" proliferation after 2 days of stimulation during priming (249), but it remains unclear if they acquire this ability later during infection. To determine if division past 6 dpi depends on Ag recognition, I labeled isolated 6 dpi effectors with CFSE, transferred to infection-matched PR8-OVA_{II}-infected, PR8-infected or uninfected hosts (as in Figure 3.5A) and assayed dilution of CFSE at 3 dpt. Only donor cells in hosts with Ag divided more than once (Figure 3.9A). To determine if this proliferation was an artifact of the transfer system, I used Ki67

staining to compare the proliferation of donor OT-II cells to that of endogenous IA^b-$NP_{311\ 325}$-specific host cells in PR8-OVA_{II}-infected hosts. I found that at 2 dpt there was a similar percentage of proliferating donor and hosts cells, and by 8 dpt neither were undergoing division, a pattern seen in the lung, spleen, and DLN (Figure 3.9B, 3.9C). Thus, division after 6 dpi is Ag-dependent, short-lived, and followed by the transition to non-dividing cells within a week. This also illustrates that the kinetics of proliferation of the transferred donor cells mimics that of the endogenous host CD4 T cell response to live IAV.

Some have reported that late Ag promotes increased effector expansion but leads to exacerbated contraction, resulting in fewer or similar numbers of long-lived memory cells (139, 263). To determine if the increased number of donor cells in the PR8-OVA_{II}-infected hosts was the result of an extended expansion of short-lived effectors, I assayed the size and phenotype of donor cells 2 and 8 dpt (Figure 3.9D, 3.9E). At 2 dpt, the donor cells where large in size (Figure 3.5D), with a high level expression of effector markers ICOS and PD-1 (Figure 3.5E), but by 8 dpt they were small (Figure 3.9D) and had downregulated ICOS and PD-1 (Figure 3.9E). Thus, by 8 dpt donor cells no longer had an effector phenotype and had mostly transitioned to resting cells.

Short-term Ag Presentation at 6-8 dpi is Sufficient to Restore CD4 T cell Responses to IAV.

Given that re-encounter with Ag was required at 6 dpi but not 14 dpi, I tested

Figure 3.9. Late Ag is required for full proliferation.

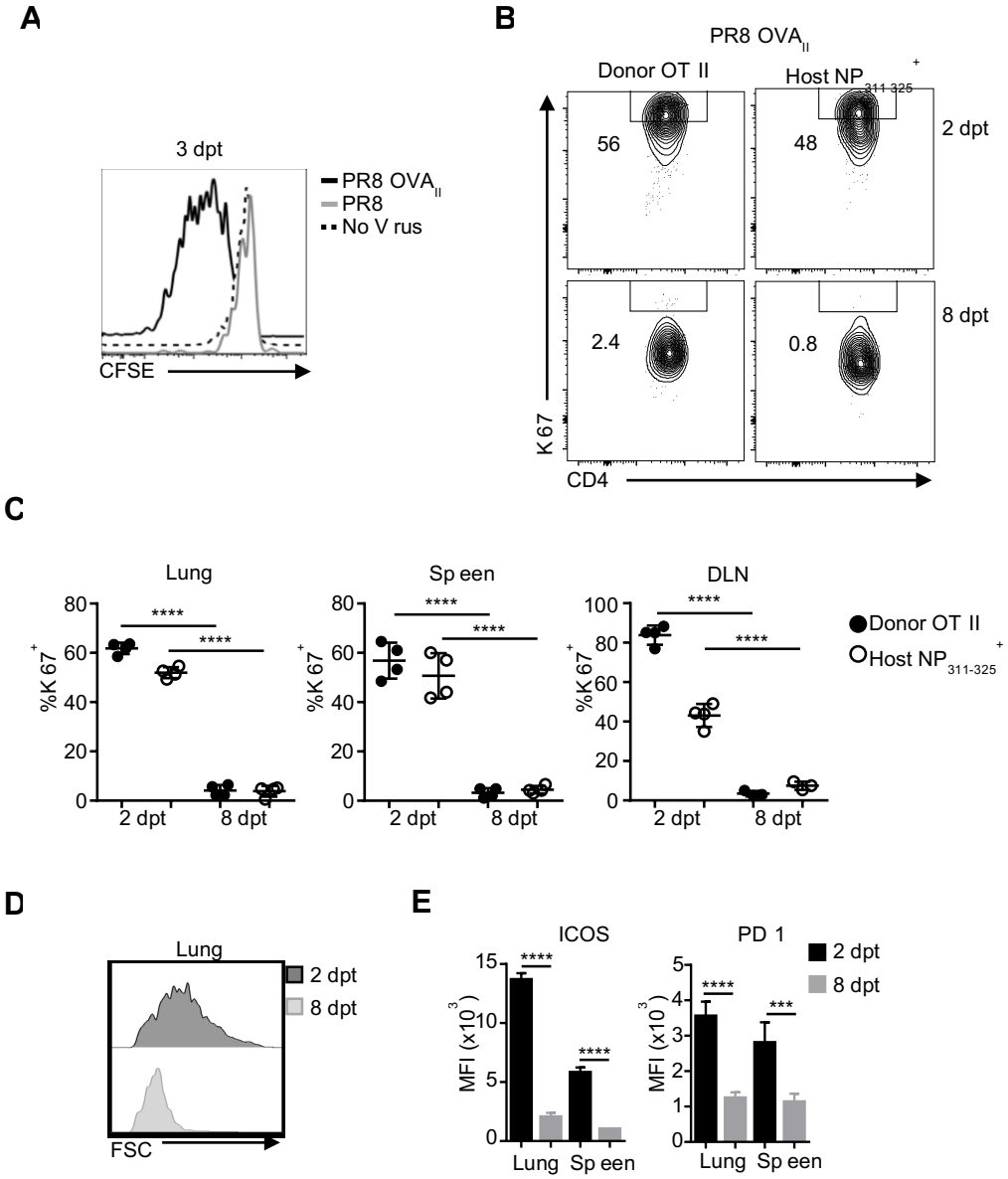


Figure 3.9. Late antigen is required for full proliferation.

(A) 6 dpi effectors were isolated, stained with CFSE, and transferred to kinetically matched PR8-OVA_{II} or PR8-infected or uninfected hosts as in Figure 3.5A. CFSE dilution was determined 3 dpt in the spleen, similar results seen in the lung. **(B-E)** Same experimental approach as in Figure 3.5A. Ki67 expression of donor OT-II and $NP_{311\ 325}$ tetramer positive host cells was determined at indicated time points following donor cell transfer. (B) Representative flow cytometry plot shown of the spleen. (C) Quantification of Ki67 staining in the lung, spleen, and DLN at 2 dpt (8 dpi) and 8 dpt (13 dpi). (D) Forward scatter and (E) ICOS and PD-1 expression of donor OT-II transferred to PR8-OVA_{II} infected hosts either 2 or 8 dpt. Representative data, n=3-5 each, 2 independent experiments, mean ± SD.

whether a short exposure of donor cells to Ag was sufficient to induce memory formation. I transferred 6 dpi OT-II donor cells to PR8-infected mice and tested if intravenously injected bone marrow-derived dendritic cells pulsed with OVA_{II}-peptide (DC-OVA_{II}) would be sufficient to restore memory formation. I found that these DC present Ag for no longer than 2 days after transfer *in vivo* by tracking their ability to induce proliferation of naïve OT-II cells (Figure 3.10A, 3.10B). Strikingly, the donor cells transferred to PR8-infected hosts that received DC-OVA_{II} were recovered at similar levels as those transferred to PR8-OVA_{II}-infected hosts out to 14 dpt (Figure 3.11A-3.11C). The kinetics of the donor cell response was very similar in the spleen and lung, with a slight reduction in the DLN of donor cells transferred to PR8-infected mice with DC-OVA_{II} (Figure 3.11C).

To examine if memory phenotype was altered when late Ag was provided by DC-OVA_{II} compared to virally produced Ag, I measured CD127, CXCR3 and TCF-1 expression. In the lung and spleen, both CD127 and CXCR3 expression were similar between cells in the PR8-OVA_{II} host and those in the PR8 host with DC-OVA_{II} (Figure 3.11D, 3.11E). However, both markers were increased in the memory cells formed in the DLN of the PR8-infected host with DC-OVA_{II}. Since fewer memory cells formed in these mice, it could be that the only cells that were able to survive were those with much higher memory cell markers. TCF-1, a transcription factor that has been shown to be required for memory cell formation in CD8 T cells (312), was similar between the two groups in all organs (Figure 3.11F). This indicates that encounter with cognate Ag for 48 hours or less, starting

Figure 3.10. Transferred OVA_{II}-DC only present Ag for 2 days *in vivo.*

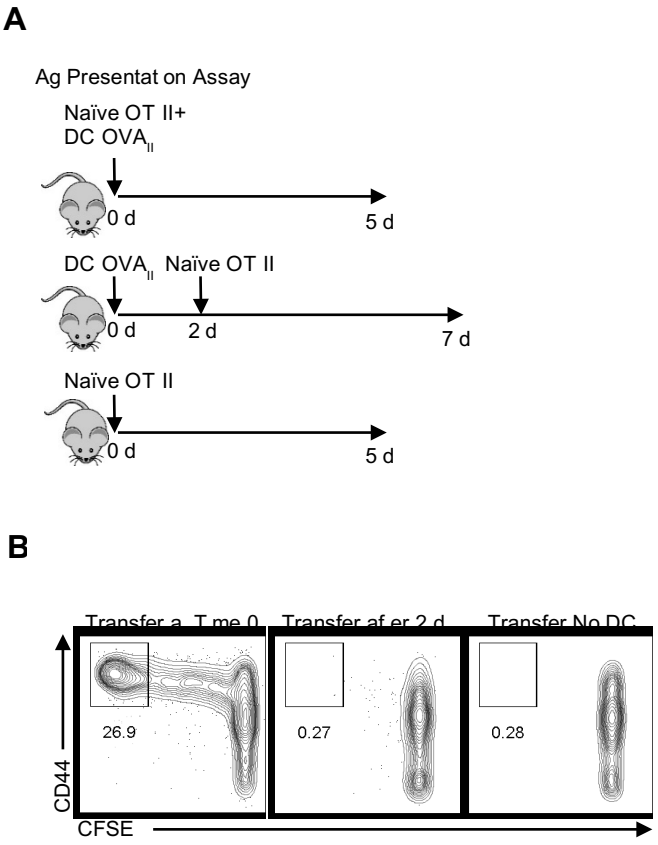


Figure 3.10. Transferred OVA_{II}-DC only present Ag for 2 days *in vivo.*

(A) Experimental schematic. $0.5x10^5$ OVA_{II}-pulsed BMDC were transferred to uninfected hosts. To determine how long BMDC present the OVA_{II}-peptide, naïve OT-II cells were CFSE labelled and transferred either at the same time as OVA_{II}-pulsed BMDC (time 0), 2 days after OVA_{II}-pulsed BMDC, or in the absence of OVA_{II}-pulsed BMDC. **(B)** Activation and proliferation were determined by analyzing CD44 expression and CFSE dilution by flow cytometry. Data is representative of an experiment with n=5.

Figure 3.11. Short-term Ag is sufficient to restore the CD4 T cell response.

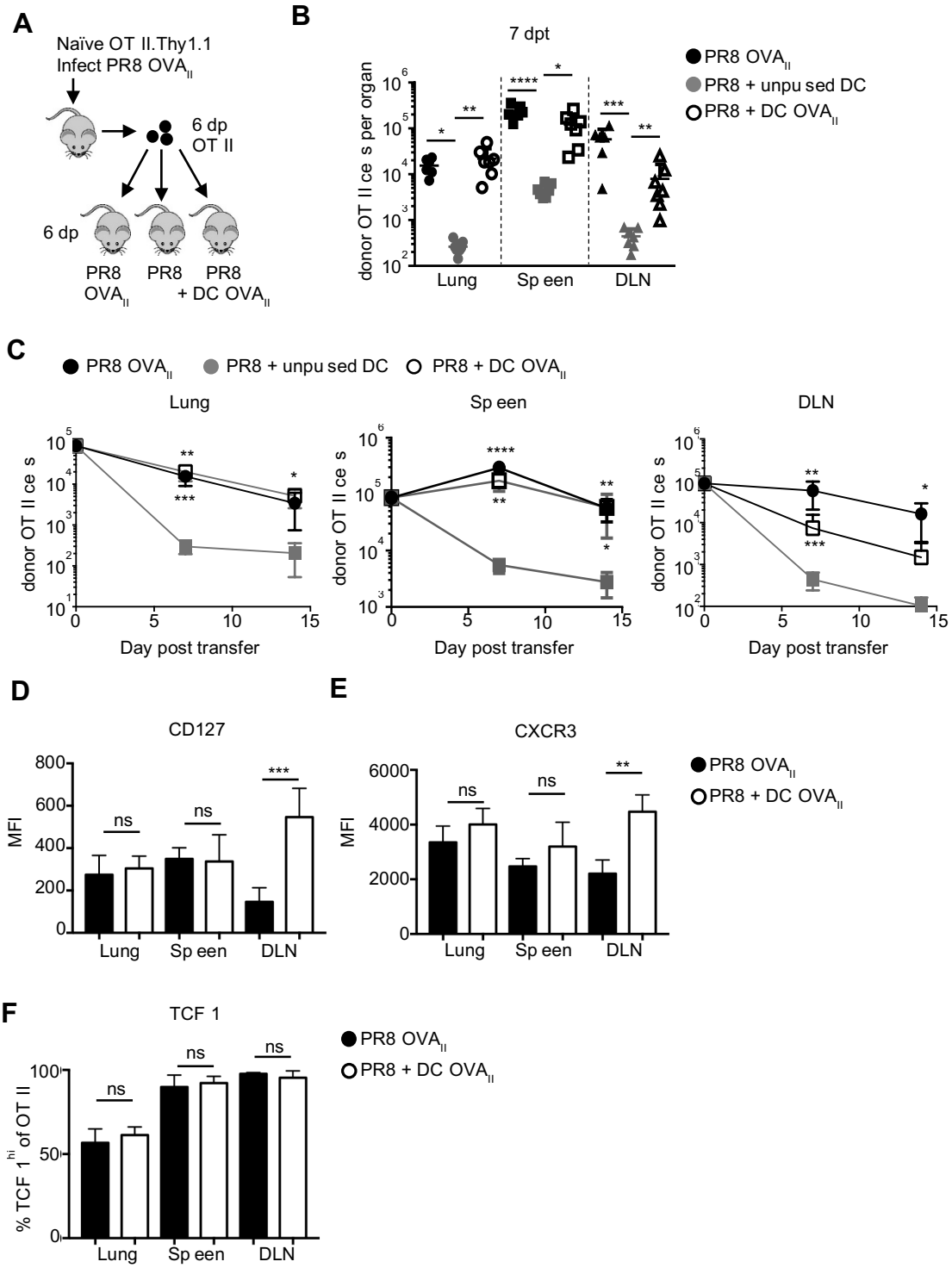


Figure 3.11. Short-term Ag is sufficient to restore the CD4 T cell response.

(A-F) $5x10^5$ naïve OT-II.Nur77^{GFP}.Thy1.1$^{+/}$ were transferred to B6 mice. Mice were infected with PR8-OVA_{II}. At 6 dpi, donor OT-II cells were isolated as described in Figure 3.5A, and transferred to kinetically-matched PR8-OVA_{II} or PR8 infected hosts along with $0.5x10^6$ BMDCs either pulsed with 10μM OVA_{II}-peptide or not. (A) Experimental schematic. (B) Donor cell recovery at 7 dpt. (C) Kinetics of cell recovery in the lung, spleen, and DLN. (D) CD127 (E) CXCR3 and (F) TCF-1 expression at 14 dpt (20 dpi). Data is representative of 2 individual experiments, n=3-5 each. Mean ± SD.

at 6 dpi, was sufficient to prevent excessive contraction and promote memory formation.

Short-term Ag Presentation at 6-8 dpi in the Absence of Viral Infection is Sufficient to Restore CD4 T cell Responses to IAV.

To determine if viral infection itself is important in promoting memory formation, I tested whether adding DC-OVA_{II} would similarly increase memory formation in uninfected hosts. 6 dpi effectors were transferred to PR8-infected hosts with DC-OVA_{II}, uninfected hosts with DC-OVA_{II}, or uninfected hosts with unpulsed DC (Figure 3.12A). Strikingly, DC-OVA_{II} strongly promoted donor recovery to a similar extent in PR8-infected and uninfected hosts (Figure 3.12B). Since Ag presentation only lasts for 2 days *in vivo,* donor cells appeared to have a resting phenotype by 7 dpt, having downregulated effector molecules PD-1 and ICOS compared to 6 dpi effectors (Figure 3.12C). Additionally, CD127 and CXCR3 expression was similar between donor cells in PR8-infected or uninfected hosts that received DC-OVA_{II} (Figure 3.12D). TCF-1 was also similar between donor cells formed with and without viral infection (Figure 3.12E). Since the TCF-1 expression appeared to be lower in the lung than the SLO, I included a comparison of host $CD4^{+}CD44^{h}CD62L^{h}$ memory cells to see if this level of TCF-1 expression was normal in lung memory cells. I found that host memory cells expressed similar levels of TCF-1 expression in both the lung and the spleen as did the donor OT-II cells (Figure 3.12E). This suggests that donor cells are phenotypically memory-

Figure 3.12. Short-term Ag can restore the CD4 T cell response in the absence of viral infection.

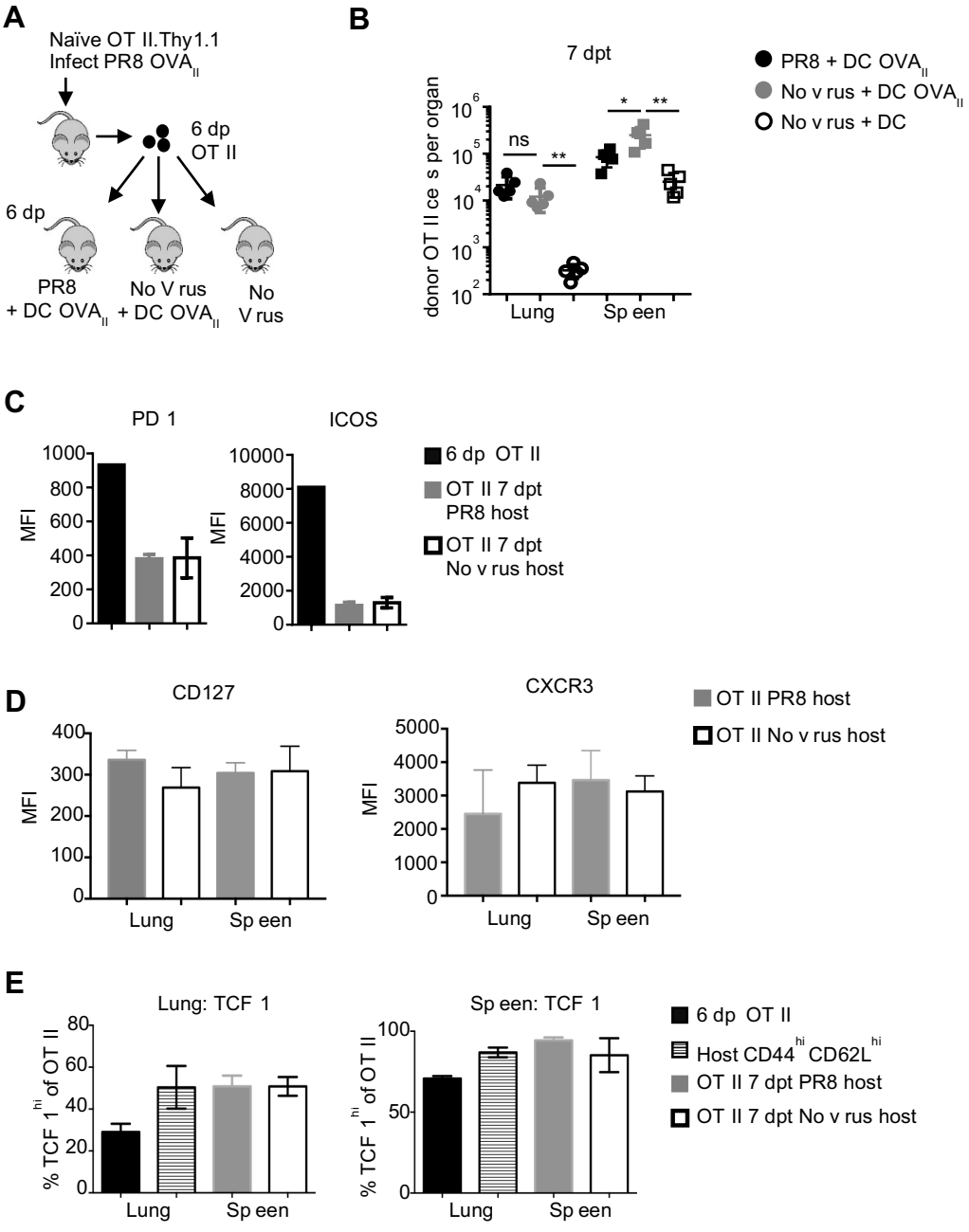


Figure 3.12. Short-term Ag can restore the CD4 T cell response in the absence of viral infection.

(A-E) 6 dpi OT-II effectors were isolated from PR8-OVA_{II}-infected mice and transferred to second hosts that were either infected with PR8 (6 dpi) or uninfected, along with BMDC that were pulsed with OVA_{II}-peptide or not. (A) Experimental schematic. (B) Cell recovery was assayed 7 dpt. (C) PD-1 and ICOS expression of donor cells at 7 dpt in the spleen compared to 6 dpi OT-II effectors. (D) CD127 and CXCR3 expression of donor cells in the spleen at 7 dpt. (E) TCF-1 expression of donor cells compared to host memory cells ($CD44^h$ $CD62L^h$) and 6 dpi OT-II effectors. All data is representative, n=3-5 each, 2-3 experiments, mean ± SD.

like by 7 dpt. Since it is unclear to what extent TCF-1 is required for tissue resident memory formation, it may be that memory cells in the lung do not express as much TCF-1 as those in the SLO. These data, combined with Figure 3.5, in which there was no difference in memory formation following transfer of 6 dpi effectors into PR8-infected and uninfected hosts, suggests that at this time Ag-independent aspects of infection, such as induction of lung inflammatory cytokines, have no discernable impact on memory formation. However, the DC I used were activated, so infection-induced viral-sensing pathways may be needed to activate *in situ* APC.

Although the use of DC-OVA_{II} to mimic the late Ag presentation of a viral infection appeared to replicate the CD4 T cell response well, it is possible that providing activated DC pulsed with cognate peptide might provide a non-physiological level of stimulation. To test this, I transferred 6 dpi effectors into infection matched PR8-OVA_{II}-infected hosts, PR8-infected hosts with DC-OVA_{II}, or uninfected hosts with DC-OVA_{II} and measured cell recovery at 2 dpt. If the DC-OVA_{II} provided a much larger boost of Ag than did viral infection, one would expect that the donor cells in the hosts with DC-OVA_{II} would be present at much greater numbers than the PR8-OVA_{II} host. However, I found that there were similar numbers of donor cells present in all three hosts in the lung, spleen, and DLN indicating that DC-OVA_{II} reliably replicate Ag presentation during viral infection (Figure 3.13).

Figure 3.13. DC-OVA$_{II}$ induces similar effector expansion as PR8-OVA$_{II}$ infection.

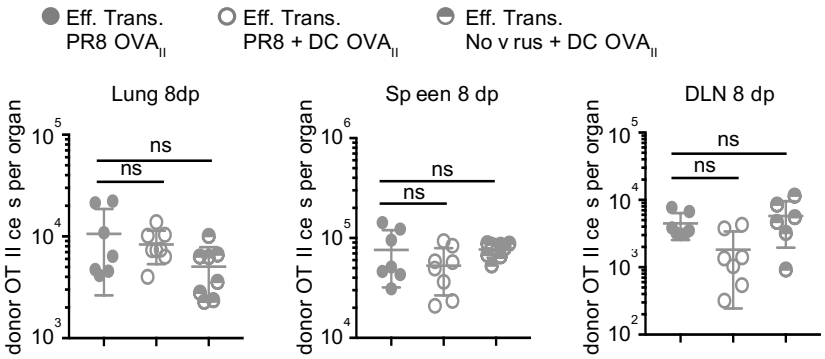


Figure 3.13. DC-OVA$_{II}$ induces similar effector expansion as PR8-OVA$_{II}$ infection.

6 dpi effectors were transferred to PR8-OVA$_{II}$-infected, PR8-infected hosts + DC-OVA$_{II}$, or uninfected hosts + DC-OVA$_{II}$. Lung, spleen, and DLN were harvested at 2 dpt. OT-II cell numbers are shown. Data is pooled from 2 independent experiments, n=7-8. Mean ± SD.

Short-term Ag is Sufficient to Restore Canonical Memory Formation.

I further examined if memory formation occurred normally when late Ag was provided by short-lived DC-OVA_{II}. I directly compared OT-II memory generated when naïve cells were transferred on day 0 and left in the same initial host (No Eff. Trans.) or when 6 dpi OT-II effectors were isolated and transferred (Eff. Trans.) to kinetically-matched PR8-OVA_{II}-infected, PR8-infected with DC-OVA_{II}, or uninfected hosts with DC-OVA_{II}. To highlight the changes that distinguish memory cells from effectors, I included 6 dpi OT-II effectors for comparison.

One functionally important characteristic of memory cells is their ability to produce multiple cytokines upon re-stimulation (2). I found that the memory cells generated following transfer (either to hosts with virally produced Ag or with short-lived Ag provided by DC-OVA_{II}) had regained the ability to produce multiple cytokines to a similar extent as those generated without transfer (Figure 3.14A, 3.13B). Additionally, memory cells generated both with and without transfer had upregulated the critical memory marker CD127 that is necessary for their persistence (Figure 3.14C). Interestingly, when comparing memory cells generated in PR8-infected or uninfected hosts with late transfer of DC-OVA_{II}, there was a decrease in CD127 expression in the uninfected hosts (Figure 3.14C). This suggests that although systemic virus-induced inflammatory cytokines may not be needed for memory cell numbers, function, or subset differentiation, it may be that virus-induced inflammatory cytokines are required for full CD127 upregulation.

Figure 3.14. Short-term Ag is sufficient to restore canonical memory formation.

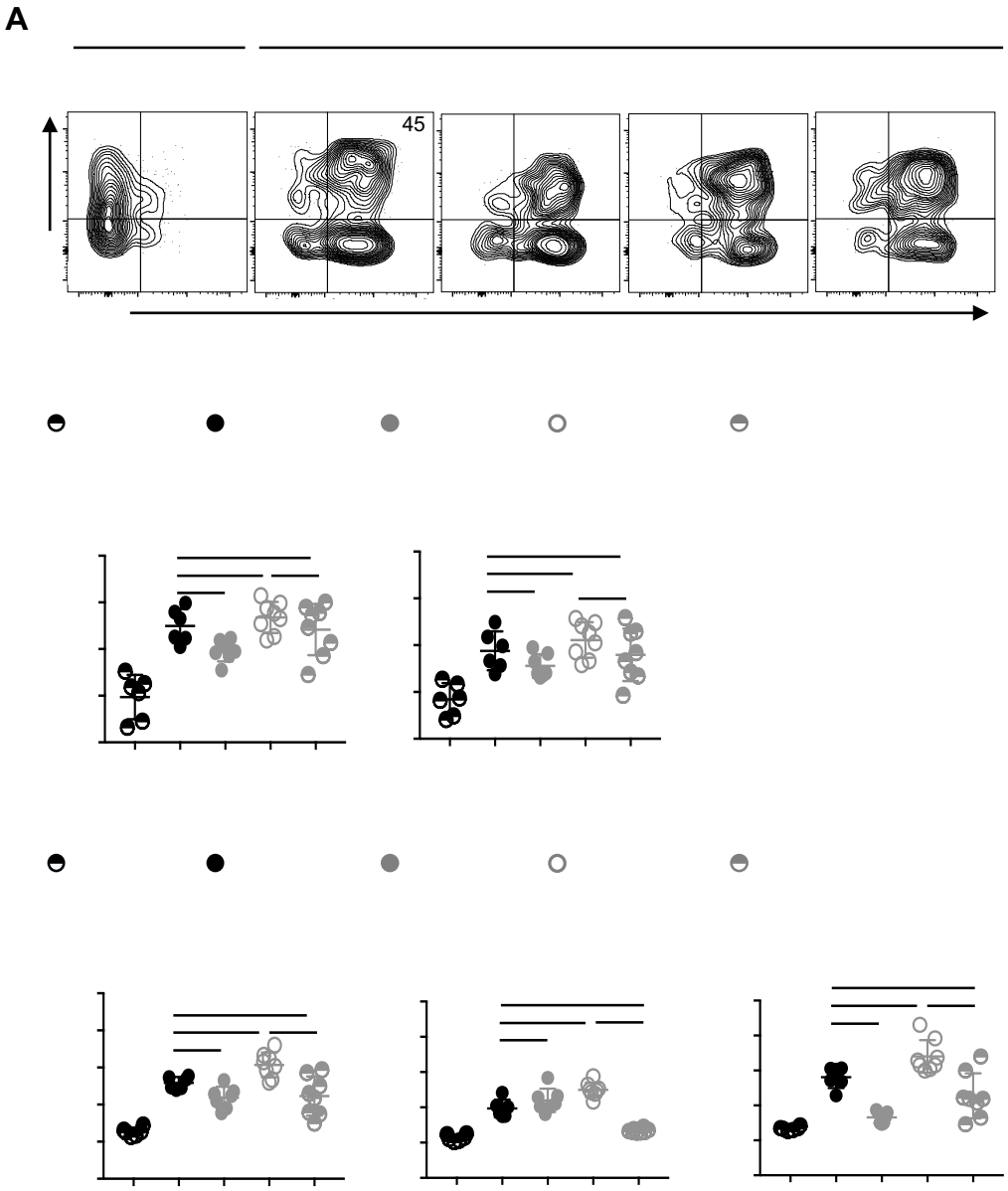


Figure 3.14. Short-term Ag is sufficient to restore canonical memory formation.

(A-C) Comparison of memory cells generated following transfer of naïve OT-II at day 0, or transfer of 6 dpi OT-II effectors into PR8-OVA_{II}-infected, PR8-infected with DC-OVA_{II}, or uninfected hosts with DC-OVA_{II}. Memory cells harvested 14 dpt (20 dpi) were compared to 6 dpi effectors generated *in vivo*. (A) Representative flow cytometry plots showing intracellular cytokine staining of IFNγ and TNFα following 4 hours of PMA + Ionomycin stimulation in memory or 6 dpi effector OT-II cells. (B) Percentage of IFNγ^+TNFα^+, IFNγ^+TNFα^+IL-2^+ producing cells in memory or effector OT-II populations in the spleen. (C) CD127 MFI of effectors and all memory groups in the lung, spleen, and DLN. Data is pooled, n=6-8, 2 experiments, mean ± SD.

However, the equivalent recovery of memory cells argues that DC-OVA_{II} exposure induced sufficient levels of CD127 for persistence.

I next examined CD4 memory subset differentiation. The tissue resident memory (T_{rm}) population identified by CD69 expression (5, 143) in the lung was similar with and without transfer (Figure 3.15A). IFNγ production, an indicator of Th1 differentiation (313, 314), was also produced to a similar extent in all memory groups (Figure 3.15A). CXCR5 has been shown to mark a memory subset that is thought to be T follicular helper (Tfh) or central memory (Tcm)-like (148, 149). CXCR5 expression was also similar among all memory groups (Figure 3.15B). Although there appeared to be a slight increase in CXCR5 expression in groups receiving DC-OVA_{II} in the spleen, this pattern was not found in the DLN. Therefore, the limited Ag provided by DC-OVA_{II} at 6 dpi appears to be sufficient to generate canonical memory formation.

Late Ag Promotes Survival of Responding CD4 T cells.

After viral clearance, most effector T cells undergo apoptosis leading to contraction, while a cohort survives to become memory. This suggests that avoiding apoptosis is a key step in the transition to memory. I therefore propose that a cohort of effector CD4 T cells recognize Ag/APC which drives them to make and respond to IL-2, which drives their survival and supports their transition to memory (1). I evaluated several components of this hypothesis.

Figure 3.15. Short-term Ag is sufficient to restore memory differentiation.

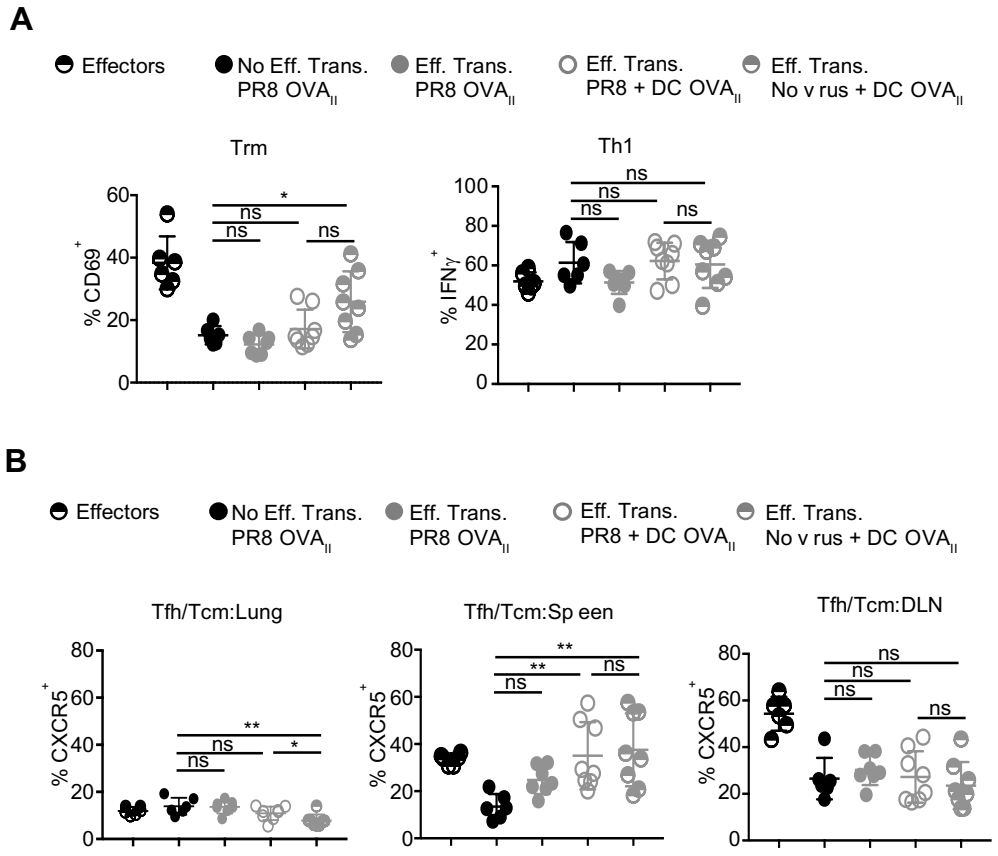


Figure 3.15. Short-term Ag is sufficient to restore memory differentiation.

(A, B) Comparison of memory cells generated following transfer of naïve OT-II at day 0, or transfer of 6 dpi OT-II effectors into PR8-OVA_{II}-infected, PR8-infected with DC-OVA_{II}, or uninfected hosts with DC-OVA_{II}. Memory cells harvested 14 dpt (20 dpi) were compared to 6 dpi effectors generated *in vivo*. (A) Quantification of Trm via CD69 expression (Lung), Th1 via IFNγ production (Spleen). (B) Quantification of Tfh/Tcm via CXCR5 expression of memory or effector OT-II cells in the lung, spleen, and DLN. Data is pooled, n=6-8, 2 experiments, mean ± SD.

To test if Ag recognition at the checkpoint promoted enhanced survival of effector CD4 T cells, I transferred naive OT-II.Nur77^{GFP} cells to hosts and infected with PR8-OVA$_{II}$. At 7 dpi, donors that had seen Ag during the first 1-2 days of the checkpoint are GFP$^+$ while those that did not, are GFP$^-$. I analyzed donor CD4 T cells from the lung, spleen, and DLN directly *ex vivo*, gating on GFP$^+$ and GFP$^-$ cells. To detect cell death directly *ex vivo* I measured 7-Aminoactinomycin D (7-AAD) staining (Figure 3.16). In each organ, 7-AAD staining was significantly greater in GFP$^-$ cells than in GFP$^+$ cells, indicating that more effector cells that recognized Ag between 5-6 dpi survived than those that did not recently encounter Ag.

To further dissect the mechanisms involved in this survival, I developed an *in vivo* to *in vitro* model to better control the signals that the effectors receive. I isolated 6 dpi OT-II effectors and co-cultured them with T-depleted splenocytes isolated from PR8-infected mice, a physiologically relevant APC, either with or without OVA$_{II}$ peptide. To mimic the short-term Ag presentation that occurs *in vivo*, I irradiated the APC, ensuring Ag presentation was restricted to the first 2 days of culture (Figure 3.17A) (245). As I found *in vivo,* in this *in vitro* model when I measured 7-AAD staining after 2 days in culture, I found that the cells that did not receive late Ag stimulation had significantly increased 7-AAD staining compared to the cells that received late Ag stimulation (Figure 3.17B).

To test if this was true of polyclonal 6 dpi IAV-specific CD4 T cells, I modified the *in vivo* to *in vitro* model by isolating total CD4 T cells at 6 dpi and culturing with

Figure 3.16. Recent Ag encounter correlates with reduced cell death *in vivo.*

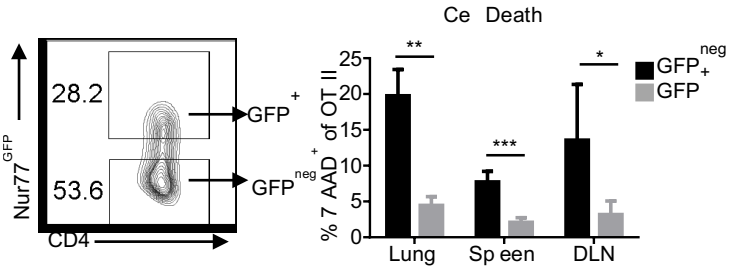


Figure 3.16. Recent Ag encounter correlates with reduced cell death *in vivo.*

OT-II.Nur77^{GFP}.Thy1.1$^{+/}$ naïve T cells were transferred to B6 mice followed by infection with PR8-OVA$_{II}$. Lung, spleen, and DLN were harvested 7 dpi. Left: Representative plot of GFP^{+} vs. GFP OT-II.Nur77^{GFP} cells. Right: Quantification of 7-AAD^{+} of GFP^{+} vs. GFP OT-II Nur77^{GFP} cells on 7 dpi. Representative data, 2 experiments, n=3-5 each, mean ± SD.

Figure 3.17. Short-term Ag at 6 dpi promotes cell survival.

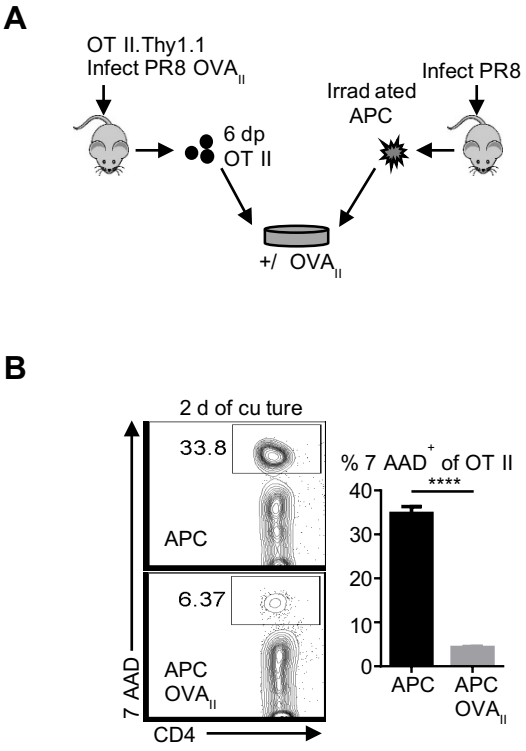


Figure 3.17. Short-term Ag at 6 dpi promotes cell survival.

(A,B) $5x10^5$ naïve OT-II.Thy1.1$^{+/}$ were transferred to B6 mice followed by infection with PR8-OVA_{II}. At 6 dpi, OT-II effectors were isolated and co-cultured with irradiated Thy-depleted splenocytes from infection-matched PR8-infected mice either with or without OVA_{II}-peptide. (A) Experimental schematic. (B) 7-AAD staining of OT-II cells after 2 days in culture. Left: Representative staining. Right: Quantification of 7-AAD$^+$ cells. All data is representative, n=3-4 each, 3 experiments, mean ± SD.

or without $NP_{311\ 325}$ peptide using *in vitro*-activated Thy-depleted splenocytes as APC (Figure 3.18A). After 6 days of culture, I found that polyclonal effectors that were exposed to late Ag were present at much higher levels than those that were not (Figure 3.18B, 3.18C). Similar to OT-II cells, when I measured 7-AAD staining, I found that cells that were cultured in the absence of late Ag had much greater cell death compared to those that were cultured with late Ag (Figure 3.18D). Interestingly, the MFI of the tetramer bound to cells that received late Ag was much higher than that of the cells that did not receive late Ag (Figure 3.18E). Since tetramer binding can be used to measure TCR affinity (315), this suggests that the presence of late Ag may have introduced some selection pressure favoring clones that recognized IA^b-$NP_{311\ 325}$ with greater avidity for enhanced survival (Figure 3.18E). However, I did not measure TCR expression which could also explain the differences in tetramer MFI. Although, after 6 days of culture, all cells are quiescent and are unlikely to have large differences in their TCR expression. Given that memory cells have increased functional avidity compared to effectors (304), it is an intriguing hypothesis that this late Ag might promote memory formation of effectors with high affinities.

To determine how Ag dose during this late time point impacted survival, I titrated the concentration of OVA_{II} peptide used to pulse *in vitro*-activated Thy-depleted splenocytes and measured cell recovery after 6 days of culture. Cell recovery appeared to plateau around 0.01μM of peptide (Figure 3.19). Given that naïve cells are generally stimulated with 10μM, 6 dpi effectors are more sensitive

Figure 3.18. Late Ag promotes survival of polyclonal CD4 T cells.

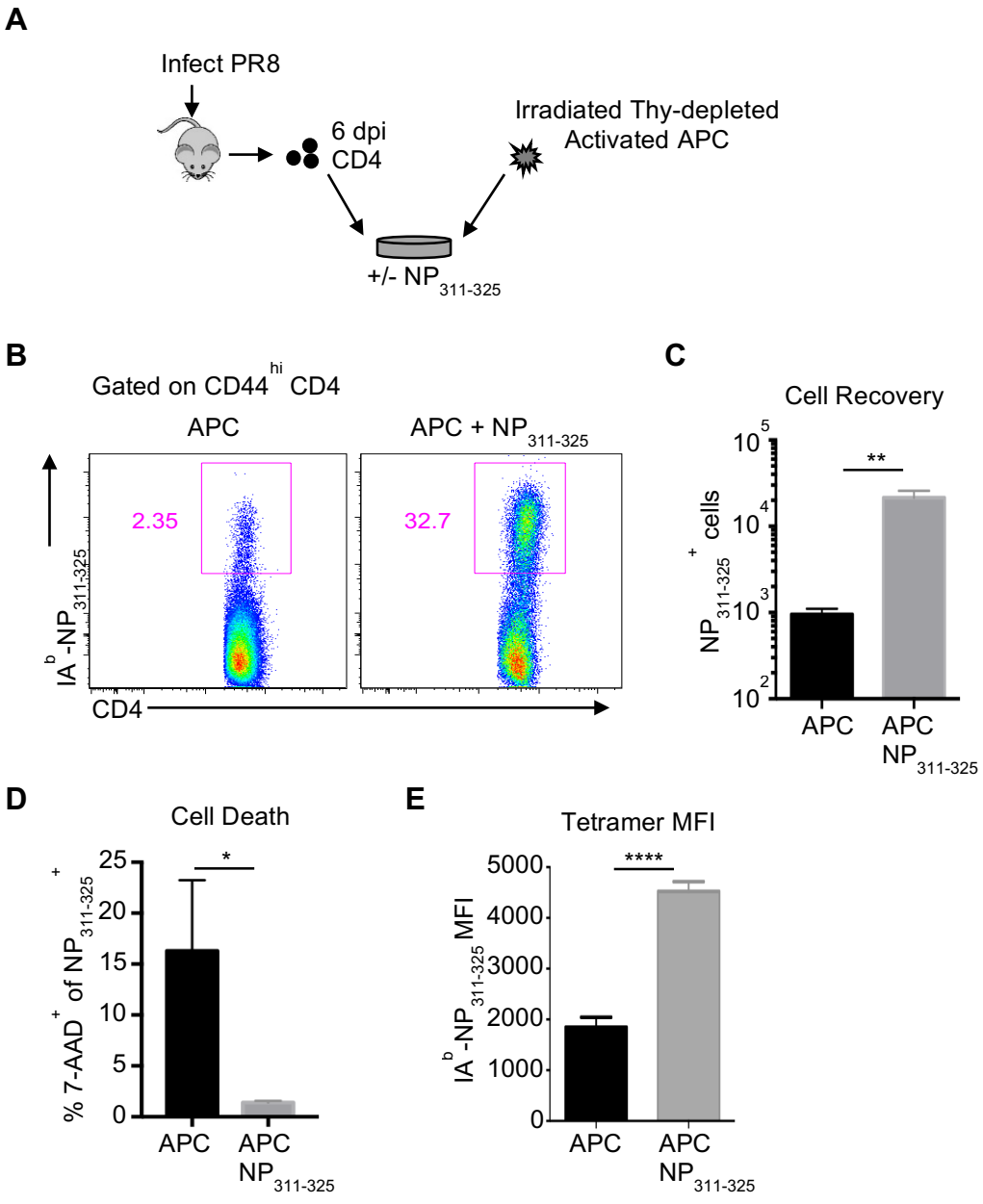


Figure 3.18. Late Ag promotes survival of polyclonal CD4 T cells.

(A-E) B6 were infected with a sublethal dose of PR8. At 6 dpi total CD4 T cells were isolated and co-cultured with irradiated APC (activated with LPS and dextran sulfate) with or without $NP_{311\ 325}$ peptide and cultured for 6 days. (A) Experimental schematic. (B) Representative flow cytometry plots of IA^b-$NP_{311\ 325}$ tetramer staining of $CD4^+CD44^h$ cells. (C) Cell recovery of IA^b-$NP_{311\ 325}$ tetramer positive cells. (D) Percentage 7-AAD^+ of $NP_{311\ 325}$-specific cells. (E) Tetramer MFI of IA^b-$NP_{311\ 325}$ tetramer positive cells with and without late Ag. Data is representative of 2 experiments, n=3 each, mean ± SD.

Figure 3.19. Increased cell recovery with increased Ag concentration.

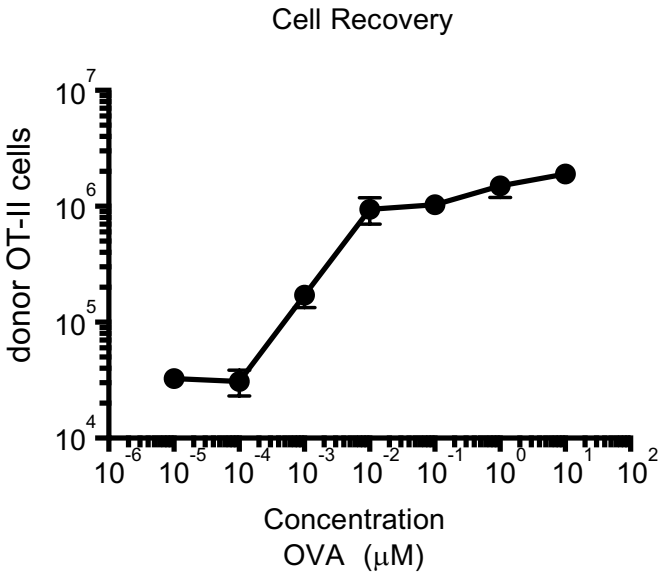


Figure 3.19. Increased cell recovery with increased Ag concentration.

6 dpi OT-II.Thy1.1$^{+/}$ effectors were isolated as in (Figure 3.17A) and cultured with irradiated, activated APC that were previously pulsed with varying concentrations of OVA_{II} peptide for 1 hour at 37 degrees Celsius. Cell recovery was measured by flow cytometry after 6 days of culture. Data is from an experiment with n=3, mean ± SD.

to lower doses of Ag. Additionally, it did not seem that higher concentrations of Ag were deleterious at this late time point (Figure 3.19).

Next I tested if the pro-survival effects of late Ag were sustained *in vitro.* The differences in cell recovery between cells that received late Ag for just the first 2 days of culture and those that had not were maintained out to 14 days of culture in both OT-II and polyclonal cells (Figure 3.20A, 3.20C). Additionally, although CD127 expression started out fairly similar between the two populations, the cells that received late Ag, for just the first 2 days of culture, gradually upregulated CD127, while those that did not receive late Ag failed to do so (Figure 3.20B, 3.20D). The differences in CD127 expression were most apparent after 14 days of culture, consistent with the idea that unlike CD8 T cells, primary CD4 T cells upregulate CD127 as they transition to memory (205). Thus, instead of re-encounter with Ag inducing widespread cell death, it promoted survival of *in vivo*-generated 6 dpi effector T cells.

Late Ag Promotes Survival of Responding CD4 T cells by Reducing Bim Expression.

To determine what might be responsible for the survival of cells exposed to late Ag, I performed a microarray experiment on 6 dpi effectors cultured for 2 days either with αCD3 and αCD28 or without and compared the gene expression of those two groups with that of the population of 6 dpi effectors I plated. A few apoptosis-related genes were upregulated above the 2-fold cutoff in the media

Figure 3.20. Short-term Ag promotes long term survival and upregulation of CD127 in OT-II and polyclonal CD4 T cells.

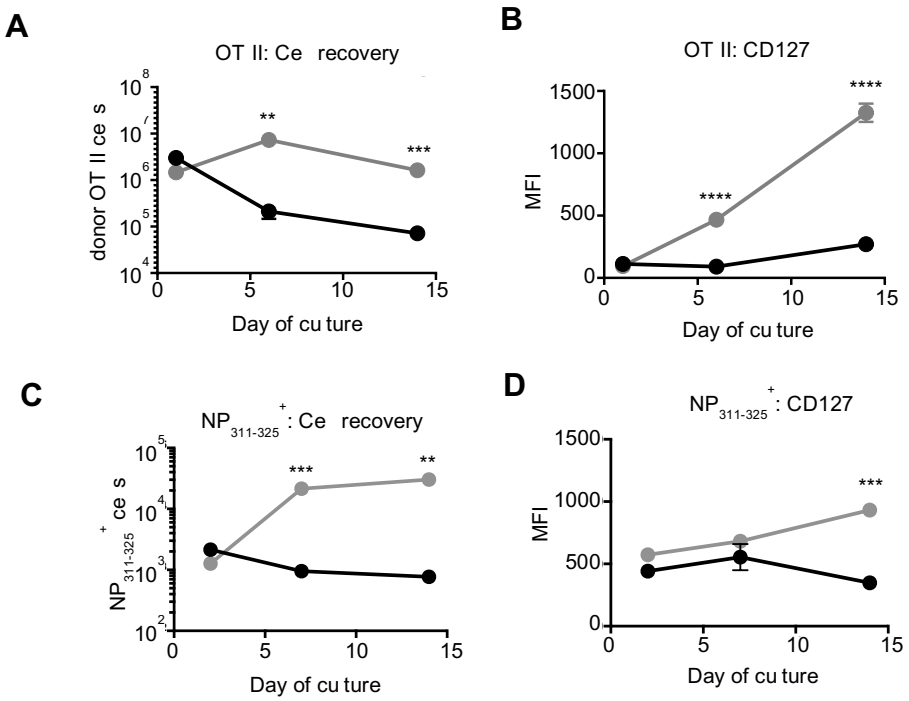


Figure 3.20. Short-term Ag promotes long term survival and upregulation of CD127 in OT-II and polyclonal CD4 T cells.

(A-B) 6 dpi OT-II.Thy1.1$^{+/}$ effectors were isolated and co-cultured with irradiated APC with or without OVA_{II} peptide as in (Figure 3.17A). Cell recovery (A) and CD127 expression (B) were measured after 1, 6, and 14 days of culture. **(C-D)** Total CD4 T cells were isolated at 6 dpi and co-cultured with irradiated APC with or without $NP_{311\ 325}$ peptide as in (Figure 3.18A). Cell recovery (C) and CD127 expression (D) were measured after 2, 6, and 14 days of culture. Data is representative of 2-3 experiments, n=3 each, mean ± SD.

alone group (Figure 3.21A). One of the genes that came up was *Bcl2l11* (which encodes Bim), a pro-apoptotic protein known to mediate cell death during T cell contraction (171, 190). When I measured Bim protein expression following 2 days of culture with or without late Ag/APC, I found that cells cultured without Ag had significantly increased Bim expression (Figure 3.21B).

I next tested if the reduced level of Bim seen in the Ag-exposed effectors was responsible for their increased survival. I co-transferred WT GFP *Bcl2l11*$^{+/+}$ or *Bcl2l11*$^{+/}$ [which express half the WT levels of Bim (187)] OT-II cells mixed at a 1:1 ratio into B6.Thy1.1$^{+/}$ mice and infected with PR8-OVA$_{II}$. I harvested total effector CD4 T cells at 6 dpi and stimulated them *ex vivo* with APC with or without OVA$_{II}$-peptide (Figure 3.21C). *Bcl2l11*$^{+/}$ OT-II and WT OT-II were still present at similar ratios at 6 dpi, indicating that *Bcl2l11*$^{+/}$ OT-II were expanded normally (Figure 3.21D). Additionally, both WT OT-II and *Bcl2l11*$^{+/}$ OT-II produced similar levels of IFNγ upon *ex vivo* re-stimulation at 6 dpi, suggesting that *Bcl2l11*$^{+/}$ OT-II were activated and functional (Figure 3.21E).

Next I measured cell recovery after 14 days of culture. I found that when no Ag was present *in vitro*, the *Bcl2l11*$^{+/}$ OT-II cells survived much better than WT OT-II cells (Figure 3.21F), implicating high levels of Bim in the death and contraction of 6 dpi effectors in the absence of Ag. In contrast, in the presence of Ag, the *Bcl2l11*$^{+/}$ OT-II and WT OT-II cells survived comparably (Figure 3.21F), consistent with the hypothesis that Ag acts to counteract apoptosis by causing Bim reduction. Indeed, in the absence of Ag, the *Bcl2l11*$^{+/}$ OT-II cells expressed less

Figure 3.21. Survival following late Ag stimulation is mediated by Bim reduction.

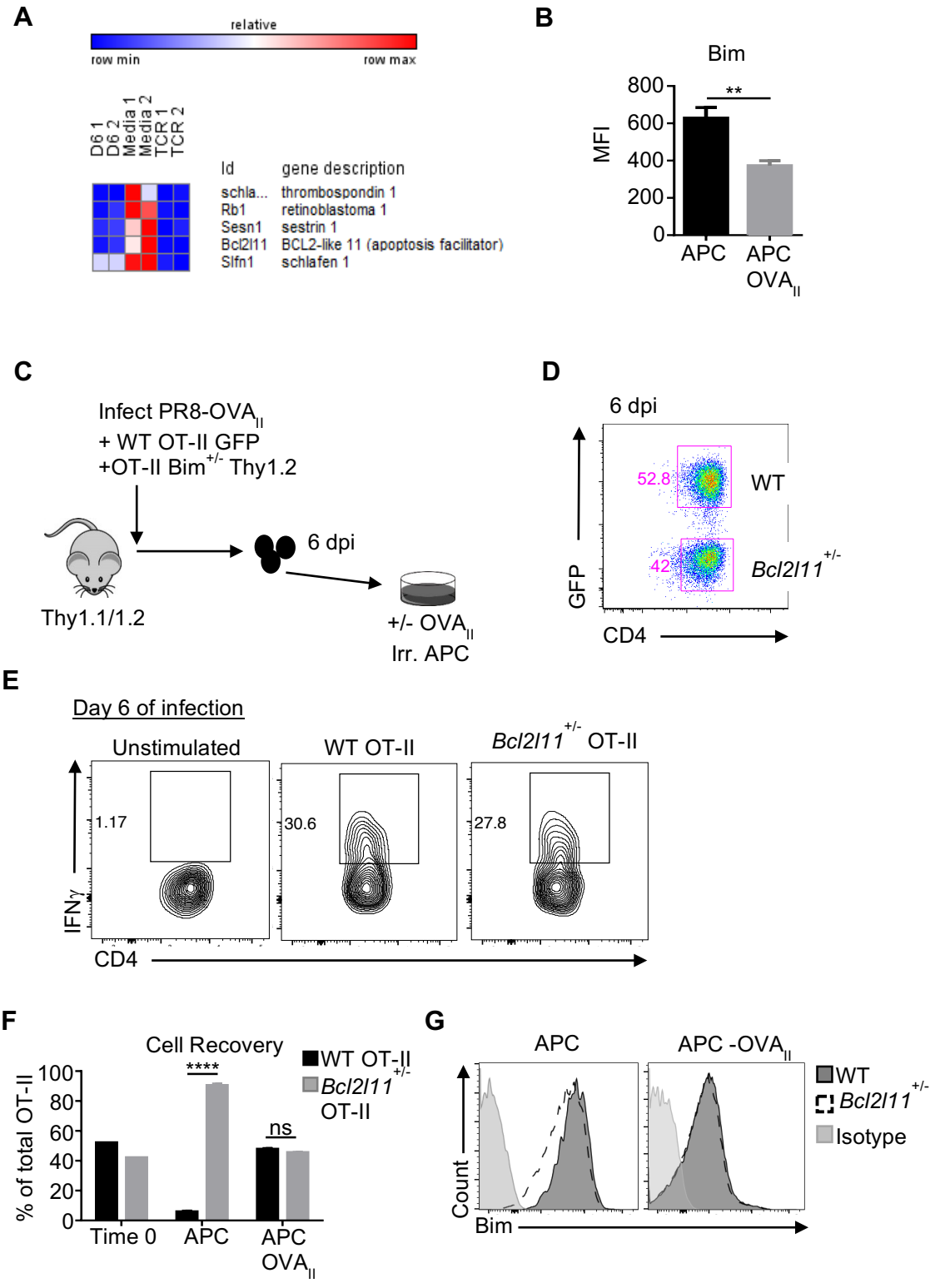


Figure 3.21. Survival following late Ag stimulation is mediated by Bim reduction.

(A) 6 dpi OT-II.Thy1.1$^{+/}$ effectors were isolated as in (Figure 3.17A), and cultured with 1μg/ml αCD3 and 5μg/ml αCD28 or in media alone for 2 days. Live OT-II.Thy1.1$^{+/}$ cells were then sorted and RNA was isolated from the media alone group, the TCR stimulated group, and sorted 6 dpi effectors. Microarray analysis was then performed on each sample. Shown are the apoptosis-related genes that were increased over 2-fold in the media alone group over both the TCR and 6 dpi effector groups. **(B)** 6 dpi OT-II.Thy1.1$^{+/}$ effectors were isolated and co-cultured with irradiated APC with or without OVA_{II} peptide as in (Figure 3.17A). After 2 days in culture, Bim protein expression was measured by flow cytometry. **(C-G)** WT GFP or *Bcl2l11*$^{+/}$ OT-II cells were mixed at a 1:1 ratio and co-transferred into B6.Thy1.1$^{+/}$ mice followed by infection with PR8-OVA_{II}. 6 dpi effectors were isolated and cultured with APC with and without OVA_{II} peptide. (C) Experimental schematic. (D) Representative flow cytometry plot showing the percentage of WT and *Bcl2l11*$^{+/}$ OT-II cells at 6 dpi, gated on donor cells. (E) IFNγ production of donor WT and *Bcl2l11*$^{+/}$ OT-II in the spleen at 6 dpi after 4 hours of αCD3 and αCD28 stimulation. (F) Relative recovery of WT or *Bcl2l11*$^{+/}$ OT-II cells after 14 days of culture. (G) Bim expression of WT and *Bcl2l11*$^{+/}$ OT-II after 2 days of culture. (A) Data is pooled from 2 independent experiments. (B-G) Data is representative of 3 experiments, n=3 each, mean ± SD.

Bim than WT, but with Ag, Bim levels were similar (Figure 3.21G). This supports the hypothesis that Ag recognition by effectors at the checkpoint acts in part through reduction of Bim expression, which prevents apoptosis and promotes survival.

IL-2 is Required for the Pro-survival Effects of Late Ag.

Since our previous studies found that autocrine IL-2 was required for CD4 effector survival (1), I tested whether Ag stimulation of 6 dpi effectors *ex vivo* would promote IL-2 production and if that IL-2 was necessary for enhanced survival. Indeed, the *ex vivo* effector CD4 T cells produced IL-2 only when cultured with Ag/APC (Figure 3.22A). I cultured 6 dpi effectors with APC, Ag/APC, or Ag/APC plus Ab specific for both CD25 (IL-2Rα) and CD122 (IL-2Rβ) to block IL-2 function. The exposure to Ag/APC enhanced donor cell recovery after 6 days, and blocking IL-2 signaling reduced that recovery (Figure 3.22B). Notably, blocking IL-2 only slightly inhibited Ag/APC-induced proliferation (Figure 3.22C), but dramatically increased cell death as measured by 7-AAD staining (Figure 3.22D).

Next, I stained Bim after 2 days of culture to see if IL-2 aided in the Bim reduction seen in groups receiving late Ag. I found that blocking IL-2 did significantly increase Bim expression (Figure 3.22E). Finally, given the role of IL-2 signaling in promoting CD127 expression (1, 206), I measured CD127 expression after 14 days of culture. As expected, blocking IL-2 significantly reduced CD127 expression following late Ag recognition (Figure 3.22F). Thus, *in vitro* Ag/APC

stimulation of 6 dpi effectors induces IL-2 production that prevents apoptosis and enhances the survival necessary for memory formation. Partial effects seen on cell recovery (Figure 3.22B), cell proliferation (Figure 3.22C), and upregulation of CD127 (Figure 3.22F) imply that factors beyond IL-2 also play a role in the effects of Ag seen at the late checkpoint.

To determine if polyclonal cells are also dependent on IL-2 signaling for survival, I used the same experimental setup as in Figure 3.22 using total CD4 T cells isolated at 6 dpi. I found significantly reduced cell recovery after 6 days of culture in cells receiving late Ag where IL-2 signaling was blocked (Figure 3.23A, 3.23B). As I saw with OT-II cells, cell recovery was not completely inhibited when IL-2 was blocked suggesting that other factors likely have a role in promoting survival at this late time point. Proliferation of polyclonal cells did seem to be a bit more impacted when IL-2 was blocked than I saw with OT-II cells (Figure 3.23C). Interestingly, in the absence of late Ag, polyclonal cells underwent significantly more proliferation than do OT-II cells in the absence of late Ag. Given that the polyclonal cells identified by tetramer are composed of clones of varying affinities they are likely not as synced as transgenic cells. Therefore, it is possible that some clones may be still undergoing some “autopilot” proliferation. Additionally, blocking IL-2 led to increased cell death, though not as robust as in OT-II cells (Figure 3.23D).

When I stained for Bim after 2 days of culture, I found that while late Ag stimulation did result in a reduction in Bim, the level of Bim expression was not

Figure 3.22. IL-2 is required for the pro-survival effects of late Ag.

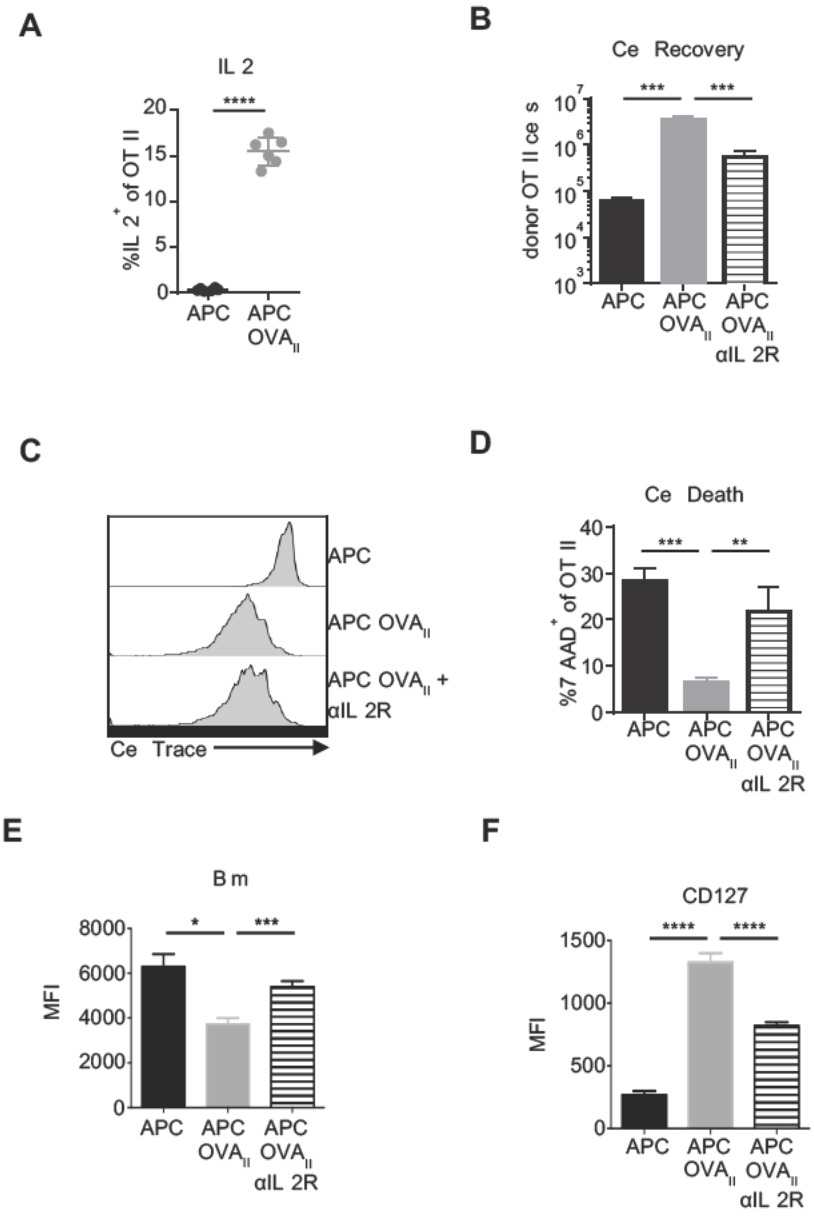


Figure 3.22. IL-2 is required for the pro-survival effects of late Ag.

(A) 6 dpi OT-II.Thy1.1$^{+/}$ effectors were isolated and co-cultured with irradiated APC with or without OVA_{II} peptide as in (Figure 3.17A). (A) After 4 hours of culture IL-2 production was assayed by intracellular staining. **(B-F)** 6 dpi effectors were co-cultured with APC without Ag, with Ag, or with Ag plus αCD25 + αCD122. Cells were stained with CTV. (B) Cell recovery was determined after 6 days of culture. (C) Dilution of CTV after 2 days of culture. (D) 7-AAD^{+} after 14 days of culture. (E) Bim expression after 2 days of culture. (F) CD127 expression after 14 days of culture. All data is representative, n=3-4 each, 3 experiments, mean ± SD.

affected by blocking IL-2 (Figure 3.23E). Blocking IL-2, however, did substantially reduce CD127 expression after 14 days of culture (Figure 3.23F). This was in contrast to the OT-II results (Figure 3.22E) suggesting that in the more heterogeneous polyclonal population IL-2 may have less of a dominant role. The fact that blocking IL-2 did not completely diminish the increased cell survival following late Ag also suggests that other factors may play a role.

Co-stimulation is Required for the Pro-survival Effects of Late Ag.

Although naïve T cells require co-stimulation for effective stimulation, it was unclear if effector CD4 T cells had a similar requirement. To test this, I isolated 6 dpi OT-II effectors and co-cultured with irradiated Thy1-depleted splenocytes from PR8-infected mice in the presence or absence of OVA_{II}. I included conditions with OVA_{II} and blocking Ab against CD80, CD86, CD70, 41BBL, OX40, CD40, or isotype controls. I found that after 6 days of culture, cell recovery was significantly reduced in groups where CD86 was blocked (Figure 3.24A). Blocking CD40 led to a consistent decrease in cell recovery across experiments but it did not reach statistical significance. Additionally, cell death indicated by 7-AAD staining was increased most substantially in the groups where CD86 was blocked, but it was also increased in groups where OX40L and CD40 were blocked (Figure 3.24B).

To further examine the role of co-stimulation during this late checkpoint, I isolated 6 dpi effectors and co-cultured with irradiated splenocytes from PR8-infected mice *in vitro* with or without Ag, or with Ag plus CD80 and CD86 blocking

Figure 3.23. IL-2 is required for the pro-survival effects of late Ag in polyclonal cells.

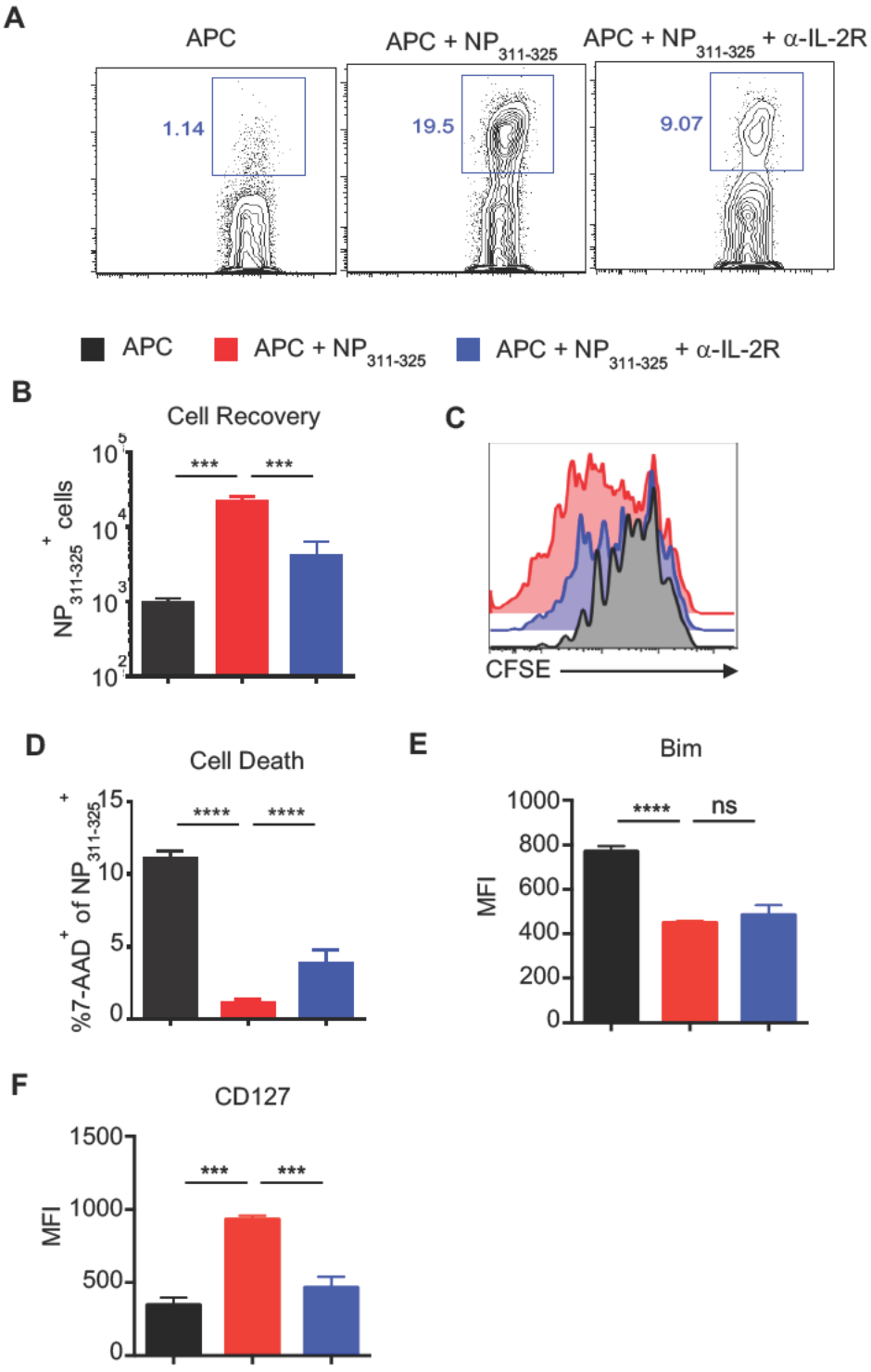


Figure 3.23. IL-2 is required for the pro-survival effects of late Ag in polyclonal cells.

(A-F) Total CD4 T cells were isolated at 6 dpi and co-cultured with irradiated APC with or without $NP_{311\ 325}$ peptide as in (Figure 3.18A) including a condition with Ag plus αCD25 + αCD122. Cells were stained with CFSE. (A) Representative flow cytometry plots of IA^b- $NP_{311\ 325}$ tetramer staining after 6 days of culture. (B) Cell recovery after 6 days of culture. (C) CFSE dilution of $NP_{311\ 325}$-specific cells after 6 days in culture. (D) Percentage of $NP_{311\ 325}$-specific cells that were 7-AAD^+ after 6 days of culture. (E) Bim expression of $NP_{311\ 325}$-specific cells after 6 days of culture. (F) CD127 expression of $NP_{311\ 325}$-specific cells after 14 days of culture. Data is representative of 2 experiments, n=3 each, mean ± SD.

Figure 3.24. CD86 co-stimulation promotes survival during late Ag stimulation.

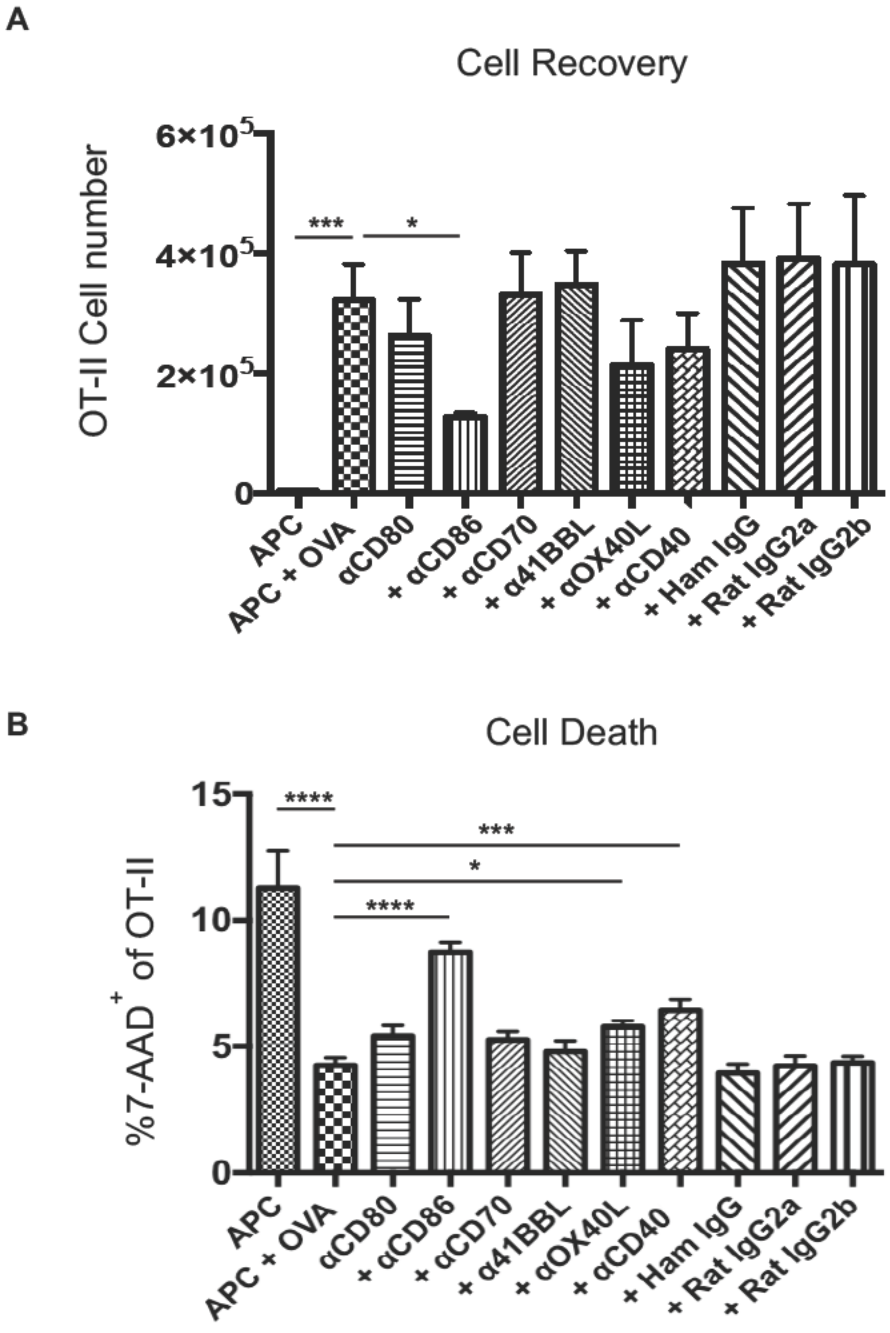


Figure 3.24. CD86 co-stimulation promotes survival during late Ag stimulation.

(A, B) 6 dpi OT-II.Thy1.1$^{+/}$ effectors were isolated and co-cultured with irradiated APC with or without OVA_{II} peptide as in (Figure 3.17A), including conditions in which OVA_{II} peptide was provided in addition to blocking Ab against CD80, CD86, CD70, 41BBL, OX40L, CD40 or isotype controls. Cells were cultured for 6 days. (A) Cell recovery is shown. (B) Cell death as measured by 7-AAD staining is shown. Data is representative of 2 experiments, n=3 each, mean ± SD.

Ab. I chose to block both CD80 and CD86 since both are ligands for CD28 co-stimulation. Again, after 6 days of culture there was a significant reduction in cell recovery when CD28 co-stimulation was blocked (Figure 3.25A). This did not appear to be due to proliferation, as cells stimulated with Ag proliferated to a similar extent whether or not they received CD28 co-stimulation after 2 days of culture (Figure 3.25B). However, when CD28 co-stimulation was blocked, cell death was significantly increased as measured by 7-AAD staining (Figure 3.25C). Given that CD28 stimulation can promote IL-2 production, I stained for IL-2 after 4 hours of culture and found that blocking CD28 co-stimulation did result in a decrease in the amount of IL-2 produced (Figure 3.25D). Since blocking CD28 co-stimulation did not result in a complete abrogation of IL-2 production, it is likely that it has IL-2 independent effects on survival. These findings suggest that co-stimulation at this late time has minimal if any impact on proliferation of effectors, but has a large impact on cell survival following late Ag stimulation.

Ag Recognition at the Effector Phase Promotes the Immediate Expression of Molecules Linked to Memory Formation.

Since Ag/APC exposure of 6 dpi effectors at the effector phase promotes the formation of a larger cohort of memory cells, as opposed to driving terminal differentiation, I tested if it also promoted expression of known memory-associated markers. I transferred OT-II.Nur77^{GFP}.Thy1.1$^{+/}$ naive cells to hosts, infected with PR8-OVA$_{II}$, and harvested lung, spleen, and DLN at 5,7, and 9 dpi. I analyzed

Figure 3.25. CD86 co-stimulation promotes survival during late Ag stimulation and reduces IL-2 production.

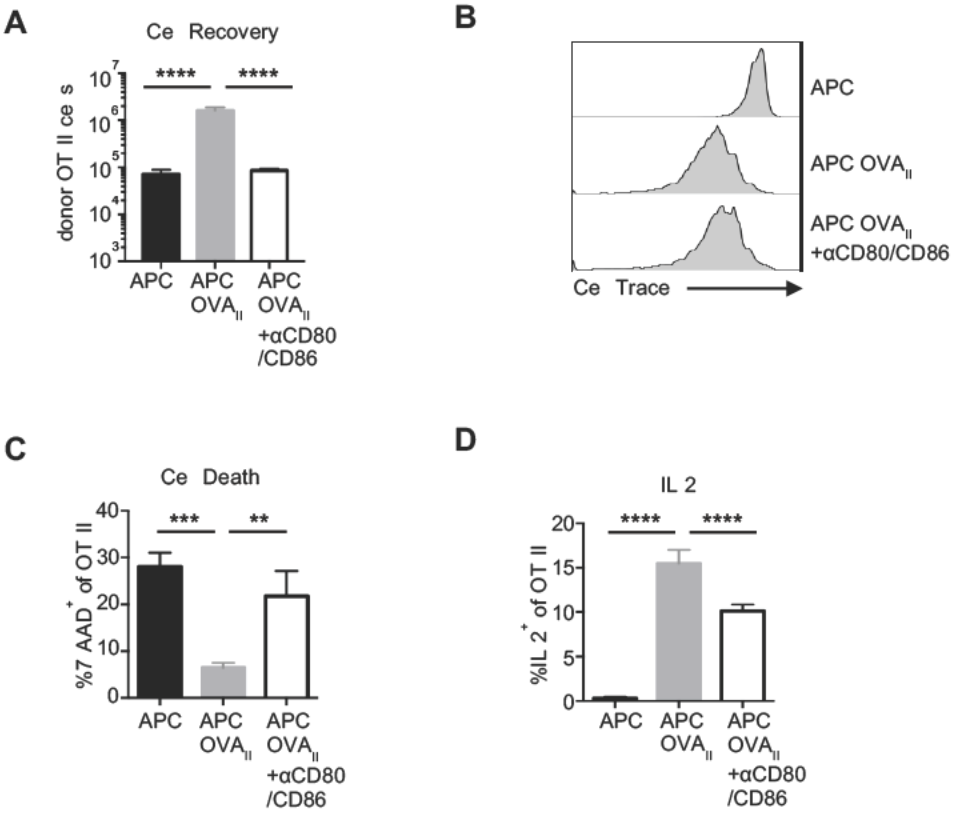


Figure 3.25. CD86 co-stimulation promotes survival during late Ag stimulation and reduces IL-2 production.

(A-D) 6 dpi OT-II.Thy1.1$^{+/}$ effectors were isolated and co-cultured with irradiated APC with or without OVA_{II} peptide as in (Figure 3.17A). A condition was included with OVA_{II} peptide and αCD80 and αCD86 blocking Ab. Cells were stained with cell trace violet (CTV) (A) Cell recovery after 6 days of culture. (B) Dilution of CTV after 2 days of culture. (C) Cell death as measure by 7-AAD staining after 2 days of culture. (D) IL-2 production after 4 hours of culture. Data is representative of 3 experiments, n=3 each, mean ± SD.

donor GFP^+ cells (recent Ag exposure) vs. GFP cells (no recent Ag exposure). I found that GFP^+ cells expressed higher levels of CD25 at 5-7 dpi in the lung compared to GFP cells (Figure 3.26A). The timing of this CD25 expression matches when IL-2 signals are required for CD4 memory formation (1). Additionally, CD27, a costimulatory molecule thought to be important for memory formation (1), was increased in GFP^+ cells in the lung at 7 dpi (Figure 3.26B). Other effector markers such as PD-1 were not different between GFP^+ and GFP cells in the lung at any time point (Figure 3.26C). This confirms that both GFP^+ and GFP effectors are all fully activated at these time points. Both CD25 and CD27 were also upregulated in GFP^+ polyclonal $NP_{311\ 325}{}^+$ cells in the lung at 7 dpi in $Nur77^{GFP}$ mice (Figure 3.26D). Although the CD25 upregulation was no longer apparent in OT-II cells by 7 dpi, there was still a difference in $NP_{311\ 325}{}^+$ cells. By harvesting at 7 dpi, I may have missed the peak of CD25 expression in the polyclonal population. However, at earlier time points tetramer detection of polyclonal IAV cells in the lung is very difficult. Further, I found that following 2 days of culture using the *in vivo* to *in vitro* system described in Figure 3.17 and Figure 3.18, CD25 expression was increased in both OT-II and polyclonal $NP_{311\ 325}{}^+$ cells (Figure 3.26E).

At 7 dpi, both GFP^+ OT-II and polyclonal $NP_{311\ 325}{}^+$ cells in lung, spleen and DLN also expressed higher levels of Bcl-6, a transcription factor implicated in memory formation (232, 235) (Figure 3.27A, 3.27C). In contrast, expression of T-bet, a transcription factor thought to promote terminal differentiation (205, 285, 286), was equivalently expressed between GFP^+ and GFP cells in both OT-II and

Figure 3.26. CD25 and CD27 upregulated in following late Ag encounter in the lung.

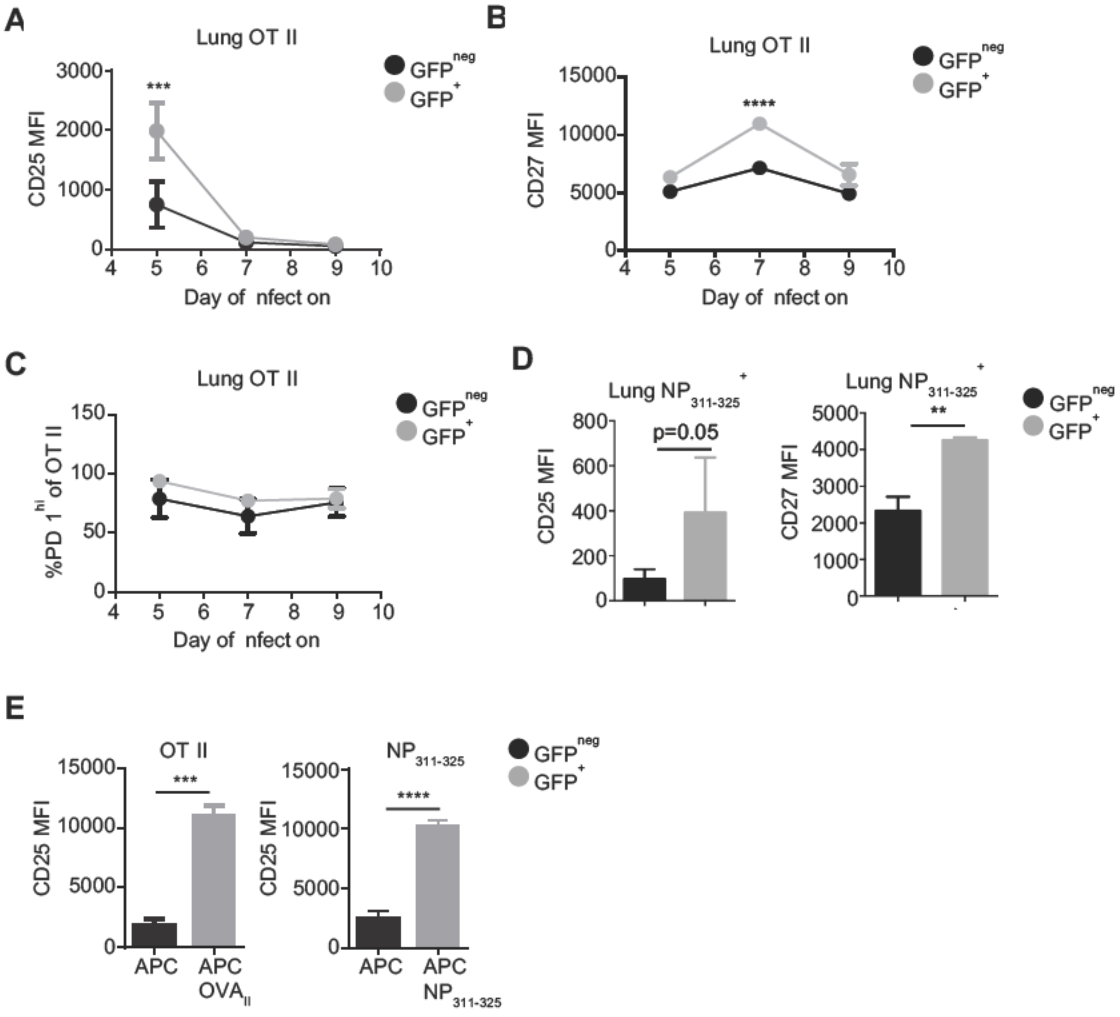


Figure 3.26. CD25 and CD27 upregulated in following late Ag encounter in the lung.

(A-C) $5x10^5$ naïve OT-II.Nur77^{GFP} were transferred to B6 mice. Mice were infected with PR8-OVA_{II}. Lung cells were harvested at 5, 7, and 9 dpi. GFP^+ and GFP donor cells were analyzed for CD25 expression (A), CD27 expression (B), and PD-1 expression (C). **(D)** Nur77^{GFP} mice were infected with PR8-OVA_{II}. At 7 dpi, GFP^+ and GFP $NP_{311\ 325}$-specific cells were analyzed for CD25 and CD27 expression in the lung. **(E)** 6 dpi OT-II.Thy1.1$^{+/}$ effectors or total CD4 T cells were isolated and co-cultured with irradiated APC with or without OVA_{II} or $NP_{311\ 325}$ peptide respectively as in (Figure 3.17A, 3.18A). After 2 days of culture, CD25 expression was measured in OT-II (left) and $NP_{311\ 325}$-specific cells (right). Data is representative of 3-4 experiments, n=3-5 each, mean ± SD. Data is representative of 3 experiments, n=3 each, mean ± SD.

polyclonal cells (Figure 3.27B, 3.27D). I confirmed the upregulation of Bcl-6 following short-term late Ag presentation using the *in vivo* to *in vitro* model described in Figure 3.17 and Figure 3.18. After 2 days of culture, Ag/APC had induced both OT-II and NP_{311}^{+} cells to upregulate Bcl-6 compared to culture with APC alone (Figure 3.28A).

Although, Bcl-6 expression is promoted by phosphorylated STAT3 (pSTAT3) (316) and IL-2 is not generally thought of as a STAT3 activating cytokine, it has been shown to promote phosphorylation of STAT3 (317, 318). I therefore tested if blocking IL-2 effected Bcl-6 expression after 2 days of culture. I found that blocking IL-2 had no effect on Bcl-6 expression (Figure 3.28B). This finding is not surprising given their largely antagonistic nature (227, 235). In addition to promoting Bcl-6 expression, pSTAT3 also promotes CD8 memory T cell formation (316, 319). I tested if late Ag promoted STAT3 phosphorylation and found pSTAT3 was indeed substantially increased following Ag stimulation of 6 dpi effectors after 4 hours of culture (Figure 3.28C). IAV-specific CD4 T cells are known to produce high levels of IL-10 during the effector stage (4).

Additionally, IL-10 is known to induce STAT3 phosphorylation (319, 320). Therefore, I tested if blocking IL-10 would have an effect on cell survival following late Ag. I found that while polyclonal CD4 T cells had reduced cell survival after 6 days of culture when IL-10 was blocked, OT-II cells did not (Figure 3.28D, 3.28E). Since the polyclonal culture contained all CD4 T cells isolated at 6 dpi, while the OT-II culture contained only donor OT-II cells, it is likely that the polyclonal

Figure 3.27. Bcl-6 is upregulated following late Ag encounter in all organs in both OT-II and polyclonal cells.

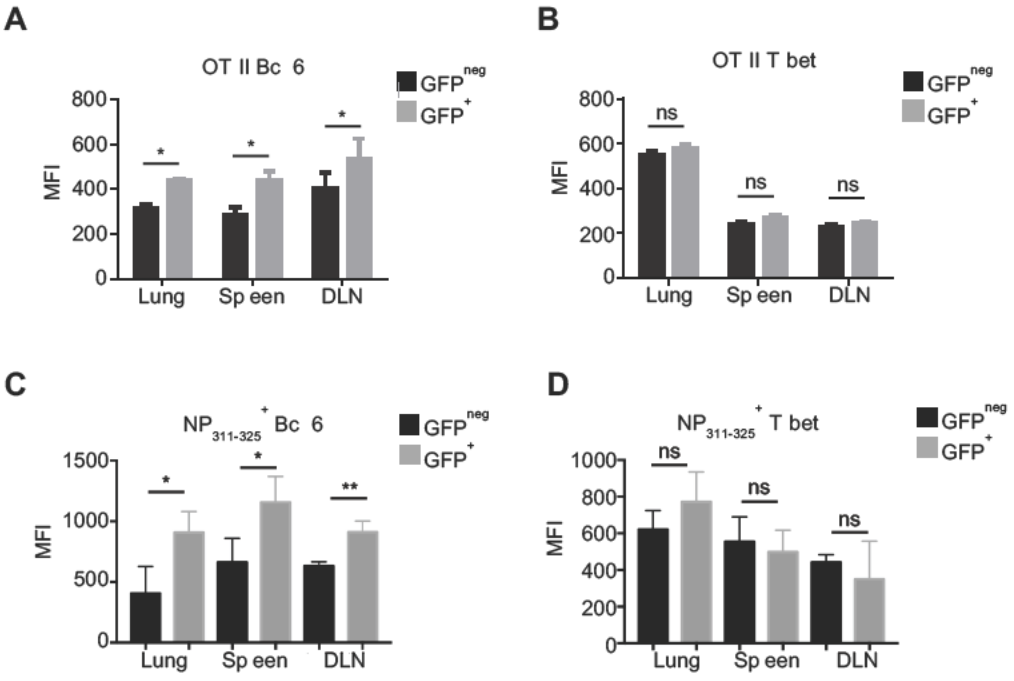


Figure 3.27. Bcl-6 is upregulated following late Ag encounter in all organs in both OT-II and polyclonal cells.

(A,B) $5x10^5$ naïve OT-II.Nur77^{GFP} were transferred to B6 mice. Mice were infected with PR8-OVA_{II}. Lung, spleen, and DLN were harvsted at 7 dpi. GFP^+ and GFP donor OT-II cells were analyzed for Bcl-6 (A) and T-bet (B) expression. **(C-D)** Nur77^{GFP} mice were infected with PR8-OVA_{II}. At 7 dpi, GFP^+ and GFP $NP_{311\ 325}$-specific cells were analyzed for Bcl-6 (C) and T-bet (D) expression. Data is representative of 3-4 experiments, n=3-5 each, mean ± SD.

Figure 3.28. A Stat3 inducing cytokine may play a role in survival following late Ag stimulation.

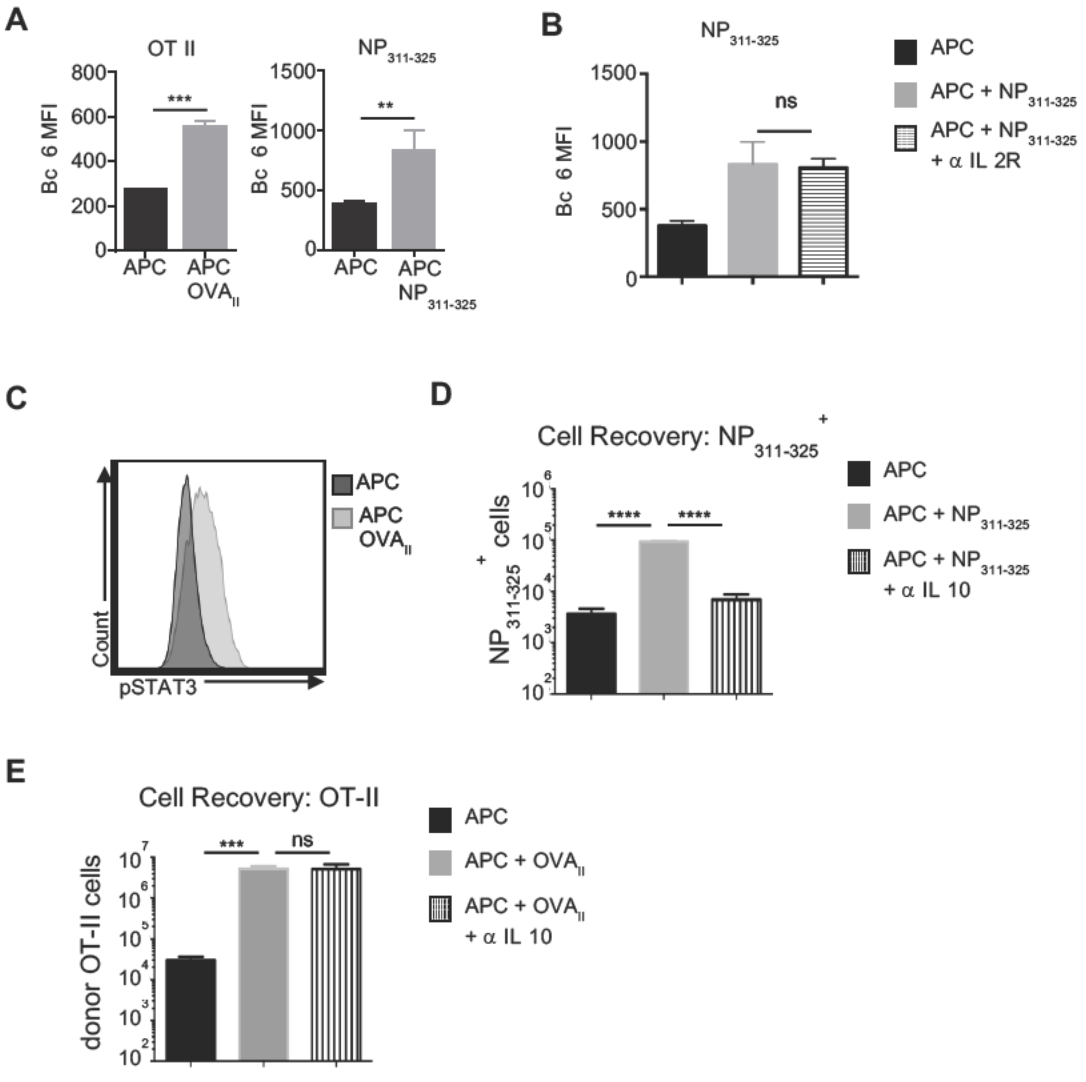


Figure 3.28. A Stat3 inducing cytokine may play a role in survival following late Ag stimulation.

(A-E) 6 dpi OT-II.Thy1.1$^{+/}$ effectors or total CD4 T cells were isolated and co-cultured with irradiated APC with or without OVA_{II} or $NP_{311\ 325}$ peptide respectively as in (Figure 3.17A, 3.18A). (A) Bcl-6 expression was measured in OT-II (left) and $NP_{311\ 325}$-specific cells (right) after 6 days of culture. (B) Bcl-6 expression was measured in $NP_{311\ 325}$-specific cells after 2 days of culture without Ag, with Ag, and with Ag plus αCD25 and αCD122 blocking Ab. (C) Phosphorylated STAT3 staining of OT-II cells after 4 hours of culture. (D, E) Cell recovery of $NP_{311\ 325}$-specific (D) or OT-II (E) cells after 6 days of culture without Ag, with Ag, and with Ag plus αIL-10 blocking Ab. Data is representative of 3 experiments, n=3 each, mean ± SD.

population included more IL-10 producing cells, possibly including regulatory T cells (Treg). Therefore, more work is needed to fully elucidate the role of STAT3 inducing cytokines on promoting the transition to memory following Ag recognition at the effector phase of the CD4 T cell response. These findings demonstrate that when Ag is recognized by fully activated effector cells, even in the presence of activated APC, the cells do not upregulate molecules associated with further terminal differentiation (T-bet or PD-1) but instead upregulate molecules that promote the transition to memory.

To further investigate what genes are upregulated by 6 dpi effectors that re-encounter Ag, I isolated 6 dpi OT-II effectors and cultured them either in media alone or in the presence of plate bound αCD3 and soluble αCD28 for 2 days. I then sorted live donor cells and isolated RNA from the group cultured in media alone (Media), the group cultured with αCD3 and αCD28 (TCR) and cells isolated at 6 dpi (6 dpi effectors) and performed a microarray experiment. I then identified genes that were upregulated by 2 fold or more in the TCR group compared to both the media group and 6 dpi effectors group. Since, in this case, the media alone group is not a true “control” group, I thought it was important to identify the changes as compared to the “time 0”, which in this experiment, is the freshly isolated 6 dpi effectors. This comparison ensured that the genes I identified were induced following late Ag stimulation and did not include genes that remained the same as 6 dpi effectors but had decreased in the media group.

I then sorted genes meeting these criteria into various functional groups as defined using The Database for Annotation, Visualization and Integrated Discovery (DAVID), a tool provided by the National Institute of Allergy and Infectious Diseases (NIAID). To provide perspective on the gene induction following TCR stimulation of activated effectors compared to that of naïve cells, I included the fold induction seen when naïve T cells are stimulated with αCD3 and αCD28 for 2 days in culture, this data was generated by Rabenstein et al. and I obtained it from the GEO database (Table 3.1-3.4).

Table 3.1 includes a list of mostly cytokines, chemokines, and various cell surface receptors. Many of these that were induced by 6 dpi effectors were also induced by naïve cells following TCR stimulation, including *Ccl3*, *Ccl4*, *Socs2*, *Ifng*, *Il2ra*, among others. However, there were a few genes uniquely upregulated in 6 dpi effectors, including *Il13*, *Il10*, *Il4*, and *Tnfrsf8*. IL-13, although largely thought to be a Th2 cytokine, can be produced during Th1 and Th17 driven responses (321). In these settings, it was shown to have an anti-inflammatory effect on responding T cells (321). As mentioned previously, IL-10 induces STAT3 phosphorylation and has recently been shown to act late during LCMV infection to promote memory CD8 T cell formation (322).

IL-4 is a Tfh-associated cytokine, its induction following TCR stimulation of effectors is consistent with findings that show a skewing in the CD4 T cell response to a more Tfh phenotype following persistent Ag (311). Although this study used a model of chronic infection, the fact that Tfh express many markers of central

memory cells [including Bcl-6, TCF-1, and a greater reliance on oxidative phosphorylation (149, 239, 323)] leads to the intriguing possibility that late Ag recognition results in a skewing away from the Th1 effector path to a more memory-like Tfh path. *Tnfrsf8* encodes CD30 which is a co-stimulatory molecule that binds a ligand expressed by lymphoid tissue inducer cells which are thought to be critical for CD4 T cell memory maintenance (324, 325).

Many of the transcription factors and signaling molecules were upregulated similarly in 6 dpi effectors and naïve CD4 T cells (Table 3.2). However, *Nedd4* and *Pparg* were uniquely upregulated in 6 dpi effectors. *Nedd4* encodes a E3 ubiquitin ligase which has been shown to promote proliferation and IL-2 production in CD4 T cells (326). *Pparg* is a fatty acid metabolite driven transcription factor which has been shown to suppress effector functions in T cells (327). *Rbpj* was upregulated to a greater extent in 6 dpi effectors than in naïve T cells. Rbpj is a Notch signaling protein that has been shown to be required for memory CD4 T cell survival (328). Of interest are the transcription factors that are largely upregulated in naïve T cells but not upregulated following TCR stimulation of 6 dpi effectors, these include *Tbx21* (encodes T-bet) and *Irf8*, two genes implicated in effector differentiation (285, 286, 329). Surprisingly, *Bcl6* did not reach the 2-fold cutoff. It may be that the microarray was not sensitive enough to detect the upregulation of Bcl-6 or that Bcl-6 protein expression is regulated post-translationally in 6 dpi effectors receiving TCR stimulation.

6 dpi effectors upregulated a variety of adhesion molecules that were not similarly upregulated in naïve T cells (Table 3.3). Additionally, many regulators of metabolism were upregulated in 6 dpi effectors and not upregulated in naïve T cells (Table 3.4). One of which, *Olr1*, is upregulated by PPARγ and is a receptor that mediates increased fatty acid uptake in adipocytes (330). Since memory cells transition to fatty acid oxidation as their primary energy source, it is possible that increased fatty acid uptake would be advantageous (331).

Next, I identified genes that were upregulated following culture in media compared to the TCR group and 6 dpi effectors group (Table 3.5-3.8). These were also separated into functional groups. For consistency, the fold change column in these tables still indicates the fold change in TCR/media, therefore values in these tables are negative. Table 3.5 includes a list of cell cycle arrest and apoptosis genes that are upregulated in the media alone condition. None of these genes were similarly upregulated in the media alone condition of naïve cells. This is not surprising given that the 6 dpi effector media alone condition is composed of highly activated cells that had recently undergone several rounds of proliferation. Interestingly, quite a few cell cycle arrest genes were upregulated which is consistent with my findings and others that responding CD4 T cells need continuous TCR stimulation for continued proliferation (Figure 3.6C, 3.9A) (262). Additionally, many metabolism genes were upregulated in the media alone condition of 6 dpi effectors that were not similarly upregulated in the media condition of naïve T cells (Table 3.6).

Tables 3.1-3.4 Genes upregulated in TCR vs. Media and 6 dpi effectors

6 dpi OT-II.Thy1.1$^{+/}$ effectors were isolated as in (Figure 3.17A), and cultured with 1μg/ml αCD3 and 5μg/ml αCD28 or in media alone for 2 days. Live OT-II.Thy1.1$^{+/}$ cells were then sorted and RNA was isolated from the media alone group, the TCR stimulated group, and sorted 6 dpi effectors. Microarray analysis was then performed on each sample. Genes that were upregulated in the TCR group by greater than 2-fold over both the media group and the 6 dpi effector group were identified. Genes were then categorized into different functional groups using the NIAID DAVID platform. The third column in every table reflects the fold change between the TCR group over the media group. The last column of every table includes the fold change seen when naïve CD4 T cells are cultured with αCD3 and αCD28 or in media alone for 2 days. This data was obtained from GEO database deposit of Rabenstein et al. (249). Data is pooled from 2 independent experiments.

Table 3.1 Genes upregulated in TCR vs. Media and 6 dpi effectors: Cytokines and Chemokines

Gene	Description	Fold Change TCR/media	P-value	FC 2d eff /naive
Il13	interleukin 13	121.96	0.000457	-
Ccl3	chemokine (C-C motif) ligand 3	126.92	0.043479	5.77
Il10	interleukin 10	91.04	0.000083	-
Tnfrsf8	tumor necrosis factor receptor superfamily, member 8	66.08	0.004121	-
Ifng	interferon gamma	57.6	0.015204	3.87
Socs2	suppressor of cytokine signaling 2	38.97	0.000829	2.84
Tnfrsf9	tumor necrosis factor receptor superfamily, member 9	35.4	0.041042	5.43
Il24	interleukin 24	33.23	0.000567	-
Ccl4	chemokine (C-C motif) ligand 4	32.05	0.016975	4.05
Ccr8	chemokine (C-C motif) receptor 8	31.55	0.003538	2.1
Il12rb2	interleukin 12 receptor, beta 2	19.96	0.036685	2.1
Lag3	lymphocyte-activation gene 3	19.47	0.013728	2.1
Il2ra	interleukin 2 receptor, alpha chain	18.77	0.020855	5.14
Inhba	inhibin beta-A	18.24	0.014225	-
Il4	interleukin 4	16.9	0.041863	-
Lif	leukemia inhibitory factor	13.37	0.000157	3.74
Tfrc	transferrin receptor	9.12	0.007582	-
Wisp1	WNT1 inducible signaling pathway protein 1	7.11	0.01151	2.36
Prf1	perforin 1 (pore forming protein)	6.35	0.00574	-
Spp1	secreted phosphoprotein 1	5.49	0.031968	-
Gzmb	granzyme B	5.49	0.002223	3.86
Csf1	colony stimulating factor 1 (macrophage)	5.22	0.03436	-
Il12rb1	interleukin 12 receptor, beta 1	4.24	0.005519	4.8
Il5	interleukin 5	3.95	0.011827	-

Table 3.2 Genes upregulated in TCR vs. Media and 6 dpi effectors: Transcription factors / Signaling

Gene	Description	Fold Change TCR/media	P-value	FC 2d eff /naive
Irf4	interferon regulatory factor 4	29.33	0.005554	7.99
Nfil3	nuclear factor, interleukin 3, regulated	11.6	0.03192	3.11
Nedd4	neural precursor cell expressed, developmentally down-regulated 4	8.66	0.014427	-
Atf3	activating transcription factor 3	7.17	0.025161	4.93
Rhoq	ras homolog gene family, member Q	6.87	0.020722	1.92
Ell2	elongation factor RNA polymerase II 2	6.35	0.029161	3.24
Pparg	peroxisome proliferator activated receptor gamma	6.18	0.047165	-
Osr2	odd-skipped related 2	6.15	0.006819	-
Rbpj	recombination signal binding protein for immunoglobulin kappa J region	5.99	0.007683	2.07
Batf3	basic leucine zipper transcription factor, ATF-like 3	5.6	0.019747	2.52
Sap30	sin3 associated polypeptide	5.4	0.019015	4.18
Smyd2	SET and MYND domain containing 2	4.11	0.012171	4.16
Junb	Jun-B oncogene	3.7	0.001306	3.87
Cask	calcium/calmodulin-dependent serine protein kinase (MAGUK family)	3.37	0.00166	-
Vdr	vitamin D receptor	3	0.017145	-
Tirap	toll-interleukin 1 receptor (TIR) domain-containing adaptor protein	2.91	0.025587	1.88
Klf8	Kruppel-like factor 8	2.84	0.031397	n/a
Stat5a	signal transducer and activator of transcription 5A	2.58	0.018008	3.12
Gata3	GATA binding protein 3	2.53	0.014909	1.8
Phf10	PHD finger protein 10	2.4	0.017691	3.42

Table 3.3 Genes upregulated in TCR vs. Media and 6 dpi effectors: Adhesion

Gene	Description	Fold Change TCR/media	P-value	FC 2d eff /naive
Mt2	metallothionein 2	76.62	0.031709	-
Adam8	a disintegrin and metallopeptidase domain 8	14.5	0.045268	-
Lamc1	laminin, gamma 1	6.9	0.031262	-
Ctnna1	catenin (cadherin associated protein), alpha 1	3.72	0.008558	5.15
Vcl	vinculin	3.68	0.012345	-
Lama5	laminin, alpha 5	3.36	0.028365	-
Adam9	a disintegrin and metallopeptidase domain 9 (meltrin gamma)	3.12	0.030545	-
Alcam	activated leukocyte cell adhesion molecule	2.79	0.039221	-
Itgav	integrin alpha V	2.66	0.031653	-
Cercam	cerebral endothelial cell adhesion molecule	2.25	0.009599	n/a

Table 3.4 Genes upregulated in TCR vs. Media and 6 dpi effectors: Metabolism

Gene	Description	Fold Change TCR/media	P-value	FC 2d eff /naive
Akr1c18	aldo-keto reductase family 1, member C18	361.96	0.000561	-
Olr1	oxidized low density lipoprotein (lectin-like) receptor 1	12.47	0.032304	-
Cth	cystathionase (cystathionine gamma-lyase)	9.93	0.000977	8.09
Asns	asparagine synthetase	17.71	0.001103	7.59
Fads2	fatty acid desaturase 2	21.57	0.003746	-
Ak4	adenylate kinase 4	11.19	0.021126	-
Gstt3	glutathione S-transferase, theta 3	4.83	0.01601	-
Gpt2	glutamic pyruvate transaminase (alanine aminotransferase) 2	9.17	0.003119	6.19
Got1	glutamate oxaloacetate transaminase 1, soluble	4.29	0.007753	-

Many cytokines and cell surface receptors were similarly regulated in the 6 dpi media condition and the naïve T cell media condition (Table 3.7). However, a few genes were uniquely upregulated in the media condition of 6 dpi effectors including *CD96. CD96* encodes the T cell-activated increased late expression protein (Tactile), a protein found to inhibit IFNγ production of NK cells (332). Among the transcription factors uniquely upregulated in the media condition of 6 dpi effectors is *Zbtb20* a transcription factor shown to inhibit Foxo1 expression in lung cancer cells (333) (Table 3.8). Given the role of Foxo1 in promoting the transition to memory in CD8 T cells (334, 335), this may be a mechanism by which these cells fail to form memory. While this microarray provides some promising leads into which genes may play a role in promoting memory formation following late Ag recognition, more work is needed to fully expand upon these potential mechanisms. However, it is clear that Ag recognition at the effector stage differs from Ag recognition at the naïve stage. Instead of driving terminal differentiation, it may promote genes with a slightly suppressive effect, limiting T cell activation and driving a memory phenotype.

Memory cells Receiving Short-term Late Ag have an Enhanced Memory Phenotype, Function, and Protective ability.

In the transfer model (Figure 3.5), 6 dpi effector cells transferred to hosts without Ag underwent extensive contraction and were often at or below the limit of detection within 7 dpt. This low number of memory cells in hosts without Ag

Tables 3.5-3.8 Genes upregulated in Media vs. TCR and 6 dpi effectors

6 dpi OT-II.Thy1.1$^{+/}$ effectors were isolated as in (Figure 3.17A), and cultured with 1μg/ml αCD3 and 5μg/ml αCD28 or in media alone for 2 days. Live OT-II.Thy1.1$^{+/}$ cells were then sorted and RNA was isolated from the media alone group, the TCR stimulated group, and sorted 6 dpi effectors. Microarray analysis was then performed on each sample. Genes that were upregulated in the media group by greater than 2-fold over both the TCR group and the 6 dpi effector group were identified. Genes were then categorized into different functional groups using the NIAID DAVID platform. The third column in every table reflects the fold change of the TCR group over the media group. The last column of every table includes the fold change seen when naïve CD4 T cells are cultured with αCD3 and αCD28 or in media alone for 2 days. This data was obtained from GEO database deposit of Rabenstein et al. (249). Data is pooled from 2 independent experiments.

Table 3.5 Genes upregulated in Media vs. TCR and 6 dpi effectors: Cell Cycle Arrest / Apoptosis

Gene	Description	Fold Change TCR/media	P-value	FC 2d eff /naive
Slfn1	schlafen 1	-7.55	0.014622	-
Bcl2l11	BCL2-like 11 (apoptosis facilitator)	-5.68	0.011702	-
Rb1	retinoblastoma 1	-5.67	0.008847	-
Thbs1	thrombospondin 1	-5.66	0.03256	-
Sesn1	sestrin 1	-5.51	0.026238	-
Crlf3	cytokine receptor-like factor 3	-4.82	0.026603	-

Table 3.6 Genes upregulated in Media vs. TCR and 6 dpi effectors: Metabolism

Gene	Description	Fold Change TCR/media	P-value	FC 2d eff /naive
Ddx60	DEAD (Asp-Glu-Ala-Asp) box polypeptide 60	-10.69	0.019595	-
Atp1b1	ATPase, Na+/K+ transporting, beta 1 polypeptide	-9.87	0.038446	-
Mgst2	microsomal glutathione S-transferase 2	-8.78	0.005582	-
Nt5e	5' nucleotidase, ecto	-8.2	0.018565	-
Acss1	acyl-CoA synthetase short-chain family member 1	-6.7	0.02737	-
Pnpla7	patatin-like phospholipase domain containing 7	-6.07	0.004	-
Camk1d	calcium/calmodulin-dependent protein kinase ID	-4.37	0.008793	-
Acpp	acid phosphatase, prostate	-3.98	0.026189	-
Ldlrap1	low density lipoprotein receptor adaptor protein 1	-3.93	0.028983	-
St6galnac3	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 3	-3.6	0.007796	-
Kat2b	K(lysine) acetyltransferase 2B	-2.98	0.005265	-
Kif1b	kinesin family member 1B	-2.81	0.009955	-
Ccs	copper chaperone for superoxide dismutase	-2.18	0.004578	-

Table 3.7 Genes upregulated in Media vs. TCR and 6 dpi effectors: Cytokines, Chemokines, Surface Receptors

Gene	Description	Fold Change TCR/media	P-value	FC 2d eff /naive
Klrd1	killer cell lectin-like receptor, subfamily D, member 1	-13.14	0.006026	-5.3
Tlr1	toll-like receptor 1	-12.27	0.001316	-2.09
Cd96	CD96 antigen	-8.39	0.001298	-
Itga6	integrin alpha 6	-6.88	0.004624	-
Il18r1	interleukin 18 receptor 1	-5.99	0.03186	-2.59
S1pr1	sphingosine-1-phosphate receptor 1	-5.88	0.030589	-2.97
Ctsw	cathepsin W	-5.53	0.026026	
Tbxa2r	thromboxane A2 receptor	-4.01	0.004048	-
Ccl5	chemokine (C-C motif) ligand 5	-3.46	0.01043	-2.12
Ltb	lymphotoxin B	-3.02	0.000318	-2.55

Table 3.8 Genes upregulated in Media vs. TCR and 6 dpi effectors: Transcription / Signaling

Gene	Description	Fold Change TCR/media	P-value	FC 2d eff /naive
Pde2a	phosphodiesterase 2A, cGMP-stimulated	-31.89	0.009852	-2.3
Sp100	nuclear antigen Sp100	-14.34	0.012502	-
Trib2	tribbles homolog 2 (Drosophila)	-8.81	0.015748	-
Card6	caspase recruitment domain family, member 6	-8.22	0.025918	n/a
Scml4	sex comb on midleg-like 4 (Drosophila)	-5.82	0.007824	-
Sla2	Src-like-adaptor 2	-4.91	0.009253	-
Zfp652	zinc finger protein 652	-3.89	0.013654	-
Zbtb20	zinc finger and BTB domain containing 20	-3.71	0.006166	-
Ssbp2	single-stranded DNA binding protein 2	-3.14	0.002102	-
Cbx7	chromobox 7	-2.8	0.011833	-
Atxn7	ataxin 7	-2.49	0.000105	-
Zfp287	zinc finger protein 287	-2.33	0.007639	-
Clock	circadian locomotor output cycles kaput	-2.23	0.000791	-

hampered my ability to determine the long-term phenotypic and functional differences between memory cells generated with or without Ag at the checkpoint. To increase the recovery of memory cells that develop without Ag at the checkpoint, I cultured *in vivo*-generated effector CD4 T cells with or without Ag for 2 days *in vitro* (as in Figure 3.17), transferred equal numbers of each to uninfected mice, and allowed the cells to transition to memory for 7 days (Figure 3.29A). *In vivo*, 3 days without Ag is sufficient for effector CD4 T cells to become virtually identical to memory (203). I then assayed cell recovery, phenotype, and cytokine production.

As expected, the donor cells that had been exposed to Ag/APC *in vitro*, formed a significantly larger memory population after transfer to uninfected hosts even though their numbers were equivalent at the time of transfer, with 18-fold more in lung and 5-fold more in spleen (Figure 3.29B). This indicates that the 2 days of exposure to Ag was sufficient to confer significantly greater survival. Compared to APC without Ag, the donor effector cells exposed to Ag/APC *in vitro*, expressed increased levels of CD127 and CXCR3, a memory marker needed for homing and protective function (216, 226) (Figure 3.29C). Moreover, they secreted more IFNγ and had a higher frequency of IFNγ/TNFα double producers after re-stimulation (Figure 3.29D, 3.29E). These results indicate that even short-term Ag recognition at 6 dpi results in both a much larger, and a functionally superior memory population.

Figure 3.29. Short-term late Ag promotes enhanced memory phenotype and function.

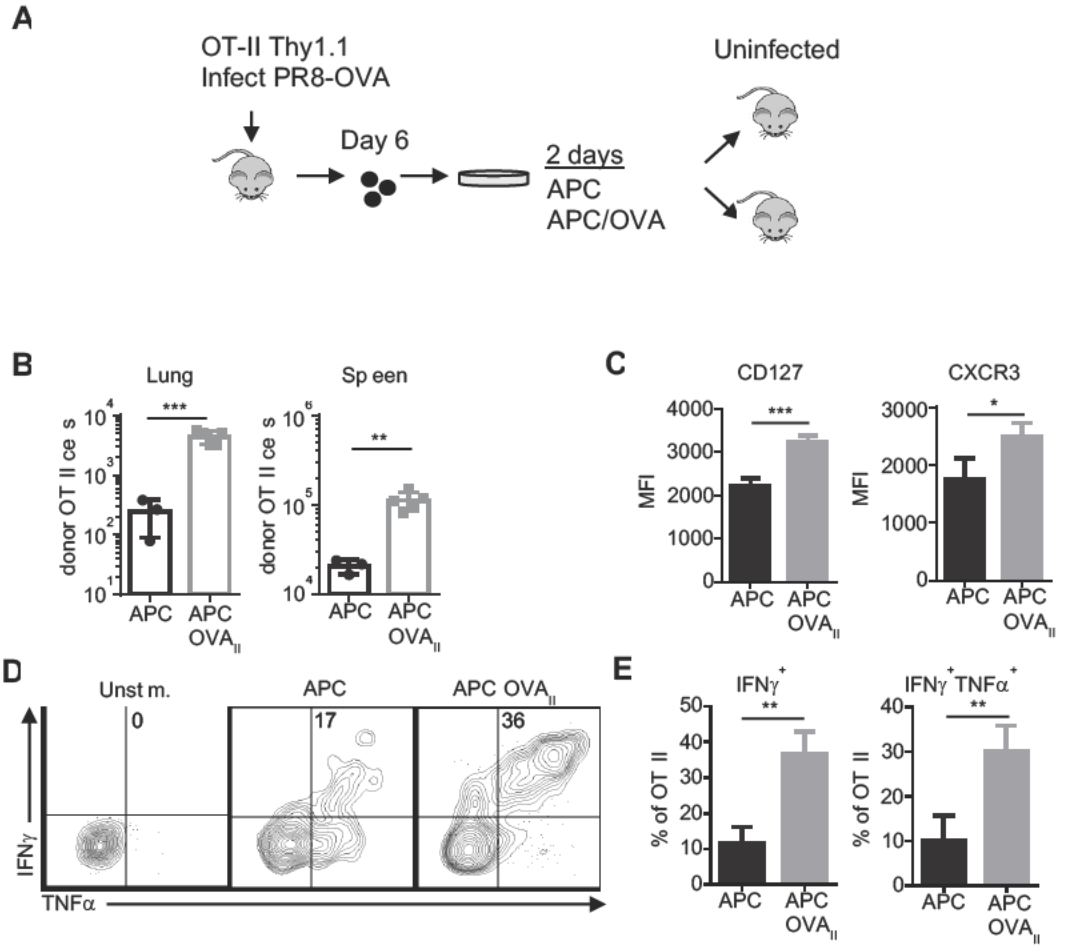


Figure 3.29. Short-term late Ag promotes enhanced memory phenotype and function.

(A-E) 6 dpt OT-II *in vivo*-generated effectors and PR8-activated APC were co-cultured *ex vivo* as described in (Figure 3.17A). After 2 days of culture, live cells were isolated using Lympholyte and $2x10^6$ cells were transferred to uninfected B6 mice. (A) Experimental schematic. (B) Cell recovery was determined 7 dpt in the lung and the spleen of host mice. (C) CD127 and CXCR3 expression was assayed in the spleen at 7 dpt. (D) Representative flow cytometry plots of intracellular cytokine staining of cells harvested from the spleen 7 dpt and re-stimulated for 4 hours with PMA + Ionomycin. (E) Percentage of $IFN\gamma^+$ and $IFN\gamma^+TNF\alpha^+$ donor cells. Representative data, n=3-4 each, 3 experiments, mean ± SD.

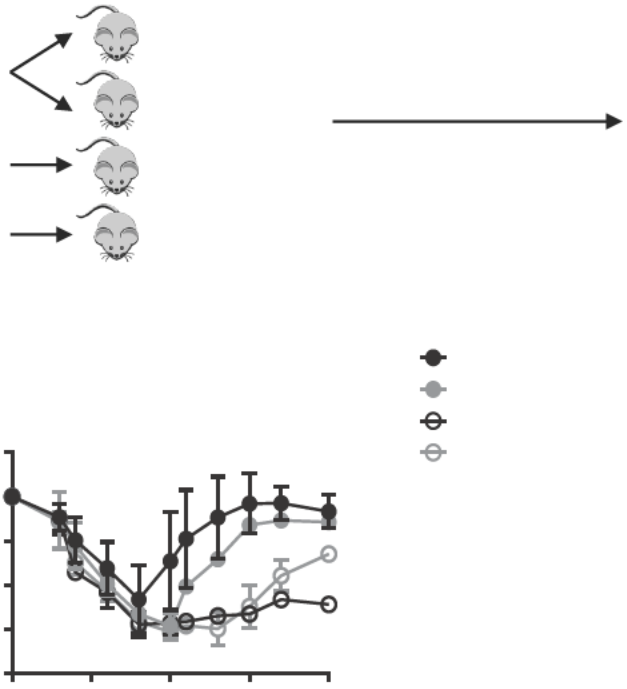
To evaluate if the differences in memory formation with or without Ag at the checkpoint would lead to differences in protection against a lethal challenge of IAV, I transferred 6 dpi OT-II effectors into uninfected B6 mice along with OVA_{II}-pulsed or un-pulsed APC. To account for a potential host naïve CD4 T cell response, I included a group of mice that received OVA_{II}-pulsed APC without transfer of 6 dpi effectors. I also included a group that received naïve OT-II cells to control for the possibility that a similar number of naïve donor OT-II could provide enhanced protection. Hosts were rested for 2-3 weeks to ensure memory generation, and then challenged with a lethal dose of PR8-OVA_{II} (Figure 3.30A).

Despite the fact that the only memory cells in the hosts were the donor 6 dpi effectors, the hosts that received effectors plus APC-OVA_{II} were mostly protected against lethal infection (12/15), whereas those that received naïve OT-II, OT-II 6 dpi effectors without Ag, or APC-OVA_{II} alone were largely unprotected (Figure 3.30B, 3.30C). Thus, providing effectors with only short-term *in vivo* Ag stimulation at the checkpoint drove the formation of protective memory cells. Since the hosts were not previously infected, I conclude that short-term Ag stimulation by activated APC, without any viral infection, is sufficient to promote the transition of 6 dpi effectors to become protective memory.

Late Ag Enhances Memory Formation in a Cold-adapted Vaccination Model.

My findings establish a checkpoint that occurs at 6-8 dpi following IAV infection where Ag recognition drives functional memory CD4 T cell formation.

Figure 3.30. Short-term late Ag is required for the formation of a protective memory CD4 T cell response.



C

Figure 3.30. Short-term late Ag is required for the formation of a protective memory CD4 T cell response.

(A-C) 6 dpi OT-II effector cells were generated *in vivo* as described in (Figure 3.5A) and transferred to uninfected mice along with either OVA_{II}-pulsed APC (APC-OVA_{II}) or unpulsed APC. One group of mice received OVA_{II}-pulsed APC alone (no effectors). Another group received $5x10^5$ naïve OT-II cells. After 2-3 weeks, hosts were challenged with $2LD_{50}$ PR8-OVA_{II}. (A) Experimental schematic. (B) Weight loss curves. (C) The survival of mice was plotted. Pooled data, n=14-15, 3 experiments. Significance for Figure 3.30C was determined using the Log-rank (Mantel-Cox) test.

Since many standard vaccinations likely do not induce the persistent levels of Ag that live virus does, I postulate that memory CD4 T cell formation following vaccination is normally constrained by a lack of Ag at the checkpoint. Therefore, the addition of Ag/APC at this time may enhance vaccine-induced memory. To test this premise, I immunized with a live attenuated, cold-adapted (ca) influenza vaccine (ca.IAV). Replication of ca.IAV is limited to the upper respiratory tract, potentially limiting the duration of Ag presentation. The ca.IAV vaccine LAIV has been shown to induce enhanced T cell responses when compared to inactivated vaccines suggesting that it had the greatest potential for persisting Ag (18). To determine if Ag persisted into the effector phase following ca.IAV inoculation, I immunized Nur77^{GFP} mice with ca.A/Alaska/6/77CR29 (ca.Alaska) and measured GFP expression in immunization-induced effector T cells. Previous work in our lab showed that ca.Alaska induces a strong heterosubtypic response to PR8 and that the $NP_{311\ 325}$ is a dominant CD4 epitope shared between these two viruses (83). At 7 dpi, effector $NP_{311\ 325}$-specific cells expressed no GFP after ca.Alaska immunization indicating no recent Ag recognition (Figure 3.31), while in mice infected with PR8, or a non-ca H3N2 strain (A/Philippines/2/82/x-79), a cohort of $NP_{311\ 325}^{+}$ cells were GFP^{+}, indicating recent Ag recognition in the live infections.

To determine if the addition of Ag during the checkpoint could boost memory following ca.Alaska immunization, I added $NP_{311\ 325}$-pulsed APC at 6 dpi to ca.Alaska-immunized mice and assayed memory CD4 T cell formation by enumerating $NP_{311\ 325}$ tetramer positive cells after 33-44 dpi (Figure 3.32A). I found

Figure 3.31. Cold-adapted virus fails to present Ag during the effector phase of the response.

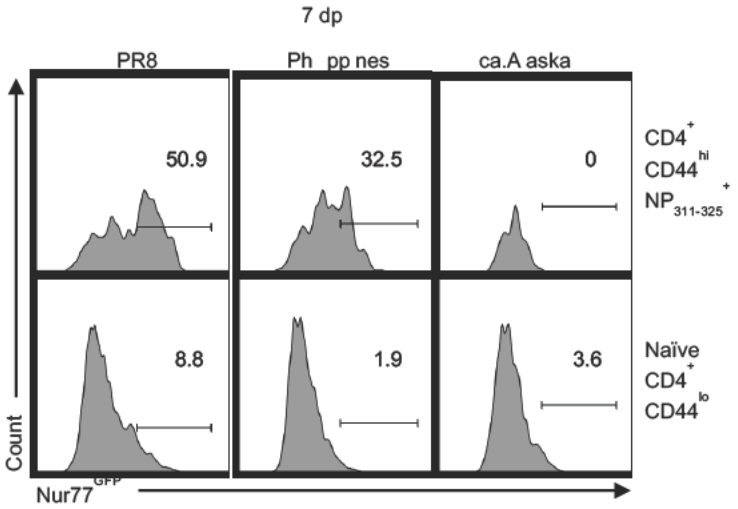


Figure 3.31. Cold-adapted virus fails to present Ag during the effector phase of the response.

Nur77^{GFP} expression of CD4^{+}CD44^{h} NP$_{311\ 325}$ tetramer^{+} cells or CD4^{+}CD44^{o} naïve cells in the lung on day 7 following PR8 (H1N1) infection, Philippines (H3N2) infection, or ca.Alaska (H3N2) immunization. Representative data, 2-3 experiments, n=3-4 mean ± SD.

significantly more $NP_{311\ 325}^{+}$ CD4 T cells in the lung and spleen, although there was no difference in the number of donors found in the DLN (Figure 3.32B). This finding suggests that the memory checkpoint exists for effectors generated by attenuated as well as live WT influenza infection. Additionally, it shows that the introduction of Ag/APC at the checkpoint can promote effector CD4 T cells induced by attenuated virus immunization to form more memory without the need for persisting live virus.

Figure 3.32. Memory CD4 T cell formation is enhanced following cold-adapted IAV vaccination with the addition of short-term Ag at 6 dpi.

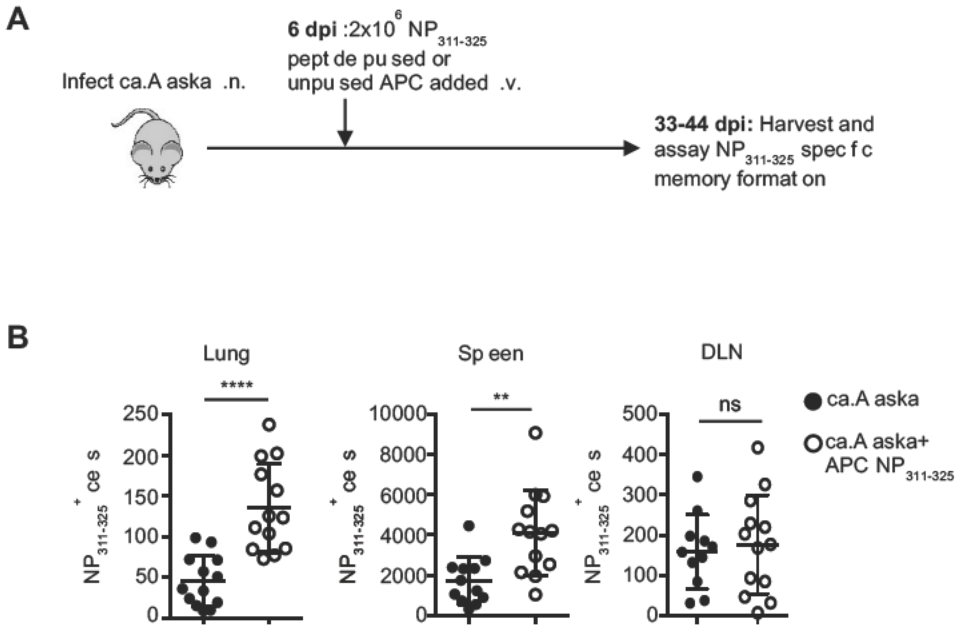


Figure 3.32. Memory CD4 T cell formation is enhanced following cold-adapted IAV vaccination with the addition of short-term Ag at 6 dpi.

(A, B) B6 mice were immunized with 2500 $TCID_{50}$ ca.Alaska intranasally. 6 days later, $2x10^6$ $NP_{311\ 325}$-pulsed APC were added via intravenous injection. (A) Experimental schematic. (B) Quantification of $NP_{311\ 325}$ tetramer$^+$ memory $CD4^+CD44^h$ T cells in the lung, spleen, and DLN 33-44 days following immunization. Representative FACS plots shown, 2-4 independent experiments. Pooled cell recovery data, 3 independent experiments, n=4-5 each, mean ± SD.

CHAPTER IV: DISCUSSION

Overview

The mechanisms that govern the selection of memory cells from the effector population remain unclear. Some have proposed that the initial interaction with cognate Ag during priming programs cells toward either effector or memory differentiation (283, 284, 288–290). While these models certainly inform the heterogeneity seen at the effector stage of the T cell response, they do not exclude the possibility that events occurring at later stages may influence memory T cell formation.

Studies from our laboratory and others suggest that signals occurring at the effector stage can enhance memory T cell formation (1, 138, 139, 211). McKinstry et al. demonstrated that CD4 T cells required autocrine IL-2 signals between 5-7 dpi to form memory (1). Given that Ag recognition is a main driver of IL-2 production in CD4 T cells, the aim of my thesis project was to determine if Ag at this time point was also required for memory formation. While much work has been done examining the role of Ag in memory CD4 T cell formation, virtually all studies have focused on the priming phase of the response (260, 305). This was likely a result of the pervasive concept of AICD (151, 159, 160) and a few findings that demonstrated that prolonged Ag presentation can be deleterious to memory formation (246, 263).

This thesis work demonstrates that CD4 T cells require Ag recognition during the effector phase of the response to continue undergoing proliferation and

to form a functional and protective memory population. These findings suggest that CD4 memory T cell population size and function is not predetermined at the priming stage. In fact, it requires additional signals including TCR stimulation, IL-2 signaling, and co-stimulation during the effector phase immediately preceding the onset of contraction. I found that, at this time, these signals promoted a transcriptional program of memory-associated genes including CD25 and Bcl-6. The long-term effects of Ag recognition at this time point included upregulation of the memory markers CD127 and CXCR3, as well as the formation of more multiple cytokine producing and protective memory CD4 T cells (Figure 4.1). Further, I found that short-term Ag stimulation without systemic inflammatory cytokines at this late time point were sufficient to fulfill the requirements of memory formation which may have important implications for vaccine design.

Ag Recognition during the Course of an Immune Response.

The finding that Ag recognition during the course of the effector response was limited provides great insight into how Ag may shape the ongoing immune response. The Nur77^{GFP} experiments clearly demonstrate that at 3 dpi virtually all the cells in the DLN and spleen had recently encountered Ag and were therefore GFP^{+} following isolation (Figure 3.2B). Just 2 days later, the vast majority of T cells in the SLO were GFP indicating that they likely did not receive additional TCR stimulation after that initial signal at 3 dpi. These GFP cells were all CD44^{h} indicating that they were not “late comers” and were sufficiently primed. In

Figure 4.1. Late Ag Checkpoint model for memory formation.

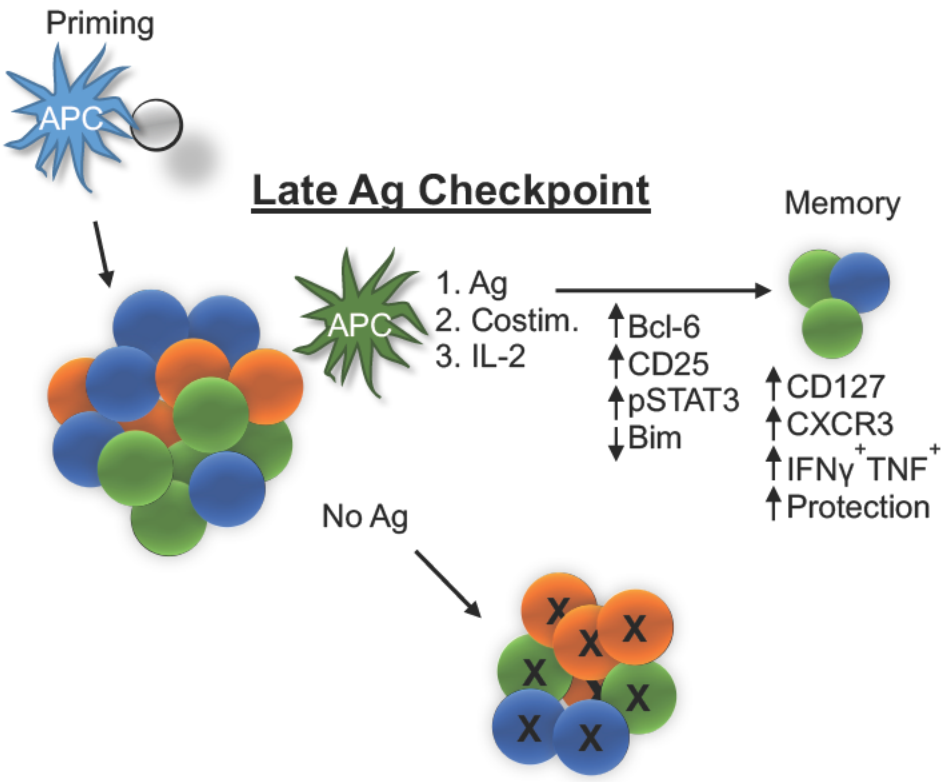


Figure 4.1. Late Ag Checkpoint model for memory formation.

Model summarizing the central findings presented in this thesis. I find that responding CD4 T cells require Ag, IL-2, and co-stimulation during the effector stage of the response to become memory. Late Ag increases Bcl-6, CD25, and phosphorylated STAT3, and decreases Bim expression. Memory cells generated with late Ag recognition have increased CD127 and CXCR3 expression, increased multiple cytokine producing ability, and protective function.

fact, studies have demonstrated that the recruitment of Ag-specific cells during priming is quite efficient (336). This pattern of GFP expression is consistent with the model of priming in which T cells engage in prolonged contact with Ag/APC, followed by a phase of Ag-independent proliferation (245, 266–268). However, studies suggest that CD4 T cells were capable of and even required additional Ag contacts during this phase of proliferation (269, 272). Given that some cells do express GFP at 5 dpi, these additional contacts are likely to occur but they do not appear to be the norm. A caveat of these early time points is that since polyclonal cells are present a such low numbers early during infection, I was unable to confirm a similar pattern of GFP expression in endogenous cells at this time. However, the microscopy studies detailing the phases of T cell priming, including the studies demonstrating a CD4 T cell requirement for additional contacts were also conducted with transgenic T cells (266–269, 337).

Another interesting finding of the Nur77^{GFP} studies was that many of the responding CD4 T cells in the lung of infected mice do not re-encounter Ag between 5-9 dpi. This is despite the continued presence of Ag at these time points (306). Additionally, there is still a significant amount of virus present in the lung between 5-9 dpi (Figure 1.2). These findings are in agreement with a two-photon microscopy study in which *in vitro*-generated rested effectors were transferred into PR8-OVA$_{II}$-infected mice at 7 dpi and 24 hours later, around 35% of transferred cells were arrested indicating Ag recognition (307). It is likely that the immune system generates a CD4 T cell response far bigger than what is needed and since

CD4 T cell cytokine production appears to be focused around sites of significant Ag burden (307), the immunopathology costs of doing so are limited.

Additionally, the phenomenon of CD4 T cells not recognizing Ag despite its presence has been observed with microscopy studies. These studies found that PD-1 interactions promoted T cell mobility which may limit their ability to receive strong TCR signals (338, 339). Effector CD4 T cells in the lung express high levels of PD-1, so this may be a relevant mechanism by which effectors receive limited TCR signals. Others have found that activated CD4 T cells undergo a programmed downregulation of TCR beginning at the peak of the T cell response (340) which may explain why there is so little Ag recognition between 9-14 dpi. This may be a mechanism to limit immunopathology and shut down the response even in the presence of continuing Ag presentation (306).

The Nur77^{GFP} kinetics experiment in Figure 3.2 also demonstrates that a few responding CD4 T cells still recognize Ag at 14 dpi. Despite this, 14 dpi effectors do not proliferate either following transfer (Figure 3.6C) or *in situ* (140). In Brdu labelling studies, Roman et al. demonstrated that proliferation in CD4 T cells during IAV infection ceases around 12 dpi (findings I have confirmed in my experiments) especially in the SLO which is where the most Ag recognition is occurring at this time (Figure 3.2). It may be interesting to determine the context of this Ag presentation, if it is occurring in germinal centers it may be that T follicular regulatory cells inhibit proliferation of these cells (341, 342). Microscopy studies identifying where effectors cells localize late in the response compared to

transferred naïve cells might be informative in discovering any potential niche or migration-dependent regulation of Ag recognition of responding T cells during contraction.

Short-term Ag Requirement.

Previous work from our lab has demonstrated that responding CD4 T cells require IL-2 signals between 5-7 dpi to form memory. Similarly, the transfer experiments using short-term Ag/APC at 6 dpi have demonstrated that Ag presentation during this limited window is sufficient for optimal memory formation (Figure 3.11, 3.12, and 3.14). Additionally, if 6 dpi effectors do not receive TCR signals at this time they undergo a sharp contraction (Figure 3.5E) and the memory cells that do form have limited functional ability (Figure 3.29D, 3.29E). Ag recognition at the effector phase appears to be critical for many aspects of memory T cell formation. It downregulates Bim expression promoting cell survival (Figure 3.21). It induces CD25 expression which enhances the ability of cells to receive IL-2 signals (Figure 3.26). It induces memory-associated molecules CD27 (Figure 3.26B) and Bcl-6 (Figure 3.27A, 3.27C) which may play a critical role in the necessary metabolic switch for a successful transition to memory (235). It programs the gradual upregulation of memory markers CD127 and CXCR3 (Figure 3.20B, 3.20D, 3.29C). Finally, it programs their ability to become memory with multi-cytokine producing potential (Figure 3.29D, 3.29E). Therefore, 6-8 dpi is

certainly a critical time in the formation of the heterogeneous, multi-functional memory population that is formed following influenza infection.

Future work may address whether or not this time point is similarly important during other viral infections. It is likely that acute infections that exhibit a similar T cell kinetics may be governed by the same principles as influenza infection. Alternatively, different inflammatory environments present during priming may alter how effectors interpret subsequent Ag encounter. The findings in this thesis demonstrate that PR8 infection generates CD4 effectors that are highly activated and will undergo apoptosis if they do not receive Ag stimulation at the effector phase. It may be that priming conditions that do not drive such extensive differentiation may not generate effectors that have such a requirement. However, the fact that effectors generated with cold-adapted virus also formed an enhanced memory population with the addition of Ag at 6 dpi suggests that this phenomenon is not restricted to the highly differentiated effectors generated by live viral infection. However, the results seen in the cold-adapted model were not as impressive as those seen with effectors generated by live viral infection, suggesting that either these cells were less dependent on the late Ag checkpoint or the administration of Ag was not as efficient at reaching effector cells as it is in the transfer models. Given that the numbers of memory cells generated was fairly low in the cold-adapted model (Figure 3.32B), it is likely that the Ag/APC were not able to reach enough $NP_{311\ 325}$-specific effector cells to generate a robust memory population.

This thesis does not examine at what point Ag recognition becomes deleterious. CD4 T cells are critical for controlling chronic viral infections (343, 344) and studies suggest that CD4 T cell exhaustion is quite different from CD8 T cell exhaustion including both the loss and gain of effector function (345). During chronic viral infection, CD4 T cells appear to skew away from a Th1 phenotype to a Tfh phenotype (311, 346). However, in a mouse model with induced Ag presentation on DC, continuous Ag stimulation for 10 days resulted in permanent loss of IL-2 and TNFα production (300). Interestingly, in this study survival was not affected by continuous Ag signaling again contradicting a potential role for AICD in regulating the contraction of CD4 T cells (300). It is therefore unclear to what extent prolonged TCR stimulation drives CD4 T cell dysfunction.

There is a precedent for late Ag having opposing effects at different times during an immune response. A CD8 T cell study found that following immunization with peptide and adjuvant, addition of Ag at 4 dpi prevented apoptosis, whereas addition of Ag at 7 dpi increased apoptosis. In this study, the peak of the response occurred at 5 dpi (3-4 days earlier than in the IAV model) (177) (Figure 1.2). Therefore, it is possible that Ag recognition during the contraction phase may have a negative impact on responding T cells. Whether or not this is the case would depend on several factors. For instance, if the Ag stimulation is continuous or includes intermittent breaks, if the Ag is provided exogenously or from an endogenous persisting source, if the Ag is administered in the context of

inflammatory cytokines or not are all likely to play a role on how long Ag is beneficial to responding T cells.

Another question is where is this critical Ag recognition occurring. As the Nur77^{GFP} studies demonstrate, a substantial number of GFP^{+} cells are present at this time in both the lung and the SLO (Figure 3.2E). However, since the GFP signal lasts for about 24 hours, it cannot be ruled out that cells see Ag in one location and quickly migrate to another. Ballesteros-Tato et al. found that CD11b^{h} DC peak around 5-7 dpi in the DLN, they suggest the DLN is the dominant site for late Ag recognition (137). On the other hand, microscopy studies have shown CD4 T cell migration arrest around sites of high Ag density in the lungs of PR8-infected mice (307). It is likely that Ag recognition occurs in both the lung and the SLO during the effector phase of the response and each contribute to the memory population as a whole. An intriguing hypothesis is that Ag recognition in the SLO promotes a central memory or Tfh-like memory population, while Ag recognition in the lung promotes Trm and Th1-like memory.

Memory cell Selection

A requirement of Ag recognition at the effector stage to generate CD4 T cell memory makes teleological sense. First, it would ensure that a substantial memory population is only formed when Ag, indicating a continuing threat, persists. If the pathogen were rapidly cleared, the generation of T cell memory would be a waste of resources. Second, a late Ag-dependent checkpoint may serve to select T cells

with high affinity to epitopes that continue to be presented late in the response. Studies have shown that memory CD4 T cells have greater functional avidity than do the CD4 effectors present at the peak of the response (304), suggesting that the cells that become memory include effector cells with the highest TCR avidity. Finally, the additional round of Ag-dependent selection may help select a memory pool with greater multi-functionality. Many recent studies have demonstrated that memory CD4 T cells retain a significant level of the differentiation acquired during the effector phase (146, 148, 149, 209). An intriguing hypothesis is that, via the memory checkpoint described here, this late Ag interaction may be responsible for the selection and formation of more specialized subsets of effectors that become memory cells particularly tailored to combat the given pathogen upon re-encounter.

The memory cell population only consists of only about ~10% of the effector cell population. Therefore, any model proposing a mechanism for selecting memory cells must describe how that model could account for such a narrow selection of cells to become memory. I believe the proposed model provides a mechanism for the selection of a limited number of effector cells to become memory (Figure 4.2). First, the Nur77^{GFP} studies demonstrated that Ag recognition by effectors is limited (Figure 3.2). Second, experiments presented in this thesis (Figure 3.22, 3.23) as well as in a previous publication (1), demonstrate that autocrine IL-2 signals are required for the pro-survival effects of late Ag and the upregulation of CD127. Given that as effector cells differentiate they can lose the

ability to produce IL-2 (4, 347), this may only allow a percentage of the cells that re-encounter Ag to go on to form memory. The role of T cell intrinsic factors like this are where some of the influences of priming may come into play. While the ability to make IL-2 is likely one limiting factor, there may be others that would prohibit cells that have undergone extensive differentiation from receiving the pro-survival, memory programming benefit of late Ag recognition. Finally, the requirement for co-stimulation, CD28 in the current work (Figure 3.24, 3.25) and CD27 found in our previous study (1), suggests that in order for late Ag recognition to promote memory formation it likely must be presented by a professional activated APC. Since lung epithelial cells upregulate MHC-II following IAV infection (14), it is unclear whether or not all Ag recognition is presented by a professional, activated APC. Therefore, it is feasible that via the mechanism described here, late Ag recognition could act to select high affinity clones against dominant pathogen epitopes that have retained their functional capacity (Figure 4.2).

Memory-associated Genes

The experiments in this thesis demonstrate that Ag recognition at the memory checkpoint initiates a program of memory-associated changes that results in a larger, long-lived memory population with increased CD127 and CXCR3 expression as well as increased cytokine production. Our Nur77^{GFP} experiments highlight that the early signaling events that occur following Ag recognition at the checkpoint include an upregulation of CD25, Bcl-6, and pSTAT3. CD25 expression

Figure 4.2. Model for selective memory formation of CD4 effector T cells.

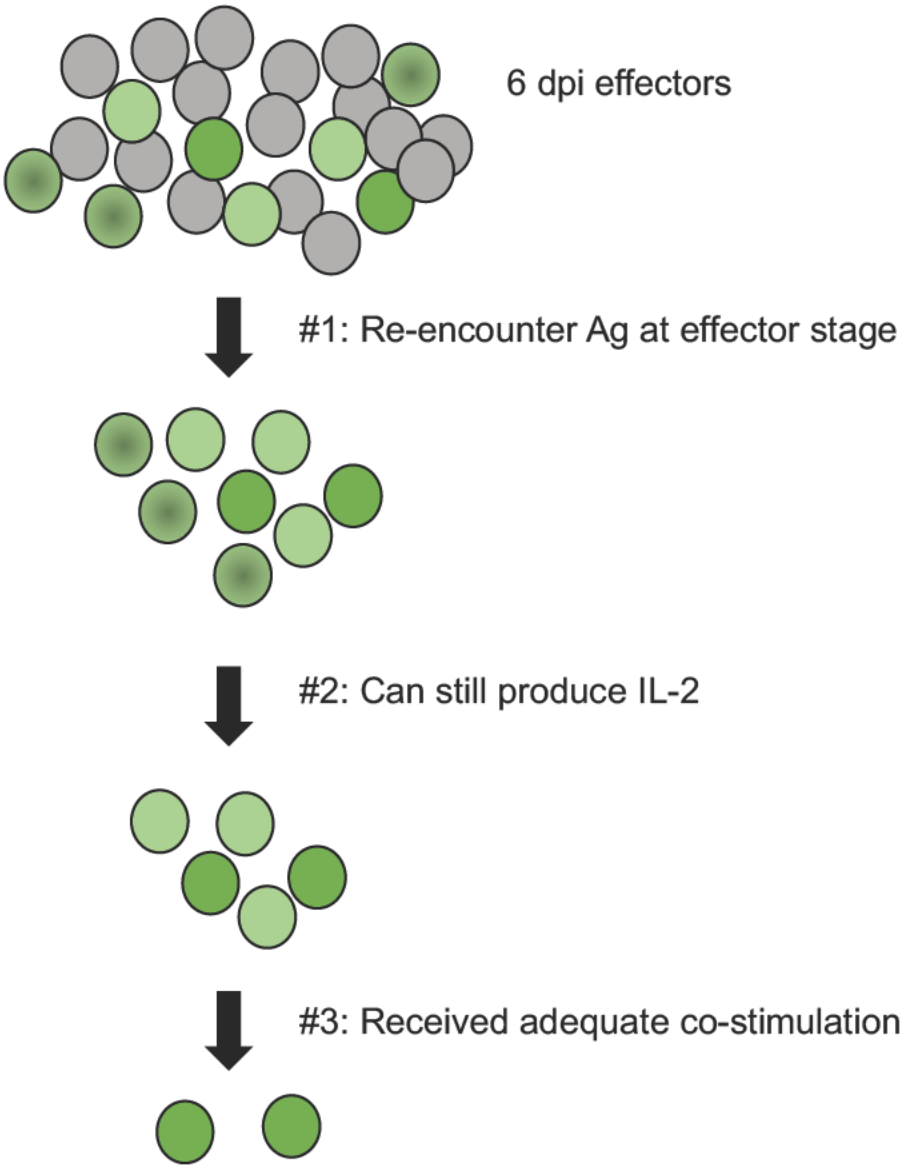


Figure 4.2. Model for selective memory formation of CD4 effector T cells.

This model describes how a late Ag checkpoint may result in the selection of only a few effector CD4 T cells to become memory. First, only a small percentage of cells will re-encounter Ag at the effector stage. Second, of those that recognize Ag, some may be extensively differentiated and therefore unable to produce IL-2, or otherwise unable to respond positively to Ag. Finally, out of the cells that recognize Ag, and are not terminally differentiated, only a limited number may have received co-stimulation in addition to TCR stimulation. This model provides at least three levels of selection, and therefore may severely limit the number of effector cells that can become memory.

is generally heterogeneous at the effector time point and one recent study found that $CD25^{h}$ effector T cells present late in the response preferentially form memory (210). Increased expression of the IL-2 receptor late in the response may ensure the cells that encountered late Ag would effectively use the autocrine IL-2 required for memory formation (1). Bcl-6 was recently shown to promote the metabolic switch required for memory formation (235). Ag at the memory checkpoint may therefore serve to selectively upregulate Bcl-6 late in the response as cells destined to become memory must transition to a self-renewing, resting population. The regulatory effect of Bcl-6 in Th1 cells has been shown to reflect the relative levels of Bcl-6 and T-bet (230). Since no significant increase in T-bet occurred following late Ag stimulation, even a modest increase in Bcl-6 expression may tip the balance in favor of a Bcl-6 mediated gene expression program. The main known promoter of Bcl-6 expression is pSTAT3, which was also induced following late Ag recognition. Future work will determine whether TCR stimulation alone promotes Bcl-6 transcription or if a STAT3-inducing cytokine either produced by the responding T cell or the APC is responsible for increased Bcl-6 expression.

Another interesting avenue of study will be to fully elucidate the differences in downstream signaling in naïve versus effector T cells following Ag stimulation. I chose to assay changes in gene expression after 2 days of culture for several reasons. First, an extensive amount of cell death occurs in the first 2 days of culture without Ag (Figure 3.17B). I reasoned that eliminating many cells that were close to death from analysis might provide insight into the transcription factors and

cytokines responsible for the phenotypic and functional differences seen between memory populations formed with or without late Ag. Second, I did not want to perform the microarray too long after TCR stimulation because I wanted to catch early mediators that initiate memory formation. However, I believe that a microarray conducted 12-24 hours after stimulation might be very informative in highlighting the differences between effector and naïve TCR stimulation.

With the caveat that it certainly does not provide the complete picture, the microarray analysis presented in this thesis does identify some differentially regulated genes that could be promising targets for further study. A couple of cytokines that were uniquely upregulated in 6 dpi effectors receiving Ag stimulation included *IL-10* and *IL-13,* both encoding cytokines that can have anti-inflammatory functions (321, 348). Inhibitory cytokines at this late effector stage may prevent terminal differentiation and aid in the transition to a resting state required for memory formation. Also, IL-10 may promote STAT3 phosphorylation and aid in memory formation as it has been shown to do in CD8 T cells (322). Although, I didn't see an effect when blocking IL-10 in the OT-II cell culture (Figure 3.28E), there was a significant effect in the polyclonal cell culture (Figure 3.28D). The microarray data suggest that OT-II cells are capable of producing IL-10 (Table 3.1) so more work is needed to determine if they in fact secrete IL-10 and if it promotes memory formation of CD4 T cells. Additionally, the TNF receptor family member CD30 was upregulated specifically in effector T cells following Ag stimulation. The ligand for this receptor CD30L is expressed by lymphoid tissue inducer cells which

have been shown to be essential for CD4 memory T cell maintenance (324, 325). The literature on this receptor is not extensive so further work is needed to determine if it potentially plays a role in CD4 memory T cell formation during influenza infection.

Potential Implications for Vaccine Design

It is well established that live infections (and vaccines mimicking them) generate the best immunity (small pox and others) while newer vaccines containing purified proteins with little or no adjuvant induce weak T cell immunity (18, 293). My results suggest that one key reason such vaccines may generate poor memory is because they do not induce sufficient Ag presentation at the effector stage. I tested this using ca.IAV because it is an attenuated virus that is capable of replication in the cooler upper respiratory tract making it the best vaccine candidate for prolonged Ag presentation. However, by 7 dpi with ca.IAV, there was no evidence of Ag presentation and when I introduced Ag/APC 6 days after ca.IAV vaccination it significantly improved memory CD4 T cell generation. This suggests that strategies to provide Ag/APC at a relevant checkpoint for each vaccine may often enhance memory CD4 T cell formation. Indeed in another scenario, an early “boost” strategy efficiently promoted CD8 T cell memory (302). Importantly since I find no need for live virus at the checkpoint, it is possible that an optimal vaccine response could be achieved without the destructive inflammatory milieu generated by replicating live virus or systemic adjuvants.

Although the exact timing and optimum approach for providing Ag at the checkpoint may need to be tailored to the specific vaccine, I anticipate such an approach could be developed to improve vaccines in humans (301) (Figure 4.3).

A potential “early boost” model for vaccination would include the use of either an infectious agent or protein with pattern recognition receptor agonists as the primary immunization. Infectious agents such as LAIV or MVA are most likely to provide pattern recognition receptor ligands to ensure full activation of APC required for the generation of adequate T cell responses (53, 301). The secondary boost could be attained either by targeting Ag to DC populations (349) or providing a large bolus of recombinant Ag with adjuvant to ensure activation of APC (Figure 4.3). Future work in our lab is seeking to determine the efficacy of these vaccination strategies.

Figure 4.3. Model for a Two-Step Vaccine Approach.

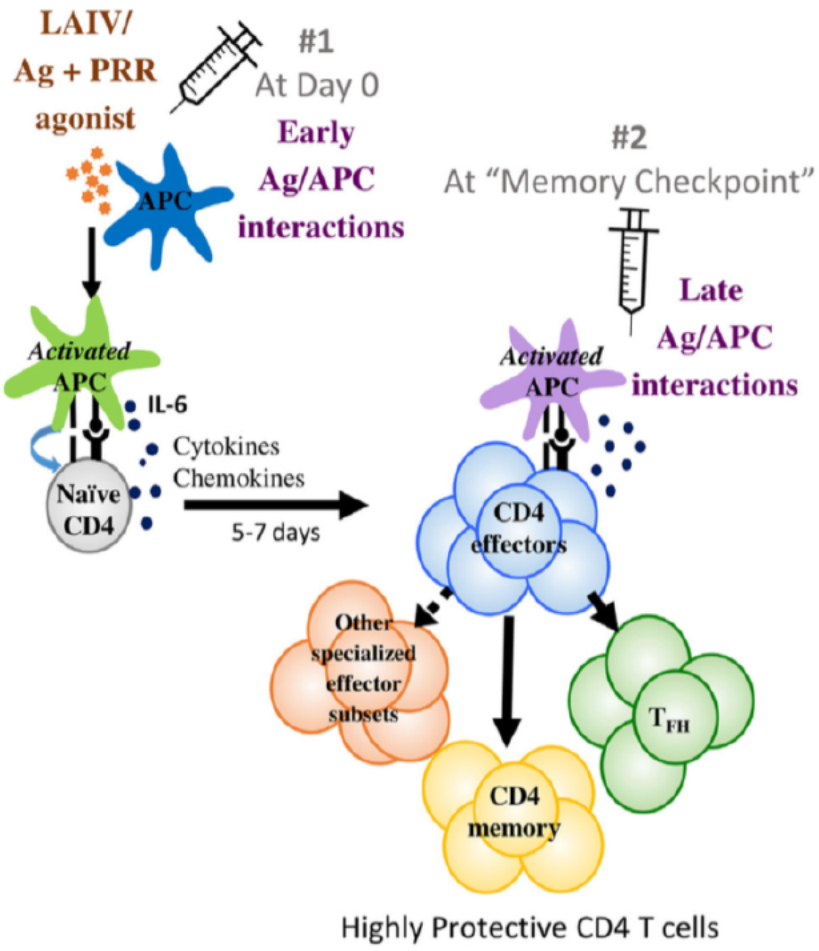


Figure 4.3. Model for a Two-Step Vaccine Approach.

Model outlining a potential two-step vaccine approach. Step one would ideally include immunization with an infectious, attenuated agent that sufficiently activates APC and leads to substantial T cell activation. Step two would include a second immunization about 5-7 days later, specific timing would have to be empirically tested.

Figure adapted from: Devarajan, P., B. Bautista, A. M. Vong, K. K. McKinstry, T. M. Strutt, and S. L. Swain. 2016. New Insights into the Generation of CD4 Memory May Shape Future Vaccine Strategies for Influenza. *Front. Immunol.* 7: 136. Creative Commons Attribution license.

CHAPTER V: REFERENCES

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