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The Role of Signal 3 Cytokine Timing in CD8 T Cell Activation: A Dissertation

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THE ROLE OF SIGNAL 3 CYTOKINE TIMING IN CD8 T CELL ACTIVATION

A Dissertation Presented

By

STINA LISA URBAN

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
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July 16, 2015

IMMUNOLOGY AND MICROBIOLOGY

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List of Publications

Marshall HD, **Urban SL**, Welsh RM. 2011. Virus-induced transient immune suppression and the inhibition of T cell proliferation by type I interferon. *J. Virol.* 85: 5929-39

Welsh RM, Bahl K, Marshall HD, **Urban SL**. 2012. Type 1 interferons and antiviral CD8 T-cell responses. *PLoS Pathog* 8: e1002352

Urban SL, Welsh RM. 2014. Out-of-sequence signal 3 as a mechanism for virus-induced immune suppression of CD8 T cell responses. *PLoS Pathog* 10: e1004357

Urban SL, Berg LJ, Welsh RM. Type 1 interferon primes naïve CD8 T cells to respond to lower concentrations and affinities of cognate ligands. *Manuscript in preparation.*

Abstract

During an acute virus infection, antigen-specific CD8 T cells undergo clonal expansion and differentiation into effector cells in order to control the infection. Efficient clonal expansion and differentiation of CD8 T cells are required to develop protective memory CD8 T cells. Antigen specific cells require 3 distinct signals for their activation: TCR engagement of peptide-MHC (signal 1), costimulation between B7 and CD28 (signal 2), and inflammatory cytokines including IL-12 or type 1 IFN (signal 3). CD8 T cells that encounter antigen and costimulation undergo programmed cell division, but these two signals alone are not sufficient for full effector cell differentiation and survival into memory. CD8 T cells need a third signal for efficient clonal expansion, differentiation into various effector populations, acquisition of cytolytic effector functions, and memory formation. The requirements for signal 3 cytokines in CD8 T cell activation have only been recently described; however, the timing of exposure to these signals has yet to be investigated. During the course of an immune response not all T cells will see antigen, costimulation, and inflammatory cytokines at the same time or in the same order. I sought to examine how the timing of signal 3 cytokines affected CD8 T cell activation. I questioned how the order of these signals effected CD8 T cell priming and subsequent activation, expansion and differentiation.

In order to study the *in vivo* effects of out-of-sequence signaling on CD8 T cell activation, I utilized poly(I:C), a dsRNA analogue, which is known to induce a strong type 1 IFN response. Through the use of various congenic transgenic and polyclonal CD8 T cell populations, in conjunction with adoptive transfer models, specific T cells which had been exposed to poly(I:C) induced environments could be identified and tracked over time. I wanted to characterize how out-of-sequence signaling affected T cell activation immediately after cognate antigen stimulation (4-5hours), and after prolonged exposure to cognate antigen (days-weeks).

Considering type 1 IFN can have both inhibitory and stimulatory effects on CD8 T cell proliferation, and when type 1 IFN provides signal 3 cytokine activity, it has positive effects on CD8 T cell expansion, I wanted to investigate the role of type 1 IFN as an out-of-sequence signal during CD8 T cell activation. We identified a transient defect in the phosphorylation of downstream STAT molecules after IFNβ signaling within poly(I:C) pretreated CD8 T cells. The inability of poly(I:C) pretreated CD8 T cells to respond to IFNβ signaling makes these cells behave in a manner more similar to T cells that only received 2 signals, rather than ones that received all 3 signals in the appropriate order. Consequently, poly(I:C) pretreated, or out-of-sequence, CD8 T cells were found to have defects in clonal expansion, effector differentiation and function as well as memory generation resulting in reduced efficacy of viral clearance.

Out-of-sequence CD8 T cells showed suppression of CD8 T cell responses after prolonged exposure to cognate antigen, but naïve CD8 T cells pre-exposed to poly(I:C) exhibited immediate effector function within hours of cognate antigen stimulation, prior to cell division. Poly(I:C) pretreated naïve CD8 T cells acquired an early activated phenotype associated with alterations of transcription factors and surface markers. Changes in naïve CD8 T cell phenotype are thought to be mediated by poly(I:C)-induced upregulation of self-MHC and costimulatory molecules on APCs through direct type 1 IFN signaling. Inoculating with poly(I:C) enabled naive CD8 T cells to produce effector functions immediately upon stimulation with high density cognate antigen, reduced affinity altered peptide ligands (APLs), and in response to reduced concentrations of cognate antigen. Unlike conventional naïve CD8 T cells, poly(I:C) pretreated naïve CD8 T cells acquired the ability to specifically lyse target cells. These studies identified how the timing of activation signals can dramatically affect the acquisition of CD8 T cell effector function.

This thesis describes how CD8 T cell exposure to activation signals in an unconventional order may result in altered response to antigen stimulation. Exposure of naïve CD8 T cells to type 1 IFN and costimulatory molecules in the presence of self-peptides enabled them to respond immediately upon antigen stimulation. Primed naïve CD8 T cells produced multiple cytokines in response to low-affinity, and low-density antigens, and gained ability to specifically lyse target cells. However, immediate effector function may come at the expense of clonal

expansion and effector cell differentiation in response to prolonged antigen exposure as out-of-sequence CD8 T cells showed reduced proliferation, effector function and memory formation. The findings presented here may seem contradictory because out-of-sequence signaling can prime T cells to produce immediate effector functions and yet cause defects in T cell expansion and effector differentiation. However, these two models ascertained T cell function at different points after antigen exposure; one where functions were evaluated within hours after seeing cognate antigen, and the other showing T cell responses after days of antigen stimulation.

Studies described in this thesis highlight the growing complexity of CD8 T cell activation. Not only do the presence or absence of signals 1-3 contribute to T cell activation, but the timing of these signals also proves to be of great importance. These studies may describe how both latecomer and third party antigen specific T cells behave when and if they encounter cognate antigen in the midst of an ongoing infection. Out-of-sequence exposure to IFN initially stimulates effector function but at the expense of efficient clonal expansion and subsequent memory formation. The immediate effector function that naïve T cells gain during out-of-sequence priming may explain how some individuals are more resistant to superinfections, whereas the impairment in proliferation describes a universal mechanism of virus-induced immune suppression, explaining how other individuals can be more susceptible to secondary infections. Ultimately, results identified here can be applied to developing better and more effective vaccines.

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List of Abbreviations

Ab	antibody
APC	antigen presenting cell
BATF	basic leucine zipper transcription factor
Bcl-2	B cell lymphoma 2
Bcl-3	B cell lymphoma 3
Bcl-6	B cell lymphoma 6
Bcl-xl	B cell lymphoma extra large
Blimp-1	B lymphocyte induced maturation protein 1
CDR	complementarity determining regions
cGAS	Cyclic GMP-AMP synthase
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
DAI	DNA-dependent activator of IFN-regulatory factors
DC	dendritic cell
EEC	early effector cell
EIF2a	eukaryotic translation initiation factor 2a
Eomes	Eomesodermin
ER	endoplasmic reticulum
HCV	hepatitis C virus
HIV	human immunodeficiency virus
IAV	influenza virus
Id	inhibitor of DNA-binding

IFN	interferon
IFNAR	type 1 IFN receptor
IRF	IFN regulatory factor
ISG	IFN-stimulated genes
ISG15	IFN-stimulated protein of 15kDa
ISGF3	IFN-stimulated gene factor 3
ISRE	IFN-stimulated response elements
IκB	inhibitor of κB
JAK	Janus kinase
KLRG1	killer cell lectin-like receptor subfamily G member 1
KO	knock out
LCMV	lymphocytic choriomeningitis virus
LN	lymph node
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MAVS	mitochondrial antiviral signaling protein
MDA5	melanoma differentiation-associated gene 5
MHC	major histocompatibility complex
MPEC	memory precursor effector cell
MS	multiple sclerosis
MTb	*Mycobacterium tuberculosis*
mTOR	mammalian target of rapamycin

Mx	Myxoma resistance GTPases
MyD88	myeloid differentiation primary response gene
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer
NLR	nucleotide-binding oligomerization domain like receptors
NOD	nucleotide-binding oligomerization domain
OAS	2'5'-oligoadenylate synthase
PAMP	pathogen associated molecular patterns
PD-1	programmed cell death 1
pDCs	plasmacytoid DCs
PD-L1	programmed death-ligand 1
PI3K	phosphoinositide 3-kinase
PKR	protein kinase R
pMHC	peptide-MHC
PRR	pattern recognition receptors
PV	Pichinde virus
RIG-I	RNA helicases retinoic acid-inducible gene I
RLR	RIG-I like receptors
SLE	systemic lupus erythematosus
SLEC	short lived effector cell
SOCS	suppressor of cytokine signaling
STAT	signal transducer and activator of transcription

STING	stimulator of IFN genes
TAP	transporters associated with antigen processing
TBK1	TANK-binding kinase 1
Tc	T cytotoxic
TCF-1	T cell factor 1
T_{CM}	T central memory
TCR	T cell receptor
T_{EM}	T effector memory
TF	transcription factors
Th	T helper
TLR	toll like receptors
TNF	tumor necrosis factor
TNFR	TNF receptor
TRAIL	TNF related apoptosis inducing ligand
TRAP	trogocytosis analysis protocol
Treg	T regulatory
TRIF	TIR domain containing adapter inducing IFN β
T_{RM}	T resident memory
USP18	ubiquitin carboxy-terminal hydrolase 18
VACV	vaccinia virus
VSV	vesicular stomatitis virus

CHAPTER I: Introduction

Type 1 IFN

A. Type 1 IFN – an overview

Interferons (IFNs) were the first cytokines to be discovered in 1957, and they were found by their ability to "interfere" with viral replication (1); however, it would be more than 20 years before IFNs could be purified and further characterized (2). IFNs are divided into 3 main categories: Type 1, Type 2 and Type 3 (3-11). Type 1 IFN includes 13 different IFNα genes in humans (and 14 in mice), 1 IFNβ gene, and several other recently identified subtypes (3). Type 2 IFN is comprised of a single type, IFNγ (3, 5, 7). Type 3 IFNs comprise 4 different IFNλ genes but will not be discussed further (3, 4, 7). Each class of IFN binds to its own unique receptor (3, 5, 7, 10-12).

Type 1 IFN is induced in response to a wide range of pathogens including viruses, bacteria and parasites. Type 1 IFN is produced in response to pathogen components called pathogen associated molecular patterns (PAMPs), which are recognized collectively by receptors known as pattern recognition receptors (PRRs) (3, 4, 8, 10, 11, 13). The majority of type 1 IFN is induced by nucleic acids where both activation of RNA and DNA sensors leads to type 1 IFN production (3, 4). The production of IFN during infections is widely accepted to be mostly beneficial for the host, inducing a strong anti-viral response associated with upregulation of a number of different IFN-stimulated genes (ISG) which

develops an anti-viral state (3-5, 7, 10, 12, 13). However, the identification and mechanistic nature of many of these ISGs remain to be elucidated. Many viruses have evolved mechanisms to inhibit type 1 IFN induction and ISGs, including production of decoy receptors, inhibiting PRRs, and blocking IFN signaling pathways (6).

It is established that in addition to beneficial effects, type 1 IFN can also have detrimental effects on the host. Type 1 IFN induction in some virus infections can lead to severe immunopathology (3, 11). Type 1 IFN can also be induced in response to bacterial infections, and similar to virus infections, the induction is not always beneficial. IFN has been shown to mediate pathology, to be detrimental to pathogen clearance, and to reduce survival of hosts in response to different bacterial infections (3-5, 11, 12, 14). Type 1 IFN has also been shown to play a role in several autoimmune disorders and cancer models and similar to the effects type 1 IFN has during viral and bacterial infections, it can also have a positive or negative role (3, 4, 11, 12). The role type 1 IFN plays in diseases and disorders is discussed in further detail in Section D.

Although originally discovered for its ability to inhibit viral replication, it is now appreciated that IFN not only has anti-viral properties but also has a number of immunoregulatory roles on cells of the immune system. Through both direct and indirect effects mediated by other cytokines, type 1 IFN regulates many aspects of the immune system. IFN has a profound effect on antigen presenting cells (APCs) (3, 8). IFN induces maturation of immature dendritic cells (DCs) and

also plays an important role in natural killer (NK) cell activation (3, 8, 15). Further, IFN can have direct effects on CD4 T cells and B cells which can result in lymphocyte differentiation and enhanced antibody production (3). Notably, IFN can have both positive and negative effects on CD8 T cell activation and proliferation (3, 8). Effects type 1 IFN has on cells of the immune system will be discussed in more detail in Sections E, F, and G.

B. Induction and production of type 1 IFN

Type 1 IFN can be produced by most cells in the body, but the PRRs that cells use to induce IFN are different based on pathogen and cell type. There are several types of PRRs: toll like receptors (TLR), retinoic acid-inducible gene I (RIG-I) like receptors (RLRs), and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (3, 4). All cells have the ability to detect PAMPs via PRRs located throughout the cell, but not all cell types express all PRRs (3, 4). TLR4 is expressed on the surface of cells, TLRs 3, 7, 8, and 9 are expressed within the endosomal compartments, and RLRs and NLRs are located in the cytosol (3, 4). Each of these PRRs detects different microbial products. TLR4 recognizes lipopolysaccharide (LPS), a component expressed by bacteria (3, 4). The endosomal TLRs, TLR3, 7, and 8, and 9 detect dsRNA, ssRNA, and CpG DNA, respectively (3, 4). RLRs including RIG-I and melanoma differentiation-associated gene 5 (MDA5) reside in the cytosol and recognize RNA products (3, 4). NLRs recognize different viral, bacterial, and fungal components (3, 4). Cytosolic DNA sensors are the most recently described PRRs and include DNA-

dependent activator of IFN-regulatory factors (DAI) and cytosolic GAMP synthase (cGAS) (3, 4, 16).

An important tool in studying the production of type 1 IFN *in vivo* and *in vitro* is the dsRNA analogue, polyinosinic:polycytidylic acid (poly(I:C)) (17-19). Poly(I:C) triggers and actives TLR3 and MDA5 to a greater extent than other RNA sensors like RIG-I (4, 6, 20).

PRRs utilize different downstream signaling components but all pathways converge upon two transcription factors (TFs) that induce IFN expression, IFN regulatory factor (IRF)3 and/or IRF7 (3, 4, 21). TANK-binding kinase 1 (TBK1) phosphorylates IRF3/7, inducing dimerization and subsequent translocation into the nucleus, allowing it to act on downstream target genes, including IFNα/β (3, 4, 22). Many PRRs, through different downstream adaptor molecules, activate TBK1 to induce IRF3/7 phosphorylation. The endosomal TLRs 7, 8 and 9 utilize the adaptor molecule myeloid differentiation primary response gene (MyD88) and can directly associate with IRF3/7, independent of TBK1 (3, 4). However, the endosomal TLR3 and the surface TLR4 utilize a different adaptor molecule, TIR domain containing adapter inducing IFN β (TRIF), which induces activation of the kinase TBK1 and subsequent phosphorylation of IRF3/7 (3, 4). The cytosolic sensors also activate TBK1 but do so using alternative adaptor molecules. The RLRs use the mitochondria-associated protein mitochondrial anti-viral signaling protein (MAVS) that converges on TBK1 (3, 4). The recognition of cytosolic DNA

utilizes the downstream adaptor stimulator of IFN genes (STING) to help mediate IRF3/7 activation (3, 4).

The majority of cells constitutively express IRF3 but not IRF7 (23). When PRRs are engaged, type 1 IFN including IFNβ and IFNα4 are upregulated by activation of IRF3 (3). IRF7 is also upregulated during the first wave of IFN production where it can subsequently regulate other IFNα subtypes upon further PRR stimulation (3, 23, 24). TLR and RLR expression can also be upregulated by IRF3, which further promotes type 1 IFN production. The upregulation of PRR signaling components is important for type 1 IFN production.

During virus infections, type 1 IFN is produced by a variety of cell types. Most cells that are productively infected produce type 1 IFN and do so through activation of the cytosolic RLRs and DNA sensors. Upon systemic infections, plasmacytoid DCs (pDCs) can produce up to 95% of IFN (6, 25). Uninfected pDCs produce high levels of type 1 IFN through activation of endosomal TLRs 7 and 9 (9, 23, 26, 27). Unlike other cell types, pDCs constitutively express IRF7; therefore pDCs have the ability to produce multiple subtypes of type 1 IFN without having to first induce IRF7 expression (25, 26). Once IFN is produced, it can act in both an autocrine and paracrine fashion. Because many components of the type 1 IFN production signaling pathway(s) are ISGs themselves, type 1 IFN production induces a positive feedback loop, which allows for the production of large amounts of IFN in a short period of time.

C. Type 1 IFN signaling

Canonical cytokine signaling, including that of IFN, generally involves activation of Janus kinase (JAK) - signal transducer and activator of transcription (STAT) pathways (10, 12, 28-30). Cytokine receptor subunits are associated with 1 of 4 different members of the JAK family (30). Upon ligand binding to the receptor, conformation changes induce auto-phosphorylation of JAK proteins resulting in their activation and subsequent phosphorylation of downstream STAT molecules (10, 30). The STAT proteins can then dimerize and translocate into the nucleus to act as TFs (10, 30). Over-activation of the immune system can result in detrimental effects including immunopathology, and therefore, cytokine signaling must be tightly regulated. Cytokine signaling is regulated at every step including cytokine receptor expression, JAK function, and STAT expression and activation (10, 30). JAK proteins must be functional in order to transmit the signals and activate downstream STATs. JAK proteins are regulated by a number of different proteins including the suppressor of cytokine signaling (SOCS) family of proteins (10, 28, 30). SOCS family members can inhibit cytokine signaling through three major mechanisms: 1) binding directly to the receptor and inhibiting STAT molecules, 2) binding to JAK proteins and inhibiting their function, 3) targeting cytokine receptors or JAK proteins for proteasome degradation (10, 28, 31). STAT molecules must also be present in order for cytokine signaling to occur. Therefore, without any one of these components (receptor, JAK and STAT), cytokine signaling can be inhibited.

Even though type 1 IFN comprises multiple types including α, β, ε, κ, ω and others, all type 1 IFN types signal through the same receptor. The type 1 IFN receptor (IFNAR) is comprised of two different subunits: IFNAR1, and IFNAR2 (8-10, 32, 33) (**Figure 1.1**). The type 1 IFN receptor is ubiquitously expressed on cells throughout the body, and therefore, all cells can respond to type 1 IFN signals (33). However, receptor expression is not uniform across all cell types. For example, CD44hi CD8 T cells were found to exhibit higher expression levels of IFNAR1 than CD44lo CD8 T cells (34). Receptor expression can be upregulated or downregulated by many different mechanisms. One study found that IFNγ could actually induce IFNAR1 and IFNAR2 mRNA induction in a cell line (35). However, more is known about downregulation of the type 1 IFN receptor. Upon ligand binding to the receptor, the IFNAR complex is internalized, ubiquitinated and degraded (36).

Each receptor component has an associated JAK protein, through which it transmits signals. IFNAR1 is associated with TYK2, and IFNAR2 is associated with JAK1 (8, 12, 30, 32, 33) (**Figure 1.1**). In addition, IFNAR1 is stabilized by its associated JAK protein TYK2 (33, 37). Therefore, without TYK2 expression, IFNAR1 expression levels are really low. JAK1 and TYK2 are important components in the canonical IFN signaling pathway. IFN binding to its receptor results in receptor dimerization, resulting in auto phosphorylation of JAK proteins, thus allowing STAT molecules to dock. The activated JAKs phosphorylate

Figure 1.1: Type 1 IFN Receptor signaling
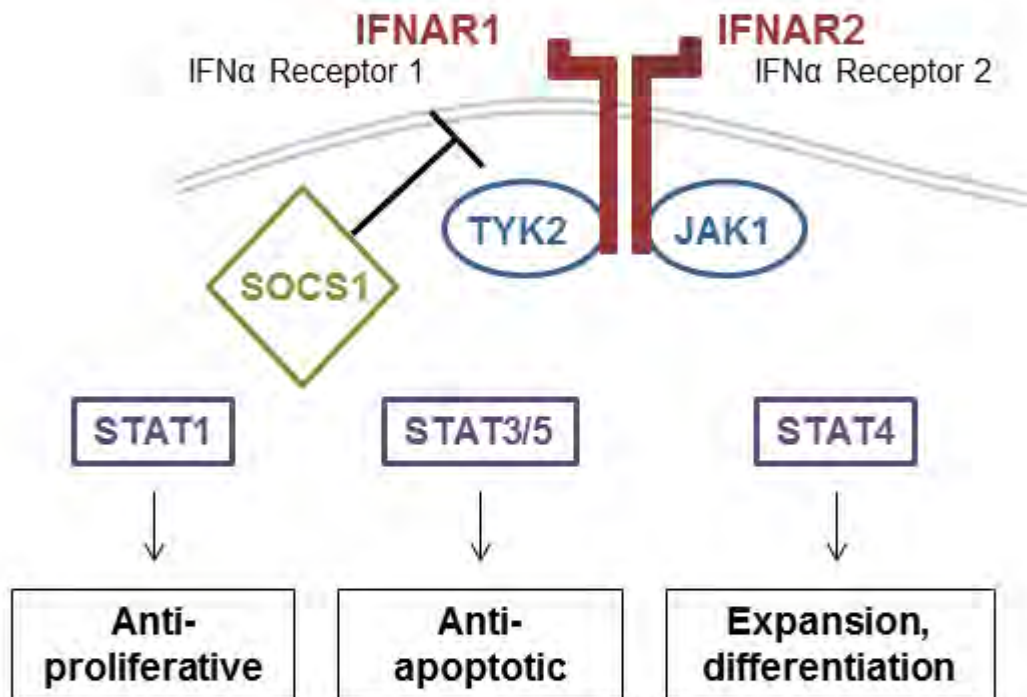
Figure 1.1: Type 1 IFN Receptor signaling

The type 1 IFN receptor is comprised of two subunits: IFNAR1 and IFNAR2. Each receptor component is associated with a specific intracellular Jak protein. IFNAR1 is associated with TYK2 and IFNAR2 is associated with JAK1. Type 1 IFN receptor signaling is inhibited by SOCS1. IFN signaling can act through different downstream STAT proteins ultimately affecting cell fate.

associated STATs, inducing their dimerization and subsequent translocation into the nucleus and allowing for regulation of gene transcription.

The most widely recognized pathway type 1 IFN signaling acts on is through activation of STAT1 and STAT2. This canonical pathway results in dimerization of STAT1 and STAT2 allowing for association with a third transcription factor IRF9, which together comprise the transcriptional regulator, IFN-stimulated gene factor 3 (ISGF3) (3, 4, 9, 10, 12, 30, 32, 33). ISGF3 has been shown to be important for the regulation of multiple ISGs by binding to IFN-stimulated response elements (ISRE) in the ISG promoters (3, 4, 10, 12, 30, 32, 38). Therefore, type 1 IFN signaling through the canonical JAK/STAT pathway induces activation of ISGF3, allowing for the regulation of hundreds of genes that initiate an anti-viral state.

However, STAT1/2 are not the only STATs that have been shown to be activated downstream of the type 1 IFN receptor. In fact, almost all STATs can be activated in response to IFN stimulation and depending on their binding partner, each STAT can have very different effects on cells (3, 8, 32) (**Figure 1.1**). Moreover, it has recently been appreciated that the JAK/STAT pathways are not the only signaling pathways induced by type 1 IFN. The mitogen-activated protein kinase (MAPK) pathway, phosphoinositide 3-kinase (PI3K) and mammalian target of rapamycin (mTOR) pathways have all been linked to IFN signaling (3, 9, 32).

Cytokine signaling is tightly regulated to prevent over-activation of the immune system and needs to be induced and inhibited at the right times. Type 1 IFN signaling is augmented by the upregulation of STAT1 and IRF9, both of which are ISGs themselves (12, 39). Small amounts of IFN can act on cells to activate ISGF3, which can upregulate components of itself thus augmenting IFN signaling. Therefore, type 1 IFN signaling can positively regulate type 1 IFN signaling.

On the other hand, there are many mechanisms to dampen IFN signaling. Defects in ability to inhibit cytokine signaling can lead to over activation of cells and may cause immunopathology due to chronic inflammation. There are a number of different mechanisms that suppress type 1 IFN signaling, including downregulation of the receptor, by internalization and degradation, and upregulating proteins that negatively regulate signaling (10, 28, 36, 37). Some other of these negative regulators include SOCS family members, ubiquitin carboxy-terminal hydrolase 18 (USP18) and miRNAs (9, 10, 12). SOCS1, SOCS3 and USP18 all negatively regulate IFN signaling. SOCS1 inhibits type 1 IFN signaling by binding to the receptor associated JAK protein TYK2, while other suppressor proteins, including USP18, displace JAK proteins (10, 12, 33, 37). These suppressors are induced downstream of type 1 IFN signaling. Thus, in addition to IFN inducing positive feedback mechanism, type 1 IFN signaling also induces negative feedback mechanisms. Therefore a fine line exists

between increasing and deceasing responsiveness to IFN signals and aberrant signaling can have detrimental effects.

D. Type 1 IFN function in diseases and disorders

Type 1 IFNs were originally identified for their ability to interfere with virus replication. IFNs have been shown to inhibit viral replication mainly through induction of ISGs. ISGs inhibit all stages of viral replication, including attachment, fusion, transcription, translation, and release (6, 7, 10, 38). Some of the most well-known ISGs include IFN-stimulated protein of 15kDa (ISG15), Myxoma resistance GTPases (Mx), 2'5'-oligoadenylate synthetase (OAS) proteins and protein kinase R (PKR) (7, 9, 38). ISG15 is a protein that, like ubiquitin, attaches to other proteins, which may affect protein function (7, 10). This is an IFN-regulated response and is referred to as ISGylation. ISGylation does not promote protein degradation like K48 linked ubiquitination does but instead can prevent degradation of proteins or alter their function (7, 10). Several targets of ISG15 have been identified and include many proteins in the type 1 IFN signaling pathway, such as JAK1, STAT1, and RIG-I (7). Mx proteins block virus replication at multiple steps including transcription and translation (7, 40). The inhibitory mechanisms of these proteins are still being investigated and may be different based on the location of the Mx protein and specific virus infection (7, 40). OAS proteins activate RNaseL, which degrades RNA. Degradation of viral RNA inhibits replication and can ultimately contribute to activation of PRRs including RIG-I and MDA5, further promoting IFN production and an anti-viral

state (7, 16). PKR inhibits translation of both viral and host proteins by inhibiting eukaryotic translation initiation factor 2a (EIF2a) via phosphorylation (7). This is by no means a comprehensive list of ISGs with anti-viral activity but just names a few of the most well-known.

Because of the potent anti-viral effects that IFN and ISGs can have, it is no surprise that viruses have developed mechanisms to inhibit IFN production and ISG functions. Similar to the ability of ISGs to inhibit almost every part of the virus life cycle, viruses have developed ways to inhibit almost every part of the type 1 IFN pathway, from its induction and production, to the specific anti-viral effects of some ISGs (6, 41). Viral proteins can inhibit the PRRs and their associated adaptors, block TBK1 kinase activity, and inhibit IRF3/7 to prevent or reduce type 1 IFN production (6). Viruses can also inhibit IFN signaling by affecting IFN receptor expression, associated JAK/STAT proteins, and other transcription factors including IRF9, to inhibit IFN-induced genes (6). Even after ISGs are produced, viruses have developed mechanism to inhibit specific ISGs, including the aforementioned PKR and ISG15.

Although known for anti-viral properties, type 1 IFN can have either beneficial or detrimental effects on the host depending on the infection. During most acute virus infections, type 1 IFN production has positive effects on both viral clearance and survival of the host; however, that is not always the case. Influenza (IAV) infections can cause a wide range of symptoms ranging from mild effects to fatality. Mouse strains have a wide range of susceptibility to IAV

infections and in one study, higher levels of type 1 IFN correlated with increased susceptibility of mice to IAV infections (42). pDCs were found to produce excessive type 1 IFN, which caused increased inflammation into the lungs during infection, causing damage to the lung epithelia and increased mortality (42). These studies suggest that although IFN primarily has anti-viral effects, that too much IFN can be detrimental to the host.

In addition to IFN contributing to susceptibility of mice to an acute lethal IAV infection, IFN has also been linked to detrimental effects in a number of chronic viral infections (3, 5, 9, 12). IFN-induced increased immunosuppressive effects can inhibit control of the virus, or over-activation of the immune response may lead to excessive inflammation and immunopathology (3). Examples are seen in individuals infected with human immunodeficiency virus (HIV), where rapid progressors showed an increased IFN signature compared to non-progressors; however, the mechanism has yet to be identified. One explanation for the difference could be from increased production of IFN-induced immunosuppressive cytokines like IL-10 (3, 9). Recent studies using the lymphocytic choriomeningitis virus (LCMV) clone 13 model system identified IFN as having detrimental effects during chronic infection. IFNAR knock out (KO) animals and anti-IFNAR1 antibody blocking studies found reduced expression of inhibitory molecules, including IL-10 and programmed death-ligand 1 (PD-L1), resulting in viral clearance and restoration of exhausted T cells (9, 43-45). Other chronic infections including hepatitis C virus (HCV) and *Mycobacterium*

*tuberculosi*s (MTb) are also associated with increased IFN signatures, which correlate with disease severity (3, 5, 9, 12).

Type 1 IFN is also produced during bacterial infections by activation of TLRs, but its role is not always beneficial. Type 1 IFN impaired clearance of *Listeria monocytogenes* and MTb (3, 5, 11). Although the exact mechanisms as to how IFN production can be detrimental during bacterial infection is still being studied, and may have multiple and unique roles based on the infecting pathogen, a few observations have been made. The detrimental effects of type 1 IFN may be attributed to the induction of lymphocytes apoptosis, thus suppressing the adaptive response to the bacterial infection. Another hypothesis that is described identifies type 1 IFN inhibiting the IL-17 production, resulting in defective neutrophil recruitment and bacterial clearance (3, 4). Type 1 IFN-induced upregulation of immunosuppressive cytokines and inhibitory molecules like IL-10 and PD-L1, may contribute to detrimental effects during some bacterial infections (3, 4, 12). IL-10 and PD-L1 may suppress T cell function, which could result in reduced pathogen clearance.

In some cases, type 1 IFN production is beneficial during bacterial infections. IFNAR KO mice showed increased bacterial load and reduced survival in response to infection with *Streptococcus pneumonia* and *Escherichia coli* (3, 4). Type 1 IFN can have beneficial effects during bacterial infections by upregulating some pro-inflammatory cytokines, including tumor necrosis factor (TNF), which has been shown to play a role in controlling infections (3, 14). The

diverse functions of IFN are not limited to bacterial infection, as fungal infections and certain parasitic infections also show different requirements for type 1 IFN in pathogen clearance (3, 9). Therefore, type 1 IFN can have either detrimental or protective roles in response to pathogenic infections.

Type 1 IFN has been used to treat many types of cancers, including some types of leukemia, lymphomas, and some solid tumors (11, 46). In this case, IFN acted as a positive regulator of the immune system, in part by acting on DCs to enhance their ability to cross present antigens and activate CD8 T cells. Type 1 IFN activation of NK cells may also contribute to control of tumor.

Type 1 IFN plays a profound role in autoimmune disorders, and similar to effects seen during pathogenic infections, it can have a positive or negative effect. Some autoimmune disorders are improved if type 1 IFN is inhibited, whereas others will benefit from type 1 IFN treatment (4, 12). Patients affected with systemic lupus erythematosus (SLE) have increased levels of type 1 IFN in the blood compared to healthy donors (11). Type 1 IFN can positively activate T cells and B cell responses, which can ultimately contribute to autoimmune disorders associated with autoantibody production, similar to what is seen in SLE. SLE is not the only autoimmune disorder in which IFN has shown to play a pathogenic role. The skin inflammatory disorder psoriasis has also been linked to high levels of type 1 IFN (4). Similar to what is seen with patients with SLE, IFN is thought to activate the pathogenic T cells, which contribute to the immune-mediated skin lesions (4). Blocking type 1 IFN in both SLE and in psoriasis

patients reduced severity of the diseases; however, IFN is not the only thing that contributes to these disorders and additional work needs to be done in order to develop new therapies for these disorders.

Type 1 IFN therapy is prescribed for patients with relapsing-remitting multiple sclerosis (MS) and has generally shown positive effects with treatment (9, 11). In this situation, IFN is thought to have a suppressive role on the pathogenic T cells that mediate the disease, possibly by inhibiting the recruitment of T cells to the brain (4, 9). Additionally, type 1 IFN induction of IL-10 may contribute to suppression of the immune response thus limiting the auto-reactive cells and subsequent pathological consequences (4, 11). Because type 1 IFN can have both positive and negative effects on lymphocyte activation, it is conceivable that IFN may play different roles during various stages of autoimmune disorders.

Even though type 1 IFN was discovered for anti-viral properties, it has a strong role in many other diseases and disorders. Depending on the infecting pathogen, or the autoimmune disorder, IFN can have detrimental or protective effects. The ability of IFN to have both stimulatory and inhibitory effects on lymphocytes allows for the wide range of results seen during disease.

E. Effects of type 1 IFN on cells of the immune system

Type 1 IFN regulates immune cell function and can have both direct and indirect effects on lymphocyte populations. Type 1 IFN is important in activation

of immature DCs, whereby it will promote the upregulation of Class I and Class II major histocompatibility complex (MHC) molecules and the costimulatory molecules CD80 and CD86 (B7.1 and B7.2) (32). Moreover, IFN increases the ability of DCs to present antigen, including cross presentation (32, 47). Migration of DCs to the lymph nodes is also affected by type I IFN. Section F, Type 1 IFN and dendritic cells, will discuss these details further.

IFN can play both stimulatory and inhibitory roles in CD8 T cell activation. It can induce T cell apoptosis, but it can also promote development of effector function and clonal expansion. Section G, Type 1 IFN effects on CD8 T cells, provides further detail on this subject.

Both indirect and direct effects of type 1 IFN are important for NK cell activation. Direct effects of IFN increase the cytolytic capability of NK cells by inducing cytotoxic effector molecules like perforin and granzymes, which promote cytolytic capability (8, 15, 32, 48-51). Some studies identified IL-12 production early after viral infections as the main driver of NK cell production of IFNγ, while type 1 IFN contributed more to NK cell cytotoxicity (52). However, studies with other infection models, including influenza, identified a direct role for IFN in NK cell production of IFNγ, not IL-12 (53). As mentioned earlier, IFN can mediate distinct functions in cells by acting through different downstream pathways. STAT1 was required for IFN mediated acquisition of cytolytic effector function, whereas STAT4 induced granzyme B and IFNγ expression in IFN-activated NK cells (52-54).

Type 1 IFN has indirect effects on NK cell activation. For example, IFN induces production of IL-15, which has positive effects on NK cell accumulation/proliferation (9). The positive effects of IFN-induced IL-15 mediated survival of NK cells were also shown to require STAT1 signaling (51). Activation of NK cells was also associated with increased blastogenesis, which was mediated by type 1 IFN acting through IL-15 (55).

NK cells are widely recognized for their ability to kill virus-infected and tumor cells. However, recent studies showed that NK cells can play a regulatory role during virus infections by specifically lysing activated T cells (56, 57). Type 1 IFN activation of NK cells was important for this regulatory phenomena. On the other hand, type 1 IFN was shown to act on CD8 T cells to prevent early NK cell mediated lysis (58, 59). The ability of IFN to both activate NK cells and inhibit T cells from NK cell lysis have important implications during infections. Although activated NK cells have the ability to specifically lyse activated T cells, killing of T cells may not be in the best interest of the host during infection.

Both CD4 T cells and B cells are affected by direct type 1 IFN signaling. When T cells lacked the type 1 IFN receptor, mice immunized with chicken gamma globulin showed significant reductions in the antibody (Ab) response, suggesting that IFN signaling of T cells promotes B cell help (60). Type 1 IFN affected the differentiation of CD4 T cells where it promoted T helper (Th1) differentiation of CD4 T cells, resulting in increased IFNγ production (32, 61) and it suppressed differentiation of Th2 and Th17 cells (62). Type 1 IFN did not

always enhance CD4 T cells proliferation. Direct IFN promoted CD4 T cell survival in response to LCMV infection, but bacterial infections did not show IFN-induced enhanced clonal expansion of CD4 T cells (62, 63). In addition to CD8 T cells, IFN induced apoptosis of memory phenotype (CD44hi) CD4 T cells (64). Type 1 IFN signaling inhibited proliferation and activation of regulatory T cells (Tregs) during acute LCMV infections (65). Thus, IFN can have both stimulatory or inhibitory effects on CD4 T cell survival and clonal expansion, and either promote or suppress differentiation of T-helper subsets. The ability of IFN to mediate these differential effects on CD4 T cells may be attributed to the ability of type 1 IFN to activate multiple signaling pathways including JAK/STAT, MAPK and mTOR (3, 32).

Although there has been evidence that IFN can decrease the survival of B cells, there is growing evidence that IFN also has stimulatory effects on B cells. Early work indicated that increased B cell function was due to indirect effects of IFN via activation of DCs (66). However, as stated earlier, type 1 IFN increased B cell responses by acting directly on CD4 T cells and B cells (60, 67). Antigen specific B cells showed increased activation and early activation markers upon type 1 IFN signaling (67). IFNAR KO B cells showed reduced activation and expansion compared to WT B cells in response to vesicular stomatitis virus (VSV) infection (68). Moreover, type 1 IFN increased antibody production and promoted class switching in B cells during IAV and VSV infection (68). Together,

these data indicate IFN may have more B cell stimulatory properties than previously thought.

F. Type 1 IFN and dendritic cells

DCs are a type of professional APC that presents antigens to both CD4 and CD8 T cells. DCs play an important role during viral infections, as they serve as a bridge between the innate and adaptive immune system.

DCs have the ability to present peptides on MHC Class I and Class II proteins, to provide recognition for CD8 and CD4 T cells respectively. DCs are one of only a few types of APCs, which have the ability to present antigens to both CD4 and CD8 T cells. Most cells have the ability to present antigens to CD8 T cells, but only specialized APCs present to CD4 T cells. Class I and II MHC proteins present peptides from different compartments within the cell. Class I presented peptides are derived from the cytosol, whereas Class II presented peptides are from endocytic vesicles. Pathogens that replicate within the cytosol will have peptides presented on Class I MHC molecules to be presented to CD8 T cells. Activated CD8 T cells that recognize cognate peptide-MHC (pMHC) complexes have the ability to specifically lyse those target cells. Extracellular pathogens are endocytosed and form vesicles where antigen can be loaded onto Class II MHC molecules for presentation to CD4 T cells. CD4 T cells help other cells, including B cells, to produce antibodies, and help CD8 T cells, often indirectly, by activating DCs. A specialized pathway called cross-presentation is a

way in which extracellular antigens can be processed and presented on MHC Class I molecules for CD8 T cell recognition.

Professional APCs, including DCs, macrophages and B cells, utilize MHC Class II molecules to present peptides derived from intracellular vesicles. Newly synthesized MHC Class II molecules from the endoplasmic reticulum (ER) will be transported and will associate with endosomal compartments (69). Antigens that are endocytosed are broken down by different proteases such that they can bind to MHC Class II molecules to be efficiently presented (69). MHC complexes that do not have a bound peptide are less stable and are rapidly degraded. Once peptide is bound, the pMHC complex can be presented on the surface of cells.

Class I MHC molecules present peptides derived from the cytosol. Pathogens and self-proteins in the cytosol are broken down by the proteasome, a large multiple subunit complex with protease activity (69). Essentially, proteins are fed into the active site of the proteasome and come out as short peptides. There are two distinct forms of the proteasome, the constitutive proteasome, which is present in all cells, and the immunoproteasome, which is only induced in cells stimulated by IFN. The immunoproteasome has three subunits that are induced upon IFN stimulation, β1i, β2i, and β5i (also known as LMP2, LMP7, and MECL-1 respectively) (70). By altering these catalytic subunits, the specificity of the peptide cleavage is altered, allowing the peptides that are released to be more efficiently presented on Class I MHC (70). Upon protein degradation, peptides derived from the cytosol are transported into the ER, mediated by two

enzymes, transporter associated with antigen processing 1 and 2 (TAP1 and TAP2). Peptides can then be loaded onto Class I MHC molecules for presentation on the surface of cells. Without associated peptides bound, empty Class I complexes will be retained in the ER and if a peptide does not bind, eventually Class I molecules will be degraded (69). Cells deficient in TAP1 and/or TAP2 show markedly reduced level of Class I MHC on the surface of cells. Only peptides that do not require TAP-mediated transport are able to be presented.

MHC Class I complexes also have the ability to present peptides from exogenous sources. This is referred to as cross-presentation, where exogenous proteins can be taken up via endocytosis, transferred into the cytosol for degradation by the proteasome, transferred to the ER via TAP and loaded onto Class I (69). Cross-presentation occurs in a number of cell types, but a subset of DCs, CD8α+ DCs, are specialized in the process (71). Cross-presentation is important for CD8 T cell activation in response to pathogens that do not productively infect antigen presenting cells (71).

DCs were originally placed into 2 different types; conventional DCs and non-conventional or plasmacytoid DCs (72). However, now there is evidence for significantly more DC heterogeneity in terms of function, location, and phenotype. Within conventional DC populations, there are migratory and lymphoid DCs. Migratory DCs are found in many locations in the body, including the skin, lungs, gastrointestinal tract and liver, and have the ability to migrate into lymphoid tissues (73). Lymphoid DCs are only found in lymphoid organs and are divided

further into subsets based on their expression of CD4 and CD8 (72, 73). All DCs express CD11c to some degree, and, based on expression of several key markers including CD4 and CD8α, DCs can be distinguished further (72, 73). DCs that express CD8α, are generally low in CD11b expression, and are termed CD8α DCs (71). As eluded to earlier, CD8α+ DCs have the ability to cross-present antigens, thus promoting activation of CD8 T cells. CD8α+ DCs are also major producers of IL-12 as compared to some other DC subsets (71). pDCs, as previously mentioned, are major producers of type 1 IFN via activation of TLRs 7 and 9. pDCs can be distinguished from other DC subsets by expressing lower levels CD11c, B220, and PDCA-1 (also known as BST2 and Tetherin) (47).

Upon pathogen infection, DCs are activated by encountering PAMPs and take up and process antigens for presentation to T cells. The activation of PRRs results in increased antigen processing and presentation, upregulation of costimulatory molecules, and upregulation of chemokine receptors that are utilized for DC migration into secondary lymphoid organs (74, 75). After antigen is taken up at the site of infection, DCs migrate to the nearest peripheral lymphoid organ where they present antigen to T cells (75, 76). In some cases the original DC that picks up antigen may not be the DC that presents it to the T cell, as migratory DCs can transfer antigen to resident lymphoid DCs (75). As early as 24 hours after infection with LCMV, CD11c+ DCs were found in T cell areas of the spleen (74).

As stated before, DCs are specialized APCs that can bridge the innate and adaptive immune system by activating T cells, both helper and cytotoxic. Naïve T cells require 3 distinct signals for their full activation: pMHC–T cell receptor (TCR) interaction (signal 1), costimulation between B7-CD28 (signal 2) and inflammatory cytokines (signal 3). DCs facilitate naïve T cell activation by expressing both Class I and Class II MHC molecules and by expressing a range of costimulatory molecules, including the canonical signal 2 molecules B7.1 (CD80) and B7.2 (CD86). However, costimulatory molecules are not constitutively expressed by immature DCs; instead DCs must be activated to upregulated these molecules. The triggering of PRRs and subsequent production of IFN induces a positive feedback autocrine loop signaling through IFNAR leading to expression of costimulatory molecules like B7 and upregulation of both MHC Class I and Class II expression on the surface of cells. IFN can induce expression of immunoproteasome catalytic subunits to increase the likelihood of MHC Class I peptide presentation. Type 1 IFN, therefore, plays an important role in activating DCs, inducing differentiation of immature DCs into mature DCs, capable of presenting antigen, providing costimulatory signals and activating T cells.

The costimulatory molecules DCs present can affect T cell activation in both positive and negative ways. B7:CD28 and CD40:CD40L interactions provide activation signals to T cells, whereas B7 interaction with cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), PD-L1 and/or PD-L2 interacting with programmed

cell death 1 (PD-1) inhibit T cell function (77). In addition, when a naïve T cell encounters its cognate pMHC expressed on an APC without B7:CD28 costimulation, T cell inactivation or deletion can occur. Therefore, the costimulatory molecules that APCs express, or lack of costimulatory molecule expression, can have dramatic effects on T cell activation.

G. Type 1 IFN effects on CD8 T cells

Type 1 IFN can exert effects on CD8 T cells both directly and indirectly. Indirectly, IFN acts on DCs to increase Class I presentation, increase costimulatory molecules and produce inflammatory cytokines, all of which help to promote CD8 T cell activation. On the other hand, type I IFN acts directly on CD8 T cells as a signal 3 cytokine and promotes clonal expansion and differentiation (62, 78-81). Type 1 IFN can have both pro- and anti-apoptotic and proliferative effects on CD8 T cells.

Type 1 IFN induced during the early stages of a virus infection will induce apoptosis of memory phenotype CD4 and CD8 T cells resulting in T cell attrition (64, 82-84). Naïve CD8 T cells are also susceptible to early attrition seen after viral infections. The inflammatory environment induced by administration of poly(I:C), mimicking an acute virus infection, also showed reduction in T cell number. Although the mechanism has not been fully identified, IFNAR KO mice were largely resistant to poly(I:C)-induced attrition of CD44hi CD8 T cells, implicating a role for type 1 IFN (82). IFN-induced TNF or TNF-related apoptosis

inducing ligand (TRAIL) upregulation was not contributing to early attrition of T cells. However, it was discovered that the IFN-induced attrition was partially mediated by Bim, as day 3 LCMV-infected Bim KO mice showed lower reduction in CD44hi CD8 T cells compared to WT mice (64). The loss of memory phenotype cells during the beginning of an infection may allow for a more diverse T cell response.

Type 1 IFN has been shown to exert both pro-proliferative and anti-apoptotic effects in CD8 T cells. Depending on how one defines proliferation, the meaning can be very different. Proliferation may refer to an increase in cell number, or simply as cell division, which does not always result in an increase in cell number. The literature showing IFN having pro-proliferative effects on T cells, mostly refers to proliferation as an increase in number. CD8 T cells that lacked IFNAR did not show defects in division, but rather in accumulation, suggesting that rather than promoting cell division, IFN has pro-survival effects on CD8 T cells (79).

TCR and costimulation upregulate the anti-apoptotic molecules B-cell lymphoma 2 (Bcl-2) and B-cell lymphoma extra large (Bcl-xL) in T cells. Investigating the role of type 1 IFN in survival of activated CD8 T cells, *in vitro* studies revealed similar levels of Bcl-2 and Bcl-xl comparing CD8 T cells treated with or without IFN (85). Addition of adjuvants or IL-12 to activated T cells *in vitro* induced upregulation of B-cell lymphoma 3 (Bcl-3) expression (86, 87). Adjuvant–induced survival of activated T cells correlated more with Bcl-3 expression than

with Bcl-2 or Bcl-xL expression (88). Therefore, the increase in Bcl-3 is thought to promote the increased accumulation of T cells stimulated with IFN vs. those without IFN signals. One study, however, suggests that IFN increases the number divisions activated CD8 T cells undergo by maintaining expression of the high affinity IL-2 receptor (89), thus contributing to enhanced accumulation.

Interestingly, IFN can affect CD8 T cell expansion by acting through other T cell subsets. Type 1 IFN signaling in Tregs was found to be important for the expansion and function of virus-specific CD4 and CD8 T cells during LCMV infection, as IFN inhibited Treg proliferation and activation. IFN inhibition of Tregs resulted in reduced regulatory function, allowing for greater expansion of other T cell subsets. These data indicate that inhibition of Tregs by type 1 IFN during acute infections is important for generation of efficient T cell responses (65).

These distinct and contradictory effects that type 1 IFN can have on CD8 T cells are most likely mediated by the ability of IFN to activate multiple downstream signaling pathways, including JAK/STAT, PI3K, and mTOR (3, 32). The combination of STAT molecule(s) that are phosphorylated and translocated into the nucleus may control the outcome of CD8 T cell activation. Activation of STAT1 downstream of type 1 IFN receptor signaling generally has anti-proliferative effects on CD8 T cells (54, 90-92). In contrast, type 1 IFN-mediated activation of STAT3 and/or STAT5 has anti-apoptotic and pro-mitogenic effects (93, 94). Type 1 IFN signaling via STAT4 promotes both the acquisition of effector function, including IFNγ production, and clonal expansion (52, 81, 95).

STAT1 is an IFN-inducible gene whose function in CD8 T cells is anti-proliferative (39, 91). Therefore, how can type 1 IFN, a signal 3 cytokine, exert positive clonal expansion effects if it induces STAT1 and anti-proliferative effects? Recent studies showed that during LCMV infection, virus-specific CD8 T cells had decreased total STAT1 levels and increased STAT4 levels, thereby promoting effector T cell differentiation and clonal expansion over anti-proliferative effects (95). Thus, type 1 IFN can have both inhibitory and stimulatory effects on CD8 T cell proliferation, and when type 1 IFN provides signal 3 cytokine activity, it has positive effects on CD8 T cell expansion.

CD8 T cells

H. CD8 T cells and virus infections – an overview

During the course of an immune response to a pathogen, the innate and adaptive immune systems work together in order to control infections. The innate immune system initiates an inflammatory response, which helps to activate the adaptive immune response. CD8 T cells are especially important for clearing intracellular pathogens, including viruses and certain bacteria.

There are several distinct phases of CD8 T cell activation during viral infections. Once naïve CD8 T cells see signals needed for full activation they undergo several rounds of division resulting in clonal expansion and differentiation into effector cells. CD8 T cell effectors comprise a heterogeneous

population of cells, most of which have the ability to produce multiple effector functions upon encountering cognate ligand. Effector CD8 T cells will produce multiple cytokines including IFNγ, TNF, and sometimes IL-2 (96). Effector CD8 T cells can also specifically lyse target cells through the activation and secretion of effector molecules including perforin and granzymes (97). After antigen is cleared, the majority of T cells die during the contraction phase, but there is a small but generally stable population that remains and forms memory. Memory T cells have reduced requirements for activation, in that they can respond immediately to encountering antigen again, without the need for costimulation or signal 3 cytokines, and produce effector functions prior to cell division. Rapid activation allows CD8 T cells to control and clear the infection faster, resulting in reduced pathology (98-103).

I.   Generation of CD8 T cells

Similar to other hematopoietic cells, T cell development begins in the bone marrow. Progenitors develop in the bone marrow and migrate to the thymus where they proliferate and differentiate into mature T cells. TCRs are generated by V, D and J gene segment rearrangement with insertions or deletions at the junctions, resulting in a variety of distinct TCRs (104). The junctions form the highly variable regions of the TCR, termed complementarity determining regions (CDR), which bind the pMHC complex. T cells are positively selected in the thymus based on their ability to bind to self-peptide MHC complexes with a certain degree of affinity. During positive selection, CD4 and CD8 T cell lineage

commitment occurs based on the ability of TCRs to bind to Class I or Class II MHC molecules (105, 106). TCRs that bind to self-peptide MHC complexes with too high affinity will be deleted in a process termed negative selection (105, 106). The deletion of T cell clones that react with self with too high affinity result in self-tolerance and therefore limit the potential for T cells to induce autoimmunity once T cells exit the thymus and migrate into the periphery. The majority of T cells die during thymic selection, but the select few that survive through positive and negative selection migrate to the periphery as a mature naïve T cells (105, 106) .

The end result of thymic selection is the generation of a wide range of T cells with different specificities. T cells have different affinities for both self- and foreign-ligands. In the mouse, there are estimated to be $\sim 2\text{x}10^6$ clones (of about 10 cells each) per spleen (107). Although this number is far lower than the possible receptor combinations, it still allows for a wide diversity. Studies identified that the number of precursors specific to an individual peptide to be anywhere from 80-1200 cells/mouse (108). Further studies identified that for a specific pathogen, containing multiple antigens, the precursor frequency was found to be quite high (~1 in 1,500 for vaccinia virus (VACV) and ~1 in 3000 for LCMV) (109). This suggests that there are ~14,000 T cells specific for VACV and ~7,000 for LCMV in a naïve mouse. However, most of these studies may not be sensitive enough to detect lower affinity clones, and thus these numbers may be underestimates. Although TCRs are said to be specific for one antigen, they are in fact specific for multiple pMHC complexes, but with a varying degree of affinity

(110). T cells show "polyspecificity," and therefore a larger number of them may be considered cross-reactive than once previously thought (111).

The affinity of the TCR for the self-selecting pMHC is much lower than the affinity of the TCR for cognate pMHC. CD5 expression was shown to positively correlate with the strength of TCR reaction with self-pMHC during selection (112). In other words, the stronger a T cell reacts with self-peptide MHC, the more CD5 expression on the surface. TCR sensitivity to self-peptide affects its ability to respond to foreign antigen (113). It was shown that T cells with higher CD5 expression will preferentially expand in response to antigen over the CD5 low expressing CD8 T cells. Consequently, the increased responsiveness to foreign antigen of CD5 high T cells, which have stronger reactivity to self pMHC, may have implications in autoimmunity.

J. CD8 T cell priming and activation

Naïve CD8 T cells require 3 distinct signals for their full activation and expansion: TCR engagement of pMHC (signal 1), costimulation CD28:B7 (signal 2), and inflammatory cytokines (signal 3). The process in which naïve CD8 T cells first encounter antigen is often termed priming and is used to distinguish it from effector CD8 T cell encountering antigen presented on target cells. Naïve T cells are constantly circulating throughout the body from the blood to lymph nodes (LN) and spleen. Naïve CD8 T cells express CD62L, which allows them to enter lymphoid structures (114). T cells enter secondary lymphoid organs in

search of their cognate antigen presented on DCs (75). T cells that do not engage cognate antigen leave the LN and continue circulating. When a naïve T cell encounters cognate antigen on DCs and other appropriate activation signals, instead of re-entering circulation, it remains in the LN where it divides, clonally expands, and differentiates into effector subsets before recirculating in search of peripheral antigen (115-117). However, should a naïve T cell only encounter cognate antigen on APCs, without costimulation, it either becomes anergic or dies via apoptosis (98, 103). Therefore, cognate antigen encounter alone is not sufficient for T cells to divide, as costimulation and inflammatory cytokines are also required.

The classical costimulatory molecules are between B7 on APCs and CD28 on T cells; however, some additional factors have been shown to either compensate in the absence of B7 or contribute to costimulatory effects on T cells (77). CD28 costimulation induces production of IL-2 and expression of the high-affinity IL-2 receptor, thus allowing for autocrine signals to promote growth and survival. Multiple members of the TNF superfamily have been shown to contribute to T cell activation. CD27, a TNF receptor family member, is constitutively expressed on T cells, and when engaged by its ligand, CD70, on DCs, it provides stimulatory signals to T cells (77, 103). CD40:CD40L interaction between DCs and T cells provide activating signals for both the APC and the T cell (77). There has even been evidence that without CD28 and IL-2, other cytokine signals like IL-6 and TNF can compensate and still provide CD8 T cells

with sufficient costimulatory signals for full activation (118). The costimulatory pathways that are engaged contribute to the outcome of CD8 T cell activation. A CD28-related protein, CTLA-4, also binds to B7 expressed on APCs, but instead of providing stimulatory signals like CD28, it inhibits T cell activation (77).

CD8 T cells that encounter antigen and costimulation undergo programmed cell division, but these two signals alone are not sufficient for full effector cell differentiation and survival into memory (98, 100). CD8 T cells need a third signal, provided by cytokines, including IL-12 or type 1 IFN, for efficient clonal expansion, differentiation into various effector populations, acquisition of cytolytic effector functions, and memory formation (81, 119-122). The most well-known cytokines to induce signal 3 cytokine activity are IL-12 and type 1 IFN (98, 100). One *in vitro* study showed that without IL-12, CD8 T cells did not proliferate (defined as an increase in number) well or develop full effector function (123). Type 1 IFN, however, can evidently substitute for IL-12 as a signal 3 cytokine (81, 122). Additional cytokines have also been described to have similar stimulatory effects on CD8 T cell expansion and effector function, including IFNγ and IL-21 (124-128).

Signal 3 cytokines are required for efficient clonal expansion in response to antigen, and the infecting pathogen and resulting inflammatory environment determine which cytokine(s) provide signal 3 activity (78, 129-131). LCMV-specific CD8 T cells use type 1 IFN as the signal 3 cytokine for effective primary T cell expansion, whereas Listeria and VSV depend on both type 1 IFN and IL-12

(78-80, 129, 132, 133). Studies showed that IFNAR KO LCMV-specific transgenic P14 CD8 T cells divided similarly to WT P14 cells but had reduced survival, thereby limiting their overall clonal expansion (79). Costimulation induces expression of certain survival factors, including anti-apoptotic molecules like Bcl-2 and Bcl-xl, but in the presence or absence of signal 3 cytokines, levels of Bcl-2 and Bcl-xl were found to be similar between the two groups (88). This indicated that the enhanced survival was mediated by additional mechanisms. In other systems, the addition of adjuvants or IL-12 to activated CD8 T cells promoted their expansion by upregulating the inhibitor of κB (IκB) family member Bcl-3, which was found to prolong activated T cell survival (86-88).

It is possible that naïve CD8 T cells acquire all three signals from an activated DC. In fact, in some infection models, CD4 T cells have been proven dispensable for efficient CD8 T cell expansion and effector function (103, 134, 135). In the absence of CD4 help, "helpless" CD8 T cells function during the primary response, but have been found to have defects in secondary memory expansion (136). Thus, it is thought that under conditions where DCs can be activated directly by the pathogen, CD8 T cells do not require CD4 help; however under other conditions, CD4 T cells have to activate DCs for them to provide efficient stimulatory functions to mediate naïve CD8 T cell responses (128, 135).

K. CD8 T cell differentiation

CD4 T cells are known for their ability to differentiate into various subsets upon activation, and CD8 T cells also generate a heterogeneous population of effector cells upon activation and expansion. After priming, changes in CD8 T cell phenotype occur during various stages of T cell activation and expansion. CD69, one of the earliest activation markers induced in CD8 T cells helps retain lymphocytes in lymphoid organs (137, 138). CD25, the high affinity IL-2 receptor is also induced early after T cell activation (139). Antigen experienced CD8 T cells upregulate the cellular adhesion molecule CD44 and high expression levels are retained throughout the remainder of the T cells life (140). Early activated T cells will also downregulate CD62L, which may serve to keep lymphocytes that have been sufficiently activated out of secondary lymphoid organs, increasing probability of encounter with antigen at the site of infection (141). After the transient upregulation of CD69 and CD25, activated CD8 T cells continue to differentiate into various effector populations.

Based on expression of two different markers, killer cell lectin-like receptor subfamily G member 1 (KLRG1) and CD127, distinct effector populations including early effector cells (EEC), short lived effector cells (SLEC) and memory precursor effector cells (MPEC) can be identified (78, 100, 142-145). Naïve CD8 T cells are CD127hi and KLRG1lo, and upon activation, many cells will downregulate CD127 and some upregulate KLRG1. T cells that are negative for both markers are termed EECs and retain the ability to differentiate into either SLECs or MPECs (100, 146). CD127lo, KLRG1hi cells are termed SLECs, and

are terminal effectors. The majority of this population die after antigen is cleared during the contraction phase. MPEC is a population of cells that has reacquired CD127 and preferentially survive the contraction phase and develop into memory cells (144, 147). Many of these effector populations were identified by sorting individual populations and determining their fate after transfer into new hosts.

A number of different factors have been associated with differentiation of CD8 T cells into effectors vs. memory precursors. TCR signal strength, TF induction, cytokine stimulation, chromatin accessibility, and metabolic regulation all contribute to T cell differentiation (77, 102, 103, 144, 148-151). Many models suggest that the balance between different TFs is important in differentiation. Two T-box TFs, Tbet and Eomesodermin (Eomes), regulate many of the same genes and are important for effector functions in CD8 T cells, including IFNγ production (103, 152-154). However, their roles are not redundant, as expression of Tbet is associated with terminal differentiation, and Eomes associates with memory formation (152, 153). The balance between other TFs are also differentially associated with the SLEC vs MPEC phenotype including inhibitor of DNA-binding (Id)2 and Id3, B lymphocyte induced maturation protein 1 (Blimp-1) and B-cell lymphoma 6 (Bcl-6), and STAT4 and STAT3, respectively (77, 102, 149, 151, 155). T-cell factor 1 (TCF-1) is a TF that was found to be particularly important for memory cell formation (102, 151). Interestingly, expression of some of these TFs will actually negatively regulate the other. High expression of Blimp-1 will suppress Bcl-6 expression, and vice-versa. The reciprocal nature of some

of these TFs suggests that once a cell is driven into a particular differentiation pathway, the TFs induced will only solidify the pathway, by actively repressing the other (102, 151).

Stronger TCR signaling has been associated with terminal effector differentiation (SLEC) rather than memory precursors (148). This may be partially due to the upregulation of the TF IRF4, which is induced and differentially regulated based on strength of TCR stimulation, where stronger TCR signals resulted in increased IRF4 expression (148). A recently identified TF, basic leucine zipper transcription factor, ATF-like (BATF), is required for effector differentiation of CD8 T cells in response to LCMV infection (156). IRF4 and BATF associate with one another, have similar downstream targets, and regulate many of the same genes (157, 158).

TCR signal strength is not the only thing that governs effector fate; inflammatory cues also contribute to T cell effector differentiation. Signal 3 cytokines play an important role in CD8 T cell differentiation into various phenotypic and functional effector populations (143, 145). Differences in CD8 T cell exposure to costimulatory molecules and cytokines can alter their differentiation into EECs, SLECs and MPECs (77). Recent studies investigating the role for signal 3 cytokines in primary CD8 T cell responses have shown that loss of IL-12R, IFNAR, or both receptors can alter CD8 T cell differentiation, showing reduced SLEC and increased MPEC formation (78, 100, 142, 143, 145). Increased inflammation, in the form of IL-12 production, induced higher levels of

Tbet and promoted terminal differentiation (143, 153). Conversely, IL-12 has been shown to inhibit Eomes induction thus inhibiting effector memory formation (159). This suggests that once a programming event is starting to take place, there are mechanisms that may result in the continuation of a specific pathway.

L. CD8 T cell effector function

In addition to cytolytic capability, CD8 T cells also can have immunoregulatory roles during infections, in part by secreting cytokines, which can activate other cells. Similar to CD4 T cells, CD8 T cells can also be distinguished as T cytotoxic (Tc)1, Tc2 and Tc17 cells, based on the cytokines produced (160). Although they may produce different cytokines, studies showed that they still retain cytolytic capability.

Naïve CD8 T cells are functionally distinct from effector and memory CD8 T cells. Naïve CD8 T cells stimulated with cognate antigen have the ability to produce TNF but not IFNγ (161, 162). After activation, division, and differentiation, naïve T cells gain the ability to produce IFNγ in response to antigen stimulation (141). Some activated CD8 T cells also retain the ability to produce TNF, and therefore will produce multiple cytokines. A small proportion of effector CD8 T cells have the capability of producing IL-2 in addition to IFNγ and TNF, although this function is seen more in memory CD8 T cells than effectors. IFNγ has anti-viral properties and increases Class I MHC on the surface of cells, making it easier for CD8 T cells to identify infected targets. IFNγ also actives

macrophages, which play an important role in pathogen clearance. TNF has the ability to bind to both TNF receptor (TNFR) 1 and TNFR2, and thus has differential effects on cells. TNFR1 is widely expressed, but TNFR2 is restricted to hematopoietic cells. TNF engagement of TNFR1 can induce apoptosis, but TNF also activates nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and MAPK pathways and thus serves an immunoregulatory role. In fact, the anti-apoptotic effects of NFκB will often dominate over the apoptotic effects TNFR1 signaling can induce (77, 163).

Activated CD8 T cells are known for their ability to specifically lyse antigen-specific target cells. Once activated, CD8 T cell upregulate effector molecules including perforin and granzymes, thus priming the T cell for rapid elimination of target cells (97). Studies have shown that activated effector CD8 T cells can lyse target cells in less than 5 minutes. Cytolytic function requires both perforin and granzymes, where perforin helps granzymes translocate into the cell (164). Granzymes are proteases that can cleave and activate effector caspase 3, thus inducing apoptosis (164). Effector CD8 T cells can also trigger apoptosis through TNF family members like FasL and TRAIL (164). Activated CD8 T cells upregulate FasL, which, upon engagement with Fas on target cells, induces cell death (164). The Fas:FasL mediated killing is important for immune cell homeostasis as mice deficient in either component develop severe lymphoproliferative disorders. TRAIL-mediated lysis of virus-infected cells is important for control of infections including IAV (165).

Many of the effector molecules that activated CD8 T cells express, including IFNγ, perforin and granzyme B, can be regulated by the T-box TFs Tbet and Eomes (153, 154, 166-168). Signal 3 cytokines, including IL-12, IL-21, and type 1 IFN, will augment the acquisition of CD8 T cell effector functions, including production of cytokines (IFNγ and TNF) and cytolytic activity (169). *In vitro* studies identified that deficiencies in type 1 IFN, IL-12 or IL-21, resulted in decreased lytic ability and low levels of granzyme B expression in CD8 T cells (79, 81, 169, 170). Additional *in vivo* studies showed reduced granzyme B expression in IFNAR KO transgenic P14 CD8 T cells compared to WT P14 CD8 T cells (79). However, not all infection models showed the same requirements for the specific signal 3 cytokines in driving these effector functions (78, 79).

M. Memory CD8 T cells

After antigen is cleared the majority of the terminal effectors die during the contraction phase while a subset of effectors that expresses the IL-7R preferentially make it into the memory pool (147). CD8 T cell memory cells can be divided into three main categories, central memory (T$_{CM}$), effector memory (T$_{EM}$), and resident memory (T$_{RM}$) (102, 149). Subsets can be distinguished based on phenotypic markers, functions, and their location within the body. Because memory T cell subsets are antigen experienced, they all express high levels of CD44. Central memory cells are CD62Lhi, which enables them to enter into secondary lymphoid organs. T$_{CM}$ generally proliferate more than other memory subsets and produce more IL-2, but have reduced effector functions

(102). T_{EM} cells express low levels of CD62L and are mostly found in non-lymphoid organs and can immediately produce cytolytic effector functions upon encountering antigen. Unlike T_{CM}, T_{EM} have reduced proliferative potential. The most recently identified subset of memory cells is the T_{RM} population, also known as tissue-resident memory cells (102). These cells can be found in organs such as the skin, lungs, and gut, but are not found in the circulation or in any lymphoid organs. T_{RM} cells are phenotypically distinct and show high expression of CD103, CD69, and low CD62L levels (102). T_{RM} have very little proliferative and migratory potential; however, they have enhanced immediate effector function and some have been shown to express high levels of granzyme B (102).

The effectiveness of memory is associated with increased frequencies of antigen specific cells and the ability to respond faster during subsequent antigen encounter. Besides differences in phenotype, memory CD8 T cells have differential requirements for activation when compared to naïve CD8 T cells. Where naïve T cells require 3 distinct signals for full acquisition of effector function and efficient clonal expansion, memory T cells have a reduced threshold for activation (171). Indeed, memory CD8 T cells were shown to have increased cytolytic effector function over naïve CD8 T cells, and produced cytokines (IFNγ and IL-2) much earlier than naïve CD8 T cells (172, 173). Additionally, memory CD8 T cells were activated in response to peptide concentrations 10-50 fold lower than naïve CD8 T cells (174). Unlike naïve CD8 T cells, which require cell

division prior to effector function acquisition, memory CD8 T cells do not need to divide before exhibiting effector function (123, 141, 154).

Thesis objectives

Efficient clonal expansion and differentiation of CD8 T cells are required to develop protective memory CD8 T cells. Efficient activation of CD8 T cells involves cell division, clonal expansion, differentiation into heterogeneous effector populations, and formation of a stable functional memory population. Priming of naïve T cells is therefore extremely important because it can affect all aspects of CD8 T cell activation. The priming of naïve T cells requires three distinct signals: a cognate peptide MHC-TCR interaction (signal 1), costimulation (signal 2), and infection-induced cytokines (signal 3). The requirements for signal 3 cytokines have only been recently identified. However, the timing of exposure to these signals has yet to be investigated. T cell priming is generally described as a linear event, where T cells always encounter antigen first, then receive costimulation, and finally signal 3 cytokines (canonical T cell priming). In reality, some T cells will be exposed to signals 1, 2 and 3 out of order. If T cells are primed out-of-sequence, do they behave the same as canonical CD8 T cells?

I sought to investigate how the timing of signal 3 cytokines affects CD8 T cell activation. During the course of an immune response, not all T cells see antigen, costimulation, and inflammatory cytokines at the same time. I

questioned how the order of these signals affects CD8 T cell priming and subsequent activation, expansion and differentiation. I investigated how out-of-sequence signaling affects T cell activation immediately after cognate antigen stimulation (4-5 hours), and after prolonged exposure to cognate antigen (days-weeks).

CD8 T cells pre-exposed to type 1 IFN and costimulatory molecules in the presence of self-peptides gained the ability to respond immediately to cognate antigen stimulation. These T cells were termed primed because they produced immediate effector functions in response to antigen stimulation prior to cell division. Primed naïve CD8 T cells produced multiple cytokines in response to low-affinity, and low-density antigens, and gained ability to specifically lyse target cells. However, CD8 T cells that are pre-exposed to signal 3 cytokines and then exposed to cognate antigen stimulation in the context of a virus infection showed defects in clonal expansion, effector differentiation, effector function, and memory formation.

The findings presented here may seem contradictory because out-of-sequence signaling can prime T cells to produce immediate effector functions and cause defects in T cell expansion and effector differentiation. However, these two models assessed T cell function at different points after antigen exposure; one where functions were evaluated within hours after seeing cognate antigen, and the other showing T cell responses after days of antigen stimulation. Identifying how the order of activation signals during T cell priming can affect

subsequent T cell function is important to study. The discoveries presented here will enhance our understanding of T cell responses during infections and will provide useful information in developing better and more effective vaccines.

CHAPTER II: Materials and Methods

A. Mice

C57BL/6J (Ly5.2+), B6.129P2-*B2m^{tm1Unc}*/J (β2Microglobulin KO) male mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6.IFNαβR KO (Muller 1994), TAP KO, B7 KO, 3 KO (70), and congenic B6.SJL (Ly5.1+) mice were bred in the Department of Animal Medicine at the University of Massachusetts Medical School (UMMS). Congenic Ly5.1 OT-I (106), P14 (175), SMARTA (176) and Thy1.1 P14 TCR-transgenic mice were bred in the Department of Animal Medicine at the University of Massachusetts Medical School (UMMS). The P14 transgenic mice were bred onto the B6.IFNαβR KO background to generate P14 CD8 T cells that lacked IFNαβR.

B. Viruses and inoculations

Lymphocytic choriomeningitis virus (LCMV), strain Armstrong, was propagated in baby hamster kidney (BHK21) cells, as previously described (177, 178). Mice were injected intraperitoneally (i.p.) with 5×10^4 pfu of LCMV. Mice were injected with 5×10^6 pfu of VACV-GP (recombinant vaccinia virus expressed glycoprotein from LCMV) i.p. Organ homogenate viral titers were determined by plaque assay using Vero cells.

Poly(I:C) was purchased from InvivoGen (San Diego, CA) and diluted in HBSS for a concentration of 1 µg/µl. Mice were either inoculated with 200 µl HBSS or 200 µg poly(I:C) i.p. Anti-CD40L antibody (clone MR1) was a gift from

Dr. Michael Brehm, and mice received 500 µg/mouse in 200 µl i.p. To activate P14 CD8 T cells without a virus-induced inflammatory response, mice were inoculated intravenously (i.v.) with 5 µg (diluted in HBSS) of a 13-mer peptide (GP$_{33-45}$) (KAVYNFATCGIFA) from the LCMV glycoprotein.

C. Cytokines and peptides

Mouse IFNβ was purchased from PBL Interferon Source. Cytokines: IL-2 was purchased from BD Biosciences, IL-6 and IL-12 were purchased from R&D, and IL-7 and IL-15 were purchased from PeproTech, INC. Splenocytes were stimulated *ex vivo* with cytokine concentrations of 1000 U/ml IFNβ, or 10 µg/ml of IL-2, IL-6, IL-7, IL-12, or IL-15 at 37°C for ~30 minutes. For intracellular cytokine staining (ICS) P14 cells were stimulated with 1 µM of GP33 peptide (KAVYNFATC) (175) or altered peptide ligand (APL) F6L (KAVYNLATC) (179), OT-I cells were stimulated with 1 µM of OVA (SIINFEKL) (106) or OVA- APLs including Y3, T4, V4, and G4 (SIYNFEKL, SIITFEKL, SIIVFEKL, and SIIGFEKL) (180) and polyclonal CD8 T cells were stimulated with 1 µM of GP33, NP396 (FQPQNGQFI), GP276 (SGVENPGGYCL), or NP205 (YTVKYPNL). Whole splenocytes or RMA cells were labeled with 1 µM of the minimal GP33 epitope or the vaccinia virus K3L epitope (YSLPNAGDVI). Peptides purchased from 21st Century Biochemicals (Marlboro, MA) unless otherwise noted. APLs were generously provided by Dr. Leslie Berg.

D. Adoptive transfers

Spleens were harvested, single cell suspensions were made, and RBCs were lysed using 0.84% ammonium chloride. Where described, cells were resuspended at 1×10^7 cells/ml of 5 mM stock of CellTrace Violet (Molecular Probes) and incubated for 15 min in a 37°C water bath, mixing every 5 minutes. HBSS was used to wash and resuspended cells for transfer into recipients, each receiving 200 µl i.v. When transferring a specific number of P14 CD8 T cells, frequencies were determined via flow cytometry by staining for CD8α, Vα2, and the congenic marker Ly5.1 or Thy1.1 if applicable. The total number of cells was calculated to transfer equal numbers into each group.

E. Surface staining

Single cell suspensions were prepared by filtering cells through 190 micron mesh. Red blood cells were lysed using 0.84% ammonium chloride and $2\text{-}4\times10^6$ cells were plated per well in a 96 well round bottom plate in complete media. Fc receptors were blocked using anti-Fc (2.4G2) in FACS buffer (2% heat-inactivated FCS in HBSS) for 5 min at 4°C. Cells were washed with FACS buffer and stained with various combinations of fluorescently conjugated antibodies against surface proteins for 20 min at 4°C. Antibodies used include CD3ε (145-2C11), CD4 (RM4-5), CD8α (53-6.7), CD8β (YTS156.7.7) CD8β (eBioH35-17.2), CD44 (IM7), CD62L (MEL-14), CD127 (A7R34), CD27 (LG.3A10), CD25 (PC61), CD43 (1b11), CD126 (D7715A7), CD132 (TUGm2),

CD122 (TM-BETA1), CD69 (H1.2F3), CD70 (FR70), CD28 (E18), ST2 (RMST2-2), CD86 (GL1), CD80 (16-10A1), H2-Kb (AF6-88.5), H2-Db (KH95), I-Ab (AF6-120.1), NK1.1 (PK136), IFNGR1 (2E2), IFNAR1 (MAR1-5A3), CD11a (M17/4), CD11b (M1/70), CD11c (HL3), B220 (RA3-6B2), CD19 (ID3), F4/80 (BM8), BST2 (CD317) (eBio927), Ly5.1 (A20), Thy1.1 (OX-7), Thy1.1 (HIS51), CXCR3 (CXCR3-173), CCR7 (4B12), KLRG1 (2F1), CD124 (mIL4R-M1) and Vα2 (B20.1). After surface staining, cells were washed with FACS buffer and fixed using BD cytofix (BD bioscience) for 5 min at RT. After washing, cells were resuspended in FACS buffer and collected via flow cytometry. All antibodies used were from BD bioscience (San Jose, CA), eBioscience (San Diego, CA), or BioLegend (San Diego, CA) unless otherwise noted.

F. Transcription factor and intracellular staining

Cells were isolated, prepared and stained as described in surface staining section. Cells were permeablized using the Foxp3 staining buffer kit (eBioscience) or BD perm buffer III (BD bioscience) per manufactures instructions. A variety of fluorescently conjugated antibodies were used to stain for intracellular proteins including Eomes (Dan11mag), Tbet (eBio4B10), IRF4 (3E4), BATF (MBM7C7), Ki67 (B56), Bcl-2 (3F11), and STAT1 (1/STAT1). Unconjugated antibodies including TCF1 (C63D9), Bcl-xl (54H6), Bcl-3 (C-14) (Santa Cruz Biotechnology Inc., Dallas, TX) Bim (C34C5), STAT3 (79D7), STAT4 (C46B10), STAT5 (3H7) and SOCS1 (A156) were stained with a secondary antibody FITC Donkey anti-rabbit IgG (Poly4046). Antibodies used

were from BD bioscience (San Jose, CA), eBioscience (San Diego, CA), BioLegend (San Diego, CA), or Cell Signaling Technology, Inc. (Danvers, MA).

G. Intracellular cytokine staining

Single cell suspensions were prepared, treated and plated as described in section E (surface staining). Cells were stimulated for 4-5 hours at 37°C 5% CO_2 in the presence of 1 µl/ml Golgi Plug (BD Bioscience), and 10 U/ml human rIL-2 with either 250 ng/ml anti-CD3ε (145-2C11, BD Bioscience), 1 µM GP33, 50 ng/ml PMA and 500 ng/ml ionomycin, or without additional stimulation. After stimulation, cells were stained for surface expression as described in section E. After surface staining, cells were permeabilized using either BD Cytofix/Cytoperm (BD bioscience), or Foxp3 Staining Buffer kit (eBioscience) per manufacturer's instructions. After permeabilization, cells were stained with a combination of antibodies conjugated with different fluorophores including IFNγ (XMG1.2), TNF (MP6-XT22), IL-2 (JES6-5H4), and granzyme B (GB11, Invitrogen). To test if cells were actively degranulating, antibodies to CD107a (1D4B) and CD107b (ABL-93) were added during stimulation at 37°C. Cells were washed and resuspended in FACS buffer for acquisition. All antibodies used were from BD bioscience (San Jose, CA), eBioscience (San Diego, CA), or BioLegend (San Diego, CA) unless otherwise noted.

H. Phosflow staining

To identify phospho-specific STATs, the BD Phosflow Alternative Protocol 1 was used and slightly modified. Spleen leukocytes were isolated, stimulated (where appropriate), fixed, stained for surface molecules, permeabilized, and finally stained for intracellular proteins. Spleens were isolated, and single cell suspensions were prepared as described in Section E. Cells were incubated at 37°C in 100 µl of media for the indicated times in the presence (stimulated) or absence (unstimulated) of cytokines. Cytokines tested included IL-2, IL-6, IL-7, IL-12, IL-15, IFNγ, and IFNβ. Total volume was brought up to 200 µl before spinning. Cells were fixed with BD cytofix (BD Bioscience) on ice for 20 min, and surface molecules were stained using the protocol described in Surface Staining. After surface staining, cells were permeablized with BD Perm buffer III (BD Bioscience) for 30 min on ice. Splenocytes were washed and then stained with a combination of fluorescently labeled Abs pY701 STAT1 (BD Bioscience), pY705 STAT3 (BD Bioscience), pY693 STAT4 (BD Bioscience), or pY694 STAT5 (BD Bioscience) for 30 min at RT in the dark. After intracellular staining, splenocytes were washed, resuspended in FACS buffer and acquired.

I. Annexin V staining

Single cell suspensions were made, and surface molecules were stained as described in section E. Cells were washed with Annexin binding buffer (eBioscience) and stained for Annexin V and 7AAD for 20 min at 4°C. Samples were collected with flow cytometer directly after staining.

J. RNA flow

To detect RNA expression in a heterogeneous population of cells, we utilized QuantiGene FlowRNA (affymetrix, eBioscience). Overall procedure includes steps 1. Fixation and permeabilization, 2. Target probe hybridization, and 3. Signal amplification and detection. Single cell suspensions were prepared and stained according to protocol stated above. FlowRNA assay was performed according to manufactures protocol using type 1 probe SOCS1 (VB1-16311), and type 6 probe Rpl13a (VB1-15315). Samples were run on day 2 of the assay, immediately after the signal amplification and detection step.

K. Flow cytometry

Previously fixed samples (Cytofix, Cytofix/Cytoperm, and Foxp3 staining buffer kit) were run within 1 week of staining. Phosflow, Annexin V, Trogocytosis, and RNA flow staining procedures were acquired immediately after staining cells. All samples were acquired on a BD bioscience LSRII using FACS Diva software for data collection and FlowJo (Tree Star Inc., Ashland, OR) for data analysis.

L. Trogocytosis assay

Effector P14 cells were generated using adoptive transfers and poly(I:C) inoculation described in sections B and D. Targets were RMA cells cultured in complete RPMI and were un-pulsed, pulsed with an irrelevant peptide, K3L, or pulsed with cognate peptide, GP33, at 1 µM for ~ 90-120 minutes at 37°C 5% CO_2. After incubation, target cells were labeled with fluorescent lipids SP-

DiIC$_{18}$(3) (Molecular probes) and diluted in Diluent C (Sigma Aldrich) using the protocol adapted from Daubeuf S. et al (181). Briefly, cells were incubated at room temp with 40 mM SP-DiIC$_{18}$(3) lipid in Diluent C buffer for 5 min. Labeling was quenched by addition of FCS, and cells were washed a minimum of 3 times with complete media. Target cells (~7×10^5) were co-cultured with effector cells (1.5×10^6 total splenocytes) per well for 1 hour at 37°C 5% CO$_2$. Cells were stained with surface antibodies of interest, and samples were acquired directly after staining.

M. *In vivo* cytotoxicity assay

Congenic P14 CD8 T cells were adoptively transferred into B6 mice as described in Section D. Mice were either inoculated with HBSS or poly(I:C) for ~ 1 day followed by target cell transfer. Splenocytes from B6 mice were isolated, single cell suspensions were prepared as described earlier. Cells were pulsed with peptide at 37°C, 5% CO$_2$ for 1 hour. After peptide labeling, cells were dual labeled with 1 µM CellTrace Far Red DDAO (Molecular Probes), and various concentrations of CellTrace violet (Molecular probes) to differentiate cells labeled with different peptides. Peptide-pulsed target cells were adoptively transferred into recipient mice, and splenocytes were harvested ~20 hours post transfer. Specific lysis was calculated based on the following formula:

% specific lysis = 100 - ((Experimental/Control)/Control x 100)

N. Plaque assay

Organs were harvested, ground, and supernatants were collected by spinning at 2000xg for ~20 minutes. Samples were stored at -80°C until plaque assay was performed. LCMV titers were determined by plaque assay using Vero cells. Vero cells, ~$2x10^5$ in 2 ml per well, were plated in 6 well plates on day -1 and incubated overnight at 37°C, 5% CO_2. Serial dilutions of organ supernatants were used with a minimum of 5 dilutions per organ. 100 µl of each virus dilution was added to 1 ml of media per well. Plates were rocked to distribute virus evenly and incubated at 37°C 5% CO_2 for 1.5 hours, rocking plates every 45 minutes. Plates were overlaid with 4 ml of 1:1 mix of 1% SeaKem-ME agarose and complete EMEM and left in incubator until stained 4 days later. Plates were stained with 2 ml of 1:1 mixture of 1% SeaKem-ME agarose and complete EMEM with ~15 µl of 1% Neutral Red per ml of mixture. Plates were counted for plaques the following day.

O. Microarray and quantitative real time PCR

Single cell suspensions of splenocytes were made, and red blood cells were lysed using 0.84% ammonium chloride. Naïve CD44lo CD8 T cells were sorted to 98-99% purity using MACS Naïve CD8α+ T cell Isolation Kit (Miltenyi Biotech). RNA was isolated from sorted naïve CD8 T cells with an RNeasy mini kit (Qiagen). cDNA was generated using the RT^2 Easy First Strand Kit (Qiagen), and QuantiFast SYBR Green PCR Kit (Qiagen) was used to determine the relative mRNA concentrations by quantitative real-time PCR. Primers for *Socs1* (RefSeq Accession number NM_009896.2) and *Actb* (RefSeq Accession number

NM_007393.3) were used. MoGene 2.0 ST Array (Affymetrix) gene chip was used to identify differences in gene expression between naïve CD8 T cells from HBSS-treated mice and poly(I:C)-treated mice.

P. Statistical analysis

Where appropriate, Students t test or linear regression were calculated using GraphPad InStat software. Significance was set at a P value of 0.05; * indicates a P of <0.05, ** a P of <0.01, *** a P of <0.001, and **** a P of < 0.0001. All results are expressed as means of +/- standard deviations (SD), unless otherwise noted.

CHAPTER III: Out-of-sequence signal 3 suppresses CD8 T cell responses _in_ _vivo_

A. Introduction

Efficient clonal expansion and differentiation of CD8 T cells are essential to develop protective memory CD8 T cells. This requires three signals: a cognate peptide MHC-TCR interaction (signal 1), costimulation (signal 2), and infection-induced cytokines (signal 3) (98-100). CD8 T cells that encounter antigen and costimulation undergo programmed cell division, but these two signals alone are not sufficient for full effector cell differentiation and survival into memory (98, 100, 142). CD8 T cells need a third signal, provided by cytokines, including IL-12 or type 1 IFN, for efficient clonal expansion, differentiation into various effector populations, acquisition of cytolytic effector functions, and memory formation (81, 119-122).

Signal 3 cytokines are required for efficient clonal expansion in response to antigen, and the infecting pathogen and resulting inflammatory environment determine which cytokine(s) provide signal 3 activity (78, 129-131, 133). LCMV-specific CD8 T cells, for example, use type 1 IFN as the signal 3 cytokine for effective primary T cell expansion (78-80). Signal 3 cytokines also play an important role in CD8 T cell differentiation into various phenotypic and functional effector populations. Differences in CD8 T cell exposure to costimulatory molecules and cytokines can alter their differentiation into EECs, SLECs and

MPECs (78, 100, 143, 145). Signal 3 cytokines have also been shown to augment the acquisition of CD8 T cell effector functions, including production of cytokines (IFNγ and TNF) and cytolytic activity (79, 81, 170). However, not all infection models show the same requirements for the specific signal 3 cytokines in driving these effector functions (78, 79).

Type 1 IFN can have both inhibitory and stimulatory effects on CD8 T cell proliferation, and when type 1 IFN provides signal 3 cytokine activity, it has positive effects on CD8 T cell expansion. Type 1 IFN signaling is complex in that it can activate multiple downstream pathways, including the JAK/STAT pathway. Engagement of the type 1 IFN receptor promotes phosphorylation of downstream STAT molecules, including STAT1, 3, 4, and 5 (32). The combination of STAT molecule(s) that are phosphorylated and translocated into the nucleus controls the outcome of CD8 T cell activation.

Recent *in vivo* studies from our laboratory showed that type 1 IFN can be a profound and universal factor inducing suppression of T cell proliferation during viral infections if the T cells are exposed to type 1 IFN prior to encountering their cognate ligand (34, 182). The fact that virus infections can induce a transient state of immune suppression was first described over a century ago, and since then, infections with a number of other viruses have been shown to induce a transient state of immune suppression in humans and animal models (183-190). Although virus-induced immune suppression can affect many aspects of the immune system, it is often associated with a reduced ability of T cells to

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proliferate in response to mitogens or antigen-specific stimulation (191, 192). It should be noted that the timing of exposure of CD8 T cells to activation signals is very important, as T cells exposed to virus-induced inflammatory environments prior to cognate antigen respond differently to signals 1 and 2 compared to CD8 T cells from naïve environments (34, 182, 193). Under circumstances when CD8 T cells see antigen and costimulation prior to or at the same time as inflammatory cytokines, IL-12 or type 1 IFN have positive effects on T cell differentiation and expansion. However, recent studies from our lab have shown that CD8 T cells pre-exposed to virus-induced inflammatory environments showed reduced proliferation when exposed to cognate antigen (182).

Considering type 1 IFN can have both positive and negative effects on CD8 T cell activation, I sought to investigate the role type 1 IFN was playing in virus-induced immune suppression. Virus-induced impaired proliferation could be mimicked by the type 1 IFN-inducer poly(I:C). To eliminate any off target effects that an actively replicating virus could induce, I chose to use poly(I:C) to study the mechanism of virus-induced T cell suppression. Through these studies, several interesting features of the mechanism of virus-induced immune suppression of CD8 T cells were identified: 1) CD8 T cells pre-exposed to poly(I:C) show a transient state of immune suppression, in terms of clonal expansion. 2) There is a marked defect in the ability of poly(I:C)-pretreated CD8 T cells to respond to IFNβ signaling in terms of phosphorylating downstream STAT proteins. 3) The inability of poly(I:C)-pretreated CD8 T cells to respond to

IFNβ signaling makes these cells behave more similar to T cells that only receive 2 signals, rather than ones that receive all 3 signals in the appropriate order. Consequently, poly(I:C)-pretreated, or out-of-sequence, CD8 T cells were found to have defects in clonal expansion, effector differentiation and function, and memory generation, resulting in reduced efficacy of viral clearance.

B. Poly(I:C)-induced suppression of CD8 T cell proliferation

1. Impaired clonal expansion of poly(I:C)-pretreated CD8 T cells is a transient and intrinsic defect.

Our lab showed that CD8 T cells exposed to exogenous cognate antigen after initiation of a virus infection proliferated poorly in response to a cognate antigen stimulus (182). To investigate the mechanism of this virus-induced suppression of T cell proliferation, the IFN-inducer poly(I:C) was used to prime CD8 T cells. Congenic transgenic LCMV-specific P14 CD8 T cells were used here to study virus-specific T cells exposed to the IFN-inducer poly(I:C) prior to infection. Ly5.1 or Thy1.1 P14 splenocytes were transferred into Ly5.2/Thy1.2 B6 hosts. Recipients were untreated (control) or inoculated with poly(I:C), and spleens were harvested 1 day later. Equal numbers of P14 cells that were Ly5.1-control and Thy1.1-poly(I:C) treated (**Figure 3.1A-C)** or Thy1.1-control and Ly5.1-poly(I:C) treated (**Figure 3.1D-F**) were transferred into recipients which were immediately infected with LCMV. Spleens and PECs were harvested at the peak of transgenic CD8 T cell expansion, day 7 post infection, and the proportion

Figure 3.1: Impaired clonal expansion of poly(I:C)-pre-exposed P14 cells when in direct competition with control-treated P14 cells
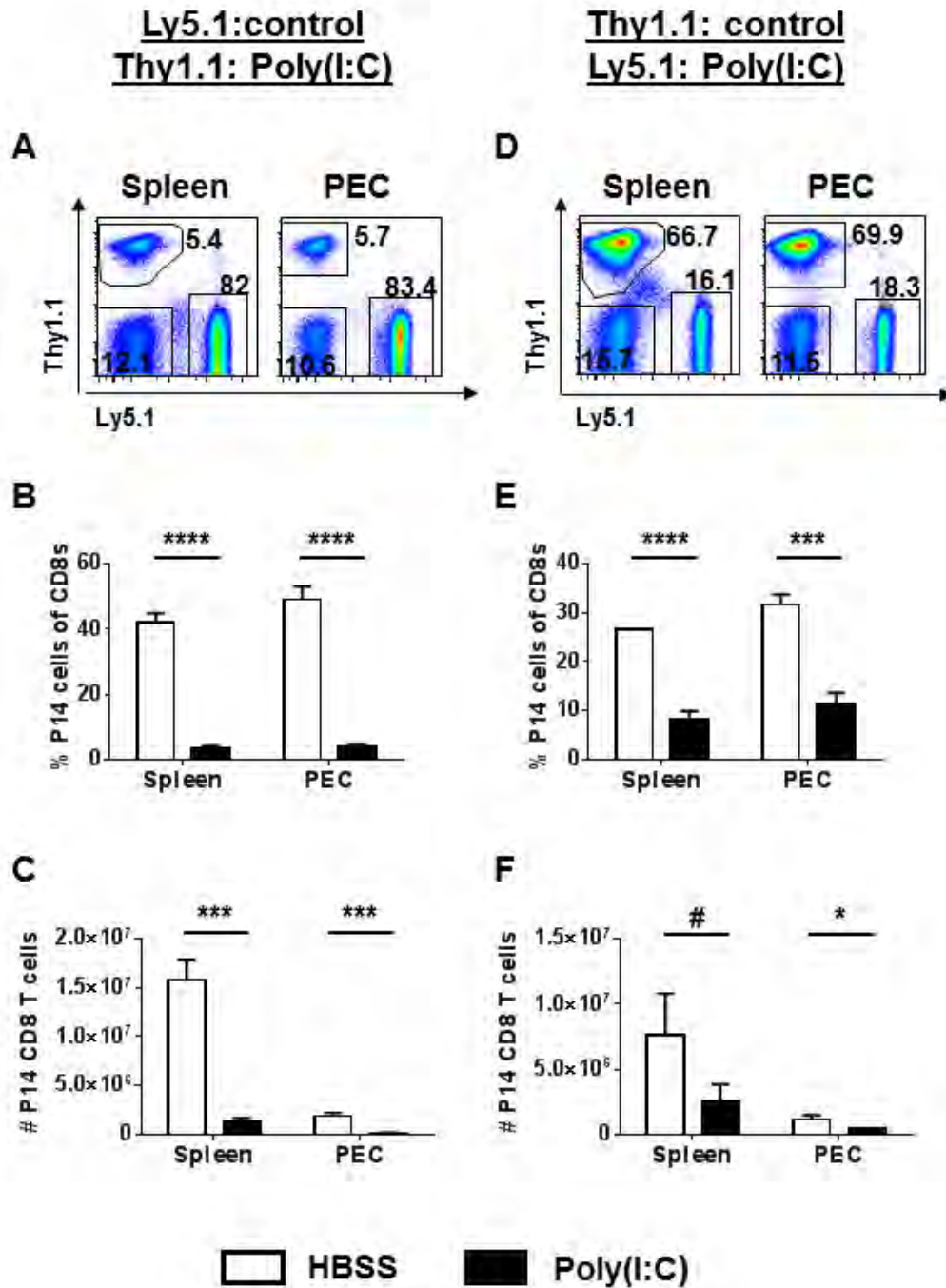
Figure 3.1: Impaired clonal expansion of poly(I:C)-pre-exposed P14 cells when in direct competition with control-treated P14 cells

Congenic P14 cells (Ly5.1 or Thy1.1) were adoptively transferred into B6 mice, which were subsequently inoculated with poly(I:C) or remained untreated. One day after treatment, splenocytes were isolated and similar numbers of P14 CD8 T cells that were untreated or poly(I:C) treated were transferred into the same WT B6 recipients. Immediately after P14 cell transfer, mice were infected with 5×10^4 pfu LCMV Armstrong i.p. At day 7 post infection, spleens and PECs were harvested and frequency and number of donor P14 cells were determined**. (A-C)** Ly5.1 P14 cells were control treated and Thy1.1 P14 cells were poly(I:C) treated. (**D-F**) Thy1.1 P14 cells were control treated and Ly5.1 P14 cells were poly(I:C) treated (**A, D**) representative flow cytometry plots gated on CD8α+Vα2+ from spleen or PEC. Frequency (**B, E**) and number (**C, F**) of P14 cells that were control treated (open bars) and poly(I:C) treated (black bars) is graphed. Data are representative of at least 2 experiments with n = 3 mice per group. # indicates a p value of 0.0625

(**Figure 3.1A-B and D-E**) and total number (**Figure 3.1C and F**) of donor P14 cells were determined. P14 cells pretreated with poly(I:C) (black bars) showed reduced proportions and total number of P14 cells compared to controls (open bars), regardless of congenic markers. That is, both Thy1.1 poly(I:C)-treated (**Figure 3.1A-C**) and Ly5.1 poly(I:C)-treated P14 cells (**Figure 3.1D-F**) showed suppression of proliferation when compared to control-treated P14 cells from the same mice. The reduced proportion and number of P14 cells was seen in both the PECs and the spleen, indicating defects in trafficking cannot account for the suppression seen.

I wanted to investigate whether the suppressive effects of poly(I:C) pretreatment were only seen when cells were in direct competition with control treated cells. To test this, Thy1.1 P14 cells were transferred into mice which were untreated (control) or exposed to poly(I:C) for 1 day. Equal numbers of control- or poly(I:C)-treated P14 cells were determined by flow cytometry and transferred into separate hosts, which were subsequently inoculated with LCMV. **Figure 3.2** shows the proportion (**A-B**) and number (**C**) of donor P14 cells in the spleen at day 7 post infection. These data indicate impaired clonal expansion of poly(I:C)-pretreated P14 cells occurs both with and without direct competition with control P14 cells. The reduced proliferation seen with direct competition suggests an intrinsic defect in the CD8 T cell expansion.

To determine how long the suppressive effects last for, I utilized the poly(I:C)-induced impaired CD8 T cell proliferation model. Ly5.1 P14 cells were

Figure 3.2: Impaired proliferation of poly(I:C)-pretreated P14 CD8 T cells without direct competition with control P14 CD8 T cells
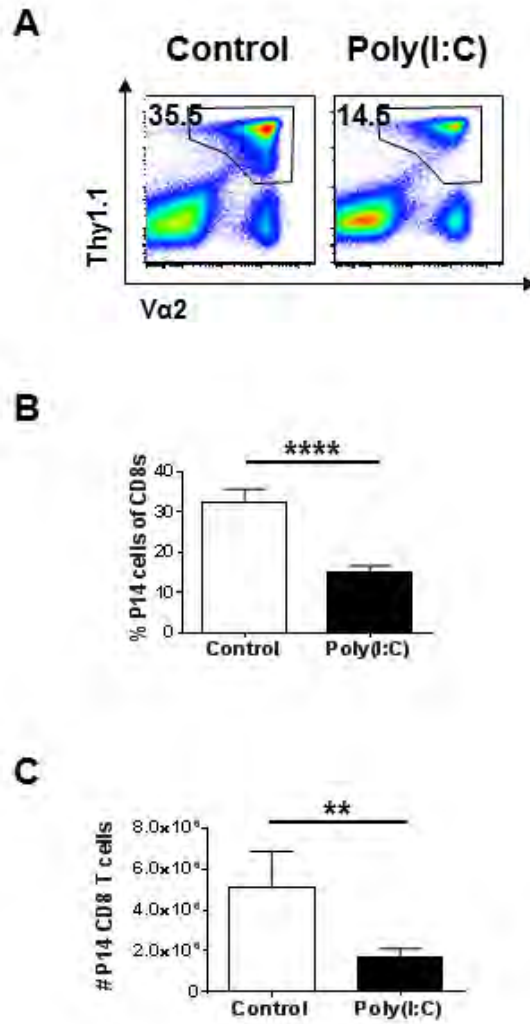
Figure 3.2: **Impaired proliferation of poly(I:C)-pretreated P14 CD8 T cells without direct competition with control P14 CD8 T cells**

Thy1.1 P14 CD8 T cells were transferred into WT B6 mice. Mice were untreated or inoculated with poly(I:C), splenocytes harvested 1 day later and equal numbers of P14 cells from each group were transferred into different recipients. Mice were infected with $5x10^4$ pfu LCMV i.p. and spleens were harvested at day 7 post infection. (**A**) Representative flow plots gated on CD8α+ cells are depicted. Frequency (**B**) and number (**C**) of P14 cells pretreated with poly(I:C) (black bars) or untreated (open bars) is plotted. Data are from a representative experiment with n=4 mice per group.

transferred into B6 hosts that were inoculated with either HBSS (control) or poly(I:C). One to 3 days later, splenocytes were isolated, and equal numbers of P14 cells (enumerated by flow cytometry staining) were transferred into recipients that were immediately infected with LCMV. Spleens from recipient mice were harvested at day 7 post infection, and the proportion (**Figure 3.3A-B**) and total number (**Figure 3.3C**) of transgenic P14 cells were determined. Suppression of proliferation of poly(I:C)-pretreated P14 cells (black bars) was greatest at 1 and 2 days of treatment compared to control-treated cells (open bars). After 3 days of pre-treatment, clonal expansion of poly(I:C)-pretreated P14 cells was almost comparable to that of the HBSS-pretreated control P14 cells, indicating that poly(I:C)-mediated suppression of proliferation is a transient effect.

1. **Direct effects of type 1 IFN are required for poly(I:C)-induced suppression of proliferation**

Because type 1 IFN is required for efficient clonal expansion of LCMV-specific CD8 T cells, I investigated its role in the reduced proliferation seen in poly(I:C)-pretreated CD8 T cells. Using a similar experimental set up as described previously, congenic Thy1.1 IFNAR KO P14 CD8 T cells were transferred into Thy1.2 mice and 1 day later they were inoculated with either HBSS or poly(I:C). One day after inoculation, equal numbers of transgenic T cells were transferred into mice prior to LCMV infection. The proportion (**Figure 3.4A**) and total number (**Figure 3.4B**) of donor IFNAR KO P14 cells in recipient host

Figure 3.3: Poly(I:C)-induced suppression of CD8 T cell proliferation is transient, with 1 and 2 days of pre-treatment showing the greatest impairment
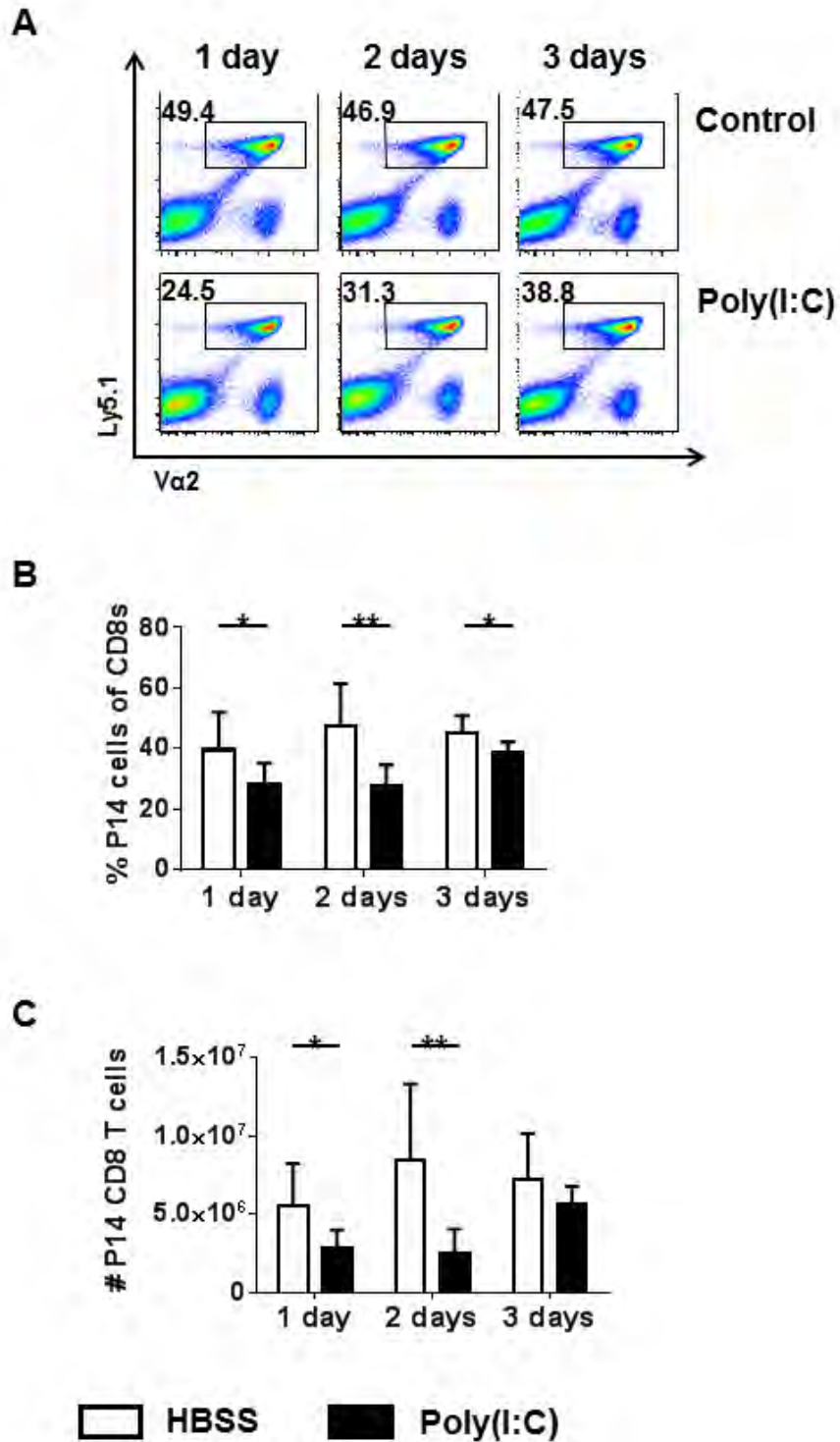
Figure 3.3: Poly(I:C)-induced suppression of CD8 T cell proliferation is transient, with 1 and 2 days of pre-treatment showing the greatest impairment

Ly5.1 P14 splenocytes were transferred into WT B6 mice, which were untreated or inoculated with poly(I:C). Splenocytes were harvested 1, 2 or 3 days after inoculation, and equal numbers of control- or poly(I:C)-treated P14 cells were transferred into separate WT B6 mice and subsequently infected with $5x10^4$ pfu LCMV. (**A**) Representative flow plots gated on CD8α+ cells are shown. Frequency (**B**) and total number (**C**) of donor P14 cells that were untreated (open bars) or poly(I:C) pretreated (black bars) is graphed. Data combined from 2 independent experiments for a total n of 8 mice per group.

Figure 3.4: IFNαβR KO P14 cells are resistant to poly(I:C)-induced suppressive effects
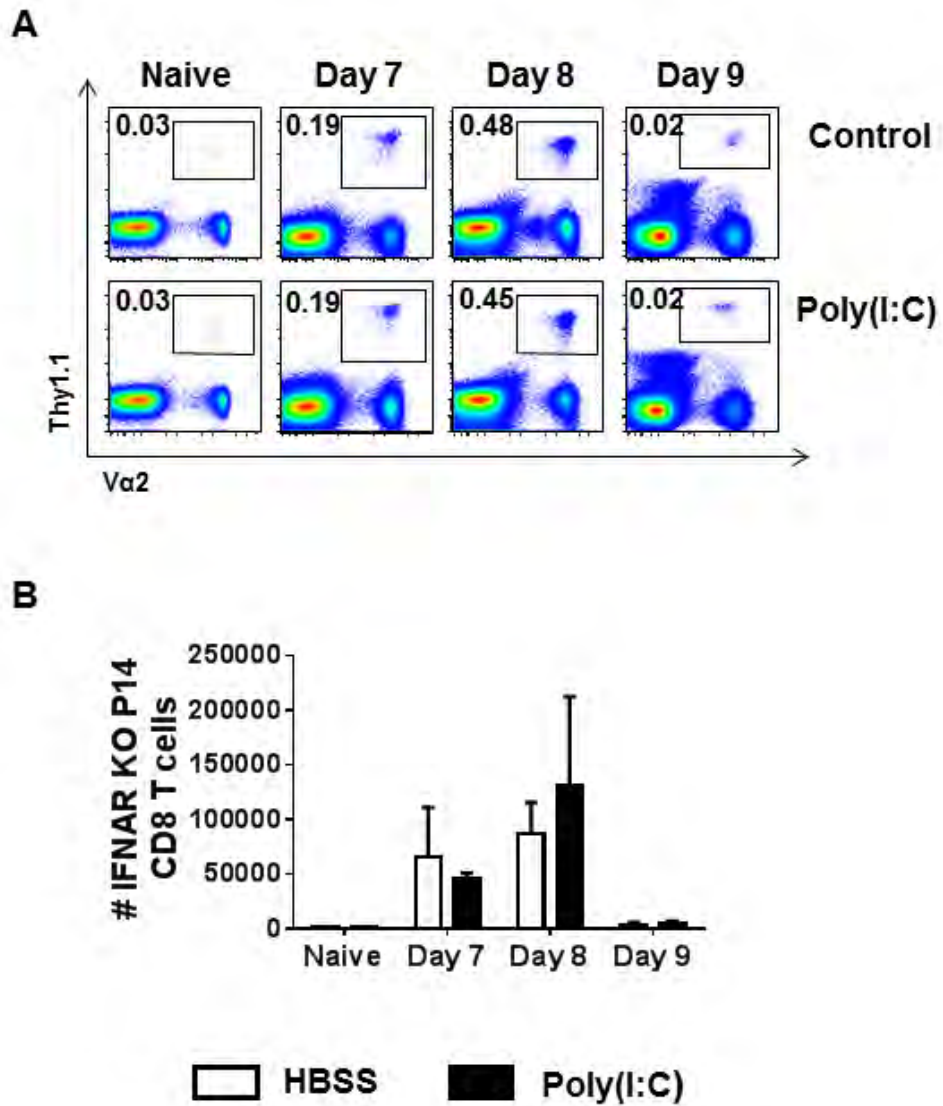
Figure 3.4: IFNαβR KO P14 cells are resistant to poly(I:C)-induced suppressive effects

Thy1.1 IFNαβR KO P14 cells were transferred in WT B6 hosts, which were inoculated with poly(I:C) or remained untreated (control). One day later, splenocytes were isolated, and equal numbers of IFNαβR KO P14 cells were transferred into separate mice, which were subsequently infected with 5×10^4 pfu LCMV. At days 7, 8, or 9 post infection spleens were isolated and total numbers of donor IFNαβR KO P14 cells were determined. (**A**) Representative flow plots gated on CD8α+ cells are shown. (**B**) Total number of IFNαβR KO P14 cells from control- (open bars) or poly(I:C)-pretreated mice (black bars) is graphed. Data combined from 3 individual experiments with n of 2-4 mice per group.

mice were determined at various times after LCMV infection. Poly(I:C)-pretreated IFNAR KO P14 cells proliferated to similar numbers as the control-treated counterparts at both day 7 and 8 post LCMV infection. The fact that these CD8 T cells lacked expression of the IFNAR and showed no difference in proliferation between HBSS- and poly(I:C)-primed groups suggested that there was a direct role for type 1 IFN on the CD8 T cells in this model of immune suppression.

Early detection of viral RNA is essential for efficient control of pathogenic infections. TLR3, an innate immune receptor that recognizes dsRNA, is known to play a major role during infections and when triggered can induce type 1 IFN through downstream pathways (3, 4, 12, 23). To determine if TLR3-mediated IFN induction was required for the poly(I:C)-induced suppression of CD8 T cell expansion, I utilized TLR3 KO host mice during the priming period. Congenic P14 splenocytes were transferred into WT B6 or TLR3 KO host mice, which were primed with poly(I:C) or HBSS (control) 1 day later. One day after inoculation, equal numbers of P14 cells from each treatment group were determined and transferred into WT B6 mice, which were subsequently infected with LCMV. At day 7 post infection, spleens were harvested and the total proportion (**Figure 3.5A**) and number (**Figure 3.5B**) of donor cells were enumerated. Similar to WT controls, suppression of T cell expansion was also seen from the TLR3 KO-primed cohort, indicating that TLR3 is not required for poly(I:C)-induced inhibition of CD8 T cell proliferation. However, because the donor P14 cells were TLR3

Figure 3.5: TLR3 is not required for poly(I:C)-induced suppression of CD8 T cell proliferation
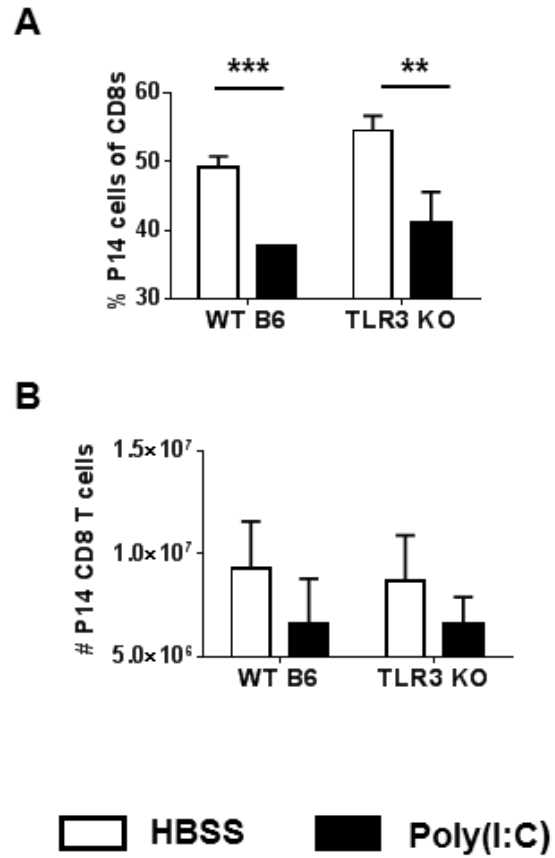
Figure 3.5: TLR3 is not required for poly(I:C)-induced suppression of CD8 T cell proliferation

Congenic P14 cells were transferred into WT B6 or TLR3 KO mice, which were treated with HBSS or poly(I:C) i.p. One day after treatment, equal numbers of P14 cells from each group were transferred into WT B6 hosts, which were subsequently infected with 5×10^4 pfu LCMV. Splenocytes were harvested at day 7 post infection. Frequency (**A)** and total number (**B**) of donor P14 cells from HBSS- (open bars) or poly(I:C)- (black bars) pretreated mice are graphed. Data from experiment with n of 2-4 mice per group.

sufficient, this experiment does not determine if CD8 T cells themselves are responding to poly(I:C) inoculation and producing type 1 IFN.

2. The priming hosts do not require Class I MHC to mediate poly(I:C)-induced CD8 T cell suppression of proliferation.

Previous studies from our lab investigating bystander sensitization to rapid effector function indicated there was a direct requirement of Class I MHC to mediated sensitization (193). Prior studies showed that P14 mice could not be directly sensitized, which we attributed to the competition for self-MHC. We hypothesized that because of the overwhelming number of P14 cells in the P14 host, the chance a particular T cell could see the self-MHC required for sensitization would be greatly reduced.

Therefore, we questioned whether the poly(I:C)-induced suppression of proliferation also required Class I MHC. Ly5.1 P14 splenocytes were transferred into WT B6 or β2M KO hosts and primed with poly(I:C) for 1 day prior to transfer into WT hosts. Recipient mice were infected with LCMV, and splenocytes were harvested at day 7 post infection. Representative flow plots gated on CD8α+ lymphocytes (**A**) and total number of donor P14 cells (**B**) are shown in **Figure 3.6**. Probably due to the large variability in this particular experiment, the difference in total number of poly(I:C)- (black bars) and HBSS- (open bars) pretreated P14 cells from β2M KO hosts does not reach statistical significance (# p=0.0901). However, there is a trend of poly(I:C)-induced suppression of CD8 T

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Figure 3.6: Class I MHC is not required for poly(I:C)-induced suppression of proliferation
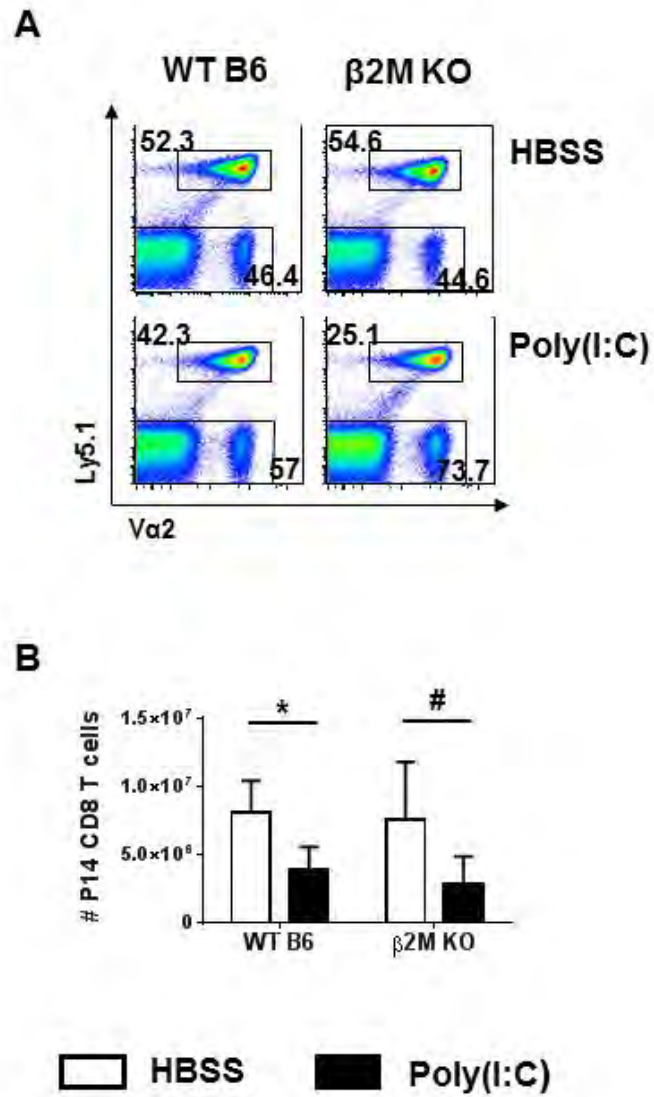
Figure 3.6: Class I MHC is not required for poly(I:C)-induced suppression of proliferation

Ly5.1 P14 splenocytes were transferred into WT B6 or β2M KO mice, which were inoculated with HBSS or poly(I:C) for 1 day. Equal numbers of poly(I:C) or control P14 cells were transferred into separate WT B6 hosts and infected with 5×10^4 pfu LCMV. Spleens were harvested at day 7 post infection. (**A**) Representative flow plots gated on CD8α+ cells are shown. (**B**) Total number of donor P14 cells is graphed. Data from experiment with n of 4 mice per group. # represents p value of 0.0901.

cell expansion, a trend similar to what is seen during poly(I:C)-priming in WT hosts. Since this phenotype is similar to what is seen when P14 cells are primed in a WT host, it suggests that Class I MHC is not required for poly(I:C)-induced suppression of CD8 T cell expansion, unlike IFN-induced sensitization of bystander CD8 T cells to acquire rapid effector function.

After determining Class I MHC was not required for poly(I:C)-induced suppression of T cell responses, I questioned whether P14 mice could be primed directly, without the dual transfer model used in previous studies. I hypothesized that since Class I was not required, self-MHC would not be a limiting factor and P14 mice could therefore be sensitized directly, without the need for P14 CD8 cells to be transferred into other hosts. Ly5.1 P14 mice were directly treated with HBSS (control) or poly(I:C) for 1 day prior to equal numbers of the transgenic cells being transferred into WT recipient hosts, which were subsequently infected with LCMV. **Figure 3.7** shows that P14 mice can be directly treated with poly(I:C) to induce suppressive effects on T cell expansion. Reduced proportions (**Figure 3.7A-B**) and total numbers (**Figure 3.7C**) of poly(I:C)-treated (black bars) P14 cells at day 7 post LCMV infection compared to the HBSS controls (open bar) were found. Together with **Figure 3.6**, these data suggest that Class I and excess of specific self-antigen is not required for poly(I:C)-induced suppressive effects on CD8 T cells.

Figure 3.7: Suppression of proliferation occurs with direct treatment of P14 mice with poly(I:C)
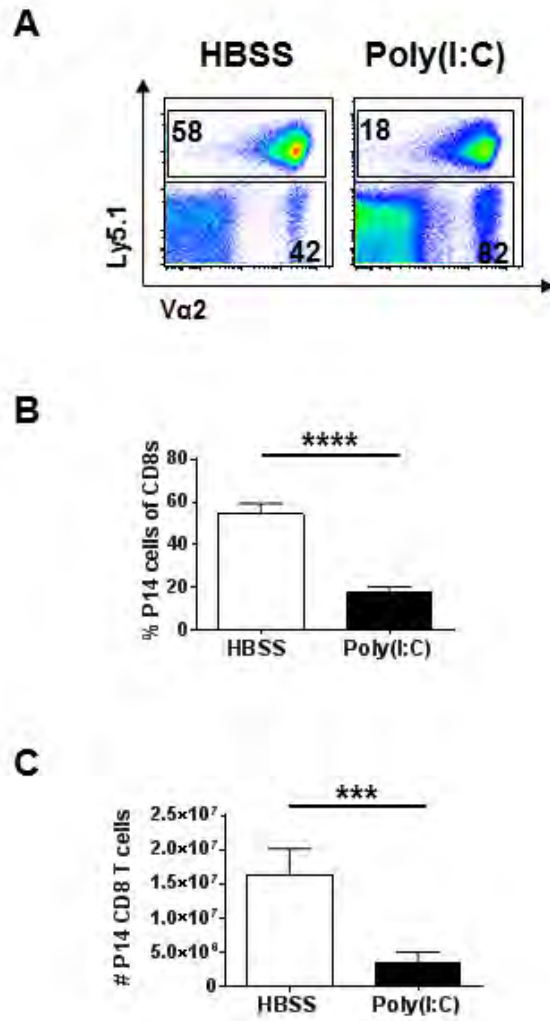
Figure 3.7: Suppression of proliferation occurs with direct treatment of P14 mice with poly(I:C)

Ly5.1 P14 mice were directly treated with HBSS or poly(I:C) for 1 day. Equal numbers of P14 transgenic cells were transferred into WT B6 hosts, which were inoculated with $5x10^4$ pfu LCMV i.p. Spleens were harvested at day 7 post infection. (**A**) Representative flow plots gated on CD8α+ cells are depicted. Frequency (**B**) and total number (**C**) of P14 cells are graphed. Data are representative of at least 5 experiments with n of 3-5 mice per group.

3. Poly(I:C)-induced suppression of CD8 T cell expansion is seen in other models.

Up until this point, poly(I:C)-induced suppression of proliferation had only been shown in P14 CD8 T cells in response to LCMV infection. I questioned whether or not poly(I:C) had suppressive effects on CD4 T cell expansion, or if it was specific to CD8 T cells. Therefore, I utilized transgenic SMARTA CD4 T cells, specific for the GP61 epitope of LCMV, to identify the effects of poly(I:C) pretreatment on CD4 T cell expansion (176). Congenic Ly5.1 SMARTA mice were inoculated with HBSS or poly(I:C) for 1 day. Equal numbers of CD4 transgenic T cells, determined by flow cytometry, were transferred into separate host mice, which were subsequently infected with LCMV, and spleens were harvested at day 9 post infection. The HBSS- and poly(I:C)-treated SMARTA cells expanded to similar levels, in terms of both proportion (**Figure 3.8A-B**) and number (**Figure 3.8C**). Thus, CD4 T cells seem to be resistant to the suppressive effects of poly(I:C), indicating it does not have inhibitory effects on all T cell populations but instead may be more specific to CD8 T cells.

I also wanted to determine if suppression of proliferation could be seen using other virus infection models in addition to LCMV. Transgenic P14 CD8 T cells were still utilized, but in addition to looking at the proliferative response to LCMV, the ability of poly(I:C)-pretreated cells to expand in response to VACV-GP infection was also investigated. VACV-GP is a recombinant vaccinia virus that

Figure 3.8: SMARTA CD4 T cells do not show suppression of clonal expansion if pre-exposed to poly(I:C)
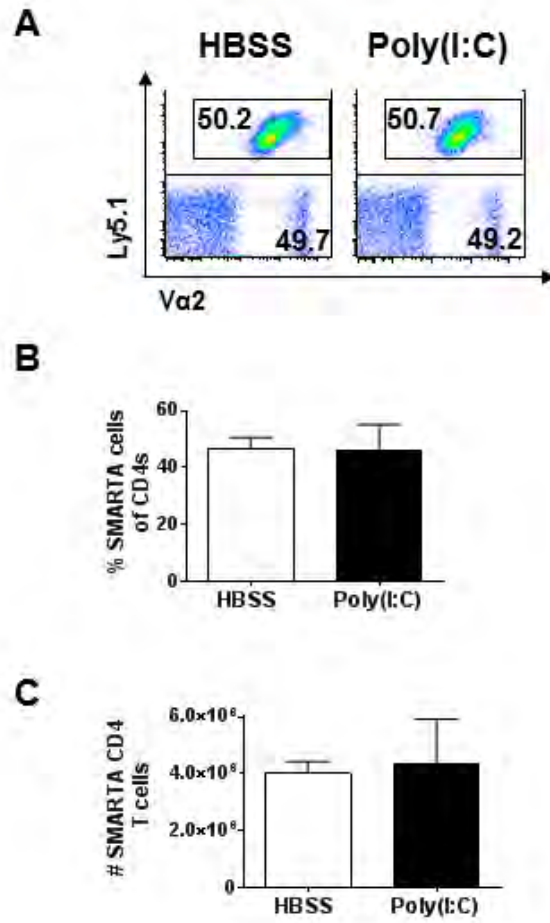
Figure 3.8: SMARTA CD4 T cells do not show suppression of clonal expansion if pre-exposed to poly(I:C)

Ly5.1 SMARTA mice were HBSS or poly(I:C) treated for 1 day. Equal numbers of SMARTA transgenic cells were transferred into WT B6 mice, which were subsequently infected with 5×10^4 pfu LCMV. Spleens were harvested at day 9 post infection, with representative flow plots gated on CD4+ cells shown in (**A**). Frequency (**B**) and total number (**C**) of donor SMARTA cells are graphed. Data from experiment with n of 5 mice per group.

expresses the glycoprotein of LCMV. Ly5.1 P14 mice were inoculated with HBSS or poly(I:C) for 1 day, and equal numbers of P14 cells were transferred into mice, which were either infected with LCMV or VACV-GP. Because the peak of the CD8 T cell response is earlier in VACV infection (194), splenocytes were harvested and stained at day 6 post infection. The representative flow plots, gated on CD8α+ lymphocytes in **Figure 3.9A** show increased proportions of the HBSS- (control) treated P14 cells compared to the poly(I:C)-treated P14 cells in response to both LCMV and VACV-GP infection. The magnitude of P14 T cell expansion was much lower in the VACV-GP-infected animals compared to the LCMV-infected animals (**Figure 3.9B-C**), note the difference in y-axis in **Figure 3.9B,** but suppression of clonal expansion was still seen in poly(I:C)-pretreated P14 cells. The suppression seen in response to both infections suggests the distinct inflammatory environments induced by each virus is not mediating the inhibition, and may be more intrinsic to the CD8 T cells.

I wanted to determine if the suppression of proliferation was also seen in a polyclonal population and not just seen using a transgenic T cell system. In the present study, Ly5.1 mice were HBSS or poly(I:C) treated for 1 day, and total splenocytes were transferred into congenic WT B6 mice, which were subsequently infected with LCMV. At day 7 post infection, spleens were harvested and cells were stimulated *ex vivo* with LCMV specific peptides including GP276, NP205 and the co-dominant NP396 and GP33 epitopes. **Figure 3.10** shows the Ly5.1 donor population on the left (**A-E**) and the host CD8

Figure 3.9: Impaired clonal expansion of poly(I:C)-pretreated P14 CD8 T cells in response to VACV-GP infection
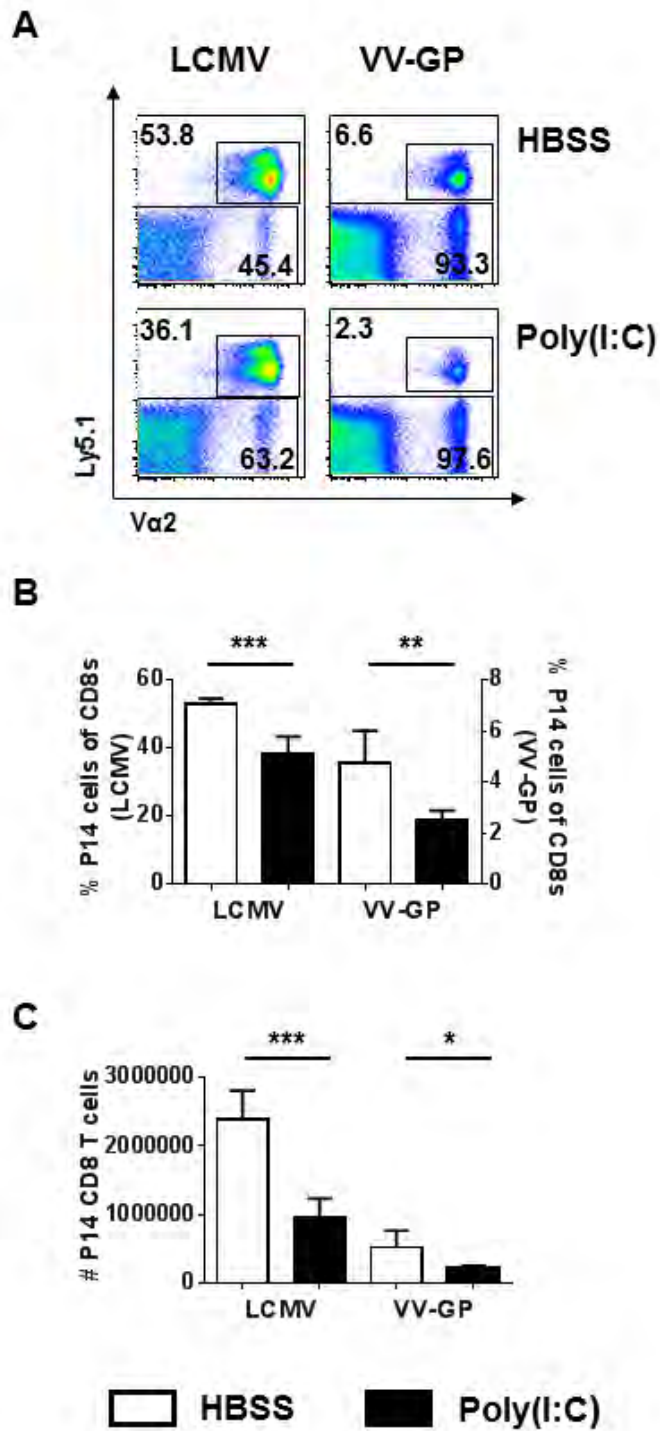
Figure 3.9: Impaired clonal expansion of poly(I:C)-pretreated P14 CD8 T cells in response to VACV-GP infection

Ly5.1 P14 mice were inoculated with HBSS or poly(I:C) i.p. One day later, splenocytes were harvested and similar numbers of HBSS- or poly(I:C)-pretreated P14 cells were transferred into WT B6 mice, which were subsequently infected with $5x10^4$ pfu LCMV or $5x10^6$ VACV-GP i.p. Splenocytes were harvested at day 6 post infection. (**A**) Representative flow plots gated on CD8α+ cells are depicted. Frequency (**B**) and total number (**C**) of P14 CD8 T cells from HBSS- (open bars) or poly(I:C)-treated (black bars) mice are graphed. Data from experiment with n of 5 mice per group.

population on the right (**A, and F-I**). The total number of donor and host CD44hi CD8 T cells was calculated from HBSS-treated (open bars) and poly(I:C)-treated (black bars) groups and are shown in **Figure 3.10A**. Host mice receiving HBSS- or poly(I:C)-treated splenocytes showed similar numbers of CD44hi CD8 T cells (right), whereas donor cells (left) showed reduced numbers of poly(I:C)-pretreated CD44hi CD8 T cells when compared to the HBSS-control CD44hi CD8 T cell number. To identify if the donor population had a skewed immunodominance hierarchy that may have been contributing to reduced number of CD44hi CD8 T cells seen in poly(I:C)-pretreated cells, multiple epitopes were tested. The donor population, regardless of the pretreatment regime, had similar proportions of the co-dominant NP396 (red) and GP33 (yellow) specific cells compared to the host populations (**B, C vs F, G**). The donor population had slightly higher proportions of the sub-dominant epitopes GP276 (green) and NP205 (blue) compared to the host cells. However, the over-all immunodominance hierarchy was not significantly skewed when comparing donor and host populations, regardless of the pretreatment regime. What was most striking was the absolute number of peptide-specific CD8 T cells from comparing HBSS- and poly(I:C)-pretreated donor populations (**Figure 3.10D-E**). Donor cells pretreated with poly(I:C) showed significantly lower numbers of peptide specific cells compared to the control-treated donor CD8 T cells. This is unlike what is seen in the host population, where both sets of mice receiving HBSS- or poly(I:C)-treated splenocytes showed similar numbers of peptide-

Figure 3.10: Polyclonal populations are also susceptible to poly(I:C)-induced suppression of proliferation
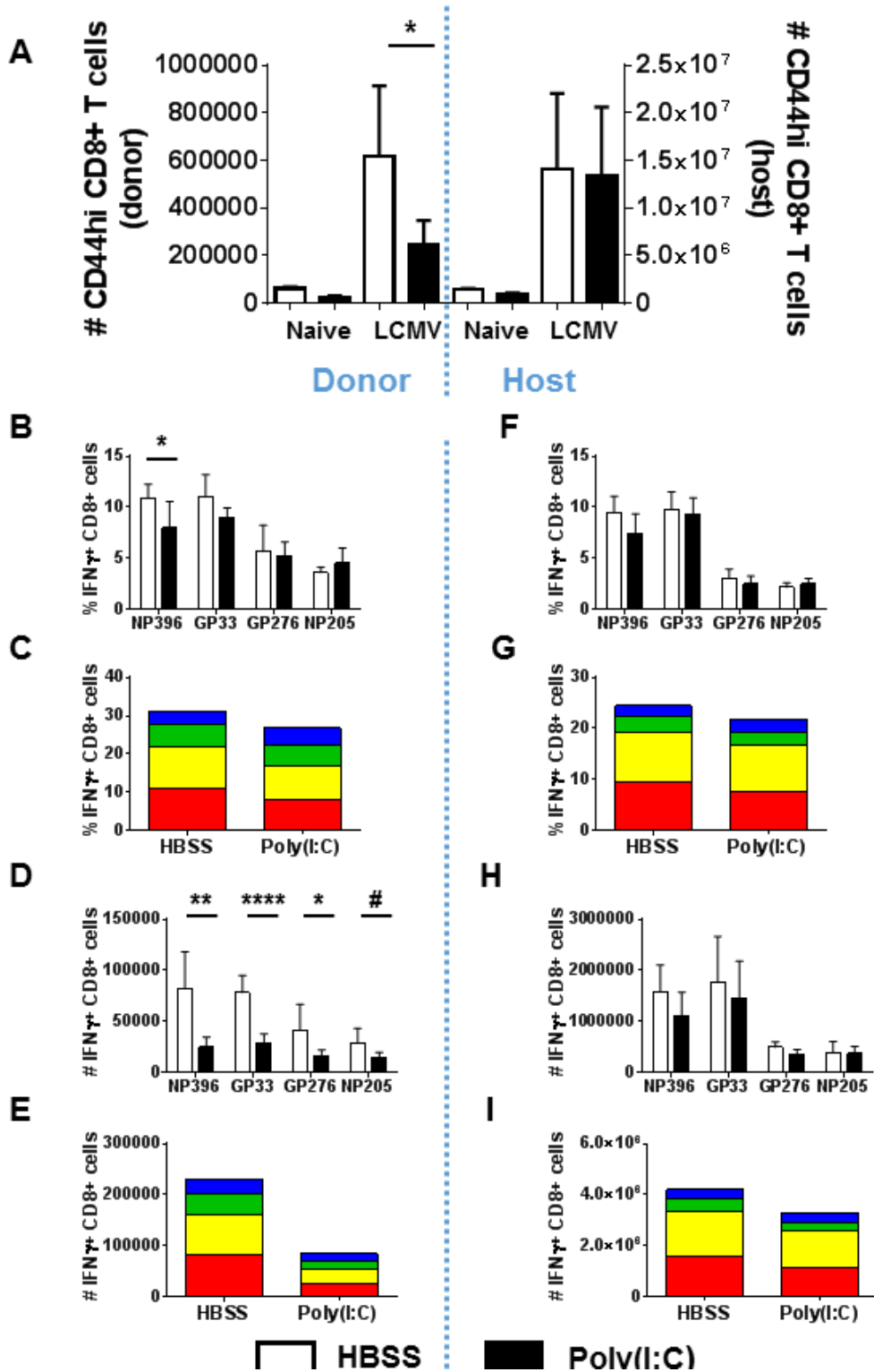
Figure 3.10: Polyclonal populations are also susceptible to poly(I:C)-induced suppression of proliferation

Ly5.1 mice were treated with HBSS or poly(I:C) for 1 day. Similar numbers of CD44lo CD8 T cells were transferred into WT B6 mice, which were infected with 5×10^4 pfu LCMV. At day 7 post infection, spleens were harvested and intracellular cytokine staining assay was performed. Mice receiving poly(I:C)-treated splenocytes are shown as black bars, and control-treated splenocytes are shown as open bars. (**A**) Total number of CD44hi CD8 T cells per spleen from donors vs. host mice is graphed. Frequency (**B-C, F-G**) and total number (**D-E, H-I**) of CD44+IFNγ+ CD8 T cells after peptide stimulation (NP396 (red), GP33 (yellow), GP276 (green) or NP205 (blue)) from donor (**B-E**) and hosts (**F-G**) are graphed. Frequency (**C and G**) and number (**E and I**) of peptide-stimulated CD44+IFNγ+ CD8 T cells from HBSS or poly(I:C) cohorts are graphed (additive). Data from experiment with n of 6 mice per group. # indicates p=0.0510.

specific CD8 T cells (**Figure 3.10H-I**). If poly(I:C)-pretreated cells were releasing suppressive factors, we would expect to see reduced expansion in the host population as well, but since we see no defect in host expansion it suggests that there is an intrinsic defect in clonal expansion in poly(I:C)-treated CD8 T cells, rather than poly(I:C) treatment inducing suppressive effects on other cell types.

4. Summary

Virus infections have been shown to induce a transient state of immune suppression, which can be mimicked by the dsRNA analog poly(I:C) (182). Poly(I:C) can induce transient suppressive effects on CD8 T cell clonal expansion in response to prolonged cognate antigen encounter. The greatest suppressive effects were seen at 1 day post poly(I:C) treatment. By 3 days post poly(I:C) treatment, the suppressive effects of pre-exposure are diminished (**Figure 3.3).**

Our lab has previously shown that acute virus infections can affect bystander CD8 T cells whereby they acquire immediate effector function upon stimulation with cognate antigen (193). The mechanism of bystander sensitization was shown to require indirect effects of type 1 IFN and self-MHC. However, the poly(I:C)-induced CD8 T cell suppression shown here is mediated by a distinct mechanism. Impaired clonal expansion does not require Class I MHC in the sensitizing environment to mediate effects **(Figure 3.6**). Suppression of proliferation does, however, require type 1 IFN, but unlike what is seen in the

bystander sensitization to rapid effector function, the poly(I:C)-mediated impaired proliferation requires direct effects of type 1 IFN on CD8 T cells (**Figure 3.4**). P14 mice directly treated with poly(I:C) may induce suppression (**Figure 3.7**) of proliferation but not rapid effector function because of the direct vs. indirect requirements for type 1 IFN, respectively.

Although poly(I:C) induces suppressive effects on CD8 T cell expansion, not all lymphocyte subsets were susceptible to poly(I:C)-induced impaired proliferation, as CD4 T cells remained unaffected by IFN pretreatment (**Figure 3.8**). Given that P14 T cells are a monoclonal population of GP33-specific cells, we wanted to identify if poly(I:C) induced suppressive effects on other polyclonal populations. Indeed, poly(I:C)-pretreated Ly5.1 splenocytes transferred into congenic hosts infected with LCMV showed reduced magnitude of antigen specific responses without skewing or altering the immunodominance hierarchy, or proportions of antigen-specific cells tested (**Figure 3.10**). These data suggest that poly(I:C) pretreatment affects more than just a monoclonal transgenic population, but also has suppressive effects on a polyclonal population. Data indicate that during an acute viral infection, CD8 T cells may be exposed to these inflammatory-induced environments and this may be one mechanism that contributes to the transient state of immune suppression seen during acute viral infections.

C. Poly(I:C)-induced lymphocyte refractoriness to IFN stimulation

1. Poly(I:C)-pretreated CD8 T cells are transiently refractory to IFNβ stimulation in terms of STAT phosphorylation

Knowing that type 1 IFN delivered at an optimal time can provide a positive signal 3 to CD8 T cells and enhance their proliferation, I questioned whether an out of sequence early exposure to IFN would interfere with later attempts at IFN signaling, as seen in poly(I:C)-induced suppression of T cell proliferation. Type 1 IFN can activate multiple downstream STAT molecules, including STAT1, 3, 4, and 5 (32). Because type 1 IFN can have both positive and negative effects on T cell expansion, where recent studies have shown that the specific STAT(s) activated dictate the outcome, all of the aforementioned STAT molecules were tested (52, 54, 90, 93, 94). The phosphorylation of STAT molecules downstream of the type 1 IFN receptor was thus examined in CD8 T cells from mice pretreated with either HBSS or poly(I:C). Mice were inoculated with either HBSS or poly(I:C) for 1 day, and their splenocytes were isolated and stimulated *ex vivo* with murine recombinant IFNβ for ~30 min, followed by phosflow to examine downstream STAT phosphorylation (**Figure 3.11**). In unstimulated (non-IFNβ-treated) CD44lo CD8 T cells, there was very little phosphoSTAT staining, regardless of the pretreatment regimen (**Figure 3.11A,** shaded histograms). In T cells from HBSS-treated mice, the phenotypically naïve CD44lo CD8 T cells responded strongly to IFNβ stimulation and showed phosphoSTAT 1, 3, 4 and 5 staining well above the unstimulated controls (solid

Figure 3.11: Poly(I:C)-pretreated naïve CD44lo CD8 T cells are refractory to IFNβ stimulation as measured by STAT phosphorylation
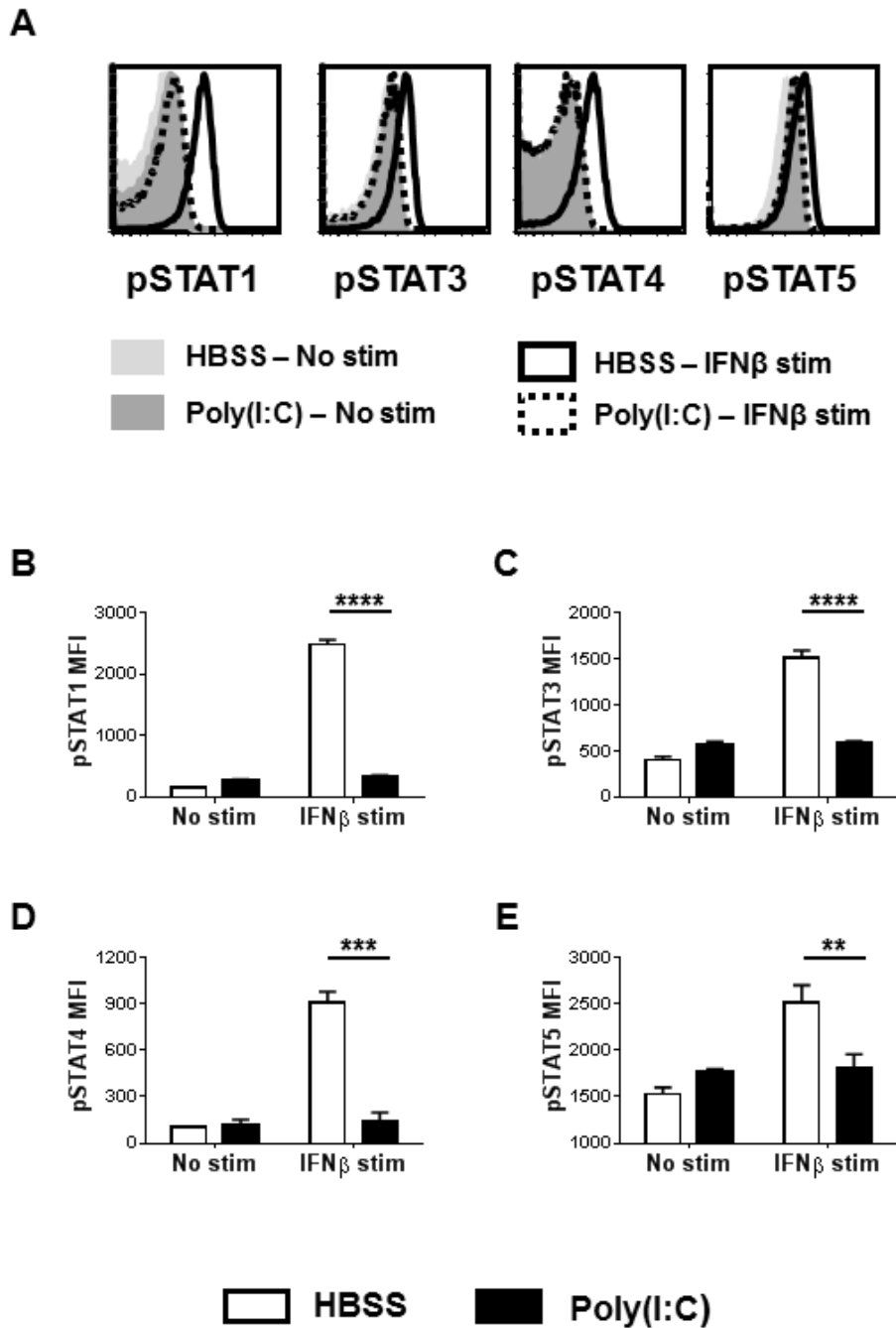
Figure 3.11: Poly(I:C)-pretreated naïve CD44lo CD8 T cells are refractory to IFNβ stimulation as measured by STAT phosphorylation

Mice were HBSS or poly(I:C) treated for 1 day. Splenocytes were isolated and either remained unstimulated or were stimulated *ex vivo* with IFNβ for 30 min and then stained for pSTAT1 (**B**), pSTAT3 (**C**), pSTAT4 (**D**) and pSTAT5 (**E**). (**A**) shows representative histograms gated on CD44lo CD8α+ (naïve) T cells showing pSTAT1, 3, 4 or 5 staining in HBSS– or poly(I:C)-pretreated naïve CD8 T cells unstimulated (shaded histograms) or IFNβ stimulated (open histograms). HBSS-pretreated naïve CD8 T cells stimulated with IFNβ shown as solid line histograms, and poly(I:C)-pretreated naïve CD8 T cells stimulated with IFNβ shown as dotted line histogram. (**B-E**) pSTAT MFI of naive T cells from unstimulated vs. IFNβ-stimulated cells from HBSS- (open bars) or poly(I:C)- (black bars) pretreated mice are graphed. Data are representative of at least 4 independent experiments with n of 3 mice per group.

line, open histograms in **Figure 3.11A**; open bars in **Figure 3.11B-E**). However, CD44lo CD8 T cells from mice pre-exposed to poly(I:C) for 1 day were unable to respond to IFNβ stimulation and did not phosphorylate any downstream STAT molecules tested (dashed line open histogram in **Figure 3.11A**; black bars in **Figures 3.11B-E**). Similar to naïve CD8 T cells, which represent most of the T cells and are the focus of this study, CD44hi memory phenotype CD8 T cells from poly(I:C)-pretreated mice also showed a reduced response to IFNβ stimulation in terms of downstream STAT phosphorylation (**Figure 3.12**). Similar to the naïve phenotype CD8 T cells, memory phenotype CD8 T cells did not phosphorylate any of the downstream STATs tested (STAT 1, 3, 4 or 5 (**Figure 3.12 A, B, C, and D**) respectively) if pre-exposed to poly(I:C). Since STAT phosphorylation is a transient event, a kinetic analysis of STAT phosphorylation in cells from HBSS- or poly(I:C)-inoculated mice stimulated with IFNβ for times ranging from 5 minutes to 2 hours was performed. The poly(I:C)-pretreated CD8 T cells did not phosphorylate downstream STATs above unstimulated controls at any time point tested (data not shown). The lack of IFNβ-induced phosphoSTAT staining in poly(I:C)-pretreated CD8 T cells suggests that the T cells are unable to respond to further IFN signals and therefore do not receive either the positive or the negative effects that type 1 IFN can have on lymphocytes.

To test the duration of this unresponsiveness to IFNβ stimulation, mice were inoculated with HBSS or poly(I:C) and after 1, 2, or 3 days, their splenocytes were stimulated *ex-vivo* with IFNβ for ~30 min before staining for

Figure 3.12: Poly(I:C)-pretreated memory phenotype CD8 T cells are refractory to IFNβ-induced STAT phosphorylation
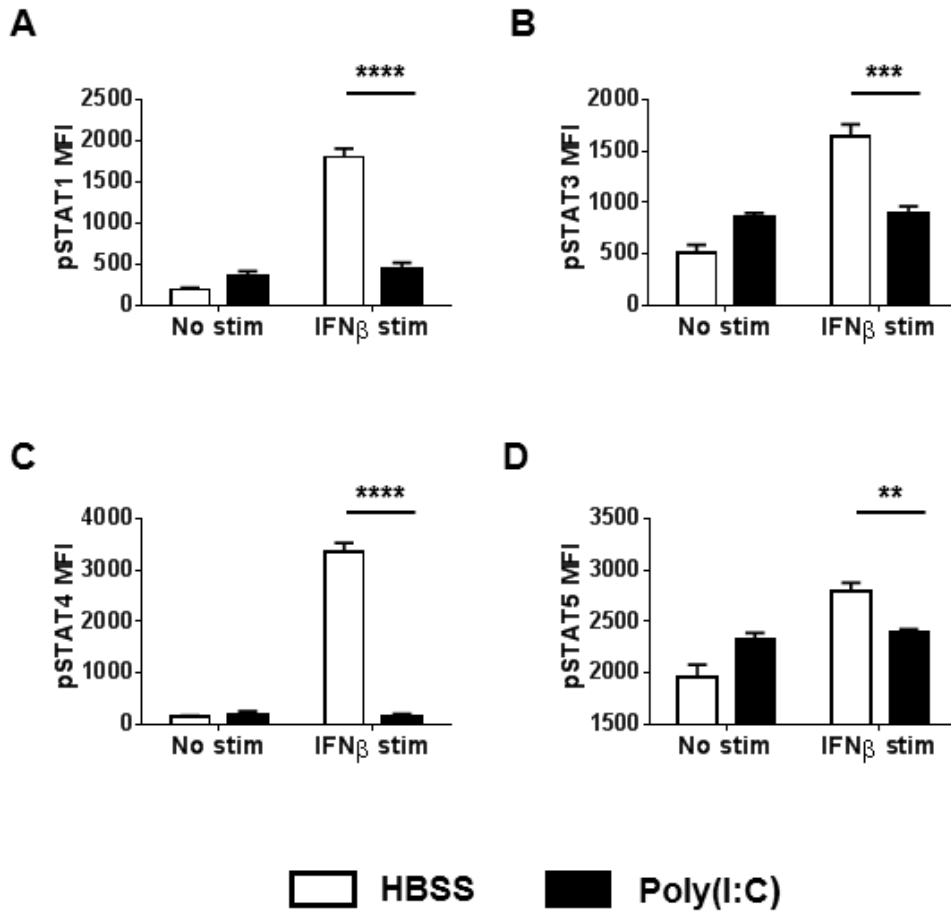
Figure 3.12: Poly(I:C)-pretreated memory phenotype CD8 T cells are refractory to IFNβ-induced STAT phosphorylation

Mice were HBSS (open bars) or poly(I:C) (black bars) treated for 1 day. Splenocytes were isolated and either remained unstimulated or were stimulated *ex vivo* with IFNβ for 30 min and then stained for pSTAT1 (**A**), pSTAT3 (**B**), pSTAT4 (**C**) and pSTAT5 (**D**). Cells were gated on CD44hi CD8α+ lymphocytes, and the MFI of each respective pSTAT is graphed. Data are representative of at least 4 independent experiments with n of 3 mice per group.

phosphoSTATs. As shown in **Figure 3.11**, the phenotypically naive CD8 T cells from mice pretreated with poly(I:C) for 1 day did not respond to IFNβ stimulation in terms of STAT phosphorylation and this is also shown in **Figures 3.13A-D** (black bars). Similarly, naïve CD8 T cells from poly(I:C)-pretreated mice were also less responsive to IFN stimulation when treated 2 days previously compared to controls. However, by 3 days after pretreatment, the CD44lo CD8 T cells from poly(I:C)-treated mice started to regain the ability to respond to IFNβ stimulation and showed downstream STAT phosphorylation above unstimulated controls. In this particular experiment, at 3 days, the IFNβ-stimulated poly(I:C)-pretreated naïve CD8 T cells phosphorylated downstream STATs above unstimulated controls but were still significantly lower than pSTAT levels from IFNβ-stimulated HBSS-pretreated CD44lo CD8 T cells. However, in a separate experiment, at 3 days, the poly(I:C)-pretreated CD8 T cells phosphorylated downstream STATs to similar levels as HBSS-pretreated CD8 T cells for all STATs tested except STAT4. Despite the slight variation from experiment to experiment, the data show that poly(I:C)-induced refractoriness to IFNβ stimulation is transient.

Memory phenotype CD44hi CD8 T cells pretreated with poly(I:C) showed a similar transient unresponsiveness to IFNβ stimulation (**Figure 3.13E-H)** as the naïve CD44lo CD8 T cells did (**Figure 3.13A-D)**. However, unlike the other STATs tested, CD44hi CD8 T cells did not show a strong reduction in phosphoSTAT5 staining in poly(I:C)-pretreated populations stimulated with IFNβ. That being said, pSTAT5 staining had the greatest background staining among

Figure 3.13: Poly(I:C)-mediated refractoriness to IFNβ-induced STAT phosphorylation is transient
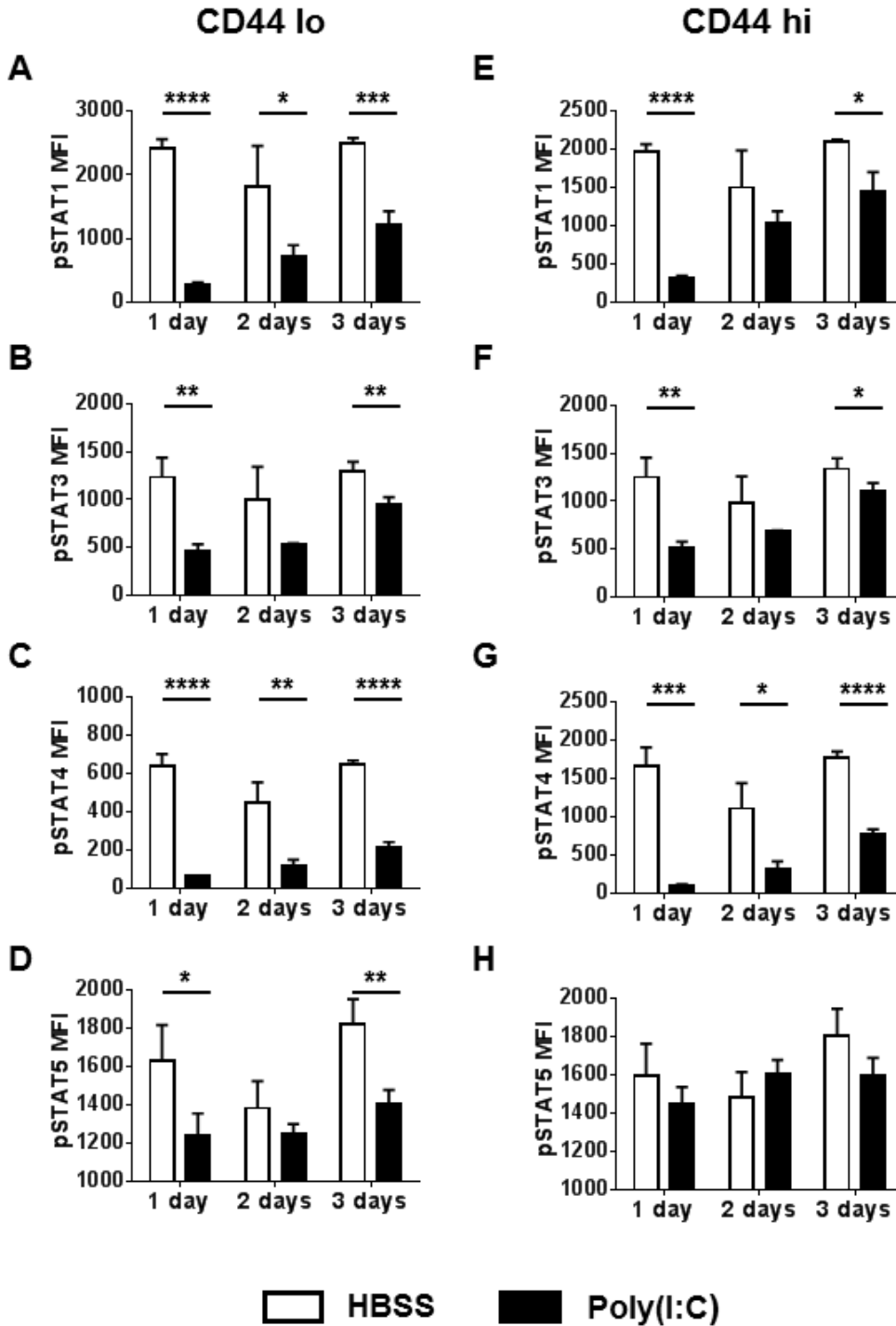
Figure 3.13: Poly(I:C)-mediated refractoriness to IFNβ-induced STAT phosphorylation is transient

Mice were HBSS (open bars) or poly(I:C) (black bars) treated once 1, 2, or 3 days prior to isolation. Splenocytes were isolated, stimulated *ex vivo* with IFNβ for 30 min and then stained for phosphoSTATs. Cells were gated on CD44lo CD8α+ T cells (**A-D**) or CD44hi CD8α+ T cells (**E-G**) plotting pSTAT MFI after IFNβ stimulation. pSTAT1 (**A, E**), pSTAT3 (**B, F**), pSTAT4 (**C, G**) or pSTAT5 (**D, H**) MFI is graphed. Data are representative of 2 independent experiments with n of 3 mice per group.

all of the pSTATs tested (This can be seen in the representative histograms in **Figure 3.11A**) and therefore is the hardest to detect small changes in pSTAT levels. Combined, **Figure 3.13** show that the refractoriness to IFNβ stimulation is transient, with kinetics similar to that of the poly(I:C)-induced impaired proliferation (**Figure 3.3**).

To make sure that STAT molecules were available to be phosphorylated, total STAT protein levels in CD8 T cells after different days post HBSS or poly(I:C) inoculation were determined (**Figure 3.14**). After 1, 2, and 3 days of treatment, total STAT 1, 3, 4 and 5 levels in poly(I:C)-pretreated CD44lo (**Figure 3.14A-D**) and CD44hi (**Figure 3.14E-H**) CD8 T cells were similar to, if not higher than, the control-treated cells. Total STAT1 expression was higher in poly(I:C)-pretreated naïve CD8 T cells after 1 day and stayed high through day 3 of treatment, as compared to STAT1 levels in HBSS-treated CD8 T cells (**Figure 3.14A and E**). Since STAT1 is an IFN-inducible gene (12, 39), higher STAT1 protein expression in poly(I:C)-pretreated CD8 T cells was expected. These data indicate that the reduced phosphoSTAT staining found in IFNβ-stimulated poly(I:C)-pretreated CD8 T cells was not due to lower levels of total STAT protein.

2. **Poly(I:C)-pretreated lymphocytes are responsive to cytokines other than type 1 IFN**

Figure 3.14: CD8 T cells express similar or increased levels of total STAT protein after poly(I:C) treatment
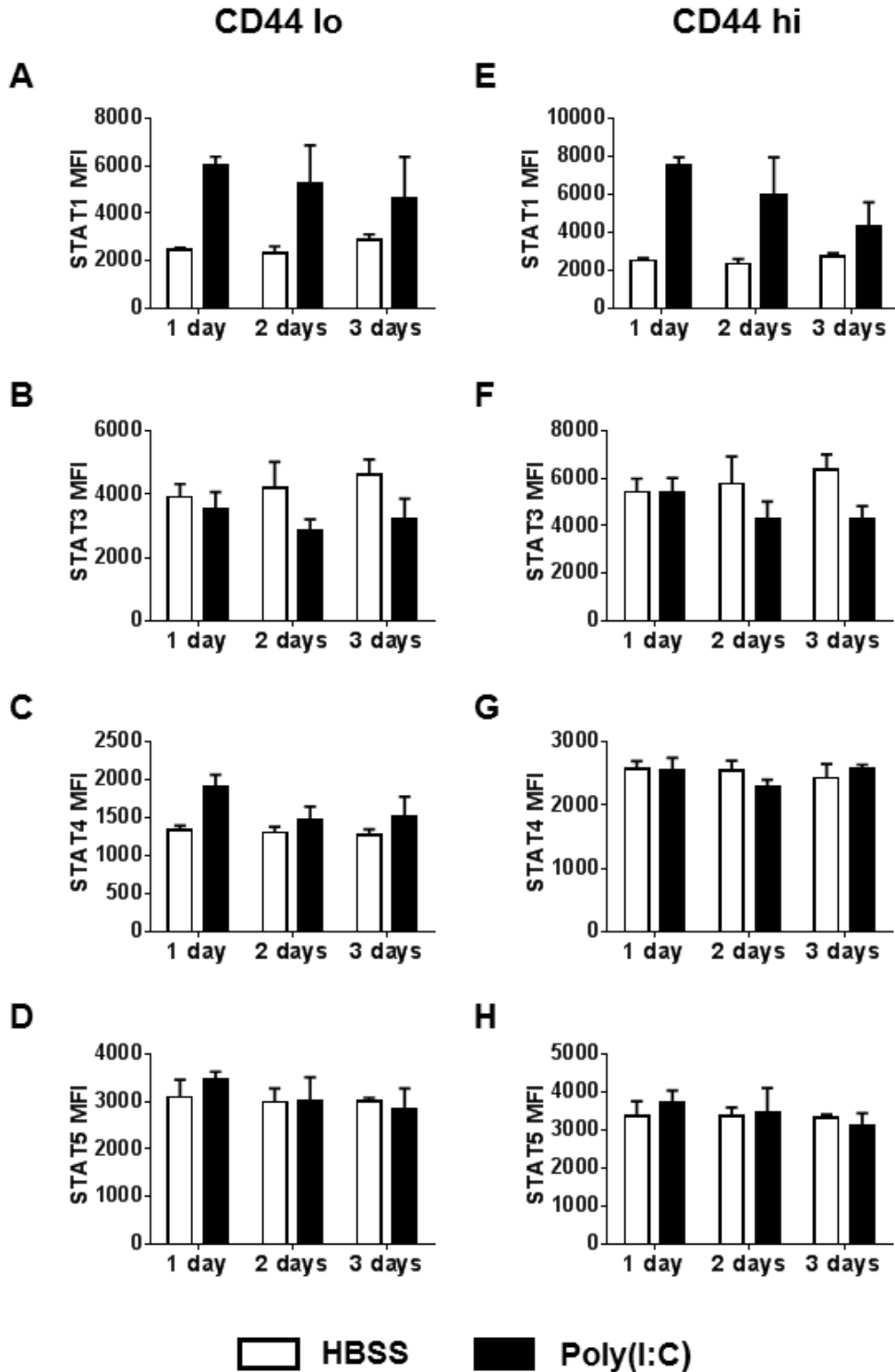
Figure 3.14: CD8 T cells express similar or increased levels of total STAT protein after poly(I:C) treatment

Mice were HBSS (open bars) or poly(I:C) (black bars) treated once 1, 2, or 3 days prior to isolation. Cells were gated on CD44lo CD8α+ T cells (**A-D**) or CD44hi CD8α+ T cells (**E-H**). STAT1 (**A, E**), STAT3 (**B, F**), STAT4 (**C, G**) or STAT5 (**D, H**) MFI is graphed. Data are representative of 2 independent experiments with n of 3 mice per group.

To test if the poly(I:C)-primed CD8 T cells were unresponsive to other cytokines, splenocytes were stimulated *ex vivo* with various cytokines for ~30 min before staining for the appropriate downstream phosphoSTATs. I tested IFNβ, IL-2, IL-6, IL-7, IL-12, and IL-15, because these cytokines have positive effects on lymphocyte survival or proliferation. It should be noted that CD8 T cells are not the only lymphocyte populations that are affected by poly(I:C) pretreatment. CD4 T cells and NK cells were also found to respond differently to cytokine stimulation induced STAT phosphorylation. **Tables 3.1-3.4** show cytokine-induced pSTAT MFIs +/- SD for different cell types, including CD4, CD8 and NK cells. Values in bold represent a pSTAT MFI above the associated unstimulated control without any overlap in SD.

Table 3.1 shows pSTAT1 MFI of different lymphocyte populations after *ex vivo* stimulation without additional cytokine, with IFNβ, IL-2, or with IL-6. Not all lymphocyte populations phosphorylated STAT1 in response to all cytokine stimulations, regardless of the pretreatment regimen. As an example, CD4, CD8 and NK cells did not phosphorylate STAT1 in response to IL-2 stimulation above the unstimulated control, but some cells did show response to IFNβ and IL-6. The variability in lymphocyte cytokine responsiveness was also seen looking at other STAT molecules, including STAT3 (**Table 3.2**), STAT4 (**Table 3.3**), and STAT5 (**Table 3.4**). As an example, T cells responded to IL-6 stimulation and phosphorylated STAT3, but NK cells did not (**Table 3.2**).

Table 3.1 Cytokine-induced phosphorylation of STAT1 in lymphocytes after poly(I:C) treatment

CD4 T cells		No stim	IFNβ	IL-2	IL-6
CD44hi	HBSS	280 ± 13	**3289 ± 544**	296 ± 19	**1447 ± 363**
	Poly(I:C)	535 ± 155	488 ± 17	445 ± 18	741 ± 71
CD44lo	HBSS	269 ± 88	**3574 ± 337**	279 ± 122	**3922 ± 649**
	Poly(I:C)	474 ± 100	452 ± 14	390 ± 8	**1461 ± 297**

CD8 T cells		No stim	IFNβ	IL-2	IL-6
CD44hi	HBSS	348 ± 33	**3880 ± 289**	319 ± 27	**1371 ± 189**
	Poly(I:C)	739 ± 277	666 ± 32	549 ± 34	1194 ± 248
CD44lo	HBSS	311 ± 82	**4712 ± 494**	296 ± 86	**4175 ± 673**
	Poly(I:C)	642 ± 194	586 ± 23	500 ± 17	**2408 ± 491**

NK cells		No stim	IFNβ	IL-2	IL-6
	HBSS	266 ± 21	**1523 ± 63**	241 ± 3	256 ± 13
	Poly(I:C)	879 ± 170	833 ± 49	840 ± 46	832 ± 57

Table 3.1 Cytokine-induced phosphorylation of STAT1 in lymphocytes after poly(I:C) treatment

B6 mice were inoculated with HBSS or poly(I:C) and spleens were harvested 1 day later. Splenocytes were stimulated individually *ex vivo* with various cytokines (none, IFNβ, IL-2 and IL-6) for ~30 min. Samples were fixed and stained for phosphoSTAT1. The MFI of pSTAT1 +/- SD for CD44hi vs CD44lo CD4 and CD8 T cells and NK cells are shown. Numbers in bold indicate a MFI above respective unstimulated control without overlapping SD.

Table 3.2 Cytokine-induced phosphorylation of STAT3 in lymphocytes after poly(I:C) treatment

CD4 T cells		No stim	IFNβ	IL-2	IL-6	IL-7	IL-15
CD44hi	HBSS	588 ± 196	**1647 ± 42**	537 ± 137	**2309 ± 276**	509 ± 140	625 ± 152
	Poly(I:C)	741 ± 40	753 ± 84	767 ± 11	**1575 ± 105**	554 ± 97	748 ± 30
CD44lo	HBSS	792 ± 421	**1706 ± 133**	650 ± 295	**2686 ± 354**	649 ± 260	775 ± 278
	Poly(I:C)	742 ± 29	728 ± 65	700 ± 7	**2089 ± 66**	531 ± 117	695 ± 17

CD8 T cells		No stim	IFNβ	IL-2	IL-6	IL-7	IL-15
CD44hi	HBSS	834 ± 240	**2165 ± 62**	711 ± 187	**2570 ± 408**	726 ± 185	1007 ± 201
	Poly(I:C)	1013 ± 23	1054 ± 74	1044 ± 30	**2062 ± 158**	782 ± 149	**1250 ± 16**
CD44lo	HBSS	835 ± 391	**2086 ± 122**	687 ± 287	**3116 ± 402**	698 ± 268	924 ± 275
	Poly(I:C)	881 ± 30	872 ± 70	833 ± 8	**2630 ± 134**	647 ± 131	**975 ± 25**

NK cells		No stim	IFNβ	IL-2	IL-6	IL-7	IL-15
	HBSS	406 ± 18	**1295 ± 90**	368 ± 13	416 ± 12	389 ± 23	**589 ± 65**
	Poly(I:C)	710 ± 40	741 ± 40	775 ± 72	766 ± 81	641 ± 48	**838 ± 17**

Table 3.2 Cytokine-induced phosphorylation of STAT3 in lymphocytes after poly(I:C) treatment

B6 mice were inoculated with HBSS or poly(I:C) and spleens were harvested 1 day later. Splenocytes were stimulated individually *ex vivo* with various cytokines (none, IFNβ, IL-2, IL-6, IL-7 and IL-15) for ~30 min. Samples were fixed and stained for phosphoSTAT3. The MFI of pSTAT3 +/- SD for CD44hi vs CD44lo CD4 and CD8 T cells and NK cells are shown. Numbers in bold indicate a MFI above respective unstimulated control without overlapping SD.

Table 3.3 Cytokine-induced phosphorylation of STAT4 in lymphocytes after poly(I:C) treatment

CD4 T cells		No stim	IFNβ	IL-12
CD44hi	HBSS	169 ± 9	**809 ± 78**	**253 ± 18**
	Poly(I:C)	173 ± 8	178 ± 3	**286 ± 20**
CD44lo	HBSS	127 ± 4	**383 ± 20**	137 ± 12
	Poly(I:C)	123 ± 3	121 ± 4	**136 ± 8**

CD8 T cells		No stim	IFNβ	IL-12
CD44hi	HBSS	240 ± 8	**2257 ± 267**	**512 ± 9**
	Poly(I:C)	235 ± 5	252 ± 16	**518 ± 39**
CD44lo	HBSS	159 ± 6	**820 ± 78**	**189 ± 11**
	Poly(I:C)	150 ± 3	154 ± 1	**211 ± 8**

NK cells		No stim	IFNβ	IL-12
	HBSS	265 ± 10	**2402 ± 243**	**2925 ± 313**
	Poly(I:C)	844 ± 190	1003 ± 169	**3100 ± 238**

Table 3.3 Cytokine-induced phosphorylation of STAT4 in lymphocytes after poly(I:C) treatment

B6 mice were inoculated with HBSS or poly(I:C) and spleens were harvested 1 day later. Splenocytes were stimulated individually *ex vivo* with various cytokines (none, IFNβ and IL-12) for ~30 min. Samples were fixed and stained for phosphoSTAT4. The MFI of pSTAT4 +/- SD for CD44hi vs CD44lo CD4 and CD8 T cells and NK cells are shown. Numbers in bold indicate a MFI above respective unstimulated control without overlapping SD.

Table 3.4 Cytokine-induced phosphorylation of STAT5 in lymphocytes after poly(I:C) treatment

CD4 T cells		No stim	IFNβ	IL-2	IL-7	IL-15
CD44hi	HBSS	1124 ± 34	1984 ± 186	2379 ± 103	1120 ± 47	4066 ± 320
	Poly(I:C)	1510 ± 66	1676 ± 64	2366 ± 142	1648 ± 159	4061 ± 134
CD44lo	HBSS	1274 ± 118	2249 ± 221	1270 ± 131	1536 ± 78	1920 ± 136
	Poly(I:C)	1502 ± 67	1616 ± 53	1552 ± 90	2010 ± 111	2719 ± 203

CD8 T cells		No stim	IFNβ	IL-2	IL-7	IL-15
CD44hi	HBSS	2090 ± 133	2833 ± 233	2076 ± 100	2097 ± 43	6439 ± 291
	Poly(I:C)	2180 ± 108	2394 ± 56	2540 ± 129	2419 ± 169	6797 ± 198
CD44lo	HBSS	1775 ± 139	2802 ± 321	1622 ± 129	1953 ± 31	6284 ± 480
	Poly(I:C)	1811 ± 130	1950 ± 109	1843 ± 125	2266 ± 167	6727 ± 209

NK cells		No stim	IFNβ	IL-2	IL-7	IL-15
	HBSS	1101 ± 29	1324 ± 69	1238 ± 43	1043 ± 35	3683 ± 163
	Poly(I:C)	1511 ± 276	1592 ± 49	1849 ± 146	1380 ± 267	2806 ± 201

Table 3.4 Cytokine-induced phosphorylation of STAT5 in lymphocytes after poly(I:C) treatment

B6 mice were inoculated with HBSS or poly(I:C) and spleens were harvested 1 day later. Splenocytes were stimulated individually *ex vivo* with various cytokines (none, IFNβ, IL-2, IL-7, and IL-15) for ~30 min. Samples were fixed and stained for phosphoSTAT5. The MFI of pSTAT5 +/- SD for CD44hi vs CD44lo CD4 and CD8 T cells and NK cells re shown. Numbers in bold indicate a MFI above respective unstimulated control without overlapping SD.

It should be noted that the magnitude of pSTAT1 staining in response to cytokine stimulation was not equal in all lymphocyte populations; CD8 T cells induced more pSTAT1 staining in response to IFNβ stimulation than did CD4 T cells and NK cells (**Table 3.1**). Additionally, CD4 and CD8 T cells responded to IL-6 stimulation in terms of phosphorylating downstream STAT1 (**Table3.1**) and STAT3 (**Table 3.2**), but NK cells did not respond to IL-6 stimulation. On the other hand, NK cells responded robustly and phosphorylated STAT4 in response to IL-12 stimulation whereas CD4 and CD8 T cells had a very low response (**Table 3.3**). Not only did different lymphocyte populations respond to the same cytokine to a varying degree, but specific subsets of cells did not respond to all cytokine stimulations to the same extent. For example, CD4 T cells showed pSTAT5 staining above unstimulated background in response to IFNβ, IL-2, and IL-15, but pSTAT5 MFI was greatest in response to IL-15 stimulation (**Table 3.4**). Different cell types respond to distinct cytokines to various extents. This may partially account for how one cytokine can affect diverse cell types in different ways.

Poly(I:C) treatment altered the ability of some cell subsets to respond to selected cytokines. Shown in bold in **Table 3.1,** all HBSS-treated lymphocyte subsets (CD44hi/lo CD4, CD44hi/lo CD8, and NK cells) responded to IFNβ stimulation and showed phosphoSTAT1 staining above their unstimulated controls, unlike the poly(I:C)-treated lymphocyte subsets stimulated with IFNβ, which did not show positive pSTAT1 staining above unstimulated controls. On the other hand, naive T cell populations induced phosphorylation of STAT1

above controls in response to IL-6 stimulation regardless of pretreatment-regime. Both CD44lo and CD44hi poly(I:C)-pretreated T cells stimulated with IL-6 (black bars, **Figure 3.15A-B**) or IL-15 (black bars, **Figure 3.15C-D**) responded just as well, in terms of phosphorylating downstream STAT3 and STAT5, respectively, as their control-treated counterparts. These results indicate that poly(I:C) pretreatment does not induce refractoriness to all cytokines, as CD4 and CD8 T cells were shown to respond to IL-6 and IL-15 stimulation and NK cells responded to IL-12 stimulation. Data shown here provide only a limited view on how complex cytokine receptor signaling can be, especially in terms of what cell types respond to what cytokines, the magnitude of the response of each cell type has to different cytokines, and how the pre-exposure of cytokines effects subsequent exposure to cytokine stimulation.

3. Poly(I:C)-pretreated CD8 T cells have decreased IFNAR1 and increased SOCS1 expression

Cytokine signaling must be tightly regulated in order to prevent over-active and prolonged immune activation. A number of different mechanisms are in place to limit cytokine signaling, including reducing cytokine receptor expression, downregulating expression of signaling protein components (JAK/STATs), and upregulating the expression of SOCS proteins (28, 31). I've shown that reduced STAT protein levels cannot account for the refractoriness to IFNβ simulation seen in the poly(I:C)-pretreated CD8 T cells (**Figure 3.14**).

Figure 3.15: Poly(I:C)-pretreated T cells can respond to other cytokines, including IL-6 and IL-15
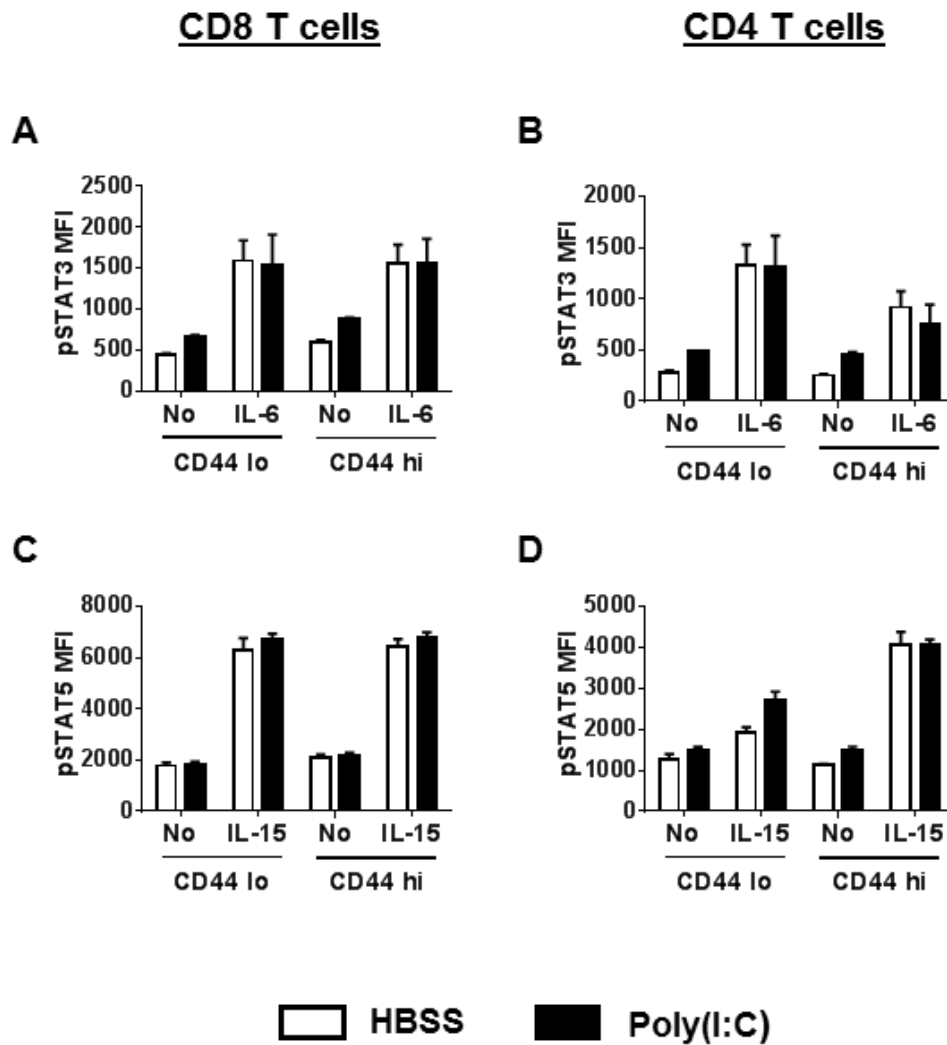
Figure 3.15: Poly(I:C)-pretreated T cells can respond to other cytokines, including IL-6 and IL-15

Mice were inoculated with HBSS (open bars) or poly(I:C) (black bars) for 1 day. Splenocytes were isolated and either unstimulated, stimulated with IL-6 (**A-B**) or IL-15 (**C-D**) and then stained for downstream pSTAT3 (**A-B**) or pSTAT5 (**C-D**). Splenocytes were gated on CD44lo or CD44hi CD8α+ T cells (**A, C**) or CD44lo or CD44hi CD4+ T cells (**B, D**) and pSTAT MFI was graphed. Data are representative of at least 2 independent experiments with n of 3 mice per group.

To investigate why naïve CD8 T cells pre-exposed to poly(I:C) were unresponsive to type 1 IFN, but not all cytokines, cytokine receptor expression was determined. At various days post HBSS or poly(I:C) treatment, naïve CD44lo CD8 T cells were assessed for cytokine receptor signaling components, including portions of the IL-2 (**Figure 3.16A, B, E**), IL-6 (**3.16C**), IL-7 (**3.16D, E**), IL-15 (**3.16 B, F**), and IFNγ (**3.16F**) receptor complexes. Of the receptors tested, the majority of cytokine receptor expression did not change after poly(I:C) treatment. However, CD127, IL-7 receptor alpha chain, was decreased in naïve CD8 T cells 1 day after poly(I:C) treatment but returned to control treated levels by 2 days post poly(I:C) treatment (**Figure 3.16D**). CD132, the common gamma chain, increased 1 day after inoculation with poly(I:C) in CD44lo CD8 T cells but this too was transient, as receptor levels were back to control treated levels after 2 days (**Figure 3.16E**). The minimal changes in cytokine receptor expression after poly(I:C) treatment in CD44lo CD8 T cells may explain why naïve CD8 T cells were able to phosphorylate downstream STATs in response to stimulation with some of these cytokines.

To see if a decrease in the type 1 IFN receptor level contributed to reduced responsiveness of poly(I:C)-pretreated CD8 T cells to phosphorylate downstream STATs in response to further IFNβ signaling, IFN receptor expression was stained for. The type 1 IFN receptor is comprised of two components, IFNAR1 and IFNAR2 (32). Naïve CD8 T cells from mice inoculated with poly(I:C) (dashed line open histograms and black bars) for 1 day had

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Figure 3.16: Cytokine receptor expression on naïve CD8 T cells after poly(I:C) treatment
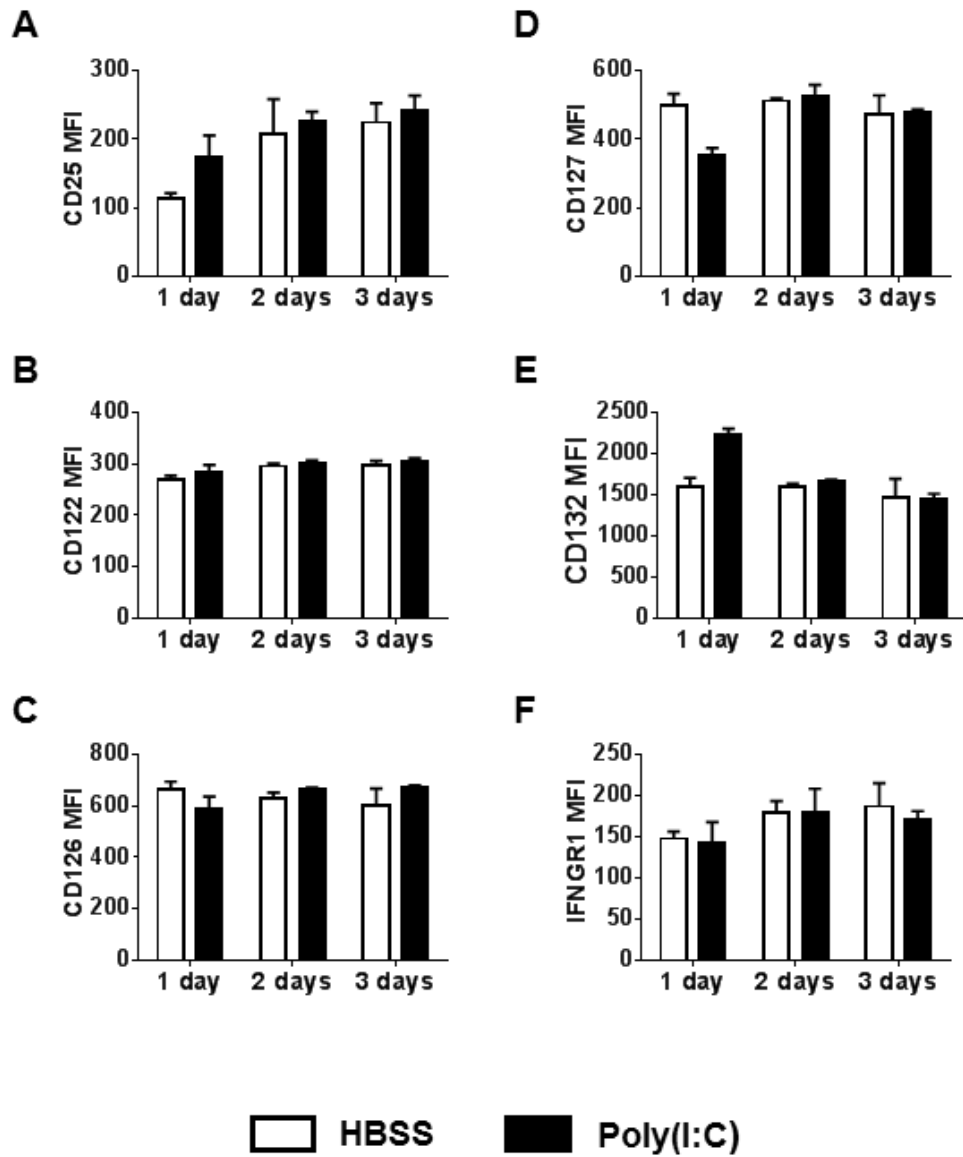
Figure 3.16: Cytokine receptor expression on naïve CD8 T cells after poly(I:C) treatment

Mice were HBSS (open bars) or poly(I:C) (black bars) treated once 1, 2, or 3 days prior to isolation. Cells were gated on CD44lo CD8α+ T cells and cytokine receptor MFI is plotted. CD25 (**A**), CD122 (**B**), CD126 (**C**), CD127 (**D**), CD132 (**E**) and IFNGR1 (**F**) is shown. Data are representative of 2 independent experiments with n of 3 mice per group.

much lower expression levels of IFNAR1, compared to the HBSS-treated cells (solid line open histograms and open bars) **(Figure 3.17A-B)**. However, IFNAR1 expression levels returned to control-treated levels by 2 days post treatment. The CD44hi CD8 T cells had similar kinetics of IFNAR1 expression as the naïve CD8 T cells, showing slightly reduced receptor expression with 1 day treatment of poly(I:C) but not 2 or 3 days of treatment **(Figure 3.17C).** In fact, IFNAR1 expression levels were modestly increased at later time points post poly(I:C) treatment in CD44hi and CD44lo CD8 T cells. Thus, unresponsiveness to type 1 IFN at day 1 post poly(I:C) treatment may be partially due to the reduced expression of the type 1 IFN receptor.

Since poly(I:C)-pretreated CD8 T cells were still less responsive to IFNβ stimulation (as measured by STAT phosphorylation) 2 days after poly(I:C) treatment (**Figure 3.13**), there were likely other suppressive mechanisms to limit IFNβ responsiveness in addition to the reduced receptor expression shown in **Figure 3.17**. SOCS proteins are known to inhibit cytokine receptor signaling by acting at many different steps in the JAK/STAT signaling pathway (28). SOCS1 inhibits type 1 IFN signaling by binding to the receptor-associated JAK protein TYK2, thus blunting IFN receptor signaling (37). The well-established protocol for staining for phosphorylated proteins was utilized to identify intracellular levels of SOCS1. SOCS1 expression was determined in CD8 T cells from poly(I:C)- or HBSS-inoculated mice 1, 2, or 3 days after treatment (**Figure 3.18**). Indeed, at both 1 and 2 days after poly(I:C) treatment (dashed line open histograms and

Figure 3.17: CD8 T cells downregulate IFNAR1 expression 1 day after poly(I:C) exposure
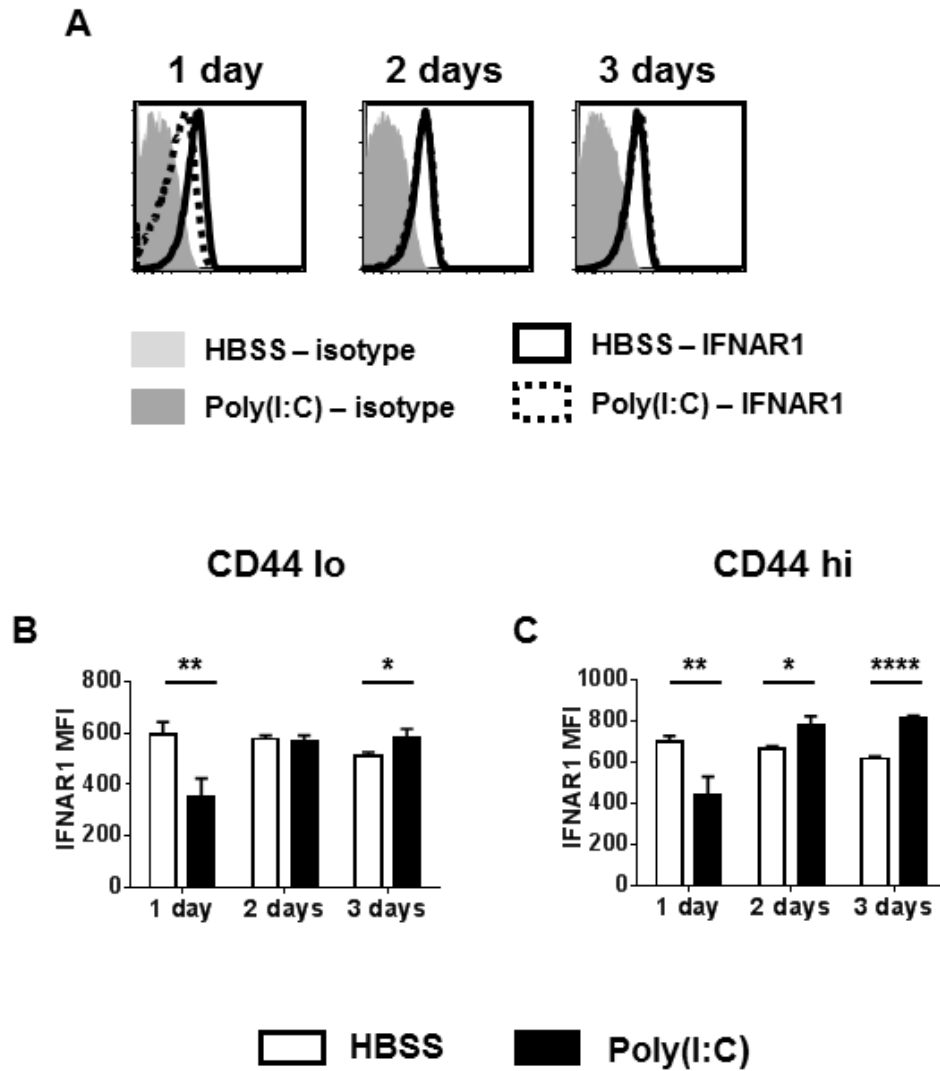
Figure 3.17: CD8 T cells downregulate IFNAR1 expression 1 day after poly(I:C) exposure

Mice were HBSS (open bars) or poly(I:C) (black bars) treated once 1, 2, or 3 days prior to isolation. Cells were gated on CD44lo CD8α+ T cells (**A-B**) or CD44hi CD8α+ T cells (**C**). Representative histograms (**A**) and MFI of IFNAR1 (**B-C**) are shown. Data are representative of at least 4 independent experiments with n of 3 mice per group.

black bars), naïve CD44lo CD8 T cells had higher expression of SOCS1 compared to the control-treated cells (solid line open histograms and open bars) (**Figure 3.18A-B**). However, by 3 days of pretreatment, there was no longer a difference in SOCS1 expression between control- and poly(I:C)-treated CD8 T cells, although it was close to reaching statistical significance (p=0.0789). CD44hi CD8 T cells also showed increased SOCS1 protein levels (**Figure 3.18C**), with similar kinetics as CD44lo CD8 T cells. These data suggest that a combination of a decrease in IFNAR expression and an increase in SOCS1 expression may account for the observed refractoriness to IFNβ stimulation in poly(I:C)-pretreated CD8 T cells.

4. Summary

Knowing that type 1 IFN can provide stimulatory signals for CD8 T cells during acute viral infections and augment their proliferation, I investigated the role type 1 IFN had in the suppression of clonal expansion seen in poly(I:C)-pretreated CD8 T cells. I tested the ability of poly(I:C)-pretreated CD8 T cells to respond to IFNβ stimulation and measured the phosphorylation status of downstream STATs. Both naïve (CD44lo) and memory (CD44hi) phenotype CD8 T cells were refractory to IFNβ-induced STAT phosphorylation if pre-exposed to poly(I:C) (**Figure 3.11-12**). T cells were unresponsive to IFNβ stimulation and did not phosphorylate any of the STATs tested, including STAT 1, 3, 4, and 5. However, IFNβ refractoriness was not permanent, as CD8 T cells exposed to

Figure 3.18: CD8 T cells upregulate SOCS1 after poly(I:C) treatment
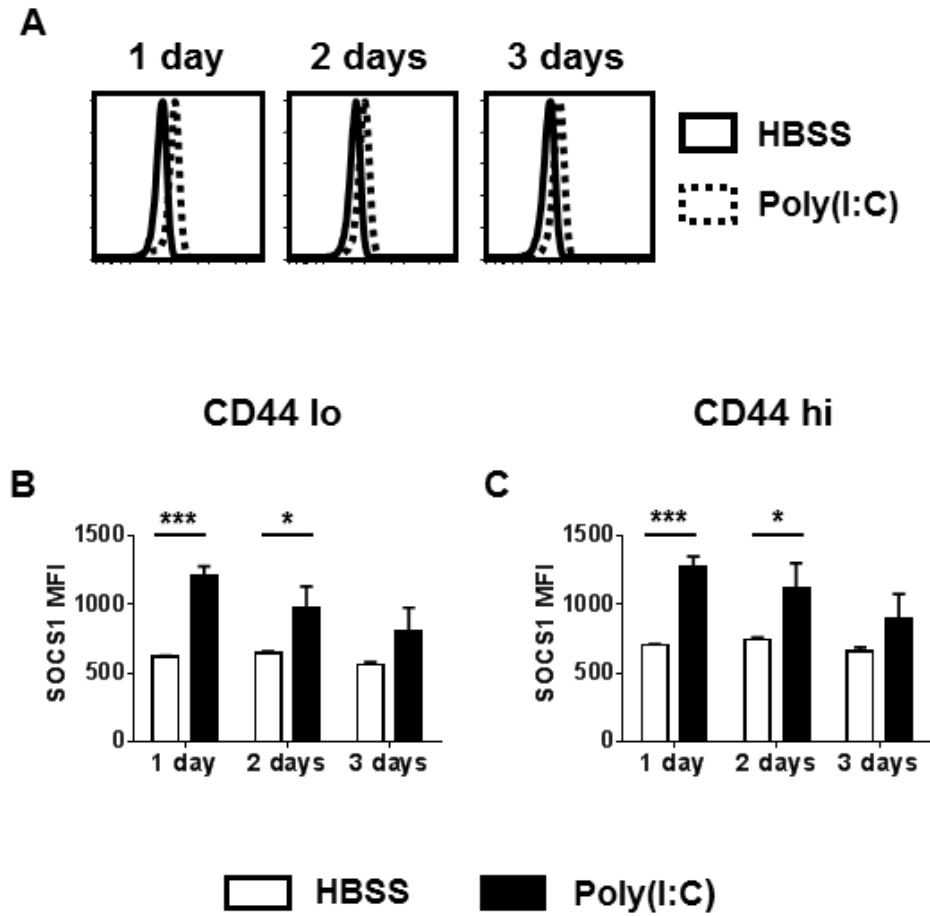
Figure 3.18: CD8 T cells upregulate SOCS1 after poly(I:C) treatment

Mice were HBSS (open bars) or poly(I:C) (black bars) treated once 1, 2, or 3 days prior to isolation. Cells were gated on CD44lo CD8α+ T cells (**A-B**) or CD44hi CD8α+ T cells (**C**). Representative histograms (**A**) and MFI of SOCS1 (**B-C**) are shown. Data are representative of 2 independent experiments with n of 3 mice per group.

poly(I:C) 3 days prior to *ex vivo* IFNβ stimulation were able to phosphorylate STATs above the unstimulated controls, indicating the IFN unresponsiveness was a transient event (**Figure 3.13**). CD8 T cells were not the only cell types found to be refractory to IFNβ-induced STAT phosphorylation after poly(I:C) treatment, as both CD4 T cells and NK cells also had a nonresponsive phenotype (**Tables 3.1-3.4).** Poly(I:C) pretreatment did not, however, render all cell types tested unresponsive to all cytokine stimulations; instead it was more specific to type 1 IFN.

Poly(I:C)-pretreated CD8 T cells had reduced IFNAR1 expression levels 1 day after inoculation but by 2 days post treatment, the receptor levels were back to control-treated levels (**Figure 3.17**). This indicated that a decrease in receptor expression level could not be the only factor contributing to the refractoriness to IFNβ-induced STAT phosphorylation because unresponsiveness was seen at 2 and (to a small extent) 3 days post poly(I:C) treatment. SOCS1 expression was induced to the greatest degree at 1 day post treatment, but remained higher than controls through 3 days post poly(I:C) inoculation (**Figure 3.18**). These results correlated kinetically with refractoriness to IFNβ stimulation (**Figure 3.11-3.12**) and to the suppressed proliferation seen in poly(I:C)-pretreated CD8 T cells (**Figure 3.3**).

These data suggest that poly(I:C)-pretreated CD8 T cells, when put into hosts subsequently infected with LCMV, are not able to respond to the type 1 IFN induced by the virus, and thus are unable to receive positive signal 3 cytokine

signals. The reduced response to postitive type 1 IFN-induced signals may contribute to suppression of proliferation seen in T cells pre-exposed to poly(I:C)-induced environments.

D. Poly(I:C)-pretreated CD8 T cells behave similarly to 2-signal-stimulated rather than 3-signal-stimulated CD8 T cells

1. Defects in clonal expansion are due to lack of receiving signal 3

Because the suppression of proliferation of poly(I:C)-pretreated CD8 T cells correlated with refractoriness to IFNβ stimulation, I hypothesized that poly(I:C)-pretreated CD8 T cells were unable to receive the positive effects that type 1 IFN exerts as a signal 3 cytokine when delivered in the proper sequence. This hypothesis would suggest that poly(I:C)-pretreated P14 cells would behave similarly to 2-signal only CD8 T cells, rather than 3-signal CD8 T cells. Thus, I examined their effector phenotype and their abilities to divide, produce cytokines, degranulate, and express the survival protein Bcl-3 in response to antigen exposure.

Studies have shown that 2-signal CD8 T cells divide similarly to 3-signal CD8 T cells but have defects in survival (79). I tested whether the impairment in proliferation seen in poly(I:C)-pretreated CD8 T cells was due to division or survival defects. A similar experimental setup as described previously was used, but after inoculation with HBSS or poly(I:C), congenic P14 CD8 T cells were labeled with CellTrace Violet and transferred together into the same recipients to

track cell division during LCMV infection. Cells were harvested at days 3 and 4 post infection, and CellTrace Violet dilution was measured. Because we were looking at early days post infection, a larger number of transgenic cells was transferred than what would normally be considered physiologically relevant in order to quantify early cell division. Neither the poly(I:C)- nor the HBSS-pretreated P14 cells diluted CellTrace Violet in naïve mice, indicating that they did not divide (**Figure 3.19A**). Two representative histograms are shown for day 3 and day 4 post infection. The division profiles for each day post infection appear very different from one another despite being infected via the same route and with the same dose of virus and harvested at the same time. This is why it is important to have the HBSS- and poly(I:C)-pretreated P14 populations transferred into the same recipients when looking at cell division. At day 3 post infection, the control-treated P14 cells (Blue histograms) diluted more CellTrace Violet, indicating they had divided more compared to the poly(I:C)-pretreated P14 cells (Red histograms) (**Figure 3.19A**). By day 4 post LCMV infection, the division profiles of both HBSS-and poly(I:C)-pretreated P14 cells appeared more similar. However, the day 4 LCMV example number 2 does show an increased HBSS-treated population on the left side of the histogram compared to poly(I:C)-treated cells, where the CellTrace Violet has been diluted out. This may be due to an increased accumulation of this population, but because of the low division resolution, it cannot be said for sure that poly(I:C)-treated P14 cells divided less. The percentage of cells that divided was statistically similar between the two

Figure 3.19: Poly(I:C)-pretreated CD8 T cells have a delay in cell division
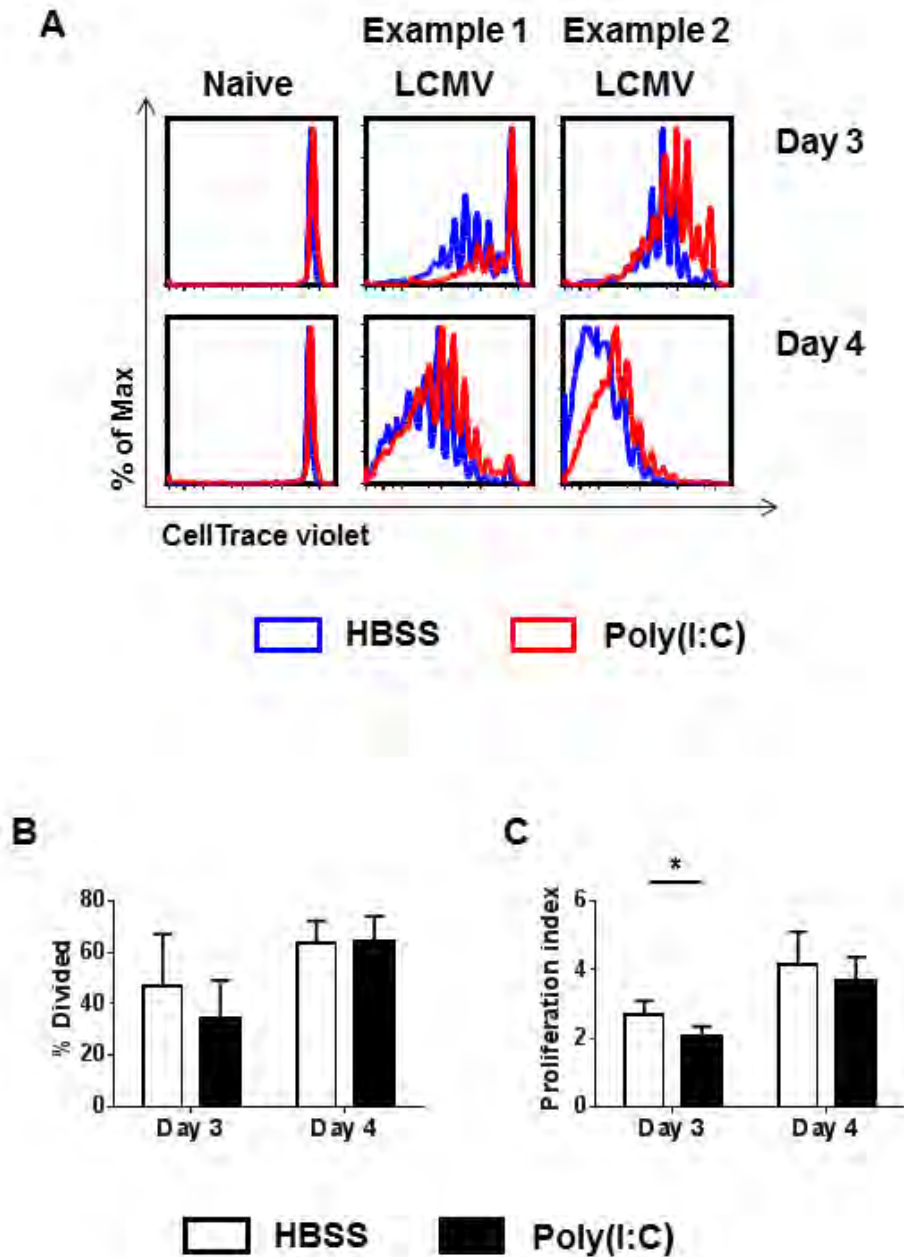
Figure 3.19: Poly(I:C)-pretreated CD8 T cells have a delay in cell division

Congenic transgenic P14 mice were HBSS or poly(I:C) treated. One day after treatment P14 cells were labeled with CellTrace Violet, and similar numbers of HBSS- and poly(I:C)-pretreated cells were transferred into the same recipients, which were subsequently inoculated with 5×10^4 pfu LCMV. (**A**) Representative CellTrace Violet dilution profiles shown for different days post infection. Percent divided (**B**) and proliferation index (**C**) of P14 cells pretreated with HBSS (open bars) or poly(I:C) (black bars) at day 3 and 4 post infection are graphed. Data are representative of 2 independent experiments with n of 4-6 mice per group.

groups (**Figure 3.19B**), but the proliferation index of poly(I:C)-pretreated P14 cells was lower compared to HBSS-treated cells at day 3 post infection (**Figure 3.19C**). The proliferation index represents the average number of divisions of the cells that have undergone at least one division. These data indicate that the frequency of cells that have undergone division is similar, but poly(I:C)-treated cells divided, on average, fewer times compared to controls. These results show that poly(I:C)-pretreated CD8 T cells have a delay in cell divisions as compared to HBSS-treated CD8 T cells.

If a delay in cell division were the only factor contributing to the suppression of proliferation, at later time points post infection the expansion of poly(I:C)-pretreated CD8 T cells might eventually reach the same level as the control-treated cells. Therefore, a time course of HBSS- or poly(I:C)-pretreated P14 CD8 T cell expansion in response to LCMV infection was performed. The peak of expansion of poly(I:C)-pretreated P14 CD8 T cells was delayed (day 9) compared to HBSS-pretreated P14 CD8 T cells (day 7), but the magnitude of the response was still reduced in the poly(I:C)-pretreated cells (**Figure 3.20A-B**). The poly(I:C)-pretreated, also described as "out-of-sequence," P14 cells showed decreased memory proportions (**Figure 3.20A**) and number (**Figure 3.20B**) compared to their control-treated counterparts at multiple time points tested.

When tracking congenic donor P14 populations at late time points post infection, there were some problems with P14 cells being rejected from the hosts.

Figure 3.20: Poly(I:C)-induced suppression of CD8 T cell proliferation occurs at multiple time points post infection
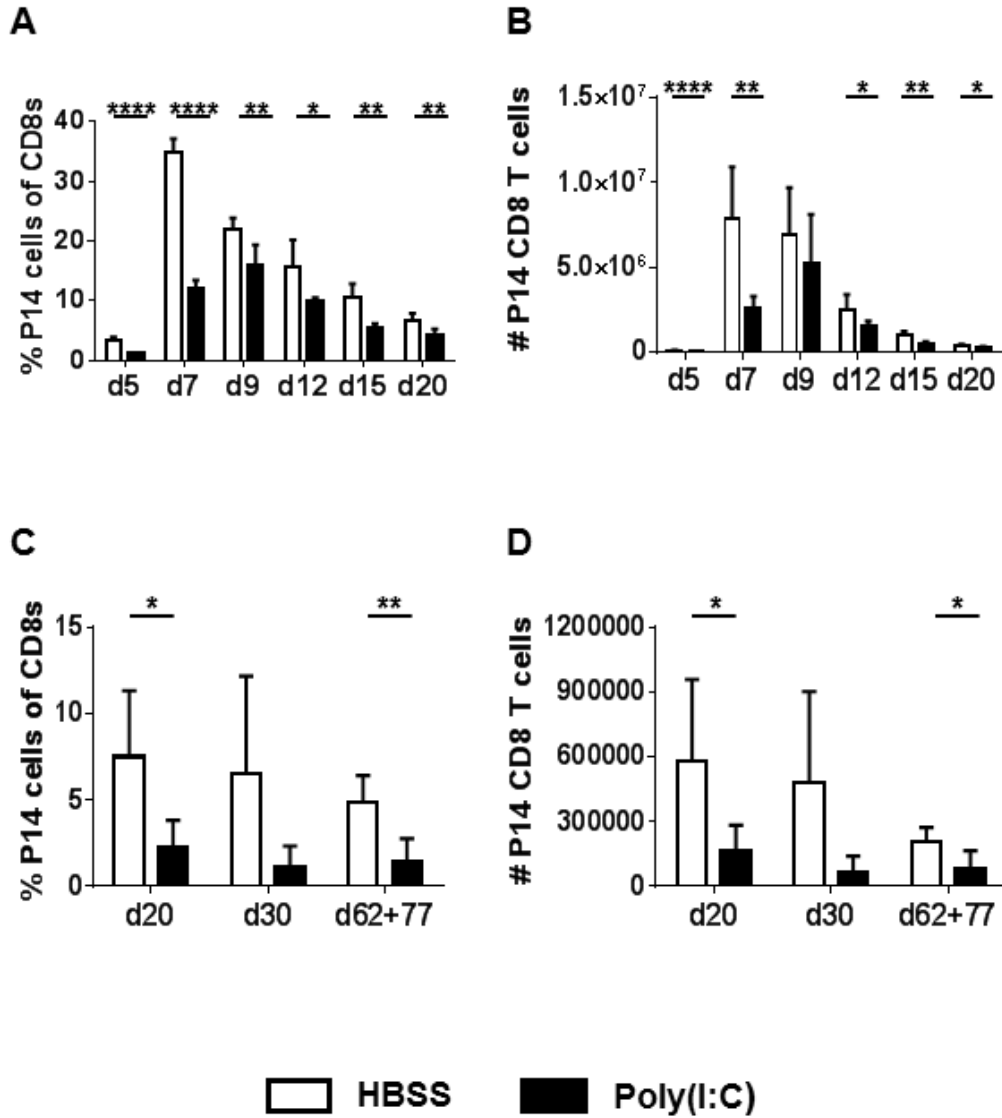
Figure 3.20: Poly(I:C)-induced suppression of CD8 T cell proliferation occurs at multiple time points post infection

Equal numbers of HBSS- (open bars) and poly(I:C)- (black bars) pretreated P14 CD8 T cells were transferred into the same or different recipients, which were subsequently infected with 5×10^4 pfu LCMV. Spleens were harvested at days 5, 7, 9, 12, 15, 20, 30, 62, or 77 post infection, and the frequency (**A, C**) and number (**B, D**) of donor P14 cells are graphed. P14 cells were transferred into the same hosts when spleens were harvested from day 5 to day 20 post infection and different hosts when harvested at day 30 or later. Data are representative of 2 individual experiments (**A-B**) or 3 experiments combined (**C-D**) with n of 3-5 mice per group.

Rejections occurred in similar numbers between the control- and poly(I:C)-treated groups (data not shown), but because of this, analyzing memory formation was difficult. However, with the few experiments looking at time points after 4 weeks post infection, poly(I:C)-pretreated P14 cells still showed reduced frequencies (**Figure 3.20C**) and numbers (**Figure 3.20D**) compared to control-treated counterparts. The difference in expansion seemed to hold steady as time elapsed, suggesting that contraction rates are likely similar between both treatment groups. Thus, the relative difference seen at the peak of expansion can be carried through to memory formation. Because the magnitude of out-of-sequence CD8 T cells never reached control-treated levels, it suggests that the defects in clonal expansion were not solely due to a delay in cell division but may also be due to other factors such as defects in cell survival.

In vitro and *in vivo* studies by others found that survival of activated T cells in response to signal 3 cytokines and adjuvants was in part due to an increase in the IκB family member Bcl-3 and that cells lacking signal 3 cytokines have reduced expression of Bcl-3 (86-88). To determine if the poly(I:C)-treated virus-stimulated T cells resembled 2-signal-only T cells in this respect, the expression of Bcl-3 was determined in HBSS- (control) or poly(I:C)-pretreated P14 CD8 T cells after LCMV infection. Indeed, a lower percent of poly(I:C)-pretreated P14 cells upregulated Bcl-3 than HBSS-treated P14 cells at days 4, 5 and 6 post LCMV infection (**Figure 3.21 A-B**). Additionally, the Bcl-3 MFI of poly(I:C)-pretreated P14 CD8 T cells was lower than in control-treated cells (**Figure**

Figure 3.21: Out-of-sequence P14 CD8 T cells have reduced Bcl-3 expression at multiple time points post infection compared to controls
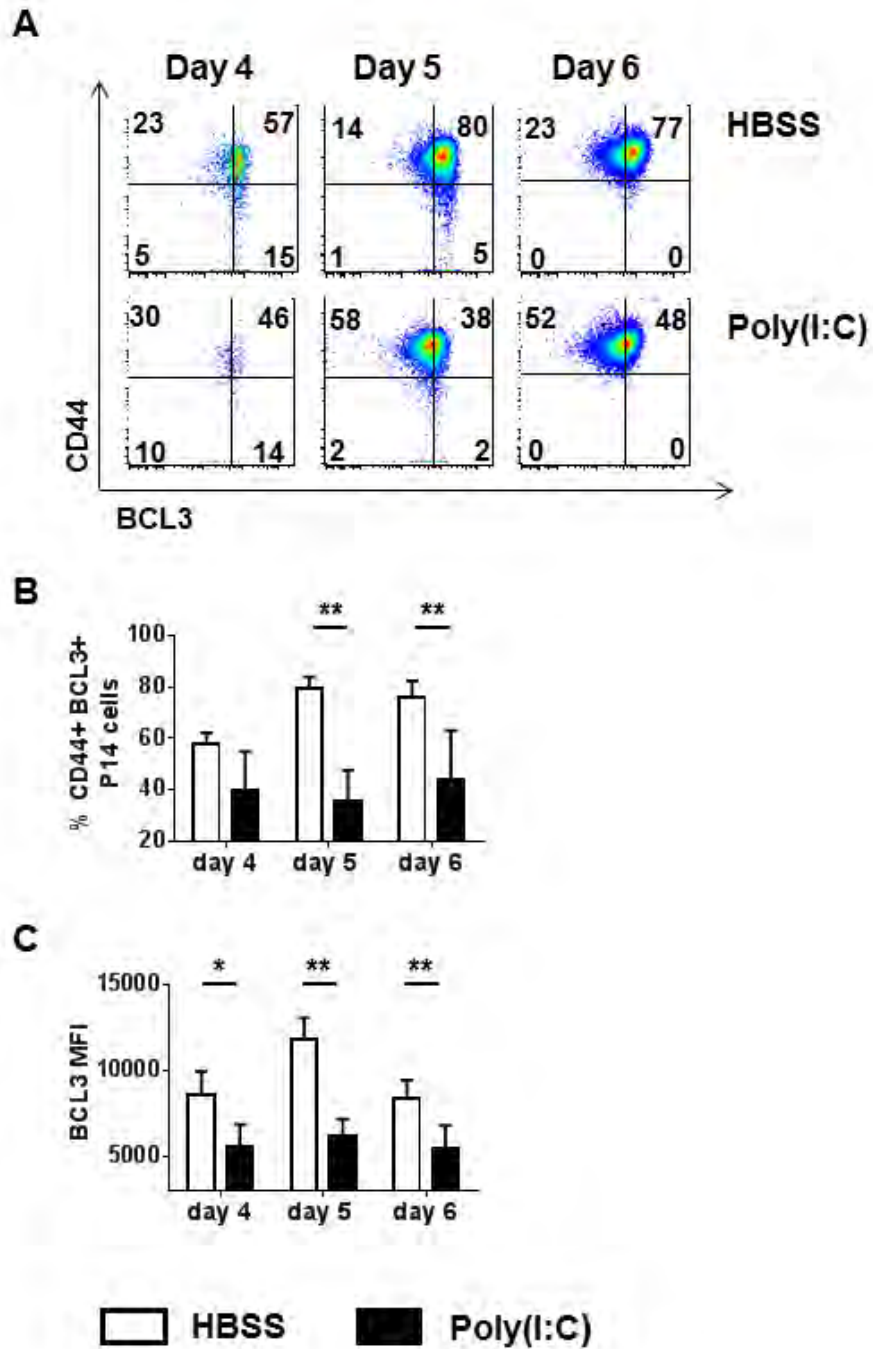
Figure 3.21: Out-of-sequence P14 CD8 T cells have reduced Bcl-3 expression at multiple time points post infection compared to controls

P14 mice were inoculated with HBSS (open bars) or poly(I:C) (black bars), spleens were harvested 1 day later, and equal numbers of transgenic T cells were adoptively transferred into B6 mice, which were subsequently infected with 5×10^4 pfu LCMV. Spleens were harvested at days 4, 5, and 6 post infection. (**A**) Representative flow plots gated on donor P14 cells. The frequency of CD44hi P14 cells expressing Bcl-3 (**B**) and MFI of Bcl-3 expression gated on donor P14 cells (**C**) are graphed. Representative of 2-4 experiments with n of 3-5 mice per group.

3.21C). Together, these results showed that, similar to 2-signal CD8 T cells, out-of-sequence signal 3-stimulated P14 CD8 T cells had a delay in cell division when compared to CD8 T cells that receive all 3 signals in the correct order. Moreover, out-of-sequence P14 cells had defects in a survival protein that may limit the ability of these cells to clonally expand.

To further support the hypothesis that out-of-sequence signal 3 CD8 T cells do not receive the positive effects that type 1 IFN can have as a signal 3 cytokine during acute virus infection, and thereby contribute to suppression of proliferation, I determined the frequency of poly(I:C)- or HBSS-pretreated P14 cells after cognate peptide stimulation. First, I wanted to make sure that WT cells did not have a proliferative advantage over IFNAR KO cells in response to peptide stimulation. WT B6 mice received equal numbers of Ly5.1 WT P14 cells and Thy1.1 IFNAR KO P14 cells in the presence or absence of the 13mer GP_{33-45} peptide. Two and 3 days post peptide inoculation, splenocytes were harvested and the frequency and number of donor P14 cells were determined. **Figure 3.22** shows IFNAR KO (grey bars) expand to similar levels in terms of frequency of total CD8 T cell response (**3.22A**) and total number (**3.22B**) compared to WT P14 cells (open bars). The stimulatory effects type 1 IFN can have on CD8 T cell expansion during LCMV infection have been shown by others, but this is also seen in **Figure 3.22C**, where WT P14 cells (open bars) expand >200 fold over the IFNAR KO P14 cells (grey bars) at day 7 post infection (note the log scale) (79). Type 1 IFN can have stimulatory effects on CD8 T cell expansion during

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Figure 3.22: IFNAR KO P14 cells and out-of-sequence P14 CD8 T cells expand to similar levels as control P14 cells in response to GP33 peptide
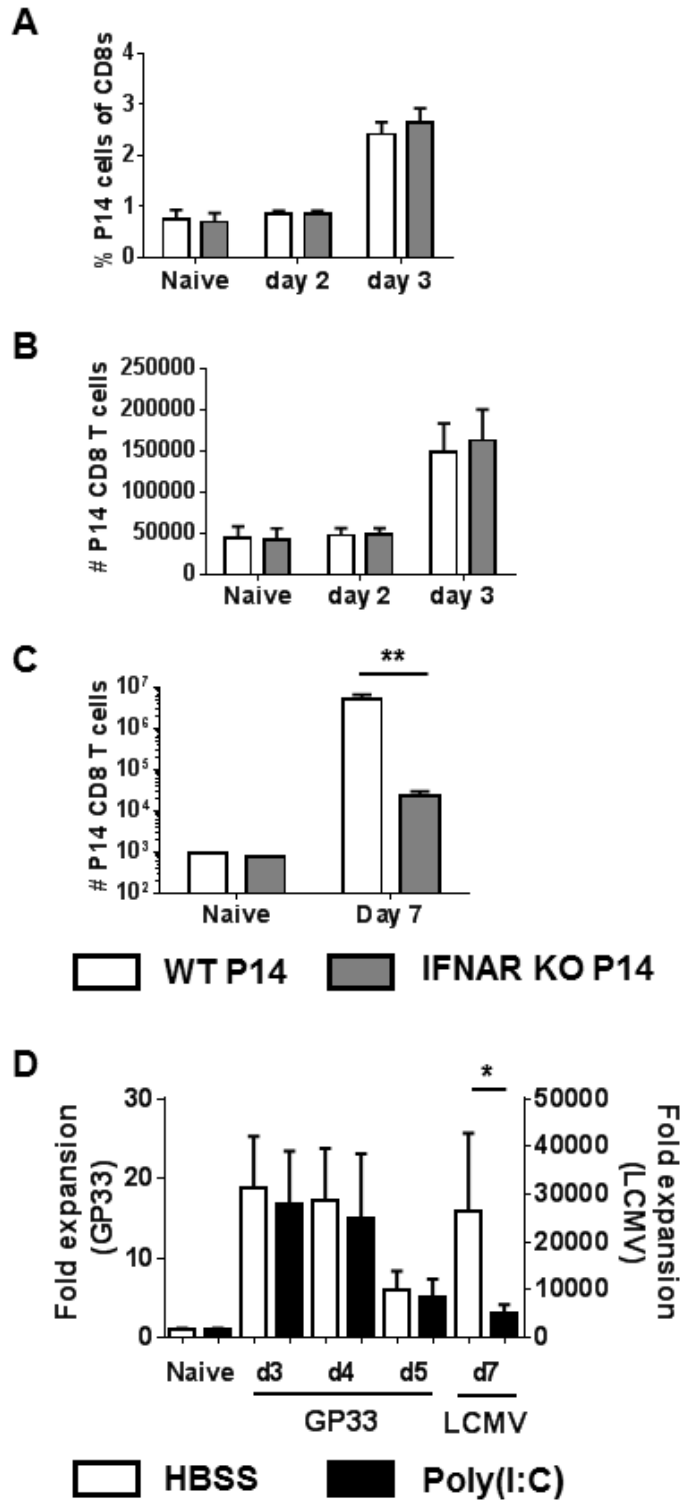
Figure 3.22: IFNAR KO P14 cells and out-of-sequence P14 CD8 T cells expand to similar levels as control P14 cells in response to GP33 peptide

(**A-B**) Ly5.1 WT P14 cells (open bars) and Thy1.1 IFNAR KO P14 cells (grey bars) were transferred into the same recipients. Mice received 5 µg GP33 13mer peptide i.v. or no peptide, and spleens were harvested at days 2 and 3 post peptide administration. The frequency (**A**) and number (**B**) of donor P14 cells were determined and graphed. (**C**) WT P14 cells (open bars) or IFNAR KO P14 cells (grey bars) were transferred into WT recipients, which were inoculated with $5x10^4$ pfu LCMV i.p. Splenocytes were harvested at day 7 post infection and total number of donor P14 cells is graphed. (**D**) Congenic P14 mice were HBSS treated (open bars) and Thy1.1 P14 mice (black bars) were poly(I:C) treated for 1 day. Equal numbers of each P14 population were transferred into the same recipient. Mice remained untreated (naïve), received 5 µg GP33 peptide i.v. or were inoculated with $5x10^4$ pfu LCMV i.p. At various time points post inoculation, spleens were harvested and fold expansion (over naïve) was calculated and graphed (**A-B**). Data from 1 experiment with 5 mice per group (**C**) Data representative of 2 individual experiments with 1 naive or 4-5 LCMV mice per group (**D**) Data were combined from 3 individual experiments with n of 4-5 mice per group.

acute infections, but during settings without type 1 IFN production, IFNAR-competent T cells do not have any advantage over IFNAR-deficient T cells in response to cognate antigen stimulation, and this is represented in **Figures 3.22A-C**.

To test whether or not out-of-sequence CD8 T cells expanded to similar levels as control-treated CD8 T cells, congenic P14 mice were directly treated with HBSS or poly(I:C) for 1 day. Splenocytes were transferred together into the same recipients that were naive, that received 13mer GP$_{33-45}$ peptide or that were inoculated with LCMV. Here the LCMV infection should induce high levels of type 1 IFN, whereas the peptides would be poor type 1 IFN inducers. **Figure 3.22D** shows that at all time points tested, poly(I:C)-pretreated P14 cells (black bars) expanded to similar levels as HBSS-pretreated P14 cells (open bars) in mice that only saw antigen (13mer GP$_{33-45}$) and did not have a major inflammatory response. However, poly(I:C)-pretreated P14 cells had defects in expansion in response to the IFN-inducing LCMV infection compared to control treated cells (note the different axis for GP33 peptide or LCMV inoculation). Given that poly(I:C)-pretreated P14 cells expanded to similar levels in response to antigen only but had defects in expansion in response to antigen and inflammation (i.e. live virus infection), and IFNAR KO P14 cells expand to similar levels as WT P14 cells in response to antigen, but have defects in clonal expansion in response to infection, these results further support the hypothesis that out-of-sequence CD8

T cells are unable to receive positive effects of signal 3 cytokines (i.e. type 1 IFN) during acute infections.

2. Poly(I:C)-pretreated CD8 T cells have defects in short lived effector cell differentiation

Signal 3 cytokines have been shown to regulate the differentiation of CD8 T cells into distinct effector populations including EEC, SLEC and MPEC (78, 100, 143-145). Therefore, I examined the ability of poly(I:C)-pretreated CD8 T cells to differentiate into EEC, SLEC and MPEC populations, which can be distinguished based on expression of KLRG1 and CD127 (100, 146, 147). A similar experimental model was used as described previously, where WT or IFNAR KO P14 cells were transferred into mice for 1 day of treatment with poly(I:C) or HBSS prior to a second transfer into congenic hosts that were subsequently inoculated with LCMV. At different days post infection, splenocytes were isolated and stained for KLRG1 and CD127. At day 7 post infection, the IFNAR KO P14 CD8 T cells had similar proportions of SLEC (KLRG1hi, CD127lo), MPEC (KLRG1lo, CD127hi) and EEC (KLRG1lo, CD127lo), regardless of the pretreatment regimen (**Figure 3.23A**). Importantly, control IFNAR KO P14 cells had reduced SLEC proportions compared to control WT P14 cells (open bars). This is consistent with other results showing that type 1 IFN is important for SLEC differentiation in various infection models (78, 100, 143, 145). Consistent with our hypothesis, poly(I:C)-pretreated WT P14 CD8 T

Figure 3.23: Poly(I:C)-pretreated P14 cells and IFNAR KO P14 cells have similar proportions of SLECs at day 7 post LCMV infection
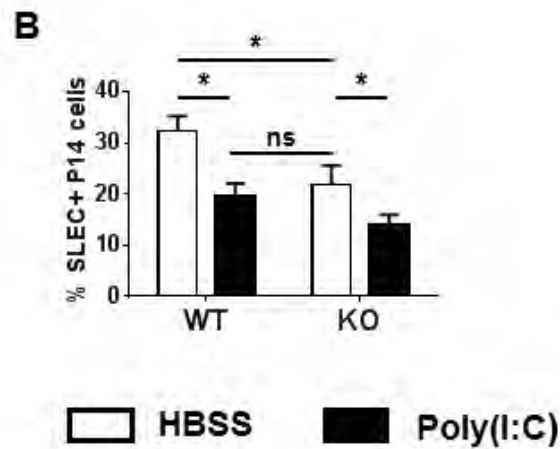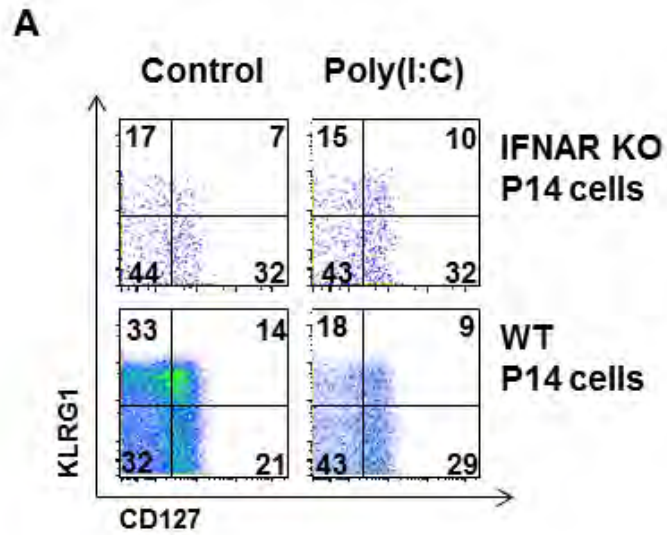
Figure 3.23: Poly(I:C)-pretreated P14 cells and IFNAR KO P14 cells have similar proportions of SLECs at day 7 post LCMV infection

WT or IFNAR KO P14 cells were transferred into mice, which were inoculated with poly(I:C). One day later, splenocytes were isolated and equal numbers of WT or IFNAR KO P14 cells were transferred into mice subsequently infected with $5x10^4$ pfu LCMV. Spleens were harvested at day 7 post infection. (**A**) Representative flow plots gated on donor P14 cells. (**B**) Frequency of control- (open bars) or poly(I:C)- (black bars) treated WT or IFNAR KO P14 cells expressing SLEC phenotype is graphed. Data are representative of 2 individual experiments with n of 2-4 mice per group.

cells (black bars) had reduced proportions of SLEC populations compared to the HBSS-pretreated WT P14 CD8 T cells (open bars) (**Figure 3.23**). Moreover, the poly(I:C)-pretreated WT P14 cells (black bars) had similar proportions of SLECs as compared to control IFNAR KO P14 cells (open bars) (**Figure 3.23B**). These data support the idea that the out-of-sequence CD8 T cells behave more similar to 2-signal only CD8 T cells (IFNAR KO P14 cells) in terms of effector cell differentiation.

The defect in SLEC differentiation in poly(I:C)-treated cells can be seen as early as day 5 post infection. However, because effector cell differentiation is only beginning, and antigen-specific CD8 T cells have only started to expand, it can be difficult to see differences at this early time point. The defect in differentiation is more dramatic at days 6 and 7 post infection and is seen in both proportion of the donor P14 response (**Figure 3.24B**) as well as total number of P14 SLECs (**Figure 3.24C**). The proportion of MPECs were generally similar to or slightly elevated in poly(I:C)-pretreated P14 cells as compared to control-treated counterparts at days 5-7 post infection (**Figure 3.24D**). However, despite having increased proportions of MPECs, the reduced clonal expansion seen in out-of-sequence CD8 T cells was still seen. Consequently, the total number of P14 MPECs was still lower in the poly(I:C)-pretreated cohort as compared to the control-treated P14 population despite the higher proportions (**Figure 3.24E**).

Not only did IFNAR KO P14 cells show defects in SLEC differentiation at day 7 post infection, consistent with what others have shown for 2-signal only T

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Figure 3.24: Out-of-sequence CD8 T cells have defects in short-lived effector cell differentiation
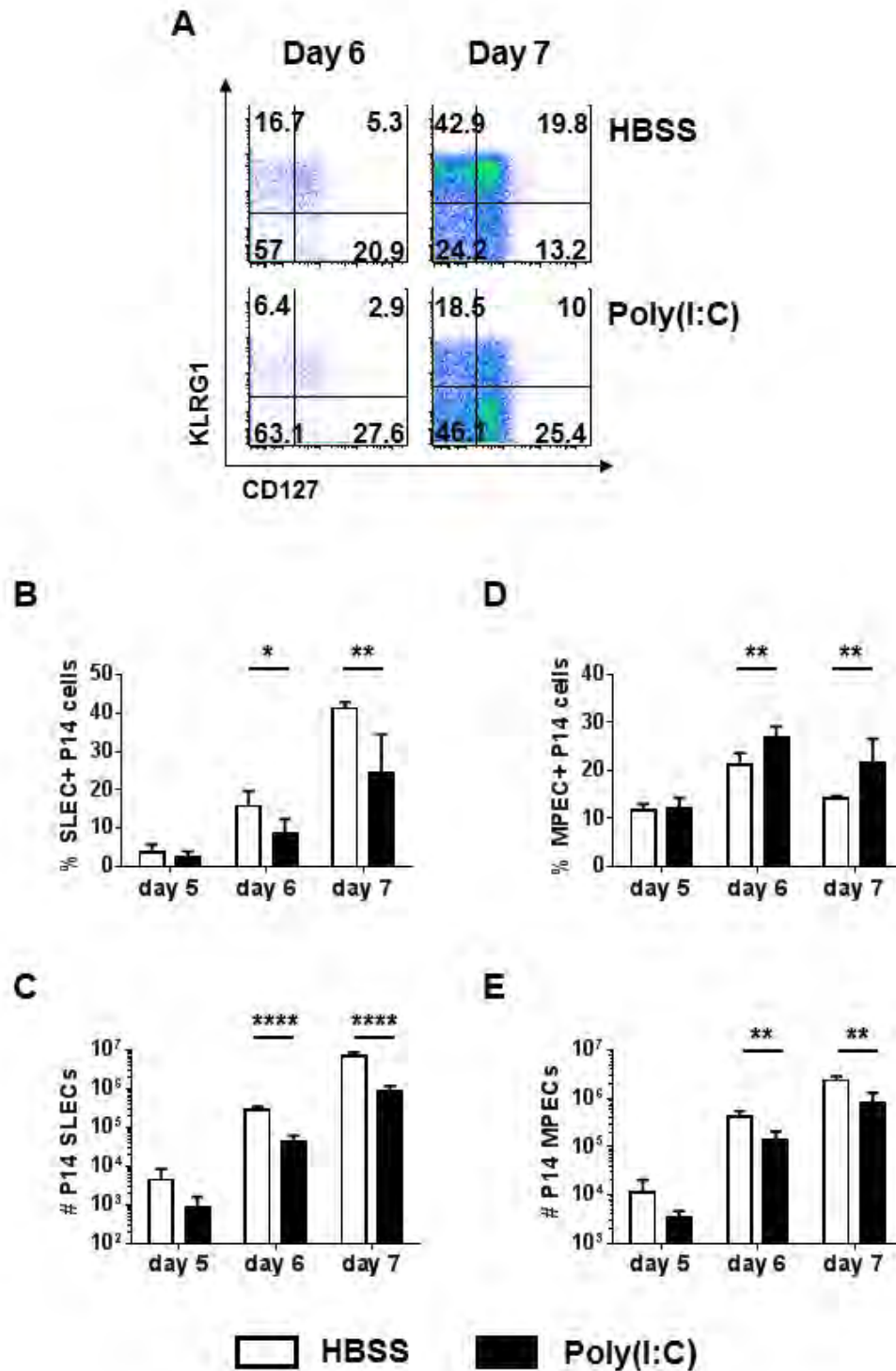
Figure 3.24: Out-of-sequence CD8 T cells have defects in short-lived effector cell differentiation

Congenic P14 mice were inoculated with HBSS (open bars) or poly(I:C) (black bars), and spleens were harvested 1 day later. Equal numbers of P14 cells were transferred into congenic WT host mice, which were subsequently infected with 5×10^4 pfu LCMV. Spleens were harvested at days 5, 6, and 7 post infection. (**A**) Representative flow plots gated on donor P14 cells showing SLEC/MPEC profiles at days 6 and 7 post infection. Frequency (**B**) and number (**C**) of P14 SLECs at days 5-7 post infection are graphed. Frequency (**D**) and number (**E**) of P14 MPECs at days 5-7 post infection are graphed. Data are from a representative experiment with 5 mice per group from more than 5 individual experiments with 3-5 mice per group.

cells, but out-of-sequence CD8 T cells also show defects in differentiation. Defects were seen early and continued through the peak of expansion. These data show that in addition to CD8 T cells requiring signal 3 cytokines for proper effector cell differentiation into various subsets, they also need to see the signals in the appropriate order.

3. Out-of-sequence CD8 T cells have defects in effector function

In some infection models, 2-signal CD8 T cells can produce similar proportions of cytokines as compared to 3-signal CD8 T cells (VSV), but other infection models show reduced cytokine production (Listeria) (78, 79). Therefore, I compared poly(I:C)- and HBSS-treated cells for their ability to produce the effector cytokines TNF and IFNγ. A similar experimental model was used as described previously, where P14 cells were transferred into mice for 1 day of treatment with poly(I:C) or HBSS or P14 mice were treated directly with poly(I:C) or HBSS prior to transferring cells into congenic hosts that were subsequently inoculated with LCMV. At day 5 post infection, splenocytes were isolated and stimulated *ex vivo* with or without cognate peptide (GP33) for 5 hours. Using naïve CD8 T cells, isotype controls and fluorescent minus one (FMO) staining to distinguish positive vs. negative staining, the poly(I:C)-pretreated P14 cells produced similar proportions of TNF and IFNγ compared to control-treated P14 cells after *in vitro* GP33 peptide stimulation (**Figure 3.25A**). Plotting the proportion of single cytokine producers (IFNγ- **3.25B**, TNF- **3.25D**) and double

Figure 3.25: Poly(I:C)-pretreated P14 cells produce similar proportions of IFNγ and TNF as control-treated P14 cells
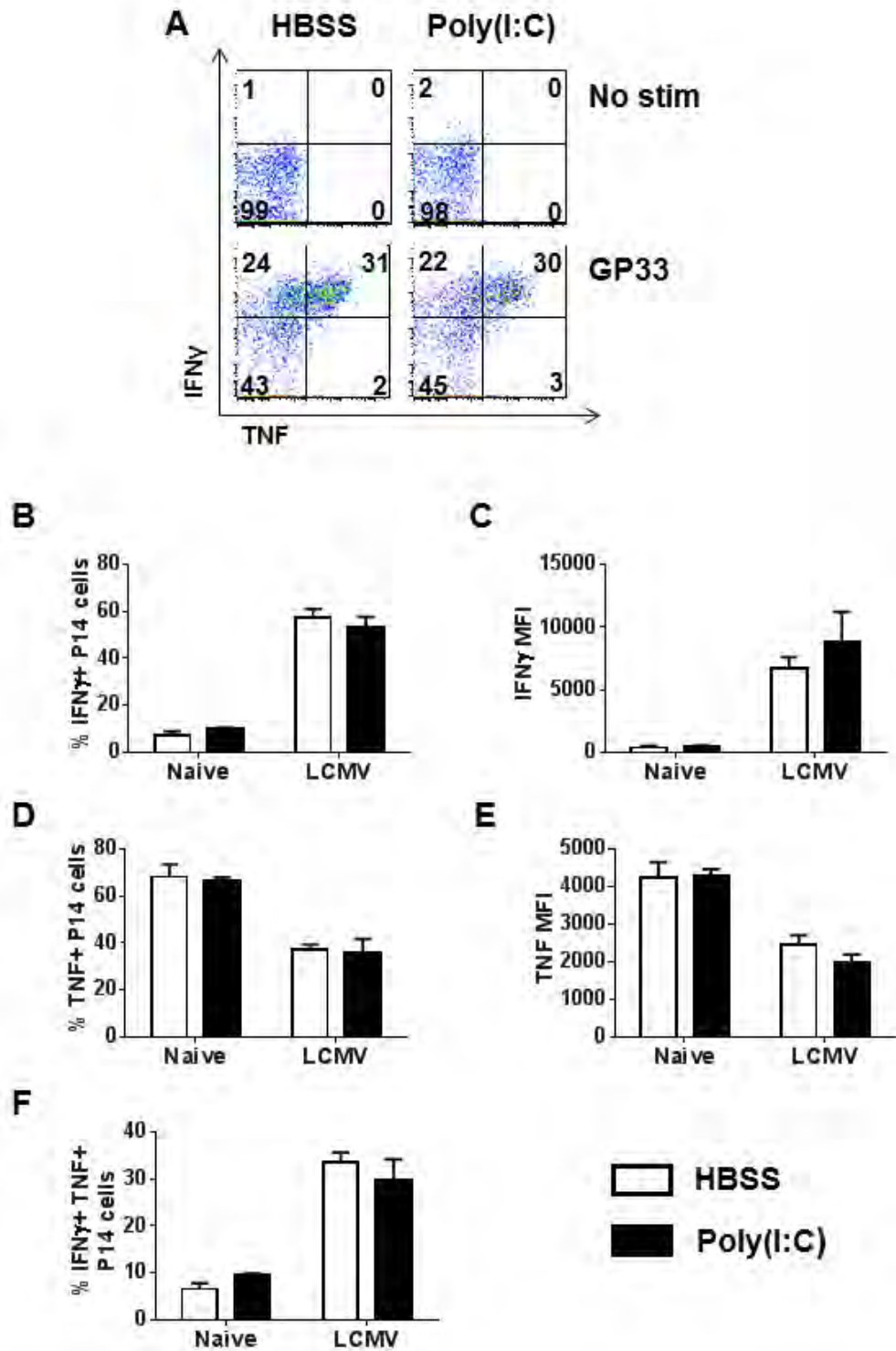
Figure 3.25: Poly(I:C)-pretreated P14 cells produce similar proportions of IFNγ and TNF as control-treated P14 cells

P14 mice were inoculated with HBSS (open bars) or poly(I:C) (black bars), and splenocytes were harvested 1 day later. Equal numbers of P14 cells were transferred into mice, which were subsequently infected with 5×10^4 pfu LCMV. At day 5 post infection, splenocytes were stimulated *ex vivo* with GP33 in an ICS. (**A**) Representative flow plots gated on donor P14 cells are depicted. The frequency of P14 cells producing IFNγ (**B**), TNF (**D**), or both IFNγ and TNF (**F**) after GP33 stimulation are graphed. The MFI of IFNγ (**C**) and TNF (**E**) of P14 cells are graphed. Data are representative of 3 individual experiments with n of 2-5 mice per group.

cytokine producers (TNF and IFNγ) (**Figure 3.25F**) revealed no significant difference in the ability of poly(I:C)-pretreated P14 cells (black bars) to produce effector cytokines. The MFI of IFNγ (**3.25C**) and TNF (**3.25E**) of HBSS- (open bars) and poly(I:C)-pretreated (black bars) P14 cells indicates that on a per cell basis there is no difference in the ability of these cells to produce effector cytokines. However, because of impaired clonal expansion, the number of cells producing IFNγ, TNF or both is reduced in out-of-sequence CD8 T cells compared to control CD8 T cells.

Using CD107a and b as markers for degranulation, poly(I:C)-pretreated P14 cells stained to a similar extent, if not slightly more, than HBSS-treated cells in response to GP33 peptide stimulation (**Figure 3.26A-B**). However, poly(I:C)-pretreated P14 CD8 T cells had substantially lower levels of granzyme B expression than control-treated cells at day 5 post infection (**Figure 3.26A, C)**. Reduced granzyme B expression in the out-of-sequence CD8 T cells was seen as early as day 4 post infection and lasted at least until day 6 post infection (**Figure 3.26D).** Out-of-sequence CD8 T cells were shown to have defects in ability to differentiate into SLECs and now showed reduced granzyme B expression, and I questioned whether these characteristics correlated with one another. KLRG1 expression in CD8 T cells is often associated with effector function, and indeed, there was a positive correlation between KLRG1 expression and granzyme B expression (R square = 0.8563, p < 0.0001) (**Figure 3.26E**). These data showing similar cytokine production but reduced granzyme B

Figure 3-26: Out-of-sequence P14 CD8 T cells have reduced granzyme B expression after LCMV infection
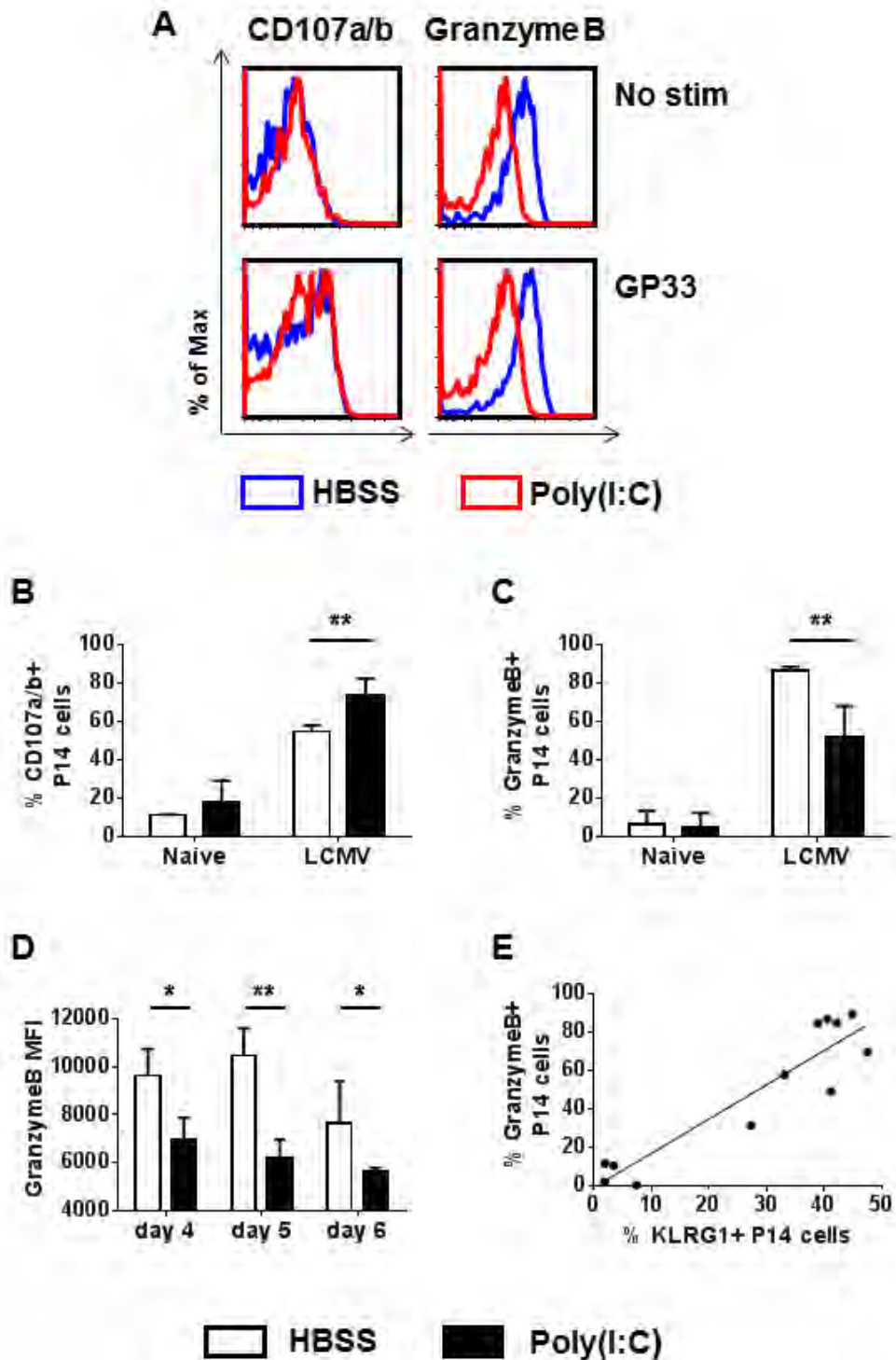
Figure 3-26: Out-of-sequence P14 CD8 T cells have reduced granzyme B expression after LCMV infection

P14 mice were inoculated with HBSS (open bars) or poly(I:C) (black bars), and splenocytes were harvested 1 day later. Equal numbers of P14 cells were transferred into mice, which were subsequently infected with $5x10^4$ pfu LCMV. Spleens were harvested at day 5 post infection (**A-D**) and days 4, 5 and 6 post infection (**E**). Splenocytes were stimulated *ex vivo* with or without GP33 peptide (**A-D**) or stained directly *ex vivo* (**E**). Data are representative of at least 3 Individual experiments with n of 3-5 mice per group.

expression are consistent with published phenotypes for CD8 T cells that only receive 2 signals (78, 79).

Since granzyme B expression has been used as a correlative marker for cytotoxic capability, data shown here suggest that poly(I:C)-pretreated CD8 T cells have reduced cytolytic function compared to HBSS-treated CD8 T cells. A new method to study effector cell function is the trogocytosis assay, whereby target cells are labeled with a membrane dye, mixed with cytotoxic effector cells for 1 hour, and then examined for the transfer of dye to a flow cytometry-defined effector cell population (181, 195). Trogocytosis is the process by which plasma membrane segments are transferred from one cell to another through cell-cell interactions. T cell engagement and formation of immunological synapse with target cells and APCs can result in trogocytosis, where T cells acquire segments of the plasma membrane of the target cell. The trogocytosis analysis protocol (TRAP) assay can measure the amount of lipid bilayer exchange between T cells and targets, where an increase in acquisition may be due to interacting with more targets, or with a single target more aggressively.

HBSS- or poly(I:C)-pretreated P14 cells were transferred into congenic hosts, which were subsequently inoculated with LCMV. At day 5 post infection, splenocytes were harvested and a TRAP assay was performed using RMA cells pulsed with various peptides as targets, including a no peptide control, irrelevant K3L peptide, and cognate GP33 peptide. Representative flow plots gated on donor P14 cells showing dye incorporation in response to different targets is

shown in **Figure 3.27A**. The MFI of P14 cells pretreated with HBSS (open bars) or poly(I:C) (black bars) expressing Sp-DiI$_{18}$(3) was normalized to control-treated cells for either K3L targets or GP33-pulsed targets is graphed (**Figure 3.27B**). The poly(I:C)-pretreated P14 cells, 5 days after LCMV infection, had modest but statistically significant reduced ability to acquire the dye from GP33-pulsed RMA cells, when compared to the HBSS-pretreated donor T cells. Reduced incorporation of the lipid dye can be an indicator of reduced effector cell function (196, 197).

I questioned whether these donor poly(I:C)-pretreated T cell responses, which were dramatically reduced in number and modestly reduced in effector function, would affect viral load differently than that in mice receiving HBSS-treated cells. Mice receiving either HBSS-pretreated or poly(I:C)-pretreated P14 CD8 T cells were subsequently infected with LCMV, and viral titers were examined at different time points post infection. As early as day 4 post infection, mice receiving poly(I:C)-pretreated P14 cells had modest but statistically significant increased viral titers compared to mice receiving control-treated P14 cells in the fat pad (4.7 ± 0.1 vs. 4.3 ± 0.08 log pfu, two independent experiments combined for n=10 per group) respectively (p = 0.0082). One individual experiment determining viral titers on day 6 post infection showed modest but significant increased viral loads in the fat pad and spleen in mice receiving poly(I:C)-pretreated P14 CD8 T cells compared to mice receiving HBSS-pretreated P14 cells (**Figure 3.28A**). Viral titers in the liver did not reach

Figure 3.27: Reduced trogocytosis of poly(I:C)-pretreated P14 cells co-cultured with GP33 pulsed RMA cells
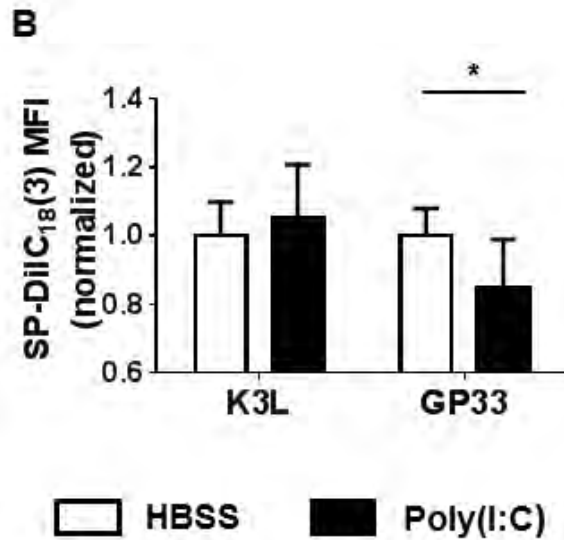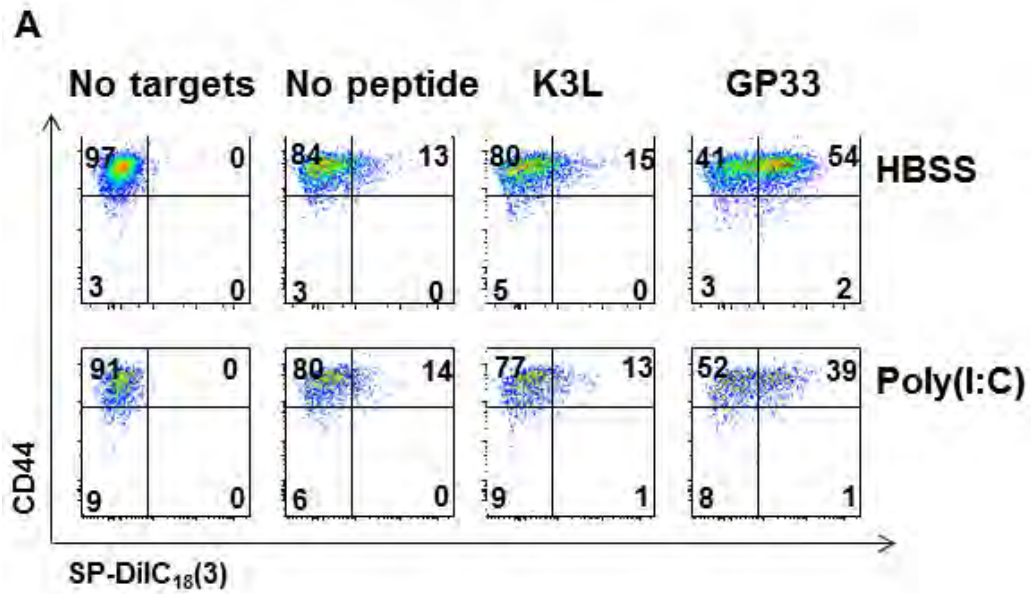
Figure 3.27: Reduced trogocytosis of poly(I:C)-pretreated P14 cells co-cultured with GP33 pulsed RMA cells

A TRAP assay was performed using day 5 HBSS- or poly(I:C)-pretreated P14 CD8 T cells as effectors and RMA cells pulsed with peptides as targets. Effectors were developed by poly(I:C) or HBSS treating a P14 transgenic mouse, transferring ~10,000 P14 cells from each group into separate mice 1 day after treatment and infecting the recipient mice with LCMV. At day 5 post infection, splenocytes containing the donor P14 CD8 T cells were isolated and used as effectors. Target cells were RMA cells that were not pulsed with peptide (no peptide), pulsed with an irrelevant peptide (K3L), or pulsed with the specific peptide (GP33). Target cells were labeled with fluorescent lipid molecule SP-DiIC$_{18}$(3) that can be detected if it is transferred to a different cell through trogocytosis. Target cells were in excess and were co-incubated with effectors for 1 hour, stained with surface antibodies and ran on a flow cytometer. (**A**) Representative flow plots gated on donor P14 cells that were HBSS or poly(I:C) pretreated co-incubated with 1. No targets, 2. No peptide pulsed targets, 3. K3L pulsed targets, or 4. GP33 pulsed targets, looking at P14 cell incorporation of SP-DiIC$_{18}$(3) are depicted. Data are representative of 2 independent experiments with n of 3-5 mice per group. (**B**) MFI of SP-DiIC$_{18}$(3) gated on donor P14 cells, normalized to HBSS control for K3L and GP33 pulsed targets. HBSS-pretreated P14 cells are in the open bars and poly(I:C)-pretreated P14 cells represented as black bars. Data are combined from 2 independent experiments with a total n of 8 mice per group.

statistical significance (p=0.0711), but still showed a similar trend as titers in the other organs tested, where mice receiving poly(I:C)-pretreated P14 cells had increased titers as compared to mice who received HBSS-treated P14 cells. These differences in viral titer are admittedly small, but they are statistically significant and occur in environments where normal endogenous host T cell responses are simultaneously occurring. By day 8 post infection the viral titers were reduced to the limit of detection in all organs tested in both treatment groups.

Since out-of-sequence P14 cells were found to have reduced KRLG1 expression compared to controls, I questioned whether KLRG1 expression correlated with viral load. After plotting the frequency of KLRG1+ P14 cells at days 4, 6, and 8 post infection vs. corresponding viral titer, a negative correlation between viral titer and frequency of KLRG1+ P14 cells in all three organs tested was revealed (**Figure 3.28B**). The greater the frequency of KLRG1+ P14 cells, the lower the viral load. In addition, and perhaps not surprising, there was also a negative correlation between viral titer and total number of P14 cells per spleen (**Figure 3.28C**). Based on these data, a combination between reduced effector function, and impaired clonal expansion may have negative consequences on the ability to control antigen load.

Figure 3.28: Mice receiving poly(I:C)-pretreated P14 cells have higher viral titers compared to mice receiving HBSS-pretreated P14 cells
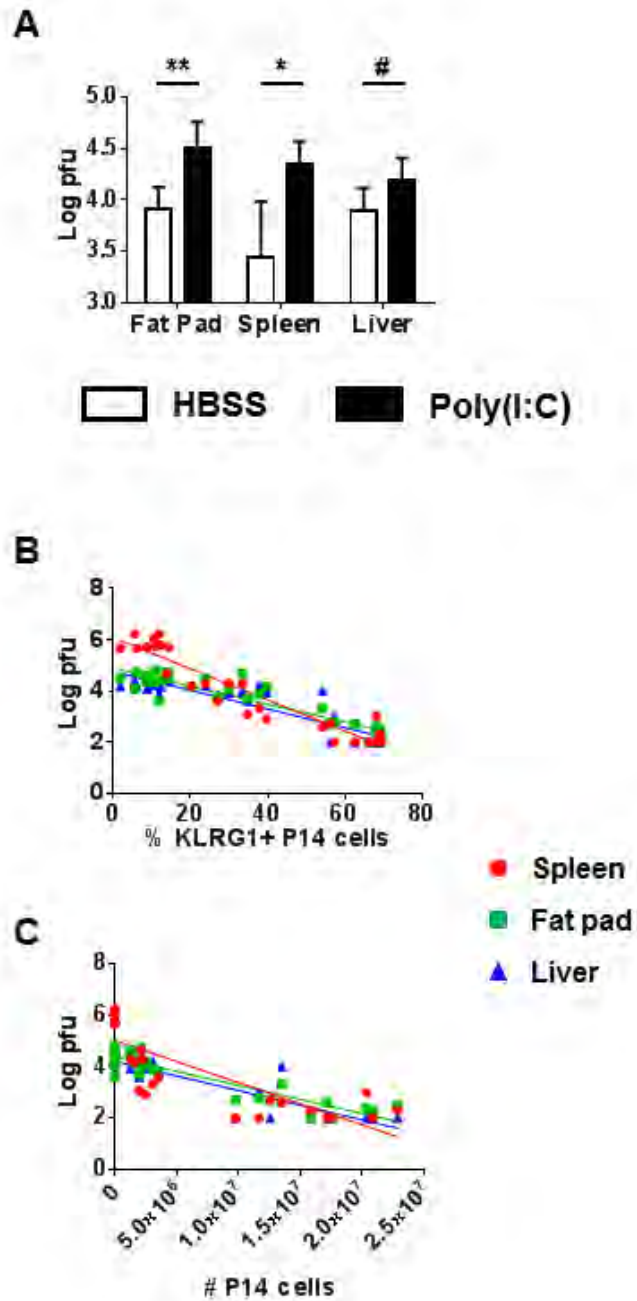
Figure 3.28: Mice receiving poly(I:C)-pretreated P14 cells have higher viral titers compared to mice receiving HBSS-pretreated P14 cells

Mice received equal numbers of HBSS- (open bars) or poly(I:C)- (black bars) pretreated P14 cells and were infected with 5×10^4 pfu LCMV. (**A-C**) At days 4, 6 and 8 post infections, spleens, fat pad and livers were harvested, viral titers were determined, and splenocytes were stained. (**A**) Viral titers at day 6 post infection are graphed. Viral titers from spleen, fat pad and liver from days 4, 6, and 8 were correlated with frequency of KLRG1+ P14 cells (**B**) or total number of P14 CD8 T cells (**C**) per spleen. Data representative of 2-3 individual experiments per time point with n of 4-6 mice per group.

4. Summary

Because the suppression of proliferation of poly(I:C)-pretreated CD8 T cells correlated with refractoriness to IFNβ stimulation, I hypothesized that poly(I:C)-pretreated CD8 T cells were unable to receive the positive effects that type 1 IFN exerts as a signal 3 cytokine when delivered in the proper sequence. This hypothesis would suggest that poly(I:C)-pretreated P14 cells would behave similarly to 2-signal only CD8 T cells, rather than 3-signal CD8 T cells.

Indeed, I found that out-of-sequence CD8 T cells behaved more like to 2-signal only CD8 T cells rather than T cells that received all 3 signals in the appropriate order. Poly(I:C)-pretreated CD8 T cells were found to have a delay in their ability to divide at 3 days post infection, but division profiles eventually mimicked that of control-treated cells by day 4 post infection (**Figure 3.19**). If a delay in cell division was the only factor contributing to the suppression of clonal expansion seen, we would have expected to see the out-of-sequence CD8 T cells catch up to control-treated cells at late enough time. However, despite a delay in the peak expansion of poly(I:C)-pretreated CD8 T cells, the magnitude of the response never reached the same levels as controls (**Figure 3.20**), suggesting that additional factors were contributing to the reduced expansion seen in poly(I:C)-pretreated CD8 T cells.

Signal 3 cytokines had been shown to prolong the survival of activated CD8 T cells by upregulating the IκB family member Bcl-3 (86-88). Although the

exact mechanism of how Bcl-3 mediates survival is not known, loss of Bcl-3 resulted in reduced clonal expansion of CD8 T cells (86-88, 198). Reduced Bcl-3 expression is another hallmark of 2-signal CD8 T cells, and here we show that the out-of-sequence T cells behave more like to the 2-signal CD8 T cells in this regard. We identified lower levels of Bcl-3 in the out-of-sequence CD8 T cells at multiple time points post infection when compared to control-treated cells (**Figure 3.21**). Therefore it is conceivable that lower Bcl-3 levels caused the poorer survival of out-of-sequence CD8 T cells, resulting in reduced clonal expansion.

I showed that in response to peptide only, IFNAR KO P14 cells expanded to similar levels as WT P14 cells. Likewise poly(I:C)-pretreated CD8 T cells expanded to similar levels as HBSS-treated CD8 T cells in response to peptide only. However, IFNAR KO P14 cells and poly(I:C)-pretreated P14 CD8 T cells both showed impaired expansion compared to their appropriate controls in response to an LCMV infection (**Figure 3.22**). This shows how type 1 IFN can have such positive effects on T cell expansion. Results here suggest it is a lack of the response to signal 3 cytokines that contributes to suppression of proliferation seen in out-of-sequence CD8 T cells.

Previously studies identified CD8 T cells lacking signal 3 cytokines signals had defects in T cell differentiation where they showed reduced SLEC proportions compared to T cells which received all 3 signals (78, 100, 143, 145). Consistent with these results, I have shown here that IFNAR KO P14 cells had lower proportions of SLECs and increased MPECs compared to WT P14 cells in

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response to LCMV infection (**Figure 3.23**). Poly(I:C)-pretreated P14 cells had comparable changes as the IFNAR KO P14 cells, showing reduced SLEC proportions, and increased MPEC proportions as compared to control-treated P14 cells after LCMV infection (**Figure 3.24**).

In addition to signal 3 cytokines being important for T cell subset differentiation, CD8 T cell function has also been shown to be affected. Each infection model may require different factors for efficient T cell activation and acquisition of effector function. My findings show that out-of-sequence T cells behave in a consistent manner with those of 2-signal only T cells. The poly(I:C)-pretreated CD8 T cells could produce similar proportions of IFNγ and TNF, and similar levels on a per cell basis (**Figure 3.25**); however poly(I:C)-pretreated CD8 T cells expressed less granzyme B (**Figure 3.26**). The combination of reduced granzyme B expression and impaired clonal expansion may have contributed to the higher viral titers seen in mice, which received out-of-sequence P14 CD8 T cells (**Figure 3.28**).

Thus, studies here have shown that not only do CD8 T cells require distinct signal 3 cytokines for efficient activation, acquisition of effector function, differentiation into effector subsets, and efficient clonal expansion, but CD8 T cells also need to see these signals in a specific order. Out-of-sequence CD8 T cells that receive signal 3 first actually behave more like a 2-signal only CD8 T cell rather than one that receives all 3 signals in the more conventional order.

E. Conclusions

Demonstrated in this chapter are three main findings: 1) poly(I:C)-pretreated CD8 T cells have reduced clonal expansion, 2) pretreatment with poly(I:C) renders T cells transiently refractory to IFNβ induced STAT phosphorylation, and 3) out-of-sequence CD8 T cells behave more similar to 2 signal only CD8 T cells. A model is shown in **Figure 3.29**.

Acute virus infections induce a transient state of immune suppression often associated with impaired T cell responses to antigen exposure. We demonstrate here a universal mechanism of IFN-induced suppression of T cell response associated with the inability to receive positive effects of IFN acting as a signal 3 cytokine, resulting in impaired T cell responses. Virus-induced suppression of T cell responses could be mimicked by pre-exposure to the dsRNA analogue, poly(I:C) (**Figures 3.1-3.10**). The suppression of clonal expansion of poly(I:C)-pretreated CD8 T cells was found to require direct effects of type 1 IFN on the CD8 T cells (**Figure 3.4**).

Because the poly(I:C)-induced suppression of CD8 T cell expansion to required direct effects of type 1 IFN, I hypothesized that poly(I:C)-pretreated CD8 T cells may have had a different pattern of STAT phosphorylation in response to IFNβ signals. It could have been conceived that poly(I:C)-pretreated CD8 T cells may phosphorylate more STAT1 than STAT4, considering that type 1 IFN signaling through STAT1 has more anti-proliferative effects, and acting through

161

Figure 3.29: Model: Out-of-sequence signal 3 suppresses CD8 T cell responses *in vivo*
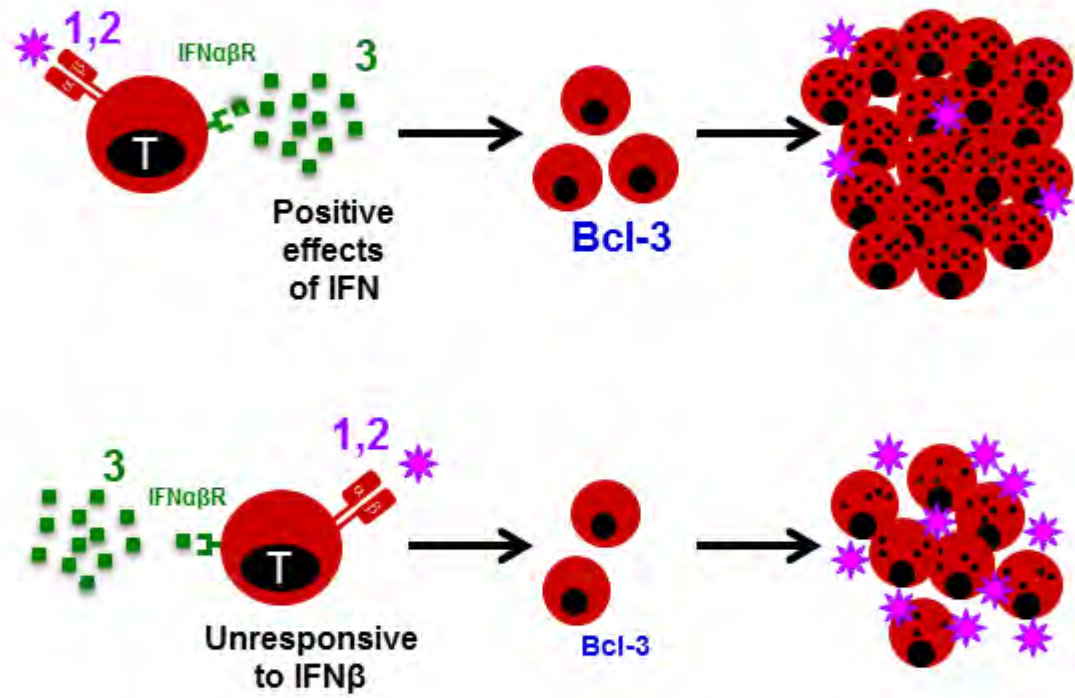
Figure 3.29: Model: Out-of-sequence signal 3 suppresses CD8 T cell responses *in vivo*

Under normal circumstances when naïve T cells see antigen and costimulation first (top), type 1 IFN will have positive effects as a signal 3 cytokine. IFN augments clonal expansion by promoting survival of activated CD8 T cells, in part by upregulating Bcl-3. Efficient clonal expansion and differentiation of T cells results in control of antigen load. Naïve CD8 T cells that see signal 3 cytokines prior to antigen and costimulation (bottom) are refractory to further IFN stimulation and do not receive the positive effects IFN normally has as a signal 3 cytokine. Out-of-sequence T cells have a delay in cell division and lower Bcl-3 levels, which may reduce survival of T cells, resulting in impaired clonal expansion. Impaired clonal expansion and differentiation may lead to reduced ability to control antigen load.

STAT4 promotes more clonal expansion (90, 91, 95). However, this was not the case, as poly(I:C)-pretreated CD8 T cells did not phosphorylate any downstream STATs tested (**Figures 3.11-3.12**). CD8 T cells eventually gained the ability to respond to further IFN signals, indicating the refractoriness to IFN-induced STAT phosphorylation is a transient event (**Figure 3.13**). Downregulation of the type 1 IFN receptor and upregulation of the suppressor protein SOCS1 were identified in poly(I:C)-pretreated CD8 T cells (**Figures 3.17-3.18**) and are thought to be contributing to the refractoriness to IFNβ stimulation. The refractoriness to IFNβ stimulation correlated kinetically with the poly(I:C)-induced impairment in clonal expansion seen (**Figures 3.13 and 3.3**). Since IFN has been shown to have such a profound effect on T cell expansion during acute infections, the correlations suggest that the lack of responsiveness to IFN signals may contribute to the suppression of clonal expansion seen in poly(I:C)-pretreated CD8 T cells.

We show here that poly(I:C)-pretreated CD8 T cells are refractory to IFNβ signaling in terms of downstream STAT phosphorylation, suggesting that they are unable to receive positive effects that signal 3 cytokines normally provide during acute infections. Indeed, these out-of-sequence signal 3 CD8 T cells were found to behave more like 2-signal only CD8 T cells rather than T cells that receive all 3 signals in the proper order. The poly(I:C)-pretreated CD8 T cells were shown to behave similar to 2-signal T cells in a number of ways; they were associated with impaired expansion, which may have been attributed to a combination between delayed cell division (**Figure 3.19)** and reduced levels of

the survival protein Bcl-3 (**Figure 3.21**). Poly(I:C)-pretreated T cells also had defects in effector cell differentiation, showing reduced proportions of SLECs (**Figures 3.23-3.24**), and were also associated with reduced effector function (**Figure 3.25-3.27**). Consequently, the combination between reduced effector function and impaired clonal expansion resulted in increased viral loads during the acute infection (**Figure 3.28**), and reduced memory formation (**Figure 3.20**). Therefore, the inability to respond to signal 3 cytokines, as seen in out-of-sequence T cells, limits CD8 T cell expansion and suggests a causative mechanism for reduced vaccine efficacy when administered during acute infections.

CHAPTER IV: Interferon primes naïve T cells to exhibit immediate effector function upon antigen stimulation

A. Introduction

During an acute virus infection, antigen-specific CD8 T cells undergo clonal expansion and differentiation into effector cells in order to control the infection. Antigen-specific cells require 3 distinct signals for their full activation: TCR engagement of pMHC (signal 1), costimulation resulting from interaction between B7 and CD28 (signal 2), and inflammatory cytokines including IL-12 or type 1 IFN (signal 3) (98-100). Without any one of these signals, dramatic differences in CD8 T cell activation can occur. Without signal 3 cytokines, CD8 T cells can divide but with defects in clonal expansion and/or effector function (79). If CD8 T cells are only exposed to pMHC without costimulation or inflammatory cytokines, they can be driven into apoptosis or become anergic (98). Identified in the previous chapter, out-of-sequence signal 3 was found to have a profound effect on CD8 T cell clonal expansion and effector differentiation when exposed to antigen for a prolonged period of time. We questioned if there might be an effect of out-of-sequence signaling on CD8 T cells prior to cell division and clonal expansion.

During the course of an immune response there are multiple stages of CD8 T cell activation: expansion and differentiation, contraction, and memory formation. Much work has been done trying to identify and differentiate naïve vs.

effector vs. memory CD8 T cell populations based on their phenotype. A heterogeneous population of CD8 T cells exists at each stage of activation. Nevertheless, the general activation status of CD8 T cells can be identified using several key molecules. Naïve CD8 T cells are generally classified as being CD44lo, CD62Lhi, and CD127hi (199). During the earliest stages of activation, CD8 T cells will become CD44hi, upregulate CD69 and CD25 and downregulate CD127 and CD62L (138, 172, 200). Closer to the peak of CD8 T cell expansion, T cells lose expression of CD25 and CD69, which are early activation markers, but remain CD44hi (140). A small proportion of CD8 T cells will upregulate KLRG1, an effector-associated surface marker. A specific population of effectors will re-express CD127; these cells, have been shown to preferentially transition into the memory pool (147). Memory T cells, although a heterogeneous population, remain CD44hi, and while the majority are CD127hi, some express CD62L, depending on the subset (199). Therefore, the relative differentiation state of CD8 T cells can be identified by staining for a subset of surface markers.

Recent work has attempted to identify the specific TFs that are associated with effector vs. memory T cells. Numerous TFs are associated with T cell differentiation, but two primary TFs play a major role in effector CD8 T cells are the T-box TFs, Tbet and Eomes (151-154). These TFs regulate a number of effector genes in CD8 T cells including IFNγ, granzyme B, and perforin (154, 168). These two TFs do not have completely redundant roles, as Tbet is more associated with effector T cells and Eomes with memory T cells (153, 154). In

addition, effector CD8 T cells are associated with Tbet and Blimp-1 expression, whereas memory CD8 T cells are associated with Eomes and TCF1 expression (152, 154). More recently, the TFs BATF and IRF4 have been shown to be important for CD8 T cell effector differentiation and proliferation. BATF is upregulated in effector cells in response to LCMV infection, and (156) IRF4 is induced through TCR stimulation. IRF4 and BATF cooperate with each other during CD8 T cell differentiation and, in fact, regulate many of the same genes including effector-associated TFs, such as Tbet, Eomes, and Blimp-1 (157, 158).

Once activated, CD8 T cells have the ability to produce multiple cytokines in response to cognate antigen and acquire ability to specifically lyse target cells. Naïve CD8 T cells require all 3 signals in order to acquire multiple effector functions. Without signal 3 cytokine stimulation, T cells can have defects in cytotoxic capability, and cytokine production (78, 79, 98). If CD8 T cells receive all the appropriate signals, they can produce multiple cytokines including TNF, IFNγ, and sometimes IL-2 in response to cognate antigen. Activated CD8 T cells will upregulate granzyme B and perforin, which enables them to specifically lyse target cells (97). However, CD8 T cells normally need to divide at least once before they acquire specific effector functions, including the ability to directly lyse target cells and rapidly produce certain cytokines (141). Naïve CD8 T cells have the capacity to produce TNF upon stimulation with cognate ligand or anti-CD3, but they do not produce IFNγ (161, 162). Traditionally, acquisition of IFNγ production upon stimulation with ligand is seen only after CD8 T cells divide

several times (141, 154, 162). Other studies have found that naïve T cells produce a short burst of IFNγ within the first 24 hours after cognate antigen stimulation and costimulation or infection, but this may have more regulatory effects (201, 202).

Recent discoveries from our lab have shown that bystander CD8 T cells, which are non-antigen specific for the infecting pathogen, can acquire rapid effector function if pre-exposed to type 1 IFN-inducing environments (193). Bystander CD8 T cell sensitization to rapid effector function required indirect effects of type 1 IFN and self-MHC. This suggested that if CD8 T cells are exposed to activation signals in an unconventional order, they may behave differently in response to antigen stimulation. We sought to determine how CD8 T cells responded to cognate antigen stimulation if they were pre-exposed to signal 3 cytokines. By using the dsRNA analog, poly(I:C), to induce type 1 IFN, we discovered poly(I:C)-induced naïve CD8 T cells to acquire immediate effector function, prior to cell division and prior to acquiring an effector cell phenotype. Through these studies, several distinct discoveries were made: 1) Poly(I:C)-pretreated naïve CD8 T cells acquired an early-activated phenotype associated with alterations of transcription factors and surface markers; 2) Priming with poly(I:C) induced naive CD8 T cells to exhibit effector functions immediately upon stimulation with high-density cognate antigen, reduced affinity APLs, and in response to reduced concentrations of cognate antigen; 3) Poly(I:C) priming requires hematopoietic cells, self-MHC, and costimulatory B7 molecules; 4)

Primed naïve CD8 T cells specifically lyse target cells; 5) Acquisition of immediate effector function in poly(I:C)-primed naïve CD8 T cells occurs before cell division. These studies identified how the timing of activation signals can dramatically affect the acquisition of CD8 T cell effector function.

B. Poly(I:C)-primed naïve CD8 T cells acquire immediate effector function

1. Naïve CD8 T cells adopt an early-activated phenotype after poly(I:C) priming *in vivo*.

Naïve CD8 T cells are phenotypically distinct from effector T cells and memory T cells. Naïve CD8 T cells can be identified as being CD44lo, CD127hi, and CD62Lhi (100). Upon cognate antigen stimulation, CD8 T cells undergo clonal expansion, differentiation and memory formation associated with alterations in CD8 T cell phenotype. In the present study, the phenotype of naïve CD8 T cells changed in the absence of cognate antigen. Upon *in vivo* stimulation with the dsRNA analog, poly(I:C), naïve CD44lo CD8 T cells adopt an early, intermediate-activated phenotype. Antigen-specific cells were tracked by using CD8 T cell congenic Ly5.1 transgenic cells, including OT-I and P14 CD8 T cells. Transgenic CD8 T cells were adoptively transferred into Ly5.2 hosts, which were inoculated with HBSS or poly(I:C). Representative histograms in **Figure 4.1A** show naïve CD8 T cells (host, donor OT-I and donor P14) upregulated the early activation markers CD69 and CD86, and downregulated IFNAR1, CD127, with

some downregulating   CD62L, one day after poly(I:C) treatment (open

histograms). The HBSS-treated controls (open bars) were used to normalize the

Figure 4.1: Poly(I:C) induces naive CD8 T cells to adopt an early-partial activated phenotype
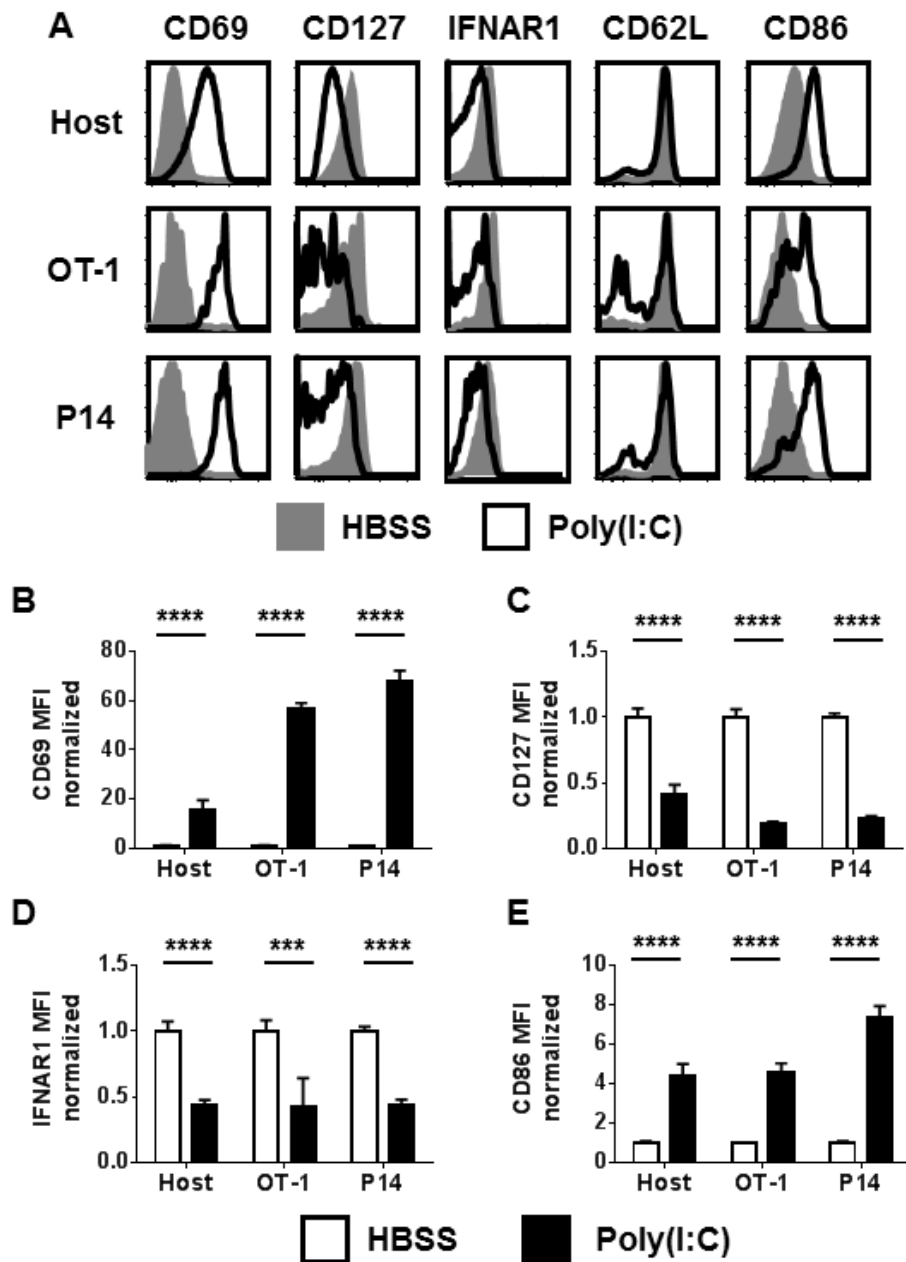
Figure 4.1: Poly(I:C) induces naive CD8 T cells to adopt an early-partial activated phenotype

Ly5.1 OT-1 or P14 T cell transgenic splenocytes were adoptively transferred into WT B6 mice, which were inoculated with HBSS or poly(I:C). Spleens were harvested 1 day later and stained for activation markers. **A**) Representative histograms gated on CD44lo host, CD44lo OT-I, or CD44lo P14 CD8 T cells from HBSS- (shaded histograms) or poly(I:C)- (open histograms) inoculated mice. **B-E**) MFI of poly(I:C)- (black bars) treated CD44lo CD8 T cell populations were normalized to HBSS- (open bars) treated CD44lo CD8 T cell populations depicting CD69 (**B**), CD127 (**C**), IFNAR1 (**D**), and CD86 (**E**). Data are representative of 2 individual experiments (OT-1) or at least 5 individual experiments (P14) with n of 2-5 mice per group.

MFI of poly(I:C)-pretreated naïve CD8 T cells (black bars). The normalized MFI of CD69 (**B**), CD127 (**C**), IFNAR1 **(D)** and CD86 (**E**) of the naïve host, OT-I and P14 CD8 T cell populations are graphed (**Figure 4.1**). Irrespective of the specificity of the naïve T cell population, each adopted a similar change in phenotype. However, both of the transgenic populations showed higher expression of CD69 when compared to the endogenous polyclonal population. Nevertheless, the poly(I:C)-treated host population still showed CD69 MFI ~20 fold higher compared to HBSS-treated controls.

These results were further corroborated by microarray data. CD44lo CD8α+ T cells were isolated from HBSS- or poly(I:C)-inoculated mice after 1 day. Naïve T cells were purified to >98.5% purity, and a microarray analysis was performed. The fold expression of poly(I:C)-treated/HBSS-treated T cells of selected genes are shown in **Table 4.1.** Both CD86 and CD69 were found to be upregulated in naïve CD8 T cells from poly(I:C)-treated over control-treated cells in microarray data, and this was confirmed with protein expression shown in **Figure 4.1**.

The upregulation of both CD69 and CD86 required type 1 IFN signaling. Splenocytes from Ly5.1 P14 mice were transferred into WT B6 or IFNAR KO hosts followed by inoculation with HBSS or poly(I:C) for 1 day. Donor CD44lo P14 cells showed reduced upregulation of the early activation marker CD69 (**Figure 4.2A**), and this upregulation was completely abrogated in the host CD44lo CD8 T cell population (**Figure 4.2C**). This indicated that type 1 IFN must

Table 4.1: Microarray analysis of selected genes differentially expressed from CD44lo CD8α+ T cells after 1-day inoculation with HBSS or poly(I:C)

FC (abs) Exp. vs Control	Exp. vs control (up or down)	Gene symbol	Gene description
25.33	up	Gzmb	granzymeB
17.52	up	Cxcl10	chemokine(C-X-Cmotif)ligand10
8.66	up	Irf7	interferonregulatoryfactor7
6.85	up	Tlr7	toll-likereceptor7
6.71	up	Stat2	signaltransducerandactivatoroftranscription2
6.02	up	Il1b	interleukin1beta
5.22	up	Bst2	bonemarrowstromalcellantigen2
4.86	up	Tlr3	toll-likereceptor3
3.73	up	Cd274	CD274antigen
3.53	up	Gzma	granzymeA
2.93	up	Eomes	eomesoderminhomolog(Xenopuslaevis)
2.87	up	Il12rb2	interleukin12receptor,beta2
2.87	up	Grn	granulin
2.85	up	Cd86	CD86antigen
2.59	up	Cd69	CD69antigen
2.54	up	Bcl3	Bcellleukemia/lymphoma3
2.53	up	Il1r2	interleukin1receptor,typeII
2.39	up	Jun	
2.29	up	Tnfsf10	tumornecrosisfactor(ligand)superfamily,member10
2.18	up	Il12rb1	interleukin12receptor,beta1
2.18	up	Unc93b1	unc-93homologB1(C.elegans)
2.10	up	Socs3	suppressorofcytokinesignaling3
2.09	up	Socs1	suppressorofcytokinesignaling1
3.01	down	Itgax	integrinalphaX
2.55	down	Cxcr6	chemokine(C-X-Cmotif)receptor6
2.20	down	Klrd1	killercelllectin-likereceptor,subfamilyD,member1

Table 4.1: Microarray analysis of selected genes differentially expressed from CD44lo CD8α+ T cells after 1-day inoculation with HBSS or poly(I:C)

WT B6 mice were inoculated with HBSS or poly(I:C). One day later, splenocytes were harvested, and CD44lo CD8α+ T cells were isolated to > 98.5% purity. MoGene 2.0 ST Array (Affymetrix) gene chip was used. Data were analyzed using Genespring software. Fold change of poly(I:C)-treated CD8 T cells relative to HBSS-treated CD8 T cells of selective genes are listed in the table. Data are from an individual experiment.

Figure 4.2: Early activated phenotype requires type 1 IFN
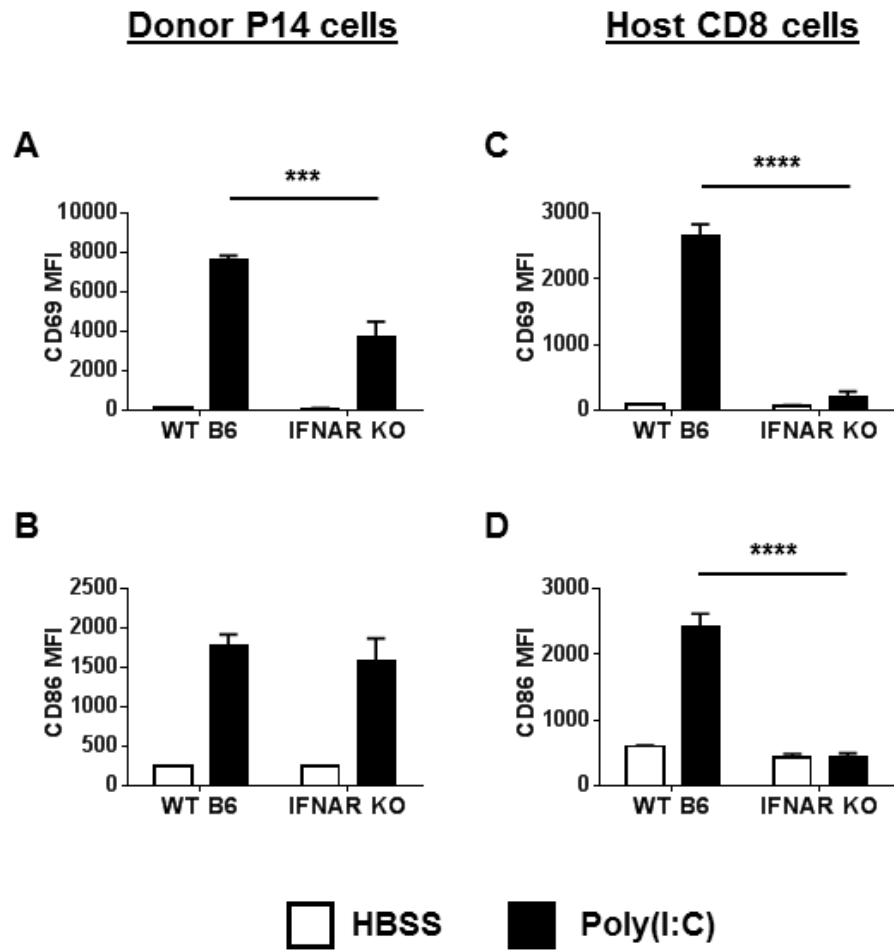
Figure 4.2: Early activated phenotype requires type 1 IFN

Congenic P14 cells were transferred into WT B6 hosts or IFNAR KO hosts, which were inoculated with HBSS or poly(I:C). One day later splenocytes were harvested and stained. MFI of CD44lo donor P14 cells expressing CD69 (**A**) and CD86 (**B**) is graphed. MFI of CD44lo host CD8 T cells expressing CD69 (**C**) and CD86 (**D**) is graphed. Individual experiment with n of 3-4 mice per group.

act directly on the CD8 T cell for its upregulation of CD69. CD86 was similarly found to require IFN signaling on CD8 T cells, as host CD44lo CD8 T cells from IFNAR KO mice did not upregulate CD86 after poly(I:C) treatment (**Figure 4.2D**), but donor P14 cells upregulated CD86 after poly(I:C) treatment (**Figure 4.2B**).

Previous studies from our lab found that P14 cells in a bystander setting maintained a naïve phenotype and remained CD44 and CD43 low at day 5 post Pichinde virus (PV) infection (non-cross-reactive with the P14 cells) and modestly increased CD122 expression (193). However, in the system shown here, 1 day after poly(I:C) treatment, CD122 expression was not increased over controls. Additional effector molecules were surveyed, including a number of different cytokine receptors, activation markers, and adhesion molecules, most of which showed no significant difference between control and poly(I:C)-pretreated groups. CD25 is one of the earliest markers upregulated after naïve CD8 T cell activation, but its expression was unaffected by poly(I:C) treatment. Another important effector CD8 T cell activation marker, KLRG1, was also unaltered by pretreatment with the dsRNA analogue.

To identify when poly(I:C)-primed naïve CD8 T cells alter their phenotype, mice were inoculated with poly(I:C) for 12, 18 or 24 hours. CD44lo P14 cells upregulated CD69 within the first 12 hours after exposure to poly(I:C)-induced environments (black bars) (**Figure 4.3A**). However, the downregulation of both CD127 (**Figure 4.3B**) and CD62L (**Figure 4.3C**) occurred in a stepwise fashion over time. There were very few CD44lo P14 cells that had downregulated CD127

Figure 4.3: Early activated phenotype of naïve CD8 T cells seen as early as 12 hours post poly(I:C) inoculation
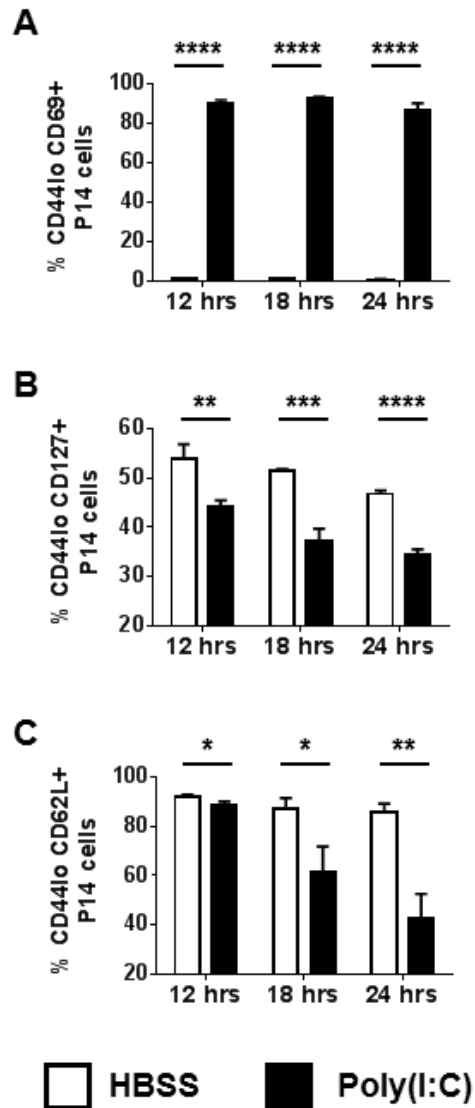
Figure 4.3: Early activated phenotype of naïve CD8 T cells seen as early as 12 hours post poly(I:C) inoculation

Congenic P14 cells were transferred into WT B6 mice, which were inoculated with HBSS or poly(I:C) for 12, 18, or 24 hours. Splenocytes were harvested and stained *ex vivo*. Frequencies of CD44lo P14 cells expressing CD69 (**A**), CD127 (**B**), and CD62L (**C**) are graphed. Data are representative of 2 individual experiments with n of 3 mice per group.

or CD62L at 12 hours post poly(I:C) treatment (black bars), but by 24 hours, a significant frequency of naïve P14 cells had reduced expression of these surface proteins when compared to controls (open bars) (**Figure 4.3B-C**).

The duration over which the altered phenotype of naïve CD8 T cells pre-exposed to poly(I:C)-induced environments was also determined. The modified phenotype, showing CD127 and CD62L downregulation and CD69 upregulation in CD44lo CD8 T cells, occurred through 2 days post-inoculation, but by 3 days post-treatment, the phenotype mostly resembled that of the control-treated counterparts (data not shown). These data indicate that poly(I:C) transiently induces naïve CD8 T cells to acquire an intermediately activated phenotype.

2. Poly(I:C)-primed naïve CD8 T cells upregulate effector-associated transcription factors

Upon activation, CD8 T cells express various transcription factors, which will allow for their differentiation into effector cells and ability to produce effector functions upon stimulation with cognate antigen. In the present study, I sought to determine the TF phenotype of poly(I:C)-primed naïve CD8 T cells. Representative histograms gated on CD44lo CD8α+ T cells are shown in **Figure 4.4A**, with HBSS-treated cells shown by shaded histograms and poly(I:C) by open histograms. The TCR-stimulated transcription factor IRF4 was found to be upregulated in CD44lo CD8 T cells after poly(I:C) treatment (**Figure 4.4B**). However, the CD8 effector-associated T-box TF Tbet remained largely

Figure 4.4: Naïve CD44lo CD8 T cells upregulate effector-associated transcription factors after poly(I:C) inoculation
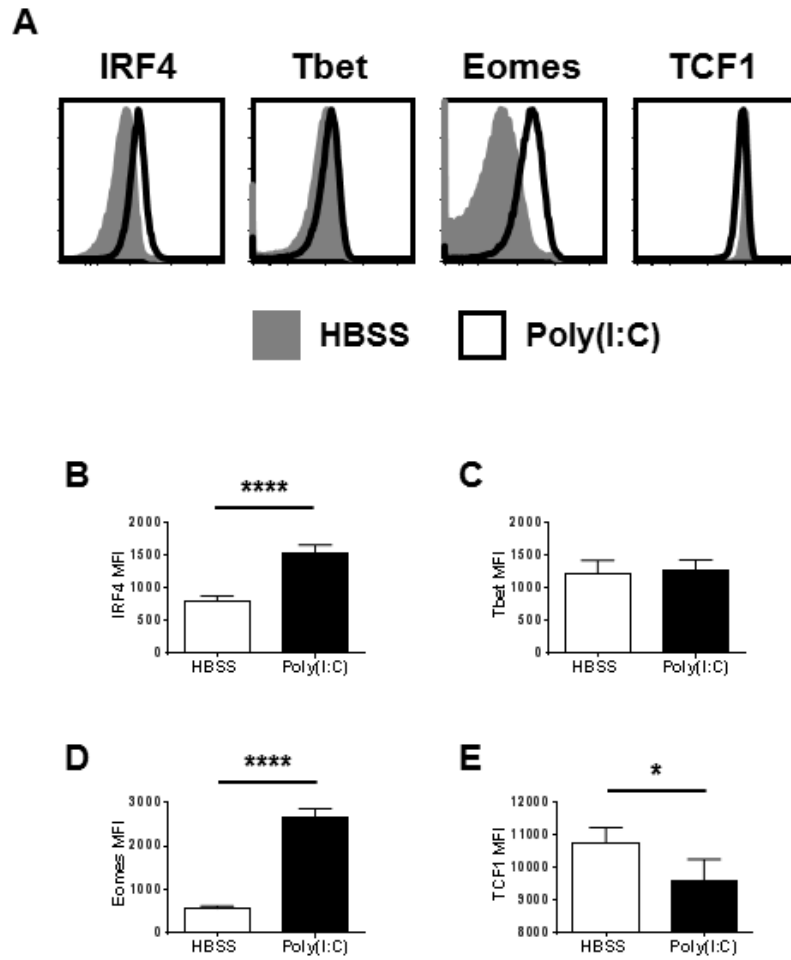
Figure 4.4: Naïve CD44lo CD8 T cells upregulate effector-associated transcription factors after poly(I:C) inoculation

WT B6 mice were inoculated with HBSS (shaded histograms) or poly(I:C) (open histograms), and splenocytes were harvested and stained for transcription factors 1 day later. **A**) Representative histograms gated on CD44lo CD8 T cells depicting IRF4, Eomes, Tbet, or TCF1 expression. **B-E**) CD44lo CD8 T cell MFI of IRF4 (**B**), Tbet (**C**), Eomes (**D**) and TCF1 (**E**). Data are representative from 2-5 individual experiments with n of 3-5 mice per group.

unchanged (**Figure 4.4C**), while the memory-associated TF Eomes was dramatically upregulated in naïve CD44lo CD8 T cells after poly(I:C) treatment (**Figure 4.4D**). Microarray data also showed expression of Eomes transcripts from poly(I:C)-treated CD44lo CD8 T cells was increased 3-fold over HBSS-treated controls (**Table 4.1**). Poly(I:C) treatment also induced a small reduction in TCF1 expression in naïve CD8 T cells (**Figure 4.4E**).

Our lab previously showed that indirect effects of type 1 IFN were required for bystander T cell sensitization to rapid effector function. Therefore, I wanted to identify if the upregulation of effector-associated TFs had similar requirements for IFN. Congenic P14 CD8 T cells were transferred into WT or IFNAR KO host mice, which were subsequently inoculated with HBSS or poly(I:C). After 1 day of *in vivo* treatment, the CD44lo donor P14 cells transferred into the IFNAR KO host did not upregulate IRF4 and Eomes to the same extent as P14 cells that were poly(I:C)-primed (black bars) in WT hosts (**Figure 4.5 A-B**). Moreover, host naïve CD8 T cells showed no increase in frequency of cells expressing the effector-associated TFs IRF4 or Eomes after poly(I:C) inoculation (**Figure 4.5 C-D**). These data indicate that IFN acts directly on CD8 T cells to upregulate IRF4 and Eomes. That being said, interpretations of these results may be more complicated, considering that in the IFNAR KO hosts, less IFN is being induced, and this may be a contributing factor to the observed phenotype.

Combined, these results are somewhat perplexing, considering that IRF4 has been shown to promote Tbet expression and inhibit Eomes induction

Figure 4.5: Type 1 IFN is required for upregulation of effector-associated transcription factors IRF4 and Eomes
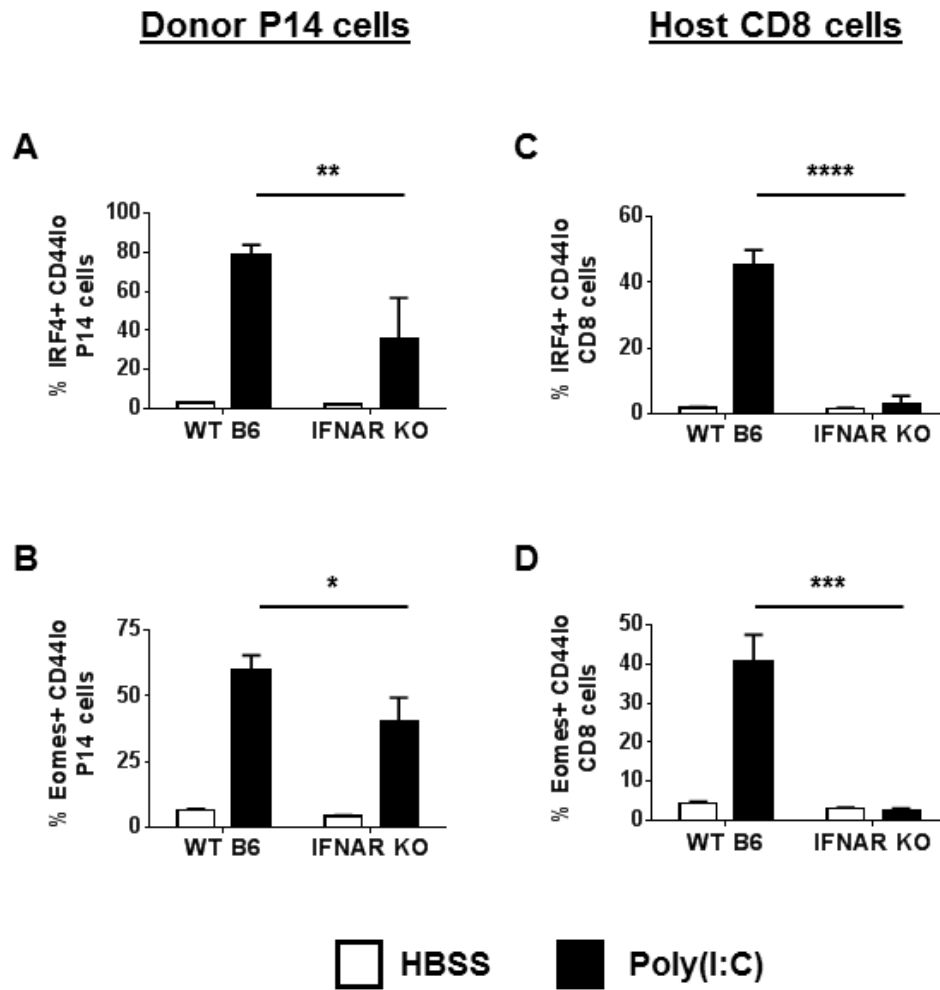
Figure 4.5: Type 1 IFN is required for upregulation of effector-associated transcription factors IRF4 and Eomes

Congenic P14 cells were transferred into WT B6 hosts or IFNAR KO hosts, which were inoculated with HBSS or poly(I:C). One day later splenocytes were harvested and stained. Frequencies of CD44lo P14 cells expressing IRF4 (**A**) or Eomes (**B**) and frequencies of CD44lo host CD8 T cells expressing IRF4 (**C**) or Eomes (**D**) are graphed. Individual experiment with n of 3-4 mice per group.

(148, 151, 153, 156). Therefore, I wanted to determine if IRF4 was upregulated before or after Eomes induction. Mice that had received congenic P14 cells were inoculated with HBSS (open bars) or poly(I:C) (black bars), and splenocytes were harvested at 12, 18 and 24 hours post-treatment. In donor CD44lo P14 CD8 T cells, Eomes expression was greatest at the earliest time point tested, whereas IRF4 expression, albeit upregulated, was lowest at the 12-hour time point (**Figure 4.6 A-B**). The kinetics of Eomes and IRF4 expression in donor naïve P14 cells was recapitulated in the endogenous host CD44lo CD8 T cell population (**Figures 4.6C-D**), where at 12 hours post poly(I:C) treatment, the lowest frequencies of IRF4+ cells were observed, but the highest frequencies of Eomes+ cells were observed. Considering that the frequency of Eomes+ cells declined slightly at later time points, but IRF4 levels increased, these data are consistent with previous findings that IRF4 expression can repress Eomes expression (203). However, because we did not see a difference in Tbet expression, results are inconsistent with IRF4 regulating Tbet in this setting. However, some experiments performed showed that a small proportion of CD44lo CD8 T cells upregulated Tbet after poly(I:C) treatment, but results were not very consistent, and expression levels were very low. However, it should be noted, that IRF4 is not the only TF that has been linked to Eomes and Tbet expression.

3. **Poly(I:C)-primed naïve CD8 T cells produce immediate effector functions upon stimulation with cognate antigen stimulation**

Figure 4.6: CD44lo CD8 T cells upregulate IRF4 and Eomes as early as 12 hours post poly(I:C) priming
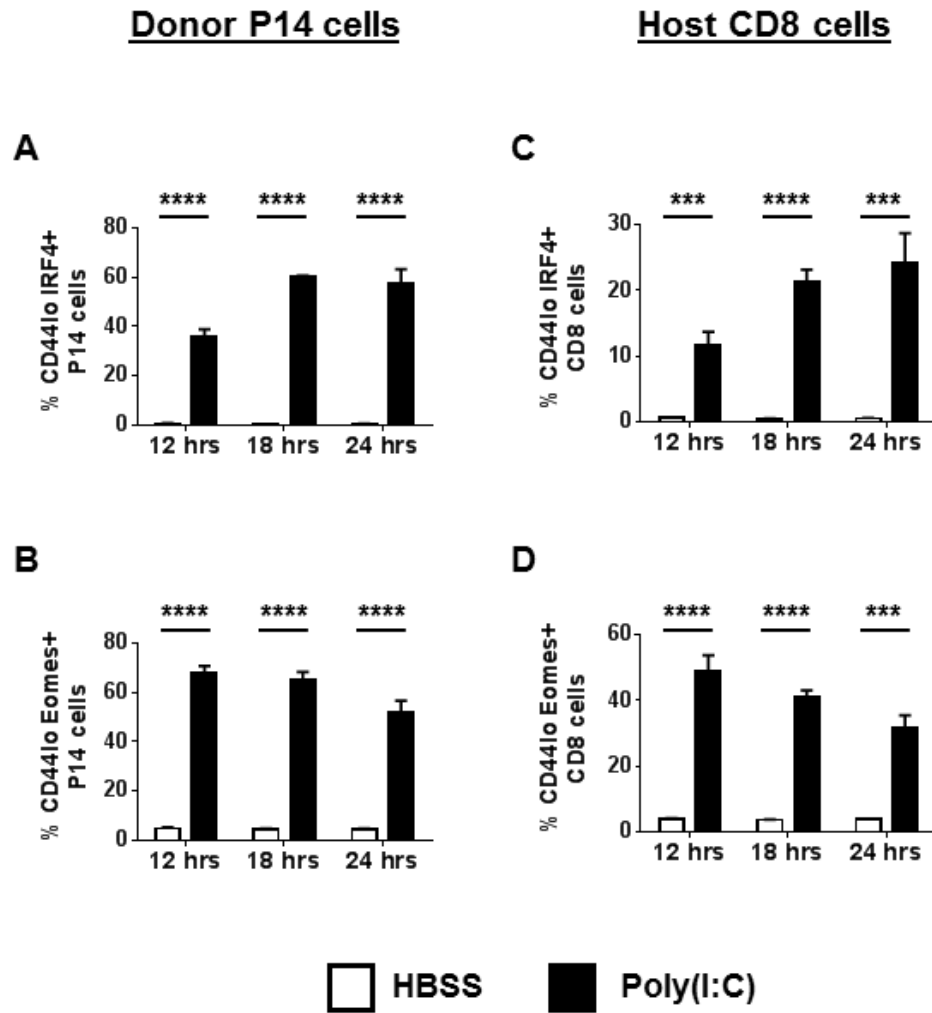
Figure 4.6: CD44lo CD8 T cells upregulate IRF4 and Eomes as early as 12 hours post poly(I:C) priming

Congenic P14 cells were transferred into WT B6 mice, which were inoculated with HBSS (open bars) or poly(I:C) (black bars). Splenocytes were harvested and stained 12, 18, or 24 hours later. Frequencies of CD44lo IRF4- (**A**) or Eomes- (**B**) expressing donor P14 cells are graphed. Frequencies of CD44lo IRF4- (**C**) or Eomes- (**D**) expressing host CD8 T cells are graphed. Data are from at least 1 experiment with 3 mice per group.

Recent studies from our lab showed that bystander CD8 T cells could rapidly produce effector functions in response to cognate antigen stimulation if pre-exposed to virus-induced inflammatory environments (193). The present study confirms previous results where P14 CD8 T cells produced immediate effector function in response to cognate antigen stimulation if pre-exposed to poly(I:C) *in vivo*. First, microarray data show that granzyme B expression is highly induced (>25 fold) in naïve CD8 T cells after poly(I:C) inoculation compared to control-treated naïve CD8 T cells (**Table 4.1**). Additional cytolytic-associated effector molecules were found to be increased in poly(I:C)-treated cells over controls including granzyme A, granulin, and TRAIL (or Tnfsf10). However, not all of the changes observed at the transcript level have been corroborated at the protein level, but granzyme B protein levels were elevated. Congenic P14 splenocytes were transferred into B6 mice, which were inoculated with HBSS or poly(I:C) for 1 day, and stimulated *ex vivo* with GP33 for 4-5 hours during an ICS assay. **Figure 4.7A-C** depicts poly(I:C)-treated CD44lo P14 CD8 T cells degranulating and secreting granzyme B upon *ex vivo* stimulation with their cognate ligand GP33. Representative histograms of poly(I:C)-treated CD44lo P14 cells (open histograms) show increased degranulation, as measured by CD107a/b staining, and release of granzyme B, over control-treated cells (shaded histograms) (**Figure 4.7A**). Quantification of the frequency of CD44lo P14 cells (control-treated represented as open bars and poly(I:C)-treated represented as black bars) producing CD107a/b and granzyme B are shown in

Figure 4.7: Poly(I:C)-pretreated CD44lo P14 CD8 T cells produce immediate effector functions upon stimulation with cognate ligand
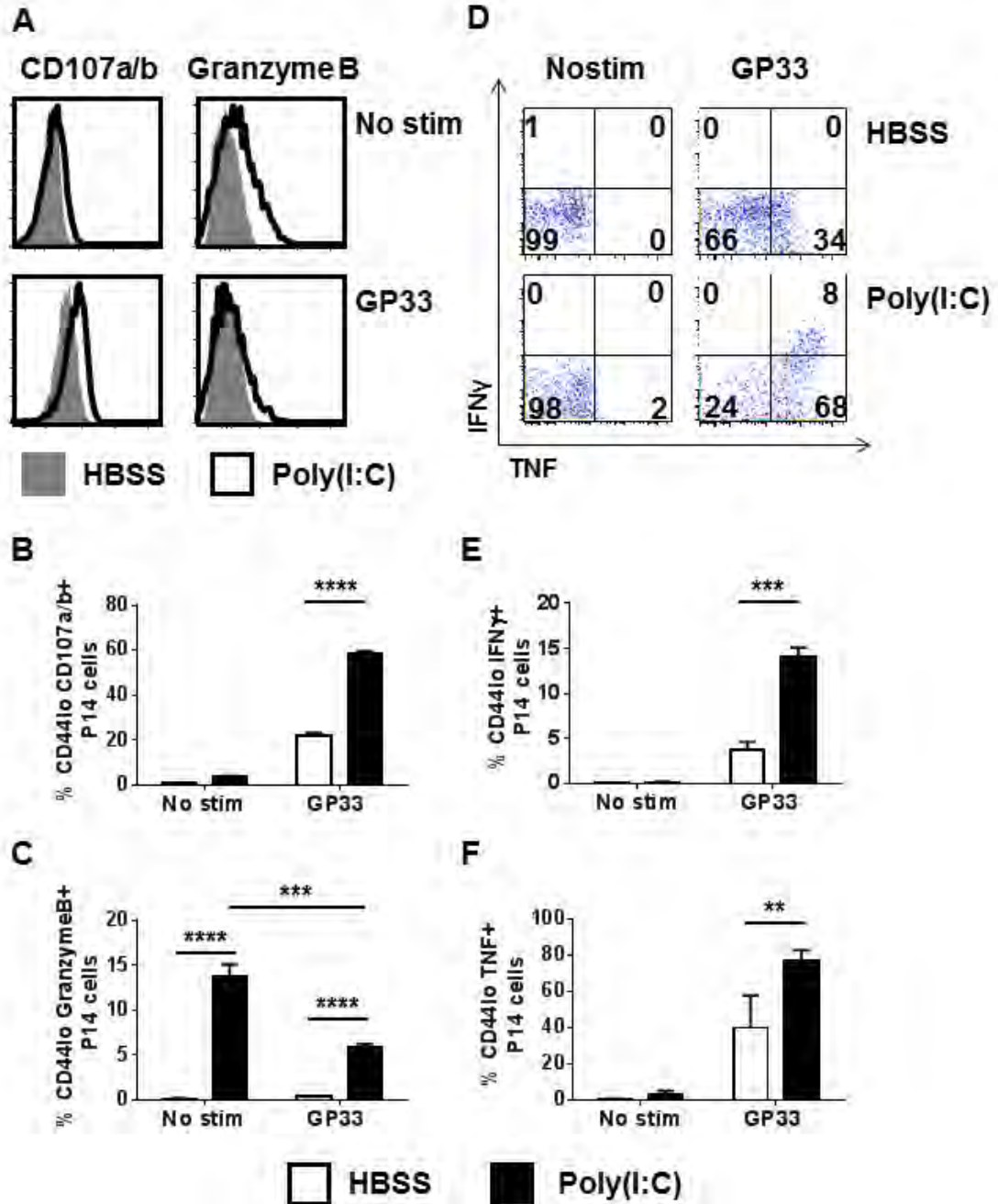
Figure 4.7: Poly(I:C)-pretreated CD44lo P14 CD8 T cells produce immediate effector functions upon stimulation with cognate ligand

Congenic P14 splenocytes were transferred into WT B6 mice, which were subsequently inoculated with HBSS or poly(I:C). One day later spleens were harvested, and cells were stimulated *ex vivo* with no peptide or GP33 for ~4-5 hours, then stained for effector molecules. **A**) Representative flow plots gated on donor CD44lo P14 CD8 T cells from HBSS- (shaded histograms) or poly(I:C)- (open histograms) treated mice showing CD107a/b and granzyme B expression. Frequencies of CD44lo P14 T cells producing CD107a/b (**B**) and granzyme B (**C**) from poly(I:C)- (black bars) and HBSS- (open bars) treated mice are graphed. **D**) Representative flow plots gated on CD44lo P14 T cells are depicted. Frequencies of CD44lo P14 T cells from HBSS- (open bars) and poly(I:C)- (black bars) treated mice producing IFNγ (**E**) and TNF (**F**) are graphed. Data are representative of 3-7 individual experiments with n of 3-5 mice per group.

Figures 4.7B and C, respectively. It should be noted that poly(I:C)-treated naïve P14 cells without *ex vivo* stimulation (No stim) show enhanced granzyme B expression, and upon stimulation with high-affinity ligand show a significant loss in granzyme B staining. Combined with the increased degranulation markers, this suggests that granzyme B is being released or secreted.

In addition to degranulation, poly(I:C)-pretreated CD44lo P14 cells also produced IFNγ and enhanced TNF in response to *ex vivo* stimulation with cognate ligand (**Figure 4.7 D-E**). Representative flow plots gated on CD44lo P14 cells from HBSS- or poly(I:C)-pretreated mice that remained unstimulated, or stimulated with GP33 *ex vivo*, are illustrated (**Figure 4.7D**). The proportion of CD44lo P14 cells expressing IFNγ (**Figure 4.7E**) or TNF (**Figure 4.7F**) from control- (open bars) or poly(I:C)- (black bars) pretreated mice are graphed. The pre-exposure of naïve CD8 T cells to IFN-induced environments primes them to be immediate effectors upon stimulation with high affinity cognate ligand such that they produce multiple cytokines.

To ensure that the enhanced effector function of naïve CD8 T cells from inflammatory environments was not due to an artifact of the P14 transgenic population, other transgenic T cells and endogenous polyclonal T cells were observed. Congenic OT-I splenocytes were transferred into B6 hosts and were assessed in a similar manner as P14 cells in **Figure 4.7**. Upon stimulation with high affinity cognate ligand (OVA), increased frequencies of poly(I:C)-pretreated CD44lo OT-I cells (black bars) produced IFNγ **(Figure 4.8A)** and enhanced TNF

(**Figure 4.8B**) compared to their control-treated counterparts (open bars). OT-I cells are another transgenic monoclonal population, therefore, I wanted to determine if polyclonal CD44lo CD8 T cells also showed immediate effector function. The endogenous host naïve CD44lo CD8 T cells were stimulated *ex vivo* with anti-CD3 and assessed for IFNγ and TNF production. **Figure 4.8C** shows increased frequencies of CD44lo CD8 T cells producing IFNγ in response to anti-CD3 stimulation in poly(I:C)-pretreated populations (black bars) compared to controls (open bars). Naïve CD8 T cells had already been shown to produce TNF in response to anti-CD3 stimulation or cognate Ag stimulation *ex vivo* (161). Data shown here depict increased proportions of TNF-producing CD44lo CD8 T cells from poly(I:C)- vs HBSS-pretreated mice (**Figure 4.8D**). Combined, these data show that transgenic P14 and OT-I CD8 T cells along with polyclonal CD44lo CD8 T cells are susceptible to poly(I:C)-induced priming and acquire the ability to produce effector functions immediately upon cognate antigen stimulation.

Previous studies in our lab showed that indirect effects of type 1 IFN were important for P14 cell production of IFNγ after poly(I:C) inoculation. To recapitulate these studies, and also to identify if degranulation and/or additional effector functions have similar requirements, WT P14 cells were transferred into WT B6 or IFNAR KO hosts and primed with poly(I:C). After *in vivo* treatment, splenocytes were harvested, and an ICS was performed. Unstimulated CD44lo P14 cells primed in WT host mice showed increased frequencies of granzyme B+

Figure 4.8: Naïve CD44lo CD8 T cells acquire immediate effector function after poly(I:C) priming
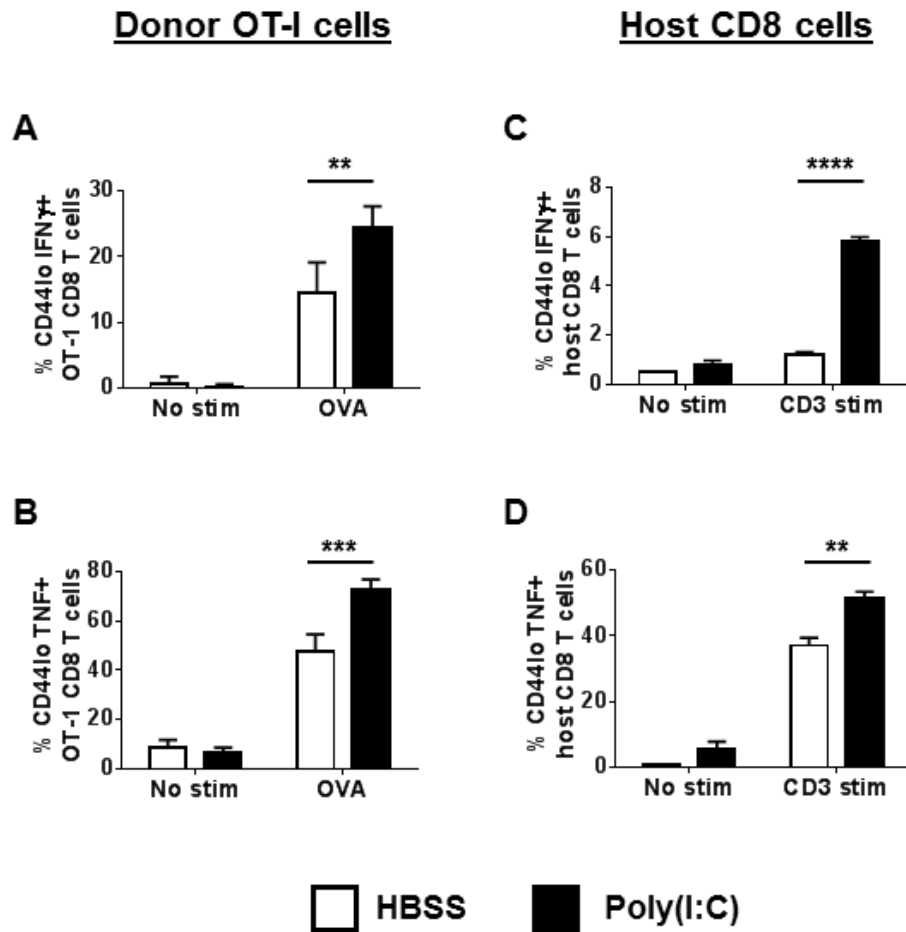
Figure 4.8: Naïve CD44lo CD8 T cells acquire immediate effector function after poly(I:C) priming

OT-I splenocytes were transferred into WT B6 mice, which were subsequently inoculated with HBSS or poly(I:C). One day later, spleens were harvested and cells were stimulated *ex vivo* with no peptide, anti-CD3, or OVA for ~4-5 hours and stained for cytokines. Frequency of donor CD44lo OT-I CD8 T cells from HBSS- (open bars) and poly(I:C)- (black bars) inoculated animals producing IFNγ (**A**) and TNF (**B**) are graphed. **C-D**) Frequencies of host CD44lo CD8 T cells from HBSS- (open bars) and poly(I:C)- (black bars) inoculated animals producing IFNγ (**C**) and TNF (**D**) are graphed. Data are representative of at least 4 experiments with n of 3-5 mice per group.

P14 cells, but if P14 cells were primed in the IFNAR KO host, granzyme B was not upregulated (**Figure 4.9A**). The ability of CD44lo P14 cells to degranulate, as measured by CD107a/b staining, and to produce IFNγ was also reduced if T cells were primed in IFNAR KO hosts compared to WT hosts (**Figure 4.9B-C**). Because there was still some degranulation and IFNγ production from poly(I:C)-primed CD44lo P14 cells from IFNAR KO hosts, this suggests that in addition to type 1 IFN indirectly activating cells, that it may also have some direct effects. However, these studies may be difficult to interpret because of the assumed reduced levels of type 1 IFN in the KO hosts.

We then questioned how early naïve CD8 T cells acquire the immediate effector function after exposure to poly(I:C)-induced inflammatory environments. P14 cells were transferred into mice that were inoculated with HBSS or poly(I:C) and harvested at 12, 18, or 24 hours post-inoculation. Directly *ex vivo*, poly(I:C)-expressed high levels of granzyme B compared to control-treated P14 cells (open bars) at all time points tested (**Figure 4.10A**). Upon stimulation with high affinity cognate ligand during an ICS assay, poly(I:C)-pretreated P14 cells (black bars) produced significant amounts of IFNγ as early as 12 hours post-inoculation (**Figure 4.10B**).

Previous studies conducted by our lab determined that the poly(I:C) priming of naïve T cells into immediate effectors is a transient effect (193). T cells were in the poly(I:C)-primed environment for 1, 2, or 3 days before assessing their capability of producing IFNγ to cognate antigen stimulation. Effector function

Figure 4.9: Poly(I:C)-induced immediate effector function requires type 1 IFN signaling in the priming environment
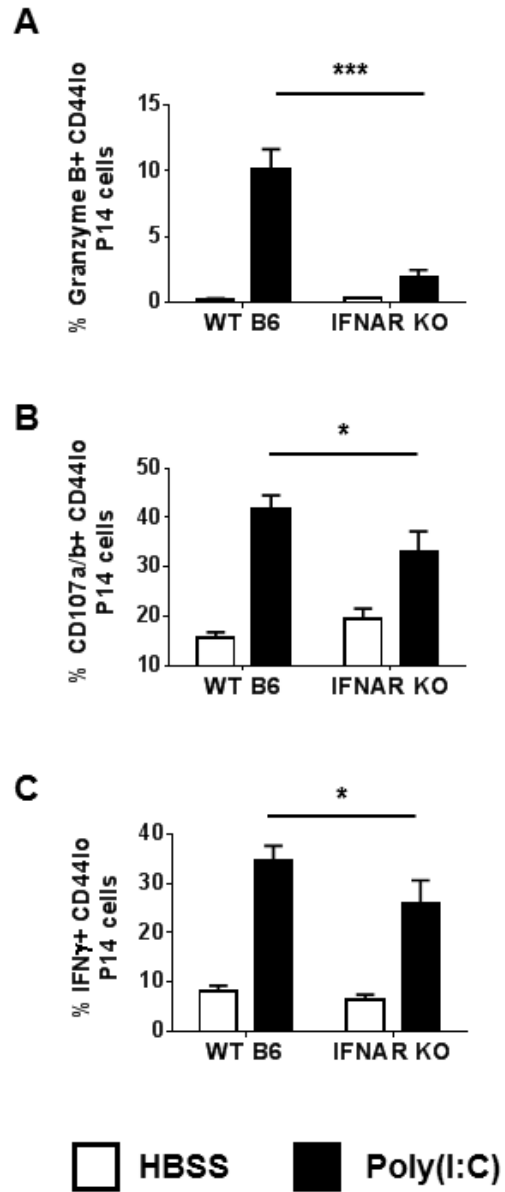
Figure 4.9: Poly(I:C)-induced immediate effector function requires type 1 IFN signaling in the priming environment

Congenic P14 cells were transferred into WT B6 hosts or IFNAR KO hosts, which were inoculated with HBSS or poly(I:C). One day later splenocytes were harvested and were either stimulated without peptide (**A**) or with GP33 (**B-C**) for ~5 hours. Frequency of CD44lo P14 cells expressing granzyme B (**A**), CD107a/b (**B**), or IFNγ (**C**) are graphed. Individual experiment with n of 3-4 mice per group.

Figure 4.10: Naïve CD8 T cells acquire immediate effector function as early as 12 hours post poly(I:C) inoculation
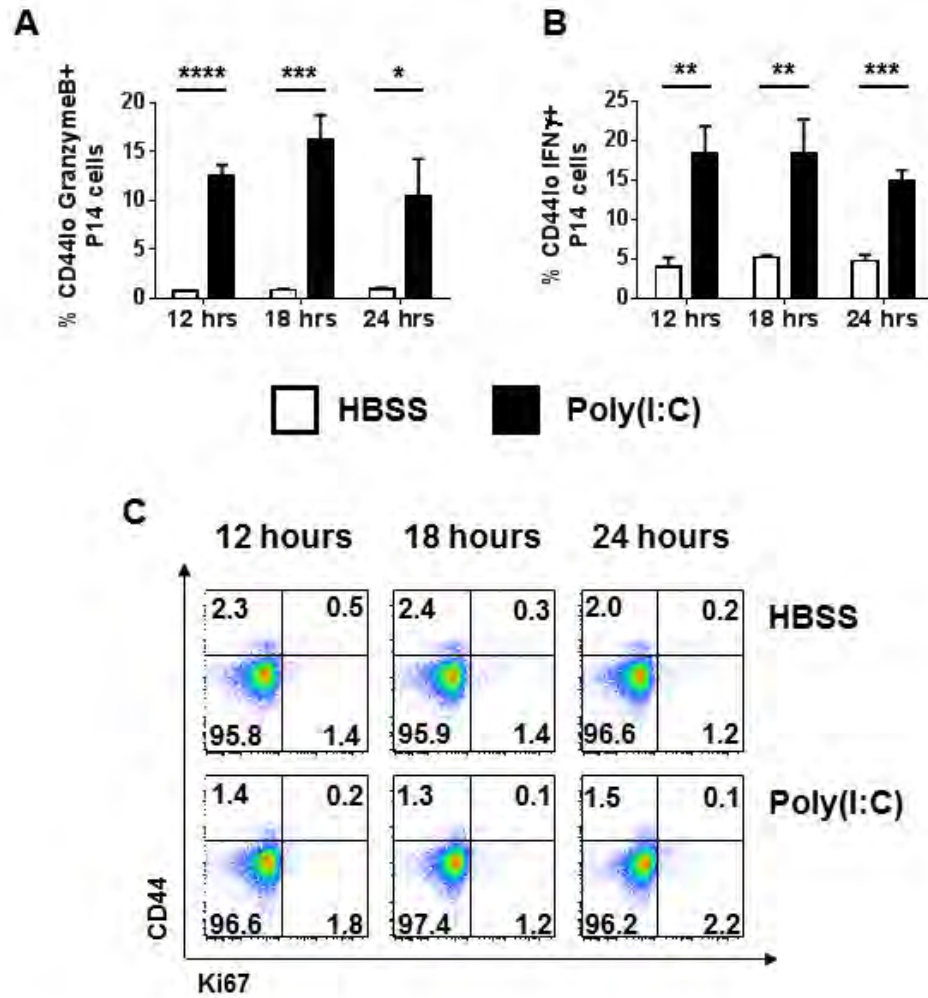
Figure 4.10: Naïve CD8 T cells acquire immediate effector function as early as 12 hours post poly(I:C) inoculation

Congenic P14 cells were transferred into WT B6 mice, which were inoculated with HBSS or poly(I:C) for 12, 18, or 24 hours. Splenocytes were harvested and stained *ex vivo* (**A, C)** or stimulated with GP33 for an ICS assay (**B**). Frequencies of CD44lo P14 cells expressing granzyme B (**A**), or IFNγ (**B**) from HBSS- (open bars) or poly(I:C)- (black bars) treated mice are graphed. Representative flow plots gated on P14 CD8 T cells expressing Ki67 from poly(I:C)- or HBSS-treated mice are graphed (**C**). Data are representative of 1-2 individual experiments with 3 mice per group.

was greatest with 1 day of priming, and decreased thereafter, where by day 3 post priming with poly(I:C), donor P14 cells only modestly induced IFNγ in response to GP33 stimulation *ex vivo*.

Our lab also identified that bystander P14 CD8 T cells did not divide during a PV infection shown by using CFSE dilution assays. To identify if poly(I:C) treatment induced division of CD8 T cells, the proliferation marker Ki67 was used. Ki67 can be detected in cells that are in a non-resting state or that are actively at some point in the cell cycle. However, the phase of the cell cycle in which a Ki67 positive cell exists is not known. **Figure 4.10C** shows that poly(I:C)-treated P14 cells do not upregulate Ki67 at 12, 18, or 24 hours post treatment, indicating that they are not dividing, nor have they exited the resting G_0 state.

4. Summary

During the course of an immune response, antigen-specific CD8 T cells will divide, clonally expand, and differentiate into effector cells. Effector cells have the capability of producing multiple cytokines in response to antigen simulation. However, the acquisition of those effector functions is thought to require CD8 T cell division. In the present study, we find that naïve CD8 T cells gain the ability to respond immediately to cognate antigen stimulation and produce effector functions if they are pre-exposed to a poly(I:C)-induced inflammatory environment. Naïve CD8 T cells exposed to poly(I:C) for as little as 12 hours will upregulate surface proteins associated with early activated T cells including

CD69 and CD86 while remaining CD44lo (**Figure 4.1-4.3**). Moreover, poly(I:C)-pretreated CD44lo CD8 T cells downregulated IFNAR1, CD127 and to a lesser extent CD62L (**Figure 4.1-4.3**). This change in phenotype of poly(I:C)-primed CD8 T cells resembles an early, intermediately activated CD8 T cell, whilst still remaining CD44lo, and having never seen cognate antigen.

In addition to altering the phenotype of naïve CD8 T cells, pre-exposure to poly(I:C) alters the TF profile of naïve CD8 T cells (**Figure 4.4**). IRF4 was upregulated in poly(I:C)-treated naïve CD8 T cells, indicating TCR signaling occurred, even without the presence of cognate antigen (**Figure 4.4-4.6**). The T-box TF Eomes, which is normally associated with memory CD8 T cells, was upregulated in poly(I:C)-primed CD44lo CD8 T cells as early as 12 hours post poly(I:C) treatment (**Figure 4.6**). Upregulation of Eomes is thought to be one factor that allows for the immediate cytokine secretion in primed naïve CD8 T cells.

Poly(I:C)-pre-exposed naïve CD8 T cells gained the ability to respond immediately to cognate antigen stimulation and showed signs of degranulation, release of granzyme B, production of IFNγ, and enhanced TNF production (**Figure 4.7-4.10**). Poly(I:C) "primes" naïve CD44lo CD8 T cells to respond to cognate antigen immediately, without the need to divide (**Figure 4.10**). The poly(I:C)-pretreated CD8 T cells maintained a naïve phenotype but behaved more similar to a memory T cell rather than a naïve CD8 T cell.

C. Cell types that mediate IFN priming

1. Hematopoietic cells mediate poly(I:C)-induced priming

The present study addressed the cell type that was mediating poly(I:C)-induced priming. Bone marrow chimeras were generated in which either WT B6, or H2-Db KO mice were lethally irradiated (950rads) and then received BM from the opposite recipient. WT B6 mice received H2-Db KO BM (KO BM), so that in these mice only the radio-resistant cells will have H2-Db on the surface, and cells of the hematopoietic compartment will lack surface H2-Db. H2-Db KO mice receiving WT BM (WT BM), conversely, will have surface H2-Db on cells of the hematopoietic lineage. After ~6 weeks of re-constitution, congenic transgenic P14 cells were transferred into each of the chimeric mice, which were inoculated with either HBSS or poly(I:C) the following day. After 1 day of *in vivo* treatment, splenocytes were harvested and stained. P14 cells from the mice that received KO BM did not upregulate the early activation marker CD69 to the same extent as P14 cells from mice that received WT BM (**Figure 4.11A**). Since CD69 was one of the phenotypic markers associated with primed naïve effectors, this suggested that self-MHC was required in the hematopoietic compartment to mediate priming. Additionally, P14 cells from mice receiving KO BM also showed lower upregulation of the T-box TF Eomes (**Figure 4.11B**), a TF shown to be upregulated in primed naïve T cells. Together, these data suggest that H2-Db

Figure 4.11: WT hematopoietic cells mediate poly(I:C)-induced CD8 T cell priming
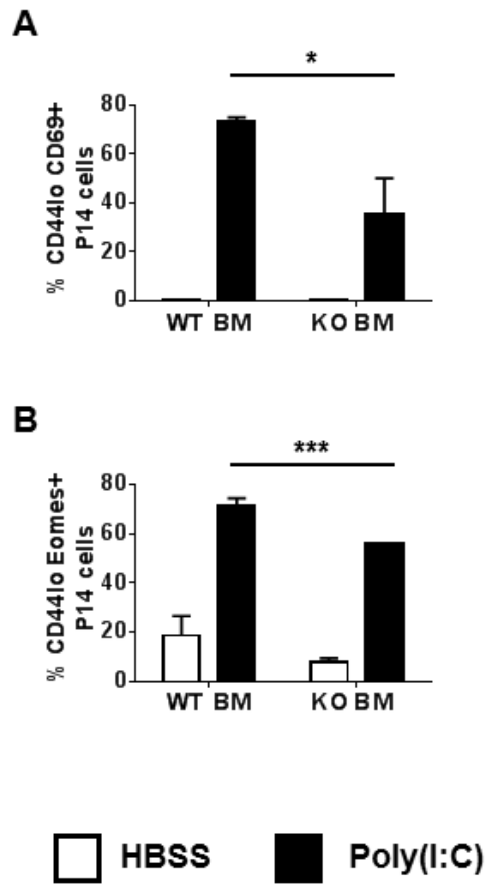
Figure 4.11: WT hematopoietic cells mediate poly(I:C)-induced CD8 T cell priming

Bone marrow chimeras were generated in which either WT BM was transferred into irradiated β2M KO hosts, or β2M KO BM was transferred into irradiated WT B6 hosts. After reconstitution for >6 weeks, congenic P14 cells were transferred into chimeras, which were control-treated with HBSS, or treated with poly(I:C). One day later splenocytes were harvested and stained. The frequency of CD44lo P14 cells expressing CD69 (**A**) and Eomes (**B**) from HBSS- (open bars) or poly(I:C)- (black bars) treated BM chimeras is graphed. Data are representative of 2 individual experiments with n of 2-3 mice per group.

expression on cells of the hematopoietic compartment mediates poly(I:C)-induced priming of naïve CD8 T cells.

2. B7 costimulation is required for poly(I:C)-induced priming of naïve CD8 T cells

After identifying that self-MHC on hematopoietic cells is important, we wanted to identify the cell type that was mediating poly(I:C)-induced priming. We hypothesized that professional APCs were required for poly(I:C)-induced priming. Because expression of costimulatory molecules can distinguish APCs from other cell types that express class I MHC, I determined whether or not the CD40-CD40L interaction was required for T cell priming. P14 cells were transferred into WT B6 hosts and mice either received an anti-CD40L blocking antibody (MR1) or did not receive the antibody treatment. After 1 day, mice were primed with poly(I:C) or control treated with HBSS for 1 day prior to splenocytes isolation and staining. The frequencies of CD44lo P14 cells that upregulate Eomes were similar between mice irrespective of blocking the CD40-CD40L interaction after poly(I:C) treatment (**Figure 4.12A**). In addition, similar frequencies of CD44lo P14 cells upregulated granzyme B after priming with poly(I:C), whether or not they were treated with MR1 blocking antibody (**Figure 4.12B**). Lastly, upon stimulation with cognate antigen, poly(I:C)-primed naïve P14 cells degranulated (**Figure 4.12C**), produced IFNγ (**Figure 4.12D**), and enhanced TNF (**Figure 4.12E**) despite blocking the CD40-CD40L interaction. Together these results

Figure 4.12: Blocking CD40-CD40L does not affect poly(I:C)-induced naïve CD8 T cell acquisition of immediate effector function
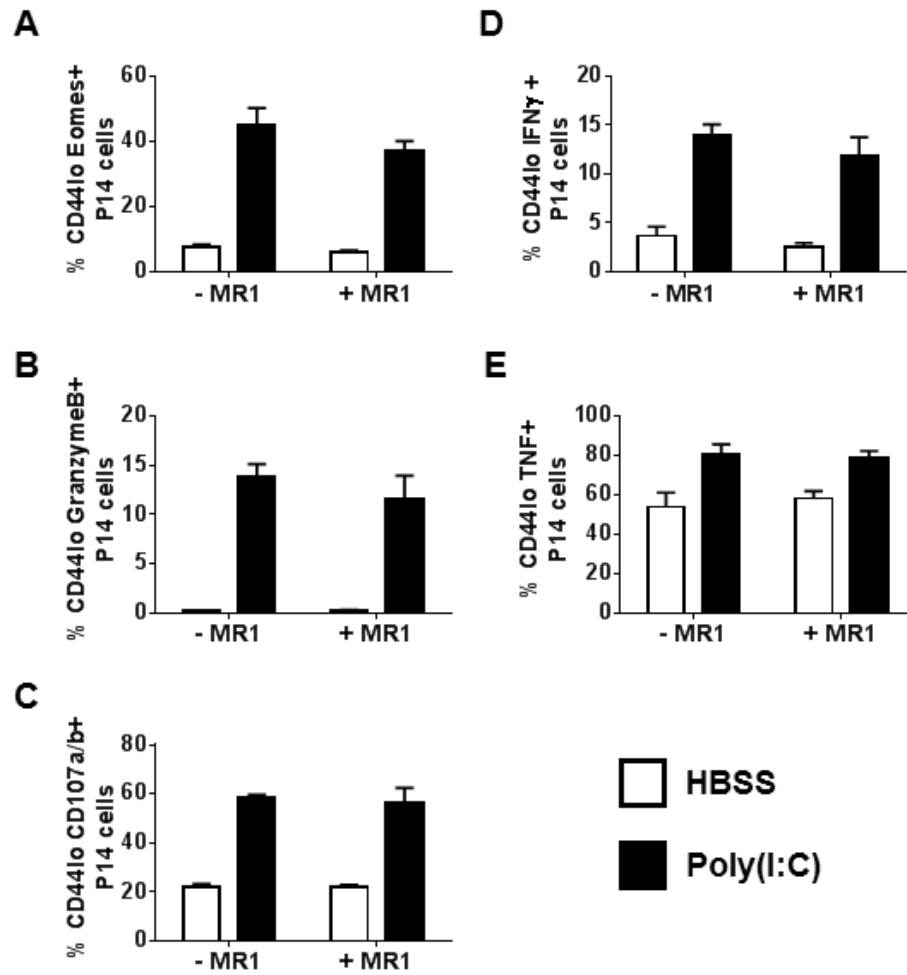
Figure 4.12: Blocking CD40-CD40L does not affect poly(I:C)-induced naïve CD8 T cell acquisition of immediate effector function

P14 CD8 T cell splenocytes were transferred into WT B6 mice, which either did or did not receive anti-CD40L blocking antibody (MR1). One day after treatment, mice were control treated (HBSS) or inoculated with poly(I:C), and spleens were harvested 1 day later. Splenocytes remained unstimulated *ex vivo* (**A-B**) or were stimulated *ex vivo* with GP33 for ~4-5 hours (**C-E**). Frequencies of donor CD44lo P14 cells from HBSS-treated (open bars) or poly(I:C)-treated (black bars) mice expressing Eomes (**A**), granzyme B (**B**), CD107a/b (**C**), IFNγ (**D**) or TNF (**E**) are depicted. Data are representative from 4 individual experiments with n of 3-4 mice per group.

indicate that the costimulation between CD40 and CD40L is not required for naïve T cell priming.

Since naïve CD8 T cells require both TCR and costimulation in order to gain effector function and to avoid becoming anergic (99), I questioned whether the canonical signal 2 costimulation was required for naive CD8 T cell priming. To determine if B7-CD28 costimulation is required for poly(I:C)-induced T cell priming, WT congenic P14 cells were transferred into B7.1/B7.2 KO mice (referred to as B7 KO) or WT B6 mice. Mice were inoculated with HBSS or poly(I:C) and harvested 1 day later. Naïve P14 CD8 T cell upregulation of Eomes was variable from experiment to experiment. In one experiment (exp #1), P14 cells from B7 KO showed no difference in Eomes upregulation compared to P14 cells from WT host after poly(I:C) treatment. However, in other experiments (exp #2), poly(I:C)-treated P14 cells from B7 KO hosts had lower levels of Eomes compared to poly(I:C)-treated P14 cells from WT hosts (**Figure 4.13A**). CD44lo P14 cells primed in the B7 KO environment did not produce IFNγ like cells primed in the WT environment (**Figure 4.13B**). To confirm that lack of costimulation during the *ex vivo* stimulation was not the reason for reduced IFNγ production in poly(I:C)-primed cells from KO mice, B7-sufficient cells were added to the *ex vivo* stimulated cultures. The frequencies of CD44lo IFNγ+ P14 cells from WT or KO priming environments that either did not receive any additional cells during ICS (No cells), received HBSS-treated splenocytes, or received poly(I:C)-treated splenocytes during the ICS assay are shown in **Figure 4.13C**.

Figure 4.13: Poly(I:C) priming of naive CD8 T cells requires B7 costimulation
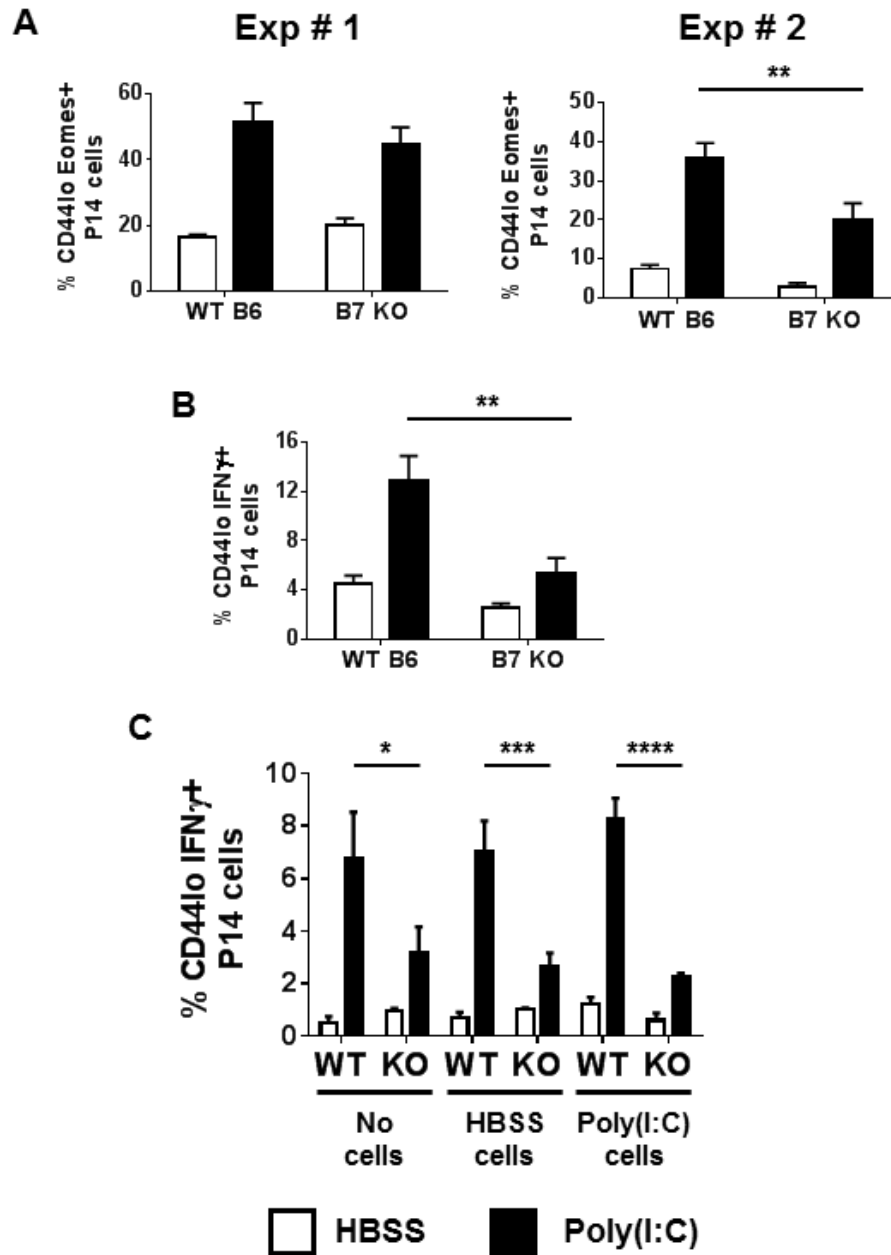
Figure 4.13: Poly(I:C) priming of naive CD8 T cells requires B7 costimulation

Congenic P14 cells were transferred into WT B6 mice or B7 KO mice, which were inoculated with HBSS or poly(I:C). One day later, splenocytes were isolated and stained directly *ex vivo* (**A**) or stimulated with GP33 (**B-C**). Frequency of CD44lo P14 cells expressing Eomes (**A**) or IFNγ (**B-C**) is graphed. (**C**) Splenocytes were stimulated in the absence of extra cells, the presence of WT HBSS-pretreated splenocytes or WT poly(I:C)-pretreated splenocytes. Data are representative of 3 Experiments (**A-B**) with n of 3-4 Mice per group or an individual experiment with n of 3-4 mice per group (**C**).

The presence of B7-sufficient cells during *ex vivo* stimulation did not allow for P14 cells primed in the B7 KO host to immediately synthesize IFNγ. Thus, B7 costimulation may play a minor role in Eomes upregulation, but it is required for IFNγ production from poly(I:C)-primed naïve CD8 T cells. Therefore, Eomes upregulation may be only part of the equation that allows naïve CD8 T cells to elicit immediate effector function.

3. Poly(I:C)-induced priming of naïve CD8 T cells requires TAP

Self-MHC was determined to be required for sensitization of T cells to exert rapid effector function (193). However, the specificity of the particular peptides presented was never addressed. I questioned whether any peptide could mediate poly(I:C)-induced priming or whether more specific peptides were required for this process. I studied two different KO strains, which limited the peptide repertoire that was presented. First, I made use of immunoproteasome KO mice (3 KO), in which the 3 catalytic subunits of the Immunoproteasome (LMP2, LMP7, and MECL-1) are knocked out (70). Therefore, any peptide that requires these specific catalytic subunits to be processed and presented will not be expressed. 3 KO mice have ~50% reduced MHC Class I expression on the cell surface and a different peptide repertoire compared to WT mice (70). If a specific peptide that is processed by the immunoproteasome was required for poly(I:C)-induced priming, CD8 T cells would not be primed in the 3 KO environment. To limit the peptide repertoire even further, TAP1/TAP2 KO (TAP

KO) mice were used. During MHC Class I presentation, TAP assists in peptide trafficking into the ER for loading onto MHC Class I complexes (204). Therefore, without TAP, only peptides that can be transported and loaded in the absence of TAP will be presented on the cell surface. Since the majority of MHC Class I peptides require TAP for presentation, this severely limits the repertoire of peptides presented and also limits the total amount of Class I on the cell surface.

First, I determined if CD44lo P14 cells would upregulate the priming-associated early activation markers and the transcription factors shown in Chapter IV, Section B. Representative histograms gated on CD44lo P14 cells from WT, 3 KO, and TAP KO mice treated with HBSS (shaded histograms) or poly(I:C) (open histograms) expressing CD69, Eomes, and IRF4 are depicted in **Figure 4.14A.** The frequency of CD44lo P14 cells expressing these poly(I:C)-induced markers are shown in **Figures 4.14B-D,** where poly(I:C)-pretreated cells are shown as black bars, and HBSS-treated cells are shown as open bars. CD44lo P14 cells primed in 3 KO hosts upregulated CD69, Eomes, and IRF4 to a similar extent as those primed in WT hosts. However, priming in TAP KO hosts resulted in impaired upregulation of CD69 (**Figure 4.14B**), Eomes (**Figure 4.14C**), and IRF4 (**Figure 4.14D**) compared to P14 cells primed in the WT hosts (statistics comparing WT B6 poly(I:C) vs. TAP KO poly(I:C)).

An ICS assay using GP33 peptide stimulation was performed to determine if poly(I:C)-primed CD44lo P14 cells could produce immediate effector function if primed in either the 3 KO or TAP KO environments. Representative flow plots

Figure 4.14: Poly(I:C)-induced early activation phenotype requires TAP
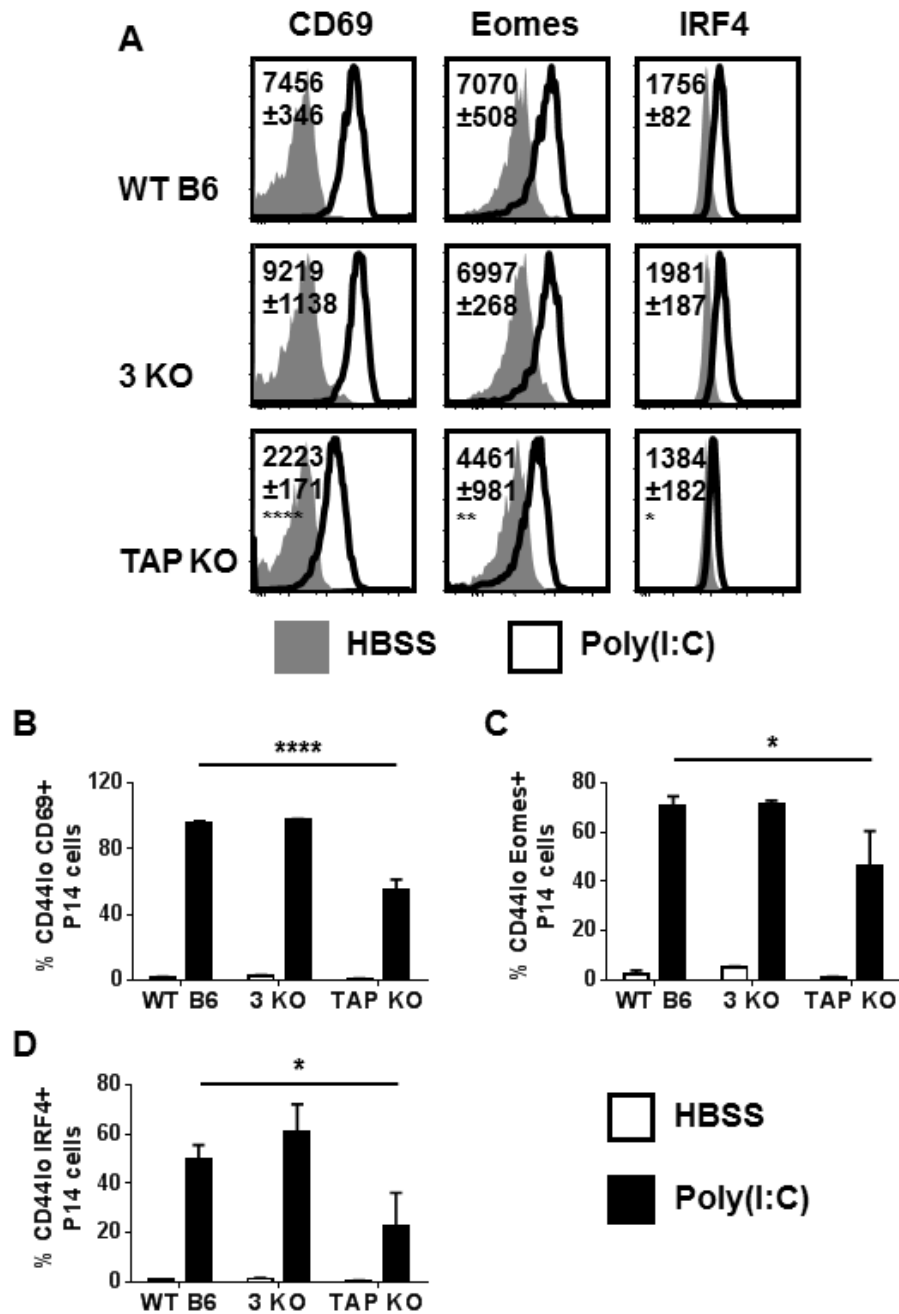
Figure 4.14: Poly(I:C)-induced early activation phenotype requires TAP

Congenic P14 cells were transferred into WT B6, 3 KO, or TAP KO mice, which were inoculated with HBSS or poly(I:C). One day later, splenocytes were isolated and stained. (**A**) Representative histograms gated on CD44lo P14 cells are shown with MFI ± SD of poly(I:C) treated group. Significance comparing poly(I:C)-treated WT to poly(I:C)-treated TAP KO. Frequencies of CD44lo P14 cells expressing CD69 (**B**), Eomes (**C**), and IRF4 (**D**), are graphed. Data are representative of 2-3 individual experiments with n of 2-4 mice per group.

gated on CD44lo P14 cells from each group are depicted in **Figure 4.15A**. Frequencies of CD44lo P14 cells producing IFNγ (**Figure 4.15B**) or TNF (**Figure 4.15C**) after poly(I:C) treatment (black bars) in WT, 3 KO or TAP KO hosts are graphed. Naïve P14 cells inoculated with poly(I:C) from TAP KO hosts did not exhibit immediate effector functions, whereas poly(I:C)-pretreated naïve P14 cells from WT or 3 KO hosts did. The frequency of CD44lo IFNγ+ T cells was significantly lower if from TAP KO hosts than if from WT hosts (**Figure 4.15B**). P14 cells from TAP KO hosts treated with poly(I:C) did exhibit enhanced production of TNF over HBSS controls from TAP KO hosts, but had slightly lower levels when compared to P14 cells treated with poly(I:C) from WT mice (**Figure 4.15C**).

These results could be interpreted in a few different ways. TAP KO mice may not have enough Class I on the surface to prime naïve CD8 T cells, suggesting that the amount of self-MHC on the surface, not the specificity of the peptides, is the important factor. On the other hand, the specific peptides presented may not be those that are required for T cell priming to immediate effector function, suggesting that in order for naïve T cells to be primed, they need to see specific self-peptides, and the amount may not be as crucial.

After identifying that P14 cells are not primed in B7 KO or TAP KO host mice, I wanted to quantify levels of self-MHC and costimulatory molecules after poly(I:C) priming. We hypothesized that professional APCs were mediating the priming of naïve T cells to become immediate effectors and chose to focus on

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Figure 4.15: Poly(I:C) priming of CD8 T cells to immediate effector function requires TAP
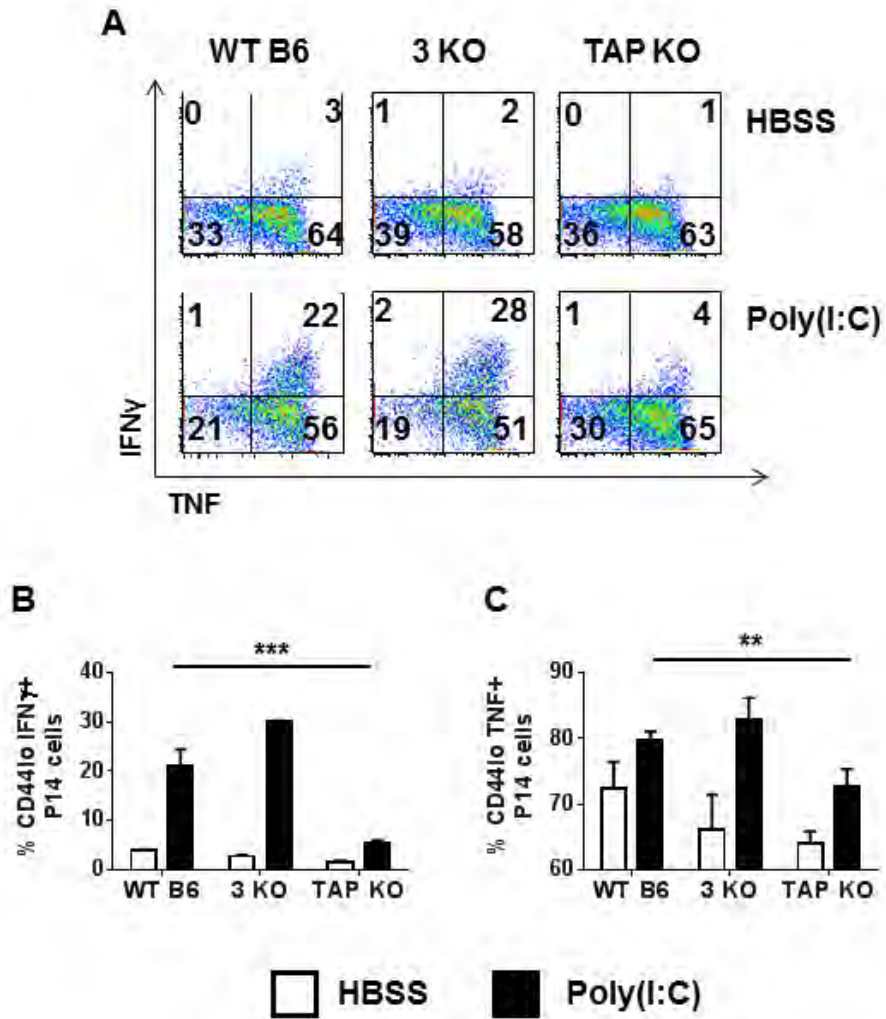
Figure 4.15: Poly(I:C) priming of CD8 T cells to immediate effector function requires TAP

Congenic P14 cells were transferred into WT B6, 3 KO, or TAP KO mice, which were inoculated with HBSS or poly(I:C). One day later, splenocytes were isolated and stimulated with GP33 *ex vivo*. (**A**) Representative flow plots gated on CD44lo P14 cells are shown. Frequencies of CD44lo P14 cells expressing IFNγ (**B**), and TNF (**C**) are graphed. Data are representative of 2-3 individual experiments with n of 2-4 mice per group.

DCs. I assessed expression levels of Class I (H2-Db) and Class II (I-Ab) expression and of the costimulatory molecules CD80 and CD86 on DCs from WT B6, IFNAR KO and TAP KO host mice after HBSS or poly(I:C) treatment (**Figure 4.16**). Although a number of different subsets of DCs have been described, each with their own unique properties, for simplification data from heterogeneous bulk DCs (identified as CD3e- and CD11c+) are shown.

It has been shown by others that type 1 IFN can upregulate both MHC Class I and Class II expression, and indeed this is also seen in our poly(I:C)-priming model (32, 74, 205). DCs from WT B6 mice upregulate H2-Db and I-Ab expression after poly(I:C) treatment. The expression level of MHC Class II I-Ab was similarly found to increase after poly(I:C) treatment on DCs from the TAP KO hosts (**Figure 4.16B**). However, the same cannot be said for Class I H2-Db expression. Although there is a slight upregulation of H2-Db on DCs from IFNAR KO mice, levels were still significantly lower than those from WT DCs. As expected, H2-Db expression levels on DCs from TAP KO mice were extremely low, and they did not increase after poly(I:C) treatment (**Figure 4.16A**). In terms of upregulating costimulatory molecules, DCs from each host - WT, IFNAR KO, and TAP KO - upregulated B7.1 (CD80) after 1 day of *in vivo* poly(I:C) treatment (**Figure 4.16C**). However, B7.2 (CD86) expression (similar to what was found on CD44lo CD8 T cells from IFNAR KO hosts) did not show poly(I:C)-induced upregulation in the IFNAR KO host DCs, as opposed to DCs from WT and TAP KO mice (**Figure 4.16D**). It should be noted that various subsets of DCs followed

Figure 4.16: Poly(I:C)-induced MHC Class I and costimulatory CD86 upregulation are mediated by type 1 IFN
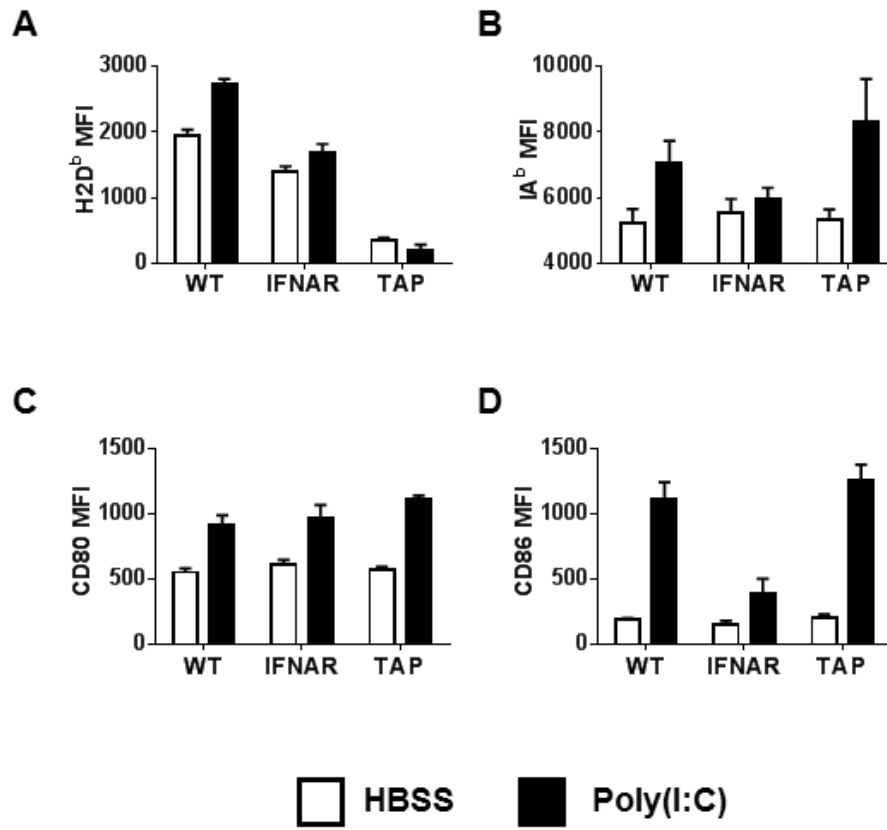
Figure 4.16: Poly(I:C)-induced MHC Class I and costimulatory CD86 upregulation are mediated by type 1 IFN

Phenotype of CD11c+ DCs from WT B6, IFNAR KO, and TAP KO hosts was assessed after HBSS or poly(I:C) treatment for 1 day. Gated on CD3e-, CD11c+ DCs, the MFI of H2-Db (**A**), I-Ab (**B**), CD80 (**C**), and CD86 (**D**) is graphed. Individual experiment with n of 3-4 mice per group.

similar changes in MHC and costimulatory molecule expression, as shown in **Figure 4.16**. That being said, some DC subsets showed stronger requirements for IFN signaling with regard to upregulation of I-Ab and CD80 (data not shown). In summary, type 1 IFN is important for the upregulation of MHC Class I and CD86, but is not as important for Class II and CD80 after poly(I:C) treatment in DCs. TAP KO DCs do not show defects in upregulating costimulatory molecules or Class II MHC after poly(I:C) treatment, but, as expected, had extremely low levels of MHC Class I.

4. Summary

We wanted to look further into the mechanism that mediates naïve T cell priming. BM chimeras where self-MHC was absent on hematopoietic cells did not induce the early activation marker CD69 or TF Eomes associated with poly(I:C)-induced priming of naïve CD8 T cells (**Figure 4.11**). These data do not directly identify a role for professional APCs but are consistent with the idea that hematopoietic cells are important for poly(I:C)-induced priming.

P14 cells primed in B7 KO hosts did not exhibit immediate effector function (i.e. IFNγ production) upon stimulation with cognate ligand *ex vivo* (**Figure 4.13**) indicating that B7-CD28 costimulatory effects are required for poly(I:C)-induced priming of naïve T cells. A deficiency in costimulation during the ICS could not account for the absence of IFNγ production in poly(I:C)-pretreated P14 cells from B7 KO hosts, because when B7-sufficient cells were

added to the cultures, IFNγ production was still lacking. Costimulatory molecules, including B7, are found on APCs, supporting the hypothesis that professional APCs are mediating naïve T cell priming.

Lastly, studies depicted here identify the requirement of either TAP-dependent peptides to be presented, or a certain amount of self-MHC on the sensitizing cells to mediate poly(I:C)-induced T cell priming (**Figures 4.14-4.15**). Our working hypothesis is that poly(I:C) induces upregulation of Class I MHC and costimulatory molecules on APCs, which can interact with naïve CD8 T cells. The reduced Class I on the surface of DCs in TAP KO host mice contributes to the idea that both MHC and costimulatory molecules are necessary for priming of naïve CD8 T cells to immediate effectors (**Figure 4.16**). DCs did not upregulate CD86 from IFNAR KO host mice after poly(I:C) inoculation and showed a reduced ability to prime naïve T cells. Combined, data presented here suggest that both MHC Class I and costimulation contribute to poly(I:C)-induced priming.

D. Poly(I:C)-primed naïve T cells have a reduced threshold required for effector function production

1. Poly(I:C)-primed naïve CD8 T cells produce immediate effector functions in response to lower concentrations of high affinity ligand

I questioned whether poly(I:C)-primed naïve CD8 T cells only responded to high-affinity, high-density antigen, or if they were able to respond to reduced

affinities or densities. Congenic P14 cells were transferred into WT B6 mice, which were HBSS- or poly(I:C)-treated for 1 day. Splenocytes were harvested and stimulated *ex vivo* with different concentrations of GP33 peptide and assayed for effector function. Due to the fact that CD69 is one of the first activation markers to be induced in activated T cells, I determined the frequency of CD44lo P14 cells that expressed CD69 after stimulation with various concentrations of GP33 (**Figure 4.17A**). The majority of poly(I:C)-pretreated P14 cells already expressed CD69, but only a small proportion of the HBSS-treated P14 cells upregulated CD69, even when stimulated with the highest concentrations of cognate ligand. Because T cell receptor signaling is essential for CD8 T cell activation, IRF4, a TF known to be induced downstream of T cell receptor signaling was measured (**Figure 4.17B**). Poly(I:C)-pretreated naïve P14 cells (black bars) showed higher proportions of IRF4+ T cells in the absence of exogenous *ex vivo* stimulation, compared to HBSS-treated P14 cells (open bars) (NO stim). Upon *ex vivo* stimulation with the lowest concentrations of cognate ligand tested, poly(I:C)-primed CD44lo P14 cells upregulate significantly more IRF4 than their control treated counterparts (16 nM and 64 nM). IRF4 expression has been associated with many aspects of CD8 T cell effector function. These results show that poly(I:C)-pretreated naïve CD8 T cells upregulate IRF4 in response to a lower density antigen compared to control-treated CD8 T cells, and this increased IRF4 expression may play a role in the acquisition of immediate effector function.

Figure 4.17: CD44lo P14 CD8 T cells primed with poly(I:C) produce immediate effector functions in response to lower concentrations of cognate ligand
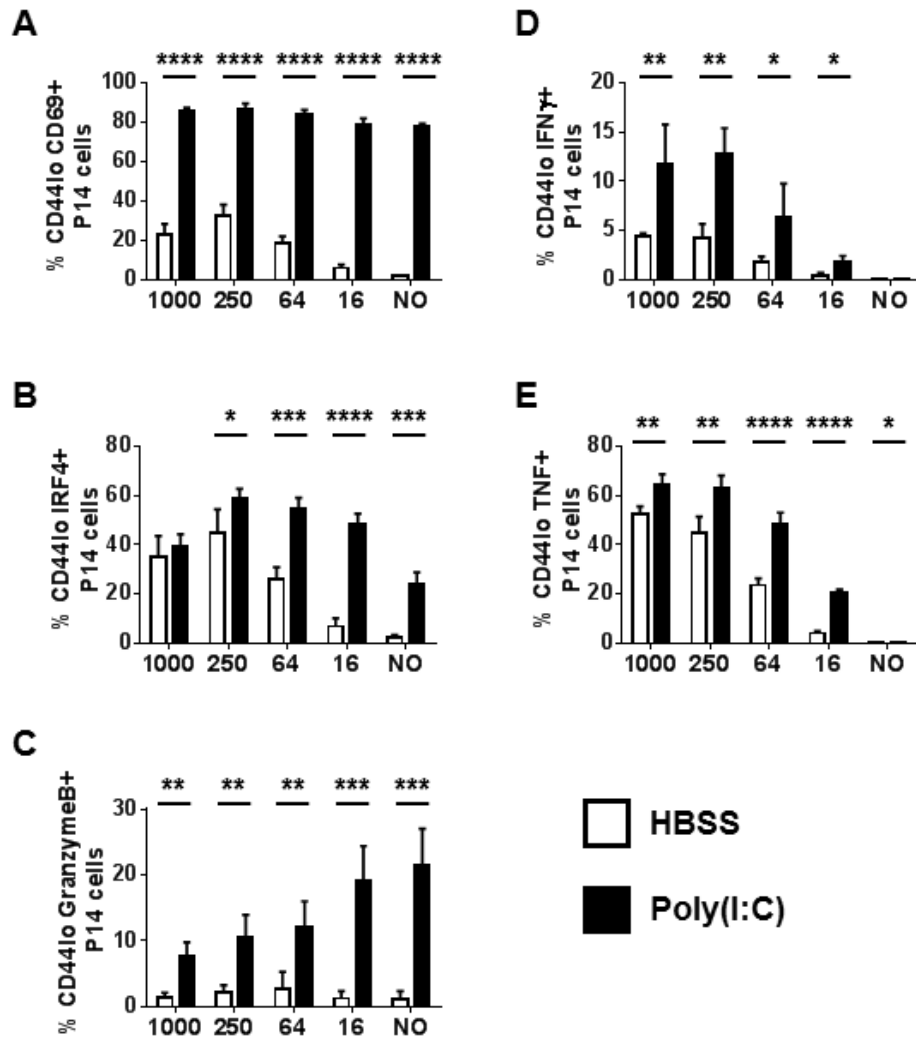
Figure 4.17: CD44lo P14 CD8 T cells primed with poly(I:C) produce immediate effector functions in response to lower concentrations of cognate ligand

P14 splenocytes were transferred into B6 mice, which were inoculated with HBSS (open bars) or poly(I:C) (black bars). Splenocytes were harvested 1 day later and stimulated *ex vivo* with different concentrations of GP33 peptide. The frequencies of CD44lo P14 cells expressing CD69 (**A**), IRF4 (**B**), granzyme B (**C**), IFNγ (**D**), or TNF (**E**) are graphed. Data are representative of 2 individual experiments with n of 4-5 mice per group.

To assess effector function, granzyme B expression levels were determined in poly(I:C)- and HBSS-pretreated naïve P14 cells after *ex vivo* stimulation with different concentrations of GP33 (**Figure 4.17C**). The frequency of poly(I:C)-primed CD44lo P14 cells expressing granzyme B was greatest without any *ex vivo* stimulation (NO), and upon increasing concentrations of GP33 stimulation, the proportion of granzyme B+ P14 cells decreased. Based on work shown earlier (**Figure 4.7**), in which primed CD8 T cells were shown to degranulate in response to cognate antigen, these data suggest that granzyme B is secreted upon stimulation with cognate ligand. It should be noted that at low concentrations, such as 64 nM (compared to 1 µM of stimulation), significant changes in granzyme B expression were seen in the poly(I:C)-primed groups, suggesting release of granzyme B with lower densities of high-affinity cognate ligands. In contrast, control-treated naïve P14 cells (HBSS-open bars) did not express granzyme B. The frequencies of IFNγ producing (**Figure 4.17D**) and TNF producing (**Figure 4.17E**) CD44lo P14 cells stimulated with varying concentrations of GP33 peptide were also determined. As shown previously, poly(I:C)-primed naïve P14 cells can produce IFNγ and enhanced TNF in response to high-affinity, high-density antigen (1000 nM). Moreover, primed CD44lo P14 cells were also found to produce these effector cytokines in response to lower concentrations of ligands (250 nM and 64 nM) to a significant level over HBSS-treated controls. At the lowest concentration tested (16 nM), a

small, but significant difference was observed in IFNγ and TNF production, when comparing poly(I:C)-primed and unprimed counterparts.

2. CD8 T cells primed with poly(I:C) produce effector functions in response to lower affinity ligands

After showing that poly(I:C)-primed naïve T cells could produce immediate effector functions in response to low-density cognate antigen, I questioned whether they could also produce immediate effector function in response to lower affinity antigens. P14 transgenic CD8 T cells were used in a similar poly(I:C)-priming experimental design as used previously, but peptides used for ICS included an APL (F6L) which reduces the affinity of the TCR for the pMHC complex ~5 fold compared to the high affinity GP33 pMHC complex. Representative flow plots gated on CD44lo P14 cells from HBSS- or poly(I:C)-treated mice stimulated *ex vivo* without peptide, APL (F6L), or cognate ligand (GP33) are shown in **Figure 4.18A.** Poly(I:C)-primed naïve P14 cells produced TNF (quantified in **Figure 4.18B**) and IFNγ (quantified in **Figure 4.18C**) in response to cognate antigen stimulation. Additionally, upon stimulation with the lower affinity APL, F6L, poly(I:C)-primed naïve P14 cells also produced significantly more IFNγ and TNF than HBSS treated naïve counterparts.

To identify if increased response to APLs occurs in other systems, a similar experimental design was performed with the OT-I transgenic T cells. Congenic OT-I cells were transferred into B6 mice that were inoculated with

Figure 4.18: Poly(I:C)-primed naïve P14 cells produce IFNγ and TNF in response to low-affinity altered peptide ligand
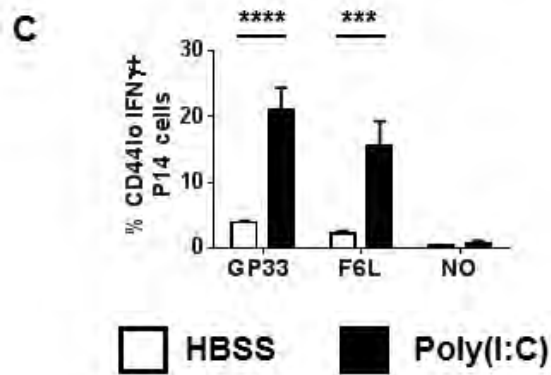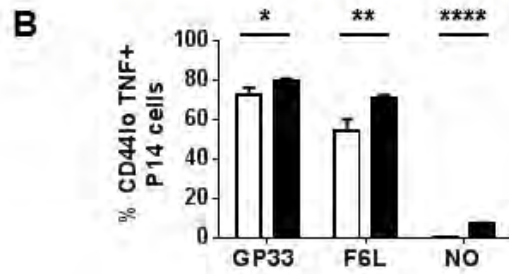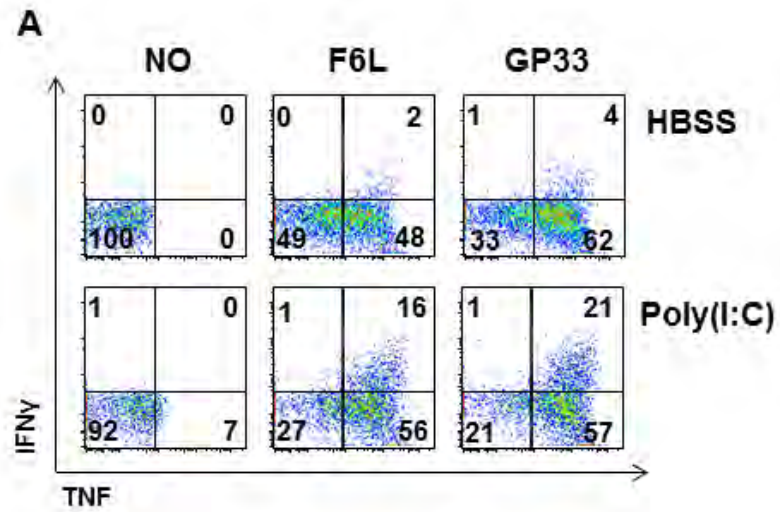
Figure 4.18: Poly(I:C)-primed naïve P14 cells produce IFNγ and TNF in response to low-affinity altered peptide ligand

Congenic P14 cells were transferred into B6 hosts. Mice were treated with HBSS or poly(I:C) for 1 day, and splenocytes were stimulated with high-affinity cognate ligand (GP33) or altered peptide ligand (F6L). **A**) Representative flow plots gated on donor P14 cells expressing IFNγ and TNF are shown. The frequencies of CD44lo P14 cells producing TNF (**B**) or IFNγ (**C**) are graphed. Data from experiment with n of 4 mice per group.

HBSS or poly(I:C) for 1 day. Cells were stimulated with a variety of APLs for which OT-I T cells have a varying degree of affinity (180). The order of peptide affinity is as follows from highest affinity to lowest affinity: OVA>Y3>T4>V4>G4. Representative flow plots gated on CD44lo OT-I T cells from HBSS- or poly(I:C)-treated mice, stimulated *ex vivo* with different peptides are shown in **Figure 4.19A**. The frequency of CD44lo TNF+ OT-I (**Figure 4.19B**), IFNγ+ OT-I (**Figure 4.19C**) and granzyme B+ OT-I (**Figure 4.19D**) from HBSS-treated (open bars) or poly(I:C)-treated (black bars) mice stimulated *ex vivo* with different peptides are graphed. Similar to results shown in **Figure 4.18** with naïve P14 T cells and the F6L APL, naïve OT-I CD8 T cells also produced multiple effector functions in response to APL stimulation to a significant level over control-treated cells stimulated *ex vivo* with the same APL. Poly(I:C)-priming induced more naïve OT-I cells to produce TNF to all of the APLs tested, including G4 which has a > 200 fold reduction in TCR affinity for the pMHC compared to OVA (180), when compared to HBSS-treated naïve OT-I T cells (**Figure 4.19B**). Not only was there an increased proportion of poly(I:C)-pretreated naïve OT-I T cells that produced TNF in response to APLs, but of the cells that produced TNF, poly(I:C)-pretreated OT-I T cells had higher MFIs than HBSS-treated OT-I cells, indicating they produced more TNF on a per cell basis (data not shown). This also holds true for IFNγ production, where an increased frequency of naïve OT-I T cells produce IFNγ to all of the APLs tested, including the lowest affinity, albeit to a very low level, if primed with poly(I:C), compared to control OT-1 T cells (**Figure**

Figure 4.19: Poly(I:C)-primed CD44lo OT-I cells produce effector functions in response to low-affinity altered peptide ligands
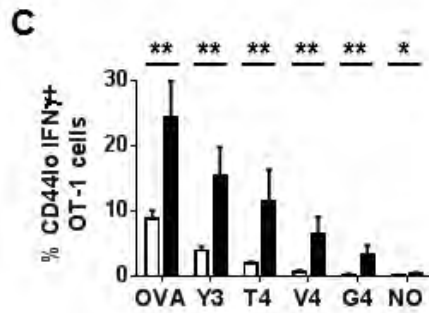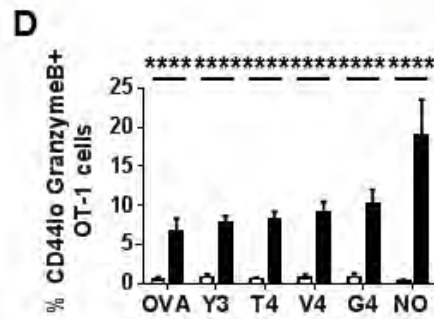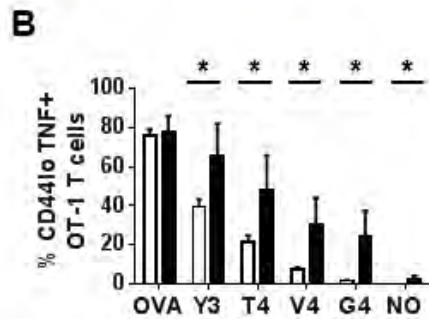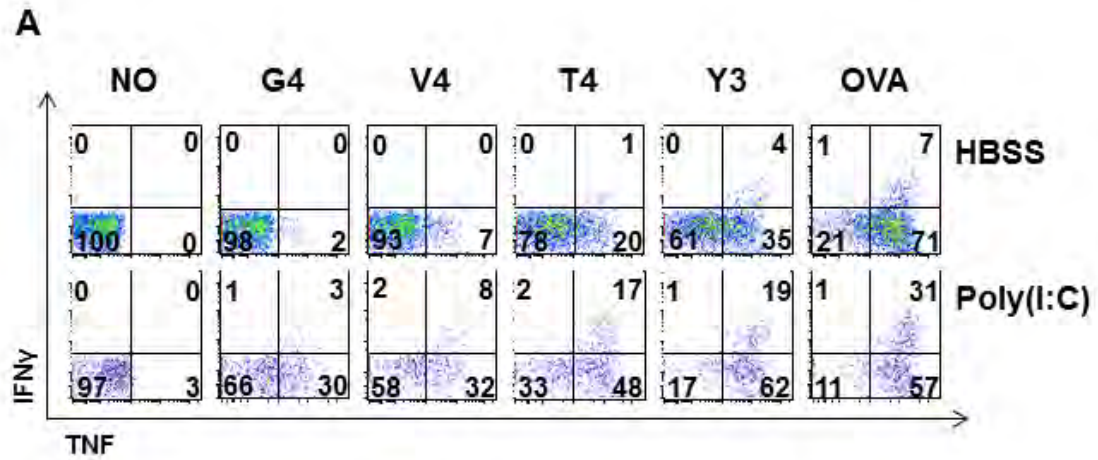
Figure 4.19: Poly(I:C)-primed CD44lo OT-I cells produce effector functions in response to low-affinity altered peptide ligands

Congenic OT-I splenocytes were transferred into B6 hosts, which were inoculated with HBSS or poly(I:C). Splenocytes were isolated 1 day later and stimulated without peptide (NO), with high affinity cognate ligand (OVA), or an altered peptide ligand (Y3, T4, V4, or G4). **A**) Representative flow plots gated on donor CD44lo OT-1 cells expressing IFNγ and TNF are shown. The frequencies of CD44lo OT-I cells producing TNF (**B**), IFNγ (**C**) or granzyme B (**D**) are graphed. Data are representative of 2 independent experiments with n of 3-5 mice per group.

4.19C). Moreover, of the cells that produced IFNγ in response to peptide stimulation with the lowest affinity APL, G4, poly(I:C)-pretreated naïve OT-1 T cells showed increased IFNγ MFI than their HBSS-treated counterparts (1810±231 vs. 615±79, p<0.0001), indicating more IFNγ production on a per cell basis.

The loss of granzyme B staining in the poly(I:C)-pretreated CD44lo OT-I T cells after APL stimulation, as compared to unstimulated counterparts, indicates that granzyme B is secreted and/or released in response to even the lowest affinity APL tested here (**Figure 4.19D**). Together, these data demonstrate that priming with poly(I:C) enables naïve T cells to produce immediate effector functions, even in response to lower affinity ligands. Thus, the poly(I:C)-primed naïve CD8 T cells appear to have a reduced threshold for production of cytokines and release of granzyme B compared to naive T cells that are not primed.

3. Poly(I:C)-primed naïve CD8 T cells specifically lyse target cells

The process of trogocytosis can be a surrogate for functional activation, in terms of cytolytic effector function. CD8 T cells engaging in trogocytosis have been found to be more active and may have increased cytolytic capability (196, 197). To determine if trogocytosis is similar between primed T cells and control T cells, a TRAP assay using fluorescently-labeled RMA peptide-pulsed targets was performed. P14 cells were transferred into B6 recipients that were inoculated with HBSS or poly(I:C) for 1 day prior to harvesting spleens. Single cell suspensions

were prepared, and P14 effectors were incubated with either: 1) No target cells, 2) K3L-pulsed RMA cells, or 3) GP33-pulsed RMA cells. Target cells were labeled with a fluorescent lipid that can be detected on effector cells if trogocytosis has occurred. Poly(I:C)-primed CD44lo P14 cells (black bars) incorporated more of the fluorescent dye (**Figure 4.20A**) compared to control-treated P14 cells (open bars) during incubation with GP33-pulsed target cells. Although the difference is small, it is statistically significant. It should be noted that there is a difference between dye incorporation of poly(I:C)-treated P14 cells when co-incubated with the irrelevant peptide K3L vs. the specific peptide GP33, demonstrating the specificity of the assay.

I questioned whether during this short 1 hour incubation with GP33 target cells, if primed cells could also downregulate CD62L. Indeed, there were increased frequencies of CD44lo CD62Llo P14 cells from poly(I:C)-inoculated host mice compared to P14 cells from control-treated host mice when incubated with GP33-pulsed target cells for only 1 hour (**Figure 4.20B**). This downregulation of CD62L is specific, as there is not a significant difference if we examine P14 cells when incubated with K3L-pulsed targets.

The increase in trogocytosis and release of granzyme B in poly(I:C)-primed naïve T cells lead us to question whether or not naïve CD8 T cells primed with poly(I:C) could exert cytolytic effector function. The current study utilizes the *in vivo* cytotoxic assay to determine if poly(I:C)-pretreated P14 cells could specifically lyse target cells. Congenic P14 cells were transferred into B6 hosts

Figure 4.20: Increased trogocytosis of poly(I:C)-primed P14 cells
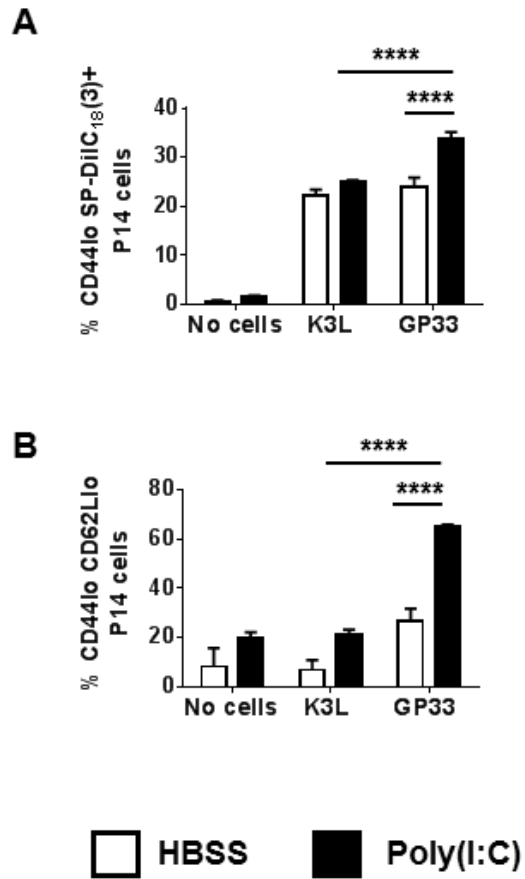
Figure 4.20: Increased trogocytosis of poly(I:C)-primed P14 cells

A TRAP assay was performed using HBSS- or poly(I:C)-pretreated P14 CD8 T cells as effectors and RMA cells pulsed with peptides as targets. Effectors were developed by poly(I:C) or HBSS treating B6 mice that had previously received congenic P14 cells. One day after inoculation, splenocytes containing the donor P14 CD8 T cells were isolated and used as effectors. Target cells were RMA cells that were pulsed with an irrelevant peptide (K3L) or pulsed with the specific peptide (GP33). Target cells were labeled with fluorescent lipid molecule SP-DiIC$_{18}$(3), which can be detected when transferred to a different cell through trogocytosis. Target cells were in excess and were co-incubated with effectors for 1 hour, stained with surface antibodies and ran on a flow cytometer. Frequency of CD44lo P14 cells incorporating the fluorescent lipid SP-DiIC$_{18}$(3) from HBSS- (open bars) or poly(I:C)-treated (black bars) mice incubated with separate targets are graphed in (**A**). Frequency of CD44lo P14 cells downregulating CD62L from each group is graphed in (**B**). Data are representative of 3 independent experiments with n of 3-5 mice per group.

that were inoculated with HBSS or poly(I:C). One day later, mice received a mixture of splenocytes that were pulsed without peptide, with K3L peptide, or with GP33 peptide. All splenocytes were labeled with CellTrace Far Red DDAO to distinguish donor from hosts, and each peptide-pulsed target was labeled with a different concentration of CellTrace Violet. After a ~20 hour *in vivo* incubation, spleens were harvested and stained, and specific lysis was determined.

I wanted to identify if the poly(I:C)-treated donor P14 cells still maintained a naïve phenotype after the 20 hour *in vivo* cytotoxicity assay. The phenotype of the donor P14 cells is shown in **Figure 4.21A**, where representative flow plots are gated on donor P14 cells. The majority of the P14 cells remained CD44lo, showing they are naïve in phenotype. Poly(I:C)-pretreated CD44lo P14 cells showed significant upregulation of CD69 as compared to their HBSS-treated counterparts. In addition, CD44lo P14 cells downregulated CD62L if pre-exposed to poly(I:C) to a greater extent over controls.

Representative histograms gated on donor CellTrace Far Red DDAO cells are shown in **Figure 4.21B**. Control-treated mice did not receive donor P14 cells, nor did they receive poly(I:C). The HBSS and poly(I:C) groups refer to the inoculation received after donor P14 cells were transferred. Three separate populations are depicted, where the lowest concentrations of CellTrace Violet are splenocytes which were not pulsed with any peptide (red), the middle concentration represents splenocytes pulsed with the irrelevant K3L peptide (green), and the highest concentration of CellTrace Violet represents splenocytes

Figure 4.21: Primed P14 cells specifically lyse GP33 peptide pulsed target cells *in vivo*
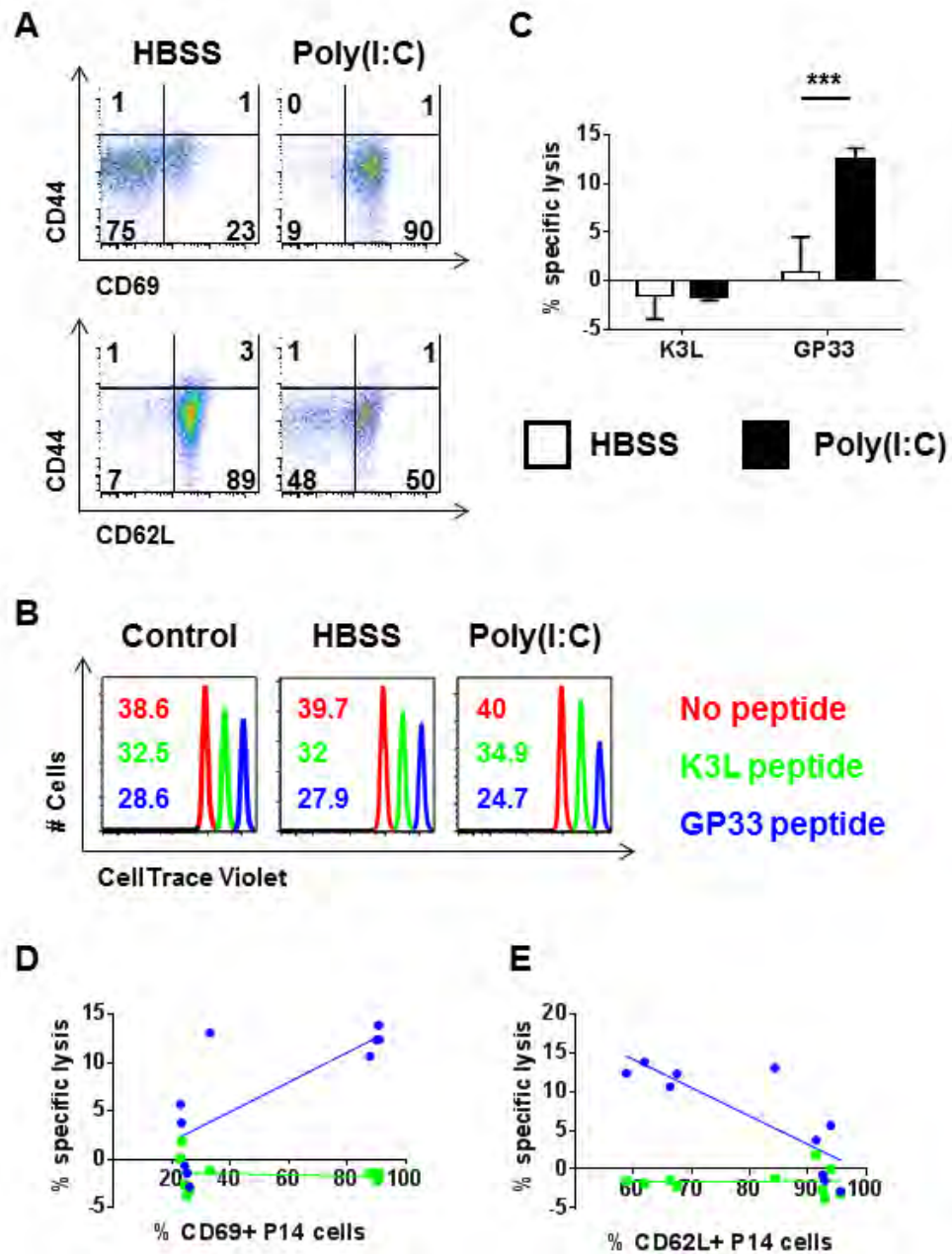
Figure 4.21: Primed P14 cells specifically lyse GP33 peptide pulsed target cells *in vivo*

Congenic P14 cells were transferred into WT B6 mice, which were inoculated with HBSS or poly(I:C). One day later, mice received splenocytes which were pulsed with either no peptide, K3L, or GP33, each labeled with DDAO far red and a different concentration of CellTrace Violet. Approximately 1 day later, spleens were harvested, and phenotype of donor P14 cells and frequency of peptide-pulsed target cells were determined. (**A**) Representative flow plots gated on donor P14 cells expressing CD69 or CD62L from HBSS- and poly(I:C)-treated mice are shown. (**B**) Representative histograms gated on donor DDAO Far red+ splenocytes with the lowest concentration of CellTrace Violet representing no peptide-pulsed (Red), middle concentration, K3L peptide (Green), or highest concentration, GP33 peptide (Blue). **C**) Percent specific lysis was calculated for K3L- and GP33- specific cells. Correlation between % specific lysis and frequency of P14 cells expressing CD69 (**D**) or CD62L (**E**) is graphed. Data are representative of 2 individual experiments with n of 4-5 mice per group.

pulsed with GP33 peptide (blue). Both the unlabeled control and the nonspecific peptide targets were used to ensure there were no off target effects of poly(I:C) treatment, and that the donor P14 populations are indeed mediating the effect. The control and HBSS histogram profiles look very similar to one another, showing comparable frequencies of each of the donor populations between the two groups. However, the poly(I:C)-treated group shows reduced frequencies of GP33 peptide-pulsed targets, and an increase in both the unlabeled and K3L-labeled group when compared to both of the controls. The percent specific lysis was calculated for both the K3L-pulsed and the GP33-pulsed targets using the un-pulsed peptide as the control by using the following equation:

$$\text{\% specific lysis} = 100 - ((\text{Experimental/Control})/\text{Control} \times 100)$$

K3L-pulsed target cells were not preferentially lysed in either the HBSS- or poly(I:C)-treated hosts (**Figure 4.21C**). However, GP33-pulsed splenocytes were found to be specifically lysed in poly(I:C)-treated (black bars) mice to a significant extent over HBSS-treated mice (open bars). When we plot the % specific lysis of GP33- (blue) or K3L- (green) pulsed targets vs. CD69 or CD62L expression on P14 cells, we find that only GP33-pulsed targets correlate with P14 cell phenotype. **Figure 4.21D** shows a positive correlation between P14 CD69 upregulation and % GP33 specific lysis, such that as the frequency of CD69+ P14 cells increases, so does the % GP33-specific lysis. On the other hand, there is a negative correlation with CD62L expression, where the higher the CD62L expression, the lower the GP33-specific lysis is **(Figure 4.21E)**. There is no

correlation between P14 phenotype and % K3L-specific lysis, indicating specificity of the P14 cells for GP33-pulsed targets.

Immediate effector function exhibited by poly(I:C)-primed naïve CD8 T cells was not associated with any cell division during this time period (previously shown **Figure 4.10**). Because P14 cells persisted in the donor hosts during the *in vivo* cytotoxicity assay for such a long period of time (**Figure 4.21**), I questioned whether they were dividing during this time. Congenic P14 cells were adoptively transferred into WT host mice that were inoculated with HBSS or poly(I:C) for 1 day. A similar experimental design as the *in vivo* cytotoxicity assay was performed, but instead of mice receiving a combination of peptide-pulsed target cells, they only received one target population. Representative flow plots gated on donor P14 cells are shown in **Figure 4.22A,** and the frequencies of CD44lo Ki67+ P14 cells are graphed in **Figure 4.22B**. P14 cells from HBSS-treated mice that received un-pulsed splenocytes or the nonspecific K3L-pulsed splenocytes did not induce Ki67 expression. Upon receiving GP33-pulsed splenocytes, P14 cells from HBSS-treated mice showed upregulation of the proliferation marker Ki67, indicating they were no longer in a G_0 resting state. Interestingly, P14 cells from poly(I:C)-treated mice that received un-pulsed or the nonspecific K3L-pulsed splenocytes showed significantly higher frequencies of Ki67+ cells compared to their control-treated counterparts. Moreover, poly(I:C)-pretreated P14 cells showed increased Ki67 staining over controls if they received the GP33-specific peptide-pulsed targets.

Figure 4.22: Poly(I:C)-primed P14 T cells increase effector transcription factors over controls in response to cognate antigen stimulation
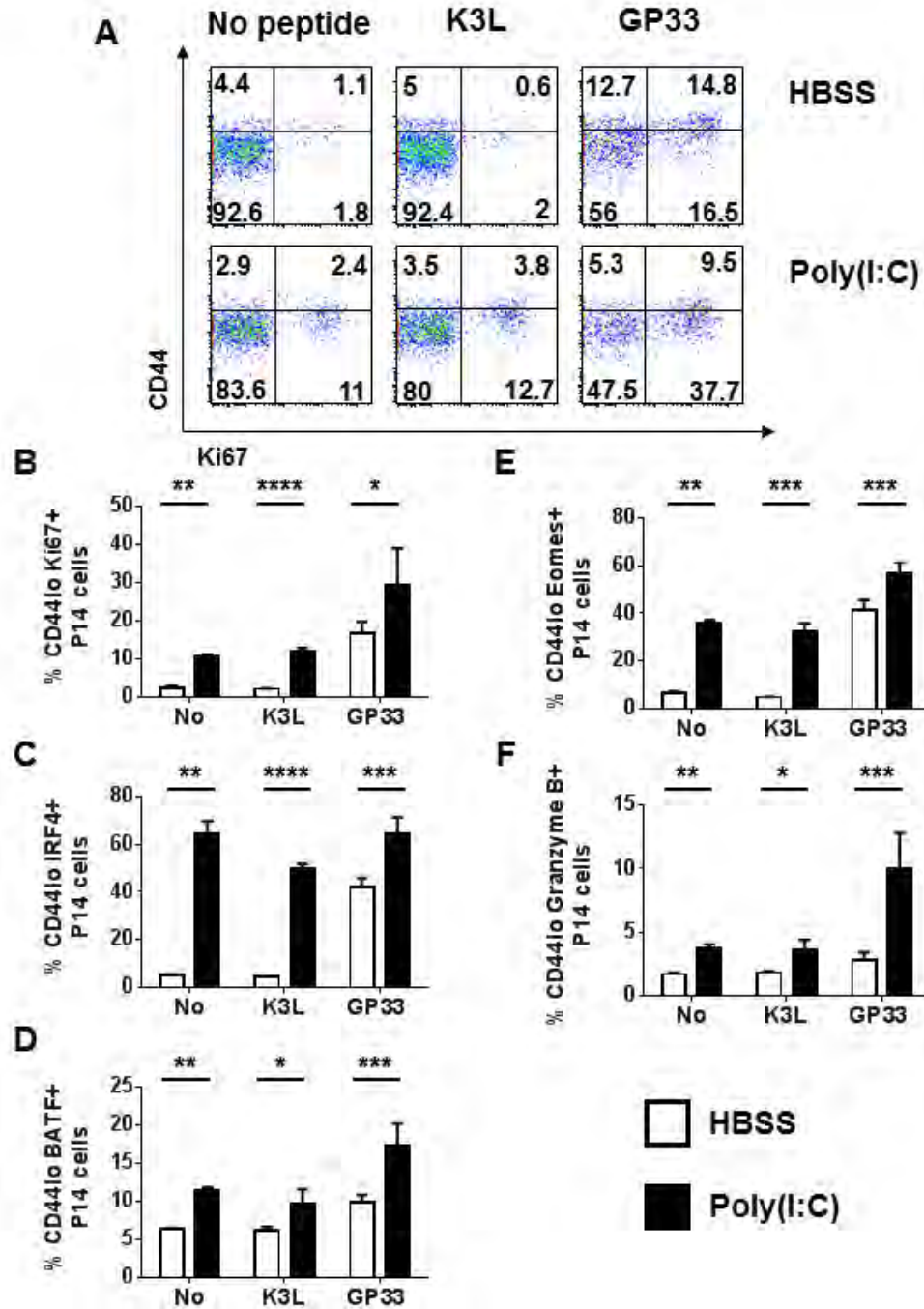
Figure 4.22: Poly(I:C)-primed P14 T cells increase effector transcription factors over controls in response to cognate antigen stimulation

Congenic P14 cells were transferred into WT B6 hosts, which were inoculated with HBSS or poly(I:C). One day after inoculation, mice received splenocytes that were pulsed with K3L peptide, GP33 peptide, or no peptide. One day after transfer, spleens were harvested and stained. Representative flow plots gated on donor P14 cells expressing the proliferation marker Ki67 (**A**), and frequencies of P14 cells expressing Ki67 (**B**) are shown. P14 cells expressing transcription factors IRF4 (**C**), BATF (**D**), and Eomes (**E**) are graphed. Frequency of P14 cells expressing granzyme B is shown in (**F**). Experiment with 2-5 mice per group.

Results from this experiment were somewhat surprising due to the fact that induction of cell cycle in CD8 T cells prior to any antigen stimulation is indicated. Results from **Figure 4.10C** show no upregulation of Ki67 expression after 1 day of poly(I:C) treatment, but **Figure 4.22B** shows Ki67+ cells at ~48 hours post-poly(I:C) inoculation. Therefore, Ki67 is being induced between 24-48 hours post-treatment in the absence of cognate antigen.

To determine if the longer exposure to cognate antigen stimulation had an effect on poly(I:C)-pretreated P14 cells, effector-associated TFs were quantified during this experiment. As shown in multiple figures previously in this chapter, CD44lo P14 cells upregulate IRF4 after poly(I:C) treatment. In the present experiment, IRF4 was similarly upregulated in naive P14 cells from poly(I:C)-pretreated mice that received any of the pulsed target populations (**Figure 4.22C**). Moreover, higher frequencies of CD44lo P14 cells upregulated IRF4 after poly(I:C) treatment than after HBSS treatment, regardless of the specificity of the peptide-pulsed splenocytes they received. However, it should be noted that CD44lo P14 cells from HBSS-treated mice induced IRF4 if they received GP33-pulsed splenocytes but not the un-pulsed or K3L-pulsed targets. These data indicate that IRF4 upregulation in HBSS-treated P14 cells was specific.

The levels of two additional TFs, BATF and Eomes, were then determined. BATF is a transcription factor known to associate with IRF4 and is important in effector CD8 T cells (158). The frequency of CD44lo BATF+ P14 cells are graphed in **Figure 4.22D**, and CD44lo Eomes+ P14 cells are graphed in

Figure 4.22E. Both BATF and Eomes showed similar patterns and changes as IRF4. Poly(I:C)-pretreated mice had increased proportions of CD44lo BATF+ P14 cells and CD44lo Eomes+ P14 cells compared to HBSS-treated mice, regardless of the splenocytes that were transferred. Furthermore, the frequency of HBSS-treated CD44lo P14 cells upregulating BATF and Eomes increased after receiving GP33-pulsed targets, but the poly(I:C)-treated CD44lo P14 cells showed more BATF and Eomes than controls. Together, these data reveal that poly(I:C) pretreatment induces upregulation of TFs associated with CD8 T cell effector function prior to cognate antigen exposure, and upon antigen exposure, they will further increase levels of these TFs to a significant extent over controls.

Because acquiring effector-associated TFs does not necessarily correspond to effector function, expression of granzyme B was also determined in the present study. There were increased proportions of CD44lo granzyme B+ P14 cells in poly(I:C)-pretreated mice compared to HBSS-treated mice (**Figure 4.22F**), and these were further increased if recipients received GP33-pulsed splenocytes. It should be noted that P14 cells did not upregulate granzyme B from any of the HBSS-treated groups, even those receiving GP33-pulsed target cells. This is consistent with the literature that shows that costimulation and signal 3 cytokines are important for granzyme B expression (170).

4. Summary

These results demonstrate that pretreatment with poly(I:C) reduces the requirements or threshold required for primed naïve CD8 T cells to produce effector functions compared to control T cells. Not only were poly(I:C)-pretreated naïve T cells able to produce immediate effector function to high-affinity high-density antigen, but they also produced these effector functions in response to reduced concentrations of cognate ligand (**Figure 4.17**) Moreover, poly(I:C)-primed naïve T cells were able to respond to a number APLs and were able to produce immediate effector functions, when conventional naïve T cells were not (**Figure 4.18-4.19**).

Poly(I:C)-primed naïve CD8 T cells had increased levels of effector-associated TFs including IRF4, BATF, and Eomes and could induce further expression upon stimulation with high-affinity ligand *in vivo*. Poly(I:C)-primed CD44lo P14 cells upregulated granzyme B levels after *in vivo* stimulation with GP33-pulsed splenocytes (**Figure 4.22**). This may have enabled them to exhibit immediate cytolytic effector function, because in addition to being able to produce multiple cytokines in response to antigen stimulation, poly(I:C)-primed naïve P14 cells were also found to specifically lyse target cells (**Figure 4.21**). This is compared to conventional naïve P14 CD8 T cells, which did not upregulate granzyme B and showed no cytolytic ability.

Primed naïve CD8 T cells acquired immediate effector function without cell division. However, during a prolonged exposure to cognate antigen *in vivo*, both HBSS and poly(I:C)-treated P14 cells upregulated the proliferation marker Ki67

(**Figure 4.22**). Pretreatment with poly(I:C) showed increased frequencies of Ki67+ P14 cells compared to controls, but the upregulation of Ki67 does not necessarily indicate that the cells have divided, as it is a marker that is expressed on all non-resting cells. Therefore, we do not know in which stage of the cell cycle primed and non-primed cells are when stained positive for Ki67, nor can we discern the potential fate of Ki67-expressing cells.

E. Conclusions

This chapter describes how out-of-sequence priming differs from conventional naïve T cell priming. Upon activation, acquisition of naïve T cell effector function usually requires cell division (141). In some instances, however, naïve T cells will acquire effector functions. For example, naïve T cells were found to produce TNF in response to anti-CD3 stimulation and/or cognate antigen stimulation *in vivo* and *in vitro* (161, 162). However, production of other cytokines and cytolytic effector function requires additional signals. Using adoptive transfers, I discovered that naive T cells acquired an early intermediately activated phenotype after being exposed to a poly(I:C)-induced inflammatory environment for as little as 12 hours (**Figures 4.1-4.3**). This phenotype was associated with upregulation of the activation markers CD69 and CD86, as well as downregulation of the IL-7 and type 1 IFN receptors. Poly(I:C)-primed naïve T cells also upregulated effector-associated transcription factors including IRF4 and Eomes (**Figures 4.4-4.6**).

Primed naïve CD8 T cells have a reduced threshold required for production of multiple effector functions compared to conventional naïve T cells. Compared to control naïve cells, poly(I:C)-primed CD8 T cells responded to lower density cognate antigen, and produced IFNγ and TNF and released granzyme B (**Figure 4.17**). In addition, primed CD44lo CD8 T cells responded to a number of different APLs, of which the TCR has a lower affinity for compared to the higher affinity cognate ligand (**Figure 4.18-4.19**). Naïve P14 cells primed with poly(I:C) also gained cytolytic capability, as they specifically lysed GP33-pulsed target cells in an *in vivo* cytotoxicity assay (**Figure 4.20-4.21**). These immediate effector functions produced by poly(I:C)-primed naïve T cells are distinct from conventional naïve T cells, which do not exert these functions in response to the same stimuli. Therefore, primed T cells behaved more similarly to memory CD8 T cells rather than naïve T cells upon antigen encounter.

It is postulated that type 1 IFN acts on the APC to upregulate self-MHC and costimulatory molecules and, through TCR stimulation, will induce an early activated phenotype of naïve CD8 T cells. BM chimeras made using WT or H2-Db KO BM suggested that self-MHC on hematopoietic cells were required to mediate priming of naïve T cells (**Figure 4.11)**. To support the hypothesis that professional APCs mediated these effects, B7 costimulation was found to be important for T cell acquisition of immediate effector function (**Figure 4.13**). The specificity of the peptides and/or amount of Class I being expressed may be important as well (**Figure 4.14-4.15**). T cells are not primed in TAP KO hosts,

which exhibit very low Class I but, upregulated costimulatory molecules, and T cells are not primed as efficiently in IFNAR KO hosts (compared to WT hosts), which show relatively high levels of Class I, but reduced costimulatory molecule expression.

A model is depicted in **Figure 4.23**. Taken together, our working hypothesis is that poly(I:C)-induced IFN causes APCs to upregulate both self-MHC and costimulatory molecules, including B7. The specific of the amount of Class I or specific peptide(s) on the surface may play an important role. Nevertheless, Class I MHC and costimulatory molecules will trigger TCR stimulation and induce an early partially activated phenotype coupled with an increase in effector-associated TFs. These T cells are now primed and ready to go, such that upon stimulation even with low-density or low-affinity ligands, due to their reduced threshold for induction of effector function, the T cells will produce multiple cytokines, degranulate and exert cytotoxic effector function. This suggests that during an acute infection, T cells that are primed in an out-of-sequence manner may be able to elicit immediate effector functions and contribute to controlling initial pathogen load prior to clonal expansion.

Figure 4.23: Model: Interferon primes naïve T cells to exhibit immediate effector function upon antigen stimulation
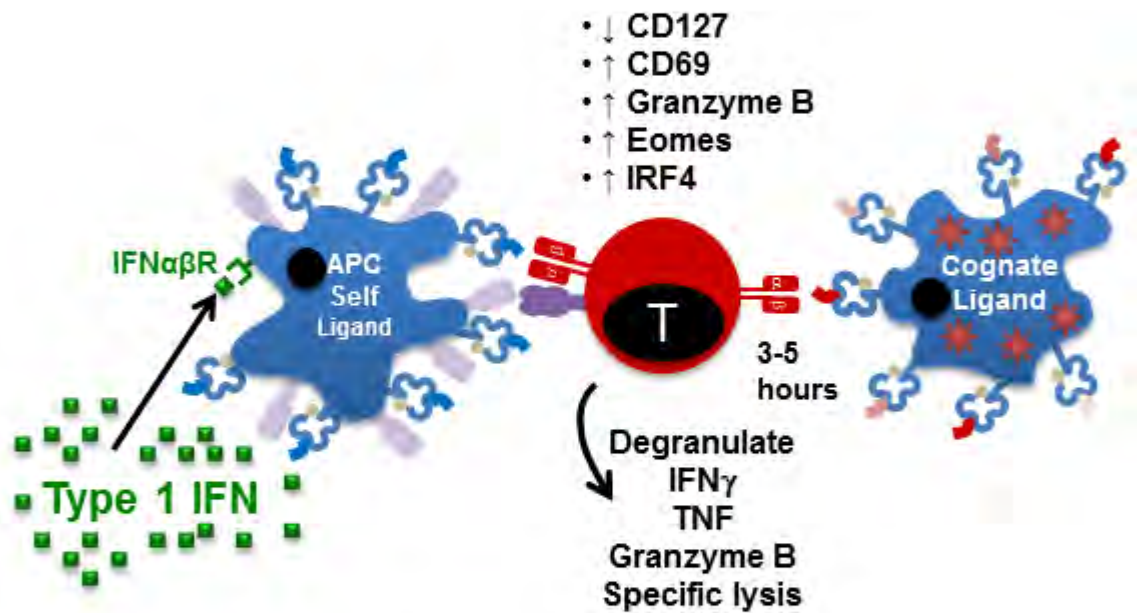
Figure 4.23: Model: Interferon primes naïve T cells to exhibit immediate effector function upon antigen stimulation

Type 1 IFN acts on APCs to upregulate Class I MHC and costimulatory molecules. Naïve T cells exposed to self-MHC, costimulation, and IFN adopt an early activated phenotype associated with the upregulation of CD69, granzyme B, Eomes and IRF4. These T cells are armed with the ability to elicit immediate effector functions in response to lower affinities and densities of cognate antigen prior to cell division.

CHAPTER V: Discussion

A. Dichotomy of out-of-sequence signaling effects

Studies described in this thesis highlight the growing complexity of CD8 T cell activation. Not only does the presence or absence of signals 1, 2 and 3 contribute to T cell activation, but the timing of these signals also proves to be of great importance. The findings presented here may seem somewhat contradictory, because in one situation we find that naïve T cells that are pre-exposed to inflammatory environments gain the ability to immediately respond to antigen and produce multiple effector functions. However, in another situation the pre-exposure of naïve T cells to IFN caused reduced CD8 T cell expansion, differentiation and development of effector function. How can the same out-of-sequence exposure of signal 3 cytokines cause such different outcomes concerning CD8 T cell activation? The timing at which CD8 T cell activation was assessed can explain the contradictory results. The ability of naïve T cells to produce immediate effector function, if pre-exposed to IFN-induced environments, was assessed within a few hours of cognate antigen stimulation, whereas suppression of CD8 T cell responses was investigated several days after initial exposure to antigen.

During an acute infection, not all T cells will see antigen, costimulation and inflammatory cytokines in the same order. CD8 T cells that come into play later in

infection, so called latecomer T cells, will have been exposed to inflammatory environments prior to antigen. Therefore, identifying how latecomer T cells respond to antigen is of great importance. A model depicting conventional T cell activation, expansion and differentiation (blue), vs. out-of-sequence activation, expansion and differentiation (green) is depicted in **Figure 5.1.**

Under circumstances where naïve T cells encounter antigen, costimulation and signal 3 cytokines in the conventional order, T cell acquisition of effector function requires cell division (141, 161, 162). Conventionally-primed naïve CD8 T cells will efficiently clonally expand and differentiate into a heterogeneous population of effectors that have the capacity to elicit multiple effector functions, including production of cytokines and cytotoxic capability (96). After antigen is cleared, the majority of effector cells die, but a subset of the effector population survives to form memory cells, which have the ability to respond rapidly upon encountering the same pathogen. We show here that out-of-sequence-primed T cells, such as latecomer T cells, adopt an early-activated phenotype associated with the ability to elicit effector functions immediately upon stimulation with cognate ligand, prior to cell division (Chapter 4). As described in Chapter 3, out-of-sequence CD8 T cells show impairment in clonal expansion and defects in effector cell differentiation and function. Moreover, CD8 T cells pre-exposed to IFN-induced environments had reduced memory formation. Therefore, out-of-sequence exposure to IFN may initially stimulate effector function, but this may come at the expense of clonal expansion and subsequent memory formation.

Figure 5.1: Conventional CD8 T cell activation vs. out-of-sequence CD8 T cell activation
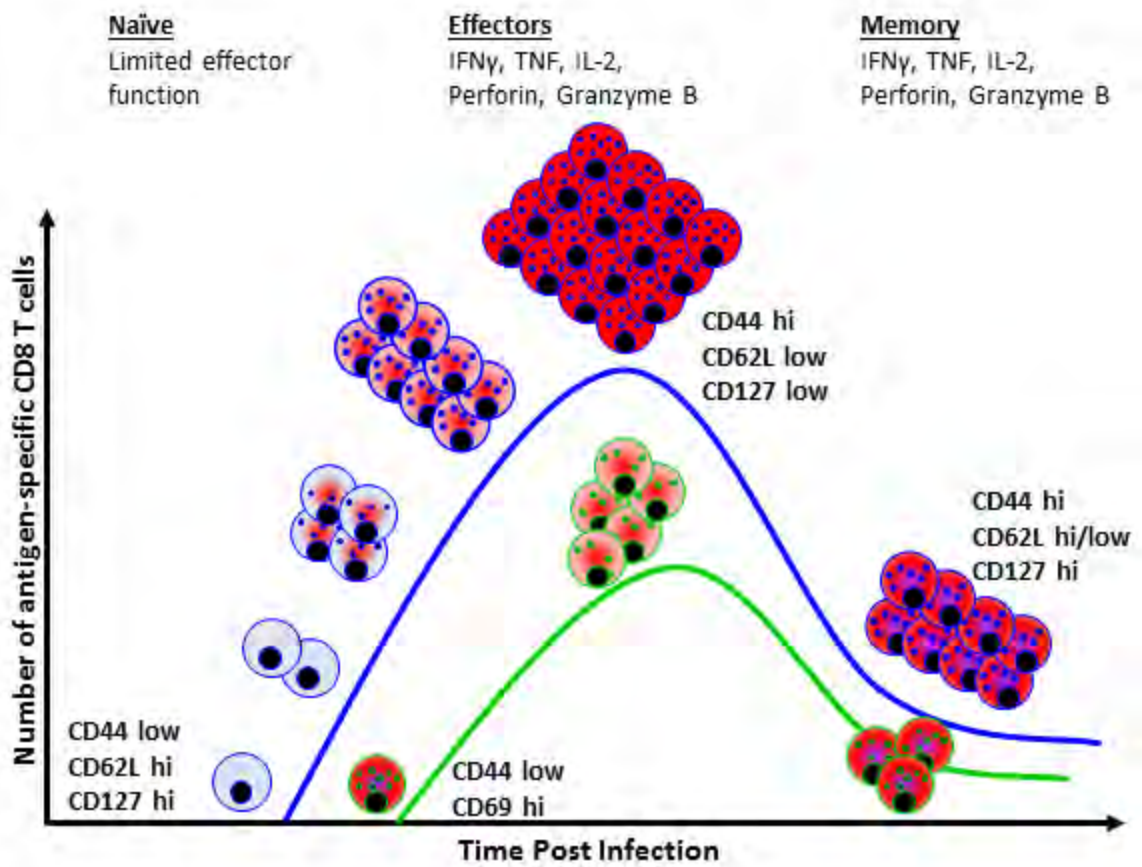
Figure 5.1: Conventional CD8 T cell activation vs. out-of-sequence CD8 T cell activation

During conventional priming (blue), naïve CD8 T cells exhibit limited effector function prior to cell division. Once antigen-specific CD8 T cells have divided, they acquire the ability to produce effector functions, such that upon encountering antigen, effector cells can produce multiple cytokines and can specifically lyse target cells. After antigen is cleared, the majority of effector T cells die but the ones that survive form a memory population which has the ability to respond immediately to cognate antigen, without the need to divide. During out-of-sequence CD8 T cell activation (green), cells that are exposed to inflammatory environments prior to antigen and costimulation have the capability of producing effector functions immediately in response to antigen, without the need to divide. However, out-of-sequence CD8 T cells showed reduced clonal expansion and effector function at the peak of the T cell response. Defects in memory cell formation were also seen in out-of-sequence-primed CD8 T cells compared to conventionally-primed memory formation.

The dichotomy between the major findings here may help to explain how individuals can be either more or less susceptible to secondary or super infections during acute virus infections. During acute infections, third-party antigen-specific T cells will be primed in an out-of-sequence manner. Therefore, T cells may adopt one of the two phenotypes described here: acquisition of immediate effector function or suppression of CD8 T cell responses. If naïve T cells are armed to immediately respond to antigen encounter, individuals may be more resistant to secondary infections. On the other hand, third-party antigen-specific CD8 T cells may not respond as robustly, showing limited expansion and effector function, thus enhancing susceptibility to secondary infections. The timing of exposure to a secondary infection in the midst of an ongoing immune response may play an important role in determining whether or not individuals are more or less susceptible to secondary infections.

B. Stimulatory effects of out-of-sequence signaling

Efficient clonal expansion and differentiation of CD8 T cells are required to develop protective memory CD8 T cells. Conventional naïve T cell activation is associated with changes in expression of a number of different activation markers including CD62L, CD69, and CD127 (137, 138, 141, 200, 206). The upregulation or downregulation of these specific activation markers is thought to be antigen driven. However, we show here that poly(I:C) priming of naïve T cells in the absence of cognate antigen induced an early-activated phenotype characterized by the upregulation of CD69 and CD86 and downregulation of

CD62L and CD127. This phenotype is normally associated with recently-activated antigen-experienced T cells.

In conventionally activated naïve T cells, CD69 is one of the earliest activation markers induced, but its role in T cell function has not been fully elucidated (138). CD69 signaling may contribute to T cell proliferation and effector acquisition by increasing intracellular Ca^{2+} levels and upregulating CD25, (138, 207). That being said, one study determined that lack of CD69 expression on DCs or CD8 T cells did not dramatically affect CD8 T cell expansion (206). We have identified cognate-antigen-independent upregulation of CD69 on naïve T cells after poly(I:C) inoculation (**Figure 4.1-4.3**) and a strong positive correlation between CD69 upregulation and cytotoxic capability (**Figure 4.21**). Intracellular Ca^{2+} is an important signaling component in T cell activation; therefore, CD69 signaling may be contributing to immediate effector functions seen in poly(I:C)-primed naïve T cells upon stimulation with antigen (208).

Another interesting aspect of primed naïve CD8 T cells is the upregulation of CD86. Although CD86 is normally found on activated macrophages, DCs, and B cells, it has recently been shown to be expressed on T cells from some chronic infections, including HIV and HCV (209, 210). Studies have shown that IL-2 signaling can induce the upregulation of CD86 on T cells, yet the function of CD86 on T cells has not been well characterized (209, 210). Both naïve CD8 T cells (**Figure 4.1-4.3)** and CD4 T cells (data not shown) primed with poly(I:C)

rapidly upregulate CD86. Although a role for CD86 on T cells hasn't been identified, it is tempting to speculate that it could provide costimulatory (or inhibitory) signals to other T cells. This might suggest that poly(I:C)-primed naïve T cells could interact with one another via CD4:CD8 or CD8:CD8 and contribute to their immediate effector function. We know that costimulatory molecules CD80 and CD86 in the priming host are required for poly(I:C)-induced arming of T cells, so it is not outrageous to postulate that costimulatory signals could be coming from other T cells.

Priming of naïve T cells with poly(I:C) induces expression of the effector-associated TFs IRF4 and Eomes. TCR stimulation regulates IRF4 expression in a positive manner, where stronger TCR signaling results in more IRF4 expression (148). Therefore, it was quite surprising to see IRF4 upregulation in poly(I:C)-inoculated naïve T cells that have not seen cognate antigen (**Figure 4.4-4.6**). If IRF4 expression is regulated by strength of TCR signals, then why is there such a strong upregulation in primed naïve T cells? Although poly(I:C)-primed naïve T cells do not see cognate antigen, they do encounter self-MHC. A combination of self-MHC:TCR signaling and other inflammation-induced signals, like costimulation and/or cytokines may drive IRF4 expression in primed naïve T cells. IRF4 is important for CD8 T cell effector differentiation. Therefore, the upregulation of IRF4 in primed T cells may give them an advantage over un-primed cells, such as reducing the amount of additional TFs needed to induce effector functions.

Type 1 IFN signaling is required for the early-activated phenotype and upregulation of effector-associated TFs in poly(I:C)-primed naïve T cells. Naïve T cells that lacked the IFNAR did not upregulate CD69 or CD86 (**Figure 4.2**), nor did IFNAR KO naïve T cells induce expression of TFs IRF4 and Eomes (**Figure 4.5**) after poly(I:C) inoculation. Consistent with this, a recent study identified a role for type 1 IFN in inducing Eomes expression in CD8 T cells (211). Expression of Class I MHC and B7 costimulatory molecules from the priming environment also played an important role in naïve T cell priming. Naïve T cells upregulated Eomes and CD69 when primed in B7 KO hosts but did not produce IFNγ when stimulated and assessed for immediate effector functions (**Figure 4.13**).

The upregulation of Eomes in primed naïve T cells was perplexing, because Eomes is associated with memory T cells, not naïve T cells. Eomes expression is known to be high in memory CD8 cells and in a distinct population of cells termed innate-like-lymphocytes, both of which exhibit immediate effector function in response to cognate antigen stimulation (152, 203, 211, 212). Given the role Eomes plays in memory CD8 T cells and innate-like lymphocytes, the presence of Eomes in primed naïve T cells may be one component regulating immediate effector functions. We hypothesized that Eomes upregulation in poly(I:C)-primed naïve T cells could also serve this type of role. Therefore, it was interesting to see that in B7 KO hosts, poly(I:C) induced upregulation of Eomes in naïve T cells, but these primed CD8 T cells did not exhibit immediate effector

function. Virtual memory (VM) cells, are antigen-inexperienced memory-phenotype (CD44hi CD122hi) CD8 T cells generated by homeostatic proliferation. Although VM cells express high levels of both Tbox TFs, Tbet and Eomes, analysis of these cells showed they did not produce significant amounts of IFNγ in response to cognate antigen like conventional memory cells (211, 213-216). This indicated that the presence of Eomes alone is not always sufficient for IFNγ production. In our poly(I:C)-priming model, events required for Eomes upregulation may contribute to, but are not sufficient for, immediate IFNγ production.

IFNγ is regulated by a number of TFs but can also be regulated by epigenetic modifications (217, 218). The differences in epigenetic modifications between naïve CD8 T cells, which do not produce IFNγ, and effector and memory CD8 T cells, which do produce IFNγ, are still being investigated. One thing that has been fairly consistent within the literature is that naïve CD8 T cells show increased CpG methylation of the IFNγ promoter compared to effector and memory CD8 T cells, and this may result in repression or silencing of the gene (218). Therefore, B7 costimulation in poly(I:C)-primed naïve T cells may alter epigenetic modifications of the IFNγ promoter (i.e. demethylation), thus contributing to increased transcription in the presence of appropriate TFs (i.e. Eomes). Naïve T cells primed with cognate antigen in the absence of B7:CD28 costimulation show reduced cytokine production compared to cells that receive costimulation (219). Interestingly, some cytokines, including IL-6 and TNF, have

been shown to compensate for B7 costimulation, in terms of T cell acquisition of cytotoxic effector function. However, these CD28-independent cytokine-stimulated CD8 T cells showed limited cytokine production in response to peptide re-stimulation (118). These observations support the idea that costimulation may be important for epigenetically regulation the IFNγ locus.

We and others have shown that type 1 IFN induces upregulation of Class I MHC and costimulatory molecules (**Figure 4.16**) (32, 74, 205). The combination of signals from self-MHC, costimulation molecules, and IFN during priming may enable naïve T cells to produce immediate effector functions upon stimulation with antigen, but only when a certain threshold of activation signals is reached. Without one of these signals, the ability of primed T cells to elicit immediate effector function may be reduced. It is plausible that there is a specific threshold of signals needed for the T cell to acquire activation markers or proliferate, and it may not matter so much as to where those signals originate as long as the T cell reaches some critical threshold. The combination of signals from self-antigen, costimulation and cytokines may provide sufficient activation of naïve T cells for acquisition of immediate effector function, but the proliferative capability and subsequent effector differentiation may have a higher threshold or require additional signals.

Type 1 IFN induces activation of immature DCs, resulting in upregulation of Class I MHC, and costimulatory molecules (**Figure 4.16**) (32). The activation of immature DCs results in trafficking to LNs and other secondary lymphoid

organs (76, 220, 221). Therefore, upon poly(I:C) treatment *in vivo*, although there is no exogenous antigen present, immature DCs are activated and migrate to LNs, where they encounter naïve T cells. Naïve T cells are constantly trafficking throughout the body in search of cognate antigen (114, 221). Upon exposure to cognate antigen, T cells arrest, but if cognate antigen is not encountered, T cells continue moving (221). In the priming to immediate effector function model described here, it is unknown whether or not T cells are arresting. Are the IFN signals and contact with self-MHC and costimulation enough for a naïve T cell to stop and begin programmed division? I suspect not; I do think, however, that primed naïve T cells might stay in contact with APCs for a longer period of time than conventional naïve T cells do. This longer interaction may not be sufficient for full activation and induction of programmed division, but it may be enough to arm T cells with the ability to immediately produce effector functions in response to cognate antigen.

Chapter 4 identifies how out-of-sequence signaling can affect CD8 T cell function immediately after antigen stimulation. We determined that T cells that are pre-exposed to type 1 IFN-induced environments in the presence of costimulation and self-MHC can manifest immediate effector functions upon stimulation with cognate antigen (**Figures 4.7-4.10**). These armed T cells have the ability to produce effector functions in response to lower concentrations of high-affinity ligands and lower affinity ligands, all without the need to divide

(**Figures 4.17-4.19**). Moreover, T cells gain the ability to specifically lyse target cells, while still maintaining a naïve phenotype (**Figures 4.20-4.21**).

In vitro studies have shown that VACV epitopes can be presented as early as 30 minutes post infection (222). The amount of viral proteins expressed within the cell often correlates with the peptides presented. That being said, not all viral proteins are induced at the same time and expressed to the same levels. Immediately after infection, when viral antigen presentation is low, primed naïve T cells may be able to recognize these recently infected cells because of their ability to respond to lower density antigens. In addition, epitopes from viral proteins that are not expressed as abundantly may be more efficiently recognized by primed naïve T cells. Many viruses have evolved mechanisms to reduce levels of Class I MHC in order to avoid recognition by the adaptive immune system. Therefore, the ability of primed naïve T cells to respond to lower density antigens may also be advantageous to the host, when infected with viruses that employ these evasion strategies (6). Primed naïve T cells may help to initially control infections prior to antigen-specific clonal expansion and may also have the ability to prevent, or control secondary infections.

So far, I have described primed naïve T cells as being an important component in the anti-viral T cell response that may contribute to reduced susceptibility to secondary infections. On the other hand, priming of naïve T cells may have a potentially negative implication and help to explain an association between onset of autoimmune disorders and virus infections. Infections are

thought to promote autoimmune disorders by a number of different mechanisms. Superantigen-induced and so-called bystander-induced activation of self-reactive T cells may contribute to autoimmune disorders. Molecular mimicry may also serve as a potential mechanism of antigen-induced autoimmune disorders (179, 223-225). Virus infections and resulting inflammatory environments may prime self-reactive T cells to become more sensitive to antigen stimulation. I have shown that primed naïve T cells can respond to lower concentrations of antigen and lower affinities of antigen, so self-reactive T cells may be more prone to activation by self-antigen. Poly(I:C) priming may break tolerance to self-antigens, thus allowing naïve auto-specific CD8 T cells to exert effector activity and contribute to induction of autoimmune disorders.

It is possible that the poly(I:C)-induced priming of naïve CD8 T cells by self-antigen, costimulation and IFN may have regulatory effects, rather than anti-viral effects. IFNγ has been shown to induce many of the same effects as type 1 IFN and can even act as a signal 3 cytokine, promoting clonal expansion of CD8 T cells (11). Primed T cell production of multiple cytokines, including IFNγ and TNF, may activate surrounding cells and could even act in an autocrine manner. However, the function of primed naïve T cells production of IFNγ and TNF during viral infections has yet to be determined.

C. Inhibitory effects of out-of-sequence signaling

Transient states of immune suppression occur during many acute viral infections, and it has long been known that individuals should not get vaccinated when they are sick. Virus-induced immune suppression was first noted over 100 years ago. More recent studies have shown immune suppression to be a common element of many viral infections and often to be associated with suppressed T cell proliferation in response to antigens and mitogens (183-189, 191, 192). While studying viral infection models, we recently found that a general mechanism of virus-induced immune suppression could be linked to type 1 IFN, normally induced at high quantities in most viral infections (182). This was somewhat surprising, given that type 1 IFN has been described as a signal 3 cytokine, which drives the expansion and differentiation of T cells after they have encountered cognate ligand (signal 1) and costimulation (signal 2). The primary observation of Chapter 3 is that if T cells are exposed to type 1 IFN inducers before exposure to cognate ligand, they lose their sensitivity to further IFN stimulation and do not receive the benefits of a signal 3 cytokine. Instead, they behave like T cells receiving only two signals, with defects in effector cell differentiation, effector function, expression of a pro-survival protein, and clonal expansion.

Type 1 IFN, like many other cytokines, can render treated cells resistant to further IFN stimulation by down regulating the IFN receptor and by inducing

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factors like SOCS1 that impair IFN-induced signal transduction (28, 30). We show here that T cells pre-exposed to IFN fail to derive the benefit of the positive signal 3 effects of IFN signaling. The implications of this phenomenon are widespread. Because IFN is induced so rapidly during viral infections, one can deduce that the T cells that encounter antigen in the first day or two of infection would respond more impressively than latecomer virus-specific T cells stimulated later in infection. Thus, the dynamics of how much antigen is synthesized and presented vs. how much and how quickly IFN is induced may dictate the efficacy of the host response. Secondly, the T cell response to many acute infections, at least in mouse models, is relatively ordered and undergoes a rather synchronized contraction from 6-9 days post-infection. How can this occur when the amount of T cell proliferation is a programmed event and when different T cells should encounter antigen at different time periods (115-117, 226)? The latecomer T cells, because of their previous exposure to IFN, would not undergo as many divisions and possibly have lower survival properties, thereby enabling them to contract when the rest of the T cells do. Third, we would argue that naïve or memory T cells specific to third party antigens would not respond well to a cognate antigen stimulus if they were first exposed to the IFN milieu of a viral infection and then stimulated with antigen. This failure to respond to recall antigens, such as tetanus toxoid or tuberculin, is a common feature of virus-induced immune suppression in humans, and the weak efficacy of vaccines in already infected individuals may well be a function of the same problem (227,

228). Finally, under conditions when a host develops a persistent viral infection there would be a chronic stimulation of the type 1 IFN response, and such hosts would probably not immunologically respond well to either the antigens of the infecting virus or to third party antigens on challenge. This weak response to third party antigens is not only seen during persistent viral infections but also during chronic autoimmune diseases, such as lupus erythematosus, where signal 3 cytokines may be chronically produced (229, 230). We therefore suggest that the elimination of signal 3 stimulation by out-of-sequence exposure to the signal 3 stimulant, in this case IFN, would be a common factor disrupting T cell responses in the context of acute or persistent viral infections.

Type 1 IFN can have both stimulatory and inhibitory effects on CD8 T cell proliferation, but here it was initially unclear if poly(I:C)-pretreated CD8 T cells were receiving direct inhibitory signals or fewer stimulatory signals from IFN. Since type 1 IFN signaling can act through multiple STATs, each capable of altering cell fate, it might have been expected that poly(I:C)-pretreated CD8 T cells would have had different STAT phosphorylation in response to IFNβ stimulation. Recent work has shown that virus-specific CD8 T cells downregulate total STAT1 and upregulate STAT4, so that, when IFN signals through the IFN receptor, the anti-proliferative effects of STAT1 will be overcome by the positive effects mediated through STAT4 (90, 91, 95). Therefore, poly(I:C)-pretreated CD8 T cells could have had more pSTAT1 and less pSTAT4 than control-treated cells after IFNβ stimulation. However, this was not the case at the time points

studied, as phosphorylation of all tested STATs was reduced (**Figures 3.11-3.13**). The fact that all pSTATs were reduced in poly(I:C)-pretreated CD8 T cells suggested that IFN was not having a direct negative effect other than by desensitizing cells to the positive effects that a later exposure to IFN could mediate.

It should be noted that not only were naïve CD8 T cells unresponsive to IFNβ stimulation after poly(I:C) treatment, but CD4 T cells and NK cells were also refractory to further IFNβ stimulation in terms of STAT phosphorylation (**Tables 3.1-3.4**). Type 1 IFN has been shown to act directly on CD4 T cells, NK cells and B cells to promote effector function, and these results may indicate that in addition to poly(I:C) inducing inhibitory effects on CD8 T cell proliferation, it may also have suppressive effects on other lymphocyte populations that utilize IFN at another time for their activation (15, 48, 49, 60, 67). Indeed, reduced antibody production by B cells and lower NK cell cytotoxicity have been seen under conditions of virus-induced immune suppression (60).

Antigen and costimulatory molecules provide proper signals for T cell activation and differentiation, but more recent studies have focused on the role for inflammatory cytokines in these processes. We find here an additional layer of complexity in that the timing of T cell exposure to signal 3 cytokines is extremely important. If CD8 T cells are unable to receive the positive effects of type 1 IFN, as shown in this study, they should behave more like T cells that only received 2

signals, rather than 3 signals. This was the case, as the out-of-sequence signal 3 CD8 T cells had defects in SLEC differentiation and effector function. Poly(I:C)-pretreated CD8 T cells degranulated, as shown by CD107a/b staining, but they had reduced granzyme B expression (**Figures 3.23-3.25**), suggesting that poly(I:C)-pretreated CD8 T cells have lower cytolytic capabilities at day 5 post-infection. Indeed, reduced T cell responses correlated with increased viral titers at day 6 post infection (**Figure 3.28**).

Another hallmark of 2-signal only CD8 T cells is limited clonal expansion, which in many cases is attributed to decreased survival. Although the exact mechanism is unknown, Bcl-3 prolongs the survival of activated CD8 T cells after signal 3 cytokine addition or CpG adjuvant administration (86-88, 198). The IFN-induced suppression of proliferation seen here may also have been due to a decrease in survival. This idea was supported by poly(I:C)-pretreated CD8 T cells having lower expression of the pro-survival protein Bcl-3 (**Figure 3.21**). We show here that poly(I:C)-pretreated P14 cells also had a delay in cell division compared to HBSS-treated cells in response to LCMV infection (**Figure 3.19-20**). Interestingly, the delay in cell division of poly(I:C)-pretreated P14 cells is seen at day 3 post infection, but not at day 4 post infection, matching the kinetics of the timing of the ability of CD8 T cells to respond to IFNβ signals by phosphorylating downstream STATs (**Figure 3.3**). The positive effects that an inflammatory environment can have on CD8 T cell expansion are also shown here, whereby P14 T cells expanded ~ 20 fold in response to GP33 peptide, but they expanded

more than 30,000 fold in response to LCMV infection. The facts that poly(I:C)-pretreated P14 cells are suppressed in proliferation in response to LCMV infection but not to GP33 peptide stimulation support the idea that the refractoriness to IFN stimulation contributes to reduced expansion (**Figure 3.22**). This mechanism of IFN-induced immune suppression may explain how many virus infections can inhibit T cell responses, by limiting the ability of T cells to receive stimulatory effects from the environment.

As described in chapter 3, if CD8 T cells see signal 3 first, they become refractory to further IFN stimulation and are unable to receive the positive signals that type 1 IFN can provide when delivered at the proper time after antigen and costimulation. This limits their ability to clonally expand, to sustain cytolytic capabilities, and possibly to form effective memory. Our studies show lower proportions and numbers of out-of-sequence CD8 T cells at early memory stages (**Figure 3.20**) and as late as 11 weeks post infection. The effectiveness of the memory cells that do form has yet to be investigated, but this is important to determine because of implications it can have in vaccine design and administration.

D. Conclusions

Studies described in this thesis clarify some complex elements of CD8 T cell activation. Not only does the presence or absence of signals 1-3 contribute to T cell activation, but the timing of these signals also proved to be of great

importance. Thus, under circumstances when CD8 T cells can receive positive signals, such as during an infection or vaccination with adjuvants, out-of-sequence signals can have a profound effect on CD8 T cell expansion and activation. This generalized IFN-induced impairment of proliferation is one example of how out-of-sequence signaling can alter responses to cognate antigen exposure. Another example described here is the ability of out-of-sequence signaling to arm naïve T cells with the ability to produce immediate effector functions in response to reduced antigen densities and specificities. We do not know if the CD8 T cells sensitized to rapid effector function are in fact the same cells that ultimately are suppressed in proliferation. However, we do know that these two changes in T cell response to cognate antigen stimulation occur by very distinct mechanisms (**Figure 5.2**) and occur in virus-induced inflammatory environments; consequently, the impairment of proliferation may contribute to generalized IFN-induced immune suppression, even though there may be an initial transient activation of the T cells.

The discoveries presented here may be extrapolated to many different aspects of the immune system, including a profound and universal mechanism of virus-induced immune suppression seen during both acute and chronic infections. In addition, these studies may describe how both latecomer and third-party antigen-specific T cells behave when and if they encounter cognate antigen in the midst of an ongoing infection. Although they may exhibit the ability to

Figure 5.2 Model: Direct vs indirect effects of IFN in out-of-sequence signaling of naïve T cells
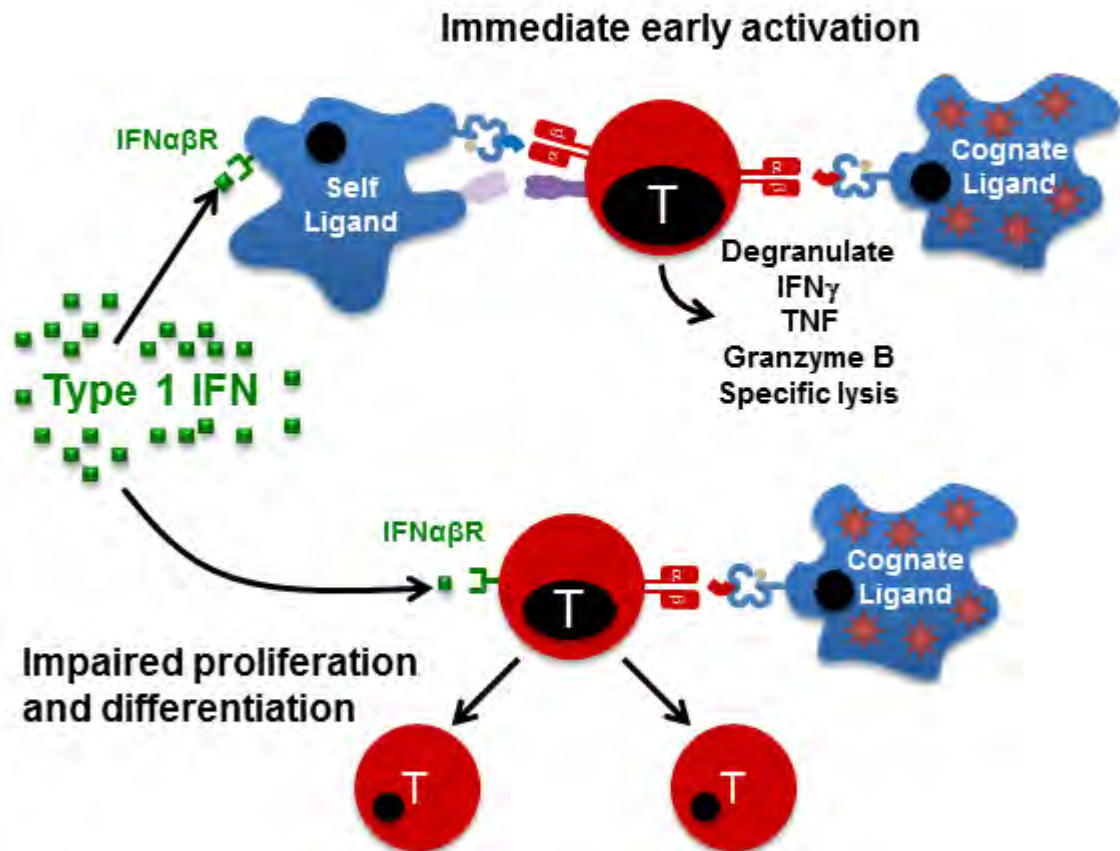
Figure 5.2 Model: Direct vs indirect effects of IFN in out-of-sequence signaling of naïve T cells

IFN plays a role in both mechanisms of out-of-sequence signaling. However, IFN acts indirectly through APCs to mediate priming of T cells to early activation, but directly on T cells to mediate impaired proliferation and differentiation.

respond immediately to antigen encounter, it may be at the expense of clonal expansion, differentiation and memory formation.

These studies may help explain why vaccines are not as effective when administered to individuals with ongoing infections and other immune-mediated disorders. However, the immediate effector function naïve T cells gain during similar out-of-sequence priming may explain how individuals are more resistant to superinfections. Importantly, results identified here can be applied to developing better and more effective vaccines.

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