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The role of type I interferon signaling on T cells during acute viral infections

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“Never allow the fear of striking out keep you from playing the game.”

Table of Contents

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Chapter 1

1. General Summary

1.1 Summary

The proper activation and establishment of a T cell response is important for the clearance of intracellular pathogens, such as viruses. Activation of T cells requires the coordinated effort of multiple signals. Of great importance for the full activation and proper expansion of T cells are the signal three cytokines. These secreted cytokines, such as IL-12 and type I interferons (IFN) can signal directly on activated T cells to ensure appropriate development of an effector T cell response. Viral infections often trigger robust type I IFN production, which plays a key role in the establishment of an innate anti-viral state as well as serving as a signal three cytokine. This thesis describes the role of type I IFN signaling directly on T cells during an acute infection associated with high levels of type I IFNs.

In a first part, the role of type I IFN signaling on T cell differentiation was examined and an important role for type I IFNs in the differentiation of effector T cells was delineated. T cells lacking the ability to sense type I IFNs ($IFNAR^{-/-}$) fail to differentiate into short lived effector cells (SLEC) but retain the ability to differentiate into memory precursor effector cells and to form a fully functional memory cell population. Mechanistically direct type I IFN signaling on T cells was shown to drive the upregulation of the fate determining transcription factor T-bet, thereby revealing a connection between type I IFN signaling and the regulation of T cell differentiation.

In a second part, we demonstrate that the curtailed expansion of $IFNAR^{-/-}$ T cells following an infection associated with high levels of type I IFNs is in part due to natural killer (NK) cell mediated killing of T cells lacking the ability to sense type I IFNs. We show that the inability of T cells to sense type I IFNs results in increased expression of NK cell activating ligands, with a dominant role of activating ligands for natural cytotoxicity triggering receptor 1 (NCR1). Recognition of $IFNAR^{-/-}$ T cells by NCR1 leads to perforin mediated killing of these T cells. These findings expand on our current knowledge of NK cell function and demonstrate a possible immunoregulatory role for NK cells. Importantly, these findings reveal an important role of type I IFNs in the protection of early activated T cells, demonstrating the multifaceted role of type I IFNs during viral infections where they are involved in both the initial innate response and in establishing a proper T cell response.

1.2 Zusammenfassung

Die vollständige Aktivierung und Erzeugung einer T-Zell Antwort ist wichtig für die Bekämpfung von intrazellulären Pathogenen wie Viren. Die effiziente Aktivierung von T-Zellen erfordert den koordinierten Einsatz dreier Signale. Von grosser Bedeutung für die vollständige Aktivierung und Vermehrung von T-Zellen sind Zytokine, die das dritte Signal ausmachen. Diese sekretierten Zytokine, wie IL-12 und Typ I Interferone (IFN), können direkt auf aktivierte T-Zellen wirken, um die Ausbildung einer vollständigen Effektor T-Zell Antwort sicher zu stellen. Viele virale Infektionen induzieren eine starke Typ I IFN Antwort, welche eine wichtige Rolle spielen bei der Etablierung eines angeborenen antiviralen Zustands und auch als Signal drei Zytokin wirken. Diese Dissertation beschreibt die Rolle von Typ I IFN und deren direkte Wirkung auf T-Zellen während einer akuten Virusinfektion, die durch einen hohen Spiegel von Typ I IFN charakterisiert ist.

Im ersten Teil dieser Arbeit untersuchen wir die Rolle von Typ I IFN Signalen und deren Auswirkung auf die T-Zell Differenzierung und zeigen damit die zentrale Rolle von Typ I IFN für die Differenzierung von Effektor T-Zellen. Wir zeigen, dass T-Zellen welche Typ I IFN nicht wahrnehmen können ($IFNAR^{-/-}$), die Fähigkeit verlieren in kurzlebige Effektor Zellen (SLEC) zu differenzieren, nicht aber die Fähigkeit in Gedächtnis Vorläufer Effektor Zellen (MPEC) zu differenzieren und eine voll funktionale Gedächtnis T-Zell Population zu bilden. Mechanistisch zeigen wir, dass direkte Typ I IFN Signale auf T-Zellen die Hochregulierung des schicksalsbestimmenden Transkriptionsfaktors T-bet induzieren kann. Dabei stellen wir eine Verbindung zwischen Typ I IFN Signalen und der Steuerung der T-Zell Differenzierung dar.

Im zweiten Teil dieser Arbeit zeigen wir, dass die eingeschränkte Vermehrung von $IFNAR^{-/-}$ T-Zellen nach einer Virusinfektion, die mit einem hohen Spiegel von Typ I IFN assoziiert ist, zum Teil direkt durch Natürliche Killer Zellen (NK)-vermittelte Eliminierung von T-Zellen gesteuert ist, die Typ I IFN nicht wahrnehmen können. Zusätzlich zeigen wir, dass die Unfähigkeit von T-Zellen Typ I IFN zu erkennen, zu einer erhöhten Expression von NK-Zell aktivierenden Liganden führt. Unsere Arbeit weist auf eine dominante Rolle der NK-Zell-aktivierenden Liganden (NCR1 Liganden) in diesem Prozess hin. Die Erkennung von $IFNAR^{-/-}$ T-Zellen durch NCR1 führt zur Perforin-vermittelten Eliminierung dieser T-Zellen. Unsere Ergebnisse erweitern die aktuellen Kenntnisse der NK-Zell-Funktion und deuten auf eine mögliche immunregulatorische Rolle von NK-Zellen hin. Wichtig ist, dass diese Ergebnisse eine bedeutende Rolle von Typ I IFN im Schutz von aktivierten T-Zellen zeigen. Zusammen

beweisen die Resultate die vielfältige Rolle von Typ I IFN während einer Virusinfektion, wo Typ I IFN nicht nur zu Beginn der angeborenen Antwort wichtig ist, sondern auch bei der Entwicklung einer effektiven T-Zellantwort.

Chapter 2

2. General Introduction

2.1 Antiviral immune response

Despite the advancements in antivirals and vaccine development, viral infections remain a top health concern for people worldwide. Our bodies are faced with daily contact with infectious agents from the environment around us and must be armed and ready to defend itself at any time. One of the more abundant and harmful infectious agents in our surrounding environment are viruses. Viruses are small, obligate parasites consisting of a nucleic acid genome, either DNA or RNA and a protein coating. Being strictly dependent on host cells for replication, viruses are not considered living organisms, but instead are programmed to hijack and exploit various aspects of host cells for their benefit. Viruses come in various shapes and sizes, some viruses consist of only a nucleic acid genome surrounded by a protein shell whereas other viruses are more complex and have a lipid bilayer covering the viral capsid (enveloped virus). The complexity of viruses varies greatly, with certain viruses containing only a few genes and others carrying up to hundreds of genes with them. The high diversity and adaptability of viruses makes them very difficult to defend against. Being intracellular pathogens, elimination of viral infections requires the coordinated effort of the body's defense system. Tasked with this challenge is a complex, multicellular system known as the immune system, which protects our bodies from foreign invaders. Although the immune system contains many diverse cellular players, the immune response can be divided into two key branches, the innate and the adaptive immune responses, which work together and have essential and specific functions in protecting the host.

2.1.1 Innate Immune response

The innate or non-specific immune response is the first line of defense against invading pathogens. Requiring no prior activation, cells of the innate system can respond very quickly, often needing only minutes to become activated. This response consists of physical barriers such as the skin and mucus membranes as well as various chemical and cellular barriers. The first line of defense against environmental viruses is our skin, which builds a thick impenetrable barrier for viruses. However if the skin is compromised, such as a cut or burn, viruses may enter the body. Another initial barrier against foreign substances are the mucus membranes, which serve to trap pathogens and harbor numerous chemicals to help protect from foreign invasion. Mucus membranes are very important as they cover some of the primary entry points for viruses such as the gut and respiratory tract. Although these anatomical barriers are very efficient, sometimes invading pathogens find a way into the body, in this case the next line of innate

defense is waiting and ready. The next level includes numerous cellular players and cytokines, with the main purpose to initiate activation of the immune response.

2.1.1.1 Innate recognition of viruses and induction of inflammation

A crucial aspect of the innate immune response is the ability of responding cells to recognize a viral particle as foreign. This recognition leads to the maturation of responding cells and induction of inflammation which initiates a response against the virus by activating and recruiting various cell types (such as neutrophils, monocytes and NK cells). Viruses are sensed as foreign by their recognition by germ line encoded pattern recognition receptors (PRRs) found on cells at the site of infection. PRRs can sense patterns on pathogens that are not found on host cells, known as pathogen associated molecular patterns (PAMPs). Important among the PRRs are the family of toll like receptors (TLR), which consist of various membrane bound PRRs, each with a specificity for unique molecular components of pathogens that identifies them as being foreign. Recognition of viral particles poses a difficult task for the immune system as a great deal of a viral particle is derived from a host cell. Therefore many TLRs are specialized at sensing viral nucleic acids (Thompson and Locarnini 2007). TLR3 recognizes double-stranded RNA, which is often produced during viral replication. TLR7,8 recognize single-stranded RNA, while TLR9 recognizes unmethylated CpG motifs in DNA, often found in viral and bacterial genomes. However, to ensure that TLRs are responding to foreign nucleic acids and not self-nucleic acids additional special measures have evolved. One way which this is achieved is through the compartmentalization of the TLRs. TLR9 for example, which detects unmethylated CpG motifs in DNA is localized to the late endosomal and lysosomal compartments which ensures that it only senses viral nucleic acid and not self-nucleic acid (Barton, Kagan et al. 2006). Another important viral detection system are the cytosolic RNA receptors retinoic acid inducible gene I (RIG-I) and melanoma differentiation associated gene 5 (MDA5), both of which are expressed ubiquitously and detect viral RNA (Loo and Gale 2011). Both receptors recognize viral dsRNA products in the cytosol however RIG-I can recognize ssRNA as well by sensing the uncapped 5`-triphosphate end of viral RNA, which is not found on capped host RNA (Hornung, Ellegast et al. 2006). In addition to cytosolic RNA receptors, cytosolic DNA sensors are important for detection of viral nucleic acids. Among the cytosolic DNA sensors is absent in melanoma 2 (AIM2) which can bind to both dsDNA and to the adaptor protein apoptosis-related speck-like protein (ASC) (Hornung and Latz 2010). Formation of this AIM2 inflammasome leads to the activation of caspase-1 and the activation and release of the proinflammatory cytokines IL-1β and IL-18. Another important cytosolic

DNA sensor is cGAMP synthase (cGAS) (Panne 2013). Sensing of DNA in the cytosol by cGAS leads to the synthesis of the signaling molecule cGAMP which binds to and activates stimulator of interferon genes (STING) leading to a signaling cascade inducing interferon genes.

Sensing of viral particles by PRRs leads to the activation of signaling cascades that leads to the production of numerous cytokines involved in immune system activation (**Figure 2.1**). The exact composition of the produced cytokines is determined by the nature of the invading pathogen, the PRR signaling pathway initiated and the responding cell type, however among the most prominently produced cytokines are; IL-6, IL-12, TNFa, various chemokines and particularly important to viral infections, type I IFNs. These signals are responsible for the initial activation of the immune response against the invading pathogen and the priming of the immune system for full activation.

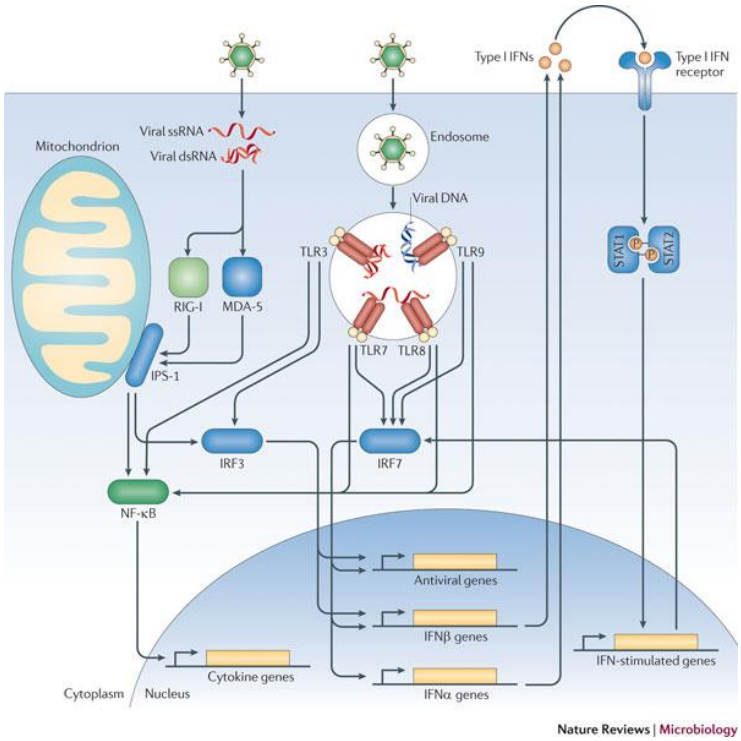


Figure 2.1 Innate sensing of viruses leads to the activation of the immune response (adapted from (Foxman and Iwasaki 2011))

Identification of invading pathogens as being foreign is a hallmark of the immune system. Innate sensing of viruses is often achieved through the recognition of viral nucleic acids. PRRs specialized in sensing different types of viral RNA can be found both in the cytosol, such as RIG-I and MDA-5 which recognize viral double stranded RNA, and in endosomal compartments. TLR compartmentalization into endosomal compartments ensures not only that they are present at a site of viral uptake but also that the TLRs are hidden from host nucleic acids. Signaling through PRRs induces a signaling cascade leading to the activation of genes involved in immune response initiation, important among the anti-viral response is the production of type I IFNS.

2.1.1.2 Type I Interferons

Among the cytokines produced during viral infections following PRR signaling are the type I interferons. Type I IFNs are a family of pro-inflammatory cytokines found conserved throughout the animal kingdom, where all known vertebrates express at least 3 different types of type I IFNs. This family of pro-inflammatory cytokines possess strong antiviral properties and can be induced at high levels following viral infections. This family includes 7 classes: IFN-α (13 subtypes), IFN-β, IFN-ε, IFN-κ, IFN-τ, IFN-δ, and IFN-ω; however, following a viral infection IFN alphas and beta are the predominate subtypes. Type I interferons can be produced by all nucleated cells, however the principle producers during a viral infection are plasmacytoid DCs (Colonna, Trinchieri et al. 2004). All members of the type I IFN family signal through the same signaling complex, which consists of the IFNAR1 and IFNAR2 subunits (Stark, Kerr et al. 1998). Binding of type I IFNs leads to the dimerization of the two receptor subunits, this dimerization brings Jak1, which is associated with IFNAR2 into close contact with the tyrosine kinase Tyk2, which is associated with IFNAR1 leading to the phosphorylation of Tyk2. This leads to the activation of Tyk2 and cross phosphorylation of Jak1 and the subsequent phosphorylation of IFNAR1, providing a docking site for signal transducer and activator of transcription (STAT) proteins. Binding of STATs leads to their phosphorylation and dissociated from the receptor complex and translocation to the nucleus leading to regulation of IFN induced genes. Although all members of the type I IFN family signal through the same receptor complex, their signaling can have various effects on different cell types depending on the responding cell, the timing of the signaling and the engaged type I IFN. The nature of the signal is in part determined by the stability of the binding of the different types of type I IFNs due to distinct receptor binding chemistries (Thomas, Moraga et al. 2011). Type I IFNs were initially discovered for their role in interfering with an infection (leading to their name), where it was shown that an infected cell could secrete a molecule that could prime neighboring cells to be resistant against viral infection (Isaacs and Lindenmann 1957, Isaacs, Lindenmann et al. 1957). Type I IFN signaling leads to the establishment of an antiviral state (Goodbourn, Didcock et al. 2000) (**Figure 2.2**) and induces the expression of various interferon stimulated genes (ISGs). These genes lead to expression of proteins involved in preventing infection such as 2′-5′ oligoadenylate synthases (OAS) which are activated by viral double stranded RNA and lead to the activation of the nuclease, RNase L which cleaves and degrades both viral and host RNA transcripts. Another important interferon stimulated gene is dsRNA-dependent protein kinase (PKR), which is activated by viral double stranded RNA. Upon binding to dsRNA this serine/threonine kinase leads to the blockade of translation by

phosphorylating the eukaryotic translation initiation factor alpha subunit (elF2a), thereby preventing viral protein synthesis. A third important interferon stimulated gene family are the Mx proteins. These large GTPase proteins are found conserved through vertebrates and interfere with virus infection, including several RNA virus family members such as Orthomyxoviruses, Rhabdoviruses and Paramyxoviruses. Although these responses do not discriminate between host and virus, RNaseL is only activated locally due to the instability of the OAS produced signaling molecules, however altogether the overall overcome is the inhibition of viral replication, often at the expense of the host. In addition to the well characterized general responses there are hundreds of other antiviral ISGs (Schoggins, Wilson et al. 2011). In addition to their role in intracellularly limiting viral replication, type I IFN signaling can also lead to the apoptosis of infected cells, which thereby helps clear the virus by removing its host. Although this does lead to the destruction of the host cell, this collateral damage is often necessary to prevent viral spread.

Importantly, type I IFN signaling can also lead to the activation of other immune cells, including phagocytes, NK cells and T cells. Type I IFNs can act directly on T cells and NK cells, leading to their proper activation and acquisition of subsequent effector functions resulting in elimination of infection. Additionally, type I IFNs can act on antigen presenting cells to promote their full activation and maturation. Type I IFN signaling on DCs leads to better antigen presentation (Santini, Di Pucchio et al. 2002), including cross presentation and cross priming of $CD8^+$ T cells, ensuring the efficient induction of antiviral T cell responses against viruses that do not directly infect antigen presenting cells (Le Bon, Etchart et al. 2003). The importance of type I IFNs during viral infections is demonstrated by the fact that infection of mice lacking the type I IFN receptor fail to properly control viral replication of Vesicular Stomatitis virus (VSV), Semliki Forest virus (SFV), Vaccinia Virus (VV) and lymphocytic choriomeningitis virus (LCMV) (Muller, Steinhoff et al. 1994). Together these functions of type I IFNs contribute not only to the establishment of an initial antiviral response but also ensure that the following immune response is properly activated.

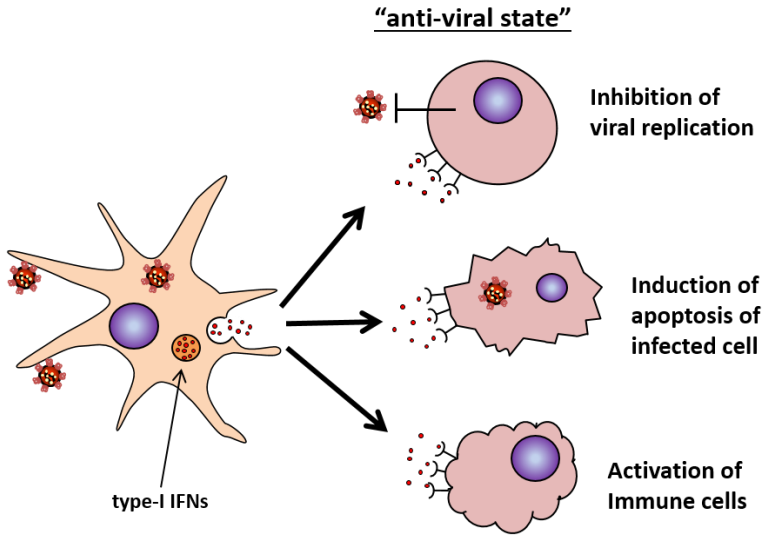


Figure 2.2 Type I IFN effects on neighboring cells

Following production of type I IFNs induced by PRR recognition of viral particles, type I IFNs signal to neighboring cells to establish an antiviral state. This includes the induction of many genes involved in the inhibition of viral replication, such as OAS and PKR. Additionally type I IFNs can induce the apoptosis of infected cells to limit virus spread. Importantly, type I IFNs can lead to the activation of numerous immune cell type, including antigen presenting cells, NK cells and T-cells.

2.1.1.3 Natural Killer cells

Among the innate immune cells activated by type I IFNs are the natural killer (NK) cells (Gidlund, Orn et al. 1978, Biron, Sonnenfeld et al. 1984). NK cells are natural born killers, armed with the ability to eliminate infected or altered host cells. NK cells were initially discovered as a cell type with cytotoxicity against certain tumor target cells without prior sensitization to the targets (Herberman, Nunn et al. 1975, Herberman, Nunn et al. 1975, Kiessling, Klein et al. 1975, Kiessling, Klein et al. 1975). Due to their ability to eliminate target cells without prior activation NK cells play a central role in the innate immune response and are important contributors to the clearance of certain viral infections such as MCMV and VV and contribute to the elimination of cancerous cells (Bukowski, Woda et al. 1983, Wu and Lanier 2003). NK cell cytotoxicity of target cells can be mediated either via the secretion of perforin/granzyme containing vacuoles, similar to cytotoxic T cell killing or via signaling through the death receptor pathway through tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and Fas ligand up-regulation on NK cells (Smyth, Cretney et al. 2005). Due to the ability of NK cells to eliminate host cells they need to be tightly regulated to protect the host from immunopathology and ensure that only altered cells are eliminated. Therefore NK cells recognize target cells through an array of germ line encoded activating and inhibitory receptors on their cell surface (Cerwenka and Lanier 2001). NK cells use these receptors to constantly scan host cells for "warning" signals, sensed in the form of an alteration of activating

or inhibitory ligands on host cells. When signals from activating ligands outweigh those of inhibitory signals NK cell effector functions are triggered (**Figure 2.3**).

In healthy steady state a host cell with display more inhibitory signals on its surface, signaling to the NK cells that nothing is wrong, leading to NK cell tolerance. Triggering of inhibitory receptors on NK cells leads to the phosphorylation of immunoreceptor tyrosine-based inhibitory motifs (ITIMs) found on the cytoplasmic tails of inhibitory receptors. This phosphorylation leads to the recruitment of phosphatases, resulting in dampening of NK cell activating signals (Lanier 2005). Inhibitory ligands are often host molecules that are constitutively expressed and sensed by NK cells as a loss of expression ("missing self"). One of the best described and most important NK cell inhibitory receptor families are the Ly49 receptors (KIRs in humans) which recognize MHC-I molecules (Karlhofer, Ribaudo et al. 1992) and transmit a negative signal to NK cells. Inhibitory ligands, such as MHC class I, are important for maintaining NK cell self-tolerance and preventing autoimmunity (Yokoyama 1995). Other inhibitory receptors include but are not limited to: 2B4 which recognizes CD48 (Lee, McNerney et al. 2004), the NK-cell receptor protein 1 (NKR-P1B and D) which recognize C-type lectin-related B (CLR-b) (Carlyle, Jamieson et al. 2004) and CD94-NKG2A which binds the MHC like molecule Qa1 (Houchins, Lanier et al. 1997). The importance of NK cell inhibitory receptors is demonstrated by the fact that many viruses have evolved mechanisms to down regulate or block MHC-I presentation, which prevents presentation of viral antigens to T cells and thereby protects the infected cells from T cell recognition and elimination (Tortorella, Gewurz et al. 2000). However, NK cells can then recognize this "missing self" and clear the infected cell.

Lacking antigen specificity, NK cells, in addition to the above mentioned inhibitory receptors, express multiple activating receptors that trigger NK cell effector functions following sensing of cellular stress ligands or direct recognition of viral products. During an event of cellular stress such as a viral infection, cells will shift this balance in a way that NK cells recognize something is wrong and eliminate the altered cell. In such a case the expression of activating ligands can override the inhibitory signals. NK cell activating receptors associate with adapter proteins that contain immunoreceptor tyrosine-based activation motifs (ITAMs). These adapter proteins such as FcεRIγ, CD3ζ and DAP12 then transmit the activating signal to trigger NK cell effector functions (Lanier 2005). Among these activating receptors are CD16, a low affinity receptor for IgG which is involved in antibody-dependent cell cytotoxicity (Perussia, Tutt et al. 1989), the activating receptor NKG2D which recognizes the stress induced MHC-I like ligands Rae1, Mult1 and H60 (ULBP and MIC in humans) (Eagle and Trowsdale 2007), and the natural

cytotoxicity receptors (NCRs). NCRs consist of three members in humans (NKp30, NKp44, NKp46) and one in mice (NKp46, NCR1) (Kruse, Matta et al. 2014). Some activating receptors such as Ly49H and NCR1 (NKp46) can directly bind viral components such as the MCMV viral protein m157 and influenza hemagglutinin, respectively, which are important for protection against these viral infections (Arase, Mocarski et al. 2002, Smith, Heusel et al. 2002, Gazit, Gruda et al. 2006), In such a situation the upregulation of activating ligands can overcome the inhibitory signal given by MHC-I.

To ensure protection of the host, NK cells should remain tolerant to normal self, therefore NK cells undergo education or "licensing" during their development (Orr and Lanier 2010). NK cell tolerance was initially thought to be controlled by inhibitory receptors that bind MHC-I, however NK cells from MHC-I deficient hosts are unresponsive to cells lacking MHC-I, indicating that the environment present during NK cell development shapes NK cell activity. Similarly, NK cells from mice, which do not express NKG2D ligands have the ability to reject cells expressing NKG2D ligands, however NK cells from mice constitutively expressing NKG2D ligands no longer have the ability to reject NKG2D ligand expressing cells. Demonstrating that NK cell development in the presence of ligands for activating receptors leads to tolerance to these ligands. The mechanisms of how NK cell licensing occurs is a current topic of research and multiple models have been developed to explain this process (Hoglund and Brodin 2010). One hypothesis states that NK cells are initially found in an unresponsive state and engagement of MHC-I leads to the "arming" of NK cells. Whereas another theory is based on the idea that NK cells are initially armed due to signaling through activation receptors but binding to MHC-I "disarms" the cells and a total lack of MHC-I binding would lead to overstimulation by host cells leading to NK cell anergy. A third model takes the first two into account and is based on the strength and frequency of engagement of inhibitory receptors. This model termed "rheostat model" claims that the quantitative signal determines the education process. Of note, unlike T cell development, NK cell education does not lead to the clonal deletion of NK cells but instead for example, NK cells that lack MHC-I receptors remain in an unreactive state.

Although NK cells are specialized killers they additionally have the ability to secrete various cytokines that act on immune cells (Biron, Nguyen et al. 1999). Important among the NK cell secreted cytokines during viral infections is IFN-γ, a type II interferon which can lead to the activation of certain phagocytic cells and induce differentiation of helper T cells (Martin-Fontecha, Thomsen et al. 2004). Therefore, due to the ability of NK cells to both carry out direct

cytotoxicity and cytokine secreting ability, NK cells play an important role not only in innate killing of target cells but also in the regulation of the immune response.

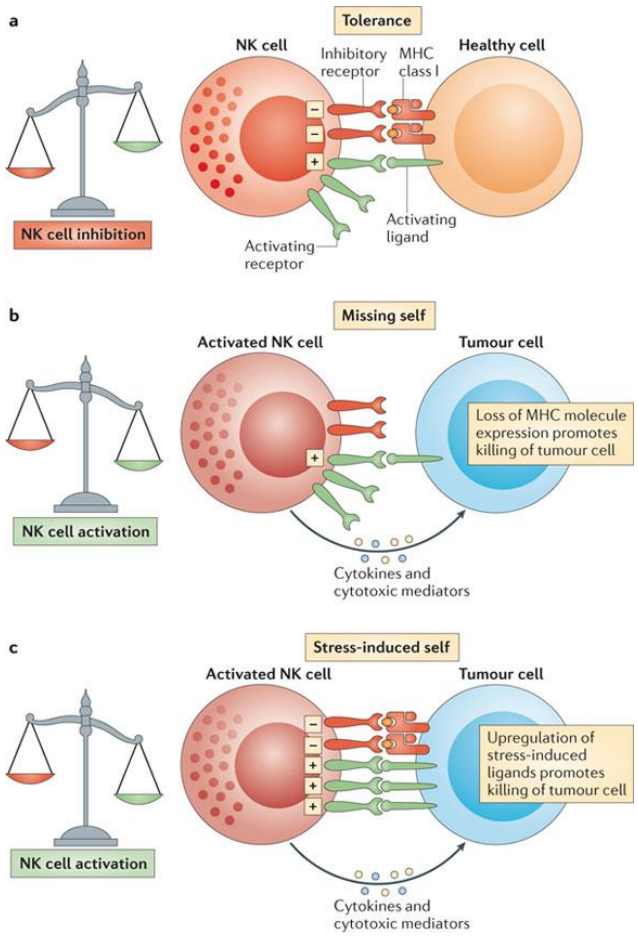


Figure 2.3 NK cell activation

Lacking antigen specificity NK cell recognize target cells through the expression of an array of activating and inhibitor receptors. NK cells use these receptors to sense whether a host cell is healthy or altered, such as a tumor cell or infected cell. A healthy cell will express on its surface more inhibitory ligands than it does activating which leads to NK cell tolerance (a). However if a host cell loses its expression of MHC-I (b) or gains expression of activating ligands (c) the balance of signaling is shifted and NK cell effector function are triggered leading to the elimination of the target cell. (adapted from (Vivier, Ugolini et al. 2012))

2.1.2 Adaptive Immune response

Although the innate immune response is very efficient at protecting the body against invading pathogens, many infectious agents can penetrate the body and overwhelm the innate immune response. In such a situation the body's specific second line of defense becomes activated to remove the invading pathogen. This second line of defense, known as the adaptive, acquired or specific immune response is activated in an antigen specific manner and requires around a week to reach full activation. A hallmark of the adaptive immune response is the ability to generate immunological memory, which leads to protection of the host from reinfection and is the basis of vaccinations. The key cellular players of the adaptive immune response are the B cells and

T cells. B cells are responsible for the production of antibodies, which upon secretion can lead to the neutralization of viral particles. The other important adaptive immune cell type are the T cells, which can be divided into two subtypes; helper T cells and cytotoxic T cells.

2.1.2.1 Antiviral cytotoxic T cells

Cytotoxic T cells, also known as $CD8^+$ T cells based on their expression of the co-receptor CD8 are critically important for protection against viral infections. Cytotoxic T cells, also known as killer T cells are specialized at recognizing and killing infected host cells. This feature of cytotoxic T cells makes them critical for the protection from intracellular infections such as viral infections. Unlike the cells of the innate immune response T cells need to be antigenically activated before they gain effector functions. Proper activation, expansion and differentiation of T cells is critical for the clearance of viral infections and this activation is dependent on three key signals; antigen presentation, co-stimulation and cytokine signaling (Mescher, Curtsinger et al. 2006) (**Figure 2.4A**). The first signal, antigen presentation determines the specificity of the response. T cells with a specific T cell receptor (TCR) for a particular antigen can recognize this antigen in association with an MHC-I molecule on the surface of an antigen presenting cell. Antigen presentation can occur on any nucleated cell, however there are specialize cells known as antigen presenting cells (APCs) which have the ability to not only process and present antigen but also to induce the other necessary signals for proper T cell activation. The second important signal for T cell activation comes in the form of co-stimulatory molecules found on the surface of APCs. These molecules are up-regulated upon APC stimulation through PRRs and signal to T cells to augment TCR signaling. A key costimulatory receptor found on all T cells is the immunoglobulin superfamily member CD28 which binds the costimulatory ligands CD80 (B7-1) and CD86 (B7-2) on activated APCs. At steady state both of these ligands are found expressed at low levels, however following APC activation these ligands are rapidly upregulated (Sharpe and Freeman 2002). Where the first two signals come from contact dependent interactions between the responding T cell and APC, signal three comes in the form of secreted cytokines. These secreted cytokines are induced by APC activation and can signal directly on T cells. The importance of signal three cytokine signaling for sustained expansion and differentiation of T cells has been demonstrated in various infection models (Kolumam, Thomas et al. 2005, Aichele, Unsoeld et al. 2006, Cui, Joshi et al. 2009, Keppler, Theil et al. 2009). The two most studied signal three cytokines are IL-12 and type I IFNs. IL-12 and type I IFNs are often expressed in an almost mutually exclusive manner, as type I IFNs can repress the expression of IL-12 (Cousens, Orange et al. 1997), with the nature of the pathogen

determining which cytokine is more predominantly expressed. Although regulated differently and signaling through distinct signaling pathways, these two cytokines can signal in a redundant manner. In the case of LCMV infection T cell responses are critically dependent on type I IFNs, where the inability to directly sense type I IFNs leads to dramatically curtailed expansion of T cells (Kolumam, Thomas et al. 2005, Aichele, Unsoeld et al. 2006); whereas during a *Listeria monocytogenes* infection the absence of IL-12 signaling leads to impaired expansion of T cells (Keppler, Theil et al. 2009). This three signal system ensures that multiple checkpoints are operating for the proper activation of T cells. This ensures that a T cell is indeed being activated by an invading pathogen and not becoming activated to a self-antigen which could lead to autoimmunity.

Following activation, antigen specific T cells undergo a program of clonal expansion resulting in a large pool of specific effector T cells. This process is necessary to produce enough antigen specific effector T cells to fight the infection, as it has been estimated that the precursor frequency of naïve antigen specific T cells is about 1 in every 10^5 T cells, which corresponds to about 50-100 T cells in the spleen of a mouse (Blattman, Antia et al. 2002). During this expansion phase T cells begin to rapidly divide and at the peak can reach approximately 10^7 antigen specific T cells, this roughly 10^4 fold expansion demonstrates that during clonal expansion T cells can divide around once every 6 to 8 hours (Murali-Krishna, Altman et al. 1998). This large pool of effector cells is armed with cytolytic and cytokine secreting activity which contributes to the elimination of infected cells. $CD8^+$ T cell killing occurs by the recognition of infected cells via MHC-I presentation of viral peptides, followed by the directed release of cytotoxic granules containing perforin and granzymes or signaling through the death receptor pathway (Berke 1995). Following clearance of an infection the majority of T cells undergo apoptosis leaving behind a population of T cells that form a memory compartment, armed with the ability to protect the host against reinfection due to their ability to respond quicker to antigen re-exposure (Wherry and Ahmed 2004).

However at the peak of the T cell response the large pool of effector cells is functionally and phenotypically heterogeneous and can be divided into two main subsets (**Figure 2.4B**). These subsets can be distinguished according to their ability to form terminally differentiated effector cells or long-lived memory cells; referred to as short-lived effector cells (SLECs) and memory precursor effector cells (MPECs) respectively (Lefrancois and Obar 2010). These two populations can be phenotypically distinguished by their expression of the surface markers killer cell lectin-like receptor G1 (KLRG1) and CD127 (Joshi, Cui et al. 2007). In general,

functionally, SLECs carry out the majority of cytolytic effector functions and mainly die as T cells undergo contraction following viral clearance, whereas MPECs perform limited effector functions and survive the contraction phase to form the memory cell population; however there is plasticity in these differentiation pathways.

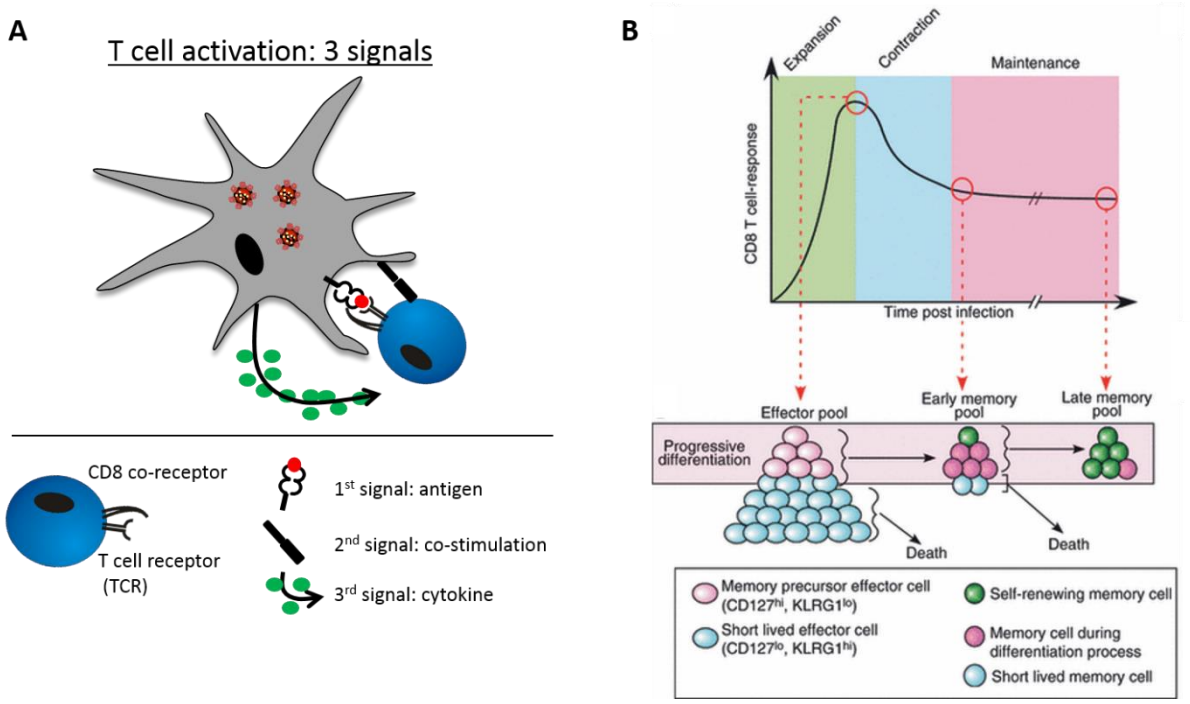


Figure 2.4 Antiviral CD8$^+$ T cell activation and differentiation

(a) Proper activation of T cells requires three signals. The first coming from antigen presentation by MHC-I molecules, recognized by the TCR, this determines the specificity of the response. The second signal comes in the form of membrane bound molecules that are upregulated upon infection. Lastly the third signal comes in the form of secretion cytokines which can signal directly on the T cells to ensure proper T cell activation. (b) T cell activation leads to the clonal expansion of antigen specific T cells. These cells are armed with the ability to clear the infection. Following clearance of the infection, the majority of T cells undergo a contraction phase leaving behind a population of memory T cells. The differentiation of T cells leads to the formation of two main populations, the SLECs and MPECs each specialized with specific roles in the T cell response (adapted from (Araki, Youngblood et al. 2010)).

There is strong evidence that inflammatory cytokines present during CD8$^+$ T cell priming play a key role in the effector and memory fate decision process (Cui and Kaech 2010). In support of this notion it has been shown that IL-12 signaling is mandatory for driving activated CD8$^+$ T cells towards a SLEC phenotype upon infection with *Listeria monocytogenes* but not vesicular stomatitis virus (VSV), vaccinia virus (VV) or lymphocytic choriomeningitis virus (LCMV) (Keppler, Theil et al. 2009). Similar to IL-12, type I IFN signaling has been shown to support the proliferation and development of cytolytic activity of CD8$^+$ T cells *in vitro* (Schmidt and Mescher 2002, Curtsinger, Johnson et al. 2003, Curtsinger, Lins et al. 2003, Agarwal, Raghavan et al. 2009, Xiao, Casey et al. 2009) and can act as an adjuvant *in vivo* to a variety of stimuli (Curtsinger, Schmidt et al. 1999, Le Bon, Etchart et al. 2003, Pearce and Shen 2007,

Cui, Joshi et al. 2009, Sikora, Jaffarzad et al. 2009). IL-12 and type I IFNs were shown to support programming of memory $CD8^+$ T cells in response to *Listeria monocytogenes* and VV infection (Xiao, Casey et al. 2009). Moreover, it was recently shown that prolonged IL-2 signaling on $CD8^+$ T cells during the priming with LCMV promotes SLEC differentiation (Kalia, Sarkar et al. 2010, Pipkin, Sacks et al. 2010). Thus, depending on the nature of the infection, the associated cytokine milieu critically regulates effector and memory $CD8^+$ T cell development.

2.1.2.2 Antiviral helper T cells

Helper T cells, also known as $CD4^+$ T cells based on their expression of the co-receptor CD4 are coordinators of the adaptive immune response and exert the majority of their effector functions by providing "help" to other immune cell types. Like $CD8^+$ T cells, helper T cells are activated in an antigen dependent manner, requiring the previously described three signals however $CD4^+$ T cells recognize antigen presented by APCs on MHC-II molecules. In contrast to the killer T cells, helper T cells are specialized at providing activating and differentiation signals to various other immune cell types, including B cells and $CD8^+$ T cells. This help provided by $CD4^+$ T cells can come in a contact dependent manner or via the secretion of chemokines and cytokines. In order to exert the various different effector functions naïve $CD4^+$ T cells can differentiate into various specialized helper subsets based on the type of the invading pathogen and associated inflammatory signals (Swain, McKinstry et al. 2012).

Among the helper subsets that are particularly important during viral infections are the T_{H1} cells. The production of inflammatory cytokines associated with viral infections such as IL-12 and IFN-γ induce the differentiation of naïve $CD4^+$ T cells into T_{H1} cells (Seder, Gazzinelli et al. 1993) through the activation of the transcription factor T-bet (Szabo, Kim et al. 2000). At the same time viral associated inflammatory cytokines can repress the differentiation into T_{H2} cells, which being induced by IL-4 (Swain, Weinberg et al. 1990) are more important during allergic and parasitic responses. Upon activation and differentiation T_{H1} cells can produce inflammatory cytokines such IFN-γ and TNF which are important for the recruitment and activation of phagocytes such as macrophages to sites of infections. Additionally these helper T cells are important for providing help to $CD8^+$ T cells (**Figure 2.5**).

Help to $CD8^+$ T cells is often provided in an indirect manner via the "licensing" of APCs by CD40-CD40L interactions between APCs and activated helper T cells (Bevan 2004). These activated APCs have higher expression levels of co-stimulatory molecules such as CD80 and CD86, and can also produce cytokines to augment $CD8^+$ T cell activation. Another mechanism

of help provided to $CD8^+$ T cells can come from the secretion of chemokines following $CD4^+$ T cell-DC interactions which can recruit naïve $CD8^+$ T cells to activated DCs (Castellino, Huang et al. 2006). Certain viruses are more dependent on help than others (Wiesel and Oxenius 2012) and it is thought that viruses such as LCMV which induce a strong activation of APCs via PRRs "licenses" them and allows for the bypass of the need for $CD4^+$ T cell help. However following a VV infection $CD4^+$ T cells help is needed for the proper survival of responding $CD8^+$ T cells and this help is provided by CD40L dependent induction of IL-12 production by DCs which signals on $CD8^+$ T cells to upregulate the IL-2 receptor to sense IL-2 provided by the helper T cells (Wiesel, Joller et al. 2010). Interesting, it has been shown that type I IFNs can replace the help dependence of $CD8^+$ T cells during a VV infection (Wiesel, Kratky et al. 2011).

Although $CD8^+$ T cells are important for the clearance of intracellular pathogens, most effective vaccines induce high levels of neutralizing antibodies. During viral infections $CD4^+$ T cell help provided to B cells is necessary for the class switching and production of high-affinity neutralizing antibodies. $CD4^+$ helper T cells that are specialized at establishing the germinal center reaction and providing help to B cells are called follicular helper T cells (T_{FH}). This subset of helper cells is induced by the cytokines IL-6 and IL-21 which lead to the activation of the transcriptional repressor Bcl-6 (Nurieva, Chung et al. 2009). These activated T_{FH} cells can produce cytokines such as IL-4 and IL-21. The importance for $CD4^+$ T cells in the production of antibodies has been shown for various virus infection models (Thomsen, Nansen et al. 1997, Liu and Chambers 2001, Sette, Moutaftsi et al. 2008, Chen, Lau et al. 2010); however, the exact subset of helper cell responsible for these effects remains a question. Due to the plasticity of differentiation of helper T cell subsets it is possible that T helper subsets such as T_{H1}, T_{H2} and T_{H17} can be reprogrammed to gain T_{FH} function and thereby provide help for B cells (Lu, Kanno et al. 2011). Similar to the help provided to $CD8^+$ T cells, $CD4^+$ T cells can also provide help to B cells via CD40-CD40L signaling, which promotes B cell activation and antibody production. Other molecules such as inducible T cell costimulatory (ICOS) and SLAM-associated protein (SAP) are important for the formation of germinal centers (Crotty 2011). Together, depending on the signals given to B cells, different antibody classes can be produced, leading to opsonization and neutralization of the virus leading to the control of infection.

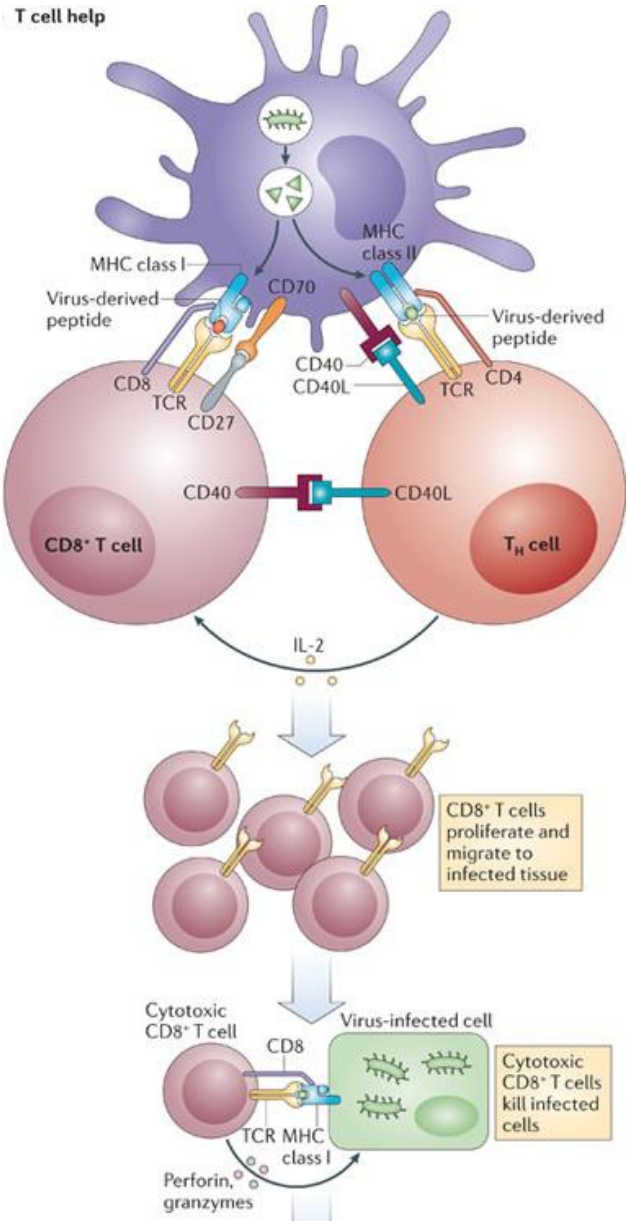


Figure 2.5 CD4$^+$ T cell help to CD8$^+$ T cells

CD4$^+$ helper T cells provide activating signals both directly to CD8$^+$ T cells and indirectly through the licensing of APCs. CD40-CD40L interactions between activated CD4$^+$ T cells and DCs licenses these DCs with the ability to activate CD8$^+$ T cells. (adapted from (Swain, McKinstry et al. 2012))

2.1.3 NK cell regulation of T cell responses

NK cells, being early activated innate lymphoid cells armed with not only cytotoxic effector functions but also cytokine secretion ability are placed in a central position in the immune response to viral infections. In addition to their direct role in viral clearance and control of "altered" self-cells, NK cells also regulate T cell responses in a positive and negative manner. Such regulation can occur in an indirect manner, for instance via the elimination of antigen-presenting dendritic cells, which has a negative impact on the induction of T cell responses and

the success of vaccination (Hayakawa, Screpanti et al. 2004, Andrews, Estcourt et al. 2010). In a direct manner, NK cells were shown to regulate T cell responses through cytokine secretion or direct cytolysis. NK cell production of IL-10 following MCMV infection has a negative impact on MCMV-specific T cell numbers (Lee, Kim et al. 2009) whereas NK cell secretion of IFN-γ can promote $CD4^+$ T_{H1} cell polarization (Martin-Fontecha, Thomsen et al. 2004). Importantly, there is growing evidence implicating a direct regulatory role, whereby regulatory NK cells can directly kill $CD8^+$ and $CD4^+$ T cells (Lu, Ikizawa et al. 2007, Waggoner, Taniguchi et al. 2010, Soderquest, Walzer et al. 2011, Lang, Lang et al. 2012, Waggoner, Cornberg et al. 2012, Waggoner, Daniels et al. 2014), thereby affecting the overall size of the antigen-specific T cell pool and consequently impacting on the outcome of an infection. Multiple NK ligands have been proposed to play a role in this killing process; blockade of the activating receptor NKG2D leads to enhanced $CD8^+$ T cell responses in the context of peptide vaccination (Soderquest, Walzer et al. 2011) and high dose LCMV infection (Lang, Lang et al. 2012). Furthermore, expression of the inhibitory ligands Qa1 and CD48 on T cells can confer protection against NK cell mediated lysis (Lu, Ikizawa et al. 2007, Waggoner, Taniguchi et al. 2010). Once considered simply innate killer cells, recent findings have expanded our knowledge on the role of NK cells in regulating the immune response where they can act as important contributors to both the innate and adaptive responses.

2.1.4 Lymphocytic choriomeningitis virus

Lymphocytic choriomeningitits virus (LCMV) is the prototypic virus of the *Arenaviridae* family. LCMV is a spherical enveloped virus with a diameter of around 100 nm. LCMV contains an ambisense bi-segmented RNA genome containing two negative sense ssRNA segments encoding 4 proteins. The S segment encodes a nucleoprotein and glycoprotein which is posttranslationally cleaved into the two glycoproteins GP-1 and GP-2 (Riviere, Ahmed et al. 1985), whereas the L segment encodes an RNA dependent RNA polymerase (Salvato, Shimomaye et al. 1989) and the Z polypeptide (Salvato and Shimomaye 1989). LCMV spreads naturally in *Mus musculus*, the common house mouse where it can be transmitted through urine or vertical transmission from mother to offspring. Although rare, human infection can occur where the infection can lead to fever, headache and nausea. Less frequently the infection can spread to the central nervous system and cause meningitis or encephalitis. Different strains of LCMV isolated from different organs can cause different infectious outcomes. Low dose infection with LCMV-WE or LCMV Armstrong leads to an acute infection which is cleared after approximately one week. However infection with LCMV clone 13 (isolated from the

spleen of an Armstrong infection) or LCMV docile can lead to a persistent infection accompanied by T cell exhaustion. These differences in LCMV Armstrong and Clone 13 can be accounted for by two amino acid differences affecting the viral polymerase and glycoprotein (Salvato, Borrow et al. 1991).

LCMV is a non-lytic virus which primary infects non-lymphocytic cells such as macrophages, dendritic cells, endothelial cells and hepatocytes. A hallmark of LCMV infection is the induction of a dominant type I IFN cytokine environment (Merigan, Oldstone et al. 1977). The induction of type I IFNs following LCMV infection is due to sensing of viral RNA by the PRRs such as RIG-I and MDA5 (Zhou, Cerny et al. 2010). These high levels of type I IFNs lead to the activation of NK cells following infection with a peak of NK cell activity, mirroring the levels of type I IFNs found at day three post infection (Welsh 1978). Accordingly, NK cells lacking the ability to sense type I IFNs following an LCMV infection are unable to lyse Yac-1 target cells, demonstrating the importance for type I IFNs in the activation of NK cells following an LCMV infection (Biron, Nguyen et al. 2002). Paradoxical, although LCMV induces a strong activation of NK cells, clearance of the infection is not dependent on NK cell mediated cytolysis (Bukowski, Woda et al. 1983). However, clearance of LCMV infection is dependent on $CD8^+$ T cell mediated perforin dependent cytotoxicity (Fung-Leung, Kundig et al. 1991, Kagi, Ledermann et al. 1994, Walsh, Matloubian et al. 1994). $CD8^+$ T cells robustly expand following LCMV infection with a peak around day 7 - 8. Two main specificities of $CD8^+$ T cell are observed at the peak of the response, gp33 and np396 specific T cells, which correspond to immunodominant epitopes in the glycoprotein and nucleoprotein respectively. These activated T cells are armed with strong effector functions, including secretion of immune cytokines such as IFN-γ and TNF and cytolytic capacity following an acute LCMV infection. However during a persistent LCMV infection $CD8^+$ T cells lose their effector functions hierarchically, where the ability to produce IL-2 and to proliferate *in vitro* are lost first followed by loss of the ability to produce TNF and IFN-γ and lastly leading to physical deletion of the cells (Shin and Wherry 2007).

Due to the strong induction of T cell responses during LCMV infection and the presence of various isolates, LCMV has been used to discover multiple fundamental concepts of the immune system including MHC restriction (Zinkernagel and Doherty 1974, Doherty and Zinkernagel 1975), T cell exhaustion (Moskophidis, Lechner et al. 1993) and persistent viral infection (Traub 1936). These key findings have significantly added to the fields of

immunology and virology and some findings have been extended to human viral infections such as HIV and hepatitis C.

2.1.5 Vaccinia virus

Vaccinia virus (VV) is the prototypic virus of the poxvirus family. VV is a large enveloped virus containing a DNA genome. Due to its large size (400x250x150 nm) VV was the first virus observed by microscopy. VV contains a linear DNA genome that encodes around 250 proteins. Unusual for DNA viruses, VV replicates entirely in the cytoplasm of host cells and therefore carries with it all the necessary transcriptional machinery. Historically, VV was used as a live vaccine against small pox (variola virus) and due to the efforts of the world health organization led to the complete eradication of smallpox by 1977, to date the only human viral disease to be eradicated. Due to this success, the use of VV as a transport vector for other vaccines has been of great interest.

Similar to LCMV infection, VV infection leads to a strong expansion of $CD8^+$ T cells, with a peak of T cell expansion around day 5 post infection. However unlike LCMV, VV induces predominantly an IL-12 cytokine environment, with very little type I IFNs present (Keppler, Rosenits et al. 2012). This difference in cytokine environment is partially due to the differential recognition of VV by PRRs but also due to VV mediated inhibition type I IFN induction and signaling, which can occur via the secretion of type I IFN binding proteins, interference with type I IFN signal transduction or minimization of PRR-induced signaling that leads to type I IFN production (Smith, Benfield et al. 2013). In addition to the induction of $CD8^+$ T cell responses, NK cells play an important role in the elimination of VV infection. VV infection leads to the proliferation and accumulation of NK cells at the site of infection (Natuk and Welsh 1987) and depletion of NK cells leads to an increased susceptibility to VV (Bukowski, Woda et al. 1983).

Chapter 3

3. Aims of the Thesis

The proper activation and induction of a specific T cell response is critical for the clearance of viral infections. Activation of anti-viral T cells leads to their expansion and acquisition of effector functions which help clear infections. Due to the ability of cytotoxic T cells to mediate elimination and protection from intracellular pathogens and antigens they have great potential for use in vaccination strategies against both viral infections and tumor cells. In order to utilize the potential of T cell based vaccinations it is necessary to fully understand the factors involved in inducing a potent, long lived T cell response.

The aim of this thesis was to characterize the role of type I IFN signaling directly on $CD8^+$ T cells during an acute LCMV infection. Type I IFNs are secreted following viral infections and play an important role in not only the innate immune response but also in establishing the adaptive immune response. The importance of type I IFN signaling is demonstrated by the fact that T cells lacking the ability to sense type I IFNs fail to properly expand following infection with viruses that induce high levels of type I IFNs such as LCMV. Due to the fundamental role of type I IFN signaling in protection against viral infections it is necessary to fully understand its role on T cell activation, expansion and memory cell development.

Therefore we set out to characterize the T cell response in the presence and absence of type I IFN signaling, focusing on the differentiation, survival and expansion of virus-specific T cells. In a first part we experimentally examined the role of type I IFN signaling on the differentiation of effector versus memory T cell precursors to better understand this key aspect of T cell biology and to further clarify the role of signal three cytokines in determining T cell fate.

Secondly, we set out to delineate the mechanisms responsible for the impaired expansion of T cells that lack the ability to sense type I IFNs. Since the discovery of the importance of type I IFNs for the proper expansion of T cells, this has been a key question that has remained unanswered. Understanding the underlying mechanisms responsible for the inability of $IFNAR^{-/-}$ T cells to properly expand will help to better understand the role of type I IFNs and signal three cytokine signaling on the establishment of an anti-viral T cell response.

Chapter 4

4. Results

4.1 The role of type I IFNs on the differentiation of CD8$^+$ T cells

4.1.1 Impaired expansion of CD8$^+$ T cells in the absence of direct type I IFN signaling

To investigate the role of direct type I IFN signaling on CD8$^+$ T cell differentiation and the SLEC versus MPEC fate decision, we used an established LCMV8.7 and VVG2 co-infection model (Wiesel, Kratky et al. 2011) combined with adoptive transfer of LCMV gp33-specific TCR-transgenic CD8$^+$ T cells (P14) which were either sufficient (WT P14) or deficient (IFNAR$^{-/-}$ P14) for type I IFN signaling. Using this system we were able to generate a type I IFN dominated inflammatory environment induced by LCMV8.7 infection however since LCMV8.7 harbors a mutation in the gp33 (V35L) epitope and is therefore not recognized by P14 cells, antigen presentation comes exclusively derived from the recombinant Vaccinia virus expressing the LCMV glycoprotein (VVG2). We chose this co-infection system as it avoids the LCMV-inherent abundant antigen presentation and hence puts more emphasis on the role of the cytokine milieu in CD8$^+$ T cell priming and differentiation. Consistent with previous findings upon single infection with wild type LCMV (Kolumam, Thomas et al. 2005, Aichele, Unsoeld et al. 2006, Wiesel, Kratky et al. 2011), WT and IFNAR$^{-/-}$ P14 cells underwent substantial expansion during the first three days after LCMV8.7 and VVG2 co-infection. However, IFNAR$^{-/-}$ P14 cells were already 5-fold reduced in frequency by day 3, and by day 6 post infection the expansion of IFNAR$^{-/-}$ P14 cells was found to be drastically reduced in frequency and total numbers in the spleen compared with the frequency and number of WT P14 cells (**Figure 4.1A and B**). Of note, although at low frequencies, IFNAR$^{-/-}$ P14 cells were still detectable at day 37 post infection in the blood, indicating that memory T cells developed and were maintained over a long time period, as also observed for single LCMV infection (Kolumam, Thomas et al. 2005). This finding could be confirmed by monitoring the total number of IFNAR$^{-/-}$ P14 cells in the spleen and lymph node 45 days post infection (**Figure 4.1B and Figure 4.8**). For further functional analysis we focused on day 3 and day 6 post infection, as at these time points the numbers of IFNAR$^{-/-}$ P14 cells were sufficient for detailed analysis and day 3 represents the peak of IFNAR$^{-/-}$ P14 expansion in this system. To determine whether impaired expansion of IFNAR$^{-/-}$ P14 cells was accompanied by altered effector functions, we measured their capacity to secrete IFN-γ upon *in vitro* peptide restimulation. In accordance with our recent studies (Wiesel, Kratky et al. 2011), we found that cells lacking type I IFN signaling showed less capacity to secrete IFN-γ as well as to degranulate (measured by cell surface CD107a mobilization) compared with WT P14 cells (**Figure 4.1C and D**) while expressing

comparable levels of perforin and granzyme B (**Figure 4.1D**). Thus, although IFNAR$^{-/-}$ CD8^{+} T cells initially expanded and gained effector functions, albeit at reduced levels, type I IFN signaling was a major promoter of their expansion, survival and effector differentiation under inflammatory conditions of an LCMV infection.

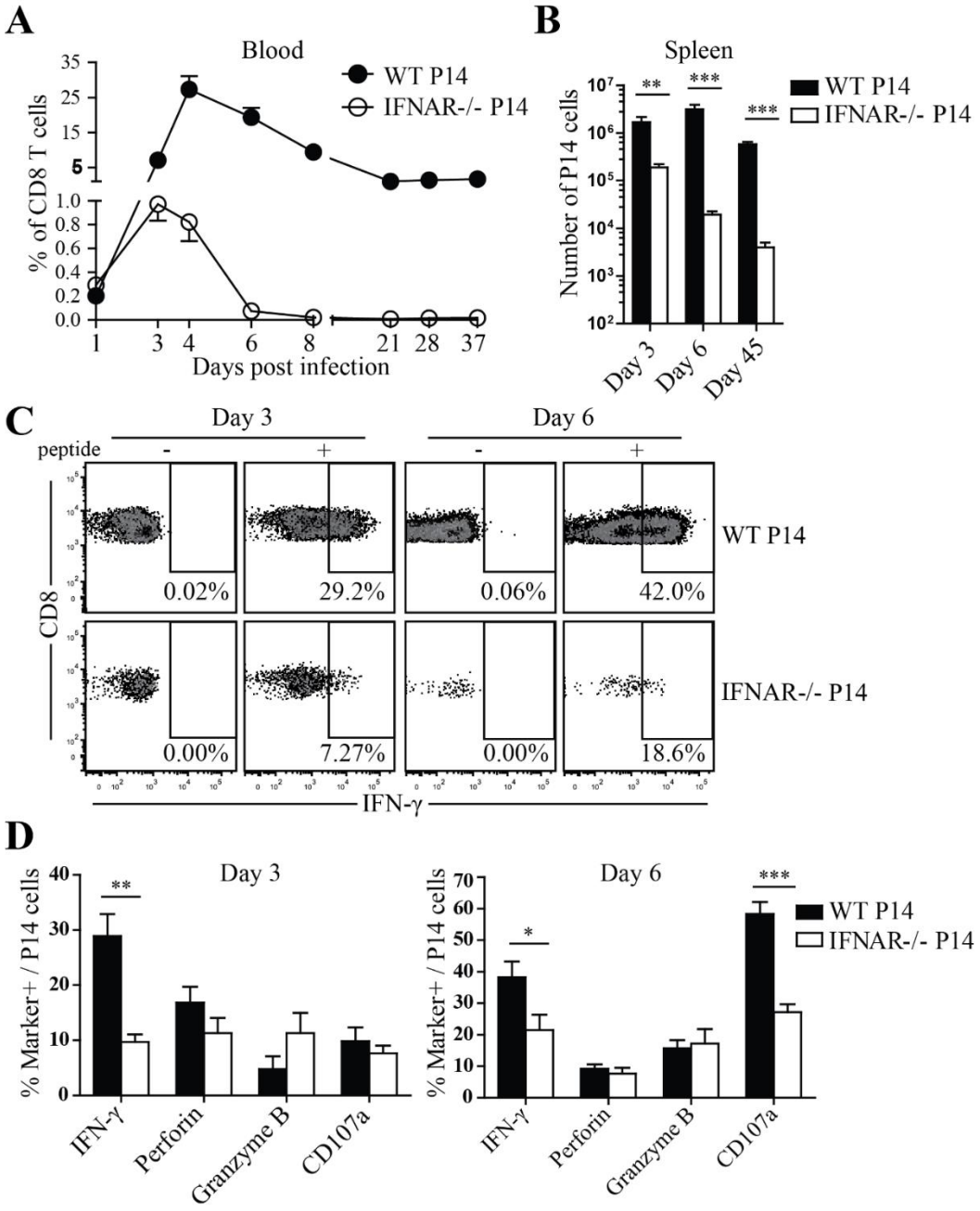


Figure 4.1 Impaired expansion of CD8^{+} T cells in the absence of direct type I IFN signaling
(A) WT and IFNAR$^{-/-}$ P14 cells (10^6) were co-transferred into naïve C57BL/6 recipients and infected with LCMV8.7 and VVG2. The frequency of WT (filled circles) and IFNAR$^{-/-}$ P14 cells (open circles) among CD8^{+} T cells were analyzed in the blood of infected mice at the indicated time points post infection. (B) Total cell numbers of WT (filled bars) and IFNAR$^{-/-}$ P14 cells (open bars) in the spleen of infected mice at the indicated time points post infection. (A and B) Data are shown as mean ± SEM of n=8 mice pooled from 2 independent experiments. **p<0.01, ***p<0.001, two-tailed Student's t-test. (C) WT (CD8^{+}, Ly5.1^{+}, top) and IFNAR$^{-/-}$ P14 cells (CD8^{+}, Thy1.1^{+}, bottom) were examined for the frequency of IFN-γ producing cells amongst P14 cells after 5 hours peptide restimulation *in vitro*. Plots shown are representative of 3 independent experiments (n=4 mice each). (D) The percentage of the indicated marker positive cells among the WT (filled bars) or IFNAR$^{-/-}$ P14 cell populations at the indicated time points post infection (open bars). Data shown are mean ± SEM of n=12 mice pooled from 3 independent experiments. *p<0.05, **p<0.01, ***p<0.001, two-tailed Student's t-test.

4.1.2 Type I IFN signaling drives the differentiation of SLEC

It is well established that type I IFNs and IL-12 have redundant functions in their role as a third signal during $CD8^+$ T cell activation; both pro-inflammatory cytokines can promote expansion as well as survival of activated $CD8^+$ T cells *in vivo* (Kolumam, Thomas et al. 2005, Aichele, Unsoeld et al. 2006, Thompson, Kolumam et al. 2006, Pearce and Shen 2007). Additionally, there is abundant evidence that IL-12 signaling during $CD8^+$ T cell priming promotes the terminal differentiation of short-lived effector cells (Joshi, Cui et al. 2007, Cui, Joshi et al. 2009, Keppler, Theil et al. 2009). However, a direct role of type I IFNs in SLEC formation *in vivo* has not been studied to date. Thus, we examined *in vivo* the expression of cell surface markers which have been described to identify SLECs ($CD44^{high}$, $CD127^{low}$, $KLRG1^{high}$) and MPECs ($CD44^{high}$, $CD127^{high}$, $KLRG1^{low}$) 3 and 6 days post LCMV8.7 and VVG2 co-infection. Notably, WT and $IFNAR^{-/-}$ P14 cells showed comparable naïve phenotypes ($CD44^{low}$, $CD25^{low}$, $CD127^{high}$, $KLRG1^{low}$ and $CD62L^{high}$) (**Figure 4.2A and data not shown**). WT P14 cells exhibited a pronounced upregulation of CD25 as early as day 3 post infection (**Figure 4.2A and B**), whereas $IFNAR^{-/-}$ P14 cells in the same recipients only slightly increased CD25 expression. By day 3 post infection, WT P14 cells could be divided into two populations with respect to CD62L expression ($CD62L^{high}$ and $CD62L^{low}$) and by day 6 the majority of the WT P14 cells showed low expression of CD62L. Although a subset of $IFNAR^{-/-}$ P14 cells started to downregulate CD62L early upon infection, by day 6 the population of $IFNAR^{-/-}$ P14 cells had re-acquired CD62L expression to comparable levels of naïve controls. This early transient downregulation of CD62L in $IFNAR^{-/-}$ P14 cells may be explained by the fact that surface CD62L is shed rapidly upon activation (Kahn, Walcheck et al. 1998) without reduction of CD62L transcripts which would lead to CD62L re-expression after initial surface shedding. Consistent with the MPEC phenotype, $IFNAR^{-/-}$ P14 cells failed to downregulate CD127 and to upregulate KLRG1 by day 6 of infection and were antigen-experienced since they uniformly expressed high levels of CD44 (data not shown).

Similar results were obtained for WT and $IFNAR^{-/-}$ P14 cells in the draining LN (**Figure 4.3**). Where $IFNAR^{-/-}$ P14 cells display a phenotype representative of MPEC cells. Analysis of the relative SLEC and MPEC composition of the WT and $IFNAR^{-/-}$ P14 cell populations from the spleen confirmed that $IFNAR^{-/-}$ P14 cell differentiation was strongly biased towards the MPEC phenotype by day 6 post infection, whereas WT P14 cells were distributed between a SLEC and MPEC phenotype (**Figure 4.2C and D**). However, by day 60 post infection, when memory P14 cells had formed, there was no longer a phenotypic difference between WT and $IFNAR^{-/-}$

P14 cells, supporting the notion that MPECs, giving rise to the memory population, were qualitatively not affected by the absence of type I IFN signaling (**Figure 4.8C**).

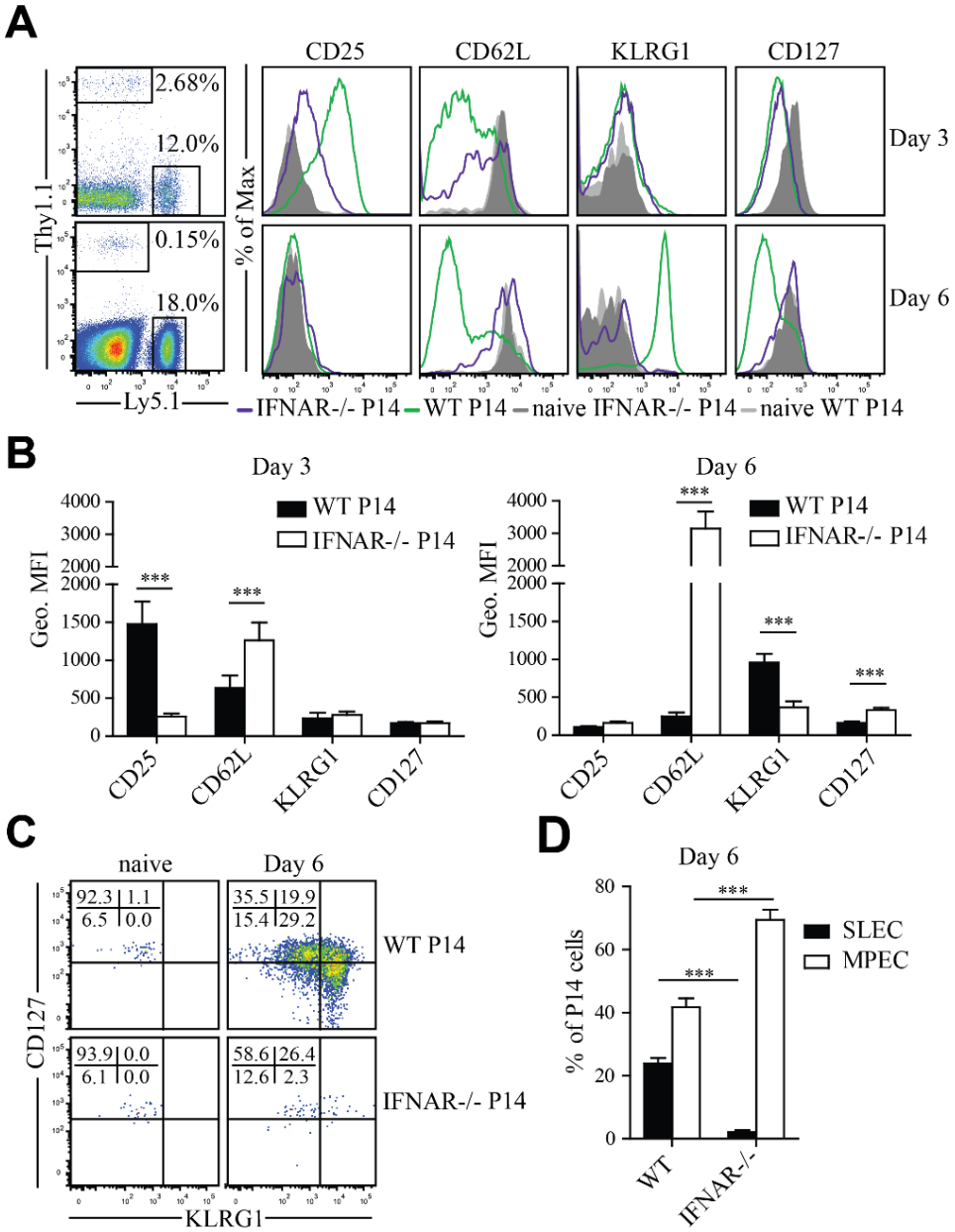


Figure 4.2 Type I IFN signaling drives the differentiation of SLECs

(A) WT and IFNAR$^{-/-}$ P14 cells (10^6) were co-transferred into naïve C57BL/6 recipients and infected with LCMV8.7 and VVG2. The frequency and phenotypic analysis of WT (Ly5.1$^+$; green line) and IFNAR$^{-/-}$ P14 cells (Thy1.1$^+$; purple line) at day 3 (top) and day 6 (bottom) after co-infection in the spleen. The numbers in the dot plots indicate the frequency of WT and IFNAR$^{-/-}$ P14 cells among total CD8$^+$ T cells. Representative overlay histograms of 3 independent experiments (n=4 mice each) are shown. Naïve samples represent mice that received transferred P14 and IFNAR$^{-/-}$ P14 cells but were left uninfected, light and dark gray filled histograms represent naïve WT and IFNAR$^{-/-}$ P14 respectively. (B) Bar graphs depict the geometric mean fluorescent intensity (Geom. MFI) of the indicated markers at day 3 (left) and day 6 (right) post infection summarizing 3 independent experiments (n=4 mice each); error bars represent SEM. ***p<0.001, two-tailed Student's *t*-test. (C) Plots show the percentage of KLRG1 and CD127 expressing P14 cells in WT (top) or IFNAR$^{-/-}$ P14 (bottom). In this plot the bottom right quadrant (KLRG1^{high}, CD127^{low}) represent SLECs, whereas the top left quadrant (KLRG1^{low}, CD127^{high}) represent MPECs. Naïve samples represent mice that received transferred P14 and IFNAR$^{-/-}$ P14 cells but were left uninfected. The data are representative for 2 independent experiments (n=4 mice each). (D) Quantification of the percentages of MPECs and SLECs among the P14 population at day 6 post infection. MPECs are defined as the KLRG^{low}, CD127^{high} population and SLECs are defined as the KLRG1^{high}, CD127^{low} population. The data shown summarize 3 independent experiments (n=4 mice each); error bars represent SEM. ***p<0.001, two-tailed Student's *t*-test.

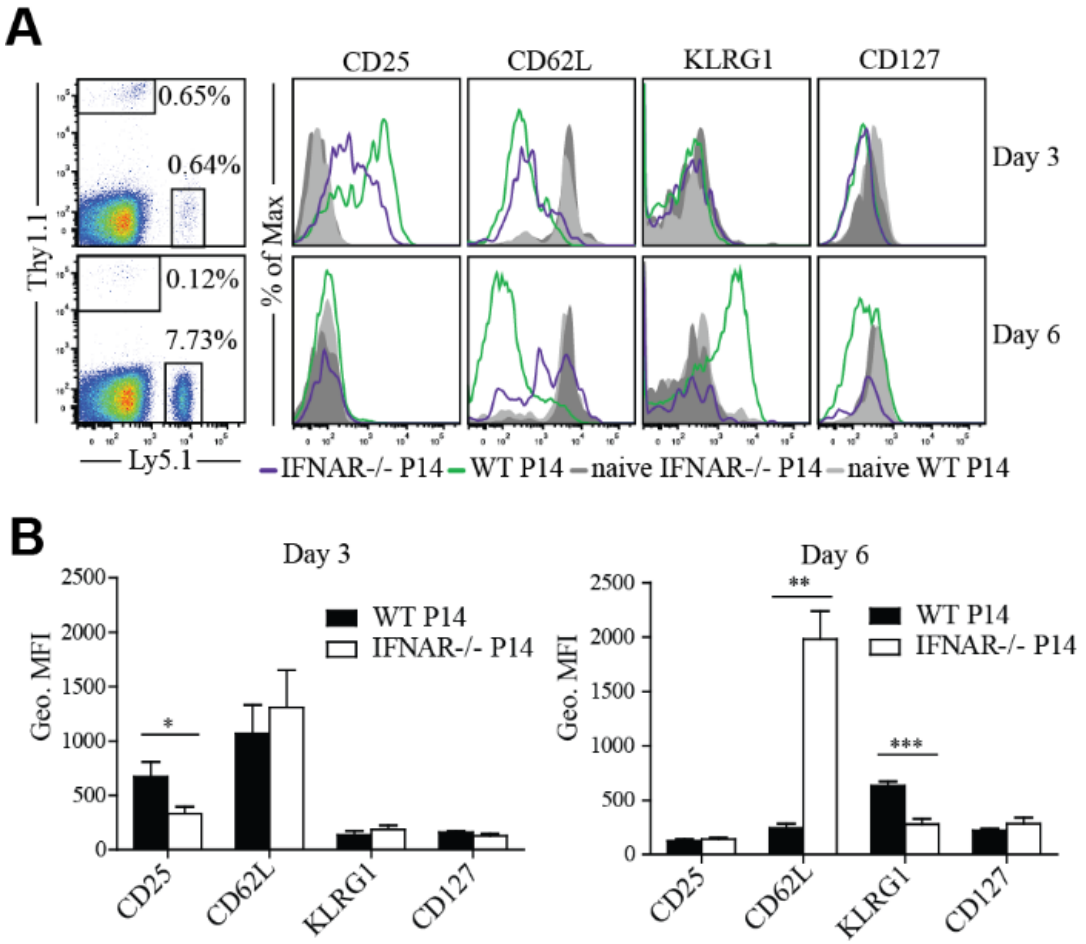


Figure 4.3 Phenotype of WT and IFNAR-/- P14 cells in the draining LN

(A) WT and IFNAR$^{-/-}$ P14 cells (10^6) were co-transferred into naïve Bl6 recipients and infected with LCMV8.7 and VVG2. The frequency and phenotypic analysis of WT (Ly5.1$^+$; green line (dashed line)) and IFNAR$^{-/-}$ P14 cells (Thy1.1$^+$; purple line (solid line)) at day 3 (upper panel) and day 6 (lower panel) post co-infection in the draining LN. The numbers in the dot plots indicate the frequency of WT and IFNAR$^{-/-}$ P14 cells among total CD8$^+$ T cells. Representative overlay histograms of 3 independent experiments (n=4 mice each) are shown. Naïve samples represent mice that received transferred P14 and IFNAR$^{-/-}$ P14 cells but were left uninfected, light and dark gray filled lines represent naïve WT and IFNAR$^{-/-}$ P14, respectively. (B) Bar graphs depict the geometric mean fluorescent intensity (Geom. MFI) of the indicated markers at day 3 and day 6 post infection summarizing 3 independent experiments (n=4 mice each) and error bars represent SEM (***p<0.001). (C) T-bet expression levels in WT (dashed line) and IFNAR$^{-/-}$ (solid line) P14 cells were analyzed by intracellular staining at day 3 (top row) and day 6 (bottom row) post infection. As in (A) naïve samples represent transferred cells that were left uninfected. The bar graph summarizes the geometric mean fluorescent intensities (Geom. MFI) of 3 independent experiments (n=4 mice each) and error bars represent SEM, **p<0.01.

Thus, IFNAR$^{-/-}$ P14 cells exhibited an augmented and accelerated MPEC phenotype (KLRG1^{low} and CD127^{high}) in sharp contrast to the pronounced effector phenotype (KLRG1^{high} and CD127^{low}) displayed by WT P14 cells (**Figure 4.2C**). Taken together these data suggest that type I IFN signaling is an important factor that promotes the transition of CD8$^+$ T cells towards an SLEC phenotype.

4.1.3 IFNAR$^{-/-}$ P14 cells also fail to differentiate in SLECs during a VSV infection

Having found that T cells lacking the ability to directly sense type I IFNs fail to differentiate into SLECs during the setting of LCMV infection we set out to see if this was an LCMV specific phenotype or whether another infection associated with high levels of type I IFNs would lead to the same T cell fate. Therefore we used a recombinant VSV virus expressing the LCMV glycoprotein (VSVGP), as VSV has been shown to be a potent inducer of type I IFNs. Much like during an LCMV infection IFNAR$^{-/-}$ P14 cells displayed an impaired expansion potential compared to WT P14 cells (**Figure 4.4A**). IFNAR$^{-/-}$ P14 cells initially began to expand but peaked much lower than WT P14 cells. Analyzing the phenotype of the P14 cells at day 7 post VSVGP infection revealed that IFNAR$^{-/-}$ P14 cells also failed to differentiate into SLECs following a VSV infection (**Figure 4.4B and C**). As seen during an LCMV infection IFNAR$^{-/-}$ P14 cells almost completely fail to form the population of SLECs whereas their ability to form MPECs is unaltered. These results demonstrate that failure of IFNAR$^{-/-}$ P14 cells to differentiate properly is not an LCMV specific issue but rather a phenotype that is found in another infection associated with high levels of type I IFNs.

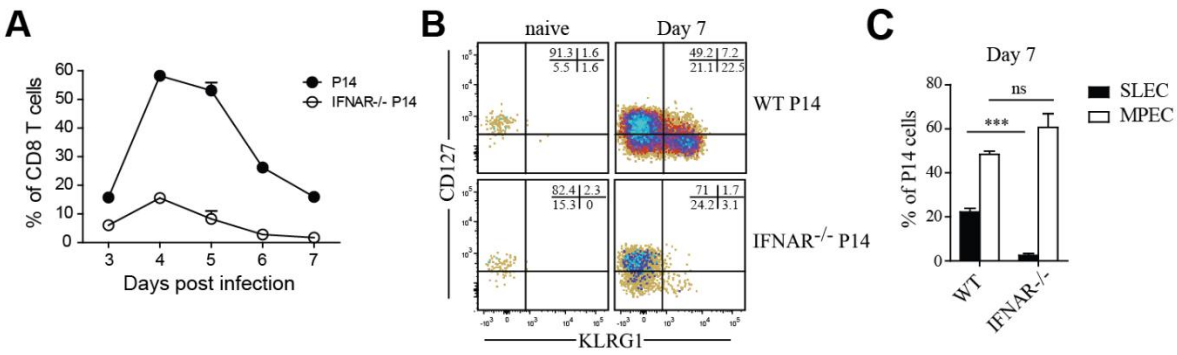


Figure 4.4 IFNAR$^{-/-}$ P14 cells to differentiate properly during a VSV infection

WT and IFNAR$^{-/-}$ P14 cells (10^6) were co-transferred into naïve C57BL/6 recipients and infected with VSV-GP. The frequency of WT (filled circles) and IFNAR$^{-/-}$ P14 cells (open circles) among CD8$^+$ T cells were analyzed in the blood of infected mice at the indicated time points post infection. (B) Dot blot representation of differentiation at day 7 post VSV infection. The plots show the percentage of KLRG1 and CD127 expressing P14 cells in WT (top) or IFNAR$^{-/-}$ P14 (bottom). In these plots the bottom right quadrant (KLRG1^{high}, CD127^{low}) represent SLECs, whereas the top left quadrant (KLRG1^{low}, CD127^{high}) represent MPECs. Naïve samples represent mice that received transferred P14 and IFNAR$^{-/-}$ P14 cells but were left uninfected. The data are representative for 2 independent experiments (n=4 mice each). (C) Quantification of the percentages of MPECs and SLECs among the P14 population at day 7 post infection. MPECs are defined as the KLRG^{low}, CD127^{high} population and SLECs are defined as the KLRG1^{high}, CD127^{low} population. The data shown summarize 2 independent experiments (n=4 mice each); error bars represent SEM. ns, not significant; ***p<0.001, two-tailed Student's *t*-test.

4.1.4 Early and abundant type I IFN levels drive the fate decision of CD8$^+$ T cells

Based on the finding that type I IFN signaling is a major regulator of the expansion and survival of CD8$^+$ T cells during LCMV infection (Kolumam, Thomas et al. 2005, Aichele, Unsoeld et al. 2006, Thompson, Kolumam et al. 2006), we aimed to exclude the possibility that IFNAR$^{-/-}$ P14 cells may initially form SLECs, which due to a lack of survival signals, are preferentially prone to undergo apoptosis. To this end, equal numbers of WT and IFNAR$^{-/-}$ P14 cells were CFSE labeled and transferred to WT hosts prior to co-infection with LCMV8.7 and VVG2 and their ability to divide and differentiate was analyzed in the spleen 2.5 days later. Both WT and IFNAR$^{-/-}$ P14 cells were initially activated and exhibited equal capacity to divide as shown by their CFSE dilution profile (**Figure 4.5A**). Furthermore, by analyzing the phenotype of cells that have only undergone a few cell divisions (CFSE high) compared with cells that have undergone intermediate (CFSE mid) or high (CFSE low) numbers of cell divisions, we found that CD25 was significantly higher expressed on WT P14 cells in the CFSE high population compared with IFNAR$^{-/-}$ P14 cells, with these differences increasing with cell division. The opposite was observed for CD62L, where CD62L expression was higher on IFNAR$^{-/-}$ P14 cells compared with that of WT P14 cells in all stages of cell divisions (**Figure 4.5B**). As the differences in the expression of CD25 and CD62L were already apparent during the initial cell divisions, we reasoned that very early following infection activated CD8$^+$ T cells undergo a lineage choice and that type I IFNs play a crucial role in this decision.

Having analyzed the very early stages of this differentiation process we next looked at the long term development of memory cells by phenotypically analyzing cell surface marker expression profiles on WT and IFNAR$^{-/-}$ P14 cells in the blood of LCMV8.7 and VVG2 co-infected mice (**Figure 4.5C**). This longitudinal analysis revealed that IFNAR$^{-/-}$ P14 cells initially began to down-regulate surface CD62L expression but after day 3 the level of CD62L is gradually regained on the population of IFNAR$^{-/-}$ P14 cells. This same trend is seen for the expression of CD127, and the opposite is seen for KLRG1 and CD25 expression where the level of expression of these two molecules remains low compared to WT P14 cells throughout the time course (**Figure 4.5C**).

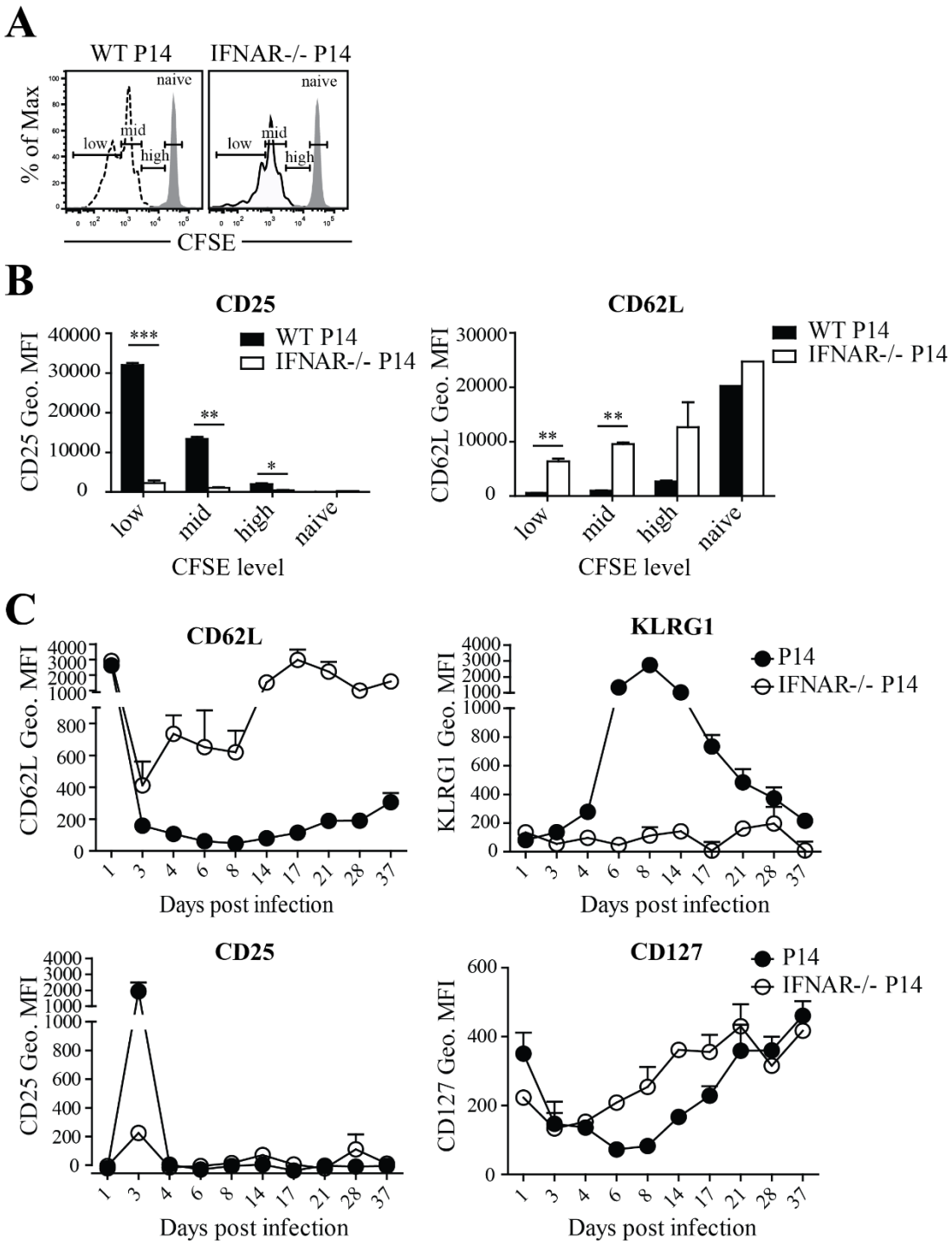


Figure 4.5 Surface activation marker kinetics

(A) CFSE labeled WT and IFNAR$^{-/-}$ P14 cells (10^6) were co-transferred into naïve recipients and infected with LCMV8.7 and VVG2. The CFSE dilution profiles of WT (dashed line) and IFNAR$^{-/-}$ (solid line) P14 cells were analyzed day 2.5 after infection in the spleen. The cells could be divided into 3 groups (high, mid, low) based on the level of CFSE left in the cells; naïve cells (gray filled histogram) representing transferred cells in uninfected recipients. (B) The surface activation markers CD25 and CD62L were analyzed in these three groups 2.5 days post infection comparing WT (filled bars) and IFNAR$^{-/-}$ (open bars) P14 within each group The data shown summarize 2 independent experiments (n=4 mice each); error bars represent the SEM; *p<0.05, **p<0.01, ***p<0.001, two-tailed Student's t-test. (C) The kinetics of CD62L, KLRG1, CD25, and CD127 expression were analyzed in the blood of infected mice at the indicated time points following co-transfer of WT and IFNAR$^{-/-}$ P14 cells and co-infection with LCMV8.7 and VVG2. The geometric mean fluorescent intensities of WT (filled circles) and IFNAR$^{-/-}$ (open circles) P14 cells for the indicated surface activation markers represent pooled data from 2 independent experiments (n=4); error bars represent the SEM.

Of note, a comparable MPEC phenotype of IFNAR$^{-/-}$ P14 cells could be observed upon single LCMV-WE infection (**Figure 4.6A**), indicating that although the antigen load seen by P14 cells profoundly differs between an infection with VVG2 or LCMV, type I IFNs are the main regulator of the fate decision towards the SLEC subset. Importantly, SLEC differentiation of IFNAR$^{-/-}$ was similar to that of WT P14 cells in the context of a VVG2 only infection (**Figure 4.6B**) (Keppler and Aichele 2011), where high levels of IL-12 are produced at the expense of type I IFNs (Wiesel, Kratky et al. 2011). These results strongly suggest that depending on the type of infection and the predominant cytokines induced, different inflammatory signals instruct effector phenotype differentiation. Thus, in the context of VV infection, the high levels of IL-12 induced upon infection are sufficient to drive the differentiation of IFNAR$^{-/-}$ P14 cells into SLECs (Wiesel, Joller et al. 2010) and type I IFNs are not required for this process. Importantly, this finding shows that CD8^{+} T cells lacking type I IFN signaling are not inherently impaired in their capacity to gain an SLEC phenotype (Keppler and Aichele 2011).

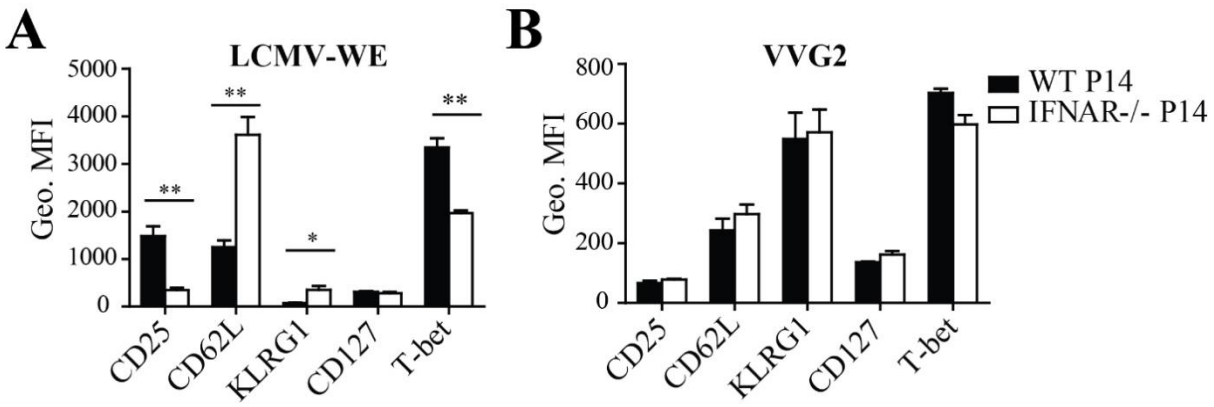


Figure 4.6 Phenotype during LCMV-WE and VVG2 infection

WT and IFNAR$^{-/-}$ P14 cells (10^6) were transferred into naïve hosts which were subsequently infected with either VVG2 or LCMV-WE. Phenotypic analysis of WT (filled bars) and IFNAR$^{-/-}$ P14 cells (open bars) at (A) day 3 following LCMV-WE infection and (B) day 6 following VVG2 infection in the spleen. The bar graphs depict the geometric mean fluorescent intensities of the indicated markers summarizing at least 2 independent experiments (n=4 mice each) and error bars represent SEM with *p<0.05, **p<0.01, two-tailed Student's *t*-test.

4.1.5 T-bet levels are regulated directly by type I IFNs

Based on these phenotypic results we reasoned that the amount of T-bet, an important transcription factor that is more abundantly expressed in SLECs compared with MPECs (Intlekofer, Takemoto et al. 2005, Joshi, Cui et al. 2007), might also differ in WT and IFNAR$^{-/-}$ P14 cells. Upon *in vivo* activation, WT and IFNAR$^{-/-}$ P14 cells upregulated T-bet expression independent of their phenotype (**Figure 4.7A**). However, WT P14 cells expressed significantly higher T-bet levels than IFNAR$^{-/-}$ P14 cells at day 3 and even more pronounced at day 6 post infection (**Figure 4.7A and B**). As terminal effector differentiation is accompanied by high

levels of T-bet whereas low amounts of T-bet rather promote MPEC development (Joshi, Cui et al. 2007), we reasoned that in a type I IFN biased cytokine milieu direct signaling via the type I IFN receptor might regulate T-bet expression and thereby drive the fate decision toward an SLEC phenotype. We therefore examined the ability of type I IFNs to directly regulate the expression of T-bet. To this end, IFN-β was added to $CD8^+$ T cells during *in vitro* activation with anti-CD3/CD28 and the relative expression levels of T-bet mRNA were monitored after 24 and 48 hours (**Figure 4.7C**). IL-12 was used as a positive control as it was previously shown to induce T-bet expression and thereby drive SLEC differentiation (Joshi, Cui et al. 2007). Indeed, IFN-β addition led to an upregulation of T-bet expression to comparable levels as IL-12 by 48 h post activation, indicating that type I IFN signaling on activated $CD8^+$ T cells directly regulates T-bet expression. Thus, under priming conditions with abundant type I IFN levels, the initial differentiation of $CD8^+$ T cells towards a SLEC phenotype is driven by T-bet that is directly induced by type I IFN signaling.

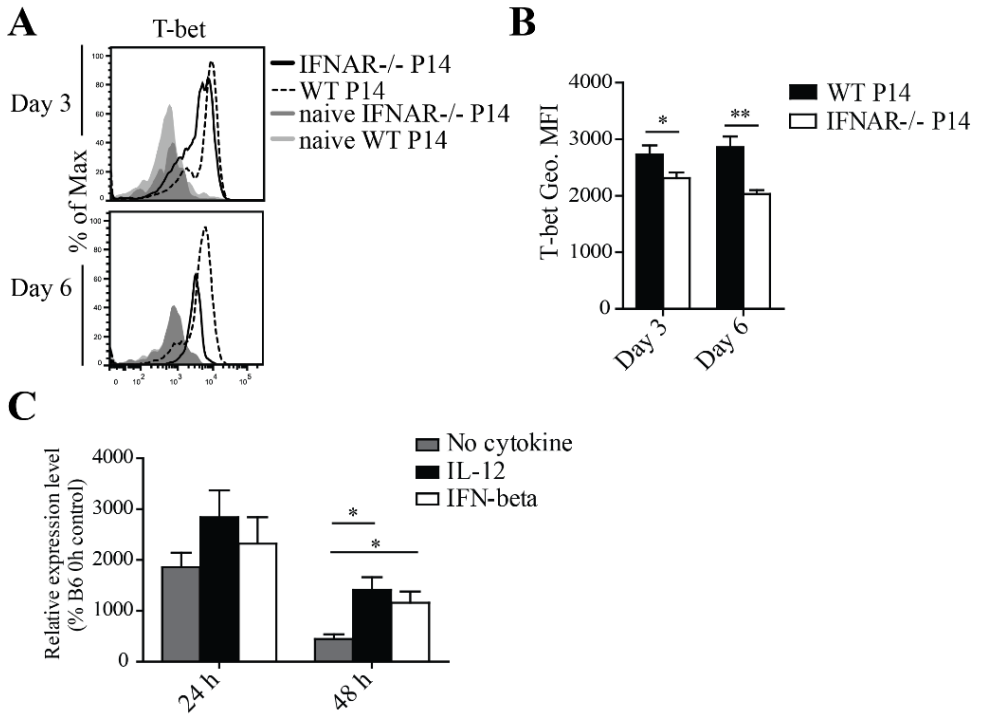


Figure 4.7 T-bet expression in $CD8^+$ T cells is directly regulated by type I IFNs

(A) WT (dashed line) and $IFNAR^{-/-}$ P14 cells (solid line) (10^6) were co-transferred into naïve mice which were then co-infected with LCMV8.7 and VVG2. Naïve samples represent mice that received transferred WT and $IFNAR^{-/-}$ P14 cells but were left uninfected (light and dark gray filled histograms respectively). T-bet expression levels were analyzed by intracellular staining at day 3 (top) and day 6 (bottom) post infection. (B) The bar graph summarizes the geometric mean fluorescent intensities of 3 independent experiments (n=4 mice each) and error bars represent SEM. *p<0.05, **p<0.01, two-tailed Student's *t*-test. (C) The relative expression levels of T-bet upon in vitro activation with anti-CD3/CD28 (light gray bar) in the presence of IL-12 (black bar) and IFN-β (white bar) after 24 h and 48 h are shown. The expression levels of T-bet were calculated relative to the T-bet levels obtained in the unstimulated 0 h control sample. Data pooled from 4 independent experiments, error bars represent SEM. *p<0.05, two-tailed Student's *t*-test.

4.1.6 Functional memory CD8$^+$ T cells in the absence of type I IFN signaling

Finally, as IFNAR$^{-/-}$ P14 cells appear to differentiate into a MPEC phenotype we addressed the ability WT and IFNAR$^{-/-}$ P14 cells to give rise to functional memory CD8$^+$ T cells with recall potential in the context of LCMV8.7 and VVG2 co-infection. Analysis of the tissue distribution of memory WT and IFNAR$^{-/-}$ P14 cells at day 45 post infection revealed that both WT and IFNAR$^{-/-}$ P14 cells could be found in the spleen and lymph nodes but only WT P14 cells could be found in liver (**Figure 4.8A**), as opposed to an equal tissue distribution of IFNAR$^{-/-}$ P14 cells seen in the spleen and liver on day 6 post infection (data not shown and [19]). To evaluate the quality of the generated memory cells, their ability to produce IFN-γ and their capacity to degranulate upon *in vitro* antigen recognition was determined. At day 45 post priming, WT and IFNAR$^{-/-}$ memory P14 cells produced comparable levels of IFN-γ and WT P14 cells showed only slightly increased levels of CD107a compared with IFNAR$^{-/-}$ memory P14 cells (**Figure 4.8B**). Thus, although the frequency of the IFNAR$^{-/-}$ memory P14 cells was strongly reduced, their per-cell functional properties did not differ from WT P14 cells. In addition to the equivalent *ex vivo* functional capacity, the proportion of P14 cells exhibiting a CD127^{high} KLRG1^{low} phenotype at day 60 post infection was comparable between WT and IFNAR$^{-/-}$ P14 cells (**Figure 4.8C**). To ascertain that the memory IFNAR$^{-/-}$ P14 cell population represented indeed memory cells and not naïve cells which had not been recruited into the primary response, we measured CD44 expression on the IFNAR$^{-/-}$ P14 cells. As all IFNAR$^{-/-}$ P14 cells uniformly expressed high levels of CD44, we conclude that these cells are indeed antigen-experienced memory cells (data not shown).

To further validate the functionality of IFNAR$^{-/-}$ memory P14 cells, we determined their potential to re-expand and to produce effector cytokines upon viral re-challenge. We chose a challenge with VVG2 as it has been shown that CD8$^+$ T cell expansion is only marginally dependent on direct type I IFN signaling during VVG2 infection (Xiao, Casey et al. 2009, Wiesel, Kratky et al. 2011). Thus, memory WT and IFNAR$^{-/-}$ P14 cells were isolated from the spleen 45 days post LCMV8.7 and VVG2 infection and transferred into naïve WT mice, which were subsequently challenged with VVG2. The fold expansion of both subsets 6 days post challenge was calculated according to the frequency of cells before and after challenge. Consistent with the comparable cytokine and degranulation data, WT and IFNAR$^{-/-}$ P14 cells expanded equally well (roughly 15-fold) and were both potent producers of IFN-γ upon *in vitro* restimulation (**Figure 4.8D and E**).

This finding shows that MPECs formed in the absence of type I IFN signaling differentiated into functional memory $CD8^+$ T cells. Thus, type I IFN signaling influences the overall frequency but not the functionality of memory $CD8^+$ T cells. Taken together these data demonstrate a key role for type I IFN signaling on T cells for the proper differentiation of $CD8^+$ T cells into short lived effector cells but even in the absence of type I IFN signaling T cells have the ability to differentiate into quantitatively equivalent functional memory cells, albeit at a low frequency.

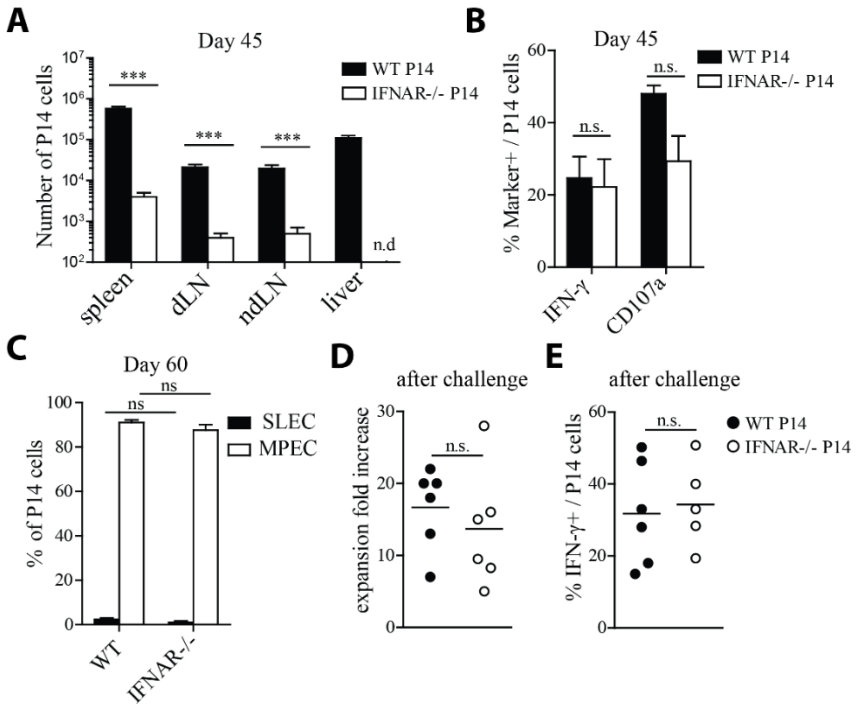


Figure 4.8 Generation of functional memory $CD8^+$ T cells in the absence of type I IFN signaling

45 days after LCMV8.7 and VVG2 infection, the functionality of memory WT and $IFNAR^{-/-}$ P14 cells was analyzed. (A) Total cell numbers of WT (filled bars) and $IFNAR^{-/-}$ (open bars) P14 cells were analyzed in the indicated organs in mice 45 days post infection. (B) Shown are the percentages of CD107a or of IFN-γ positive cells among WT P14 (filled bars) or $IFNAR^{-/-}$ P14 (open bars) cells following 5 h *in vitro* peptide restimulation. (C) Quantification of the percentages of MPECs and SLECs among the P14 population at day 60 post infection. MPECs are defined as the $KLRG1^{low}$, $CD127^{high}$ population and SLECs are defined as the $KLRG1^{high}$, $CD127^{low}$ population. (A-C) The data shown summarize 2 independent experiments (n=4 mice each); error bars represent SEM; n.d., not detectable; n.s. indicates data sets that are not significant; ***$p<0.001$, two-tailed Student's *t*-test. (D) MACS purified memory $CD8^+$ T cells from the spleen of infected mice at day 45 were transferred ($1x10^6$) into naïve hosts, which were subsequently challenged with VVG2. The fold expansion of WT (filled circles) and $IFNAR^{-/-}$ P14 cells (open circles) 6 days post challenge is shown. (E) The percentage of IFN-γ positive cells among the WT (open circles) or $IFNAR^{-/-}$ (filled circles) P14 population following 5 h *in vitro* peptide restimulation is shown. (D and E) pooled data from 2 independent experiments (n=3 mice each) are depicted. n.s. indicates data that is not-significant.

4.2 The role of type I IFN signaling on the survival and expansion of T cells

4.2.1 Microarray analysis reveals distinct gene expression profiles in IFNAR$^{-/-}$ and WT P14 cells

Having described the key role of type I IFNs in the differentiation of T cells we sought out to analyze the role of type I IFN signaling on T cells with respect to their survival and expansion. To investigate the reason(s) for the massively compromised expansion of CD8^{+} T cells lacking the ability to sense type I IFNs during LCMV infection, we set out to perform a whole genome microarray comparing type I interferon receptor sufficient (WT) and deficient (IFNAR$^{-/-}$) LCMV-specific CD8^{+} T cells. To this end WT and IFNAR$^{-/-}$ P14 cells were co-transferred into naïve C57BL/6 (Bl6) mice followed one day later by LCMV infection. To increase the low IFNAR$^{-/-}$ P14 T cell numbers recovered following LCMV infection, and to recover a sufficient number of cells for reliable analysis, we utilized the previously described LCMV8.7 and VVG2 co-infection system (Wiesel, Kratky et al. 2011, Wiesel, Crouse et al. 2012), where the inflammatory environment is provided by the LCMV8.7 mutant which is not recognized by P14 cells and antigen is provided by the LCMV-GP recombinant Vaccinia virus (VVG2). This setup leads to greater expansion of both IFNAR$^{-/-}$ and WT P14 T cells compared to LCMV, allowing for superior analysis of IFNAR$^{-/-}$ P14 cells at early time points. Importantly, the low ratio between IFNAR$^{-/-}$ and WT cells is comparable in the co-infection and single infection models, where there is an impaired expansion of IFNAR$^{-/-}$ P14 cells seen in both systems (**Figure 4.9A and B**). Furthermore, it has been previously shown that there is no difference in the phenotype or differentiation of P14 cells following the two infection models allowing for comparison of the two systems (Wiesel, Crouse et al. 2012). For microarray analysis, WT and IFNAR$^{-/-}$ P14 cells were FACS sorted to high purity based on their expression of the congenic markers Ly5.1 (WT) and Thy1.1 (IFNAR$^{-/-}$) at day 3 post co-infection, the time point corresponding to the peak of IFNAR$^{-/-}$ P14 expansion before they begin to decrease in numbers (**Figure 4.9C**). Analysis of genes more than 2-fold differentially regulated revealed 631 genes that were differentially expressed in activated IFNAR$^{-/-}$ and WT P14 cells. Of these genes, 374 were up-regulated in IFNAR$^{-/-}$ compared to WT P14 cells and 257 were down-regulated, the 50 most upregulated (**appendix table 1.1**) and downregulated (**appendix table 1.2**) are shown in the appendix section. Pathway analysis of the 631 genes revealed that many genes grouped together into distinct functional pathways (**Figure 4.9D**). The pathways with the greatest number of differentially expressed genes were those related to signaling and signal transduction, metabolic processes, cell death and apoptosis, IFN signaling and cell cycle

regulation. Of particular interest were those genes associated with cell death regulation, as these could be of direct relevance for the abortive expansion of IFNAR$^{-/-}$ P14 cells. Further analysis of genes involved in cell death regulation revealed differential expression of many genes encoding ligands for NK cell activating or inhibitory receptors (**Figure 4.9E**). In combination with recent reports demonstrating NK cell regulation of T cell responses (Lu, Ikizawa et al. 2007, Waggoner, Taniguchi et al. 2010, Soderquest, Walzer et al. 2011, Lang, Lang et al. 2012, Waggoner, Cornberg et al. 2012), we decided to further examine a potential role of NK cells in the negative regulation of expansion of IFNAR$^{-/-}$ P14 cells.

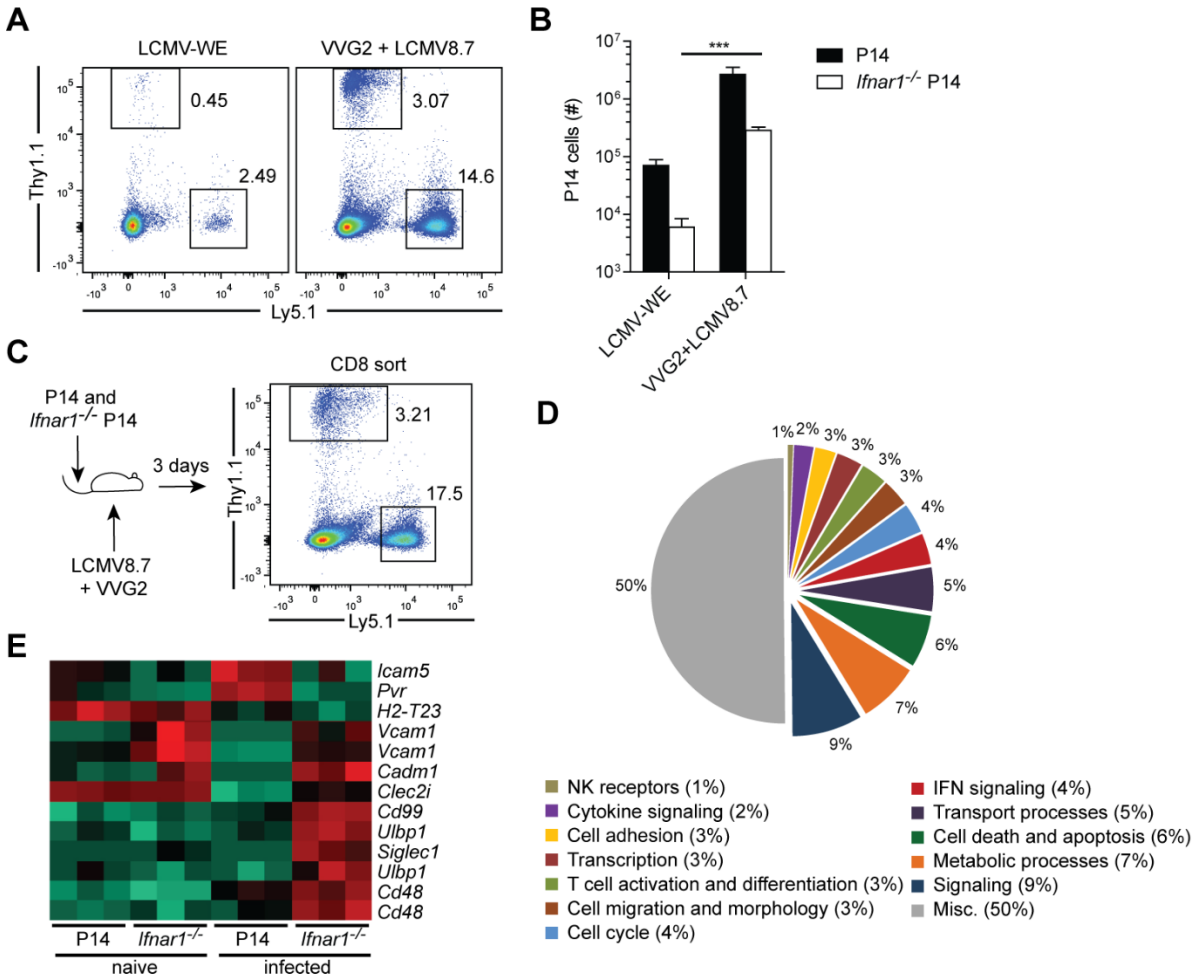


Figure 4.9 Microarray analysis reveals distinct gene expression profiles in IFNAR$^{-/-}$ and WT P14 cells

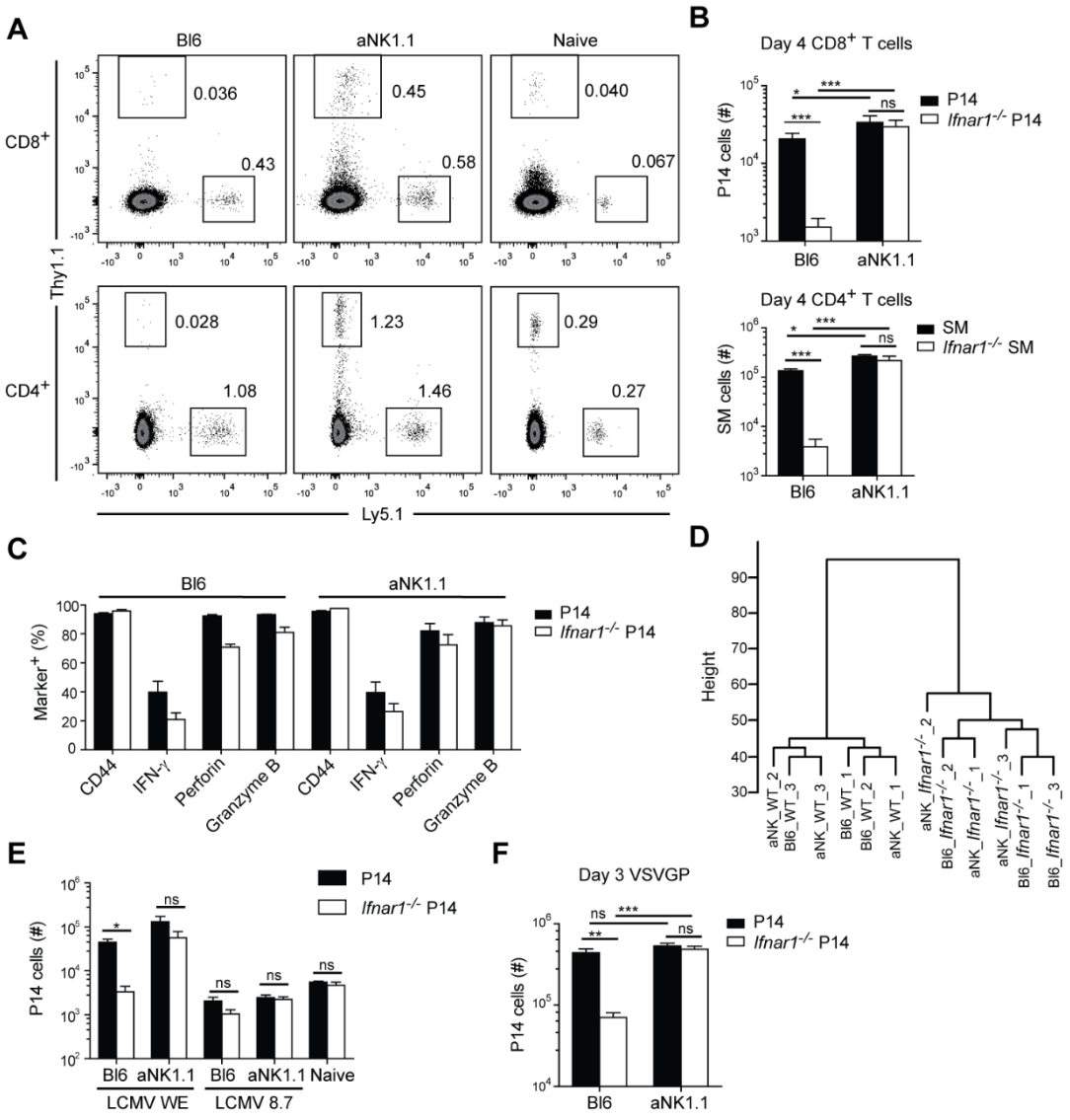
(a and b) Comparison of LCMV-WE infection and LCMV8.7 + VVG2 co-infection following co-transfer of 106 WT and IFNAR-/- P14 cells. (a) Representative flow cytometric dot plots of WT (Ly5.1+) and IFNAR-/- (Thy1.1+) P14 cells 3 days post infection in the spleen, numbers indicate the percentage of total CD8$^+$ T cells. (b) Total P14 cell numbers in the spleen 3 days post infection are shown. Data are shown as mean ± SEM of n=12 mice pooled from 3 independent experiments. ***p<0.001 (unpaired two-tailed t-test) (c) Experimental setup for microarray analysis; 106 WT and IFNAR-/- P14 were co-transferred into naïve Bl6 mice followed one day later by LCMV8.7 + VVG2 co-infection. 3 days post infection WT (Ly5.1+) and IFNAR-/- (Thy1.1+) P14 cells were FACS sorted to purity and used for microarray analysis. (d) Pie chart representing functional pathways enriched amongst genes over 2-fold differentially expressed between WT and IFNAR-/- P14 cells. Numbers represent the percentage of genes found in the respective pathways. (e) Heat map expression pattern of genes involved in NK cell activation and inhibition. Expression ranges from low expression (green) to high expression (red). Each box represents a group from the microarray analysis; each group was performed in triplicates.

4.2.2 NK cell depletion restores IFNAR$^{-/-}$ P14 cell expansion

To test for a regulatory role of NK cells in the impaired expansion of IFNAR$^{-/-}$ P14 cells, we co-transferred WT and IFNAR$^{-/-}$ P14 cells into NK cell depleted (aNK1.1) or un-depleted (Bl6) mice followed by an acute LCMV-WE infection. Interestingly, we found that depletion of NK cells led to the complete recovery of IFNAR$^{-/-}$ P14 cell expansion at day 4 post infection, both in percentage and total cell numbers (**Figure 4.10A and B, top row**). This ~20 fold increase in expansion was observed in the spleen and lymph nodes (data not shown). Consistent with previous reports (Lu, Ikizawa et al. 2007, Waggoner, Taniguchi et al. 2010, Soderquest, Walzer et al. 2011, Lang, Lang et al. 2012, Waggoner, Cornberg et al. 2012), we observed a moderate increase (~1.5 fold) in expansion of WT P14 cells in NK cell depleted mice. Furthermore, previous reports found that IFNAR$^{-/-}$ CD4^{+} T cells also show a strong reduction in expansion following LCMV infection (Havenar-Daughton, Kolumam et al. 2006). Therefore, we generated IFNAR$^{-/-}$ Smarta (SM) cells, being transgenic CD4^{+} T cells specific for the LCMV glycoprotein gp_{61-80} epitope. Co-transfer of WT and IFNAR$^{-/-}$ SM cells into NK cell depleted mice followed by LCMV infection revealed that IFNAR$^{-/-}$ SM cell expansion could also be completely recovered to WT levels by NK cell depletion (**Figure 4.10A and B, bottom row**). Furthermore, we found a relation between the NK cell depletion efficiency and the recovery of IFNAR$^{-/-}$ P14 cell expansion, where in general the better the NK cell depletion efficiency, the closer IFNAR$^{-/-}$ P14 expansion got to WT P14 levels (data not shown). In addition, IFNAR$^{-/-}$ P14 cell expansion was also recovered using the anti-Asialo GM1 NK cell depleting antibody, which targets an epitope not expressed on NKT or T cells (**Figure 4.11**). Analysis of the functionality of P14 cells at day 4 post infection revealed that NK depletion had no significant effect on the activation (CD44) and effector functions of WT and IFNAR$^{-/-}$ P14 cells, as shown by percentage of IFN-γ, perforin and granzyme B positive T cells (**Figure 4.10C**). Furthermore, comparing the global gene expression profiles of P14 cells isolated from Bl6 and NK cell depleted mice by cluster analysis revealed that NK cell depletion had little effect on the overall expression profile of WT or IFNAR$^{-/-}$ P14 cells, indicating little cross talk between the T cells and NK cells (**Figure 4.10D**). In fact, comparison of genes that were at least 2 fold differentially regulated revealed that there were 601 genes that were differentially regulated between WT and IFNAR$^{-/-}$ P14 cells from NK cell depleted mice. Of the 601 genes, 334 were upregulated (**appendix table 1.3**) in IFNAR$^{-/-}$ P14 cells compared to WT P14 and 267 were downregulated (**appendix table 1.4**). These numbers are very similar to the comparisons from the undepleted mice. In addition, comparison of differentially expressed genes between IFNAR$^{-/-}$ P14 cells

from undepleted and NK cell depleted mice and WT P14 cells from undepleted and NK cell depleted mice revealed that no genes were more than 2 fold differentially regulated comparing T cells from undepleted and depleted mice. Further indicating that NK cells have no impact on the overall gene expression of the T cells and are exerting their regulatory effector in a different manner.

Figure 4.10 NK cell depletion restores IFNAR$^{-/-}$ P14 cell expansion



(**a** and **b**) The percentage and total cell numbers of T cells from day 4 LCMV infected mice following co-transfer of WT and IFNAR$^{-/-}$ P14 or SM cells into NK cell depleted (aNK1.1) or undepleted (Bl6) mice. Naïve mice received cell co-transfers but were left uninfected. (**a**) Representative flow cytometric dot plots pre-gated on CD8^{+} (top row) or CD4^{+} (bottom row) T cells. The numbers represent the percentage of total CD8^{+} or CD4^{+} T cells in the spleen. (**b**) Total cell numbers of WT and IFNAR$^{-/-}$ P14 and SM cells 4 days post infection are shown. (**c**) Functionality of T cells from NK depleted (aNK1.1) and un-depleted (Bl6) mice 4 days post infection. Shown is the % of cells positive for the indicated marker. No significant differences were found when comparing activation markers of IFNAR$^{-/-}$ P14 cells from Bl6 and NK depleted mice. (**d**) Hierarchical clustering of overall gene expression patterns of WT and IFNAR$^{-/-}$ (KO) P14 cells from un-depleted (Bl6) and depleted (aNK) mice. (**e**) Day 4 post infection P14 cell numbers of WT and IFNAR$^{-/-}$ P14 cells in the spleen as in (a) from mice infected with LCMV-WE or LCMV8.7. (**f**) Total cell numbers of P14 cells 3 days following VSVGP infection is shown. (**b,d,e,f**) Data shown are mean ± SEM of n= 4 mice representative of at least 3 experiments. ns, not significant; *p<0.05; **p<0.01; ***p<0.001 (unpaired two-tailed *t*-test).

Next, we addressed the question whether naïve and activated IFNAR$^{-/-}$ P14 cells are similarly sensitive to NK cell mediated killing. To this end we infected mice with LCMV8.7 (which is not recognized by P14 cells), following co-transfer of WT and IFNAR$^{-/-}$ P14 cells. In this setting P14 cells remain antigen inexperienced, but the inflammatory environment and NK cell activation remains the same as in LCMV-WE infection. WT and IFNAR$^{-/-}$ P14 cell numbers were comparable during LCMV8.7 infection with or without NK depletion (**Figure 4.10E**), leading to the conclusion that T cells need to be activated to be negatively regulated by NK cells. Finally, infection with VSV expressing the LCMV glycoprotein (VSV-GP) following co-transfer of WT and IFNAR$^{-/-}$ P14 cells in NK cell depleted mice revealed that IFNAR$^{-/-}$ P14 cell expansion could also be recovered to WT levels in another infection associated with high levels of type I IFNs (**Figure 4.10F**). Taken together, we found that both CD8^{+} and CD4^{+} T cells lacking the ability to sense type I IFNs are highly susceptible to negative regulation by NK cells.

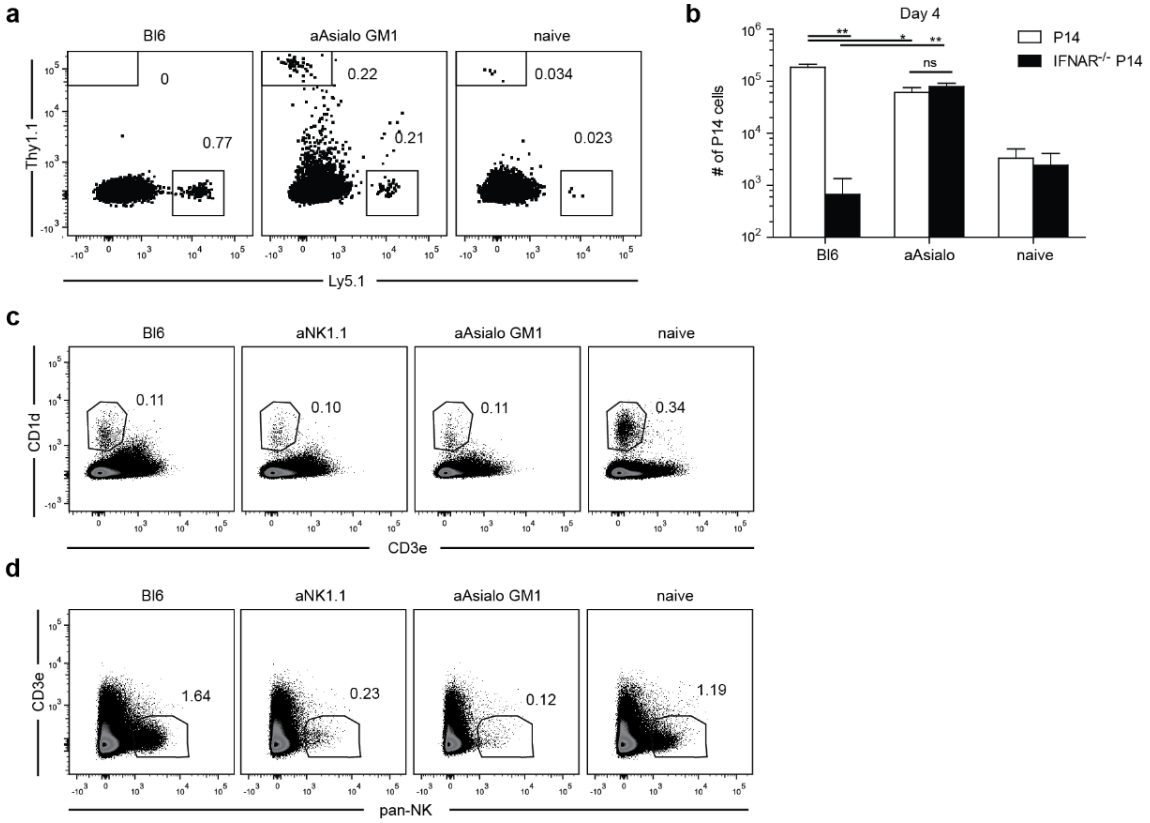


Figure 4.11: Asialo GM1 NK cell depletion restores IFNAR$^{-/-}$ P14 cell expansion

(**a** and **b**) Co-transfer of WT and IFNAR$^{-/-}$ P14 cells into NK cell depleted (aAsialo) or un-depleted (Bl6) mice followed one day later by LCMV infection. Naïve mice received cell co-transfers but were left uninfected. On day 4 post infection total cell numbers were determined. (**a**) Representative flow cytometric dot plots pre-gated on CD8^{+} T cells are shown. The numbers represent the percentage of total CD8^{+} T cells in the spleen. (**b**) Total cell numbers of WT and IFNAR$^{-/-}$ P14 cells at day 4 post LCMV infection. Data shown are mean ± SEM of n= 4 mice representative of at least 3 experiments. ns, not significant; * $p<0.05$; ** $p<0.01$ (unpaired two-tailed *t*-test). (**c,d**) aAsialo treatment leads to efficient depletion of NK cells (**d**) but not of NKT cells (**c**), numbers indicate the percentage gated amongst B220^{-} lymphocytes. Shown are representative dot plots of 3 independent experiments.

4.2.3 NK cell depletion leads to enhanced memory IFNAR$^{-/-}$ P14 cell formation

NK cell depletion leads to a full recovery of expansion of IFNAR$^{-/-}$ P14 cell up to day 4 post infection, which is the time point where IFNAR$^{-/-}$ P14 cells peak following LCMV infection, but at day 7 post infection, IFNAR$^{-/-}$ P14 cells exhibited greatly reduced expansion compared to WT P14 cells which could only partially be recovered by NK cell depletion (**Figure 4.12A and B, top row**). Nonetheless, NK depletion led to a ~100 fold increase in total IFNAR$^{-/-}$ P14 cell numbers compared to un-depleted controls. Similar to IFNAR$^{-/-}$ P14 cells, IFNAR$^{-/-}$ SM cells also exhibited a partial recovery of expansion at day 7 post infection when NK cells were depleted (**Figure 4.12A and b, bottom row**). Comparable results were obtained with aNK1.1 and anti-Asialo-GM1 targeted NK cell depletion (**Figure 4.13**). To examine the effect of NK cells on the endogenous IFNAR$^{-/-}$ T cell response we utilized mice in which only T cells specifically lack the type I IFN receptor (CD4^{cre+}IFNAR$^{f/f}$) (Kamphuis, Junt et al. 2006). Consistent with the results obtained by adoptive transfer of IFNAR$^{-/-}$ P14 cells, we found a substantial recovery in the expansion of total activated (CD44^{hi}), of gp33 tetramer and np396 tetramer specific CD8^{+} T cells in NK cell depleted (aNK1.1) CD4^{cre+}IFNAR$^{f/f}$ mice compared to un-depleted (Ø) mice at day 7 post infection (**Figure 4.12C**). This recovery at day 7 was even greater than the one seen in the adoptive transfer experiments using transgenic T cells. As previously reported, IFNAR$^{-/-}$ P14 cells failed to differentiate into short lived effector cells (SLECs, CD44^{hi}, KLRG1^{hi}, CD127^{low}) and were skewed towards a memory precursor effector cell (MPEC) phenotype (CD44^{hi}, KLRG1^{low}, CD127^{hi}). Depletion of NK cells had no effect on acquisition of this differential phenotype between WT or IFNAR$^{-/-}$ P14 cells (**Figure 4.12D**). As NK cell depletion had no impact on the differentiation of IFNAR$^{-/-}$ P14 cells but did have a strong impact on the size of the response at day 7, we examined memory formation of WT and IFNAR$^{-/-}$ P14 cells with and without NK cell depletion. Depletion of NK cells during priming led to a substantial increase in the percentage and number (**Figure 4.12E and F**) of IFNAR$^{-/-}$ memory P14 cells compared to the un-depleted situation at day 80 post infection. Phenotypically, NK cell depletion during priming had no effect on the differentiation of memory cells into central memory (CD44^{hi}, CD62L^{hi}) and effector memory (CD44^{hi}, CD62L^{low}) cells at day 80 post infection and VVG2 challenge revealed that WT and IFNAR$^{-/-}$ P14 cells were equally functional with respect to recall potential and exertion of effector functions, regardless of whether they were primed in the presence or absence of NK cells (**Figure 4.14**). Taken together, NK cell depletion leads to a partial recovery of IFNAR$^{-/-}$ T cell

expansion at day 7 post infection but dramatically increased formation of functional memory cells.

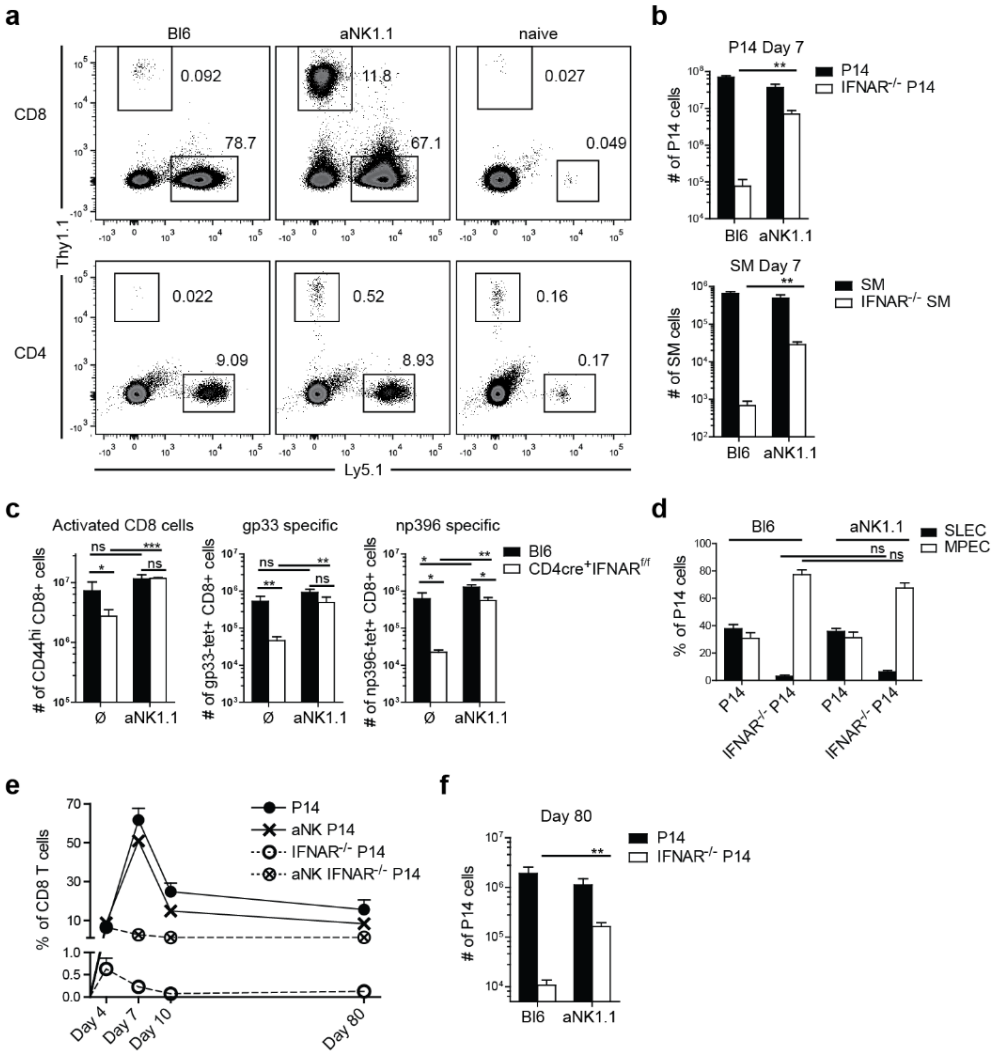


Figure 4.12 NK cell depletion leads to enhanced memory IFNAR$^{-/-}$ P14 cell formation

(**a** and **b**) The percentage and total cell numbers of T cells from day 7 LCMV infected mice following co-transfer of WT and IFNAR$^{-/-}$ P14 or SM cells into NK cell depleted (aNK1.1) or undepleted (Bl6) mice. Naïve mice received cell co-transfers but were left uninfected. (**a**) Representative flow cytometric dot plots pre-gated on CD8^{+} (top row) or CD4^{+} (bottom row) T cells are shown. The numbers represent the percentage of total CD8^{+} or CD4^{+} T cells in the spleen. (**b**) Total cell numbers of WT and IFNAR$^{-/-}$ P14 and SM cells 7 days post LCMV infection. (**c**) To examine the endogenous T cell response WT (Bl6) or CD4^{cre}IFNAR$^{flox/flox}$ mice were depleted of NK cells (aNK1.1) or left un-depleted (Ø) and infected with LCMV-WE. At day 7 post infection, total activated CD8^{+} T cells were analyzed by gating on CD44^{hi}CD8^{+} cells. In addition the gp33 and np396 specific responses were analyzed by tetramer staining of the indicated specificities. Shown are the total numbers of the indicated cells in the spleen. Data shown are representative graphs with n=4 mice. ns, not significant; * p<0.05; ** p<0.01 (unpaired two-tailed *t*-test). (**d**) Differentiation phenotype of WT and IFNAR$^{-/-}$ P14 cells as in (a) in NK cell depleted (aNK1.1) and un-depleted (Bl6) mice, shown are the % of short lived effector cells (CD44^{hi}, KLRG1^{hi}, CD127^{low}) and memory precursor effector cells (CD44^{hi}, KLRG1^{low}, CD127^{hi}) amongst WT or IFNAR$^{-/-}$ P14 cells 7 days post LCMV infection. Data shown are mean ± SEM of n= 12 mice pooled from 3 independent experiments. ns, not significant (unpaired two-tailed *t*-test). (**e** and **f**) Time course analysis of LCMV infected mice following co-transfer of WT and IFNAR$^{-/-}$ P14 cells into NK cell depleted (aNK) and un-depleted (Bl6) mice. (**e**) Percentage of P14 cells amongst total CD8^{+} T cells in blood is shown. (**f**) Total P14 cell number day 80 post infection in the spleen of un-depleted (Bl6) and NK cell depleted (aNK1.1) mice. (**b,d,e,f**) Data shown are mean ± SEM of n=4 mice representative of 3 independent experiments. ns, not significant; ** p<0.01 (unpaired two-tailed *t*-test).

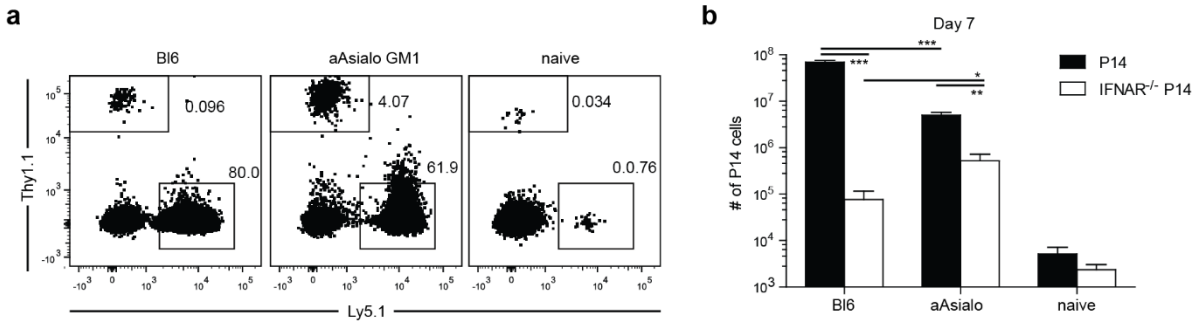


Figure 4.13 Asialo GM1 NK cell depletion partially restores IFNAR$^{-/-}$ P14 cell expansion at day 7 post infection

(**a** and **b**) Co-transfer of WT and IFNAR$^{-/-}$ P14 cells into NK cell depleted (aAsialo) or un-depleted (Bl6) mice followed one day later by LCMV infection. Naïve mice received cell co-transfers but were left uninfected. On day 7 post infection total cell numbers were determined. (**a**) Representative flow cytometric dot plots pre-gated on CD8$^+$ T cells are shown. The numbers represent the percentage of total CD8$^+$ T cells in the spleen. (**b**) Total cell numbers of WT and IFNAR$^{-/-}$ P14 cells at day 7 post LCMV infection. Data shown are mean ± SEM of n= 4 mice representative of at least 3 experiments. * p<0.05; ** p<0.01; *** p<0.001 (unpaired two-tailed *t*-test).

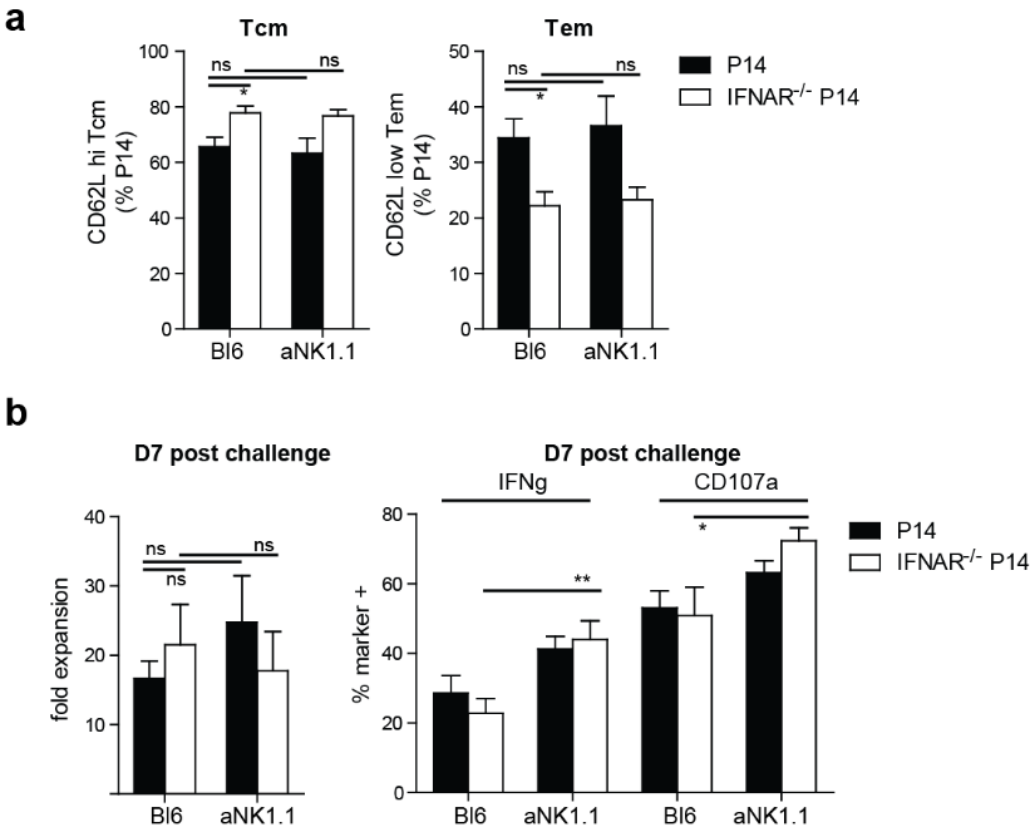


Figure 4.14: Memory P14 cell functionality is not affected by NK depletion

(a) Memory cell differentiation phenotype at day 80 post LCMV infection from un-depleted (Bl6) and NK cell depleted (aNK1.1) mice. Shown is the percentage of central memory T cells (CD44^{hi}, CD62L^{hi}) and effector memory T cells (CD44^{hi}, CD62L^{low}) amongst total WT or IFNAR$^{-/-}$ P14 cells. (**b**) Memory cell functionality was tested by isolation of CD8$^+$ T cells from day 80 post infected mice from un-depleted (Bl6) or NK cell depleted (aNK1.1) mice, MACS purification of CD8$^+$ T cells followed by transfer into naïve hosts and challenge one day later with VVG2. Fold expansion of T cells from un-depleted (Bl6) or NK cell depleted (aNK1.1) mice at day 7 post challenge was calculated and the percentage of IFN-γ and CD107a positive cell was determined after in vitro peptide restimulation. Data shown are mean ± SEM of n=4 mice representative of 3 independent experiments. ns, not significant; *p<0.05; **p<0.01 (unpaired two-tailed t-test).

4.2.4 Memory IFNAR$^{-/-}$ P14 cell expansion is also curtailed by NK cells during LCMV infection

Similar to primary T cell responses, memory recall of CD8^{+} T cells is also dependent on signal 3 cytokines and memory CD8^{+} T cell responses to LCMV are dependent on type I IFN signaling, with IFNAR$^{-/-}$ CD8^{+} T cells showing greatly reduced recall expansion (Keppler and Aichele 2011). Therefore, we examined whether the memory response to LCMV is also negatively regulated by NK cells in the absence of type I IFN signaling on CD8^{+} T cells. To generate phenotypically equivalent memory WT and IFNAR$^{-/-}$ P14 cells, WT and IFNAR$^{-/-}$ P14 cells were co-transferred into naïve mice followed by VVG2 infection, which leads to an equal expansion of WT and IFNAR$^{-/-}$ P14 cells (**Figure 4.15A**). At day 80 post infection WT and IFNAR$^{-/-}$ P14 cells had differentiated into phenotypically equivalent memory cells (**Figure 4.15B**). At this time point, memory CD8^{+} T cells were purified and transferred into either NK cell depleted or un-depleted mice, followed by challenge with LCMV or VVG2. At day 4 post challenge, analysis of T cell expansion in the blood revealed that the reduced expansion of IFNAR$^{-/-}$ P14 cells was also apparent during LCMV challenge and that this defect could be completely restored by NK cell depletion during the challenge phase (**Figure 4.15C**). NK cell depletion during LCMV challenge led to expansion of IFNAR$^{-/-}$ P14 cells equivalent to those with VVG2 challenge. At day 7 post LCMV challenge, as during the primary response, NK cell depletion led to a partial recovery of IFNAR$^{-/-}$ P14 cell expansion (**Figure 4.15D**). These data demonstrate that both primary and secondary CD8^{+} T cell responses to LCMV are dependent on type I IFN signaling and that depletion of NK cells leads to a markedly enhanced expansion of both naïve and memory IFNAR$^{-/-}$ P14 cells.

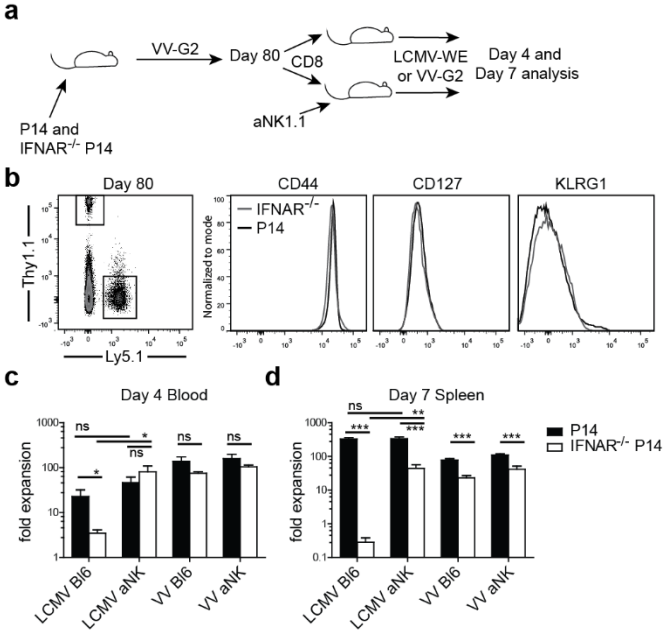


Figure 4.15 Memory IFNAR-/- P14 cell expansion is curtailed by NK cells during LCMV infection

(**a**) Functionally equivalent WT and IFNAR$^{-/-}$ P14 cells were generated by infection with VVG2 one day after co-transfer of 10^{6} WT and IFNAR$^{-/-}$ P14 cells. At day 80 post infection CD8^{+} T cells were MACS purified and transferred into naïve NK cell depleted (aNK1.1) or un-depleted (Bl6) hosts followed one day later by challenge with LCMV or VVG2. (**b**) Phenotypic analysis of P14 memory T cells at day 80 post VVG2 infection. Flow cytometric dot plots and histograms of the indicated markers are shown and are representative of 3 independent experiments with n=4 mice each. (**c**) Fold expansion of WT and IFNAR$^{-/-}$ P14 cells at day 4 post challenge and (**d**) day 7 post challenge is shown. (**c,d**) Data shown are mean ± SEM of n=12 mice pooled from 3 independent experiments. ns, not significant; * $p<0.05$; ** $p<0.01$; *** $p<0.001$ (unpaired two-tailed *t*-test).

4.2.5 Negative regulation of IFNAR$^{-/-}$ T cell expansion by NK cells is perforin dependent

Since negative regulation of IFNAR$^{-/-}$ T cell expansion was NK cell dependent we investigated the mechanisms of how this regulation occurs, focusing initially on a potential role of perforin mediated cytotoxicity. To this end, we co-transferred WT and IFNAR$^{-/-}$ P14 cells into perforin knockout mice followed by LCMV infection. Analysis at day 4 post infection revealed that IFNAR$^{-/-}$ P14 cell priming in a perforin deficient host led to the complete recovery of early expansion, comparable to WT P14 cells (**Figure 4.16A and B, upper row**). The expansion of IFNAR$^{-/-}$ P14 cells in perforin deficient hosts was comparable to the NK cell depleted situation and additional NK cell depletion in perforin knockout mice had no added effect (**Figure 4.17**). Furthermore, at day 7 post infection, expansion of IFNAR$^{-/-}$ P14 cells in perforin knockout mice was also only partially recovered (**Figure 4.16A and B, bottom row**), in analogy to NK cell depleted hosts. Similar results were obtained when IFNAR$^{-/-}$ SM cells were transferred into perforin deficient hosts, with complete recovery of IFNAR$^{-/-}$ SM cell expansion by day 4 post infection and partial recovery by day 7 in NK cell depleted hosts, and a full recovery in perforin deficient mice (**Figure 4.16C and D**). The reduced expansion of WT SM cells at day 7 in perforin deficient compared to perforin sufficient mice is in line with an increased viral burden in the former, as control of LCMV is dependent on perforin-mediated CD8^{+} T cell effector function. The total number of IFNAR$^{-/-}$ SM cells was comparable between PKOB mice and NK cell depleted B6 mice. Taken together, we show that activation of IFNAR$^{-/-}$ T cells in a perforin deficient host leads to a recovery in expansion similar to the expansion in NK cell depleted animals, indicating that the early negative regulation by NK cells is perforin dependent, implicating a direct killing mechanism.

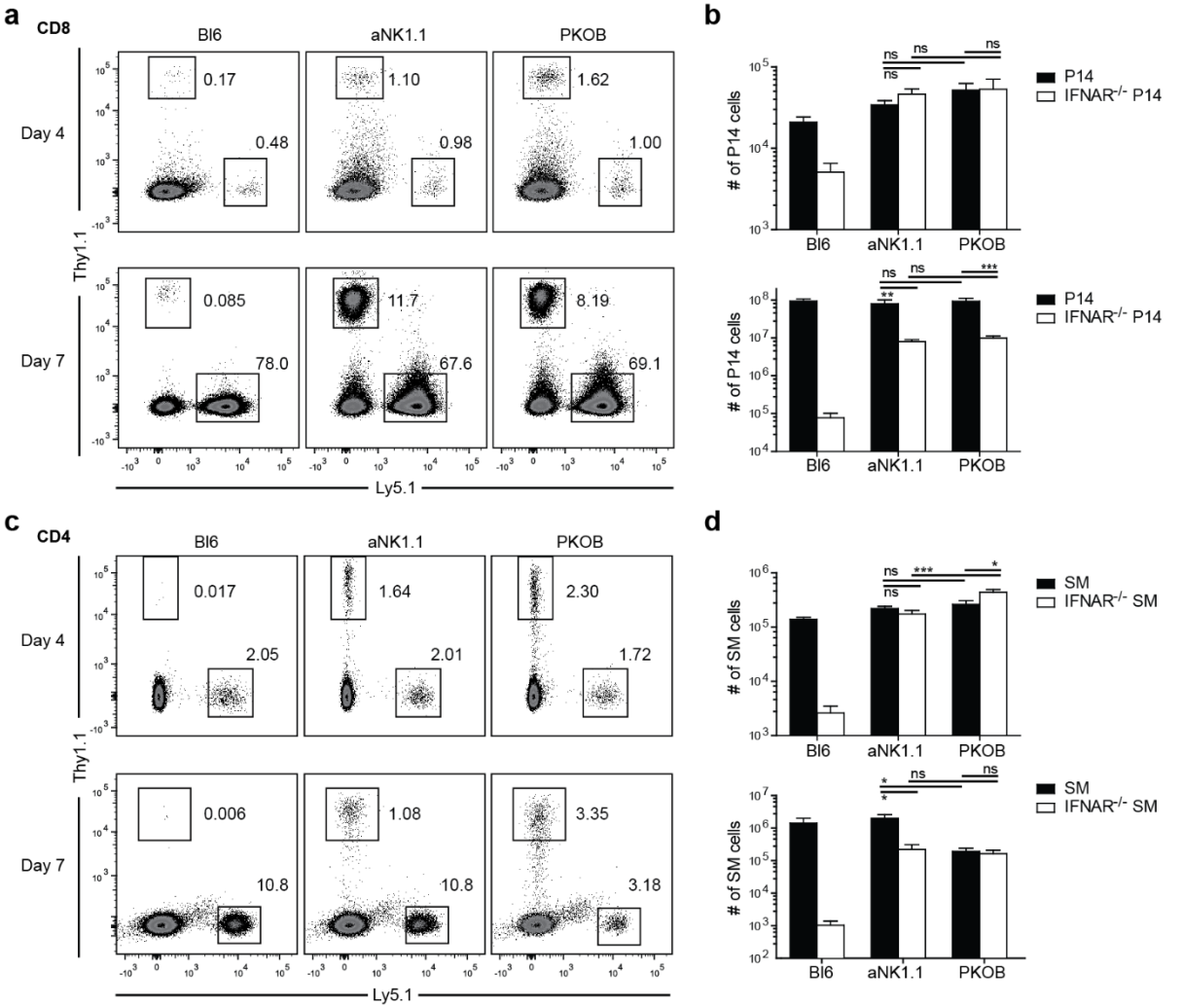


Figure 4.16 Negative regulation of IFNAR-/- T cell expansion by NK cells is perforin dependent

(**a-d**) Co-transfer of WT and IFNAR$^{-/-}$ P14 or SM cells into NK cell depleted (aNK1.1), perforin deficient (PKOB) or un-depleted (Bl6) mice followed one day later by LCMV infection. On day 4 (top row) and day 7 (bottom row) post infection the percentage and total cell numbers were determined. (**a,c**) Representative flow cytometric dot plots pre-gated on CD8$^+$ (**a**) or CD4$^+$ (**c**) T cells, the numbers represent the percentage of total CD8$^+$ (**a**) or CD4$^+$ (**c**) T cells in the spleen. (**b,d**) Total cell numbers of WT and IFNAR$^{-/-}$ P14 or SM cells at day 4 (top) and day 7 (bottom) post LCMV infection. Data shown are mean ± SEM of n=12 mice pooled from 3 experiments. ns, not significant; **p<0.01; ***p<0.001 (unpaired two-tailed *t*-test).

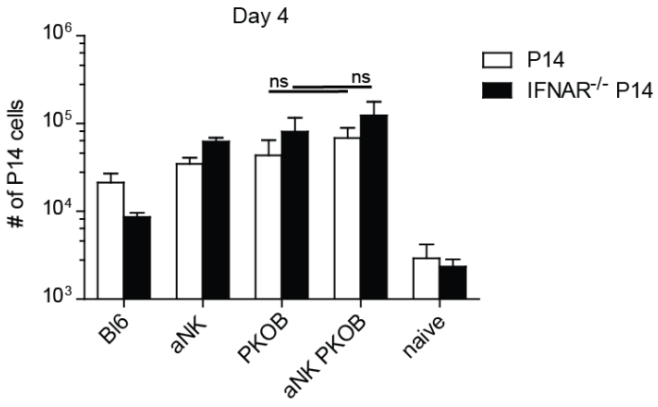


Figure 4.17 NK depletion in perforin deficient mice has no additional impact on recovery of P1 IFNAR-/- cells

Co-transfer of WT and IFNAR$^{-/-}$ P14 cells into NK cell depleted (aNK1.1), perforin deficient (PKOB), NK cell depleted perforin deficient (aNK PKOB) or un-depleted (Bl6) mice followed one day later by LCMV infection. Naïve mice received cell co-transfers but were left uninfected. On day 4 post infection the total cell numbers were determined. Data shown are mean ± SEM of n=4 mice representative of 3 independent experiments. ns, not significant (unpaired two-tailed *t*-test).

4.2.6 NK cell depletion has no effect on DC function, viral titre or type I IFN levels

As NK cell depletion leads to a complete recovery of IFNAR$^{-/-}$ P14 and SM cells up to day 4 post infection we decided to see if this negative regulation by NK cells was due to an indirect effect such as altered DC numbers or function, altered viral load or altered type I IFN levels. First to analyze the effect of NK cell depletion on DCs we infected Bl6 or NK cell depleted mice with LCMV-WE and analyzed the number of DCs and their phenotype. We found that NK cell depletion had no effect on either the number or the phenotype of the DCs (**Figure 4.18A and B**). Next we analyzed the ability to DCs from Bl6 and NK cell depleted mice to activate and induce proliferation of naïve WT or IFNAR$^{-/-}$ P14 cells. DCs were taken from the spleen of day 2 post LCMV infected mice isolated and cultured together with CFSE labelled naive P14 cells. Three days after co-culture the CFSE dilution profile of the T cells was analyzed and revealed no functional difference in the ability of DCs to activate T cells regardless of whether the DCs came from an NK sufficient or deficient environment (**Figure 4.18C and D**). An additional indirect effect NK cell depletion could have on T cells would be a change in the type I IFN levels, however at day 2 post infection no significant differences were seen between Bl6 and NK cell depleted mice (**Figure 4.18E**). Lastly a depletion of NK cells could have an impact on the viral load in the mice which could have an impact on the T cell response, however we found only moderate differences in the viral load from Bl6, NK cell depleted or perforin knockout mice (**Figure 4.18F**). Although the minor differences observed are statistically significant, all values are in the same range, with a slight decrease in titer in NK cell depleted mice and a slight increase in titer in PKOB mice (Bl6-470000, aNK-350000, PKOB-700000). At day 7 virus has largely been cleared from the spleen of Bl6 and NK cell depleted mice, whereas a small amount of virus is still present in PKOB mice (270 pfu). These minor differences reflect the T cell expansion seen as NK cell depleted mice have a stronger T cell response which leads to better clearance. Perforin deficient mice show delayed clearance of the infection, as the only cells present with perforin in this setup are the transferred T cells, and LCMV is dependent on perforin-mediated CD8^{+} T cell effector function for clearance. Taken together the results indicate a direct role for NK cell regulation of the T cells response.

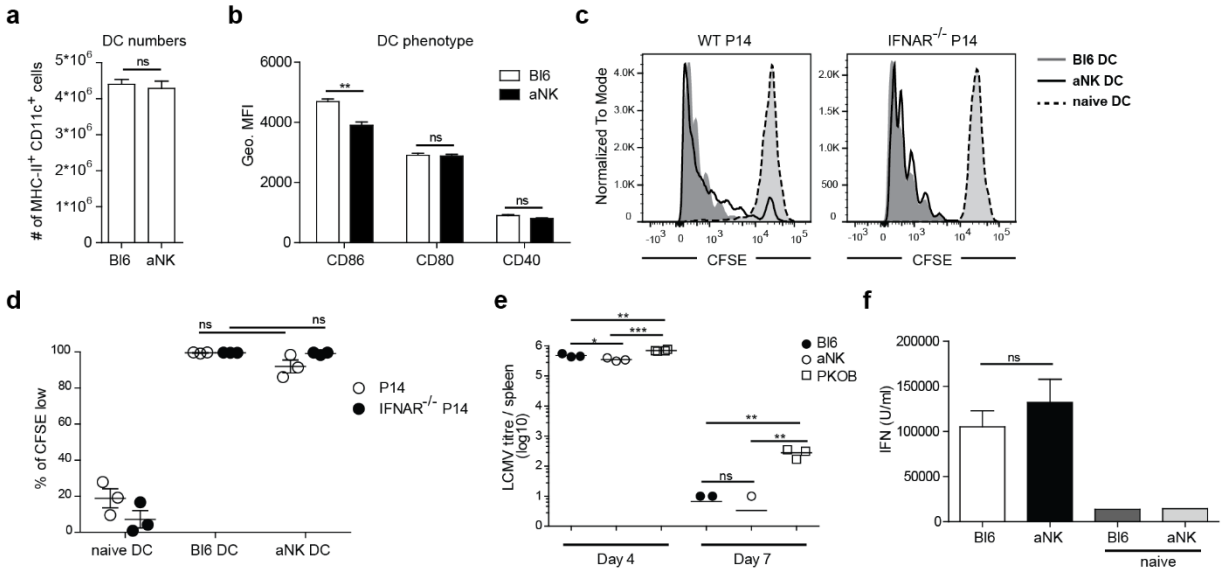


Figure 4.18: DCs, viral titre and type I IFN levels are not affected by NK cell depletion

(a) Total DC numbers (MHCII$^+$, CD11c$^+$) in the spleen at day 2 following infection of NK1.1 depleted (aNK) or un-depleted (Bl6) mice with LCMV-WE. (**b**) Phenotype (CD86, CD80, CD40) of DCs (MHCII$^+$, CD11c$^+$) in the spleen at day 2 following infection of NK1.1 depleted (filled bars) or un-depleted (open bars) mice with LCMV-WE in shown. Data shown are mean SEM of n=4 mice representative of 2 independent experiments. ns, not significant; ** p<0.01 (unpaired two-tailed t-test). (**c**) CFSE dilution profiles of WT P14 (left) and IFNAR$^{-/-}$ P14 (right) cultured together with day 2 post infection CD11c MACSed DCs. DCs were isolated from un-depleted (Bl6, dark gray filled), NK depleted (black line) or naïve (dashed line, light gray filled) mice. (**d**) Summary graph showing the percentage of CFSE low WT (open circles) and IFNAR$^{-/-}$ (filled circles) P14 cells cultured together with the indicated DCs. (e) LCMV titers in spleen were measured in Bl6, NK depleted Bl6 (aNK) and perforin deficient (PKOB) mice that were adoptively transferred with WT and IFNAR$^{-/-}$ P14 cells followed by infection with 200 pfu LCMV-WE. At day 4 and day 7 post infection, infectious viral particles were enumerated by focus forming assay.(f) C57BL/6 (Bl6) mice were either depleted of NK cells (aNK) or left untreated (Bl6), followed by infection with 200 pfu LCMV WE. Serum type I IFN levels were measured in the serum of infected mice as well as in uninfected Bl6 or NK depleted Bl6 mice by a type I IFN bioassay at day 2 post infection. Data shown are mean SEM of n=3 representative of 2 independent experiments. ns, not significant; * p<0.05; ** p<0.01; *** p<0.001 (unpaired two-tailed t-test).

4.2.7 NK cells preferentially kill IFNAR$^{-/-}$ T cells

Since NK cells negatively regulate IFNAR$^{-/-}$ T cell expansion in a perforin dependent manner we investigated whether NK cells were preferentially killing IFNAR$^{-/-}$ P14 cells. To demonstrate direct killing of CD8$^+$ T cells by NK cells we established an *in vivo* killer assay. In this assay, WT and IFNAR$^{-/-}$ P14 cells were activated *in vivo* by a VVG2 and LCMV8.7 co-infection, to increase T cell recovery. Activated CD8$^+$ T cells were isolated at day 3 post infection and transferred into naive or infection matched hosts which had been depleted of NK cells or left untreated (**Figure 4.19A**). After 6 hours, spleens from host mice were isolated and P14 cell recovery was analyzed. The number of recovered P14 cells in infected hosts was normalized to the number recovered in naïve hosts and the percentage of recovery was determined (**Figure 4.19B**). We found a significant reduction in the recovery of IFNAR$^{-/-}$ P14

cells in un-depleted hosts compared to WT P14 cells, and this reduced recovery was abolished in NK cell depleted hosts. Also, by examining the ratio of IFNAR$^{-/-}$ to WT P14 cells in the different hosts we found a specific reduction in IFNAR$^{-/-}$ P14 cells in un-depleted hosts which was abolished by NK cell depletion (**Figure 4.19C**). These results indicate a preferential killing of IFNAR$^{-/-}$ compared to WT P14 cells, but due to the limitations and complexity of this *in vivo* killer assay we next developed an *ex vivo* killer assay to directly examine the killing of P14 cells by NK cells. In this assay, WT and IFNAR$^{-/-}$ P14 cells were activated in NK cell depleted mice *in vivo* by co-infection with VVG2 and LCMV8.7. At day 3 post infection WT and IFNAR$^{-/-}$ P14 cells were FACS sorted to high purity and placed together with day 2 *in vivo* LCMV activated NK cells (**Figure 4.19D**). Following a 6 hour incubation period, T cell killing was visualized by CD8^{+} T cell staining with 7-AAD and Annexin V, where double positive cells represent late apoptotic cells. We observed a strong increase in the percentage of double positive IFNAR$^{-/-}$ P14 cells with increasing ratios of effector NK to CD8^{+} T cell target ratios (**Figure 4.19E**). This increase in apoptotic IFNAR$^{-/-}$ P14 cells was reduced to background levels in the presence of naive NK cells or activated NK cells from perforin knockout mice. As we observed an increase of apoptotic IFNAR$^{-/-}$ P14 cells but no increase in apoptotic WT P14 cells, we concluded that IFNAR$^{-/-}$ P14 cells are specifically rendered susceptible to NK cell mediated killing, whereas T cells that can sense type I IFNs are protected.

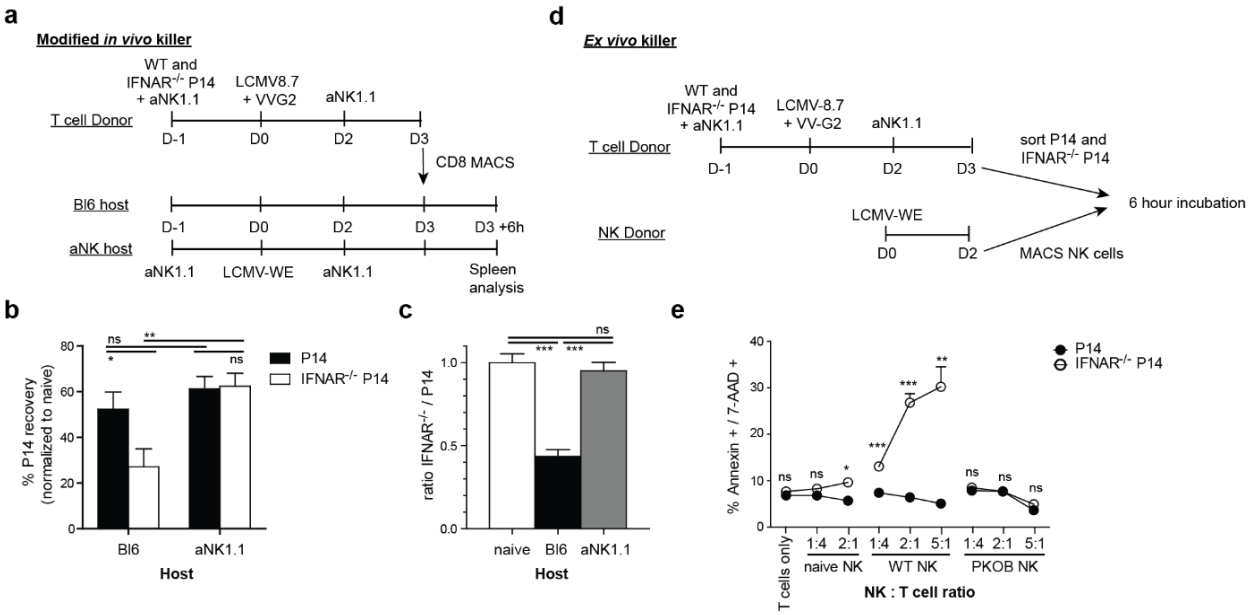


Figure 4.19 NK cells preferentially kill IFNAR$^{-/-}$ T cells

(a) Experimental setup of the *in vivo* killer assay used in **b** and **c**. T cell donors were NK cell depleted and co-transferred WT and IFNAR$^{-/-}$ P14 cells followed one day later by LCMV8.7 + VVG2 co-infection. Day 3 post infection P14 cells were isolated, MACS purified and transferred into infection matched (LCMV-WE) un-depleted (Bl6) or NK cell depleted (aNK1.1) hosts. 6 hours later P14 cell recovery was determined and normalized to the recovery in naïve hosts. (**b**) WT and IFNAR$^{-/-}$ P14 cell recovery relative to the amount recovered in naïve mice is shown. Data shown are mean ± SEM of n=12 mice pooled from 3 experiments. ns, not significant; *p<0.05; **p<0.01 (unpaired two-tailed t-test). (**c**) Ratio of IFNAR$^{-/-}$ to WT P14 cells from the indicated hosts, data shown are mean ± SEM of n=4 mice representative of 3 independent experiments. ns, not significant; ***p<0.001 (unpaired two-tailed t-test). (**d**) Experimental setup of the *ex vivo* killer assay used in **e**. Activated T cells and NK cells were placed together in round bottom 96 well plates. T cell donors were NK cell depleted and co-transferred WT and IFNAR$^{-/-}$ P14 cells followed by LCMV8.7 + VVG2 co-infection. At day 3 post infection WT (Ly5.1^{+}) and IFNAR$^{-/-}$ (Thy1.1^{+}) cells were FACS sorted to high purity. NK cells were isolated from LCMV-WE infected mice at day 2 post infection and MACS purified. 2x10^{4} WT or IFNAR$^{-/-}$ P14 cells were placed together in triplicates with the indicated amounts of NK cells for 6 hours followed by flow cytometric analysis. (**e**) The percentage of apoptotic Annexin V and 7-AAD double positive cells is depicted as percentage of total WT or IFNAR$^{-/-}$ P14 cells. Data shown are mean ± SEM of n=3 representative from at least 3 experiments ns, not significant; * p<0.05; ** p<0.01; *** p<0.001 (unpaired two-tailed *t*-test).

4.2.8 Activated IFNAR$^{-/-}$ CD8^{+} T cells are killed by NK cells in an NCR1 dependent manner

As IFNAR$^{-/-}$ P14 cells were more susceptible to NK cell mediated killing we examined the mechanisms of how IFNAR$^{-/-}$ P14 cells are recognized and killed whereas WT P14 cells are left largely unharmed. From the microarray analysis we found differential regulation of various molecules involved in NK cell activation or inhibition. To reconfirm differential expression of activating or inhibiting ligands for NK cells, expression of a selection of ligands was analyzed by quantitative real time PCR (**Figure 4.20A**). Amongst the reconfirmed ligands we found a 3-fold up-regulation of the NKG2D activating ligand Mult-1 (Carayannopoulos, Naidenko et al. 2002, Diefenbach, Hsia et al. 2003) on IFNAR$^{-/-}$ compared to WT P14 cells. IFNAR$^{-/-}$ P14 cells had a 2-fold up-regulation of Clec2i (Clr-g), the activating ligand for NKR-P1F (Iizuka, Naidenko et al. 2003) and a 3-fold up-regulation of CD99 which has been shown to have both activating and inhibitory functions, depending on the receptor it binds to (Shiratori, Ogasawara et al. 2004). The inhibitory ligand CD48, which binds 2B4 (McNerney, Guzior et al. 2005, Waggoner, Taniguchi et al. 2010), was expressed at similar levels on IFNAR$^{-/-}$ and WT P14 cells. Strongly up-regulated on IFNAR$^{-/-}$ compared to WT P14 cells was the adhesion molecule Vcam1, but no difference was seen in the expression of the activating ligand CD155 (Bottino, Castriconi et al. 2003, Fuchs, Cella et al. 2004). Of note, the classical inhibitory ligand, MHC class I, was not differentially expressed on WT or IFNAR$^{-/-}$ P14 cells (data not shown). To investigate a possible role of the mentioned activating ligands, we utilized the *ex vivo* killer assay together in combination with various blocking antibodies. Blocking of NKG2D, Vcam1, CD48 or CD7, a ligand involved in T cell apoptosis (Pace, Hahn et al. 2000), had no impact on

preferential NK cell mediated killing of IFNAR$^{-/-}$ compared to WT P14 cells (**Figure 4.20B**). Confirming the *ex vivo* killer analysis we found no role for NKG2D *in vivo* when co-transferring WT and IFNAR$^{-/-}$ P14 cells into NKG2D$^{-/-}$ mice followed by LCMV infection (data not shown). Surprisingly, we found that *in vitro* blocking of the NK cell activating receptor NCR1, of which the cellular ligands are unknown, led to a complete abrogation of apoptosis induction in IFNAR$^{-/-}$ P14 cells. Consequently, we examined the expression of NCR1 ligands on WT and IFNAR$^{-/-}$ P14 cells at day 4 post infection, using an NCR1-IgG fusion protein, recognizing the unknown NCR1 ligands (Mandelboim, Lieberman et al. 2001). NCR1-IgG fusion protein staining revealed a significantly increased level of NCR1 ligand expression on IFNAR$^{-/-}$ compared to WT P14 cells following LCMV infection, where around 40% of the IFNAR$^{-/-}$ cells expressed NCR1 ligands (**Figure 4.20C and D**). Interestingly, following infection with VVG2, IFNAR$^{-/-}$ P14 cells expressed NCR1 ligands to comparable levels as WT P14 cells during LCMV infection (data not shown), indicating that the inflammatory environment associated with VV could possibly compensate for the lack of type I IFN signaling to protect the T cells from expressing NCR1 ligands. As IFNAR$^{-/-}$ CD4^{+} Smarta cells are also killed in an NK cell dependent manner, we examined the NCR1 ligand expression levels on WT and IFNAR$^{-/-}$ Smarta cells four days post LCMV infection. Similar to IFNAR$^{-/-}$ P14 cells we found an increased level of NCR1 ligand expression on IFNAR$^{-/-}$ Smarta cells compared to WT Smarta cells (**Figure 4.20C and D**). Additionally, using CD4^{cre+}IFNAR$^{f/f}$ mice, which lack IFNAR expression specifically on T cells, we found that endogenous T cells specific for gp33 and np396 also showed an increased expression of NCR1 ligands on their surface at day 7 post infection compared to T cells from Bl6 mice (**Figure 4.21A**). Furthermore, infection with the LCMV mutant LCMV8.7 following co-transfer of WT and IFNAR$^{-/-}$ P14 revealed that the up-regulation of NCR1 ligands on IFNAR$^{-/-}$ P14 cells is antigen activation dependent, as no increase was seen in the expression levels of NCR1 ligands in IFNAR$^{-/-}$ P14 cells upon LCMV8.7 infection (**Figure 4.21B**).

To functionally test the role of NCR1 *in vivo*, WT and IFNAR$^{-/-}$ P14 cells were co-transferred into NCR1$^{iCre/iCre}$ knock-in mice, which exhibit severe impairment of NCR1 expression, followed by LCMV infection. Four days post infection we found a significant recovery of IFNAR$^{-/-}$ P14 cell expansion compared to the expansion in WT hosts or heterozygous NCR1$^{iCre/WT}$ mice, despite the reported hyperactivity of NK cells in the NCR1$^{iCre/iCre}$ knock-in mice (Narni-Mancinelli, Jaeger et al. 2012) (**Figure 4.20E**). This recovery in expansion of IFNAR$^{-/-}$ P14 cells was comparable to NK cell depleted Bl6 hosts, indicating a strong role for NCR1 dependent killing of IFNAR$^{-/-}$ T cells *in vivo*. Similarly, following transfer of WT and

IFNAR$^{-/-}$ Smarta cells into NCR1$^{icre/icre}$ mice, followed by LCMV infection, we found that the expansion of IFNAR$^{-/-}$ Smarta cells could also be recovered to levels similar to NK cell depleted Bl6 mice when NK cells specifically lack the expression of NCR1 (**Figure 4.20F**). Taken together, the significant recovery of IFNAR$^{-/-}$ P14 and Smarta cell expansion in NCR1 deficient mice, together with the results from *ex vivo* NCR1 blocking in NK-mediated T cell killing assays, led us to conclude that activated NK cells recognize IFNAR$^{-/-}$ T cells via increased NCR1 ligand expression on those cells, culminating in perforin dependent killing of T cells that lack the ability to sense type I interferons.

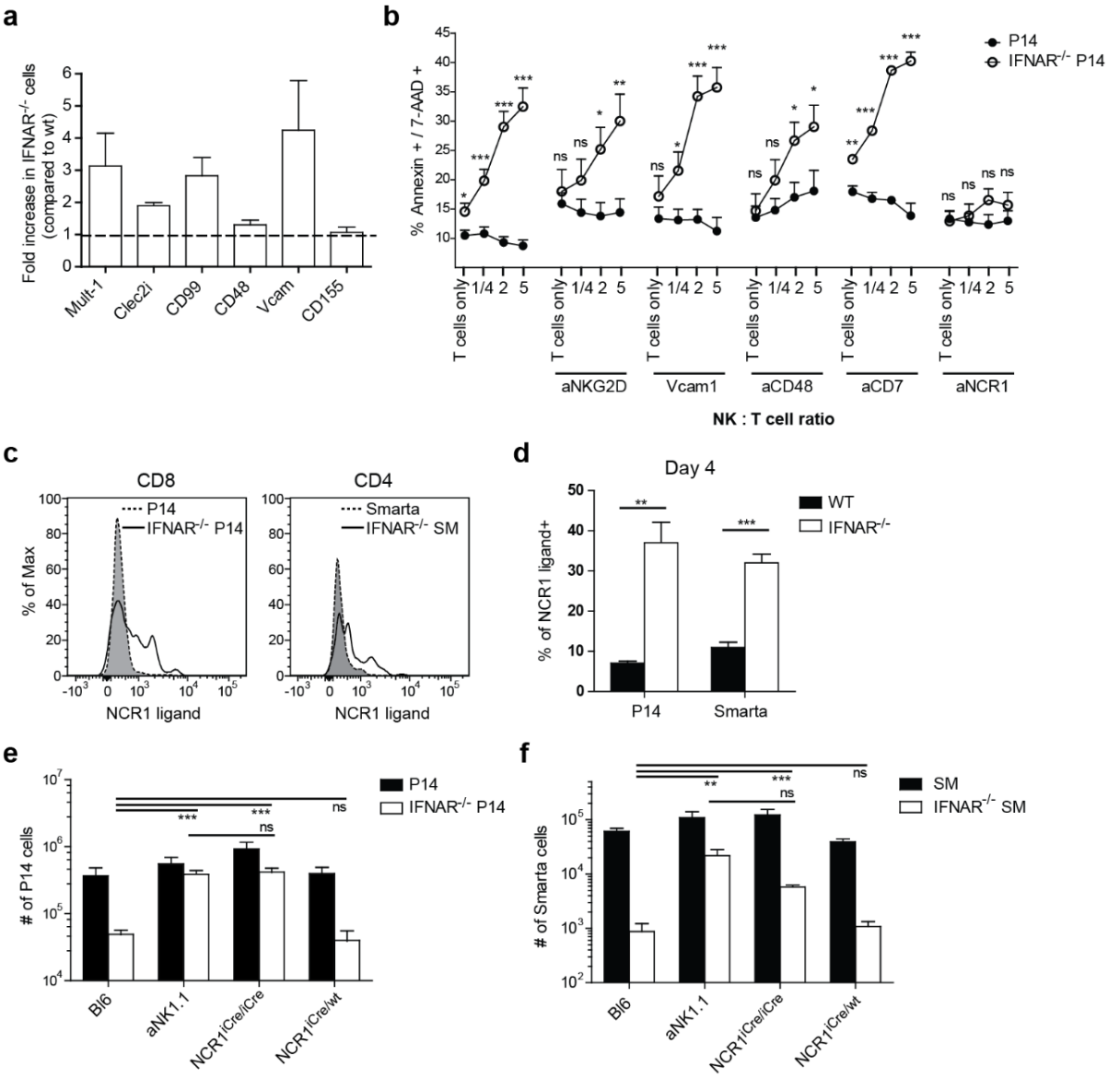


Fig. 4.20: Activated IFNAR$^{-/-}$ CD8^{+} T cells are killed by NK cells in a NCR1 dependent manner

(a) Quantitative real time PCR of the indicated NK cell activating or inhibitory ligands. RNA was isolated from WT and IFNAR-/- P14 cells on day 4 post LCMV. Shown is the fold increase in expression in IFNAR-/- P14 cells compared to WT P14 cells where a value over 1 (dashed line) indicates more expression in IFNAR-/- P14 cells. (b) Ex vivo killer assay as described in Fig. 6d in combination with blocking reagents for the indicated molecules. Shown is the percentage of Annexin V and 7-AAD double positive cells amongst the total P14 or IFNAR-/- P14 cells. Data shown are mean ± SEM of n=12 pooled from at least 3 experiments. (c,d) NCR1 ligand expression on IFNAR-/- and WT P14 and Smarta cells, stained by NCR1-IgG fusion protein on day 4 post LCMV infection. (c) Overlay histogram of WT (dashed, shaded line) and IFNAR-/- (black line) P14 cells (left) and WT (dashed, shaded line) and IFNAR-/- (black) Smarta cells (right) stained with 1 g NCR1-IgG fusion protein. (d) Percentage of NCR1 positive cells amongst WT or IFNAR-/- P14 or Smarta cells at day 4 post LCMV infection stained with 1 g NCR1-IgG fusion protein. (c,d) Data shown are mean ± SEM of n=4 representative from at least 3 experiments (e) Co-transfer of WT and IFNAR-/- P14 cells into NCR1 deficient (NCR1iCre/iCre), litter mate heterozygous controls (NCR1iCre/wt), NK1.1 depleted (aNK1.1) or un-depleted (Bl6) mice followed one day later by LCMV-WE infection. On day 4 post infection total cell numbers were determined. Data shown are mean ± SEM of n=12 mice from 3 pooled independent experiments. ns, not significant; *** p<0.001 (unpaired two-tailed t-test). (f) Co-transfer of WT and IFNAR-/- Smarta cells into NCR1 deficient (NCR1iCre/iCre), litter mate heterozygous controls (NCR1iCre/wt), NK1.1 depleted (aNK1.1) or un-depleted (Bl6) mice followed one day later by LCMV infection. On day 4 post infection total cell numbers were determined. Data shown are mean ± SEM of n=8 mice from 2 pooled independent experiments. ns, not significant; ** p<0.01; *** p<0.001 (unpaired two-tailed t-test).

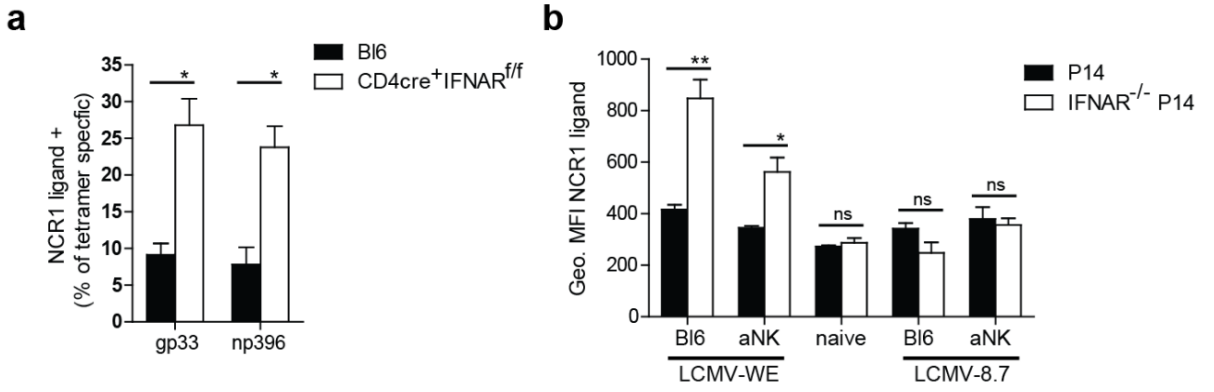


Figure 4.21 NCR1 ligand is expression on endogenous T cells and is activation dependent

(**a**) NCR1 ligand expression on endogenous T cells was analyzed day 7 post LCMV infection of Bl6 or CD4^{cre}IFNAR$^{f/f}$ mice. Shown is the percentage of NCR1 ligand positive T cells amongst the indicated tetramer specific CD8^{+} T cells. Data shown are mean ± SEM of n=4 representative mice. * p<0.05 (unpaired two-tailed *t*-test). (**b**) WT and IFNAR$^{-/-}$ P14 cells were co-transferred into NK cell depleted (aNK) or un-depleted mice (Bl6) followed by LCMV-WE or LCMV8.7 infection. Four days post infection NCR1 ligand expression was examined on the T cells in the spleen by staining with an NCR1-IgG fusion protein. Data shown are mean SEM of n=4 mice representative of 3 independent experiments. ns, not significant; * p<0.05; ** p<0.01 (unpaired two-tailed *t*-test).

Chapter 5

5. Discussion

5.1 Type I IFN regulation of T cell biology

Type I IFNs play a central role in the immune response to viral infections. Being induced by multiple PRRs involved in sensing viral nucleic acids (Perry, Chen et al. 2005, Keating, Baran et al. 2011), type I IFNs are secreted at high amounts very early after viral infections. Their release early following an infection parallels their role in the immune response, where type I IFNs play an important role not only in establishing an innate antiviral state but also in providing key signals for the proper development of an adaptive immune response (Seo and Hahm 2010). Focusing on the latter, we studied here the role of type I IFN signaling on T cells following an acute LCMV infection. Type I IFNs promote the induction of a proper T cell response not only indirectly through the activation and maturation of APCs but also directly where they signal directly on T cells and act as a signal 3 cytokine for T cell activation. In addition to the essential role of signal 1 (antigen presentation) and signal 2 (co-stimulation) for T cell activation, soluble signal 3 cytokines are essential for the full activation and survival of T cells, highlighted by the findings that T cells lacking the ability to senses type I IFNs following an LCMV infection display a serve impairment in expansion (Kolumam, Thomas et al. 2005, Aichele, Unsoeld et al. 2006). As type I IFN signaling on T cells is critical for the production of a proper T cell response we analyzed here the role of type I IFN signaling on T cells to better understand the underlying mechanisms of the impaired expansion of T cells lacking the ability to sense type I IFNs. Therefore we experimentally analyzed to role of type I IFN signaling on several key aspects of T cell biology including differentiation, expansion and survival of antigen specific T cells.

5.1.1 The role of type I IFNs on T cell differentiation

Following antigen encounter and activation, T cells undergo a process of differentiation which initially leads to the development of effector T cells and later the development of memory T cells. However, differentiation of T cells can lead to the production of various subsets of effector T cells depending on the signals received by the activated T cells (Cui and Kaech 2010). At the peak of effector T cell expansion two main subsets can be found, short lived effector cells (SLECs) and memory precursor effector cells (MPECs). SLECs are more terminally differentiated cells with strong cytolytic activity but shortened life span, with the majority dying during the contraction phase. MPECs on the other hand have more memory potential and often survive contraction to contribute to the memory T cell pool. Many factors and pathways have been proposed to lead to the development of these two subsets and memory T cells, including; asymmetric cell division, TCR signaling strength, timing of stimulation and inflammatory

cytokine signaling. Interestingly it was shown that a single naïve T cell has the potential to differentiate into all subsets, indicating that naïve T cells are not preprogrammed for a certain differentiation fate, but instead other factors are important in determining the fate of T cell differentiation (Stemberger, Huster et al. 2007). Here we have elucidated the role of type I IFN signaling on $CD8^+$ T cells and its ability to act as a fate determining differentiation factor *in vivo*. We found that $CD8^+$ T cells lacking the ability to sense type I IFNs failed to form terminally differentiated SLECs following an acute viral infection associated with abundant levels type I IFNs. $IFNAR^{-/-}$ P14 cells, despite demonstrating a reduced expansion potential, could form qualitatively equivalent memory cells compared with WT P14 cells, albeit at a much lower frequency than their WT counterparts. Moreover, we showed *in vivo* and confirmed *in vitro* that type I IFN signaling on $CD8^+$ T cells leads to upregulation of the transcription factor T-bet which can drive the differentiation of SLECs (**Figure 5.1**). In summary, this study identifies type I IFNs as an important factor instructing the lineage choice towards the differentiation of SLECs in the context of an infection inducing a type I IFN dominated inflammatory cytokine milieu.

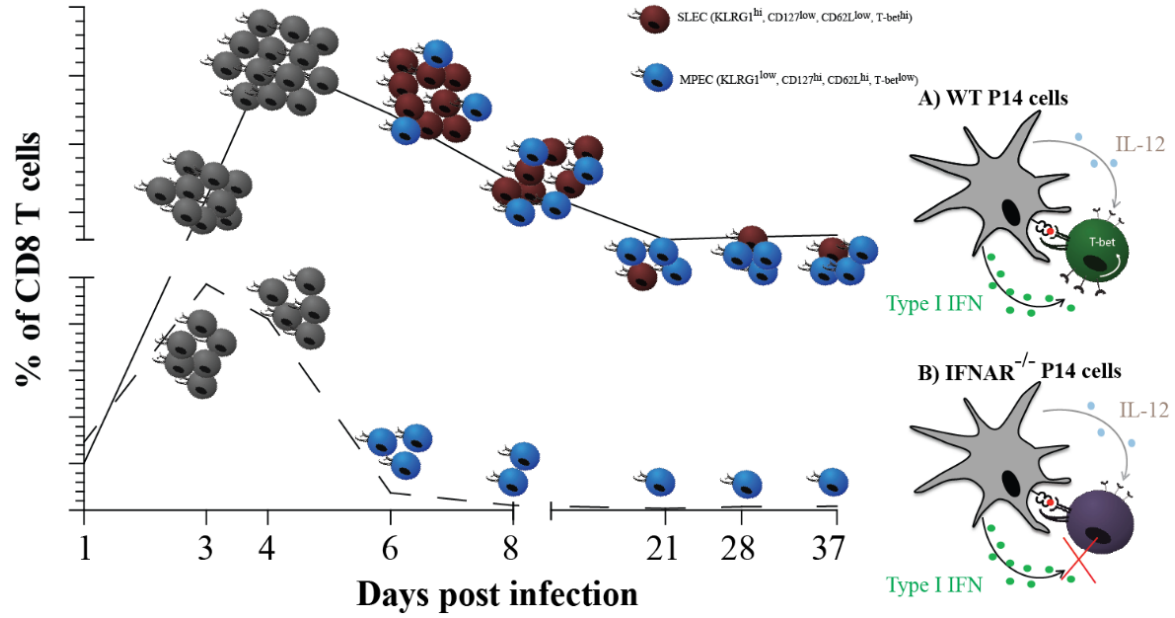


Figure 5.1. Fate decision of $CD8^+$ T cells by direct type I IFN signaling.

During an acute infection leading to an inflammatory milieu which is dominated by type I IFNs, not only the extent of $CD8^+$ T cell expansion and survival but also the differentiation pathway into SLECs and MPECs is critically influenced by type I IFN receptor expression on $CD8^+$ T cells. (A) In the presence of type I IFN receptor expression, type I IFN signaling on $CD8^+$ T cells leads to pronounced upregulation of T-bet, thereby initiating an SLEC differentiation program. (B) In the absence of type I IFN receptor expression, $CD8^+$ T cells fail to upregulate T-bet comparably to their WT counterparts, leading to preferential MPEC differentiation. Memory $CD8^+$ T cell levels are substantially reduced in absence of type I IFN signaling on $CD8^+$ T cells but not their quality with respect to undergoing secondary expansion and effector cell differentiation.

The data presented here expand and complement our current knowledge about the factors involved in the differentiation of $CD8^+$ T cells (Parish and Kaech 2009, Lefrancois and Obar 2010), which is known to involve both cell intrinsic factors such as T-bet and eomesodermin as well as cell extrinsic differentiation factors, such as IL-2 and IL-12. Much like IL-12, type I IFNs acts as a signal 3 cytokine promoting expansion, effector cell differentiation and survival of activated $CD8^+$ T cells (Curtsinger, Valenzuela et al. 2005). As both of these cytokines can serve as differentiation factors for $CD8^+$ T cells, the nature of the invading pathogen determines which cytokine is predominantly induced, often one cytokine is produced at the expense of the other (Cousens, Peterson et al. 1999, Dalod, Salazar-Mather et al. 2002). Of note, less redundancy between IL-12 and type I IFNs has been found in humans and IL-12 seems to be the main signal driving $CD8^+$ T cell effector differentiation, whereas type I IFNs enhances the development of memory $CD8^+$ T cells (Huber and Farrar 2011).

There is ample evidence in the literature that direct IL-12 signaling on activated $CD8^+$ T cells enhances expansion and promotes the transition towards an SLEC phenotype (Takemoto, Intlekofer et al. 2006, Joshi, Cui et al. 2007, Pearce and Shen 2007, Wilson, Matthews et al. 2008, Cui, Joshi et al. 2009). An elegant study by Kaech and colleagues further clarified these findings, identifying IL-12 as an important factor regulating memory $CD8^+$ T cell formation by establishing a gradient of the transcription factor T-bet. In particular, this report clearly showed that T-bet is necessary and sufficient to drive the formation of SLECs, with high T-bet expression leading to the differentiation into SLECs, and lower amounts of T-bet facilitating the formation of MPECs (Joshi, Cui et al. 2007). These finding supports our *in vivo* results showing that following an acute LCMV8.7 and VVG2 co-infection WT P14 cells strongly upregulated T-bet expression, whereas $IFNAR^{-/-}$ P14 cells also upregulated T-bet but to significantly lower levels. It should be noted, that TCR mediated activation of $CD8^+$ T cells alone in the absence of exogenous cytokines is sufficient to upregulate T-bet expression, at least to a certain extent (**Figure 4.7C** and (Joshi, Cui et al. 2007)) but only sustained high level expression of T-bet seems to be instructive for SLEC differentiation (Joshi, Cui et al. 2007). In our *in vivo* experimental setup, it is also conceivable that low levels of IL-12 induced upon LCMV8.7 and VVG2 co-infection were contributing the upregulation of T-bet in $IFNAR^{-/-}$ P14 cells. Nevertheless, the extent of T-bet upregulation was not sufficient to drive the differentiation of $IFNAR^{-/-}$ $CD8^+$ T cells into SLECs which is in agreement with the demonstration that only high levels of T-bet expression favored SLEC differentiation upon transduction of $T\text{-}bet^{-/-}$ $CD8^+$ T cells with a retroviral construct allowing for graded amounts of T-bet expression (Joshi, Cui et al. 2007).

In line with our observation that type I IFN signaling can act as an instructive signal for SLEC differentiation, it was recently reported by Mescher and colleagues that type I IFNs can induce the upregulation of certain effector molecules as well as the transcription factor T-bet in activated $CD8^+$ T cells *in vitro* (Agarwal, Raghavan et al. 2009). As many *in vitro* differentiation studies use large amounts of cytokines which might not reflect the *in vivo* situation, it is important to consolidate such *in vitro* findings by *in vivo* data. Our results identifying direct type I IFN signaling on $CD8^+$ T cells as a differentiation factor of SLECs, clearly support these *in vitro* data. Moreover, our results are in accordance with previous *in vivo* data in the context of T cell mediated tumor control, where it was shown that supplementation of IFN-α to a peptide vaccination led to increased tumor infiltration by effector $CD8^+$ T cells and preferentially promoted the differentiation of $CD8^+$ T cells with an effector memory like phenotype (Sikora, Jaffarzad et al. 2009). In line with these results, we found that $IFNAR^{-/-}$ P14 cells were undetectable in peripheral tissue 45 days after infection as opposed to WT P14 cells which were found at high numbers in the liver of infected mice, indicating that type I IFNs are necessary for the formation of effector memory cells. This further suggests that type I IFNs are not only necessary for the short term differentiation of SLECs but also play a role in the long term formation of effector memory cells. Although, qualitatively equivalent memory cells with respect to their recall proliferation potential are formed the absence of type I IFN signaling, suggestive of unaltered central memory $CD8^+$ T cell differentiation, there is a significant difference in the overall quantity of memory cells formed in the absence of type I IFN signaling.

Besides IL-12 and type I IFNs, IL-2 was found to act as a differentiation factor for $CD8^+$ T cells (Kalia, Sarkar et al. 2010, Obar and Lefrancois 2010, Obar, Molloy et al. 2010, Pipkin, Sacks et al. 2010). It was shown that LCMV-specific $CD8^+$ T cells exhibit an early transient heterogeneity in their CD25 expression which directs them into different developmental programs (Kalia, Sarkar et al. 2010) with $CD25^{high}$ P14 cells exhibiting enhanced SLEC differentiation compared with $CD25^{low}$ cells. Furthermore, $CD8^+$ T cells that lack CD25 signaling differentiate inefficiently into effector $CD8^+$ T cells (Pipkin, Sacks et al. 2010), suggesting that IL-2 is a potent factor driving SLEC differentiation. Combined with our results and the fact that type I IFN signaling can directly upregulate CD25 expression on $CD8^+$ T cells (Le Bon, Etchart et al. 2003, Wiesel, Kratky et al. 2011), we hypothesize that besides IL-2, type I IFNs are an important factor in promoting the early differentiation of $CD8^+$ T cells towards a SLEC phenotype and that type I IFN signaling, being upstream of CD25 expression, might in fact be instructive for CD25 expression levels. Furthermore, in contrast to type I IFNs and IL-12, IL-2 is not by itself sufficient to upregulate T-bet expression in activated $CD8^+$ T cells *in*

vitro (Pipkin, Sacks et al. 2010). Thus, we conclude that while type I IFN signaling induces expression of CD25 and thereby increases IL-2 sensitivity of activated $CD8^+$ T cells, IL-2 signaling is not required for the early fate decision of $CD8^+$ T cells with respect to T-bet expression. Instead, IL-2 may rather act at later time points to further promote the differentiation into SLECs.

In summary the data presented here identify direct type I IFN signaling on $CD8^+$ T cells as an important factor regulating the expression of T-bet and thereby promoting the early differentiation of short-lived effector cells. However, absence of direct type I IFN signaling on differentiating $CD8^+$ T cells showed no defects in qualitative differentiation of memory $CD8^+$ T cells which where endowed with the capacity to undergo secondary expansion. These findings may bear important practical implications for vaccine design with respect to the importance of choosing vaccine adjuvants for promoting optimal memory $CD8^+$ T cell development.

5.1.2 The role of type I IFNs on T cell expansion and survival

Type I IFN signaling on T cells is critically important for their proper expansion and survival following infections associated with high levels of type I IFNs. Both $CD8^+$ and $CD4^+$ $IFNAR^{-/-}$ T cells fail to expand properly following LCMV infection (Kolumam, Thomas et al. 2005, Aichele, Unsoeld et al. 2006, Havenar-Daughton, Kolumam et al. 2006). Interestingly we demonstrate here that T cells lacking the ability to sense type I IFNs were highly susceptible to elimination by NK cells during the early phase of LCMV infection, indicating that type I IFN signaling on T cells is necessary to protect T cells from negative regulation by NK cells. Previous findings have demonstrated that T cells can be recognized and killed by NK cells in a perforin dependent manner, but the conclusions and mechanisms are controversial. It has been proposed that NK cells can act as rheostats by killing $CD4^+$ T cells and thereby indirectly affecting the $CD8^+$ T cell response (Waggoner, Cornberg et al. 2012), whereas other groups have found that $CD8^+$ T cells themselves can be direct targets for NK cell mediated killing (Soderquest, Walzer et al. 2011, Lang, Lang et al. 2012). Our results complement these previous findings and indicate that both $CD8^+$ and $CD4^+$ T cells could be directly killed by NK cells following LCMV infection. Importantly, our results add to these previous observations by revealing a mechanism by which T cells can protect themselves from negative regulation by NK cells via the direct sensing of type I IFNs. In line with previous reports we found that WT T cells were modestly increased following NK cell depletion, whereas the massively curtailed $IFNAR^{-/-}$ T cell expansion was greatly increased (~20 fold) following NK cell depletion. We have found that $IFNAR^{-/-}$ T cells could be specifically recognized and eliminated by NK cells

in an NCR1 dependent manner, demonstrating a prominent involvement of NCR1 ligand expression on T cells as a mechanism by which NK cells could recognize improperly activated T cells and eliminate them (**Figure 5.2**). Our data clearly indicate that type I IFN signaling on T cells protects them from NK cell mediated killing by regulating the balance of activating and inhibitory signals to NK cells with a dominant contribution of NCR1 ligands.

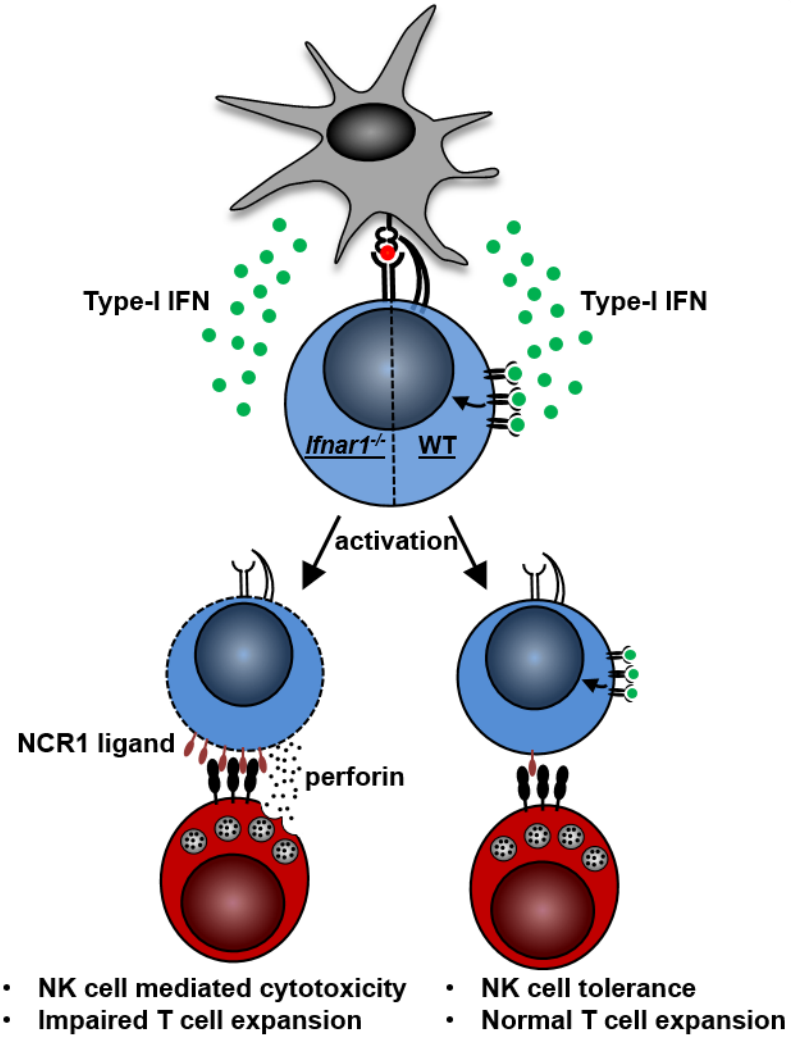


Figure 5.2 Type I IFNs protect T cells from NCR1 mediated NK cell elimination

Type I IFN signaling on T cells is necessary for the proper activation and expansion of anti-viral T cells following an infection associated with high levels of type I IFNs such as LCMV. The impaired expansion of T cells lacking the ability to sense type I IFNs (*Ifnar1*$^{-/-}$) is in part due to NK cell meditated elimination of these T cells. *Ifnar1*$^{-/-}$ T cells express elevated levels of activating ligands for the NK cell activating receptor NCR1, which renders them susceptible to NK cell mediated recognition and elimination. T cells that can sense type I IFNs are protected from this NK cell mediated elimination due to their low expression of NCR1 activating ligands, demonstrating the ability of type I IFN signaling to protect T cells from NK cell mediated regulation.

The finding that NCR1 ligands are up-regulated on IFNAR$^{-/-}$ T cells following LCMV infection implies that type I IFNs somehow block the up-regulation of NCR1 ligands following activation, implicating the possibility that NCR1 ligand up-regulation is a default program

induced following T cell activation and that additional cytokine signaling is necessary to block its up-regulation. The finding that IFNAR$^{-/-}$ P14 cells did not up-regulate NCR1 ligands following VVG2 infection leads to the possibility that the inflammatory environment associated with a VV infection could compensate for the lack of type I IFN signaling and thereby protect T cells from up-regulating NCR1 ligands and becoming targets for NK cell killing. As some redundancy between the signal 3 cytokines IL-12 and type I IFNs has been demonstrated for expansion and differentiation of T cells (Curtsinger, Valenzuela et al. 2005), it is possible that IL-12 signaling on IFNAR$^{-/-}$ P14 cells following VVG2 infection can suppress the up-regulation of NCR1 ligands and thereby protect the activated T cells. However, further studies need to be performed to address this hypothesis.

NCR1 activation on NK cells was shown to play an important role in various viral infections; *Ncr1*$^{-/-}$ mice, despite normal *in vitro* killing of tumor cells, are more susceptible to lethal influenza infection (Gazit, Gruda et al. 2006) due to the ability of NK cells to directly recognize and eliminate influenza infected cells (Mandelboim, Lieberman et al. 2001). Furthermore, in humans, low expression of NCR1 has been associated with the persistent infections hepatitis C (Nattermann, Feldmann et al. 2006) and human immunodeficiency virus (HIV) (De Maria, Fogli et al. 2003), leading to the speculation that hosts might down-regulate the expression of NCR1 on NK cells to protect antiviral T cells from elimination in a setting of persistent antigen exposure with low abundance of signal 3 cytokines. It will be interesting to experimentally address such hypothesis in future studies. Our findings expand on the role of NCR1 activation of NK cells and demonstrate that in addition to its role in direct viral clearance it also has a potent regulatory role on T cell expansion.

Despite the decisive role found for NK cells in the regulation of IFNAR$^{-/-}$ T cell expansion at early time points, at the peak of T cell expansion (day 7) IFNAR$^{-/-}$ T cell numbers were reduced compared to WT T cells, which was independent of NK cell regulation. These findings demonstrate that type I IFN signaling has additional roles in the expansion of T cells. This could be due to type I IFN dependent regulation of other cytokine receptors such as IL-7R and IL-15R which both have important roles in T cell survival (Schluns and Lefrancois 2003). In addition, the inability of IFNAR$^{-/-}$ T cells to differentiate into SLECs can affect the overall clonal burst size, as this population of cells with greater ability to expand (Sarkar, Kalia et al. 2008) is missing from the IFNAR$^{-/-}$ T cell pool. In line with this, our microarray data indicates that many cellular pathways involved in cell survival and cell division are differentially regulated in IFNAR$^{-/-}$ P14 cells, indicating that type I IFN signaling could imprint WT P14 cells

for better survival and expansion. In line with this reasoning it has been shown that type I IFNs are important for T cell survival (Marrack, Kappler et al. 1999), and both signal 3 cytokines IL-12 and type I IFNs have been found to induce chromatin remodeling in $CD8^+$ T cells, leading to a complex gene regulation program that promotes survival and expansion of $CD8^+$ T cells (Agarwal, Raghavan et al. 2009).

In conclusion, we demonstrate that NK cell mediated killing of improperly activated T cells is responsible for the abortive expansion of $IFNAR^{-/-}$ T cells early after LCMV infection and also after VSV infection, both infections being dominated by a biased type-1 IFN milieu. The results lend support to a scenario in which activated T cells need to efficiently safe-guard themselves against NK cell mediated cytotoxicity, and this protection is afforded by type I IFN driven inhibition of NCR1 ligand expression.

5.2 NK cell regulation of T cell responses

Natural killer cells (NK) are a unique population of innate lymphoid cells armed with the ability to centrally contribute to the immune response. Initially discovered for their role in the elimination of tumor cells (Herberman, Nunn et al. 1975, Herberman, Nunn et al. 1975), NK cells became a topic of great interest with the possibility of harnessing their anti-tumor activity clinically (Cheng, Chen et al. 2013). Further research has contributed to the understanding of NK cell biology, establishing a key role for NK cells not only in tumor cell killing but also in the protection against various viral infections (French and Yokoyama 2003). As part of the innate immune response NK cells are armed with the ability to quickly respond to changes in the host, placing them at the front line of the host immune response. In addition to the well characterized function of NK cells as innate killers, NK cells are also equipped with the ability to produce cytokines such as IFN-γ, expanding their role in the immune response. Being part of the innate immune response NK cells can quickly exert effector functions following an infection. The activation of NK cells has been shown to occur through direct recognition of pathogen associated molecules through toll like receptor signaling (Schmidt, Leung et al. 2004, Hart, Athie-Morales et al. 2005) and by various cytokines including type I IFNs, IL-12, IL-18, IL-2 and IL-15 (Gidlund, Orn et al. 1978, Fehniger, Cai et al. 2007, Lucas, Schachterle et al. 2007, Kang, Liang et al. 2008). Being potent killers, ensuring NK cell self-tolerance is of great importance for protection from autoimmunity. To ensure host protection NK cells undergo a

process of licensing or "education" during their development, ensuring that host NK cells are self-tolerant at steady state (Orr and Lanier 2010). Lacking antigen specificity, unlike T and B cells, NK cells are activated by target cells through their expression of various inhibitory and activating receptors which scan host cells for "warning" signs (Lanier 1998). The switch between NK cell activation and tolerance is determined by the net balance of signals received by NK cells, where a loss of inhibitory signals or an increase in activating signals can both lead to NK cell activation. Importantly, the induction of stress induced activating ligands such as NKG2D ligands can override inhibitory signals, leading to NK cell activation even in the presence of inhibitory ligands such as MHC class I (Long and Rajagopalan 2002). Due to the wide range of functions carried out by NK cells and their activation early during the immune response, NK cells have the ability to regulate not only the innate immune response but also play an important role in modulating the adaptive immune response. Here we summarize the current knowledge on the role of NK cells in modulating the T cell response and demonstrate their importance as regulators of T cell immunity.

5.2.1 NK cells - regulators of the T cell response

Being early activated innate lymphoid cells, NK cells are highly active during the initial stages of T cell activation. NK cells are found distributed throughout the body and can be activated in secondary lymphoid organs in close contact with T cells having undergone cognate activation (Lucas, Schachterle et al. 2007, Beuneu, Deguine et al. 2009). These features of NK cells endow them with the potential to regulate the T cell response. Such NK cell modulation of the T cell response can occur in both a direct and an indirect manner and this modulation can have either a positive or a negative impact on the overall T cell response and progression of infection, as summarized in **Table 5.1**.

Table 5.1 NK cell regulation of T cell responses

NK cell regulation of T cell responses can occur in different ways. Summarized here are the different mechanisms by which NK cells were shown to modulate T cell responses. The table is divided into NK cell modulation resulting in augmentation or inhibition of T cell immunity and further subdivided into direct and indirect regulation by NK cells.

Enhancement of T cell responses by NK cells		
Indirect mechanisms	**outcome**	**reference**
Dendritic cell maturation	Human NK cell produced IFN-γ induced maturation of DCs *in vitro*, leading to IL-12 production and increased costimulation on DCs	(Gerosa, 2002)
	NK cell produced IFN-γ *in vivo* promotes IL-12 production by DCs leading to an enhanced $CD8^+$ T cell response and tumor clearance	(Mocikat, 2003)
	NK cell produced IFN-γ following DC vaccination *in vivo* promotes IL-12 production by DCs leading to $CD4^+$ T cell help independent $CD8^+$ T cell induction	(Adam 2005)
Antigen cross presentation	NK cell killing of target cells releases antigen for cross presentation and activation of T cell responses	(Krebs 2009)
Direct mechanisms	**outcome**	**reference**
$CD4^+$ T cell differentiation	NK cell produced IFN-γ signaling on $CD4^+$ T cells promotes differentiation into T_{H1} helper cell	(Martin-fontecha, 2004)
	Human tonsilar NK cells secreted IFN-γ assists in T_{H1} cell polarization	(Morandi 2006)
	NK cell produced IFN-γ signaling on $CD4^+$ T cells promotes differentiation into T_{H1} helper cells and protection from *L. major* infection	(Laouar 2005)
Impairment of T cell responses by NK cells		
Indirect mechanisms	**outcome**	**reference**
Reduced antigen presentation	Ly49H dependent elimination of MCMV infected DCs by NK cells leading to reduced $CD4^+$ and $CD8^+$ T cell responses and increased viral persistence in $Ly49H^+$ mice	(Andrew, 2010)
Reduced stimulatory capacity of APCs	Reduced $CD8^+$ and $CD4^+$ T cell response to LCMV	(Cook , 2013)
Direct mechanisms	**outcome**	**reference**
Reduced $CD8^+$ T cell activation	NK cell secreted IL-10 following MCMV infection leads to a reduced $CD8^+$ T cell response and protection from immunopathology	(Lee 2009)
T cell elimination	NK cell mediated killing of $CD8^+$ T cells	(Soderquest 2011, Waggoner 2010, Lang 2012, Crouse 2014, Peppa 2012, Rabinovich 2003, Cerboni 2007, Lang 2014)
	NK cell mediated killing of $CD4^+$ T cells	(Lu 2007, Crouse 2014, Waggoner 2012, Cerboni 2007, Rabinovich 2003)

5.2.2 Indirect regulation of the T cell response

NK cells are armed with the ability to secrete cytokines as well as to eliminate target cells via direct cytolysis. Both of these effector functions of NK cells are involved in the indirect regulation of T cell responses. Indirect regulation of T cell responses occurs mainly through the interaction between NK cells and APCs. Due to the importance of APCs in T cell activation, changes in APCs can have a significant impact on the activation of T cells. This NK-DC cross talk has been demonstrated *in vitro* where human NK cells are able to induce the maturation of DCs in an *in vitro* co-culture system. In such a setting, NK cell produced IFN-γ was necessary for the induction of IL-12 production by DCs and upregulation of costimulatory molecules on DCs (Gerosa, Baldani-Guerra et al. 2002). Additionally, the ability of NK cells to induce the maturation of DCs and thus augment the T cell response has been shown *in vivo*. Similar to the human *in vitro* data NK cell produced IFN-γ secreted following NK cell recognition of MHC class I^{low} tumor cells leads to the activation and IL-12 production by DCs which in turn leads to an enhanced $CD8^+$ T cell response and tumor clearance (Mocikat, Braumuller et al. 2003). Furthermore, the interplay between DCs and NK cells has been shown to be sufficient to replace $CD4^+$ T cell help for the induction of an anti-tumor $CD8^+$ T cell response following DC vaccination (Adam, King et al. 2005). Alternatively, NK cell mediated cytotoxicity of target cells can lead to the release of antigens which can be cross presented by DCs. This release of antigen by NK cell mediated killing has a positive effect on the T cell response by promoting cross presentation by DCs (Iyoda, Shimoyama et al. 2002, Krebs, Barnes et al. 2009). These findings highlight the importance of NK cell crosstalk with DCs for the induction of a $CD8^+$ T cell response, however not all interactions between NK cells and DCs favor the augmentation of T cell responses.

NK cells can also exert an indirect negative effect on the T cell response. This negative effect can also occur through interactions between NK cells and APCs. During a high dose LCMV clone 13 infection it has been shown that NK cells can negatively regulate the stimulatory capacity of APCs, resulting in a reduced $CD8^+$ and $CD4^+$ T cell response (Cook and Whitmire 2013). Furthermore, NK cells can affect the outcome of the T cell response by limiting the availability of antigen-presenting APCs as has been shown following an MCMV infection. NK cells can eliminate infected DCs by the direct recognition of the MCMV viral protein m157 on infected APCs by the activating NK cell receptor Ly49H. This elimination of infected DCs reduces the amount of antigen presentation and leads to a reduced T cell response culminating in increased viral persistence (Andrews, Estcourt et al. 2010). Together, these findings

emphasize the importance of early NK cell - DC interactions and their impact on the subsequent T cell response.

5.2.3 Direct regulation of the T cell response

In addition to the indirect mechanisms of NK cell regulation of T cell responses though DC interactions, NK cells can regulate T cells themselves directly. NK cells have been shown to promote the differentiation the T_{H1} subset of $CD4^+$ T cells by the secretion of IFN-γ from NK cells recruited to draining lymph nodes (Martin-Fontecha, Thomsen et al. 2004). In addition, the same observation was made for human NK cells isolated from secondary lymphoid tissue, but not for NK cells from peripheral blood, as tonsilar NK cells secreted higher levels of IFN-γ than peripheral blood NK cells and this NK cell derived IFN-γ assisted in the differentiation of T_{H1} cells (Morandi, Bougras et al. 2006). The importance of NK cell mediated polarization of $CD4^+$ T cells has been demonstrated *in vivo* where it was found that the NK cell mediated differentiation of T_{H1} cells is important in the protection of *Leishmania major* infection (Laouar, Sutterwala et al. 2005).

Although NK cells can have some direct augmenting effects on the T cell response, the majority of direct NK - T cell interactions results in the impairment of T cell responses. NK cell secreted IL-10 has been shown to reduce the $CD8^+$ T cell response to MCMV, leading to reduced T cell mediated immunopathology in these mice (Lee, Kim et al. 2009). In addition to the role of NK cell secreted cytokines acting on T cells, it has been shown by numerous groups in various priming conditions that NK cells have the ability to directly recognize and kill activated T cells (Rabinovich, Li et al. 2003, Cerboni, Zingoni et al. 2007, Lu, Ikizawa et al. 2007, Waggoner, Taniguchi et al. 2010, Soderquest, Walzer et al. 2011, Lang, Lang et al. 2012, Waggoner, Cornberg et al. 2012, Cook and Whitmire 2013, Peppa, Gill et al. 2013). Although many groups have found that this process is dependent on perforin mediated cytotoxicity, the mechanisms of T cell recognition and NK cell activation are less well described and many different activating and inhibitory receptors and ligands have been described to be involved in this process of T cell killing by NK cells. **Table 5.2.** summarizes the current knowledge of the various NK cell receptors and ligands involved in this negative regulation of T cell responses.

T cell ligand	NK cell receptor	type	Perforin dependent	T cell type affected	Exp. setting	reference
H-60	NKG2D	activating	yes	CD8 and CD4	*in vitro* T cell activation LAK NK cells	(Rabinovich et. al. 2003)
NKG2D ligands	NKG2D	activating	lytic granule dependent	CD8 and CD4	PMA or SEB activated T cells IL-2 activated NK cells	(Cerboni et. al. 2007)
Qa-1	NKG2A	inhibitory	yes	CD4	Ova immunization	(Lu 2007)
CD48	2B4	inhibitory	yes	CD8	persistent LCMV clone 13 infection	(Waggoner 2010)
NKG2D ligands	NKG2D	activating	yes	CD8	Ova + LPS immunization	(Soderquest et. al. 2011)
NKG2D ligands	NKG2D	activating	yes	CD8	high dose LCMV-WE, Clone 13 and Docile	(Lang 2012)
?	?	?	yes	CD4	LCMV clone 13 (high, medium, low dose)	(Waggoner 2012)
TRAIL-R2	TRAIL	activating	no	CD8	chronic hepatitis B	(Peppa et. al. 2012)
Qa-1 / MHC-I	NKG2A / Ly49	inhibitory	yes	CD8 and CD4	acute LCMV-WE	(Xu et al. 2014)
NCR1 ligands	NCR1	activating	yes	CD8 and CD4	acute LCMV-WE and VSV	(Crouse et. al. 2014)

Table 5.2 Mechanisms of NK cell recognition of T cells

NK cell regulation of T cell responses is often achieved through the direct recognition and lysis of T cells. Summarized here are the different setting in which NK cell killing of T cells has been demonstrated. The various different activating or inhibitory ligands on T cells implicated in NK cell killing of T cells are listed.

5.2.3.1 T cell expression of NK cell inhibitory ligands

Of great importance for NK cell tolerance and protection of host cells is the expression of inhibitory ligands on the surface of host cells. These ligands, such as MHC class I, provide inhibitory signals to NK cells ensuring protection of healthy cells. As the activation of T cell responses coincides with the presence of highly activated NK cells, T cells have devised mechanisms to safe-guard themselves against NK cell attack in order to successfully undergo clonal amplification. One such mechanism is the expression of the inhibitory ligand Qa-1 on the surface of activated $CD4^+$ T cells. This MHC class Ib molecule is recognized by the CD94-NKG2A receptor on NK cells and provides protection for $CD4^+$ T cells from perforin dependent

NK cell elimination (Lu, Ikizawa et al. 2007). It was further shown that Qa-1 deficiency on myelin oligodendrocyte glycoprotein (MOG) specific $CD4^+$ T cells or blocking of Qa-1 can lead to protection from the development of experimental autoimmune encephalomyelitis (EAE) as the loss of the Qa-1 inhibitory signal on these cells leads to their elimination by NK cells. In addition to Qa-1 it has been shown that the expression of CD48 on activated $CD8^+$ T cells protects them from elimination by NK cells following a persistent LCMV clone 13 infection (Waggoner, Taniguchi et al. 2010). NK cells lacking the inhibitory receptor 2B4 have the ability to lyse activated T cells and 2B4 deficient mice, which have reduced T cell numbers following LCMV clone 13 infection fail to control the virus, leading to viral persistence. A recent finding also implicates not only the inhibitory ligand Qa-1 in NK cell regulation of T cell responses but also the classical inhibitory ligand MHC class I (Xu 2014). Following acute LCMV infection it was found that T cells susceptible to NK cell mediated killing express lower levels of MHC I on their surface, rendering them targets for NK cell mediated elimination. These findings highlight the importance of inhibitory signaling to NK cells as a mechanism to ensure protection of activated T cells.

5.2.3.2 T cell expression of NK cell activating ligands

Unlike the constitutively expressed cellular NK cell inhibitory ligands, NK cell activating ligands are often stress induced cellular ligands which serve as "warning" signals to induce NK cell activation. Although many NK cell activating ligands have been described one of the best defined are the family of NKG2D ligands. NKG2D is a homodimeric receptor found on all NK cells (Jamieson, Diefenbach et al. 2002) which upon binding its ligands induces an activating signal through the intracellular adaptors DAP10 and DAP12. NKG2D recognizes several MHC class I related molecules in mice including the retinoic acid early transcript 1 (Rae1) family members Rae1α-Raelε, histocompatibility 60 (H60) and mouse UL16-binding protein-like transcript 1 (Mult1) (Raulet 2003). In addition to NKG2D ligand expression on tumor cells (Spear, Wu et al. 2013) it has been shown that T cells can transiently upregulate NKG2D ligands following activation. This upregulation of NKG2D ligands on T cells has been shown by various groups to be involved in the NK cell dependent killing of activated T cells. One of the earlier reports demonstrating the ability of NK cells to eliminate T cells found that *in vitro* activated $CD4^+$ and $CD8^+$ T cells are sensitive to elimination by IL-2 activated lymphokine-activated killer cells (LAK). This elimination was shown to be dependent on TCR activation of T cells and on trafficking of NKG2D ligands to the surface of activated T cells (Rabinovich, Li et al. 2003). Similarly in the human setting it was found that TCR dependent *in vitro* activation

of T cells leads to the upregulation of ligands for NKG2D which renders these *in vitro* activated T cells targets for lysis by IL-2 activated NK cells in a granule exocytosis dependent manner (Cerboni, Zingoni et al. 2007). Building on these *in vitro* findings it was shown *in vivo* in mice following immunization with OVA and LPS that NK cells can kill $CD8^+$ T cells in a perforin and NKG2D dependent manner and that the depletion of NK cells during priming leads to not only an increased number of responding T cells but also increased amounts of central memory T cells (Soderquest, Walzer et al. 2011).

Also during LCMV infection NK cells were shown to negatively regulate T cells. NK cell depletion leads to an enhanced $CD8^+$ T cell response which is able to contribute to viral protection (Lang, Lang et al. 2012). This negative regulation was reversed by the blocking of NKG2D and NKG2D ligands were found to be expressed at higher levels on activated $CD8^+$ T cells compared to naïve T cells. Using the same infection setting another group found that following infection with LCMV clone 13 NK cell depletion also leads to reduced viral titers. However they found that this regulation was due to NK cell elimination of activated $CD4^+$ T cells instead of $CD8^+$ T cells and that the elimination of $CD4^+$ T cells indirectly affected the $CD8^+$ T cell response and outcome of infection (Waggoner, Cornberg et al. 2012).

In chronic hepatitis B (CHB) infection in humans reduced $CD8^+$ T cell activity in the liver is in part due to NK cell mediated regulation of HBV-specific T cells in the liver (Peppa, Gill et al. 2013). T cells isolated from CHB patients expressed elevated levels of TRAIL-R2 which rendered them susceptible to NK cell mediated regulation via TRAIL dependent activation of NK cells. Interestingly in the setting of CHB only HBV specific T cells were found to be regulated by NK cells whereas EBV or CMV specific T cells from the same host were unaffected by NK cells.

Recent findings from our group identified a pivotal role of the activating receptor NCR1 (NKp46) in the negative regulation of T cell responses following acute LCMV infection (Crouse, 2014). NCR1 is an activating receptor found on all NK cells that recognizes viral hemagglutinins (Mandelboim, Lieberman et al. 2001); the cellular ligands, however, remain unknown (Hudspeth, Silva-Santos et al. 2013). A mutation in NCR1 leading to hyperreactive NK cells has been shown to limit the T cell response following an MCMV infection (Narni-Mancinelli, Jaeger et al. 2012), suggesting a role for this activating receptor in the regulation of T cell responses. Indeed we found that T cells which lack the ability to sense type I IFNs express elevated levels of NCR1 ligands which renders them targets for NK cell mediated lysis, further demonstrating the importance of appropriate regulation activating ligand expression on T cells.

Together these results demonstrate that NK cells can regulate T cell responses in many different ways whereby the balance of activating and inhibitory signals provided by expanding T cells to activated NK cells dictates whether or not and to what extent they are subjected to NK cell regulation, with a loss of inhibitory signals or a gain in activating ligands determining whether they become targets for NK cell mediated regulation or not.

5.2.4 NK cell regulation of T cells: Friend or Foe

The ability of NK cells to regulate T cell responses proposes the question of why this occurs and whether or not this process is beneficial to the host or not. Different groups have proposed different answers to this question and it appears that depending on the infection or immunization setting the regulation of T cell responses by NK cells can be beneficial or detrimental for the host. In general the negative regulation of T cell responses seems to have a negative impact on viral clearance. This plays a particularly important role during infections which are prone to establish persistence, such as LCMV. NK cell mediated reduction of T cell responses during LCMV clone 13 infection promotes the development of viral chronicity. Depletion of NK cells during high dose LCMV clone 13 or LCMV Docile infection, which normally lead to chronic infection, leads instead to an elevated T cell response which is able to contribute to accelerated control of the infection (Lang, Lang et al. 2012). Thus, the presence of NK cells promotes viral persistence which is associated with increased liver cell damage and NK cell depletion promotes viral control and reduces liver pathology. Similarly it was shown during a medium dose LCMV clone 13 infection that NK cell activity leads to reduced levels of activated T cells, resulting in viral persistence. In this medium dose LCMV infection the amount of viral antigen is not sufficient to induce T cell exhaustion, thereby leading to a scenario in which functional T cells are confronted with large numbers of infected cells, culminating in fatal immunopathology. Depletion of NK cells in this setting leads to an increased T cell response which is able to control the infection, highlighting a negative role for NK cell regulation in host survival (Waggoner, Cornberg et al. 2012). Furthermore, NK cell mediated regulation of T cells during T cell priming has an impact on the generation of memory T cells. Priming of $CD8^+$ T cells in the absence of NK cells has been shown to lead to a higher number of central memory T cells resulting in better antitumor activity (Soderquest, Walzer et al. 2011) and the reduction of T cell responses due to hyperactive NK cells has a negative impact on protective immunity to *Listeria monocytogenes* challenge (Narni-Mancinelli, Jaeger et al. 2012).

However, regulation of T cell responses by NK cells is not always detrimental for the host. NK cell enhancement of T cell responses by maturation of APCs leads to better T cell responses,

memory formation and tumor clearance (Mocikat, Braumuller et al. 2003). Also, during a high dose LCMV clone 13 infection, NK cells act in a beneficial way by reducing the T cell response, which facilitates viral persistence and T cell exhaustion, thereby preventing fatal T cell mediated immunopathology (Waggoner, Cornberg et al. 2012).

As T cell activation occurs in the presence of cytolytic NK cells it is conceivable that T cells have devised strategies to protect themselves from NK mediated killing. It has been shown that inhibitory ligand expression such as CD48 and Qa-1 on activated T cells may protect them from NK cell mediated killing (Lu, Ikizawa et al. 2007, Waggoner, Taniguchi et al. 2010). In addition recent findings from our group show that T cells effectively safe-guard themselves against NK cell attack by down-regulating or preventing the expression of ligands for the NK activating receptor NCR1. The absent surface expression of the NCR1 ligands was strictly dependent on direct type I interferon sensing by activated T cells (Crouse, 2014). These findings demonstrate that the ability of T cells to directly sense type I IFNs following infection with viruses known to induce high levels of type I IFNs, such as acute LCMV or VSV infection, leads to the protection of T cells from NK cell mediated killing by reducing the levels of NCR1 activating ligands on expanding T cells. T cells lacking the ability to directly sense type I IFNs are rendered targets for NK cell mediated killing, opening the possibility that NK cells may act in a beneficial regulatory manner to eliminate improperly activated T cells. This hypothesis hints at a potential regulation pathway by NK cells to prevent expansion of self-reactive T cells which may sense antigen in the absence of inflammatory signals and signal three cytokines. In line with this hypothesis it has been shown that NK cell depletion can result in enhanced development of EAE due to increased numbers of autoreactive $CD4^+$ T cells (Zhang, Yamamura et al. 1997) and treatment of multiple sclerosis patients with the IL-2 receptor targeting antibody daclizumab leads to increased expansion of NK cells which correlates with decreased total circulating T cell numbers and a strong reduction in brain inflammation (Bielekova, Catalfamo et al. 2006). These studies indicate a beneficial role of NK cell regulation of T cell responses by limiting the accumulation of self-reactive T cells. However, additional studies will be needed to determine the physiological significance and the mechanism of NK cell regulation of self-reactive T cell responses.

5.2.5 Regulation of NK cell ligand expression on T cells

The underlying signaling involved in the expression of the various NK cell ligands on T cells is a topic with many open questions. Although little is known about the regulation of NK cell ligands on T cells, evidence from multiple groups suggests that the direct regulation of T cells

by NK cells is dependent on T cell activation, as naïve T cells remain largely unharmed. These findings make sense as it would be detrimental to the host to have NK cell mediated elimination of naïve T cells. In support of the notion that T cells need to be activated to upregulate NK cell activating ligands it was found that activated T cells express NKG2D ligands but not naïve T cells (Cerboni, Zingoni et al. 2007, Soderquest, Walzer et al. 2011, Lang, Lang et al. 2012). However, it remains to be determined why some T cells would upregulate these activating ligands, rendering them susceptible to NK cell mediated lysis. Interestingly, following *in vitro* superantigen SEB activation of T cells NKG2D ligands were found on CFSE low cells and very little was found on undivided T cells (Cerboni, Zingoni et al. 2007). The upregulation of NKG2D ligands was caused by the activation and phosphorylation of ATM which can activate NF-kB signaling and inhibition of ATM activation leads to reduced expression of the NKG2D ligand MICA on *in vitro* activated T cells, demonstrating a direct link between T cell activation and NKG2D ligand expression (Cerboni, Zingoni et al. 2007).

Recent findings have demonstrated that type I IFN signaling on T cells can protect them from NK cell mediated killing (Crouse, 2014; Xu, 2014). This recognition of T cells which lack the ability to sense type I IFNs can occur via increased expression of NCR1 ligands, indicating that type I IFNs play an important role in the regulation of NCR1 ligand expression on T cells. Besides absence of type I IFN signaling, TCR mediated activation was required for upregulation of NCR1 ligands on T cells (Crouse, 2014). However, how NCR1 ligand expression is regulated by TCR triggering and type I IFN signaling is still unclear. It is possible that the upregulation of activating ligands for NCR1 might be a default pathway following T cell activation and signaling by signal 3 cytokines serve to block this upregulation and protect the properly activated expanding T cells. Often the expression of activating ligands is induced in stressed or malignant cells and in the setting of T cells the activation induced proliferative program coupled to major metabolic changes (Pearce 2010, MacIver, Michalek et al. 2013) may well provide significant enough stress to the cells to lead to the upregulation of activating ligands, which, if not down regulated, could be detrimental for the expanding T cells

5.3 Concluding remarks and outlook

The results presented here build upon previous findings and demonstrate the central role that not only type I IFNs play in the innate and adaptive immune response, but also the integrated role that NK cells have in regulating the immune response. Due to their importance in the establishment of an innate anti-viral state and importance as a signal 3 cytokine, type- I IFN responses to infections prove to be a very interesting topic of research. Understanding the

various effects of type I IFNs can provide useful information for the development of anti-viral agents. Similarly, the growing field of NK cell biology has broadened our understanding of the numerous roles NK cells can play in the immune response. The finding that NK cells have the ability to regulate T cell responses has opened the question of why such a process is occurring. We found here that T cells require the signaling of type I IFNs to protect themselves from the negative regulation by NK cells, leading to the hypothesis that NK cells could possibly be eliminating T cells that are improperly activated. A setting of T cell activation in the absence of signal 3 cytokines implies T cell activation in the absence of inflammation which is a situation where T cells should not be activated. Such a situation could occur during activation of T cells specific for self-antigens. A possible elimination of such improperly activated T cells by NK cells could represent an important mechanism for protection from autoimmunity, however further research needs to be performed to further understand whether and how this process is occurring. Furthermore, the regulation of NK cell activating ligands on T cells is a topic with many open questions. Is the upregulation of activating ligands a default pathway induced during T cell activation, where T cells are undergoing a (possibly stressful) process of rapid division and differentiation? Also, when and how type I IFNs and possibly other signal 3 cytokines regulate the expression of activating ligands is a topic with many open questions. Understanding the mechanisms of this regulation will further broaden our knowledge of T cell biology and the interplay between NK cells and the adaptive immune response.

Chapter 6

6. Appendix

Gene Symbol	NCBI Entrez Gene ID	Gene Name	log2FC	adjp
Dapl1	76747	death associated protein-like 1	3.65	4.30E-07
Cd7	12516	CD7 antigen	3.54	6.23E-08
Cd5l	11801	CD5 antigen-like	3.5	8.32E-06
Slpi	20568	secretory leukocyte peptidase inhibitor	3.18	3.76E-05
Il6ra	16194	interleukin 6 receptor, alpha	3.06	3.90E-08
Spic	20728	Spi-C transcription factor (Spi-1/PU.1 related)	2.85	3.04E-05
Vcam1	22329	vascular cell adhesion molecule 1	2.83	7.77E-05
Tcf7	21414	transcription factor 7, T-cell specific	2.65	2.62E-06
C1qc	12262	complement component 1, q subcomponent, C chain	2.64	2.49E-04
Id3	15903	inhibitor of DNA binding 3	2.62	8.74E-07
Lilrb3	18733	leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 3	2.61	1.44E-05
Slc40a1	53945	solute carrier family 40 (iron-regulated transporter), member 1	2.56	2.37E-04
Sell	20343	selectin, lymphocyte	2.55	2.60E-06
Klrd1	16643	killer cell lectin-like receptor, subfamily D, member 1	2.52	1.43E-08
Fcer1g	14127	Fc receptor, IgE, high affinity I, gamma polypeptide	2.48	5.03E-05
Aif1	11629	allograft inflammatory factor 1	2.48	1.47E-04
Capn3	12335	calpain 3	2.47	1.30E-05
C1qa	12259	complement component 1, q subcomponent, alpha polypeptide	2.44	6.55E-04
Plbd1	66857	phospholipase B domain containing 1	2.43	7.62E-05
Rgs10	67865	regulator of G-protein signalling 10	2.4	4.67E-08
Tgfbi	21810	transforming growth factor, beta induced	2.36	4.13E-04
Erdr1	170942	erythroid differentiation regulator 1	2.32	2.54E-08
Timp2	21858	tissue inhibitor of metalloproteinase 2	2.3	6.33E-09
Ccr6	12458	chemokine (C-C motif) receptor 6	2.28	1.15E-06
CD83	12522	CD83 antigen	2.28	1.53E-04
S100a9	20202	S100 calcium binding protein A9 (calgranulin B)	2.23	4.41E-02
Phyhd1	227696	phytanoyl-CoA dioxygenase domain containing 1	2.22	1.84E-06
St6gal1	20440	beta galactoside alpha 2,6 sialyltransferase 1	2.19	1.54E-06
Cfb	14962	complement factor B	2.19	3.79E-06
Sirpb1b	668101	signal-regulatory protein beta 1B	2.19	9.53E-05
Ephx1	13849	epoxide hydrolase 1, microsomal	2.16	7.40E-07
Nrp2	18187	neuropilin 2	2.15	4.22E-06
AI661384	106930	expressed sequence AI661384	2.14	1.19E-07
Tyrobp	22177	TYRO protein tyrosine kinase binding protein	2.14	1.70E-03
Sirpa	19261	signal-regulatory protein alpha	2.13	2.32E-04
Lgmn	19141	legumain	2.11	3.36E-04
Hba-a2	110257	hemoglobin alpha, adult chain 2	2.11	2.96E-02
Gata3	14462	GATA binding protein 3	2.09	1.15E-08
Clec4n	56620	C-type lectin domain family 4, member n	2.09	3.83E-04
Parm1	231440	prostate androgen-regulated mucin-like protein 1	2.08	6.23E-08
Ypel3	66090	yippee-like 3 (Drosophila)	2.07	2.64E-07
Fcgr4	246256	Fc receptor, IgG, low affinity IV	2.07	1.74E-04
Plxdc2	67448	plexin domain containing 2	2.05	8.61E-08
Fxyd4	108017	FXYD domain-containing ion transport regulator 4	2.01	9.34E-07
Sepp1	20363	selenoprotein P, plasma, 1	2	3.54E-07
Ctsh	13036	cathepsin H	2	2.37E-05
Tmem141	51875	transmembrane protein 141	1.98	1.33E-05
Serpina3g	20715	serine (or cysteine) peptidase inhibitor, clade A, member 3G	1.98	9.72E-05
Nsg2	18197	neuron specific gene family member 2	1.94	3.27E-08
Slc11a1	18173	solute carrier family 11 (proton-coupled divalent metal ion transporters), member 1	1.94	2.77E-03

Table 1.1 Genes upregulated in IFNAR$^{-/-}$ P14 cells compared to WT P14

Shown are the top 50 upregulated genes in IFNAR$^{-/-}$ P14 compared to WT P14 cells. Genes are sorted by their log2 fold change were a log2 fold change of 1 corresponds to a fold change of 2.

Gene Symbol	NCBI Entrez Gene ID	Gene Name	log2FC	adjp
Usp18	24110	ubiquitin specific peptidase 18	-5.21	4.67E-08
Ctse	13034	cathepsin E	-4.63	6.07E-09
Oas1a	246730	2'-5' oligoadenylate synthetase 1A	-4.43	6.33E-09
Gm9706	677168	predicted gene 9706	-4.43	1.31E-07
Rtp4	67775	receptor transporter protein 4	-4.29	6.47E-07
Isg15	100038882	ISG15 ubiquitin-like modifier	-4.23	3.06E-08
Oas1f	243262	2'-5' oligoadenylate synthetase 1F	-4.12	1.79E-08
Ifi27l2a	76933	interferon, alpha-inducible protein 27 like 2A	-3.87	2.04E-08
Mx1	17857	myxovirus (influenza virus) resistance 1	-3.71	5.84E-06
Gm12253	624860	predicted gene 12253	-3.59	6.33E-09
Dhx58	80861	DEXH (Asp-Glu-X-His) box polypeptide 58	-3.59	2.77E-08
Klrg1	50928	killer cell lectin-like receptor subfamily G, member 1	-3.52	1.49E-09
Il10	16153	interleukin 10	-3.46	1.87E-08
Mnda	381308	myeloid cell nuclear differentiation antigen	-3.46	3.46E-08
Esm1	71690	endothelial cell-specific molecule 1	-3.46	2.44E-07
Ifi44	99899	interferon-induced protein 44	-3.43	1.22E-07
Oasl1	231655	2'-5' oligoadenylate synthetase-like 1	-3.35	3.36E-06
Isg20	57444	interferon-stimulated protein	-3.26	5.79E-07
Ifi204	15951	interferon activated gene 204	-3.12	1.43E-08
Mx2	17858	myxovirus (influenza virus) resistance 2	-3.07	4.68E-06
Il2ra	16184	interleukin 2 receptor, alpha chain	-2.96	1.20E-07
Bst2	69550	bone marrow stromal cell antigen 2	-2.95	9.30E-08
Apol9b	71898	apolipoprotein L 9b	-2.9	1.67E-05
Mt2	17750	metallothionein 2	-2.88	1.52E-03
Batf3	381319	basic leucine zipper transcription factor, ATF-like 3	-2.83	5.99E-05
BC094916	545384	cDNA sequence BC094916	-2.74	2.73E-07
Ly6c1	17067	lymphocyte antigen 6 complex, locus C1	-2.71	3.27E-08
Rsad2	58185	radical S-adenosyl methionine domain containing 2	-2.57	7.80E-05
Chac1	69065	ChaC, cation transport regulator-like 1 (E. coli)	-2.54	1.30E-05
Gm4951	240327	predicted gene 4951	-2.49	2.48E-07
Parp12	243771	poly (ADP-ribose) polymerase family, member 12	-2.46	2.32E-07
Xaf1	327959	XIAP associated factor 1	-2.46	3.25E-07
Il1rl1	17082	interleukin 1 receptor-like 1	-2.42	2.41E-08
Slfn1	20555	schlafen 1	-2.39	2.49E-06
AA467197	433470	expressed sequence AA467197	-2.38	9.85E-06
Fasl	14103	Fas ligand (TNF superfamily, member 6)	-2.36	2.52E-07
Lgals3bp	19039	lectin, galactoside-binding, soluble, 3 binding protein	-2.33	1.43E-08
Phf11	219131	PHD finger protein 11	-2.33	1.73E-07
Gzmb	14939	granzyme B	-2.32	4.81E-05
Slfn8	276950	schlafen 8	-2.31	8.25E-07
9030619P08Rik	105892	RIKEN cDNA 9030619P08 gene	-2.31	9.36E-07
Tuba8	53857	tubulin, alpha 8	-2.3	6.33E-09
Tmprss13	214531	transmembrane protease, serine 13	-2.29	1.43E-08
Rnf213	672511	ring finger protein 213	-2.26	4.90E-07
Ly6a	110454	lymphocyte antigen 6 complex, locus A	-2.26	3.11E-05
Cmpk2	22169	cytidine monophosphate (UMP-CMP) kinase 2, mitochondrial	-2.25	2.89E-07
Sdc3	20970	syndecan 3	-2.25	7.40E-04
I830127L07Rik	546643	RIKEN cDNA I830127L07 gene	-2.23	7.80E-06
Ifit1	15957	interferon-induced protein with tetratricopeptide repeats 1	-2.23	3.39E-05
Ly6g	546644	lymphocyte antigen 6 complex, locus G	-2.22	3.28E-07

Table 1.2 Genes downregulated in IFNAR$^{-/-}$ P14 cells compared to WT P14

Shown are the top 50 downregulated genes in IFNAR$^{-/-}$ P14 compared to WT P14 cells. Genes are sorted by their log2 fold change were a log2 fold change of -1 corresponds to a fold change of 2.

Gene Symbol	NCBI Entrez Gene ID	Gene Name	log2FC	adjp
Cd7	12516	CD7 antigen	3.63	5.24E-08
Cd5l	11801	CD5 antigen-like	3.48	9.20E-06
Slpi	20568	secretory leukocyte peptidase inhibitor	3.28	3.04E-05
Dapl1	76747	death associated protein-like 1	3.15	1.60E-06
Il6ra	16194	interleukin 6 receptor, alpha	2.98	5.33E-08
Spic	20728	Spi-C transcription factor (Spi-1/PU.1 related)	2.88	2.94E-05
Fcer1g	14127	Fc receptor, IgE, high affinity I, gamma polypeptide	2.83	1.69E-05
C1qc	12262	complement component 1, q subcomponent, C chain	2.83	1.55E-04
Parm1	231440	prostate androgen-regulated mucin-like protein 1	2.8	5.98E-09
Tgfbi	21810	transforming growth factor, beta induced	2.7	1.53E-04
Lilrb3	18733	leukocyte immunoglobulin-like receptor, subfamily B, member 3	2.6	1.57E-05
Hba-a2	110257	hemoglobin alpha, adult chain 2	2.6	8.80E-03
Klrd1	16643	killer cell lectin-like receptor, subfamily D, member 1	2.54	1.43E-08
Slc40a1	53945	solute carrier family 40 (iron-regulated transporter), member 1	2.5	3.14E-04
Id3	15903	inhibitor of DNA binding 3	2.44	1.70E-06
S100a9	20202	S100 calcium binding protein A9 (calgranulin B)	2.44	2.79E-02
Timp2	21858	tissue inhibitor of metalloproteinase 2	2.43	5.98E-09
C1qa	12259	complement component 1, q subcomponent, alpha polypeptide	2.43	7.31E-04
Ccdc164	381738	coiled-coil domain containing 164	2.42	9.11E-06
Tcf7	21414	transcription factor 7, T-cell specific	2.41	6.57E-06
Scn2b	72821	sodium channel, voltage-gated, type II, beta	2.35	2.34E-05
Nrp2	18187	neuropilin 2	2.34	2.29E-06
Erdr1	170942	erythroid differentiation regulator 1	2.33	2.41E-08
Plbd1	66857	phospholipase B domain containing 1	2.33	1.20E-04
Phyhd1	227696	phytanoyl-CoA dioxygenase domain containing 1	2.32	1.40E-06
Sirpa	19261	signal-regulatory protein alpha	2.29	1.44E-04
Aif1	11629	allograft inflammatory factor 1	2.24	3.58E-04
Tyrobp	22177	TYRO protein tyrosine kinase binding protein	2.24	1.27E-03
Sgms1	208449	sphingomyelin synthase 1	2.2	3.70E-06
Clec4n	56620	C-type lectin domain family 4, member n	2.2	2.81E-04
Hbb-b1	15129	hemoglobin, beta adult major chain	2.19	1.85E-02
Lgmn	19141	legumain	2.18	2.81E-04
Hba-a1	15122	hemoglobin alpha, adult chain 1	2.15	1.87E-02
Cfb	14962	complement factor B	2.14	5.18E-06
Sirpb1b	668101	signal-regulatory protein beta 1B	2.13	1.36E-04
Nr1h3	22259	nuclear receptor subfamily 1, group H, member 3	2.12	7.19E-04
Rgs10	67865	regulator of G-protein signalling 10	2.08	1.56E-07
Trf	22041	transferrin	2.03	8.95E-05
Ccr6	12458	chemokine (C-C motif) receptor 6	2.01	3.44E-06
Fcgr4	246256	Fc receptor, IgG, low affinity IV	2	2.56E-04
CD83	12522	CD83 antigen	1.98	5.01E-04
Slc11a1	18173	solute carrier family 11, member 1	1.97	2.54E-03
Ctsh	13036	cathepsin H	1.96	2.97E-05
AI661384	106930	expressed sequence AI661384	1.94	2.62E-07
Ephx1	13849	epoxide hydrolase 1, microsomal	1.93	2.01E-06
Cfp	18636	complement factor properdin	1.92	3.86E-04
C1qb	12260	complement component 1, q subcomponent, beta polypeptide	1.92	8.12E-04
Ifitm1	68713	interferon induced transmembrane protein 1	1.91	1.06E-07
Gata3	14462	GATA binding protein 3	1.9	2.00E-08
Sepp1	20363	selenoprotein P, plasma, 1	1.89	6.23E-07

Table 1.3 Genes upregulated in IFNAR$^{-/-}$ P14 cells compared to WT P14 from NK cell depleted mice

Shown are the top 50 upregulated genes in IFNAR$^{-/-}$ P14 compared to WT P14 cells. Genes are sorted by their log2 fold change were a log2 fold change of 1 corresponds to a fold change of 2.

Gene Symbol	NCBI Entrez Gene ID	Gene Name	log2FC	adjp
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Oas1a	246730	2'-5' oligoadenylate synthetase 1A	-4.48	5.05E-09
Rtp4	67775	receptor transporter protein 4	-4.37	6.14E-07
Gm9706	677168	predicted gene 9706	-4.24	1.84E-07
Isg15	100038882	ISG15 ubiquitin-like modifier	-4.23	3.52E-08
Oas1f	243262	2'-5' oligoadenylate synthetase 1F	-4.17	1.67E-08
Ifi27l2a	76933	interferon, alpha-inducible protein 27 like 2A	-3.88	1.97E-08
Ifi44	99899	interferon-induced protein 44	-3.71	6.12E-08
Dhx58	80861	DEXH (Asp-Glu-X-His) box polypeptide 58	-3.67	2.37E-08
Gm12253	624860	predicted gene 12253	-3.65	4.24E-09
Mx1	17857	myxovirus (influenza virus) resistance 1	-3.63	7.34E-06
Apol9b	71898	apolipoprotein L 9b	-3.53	3.49E-06
Il10	16153	interleukin 10	-3.5	1.78E-08
Isg20	57444	interferon-stimulated protein	-3.5	3.86E-07
Mnda	381308	myeloid cell nuclear differentiation antigen	-3.42	4.40E-08
Oasl1	231655	2'-5' oligoadenylate synthetase-like 1	-3.25	4.73E-06
Bst2	69550	bone marrow stromal cell antigen 2	-3.22	4.40E-08
Ifi204	15951	interferon activated gene 204	-3.21	1.06E-08
Mx2	17858	myxovirus (influenza virus) resistance 2	-3.14	4.20E-06
Klrg1	50928	killer cell lectin-like receptor subfamily G, member 1	-3.05	3.78E-09
BC094916	545384	cDNA sequence BC094916	-3.02	1.27E-07
Chac1	69065	ChaC, cation transport regulator-like 1 (E. coli)	-2.88	4.77E-06
Batf3	381319	basic leucine zipper transcription factor, ATF-like 3	-2.87	5.67E-05
Gm4951	240327	predicted gene 4951	-2.73	1.24E-07
Slfn1	20555	schlafen 1	-2.73	8.69E-07
Ifit1	15957	interferon-induced protein with tetratricopeptide repeats 1	-2.73	6.34E-06
Parp12	243771	poly (ADP-ribose) polymerase family, member 12	-2.64	1.27E-07
Il2ra	16184	interleukin 2 receptor, alpha chain	-2.63	3.22E-07
Ly6c1	17067	lymphocyte antigen 6 complex, locus C1	-2.61	4.13E-08
Apol9a	223672	apolipoprotein L 9a	-2.61	7.63E-07
Esm1	71690	endothelial cell-specific molecule 1	-2.56	3.22E-06
Xaf1	327959	XIAP associated factor 1	-2.54	2.07E-07
Zbp1	58203	Z-DNA binding protein 1	-2.53	2.36E-07
Rsad2	58185	radical S-adenosyl methionine domain containing 2	-2.52	1.03E-04
Oasl2	23962	2'-5' oligoadenylate synthetase-like 2	-2.52	3.16E-04
Lgals3bp	19039	lectin, galactoside-binding, soluble, 3 binding protein	-2.48	9.49E-09
Slfn8	276950	schlafen 8	-2.48	4.73E-07
Cmpk2	22169	cytidine monophosphate (UMP-CMP) kinase 2, mitochondrial	-2.47	1.38E-07
Csprs	114564	component of Sp100-rs	-2.47	4.77E-06
Irf7	54123	interferon regulatory factor 7	-2.47	2.00E-05
Tuba8	53857	tubulin, alpha 8	-2.39	4.11E-09
Rnf213	672511	ring finger protein 213	-2.39	3.24E-07
Tnp2	21959	transition protein 2	-2.36	9.57E-06
Phf11	219131	PHD finger protein 11	-2.31	1.84E-07
Gm12250	631323	predicted gene 12250	-2.3	6.33E-06
Ly6a	110454	lymphocyte antigen 6 complex, locus A	-2.29	2.95E-05
Mt2	17750	metallothionein 2	-2.28	7.78E-03
Tmprss13	214531	transmembrane protease, serine 13	-2.27	1.43E-08
Cited4	56222	Cbp/p300-interacting transactivator, 4	-2.27	4.64E-06

Table 1.4 Genes downregulated in IFNAR$^{-/-}$ P14 cells compared to WT P14 from NK cell depleted mice

Shown are the top 50 downregulated genes in IFNAR$^{-/-}$ P14 compared to WT P14 cells. Genes are sorted by their log2 fold change were a log2 fold change of -1 corresponds to a fold change of 2.

Chapter 7

7. Materials and Methods

Mice

The following mouse strains were housed and bred in specific pathogen-free facilities: C57BL/6 mice, Perforin deficient mice (Kagi, Ledermann et al. 1994). P14 transgenic ($Ly5.1^{+}$) mice expressing a TCR specific for LCMV peptide gp33-41 described previously (Pircher, Moskophidis et al. 1990). P14 mice were crossed with $IFNAR^{-/-}$ mice to generate $IFNAR^{-/-}$ P14 mice ($Thy1.1^{+}$). SM transgenic ($Ly5.1^{+}$) mice expressing a TCR specific for LCMV peptide gp61-80 were described previously (Oxenius, Bachmann et al. 1998). SM mice were crossed with $IFNAR^{-/-}$ mice to generate $IFNAR^{-/-}$ SM mice ($Thy1.1^{+}$). $NKG2D^{-/-}$ mice were a kind gift from Prof. Bojan Polic (Zafirova, Mandaric et al. 2009). $NCR1^{iCre/iCre}$ knock-in mice were described previously (Narni-Mancinelli, Jaeger et al. 2012) and were generated by breeding $NCR1^{iCre}$ mice to homozygosity (Narni-Mancinelli, Chaix et al. 2011). T cell specific $IFNAR^{-/-}$ mice ($CD4^{+cre}IFNAR^{f/f}$) mice were a kind gift from Prof. Ulrich Kalinke. All animals were used at 6 to 12 weeks of age. All animal experiments have been performed in accordance with institutional policies and have been reviewed by the cantonal veterinary office.

Viruses, infections and depletion

The LCMV isolates WE and the mutant strain LCMV-WE8.7 (LCMV8.7) (Pircher, Moskophidis et al. 1990) were provided by Dr. R.M. Zinkernagel (University Hospital, Zurich, Switzerland) and propagated at a low multiplicity of infection on L929 fibroblast cells. Recombinant VSV expressing the LCMV glycoprotein (VSVGP) was grown on L292 cells and was a kind gift from Prof. Dorothee Von Lauer (Innsbruck Medical University). Recombinant Vaccinia virus expressing the LCMV glycoprotein (VVG2) was originally obtained from Dr. D. H. L. Bishop (Oxford University, Oxford, U.K.) and was grown on BSC40 cells at low multiplicity of infection. Co-infection of LCMV8.7 and VVG2 was performed by i.p. injection of $1x10^4$ ffu LCMV8.7 and $5x10^6$ pfu VVG2. Infections with 200 ffu LCMV-WE or $2x10^6$ pfu VSVGP were performed i.v. Infection with $5x10^6$ pfu VVGP was performed i.p.

Depletion of NK cells with either aNK1.1 (clone PK136, Bio X cell) or anti-Asialo GM1 (wako chem) was performed by i.p injection of 300µg aNK1.1 or 10µl anti-Asialo GM1 one day before and after infection.

Adoptive transfer

$CD8^+$ and $CD4^+$ T cells were isolated from naïve P14 or SM ($Ly5.1^+$) or $IFNAR^{-/-}$ P14 or SM ($Thy1.1^+$) mice using anti-$CD8^+$ or anti-$CD4^+$ beads (Miltenyi Biotech) and adoptively co-transferred into naive recipient mice. To study the functionality of memory P14 cells, lymphocytes were isolated 80 days post infection and a total of 10^6 $CD8^+$ T cells were purified by anti-$CD8^+$ MACS beads and transferred into naïve recipient mice. Prior to transfer, the frequency of memory WT and $IFNAR^{-/-}$ P14 cells amongst the total $CD8^+$ T cells was determined by flow cytometry.

Flow Cytometry

Staining was performed on whole blood or single cell suspensions from the indicated organs. The following antibodies were purchased from Biolegend (San Diego, CA, USA): anti-$CD8^+$ (53-6.7), anti-CD45.1 (A20), anti-CD127 (SB/199), anti-CD25 (3C7), anti-CD44 (IM7), , CD49b (DX5), anti-MULT1 (5D10), anti-CD3e (145-2C11), CD80 (16-10A1), CD40 (3/23) anti-T-bet (4B10) and anti-CD107a (1D4B). Anti-CD62L (MEC-14), CD86 (GL1), MHCII (M5/114.15.2), NK1.1 (cPK136) and anti-IFN-γ (XMG1.2) were purchased from BD Biosciences (Switzerland). Anti-Granzyme B (16G6), anti-Perforin (eBioOMAK-D), anti-Thy1.1 (HIS51), anti-Vcam1 (429), and anti-KLRG-1 (2F1) were purchased from eBioscience (San Diego, CA, USA). Anti-human IgG1 was purchased from Jackson ImmunoResearch. Intracellular T-bet staining was performed using the Foxp3 staining kit according to the manufactures protocol eBioscience (San Diego, CA, USA). NCR1-IgG fusion protein was a kind gift from Prof. Mandelboim and was described previously (Mandelboim, Lieberman et al. 2001). Following production of NCR1-IgG fusion protein, the staining was titrated to determine the best concentration for staining. Data were acquired on a LSRII^{TM} flow cytometer (BD Bioscience, Switzerland) and analyzed using Flowjo software (Treestar, Ashland, OR, USA).

Microarray Analysis

WT and $IFNAR^{-/-}$ P14 T cells were isolated from day 3 LCMV8.7 + VVG2 co-infected mice that were either undepleted or depleted of NK cells. T cells were FACS sorted to purity, frozen and sent to Miltenyi Biotec for microarray analysis. Data obtained from Miltenyi was analyzed in collaboration with the Vital-IT Group, SIB Swiss Institute of Bioinformatics. The arrays hybridized were Agilent 8x60K mouse whole genome. Annotation files for this array were

downloaded from the Agilent website. Background subtraction and quintile normalization was performed using the Agilent preprocessing package Agi4x44PreProcess. Differentially expressed probes between different conditions were detected by fitting linear models and computing empirical Bayes moderated t statistics, using the limma package. P values were adjusted for multiple comparisons using the Benjamini Hochberg procedure. For pathway analysis Gene Set Enrichment Analysis (GSEA) was performed. Heatmaps were produced by performing hierarchical clustering on the genes (Pearson correlation, average linkage) and visualized using heatmap.2 in Bioconductor.

Quantitative real-time PCR

For in vitro T cell activation, $CD8^+$ T cells were isolated using anti-$CD8^+$ beads (Miltenyi Biotech, Germany) and stimulated with plate bound anti-CD3 (145-2C11, 2 mg/ml) and anti-CD28 (PV-1, 2 mg/ml) in the presence of 1000 U/ml IFN-β or 25 ng/ml IL-12 (both R&D Systems, Abingdon, UK). RNA was extracted with RNAeasy Mini kits (Qiagen, Valencia, CA, USA) and was analyzed by real-time PCR according to the manufacturer's instructions (Applied Biosystems, Carlsbad, CA, USA). Primers–probe mixtures were: T-bet (Mm00450960_m1) and β-actin (Mm00446968_m1).

For qPCR of *in vivo* activated T cells total RNA was isolated from FACS sorted P14 T cells 3 days after LCMV8.7 + VVG2 co-infection of NK depleted mice. RNA isolation was performed using TRIzol reagent (Invitrogen) according to the manufactures instructions and cDNA was reverse transcribed from RNA using M-MLV reverse transcriptase RNase, H minus (Promega). Real-time PCR was performed using Rotorgene 3000 (Corbett). qPCR was performed using TaqMan probes for the indicated genes together with GAPDH as internal control. Analysis was performed by determining the Ct value for each sample normalized to GAPDH (ΔCt). Fold expression was then calculated using the ΔΔCt method, briefly ΔCt from $IFNAR^{-/-}$ P14 cells was subtracted from the ΔCt of WT P14 cells giving a ΔΔCt. Fold induction was calculated using the equation $2^{-\Delta\Delta Ct}$.

In vivo killer assay

Activated T cells were isolated from VVG2 and LCMV8.7 co-infected mice following adoptive co-transfer of 10^6 WT and $IFNAR^{-/-}$ P14 cells at day 3 post infection. $CD8^+$ T cells were MACS purified and adoptively transferred into naïve or infection matched hosts. Following 6 hour

incubation, spleens were isolated and analyzed by flow cytometry. The recovery of P14 cells from infected hosts was normalized to the number recovered in the naïve hosts.

Ex vivo killer assay

Activated NK cells were isolated from day 2 post LCMV (10^6 ffu) infected mice by DX5 MACS bead purification. NK cells were added to round bottom 96 well plates together with the indicated blocking antibodies. aNCR1 and aNKG2D were used at a concentration of 5µg per well and were a kind gift of Prof. Stipan Jonjic (Rijeka, Croatia). aCD48 (Biolegend) was used a concentration of 10µg/ml as previously described (Kato, Koyanagi et al. 1992). aVCAM (Biolegend) was used at a concentration of 10µg/ml. Inhibition of CD7 was achieved by using 20µM of thiodigalactoside (Carbosynth) as previously described (Stannard, Collins et al. 2010). Activated T cells were isolated from VVG2 and LCMV8.7 co-infected mice following adoptive transfer of WT and IFNAR$^{-/-}$ P14 cells at day 3 post infection. Activated T cells were FACS sorted to purity based on the expression of CD8$^+$ and Ly5.1 (WT) or Thy1.1 (IFNAR$^{-/-}$ P14). $2x10^4$ purified P14 or IFNAR$^{-/-}$ T cells were added to the 96 well plates separately. Following a 6 hour incubation T cells were stained with 7-AAD and Annexin V according to manufactures recommendation.

CFSE labeling

CFSE labeling was performed on CD8$^+$ MACS T cells. Immediately following MACS separation, T cells were washed and resuspended in PBS. CFSE was diluted to a concentration of 2uM and diluted one to one with the T cells giving a final concentration of 1uM. The cells were incubated for 10min at 37°C following by addition of FCS to quence the CFSE labeling. Cells were then washed twice with PBS to remove any remaining CFSE. The T cells were then diluted to the appropriate concentration and used.

Dendritic cell functionality

Dendritic cell functionally and phenotype was analyzed from splenocytes of day 2 post LCMV infected mice. DCs were isolated via CD11c MACS purification from day 2 post LCMV infected mice that were either untreated or depleted of NK cells. These purified DCs were

cultured together with either naïve WT or naïve IFNAR$^{-/-}$ P14 cells that had been previously CFSE labelled. T cells were incubated with DCs for 3 days at 37°C followed by flow Cytometry analysis of CFSE dilution.

Accession Number

The microarray data discussed in this paper have been deposited in NCBI`s Gene Omnibus and are accessible through the GEO accession number GSE57355 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE57355)

Statistical Analysis

Statistical significance was determined by a 2-tailed unpaired t test using GraphPad Prism (La Jolla, CA, USA).

Chapter 7

8. References

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Chapter 8

9. Abbreviations

Ag	Antigen
APC	Antigen presenting cell
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
DC	Dendritic cell
dsRNA	Double stranded RNA
FACS	Florescence-activated cell sorting
ffu	Focus forming unit
i.p.	Intraperitoneal
i.p.	Intraperitonellay
i.v.	Intravenous
i.v.	intravenously
IFN	Interferon
IFNAR	Type I interferon receptor
Ig	Immune globulin
IL	Interleukin
ISG	interferon stimulated genes
KO	Knockout
LCMV	Lymphocytic choriomeningitis virus
LCMV8.7	LCMV strain harboring a mutation in gp33
LN	Lymph node
MACS	Magnetic activated cell sorting
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MPEC	Memory precursor effector cells
NCR	Natural cytotoxicity triggering receptor
NK	Natural killer
OAS	2′-5′ oligoadenylate synthases
P14	TCR transgenic $CD8^+$ T cells specific for lcmv gp33-41 epitope
PAMP	Pathogen associated molecular pattern
PCR	Polymerase chain reaction
PFU	Plaque forming unit
PKR	double-stranded RNA-activated protein kinase

PRR	Pattern recognition receptor
qPCR	Quantitative real time PCR
SLEC	Short lived effector cells
ssRNA	Single stranded RNA
STAT	Signal transduce and activator of transcription
Tcm	Central memory T cell
TCR	T cell receptor
Tem	Effector memory T cell
tg	Transgenic
TLR	Toll like receptor
TNF	Tumor necrosis factor
TRAIL	Tumor necrosis factor-related apoptosis inducing ligand
VSV	Vesicular stomatitis virus
VV	Vaccina virus
VVG2	Recombinant VV expressing the LCMV glycoprotein
WT	Wild type

Chapter 9

10. Publications and Presentations

10.1 Publications

Crouse, J., Bedenikovic, G., Wiesel, M., Ibberson, M., Xenarios, I., Von Laer, D., Kalinke, U., Vivier, E., Jonjic, S., Oxenius, A. (2014). "Type I Interferons protect T cells against NCR1 directed NK attack". **Immunity**. *In press*

Bedenikovic. G., **Crouse. J.,** Oxenius. A. (2014). "T-cell help dependence of memory $CD8^+$ T-cell expansion upon vaccinia virus challenge relies on CD40 signaling". **Eur J Immunol** 44(1):115-26.

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Wiesel. M., Joller. N., Ehlert. AK., **Crouse. J.,** Spörri. R., Bachmann. MF., Oxenius. A. (2010). "Th cells act via two synergistic pathways to promote antiviral $CD8^+$ T cell responses." **J Immunol**. 185(9):5188-97.

Mercer. J., Knébel. S., Schmidt. FI., **Crouse. J.,** Burkard. C., Helenius. A. (2010). "Vaccinia virus strains use distinct forms of macropinocytosis for host-cell entry." **Proc Natl Acad Sci U S A**. 107(20):9346-51.

10.2 Presentations

10.2.1 Oral Presentations

"Direct type I IFN signaling on $CD8^+$ T cells protects them from NK cell mediated killing", Annual Meeting of the Swiss Society for Allergology & Immunology and the Swiss Respiratory Society, 2013, Bern, Switzerland

"Type I interferon signaling protects T cells from NK cell mediated killing", Meeting of the Swiss Immunology PhD students, 2012. Schloss Wolfsberg, Switzerland.

10.2.2 Poster Presentations

"Direct type I IFN signaling determines the cell fate decision between short lived effector cells and memory precursor effector cells", EMBO conference on Homeostasis and perturbations of immunity. 2011. Capo Caccia, Sardinia.

Chapter 11

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