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The Role of Hepatocyte-Intrinsic NF-κB Signaling in Controlling Systemic Viral Infections

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1. SUMMARY

The liver is one of the pivotal organs in vertebrate animals, serving a multitude of functions including a predominant role in metabolism, detoxification and protein synthesis. Being located at a hemodynamic confluence of portal vein and hepatic artery, the liver can screen both systemic as well as gut-derived pathogens, and this unique positioning in the body also enables the liver to serve as a principal immune organ. The liver has the single largest population of macrophages; known as Kupffer cells, a large number of liver dendritic cells, lymphocytes such as $CD8^+$ T cells, NK cells, NKT cells and an enriched network of reticuloendothelial cells. Hepatocytes, liver sinusoidal endothelial cells and stellate cells express multiple immune receptors, co-receptors, costimulatory molecules, adhesion molecules, various TLRs and are capable of functioning as strong APCs among the non-myeloid cell types in the liver. All these cells are seen in the liver vasculature where they directly detect and capture the pathogens in circulation suggesting that every cell type in the liver can efficiently function on an immune platform. Despite being equipped with this arsenal of immune cells, prolonged exposure to PAMPs such as LPS derived from the gut induces a state of immune non-responsiveness in the liver favoring immune tolerance through multiple mechanisms. However, upon encountering live bacteria and viruses which are recognized by an increased expression of PAMPs, the liver elicits a robust immune response, aiding in the control of the infection.

The innate immune mechanisms pertaining to liver in controlling viral infections have largely been attributed to the Kupffer cells, the locally resident macrophages. However all the cells of liver are equipped with innate immune functions including in particular, the hepatocytes. Hence, our aim in this study was to elucidate the innate immune contribution of hepatocytes in viral clearance using mice lacking Ikkβ specifically in the hepatocytes, termed $Ikk\beta^{\Delta Hep}$ mice. Blockade of Ikkβ activation in $Ikk\beta^{\Delta Hep}$ mice affects the downstream signaling of canonical NF-κB signaling by preventing the nuclear translocation of NF-κB, an important step required for the initiation of innate immune responses.

Interestingly, infection of $Ikk\beta^{\Delta Hep}$ mice with LCMV-WE virus led to increased viral loads in the livers compared to virus titers from the livers of wild-type C57BL/6 mice infected with the same PFU. Furthermore, hepatocytes were observed forming clusters of cells

filled with virus, as evidenced by staining for LCMV-NP in infected Ikkβ$^{\Delta Hep}$ mice, whereas no such foci were observed in C57BL/6 mice under the same infection conditions. The increase in the viral burden in the livers of Ikkβ$^{\Delta Hep}$ mice was found to be due to a reduction in the expression of various interferon stimulated genes (ISGs) which are required to control viral replication. LCMV-WE infection of *ex vivo* cultured hepatocytes derived from Ikkβ$^{\Delta Hep}$ and C57Bl6 mice also showed an increase in the expression of viral proteins with a concomitant reduction in ISG expression confirming that the increased virus titers in the livers of Ikkβ$^{\Delta Hep}$ mice is indeed a hepatocyte intrinsic phenotype.

Further, at day 6 post-infection, we observed a reduction in the infiltration of LCMV-specific cytotoxic CD8^{+} T cell response possibly due to a reduction in the interferon responsive chemokines by the hepatocytes lacking NF-κB signaling. The phenotype of hepatocytes forming clusters of cells filled with LCMV, observed with infections using Ikkβ$^{\Delta Hep}$ was further complemented with infections using IFNAR$^{\Delta Hep}$ mice, indicating a link between NF-κB signaling in the hepatocytes and the hepatocyte interferon response. In another experimental setup, IFNAR$^{\Delta Kup}$ mice exhibited infected Kupffer cells, as well as clusters of hepatocytes filled with LCMV similar to Ikkβ$^{\Delta Hep}$ and IFNAR$^{\Delta Hep}$ mice at day 6 post-infection. Strikingly, at day 8 post-infection, the numbers of hepatic clusters were reduced, suggesting that hepatocytes can clear the virus independent of Kupffer cells especially when they are deficient in interferon signaling.

Furthermore, comparative analysis of interferon stimulated gene production at early time points following infections with LCMV-WE in C57BL/6, Ikkβ$^{\Delta Hep}$, IFNAR$^{\Delta Hep}$ and IFNAR$^{\Delta Kup}$ mice showed a severely reduced expression of ISGs in IFNAR$^{\Delta Hep}$ mice compared to IFNAR$^{\Delta Kup}$ mice and Ikkβ$^{\Delta Hep}$ mice while there is an enhanced expression of ISGs in wildtype-C57BL/6. This observation strongly suggests that the major producers of ISGs in the liver are hepatocytes and not Kupffer cells.

Collectively, these results clearly highlight a previously unknown and influential role of hepatocytes in the induction of innate immune responses leading to viral clearance during systemic viral infection with LCMV-WE.

1a. ZUSAMMENFASSUNG

Die Leber ist ein zentrales Organ in Säugetieren, das eine Vielzahl von Funktionen ausübt, unter anderem eine prägende Rolle im Metabolismus. Durch die Lokalisation im hämodynamischen Zusammentreffen von Portalvene und Leberarterie, kann die Leber sowohl systemische als auch Leber-vermittelte Pathogene überprüfen. Die einzigartige Position im Körper ermöglicht es der Leber, dass sie als wesentliches Immunorgan fungiert. Die Leber hat die größte Organ-spezifische Makrophagenpopulation - sogenannte Kupfferzellen - sowie eine große Anzahl Dendritischer Zellen, Lymphozyten wie CD8+ T Zellen, NK Zellen, NKT Zellen und ein angereichertes Netzwerk an retikuloendothelialen Zellen. Hepatozyten, LSECS und Sternzellen exprimieren zahlreiche Immunrezeptoren, Korezeptoren, costimulatorische Zelladhäsionsmoleküle sowie mehrere TLRs, welche als starke APCs innerhalb der Nicht-Myeleoiden Zellpopulationen in der Leber fungieren. Das deutet darauf hin, dass jeder Zelltyp in der Leber effizient als Immunplattform dienen kann. Sämtliche Zellen sind im Lebergefäßsystem nachweißbar, wo sie direkt Pathogene aus der Zirkulation erkennen und abfangen können. Allerdings kann eine andauernde Präsentation von PAMPs - wie beispielsweise LPS aus dem Darm – zu einer Immun-Unempfindlichkeit in der Leber führen, die in Toleranz gipfeln kann. Andererseits kann die Leber eine robuste Immunantwort durch das Zusammentreffen von Bakterien und Viren herbeiführen, um die Infektion zu kontrollieren, die durch eine erhöhte Expression von PAMPs erkannt werden kann. Ein Ausfall der Leberimmunantwort kann zum Tod des Wirts führen.

Die von der Leber bereitgestellten angeborenen Immunmechanismen, welche virale Infektionen kontrollieren können, konnte man größtenteils Kupfferzellen zuschreiben. Allerdings sind alle Leberzellen dafür ausgestattet, dass sie angeborene Immunfunktionen ausführen können, vor allem die Hepatozyten. Das Ziel in der vorliegenden Studie war es deshalb, die angeborene Immunbeteiligung von Hepatozyten in der viralen Clearance mit Hilfe von Mäusen ohne Ikkβ spezifisch in Hepatozyten - Ikkβ$^{\Delta Hep}$-Mäusen - aufzuklären. Das Blockieren von Ikkβ-Aktivierung in Ikkβ$^{\Delta Hep}$-Mäusen beeinträchtigt den nachgeschalteten NF-κB Signalweg und verhindert die nukleare Translokation von NF-κB, die wichtig für die Initiierung der angeborenen Immunantworten ist.

Interessanterweise führt die Infektion von Ikkβ$^{\Delta Hep}$-Mäusen mit LCMV-WE Virus zu einer erhöhter viraler Last in der Leber. im Vergleich zu Virustitern von Wildtyp C57Bl6 Maus Lebern. Desweiteren wurde beobachtet das Hepatozyten Cluster voller Virus bilden, wie durch Färbungen für LCMV-NP in infizierten Ikkβ$^{\Delta Hep}$ Virus nachgewiesen werden konnte. In C57Bl6 Mäusen konnten solche Herde bei gleichen Bedingungen nicht nachgewiesen werden. Der Anstieg der viralen Last konnte zurückgeführt werden auf eine Reduzierung in der Expression vorn mehreren Interferon-stimulierten Genen, die für die Kontrolle der Viruslast verantwortlich sind.

Desweiteren konnten wir eine Reduzierung in der Infiltration der LCMV-spezifischen zytotoxischen CD8+ T Zell-Antwort an Tag 6 nach Infektion beobachten, wahrscheinlich aufgrund einer Reduzierung in der Interferonexpression durch die Hepatozyten, die keinen intakten NF-κB Signalweg besitzen.

Der Phänotyp von Hepatozyten, die in Infektionen von Ikkβ$^{\Delta Hep}$ mit LCMV gefüllte Cluster bilden, wurde weiter weitergeführt mit Infektionen von IFNAR$^{\Delta Hep}$ Mäusen. Diese Experimente weisen auf eine Verbindung zwischen dem NF-κB Signalweg in Hepatozyten und der Interferon Antwort hin.

In einem weiteren Experiment, konnte gezeigt werden, dass IFNAR$^{\Delta Kup}$ infizierte Kupfferzellen sowie Cluster voller LCMV - ähnlich IkkβΔHep und IFNAR$^{\Delta Hep}$ Mäusen - an Tag 6 nach Infektion bildeten. Interessanterweise, an Tag 8 nach der Infektion war die Anzahl der hepatischen Cluster reduziert. Das weist darauf hin, dass Hepatozyten den Virus unabhängig von Kupfferzellen säubern können, vor allem wenn sie keinen intakten Interferon Signalweg besitzen.

Des Weiteren wurde eine Vergleichsstudie der Interferon-stimulierten Genproduktion von frühen Zeitpunkten nach LCMV Infektionen in C57Bl6, Ikkβ$^{\Delta Hep}$, IFNAR$^{\Delta Hep}$ und IFNAR$^{\Delta Kup}$ Mäusen durchgeführt. Diese Studie zeigte eine stark reduzierte ISG Expression in IFNAR$^{\Delta Hep}$ vergleichen zu IFNAR$^{\Delta Kup}$ und Ikkβ$^{\Delta Hep}$ Mäusen, wohingegen Wildtyp C57BL6 eine verstärkte ISG Expression zeigten. Das deutet darauf hin, dass Hepatozyten der Haupterzeuger von ISGs in der Leber die und nicht die Kupfferzellen.

2. ABBREVIATIONS

APC	Antigen Presenting Cell
BAFF	B-cell Activating Factor
CARD	Caspase Activation and Recruitment Domain
CARDIF	CARD adapter inducing Interferon-β (also called VISA, MAVS)
CCL	Chemokine (C-C motif) Ligand
CD	Cluster of Differentiation
CH25H	Cholesterol-25-Hydroxylase
CIAP	Calf Intestinal Alkaline Phosphatase
CpG DNA	Cytosin-phoshatidyl-Guanin
CTL	Cytotoxic T cell
CXCL	Chemokine (C-X-C motif) Ligand
DC	Dendritic Cell
eIF	Eukaryotic Initiation Factor
ERK	Extracellular signal Regulated Kinase
GAF	IFN-γ Activation Factor
GAS	Gamma-Activated Sequence
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HSC	Hematopoietic stem cell
IHC	Immunohistochemistry
i.v	intra venous
ICAM	Intercellular Adhesion Molecule
ICS	Intracellular Cytokine Staining
IFIT	IFN-induced proteins with Tetratricopeptide repeat
IFITM	IFN Inducible TransMembrane
IFN	Interferon

IFNAR	Interferon-alpha/beta Receptor
IFNGR	Interferon-gamma Receptor
IGR	Intergenic region
IκB	Inhibitor of κB
IKBKE	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon
IKK	IkB Kinase
IL	Interleukin
IP-10	IFN-γ-inducible protein 10
IRAK	IL-1-Receptor-Associated Kinase
IRF	Interferon Regulatory Facror
ISG	Interferon Stimulated Gene
ISGF	Interferon Stimulated Gene Factor
ISH	in situ Hybridisation
ISRE	IFN-Stimulated Response Elements
JAK	Janus Kinase
JNK	c-Jun amino N-terminal protein Kinase
KC	Kupffer Cell
LCMV	Lymphocytic Choriomeningitis Virus
LFA1	Lymphocyte Function associated Antigen 1
LGP2	Laboratory of Genetics and Physiology 2
LPS	Lippolysacharide
LSEC	Liver Sinusoidal Endothelial Cells
LT	Lymphotoxin
MAGUK	Membrane-Associated Guanylate Kinase homologue
MALT	Mucosal-Associated Lymphoid Tissue
MAPK	Mitogen Activated Protein Kinase
MAVS	Mitochondrial Antiviral-signaling protein

MDA5	Melanoma Differentiation-Associated protein 5
MEF	Mouse Embryonic Fibroblast
MEKK	MAP/ERK Kinase Kinase
MHC	Major Histocompatability Complex
Mx	Myxovirus gene
MyD88	Myeloid Differentiation primary response gene 88
NEMO	NF-kappa-B Essential Modulator
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B-cells
NIK	NF-kappa-B Inducing Kinase
NK cell	Natural Killer cell
NKT cell	Natural Killer T cell
NOD	Nucleotide-binding Oligomerization Domain
NP	Nucleoprotein
OAS	2'-5'-OligoAdenylate Synthetase
p.i	post infection
PAMP	Pathogen Associated Molecular pattern
pDC	plasmacytoid Dendritic Cell
pI:C	Polyinosinic:polycytidylic acid
PIAS	Protein inhibitors of activated STAT
PK	Protein Kinase
PKR	Protein Kinase RNA-activated
PML	Promyelocytic lleukemia
PRR	Pattern Recognition Receptor
RANK	Receptor Activator of NF-κB
RdRp	RNA-dependent-RNA polymerase
RHD	Rel Homology Domain
RIG-I	Retinoicacid Inducible Gene I

RING	Really Interesting New Gene
RIP	Receptor-Interacting Protein kinase
RLR	RIG-I-Like Receptor
RNP	Ribonucleoprotein
sDC	spleenic Dendritic Cell
ssDNA	single stranded Deoxyribo Nucleic Acid
ssRNA	single stranded Ribo Nucleic Acid
STAT	Signal Transducer and Activator of Transcription
STING	Stimulator of Interferon Genes
TAB	TAK1-Binding protein
TAD	Transactivating Domain
TAK	Tat-associated kinase
TANK	TRAF family member-associated NF-kappa-B activator
TBK	TANK-Binding Kinase
TCR	T Cell Receptor
TIR	Toll-Interleukin 1 Receptor
TIRAP	Toll-Interleukin 1 Receptor (TIR) Domain Containing Adaptor Protein
TLR	Toll Like Receptor
TNF	Tumor Necrosis factor
TNFAIP	Tumor necrosis factor alpha-induced protein
TRADD	Tumor necrosis factor Receptor type 1-Associated DEATH Domain
TRAF	TNF receptor Associated Factor
TRAM	TRIF-related Adaptor Molecule
TRIF	TIR-domain-containing adapter-Inducing interferon-β
TYK	Tyrosine Kinase
VISA	Virus-Induced Signaling Adapter (also called MAVS)
α-DG	alpha-Dystroglycan

3. INTRODUCTION

3.1 The liver

3.1.1 Structural and functional overview of the liver

The liver is the largest glandular internal organ in any vertebrate animal. The Liver performs many varied and essential functions and is one of the three pivotal organs along with heart and brain. The liver emerges first of all the organs during the formation of the fetus. Principally, the liver is involved in numerous metabolic functions of the body such as synthesis and secretion of bile which aids in digestion, protein synthesis, detoxification, glucose homeostasis, storage of metabolites and immune surveillance (Thomson et al., 2011). Owing to its positional anatomy at the convergence of the portal vein and hepatic artery, the liver is able to screen the blood for systemic as well as gut derived pathogens. Perhaps due to its versatility of functions, the liver has attained an evolutionary advantage to regenerate to its complete mass from as little as 25% of its mass (Fausto et al., 2006; Michalopoulos and DeFrances, 1997). The liver has a dual blood supply of blood coming from both portal vein and hepatic artery. Every minute, 30% of the total volume of blood in the body passes through the liver. 80% of the blood flows into the liver through portal vein and is poorly oxygenated but nutrient rich collected from the gastrointestinal tract, pancreas, spleen and gallbladder. 20% of the blood flows through the hepatic artery liver and is well oxygenated (Crispe, 2011).

In humans, the liver is positioned in the right upper quadrant of the abdomen and in case of mice; the liver spans the whole subdiaphragmatic space. The Liver is a lobular structure covered by a connective tissue layer called Glisson's capsule and is an assembly of regularly arranged type I collagen fibers, scattered type III fibers, fibroblasts, mast cells and some small blood vessels. The cells in the liver are arranged in the form

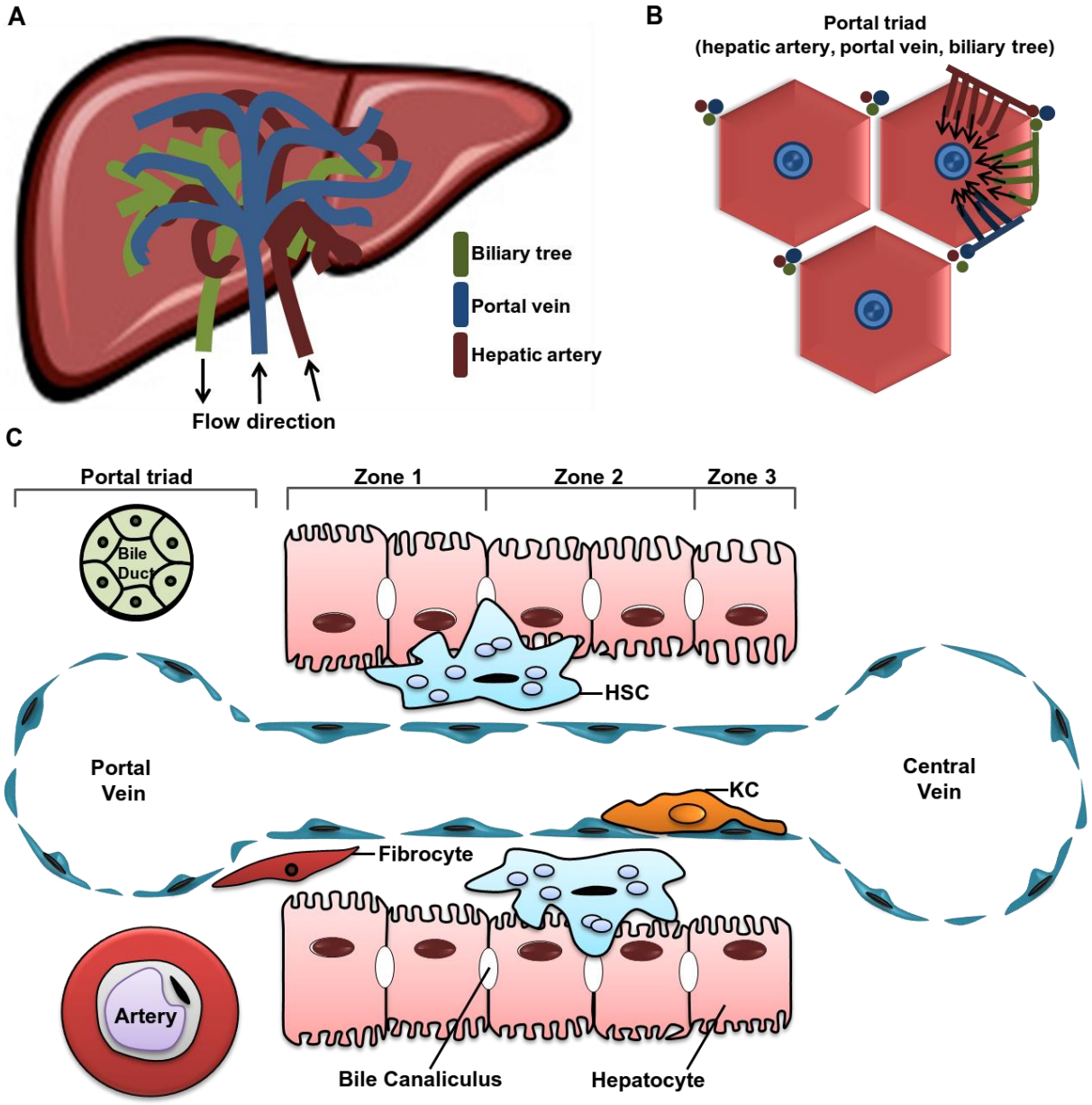

Figure 1: Structural overview of the liver. A. Hepatic artery, portal vein and biliary tree form the portal triad and they bear similar anatomical paths throughout the liver. The flow of bile through bile ducts is in opposite direction to the blood in hepatic artery and portal vein. B. Hepatic lobule is the structural and functional unit of the liver and is composed of Hepatic stellate cells (HSCs), hepatic sinusoid, cholangiocytes, hepatocytes, endothelial cells, portal fibroblasts and Kupffer cells (KC)-the liver resident macrophages. HSCs are placed in the space between hepatocytes and sinusoidal endothelium known as the space of disse. Blood flowing in the portal vein and hepatic artery enters the central vein through the sinusoids. (Adapted from (Kline et al., 2011; Xu et al., 2014b)).

of lobules called hepatic lobule, which is a structural and functional unit of the liver (Fig 1).

The liver has stacks of hexagonal arrangement of cells collectively called hepatic lobule which is considered as the structural and functional unit of the liver. Hepatic lobule constitute liver parenchymal hepatocytes, Hepatic sinusoid, non-parenchymal cells of the liver such as cholangiocytes, liver sinusoidal endothelial cells, hepatic stellate cells and Kupffer cells. A bile duct, portal vein and hepatic artery forms a hepatic triad that is separated by intervening sinusoids. Hepatic artery and portal vein are at the perimeter of a hepatic lobule. The blood from hepatic artery and portal vein enters the central vein through the sinusoids (Fig. 1).

3.1.2 Liver as an immunological organ

The spleen is known to be responsible for detecting and clearing blood borne pathogens. But experiments involving splenectomy has proven that the functions of the spleen are largely redundant except in the clearance of encapsulated bacteria (Jenne and Kubes, 2013). The liver stands as a barrier organ to the host from its external environment as it is constantly exposed to blood-borne pathogens derived from the gut due to its dual blood supply through the hepatic portal vein as well as the hepatic artery. Infection studies using a sublethal dose of *Listeria monocytogenes* and *Borrelia burgdorferi*, a non-lethal bacterium in mice depleted off Kupffer cells, the liver resident macrophages lead to an increased bacterial load, dissemination and death of mice which is unusual with normal mice (Ebe et al., 1999; Jenne and Kubes, 2013; Lee et al., 2010). These reports unequivocally stress that the immune surveillance functions mediated by the liver are non-redundant and they play a critical role in the survival of the host. All most all the cells of the liver are capable of mounting an immune response.

3.1.3 Immune tolerance in the liver

The liver maintains a default state of immune nonresponsiveness under basal conditions due to the low expression of MHC molecules and absence of costimulatory molecules on the liver resident LSECs, HSCs, KCs, DCs and hepatocytes (Schildberg et al., 2008; Wahl et al., 2008; You et al., 2008). Direct cell to cell contact among LSECs and liver DCs also promotes T cell tolerance within the liver (Schildberg et al., 2008). Gut originated PAMPs such as LPS in the portal veins results in the expression of anti-inflammatory cytokines such as IL-10 which reduce the expression of MHCs and brings about a state of nonresponsiveness under basal conditions in the liver (Knolle et al., 1999; Wahl et al., 2008).

However, the liver is also capable of inducing a robust immune response to severe bacterial and viral infections during which, elevated levels of PAMPs results in the infiltration of neutrophils and platelets in the liver (Jenne et al., 2013; McDonald et al., 2008; McDonald et al., 2012). This failure in the detection and clearance of blood borne pathogens leads to systemic infections that could be fatal which reinforces the contribution of liver specific immunity to the host (Jenne and Kubes, 2013). In cases of chronic viral infections, PAMPs such as CpG DNA induce an influx of cytotoxic effector cells, eventually clearing the viral infection from the liver and overcoming T cell tolerance (Huang et al., 2013).

3.1.4 Blood circulation and pathogen recognition in the liver

The liver being positioned at the convergence of hepatic artery and hepatic portal vein carries an extraordinary advantage to screen both systemic as well as gut derived pathogens. Blood flowing through the liver from the portal vein is derived from the pancreas, spleen and gut and so is nutrient rich but also might contain pathogens derived from gut epithelium which traverses to the portal blood (Berg, 1995). All blood

entering the liver passes through capillary-like vessels called sinusoids which slow down blood flow so that the contact between pathogens in circulation and endothelium is properly made and recognized by the liver resident immune cells. The rate of blood flow in the liver is only half compared to other capillary beds (Jenne and Kubes, 2013). The amount of pathogen associated molecular patterns (PAMPs) such as LPS are shown to drop 100-fold from portal and peripheral venous blood (Lumsden et al., 1988). Despite this constant exposure to pathogens, there are no signs of chronic inflammation which is clearly indicative that gut derived pathogens are removed from circulation by the liver.

3.1.5 Parenchymal cells of the liver

i.) Hepatocytes and hepatocytic innate immunity

Hepatocytes are the sole parenchymal cells of the liver and comprise ~80% of the entire liver cell mass. Hepatocytes are mainly involved in metabolic functions of the liver such as synthesis of bile acids from cholesterol, synthesis of enzymes involved in detoxification processes in the liver, metabolizing drugs and toxins entering the liver through portal circulation and glucose homeostasis. Up to 2000 mitochondria are seen in a single hepatocyte which accounts for 18% to 20% of its total cell volume which functions in oxidative phosphorylation and oxidation of fatty acids as well as other metabolic functions of hepatocytes. Hepatocytes are characterized by the presence of one or two nuclei and one or more nucleoli. The nucleolus is very large owing to the extensive synthesis of proteins by the hepatocytes which can be visualized by light microscopy.

Despite indulging heavily in metabolic functions, hepatocytes are considered potent immunological agents in their own right (Crispe, 2016; Zhou et al., 2016). They possess and deploy an arsenal of innate immune mechanisms upon encountering PAMPs leading to the secretion of inflammatory cytokines which eventually control the spread of

pathogens (Zhang et al., 2009). Hepatocytes play a pivotal role in inducing a systemic immune response to sepsis, a mechanism that is evolutionarily conserved in vertebrates from mammals to bony fish (Crispe, 2016; Lin et al., 2007). Bacterial infections induce an acute phase response during which circulation of cytokines such as IL-1α, TNFα and IL-6 are increased and among these IL-6 is shown to act directly on hepatocytes resulting in an elevated antibacterial lipocalin-2 protein (Xu et al., 2015).

Hepatocytes express PRRs such as cell surface TLR2, TLR4, endosomal TLR3, TLR9 and cytosolic receptors such as STING (stimulator of IFN genes), RIG-I (Retinoic acid inducible gene-1) and NOD (Nucleotide-binding oligomerization domain) family members and with these receptors, ex vivo isolated hepatocytes were directly shown to mediate immune responses to microbial products (Petrasek et al., 2011; Seki and Brenner, 2008; Vodovotz et al., 2001).

The ability of hepatocytes to exert antiviral responses is well observed in cases of HCV and HBV infections. In the case of HCV infection, viral RNA is detected by the RIG-I system within the hepatocytes and further downstream activation of IRF3 takes place through MAVS (Mitochondrial Antiviral Signaling protein) leading to the activation of type I IFNs. But, a protease expressed by HCV called NS3/4a cleaves both TRIF and MAVS, hindering IRF3 activation leading to a blockade in type I IFN production (Ferreon et al., 2005; Foy et al., 2005). In the case of HBV infection, HBV viral polymerase directly interacts with STING DNA sensor so that viral DNA is not detected (Liu et al., 2015). These data clearly show that pathogenic viruses turns down interferon signaling pathways in the hepatocytes which would otherwise hinder viral growth and replication, highlighting the importance of hepatocytes in mediating immune responses to viruses.

Hepatocytes are also involved in inducing adaptive immune responses. While hepatocytes normally express only MHC-I, they can be induced to express MHC II under

certain inflammatory conditions (Chen et al., 2005; Franco et al., 1988; Jenne and Kubes, 2013). In some cases, hepatocytes endocytose and kill CD8+ T cells recognizing them through a process called suicidal emperipolesis (Benseler et al., 2011). Hepatocytes have been shown to directly interact with naive T cells in an ICAM-I, MHC dependent manner (Warren et al., 2006) and activate naive T cells (Balam et al., 2012; Bertolino et al., 2001). Given the presence of APCs such as KCs, DCs and LSECS in the liver, the role of hepatocytes in activating T cell responses is still unclear and is a subject of active research.

3.1.6 Non-parenchymal cells of the liver

i.) Liver Sinusoidal Endothelial Cells (LSECs)

LSECs comprise ~50% of non-parenchymal cell population in the liver, twice as abundant as Kupffer cells and other lymphocytes (Racanelli and Rehermann, 2006). Hepatocytes are separated from the blood in the sinusoidal lumen by LSECs with a gap called space of Diss which is populated by Hepatic stellate cells (HSC) (Wisse et al., 1996). LSECs express various PRRs like endosomal TLRs such as TLR3, TLR4, TLR7 and TLR9 (Knolle and Limmer, 2003; Wu et al., 2010), co-receptors, costimulatory molecules, and many adhesion molecules. LSECs have been shown to constitutively express major histocompatibility complex molecules, MHC I and MHC II, CD80 and CD86 co-stimulatory molecules and Intercellular Adhesion Molecule (ICAM) in order to interact with lymphocytes (Knolle and Limmer, 2003). LSECs are among the non-myeloid APCs capable of cross presenting soluble antigen to CD8+ T cells which would lead to efficient T cell tolerance in the liver (Limmer et al., 2000).

ii.) Hepatic Stellate cells (HSCs)

HSCs, also called Ito cells are found in the space of Diss between LSECs and hepatocytes. They comprise only 5-8% of the total liver cells (Geerts, 2001). Having an

astral phenotype, they are extended and are seen wrapped around the sinusoids. Storage of lipids and vitamin A are the principal functions of HSCs under normal conditions. They transdifferentiate into fibrinogenic proliferating cells in cases of chronic liver damage causing liver fibrosis.

Under basal conditions, HSCs do not express molecules required for antigen presentation to lymphocytes but during liver inflammation and exposure to inflammatory cytokines such as IFN-γ changes they are activated and acquire a change in their phenotype (Chang et al., 2013). In the activated state, HSCs express molecules required for presenting antigens to T cells such as MHCI, MHCII and co-receptors CD80 and CD86, albeit in very low amounts (Winau et al., 2007; Winau et al., 2008). HSCs were reported to be capable of directly activating naive lymphocytes and to activate cytokine production by T cells, NK cells, NKT cells (Muhanna et al., 2007; Vinas et al., 2003).

iii.) Kupffer cells (KCs)

Named after Karl Wilhelm von Kupffer, these cells are resident macrophages of the liver. KCs comprise ~35% of the non-parenchymal cells of the liver and astonishingly, liver specific KCs represent 80-90% of the total macrophages in the body (Bilzer et al., 2006). The differences in development and lifespan of KCs in the liver indicate that they are not a homogenous population of cells (Bouwens et al., 1986; Gale et al., 1978). Unlike monocytic and macrophage cell populations in other tissues that move around the tissues searching for pathogens, KCs are stationary cells in contact with the blood. KCs express TLRs, complement receptors and antibody receptors that enable them to phagocytose pathogens. Activated KCs produce cytokines and chemokines fulfilling its role as an immune sentinel thereby keeping the immune system on alert in cases of pathogenic infections (Bilzer et al., 2006).

KCs have a remarkable capability to capture bacteria under flow conditions (Helmy et al., 2006), another contrast to the rest of tissue macrophages which phagocytose bacteria under static conditions. Being professional antigen presenting cells, they express MHC-I and MHC-II as well as costimulatory molecules needed for activating T cells. Even though KCs are well known for their phagocytic capabilities; they poorly activate adaptive immune responses and might need some activation cues from the parenchymal cells of the liver such as hepatocytes.

iv.) Dendritic cells (DCs)

Many different populations of DCs are present in the liver (Crispe, 2011). DCs enter the liver through portal blood supply and mature as they pass towards the central vein (Sato et al., 1998). Under basal conditions, DCs do not show intense antigen presenting capabilities similar to KCs and promote tolerance rather than activation of T cells (Jenne and Kubes, 2013).

In addition to the commonly found myeloid DCs ($CD11c^{hi}$) and plasmacytoid DCs ($CD123^{+}$), mouse liver is exclusively shown to contain a population of $CD11c^{+}$ $CD8^{+}$ DCs (Crispe, 2011; Pillarisetty et al., 2004) capable of inducing a robust T cell immune response. Antigen presentation by $CD11c^{+}$ $CD8^{+}$ DCs promotes T_H1-type response by producing large amounts of IL-12, whereas $CD8^{-}$ DCs promote T_H2-type responses by the production of IL-10 (Gao et al., 2010; Pillarisetty et al., 2004).

A cell type having characteristics of both DCs and NK cells has been reported in the liver called NK-DCs which express NK cell marker NK1.1 and DC marker CD11c. Functionally, they are known to present antigens efficiently and to activate T cells.

v.) Liver specific lymphocytes

There are many liver specific lymphocyte populations that are important for detecting and responding to pathogens. Almost half of these lymphocytic populations are liver specific NK cells which are at least three times more enriched than in the blood (Crispe, 2009; Doherty and O'Farrelly, 2000). They secrete cytotoxic granules containing perforin and granzyme, cytokines such as IFN-γ and importantly modulate MHC expression on hepatocytes and HSCs (Crispe, 2009). NKT cells are another important lymphocytic population in the liver also capable of secreting perforin and granzyme to target and kill the cells like NK cells (Matsuda et al., 2008). The unique feature of NKT cells is that they actively patrol through the liver vasculature searching for the presence of any potential pathogens rather than waiting for the antigens to come to them. Interestingly, hepatic NKT cells have been reported to respond to distal injury resulting in the production of anti-inflammatory cytokines, induction of an immunosuppressed state and protecting the brain. (Jenne and Kubes, 2013; Wong et al., 2011).

The liver is particularly enriched with $CD8^+$ T cells in comparison to the blood where there are at least double the numbers of $CD4^+$ T cells compared to $CD8^+$ T cells (Parker and Picut, 2005). The liver is also an abode for the largest population of γδT cells comprising about 15-25% of the total lymphocytes in the liver where as they are less than 3% in the blood (Abo et al., 2000; Parker and Picut, 2005). γδT cells are capable of binding to many ligands, both foreign and self in an MHC dependent and independent manner (Vantourout and Hayday, 2013) which is a striking contrast to conventional T cells that recognize a single antigenic peptide presented by a particular MHC molecule (Jenne and Kubes, 2013).

3.2 NF-κB Signaling

NF-κB (Nuclear transcription Factor of light chain Kappa immunoglobulins of activated B lymphocytes) was initially identified as a transcriptional enhancer of immunoglobulin κ light chain gene in the late 80's (Hoffmann and Baltimore, 2006; Sen and Baltimore, 1986; Sen and Baltimore, 2006). Extensive further research into the area of NF-κB signaling has established that NF-κB is not truly a nuclear factor and is also not B-cell specific but present in all the cell types shuttling between the cytoplasmic and nuclear compartments. In principle, NF-κB signaling involves a stimulus-driven activation and translocation of a set of NF-κB family transcription factors from the cytoplasm to the nucleus where they bind to the kappa B sites of the respective target genes, thereby modulating their transcription and expression.

3.2.1 NF-κB Transcription factor family

The mammalian NF-κB transcription factor family includes RELA (p65), c-REL, RELB, NF-κB1 (p105:p50) and NF-κB2 (p100:p52), all of which share a common structural feature, the presence of an amino terminal Rel homology domain (RHD) which is required for DNA binding, nuclear localization and dimerization with different NF-κB family members. RELA, c-REL and RELB has a non-homologous transactivation domain (TAD) at their carboxy terminus which aids in the recruitment of transcriptional coactivators to the DNA, thereby activating transcription from NF-κB binding sites in target genes. The absence of TAD in p50 and p52 enables them to function as transcriptional repressors unless being dimerized with other TAD containing REL member, thus p50 and p52 homo-dimers act as transcriptional repressors (Fig. 2). Excluding RELB, all the members of NF-κB family can form homo and heterodimers with one another (Li and Verma, 2002).

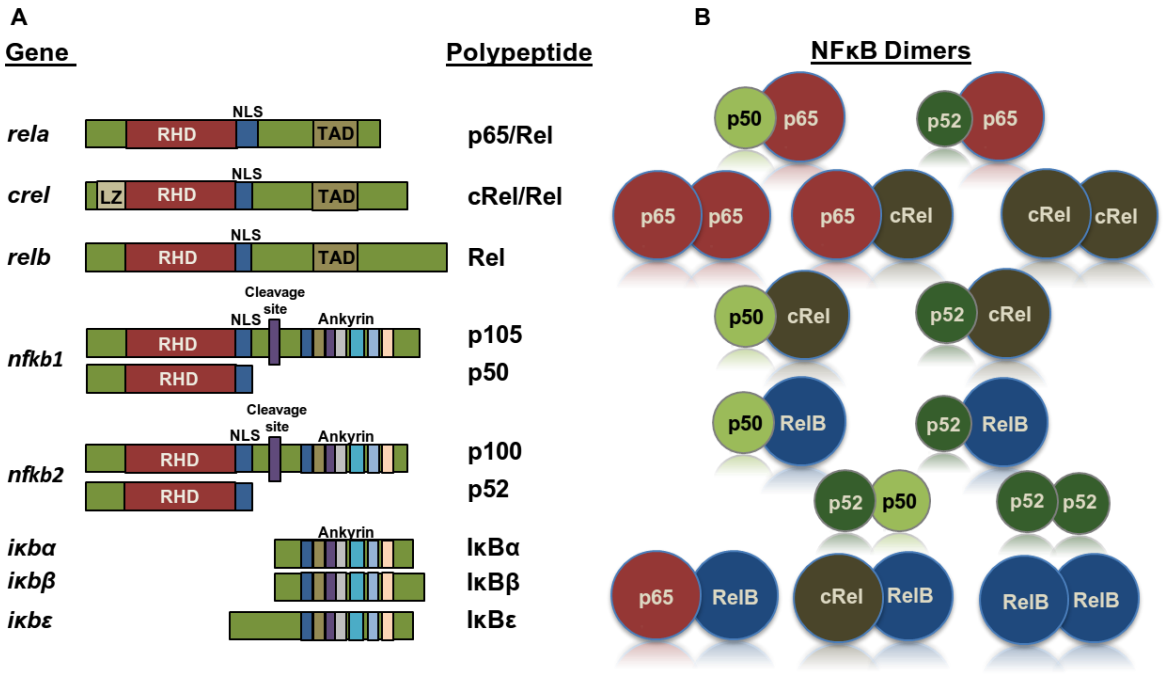

Figure 2: Members of the Nuclear factor (NF)-κB family. (A) Five mammalian NF-κB family genes express five transcription factors proteins namely RelA, RelB, cRel, p50 and p52 all of them share a highly conserved Rel-homology domain (RHD)- responsible for DNA binding, dimerization, nuclear translocation, and interaction with IκB molecules. c-Rel, RelA and RelB contain acidic transcriptional activation domain (TAD) which initiate transcription from NF-κB binding sites in target genes. RelB has an additional N-terminal leucine Zipper (LZ) motif which aids in protein interactions. p100 and p105 contains a nuclear localization signal (NLS) masked by IkB proteins and a C-terminal ankyrin repeat domains which is a hallmark of the inhibitory three IκB proteins, IκBα, IκBβ and IκBε. Proteolytic processing of p100 and p105 at specific cleavage sites results in p52 and p50 proteins. **(B)** Five NF-κB polypeptides by homo- and hetero- dimerization form transcription factors. Among them; 9 dimers function as transcriptional activators (top four rows), 3 dimers lack transcriptional activation domain and act as repressors (fifth row) and 3 dimers do not have the ability to bind DNA (sixth row). The presence of a given subset of these dimers depends on the cell type, stage and other environmental cues. Adapted from (Hoffmann and Baltimore, 2006).

NF-κB proteins are held in the cytoplasm in an inactive state by binding to an inhibitory protein called Inhibitor of kappaB (IκB). Three IκB proteins, IκBα, IκBβ, IκBε having a 33 amino-acid ankyrin repeat motif mediates protein-protein interactions associated with Rel-A, c-Rel and RelB in the inactive state. Ubiquitination of these inhibitory proteins leads to their activation. In contrast to binding, ankyrin repeats present in the precursor

proteins; p105 and p100 undergo a proteolytic cleavage and degradation resulting in the active forms of p50 and p52 (Li and Verma, 2002). While the expression of p50 and p65 is abundant in many cell types, RELB expression is restricted to Peyer's patches, lymph nodes and thymus whereas the expression of c-REL is restricted to the lymphocytes and hematopoietic cells.

3.2.2 Canonical NF-κB signaling pathway

Physiologically, canonical NF-κB signaling is activated by a wide variety of stimuli such as- binding of pathogen associated molecular patterns (PAMPs) like double-stranded RNA molecules from viruses or LPS from bacteria to the Pattern recognition receptors (PRRs) like Toll-like Receptors (TLRs); signaling by pro-inflammatory cytokines such as IL-1, TNFα through their cognate receptors and signaling mediated by antigen presentation to the T cell receptors (TCRs) by antigen presenting cells (APCs). Owing to its involvement in the regulation of inflammatory responses, canonical NF-κB signaling is activated in as little as 10 min (Hoffmann and Baltimore, 2006). While the downstream signaling starting from the receptors of these stimuli might vary, in most of the cases, they all converge on the activation of IKK (IκB kinase) complex (Fig. 3). IKK complex consists of two catalytic kinase subunits, Ikkα and Ikkβ with an intrinsic kinase activity and a regulatory subunit, Ikkγ also known as NF-κB essential modulator (NEMO) with helix-loop helix and leucine-zipper motifs that mediate protein-protein interactions (Karin and Ben-Neriah, 2000). Gene targeting experiments highlighted the importance of this complex in NF-κB activation. MEFs lacking Ikkα and Ikkβ or NEMO showed a complete lack of NF-κB activation to various stimuli (Li et al., 2000; Rudolph et al., 2000). It has been shown that IKKα and Ikkβ can phosphorylate all three known IκBs namely, IκBα, IκBβ and IκBε *in vitro* (Karin and Ben-Neriah, 2000). Mice lacking Ikkβ die from liver apoptosis as soon as 14 days post gestation similar to mice lacking p65 due to TNF

induced liver apoptosis (Li et al., 1999). Absence of Ikkβ results in a significant decrease in the activation of IκB and subsequent NF-κB translocation into the nucleus. Mice having mutations in NEMO shows embryonic lethality due to massive hepatic apoptosis (Rudolph et al., 2000).

A variety of stimuli at the cell surface induces the activation of IKK complex resulting in degradation of IκB proteins culminating in the nuclear translocation of NF-κB. LPS signals by binding to TLR4, CD14 and MD-2 protein complexes. This binding leads to the recruitment of MYD88 (myeloid differentiation primary response gene 88) which further interacts with IRAK (IL-1-receptor-associated kinase) leading to the activation of TRAF6 (TNF-receptor-associated factor 6). A complex of TAK (transforming-growth-factor-β-activated kinase 1), TAB1 (TAK1-binding protein 1) and TAB2 (TAK1-binding protein 2) further relays the signal leading to the phosphorylation of IKK complex. Binding of TNFα to its cognate receptor results in receptor trimerization and recruitment of TRADD adaptor protein which further interacts with TRAF2. Affinity of TRAF2 to downstream signaling proteins such as receptor-interacting serine/threonine (RIP) kinase and MAP/ERK kinase kinase 3 (MEKK3) relays TNF signaling to activate IKK complex. In the context of T cell immunity, response to APCs or anti-TCT-CD3 antibodies leads to a rapid translocation of protein kinase Cθ (PKCθ) to the plasma membrane (Fig3). Further components that connect PKCθ to the activation of IKK complex are yet to be identified but a trimolecular complex involving membrane-associated guanylate kinase homologue (MAGUK) and mucosal-associated lymphoid tissue (MALT)-lymphoma-associated proteins BCL-10 and MALT1 have been reported to play a role (Li and Verma, 2002).

In the inactive state p50:p65 dimers are held in the cytoplasm in association with the inhibitory IκB proteins which mask the nuclear localization signal. Activated IKK complex phosphorylates IκB proteins at the amino terminal serine residues and mark them for

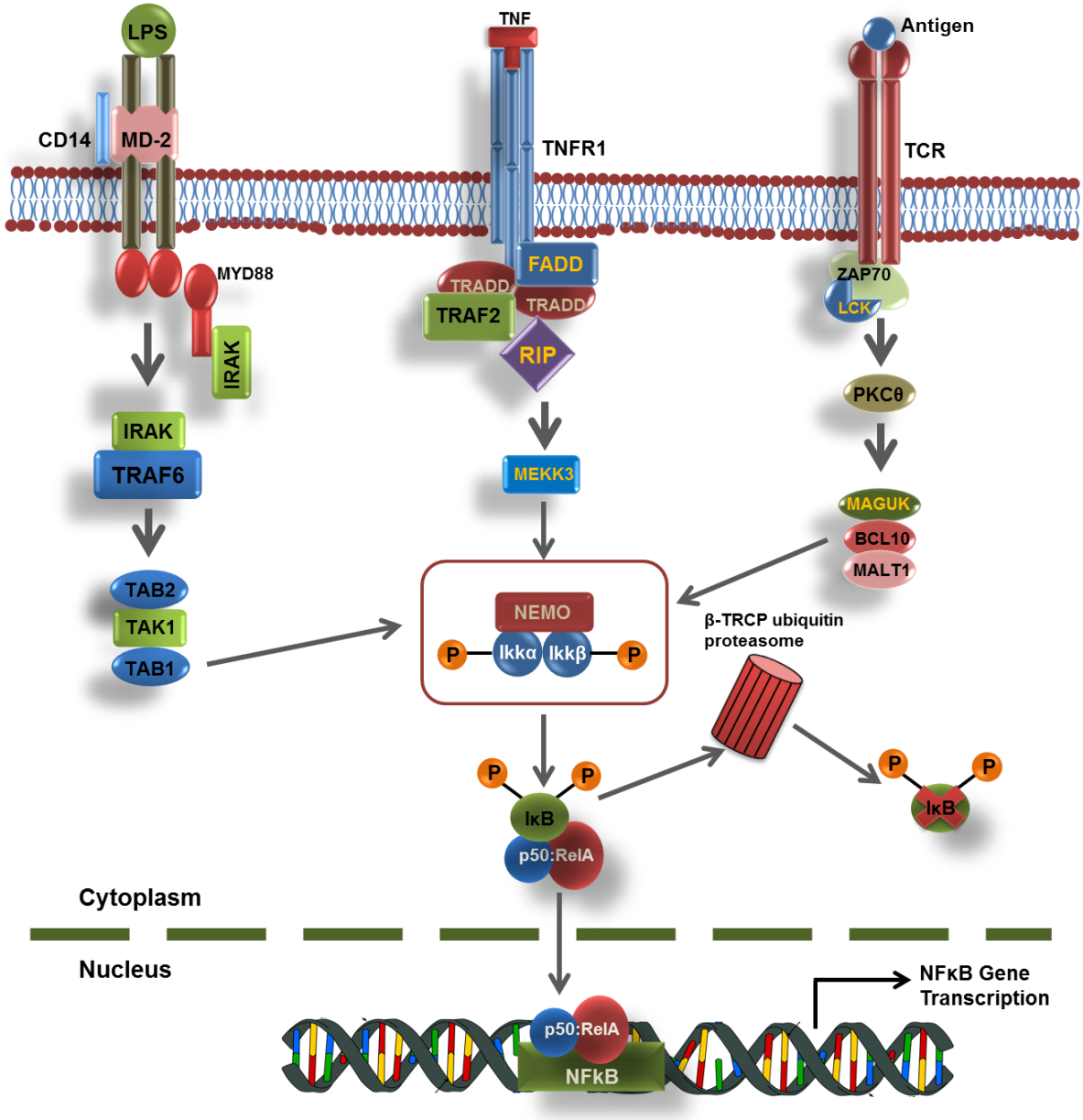

Figure 3: Activation of Canonical NF-κB signaling pathway: NF-κB is activated by multiple stimuli such as LPS, TNF or activation of T cell receptors. All of the various stimuli transduce the signals from the plasma membrane culminating in the activation of IKK complex, which is composed of two kinases Ikkα, Ikkβ and a regulatory subunit called Ikkγ (also called NEMO). Activation of IKK complex further phosphorylates inhibitory IκB proteins and target them for ubiquitin mediated proteasomal degradation and exposing the nuclear localization signals on NF-κB proteins aiding in their nuclear translocation, binding to DNA sequences and transcription of NF-κB associated proteins. TRADD, TNF receptor type 1-associated DEATH domain protein IRAK, IL-1-receptor-associated kinase; TRAF, TNF-receptor-associated factor; TAK, Transforming-growth-factor-β-Activated Kinase 1; TAB, TAK1-Binding Protein; PKCθ, protein kinase Cθ. Adapted from (Li and Verma, 2002).

ubiquitin mediated proteosomal degradation following which NF-κB dimers translocate to the nucleus to bind DNA and enable gene transcription.

3.2.3 Noncanonical NF-κB signaling pathway

Noncanonical NF-κB signaling is generally induced by the members of TNF cytokine family such as CD40 ligand, BAFF (B-cell-activating factor) and lympohotoxin-β (LTβ) (Bauer et al., 2012; Hayden and Ghosh, 2012; Sun, 2011). In contrast to the canonical pathway which is Ikkβ and NEMO dependent, noncanonical NF-κB is dependent on Ikkα. Another distinctive feature of noncanonical signaling compared to canonical is that it requires de novo protein synthesis (Dejardin et al., 2002) which delays its activation. Since noncanonical NF-κB signaling is involved in mediating long term effects, it is persistent and also found to be lacking a strong feedback mechanism. This long lasting NF-κB activity might be required owing to its involvement in cellular differentiation, survival and organogenesis (Hoffmann and Baltimore, 2006). Noncanonical NF-κB signaling is activated by BAFF in B cells and by LTβ in splenic stromal cells and lasts over a few hours to days (Claudio et al., 2002; Dejardin et al., 2002).

The existence of non-canonical NF-κB pathway was discovered while studying mechanisms involved in the processing of p100 (Xiao et al., 2001). p100 is a precursor molecule of p52 and acts as an IκB like molecule by inhibiting RelB translocation to the nucleus in non-canonical NFκB signaling (Solan et al., 2002). The initiation of non-canonical NF-κB signaling is a result of *de novo* synthesis of NFκB inducible kinase (NIK), its stabilization and accumulation following receptor cross linking at the cell membrane.Under inactivated conditions, de novo synthesized NIK is bound to TRAF3 (TNF receptor-associated factor 3) and recruited to TRAF-cIAP E3 ubiquitin ligase complex by the formation of TRAF3-TRAF2 dimers. cIAP1/2 catalyzes K48 ubiquitination of NIK leading to its degradation in the proteasome (Liao et al., 2004).

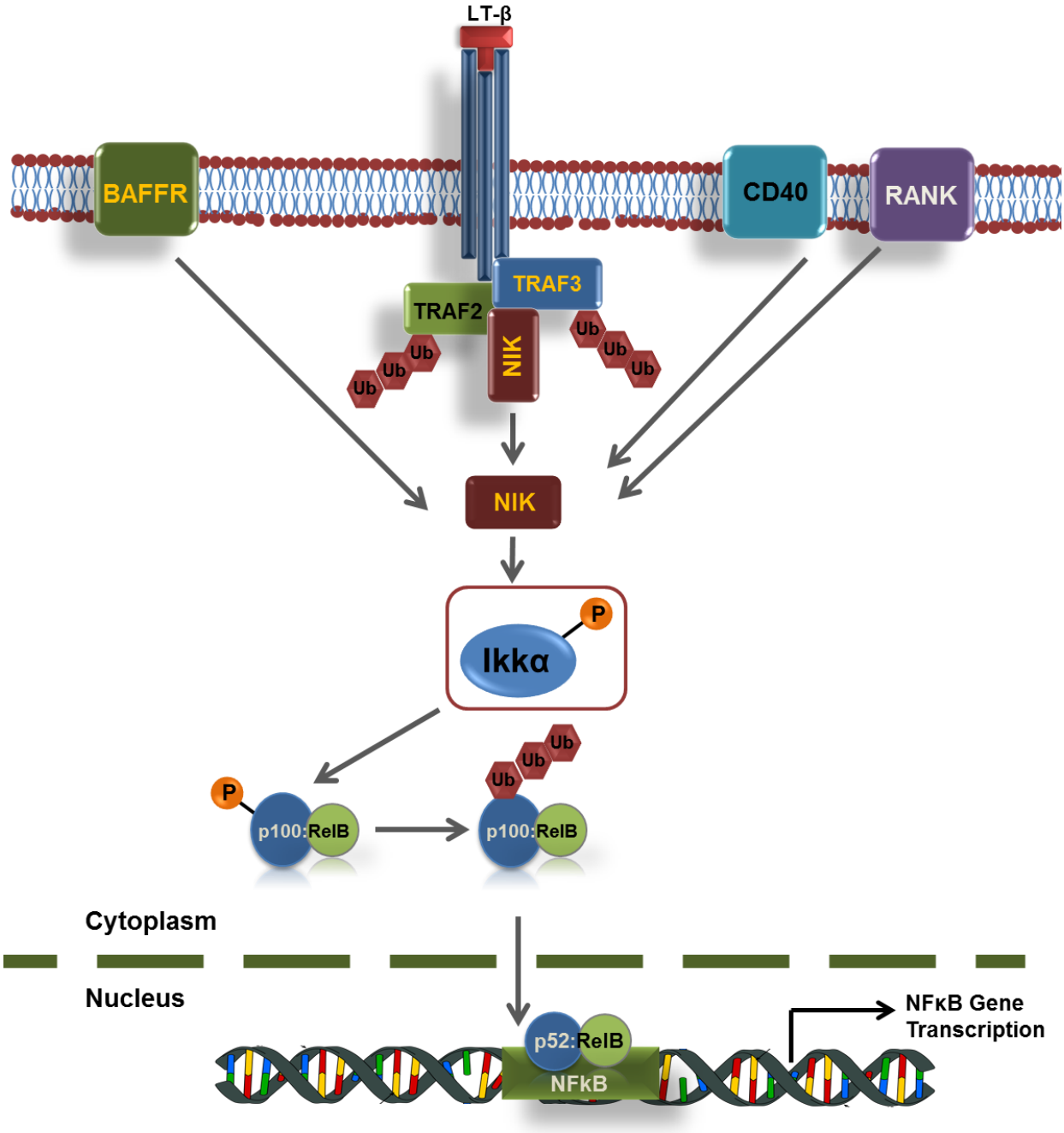

Figure 4: Non-canonical NF-κB signaling pathway: Non-canonical pathway is activated by a subset of TNF family members such as CD40L, BAFF and LTβ which leads to the dissociation of NIK and its stabilization involving TRAF3, TRAF2 and ubiquitin ligases. NIK phosphorylates and activate IKKα and further, IKKα phosphorylates p100 and resulting in its proteolytic cleavage and generation of transcriptionally active p52-RelB complexes. LTβ, Lymphotoxin- β, BAFFR, B-Cell Activating factor Receptor; RANK, Receptor Activator of NF-κB; TRAF, TNF-receptor-associated factor; NIK, NF-κB inducible kinase.

Thus, TRAF3-TRAF2-cIAP1/2 complex ensures a constant degradation of *de novo* synthesized NIK preventing non-canonical NF-κB activation.

Following activation by TNF cytokine family such as CD40 or LTβ (Coope et al., 2002; Derudder et al., 2003), NF-κB stimulatory receptors stimulates recruitment of TRAF-cIAP1/2 to the receptor complex resulting in cIAP1/2 mediated ubiquitination and degradation of TRAF3. Lack of TRAF3 leads to stabilization of NIK and facilitates its accumulation which in turn phosphorylates and processes p100, resulting in the formation of p52 and nuclear translocation of p52:RelB complex (Fig.3) (Sun, 2011).

3.2.4 Role of NF-κB signaling in mediating immune responses

NF-κB signaling is principally involved in the induction of immune responses such as induction of pro-inflammatory cytokines, chemokines, expression of adhesion molecules, matrix metalloproteinases, cyclooxygenase 2 and production of inducible nitric oxide synthase. Activation of NF-κB during inflammation is often marked by an increased expression of cytokines such as TNF, IL-1, IL-6 and IL-8 (Li and Verma, 2002). Involvement of NF-κB signaling in the induction of acute phase responses to pathogens has been well established. Defective NF-κB signaling in both humans and mice results in susceptibility to infections. Furthermore, transgenic mice lacking NF-κB family proteins are reported to be deficient in activation, proliferation of B and T cells as well as cytokine production. p65 deficient mice normally die during fetal development. Adoptive transfer of fetal liver stem cells derived from p50/p65, Ikkβ$^{-/-}$ or wild-type mice in to lethally irradiated mice showed a deficiency of fetal liver-derived lymphocytes, abnormal lymphoid organs indicating the role of NF-κB proteins in lymphopoiesis (Horwitz et al., 1997; Senftleben et al., 2001). NF-κB has been shown to be involved in the production of cytokines such as IL-18 and IFN-γ that are required for a functional T_H1 responses associated with cellular immunity (Kojima et al., 1999). Development of $CD8^+$ T cell responses which are

essential in controlling chronic and acute viral infections has been also shown to be dependent on p65 (Ouaaz et al., 1999). Collectively, these findings signify the importance of NF-κB signaling in the induction of immune responses to pathogens.

3.3 Innate Immune Signaling Pathways

There is a multitude of evolutionally conserved signaling pathways deployed by the host immune system to recognize pathogenic microbes and prevent them from causing their deleterious effects. In the late 90's Charles Janeway postulated the pattern recognition theory suggesting a general principle of innate immune recognition. Ever since, there has been tremendous progress into the research of how pathogen associated molecular patterns (PAMPs) are detected by the host immune system through various pattern recognition receptors (PRRs) present on the cell surface (Medzhitov, 2009). This initial recognition leads to the induction of various immune signaling cascades resulting in the expression of NF-κB induced inflammatory cytokine effectors and interferons which act by activating innate and adaptive immunity thereby controlling the viral replication and spread in the host.

3.3.1 TLR signaling pathway

The Toll gene was originally identified in Drosophila as a gene responsible for establishing dorsoventral polarity during embryogenesis (Hashimoto et al., 1988). Over a decade later, toll was also found to be associated with the fruit-fly's innate immunity in controlling fungal infections (Lemaitre et al., 1996). After a year, mammalian homologues of Drosophila Toll were detected and aptly named as Toll-like receptors (Medzhitov et al., 1997). Since then, at least 10 different TLRs were identified in humans and 12 in mice.

The TLR signaling pathway comprises a family of membrane spanning proteins composed of an ectodomain with leucine-rich repeats, a transmembrane domain and a

cytoplasmic domain called Toll/IL-1 receptor (TIR) domain. TLRs recognize and bind PAMPs through their leucine-rich ectodomain. Different TLRs are engaged in binding to a variety of PAMPs. Bacterial cell wall component lipopolysaccharide (LPS) serves as a ligand for TLR4 (Poltorak et al., 1998), TLR5 detects bacterial flagellin (Hayashi et al., 2001), TLR2 complexed with TLR1 or TLR6 recognizes lipoprotein and peptidoglycan (Aliprantis et al., 1999; Gilliet et al., 2008; Schwandner et al., 1999). Microbial nucleic acids are produced in a cell once they start to replicate and the extracellular TLRs cannot detect them. To enable their detection, TLRs such as TLR3, TLR7, TLR8 and TLR9 are located in the intracellular endosomal-lysosomal compartments (Alexopoulou et al., 2001; Heil et al., 2004; Hemmi et al., 2000). With the exception to TLR4 which localize both at plasma membrane and the endosomes, all the rest of the TLRs function at either the cell membrane or the endosomes intracellularly.

i.) TLR signaling at the plasma membrane - TLR2-TLR1/TLR6, TLR4, TLR5

Activation of a particular TLR by its ligand results in the recruitment of cellular adaptor molecules having TIR domains resulting in the induction of signal transduction complexes in the cytoplasm. During this signal transduction, four key adaptor protein molecules namely, MyD88 (Myeloid Differentiation primary-response gene 88), TIRAP (TIR domain containing adaptor protein), TRIF (TIRAP inducing IFNβ) and TRAM (TRIF-related adaptor molecule) bind to the TLRs directly in several combinations (Akira and Takeda, 2004; Kawai and Akira, 2006; O'Neill et al., 2013). Binding of these adaptor molecules to TLRs activates a multitude of signaling pathways involving NF-κB, IRFs (Interferon Regulatory Factors) and MAPKs (Mitogen Activated Protein Kinases) resulting in the transcription of innate immune players such as interferons, cytokines, chemokines and co-stimulatory molecules which would form the basis for the further development of an efficient adaptive immune response (Fig. 5).

ii.) Endosomal TLR signaling - TLR7/TLR8/TLR9-MyD88

TLRs 7, 8 and 9 have a high sequence homology and all of them function through the adapter protein MyD88. The endosomal TLRs are mainly involved in recognizing RNA and DNA of bacteria and viruses. TLR7 and TLR8 detects guanosine or uridine rich single stranded RNA (ssRNA) of viruses (Heil et al., 2004; Lund et al., 2004). TLR9 is involved in detecting ssDNA with unmethylated CpG containing motifs that are commonly found in the genomes of bacteria and viruses (Hemmi et al., 2000). Endosomal TLRs are activated after binding to their ligands and interact with MyD88 which contains a TIR domain and a death domain (Fig. 5). The adapter molecule MyD88 serves in recruiting IRAKs (IL-1 receptor associated kinase) to all the TLRs except TLR3. TLR4 uses both MyD88 and TIRAP as adaptors and signal through MyD88, TIRAP, TRIF and TRAM (Akira and Takeda, 2004; Gilliet et al., 2008).

iii.) Endosomal TLR signaling - TLR3-TRIF

Endosomal TLR3 is involved in recognizing viral dsRNA and transducing downstream signals by interacting with TRIF and activating IRF3, an important transcription factor required for the production of interferon-β. TLR3 is exclusive in its interaction with TRIF to transmit signals to the nucleus whereas TLR7, TLR8 and TLR9 signal via MyD88 .

3.3.2 RIG-I and MAVS signaling pathway

All nucleated cells are capable of mounting an interferon response even in the absence of endosomal TLRs and their adaptors during a viral infection or the presence of dsRNA in the cytosol. This observation led to the discovery of an alternative signaling system to TLRs. Three proteins, RIG-I (retinoic-acid-inducible gene), MDA5 (melanoma differentiation-associated antigen 5), LGP2 (laboratory of genetics and physiology 2) collectively known as RIG-I-like receptors (RLRs) have been identified in mice and humans (Stetson and Medzhitov, 2006).

Structurally, RLRs are DExD box RNA helicases. An N-terminal caspase-recruitment domain (CARD) is found in RIG-I and MDA5. RLRs recognize dsRNA poly(rI:C), poly(rA:rU) (Yoneyama et al., 2005). 5'-triphosphate RNA present in viral genomes or found as viral replication intermediates are detected by RIG-I (Hornung et al., 2006; Pichlmair et al., 2006).

RLRs signal by interacting with VISA (Virus-Induced Signaling Adapter - also known as MAVS, an adapter protein also known as VISA, CARDIF or IPS1) (Kawai et al., 2005; Meylan et al., 2005; Seth et al., 2005). This leads to the assembly of a signaling complex containing TRAF3 (TNF Receptor-Associated Factor 3), TBK1 (TANK-binding kinase 1) and IKBKE (inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon) which signal through IRF3 to induce the production of type-I interferons (Matsui et al., 2006; Yoneyama and Fujita, 2007).

3.3.3 Interferon signaling pathway and its antiviral effectors

During the late 50's, Isaacs and Lindenmann discovered that heat-inactivated influenza virus induces a soluble factor in cell culture that is capable of inhibiting replication of live influenza virus and they named this soluble factor as interferon (Isaacs and Lindenmann, 1957; Isaacs et al., 1957). Most of the innate immune signaling pathways detect pathogens and trigger the expression of Type I Interferons through the activation of NF-κB and IRFs. During viral infections, interferon signaling leads to the activation of a wide variety of interferon stimulated genes (ISGs) which induce an antiviral state limiting their replication and spread. Interferons modulate innate immune responses by promoting antigen presentation and activating NK cells which would also help in the initiation of the adaptive immune system leading to the development of antigen specific T and B cell responses and immunological memory. Interferons have been known to induce expression of over 2000 genes which interfere with various stages in the life cycle of

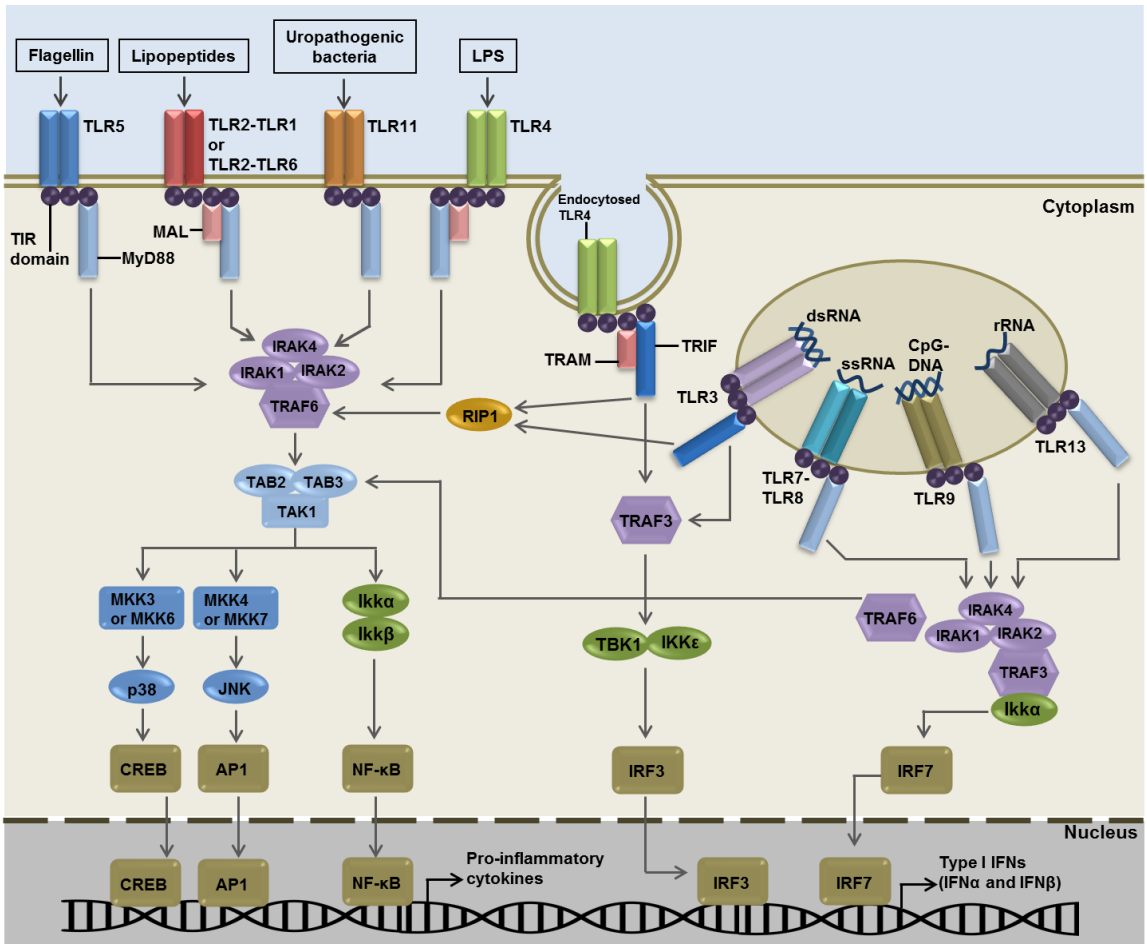

Figure 5: TLR signaling pathways. At the cell surface, TLR4, TLR5, TLR11 and heterodimers of TLR2-TLR1 or TLR2-TLR6 bind to various ligands. TLRs such as TLR3, TLR7-TLR8, TLR9 and TLR13 are localized to the endosomes so that they can sense microbial as well as host-derived nucleic acids. TLR4 is localized both at the plasma membrane as well as to the endosomes. Ligand-induced dimerization of receptors at the cell surface or endosomes initiates TLR signaling. Later, TIR domains of TLRs associate with TIR domain containing adaptor proteins MyD88, MAL, TRIF and TRAM. TLR4 switches its adaptor protein from MyD88 to TRIFF while switching localization from cytoplasm to endosome. Engagement of TLRs activate downstream signaling pathways involving interactions between IRAKs and TRAF that lead to the activation of MAPKs such as p38 and JNK leading to the activation of transcription factors. NF-κB, IRFs, CREB, AP1 are the important transcription factors activated by TLR signaling which mediate induction of pro-inflammatory cytokines in the case of plasma membrane TLRs and induction of type I interferons in the case of endosomal TLRs. TLR, toll like receptor; TIR, Toll-IL-1-resistance; MyD88, myeloid differentiation primary-response protein 88; MAL, MYD88-adaptor-like protein; TRIF, TIR domain-containing adaptor protein inducing IFNβ; TRAM, TRIF-related adaptor molecule; IRAK, IL-1R-associated kinases; TRAF, TNF receptor-associated factors; MAPK, mitogen-activated protein kinases; JNK, JUN N-terminal kinase; NF-κB, nuclear factor-κB; IRFs, interferon-regulatory factors; CREB, cyclic AMP-responsive element-binding protein; AP1, activator protein 1; IKK, inhibitor of NF-κB kinase; LPS, lipopolysaccharide; MKK, MAP kinase kinase; RIP1, receptor-interacting protein 1; TAB, TAK1-binding protein; TAK, TGFβ-activated kinase; TBK, TANK-binding kinase 1. Adapted from (O'Neill et al., 2013)

viruses leading to their inhibition and spread in the host. All interferons bind to heterodimeric receptors on the cell surface, one receptor having a low affinity and one receptor with a high affinity for binding.

i.) Type I IFNs and IFNARs

Type I IFNs are the largest amongst all interferon classes comprising IFNα and IFNβ. While IFNβ is expressed by almost all the cells following viral infection, IFNα is expressed exclusively by the cells of haematopoietic origin, such as macrophages and predominantly plasmacytoid dendritic cells (Barchet et al., 2002; Crouse et al., 2015; Reizis et al., 2011). A single IFNB gene encodes IFNβ whereas at least 14 distinct genes have been identified to encode various IFNα isoforms.

Sensing viral nucleic acids and other PAMPs by PRRs of the host results in the production of type I IFNs. Both IFNα and IFNβ bind to IFNα receptor (IFNAR) which comprises IFNAR1 and IFNAR2 subunits. IFNAR engagement activates receptor-associated protein tyrosine kinases, JAK1 (Janus Kinase 1) and TYK2 (Tyrosine Kinase2) which further phosphorylate the latent STAT1 (Signal Transducer and Activator of Transcription 1) and STAT2 (Signal Transducer and Activator of Transcription 2) transcription factors present in the cytoplasm. Tyrosine phosphorylated STAT1 and STAT2 form heterodimers and in turn recruits IRF9 (Interferon Regulatory Factor 9) to form the ISGF3 (Interferon Stimulated Gene Factor 3) complex which further translocate to the nucleus and binds to its cognate DNA sequences called IFN-stimulated response elements (ISRE) resulting in the expression of Interferon Stimulated Genes (ISGs) (Fig. 6), there by facilitating an antiviral state (Ivashkiv and Donlin, 2014).

ii.) Type II IFN and IFNGRs

Type II IFN comprises IFN-γ alone and is produced predominantly by the cells of immune system. However, all the cells expressing IFNGR1/2 proteins are capable of responding to IFN-γ.

IFN-γ signals by forming a homodimer and binding to IFN-γ receptor complex (IFNGR). Two IFNGR1 subunits interact resulting in the binding of two additional IFNGR2 subunits resulting in receptor activation and leads to the phosphorylation of JAK1 and JAK2 tyrosine kinases and transphosphorylation of these receptor chains results in the recruitment and phosphorylation of STAT1 leading to the formation of IFN-γ activation factor (GAF) which is a homodimer of phosphorylated STAT1. GAF translocates to the nucleus and binds to gamma-activated sequence (GAS) promoter elements resulting in the expression of ISGs (Fig. 6) (Schneider et al., 2014).

iii.) Type III IFN, IFNGRs and IL-10

Type III IFNs comprise of IFN-λ1, IFN-λ2, and IFN-λ3 also known as interleukin (IL)-29, IL-28A and IL-28B (Kotenko et al., 2003; Sheppard et al., 2003). This family of cytokines is structurally found to be similar to IL-10 and the members of IL-10 cytokine family and have been shown to utilize IL-10 receptor 2 like IL-10, IL-22 and IL-26.

Binding of type III IFNs to IL-10R2 (interleukin-10 receptor 2) and IFNLR1 (IFN-λ receptor 1) heterodimers results in the phosphorylation of JAK1 and Tyk2 leading to a signal transduction cascade similar to that of type-I IFNs (Fig. 6).

iv.) Interferon stimulated genes leading to control of viral replication

In order to complete a successful lifecycle, every virus needs to *enter* in to the host cell, *translate* its genome in order to *replicate* and finally *exit* from the host cell. Interferons

trigger transcription of hundreds of interferon stimulated genes which interfere with all these stages of the viral life cycle, leading to the control of viral spread.

Myxovirus resistance (Mx). Two different Mx proteins are known to be expressed in humans, Mx1 and Mx2, whereas there is only one gene product reported in mice, Mx1. Mx1 has been shown to inhibit viral genome replication by trapping viral components such as nucleocapsids so as to block them from reaching their cellular destination (Schneider et al., 2014).

Cholesterol-25-hydroxylase (CH25H): CH25H is an enzyme known to convert cholesterol into 25-hydroxycholesterol (25HC). Converted 25HC is capable of directly blocking virus fusion to the host cell membrane (Liu et al., 2013).

IFN-inducible transmembrane (IFITM) proteins. The IFITM family comprises four members, IFITM1, IFITM2, IFITM3 and IFITM4 in mice and their orthologous counterparts are also found in mice. IFITM proteins are known to prevent viral infection even before a virus traverses the lipid bilayer there by blocking its entry in to the cell. IFITM1, 2 and 3 proteins are reported to be early players in the replication of influenza A, flaviviruses such as dengue virus and West Nile Virus (Brass et al., 2009)

IFN-induced protein with tetratricopeptide repeat (IFIT) proteins. IFIT family proteins are induced by interferon following a viral infection. Four IFIT family members have been identified in humans namely IFIT1, IFIT2, IFIT3 and IFIT5 and three members in mice namely IFIT1, IFIT2 and IFIT3. Only cells of myeloid origin are known to express basal levels of IFIT proteins but their expression is rapidly enhanced in all cell types following a viral infection. IFIT proteins are reported to identify viral uncapped 5'-ppp RNA during infections with –ssRNA genome containing viruses such as LCMV (Lymphocytic Choriomeningitis Virus), VSV (vesicular stomatitis virus), RVFV (Rift Valley fever virus) and influenza virus. This recognition leads to the formation of a complex

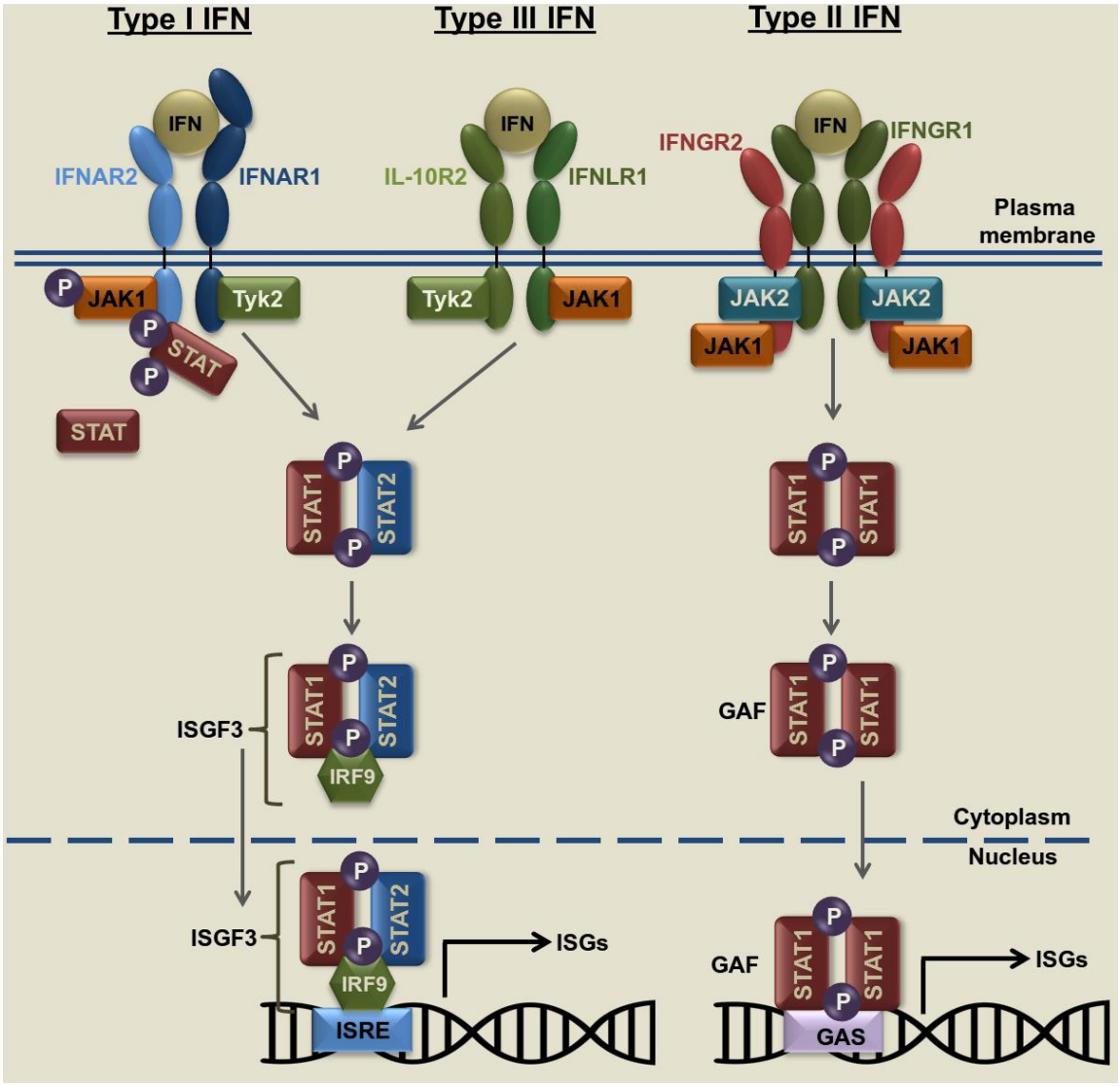

Figure 6: The interferon signaling cascade. Three distinct classes of interferons signal through specific receptor complexes at the plasma membrane. Type I IFNs act through IFANR1 and IFNAR2 heterodimer complexes, type III IFNs act through IL-10R2 and IFNLR1 heterodimers whereas type II IFNs signal through heterodimers of IFNGR1 and IFNGR2. Binding of type I and type III IFNs to their cognate receptors results in the phosphorylation of preassociated JAK1 and TyK2 which in turn phosphorylate the receptors at specific intracellular tyrosine residues. This results in the recruitment and phosphorylation of STAT1 and 2 which associate to form a heterodimer and in turn recruit IRF9 resulting in the formation of ISGF3. Binding of type II IFNs to their cognate receptors results in the phosphorylation of preassociated JAK1 and 2 following which, transphosphorylation of receptor chains leads to the recruitment and phosphorylation of STA1 homodimers which form GAF. ISGF3 and GAF translocate to the nucleus to transcriptionally induce genes regulated by ISRE and GAS promoter elements resulting in the expression of antiviral interferon stimulated genes. IFNAR, IFN-α receptor 1; IL-10R, interleukin-10 receptor 2; IFNLR1, IFN-λ receptor 1;IFNGR1, IFN-γ receptor 1;JAK, Janus kinase; TYK, tyrosine kinase; STAT, signal transducers and activators of transcription ; IRF9, IFN-regulatory factor 9; ISGF3, IFN-stimulated gene factor 3; GAF, IFN-γ activation factor; ISRE, IFN-stimulated response elements; GAS, gamma-activated sequence. Adapted from (Schneider et al., 2014).

involving IFIT2 and IFIT3 which helps in sequestering viral nucleic acids thereby inhibiting viral infection.

2′-5′-oligoadenylate synthetase (OAS)/RNase L pathway. The interferon-induced OAS/RNase L pathway was one of the first antiviral pathways discovered. A unique feature of this pathway is that they block viral infections by cleaving both viral and host cytosolic RNA indiscriminately (Abernathy and Glaunsinger, 2015; Chakrabarti et al., 2011). Cleavage of RNA further reinforces the innate immune response by the production of additional PAMPS. dsRNA replicative intermediates of ssRNA containing viruses such as members of Arenaviridae, Picornaviridae serves as a PAMP for OAS which results in the production of 5’ phosphorylated, 2’-5’ linked oligoadenylates known as 2-5A which are generally represented with the formula, ppp(A2’5’)nA (n≥2). 2-5A activates latent RNase L which cleaves single stranded RNA resulting in the inhibition of viral replication as well as cellular proliferation (Liang et al., 2006; Silverman, 2007). 2-5A has a very short life span and is degraded within minutes by 2’-phosphodiesterase and 5’-phosphatase activities (Kubota et al., 2004; Schmidt et al., 1979). Thus, 2-5A serves as an early alarmone to control viral replication.

Protein Kinase R (PKR) antiviral response pathway. PKR is a ubiquitously expressed serine-threonine kinase induced by interferon expression. Activation of PKR upon its encounter with dsRNA leads to autophosphorylation of PKR and further PKR induced phosphorylation of eIF2α (eukaryotic initiation factor 2) resulting in the blockade of translation and thereby inhibiting viral protein synthesis (Dauber and Wolff, 2009; Williams, 1999).

Even through it is established that the products of ISGs exert a variety of antiviral effector functions, the functional mechanism of all known ISGs is still unclear and is an area of intense research (Kane et al., 2016; Schoggins and Rice, 2011)

v.) Interferon mediated regulation of T cell responses

Interferons aid in T cell mediated immunity by inducing maturation of APCs which serve as a connecting cellular link between innate and adaptive immunity. Type I interferon signaling on APCs initiates processing and presentation of antigens, migration from the site of antigen detection to the sites of T cell activation as well as expression of co-stimulatory molecules and inflammatory cytokines on APCs (Crouse et al., 2015). T cells activated as a response to infection has to incorporate three signals so that they clonally expand; survive and later acquire effector and memory formation. While TCR engagement by peptide-MHC complexes serves as *signal-1*, ligation of co-stimulatory receptors serves as *signal-2*. Type-I interferons serve as *signal-3* in the activation of $CD8^+$ T cell responses. It has been shown *in vitro* that $CD8^+$ T cells receiving only signals 1 and 2 has a limited potency to proliferate and survive (Curtsinger et al., 2003b). Type I IFNs directly upregulate expression of transcription factors such as T-bet which are involved in effector T cell differentiation, and thus enabling sustained expression of CD25 on T cells. In cases of acute viral infections such as LCMV, Type I interferons directly inhibit regulatory T (T_{Reg}) cells to induce optimal antiviral T cell responses (Srivastava et al., 2014).

vi.) Cessation of interferon signaling

It has been observed *in vitro* that induction of transcriptional response by interferons begins as soon as 30 minutes to 2 hours and later cultured cells enter an IFN-desensitized state which could last for several days (Larner et al., 1986). This desensitized state is important for the cells to recover from interferon signaling and a dysregulation in this process results in autoimmune disorders such as Sjögren's syndrome, systemic lupus erythematosus (Ronnblom, 2011).

Cessation of interferon responses is equally important as its induction and is achieved

by multiple mechanisms. Protein inhibitors of activated STAT (PIAS) family of proteins attune STAT activity by inhibiting the binding of these transcription factors to DNA (Schneider et al., 2014). Certain protein tyrosine phosphatases negatively regulates JAK-STAT pathway by dephosphorylating activated STAT proteins (Irie-Sasaki et al., 2001; ten Hoeve et al., 2002). Suppressor of cytokine signaling (SOCS) proteins are induced early during the interferon responses and play a crucial role in inducing desensitization of interferon signaling by directly binding to phosphorylated tyrosine residues of IFN receptors or JAK proteins leading to inhibition of STAT binding and JAK activity. USP18 (also known as UBP43 or ISG15 isopeptidase), a member of ubiquitin-specific peptidases (USPs) is well known negative regulator of type I and type III interferon signaling. USP18 has two functional domains. While one domain is functionally associated with its isopeptidase activity, the other domain inhibits type I interferon signaling by blocking the type I interferon receptor 2 (IFNAR2) subunit (Burkart et al., 2013; Francois-Newton et al., 2011; Honke et al., 2016)

3.4 Arenaviridae and Lymphocytic Choreomeningitis Virus

LCMV is a prototypic member of the Arenaviridae family commonly infecting rodents worldwide. Based on their geographical distribution, Arenaviruses are classified into Old World and New World viruses. While rodents are typically affected by the members of Arenaviridae family, they are mostly benign. But a few arenaviruses such as LASV and LCMV occasionally infects humans causing severe viral hemorrhagic fever (VHF). LCMV is also known to be a fetal teratogen in cases of congenital infection leading to hydrocephalus, mental retardation and chorioretinitis in infants (Barton et al., 2002; de la Torre, 2009) Transmission of LCMV and cases of fatalities arising due to cerebral hemorrhage after organ transplantation have also been reported (Palacios et al., 2008).

In light of these compelling evidences and its distribution worldwide, LCMV is considered a neglected human pathogen of clinical significance (de la Torre, 2009).

The biology of LCMV enables it to be most suitable organism to study immunology. LCMV is non-cytolytic in its natural host, the mouse which allows a clear separation of effects caused by the virus from that of host immune system upon infection (Oldstone, 2002). Many of the important discoveries in immunology were made using LCMV such as elucidation of MHC (Major Histocompatibility Complex) restriction, persistent viral infections, T cell memory, T cell exhaustion (Zhou et al., 2012; Zinkernagel, 2002).

3.4.1 LCMV Genome organization and Life cycle.

All the members of Arenaviridae including LCMV are enveloped with a bisegmented negative single stranded RNA as their genome with a life cycle restricted to the cytoplasm (Meyer et al., 2002). A characteristic feature of LCMV, like other negative stranded RNA viruses is the lack of infectivity of isolated virion RNAs and the presence of RNA-dependent-RNA polymerase (RdRp). Purified virion preparations have been shown to contain electron-dense granules that are identified as host ribosomes. The term arena which translates to sandy is accounted for this granulated appearance.

LCMV is structurally quite simple with only four primary gene products encoded on two genomic RNA segments of unequal length; an L RNA segment of 7.2 kb and an S RNA segment of 3.5 kb. S RNA encodes the viral glycoprotein precursor, GP-C (75 kDa) which is post-translationally cleaved resulting in mature glycoproteins, GP-1 and GP-2 (Buchmeier et al., 1987) and nucleoprotein, NP (63kDa). The L RNA encodes RNA-dependent-RNA polymerase (200 kDa) and a small RING finger protein Z (11 kDa).

α-Dystroglycan (α-DG) is identified as the receptor for LCMV utilized to gain entry into the infected cells (Cao et al., 1998). α-Dystroglycan is non-covalently attached to β-

Dystroglycan which is a transmembrane protein associated with proteins of extracellular matrix such as laminin-1 and 2 (Henry and Campbell, 1998). α-DG is involved in interactions between cells and the extracellular matrix. Affinity of different LCMV strains in binding to α-DG determines viral tropism and the outcome of infection. Strains with a higher affinity to bind α-DG causes a persistent chronic infection and the strains with a lower affinity leads to an acute infection within the host (Smelt et al., 2001).

Following binding to α-Dystroglycan, LCMV virions are internalized by uncoated vesicles and are released into the cytoplasm by a pH-dependent membrane fusion step mediated by GP-2. This fusion between viral and cellular membranes releases the viral ribonucleoprotein (RNP) complex into the cytoplasm, which is followed by viral RNA synthesis. LCMV uses ambisense coding strategy to direct synthesis of two polypeptides in opposite orientation separated by a non-coding intergenic region (IGR) also which aids in transcription termination. NP and L coding regions are transcribed directly into genomic complementary mRNA while GPC and Z coding regions are not directly translated from genomic RNA but from genomic sense mRNAs which are transcribed from the corresponding antigenome RNA (agRNA) species as templates agRNA also serve as a replicative intermediate (Emonet et al., 2011). Assembly and release of infectious LCMV progeny occurs by budding at the surface of infected cells (Figs. 7 and 8).

3.4.2 Immune responses to Arenaviral infections

Both acute and chronic infections result in the early induction of interferon responses, marked by the activation of innate immune cells, induction of interferon responses and further expression of interferon stimulated genes and subsequent cell-mediated adaptive immunity. Infections with acute strains such as LCMV ARM have been shown to activate splenic dendritic cells (sDC) within 24h post-infection, marked by an elevated expression

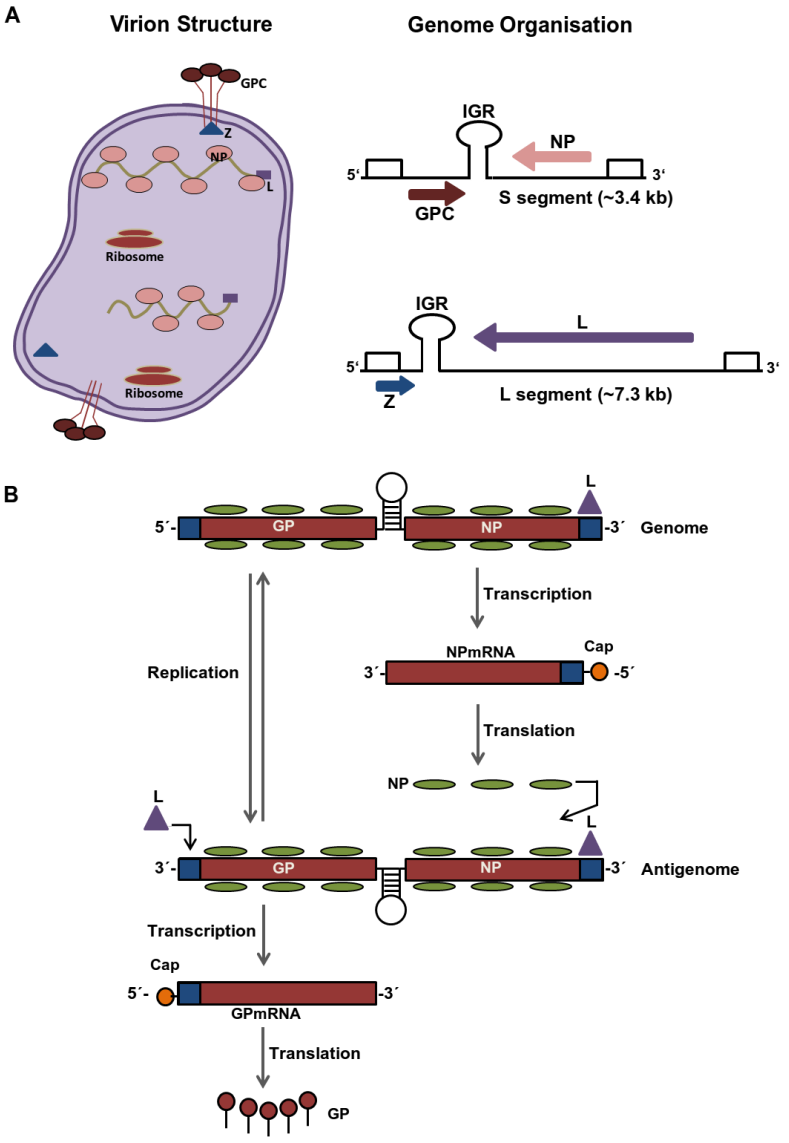

Figure 7: LCMV genome organization, RNA replication and gene expression: A. LCMV-WE is an enveloped virus with a bi-segmented –ssRNA genome. The S segment encodes viral nucleoprotein (NP) as well as the glycoprotein precursor (GPC) which is postranslationally processed to GP1 and GP2. The L segment encodes RNA dependent RNA polymerase (RdRp) and a small RING finger protein (Z). B. After the viral ribonucleoprotein (RNP) is released into the cytoplasm of an infected cell, RdRp associated with RNP initiated transcription from the genome promoter at the 3' end. This primary transcription leads to the synthesis of NP and L mRNA from the S and L segments. Successive to that, a full length copy of antigenome RNA (agRNA) is generated by the viral polymerase after it adopts a replicase mode and moves across the IGR. Successive to that, viral RdRp adopts a replicase mode and traverse the IGR generating full length copy of antigenome RNA (agRNA) which serve as a template for the synthesis if GP (agS) and Z (agL). agRNA also act as a template for the further amplification of corresponding genomic RNA. Adapted from (Emonet et al., 2011).

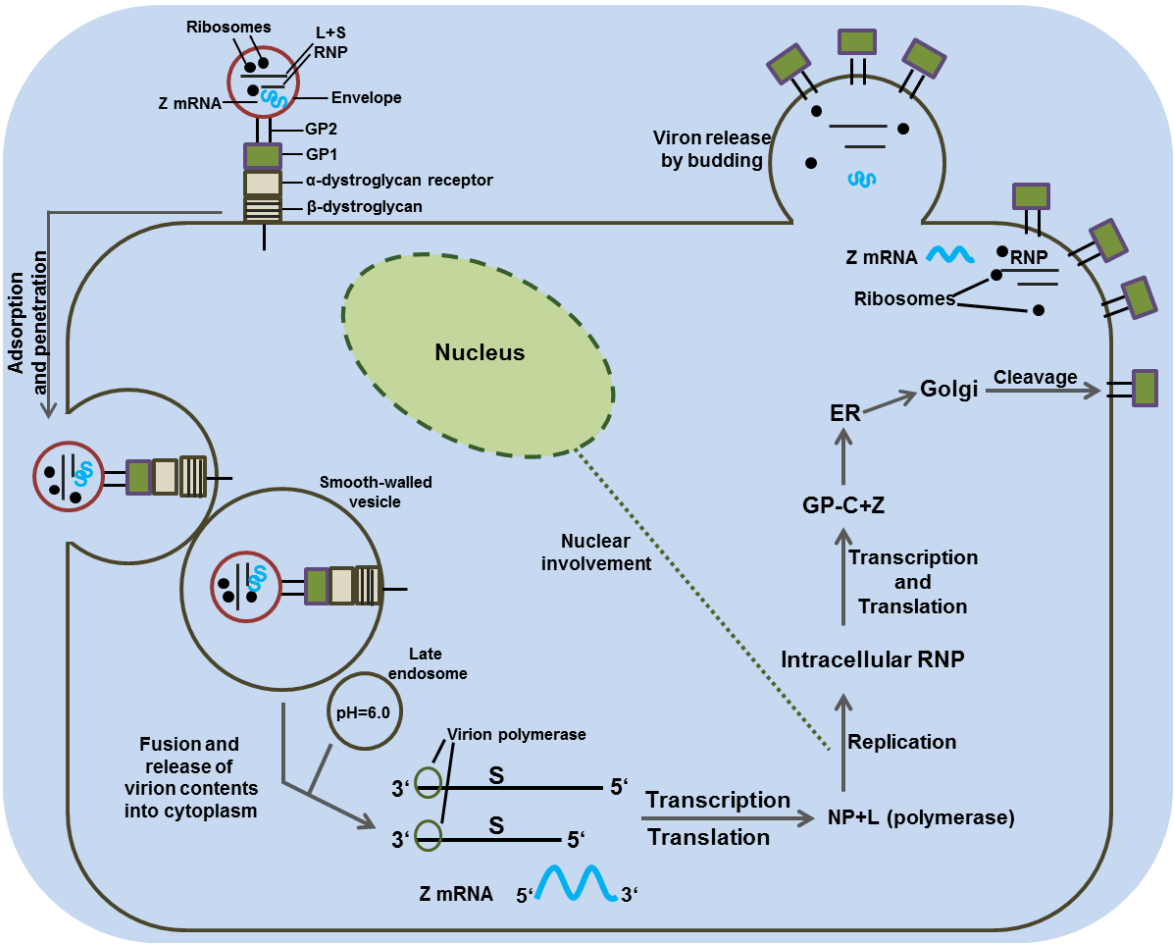

Figure 8: Lifecycle of LCMV-WE. LCMV virions bind to α-Dystroglycan on the cell membrane and internalized into the cytoplasm. LCMV-WE genomic organization, patterns of transcription, translation and replication and further budding from the cell membrane are depicted. Adapted from (Meyer et al., 2002).

of co-stimulatory molecules such as CD40, CD80 and CD86, as well as increased surface expression of MHC class I and II molecules and proinflammatory cytokines such as IL-12 and TNFα. These activated sDCs are capable of stimulating naive LCMV-specific CD8+ T cells *ex vivo* (Montoya et al., 2005). Mice lacking sDCs are defective in mounting an appropriate CTL response to LCMV despite the presence of LCMV infected macrophages and B cells in the spleen (Probst and van den Broek, 2005). Acute infections with LCMV WE and ARM results in the induction of interferon responses leading to interferon stimulated gene expression and antiviral effectors followed by a

vigorous cytotoxic $CD8^+$ T cell mediated lysis of infected cells as early as 4 days post infection leading to viral clearance within 10-14 days.

In the case of chronic infections, despite a similar course of activation early during infection, marked by activation of DCs and myeloid cell population, the population of $Ly6C^{hi}$ monocytes and $Gr\text{-}1^{hi}$ neutrophilic cells resembling myeloid-derived suppressor cells are increased in lymphoid organs and blood at a later phase- seven days post infection, resulting in a sustained chronic LCMV C13 infection (Norris et al., 2013). In addition, chronic infections marked by persistent viral replication, lead to negative effects of interferon signaling, resulting in tissue destruction, immunosuppression and chronic inflammation. Infections with chronic strains such as LCMV C13 induces a delayed $CD8^+$ T cell responses seen after 7 days post-infection which are characterized by a reduced effector function (Zajac et al., 1998) and an eventual functional exhaustion resulting in higher viremia that lasts for several months (Wherry et al., 2003). During such chronic viral infections, blockade of interferon signaling lead to a hindered immune activation and immune suppression followed by a restored lymphoid architecture. This enhanced control of viral replication was CD4 T cell-dependent and coincided with increased secretion of IFN-γ (Teijaro et al., 2013; Wilson et al., 2013). In another study involving chronic infections with HIV and LCMV, it was reported that elevated concentration of TNF leads to dysfunctional $CD4^+$ T cell response and blockade of TNFR signaling in $CD4^+$ T cells reversed T cell dysfunction and restored virus-specific immunity leading to virus control (Beyer et al., 2016).

While the involvement of innate immune cells of the spleen in the induction of immune responses has been well-established, the contribution of liver specific innate immunity to viral infections is mainly attributed to Kupffer cells and it has been argued that in the absence of macrophages, LCMV disseminates to hepatocytes, resulting in enhanced

viral replication (Lang et al., 2010). Liver endothelial cells were also shown to be involved in antiviral innate immune response by secreting cytokines and have the ability to respond to IFN-I enabling suppression of viral replication (Khaiboullina et al., 2005; Krug et al., 2004).

The liver is an organ not only charged with dynamic metabolic activities but is also a site for generation of acute phase immune responses. Hepatocytes, the parenchymal cells of the liver, are cells with potent innate immune functions, in addition to their role in performing metabolic functions of the liver (Crispe, 2016; Volpes et al., 1992; Zhou et al., 2016). Using mouse models that lack innate immune signaling specifically in the hepatocytes would enable a better understanding of the role played by hepatocytes following systemic viral infections which is the prime focus of this study.

4. MATERIALS AND METHODS

4.1 Materials

4.1.1 Chemical Reagents

Chemical	Manufacturer
Agarose	Peqlab
Alexa Flour 488	Life Technologies
Ammonium Chloride (NH_4Cl)	Sigma, Munich
BD Cytofix/Cytoperm™ Solution	BD Bioscience, San Jose, USA
BD Perm/Wash™ Buffer	BD Bioscience, San Jose, USA
Bovine serum Albumin	Sigma, Munich
Brefeldin A (Golgi stop)	Sigma, Munich
$CaCl_2$	Sigma, Munich
Collagen Type I from rat tail (Cat. C3867)	Sigma, Munich
Collagenase A	Roche, Switzerland
Collagenase D	Roche, Switzerland
DMEM	Life Technologies
EGTA	Roche, Switzerland
Fetal Calf Serum	Life Technologies
Flow cytometry buffer	1x PBS, 20mM EDTA, 2% FBS
Fluoromount-G® Mounting Media (+/-Dapi)	Southernbiotech
Gentamycin	Delta Select
Glutamin	Gibco
HBSS	Life Technologies
Heparin	Ratiopharm

Hepes	Gibco
HRP-Substrate	Thermo Scientific
Hydrocortison	Rotexmedica
Insulin	Novo Nordisk
Isopropanol	Roth
Methanol	Roth
Methyl cellulose	Sigma, Munich
Non-Essential Amino Acids 100x	Gibco
PBS	Life Technologies
Penicillin/Streptomycin	Biochrom AG
Pen-Strep	Biochrom KO282
Ponceau S	Sigma
Sucrose	Roth
Tissue-Tek O.C.T.	Sakura
Triton X-100	Roth
Williams medium E	Gibco

4.1.2 Buffer Recipes

Buffer	Composition
Western blotting buffers	
1x PBS	135mM NaCl, 2.5 mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4
1x SDS buffer	62.5 mM, 50 mM DTT, 2% (w/v) SDS, 10% (v/v) gycerol 0.01% (w/v) bromphenol blue
1x TBST washing buffer	0.1M Tris-Cl (pH 8.0), 1.5M NaCl, 0.5% Tween-20
Acrylamide solution	40% (w/v) acrylamide / Bis 19:1 (Ambion)
Blocking buffer	TBST + 5% BSA

Electrophoresis buffer	25 mM Tris-HCl, 250 mM Glycin, 0.1% (w/v) SDS pH 8.3
Ponceau S stain	0.1% PONCEAU-S (w/v) in 5% acetic acid solution
Stacking gel	125 mM Tris-HCl pH 6.8, 0.1% SDS, 0.1% APS 0.1% TEMED
Separating gel	375 mM Tris-HCl pH 8.8, 0.1% SDS, 0.1% APS 0.04-0.06% TEMED
Transfer buffer	25 mM Tris-HCl, 190 mM Glycin, 0.01% (w/v) SDS, 20% ethanol
RIPA buffer	50 mM TRIS-HCL (pH 7,4), 150 mM NaCl, 0.25% DOC 1 mM EDTA, 1% NP-40 (Igepal)
Tissue lysis buffer for protein extraction	RIPA buffer plus Roche complete protease inhibitor cocktail
RNA isolation buffers	
Tissue lysis buffer for RNA extraction	RLT buffer + 1% β-mercaptoethanol
FACS buffers	
TAC buffer	90% v/v NH_4Cl (0.16M stock) 10% v/v Tris pH 7.65 (0.17M stock)
Trypan blue	0.2% (w/v) Trypan blue in 1x PBS
FACS buffer pH7.4 (1x)	0.14M NaCl, 2.7mM KCl, 3.2mM Na_2HPO_4, 1.5mMKH_2PO_4
Propidium iodide (PI)	10 mg/ml in water (stock), 1 µg/ml (working conc.)
Ethidium Monoazide Bromide (EMA)	2mg/ml in DMF (stock), 1 µg/ml (working conc.)
LCMV Plaque assay buffers	
OPD substrate solution	Stock A: 0.2 M Na_2HPO_4 x $2H_2O$ (35.6 mg/ml)

	Stock B: 0.1 M citric acid (19.2 mg/ml)
Working buffer (50ml)	OPD: 20 mg/table (Sigma P3888) Hydrogenperoxide (H_2O_2), 30%
	Stock A 12.5 ml Stock B 12.5 ml dd$H_2$0 25 ml OPD 20mg H_2O_2 50 µl
Hepatocyte isolation buffers	
EGTA	0.1M in d$H_2$0, pH 7.4 (with NaOH), sterile filtered or autoclaved
Heparin	5000U/ml with sterile PBS
$CaCl_2$	1M in d$H_2$0, sterile filtered or autoclaved
Collagenase A	60 mg ColA in 6 ml HBSS - aliquoted in 1.5 ml quantities working stock - 1.5 ml of above solution in 50 ml Buffer B
Collagenase D	18 mg ColD in 6 ml HBSS - aliquoted in 1.5 ml quantities working stock - 1.5 ml of above solution in 50 ml Buffer B
Hydrocortison	100 mg in 20.6 ml HBSS with 10mM Hepes pH 7.4 dissolved, filtered with 0.45 um filters and aliquoted in 0.6 ml quantities.
Insulin	320 µl = 0.45 mg Actrapid (aliquoted)
Inosin	2.5mg/ml in PBS, 0.2µm filtered and stored as 20ml aliquotes
5% Glucose	5g/100 ml in d$H_2$0, 0.2µm filtered
Gentamycin	Gencin 80mg Delta Select N3; 10 ampules of 2 ml pool the ampules and store as 1.25 ml (50mg/ml) aliquots.

4.1.3 Cell culture medium

DMEM complete medium	
DMEM	500 ml
FCS (10%)	50 ml

Pen/Strep (5000 I.U/ml)	5 ml
L-Glutamine	5ml
Non Essential Aminoacids (100x)	5ml
RPMI 1640 medium	
RPMI 1640 medium	500 ml
FCS (10%)	50 ml
Pen/Strep (5000 I.U/ml)	5 ml
Non Essential Aminoacids (100x)	5ml
Hepatocyte culture medium	
Perfusion medium: Buffer A	
HBSS (calcium & Magnesium free)	500 ml
EGTA (100mM)	2.5 ml
Heparin (5000U/0.2 ml)	1 ampule
Collagenase medium: Buffer B	
Williams medium E	250 ml
Calcium Chloride ($CaCl_2$) - 2,5 M	0.36 ml
Gentamycin (50mg/ml)	0.5 ml
Collagenase type IV (280U/mg)	200 mg
Wash medium:	
Williams medium E	500 ml
Glutamine (200mM)	5.6 ml
Glucose (5%)	6 ml
Hepes pH 7,4 (1M)	11.5 ml
Pen/Strep (10000 I.U/ml)	2.8 ml
Maintainance medium: Buffer C	
Williams medium E	500 ml

Glutamine (200mM)	5.6 ml
Glucose (5%)	6 ml
Hepes pH 7,4 (1M)	11.5 ml
Pen/Strep (10000 I.U/ml)	2.8 ml
Gentamycin (50mg/ml)	1 ml
Hydrocortison	0.5 ml
Insulin	0.32 ml
DMSO (100%)	8.7 ml
Inosine (25mg/ml)	0.28 ml

4.1.4 Antibodies

Antibody	Source	Dilution	Clone	Manufacturer
Immunohistochemistry				
B220/CD45R	mouse	1:3000	RA36B2	BD Biosciences, USA
MHC-II	rat	1:500	monoclonal	Novus Biologicals, USA
F4/80	rat	1:120	BM8	BMA Biomedicals, Switzerland
CD8-PE	Rat	1:100	53-6.7	eBioscience
NF-κB p65 (RelA)	rabbit	1:500	polyclonal	Lab vision, Neomarkers, USA
LCMV-NP				Prof.Karl Lang, Essen.
Ki67	rabbit	1:200	SP6	Lab vision, Neomarkers, USA
CD3	rabbit	1:300	SP7	Lab vision, Neomarkers, USA
Ly6G	rat	1:600	monoclonal(1A8)	BD Pharminogen, USA
NKp46	goat	1:60	polyclonal	R&D systems, USA
TNFα	rabbit	1:300	polyclonal	abcam, USA
pSTAT1	rabbit	1:100	monoclonal	Cell signaling, USA
Immunoblotting				

pSTAT1	rabbit	1:1000	monoclonal	Cell signaling, USA
pSTAT3	rabbit	1:1000	D387	Cell signaling, USA
STAT1	rabbit	1:1000	polyclonal	Cell signaling, USA
pJAK	rabbit	1:1000	polyclonal	Cell signaling, USA
JAK	rabbit	1:1000	polyclonal	Cell signaling, USA
pTyk	rabbit	1:1000	polyclonal	Cell signaling, USA
Tyk	rabbit	1:1000	polyclonal	Cell signaling, USA
GAPDH	rabbit	1:1000	14C10	Cell signaling, USA
β-actin	rabbit	1:10000	13E5	Cell signaling, USA
FACS				
CD8α-PE	Rat	1:200	53-6.7	eBioscience, Germany
IFN-γ-APC	Rat IgG1	1:200	XMG1.2	eBioscience, Germany

4.1.5 Primers

Primer	Sequence
OAS1 Forward	TGGGTGGCTGCTACAATGAAT
OAS1 Reverse	CACTACCTGTCCGTTGAACTG
OAS2 Forward	TTGAAGAGGAATACATGCGGAAG
OAS2 Reverse	GGGTCTGCATTACTGGCACTT
OAS3 Forward	TCTGGGGTCGCTAAACATCAC
OAS3 Reverse	GATGACGAGTTCGACATCGGT
STAT1 Forward	TCACAGTGGTTCGAGCTTCAG
STAT1 Reverse	GCAAACGAGACATCATAGGCA
STAT2 Forward	TCCTGCCAATGGACGTTCG
STAT2 Reverse	GTCCCACTGGTTCAGTTGGT
STAT3 Forward	AGCTGGACACACGCTACCT
STAT3 Reverse	AGGAATCGGCTATATTGCTGGT

IRF7 Forward	AGCTGGACACACGCTACCT
IRF7 Reverse	AGGAATCGGCTATATTGCTGGT
IL-6 Forward	CCACACAGACAGCCACTCAC
IL-6 Reverse	AGGTTGTTTTCTGCCAGTGC
IFITM1 Forward	TCCCTGTTCAACACCCTCTTCT
IFITM1 Reverse	GTCACGTCGCCAACCATCTT
Mx1 Forward	CAGCACCTGATGGCCTATCA
Mx 1 Reverse	ACGTCTGGAGCATGAAGAACTG
Mx2 Forward	CCAGTTCCTCTCAGTCCCAAGATT
Mx2 Reverse	TACTGGATGATCAAGGGAACGTGG
IRF3 Forward	GAGAGCCGAACGAGGTTCAG
IRF3 Reverse	CTTCCAGGTTGACACGTCCG
Albumin Forward	TGCTTTTTCCAGGGGTGTGTT
Albumin Reverse	TTACTTCCTGCACTAATTTGGCA
IFNAR1 Forward	GGTCATTACTGTCACCGCCA
IFNAR1 Reverse	ACACAGTACACAGTCAGCGG
IFNAR2 Forward	GAGAACAAGTCTGGCCCACC
IFNAR2 Reverse	ACGATGTAGTCTGAGCAGCG
IFNAGR1 Forward	AACTCCGACACAAAGACGCA
IFNAGR1 Reverse	AGTGATAGGCGGTGAGGCTA
IFNAGR2 Forward	CACCTTCCAGCAATGACCCA
IFNAGR2 Reverse	GATGTCCGTACAGTTCGGCT
IFNα Forward	GACCTGCAAGGCTGTCTGAT
IFNα Reverse	AGACAGGGCTCTCCAGACTT
IFN-α9 Forward	CCTGATGGTCTTGGTGGTGATAA
IFN-α9 Reverse	CAGTTCCTTCATCCCGACCAG
IFNβ Forward	CAGCTCCAAGAAAGGACGAAC

IFNβ Reverse	GGCAGTGTAACTCTTCTGCAT
IFNγ Forward	ACACTGCATCTTGGCTTTGC
IFNγ Reverse	GCTTTCAATGACTGTGCCGT
IL-10 Forward	TTCCTTCTGGTGCCAGCTCTA
IL-10 Reverse	AAGCAGGTACCTCCCACTGT
IRF1 Forward	CCCACAGAAGAGCATAGCAC
IRF1 Reverse	AGCAGTTCTTTGGGAATAGG
IRF7 Forward	GTCTCGGCTTGTGCTTGTCT
IRF7 Reverse	CCAGGTCCATGAGGAAGTGT
Mx1 Forward	GGCAGACACCACATACAACC
Mx1 Reverse	CCTCAGGCTAGATGGCAAG
STAT1 Forward	CAGATATTATTCGCAACTACAA
STAT1 Reverse	TGGGGTACAGATACTTCAGG
IL-1β Forward	ACATCAGCACCTCACAAGCA
IL-1β Reverse	TTAGAAACAGTCCAGCCCATA
TNFα Forward	GTCAGGTTGCCTCTGTCTCA
TNFα Reverse	TCAGGGAAGAGTCTGGAAAG
LCMV-NP Forward	CAGAAATGTTGATGCTGGACTGC
LCMV NP Reverse	CAGACCTTGGCTTGCTTTACACAG
LCMV-GP Forward	CATTCACCTGGACTTTGTCAGACTC
LCMV GP Reverse	GCAACTGCTGTGTTCCCGAAAC
CXCL9 Forward	TTTGGGGTTCTACAGTGGAG
CXCL9 Reverse	GAAGATGGGATCAAGTTAATA
CXCL10 Forward	ATTCTTTAAGGGCTGGTCTGA
CXCL10 Reverse	CACCTCCACATAGCTTACAGT
CXCL11 Forward	ACACTCCACGCTACCTTCTG
CXCL11 Reverse	TGTGCCTCGTGATATTTGG

IFIT1 Forward	CTCCACTTTCAGAGCCTTCG
IFIT1 Reverse	TGCTGAGATGGACTGTGAGG
IFIT2 Forward	AAATGTCATGGGTACTGGAGTT
IFIT2 Reverse	ATGGCAATTATCAAGTTTGTGG
ISG15 Forward	GAGCTAGAGCCTGCAGCAAT
ISG15 Reverse	CTTCTGGGCAATCTGCTTCT

4.1.6 Commercial kits

Kit	Manufacturer
BCA protein Assay	Thermo Scientific, Germany
BD Cytofix/cytoperm	BD Pharminogen, Hamburg, Germany
BondMax	Leica Novocastra
Immun-Star™WesternC™Chemiluminescent Kit	BioRad
PeqGold HP Kit	VWR International
QuantiTec Reverse Transcription Kit	Qiagen, Hilden, Germany
RNAscope 2.0 brown FFPE Assay kit	Advanced cell diagnostic

4.1.7 Consumables

Product	Company
384 well qPCR plates colourless, ABI type	Biozym Scientific GmbH, Oldendorf, Germany
15mL; 50 mL	
96-,24-,12-,6-well plates polystyrene cell culture	Corning Life Sciences, Tewksbury, USA
Adhesive Microplate Seal, 135 x 80 mm, Sheets	Biozym Scientific GmbH, Oldendorf, Germany
Cell-Star Serological Pipets 5mL, 10mL, 25mL	Greiner Bio-one, Frickenhausen, Germany
Conical Sterile Polypropylene Centrifuge Tubes	Thermo Scientific, Ulm, Germany
Cryotubes 1.8mL	Thermo Scientific, Ulm, Germany

Disposal Bags, autoclavable	Roth, Karlsruhe, Germany
Drigalski spatula, polypropylene	A. Hartenstein, Marburg, Germany
GentleMACS M-tubes	Miltenyi, Bergisch-Gladbach , Germany
Kimtech Science Professional tissues	Kimberly Clarke, Reigate Surrey, UK
Nitrex Nextgen examination gloves	Rösner-Mautby Meditrade, Germany
NuPAGE® Novex® Bis-Tris Protein Gels	Life Technologies (Invitrogen), Germany
Omnican single use fine dosage syringe	Braun-Melsungen, Melsungen, Germany
Omnifix syringes 5 mL /10 mL	Braun-Melsungen, Melsungen, Germany
PCR tube stripes and lid stripes	Biozym Scientific GmbH, Oldendorf, Germany
Pipette filter tips (10µl; 20µl; 200µl; 1000µl)	VWR International GmbH, Erlangen, Germany
Pipette tips (20-200µl; 1000µl)	VWR International GmbH, Erlangen, Germany
Preparation Sets (scissors, foreceps…)	Omnilab, München, Germany
Reaction tubes (0.5 mL; 1.5mL; 2mL)	Eppendorf, Hamburg, Germany
Rotilab Syringefilters 0.45 µm, 0.22µm	Roth, Karlsruhe, Germany
Superfrost Plus Microscope Slides	Thermo Scientific, Ulm, Germany
Surgical blades	Schreiber, Fridingen, Germany
Surgical disposable scalpels	Braun-Melsungen, Melsungen, Germany
Tissue culture flasks with filter lid (T15;T75)	Greiner Bio-one, Frickenhausen, Germany

4.1.8 Equipment

Equipment	Manufacturer
-20°C fridge	Liebherr, Switzerland
4°C fridge	Bauknecht, Stuttgart, Germany
7900HT Fast Real Time PCR System	Applied Biosystems, Darmstadt, Germany

-80°C fridge Herafreeze	Heraeus, Hanau, Germany
AG Protect Cryostat	Leica Microsystems, Wetzlar, Germany
Automatic Ice Machine AF10	Scotsman, USA
Biofuge Fresco Microcentrifuge	Heraeus, Hanau, Germany
BondMaxII	Leica Microsystems, Wetzlar, Germany
gentleMACS™ Dissociator	Miltenyi, Bergisch Gladbach, Germany
Hybaid Shake'n Stack Hybridization oven	Thermo Scientific, Ulm, Germany
HybEZ oven	Advanced cell diagnostic, Hayward CA, USA
Hyrax M25 Microtome	Zeiss, Jena, Germany
IKAMAG RCT magnetic stirrer	IKA Werke, Staufen, Germany
Megafuge 1.0	Heraeus, Hanau, Germany
Micro centrifuge MiniStar Silverline	VWR, Darmstadt, Germany
Microscope Axiovert 25	Zeiss, Jena, Germany
Microscope BX53	Olympus, Hamburg, Germany
Milli-Q® A10 Ultrapure Water Purification System	Merck Millipore, Darmstadt , Germany
Molecular Imager® ChemiDoc™ XRS	Bio Rad, München, Germany
Nanodrop 2000 Spectrophotometer	Peqlab, Erlangen, Germany
Novex® NuPAGE® SDS-PAGE Gel System	Life Technologies, Frankfurt, Germany
PCR-cycler	Biometra, Göttingen, Germany
PipetBoy	Integra Bioscience, Zizers, Switzerland
Pipettes 10µl; 20µl; 200µl; 1000µl	Eppendorf, Hamburg, Germany
Power 300 Power Supply	Peqlab, Erlangen, Germany
SCN400 slide scanncer	Leica Microsystems, Wetzlar, Germany
Subcell Agarose Electrophoresis System	Bio Rad, München, Germany
Tetra Blotting Module	Bio Rad, München, Germany

Thermomixer comfort	Eppendorf, Hamburg, Germany
Tissue drying oven TDO66	Medite, Burgdorf, Germany
TransBlot Turbo	Bio Rad, München, Germany
UNITWIST RT shaker	UniEquip, Planegg, Germany
Varioklav autoclave	HP-Medizintechnik, Oberschleißheim, Germany
Vortexer Reax 200	Heidolpf, Kelheim, Germany

4.1.9 Software

Software	Providing Company
Adobe Photoshop CS5	Adobe
Adobe Illustrator	Adobe
BD FACS Diva™	BD Biosciences
Digital Image Hub (DIH)	Leica Biosystems
DIH-TissueIA	Leica Biosystems
Endnote	Microsoft Corporation
GraphPad Prism	GraphPad Software Inc.
Image Lab™	BioRad
LabImage 1D	INTAS
Magellan™ - Data Analysis Software	Tecan
Microsoft Office	Microsoft Corporation
NanoDrop2000	Thermo Scientific
PrimerBlast	Open Source (NCBI)
Quantity One™	BioRad
SCN400 viewer	Leica Biosystems
SDS 2.4 ™	Applied Biosystems
Tree Star	FlowJo, LLC
Vector NTI	invitrogen

4.2 Methods

4.2.1 Experimental mice and viral infections

Mice used for the experiments were maintained in single ventilated cages and under specific pathogen free conditions. All the mice used were on the C57BL/6 genetic background. LCMV-WE was originally obtained from Pal Johansen and Rolf Zinkernagel (University hospital, Zurich, Switzerland) and propagated by infection on L929 fibroblast cells. Infection experiments were performed in Zurich under the licenses ZH 200/2006 and ZH 69/2012 as approved from the cantonal veterinary office of Zürich. Infection doses were given as indicated under each experiment. At least 3 mice were used in each experimental group.

4.2.2 Measurement of viral titers through plaque forming unit assay

Preparation of 48-well plate with murine fibroblast MC57 cells (H-2b): MC57 cells were grown in RPMI1640 with 5% FCS, Glu and and P/E. The adherent cells were trypsinised and the concentration was adjusted to 1×10^6 cells/ml in RPMI. 100 µl of the cell suspension (~1×10^5 cells/well) was plated to each well of a 48-well plate. The cells were allowed to grow overnight to a confluence of approx. 60-70%.

Preparation of organs infected with LCMV: Liver and spleen samples were weighed and snap-frozen in 1 ml DMEM/FCS in 2 ml eppendorf tubes. During the assay, samples were thawed, DMEM is replaced with HBSS and homogenised using stainless steel balls with Qiagen TissueLyser for 3 min at 30 Hz. Samples were vortexed and centrifuged at 4°C and 3000 rpm for 10 min. Eight 1/10 dilutions of the samples were prepared with cold medium in a 96-well round bottom plate (keep on ice). The wells were prefilled with 180 µl medium and 20 µl of the homogenate was transferred to the first well (A).

Subsequently 20 µl of homogenate was added from wells A to H while changing the tip for each new dilution and the place is kept on ice.

Plaque assay: The medium was removed from the 48-well plate with cells and replaced with 100 µl of the virus samples using the dilutions 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}. One well of the row was kept with medium only. The plates were incubated at 37°C for 2-4 hours while rocking the plates every now and then to allow good distribution of the virus. An overlay of 200 µl with 1:1 mixture of 2% methyl cellulose in empty medium was added along with 2x medium (double FCS, Glu, and antibiotics). The polymer solution was microwaved and cooled down to ca. 50°C in a water bath and the medium is pre-warmed. The temperature of the mix was kept at ca. 40-44°C to ensure that the cells are not killed due to high temperature and prevent the polymer solution from solidification. The plate was left at RT for ca. 15 min to allow solidification of polymer and further incubated for 2 days and the plates were stained (if cells are not confluent, incubate for another day).

Staining: A confluent monolayer of cells was ensured by microscopy. The overlay/medium was gently flicked off into a biohazard waste bin (inside the hood as to protect from virus) from each plate, one at a time. 250 µl of 4% Formalin in PBS was added to each well and incubated for > 30 min at RT. Formalin was gently flicked off into waste bin and washed twice with PBS. All the subsequent washing cycles were flicked down the drain. 200 µl of Triton X solution (1% in PBS) was added and incubated for 20 min at RT. Plates were again washed twice and after adding 200 µl PBS-FCS 10%, the were incubated for 60 min at RT. After washing once, 100 µl of VL-4 rat anti-LVMC mAb (1:10000; aliquots pre-diluted 1:1000 in PBS-FCS 1%) was added and left to incubate for 60 min at RT. After washing twice, 100 µl of secondary antibody (goat anti-rat IgG-HRP) in PBS-FCS 1% (1:400) was added and incubated at RT for 60 min. Later, 250 ul OPD substrate solution was added and incubated for 15-20 min until a good colour was

produced. The colour reaction was flicked off into a waste bin (not drained). A gentle wash performed with running tap water to set the colour and the plates were dried inverted on paper towels and finally the plaques were visually counted.

4.2.3 RNA isolation from the livers and cDNA synthesis

Total RNA was isolated from approximately 20 mg of the liver tissue lysed in RTL buffer, part of RNeasy mini kit (Qiagen) using gentleMACS™ Dissociator (Miltenyi Biotec). Spectrophotometric analysis of the quality and quantity of isolated RNA was performed using nanodrop (Thermo Scientific). 1 µg of RNA was reverse transcribed to cDNA using Quantitect Reverse Transcription Kit (Qiagen) following instructions from the manufacturer.

4.2.4 Quantitative Real-time PCR analysis

Relative mRNA expression was analyzed in duplicates on 384-well PCR plates (Thermo Fisher Scientific) using FastStart Universal SYBR Green Master (Rox) and the qPCR was run on 7900 HT qRT-PCR system (Applied Biosystems, Life Technologies Darmstadt, Germany). Relative mRNA levels were calculated through ΔΔCT relative quantification method and the obtained values were normalized to housekeeping genes albumin (for liver), β-actin and GAPDH.

4.2.5 Immunofluorescence microscopy

LCMV-NP (nuclear protein) Immunofluorescence

5 µm liver cryo-sections on glass coverslips (Thermofisher) from LCMV-infected mice were fixed in ice-cold 100% acetone for 10 minutes. Sections were then blocked for 15 min. in 2% fetal calf serum (FCS; Hyclone) in 1x PBS. Sections were then incubated overnight at 4° C in anti-LCMV NP supernatant (kind gift from Prof. Karl Lang, Germany)

diluted 1:4 in 2% FCS in 1x PBS. Sections were washed in 1x PBS and incubated for 1 hr. in 2 µg/mL AlexaFluor 488-conjugated goat anti-rat secondary antibody (Invitrogen) containing 1:10,000 DAPI in 2% FCS in 1x PBS. Sections were then washed in 1x PBS and mounted with fluorescent mounting medium (Dako), coverslipped, and imaged using an Olympus BX53F fluorescent microscope.

RelA immunofluorescence

5 µm liver cryo-sections on glass coverslips from LCMV-infected mice were fixed in 4% formalin for 10 minutes, washed in 1x PBS for 2 min. and then washed for 10 min. in 1x PBS with 0.1% Tween 20 (PBST). Sections were then blocked for 1 hr. in 5% FCS, 0.25% Triton X-100 in 1x PBS. Sections were then incubated overnight at 4° C in anti-RelA (NeoMarkers # RB-1638-P0) diluted 1:100 in 1% bovine serum albumin (BSA), 0.25% Triton-X 100 in 1x PBS. Sections were then washed in 1x PBS for 2 min., followed by 1x PBST for 2 min., and incubated for 1 hr. in 4 µg/mL AlexaFluor 488-conjugated goat anti-rabbit secondary antibody containing 1:10,000 DAPI in 1% BSA, 0.25% Triton-X 100 in 1x PBS. Sections were then washed in 1x PBS followed by 1x PBST, mounted with fluorescent mounting medium, coverslipped, and imaged using an Olympus BX53F fluorescent microscope.

$CD8^+$ T cell immunoflouresence

5 µm liver cryo-sections on glass coverslips from LCMV-infected mice were fixed in ice-cold 100% acetone for 10 minutes. Sections were then blocked for 10 min. in 2% fetal calf serum (FCS; Hyclone) in 1x PBS. Sections were then incubated for 60 min. in anti-CD8-PE diluted 1:100 in 1% BSA, 0.25% Triton-X 100 in 1x PBS. Sections were then washed in 2% FCS-PBS solution and mounted with fluorescent mounting medium, coverslipped, and imaged using an Olympus BX53F fluorescent microscope.

4.2.6 Immunohistological stainings.

Liver tissue samples were fixed in 4% paraformaldehyde for 5 days at room temperature and embedded in paraffin and ~2 µm tissue sections were made using a microtome. The sections were later deparaffinized, rehydrated and boiled at 100^0C with EDTA to facilitate antigen retrieval. IHC staining was done on Leica automated BOND-MAX stainer using Bond Polymer Refine Detection kit (Leica, Catalog #DS9800). Staining was performed using antibodies purchased from Cell signaling for pSTAT1-pY701 (1:100 dilution), pSTAT3 (1:100 dilution) and B220 (1:300 dilution)

4.2.7 CXCL10 RNA *In situ* hybridization (ISH)

RNA *In situ* hybridization was performed on the liver tissues by following manufacturer instructions for RNAscope 2.0 FFPE Assay kit –BROWN, purchased from Advanced Cell Diagnostics. Briefly, 2 µm paraffin sections mounted on glass slides were boiled at 75^0C in EZprep buffer for 20 minutes followed by a protease treatment at 37^0C for 30 minutes. Probes specific to mouse CXCL10 (Advanced Cell Diagnostics) were hybridized at 48^0C for 2 hours using HybEZ Hybridization oven (Advanced Cell Diagnostics) followed by a subsequent series of washing and signal amplification steps. Mouse specific probe for Ubiquitin C, a common housekeeping gene is used as a positive control along with a probe for bacterial gene dapB which served as a negative control. In the end, hybridization signals were detected by DAB staining followed by counterstaining with hematoxylin. Stained tissues were digitally scanned using Leica SCN400 scanner (Leica) and hyridization signals were analyzed at 40x magnification using DIH software (Leica).

4.2.8 Immunoblotting

Liver tissue homogenates were obtained by grinding for 3 minutes using gentleMACS Dissociator (Miltenyi Biotec) by placing the tissue in RIPA buffer (50 mM Tris; 1% NP40;

0.25% Deoxycholic acid sodium salt; 150 mM NaCl; 1 mM EGTA) containing Protease and Phosphatase Inhibitor Cocktail (Roche). Supernatants from the lysed homogenates were collected and quantified with a BCA protein assay kit (Thermo Scientific) according to the instructions provided. 50 µg of protein was denatured in Laemmli buffer (Bio-Rad) containing 5% β-mercaptoethanol and separated by gel electrophoresis followed by blotting on to nitrocellulose membranes using wet blotting equipment from Bio Rad. Transfer of proteins on to the blot was confirmed by briefly staining with Ponceau S (Sigma). Membranes were then blocked in 5% BSA/TBS-T for 1 hour at RT. Primary antibodies against pSTAT1, STAT1, pTyk, Tyk, pJAK, JAK and GAPDH (all obtained from Cell Signaling) were used at 1:1000 dilution and were incubated at 4°C overnight while shaking. Incubation with the secondary antibody (HRP-anti rabbit IgG, Cell signaling) was carried out while shaking for 1 hour at room temperature. Expression of interested proteins was detected by soaking the blot briefly in Clarity Western ECL Substrate (Bio Rad) and the resulting chemiluminescent signal was captured on Stella 3200 imaging system (Bio Rad). To ensure equal loading, membranes were reprobed with anti-GAPDH antibody (Cell Signaling) and detected following the protocol described above.

4.2.9 BD cytokine ELISA

Serum levels of TNFα, IL-6, IL-12p70 were measured using BD Cytometric Bead Array (CBA) Mouse Enhanced Sensitivity Master Buffer Kit from BD Biosciences and following sequential steps as instructed by the manufacturer and the expression levels of cytokines were detecting by flow cytometry.

4.2.10 Intracellular Cytokine Staining from the liver

Preparation of single cells suspension of liver cells: Whole livers were excised and mashed through a wired mesh into 5ml of 1% RPMI medium using a syringe plunger. To create a single cell suspension and ensure no clumps, the lysate, was filtered through a 100 µm cell strainer and centrifuged at 1500 rpm for 5 min at 4^0C. Pellets were resuspened in 3ml of TAC buffer and incubated for 2 minutes at 37^0C to facilitate erythrolysis and the reaction was stopped by adding 40 ml of 1% RPMI medium. Cells were again filtered through a 100 µm cell strainer and centrifuged at 1500 rpm for 5 min at 4^0C. Viable cells were counted at 1:40 dilution with Typan blue and using a Neubauer hemocytometer.

Peptide restimulation of liver lymphocytes: Single cell suspension with ~ $4x10^6$ were transferred to a 96 well U-bottom plate and centrifuged for 5 minutes at 1500 rpm and resulting pellets were resuspended in 200 µl of 10% RPMI for *ex vivo* peptide stimulation. 50 µl of 5x mastermix containing 5 µg/ml of LCMV gp33 peptide and another master mix of a non-specific peptide with same concentration and 5 µg/ml of Brefeldin A (Golgi-plug; 1mg/ml stock) was added to each well to obtain a final working concentration of 1 µg/ml of peptide and Brefeldin A. Plates were later incubated for 5 hours at 37^0C.

Fc receptor blockade and live-dead staining: Cell suspensions from above were transferred to a 96 well V-bottom plate and spun centrifuged for 2 min at 4^0C and 1500 rpm. Resulting pellets were resuspended in 100 µl FACS buffer containing 2µg/ml EMA-1mg/ml stock (dead cells incorporate EMA into their nucleic acid by covalent bonding that is detected through excitation by 488nm laser; emission peak at 625 nm) and 1 µl of anti CD16/CD32 antibody (to block $F_{c\gamma}$ receptor and prevent unspecific binding of IgG antibodies to Fc receptors) and incubated on ice for 15 min under constant illumination to facilitate photo activation of EMA.

Cell surface and intracellular cytokine staining: After staining with EMA and blocking Fc receptor, cells were twice washed with FACS buffer and later resuspended in 50 µl of FACS buffer with CD8α surface antibody and left for 30 min on ice in dark. Later to that, cells were washed twice with 200 µl of FACS buffer centrifuged for 2 min at 4^0C and 1400 rpm. Following this, cells were permeabilized and fixed with 50 µl cytofix/Cytoperm solution for 15 min on ice in the dark. Subsequently, cells were washed twice with 200 µl BD 1x Perm/Wash buffer (in distilled water) and stained with 50 µl IFN-γ APC antibody constituted in BD 1x Perm/Wash buffer for 30 min on ice and in dark. After this, stained cells were washed twice with 200 µl 1x Perm/Wash buffer and transferred to FACS tubes containing 340 µl FACS buffer and 70 µl 2% PFA (0.4% of final PFA concentration) was added to fix the cells. Samples were stored at 4^0C and analyzed by FACS.

4.2.11 Primary murine hepatocyte isolation and culture

Coating the plates and preparing the media: On the day prior to isolation, 6 well plates were coated with 1.5 ml of Type I collagen (final conc. 20 µg/ml) and left in 37^0C incubator overnight. On the day of isolation, the plates were washed twice with distilled water and air dried briefly under the UV light.

Buffer A (10 mM EGTA 1.5 ml + 48.5 ml Hank's balanced salt solution) and buffer B (HBSS with 10 mM Calcium & Collagenase A+D, freshly prepared) along with wash and maintenance/ suspension medium (Williams' E) were pre warmed to 42^0C. The pump for cannulation was filled with Buffer A and the flow rate was adjusted to 2 ml/min.

Isolation procedure: The mouse was anesthised with 120 ul Ketamin and xylazin (Ketamin 5%, Xylazin 20mg/ml). The mouse was teared open and perfusion with buffer A is carried out through the portal vein for 5 min. Vena cava was cut immediately as soon

as the perfusion was started and the perfusion was switched to buffer B with collagenase and was carried out till the liver turns to pale color (5-8 min).

The liver was later removed and placed on a 100µm Cell Strainer in a pertri dish containing buffer C. The outer membrane of the liver was removed using a fine forceps and the liver was gently scratched and shaken in buffer C to let the cells come out into the solution. The resulting solution was passed through a 100µm (BD) cell strainer and collected into a 50 ml falcon filled with 40 ml of buffer C. The cell suspension was centrifuged at 50g (450rpm) for 2 min. The resulting pellet was washed again twice in 40 ml of buffer C followed by centrifugation after discarding the supernatant. After the final wash step, the pellet was resuspended in 10 ml of buffer C and viability of isolated cells was measured using Trypan blue at 1:10 dilution. A viability rate above 80% was considered appropriate for the experimental usage. 0.75×10^6 cells were plated on to 6 well plates in required quantities and used for the experiments.

4.2.12 Statistical Analysis

A statistical analysis of the data was performed using Prism software (Graphpad Prism version 5.0a). The standard error of the mean was calculated from the average of at least 3 independent samples in a given treatment condition. To evaluate statistical significance, obtained results were subjected to Student's t-test (unpaired, two-tailed test) and a p-value of less than 0.05 was considered significant.

5. RESULTS

5.1 Kinetics of viral replication following infection with LCMV-WE

In order to examine the kinetics of viral replication in the liver following viral infection, C57BL/6 mice were infected with a very low dose of LCMV-WE through the i.v route. Infection of LCMV is route and strain-specific and an intra venous injection would lead to a systemic infection in visceral organs such as spleen, kidney, lungs and especially the liver. Virus titers from the livers of infected mice were measured on day 2, day 4, day 6 and day 9 post infections. Maximal virus titers were found on day 4 and day 6 (Fig. 9). LCMV being a non-cytopathic virus induced no pathology in the livers as evidenced by H&E stainings on the livers from infected mice compared to uninfected C57BL/6 mice.

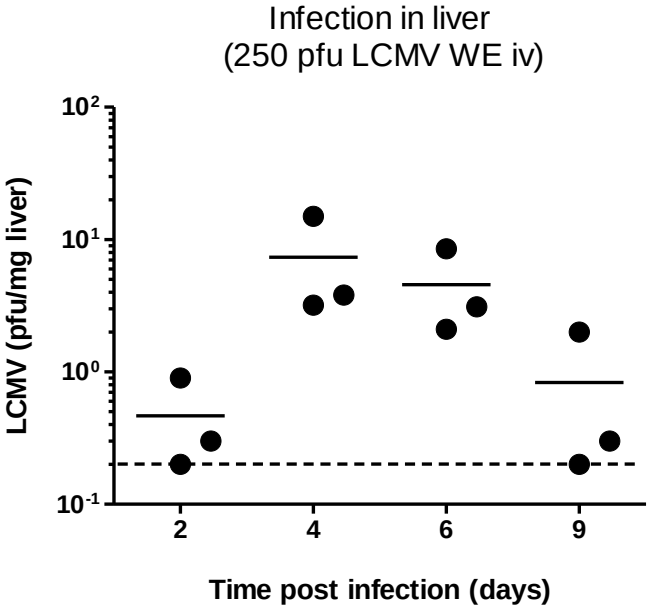

Figure 9: Kinetics of LCMV-WE replication. C57BL/6 mice were intravenously infected with 250 pfu of LCMV-WE. Hepatectomy was performed at indicated time points and analyzed for infectious virus using a virus plaque forming assay (n=3). Virus titers were normalized to per mg of liver. Higher virus titers were found on Day 4 and 6 without any visible pathology (data not shown). Each circle indicates measurement from single mouse.

5.2 Nuclear RelA translocation in hepatocytes following LCMV-WE infection controls viral replication in the liver

5.2.1 Infection with LCMV-WE leads to translocation of RelA in hepatocytes of C57BL/6 mice

LCMV-WE is a negative-sense single-stranded RNA virus belonging to the Arenaviridae family. The replication intermediates of this virus involve formation of dsRNA and 5′-PPP structures which are recognized by the PRRs of the innate immune system such as RIG-I and MDA5 which activate NF-κB-mediated interferon responses (Marq et al., 2011; Pichlmair et al., 2006). Response to NF-κB activation is typically associated with professional immune cells. However, given the central role that hepatocytes play in the viral response, we wanted to test whether LCMV-WE infection in mice would activate NF-κB signaling in hepatocytes of C57BL/6 mice. To this end, C57BL/6 mice were intravenously infected with $2x10^6$ PFU of LCMV and livers of infected mice were stained for RelA at 24h p.i. Notably, we could observe strong RelA translocation in hepatocytes, whereas non-infected C57BL/6 mice lacked detectable nuclear RelA translocation in hepatocytes (Fig. 10).

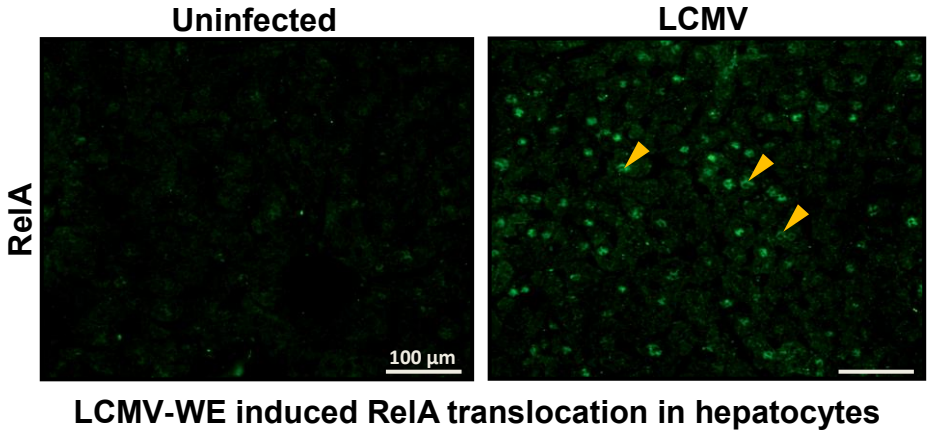

Figure 10: LCMV-WE induce RelA translocation. C57BL/6 mice were intravenously infected with $2x10^6$ PFU of LCMV-WE. 24h p.i, livers were harvested from mice and frozen sections were stained for RelA which is found to be localized in the hepatocytic nuclei, indicated by yellow triangles (n=3). Uninfected C57BL/6 mice were used as controls.

5.2.2 Hepatocyte specific Ikkβ deletion results in a marked reduction in RelA translocation leading to an enhanced viral replication

To elucidate the role of hepatic NF-κB signaling in conferring immunity to viral infections, a mice having hepatocyte specific deletion of Ikkβ, termed Ikkβ$^{\Delta Hep}$ mice was used. In these mice, the exon 3 of Ikkβ gene carrying the phosphorylation site required for its activation is flanked by loxp sites and cre recombinase driven by albumin promoter deletes this activation site resulting in the blockade of nuclear translocation of p50:RelA subunits to the nucleus upon stimulation (Maeda et al., 2005). The floxing efficiency of Ikkβ is evaluated by isolating genomic DNA from the hepatocytes of Alb Cre Ikkβ$^{\Delta Hep}$ mice and confirmed to be of greater than 85% through qPCR.

C57BL/6 and Alb Cre Ikkβ$^{\Delta Hep}$ mice were intravenously infected with 2x10^6 PFU of LCMV and the livers of infected mice were stained for RelA starting from 3h to 24h p.i. Notably, we could observe strong RelA translocation in hepatocytes with a peak at 12h p.i., whereas non-infected C57BL/6 mice lacked detectable nuclear RelA translocation in hepatocytes (Fig. 11) In contrast, Ikkβ$^{\Delta Hep}$ mice infected with an identical dose of LCMV-WE displayed a strong reduction in RelA translocation in hepatocytes at all the time points observed.

Translocation of RelA following a viral infection is an important event required to initiate interferon responses. IKKβ has been shown to be an essential factor in the induction of type I IFN responses leading to viral clearance (Chu et al., 1999). Also, activation of NF-κB alone can confer an antiviral response in an interferon independent manner (Bose et al., 2003). In light of these reports, we proceeded to evaluate the functional consequence of this impediment in RelA translocation; we wanted to compare the virus titers from the livers of Ikkβ$^{\Delta Hep}$ mice and C57BL/6 mice, particularly at the time points where the viral replication was at the peak (Fig. 9). An infection time course on C57BL/6

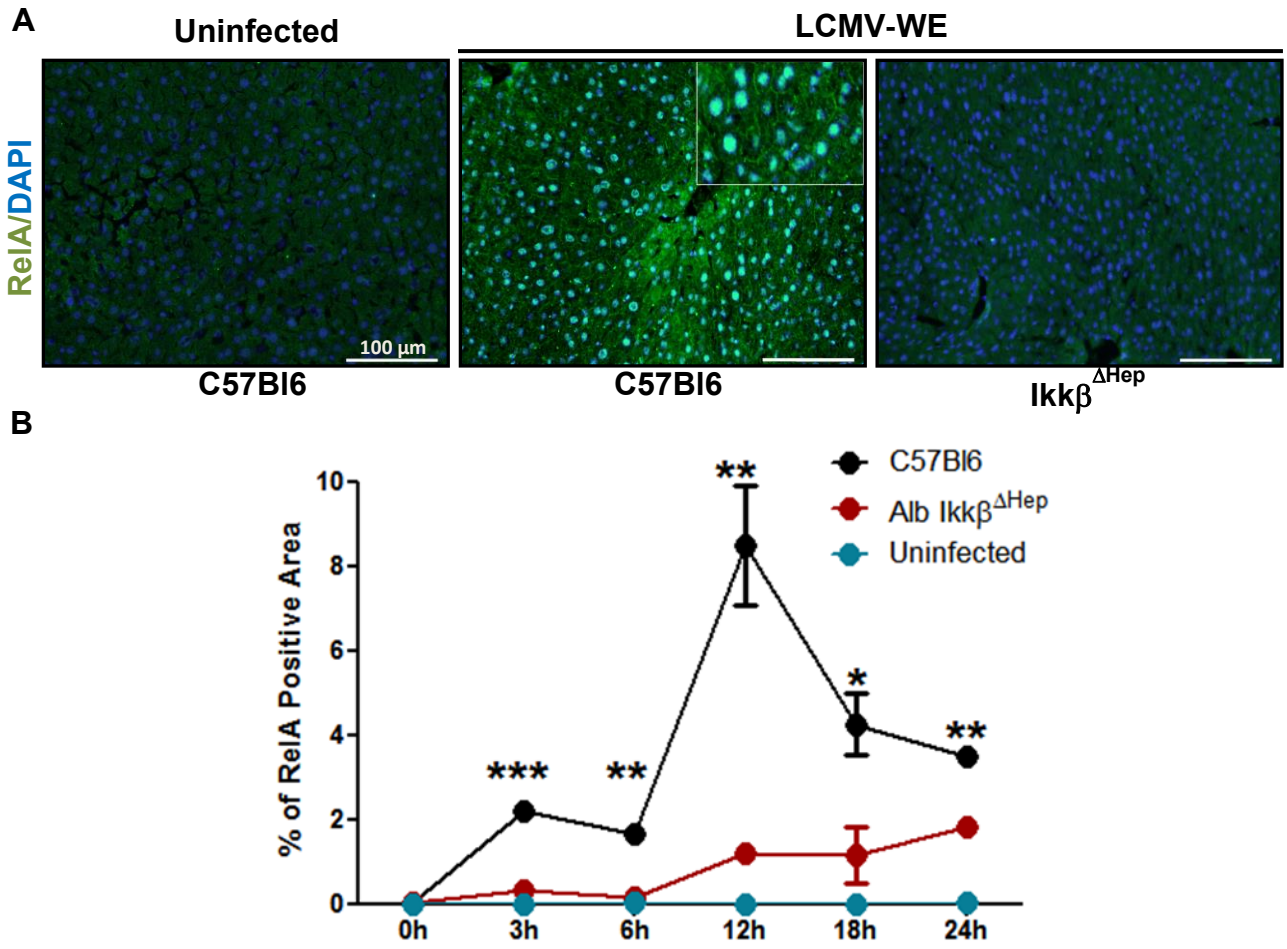

Figure 11: Comparative RelA translocation in C57BL/6 and Ikkβ$^{\Delta Hep}$ mice. **(A)** C57BL/6 and Ikkβ$^{\Delta Hep}$ mice were intravenously infected with 2x10^{6} PFU of LCMV-WE (n=3). Hepatectomy was performed starting from 3h until 24h p.i and frozen liver sections of infected mice were stained for RelA (green) and DAPI (blue) to stain nuclei. Representative images from 12h are shown. Uninfected C57BL/6 mice were used as controls. **(B)** Quantification of RelA staining in C57BL/6 and Ikkβ$^{\Delta Hep}$ mice.

and Ikkβ$^{\Delta Hep}$ mice and was carried out using 1x10^{5} PFU of LCMV-WE. Virus titers from the infected mice were analyzed on day 5, 8 and 15.

Strikingly, there was almost a 2 log increase of in the virus titers from Ikkβ$^{\Delta Hep}$ mice indicating that impairment in hepatic NF-κB signaling confers a selective advantage for the virus to replicate uninterruptedly amidst the professional immune cells of liver such as KCs, pDCs and also highly immune competent non-parenchymal cells such as

LSECs. This finding might imply that there occurs a cross-talk between the hepatocytes and professional immune cells through factors activated by hepatic NF-κB signaling.

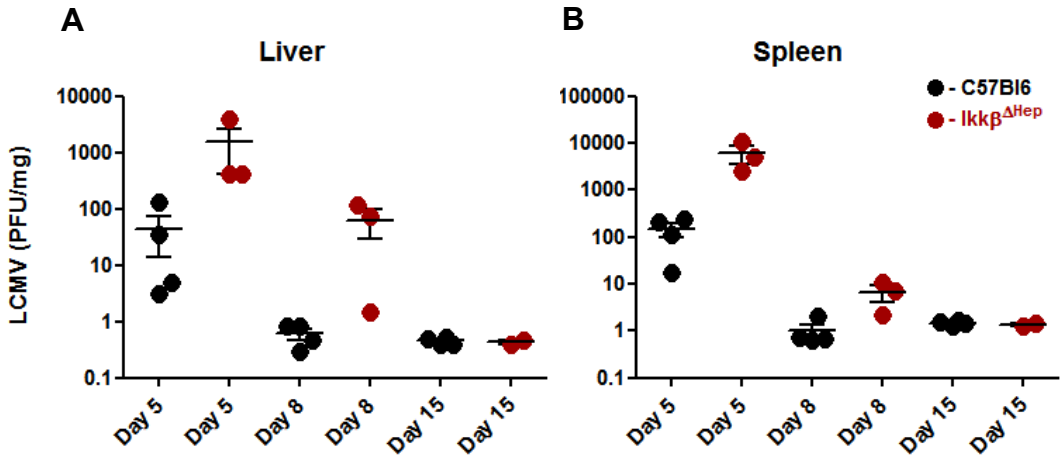

Figure 12: Ikkβ depletion in hepatocytes leads to enhanced viral replication: Analysis with lower dose of LCMV. C57BL/6 and Ikkβ$^{\Delta Hep}$ mice were intravenously infected with 1x10^5 PFU of LCMV-WE (n=3). Hepatectomy and splenectomy was performed at indicated time points and analyzed for infectious virus using a virus plaque forming assay (n=3). Virus titers were normalized to per mg of liver.

Another striking observation was that the increase in virus titers was not restricted to the liver (Fig 12A) but also these differences were seen in the spleen suggesting a systemic effect of innate immune control monitored by the hepatocytes (Fig 12B).

We further went on to see how the virus titers would differ in Ikkβ$^{\Delta Hep}$ mice would with an increase in the dose of LCMV. A higher dose of 2 x 10^5 PFU of LCMV-WE was used to infect C57BL/6 and Ikkβ$^{\Delta Hep}$ mice and titers from infected mice were analyzed through plaque assay. Consistent with the earlier observations, there was a significant increase in virus titers from the livers and spleens of Ikkβ$^{\Delta Hep}$ mice compared to C57BL/6.

LCMV-WE virus gains entry in to the host cells by targeting α-Dystroglycan, a protein involved in interactions between the cells and extracellular matrix (Cao et al., 1998). The lifecycle of LCMV occurs within the cytoplasm of acutely infected cells and within 16-24h

post initiation of infection, progeny virions are released by budding from the plasma membrane (Meyer et al., 2002). To know the earliest time, by which the differences in virustiters start to be appear between the C57BL/6 and Ikkβ$^{\Delta Hep}$ mice, plaque forming

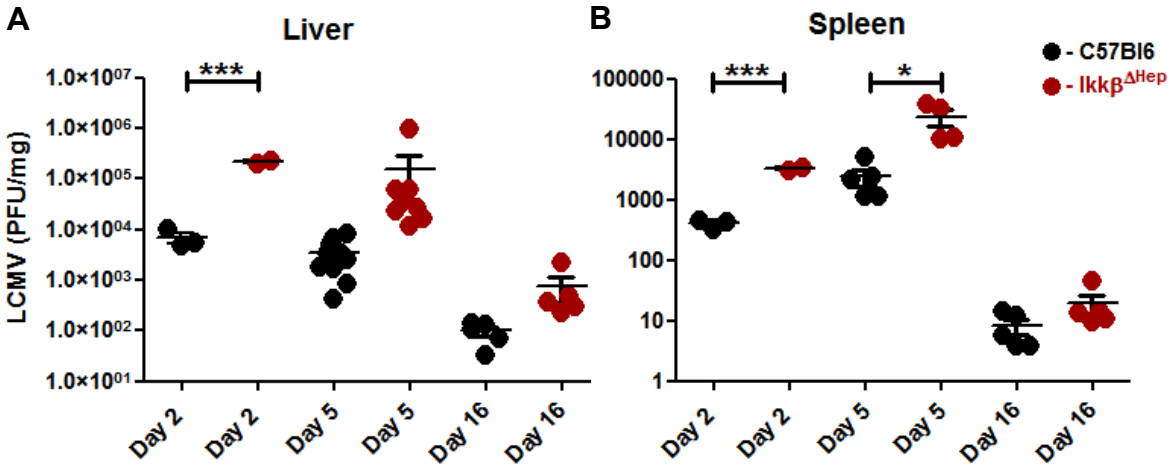

Figure 13: Ikkβ depletion in hepatocytes leads to enhanced viral replication: Analysis with lower dose of LCMV. C57BL/6 and Ikkβ$^{\Delta Hep}$ mice were intravenously infected with 2x10^5 PFU of LCMV-WE (n=3). Hepatectomy and splenectomy was performed at indicated time points and analyzed for infectious virus using a virus plaque forming assay (n=3). Virus titers were normalized to per mg of liver.

assay was performed at 6h and 24h post infection with LCMV-WE. Logically, no differences were observed in virus titers were observed at 6hrs p.i, when an the infection is not initiated but a significant increase in viral replication is noticed at 24h post infection by which time virions are normally released by budding. This dramatic increase in the increased virus titers as early as 24h in mice lacking Ikkβ in hepatocytes is clearly indicative of its importance in mediating early innate immune responses required to control viral replication and spread.

The observed phenotype of increased virus titers in the livers of Ikkβ$^{\Delta Hep}$ mice was confirmed through a number of independent infection experiments with varying doses of LCMV-WE viral load and found to be consistent (Fig. 15) establishing that lack of canonical NF-κB signaling selectively in the hepatocytes confers an advantage for the

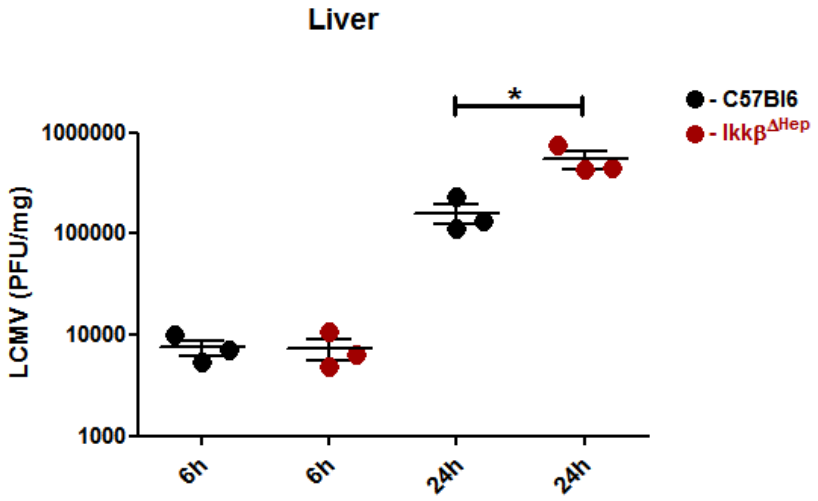

Figure 14: Increased viral load in Ikkβ$^{\Delta Hep}$ mice correlates with LCMV-WE viral replication. C57BL/6 and Ikkβ$^{\Delta Hep}$ mice were intravenously infected with 2x10^5 PFU of LCMV-WE (n=3). Livers from infected mice were harvested at indicated time points and analyzed for infectious virus using a virus plaque forming assay (n=3). Virus titers were normalized to per mg of liver. The measured viral titers were similar in C57BL/6 or Ikkβ$^{\Delta Hep}$ mice before the virus start to replicate at 6h p.i. The differences are visible as soon as 24h p.i corresponding to the doubling time of LCMV.

virus to replicate unrestricted even amidst other professional immune cells of the liver having no defects in NFκB signaling.

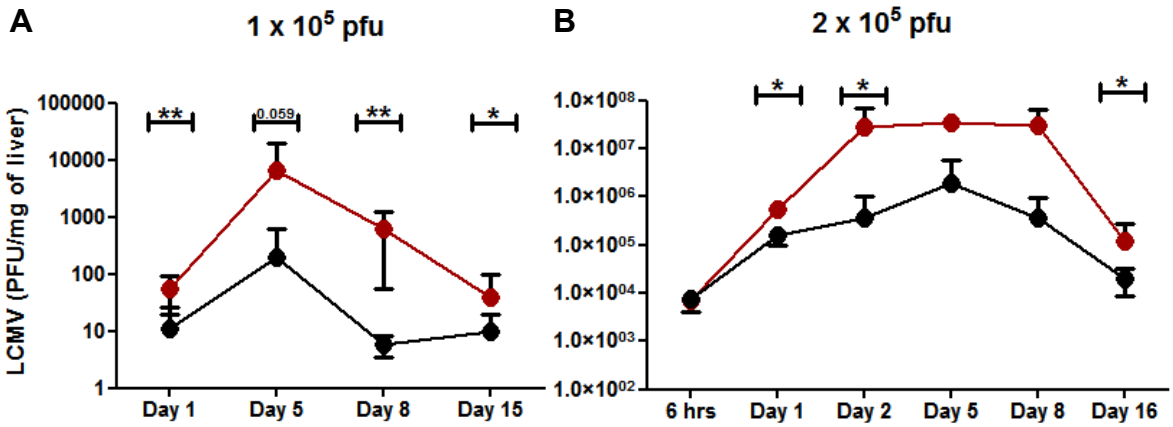

Figure 15: Quantification of viral load in C57BL/6 and Ikkβ$^{\Delta Hep}$ mice. Pool of 3 independent experiments with wither a low dose (1x10^5 PFU) or higher dose (2x10^5 PFU) of LCMV infection indicates is a 2 log increase in the viral load from the livers of Ikkβ$^{\Delta Hep}$ mice compared to C57BL/6.

5.3 LCMV-WE is predominantly localized in hepatocytes of Ikkβ$^{\Delta Hep}$ mice compared to C57BL/6 mice post infection

5.3.1 Histological staining for LCMV-NP complements viral plaque assay

Following our initial observation that lack of hepatic NF-κB signaling leads to an increase in virus titers even when other professional immune cells are having an intact NF-κB signaling, we went on to verify the localization of LCMV-WE in liver sections of C57BL/6 and Ikkβ$^{\Delta Hep}$ mice post infection with 2x10^6 PFU of LCMV starting from Day 2 until Day 20 p.i by which time the acute LCMV infection is expected to be cleared in mice. While LCMV was localized in cells exhibiting Kupffer cell like morphology as early as day 2 p.i, LCMV was only sporadically in hepatocytes by day 6 and 8 p.i indicating that the LCMV-WE also disseminates to the hepatocytes at later stages of infection in C57BL/6 mice (Fig. 16). Strikingly, we observed that hepatocytes from Ikkβ$^{\Delta Hep}$ mice were severely impaired in clearing LCMV and tended to form large hepatocyte clusters filled with virus, particularly at days 6 and 8 dpi, in some cases already even at earlier time points (data not shown) corroborating the virus titers from plaque assay. The overall area covered by cells infected with LMCV was quantified and plotted indicating a significant, strong rise (> 10 fold difference) in the hepatic area covered by LCMV-WE infected cells.

5.3.2 Cellular localization of LCMV-WE post infection

While deciphering the localization of LCMV-WE post infection, Kupffer cells were shown to be the responsible cell type for the capture and elimination of virus through interferon dependent mechanisms in the liver (Lang et al., 2010). In light of enhanced viral replication at day 6 and 8 and hepatocytes forming clusters, we further investigated if the capture of viral particles by the Kupffer cells is influenced in Ikkβ$^{\Delta Hep}$ mice and where it is localized in the liver. To this end, Ikkβ$^{\Delta Hep}$ mice along with C57BL/6 mice were infected

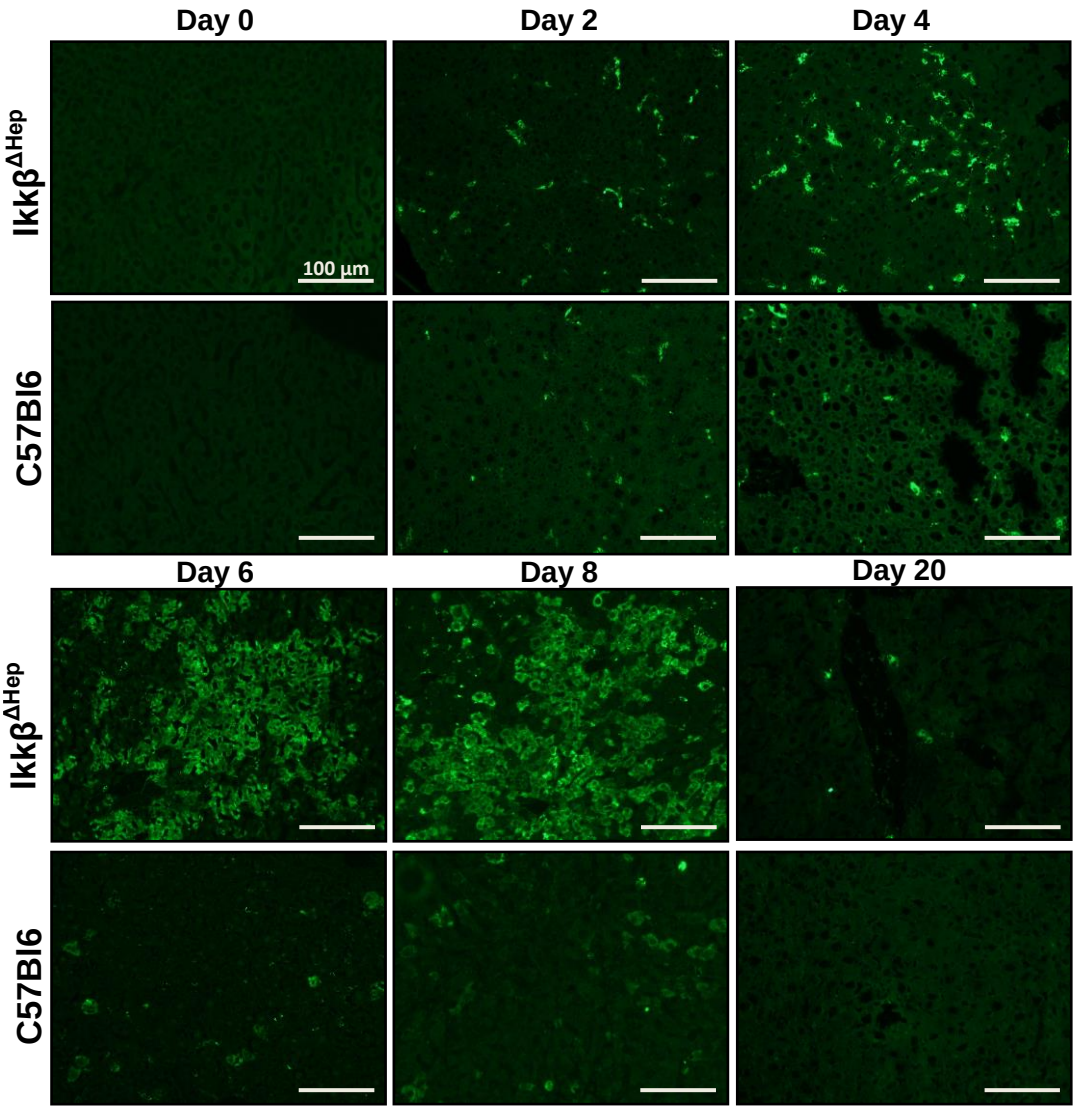

Figure 16: Enhanced viral replication in the livers of Ikkβ$^{\Delta Hep}$ mice. C57BL/6 and Ikkβ$^{\Delta Hep}$ mice were intravenously infected with 2x10^{6} PFU of LCMV-WE (n=3). Hepatectomy was performed starting from day 2 until day 20 p.i. Frozen liver sections from infected mice were stained for LCMV-NP (green). Representative images from indicated time points are shown.

with LCMV and the livers were stained using antibody against LCMV nucleoprotein and F4/80 antibody for Kupffer cells.

The localization of LCMV was measured using algorithms developed through Leica software and the results were quantified. Interestingly, the number of Kupffer cells that were infected with LCMV also turned out to be higher in Ikkβ$^{\Delta Hep}$ mice compared to

C57BL/6, strongly supporting the requirement of a crosstalk between the hepatocytes and Kupffer cells.

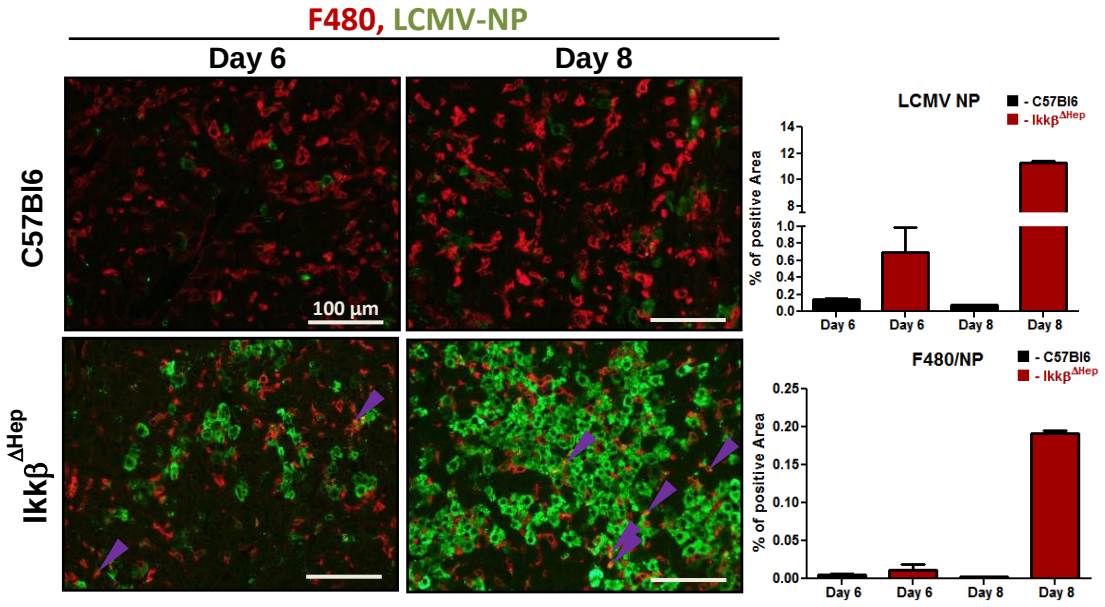

Figure 17: Localization of LCMV in C57Bl6 and Ikkβ$^{\Delta Hep}$ mice. C57BL/6 and Ikkβ$^{\Delta Hep}$ mice were intravenously infected with 2x10^6 PFU of LCMV-WE (n=3). Hepatectomy was performed at day 6 and day 8 p.i. Frozen liver sections from infected mice were co-stained for LCMV-NP (green) and F4/80 (red). Yellow colored KCs (purple triangles) indicative of viral infection were quantified against LCMV-Np (green) using Leica software. Representative images from indicated time points are shown.

The enhanced LCMV replication in livers of Ikkβ$^{\Delta Hep}$ mice and its predominant localization in hepatocytes, forming clonal areas are intriguing as the professional immune cells such as Kupffer cells and dendritic cells and the main antigen-presenting cells of the liver such as LSECs all possess functional NF-κB signaling. While all the cells are capable of mounting an interferon response upon viral infection, the type of interferon secretion is cell type restricted. Whereas all cells can produce IFN-β, secretion of IFN-α is restricted to professional immune cells such as plasmacytoid dendritic cells and Kupffer cells of the liver (Barchet et al., 2002; Crouse et al., 2015). For the professional cells to secrete IFN-α, IFN-β secretion from cells that are infected with a

virus might be required- in this case the hepatocytes. A lack of canonical NFκB signaling in the hepatocytes renders them less effective in producing the levels of IFN-β required to trigger an efficient response from KCs and pDCs. These results demonstrate that Ikkβ from the hepatocytes is indispensable either for responding to the paracrine interferon signaling cues from the neighboring pDCs and Kupffer cells or to initiate an autocrine and paracrine antiviral response required for viral clearance in hepatocytes.

5.3 Clodronate mediated depletion of Kupffer cell population does not affect RelA translocation in the hepatocytes post infection with LCMV

In the context of liver viral infections, Kupffer cells are known to capture the virus at early time points and thus prevent virus from disseminating into the hepatocytes (Lang et al., 2010). But we have verified through localization studies following LCMV infection that the virus disseminates into hepatocytes when they have defective NF-κB signaling even in the presence of intact NF-κB signaling in Kupffer cells. Kupffer cells readily secrete TNFα in their activated state which could act as a trigger to induce NF-κB signaling in the neighboring hepatocytes. Apart from that, the replication intermediates such as dsRNA following LCMV infection with in a virus infected cell could also activate NF-κB signaling.

In order to identify the initial molecular cues that activate NF-κB signaling within hepatocytes following LCMV infection, we depleted Kupffer cells by treating mice with clodronate liposomes 2 days prior to infection and stained the infected livers for RelA translocation 24h p.i. PBS liposome-treated mice receiving similar amounts of LCMV were used as controls for comparing RelA translocation. The absence of Kupffer cells in clodronate treated mice was confirmed by staining using F4/80 antibody that is specific to Kupffer cells, whereas the mice receiving PBS liposomes showed the presence of normal Kupffer cells (Fig. 18). Despite the absence of Kupffer cells, there is a clear RelA

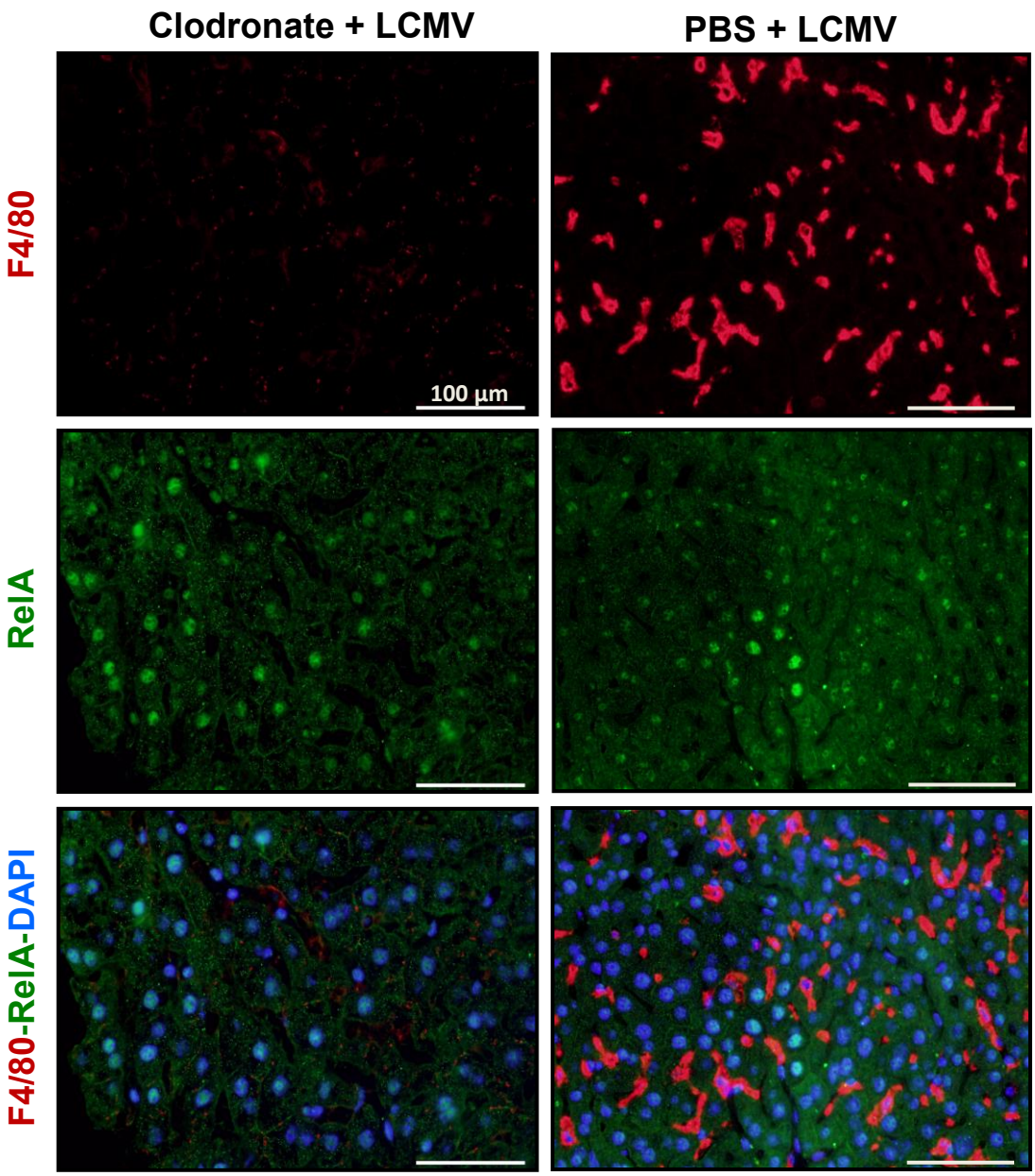

Figure 18: Hepatocytic RelA translocation is independent of Kupffer cell involvement. C57BL/6 mice were injected intravenously with clodronate or with PBS liposomes on day -2. At day 0, mice were intravenously infected with $2x10^6$ LCMV-WE. On day 1 post infection, hepatectomy was performed and frozen sections of the liver were stained for F4/80 (red), RelA (green) and DAPI (blue) to stain nuclei.

translocation in the hepatocytes of clodronate-treated mice similar to translocation of RelA observed in the livers in which Kupffer cells are intact, which indicates that RelA translocation occurs in hepatocytes independent of inflammatory signals by the Kupffer

cells, and detection of viral replication intermediates by the hepatocytes alone can activate NF-κB signaling.

A further additional experiment to characterize the outcome of viral replication in the livers 4 days p.i in the clodronate-treated mice in comparison to PBS-treated mice indicated that there is enhanced viral replication in the livers of clodronate treated mice compared to PBS treated mice infected with LCMV (data not shown).

5.4 Defective interferon response causes increased virus titers in the livers of Ikkβ$^{\Delta Hep}$ mice

5.4.1 Loss of NF-κB signaling in hepatocytes leads to an impaired interferon response in the liver

Expression of interferons and ISGs is a primary and immediate innate immune response induced by the host immune system following a viral infection. As soon as four hours following a viral infection, initiation of IFN-β transcription begins by the formation of the interferon enhanceosome complex (Maniatis et al., 1998; Panne et al., 2007). Binding of IFN to the cell surface and subsequent signal transduction leads to rapid changes in the cellular properties. The necessary components of the IFN signaling pathway do not require *de novo* synthesis so that the interferon response is rapid; occurring within 30 minutes post-induction (Larner et al., 1986; Larner et al., 1984). A failure in the timely induction of these responses leads to enhanced viral replication and a consequent inability of the host to clear the virus. It is well-established that Ikkβ plays a pivotal role in the induction of interferon mediated innate immune responses following the detection of viral replication intermediates such as dsRNA (Chu et al., 1999) which are also formed during the life cycle of LCMV. Ikkβ has been shown to be essential for the production of type 1 interferons by plasmacytoid dendritic cells (Pauls et al., 2012) and RelA, which is translocated following Ikkβ activation, is essential for the basal

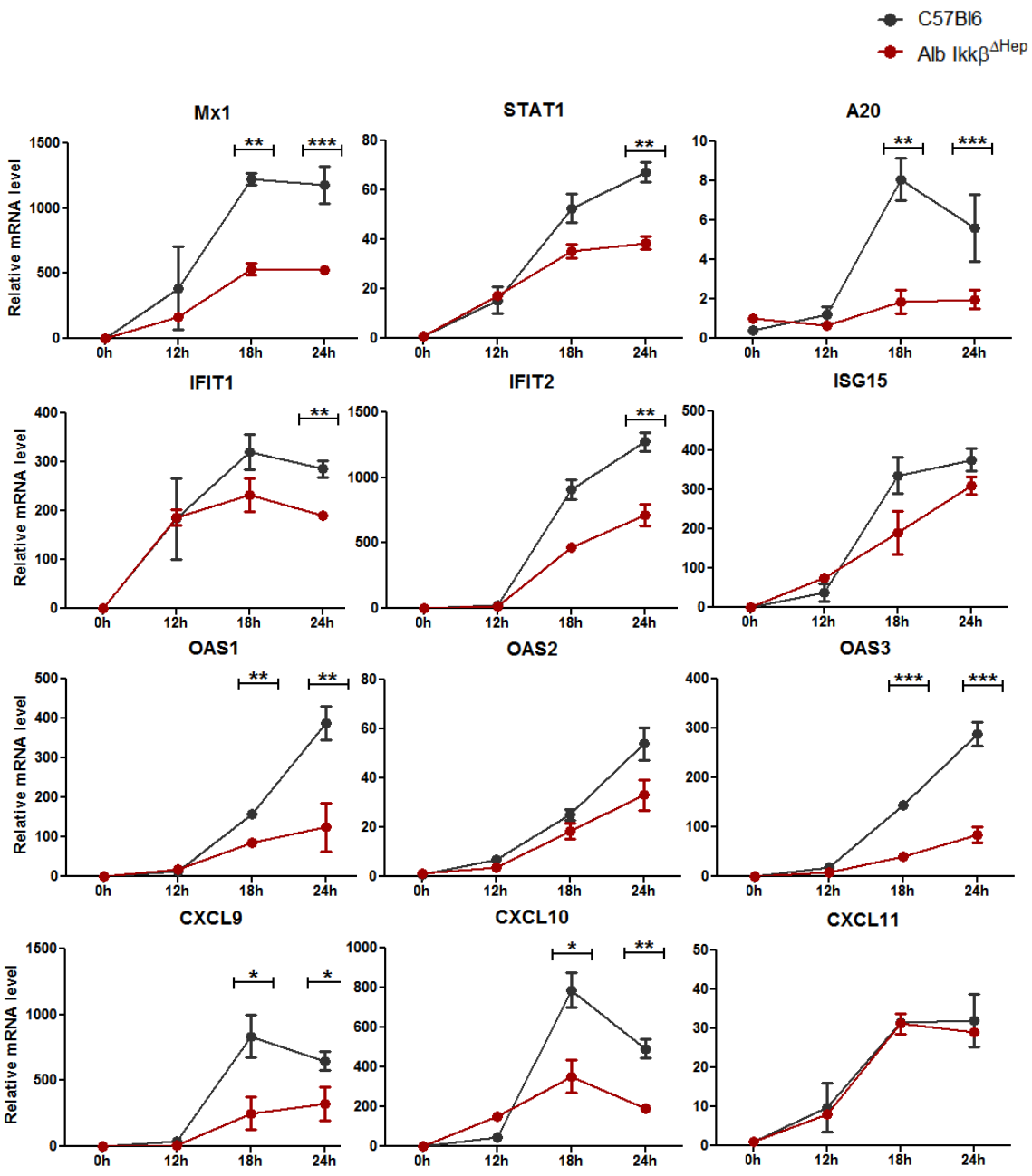

Figure 19. Reduced expression of ISGs in the livers Ikkβ$^{\Delta Hep}$ mice. C57BL/6 and Ikkβ$^{\Delta Hep}$ mice were intravenously infected with 2x10^6 PFU of LCMV-WE (n=3). Hepatectomy was performed at indicated time points and the livers were analyzed for the expression of interferon stimulated genes through qRT-PCR.

expression of interferon genes (Basagoudanavar et al., 2011; Taniguchi and Takaoka, 2001). It has been also established that an interferon-independent antiviral state following viral infection can be modulated solely by Ikkβ through activation of NF-κB

signaling by secreted cytokines such as TNFα and IL-1β during an infection (Bose et al., 2003).

Considering the pivotal involvement of Ikkβ in the induction of interferon responses, we wanted to determine whether Ikkβ$^{\Delta Hep}$ mice were defective in the early induction of interferon responses following infection with LCMV. To this end, C57BL/6 and Ikkβ$^{\Delta Hep}$ mice were infected with 2x10^{6} PFU of LCMV, and the expression of ISGs was measured in whole liver homogenates of infected mice by qPCR starting from 12h to 24h p.i. A significant reduction in the expression of ISGs was observed in the livers of Ikkβ$^{\Delta Hep}$ mice compared to livers from C57BL/6 mice starting from 18h, and the largest differences were found at 24h p.i. (Fig 19).

It is interesting to note that among the differentially expressed genes, Mx1, which is an early expressed antiviral ISG that acts prior to the start of viral genome replication (Haller et al., 2007; Sadler and Williams, 2008) in the host, is significantly reduced. IFIT proteins that bind to eukaryotic initiation factor (eIF3), thereby inhibiting the start of translation (Hui et al., 2003; Terenzi et al., 2006; Wang et al., 2003), members of the 2-5A synthetase family involved in the degradation of endogenous RNA, thereby limiting viral replication (Silverman, 2007), also had reduced expression in the livers of Ikkβ$^{\Delta Hep}$ mice. Expression of interferon-dependent chemokines such as CXCL9, CXCL10, and CXCL11, important for the chemo-attraction of monocytes, T cells and NK cells, was also significantly reduced in the liver due to hepatocyte-specific loss of NF-κB signaling.

The production of type-I interferons is observed even in the absence of viral infections at very low levels (Gresser, 1990; Taniguchi and Takaoka, 2001). This spontaneous weak signal of interferons is essential to induce a further robust response in cases of viral infection and for enhancing the activation of CD8^{+} T cells (Hida et al., 2000). It has been shown that the low level spontaneous production of type-I interferons is NF-κB

dependent (Wang et al., 2010). It is possible that a failure in the basal expression of interferons might lead to the initial lag in the production of interferon responses resulting in reduced interferon stimulated gene expression in Ikkβ$^{\Delta Hep}$ mice. Tumor necrosis factor alpha-induced protein 3 (TNFAIP3) or A20 known as a feedback regulatory gene controlling NF-κB activation is found to be expressed in the livers of C57BL/6 mice at 18h p.i as compared to the livers of Ikkβ$^{\Delta Hep}$ mice where it is weakly expressed. This indicates that the reduction in the induction of interferon responsive genes in Ikkβ$^{\Delta Hep}$ is indeed due to a failure in the induction of interferon responses mediated by activation of NFκB signaling.

CXCL10, also known as IFN-γ-inducible protein 10 (IP-10) is a chemokine abundantly expressed in the liver following type I and II interferon induction and is known to be an important chemokine in recruiting activated T cells to the sites of inflammation in cases of viral and bacterial infections (Dufour et al., 2002; Khan et al., 2000; Liu et al., 2000; Padovan et al., 2002). It was also shown that during HCV infections, CXCL10 is activated directly by NF-κB and IRF3 independent of interferons (Brownell et al., 2014). We hence used it as a marker to monitor the antiviral responses following infection with LCMV via *in situ* hybridization in livers of Ikkβ$^{\Delta Hep}$ and C57BL/6 mice (Figure 2B).

There was no induction in the expression of CXCL10 at 6h p.i in the livers from either C57BL/6 or Ikkβ$^{\Delta Hep}$ mice. But from 12h p.i and at 18h p.i, expression of CXCL10 in Ikkβ$^{\Delta Hep}$ livers was markedly reduced specifically in the hepatocytes, whereas the non-parenchymal compartment (black triangle) still strongly expressed CXCL10. In the livers of C57BL/6 mice, both the hepatocytes (red triangle) and non-parenchymal cells (black triangle) expressed CXCL10 at high levels following infection corroborating the reduction in ISG expression analyzed through qPCR.

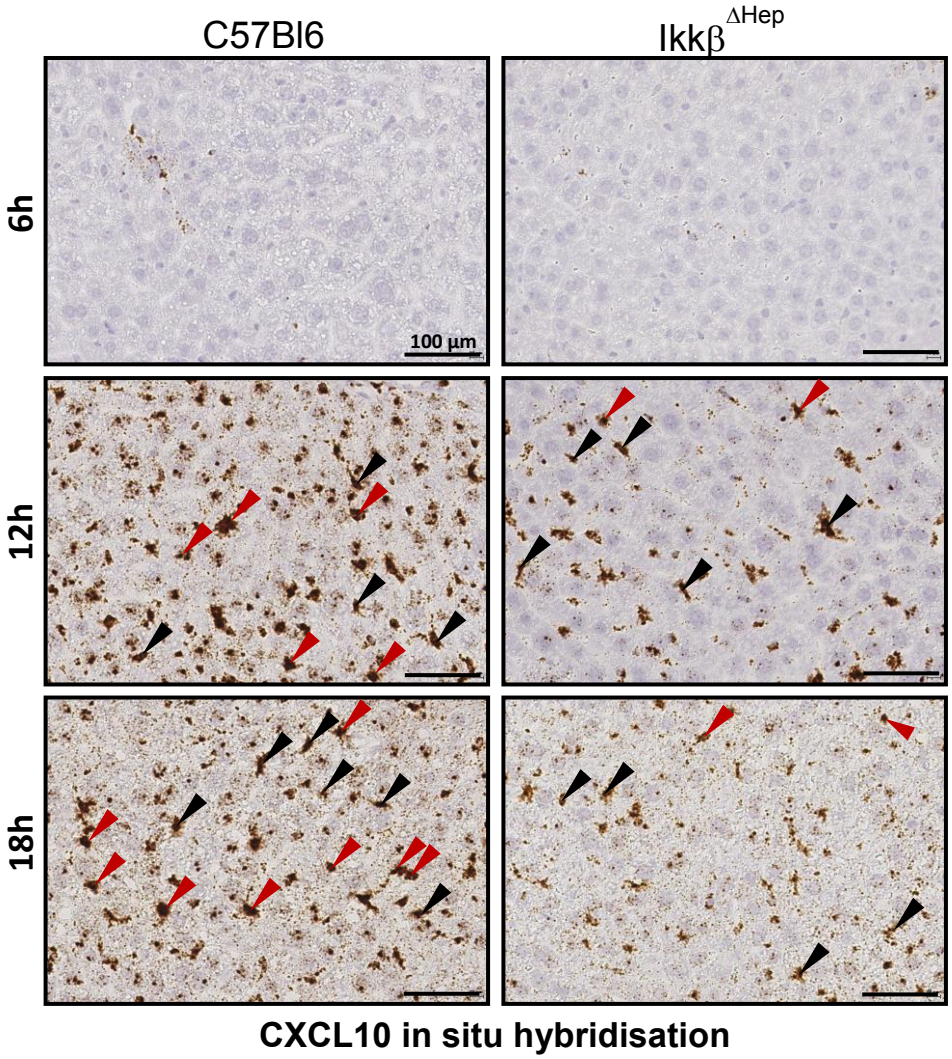

Figure 20: CXCL10 ISH from the livers of Ikkβ$^{\Delta Hep}$ and C57BL/6 mice. C57BL/6 and Ikkβ$^{\Delta Hep}$ mice were intravenously infected with 2x10^{6} PFU of LCMV-WE (n=3). Hepatectomy was performed at indicated time points and the livers were fixed in 4% PFA for 3 days. Expression of CXCL10 from the livers was observed by ISH. Red triangle = Parenchymal hepatocytes. Black triangles = Non-parenchymal cells.

Interferon signaling is propagated to the nucleus through the JAK-STAT pathway. Janus Kinases 1, 2 (JAK1, JAK2) and Tyrosine kinase 2 (Tyk2) are ubiquitously expressed proteins playing a major role in IFN signaling by binding to receptor chains on the inner side of the membrane, providing receptors with stability and facilitating their cell surface localization(Gauzzi et al., 1997; Haan et al., 2006; Ragimbeau et al., 2003;

Schneider et al., 2014). Activated JAKs phosphorylate IFN receptor chains on their tyrosine residues resulting in the binding of STAT proteins by their Src homology 2 8SH2) domains (Heim et al., 1995). STATs are further phosphorylated leading to conformational changes resulting in exposure of their nuclear translocation signal and entry into the nucleus where they act as transcriptional activators driving ISG expression (McBride et al., 2002; Schindler et al., 1992).

STAT1 and STAT2 are the most important transcription factors among all the seven known STATs in mammals. Type II interferon signaling results in the phosphorylation, homodimerisation and nuclear translocation of STAT1 where they bind to DNA at GAS(gamma activated sequence) elements resulting in the transcriptional activation of IFN-γ induced genes (Decker et al., 1997; Shuai et al., 1993). Upon activation by Type I and Type III interferons, STAT1 and STAT2 forms heterodimers and forms ISG factor 3 (ISGF3) complex upon binding to interferon regulatory factor 9 (IRF9) (Fu et al., 1990; Sadler and Williams, 2008). ISGF3 later translocate to the nucleus and bind to IFN stimulated regulatory elements (ISREs) resulting in the transcription of interferon stimulated genes (ISGs).

We went on to further analyze if a reduction in the expression of ISGs in the livers of Ikkβ$^{\Delta Hep}$ mice correlates with a reduction in the phosphorylation of STAT1 which in turn would be indicative of a reduced interferon response. Immunohistochemical analysis for STAT1 nuclear translocation in the livers of Ikkβ$^{\Delta Hep}$ and C57BL/6 mice revealed that hepatocytes from C57BL/6 mice (red triangle) were strongly positive for pSTAT1, whereas hepatocytes from Ikkβ$^{\Delta Hep}$ mice, by comparison, were only faintly positive for pSTAT1 immunohistostaining (Fig 2C). In contrast, the non-parenchymal cells (black triangle) are equally positive for pSTAT1 in the livers of both genotypes.

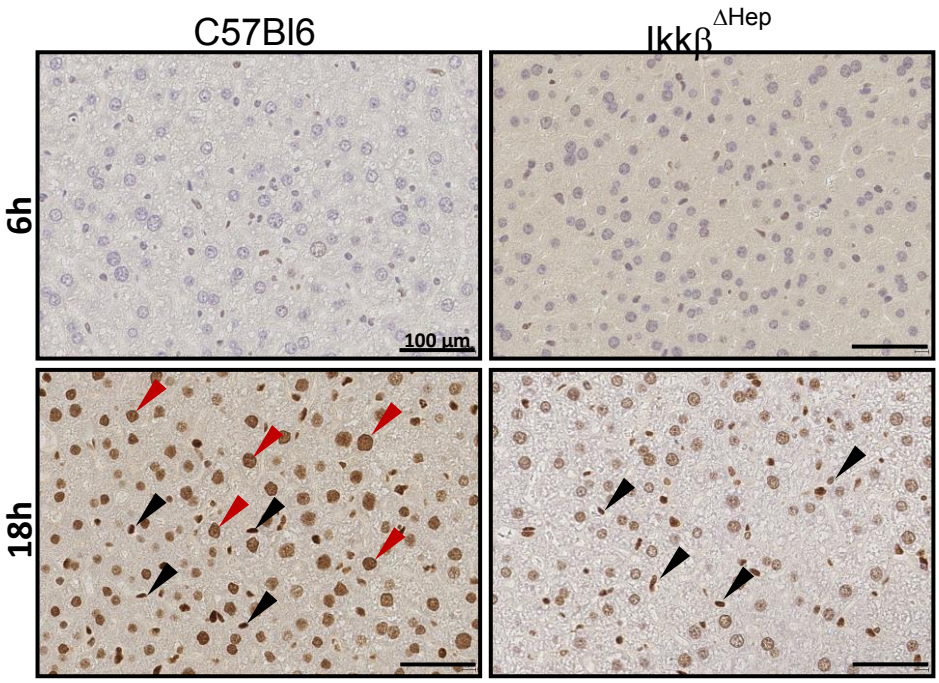

Figure 21: Reduced pSTAT1 expression in the livers of Ikkβ$^{\Delta Hep}$ mice. C57BL/6 and Ikkβ$^{\Delta Hep}$ mice were intravenously infected with 2x10^6 PFU of LCMV-WE (n=3). Hepatectomy was performed at indicated time points and the livers were fixed in 4% PFA for 3 days. Expression of pSTAT1 from the livers was observed by IHC. Red triangles indicate parenchymal hepatocytes expressing pSTAT1 and black triangles are indicative of non-parenchymal cells expressing pSTAT1.

Interferon signaling is a cell type-specific response (van Boxel-Dezaire et al., 2006) and its activation results in different responses in addition to directly conferring immunity to pathogens. STAT3 is a transcription factor with diverse roles depending on the cell type in which it is expressed. In B cells, apoptosis mediated by IFN-β is dependent on the activation of TYK2 and STAT3 (Gamero et al., 2006). Activation of STAT3 along with STAT1 and STAT4 by IFN-α in primary human T cells results in the upregulation of proteins involved in proliferation and survival of T cells (Matikainen et al., 1999). Even though type I interferons activate STAT3; its expression is not involved in the expression of ISGs. When we tested the translocation of phosphorylated STAT3 following LCMV

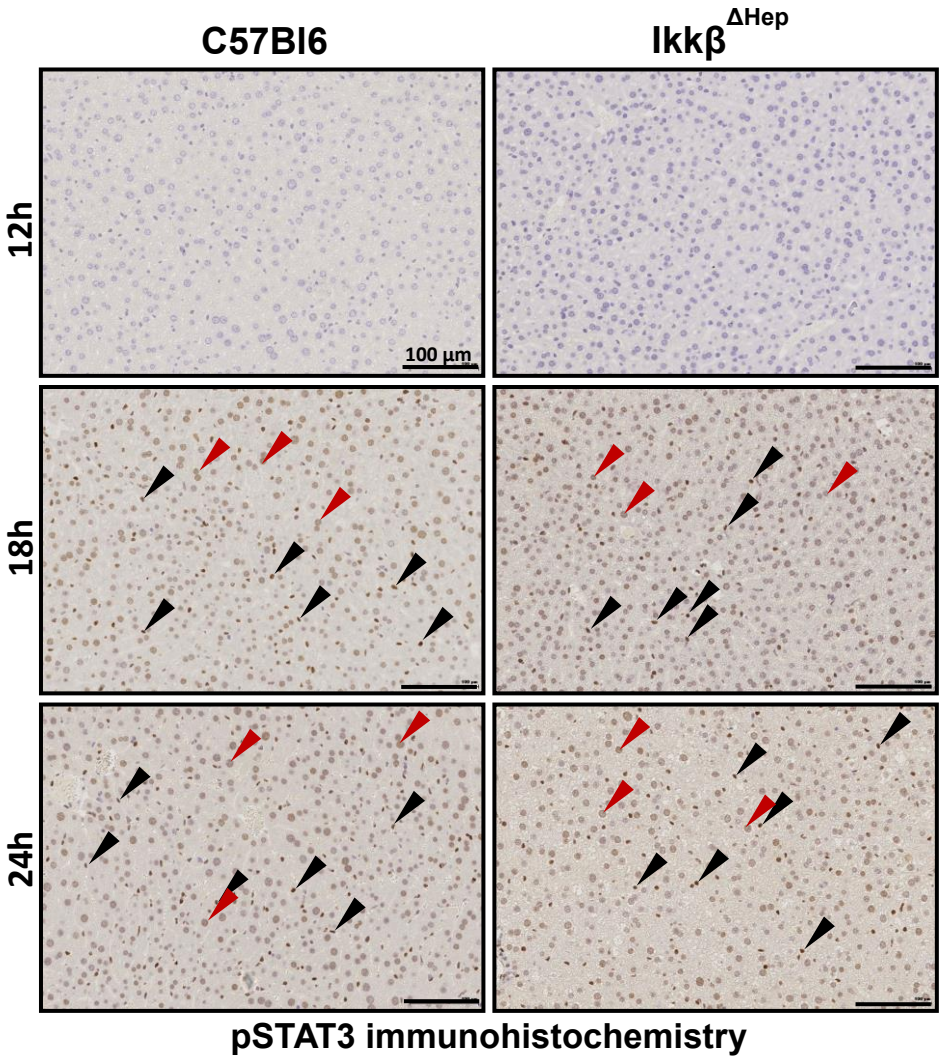

Figure 22: pSTAT3 expression in the livers of C57BL/6 and Ikkβ$^{\Delta Hep}$ mice. C57BL/6 and Ikkβ$^{\Delta Hep}$ mice were intravenously infected with 2x10^{6} PFU of LCMV-WE (n=3). Hepatectomy was performed at indicated time points and the livers were fixed in 4% PFA for 3 days. Expression of pSTAT3 from the livers was observed by IHC. Red triangles indicate parenchymal hepatocytes expressing pSTAT1 and black triangles are indicative of non-parenchymal cells expressing pSTAT1. (__=100 µm)

LCMV infection in the livers of C57BL/6 and Ikkβ$^{\Delta Hep}$ mice, we noticed that pSTAT3 is only expressed in minimal quantities in the hepatocytes compared to the non-parenchymal cells and it is not differentially expressed in the livers of C57BL/6 and Ikkβ$^{\Delta Hep}$ mice (Fig. 22).

Taken together, these experiments indicate that hepatocyte-specific blockade of canonical NF-κB signaling leads to strongly reduced or even impaired activation of STAT1-dependent interferon responses and downstream ISG induction in hepatocytes of Ikkβ$^{\Delta Hep}$ mice infected with LCMV.

5.5 Differential responsiveness to IFNα treatment by C57BL/6 and Ikkβ$^{\Delta Hep}$ mice

Reduced expression of ISGs in the livers of Ikkβ$^{\Delta Hep}$ mice might be due to a reduced interferon response with in the hepatocytes or reduced interferon responsiveness to paracrine interferon signals from the Kupffer cells acting on hepatocytes. In order to be clear off this ambiguity, we injected C57BL/6 and Ikkβ$^{\Delta Hep}$ mice with either a low dose of 1µg or high dose of 10µg of IFNα through subcutaneous route and analyzed the activation of JAK-STAT pathway 15 hours post infection. We performed immunoblotting analysis on the liver lysates and observed a significant decrease in the phosphorylation of STAT1, JAK and Tyk in the lysates from Ikkβ$^{\Delta Hep}$ mice compared to C57BL/6 when a low dose of 1µg was used (Fig. 23). The phosphorylation activity of each of the signaling molecules analyzed were minimal when a high dose of IFN was used compared to the low dose perhaps due to interferon desensitization following a high dose of interferon stimulation.

We then went on to analyze the expression levels of interferon stimulated genes by injecting IFNα into C57BL/6 and Ikkβ$^{\Delta Hep}$ mice through the i.v route so as to directly target the liver. Even though there is a minor decrease in the expression of few interferon stimulated genes such as ISG15 in Ikkβ$^{\Delta Hep}$ mice, expression of other molecules such as IFIT, OAS genes and STAT was similar to that of C57BL/6 mice (data not shown) and so it could not be conclusively established that the hepatocytes from Ikkβ$^{\Delta Hep}$ mice are unable to respond to the externally given interferon. We confirmed this

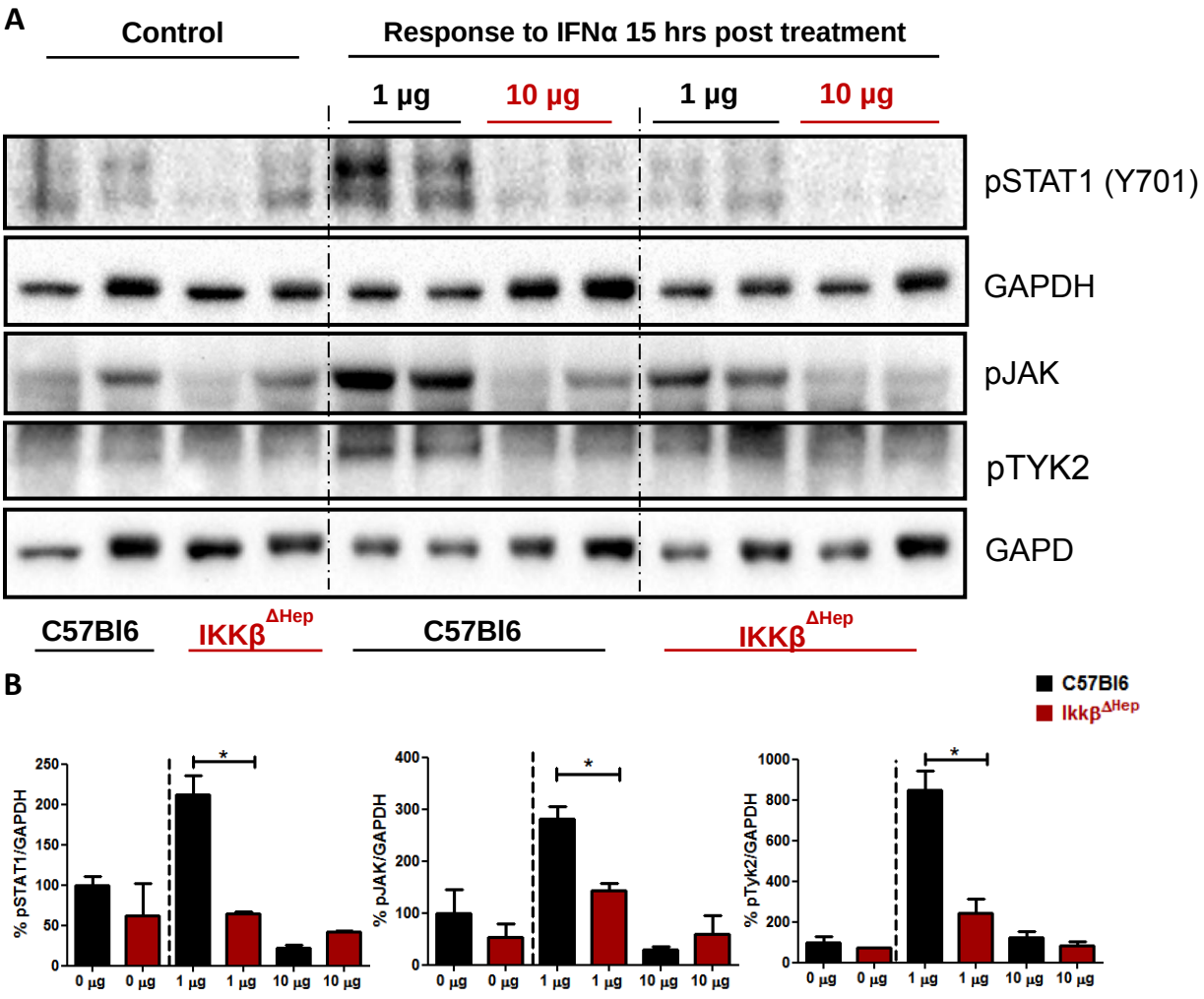

Figure 23: Measurement of interferon responsiveness in the livers of C57BL/6 and Ikkβ$^{\Delta Hep}$ mice. (A) C57BL/6 and Ikkβ$^{\Delta Hep}$ mice were subcutaneously infected with 2x10^{6} PFU of LCMV-WE (n=3). Hepatectomy was performed at indicated time points and western blotting was performed on the liver lysates using indicated antibodies. **(B)** Quantification of the immunoblots.

observation by repeating the treatments in three independent experiments and verified that hepatocytes from Ikkβ$^{\Delta Hep}$ mice are not losing their responsiveness to interferon which suggests that defective autocrine interferon signaling within the hepatocytes of Ikkβ$^{\Delta Hep}$ mice is the cause for observed decreases in interferon stimulated gene expression following LCMV infection.

5.6 Inability to control viral replication in the livers of Ikkβ$^{\Delta Hep}$ mice is hepatocyte-intrinsic

In order to determine whether the increase in virus titers in the livers of Ikkβ$^{\Delta Hep}$ mice was due to an inability to integrate signals from other cells or an intrinsic defect in the ability of Ikkβ$^{\Delta hep}$ hepatocytes to control viral replication, hepatocytes from Ikkβ$^{\Delta Hep}$ and C57BL/6 mice were isolated and infected with LCMV *ex vivo*. Supernatants from infected cells were tested for virus titers using plaque assays, and cell lysates were analyzed for the expression of viral proteins and ISGs. Interestingly, there was a significant increase in the expression of LCMV-NP in the hepatocytes derived from Ikkβ$^{\Delta Hep}$ mice accompanied by a decrease in the expression of ISGs (Fig. 24), which proves that hepatocyte intrinsic canonical NFκB signaling in hepatocytes is indispensable in controlling the viral infection.

5.7 Diminished adaptive immune responses in the livers of Ikkβ$^{\Delta Hep}$ mice

Efficient innate immune activation upon encountering a pathogen is an essential prerequisite for the further development of adaptive immune responses. All the infected cells including immune cells such as Kupffer cells readily secrete elevated levels of cytokines such as TNFα which is indicative of a systemic inflammation and enhance the host immune responses. In the context of viral infections, many cytokines dependent on NF-κB signaling such as IL-6 and IL-12 activate cell mediated cytotoxic responses (Orange and Biron, 1996; Pulliam et al., 1995).

When we tested the inflammatory parameters in the mouse by measuring the serum concentration for TNFα, we found it be significantly reduced in Ikkβ$^{\Delta Hep}$ mice compared to C57BL/6 starting from 18h p.i with LCMV (Fig. 25). Since Kupffer cells are thought to be the major TNFα–secreting source following LCMV infection in mice, a reduction in

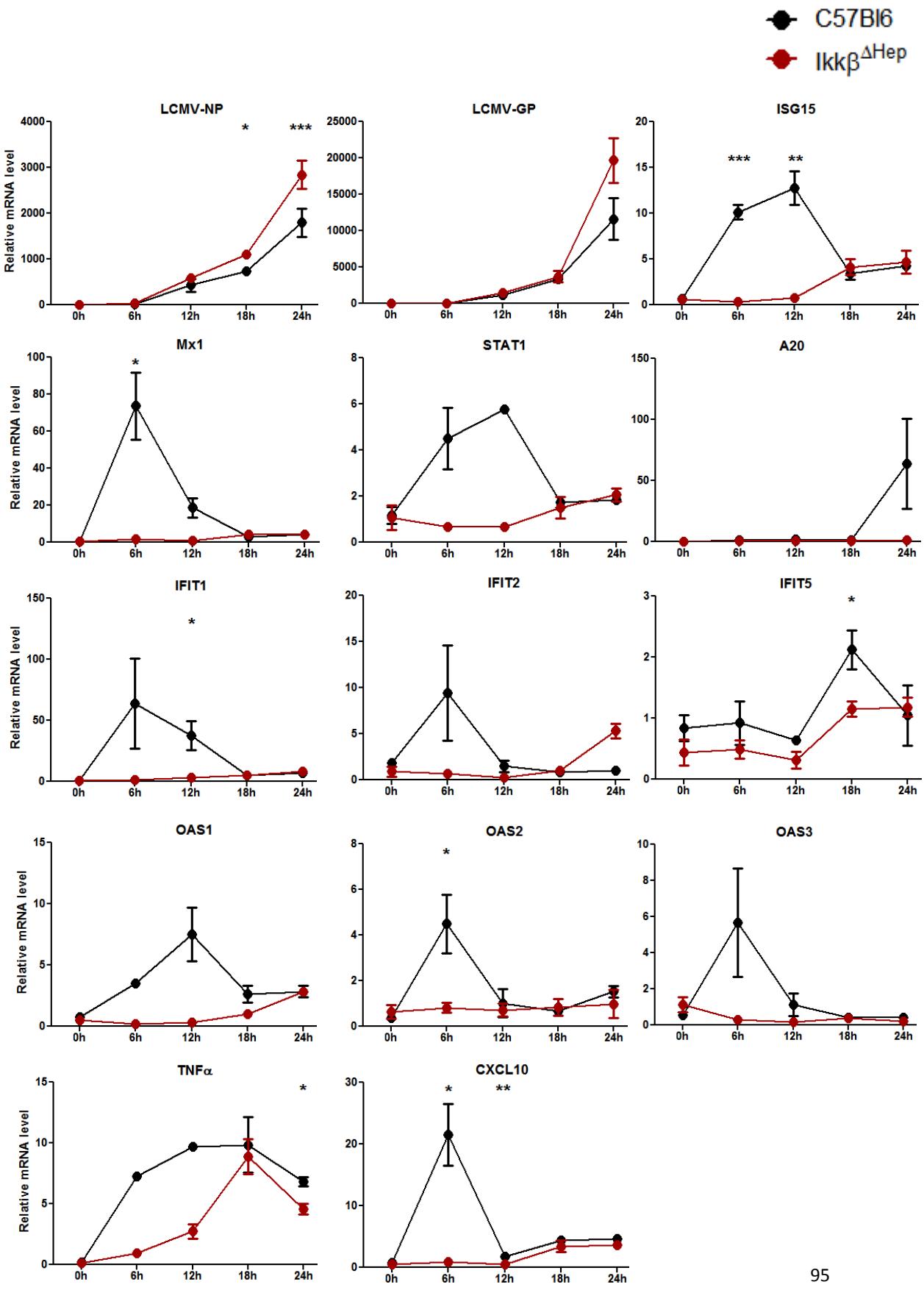
C57Bl6
Ikkβ ΔHep
LCMV-NP
LCMV-GP
ISG15
Mx1
STAT1
A20
IFIT1
IFIT2
IFIT5
OAS1
OAS2
OAS3
TNFα
CXCL10
Relative mRNA level
0h
6h
12h
18h
24h

Figure 24: Inability to control viral replication in the livers of Ikkβ$^{\Delta Hep}$ mice is hepatocyte intrinsic. Hepatocytes were isolated from C57BL/6 and Ikkβ$^{\Delta Hep}$ mice and infected *ex vivo* with LCMV-WE (MOI=1). RNA was extracted at the indicated time points from the cells and analyzed for the expression of LCMV NP and GP and other ISGs.

TNFα levels in Ikkβ$^{\Delta Hep}$ mice might indicate that hepatocytes themselves are capable of producing TNFα following infection or that an interaction between Kupffer cells and hepatocytes is essential for the proper initiation of the inflammatory reaction following a viral infection.

IL-6 is an important cytokine known to exhibit pleotropic functions such as mediating innate immune responses to pathogens, liver regeneration and metabolic control of the body (Hunter and Jones, 2015). IL-6 along with TNFα and IL-1 are classically elevated cytokines following inflammation. IL-6 expression following an inflammation binds to IL-6 receptor (IL-6R) that is expressed only on specific cells such as hepatocytes, macrophages, neutrophils and certain leukocytes (Norris et al., 2014). Later, IL-6 and IL-6R complex associate with gp130 protein resulting in intracellular signaling (Rose-John, 2012; Scheller et al., 2011). While the expression of IL-6R is limited to certain cell types, gp130 is expressed on most of the cell types but gp130 alone doesn't have a measurable affinity for IL-6. Certain cells such as monocytes, endothelial cells and hepatocytes secrete soluble form of IL-6R, sIL-6R that is comprised of the extracellular portion of IL-6R along with IL-6 has greater affinity to gp130 that is present on cells not expressing IL-6R (Wolf et al., 2014). The expression and propagation of IL-6 cytokine signaling to cells not expressing IL-6R is known as trans-signaling and is known to play a crucial role in mediating responses to viruses (Wang et al., 2015; Wang et al., 2013).

When we analyzed the levels of IL-6 in the serum of Ikkβ$^{\Delta Hep}$ mice compared to C57BL/6, we found a significant decrease in expression 18h p.i with LCMV indicating a decreased inflammatory status post infection (Fig. 25). IL-6 is also known to play an

important role in relaying the transition phase of innate to adaptive immunity. IL-6 along with TNFα modulates attraction of neutrophils in the initial phase of an acute inflammation. The proteolytic processing of IL-6R by the invading neutrophils induces IL-6 trans-signaling leading to the suppression of chemokines attracting neutrophils and enhancement of chemokines attracting monocytes such as CCL2, CXCL5 and T cell-attracting chemokines such as CCL4, CCL5, CXCL10 within a span of 24 to 48h (McLoughlin et al., 2005).

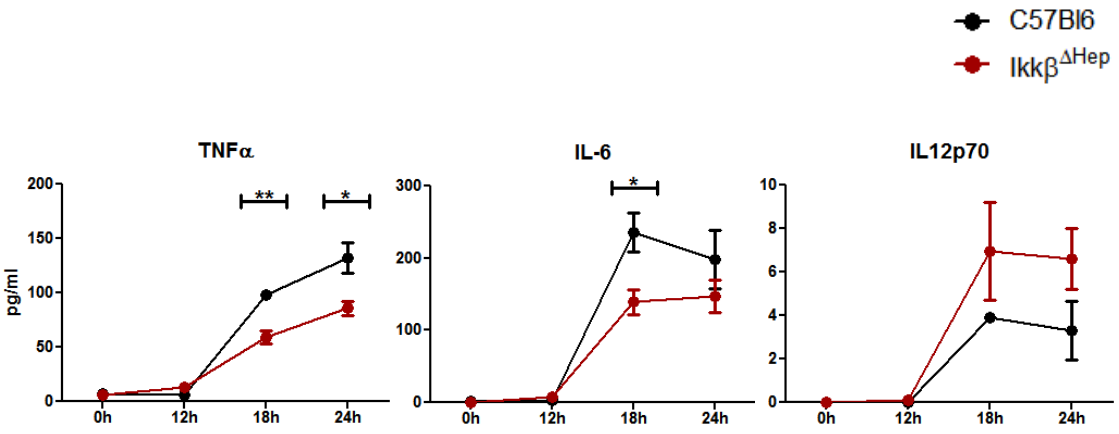

Figure 25: Expression of acute phase proteins in the serum of C57BL/6 and Ikkβ$^{\Delta Hep}$ mice. C57BL/6 and Ikkβ$^{\Delta Hep}$ mice were intravenously infected with 2x10^6 PFU of LCMV-WE (n=3). Serum was concentration of TNFa, IL-6 and IL-12p70 was analyzed through ELISA.

IL-12 is another important cytokine produced by activated macrophages, dendritic cells and neutrophils following activation by bacterial products such as LPS and intracellular pathogens. The active form of IL-12 also known as IL-12p70 is a heterodimer composed of p40 and p35 subunits. IL-12 is capable of inducing IFN-γ production, stimulating cell proliferation and inducing cytotoxicity mediated by NK cells and T cells and thus also involved in linking innate and adaptive immunity. In some cases of viral infections, type – I interferon are shown to inhibit the expression of IL-12 (Byrnes et al., 2001; Cousens et al., 1997) even though both interferons and IL-12 promote T cell proliferation.

When we verified the levels of IL-12p70 in the serum of Ikkβ$^{\Delta Hep}$ mice compared to C57BL/6, we could see that the levels of IL-12p70 are decreased in C57BL/6 mice at 18h and 24h p.i with LCMV (Fig. 25), probably by the inhibitory action of interferons following infection and the levels were found to be increased in Ikkβ$^{\Delta Hep}$ mice in which we observed a reduction in the interferon stimulated genes.

Apart from playing a crucial role in shaping innate immunity to viral infections by the production of ISGs, inducing APC maturation, activating other immune cells with cytotoxic functions such as NK cells, interferons are heavily involved in shaping adaptive immune responses. Type I IFNs enhance differentiation of monocytes to mature DCs mediated by GM-CSF (granulocyte-macrophage colony stimulating factor) (Paquette et al., 1998; Santini et al., 2000). Exposure of differentiated dendritic cells *in vitro* to type I IFNs results in the phenotypic maturation of DCs as evidenced by an increase in the expression of MHC class I and MHC class II molecules, an increase in the expression of co-stimulatory molecules such as CD40, CD80, CD83 and CD86 and an increase in the expression of CCR5, CCR7 and lymphocyte function associated antigen 1 (LFA1) known to facilitate DC migration into the draining lymph nodes (Crouse et al., 2015; Parlato et al., 2001; Rouzaut et al., 2010). IFNα specifically has also been shown to induce production of CXCL9 and CXCL10 in DCs which act as chemoattractants for T cells (Padovan et al., 2002). Interferons have also been shown to increase antigen presentation by extending MHC class II synthesis and antigen presentation (Simmons et al., 2012).

In view of these effector functions modulated by interferons, we tested if the expression of MHC-II is disturbed in the livers of Ikkβ$^{\Delta Hep}$ mice where we observed increased virus titers followed by decreased ISG expression upon infection with LCMV. We have observed that MHC-II expression is significantly reduced in the livers of Ikkβ$^{\Delta Hep}$ mice

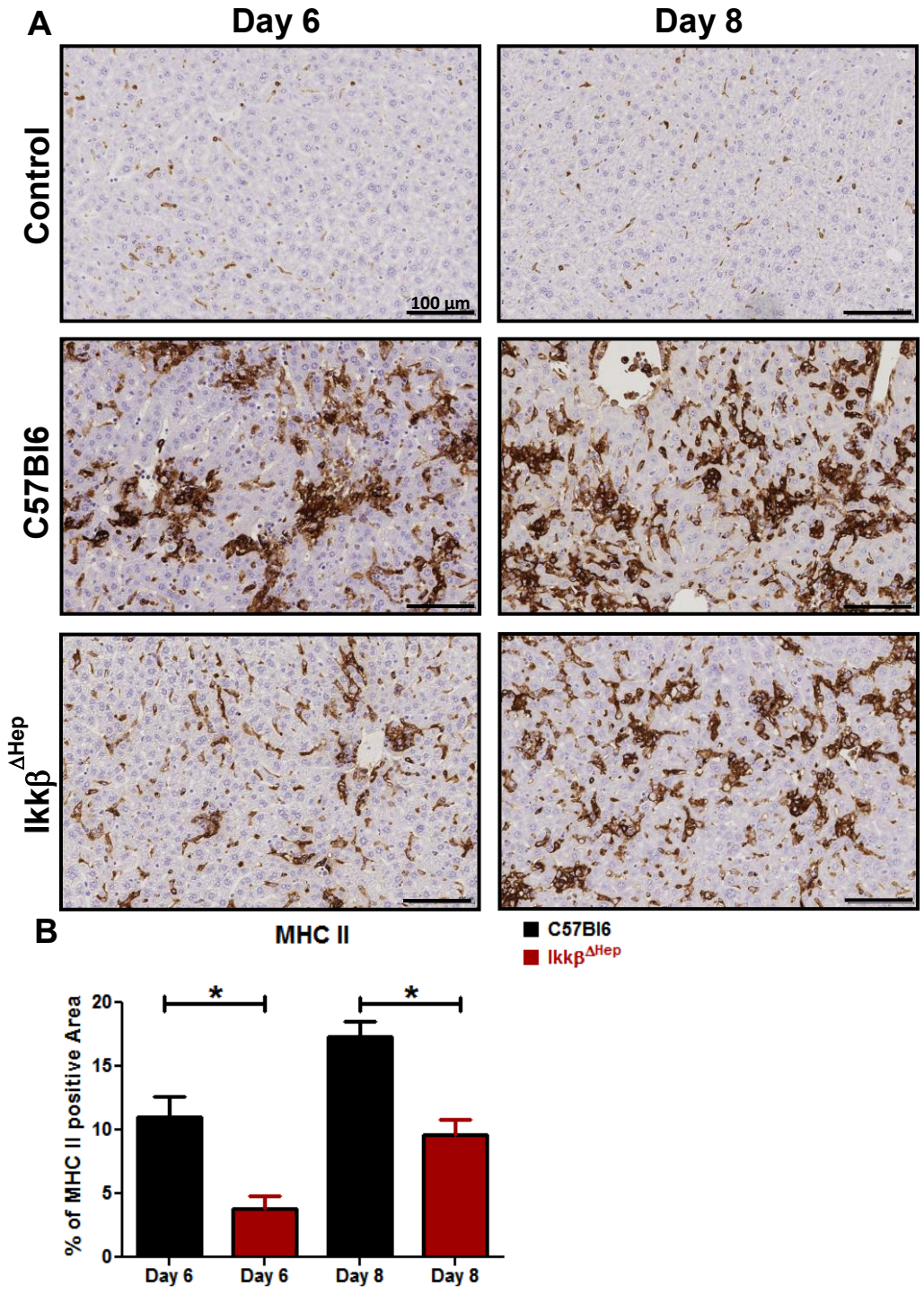

Figure 26: Reduced MHC-II expression in the livers of Ikkβ$^{\Delta Hep}$ mice. (A) C57BL/6 and Ikkβ$^{\Delta Hep}$ mice were intravenously infected with 2x10^{6} PFU of LCMV-WE (n=3). Hepatectomy was performed at indicated time points and paraffin embedded liver sections were subjected to Immunohistochemical analysis to observe the expression of MHC-II. (B) Quantification of MHC-II staining.

compared to C57BL/6 at both day 6 and day 8 (Fig. 26) indicating that NF-κB induced production of interferon expression within the hepatocytes facilitates a cross talk with innate immune cells of the liver such as Kupffer cells and dendritic cells.

Even though hepatocytes express only MHC-I under normal conditions, they also express MHC-II under the stress of inflammation. We wanted to verify if there is a defective infiltration in the number of F4/80 positive Kupffer cells in Ikkβ$^{\Delta Hep}$ mice which might be the cause of reduced in MHC-II levels. Through immuno histological stainings, we found that there was no change in the expression of F4/80-positive cells (Fig. 27) which might indicate that there is a reduced expression of MHC-II molecules by the hepatocytes in Ikkβ$^{\Delta Hep}$ mice as hepatocytes can express MHC-II under certain inflammatory conditions (Jenne and Kubes, 2013).

We further went on to verify if there are any changes in the humoral or cell mediated adaptive immune responses in Ikkβ$^{\Delta Hep}$ mice owing to a reduced interferon mediated expression of chemokines and ISGs. To this end, we verified the expression of B cell marker, B220, in the livers of C57BL/6 and Ikkβ$^{\Delta Hep}$ mice following LCMV infection. Interestingly, there is a significant difference in B cell infiltration in the livers from Ikkβ$^{\Delta Hep}$ mice (Fig. 28).

Owing to their adverse cytotoxic effects and the capacity to induce robust inflammation, activation of naive T cells is regulated at multiple steps and thus, proper activation of T cells requires three important signals. While T cell receptor engagement by the MHC molecules on APCs serves as *signal 1*, ligation of co-stimulatory receptors acts as *signal 2* which initiates the proliferation of naive T cells. However, for these cells to expand cells requires three important signals. However, for these cells to expand clonally, survive, differentiate to effector T cells and finally to memory T cells, they require specific cytokine signals known as *signal 3*. IL-12 and Type I interferons are

known to be the prominent cytokines acting as signal-3 to modulate $CD8^+$ T cell responses. Mouse derived naive $CD8^+$ T cells that are stimulated with an antigen (signal1) and co-stimulatory signals (signal 2) resulted in their limited proliferation and survival (Crouse et al., 2015; Curtsinger et al., 2003b) whereas the addition of IL-12 or type I interferons as a third signal is shown to promote clonal expansion and differentiation to effector $CD8^+$ T cells (Curtsinger et al., 2003a; Curtsinger et al., 2005).

A direct role for the type I interferons in survival and differentiation of antiviral T cells is established using wild-type and IFNAR-deficient mice in which LCMV specific $CD8^+$ and $CD4^+$ T cells were adoptively transferred to wild-type mice which were later infected with LCMV. Despite a similar early division, IFNAR-deficient T cells failed during the expansion phase compared to T cells derived from C57BL/6 mice (Crouse et al., 2014; Crouse et al., 2015; Keppler et al., 2012; Kolumam et al., 2005; Xu et al., 2014a). IFNα has also been shown to exert its signal-3 cytokine effect during the priming of human naive and antigen experienced CD8+ T cells (Hervas-Stubbs et al., 2010).

Based on these compelling data on the important role played by interferons in the control of cytotoxic T cell infiltration, we went further verified if the loss of hepatic NF-κB signaling would affect $CD8^+$ T cell infiltration by staining the livers of $Ikk\beta^{\Delta Hep}$ versus C57BL/6 mice following LCMV infection (Fig. 29). Quantification of these immuno histological analyses indicated a trend towards a decrease in $CD8^+$ area at Day 6 and a significantly reduced CD8+ area at 8 days post-LCMV infections in the livers of $Ikk\beta^{\Delta Hep}$ mice.

Cytolytic effector functions and IFN-γ secretion in $CD8^+$ T cells are induced by type I interferons (Curtsinger et al., 2005; Nguyen et al., 2002). The timing of IFNAR signaling

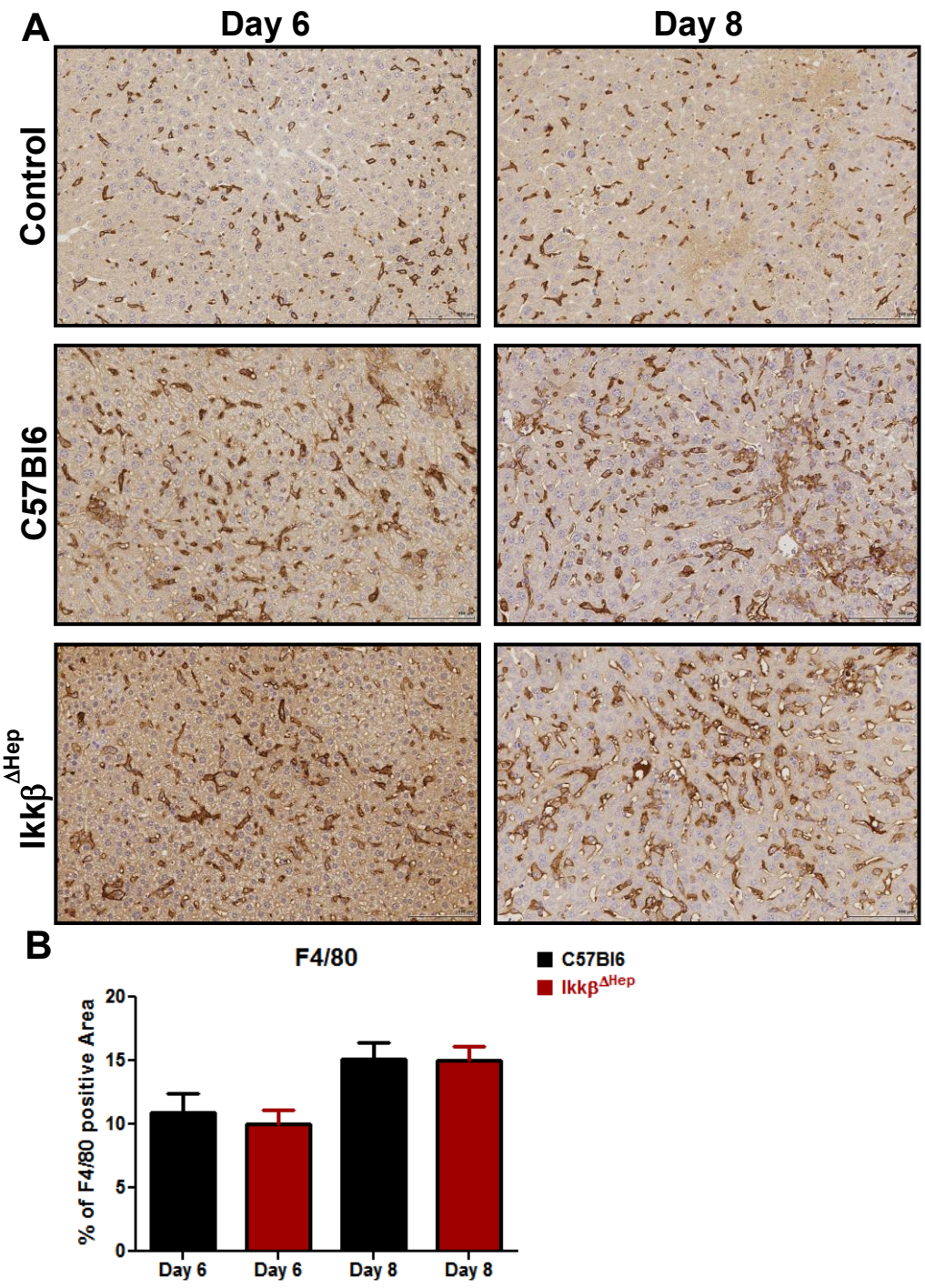

Figure 27. Abundance of Kupffer cell infiltrates in the livers of C57BL/6 and Ikkβ$^{\Delta Hep}$ mice. (A) C57BL/6 and Ikkβ$^{\Delta Hep}$ mice were intravenously infected with 2x10^{6} PFU of LCMV-WE (n=3). Hepatectomy was performed at indicated time points and paraffin embedded liver sections were subjected to Immunohistochemical analysis to observe the expression of F4/80. (B) Quantification of F4/80 staining.

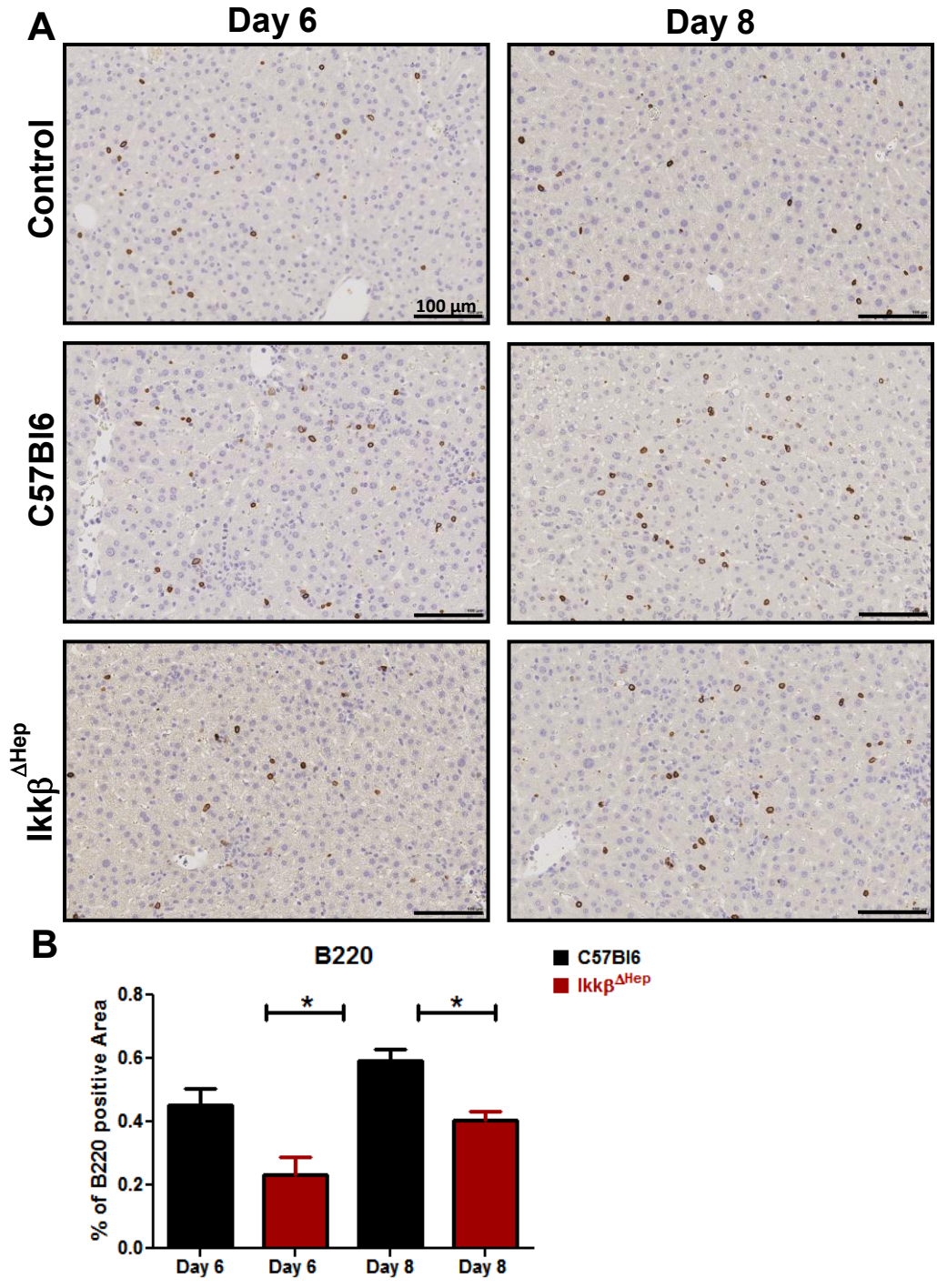

Figure 28. (A) **Reduced infiltration of B cells in the livers of Ikkβ$^{\Delta Hep}$ mice**. (A) C57BL/6 and Ikkβ$^{\Delta Hep}$ mice were intravenously infected with 2x10^6 PFU of LCMV-WE (n=3). Hepatectomy was performed at indicated time points and paraffin embedded liver sections were subjected to Immunohistochemical analysis to observe the expression of B220. (B) Quantification of B220 staining.

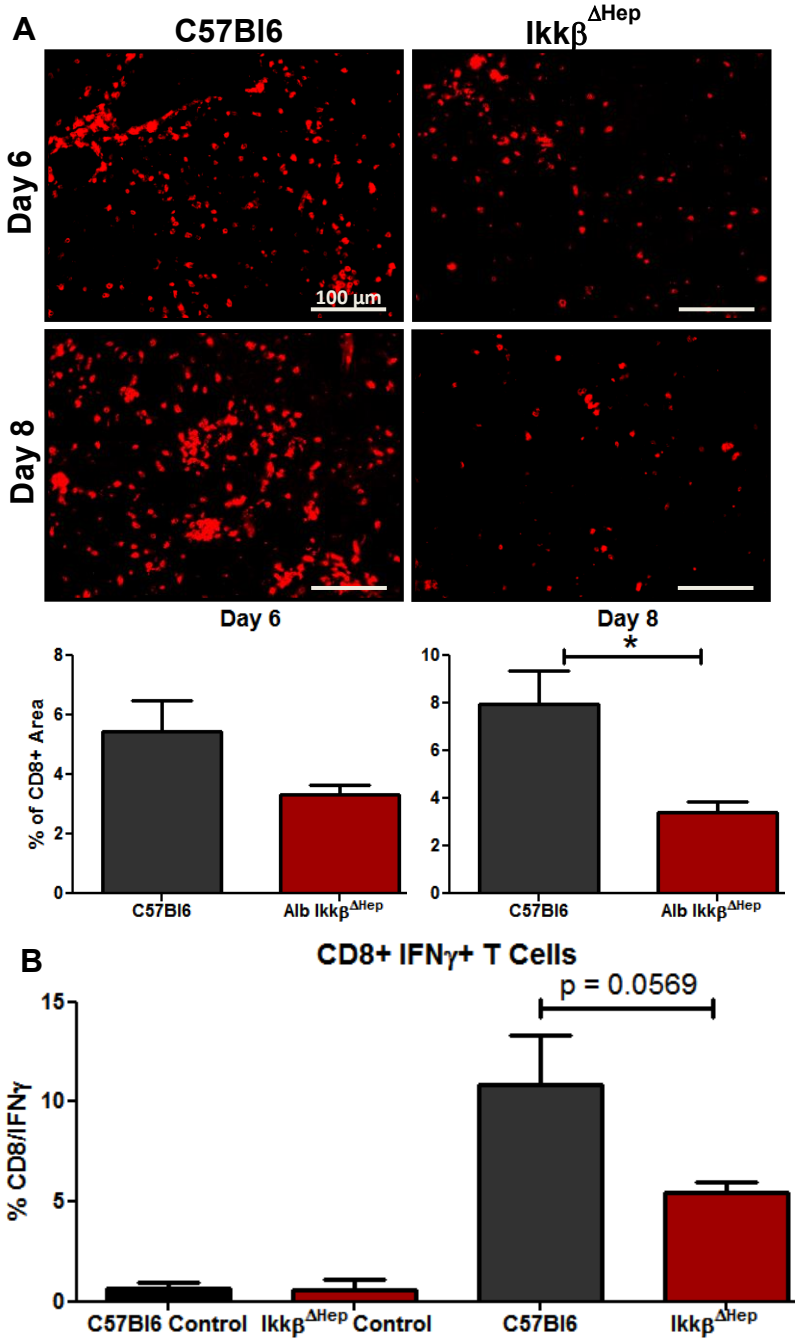

Figure 29. Dampened interferon responses in the livers of Ikkβ$^{\Delta Hep}$ mice results in diminished CD8^{+} T cell infiltration. (A) C57BL/6 and Ikkβ$^{\Delta Hep}$ mice were intravenously infected with 2x10^{6} PFU of LCMV-WE (n=3). Hepatectomy was performed at indicated time points and frozen liver sections were stained for CD8^{+} T cells. Representative images from indicated time points are shown. (B) Intracellular cytokine staining for IFNγ secreting activated T cells following LCMV specific gp33 peptide stimulation.

within $CD8^+$ T cells also act as signal-3 enabling T cells to acquire cytotoxic capabilities and cytokine secretion function, qualifying them to be effector T cells and subsequently attain memory cell formation (Curtsinger et al., 2003a; Xiao et al., 2009). In order to corroborate the reduction in $CD8^+$ T cell infiltration observed through immunohistochemistry, we did a FACS to measure the percentage of activated IFN-γ secreting effector $CD8^+$ T cells following LCMV specific gp33 peptide stimulation on the liver lysates of LCMV infected $Ikk\beta^{\Delta Hep}$ and C57BL/6 mice. Consistent with the results from immunohistochemistry, there was a clear difference in the percentage of effector $CD8^+$ T cells (Fig. 29).

These experiments identify a novel role for hepatocyte-specific NF-κB signaling in integrating parenchymal and immune cell crosstalk in the liver following a viral infection.

5.8 LCMV accumulates in hepatocytes from mice with defective hepatocyte-specific interferon signaling

The results we observed so far indicated that there is defective RelA translocation in $Ikk\beta^{\Delta Hep}$ mice following infection with LCMV compared to C57BL/6 mice. Furthermore, this defect in NF-κB signaling led to diminished interferon responses allowing a replicative advantage to the virus as evidenced by the increased virus titers in the livers of $Ikk\beta^{\Delta Hep}$ mice compared to C57BL/6. Corroborating the increased virus titers, we also observed that hepatocytes in $Ikk\beta^{\Delta Hep}$ mice form clusters filled with LCMV as evidenced by immunohistological staining for LCMV nucleoprotein. To further attribute these phenotypical differences in $Ikk\beta^{\Delta Hep}$ mice to defective hepatocytic NF-κB signaling and subsequent failure in interferon signaling, we compared the phenotype of livers of mice in which hepatocytes are lacking interferon signaling with an intact canonical NF-κB signaling. We hypothesized that defective hepatocytic NF-κB signaling and defective hepatocytic interferon signaling should yield a similar phenotype if hepatocyte-intrinsic

NF-κB signaling is crucial for modulating interferon responses, as indicated by our earlier experiments. In addition to that, we also wanted to verify the involvement of interferon signaling by the Kupffer cells in viral clearance.

To test this hypothesis, mice lacking IFNAR1 receptor in the hepatocytes, Alb-Cre x IFNAR$^{fl/fl}$ (IFNAR$^{\Delta Hep}$) or in Kupffer cells LysM-Cre x IFNARfl$^{/fl}$ (IFNAR$^{\Delta Kup}$), as well as C57BL/6 WT control mice were infected with LCMV. A staining for LCMV nucleoprotein (NP) in the livers of C57BL/6 has shown that the virus is efficiently cleared at day 6 and day 8 in C57BL/6 mice and we could hardly find any hepatocytes forming clusters (Fig. 30).

Consistent with our earlier observations, hepatocytes from Ikkβ$^{\Delta Hep}$ mice were not able to clear the virus, and we could observe hepatocytes forming clusters filled with LCMV (Fig. 30). Intriguingly, a LCMV-NP staining in the livers of IFNAR$^{\Delta Hep}$ mice showed a phenotype similar to Ikkβ$^{\Delta Hep}$ mice, marked by the formation of hepatocyte clusters filled with LCMV by day 6 p.i., supporting our hypothesis and earlier observations that hepatocyte intrinsic NF-κB signaling is required to induce an efficient early interferon response in mice leading to a viral clearance.

In the IFNAR$^{\Delta Kup}$ mice, both Kupffer cells and hepatocytes were strongly positive for LCMV NP at day 6, suggesting a total failure to control viral replication as Kupffer cells are not able to respond to the interferon signaling that they produce. However, the intensity of LCMV-NP in IFNAR$^{\Delta Kup}$ mice dramatically decreased in the hepatocytes by day 8 p.i, indicating that hepatocytes respond to the paracrine interferon signaling coming from the Kupffer cells or to the autocrine interferon signaling within the hepatocytes to clear virus infection (Fig. 30). This experiment highlights the role played by hepatocytes in controlling viral replication even when the Kupffer cells are defective in their responsiveness to interferon signaling.

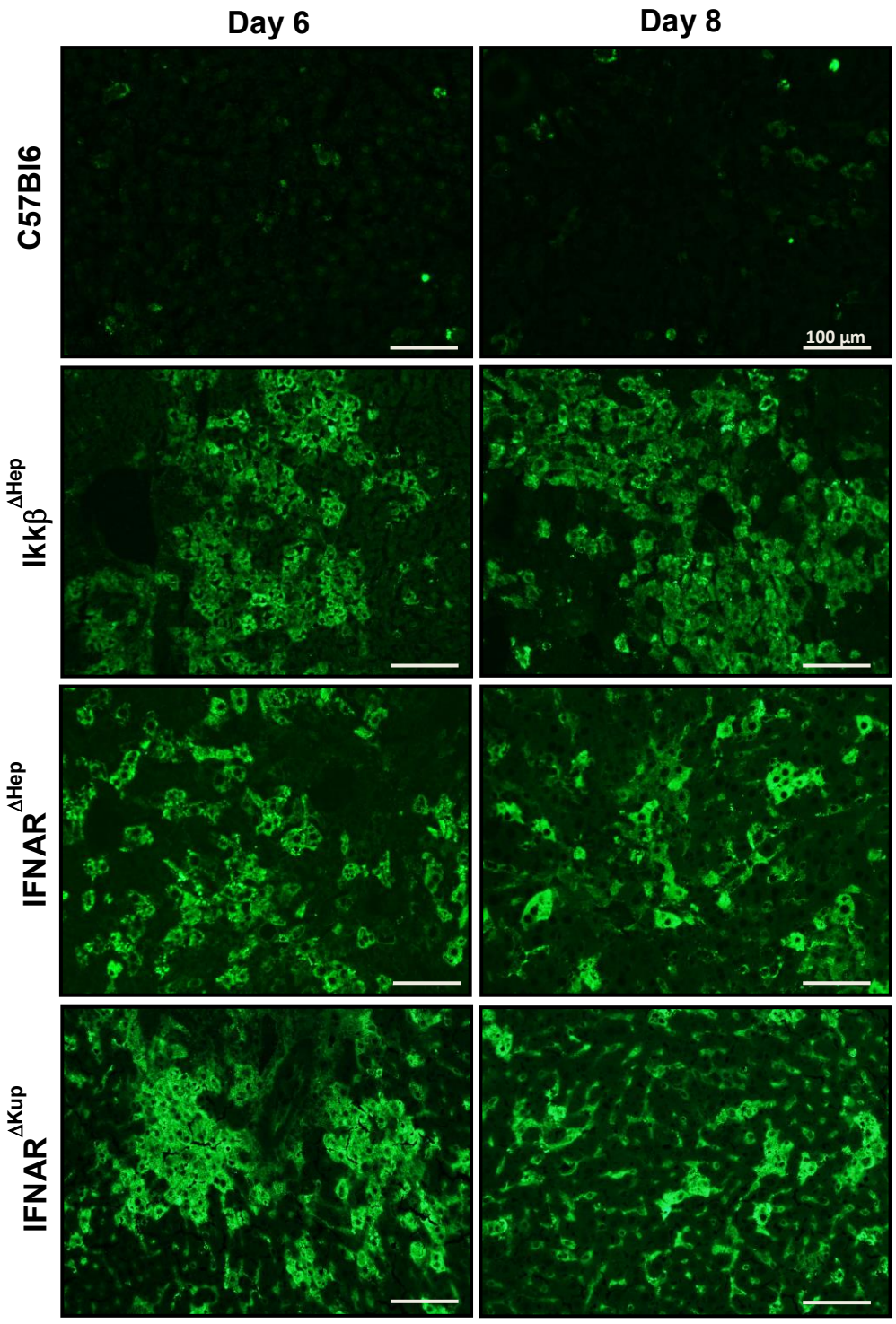

Figure 30. Comparative localization of LCMV in C57BL/6, Ikkβ$^{\Delta Hep}$, IFNAR$^{\Delta Hep}$ and IFNAR$^{\Delta Kup}$ mice. Mice with indicated genotype were intravenously infected with 2x10^6 PFU of LCMV-WE (n=3). Hepatectomy was performed at indicated time points and frozen liver sections were stained for LCMV-NP. Representative images from indicated time points are shown.

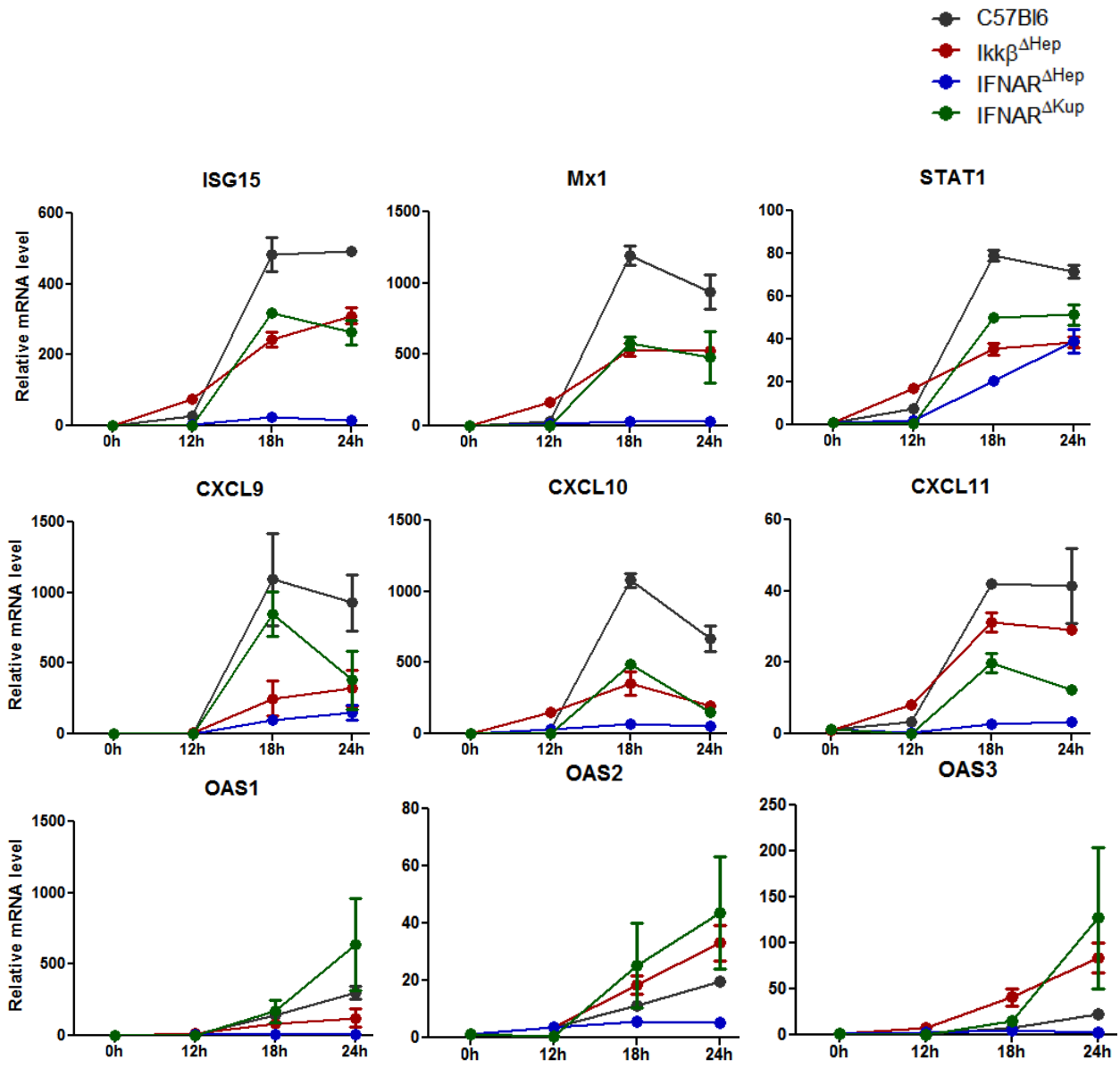

Figure 31. Hepatocytes are the major producers of ISGs in the liver. C57BL/6, Ikkβ$^{\Delta Hep}$, IFNAR$^{\Delta Hep}$ and IFNAR$^{\Delta Kup}$ mice were intravenously infected with 2x10^6 PFU of LCMV. Hepatectomy was performed at indicated time points and the livers were analyzed for the expression of interferon stimulated genes through qRT-PCR.

To further delineate the functional importance of hepatocyte-versus Kupffer cell-mediated interferon responses, whole liver lysates from C57BL/6 mice along with Ikkβ$^{\Delta Hep}$, IFNAR$^{\Delta Hep}$ and IFNAR$^{\Delta Kup}$ mice were analyzed for the expression of ISGs as early as 12h up to 24h post-infection with LCMV. The responses to early-induced ISGs

such as ISG15, Mx1 and STAT1 as well as interferon-induced chemokines such as CXCL10 are elevated by 18h p.i in C57BL/6 mice, whereas this upregulation is significantly blunted in Ikkβ$^{\Delta Hep}$ mice. Interestingly, ISG expression in IFNAR$^{\Delta Kup}$ mice decreased significantly compared to C57BL/6 mice, and the levels were similar to that of Ikkβ$^{\Delta Hep}$ mice for most of the genes tested (Fig. 31). Quite surprisingly, the extent of ISG expression in IFNAR$^{\Delta Hep}$ mice was negligible, as the hepatocytes in these mice are not responsive to the interferons. This clearly suggests that hepatocytes are the major cell type contributing for the expression of interferon stimulated genes following viral infections such as LCMV.

6. DISCUSSION

6.1 Prevalence of liver diseases and liver-mediated immunity

Persistent pathogenic infections of the liver pose a significant threat to global health. The liver, apart from being principally a metabolic organ is also fully engaged in modulating immune responses that are essential for the overall health of the body. Many pathogens such as malaria, HBV and HCV establish chronic hepatic infections by bypassing the classical T cell-mediated immunity and residing in the hepatocytes, causing chronic infections in the liver.

According to WHO reports in 2015, 95 countries across the world have an ongoing malaria transmission, and almost half of the world's population of about 3.5 billion people are at risk of malaria. About 3 million people die from malaria every year, including around 3000 children per day. Liver viral infections is also a global concern with more than 350 million people chronically infected with HBV and 1 million deaths per year arising from HBV-induced cirrhosis and liver cancer. Over 130 million people are chronically infected with HCV and are at risk of developing cirrhosis and liver cancer. Effective vaccination against HCV and malaria are still not available leading to an increase in the mortality rate in underdeveloped countries. In this regard, there is an increased demand to reexamine the mechanisms of liver disease, so that novel therapeutic strategies can be achieved.

The liver is constantly supplied with blood coming from both the hepatic artery and portal vein. Every minute, 30% of the total blood volume of the body passes through the liver. Blood coming through the portal vein flows directly to the liver, by bypassing the spleen and lymph nodes, the classical immune sentinel tissues of our immune system. Despite this bypassing, absence of chronic inflammation and infection by pathogen

derived molecules in the portal blood is a direct proof to the ability of the liver to immunologically screen for pathogens and filter the blood (Jenne and Kubes, 2013).

The initiation of innate immune responses in the liver requires the presence of pattern recognition receptors such as TLRs, endosomal cytosolic helicases and several other PRRs. In the liver, these immune receptors are not only found in the bone-marrow derived professional immune cells such as Kupffer cells, hepatic DCs, but also among the liver resident cells such as LSECS, stellate cells, and hepatocytes (Protzer et al., 2012) which have been shown to express multiple immune receptors, co-receptors, costimulatory, adhesion molecules, various TLRs. LSECS of the liver are important immune-sentinels acting as antigen presenting cells of non-myeloid origin. They constitutively express MHC I and MHC II molecules, co-stimulatory CD80 and CD86 molecules as well as adhesion molecules such as ICAM which enables them to interact with lymphocytes and present antigens directly to the T cells (Knolle and Limmer, 2003; Knolle et al., 1998; Limmer et al., 2000). LSECs also display phagocytic properties and have been shown to directly internalize antigens and cellular debris (Steffan et al., 1986). HSCs are another liver resident cell playing a crucial role on the immune platform with the expression of MHC I, MHC II, CD80 and CD86 capable of presenting antigens to T cells (Winau et al., 2007). HSCs can also directly activate naive lymphocytes (Muhanna et al., 2007). In addition to that, the liver has the largest population of Kupffer cells accounting for 80-90% of the total macrophages present in the body (Bilzer et al., 2006), as well as a large population of liver-specific dendritic cells and liver resident lymphocytes.

Hepatocytes are the parenchymal cells of the liver, accounting for 80% of all the liver cells (Racanelli and Rehermann, 2006) and primarily known to fulfil most of the metabolic functions associated with the liver. Hepatocytes express numerous innate

immune receptors and are functionally capable of inducing a potent immune response upon encountering pathogen associated molecular patterns (Seki and Brenner, 2008). Hepatocytes have also been shown to physically interact with naive T cells in an ICAM-I and MHC dependent manner and activate naive T cells. Hepatocytes constitutively express MHC-I, accessory molecules and under certain inflammatory conditions also express MHC-II and have been shown to play a clear role in the initiation of adaptive responses. (Bertolino et al., 2001; Bode et al., 2012; Franco et al., 1988; Warren et al., 2006). In the liver microenvironment, the role of hepatocyte-mediated activation of T cells is still unclear amidst the presence of APCs such as KCs, DCs and LSECs.

Immunity conferred by hepatocytes is not just restricted to the liver. By synthesizing acute phase proteins and complement components (Bode et al., 2012) hepatocytes induce a systemic immune response that is conserved in all vertebrates from mammals to fish (Lin et al., 2007). The ability of hepatocytes to fight against bacteria is well recognized by their ability to secrete antibacterial molecules such as lipocalin-2, hepcidin (Park et al., 2001; Xu et al., 2015).

Hepatocytic responses to viral infections have been intensively studied in cases of major human pathogens such as HCV. During HCV infection, viral RNA activates the hepatocyte-specific RIG-I system leading to the activation of MAVS which further activates IRF3 leading to type-I IFN production. At the same time, dsRNA of HCV is detected by TLR3 in hepatocytes leading to the activation of IRF3 mediated by TRIF. These antiviral mechanisms of hepatocytes are countered by NS3/4a protein of HCV that cleaves both TRIF and MAVS preventing IRF3 activation and subsequent IFN production (Ferreon et al., 2005; Foy et al., 2005). In such cases of viral immune subversion, innate immune functions of hepatocytes seem to be curtailed partially, increasing the importance of non-parenchymal cells (Crispe, 2016). Viral polymerase of HBV directly

inhibits hepatocyte STING DNA function (Liu et al., 2015). But *in vitro*, a direct stimulation of hepatocyte STING using a synthetic agonist has been shown to suppress HBV replication via type-I IFN secretion (Guo et al., 2015) highlighting the capability of hepatocytes to control viral infections. Also in cases of other Hepadnaviridal infections, primary woodchuck hepatocytes (PWHs) have been shown to downregulate woodchuck hepatitis virus (WHV) replication via interferon-independent pathways activated by TLR signaling (Zhang et al., 2009).

6.2 Ikkβ$^{\Delta Hep}$ mice establish that hepatocytes contribute to the clearance of systemic viral infections by eliciting interferon responses

A clear understanding of hepatocyte-specific immune responses during systemic viral infections would be helpful in developing novel antiviral therapeutics. While studying the innate immune responses to LCMV infections pertaining to the liver in mice, Kupffer cells have been shown to be the responsible cell type for capturing the virus and preventing spread to neighboring hepatocytes, which involved IFN-dependent mechanisms, and it was determined that hepatocytes are not essential for controlling LCMV replication (Lang et al., 2010).

Considering that the molecular signaling pathways involved in viral control operating within hepatocytes are similar to those in other immune cells, we aimed to elucidate the functional importance of hepatocytes in the context of systemic viral infections by using Ikkβ$^{\Delta Hep}$ mice in which stimulus-driven canonical NF-κB signaling, one of the major innate immune signaling pathway, is specifically blocked in the hepatocytes.

We initially compared the activation of RelA following infection using LCMV-WE in Ikkβ$^{\Delta Hep}$ mice and C57BL/6 mice and verified that hepatocytes from Ikkβ$^{\Delta Hep}$ mice with depleted Ikkβ, failed to induce RelA translocation following infection with LCMV-WE virus

whose replication intermediates are normally recognized by the PRRs like RIG-I (Hornung et al., 2006; Marq et al., 2011) as seen in wildtype C57BL/6 mice with normal hepatocytes. The requirement of Kupffer cells for inducing RelA translocation in hepatocytes was ruled out by clodronate-mediated depletion of Kupffer cells in C57BL/6 mice and verifying RelA translocation post-LCMV infection. In both Kupffer cell-depleted as well as control mice which received PBS liposomes, there was no change in the RelA translocation, clearly indicating that hepatocytes are independent of molecular cues from Kupffer cells to recognize the replication intermediates of viruses and activate NF-κB signaling, as in the case of other professional innate immune cells.

Looking into the functional consequences of lacking RelA translocation, we quantified the viral load in the livers and spleen of Ikkβ$^{\Delta Hep}$ mice compared to C57BL/6 following LCMV infection. We quantified a 2 log increase in the virus titers from the livers and spleens of Ikkβ$^{\Delta Hep}$ mice compared to C57BL/6 which is entirely surprising as a mere blockade of NF-κB signaling in the hepatocytes conferred an enhanced replicative advantage to the virus despite the presence of an intact NF-κB signaling in all the professional immune cells such as KCs and DCs as well as non-myeloid APCs such as LSECs and HSCs of the liver (Buttmann et al., 2007; Wu et al., 2010) which highlights the previously unrecognized role played by the hepatocytes in conferring interferon mediated resistance to systemic viral infections.

In addition to the increase in viral titers, we have also observed that the hepatocytes of Ikkβ$^{\Delta Hep}$ mice showed a peculiar pattern of forming clusters that are filled with virus which is rarely seen in the hepatocytes of C57BL/6 mice at day 6 and day 8 post-infection. Furthermore, the number of Kupffer cells that were infected in the Ikkβ$^{\Delta Hep}$ mice was also increased by day 8 p.i compared to the C57BL/6 mice which indicates that Kupffer cells alone cannot influence the dissemination of viral spread as previously reported (Lang et

al., 2010) and a cross talk between the hepatocytes and Kupffer cells might be essential for an efficient control of viral spread in the liver.

Viral infection results in the transcription of IFN-β in the host as soon as 4 hours following infection. NF-κB subunits along with IRF-1 are the first to bind to the core promoter of the interferon enhanceosome finally leading to the expression of interferon β (Maniatis et al., 1998; Thanos and Maniatis, 1995). NF-κB activation alone can confer interferon-independent innate antiviral responses against cytoplasmic RNA viruses (Bose et al., 2003). Response to interferon occurs within 30 minutes post-induction (Larner et al., 1986), and a delay in the induction of these responses could lead to an increased viral replication. It is well-established that Ikkβ mediates innate immune responses involving interferon signaling following detection of dsRNA in the cells (Chu et al., 1999). Ikkβ is essential to induce production of type-1 IFNA by pDCs (Pauls et al., 2012). RelA translocation is a prerequisite for basal expression of interferon genes which is crucial for the further enhancement of interferon signals following infection (Basagoudanavar et al., 2011) .

Considering these pivotal roles played by Ikkβ in inducing interferon responses, we verified the expression of interferon-stimulated genes from the livers of $Ikk\beta^{\Delta Hep}$ mice compared to C57BL/6. Interestingly, expression of antiviral ISGs such as Mx1 which through its GTPase activity counters viral growth even before the start of viral genomic replication (Haller et al., 2007; Sadler and Williams, 2008; Verhelst et al., 2012), IFIT proteins, members of the 2-5 A synthetase family, expression of interferon-induced chemokines such as CXCL9, CXCL10 and CXCL11 important for the chemo-attraction of monocytes, and other immune cells were all significantly reduced in the livers of $Ikk\beta^{\Delta Hep}$ mice compared to C57BL/6. The reduction in ISG expression verified through qPCRs was corroborated with a reduction in the expression of CXCL10 as evidenced by *in situ*

hybridization in the livers of Ikkβ$^{\Delta Hep}$ mice compared to C57BL/6. CXCL10 recruits activated T cells to the site of inflammation during bacterial and viral infections (Dufour et al., 2002; Khan et al., 2000; Liu et al., 2000) which supports our observation that there are a reduced number of effector T cells in Ikkβ$^{\Delta Hep}$ mice following LCMV-WE infection.

STAT1, along with STAT2 propagates the interferon signal to the nucleus leading to the production of interferon stimulated genes. In the livers of Ikkβ$^{\Delta Hep}$ mice, interferon induced phosphorylation of STAT1, as observed by immunohistochemical analysis, was found to be reduced in the hepatocytic compartment, whereas it was still strongly immune-positive expressed in the non-parenchymal cell compartment as compared to the livers if wildtype-C57BL/6 mice in which both hepatocytes and non-parenchymal cells exhibited equal levels of pSTAT1. pSTAT3, another interferon induced molecule which is not involved in the expression of ISGs is similarly expressed in the livers of both Ikkβ$^{\Delta Hep}$ and C57BL/6 mice indicating that the reduction in ISG expression in Ikkβ$^{\Delta Hep}$ mice is specific to the loss of Ikkβ in the hepatocytes.

Taken together, we could conclude that the major cell type that is contributing to the production of cytokines, chemokines and interferon-stimulated genes is the hepatocytes. Further infection of hepatocytes *ex vivo* derived from Ikkβ$^{\Delta Hep}$ mice and C57BL/6 resulted in a decreased interferon-stimulated gene expression with an increase in the expression of LCMV NP and GP subunits which establishes that the phenotype of reduced interferon responses and increased viral replication is the consequence of a hepatocyte intrinsic defect in canonical NF-κB signaling.

6.3 Reduced infiltration of CD8^{+} T cells in the livers of Ikkβ$^{\Delta Hep}$ mice proves the involvement of hepatocytes in mediating adaptive immune responses

Adaptive responses are shaped by the initial innate responses developed in the host upon encountering a pathogen. We found that as soon as 18h following infection with LCMV, there is a reduction in IL-6 expression, one of the important acute phase proteins in the serum of Ikkβ$^{\Delta Hep}$ mice compared to the serum from infected wildtype-C57BL/6 mice. Expression of IL-6 facilitates enhanced expression of chemokines such as CCL2, CXCL5 which attracts monocytes and chemokines such as CCL4, CCL5 and CXCL10 which are T cell-attracting chemokines within a span of 24h following infection. (McLoughlin et al., 2005). TNFα, another classically elevated cytokine following inflammation was also found to be decreased in the serum of Ikkβ$^{\Delta Hep}$ mice 18h p.i, indicating a poor induction of inflammation following infection with LCMV. Kupffer cells in the liver are considered to be the sole source of TNFα, but cultured rat hepatocytes have been shown to secrete TNFα in response to the pro-inflammatory cytokine IL-1β, elucidating a new source of TNFα in the liver (Yoshigai et al., 2014). These reports suggest that reduced production of TNFα in Ikkβ$^{\Delta Hep}$ mice when the professional immune cells are intact may be due to defective production of TNFα by hepatocytes. Interferon-stimulated gene production was also lower in Ikkβ$^{\Delta Hep}$ mice at earlier time points compared to WT-C57BL/6 mice which could lead to reduced maturation of APCs, activation of other immune cells such as cytotoxic T cells. Interferons are known to enhance the differentiation of monocytes to mature DCs and increases MHC-II expression and antigen presenting potential (Paquette et al., 1998; Santini et al., 2000; Simmons et al., 2012). Type I IFNs also act as signal 3 molecules in the activation of naive T cells leading their differentiation to effector and memory phases (Curtsinger et al., 2005).

In order to assess the adaptive immune responses at later time points, we verified the amounts of activated immune cells involved in antigen presentation by measuring the expression of MHC-II molecules and infiltration of $CD8^+$ T cells by immunohistochemistry. Sequential to the decrease in pro-inflammatory parameters and interferon stimulated genes, we observed a reduced expression of MHC-II and infiltration of $CD8^+$ T cells at day 6 and day 8 p.i. This decrease in $CD8^+$ T cell infiltration observed through staining was further corroborated by measuring the percentage of activated IFN-γ-secreting effector $CD8^+$ T cells following LCMV specific gp33 peptide stimulation on the livers lysates of LCMV infected $Ikk\beta^{\Delta Hep}$ and C57BL/6 mice.

These results confirm that Ikkβ deletion in the hepatocytes leads to a decreased inflammatory response and interferon stimulated gene expression early during infection culminating in a reduced adaptive immune responses and a subsequent increase in the virus titers in the livers of $Ikk\beta^{\Delta Hep}$ mice compared to C57BL/6.

6.4 Hepatocytes with a defective NF-κB signaling or a defective interferon response display a common phenotype following infection with LCMV

After observing that lack of Ikkβ would lead to an enhanced viral replication as a consequence of diminished IFN responses resulting in enhanced viral replication in the livers, we hypothesized that mice lacking defects in interferon signaling with an intact NF-κB signaling should yield a similar phenotype. Indeed, $IFNAR^{\Delta Hep}$ mice which lack interferon receptor selectively in the hepatocytes showed a similar phenotype of increased virus titers at day 6 and day 8 p.i with LCMV, as evidenced by staining for LCMV-NP. To further verify the role of Kupffer cells in conferring interferon signals to hepatocytes, we infected $IFNAR^{\Delta Kup}$ mice and observed that Kupffer cells as well as hepatocytes were unable to clear the virus initially at day6 and we could observe the

hepatocytes forming clusters as seen in Ikkβ$^{\Delta Hep}$ and IFNAR$^{\Delta Hep}$ mice which indicates that interferon responses from Kupffer cells are indispensable for controlling viral replication. Quite strikingly, we also observed that the number of hepatocytes forming clusters filled with LCMV were remarkably reduced by day 8 p.i in IFNAR$^{\Delta Kup}$ mice, indicating that hepatocytes respond to the paracrine cues coming from the Kupffer cells or were still able to respond to its own cell-intrinsic viral clearance mechanisms which highlights the contribution of hepatocytes in viral clearance even when the Kupffer cells are in a defective interferon responsive state.

6.5 Hepatocytes are the major cell type in the liver producing interferon stimulated genes following infection with LCMV-WE

When we analyzed the ISG expression following infection with LCMV in C57BL/6, Ikkβ$^{\Delta Hep}$, IFNAR$^{\Delta Hep}$ and IFNAR$^{\Delta Kup}$ mice with the aim of functionally delineating the requirement for interferon responses from the hepatocyte versus Kupffer cell compartment, we found that the ISGs such as such as Mx1, ISG15 and STAT1 required for controlling viral replication were elevated by 18h p.i in C57BL/6 mice but the expression was blunted in Ikkβ$^{\Delta Hep}$ and IFNAR$^{\Delta Kup}$ mice. However, quite strikingly, there was almost no expression of ISGs in the livers of IFNAR$^{\Delta Hep}$ mice where hepatocytes are lacking interferon receptors and thus are unresponsive to interferons, which implicates hepatocytes as the major cell type contributing to the expression of interferon-stimulated genes following viral infections such as LCMV.

The results in this study prove that, while the presence of Kupffer cells is required for optimal viral control, the NF-κB-induced interferon response within hepatocytes is crucial for triggering the downstream pathways required for the induction and maintenance of the interferon-mediated antiviral state. A defect in either canonical NF-κB signaling or interferon signaling in the hepatocytes leads to a decreased interferon-mediated antiviral

state in the whole organ resulting in enhanced viral replication despite the presence of functional Kupffer cells and its initial interferon cues. While Kupffer cells might be essential in responding to the viral infection early during infection, hepatocytes further enhance the expression of ISGs enabling efficient viral clearance as observed in Ikkβ$^{\Delta Hep}$ mice or IFNAR$^{\Delta Hep}$ mice.

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8. CURRICULUM VITAE

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Publications List	Enclosed in the list of publications.

List of publications

1. **Sukumar Namineni**, Tracy O'Connor, Pal Johannsen, Prashant Shinde, Aleks Pandyra, Piyush Sharma, , Zeinab Abdullah, Dirk Wohlleber, Percy Knolle, Philipp Lang, Karl Lang, Mathias Heikenwalder.

   The Role of Hepatic NF-κB Signaling in Viral Clearance. (Manuscript under preparation, to be submitted to The Journal of Experimental Medicine)

2. **IFN-γ Hinders Recovery from Mucosal Inflammation during Antibiotic Therapy for Salmonella Gut Infection.** Cell Host Microbe. 2016 Aug 10;20(2):238-49.

3. **Lymphotoxin, NF-κB, and Cancer: The Dark Side of Cytokines.** (Dig Dis. 2012;30(5):453-68)

4. **The multifunctional PE_PGRS11 protein from Mycobacterium tuberculosis plays a role in regulating resistance to oxidative stress.** J Biol Chem. 2010 Oct 1;285(40):30389-403.

5. Halime Kalkavan, Piyush Sharma, Stefan Kasper, Iris Helfrich, Aleksandra A. Pandyra, Asmae Gassa, Isabel Virchow, Lukas Flatz, Tim Brandenburg, **Sukumar Namineni**, Mathias Heikenwalder, Bastian Höchst, Percy Knolle, Guido Wollmann, Dorothee von Laer, Ingo Drexler, Jessica Rathbun, Paula Cannon, Stefanie Scheu, Jens Bauer, Dieter Häussinger, Gerald Willimsky, Max Löhning, Dirk Schadendorf, Sven Brandau, Martin Schuler, Philipp A. Lang and Karl S. Lang.

   Spatiotemporal restricted arenavirus replication induces immune surveillance and type I interferon-dependent tumor regression. (Manuscript submitted to Nature Medicine).

6. Prashant Shinde, Haifeng C. Xu, Sathish Kumar Maney, Andreas Kloetgen, **Sukumar Namineni**, Nicolas Bellora, Mirko Trilling, Vitaly I Pozdeev, Nico van Rooijen, Klaus Pfeffer, Sujitha Duggimpudi, Jessica Höll, Arndt Borkhardt, Percy Knolle, Mathias Heikenwalder, Jürgen Ruland, Tak W. Mak, Dirk Brenner, Aleksandra A. Pandyra, Dieter Häussinger, Karl S. Lang, and Philipp A. Lang.

   TNF mediated survival of CD169^{+} cells mediate innate and adaptive immune activation during viral infection. (Manuscript submitted to Nature Communications)**.**

7. Katharina Borst, Theresa Frenz, Stefan Lienenklaus, Mario Köster, **Sukumar Namineni**, Mathias Heikenwälder, Gerd Sutter, Ulrich Kalinke.

   Local IFN-I responses orchestrate cytokine responses and confer protection upon vaccinia virus infection. (Manuscript under preparation)

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