Elucidating the molecular mechanisms of oxidative damage in viral hepatitis

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Submitted by

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Declaration

The following thesis has been written in a cumulative format and includes one shared first author publication. *In vitro* experiments were performed by me at the CeMM Research for Molecular Medicine of the Austrian Academy of Sciences. I carried out the animal experiments at the Department for Biomedical research of the Medical University of Vienna.

The contributions of other people is described as follows:

Ahmed N. Hegazy carried out *in vivo* experiments at the Charité animal facility in Berlin and at the animal facility of the Max Planck Institute for Infection Biology in Berlin, Germany. He also contributed in analyzing FACS data and writing the manuscript. Nikolaus Deigendesch, Lindsay Kosack, Andrea Hildebrandt, Bojan Vilagos, Christopher Schliehe, Kseniya Khamina, Hatoon Baazim, Isabelle Arnold, Chris Haifeng Xu, Philipp A. Lang and Isabel Panse conducted *in vitro* or *in vivo* experiments and/or provided technical assistance. Richard Kumaran Kandasamy performed the bio-informatical analysis. Jovana Cupovic and Burkhard Ludewig performed the Mouse Hepatitis Virus experiments at Cantonal Hospital, St. Gallen, Switzerland. Doron Merkler and Anja A. Kühl carried out the histology. Giulio Superti-Furga, Jacques Colinge, Akinori Takaoka, Lukas Flatz and Max Löhning provided reagents and/or contributed in critically reviewing the manuscript.

The shared first author, Ahmed N. Hegazy is currently a Post-doctoral Fellow at University of Oxford and will/did not use any content of this thesis for the purpose of Dissertation.

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Abstract

Viral hepatitis is a primary cause of mortality due to liver diseases worldwide. It manifests with a complex pathogenesis that involves immune-related events and altered cellular and tissue homeostasis, both of which eventually drive liver damage. The immunological response of the host to the viral infection involves various cellular populations and mediators. The altered cellular homeostasis, on the other hand, refers to disruptions in the physiological state of the cell. This includes, for example, an imbalance in the cellular redox state, a consequence of which would be oxidative stress. One of the important factors implicated in the pathology of viral hepatitis is oxidative stress. However, there are several aspects till date that are not clearly understood, for example, what molecular mechanisms are responsible for initiating oxidative stress and what roles do the host anti-oxidative systems such as superoxide dismutases (SODs) play in protecting the host from liver damage.

We describe a new concept of how type I interferon (IFN-I), a branch of the innate immune system induces oxidative stress and subsequently liver damage. We further identified the key antioxidant enzyme superoxide dismutase 1 (SOD1) as an essential host factor that prevented oxidative stress and hepatitis. Upon viral infection of the liver, we observed dysregulation of redox pathways, which included downregulation of SOD1. *Sod1*$^{-/-}$ mice suffered from exacerbated hepatitis compared to wild type mice post viral infection, which was ameliorated upon administration of antioxidant. Type I interferon (IFN-I) downregulated *Sod1* in wild type mice on a transcriptional level and was sufficient to cause oxidative damage in the livers of *Sod1*$^{-/-}$ and wild type mice in the absence of infection. Moreover, both WT and *Sod1*$^{-/-}$ mice were protected against virus-induced hepatitis upon blocking IFN-I signaling. These results provide a new concept of innate immunity-driven immunopathology, connecting IFN-I signaling with redox homeostasis and tissue damage.

Zusammenfassung

Virale Hepatitis zählt zu den häufigsten Todesursachen, die durch Lebererkrankungen verursacht werden. Sie zeichnet sich durch eine komplexe Pathogenese aus, die sowohl durch Mechanismen des Immunsystems als auch durch Veränderungen in der zellulären und gewebsspezifischen Homöostase ausgelöst wird und letzten Endes zu einem schweren Leberschaden führt. Die durch das Virus ausgelöste Immunpathologie wird in erster Linie durch Zellpopulationen und Botenstoffe ausgelöst, die ein natürlicher Bestandteil der antiviralen Immunantwort sind. Eine Veränderung in der Homöostase entsteht durch Ungleichgewichte im physiologischen Zustand der beteiligten Zellen. Beispielsweise kann das intrazelluläre Redox-Gleichgewicht verändert werden, was zu einer erhöhtem oxidativen Stress führen kann. Es wurde gezeigt, dass solch oxidativer Stress ein wichtigen Parameter ist, der zur Pathologie einer viralen Hepatitis beiträgt. Trotz dieser Erkenntnis sind die molekularen Mechanismen, die zu einer Zunahme an oxidativem Stress während einer viralen Infektion führen, sowie die Rolle der anti-oxidativen Schutzmechanismen des Wirtes, beispielsweise den Superoxid-Dismutasen (SODs), bisher wenig verstanden.

In dieser Arbeit zeigen wir einen bisher nicht beschriebenen Mechanismus, wie Typ I Interferon (IFN-I), welches ein Botenstoff des angeborenen Immunsystems ist, zu oxidativem Leberschaden führen kann. Wir haben außerdem das anti-oxidative Enzym Superoxid-Dismutase 1 (SOD1) als den essentiellen Wirtsfaktor identifiziert, der die Leber während einer viralen Infektion vor oxidativem Stress schützt. Nach einer viralen Infektion der Leber haben wir beobachtet, dass dies zu einer Herunterregulation von SOD1 führt. *Sod1*$^{-/-}$ Mäuse zeigten nach einer viralen Infektion erhöhte Entzündungsreaktionen und erhöhten Leberschaden. Dieser konnte durch Behandlung der Mäuse mit einem Antioxidans rückgängig gemacht werden. Die Behandlung von Wildtyp-Mäusen mit IFN-I verringerte die Expression von SOD1 in der Leber und führte in nichtinfizierten *Sod1*$^{-/-}$ und Wildtyp-Mäusen zu einem oxidativen Leberschaden. Zusätzlich hat eine Blockade der IFN-I-Signalwege dazu geführt, dass sowohl Wildtyp- als auch *Sod1*$^{-/-}$ Mäuse nach viraler Infektion vor Leberschaden geschützt waren. Aus den Ergebnissen dieser Arbeit leitet sich ein neues Konzept einer vom angeborenen Immunsystem-induzierten Immunpathologie ab, welches IFN-I-Signalwege auf neuartige Weise mit der Redox-Homöostase und Gewebeschaden in der Leber in Zusammenhang bringt.

Publication arising from this thesis

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ABBREVIATIONS

8-OHdG	8-hydroxy-2'-deoxyguanosine
ALT	Alanine Aminotransferase
ATM	Ataxia Telangiectasia mutated
cGAS	cytosolic GAMP synthase
CMV	Cytomegalovirus
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DAI	DNA-dependent activator of IFN-regulatory factors
DNA	Deoxyribonucleic Acid
EBV	Epstein Barr Virus
ERO1	Endoplasmic Reticulum oxidoreductin1
GAF	Interferongamma-activating factor
GP	Glycoprotein
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HCC	Hepatocellular carcinoma
HIV	Human Immunodeficiency Virus
HSV	Herpes Simplex Virus
IFN-I	Type I interferon
IFNAR	Interferon-alpha/beta receptor
IL-2	Interleukin 2
IL-10	Interleukin 10
IRF	Interferon regulatory factor
ISG	Interferon stimulated gene
ISGF3	Interferon stimulated gene factor 3
JAK	Janus kinase
LCMV	Lymphocytic Choriomeningitis Virus
LSECs	Liver Sinusoidal Endothelial Cells
MAPK	Mitogen Activated Protein Kinase
MAVS	Mitochondrial Antiviral Signaling Protein
MDA-5	Melanoma Differentiation Associated protein 5
MHC	Major Histocompatibility Complex
mTOR	mammalian Target Of Rapamycin
NADPH	Nicotinamide Adenine Dinucleotide phosphate
NK cells	Natural Killer cells
NOD	Nucleotide-binding oligomerization domain

NP	Nucleoprotein
PAMP	Pathogen Associated Molecular Pattern
PD-1	Programmed cell death protein 1
PDL-1	Programmed Death Ligand 1
PI3K	Phosphoinositide 3-kinase
PRR	Pattern Recognition Receptor
PTEN	Phosphatase and Tensin Homolog
RIG-I	Retinoic acid Inducible Gene I
ROS	Reactive Oxygen Species
SOD	Superoxide dismutase
STAT	Signal Transducer and Activator of Transcription
STING	Stimulator of Interferon Genes
TCR	T cell receptor
TGF-β	Transforming Growth Factor beta
Tim-3	T-cell immunoglobulin and mucin-domain containing-3
TLR3	Toll-Like Receptor 3
TYK	Tyrosine Kinase
VZV	Varicella Zoster Virus

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1. Introduction

Infectious diseases pose a great burden on health worldwide (Fauci, 2001). They bring about tissue damage in patients and thereby cause significant mortality and morbidity (Fauci & Morens, 2012). The host tries to protect oneself against invading pathogens by mounting an immune response. Often, these immune responses comprise inflammatory events, which ideally subside once the pathogen is cleared by the host. Inflammation, thus, when self-limited, is beneficial to the host. On the other hand, if unchecked inflammation continues, the host suffers from tissue damage. Inflammation can thus be described as a two-edged sword, which can have both beneficial and detrimental effects on the host and therefore needs to be tightly regulated (Chovatiya & Medzhitov, 2014; Kotas & Medzhitov, 2015; Okin & Medzhitov, 2012).

Viral hepatitis is one such example of inflammation of the liver, which can manifest either as an acute or a chronic infection (Rehermann & Nascimbeni, 2005). Hepatitis B or Hepatitis C viruses can cause chronic viral hepatitis in man. The liver damage inflicted upon chronic inflammation could eventually progress towards liver fibrosis, cirrhosis or liver cancer, also known as hepatocellular carcinoma (Rehermann & Nascimbeni, 2005). The pathogenesis has been attributed to several factors emanating from the virus itself or the host (Park & Rehermann, 2014; Rehermann, 2013). Yet, a detailed thorough understanding of the molecular mechanisms behind the pathology of viral hepatitis is lacking. This thesis describes a hitherto unknown phenomenon whereby type I interferon signaling, a branch of the innate immune system leads to liver damage by inducing oxidative stress. Therefore, the introduction section of this thesis has been divided into the following sub-chapters:

1.1 The liver: the main organ of interest in this study

1.2 Oxidative stress: the factor responsible for liver pathology in this study

1.3 Viral hepatitis: the disease of interest and relevance in this study

1.4 Type I interferon: the primary player that induces oxidative stress and therefore leads to subsequent liver damage

1.5 Lymphocytic choriomeningitis virus: the disease model used in this study

1.1 The liver

The liver is a vital organ performing important metabolic, detoxification and immunoregulatory functions in the body (Knolle & Thimme, 2014; Protzer et al, 2012). It acts as a site for break down as well as synthesis of complex macromolecules like carbohydrates, proteins and lipids. Additionally the liver is rich in

several enzymes such as the cytochrome P450 enzymes that help in eliminating toxins and other foreign substances like drugs from the body (Taub, 2004).

The liver can carry out these functions efficiently owing to its microstructure (**Figure 1**) (Heymann & Tacke, 2016; Knolle & Thimme, 2014; Robinson et al, 2016). A major part of the liver comprises cells called hepatocytes to which its metabolic functions can be largely attributed. The other cell populations include the Kupffer cells or the liver resident macrophages, the hepatic stellate cells (HSCs) and the Liver Sinusoidal Epithelial Cells (LSECs) (Heymann & Tacke, 2016; Malarkey et al, 2005; Racanelli & Rehermann, 2006; Robinson et al, 2016). The liver is richly supplied with blood vessels and a network of sinusoids that bring in blood from the gut. This, therefore, allows continuous interactions between circulating cells in the bloodstream, primarily lymphocytes and cell populations located *in situ* in the liver or in the hepatic sinusoids (Hotamisligil, 2006; Jenne & Kubes, 2013; Knolle & Thimme, 2014). The liver sinusoids are structured such that the blood flowing through the sinusoids does not come directly in contact with the hepatocytes. The Kupffer cells and the LSECs are the initial cell populations that encounter molecules borne by the bloodstream and deliver them to the hepatocytes via transcytosis. The HSCs on the other hand play an important role in liver fibrosis and produce cytokines such as Transforming Growth Factor β (TGFβ) (Knolle & Thimme, 2014; Malarkey et al, 2005; Protzer et al, 2012; Weiskirchen & Tacke, 2014).

The leukocytes present in the blood flowing through the sinusoids get ample time to interact with the endothelial cells or the Kupffer cells owing to the narrow width of the liver sinusoids. The leukocytes can carry out their effector function directly at the site. They can also extravasate and migrate into the parenchyma via the Space of Dissé. Thus the liver acts as a site for close cross talk between immune and metabolic responses (Hotamisligil, 2006; Robinson et al, 2016).

The liver is continuously exposed to products of microbial origin owing to the blood supply via the portal vein. It therefore possesses immunoregulatory functions to prevent tissue damage due to unchecked inflammation and overt immune activation (Holz et al, 2008; Jenne & Kubes, 2013; Thomson & Knolle, 2010). All in all, the liver microenvironment and the diverse cell populations comprising it govern the hepatic immune responses. This is clinically relevant with respect to infections in the liver such as chronic viral hepatitis caused by Hepatitis B virus (HBV) or Hepatitis C virus (HCV) in humans (Holz et al, 2008; Protzer et al, 2012; Racanelli & Rehermann, 2006; Robinson et al, 2016).

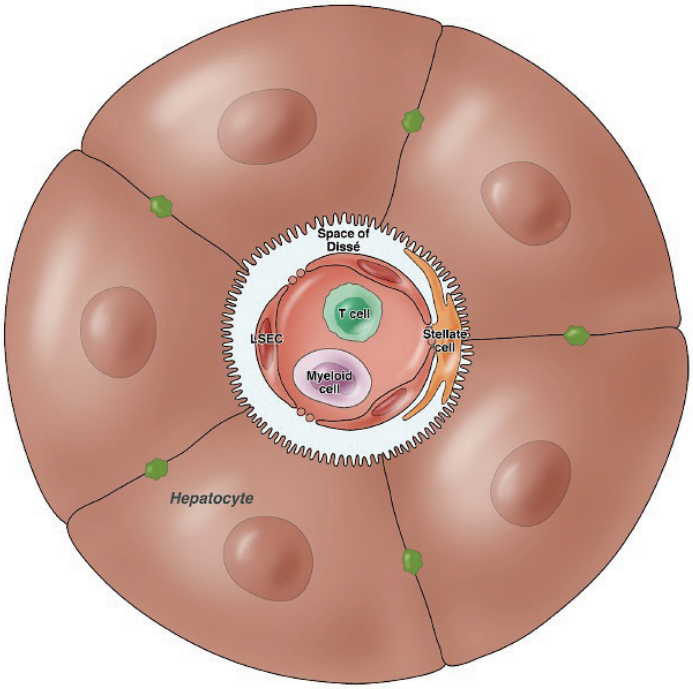

Figure 1: Microarchitecture of the liver. Schematic representation of the microarchitecture of the liver sinusoid is shown. Hepatocytes are the major cell types comprising the liver. Blood passing through the sinusoids is separated from the hepatocytes by stellate cells, Kupffer cells and LSECs. Image taken from (Knolle & Thimme, 2014)

1.2 Oxidative Stress

Oxidative stress refers to the phenomenon where excessive accumulation of highly reactive radicals, ions or molecules collectively called as Reactive Oxygen Species (ROS) takes place when the host is incapable of efficiently eliminating them. ROS comprise diverse kinds of radicals and ions such as superoxide anion, hydroxyl radical, singlet oxygen, hypohalous acids, hydrogen peroxide and organic peroxides (Brieger et al, 2012; Finkel, 2011; Jomova et al, 2010; Lugrin et al, 2014; Nathan & Ding, 2010; Sies, 2007). ROS can be generated in the cell due to exogenous and endogenous sources. Exogenous sources include electromagnetic radiations such as gamma rays, ultraviolet radiation, air pollutants, several drugs and smoke. Endogenous ROS can be generated within the cell as a by-product of aerobic respiration. The two major endogenous sources of ROS are the NADPH oxidases (NOXs) and the mitochondrial electron transport chain (ETC) (Brunelle et al, 2005; Finkel, 2012; Lambeth, 2004). Other endogenous sources of ROS are the

flavoenzyme Endoplasmic Reticulum oxidoreductin1 (ERO1), xanthine oxidase, lipoxygenases, cyclooxygenases, cytochrome P450 enzymes and nitric oxide synthases (Brieger et al, 2012; Droge, 2002; Finkel, 2011; Nathan & Cunningham-Bussel, 2013). Previously, the term ROS was used almost interchangeably with oxidative stress. But it is getting clearer to us with each passing day that two different aspects of ROS exist. They play a wide range of roles in signaling and homeostasis, which is currently known as redox biology or redox signaling. However, if their production is uncontrolled and they accumulate at a wrong location over a long period of time, they can cause damage to lipids, proteins and DNA as a consequence of oxidative stress (**Figure 2**) (Alfadda & Sallam, 2012; Bae et al, 2011; Droge, 2002; Nathan, 2003; Schieber & Chandel, 2014).

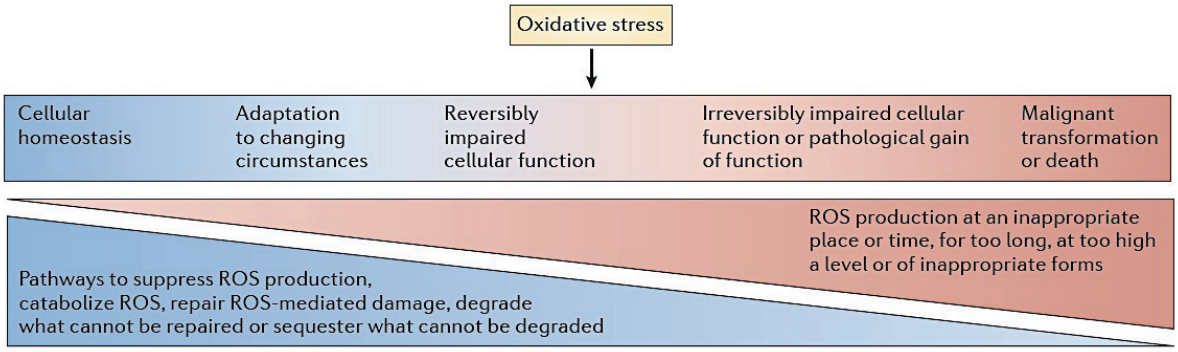

Figure 2: The production and catabolism of Reactive Oxygen Species (ROS) needs to be tightly regulated and this balance is crucial for its signaling and homeostasis. ROS production when confined to appropriate subcellular locations, levels and time maintains homeostasis and physiological cell activation. However, high levels or sustained production of ROS, which can not be efficiently counteracted by the cell could lead to impaired cellular function, cell death or malignant transformation. Image taken from (Nathan & Cunningham-Bussel, 2013)

ROS help the host to defend against invading pathogens (Alfadda & Sallam, 2012; Lugrin et al, 2014). As an example, phagocytes like neutrophils and macrophages resort to oxidative burst to combat bacterial infections (Droge, 2002). ROS can also activate proteins and gene transcription, promote or suppress inflammation, bring about mutagenesis and carcinogenesis (Alfadda & Sallam, 2012; Lugrin et al, 2014; Nathan & Cunningham-Bussel, 2013; Sen & Packer, 1996). There are several proteins which are regulated by ROS e.g. phosphatases like ATM and PTEN (Guo et al, 2010; Ito et al, 2006; Kuiper et al, 2011; Okuno et al, 2012). The active site of

most phosphatases contains cysteine sulphydryl groups. ROS can oxidize these residues and thereby can regulate their enzymatic activity (Barrett et al, 1999; Denu & Tanner, 1998; Karisch et al, 2011). ROS can additionally affect transcription by either directly oxidizing bases making up the DNA or by phosphorylation of transcription factors (Amente et al, 2010; Perillo et al, 2008; Storz et al, 1990). ROS act as signaling molecules for immune cells too e.g. H_2O_2 itself can act as chemotactic factor and attract leukocytes to the site of tissue damage (Hattori et al, 2010; Niethammer et al, 2009; Yoo et al, 2011). In the case of adaptive immunity, it has been documented that once T-cells or B-cells are activated upon ligation of their receptors, ROS production takes place via different pathways, which further influences downstream signaling (Angelini et al, 2002; Devadas et al, 2002; Hara-Chikuma et al, 2012; Singh et al, 2005; Yan et al, 2009). Further, if sustained T cell receptor (TCR) signaling takes place, excess of ROS accumulate which mediate hypo-responsiveness and could even lead to activation induced cell death via the Fas-FasL axis in T cells (Efimova et al, 2011; Fisher & Bostick-Bruton, 1982; Gelderman et al, 2006). Thus, taken together, the redox status within the T cells and their surrounding environment together influence their survival, activity and functioning.

ROS can be harmful to the host if its production is unchecked. To bolster this statement, excessive ROS has been associated with the pathology of different diseases such as Alzheimer's disease, Parkinson's disease, diabetes mellitus, cardiovascular diseases and even cancer (Alfadda & Sallam, 2012; Barnham et al, 2004; Droge, 2002). The host, therefore, has evolved ways to counteract the excessive production of ROS. They comprise mostly enzymatic systems with antioxidant functions. The major antioxidant enzymes are superoxide dismutases (SODs), glutathione peroxidases, thioredoxins, catalases and peroxidredoxins. In addition to this, non-enzymatic scavengers of ROS primarily from dietary origin also exist such as carotenoids, Vitamin E and ascorbic acid (Gelain et al, 2009; Nathan, 2003; Nathan & Cunningham-Bussel, 2013; Schieber & Chandel, 2014).

Superoxide dismutases are a class of enzymes that convert the highly toxic superoxide radical into hydrogen peroxide (**Figure 3**). The hydrogen peroxide is further catalyzed into a harmless product like water by the downstream enzyme catalase. There are three different members in the family of superoxide dismutases: SOD1 (or Cu/Zn SOD); SOD2 (or Mn SOD) and SOD3 (or extracellular SOD). These three members perform the same enzymatic reaction but differ in their cellular location. SOD1 is found abundantly in the cytoplasm but it can also be found to a lesser extent in the intermembrane space of the mitochondria and in the nucleus.

SOD2 is localized in the mitochondria and SOD3 is present in the extracellular matrix (Fridovich, 1989; Miao & St Clair, 2009; Zelko et al, 2002).

Interestingly, mutations in the *Sod1* gene in humans has been linked to Amyotrophic Lateral Sclerosis (ALS) (Andersen, 2006; Kaur et al, 2016; Renton et al, 2014). The characteristic feature of ALS is the deposition of abnormal protein aggregates. Although the detailed mechanisms are still unknown, several studies implicate the improperly folded mutant SOD1 to be responsible for the above-mentioned feature of ALS. (Brown, 1998; Pasinelli & Brown, 2006; Silverman et al, 2016)

Moreover, decreased levels of SOD1 have been reported in patients suffering from chronic viral hepatitis and HBV or HCV mediated hepatocellular carcinoma (Diamond et al, 2012; Dillon et al, 2013; Kim et al, 2003; Levent et al, 2006; Megger et al, 2013). This finding coincides with the fact that oxidative stress acts as one of the important players behind the pathology of viral hepatitis (Bolukbas et al, 2005; Dionisio et al, 2009; Esrefoglu, 2012; Ha et al, 2010; Hino et al, 2014; Koike & Moriya, 2005; Okuda et al, 2002). However, what initiates and drives the oxidative damage is not well described or studied.

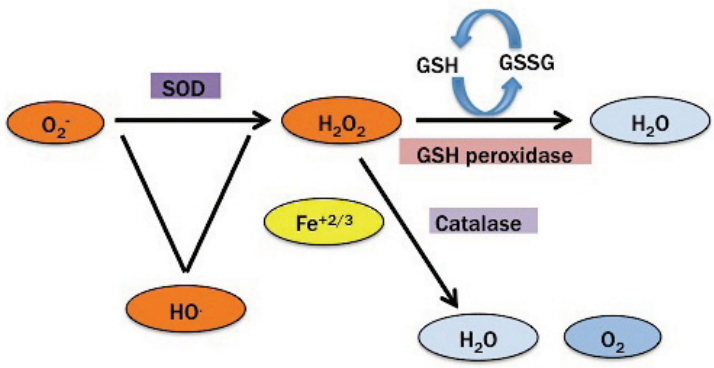

Figure 3: Schematic representation depicting the function of Superoxide dismutase. Superoxide dismutase converts superoxide radical to hydrogen peroxide and thereby serves as an important antioxidant enzyme. Figure adapted from (Brieger et al, 2012)

1.3 Viral Hepatitis

The term hepatitis means inflammation of the liver. It can be caused by several factors in man such as ingestion of excessive alcohol, drugs, toxic substances or viral infections (Beckett et al, 1961; Popper, 1972; Popper et al, 1965; Popper & Schaffner, 1971). The viruses classically known to target the liver and cause hepatitis

are Hepatitis A, B, C, D and E viruses (Protzer et al, 2012; Rehermann & Nascimbeni, 2005). These viruses belong to different families. However, the clinical manifestations of hepatitis are most often indistinguishable from each other. Hepatitis B and C viruses can cause chronic infections in man out of the above-mentioned viruses (**Figure 4**). There are more than 500 million people in the world chronically infected with HBV or HCV (El-Serag, 2012; Rehermann & Nascimbeni, 2005). Chronic viral hepatitis accounts for 57% of the cases of liver cirrhosis and 78% of the cases of liver cancer or hepatocellular carcinoma (Arzumanyan et al, 2013; El-Serag, 2012; Hajarizadeh et al, 2013; Rehermann, 2013; Thomas, 2013). It is also responsible for about a million deaths per year. However, the rate at which disease progression takes place to cirrhosis and liver cancer shows great variability among patients and the factors underlying this are poorly understood (Rehermann, 2013; Yang & Roberts, 2010).

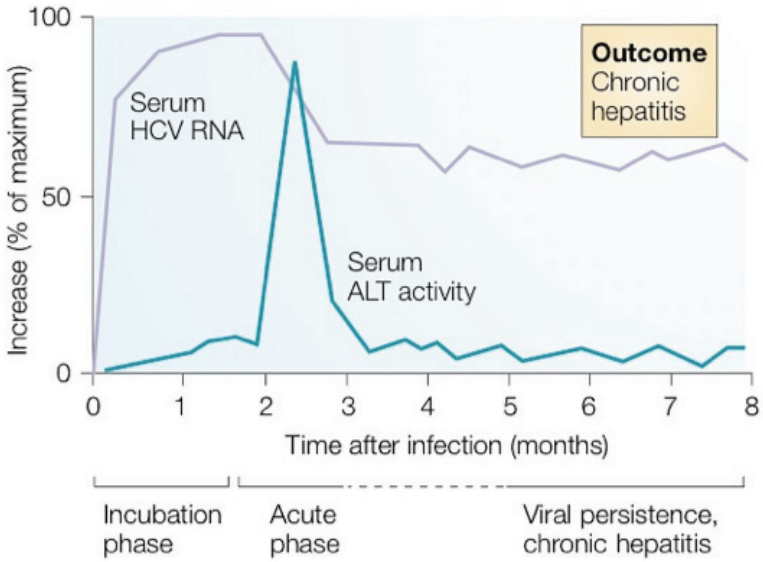

Figure 4: Schematic depiction of the course of disease in a chronic Hepatitis C infection. HCV persists several months after infection in the case of a chronic infection. Image taken from (Rehermann & Nascimbeni, 2005)

Viral hepatitis manifests with a complex pathogenesis and involves different cell populations and mediators originating from the host that collectively contribute to the pathology (Guidotti & Chisari, 2006; Heymann & Tacke, 2016; Park & Rehermann, 2014; Rehermann, 2013). HBV and HCV are non-cytopathic viruses. Thus, the pathology is majorly determined by the immune responses that the host mounts

against the viral infection rather than the virus itself (Park & Rehermann, 2014; Rehermann, 2013; Trepo et al, 2014). A brief overview of how the various aspects of the host immune system influence the outcome of viral hepatitis is described below.

A lot of studies have focused on the role of the adaptive immune system with respect to chronic viral hepatitis (Bertoletti & Gehring, 2006; Boonstra et al, 2008; Bowen & Walker, 2005; Chang, 2003; Das & Maini, 2010; Guidotti & Chisari, 2006; Knolle & Thimme, 2014; Rehermann, 2013). Virus specific $CD8^+$ T cells are one of the major immune cell populations held responsible for driving the immunopathology (Guidotti & Chisari, 2006; Park & Rehermann, 2014; Rehermann, 2013; Thimme et al, 2003). They recognize hepatocytes infected with virus and kill them, thereby causing liver damage. In the case of chronic hepatitis, the host fails to clear the virus owing to inefficient T cell responses (Guidotti & Chisari, 2006; Rehermann, 2013). Virus specific T cells are functionally exhausted in the chronic phase, which could progressively lead to their deletion. This downregulation of T cell responses therefore gives an opportunity to the virus to establish a persistent infection (Boni et al, 2007; Penna et al, 2007; Radziewicz et al, 2007; Wedemeyer et al, 2002). Additional factors identified recently could further explain the unsuccessful immune response of the host during chronic viral hepatitis e.g. a shift in the cytokine milieu occurs in the chronic phase from a T cell activity sustaining environment with presence of cytokines like IL-2 to a more suppressive cytokine environment with predominance of cytokines like IL-10 and transforming growth factor-β (TGF-β) (Alatrakchi et al, 2007; Das et al, 2012; Dunn et al, 2007; Radziewicz et al, 2010). Also, regulatory T cells increase in numbers in the liver and T cells express inhibitory molecules such as PD-1, CTLA-4, Tim-3 and their respective ligands, which further dampen T cell responses of the host (Accapezzato et al, 2004; Das et al, 2008; McMahan et al, 2010; Stoop et al, 2005; Xu et al, 2006).

Moreover, on the viral side, the virus itself undergoes mutations in dominant T cell epitopes to evade recognition by the host, thereby being able to persist (Bertoletti et al, 1994; Chang et al, 1997; Grakoui et al, 2003; von Hahn et al, 2007; Wolfl et al, 2008). Taken together, constant low-level cellular damage eventually add up and may lead to the development of cirrhosis and even hepatocellular carcinoma.

The innate immune system on the other hand senses invading pathogens and acts as the first line of defense against them. In the case of viral hepatitis too, it plays a role in the recognition of the virus and the signaling events following it. The HBV DNA, for example, is recognized by cGAS, which leads to downstream signaling via Stimulator of Interferon Genes (STING) and ultimately results in production of the potent antiviral cytokine type I interferon and induction of Interferon Stimulated

Genes (ISGs) such as ISG15 and ISG56 (Dansako et al, 2016). Additionally, the HBV pre-genomic RNA is recognized by Retinoic acid Inducible Gene I (RIG-I), which is followed by induction of type III interferon (Sato et al, 2015). HCV too leads to a potent interferon production after being recognized by the host via Pattern Recognition Receptors like RIG-I and TLR3 (Gale & Foy, 2005; Li et al, 2005; Rehermann, 2009; Sumpter et al, 2005; Yoneyama et al, 2004). The interferons and ISGs collectively try to hinder viral replication (Dash et al, 2005; Gale & Foy, 2005; Guo et al, 2001; Shimazaki et al, 2002; Wang et al, 2003). But the viruses have successfully evolved to interfere with the host systems that counteract viral replication and thereby, evade elimination. To cite a few examples, the X protein of HBV degrades Mitochondrial Antiviral Signaling protein (MAVS), the protein downstream of RIG-I pathway and therefore inhibits interferon induction via this pathway (Rehermann, 2009; Seth et al, 2005; Wei et al, 2010). HBV also inhibits activation of IRF3 along the STING axis and translocation of STAT1 to the nucleus, both of which are important events necessary for potent ISG induction (Luangsay et al, 2015; Lutgehetmann et al, 2011; Yu et al, 2010). The HCV also developed strategies to interfere with the IFN-I signaling pathway at multiple levels. The HCV core protein blocks JAK-stat signaling and the NS3/4A protease blocks the RIG-I signaling pathway (Blindenbacher et al, 2003; Bode et al, 2003; Duong et al, 2004; Foy et al, 2005; Heim et al, 1999; Rehermann, 2009). Surprisingly, recent studies show that HCV patients with already activated endogenous interferon signaling respond poorly to the pegylated interferon therapy regime (Bieche et al, 2005; Sarasin-Filipowicz et al, 2008; Sarasin-Filipowicz et al, 2009). Although a thorough explanation is not yet available, refractoriness of the IFN signaling pathway in these patients has been cited as a possible explanation (Sarasin-Filipowicz et al, 2009). HCV proteins also disrupt the functional activity of STAT1 and block ISG expression (Duong et al, 2004; Heim et al, 1999; Wang et al, 2003).

Natural Killer (NK) cells and Dendritic Cells (DCs) have been reported as important cell populations contributing to innate immune response against HBV and HCV infections (Bain et al, 2001; Fisicaro et al, 2009; Golden-Mason & Rosen, 2013; Guidotti & Chisari, 2001; Kanto et al, 1999; Khakoo et al, 2004; Longman et al, 2004; Maini & Peppa, 2013; Zhang et al, 2008). NK cells are known to kill virus-infected hepatocytes either by releasing their characteristic enzymes like perforin and granzyme or by receptor mediated cell lysis. They also produce cytokines like IFNγ and TNFα (Kramer et al, 2012; Radaeva et al, 2006). Plasmacytoid DCs have been identified as potent producers of IFNα against HCV and activator of NK cell and T cell responses (Martinet et al, 2012b; Yoshio & Kanto, 2016; Zhang et al, 2008).

Importantly, impaired activity of NK cells DCs have been reported in chronic HBV and HV patients, implying that these cells may have a protective role against these infections (Dolganiuc et al, 2003; Maini & Peppa, 2013; Martinet et al, 2012a; Rehermann, 2013; Rehermann & Nascimbeni, 2005; Schuch et al, 2014; Xie et al, 2009).

Apart from the above-mentioned classical immunological events, another factor involved in the pathogenesis of viral hepatitis is oxidative stress (Bhargava et al, 2011; Cardin et al, 2014; Czaja, 2014; Fujinaga et al, 2011; Higgs et al, 2014; Paracha et al, 2013; Ripoli et al, 2010; Stauffer et al, 2012; Yang et al, 2008). This is supported by clinical evidence ranging from decreased levels of antioxidant enzymes in the sera of chronic hepatitis patients to liver biopsies from the latter that stain positively for oxidized residues of guanine, indicating increased oxidative damage (Cardin et al, 2014; Cardin et al, 2001; Koike et al, 2002; Levent et al, 2006; Shimoda et al, 1994). Excessive ROS in the liver can promote fibrogenesis by activating hepatic stellate cells or could also lead to development of liver cancer (Czaja, 2014). Some viral proteins like the HCV core protein or the HBV X protein have been associated with increased oxidative stress, particularly in the early phases of hepatocellular carcinoma (Koike, 2007; Koike et al, 2002).

Keeping this in mind, several clinical trials have taken place whereby chronic viral hepatitis patients were administered with antioxidants (Andreone et al, 2001; Gerner et al, 2008; Melhem et al, 2005; Murakami et al, 2006). While some studies report that there was a beneficial effect, others refute this claim. The conclusions therefore drawn from these studies were debatable.

1.4 Type I interferon

Interferons are a class of cytokines mostly known for their potent antiviral activity. However, in the recent years, their other functional attributes have been discovered e.g. interferons can act as anti-proliferative agents and/or can have anti-tumor functions (Lengyel, 1982; Lopez de Padilla & Niewold, 2016; Pestka et al, 1987). They also can regulate immune responses, thereby acting as immunomodulatory molecules. Three major classes of interferons have been identified till date: type I interferon (IFN-I), type II interferon and type III interferon (Lopez de Padilla & Niewold, 2016; Pestka et al, 2004). The type I interferon family mainly comprises interferon alpha and interferon beta. In humans, they are encoded by 13 IFNα genes and one IFNβ gene. In mice, 14 IFNα genes exist (Pestka et al, 2004). IFN-I are mainly known for their potent antiviral activity. They induce the expression of a set of

genes called as Interferon Stimulated Genes (ISGs) in infected cells, which target viral replication. In addition, they also prepare the neighboring bystander uninfected cells to combat viral infection by inducing an antiviral state in them through paracrine signaling (Yan & Chen, 2012). Recently, additional members belonging to the IFN-I family have been discovered, namely IFNε, IFNτ, IFNκ, IFNω, IFNδ and IFNζ (Pestka et al, 2004). They have been however functionally poorly described. IFN-I bind to the type I interferon receptor, namely interferon alpha/beta receptor (IFNAR). IFNAR is made up of two subunits: IFNAR1 and IFNAR2 (Pestka et al, 2004; Uze et al, 2007). The IFN-II family on the other hand comprises a single member IFNγ. T cells and NK cells are the main producers of IFNγ. This cytokine can act on diverse cell types that express the IFNγ receptor (Schoenborn & Wilson, 2007). The IFN-III family consists of IFNλ1, IFNλ2, IFNλ3 (also known as IL29, IL-28A and IL-28B) and the recently identified IFNλ4 (O'Brien et al, 2014). Functionally, they are quite similar to the IFN-I family. However, their receptors are expressed on distinct cell populations localized on epithelial surfaces. Therefore, their activity is restricted to specific tissues (Witte et al, 2010).

1.4.1 IFN-I production

The host senses invading pathogens by recognizing specific molecular motifs or features present in the latter known as Pathogen Associated Molecular Patterns (PAMPs) via specialized receptors present on the surface or inside the cell in endosomal compartments called as Pattern Recognition Receptors (PRRs) (Takeuchi & Akira, 2010). PAMPs include products of microbial origin such as lipopolysaccharides, foreign nucleic acids, peptidoglycans and glucans. IFN-I production is strongly induced upon viral infections (Lopez de Padilla & Niewold, 2016; Pestka et al, 1987). Viral RNA is mainly recognized by RIG-I and MDA5 (Goubau et al, 2013; Kato et al, 2006). The signaling cascade downstream of these two receptors involve Mitochondrial Antiviral Signaling protein (MAVS), which activates the kinase TBK1, resulting in phosphorylation of two important transcription factors, IRF3 and IRF7 (Kato et al, 2006; Seth et al, 2005; Xu et al, 2005). The phosphorylation of these two transcription factors leads to induction of IFN-I production (Honda et al, 2005; Sato et al, 2000). The host also possesses other PRRs, e.g. DNA-dependent activator of IFN-regulatory factors (DAI), the DEAD box and DEAH box (DEXD/H box) helicases, and the cytosolic GAMP synthase (cGAS), which recognize cytosolic DNA (Goubau et al, 2013; Paludan & Bowie, 2013). Engagement of these receptors can also lead to IFN-I production through different

signaling pathways. cGAS, after binding to its ligand, signals through STING and TBK1 to induce IRF3 dimerization and consequently leads to IFN-I production (Ma & Damania, 2016). Additionally, several Toll-like receptors (TLRs) and cytosolic molecular sensors NOD-containing protein 1 (NOD1) and NOD2 can also lead to IFN-I induction (Moreira & Zamboni, 2012; Moynagh, 2005). TLR 3 and TLR 4 signal through TRIF whereas the rest of the TLRs signal through MyD88. This signaling cascade ultimately converges at the IRFs (mainly IRFs 3, 7 and 5) and results in IFN-I production (**Figure 5**). Most of the above-mentioned signaling cascades rely on IRF3 for the first wave of transcription of IFN-I encoding genes, followed by IRF7 which creates a positive feedback loop and leads to transcription of more IFN-I encoding genes (Honda et al, 2006; Tamura et al, 2008).

1.4.2 IFN-I signaling

IFN-I bind to the receptor, IFNAR. IFNAR is a transmembrane receptor made up of two subunits, IFNAR1 and IFNAR2 (Uze et al, 2007). The canonical type I interferon signaling pathway involves activation of the kinases JAK1 and TYK2 downstream of IFNAR engagement (Ivashkiv & Donlin, 2014). This is followed by phosphorylation of tyrosine residues of the adaptor molecules STAT1 and STAT2 (Stark & Darnell, 2012). Upon phosphorylation, these two proteins form a dimer and further bind to IRF9 to form the ISGF3 complex, which translocates to the nucleus. As a next step, the complex binds to interferon stimulated response elements (ISRE) present in the promoter regions of ISGs, leading to ISG production (**Figure 5**) (Schoggins et al, 2011). The canonical pathway can also lead to formation of STAT1 homodimers, which bind to interferongamma-activating factor (GAF) and can also induce ISG production (David, 2002).

Apart from the canonical pathway, IFN-I signaling can also occur through other pathways e.g. along the PI3K-mTOR axis or the MAPK pathway (Ivashkiv & Donlin, 2014; Kaur et al, 2008; Kaur et al, 2012; Rani et al, 2002). This could therefore partially explain the diverse roles that interferons play since all these diverse pathways not only lead to ISG induction but also could lead to production of several other cytokines, mediators or metabolically active molecules (Rauch et al, 2013). Thus, although the most well described function of interferons has been their antiviral activity, yet their roles are not just limited to this. There is always a constitutive low-level interferon signaling existing which is highly induced in response to an infection (Gough et al, 2012).

Other than viral infections, IFN-I have also been demonstrated to play a role in bacterial infections such as *Listeria monocytogenes*, *Mycobacterium tuberculosis*, *Salmonella* and *Staphylococcus aureus* (Stifter & Feng, 2015). They can mediate immunosuppression, induce apoptosis or interfere with protective host responses during these infections such as neutrophil migration or IFN-II signaling in the case of *Mycobacterium tuberculosis* (Desvignes et al, 2012; Kearney et al, 2013). Unchecked interferon signaling has also been associated with autoimmune conditions or severe tissue damage. Along similar lines, a recent study in our laboratory, where I contributed as a co-author, describes how a methyltransferase Setdb2, which is induced upon IFN-I signaling, can make the host susceptible to bacterial superinfection post challenge with a viral infection (Schliehe et al, 2015). A similar finding holds true in the case of chronic Lymphocytic choriomeningitis virus (LCMV) infections whereby IFN-I induce a immunosuppressive state by inducing inhibitory molecules like PDL-1 and IL-10 which impair viral clearance by the host (Teijaro et al, 2013; Wilson et al, 2013). IFN-I´s role in HIV progression has also come to the forefront where it promotes $CD4^+$ T cell apoptosis leading to impaired T cell function and viability (Herbeuval et al, 2005; Herbeuval & Shearer, 2007; Hosmalin & Lebon, 2006). The beneficial and detrimental effects of IFN-I are complex to understand and depend on the combination of pathogen and host responses (Stifter & Feng, 2015).

Further studies are required to have a deeper and better understanding of the pleiotropic roles of IFN-I and thereby identifying targets for therapeutic purposes.

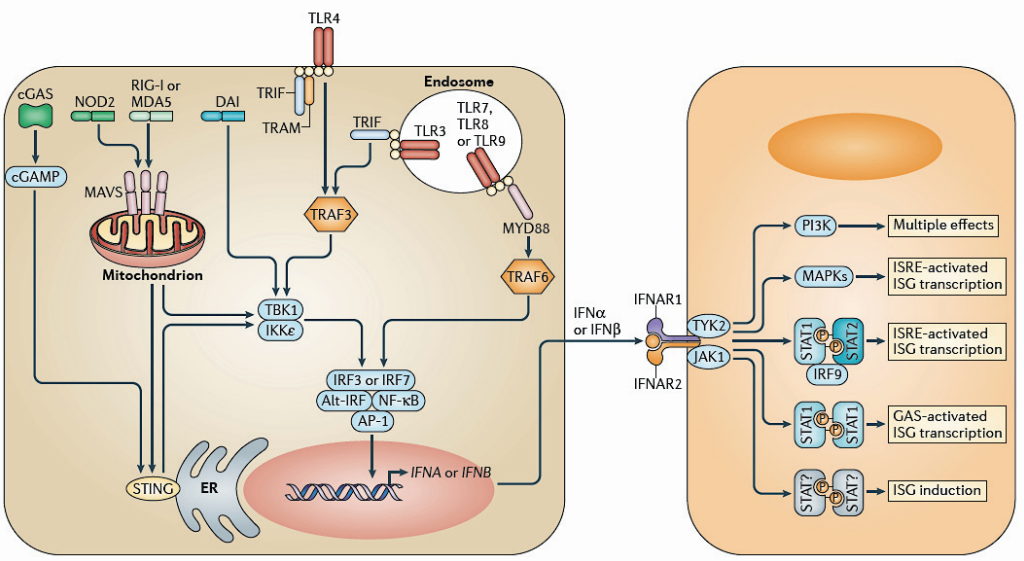

Figure 5: Type I interferon production and signaling pathway. Several pattern recognition receptors recognize microbial products, which leads to induction of type I interferon. Upon binding of type I interferon to its receptor, several signaling pathways can be activated downstream, leading to diverse biological outcomes. Image taken from (McNab et al, 2015).

1.5 Lymphocytic choriomeningitis virus

The lymphocytic choriomeningitis virus (LCMV) is a RNA virus belonging to the family *Arenaviridae* (Bergthaler et al, 2010; Bergthaler et al, 2007). Its genome consists of single stranded negative sense RNA. The genome has two segments: the long segment (L) and the short segment (S). The long segment (L) codes for the RNA dependent RNA polymerase and the matrix protein Z. The virus requires the RNA dependent RNA polymerase for replication and the matrix protein for budding out of the cell. The S segment encodes the nucleoprotein (NP) and the glycoprotein (GP). The viral RNA is enclosed by the nucleoprotein (NP). Thus, the latter holds the viral genome together and makes it accessible to the polymerase for replication. The glycoprotein (GP) is required by the virus to attach to host cells and enter them (Bergthaler et al, 2010). The common mouse *Mus musculus* is the natural host of the virus.

The LCMV model provides an excellent tool to study various important immunological aspects pertaining to host-virus interactions e.g. an acute infection resulting in pathogen clearance, viral persistence, immunopathology and death (Bocharov, 1998; Doherty & Zinkernagel, 1974; Zinkernagel, 2002). The outcome of the infection depends on several factors. They include the strain and dose of the virus, the route of inoculation and the MHC haplotype of the host (Bergthaler et al, 2007; Zinkernagel, 1986). T cell dependence is one of the characteristic features of this model (Doherty & Zinkernagel, 1974; Zinkernagel et al, 1986). Intravenous infection of wild type mice with the strain LCMV cl13 results in a systemic persistent infection whereby the virus can persist up to more than 60 days (**Figure 6**) (Moskophidis et al, 1993). Wild type mice infected with LCMV intravenously suffer from hepatitis, which peaks between 8 to 10 days post infection (Bergthaler et al, 2007). This is a classical example of immunopathology whereby the infected liver cells or hepatocytes are killed by the virus specific T cells of the host (Zinkernagel et al, 1986).

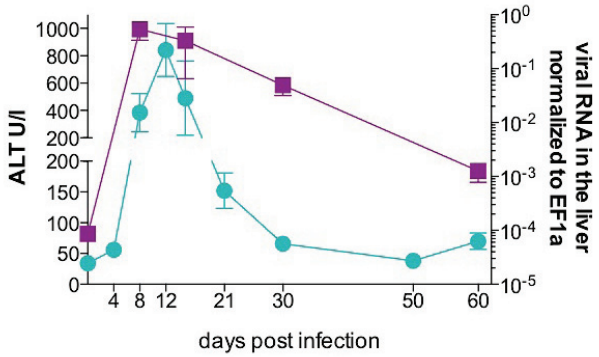

Figure 6: The course of infection in wild type mice upon intravenous infection with LCMVcl13 (unpublished data). Intravenous injection of wild type mice with LCMV cl13 results in a persistent infection whereby the virus can persist up to 60 days post infection. The mice suffer from T-cell mediated hepatitis, which peaks at around 8-12 days post infection as indicated by the elevated levels of alanine aminotransferase (ALT).

2. Aims

The aims of the study were as follows:

2.1 To investigate the role of the antioxidant superoxide dismutase 1 (SOD1) in LCMV mediated viral hepatitis

2.2 To elucidate the molecular mechanisms of oxidative stress in LCMV mediated viral hepatitis

3. Results

3.1 Prelude

Viral hepatitis poses a great challenge to the field of medical science since it causes substantial mortality and morbidity. Clinical studies have reported alterations in several aspects of the metabolic status of patients suffering from chronic hepatitis mediated by HBV or HCV. A few examples include dysregulation of lipid metabolism, development of insulin resistance and disturbance of redox homeostasis, resulting in increased oxidative stress. Oxidative stress has been associated with increased risk of disease progression towards liver fibrosis and even hepatocellular carcinoma. Although there is strong correlative evidence that oxidative stress contributes to the pathology of viral hepatitis, the detailed molecular mechanisms that initiate oxidative stress are currently unclear.

In the following research article entitled “Superoxide dismutase 1 Protects Hepatocytes From Type I Interferon-Driven Oxidative Damage” published in *Immunity* in November 2015, we described a novel mechanism by which type I interferon signaling, a branch of the innate immune system, causes oxidative stress in hepatocytes and leads to hepatitis using the mouse hepatitis model of lymphocytic choriomeningitis virus. Additionally, we discovered that the antioxidant enzyme superoxide dismutase 1 (SOD1) allows the host to counteract the IFN-I signaling mediated hepatitis. This study, therefore, provides a new concept of how the innate immune system can drive liver pathology.

3.2 Superoxide dismutase 1 protects hepatocytes from Type-I interferon driven oxidative damage, *Immunity* 43: 974-986, November 17, 2015

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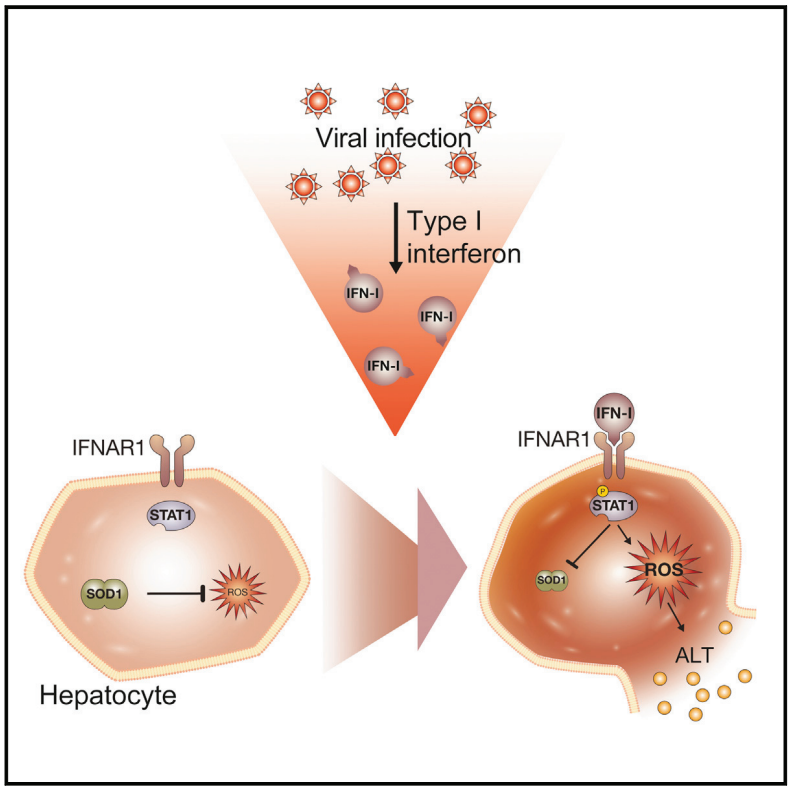
Article

Immunity

Superoxide Dismutase 1 Protects Hepatocytes from Type I Interferon-Driven Oxidative Damage

Graphical Abstract



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In Brief

Bergthaler and colleagues show that superoxide dismutase 1 protects the liver from type I interferon-driven oxidative damage in viral hepatitis. Liver damage was mediated by hepatocyte-intrinsic IFNAR1-STAT1 signaling.

Highlights

- Viral infection leads to redox dysregulation including the downregulation of SOD1
- *Sod1*$^{-/-}$ mice exhibit aggravated viral hepatitis, which is rescued by antioxidants
- IFN-I signaling via STAT1 drives SOD1 downregulation and early liver damage
- Ablation of IFN-I signaling ameliorates viral hepatitis in *Sod1*$^{-/-}$ and WT mice

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Article

Superoxide Dismutase 1 Protects Hepatocytes from Type I Interferon-Driven Oxidative Damage

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SUMMARY

Tissue damage caused by viral hepatitis is a major cause of morbidity and mortality worldwide. Using a mouse model of viral hepatitis, we identified virus-induced early transcriptional changes in the redox pathways in the liver, including downregulation of superoxide dismutase 1 (*Sod1*). *Sod1*$^{-/-}$ mice exhibited increased inflammation and aggravated liver damage upon viral infection, which was independent of T and NK cells and could be ameliorated by antioxidant treatment. Type I interferon (IFN-I) led to a downregulation of *Sod1* and caused oxidative liver damage in *Sod1*$^{-/-}$ and wild-type mice. Genetic and pharmacological ablation of the IFN-I signaling pathway protected against virus-induced liver damage. These results delineate IFN-I mediated oxidative stress as a key mediator of virus-induced liver damage and describe a mechanism of innate-immunity-driven pathology, linking IFN-I signaling with antioxidant host defense and infection-associated tissue damage.

INTRODUCTION

More than 500 million people worldwide are infected with Hepatitis B virus (HBV), Hepatitis C virus (HCV), or other hepatotropic viruses. These viral infections often lead to liver damage and associated complications such as advanced liver fibrosis, cirrhosis, and hepatocellular carcinoma, which cause substantial morbidity and mortality (Guidotti and Chisari, 2006; Park and Rehermann, 2014). The complex pathology of viral hepatitis is driven by multiple viral and host factors interacting with various immune cell populations and cytokines such as type I interferon-I (IFN-I) (Park and Rehermann, 2014; Schoggins et al., 2011). Together, these determinants mediate the antiviral response, but they also lead to subsequent immunopathology and tissue damage (Guidotti and Chisari, 2006; Medzhitov et al., 2012; Rouse and Sehrawat, 2010). Yet, the mechanisms involved are largely unknown.

Perturbations in several metabolic and cellular stress pathways induced by viral infections have been associated with liver disease (Drakesmith and Prentice, 2008; Koike and Moriya, 2005; Sheikh et al., 2008; Stauffer et al., 2012). Such imbalance in the host redox system resulting from infections such as HBV and HCV affects many processes governing intracellular homeostasis and signaling (Bolukbas et al., 2005; Nathan and Cunningham-Bussel, 2013; Okuda et al., 2002; Schieber and Chandel, 2014). Cells have evolved dedicated antioxidant enzymatic systems

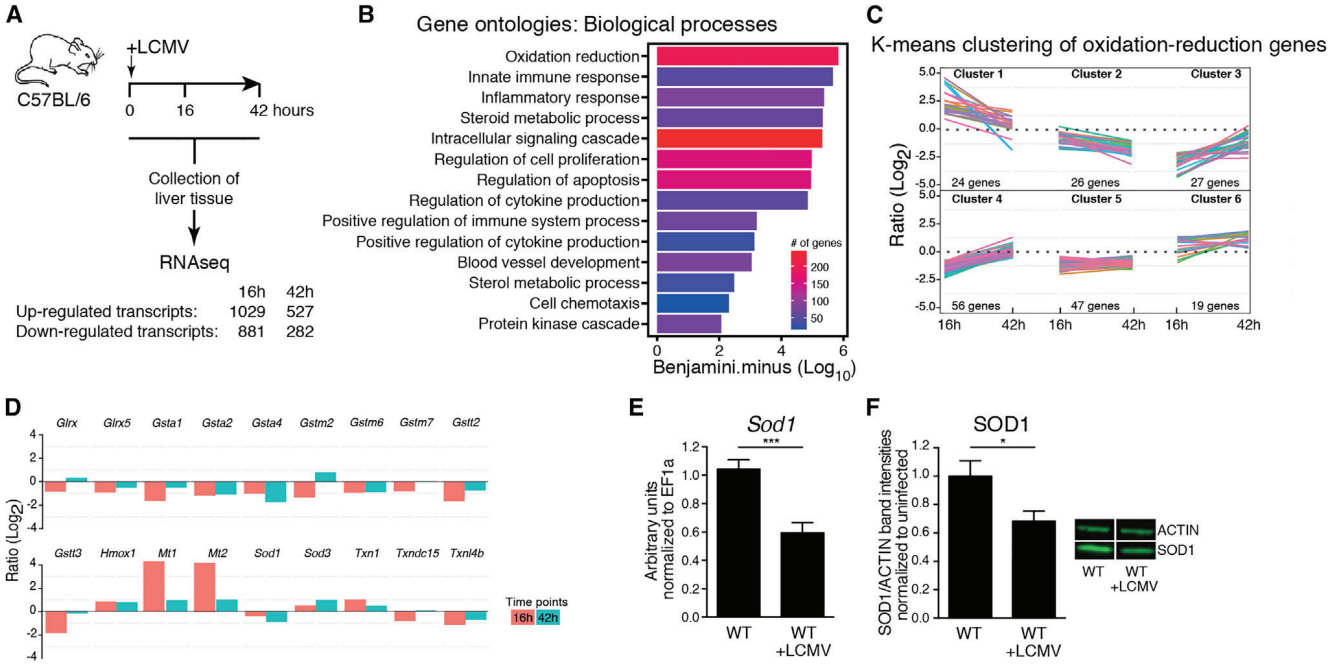

Figure 1. Viral Infection Results in Transcriptional Regulation of Oxidation-Reduction Pathways in the Liver

(A–D) Wild-type (WT) mice were infected with LCMV. Profiling of liver tissue harvested at the indicated time points was performed by RNA-seq (n = 3 mice).

(A) Workflow and summary of up- and downregulated transcripts. See also Table S1.

(B) Gene ontology enrichment analysis of significantly regulated transcripts from RNaseq data.

(C) K-means clustering of gene regulatory profiles from the GO term oxidation reduction. Individual genes are represented by different colored lines. See also Table S2.

(D) Selection of differentially regulated genes involved in oxidation-reduction related processes (Experimental Procedures).

(E) mRNA expression of *Sod1* was determined by real-time PCR from liver tissue of WT mice that were either left uninfected or infected with LCMV 44 hr previously (n = 7–12 mice per group pooled from three independent experiments).

(F) Western blot for SOD1 and actin were performed from liver lysates of WT mice that were either left uninfected or infected with LCMV 44 hr previously (representative results are shown). Relative protein ratios of SOD1 to actin were quantified by LI-COR (n = 11 mice per group pooled from three independent experiments). Statistical significance was calculated by unpaired t test (E and F). Symbols represent the mean ± SEM.

including superoxide dismutases (SODs), catalases, peroxidases, and reductases, which act as rheostats to counteract redox imbalances (Miao and St Clair, 2009; Nathan and Cunningham-Bussel, 2013; Schieber and Chandel, 2014). However, the mechanisms initiating and promoting oxidative stress and the subsequent tissue damage in viral hepatitis remain unclear.

In this study, we employed two unrelated mouse infection models to dissect host determinants of viral hepatitis, i.e., the noncytolytic lymphocytic choriomeningitis virus (LCMV) (Zinkernagel et al., 1986) and the cytolytic mouse hepatitis virus (MHV) (Cervantes-Barragan et al., 2007). We uncovered an essential role for the antioxidant SOD1 in protecting hepatocytes from virus-induced oxidative stress and cell death. Further, our data identifies IFN-I signaling as a key inducer of virus-mediated oxidative liver damage, exposing innate immunity as a driver of liver pathology. These results provide insights into the molecular pathogenesis of viral hepatitis and infection-associated tissue damage.

RESULTS

Viral Infection Results in Transcriptional Regulation of Redox Pathway-Related Genes

To obtain an unbiased view of global gene expression in the liver in the early phase of a chronic viral infection, we infected wild-type (WT) mice with LCMV strain clone 13 and performed transcriptional profiling of liver tissue at different time points by RNA-seq (Figure 1A, Table S1). The differentially up- or downregulated transcripts were subjected to gene ontology (GO) analysis. As expected, genes involved in innate immune and inflammatory responses were significantly overrepresented (Figure 1B). The most highly enriched GO term was related to oxidation-reduction processes. Further analysis by k-means clustering revealed distinct patterns of transcriptional up- and downregulation among this group of transcripts (Figure 1C, Table S2), which included genes with antioxidant function such as glutathione S-transferases, hemoxygenase, and metallothioneins, as well as SODs (Figure 1D, Table S1). The SOD family members SOD1, SOD2, and SOD3 are crucial scavengers of O_2^- (Miao and St Clair, 2009). SOD1 (also known as Cu/Zn-SOD) is ubiquitously expressed, localized in the cytoplasm, nucleus and mitochondrial intermembrane space and has been linked to human diseases such as amyotrophic lateral sclerosis (Miao and St Clair, 2009). Yet, little is known about the role of SOD enzymes in the context of infection. Decreased levels of SOD1 were found in patients chronically infected with HCV (Diamond et al., 2012; Levent et al., 2006), in HCV-induced hepatocellular carcinoma (Dillon et al., 2013; Megger et al., 2013), as well as in HBV-associated cancer tissue (Kim et al., 2003). This

coincided with our observation that infection with LCMV resulted in a downregulation of SOD1 expression at the RNA (Figures 1D and 1E) and protein level (Figure 1F).

SOD1 Deficiency Leads to Aggravated Liver Damage upon LCMV Infection

To investigate whether SOD enzymes contribute to viral hepatitis, we infected *Sod1*$^{-/-}$, *Sod2*$^{+/-}$—a commonly used model for *Sod2* deficiency (Boelsterli and Hsiao, 2008)—and *Sod3*$^{-/-}$ mice with LCMV and monitored the course of disease. *Sod1*$^{-/-}$ but not *Sod2*$^{+/-}$ or *Sod3*$^{-/-}$ mice lost more body weight compared to WT mice, which started in the early phase of infection (Figure 2A, Figure S1A). Next, we assessed serum concentrations of alanine aminotransferase (ALT), a routinely used clinical parameter of hepatitis. Again, *Sod1*$^{-/-}$ mice, but neither *Sod2*$^{+/-}$ nor *Sod3*$^{-/-}$ mice, showed highly elevated concentrations of ALT (Figure 2B, Figure S1B) within the first 2 days of infection. We also measured two alternative parameters for hepatitis, aspartate aminotransferase (AST) and alkaline phosphatase (AP), and found elevated concentrations in *Sod1*$^{-/-}$ compared to WT mice (Figure 2C). In addition, we observed an early increase of ALT in WT mice upon LCMV infection (Figure 2B). The serum concentrations of blood urea nitrogen and creatinine, which represent parameters of kidney damage, were comparable between *Sod1*$^{-/-}$ and WT mice upon LCMV infection (Figure S1C), suggesting a non-generalized pathogenesis that is primarily affecting the liver. SOD2 and SOD3 were dispensable for liver protection in our experiments, which might be due to lower expression in the liver (Marklund, 1984) or different biological properties including metal cofactors and subcellular localization. To study the effects of the virus infection dose on the observed pathology, we infected *Sod1*$^{-/-}$ and WT mice with either a low dose of LCMV strain clone 13 or with another LCMV strain ARM that is usually cleared within 8 days. In either case we observed increased hepatitis in *Sod1*$^{-/-}$ mice compared to WT mice (Figure S1D), suggesting that the pathology is independent of the infection inoculum and LCMV strain. Together, our results indicate that SOD1 plays a non-redundant protective role in viral hepatitis and liver damage.

Upon infection *Sod1*$^{-/-}$ and WT mice showed comparable viral loads in blood (Figure 2D), liver (Figure 2E), and spleen and kidney (Figure S1E), which argued against a role for SOD1 in virus control. Histological analysis of infected liver tissue revealed pathologic lesions in infected *Sod1*$^{-/-}$ mice, which were absent in infected WT mice and in uninfected *Sod1*$^{-/-}$ and WT mice (Figure 2F). This was associated with increased expression of cell-death-associated genes (Figure 2G) and more cleaved caspase-3 positive hepatocytes in the liver at 16 hr after infection (Figure 2H), indicating the rapid activation of apoptotic pathways in infected *Sod1*$^{-/-}$ mice. Thirty days after infection with LCMV strain clone 13 *Sod1*$^{-/-}$ mice showed fibrotic processes in the liver tissue as indicated by increased *Col1a1* mRNA expression (Figure 2I). A similar increase of *Col1a1* expression was observed also upon infection with the acute LCMV strain ARM (Figure S1F). More than 100 days after infection, *Sod1*$^{-/-}$ mice showed recovered body weight compared to WT mice (Figure S1G) and comparable residual viral RNA in the liver (Figure S1H). Histopathological analyses of liver tissue for H/E and cleaved caspase 3 revealed no differences between *Sod1*$^{-/-}$ and WT mice (Figure S1I). Yet, we found increased levels of 8-oxoguanine (8-oxoG), a marker for oxidative damage, in the liver tissue of *Sod1*$^{-/-}$ mice at 123 days after infection (Figure S1I). Oxidative stress is considered to play a pathogenic role in liver fibrosis (Parola and Robino, 2001; Sánchez-Valle et al., 2012). In line with this and our *Col1a1* expression data, *Sod1*$^{-/-}$ mice showed an accumulated deposition of collagen fibers compared to WT mice as detected by Elastica-van Gieson (Figure 2J) and Sirius Red staining (Figure 2K). Together, this indicated that *Sod1*$^{-/-}$ mice exhibited increased fibrotic changes during the late phase of infection compared to WT mice.

To investigate whether the SOD1-mediated protection of the liver constituted a general host mechanism during viral infections, we infected *Sod1*$^{-/-}$ and WT mice with the cytolytic murine coronavirus MHV. Similar to our previous findings with the noncytolytic LCMV, the lack of SOD1 resulted in increased body weight loss (Figure S1J) and elevated concentrations of ALT (Figure S1K). Further, we observed more histopathological lesions after infection (Figure S1L) despite similar viral loads in the liver (Figure S1M). Together, these results suggest that SOD1 plays a general protective role in the liver during viral infection.

SOD1 Deficiency Results in Oxidative Stress-Induced Liver Damage upon Viral Infection

To determine whether oxidative damage was responsible for the exacerbated liver pathology observed in infected *Sod1*$^{-/-}$ mice, we adopted several complementary approaches. First, we stained liver tissue of uninfected *Sod1*$^{-/-}$ mice for 8-oxoG and found no differences in 8-oxoG staining compared to uninfected WT mice (Figure 3A). LCMV infection, however, led to elevated staining of 8-oxoG in hepatocytes of *Sod1*$^{-/-}$ mice at 16 hr after infection (Figure 3A), confirming increased virus-induced oxidative damage in the liver. Likewise, we detected elevated mRNA expression of *Atf3* (Figure 3B), encoding a transcription factor that is induced by reactive oxygen species (ROS) and plays an important role in immunoregulation (Gilchrist et al., 2006; Hoetzenecker et al., 2012). The observed transient cellular damage as seen by histological staining for 8-oxoG and cleaved caspase 3 at 16 hr after infection might be due to refractoriness of JAK-STAT signaling after sustained IFN-I signaling in liver tissue during viral infection (Sarasin-Filipowicz et al., 2009). Thus, SOD1 is required to prevent oxidative damage in the liver upon viral infection.

We next aimed to identify the cellular compartments that require SOD1 to protect against oxidative damage. Bone-marrow-chimeric mice were generated by reciprocal transfer of *Sod1*$^{-/-}$ and WT genotypes followed by administration of liposomal clodronate to deplete remaining radioresistant macrophages. Chimerism was confirmed in liver and spleen by using a congenic marker (Figures S2A and S2B). Upon LCMV infection, *Sod1*$^{-/-}$ → *Sod1*$^{-/-}$ and WT → *Sod1*$^{-/-}$, but not WT → WT nor *Sod1*$^{-/-}$ → WT chimeric mice exhibited elevated concentrations of ALT (Figure 3C). This result indicates an essential role for SOD1 in the non-hematopoietic compartment, of which hepatocytes comprise the major cell population in the liver.

In support of these findings, ROS production was also observed in vitro in primary mouse hepatocytes upon LCMV infection by staining with the oxidation-sensitive fluorogenic probe CellROX Deep Red Reagent (CellROX) (Figure 3D, Figures S2C and S2D), which was reversed by treatment with the antioxidant

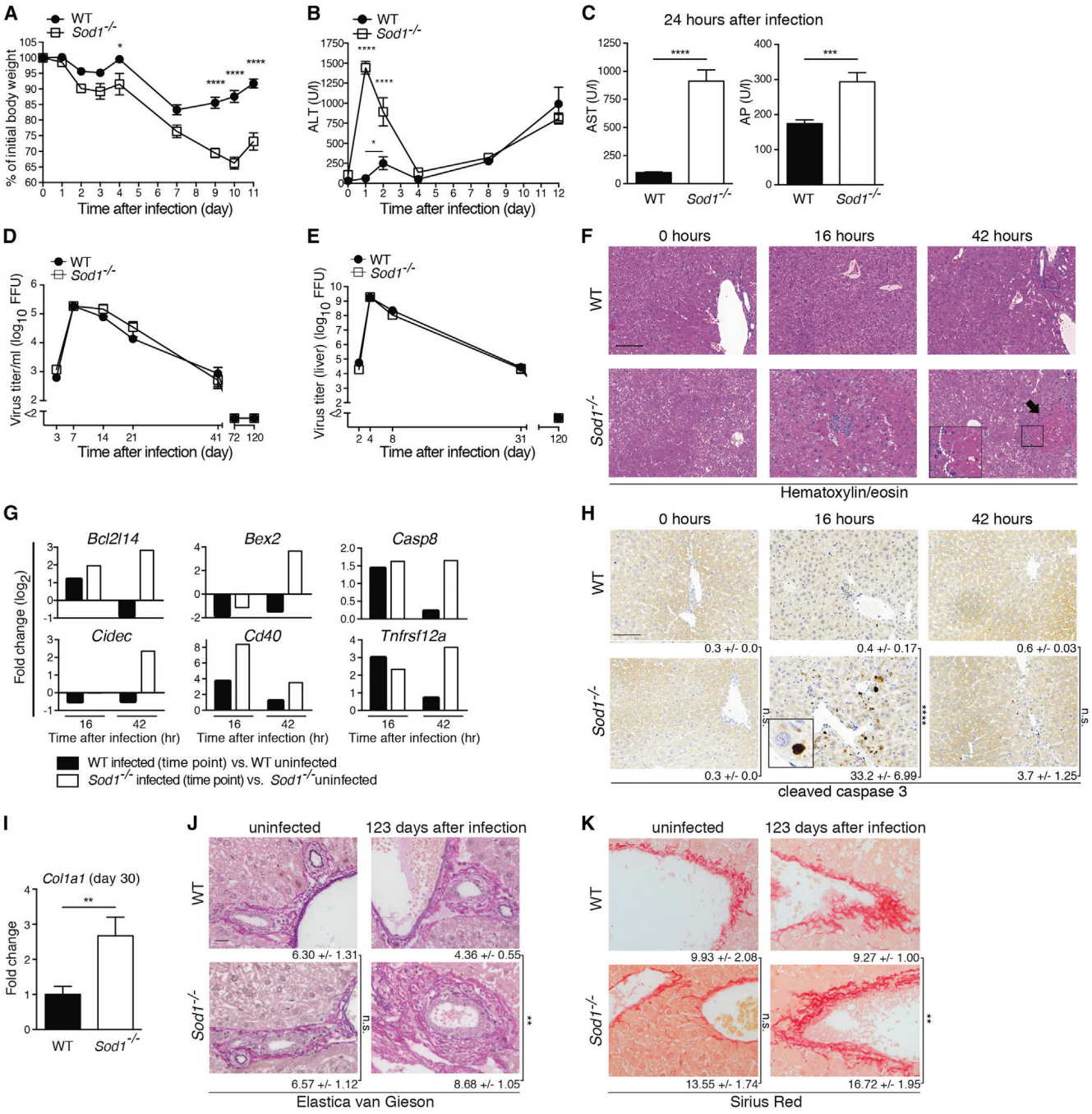

Figure 2. SOD1 Deficiency Leads to Aggravated Liver Damage upon LCMV Infection

(A–K) WT and *Sod1*$^{-/-}$ mice were infected with LCMV.

(A) Body weight was monitored after LCMV infection (n = 4 mice per group).

(B) Serum kinetics of alanine aminotransferase (ALT) was measured after LCMV infection (n = 10 mice per group).

(C) Aspartate aminotransferase (AST) and alkaline phosphatase (AP) were analyzed 24 hr after infection (n = 10 mice per group). One out of ≥ two similar experiments is shown.

(D and E) Viral loads from blood (D) and liver (E) were determined by focus-forming assay (n = 3–7).

(F) Liver sections were stained for hematoxylin/eosin (H/E) (n = 3 mice per group, scale bar represents 200 μm). Representative images are shown. Pathologic lesions are highlighted by arrow and insert.

(G) Comparison of a subset of RNA-seq-derived significantly differentially regulated genes involved in cell death is shown from infected versus uninfected WT or *Sod1*$^{-/-}$ mice (n = 3 mice per group).

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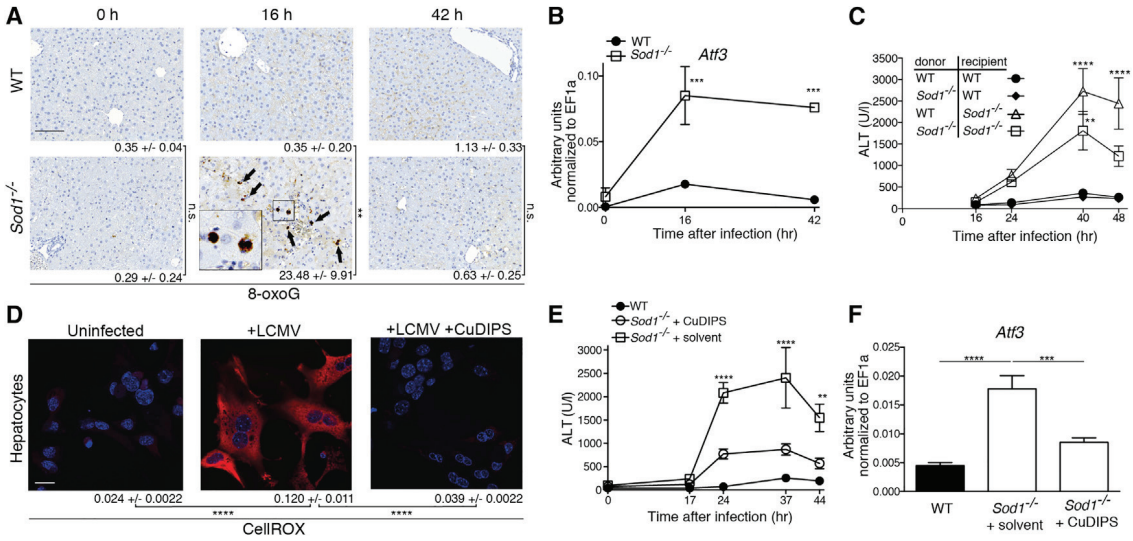

Figure 3. SOD1 Deficiency Leads to Oxidative Stress-Induced Liver Damage upon Viral Infection

(A) Liver sections were stained for 8-oxoguanine (8-oxoG) from uninfected or LCMV infected WT and *Sod1*$^{-/-}$ mice. Arrows indicate 8-oxoG positive cells. Numbers of positive nuclei are shown as mean ± SEM (n = 3 mice per group, scale bar represents 200 μm). Representative images are shown.

(B) WT and *Sod1*$^{-/-}$ mice were infected with LCMV. *Atf3* mRNA levels were determined in liver tissue before and after infection by real-time PCR (n = 3 mice per group). One out of ≥ two similar experiments is shown.

(C) Bone marrow-chimeric mice were generated by reciprocal transfer of *Sod1*$^{-/-}$ and WT genotypes. Serum ALT levels of mice infected with LCMV are shown (n = 5-8 mice per group pooled from two experiments). p values are derived from the comparison to the control group of WT→WT mice.

(D) Primary hepatocytes from WT mice were left uninfected or infected with LCMV (MOI 5) +/− treatment with 10 μM CuDIPS before staining with CellROX. Scale bar represents 20 μm. Representative images are shown. Quantification was performed by CellProfiler and numbers represent mean ± SEM. One out of ≥ two similar experiments is shown.

(E and F) WT and *Sod1*$^{-/-}$ mice, which received either 10 mg/kg body weight CuDIPS or solvent, were infected with LCMV and (E) serum levels of ALT (n = 12 mice per group, pooled from three independent experiments) and (F) *Atf3* mRNA in liver tissue were measured 44 hr after infection (n = 8 mice per time point, pooled from two independent experiments). Statistical significance was calculated by Two-way (A–C, E) or one-way (D and F) ANOVA with Bonferroni correction. Symbols represent the mean ± SEM.

copper(II) (3,5-diisopropyl salicylate)4 (CuDIPS), a non-peptide O_2^- scavenger that mimics SOD1 activity (Laurent et al., 2004). To test the potential effects of antioxidant treatment on virus-induced liver damage, we assessed the effect of CuDIPS in vivo. This ameliorated the virus-induced increase in concentrations of ALT in *Sod1*$^{-/-}$ mice upon infection (Figure 3E) and led to a reduction of *Atf3* mRNA expression (Figure 3F). Together, these data reveal that oxidative stress in hepatocytes plays a fundamental role in the observed virus-induced liver pathology.

T Cells and NK Cells Are Not Involved in the Virus-Induced SOD1-Dependent Liver Pathology

T cells play a major immunopathological role in HBV and HCV infection, as well as in the hitherto-described model of LCMV hepatitis (Guidotti and Chisari, 2006; Lang et al., 2013; Park and Rehermann, 2014; Zinkernagel et al., 1986). We found comparable numbers of infiltrating CD8$^+$ and CD4$^+$ T cells in the liver tissue of infected *Sod1*$^{-/-}$ and WT mice at 24 hr after infection (Figures 4A and 4B). Next, we assessed T cell responses in *Sod1*$^{-/-}$ and WT mice after LCMV infection and found no major differences in virus-specific CD8$^+$ T cells (Figures S3A–S3I) and CD4$^+$ T cells (Figures S3J–S3M). In addition, T cell receptor beta chain (*Tcrb*)$^{-/-}$ → *Sod1*$^{-/-}$ (Figure 4C) and perforin 1 (*Prf1*)$^{-/-}$ → *Sod1*$^{-/-}$ bone marrow chimeric mice (Figure 4D), lacking either αβ T cells or the hematopoietically-expressed cytolytic effector protein PRF1 respectively, exhibited liver damage similar to controls upon infection. Thus, the SOD1-dependent pathology occurs independently of T cells.

NK cells are important regulators of T cell function as well as liver inflammation (Crouse et al., 2014; Rehermann, 2013; Waggoner et al., 2012; Xu et al., 2014) and we, therefore, investigated the potential role of NK cells in the observed liver pathology. Yet, we did not detect any differences in liver-infiltrating NK cells (Figure 4E) nor found any change of pathology upon the depletion of NK cells (Figures 4F and 4G). To study the potential involvement of leucocyte populations other than T cells and NK cells, we

(H) Liver sections were stained for cleaved caspase-3 and numbers of positive cells are shown as mean ± SEM (n = 3 mice per group, scale bar represents 200 μm). Representative images are shown. Insert shows a hepatocyte positive for cleaved caspase-3.

(I) *Col1a1* mRNA was determined by real-time PCR in liver tissue 30 days after infection and fold-change was calculated between infected WT and *Sod1*$^{-/-}$ mice (n = 10 mice per group, pooled from three experiments). Liver tissue was stained by Elastica van Gieson (J) and Sirius Red. (K). Pictures are representative of 9 or 10 infected mice per genotype (pooled from two independent experiments collected on day 103 and day 123, respectively, after LCMV infection) and n = 5 uninfected mice per genotype (scale bars represent 20 μm). Numbers represent the means ± SEM derived from automated quantification (% of area) based on the analysis of 5 high-power fields per sample. Statistical significance was calculated by two-way ANOVA (A, B, D, E, H) or by unpaired t test (C, I–K). Symbols/bars represent the mean ± SEM.

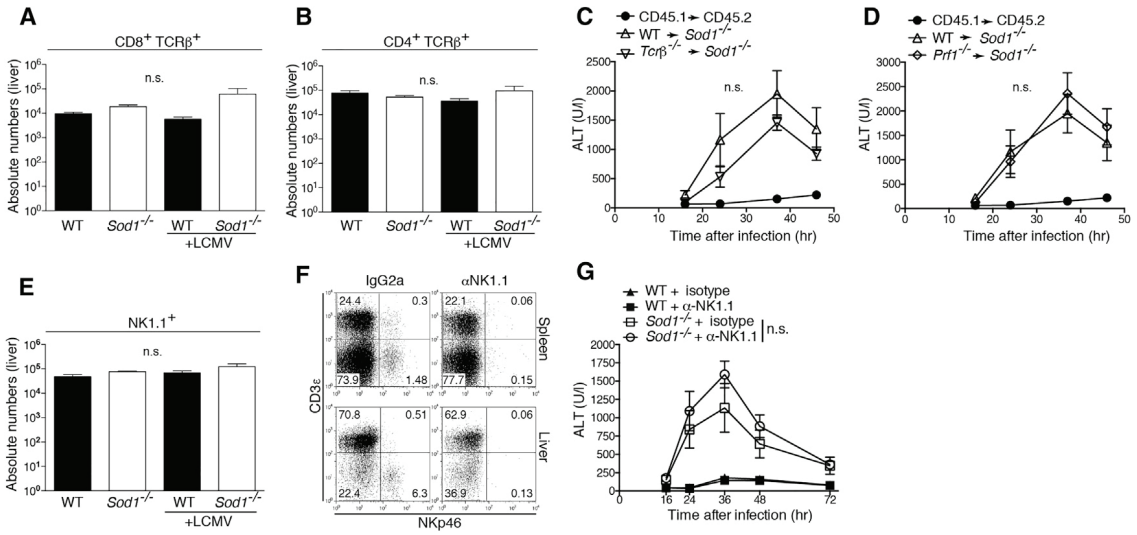

Figure 4. T and NK Cell Independent Liver Pathology in *Sod1*$^{-/-}$ Mice upon Viral Infection

(A and B) WT and *Sod1*$^{-/-}$ mice were infected with LCMV. 24 hr after infection, (A) CD8 T cells (CD8$^+$ TCRβ$^+$) and (B) CD4 T cells (CD4$^+$ TCRβ$^+$) were enumerated in the liver (n = 4-5 mice per group). Absolute numbers are shown.

(C) *Tcrb*$^{-/-}$ or (D) *Prf1*$^{-/-}$ (*perforin 1*) as well as WT bone marrow was transferred into irradiated *Sod1*$^{-/-}$ donor mice (n = 6 mice per group from two pooled experiments). Serum levels of alanine aminotransferase (ALT) were measured upon infection with LCMV.

(E) Natural killer cells (NK1.1$^+$) were quantified in the liver 24 hr after LCMV infection (n = 4-5 mice per group).

(F) NK cells were depleted in WT and *Sod1*$^{-/-}$ mice with the anti-NK1.1 specific antibody. Depletion of NK cells was confirmed by flow cytometry in spleen and liver.

(G) *Sod1*$^{-/-}$ and WT mice, either NK1.1 depleted or treated with isotype, were infected with LCMV and serum levels of ALT were determined (n = 4 mice per group).

Statistical significance was calculated by one-way (A, B, and E) ANOVA or by two-way (C, D, and G) ANOVA with Bonferroni correction. Symbols represent mean ± SEM.

performed a cellular profiling of liver and spleen tissue and detected comparable infiltration of inflammatory monocytes, plasmacytoid dendritic cells, neutrophils, and eosinophils at 24 hr after infection (Figures S3N–S3Q). Together, these data indicate that T and NK cells are unlikely to be causally involved in the SOD1-dependent pathology and exclude a profoundly altered recruitment of inflammatory myeloid populations to the liver, arguing in favor of a hepatocyte-intrinsic defect in infected *Sod1*$^{-/-}$ mice.

Type I Interferon Drives Oxidative Damage in the Liver

In the early phase of infection, we found a more pronounced upregulation of interferon-stimulated genes in *Sod1*$^{-/-}$ compared to WT mice (Figure 5A, Table S1). We also detected increased phosphorylation of signal transducer and activator of transcription 1 (STAT1), a downstream effector of IFN-I, in hepatocytes of *Sod1*$^{-/-}$ compared to WT mice (Figure 5B). This prompted us to further investigate the potential role of IFN-I signaling in SOD1-dependent liver damage upon viral infection. We found that LCMV-infected *Sod1*$^{-/-}$ mice exhibited higher serum concentrations of IFN-α compared to WT mice (Figure 5C), which might itself be driven by the increased phosphorylation of STAT1 upon oxidative stress (Kim and Lee, 2005). Furthermore, we infected *Sod1*$^{-/-}$ mice with a replication-defective recombinant LCMV vector (rLCMV), which is capable of only a single round of infection (Flatz et al., 2010). rLCMV hardly induced any serum IFN-α (Figure 5C) and did not lead to a marked increase of ALT (Figure 5D), despite exhibiting high viral RNA loads in the liver (Figure S4A). Together, these findings demonstrate that propagating virus results in excessive oxidative damage in hepatocytes of *Sod1*$^{-/-}$ mice, which was associated with the induction of IFN-I.

To address whether IFN-I induces oxidative stress, we first treated cells in vitro with rIFN-α and observed accumulation of ROS (Figure 5E). Next, we treated mice with rIFN-α, which resulted in the upregulation of the IFN-stimulated gene *Ifit1* in both *Sod1*$^{-/-}$ and WT mice (Figure S4B). Importantly, rIFN-α led to increased expression of *Atf3* mRNA (Figure 5F) and was sufficient to induce hepatitis as measured by ALT in *Sod1*$^{-/-}$ mice in the absence of infection (Figure 5G). In agreement with this, elevation of IFN-α induced by the neurotropic vesicular stomatitis virus (VSV) (Figure S4C) also resulted in increased concentrations of ALT in *Sod1*$^{-/-}$ compared to WT mice (Figure S4D), indicating that systemic IFN-I is sufficient to induce liver damage in the absence of SOD1. Moreover, treatment with rIFN-α downregulated *Sod1* expression in WT mice (Figure 5H), similar to what we observed upon viral infection (Figures 1D–1F), suggesting a direct involvement of IFN-I signaling in the regulation of *Sod1* expression.

Hepatocyte-Intrinsic Type I Interferon Signaling Drives Liver Damage

To further dissect the role of IFN-I signaling in virus-induced oxidative liver damage, we transplanted bone marrow of WT or *Irf7*$^{-/-}$ mice, which lack the master regulator of IFN-I dependent immune responses, into *Sod1*$^{-/-}$ recipient mice. Upon viral infection, we observed reduced serum concentrations of IFN-α and ALT in *Irf7*$^{-/-}$→*Sod1*$^{-/-}$ mice compared to WT→*Sod1*$^{-/-}$ mice (Figure 6A). This demonstrates that IFN-α derived from hematopoietic cells contributes to tissue damage. Previous studies have shown that a lack of phagocytes resulted in reduced concentrations of serum IFN-α (Louten et al., 2006). Indeed,

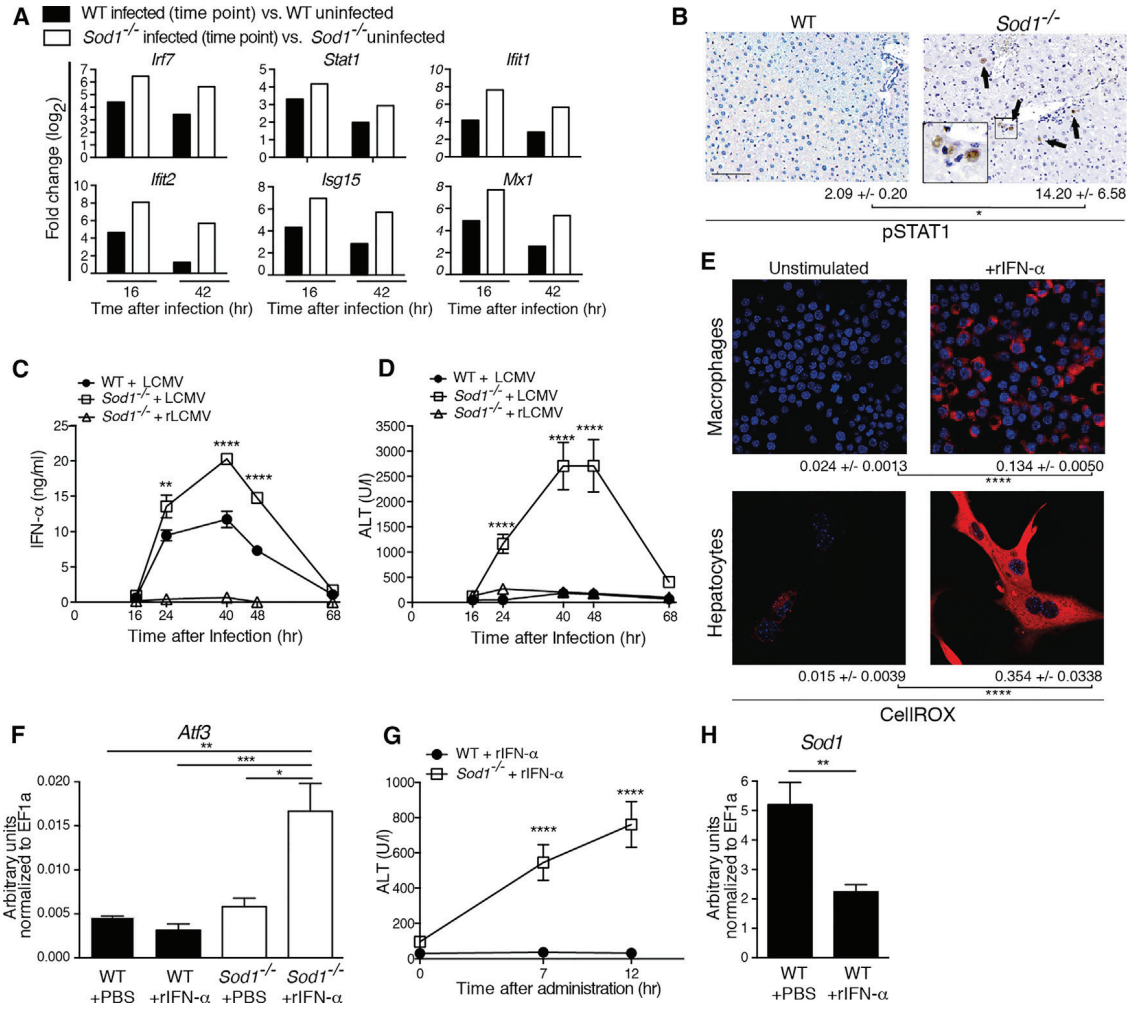

Figure 5. Type I Interferon Drives Oxidative Damage in the Liver

(A) Comparison of a subset of RNA-seq-derived significantly differentially regulated interferon-stimulated genes from LCMV infected versus uninfected WT and *Sod1*$^{-/-}$ mice (n = 3 mice per group). See also Table S1.

(B) Liver sections were stained for STAT1 phosphorylation 16 hr after infection with LCMV. Arrows highlight positive nuclei. Numbers of phospho-STAT1 positive cells per mm^2 are shown as mean ± SEM (n = 3 mice per group, scale bar represents 200 μm). Representative images are shown.

(C and D) WT and *Sod1*$^{-/-}$ mice were infected with LCMV and, in addition, *Sod1*$^{-/-}$ mice with a rLCMV vector. (C) IFN-α and (D) ALT were measured in the serum (n = 6 mice per group).

(E) RAW264.7 macrophages and primary hepatocytes from WT mice were stimulated with 2000 U/ml recombinant mouse IFN-α (rIFN-α) or left unstimulated. 24 hr later cells were stained with CellROX. Scale bar represents 20 μm. Representative images are shown. Quantification was performed by CellProfiler and numbers represent mean ± SEM.

(F–H) WT respectively *Sod1*$^{-/-}$ mice were treated with 100ng of rIFN-α. (F) *Atf3* and (H) *Sod1* mRNA were determined by real-time PCR in the liver 12 hr later, and (G) serum levels of ALT were measured (n = 4–6 mice per group). (C–H) one out of ≥ two similar experiments is shown. Statistical significance was calculated by two-way (B, C, D, and G) ANOVA, one-way (F) ANOVA with Bonferroni correction, or by unpaired t test (E and H). Symbols and bars represent the mean ± SEM.

Sod1$^{-/-}$ mice treated with liposomal clodronate to deplete phagocytic cells had reduced concentrations of serum IFN-α upon infection (Figure 6B), which was accompanied by lower concentrations of ALT (Figure 6C).

To study the causative role of IFN-I in virus-induced oxidative tissue damage, we infected mice lacking IFN-α/β receptor 1 (IFNAR1) with LCMV. *Ifnar1*$^{-/-}$ mice exhibited reduced concentrations of ALT compared to WT mice (Figure 6D) and a decreased induction of *Atf3* mRNA in the liver after infection (Figure 6E), confirming the central role of IFN-I signaling in mediating liver damage. In line with these findings, infected *Stat1*$^{-/-}$ mice were also protected from liver damage (Figure 6F). This correlated with the absence of *Sod1* mRNA downregulation in infected *Stat1*$^{-/-}$ mice (Figure 6G), suggesting that STAT1 signaling negatively regulates the expression of *Sod1*.

To further investigate the role of IFN-I signaling in mediating liver damage in *Sod1*$^{-/-}$ mice, we crossed *Sod1*$^{-/-}$ mice to *Stat1*$^{-/-}$ mice. Indeed, these double gene-deficient mice were protected from virus-induced early hepatitis (Figure 6H). The proximity of the *Sod1* and *Ifnar1* genes (1.42cM) prevented us from generating double gene-deficient mice of this combination.

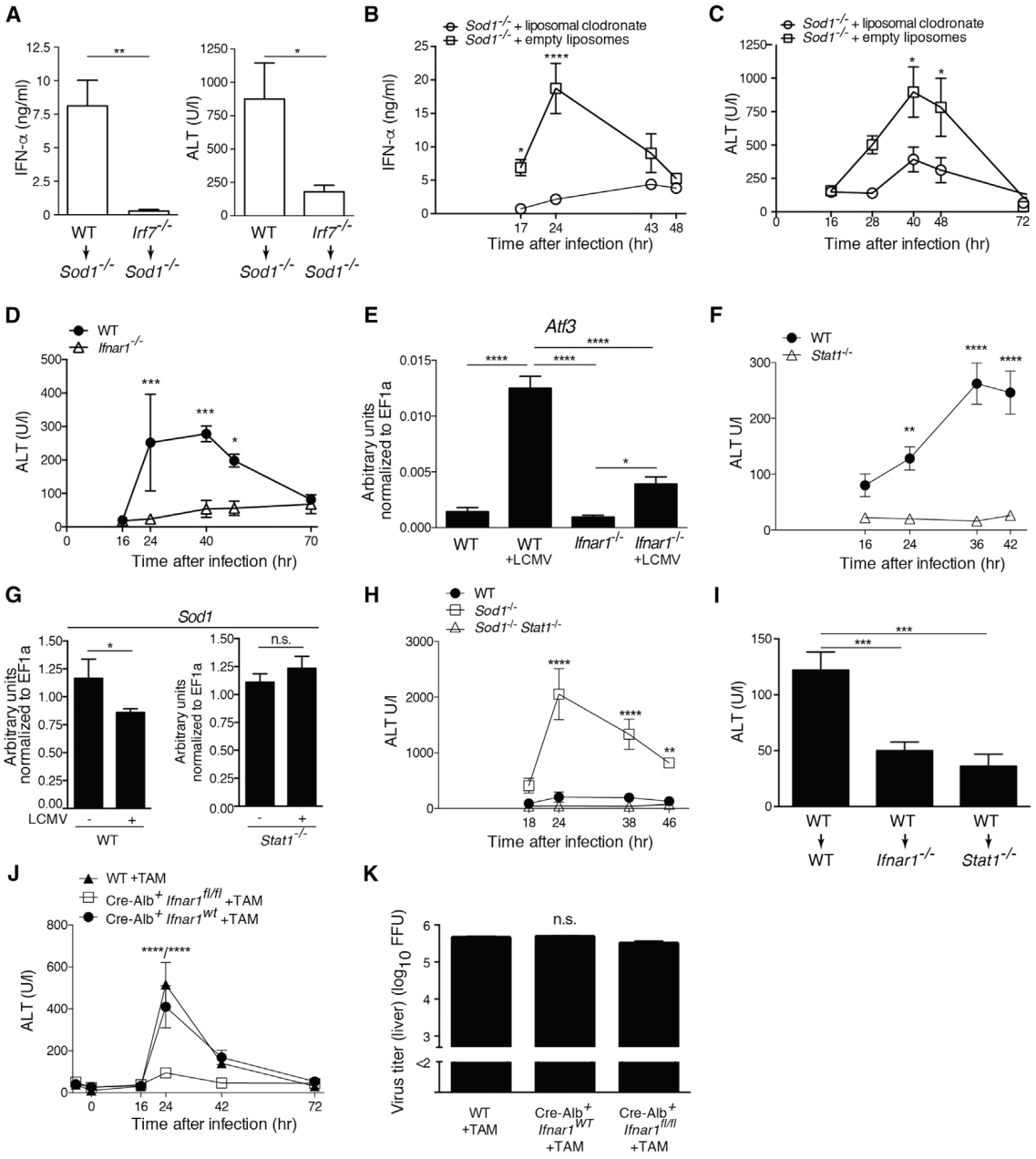

Figure 6. Non-hematopoietically Derived Type I Interferon Signals to IFNAR1 Expressed on Hepatocytes and Causes Liver Pathology

(A) Serum levels of IFN-α (n = 6 mice) and ALT (n = 10 or 11 mice pooled from three independent experiments) of WT→*Sod1*$^{-/-}$ and *Irf7*$^{-/-}$→*Sod1*$^{-/-}$ chimeric mice 24 hr after infection with LCMV.

(B) IFN-α and (C) ALT of *Sod1*$^{-/-}$ mice infected with LCMV upon treatment with liposomal clodronate or empty liposomes (n = 4 mice per group). One out of ≥ two similar experiments is shown.

(D and E) WT and *Ifnar1*$^{-/-}$ mice were infected with LCMV. Levels of (D) serum ALT and (E) *Atf3* mRNA in the liver were measured at 42 hr after infection (D and E, n = 4 mice). One out of ≥ two similar experiments is shown.

(F) WT and *Stat1*$^{-/-}$ mice were infected with LCMV and levels of serum ALT (n = 4 mice) were measured. One out of ≥ two similar experiments is shown.

(G) *Sod1* mRNA (n = 4–9 mice pooled from three experiments) in the liver were measured at 42 hr after infection.

(H) WT, *Sod1*$^{-/-}$ and *Stat1*$^{-/-}$ *Sod1*$^{-/-}$ mice were infected with LCMV and levels of serum ALT were measured (n = 4 mice per group).

(I) WT→WT, WT→*Ifnar1*$^{-/-}$, and WT→*Stat1*$^{-/-}$ chimeric mice were infected with LCMV and levels of serum ALT at 36–39 hr after infection were measured (n = 8 mice pooled from two experiments).

(J and K) WT mice, Cre-Alb ERT2 x *Ifnar1*$^{fl/WT}$ or Cre-Alb ERT2 x *Ifnar1*$^{WT/WT}$ (designated as Cre-Alb ERT2 × *Ifnar1*^{WT} in the graph) and Cre-Alb ERT2 x *Ifnar1*$^{fl/fl}$ mice were administered 1 mg tamoxifen in sunflower oil i.p. each for 5 consecutive days, subsequently infected with LCMV and (J) levels of serum ALT and (K) viral loads in the liver 72 hr after infection were measured (n = 4 or 5 mice per group).

Statistical significance was calculated by unpaired t test (A and G), two-way (B–D, F, H, J) or one-way (E, I, and K) ANOVA with Bonferroni correction. Symbols and bars represent the mean ± SEM.

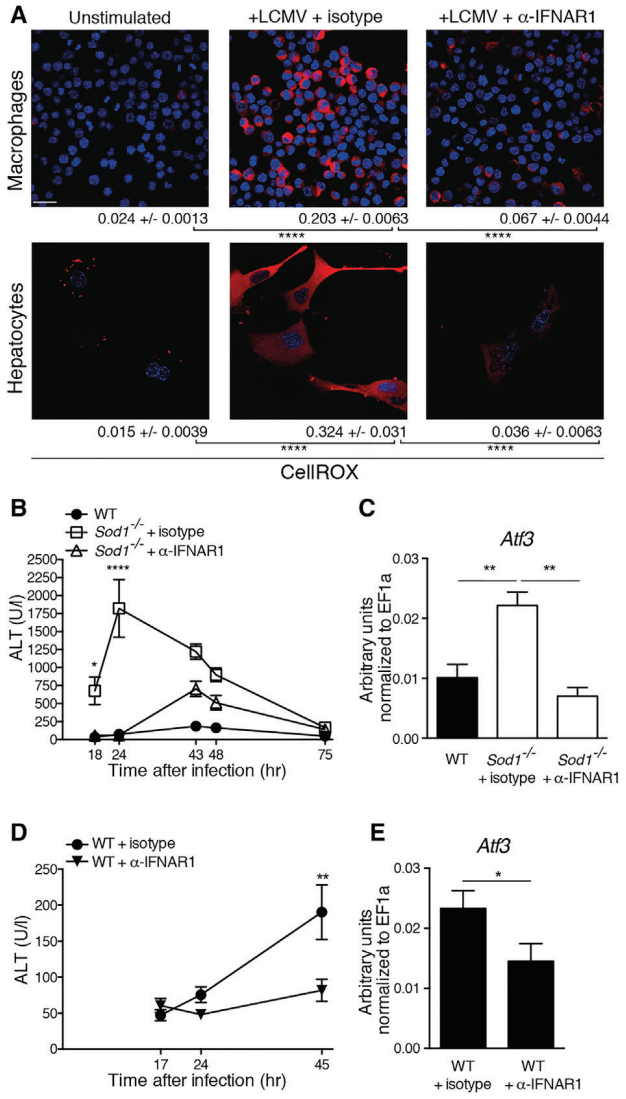

Figure 7. Blockade of Type I Interferon Signaling Ameliorates Oxidative Stress-Induced Liver Pathology upon Viral Infection

(A) RAW264.7 macrophages and primary hepatocytes from WT mice were infected with LCMV (MOI 5) and co-treated with 20 μg/ml blocking antibody α-IFNAR1 or isotype control. 24 hr later cells were stained with CellROX. Scale bar represents 20 μm. Representative images are shown. Quantification was performed by CellProfiler and numbers represent mean ± SEM. The group of unstimulated hepatocytes is the same as used for Figure 5E. One out of ≥ two similar experiments is shown.

(B) WT mice respectively *Sod1*$^{-/-}$ mice, which received either isotype control or α-IFNAR1 blocking antibody, were infected with LCMV. Serum kinetics of ALT was measured (n = 4 mice per group). One out of ≥ two similar experiments is shown.

(C) *Atf3* mRNA in the liver was measured 72 hr after LCMV infection from mice treated as described in (B).

(D) WT mice received either isotype control or α-IFNAR1 blocking antibody and were infected with LCMV. Serum kinetics of ALT was measured (n = 9 or 10 mice pooled from two experiments).

(E) *Atf3* mRNA in the liver was measured 45 hr after LCMV infection from mice treated as described in (D).

Statistical significance was calculated by one-way (A and C) or by two-way ANOVA (B and D) with Bonferroni correction and (E) with unpaired t test. Symbols and bars represent the mean ± SEM.

Further, we also observed that WT→*Ifnar1*$^{-/-}$ and WT→*Stat1*$^{-/-}$ bone marrow chimeric mice were protected from early hepatitis upon LCMV infection (Figure 6I), suggesting that the liver damage is mediated by nonhematopoietic IFNAR1-STAT1 signaling. Finally, genetic ablation of *Ifnar1* specifically in hepatocytes was sufficient to confer protection (Figure 6J) despite comparable viral loads (Figure 6K). Collectively, these results provide evidence that the death of hepatocytes is mediated by cell-intrinsic IFN-I signaling through the IFNAR1-STAT1 axis.

Blockade of Type I Interferon Signaling Ameliorates Oxidative Stress-Induced Pathology

Next, we investigated whether the pharmacological blockade of the IFN-I signaling pathway has a beneficial effect on the virus-induced oxidative tissue damage. Antibody blockade of IFNAR1 abrogated the virus-induced generation of ROS in hepatocytes and macrophages in vitro (Figure 7A), highlighting a broader relevance for IFN-I induced oxidative stress in different cell types. In line with these in vitro experiments, blockade of IFNAR1 prevented the early elevation of ALT (Figures 7B and 7D) and decreased the expression of *Atf3* mRNA in *Sod1*$^{-/-}$ and WT mice upon LCMV infection (Figures 7C and 7E).

To investigate the long-term effects of transient blockade of IFNAR1, we administered the IFNAR1-specific antibody on day −1, 0, and 1 after infection as performed previously and monitored the course of chronic viral infection for 30 days. The blockade of IFNAR1 resulted in increased viremia in the early phase of infection (Figure S5A), but within 12 days the viral loads in the blood decreased to levels found in mice that had not received the blocking antibody. In line with this, comparable viral loads were found in organs 30 days after infection (Figure S5B). As shown previously in Figure 7, the blockade of IFNAR1 resulted in protection from early hepatitis, yet it subsequently led to exacerbated concentrations of ALT 12 days after infection, coinciding with improved virus control that was likely driven by T cells (Figure S5C) (Teijaro et al., 2013; Wilson et al., 2013). The transient blockade of IFNAR1 did not affect the increased expression of *Col1a1* mRNA in the liver tissue of infected *Sod1*$^{-/-}$ mice on day 30 (Figure S5D), suggesting that early IFN-I signaling might be insufficient to drive late liver pathology. The increased *Col1a1* levels correlated with elevated *Ifit1* expression in the liver tissue of *Sod1*$^{-/-}$ mice (Figure S5E), which might indicate a contributive role of sustained IFN-I signaling to the observed fibrotic processes. Together, these results reveal an early tissue-protective effect for the blockade of IFNAR1 and highlight the role of IFN-I in driving oxidative liver damage induced by viral infections both in *Sod1*$^{-/-}$ and in WT mice.

DISCUSSION

These findings demonstrate that (1) SOD1 is essential in protecting hepatocytes from virus-induced damage, (2) IFN-I decreases the expression of SOD1, (3) IFN-I is necessary and sufficient to promote oxidative damage in the liver, and (4) blockade of IFN-I signaling protects from virus-induced oxidative liver damage. This functional circuit of IFN-I, SOD1, and oxidative stress provides mechanistic insights into the inflammatory and tissue-damaging processes in the liver upon viral infection, as well as a deeper understanding of the role of oxidative stress in viral

hepatitis. Our data suggest a similar role for IFN-dependent processes occurring in human infections with hepatotropic and non-hepatotropic viruses, which can also result in clinically apparent liver injury (Lalazar, 2014). The observed SOD1-dependent pathology appeared to be localized predominantly to the liver, which might be due to its delicate redox status as the major organ for iron transport and storage resulting in the production of large amounts of ROS (Crichton et al., 2002).

Our study shows that the expression of SOD1 is downregulated by IFN-I signaling during viral infection and that loss of SOD1 results in oxidative damage in the liver. Together with similar observations of SOD1 downregulation in viral hepatitis in man (Diamond et al., 2012; Dillon et al., 2013; Kim et al., 2003; Levent et al., 2006; Megger et al., 2013), this implies a likely role for SOD1 in virus-driven liver pathogenesis. The molecular mechanism of how IFN-I signaling induces downregulation of *Sod1* needs further investigations and is expected to involve post-translational regulation of transcription factors such as NF-κB, AP-1, and SP1, which bind to the *Sod1* promoter (Chang and Hung, 2012; Miao and St Clair, 2009; Radaeva et al., 2002; von Marschall et al., 2003).

Liver fibrosis in chronic hepatitis C patients was found to be associated with elevated endogenous IFN-I signatures (Bièche et al., 2005; Sarasin-Filipowicz et al., 2008) and increased oxidative stress (Parola and Robino, 2001; Sánchez-Valle et al., 2012), which correlates with the data of our study. Yet, the LCMV model might not recapitulate all features of chronic liver fibrosis seen in humans, and studies with other models and/or patient samples will be required to provide further insights into the contribution of SOD1 in disease pathogenesis.

It remains to be determined whether there is a benefit to the host or whether, alternatively, the pathogen-induced oxidative stress represents simply a metabolic by-product of the IFN-I driven response. We speculate that this process might bear relevance for the metabolic rewiring of the cell, whereby the IFN-I driven transient changes in the redox status contribute to the rapidly changing bioenergetic and signaling demands as part of the antiviral state and/or of mechanisms of disease tolerance (Everts et al., 2014; Medzhitov et al., 2012; Pantel et al., 2014; Schieber and Chandel, 2014; Weinberg et al., 2015). The molecular understanding of such crosstalk between metabolic and inflammatory processes might also contribute to a better understanding of the mechanism(s) of action and side effects of IFN-I therapies in non-infectious diseases like multiple sclerosis and cancer (Reder and Feng, 2014; Sistigu et al., 2014).

Insights into this innate immunity-driven immunopathology provide a paradigm for infection-associated tissue damage by uncovering the redox system as a crucial effector downstream of the IFN-driven innate immune response. This establishes a molecular connection between cellular homeostasis, metabolism, and tissue damage in the context of viral infection and adds to the ongoing efforts to understand the pleiotropic antiviral and immunomodulatory effects of IFN-I (McNab et al., 2015; Schoggins et al., 2011; Teijaro et al., 2013; Wilson et al., 2013). Administration of targeted antioxidants and/or transient blockade of IFN-I signaling might bear liver-protective potential in the context of IFN-I responses in infectious and inflammatory diseases.

EXPERIMENTAL PROCEDURES

Mice

CD45.1 (Janowska-Wieczorek et al., 2001), Cre-Alb ERT2 (Schuler et al., 2004), *Irf7*$^{-/-}$ (Honda et al., 2005), *Ifnar1*$^{fl/fl}$ (Kamphuis et al., 2006), *Ifnar1*$^{-/-}$ (Müller et al., 1994), *Prf1*$^{-/-}$ (Kägi et al., 1994), *Sod1*$^{-/-}$ (Matzuk et al., 1998), *Sod2*$^{+/-}$ (Lebovitz et al., 1996), *Sod3*$^{-/-}$ (Carlsson et al., 1995), *Stat1*$^{-/-}$ (Durbin et al., 1996), *Tcrb*$^{-/-}$ (Mombaerts et al., 1992) (all on a C57BL/6J genetic background), and C57BL/6J mice were bred under specific pathogen-free conditions at the Institute for Molecular Biotechnology of the Austrian Academy of Sciences in Vienna, Austria, at the Charité animal facility in Berlin, Germany, and at the animal facility of the Max Planck Institute for Infection Biology in Berlin, Germany. Experiments were performed in individually ventilated cages at the Department for Biomedical Research of the Medical University of Vienna in Vienna, Austria, at the Charité animal facility in Berlin, Germany, and at the Institute of Immunobiology, Kantonal Hospital St. Gallen in St. Gallen, Switzerland, in compliance with the respective animal experiment licenses approved by the institutional ethical committees and the institutional guidelines.

For the generation of chimeric mice, bone marrow cells were obtained from respective donor mice by flushing the femur, tibia, and fibula bones with PBS/BSA/EDTA. Recipient mice were subjected to a total irradiation of 11Gy using a Gammacell 40 B(U) (Nordion International Inc.). 1×10^7 bone marrow cells from the respective donor were transferred to the recipient mice 1 day post irradiation. 4 weeks following this procedure, the chimeric mice received 200 μg of anti-CD90 antibody intraperitoneally (i.p.) to deplete any remaining peripheral T cells from the recipient. Approximately 3 weeks later, the mice received intravenously 100 μl liposomal clodronate (clodronateliposomes.com) per 10 g of bodyweight to deplete any remaining macrophages from the recipient. These mice were taken in experiment 3 weeks later to allow repopulation of macrophages. No depletions were done for the chimeric experiments shown in Figure 6I.

Viruses

As the standard LCMV protocol, we infected mice intravenously (i.v.) with 2×10^6 focus-forming units (FFU) of LCMV strain clone 13 (Bergthaler et al., 2010). In addition, we infected mice with either 2×10^3 FFU (low dose) of strain clone 13 or 2×10^6 FFU of strain ARM (Bergthaler et al., 2010; Bergthaler et al., 2007). For experiments with the propagation-deficient vector "rLCMV," we infected mice i.v. with 2×10^5 FFU of rLCMV/OVA (Flatz et al., 2010). Infectious titers of LCMV were determined by focus-forming assay (Bergthaler et al., 2010). For MHV experiments, mice were infected intraperitoneally with 1×10^3 PFU of strain A59 (Cervantes-Barragán et al., 2009). MHV titers were determined by plaque assay (Cervantes-Barragan et al., 2007). For VSV experiments, we infected mice i.v. with 2×10^6 PFU of strain Indiana. VSV titers were determined by plaque assay (Bonilla et al., 2002).

Statistical Analysis

Results are displayed as mean ± SEM and were statistically analyzed as detailed in the figure legends using GraphPad Prism version 5 or 6. Statistically significant p values were indicated as follows: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

ACCESSION NUMBER

The raw data from our RNaseq data are deposited at ArrayExpress (http://www.ebi.ac.uk/arrayexpress) with accession number E-MTAB-2351.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2015.10.013.

AUTHOR CONTRIBUTIONS

A. Bhattacharya and A.N.H. designed experiments, performed in vitro and in vivo studies, and wrote the manuscript. N.D., L.K., A.H., B.V., C.S., I.P.,

K.K., H.B., I.A., H.C.X., and P.A.L. performed in vitro and/or in vivo experiments. N.D. and L.K. contributed equally to this work. R.K.K. did bioinformatical analyses. J.C. and B.L. performed MHV experiments. D.M. and A.A.K. did histological analyses. L.F., G.S.-F., J.C., A.T., and M.L. provided reagents, analyzed data, and/or contributed to the experimental design. A. Bergthaler supervised the study, designed experiments, performed in vitro and in vivo experiments, and wrote the manuscript.

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Immunity

Supplemental Information

Superoxide Dismutase 1 Protects Hepatocytes from Type I Interferon-Driven Oxidative Damage

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Supplemental Experimental Procedures

Blood chemistry

Activities of Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (AP), blood urea nitrogen and creatinine were analyzed using a Cobas C311 Analyzer (Roche) or a 747 Automatic-Analyzer (Hitachi).

RNA isolation and real-time PCR

Total RNA was extracted from cells or homogenized tissue lysates with QIAzol lysis reagent according to the manufacturer`s instructions (Qiagen). RNA was reverse transcribed to cDNA using random primers and the First Strand cDNA Synthesis Kit (Fermentas). Real-time PCR was performed with Taqman Fast Universal PCR Mastermix (Life Tech) using the Taqman GeneExpression Assays *Atf3* (Mm00476032, Life Tech) and *Sod1* (Mm01700393, Life Tech). LCMV NP and EF1a were detected by corresponding probe and primer sets as described previously (Gilchrist et al., 2006; Pinschewer et al., 2010). Expression values are expressed as ΔCt.

RNAseq

RNA was extracted from livers of *Sod1*$^{-/-}$ and WT mice harvested 16h and 42h after infection with LCMV clone 13 (in triplicates for each time-point). For the library preparation, the TruSeq RNA sample preparation kit v2 (Illumina) was used according to the manufacturer`s protocol. Quality control analysis was performed on all samples of the cDNA library using Experion DNA Analysis chip (Biorad) and Qubit Fluorometric quantitation (Life Tech). 6 samples were pooled per lane and run on a 50bp single-end flow cell in a Hiseq2000 sequencer (Illumina).

The RNA-Seq data was processed by aligning the fastq files containing the read sequences to mouse genome (build mm9) using TopHat2. Relative fold changes were computed using Cuffdiff at an FDR of 0.05. Genes with a corrected P-value ≤ 0.05 and absolute robust Z score ≥ 1.5 were marked as differentially regulated genes. The raw data from our RNAseq data are deposited at ArrayExpress (http://www.ebi.ac.uk/arrayexpress/) with accession number E-MTAB-2351.

Bioinformatical Analysis

Enrichment for Gene Ontology – Biological Processes was carried out using DAVID analysis tool (david.abcc.ncifcrf.gov). Terms with a Benjamini-corrected p-value ≤ 0.01 were

considered as significantly enriched processes. Genes involved in oxidation-reduction were clustered into 6 distinct groups using k-means clustering method implemented using TM4 Microarray Expression Viewer (www.tm4.org). The significantly differentially regulated genes shown in **Figure 1D** were selected based on an intersection with a published list of human genes with antioxidant function (Gelain et al., 2009).

***In vitro* staining for oxidative stress**

To detect ROS production, primary mouse hepatocytes were seeded on cover slips pre-coated with collagen and RAW264.7 macrophages were seeded on Chamber Slides Lab-Tek II (Thermo Scientific).

At the indicated time point, the cells were stained with 5µM CellROX Deep Red Reagent (Life Tech, Cat. No. C10422) for 30 min at 37 °C, washed with PBS, fixed in 4 % paraformaldehyde respectively 4 % formaldehyde for 20 min at 4 °C and counterstained with 5 µg/ml Hoechst 33258 for 7 min. Finally, the coverslips were mounted onto microscopy slides using Fluoro Mounting Medium. Quantification of immunofluorescence images with CellROX was performed based on the mean fluorescence intensity of cytoplasmic area defined by the distance from the nuclei using the CellProfiler cell image analysis software v2.0 (Carpenter et al., 2006). The size range for the pipelines was adjusted depending on the cell type.

Histology

Tissue specimens (liver) were fixed (12-48 hours at 4 °C) with 4% formaldehyde and subsequently embedded in paraffin as described previously (Bergthaler et al., 2007). Microtome sections (3 µm thick) were stained with Hematoxylin and Eosin or prepared for immunohistochemistry as follows: Endogenous peroxidases (PBS/3% H_2O_2) were neutralized and unspecific binding blocked (PBS/10% FCS). Sections were stained with primary antibodies: rabbit α-Phospho-STAT1 (detecting only phosphorylated STAT1 at position Tyrosine 701; Cell Signaling #9167), rabbit α-cleaved caspase 3 (BD Pharmingen or Cell Signalling) and mouse α-8-oxoguanine (8-oxoG) (Millipore, MAB3560) (Kitada et al., 2001) antibody. Bound primary antibodies were visualized using ready-to-use, peroxidase-based EnVision™ (Dako) secondary detection system with 3,3′-diaminobenzidine (DAB) as chromogen (haemalaun counterstaining of nuclei). Immunostained sections were registered using the slide scanner Pannoramic 250 Flash (3DHistech) at 200x magnification. Immunostained liver sections were quantified using Tissue Studio 3 software (Definiens AG, Munich, Germany). DAB positive structures were expressed as cells/nuclei per mm^2. Phospho-

STAT1 stained sections were scanned using panoramic 250 flash slide scanner (3D Histech Ltd, Hungary). Phospho-STAT1 positive nuclei in the liver were quantified using Definiens Tissue Studio® 3 software (Definiens AG, Munich, Germany) and detected nuclei were expressed as cells per mm^2.

For fibrosis stainings, 1-2 µm paraffin sections of livers were de-waxed, hydrated and stained for one hour at room temperature with Sirius Red (Polyscience, USA) and picric acid (Merck, Darmstadt). Sections were washed in acidified water (0.01N HCl), dehydrated, cleared in xylol (Roth, Karlsruhe) and mounted with corbit balsam (Hecht, Kiel). Additionally, liver sections were stained for elastic fibres with Elastica van Gieson staining kit (Merck, Darmstadt) according to manufacturer´s instructions. Nuclei appear black, elastic fibres purple and collagen red. Images were acquired using an AxioImager Z1 microscope (Carl Zeiss MicroImaging, Inc., Jena, Germany). For quantification of collagen content, five high power fields (hpf; 0.237 mm^2) per section were analysed using the module AutMess (Carl Zeiss MicroImaging, Inc.).

Histo-pathological scoring for MHV infection was performed evaluating the following criteria: i) lobular area inflammation (0 no infiltrating cells, 1 <100 infiltrating cells, 2 >100 infiltrating cells, 3 > 300 infiltrating cells), ii) lobular disarray and hepatocyte ballooning (0 nil, 1 <60%, 2 >60%) and iii) hepatocyte death (0 nil, 1 <20, 2 >20 cells).

Western blot

Protein concentration of liver lysates were determined by Coomassie Protein Assay (Thermo Scientific). Proteins were analyzed by SDS-Page using NuPAGE® Novex 4-12% Bis-Tris Gels (Life Tech), Westran® Clear signal PVDF membranes (Whatman) and the following antibodies: anti-SOD1 (Abcam #ab16831) and anti-ACTIN (Sigma #A2066). The protein size was determined with the PageRuler^{TM} Prestained Protein Ladder (Thermo Scientific). For protein quantification, the secondary antibody donkey anti-rabbit IgG-IRDye800 (Rockland, #611-732-128) was used. The bands were quantified on an Odyssey Imager (model #9120, LI-COR) using the software Image Studio v3.1 (LI-COR).

Cytokine determination

For detection of IFNα, sera were 1:10 prediluted and analysed by ELISA using rat α-mIFN-α capture antibody (PBL Interferon Source 22100-1), rabbit α-mIFN-α detection antibody (PBL Interferon Source 32100-1), α-rabbit HRP secondary antibody (Jackson ImmunoResearch 711-036-152) and TMB solution (Life Tech 002023).

Primary mouse hepatocyte isolation

Primary hepatocytes were isolated from anesthetized mice (Ketamine/Xylazine: 1:3, 0.1 ml/10 g mouse) upon perfusion via the portal vein with washing buffer followed by digestion of the liver using liberase (Roche). Next, the liver was detached and put in a petridish containing liberase buffer. Cells were shaken out of the liver, filtered using a 70 μm cell strainer (BD Falcon) and suspended in William's E Medium GlutaMAX™ (Invitrogen) containing 10 % FCS and 1 % PSQ. The cells were allowed to settle for 20 min on ice, before the upper 25 ml containing dead cells and debris were discarded. The remaining 25 ml were spun down for 10 min at 500 rpm at 4 °C. The pellet was washed in William`s E Medium/10 % FCS/1 % PSQ and centrifuged at 500 rpm for 3 min. Finally, cells were resuspended in William`s E Medium/10 % FCS/1 % PSQ and seeded at a density of 0.75-1x10^6 cells/well on 6-well plates precoated with 50 mg/l rat tail collagen type I (BD, Cat. No. 354236) containing 8.3 % glacial acetic acid (Merck, Cat. No. 1000632500). After 3 h the medium was changed to Will E/0.5 % FCS/1 % PSQ and maintained in this medium.

Flow-cytometric analysis

Single cell suspensions of lymphoid and nonlymphoid organs were prepared by mechanical disruption. Lymphocytes were purified using Histopaque 1083 (Sigma-Aldrich) and density centrifugation (400 g at 20°C for 30 min). To prevent unspecific binding of mAb, 50 μg/ml of purified mouse and rat IgG were added to the staining mixture (Jackson Immunoresearch). Dead cells were excluded using fixable viability dye eFluor 780 (eBioscience). Subsequently, the cells were stained with the following mAbs in PBS containing 0.2% BSA and 2 mM EDTA: α-CD45 (30-F11), α-CD8a (53-6.7), α-CD11c (N418), α-F4/80 (BM8), α-MHCII (M5/114.15.2), α-NK1.1 (PK136), α-PDCA-1 (eBio927) and α-TCRβ (H57-597) were from eBioscience; α-CD45.1 (A20), α-CD45.2 (104), α-CD4 (RM4-5), α-Ly6G (1A8) and α-Ly6C (HK1.4) were from BioLegend. α-CD11b (M1/70) and α-siglec-F (E50-2440) were from BD Bioscience. Samples were acquired on a FACS LSR Fortessa or FACSCanto II (Becton Dickinson), data was analyzed with FlowJo (Tree Star).

Pharmacological treatments

Cells were treated *in vitro* with 10μM Copper (II) 3,5-diisopropylsalicylate hydrate (CuDIPS) (Sigma Aldrich, Cat. No. 341649) (Laurent et al., 2004). Mice were administered i.p. with 10mg of CuDIPS per kg body weight one day before infection, and received two additional

shots of 10mg/kg each at zero and one day after infection. Mice were administered 100ng recombinant mouse IFN-α4 i.v. (PBL Interferon Source 12115-1).

***In vivo* depletions/blockades**

To neutralize type I IFN signaling, 250μg of α-IFNAR1 antibody (clone MAR1-5A3, BioXCell) was administered i.p. one day prior to infection with two additional shots of 250μg each at zero and one day after infection. The control group was administered with a matched isotype control (clone MOPC21, BioXCell). Natural Killer (NK) cells were depleted by administration of 25 μg of α-NK1.1 (clone PK136, BioXCell) i.p. one day prior and one day after infection. The control group received mouse IgG2a isotype (clone C1.18.4, BioXCell). Phagocytic cells were depleted two days prior to infection by i.v. administration of 100μl liposomal clodronate or empty liposomes (ClodronateLiposomes.com) per 10g body weight. For tamoxifen treatment, mice were administered 1mg tamoxifen (Sigma Aldrich, Cat. No. T5648) in sunflower oil (Sigma Aldrich, Cat. No. S5007) i.p. each for 5 consecutive days.

Supplemental References

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Supplemental Figures

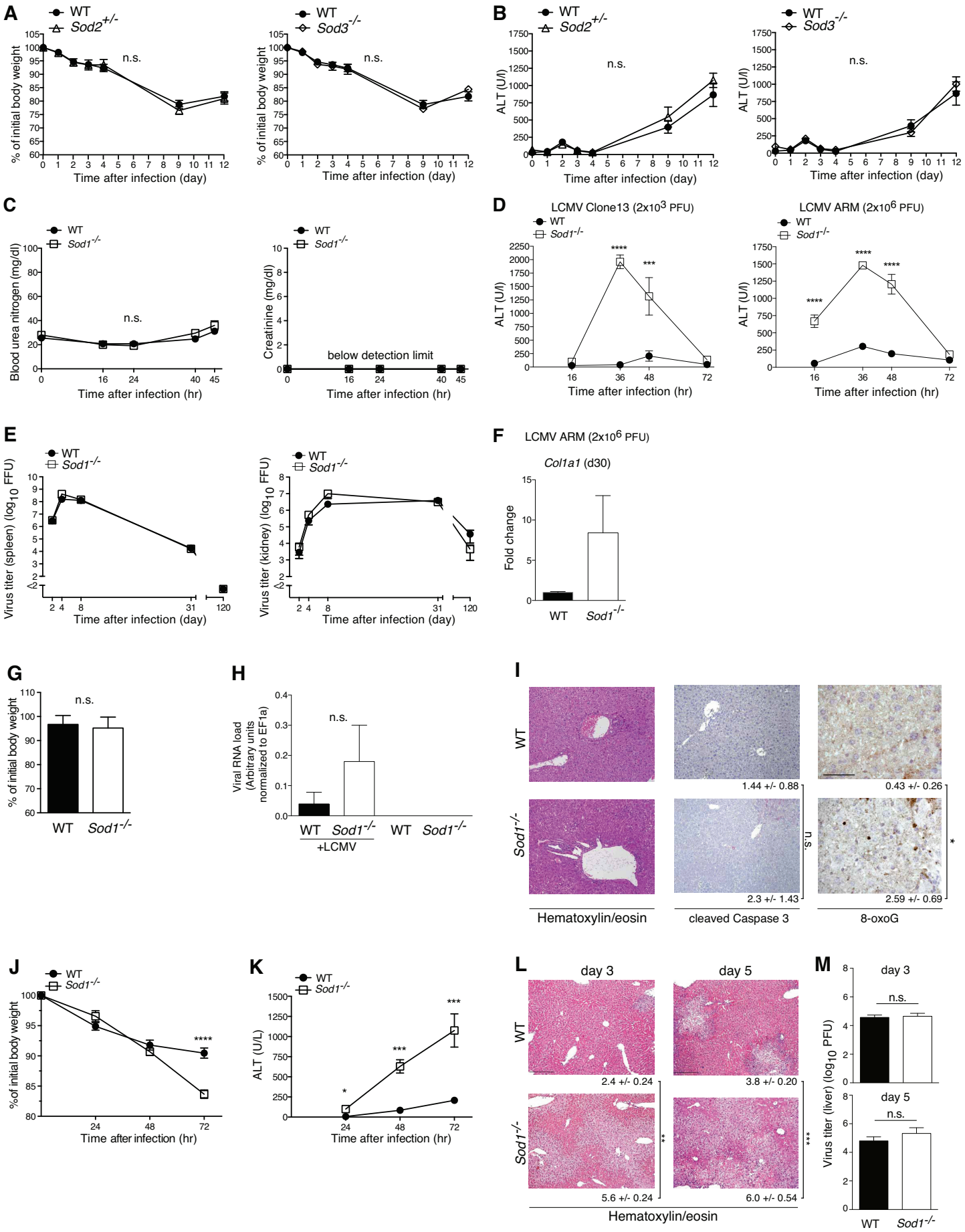
A
WT
Sod2+/-
% of initial body weight
n.s.
Time after infection (day)
WT
Sod3-/-
% of initial body weight
n.s.
Time after infection (day)
B
WT
Sod2+/-
ALT (U/l)
n.s.
Time after infection (day)
WT
Sod3-/-
ALT (U/l)
n.s.
Time after infection (day)
C
WT
Sod1-/-
Blood urea nitrogen (mg/dl)
n.s.
Time after infection (hr)
WT
Sod1-/-
Creatinine (mg/dl)
below detection limit
Time after infection (hr)
D
LCMV Clone13 (2x10^3 PFU)
WT
Sod1-/-
ALT (U/l)
****
***
Time after infection (hr)
LCMV ARM (2x10^6 PFU)
WT
Sod1-/-
ALT (U/l)
****
****
****
Time after infection (hr)
E
WT
Sod1-/-
Virus titer (spleen) (log10 FFU)
Time after infection (day)
WT
Sod1-/-
Virus titer (kidney) (log10 FFU)
Time after infection (day)
F
LCMV ARM (2x10^6 PFU)
Col1a1 (d30)
Fold change
WT
Sod1-/-
G
% of initial body weight
n.s.
WT
Sod1-/-
H
Viral RNA load (Arbitrary units normalized to EF1a)
n.s.
WT
Sod1-/-
+LCMV
WT
Sod1-/-
I
WT
Sod1-/-
1.44 +/- 0.88
0.43 +/- 0.26
2.3 +/- 1.43
2.59 +/- 0.69
n.s.
*
Hematoxylin/eosin
cleaved Caspase 3
8-oxoG
J
WT
Sod1-/-
%of initial body weight
****
Time after infection (hr)
K
WT
Sod1-/-
ALT (U/L)
*
***
***
Time after infection (hr)
L
day 3
day 5
WT
Sod1-/-
2.4 +/- 0.24
3.8 +/- 0.20
5.6 +/- 0.24
6.0 +/- 0.54
**
***
Hematoxylin/eosin
M
day 3
n.s.
day 5
n.s.
Virus titer (liver) (log10 PFU)
WT
Sod1-/-

Figure S1

Figure S1 (related to Figure 2)

(**A-B**) Comparable body weight and concentration of serum ALT upon LCMV infection of *Sod2*$^{+/-}$, *Sod3*$^{-/-}$ and WT mice. (**A**) body weight and (**B**) serum concentration of ALT were determined (n = 5 mice per group). One out of two similar experiments is shown. Statistical significance was calculated by Two-way ANOVA with Bonferroni correction. Symbols represent the mean ± S.E.M.

(**C**) Unaltered kidney-specific blood parameter in *Sod1*$^{-/-}$ mice. WT and *Sod1*$^{-/-}$ mice were infected with LCMV and serum concentration of blood urea nitrogen and creatinine were determined (n = 3 to 9 mice, pooled from two experiments). Statistical significance was calculated by Two-way ANOVA with Bonferroni correction. Symbols represent the mean ± S.E.M.

(**D**) Virus-induced hepatitis in *Sod1*$^{-/-}$ mice is independent of inoculum dose and virus strain. WT and *Sod1*$^{-/-}$ mice were infected with either $2x10^3$ FFU of LCMV strain clone 13 or $2x10^6$ FFU of LCMV strain ARM. Serum concentration of ALT were determined (n= 3-4 mice per group). Statistical significance was calculated by Two-way ANOVA with Bonferroni correction. Symbols and bars represent the mean ± S.E.M.

(E) Comparable viral loads in *Sod1*$^{-/-}$ and WT mice. WT and *Sod1*$^{-/-}$ mice were infected with LCMV and viral loads were determined by focus-forming assay from spleen and kidney (n = 3 - 7 mice per time point pooled from four experiments).

(**F**) *Col1a1* mRNA was determined by real-time PCR in liver tissue of mice infected $2x10^6$ FFU ARM 30 days previously and fold-change was calculated between infected WT and *Sod1*$^{-/-}$ mice (n = 4-5 mice per group). Bars represent the mean ± S.E.M.

(G-I) Characterization of WT and *Sod1*$^{-/-}$ mice in the late phase of infection. WT and *Sod1*$^{-/-}$ mice were infected with LCMV. (**G**) Body weight of mice infected 103 or 123 days previously (n = 9-10, pooled from two experiments). (**H**) *Sod1*$^{-/-}$ and WT mice were infected with LCMV or left uninfected, and viral RNA loads were determined in liver tissue 123 days after infection by real-time PCR (n= 3-7 mice per group). (**I**) Histological analysis of liver tissue sections of mice infected 103 or 123 days previously: H/E staining, Staining for cleaved caspase 3 positive hepatocytes (n = 9-10, pooled from two experiments), 8-oxoG staining (n = 5-7 mice per group, scale bar = 50μM). Positive cells were counted in 10 high power fields. Statistical significance was calculated by unpaired t-test (**G**, **I**) or One-way ANOVA with Bonferroni correction (**H**). Bars and values represent the mean ± S.E.M.

(J-M) SOD1 deficiency leads to aggravated liver damage upon MHV infection. WT and *Sod1*$^{-/-}$ mice were infected with MHV and (**J**) body weight and (**K**) serum concentration of ALT were

determined (n = 9 - 10 mice per group of two pooled experiments). (**L**) Liver sections 3 and 5 days after infection were stained for hematoxylin/eosin (n = 5 mice per group, scale bar = 200μm). Representative images are shown. Histopathological scores for liver damage were determined as described in the Supplemental Information. (**M**) Viral loads in the liver for 3 and 5 days after infection were determined by plaque assay (n = 5 mice per group). Statistical significance was calculated by Two-way ANOVA with Bonferroni correction (**J-K**) or by unpaired t-test (**L-M**). Symbols and bars represent the mean ± S.E.M.

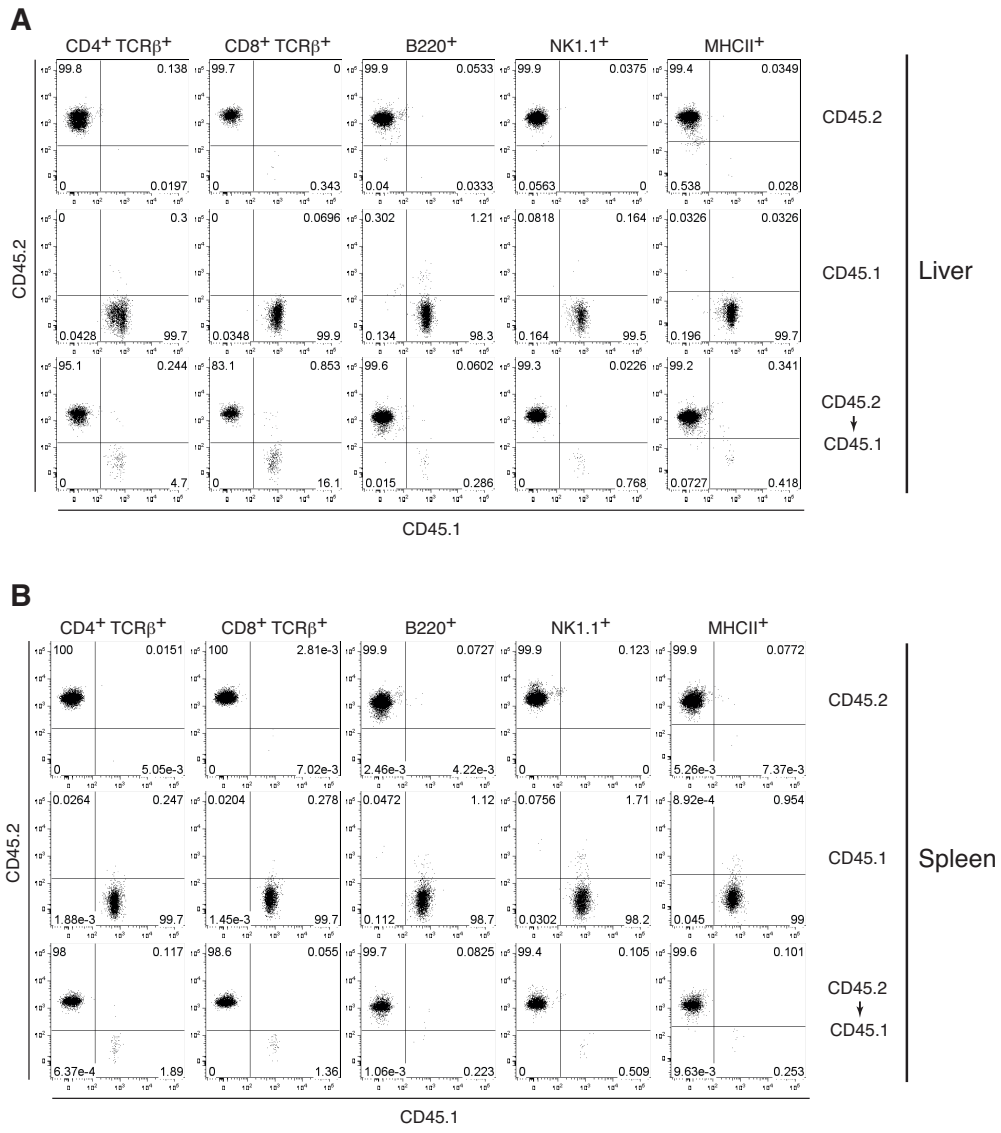
A
CD4+ TCRβ+
CD8+ TCRβ+
B220+
NK1.1+
MHCII+
CD45.2
CD45.1
CD45.2 → CD45.1
Liver
CD45.2
CD45.1
B
Spleen
C
D
Viral RNA load (Arbitrary units normalized to EF1a)
Time after infection (hr)

Figure S2

Figure S2 (related to Figure 3)

(A-B) Successful chimerism in liver and spleen tissue. CD45.2 bone marrow was transferred into congenic CD45.1 recipient mice. Chimerism in (**A**) liver and (**B**) spleen was determined for CD4 T cells ($CD4^+$ $TCR\beta^+$), CD8 T cells ($CD8^+$ $TCR\beta^+$), B cells ($B220^+$), NK cells ($NK1.1^+$) and antigen-presenting cells ($MHCII^+$) by using the surface markers CD45.1 and CD45.2. Graphs are representative of three CD45.2→CD45.1 chimeric mice.

(**C-D**) Primary mouse hepatocytes are permissive to LCMV infection. (**C**) Phase contrast picture of primary mouse hepatocytes at day one of culture (scale bar = 40μm). (**D**) Primary mouse hepatocytes were infected with LCMV at a MOI of 0.02 and intracellular viral RNA loads were determined by real-time PCR (n = 3 replicate wells per time point). Symbols represent the mean ± S.E.M.

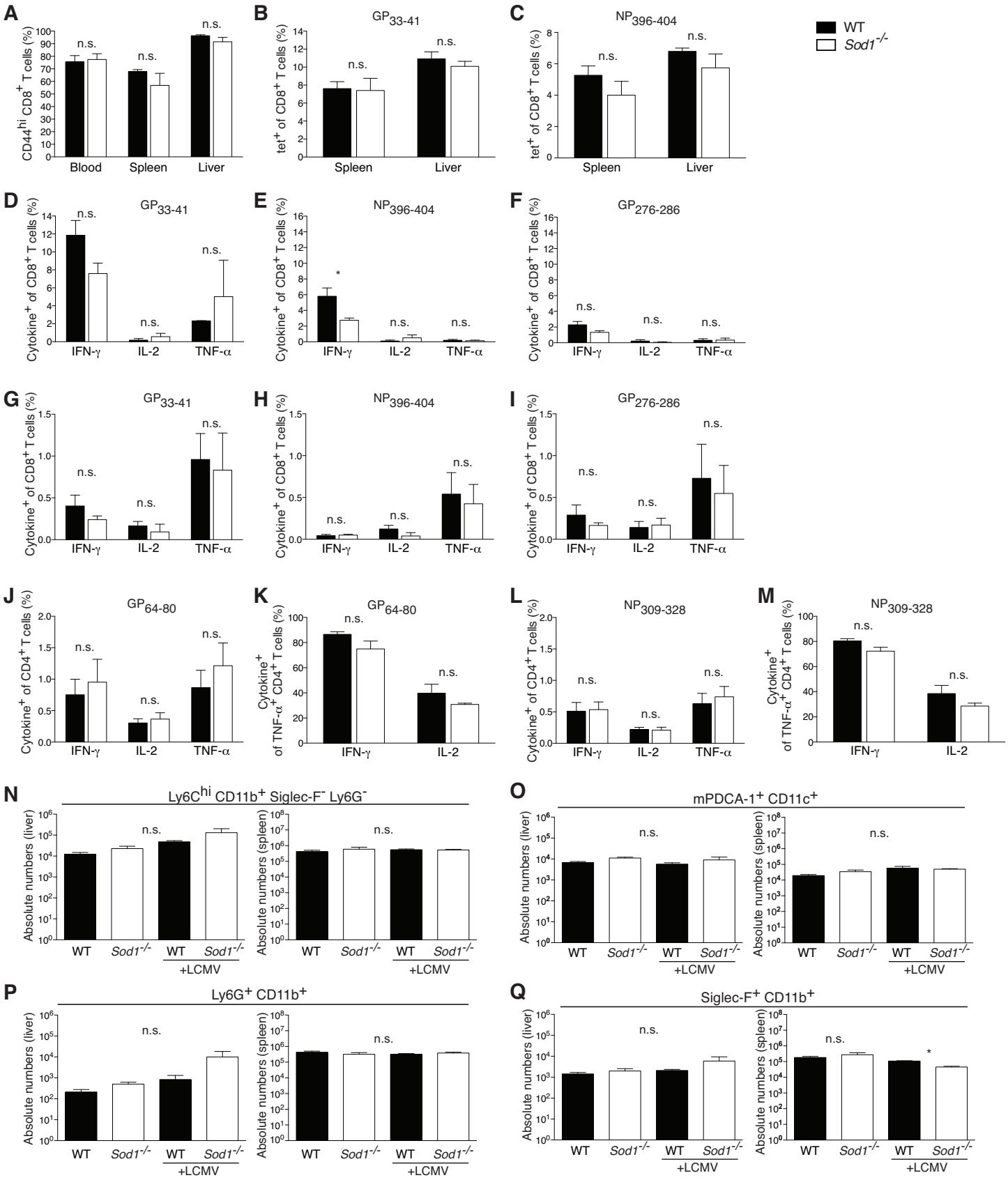
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
WT
Sod1-/-
GP33-41
NP396-404
GP276-286
GP64-80
NP309-328
CD44hi CD8+ T cells (%)
tet+ of CD8+ T cells (%)
Cytokine+ of CD8+ T cells (%)
Cytokine+ of CD4+ T cells (%)
Cytokine+ of TNF-α+ CD4+ T cells (%)
Blood
Spleen
Liver
IFN-γ
IL-2
TNF-α
n.s.
*
Ly6Chi CD11b+ Siglec-F- Ly6G-
mPDCA-1+ CD11c+
Ly6G+ CD11b+
Siglec-F+ CD11b+
Absolute numbers (liver)
Absolute numbers (spleen)
+LCMV

Figure S3

Figure S3 (related to Figure 4)

(A-M) WT and *Sod1*$^{-/-}$ mice develop comparable CD8 and CD4 T cell responses. WT and *Sod1*$^{-/-}$ mice were infected with LCMV and CD8 T cell **(A-F; G-I)** and CD4 T cell **(J-M)** responses were enumerated by flow cytometry 8 days (**A-F**, **J-M**; n = 3 mice per group) and 31 days (**G-I**; n = 5-6 mice per group), respectively, after infection. (**A**) Total CD44^{hi} CD8 T cells and virus-specific CD8 T cell responses were measured in the indicated organs by MHC class I tetramers for the viral epitopes (**B**) GP_{33-41} and (**C**) $NP_{396-404}$. Intracellular cytokine staining of splenic CD8 T cells for IFN-γ, IL-2 and TNF-α was done following peptide stimulation *in vitro* for the viral epitopes GP_{33-41} (**D**, **G**), $NP_{396-404}$ (**E**, **H**) and $GP_{276-286}$ (**F**, **I**). Values of medium control were subtracted. Intracellular cytokine staining of splenic CD4 T cells was done following peptide stimulation *in vitro* for the viral epitopes GP_{64-80} (**J**, **K**) and $NP_{309-328}$ (**L**, **M**). Statistical significance was calculated by unpaired t-test. Bars represent the mean ± S.E.M.

(N-Q) Similar leucocyte infiltration in liver of WT and *Sod1*$^{-/-}$ mice upon LCMV infection. WT and *Sod1*$^{-/-}$ mice were infected with LCMV. 24 hours after infection the following CD45^{+} leucocyte populations were enumerated: (**N**) monocytes (Ly6C^{hi} CD11b^{+} Siglec-F^{-} Ly6G^{-}), (**O**) plasmacytoid dendritic cells (mPDCA1^{+} CD11c^{+}), (**P**) neutrophils (Ly6G^{+} CD11b^{+}) and (**Q**) eosinophils (Siglec-F^{+} CD11b^{+}). Absolute cell numbers are shown (n = 4-5 mice per group). Statistical significance was calculated by One-way ANOVA with Bonferroni correction. Bars represent the mean ± S.E.M.

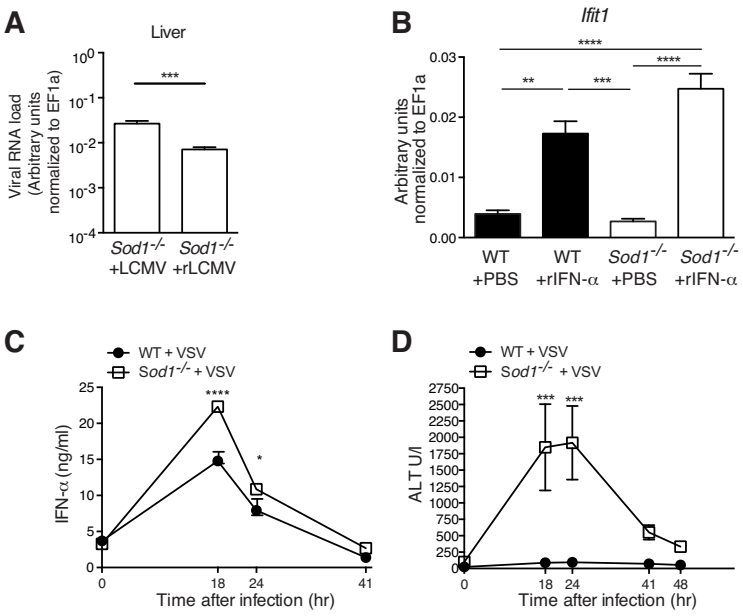
A
Liver
Viral RNA load (Arbitrary units normalized to EF1a)
***
Sod1-/- +LCMV
Sod1-/- +rLCMV
B
Ifit1
Arbitrary units normalized to EF1a
WT +PBS
WT +rIFN-α
Sod1-/- +PBS
Sod1-/- +rIFN-α
C
WT + VSV
Sod1-/- + VSV
IFN-α (ng/ml)
Time after infection (hr)
D
WT + VSV
Sod1-/- + VSV
ALT U/l
Time after infection (hr)

Figure S4

Figure S4 (related to Figure 5)

(A) Viral RNA loads in liver tissue of rLCMV-infected *Sod1*$^{-/-}$ mice. *Sod1*$^{-/-}$ mice were infected with LCMV or rLCMV, and viral RNA loads were determined in liver 72 hours after infection by real-time PCR (n= 6 mice per group). The results are representative of two similar experiments. Statistical significance was calculated by unpaired t-test. Bars represent the mean ± S.E.M.

(B) Treatment with rIFNα induces expression of *Ifit1* in the liver. WT and *Sod1*$^{-/-}$ mice were administered with 100ng of rIFNα, and levels of *Ifit1* mRNA in the liver were determined 12 hours after administration by real-time PCR (n = 4-6 mice per group). Statistical significance was calculated by One-way ANOVA with Bonferroni correction. The results are representative of two similar experiments. Bars represent the mean ± S.E.M.

(C-D) SOD1 deficiency leads to aggravated liver damage upon infection with vesicular stomatitis virus (VSV). WT and *Sod1*$^{-/-}$ mice were infected with VSV and serum concentrations of (**C**) IFNα and (**D**) ALT were determined (n = 6 mice per group). Statistical significance was calculated by Two-way ANOVA with Bonferroni correction. The results are representative of two similar experiments. Symbols represent the mean ± S.E.M.

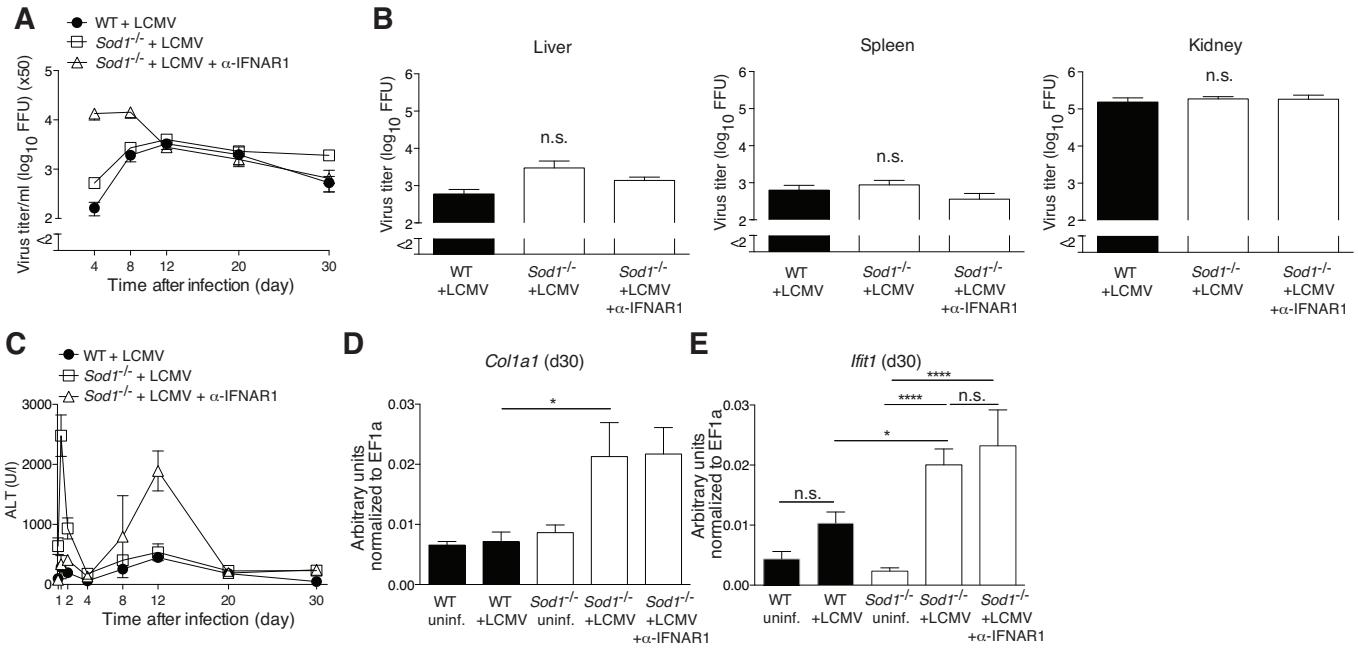
A
WT + LCMV
Sod1-/- + LCMV
Sod1-/- + LCMV + α-IFNAR1
Virus titer/ml (log10 FFU) (x50)
Time after infection (day)
B
Liver
Spleen
Kidney
Virus titer (log10 FFU)
n.s.
WT +LCMV
Sod1-/- +LCMV
Sod1-/- +LCMV +α-IFNAR1
C
ALT (UI)
D
Col1a1 (d30)
Arbitrary units normalized to EF1a
WT uninf.
WT +LCMV
Sod1-/- uninf.
Sod1-/- +LCMV
Sod1-/- +LCMV +α-IFNAR1
E
Ifit1 (d30)

Figure S5

Figure S5 (related to Figure 7)

(A-E) Long-term impact of transient IFNAR1 blockade in *Sod1*$^{-/-}$ mice upon viral infection. *Sod1*$^{-/-}$ mice either with or without administration of α-IFNAR1 antibody (250 μg i.p. each on day -1, 0 and 1) and WT mice were infected with LCMV. (**A, B**) Viral loads were measured by focus forming assay in (**A**) blood and (**B**) in liver, spleen and kidney 30 days after infection. (**C**) Serum concentrations of ALT. (**D)** *Col1a1* mRNA and **(E)** *Ifit1* mRNA were determined by real-time PCR in liver tissue 30 days after infection. N = 3-8 mice per group. Statistical significance was calculated by (**A, C**) Two-way or (**B, D, E**) One-way ANOVA with Bonferroni correction. Symbols represent the mean ± S.E.M.

4. Discussion

Viral hepatitis is a medical challenge globally. Not only can it result in a chronic infection that damages the liver, but also it can lead to systemic effects (Gill et al, 2016; Tang et al, 2016). Viral hepatitis can thus be described as a disease with multiple aspects.

Perturbed metabolism is a characteristic feature of patients suffering from chronic hepatitis (Bugianesi et al, 2012; Chang, 2016; Ivanov et al, 2013; Koike, 2007). Clinical studies describe alterations in lipid and glucose metabolism, development of insulin resistance resulting in type 2 diabetes mellitus and dysregulated redox status associated with viral hepatitis (Adinolfi et al, 2016; Kralj et al, 2016). To cite an example, almost 55% of patients suffering from HCV infection end up with NAFLD (Non-Alcoholic Fatty Liver Disease) and about 4%-10% of the patients suffer from NASH (Non-Alcoholic Steatohepatitis) (Adinolfi et al, 2011). Moreover, this influences the disease severity and progression. Several studies associate steatosis and insulin resistance to progression towards cirrhosis and poor prognosis in HCV patients (Adinolfi et al, 2016; Gonzalez-Reimers et al, 2015; Negro et al, 2015).

Additional factor associated with increased risk towards disease progression is oxidative stress. Increased oxidative stress damages essential cellular components like DNA and proteins (Cardin et al, 2014; Ha et al, 2010). It also activates hepatic stellate cells, for example, and induces Kupffer cells to produce fibrogenic mediators, thereby leading to liver fibrosis (Czaja, 2014; Koike et al, 2002). Although this strong correlation between oxidative stress and viral hepatitis has been existent for years, the factors that initiate and drive oxidative stress have not been well described.

Our study describes how type I interferon signaling downregulates a key antioxidant enzyme Sod1, thereby leading to oxidative stress in hepatocytes and eventually their death, resulting in hepatitis as a clinical manifestation (Bhattacharya et al, 2015). Thus, this study illustrates how the immune system can influence host redox homeostasis and lead to pathology.

Interestingly, viral infections other than the classical hepatotropic viruses such as HBV and HCV can also lead to liver damage (Lalazar, 2013). To cite some examples, infections with Epstein Barr Virus (EBV), Herpes Simplex Virus (HSV), Cytomegalovirus (CMV), Varicella Zoster Virus (VSV) and adenoviruses can cause elevation of serum transaminases for a transient period, indicating death of hepatocytes and therefore liver injury (Kunno et al, 1997; Peters et al, 2000; Petrova & Kamburov, 2010). In some cases, it might also lead to acute hepatitis and liver failure. Our study demonstrates that systemic interferon itself is sufficient to cause

liver damage. This could possibly explain the above-mentioned phenomenon whereby an individual may present clinical symptoms similar to hepatitis in spite of not being infected with a classical hepatotropic virus. Our study therefore holds relevance to not only viral hepatitis but also to the context of other infectious diseases or inflammatory conditions involving IFN-I signaling.

Additionally, interferon therapy is used in the clinics to treat several diseased conditions such as multiple sclerosis and some forms of cancer (Killestein & Polman, 2011; Ortiz & Fuchs, 2016; Shirani et al, 2015). In the recent years, treatment of chronic hepatitis has improved considerably, especially with respect to HCV infection, after the approval of Direct Acting Antivirals (DAA), which directly target viral proteins essential for viral replication at various stages of its life cycle (Gotte & Feld, 2016). Until then, pegylated interferon-α in combination with ribavirin was used as the treatment regime for HCV infection. There are several studies that report that a large fraction of the patients did not respond to the interferon therapy (Asselah et al, 2010). It is known that HCV induces a robust interferon response in the host. However, counter-intuitively, chronically infected HCV patients with a pre-activated endogenous interferon system have been reported to respond poorly to the interferon treatment (Bellecave & Moradpour, 2008; Sarasin-Filipowicz et al, 2008). Refractoriness of the interferon-signaling pathway has been implied as a possible explanation for this observation. The findings of our study could also help explain the observed correlation between pre-activated endogenous interferon signaling and poor reponse to interferon therapy.

We observed that the antioxidant Sod1 is downregulated upon viral infection in the liver in a IFN-I signaling dependent manner. However, we do not fully understand how is IFN-I modulating Sod1 expression. Interestingly the proximal region of the Sod1 promoter has binding sites for several transcription factors such as NF-κB, AP-1, and SP1 (Chang & Hung, 2012; Radaeva et al, 2002; von Marschall et al, 2003). We speculate that the post translation modifications of these transcription factors could be influenced by IFN-I, which in turn could regulate the expression of *Sod1*.

Our study helps to appreciate the pleiotropic nature of IFN-I, whereby the function of IFN-I is not just limited to its potent antiviral activity but also to its role in regulating immune responses. This has also led to several exciting questions that we do not fully understand.

One such question that arises is what is the rationale behind the self-afflicted damage that the host undergoes due to oxidative stress upon viral infection. In other words, why would the host expend energy to generate oxidative stress and kill its own cells? Is there an evolutionary benefit behind this phenomenon? Alternatively,

the oxidative stress is possibly a metabolic by-product of the processes the host undergoes in order to meet the increasing energy demands of the cell in terms of signaling, inducing an antiviral state in the cell or development of disease tolerance (Schieber & Chandel, 2014; Weinberg et al, 2015). Details pertaining to meeting increasing energy demands upon infection or development of disease tolerance have been described below.

Intriguingly, the $Sod1^{-/-}$ mice had viral loads comparable to those of WT mice. This implies the increased oxidative stress in the $Sod1^{-/-}$ mice did not affect the pathogen directly, reminding us of the concept of disease tolerance. The host may suffer from two types of tissue damage during infection: either by direct damage caused by the pathogen or by immunopathology where tissue damage occurs as a consequence of the host's own immune defenses. Thus, the regulation of efficient pathogen clearance and extent of tissue damage determines the optimal immune response (Medzhitov et al, 2012). The host tries to achieve this by employing two types of tolerance mechanisms; either by reducing direct pathogen induced damage or by decreasing immunopathology. SOD1 could be therefore implicated to contribute to disease tolerance to viral infection.

Recent studies in the field of immunometabolism have highlighted the importance of "metabolic reprogramming" that immune cells have to undergo upon activation in order to meet the increased demand of cellular energy (Chandel, 2014; Weinberg et al, 2015). In the context of our study, the host oxidative metabolism could serve as one such step towards such reprogramming. Furthermore, we do not know the source of ROS in our model. There are two major endogenous sources of ROS: the NADPH oxidases and the mitochondrial ETC (Electron Transport Chain) (Nathan & Cunningham-Bussel, 2013). Oxidative stress has been linked to aberrant mitochondrial function (Cardin et al, 2014). Moreover, mitochondria are gaining importance as a site at the interface of metabolism and immune response, especially after the discovery of immune signaling pathways whose important signaling components are located within the mitochondria (Chandel, 2014). For example, IFN-I induction can take place via the MAVS-TBK1 axis upon sensing viral RNA by the PRRs RIG-I or MDA5, whereby MAVS is a mitochondrial membrane protein (Seth et al, 2005). Preliminary unpublished data from our laboratory indicate that mitochondria could be one of the possible sources of ROS in our model but further investigations are required to confirm this finding.

Concludingly, our study opens up newer avenues for therapeutic interventions not only for viral hepatitis but potentially also for other diseases or inflammatory conditions involving IFN-I signaling.

5. Materials and Methods

Please refer to the section named “Experimental Procedures” in the main text of the manuscript as well as to the “Supplemental Experimental Procedures” in the supplemental information.

6. References

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Zinkernagel RM, Haenseler E, Leist T, Cerny A, Hengartner H, Althage A (1986) T cell-mediated hepatitis in mice infected with lymphocytic choriomeningitis virus. Liver cell destruction by H-2 class I-restricted virus-specific cytotoxic T cells as a physiological correlate of the 51Cr-release assay? *J Exp Med* **164:** 1075-1092

Curriculum vitae

Personal Data

Name:	Anannya Bhattacharya
Date of birth:	05.09.1986
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Address:	Klährgasse 5/5 1120 Vienna, Austria
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Current position:	Predoctoral fellow CeMM - Research Center for Molecular Medicine of the Austrian Academy of Sciences Bergthaler Group

Education

Oct 2011 – Present	Pre-doctoral fellow at CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, Bergthaler Group
Nov 2010 – Aug 2011	Junior Research Fellow (fellowship awarded by the Indian Council of Medical Research), Department of Molecular Virology, National Institute of Cholera and Enteric Diseases (NICED), Kolkata, India (WHO Collaborating Center for Diarrheal Diseases in South East Asia)
May 2008 - May 2010	Master of Science in Virology at National Institute of Virology, University of Pune, India First class with distinction, Grade "O" (Outstanding), CGPA 5.34 on a scale of 6
Dec 2009- May 2010	Master's Thesis: Rapid Molecular Detection of H275Y Oseltamivir Resistance Mutation in seasonal and pandemic A/H1N1 Supervision: Dr. M.S. Chadha and Dr. Varsha Potdar, Department of Influenza, National Institute of Virology, Pune, India
May 2005 – May 2008	Bachelor of Science in Microbiology/Biochemistry (Double Major) at St. Xavier's College, Mumbai, University of Mumbai, India First Class with Distinction, Aggregate 83%
May 2006 – June 2006	Internship: Department of Bacteriology, Serology and Biochemistry of Burnpur Hospital, SAIL (Steel Authority of India Limited), West Bengal, India
March 2005	Indian School Certificate Examination, G.D. Birla Center for Education, Kolkata Secured 93.6%

Technical Expertise

Mouse handling: Injection via intravenous and intra-peritoneal routes, generation of bone marrow chimeras, depletion of cell populations *in vivo*, isolation of primary cells

Imaging: Confocal microscopy using Zeiss LSM 700 and LSM 780

Next generation sequencing: Library preparation for RNAseq using Illumina kit

Cell Culture and Molecular Biology techniques

Bioinformatics: Enrichment analyses by DAVID, GORILLA, REVIGO

Awards/Fellowships/Scholarships

June 2016	Young Scientist Association Publication Award 2016
March 2015	Joint Research Program Fellowship of Hokkaido University
Jan 2013 – Dec 2015	DOC stipendium of the Austrian Academy of Sciences
Nov 2010 – Aug 2011	Junior Research Fellowship of Indian Council of Medical Research
2005 – 2006	Mahindra Search for Talent Scholarship for obtaining the highest aggregate marks in First Year BSc
2005 – 2006	Meenal Dinkar Rao Mugbe Scholarship for obtaining the highest marks in Chemistry in First Year BSc
2006 - 2007	Prof. J.V. Bhat Scholarship obtaining the second highest marks in Microbiology in Second Year BSc

Publications and Conferences

Publications

Bhattacharya A, Hegazy AN, Deigendesch N, Kosack L, Cupovic J, Kandasamy RK, Hildebrandt A, Merkler D, Kühl AA, Vilagos B, Schliehe C, Panse I, Khamina K, Baazim H, Arnold I, Flatz L, Xu HC, Lang PA, Aderem A, Takaoka A, Superti-Furga G, Colinge J, Ludewig B, Löhning M, Bergthaler A. Superoxide dismutase 1 protects hepatocytes from type I interferon-driven oxidative damage. ***Immunity***, 2015 Nov 17; 43(5): 974-86

Schliehe, C., Flynn EK, Vilagos B, Richson U, Swaminathan S, Bosnjak B, Bauer L, Kandasamy RK, Griesshammer IM, Kosack L, Schmitz F, Litvak V, Sissons J, Lercher A, **Bhattacharya A**, Khamina K, Trivett AL, Tessarollo L, Mesteri I, Hladik A, Merkler D, Kubicek S, Knapp S, Epstein MM, Symer DE, Aderem A, Bergthaler A. The methyltransferase Setdb2 mediates virus-induced susceptibility to bacterial superinfection. ***Nature Immunology***, 2015 Jan; 16(1): 67-74.

Bhattacharya A, Popa A, Lercher A, Vitko D, Parapatics K, Bennett K, Colinge J, Bergthaler A. Dynamics of global changes in the liver upon chronic viral infection. Manuscript under preparation.

Conferences

4th European Congress of Immunology (ECI 2015)
6-9/09/2015 Vienna, Austria; **Oral Presentation**

Viral Infection, Immunity and Autoimmunity
11-14/08/2015 Düsseldorf, Germany; **Oral presentation**

Annual Meeting of the Society of Allergology and Immunology
6-8/11/2014 Salzburg, Austria; Poster Presentation (**Awarded Best Poster Prize**)

Viral Infection, Immunity and Autoimmunity
03-05/07/2014 Düsseldorf, Germany; **Oral presentation**

17th International Summer School on Immunology
14-21/09/2013 Rabac, Croatia; **Poster presentation**

Teaching and Supervision

Apr 2012 – Sep 2013 Supervision of Master's Thesis by Andrea Hildebrandt
Title: "Oxidative stress in viral hepatitis"

Language skills

English: fluent in spoken and written

German: A1 (Basic reading, writing and speaking skills)

Bengali: Mother Tongue

Hindi: fluent in spoken and written

Extra-curricular activities and Hobbies

Trained in Indian Classical Dance Form "Odissi"

Contributed as a writer and speaker in a video abstract of our *Immunity* study
https://www.youtube.com/watch?v=opTWG8QZfzQ

Acted in a documentary film shot by the Government of Japan which is displayed at Okinawa Peace Museum, Japan

Participated and won many prizes in dance competitions, collage competitions and essay writing competitions at school

Reading and listening to music

Photography

Anannya Bhattacharya
Vienna, 23.06.2016

References

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2. Dr. Giulio Superti- Furga
   Scientific Director CeMM
   Professor for Medical Systems Biology, Medical University of Vienna
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3. Dr. Sylvia Knapp
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   Department of Medicine I, Laboratory of Infection Biology, Medical University of Vienna
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