# Tumor necrosis factor mediated augmentation of immunity protects host against Vesicular stomatitis virus infection

Inaugural dissertation

for the attainment of the title of doctor
in the Faculty of Mathematics and Natural Sciences
at the Heinrich Heine University Düsseldorf

presented by

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Düsseldorf, September 2018

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Date of the oral examination: 11th December, 2018

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**Declaration and statement of contribution**

I, Prashant Vishwas Shinde, declare that the content in this dissertation is original. I have cited appropriate texts and figures wherever necessary. No part of this dissertation is submitted somewhere else for consideration of a degree. Part of the dissertation is published in the Journal of Virology entitled "*Tumor Necrosis Factor-Mediated Survival of CD169+ Cells Promotes Immune Activation during Vesicular Stomatitis Virus Infection*. Journal of Virology 17; 92(3), 2018 Jan."

I was involved in execution of the experiments, analysis, writing and preparation of the manuscript which consists ≈25% of the published manuscript.

I have contributed in the preparation of following figures which are included in the thesis are as follows,

Fig 1A, 1B, 1E-G; Fig 2C-E; Fig 3A-D; Fig 4A-H; Fig 5A-C; Fig 6A-F; Fig 7A-D; Fig 8A-H; Fig 9B-E; Fig 10A-C; Fig 11C-D; Fig 12A-D; Fig 13C-D; Fig 14A-C; Fig 15A-G and Graphical Figures 1-5.

Contribution in the other published manuscripts is mentioned in the list of the publications.

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# Publication list and contribution

1. Lang E*, Pozdeev VI*, **Shinde PV***, Xu HC, Sundaram B, Zhuang Y, Poschmann G, Huang J, Stuhler K, Pandyra AA, Keitel V, Haussinger D, Lang KS, Lang PA. 2018. **Cholestasis induced liver pathology results in dysfunctional immune responses after arenavirus infection**. Sci Rep 8:12179. *shared authorship

   **Contribution to the publication: ≈ 8%**

2. Behnke K, Zhuang Y, Xu HC, Sundaram B, Reich M, **Shinde PV**, Huang J, Modares NF, Tumanov AV, Polz R, Scheller J, Ware CF, Pfeffer K, Keitel V, Haussinger D, Pandyra AA, Lang KS, Lang PA. 2018. **B Cell-Mediated Maintenance of Cluster of Differentiation 169-Positive Cells Is Critical for Liver Regeneration**. Hepatology 68:2348-2361.

   **Contribution to the publication: ≈ 2%**

3. **Shinde PV**, Xu HC, Maney SK, Kloetgen A, Namineni S, Zhuang Y, Honke N, Shaabani N, Bellora N, Doerrenberg M, Trilling M, Pozdeev VI, van Rooijen N, Scheu S, Pfeffer K, Crocker PR, Tanaka M, Duggimpudi S, Knolle P, Heikenwalder M, Ruland J, Mak TW, Brenner D, Pandyra AA, Hoell JI, Borkhardt A, Haussinger D, Lang KS, Lang PA. 2018. **Tumor Necrosis Factor-Mediated Survival of CD169$^+$ Cells Promotes Immune Activation during Vesicular Stomatitis Virus Infection**. J Virol 92.

   **Contribution to the publication: ≈ 25%**

4. Pozdeev VI, Lang E, Gorg B, Bidmon HJ, **Shinde PV**, Kircheis G, Herebian D, Pfeffer K, Lang F, Haussinger D, Lang KS, Lang PA. 2017. **TNF alpha induced up-regulation of Na(+), K(+),2Cl(-) cotransporter NKCC1 in hepatic ammonia clearance and cerebral ammonia toxicity**. Sci Rep 7:7938.

   **Contribution to the publication: ≈ 2 %**

5. Lang E, Pozdeev VI, Xu HC, **Shinde PV**, Behnke K, Hamdam JM, Lehnert E, Scharf RE, Lang F, Haussinger D, Lang KS, Lang PA. 2016. **Storage of Erythrocytes Induces Suicidal Erythrocyte Death**. Cell Physiol Biochem 39:668-676.

**Contribution to the publication: ≈2 %**

6. Beyer M, Abdullah Z, Chemnitz JM, Maisel D, Sander J, Lehmann C, Thabet Y, **Shinde PV**, Schmidleithner L, Kohne M, Trebicka J, Schierwagen R, Hofmann A, Popov A, Lang KS, Oxenius A, Buch T, Kurts C, Heikenwalder M, Fatkenheuer G, Lang PA, Hartmann P, Knolle PA, Schultze JL. 2016. **Tumor-necrosis factor impairs CD4(+) T cell-mediated immunological control in chronic viral infection.** Nat Immunol 17:593-603.

   **Contribution to the publication: ≈ 2%**

7. Xu HC, Huang J, Khairnar V, Duhan V, Pandyra AA, Grusdat M, **Shinde P**, McIlwain DR, Maney SK, Gommerman J, Lohning M, Ohashi PS, Mak TW, Pieper K, Sic H, Speletas M, Eibel H, Ware CF, Tumanov AV, Kruglov AA, Nedospasov SA, Haussinger D, Recher M, Lang KS, Lang PA. 2015. **Deficiency of the B cell-activating factor receptor results in limited CD169+ macrophage function during viral infection**. J Virol 89:4748-4759.

   **Contribution to the publication: ≈ 2%**

**Previous publications:**

1. Pawar SD, Murtadak VB, Kale SD, **Shinde PV**, Parkhi SS. 2015. Evaluation of different inactivation methods for high and low pathogenic avian influenza viruses in egg-fluids for antigen preparation. J Virol Methods 222:28-33.
2. **Shinde PV**, Koratkar SS, Pawar SD, Kale SD, Rawankar AS, Mishra AC. 2012. Serologic evidence of avian influenza H9N2 and paramyxovirus type 1 infection in emus (*Dromaius novaehollandiae*) in India. Avian Dis 56:257-260.

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# Abbreviations

**APCs**: Antigen presenting cells

**ASCs:** Antibody secreting cells

**BCR**: B cell receptor

**CARD:** Caspase activation and recruitment domains

**CD**: Cluster of differentiation

**CNS:** Central nervous system

**CTD:** C terminal domain

**cDCs:** Conventional dendritic cell

**FOB:** Follicular B cells

**GC:** Germinal center

**IFIT2:** Interferon induced protein with tetratricopeptide repeats 2

**IFN:** Interferon

**IKK:** inhibitor of nuclear factor kappa-B kinase subunit

**IL:** Interleukin

**IRF 3:** Interferon regulatory factor 3

**iRhom2:** Inactive Rhomboid 2

**ISGs**: IFN stimulated genes

**ISRE**: IFN-stimulated response elements

**JAK1:** Janus kinase 1

**LN:** Lymph node

**LPS**: Lipopolysaccharide

**Lt:** Lymphotoxin

**MALT1:** Mucosa associated lymphoid tissue 1

**MDA5**: Melanoma Differentiation-Associated protein 5

**MMM:** Metallophilic marginal zone macrophages

**MyD88**: Myeloid differentiation primary response 88

**MMZ:** Metallophilic marginal zone macrophages

**MZB:** Marginal zone B cells

**MZM:** Marginal zone macrophages

**Mx1:** Myxovirus resistance 1

**NEMO:** NF-kappa-B essential modulator

**NIK:** NF-κB inducing kinase

**OAS:** 2'-5' oligoadenylate synthetase-

**OASL1:** 2'-5' oligoadenylate synthetase-like 1

**PAMP**: Pathogen associated molecular pattern

**pDCs:** Plasmacytoid dendritic cells

**PRR:** pattern recognition receptors

**RAG1:** Recombination activating gene

**RIG-I**: Retinoic acid inducible gene 1

**RIPK1:** Receptor-interacting serine/threonine-protein kinase 1

**SODD:** Silencer of death domain

**STAT:** Signal activator and transducer

**TACE:** TNF alpha converting enzyme

**TAK1:** Transforming growth factor beta-activated kinase 1 (TAK1)

**TANK:** TRAF family member-associated NF-kappa-B activator

**TBK1:** Tank Binding Kinase 1

**TCR:** T cell receptor

**TLR**: Toll-like receptor

**TNF:** Tumor necrosis factor

**TRADD:** TNF receptor type-1 associated death domain

**TRAF:** TNF receptor-associated factor

**TRIF**: TIR-domain-containing adapter-inducing interferon-β (TRIF)

**TYK2:** Tyrosine kinase 2

**VSV**: Vesicular stomatitis virus

# Foreword

It all began 3 billion years ago, give or take a few million years when a few carbon, hydrogen, nitrogen and company decided to make something of themselves on this forsaken planet. Thus, came together to form what is known as organic life forms. Although it is a well-evolved ecosystem of the plant, animals, primitive and advanced microbes; the complexity of the organic life is not only enthralling but also difficult to comprehend in its given form.

All the life forms which are coexisting on earth are directly or indirectly dependent on each other. Be it a massive elephant in African and Asian jungles or blue whales in Pacific oceans or a measly bacteria or protozoans in a pond, all are interdependent.

Most fascinating of all the relationship is of host and a parasite, which happens to be the topic of this manuscript.

# Summary

Innate immune activation is essential to mount an effective antiviral response and to prime adaptive immunity. Over the last decade, strategically placed $CD169^+$ metallophilic macrophages in the marginal zone of the murine spleen and subcapsular sinus macrophages in the LN have been shown to play a very important role in host defense against viral pathogens. $CD169^+$ macrophages are shown to activate innate and adaptive immunity via "enforced virus replication" a controlled amplification of virus particles. Although a crucial role of $CD169^+$ macrophages during vesicular stomatitis virus (VSV) infections are increasingly recognized, factors regulating $CD169^+$ macrophages during viral infections remain unclear. Here we show that tumor necrosis factor (TNF) is produced by $CD11b^+$ $Ly6C^+Ly6G^+$ cells following infection with VSV. The absence of TNF or TNF receptor 1 (TNFR1) resulted in reduced numbers of $CD169^+$ macrophages and in reduced IFN-I production during VSV infection, with a severe disease outcome. Specifically, TNF triggered RelA translocation into the nucleus of $CD169^+$ macrophages in the spleen to initiate NF-κB. This translocation was inhibited when paracaspase MALT-1 was absent. Consequently, MALT1 deficiency resulted in reduced VSV replication, defective innate immune activation, and severe disease development. These findings indicate that TNF mediates the maintenance of $CD169^+$ macrophages and thereby promotes "enforced virus replication" in $CD169^+$ macrophages.

# Zusammenfassung

Das angeborene Immunsystem ist ein zentrales Element, um eine wirksame antivirale Immunreaktion zu realisieren, nicht zuletzt indem es das adaptive Immunsystem induziert. In den vergangenen Jahrzehnten konnte gezeigt werden, dass die strategisch gut lokalisierten metallophilen CD169$^+$ Makrophagen in der Marginal zone der murinen Milz und die subkapsulären Sinus-Makrophagen in den Lymphknoten eine zentrale Rolle bei der Abwehr von Virusinfektionen besitzen. So können CD169$^+$ Makrophagen über den Mechanismus der "verstärkten Virusreplikation", welcher eine kontrollierte Amplifikation von Viruspartikeln beschreibt, sowohl das angeborene als auch das adaptive Immunsystem aktivieren.

Obwohl den CD169$^+$ Makrophagen während einer vesikulären Stomatitis-Virus (VSV) -Infektionen zunehmend eine zentrale Rolle zugeschrieben wird, ist der genaue Mechanismus, wie CD169 $^+$ Makrophagen eine virale Infektion regulieren, unklar. In der vorliegenden Arbeit konnte gezeigt werden, dass der Tumor-Nekrose-Faktor (TNF), exprimiert von CD11b$^+$ Ly6C$^+$ Ly6G$^+$ Zellen, nach der Infektion mit VSV von zentraler Bedeutung ist. Demzufolge konnte in Abwesenheit von TNF oder TNF-Rezeptor 1 (TNFR1) ein vermindertes Aufkommen von CD169$^+$ Makrophagen sowie eine reduzierte Expression von Typ-I IFN detektiert werden, welches mit einer verstärkten Pathologie während der VSV-Infektion einherging.

Ferner wurde deutlich, dass TNF die Translokation von RelA in den Zellkern von CD169$^+$ Makrophagen in der Milz induziert, um NF-κB zu initiieren. In Übereinstimmung fand keine Translokation nach der Inhibition der Paracaspase MALT-1 statt. Folglich führt die Defizienz von MALT1 zu einer verminderten VSV-Replikation, einer defekten Induktion des angeborenen Immunsystems welches mit einer verstärkten Pathologie einhergeht. Zusammenfassend weisen diese Resultate daraufhin, dass TNF die Präsenz von CD169$^+$ Makrophagen begünstigt wodurch eine "verstärkte Virusreplikation" in CD169$^+$ Makrophagen induziert.

# Chapter 1.

# Introduction

# 1. Introduction

An estimation of the global burden of disease puts ischemic heart diseases and infectious diseases as major contributors to human morbidity and mortality (1) (2). Since the discovery of antibiotics and vaccines in the 20$^{th}$ century, the mortality and morbidity rates in humans and animals resulting from infections have fallen drastically and contributed towards saving countless lives and billions of dollars (3). More than a century ago the importance of vaccination was put forward into a perspective of global health by Eugene Foster (4).

While the humongous task of eradicating smallpox in 1977 was achieved through well-coordinated efforts by various nations, till today many of the viral and bacterial pathogens remain a public nuisance. Eradicating poliovirus and other diseases is the next task for the world health authorities (5). The spread of new pandemic strains of influenza and Ebola virus in the last few decades have caused severe panic among nations. It's not only the pandemic strains, but also the sporadic infections caused by viruses remain a major risk factor.

The host immunity being the major contributor along with external preventive measures is the definite driving factor in controlling the diseases caused by viruses.

As a result of an interaction between viral pathogens and the host, both have evolved simultaneously over the time. This evolution has revealed best of each other in terms of antiviral immunity and pathogenicity (6, 7). Over the course of evolution viral pathogens have found a way to overcome the resistance either through molecular mimicry or rapid genetic mutations that alter the infectivity (8).

Apart from cardiovascular diseases, bacterial, viral and fungal infections are the major contributors towards morbidity and mortality in humans. Antibiotics, synthetic drugs, and vaccines have led to effective treatments. However, in recent years, we have seen the emergence of resistant strains of bacteria and viruses due to excessive use of antibiotics and drugs, that has resulted into a public menace.

Additionally, rapid urbanization has led to sporadic outbreaks of deadly viruses such as Ebola, Zika virus and recently Nipha virus in the Indian sub-continent. Studying host and pathogen interaction gives us novel insights into underlying concepts of infection as a key factor in developing therapeutic approaches.

## 1.1 Pathogen

A pathogen can be of many origins such as bacterial, fungal, protozoan or viral. Many viral pathogens in recent years have caused havoc in humans as well as live stocks such as pandemic flu; avian flu, foot and mouth disease and recently Ebola virus infections.

### 1.1.1 Pathogen entry and recognition

Pathogens enter hosts when there is a breach of the first barrier - skin or mucosal surfaces (9). Usually, viruses and bacteria replicate at the site of the infection and Then rapidly spread to other organs. This widespread dissemination of the pathogen generates a lot of attention at the site of infection by dendritic cells (DCs), Inflammatory monocytes and granulocytes are the first responders at the site of infection (10). Viral pathogens are diverse in their replication methods having DNA and RNA as their genomes. Pathogen-associated molecular patterns (PAMPs) known as virulence factors are derived from the pathogens (9, 11). Seminal observations by Prof. Janeway lead to the foundation of pathogen recognition as self/non self-discrimination mechanism in the late 1980s (12, 13).

Recognition of these viruses and bacteria have been conferred to pattern recognition receptors (PRR). These receptors are conserved throughout evolution where humans share 90% homology with *Drosophila* and *C. elegans* (14). PRR involved in virus recognition can be classified into two groups cytosolic and endosomal receptors. Shortly after Toll-like receptors (TLRs) were discovered in *Drosophila*; Medzitov et al. and Rock et al., were able to clone human TLRs (15, 16). Hence opening new avenues in pathogen recognition by the host immune system. PAMPs recognition by PRRs leads to activation of various pathways leading to the induction of inflammatory cytokines (17, 18).

Various classes of PRRs are described in mammals which consist of TLRs, RIG-I-like receptors (RLRs), NOD-like receptors (NLRs), AIM2-like receptors (ALRs), C-type

lectin receptors (CLRs), and intracellular DNA sensors such as cyclic GMP-AMP synthase (cGAS) (19) (9, 11, 20, 21).

Distribution of these receptors in various cellular compartments such as the cytoplasm, endosomal and the plasma membrane is unique in pathogen recognition (9). In the cytoplasm, RNA viruses are primarily recognized by RLRs which consist of retinoic acid inducible gene I (RIG-I), melanoma differentiation-associated protein 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2) induce IFN-I and IFN-II (22), whereas recognition by NLRs which consist of 20 members that trigger potent IL-1β secretion (23-25).

### 1.1.2 Receptor Signaling

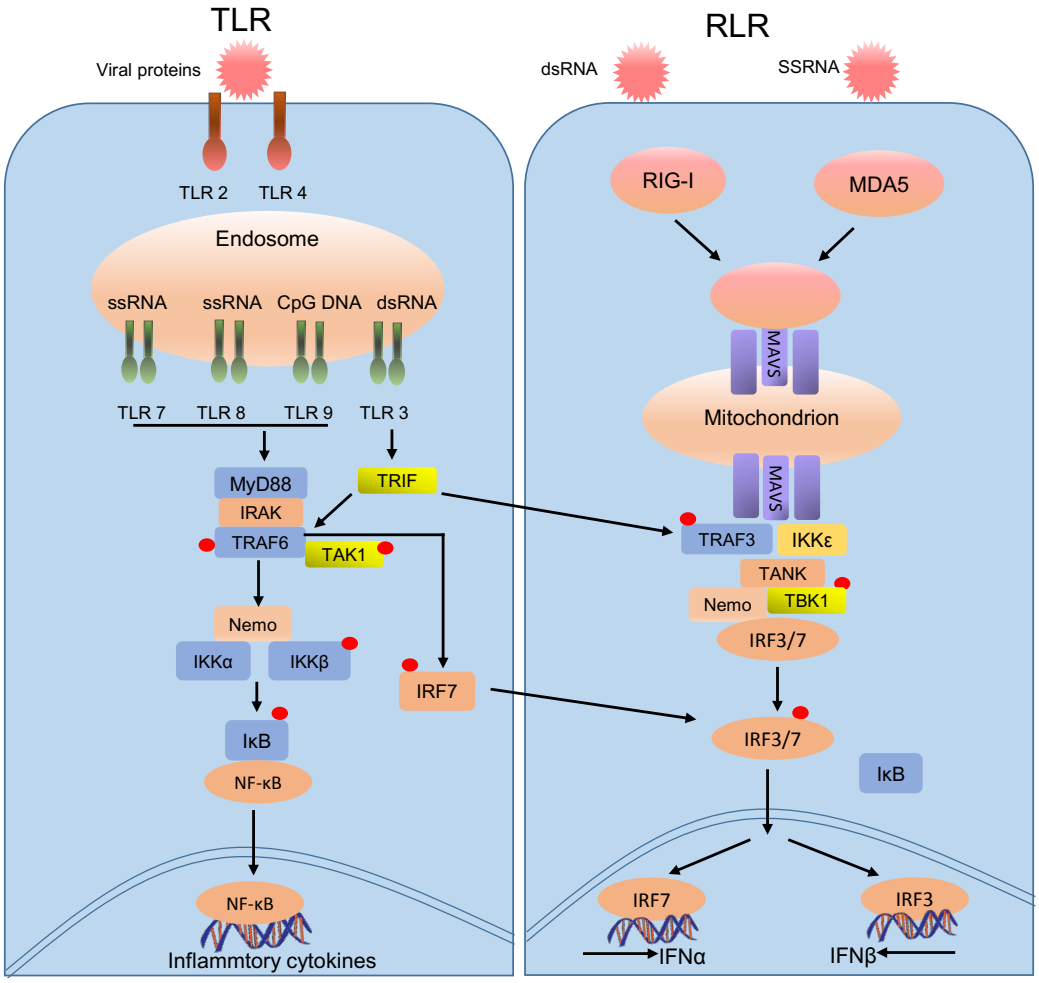
In humans, there are 10 TLRs involved in PRR recognition (TLR1-10) and 12 TLRs in mice (TLR1-9, TLR11-13). TLR1, 2, 4, 5, 6 and 11 are expressed on the cell surface whereas TLR3, 7, 8 and 9 are distributed in endosomes, lysosomes and endoplasmic reticulum (11). TLRs form a single transmembrane protein which along with TIR-domain-containing adapter-inducing interferon-β (TRIF) and Myeloid differentiation primary response 88 (MYD88) as an adaptor molecule carry out the signaling cascade (11, 21). They contain leucine-rich domains for signal transduction via cytosolic Toll-interleukin like 1 (IL-1) receptor (22).

TLR3 primarily activates interferon regulatory factor 3 (IRF3) via TRIF and TLR7, whereas TLR 8 and 9 activates IRF7 via MyD88 (11). However, they both merge in signaling by activating NF-κB (11). These TLR functions were elucidated using mice deficient in different TLRs which paved a way to understand their role in antiviral signaling (11, 26-28). TLR3, as an important player in antiviral response, was identified using synthetic double-stranded RNA, poly I:C which mimics viral RNA (29, 30). TRIF, which comes downstream of TLR3, associates with TNF receptor-associated factor 3 (TRAF3) and TRAF6. TRAF3 recruits TANK Binding Kinase 1 (TBK1) and inhibitor of nuclear factor kappa-B kinase subunit epsilon (IKKi/IKKε) which in turn phosphorylates IRF3. Ultimately, IRF3 forms a

dimer and translocates to the nucleus to initiate IFN-I transcription (9, 11). In contrast, TRAF6 recruits Transforming growth factor beta-activated kinase 1 (TAK1) which leads to the activation of NFκB and MAPK, that in turn results in inflammatory cytokine production (31).

However, ssRNA and dsRNA are both parts of the life cycle of a virus. How cells discriminate between self and non-self-nucleic acids remained unanswered. Recently it was shown that cytoplasmic RIG-I is able to bind RNA only containing 5'-triphosphate but not 5'-di or 5'-monophosphate RNA (32, 33). This selective recognition might explain why recognition is attributed to conformational changes in the respective PRR proteins upon binding of ssRNA and dsRNA (34-36). RIG-I and MDA-5 have shared homology and are composed of three units, the helicase domains (Hel-1 and Hel-2), C terminal domain (CTD) and N terminal caspase activation and recruitment domain (CARD) (37-40). Recognition by RIG-I and MDA-5 is differential owing to its binding efficiency to different sizes of nucleic acids such as poly(I:C). RIG-I binds to shorter lengths ~300bp whereas MDA-5 binds to longer forms, ~4000bp (41). It was recently shown that viral RNA degraded by RnaseL and viral negative-strand genomic transcripts can also result in activation of RIG-I but not the short replication intermediates (42-44).

At resting state RLRs are auto repressive that restricts activation of CARD domains. This is achieved by binding of a CARD2 domain with Hel-2i (45, 46). Binding of dsRNA to CTD, brings CTD loser to a Hel-2 domain and helps in releasing CARD domains after hydrolysis for further signaling (46-49). This conformational change in the CARD domain results in interaction with the mitochondrial antiviral signaling (MAVS), also known as IPS-1, CARDIF or VISA (50-52). However, before interacting with MAVS, RIG-I binds to K63 ubiquitins via tandem CARD domains (53).




***Graphical figure 1.*** *Pathogen recognition via different pattern recognition sensors in the cell. Figure was adapted from Heaton et al.,(54).*

Moreover, CARD domains of MAVS, upon binding with CARD domains of RIG-I and MDA-5 forms an aggregate, which further leads to activation of IRF3 (53). MAVS recruit two essential elements after binding of RIG-I, TNF receptor associated family of proteins (TRAF3) and TBK1 which activates IKKe or IRF3/7 which leads to induction of IFN regulated genes (Graphical Figure 1) (21).

### 1.1.3 Effector genes:

Classical TLR signaling leads to the induction of proinflammatory cytokines, which are important in clearing the pathogen (31, 55). Since TLRs and RLRs are specific to the pathogens, cytokines produced during infection are also quite unique. Bacterial infection primarily induces production of TNF and IL6, viral infection would result in the production of Type 1 and Type 2 interferon (IFN-I and IFN-II) (56).

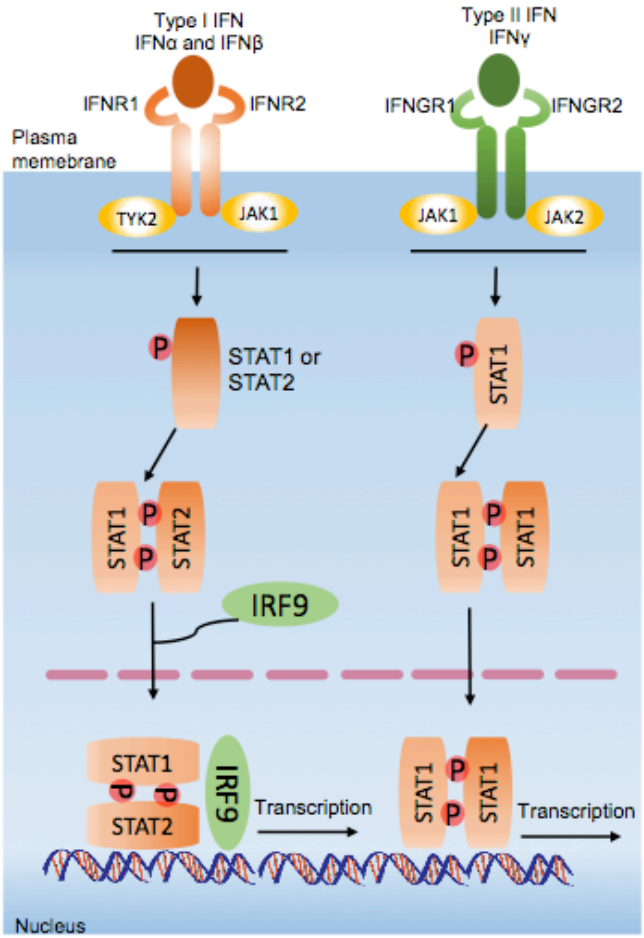
#### 1.1.3.1 Interferon signaling

Brilliant experiments by Alick Issacs and Jean Lindenmann in the late 1950s involving inactivated influenza virus conferring protection against subsequent infection by live influenza or vaccinia virus lead to the foundation of interferons (57-59). Its direct role via induction of antiviral response was shown using mice deficient in IFNAR (60). IFN response elements consist of two classes of interferon; IFN-I and IFN-II and newly recognized IFN-III (61). IFN-I have 14 members of IFN-α, IFN-β and IFN-ω, IFN-II has only one-member IFN-γ and IFN-III contains IFN-λ (62, 63). Although, such diverse composite of cytokines; its signaling is limited to only two receptors; IFN receptor I which is composed of two subunits (IFNαβ) (IFNR1 and IFNR2) and IFN gamma receptor (IFNGR) (64-66). As speculated in earlier work, studies using cloning and soluble IFNαβ receptor was shown to be the only receptor for IFN-I and IFNGR for IFN-II (67, 68)

Only a handful of cells are specialized to respond to the viral infections. Inflammatory monocytes, plasmacytoid dendritic cells (PDCs), conventional dendritic cells (CDCs) are the major contributors to systemic IFN levels (29, 69, 70). Termed as interferon-producing cells (IPCs), are present in blood circulation and secondary lymphoid organs (71, 72) and are specialized in secreting large amounts of interferons after viral stimulation (73). IFNAR, Janus kinase (JAK1), tyrosine kinase 2 (TYK2), signal activator and transducer (STAT1) STAT1 and STAT2 are the core components of the IFN signaling (Graphical figure 2) (55).

IFNAR engagement via binding of IFN-I activates JAK1 and TYK2, which in turn phosphorylate STAT1 and STAT2 (74, 75). This phosphorylated heterodimer translocates into the nucleus, which forms a trimeric complex along with IFN-regulatory factor (IRF9) called interferon stimulated gene factor (ISGF) (55). This ISGF complex binds to a specific DNA sequence called IFN-stimulated response elements (ISRE) thereby activating various

ISGs (76-78). A novel approach of using overexpression of ISGs, Schoggins *et al.,* showed that ISGs such as IRF1, RIG-I, MDA-5, and HPSE have broader antiviral effect, whereas IFI6, IFIT2, MAP3K14, OASL and others have antiviral activity against select viruses (78) which were speculated to play role in antiviral defense (79, 80).




***Graphical figure 2.*** *IFN-I signaling cascade leading to expression of interferon stimulated genes (ISGs). Figure was adapted from Platanias et al., (81).*

Antiviral genes act at different level of a virus life cycle, e.g. Virus entry, uncoating, replication, assembly and budding process (82). GBP1, MX1, OAS1, PKR, and viperin have shown to inhibit replication activity (83, 84) whereas IFITM proteins thought to be interfering with binding, entry and uncoating process of a virus life cycle (85).

#### 1.1.3.2 Regulation of interferon signaling

Although potent IFN responses are induced after pathogen recognition, it's the basal IFN-I production with the help of commensal bacteria in the intestine results in a quicker response (86, 87). Even though IFN signaling is beneficial to host after infection, chronic activation of IFN is as much deleterious in case of bacterial infections and certain autoimmune diseases (88). It was reported that IFN-I gene signature was responsible for suppression of IFN-II, which resulted in the severity of mycobacterial disease such as leprosy and tuberculosis (89, 90). These immunosuppressive modes of action are partly due to induction of IL-10 which antagonizes antibacterial activity of IFNγ (90). Moreover, persistent LCMV infections can also be controlled by blocking IFN-I signaling using neutralizing antibody (91, 92).

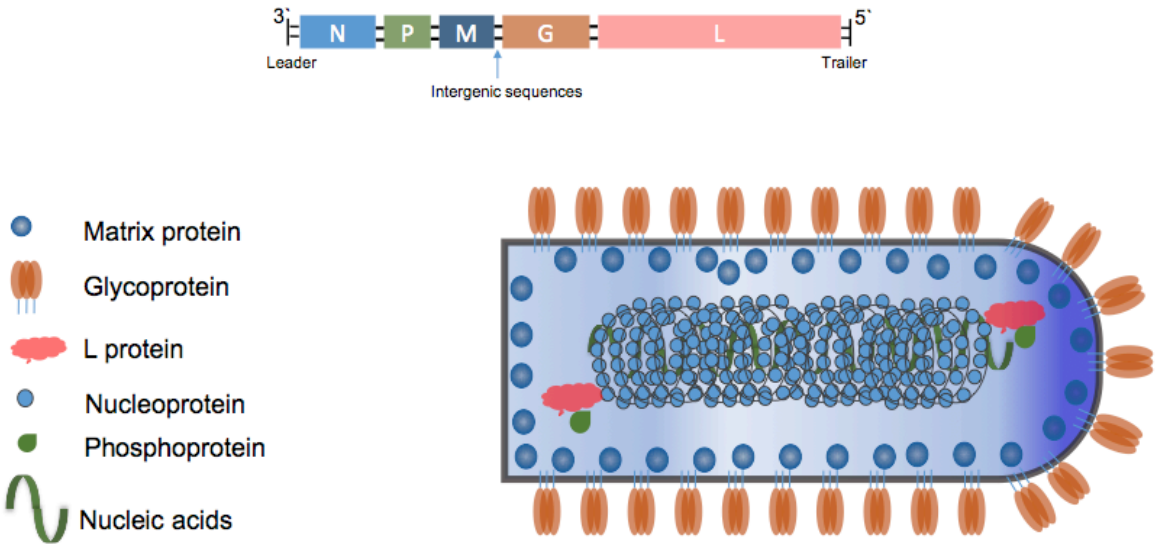
Hence, IFN signaling is tightly controlled via a negative feedback loop which involves suppression of IRF7 translation by OASL1 and inhibition of eukaryotic transcription initiating factor (eIF3) by interferon-induced protein with tetratricopeptide repeats 1 (IFIT1) and IFIT2 (93, 94). IFN signaling also induces expression of suppressor of cytokine signaling (SOCS1), SOCS3 and ubiquitin-specific proteases 18 (USP18) which negatively regulates the IFN pathway (95, 96). SOCS proteins exert their inhibitory action by competing with STATs whereas USP18 interacts with JAK1 and suppress the downstream IFN signals (55).

### 1.1.4 Vesicular Stomatitis virus

Vesicular stomatitis virus (VSV) infection in rodent models is classically used to study innate and adaptive immune responses. VSV belongs to family *Rhabdoviridae*. Its close relative rabies virus causes fatal encephalitis in humans and other mammals. It is primarily transmitted through the bite of an infectious individual, whereas transmission of VSV depends on a bite of a tick and other parasites living on the mammal's skin (97). VSV contains a single-stranded RNA genome with a lipid envelope which contains glycoprotein

(VSV-GP) (98). Two serotypes of the virus have been isolated, VSV Indiana and New Jersey (98) along with various other strains such as Mudd-Summers isolate.

VSV has a 11kb, single negative sense RNA genome (98). Around 95-98% of the genome encodes for 5 different proteins namely L protein (RNA dependent RNA polymerase), G protein (glycosylated envelope glycoprotein), M protein (non-glycosylated Matrix protein), phosphoprotein and N protein (nucleoprotein) (Graphical figure 3) (99-101).




***Graphical Figure 3***. *Figure depicting internal components of VSV structure. Figure was adapted from Fuchs et al., (102).*

VSV has been used as a murine model of viral infections to study the innate immune response, virus replication in secondary lymphoid organs and the central nervous system (CNS) (103-105). Pathology of the VSV heavily depends on the dose of the virus and route of infection. Since VSV is highly neurotropic, intranasal infection leads to higher morbidity and mortality rate compared to subcutaneous infections (106). VSV infection leads to rapid induction of Type I interferon (IFN-I) and clearance depends heavily on IFN-I and the presence of neutralizing antibodies (60, 107). Accordingly, mice deficient in IFN-α/β

receptor (IFNAR) signaling exhibit paralysis and the presence of VSV in the CNS (60). IFN-I is a potent inhibitor of VSV replication in neurons, and defects in IFN-stimulated genes in the CNS tissue trigger pathology during VSV infection (108, 109). During infection with low doses of VSV, replication of VSV in $CD169^{+}$ cells in the spleen and lymph node tissue is important for inducing protective immunity and preventing CNS infection (110, 111). The VSV backbone is also used during vaccination to Induce protective immunity against viruses such as the Ebola virus (112). VSV has played a key role in identifying various immunological phenomenon. Class switch, IFN-I, role of $CD169^{+}$ subcapsular sinus macrophages (SNS) and enforced virus replication in splenic $CD169^{+}$ cells (60, 107, 110).

## 1.3 Immune system:

### 1.3.1 Lymphoid organs: the production and battling grounds

Spleen and lymph nodes (LN) are considered secondary lymphoid organs of the immune system. Blood and lymph flow in the spleen and LN respectively enables them to act as a filtering agent to filter and sample pathogens, antigens and foreign substances (113-115). Spleen microvasculature carries the red blood cells, platelets and bloodborne pathogens in the red pulp area of the spleen (116). Elaborate splenic reticuloendothelial system (RES) allows carrying out filtering of the blood. Marginal zone (MZ) macrophages and red pulp (RP) macrophages comprise the system (115, 117). Origin of these macrophages is approximately 55% from monocyte influx whereas 45% is a result of local differentiation of monocytes (118).

### 1.3.2 Cells of Innate immunity

#### 1.3.2.1 Dendritic cells

Dendritic cells (DCs) as we know of today were discovered five decades ago by Steinman and colleagues. These specialized cells display various functions during antigen uptake, antigen presentation, and induction of activation or tolerance in the immune system (119, 120). A role of DCs as cross-priming T cells was uncovered using diphtheria toxin (DT) inducible depletion of DCs (121). DCs can be broadly divided into two classes conventional DCs (CDC) and plasmacytoid DCs (PDC) (122). They both differ significantly in their antigen presentation and IFN-I production capacity (69, 123). One of the fundamental difference in both types of DCs is that pDCs have a longer lifespan and show D-J rearrangements of IgH genes (124). CDCs present in spleen and LN and comprised of $CD8^{+}CD11b^{-}$ and $CD8^{-}CD11b^{+}$ DCs (125). Presence of CD8 was thought to be helpful in the accessory function of antigen presentation hence $CD8^{+}$ DCs were more efficient than $CD8^{-}$ (126).

Viral nucleic acids are differentially recognized by pDCs which largely rely on the TLR system, namely TLR7 and TLR9. CDCs mainly use the cytoplasmic RIG-I and MDA5 (127). However, it is known that $CD8^+$ DCs express TLR3 but not TLR7, they lack the receptor for the recognition of certain viruses (128). pDCs respond and secrete large amount of IFN-I via TLR7 and TLR9. This signaling is carried out via MyD88 (129). Since pDCs lack TLR3, they do not respond to poly(I:C) whereas, in case of ssRNA viruses (VSV, influenza and synthetic ssRNA) pDCs secrete copious amounts of IFN-I (27, 130).

#### 1.3.2.2 NK cells

Natural killer (NK) cells were first identified in by incubating tumor cells with cytotoxic cells (131, 132). NK cells are part of one of the first lines of defense in viral infections and comprise of 5 to 10% of total lymphocyte population (133). The main function of NK cells is to eliminate cells lacking MHC-I molecules was termed as "missing self-hypothesis". This hypothesis was supported by two studies where syngeneic mice rejected bone marrow cells of β2-microglobulin-deficient syngeneic mice, which lack MHC-I expression (134, 135). Thus, when the MHC-I is present on the cells NK cell activity is inhibited.

IFN-I produced after virus infections are potent activators of NK cells (136). NK cells share homology with $CD8^+$ cytotoxic T cells (CTL), NK cells use granzyme and perforin to carry out the killing (137). NK cells are major producers of IFNγ, TNF and also secrete other cytokines along with many chemokines, including CCL2 (MCP-1), CCL3 (MIP1-α), CCL4 (MIP1-β), CCL5 (RANTES), XCL1 (lymphotactin), and CXCL8 (IL-8) (138, 139). Hence, regulation of NK cell activity is tightly controlled by expressing activating and inhibitory receptors on the surface (140). NK cell receptors have immunoreceptor tyrosine-based inhibitory motifs (ITIM) and immunoreceptor tyrosine-based activation motif (ITAM) (141, 142). NK cells are able to modulate immune response via killing of infected cells and antiviral T cells (143-145). The murine cytomegalovirus (MCMV) infection model has been used to study

the killing of virus-infected cells and long-lasting NK cell memory functions, usually attributed to adaptive immunity (146, 147).

#### 1.3.2.3 Red pulp and Marginal zone macrophages

The murine spleen can be divided into red pulp and white pulp. Red pulp is mostly consisting of macrophages which express F4/80, vascular adhesion molecule and α9 integrins (148, 149). The white pulp contains B cells, T cells, and DCs. A marginal zone (MZ) demarcates both the areas which contain macrophages. MZ macrophages can be further subdivided according to the expression of surface markers, ER-TR9, SIGN-R1 and $CD169^+$ metallophilic MZ macrophages (MMM) (150). Resident lymphoid-tissue inducer (LTi) cells and stromal lymphoid tissue organizer (LTo) cells are crucial for the development of lymph nodes and splenic architecture (148). Importance of lymphoid and parenchymal cells in developing secondary lymphoid organs (spleen, LN and Payer's patches) was shown by using mice deficient in lymphotoxin beta receptor (LTBR) (151, 152). Although macrophage colony stimulating factor (M-CSF) helps in MZ development is heavily dependent on CCL21 and some extent on CCL19, both of which are a product of LTBR singling of B cell and stromal cell interaction (153, 154).

#### 1.3.2.4 $CD169^+$ macrophages

$CD169^+$ macrophages were identified over three decades ago, however, their role in pathogen defense and anti-tumor immunity have just come into a limelight. CD169 macrophages are also known as metallophilic marginal zone (MMZ) macrophages for their ability to process ammonical silver which was originally used to stain macrophages in spleen (Marshal et al, 1956). They express surface marker CD169 also known as Siglec-1 (155-157). $CD169^+$ Macrophages are strategically placed in the marginal zone of the spleen and sinusoidal area in the lymph nodes (LN) which enables them to sample out pathogens and foreign antigens (149).

$CD169^+$ macrophages not only play role in antiviral defense but also effectively suppresses bacterial infections such as *Listeria monocytogenes* and *Plasmodium*

infections (158, 159). CD169 express a variety of adhesion molecules as well as surface molecules involved in antigen capture and presentation. Its most important role in antiviral immunity is widely recognized. *Junt et al* showed that subcapsular sinus (SCS) macrophages help to initiate adaptive immunity (160). Whereas *Honke et al.,* with the focus on different site of infection in the spleen discovered that $CD169^+$ cells enforced the virus replication. CD169 macrophages express a peptidase USP18 which cleaves the adducts JAK1 and JAK2 of the cytoplasmic domain of IFNR2 upon IFN stimulation (161). This hindrance in the in IFN-I signaling renders the CD169 macrophages to support VSV replication (162).

Although the marker CD169 is used only to identify MMM macrophages, recent studies show that CD169 itself is involved in transferring antigen to antigen presenting cells (APCs) (163). MMMs were shown to be extensively involved in uptake of pathogens and able to present antigens to $CD8^+$ T cells and contribute to antiviral immunity (164, 165).

### 1.3.3 Cells of adaptive the adaptive immune system

Somatic hypermutation (SHM), allelic exclusion and antigen-specific immunological memory response are the hallmark of adaptive immunity. All the jawed vertebrates share the evolutionary origins of the DNA rearrangement using recombination activating gene (RAG) (166). Diversity in the immunoglobulins and T cell receptors is mediated by shuffling or recombination of Variable (V), diversity (D) and joining (J) gene segments (167). Foundations were laid by Burnet, who in the 1960s speculated that genetic diversity could account for diversity observed in immunoglobulin production. V(D)J DNA recombination is initiated by lymphoid cell-specific RAG1 and RAG2 proteins (168). B cells and T cells comprise the lymphocytes responsible for the adaptive immune response.

#### 1.3.3.1 B cells

The existence of cells producing antitoxins was proposed in the late 19$^{th}$ century by Behring and Kitasato, using Tetanus and Diphtheria toxoids (169). Paul Ehrlich further advanced the idea of specific cells responsible for antitoxin production.

Production of antibodies against cognate antigens of bacterial and viral origins are arguably the best-known function of B cells, maturing B cells were found in Bursa of Fabricius in birds (169, 170). Mature B cells can be traced to hematopoietic cells of bone marrow which gives rise to cells expressing surface B cell receptor (BCR) (171).

##### 1.3.3.1.1 Lineage development

B cells arise from pluripotent hematopoietic stem cells (pHSCs) in the bone marrow. From earlier progenitors to immature B cells. Egress of B cells into secondary lymphoid organs (SLO), marks the maturation of the B cell life cycle. It encounters the various antigens in the SLOs, and via gene arrangement generates a continuous repertoire of B cells capable of binding to vast amounts of antigens (169, 171-173). These multipotent progenitor cells (MPP) gives rise to common progenitor cells (CLP). Developing B cells express IgM on the cell surface and are classified as immature B cells (174, 175). But how it is ensured that the B cell repertoire has minimum reactivity towards self-antigens?

The question puzzled scientist for many years, even though the initial clonal selection theory was put forward by Burnet (176). A few decades later it was shown that B cells recognizing autoantigens with high avidity undergo apoptosis in the peripheral lymphoid tissue (177-179). However further studies led to another radical hypothesis that not only clonal deletion of autoreactive B cells contributes to B cell tolerance but receptor editing as well. Autoreactive B cells were shown to edit the L segment and produce new L segments which bind to pre-existing H segment to form a new non-autoreactive B cell. Here the B cells reactive against self-antigens undergo negative selection (180-182).

Non-autoreactive B cells further develop into transitional/mature B cells via tonic BCR activation and extracellular signal-regulated kinase (Erk) phosphorylation and progressively acquire more specialized surface receptors such as IgD, CD21, and CD23 (183-185). Experiments with mice lacking Ekr1 and Erk2 show that these mice have a complete block of pre-B cells which was dependent on tonic BCR signaling (186). Once the non-autoreactive B cells are selected they home into peripheral lymphoid organs for further development (187, 188). Once in the periphery, B cells are in the transitionary stage where they can be divided into three subsets depending on the expression of IgM and CD23 (183). Immature B cells express surface receptors for sphingosine-1-phosphate (S1P) and Cannabinoid receptor 2 (CNR2) which are secreted by platelets, vascular endothelial cells, positions them into lymphoid organs (189, 190).

Cytokines play a very important role in the development of the B cells. It was reported that bone marrow stromal cells producing CXCL12, IL-7 and galectin-1 interact with pre-B cells and directs proper development (191, 192). Peripheral B cells survive owing to B-cell activator of the TNF-α family (BAFF) binding to BAFF receptor (BAFF-R) which was recently identified (193, 194). BAFF can also bind to two other receptors, transmembrane activator, calcium modulator, and cyclophilin ligand interactor (TACI) and B-cell maturation factor (BCMA) (195). BAFF mainly play role in the survival of the B cells which was confirmed by using transgenic mice overexpressing *Bcl-2,* which lack BAFFR (196).

#### 1.3.3.1.2 B cell maturation and antibody secretion

Antibody generation by terminally differentiated plasma B cells is an essential part of the B cell life cycle. Immature B cells egress in the peripheral lymphoid organs where it interacts with the antigens mature into Follicular B cells (FO), Marginal zone B (MZB) cells and B1 cells (197, 198). FO B cells display higher amounts of B220 and IgD but low levels of IgM, lack CD5 and low level of CD21; MZB cells show high levels of IgM and CD21 in contrast to FOB cells and IgD and CD23 low. On the other hand, B1 B cells show lower B220 and IgD levels but higher surface IgM (199-201).

Generation of antibody secreting cells (ASCs) can be broadly classified into T cell dependent and T cell independent (202). Role of B cells in antiviral defense was established using passive immunization of immunodeficient mice. In the case of antibody production against the viral pathogen, lymph node (LN) and spleen play an important part. The neutralization of the virus particles is defined as the inhibition of the infectivity of the virus particles (203). The proliferation of ASCs occurs in two steps in TD antigens (204).

First response known as extrafollicular response leads to short-lived plasma blast which shows low amount somatic hypermutation (SHM), hence the response is moderate (205). In the second stage, some of the surviving short-lived activated B cells with the help of follicular helper $CD4^+$ T cells ($T_{FH}$) forms actively replicating germinal center (GC). At the GC, B cells rapidly develop into antibody-secreting plasma blasts producing high-affinity antibodies on a larger scale (206, 207). In case of antibodies produced against virus antigens, they interfere with the receptor binding and cell entry of virus thereby protecting the subsequent spread of the virus particles (107, 208).

To actively start secreting antibodies, B cells must encounter the antigens, coordinated migration of $T_{FH}$ and DCs in the niche of lymph nodes or follicular region gives rise to plasma blasts. This process is facilitated by follicular dendritic cells (FDCs) and dependent on the expression of B cell lymphoma 6 (BCL-6), the master regulator of GC formation (209, 210). GCs are composed of light and dark areas, where a light area is mostly loosely packed B cells, $T_{FH}$ and DCs, and dark area composed of densely

packed plasma blast. This differentiation was mostly attributed to the expression of CXCR4 and CXCR5 (211). Intravital imaging was instrumental in determining the exact role of $T_{FH}$ cells. B cells capture the antigens via BCR and present it via MHC class II to T cells and help in enriching IL4 and IL21 in $T_{FH}$ cells which further leads to upregulation of CD40 to stimulate the CD40 receptor on B cells in the GCs (212-214). This interaction is a central process of immunoglobulin class switch and high-affinity antibody production (215).

MZB cells mount T cell-independent response in the initial stages of infection of bloodborne pathogens (216). In the first three days of infection, MZB cells differentiate into IgM producing plasma blasts (217). Secretion of antibody starts with somatic rearrangement of two part of an antibody, heavy and light chain (218). Creation of light chin involves joining of V (variable) and J (joining) chain whereas heavy chain involves V, D (diversity) and J joining (219, 220). The μ-heavy chains expressed by pre-B cells on the cell surface and then secreted in the form of IgM is due to alternative splicing of heavy gene transcripts (221). In the course of cellular immunity, secreted antibodies change their form, called as isotype switch. Antibody production changes from earlier IgM response to either IgA, IgG, by deletion of a constant region of μ-heavy chains and replacing other segments (222-224). This class switch is initiated by recombination activating gene (RAG1) and RAG2 (225, 226) and activation-induced cytidine deaminase (AID) which is essential for heavy chain switching (227). Memory B cells have isolated from the survivors of the 1918 influenza pandemic (Spanish flu); circulating B cells were able to react with HA protein of the influenza virus (228). In murine settings, it is reported that antiviral plasma B cells can survive up to 175 days in the spleen and 90 days in the bone marrow (229).

#### 1.3.3.2 T cells

T cells play cells play a vital and diverse role in the eliminating pathogens. Memory, providing help to other cells and the direct killing of infected cells are one of the few functions T cells carry out in their lifespan.

T cells are developed and matured in the thymus from hematopoietic progenitors trafficked from bone marrow (230, 231). Egress from bone marrow depends on the surface expression of α4β1 (VLA-4), α5β1 integrins and cytokines such as IL-8, macrophage inflammatory protein (MIP) (232, 233). The decrease of surface expression of integrins or use of antibodies against VCAM-1 increases the mobilization of HSCs from the BM (234).

Once in the thymus, various T cell lineages are developed, $CD4^+$ αβ T cells, $CD8^+$ αβ T cells and γδ T cells as well as other subsets such as regulatory T cells and NK cells (231). Thymic selection ensures T cells reactive to self-antigens displayed on MHC are eliminated and only the low-affinity T cell receptor (TCR)- MHC interaction results in survival (235).

Antiviral T cell response is paramount in clearing virus infection and induction of virus-specific T cells can be detected as early as 4-5 days and reaches at peak around 7-14 days (236). How do these $CD8^+$ T cells get activated? It is known that T cells encounter antigen-loaded APCs in secondary lymphoid organs such as the LN and the spleen. But with the advent of the multiphoton microscope, it was revealed that naive $CD8^+$ T cells stroll randomly along with fibroblastic reticular networks (237). They are found mostly in the T cell zones and in close contact with DCs. Soon after infection DCs migrate and populate the LN where the inflow of antigens and pathogens via subcapsular sinus takes place and activate naive $CD8^+$ T cells (238, 239). These naïve $CD8^+$ T cells express chemokine receptors CCR4 and CCR5 which gets attracted to its ligands CCL3, CCL4 and CCL17 produced by DCs (240, 241).

To meet high demands of the rapid division after the antigen stimulation $CD8^+$ T cells must undergo a change in the metabolism. Cells switch from oxidative phosphorylation to aerobic glycolysis which was shown to be dependent on PI-3K-Akt-mTOR which is induced by CD28 and TCR engagement (242, 243). To further the expansion, $CD8^+$ T cells must rely on external stimuli by cytokines which are provided by IL-2 and IFN-I (244, 245). $CD4^+$ T cells which migrate before $CD8^+$ T cells at the site of infection secrete IFNγ and induce epithelial cells to secrete chemokines. Once activated, $CD8^+$ T cells

express CXCR3 which allows them to migrate to the peripheral site of infection where epithelial cells secrete CXCL9 and CXCL10 which in turn is secreted by $CD4^+$ T cells (246, 247).

Cytotoxic T cell (CTL) response is essential in eliminating intracellular pathogens and tumor cells and it is achieved by the interaction of TCR and peptide-MHC-I complex termed as immunological synapse (248). Once the synapse is complete, cellular machinery involved in target cell killing is mobilized. Perforin and granzyme B (GrzB) are the effector molecules which induce cell killing (249, 250). High-resolution microscopy has revealed that these molecules are polarized within few minutes towards target cell and initiate killing (251). Perforin forms membrane pores and helps to deliver GrzB which initiates apoptosis in the target cell (252, 253). This process eliminates virus-infected cells and tumor cells thereby protecting the host of further spread of pathogens.

## 1.4 TNF signaling:

Before the end of the 19th century, spontaneous tumor regression observed after infections led to the treatment of tumors with deliberate infections of the tumors (254, 255). It was William Coley, whose efforts led to the first refined approach to treat tumors of various origins with bacterial extracts (256). Known as the father of cancer immunotherapy, he used S*treptococcus pyogenes* and S*erratia marcescences* to concoct Coley's toxins (255). It was not until *Granger et al.,* and *Carswell et al.*, who showed that the administration of bacterial products such as Bacillus of Calmette and Guerin (BCG) and endotoxins/lipopolysaccharides (LPS) derived from *Escherichia coli* produced a factor responsible for reducing tumors or causing hemorrhagic necrosis, termed tumor necrosis factor (TNF) (257-259). Serum obtained from mice which were administered with endotoxins, when injected into tumor-bearing mice could reduce the tumor size. It was hypothesized and proved to be produced by macrophages also known as cachectin which causes weight loss in chronic infections (257, 260, 261). Since earlier preparations of cytokines were crude mixtures of multiple cytokines it was not until the mid-80s, *Aggarwal et al.,* who showed that lymphotoxin (LT) depletion using a neutralizing antibody still retained the anti-tumor activity (262, 263). Soon after they were able to successfully clone and characterize the protein (262). That led to the discovery of TNF as a causative agent of cachexia and inflammation as well (264, 265).

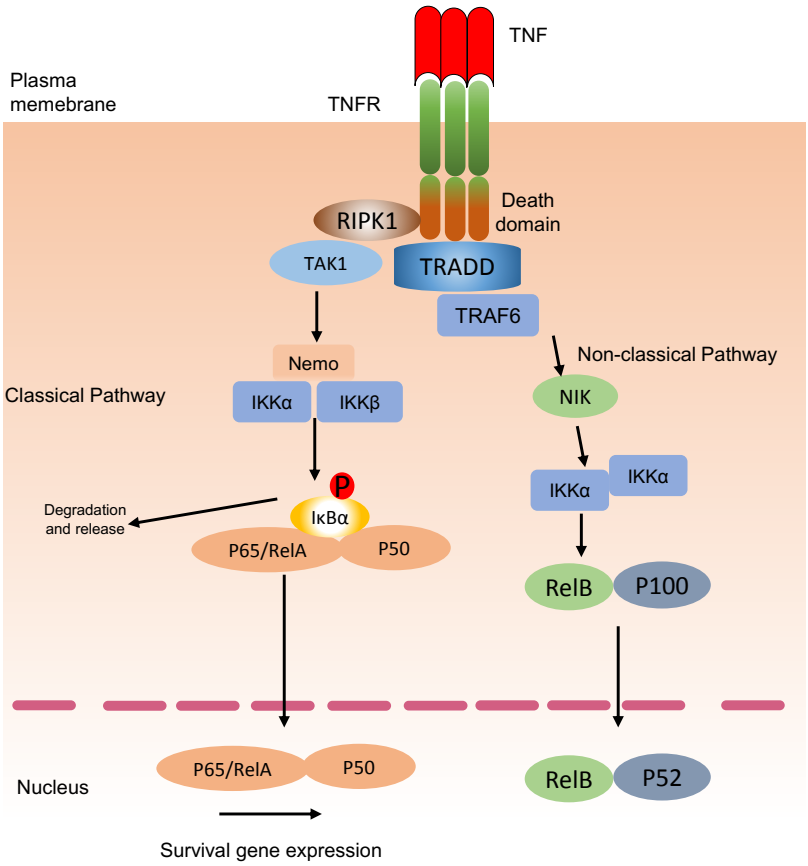
### 1.4.1 TNF structure and function:

TNF is located in chromosome 6p21.3 region translated as 17kD monomer protein forms a homotrimer 157 amino acid each (266). TNF is a transmembrane protein which is secreted when cleaved by the TNF-converting enzyme (TACE) or called ADAM17 (267, 268). This process, in turn, is heavily regulated by iRhom2 (269, 270). iRhom2 is involved in the trafficking of TACE from the endoplasmic reticulum to plasma membrane. Mice deficient in iRhom2 were unable to clear *Listeria monocytogenes* infection and were less susceptible to lethal LPS shock (270). TNF binds to two knows receptors, 55kD TNF receptor 1 (TNFR1) and 75kD TNF receptor 2 (TNFR2) and

activates two major transcription factors, nuclear factor-κB (NF- κB) and c-Jun (271-273).

### 1.4.2 TNF signaling

TNF is produced in response to various immune receptor stimuli of bacterial and viral pathogens (274-276). Termed as a double-edged sword for its role in the survival and apoptosis of cells, TNF exerts its effects via TNFR1 and TNFR2 (277). NF-κB signaling composed of multiple proteins and complexes namely p50, p52, RelA, RelB and c-Rel and can be activated by TNF-TNFR axis (278). These proteins bind and form up to 15 homo and heterodimeric protein complexes and initiate DNA binding (279). NF-κB signaling can be subdivided into two types, classical (canonical) and the alternative (non-canonical) pathway (280, 281). The classical pathway is induced via proinflammatory cytokines and PAMPs which is rapid, whereas alternative pathway is engaged by cytokines belonging to TNF superfamily of proteins such as lymphotoxin (LT), receptor activator of NF-κB ligand (RANKL), CD40 ligand and B cell activating factor (BAFF) (277, 282, 283).




***Graphical Figure 4***. *Figure depicting signaling cascade after interaction of TNF with its receptors. Figure was adapted from Brenner et al.,* (284).

Hallmark of classical NF-κB is the nuclear translocation of p50-p65 dimer whereas in the alternative pathway p52-RelB dimer translocate to the nucleus (281). Activation of the alternative pathway depends on NF-κB inducing kinase (NIK), which requires longer time compared to the classical pathway (276). TNF induced NF-κB activation is a series of events which involves TGFβ activated kinase (TAK1) activation which phosphorylates trimeric complex of IKKα and IKKβ along with regulatory unit IKK-γ. This complex after activation phosphorylates IκBα which undergoes ubiquitination and

degradation releasing p50-RelA (283, 285). IKKγ and IKKβ are considered central regulators of NF-κB by degrading IκBα, where IKKα plays only a supporting role (286).

Alternative NF-κB signaling is induced via the processing of p100 unit by NIK (287). Phosphorylation of p100 mediated by NIK is dependent on IKKα which plays a minor role in the classical NF-κB pathway but is an essential component in an alternative pathway (Graphical figure 4) (288).

### 1.4.3 Functional role of NF-κB regulated genes

The chromatin structures become permissible for the attachment of the transcription factors such as NF-κB after the activation and along with lineage-specific gene expression is determined activators or repressors of transcription factors. For example, feed-forward loop which is essential for proinflammatory target gene activation is achieved via histone methylation and other modifications (289-291). Along with histone modifications, other mechanisms such as nucleosome remodeling may also play a role in differential gene expression (292). It is proposed that a synergy exists between NF-κB and other transcription factors such as IRF3/IRF7, ATF2/c-Jun (293). Temporal activation kinetics of activation of NF-κB activated genes is also an essential factor for differential gene expression (294-296). Periodicity in the gene expression was determined by a short stimulus of TNF to cells and tracked using single cell high throughput microfluidic chambers and fluorescence microscopes (294, 296). Short and less frequent TNF pulses led to proper activation of genes whereas more frequent pulses don't restore the normal gene expression, indicating that a negative feedback mechanism is essential (294-296).

### 1.4.4 TNF and apoptosis

Binding of TNF to TNFR1 induces activation TNF receptor-associated death domain (TRADD) via the release of silencer of death domain (SODD) (297-299). The cytoplasmic domain of TNFR1 recruits adaptor molecule TRADD which in turn recruits TNFR associated factor 2 (TRAF2) and receptor interacting protein kinase 1 (RIPK1) and inhibitor of NF-κB kinase (IKK) (300) (297). Whereas binding to TNFR2, which lacks death domain signals via TRAF1 and TRAF2 (301). RIP1 which is a serine-threonine kinase is recruited by TRADD to induce NF-κB activation and apoptosis (300). Although RIP1 was known to interact with Fas-associated death domain (FADD), cells lacking RIP1 failed to activate NF-κB and underwent apoptosis, suggesting the role of RIP1 in activation of NF-κB and survival of cells (302). Binding of TNFR1, TRADD and RIPK1 complex initiates the downstream TNF signaling (275).

### 1.4.5 TNF involvement in multitudes of viral infections

The role of tumor necrosis factor (TNF) in marginal zone development and marginal zone function is controversial. Although reports show that marginal zone development is impaired and fewer marginal zone macrophages are present in TNF-deficient and p55-TNFR (tumor necrosis factor receptor 1 [TNFR1])-deficient mice (303), other reports suggest that TNF triggers marginal zone macrophage depletion after infection (304, 305). It has also been shown that TNFR1 deficient mice are less susceptible to West Nile virus infection as a result of uncompromised blood-brain-barrier (306). However, these findings are contradicting other studies utilizing Herpes simplex virus-1 as infection model where it is shown that TNFR1 deficient mice are more susceptible to virus infection (307, 308).

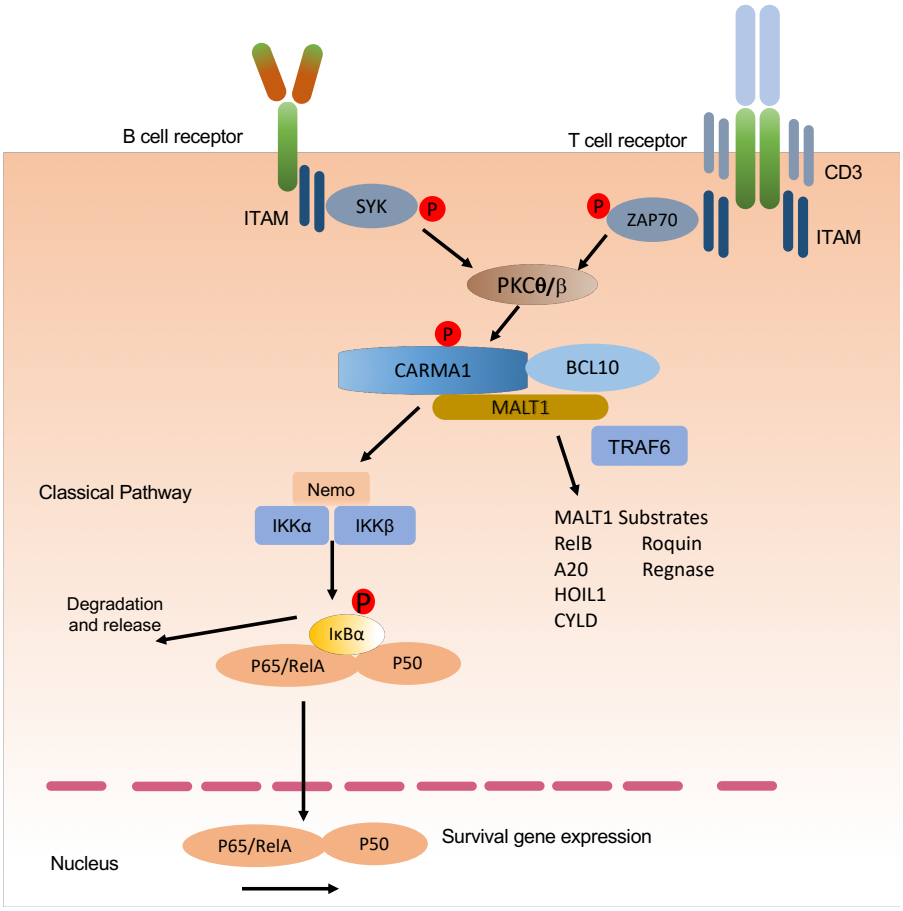
It is clear that TNF-deficient mice exhibit $CD169^{+}$ cells in the spleen, whereas this cell population is absent in $Lt\alpha^{-/-}$ mice (303, 304). Furthermore, the production of neutralizing antibodies and the proliferation of antiviral T cells can be induced in TNF-deficient animals (305, 309). These findings suggest that TNF, which is crucial for overcoming bacterial infections plays a minor role in antiviral immunity (310-313).

### 1.4.6 MALT1 signaling

The NF-κB pathway have been implicated to be under control of Mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1). Although first identified in lymphoma in the mucosal lymphoid tissue, if is shown to play important role in CD8 T cell, B cell and macrophage functions (314-317). MALT1 forms heteromeric complex with B-cell lymphoma/leukemia (BCL10) and CARMA proteins known as CBM complex. Cells of immune system T cells and B cells upon activation with its respective receptors (TCR and BCR) activates cascade of reactions, which includes activation of NF-κB. Germline mutations in any of the complex proteins causes severe immunodeficiencies which are hall mark of abnormal NF-κB or B cell deficiencies (318).

In early studies it was known that MALT1 and BCL10 formed a complex and was responsible for NF-κB activation via phosphorylation of IKK (319, 320). Specifically, immunoreceptor tyrosine-based activation motif (ITAM) once engaged with its corresponding ligand gets phosphorylated this in turn phosphorylates spleen tyrosine kinase (SYK) for B cell receptor and zeta chain associated protein kinase (ZAP70) for TCR (321). The Protein Kinase C (PKC) family of kinases are downstream of SYK or ZAP70, after the activation of PKC which phosphorylates inhibitory domain of CARD11 and open up the space for the docking of BCL10 and MALT1 complex. This complex further recruits TRAF6 and gets ready for ubiquitination and phosphorylation of IKK complexes (321).

MALT1, owing its ability to act as a paracaspase (cleaving substrates at basic or uncharged amino acids instead of acidic amino acid as in the case of caspases), cleaves BCL10, Regnase, Roquine, HOIL1 and A20 proteins (a known inhibitor of NF-κB) (322). This cleavage of inhibitor proteins paves way for NF-κB activation once the receptor is engaged with its corresponding ligand. BCL10 cleavage is important for signaling induced T cell adhesion to fibronectin and activation of NF-κB. Purified CD8 T cells when stimulated in vitro showed BCL10 cleavage products and activation of NF-κB (323).




***Graphical Figure 5***. *Figure depicting MALT1 signaling cascade after activation of B cell receptor (BCR) or T cell receptor (TCR). Figure was adapted from Lu et al., (324).*

This indirect role in NF-κB activation, puts MALT1 in very important position. Somatic mutations in BCL10 or MALT1 leads to aberrant NF-κB activation ultimately results into B cell lymphoma (325). Many human patients with loss of function mutations in MALT1 suffers from infections of bacterial, fungal and viral origin in multiple organs, whereas transplantations with healthy hematopoietic stem cells in these patients restores their immunity against the pathogens (326, 327). This phenomenon occurs due to defective marginal zone B cells, peritoneal B1 B cells and germinal centre formation in spleen, which are important in defence against pathogens. (328, 329).

Since, pivotal role played by MALT1 in the host defence in B cell and T cell compartment is well studied, warrants its further investigation in macrophages and other myeloid cells.

# Chapter 2.

## Materials and methods

## 2. Materials and Methods:

Part of the materials and methods have been adapted from a published manuscript,

**Shinde PV, Xu HC, Maney SK, Kloetgen A, Namineni S, Zhuang Y et al.,** "Tumor Necrosis Factor-Mediated Survival of CD169+ Cells Promotes Immune Activation during Vesicular Stomatitis Virus Infection" *J Virol. 2018 Jan 17;92(3).*

### 2.1 Mice, viruses, virus titration:

All mice were maintained under specific pathogen-free conditions at the authorization of the Landesamt für Natur, Umwelt und Verbraucherschutz of North Rhine-Westphalia (LANUV NRW) in accordance with the German laws for animal protection.

*Tnfa*$^{-/-}$ mice lack TNF secretion and have been described earlier (330). *Tnfrsf1a*$^{-/-}$ mice lack TNFR1 on all the cells, have been previously described (311). *Tnfrsf1b*$^{-/-}$ mice lack TNFR2 on all the cells have previously been described (331). *Cd8*$^{-/-}$ mice lacks functional CD8$^{+}$ T cells (332), *Rag1*$^{-/-}$ mice lack B cell and T cells. The mouse was generated by deleting rearrangement gene (*Rag1*) which codes for a protein involved in heavy and light chain arrangement (333), this strain was purchased from Jackson Laboratories (United States). *Malt1*$^{-/-}$, mice lack the gene encoding for mucosal-associated lymphoid tissue (MALT) adaptor (121). *CD169*$^{-/-}$, CD169-DTR, and CD11c-DTR mice have also been previously described (121, 316, 334).

For chimera experiments, mice were lethally irradiated with 10.2 Gy. After 24 h, mixed bone marrow from WT and CD169-DTR, CD11c-DTR, *Jh*$^{-/-}$, and *Rag1*$^{-/-}$ mice was transplanted into the irradiated mice. CD169 cells were depleted by injecting 2 doses of 100ng diphtheria toxin (DT) (Sigma) before the infections. To inhibit caspase activity *in vivo*, we administered three doses (2 µg/g each) of zVAD-FMK (Abcam, Cambridge, UK) (335, 336).

### 2.2 Virus

VSV Indiana strain (VSV-IND, strain Mudd-Summers) was originally obtained from Prof. D. Kolakofsky (University of Geneva, Switzerland). VSV was propagated and titrated as previously described (337). The virus was inoculated via tail vein injection.

#### 2.2.1 Virus titration

Briefly, Vero cells were cultured in 5% FCS and Alpha-MEM in T-150 flasks until 90 % growth was observed. A single cell suspension was prepared using 0.025% Trypsin-EDTA and seeded onto 24 well plate at a density of $10^5$ cells per well. Plates were incubated overnight and checked for complete monolayer formation next day. Tissue samples were homogenized using TissueLyser (Qiagen, Germany) and were kept on ice until processed. U bottom 96 well plate was seeded with 130 ul of 2% FCS Alpha-MEM, from well 2-12. 90 µl of the sample was added to well number 1 in duplicate. 60µl sample was pipetted into next well and mixed repeatedly. The process was continued until well number 7, tips were discarded and with fresh tips pipetting was carried out until well number 12. 100 µl of the sample from well number 2, 4, 6, 8, 10 and 12 were transferred to a 24 well plate with a Vero cell monolayer. After 2-3 hr incubation at 37°C, 200µl of an overlay medium (a mixture of 1:1, 2% methylcellulose + 2X IMDM) was added gently onto the cells. Plates were incubated at 37°C overnight for 20-24 hr and checked for regularly for virus plaque formation. Once plaques were visible, the supernatant was discarded and cells were stained with 0.5% crystal violet solution. Plates were washed and dried.

#### 2.3.2 Neutralizing antibody titration:

Briefly, Vero cells were cultured as described above, single cell suspension was seeded onto flat bottom 96 well plate appr. 15000 cells per well. Plates were incubated overnight until complete monolayer was formed.

To determine total immunoglobulins (Ig) serum was diluted 1:40 (10µl serum + 390µl MEM) and heated at 56°C to inactivate the complement factors. To determine IgG levels, serum was incubated with 50µM β-mercaptoethanol in 0.9% NaCl at RT for 1hr. After

the treatment serum was diluted using 2% Alpha-MEM to 1:40 and heated at 56°C to inactivate the complement factors. Afterwards, samples were kept on ice until processed.

100 µl medium was added to U bottom 96 well plate from well 2-12. 200µl sample was added to well number 1. 100µl from first well was transferred to well 2 and serial dilution was performed. VSV virus suspension was prepared using virus stock at a concentration of 5000 PFU/ml. Virus solution was mixed in the 96 well plate along with serially diluted serum at 37°C for 1hr. 100µl solution from virus mixture was transferred on to Vero cell monolayer and incubated at 37°C for 1-2hr followed by addition of an overlay medium.

Plates were stained with crystal violet solution as described above after incubation of 20-24hr.

**2.3 Histology and ELISA:**

Histological analysis of snap-frozen tissue was performed as previously described. Briefly, Snap-frozen tissue sections were air dried and fixed with acetone for 10 min. Tissue sections were blocked with 10 % fetal calf serum (FCS) for 1 hour. Then stained with primary antibodies for 1hr at RT. Slides were washed with 0.05% Tween 20 with a squirt bottle. Tissue sections were then incubated with secondary antibodies at indicated dilutions (Table 1).

**Table 1: List of antibodies used in immunofluorescence (IF), flow cytometry (FC) and western blot (WB)**

| Antibody | Company | Catalog number | Dilutions | Application |
|---|---|---|---|---|
| Anti-CD169-Biotin | Acris | SM066B | 1:200 | IF |

| | | | | |
|---|---|---|---|---|
| Anti-CD169-FITC | ABD Serotec | MCA947GA | 1:50 | IF |
| Anti-CD11b-APC | Thermofisher | | 1:200 | IF |
| Anti-GAPDH | Cell Signalling | 8884S | 1:4000 | WB |
| Anti-p100 | Cell Signalling | 4882S | 1:1000 | WB |
| Anti-RelA | Santa-Cruz | sc-109 | 1:200 | IF, WB |
| Anti-RelB | Santa-Cruz | sc-166417 | 1:200 | IF, WB |
| Anti-RelB | Cell Signalling | 4954 | 1:1000 | WB |
| Anti-VSV-G | Clone Vi10 | in house | 1:200 | IF |
| Anti-Rabbit | Cell Signalling | 7074 | 1:5000 | WB |
| Histone | Cell Signalling | 5192S | 1:5000 | WB |
| PE-streptavidin | Thermofisher | | 1:300 | IF |
| anti-CD3 | Thermofisher | | 1:300 | FC |
| anti-CD8 | Thermofisher | | 1:300 | FC |
| anti-MHC-II | Thermofisher | | 1:300 | FC |
| anti-CD11c | Thermofisher | | 1:300 | FC |
| anti-CD19 | Thermofisher | | 1:300 | FC |
| anti-NK1.1 | Thermofisher | | 1:300 | FC |

| | | | |
|---|---|---|---|
| Anti-CD5 | Thermofisher | 1:300 | FC |
| Anti-CD21 | Thermofisher | 1:300 | FC |
| Anti-CD23 | Thermofisher | 1:300 | FC |

### 2.3.2. Caspase 3 activity

To detect caspase activity, *in-vitro* fluorescence assay was used according to the manufacturer's instructions (Cell Signaling). Briefly, spleen tissue was homogenized using Qiagen TissueLyser in the presence of 1% TritonX and protease inhibitors. The clear lysate was prepared after samples were spun at 2000g. Protein concentration was measured using Bradford assay (Cytoskeleton Inc, USA) and was adjusted to have an equal amount.

### 2.3.3 TUNEL assay:

To detect in-situ apoptosis, Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) staining was performed on formalin-fixed spleen sections according to the manufacturer's instructions (Thermo Scientific, Massachusetts, USA).

Briefly, spleen tissue sections were fixed in 4% formalin. Sections were incubated with TritonX at 37°C for 1hr. Sections were incubated with the reagent for 37°C for 1hr. Sections were mounted using a mounting medium. Fluorescence images were obtained with an LSM510 confocal microscope and Axio Observer Z1 fluorescence microscope (Zeiss, Germany). Analysis of the fluorescence images was performed with ImageJ software.

### 2.3.4 ELISA:

Whole blood was collected via intra-orbital vein puncture into tubes. Mice were anesthetized for the entire duration of the procedure using Isoflurane. Serum was isolated by spinning the tubes at 5000 RPM at 4°C. Serum dilutions from 1:10 to 1:100 were used to perform enzyme-linked immunosorbent assay (ELISA). IFN-γ (eBioscience, San

Diego) and IFN-α (PBL Biosciences, New Jersey, USA) concentrations were determined using ELISA according to the manufacturers' instructions.

**2.4 Immunoblotting:**

*Malt1*$^{+/-}$ and *Malt1*$^{-/-}$ mouse embryonic fibroblasts (MEFs) were stimulated with 100 ng/ml murine-soluble TNF (mTNF; R&D Systems). Cytoplasmic and nuclear extracts were prepared according to the manufacturer's instructions (Active Motif, Belgium). Immunoblots were probed with primary antibodies (Table 1).

Briefly, total protein concentration was measured in the samples using the Bradford assay kit (Cytoskeleton Inc, USA). 50μg of total protein was heated to 95°C for 10 min along with SDS page- sample buffer (Invitrogen). Samples were loaded on pre-casted SDS page gels. After the sample run, gels were transferred to nitrocellulose membranes (Amersham, GE lifesciences). Membranes were blocked with 5% BSA (Roche) in TBST for 30 min. Membranes then were incubated overnight at 4°C with the primary antibody. Next day membranes were washed with 0.05% Tween 20 in TBST and incubated with the secondary antibody tagged with horseradish peroxidase (HRP) (Jackson). Membranes were washed and incubated with ECL for 2 min and developed using X-ray films (Amersham, GE lifesciences).

**2.5 Sorting of CD169$^{+}$ cells:**

For CD169$^{+}$ macrophage isolation, spleen tissue was incubated with tissue digestion cocktail containing liberase () and DNAse () for 20 min at 37°C for obtaining single-cell suspended splenocytes. After lysis of red blood cells, splenocytes were presorted with a CD19$^{+}$ and a CD5$^{+}$ magnetic cell separation (MACS) kit (Miltenyi Biotec, Germany). The remaining cell suspension was stained with anti-CD11b$^{+}$ (eBioscience) and anti-CD169$^{+}$ (ABD Serotec, Germany) antibodies for fluorescence-activated cell sorting (FACS Aria III; San Jose, USA).

**2.6 Flow cytometry:**

For intracellular cytokine staining, single-cell suspended splenocytes were incubated with Brefeldin A (eBioscience), followed by an additional 5h of incubation at 37°C. After surface staining with anti-CD3, anti-CD8, anti-CD11b, anti-CD11c, anti-CD19, anti-MHC-II, and anti-NK1.1 antibodies (eBioscience), cells were fixed with 2% formalin, permeabilized with 0.1% saponin, and stained with anti–IFN-γ and anti-TNF antibodies (eBioscience) for 30 min at 4°C. B-cell subsets were detected in single-cell suspensions of splenocytes with anti-CD5, anti-CD19, anti-CD21, anti-CD23, and anti–immunoglobulin M (IgM) antibodies (all from eBioscience).

**2.7 RNAseq and RT-PCR analyses:**

RNA purification and RT-PCR analyses were performed according to the manufacturer's instructions (Qiagen RNeasy Kit, Germany and Arcturus Picopure RNA isolation kit, Applied Biosystems). Expression of various genes (Table 2) was detected with FAM/VIC probes (Applied Biosystems) on AB systems HT9500 or AB systems QuantStudio.

Briefly, organ samples were collected in Trizol. Organs were homogenized using metal beads in Tissuelyser (Qiagen). 200µl of the sample was further diluted with 800µl of fresh TRIzol® (Ambion) and 200 µl of chloroform. Samples were vortexed for 1 min and incubated for 15 min at room temperature (RT). Tubes were spun down at 14000 RPM for 10 min at 4°C. Top clear aqueous phase was collected and mixed with an equal amount of isopropanol. Samples were incubated at RT for 1hr and spun at 14000RPM for 10 min to collect the RNA precipitate. RNA pellet was washed twice using 70% ice-cold ethanol to remove salts and other debris. Pellet was air dried and dissolved in DEPC treated water (Ambion). RNA concentration was measured using NanoDrop™ (Thermo) and final concentration was adjusted to 100 ng/µl. 200ng per reaction was used in all the RT-PCR tests.

**Table 2: List of TaqMan® probes used in the real time PCR**

| Primer | Probe | Catalogue number |
|---|---|---|
| *b-actin* | FAM | Mm00607939_s1 |
| *Eif2aka* | FAM | Mm00440966_m1 |
| *GAPDH* | VIC | 4352339E-0806018 |
| *Ifn4a* | FAM | Mm00833969_s1 |
| *Ifit1* | FAM | Mm00515153-m1 |
| *Ifitt2* | FAM | Mm00492606_m1 |
| *Ifnb1* | FAM | Mm00439552_m1 |
| *Isg15* | FAM | Mm01705338_s1 |
| *Irf7* | FAM | Mm00516788_m1 |
| *Oasl1* | FAM | Mm00455081_m1 |
| *Tnfa* | FAM | Mm0043260_g1 |
| *Tnfrsf1a* | FAM | Mm00441883_g1 |
| *Tnfrsf1b* | FAM | Mm00441889_m1 |
| *Cxcl9* | FAM | Mm00434946_m1 |
| *Cxcl10* | FAM | Mm00445235_m1 |
| *Fas* | FAM | Mm01204974_m1 |
| *Il6* | FAM | Mm00446190_m1 |
| *Il10* | FAM | Mm01288386_m1 |
| *Mda5* | FAM | Mm00459183_m1 |

| | | |
|---|---|---|
| *Oasl1a* | FAM | Mm00455081_m1 |
| *RanseL* | FAM | Mm00712008_m1 |
| *Usp18* | FAM | Mm00449455_m1 |

For analysis, the expression levels of all target genes were normalized to β-actin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression (ΔCt). Gene expression values were then calculated with the ΔΔCt method, with naive WT mice as controls to which all other samples were compared. Relative quantities (RQ) were determined with the equation $RQ = 2^{-\Delta\Delta Ct}$. Lib raries were prepared according to the manufacturer's instructions with the TruSeq Total RNA sample preparation kit (Illumina) and were further sequenced with the Illumina HiSeq 2500 platform (single end, 100 cycles).

**2.8 Bioinformatics analysis of RNA-Seq data:**

We obtained a total of 203,611,610 sequencing reads for 6 RNA-Seq samples. First, adapter sequences and low-quality ends were trimmed off with seqtk version 1.0 (https://github.com/lh3/seqtk) and cutadapt version 1.4.1, keeping all reads longer than 25 for further analysis. Next, the remaining reads were aligned against the reference genome sequence of GRCm38 with TopHat2 version 2.0.13(338) with standard parameters. Relative transcript abundances were measured by counting all uniquely aligned reads mapping to mouse gene annotations downloaded from Ensembl genome browser 78(339) with HTSeq version 0.5.4p5 (340). This measurement resulted in 128,972,294 sequencing reads (63.34%) mapping to annotated genes. Before performing differential gene expression analysis, we normalized read counts per sample regarding sequencing depth using the Bioconductor package edgeR version 3.8.6 (341). This analysis resulted in so-called counts per millions (CPM). Differential gene expression was performed with edgeR; multiple testing corrections were applied, and the FDR per gene was reported.

**2.9 Pathway analysis:**

Mouse-specific pathway maps were downloaded from KEGG (342). We performed an enrichment analysis of all pathways against differentially expressed genes (FDR ≤ 0.05; logFC ≥ ± 1.5) and calculated the statistical significance for overlaps per pathway with Fisher's exact test and multiple testing correction (FDR). The size of the entire gene population was set to the union of all genes available in all annotated KEGG pathways, resulting in a population of 4,594 genes. The enrichment score was calculated with the following formula:

Enrichment = #overlap / (#DE genes * #pathway genes/ #population genes).

**2.10 Promoter Analysis:**

Proximal promoter regions spanning nucleotides −600 to +100 bp and nucleotides -1000 to +500 bp relative to the transcription start site were retrieved from the mouse genome. The GRCm38/mm10 assembly was downloaded from the University of California, Santa Cruz (UCSC) genome browser with Ensembl genome browser 78. Matches to putative TF binding sites were obtained with Fimo ($P < 0.0001$)(343) from the library of 784 mouse position frequency matrices in the Catalog of Inferred Sequence Binding Preferences (CIS-BP) database (344). Overrepresentation and underrepresentation of promoters with specific motifs in deregulated genes or pathway-related genes were determined by comparison to the total interrogated genes with a two-tailed Fisher's exact test. The Benjamini-Hochberg FDR multiple test correction procedure was applied to the *P* values.

**2.11 Statistical analyses:** Data are expressed as mean ± S.E.M. Statistically significant differences between two groups were determined with Student's *t*-test. Statistically significant differences between several groups were determined with one-way analysis of variance (ANOVA) with additional Bonferroni or Dunnett post hoc tests. Statistically significant differences between groups in experiments involving more than one time-point were determined with two-way ANOVA (repeated measurements).

# Chapter 3.

## Aim of the study

# 3. Aim of the study

To prevent infections, novel vaccine delivery strategies are being developed such as Nanopatch and mRNA vaccines to name a few.

In recent years the role of $CD169^+$ cells in virus infection has led to considerable advancement in understanding virus infections such as enforced virus replication. Moreover, in recent studies, these cells are being exploited for targeted delivery of vaccines for effective therapy. So, it is important to elucidate the factors governing functions of these cells.

Almost a decade ago Subcapsular sinus macrophages present in peripheral secondary lymphoid organs such as lymph node were shown to be important in host defence. Specifically, $CD169^+$ cells helped to clear peripheral and systemic VSV infection in the mice. Production of IFN-I and sufficient antiviral antibody production was ensured by functional $CD169^+$ cells (111, 160, 345).

Although indisputable amount of evidence of role $CD169^+$ cells in antiviral response is exciting, it demands further investigation into specific mechanism involved in enforced virus replication.

In this study, we utilize the VSV infection model in C57Bl/6 mice to understand the role of cytokines in regulating innate and adaptive immune responses. We uncover a critical role of TNF in regulating virus infection.

**Specific objectives of the study:**

1. Determine factors regulating enforced virus replication in $CD169^+$ cells Using RNAseq
2. Elucidate the role of TNF during virus infection using TNF and its receptor-deficient mice
3. Mechanism of MALT1 in enforced virus replication via activation of NF-κB pathways

# Chapter 4.

# Results

# 4. Results

## 4.1 CD169$^+$ not only capture and enforce virus replication but act as early responder and producer of type 1 interferon response

CD169$^+$ cells allow viral replication after infection with VSV to promote both antigen presentation and innate and adaptive antiviral immunity (110). To gain more insight into their regulation, we sorted CD169$^+$ cells from spleen tissue before and after intravenous infection with VSV (Fig. 1A). To confirm sorting efficiency, we determined RNA levels of *Cd169* in the sorted fraction. When compared with the whole spleen we detected significant increase CD169 RNA in the sorted fraction (Figure 1B). Sequencing of RNA isolated from CD169$^+$ cells, which were harvested from infected and uninfected splenocytes, detected 1,070 significantly regulated genes (Figure 1C; false discovery rate (FDR) $\leq 0.05$; log fold change (logFC) $\geq 1.5$). Interestingly, genes encoding for IFN-I were highly upregulated indicating that CD169$^+$ cells contribute to IFN-I production during viral infection (Figure 1D). These findings were verified by Reverse transcription polymerase chain reaction (RT-PCR) analysis of samples containing isolated CD169$^+$ cells from spleen tissue before and after infection with VSV (Figure 1E). To further confirm IFN-I signature, we stained spleen tissue sections for IFN alpha, and we could detect IFN alpha production co-localized with CD169$^+$ cells along with CD11c$^+$ dendritic cells (Figure 1F). Previous findings show that CD169$^+$ cells contribute to innate immune activation not only by allowing viral replication but also by producing IFN-I in mice (111, 346). Furthermore, when we depleted CD169 diphtheria toxin receptor (CD169-DTR) cells by administering diphtheria toxin (347), we observed a reduction in IFN-I concentrations in the serum of infected animals (Figure 1G).

These and previous findings show that CD169$^+$ cells contribute to innate immune activation not only by allowing viral replication but also by producing IFN-I in mice (111, 346).

# Figure. 1

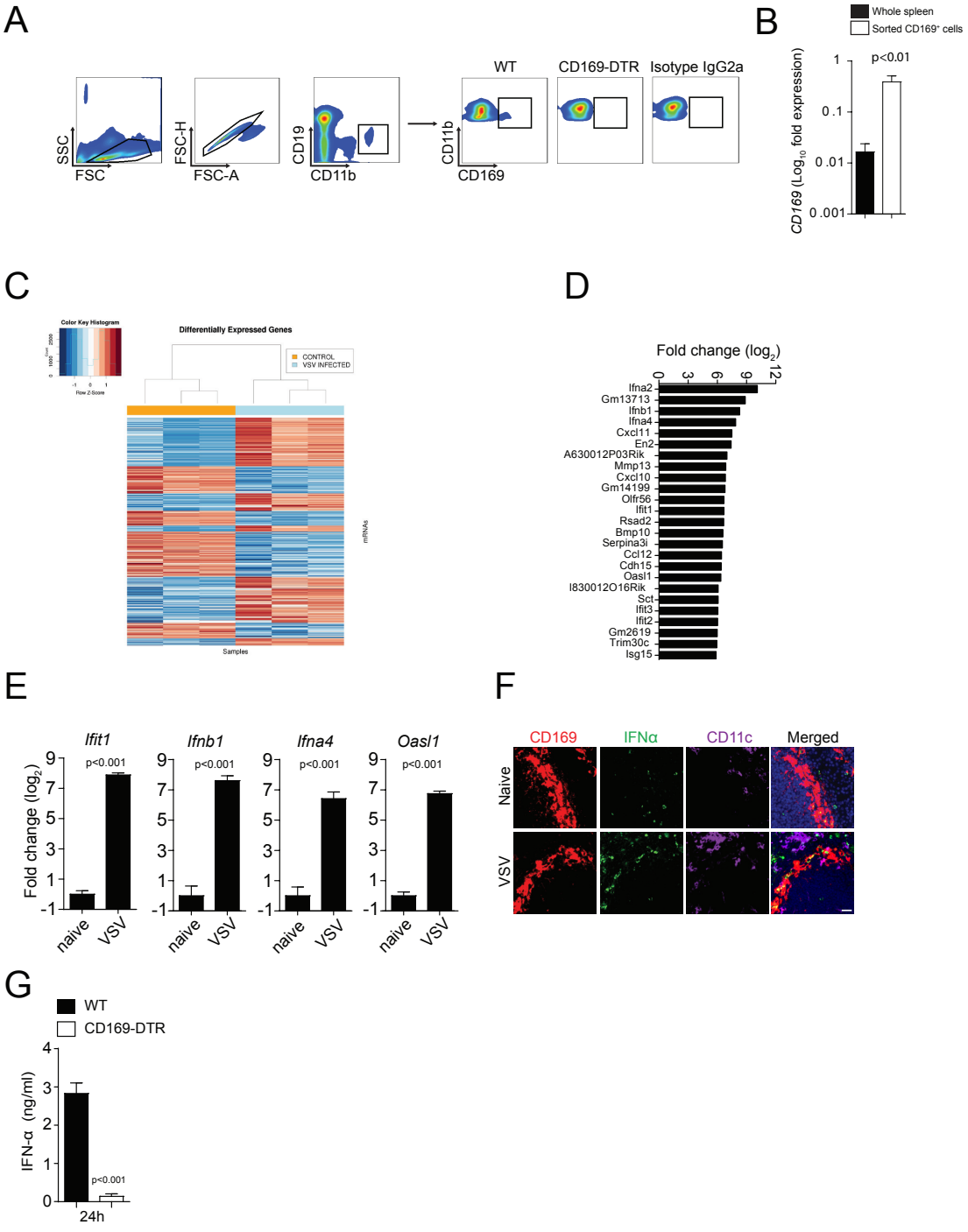

A
SSC
FSC
FSC-H
FSC-A
CD19
CD11b
CD11b
CD169
WT
CD169-DTR
Isotype IgG2a
B
Whole spleen
Sorted CD169+ cells
p<0.01
CD169 (Log10 fold expression)
1
0.1
0.01
0.001
C
Color Key Histogram
Differentially Expressed Genes
CONTROL
VSV INFECTED
Row Z-Score
mRNAs
Samples
D
Fold change (log2)
0
3
6
9
12
Ifna2
Gm13713
Ifnb1
Ifna4
Cxcl11
En2
A630012P03Rik
Mmp13
Cxcl10
Gm14199
Olfr56
Ifit1
Rsad2
Bmp10
Serpina3i
Ccl12
Cdh15
Oasl1
I830012O16Rik
Sct
Ifit3
Ifit2
Gm2619
Trim30c
Isg15
E
Ifit1
Ifnb1
Ifna4
Oasl1
p<0.001
Fold change (log2)
naive
VSV
F
CD169
IFNα
CD11c
Merged
Naive
VSV
G
WT
CD169-DTR
IFN-α (ng/ml)
p<0.001
24h

**Figure 1. Systemic infection with VSV induces IFN-I signature in CD169$^+$ cells.**

***(A)*** *CD169$^+$ cells were extracted from single-cell suspended, B-cell depleted splenocytes. Rat immunoglobin G 2a (IgG2a) served as an isotype control; diphtheria toxin-treated CD169–diphtheria toxin receptor (DTR) animals served as a negative control.* ***(B)*** *Cd169 mRNA expression was determined in sorted CD169$^+$ cells and was compared to whole-spleen RNA with RT-PCR (n=5).* ***(C and D)*** *RNA was extracted from sorted CD169$^+$ cells before and after infection with 2 × 10$^8$ plaque-forming units (PFU) vesicular stomatitis virus (VSV) for 4 h, followed by RNA sequencing. Gene expression patterns are illustrated and show significantly different gene expression levels (false discovery rate [FDR] ≤ 0.05; log fold change [logFC] n=3). The top 25 increased gene expression levels of CD169$^+$ cells after infection with VSV are shown relative to naïve CD169$^+$ cells (n=3).* ***(E)*** *Ifit1, Ifnb1, Ifna4, and Oasl1 expression levels in naïve and sorted CD169$^+$ cells were confirmed by reverse transcription polymerase chain reaction (RT-PCR) (n=5).* ***(F)*** *Wild-type (WT) mice were infected with 2 × 10$^8$ PFU VSV. Snap-frozen spleen sections were stained with anti–IFN-α antibodies 6 h after infection (n= 6; scale bar= 20 μm).*

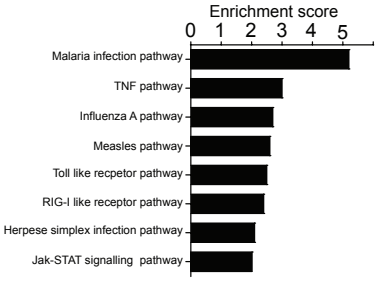
## 4.2 Tumor necrosis factor alpha (TNF) drives major pathways upon VSV infection

Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway analysis of the differentially regulated genes showed that the TNF pathway in $CD169^+$ cells was significantly regulated after infection with VSV (Figure 2A+B). We also found that TNF expression levels were consistently higher in the spleen after infection with VSV (Figure 2C). To further analyze which cell types are producing TNF we performed intracellular cytokine staining (ICS) for TNF. Back-gating of $TNF^+$ cells showed that TNF-producing cells are a heterogeneous $CD11b^+$ population, partially expressing CD11c (Figure 2D+E). Therefore, TNF was probably not expressed by B or T cells, a finding consistent with still increased TNF RNA expression levels in *Cd8*$^{-/-}$, B cell-deficient *Jh*$^{-/-}$, and *Rag1*$^{-/-}$ mice (Figure 3A). However, clodronate depletion reduced TNF expression after VSV infection, in contrast to depletion of $CD169^+$ cells and $CD11c^+$ cells or in IFN-I receptor-deficient mice (Figure 3B). We concluded that phagocytes were responsible for TNF production, we further employed flow cytometry staining for detailed analysis for cell populations. TNF producing cells could be predominantly characterized as $CD11b^+CD11c^-Ly6C^+Ly6G^+MHCII^-$ (Figure 3C). Consistent with reports that neutrophils (348, 349) and $CD11b^+Ly6C^+Ly6G^+$ cells (350) are important during early defense against bacterial and viral infections via production of proinflammatory cytokines such as IL1β, IL6, TNF, and IFN-I, we found a significant increase of $TNF^+CD11b^+Ly6C^+Ly6G^+$ cells (Figure 3D).

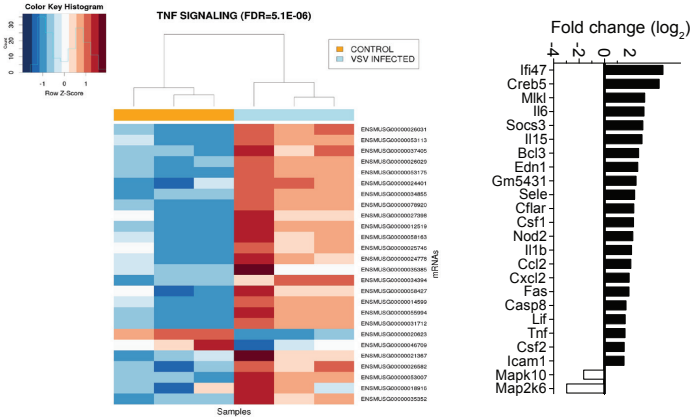
Taken together, these findings indicate that $CD169^+$ cells produce IFN-I after infection with VSV and that TNF is derived from $CD11b^+CD11c^-Ly6C^+Ly6G^+$ phagocytes.

**Figure. 2**

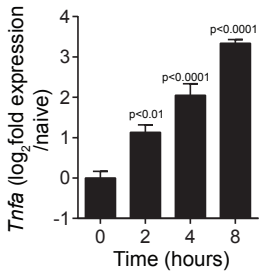
A



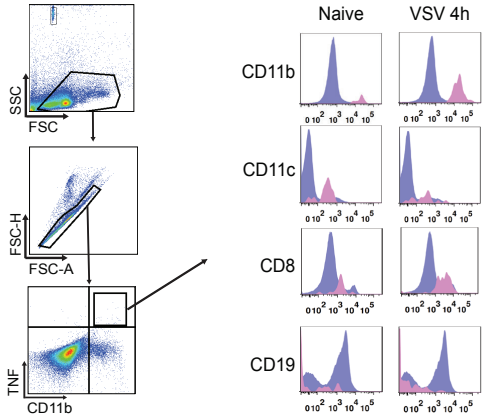

B




C




D




E

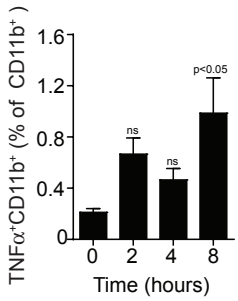


**Figure 2. KEGG pathway analysis reveals induction of TNF pathway after VSV infection.**

***(A)*** *Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis shows significantly overrepresented signaling pathways in infected CD169$^{+}$ cells compared to naïve cells.* ***(B)*** *Heat map (left panel) and expression levels (right panels) of tumor necrosis factor (TNF)-regulated genes are shown.* ***(C)*** *Tumor necrosis factor (TNF)-α mRNA expression levels in WT spleen tissue were determined at the indicated time points after infection (n=6).* ***(D)*** *Surface molecule expression of CD11b, CD11c, CD8, and CD19 on TNF$^{+}$ cells is shown 4h after infection (purple gate, whole spleen; pink gate, TNF$^{+}$ cells) (one result representative of 5 experiments is shown).* ***(E)*** *Intracellular TNF production by CD11b$^{+}$ cells from splenocytes of WT mice was determined at indicated time points (n=5). Figure 2C, D and E were taken from Shinde et al., J Virol. 2018 Jan 17;92(3).*

# Figure. 3

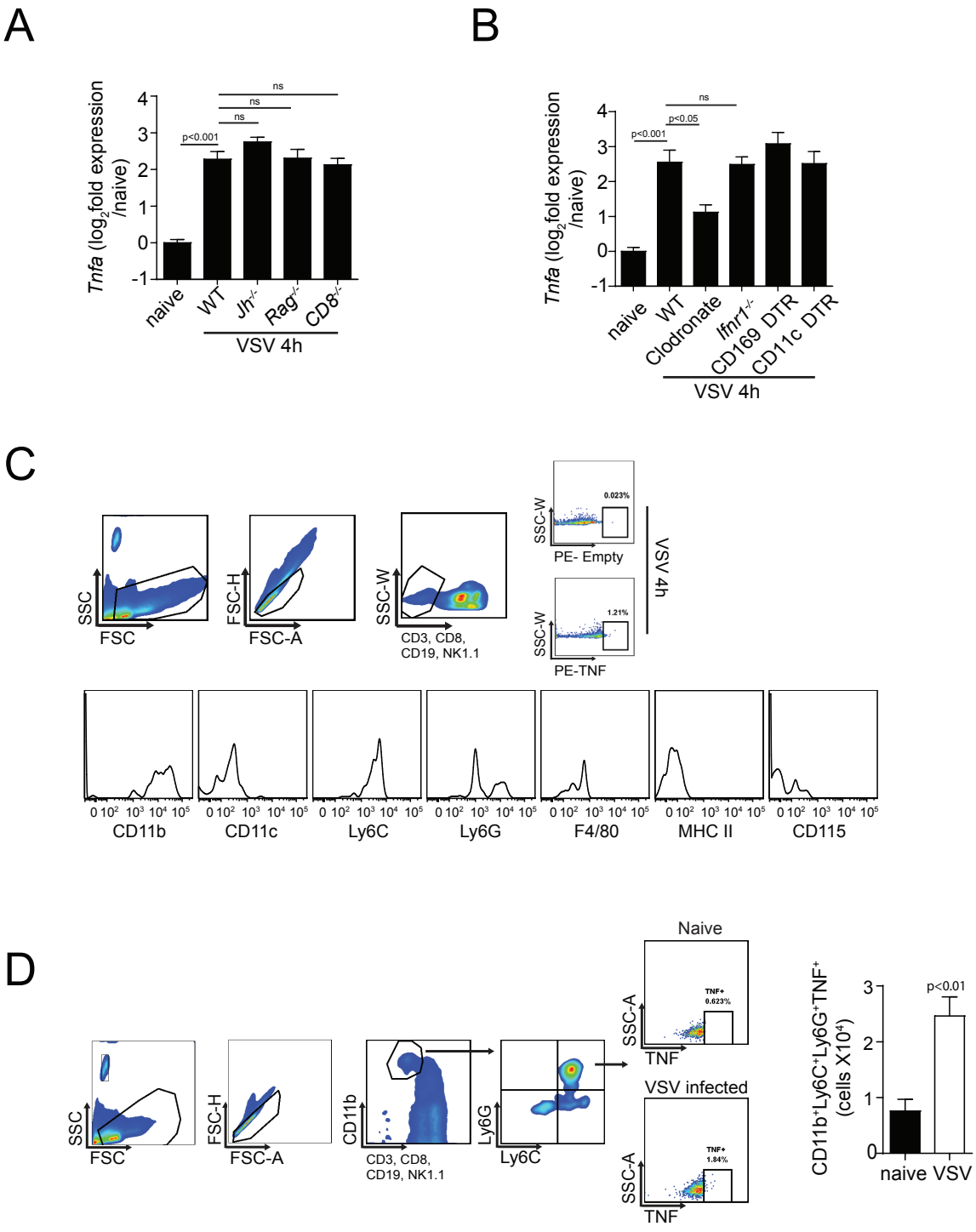

A
B
C
D
Tnfa (log2 fold expression /naive)
p<0.001
ns
naive
WT
Jh-/-
Rag-/-
CD8-/-
VSV 4h
p<0.05
Clodronate
Ifnr1-/-
CD169 DTR
CD11c DTR
SSC
FSC
FSC-H
FSC-A
SSC-W
CD3, CD8, CD19, NK1.1
PE- Empty
PE-TNF
0.023%
1.21%
CD11b
CD11c
Ly6C
Ly6G
F4/80
MHC II
CD115
Naive
VSV infected
TNF
SSC-A
TNF+ 0.623%
TNF+ 1.84%
CD11b+Ly6C+Ly6G+TNF+ (cells X10⁴)
naive VSV

**Figure 3. Infiltrating granulocytes in the spleen after VSV infection drives induction of TNF pathway.**

***(A)*** *TNF-α mRNA expression was determined in the spleen of WT Jh$^{-/-}$, Rag$^{-/-}$, and CD8$^{-/-}$ mice 4 h after infection (n=5-6).* ***(B)*** *TNF-α mRNA expression was determined in the spleen of WT, clodronate-treated WT, Ifnar$^{-/-}$, DT-treated CD169-DTR, and CD11c-DTR mice 4 h after infection (n=6). Data are shown as mean ± SEM.* ***(C)*** *Surface molecule expression of TNF producing cells is shown 4h after infection. Lineage-negative population (CD3$^{-}$CD8$^{-}$CD19$^{-}$NK1.1$^{-}$) was further characterized for expression of CD11b, CD11c, Ly6C, Ly6G, F4/80, MHC II, and CD115 on TNF$^{+}$ cells (n=6).* ***(D)*** *CD11b$^{+}$Ly6C$^{+}$ Ly6G$^{+}$ cells were gated on lineage negative population (CD3$^{-}$CD8$^{-}$CD19$^{-}$NK1.1$^{-}$) in the spleen were quantified for the expression of TNF 6h after infection (n=6).*

*Figures were taken from Shinde et al., J Virol. 2018 Jan 17;92(3).*

### 4.3 TNF triggers the maintenance of CD169$^+$ cells during viral infection to protect animals against the development of fatal disease

To determine whether TNF affects the outcome after VSV infection, we infected wild-type (WT) and TNF-deficient mice. TNF-deficient mice developed severe VSV infection in comparison to WT mice (Figure 4A). Neutralizing antibody titer was achieved later in TNF-deficient mice than in WT mice after infection with low doses of VSV (Figure 4B). Since IFN-I is critical to overcoming an infection with VSV (60), we measured IFN alpha and IFN beta in the serum of infected animals. IFN alpha production was impaired in TNF-deficient mice when compared to control animals (Fig. 4C). However, IFN beta was undetectable in the serum of infected animals when infected with $10^5$ PFU VSV (Fig. 4D). Although, impaired IFN-I production was found in TNF-deficient mice, after injection of the Toll-like receptor 3 (TLR3) agonist poly I:C, IFN-I production was intact in both WT and TNF-deficient mice (Figure 4E). To determine whether TNF affects CD169$^+$ cells after VSV infection, we infected wild-type (WT) and TNF-deficient mice. Notably, CD169$^+$ cells were slightly reduced in number but were present in spleen tissue of TNF deficient mice before infection (Figure 4F). However, shortly after infection with VSV, the number of CD169$^+$ cells in spleen tissue decreased in TNF-deficient mice (Figure 4F). Furthermore, the expression of VSV glycoprotein (VSV-G), which was found in CD169$^+$ cells of WT mice after VSV infection, was not detected in spleen tissue harvested from TNF-deficient animals (Figure 4G+H).

These findings indicate that TNF is necessary to sustain virus replication but is dispensable for sterile innate immunity.

# Figure. 4

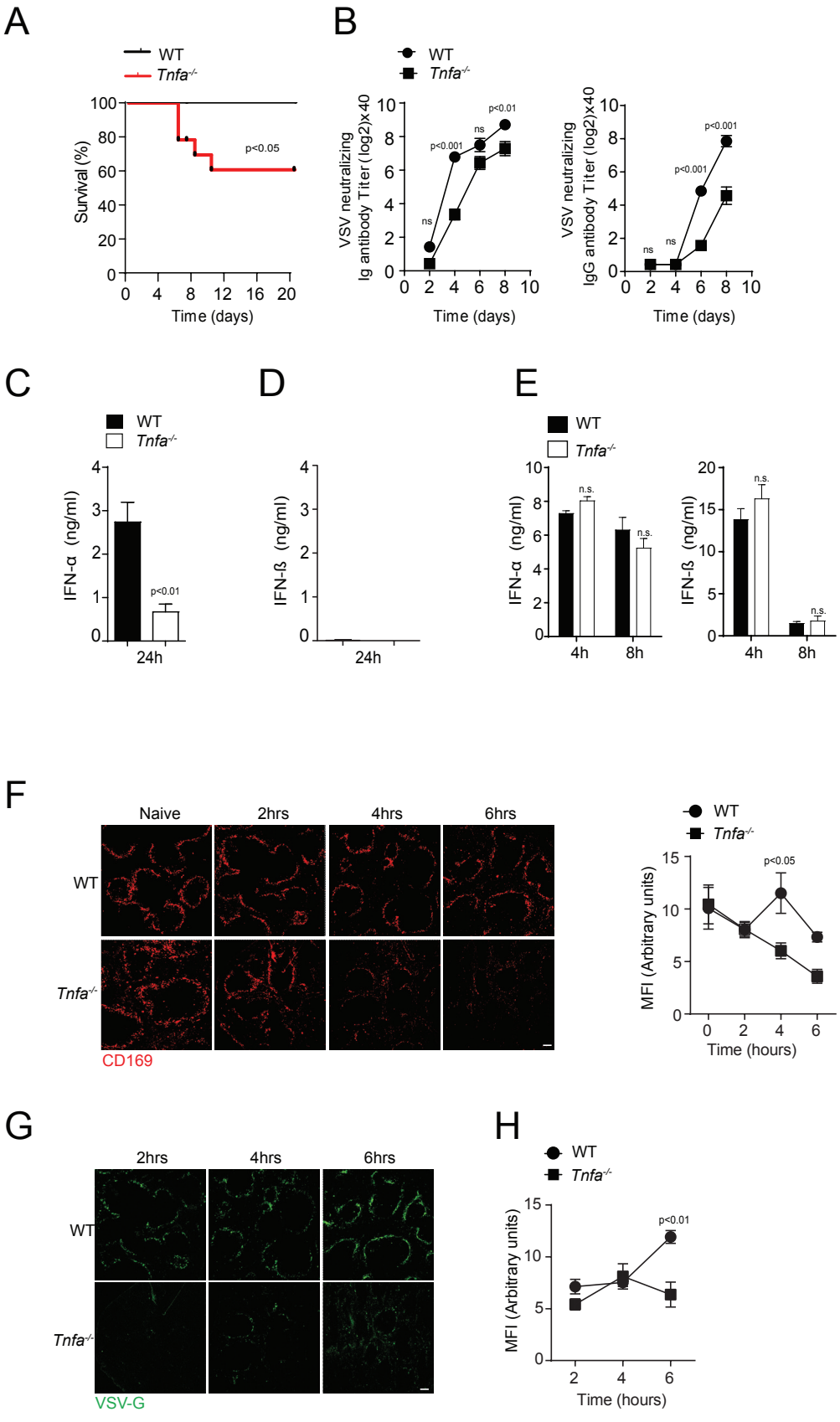

A
WT
Tnfa-/-
Survival (%)
p<0.05
Time (days)
B
WT
Tnfa-/-
VSV neutralizing Ig antibody Titer (log2)x40
ns
p<0.001
p<0.01
Time (days)
VSV neutralizing IgG antibody Titer (log2)x40
p<0.001
Time (days)
C
IFN-α (ng/ml)
p<0.01
24h
D
IFN-ß (ng/ml)
24h
E
IFN-α (ng/ml)
n.s.
4h
8h
IFN-ß (ng/ml)
F
Naive
2hrs
4hrs
6hrs
WT
Tnfa-/-
CD169
MFI (Arbitrary units)
p<0.05
Time (hours)
G
2hrs
4hrs
6hrs
VSV-G
H
p<0.01

**Figure 4. TNF deficiency leads to increased disease severity after VSV infection in mice.**

*(A to D) Mice were infected with $10^5$ PFU VSV.* ***(A)*** *Survival of Wild-type (WT) and tumor necrosis factor-α null ($Tnfa^{-/-}$) mice was monitored for 20 days after infection (n=9-12).* ***(B)*** *Titers of neutralizing total immunoglobulin (Ig; left) and IgG (right) antibody were determined in WT and $Tnfa^{-/-}$ mice at indicated time points after infection (n=7).* ***(C)*** *Interferon (IFN)-α and* ***(D)*** *IFN-β concentrations were determined in the sera of WT and $Tnfa^{-/-}$ mice 24 h after infection (n=6-9).* ***(E)*** *IFN-α and β concentration were determined in the sera of WT and $Tnfa^{-/-}$ mice injected with 200μg of polyinosinic:polycytidylic acid (polyI:C) at indicated time points (n=3).* ***(F)*** *WT and $Tnfa^{-/-}$ mice were infected with $2 \times 10^8$ plaque-forming units (PFU) of VSV. Snap-frozen spleen sections were stained with anti-CD169 antibodies (clone: MOMA-1) at indicated time points (one result representative of 6 mice is shown; scale bar = 100 μm). Mean fluorescence intensity (MFI) of CD169 was quantified across spleen section form naïve and VSV infected WT and $Tnfa^{-/-}$ mice using ImageJ (n=3-4).* ***(G)*** *Snap-frozen spleen sections from WT and $Tnfa^{-/-}$ mice were stained for VSV glycoprotein (VSV-G) expression (clone: Vi10) after infection with $2 \times 10^8$ PFU VSV at indicated time points (one results representative of 6 mice is shown; scale bar = 100μm).* ***(H)*** *MFI of VSV-G expression was quantified across spleen section form naïve and VSV infected WT and $Tnfa^{-/-}$ mice using ImageJ (n=3-4). Figures were taken from Shinde et al., J Virol. 2018 Jan 17;92(3).*

### 4.4 TNF plays important role in virus production

VSV propagation in $CD169^{+}$ cells promote B-cell activation and production of neutralizing antibodies (110). To determine the actual role of TNF in virus production which ultimately leads to a better immune response, we measured VSV titers 8h after infection. VSV titers were lower in *Tnfa*$^{-/-}$ mice than in control mice, a condition that negatively affects antiviral immune activation (Figure 5A). We wondered if ultraviolet light inactivated VSV (UV-VSV) could induce TNF production in the spleen? Surprisingly we found that UV-VSV was able to induce TNF expression but failed to reduce $CD169^{+}$ cells in *Tnfa*$^{-/-}$ mice (Figure 5B+ C).

The question arised if the $CD169^{+}$ is down-regulated after infection. Notably, *CD169*$^{-/-}$ mice exhibited VSV-G expression in spleen tissue, a finding indicating that downregulation of the protein CD169 would not cause absence of virus replication (Figure 5D).

Taken together, these findings indicate that the absence of TNF results in defective antiviral innate immune activation after infection with VSV.

# Figure. 5

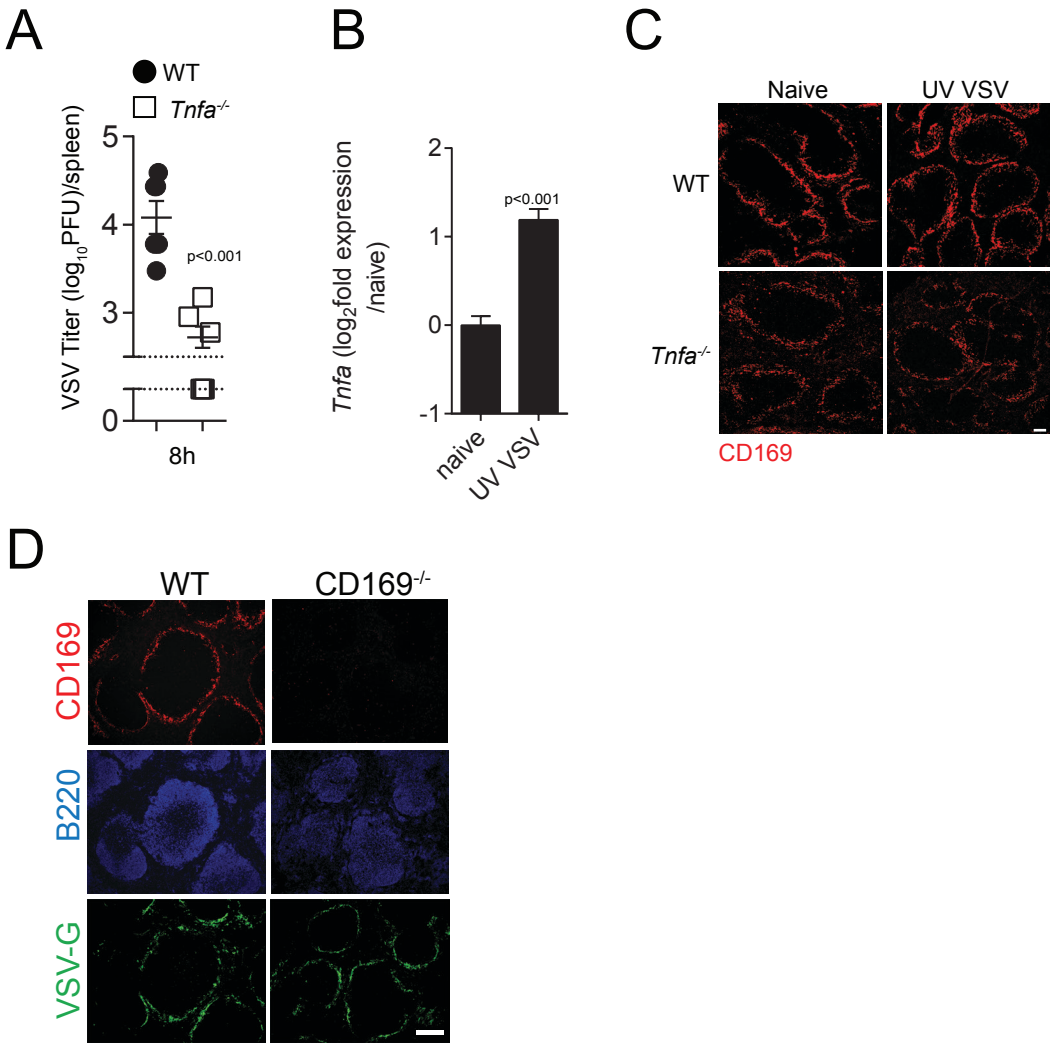



**Figure 5. Surface expression of CD169 is dispensable for VSV infection but TNF is essential to initiate the VSV replication.**

*(A) WT and Tnfa$^{-/-}$ mice were infected with 10$^5$ PFU VSV. Viral titers were measured in the spleen of WT and Tnfa$^{-/-}$ mice 8 h after infection with VSV (n=6).* ***(B)*** *TNF-α mRNA expression was determined in the spleen of WT mice 4 h after injection with UV-inactivated VSV (n=4).* ***(C)*** *Spleen tissue sections were stained with anti-CD169 antibodies in WT and Tnfa$^{-/-}$ mice 8h after infection with 2×10$^8$ PFU of VSV ultraviolet (UV)-inactivated VSV (one result representative of 6 experiments is shown).* ***(D)*** *Snap-*

*frozen spleen tissue sections from WT and CD169$^{-/-}$ mice were stained for CD169, B220, and VSV-G 7h after infection with 2×10$^{8}$ PFU VSV (n=3; scale bar = 100 µm). Figures were taken from Shinde et al., J Virol. 2018 Jan 17;92(3).*

### 4.5 TNFR1 on CD169$^+$ cells is essential for survival after VSV infection

Since VSV replication was dependent on TNF we opted to study further, which receptors are involved in the observed phenotype. First, we assessed whether CD169$^+$ cells express TNFRs. We employed real-time PCR to check for the expression of TNFRs. CD169$^+$ cells exhibited expression of mRNA from TNF receptor superfamily member TNFR1 (*Tnfrsf1a*) and TNFR2 (*Tnfrsf1b*) (Figure 6A). To further characterize the role of TNF during viral infection, we infected TNFR1 and TNFR2-deficient mice with VSV. To elucidate proinflammatory and antiviral response, we measured various genes involved in the process. We observed that there was no gross difference between different genes characterized (Figure 6B). However, in line with findings from TNF-deficient animals, the absence of TNFR1 but not that of TNFR2 resulted in a decrease in the number of CD169$^+$ cells in spleen tissue (Figure 6C). Furthermore, VSV-G production was lower in *Tnfrsf1a*$^{-/-}$ animals than in WT or *Tnfrsf1b*$^{-/-}$ mice (Figure 6D). Consistently, VSV titers were reduced in spleen tissue shortly after infection in *Tnfrsf1a*$^{-/-}$ animals, in sharp contrast to the findings in WT and *Tnfrsf1b*$^{-/-}$ mice (Figure 6E). Interestingly, IFN-I production was also defective in *Tnfrsf1a*$^{-/-}$ mice but was also lower in *Tnfrsf1b*$^{-/-}$ animals than in WT control mice (Figure 6F). These findings were also observed after intraperitoneal infection of TNFR1-deficient mice and control mice (Figure 6F). Taken together, these findings suggest that TNFR1 contributes to antiviral defense by promoting CD169$^+$ cell survival.

# Figure. 6

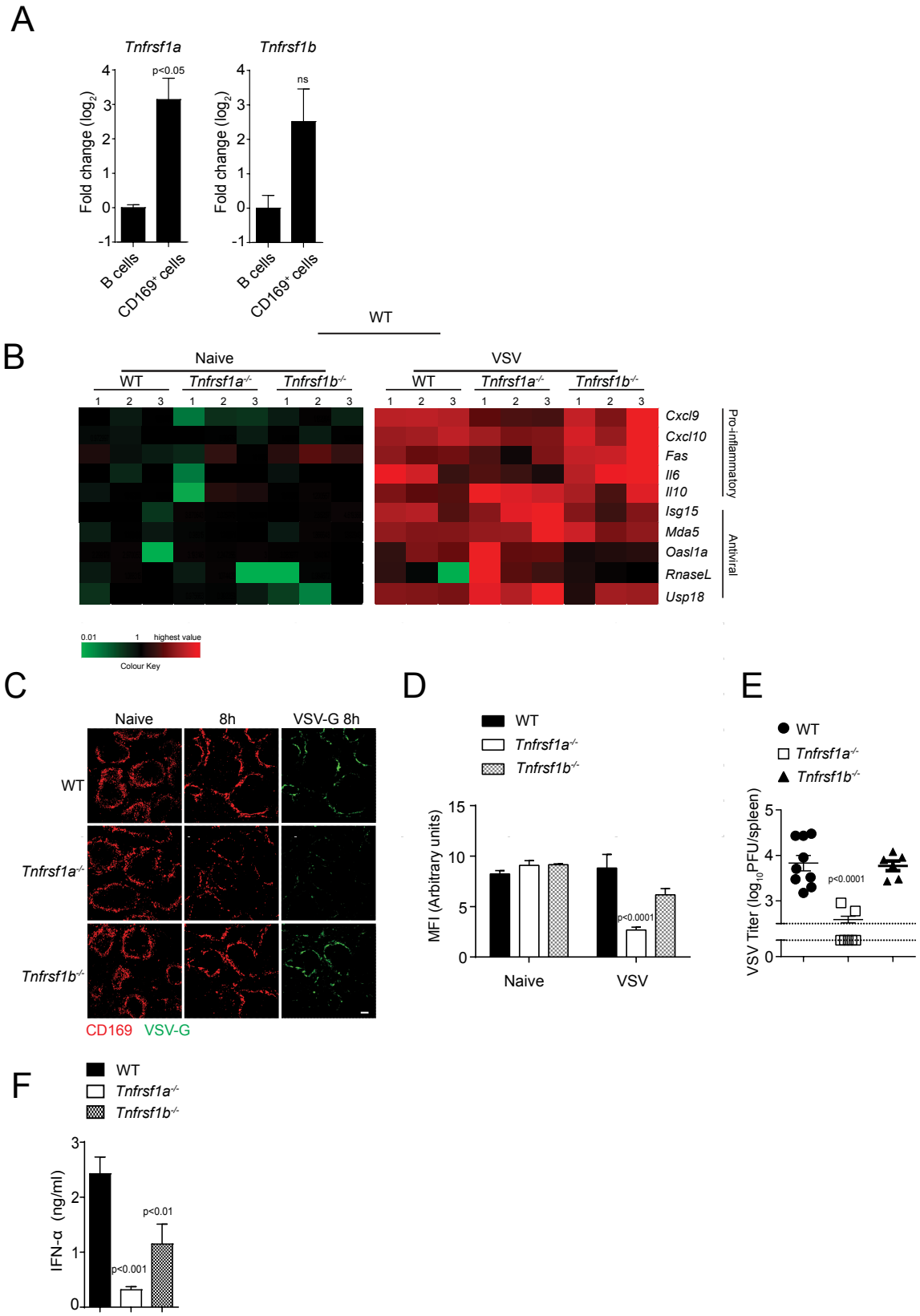

A
Tnfrsf1a
Tnfrsf1b
Fold change (log2)
p<0.05
ns
B cells
CD169+ cells
WT
B
Naive
VSV
WT
Tnfrsf1a-/-
Tnfrsf1b-/-
Cxcl9
Cxcl10
Fas
Il6
Il10
Isg15
Mda5
Oasl1a
RnaseL
Usp18
Pro-inflammatory
Antiviral
0.01
1
highest value
Colour Key
C
Naive
8h
VSV-G 8h
CD169
VSV-G
D
MFI (Arbitrary units)
p<0.0001
Naive
VSV
E
VSV Titer (log10PFU/spleen)
p<0.0001
F
IFN-α (ng/ml)
p<0.001
p<0.01

**Figure 6: TNF acts via TNFR1 to support virus replication in CD169$^{+}$ cells and contributes to induction of innate immunity.**

***(A)*** *Expression of tumor necrosis factor receptor superfamily 1a (Tnfrsf1a) and Tnfrsf1b mRNA was measured in the sorted CD11b$^{+}$CD169$^{+}$ cell population and compared to expression in sorted wild-type (WT) splenic B cells (n=3-5).* ***(B)*** *Wild-type (WT), Tnfrsf1a$^{-/-}$ (tumor necrosis factor receptor 1 [TNFR1]), and Tnfrsf1b$^{-/-}$ (TNFR2) mice were infected with $10^{5}$ PFU VSV. Spleen tissue was harvested; mRNA expression levels of indicated genes were determined 4h after infection (n=3)* ***(C)*** *Spleen tissue sections from wild-type (WT), Tnfrsf1a$^{-/-}$ (tumor necrosis factor receptor 1 [TNFR1]), and Tnfrsf1b$^{-/-}$ (TNFR2) mice were stained with anti-CD169 and VSV-G antibodies 8 h after infection with 2 × $10^{8}$ PFU of VSV (One representative result of n=6 mice is shown; scale bar = 100µm).* ***(D)*** *MFI of CD169 was quantified across spleen sections from WT, Tnfrsf1a$^{-/-}$, and Tnfrsf1b$^{-/-}$ infected mice, using ImageJ (1-3 images per spleen from 3-4 mice were analyzed). (C-G) WT, Tnfrsf1a$^{-/-}$, and Tnfrsf1b$^{-/-}$ mice were infected with $10^{5}$ PFU VSV.* ***(E)*** *Viral titers were measured in spleen tissue 8h after infection in WT, Tnfrsf1a$^{-/-}$, and Tnfrsf1a$^{-/-}$ mice (n=6-9).* ***(F)*** *IFN-α concentration was determined in the sera of WT, Tnfrsf1a$^{-/-}$, and Tnfrsf1a$^{-/-}$ mice 24 h after infection with VSV (n=6-9). Figures 4C, D, E and F were taken from Shinde et al., J Virol. 2018 Jan 17;92(3).*

### 4.6 Essential antiviral response against VSV is mediated via TNFR1 which leads to protection of the host

IFN-I is necessary for the expression of antivirally active IFN-stimulated genes (ISGs) (56). Since there was less IFN-I production, as expected, we found reduced expression of ISGs in the CNS of *Tnfrsf1a*$^{-/-}$ mice after infection with VSV (Figure 7A). Defective ISG expression was not found to the same extent in *Tnfrsf1b*$^{-/-}$ CNS tissue (Figure 7B). Since, VSV can drive neuropathological symptoms by infecting the CNS (106), when we measured the viral titer in the spinal cord and brain tissue of mice exhibiting disease symptoms, we found infectious VSV in tissue from TNFR1-deficient mice (Figure 7C). Consequently, *Tnfrsf1a*$^{-/-}$ mice succumbed to VSV infection, unlike WT and *Tnfrsf1b*$^{-/-}$ mice (Figure 7D). These findings indicate that early IFN-I response is essential for controlling VSV infection which is dependent on TNFR1 and partially on TNFR2.

# Figure. 7

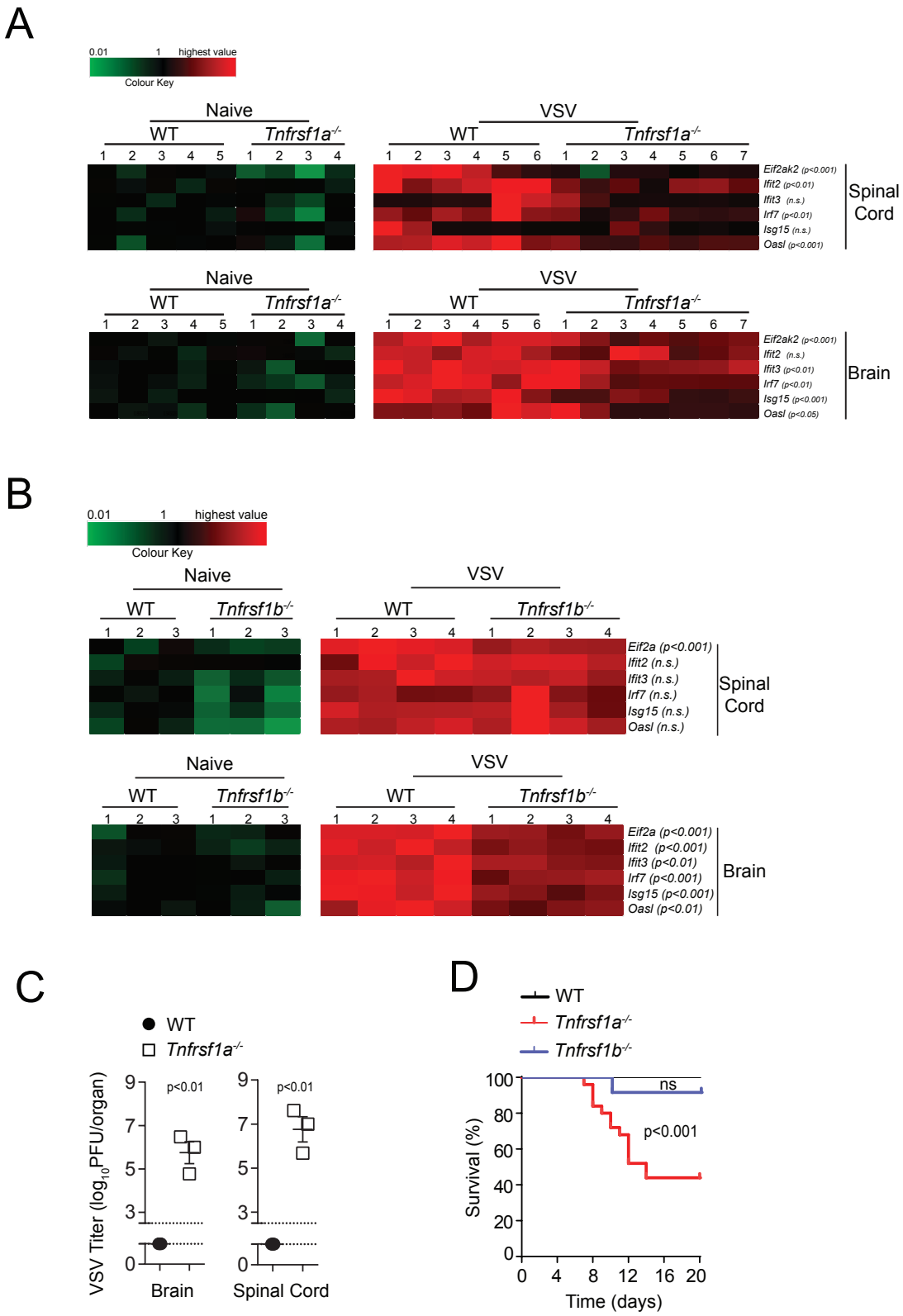

A
0.01
1
highest value
Colour Key
Naive
VSV
WT
Tnfrsf1a-/-
Eif2ak2 (p<0.001)
Ifit2 (p<0.01)
Ifit3 (n.s.)
Irf7 (p<0.01)
Isg15 (n.s.)
Oasl (p<0.001)
Spinal Cord
Eif2ak2 (p<0.001)
Ifit2 (n.s.)
Ifit3 (p<0.01)
Irf7 (p<0.01)
Isg15 (p<0.001)
Oasl (p<0.05)
Brain
B
0.01
1
highest value
Colour Key
Naive
VSV
WT
Tnfrsf1b-/-
Eif2a (p<0.001)
Ifit2 (n.s.)
Ifit3 (n.s.)
Irf7 (n.s.)
Isg15 (n.s.)
Oasl (n.s.)
Spinal Cord
Eif2a (p<0.001)
Ifit2 (p<0.001)
Ifit3 (p<0.01)
Irf7 (p<0.001)
Isg15 (p<0.001)
Oasl (p<0.01)
Brain
C
WT
Tnfrsf1a-/-
VSV Titer (log10PFU/organ)
p<0.01
p<0.01
Brain
Spinal Cord
D
WT
Tnfrsf1a-/-
Tnfrsf1b-/-
Survival (%)
ns
p<0.001
Time (days)

**Figure 7: Ample activation of antiviral immune response against VSV results in protection of host via TNFR1 signaling.**

*(A) WT and Tnfrsf1a$^{-/-}$ mice were infected with 10$^5$ PFU VSV. RNA expression levels of indicated genes were determined in brain and spinal cord 24 h after infection (n=4-7, highest relative expression values brain/spinal cord: Eif2ak2, 13.72/7.98; Ifit2, 5.41/6.13; Ifit3, 35.99/34.15; Irf7, 68.80/54.55; Isg15, 42.54/51.23; Oasl1, 70.43/84.94).* ***(B)*** *WT and Tnfrsf1b$^{-/-}$ mice were infected with 10$^5$ PFU VSV. RNA expression levels of indicated genes were determined in brain and spinal cord 24h after infection (n=3-4, highest relative expression values brain/spinal cord: Eif2ak, 29.84/18.21; Ifit2, 7.99/10.24; Ifit3, 41.05/51.25; Irf7, 166.79/88.58; Isg15, 29.78/52.99; Oasl1, 75.60/114.39).* ***(C)*** *Viral titers were measured in brain and spinal cord tissue of WT and Tnfrsf1a$^{-/-}$ mice, once Tnfrsf1a$^{-/-}$ mice exhibited hind limb paralysis (n=3).* ***(D)*** *Survival of WT, Tnfrsf1a$^{-/-}$, and Tnfrsf1b$^{-/-}$ mice was monitored over time after infection with VSV (n=15-24). Figures were taken from Shinde et al., J Virol. 2018 Jan 17;92(3).*

### 4.7 TNFR1 triggers the survival of CD169$^+$ cells

Even though TNFR1 deficient mice were unable to mount sufficient immune response against VSV, we wanted to confirm if there were any other defects in the transgenic mice which could interfere with immunity. Hence, we opted to characterize the knockout mice in a naïve and an infected state. B cell-mediated Ltβ production is important for splenic CD169$^+$ cells. Hence, we wondered whether defects in TNF signaling of B cells might be important for the observed defects. Notably, we did not observe any major phenotypes on B-cell subsets in TNF, TNFR1 or TNFR2-deficient mice (Figure 8A). Consistently, we did not see a differential expression of *Lt*α, *Lt*β, or Ltβ receptor (*LtbR*) in TNFR1-deficient mice (Figure 8B). Additionally, we found no important differences in B-cell subsets between WT and TNFR1-deficient mice after infection (Figure 8C). Furthermore, we reconstituted lethally irradiated C57BL/6 mice with mixed bone marrow from *Rag1*$^{-/-}$ and *Tnfrsf1a*$^{-/-}$ donors at a ratio of 1:1. Compared to their corresponding control mice, mice reconstituted with *Rag1*$^{-/-}$: *Tnfrsf1a*$^{-/-}$ bone marrow exhibited no significant reduction in IFN-α in the serum (Figure 8D). Furthermore, there was no difference between these mice in neutralizing antibody production (Figure 8E). In contrast, when we reconstituted lethally irradiated C57BL/6 mice with mixed bone marrow from CD169-DTR$^+$ and *Tnfrsf1a*$^{-/-}$ donors at a ratio of 1:1, we found that the production of IFN-α was lower in these mice than in the corresponding WT control reconstituted with CD169-DTR$^+$ and WT bone marrow (Figure 8F). Furthermore, we found slight, but significant differences in VSV neutralizing antibody titers in CD169-DTR: *Tnfrsf1a*$^{-/-}$ recipients when compared to corresponding DTR: WT recipient controls (Figure 8G). Furthermore, CD169$^+$ cells can be depleted with CD11c-DTR mice, because they exhibit an intermediate expression of CD11c (111, 351). Consistently, lethally irradiated mice reconstituted with mixed bone marrow from CD11c-DTR$^+$ and *Tnfrsf1a*$^{-/-}$ mice exhibited reduced concentrations of IFN-α after VSV infection (Figure 8H). These findings suggest that TNFR1 triggers cell-intrinsic effects on CD169$^+$ cells.

# Figure. 8

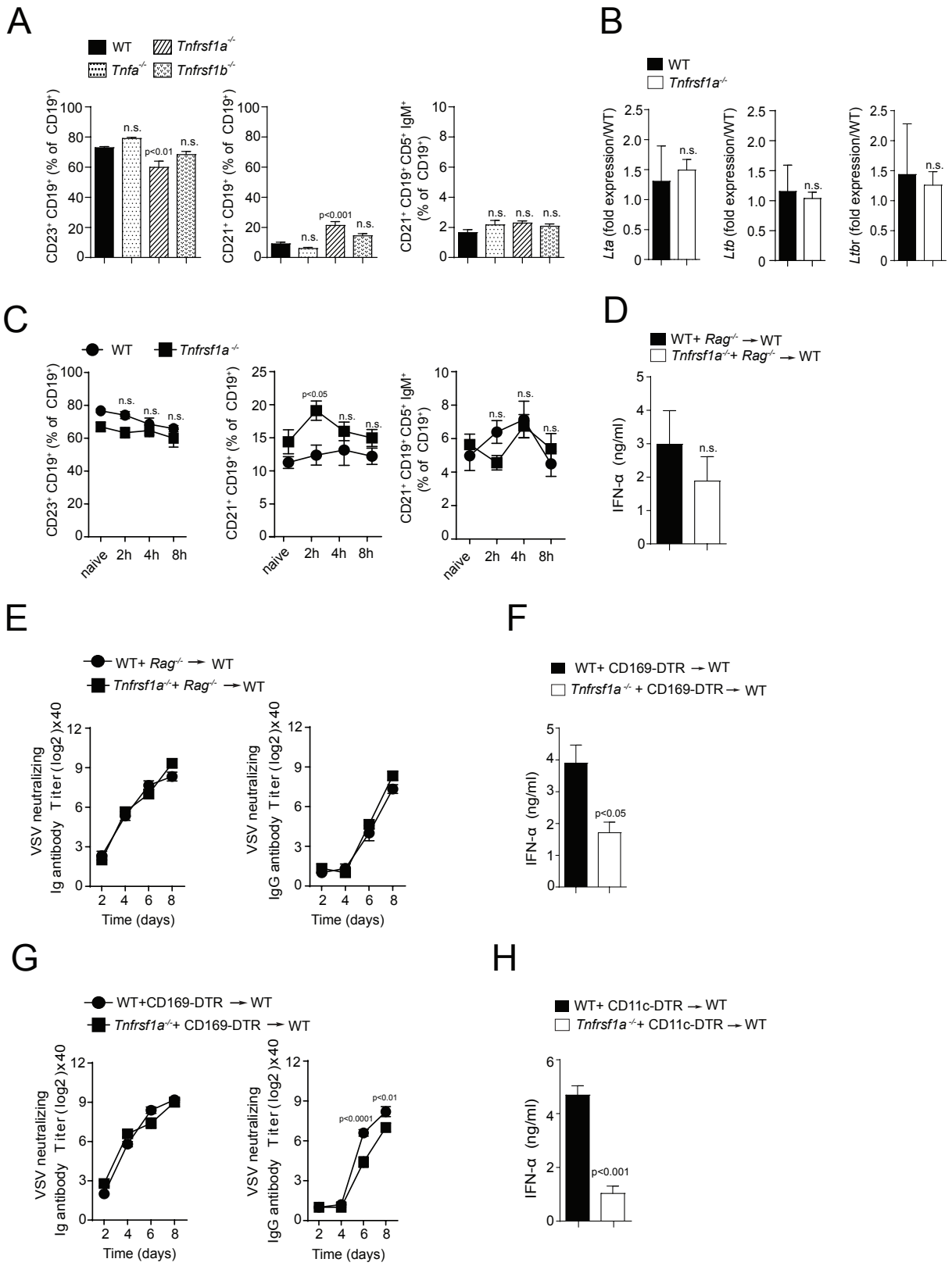

A
WT
Tnfrsf1a-/-
Tnfa-/-
Tnfrsf1b-/-
CD23+ CD19+ (% of CD19+)
CD21+ CD19+ (% of CD19+)
CD21+ CD19+ CD5+ IgM+ (% of CD19+)
n.s.
p<0.01
p<0.001
B
WT
Tnfrsf1a-/-
Lta (fold expression/WT)
Ltb (fold expression/WT)
Ltbr (fold expression/WT)
C
WT
Tnfrsf1a-/-
naive
2h
4h
8h
p<0.05
D
WT+ Rag-/- → WT
Tnfrsf1a-/-+ Rag-/- → WT
IFN-α (ng/ml)
E
WT+ Rag-/- → WT
Tnfrsf1a-/-+ Rag-/- → WT
VSV neutralizing Ig antibody Titer (log2)x40
VSV neutralizing IgG antibody Titer (log2)x40
Time (days)
F
WT+ CD169-DTR → WT
Tnfrsf1a-/- + CD169-DTR → WT
IFN-α (ng/ml)
p<0.05
G
WT+CD169-DTR → WT
Tnfrsf1a-/-+ CD169-DTR → WT
p<0.0001
p<0.01
H
WT+ CD11c-DTR → WT
Tnfrsf1a-/-+ CD11c-DTR → WT
p<0.001

**Figure 8: TNFR1 specifically on CD169$^+$ cells is essential for the adequate antiviral immune response.**

***(A)*** *Follicular B cells (CD19$^+$CD23$^+$) (FB), marginal zone B cells (CD19$^+$CD21$^+$ CD23$^-$) (MZB), and regulatory B cells (CD19$^+$CD21$^+$ CD5$^+$IgM$^+$) (RB) were analyzed in naïve WT, Tnfa$^{-/-}$, Tnfrsf1a$^{-/-}$, and Tnfrsf1b$^{-/-}$ deficient mice (n=6).* ***(B)*** *Lymphotoxin α (LTα), LTβ, and lymphotoxin β receptor (LtβR) gene expression were determined in spleen tissue from WT and Tnfrsf1a$^{-/-}$ mice by reverse-transcription polymerase chain reaction (RT-PCR) (n=3).* ***(C)*** *Splenic B-cell populations FB, MZB, and RB were analyzed after infection with 2×10$^8$ PFU of VSV in WT and Tnfrsf1a$^{-/-}$ mice at indicated time points (n=5).* ***(D)*** *IFN-α concentration was determined 24 h after infection with 10$^5$ PFU VSV in the sera of lethally irradiated mice reconstituted with either WT: Rag$^{-/-}$ or Tnfrsf1a$^{-/-}$: Rag$^{-/-}$ bone marrow at a ratio of 1:1 (n=4).* ***(E)*** *Neutralizing total immunoglobulin (Ig; left) and IgG (right) antibody titers were determined in the sera of WT: Rag$^{-/-}$ or Tnfrsf1a$^{-/-}$: Rag$^{-/-}$ reconstituted animals (n=4).* ***(F-H)*** *Lethally irradiated WT mice were reconstituted with bone marrow (BM) from WT or Tnfrsf1a$^{-/-}$ mice mixed with BM from* ***(F)*** *CD169-DTR and* ***(H)*** *CD11c-DTR at a 1:1 ratio. After 40 days, mice were infected with 10$^5$ PFU of VSV. Before the infected mice were treated with 2 doses of 100 ng DT via intraperitoneal injection* ***(F)*** *IFN-α concentration was determined 24h after infection in the sera of WT:CD169-DTR and Tnfrsf1a$^{-/-}$: CD169-DTR reconstituted animals (n=4-5).* ***(G)*** *Neutralizing total immunoglobulin (Ig; left) and IgG (right) antibody titers were determined in the sera of WT:CD169-DTR and Tnfrsf1a$^{-/-}$: CD169-DTR reconstituted animals after infection with 10$^5$ PFU VSV at indicated time points (n=4).* ***(H)*** *IFN-α concentration was determined 24 h after infection in the sera of WT: CD11c-DTR and Tnfrsf1a$^{-/-}$: CD11c-DTR reconstituted mice (n=4-5). Figures were taken from Shinde et al., J Virol. 2018 Jan 17;92(3).*

### 4.8 Absence of TNF signaling leads to increased apoptosis in CD169$^+$ cells after VSV infection

We speculated that TNF delivers an important survival signal for CD169$^+$ cells. To determine if TNF is involved in protection against VSV induced apoptosis, we measured caspase 3 activity on whole spleen tissue lysates. After VSV infection caspase 3 activity was significantly higher in spleen tissue of *Tnfa*$^{-/-}$ mice compared to control animals (Figure 9A). VSV is known to induce apoptosis and inactivates Mcl-1 and Bcl-Xl (352). To elucidate if TNF plays a role in promoting expression of anti-apoptotic genes, we measured mRNA expression of *Bcl2*, *Bcl-Xl* and *xIAP* in spleen tissue of mice after VSV infection. After VSV infection, *Bcl2* and *Bcl-Xl* expression were significantly reduced in *Tnfa*$^{-/-}$ mice compared to WT mice (Figure 9B). To enumerate the mechanism which reduces CD169$^+$ cells in TNF deficient mice after infection we made use of terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay. The number, as well as the mean fluorescence intensity of TUNEL-positive CD169$^+$ cells, was higher in spleen tissue from TNF deficient mice than in tissue from corresponding WT control mice (Figure 9C+D). The proportion of CD169$^+$ cells that stained positive for 7-amino actinomycin D (7-AAD) was higher in TNFR1-deficient mice than in WT control mice 8h after infection (Figure 9E). Next, we wondered if we can rescue the CD169$^+$ cells by injecting the pan-caspase inhibitor Z-Val-Ala-Asp-fluoromethylketone (zVAD-FMK). Z-VAD treatment restored the presence of CD169$^+$ cells in TNF-deficient animals, a finding indicating that CD169$^+$ cells depend on TNF-mediated survival (Figure 10A+B). Although treatment of TNF-deficient mice with Z-VAD rescued CD169$^+$ cells, it failed to rescue the IFN-I response suggesting the role of TNF signaling is not only essential to prevent apoptosis, but also for IFN-I production (Figure 10C).

In summary, these findings indicate that TNF delivers a survival signal that is important for the maintenance of CD169$^+$ cells in the spleen after viral infection.

## Figure. 9

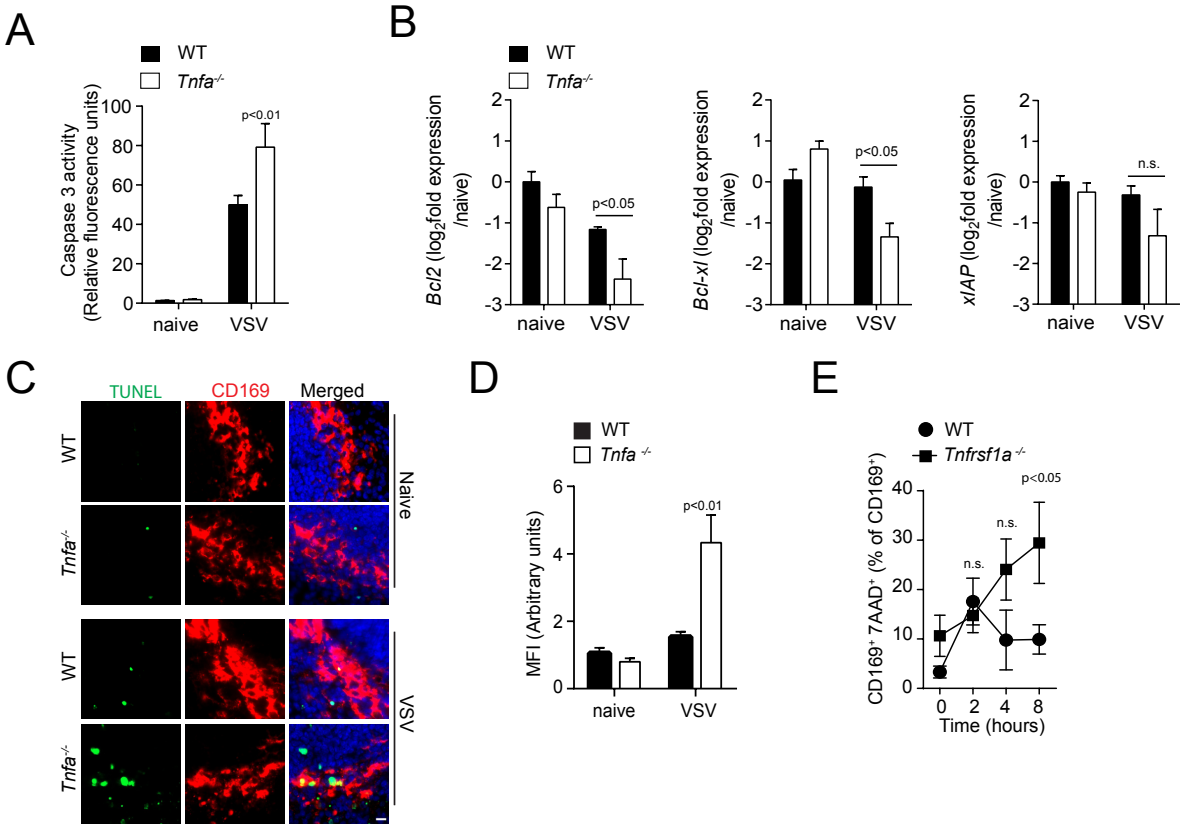



**Figure 9: Activation of TNF-TNFR1 signaling axis protects CD169$^{+}$ cells from VSV induced apoptosis.**

***(A-E)*** *Mice were infected with 2 × 10$^{8}$ PFU VSV.* ***(A)*** *Caspase 3 activity was determined in spleen tissue harvested from WT and Tnfa$^{-/-}$ mice 6h after infection with 2×10$^{8}$ PFU VSV (n=4-7, RFU = relative fluorescence units).* ***(B)*** *Bcl2, Bclxl, Xiap RNA expression was determined in spleen tissue from WT and Tnfrsf1a$^{-/-}$ mice 8h after infection (n=3).* ***(C)*** *Tissue sections from WT and Tnfa$^{-/-}$ mice were stained with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) 5 h after infection (one result representative of 3 or 4 mice is shown; scale bar = 10 µm).* ***(D)*** *Mean fluorescence intensity (MFI) of TUNEL was quantified across spleen sections from naïve and VSV infected WT and Tnfa$^{-/-}$ mice using ImageJ (1-2 images per spleen from 3-4 mice were analyzed).* ***(E)*** *At indicated time points, the proportion of 7 amino*

*actinomycin D–positive (7AAD$^{+}$) cells among CD11b$^{+}$CD169$^{+}$ cells were determined (n=5) in WT and Tnfrsf1a$^{-/-}$ mice. Figures were taken from Shinde et al., J Virol. 2018 Jan 17;92(3).*

## Figure. 10

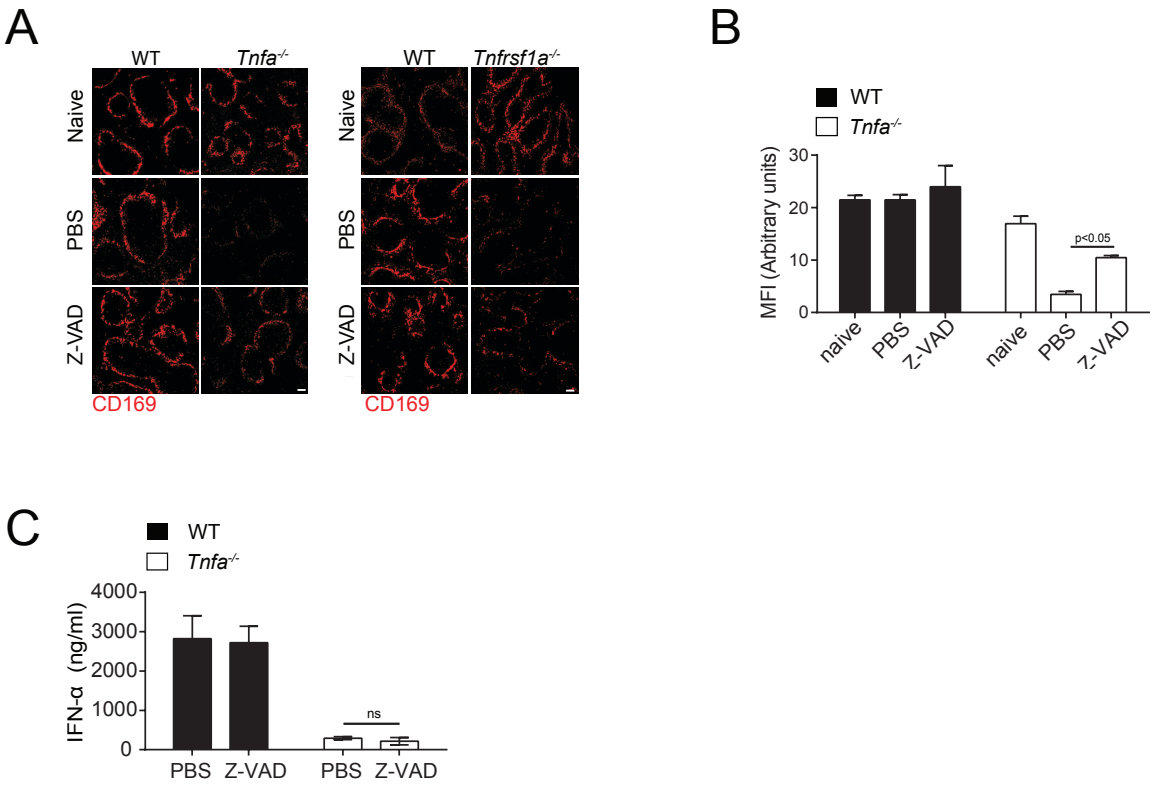



**Figure 10: VSV induced apoptosis can be partially rescued using apoptosis inhibitors.**

***(A)*** *WT, Tnfa$^{-/-}$ and Tnfrsf1a$^{-/-}$ mice were treated with Z-Val-Ala-Asp-fluoromethylketone (zVAD-FMK) and infected with 2 × 10$^{8}$ PFU VSV. Spleen tissue sections were stained with anti-CD169 antibodies 8 h after infection (one result representative of 3-4 mice is shown; scale bar = 100 µm).* ***(B)*** *MFI of CD169 was quantified across spleen sections from naïve and VSV infected WT and Tnfa$^{-/-}$ mice treated with Z-VAD using ImageJ (1-3 images per spleen from 3-4 mice were analyzed).* ***(C)*** *IFN-α concentration was determined 24 h after infection in the sera of Z-VAD treated WT and Tnfa$^{-/-}$ mice after infection with 10$^{5}$ PFU of VSV (n=3). Figures were taken from Shinde et al., J Virol. 2018 Jan 17;92(3).*

### 4.9 Transcription factor RelA associated gene signature upregulates after VSV infection in CD169$^+$ cells

TNF can induce NF-κB activation via TNFR1 and can promote the expression of genes driving survival and of proinflammatory cytokines (284). As expected, genes under the transcriptional control of transcription factors (TF) regulated by IFN-I were significantly overrepresented (Figure 11A). Interestingly, after VSV infection of CD169$^+$ cells, one crucial TF exhibiting the potential to bind approximately 28% of the NF-κB pathway genes was *RelA* (Figure 11A). Hence, we opted to further investigate the role of NF-κB. Using KEGG pathways, we found that *RelA* was involved in multiple pathways regulating infectious diseases (Figure 11B). Consistently, RelA expression was increased in the marginal zone of spleen tissue after VSV infection (Figure 11C). Furthermore, nuclear expression of RelA in CD169$^+$ cells was higher in VSV-infected mice than in naïve control mice (Figure 11D).

**Figure. 11**

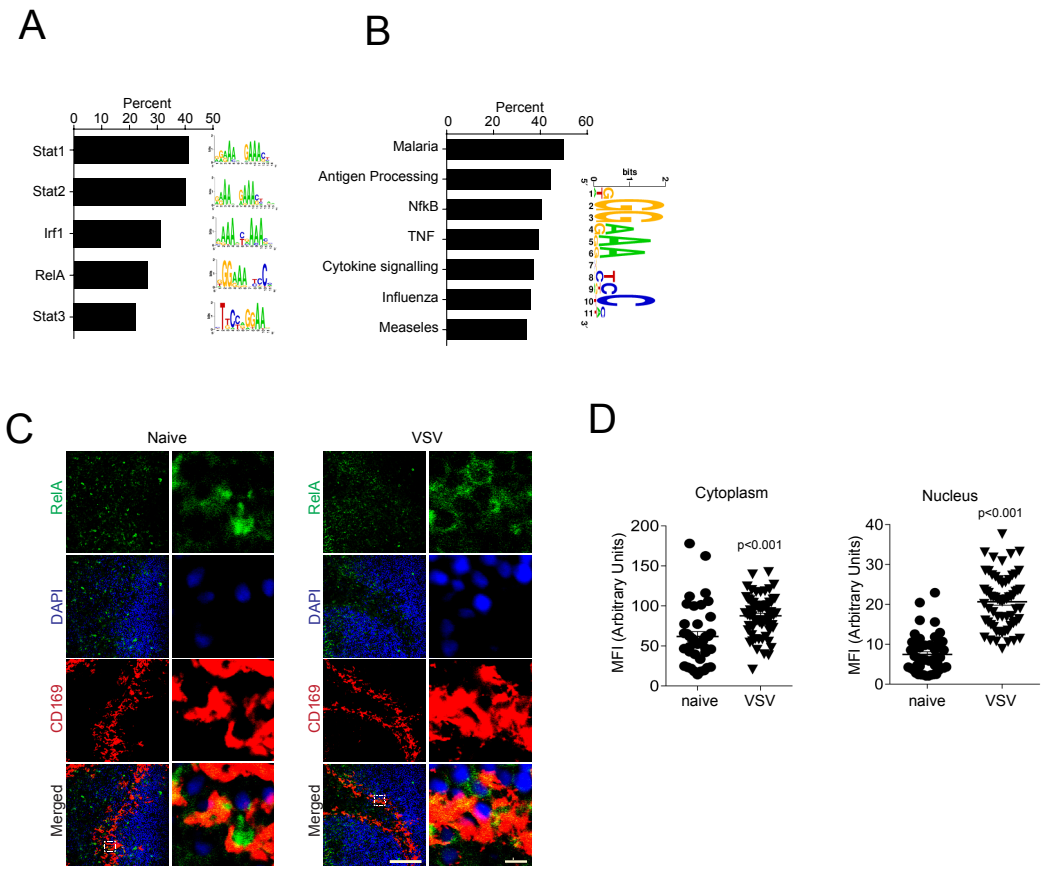



**Figure 11: VSV infection leads to increase in RelA translocation in the nucleus indicating activation of NF-κB in CD169$^{+}$ cells**

***(A)*** *Overrepresentation of transcription factor (TF) binding sites of Type I interferon (IFN-I)-regulated TFs. Percentage of up-regulated gene promoter regions containing significant binding motifs (Fisher's exact test; false discovery rate [FDR] < 0.001).* ***(B)*** *Promoter analysis for the RelA binding motif. The percentage represents promoters of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway genes containing the significant motif (Fisher's exact test; FDR < 0.05). (C-D) Sections of snap-frozen spleen tissue were analyzed after infection with 2 x $10^{8}$ PFU VSV.* ***(C)*** *Sections were stained for RelA before and after infection (one result representative of 3 experiments is shown; scale bar = 100 µm; side panel shows the cropped image; scale bar = 10 µm).* ***(D)*** *Mean fluorescence intensity (MFI) of RelA in the nucleus and*

*the cytoplasm was quantified in CD169$^{+}$ cells from WT mice with ImageJ software (n = 48-63 are shown). Figures were taken from Shinde et al., J Virol. 2018 Jan 17;92(3).*

### 4.10 Nuclear translocation of RelA, a hallmark of signaling via TNFR1 after VSV infection

Next, we wondered whether nuclear *RelA* expression was dependent on TNFR1. As expected, compared with WT control mice, VSV-infected mice exhibited reduced expression of RelA in the nuclear compartment of $CD169^{+}$ cells in absence of TNF (Figure 12A). Notably, the presence of RelA was reduced in TNFR1 deficient mice, but we observed no difference in RelA expression between TNFR2-deficient mice and corresponding control mice (Figure 12C).

**Figure. 12**

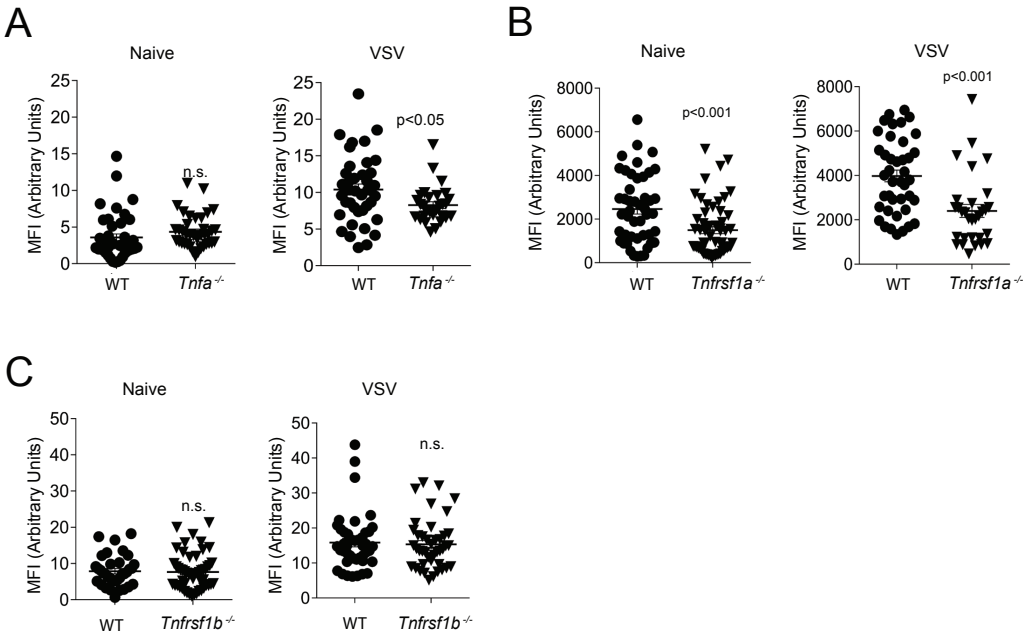



**Figure 12: TNFR1 dependent nuclear RelA translocation hints at the involvement of TNF signaling.**

***(A to C)*** *Sections from snap-frozen spleen tissue harvested from WT and $Tnf^{-/-}$, $Tnfrsf1a^{-/-}$ and $Tnfrsf1b^{-/-}$ mice of naive and infected with 2 x $10^{8}$ PFU VSV were stained with anti-RelA antibodies, and RelA MFI in the nucleus of $CD169^{+}$ cells was determined. Figures were taken from Shinde et al., J Virol. 2018 Jan 17;92(3).*

### 4.11 The NF-κB regulator MALT1 promotes canonical NF-κB expression, VSV replication in CD169$^+$ cells, and immune activation during viral infection

RelA signaling can be reduced by RelB through sequestration of RelA and competitive binding of DNA (353). We used immune histochemistry to determine the presence of RelB in splenic tissue sections. We found that RelB can be detected in the marginal zone of spleen in mice (Figure 13A). Whereas, once CD169$^+$ are depleted in CD169-DTR mice, RelB staining in the marginal zone is decreased significantly (Figure 13A). Since the paracaspase MALT1 is known to promote canonical NF-κB signaling by cleaving RelB (354, 355), ablation of MALT1 resulted in increased levels of RelB in CD169$^+$ cells in the marginal zone of the spleen (Figure 13B). Using immunofluorescence, we quantified the presence of RelB in the cytoplasm and nucleus of MALT deficient mice. As expected, RelB levels were significantly higher in *Malt1*$^{-/-}$ mice compared to control mice (Figure 13C+D).

In turn, nuclear RelA levels were lower in CD169$^+$ cells in *Malt1*$^{-/-}$ spleen tissue than in control tissue (Figure 14A). Consistently, mouse embryonic fibroblasts (MEFs) derived from *Malt1*$^{-/-}$ mice showed reduced translocation of p65 into the nucleus after stimulation with TNF and increased expression of RelB (14B+C). These findings indicate that MALT1 destabilizes RelB in the marginal zone to promote canonical NF-κB signaling. The presence of CD169$^+$ cells in spleen tissue was not affected by *Malt1* before or after infection with VSV (Figure 15A). However, the expression of VSV-G was much lower in *Malt1*$^{-/-}$ mice than in control mice (Figure 15B+C). Consistently, the numbers of infectious VSV particles were lower in spleen tissue harvested from *Malt1*$^{-/-}$ mice than in corresponding tissue from control mice (Figure 15D). Hence, IFN-I serum concentrations after VSV infection were lower in MALT1 deficient mice than in control mice (Figure 15E). This finding was probably dependent on defective VSV replication early during infection because injection with poly I:C did not result in variations in IFN-I levels in the serum (Figure 15F), a finding consistent with previous findings (356). Consequently, although control mice could overcome VSV infection, *Malt1*-deficient mice succumbed to the infection (Figure 15G).

Taken together, these findings indicate that absence of MALT1 results in reduced canonical NF-κB signaling in response to infection. *Malt1*-deficient mice exhibit reduced viral replication and immune activation.

**Figure. 13**

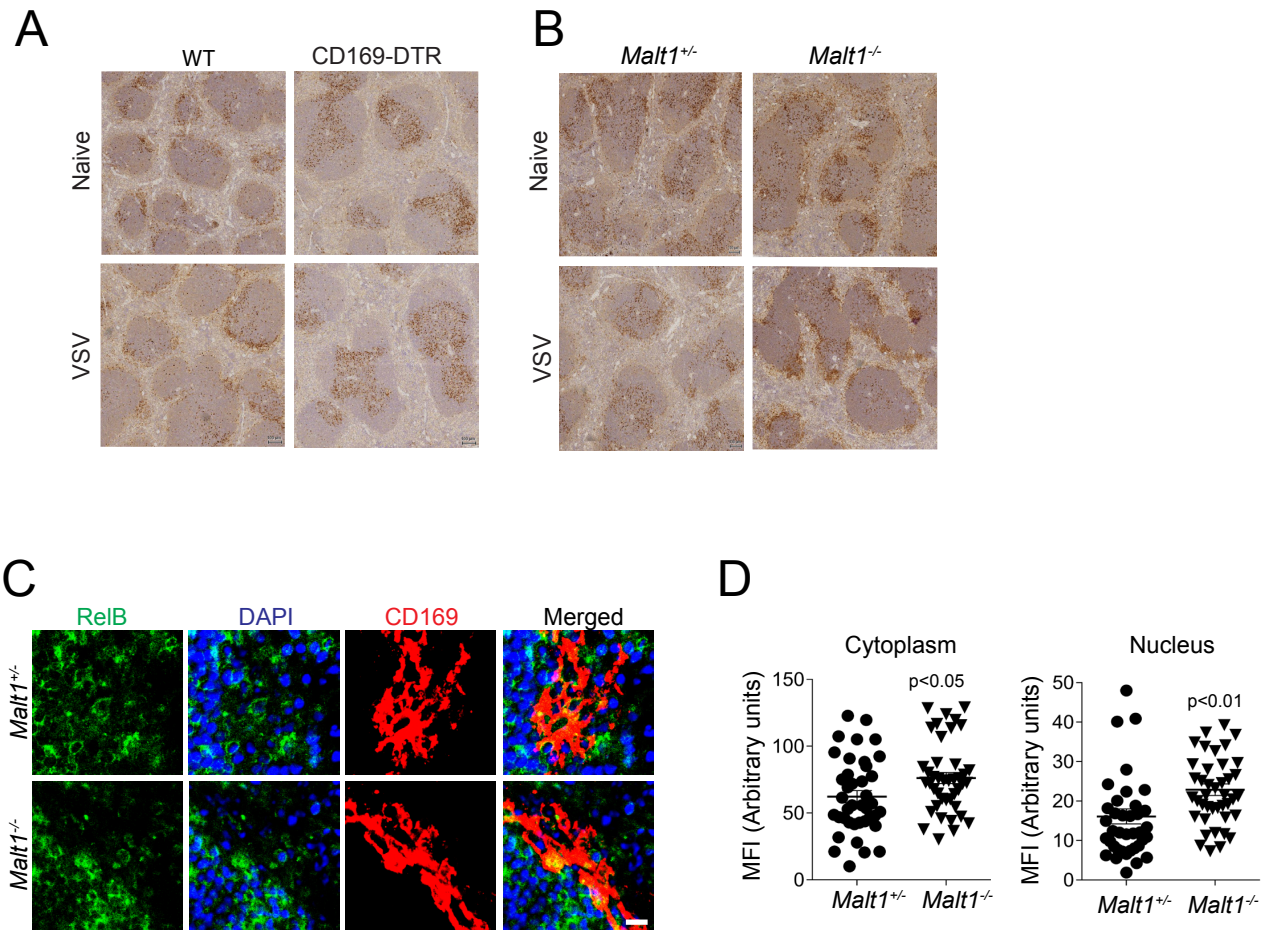

A
WT
CD169-DTR
Naive
VSV
B
Malt1+/-
Malt1-/-
Naive
VSV
C
RelB
DAPI
CD169
Merged
Malt1+/-
Malt1-/-
D
Cytoplasm
Nucleus
MFI (Arbitrary units)
p<0.05
p<0.01
Malt1+/-
Malt1-/-

**Figure 13: MALT1 deficiency leads to accumulation of RelB in the cytoplasm of splenic CD169$^+$ cells.**

***(A)*** *Wild-type (WT) and CD169–diphtheria toxin receptor (CD160-DTR) mice were infected with 2 × $10^8$ plaque-forming units (PFU) of vesicular stomatitis virus (VSV). Formalin-fixed spleen tissue was stained for RelB (one result representative of 3 experiments is shown; scale bar = 100 µm).* ***(B)*** *Snap-frozen spleen tissue sections from Malt1$^{+/-}$ and Malt1$^{-/-}$ mice were stained with anti-RelB antibody 4 h after infection with 2 × $10^8$ PFU VSV (one result representative of 3 experiments is shown; scale bar = 100 µm).* ***(C)*** *Sections from snap-frozen spleen tissue harvested from naive Malt1$^{+/-}$ and Malt1$^{-/-}$ mice were stained with anti-RelB antibodies (one representative result of n=3 is shown; scale bar = 10 µm).* ***(D)*** *MFI of cytoplasmic and nuclear RelB was quantified in CD169$^+$ cells using ImageJ (n=39-42).*

## Figure. 14

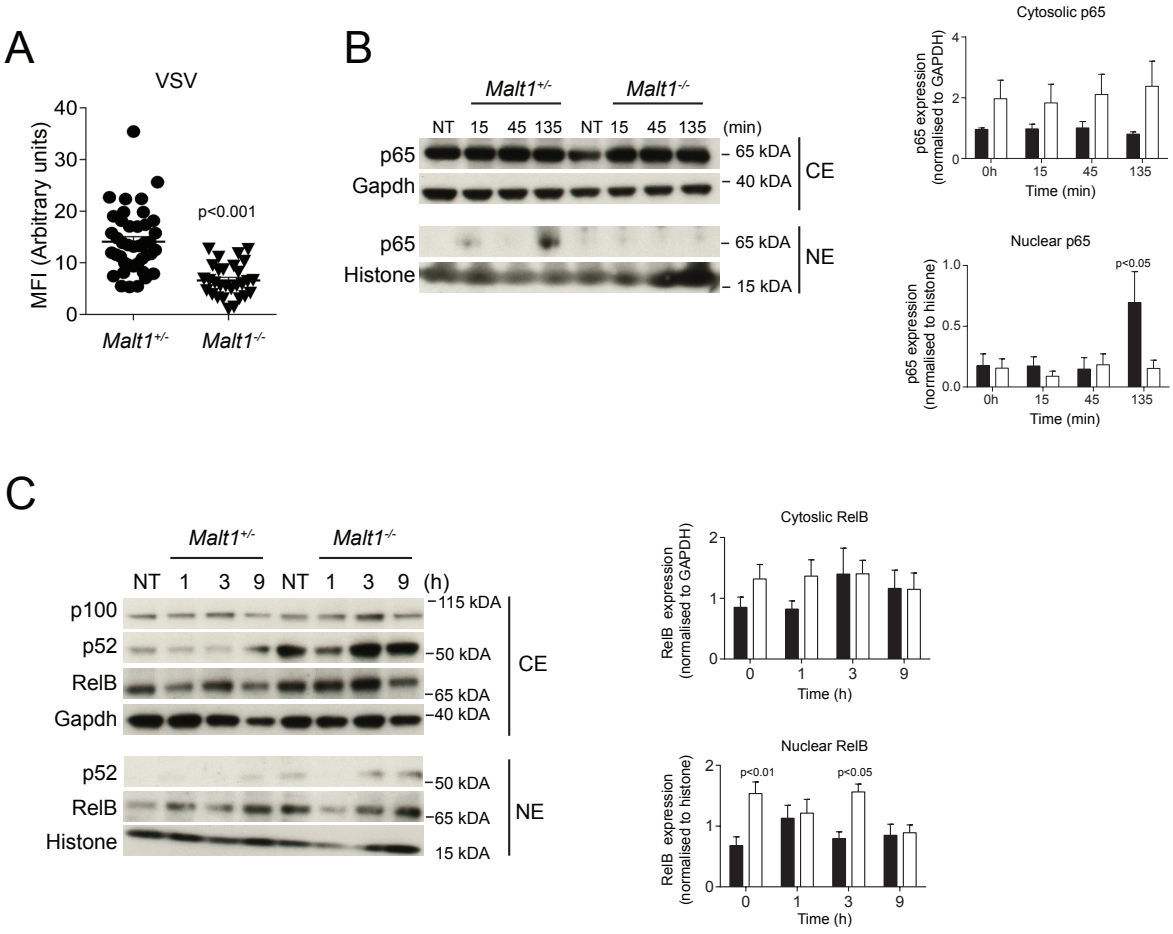



**Figure 14: MALT1 facilitates NF-κB signaling via degrading RelB and translocation of RelA (p65).**

***(A)*** *Sections of snap-frozen spleen tissue from Malt1$^{+/-}$ and Malt1$^{-/-}$ mice were stained with anti-RelA antibodies 4h after infection with 2 x 10$^{8}$ PFU VSV. The MFI in the nucleus of CD169$^{+}$ cells was quantified (n=29-41).* ***(B+C)*** *Malt1$^{+/-}$ and Malt1$^{-/-}$ mouse embryonic fibroblasts (MEFs) were stimulated with 100 ng/ml recombinant mouse tumor necrosis factor (rmTNF) at indicated time points. Cytosolic (CE) and nuclear extracts (NE) were harvested and probed for p65. Densitometry analysis of p65 and RelB was performed on the WB images from cytosolic and nuclear fractions at indicated time points. Proteins were normalized to GAPDH or histone (n=4). Figures were taken from Shinde et al., J Virol. 2018 Jan 17;92(3).*

**Figure. 15**

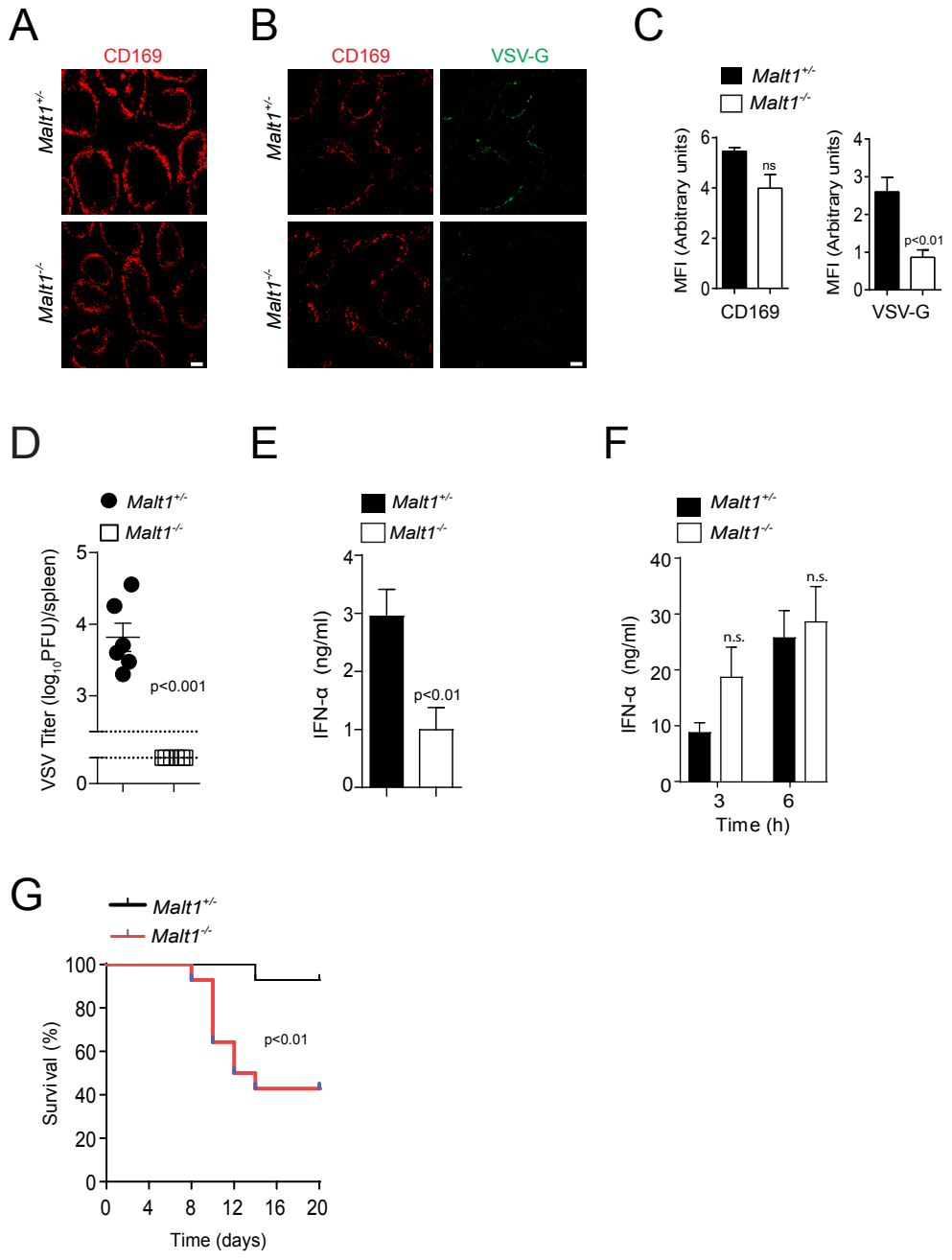

A
CD169
Malt1+/-
Malt1-/-
B
CD169
VSV-G
Malt1+/-
Malt1-/-
C
Malt1+/-
Malt1-/-
MFI (Arbitrary units)
ns
CD169
p<0.01
VSV-G
D
Malt1+/-
Malt1-/-
VSV Titer (log10PFU)/spleen)
p<0.001
E
Malt1+/-
Malt1-/-
IFN-α (ng/ml)
p<0.01
F
Malt1+/-
Malt1-/-
IFN-α (ng/ml)
n.s.
n.s.
Time (h)
G
Malt1+/-
Malt1-/-
Survival (%)
p<0.01
Time (days)

**Figure 15: MALT 1 plays an important role in survival of mice via induction of antiviral response.**

***(A)*** *Spleen sections from naïve Malt1$^{+/-}$ and Malt1$^{-/-}$ mice were stained with anti-CD169 (one representative result of n=3 is shown; scale bar = 100 µm).* ***(B)*** *Sections of snap-frozen spleen tissue from Malt1$^{+/-}$ and Malt1$^{-/-}$ mice were analyzed 8h after infection with 2 x 10$^{8}$ PFU VSV, stained with anti-CD169 and anti–vesicular stomatitis virus glycoprotein (VSV-G) (one representative result of n=3 is shown; scale bar = 100 µm).* ***(C)*** *MFI of CD169 and VSV-G was quantified across spleen sections from VSV infected Malt1$^{+/-}$ and Malt1$^{-/-}$ mice using ImageJ (n=4). (D and E) Mice were infected with 10$^{5}$ PFU VSV.* ***(D)*** *Viral titers were measured in spleen tissue of Malt1$^{+/-}$ and Malt1$^{-/-}$ mice 8h after infection (n=6).* ***(E)*** *IFN-α concentration was determined in the sera of Malt1$^{+/-}$ and Malt1$^{-/-}$ mice 24 h after infection (n=6).* ***(F)*** *IFN-α concentration was determined in the sera of Malt1$^{+/-}$ and Malt1$^{-/-}$ mice injected with 200µg polyinosinic:polycytidylic acid (poly I:C) at indicated time points (n=3-4).* ***(G)*** *Survival of Malt1$^{+/-}$ and Malt1$^{-/-}$ animals were monitored for 20 days after infection (n=13-14). Figures were taken from Shinde et al., J Virol. 2018 Jan 17;92(3).*

# Chapter 5.

# Discussion

## 5. Discussion

In this study, we found that TNF plays a crucial role in the maintenance of $CD169^+$ cells early after infection with VSV. Consequently, TNF, TNFR1, and MALT1 deficient animals exhibited reduced immune activation and limited IFN-I production, which consequently led to a severe VSV infection and mortality (357).

VSV is used as a model of virus infection to study IFN-I and tumor biology (358). Oncolytic activity of VSV can be attributed to defective IFN-I signaling in most of the tumor cells, as it was shown that phosphorylation of eIF2 alpha was aberrant due to defective eIF2B activity (359). eIF2 alpha phosphorylation is an essential step to produce antiviral genes. At the cellular level, protein kinase R (PKR) is a catalyst for eIF2 alpha phosphorylation leading to inhibition of viral protein synthesis, but it is not sufficient because STAT1- deficient mice succumbed to infection as well, even in the presence of PKR (360). Although VSV is highly neuropathic (60), it's rapid clearance depends heavily on neutralizing antibodies (361). However, long-term survival was shown to depend on T cells, as T cell-deficient mice succumbed to infection in 30 days (361). Hence, it can be concluded that the multilayered protection of IFN-I, neutralizing antibodies and T cell response is essential to prevent fatal VSV infection.

Viral infections activate a number of pathways by induction of innate immunity by NK cells, DCs, macrophages. Virus recognition by PRRs such as RIG-I, MDA5, and TLRs leads to activation of NF-κB, which in turn leads to secretion of various pro-inflammatory cytokines (362, 363). TNF, IFNγ, IL2, IL6, IL12 are some of the primary cytokines released after viral infections (362, 364, 365). Cytokines produced after the infection leads to upregulation of MHC-I and II molecules, costimulatory molecules on DCs which confer their maturation and increases their efficacy in antigen presentation (366, 367). VSV infection, which was used in this study, has a different outcome depending on the route of infection. It is reported that intranasal infection of MyD88 deficient mice are susceptible to infection whereas intravenous infection of MyD88 deficient mice does not show any signs of morbidity and mortality (368). However, intravenous route of infection with VSV has been used to study a number of antiviral mechanisms, such as IFN-I response and the role of $CD169^+$

macrophages in the spleen and LN (60, 110, 337, 345). In the recent years, CD169$^+$ macrophages have received considerable attention in their ability to capture, process and present antigen to APCs or directly to T cells (163, 165). Furthermore, CD169$^+$ cells have been shown not only to present antigens to B cells in the lymph nodes but also to prime T cells (160, 164). Moreover, CD169$^+$ cells are important for virus-mediated IFN-I production, which prevents severe CNS infection in mice (345). Since CD169$^+$ cells in the marginal zone are in close contact with pathogens and are ideally situated to induce an immune response (369). Hence it is imperative to study further the mechanisms involved in CD169$^+$ macrophage functions.

In our study, we used a similar approach of intravenous infection of VSV in wild-type C57Bl/6 mice and checked for gene expression of the whole transcription genome level using RNAseq. As expected, several antiviral genes were upregulated after the infection such as *Oasl1, Ifit1*, *Ifit2* and *Rsad2*. We observe one of the transcription factor in NF-κB, RelA to be significantly upregulated in CD169$^+$ macrophages after VSV infection. It is well known, that NF-κB is a major contributor to immunity (370), it prompted us to study further the role of cytokines which induce NF-κB.

Splenic TNF RNA expression was increased after the VSV infection at early time points (Within a few hours). This viral replication in CD169$^+$ cells, which is promoted by TNF, contributes to improved antigen production following VSV infection (357). Furthermore, our findings show that the translocation of RelA to the nuclei of CD169$^+$ cells after VSV infection is dependent on TNF. It has been postulated that canonical NF-κB can contribute to the production of IFN-α (371, 372). However, RelA-deficient and p50-deficient MEFs can produce IFN-α after viral infection, whereas only early IFN-I transcription is reduced (373, 374). Furthermore, RelA-deficient plasmacytoid dendritic cells (pDCs) exhibited reduced IFN production after exposure to Sendai virus (375). Our findings indicate that canonical NF-κB activation can also promote early viral replication and consequently contribute to the production of IFN-I. This increased production of IFN-I induces an antiviral state in the cells which become resistant to subsequent viral infections. In our setting we found that TNF deficient mice produced less amount of virus particles which led to reduced IFN-I production and succumbed to the virus infection (357).

The role of TNF during viral infection is complicated depending on the site and the cell types involved in the infection. Although reports describe activating polymorphisms in TNF, which are associated with the establishment of a chronic viral infection (376), other reports state that the same mutations are protective against chronic hepatitis B virus infection (HBV) (377). *In vitro*, TNF can propagate the viral replication of HCV (378) although HCV increases the incidence of TNF-induced apoptosis (379). On the other hand, TNF strongly inhibits influenza virus replication in porcine lung epithelial cells (380). Consistently, the attenuation of TNF signaling in a murine T cell-independent model of HBV infection results in viral persistence (381). In turn, the application of Smac mimetics enhances TNF signaling and is associated with increased clearance of HBV in this model system (382).

All these studies point towards the conclusion that TNF plays a wide variety of roles depending upon the type of infection. In our VSV model of infection, VSV replicates in $CD169^+$ owing to increased expression of USP18, which interferes with IFN-I signaling by cleaving ISG15 complexes and binding to IFNAR2 (161, 383). However, VSV is a cytopathic virus, hence there must be a mechanism involved in the survival of the infected $CD169^+$ cells. Our observations suggest that TNF produced by the $CD11b^+$ $Ly6C^+Ly6G^+$ neutrophil-like granulocytes, provide an essential signal via TNFR1 for the survival of $CD169^+$ cells (357). These granulocytes home into the infection sites by sensing the gradient of various proinflammatory cytokines (IL1, IL6, and TNF) and chemokines (CXCL1 and CXCL8) (384). Although short-lived, they show a potent signature of acute phase response genes, induction of TNF, IL6 and NF-κB, MAPK and JAK-STAT once they are at the site of infection (348). Granulocytes lack TLR3 but express TLR7, MDA-5, and RIG-I which helps in pathogen recognition and contribute to secretion of TNF, CXCL10, CCL4, IL-8 as well as IFN responsive genes (385, 386).

Our findings that TNF is crucial for the maintenance of $CD169^+$ cells in the spleen tissue may be important for infections with lower doses of virus because allowing viral replication in $CD169^+$ cells is particularly important for protective adaptive immunity (110, 337). This may be crucial for the maintenance of $CD169^+$ cells in spleen tissue during vaccination with attenuated virus strains or VSV vector-based vaccines (112). However, these findings may not only be specific for splenic $CD169^+$ cells, since intranasal infection

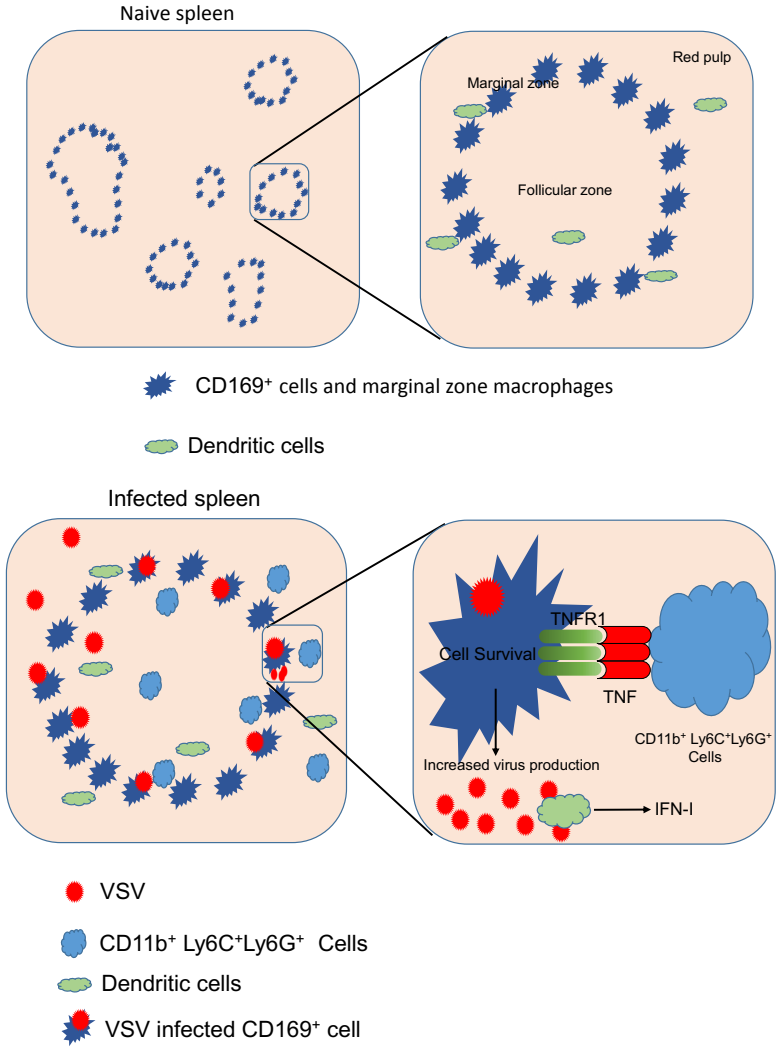
with recombinant TNF overexpressing Rabies virus (RV), reduced RV load and mortality (387). On the similar grounds, TNF and IFNγ have been shown to clear HBV and LCMV from hepatocytes independent of cytotoxic $CD8^+$ T cells mediated killing by activating intracellular antiviral genes (388, 389).

Our findings show that TNF promotes maintenance of $CD169^+$ cells and IFN-I.

Regulation of NF-κB is essential and which can be achieved by ubiquitination and deubiquitination of various components of NF-κB (390). Deubiquitinating enzymes such as cylindromatosis (CYLD) and A20 are one of the best characterized (391). CYLD interacts with the NF-kappa-B essential modulator (NEMO) and inhibits its activity, whereas it removes K63 -linked ubiquitins from certain proteins of the TNF signaling pathway (TRAF2, TRAF6, RIP1, and TAK1) (391). A20 regulates RIP1 by ligating it with K48- linked ubiquitin and sending it off to proteasomal degradation (392). RelA, a part of heterodimer is phosphorylated and acetylated after the activation which is essential for its nuclear transport and DNA binding activity (275, 393).

Recently another system of regulation of NF-κB has been discovered; RelB a member of the non-canonical NF-κB pathway where it forms a complex with p52 and translocates to the nucleus. RelB forms a complex with RelA in the cytoplasm and renders its DNA binding capacity to null (353) reducing NF-κB regulated gene expression. Furthermore, RelB itself can be cleaved by the paracaspase MALT1 and consequently promote canonical NF-κB signaling (354, 355). Our results show that VSV replication also depends on MALT1, which concurs with previous studies. As we see reduced VSV replication in the spleen of MALT1 deficient mice, which hints at the regulation of negative NF-κB in $CD169^+$ cells (357). Soon after infection $CD169^+$ cells underwent apoptosis in TNF or TNFR1 deficient mice, which points to an important role of TNF in preventing apoptosis of virus-infected cells. VSV is known to induce apoptosis via the mitochondrial pathway and caspase 9 (352, 394). The Bak and Bax proteins play a role in permeabilization of the mitochondrial membrane and antiapoptotic BCL-2, BCL-XL, and MCL1 proteins can inhibit their activity and apoptosis (395). In our study, we found less BCL2 and BCL-XL in TNF deficient mice after VSV infection which points to an essential role of TNF in virus infection.

In summary, upon systemic VSV infection virus particles captured by CD169$^{+}$ cells, attracts CD11b$^{+}$ Ly6C$^{+}$Ly6G$^{+}$ neutrophil-like granulocytes at the site of infection, which provide TNF to CD169$^{+}$ cells which activate the canonical NF-κB signaling (357). This activation results in survival of infected cells which further leads to controlled amplification of VSV. This increased virus load is recognized by the bystander DCs which ultimately secrets copious amounts of IFN-I resulting in antiviral defense (Graphical Figure 5). Taken together, we have found that TNF-TNFR1 signaling is crucial for protecting CD169$^{+}$ cells and their function in innate immune activation during VSV infection.




***Graphical Figure 5***. *Figure depicting signalling cascade and the events in the spleen after VSV infection.*

# Chapter 6.

# Bibliography

## 6. References


1. **Anonymous.** 2017. Global, regional, and national under-5 mortality, adult mortality, age-specific mortality, and life expectancy, 1970-2016: a systematic analysis for the Global Burden of Disease Study 2016. Lancet **390:**1084-1150.
2. **Murray CJ, Lopez AD.** 2013. Measuring the global burden of disease. N Engl J Med **369:**448-457.
3. **Bloom DE.** 2011. The value of vaccination. Adv Exp Med Biol **697:**1-8.
4. **Foster E.** 1883. Report of the Committee on Compulsory Vaccination; also, a Supplementary Report on the Efficiency and Safety of Vaccination. Public Health Pap Rep **9:**238-289.
5. **Hopkins DR.** 2013. Disease eradication. N Engl J Med **368:**54-63.
6. **Cobey S.** 2014. Pathogen evolution and the immunological niche. Ann N Y Acad Sci **1320:**1-15.
7. **tenOever BR.** 2016. The Evolution of Antiviral Defense Systems. Cell Host Microbe **19:**142-149.
8. **Koonin EV, Dolja VV.** 2013. A virocentric perspective on the evolution of life. Curr Opin Virol **3:**546-557.
9. **Akira S, Uematsu S, Takeuchi O.** 2006. Pathogen recognition and innate immunity. Cell **124:**783-801.
10. **Goldszmid RS, Dzutsev A, Trinchieri G.** 2014. Host immune response to infection and cancer: unexpected commonalities. Cell Host Microbe **15:**295-305.
11. **Kawai T, Akira S.** 2010. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol **11:**373-384.
12. **Janeway CA, Jr.** 1989. Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harb Symp Quant Biol **54 Pt 1:**1-13.
13. **Janeway CA, Jr.** 1992. The immune system evolved to discriminate infectious nonself from noninfectious self. Immunol Today **13:**11-16.
14. **Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA.** 1996. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell **86:**973-983.
15. **Medzhitov R, Preston-Hurlburt P, Janeway CA, Jr.** 1997. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature **388:**394-397.
16. **Rock FL, Hardiman G, Timans JC, Kastelein RA, Bazan JF.** 1998. A family of human receptors structurally related to Drosophila Toll. Proc Natl Acad Sci U S A **95:**588-593.

17. **Mariathasan S, Monack DM.** 2007. Inflammasome adaptors and sensors: intracellular regulators of infection and inflammation. Nat Rev Immunol **7:**31-40.
18. **Trinchieri G, Sher A.** 2007. Cooperation of Toll-like receptor signals in innate immune defence. Nat Rev Immunol **7:**179-190.
19. **Yoneyama M, Onomoto K, Fujita T.** 2008. Cytoplasmic recognition of RNA. Adv Drug Deliv Rev **60:**841-846.
20. **Cai X, Chiu YH, Chen ZJ.** 2014. The cGAS-cGAMP-STING pathway of cytosolic DNA sensing and signaling. Mol Cell **54:**289-296.
21. **Wu J, Chen ZJ.** 2014. Innate immune sensing and signaling of cytosolic nucleic acids. Annu Rev Immunol **32:**461-488.
22. **Yoneyama M, Fujita T.** 2009. RNA recognition and signal transduction by RIG-I-like receptors. Immunol Rev **227:**54-65.
23. **Ferrero-Miliani L, Nielsen OH, Andersen PS, Girardin SE.** 2007. Chronic inflammation: importance of NOD2 and NALP3 in interleukin-1beta generation. Clin Exp Immunol **147:**227-235.
24. **Franchi L, Eigenbrod T, Munoz-Planillo R, Nunez G.** 2009. The inflammasome: a caspase-1-activation platform that regulates immune responses and disease pathogenesis. Nat Immunol **10:**241-247.
25. **Martinon F, Mayor A, Tschopp J.** 2009. The inflammasomes: guardians of the body. Annu Rev Immunol **27:**229-265.
26. **Alexopoulou L, Holt AC, Medzhitov R, Flavell RA.** 2001. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature **413:**732-738.
27. **Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C.** 2004. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science **303:**1529-1531.
28. **Negishi H, Osawa T, Ogami K, Ouyang X, Sakaguchi S, Koshiba R, Yanai H, Seko Y, Shitara H, Bishop K, Yonekawa H, Tamura T, Kaisho T, Taya C, Taniguchi T, Honda K.** 2008. A critical link between Toll-like receptor 3 and type II interferon signaling pathways in antiviral innate immunity. Proc Natl Acad Sci U S A **105:**20446-20451.
29. **Bell JK, Askins J, Hall PR, Davies DR, Segal DM.** 2006. The dsRNA binding site of human Toll-like receptor 3. Proc Natl Acad Sci U S A **103:**8792-8797.
30. **Choe J, Kelker MS, Wilson IA.** 2005. Crystal structure of human toll-like receptor 3 (TLR3) ectodomain. Science **309:**581-585.
31. **Kawasaki T, Kawai T.** 2014. Toll-like receptor signaling pathways. Front Immunol **5:**461.
32. **Hornung V, Ellegast J, Kim S, Brzozka K, Jung A, Kato H, Poeck H, Akira S, Conzelmann KK, Schlee M, Endres S, Hartmann G.** 2006. 5'-Triphosphate RNA is the ligand for RIG-I. Science **314:**994-997.

33. **Pichlmair A, Schulz O, Tan CP, Naslund TI, Liljestrom P, Weber F, Reis e Sousa C.** 2006. RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates. Science **314:**997-1001.
34. **Kato H, Takeuchi O, Akira S.** 2006. [Cell type specific involvement of RIG-I in antiviral responses]. Nihon Rinsho **64:**1244-1247.
35. **Kato H, Takeuchi O, Sato S, Yoneyama M, Yamamoto M, Matsui K, Uematsu S, Jung A, Kawai T, Ishii KJ, Yamaguchi O, Otsu K, Tsujimura T, Koh CS, Reis e Sousa C, Matsuura Y, Fujita T, Akira S.** 2006. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. Nature **441:**101-105.
36. **Saito T, Hirai R, Loo YM, Owen D, Johnson CL, Sinha SC, Akira S, Fujita T, Gale M, Jr.** 2007. Regulation of innate antiviral defenses through a shared repressor domain in RIG-I and LGP2. Proc Natl Acad Sci U S A **104:**582-587.
37. **Jiang X, Kinch LN, Brautigam CA, Chen X, Du F, Grishin NV, Chen ZJ.** 2012. Ubiquitin-induced oligomerization of the RNA sensors RIG-I and MDA5 activates antiviral innate immune response. Immunity **36:**959-973.
38. **Kolakofsky D, Kowalinski E, Cusack S.** 2012. A structure-based model of RIG-I activation. RNA **18:**2118-2127.
39. **Luo D, Kohlway A, Pyle AM.** 2013. Duplex RNA activated ATPases (DRAs): platforms for RNA sensing, signaling and processing. RNA Biol **10:**111-120.
40. **Yoneyama M, Fujita T.** 2010. Recognition of viral nucleic acids in innate immunity. Rev Med Virol **20:**4-22.
41. **Kato H, Takeuchi O, Mikamo-Satoh E, Hirai R, Kawai T, Matsushita K, Hiiragi A, Dermody TS, Fujita T, Akira S.** 2008. Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5. J Exp Med **205:**1601-1610.
42. **Rehwinkel J, Tan CP, Goubau D, Schulz O, Pichlmair A, Bier K, Robb N, Vreede F, Barclay W, Fodor E, Reis e Sousa C.** 2010. RIG-I detects viral genomic RNA during negative-strand RNA virus infection. Cell **140:**397-408.
43. **Malathi K, Saito T, Crochet N, Barton DJ, Gale M, Jr., Silverman RH.** 2010. RNase L releases a small RNA from HCV RNA that refolds into a potent PAMP. RNA **16:**2108-2119.
44. **Malathi K, Dong B, Gale M, Jr., Silverman RH.** 2007. Small self-RNA generated by RNase L amplifies antiviral innate immunity. Nature **448:**816-819.
45. **Yoneyama M, Kikuchi M, Natsukawa T, Shinobu N, Imaizumi T, Miyagishi M, Taira K, Akira S, Fujita T.** 2004. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat Immunol **5:**730-737.
46. **Kowalinski E, Lunardi T, McCarthy AA, Louber J, Brunel J, Grigorov B, Gerlier D, Cusack S.** 2011. Structural basis for the activation of innate immune pattern-recognition receptor RIG-I by viral RNA. Cell **147:**423-435.

47. **Civril F, Bennett M, Moldt M, Deimling T, Witte G, Schiesser S, Carell T, Hopfner KP.** 2011. The RIG-I ATPase domain structure reveals insights into ATP-dependent antiviral signalling. EMBO Rep **12:**1127-1134.
48. **Jiang F, Ramanathan A, Miller MT, Tang GQ, Gale M, Jr., Patel SS, Marcotrigiano J.** 2011. Structural basis of RNA recognition and activation by innate immune receptor RIG-I. Nature **479:**423-427.
49. **Luo D, Ding SC, Vela A, Kohlway A, Lindenbach BD, Pyle AM.** 2011. Structural insights into RNA recognition by RIG-I. Cell **147:**409-422.
50. **Kawai T, Takahashi K, Sato S, Coban C, Kumar H, Kato H, Ishii KJ, Takeuchi O, Akira S.** 2005. IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. Nat Immunol **6:**981-988.
51. **Meylan E, Curran J, Hofmann K, Moradpour D, Binder M, Bartenschlager R, Tschopp J.** 2005. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. Nature **437:**1167-1172.
52. **Seth RB, Sun L, Ea CK, Chen ZJ.** 2005. Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. Cell **122:**669-682.
53. **Zeng W, Sun L, Jiang X, Chen X, Hou F, Adhikari A, Xu M, Chen ZJ.** 2010. Reconstitution of the RIG-I pathway reveals a signaling role of unanchored polyubiquitin chains in innate immunity. Cell **141:**315-330.
54. **Heaton SM, Borg NA, Dixit VM.** 2016. Ubiquitin in the activation and attenuation of innate antiviral immunity. J Exp Med **213:**1-13.
55. **Ivashkiv LB, Donlin LT.** 2014. Regulation of type I interferon responses. Nat Rev Immunol **14:**36-49.
56. **McNab F, Mayer-Barber K, Sher A, Wack A, O'Garra A.** 2015. Type I interferons in infectious disease. Nat Rev Immunol **15:**87-103.
57. **Isaacs A, Lindenmann J.** 1957. Virus interference. I. The interferon. Proc R Soc Lond B Biol Sci **147:**258-267.
58. **Isaacs A, Lindenmann J, Valentine RC.** 1957. Virus interference. II. Some properties of interferon. Proc R Soc Lond B Biol Sci **147:**268-273.
59. **Lindenmann J, Burke DC, Isaacs A.** 1957. Studies on the production, mode of action and properties of interferon. Br J Exp Pathol **38:**551-562.
60. **Muller U, Steinhoff U, Reis LF, Hemmi S, Pavlovic J, Zinkernagel RM, Aguet M.** 1994. Functional role of type I and type II interferons in antiviral defense. Science **264:**1918-1921.
61. **Kotenko SV, Gallagher G, Baurin VV, Lewis-Antes A, Shen M, Shah NK, Langer JA, Sheikh F, Dickensheets H, Donnelly RP.** 2003. IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. Nat Immunol **4:**69-77.

62. **Galani IE, Koltsida O, Andreakos E.** 2015. Type III interferons (IFNs): Emerging Master Regulators of Immunity. Adv Exp Med Biol **850:**1-15.
63. **Kotenko SV.** 2011. IFN-lambdas. Curr Opin Immunol **23:**583-590.
64. **Cleary CM, Donnelly RJ, Soh J, Mariano TM, Pestka S.** 1994. Knockout and reconstitution of a functional human type I interferon receptor complex. J Biol Chem **269:**18747-18749.
65. **Flores I, Mariano TM, Pestka S.** 1991. Human interferon omega (omega) binds to the alpha/beta receptor. J Biol Chem **266:**19875-19877.
66. **Merlin G, Falcoff E, Aguet M.** 1985. 125I-labelled human interferons alpha, beta and gamma: comparative receptor-binding data. J Gen Virol **66 ( Pt 5):**1149-1152.
67. **Novick D, Cohen B, Rubinstein M.** 1994. The human interferon alpha/beta receptor: characterization and molecular cloning. Cell **77:**391-400.
68. **Uze G, Lutfalla G, Gresser I.** 1990. Genetic transfer of a functional human interferon alpha receptor into mouse cells: cloning and expression of its cDNA. Cell **60:**225-234.
69. **Perussia B, Fanning V, Trinchieri G.** 1985. A leukocyte subset bearing HLA-DR antigens is responsible for in vitro alpha interferon production in response to viruses. Nat Immun Cell Growth Regul **4:**120-137.
70. **Siegal FP, Kadowaki N, Shodell M, Fitzgerald-Bocarsly PA, Shah K, Ho S, Antonenko S, Liu YJ.** 1999. The nature of the principal type 1 interferon-producing cells in human blood. Science **284:**1835-1837.
71. **Fitzgerald-Bocarsly P.** 1993. Human natural interferon-alpha producing cells. Pharmacol Ther **60:**39-62.
72. **Kadowaki N, Antonenko S, Lau JY, Liu YJ.** 2000. Natural interferon alpha/beta-producing cells link innate and adaptive immunity. J Exp Med **192:**219-226.
73. **Liu YJ.** 2005. IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. Annu Rev Immunol **23:**275-306.
74. **Levy DE, Darnell JE, Jr.** 2002. Stats: transcriptional control and biological impact. Nat Rev Mol Cell Biol **3:**651-662.
75. **Stark GR, Darnell JE, Jr.** 2012. The JAK-STAT pathway at twenty. Immunity **36:**503-514.
76. **MacMicking JD.** 2012. Interferon-inducible effector mechanisms in cell-autonomous immunity. Nat Rev Immunol **12:**367-382.
77. **Rusinova I, Forster S, Yu S, Kannan A, Masse M, Cumming H, Chapman R, Hertzog PJ.** 2013. Interferome v2.0: an updated database of annotated interferon-regulated genes. Nucleic Acids Res **41:**D1040-1046.
78. **Schoggins JW, Wilson SJ, Panis M, Murphy MY, Jones CT, Bieniasz P, Rice CM.** 2011. A diverse range of gene products are effectors of the type I interferon antiviral response. Nature **472:**481-485.

79. **de Veer MJ, Holko M, Frevel M, Walker E, Der S, Paranjape JM, Silverman RH, Williams BR.** 2001. Functional classification of interferon-stimulated genes identified using microarrays. J Leukoc Biol **69:**912-920.
80. **Der SD, Zhou A, Williams BR, Silverman RH.** 1998. Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays. Proc Natl Acad Sci U S A **95:**15623-15628.
81. **Platanias LC.** 2005. Mechanisms of type-I- and type-II-interferon-mediated signalling. Nat Rev Immunol **5:**375-386.
82. **Schoggins JW, Rice CM.** 2011. Interferon-stimulated genes and their antiviral effector functions. Curr Opin Virol **1:**519-525.
83. **Itsui Y, Sakamoto N, Kurosaki M, Kanazawa N, Tanabe Y, Koyama T, Takeda Y, Nakagawa M, Kakinuma S, Sekine Y, Maekawa S, Enomoto N, Watanabe M.** 2006. Expressional screening of interferon-stimulated genes for antiviral activity against hepatitis C virus replication. J Viral Hepat **13:**690-700.
84. **Jiang D, Guo H, Xu C, Chang J, Gu B, Wang L, Block TM, Guo JT.** 2008. Identification of three interferon-inducible cellular enzymes that inhibit the replication of hepatitis C virus. J Virol **82:**1665-1678.
85. **Jiang D, Weidner JM, Qing M, Pan XB, Guo H, Xu C, Zhang X, Birk A, Chang J, Shi PY, Block TM, Guo JT.** 2010. Identification of five interferon-induced cellular proteins that inhibit west nile virus and dengue virus infections. J Virol **84:**8332-8341.
86. **Gough DJ, Messina NL, Clarke CJ, Johnstone RW, Levy DE.** 2012. Constitutive type I interferon modulates homeostatic balance through tonic signaling. Immunity **36:**166-174.
87. **Kawashima T, Kosaka A, Yan H, Guo Z, Uchiyama R, Fukui R, Kaneko D, Kumagai Y, You DJ, Carreras J, Uematsu S, Jang MH, Takeuchi O, Kaisho T, Akira S, Miyake K, Tsutsui H, Saito T, Nishimura I, Tsuji NM.** 2013. Double-stranded RNA of intestinal commensal but not pathogenic bacteria triggers production of protective interferon-beta. Immunity **38:**1187-1197.
88. **Trinchieri G.** 2010. Type I interferon: friend or foe? J Exp Med **207:**2053-2063.
89. **Berry MP, Graham CM, McNab FW, Xu Z, Bloch SA, Oni T, Wilkinson KA, Banchereau R, Skinner J, Wilkinson RJ, Quinn C, Blankenship D, Dhawan R, Cush JJ, Mejias A, Ramilo O, Kon OM, Pascual V, Banchereau J, Chaussabel D, O'Garra A.** 2010. An interferon-inducible neutrophil-driven blood transcriptional signature in human tuberculosis. Nature **466:**973-977.
90. **Teles RM, Graeber TG, Krutzik SR, Montoya D, Schenk M, Lee DJ, Komisopoulou E, Kelly-Scumpia K, Chun R, Iyer SS, Sarno EN, Rea TH, Hewison M, Adams JS, Popper SJ, Relman DA, Stenger S, Bloom BR, Cheng G, Modlin RL.** 2013. Type I interferon suppresses type II interferon-triggered human anti-mycobacterial responses. Science **339:**1448-1453.

91. **Teijaro JR, Ng C, Lee AM, Sullivan BM, Sheehan KC, Welch M, Schreiber RD, de la Torre JC, Oldstone MB.** 2013. Persistent LCMV infection is controlled by blockade of type I interferon signaling. Science **340:**207-211.
92. **Wilson EB, Yamada DH, Elsaesser H, Herskovitz J, Deng J, Cheng G, Aronow BJ, Karp CL, Brooks DG.** 2013. Blockade of chronic type I interferon signaling to control persistent LCMV infection. Science **340:**202-207.
93. **Lee MS, Kim B, Oh GT, Kim YJ.** 2013. OASL1 inhibits translation of the type I interferon-regulating transcription factor IRF7. Nat Immunol **14:**346-355.
94. **Terenzi F, Hui DJ, Merrick WC, Sen GC.** 2006. Distinct induction patterns and functions of two closely related interferon-inducible human genes, ISG54 and ISG56. J Biol Chem **281:**34064-34071.
95. **Sarasin-Filipowicz M, Wang X, Yan M, Duong FH, Poli V, Hilton DJ, Zhang DE, Heim MH.** 2009. Alpha interferon induces long-lasting refractoriness of JAK-STAT signaling in the mouse liver through induction of USP18/UBP43. Mol Cell Biol **29:**4841-4851.
96. **Yoshimura A, Naka T, Kubo M.** 2007. SOCS proteins, cytokine signalling and immune regulation. Nat Rev Immunol **7:**454-465.
97. **Fields BN, Knipe DM, Howley PM.** 2007. Fields virology, 5th ed. Wolters Kluwer Health/Lippincott Williams & Wilkins, Philadelphia.
98. **Pringle CR.** 1982. The genetics of vesiculoviruses. Arch Virol **72:**1-34.
99. **Keene JD, Schubert M, Lazzarini RA.** 1980. Intervening sequence between the leader region and the nucleopcapsid gene of vesicular stomatitis virus RNA. J Virol **33:**789-794.
100. **McGeoch DJ.** 1979. Structure of the gene N:gene NS intercistronic junction in the genome of vesicular stomatitis virus. Cell **17:**673-681.
101. **Rose JK.** 1980. Complete intergenic and flanking gene sequences from the genome of vesicular stomatitis virus. Cell **19:**415-421.
102. **Fuchs JD, Frank I, Elizaga ML, Allen M, Frahm N, Kochar N, Li S, Edupuganti S, Kalams SA, Tomaras GD, Sheets R, Pensiero M, Tremblay MA, Higgins TJ, Latham T, Egan MA, Clarke DK, Eldridge JH, Group HS, the National Institutes of A, Infectious Diseases HIVVTN, Mulligan M, Rouphael N, Estep S, Rybczyk K, Dunbar D, Buchbinder S, Wagner T, Isbell R, Chinnell V, Bae J, Escamilla G, Tseng J, Fair R, Ramirez S, Broder G, Briesemeister L, Ferrara A.** 2015. First-in-Human Evaluation of the Safety and Immunogenicity of a Recombinant Vesicular Stomatitis Virus Human Immunodeficiency Virus-1 gag Vaccine (HVTN 090). Open Forum Infect Dis **2:**ofv082.
103. **Bi Z, Barna M, Komatsu T, Reiss CS.** 1995. Vesicular stomatitis virus infection of the central nervous system activates both innate and acquired immunity. J Virol **69:**6466-6472.

104. **Chauhan VS, Furr SR, Sterka DG, Jr., Nelson DA, Moerdyk-Schauwecker M, Marriott I, Grdzelishvili VZ.** 2010. Vesicular stomatitis virus infects resident cells of the central nervous system and induces replication-dependent inflammatory responses. Virology **400:**187-196.
105. **Christian AY, Barna M, Bi Z, Reiss CS.** 1996. Host immune response to vesicular stomatitis virus infection of the central nervous system in C57BL/6 mice. Viral Immunol **9:**195-205.
106. **Huneycutt BS, Bi Z, Aoki CJ, Reiss CS.** 1993. Central neuropathogenesis of vesicular stomatitis virus infection of immunodeficient mice. J Virol **67:**6698-6706.
107. **Hangartner L, Zinkernagel RM, Hengartner H.** 2006. Antiviral antibody responses: the two extremes of a wide spectrum. Nat Rev Immunol **6:**231-243.
108. **Fensterl V, Wetzel JL, Ramachandran S, Ogino T, Stohlman SA, Bergmann CC, Diamond MS, Virgin HW, Sen GC.** 2012. Interferon-induced Ifit2/ISG54 protects mice from lethal VSV neuropathogenesis. PLoS Pathog **8:**e1002712.
109. **Trottier MD, Jr., Palian BM, Reiss CS.** 2005. VSV replication in neurons is inhibited by type I IFN at multiple stages of infection. Virology **333:**215-225.
110. **Honke N, Shaabani N, Cadeddu G, Sorg UR, Zhang DE, Trilling M, Klingel K, Sauter M, Kandolf R, Gailus N, van Rooijen N, Burkart C, Baldus SE, Grusdat M, Lohning M, Hengel H, Pfeffer K, Tanaka M, Haussinger D, Recher M, Lang PA, Lang KS.** 2012. Enforced viral replication activates adaptive immunity and is essential for the control of a cytopathic virus. Nature Immunology **13:**51-57.
111. **Iannacone M, Moseman EA, Tonti E, Bosurgi L, Junt T, Henrickson SE, Whelan SP, Guidotti LG, von Andrian UH.** 2010. Subcapsular sinus macrophages prevent CNS invasion on peripheral infection with a neurotropic virus. Nature **465:**1079-1083.
112. **Jones SM, Feldmann H, Stroher U, Geisbert JB, Fernando L, Grolla A, Klenk HD, Sullivan NJ, Volchkov VE, Fritz EA, Daddario KM, Hensley LE, Jahrling PB, Geisbert TW.** 2005. Live attenuated recombinant vaccine protects nonhuman primates against Ebola and Marburg viruses. Nat Med **11:**786-790.
113. **Moghimi SM, Hunter AC, Murray JC.** 2001. Long-circulating and target-specific nanoparticles: theory to practice. Pharmacol Rev **53:**283-318.
114. **Moghimi SM, Szebeni J.** 2003. Stealth liposomes and long circulating nanoparticles: critical issues in pharmacokinetics, opsonization and protein-binding properties. Prog Lipid Res **42:**463-478.
115. **Wadenvik H, Kutti J.** 1988. The spleen and pooling of blood cells. Eur J Haematol **41:**1-5.
116. **Cesta MF.** 2006. Normal structure, function, and histology of the spleen. Toxicol Pathol **34:**455-465.
117. **Humphrey JH, Grennan D.** 1981. Different macrophage populations distinguished by means of fluorescent polysaccharides. Recognition and properties of marginal-zone macrophages. Eur J Immunol **11:**221-228.

118. **van Furth R, Diesselhoff-den Dulk MM.** 1984. Dual origin of mouse spleen macrophages. J Exp Med **160:**1273-1283.
119. **Banchereau J, Steinman RM.** 1998. Dendritic cells and the control of immunity. Nature **392:**245-252.
120. **Manz MG.** 2018. Plasmacytoid dendritic cells: origin matters. Nat Immunol doi:10.1038/s41590-018-0143-x.
121. **Jung S, Unutmaz D, Wong P, Sano G, De los Santos K, Sparwasser T, Wu S, Vuthoori S, Ko K, Zavala F, Pamer EG, Littman DR, Lang RA.** 2002. In vivo depletion of CD11c+ dendritic cells abrogates priming of CD8+ T cells by exogenous cell-associated antigens. Immunity **17:**211-220.
122. **Geissmann F, Manz MG, Jung S, Sieweke MH, Merad M, Ley K.** 2010. Development of monocytes, macrophages, and dendritic cells. Science **327:**656-661.
123. **Chehimi J, Starr SE, Kawashima H, Miller DS, Trinchieri G, Perussia B, Bandyopadhyay S.** 1989. Dendritic cells and IFN-alpha-producing cells are two functionally distinct non-B, non-monocytic HLA-DR+ cell subsets in human peripheral blood. Immunology **68:**486-490.
124. **Corcoran L, Ferrero I, Vremec D, Lucas K, Waithman J, O'Keeffe M, Wu L, Wilson A, Shortman K.** 2003. The lymphoid past of mouse plasmacytoid cells and thymic dendritic cells. J Immunol **170:**4926-4932.
125. **Vremec D, Zorbas M, Scollay R, Saunders DJ, Ardavin CF, Wu L, Shortman K.** 1992. The surface phenotype of dendritic cells purified from mouse thymus and spleen: investigation of the CD8 expression by a subpopulation of dendritic cells. J Exp Med **176:**47-58.
126. **Shortman K, Heath WR.** 2010. The CD8+ dendritic cell subset. Immunol Rev **234:**18-31.
127. **Kato H, Sato S, Yoneyama M, Yamamoto M, Uematsu S, Matsui K, Tsujimura T, Takeda K, Fujita T, Takeuchi O, Akira S.** 2005. Cell type-specific involvement of RIG-I in antiviral response. Immunity **23:**19-28.
128. **Luber CA, Cox J, Lauterbach H, Fancke B, Selbach M, Tschopp J, Akira S, Wiegand M, Hochrein H, O'Keeffe M, Mann M.** 2010. Quantitative proteomics reveals subset-specific viral recognition in dendritic cells. Immunity **32:**279-289.
129. **Lee J, Chuang TH, Redecke V, She L, Pitha PM, Carson DA, Raz E, Cottam HB.** 2003. Molecular basis for the immunostimulatory activity of guanine nucleoside analogs: activation of Toll-like receptor 7. Proc Natl Acad Sci U S A **100:**6646-6651.
130. **Lund JM, Alexopoulou L, Sato A, Karow M, Adams NC, Gale NW, Iwasaki A, Flavell RA.** 2004. Recognition of single-stranded RNA viruses by Toll-like receptor 7. Proc Natl Acad Sci U S A **101:**5598-5603.
131. **Kiessling R, Klein E, Pross H, Wigzell H.** 1975. "Natural" killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell. Eur J Immunol **5:**117-121.

132. **Smith HJ.** 1966. Antigenicity of carcinogen-induced and spontaneous tumours in inbred mice. Br J Cancer **20:**831-837.
133. **Lodoen MB, Lanier LL.** 2005. Viral modulation of NK cell immunity. Nat Rev Microbiol **3:**59-69.
134. **Bix M, Liao NS, Zijlstra M, Loring J, Jaenisch R, Raulet D.** 1991. Rejection of class I MHC-deficient haemopoietic cells by irradiated MHC-matched mice. Nature **349:**329-331.
135. **Hoglund P, Ohlen C, Carbone E, Franksson L, Ljunggren HG, Latour A, Koller B, Karre K.** 1991. Recognition of beta 2-microglobulin-negative (beta 2m-) T-cell blasts by natural killer cells from normal but not from beta 2m- mice: nonresponsiveness controlled by beta 2m- bone marrow in chimeric mice. Proc Natl Acad Sci U S A **88:**10332-10336.
136. **Biron CA, Nguyen KB, Pien GC, Cousens LP, Salazar-Mather TP.** 1999. Natural killer cells in antiviral defense: function and regulation by innate cytokines. Annu Rev Immunol **17:**189-220.
137. **Lanier LL.** 2005. NK cell recognition. Annu Rev Immunol **23:**225-274.
138. **Trinchieri G.** 1989. Biology of natural killer cells. Adv Immunol **47:**187-376.
139. **Walzer T, Dalod M, Robbins SH, Zitvogel L, Vivier E.** 2005. Natural-killer cells and dendritic cells: "l'union fait la force". Blood **106:**2252-2258.
140. **Lanier LL.** 1998. NK cell receptors. Annu Rev Immunol **16:**359-393.
141. **Muta T, Kurosaki T, Misulovin Z, Sanchez M, Nussenzweig MC, Ravetch JV.** 1994. A 13-amino-acid motif in the cytoplasmic domain of Fc gamma RIIB modulates B-cell receptor signalling. Nature **368:**70-73.
142. **Reth M.** 1989. Antigen receptor tail clue. Nature **338:**383-384.
143. **Andrews DM, Estcourt MJ, Andoniou CE, Wikstrom ME, Khong A, Voigt V, Fleming P, Tabarias H, Hill GR, van der Most RG, Scalzo AA, Smyth MJ, Degli-Esposti MA.** 2010. Innate immunity defines the capacity of antiviral T cells to limit persistent infection. J Exp Med **207:**1333-1343.
144. **Crouse J, Bedenikovic G, Wiesel M, Ibberson M, Xenarios I, Von Laer D, Kalinke U, Vivier E, Jonjic S, Oxenius A.** 2014. Type I interferons protect T cells against NK cell attack mediated by the activating receptor NCR1. Immunity **40:**961-973.
145. **Xu HC, Grusdat M, Pandyra AA, Polz R, Huang J, Sharma P, Deenen R, Kohrer K, Rahbar R, Diefenbach A, Gibbert K, Lohning M, Hocker L, Waibler Z, Haussinger D, Mak TW, Ohashi PS, Lang KS, Lang PA.** 2014. Type I interferon protects antiviral CD8+ T cells from NK cell cytotoxicity. Immunity **40:**949-960.
146. **Sun JC, Beilke JN, Lanier LL.** 2009. Adaptive immune features of natural killer cells. Nature **457:**557-561.
147. **Tay CH, Welsh RM.** 1997. Distinct organ-dependent mechanisms for the control of murine cytomegalovirus infection by natural killer cells. J Virol **71:**267-275.

148. **den Haan JM, Mebius RE, Kraal G.** 2012. Stromal cells of the mouse spleen. Front Immunol **3:**201.
149. **Mebius RE, Kraal G.** 2005. Structure and function of the spleen. Nat Rev Immunol **5:**606-616.
150. **Davies LC, Jenkins SJ, Allen JE, Taylor PR.** 2013. Tissue-resident macrophages. Nat Immunol **14:**986-995.
151. **Honda K, Nakano H, Yoshida H, Nishikawa S, Rennert P, Ikuta K, Tamechika M, Yamaguchi K, Fukumoto T, Chiba T, Nishikawa SI.** 2001. Molecular basis for hematopoietic/mesenchymal interaction during initiation of Peyer's patch organogenesis. J Exp Med **193:**621-630.
152. **Rennert PD, Browning JL, Mebius R, Mackay F, Hochman PS.** 1996. Surface lymphotoxin alpha/beta complex is required for the development of peripheral lymphoid organs. J Exp Med **184:**1999-2006.
153. **Ato M, Nakano H, Kakiuchi T, Kaye PM.** 2004. Localization of marginal zone macrophages is regulated by C-C chemokine ligands 21/19. J Immunol **173:**4815-4820.
154. **Nolte MA, Arens R, Kraus M, van Oers MH, Kraal G, van Lier RA, Mebius RE.** 2004. B cells are crucial for both development and maintenance of the splenic marginal zone. J Immunol **172:**3620-3627.
155. **Crocker PR, Gordon S.** 1986. Properties and distribution of a lectin-like hemagglutinin differentially expressed by murine stromal tissue macrophages. J Exp Med **164:**1862-1875.
156. **Kraal G, Janse M.** 1986. Marginal metallophilic cells of the mouse spleen identified by a monoclonal antibody. Immunology **58:**665-669.
157. **Oetke C, Kraal G, Crocker PR.** 2006. The antigen recognized by MOMA-I is sialoadhesin. Immunol Lett **106:**96-98.
158. **Gupta P, Lai SM, Sheng J, Tetlak P, Balachander A, Claser C, Renia L, Karjalainen K, Ruedl C.** 2016. Tissue-Resident CD169(+) Macrophages Form a Crucial Front Line against Plasmodium Infection. Cell Rep **16:**1749-1761.
159. **Perez OA, Yeung ST, Vera-Licona P, Romagnoli PA, Samji T, Ural BB, Maher L, Tanaka M, Khanna KM.** 2017. CD169(+) macrophages orchestrate innate immune responses by regulating bacterial localization in the spleen. Sci Immunol **2**.
160. **Junt T, Moseman EA, Iannacone M, Massberg S, Lang PA, Boes M, Fink K, Henrickson SE, Shayakhmetov DM, Di Paolo NC, van Rooijen N, Mempel TR, Whelan SP, von Andrian UH.** 2007. Subcapsular sinus macrophages in lymph nodes clear lymph-borne viruses and present them to antiviral B cells. Nature **450:**110-114.
161. **Malakhova OA, Kim KI, Luo JK, Zou W, Kumar KG, Fuchs SY, Shuai K, Zhang DE.** 2006. UBP43 is a novel regulator of interferon signaling independent of its ISG15 isopeptidase activity. EMBO J **25:**2358-2367.

162. **Honke N, Shaabani N, Cadeddu G, Sorg UR, Zhang DE, Trilling M, Klingel K, Sauter M, Kandolf R, Gailus N, van Rooijen N, Burkart C, Baldus SE, Grusdat M, Lohning M, Hengel H, Pfeffer K, Tanaka M, Haussinger D, Recher M, Lang PA, Lang KS.** 2011. Enforced viral replication activates adaptive immunity and is essential for the control of a cytopathic virus. Nat Immunol **13:**51-57.
163. **van Dinther D, Veninga H, Iborra S, Borg EGF, Hoogterp L, Olesek K, Beijer MR, Schetters STT, Kalay H, Garcia-Vallejo JJ, Franken KL, Cham LB, Lang KS, van Kooyk Y, Sancho D, Crocker PR, den Haan JMM.** 2018. Functional CD169 on Macrophages Mediates Interaction with Dendritic Cells for CD8(+) T Cell Cross-Priming. Cell Rep **22:**1484-1495.
164. **Bernhard CA, Ried C, Kochanek S, Brocker T.** 2015. CD169+ macrophages are sufficient for priming of CTLs with specificities left out by cross-priming dendritic cells. Proc Natl Acad Sci U S A **112:**5461-5466.
165. **Martinez-Pomares L, Gordon S.** 2012. CD169+ macrophages at the crossroads of antigen presentation. Trends Immunol **33:**66-70.
166. **Flajnik MF, Kasahara M.** 2010. Origin and evolution of the adaptive immune system: genetic events and selective pressures. Nat Rev Genet **11:**47-59.
167. **Tonegawa S.** 1983. Somatic generation of antibody diversity. Nature **302:**575-581.
168. **Gellert M.** 2002. V(D)J recombination: RAG proteins, repair factors, and regulation. Annu Rev Biochem **71:**101-132.
169. **Cooper MD.** 2015. The early history of B cells. Nat Rev Immunol **15:**191-197.
170. **Murphy K, Travers P, Walport M, Janeway C.** 2012. Janeway's immunobiology, 8th ed. Garland Science, New York.
171. **Kondo M.** 2010. Lymphoid and myeloid lineage commitment in multipotent hematopoietic progenitors. Immunol Rev **238:**37-46.
172. **Melchers F.** 2015. Checkpoints that control B cell development. J Clin Invest **125:**2203-2210.
173. **Pieper K, Grimbacher B, Eibel H.** 2013. B-cell biology and development. J Allergy Clin Immunol **131:**959-971.
174. **Hardy RR, Carmack CE, Shinton SA, Kemp JD, Hayakawa K.** 1991. Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow. J Exp Med **173:**1213-1225.
175. **Pelanda R, Schaal S, Torres RM, Rajewsky K.** 1996. A prematurely expressed Ig(kappa) transgene, but not V(kappa)J(kappa) gene segment targeted into the Ig(kappa) locus, can rescue B cell development in lambda5-deficient mice. Immunity **5:**229-239.
176. **Ada G.** 2008. The enunciation and impact of Macfarlane Burnet's clonal selection theory of acquired immunity. Immunol Cell Biol **86:**116-118.

177. **Hartley SB, Cooke MP, Fulcher DA, Harris AW, Cory S, Basten A, Goodnow CC.** 1993. Elimination of self-reactive B lymphocytes proceeds in two stages: arrested development and cell death. Cell **72:**325-335.
178. **Hartley SB, Crosbie J, Brink R, Kantor AB, Basten A, Goodnow CC.** 1991. Elimination from peripheral lymphoid tissues of self-reactive B lymphocytes recognizing membrane-bound antigens. Nature **353:**765-769.
179. **Nemazee DA, Burki K.** 1989. Clonal deletion of B lymphocytes in a transgenic mouse bearing anti-MHC class I antibody genes. Nature **337:**562-566.
180. **Gay D, Saunders T, Camper S, Weigert M.** 1993. Receptor editing: an approach by autoreactive B cells to escape tolerance. J Exp Med **177:**999-1008.
181. **Radic MZ, Erikson J, Litwin S, Weigert M.** 1993. B lymphocytes may escape tolerance by revising their antigen receptors. J Exp Med **177:**1165-1173.
182. **Tiegs SL, Russell DM, Nemazee D.** 1993. Receptor editing in self-reactive bone marrow B cells. J Exp Med **177:**1009-1020.
183. **Allman D, Lindsley RC, DeMuth W, Rudd K, Shinton SA, Hardy RR.** 2001. Resolution of three nonproliferative immature splenic B cell subsets reveals multiple selection points during peripheral B cell maturation. J Immunol **167:**6834-6840.
184. **Rowland SL, DePersis CL, Torres RM, Pelanda R.** 2010. Ras activation of Erk restores impaired tonic BCR signaling and rescues immature B cell differentiation. J Exp Med **207:**607-621.
185. **Su TT, Rawlings DJ.** 2002. Transitional B lymphocyte subsets operate as distinct checkpoints in murine splenic B cell development. J Immunol **168:**2101-2110.
186. **Yasuda T, Sanjo H, Pages G, Kawano Y, Karasuyama H, Pouyssegur J, Ogata M, Kurosaki T.** 2008. Erk kinases link pre-B cell receptor signaling to transcriptional events required for early B cell expansion. Immunity **28:**499-508.
187. **Halverson R, Torres RM, Pelanda R.** 2004. Receptor editing is the main mechanism of B cell tolerance toward membrane antigens. Nat Immunol **5:**645-650.
188. **Pelanda R, Schwers S, Sonoda E, Torres RM, Nemazee D, Rajewsky K.** 1997. Receptor editing in a transgenic mouse model: site, efficiency, and role in B cell tolerance and antibody diversification. Immunity **7:**765-775.
189. **Muppidi JR, Arnon TI, Bronevetsky Y, Veerapen N, Tanaka M, Besra GS, Cyster JG.** 2011. Cannabinoid receptor 2 positions and retains marginal zone B cells within the splenic marginal zone. J Exp Med **208:**1941-1948.
190. **Venkataraman K, Lee YM, Michaud J, Thangada S, Ai Y, Bonkovsky HL, Parikh NS, Habrukowich C, Hla T.** 2008. Vascular endothelium as a contributor of plasma sphingosine 1-phosphate. Circ Res **102:**669-676.
191. **Mourcin F, Breton C, Tellier J, Narang P, Chasson L, Jorquera A, Coles M, Schiff C, Mancini SJ.** 2011. Galectin-1-expressing stromal cells constitute a specific niche for pre-BII cell development in mouse bone marrow. Blood **117:**6552-6561.

192. **Tokoyoda K, Egawa T, Sugiyama T, Choi BI, Nagasawa T.** 2004. Cellular niches controlling B lymphocyte behavior within bone marrow during development. Immunity **20:**707-718.
193. **Schneider P, MacKay F, Steiner V, Hofmann K, Bodmer JL, Holler N, Ambrose C, Lawton P, Bixler S, Acha-Orbea H, Valmori D, Romero P, Werner-Favre C, Zubler RH, Browning JL, Tschopp J.** 1999. BAFF, a novel ligand of the tumor necrosis factor family, stimulates B cell growth. J Exp Med **189:**1747-1756.
194. **Thompson JS, Bixler SA, Qian F, Vora K, Scott ML, Cachero TG, Hession C, Schneider P, Sizing ID, Mullen C, Strauch K, Zafari M, Benjamin CD, Tschopp J, Browning JL, Ambrose C.** 2001. BAFF-R, a newly identified TNF receptor that specifically interacts with BAFF. Science **293:**2108-2111.
195. **Gross JA, Johnston J, Mudri S, Enselman R, Dillon SR, Madden K, Xu W, Parrish-Novak J, Foster D, Lofton-Day C, Moore M, Littau A, Grossman A, Haugen H, Foley K, Blumberg H, Harrison K, Kindsvogel W, Clegg CH.** 2000. TACI and BCMA are receptors for a TNF homologue implicated in B-cell autoimmune disease. Nature **404:**995-999.
196. **Ng LG, Sutherland AP, Newton R, Qian F, Cachero TG, Scott ML, Thompson JS, Wheway J, Chtanova T, Groom J, Sutton IJ, Xin C, Tangye SG, Kalled SL, Mackay F, Mackay CR.** 2004. B cell-activating factor belonging to the TNF family (BAFF)-R is the principal BAFF receptor facilitating BAFF costimulation of circulating T and B cells. J Immunol **173:**807-817.
197. **Allman D, Pillai S.** 2008. Peripheral B cell subsets. Curr Opin Immunol **20:**149-157.
198. **Carsetti R, Rosado MM, Wardmann H.** 2004. Peripheral development of B cells in mouse and man. Immunol Rev **197:**179-191.
199. **Hardy RR, Hayakawa K.** 2001. B cell development pathways. Annu Rev Immunol **19:**595-621.
200. **Herzenberg LA.** 2000. B-1 cells: the lineage question revisited. Immunol Rev **175:**9-22.
201. **Martin F, Kearney JF.** 2002. Marginal-zone B cells. Nat Rev Immunol **2:**323-335.
202. **Fairfax KA, Kallies A, Nutt SL, Tarlinton DM.** 2008. Plasma cell development: from B-cell subsets to long-term survival niches. Semin Immunol **20:**49-58.
203. **Burton DR.** 2002. Antibodies, viruses and vaccines. Nat Rev Immunol **2:**706-713.
204. **De Silva NS, Klein U.** 2015. Dynamics of B cells in germinal centres. Nat Rev Immunol **15:**137-148.
205. **MacLennan IC, Toellner KM, Cunningham AF, Serre K, Sze DM, Zuniga E, Cook MC, Vinuesa CG.** 2003. Extrafollicular antibody responses. Immunol Rev **194:**8-18.
206. **Shlomchik MJ, Weisel F.** 2012. Germinal center selection and the development of memory B and plasma cells. Immunol Rev **247:**52-63.

207. **Victora GD, Nussenzweig MC.** 2012. Germinal centers. Annu Rev Immunol **30:**429-457.
208. **Zinkernagel RM.** 1996. Immunology taught by viruses. Science **271:**173-178.
209. **Kerfoot SM, Yaari G, Patel JR, Johnson KL, Gonzalez DG, Kleinstein SH, Haberman AM.** 2011. Germinal center B cell and T follicular helper cell development initiates in the interfollicular zone. Immunity **34:**947-960.
210. **Kitano M, Moriyama S, Ando Y, Hikida M, Mori Y, Kurosaki T, Okada T.** 2011. Bcl6 protein expression shapes pre-germinal center B cell dynamics and follicular helper T cell heterogeneity. Immunity **34:**961-972.
211. **Allen CD, Ansel KM, Low C, Lesley R, Tamamura H, Fujii N, Cyster JG.** 2004. Germinal center dark and light zone organization is mediated by CXCR4 and CXCR5. Nat Immunol **5:**943-952.
212. **Liu D, Xu H, Shih C, Wan Z, Ma X, Ma W, Luo D, Qi H.** 2015. T-B-cell entanglement and ICOSL-driven feed-forward regulation of germinal centre reaction. Nature **517:**214-218.
213. **Shulman Z, Gitlin AD, Weinstein JS, Lainez B, Esplugues E, Flavell RA, Craft JE, Nussenzweig MC.** 2014. Dynamic signaling by T follicular helper cells during germinal center B cell selection. Science **345:**1058-1062.
214. **Victora GD, Schwickert TA, Fooksman DR, Kamphorst AO, Meyer-Hermann M, Dustin ML, Nussenzweig MC.** 2010. Germinal center dynamics revealed by multiphoton microscopy with a photoactivatable fluorescent reporter. Cell **143:**592-605.
215. **Cyster JG.** 2010. B cell follicles and antigen encounters of the third kind. Nat Immunol **11:**989-996.
216. **Martin F, Oliver AM, Kearney JF.** 2001. Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens. Immunity **14:**617-629.
217. **Balazs M, Martin F, Zhou T, Kearney J.** 2002. Blood dendritic cells interact with splenic marginal zone B cells to initiate T-independent immune responses. Immunity **17:**341-352.
218. **Hozumi N, Tonegawa S.** 1976. Evidence for somatic rearrangement of immunoglobulin genes coding for variable and constant regions. Proc Natl Acad Sci U S A **73:**3628-3632.
219. **Bernard O, Hozumi N, Tonegawa S.** 1978. Sequences of mouse immunoglobulin light chain genes before and after somatic changes. Cell **15:**1133-1144.
220. **Early P, Huang H, Davis M, Calame K, Hood L.** 1980. An immunoglobulin heavy chain variable region gene is generated from three segments of DNA: VH, D and JH. Cell **19:**981-992.

221. **Rogers J, Early P, Carter C, Calame K, Bond M, Hood L, Wall R.** 1980. Two mRNAs with different 3' ends encode membrane-bound and secreted forms of immunoglobulin mu chain. Cell **20:**303-312.
222. **Davis MM, Calame K, Early PW, Livant DL, Joho R, Weissman IL, Hood L.** 1980. An immunoglobulin heavy-chain gene is formed by at least two recombinational events. Nature **283:**733-739.
223. **Sakano H, Maki R, Kurosawa Y, Roeder W, Tonegawa S.** 1980. Two types of somatic recombination are necessary for the generation of complete immunoglobulin heavy-chain genes. Nature **286:**676-683.
224. **Yaoita Y, Honjo T.** 1980. Deletion of immunoglobulin heavy chain genes from expressed allelic chromosome. Nature **286:**850-853.
225. **Oettinger MA, Schatz DG, Gorka C, Baltimore D.** 1990. RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination. Science **248:**1517-1523.
226. **Schatz DG, Oettinger MA, Baltimore D.** 1989. The V(D)J recombination activating gene, RAG-1. Cell **59:**1035-1048.
227. **Muramatsu M, Kinoshita K, Fagarasan S, Yamada S, Shinkai Y, Honjo T.** 2000. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. Cell **102:**553-563.
228. **Yu X, Tsibane T, McGraw PA, House FS, Keefer CJ, Hicar MD, Tumpey TM, Pappas C, Perrone LA, Martinez O, Stevens J, Wilson IA, Aguilar PV, Altschuler EL, Basler CF, Crowe JE, Jr.** 2008. Neutralizing antibodies derived from the B cells of 1918 influenza pandemic survivors. Nature **455:**532-536.
229. **Slifka MK, Antia R, Whitmire JK, Ahmed R.** 1998. Humoral immunity due to long-lived plasma cells. Immunity **8:**363-372.
230. **Scollay R, Smith J, Stauffer V.** 1986. Dynamics of early T cells: prothymocyte migration and proliferation in the adult mouse thymus. Immunol Rev **91:**129-157.
231. **Petrie HT.** 2003. Cell migration and the control of post-natal T-cell lymphopoiesis in the thymus. Nat Rev Immunol **3:**859-866.
232. **Pelus LM, Horowitz D, Cooper SC, King AG.** 2002. Peripheral blood stem cell mobilization. A role for CXC chemokines. Crit Rev Oncol Hematol **43:**257-275.
233. **Whetton AD, Graham GJ.** 1999. Homing and mobilization in the stem cell niche. Trends Cell Biol **9:**233-238.
234. **Papayannopoulou T, Nakamoto B.** 1993. Peripheralization of hemopoietic progenitors in primates treated with anti-VLA4 integrin. Proc Natl Acad Sci U S A **90:**9374-9378.
235. **Gaud G, Lesourne R, Love PE.** 2018. Regulatory mechanisms in T cell receptor signalling. Nat Rev Immunol doi:10.1038/s41577-018-0020-8.
236. **Kulinski JM, Tarakanova VL, Verbsky J.** 2013. Regulation of antiviral CD8 T-cell responses. Crit Rev Immunol **33:**477-488.

237. **Bajenoff M, Egen JG, Koo LY, Laugier JP, Brau F, Glaichenhaus N, Germain RN.** 2006. Stromal cell networks regulate lymphocyte entry, migration, and territoriality in lymph nodes. Immunity **25:**989-1001.
238. **Hickman HD, Takeda K, Skon CN, Murray FR, Hensley SE, Loomis J, Barber GN, Bennink JR, Yewdell JW.** 2008. Direct priming of antiviral CD8+ T cells in the peripheral interfollicular region of lymph nodes. Nat Immunol **9:**155-165.
239. **John B, Harris TH, Tait ED, Wilson EH, Gregg B, Ng LG, Mrass P, Roos DS, Dzierszinski F, Weninger W, Hunter CA.** 2009. Dynamic Imaging of CD8(+) T cells and dendritic cells during infection with Toxoplasma gondii. PLoS Pathog **5:**e1000505.
240. **Castellino F, Huang AY, Altan-Bonnet G, Stoll S, Scheinecker C, Germain RN.** 2006. Chemokines enhance immunity by guiding naive CD8+ T cells to sites of CD4+ T cell-dendritic cell interaction. Nature **440:**890-895.
241. **Semmling V, Lukacs-Kornek V, Thaiss CA, Quast T, Hochheiser K, Panzer U, Rossjohn J, Perlmutter P, Cao J, Godfrey DI, Savage PB, Knolle PA, Kolanus W, Forster I, Kurts C.** 2010. Alternative cross-priming through CCL17-CCR4-mediated attraction of CTLs toward NKT cell-licensed DCs. Nat Immunol **11:**313-320.
242. **Marko AJ, Miller RA, Kelman A, Frauwirth KA.** 2010. Induction of glucose metabolism in stimulated T lymphocytes is regulated by mitogen-activated protein kinase signaling. PLoS One **5:**e15425.
243. **Michalek RD, Rathmell JC.** 2010. The metabolic life and times of a T-cell. Immunol Rev **236:**190-202.
244. **Mescher MF, Curtsinger JM, Agarwal P, Casey KA, Gerner M, Hammerbeck CD, Popescu F, Xiao Z.** 2006. Signals required for programming effector and memory development by CD8+ T cells. Immunol Rev **211:**81-92.
245. **Parish IA, Kaech SM.** 2009. Diversity in CD8(+) T cell differentiation. Curr Opin Immunol **21:**291-297.
246. **Groom JR, Luster AD.** 2011. CXCR3 ligands: redundant, collaborative and antagonistic functions. Immunol Cell Biol **89:**207-215.
247. **Nakanishi Y, Lu B, Gerard C, Iwasaki A.** 2009. CD8(+) T lymphocyte mobilization to virus-infected tissue requires CD4(+) T-cell help. Nature **462:**510-513.
248. **Dustin ML, Long EO.** 2010. Cytotoxic immunological synapses. Immunol Rev **235:**24-34.
249. **Lopez JA, Susanto O, Jenkins MR, Lukoyanova N, Sutton VR, Law RH, Johnston A, Bird CH, Bird PI, Whisstock JC, Trapani JA, Saibil HR, Voskoboinik I.** 2013. Perforin forms transient pores on the target cell plasma membrane to facilitate rapid access of granzymes during killer cell attack. Blood **121:**2659-2668.

250. **de Saint Basile G, Menasche G, Fischer A.** 2010. Molecular mechanisms of biogenesis and exocytosis of cytotoxic granules. Nat Rev Immunol **10:**568-579.
251. **Ritter AT, Asano Y, Stinchcombe JC, Dieckmann NM, Chen BC, Gawden-Bone C, van Engelenburg S, Legant W, Gao L, Davidson MW, Betzig E, Lippincott-Schwartz J, Griffiths GM.** 2015. Actin depletion initiates events leading to granule secretion at the immunological synapse. Immunity **42:**864-876.
252. **Froelich CJ, Orth K, Turbov J, Seth P, Gottlieb R, Babior B, Shah GM, Bleackley RC, Dixit VM, Hanna W.** 1996. New paradigm for lymphocyte granule-mediated cytotoxicity. Target cells bind and internalize granzyme B, but an endosomolytic agent is necessary for cytosolic delivery and subsequent apoptosis. J Biol Chem **271:**29073-29079.
253. **Shi L, Mai S, Israels S, Browne K, Trapani JA, Greenberg AH.** 1997. Granzyme B (GraB) autonomously crosses the cell membrane and perforin initiates apoptosis and GraB nuclear localization. J Exp Med **185:**855-866.
254. **Hoption Cann SA, van Netten JP, van Netten C, Glover DW.** 2002. Spontaneous regression: a hidden treasure buried in time. Med Hypotheses **58:**115-119.
255. **Nauts HC, Fowler GA, Bogatko FH.** 1953. A review of the influence of bacterial infection and of bacterial products (Coley's toxins) on malignant tumors in man; a critical analysis of 30 inoperable cases treated by Coley's mixed toxins, in which diagnosis was confirmed by microscopic examination selected for special study. Acta Med Scand Suppl **276:**1-103.
256. **Coley WB.** 1991. The treatment of malignant tumors by repeated inoculations of erysipelas. With a report of ten original cases. 1893. Clin Orthop Relat Res**:**3-11.
257. **Carswell EA, Old LJ, Kassel RL, Green S, Fiore N, Williamson B.** 1975. An endotoxin-induced serum factor that causes necrosis of tumors. Proc Natl Acad Sci U S A **72:**3666-3670.
258. **Granger GA, Shacks SJ, Williams TW, Kolb WP.** 1969. Lymphocyte in vitro cytotoxicity: specific release of lymphotoxin-like materials from tuberculin-sensitive lymphoid cells. Nature **221:**1155-1157.
259. **Old LJ.** 1985. Tumor necrosis factor (TNF). Science **230:**630-632.
260. **Mannel DN, Moore RN, Mergenhagen SE.** 1980. Macrophages as a source of tumoricidal activity (tumor-necrotizing factor). Infect Immun **30:**523-530.
261. **Matthews N.** 1978. Tumour-necrosis factor from the rabbit. II. Production by monocytes. Br J Cancer **38:**310-315.
262. **Aggarwal BB, Kohr WJ, Hass PE, Moffat B, Spencer SA, Henzel WJ, Bringman TS, Nedwin GE, Goeddel DV, Harkins RN.** 1985. Human tumor necrosis factor. Production, purification, and characterization. J Biol Chem **260:**2345-2354.
263. **Williams TW, Granger GA.** 1968. Lymphocyte in vitro cytotoxicity: lymphotoxins of several mammalian species. Nature **219:**1076-1077.

264. **Beutler B, Cerami A.** 1988. Tumor necrosis, cachexia, shock, and inflammation: a common mediator. Annu Rev Biochem **57:**505-518.
265. **Beutler B, Cerami A.** 1989. The biology of cachectin/TNF--a primary mediator of the host response. Annu Rev Immunol **7:**625-655.
266. **Nedwin GE, Naylor SL, Sakaguchi AY, Smith D, Jarrett-Nedwin J, Pennica D, Goeddel DV, Gray PW.** 1985. Human lymphotoxin and tumor necrosis factor genes: structure, homology and chromosomal localization. Nucleic Acids Res **13:**6361-6373.
267. **Black RA, Rauch CT, Kozlosky CJ, Peschon JJ, Slack JL, Wolfson MF, Castner BJ, Stocking KL, Reddy P, Srinivasan S, Nelson N, Boiani N, Schooley KA, Gerhart M, Davis R, Fitzner JN, Johnson RS, Paxton RJ, March CJ, Cerretti DP.** 1997. A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. Nature **385:**729-733.
268. **Moss ML, Jin SL, Milla ME, Bickett DM, Burkhart W, Carter HL, Chen WJ, Clay WC, Didsbury JR, Hassler D, Hoffman CR, Kost TA, Lambert MH, Leesnitzer MA, McCauley P, McGeehan G, Mitchell J, Moyer M, Pahel G, Rocque W, Overton LK, Schoenen F, Seaton T, Su JL, Becherer JD, et al.** 1997. Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor-alpha. Nature **385:**733-736.
269. **Adrain C, Zettl M, Christova Y, Taylor N, Freeman M.** 2012. Tumor necrosis factor signaling requires iRhom2 to promote trafficking and activation of TACE. Science **335:**225-228.
270. **McIlwain DR, Lang PA, Maretzky T, Hamada K, Ohishi K, Maney SK, Berger T, Murthy A, Duncan G, Xu HC, Lang KS, Haussinger D, Wakeham A, Itie-Youten A, Khokha R, Ohashi PS, Blobel CP, Mak TW.** 2012. iRhom2 regulation of TACE controls TNF-mediated protection against Listeria and responses to LPS. Science **335:**229-232.
271. **Brockhaus M, Schoenfeld HJ, Schlaeger EJ, Hunziker W, Lesslauer W, Loetscher H.** 1990. Identification of two types of tumor necrosis factor receptors on human cell lines by monoclonal antibodies. Proc Natl Acad Sci U S A **87:**3127-3131.
272. **Hohmann HP, Brockhaus M, Baeuerle PA, Remy R, Kolbeck R, van Loon AP.** 1990. Expression of the types A and B tumor necrosis factor (TNF) receptors is independently regulated, and both receptors mediate activation of the transcription factor NF-kappa B. TNF alpha is not needed for induction of a biological effect via TNF receptors. J Biol Chem **265:**22409-22417.
273. **Sen R, Baltimore D.** 1986. Multiple nuclear factors interact with the immunoglobulin enhancer sequences. Cell **46:**705-716.
274. **Vallabhapurapu S, Karin M.** 2009. Regulation and function of NF-kappaB transcription factors in the immune system. Annu Rev Immunol **27:**693-733.
275. **Hayden MS, Ghosh S.** 2008. Shared principles in NF-kappaB signaling. Cell **132:**344-362.

276. **Vallabhapurapu S, Matsuzawa A, Zhang W, Tseng PH, Keats JJ, Wang H, Vignali DA, Bergsagel PL, Karin M.** 2008. Nonredundant and complementary functions of TRAF2 and TRAF3 in a ubiquitination cascade that activates NIK-dependent alternative NF-kappaB signaling. Nat Immunol **9:**1364-1370.
277. **Aggarwal BB.** 2003. Signalling pathways of the TNF superfamily: a double-edged sword. Nat Rev Immunol **3:**745-756.
278. **Ghosh S, May MJ, Kopp EB.** 1998. NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. Annu Rev Immunol **16:**225-260.
279. **Smale ST.** 2012. Dimer-specific regulatory mechanisms within the NF-kappaB family of transcription factors. Immunol Rev **246:**193-204.
280. **Karin M, Greten FR.** 2005. NF-kappaB: linking inflammation and immunity to cancer development and progression. Nat Rev Immunol **5:**749-759.
281. **Sun SC, Ley SC.** 2008. New insights into NF-kappaB regulation and function. Trends Immunol **29:**469-478.
282. **Bonizzi G, Karin M.** 2004. The two NF-kappaB activation pathways and their role in innate and adaptive immunity. Trends Immunol **25:**280-288.
283. **Sun SC.** 2017. The non-canonical NF-kappaB pathway in immunity and inflammation. Nat Rev Immunol **17:**545-558.
284. **Brenner D, Blaser H, Mak TW.** 2015. Regulation of tumour necrosis factor signalling: live or let die. Nat Rev Immunol **15:**362-374.
285. **Taniguchi K, Karin M.** 2018. NF-kappaB, inflammation, immunity and cancer: coming of age. Nat Rev Immunol **18:**309-324.
286. **Israel A.** 2010. The IKK complex, a central regulator of NF-kappaB activation. Cold Spring Harb Perspect Biol **2:**a000158.
287. **Xiao G, Harhaj EW, Sun SC.** 2001. NF-kappaB-inducing kinase regulates the processing of NF-kappaB2 p100. Mol Cell **7:**401-409.
288. **Senftleben U, Cao Y, Xiao G, Greten FR, Krahn G, Bonizzi G, Chen Y, Hu Y, Fong A, Sun SC, Karin M.** 2001. Activation by IKKalpha of a second, evolutionary conserved, NF-kappa B signaling pathway. Science **293:**1495-1499.
289. **De Santa F, Totaro MG, Prosperini E, Notarbartolo S, Testa G, Natoli G.** 2007. The histone H3 lysine-27 demethylase Jmjd3 links inflammation to inhibition of polycomb-mediated gene silencing. Cell **130:**1083-1094.
290. **Saccani S, Natoli G.** 2002. Dynamic changes in histone H3 Lys 9 methylation occurring at tightly regulated inducible inflammatory genes. Genes Dev **16:**2219-2224.
291. **van Essen D, Zhu Y, Saccani S.** 2010. A feed-forward circuit controlling inducible NF-kappaB target gene activation by promoter histone demethylation. Mol Cell **39:**750-760.
292. **Ramirez-Carrozzi VR, Braas D, Bhatt DM, Cheng CS, Hong C, Doty KR, Black JC, Hoffmann A, Carey M, Smale ST.** 2009. A unifying model for the selective

regulation of inducible transcription by CpG islands and nucleosome remodeling. Cell **138:**114-128.

293. **Panne D, Maniatis T, Harrison SC.** 2004. Crystal structure of ATF-2/c-Jun and IRF-3 bound to the interferon-beta enhancer. EMBO J **23:**4384-4393.
294. **Ashall L, Horton CA, Nelson DE, Paszek P, Harper CV, Sillitoe K, Ryan S, Spiller DG, Unitt JF, Broomhead DS, Kell DB, Rand DA, See V, White MR.** 2009. Pulsatile stimulation determines timing and specificity of NF-kappaB-dependent transcription. Science **324:**242-246.
295. **Hoffmann A, Levchenko A, Scott ML, Baltimore D.** 2002. The IkappaB-NF-kappaB signaling module: temporal control and selective gene activation. Science **298:**1241-1245.
296. **Tay S, Hughey JJ, Lee TK, Lipniacki T, Quake SR, Covert MW.** 2010. Single-cell NF-kappaB dynamics reveal digital activation and analogue information processing. Nature **466:**267-271.
297. **Chen G, Goeddel DV.** 2002. TNF-R1 signaling: a beautiful pathway. Science **296:**1634-1635.
298. **Hsu H, Xiong J, Goeddel DV.** 1995. The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. Cell **81:**495-504.
299. **Tartaglia LA, Ayres TM, Wong GH, Goeddel DV.** 1993. A novel domain within the 55 kd TNF receptor signals cell death. Cell **74:**845-853.
300. **Hsu H, Huang J, Shu HB, Baichwal V, Goeddel DV.** 1996. TNF-dependent recruitment of the protein kinase RIP to the TNF receptor-1 signaling complex. Immunity **4:**387-396.
301. **Rothe M, Sarma V, Dixit VM, Goeddel DV.** 1995. TRAF2-mediated activation of NF-kappa B by TNF receptor 2 and CD40. Science **269:**1424-1427.
302. **Ting AT, Pimentel-Muinos FX, Seed B.** 1996. RIP mediates tumor necrosis factor receptor 1 activation of NF-kappaB but not Fas/APO-1-initiated apoptosis. EMBO J **15:**6189-6196.
303. **Pasparakis M, Kousteni S, Peschon J, Kollias G.** 2000. Tumor necrosis factor and the p55TNF receptor are required for optimal development of the marginal sinus and for migration of follicular dendritic cell precursors into splenic follicles. Cell Immunol **201:**33-41.
304. **Engwerda CR, Ato M, Cotterell SE, Mynott TL, Tschannerl A, Gorak-Stolinska PM, Kaye PM.** 2002. A role for tumor necrosis factor-alpha in remodeling the splenic marginal zone during Leishmania donovani infection. Am J Pathol **161:**429-437.
305. **Calzascia T, Pellegrini M, Hall H, Sabbagh L, Ono N, Elford AR, Mak TW, Ohashi PS.** 2007. TNF-alpha is critical for antitumor but not antiviral T cell immunity in mice. J Clin Invest **117:**3833-3845.

306. **Wang T, Town T, Alexopoulou L, Anderson JF, Fikrig E, Flavell RA.** 2004. Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis. Nat Med **10:**1366-1373.
307. **Lundberg P, Welander PV, Edwards CK, 3rd, van Rooijen N, Cantin E.** 2007. Tumor necrosis factor (TNF) protects resistant C57BL/6 mice against herpes simplex virus-induced encephalitis independently of signaling via TNF receptor 1 or 2. J Virol **81:**1451-1460.
308. **Vilela MC, Lima GK, Rodrigues DH, Lacerda-Queiroz N, Mansur DS, de Miranda AS, Rachid MA, Kroon EG, Vieira LQ, Campos MA, Teixeira MM, Teixeira AL.** 2010. TNFR1 plays a critical role in the control of severe HSV-1 encephalitis. Neurosci Lett **479:**58-62.
309. **Fehr T, Bachmann MF, Bluethmann H, Kikutani H, Hengartner H, Zinkernagel RM.** 1996. T-independent activation of B cells by vesicular stomatitis virus: no evidence for the need of a second signal. Cell Immunol **168:**184-192.
310. **Rothe J, Lesslauer W, Lotscher H, Lang Y, Koebel P, Kontgen F, Althage A, Zinkernagel R, Steinmetz M, Bluethmann H.** 1993. Mice lacking the tumour necrosis factor receptor 1 are resistant to TNF-mediated toxicity but highly susceptible to infection by Listeria monocytogenes. Nature **364:**798-802.
311. **Pfeffer K, Matsuyama T, Kundig TM, Wakeham A, Kishihara K, Shahinian A, Wiegmann K, Ohashi PS, Kronke M, Mak TW.** 1993. Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to L. monocytogenes infection. Cell **73:**457-467.
312. **Botha T, Ryffel B.** 2003. Reactivation of latent tuberculosis infection in TNF-deficient mice. J Immunol **171:**3110-3118.
313. **Wellmer A, Gerber J, Ragheb J, Zysk G, Kunst T, Smirnov A, Bruck W, Nau R.** 2001. Effect of deficiency of tumor necrosis factor alpha or both of its receptors on Streptococcus pneumoniae central nervous system infection and peritonitis. Infect Immun **69:**6881-6886.
314. **Akagi T, Motegi M, Tamura A, Suzuki R, Hosokawa Y, Suzuki H, Ota H, Nakamura S, Morishima Y, Taniwaki M, Seto M.** 1999. A novel gene, MALT1 at 18q21, is involved in t(11;18) (q21;q21) found in low-grade B-cell lymphoma of mucosa-associated lymphoid tissue. Oncogene **18:**5785-5794.
315. **Ruland J, Duncan GS, Elia A, del Barco Barrantes I, Nguyen L, Plyte S, Millar DG, Bouchard D, Wakeham A, Ohashi PS, Mak TW.** 2001. Bcl10 is a positive regulator of antigen receptor-induced activation of NF-kappaB and neural tube closure. Cell **104:**33-42.
316. **Ruland J, Duncan GS, Wakeham A, Mak TW.** 2003. Differential requirement for Malt1 in T and B cell antigen receptor signaling. Immunity **19:**749-758.
317. **Willis TG, Jadayel DM, Du MQ, Peng H, Perry AR, Abdul-Rauf M, Price H, Karran L, Majekodunmi O, Wlodarska I, Pan L, Crook T, Hamoudi R, Isaacson**

**PG, Dyer MJ.** 1999. Bcl10 is involved in t(1;14)(p22;q32) of MALT B cell lymphoma and mutated in multiple tumor types. Cell **96:**35-45.

318. **Turvey SE, Durandy A, Fischer A, Fung SY, Geha RS, Gewies A, Giese T, Greil J, Keller B, McKinnon ML, Neven B, Rozmus J, Ruland J, Snow AL, Stepensky P, Warnatz K.** 2014. The CARD11-BCL10-MALT1 (CBM) signalosome complex: Stepping into the limelight of human primary immunodeficiency. J Allergy Clin Immunol **134:**276-284.
319. **Lucas PC, Yonezumi M, Inohara N, McAllister-Lucas LM, Abazeed ME, Chen FF, Yamaoka S, Seto M, Nunez G.** 2001. Bcl10 and MALT1, independent targets of chromosomal translocation in malt lymphoma, cooperate in a novel NF-kappa B signaling pathway. J Biol Chem **276:**19012-19019.
320. **Uren AG, O'Rourke K, Aravind LA, Pisabarro MT, Seshagiri S, Koonin EV, Dixit VM.** 2000. Identification of paracaspases and metacaspases: two ancient families of caspase-like proteins, one of which plays a key role in MALT lymphoma. Mol Cell **6:**961-967.
321. **Meininger I, Krappmann D.** 2016. Lymphocyte signaling and activation by the CARMA1-BCL10-MALT1 signalosome. Biol Chem **397:**1315-1333.
322. **Coornaert B, Baens M, Heyninck K, Bekaert T, Haegman M, Staal J, Sun L, Chen ZJ, Marynen P, Beyaert R.** 2008. T cell antigen receptor stimulation induces MALT1 paracaspase-mediated cleavage of the NF-kappaB inhibitor A20. Nat Immunol **9:**263-271.
323. **Rebeaud F, Hailfinger S, Posevitz-Fejfar A, Tapernoux M, Moser R, Rueda D, Gaide O, Guzzardi M, Iancu EM, Rufer N, Fasel N, Thome M.** 2008. The proteolytic activity of the paracaspase MALT1 is key in T cell activation. Nat Immunol **9:**272-281.
324. **Lu HY, Bauman BM, Arjunaraja S, Dorjbal B, Milner JD, Snow AL, Turvey SE.** 2018. The CBM-opathies-A Rapidly Expanding Spectrum of Human Inborn Errors of Immunity Caused by Mutations in the CARD11-BCL10-MALT1 Complex. Front Immunol **9:**2078.
325. **Hamoudi RA, Appert A, Ye H, Ruskone-Fourmestraux A, Streubel B, Chott A, Raderer M, Gong L, Wlodarska I, De Wolf-Peeters C, MacLennan KA, de Leval L, Isaacson PG, Du MQ.** 2010. Differential expression of NF-kappaB target genes in MALT lymphoma with and without chromosome translocation: insights into molecular mechanism. Leukemia **24:**1487-1497.
326. **Punwani D, Wang H, Chan AY, Cowan MJ, Mallott J, Sunderam U, Mollenauer M, Srinivasan R, Brenner SE, Mulder A, Claas FH, Weiss A, Puck JM.** 2015. Combined immunodeficiency due to MALT1 mutations, treated by hematopoietic cell transplantation. J Clin Immunol **35:**135-146.
327. **Rozmus J, McDonald R, Fung SY, Del Bel KL, Roden J, Senger C, Schultz KR, McKinnon ML, Davis J, Turvey SE.** 2016. Successful clinical treatment and

functional immunological normalization of human MALT1 deficiency following hematopoietic stem cell transplantation. Clin Immunol **168:**1-5.

328. **Lee P, Zhu Z, Hachmann J, Nojima T, Kitamura D, Salvesen G, Rickert RC.** 2017. Differing Requirements for MALT1 Function in Peripheral B Cell Survival and Differentiation. J Immunol **198:**1066-1080.
329. **Xue L, Morris SW, Orihuela C, Tuomanen E, Cui X, Wen R, Wang D.** 2003. Defective development and function of Bcl10-deficient follicular, marginal zone and B1 B cells. Nat Immunol **4:**857-865.
330. **Pasparakis M, Alexopoulou L, Episkopou V, Kollias G.** 1996. Immune and inflammatory responses in TNF alpha-deficient mice: a critical requirement for TNF alpha in the formation of primary B cell follicles, follicular dendritic cell networks and germinal centers, and in the maturation of the humoral immune response. J Exp Med **184:**1397-1411.
331. **Erickson SL, de Sauvage FJ, Kikly K, Carver-Moore K, Pitts-Meek S, Gillett N, Sheehan KC, Schreiber RD, Goeddel DV, Moore MW.** 1994. Decreased sensitivity to tumour-necrosis factor but normal T-cell development in TNF receptor-2-deficient mice. Nature **372:**560-563.
332. **Fung-Leung WP, Schilham MW, Rahemtulla A, Kundig TM, Vollenweider M, Potter J, van Ewijk W, Mak TW.** 1991. CD8 is needed for development of cytotoxic T cells but not helper T cells. Cell **65:**443-449.
333. **Mombaerts P, Iacomini J, Johnson RS, Herrup K, Tonegawa S, Papaioannou VE.** 1992. RAG-1-deficient mice have no mature B and T lymphocytes. Cell **68:**869-877.
334. **Oetke C, Vinson MC, Jones C, Crocker PR.** 2006. Sialoadhesin-deficient mice exhibit subtle changes in B- and T-cell populations and reduced immunoglobulin M levels. Mol Cell Biol **26:**1549-1557.
335. **Iwata A, Nishio K, Winn RK, Chi EY, Henderson WR, Jr., Harlan JM.** 2003. A broad-spectrum caspase inhibitor attenuates allergic airway inflammation in murine asthma model. J Immunol **170:**3386-3391.
336. **Hotchkiss RS, Chang KC, Swanson PE, Tinsley KW, Hui JJ, Klender P, Xanthoudakis S, Roy S, Black C, Grimm E, Aspiotis R, Han Y, Nicholson DW, Karl IE.** 2000. Caspase inhibitors improve survival in sepsis: a critical role of the lymphocyte. Nature Immunology **1:**496-501.
337. **Xu HC, Huang J, Khairnar V, Duhan V, Pandyra AA, Grusdat M, Shinde P, McIlwain DR, Maney SK, Gommerman J, Lohning M, Ohashi PS, Mak TW, Pieper K, Sic H, Speletas M, Eibel H, Ware CF, Tumanov AV, Kruglov AA, Nedospasov SA, Haussinger D, Recher M, Lang KS, Lang PA.** 2015. Deficiency of the B cell-activating factor receptor results in limited CD169+ macrophage function during viral infection. J Virol **89:**4748-4759.

338. **Kim D, Pertea G, Trapnell C, Pimentel H, Kelley R, Salzberg SL.** 2013. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. Genome Biol **14:**R36.
339. **Yates A, Akanni W, Amode MR, Barrell D, Billis K, Carvalho-Silva D, Cummins C, Clapham P, Fitzgerald S, Gil L, Giron CG, Gordon L, Hourlier T, Hunt SE, Janacek SH, Johnson N, Juettemann T, Keenan S, Lavidas I, Martin FJ, Maurel T, McLaren W, Murphy DN, Nag R, Nuhn M, Parker A, Patricio M, Pignatelli M, Rahtz M, Riat HS, Sheppard D, Taylor K, Thormann A, Vullo A, Wilder SP, Zadissa A, Birney E, Harrow J, Muffato M, Perry E, Ruffier M, Spudich G, Trevanion SJ, Cunningham F, Aken BL, Zerbino DR, Flicek P.** 2016. Ensembl 2016. Nucleic Acids Res **44:**D710-716.
340. **Anders S, Pyl PT, Huber W.** 2015. HTSeq--a Python framework to work with high-throughput sequencing data. Bioinformatics **31:**166-169.
341. **Robinson MD, McCarthy DJ, Smyth GK.** 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics **26:**139-140.
342. **Kanehisa M, Sato Y, Kawashima M, Furumichi M, Tanabe M.** 2016. KEGG as a reference resource for gene and protein annotation. Nucleic Acids Res **44:**D457-462.
343. **Bailey TL, Williams N, Misleh C, Li WW.** 2006. MEME: discovering and analyzing DNA and protein sequence motifs. Nucleic Acids Res **34:**W369-373.
344. **Weirauch MT, Yang A, Albu M, Cote AG, Montenegro-Montero A, Drewe P, Najafabadi HS, Lambert SA, Mann I, Cook K, Zheng H, Goity A, van Bakel H, Lozano JC, Galli M, Lewsey MG, Huang E, Mukherjee T, Chen X, Reece-Hoyes JS, Govindarajan S, Shaulsky G, Walhout AJ, Bouget FY, Ratsch G, Larrondo LF, Ecker JR, Hughes TR.** 2014. Determination and inference of eukaryotic transcription factor sequence specificity. Cell **158:**1431-1443.
345. **Moseman EA, Iannacone M, Bosurgi L, Tonti E, Chevrier N, Tumanov A, Fu YX, Hacohen N, von Andrian UH.** 2012. B cell maintenance of subcapsular sinus macrophages protects against a fatal viral infection independent of adaptive immunity. Immunity **36:**415-426.
346. **Garcia Z, Lemaitre F, van Rooijen N, Albert ML, Levy Y, Schwartz O, Bousso P.** 2012. Subcapsular sinus macrophages promote NK cell accumulation and activation in response to lymph-borne viral particles. Blood **120:**4744-4750.
347. **Asano K, Nabeyama A, Miyake Y, Qiu CH, Kurita A, Tomura M, Kanagawa O, Fujii S, Tanaka M.** 2011. CD169-positive macrophages dominate antitumor immunity by crosspresenting dead cell-associated antigens. Immunity **34:**85-95.
348. **Brandes M, Klauschen F, Kuchen S, Germain RN.** 2013. A systems analysis identifies a feedforward inflammatory circuit leading to lethal influenza infection. Cell **154:**197-212.

349. **Petrofsky M, Bermudez LE.** 1999. Neutrophils from Mycobacterium avium-infected mice produce TNF-alpha, IL-12, and IL-1 beta and have a putative role in early host response. Clin Immunol **91:**354-358.
350. **Fischer MA, Davies ML, Reider IE, Heipertz EL, Epler MR, Sei JJ, Ingersoll MA, Rooijen NV, Randolph GJ, Norbury CC.** 2011. CD11b(+), Ly6G(+) cells produce type I interferon and exhibit tissue protective properties following peripheral virus infection. PLoS Pathog **7:**e1002374.
351. **Probst HC, Tschannen K, Odermatt B, Schwendener R, Zinkernagel RM, Van Den Broek M.** 2005. Histological analysis of CD11c-DTR/GFP mice after in vivo depletion of dendritic cells. Clin Exp Immunol **141:**398-404.
352. **Pearce AF, Lyles DS.** 2009. Vesicular stomatitis virus induces apoptosis primarily through Bak rather than Bax by inactivating Mcl-1 and Bcl-XL. J Virol **83:**9102-9112.
353. **Marienfeld R, May MJ, Berberich I, Serfling E, Ghosh S, Neumann M.** 2003. RelB forms transcriptionally inactive complexes with RelA/p65. J Biol Chem **278:**19852-19860.
354. **Hailfinger S, Nogai H, Pelzer C, Jaworski M, Cabalzar K, Charton JE, Guzzardi M, Decaillet C, Grau M, Dorken B, Lenz P, Lenz G, Thome M.** 2011. Malt1-dependent RelB cleavage promotes canonical NF-kappaB activation in lymphocytes and lymphoma cell lines. Proc Natl Acad Sci U S A **108:**14596-14601.
355. **Brustle A, Brenner D, Knobbe CB, Lang PA, Virtanen C, Hershenfield BM, Reardon C, Lacher SM, Ruland J, Ohashi PS, Mak TW.** 2012. The NF-kappaB regulator MALT1 determines the encephalitogenic potential of Th17 cells. J Clin Invest **122:**4698-4709.
356. **Poeck H, Bscheider M, Gross O, Finger K, Roth S, Rebsamen M, Hannesschlager N, Schlee M, Rothenfusser S, Barchet W, Kato H, Akira S, Inoue S, Endres S, Peschel C, Hartmann G, Hornung V, Ruland J.** 2010. Recognition of RNA virus by RIG-I results in activation of CARD9 and inflammasome signaling for interleukin 1 beta production. Nature Immunology **11:**63-69.
357. **Shinde PV, Xu HC, Maney SK, Kloetgen A, Namineni S, Zhuang Y, Honke N, Shaabani N, Bellora N, Doerrenberg M, Trilling M, Pozdeev VI, van Rooijen N, Scheu S, Pfeffer K, Crocker PR, Tanaka M, Duggimpudi S, Knolle P, Heikenwalder M, Ruland J, Mak TW, Brenner D, Pandyra AA, Hoell JI, Borkhardt A, Haussinger D, Lang KS, Lang PA.** 2018. Tumor Necrosis Factor-Mediated Survival of CD169(+) Cells Promotes Immune Activation during Vesicular Stomatitis Virus Infection. J Virol **92**.
358. **Barber GN.** 2005. VSV-tumor selective replication and protein translation. Oncogene **24:**7710-7719.
359. **Balachandran S, Barber GN.** 2004. Defective translational control facilitates vesicular stomatitis virus oncolysis. Cancer Cell **5:**51-65.

360. **Meraz MA, White JM, Sheehan KC, Bach EA, Rodig SJ, Dighe AS, Kaplan DH, Riley JK, Greenlund AC, Campbell D, Carver-Moore K, DuBois RN, Clark R, Aguet M, Schreiber RD.** 1996. Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. Cell **84:**431-442.
361. **Thomsen AR, Nansen A, Andersen C, Johansen J, Marker O, Christensen JP.** 1997. Cooperation of B cells and T cells is required for survival of mice infected with vesicular stomatitis virus. Int Immunol **9:**1757-1766.
362. **Guidotti LG, Chisari FV.** 2000. Cytokine-mediated control of viral infections. Virology **273:**221-227.
363. **Mogensen TH, Paludan SR.** 2001. Molecular pathways in virus-induced cytokine production. Microbiol Mol Biol Rev **65:**131-150.
364. **Czarniecki CW, Fennie CW, Powers DB, Estell DA.** 1984. Synergistic antiviral and antiproliferative activities of Escherichia coli-derived human alpha, beta, and gamma interferons. J Virol **49:**490-496.
365. **Mestan J, Brockhaus M, Kirchner H, Jacobsen H.** 1988. Antiviral activity of tumour necrosis factor. Synergism with interferons and induction of oligo-2',5'-adenylate synthetase. J Gen Virol **69 ( Pt 12):**3113-3120.
366. **Iwasaki A, Medzhitov R.** 2004. Toll-like receptor control of the adaptive immune responses. Nat Immunol **5:**987-995.
367. **Takeda K, Akira S.** 2005. Toll-like receptors in innate immunity. Int Immunol **17:**1-14.
368. **Zhou S, Kurt-Jones EA, Fitzgerald KA, Wang JP, Cerny AM, Chan M, Finberg RW.** 2007. Role of MyD88 in route-dependent susceptibility to vesicular stomatitis virus infection. J Immunol **178:**5173-5181.
369. **Junt T, Scandella E, Ludewig B.** 2008. Form follows function: lymphoid tissue microarchitecture in antimicrobial immune defence. Nat Rev Immunol **8:**764-775.
370. **Dev A, Iyer S, Razani B, Cheng G.** 2011. NF-kappaB and innate immunity. Curr Top Microbiol Immunol **349:**115-143.
371. **Lenardo MJ, Fan CM, Maniatis T, Baltimore D.** 1989. The involvement of NF-kappa B in beta-interferon gene regulation reveals its role as widely inducible mediator of signal transduction. Cell **57:**287-294.
372. **Hiscott J, Alper D, Cohen L, Leblanc JF, Sportza L, Wong A, Xanthoudakis S.** 1989. Induction of human interferon gene expression is associated with a nuclear factor that interacts with the NF-kappa B site of the human immunodeficiency virus enhancer. J Virol **63:**2557-2566.
373. **Wang X, Hussain S, Wang EJ, Li MO, Garcia-Sastre A, Beg AA.** 2007. Lack of essential role of NF-kappa B p50, RelA, and cRel subunits in virus-induced type 1 IFN expression. J Immunol **178:**6770-6776.

374. **Wang J, Basagoudanavar SH, Wang X, Hopewell E, Albrecht R, Garcia-Sastre A, Balachandran S, Beg AA.** 2010. NF-kappa B RelA subunit is crucial for early IFN-beta expression and resistance to RNA virus replication. J Immunol **185:**1720-1729.
375. **Wang X, Wang J, Zheng H, Xie M, Hopewell EL, Albrecht RA, Nogusa S, Garcia-Sastre A, Balachandran S, Beg AA.** 2014. Differential requirement for the IKKbeta/NF-kappaB signaling module in regulating TLR- versus RLR-induced type 1 IFN expression in dendritic cells. J Immunol **193:**2538-2545.
376. **Sghaier I, Zidi S, Mouelhi L, Dabbech R, Ghazouani E, Brochot E, Stayoussef M, Yacoubi-Loueslati B.** 2015. The relationship between TNF alpha gene polymorphisms (-238/-308), TNF RII VNTR (p75) and outcomes of hepatitis B virus infection in Tunisian population. Gene **568:**140-145.
377. **Xu J, Zhang S, Zhang Z, Fu L, Zheng Q, Wang J, Lu S, Du J.** 2013. TNF-alpha promoter region polymorphisms affect HBV virus clearance in southern Chinese. Clin Chim Acta **425:**90-92.
378. **Fletcher NF, Sutaria R, Jo J, Barnes A, Blahova M, Meredith LW, Cosset FL, Curbishley SM, Adams DH, Bertoletti A, McKeating JA.** 2014. Activated macrophages promote hepatitis C virus entry in a tumor necrosis factor-dependent manner. Hepatology **59:**1320-1330.
379. **Park J, Kang W, Ryu SW, Kim WI, Chang DY, Lee DH, Park DY, Choi YH, Choi K, Shin EC, Choi C.** 2012. Hepatitis C virus infection enhances TNFalpha-induced cell death via suppression of NF-kappaB. Hepatology **56:**831-840.
380. **Seo SH, Webster RG.** 2002. Tumor necrosis factor alpha exerts powerful anti-influenza virus effects in lung epithelial cells. J Virol **76:**1071-1076.
381. **Ebert G, Preston S, Allison C, Cooney J, Toe JG, Stutz MD, Ojaimi S, Scott HW, Baschuk N, Nachbur U, Torresi J, Chin R, Colledge D, Li X, Warner N, Revill P, Bowden S, Silke J, Begley CG, Pellegrini M.** 2015. Cellular inhibitor of apoptosis proteins prevent clearance of hepatitis B virus. Proc Natl Acad Sci U S A **112:**5797-5802.
382. **Ebert G, Allison C, Preston S, Cooney J, Toe JG, Stutz MD, Ojaimi S, Baschuk N, Nachbur U, Torresi J, Silke J, Begley CG, Pellegrini M.** 2015. Eliminating hepatitis B by antagonizing cellular inhibitors of apoptosis. Proc Natl Acad Sci U S A **112:**5803-5808.
383. **Malakhov MP, Malakhova OA, Kim KI, Ritchie KJ, Zhang DE.** 2002. UBP43 (USP18) specifically removes ISG15 from conjugated proteins. J Biol Chem **277:**9976-9981.
384. **Summers C, Rankin SM, Condliffe AM, Singh N, Peters AM, Chilvers ER.** 2010. Neutrophil kinetics in health and disease. Trends Immunol **31:**318-324.
385. **Tamassia N, Le Moigne V, Rossato M, Donini M, McCartney S, Calzetti F, Colonna M, Bazzoni F, Cassatella MA.** 2008. Activation of an immunoregulatory

and antiviral gene expression program in poly(I:C)-transfected human neutrophils. J Immunol **181:**6563-6573.

386. **Wang JP, Bowen GN, Padden C, Cerny A, Finberg RW, Newburger PE, Kurt-Jones EA.** 2008. Toll-like receptor-mediated activation of neutrophils by influenza A virus. Blood **112:**2028-2034.
387. **Faber M, Bette M, Preuss MA, Pulmanausahakul R, Rehnelt J, Schnell MJ, Dietzschold B, Weihe E.** 2005. Overexpression of tumor necrosis factor alpha by a recombinant rabies virus attenuates replication in neurons and prevents lethal infection in mice. J Virol **79:**15405-15416.
388. **Guidotti LG, Borrow P, Brown A, McClary H, Koch R, Chisari FV.** 1999. Noncytopathic clearance of lymphocytic choriomeningitis virus from the hepatocyte. J Exp Med **189:**1555-1564.
389. **Guidotti LG, Ishikawa T, Hobbs MV, Matzke B, Schreiber R, Chisari FV.** 1996. Intracellular inactivation of the hepatitis B virus by cytotoxic T lymphocytes. Immunity **4:**25-36.
390. **Nijman SM, Luna-Vargas MP, Velds A, Brummelkamp TR, Dirac AM, Sixma TK, Bernards R.** 2005. A genomic and functional inventory of deubiquitinating enzymes. Cell **123:**773-786.
391. **Sun SC.** 2008. Deubiquitylation and regulation of the immune response. Nat Rev Immunol **8:**501-511.
392. **Wertz IE, O'Rourke KM, Zhou H, Eby M, Aravind L, Seshagiri S, Wu P, Wiesmann C, Baker R, Boone DL, Ma A, Koonin EV, Dixit VM.** 2004. De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-kappaB signalling. Nature **430:**694-699.
393. **Chen LF, Greene WC.** 2004. Shaping the nuclear action of NF-kappaB. Nat Rev Mol Cell Biol **5:**392-401.
394. **Balachandran S, Roberts PC, Kipperman T, Bhalla KN, Compans RW, Archer DR, Barber GN.** 2000. Alpha/beta interferons potentiate virus-induced apoptosis through activation of the FADD/Caspase-8 death signaling pathway. J Virol **74:**1513-1523.
395. **Czabotar PE, Lessene G, Strasser A, Adams JM.** 2014. Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy. Nat Rev Mol Cell Biol **15:**49-63.

## 7. Acknowledgement

It's been five long fruitful years accompanied with immense self-discovery and adventures.

First of all, I would like to thank Philipp, my supervisor, and mentor for the most critical stage of my career. Your devotion towards work as a scientist and a mentor has been an inspiration to me the whole time. You are bestowed with unfathomable energy and a constant struggle to achieve the best which I admire the most. Thank you for accepting me as a student and letting me be a part of a wonderful lab.

I thank Prof. Hegemann to patiently listening to my lengthy talks and providing critical inputs on the projects. Also, I am greatly indebted to Dr. Inge Krümpelbeck and my graduate school, Molecules of Infection (MOI 2) from Jürgen Manchot Foundation for supporting my PhD studies.

I would like to thank Sathish, who helped me to settle in Düsseldorf even before I left India and being a wonderful lab mate. Krissi and Melanie; special thanks for all the lovely meals, night outs and of course all the German translations you helped me with. Junnat and Vitaly, I am indebted to both of you for introducing a different side of the world. The Lab was always fun when you were around. I loved the randomness of all the crazy discussions we had.

I cannot say that I knew a lot about China but both of you helped me discover the true China and her culture; Jun and Yuan I cannot thank you enough. Thanks for introducing to real Chinese food (very different than what you get in India). Bala, I always felt home when you are around, it gives a soothing feeling when another Indian as calm and determined as you are around. Chris, thank you so much for your help in the lab.

Marc, wonderful you, I am sure except lab work you can do anything. Mathias, thanks for being a great student (terrible in the beginning though) and a very good friend at the end. I admire your capacity for alcohol consumption.

Finally, I would like to thank my family Mom, Dad, and Amruta for being there all the time in need and the support for all these years.