Delayed viral propagation during initial phase of infection leads to viral persistence

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Contents

Declaration and statement of contribution

I, Ruifeng Wang, 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.

I was involved in execution of the experiments, analysis, writing and preparation of the manuscript which consists 20% of the published manuscript.

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

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

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Abbreviations

ADCC: Antibody dependent cellular cytotoxicity

APCs: Antigen presenting cells

AP-1: Activator protein 1

BCR: B cell receptor

BIRs: Baculoviral inhibitor repeats

BM: Bone marrow

BTK: Bruton tyrosine kinase

CARDs: Caspase activation and recruitment domains

CD: Cluster of differentiation

cDCs: Conventional dendritic cell

CDC1s: Conventional type 1 DCs

CDC2s: Conventional type 2 DCs

CDP: Common DC precursor

CLP: Common lymphoid progenitor

CMP: Common myeloid progenitor

CNS: Central nervous system

CTD: C terminal domain

CTL: Cytotoxic T lymphocytes

DCs: Dendritic cells

DD: Death domain

EMCV: Encephalomyocarditis virus

ERK: Extracellular signal-regulated kinases

ETP: Thymic progenitor

FasL: Fas ligand

FOB: Follicular B cells

GC: Germinal center

GP: Glycoproteins

HSC: Hematopoietic stem cell

IFN: Interferon

iNKT: Invariant NKT

IκB: Inhibitor of NF-κB

IKK: IκB kinase

IKK-γ: IκB kinase gamma

IKK-ξ: IκB kinase epsilon

IL: Interleukin

IP3: Inositol trisphosphate

IRAKs: Interleukin-1 receptor (IL-1R) associated kinases

IRF3: Interferon regulatory factor 3

IRF7: Interferon regulatory factor 7

IRG: Intergenic region

ISGs: IFN stimulated genes

ISRE: IFN-stimulated response elements

JAK1: Janus kinase 1

TYK2: Tyrosine kinase 2

JEV: Japanese encephalitis virus

LCMV: Lymphocytic choriomeningitis virus

LCs: Langerhans cells

LN: Lymph node

LRR: leucine-rich-repeat

LPS: Lipopolysaccharide

MAPK: Mitogen-activated protein kinases

MDA5: Melanoma Differentiation-Associated protein 5

MDP: Macrophage and DC precursors

MHC: Major histocompatibility complex

MHC-I: MHC class I

MHC-II: MHC class II

MKK6: MAP kinase kinase 6

MNoV: Murine Norovirus

MoDCs: Monocyte-derived DCs

MPP: Multi-Potent Progenitor

MPS: Mononuclear phagocytic system

MyD88: Myeloid differentiation primary response 88

mTOR: Mammalian target of rapamycin

Mx1: Mycovirus resistance 1

MZ: Marginal zone

NF-κB: Nuclear factor kappa-B

NKT: Natural killer T

NIK: NF-κB inducing kinase

NLRs: NOD-like Receptors

NOD: Nucleotide-binding and oligomerization domain

NP: Nucleoprotein

NTD: N terminal domain

NFAT: Nuclear factor of activated T-cells

PAMP: Pathogen associated molecular pattern

pDCs: Plasmacytoid dendritic cells

pMHC-I: Peptide-MHC-I complexes

pMHC-II: Peptide-MHC-II complexes

PRR: Pattern recognition receptors

PYD: Pyrin domain

RIG-I: Retinoic acid inducible gene 1

RIP1: Receptor-interacting serine/threonine-protein1

RIP3: Receptor-interacting serine/threonine-protein3

RLRs: RIG-I like receptors

ROS: Reactive oxygen species

SARM: Sterile-alpha and Armadillo motif containing protein

SCF: Stem cell factor

SeV: Sendai virus

STAT: Signal activator and transducer

TAB1: TGF-Beta Activated Kinase 1 (MAP3K7) Binding Protein1

TAB2: TGF-Beta Activated Kinase 1 (MAP3K7) Binding Protein2

TAB3: TGF-Beta Activated Kinase 1 (MAP3K7) Binding Protein3

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

Tfh: Follicular helper T cells

TIR: Toll/IL-1R homology

TIRAP: Toll/interleukin-1 receptor domain-containing adapter protein

TLR: Toll-like receptor

TNF: Tumor necrosis factor

TRADD: TNF receptor type-1 associated death domain

TRAF: TNF receptor-associated factor

TRAF: Translocating chain-associated membrane protein

TRAF3: Translocating chain-associated membrane protein3

TRAF6: Translocating chain-associated membrane protein6

Treg: Tegulatory T cells

TRIF: TIR-domain-containing adapter-inducing interferon-β

TSC1/2: Tuberous sclerosis proteins 1 and 2

VSV: Vesicular stomatitis virus

UTR: Untranslated region

WB: Western blot

Summary

Lymphocytic choriomeningitis virus (LCMV) is an important reason of neurologic disease in humans. Rodents are the principal host of LCMV. The model of LCMV infection is one of the widely used animal models for viral infections. Typically, LCMV infection induces the secretion of IFN-I by APCs, which are able to stimulate the expression of co-stimulatory molecules. LCMV infection also induces a series of inflammatory responses. IFN-I and co-stimulatory molecules play an important role in the activation of adaptive immune responses, which are mainly mediated by the cytotoxic T lymphocytes (CTLs). Virus-induced cytotoxic T lymphocytes (CTLs) expand in lymphoid tissues, some migrate to nonlymphoid tissues to deal with the pathogen. Various subsets of LCMV are able to induce different immune responses which lead to the formation of chronic and acute infections in mice. Thus, the activation of innate immunity to initiate and maintain adaptive immune responses is critical for the control of viral persistence.

LCMV genome consists of two single-stranded RNA segments, L and S, which use an ambisense coding strategy to complete the viral RNA replication and protein translation. The S segment encodes the structural proteins: nucleoprotein (NP), and two glycoproteins, GP-1 and GP-2. The L segment directs synthesis of the viral polymerase L and a small polypeptide Z. The viral RNA associated with the NP protein and the viral polymerase composes the viral ribonucleoprotein (RNP), which is the minimal infectious unit. Different proteins have distinct functions during viral infection. However, different point mutations in the proteins of LCMV result in the formation of acute and chronic infection,

Thus, we focus on two different strains of LCMV, WE and Docile, which are able to induce acute and chronic infection, respectively. We found that WE induced much more IFNα production

and CTL expansion, while Docile did less so. Additionally, viral replication is faster in WE infected mice and APCs at early time points. Furthermore, we have shown that viral entry is faster during infection with LCMV WE. These findings indicate that delayed viral entry and viral replication during the initial phase of infection leads to viral persistence.

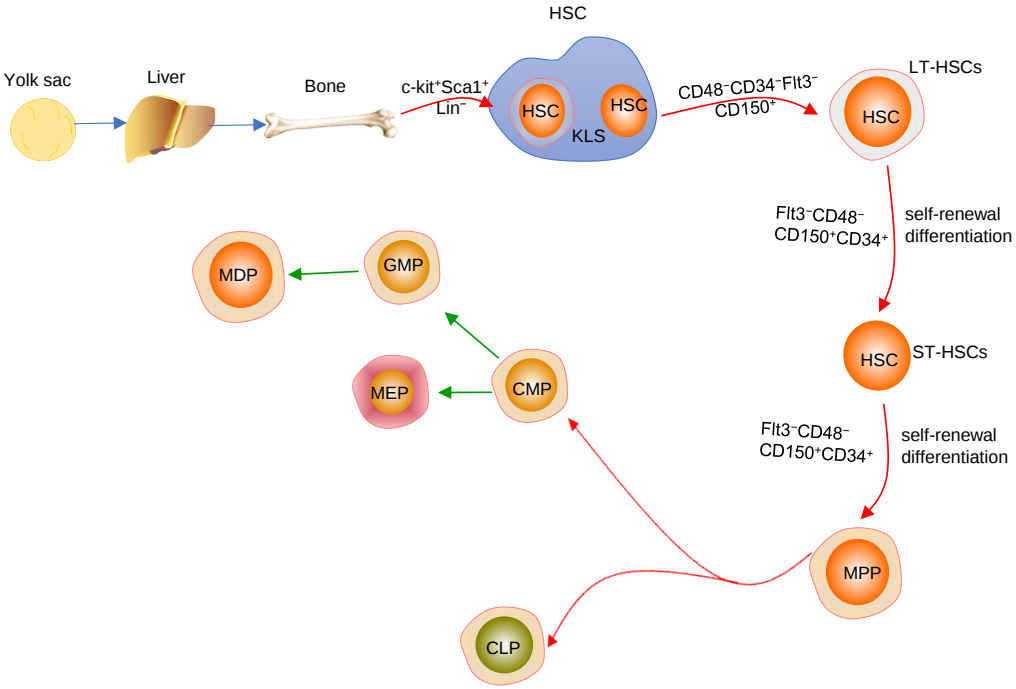
1 Introduction

1.1 Immune system

Pathogenic and non-pathogenic microbes exist in every corner of the world, and some of the microorganisms threat normal homeostasis and host functions through different mechanisms of replication and propagation [1, 2].The healthy immune system is able to eliminate pathogens and maintain tissue homeostasis to avoid potential tissue damage through complex networks [3-5]. In contrast, the dysfunctional immune system usually results in many diseases [5]. Host defense against microbes contain three levels: Natural barriers; innate immunity; and adaptive immunity. Sometimes natural barriers are also classified as part of the innate immune system. In mammals, the immune system includes two branches: innate and adaptive immunity [6].

The immune system is composed of lots of cell types. Leucocytes are the main group of cells of the immune system that are involved in host defense against microbes [4, 7]. The innate leukocytes consist of different subtypes, including macrophages, dendritic cells, neutrophils, mast cells, eosinophils, basophils, natural killer cells, and innate lymphoid cells [8, 9]. These cells secrete a number of cytokines, chemokines, and surface proteins to regulate and carry out immune functions [10, 11]. Lymphocytes are one of the types of leukocytes including B cells and T cells, which are involved in the adaptive immune system [12]. Hematopoietic stem cells (HSCs) that reside in the yolk sac, liver, and the bone marrow in adults are the original progenitors of immunity system [13-16]. Hematopoietic Stem Cells (HSCs) are the source of all blood cell lineages and possess the ability of multi-potency and self-renewal [16, 17]. Multi-potency is the ability that HSCs differentiate into all functional blood cells, while self-renewal means the ability to produce

HSCs itself without differentiation [18]. The hematopoietic stem cells were firstly purified from mouse bone marrow with the development of multi-color fluorescence-activated cell sorting technology and monoclonal antibodies in 1988 [19, 20]. Almost all HSC identification rely on the positive selection markers of c-Kit and Sca-1 and negative selection marker Lin of mature hematopoietic cell lineages (identified as c-kit^{+}Lin^{-}Sca1^{+} or KLS) of cell surface molecules. According to the surface molecule expression researchers finally identified that HSCs can be classified into two populations: Long-Term-HSCs (LT-HSCs) which are identified as KLS-CD150^{+}CD48^{-}CD34^{-}Flt3^{-} and short-Term-HSCs (ST-HSCs) which are identified as KLS-CD150^{+}CD48^{-}CD34^{+}Flt3^{-} [21]. LT-HSCs initially reside in the bone marrow and differentiate into ST-HSCs through a process of cell division, while ST-HSCs advance to Multi-Potent Progenitors (MPPs) which share the same surface molecules with ST-HSCs [22-25]. Different from HSCs, although MPPs lose the ability to self-renewal, they still maintain the differentiation potential [24, 26]. But different from LT-HSCs, both ST-HSCs and MPPs can sustain hematopoiesis in the very short term [27]. MPPs give rise to the common myeloid progenitor (CMP) that can form all subtypes of the lymphoid lineages, and common lymphoid progenitor (CLP) that can differentiate into either GMPs or MEPs. Furthermore, GMPs form the cells of the granulocytes and monocytes, while MEPs give rise to megakaryocytes and erythrocytes [28-30]. The differentiation of immune cell is summarized in *Graphic Figure 1*.



Graphical figure 1. *A schematic representation of major hematopoietic maturation pathways from HSCs. Figure was adapted from Koichi Akashi et al., [29].*

1.2 Innate Immunity

The innate immunity is the early protected line of host defense against invading pathogens, which provides essential functions for the identification and elimination of pathogens [31]. Two distinct features of innate immunity have been described: the recognition of nonself-molecules and the activation of adaptive immune responses [32]. The normal strategy of innate immune system to avoid microbial invasion is constitutive production of generic receptors which are presented in the fully functional form, by which hosts can recognize conserved patterns on different classes of pathogens and induce inflammatory responses [1, 3, 33]. The cells that involved in the innate

immune system consist of dendritic cells, macrophages, monocytes, neutrophils, and natural killer cells, etc.

Type I interferons (IFN-Is), which are involved in antiviral effects and the cellular immune responses [34], are secreted by a large array of cell types including dendritic cells, macrophages, fibroblasts, endothelial cells, osteoblasts and others, but mainly antigen presenting cells (APCs) during viral infection [35-38]. APCs can be divided into professional and nonprofessional APCs. Professional APCs consist of macrophages, dendritic cells, Langerhans cells and B cells, whereas nonprofessional APCs are found among nonlymphoid cells [39, 40]. The IFN-Is family comprises multiple IFNα subtypes, IFNβ and several uncommon subtypes including IFN-ξ, IFN-κ, IFN-ω and IFN-δ. All of type I interferons bind to a common IFNAR1-IFNAR2 receptor. Upon binding IFNAR receptor, JAK1/TYK2 are activated by IFN-I signaling [38]. Activated JAK1/TYK2 signaling triggers not only transcription of various STAT transcription factors but also the activation of p38-MAP kinase (MAPK) pathway [34, 41]. Furthermore, a multitude of different IFN-stimulatory genes (ISGs) are activated, by which viral replication is restricted through different mechanisms [38, 42]. Three functions of IFN-Is have been researched in the last decades. Firstly, IFN-Is contribute to inhibit the spread of viral pathogens. Secondly, IFN-Is regulate innate immune responses to promote antigen presentation, cytokine production and natural killer cell functions. IFN-Is activate the adaptive immune system that are involved in antigen-specific T and B cell development [36, 43, 44].

1.2.1 Dendritic cells

Dendritic cells (DCs) are a class of bone marrow derived cells that present in blood, tissues and lymphoid organs [45]. They belong to APCs and play an essential role in the induction of

adaptive immune responses with an ability to capture and transfer information from the innate immune system to adaptive immune system [46-48]. DCs were firstly identified from human skin in 1868 by Paul Langerhans and named in 1973 by *Ralph M. Steinman* and *Zanvil A. Cohn* [49, 50]. They discovered that the DCs are characterized by stellate morphology and extended veils [50]. Furthermore, they found that DCs express both major histocompatibility complex (MHC) class I and II molecules, which are the major factors for T cell activation after few years [51-53]. The MHC proteins are encoded by major histocompatibility complex (MHC) genes of vertebrates on the cell surface. MHC molecules are able to bind peptide fragments derived from pathogens and present them to the cell surface for T or B cells recognition. *Ralph Steinman* was honored with the Nobel Prize in Physiology or Medicine in 2011, due to his pioneering work in founding DC function and biology.

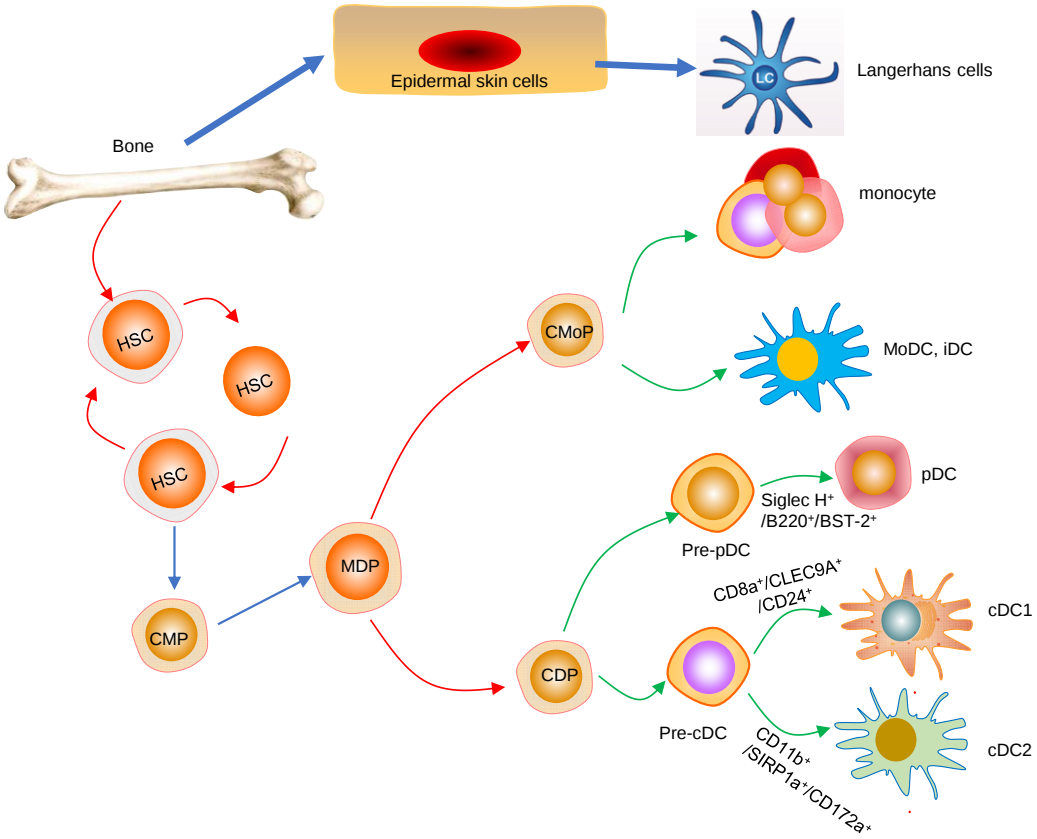
Different kinds of methods have been used to define DCs, such as functional and anatomical classification. However, a robust classification of DCs has been wildly used, which is based primarily on ontogeny [49, 54]. This method divides DCs into two subsets including steady-stage conventional DCs (cDCs) and non-conventional DCs [49]. The steady-stage conventional DCs (cDCs) have the DC form and function, which critically locate in nonlymphoid tissues and in the spleen marginal zone (MZ) [49]. Non-conventional DCs including plasmacytoid DCs (pDCs), Langerhans cells (LCs) and monocyte-derived DCs (moDCs) play an important role in response to inflammatory stimuli [45, 54-56].

The cDCs which derive from a common DC precursor (CDP) commonly exhibit a short half-life of roughly 3–6 days [49, 57]. The primary function of cDCs is to process and present endogenous and exogenous antigens through MHC-I and -II complexes to prime naive T cells [58].

Fluorescence flow cytometry has been commonly used to identify DCs. In mice, cDCs populating the lymphoid organs are subdivided into cDC1 and cDC2 cells, while the cDCs in non-lymphoid organs are subdivided into CD103+ and CD11b+ cDC cells [12, 49, 58-60]. CD8a, Clec9a and CD24 are used to identify cDC1 [49, 58]. Thus, CD8a-expressing DCs are also named cDC1s, which uniquely express Toll-like receptor 3 (TLR3), and produce more IL-12 compared with cDC2s. cDC1s are the primary subset that cross-presents antigens to CD8+ T cells in mice [54, 57, 61, 62]. In contrast, the cDC2 subset, which is notably identified by the expression of CD11b and SIRP1α/CD172a, mainly presents soluble antigens to CD4+ T cells, thereby regulating immune responses to extracellular microbes [59, 60]. CD11b-expressing cDCs are the most abundant cDCs in lymphoid organs, except for the thymus, which can also be found in nonlymphoid tissue [49]. CD11b+ DCs are characterized by the production of cytokines and transcription factor IRF4 [63-65]. But the functions of CD11b expressing DCs remain unclear [57].

pDCs that arise from CDPs were identified firstly in humans [66, 67]. Siglec-H, B220, and BST-2 normally are used to identify pDCs in mice [59, 68]. The best characters of pDCs are the property to rapid secretion of type I interferon (IFN-α/β) in response to external nucleic acids and rapid inducible Interferon regulatory factor 7 (IRF7) expression [35, 69, 70]. pDCs normally circulate in the blood and peripheral organs [69]. The development and homeostasis of pDCs are regulated by the different transcription factor like TCF4, Id2 and E2-2 during proliferation [71, 72]. Recent studies demonstrated that Leukemia inhibitory Factor (LIF) can Inhibit pDC function and development by the inhibition of IFN-I, TNF, and IL-6 responses to CpG [72]. The Langerhans cells (LCs) originate from the bone marrow [73, 74]. LCs are characterized by lower expression of MHC-II but higher C-type lectin langerin, which populate the epidermal later of the skin [49, 75]. The DCs that originate from monocyte infiltrates exist in lymphoid and nonlymphoid organs,

which are termed monocyte-derived DCs (moDCs). moDCs can be cultured with GM-CSF and IL4 in vitro [76, 77]. The differentiation of DCs is summarized in *Graphic Figure 2*.



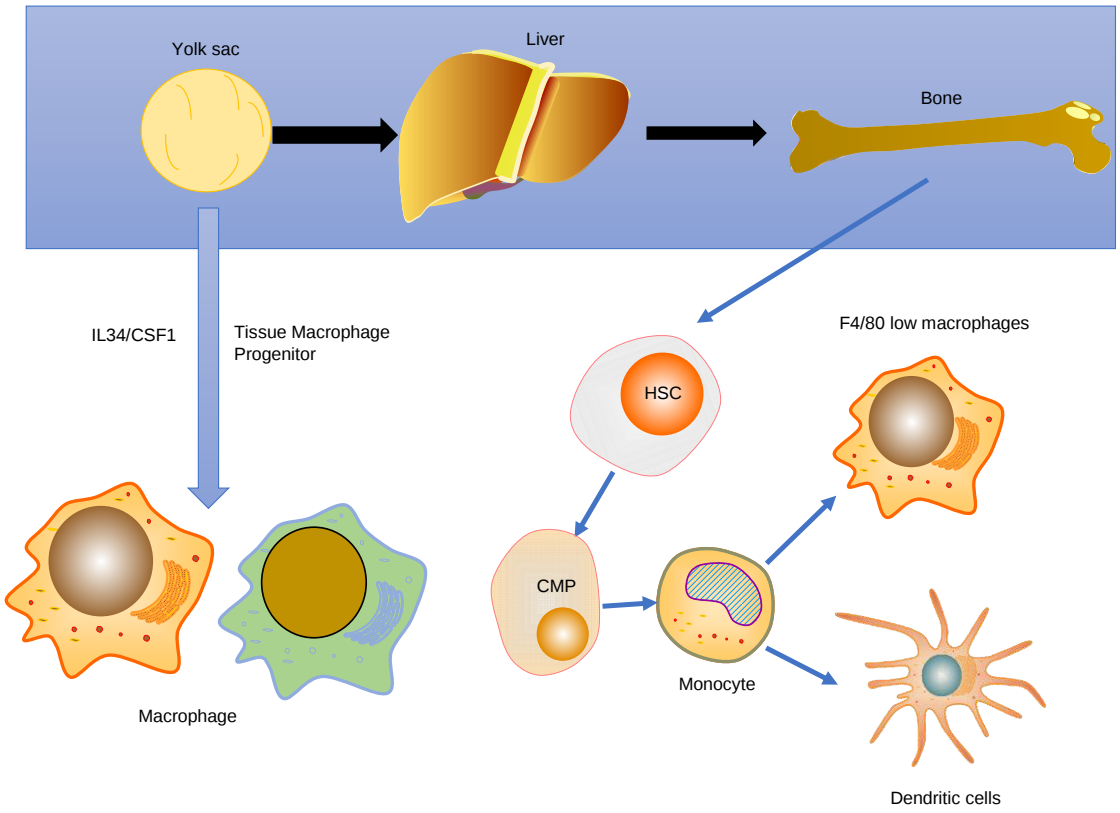
Graphical figure 2. *Functionally specialized conventional and non-conventional dendritic cell subsets and related lineages. Figure was adapted from S. C. Eisenbarth et al., [75].*

1.2.2 Macrophages

Based on the phagocytic nature of the cells, macrophages were first identified by *Eli Metchnikoff* in the 1884 [78]. Macrophages are present in almost all tissues in vertebrates, especially in adult mammals [79]. Macrophages are recognized as multifunctional cell types involved in immune responses, which have function in repair, tissue homeostasis and development

of mammals [80]. The activited macrophages are important not only for host immune response but also for a variety of protective functions, such as the removal of apoptotic cells [81, 82] .

Although a lot of attempts have been made to classify macrophages, there is no effective classification which can cover all macrophages [83]. The mononuclear phagocytic system (MPS) is the most successful definition, which guided many researchers studying the functions of macrophages over the past several decades [84]. According to the MPS, tissue resident macrophages arise from at least three sources including yolk sac macrophages, monocytes derived from fetal liver and bone marrow (BM) [80, 85]. The first is the yolk sac, which contributes to tissue macrophage populations including liver Kupffer cells and identified by F4/80 high expression [86]. The second is the fetal liver, from which the progenitors of macrophages of the skin and epidermal Langerhans cells arise [87, 88]. Both the yolk sac and fetal liver are mainly regulated by IL34 and CSF1 [87, 88]. The third lineage derives from the bone marrow (BM), which is the source of monocytes and their progeny F4/80 low macrophages and dendritic cells (DCs) [89]. Langerhans cells firstly identified in 19^{th} century, which arise from fetal liver and are restricted to the epidermal skin layer [57]. In addition to these resident macrophage populations, $CD14^{+}CD16^{-}$ and $GR1^{+}/Ly6C^{high}$ classical monocytes in mice can be recruited to spots of infection or injury and finally differentiate into macrophages [90]. The differentiation of macrophages is summarized in *Graphic Figure 3.*



Graphical figure 3. *The MPS model of macrophage lineages in mice. Figure was adapted from Wynn TA et al.,[80].*

1.2.3 Monocytes

Monocytes are a subset of blood leukocytes with a plasticity to develop into dendritic cells and macrophages in vivo, which develop in the bone marrow and then migrate to the blood and finally locate to the tissues as nondividing cells [91]. Finally, monocytes occupy 5–12% of the total leukocyte population in circulation in normal hemostasis and the ratio changes dramatically during infection or inflammation [92]. Monocytes consist of two primary subsets based on the distinct surface molecules expression, which play different roles in inflammation [93]. In mice, they are identified by the high expressed Gr-1 (Gr-1^{high}) and low expressed Gr-1 (Gr-1^{low}). While

they are $CD14^{+}CD16^{-}CCR2^{+}CX3CR1^{low}$ and $CD14^{+}CD16^{+}CCR2^{-}CX3CR1^{+}$ in humans. Monocytes play an important role in the immune response to most pathogens. In the early phase of infection, monocytes produce different cytokines, such as IL-1β, IL-6, and TNF-α, to control the virus [94]. The expression of chemokine receptor determines the migration of monocytes [95, 96]. It has been shown that MCP-1, which is induced by circulating TLR-ligands, binds to the CCR2 receptor expressed by the $Gr\text{-}1^{high}$ monocytes and promotes the transfer of monocytes from the bone marrow to the tissues [97]. It's also reported that monocytes can be recruited to infected tissues by the chemokine receptor CCR2. Moreover, the monocytes in the spleen also have a crucial role in response to injury or inflammation [98]. During the migration they can differentiate into special cells to eliminate the pathogen infection, such as DCs, macrophages and Langerhans cells (LC) [99, 100]. Some reports also shown that CCR2 produced by monocytes promote liver fibrosis facing the challenge of HBV, HCV infection or chronic alcoholism [101]. Furthermore, it was revealed that monocytes also play a role in different diseases, such as atherosclerosis, multiple sclerosis, Alzheimer's disease and tumors, by the immigration or production of cytokine and chemokine receptors to the position of inflammation and lesion [101].

1.2.4 Neutrophils

Neutrophils are a type of blood cells that develops in bone marrow from hematopoietic stem cells (HSC) and make up 70% of leukocytes [102, 103]. The terminal neutrophils from the circulation are normally cleaned up by macrophages after completing their biological functions [104]. They also have important role during acute inflammation [105]. All the functions of neutrophils are induced by the expression of cell surface molecule and the activity of transcription factors [102]. Several transcription factors are involved in the granulopoiesis of neutrophils from HSC including PU.1, C/EBPα, GFI1, and C/EBPε, which influence the development of

neutrophils by diverse signaling pathways [106, 107]. PU.1 and C/EBPα induce CMPs to differentiate into monocytes by inhibition of the *c-Myc* gene, while GFI1, and C/EBPε induce the generation of neutrophils and eosinophils [106, 108, 109]. Once neutrophils mature in the bone marrow they migrate to the circulation, in which G-CSF has an important role in the release of neutrophils [110].

1.2.5 NK cells

NK cells, also termed as natural killer cells, which are a member of the innate immune system, have a crucial role in anti-tumor properties and were firstly described in 1970s [111, 112]. As natural killer cells, they do not need to be stimulated with antigen in advance to mediate their "killer" function [113]. They can be distinguished by cell surface molecules (CD16, CD56, CD57) and occupy a large proportion of blood mononuclear cells. In mice, NK1.1 and NCR1 are common markers of subsets of NK cells and the percentage of NK cells reach to 2–5% among lymphocytes presented in the spleens and BMs [114-116]. Few papers reported that NK cells develop primarily in the bone marrow and partly in lymph nodes and liver [117, 118]. However, they reside in multiple tissues among human organs including the bone marrow (BM), lymph nodes (LNs), skin, gut, tonsils, spleen, liver, and lungs [119]. The development of NK cells undergoes a process of proliferation and differentiation from hematopoietic stem cell (HSC) to common lymphoid progenitor (CLP), then to Pre-T/early NKP lineages. After several continuous process, pre-NKP finally mature to NK cells [30, 120]. Several transcription factors are involved in the maturated process of NK cells including Ets1, Id2, Ikaros and PU.1 [121, 122].

One crucial function of NK cells is that they recognize and kill virally infected and neoplastic cells, which present in a resting stage in normal circulation but infiltrate into most tissue

that contain infected and malignant cells after activation by different cytokines [123, 124]. NK cells express multiple cytokine receptors including receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 [125]. NK cells also induce the production of cytokines and chemokines that have function in regulating immune responses including interferon gamma (IFNγ) and CC-chemokine ligand 5 (CCL5) [126, 127]. IFNγ promotes DC activation and a more efficient killing response of T cells. CCL5 plays an important role in promoting the recruitment of dendritic cells (DCs) into solid tumors [128, 129]. The function of NK cells is also controlled by the cell surface receptors, which contain both activating and inhibitory properties [130]. NK cells recognize self-MHC-I molecules by the inhibitor receptor to prevent NK cell activation. Moreover, NK cells can differentiate infected cells from normal cells by the recognition of MHC-I molecules [131]. Normally, low or absent MHC-I expression of virally infected and tumor cells allow abnormal cells to escape recognition of cytotoxic T lymphocytes (CTLs), but enhances NK-cell attack [132]. The cytotoxic ability of NK cells is mediated by two pathways: perforin and granzyme induced apoptosis and caspase-dependent apoptosis [133-136]. In the second pathway, several receptors and ligands are involved in the process such as death receptors of Fas and Fas ligand (FasL), tumor necrosis factor related apoptosis-inducing ligand (TRAIL) and antibody dependent cellular cytotoxicity (ADCC), which can express low-affinity Fc receptor for IgG (CD16) [136].

1.2.6 NKT cells

Natural killer T(NKT) cells, which are from T lineage cells, are an innate-like lymphocyte population firstly described in 1987 [137, 138]. As the name implies, they share partial morphological and functional characteristics with both T cells and NK cells [139]. Instead of the ability to recognize MHC molecules by conventional αβ T cells, the T cell receptors (TCRs) of NKT cells are able to recognize different complexes including glycolipid, glycosphingolipid, or

lipid structures presented by the CD1d molecules of APCs (including hepatocytes and endothelial cells) [137, 138, 140]. The function of NKT cells are regulating diverse immune responses and inducing the production of huge quantities of cytokines following activation.

NKT cells are composed of three types of cells: type I NKT, type II NKT, and NKT-like cells. Both murine and human Type I NKT cells recognize the ligand a-GalCer, a synthetic glycosphingolipid initially isolated from a marine sponge [141]. In mice, Type I NKT cells that also named invariant NKT (iNKT) cells are the most prominent CD1d-restricted NKT cells with a restricted TCR diversity [142, 143]. iNKT cells express an invariant Vα14-Jα18 TCRα chain paired with a limited set of TCRβ chains, including Vβ8.2, Vβ7, and Vβ2[144]. Compared with murine iNKT cells, human Type I NKT cells express an invariant Vα24-Jα18 TCRα chain paired mainly with Vβ11 [145].

In contrast to Type I NKT cells, type II NKT cells exhibit variant TCR diversity [146, 147]. Type II natural killer T cells belong to the innate-like CD1d-restricted lymphocytes that respond to lipid antigens. [146]. However, Type II NKT cells respond to antigens by sulfatide /CD1d dependent manner but not α-GalCer/CD1d [148, 149].

Unlike type I and type II NKT, NKT-like cells also exhibit more TCR variability and respond to antigens in CD1d independent manner. NKT-like cells can express NK associated receptors, present a extremely specialized effector memory phenotype, and recognize antigens in a manner similar to conventional T cells [150]. This subset is affected by ageing and performs potent antitumor effects [151].

1.3 Innate immune system recognition

Host defense against infection is initiated after hosts recognize pathogen-associated molecular patterns (PAMPs), such as LPS, CpG, and viral nucleic acids which induce inflammatory responses [31, 152]. The recognition depends on germline encoded receptors: the pattern recognition receptors (PRRs) that are expressed on any life-cycle stage of innate immune cells such as dendritic cells, macrophages and neutrophils [153, 154]. PRRs are comprised of three different classes including Toll-like receptors (TLRs), Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), NOD-like receptors (NLRs). The sensing of PAMPs induces the production of type I interferons (IFNs), proinflammatory cytokines, chemokines and antimicrobial proteins, which are involved in the modulation of PRR signaling [31].

1.3.1 TLR

TLRs are evolutionarily conserved PRRs and are the primary sensors of pathogens. TLRs belong to the subset of the type I membrane glycoproteins that are characterized by the extracellular domains of leucine-rich-repeat (LRR) motifs required for PAMP recognition and a cytoplasmic signaling domain of Toll/IL-1R homology (TIR) domain required for downstream signaling activation [155]. LRR domains are 24–29 amino acids in length that consists of a β strand and an α helix connected by the loop [156]. Based on the primary sequences of LRR domain, TLRs divide into several subsets. Toll was firstly identified as a protein of anti-fungal response of the flies [157]. In the past decades, 12 TLRs have been identified in mammals. TLR10 is expressed in humans, whereas TLR11 is expressed in mice, uniquely. Although each subset of TLRs have a special function during different pathogens infection, they also have common characteristics. TLR1, 2, 4, 5 and 6 recognize PAMPs derived from bacteria, fungi and protozoa that are primarily expressed on the cell surface, whereas TLR3, 7, 8 and 9 primarily recognize nucleic acid PAMPs

originated from various viruses and bacteria merely expressed within endocytic compartments such as endosomes [154, 158, 159].

Recognition of PAMPs by TLRs triggers the activation of host defense against mechanisms [1]. Activated TLRs recruit the TIR-domain-containing adaptor molecules to the TLRs, which is a critical step for initiating signaling [160]. There are five adaptor molecules that have been reported including Myeloid differentiation primary response 88 (MyD88), TIR-domain-containing adapter-inducing interferon-β (TRIF), Toll/interleukin-1 receptor domain-containing adapter protein (TIRAP), Translocating chain-associated membrane protein (TRAM), and Sterile-alpha and Armadillo motif containing protein (SARM) [154]. TLR signaling is divided into two distinct signaling pathways according to the function of adaptor molecules, MyD88 and TRIF. They initiate different pathways to promote the production of proinflammatory cytokines and type I IFNs, respectively [161].

MyD88 is critical for the signaling cascades of various TLRs except TLR3 that is firstly reported in 1990 [162, 163]. Two functional domains of MyD88 have been reported including the C-terminal TIR domain and the N-terminal death domain (DD). Whereas, TIRAP/Mal is also required for the connection of TLR and MyD88 in TLR2 and TLR4 [164-166]. After receptor activation, MyD88 interacts with IL-1R-associated kinase (IRAK)-4 to promote the autophosphorylation of MyD88, which is necessary for the activation of subsets of IRAK family by its N-terminal death domain [167]. The interleukin-1 receptor (IL-1R) associated kinases (IRAKs) then dissociate from MyD88 and interact with TNFR-associated factor 6 (TRAF6). TRAF6 promotes the formation of a K63-linked polyubiquitin chain on inhibitor of nuclear factor kappa-B kinase subunit gamma (IKK-γ)/ nuclear factor kappa-B (NF-κB) essential modulator

(NEMO), while the free ubiquitin chain activates and recruits TGF-β-activated kinase 1 (TAK1) and the TAK1 binding proteins TGF-Beta Activated Kinase 1 (MAP3K7) Binding Protein (TAB) subsets including TAB1, TAB2, and TAB3 to TRAF6 [168]. TAK1 then catalyzes the phosphorylation of IKK-β and MAP kinase kinase 6 (MKK6). The IκB kinase (IKK) complex induces the phosphorylation of IκBa, an NF-κB inhibitory protein resulting in inflammatory responses by translocation of NF-κB into the nucleus [154, 169]. In DCs, MyD88 also forms a complex with IRAK-1, TRAF6, TRAF3, IKK-a, which induces the phosphorylation of IRF3 or IRF7, and the translocation of p-IRF3 and p-IRF7 into the nucleus. The p-IRF3 and p-IRF7 bind to the IFN-α or IFN-β promoter to activate the type I IFNs transcription [170, 171].

In contrast to most TLRs, TLR3 evolutionally chooses TRIF as the adaptor. Whereas, TLR4 can use both MyD88 and TRIF. TLR4 needs another protein, TRAM, to combine and active TRIF [172]. TRIF interacts with Translocating chain-associated membrane protein 3 (TRAF3) and Translocating chain-associated membrane protein 6 (TRAF6) by the N-terminal TRAF-binding motifs and interacts with Receptor-interacting serine/threonine-protein 1 (RIP1) and Receptor-interacting serine/threonine-protein 3 (RIP3) via the C-terminal motif (RHIM) to initiate the TRIF-dependent signaling. Both the interaction of TRIF with TRAF3 and TRAF6 actives TAK1, and the interaction between TRIF and RIP1, which are required for NF-κB activation. The interaction of TRIF with TRAF3 promotes K63-type polyubiquitin chain formation of TRAF3, which play an important role in Tank Binding Kinase 1 (TBK1) and IκB kinase epsilon (IKK-ξ) phosphorylation [173, 174]. Phosphorylated TBK1 and IKK-ξ phosphorylate IRF3 and IRF7. Phosphorylated IRF3 and IRF7 translocate to the nucleus, resulting in the production of type I IFNs and expression of IFN-inducible genes. IFNs also induce STAT1 phosphorylation to induce the expression of a set of IFN-inducible genes including Adar1, Ifit3, and Irf7 [175].

1.3.2 RLR

The RIG-I-like receptor (RLR) family consist of three proteins: RIG-I, MDA5, and LGP2 [176]. They can recognize dsRNA in the cytoplasm of virally infected cells, which induces the production of inflammatory cytokines and type I interferons. The expression of RLRs is also significantly increased in virally infected cells [176].

RLRs are normally composed of N-terminal caspase recruitment domains (CARDs), a central DEAD box helicase/ATPase domain, and a C-terminal regulatory domain. However, the CARD domain is deficient in LGP2. It also reported that LGP2 serves as a positive regulator in RIG-I and MDA5 mediated antiviral responses [177-180]. The CARDs regulate downstream signaling presented in RIGI and MDA5. The DEAD box helicase/ATPase domain present in these three sensors is required for ligand recognition. The C-terminal regulatory domain is responsible for the binding to dsRNAs by the RNA-binding loop. Once viral RNA is transferred into the cytoplasm of the host cells through endocytosis, the binding of viral RNA with RIGI and MDA5 induces conformational changes exposing the CARD domains, which is modulated by diverse ubiquitin chains [181]. RIGI or MDA5 subsequently interact with MAVS by the N-terminal CARD domain, which is located on outer membrane of mitochondria and peroxisomes [182]. Furthermore, the interaction induces MAVS aggregation, which activates the phosphorylation of TBK1/IKKi [183]. p-TBK1/IKKi initiates downstream activation of NF-κB and IRF3/IRF7 and subsequent IFNs production.

RIG-I recognizes diverse viruses that include members of paramyxoviridae family such as Newcastle disease virus (NDV), Sendai virus (SeV), vesicular stomatitis virus (VSV), influenza virus, and Japanese encephalitis virus (JEV). At the same time RIGI also recognizes some other viruses such as respiratory syncytial virus, Ebola virus and hepatitis C virus [184]. In contrast,

MDA5 mainly responds to several members of Picornaviridae, one subset of Picornaviruses, including encephalomyocarditis virus (EMCV), Mengo virus, Polio Virus and Theiler's virus. Whereas, some viruses can be recognized by both RIGI and MDA5 including Reovirus, dengue virus and West Nile virus [185, 186]. In addition to viruses, RIGI also recognizes synthesized short dsRNA, but not ssRNA, in vitro. The 5' phosphate of dsRNA is crucial for the recognition of viral RNA by RIG-I, but dispensable for MDA5 activation [187-189]. The minimum length of 19- 21-mer dsRNA and the maximum length of 1kb dsRNA are essential for the recognition [190-192]. It's also confirmed that short length of Poly-IC can potently activate RIG-I [190]. While, Poly(U)- or poly(A)-rich sequences from the HCV RNA 3'untranslated region (UTR), as well as in vitro-transcribed RNAs with low U/A content, are also responsible for RIG-I-mediated IFN production [154, 193]. The sequences of the 5' and 3' ends of the viral RNA are partially complementary to each other to form a panhandle structure presented in several viruses that also is able to potently recognized by RIG-I [194]. In contrast to RIG-I, MDA5 recognizes long dsRNA more than 2kb such as Poly-IC and higher-order RNA structures during virus infection [186, 195]. LGP2 play a role in removing proteins from ribonucleoprotein complexes and unwinding viral RNA structures to promote the recognition of RIGI and MDA5 [180].

1.3.3 NLR

Nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) are a family of cytosolic PPRs that mediate responses to a wide range of PAMPs presented in the cytoplasm. The ligands of NLR signaling include LPS, flagellin, viral RNA, etc. [177, 196]. NLRs consist of three domains: The C-terminal domain that is involved in the recognition of microbial PAMPs and some endogenous host molecules, the central NACHT domain that are required for oligomerization of the sensors and formation of active receptor complexes for the downstream

signaling activation, and the N-terminal domain [197]. Based on the different functions of the N-terminal domain, the NLRs are divided into five subsets: NLRC subfamily with the CARD domain, NLRP subfamily with the Pyrin domain (PYD), NLRX with the death effector domain (DED), NLRB with a baculoviral inhibitor repeats (BIRs) and NLRA with an acidic activation domain [177]. NLRPs and NLRCs mediate the recruitment of Caspase-1 and release of the inflammatory cytokines IL-1β and IL-18. NLRPs play an important role in viral infection [198-200]. Whereas, NLRCs mainly consisted of NOD1 and NOD2 respond to bacterial peptidoglycans and activate both NF-κB and AP-1 signaling pathways [201]. NLRA act as a transcription factor to active the expression of MHC class II genes [202].

1.4 Adaptive immunity

Microbial infection is a widespread health problem for many species of the world, which could lead to huge challenge for public health and the global economy [203]. Although the innate immune system can eliminate most pathogens, the recognition ability of innate immunity is still limited. As pathogens evolved to avoid detection by the innate immune system, it promotes the evolution of the adaptive immune system [3, 204, 205]. Unlike the germline encoding form of innate immunity, adaptive immunity depends on somatic recombination to generate a large array of gene segments, which compose different antigen receptors for various cells [3, 206, 207]. Adaptive immunity differs from innate immunity in that it contains a large number of functional cells to target different microbes[208, 209]. Adaptive immunity is initiated when invaded pathogens escape from the innate immunity mediated defense systems [210, 211]. Adaptive immunity is initiated and executed by small numbers of antigen-specific cells after encountering the antigens, but the adaptive immunity still needs several days to become effective [3]. During this time, antigen-specific T cells and B cells locate specific foreign antigen and encounter

proliferation and differentiation to into effector cells. At the same time, part of the cells persists in a dormant state after a previous initial exposure which can expand rapidly after encountering the same antigen by clonal amplification, cellular differentiation and proliferation [3, 212, 213].

The adaptive immunity consists of two major lineages of lymphocytes, the B and T lymphocytes [12]. The B and T lymphocytes are the respective key mediators of antibody-mediated immunity and cell-mediated immunity, which can recognize potentially pathogens by the antigen-specific receptors expressed on the cell surfaces [214].

1.4.1 T cells

T-cell development occurs in the thymus which is different from other blood cell lineages in the BM. The microenvironment provided by thymus is critical for the development of T cells that initiate from HSCs [215]. The cells that present in the thymus cannot renew by proliferation so that thymus recruits hematopoietic precursors from the bone marrow (BM) to complete self-renewal [216]. During T cell development, several steps related to T cell differentiation and proliferation occur in the thymus. Firstly, lymphocytic progenitors migrate into the thymus and produce $CD4^-$ $CD8^-$ double-negative (DN) and $CD4^+$ $CD8^+$ double-positive (DP) lymphocytic cells in the outer cortex of the thymus. Secondly, DN and DP thymocytes encounter the selection to generate single-positive (SP) $CD4^+$ or $CD8^+$ T cells in the cortex of the thymus. Finally, the mature T cells export from thymus to circulation, which is regulated by different environmental signals they encounter including cytokines like stem cell factor (SCF) and Flt3L, Wnt and Notch signaling pathway [217-222]. During this process, just 1-3% of the thymocytes succeed in completing selection and export from the thymus [223-225].

The entry of T-progenitor cells to the thymus occurs in the early stage of embryonic day 11.5 (E11.5) in mice and the eighth week of gestation in humans [226, 227]. In the early stage of fetal thymus, CC-chemokine ligands CCL21 and CCL25 have partial but significant roles in the colonization of thymus. Because in the early stage of thymus development there is no vasculature in thymus, the CC-chemokine attract the lymphoid progenitor cells to the thymic primordium [226, 227]. At the same time several chemokine receptors, like CCR7 receptor for CCL21 and CCR9 receptor for CCL25, are involved in the process of immature thymocytes transfer [228, 229]. But in the postnatal thymus lymphoid progenitor cells enter the thymus from blood vessels near the corticomedullary junction [230]. After entering, the thymus T-cell precursors subsequently migrate to the cortex and begin development into DN stage through complex process. Furthermore, the DN stage can be subdivided into four stages from DN1 to DN4 [231, 232], which can be distinguished by the molecular expression of CD25 and CD44 on the cell surface [233, 234]. DN1 is the most immature T-progenitor cell in this stage of thymus development [235]. A small population of DN1 termed as early thymic progenitor (ETP) cells, which can express c-Kit, Flt3 and CCR9, is the precursor of DN2 T-progenitor cells [236-238]. With the development of DN2 cells, the expression of c-Kit significantly decreases, during which the T progenitor cells lose their ability to differentiate into non-T-cell lineage cells [239]. After DN2 cells mature to the DN3 stage, they are characterized by expression of CD27 molecule and the recombination of *Tcrb* by the process of V(D)J recombination [240, 241]. DN3 T-cell progenitors rearrange their genomic at β, γ, and δ loci to promote the γδ T cell fate specification. Finally, rearrangement of Tcrβ chains combine with an invariant pre-Tα chain to form the Pre-TCR complex [242]. At the same time αβ T cells continue their development by β-selection, during this process Notch signaling plays an important role in the homing and settling of cells [215]. After the process of β-selection,

thymocytes develop into DN4 stage with proliferation and differentiation induced by Pre-TCR, through which CD4 and CD8 double positive cells are highly enriched [243]. When the thymocytes mature to the DP stage, *Tcra* recombination is initiated leading to expression of the TCRαβ complex. This then leads to positive selection by specifically binding MHC ligands and negative selection by deficient recognition of self-antigens on the cell surface [244, 245]. Alternatively, high-affinity interaction of TCRαβ complex and MHC ligands also leads to the apoptosis of thymocytes, that is also referred to as negative selection, by which cells can avoid autoimmunity [246]. After this negative and positive selection processes, cells differentiate into single positive (SP) lineages and emerge into the periphery [247].

After the process of development, SP T cells still need a process termed as T cell activation which is induced by different antigen then migrate to diverse organs to promote pathogen clearance [248]. Naïve T cells differentiate into memory and effector T cells which have a critical role in immune responses. According to the glycoprotein types of TCR expressed on the cell surface which is composed of TCRα/β chains and CD3γ/δ/ε/ζ subunits, the mature T cells in the thymus can be classified into two types: $CD4^+$ T-helper cell or to $CD8^+$ cytotoxic T-cells. CD4 T cells contain different subsets: Th1, Th2, Th9, Th17, Th22, Treg (regulatory T cells), and Tfh (follicular helper T cells). Different subsets can be generated by different cytokines and they secrete different interleukins [249]. During the period of T cell activation, the TCR induces the recognition of antigenic peptides presented by APCs bound to MHC class I (MHC-I) and MHC class II (MHC-II) molecules to form the peptide-MHC-I and peptide-MHC-II complexes (pMHC-I/pMHC-II), while the CD3 transduces activation signals to the T cell [250]. Moreover, costimulatory molecules also provide additional signals for T cell activation. Numerous studies have shown that the costimulatory molecule CD28 is essential for T cell proliferation, cytokine production and

activation, which can bind to its ligands CD80 or CD86 on APCs [251]. $CD4^+$ and $CD8^+$ T-cells recognize antigen/MHCII and MHCI complexes respectively after the T cells differentiate [252]. $CD8^+$ T cells differentiate into cytotoxic T lymphocytes (CTL) recognizing pMHC-I, which are presented on nucleated cells, presenting 8–10 residues long peptides that are derived from pathogens or cancer cells [253]. However, $CD4^+$ T. cells are derived from a common precursor (TH0) then differentiate into different cell types. MHC-II molecules are uniquely expressed on the surfaces of APCs such as dendritic cells, B cells or macrophages, which can present 15-24 exogenous residues long peptides to the helper T cells expressing CD4 molecule [254]. Several signaling pathways are also involved in the recognition of TCRs including extracellular signal-regulated kinases (ERK) activated Activator protein 1 (AP-1) pathway, Inositol trisphosphate (IP3)-Ca2+ activated Nuclear factor of activated T-cells (NFAT) pathway, IKK involved NF-κB pathway and Tuberous sclerosis proteins 1 and 2 (TSC1/2) target mammalian target of rapamycin (mTOR) pathway [255].

1.4.2 B cells

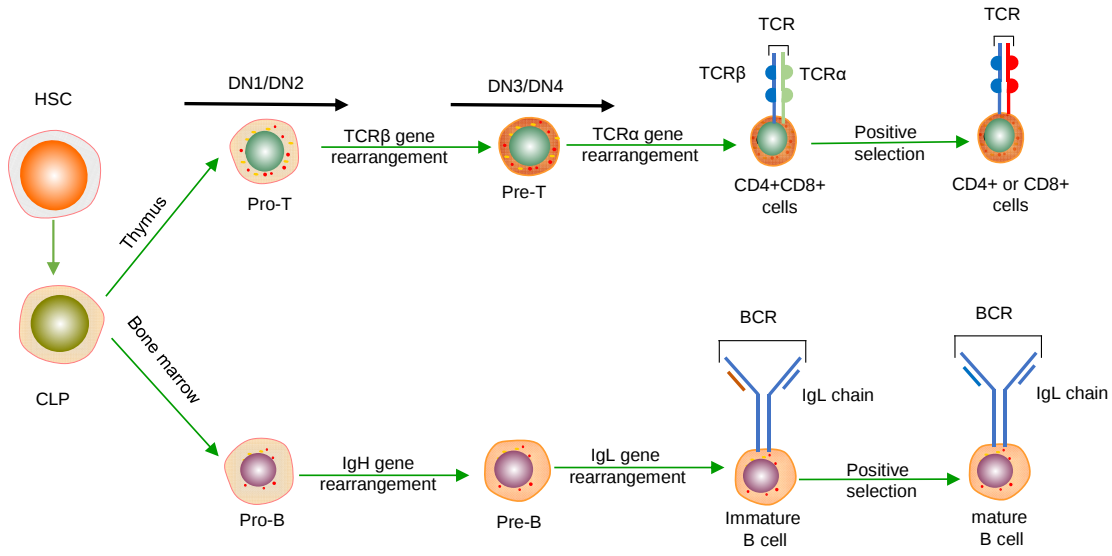
With the identification of antibody derived from gamma globulin in serum, researchers also discovered that plasma cells are the original cells of antibody production in the mid-nineteenth century [256, 257]. B cells are firstly found to the end of 1960s [258]. Because of these great discoveries in the process of B cells research, many people have won the Nobel Prize for their great contributions in the 20th century. *Niels Jerne* and *Macfarlane Burnet* contribute to the development of natural selection and the clonal selection theory. While *Gerald Edelman* and *Rodney Porter* contribute to the chemical structure of the antibody molecule [259-262].

In mammals, early B cells develop in bone marrow from hematopoietic precursor cells through rearrangement process of the V, D, and J gene segments of immunoglobulin in the heavy

chain (H chain) locus and the V and J gene segments in the light chain (L chain) loci encoding the B cell receptor (BCR) named as pro–B cells stage [263]. Ig proteins have a function in B-cell activation in this stage [264]. Pre-B cells encounter division and rearrangement encoding the κ and λ chains to form the IgM molecule and express on B cell surface [265]. The B cells in this stage are termed immature B cells which migrate to secondary lymphoid tissues and differentiate into naive, follicular, or MZ B cells. After encountering the pathogens from blood, these cells rapidly develop into extrafollicular plasma cells and secret IgM, which can directly bind the pathogens to form the IgM-Pathogen complex [111]. BCR signaling has an important role in the B cell development. Several proteins have been reported to play a critical role in the development of B cells such as IL-2 receptor common γ, the IL-7 receptor α chain, or the associated kinase Janus kinase 3 and Bruton tyrosine kinase (BTK) [266-269].

In mice, B cells are also derived from pluripotent hematopoietic stem cells (HSC) [270]. The bone marrow contains different stage of B lineage cells from earliest progenitors to mature B cells, which can be described based on the expression of cell surface molecules such as CD45R, B220 and CD19 [112, 113]. B cells initiate in the liver during embryonic life and in the bone marrow after birth. They are defined by the expression of diverse cell surface immunoglobulin (Ig) receptors recognizing specific antigenic epitopes [271]. They then mature in secondary lymphoid tissues. Finally, the matured B cells produce antibody as differentiated plasma cells [272]. B cells can be classified in B1 and B2 lymphocytes based on the anatomic localization. B1 lymphocytes originate from B1 progenitors in fetal liver and mostly occupy the peritoneal and pleural cavities after development, while B2 cells initiate from B2 progenitors in the BM with subsequent differentiation into MZ and follicular (FO) lineages in the spleen [273, 274]. B1 cells produce IgM antibodies directed against T-independent antigens presented on commensal bacteria. In contrast,

B2 lymphocytes are the source of all Ig isotypes including IgM and long-lived memory B cells and plasma cells [117-119]. FO B cells consist of the common B lymphocytes in the adaptive immune system and are the most frequent of all B cell lineages. They are primarily responsible for antigen-induced T cell–dependent B-cell activation in the germinal center (GC) of the spleen and lymph nodes. They generate of high-affinity IgG antibodies after infection and vaccination, which also have a critical role in the development of plasma cells and switching of memory B cells [275]. The development of B and T cells are summarized in *Graphic Figure 4*.



Graphical figure 4. *T cell and B cell development. Figure was adapted from Muhammad Haseeb et al., [276].*

1.5 LCMV

LCMV genome consists of two single-stranded RNA segments, L and S, which use an ambisense coding strategy to complete the viral RNA replication and protein translation. It was first isolated in the 1930s by *Muckenfuss*, *Armstrong* and *McCordock*, and named by *Armstrong*

and *Lillie* (1934) [277]. The unusual characteristic of virus persistence was recognized not long after the virus was first isolated, which are associated with congenital and neonatal infection of mice. Traub in 1936 recognized that LCMV infected mice in utero or shortly after birth neither eliminate the virus nor die [278]. The studies of LCMV infection established that rodents are the principal host of LCMV, which is able to transfer virus to next generation by intrauterine infection [279]. There are several clinical syndromes produced by LCMV infection in humans [280, 281]. It was firstly documented by Scott and Rivers (1936) with the study of two patients who presented with acute meningitis [277, 282]. The congenital LCMV infection usually happens in placental [283]. Acquired LCMV Infection was able to occur after contact with virus found in the excrement, and saliva of infected animals, including mice, hamsters, and guinea pigs [284].

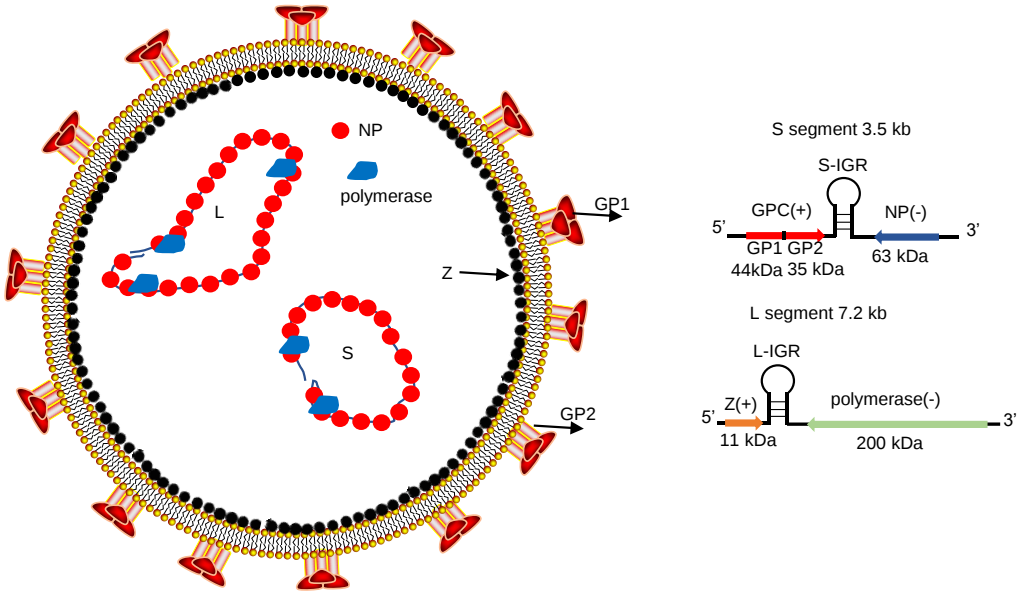
The model of LCMV infection is the most useful animal model [285]. Rowe used depletion experiment to show that acute LCMV infection in mice resulted from the immune response [280]. LCMV induced clinical symptoms are associated with initial inflammatory response and continuous adaptive immune responses, in which APCs and cytotoxic T lymphocytes (CTLs) play an important role especially [3, 286]. PRR dependent signaling has a critical role in the secretion of cytokines and chemokines which are the first line of host defense against response to infection with LCMV [1, 154, 287]. Typically, LCMV infection induces the secretion of IFN-I by APCs, which are able to stimulate the expression of co-stimulatory molecules [288, 289]. IFN-I and co-stimulatory molecules cooperate and active the adaptive immunity [36]. The response of T lymphocytes to viral antigens plays a critical role in tissue injury and death or survival of the infected animal especially [290-292]. Virus-induced CTLs expand in lymphoid tissues and transfer numerous CTLs to nonlymphoid tissues to deal with the pathogen [293, 294]. Various members of LCMV are able to induce different immune responses that leads to the formation of chronic and

acute infections in mice. Thus, the activation of innate immunity to initiate and maintain adaptive immune responses is critical for the control of viral persistence.

1.5.1 The structure of LCMV

The two genomic RNA segments of LCMV, termed L segment and S segment, are approximate sizes of 7.2kb and 3.4 kb, respectively [295-297]. Different methods have been used to identified the composition of LCMV genomic. Firstly, when investigators treated the cells with DNA viral inhibitors during viral expansion, they found that there is no effect on the virus growth. This means LCMV is not a DNA virus but an RNA virus. The conception is directly proven by PAGE analysis of viral RNA on highly purified LCMV in 1971 [297]. LCMV contains two RNPs of 23S and 31S, which have a molecular weight of 1.1×10^6 and 2.1×10^6, respectively [297, 298]. Investigators also prove that LCMV RNA can be resolved into 4 fragments after denatured with glyoxal [280]. LCMV uses an ambisense coding strategy to complete the viral RNA replication and protein translation. Finally, each RNA segment encodes two proteins in opposite orientations, which are separated by an intergenic region (IRG) [299, 300]. The S segment synthesizes the major structural proteins: nucleoprotein (NP) (63 kDa) involved with RNP complexes and a precursor polypeptide (GPC), which is post-translationally cleaved to two virion glycoproteins (GP), GP-1 (44 kDa) and GP-2 (35 kDa) that are present on the surface of the virus [299-302]. Cleavage of GPC occurs in the Golgi or post-Golgi compartment and Post-translational cleavage of GPC requires prior glycosylation [302]. The surface of the virion envelope is covered by GP-1 and GP-2, which mediate virus interaction with host cell surface receptors [303, 304]. The viral L RNA segment codes for the viral RNA-dependent RNA polymerase (200 kDa), which is required for viral transcription and replication and a small (11 kDa) RING finger protein (Z) [305, 306]. The

structure of LCMV is the best characterized of the arenaviruses [295]. The components of LCMV are summarized in *Graphical Figure 5*.



Graphical figure 5. *Schematic diagram of LCMV genome organization. Figure was adapted from Laposova K. et al., [307].*

1.5.2 LCMV family members

Over 30 strains of LCMV have been found since the 1930s [308]. In research several subsets are used including Armstrong (Arm), Clone-13 (Cl13), Traub, WE, Aggressive and Docile. These LCMV strains came from three parental strains. The first strain, LCMV-Arm 53b (often named as LCMV-Arm) was isolated from the brain by *Charles Armstrong* in 1934. LCMV-Cl13 was reported in 1984, when researchers select the genetic variants of LCMV in spleens of persistent infection. Both Arm and Cl13 initiate from a common ancestor LCMV-Arm strain [309]. The second strain, LCMV-Traub was isolated from a persistently infected mouse by *Erich Traub* in 1935 [310]. The third parental strain is the WE strain, from which a number of sub-strains have

been isolated [277]. LCMV WE was originally isolated from a patient who was exposed to the infected mice in 1935 and following the transfer of virus stocks in the 1940s, became known as LCMV-UBC [311]. After that two new sub-strains were isolated from an LCMV-UBC infected mouse, LCMV-Docile and LCMV-Aggressive [312]. However, coding information between LCMV-WE and LCMV-UBC shown that both strains of LCMV-WE and LCMV-UBC are independent sub-strains. Furthermore, two isolates of LCMV-WE have been reported, including the clones WE c54 and WE c2.2 [313].

2 Materials and Methods

2.1 Mice

All mice used on this thesis were bred on a C57BL/6 background and maintained under specific pathogen-free conditions. Experiments were performed under the authorization of the LANUV NRW in accordance with German law for animal protection. The *Ifnar1*$^{-/-}$ mice has been described earlier [314, 315].

2.2 Viruses and virus titration

LCMV strain WE was originally obtained from F. Lehmann-Grube (Heinrich Pette Institute). LCMV strain Docile was originally obtained from Dr. C.J. Pfau (Troy, NY). Viruses were expanded in L929 cells.

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 105 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 hours 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 hour 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 Viral sequence analysis

Viral RNA was reverse transcribed utilizing the ProtoScript®II First Strand cDNA Synthesis Kit (New England Biolabs GmbH, Frankfurt, Germany) and random primers according to the manufacturers protocol. LCMV-WE and LCMV-Docile cDNA was amplified with Taq DNA Polymerase (Qiagen, Hilden, Germany) and overlapping LCMV specific primer pairs to cover the S-segment and L-segment of both LCMV strains. Purified PCR products (Qia Quick PCR purification Kit) were sequenced utilizing the Big Dye™ Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems™) and analyzed on a Prism Genetic Analyzer 3130-16 (Applied Biosystems™). Terminal Sequences (5' and 3' ends) of S- and L-segments were obtained from viral RNA utilizing the Superscript™ IV First-Strand Synthesis System (Thermo Fisher Scientific) according to the manufacturers protocol and gene specific primer for first strand synthesis, followed by dA tailing and second strand synthesis with oligo-T-tailed primer. The second strand synthesis product was PCR amplified utilizing Q5® Hot Start High-Fidelity DNA polymerase (New England Biolabs GmbH, Frankfurt, Germany) oligo -T-tailed primer and nested gene specific primer. Purified PCR products were sequenced with nested gene specific primer as described above.

2.4 Bone marrow derived dendritic cell (BMDC) generation

BMDCs were generated as previously described[316]. Briefly 2 million bone marrow cells were cultured in non-tissue culture treated dishes in the presence of GM-CSF (40ng/ml). On day 3, day 6 and day 8, fresh medium containing GM-CSF was added into the BMDC cultures. Experiments were performed at day 10 post GM-CSF culture.

2.5 Viral entry assay

BHK-21 cells were kept on ice together with LCMV virus at MOI 0.5 for 1 hour. Then BHK-21 cells and LCMV virus mixture were incubated at 37°C. At 20 minutes, 60 minutes and 150 minutes post incubation, monensin was added (2.0 μM). Next day, LCMV infected cells were quantified by flow cytometry using anti-LCMV NP antibody (clone VL-4).

2.6 Western Blot assay

Immunoblot analysis: cells were lysed with RIPA lysis buffer supplemented with a protease inhibitor 'cocktail' (Sigma, P8340). Protein concentration in cell lysates was measured by Advanced Protein assay Reagent: 5 X Concentrate (Advanced, ADV01). Equal lots of cell lysates were subjected to separation by SDS–PAGE, then transferred to a Nitrocellulose Blotting Membrane (GE Healthcare Life Sciences, 10600002). After transfer, the membrane was blocked with freshly prepared Odyssey® Blocking Buffer (PBS) for 30-60min. Primary antibody was diluted with blocking buffer and the membranes were incubated for overnight at 4℃. IRDye 680RD (LI-COR, 925-68070) or IRDye® 800CW (LI-COR, 925-32211) secondary antibodies were used for developing. Band intensity was quantified by ImageJ software.

2.7 Flow cytometric analysis

For dendritic cell staining, singly suspended cells were incubated with antibodies (anti-CD19, CD3, CD8, CD11c, MHC-II, B220, Siglec-H, CD40, CD80, and CD86) for 30min at 4°C. Tetramer and intracellular cytokine staining were performed as described previously. For tetramer staining, singly suspended cells were incubated with tetramer-gp33 or tetramer-gp34 (CD8) for 15 minutes at 37°C. After incubation, surface antibodies (anti-CD8, IL-7R, KLRG1, CD44, CD62L, PD-1, TIM-3, LAG-3, CXCR5) were added for 30 minutes at 4°C. For intracellular cytokine re-stimulation, singly suspended cells were stimulated with LCMV specific peptides gp33, np396,

and gp61 for 1 hour. Brefeldin A (eBiocience) was added for another 5 hours' incubation at 37°C followed by staining with anti-CD8/anti-CD4, anti-IFN-γ, and anti-TNF-α. Experiments were performed using a FACS Fortessa and analyzed using FlowJo software.

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

ANTIBODY	SOURCE	IDENTIFIER
RIG-I (D14G6) antibody	Cell Signaling Technology	Cat #3743
MDA-5 (D74E4) antibody	Cell Signaling Technology	Cat #5321
MAVS(C-1)	Santa Cruz	Cat #365333
Phospho-TBK1/NAK(Ser172) (D52C2) antibody	Cell Signaling Technology	Cat #5483
Phospho-IKKε (Ser172) (D1B7)	Cell Signaling Technology	Cat #8766
TBK1/NAK antibody	Cell Signaling Technology	Cat #3013
IKKε antibody	Cell Signaling Technology	Cat #2690
Phospho-IRF-7 (Ser437/438) (D6M2I) antibody	Cell Signaling Technology	Cat #24129
IRF-7 antibody	Abcam	Cat #ab109255
Phospho-IκBα (Ser32/36) (5A5) antibody	Cell Signaling Technology	Cat #9246
IκBα (44D4) antibody	Cell Signaling Technology	Cat #4812
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (E10) antibody	Cell Signaling Technology	Cat #9106
p44/42 MAPK (Erk1/2) (137F5) antibody	Cell Signaling Technology	Cat #4695
Phospho-SAPK/JNK (Thr183/Tyr185) (81E11) antibody	Cell Signaling Technology	Cat #4668
SAPK/JNK antibody	Cell Signaling Technology	Cat #9252
IL-1β (D6D6T) antibody	Cell Signaling Technology	Cat #31202
Phospho-Stat1 (Tyr701) (58D6) antibody	Cell Signaling Technology	Cat #9167
Stat1antibody	Cell Signaling Technology	Cat #9172
β-Tubulin (9F3) antibody	Cell Signaling Technology	Cat #5346
FITC anti-mouse CD40 Antibody	BioLegend	Cat #124608

APC anti-mouse CD80 Antibody	BioLegend	Cat #104714
PE anti-mouse CD86 Antibody	BioLegend	Cat #159204
PE/Cyanine7 anti-mouse CD11c Antibody	BioLegend	Cat #117318
APC anti-mouse CD80 Antibody	BioLegend	Cat #104714
MHC Class II (I-A/I-E) Monoclonal Antibody (M5/114.15.2), APC-eFluor 780	eBioscience™	Cat # 47-5321-82
7-AAD Viability Staining Solution	BioLegend	Cat #420404
PE anti-mouse IFN-γ Antibody	BioLegend	Cat #505808

2.8 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. Supernatant of LCMV infected BMDCs was collected into tubes. Serum and supernatant dilutions from 1:10 to 1:100 were used to perform enzyme-linked immunosorbent assay (ELISA). IFN-α (TheroFisher), IL-6 (TheroFisher), TNF-α (TheroFisher) and IL-1β (TheroFisher) concentrations were determined using ELISA according to the manufacturers' instructions.

2.9 Northern blot analysis

5µg RNA were separated on a denaturing 1% agarose gel and capillary blotted overnight onto a positively charged nylon membrane by using 20x SSC (3M NaCl, 300mM tri-sodium-citrate). The RNA was UV cross-linked to the membrane, the large and small rRNAs were marked, and the membrane was washed twice with dH_2O. After 2h of pre-hybridization with 10 ml 1x DIG Easy Hybridization solution (Roche) at 55°C, the membrane was hybridized with specific

digoxigenin (DIG)-labeled NP-PCR probes (DIG-11-dUTP alkali-labile; Roche). Following overnight hybridization at 55°C, the membrane was washed twice with dH_2O and twice with stringent wash buffer I (2× SSC, 0.1% SDS) at room temperature, followed by two 20 min washing steps in stringent wash buffer II (0.2× SSC, 0.1% SDS) at 68°C. After two additional washing steps with dH_2O the membrane was incubated in maleic acid buffer (0.1M maleic acid, 150mM NaCl, pH 7.5) and blocked with 1x blocking solution (Roche) dissolved in maleic acid buffer for 45 min. Anti-Digoxigenin-AP, Fab fragments (Roche) were diluted 1:20,000 in 1× blocking solution and incubated for 1h at RT. The membrane was washed three times with maleic acid buffer (10min each), and the RNA bands were visualized by using CDP-Star for chemiluminescent reactions (1:100 in AP buffer [0.1M Tris HCl, 0.1M NaCl, pH 9.5]; Roche). The blots were visualized using the Lumi-Imager™ F1 (INTAS) (from Lara Walotka[2]). Band intensity was quantified by ImageJ software.

2.10 Plasmids, Cells, and Transfections

The pC-NP, pC-L-Cl13 and pI-L-Cl13(-) plasmids were obtained from Juan Carlos de la Torre (Molecular Integrative Neuroscience Department, The Scripps Research Institute). For generation of polI-S-WE and polI -S-Docile, the WE S segment and Docile S segment with a murine RNA polymerase I (pol I) promoter were synthesized for insertion into the pUC18 backbone (BioCat GmbH, Germany). In brief, after the S segments of LCMV (WE and Docile) were generated, both segments were placed under the control of a pol I expression cassette that are expressed by the pol I promoter. The details of pC-NP, pC-L-Cl13 and pI-L-Cl13(-) have been described [295, 317, 318]. BHK-21 cells were transfected by using jetPRIME® (2.5 µl/µg DNA; Polyplus-transfection® SA).

2.11RNA purification and RT-PCR

Viral RNA obtained from culture supernatant or cells was purified by Trizol according to manufacture instruction. 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 mins 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 mins 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. GP, IGR, and NP primers were designed to share 100% match between WE and Docile S-segment genome. GP, IGR, and NP expression level were analyzed using an iTaq universal SYBR Green 1-Step kit (Bio-Rad, Cat #1725151). The expression level of IFNA subtype genes was analyzed using an iTaq Universal Probes One-Step Kit (Bio-Rad, Cat #1725141).

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).

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

Primer TaqMan®	**Catalogue number**	**Probe**
Gapdh	4352339E-0806018	VIC
Ifna1	Mm03030145_gH	FAM
Ifna2	Mm00833961_m1	FAM
Ifna4	Mm00833969_s1	FAM
Ifna5	Mm00833976_s1	FAM
Ifna6	Mm01703458_s1	FAM
Ifna7	Mm02525960_s1	FAM
Ifna9	Mm00833983_s1	FAM
Ifna11	Mm04207507_gH	FAM
Ifna12	Mm00616656_s1	FAM
Ifna13	Mm00781548_s1	FAM
Ifna14	Mm01703465_s1	FAM
Ifnb1	Mm00439552_m1	FAM
Primer SYBR	**Forward sequence**	**Reverse sequence**
Actb	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT
Ifna	TCTGATGCAGCAGGTGGG	AGGGCTCTCCAGACTTCTGCTCTG
GP	GGGGCTGGGCAGGTTCAGATGGCA	TGCAGCAAGGATCATCCATTTGGT
S-IGR	TGTAAAAACTATCTGGAAAAGACGC	ACTGTGCACTCATGGACTGCATCAT
NP	TCTGATGTCATCAGAACCTTGAC	ACCACAAAATGGGCAATTCATAC
Z	CCAGACACCACCTATCTTGG	TCACTCCTCATAGGGAGGTGG
L-IGR	CCAGACACCACCTATCTTGG	AGGTTCAGACTCAAGGGGAA
L	ATGCTCACCAACCCAACAAAGAGAA	TTAGGGTTGACAAAGAAACCAAACT

2.12 Statistical analysis

Graphpad prism software (version 8) was used to calculate significance between the samples. Data are expressed as mean ± S.E.M. For analysis of statistical significance between two groups, an unpaired *student's t-test* (2 tailed) was used. For analysis of statistical significance

between multiple groups, a one-way ANOVA was used. For analysis of multiple time point experiments, two-way ANOVA with an additional Bonferroni post-test was used. $P<0.05$ was considered as statistically significant. The significance level was classified by asterisks as follows: $p < 0.05$ (*); $p < 0.01$ (**); $p < 0.001$ (***) and $p < 0.0001$ (****). Statistical test is indicated in each figure. Samples sizes(n) are provided in the respective figure legends.

3 Results

3.1 Co-infection with acute and chronic LCMV strains rescues exhausted virus-specific CD8$^+$ T cells

Virus-specific effector CD8$^+$T cells Immunology leads to the efficient clearance of acute LCMV infection. However, Chronic infection is accompanied by exhaustion of antigen specific T cells and persistence of LCMV in vivo. Consistently, when WT animals were infected with same numerous infectious units (2x10^4 pfu) of LCMV strain WE (acute) or Docile (chronic), mice infected with the Docile (chronic) strain exhibited reduced numbers of LCMV specific CD8$^+$ and CD4$^+$ T cells in both blood and spleen tissue when compared to mice infected with the acute strain (Figure 1A-B). Surprisingly, when the same host was inoculated with both the acute (2x10^4 pfu) and chronic (2x10^4 pfu) strain of LCMV, enhanced antigen specific T cell numbers were observed when compared to mice only infected with the chronic strain (Figure 1A-B). Activated CD8$^+$ T cells can be differentiated into short lived effector T cells or memory precursor cells as classified by surface KLRG1 and IL-7R expression. We observed reduced SLEC (KLRG1$^+$ IL-7R$^-$) and MPEC (KLRG1$^-$ IL-7R$^+$) expression in chronic infected hosts when compared to acute infected counterparts (Figure 1C). Consistently, co-infection resulted in rescue of SLEC and MPEC formation when compared to chronic infected animals (Figure 1C). In addition, exhaustion molecules such as PD-1 and TIM-3 were highly reduced in LCMV specific CD8$^+$ T cells derived from acute or co-infected hosts when compared to chronically infected hosts (Figure 1D). Consistently, IL-7Rs maintaining the survival of T cells were up-regulated in acute or co-infected animals compared to mice infected with LCMV Docile alone (Figure 1D).

Figure 1

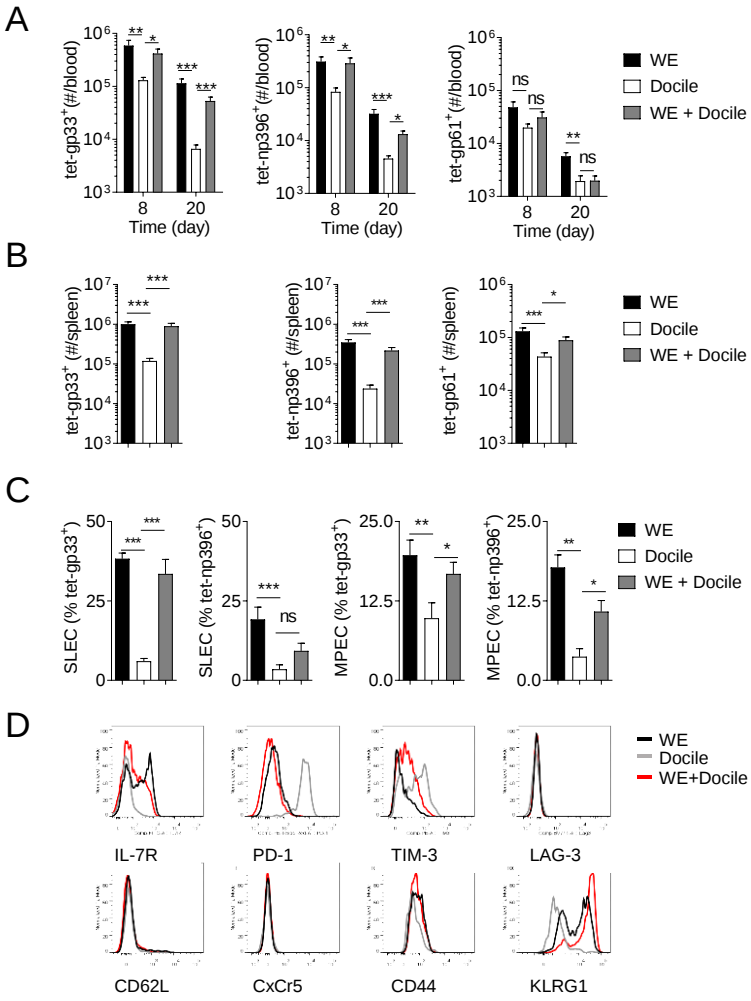


Figure 1: Co-infection rescues exhausted virus-specific CD8+ T cells.

C57BL/6 mice were infected with WE, Docile, or WE together with Docile for 20 days. ***(A-B)*** *at indicated day post infection, tet-gp33^{+} (CD8^{+}), tet-np396^{+} (CD8^{+}), and tet-gp61^{+} (CD4^{+}) were determined in the* ***(A)*** *blood tissue and in* ***(B)*** *spleen tissue (n=9-11). Frequency of* ***(C)*** *short lived effector cells (SLEC, KLRG1^{+} IL-7R^{-}) and memory precursor cells (MEPC, KLRG1^{-}, IL-7R^{+}) are shown from splenic tet-gp33^{+} and tet-np396^{+} (n=9-11).* ***(D)*** *Represented surface molecule FACS*

*blots were shown from splenic tet-gp33^{+} cells (n=9-11). For (A-C), data are plotted as mean ± SEM and are pooled data from three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001 and ns, not significant.*

3.2 Co-infection with acute and chronic LCMV strains leads to reduced viral load in mice

During acute viral infection, virus-specific effector CD8+T cells highly expand, secrete abundant number of inflammatory cytokines and exert potent cytolytic activities on infected cells. To evaluate the function of LCMV specific T cells, in blood or spleen samples from infected animal cells have stimulated with peptides derived from LCMV. We observed a significant increase of IFN-γ and TNF-α producing $CD8^+$ T cells in animals with acute- or co- infection, in sharp contrast to their chronically infected counterparts. Moreover, we also observed a mild difference in $CD4^+$ T cell response (Figure 2A-F). Consistently, 20 days post-infection, the virus titer was below the detection limit in mice infected in an acute setting, while LCMV was persistent in chronic infected animals (Figure 2G). Notably, LCMV titers of mice, which received both LCMV strains simultaneously showed reduced viral titer in organs tested (Figure 2G).

Figure 2

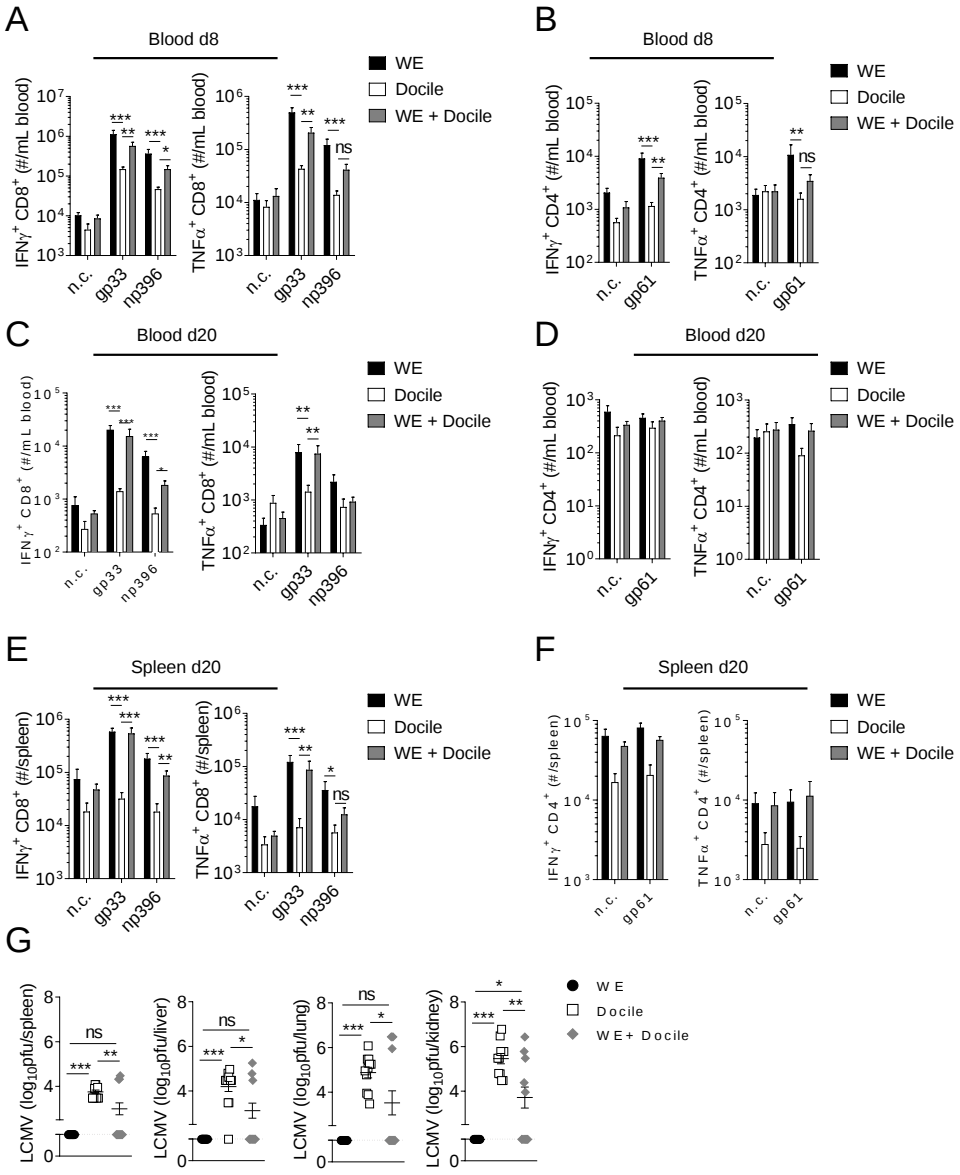


Figure 2: Co-infection induces inflammatory cytokine production and recuses chronic viral infection.

C57BL/6 mice were infected with WE, Docile, or WE together with Docile. ***(A-F)*** *at indicated time post infection, blood cells, single cell suspended splenocytes were re-stimulated with LCMV-*

specific CD8$^+$ T cell epitopes as indicated followed by staining for IFN-γ and TNF-α (n=9-11). ***(G)*** *at 20 days' post infection, virus titers were determined in spleen, liver, lung, and kidney tissue (n=9-11). Data are plotted as mean ± SEM and are pooled data from three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001 and ns, not significant.*

3.3 Adaptive immune cells are required for the IFN-I response in acute- and co- infected mice

T cell immunity is tightly regulated by antigen presenting cells (APCs), hence we hypothesized that acute or co-infection can result in enhanced activation of APCs in comparison to chronic infection. To investigate dendritic cell (DC) activation, we infected WT animals with the acute, or chronic strain of LCMV. Interestingly, WE or co-infected animals exhibited reduced numbers of cDCs ($CD11c^+$ $MHC\text{-}II^+$) and pDCs ($B220^+$ $Siglec\text{-}H^+$) (Figure 3A). Furthermore, we observed that DCs from acute or co-infected mice had increased expression levels of co-stimulatory molecules such as CD40, CD80 and CD86 when compared to the chronic infected counterpart (Figure 3B). CD11c expressing cells can contribute to IFN-α production following LCMV infection [319]. Consistently, serum from acute and co-infected animals contained increased levels of IFN-α after infection when compared to Docile infected mice (Figure 3C). However, we did not observe any difference in the pro-inflammatory cytokines IL-6, TNF-α, and IL-1β between all groups (Figure 3D-F). Furthermore, we determined that expression levels of IFN-I mRNA in spleen tissue of LCMV-infected mice was significantly induced in acute and co-infection mice (Figure 3G-H).

Figure 3

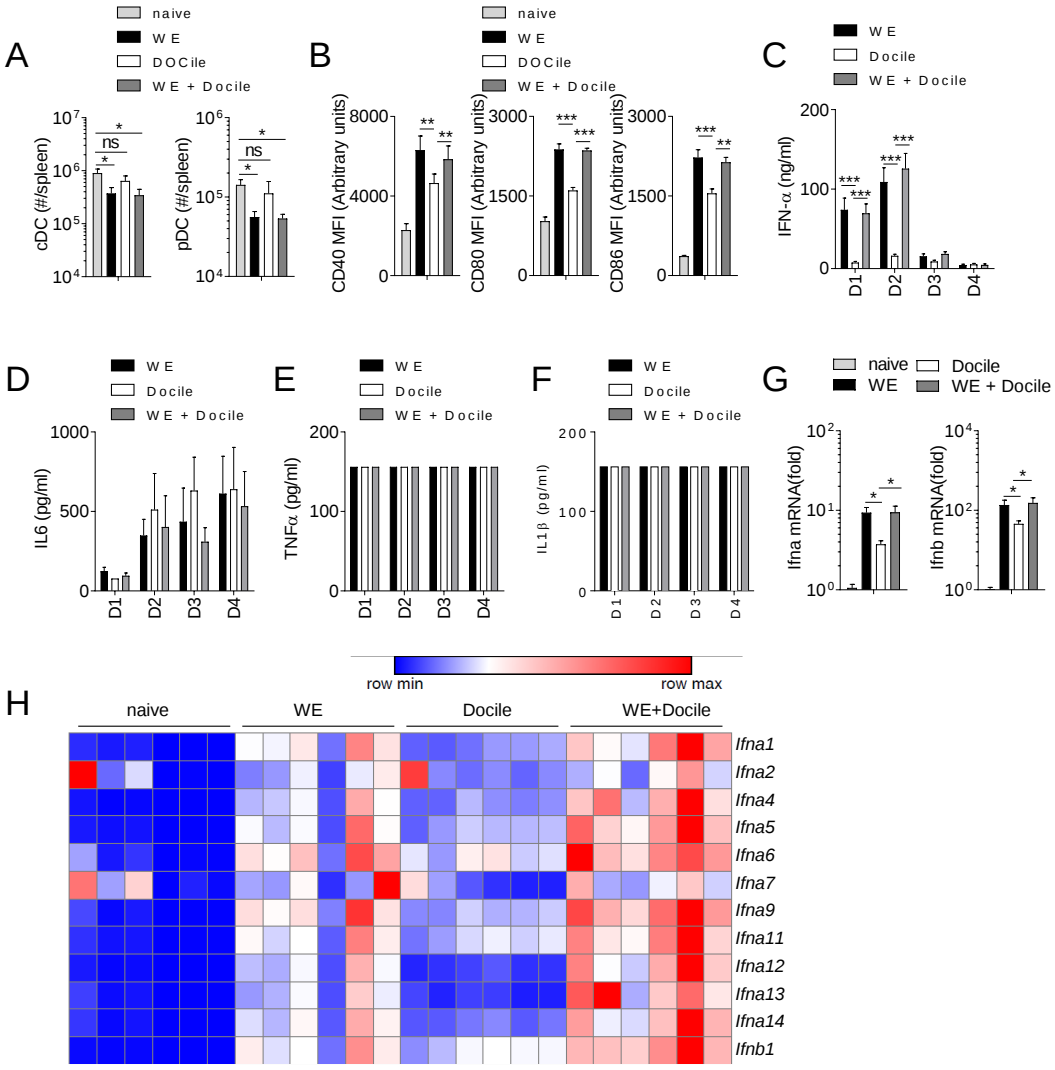


Figure 3: DCs are required for the IFN-I response in acute- and co- infected mice.

C57BL/6 mice were infected with WE, Docile, or WE together with Docile. ***(A-B)*** *at day 2 post infection, number of* ***(A)*** *splenic cDC ($CD11c^+$ $MHCII^+$) and pDC ($B220^+$ $Siglec\text{-}H^+$) were determined (n=9) by FACS.* ***(B)*** *Co-stimulatory molecules CD40, CD80, and CD86 were measured from splenic cDC (n=6) followed by staining for antibodies.* ***(C)*** *Serum IFN-α*

concentration was determined at indicated time post infection (n=9) by ELISA. ***(D)*** *Serum IL-6,* ***(E)*** *serum TNF-α,* ***(F)*** *serum IL-1β concentrations were measured at indicated time points (n=5) by ELISA.* ***(G-H)*** *pan-IFN-α, IFN-α different isoforms and pan-IFN-β mRNA transcripts were measured from spleen tissue (n=6) by RT-PCR. For (A-G), data are plotted as mean ± SEM and are pooled data from three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001 and ns, not significant.*

3.4 Acute infection induces enhanced DC activation and immune responses in BMDC

To further characterize DC activation during LCMV infection, bone marrow derived dendritic cells were infected with strain WE or Docile or both strains. Similar to our in vivo findings, we observed increased co-stimulatory molecule expression in WE or co-infected BMDCs when compared to Docile infected BMDCs (Figure 3A). Moreover, we observed highly reduced IFN-α production in Docile infected BMDCs when compared to the other groups (Figure 3B). Furthermore, the production of IL-6 and TNF-α was slightly reduced in Docile infected BMDCs (Figure 3C-D). NLRP3, which belongs to the large family of intracellular NOD-like receptors (NLRs), activates caspase-1 and cleavage of inactive cytosolic precursor pro-IL-1β to its mature form IL-1β [320]. Interestingly, the IL-1β production pattern was similar to the IFN-α production pattern in LCMV infected BMDCs (Figure 3E). Collectively, these findings showed that Docile fails to induce IFN-I and systemic cytokine (IL-1β and TNF-α) production in sharp contrast to WE infection.

Figure 4

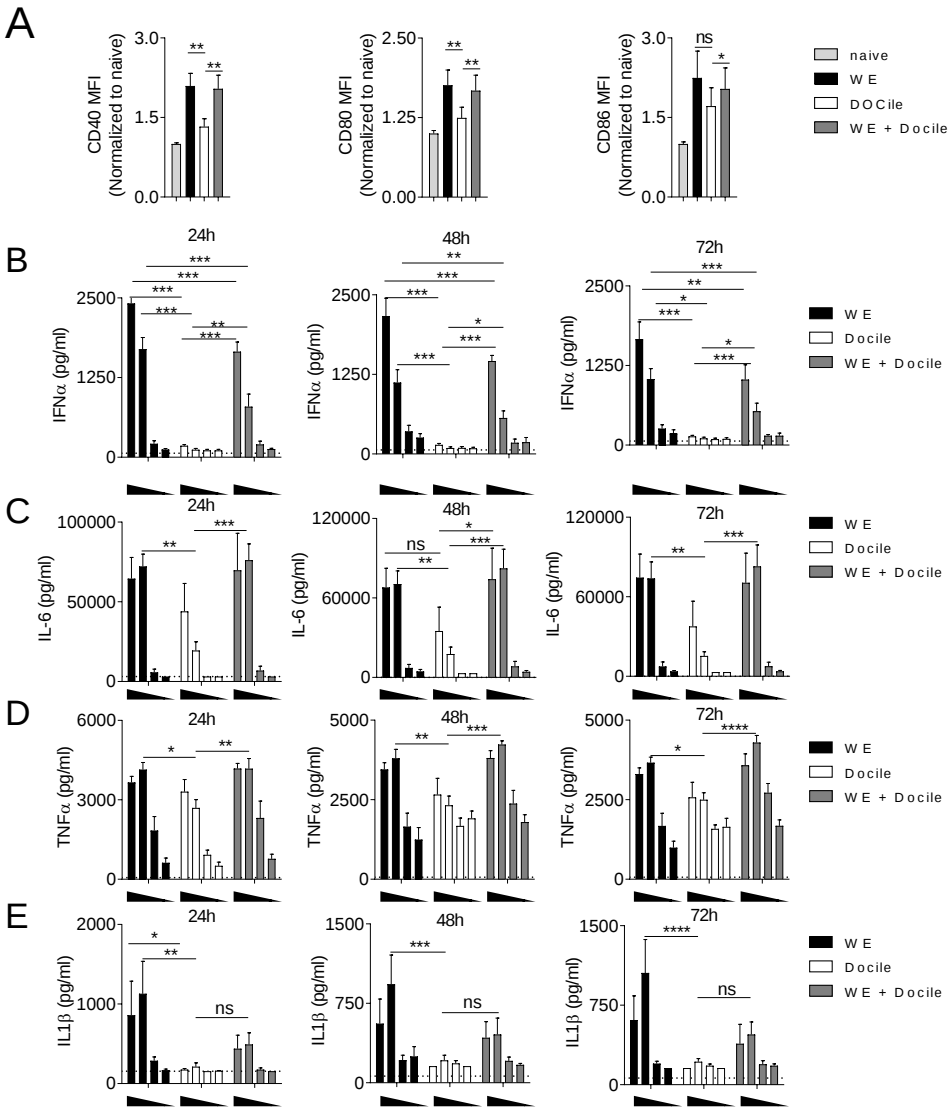


Figure 4: WE- and co- infection induces enhanced DC activation and IFN-I and cytokine production in BMDC.

GM-CSF induced BMDCs were infected with LCMV WE or Docile, or co-infected at the indicated MOI, ***(A)*** *Co-stimulatory molecules expression was monitored by flow cytometry 24 hours post infection on CD11c^{+} MHC-II^{+} BMDCs (n=6).* ***(B)*** *IFN-α concentration was determined in the*

supernatant of LCMV infected BMDCs at indicated time points (n=9). ***(C)*** *IL-6,* ***(D)*** *TNF-α,* ***(E)*** *IL-1β levels were measured in the supernatant of LCMV infected BMDCs at indicated time points (n=7). Data are plotted as mean ± SEM and are pooled data from three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001, ****p < 0.0001 and ns, not significant.*

3.5 LCMV WE infection results in effective PRR activation compared to LCMV Docile

Type I interferon production is mediated by phosphorylated IRF3 and IRF7, which can be activated by phosphorylated TBK1 and IKKε [321]. Consistently, we observed reduced activation of TBK1 and IKKε in Docile infected BMDCs when compared to WE or co-infected BMDCs (Figure 5A-C). It has been reported that direct interaction of NP protein with RIG-I and MDA-5 can suppress PRR signaling [309, 322, 323]. We hypothesized that Docile NP downregulates RIG-I or MAD5 expression and accordingly affects IFN-I production. However, we did not see major differences in the expression of RIG-I, MAD5, and MAVS protein between acute, chronic or co-infected BMDCs during early course of infection (Figure 5D-G).

Figure 5

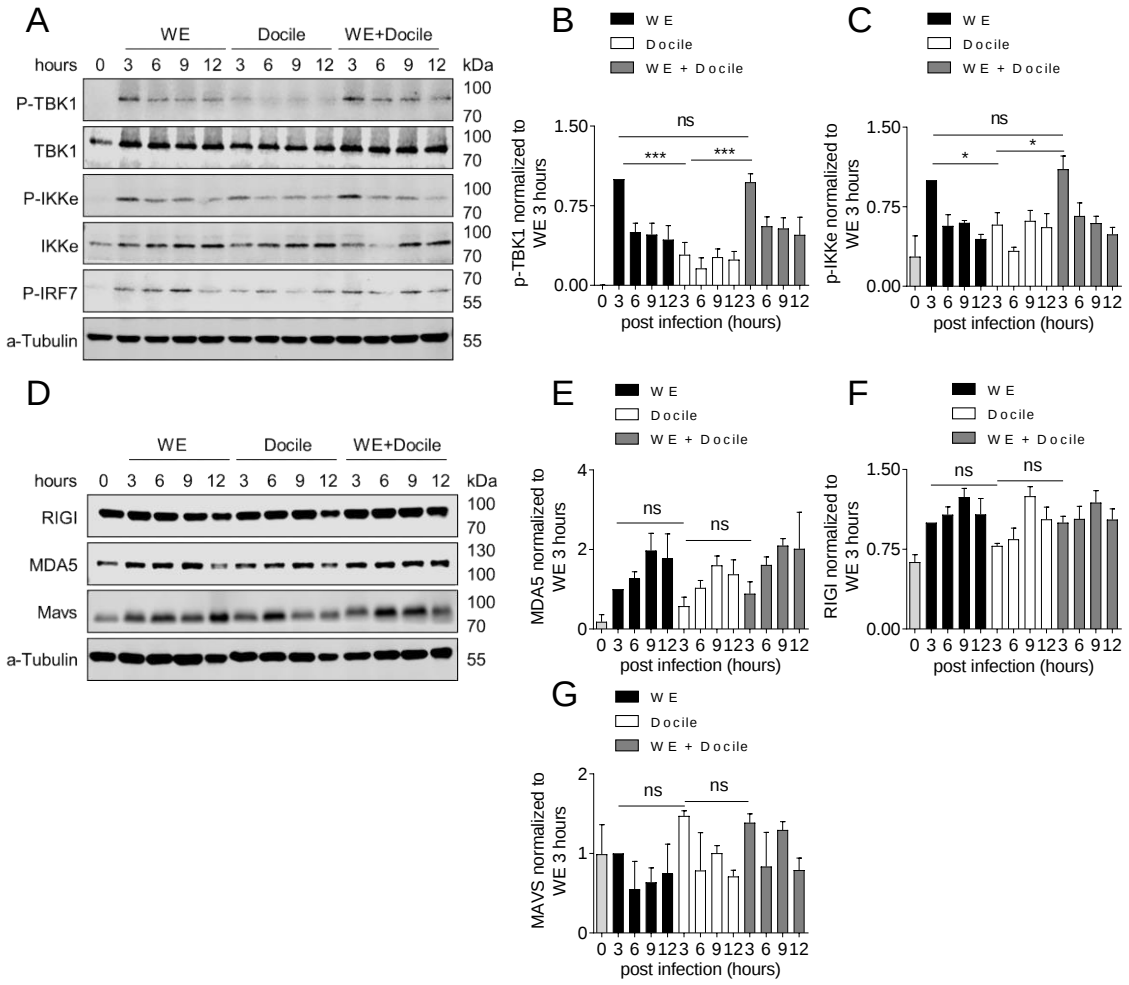


Figure 5: LCMV WE strain triggers higher PRR activation than LCMV Docile.

GM-CSF induced BMDCs were infected with LCMV WE or Docile, or co-infected at MOI 1. ***(A)*** *Whole cell lysates from LCMV infected BMDCs were blotted for p-TBK1, total TBK1, p-IKKε, total IKKε, and loading control α-Tubulin (one representative blot of n=4 was shown).* ***(B)*** *p-TBK1,* ***(C)*** *p-IKKε quantification was analyzed by first normalizing to corresponding loading control, followed by standardizing to 3 hours WE infected BMDC (n=4).* ***(D)*** *whole cell lysates from infected BMDCs were blotted for RIG-I, MDA5, MAVS and loading control Tubulin at indicated*

time point post infection. ***(E)*** *RIG-I,* ***(F)*** *MDA5,* ***(G)*** *MAVS quantification was analyzed by first normalizing to corresponding loading control, followed by standardizing to 3 hours WE infected BMDC (n=4). Data are plotted as mean ± SEM and are pooled data from three independent experiments. *p < 0.05; ***p < 0.001 and ns, not significant.*

We previously showed that LCMV WE infected BMDCs have heightened cytokine responses. To determine the contribution of BMDCs in this process, western blot (WB) examination was performed on LCMV-infected BMDC (Figure 6A-E). Following LCMV infection, little to no changes were observed in the NF-κB or MAPK pathway activation (Figure 6A-C), but the IL1beta is significantly induced in WE- and co- infected BMDC (Figure 6D-E). Collectively, these findings demonstrate that BMDCs have function in the activation of the PRR signaling pathway and prolonged inflammatory state at the early time points of LCMV post-infection.

Figure 6

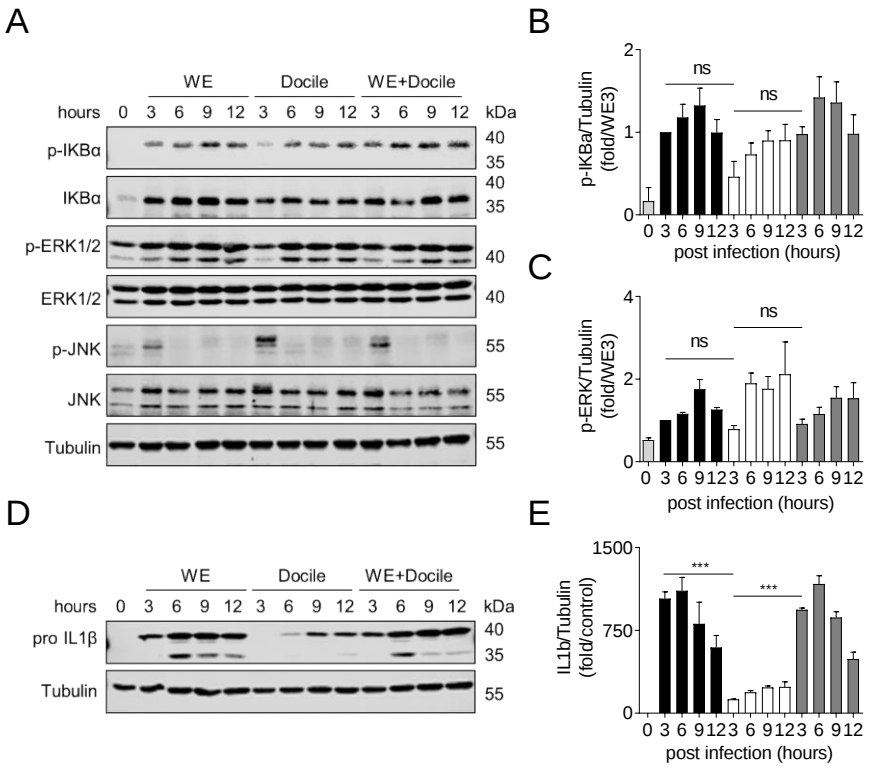


Figure 6: WE infection actives NLP signalling pathway.

GM-CSF induced BMDCs were infected with LCMV WE or Docile, or co-infected at MOI=1, ***(A)*** *at the indicated time points p-IKBα, total IKBα, pERK1/2, ERK1/2, p-JNK, total JNK, and their loading control Tubulin were measured by western blot (one of n=4 representative blot is shown).* ***(B)*** *p-IKBα,* ***(C)*** *pERK1/2 quantification was analyzed by first normalizing to corresponding loading control, followed by standardizing to 3 hours WE infected BMDC (n=4).* ***(D)*** *GM-CSF induced BMDCs were infected with LCMV WE or Docile, or co-infected at MOI=1, at the indicated time points IL-1β was determined by western blot (one of n=4 representative blot was shown),* ***(E)*** *IL-1β quantification was analyzed by first normalizing to the corresponded loading control, followed by standardizing to 3 hours WE infected BMDC (n=4). Data are plotted as mean ± SEM and are pooled data from three independent experiments. ***p < 0.001 and ns, not significant.*

3.6 LCMV Docile infection reduced immune response is independent of the IFN-I signaling pathway

We have shown that WE- and co- infection induce enhanced immune responses both in vivo and in vitro. IFN-I production can be amplified through a positive feedback loop via IFNAR signaling [36]. IFN-I signaling also induces the antiviral state that includes the production of restriction factors by which virus replication is inhibited, thereby limiting the viral load [324]. Therefore, we wondered whether LCMV Docile impaired IFNAR signaling. Accordingly, LCMV infected BMDCs were treated with recombinant IFN-α (rIFN-α) and monitored for pSTAT1. As expected, non rIFN-α treated BMDCs showed reduced levels of pSTAT1 when compared to WE or co-infected BMDCs, because of the limited IFN-I production. However, rIFN-α treated Docile infected BMDCs showed increased levels of pSTAT1. The pSTAT1 levels were similar among all rIFN-α treated LCMV infected BMDCs, indicating that Docile did not inhibit the IFNAR positive feedback loop (Figure 7A-B). Next, LCMV infected MC57 cells were infected with LCMV WE and Docile at the indicated MOI, then treated with recombinant IFN-α (rIFN-α) and monitored for virus titer. As expected, the viral load gradually decreases with the increase of rIFN-α concentration and there is no difference in both WE and Docile infected groups after treatment with rIFN-α in MC57 cells (Figure 7C). Consistently when rIFN-α was added to the LCMV infected BMDCs, LCMV Docile infected BMDCs up-regulate co-stimulatory molecules to WE or co-infected BMDC levels (Figure 7D), while virus replication was inhibited in all groups (Figure 7E). Moreover, expression of co-stimulatory molecules in BMDCs following LCMV infection was dependent on IFNAR signaling (Figure 7F), and viral load was enhanced in all groups of the IFNR1 knockout cells (Figure 7G). Taken together, reduced immune responses during LCMV Docile infection is independent of the IFN-I signaling pathway.

Figure 7

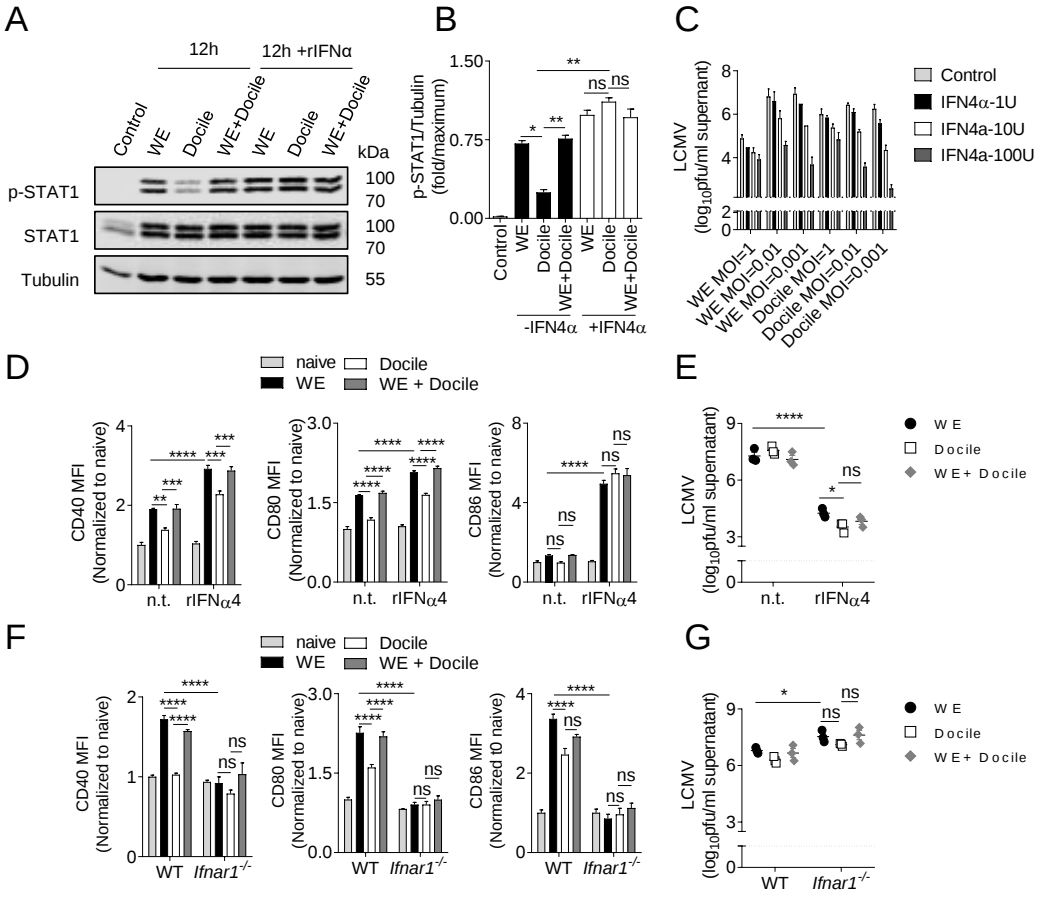


Figure 7: Reduced immunity of LCMV Docile infection is independent of the IFN-I signaling pathway.

GM-CSF induced BMDCs were infected with LCMV WE or Docile, or co-infected at MOI=1. ***(A)*** *12h post infection, BMDCs were treated with recombinant IFNα4 at 100U/ml, 30 minutes later, p-STAT1, total STAT1, and loading control Tubulin were measured by Western blot (one of n=3 representative blot was shown).* ***(B)*** *Quantification of (A) was shown (n=3).* ***(C)*** *MC57 cells were infected with LCMV WE or Docile at different MOI. 2h post infection, cells were washed with PBS and treated with recombinant IFNα4 at 100U/ml. 24h later, virus titer was determined from cell*

supernatant by plaque assay. ***(D)*** *Co-stimulatory molecule expression on LCMV infected BMDCs was monitored by flow cytometry 24h post infection(n=3),* ***(E)*** *Virus titer was determined from cell supernatant at 24h post infection in the presence or absence of 100U/ml recombinant IFNα4 (n=3).* ***(F-G)*** *C57BL/6 or Ifnar1 deficient GM-CSF induced BMDCs were infected with LCMV WE or Docile at MOI 1.* ***(F)*** *Co-stimulatory molecule expression on LCMV infected BMDC was monitored by flow cytometry 24h post infection (n=3),* ***(G)*** *Virus titer was also determined from cell supernatant at 24h post infection (n=3). Data are plotted as mean ± SEM and are pooled data from independent experiments. *p < 0.05; **p < 0.01; ****p < 0.0001 and ns, not significant.*

3.7 LCMV docile infection leads to delayed viral genomic replication compared to WE infection

IFN-I production through MDA5 and/or RIG-I sensing is important for virus control and $CD8^+$ T cell activation during LCMV infection [323, 325, 326]. While our data suggest that PRR activation by LCMV Docile was reduced, RNA harvested from LCMV Docile was able to induce IFN-I following transfection into DCs. Accordingly, we hypothesized that intracellular RNA levels might be reduced following infection with LCMV Docile. Hence, we designed primers targeting the common region of GPC, IGR, or NP of the S segment and Z, IGR, or polymerase of the L segment of LCMV WE and Docile strains (Figure 8A). RT-PCR results from LCMV infected BHK-21 cells revealed enhanced GPC and IGR RNA expression in both WE or co-infected groups when compared to Docile infected counterparts (Figure 8B-C). Interestingly, comparable or minor differences of NP RNA expression between WE and Docile infected cells was observed (Figure 8D). Thereby, enhanced Z and L-IGR RNA expression in both WE or co-infected groups are also observed when compared to Docile infected counterparts (Figure 8E-F). However, comparable or minor differences of polymerase RNA expression between WE and Docile infected cells was detected (Figure 8G). These data indicate that functional transcriptional machinery was present during both infection with LCMV WE and Docile but coding on the negative strand was highly reduced. Consistently, similar RNA expression patterns were observed for the L-segment (Figure 8A-G).

Figure 8

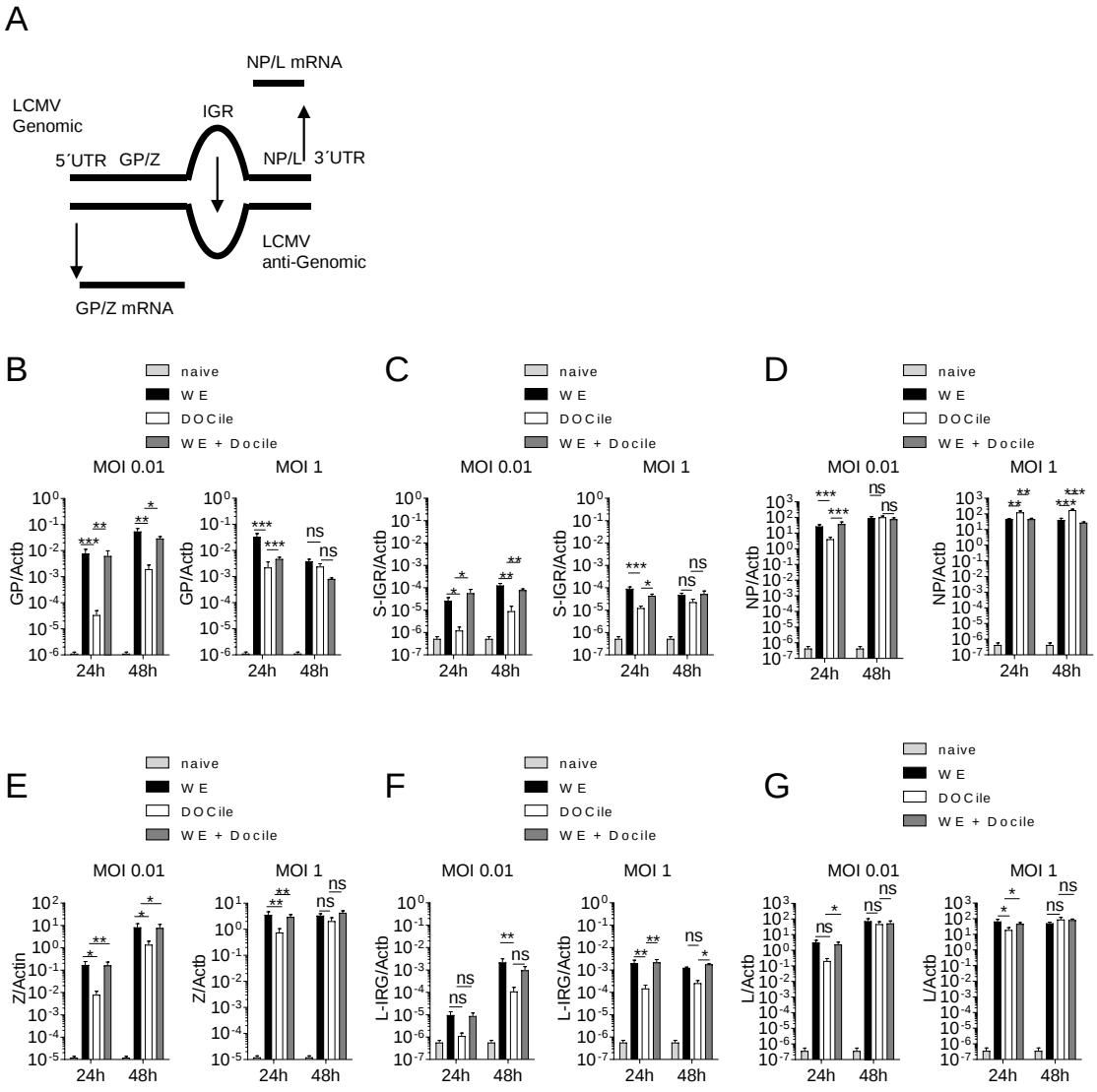


Figure 8: Docile exhibited slower viral replication than WE strain.

(A) *Schematic of LCMV S or L segment replication steps.* ***(B-G)*** *BHK-21 cells were infected with WE, Docile, or both at indicted MOI. At 24h and 48h post infection, BHK-21 cellular RNA was isolated,* ***(B)*** *GP RNA,* ***(C)*** *S-IGR RNA,* ***(D)*** *NP RNA,* ***(E)*** *Z RNA,* ***(F)*** *L-IRG RNA, and* ***(G)*** *L RNA*

*were quantified by RT-PCR (n=6). Data are plotted as mean ± SEM and are pooled data from three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001 and ns, not significant.*

Furthermore, to directly quantify the genomic/anti-genomic mRNA species during LCMV infection, we harvested cellular RNA from LCMV infected BHK-21 cells and analyzed these samples by Northern blot. LCMV WE produced more genomic /anti-genomic RNA and mRNA when compared to the LCMV Docile (Figure 9A-E). However, the ratio of mRNA to genomic/anti-genomic RNA suggested that the Docile strain had more transcription than genomic replication when compared to the WE strain (Figure 9F). Taken together, these data indicated delayed replication of LCMV Docile over LCMV WE. Consistently, the virus titer in the supernatant of Docile infected cells was highly reduced when compared to LCMV WE infected cells (Figure 9G). Overall, our results suggested a delay of viral replication and viral load in LCMV Docile infected BHK21 cells.

Figure 9

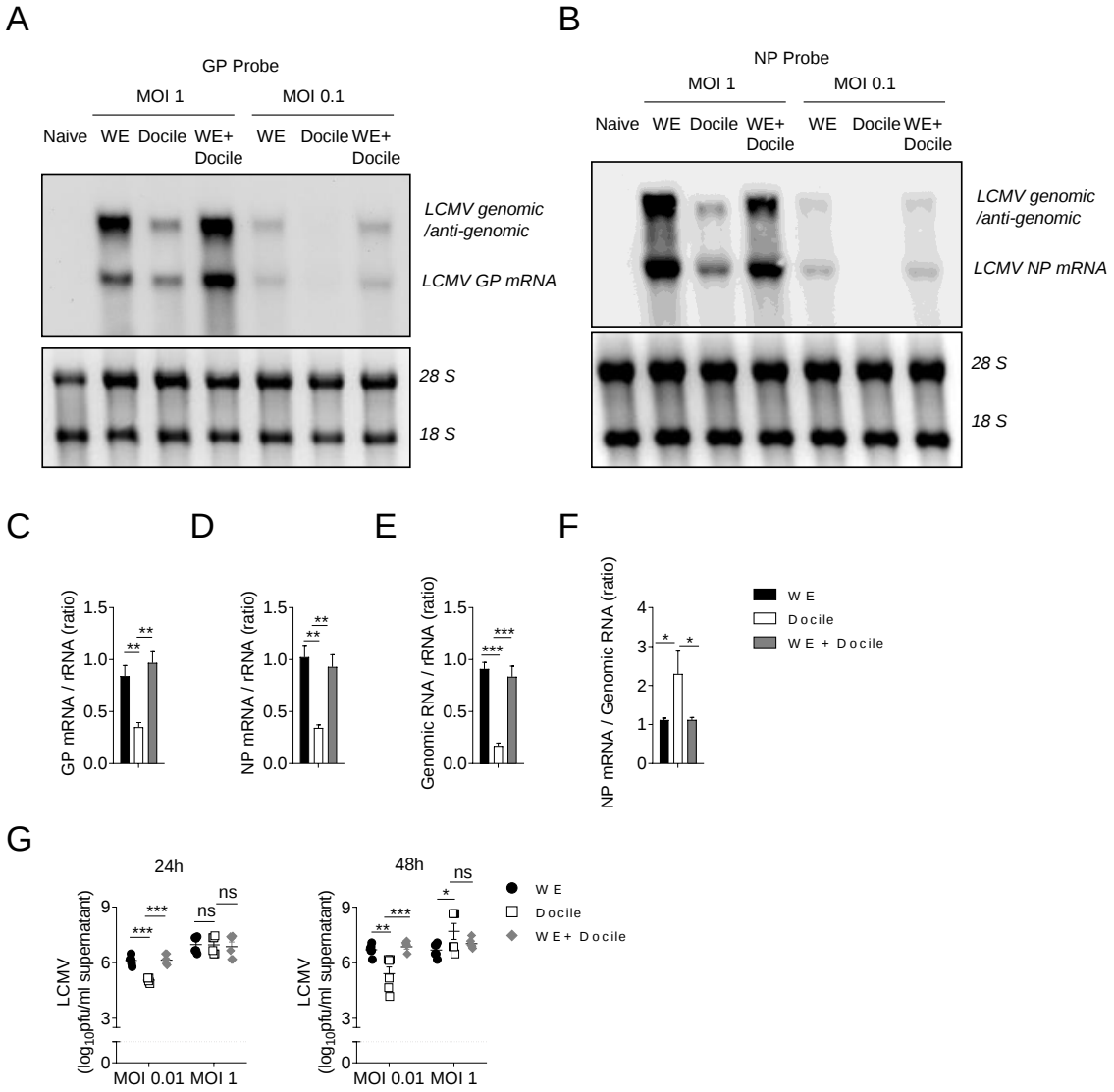


Figure 9: Docile exhibited slower viral replication and viral load than LCMV WE.

(A-B) *BHK-21 cells were infected with WE, Docile, or both at indicted MOI. At 24h post infection, BHK-21 cellular RNA was isolated and analyzed by north blot using a GP or NP specific probe (one of n=4 representative blot was shown from Lara Walotka[2]).* ***(C-E)*** *Ratio of GP mRNA/ribosomal RNA, NP mRNA/ ribosomal RNA and genomic viral RNA/ribosomal RNA were*

quantified (n=4). ***(F)*** *Ratio of NP mRNA/genomic viral RNA was determined (n=4).* ***(G)*** *Virus titers were determined from LCMV infected BMDC supernatant at 24h and 48h post infection (n=6). Data are plotted as mean ± SEM and are pooled data from three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001 and ns, not significant.*

3.8 IFNAR triggers suppression of LCMV WE replication

We wondered whether these findings were also observed in BMDCs. Real time PCR results show that GPC RNA but not NP RNA expression was significantly increased in WE and co-infected BMDCs when compared to Docile infected counterparts (Figure 10A). Interestingly, LCMV titers in the supernatant of BMDCs did not differ despite the presence of IFN-I in LCMV WE infected BMDCs (Figure 10B). However, following infection of *Ifnar1*$^{-/-}$ BMDCs, increased LCMV titers could be measured in the supernatant of LCMV WE infected cells in comparison to LCMV Docile infected cells (Figure 10B).

Figure 10

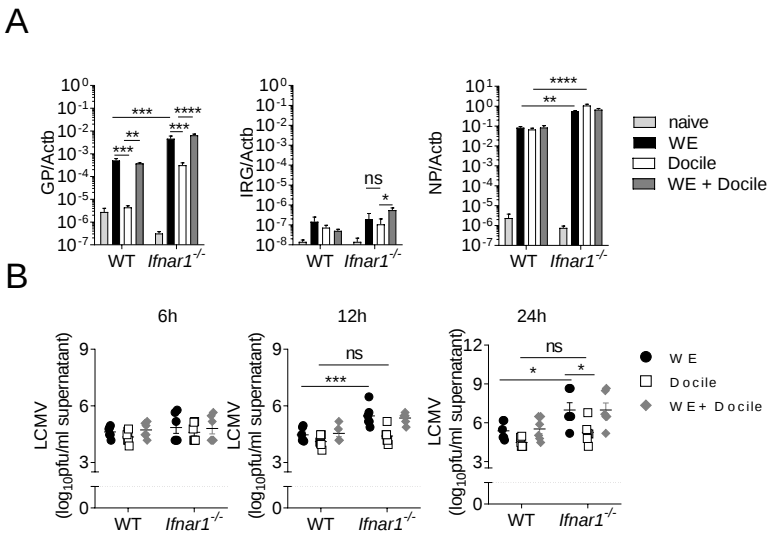


Figure 10: IFNAR trigger suppression of LCMV WE replication.

(AB) *C57BL/6 or Ifnar1 deficient GM-CSF induced BMDCs were infected with LCMV WE or Docile at MOI 1.* ***(A)*** *GP, IGR, and NP region RNA levels were quantified by RT-PCR from infected BMDC cellular RNA (n=3).* ***(B)*** *Virus titer was determined in supernatants of LCMV*

*infected BMDC cultures at 6h, 12h, and 24h post infection (n=6). Data are plotted as mean ± SEM and are pooled data from three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 and ns, not significant.*

3.9 LCMV docile infection leads to delayed viral genomic replication and viral load in vivo

Furthermore, we confirm this infection phenotype in vivo. The data show that LCMV Docile RNA expression in spleen tissue was decreased when compared to LCMV WE in infected mice (Figure 11A-B). Moreover, reduced viral titers were observed in spleen tissue of LCMV Docile infected animals when compared to WE or co-infected mice (Figure 11C). Taken together, these data indicate that LCMV WE- and co- infection exhibit faster viral replication than LCMV Docile resulting in increased viral load at early infected time points.

Figure 11

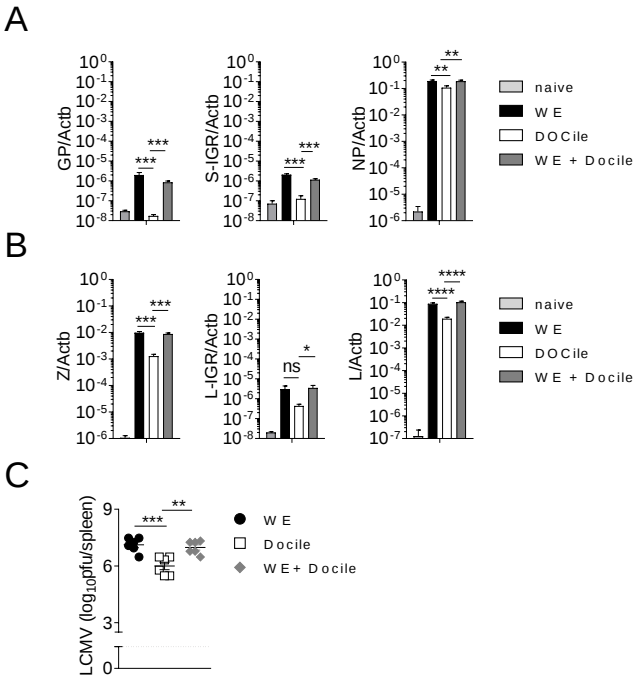


Figure 11: Slower viral replication results in reduced viral load in LCMV Docile infected mice.

(A-C) *C57BL/6 mice were infected with WE, Docile, or both for 2 days.* ***(A)*** *GP, S-IGR, and NP and* ***(B)*** *Z, L-IGR, and L RNA were determined in spleen tissue purified RNA of LCMV infected animals (n=6).* ***(C)*** *Virus titer was determined from spleen tissue of infected animals (n=6). Data are plotted as mean ± SEM and are pooled data from three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 and ns, not significant.*

3.10 Docile while exhibiting slower viral entry is still recognized by PRRs

Previous studies suggest that the C-terminal region of the NP is important for inhibition of IFN-I production and the GP is important for viral entry. Specifically, the DIEG motif (spanning residues 382 to 385) in the NP C-terminal region is critical for suppressing the activity of IKKε [327]. However, our sequence data revealed, that both, WE and Docile contained this motif (Figure 12A). But we found several mutations in the GP which function is still unknown (Figure 12B). Furthermore, we wondered whether the LCMV WE RNA can induce PRR signaling, while the LCMV Docile RNA might have a reduced affinity to PRRs. However, when DCs were transfected with WE or Docile genomic RNA, similar IFN-α production was observed, indicating the failure of IFN-α expression in Docile infected cells was not due to a failure of RIG-I/MDA5 recognition (Figure 12C). Consistently, serum from acute and co-infected animals contained increased levels of IFN-α after high dose infection when compared to Docile infected mice, but infected with Docile still shows reduced IFN-α (Figure 12D). These data indicate that the affinity of viral RNA between WE and Docile strains is similar. Our data suggested a delay of viral replication of LCMV Docile, but similar affinity to PRRs. Hence, we wondered whether viral entry was reduced in LCMV Docile when compared to LCMV WE. We infected cells with either LCMV Docile or WE and blocked further viral entry by application of monensin at different time points. After 16h we determined the number of cells infected by NP expression, since we only found little difference in NP mRNA expression between both strains. Interestingly, entry of LCMV WE was highly increased when compared to LCMV Docile into BHK-21 cells (Figure 12E). We also infected cells with either LCMV Docile or WE and harvested the cells and the supernatant in the cells to

detect the virus titer. Different virus titers were found in the cells between both strains, but not in the supernatants (Figure 12F).

Figure 12

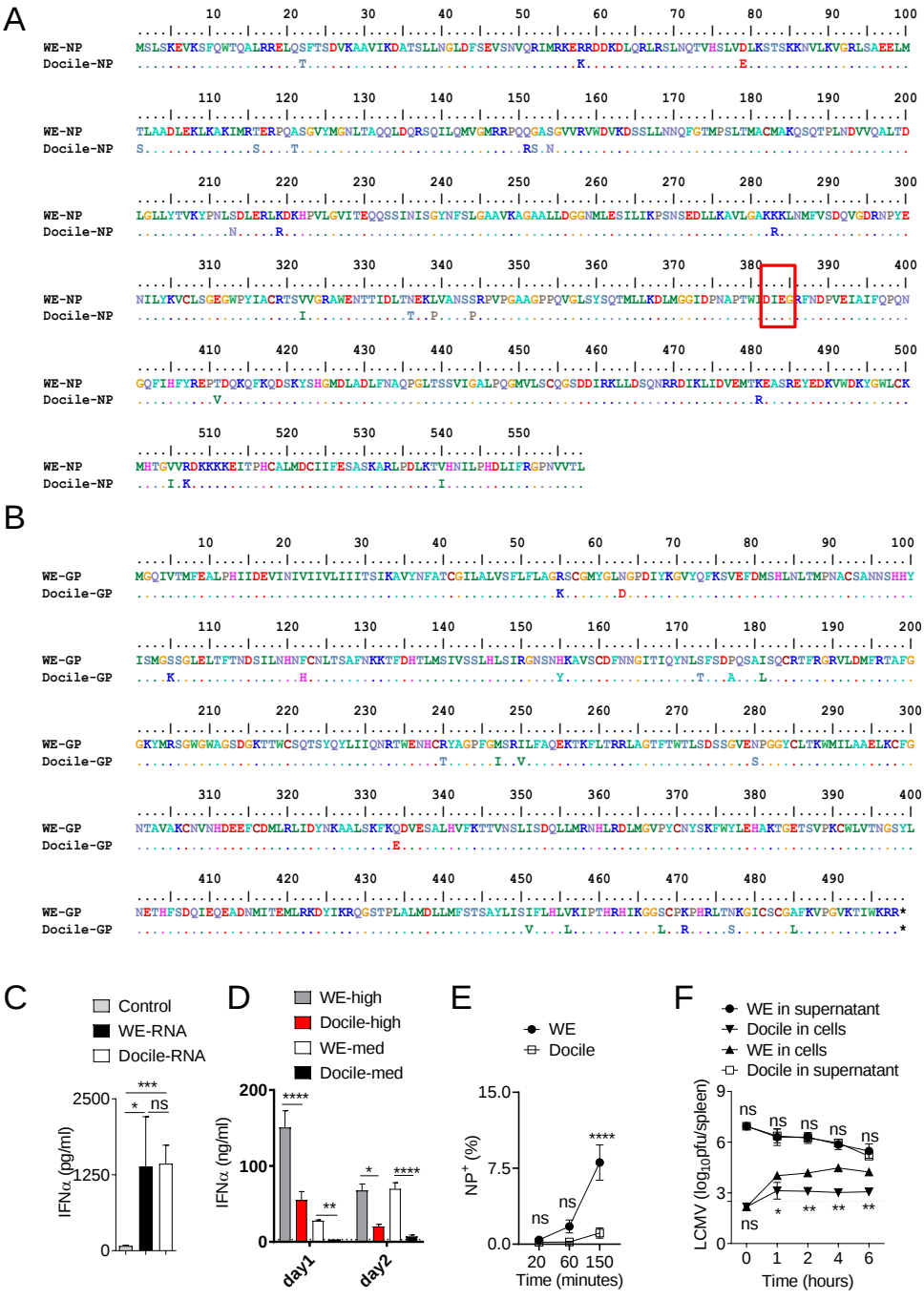


Figure 12: Docile while having slower viral entry is still recognized by the cell receptor.

(A) *NP and* ***(B)*** *GP Amino acids sequence of LCMV WE and Docile Strain is shown. The DIEG motif is highlighted in the red box.* ***(C)*** *JAWSII cells were transfected with 500 ng of WE or Docile genomic RNA, 24h post transfection, IFN-α were determined from supernatant of transfected JAWSII cells (n=12).* ***(D)*** *C57BL/6 mice were infected with $2X10^5$ pfu WE, Docile, or WE together with Docile. Serum IFN-α concentration was determined at the indicated time points post infection (n=6).* ***(E)*** *BHK-21 cells were infected with WE or Docile at MOI 0.5, at the indicated time points post infection, monensin was added. 16h later LCMV infected cells were quantified by anti-LCMV-NP staining (n=5).* ***(F)*** *BHK-21 cells were infected with WE or Docile at MOI 1. The supernatant and cells were harvested at the indicated time points (n=3). Data are plotted as mean ± SEM and are pooled data from independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001 and ns, not significant.*

3.11 The LCMV viral S segment has a critical role in viral entry and replication

To further explore whether slow viral entry and delayed viral replication played an important role in establishing chronic infection, we generated chimeric LCMV viruses utilizing the S from LCMV WE and Docile with the L segment from Clone 13, here named LCMV-S(WE) and LCMV-S(Docile) [328]. LCMV entry of the virus harboring the S(WE) into BHK-21 cells was faster than LCMV containing the S(Docile) (Figure 13A), suggesting that WE-GP can facilitate faster LCMV entry than Docile-GP. Consistently, LCMV-S(WE) showed increased replication in host cells when compared to LCMV-S(Docile) (Figure 13A-B).

Figure 13

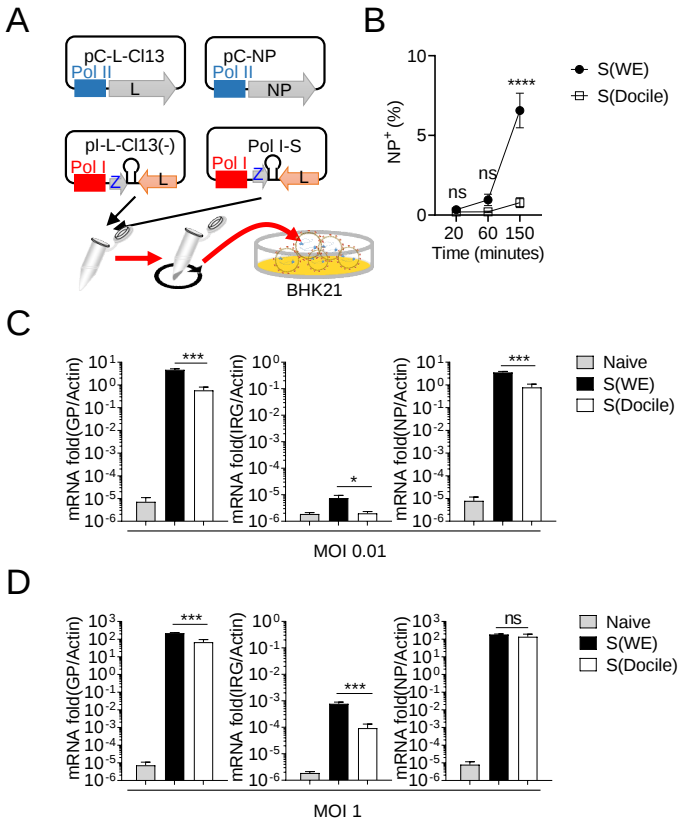


Figure 13: LCMV Docile S-segment contributes to slower viral entry and viral replication.

(A) *BHK-21 cells in M6 tissue culture wells were transfected with 0.8 μg of pC-NP, 1 μg of pC-Cl13-L, 1.4 μg of pI-L-Cl13(-), and either 0.8 μg of pol I-S-WE to recover rWE or 0.8 μg of pol I-S-Docile to recover rDoc* ***(B)*** *BHK-21 cells were infected with chimeric virus S(WE)/L(Clone 13) or S (Docile)/L(Clone 13) at MOI 0.5, at the indicated times post infection, monensin was added. 16h later LCMV infected cells were quantified by anti-LCMV-NP staining (n=5).* ***(C-D)*** *BHK-21 cells were infected with chimeric virus S(WE)/L(Clone 13) or S (Docile)/L(Clone 13) at MOI 0.01* ***(C)****, MOI 1* ***(D)****. At 24h post infection, BHK-21 cellular RNA was isolated, GP RNA, S-IGR RNA, and NP RNA were quantified by RT-PCR (n=6). Data are plotted as mean ± SEM and are pooled data from independent experiments. *p < 0.05; ***p < 0.001; ****p < 0.0001 and ns, not significant.*

3.12 Slow entry leads to impaired innate immune activation

We have already shown that LCMV S segment played an important role in viral entry and replication. Furthermore, we wondered whether LCMV-S(WE) and LCMV-S(Docile) also have the same function in inducing the IFN-I production in BMDCs *in vitro* and in mice *in vivo* (Figure 14A-B). However, infection with LCMV-S(Docile) resulted in highly reduced IFN-I in comparison with LCMV-S(WE) (Figure 14A-B). Consequently, LCMV-S(Docile) infected animals exhibited reduced anti-LCMV $CD8^+$ T cell immunity in number and function (Figure 14C-G). Notably, LCMV-S(Docile) infected animals exhibited elevated viral titers when compared to LCMV-S(WE) infected animals at d7 post infection (Figure14H). However, both chimeric viruses can be cleared at d12 post infection, suggesting both viruses to be attenuated with the L segment from another LCMV strain (Figure 14I). These data suggest that expression of the Docile S segment mediated reduced viral entry, reduced innate and adaptive immune activation, and increased viral load.

Figure 14

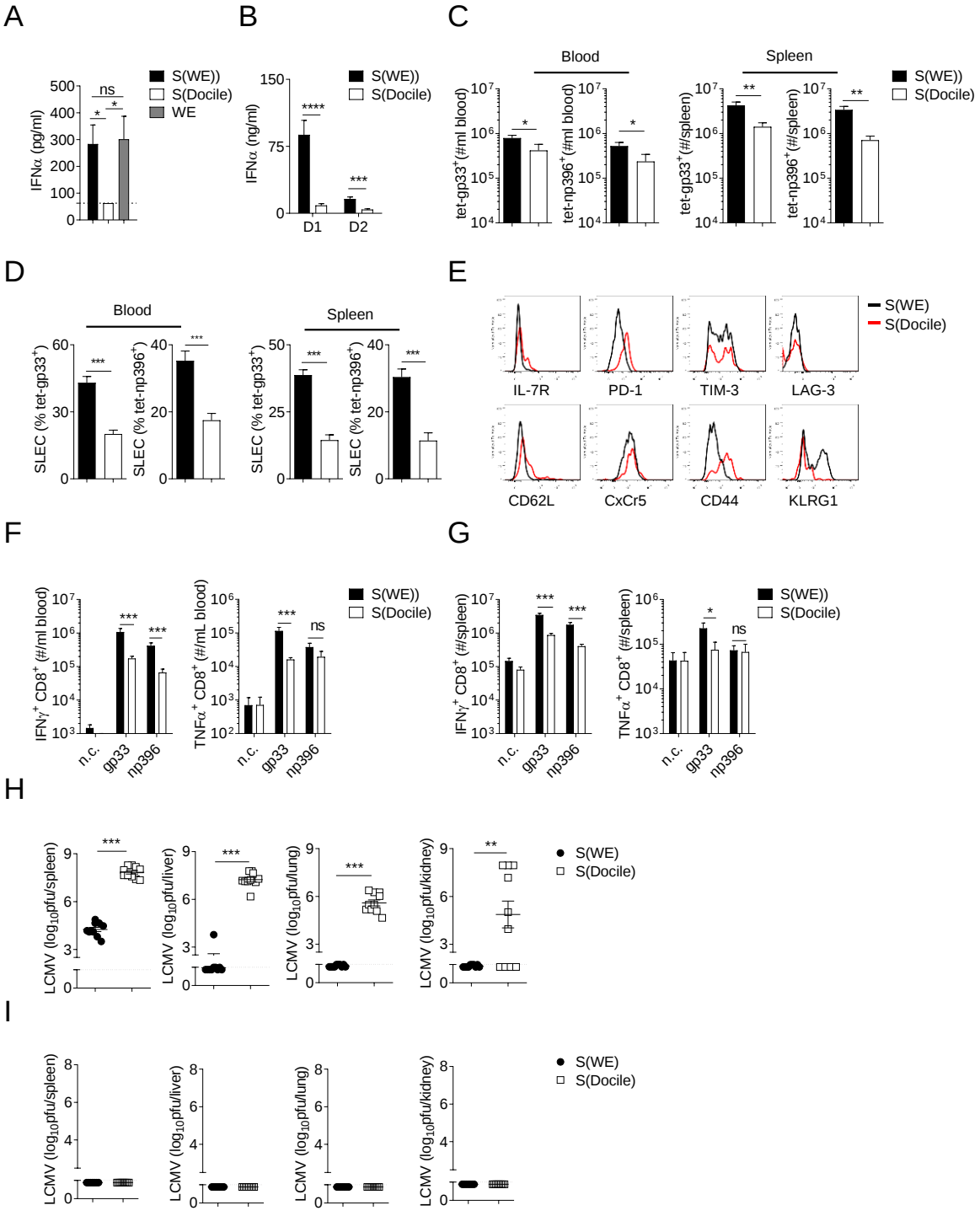

A
B
C
D
E
F
G
H
I
S(WE))
S(Docile)
WE
IFNα (pg/ml)
IFNα (ng/ml)
D1
D2
Blood
Spleen
SLEC (% tet-gp33+)
SLEC (% tet-np396+)
IL-7R
PD-1
TIM-3
LAG-3
CD62L
CxCr5
CD44
KLRG1
n.c.
gp33
np396
LCMV (log10pfu/spleen)
LCMV (log10pfu/liver)
LCMV (log10pfu/lung)
LCMV (log10pfu/kidney)

Figure 14: LCMV Docile S-segment contributes to impaired innate immune activation.

(A) *GM-CSF induced BMDCs were infected with chimeric virus S(WE)/L(Clone 13) or S (Docile)/L(Clone 13) or WE WT virus at MOI 1. 24h post infection, IFN-α levels were determined from infected cell supernatant (n=6).* ***(B-I)*** *C57BL/6 mice were infected with $2x10^5$ pfu chimeric virus S(WE)/L(Clone 13) or S (Docile)/L(Clone 13).* ***(B)*** *At day 1 and day 2 post infection serum IFN-α levels were determined (n=6).* ***(C)*** *Numbers of tet-gp33$^+$ (CD8$^+$), tet-np396$^+$ (CD8$^+$) were determined in blood tissue and spleen tissue at 7 days after infection (n=10).* ***(D)*** *Frequency of short-lived effector cells (SLEC, KLRG1$^+$ IL-7R$^-$) were quantified from blood and spleen LCMV specific CD8$^+$ T cells at 7 days post infection (n=10).* ***(E)*** *T cell surface molecules were illustrated from spleen tet-gp33$^+$ CD8$^+$ T cells (n=10).* ***(F)*** *Blood cells and* ***(G)*** *single cell suspended splenocytes were re-stimulated with LCMV-specific CD8$^+$ T cell epitopes as indicated followed by staining for IFN-γ and TNF-α (n=10).* ***(H)*** *at 7 days' post infection, virus titers were determined in spleen, liver, lung, and kidney tissue (n=10).* ***(I)*** *C57BL/6 mice were infected with $2x10^5$ pfu chimeric virus S(WE)/L(Clone 13) or S (Docile)/L(Clone 13). At day 12 post infection, virus titers were determined in spleen, liver, lung, and kidney tissue (n=7-8). Data are plotted as mean ± SEM and are pooled data from three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001 and ns, not significant.*

4 DISCUSSION

In this study we focus on the evasion mechanism of chronic infection using the LCMV model. The data show that the chronic strain Docile leads to reduced IFN-I production and virus persistence during infection with a medium dose. In contrast, infection with the acute strain LCMV WE resulted in IFN-I production and virus clearance under the same conditions. Mechanistically, infection with LCMV Docile showed reduced PRR activation, which was associated with reduced presence of viral RNA, reduced viral replication and delayed viral entry. In addition, infection of both LCMV WE and Docile strains simultaneously is able to induce IFN-I production following infection, effective T cell immunity and results in reduced viral loads compared to infection with the chronic LCMV Docile strain alone.

Although IFN-I signaling induces multiple antiviral immune responses through different downstream signaling pathways, several important questions still need to be explored. How individual IFN-I subsets play a role in specific immune responses following viral infection remains unknown, providing new research avenues for us to investigate the roles of IFN-I signaling during viral infection in the future.

Since IFN-I was reported for its ability to limit influenza virus replication in the 1950s, its best known for its potential effects in antiviral, anti-proliferative and immune modulation [34, 329, 330]. Viral persistence is normally associated with various immune dysfunctions including lymphoid disorganization, deficient ability of antigen presentation, abnormal B cell and T cell responses [38], which are associated with directly or indirectly response of IFN-I signaling. Treatment with IFN-I therefore was a standard therapy for patients with persistent viral infection such as HCV [331, 332]. However, recent studies also demonstrated that IFN-I signaling

negatively regulated the immune response, lymphoid tissue organization and $CD4^+$ T cell function during persistent viral infection [333]. During HCV infection, patients who fail to control viral loads following a course of IFN-I therapy presented with a higher expression of IFN-I prior to therapy, while, patients who present with lower IFN-I production responded to the therapy [334, 335]. The reasons for the divergence remain unknown. Moreover, numerous studies documented that early expression of IFN-I is higher in persistent clone13 infection than acute ARM infection [38, 336]. However, in our research on LCMV WE and Docile, we found the opposite. LCMV Docile induced less IFNα production compared with LCMV WE infection in mice. At the same time, the viral replication of LCMV Docile is able to be suppressed by IFN-I treatment, which was previously documented in Clone13 infected mice. Other studies have also reported that treatment of mice with IFN-I during the early stages of chronic LCMV Clone13 infection promotes rapid virus control [34, 38]. Although decreased expansion of virus-specific $CD8^+$ T cells and increased T cell exhaustion are detected in both strains of LCMV Docile and LCMV Clone13 infection, they follow different mechanisms. Enhanced IFN-I production in clone13 infected mice promote the expression of many specific subsets including IL-10 and PDL-1 which inhibit T cell responses [329, 337]. However, we have shown that decreased expansion of virus-specific $CD8^+$ T cells and increased T cell exhaustion are induced in middle dose Docile infected mice. At the same time, we also found that high dose LCMV Docile still can induce IFNα production. Previous studies and our current data show that the serum IFN-I levels peak 1-2 days after LCMV infection and then decrease independent of infected dose. Although the detectable IFN-I proteins decline, viral persistence still increased by blocking IFNAR signalling after the first few days of infection [338-340]. However, how the undetectable IFN-I in the serum has such a considerable effect on the

immune response remains unknown. In brief, diverse members of LCMV strains follow different principles in response to immune response and IFN-Is production.

Consistently, chronic viral infection may avoid immune surveillance during the initial phase of infection by slower viral replication. We wondered whether other viruses also possess the same characteristics. HCV is found worldwide, which is the most common blood-borne viral infection [341]. During Hepatitis C virus infection, pDCs or Kupffer cells can recognize HCV RNA and produce IFN-I [342, 343]. Consistently, HCV infected chimpanzee up-regulate ISG transcripts. Interestingly, the ISG transcripts peaked around 6-week post infection in chimpanzees who eventually cleared HCV, whereas in chimpanzees who developed a persistent infection, a sustained ISG transcript expression was observed only after 14 weeks [344]. Moreover, during human or chimpanzee HCV infection, the initial HCV titer positively correlates with viral clearance [344, 345]. Further, it's reported that pathologic infiltration of $Ly6C^{+}$ inflammatory monocytes into the lungs is IFN-I independent during murine SARS-CoV infection. However, some studies also highlighted the importance of delayed IFN-I induction in promoting the development and infiltration of inflammatory monocyte–macrophages in mice [346]. Importantly, SARS-CoV infected mice still possess the ability of viral control in IFN-I signaling deficient mice that exerts antiviral immune responses by the induction of IFN-λ antiviral program [347]. Considering our data, it might be possible that low HCV replication causes reduced APC activation. Accordingly, reduced expression of co-stimulatory molecules might result in reduced or impaired CTL priming, which might contribute to persistence of HCV. Consistently, delayed IFN-I response might promote severe acute respiratory syndrome (SARS)-CoV and SARS-CoV2 mediated viral pathology [348, 349].

In addition to the direct efforts in restricting the virus propagation, IFN-I also plays a potent role in antiviral immune responses. The increased expression of MHC-I is detected in multiple cells in response of IFN-I signaling, which is required for T cell function. IFN-I signaling on DCs stimulate their activation and T cell stimulatory capacity and promotes DCs mature during virus infection [350]. We found that LCMV WE infection induces enhanced MHC-I expression compared to Docile infection. At the same time, we also found that IFN-I induces initial cDC and pDC apoptosis in LCMV WE infected mice. Furthermore, IFN-I can promote adaptive immunity against viruses. IFN-I signaling promotes T cell apoptosis, and it is also required to induce IFN-γ production of NK cells [334, 351]. Moreover, IFN-I is critical for protecting antigen specific T cells from NK cell mediated regulation [352, 353]. Activated NK cells can target anti-viral T cells and promote the formation of a chronic viral infection [31-33]. Notably, we also observed increased T cell immunity following infection with both LCMV strains. In this setting, IFN-I production through LCMV WE infection might trigger protection of anti-viral T cells, which will recognize both strains. Hence, LCMV load in mice receiving both strains was reduced when compared to animals receiving LCMV Docile alone. However, excess IFN-I can result in expression of ligands triggering T cell exhaustion. PD-1 has been identified as critical player in facilitating loss of T cell function and blockade of PD-1 can restore T cell immunity during chronic viral infection [354]. Interestingly, IFN-I triggers expression of PD-1L and thus causing chronic viral infection. Specifically, LCMV clone 13 can trigger IFN-beta production, which was critical in establishing viral persistence [355]. Notably, NK cell activation during LCMV infection is also triggered by IFN-I, possibly contributing to limited T cell immunity after excess IFN-I [356]. Accordingly, viruses are able to target IFN-I in both ways to evade adaptive immunity, either by excess IFN-I production following stimulation of PD-1L expression, or by reduced entry and

slower replication resulting in limited IFN-I production and accordingly reduced adaptive immunity (Table 3).

Table 3

Immune responses of commonly used LCMV strains in C57BL/6 mice

Viral infection	**IFNα production**	**Immune response**	**Viral persistence**	**Selected References**
Armstrong 53b high dose (IV)	high	$CD8^+$ T cells response; IFN-I signaling dependent	acute	[325, 338, 357]
Clone13 high dose (IV)	middle	$CD8^+$ T cell exhaustion; increased expression of co-inhibitory receptors	chronic	[325, 338]
WE middle dose (IV)	high	$CD8^+$ T cells response; IFN-I signaling dependent	acute	[358]
Docile middle dose (IV)	low	$CD8^+$ T cell exhaustion; increased expression of co-inhibitory receptors	chronic	[338, 357]
Aggressive high dose (IV)	high	$CD8^+$ T cells response;	acute	[357]
Traub	low		chronic	[359]

In recent reports about respiratory viral infection, IFN-I signaling promotes immune pathology during acute respiratory viruses of influenza, SARS-CoV and COVID-19 infection, while several detrimental proinflammatory cytokines and chemokines are induced [360, 361]. It's reported that IFN-λ plays an important role in control of viral loads while IFN-I signaling is inhibited during respiratory viral infection. Thus, how IFN-I signaling modulates the immune

response in specific cellular subsets, and what cell types participate in the regulation should be explored. The detailed cellular and molecular mechanisms during acute and persistent viral infections also need to be researched. And further studies are required to focus on the function of IFN-λ during acute viral infection in mice and subsequently humans. However, recent research show that IFN-β negatively regulates virus control by reducing virus-specific $CD4^+$ and $CD8^+$ T cell responses during chronic infection. This suggests that selective therapeutic targeting of IFN-I species through small molecule design is necessary for diverse acute or chronic virus infections. Taken together, our data identifies that LCMV Docile exhibits slow viral entry, reduced viral replication, reduced innate and adaptive immune activation and viral persistence, which in part can be rescued by coinfection with an acute strain of LCMV.

The IFNAR1-IFNAR2 receptors are common receptors for the IFN-I subsets, which are modulated by the downstream protein of JAK1/TYK2, STAT1, STAT2 and IFN-regulatory factor 9 (IRF9). The individual subtypes of IFN-Is are able to interact with different IFNAR1 or IFNAR2 subunits of the receptor, which potentially results in their unique effects in downstream signaling. Acute LCMV Armstrong (Arm) infected IFNAR1 KO mice and neutralizing antibody treatment with ARM infected IFNAR1 sufficient mice result in high viral loads and virus persistence [340]. Dendritic cell specific deletion of IFNAR1 revealed the same conclusion after infection with CW3 strain of murine Norovirus (MNoV) [362]. In our study, we also infect IFNAR1-dificient mice with LCMV WE, Docile and WE plus Docile co-infection, respectively. The virus titer is highly increased in IFNAR1-dificient mice compared to IFNAR1-sufficient mice as well as dendritic specific deletion of IFNAR1 cells after LCMV virus infection. This means IFNAR1 is critical for virus control in LCMV infected mice. Moreover, the undetectable IFN-I in the serum still has

considerable effect on the immune response in LCMV Docile infected mice, which is important for the development of treatments and modalities to control viral associated pathologies.

Moreover, deletion of IFN-I related signaling pathways results in diverse effects when infected with diverse strains of virus with different genetic background in mice [334]. As the downstream regulators of IFN-I signaling, STAT1 is a critical protein in the regulation of IFN-I signaling. STAT1-deficient mice were susceptible to virus infection and display increased pathology [363]. In our in vitro experiments, we also confirm that STAT1 plays an important role in virus control induced by LCMV WE, which is mediated by the phosphorylation of STAT1. Furthermore, when we activate the phosphorylation of STAT1 in LCMV Docile infected cells by IFNα treatment, the virus titer decreases. Therefore, in this study we firstly focus on the IFN-I production during two different strains of LCMV. The research of the relationship between IFN-I production and viral infection has important significance for the therapy during virus infection. Indeed, IFNα is usually used to treat chronic viral infections in the clinic, but it also can cause immunopathology [364].

According to the above dissertation, we known that LCMV is a common model for the research of virus infection versus host immune response. In addition, the evidences from published paper and our study show that the ability of either acute or persistent infection is determined by the nature of virus including viral RNA segments and proteins, which contribute to the development of vaccines [338].

It’s reported that the amino-acid mutation in the viral genomic S and L RNA segments is able to affect the ability of viral persistence by different mechanisms including the change of T cell recognition, affinity of virus with viral-receptors and virus replication [357, 365]. Viral

proteins possess the specific functions in preventing IFN-I production and signaling activation [366]. The diverse phenotype of acute and chronic infection with LCMV WE and Docile separately promote the study aimed at investigating the mechanism of virus control of LCMV WE and Docile. A comprehensive screen of the entire LCMV Arm proteome for $CD8^+$ T cell epitopes was performed in 2007, in which 28 $CD8^+$ T cell epitopes of LCMV ARM were identified, nine of which are located in the GP, 4 in the NP and 15 in the L protein [367]. Different groups confirmed the diverse functions of mutation in different LCMV subsets. It has been reported that two mutations in LCMV-Cl13 contribute to its persistence compared with LCMV-Arm. The substitution from lysine to glutamine (K1079Q) in the viral polymerase (L protein) results in enhanced intracellular replication. another is a mutation of GP protein from phenylalanine to leucine (F260L), which results in a high affinity of virus with its' surface receptor, α-dystroglycan [338]. Prior evidence indicates that LCMV GP-1 is critical for cell receptor recognition and cell entry of LCMV [295]. The substitution 280N→S in the $GP2_{276-286}$ T cell epitope of Doc abolishes $CD8^+$ T cell recognition and promotes the persistence of Docile [357]. Furthermore, 494V→I of the LCMV GP eaffects virus production and interactions of GP with cellular factors at the C terminal WKRR sequence of GP2 [368]. NP plays an important role in the formation of RNPs and control of the replication and transcription of the virus genome. Notably, we found no difference of NP protein of LCMV WE and Docile in IFN-I production and signaling activation. Although LCMV WE and Docile share the common critical motif already mentioned ago, there are many mutations we didn't confirm, which may contribute to control of virus loads in mouse tissues (Table 4).

TABLE 4. Summary of LCMV-derived epitopes in different strains

Antigen name	**Peptide sequence**	**Peptide sequence**	**Peptide sequence**

(peptide position)	(ARM) [367]	(WE)	(Docile)
GP118-125	ISHNFCNL	LNHNFCNL	LNHNHCNL
GP166-173	ITIQYNLT	ITIQYNLS	ITIQYNLT
GP221-228	SQTSYQYL	SQTSYQYL	SQTSYQYL
GP276-286	SGVENPGGYCL	SGVENPGGYCL	SGVESPGGYCL
GP33-41	KAVYNFATC	KAVYNFATC	KAVYNFATC
GP34-41	AVYNFATC	AVYNFATC	AVYNFATC
GP365-372	MGVPYCNY	MGVPYCNY	MGVPYCNY
GP44-52	FALISFLLL	LALVSFLFL	LALVSFLFL
GP92-101	CSANNSHHYI	CSANNSHHYI	CSANNSHHYI
NP165-175	SSLLNNQFGTM	SSLLNNQFGTM	SSLLNNQFGTM
NP205-212	YTVKYPNL	YTVKYPNL	YTVKYPNL
NP238-248	SGYNFSLGAAV	SGYNFSLGAAV	SGYNFSLGAAV
NP396-404	FQPQNGQFI	FQPQNGQFI	FQPQNGQFI
L1189-1196	MMCPFLFL	MMCPFLFL	MMCPFLFL
L1302-1310	INYCIGVIF	INYCIGVIF	INYCIGVIF
L1369-1377	FAAEFKSRF	FAAEFKSRF	FAAEFKSRF
L1428-1435	NSIQRRTL	NCIQRRTL	NHIQKRTL
L156-163	ANFKFRDL	VNFKFRDL	VNFKFRDL
L1878-1885	GPFQSFVS	PFQSFILK	PFQSFILK
L2062-2069	RSIDFERV	STDFERIG	SLDFERIG
L313-320	TSTEYERL	ANTEYERL	ISTEYERL
L338-346	RQLLNLDVL	RQLLNLDVL	RQLLNLDVL
L349-357	SSLIKQSKF	SSLIKQSIS	SSLIKQSKL
L455-463	FMKIGAHPI	FMRAGTHPV	FMRAGTHPV
L663-671	VVYKLLRFL	VVYRLLRFL	LVYRLLRFL
L689-697	KFMLNVSYL	KFMLNVSYL	KFMLNVSYL
L743-751	VFYEQMKRF	VFYEQMKKF	VFYEQMRKF
L775-782	SSFNNGTL	SSFNNGTL	SSFNNGTL

Red color: different amino acid in sequence; Blue color: sequence shift one amine acid

The immune responses of the mice and BMDCs infected with rWE and rDocile demonstrated the critical contribution of the S segment to viral control. Mutations in the GP protein is able to modulate the affinity of the GP receptors associated with viral entry and recognition. However, specific function of the several amino-acid mutations existing throughout the entire sequence of the GP protein of WE versus Docile remain unclear. The intergenic region (IGR) present in LCMV genome, the S-IGR of S segment and the L-IGR of L segments, play important roles in the control of virus protein expression and are involved in viral packaging [369, 370]. In this study we found five nucleotide differences that exist between the IGRs of WE and Docile strains. Therefore, it is possible that these differences between the IGRs of WE and Docile strains may contribute to the viral pathology.

The development of reverse genetics to generate recombinant LCMV strains from cloned DNA promotes the investigation of virus structure, viral pathogenesis, the affinity of virus versus cellular receptor and vaccines of arenavirus [371, 372]. Notably, generation of recombinant LCMV WE and Docile viruses provide new insights to understand the accurate mechanisms of acute and chronic infection.

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