Crosstalk between Splenic CD169^{+} Macrophages and Adaptive Immune System

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All the experiments for this study have been carried out at the Institute of Immunology, Faculty of Medicine, University Hospital Essen, at the University of Duisburg-Essen or at another equivalent facility.

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Die Experimente der vorliegenden Arbeit wurden am Institut für Immunology, Medizinische Fakultät, der Universität Duisburg-Essen oder an einer anderen gleichwertigen Einrichtung durchgeführt.

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Summary

Diseases caused by viral infections are major concerns for human being. Viruses like human immunodeficiency virus (HIV), hepatitis B virus (HBV) and hepatitis C virus (HCV) that induce chronic infection, is always a risk for human life. Understanding the mechanisms how immune system combat against viral infection can help to develop better vaccines or treatment therapies to prevent from lethal viral infections.

Here in this study, we analyzed the roles of $CD169^+$ macrophages present in splenic marginal zone on adaptive immunity. Initial virus replication in splenic $CD169^+$ macrophages during systemic infection of vesicular stomatitis virus (VSV) was described earlier which activate innate and adaptive immune system. Here using Lymphocytic choriomeningitis virus (LCMV) in mouse model, we found that $CD169^+$ macrophages in spleen and lymph node produce type I interferon (IFN-I). IFN-I acts as antiviral and inhibited virus replication in different peripheral organs, and stimulated *program death ligand 1* (PD-L1) expression in liver. PD-L1 is a ligand for PD-1 expressed on activated $CD8^+$ T cells, and PD-L1/PD-1 axis signaling induces $CD8^+$ T cell exhaustion. In absence of $CD169^+$ macrophages, IFN-I production was impaired and PD-L1 expression was highly reduced. This led to overwhelming virus replication in absence of $CD8^+$ T cells exhaustion that resulted in severe immunopathology and death of mice. This study clearly shows the role of $CD169^+$ macrophages in regulating the functions of $CD8^+$ T cells.

In another study, we described the influence of virus-specific antibodies or virus-specific memory $CD8^+$ T cells on splenic $CD169^+$ macrophages during systemic recall infection of LCMV. We reported that the presence of LCMV-specific antibodies permit intracellular viral replication in $CD169^+$ macrophages of splenic marginal zone but suppressed viral replication in peripheral organs. During persistent infection with the LCMV-Docile, viral replication in $CD169^+$ macrophages of spleen in presence of virus specific antibodies was found to be essential for priming of $CD8^+$ T cells that led to viral clearance. In parallel to specific antibodies, memory $CD8^+$ T cells inhibited viral replication in $CD169^+$ macrophages and systemic IFN-I production, and failed to mount effective $CD8^+$ T cell response that resulted in viral persistence. This investigation explains the mechanism of immune activation and protection in presence of virus specific antibodies during secondary viral infection.

Over all from these two studies we can conclude that $CD169^+$ macrophages play significant roles for inducing strong antiviral innate and adaptive immunity during primary and secondary viral infection.

Zusammenfassung

Krankheiten, die durch Virusinfektionen ausgelöst werden, stellen eine große Besorgnis für die Menschheit dar. Viren wie das humane Immundefizienz-Virus (HIV), Hepatitis B Virus (HBV) und Hepatitis C Virus (HCV), die chronische Infektionen verursachen, sind immer lebensbedrohlich. Die Mechanismen zu verstehen, wie das Immunsystem Virusinfektionen bekämpft, kann helfen bessere Impfstoffe oder Therapien zu entwickeln, um tödlichen Virusinfektionen vorzubeugen.

In dieser Studie untersuchten wir die Rolle von $CD169^+$ Makrophagen, die in der Marginalzone der Milz lokalisiert sind, für der adaptiven Immunantwort. Die anfängliche Virus-Replikation in den $CD169^+$ Makrophagen der Milz bei einer systemischen Infektion mit dem vesikulären Stomatitis-Virus (VSV), welche das angeborene und adaptive Immunsystem aktiviert, wurde bereits beschrieben. Indem wir hier das lymphozytäre Choriomeningitis-Virus (LCMV) im Maus-Modell untersuchten, fanden wir, dass die $CD169^+$ Makrophagen in Milz und Lymphknoten Typ-I-Interferon (IFN-I) produzieren. IFN-I agierte antiviral, inhibierte somit die Virus-Replikation in verschiedenen peripheren Organen und stimulierte die Expression von *program death ligand 1* (PD-L1) in der Leber. PD-L1 ist ein Ligand für PD1, das auf aktivierten $CD8^+$ T-Zellen exprimiert wird. Die Signale über die PD-L1/PD1-Achse induzieren die Erschöpfung von $CD8^+$ T-Zellen. In Abwesenheit von $CD169^+$ Makrophagen war die IFN-I-Produktion und PD-L1-Expression stark reduziert. Die führte zu einer überwältigenden Virus-Replikation bei fehlender $CD8^+$ T-Zell-Erschöpfung, was in schwerer Immunpathologie und dem Tod der Mäuse endete. Diese Arbeit zeigt klar die Rolle von $CD169^+$ Makrophagen bei der funktionalen Regulierung der $CD8^+$ T-Zellen auf.

In einer zweiten Studie beschrieben wir den Einfluss Virus-spezifischer Antikörper oder Virus-spezifischer $CD8^+$ T-Zellen auf die $CD169^+$ Makrophagen der Milz während einer systemischen Zweit-Infektion mit LCMV. Wir berichteten, dass die Anwesenheit von LCMV-spezifischen Antikörpern die intrazelluläre Virus-Replikation in den $CD169^+$ Makrophagen der Marginalzone der Milz zulässt, aber die Virus-Replikation in peripheren Organen unterdrückt. Während einer persistenten Infektion mit LCMV-Docile war die Replikation in den $CD169^+$ Makrophagen der Milz bei Anwesenheit Virus-spezifischer Antikörper essentiell für die Vorbereitung der $CD8^+$ T-Zellen, was zur Beseitung des Virus führte. Parallel zu spezifischen Antikörpern inhibierten $CD8^+$ T-Gedächtnis-Zellen die Virus-Replikation in $CD169^+$ Makrophagen und die systemische IFN-I-Produktion und schafften es nicht eine effective $CD8^+$ T-Zell-Antwort aufzubauen, was in der Persistenz des Virus resultierte. Diese Untersuchung erklärt die Mechanismen der Immun-Aktivierung und des Immun-Schutzes bei Anwesenheit von Virus-spezifischen Antikörpern bei viralen Zweit-Infektionen.

Insgesamt kann man aus diesen beiden Studien schlussfolgern, dass $CD169^+$ Makrophagen signifikante Rollen für die Induzierung einer starken antiviralen angeborenen und adaptiven Immunantwort bei viralen Erst- und Zweit-Infektionen einnehmen.

Chapter 1

Introduction

1.1 Immune System

The immune system is a defense machinery of multicellular organisms to protect them from harmful invaders like viruses, bacteria, fungus and parasites. The mammalian immune system is highly evolved and consists of three different layers of host defense includes physical barrier, innate immunity and adaptive immunity[1] *(Fig.*1.1). Physical barriers act as first line of defense include skin, mucus layer in airway and intestine, low stomach pH and lysozymes in tear and saliva[1-3]. If physical barrier is breached by pathogens then innate immune system comes in to play that includes phagocytic cells like granulocytes, macrophages and dendritic cells[4]. These innate immune cells engulf, digest and process pathogens and present antigens to adaptive immune system cells like B cells and T cells to mount a highly specific immune response against them[5]. Because of specific adaptive immune response, host get rid of pathogens and memorize this identity to response fast and aggressively if same pathogen attacks the host in future[6] (Fig. 1.1).

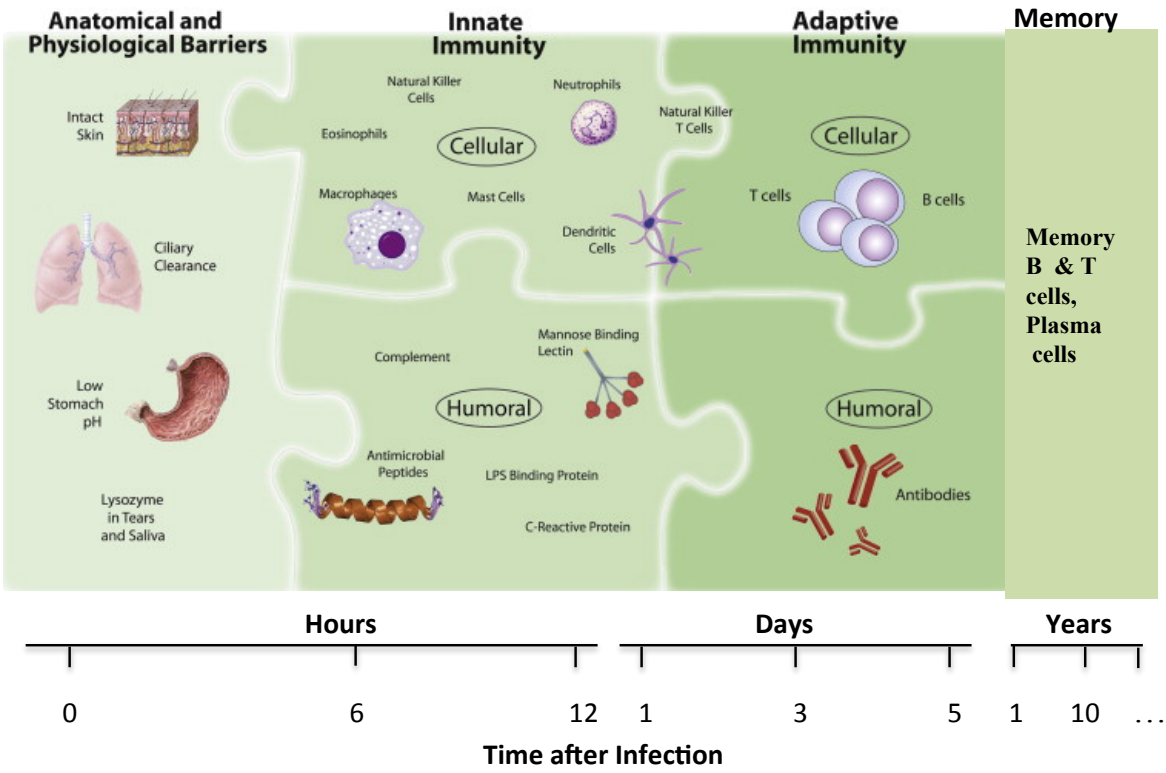


Figure 1.1: Components of immune system

Turvey S.E; et al. J Allergy Clin Immunol. Feb. 2010 (modified)

1.1.1 Structure of Immune System

The immune system of mammals is composed of complete organs, lymphatic vessels, cells and proteins[2,3]. On the basis of development and performing of immune response, there are three main organs of immune system including primary, secondary and tertiary lymphoid organs[2,3,7,8]. Primary lymphoid organs include bone marrow and thymus, mainly responsible for development of all immune cells from hematopoietic stem cells, process known as Haematopoiesis[3,9] (Fig. 1.2). Secondary lymphoid organs provide a proper microenvironment to initiate effective immune response against pathogens[3,9].

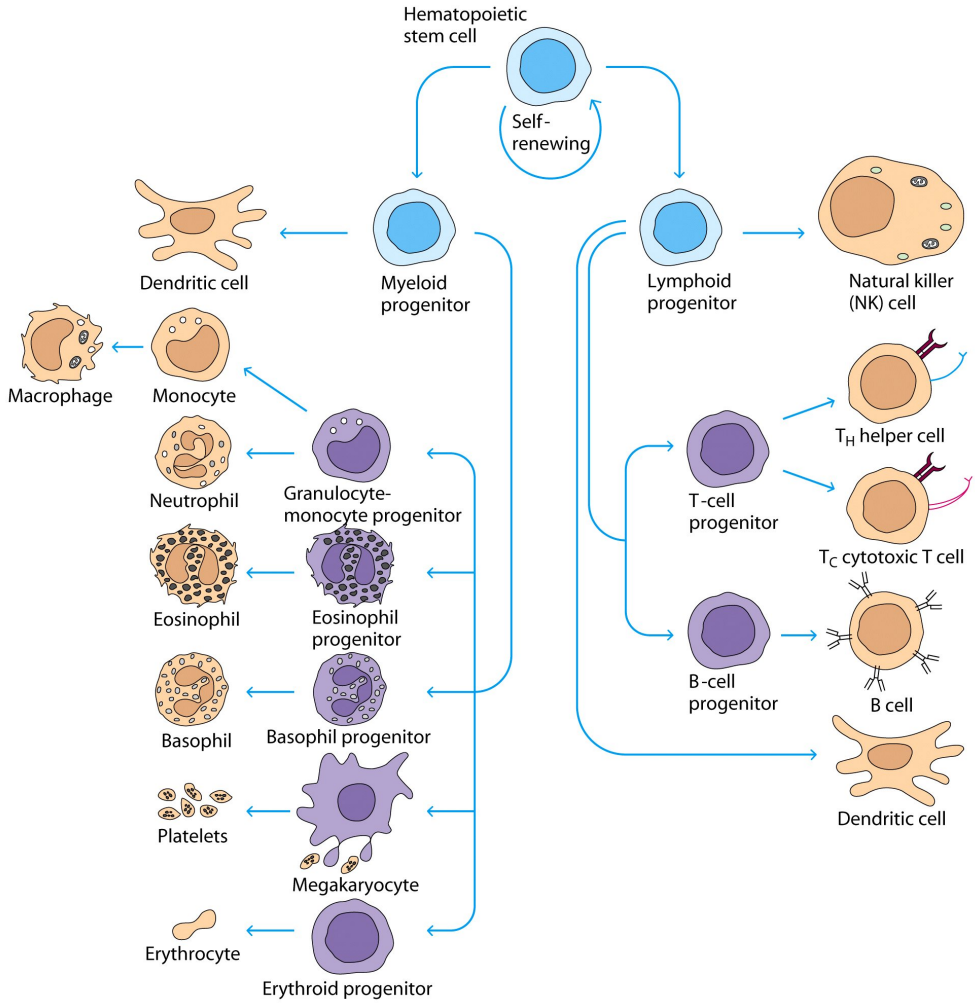


Figure 1.2: Haematopoiesis

Kindt TJ, et al Kuby immunology (2007), (6th ed.)

Spleen, lymph nodes (LNs) and mucosal associated lymphoid tissue (MALT) are part of secondary lymphoid organs[8]. Immune cells primed and matured upon antigen encounter to become effector cells in secondary lymphoid organs, when these cells return to site of infection and reside there as memory cells form a tertiary lymphoid structure[3,8]. Lymph nodes

are present all over in mammalian body collecting antigens from peripheral tissues and specialized to provide environment for mounting immune response against pathogens[3]. There is only one spleen in mammalian body which is specialized to filter pathogens entering in blood circulation[3]. For this study, we mainly focus on structure of the spleen.

1.1.1a Spleen

The spleen is a well-structured oval shaped capsule contains two main compartments, the red pulp and white pulp which are separated by specialized region called as marginal zone (MZ)[3,10,11] (Fig 1.3). The red pulp is comprised of network of sinuses populated by red blood cells (RBCs), red pulp macrophages and some lymphocytes as well. Splenic red pulp macrophages engulf and degrade old and defective RBCs. The red pulp apart from immune functions is also involved in iron metabolism, thrombocytes (platelets) storage and haematopoiesis[3,10].

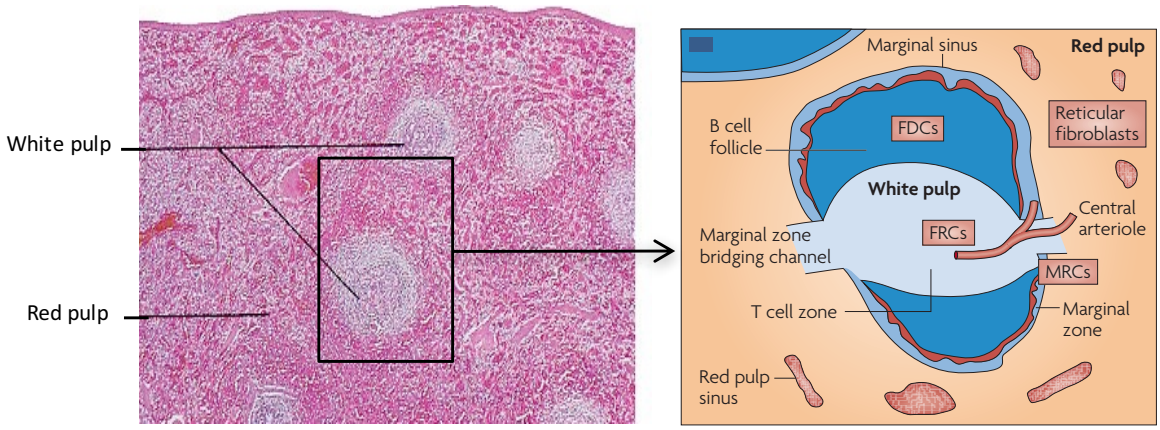


Figure 1.3: Structure of spleen

Kindt T. J; et al (2007), Kuby immunology (6th ed.)

Mueller S.N; et al. Nat Rev Immunol. 2009 (modified)

The white pulp made-up of T cell and B cell zones. T cell zone is also known as the periarteriolar lymphoid sheath (PALS) contains networks of fibroblastic reticular cells (FRCs) adjoining by central arteriole. B cell zone contains B cells follicles and a central network of follicular dendritic cells (FDCs)[10,11] (Fig 1.3).

The splenic marginal zone (MZ) surrounding the white pulp is in direct contact with white pulp B cells. MZ contains two sunsets of macrophages. The outer layer of marginal zone

contains marginal zone (MZ) macrophages are identified by expression of SIGN-R1 and MARCO markers (Fig. 1.4). The macrophages in inner layer of MZ are classified by expression of SINGLEC1/CD169 marker and known as metallophilic or marginal zone metallophilic macrophages[10-12]. There are also two subsets of other cells, the marginal zone B cells and DCs located in between these two layers of MZ macrophages[13] (Fig. 1.4).

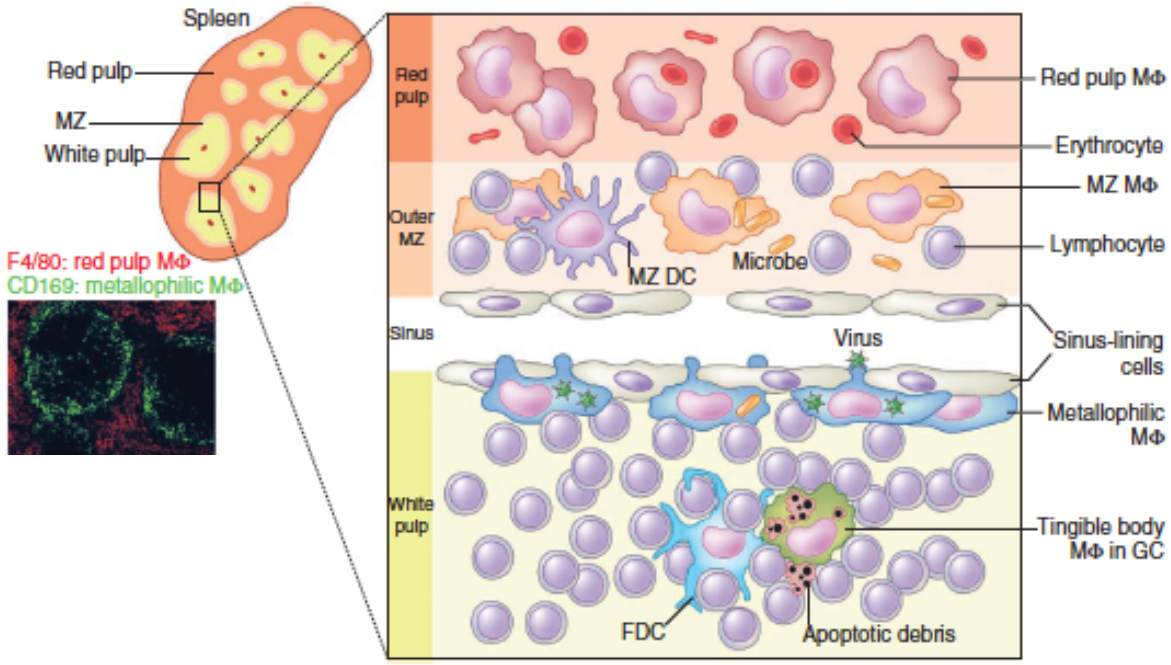


Figure 1.4: Marginal zone macrophages

Davies LC, et al. Nat Immunol. 2013

Blood circulation in spleen- Spleen receives blood from splenic artery which branches into central arterioles that are surrounded by PALS. Central arterioles further branch into smaller follicular arterioles, which cross follicles, and penicillar arterioles that cross end in marginal sinus or connected to red pulp vein[10,14] (Fig. 1.5). The blood in the marginal sinus drains into the splenic cords and then to sinuses of the red pulp makes an open circulation system. When blood from penicillar arteriole flowing directly to sinus, this makes closed circulation in spleen[10,14].

During systemic infection, pathogens or cells carrying the pathogens or other immune cells enter spleen by splenic artery and make initial interaction with MZ cells in marginal sinus (Fig. 1.5). The blood in the marginal sinus flows slowly and the cells of MZ like marginal zone macrophages, metallophilic macrophages, MZ B-cells and dendritic cells get enough

time to trap the antigens[13]. After binding with antigens, DCs migrate to T cell area and lead to antigen specific T-cell response. B-cells that bind to its specific antigen, migrate to follicles and generate germinal center and produce antigen specific antibodies[15,16].

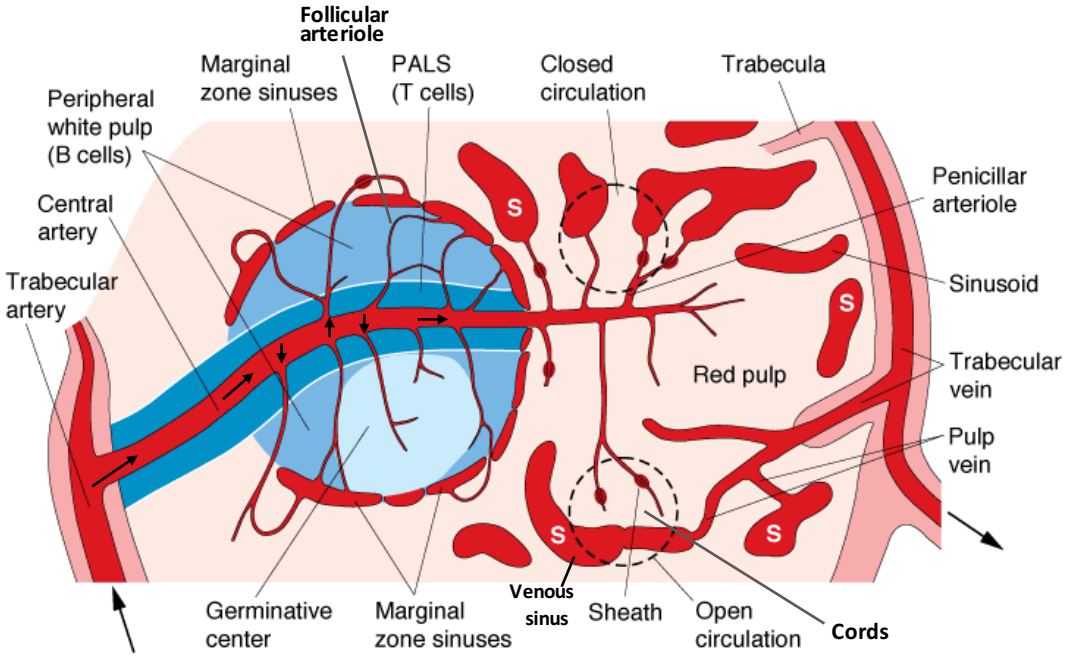


Figure 1.5: Blood circulation in spleen

Junqueira's Basic Histology Text & Atlas (12th ed.) (modified)

1.1.2 Innate immune system

Innate immune system induces non-specific immediate immune response upon entry of pathogens to host body. Innate immune cells recognize specific pattern of molecules present on pathogens called as pathogen-associated molecular patterns (PAMPs), mediated by pattern-recognition receptors (PRRs) on innate immune cells[4,5]. Due to this interaction of pathogens, immune cells get activated and produce several soluble proteins like cytokines and chemokines, which further inhibit pathogens and alert other cells. Innate immune system comprises granulocytes, dendritic cells (DCs), monocytes, macrophages, NK cells and complement molecules[3]. Here we want to focus on DCs and specially on macrophages.

1.1.2a Dendritic Cells

DCs develop from lymphoid and myeloid progenitors (Fig. 1.1), and are present at possible entry sites for pathogens like skin, mucosal tissues and in most of lymphoid and non-lymphoid organs[3,17]. DCs upon pathogen encounter promote both pro-inflammatory

and anti-inflammatory immune responses[17-19]. DCs are professional antigen-presenting cells and their main function is to process and present antigen to T cells of adaptive immune system to stimulate specific immunity to foreign antigens and tolerance to self-antigens[17-19]. The surface phenotypic markers expressed by almost all type of DCs are CD45, CD11c and MHCII[17]. There are two main types of DCs depending upon their location and functions, plasmacytoid DCs (pDCs) and conventional or classical DCs (cDCs)[17]. cDCs are very efficient in antigen processing and presentation to T cells. pDCs are mainly present in circulation and SLOs, express high level of TLR7 and TLR9, and are main producers of IFN-I upon viral infection[20].

1.1.2b Macrophages

Macrophages are located throughout the body in lymphoid and non-lymphoid organs, and are professional phagocytic innate immune cells. These cells engulf all kind of particles or cells including microbes or cancer cell which do not resemble to be a part of healthy body[21,22]. Before it was shown that macrophages develop from circulatory blood monocytes during steady state and inflammation in different tissues[23]. But recent studies showed that macrophages are derived from embryonic precursors and maintain them by self-renewal[24]. Macrophages are present in most of organs and got their name according to their location (shown in Fig. 1.6). In mice, all tissue resident macrophages can be identified by surface expression of F4/80[12]. There are some other cells types beside tissue resident macrophages that are present in lymphoid tissues and identified by different marker like SIGNR1 and CD169. Cells that express CD169 marker on cell surface are metallophilic macrophage in MZ of spleen, subcapsular sinus and medullary sinus macrophages in lymph nodes, and specific subsets of tissue resident macrophage[25]. Here we will discuss more about splenic metallophilic macrophages and liver macrophages.

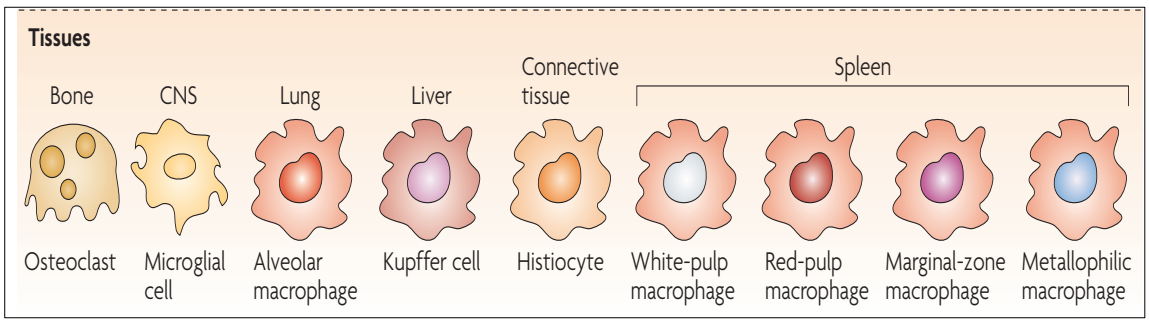


Figure 1.6: Types of Macrophages

Mosser DM, et al. Nat Rev Immunol. 2008

1.1.2b1 Metallophilic macrophages/MZ CD169^{+} macrophages

Metallophilic macrophages also known as marginal zone metallophilic macrophages are present in marginal zone of spleen (Fig. 1.4). These cells express CD169 (sialoadhesin or siglec-1; sialic acid binding Ig-like lectin-1) protein on cell surface[10,12]. Presence of B-cells or lymphotoxin signaling is crucial for development of metallophilic macrophages[26,27]. During systemic infection metallophilic macrophages capture pathogen flowing in MZ sinus and mount initial immune response. Recently it was described that these cells lack sufficient interferon signaling and experience enhanced virus replication during initial virus infection phase[28]. Studies showed that metallophilic macrophages produce type-I interferon after viral[29] and bacterial infection[30]. Targeting of antigen to metallophilic macrophages appears to facilitate the generation of cytotoxic T cells after transfer of blood-borne antigen[31].

1.1.2b2 Liver macrophage

Liver macrophages were first discovered by Karl Wilhelm von Kupffer in 1876, hence also known as Kupffer cells (KCs)[32]. KCs are seeded along sinusoidal endothelial cells all over liver (Fig. 1.7) and making largest population of macrophages among all solid tissues[32,33]. KCs present promote tissue integrity upon liver injury or infection by maintaining homeostasis, immunological tolerance, filtering all entering microbes via blood and initiation or suppressing immune responses[34-36]. KCs also contribute to progression of liver disease like hepatitis, fibrosis and cancer[34-36]. KCs originate from erythromyeloid progenitors derived from the embryonic yolk sac and/or fetal liver[37-39]. KCs are capable of self-replication during homeostasis and after liver injury[37,38]. Mouse Kupffer cells are characterized by their F4/80, CD11b$^{+/low}$, CD68 and C-type lectin domain family 4-member F (CLEC4F) surface phenotype[39-41].

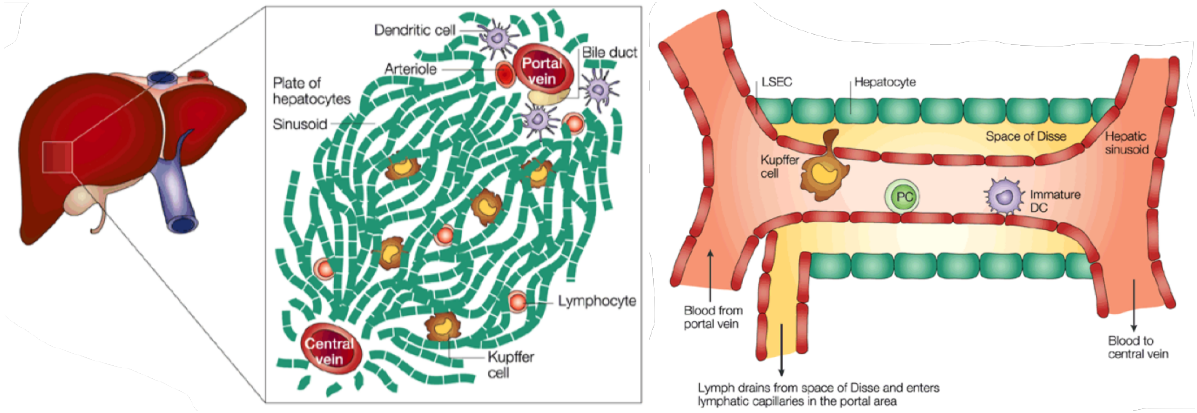


Figure 1.7: Kupffer Cells in hepatic microenvironment

Crispe IN. Nature Reviews Immunology, 2013

KCs show antiviral roles early after infection in IFN-I dependent manner[42]. Hepatitis B virus (HBV) and Hepatitis C virus (HCV) bind to KCs and activate them to inhibit virus replication[43-45]. KCs express programmed cell death ligand (PD-L1) and secret IL-10, transforming growth factor beta 1 (TGF-β1) and prostaglandin E2 (PGE2) and thereby down regulate T cell functions during virus infection[46-48].

1.1.2c Interferons

Interferons (IFNs) are secreted cytokines and have diverse effect on different cell types. They are grouped in to three classes called type I, II and III IFNs[49]. IFN-I include IFNα and IFNβ, and usually almost all cells in body can produce IFN-I upon stimulation of PRRs by PAMPs[50]. PRRs are toll like receptors (TLRs) or other receptors present on cell surface as well as in inside the cell, and can detect nucleic acid or other surface moieties of pathogens. IFN-I have diverse effects on innate and adaptive immune cells. IFN-I signaling via its receptor induces antiviral mechanisms and inhibit host cell proliferation[3]. IFN-I enhance antiviral functions of B and T cells[50]. IFN-I have been shown to cause immunopathology in influenza virus infection[51]. They can lead to immunosuppression during chronic viral infections, such as LCMV[52]. During bacterial infections, low levels of type I IFNs required to initiate cell-mediated immune responses[50].

IFN-II known as interferon-γ is produced by activated T and NK cells. IFN-II bias T cell help toward T_H1 type, and activate and enhance macrophages functions[53]. IFN-I and II are known to enhance antigen presentation[54,55]. IFN-III is interferon-λ and there are three members of this group named IFN-λ1(IL-29), IFN-λ2(IL-28A), and IFN-λ3(IL-28B)[56]. IFN-III like type IFN-I upregulate the expression of genes that control viral replication and host cell proliferation[56].

1.1.3 Adaptive immune system

Innate immune cells process and present pathogen moieties to adaptive immune system, which induces highly pathogen-specific immune response. It is mediated by T cells and B cells, and always leads to immunological memory that is a hallmark of adaptive immune system[2,3]. T cells and B cells are two main cell types of adaptive immune system and key parts of cellular and humoral immunity respectively.

1.1.3a T cells

T cells or T lymphocytes get their name as 'T' because these cells originate in bone marrow and get mature in thymus (T). T cells play central role in cell-mediated immunity and can be recognized by their T-cell receptor (TCR) expression on cell surface[57]. T cells develop from T-cell progenitor cells in thymus on the basis of their TCRs reactivity to complex of self peptides and major histocompatibility complex (MHC) molecules on surface of thymic epithelial cells[57]. This process undergoes different stages and produce mature $CD4^+$ T cells and $CD8^+$ T cells and these cells then migrate to SLOs.

1.1.3a1 T cell activation

Once APCs mainly DCs engulf pathogens or have been infected by pathogens, get activated via PRRs and migrate to LN or spleen[58]. DCs enter T cell zone and present antigen

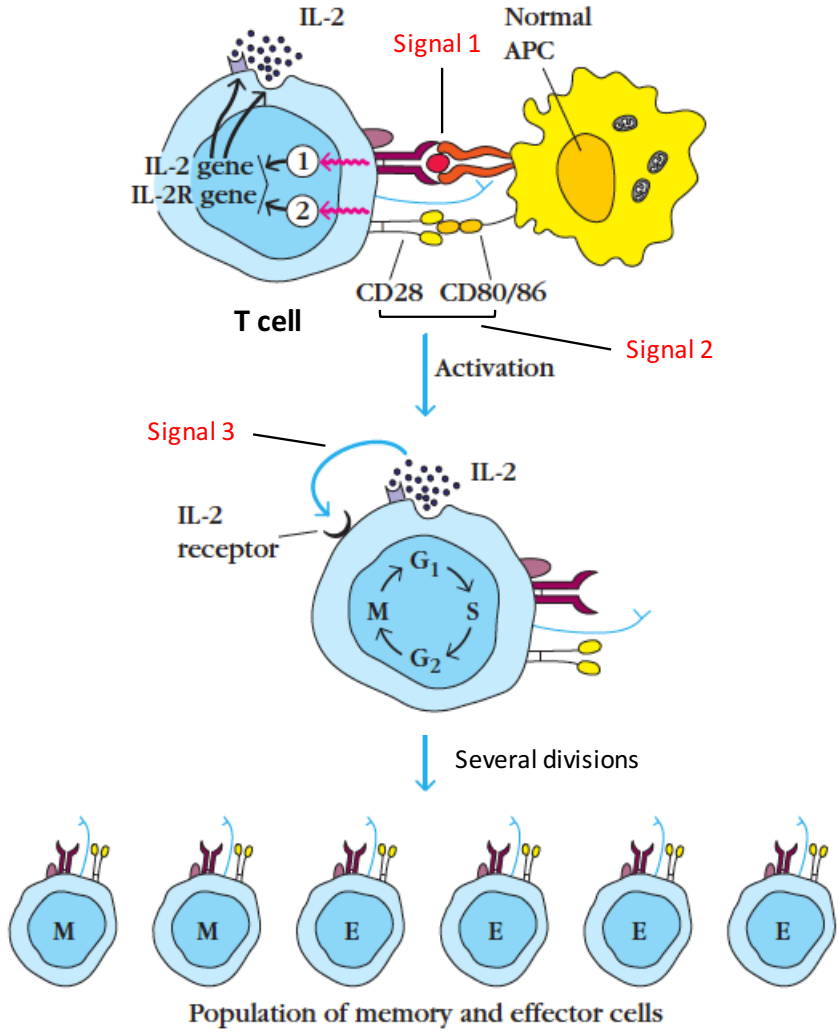


Figure 1.8: T cell activation

Owen JA, et all. Kuby Immunology, 7th edition, 2013 (modified)

in complex with MHC molecule to naïve T cells. For the activation of naïve T cells mainly three signals are required[59,60]. Signal 1 is TCR/MHC-peptide along with CD4 and CD8 coreceptors. Signal 2 is costimulation by CD28 (expressed on T cells) with CD80 or CD86 (expressed on APCs). Signal 1 and 2 initiate activation of transcription factors that induce IL-2 receptors expression and IL-2 release from T cells. IL-2 (can also be generated from helper T cells) is signal 3 and act in autocrine (stimulate same cells) or paracrine (stimulate nearby cells) manner. Signal 1 and 2 along with 3 lead to T cell proliferation and differentiation in to effector or memory cells[59-61] (Fig. 1.8).

1.1.3a2 Effector T cells

$CD8^+$ T cells that interact with antigen in complex with MHC class I via TCR lead to proliferation and differentiate in to effector $CD8^+$ T cells or cytotoxic T cell/lymphocytes (T_C cells or CTL)[58]. In the same way $CD4^+$ T cells interact with antigen in complex with MHC class II undergo proliferation and differentiation in to effector helper T cell[58] (T_H cells).

CTLs leave SLOs and circulate to site of infection where they attack target cells (pathogen infected cell). A CTL binds to target cell by recognizing antigen from pathogens bound to MHC class I expressed by infected cells that lead to formation of highly organized immunological synapse (Fig. 1.9)[3]. This immunological synapse is more supported by adhesion molecules expressed by CTL and target cell and bring them very close together.

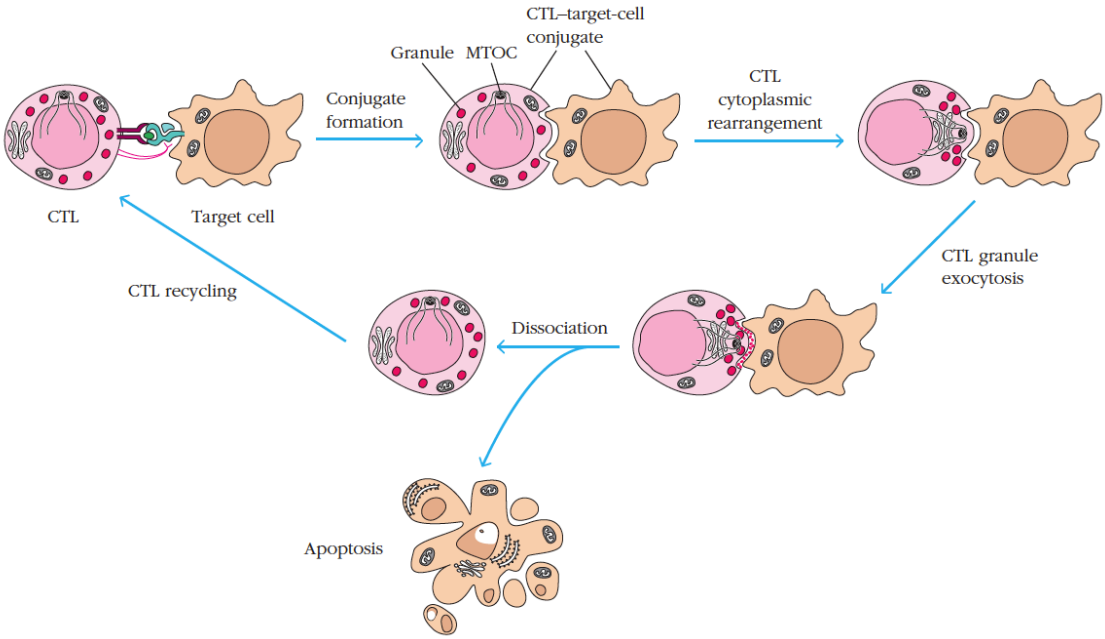


Figure 1.9: Cytotoxic T lymphocyte mediated killing

Owen JA, et all. Kuby Immunology, 7th edition, 2013

CTL have large granules filled with perforin and granzymes molecules and after conjugation with target cell these granules migrate to site of interaction and released in to space between two cells (Fig. 1.9). Perforin molecules create pore in target cell and granyzme molecules enter cell via these pores or by endocytosis that trigger apoptosis in target cell (Fig. 1.9)[3,62]. CTLs can also induces apoptosis in target cells by Fas-FasL interactions. FasL expressed on CTLs and trigger death signal via its natural receptor Fas expressed by target cells[3,62].

On the other hand, effector $CD4^+$ T cells or T_H cells secret cytokines that regulate functions of different cell types including B cells, macrophages and other T cells[3]. At the site of infection, T_H cells secrets cytokines that enhance activity of macrophages and CTLs.

Naïve $CD4^+$ T cells that are primed and activated in presence of different cytokines produced by APCs or other immune cells, become polarised and differentiate in to diverse effector helper T cells and regulate different immune functions[63]. There are several subsets of effector helper T cells with their different functions are described in figure 1.10.

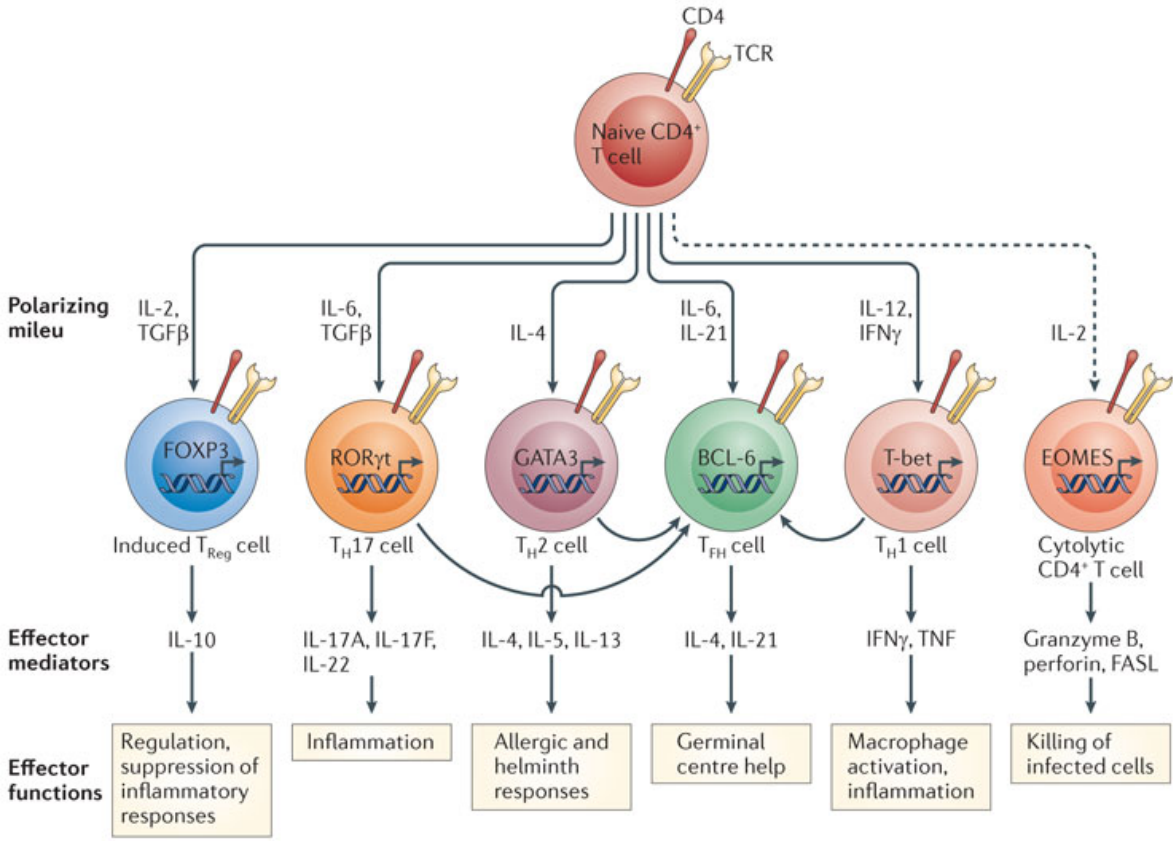


Figure 1.10: T helper subsets differentiation and functions

Swain SL, et al. Nature Reviews Immunology, 2012

1.1.3a3 Memory T cells

Once pathogen is eliminated, almost 90% of effector T cells die and leaving behind a small population of antigen specific memory T cells. Memory T cells are long lived and very sensitive cells that mount fast, robust and more effective secondary immune response with minimal symptoms upon antigen re-encounter[3,64]. Naïve T cells are mostly activated by DCs but memory T cells can be activated by macrophages, DCs and B. cells. There are two known subsets of memory T cells on the basis of their location, surface expression of markers (Fig. 1.11) and to some extent their functions[3,64]. One is central memory T cells that reside in SLOs and other is effector memory T cells which reside mainly in peripheral tissues. Surface expression molecules of memory cells in relation to naïve and effector T cells are shown in Figure 1.11.

Cell type	CD44	CD62L	CCR7
Naïve T cell	low	+	+
Effector T cell	+	low	—
Effector memory T cell	+	variable	—
Central memory t cell	+	+	+

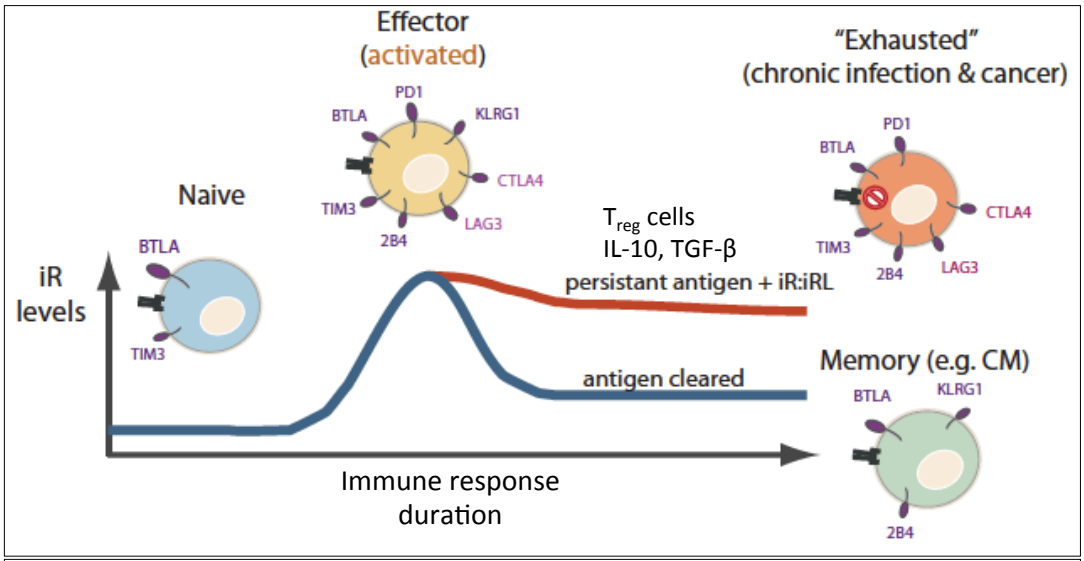
Figure 1.11: Surface expression markers of T cell subsets

Owen JA, et all. Kuby Immunology, 7th edition, 2013

1.1.3a4 Exhausted T cells

Exhausted T cells are dysfunctional effector T cells that result during chronic infection or cancer. During persistent infection with constant antigen signaling via TCRs, exhaustion can occur by 3 different negatively regulatory pathways[52,65] that include cell surface inhibitory receptors[66] (iRs) (such as PD-1, CTLA-4, Tim3 and many more) on T cells, inhibitory cytokines and immunoregulatory cell types. iRs are highly expressed on effector T cells and down regulated on memory T cells upon pathogens elimination but prolonged and high expression of multiple iRs is a key feature of T cell exhaustion[66] (Fig. 1.12). One of the well-known inhibitory receptor on activated effector T cells is PD-1. iR ligand (iRL) for PD-1 are PD-L1 expressed more broadly on non-hematopoietic cells in different tissues like SLOs, liver, lung and also on tumor cells; and PD-L2 expressed mainly on APCs[67]. Blocking of PD-

1 pathway during chronic infection lead to rescue of T cells exhaustion[68]. There are some immunoregulatory cytokines which induce T cell exhaustion. IL10[69,70] and transforming growth factor-β (TGF-β)[71] are well described cytokines involve in T cell dysfunctions. Cells are also known to inhibit T cells functions and induce exhaustion[72]. Exhausted T cells lack effector functions like cytotoxicity, IFNγ, TNFα, proliferation capacity and have apoptotic features as compare to effector and memory T cells and fail to mount effective immune response[52] (Fig. 1.12).



	IFN-γ	TNF-α	CTL	IL-2	Proliferative capacity	Apoptosis
Memory T cells	+++	+++	+++	+	+++	—
Exhausted T cells	+/—	—	—	—	—	++

Figure 1.12: T cell exhaustion scheme

Fuertes Marraco SA, et al. Front Immunol. 2015 (modified)
Wherry EJ. Nature Immunology, 2011 (modified)

1.1.3b B cells

B cells or B lymphocytes are antibody (Ab) secreting cells of adaptive immune system. Antibodies also known as immunoglobulins (Ig) are part of humoral immunity and they act directly against extracellular pathogen or involve other immune components to

eliminate pathogen or pathogen bearing cells. B cells besides producing antibodies are also professional antigen presenting cells and secret cytokines. Development of B cells start in bone marrow includes several stages such as change in surface molecules expression, immunoglobulin gene rearrangement and selection process, that lead to production of immature B cells. After that immature B cells enter spleen and complete its development to become mature B cells[2,3,73].

There are three major populations of B cells depending mainly on their surface phenotype; functions and anatomical locations include B-2 B cells (Follicular B cells), B1 B cells and marginal zone B cells[2,3] (Fig. 1.13). B-2 B cells are mostly present in lymphoid follicles of SLOs. B-1 B cells are present in peritoneal and pleural cavities, and marginal zone B cells are present in marginal zone of spleen.

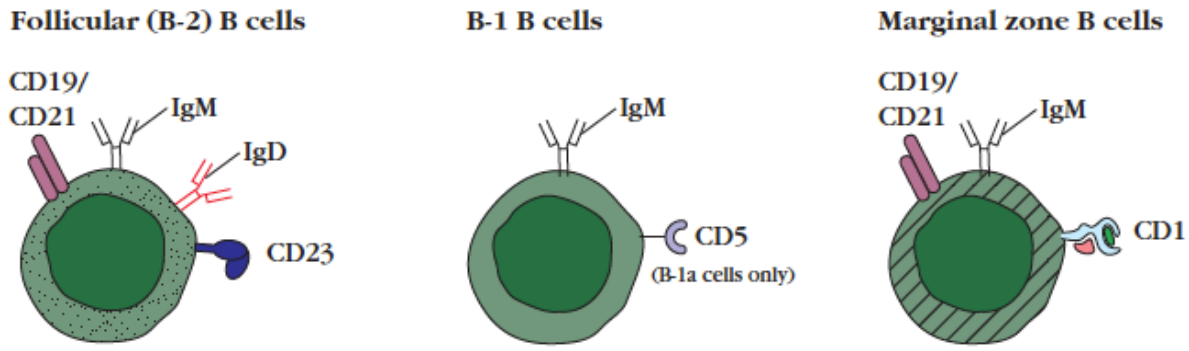


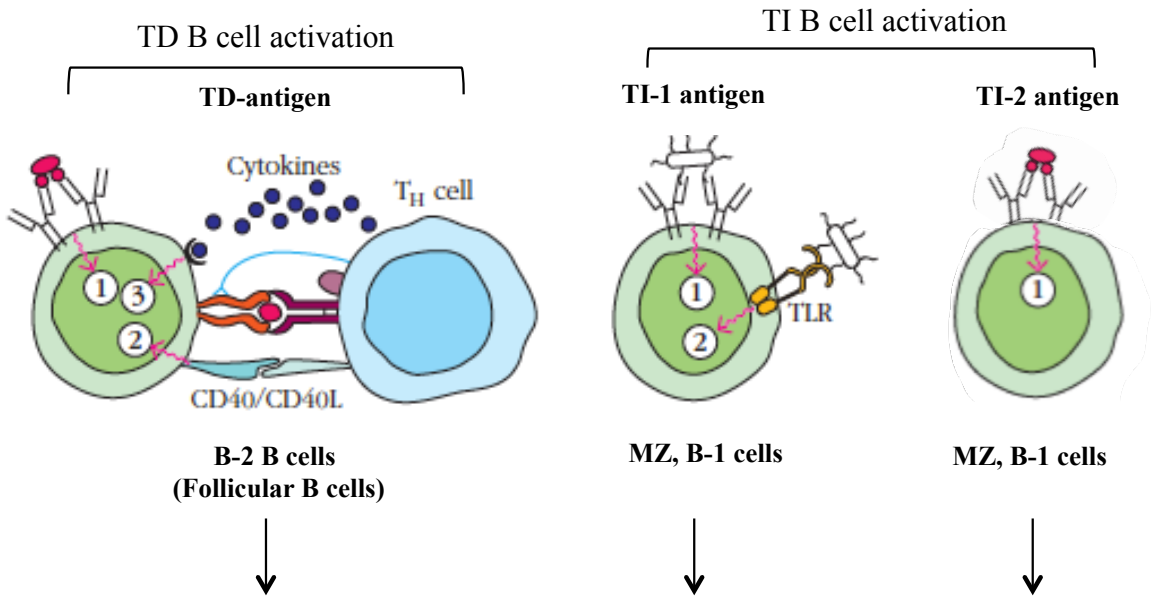
Figure 1.13: B cell subsets

Owen JA, et all. Kuby Immunology, 7th edition, 2013

1.1.3b1 B cell activation

B cell activation starts when B-cell receptors (BCRs) bind to its specific antigen. There are two main ways for B cell activation that depends on nature of antigens[2,3]. One is T cell dependent (TD) and other is T cell independent (TI) B cell activation. Non-protein microbial antigens like lipids, nucleic acids and glycoproteins cannot stimulate classical T cell response but are able to stimulate Ab production in the absence of T cells and called as thymus or T cell independent (TI) antigens[74]. On the other hand, the antibody production to protein antigens requires T cell involvement, and these antigens are described as thymus dependent (TD) antigens[75]. TI responses are often of shorter duration than T cell dependent B cell responses.

TD B cell activation- After antigen exposure, antigen bind to its specific BCR on B cell and this lead to B cell activation (signal 1, Fig. 1.14). Activated B cell then internalize and process the antigen and present it to already primed $CD4^+$ T cell with same antigen. This lead to further activation of $CD4^+$ T cell and it express CD40L, which binds to CD40 on the B cell surface and induces signaling (signal 2, Fig. 1.14). At same time, activated $CD4^+$ T cell release cytokines (Signal 3, Fig. 1.14) that leads to B cell proliferation. Some of the proliferating B cells differentiate into short-lived plasma cells and memory B cells outside the follicles (germinal center independent) form primary focus and produce Abs in initial stage of immune response which are mainly class IgM[75,76].



Cytokine Signal	B cell isotype switch
IL-4	IgG1, IgE
TGF-β	IgA, IgG2b
IL-5	IgA
IFN-γ	IgG3, IgG2a

Figure 1.14: B cell activation

Owen JA, et all. Kuby Immunology, 7th edition, 2013 (modified)

Activated B cells move to the follicle and undergo rapid proliferation and clonal expansion to form the germinal center, which is accompanied by somatic hypermutation (SHM), and affinity maturation through interaction with immune complex-coated follicular dendritic cells (FDCs) and antigen-specific T follicular helper cells (TFH cells). After that Ig

class switching take place under the influence of cytokines produced by activated $CD4^+$ T cell (Fig. 1.14). These germinal center B cells then exit the germinal center, either as memory B cells or as fully differentiated long-lived plasma cells which roam all over body and produce large amount of Abs and finally make home in bone marrow for life time[75,76].

TI B cell activation is depending on TI antigens, which are further divided in to two subclasses, TI type 1 (TI-1) and TI type 2 (TI-2) on the mechanism of B cell activation by them. TI-1 antigens are LPS, CpG or Ploy IC, act via TLRs and directly induce B cell division (Fig. 1.14) [74]. At high concentrations, TI-1 antigens induce the proliferation and differentiation of most B cells regardless of antigen specificity (polyclonal activation). However, at low concentrations, they induce antigen-specific antibody responses. However, they are not able to undergo isotype switching or affinity maturation due to lack of T cell help. TI-2 antigens are polysaccharides that cross link the specific BCR and thus induce antigen specific B cell responses (Fig. 1.14)[74]. TI-2 antigens don't have B cell activating activity and can activate only mature B cells to produce antibodies. After TI antigen challenge B cell get activated and differentiate in to plasma cells in extra follicular regions and produce low affinity mainly IgM Abs. TI antigens usually activate B-1 B and marginal zone B cells.

1.1.3b2 Functions of B cells

B cells are functional in two ways, one is antibody dependent and others are its cellular functions. Besides producing Abs, B cells are involved in antigen presentation and secrete different cytokines which lead to T cell stimulation or inhibition; help in memory formation, lymphoid tissue organogenesis, autoimmunity and tumor immunity[77,78].

Antibody secreted by B cells protects the host from pathogen by involving in different crucial functions. Abs can directly neutralize the pathogen by binding and blocking the receptors that pathogen use to get entry in to a cell. Abs can opsonize pathogen by binding to them and recruiting phagocytic cells, which engulf and digest Ab-Ag complex (Fig. 1.15). Abs bind directly to pathogen or antigen expressing infected cells that can initiate the complement cascade, which can kill pathogen and infected cells (Fig. 1.15). [79-81]. Abs bind to antigen expressing infected cells and can recruit cytotoxic natural killer (NK) cells by making interaction with FcR on NK cells that lead NK cell activation and killing of infected cell in a process called antibody- dependent cell-mediated cytotoxicity (ADCC) (Fig. 1.15) [79-81].

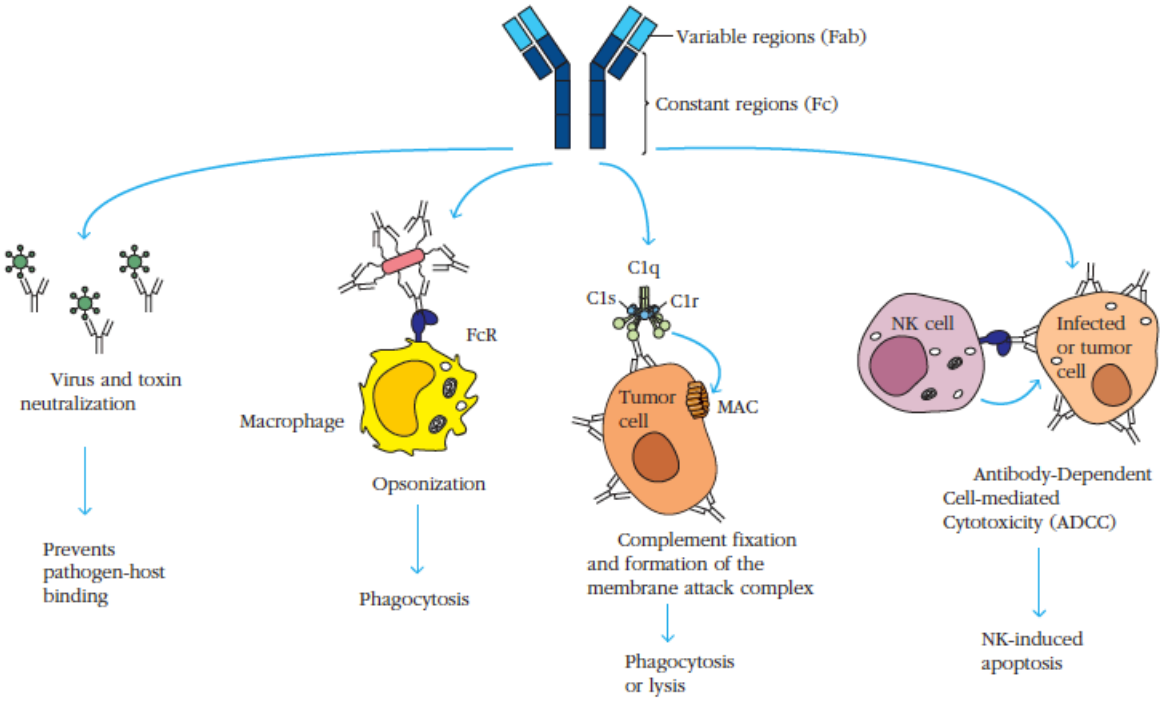


Figure 1.15: Antibody functions

Owen JA, et all. Kuby Immunology, 7th edition, 2013

1.2 Pathogens

For this study, we used different pathogens include lymphocytic choriomeningitis virus (LCMV), recombinant-LCMV (r-LCMV), recombinant listeria monocytogenes and wild type listeria monocytogenes.

1.2.1 Lymphocytic choriomeningitis virus (LCMV)

LCMV was first discovered by Charles Armstrong in 1933 during epidemic encephalitis in St. Louis. The natural host of LCMV is mouse but also infect wide range of animals including human and responsible for aseptic meningitis and encephalitis[82]. LCMV belongs to arenaviridae family and is an enveloped virus consists of glycoprotein (GP) spikes on surface and two negative sense-single stranded RNA segments named L and S, which are wrapped along nucleoproteins[83]. LCMV infect cells by binding through their glycoprotein to cell surface receptor called alpha-dystroglycan (α-DG)[84]. Alpha-dystroglycan is a receptor for extracellular matrix and widely expressed on cells[84]. Upon receptor binding LCMV is internalized in to cell vesicle where viral membrane fuse to cell and release its genome in cytoplasm[85].

LCMV is a non-cytopathic virus that replicates in host cells without harming them. The progress of LCMV infection in mice mainly depends upon dose and strain of LCMV used. There are four known strains of LCMV, two are neurotropic include Armstrong (mild) and clone13 (aggressive) and two are hepatotropic include WE (mild) and Docile (aggressive)[86,87]. Infection with mild strains of LCMV in mice induce acute infection and resolved with in 2 weeks with the help of virus specific cytotoxic T lymphocyte (CTL). But aggressive LCMV strains lead to T-cell exhaustion and virus persistent[88]. Lower infection dose of LCMV induce strong CTL response and clear virus quickly with minimal pathology, however higher LCMV dose result in clonal T cell exhaustion, viral persistent and little immunopathology. But intermediate dose partially exhaust T cells and result in massive immunopathology and high mortality[89].

Recombinant LCMV (rLCMV) express mutated form of LCMV-GP that can infect cells and viral genome can undergo transcription and replication but can't form infectious particles[90]. rLCMV have antigenic properties and can induce CTL response[90,91].

1.2.2 Listeria Monocytogenes (LM)

Listeria monocytogenes (LM) a gram-positive facultative intracellular bacterium[92]. Infection with LM generate CTL response which is important for elimination of LM from host[93]. Recombinant LM (LM-GP33) that express the GP(GP33-41) of LCMV and infection of mice with LM-GP33 result in GP33 specific CTL response[94,95]. LM-GP33 mostly used as a vaccine to generate LCMV specific T cell response[94,95].

1.3 Mouse models

- **P14 × CD45.1**

  These transgenic mice have T-cell receptor (TCR) specific for the LCMV glycoprotein epitope 33 (GP-33-41)[96]. P14 mice are crossed with CD45.1 congenic mice to track lymphocytes during adoptive transfer experiments.

- **Usp18$^{-/-}$**

  These mice lack functional Ubiquitin-specific peptidase 18 (USP18 or UBP43) protein. USP18 act as a negative regulator of interferon signaling[97].

- **Jh$^{-/-}$**

  These mice lack gene for heavy chain joining region and fail to develop functional B cells[98].

- **PD1$^{-/-}$**

  PD1$^{-/-}$ mice lack functional programmed cell death 1 protein, also known as Pdcd1. PD1 is a cell surface inhibitory molecules and negatively regulate functions of T cells[99].

- **PDL1$^{-/-}$**

  These mice are deficient of PDL1, which is a ligand for PD1. These mice exhibit higher functional activity of T cells[100].

- **CD169-DTR**

  These transgenic mice express human diphtheria toxin receptor (DTR) under the promoter of CD169 gene. In these mice, CD169^{+} macrophages can be depleted selectively by administration of diphtheria toxin[101].

- **IFNAR$^{-/-}$**

  These mice lack the receptors for interferon α and β, IFN signaling, and are highly susceptible to viral infection[102].

- **IFNβ $^{mob/mob}$**

  These are IFN-β reporter knock-in mice which express yellow fluorescent protein (YFP) under the IFN-β promoter. Activation of IFN-β promotor lead to YFP expression and visualization of IFN-β producing cells[103].

- **Prf1$^{-/-}$**

  These mice are perforin deficient, and CTL and NK cells from these mice lack cell mediated cytolysis effector functions[104].

- **B2m $^{-/-}$**

  These mice are lacking β_2-Microglobulin that is a part of MHC class I molecule, expressed on all nucleated cells and needed for development of CD8^{+} T-cell in thymus [105]. These mice are lacking CD8^{+} T cells.

- **KL25 × CD45.1**

  In these transgenic mice, heavy chains of immunoglobulins are specific for LCMV. So these mice naturally have LCMV neutralizing antibodies in serum[106].

OPEN

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CD169$^+$ macrophages regulate PD-L1 expression via type I interferon and thereby prevent severe immunopathology after LCMV infection

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Upon infection with persistence-prone virus, type I interferon (IFN-I) mediates antiviral activity and also upregulates the expression of programmed death ligand 1 (PD-L1), and this upregulation can lead to CD8$^+$ T-cell exhaustion. How these very diverse functions are regulated remains unknown. This study, using the lymphocytic choriomeningitis virus, showed that a subset of CD169$^+$ macrophages in murine spleen and lymph nodes produced high amounts of IFN-I upon infection. Absence of CD169$^+$ macrophages led to insufficient production of IFN-I, lower antiviral activity and persistence of virus. Lack of CD169$^+$ macrophages also limited the IFN-I-dependent expression of PD-L1. Enhanced viral replication in the absence of PD-L1 led to persistence of virus and prevented CD8$^+$ T-cell exhaustion. As a consequence, mice exhibited severe immunopathology and died quickly after infection. Therefore, CD169$^+$ macrophages are important contributors to the IFN-I response and thereby influence antiviral activity, CD8$^+$ T-cell exhaustion and immunopathology.

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Chronic viral infection is a serious health concern. Many viruses, such as human immunodeficiency virus (HIV), hepatitis B virus (HBV) and hepatitis C virus (HCV), lead to viral persistence and dysfunction of adaptive immunity.[1] The persistence of HCV can lead to chronic liver inflammation, resulting in liver cirrhosis, liver steatosis, end-stage liver failure or hepatocellular carcinoma. Many of these clinical problems are related to the constant activity of cytotoxic CD8$^+$ T cells. Therefore, exhaustion of CD8$^+$ T cells may be essential for preventing severe immunopathology in chronic infections. Although mechanisms of exhaustion that involve inhibitory receptors have been thoroughly studied on the T-cell side, it has not yet been determined which cell types modulate the expression of the ligands for inhibitory receptors and thereby contribute to T-cell exhaustion during chronic viral infection. Identifying such mechanisms may help explain why some patients suffer from severe immunopathology during chronic infection, whereas others do not.

Type I interferon (IFN-I) plays a dual role during viral infection. On the one hand, it limits viral replication because it directly induces antiviral factors in the infected cell.[2,3] Consequently, the absence of the interferon-α/β receptor (IFNAR) promotes viral replication and can result in persistence of virus.[4–6] On the other hand, sustained IFN-I signaling induces immunosuppressive mechanisms, including the production of interleukin-10 (IL-10) and the expression of programmed cell death ligand 1 (PD-L1).[7–9] IL-10 and PD-L1 are important inhibitors of CD8$^+$ T cells and thereby limit the function of virus-specific CD8$^+$ T cells. Programmed cell death protein 1 (PD-1) is upregulated on all activated CD8$^+$ T cells,[10] a finding suggesting that the regulation of its ligand (PD-L1) determines the fate of virus-specific CD8$^+$ T cells. Viral infection can upregulate PD-L1 expression by target cells, and this expression mediates the immune escape of these cells from killing by cytotoxic T lymphocytes (CTLs).[11] How professional immune cells regulate PD-L1 expression during an ongoing infection is not well defined.

Here we report that during infection with lymphocytic choriomeningitis virus (LCMV), CD169$^+$ macrophages prolong the IFN-I response that mediates antiviral activity. In addition, a prolonged IFN-I response induces PD-L1

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Abbreviations: ALT, alanine aminotransferase; CFSE, carboxyfluorescein succinimidyl ester; CD169-DTR, CD169 diphtheria toxin receptor; CTL, cytotoxic T lymphocytes; GzmB, granzyme B; IL-10, interleukin-10; IFNAR, interferon-α/β receptor; LCMV, lymphocytic choriomeningitis virus; PFU, plaque-forming units; PD-L1, programmed death ligand 1; RT-PCR, real-time polymerase chain reaction; IFN-I, type I interferon; WT, wild type; VSV, vesicular stomatitis virus

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Chapter 2

CD169^{+} macrophages regulate PD-L1 expression via type I interferon and thereby prevent severe immunopathology after LCMV infection

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2.1 Abstract

Upon infection with persistence-prone virus, type I interferon (IFN-I) mediates antiviral activity and also upregulates the expression of programmed death ligand 1 (PD-L1), and this upregulation can lead to $CD8^+$ T-cell exhaustion. How these very diverse functions are regulated remains unknown. This study, using the lymphocytic choriomeningitis virus, showed that a subset of $CD169^+$ macrophages in murine spleen and lymph nodes produced high amounts of IFN-I upon infection. Absence of $CD169^+$ macrophages led to insufficient production of IFN-I, lower antiviral activity and persistence of virus. Lack of $CD169^+$ macrophages also limited the IFN-I-dependent expression of PD-L1. Enhanced viral replication in the absence of PD-L1 led to persistence of virus and prevented $CD8^+$ T-cell exhaustion. As a consequence, mice exhibited severe immunopathology and died quickly after infection. Therefore, $CD169^+$ macrophages are important contributors to the IFN-I response and thereby influence antiviral activity, $CD8^+$ T-cell exhaustion and immunopathology.

2.2 Introduction

Chronic viral infection is a serious health concern. Many viruses, such as human immunodeficiency virus (HIV), hepatitis B virus (HBV) and hepatitis C virus (HCV), lead to viral persistence and dysfunction of adaptive immunity[107]. The persistence of HCV can lead to chronic liver inflammation, resulting in liver cirrhosis, liver steatosis, end-stage liver failure or hepatocellular carcinoma. Many of these clinical problems are related to the constant activity of cytotoxic $CD8^+$ T cells. Therefore, exhaustion of $CD8^+$ T cells may be essential for preventing severe immunopathology in chronic infections. Although mechanisms of exhaustion that involve inhibitory receptors have been thoroughly studied on the T-cell side, it has not yet been determined which cell types modulate the expression of the ligands for inhibitory receptors and thereby contribute to T-cell exhaustion during chronic viral infection. Identifying such mechanisms may help explain why some patients suffer from severe immunopathology during chronic infection, whereas others do not.

Type I interferon (IFN-I) plays a dual role during viral infection. On the one hand, it limits viral replication because it directly induces antiviral factors in the infected cell [108,109]. Consequently, the absence of the interferon-α/β receptor (IFNAR) promotes viral replication and can result in persistence of virus [102,110,111]. On the other hand, sustained IFN-I signaling induces immunosuppressive mechanisms, including the production of interleukin-10 (IL-10) and the expression of programmed cell death ligand 1 (PD-L1) [68,112,113]. IL-10 and PD-L1 are important inhibitors of $CD8^+$ T cells and thereby limit the function of virus-specific $CD8^+$ T cells. Programmed cell death protein 1 (PD-1) is upregulated on all activated $CD8^+$ T cells [114], a finding suggesting that the regulation of its ligand (PD-L1) determines the fate of virus-specific $CD8^+$ T cells. Viral infection can upregulate PD-L1 expression by target cells, and this expression mediates the immune escape of these cells from killing by cytotoxic T lymphocytes (CTLs) [115]. How professional immune cells regulate PD-L1 expression during an ongoing infection is not well defined.

Here we report that during infection with lymphocytic choriomeningitis virus (LCMV), $CD169^+$ macrophages prolong the IFN-I response that mediates antiviral activity. In addition, a prolonged IFN-I response induces PD-L1 expression in the liver. The absence of $CD169^+$ macrophages reduces antiviral IFN-I activity and also limits PD-L1 expression. As a result, mice exhibit overwhelming viral replication in the absence of $CD8^+$ T-cell exhaustion, and this replication results in severe immunopathology and death of mice.

2.3 Results

2.3.1 Depletion of CD169$^+$ macrophages affects a subtype of F4/80$^+$ macrophages in the liver and viral control

In order to study the role CD169$^+$ cells, we used CD169 diphtheria toxin receptor (CD169-DTR) mice that express DTR under the CD169 promoter. Treating these mice with diphtheria toxin (DT) specifically depletes CD169$^+$ cells. Interestingly, we found that after DT treatment, not only CD169$^+$ cells in lymphoid organs were depleted but also the CD169$^+$ cell number in the liver was reduced (**Figure 1a** and **Supplementary Figure S1**). Next we wondered on which cell type CD169 is expressed and whether its expression is upregulated during viral infection. To study this, we infected C57BL/6 wild type (WT) mice with LCMV strain WE and analyzed the expression of CD169 on different cell types in comparison with non-infected mice. We found that, without infection, CD169 is expressed on different cell types in the bone marrow and on F4/80$^+$ cells in the liver and spleen (**Supplementary Figure S2**). After infection, CD169 was mostly upregulated in the bone marrow, on different cell populations in the spleen and on F4/80$^+$ and Ly6C$^+$ cells in the liver, whereas we did not detect an upregulation of CD169 in the lymph nodes (LNs) (**Supplementary Figure S2** and **Figure 1b**). By analyzing the number of F4/80$^+$ cells, we found a reduction in F4/80$^+$ macrophages in the liver even in non-infected mice (**Figure 1c**). This means that a subtype of F4/80$^+$ macrophages express CD169 in the liver under naive conditions. This raised the question whether depletion of CD169$^+$ cells has an impact on phagocytic activity. We infected WT and CD169-DTR mice with LCMV-WE and measured viral titer in the blood. We detected more viruses in the blood of CD169-DTR mice after 10 min of infection in CD169-DTR mice (**Figure 1d**). However, virus was cleared from blood in both groups within 60 min post infection (**Figure 1d**). In a previous study, we showed that infecting WT mice with low dose of LCMV-WE ($\leq$ 200 PFU) leads to viral replication only in the spleen but not in the liver. The inhibition of viral replication in the liver was IFN-I dependent because lack of interferon type I receptor led to high viral titer in the liver [116]. Here we found that depletion of CD169$^+$ macrophages in CD169-DTR mice did not affect viral replication in the spleen and LNs (**Figure 1e**). Also, after 5 days we could not detect enhanced viral replication (**Figure 1e**). However, CD169$^+$macrophages were essential for controlling LCMV replication in the liver 5 days after infection (**Figure 1e**). Histological staining showed that the presence of CD169$^+$macrophages in WT mice could prevent infection of hepatocytes, whereas mice without CD169$^+$ macrophages exhibited virus-infected

hepatocytes **(Figure 1f)**. We conclude that depletion of $CD169^+$ macrophages affects a subtype of $F4/80^+$macrophages in the liver which leads to enhanced viral replication.

2.3.2 $CD169^+$ macrophages in the spleen and lymph nodes contribute to the production of IFN-I

In a previous study, we found that after viral infection with the cytopathic vesicular stomatitis virus (VSV), macrophages in liver are contributing mainly in taking up the virus. Even though viral replication is suppressed in the liver and allowed in $CD169^+$ macrophages in the spleen and LNs in an IFN-I dependent manner [117]. To define the role of $CD169^+$ macrophages during infection with the non-cytopathic LCMV, we first infected C57BL/6 wild-type (WT) mice with 2×10^6 plaque-forming units (PFU) of LCMV strain WE and analyzed the viral uptake. After 1 h, viral RNA levels in various organs were analyzed. We found that the liver initially takes up most of the virus followed by the spleen (**Figure 2a**). However, one day after infection, viral replication was suppressed in the liver and allowed in the spleen and LNs (**Figure 2b**). This suppression was IFN-I dependent because infecting IFN-I receptor-deficient mice ($Ifnar^{-/-}$) led to an increase in viral titer in the liver (**Figure 2b**). Histological staining of spleen and LNs showed that viral replication in spleen and LNs was mostly occurring in the $CD169^+$ macrophages and that LCMV nucleoprotein (LCMV-NP) partially co-localized with $CD169^+$ macrophages (**Figure 2c**). Because virus can induce IFN-I via activation of pattern recognition receptors [118], we hypothesized that replication of virus in $CD169^+$ macrophages may lead to induction of IFN-I in these cells. To test this hypothesis, we infected IFN-β reporter knock-in mice ($IFN\beta^{mob/mob}$ mice) with LCMV. In these mice the yellow fluorescent protein (YFP) is expressed after activation of the ifnb promoter. Additionally, we infected C57BL/6 mice as negative controls. Two days after LCMV infection, we analyzed the expression of YFP. Infection of $IFN\beta^{mob/mob}$ mice led to the expression of YFP in 0.008% of all splenocytes (**Figure 2d**), a finding suggesting that these were the main IFN-I producers during LCMV infection [42,103]. WT mice did not exhibit YFP expression after LCMV infection (**Figure 2d**). Gating of IFN-I-producing cells showed that approximately 10% of IFN-I producers expressed CD169. Next, we performed real-time polymerase chain reaction (RT-PCR) for the early IFN-I genes Ifn-α4 and Ifn-β1 in spleen and LNs to determine whether the depletion of $CD169^+$ macrophages can influence IFN-I production. We found that LCMV infection induced strong expression of Ifn-α4 and Ifn-β1 mRNA in the spleen and LNs of WT mice (**Figure 2e**). DT-treated CD169-DTR mice

exhibited significantly lower mRNA levels of Ifn-α4 and Ifn-β1 than WT mice, especially on day 5 after infection (**Figure 2e**). In line with these results, we found that systemic IFN-α levels were significantly lower in the serum of DT-treated CD169-depleted mice than in WT mice 2 days after infection (**Figure 2f**). In conclusion, we found that early replication of LCMV in $CD169^+$ macrophages resulted in IFN-I production. Especially at later stages of infection, $CD169^+$ macrophages were responsible for most of the systemic production of IFN-α.

2.3.3 $CD169^+$ macrophages have limited impact on $CD8^+$ T cell priming but are essential for controlling acute viral infection and prevention of immunopathology

To determine whether early priming of $CD8^+$ T cells is influenced by $CD169^+$ macrophages, we transferred carboxyfluorescein succinimidyl ester (CFSE)-labeled splenocytes from naive CD45.1 x LCMV-P14 T-cell receptor transgenic mice (CD45.1 x P14 mice) into WT or CD169-DTR mice. P14 mice express a LCMV–GP33-41–specific TCR as a transgene [96]. Infection with LCMV resulted in proliferation of virus-specific $CD8^+$ T cells in the spleen and LNs in both WT and CD169-DTR mice (**Figure 3a**). The total number of virus-specific $CD8^+$ T cells was slightly higher in the absence of $CD169^+$ macrophages (**Figure 3b**). This finding suggests that $CD169^+$ macrophages exert limited impact on $CD8^+$ T-cell priming.

Next we investigated the activation markers of GP33-specific tetramer positive $CD8^+$ T cells (Tet-$GP33^+$). We treated WT and CD169-DTR mice with DT and infected them with LCMV-WE. We found, after 8 days of viral infection, that in CD169-DTR mice, Tet-$GP33^+$ $CD8^+$ T cells are more highly activated than in WT mice, as determined with granzyme B (GzmB), CD43, PD-1 and Lag3 (**Figure 3c**). Together, our findings suggest that the absence of $CD169^+$ macrophages does not affect priming of virus-specific $CD8^+$ T cells.

The absence of $CD169^+$ macrophages was associated with a weak IFN-I response but normal $CD8^+$ T-cell priming. We next investigated the impact of $CD169^+$macrophages on overall virus control and pathology after infection with an acute virus strain. We found that LCMV persists in LNs, spleen and liver of CD169-DTR mice but is controlled by WT mice (**Figure 3d**). This finding was in line with enhanced liver cell damage, because alanine aminotransferase (ALT) activity was dramatically increased in CD169-depleted mice (**Figure 3e**) and these mice became terminally ill after acute infection, whereas WT mice survived

(Figure 3f). We conclude that absence of $CD169^+$ cells has limited impact on T-cell priming but it leads to viral persistence and immunopathology.

2.3.4 $CD169^+$ macrophages induce PD-L1 expression which prevents immunopathology

IFN-I plays two main roles during LCMV infection. First, it directly inhibits viral replication by inducing antiviral enzymes. Second, it upregulates PD-L1 expression, and this upregulation can lead to exhaustion of $CD8^+$ T cells. This finding suggested that the absence of $CD169^+$ macrophages not only limits direct antiviral effects but may also influence $CD8^+$ T-cell functions. The fact that the liver strongly responds to antiviral IFN-I, we investigated whether PD-L1 expression is regulated by IFN-I in the liver. We infected WT and $Ifnar^{-/-}$ mice with LCMV and analyzed the expression of PD-L1 in the liver. We found that during LCMV infection, Kupffer cells express high levels of PD-L1 (**Figure 4a**). The absence of IFN-I signaling in $Ifnar^{-/-}$mice inhibits the upregulation of PD-L1 on Kupffer cells during acute infection (**Figure 4a**). Next we investigated whether $CD169^+$ macrophages affect PD-L1 expression. To do so we stained the cells for PD-L1 in CD169-depleted mice after LCMV infection. PD-L1 expression was strongly reduced in the liver of DT-treated CD169-DTR mice (**Figure 4b**). This finding suggests that $CD169^+$ macrophages-derived IFN-I is essential for PD-L1 upregulation in the liver.

We speculate that higher viral replication and a reduction of $CD8^+$ T-cell exhaustion due to lack of PD-L1 expression in CD169-DTR mice are responsible for the death of the mice. Indeed depletion of $CD8^+$ T cells prevented death in 70% of the CD169-DTR mice (**Figure 4c**). In order to compensate for PD-L1 expression in CD169-DTR mice, we generated bone marrow-derived macrophages (BMDMs) from WT and PD-L1-deficient mice and treated them with IFN-I in order to increase PD-L1 expression (**Figure 4d**). Afterwards, we transferred the BMDMs into two groups of LCMV-infected CD169-DTR mice. The group that received WT cells survived after infection with LCMV, whereas the group that received PD-L1–deficient cells died of infection (**Figure 4e**). In conclusion, the absence of $CD169^+$ macrophages leads to a lethal immunopathology because of the limited expression of PD-L1.

2.3.5 CD169$^+$ macrophages prevent severe immunopathology during chronic viral infection

We found that during acute LCMV infection, the absence of CD169$^+$ macrophages results in insufficient control of virus and limited CD8$^+$ T-cell exhaustion. Both effects result in severe immunopathology. Next we analyzed how CD8$^+$ T-cell responses develop during overwhelming viral replication. To do so, we used the LCMV strain Docile, which is known to persist and is associated with severe CD8$^+$ T cell exhaustion in WT mice [119]. In WT mice and CD169-DTR mice infected with LCMV-Docile, virus persisted after infection (**Figure 5a**). CD169-DTR mice generated more virus-specific CD8$^+$ T cells in the spleen than did WT mice, whereas in both types of mice the numbers of these cells in the liver were comparable (**Figure 5b**). Moreover, CD8$^+$ T cells in CD169-DTR mice exhibited higher frequencies of IFN-γ producing CD8$^+$ T cells in the liver (**Figure 5c**). In the absence of CD169$^+$macrophages, liver cell damage was exacerbated (**Figure 5d**) and mice became terminally ill after infection (**Figure 5e**). To check whether this severe liver cell damage and clinical disease was dependent on CD8$^+$ T-cell-mediated immunopathology, we depleted CD8$^+$ T cells in both groups. We found that absence of CD8$^+$ T cells in CD169-DTR mice prevented morbidity and mortality (**Figures 5d and e**). To determine whether the immunopathology in CD169-DTR mice was due to their inability to induce PD-L1 expression, we infected PD-1-deficient mice with LCMV-Docile. Indeed, the absence of PD-1 during chronic LCMV infection led to liver damage, as determined by serum ALT activity **(Figure 5f)**; this phenotype resembled that of mice deficient in CD169$^+$ cells (**Figure 5d**). The immunopathology also led to the death of LCMV-Docile-infected PD-1-deficient mice (**Figure 5g**). From these findings we concluded that CD169$^+$ macrophages are essential for preventing severe immunopathology and death during chronic viral infection.

2.4 Discussion

The results of this study show that CD169$^+$ macrophages are important contributors to prolonged IFN-I production after LCMV infection. This prolonged production is associated with reduced viral replication in peripheral organs and with upregulation of PD-L1. Depletion of CD169$^+$ macrophages results in enhanced viral propagation and prevents CD8$^+$ T-cell exhaustion, both of which contribute to severe immunopathology.

The function of CD8$^+$ T cells must be carefully regulated to ensure the elimination of virus without the development of severe immunopathology. IFN-I has been found to be a

crucial innate cytokine that strongly regulates $CD8^+$ T cell function. First, it protects $CD8^+$ T cells from cytotoxicity induced by natural killer cells [120]. Second, IFN-I upregulates IL-10 and PD-L1, and this upregulation contributes to the exhaustion of $CD8^+$ T cells [112,113]. Genetic deletion of IL-10 or partial blockade of PD-1 enhances the control of virus during chronic infection [68,70]. Earlier studies showed that PD-1^{high} CTLs are effective and important in reducing viral titers during acute infection [114]. The factor that determines whether PD-1 expression can contribute to $CD8^+$ T cell exhaustion is the upregulation of PD-L1 on cells that contact $CD8^+$ T cells [115]. In addition to PD-1, 13 other inhibitory cell surface pathways have been shown to be overexpressed in exhausted $CD8^+$ T cells [121]. In addition to passive defects in metabolism, active suppression is needed for functional exhaustion of $CD8^+$ T cells [121]. The results of the present study show that mice lacking $CD169^+$ macrophages cannot control LCMV because of the absence of prolonged production of IFN-I. Therefore, $CD169^+$ macrophages may be the crucial cell type that balances viral suppression and $CD8^+$ T cell exhaustion.

In earlier studies using VSV, we found that $CD169^+$ macrophages are essential for enforcing viral replication in the spleen and thereby play an important part in immune activation [116,117,122]. In this study using LCMV, we did not find reduced priming of adaptive immune cells; however, we did find reduced induction of IFN-I. There may be two reasons for these diverse functions of $CD169^+$ macrophages in VSV infection and LCMV infection. First, LCMV can replicate not only in follicular dendritic cells but also in $CD169^+$ macrophages and conventional dendritic cells [116]. Therefore, enforced viral replication does not depend entirely on $CD169^+$ macrophages that will lead to early $CD8^+$ T cell activation and IFN-I production. Second, $CD169^+$ macrophages in the liver may also participate in antiviral effector functions. Therefore, the absence of $CD169^+$ macrophages may also limit local IFN-I production in the liver.

It remains to be answered how these findings can be transferred to chronic viral diseases in humans. The role of various subtypes of Kupffer cells during chronic viral infection has not been well studied. Some human macrophages express CD169 [123]. Whether these macrophage populations similarly contribute to the prolonged induction of IFN-I during chronic viral infections in humans remains to be studied.

In conclusion, we found that $CD169^+$ macrophages contribute to viral propagation and $CD8^+$ T-cell exhaustion during viral infection. $CD169^+$ macrophage-derived IFN-I is essential

for preventing viral replication in peripheral organs and for inducing PD-L1 expression so that severe immunopathology can be prevented.

2.5 Methods

2.5.1 Mice

All mice were sex, age and weight matched to their controls. CD169-DTR mice were generated in the Tanaka lab [101], and IFNβ$^{mob/mob}$ mice were generated in the Scheu lab [103]. P14 mice expressing a LCMV–Gp33-41–specific TCR as a transgene were used for adoptive transfer experiments [96]. All of these mice were maintained on a C57BL/6 background, as were PD-1$^{-/-}$, PD-L1$^{-/-}$, and Ifnar$^{-/-}$ mice. All experiments were performed with the animals housed in single ventilated cages and with the authorization of the Veterinäramt Nordrhein Westfalen (Düsseldorf, Germany) in accordance with the German law for animal protection or the institutional guidelines of the Ontario Cancer Institute.

2.5.2 Depletion of the cells

For CD8$^+$ T-cell depletion, 500 μg of anti-CD8 antibody clone YTS 169.4 (Bioxcell, West Lebanon, NH, USA) was injected intraperitoneally on days 1, 3 and 5. For the depletion of CD169$^+$ macrophages, 30 μg/kg body weight DT (Sigma Aldrich, St. Louis, MO, USA) was injected intraperitoneally on days −3, 2 and 5.

2.5.3 Generation of bone marrow–derived macrophages and transfer experiment

Primary macrophages were generated by isolating bone marrow from femurs and tibias of mice and eliminating erythrocytes. Macrophages were generated by culturing bone marrow cells in very low endotoxin Dulbecco's modified Eagle's medium (VLE-DMEM) (Biochrom, Berlin, Germany) supplemented with 10% (v/v) fetal bovine serum (Biochrom), 0.1% (v/v) β-mercaptoethanol (β-ME) (Invitrogen, Carlsbad, CA, USA) and 10 ng/ml macrophage colony-stimulating factor (made in house). Cells were treated with 100 units of IFN-α4 (PBL Assay Science, Pistcataway, NJ, USA) on day 7 for 12 h. On day 8 of harvesting, cells were washed and 8×10^6 cells were transferred into LCMV-infected mice. Cells transfer was performed twice on day 5 and day 7 of infection.

2.5.4 Plaque assay

Virus titers were measured with a focus-forming assay as previously described [124].

2.5.5 Real-time polymerase chain reaction

Total RNA was extracted with Trizol (Life Technologies, Carlsbad, CA, USA). The RNA was reverse-transcribed into cDNA with the Quantitect Reverse Transcription Kit (Qiagen, Hilden, Germany). Gene expression analysis was performed with assays from Qiagen: glyceraldehyde 3-phosphate dehydrogenase (GAPDH; QT01658692), IFN-α (QT01774353) or IFN-β (QT00249662). Relative quantities (RQs) were determined with the equation RQ=2–ddCt.

2.5.6 Lymphocyte transfer

Splenocytes from P14/CD45.1 mice were labeled with 1 μM CFSE (Invitrogen) and injected intravenously into mice. One day later, mice were infected with LCMV-WE. The proliferation of P14 T cells was assessed in the spleen and LNs with CFSE dilution by flow cytometry.

2.5.7 Flow cytometry

Tetramers were provided by the National Institutes of Health (NIH) Tetramer Facility (Emory University, Atlanta, GA, USA). Cells were stained with allophycocyanin (APC)-labeled GP-33 major histocompatibility complex class I tetramers (GP-33/H-2Db) for 15 min at 37 °C. After incubation, the samples were stained with anti-CD8 (BD Biosciences, San Diego, NJ, USA) for 30 min at 4 °C. Erythrocytes were then lysed with 1 ml BD lysing solution (BD Biosciences), washed once and analyzed by flow cytometry. Absolute numbers of GP-33-specific $CD8^+$ T cells per microliter of blood were determined by fluorescence-activated cell sorting (FACS) analysis using fluorescent beads (BD Biosciences). IFN-γ was purchased from eBiosciences (San Diego, CA, USA).

2.5.8 Enzyme-linked immunofluorescent assays

ELISAs for IFN-α were performed according to the manufacturer's protocol (PBL Assay Science).

2.5.9 Histology

Histologic analyses used snap-frozen tissue. Sections were stained with anti-PD-L1 (eBioscience, CA, USA), anti-CD169 (AbD Serotec, Oxfordshire, UK), anti-CD45R (B220)

(eBioscience, CA, USA), anti-F4/80 (eBioscience, CA, USA) or anti-LCMV-NP (made in-house).

2.5.10 Alanine aminotransferase

Biochemical analyses were performed by the Central Laboratory, University hospital, Essen, Germany.

2.5.11 Statistical analysis

Unless otherwise stated, data are expressed as mean±S.E.M. Student's t-test was used to detect statistically significant differences between groups. Significant differences between several groups were detected by two-way analysis of variance (ANOVA). The level of statistical significance was set at $P<0.05$, $P<0.01$ or $P<0.001$.

2.6 Ethics Statement

All experiments were performed with the animals housed in single ventilated cages. Experiments were conducted under the authorization of the Veterinäramt Nordrhein Westfalen (Düsseldorf, Germany) and in accordance with the German law for animal protection or the institutional guidelines of the Ontario Cancer Institute. The Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen (LANUV; State Agency for Nature, Environment, and Consumer Protection) approved all experiments (project numbers 84-02.04.2014.A242 and 84-02.04.2012.A094).

2.7 Acknowledgments

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2.8 Figure Legends

2.8.1 Figure 1: Depletion of CD169$^+$ macrophages affects a subtype of F4/80$^+$ macrophages in the liver and viral control

(**a**) Wild type (WT) and CD169 diphtheria toxin receptor (−DTR) mice were treated with diphtheria toxin (DT) day −3. On day 0, CD169$^+$ cells were analyzed in the indicated organs (n=3). (**b**) Wild-type (WT) mice were infected with 30 plaque-forming units (PFU) of lymphocytic choriomeningitis virus strain WE (LCMV-WE) or left uninfected. On day 5, mean fluorescence intensity (MFI) of CD169 was measured in different organs (n=3). (**c**) Wild type (WT) and CD169 diphtheria toxin receptor (−DTR) mice were treated with diphtheria toxin (DT) day −3. On day 0 mice were infected with 30 PFU of LCMV-WE or left uninfected. On day 5, indicated organs were analyzed for F4/80$^+$ cells (n=6 naive mice; n=3 LCMV-infected mice). (**d**) WT and CD169 diphtheria toxin receptor (−DTR) mice were treated with DT and infected intravenously with 2 × 10^6 PFU LCMV-WE. Viral titers were measured in blood at indicated time points (n=6). (**e**) WT and CD169 diphtheria toxin receptor (−DTR) mice were treated with diphtheria toxin and infected intravenously with 30 PFU LCMV-WE. Viral titers were measured in various organs at indicated time points (n=3). (**f**) Wild type and CD169-DTR mice were treated with diphtheria toxin and infected intravenously with 30 PFU LCMV-WE. Liver sections collected 5 days after infection were stained for F4/80 (green) and LCMV nucleoprotein (−NP) (red) (n=3). Scale bars, 100 μm (main images) or 50 μm (insets). NS, not significant, *P<0.05, **P<0.01, ***P<0.001. Statistical significance was detected by Student's t-test (**a** and **c**) or analysis of variance (ANOVA) (**e**).

2.8.2 Figure 2: CD169$^+$ macrophages in the spleen and lymph nodes contribute to the production of IFN-I

(**a**) Wild-type (WT) mice were infected intravenously with 2 × 10^6 PFU (plaque-forming units) lymphocytic choriomeningitis virus strain WE (LCMV-WE). Viral RNA expression in different organs was analyzed after 60 min with quantitative real-time polymerase chain reaction (qRT-PCR) (n=6). (**b**) WT and interferon-α/β receptor–null (Ifnar$^{-/-}$) mice were infected intravenously with 2 × 10^4PFU LCMV-WE. One day after infection, viral titers in various organs were measured (n=4). (**c**) WT mice were infected intravenously with 2 × 10^6 PFU of LCMV-WE. Spleen sections collected 1 day after infection or lymph node (LN)

sections collected 3 days after infection were stained for CD169 (red), LCMV nucleoprotein (NP) (green) and B220 (blue) (n=3). (**d**) WT and interferon-β (IFNβ) reporter knock-in ($IFN\beta^{mob/mob}$) mice were infected intravenously with LCMV-WE (30 PFU) for 48 h. IFN-β expression was analyzed by fluorescence-activated cell sorting (FACS) (n=3). (**e** and **f**) WT and CD169-DTR mice were treated with diphtheria toxin and infected intravenously with LCMV-WE (30 PFU). (**e**) IFN-α4 and IFN-β1 expression were measured with quantitative real-time polymerase chain reaction (qRT-PCR) at the indicated time points in spleen and LNs (n=3–4). (**f**) Levels of IFN-α in the serum were measured by enzyme-linked immunosorbent assay (ELISA) at the indicated time points (n=3–10). Scale bars, 100 μm. *P<0.05, **P<0.01, ***P<0.001. Statistical significance was detected by Student's t-test (**e**) or analysis of variance (ANOVA) (**f**).

2.8.3 $CD169^+$ macrophages have limited impact on $CD8^+$ T cell priming but are essential for controlling acute viral infection and prevention immunopathology

(**a** and **b**) Splenocytes (10^7) from P14/CD45.1 mice were labeled with carboxyfluorescein succinimidyl ester (CFSE) and transferred to WT or CD169-DTR mice. On the next day, mice were infected with 30 PFU LCMV-WE. Four days after infection, (**a**) proliferation of $CD45.1^+CD8^+$ T cells was assessed by CFSE dilution in spleen and lymph nodes (LNs) (n=5). (**b**) Total number of $CD45.1^+CD8^+$T cells in the spleen and blood 4 days after infection (n=5). (**c**) WT and CD169-DTR mice were treated with DT and infected intravenously with 30 PFU LCMV-WE. Indicated markers were measured in GP33-specific tetramer positive $CD8^+$ T cells in LNs, liver and spleen 8 days after infection (n=3). (**d**–**f**) WT and CD169 diphtheria toxin receptor (−DTR) mice were infected intravenously with 30 PFU LCMV-WE. (**d**) Viral titers were measured in LNs, spleen and liver at indicated time points (n=3). (**e**) Alanine aminotransferase (ALT) activity in the serum was measured 8 days after infection (n=3). (**f**) Survival of WT and CD169-DTR mice was monitored (n=15). NS, not significant, ***P<0.001. Statistical significance was detected by Student's t-test (**b** and **e**), analysis of variance (ANOVA) (**d**) or log-rank (Mantel-Cox) test (**f**).

2.8.4 Figure 4 $CD169^+$ macrophages induce PD-L1 expression which inhibits immunopathology

(**a**) Wild type (WT) and interferon-α/β receptor-null (Ifnar$^{-/-}$) mice were infected intravenously with 30 PFU of lymphocytic choriomeningitis virus strain WE (LCMV-WE). Three days after infection, liver sections were stained for F4/80 (green) and PD-L1 (red) (n=3). Scale bar, 100 μm. (**b**) WT and CD169 diphtheria toxin receptor (−DTR) mice were treated with diphtheria toxin and infected intravenously with 30 PFU LCMV-WE. Five days after infection, liver sections were stained for F4/80 (green) and PD-L1 (red) (n=3). Scale bar, 100 μm. (**c**) Wild type (WT) and CD169 diphtheria toxin receptor (−DTR) mice were treated with DT and anti-CD8 antibody on days −1 and 3. Mice were infected intravenously with 30 PFU LCMV-WE and survival of WT and CD169-DTR mice was monitored (n=6). (**d**) Bone marrow-derived macrophages (BMDM) from wild type (WT; red line) or programmed cell death ligand 1-null mice (PD-L1$^{-/-}$; dashed line) were treated overnight with 100 units of IFN-α4. Untreated WT macrophages were used as a control (filled line). Upregulation of PD-L1 was measured by fluorescence-activated cell sorting (FACS). (**e**) CD169-DTR mice were infected with 30 PFU LCMV-WE. 8×10^6 BMDMs generated either from WT or from programmed cell death ligand 1-null mice (PD-L1$^{-/-}$) were transferred into mice on days 5 and 7 post infection. Survival of mice was monitored (n=6). NS, not significant, *P<0.05. Statistical significance was detected by log-rank (Mantel-Cox) test (**c** and **e**).

2.8.5 Figure 5: CD169$^+$ macrophages prevent severe immunopathology during chronic viral infection

(**a**–**c**) Wild type (WT) and CD169 diphtheria toxin receptor (−DTR) mice were infected intravenously with 2×10^4 plaque-forming units (PFU) of lymphocytic choriomeningitis virus strain Docile (LCMV-Docile) for 11 days. (**a**) Viral titers were measured in various organs (n=6). (**b**) Number of virus-specific Tet-GP33$^+$ CD8$^+$ T cells was determined in the spleen and liver (n=7–8). (**c**) IFN-γ$^+$CD8$^+$ T cells were counted in the spleen and liver (n=7–8). (**d**) WT and CD169-DTR mice were infected intravenously with 2×10^4 PFU LCMV-Docile and treated with anti-CD8 depletion antibody or left untreated. Serum alanine aminotransferase (ALT) activity was measured after 13 days (n=3-4). (**e**) WT and CD169-DTR mice were infected intravenously with 2×10^4PFU LCMV-Docile and treated with anti-CD8 depletion antibody or left untreated. Survival of mice was monitored (n=8–33). (**f** and **g**) WT and programmed cell death protein 1-null (PD-1$^{-/-}$) mice were infected intravenously with 2×10^4 PFU LCMV-Docile. (**f**) Serum ALT activity was measured after 13 days (n=3). (**g**) Survival of mice was monitored (n=3). NS, not significant, *P<0.05, ***P<0.001. Statistical

significance was detected by Student's t-test (**b**, **c**, **d** and **f**) or log-rank (Mantel-Cox) test (**e** and **g**).

2.9 Supplementary Figures

2.9.1 Figure 1: Gating strategy of CD169$^+$ macrophages

Wild-type (WT) and CD169 diphtheria toxin receptor (-DTR) mice were treated with diphtheria toxin (DT) day -3. On day 0, CD169$^+$ cells were analyzed in the indicated organs (n = 3).

2.9.2 Figure 2: Expression of CD169 on immune cells

Wild-type (WT) mice were infected with 30 plaque-forming units (PFU) of lymphocytic choriomeningitis virus strain WE (LCMV-WE) or left uninfected. On day 5, the expression of CD169 was measured in different organs (dashed line: isotype control; black line: naive mice; red line: LCMV-infected mice) (n = 3).

Figure 1

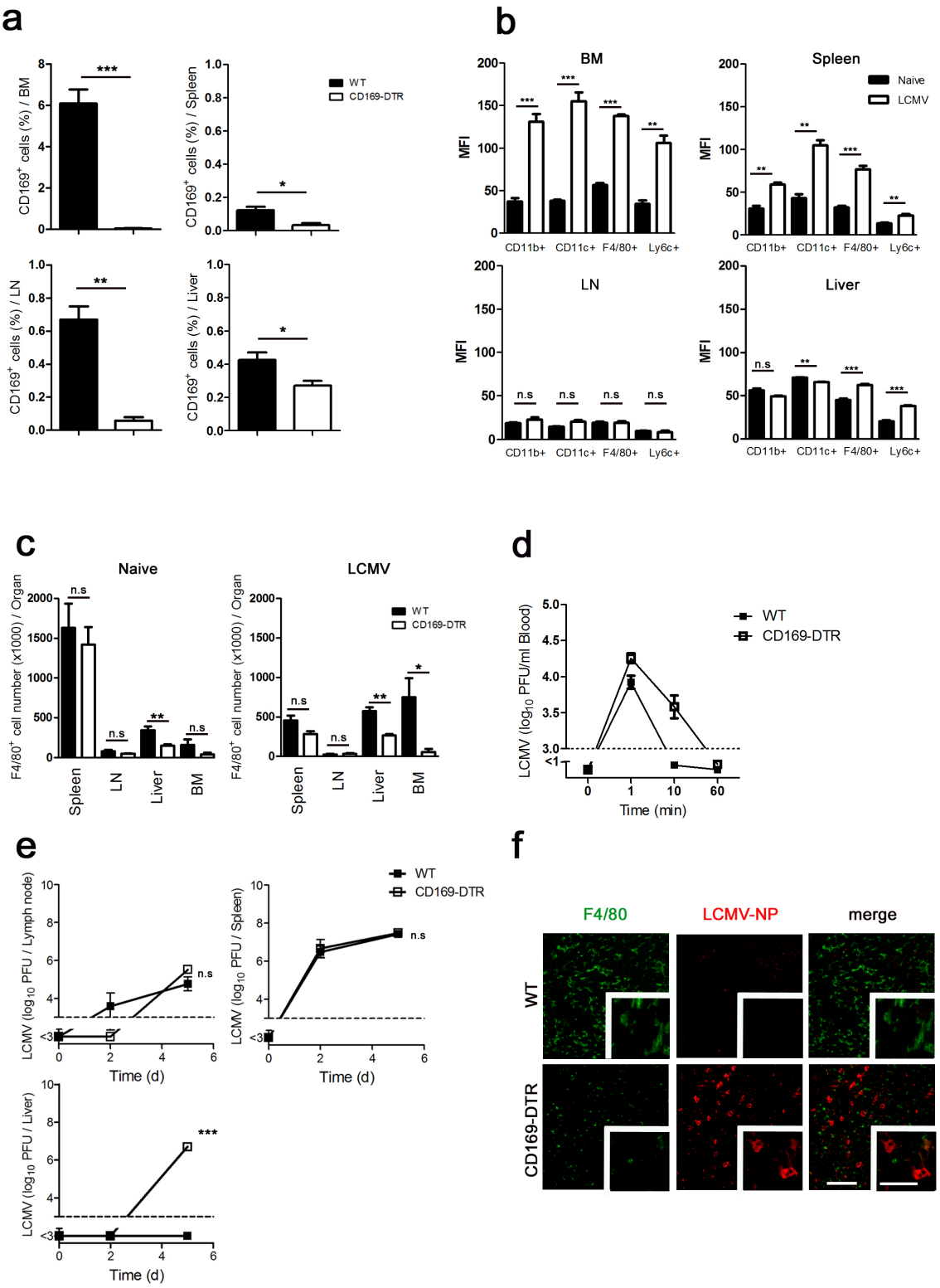


Figure 2.1: Depletion of CD169$^+$ macrophages affects a subtype of F4/80$^+$ macrophages in the liver and viral control.

Figure 2

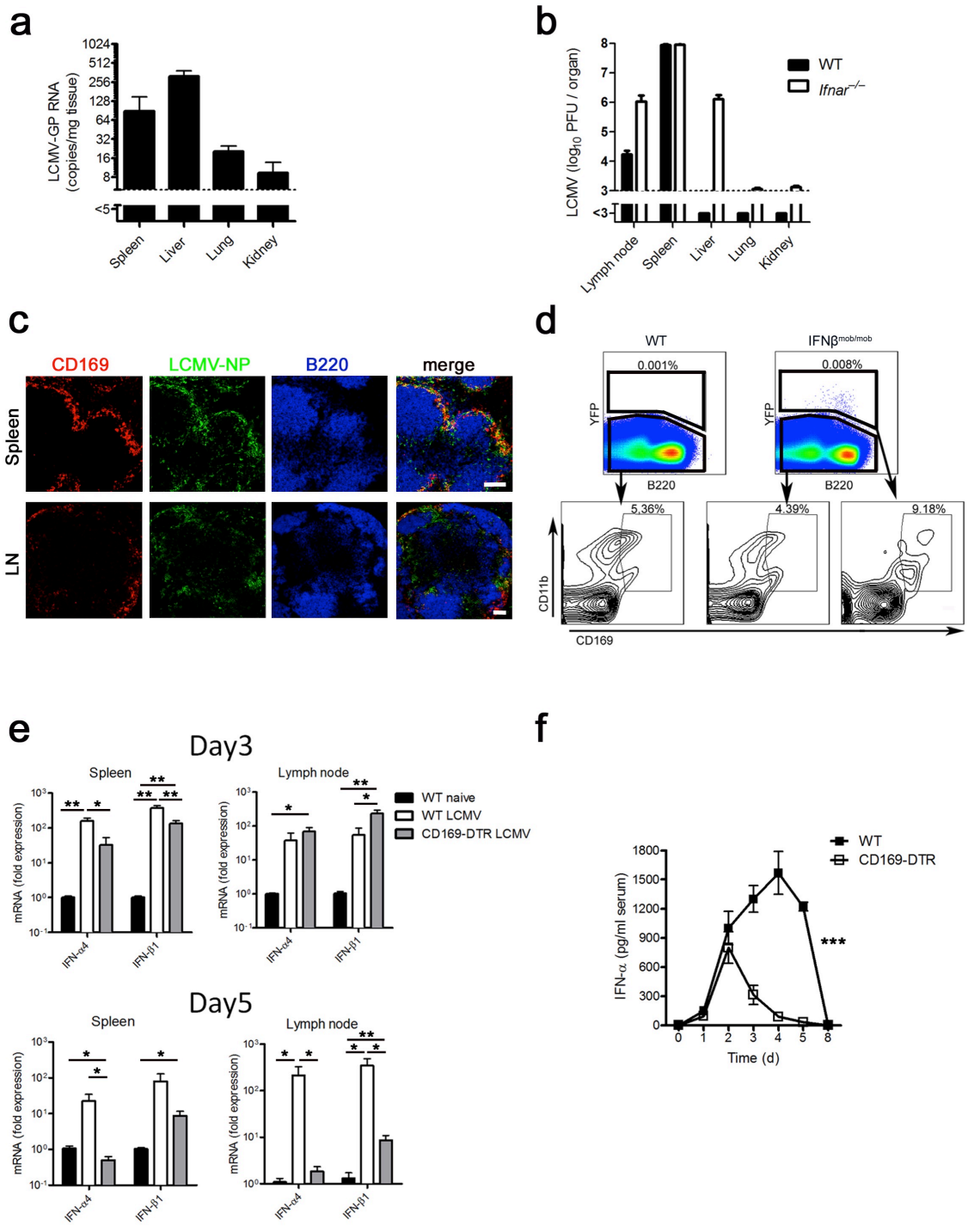


Figure 2.2: CD169^{+} macrophages in the spleen and lymph nodes contribute to the production of IFN-I.

Figure 3

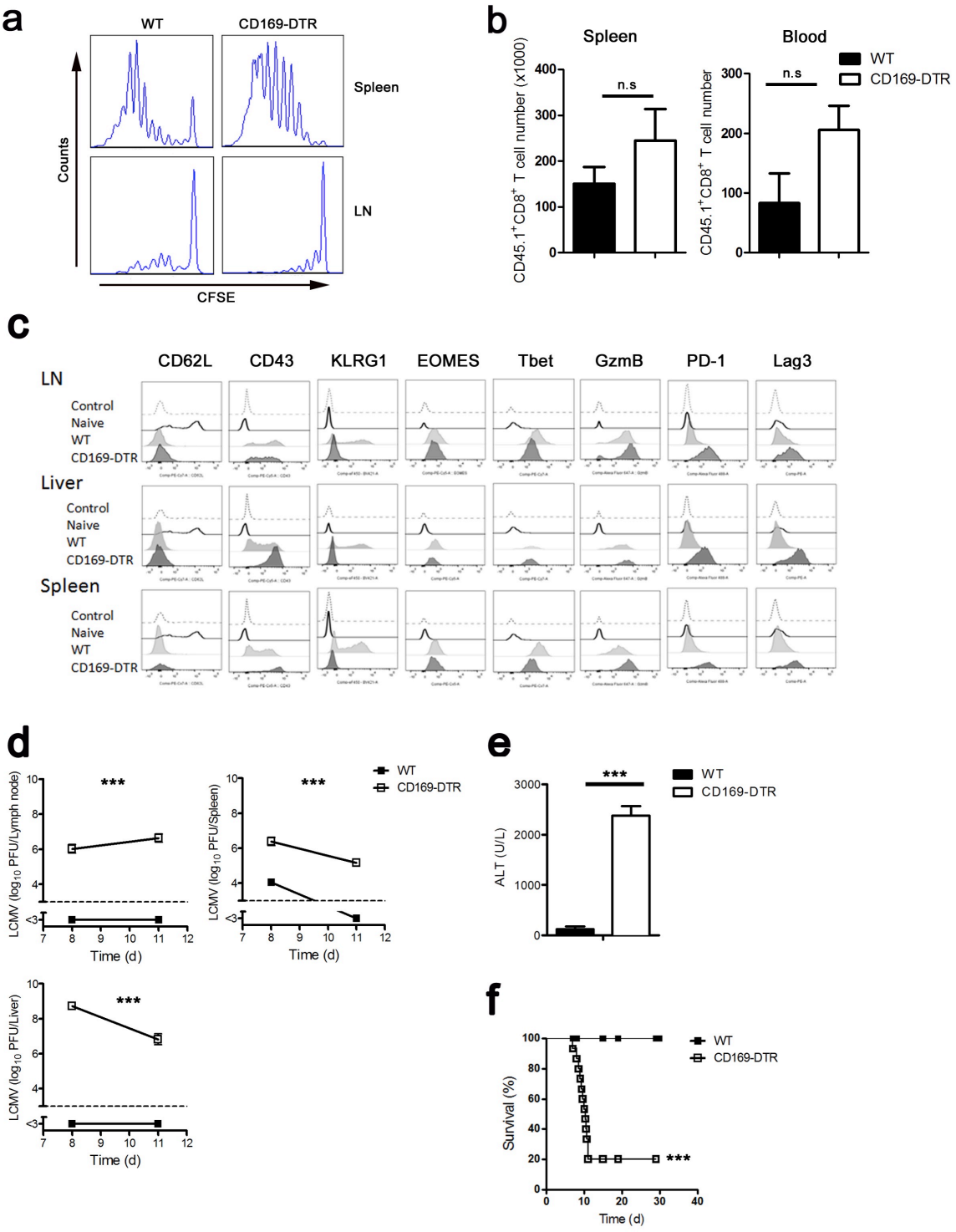


Figure 2.3: CD169^{+} macrophages have limited impact on CD8+ T cell priming but are essential for controlling acute viral infection and prevention immunopathology.

Figure 4

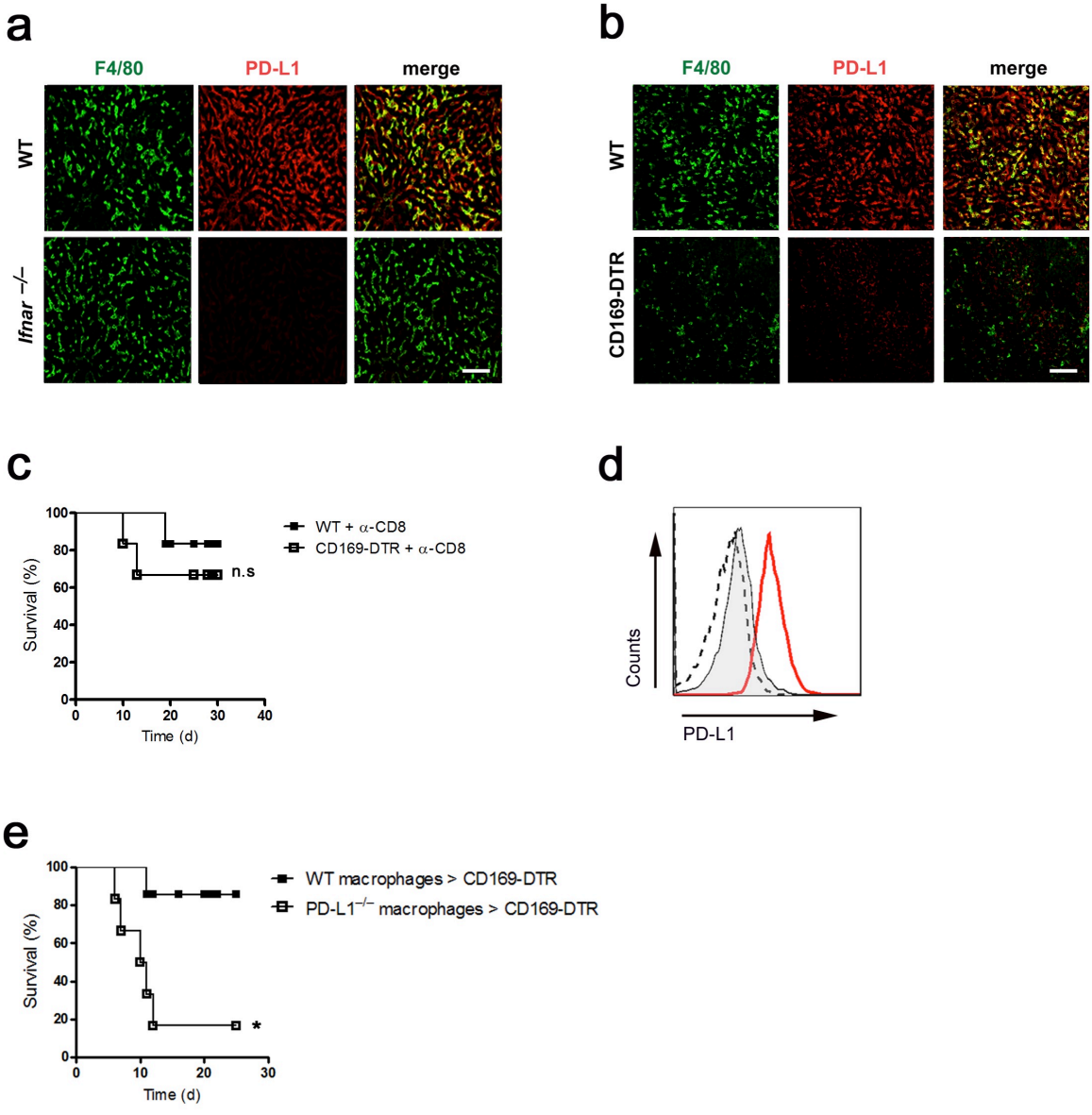


Figure 2.4: CD169^{+} macrophages induce PD-L1 expression which inhibits immunopathology.

Figure 5

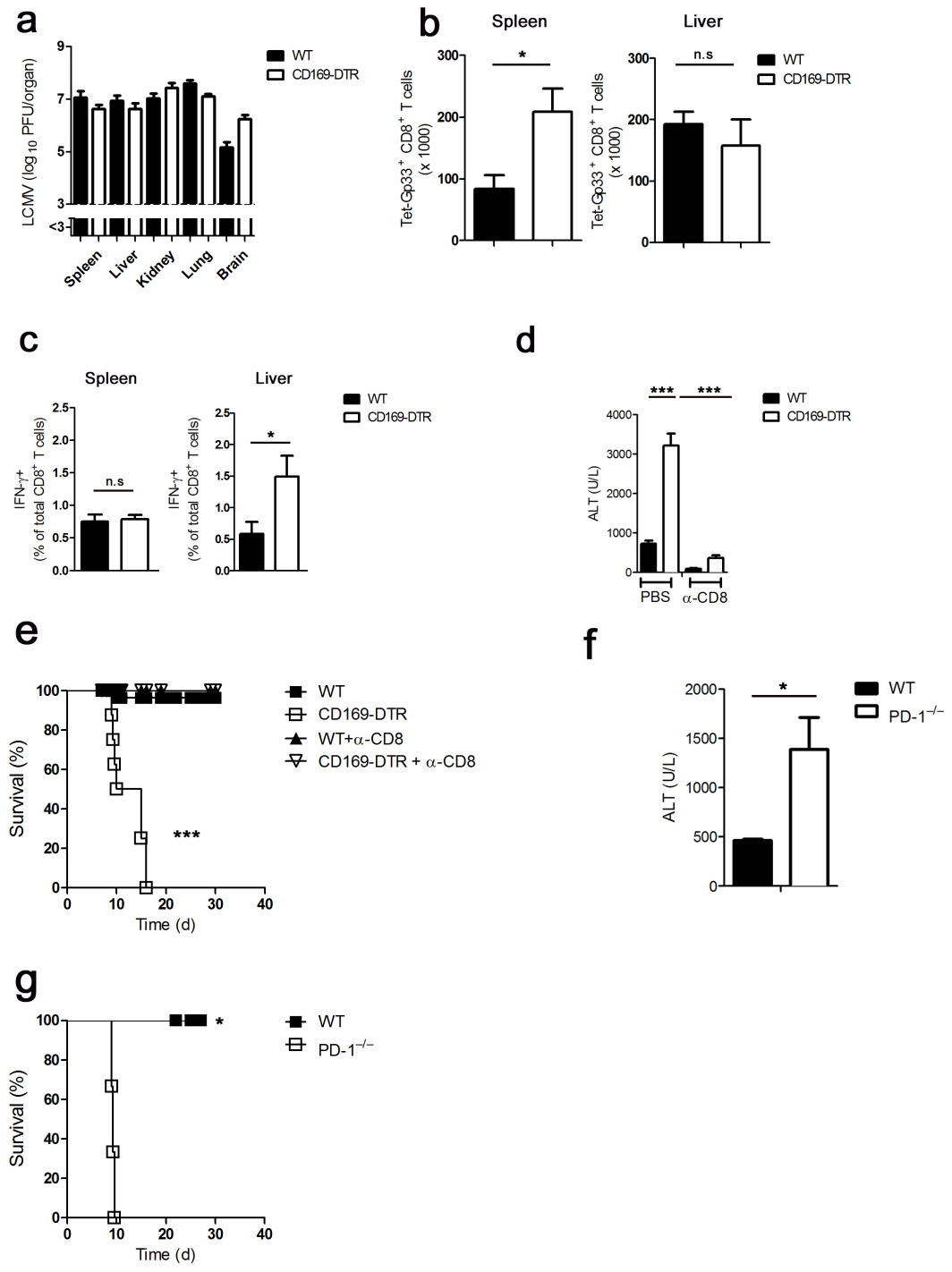
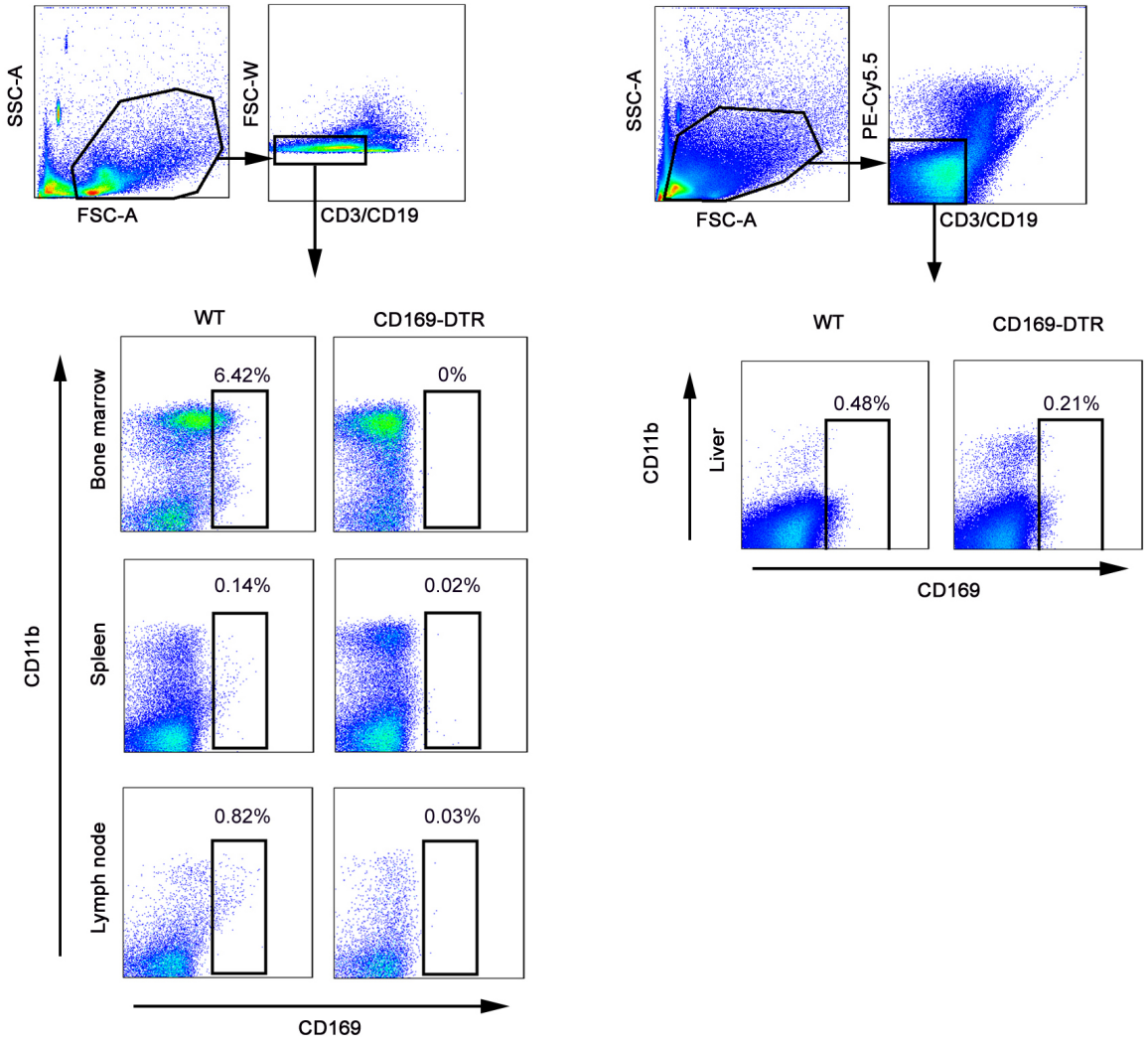


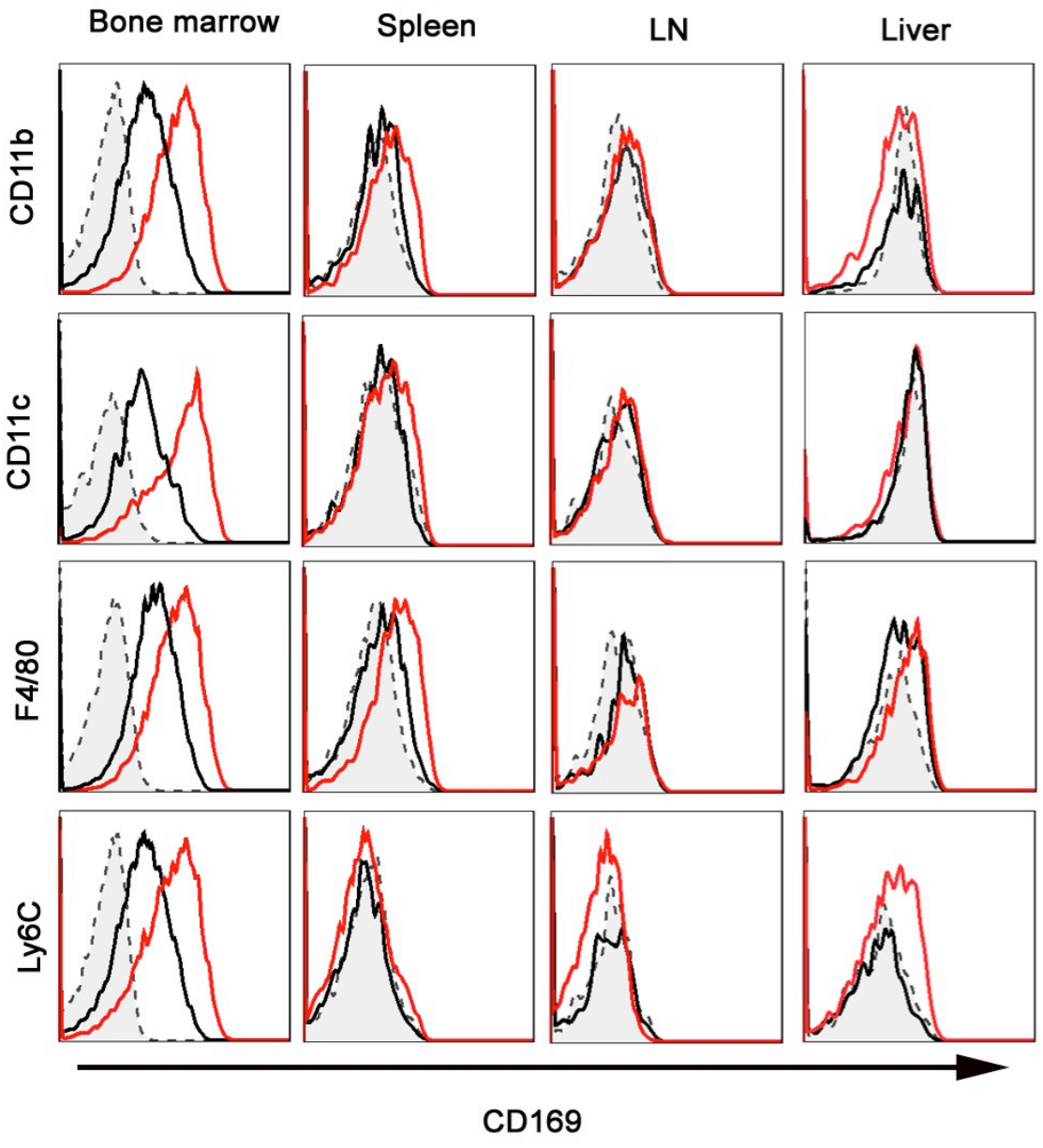
Figure 2.5: CD169^{+} macrophages prevent severe immunopathology during chronic viral infection.

Supplementary Figure 1



Supplementary Figure 2.1: Gating strategy of CD169^{+} macrophages.

Supplementary Figure 2



Supplementary Figure 2.2: Expression of CD169 on immune cells.

Article Statement

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$CD169^+$ macrophages regulate PD-L1 expression via type I interferon and thereby prevent severe immunopathology after LCMV infection

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  - Material and Methods: Writing part of materials and methods with Shaabani N.
  - Results: Planing of the experiments and execution with Shaabani N, Khairnar V, Gassa A, Ferrer-Tur R, Zelinskyy G, Scheu S and Honke N.

- Discussion: Part of discussion writing and critical reviewing with Shaabani N, Khairnar V, Häussinger D, Recher M, Zelinskyy G, Liu J, Dittmer U, Trilling M, Scheu S, Hardt C, Lang PA, Honke N and Lang KS.

- Results:
  - Fig. 1: E; D and F with Shaabani N.
  - Fig. 2: C and D with Shaabani N; E and F.
  - Fig. 3: A, B, D and E.
  - Fig. 4: A, B and C.
  - Fig. 5: D and E.

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Vikas Duhan **Prof. Dr. Karl S. Lang**

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Virus-specific antibodies allow viral replication in the marginal zone, thereby promoting CD8$^+$ T-cell priming and viral control

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Clinically used human vaccination aims to induce specific antibodies that can guarantee long-term protection against a pathogen. The reasons that other immune components often fail to induce protective immunity are still debated. Recently we found that enforced viral replication in secondary lymphoid organs is essential for immune activation. In this study we used the lymphocytic choriomeningitis virus (LCMV) to determine whether enforced virus replication occurs in the presence of virus-specific antibodies or virus-specific CD8$^+$ T cells. We found that after systemic recall infection with LCMV-WE the presence of virus-specific antibodies allowed intracellular replication of virus in the marginal zone of spleen. In contrast, specific antibodies limited viral replication in liver, lung, and kidney. Upon recall infection with the persistent virus strain LCMV-Docile, viral replication in spleen was essential for the priming of CD8$^+$ T cells and for viral control. In contrast to specific antibodies, memory CD8$^+$ T cells inhibited viral replication in marginal zone but failed to protect mice from persistent viral infection. We conclude that virus-specific antibodies limit viral infection in peripheral organs but still allow replication of LCMV in the marginal zone, a mechanism that allows immune boosting during recall infection and thereby guarantees control of persistent virus.

Memory formation after antigen challenge is one of the most important hallmarks of the adaptive immune system[1]; it protects the host from exposure to the original or a slightly modified pathogen[1]. Because of this known memory formation, vaccination with attenuated pathogens has been an important tool for preventing outbreaks of severe pathogen-mediated diseases. In the Western world, the World Health Organization recommends approximately 16 vaccinations[2], 10 of which are antiviral.

Although virus-specific CD8$^+$ T cells are known to contribute to the control of viral infections, all recommended vaccinations are aimed at inducing antibodies against a pathogen[3–7]. For example, newly designed vaccines against HIV are intended to specifically activate HIV-specific CD8$^+$ T cells[8]. However, to date, CD8$^+$ T cell–mediated vaccines have failed to protect the host from persistent infection[9]. Therefore, the role of vaccine-induced virus-specific CD8$^+$ T cells in long-term protection is still being debated[10–12]. To know in more

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Chapter 3

Virus-specific antibodies allow viral replication in the marginal zone, thereby promoting $CD8^+$ T-cell priming and viral control

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3.1 Abstract

Clinically used human vaccination aims to induce specific antibodies that can guarantee long term protection against a pathogen. The reasons that other immune components often fail to induce protective immunity are still debated. Recently we found that enforced viral replication in secondary lymphoid organs is essential for immune activation. In this study we used the lymphocytic choriomeningitis virus (LCMV) to determine whether enforced virus replication occurs in the presence of virus-specific antibodies or virus-specific $CD8^+$ T cells. We found that after systemic recall infection with LCMV-WE the presence of virus-specific antibodies allowed intracellular replication of virus in the marginal zone of spleen. In contrast, specific antibodies limited viral replication in liver, lung, and kidney. Upon recall infection with the persistent virus strain LCMV-Docile, viral replication in spleen was essential for the priming of $CD8^+$ T cells and for viral control. In contrast to specific antibodies, memory $CD8^+$ T cells inhibited viral replication in marginal zone but failed to protect mice from persistent viral infection. We conclude that virus-specific antibodies limit viral infection in peripheral organs but still allow replication of LCMV in the marginal zone, a mechanism that allows immune boosting during recall infection and thereby guarantees control of persistent virus.

3.2 Introduction

Memory formation after antigen challenge is one of the most important hallmarks of the adaptive immune system[125]; it protects the host from exposure to the original or a slightly modified pathogen[125]. Because of this known memory formation, vaccination with attenuated pathogens has been an important tool for preventing outbreaks of severe pathogen-mediated diseases. In the Western world, the World Health Organization recommends approximately 16 vaccinations[126], 10 of which are antiviral.

Although virus-specific $CD8^+$ T cells are known to contribute to the control of viral infections, all recommended vaccinations are aimed at inducing antibodies against a pathogen[127-131]. For example, newly designed vaccines against HIV are intended to specifically activate HIV-specific $CD8^+$ T cells[132]. However, to date, $CD8^+$ T cell–mediated vaccines have failed to protect the host from persistent infection[133]. Therefore, the role of vaccine-induced virus-specific $CD8^+$ T cells in long-term protection is still being debated[134-136]. To know in more detail why several vaccines produce protective antibodies but vaccines against HIV and HCV could not do so far. The mechanistic understanding may help to generate new vaccines in future.

Lymphocytic choriomeningitis virus (LCMV) is a non-cytopathic virus with the ability to persist. The acute strain LCMV-WE is usually controlled within 1 or 2 weeks, primarily by virus-specific $CD8^+$ T cells. The functions of B cells against LCMV are important for long-term control of the virus; however, $CD8^+$ T cells are necessary for early control of LCMV. Infection with the LCMV-Docile strain leads to exhaustion of $CD8^+$ T cells and therefore to persistence of the virus in the host[119].

Recently we found that antigen-presenting cells ($CD169^+$ macrophages and $CD11c^+$ dendritic cells) within the marginal zone specifically allow viral replication[137]. Enforced viral replication in the spleen is essential for activating the innate and adaptive immune systems[138]. It is still unknown whether enforced viral replication occurs after vaccination or after secondary infection and whether such replication is involved in immune boosting.

In the study reported here we found that, after systemic recall, infection-specific antibodies allow intracellular replication of the virus in the marginal zone of the spleen but limit the replication of infectious virus in liver, lungs, and kidneys. Upon recall infection with the persistent virus strain LCMV-Docile, spleen-specific viral replication is associated with sufficient priming of $CD8^+$ T cells and with viral control. In contrast to specific antibodies,

memory $CD8^+$ T cells inhibit viral replication in the marginal zone thus fail to protect mice against persistent infection.

3.3 Results

3.3.1 Replication of LCMV in the marginal zone is associated with immune activation and viral control

During primary viral infection, LCMV replicates in the marginal zone; this replication is essential for inducing adaptive immunity against the virus[138]. Histologic examination of the spleen on day 3 after infection with 2×10^4 plaque-forming units (PFU) of the acute strain LCMV-WE detected staining of LCMV along the marginal zone **(Fig. 1A)**. This finding was associated with the induction of virus-specific $CD8^+$ T cells **(Fig. 1B)** and the induction of LCMV-specific antibodies **(Fig. 1C);** these activities resulted in control of the virus within 8 days **(Fig. 1D).** For early control of the virus, virus-specific $CD8^+$ T cells are essential, as demonstrated by our finding that $B2m^{-/-}$ mice, which lack $CD8^+$ T cells, could not control the virus in the circulation **(Fig 1E).** $Jh^{-/-}$ mice, which are deficient in B cells, controlled the virus in a manner similar to that of wild-type mice **(Fig 1E).**; this finding emphasizes that early control of LCMV-WE depends primarily on virus-specific $CD8^+$ T cells. Therefore, we conclude that enforced viral replication leads to the priming of $CD8^+$ T cells, which are necessary for early viral control, whereas B cells are most likely needed for long-term protection against LCMV[139,140].

3.3.2 Virus-specific antibodies, but not virus-specific $CD8^+$ T cells, allow viral replication in the marginal zone

Recall viral infections often boost the existing immune response[141]. Whether an immune-response boost after a recall infection with LCMV requires viral replication and whether adaptive memory components allow replication of virus in the marginal zone remain unknown. To gain insights into this question we first infected mice with 200 PFU of LCMV-WE and then 50 days later challenged them with 2×10^7 PFU of LCMV-WE. We could not detect any replication of virus within the marginal zone after recall infection **(Fig. 2A).** In line with this finding, no infectious virus was detected in any organ tested **(Fig. 2B)**. These findings suggest that memory mice are well protected against LCMV recall infection.

Next we aimed to determine how various specific memory immune components limit viral replication in the marginal zone and peripheral organs. We infected wild-type (WT) mice with 2×10^6 PFU of LCMV-WE, and after 80 to 120 days of infection we transferred

various memory components from these infected mice into naïve WT mice. For control mice we transferred immune components from naïve mice to naïve mice. We focused on the transfer of serum for virus-specific antibodies, sorted splenic B cells, splenic $CD8^+$ T cells, and splenic $CD4^+$ T cells. For each memory component we transferred approximately 20% of the specific compartment of an LCMV-infected mouse (see Material and Methods). Two days after transfer we challenged mice with 2×10^6 PFU of LCMV-WE, and at days 1, 2, and 3 we analyzed viral distribution. Mice that received naïve immune components exhibited normal staining of LCMV in the marginal zone **(Fig. 2C, supplementary Figure 1A)**. Both virus-specific $CD8^+$ T cells and antibodies allowed replication of virus at day 1 **(Supplementary Figure 1A and 1B)**. After day 1, virus-specific $CD8^+$ T cells inhibited the replication of virus in the marginal zone **(Fig. 2C, Supplementary Figure 1A)**. Perforin deficient LCMV-specific $CD8^+$ T cells which were primed with recombinant LCMV (rLCMV) (see material and methods) did not effect the virus replication in marginal zone **(Supplementary Figure 1C)** suggesting that direct cytotoxicity of virus-specific $CD8^+$ T cells mediated by perforin killed virus-infected antigen-presenting cells in the marginal zone **(Supplementary Figure 1C)**. Transfer of virus-specific antibodies slightly reduced LCMV staining in the marginal zone but still allowed abundant replication at any time tested **(Fig. 2C, Supplementary Figure 1A)**. Transfer of memory $CD4^+$ T cells or memory B cells exerted no measurable influence on the replication of LCMV in the marginal zone **(Supplementary Fig. 2A)**.

Next we analyzed the role of virus-specific $CD8^+$ T cells and virus-specific antibodies on the early distribution of virus in other organs. Virus-specific $CD8^+$ T cells reduced infectious virus in the spleen alone after day 1 (**Fig. 2D and Supplementary Figure 1B**). Interestingly, although we found limited staining of virus-infected cells in the marginal zone, the levels of infectious LCMV were still easily detectable. This is probably due to the fact that virus-specific $CD8^+$ T cells target virus-bearing cells rather than free infectious virus. In peripheral organs, virus-specific $CD8^+$ T cells exerted only a limited effect on viral replication **(Fig. 2D and Supplementary Figure 1B)**. This finding suggests that virus-specific $CD8^+$ T cells exert limited influence on the early replication of virus in the spleen, lymph nodes, liver, and lungs.

Like virus-specific $CD8^+$ T cells, virus-specific antibodies reduced the amount of infectious virus in the spleen and lymph nodes but still allowed replication of virus (**Fig. 2D, Supplementary Figure 1B**). However, unlike virus-specific $CD8^+$ T cells, virus-specific antibodies completely blunted the replication of virus in all peripheral organs tested **(Fig. 2D, Supplementary Figure 1B)**. Memory B cells and $CD4^+$ T cells exerted no significant effect

on the replication of virus in any of the organs tested **(Supplementary Figure 2B)**, a finding implying that memory B and $CD4^+$ T cells have no impact on the early distribution of virus. Therefore, we conclude that virus-specific antibodies allow the replication of virus in the splenic marginal zone but protect against the replication of virus in peripheral organs. Virus-specific $CD8^+$ T cells inhibit the replication of virus in the marginal zone but have limited impact on the replication of virus in peripheral organs.

Next we determined whether antigen-specific $CD8^+$ T cells that were primed with *Listeria monocytogenes* behaved in the same manner as transferred virus-specific $CD8^+$ T cells. We infected WT mice with *L. monocytogenes* expressing the glycoprotein of LCMV (LM-GP33) or with wild-type *L. monocytogenes* (LM-WT). Mice infected with LM-GP33 generated LCMV GP33-specific $CD8^+$ T cells (**Fig. 3A and 3B**). After 30 days the mice were infected with LCMV-WE. Control mice infected with LM-WT exhibited normal replication of virus in the marginal zone (**Fig. 3C**). In contrast, mice challenged with LM-GP33 did not exhibit viral staining in the marginal zone (**Fig. 3C**), a finding indicating inhibition of virus in the marginal zone by virus-specific $CD8^+$ T cells. Virus-specific $CD8^+$ T cells generated after LM-GP33 infection reduced the replication of infectious virus in lymph nodes and lungs; however, they did not influence the replication of virus in the liver (**Fig. 3D**).

Therefore, we conclude that virus-specific antibodies allow viral replication in the marginal zone but suppress viral replication in other organs. Virus-specific $CD8^+$ T cells suppress viral replication in the marginal zone but have limited influence on viral replication in peripheral organs.

3.3.3 Virus-specific antibodies allow innate and adaptive immune activation

We found that virus-specific $CD8^+$ T cells and virus-specific antibodies exert different effects on early viral distribution. Next we examined how differences in viral replication affect innate and adaptive immune activation. We transferred virus-specific antibodies or $CD8^+$ T cells from memory mice into naïve C57BL/6 mice and infected them with LCMV-WE. Virus-specific $CD8^+$ T cells strongly reduced the induction of antiviral interferon type I (IFN-I) (**Fig. 4A**). The presence of virus-specific antibodies also reduced the IFN-I response (**Fig. 4A**) but to a lower extent than did virus-specific $CD8^+$ T cells (**Fig. 4A**).

Next we examined the antiviral $CD8^+$ T cell response after LCMV infection. Transfer of virus-specific $CD8^+$ T cells before infection exerted no effect on the total number of virus-specific $CD8^+$ T cells at day 10 after challenge with LCMV-WE (**Fig. 4B** upper panel, **4C**).

Non-transferred endogenous virus-specific $CD8^+$ T cells exhibited reduced activation in the presence of memory $CD8^+$ T cells (**Supplementary Figure 3**). In contrast, in the presence of virus-specific antibodies the expansion of virus-specific $CD8^+$ T cells was greater than that in mice receiving virus-specific $CD8^+$ T cells or in mice receiving non-specific immune components (**Fig. 4B** upper panel and **4C**). In addition, the production of IFN-γ after *in vitro* restimulation was enhanced in splenocytes derived from mice treated with virus-specific antibodies (**Fig. 4B** middle panel and **4D**). The total number of IFN-γ–producing $CD4^+$ T cells was also enhanced in the spleens of mice that had received virus-specific antibodies (Fig. **4B** lower panel and **4E**).

We conclude that virus-specific antibodies allow innate immune activation and exert a positive effect on the induction of virus-specific $CD8^+$ T cells. In contrast, the presence of virus-specific $CD8^+$ T cells is not beneficial for immune activation upon challenge infection.

3.3.4 Virus-specific antibodies protect against immunopathology and lead to control of virus

Next we investigated the influence of virus-specific $CD8^+$ T cells or virus-specific antibodies on overall outcome after infection with the persistent LCMV-Docile strain. Unlike LCMV-WE, LCMV-Docile induces persistent viral infection (**Supplementary Figure 4A**). We transferred virus-specific $CD8^+$ T cells and virus-specific antibodies to naïve mice and infected them with 2×10^4 PFU of LCMV-Docile. As was true of challenge infection with the acute LCMV-WE strain, the presence of virus-specific antibodies allowed viral replication, and virus-specific $CD8^+$ T cells almost inhibited viral replication in the splenic marginal zone (**Fig. 5A**). Unlike virus-specific $CD8^+$ T cells, virus-specific antibodies totally blunted viral load in the peripheral organs (**Supplementary Figure 4B**). Transfer of virus-specific antibodies enhanced the priming and expansion of virus-specific $CD8^+$ T cells (**Fig. 5B**). But transfer of virus-specific $CD8^+$ T cells before infection abrogated the expansion of antigen specific $CD8^+$ T cells (**Fig. 5B**). In line with these findings, our study showed that mice that received virus-specific antibodies before infection could eliminate LCMV-Docile, whereas it persisted in mice transferred with naïve immune components and in mice treated with virus-specific $CD8^+$ T cells (**Fig. 5C**). Virus-specific antibodies prevented elevation of serum alanine aminotransaminase (ALT) and lactate dehydrogenase (LDH) activity, which are signs of virus-induced immunopathology (**Fig. 5D**). Interestingly, the transfer of virus-specific $CD8^+$ T cells reduced immunopathology, a finding that is in line with reduced activation of $CD8^+$ T cells. Next we subjected mice that had been vaccinated with LM-GP33 to infection

with LCMV-Docile. As in our transfer experiments, LM-GP33–vaccinated mice could not eliminate LCMV-Docile (**Fig. 5E**).

Next we examined whether the priming of $CD8^+$ T cells in the presence of virus-specific antibodies was essential for the control of LCMV-Docile. We transferred virus-specific antibodies into $B2m^{-/-}$ mice, which lack $CD8^+$ T cells. In the absence of $CD8^+$ T cells, virus-specific antibodies did not control LCMV-Docile infection (**Fig. 5F**), a finding suggesting that secondary $CD8^+$ T-cell activation is essential for viral control after challenge infection.

3.3.5 Virus-specific antibodies enhance priming and expansion of $CD8^+$ T cells

We found that the priming and expansion of virus-specific $CD8^+$ T cells was highly improved in the presence of virus-specific antibodies. Virus-specific antibodies, which were used for the transferred experiments showed only slight neutralization capacity in in-vitro assay (**Fig. 6A**). To gain insights into the mechanism of action of virus-specific antibodies, we first determined whether these antibodies simply reduced the amount of free infectious virus immediately after infection. To do so, we examined $CD8^+$ T-cell activation after infection with various doses of LCMV-Docile with or without additional treatment with LCMV-specific antibodies (LCMV-Ab). Viral control was possible after infection with 200 PFU, 1000 PFU, or 20,000 PFU in the presence of virus-specific antibodies (**Fig. 6B**). In the absence of these antibodies, maximal $CD8^+$ T-cell priming was achieved with an infectious dose of 200 PFU LCMV-Docile (**Fig. 6C**, **D** and **E**). In the presence of virus-specific antibodies the priming of $CD8^+$ T cells was even higher than after infection of 200 PFU (**Fig. 6D** and **E**), a finding suggesting that additional mechanisms other than reducing the infectious dose contribute to enhancements in the priming of $CD8^+$ T cells.

3.3.6 Immune activation in the presence of virus-specific antibodies is essential for controlling persistent infection

We found that the transfer of virus-specific antibodies leads to strong priming of $CD8^+$ T cells, which is associated with viral control and limited immunopathology. Recently we found that the IFN-I inhibitor *Usp18* is highly expressed in $CD169^+$ marginal zone macrophages and dendritic cells and is therefore crucial for viral replication in these cells reside in spleen and lymph nodes[137,138]. The absence of either marginal zone macrophages or *Usp18* limits viral replication, and this limitation is associated with the absence of antiviral innate or adaptive immune responses[137,138]. To determine whether *Usp18*-dependent viral

replication in marginal zone macrophages is also necessary for the activation of $CD8^+$ T cells after secondary antigen challenge, we next transferred virus-specific antibodies to WT and CD169-DTR mice, in which specific $CD169^+$ marginal zone macrophages can be depleted by injecting diphtheria toxin, and infected them with LCMV-Docile. In the absence of marginal zone macrophages, virus-specific $CD8^+$ T-cell expansion (**Fig. 7A**) and IFN-γ production by $CD8^+$ T cells (**Fig. 7B**) and $CD4^+$ T cells (**Fig. 7C**) were reduced. This lack of a sufficient virus-specific $CD8^+$ T-cell response leads to viral persistence (**Fig. 7D**).

Furthermore, to examine the role of *Usp18* we transferred virus-specific antibodies to WT and *Usp18*$^{-/-}$ mice and infected the mice with LCMV-Docile. In absence of *Usp18*, viral replication was impaired in splenic marginal zone macrophages (**Fig. 8A** and **supplementary Figure 5**). The absence of *Usp18* limited the expansion of virus-specific $CD8^+$ T cells (**Fig. 8B**) and reduced IFN-γ production by $CD8^+$ T cells (**Fig. 8C**) and $CD4^+$ T cells (**Fig. 8D**). The lack of innate and adaptive immune activation in the absence of *Usp18* was associated with a problem in viral clearance (**Fig. 8E**). This finding suggests that, in the presence of virus-specific antibodies, *Usp18* is necessary for viral replication in marginal zone macrophages and also enhances the priming of virus-specific $CD8^+$ T cells.

3.4 Discussion

In the study reported here, we found that virus-specific antibodies limit the quantity of infectious virus in peripheral organs but still allow viral replication in the marginal zone. This specific distribution of virus after challenge with infection is beneficial for innate and adaptive immune activation; it limits immunopathology and leads to viral control.

Currently the World Health Organization (WHO) recommends 10 antiviral vaccinations, which clearly protect the vaccinated host from infection with the live pathogen. Natural viral infection induces plasma cells, which produce virus-specific antibodies[142]. Although specific antibodies mainly target infectious virus particles, virus-specific $CD8^+$ T cells can directly suppress viral replication in infected cells[143]. Currently available vaccinations induce measurable antibodies against the pathogen but often fail to induce virus-specific $CD8^+$ T cells[144]. Some reports suggest that, after secondary infection or vaccination, contact with the virus will further activate the immune system, which then induces protective T-cell immunity[145-147]. Indeed, in the Friend virus model, antibodies can enhance the virus-specific $CD8^+$ T-cell response[148]. The results of our study show that specific antibodies block viral replication within peripheral organs within the first days after infection but still allow

enforced viral replication in lymphoid organs. We found that the presence of antibodies limited viral replication in the liver and induced more-efficient antiviral CD8 T cells. The organ-specific antiviral capacity of virus-specific antibodies may be due to differential expression of Fc receptors in different organs. One way to induce such antiviral mechanisms could be via Fc receptor III (CD16), which either tracks virus into various vesicular compartments or induces antiviral activity[149]. In addition, complement may track virus into a separate compartment[150]. Further studies remain to be done to analyse the mechanism of Fc receptors on antiviral activity in macrophages. The production of antiviral cytokines and virus-specific $CD8^+$ T cells could be induced and could lead to rapid control of recall infection. Therefore, we suggest that antibodies are, at least for some viruses, much more potent memory components than are $CD8^+$ T cells.

Immunological memory against hepatitis C virus (HCV) is a challenge to the immune system, and the generation of vaccines against HCV has failed to date[151]. One reason for this failure is that the virus can mutate quickly during infection; when this happens, the existing immunological memory is no longer protective[152]. On the basis of our findings, we suggest that a good vaccine should still allow some viral replication in certain secondary lymphoid niches but should also inhibit the spread of virus to the susceptible organ. We suggest that, because HCV replication in antigen-presenting cells is very limited or almost absent, it is probably impossible for $CD8^+$ T cells to be primed in the presence of virus-specific antibodies. This hypothesis may at least partially explain the failure of HCV vaccines. Recently it was shown that preexposure to HCV antigen induces the production of $CD8^+$ T cells, which suppress the immune response after viremic infection with HCV[153]. Although the authors of that study explained this phenomenon by the presence of regulatory T cells, it is possible that rapid inhibition of viral replication may limit the draining of HCV antigen to secondary lymphoid organs and can thereby limit the induction of a protective antiviral immune response.

Of course, we may question whether the mechanisms we found for LCMV are also relevant to HIV. HIV-specific antibodies do not induce protective immunity[154], and cellular immunity does not lead to a protective immune response upon challenge infection[155]. The fact that HIV induces marginal zone atrophy and marginal zone lymphoma[156,157] and the fact that $CD4^+$ T cells can be activated in the marginal zone and are the main target cells of HIV infection[137] suggest that the marginal zone is a niche in which activated $CD4^+$ T cells are easily infected. In light of our findings, we suggest that allowing HIV replication in the

marginal zone of the spleen may in this special case be a disadvantage, because activated $CD4^+$ T cells are located mainly within the marginal zone. Although this hypothesis could explain the difficulties in generating an HIV vaccine[154], the generation of more data from HIV animal models is necessary before we can draw conclusions about the relationship between antibodies, $CD8^+$ T cells, and HIV replication in the marginal zone.

In conclusion, we found that specific antibodies are much more potent than $CD8^+$ T cells in protecting mice against viruses that are prone to persistence because these antibodies blunt the replication of virus in peripheral organs but allow replication of virus in the marginal zone, thereby leading to effective immune priming.

3.5 Methods

3.5.1 Mice

CD169-DTR, $Jh^{-/-}$, $Prf1^{-/-}$, and $B2m^{-/-}$ mice were maintained on a C57BL/6 background. CD45.1 congenic mice were used as wild type mice to track the cells. $Usp18^{-/-}$ mice were maintained on a mixed background, and mice were directly compared to littermate controls. KL25 mice, which express the immunoglobulin heavy chain of LCMV-neutralizing antibodies were maintained on a CD45.1 background and serum of LCMV infected KL25 mice was used as positive control for in vitro virus neutralization assay. This study was approved by the Nordrhein Westfalen Landesamt für Natur, Umwelt und Verbraucherschutz (Recklinghausen, Germany) and carried out in accordance with the German law for animal protection. All the experimental protocols were approved by the Nordrhein Westfalen Landesamt für Natur, Umwelt und Verbraucherschutz (Recklinghausen, Germany) or with the institutional guidelines of the Ontario Cancer Institute of the University Health Network and at McGill University.

3.5.2 Pathogens and plaque assays

The LCMV-WE and LCMV-Docile strains were originally obtained from F. Lehmann-Grube (Heinrich Pette Institute, Hamburg, Germany) and were propagated on L929 cells, MC57 cells, or both. Mice were infected intravenously with various doses of LCMV. LCMV viral titers were detected by plaque-forming assays on MC57 fibroblasts, as previously described[158]. A replication-deficient recombinant LCMV (rLCMV; kindly provided by Pinschewer) that express the mutated form of LCMV-GP but still has antigenic properties was produced according to standard protocols[90] and was injected intravenously into mice. The recombinant Listeria monocytogenes expressing the epitope of glycoprotein 33–41 of LCMV

(LM-GP33) and wild-type Listeria monocytogenes (LM-WT) were grown in brain-heart infusion medium diluted in phosphate buffered saline (PBS) and were injected intravenously into mice.

3.5.3 Memory cells and immune serum isolation and transfer

Six- to 8-week-old C57BL/6 naïve mice were infected intravenously with 2×10^6 PFU of LCMV-WE. After 80 to 120 days of infection, immune components were isolated from these memory mice. LCMV-immune serum was collected and pooled from a group of mice, and virus-free serum was used to inject intravenously into mice for all experiments. $CD8^+$ T cells, $CD4^+$ T cells, and $B220^+$ cells were isolated from spleen of memory mice with magnetic-activated cell sorting (MACS) isolation kit, according to the manufacturer's protocol (Miltenyi Biotec, Germany). Serum and memory-cell transfers were performed 2 days before the infection. Mice were injected once intravenously with 300 µl of immune serum, 5×10^6 memory $CD8^+$ T cells, 1×10^7 memory $CD4^+$ T cells, or 1×10^7 memory B cells. $Prf1^{-/-}$ mice were infected with 2×10^5 PFU of rLCMV and for control group C57BL/6 naïve mice got 2×10^5 PFU of rLCMV infection. After 30 days spleens of these memory mice were used as donors for LCMV-specific memory $CD8^+$ T cells.

3.5.4 Histologic analysis

Histologic analyses of snap-frozen tissues were performed with mouse monoclonal antibodies to LCMV nucleoprotein (NP; made in house), CD169 (MCA884F; AbD Serotec, Germany), or CD45R/B220 (RA3-6B2; eBioscience, Germany). Red pulp macrophages were stained with F4/80 (BM8; eBiosciences).

3.5.5 Enzyme-linked immunosorbent assays

Interferon-alpha (IFN-α) enzyme-linked immunosorbent assays (ELISA) were performed according to the manufacturer's protocol (PBL Interferon Science, Germany).

3.5.6 Flow cytometry

The Tetramer Facility of National Institutes of Health (NIH) provided LCMV-GP33 tetramer. Cells were stained with allophycocyanin (APC)-labeled GP33 MHC class I tetramer (GP33/H-2Db) for 15 minutes at 37 °C. After incubation, the samples were stained with anti-CD8 (clone 53–6.7; eBioscience) or anti-CD4 (clone GK1.5; eBioscience) antibodies for 30 minutes at 4 °C. Absolute numbers of GP33-specific $CD8^+$ T cells were calculated with fluorescent beads (BD Biosciences) by using fluorescence-activated cell sorting (FACS). For

measurement of intracellular IFN-γ, cells were fixed with 2% formaldehyde in PBS for 10 minutes, permeabilized with 1% saponin in FACS buffer at room temperature, and stained with anti–IFN-γ antibody for 30 minutes at 4 °C (clone XMG1.2; eBioscience). All stained cells were analysed with a FACS Fortessa (BD Biosciences) flow cytometer, and data were analysed with FlowJo software.

3.5.7 ALT and LDH measurement

The activity of ALT and LDH was measured in the Central Laboratory, University Hospital Essen, Germany.

3.5.8 LCMV neutralization assay

The neutralizing capability of serum was measured with plaque-forming assays according to a previously published protocol[158].

3.5.9 Statistical analysis

Data are expressed as means ± SEM. Student's t-test was used to detect statistically significant differences between groups. Significant differences between several groups were detected by one-way analysis of variance (ANOVA) with the Bonferroni or Dunnett post hoc test. The level of statistical significance was set at $P < 0.05$.

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3.7 Author Contributions

V.D. and V.K. designed and executed the experiments, evaluated the data; and wrote the manuscript. S.K.F., F.Z., A.G., N.H., N.S. and N.G. helped with the execution of experiments and were involved in data analysis. L.B. and C.K. were involved in data discussion. U.D., D.H., M.R. and C.H. discussed the data and outlined the manuscript. P.A.L. wrote and

corrected the paper. K.S.L. initiated and structured the study and wrote and completed the manuscript.

3.8 Figure Legend

3.8.1 Figure 1: Replication of lymphocytic choriomeningitis virus (LCMV) in the marginal zone is associated with immune activation and viral control

C57BL/6 mice were infected intravenously with 2×10^4 plaque-forming units (PFU) of LCMV strain WE (LCMV-WE) and were analysed for various parameters. (**A**) Representative immunofluorescence of spleen after 3 days of infection, stained for LCMV nucleoprotein (red), marginal zone macrophages (CD169, green), and red pulp macrophages (F4/80, blue). One slide representative of 6 slides is shown. Scale bar, 200 μm. (**B**) Total number of LCMV-specific T cells in the blood that were positive for the MHC class I tetramer of the glycoprotein of LCMV (Tet-GP33$^+$) and for CD8 (CD8$^+$), as measured by fluorescence-activated cell sorting (FACS) at the indicated days after infection (n = 3–7). (**C**) LCMV GP-specific antibodies in serum were analysed by enzyme-linked immunosorbent assay (ELISA) on various days after infection (n = 4). (**D**) Viral titers from spleen and inguinal lymph nodes were analysed by plaque-forming assay at the indicated time points after infection ($n = 3$). (**E**) Viral titers in serum of wild type (WT), $B2m^{-/-}$, and $Jh^{-}/^{-}$ mice on various days after infection ($n = 4$). Horizontal dotted lines designate the detection limit. Data are shown as mean ± SEM.

3.8.2 Figure 2: Virus-specific antibodies, but not virus-specific CD8+ T cells, allow viral replication in the marginal zone

(A) Naïve (non-memory) and memory C57BL/6 mice were infected intravenously with 2×10^7 plaque-forming units (PFU) of lymphocytic choriomeningitis virus strain WE (LCMV-WE). After day 1 spleen sections were stained for LCMV nucleoprotein (red), marginal zone macrophages (CD169, green), and red pulp macrophages (F4/80, blue). One slide representative of 4 slides is shown. Scale bar, 100 μm. (B) Viral titers from spleen, inguinal lymph nodes (LN), liver, and lungs of naïve and memory C57BL/6 mice infected intravenously with 2×10^7 PFU of LCMV-WE, as measured on day 1 (n = 4–6). (C, D) C57BL/6 naïve mice were injected separately with naïve CD8$^+$ T cells and non-specific antibodies (naïve serum) collected from naïve mice, and with LCMV-specific CD8$^+$ T cells and LCMV-specific antibodies (immune serum) collected from memory mice. After 2 days all mice were infected with 2×10^6 PFU of LCMV-WE. (C) Representative

immunofluorescence of spleen after 3 days of viral infection, stained for LCMV nucleoprotein (red) and marginal zone macrophages (CD169, green). One slide representative of 6 slides is shown. Scale bar, 200 μm. (D) Viral titers from spleen, inguinal lymph node (LN), liver, and lungs after 3 days of viral infection (n = 6–7). Horizontal dotted lines designate the detection limit. Data are shown as mean ± SEM and are pooled from 2 or 3 independent experiments.

3.8.3 Figure 3: Inhibition of viral replication in splenic marginal zone of mice primed with recombinant Listeria monocytogenes expressing the glycoprotein of LCMV

C57BL/6 naïve mice were infected with 1×10^6 colony-forming units (CFU) of *Listeria monocytogenes* expressing the glycoprotein of lymphocytic choriomeningitis virus (LM-GP33), and control mice were infected with a lower dose (1×10^4 CFU) of wildtype *L. monocytogenes* (LM-WT) as higher dose is lethal for mice. After 30 days mice were injected with 2×10^6 PFU of LCMV-WE. (**A**) Fluorescence-activated cell sorting (FACS) plots showing the frequency of T cells that were positive for the MHC class I tetramer of the glycoprotein of LCMV (Tet-GP33$^+$) and for CD8 (CD8$^+$) in the total number of CD8$^+$ T cells in the blood at indicated days after LM-GP33 infection. (**B**) Graph showing the total number of Tet-GP33$^+$ CD8$^+$ T cells in blood on various days after LM-GP33 infection (n = 6). (**C**) Immunohistochemical analysis of spleens from LM-GP33–primed mice after 3 days of LCMV strain WE (LCMV-WE) infection, showing LCMV nucleoprotein (red) and marginal zone macrophages (CD169, green). Scale bar, 200 μm (n = 5). (**D**) Viral titers from spleen, inguinal LN, liver, and lungs after 3 days of LCMV-WE infection (n = 5). Horizontal dotted lines designate the detection limit. Data are shown as mean ± SEM and are pooled from 2 independent experiments.

3.8.4 Figure 4. Virus-specific antibodies allow innate and adaptive immune activation

C57BL/6 naïve mice were injected separately with naïve CD8$^+$ T cells and non-specific antibodies (naïve serum) collected from naïve mice, and with lymphocytic choriomeningitis (LCMV)-specific CD8$^+$ T cells and LCMV-specific antibodies (immune serum) collected from memory mice. After 2 days all mice were infected with 2×10^6 plaque-forming units (PFU) of LCMV strain WE (LCMV-WE). (**A**) Levels of interferon (IFN)-α were measured in the serum by enzyme-linked immunosorbent assay (ELISA) after 2 days of infection (n = 6).

(**B**) Representative fluorescence-activated cell sorting (FACS) plots showing the frequency of LCMV-specific T cells in the spleen that were positive for the MHC class I tetramer of the glycoprotein of LCMV (Tet-GP33$^+$) and for CD8 (CD8$^+$) in the total number of CD8$^+$ T cells in the spleen (upper plots). Frequency of IFN-γ$^+$ CD8$^+$ T cells (middle plots) and IFN-γ$^+$ CD4$^+$ T cells (lower plots) in the spleen after in vitro stimulation with LCMV GP33 and LCMV GP64 peptide respectively after 10 days of viral infection. (**C**) Total number of LCMV-specific Tet-GP33$^+$ CD8$^+$ T cells in the spleen after 10 days of viral infection (n = 4–6). (**D**) Total number of IFN-γ$^+$ CD8$^+$ T cells in the spleen after 10 days of viral infection and after in vitro stimulation with LCMV GP33 peptide for 5 hours (n = 4–6). (**E**) Total number of IFN-γ–producing CD4$^+$ T cells in the spleen after 10 days of viral infection and after in vitro stimulation with LCMV GP64 peptide for 5 hours (n = 3–6). Data are shown as mean ± SEM and are pooled from 2 or 3 independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001 (Student's t-test).

3.8.5 Figure 5. Virus-specific antibodies protect against immunopathology and lead to control of virus

C57BL/6 naïve mice were injected separately with naïve CD8$^+$ T cells and non-specific antibodies (naïve serum) collected from naïve mice, and with lymphocytic choriomeningitis virus (LCMV)-specific CD8$^+$ T cells and LCMV-specific antibodies (immune serum) collected from memory mice. After 2 days all mice were infected with 2 × 10^4 plaque forming units (PFU) of LCMV-Docile. (**A**) Representative immunofluorescence of spleen after 3 days of infection, stained for LCMV nucleoprotein (red) and marginal zone macrophages (CD169, green). One slide representative of 3 slides is shown. Scale bar, 200 μm. (**B**) Total number of LCMV-specific T cells in the spleen that were positive for the MHC class I tetramer of the glycoprotein of LCMV (Tet-GP33$^+$) and for CD8 (CD8$^+$) after 10 days of viral infection (n = 4–7). (**C**) Viral titers from spleen, inguinal LN, liver, kidney, and lungs after 10 days of viral infection (n = 7–10). (**D**) Levels of alanine aminotransaminase (ALT) and lactate dehydrogenase (LDH) in serum were measured after 10 days of viral infection (n = 7–10). (**E**) C57BL/6 mice primed with *Listeria monocytogenes* expressing the glycoprotein of lymphocytic choriomeningitis virus (LM-gp33) were infected with 2 × 10^6 PFU of LCMV-Docile. After 10 days viral titers were measured in various organs, as indicated (n = 5–8). (**F**) C57BL/6 and *B2m*$^{-/-}$ mice were treated with virus-specific antibodies or were left untreated. After 2 days all mice were infected with 2 × 10^4 PFU of LCMV-Docile. Viral titers from spleen, inguinal LN, liver, kidney, and lungs were measured after 10 days of viral infection (n

= 3–4). Horizontal dotted lines designate the detection limit. Data are shown as mean ± SEM and are pooled from 2 or 3 independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001 (Student's t-test).

3.8.6 Figure 6: Virus-specific antibodies enhance priming and expansion of $CD8^+$ T cells

(**A**) Graph showing the total neutralizing antibodies in naïve serum (WT, naive), serum from memory C57BL/6 mice (WT, LCMV) on day 100 and from KL25 mice (KL25, LCMV) on day 10 after infection with 2×10^6 PFU of LCMV-WE and determined by in vitro neutralization assay (n = 4–5). (**B–E**) Naïve C57BL/6 mice were injected with non-specific antibodies or LCMV-specific antibodies. Mice treated with non-specific antibodies were infected separately with 2×10^2, 1×10^3, 5×10^3, or 2×10^4 PFU of LCMV-Docile. Mice treated with LCMV-specific antibodies were infected with 2×10^4 PFU of LCMV-Docile. (**B**) Viral titers in various organs after 10 days of viral infection (n = 6). (**C**) FACS plots representing the frequency of LCMV-specific Tet-$GP33^+$ $CD8^+$ T cells in blood (upper plots) and spleen (middle plots) after 10 days of infection. Lower plots show the frequency of interferon (IFN)-γ^+ $CD8^+$ T cells in spleen after 10 days of viral infection and in vitro stimulation with LCMV GP33 peptide (n = 6–9). (**D**) Total number of LCMV-specific Tet-$GP33^+$ $CD8^+$ T cells in spleen after 10 days of viral infection (n = 6–9). (**E**) Total number of IFN-γ^+ $CD8^+$ T cells in spleen after in vitro stimulation with LCMV GP33 peptide on day 10 of infection (n = 6–9). Horizontal dotted lines designate the detection limit. Data are shown as mean ± SEM and are pooled from 2 or 3 independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001 (Student's t-test).

3.8.7 Figure 7: Immune activation in the presence of virus-specific antibodies is essential for controlling persistent viral infection

Naïve C57BL/6 mice and CD169-DTR mice were treated with diphtheria toxin (30 µg/kg) on day -3 and day 2 and were injected with lymphocytic choriomeningitis (LCMV)-specific antibodies on day -2. One group of C57BL/6 mice was injected with non-specific antibodies on day -2. All mice were infected with 2×10^4 plaque-forming units (PFU) of LCMV-Docile on day 0. Ten days later mice were evaluated for various parameters. (**A**) Total number of LCMV-specific T cells in spleen that were positive for the MHC class I tetramer of the glycoprotein of LCMV (Tet-$GP33^+$) and for CD8 ($CD8^+$) (n = 6). (**B**) Total number of interferon (IFN)-γ^+ $CD8^+$ T cells in spleen was determined after in vitro stimulation with or

without LCMV GP33 peptide for 5 hours (n = 6). (**C**) Total number of IFN-γ producing $CD4^+$ T cells in spleen after in vitro stimulation with or without LCMV GP64 peptide for 5 hours (n = 6). (**D**) Viral titers in spleen, inguinal lymph nodes (LN), liver, kidney, and lungs were measured after 10 days of viral infection (n = 6). Data are shown as mean ± SEM and are pooled from 2 independent experiments. *P < 0.05; **P < 0.01 and ***P < 0.001 (Student's t-test).

3.8.8 Figure 8: Immune activation in the presence of virus-specific antibodies is Usp18 dependent

Lymphocytic choriomeningitis virus (LCMV)-specific antibodies were injected into $Usp18^{-/-}$ mice and littermate control mice. Non-specific antibodies were injected into littermate control mice to form a control group. Mice were challenged with LCMV-Docile. (**A**) Immunohistochemical analysis of spleen showing LCMV nucleoprotein (red), marginal zone macrophages (CD169, green), and follicular B cells (B220, blue) after 1 day of infection with 2×10^6 plaque-forming units (PFU) of LCMV-Docile (n = 3). Scale bar, 200 μm. (**B–E**) Mice were infected with 2×10^4 PFU of LCMV-Docile and were evaluated for various parameters after 10 days of infection. (**B**) Total number of LCMV-specific T cells in the spleen that were positive for the MHC class I tetramer of the glycoprotein of LCMV (Tet-$GP33^+$) and for CD8 ($CD8^+$) (n = 5–8). (**C**) Total number of interferon (IFN)-γ^+ $CD8^+$ T cells in the spleen was determined after in vitro stimulation with or without LCMV GP33 peptide for 5 hours (n = 5–8). (**D**) Total number of IFN-γ producing $CD4^+$ T cells after in vitro stimulation with or without LCMV GP64 peptide for 5 hours in spleen (n = 5–8). (**E**) Viral titers from spleen, inguinal lymph nodes (LN), liver, kidneys, and lungs (n = 7–8). Data are shown as mean ± SEM and are pooled from 2 or 3 independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001 (Student's t-test).

3.9 Supplementary Figures

3.9.1 Figure 1: Virus-specific antibodies, but not virus-specific $CD8^+$ T cells, allow viral replication in the marginal zone

(**A, B**) C57BL/6 naïve mice were injected with naïve $CD8^+$ T cells, non-specific antibodies (naïve serum), lymphocytic choriomeningitis (LCMV)- specific $CD8^+$ T cells, or LCMV-specific antibodies (immune serum). After 2 days all mice were infected with 2×10^6 plaque-forming units (PFU) of LCMV strain WE (LCMV-WE). (**A**) Representative immunofluorescence of spleen is shown after day 1 and 2 of infection, stained for LCMV

nucleoprotein (red) and marginal zone macrophages (CD169, green). One slide representative of 3 slides is shown. (**B**) Viral titers from spleen, inguinal lymph nodes (LN), liver, and lungs after 1 or 2 days of viral infection (n = 3). (**C,D**) Memory $CD8^+$ T cells isolated from C57BL/6 memory and $prf1^{-/-}$ memory mice were injected to CD45.1 (wild-type; WT) naïve mice. One CD45.1 (wild-type; WT) group received naïve $CD8^+$ T cells as control. After 2 days mice were injected with 2×10^4 PFU of LCMV-WE. (**C**) Representative immunofluorescence of the spleen is shown after 3 days of infection, stained for LCMV nucleoprotein (red), marginal zone macrophages (CD169, green) and transferred $CD8^+$ T cells (CD45.2, blue). One slide representative of 3 experiments is shown. (**D**) Graph shows the viral titers in the spleen after 3 days of infection. Horizontal dotted lines designate the detection limit. Data are shown as mean ± SEM. *P < 0.05; **P < 0.01; ***P<0.001 (Student's t-test).

3.9.2 Figure 2: Memory $CD4^+$ T cells and memory B cells has no effect on viral replication in the marginal zone

C57BL/6 naïve mice were injected with memory $B220^+$ B cells and memory $CD4^+$ T cells isolated from memory mice. One group of mice was left untreated. After 2 days all mice were infected with 2×10^6 plaque-forming units (PFU) of lymphocytic choriomeningitis virus strain WE (LCMVWE). (**A**) Representative immunofluorescence in spleen after 3 days of infection, stained for LCMV nucleoprotein (red), marginal zone macrophages (CD169, green), and red pulp macrophages (F4/80, blue). One representative slide of 3 is shown. (**B**) Viral titers from various organs after 3 days of viral infection (n = 3). Horizontal dotted lines designate the detection limit. Data are shown as mean ± SEM.

3.9.3 Figure 3: Memory $CD8^+$ T cells reduce the expansion of endogenous $CD8^+$ T cells

CD45.1 (wild-type; WT) congenic naïve mice were injected with lymphocytic choriomeningitis virus (LCMV)-specific $CD8^+$ T cells isolated from C57BL/6 memory mice. One group of mice was injected with naïve $CD8^+$ T cells isolated from naïve C57BL/6 mice. After 2 days all mice were infected with 2×10^6 plaque-forming units (PFU) of LCMV strain WE (LCMV-WE). Total numbers of endogenous and exogenous T cells positive for the MHC class I tetramer of the glycoprotein of LCMV (Tet-$GP33^+$) and for CD8 ($CD8^+$) in the spleen were determined after 10 days of infection (n = 3-4). Data are shown as mean ± SEM. *P < 0.05 (Student's t-test).

3.9.4 Figure 4: Virus-specific antibodies inhibit persistent LCMV-Docile replication in peripheral organs

(**A**) C57BL/6 naïve mice were infected separately with 2×10^4 plaque-forming units (PFU) of lymphocytic choriomeningitis virus strain WE (LCMVWE) and LCMV-Docile. Graph shows viral titers in serum on indicated days. (**B**) C57BL/6 naïve mice were injected with naïve $CD8^+$ T cells, nonspecific antibodies (naïve serum), LCMV-specific $CD8^+$ T cells, or LCMV-specific antibodies (immune serum). After 2 days all mice were infected with 2×10^4 PFU of LCMV-Docile. Graph shows viral titers in spleen, inguinal lymph nodes, liver, and lungs after 3 days of infection. Horizontal dotted lines designate the detection limit. Data are shown as mean ± SEM.

3.9.5 Figure 5: Usp18 promotes LCMV replication

$Usp18^{-/-}$ mice and littermate control naïve mice (wild-type; WT) were infected with 2×10^6 plaque-forming units (PFU) of lymphocytic choriomeningitis strain Docile (LCMV-Docile). Graph shows viral titers in spleen after 1 day of infection (n = 4). Horizontal dotted lines designate the detection limit. Data are shown as mean ± SEM. ***P < 0.001 (Student's t-test).

Figure 1

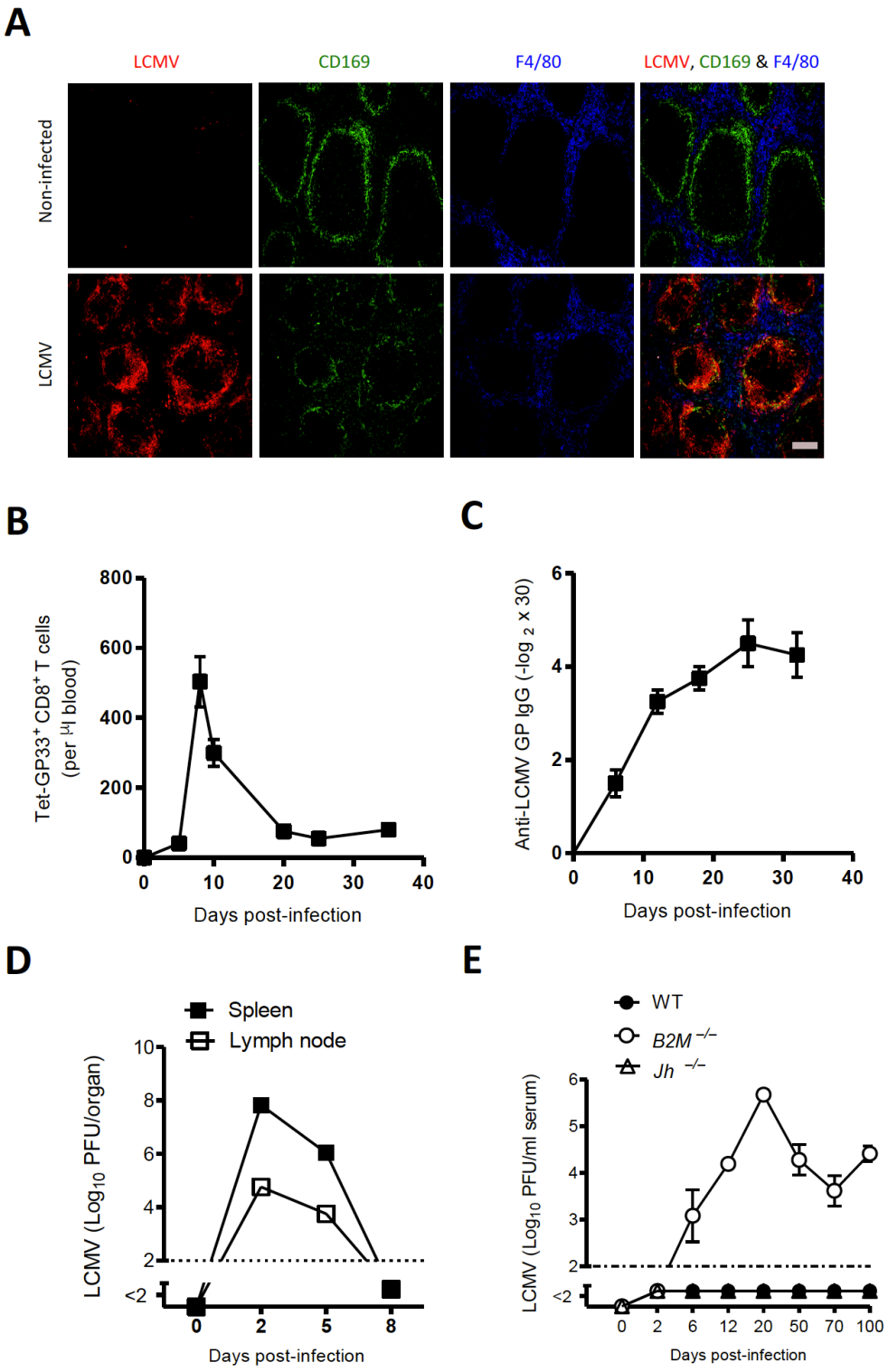


Figure 3.1: Replication of lymphocytic choriomeningitis virus (LCMV) in the marginal zone is associated with immune activation and viral control.

Figure 2

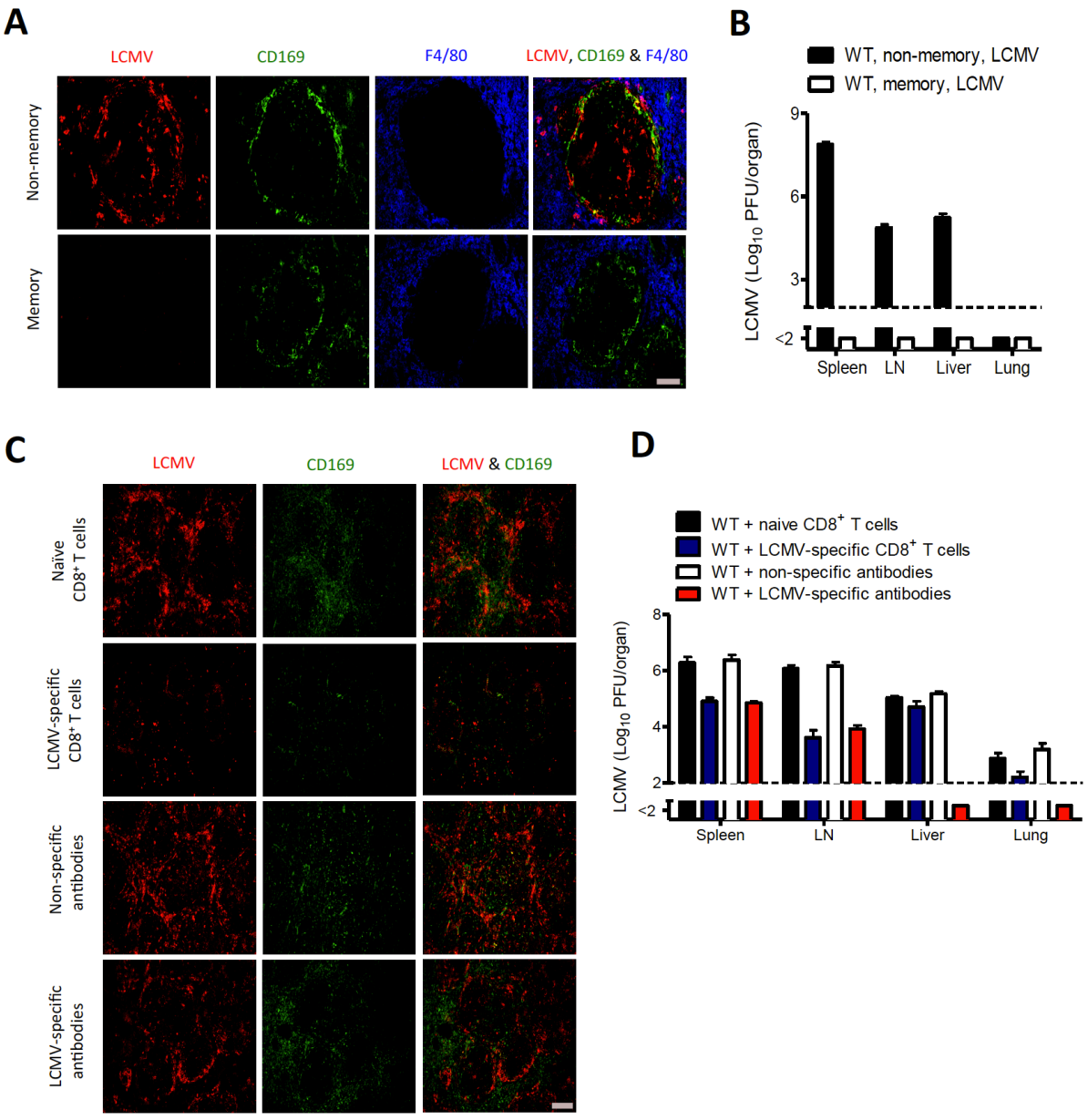


Figure 3.2: Virus-specific antibodies, but not virus-specific CD8$^+$ T cells, allow viral replication in the marginal zone.

Figure 3

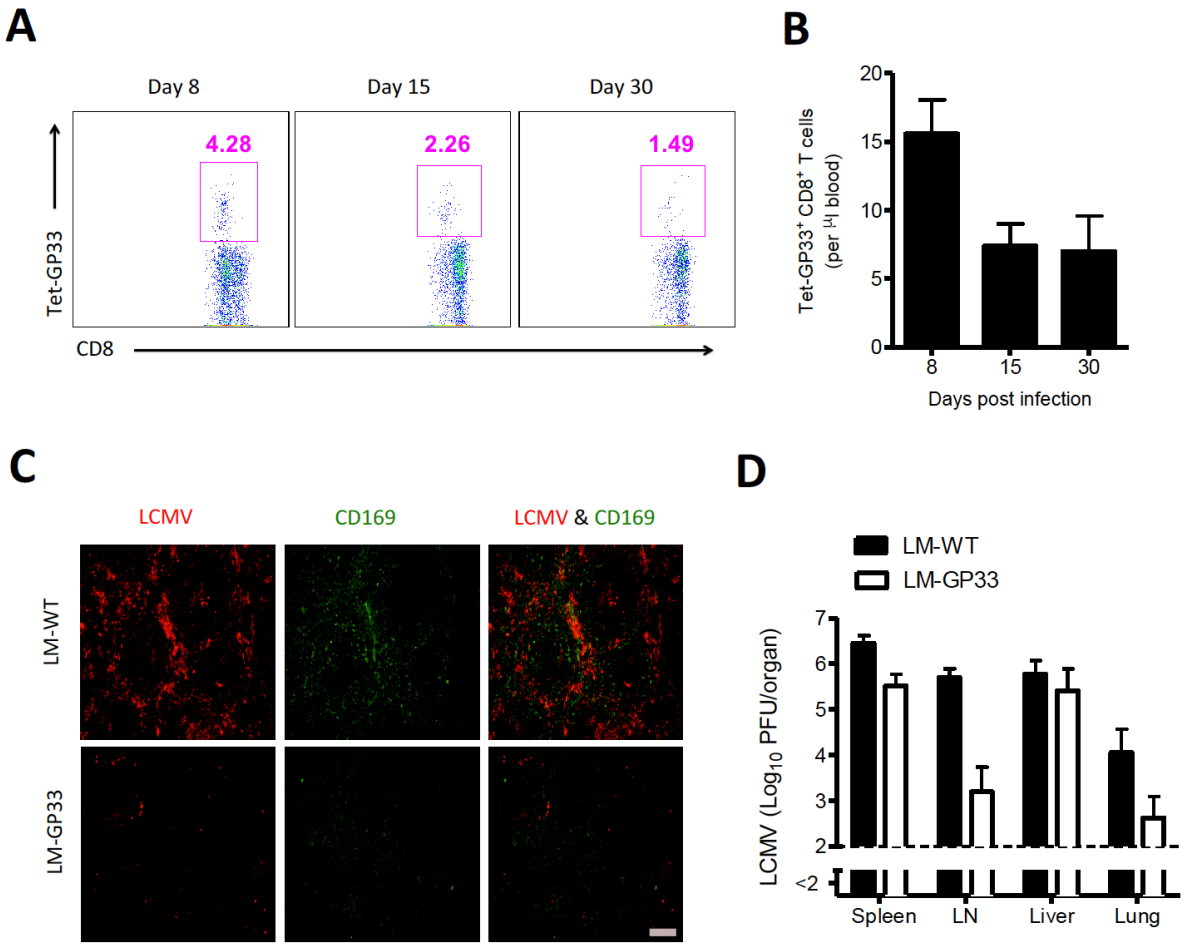


Figure 3.3: Inhibition of viral replication in splenic marginal zone of mice primed with recombinant *Listeria monocytogenes* expressing the glycoprotein of LCMV.

Figure 4

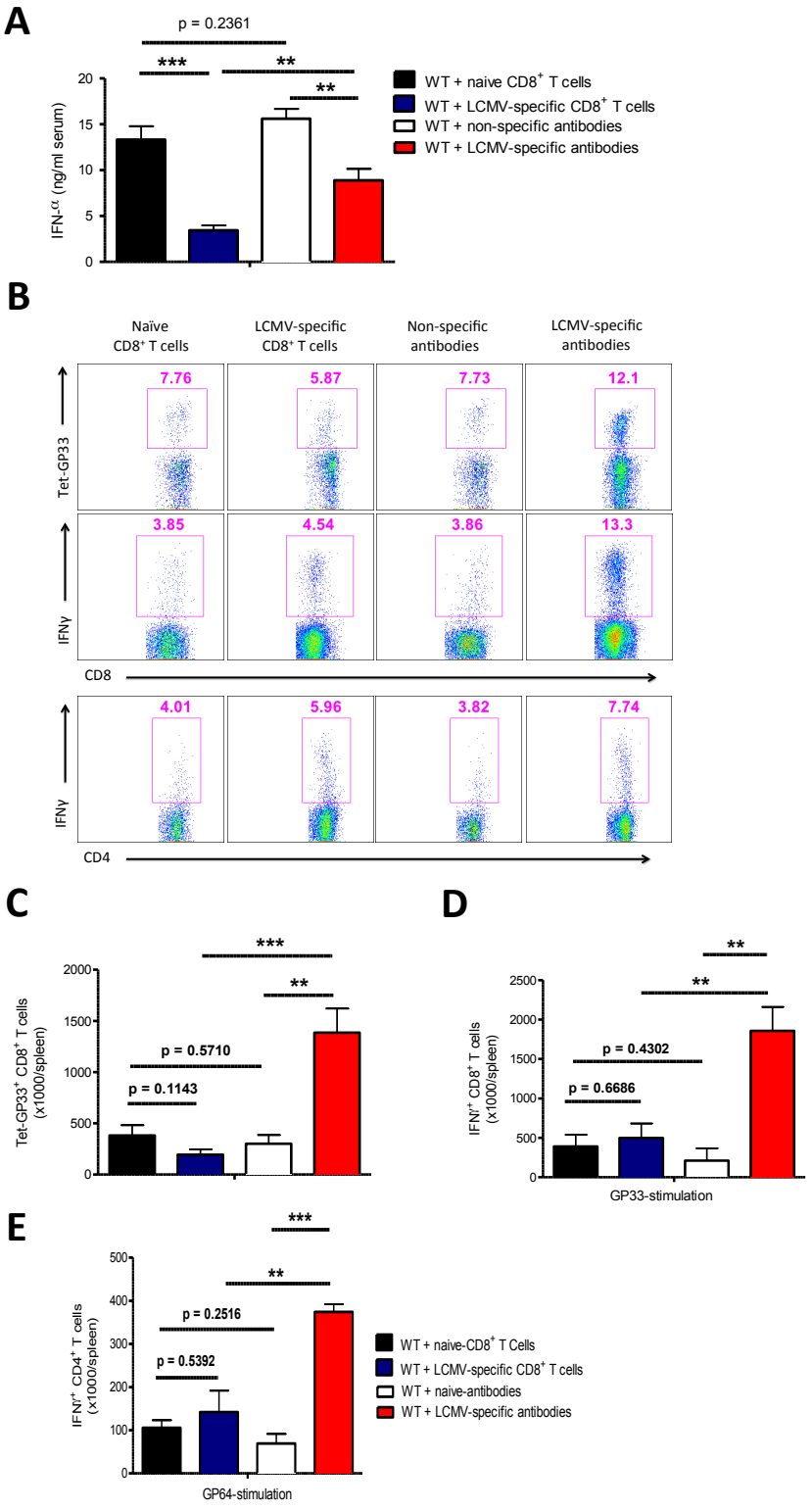


Figure 3.4: Virus-specific antibodies allow innate and adaptive immune activation.

Figure 5

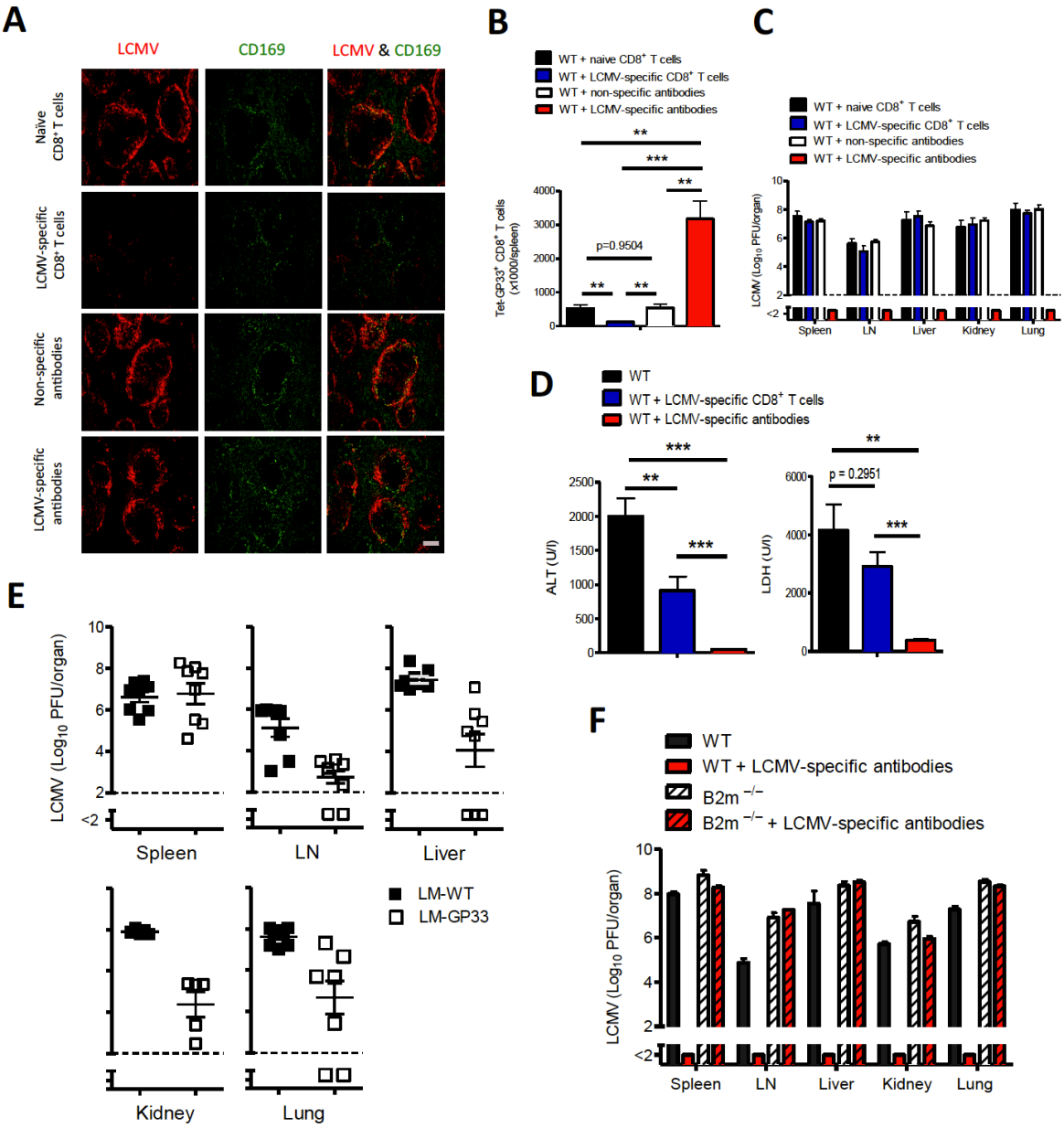


Figure 3.5: Virus-specific antibodies protect against immunopathology and lead to control of virus.

Figure 6

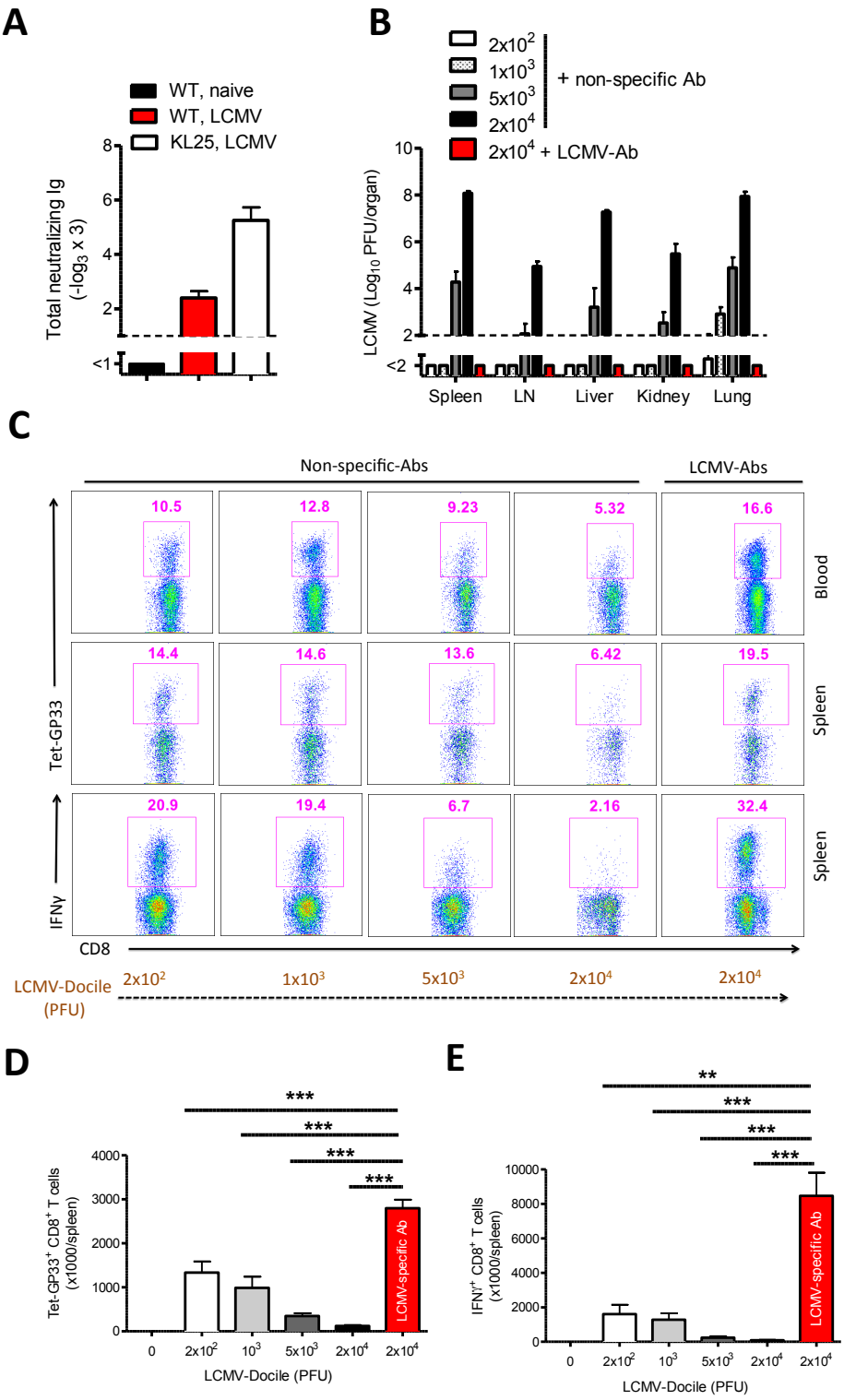


Figure 3.6: Virus-specific antibodies enhance priming and expansion of CD8$^+$ T cells.

Figure 7

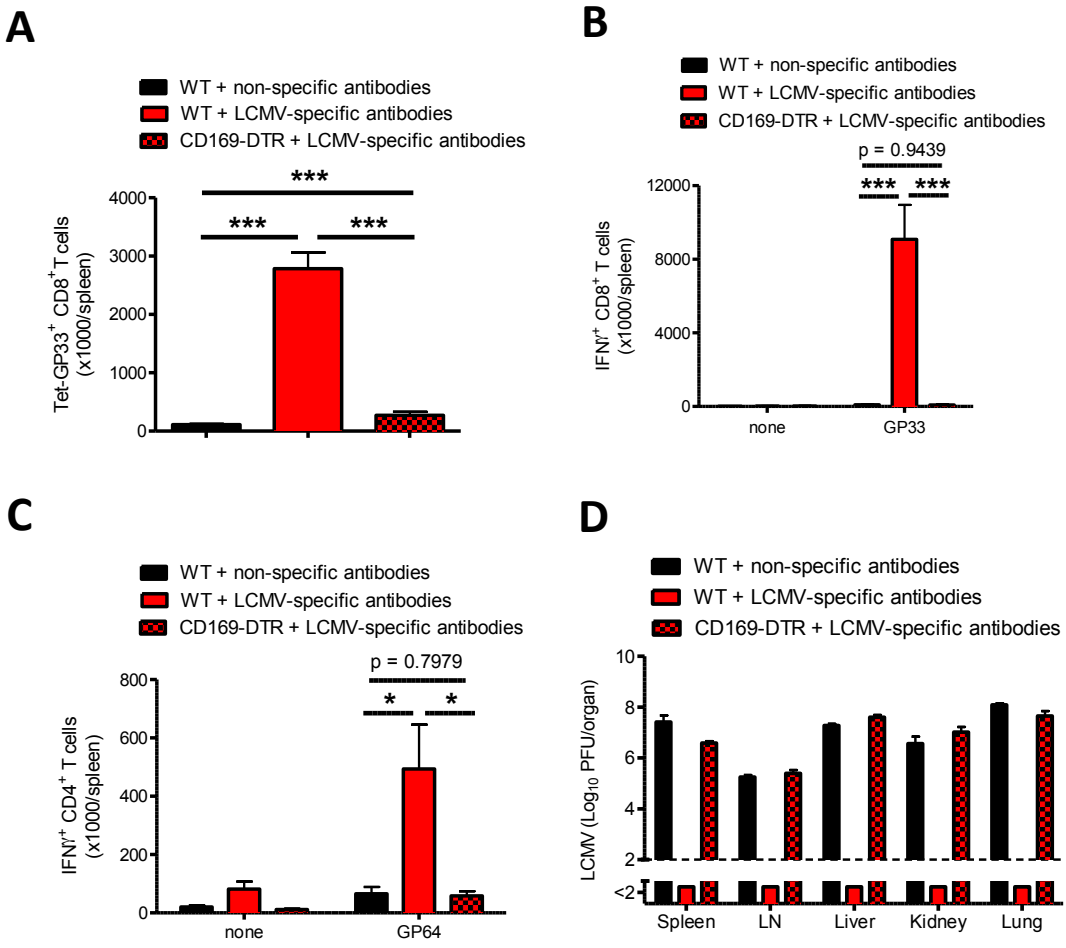


Figure 3.7: Immune activation in the presence of virus-specific antibodies is essential for controlling persistent viral infection.

Figure 8

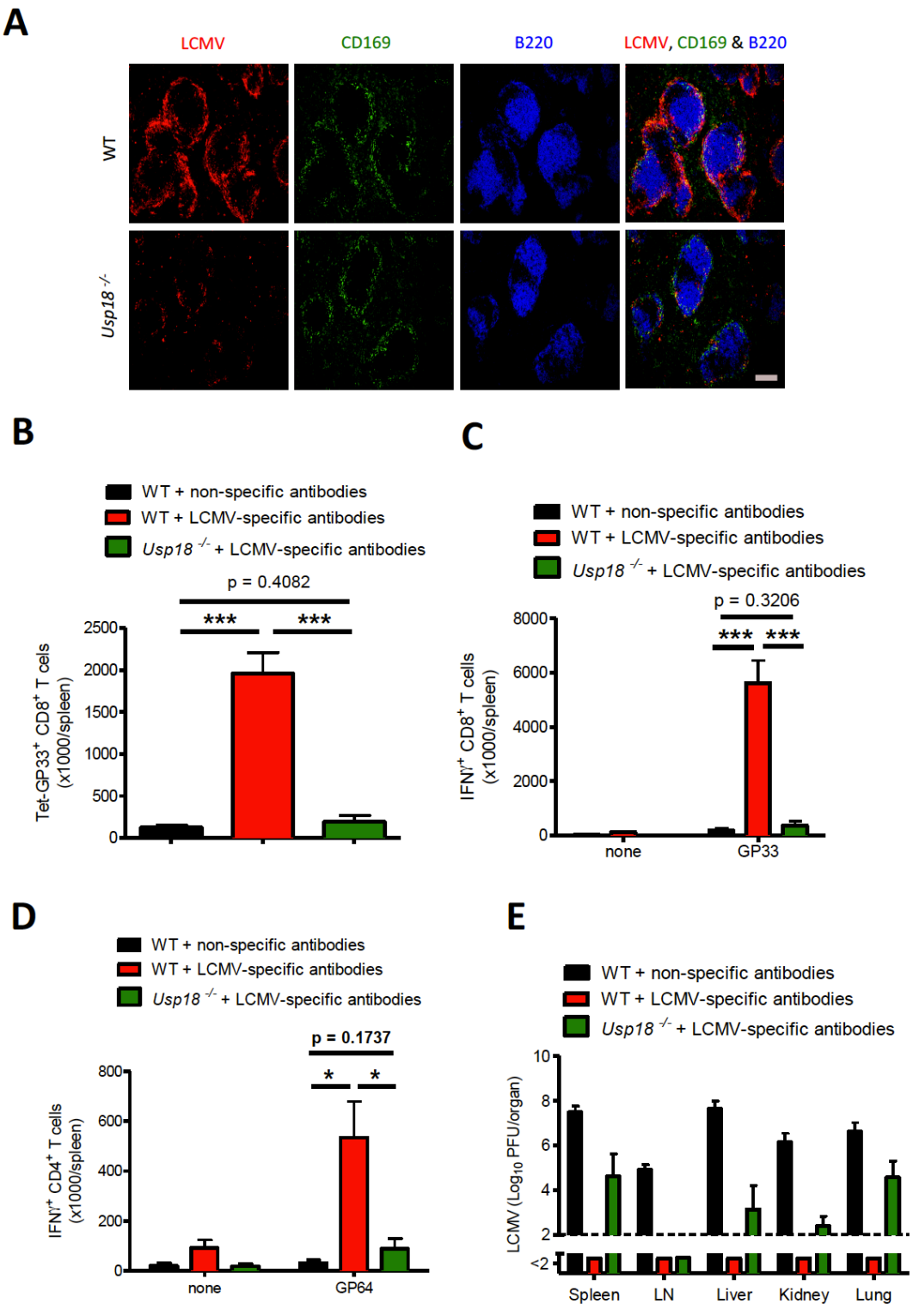
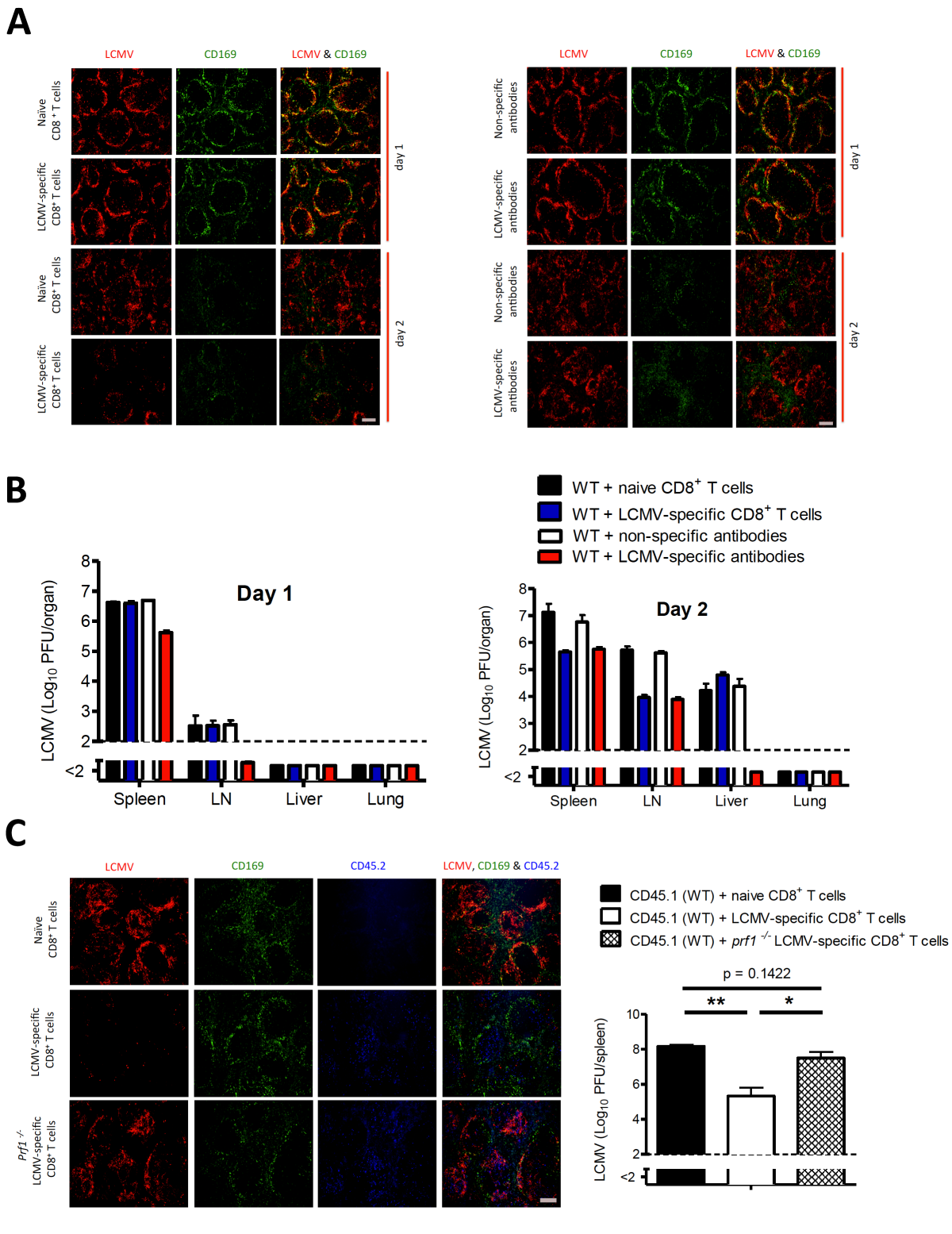


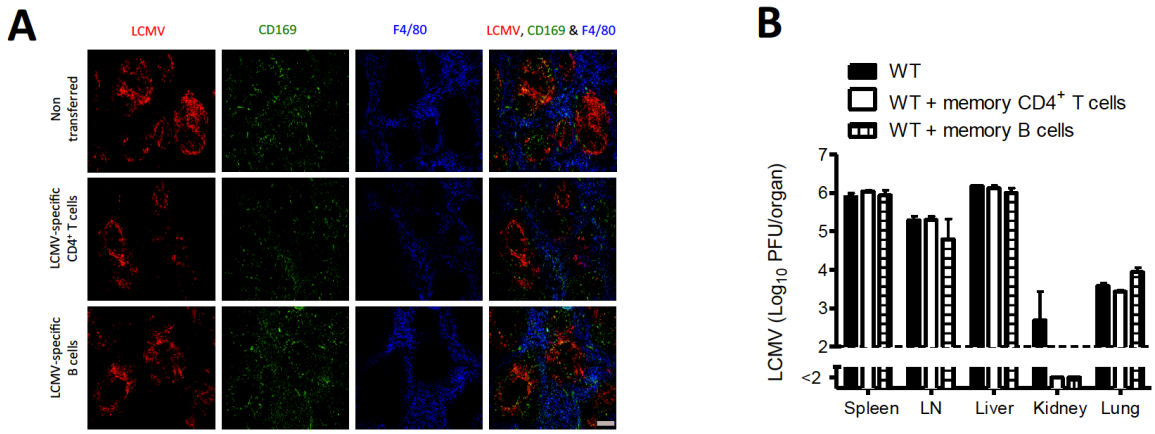
Figure 3.8: Immune activation in the presence of virus-specific antibodies is *Usp18* dependent.

Supplementary Figure 1



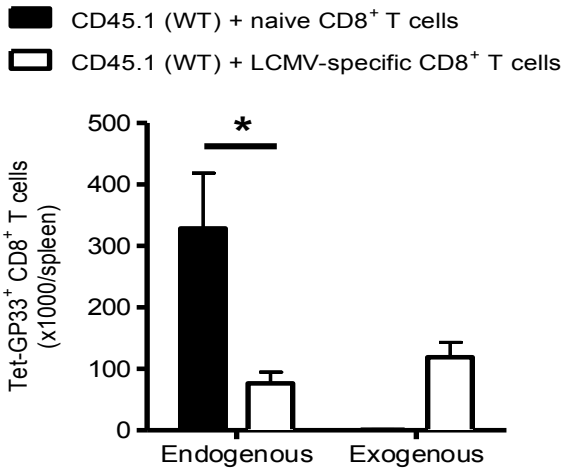
Supplementary Figure 3.1: Virus-specific antibodies, but not virus-specific CD8$^+$ T cells, allow viral replication in the marginal zone.

Supplementary Figure 2



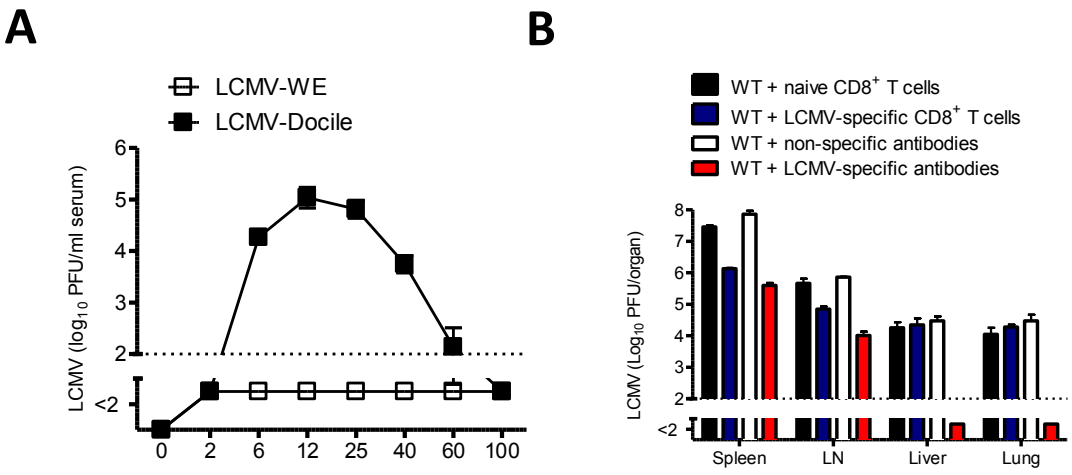
Supplementary Figure 3.2: Memory $CD4^+$ T cells and memory B cells has no effect on viral replication in the marginal zone.

Supplementary Figure 3



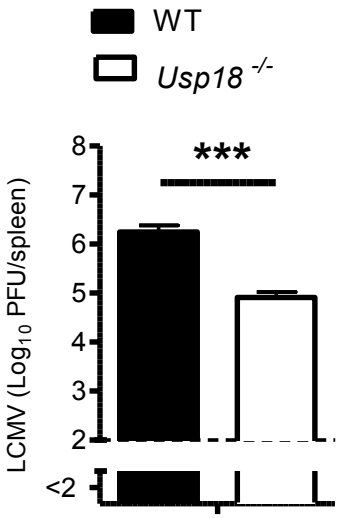
Supplementary Figure 3.3: Memory $CD8^+$ T cells reduce the expansion of endogenous $CD8^+$ T cells.

Supplementary Figure 4



Supplementary Figure 3.4: Virus-specific antibodies inhibit persistent LCMV-Docile replication in peripheral organs.

Supplementary Figure 5



Supplementary Figure 3.5: *Usp18* promotes LCMV replication.

Article Statement

Publication

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Contribution to the publication:

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  - Introduction: Part of the literature research and critical review
  - Material and Methods: Writing part of materials and methods with Khairnar V.
  - Results: Planing of the experiments and execution with Khairnar V, Friedrich SK, Zhou F, Gassa A, Honke N, Shaabani N, Gailus N, Botezatu L.
  - Discussion: Part of discussion writing and critical reviewing with Khairnar V, Honke N, Shaabani N, Botezatu L, Khandanpour C, Dittmer U, Häussinger D, Recher M, Hardt C, Lang PA & Lang KS.

- Results
  - Fig. 1: A to E with Khairnar V and Gailus N.
  - Fig. 2: B and D; A and C with Khairnar V.
  - Fig. 3: A to C; D with Friedrich SK.
  - Fig. 4: A with Gassa A; B to E.
  - Fig. 5: A with Khairnar V; B to E; F with Zhou F.
  - Fig. 6: A and B; C to E with Khairnar V.
  - Fig. 7: A to C; D with Khairnar V.
  - Fig. 8: A with Khairnar V; B to E.
  - Supplementary Figures: Fig. 1B-C, Fig. 2B and Fig. 3-5; Fig. 1A and 2A with Khairnar V.

- Revision and Proof reading

Discussion on the reviewer comments, data analysis and drafting the manuscript to it's final online version. (with Khairnar V, Honke N, Shaabani N, Botezatu L, Khandanpour C, Dittmer U, Häussinger D, Recher M, Hardt C, Lang PA & Lang KS)

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Vikas Duhan **Prof. Dr. Karl S. Lang**

Chapter 4

Discussion

In these two studies, we found the roles of splenic metallophilic marginal zone macrophages ($CD169^+$ macrophages) during primary and secondary infection of LCMV. Recently it was explained that $CD169^+$ macrophages experience early virus replication in secondary lymphoid organs during systemic VSV infection[117]. In this study using LCMV infection, we did not see significant effect of $CD169^+$ macrophages on early virus replication in secondary lymphoid organs. This could be explained by the involvement of dendritic cells in early virus replication of LCMV[116]. But $CD169^+$ macrophages present in spleen and lymph node contributed for sustained production of systemic IFN-I. This contribution to IFN-I production lead to activation of antiviral effector functions in lymphoid and non-lymphoid organs that inhibited virus replication. Lack of $CD169^+$ macrophages led to tremendous virus replication in liver hepatocytes.

Priming and expansion of virus specific $CD8^+$ T cells was found to be similar in absence of $CD169^+$ macrophages during LCMV infection. But activation status explained by surface molecules expression and functional effectors molecules of virus specific $CD8^+$ T cells were significantly high in mice deficient with $CD169^+$ macrophages. Due to IFN-I signaling in WT mice, expression of PD-L1 in liver was enhanced. PD-L1 is a negative regulator of T-cell effector functions. Presence of $CD169^+$ macrophages maintained a balanced $CD8^+$ T cells effector functions, which led to clearance of virus with minimal pathology. Due to lack of $CD169^+$ macrophages, IFN-I production was impaired that led to low level of PD-L1 expression in liver. Lack of IFN-I signaling caused virus spread in most of the organs and uncontrolled $CD8^+$ T cells effector functions. High functional virus specific $CD8^+$ T cells induced strong immunopathology and resulted in death of mice.

IFN-I is a crucial pro inflammatory cytokine produced mainly by innate immune cells and plays a dual role during viral infection. On the one hand, it limits viral replication because it directly induces antiviral molecules in the infected cell[108,109,159]. Consequently, the absence of the interferon-α/β receptor (IFNAR) promotes viral replication and can result in persistence of virus[102,159]. On the other hand, sustained IFN-I signaling induces immunosuppressive mechanisms, including the production of interleukin-10 (IL-10) and the expression of programmed cell death ligand 1 (PD-L1)[112,113,160]. IL-10 and PD-L1 are important inhibitors of $CD8^+$ T cells and thereby limit the functions of virus-specific $CD8^+$ T cells[69,70,161,162]. Programmed cell death protein 1 (PD-1) is upregulated on almost all activated $CD8^+$ T cells[114], a finding suggesting that the regulation of its ligand (PD-L1) determines the fate of

virus-specific $CD8^+$ T cells. How professional immune cells and/or virus infected cells regulate PD-L1 expression during an ongoing infection is not well defined.

Here with our study we showed viral persistent in absence of adequate IFN-I production, means there is a reduced IFN-I signature, lack of immune activation and lack of negative immune regulators expression. This type of conditions known to favor for effector functions of $CD8^+$ T-cells but with possibilities of immunopathology[160]. Our results from this study conclude that during LCMV infection, $CD169^+$ macrophages prolonged the IFN-I response. Prolonged IFN-I responses induced PD-L1 expression in the liver. The absence of $CD169^+$ macrophages reduced the antiviral IFN-I activity, but also limited PD-L1 expression. As a consequence, mice exhibited overwhelming viral replication in the absence of $CD8^+$ T-cell exhaustion, and this replication resulted in strong immunopathology and death of host.

In next study, we showed that acute infection with LCMV-WE strain lead to induction LCMV-specific $CD8^+$ T cells and LCMV-specific antibodies, which finally eliminate virus within 10 days and provides long lasting immunological memory. We found that presence of LCMV-specific antibodies during systemic recall infection allowed intracellular viral replication in $CD169^+$ macrophages of splenic marginal zone but inhibited virus replication in peripheral tissues. But presence of LCMV-specific memory $CD8^+$ T cells before infection strongly inhibited virus replication in $CD169^+$ macrophages and had limited effect on virus replication in peripheral organs. Due this strong inhibition of virus replication in $CD169^+$ macrophages, induction of systemic IFN-I was strongly reduced as compare to presence of virus-specific antibodies.

During persistent virus infection with LCMV-Docile, the presence of virus specific antibodies before infection led to enhance priming and expansion of virus specific $CD8^+$ T cells which resulted in viral control with negligible immunopathology. But presence of virus specific memory $CD8^+$ T cells inhibited priming and expansion of endogenous virus specific $CD8^+$ T cells that led to virus persistent and immunopathology in mice. The viral clearance in presence of virus specific antibodies was found $CD8^+$ T cell dependent because in absence of $CD8^+$ T cells, presence of virus specific antibodies could not eradicate viral infection. Virus replication in $CD169^+$ macrophages in presence of virus specific antibodies promoted $CD8^+$ T cell response and viral control. In absence of $CD169^+$ macrophages or absence of virus replication in $CD169^+$ macrophages, the presence of virus specific antibodies failed to induce proper $CD8^+$ T cell response which led to develop persistent infection.

Viral diseases are big threat for developing world and current vaccination policy of World Health Organization (WHO) includes more than 60% of vaccines, which target viral diseases[163]. Most of viral vaccines mediate protective efficacy by inducing virus specific antibodies[163,164] but often fail to induce virus-specific $CD8^+$ T cells[144] . Virus specific antibodies are known to clear extracellular virus particles[131] but virus specific $CD8^+$ T cells are indispensable to kill infected cells, which are reservoir and factory for virus production[165]. Secondary infection after vaccination usually leads to induction of virus specific $CD8^+$ T cells and required for virus elimination[146,147]. The induction of virus specific $CD8^+$ T cells depends on innate immune activation in secondary lymphoid organs[28,116]. So, the efficacy of a successful vaccine is at least depending on factors that help innate immune cells activation in secondary lymphoid organs during real virus infection. At least from this study we can determine that vaccines which are confined to antibody immune response can be a better option for targeting viral infections as compare to vaccines that induce only $CD8^+$ T cell response.

From our study, we can conclude that after systemic recall infection during LCMV, the presence of virus-specific antibodies allowed intracellular virus replication in $CD169^+$ macrophages of splenic marginal zone but cleared virus replication in liver, lung and kidneys. After recall infection with the persistent virus strain LCMV-Docile, viral replication in the marginal zone was essential for priming and expansion of $CD8^+$ T cells and lead to viral elimination. In contrast to specific antibodies, memory $CD8^+$ T cells inhibited viral replication in the marginal zone and failed to induce protective immune response, which resulted in immunopathology and virus persistent. In conclusion, virus-specific antibodies are better as compare to virus specific $CD8^+$ T cells in protecting against persistent prone virus infection. As antibodies inhibit virus propagation in periphery but still allow virus replication in spleen to induce effective immune response. This describes a novel mechanism of virus specific antibodies that provide protection by inhibiting virus in susceptible organs but allowing virus replication in a special niche of secondary lymphoid organs.

From these two studies, we can conclude that $CD169^+$ macrophages present in marginal zone of spleen efficiently filter virus particles from circulation, experience virus replication that induce IFN-I production and provide sufficient antigens for $CD8^+$ T cell priming during primary and secondary infection. These special roles played by marginal zone $CD169^+$ macrophages confirm the effective activation of innate and adaptive immune system that deliver protective immunity against viral infections.

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Abbreviations

ADCC	antibody- dependent cell-mediated cytotoxicity
ALT	alanine aminotransferase
APC	allophycocyanin
APC	antigen presenting cell
BMDM	generated bone marrow-derived macrophage
B2M	beta 2 microglobulin
cDC	conventional or classical dendritic cell
CFSE	carboxyfluorescein succinimidyl ester
CFU	colony-forming units
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
DC	dendritic cell
DT	diphtheria toxin
DTR	diphtheria toxin receptor
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FcR	Fc receptor
FDC	follicular dendritic cell
FRC	fibroblastic reticular cell
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GP	glycoprotein
GzmB	granzyme B
HBV	hepatitis B virus
HCV	hepatitis C virus
HIV	human immunodeficiency virus
IFN-I	type I interferon

IFN-γ	interferon gamma
IFNAR	interferon alpha/beta receptor
IFNβ	interferon beta
Ig	immunoglobulin
IL	interleukin
KC	kupffer cell
LCMV	Lymphocytic choriomeningitis virus
LCMV-Docile	lymphocytic choriomeningitis virus strain Docile
LCMV-WE	lymphocytic choriomeningitis virus strain WE
LDH	lactate dehydrogenase
LM	listeria monocytogenes
LN	lymph node
LPS	lipopolysaccharide
MACS	magnetic-activated cell sorting
MALT	mucosal associated lymphoid tissue
MHC	major histocompatibility complex
MZ	marginal zone
NK	natural killer
NP	nucleoprotein
NP	nucleoprotein
PALS	periarteriolar lymphoid sheath
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
PD-L1	program death ligand 1
PD1	program death protein 1
pDC	plasmacytoid dendritic cell
PFU	plaque-forming units
PRF1	perforin1

PRR	pattern-recognition receptors
qRT-PCR	quantitative real-time polymerase chain reaction
r-LCMV	recombinant-LCMV
RBC	red blood cells
rLM	recombinant-listeria monocytogenes
SEM	standard error mean
SHM	somatic hypermutation
SLO	secondary lymphoid organ
T_C	cytotoxic T
TCR	T-cell receptor
TD	thymus or T cell dependent
TFH	T follicular helper cell
TGF-β1	transforming growth factor beta 1
T_H	T helper
TID	T cell independent
TIM-3	T-cell immunoglobulin and mucin-domain containing-3
TLR	toll like receptor
T_{reg}	T regulatory
USP18	Ubiquitin-specific peptidase 18
VSV	vesicular stomatitis virus
WHO	world health organization
WT	wild type
YFP	yellow fluorescent protein

Erklärung

Hiermit erkläre ich, gem. § 6 Abs. 2, g der Promotionsordnung der Fakultät für Biologie zur Erlangung der Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema **'Crosstalk between Splenic CD169^{+} Macrophages and Adaptive Immune System'** zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von **Vikas Duhan** befürworte.

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Curriculum Vitae

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