Role of Lymphotoxin Beta and Cell Adhesion Molecule (CEACAM1) in Innate and Adaptive Immune Activation

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The experiments on which this work is based have been carried out at the Institute of Immunology, Faculty of Medicine, University Hospital Essen, at the University of Duisburg-Essen.

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

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Tag der mündlichen Prüfung: 23rd June 2017

Dedicated to my Parents (Aai and Aappu) ...

Should have faith in God

but more trust in yourself...

-Aappu

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Summary

The induction of the adaptive and innate immune system is essential for controlling viral infections. Balance between the innate immune system and adaptive immune responses results in better antiviral immune activation. Not enough or too much activation can lead to impairment of immune responses. In this thesis, we have investigated the role of enforced viral replication and different mechanisms by which adequate innate and adaptive immune responses are generated.

Recently, it was shown that *Usp18* dependent viral replication in splenic $CD169^{+}$ macrophages are important to induce a sufficient type I interferon response resulting in the subsequent activation of adaptive immune responses. Here, in one study, we have shown that the intracellular replication of viral particles is sufficient to generate virus specific CD8 T cell responses. On the other hand, extracellular distribution of viral particles along the splenic conduits was necessary for inducing systemic levels of type I interferon (IFN-I). We have shown that *Usp18* is important for viral replication, but that B cell-derived lymphotoxin beta is necessary for the extracellular distribution of virus along the marginal zone in spleen.

In a further study, we have demonstrated a role of CEACAM1 in B cell survival, proliferation and innate immune activation. B cells are important part of immune system because they are in close proximity of blood-borne antigen in splenic circulation, they secrete antibodies and maintain the lymphoid architecture. We have shown that cell intrinsic signalling of CEACAM1 is essential for survival of proliferating B cells. CEACAM1 is involved in B-cell receptor and signals through the Btk/Syk/NF-κB axis. Absence of CEACAM1 signalling leads to reduced number of B-cells in lymphoid organs especially marginal zone B cells and $CD169^{+}$ macrophages. Absence of CEACAM1 leads to poor anti-viral antibody secretion after systemic infection with cytopathic virus resulting in early death of *Ceacam1*$^{-/-}$ mice.

In another set of experiment, we investigated the role of virus specific antibodies on adaptive immune activation. With the use of chronic virus strain LCMV-Docile, we determined whether enforced viral replication could occur in presence of virus-specific antibodies or virus-specific CD8 T cells. We found that after systemic recall infection, virus-specific antibodies allow viral replication in splenic marginal zone but controlled in peripheral organs resulting in strong CD8 T cell priming whereas, virus-specific CD8 T cells blunted viral replication in spleen but failed to control the persistent chronic viral infection.

In conclusion, using several mouse models of noncytopathic and cytopathic viral infection, we have elucidated the functional role of lymphotoxin beta, CEACAM1 and virus-specific antibodies in immune function particularly during enforced viral replication in the spleen.

Zusammenfassung

Die Aktivierung des adaptiven und angeborenen Immunsystems ist essentiell für die Kontrolle viraler Infektionen. Ausgeglichene Antworten des angeborenen und adaptiven Immunsystems führen zu einer verbesserten antiviraler Immunantwort. Zu geringe oder zu hohe Aktivierung führt zu einer verschlechterten Immunantwort. In dieser Arbeit wurde die Rolle der ‘’enforced virus replication’’ und verschiedene Mechanismen untersucht, welche eine adäquate angeborene und adaptive Immunaktivierung garantieren.

Kürzlich wurde gezeigt, dass die Usp18 abhängige Virusreplikation in splenischen $CD169^+$ Makrophagen bedeutsam für eine hinreichende Typ I Interferonantwort ist, welche später zur Aktivierung der adaptiven Immunantwort führt. In dieser Arbeit wurde gezeigt, dass schon die intrazelluläre Replikation von Viruspartikeln genügte, um eine virusspezifische CD8 T Zell Antwort zu generieren. Des Weiteren wurde gezeigt, dass die extrazelluläre Verteilung viraler Partikel im gesamten Milzgewebe unabdingbar für die Induktion systemischer Level Typ I (IFN-I) war. Wir haben herausgestellt, dass Usp18 wichtig für die Virusreplikation ist, jedoch durch B Zellen produziertes Lymphotoxin beta notwendig für die extrazelluläre Verteilung von Virus entlang der splenisches Marginalzone ist.

In einer weiteren Studie wurde die Rolle von CEACAM1 in Hinsicht auf das Überleben, die Proliferation und die angeborene Immunantikvierung von B Zellen untersucht. B Zellen sind ein wichtiger Bestandteil des Immunsystems, weil sie in direkter Umgebung der aus dem Blut stammenden Antigene in der splenischen Zirkulation sind; sie produzieren Antikörper und erhalten die lymphoide Struktur. Es wurde gezeigt, dass zellintrinsisches CEACAM1-signaling essenziell für das Überleben proliferierender B Zellen ist. CEACAM1 ist involviert in den B Zell-Rezeptor und interagiert durch den Btk/Syk/NF-κB Signalweg. Das Fehlen von CEACAM1-signaling resultiert in einer reduzierten B Zellzahl in lymphoiden Organen, insbesondere von B Zellen und $CD169^+$ Makrophagen in der Marginalzonen. Weiterhin führte die Abwesenheit von CEACAM1 zur einer mangelhaften antiviralen Antikörpersekretion nach systemischer Infektion mit zytopathischen Virus, was zu einem frühen Versterben der *Ceacam1*$^{-/-}$ Mäuse führte.

Weitere Experimente untersuchten die Rolle virusspezifscher Antikörper auf die adaptive Immunaktivierung. Mit Hilfe des chronischen LCMV-Docile Virus wurde bestimmt, ob die ‘’enforced virus replication’’ in der Anwesenheit von virusspezifischen Antikörpern oder Virusspezifischen CD8 T Zellen stattfinden kann. Es konnte gezeigt werden, dass nach wiederholter systemischer Infektion virusspezifische Antikörper die virale Replikation in der Marginalzone der Milz erlauben, wohingegen sie die Replikation in peripheren Organen kontrollierten. Obgleich dies zu einem starken T Zell priming führte, verminderten virusspezifische CD8 T Zellen die zwar die Virusreplikation in der Milz, scheiterten jedoch die persistierende virale Infektion zu kontrollieren.

Zusammenfassend konnte auf mit Hilfe unterschiedlicher Mausmodelle, sowie nicht zytopathischer und zytopathischer viraler Infektion die Rolle von Lymphotoxin beta, CEACAM1 und virusspezifscher Antikörper in Bezug auf die Immunfunktion, insbesondere ihrer Rolle in der ‘’enforced virus replication’’ in der Milz, gezeigt werden.

1. Chapter I: *Introduction*

1.1 *Immune System*

The immune system has evolved to protect multicellular organisms from pathogens. It encompasses many biological processes and structures to protect host from invading pathogens such as viruses, bacteria, fungi, protozoa and worms.[1] The immune system not only defends the host against external pathogens but also actively eliminates self-antigens as well as tumorigenic cells. Initially many researchers characterised the active immune components in the blood as having the ability to neutralize toxins. As those components were found in the serum (body component) it was called as "humoral immunity". Other type of immunity generated by cellular response is called "cell-mediated immunity''.[2] T cells have an ability to recognise cells that are infected by pathogens and they have evolved mechanisms to distinguish between foreign and self-antigens called "tolerance".[2] There are two types of immune responses: innate and adaptive. Innate immunity is the first line of response and it is also known as the non-specific immune system whereas the adaptive immune system generates a specific response against pathogens. Adaptive immune system is an important part of the hosts' defence as it results in long term memory formation.[1]

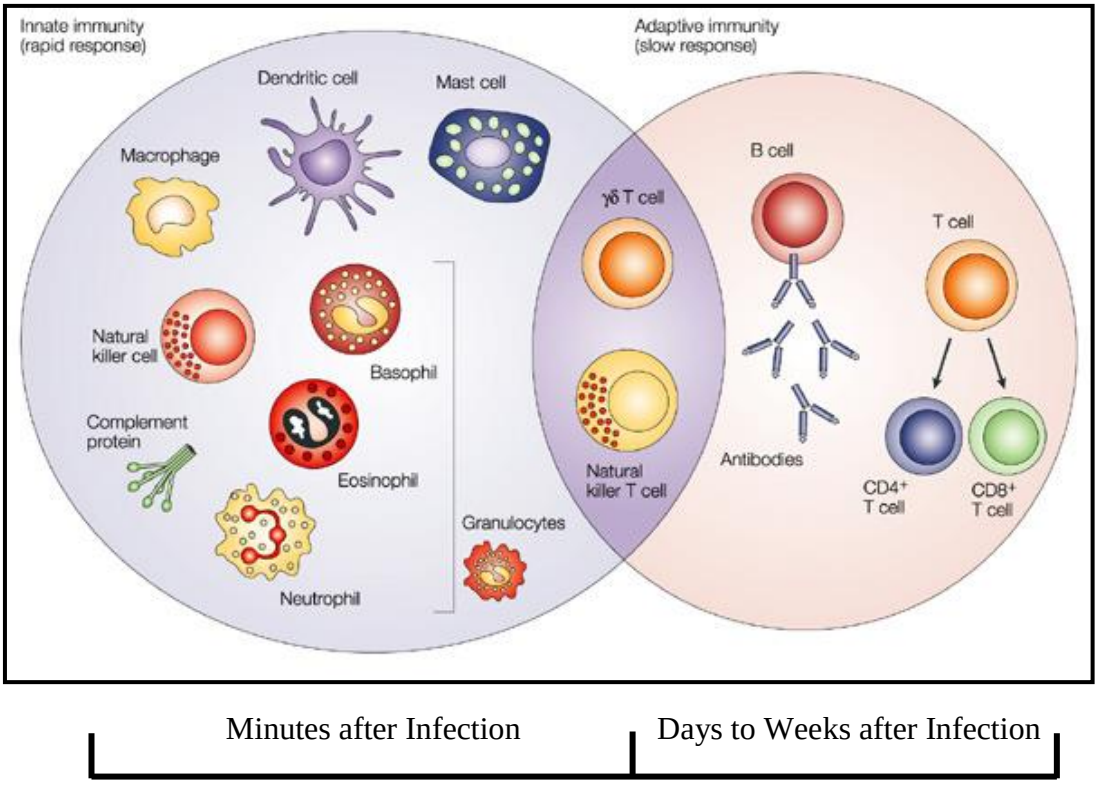


Fig 1-1: Innate Versus Adaptive Immune System (Dranoff G., *Nat. Rev. Cancer*, 2004, modified)

1.2 *Types of Immunity*

1.2.1 *Innate Immunity*

The innate immune system provides the first line of defence against infection and acts immediately after invasion by pathogens. The first line of defence includes the release of various soluble molecules in blood, antimicrobial enzymes such as lysozyme and antimicrobial peptides. Components of innate immune system don't generate long lasting immunological memory. Epithelial surfaces of body comprise skin, gastrointestinal, urogenital and respiratory tracks provide the initial defence against pathogens. Epithelia is more than just a physical barrier as it produces a range of chemical substances such as digestive enzymes, bile acids and fatty acids in the gastrointestinal track. Lysozyme present in saliva and tears breaks the peptidoglycan component of bacterial cell wall is important for antibacterial defence.[1, 3]

Apart from physical barriers two categories of lymphoid and myeloid lineages derived from the hematopoietic stem cells are precursors of the macrophages, granulocytes, dendritic cells and mast cells of the innate immune system.[3]

1.2.1.1 *Macrophages*

Macrophages are the phagocytic cells that phagocyte and digest cellular debris, foreign particles, microbes and cancer cells. Macrophages reside in almost all tissues and can play several different roles. Some macrophages are involved in tissue regeneration and repair. Other macrophages which are stimulated by encounters with pathogens called inflammatory macrophages contribute to elimination of pathogens from a tissue and can act as antigen presenting cells (APC's) that can activate T lymphocytes.[1]

M1 macrophages are considered ''killer'' macrophages and secrets high levels of IL(Interleukin)-12 upon activation by lipopolysaccharide (LPS) and IFN-γ. M2 macrophages are attributed a ''repair'' function that involve in wound healing and tissue repair by secreting high levels of anti-inflammatory cytokine such as IL-10.[4] Macrophages usually remain at strategic positions where the invasion is likely to occur. They are named according on the location in which they reside (**Table 1-1**).[5]

Anatomical Location	Name
Kupffer cells	Liver
Red pulp macrophages	Spleen
Alveolar macrophages	Lung
Monocytes	Bone marrow
Peritoneal Macrophages	Peritoneal Cavity
Sinus Histiocytes	Lymph node
Langerhans cells	Skin

Table 1-1: Different names of macrophages and their locations
(Gordon S., *et.al, Immunol. Rev.*, 2014, modified)

Apart from red pulp macrophages there are specialized type of macrophages called metallophilic macrophages or marginal zone macrophages located in marginal zone of spleen or lymph nodes. They are characterized by the expression of $CD169^{+}$ receptor and are important for viral replication and inducing adaptive immune responses.[6,7]

1.2.1.2 *Granulocytes*

Granulocytes are so named because of the dense granules in their cytoplasm and are of three types: basophils, eosinophils and neutrophils. These granules store proteins that are important for granulocytes to move to the site of infection and destroy the pathogen. At the time of infection mature basophils are released from bone marrow, travel to the site of infection and will release histamine, serine proteases and interleukins to initiate inflammatory response.[8] Eosinophils are terminally differentiated effector cells involved in clearance of parasitic infections through the release of cytotoxic proteins.[9] Neutrophils are not only the major pathogen fighting cells but also elicit immune responses against intracellular pathogens and viruses through complex cross talk with lymphocytes, dendritic cells and NK cells. The crosstalk is established by secreting lots of cytokines or by expressing several surface molecules.[10]

1.2.1.3 *Dendritic Cells (DC's)*

DC's are the most critical cells for the initiation of immune response arise from both myeloid and lymphoid lineage of hematopoietic cells. DC's are antigen presenting cells that are not only important for induction of primary immune responses but also immunological

tolerance. They help in establishing immunological memory by capturing antigen in tissues and presenting them to T cells in lymphoid organs.[11] Lymphoid DC's are localised in T cell rich areas in spleen and lymph nodes whereas myeloid DC's are present in marginal zone of spleen.

DC's can prime CD8 T cells *in vivo* and *in vitro* and strong antigen response can be elicited *in vivo* by presenting antigen bearing DC's to T cells.[12] CD11c expression on DC's can lead to the generation of distinct T cell responses. $CD11c^+$ DC's can induce T cells to generate Th1 cytokines on the other hand $CD11c^-$ DC's can induce T cells to generate Th2 type cytokine response.[13] At different stages of differentiation, DC's can activate natural killer (NK) cells or NK T-cells[11] through release of IL-12, IL-15 and IL-18.[14] Other kind of DC's called plasmacytoid dendritic cells (p-DC's) which are mainly found in circulation and lymphoid organs are important players in secreting type I interferon (IFN-α/β) in response to viruses or viral peptides.[15]

1.2.2 *Adaptive Immunity*

Adaptive immune system or acquired immune system is a specialized immune system that eliminates pathogens. All pathogens are not distinguished by innate immune system and when these pathogens escaped innate cells they are identified by a highly controlled process of adaptive immunity. Adaptive immunity mainly consists of 3 types of lymphocytes: B lymphocytes, T lymphocytes and NK cells.

After antigen binding to a B cell receptor (BCR) on B cell surface, the lymphocytes will proliferate and differentiate in to plasma cells. B cells secret antibodies against specific antigen hence they can be targeted and eliminated by antibodies. On the other hand, unlike B-cells, T cell receptor (TCR) recognizes only processed pieces of antigens or peptides which are presented to them by protein molecules on the surface of APC's called major histocompatibility complex (MHC) molecules. After antigen encounter, T cells are activated and proliferate. The hallmark of adaptive immune activation is the formation of memory and a more rapid response upon second encounter with the same pathogen.[1, 3]

1.2.2.1 *B cells*

B cells are the main antibody secreting cells that generate and mature in the bone marrow. B-cells are found to secret large variety of cytokines and are major players of

adaptive immune responses. Due to number of functions of B cells, it's important to study the factors affecting the development and function of B-cells. Therefore, in this study we investigated the development of B-cell and the role of B cells after the infection of cytopathic vesicular stomatitis virus (VSV).

1.2.2.1.a *Development of B cells*

Bone marrow of mouse contains B lineage cells at each stage of development from progenitors to mature B cells. B cells are primarily divided in two groups B-1 and B-2 B-cells. Murine B-1 cells are generated from fetal liver and are sustained in periphery by self-renewal.[16, 17] On the other hand, B-2 B cells are generated in bone marrow and are produced throughout lifelong (**Table 1-2**).

Ontogeny and function	B-1	B-2
Major roles	Immune barrier; rapid, early immune responses; natural Abs; TI responses	Surveillance; adaptive immune responses; memory; produce Ab targeted to pathogens; secondary immune responses; TI and TD responses
Anatomic locations	Coelomic cavities Mucosal interfaces Spleen	Secondary lymphoid organs Lymphatics blood
Development	Fetal liver; adult bone marrow; self-renewal in periphery	Continuous generation from bone marrow HSC pool
Major subsets	B-1a, B-1b	Transitional (TR), follicular (FO), marginal zone (MZ), germinal center (GC), memory B (MBC)
Pool size	Small	Large overall; FO B cells comprise the majority in young adult life
Primary antibody isotype (s) secreted	IgM, IgA	IgM, IgG
BCR/repertoire	Generated by somatic recombination J-proximal V_H segments Lack junctional diversity	Generated by somatic recombination; random use of entire V_H cluster; high junctional diversity Somatic mutation in GC, memory B

Table 1-2: Overview of B-1 and B-2 B cells. (Naradikian MS., *et.al*, 2014)

The expression of B cell receptor is fundamental to mature B-cell. Both B-1 and B-2 cell lineage undergo V(D)J rearrangement. The V(D)J rearrangement provides the basis for classifying the development of B-2 cells in bone marrow (**Table 1-3**).

Developmental stages			
Osmond	Melchers and Rolink	Hardy	Status of Ig loci
Pro-B	Pre-pro B	A	Germline
	Pro-B	B	D–J_H rearrangement
		C	V_H–DJ_H rearrangement
Pre-B	Large pre B	C′	V_H DJ_H pairs with λ5-Vpre-B Pre-BCR surface expression
	Small pre B	D	V_κ–J_κ or V_λ–J_λ rearrangement
Immature B	Immature B	E	Complete BCR (receptor editing can occur)

Table 1-3: Developmental stages of B-2 cells in bone marrow according to different research groups. (Naradikian MS., *et.al*, 2014)

After reaching the immature stage, the developing B-2 cells will leave the bone marrow and will enter the circulation as transitional (TR) B cells. Transitional zone B cells are divided in three subsets T1, T2 and T3 before they develop in to follicular (FO) or marginal zone (MZ) B subset (**Table 1-4**).

Location	B cell subset	Phenotype
Bone marrow	Newly formed, immature	$AA4^+$ $CD23^-$ $sIgM^{high}$ $sIgD^{-/low}$ HSA^{high} $CD62L^-$ $CD21/35^{-/low}$
	T2-like	$AA4^+$ $CD23^+$ $sIgM^{high}$ $sIgD^{high}$ HSA^{high} $CD62L^+$ $CD21/35^{low}$
	Mature	$CD23^+$ $AA4^-$ $sIgM^{low}$ $sIgD^{high}$ HSA^{low} $CD62L^+$ $CD21/35^{low}$
Spleen	Transitional T1	$CD23^-$ $AA4^+$ $sIgM^{high}$ $sIgD^{-/low}$ HSA^{high} $CD62L^-$ $CD21/35^{-/low}$
	Transitional T2	$CD23^+$ $AA4^+$ $sIgM^{high}$ $sIgD^{high}$ HSA^{high} $CD62L^+$ $CD21/35^{low}$
	Transitional T3	$CD23^+$ $AA4^+$ $sIgM^{low}$ $sIgD^{high}$ HSA^{high} $CD62L^+$ $CD21/35^{low}$
	Follicular type I	$CD23^+$ $AA4^-$ $sIgM^{low}$ $sIgD^{high}$ HSA^{low} $CD62L^+$ $CD21/35^{int}$
	Follicular type II	$CD23^+$ $AA4^{-/low}$ $sIgM^{high}$ $sIgD^{high}$ HSA^{low} $CD62L^+$ $CD21/35^{int}$
	MZP	$CD23^+$ $AA4^{-/low}$ $sIgM^{high}$ $CD1d^+$ $sIgD^{high}$ HSA^+ $CD21/35^{high}$
	MZ	$CD23^-$ $AA4^-$ $sIgM^{high}$ $CD1d^+$ $sIgD^{low}$ HSA^+ $CD21/35^{high}$

Table 1-4: Cell surface markers for identification of bone marrow and splenic B cell sub-populations. (Allman D., Pillai S., *Curr Opin Immunol*., 2008, modified)

The fate of immature B-cell to develop into follicular or marginal depends on the extent of the BCR signal. Mouse studies have revealed that mature follicular zone B-cell numbers were increased as a result of increased BCR signal strength when a zinc finger protein Aiolos was knocked down.[18] On the other hand, low or weak strength of BCR signal favours the development of marginal zone B cells.[18] The mature B cells (IgD^{high}) from spleen will leave in to circulation and travel back to the bone marrow. Various *in vivo* mouse studies

have proven that knocking out one or more functional protein in BCR signalling pathway leads to abnormal or no development of mature follicular or marginal zone B cells.

1.2.2.1.b *Functional role of B cells*

B cells are not only antibody secreting cells but also serve many important functions in maintaining the structure of secondary lymphoid organs. In addition to antibody secretion, there are investigations showing that B cells are involved in antigen presentation, cytokine secretion and secretion of interleukin 10 (IL-10).[19] B cell-deficient mice are also susceptible to autoimmune encephalomyelitis (EAE) highlighting the importance of B cells in autoimmune disease.[20] Genaral B cell functions are summerised below (**Fig. 1-2)**.

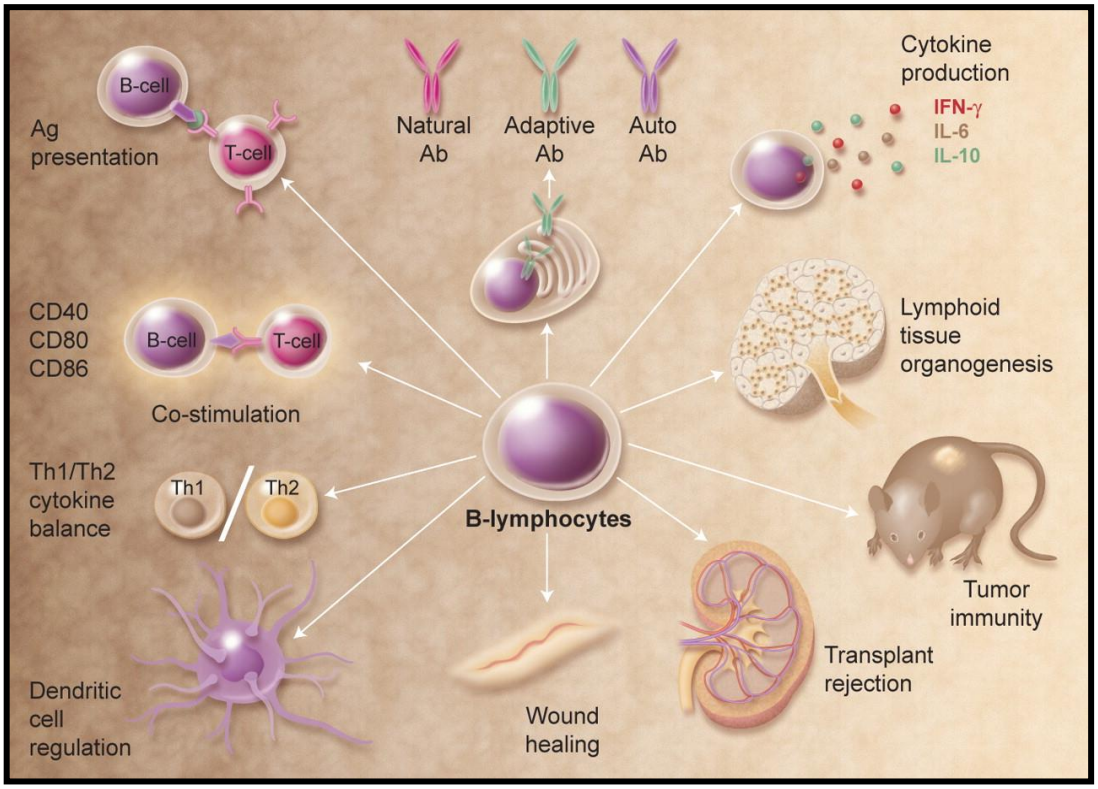


Fig.1-2: Multifunction of B cells. (LeBien TW., Tedder TF., *Blood*, 2008)

B cells are also required for the priming of CD4 T cells. In humans, abnormalities in B-cell development can lead to immunodeficiency's like X-linked aggamaglobulinemia (XLA).[21, 22] B-cell production of proinflammatory cytokines can also lead to autoimmunity by presentation of self-antigens to T cells.[23] The splenic marginal zone separates white pulp from red pulp in the spleen. Due to the presence of marginal zone macrophages and marginal zone B cells in the white pulp area it plays an important role in up-taking blood born antigens

and gives the spleen antibacterial immunity. Follicular zone B-cells which are localized in follicles and the location of marginal zone B-cells around the white pulp are not only important for development but also for the maintenance of splenic marginal zone structure.[24] Marginal zone B-cells by their pre-activated state and location, joins B-1 B cells in the initial days of antigen response to generate massive IgM producing plasmablasts, providing a bridge between early innate and later adaptive immune responses.[25] In addition to T-independent responses, MZ B-cells can also participate in T-dependent responses to protein antigens by helping to deliver these antigens to follicular B cells. Furthermore, they may be activated by T-dependent antigens and receive T cell help.[26] Also high levels of CD1d expression on MZ B-cells could lead to presentation of lipid antigens to NKT cells via the CD40-CD40 interactions giving rise to rapid class switched and somatically mutated antibody responses.[27] In this study, we identified carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) as being important for B cell development and maturation. Lack of CEACAM1 was linked to loss of marginal zone B cells in the spleen which resulted in less antigen presentation, antiviral antibody production and early death of *Ceacam1*$^{-/-}$mice following cytopathic vesicular stomatitis virus (VSV) infection.

1.2.2.2 *T cells*

T cells derive their name from their site of maturation the thymus. Like B-cells, T cells also express unique antigen binding receptor called the T-cell receptor (TCR). However, compared to B-cells which can detect soluble or particulate antigen by membrane bound antibodies, T-cells receptors recognize only processed antigens by APC's bound to their MHC molecules.[1] T cells are generally divided in two major types either T helper cell (T_H) or T cytotoxic (T_C) cell. Both cell types can be differentiated by presence of CD4 or CD8 molecules on their surface respectively. CD4 T cells generally function as T_H cells whereas CD8 T cells function as T_C cells recognising antigen in complex with MHC-II and MHC-I respectively.[1]

DC's present antigenic peptides to naïve T cells in secondary lymphoid organs. Activated T cells rapidly expand and will migrate to the sites of antigen presence, and perform effector function such as cell mediated cytotoxicity and production of various cytokines. Cytotoxic CD8 T cells will directly lyse the infected cell containing the antigen, while CD4 T helper cells will produce cytokines which are cytolytic to target cells or

stimulate other T cell effector functions. CD4 T helper cells can also activate B cell antibody production.[28]

1.2.2.2.a *CD4 T cells*

Upon antigen activation CD4 T cells differentiate into different subsets namely Th1, Th2, Th9, Th17, Th22, Treg (regulatory T cells) and Tfh (follicular helper T cells), which secret distinct cytokines.[29]

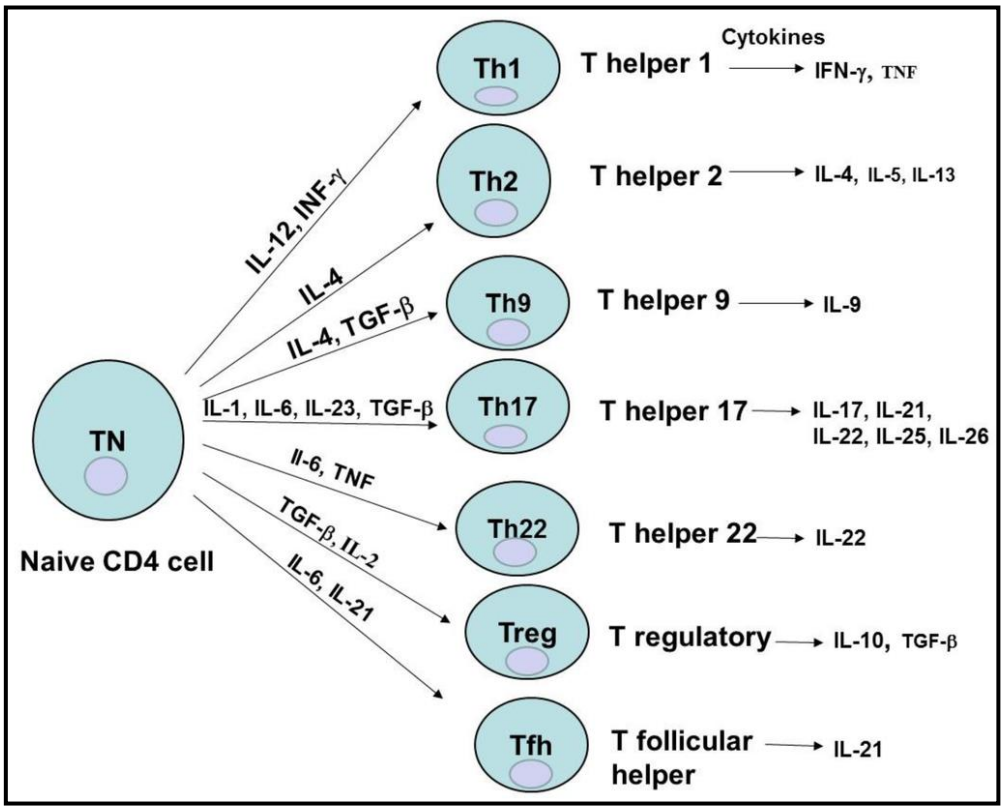


Fig. 1-3: Different CD4 subsets generated from naïve CD4 T cell by different cytokines and interleukins produced by them. (Golubovskaya V., Wu L., *Cancers*, 2016)

All CD4 T cell subsets are differentiated from naïve CD4 T cell by specific cytokines and each CD4 T cell subset secretes specific cytokines that have either pro- or anti-inflammatory functions, survival or protective functions (**Fig. 1-3**).[30]

1.2.2.2.b *CD8 T cells*

CD8 T cells also called cytotoxic T lymphocytes (CTL's), T-killer cells, killer T cells, destroy virus infected cells in acute and chronic viral infections.[31] CD8 T cells can also infiltrate solid tumors such as primary melanoma[32], ovarian cancers[33], bladder cancer[34], renal

cell carcinomas (RCC)[35] and several other cancers. CD8 T cells are also major players in the rejection of Graft in host transplant.[36, 37] These cells recognize the target by binding to the antigen associated with MHC-I molecule on antigen presenting cells. After antigen challenge these cells differentiate into stem cell memory cells (T_{SCM}), T central memory cell (T_{CM}), T effector memory cell (T_{EM}) and T effector cells (T_{EFF}).[30]

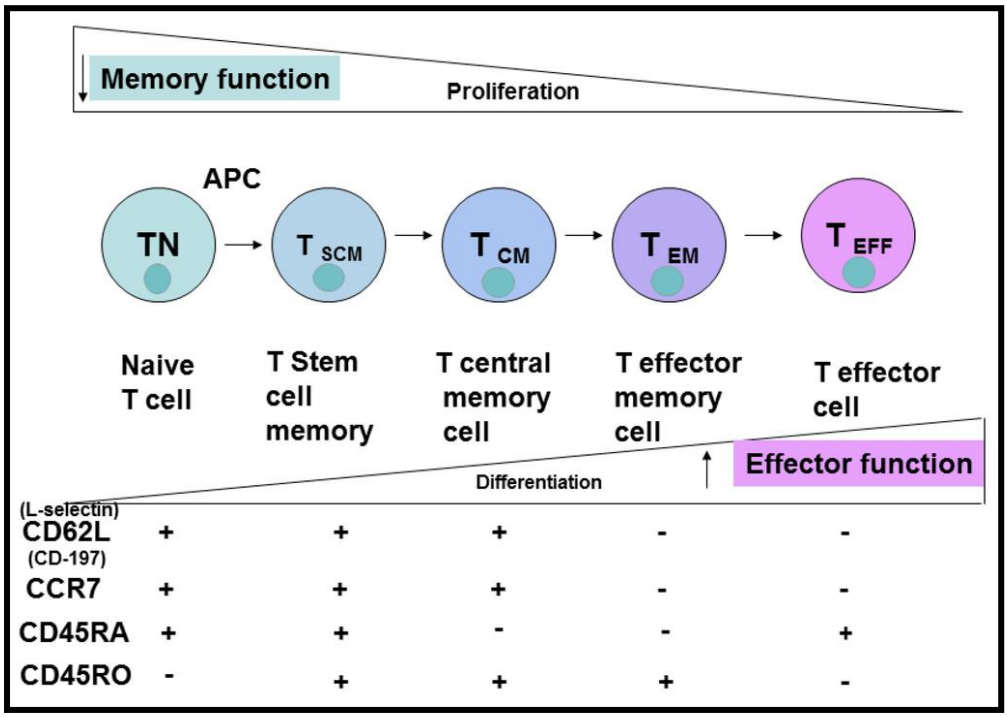


Fig. 1-4: Differentiation of CD8 T cell subset and different CD8 subsets.
(Golubovskaya V., Wu L., *Cancers*, 2016)

1.2.2.2.c *Memory T cells*

Memory T cells are a subset of T cells that persist for a long time even after the infection has been cleared. Memory T cells expand quickly to generate a large number of effector T cells after re-infection with the same antigen. CD4 as well as CD8 T cells can form memory which are characterized by expression of CD45RO marker on their surface (**Table 1-5**).[38] Memory cells can be located in secondary lymphoid organs or recently infected tissues.[30] Memory cells generally have several features: 1) Presence of previous expansion and activation, 2) persistence in the absence of antigen, 3) increased and quick activity upon re-exposure to antigen.[39]

1.2.2.2.d *Effector T cells*

During primary immune response, T cells differentiate into effector cells in lymphoid organs. They must immediately home to peripheral tissue containing pathogen to generate inflammation by stimulating immune cells.[40] Therefore, effector cells up-regulate the expression of receptors for inflammation-induced endothelial adhesion molecules and inflammatory chemokines (**Table 1-5**).[41] However, different pathogens can induce different effector responses generated by Th1 or Th2 cells.

	Naïve	Effector	T_{EM}	T_{CM}
CCR7	+++	_	_	+++
CD62L	+++	_	+/-	+++
CD45RO	+	+++	+++	+
CD45RA	+++	_	+	++
CD95	+/-	+++	++	+/-
Granzyme B	_	+++	+/-	_
CD25	_	+	_	_
CD127	++	+/-	+	+++
CD28	++	_	+	++

Table 1-5: Phenotypic markers associated with naïve, effector and memory cells. (Nijkamp FP., Parnham MJ., *Principles of Immunopharmacology*, 2011, modified)

1.3 *Lymphotoxins*

Lymphotoxin is a protein produced by Th1 T-cells which induces surface re-arrangement of vascular endothelial cells to change the structure of cell adhesion molecules to recognise the pathogens by phagocytic cells. The lymphotoxin-α (LT-α) and tumor necrosis factor alpha (TNF-α) cytokines belongs to the TNF superfamily. Following the discovery of lymphotoxin-β (LT-β), it became apparent that these molecules form a heterodimer complex $LT\alpha_1\beta_2$ which binds to a unique lymphotoxin-β receptor (LTβR).[42] Several remarkable immunological processes are carried out by lymphotoxins, including development of the lymph node, embryogenesis, generation and activation of dendritic cells (DC's), and homeostatic control of chemokine expression in lymphoid organs.[43, 44, 45] Lymphotoxin-β also plays an important role in development of Payer's patches and colon-associated lymphoid tissue.[46] B cells expressing LTα1β2 control splenic macrophage

phenotype and important in maintaining subcapsular sinus macrophages recruiting $CD169^+$ macrophages in marginal zone.[7]

LTαβ is expressed on activated T, B and Natural Killer (NK) cells as well as on subsets of resting B cells.[47] Inducer cells characterised by $CD4^+CD3^-$ expression, which are important in lymph node organogenesis express LT.[48] During the developmental process lymphocytes that express LTαβ communicate with the cells that express the LTβR, triggering the secretion of chemokines such as CXCL13, CCL19 and CCL21 and upregulation of cell adhesion molecule.[49, 50]

There are several reports showing that LTβR signalling is involved in autoimmunity. Treatment with anti-LTβ monoclonal antibody (mAb) showed effective preventiion of autoimmune encephalomyelitis (EAE) in rat models.[44] Different roles played by LTα1β2+ B cells are summerised below (**Fig. 1-5**).

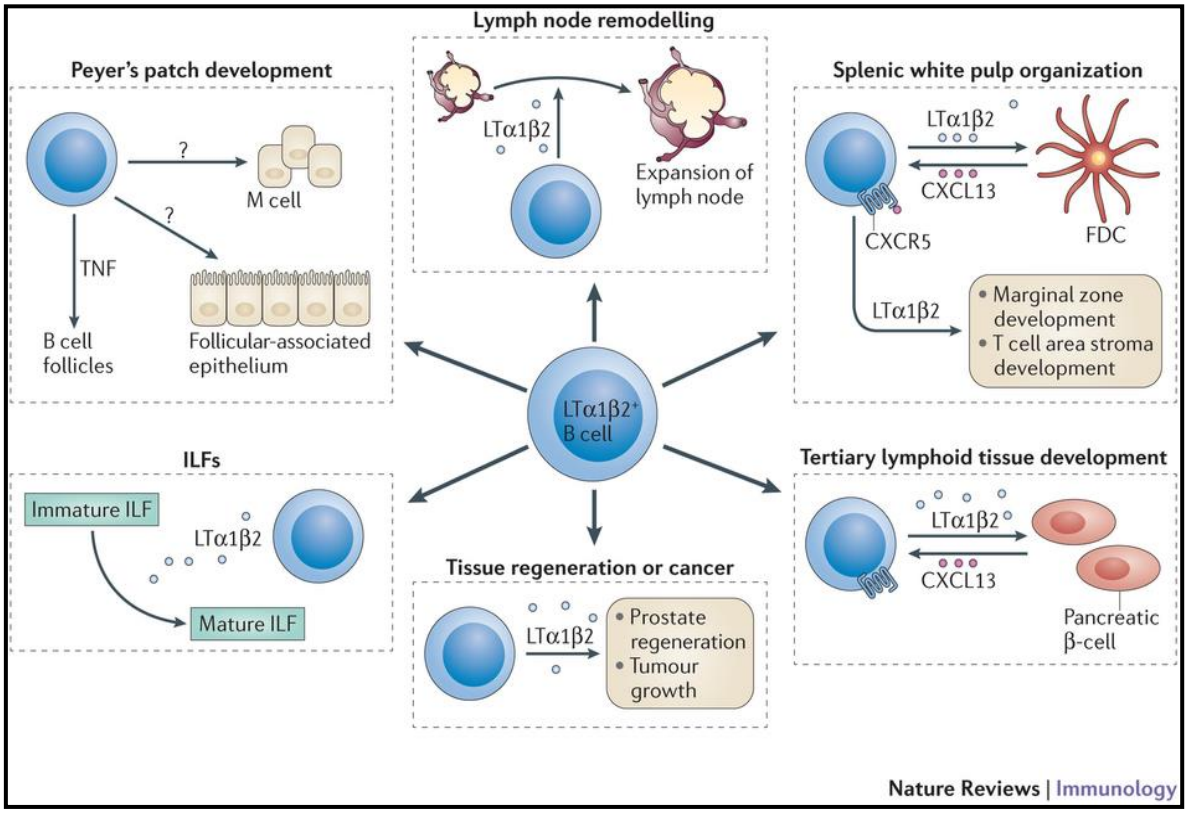


Fig. 1-5: The role and function of LTαβ secreting B cells in lymphoid organ development. (Shen P., Fillatreau S., *Nat Rev Immunol*, 2015)

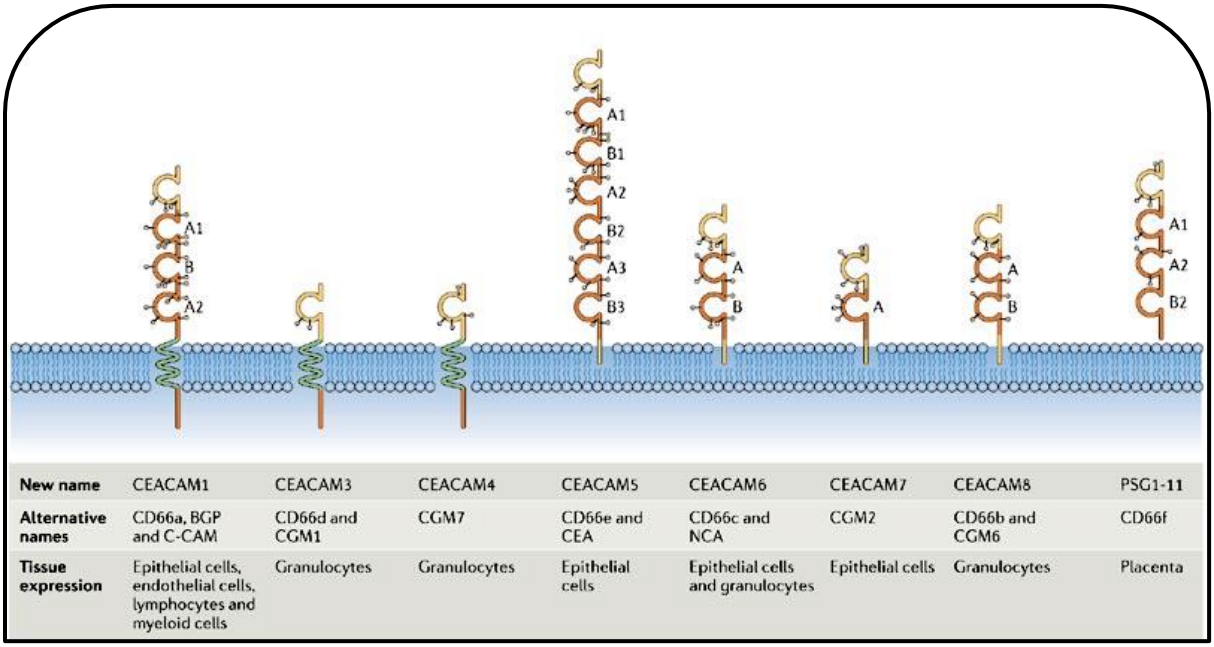
There is evidence to indicate that LTβ can influence CD8 T cell priming to viral infection. In case of LCMV challenge, LTβ deficient mice ($Lt\beta^{-/-}$) showed poor CD8 T cell activity and failed to generate virus specific CD8 T cell response as well as memory CD8 T

cell response.[51] As a result of insufficient CD8 T cell priming *Ltβ*$^{-/-}$ mice failed to generate IFN-γ and showed delayed viral clearance.[51]

In line with these results, we in our study found that viral replication of initial inoculum is essential for CD8 T-cell activation and priming. On the other hand, extracellular distribution of virus along the splenic conduits and virus replication in marginal zone is necessary for inducing type I interferon (IFN-I) response. The presence of LTβ is important for distribution of virus along the marginal zone; therefore, the lack of LTβ resulted in limited systemic production of IFN-I but in normal CD8 T-cell responses.

1.4 *Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1)*

Carcinoembryonic antigen (CEA) family is involved in intercellular binding interactions important for cellular growth and differentiation. These are subdivided into the-CEA-related-cell adhesion molecules (CEACAM's) and pregnancy specific glycoproteins (PSG's) (**Fig. 1-6**).[52] CEACAM's usually mediate homophilic and/or heterophilic interactions with other CEACAM's.[53]



New name	CEACAM1	CEACAM3	CEACAM4	CEACAM5	CEACAM6	CEACAM7	CEACAM8	PSG1-11
Alternative names	CD66a, BGP and C-CAM	CD66d and CGM1	CGM7	CD66e and CEA	CD66c and NCA	CGM2	CD66b and CGM6	CD66f
Tissue expression	Epithelial cells, endothelial cells, lymphocytes and myeloid cells	Granulocytes	Granulocytes	Epithelial cells	Epithelial cells and granulocytes	Epithelial cells	Granulocytes	Placenta

Fig. 1-6: Different types of CEACAM's in human and their tissue expression (Grey-Owen SD., Blumberg R., *Nat Rev Immunol.*, 2006, modified)

CEACAM1 is expressed by epithelial, endothelial, B and T lymphocytes and myeloid cells.[54, 55] CEACAM1 consists of amino terminal, immunoglobulin-variable-region (IgV-like) like domain followed by immunoglobulin constant-region-type-2-(IgC2-like) like domain.[52] CEACAM1 also contains cytoplasmic and transmembrane domain. Isoforms with long cytoplasmic domain (CEACAM1-4L) contains two immunoreceptor tyrosine-based inhibitory motifs (ITIMs), which usually transmit inhibitory signals.[52] On the other hand, isoforms with short cytoplasmic tail (CEACAM1-4S) lacks ITIMs, but contains sequences that can bind to calmodulin[56], tropomyosin and globular actin showing interaction with the cytoskeleton (**Fig. 1-7**).[57]

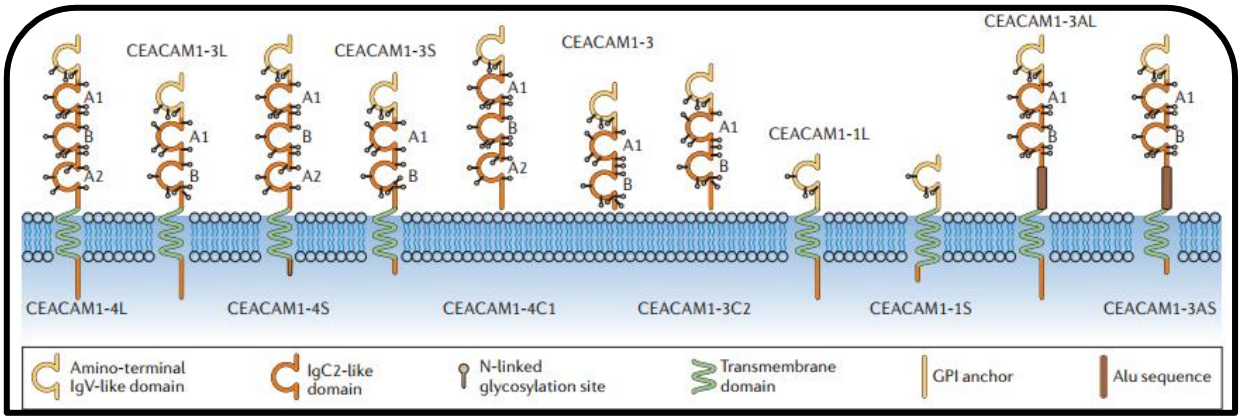


Fig. 1-7: Different isotypes of CEACAM1 in human (Grey-Owen SD., Blumberg R., *Nat Rev Immunol.,* 2006, modified)

CEACAM1 has been shown to be involved in a variety of immune functions. CEACAM1 is expressed on almost all immune cells including B cells, T cells, NK cells, neutrophils, dendritic cells, granulocytes and monocytes. CEACAM1-L recruits SHP-1 and SHP-2 to its cytoplasmic tail in an ITIM dependent manner and has been shown to be important for inhibition of mouse epithelial tumour-cell growth. Whereas CEACAM1-4S has been shown to induce apoptosis in epithelial cells.[58] CEACAM1 is also involved in B cell-receptor mediated complex activation. Anti-CEACAM1 antibody (mAb) strongly triggered mouse B cells together with IgM crosslinking.[59] Recently, it has been shown that CEACAM1 expression is increased on B-cells and CEACAM1^{+} B cells were present in brain infiltrates of multiple sclerosis (MS) patients. Treatment with anti-CEACAM1 antibody resulted in blocking aggregation of B cell derived from MS patients.[60] T cells also express CEACAM1 *in vivo* during coeliac disease[61] and in large intestine during inflammatory bowls disease.[62] Similarly, down regulation of CEACAM1expression in a non-tumorigenic cell line increased

its tumorigenic capacity[63]. Conversely, over expression of CEACAM1 in cancer cells supress their tumorigenic phenotype both *in vitro* and *in vivo*.[63, 64]

In this study, we showed that intrinsic signalling of CEACAM1 is essential for generating efficient B-cell responses. Expression of CEACAM1 induced survival of proliferating B cells via the BTK/Syk/NF-κB-axis. Lack of this BCR signalling cascade in naive *Ceacam1*$^{-/-}$ mice limited the survival of B cells and hence total B cell numbers. Hence, when the *Ceacam1*$^{-/-}$ mice were challenged with cytopathic VSV, they could not induce neutralizing antibody responses and died early after infection. We found that CEACAM1 is a crucial regulator of B-cell survival, influencing B-cell numbers and is important for generating antiviral antibody responses.

1.5 *Viruses*

In this study, we used different strains of noncytopathic virus called lymphocytic choriomeningitis virus (LCMV) to check the role of enforced viral replication in innate and adaptive immune activation. We used LCMV strain WE (LCMV-WE), an acute virus strain and recombinant LCMV strain (rLCMV), to investigate the role of IFN-I in CD8 T-cell priming. To investigate the role of virus-specific antibodies on enforced virus replication and CD8 T-cell priming in addition to LCMV-WE we used LCMV strain Docile (LCMV-Docile) which induces chronic infection and persists longer in the host system.

We also used cytopathic virus, vesicular stomatitis virus (VSV) to understand the role of CEACAM1 in antibody responses.

1.5.1 *Lymphocytic choriomeningitis virus (LCMV)*

The first strain used in our study is LCMV-WE. LCMV was discovered in 1934 by Charles Armstrong. It's a noncytopathic arenavirus responsible for aseptic meningitis and encephalitis. The LCMV genome consist of two negative sense-single stranded RNA designated L and S.[65, 66] LCMV infection is initiated by attachment of virus to the host receptors through the glycoproteins. It is then endocytosed and fusion of virus and vesicle membrane is formed followed by release of the ribonucleocapsid into the cytoplasm.

Different strains of LCMV generate different T cell responses and the clearance is also strain-dependent. So far four strains have been described. Neurotropic ''Clone 13'' is a derivative of ''Armstrong'' strain and hepatotropic ''docile'' is a derivative of WE strain.[67] WE and Armstrong infection cause acute infection where the viral particles are cleared

within few days. On the other hand, docile and clone 13 cause chronic infection and it can take up to few weeks to clear the viral particles. Mice infected with lower dose such as 100 Plaque forming units (PFU) of LCMV-Docile cleared virus within 2-4 weeks because of normal cytotoxic T cell response (CTL), whereas mice infected with higher doses (1×10^7 PFU) generate weak CTL responses and the viral particles are detectable until day 280 post infection.[68] On the other hand with an intermediate dose of 1×10^4 PFU of LCMV-Docile mice failed to cleared the virus and exhibited high immunopathology.[68] The balance between the viral load and T cell response is shown below (**Fig. 1-8**).

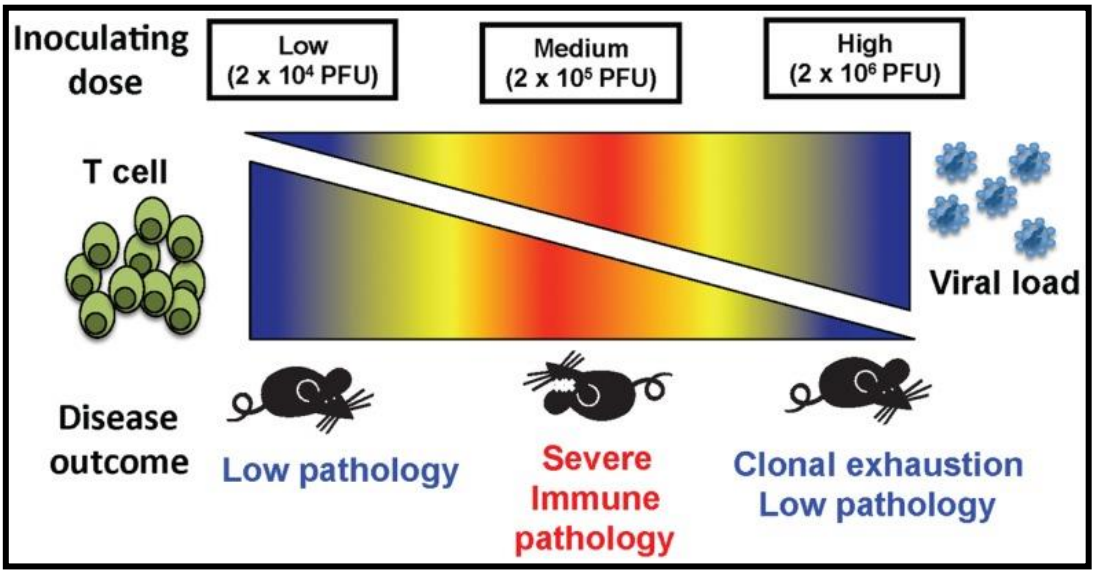


Fig. 1-8: Balance between the viral load and T cell response in terms of disease outcome. (Cornberg M. *et al*., *Front. Immun.*, 2013, modified)

Disease progression in LCMV-Docile or LCMV-Clone13 infection is due to dysfunction of T cells. Exhaustion is a state of T-cell where the ability to produce cytokines such as IL-2 and higher proliferative and *ex vivo* capacities are lost. The production of antiviral cytokines such as TNF-α and IFN-γ are abolished and in final stage the CD8 T cells are deleted.[69] As a result of high activation due to viral load the hepatocytes in liver are targeted by CD8 T cells which results in high levels of immunopathology in the infected organism.

1.5.2 *Vesicular Stomatitis Indiana Virus (VSV)*

Another virus we used in our study is Vesicular Stomatitis Virus (VSV) belonging to the family *Rhabdoviridae*. VSV is a cytopathic virus and the control of the virus is highly dependent on antiviral antibodies generated by B cells. The genome of VSV is single

negative sense-RNA that encodes five major proteins: G protein (G), large protein (L), phosphoprotein, matrix protein (M) and nucleoprotein.

VSV exhibits exclusive neurotropism, and if not controlled early enough by immune responses can cause paralytic disease after 5 to 6 days of infection invading the central nervous system by breaching the blood-brain barrier reulting in death.[70]

In this study, we used VSV to check the role of CEACAM1 in antiviral antibody generation. We injected VSV to Wild type (WT) and *Ceacam1*$^{-/-}$ mice and found that lack of CEACAM1 hinders the production of anti-VSV neutralizing antibodies. Total neutralizing antibody titers in *Ceacam1*$^{-/-}$ mice were significantly lower than WT mice. Interestingly, neutralizing IgG antibody generation was completely missing in the *Ceacam1*$^{-/-}$ mice. To check if the viral replication in the spleen was the main reason to generate anti-VSV antibodies we used ultraviolet inactivated (UV) VSV and measured the antibody response and we did not see any secretion of total (IgM + IgG) as well as IgG neutralizing antibodies.

In the first part of the thesis, using LCMV-WE and rLCMV we showed how viral replication of the initial inoculum is essential for activating virus-specific CD8 T cells. In contrast, extracellular distribution of virus along the splenic conduits is necessary for induction of systemic levels of type I interferon (IFN-I). In second part, we investigated the role of CEACAM1 on B-cell survival and antiviral antibody secretion using the VSV model. In the last part of the thesis we show that upon recall infection virus specific antibodies allow viral replication in the splenic marginal zone. This replication was necessary for priming CD8 T cells and control of chronic virus infection. In contrast, memory CD8 T cells inhibited virus replication in marginal zone but failed to protect the mice upon chronic virus infection.

1.6 *Mouse models used*

- **P14 × CD45.1**

  These mice express a T-cell receptor (TCR) specific for the LCMV glycoprotein 33 to 41 (GP-33-41) as a transgene. Splenocytes from these mice were used in adaptive transfer experiments. Because T-cells from these mice express TCR specific against LCMV-GP-33-41, the T cell response is initiated immediately and can be measured by staining against the congenic marker CD45.1.[71]

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

  In these mice, the Ubiquitin-specific peptidase 18 (USP18), is knocked out. USP18 is also known as ISG15 and it is a negative regulator of Type I and Type III Interferon signalling.[72]

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

  These mice carry a deletion of the endogenous murine J segments of the Ig heavy chain locus. Lack of all four JH gene segment results in cells that cannot produce a complete, recombined version of the variable region of the heavy chain. These mice lack mature B cells in bone marrow and peripheral organs as well. However, these mice have normal T cell response.[73]

- ***Ltbr*$^{-/-}$**

  Lymphotoxin beta receptor (*Ltbr*$^{-/-}$) mice lack Payer's patches, colon associated lymphoid tissues and lymph nodes. These mice do not develop normal splenic architecture and marginal zone in spleen is absent as well.[46]

- ***Ltb*$^{fl/fl}$ × *CD19*^{cre}**

  These mice lack lymphotoxin solely on B cells. Only the splenic architecture of the mice is affected but these mice develop normal Payer's patches and have functional lymph nodes.[74]

- ***CD169-DTR***

  These transgenic mice have human diphtheria toxin receptor (DTR) under the promoter of CD169 gene. Hence the CD169^{+} marginal zone macrophages can be selectively depleted by administration of diphtheria toxin.[75]

- ***Ceacam1*$^{-/-}$**

  These mice lack CEACAM1 on all cells and is used to study the effect of CEACAM1 on B cell development and antiviral antibody production.[76]

- ***Tcrab*$^{-/-}$**

  These transgenic mice lack alpha beta T-cell receptor. There are normal number of $CD4^{-}CD8^{-}$ T cells but lack mature CD4 and CD8 T cells.[77]

- ***Aid*$^{-/-}$**

  The activation induced cytidine deaminase is expressed in germinal centre B cells. These mice lack immunoglobulin class switch recombination, immunoglobulin somatic hypermutations and lymph node hyperplasia.[78]

- ***sIgM*$^{-/-}$**

  These mice lack the secreted form of IgM in serum.[79]

- ***Myd88/Trif*$^{-/-}$**

  There mice are defective in both TLR-3 and TLR-4 mediated expression of IFN-β and activation of IRF-3.[80]

- ***Baffr*$^{-/-}$**

  These transgenic mice lack receptor for B-cell activating factor (BAFF). These mice exhibit defective splenic architecture, shows abnormal B cell development and impaired T-cell dependent antibody formation.[81]

- ***Fcgr2b*$^{-/-}$**

  These transgenic mice shows elevated levels of immunoglobulin in response to thymus dependent and independent antigens.[82]

- **Vi10 × CD45.1**

  These transgenic mice express VSV-specific BCR as a transgene. Splenocytes from these mice were used for transfer experiments. The antibody response can be measured by neutralization assay from serum.[83]

❖ ***Prf1*** $^{-/-}$

Mice transgenic for Perforin do not lyse virus-infected or allogeneic fibroblasts or natural killer target cells *in vitro.* These mice also failed to clear LCMV. This molecule is important for T-cell and NK-cell mediated killing.[84]

❖ ***B2m*** $^{-/-}$

These mice are devoid of β_2-Microglobulin which is important for MHC-I expression and $CD8^+$ T-cell development. Hence in antigen challenge these mice lack cytotoxic T-cell function.[85]

❖ ***KL25 × CD45.1***

These transgenic mice have LCMV specific B-cell response. These mice were used for adoptive transfer experiments.[86]

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Two separate mechanisms of enforced viral replication balance innate and adaptive immune activation

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ABSTRACT

The induction of innate and adaptive immunity is essential for controlling viral infections. Limited or overwhelming innate immunity can negatively impair the adaptive immune response. Therefore, balancing innate immunity separately from activating the adaptive immune response would result in a better antiviral immune response. Recently, we demonstrated that *Usp18*-dependent replication of virus in secondary lymphatic organs contributes to activation of the innate and adaptive immune responses. Whether specific mechanisms can balance innate and adaptive immunity separately remains unknown. In this study, using lymphocytic choriomeningitis virus (LCMV) and replication-deficient single-cycle LCMV vectors, we found that viral replication of the initial inoculum is essential for activating virus-specific $CD8^+$ T cells. In contrast, extracellular distribution of virus along the splenic conduits is necessary for inducing systemic levels of type I interferon (IFN-I). Although enforced virus replication is driven primarily by *Usp18*, B cell–derived lymphotoxin beta contributes to the extracellular distribution of virus along the splenic conduits. Therefore, lymphotoxin beta regulates IFN-I induction independently of $CD8^+$ T-cell activity. We found that two separate mechanisms act together in the spleen to guarantee amplification of virus during infection, thereby balancing the activation of the innate and adaptive immune system.

1. Introduction

During infection with a persistence-prone virus, type I interferon (IFN-I) and $CD8^+$ T cells mediate the control of virus in infected cells. $CD8^+$ T cells are activated early by viral replication; however, prolonged viral replication results in $CD8^+$ T-cell exhaustion and persistence of virus. Recently, striking data showed that IFN-I is like a yin and yang for controlling virus [1–3]. On the one hand, IFN-I is clearly antiviral, and it can influence the very early viral replication that can affect the course of persistent infection [4]. On the other hand, it induces programmed death ligand 1 (PD-L1) and interleukin IL-10 and thereby drives $CD8^+$ T-cell exhaustion [1,2]. Therefore limited production of IFN-I in the presence of highly activated $CD8^+$ T cells results in severe immunopathology [5].

Lymphotoxins play an important role in several immunological processes, including lymph node development during embryogenesis, generation and activation of dendritic cells (DCs), and homeostatic control of chemokine expression in lymphoid organs [6–8]. Lymphotoxin beta (Ltb) plays a role in splenic architecture, developing conduits along the marginal zone and recruiting

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2. Chapter II:
Two separate mechanisms of enforced viral replication balance innate and adaptive immune activation

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2.1 *Abstract*

The induction of innate and adaptive immunity is essential for controlling viral infections. Limited or overwhelming innate immunity can negatively impair the adaptive immune response. Therefore, balancing innate immunity separately from activating the adaptive immune response would result in a better antiviral immune response. Recently, we demonstrated that Usp18-dependent replication of virus in secondary lymphatic organs contributes to activation of the innate and adaptive immune responses. Whether specific mechanisms can balance innate and adaptive immunity separately remains unknown. In this study, using lymphocytic choriomeningitis virus (LCMV) and replication-deficient single-cycle LCMV vectors, we found that viral replication of the initial inoculum is essential for activating virus-specific $CD8^+$ T cells. In contrast, extracellular distribution of virus along the splenic conduits is necessary for inducing systemic levels of type I interferon (IFN-I). Although enforced virus replication is driven primarily by Usp18, B cell-derived lymphotoxin beta contributes to the extracellular distribution of virus along the splenic conduits. Therefore, lymphotoxin beta regulates IFN-I induction independently of $CD8^+$ T-cell activity. We found that two separate mechanisms act together in the spleen to guarantee amplification of virus during infection, thereby balancing the activation of the innate and adaptive immune system.

2.2 *Introduction*

During infection with a persistence-prone virus, type I interferon (IFN-I) and $CD8^+$ T cells mediate the control of virus in infected cells. $CD8^+$ T cells are activated early by viral replication; however, prolonged viral replication results in $CD8^+$ T-cell exhaustion and persistence of virus. Recently, striking data showed that IFN-I is like a yin yang for controlling virus [1,2]. On the one hand, IFN-I is clearly antiviral, and it can influence the very early viral replication that can affect the course of persistent infection [3]. On the other hand, it induces programmed death ligand 1 (PD-L1) and interleukin IL-10 and thereby drives $CD8^+$ T-cell exhaustion [1,2]. Therefore, limited production of IFN-I in the presence of highly activated $CD8^+$ T cells results in severe immunopathology [4].

Lymphotoxins play an important role in several immunological processes, including lymph node development during embryogenesis, generation and activation of dendritic cells (DCs), and homeostatic control of chemokine expression in lymphoid organs [5,6,7]. Lymphotoxin beta (Ltb) plays a role in splenic architecture, developing conduits along the marginal zone and recruiting $CD169^+$ macrophages [8,9].

Recently we found that, during acute viral infection, viral replication in the marginal zone of the spleen and lymph nodes is essential for activating the innate and adaptive immune responses. This replication is dependent on ubiquitin-specific peptidase 18 (Usp18); therefore, the lack of Usp18 blunts the innate and adaptive immune responses [10,11]. Although Ltb obviously enhances replication in secondary lymphatic organs [9], the interplay between Ltb and Usp18 remains unknown.

In the present study, using lymphocytic choriomeningitis virus (LCMV), we found that extracellular distribution of virus along the splenic conduits is necessary for inducing systemic levels of IFN-I but not for inducing virus-specific $CD8^+$ T cells. Only the presence of Ltb-induced conduits allows the distribution of virus along the marginal zone; therefore, the lack of Ltb results in limited systemic production of IFN-I but in normal $CD8^+$ T-cell responses.

2.3 *Methods*

2.3.1 *Mice*

P14/CD45.1 mice expressing a T-cell receptor (TCR) specific for LCMV glycoproteins 33 to 41 (LCMV-GP33-41) as a transgene were maintained on a C57BL/6 background and were used for adoptive transfer experiments [13]. *Usp18*$^{-/-}$ mice were generated in the Dong-ER Zhang laboratory (University of California san Diego, La jolla CA, USA) and were bred heterozygously on a Sv129 × C57BL/6 background, generation F4; these mice were directly compared with littermate control animals. Heavy chain joining gene null (*Jh*$^{-/-}$) mice were maintained on a C57BL/6J background; lymphotoxin beta receptor null (*Ltbr*$^{-/-}$) mice were maintained on a C57BL/6N background. Conditionally targeted Ltb (*Ltb*$^{fl/fl}$) mice were crossed with CD19-Cre transgenic mice, and CD19-Cre-negative mice were used as littermate controls [14]. CD169-diphtheria toxin receptor (DTR) mice were maintained on a C57BL/6J background.

2.3.2 *Virus and plaque assays*

LCMV strain WE was originally obtained from F. Lehmann- Grube (Heinrich Pette Institute, Hamburg, Germany) and was propagated in L929 cells. Mice were infected intravenously with LCMV-WE at the indicated doses. Viral titers were measured with a plaque-forming assay using MC57 cells, as previously described [15]. We used recombinant LCMV (rLCMV; kindly provided by Daniel Pinschewer, University of Basel, Switzerland) to produce rLCMV vectors expressing a mutated version of LCMV-GP, which fails to confer viral infectivity but remains antigenic. We titrated these vectors according to standard procedures [16].

2.3.3 *Lymphocyte transfer*

Splenocytes from P14/CD45.1 mice were injected intravenously into *Usp18*$^{-/-}$ mice and littermate control mice. One day later, mice were infected with rLCMV (6×10^4 plaque-forming units [PFU]), and the percentage of P14/CD45.1 T cells in the spleen was assessed by flow cytometry.

2.3.4 *Diphtheria toxin*

Diphtheria toxin was purchased from Sigma (ST Louis, Missouri, USA) and was injected intraperitoneally on day −3 and day 2 at a dose of 30 µg/kg.

2.3.5 *Cell culture and generation of bone marrow − derived macrophages*

Primary macrophages were generated by isolating bone marrow cells from femurs and tibias of mice. Macrophages were generated by culturing bone marrow cells in very low endotoxin Dulbecco's Modified Eagle's Medium (VLE-DMEM) supplemented with 10% (v/v) fetal calf serum (FCS), 0.1% (v/v) b-mercaptoethanol (b-ME), and 10 ng/ml macrophage colony-stimulating factor (M-CSF). After 9 days, macrophages were harvested for use in subsequent experiments. Recombinant mouse lymphotoxin α2/β1 was purchased from R&D Systems (Minneapolis, USA).

2.3.6 *Flow cytometry*

Tetramers were provided by the National Institutes of Health (NIH) Tetramer Core Facility (Emory University, Atlanta, GA, USA). Cells were stained with allophycocyanin (APC)-labelled GP33 major histocompatibility complex (MHC) class I tetramer (GP33/H-2Db) for 15 min at 37 °C. After incubation, the samples were stained with anti-CD8 (eBiosciences, San Diego, USA) for 30 min at 4 °C. Absolute numbers of GP33-specific $CD8^+$ T cells were calculated by fluorescence-activated cell sorting (FACS) analysis using fluorescent beads (BD Biosciences, San Jose, CA, USA). For measurement of intracellular IFN-γ, cells were stimulated with glycoprotein 33 (GP33) or nucleoprotein 396 (NP396), fixed with 2% formaldehyde for 10 min followed by permeabilization with saponin, and stained with anti-IFN-γ or tumor necrosis factor (TNF-α) antibodies (eBioscience). For MHC-I expression, Blood was stained on day 4 with anti CD8, anti CD4 and anti MHC-I (eBioscience) antibodies.

2.3.7 *ELISA*

Enzyme-linked immunosorbent assays (ELISA) for IFN-α were performed according to the manufacturer's protocol (PBL Interferon Source, New Jersey, USA).

2.3.8 *Histology*

Conventional staining was performed as previously described [11]. Briefly, snap-frozen tissue was stained with rat anti-mouse polyclonal antibody to LCMV NP (VL4; made in house). Polyclonal anti-rat biotin antibody (eBioscience) and anti-biotin streptavidin peroxidase antibody (Thermo Scientific, Fremont, CA, USA) were then added before visualization with a 2-solution diaminobenzidine (DAB) staining kit (Invitrogen, Carlsbad, CA, USA), after which the tissue was stained with hematoxylin. Immunofluorescence

analyses of snap-frozen tissue were performed with anti-F4/80 (eBioscience), and anti-CD169 (Acris) antibodies. Mouse nonspecific goat anti-guinea pig immunoglobulin (IgG; Jackson ImmunoResearch, West Grove, PA, USA) antibody was injected into mice to check *in vivo* the early distribution of the antibody.

2.4 *Results*

2.4.1 *Viral amplification is suppressed in peripheral organs but is allowed in spleen and lymph nodes*

During systemic infection, macrophages in the liver (Kupffer cells) capture most of the virus. This activity is observed when real time polymerase chain reaction (RT-PCR) for LCMV is performed 30 min after infection (**Fig. 1A**). If Kupffer cells are depleted with Clodronate-Liposome, the uptake of virus by the liver is reduced, whereas viral uptake by spleen, kidney and lung is increased (**Fig.1A**). In normal case, without clodronate treatment, virus that is captured in the liver cannot amplify to detectable levels (**Fig. 1B**); however, virus can replicate only in spleen and lymph nodes (**Fig. 1B**). Histologic analysis of the spleen demonstrates that virus spreads along the conduits of the marginal zone; thus, once a lymph follicle is infected, the virus is quickly distributed along the marginal zone in the spleen (**Fig. 1C**). These findings indicate that the spread of virus along marginal zone conduits is essential for viral replication in the spleen.

2.4.2 *Lack of lymphotoxin beta limits the flow along the marginal zone*

Next, we questioned whether spread of the virus is regulated independently of viral replication and how these diverse mechanisms are regulated. We speculated that LTb is an important contributor to viral spread along the marginal zone; therefore, we infected wild-type (WT) and $Ltb^{fl/fl}$CD19-Cre (which lack LTb specifically on B cells) mice with LCMV and performed histologic analysis of the spleens. $Ltb^{fl/fl}$CD19-Cre mice exhibited several spots of infected cells but no distribution along the marginal zone (**Fig. 2A**). This finding suggests that Ltb is indeed necessary for viral distribution within the marginal zone of the spleen. We speculated that the lack of conduits in mice lacking the Ltb receptor ($Ltbr^{-/-}$) limits flow along the marginal zone, a factor that could explain the limited spread of virus. To explore this speculation, we injected fluorescent beads into WT and $Ltbr^{-/-}$ mice. Histologic staining detected only limited numbers of beads in the marginal zone area of *Ltbr*$^{-}$

$^{-/-}$ mice compared to WT mice (**Fig. 2B**), a finding supporting the hypothesis that flow of the virus along the marginal zone of the spleen is limited in the absence of Ltb. To validate our finding we then injected mice with xenogenous nonspecific antibody labeled with fluorescent cyanine dye into WT and Ltbr$^{-/-}$ mice and analyzed its distribution after 2 min. In WT mice, antibody stained the conduits along the marginal zone of the spleen (**Fig. 2C**). In contrast, Ltbr$^{-/-}$ mice exhibited limited staining along the marginal zone area (**Fig. 2C**). Liver vessels were not affected by Ltb, a finding suggesting that the formation of conduits is specifically sensitive to Ltb (**Fig. 2C**) and are important for the cell to cell distribution of the viral particles. In conclusion, we found that the absence of Ltb limits flow of the antigen along the marginal zone, and this limitation in turn reduces the distribution of virus in the spleen.

2.4.3 *Usp18 and lymphotoxin beta allow viral replication in the spleen*

Next, we questioned how the limited viral spread in the marginal zone influences the production of virus in the spleen. First, we infected bone marrow derived macrophages of WT and Usp18 deficient mice with LCMV and measured the virus titer in the supernatant after 24 h. We found that Usp18 enhances LCMV replication on a cellular level, as demonstrated by *in vitro* study (**Fig. 3A**). Similarly, *in vivo* study showed that Usp18 enhances viral replication as WT mice have higher titer of LCMV relative to *Usp18*$^{-/-}$ mice (**Fig. 3B**). In contrast, the lack of Ltb does not reduce viral replication *in vitro* (**Fig. 3C**). Moreover, treating cells with recombinant LTα2/β1 did not reduce viral titer in the supernatant (**Fig. 3C**). Interestingly, *in vivo*, *Ltbr*$^{-/-}$ mice exhibit limited viral replication (**Fig. 3D**). To see whether this limited viral replication was due to reduction of CD169^{+} macrophages, we depleted CD169^{+} cells in CD169-DTR mice and infected them with LCMV. The depletion of CD169^{+} macrophages enhances viral replication (**Fig. 3E** and **Supplementary Figure 1**) which means that the limited replication in *Ltbr*$^{-/-}$ mice is not caused by the reduction in the number of CD169^{+} macrophages. Instead, viral replication is limited by Ltb-derived B cells, because viral replication is similarly reduced in *Jh*$^{-/-}$ and *Ltb*$^{fl/fl}$CD19-Cre mice (**Fig. 3F**). Therefore, we conclude that both Usp18 and Ltb are essential for enforced viral replication in the spleen.

2.4.4 *Usp18 and lymphotoxin beta are essential for inducing systemic type I interferon production, but only Usp18 influences CD8$^+$ T-cell priming*

We found that Usp18 influences viral replication, whereas Ltb guarantees viral spread along the marginal zone. Both mechanisms are necessary for viral replication in the spleen. To determine how Usp18 and Ltb influence the innate and adaptive immune responses, we infected Usp18$^{-/-}$ mice with 200 PFU of LCMV and measured IFN-I in the serum. We found that early viral replication is essential for IFN-I production (**Fig. 4A**). Similarly, IFN-I production is strongly impaired in *Ltb*$^{fl/fl}$CD19-Cre mice (**Fig. 4B**). Although viral replication was not completely inhibited in both Usp18$^{-/-}$ and *Ltb*$^{fl/fl}$CD19-Cre mice, this replication was not enough to trigger IFN-I production. Next, we wanted to examine the effect of Usp18 and Ltb on adaptive immune responses. As expected, the lack of Usp18 limits the magnitude of CD8$^+$ T-cell responses (**Fig. 4C**). Lately, it was shown that IFN-I can protect CD8$^+$ T cells from killing by NK cells through upregulation of MHC-I [17]. We wondered whether the reduction of CD8$^+$ T cell numbers was due to lack of IFN-I production. To check this, we measured MHC-I expression on T cells on day 4 after infection. We found that Usp18-deficient T cells showed higher MHC-I expression than WT T cells (**Supplementary Figure 2**) which can be explained due to a stronger IFN-I signalling in these cells.

In contrast to Usp18$^{-/-}$ mice, *Ltb*$^{fl/fl}$CD19-Cre mice exhibit CD8$^+$ T-cell responses similar to those of WT mice (**Fig. 4D**). This finding suggests that viral spread along the marginal zone is essential for IFN-I production but not for CD8$^+$ T-cell priming.

2.4.5 *Extracellular spread of virus is essential for inducing systemic type I interferon production but not for inducing CD8$^+$ T-cell responses*

To determine whether the spread of virus along the marginal zone is essential for activating the innate but not the adaptive immune response, we used a single-cycle virus (rLCMV). Because this virus does not express the full LCMV-GP, cells infected with rLCMV do not produce infectious virus particles. However, viral genome transcription and replication are allowed within the cells that were initially infected. We infected C57BL/6 mice with LCMV or rLCMV. We found that, LCMV induces large amounts of serum IFN-I at almost all infectious doses tested, whereas rLCMV does not induce detectable levels of systemic IFN-I (**Fig. 5A**). Therefore, we conclude that the spread of virus is essential for inducing IFN-I.

We next examined the role of rLCMV in activating $CD8^+$ T cells. We found that, compared to LCMV infection, infection with the rLCMV vector induces weaker but still potent virus-specific $CD8^+$ T-cell responses (**Fig. 5B**). As few as 100 infectious units produce measurable $CD8^+$ T-cell responses. Immunization with 10^5 PFU of vector results in frequencies of epitope-specific $CD8^+$ T cells that are similar to those produced by WT virus (**Fig. 5B**). To check whether single-cycle rLCMV exhibits limited $CD8^+$ T-cell expansion in the absence of Usp18, we transferred splenocytes from P14/ CD45.1 mice into WT and *Usp18*$^{-/-}$ mice, These mice express a T-cell receptor (TCR) specific for LCMV glycoproteins 33 to 41 (LCMV-GP33-41). We infected both groups with rLCMV and measured the number of transferred $CD8^+$ T cells. We found that $CD8^+$ T cells expand much less in the absence of Usp18 compared to WT mice (**Fig. 5C**). These findings led us to conclude that the induction of systemic IFN-I depends on extracellular transfer and dissemination of virus. In contrast, replication of the initial virus inoculum is sufficient for inducing the production of virus-specific $CD8^+$ T cells.

2.5 *Discussion*

The results of this study show that both Usp18 and Ltb contribute to viral replication in the spleen. Whereas Usp18 directly influences virus replication, Ltb guarantees the spread of LCMV along the marginal zone. Although LCMV replication is sufficient for $CD8^+$ T-cell activation, both effects, i.e., virus replication and extracellular virus transfer, are necessary for inducing systemic IFN-I.

These two mechanisms of virus propagation may influence the outcome of viral infection. This possibility is of special interest because both mechanisms influence replication separately. Usp18 is strongly induced by IFN-I, whereas Ltb is produced constantly in the spleen and is mainly influenced by the number of B cells in a lymph follicle. Therefore, various infections could regulate both mechanisms separately. In the presence of B cells and high levels of Ltb, IFN-I is induced more efficiently.

We found that the spread of virus along the marginal zone is essential for the induction of systemic IFN-I. During LCMV infection, plasmacytoid dendritic cells (pDCs) produce large amounts of IFN-I [18,19], for which the IFN-I-activating receptor retinoic acid-inducible gene-I (RIG-I) is at least partially responsible [20]. We determined that B cell-derived Ltb is an important contributor to extracellular distribution of virus along the

marginal zone. However, it remained unclear why this distribution of virus is essential for IFN-I induction. One explanation could be that only large amounts of viral RNA can sufficiently activate pDCs. A second explanation could be that the composition of the cell membrane of infected cells along the marginal zone is different from that of other cell types; therefore, virus particles received from these cells could be more likely to infect pDCs. Indeed, recent findings show that the composition of the cell membrane can influence viral tropism [21]. A third explanation could be that this distribution of virus is important for infecting cells that are near pDCs. Consequently, those pDCs may sense the presence of infected cells and produce IFN-I. Recent findings have shown that uninfected pDCs can produce IFN-I by sensing infected cells; this mechanism is independent of intrinsic viral replication in pDCs [22]. However, more studies are needed for determining which of these explanations may be correct.

It remains unclear why $CD8^+$ T cells but not IFN-α-producing cells can be activated by a single-cycle virus. We consider that the amount of viral protein and viral RNA within an infectious particle is limited compared to that in a virus-infected cell. Therefore, if one infectious rLCMV particle infects a DC, this virus-infected DC can probably generate a sufficient virus-specific $CD8^+$ T-cell response.

Recent publications reported that an overwhelming IFN-I response can induce IL-10 and PD-L1, which then affect the exhaustion of $CD8^+$ T cells [1,2]. Another supporting study showed a negative role of IFN-b in controlling persistent viral infection [3]. In light of our findings, we suggest that inhibiting the extracellular spread of virus may limit the virus-induced production of IFN-I and thereby may prevent the exhaustion of $CD8^+$ T cells. Various antiviral drugs target separate virus pathways. We suggest that antiviral drugs, which allow viral replication but block the release of virus particles, may inhibit viral propagation but still allow the priming and activation of antiviral $CD8^+$ T cells. If so, treatment with these drugs may provide the most effective long-term protection against virus.

Further studies are needed to be done to show whether these two mechanisms may influence additionally the peripheral IFN-II production and thereby contribute to relapse of lupus erythematosus [23,24].

In conclusion, we found that viral replication and extracellular viral spread are two separate mechanisms of enforced viral replication. Whereas viral replication is essential for activation of the innate and adaptive immune system, extracellular viral spread primarily affects systemic IFN-I production.

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: Viral amplification is suppressed in peripheral organs but allowed in spleen and lymph nodes.*

(A) C57BL/6 mice were injected with empty liposomes (as controls) or were depleted of macrophages with clodronate liposomes. After one day, mice were infected with lymphocytic choriomeningitis virus (LCMV; 2×10^6 plaque-forming units [PFU]), and after 30 min copies of LCMV were quantified by real time polymerase chain reaction (RT-PCR) (n = 6). **(B)** C57BL/6 mice were infected with LCMV (200 PFU), and viral titers were determined in various organs at indicated time points (n = 4). **(C)** Histologic staining for LCMV nucleoprotein (NP) was performed on splenic sections from C57BL/6 mice infected intravenously with LCMV (200 PFU) for three days (n = 3; scale bar in main images, 500 µm; inlets, 100 µm).

2.8.2 *Figure 2: Lack of lymphotoxin beta limits flow along the marginal zone.*

(A) Results of conventional histologic analysis of splenic sections from wild-type (WT) mice and conditionally targeted lymphotoxin beta (*Ltb*$^{fl/fl}$) CD19-Cre mice infected with lymphocytic choriomeningitis virus strain WE (LCMV-WE; 200 plaque-forming units [PFU]) for 3 days. LCMV, brown (n = 3; scale bar main images, 500 µm; inlets, 100 µm). **(B)** Immunofluorescent staining of spleen sections from C57BL/6 mice 10 min after being injected with fluorescent beads. Beads, green; CD169, red; F4/80, blue (n = 3; scale bar, 100 µm). **(C)** Immunofluorescent staining of spleen and liver sections from WT and lymphotoxin beta receptor deficient (*Ltbr*$^{-/-}$) mice 2 min after injection with anti-guinea pig immunoglobulin (IgG), red (n = 5; scale bar, 100 µm). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.8.3 *Figure 3: Ubiquitin-specific peptidase 18 and lymphotoxin beta allow viral replication in the spleen.*

(A) Bone marrow-derived macrophages from ubiquitin-specific peptidase 18 deficient (*Usp18*$^{-/-}$) mice and littermate control mice were infected with lymphocytic choriomeningitis virus strain WE (LCMV-WE; multiplicity of infection [MOI], 1). After 24 h, viral titer in the supernatant was determined (n = 4). **(B)** *Usp18*$^{-/-}$ and littermate control mice were infected intravenously with LCMV-WE (200 plaque-forming units [PFU]). After 3 days, viral titers in the spleen were determined (n = 3). **(C)** Bone marrow-derived macrophages from wild-type (WT) and lymphotoxin beta receptor deficient (*Ltbr*$^{-/-}$) mice were infected with LCMV-WE (MOI, 1) and treated with interferon alpha (IFN-α; 50 U/ml), (lymphotoxin α2/β1; 100 ng/ml), or both, or were left untreated. After 24 h, viral titer in the supernatant was determined (n = 3 - 6). **(D)** WT and *Ltbr*$^{-/-}$ mice were infected intravenously with LCMV-WE (200 PFU). After 3 days, viral titers in the spleen were determined (n = 3 - 4). **(E)** C57/BL6 or CD169 diphtheria toxin receptor (CD169-DTR) mice were treated with diphtheria toxin and infected with LCMV (200 PFU). After 2 days, viral titers in the spleen were determined (n = 5). **(F)** Heavy chain joining gene deficient (*Jh*$^{-/-}$), conditionally targeted Ltb (*Ltb*$^{fl/fl}$) CD19-Cre, and control mice were infected intravenously with LCMV-WE (200 PFU). After 3 days, viral titers in the spleen were determined (n = 3 - 4).

2.8.4 *Figure 4: Ubiquitin-specific peptidase 18 and lymphotoxin beta are essential for inducing systemic type I interferon, but only ubiquitin-specific peptidase 18 influences the priming of CD8$^+$ T cells.*

(A) Ubiquitin-specific peptidase 18 deficient (*Usp18*$^{-/-}$) mice and littermate control mice were infected intravenously with 200 PFU of lymphocytic choriomeningitis virus (LCMV). Interferon alpha (IFN-α) levels in serum were determined by enzyme-linked immunosorbent assay (ELISA) at the indicated time points (n = 4). **(B)** Wild-type (WT) and conditionally targeted lymphotoxin beta (*Ltb*$^{fl/fl}$)CD19-Cre mice were infected intravenously with 200 plaque-forming units (PFU) of LCMV strain WE. IFN-α levels were measured in the serum by ELISA at the indicated time points (n = 3-7). **(C)** Fluorescence-activated cell sorting (FACS) analysis of CD8$^+$ T cells positive for glycoprotein (GP33), nucleoprotein 396 (NP396), and IFN-γ in splenocytes from *Usp18*$^{-/-}$ and littermate control mice on day 30 after infection with 200 PFU LCMV-WE and six hours after re-stimulation with GP33 peptide or NP396 peptide (n = 4). **(D)** FACS analysis of GP33$^+$ IFN-γ$^+$ CD8$^+$ T cells and GP33$^+$ tumor necrosis factor TNF-α$^+$ CD8$^+$ T cells in splenocytes from (*Ltb*$^{fl/fl}$)CD19- Cre mice and control mice on day 14 after infection with 200 PFU LCMV-WE and six hours after re-stimulation with GP33 peptide (n = 5-8). n.s., not statistically significant.

2.8.5 *Figure 5: Extracellular spread of virus is essential for inducing systemic type I interferon but not for inducing a CD8$^+$ T-cell response.*

(A) C57BL/6 mice were infected intravenously with the indicated doses of lymphocytic choriomeningitis virus strain WE (LCMV-WE) or recombinant LCMV (rLCMV). Interferon alpha (IFN-α) in the serum was measured by enzyme-linked immunosorbent assay (ELISA) at the indicated time points (n = 3). **(B)** Percentages of nucleoprotein 396 (NP396)-specific and glycoprotein 33 (GP33)-specific CD8$^+$ T cells in blood were determined on day 9 after infection with the indicated doses of LCMV or rLCMV (n = 3-5, (virus dose 20,000 PFU; Tet-GP33$^+$ n = 2; one of two experiment is shown)). **(C)** We adoptively transferred 10^7 splenocytes from P14/CD45.1 mice into Ubiquitin-specific peptidase 18 deficient (*Usp18*$^{-/-}$) or wild-type (WT) littermate control mice. After 24 h, mice were infected intravenously with rLCMV (6×10^4 plaque-forming units [PFU]). Absolute number of CD45.1$^+$ CD8$^+$ T cells in total lymphocytes in blood and spleen was determined (n = 3-6).

2.8.6 *Supplementary Fig. 1: Lymphocytic choriomeningitis virus replicates in the absence of CD169^{+} macrophages.*

Wild-type (WT) C57BL/6 and CD169 diphtheria toxin receptor (CD169-DTR) mice were treated with diphtheria toxin (30 μg/kg) three days before being infected with lymphocytic choriomeningitis virus strain WE (LCMV-WE; 2 × 10^6 PFU). After 24 hours, immunohistologic staining for LCMV nucleoprotein was performed on splenic sections (n = 3; scale bar main images, 500 μm; inlets, 100 μm).

2.8.7 *Supplementary Fig. 2: Usp18 deficient T cells have higher MHC-I expression after LCMV infection.*

Ubiquitin-specific peptidase 18 deficient (*Usp18−/−*) mice (continuous line) and littermate control mice (dotted line) were infected intravenously with 200 PFU of lymphocytic choriomeningitis virus (LCMV). On day4, MHC-I expression was measured on T cells in the blood.

2.9 *Reference*

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Figure 1

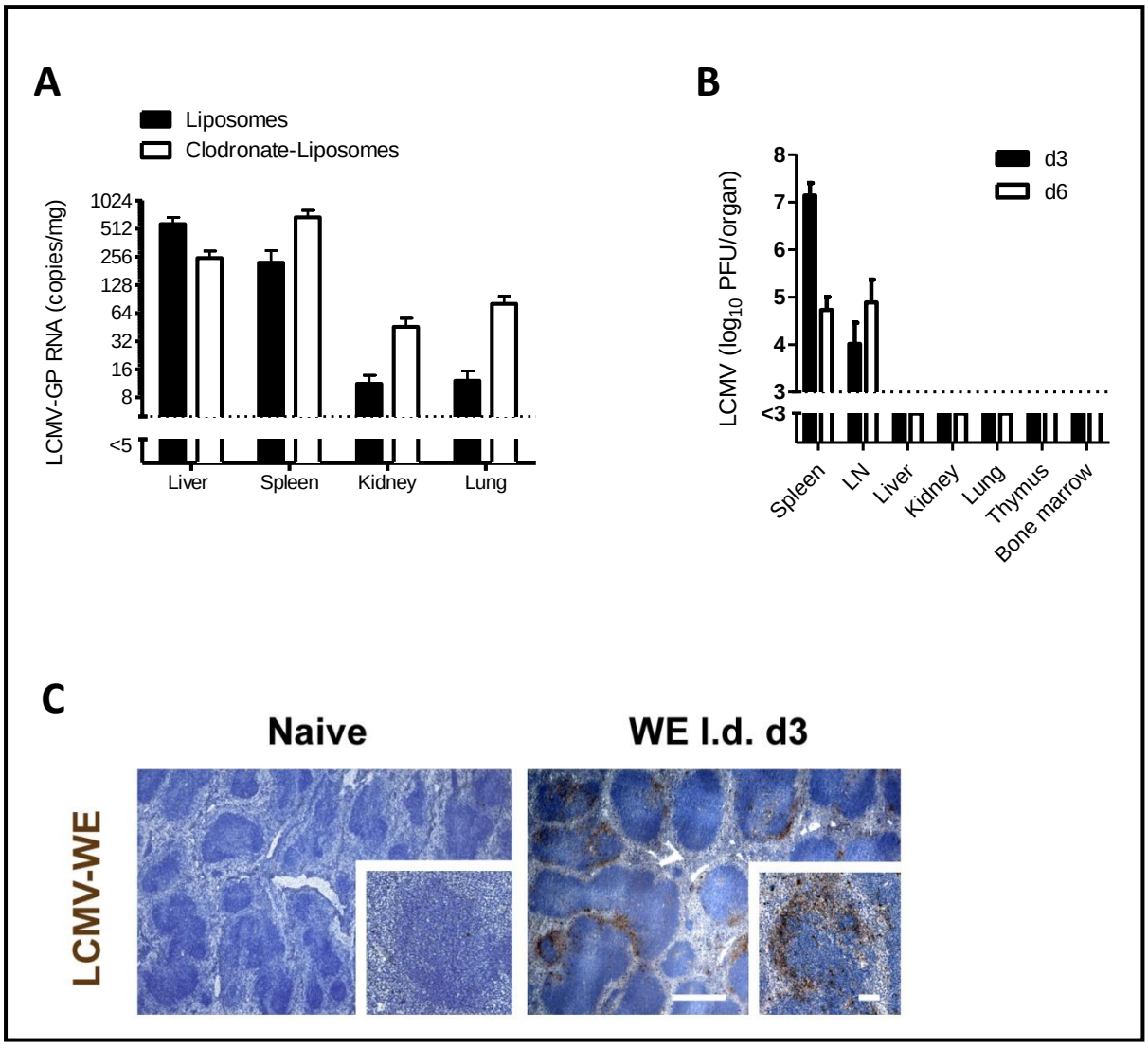


Figure 2-1: ***Viral amplification is suppressed in peripheral organs but allowed in spleen and lymph nodes.***

Figure 2

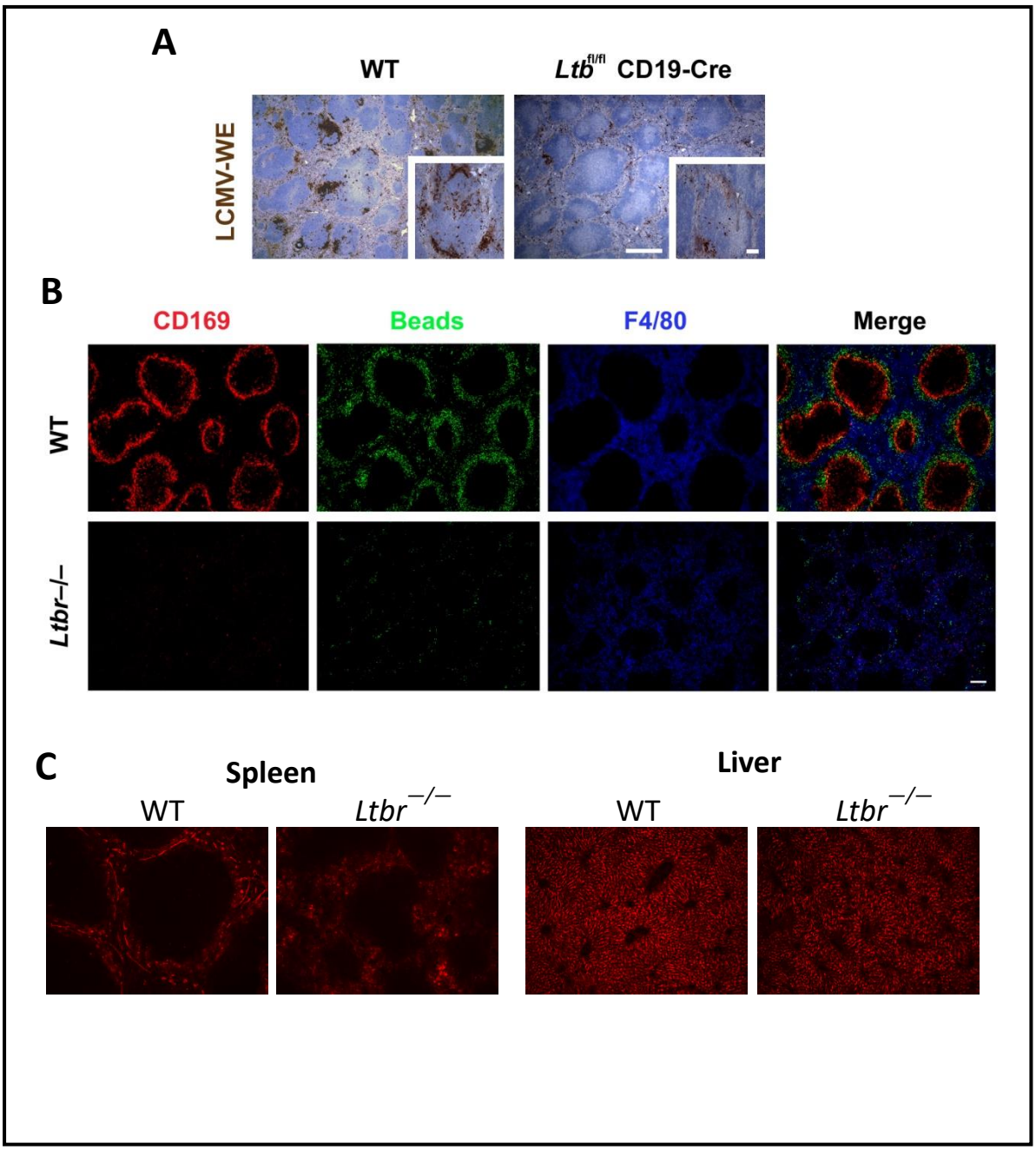


Figure 2-2: ***Lack of lymphotoxin beta limits flow along the marginal zone.***

Figure 3

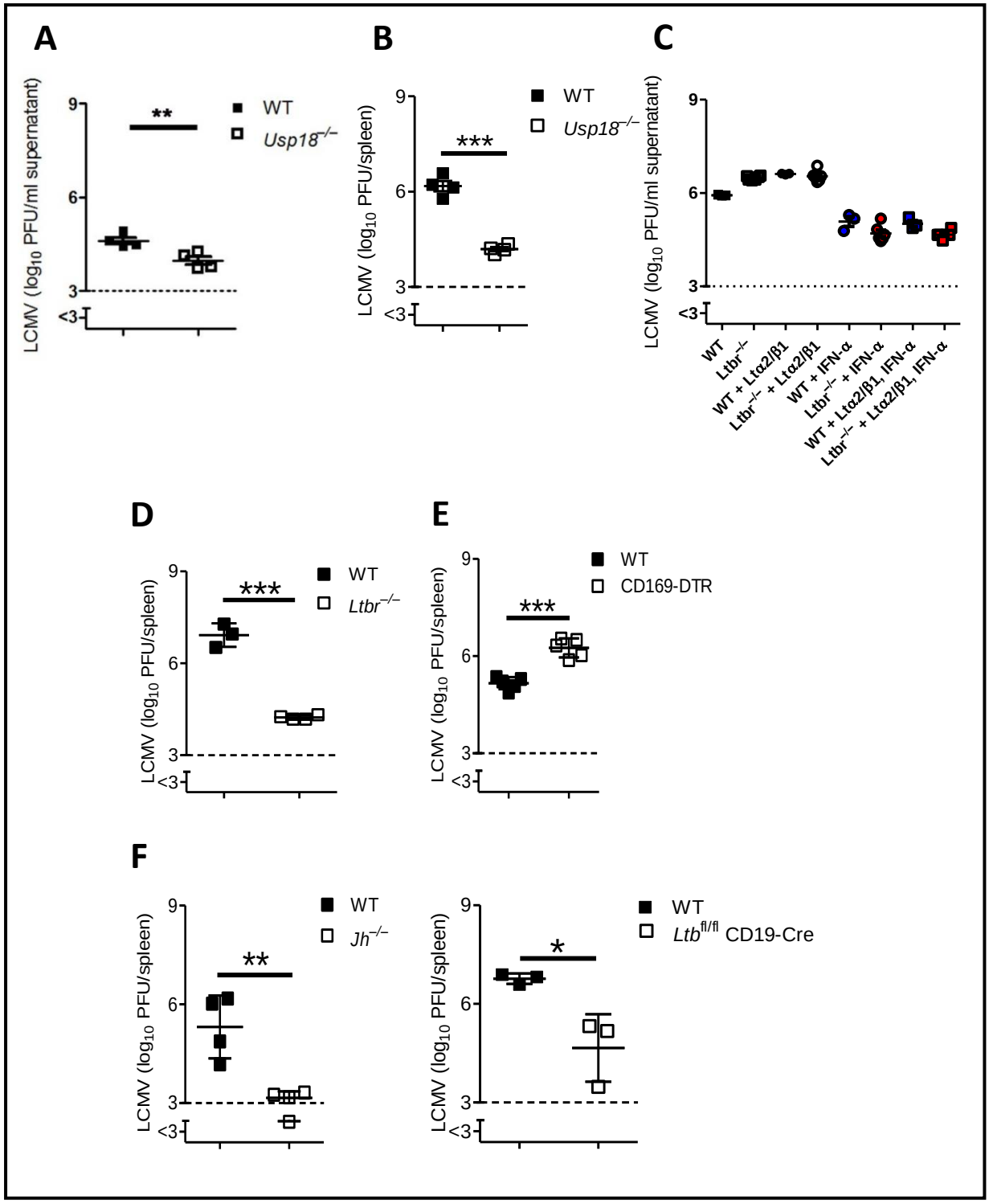


Figure 2-3: ***Ubiquitin-specific peptidase 18 and lymphotoxin beta allow viral replication in the spleen.***

Figure 4

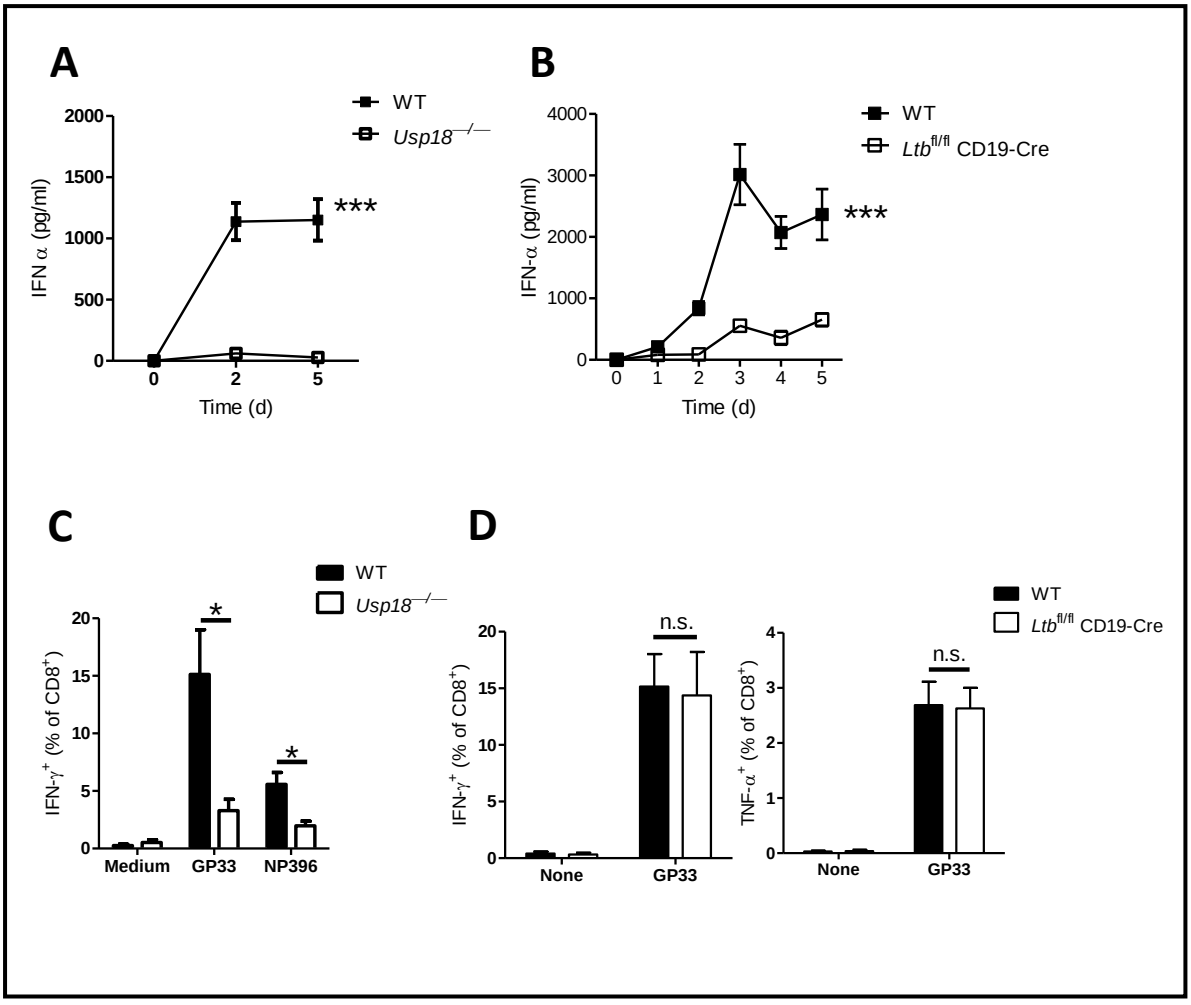


Figure 2-4: ***Ubiquitin-specific peptidase 18 and lymphotoxin beta are essential for inducing systemic type I interferon, but only ubiquitin-specific peptidase 18 influences the priming of CD8$^+$ T cells.***

Figure 5

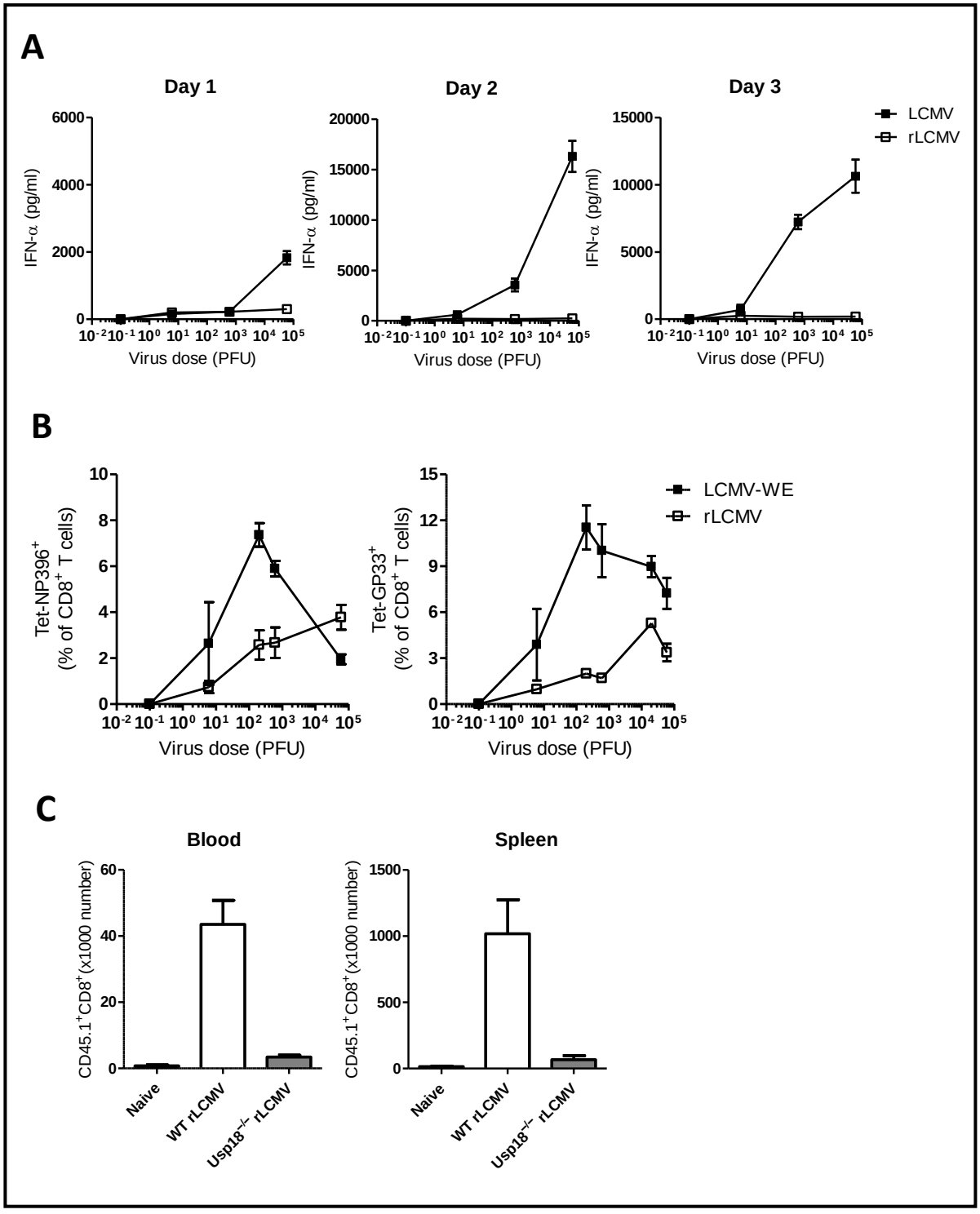
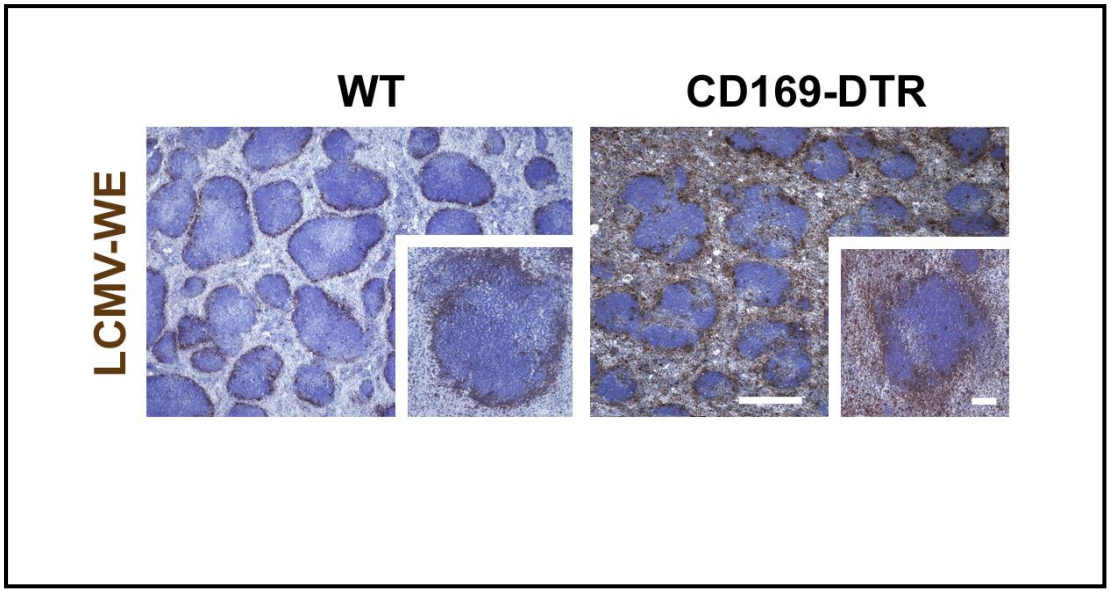


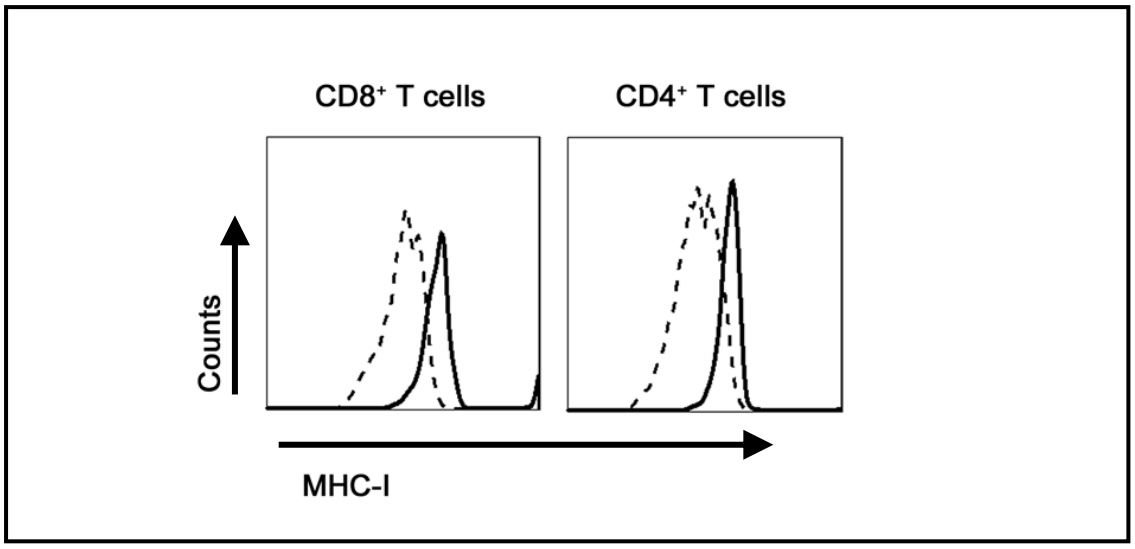
Figure 2-5: ***Extracellular spread of virus is essential for inducing systemic type I interferon but not for inducing a CD8⁺ T-cell response.***

Supplementary Figure 1



Supplementary Figure 2-1: The depletion of CD169^{+} macrophages enhances viral replication

Supplementary Figure 2



Supplementary Figure 2-2: ***Usp18-deficient T cells showed higher MHC-I expression than WT T cells***

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  - Results: Contributing to Fig. 2B, 2C, 3D, 3F, 4B 4D, ELISA for 5A and part of 5B. Writing results with *Shaabani N.*, *Duhan V.* and *Honke N.* and critical reviewing.
  - Discussion: Writing part of discussion and reviewing with *Shaabani N.*, *Duhan V.* and *Honke N.*

- Results (with *Shaabani N.*, *Duhan V.* and *Honke N.*)
    - Fig. 2B: Injection of FITC-dextran beads and analysis of its distribution in the spleen over the time period with Immunohistochemistry.
    - Fig. 2C: Immunohistochemistry of spleen and liver section after injection of anti-guinea pig antibody.
    - Fig. 3D & 3F: Virus infection, organ collection and plaque assay for the detection of viral replication in spleen of indicated mice and analysis.
    - Fig. 4B: Virus infection, serum collection and IFN-α ELISA for the mice and analysis
    - Fig. 4D: Intercellular cytokine staining after viral infection and data analysis
    - Fig. 5A: ELISA from the serum samples for indicated days
    - Fig. 5B: Blood collection and tetramer staining from the samples with FACS

- Revision and Proof reading (with *Shaabani N.*, *Duhan V.* and *Honke N.*)
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Vishal Khairnar **Prof. Dr. Karl S. Lang**

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CEACAM1 induces B-cell survival and is essential for protective antiviral antibody production

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B cells are essential for antiviral immune defence because they produce neutralizing antibodies, present antigen and maintain the lymphoid architecture. Here we show that intrinsic signalling of CEACAM1 is essential for generating efficient B-cell responses. Although CEACAM1 exerts limited influence on the proliferation of B cells, expression of CEACAM1 induces survival of proliferating B cells via the BTK/Syk/NF-κB-axis. The absence of this signalling cascade in naive *Ceacam1*$^{-/-}$ mice limits the survival of B cells. During systemic infection with cytopathic vesicular stomatitis virus, *Ceacam1*$^{-/-}$ mice can barely induce neutralizing antibody responses and die early after infection. We find, therefore, that CEACAM1 is a crucial regulator of B-cell survival, influencing B-cell numbers and protective antiviral antibody responses.

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3. Chapter III:
CEACAM1 induces B-cell survival and is essential for protective antiviral antibody production

Vishal Khairnar*, Vikas Duhan*, Sathish Kumar Maney, Nadine Honke, Namir Shaabani, Aleks Pandyra, Marc Seifert, Vitaly Pozdeev, Haifeng C. Xu, Piyush Sharma, Fabian Baldin, Florian Marquardsen, Katja Merches, Elisabeth Lang, Carsten Kirschning, Astrid M. Westendorf, Dieter Häussinger, Florian Lang, Ulf Dittmer, Ralf Küppers, Mike Recher, Cornelia Hardt, Inka Scheffrahn, Nicole Beauchemin, Joachim R. Göthert*, Bernhard B. Singer*, Philipp A. Lang* and Karl S. Lang*

3.1 *Abstract*

B cells are essential for antiviral immune defence because they produce neutralizing antibodies, present antigen and maintain the lymphoid architecture. Here we show that intrinsic signalling of CEACAM1 is essential for generating efficient B-cell responses. Although CEACAM1 exerts limited influence on the proliferation of B cells, expression of CEACAM1 induces survival of proliferating B cells via the BTK/Syk/NF-κB-axis. The absence of this signalling cascade in naive *Ceacam1*$^{-/-}$ mice limits the survival of B cells. During systemic infection with cytopathic vesicular stomatitis virus, *Ceacam1*$^{-/-}$ mice can barely induce neutralizing antibody responses and die early after infection. We find, therefore, that CEACAM1 is a crucial regulator of B-cell survival, influencing B-cell numbers and protective antiviral antibody responses.

3.2 *Introduction*

B cells are central players in initiating a rapid antiviral immune response. Their main functions are producing virus-specific antibodies, presenting antigen and participating in building the splenic architecture[1–4]. Three subsets of B cells are important contributors to immune responses against pathogens: B1, marginal zone (MZ) and follicular B cells[5,6]. B1 B cells usually seed the peritoneal and pleural cavities and develop T-cell-independent antibody responses against bacterial antigens[7]. B1 B cells are also responsible for generating the so called natural antibodies that are detectable in naïve mice that have not experienced antigen[7]. MZ B cells are located in the splenic MZ, where they have direct contact with blood-borne pathogens. Therefore, antigen-activated MZ B cells usually respond hours after infection and build the specific antibody response early after infection[5]. Antigen-activated follicular B cells move to germinal centres, where the antibody's affinity matures, and switch classes by recombining to mount long-lasting high affinity immunoglobulin G (IgG) antibody responses against pathogens[5].

Once B cells leave the bone marrow, two important signals determine their fate. First, tonic signalling by the B-cell receptor (BCR) in the absence of antigen is essential for the further differentiation and survival of mature B cells[8]. Second, signalling via the B-cell-activating factor (BAFF) receptor strongly contributes to B-cell survival[9]. BCR activation of B cells leads to phosphorylation of Bruton's tyrosine kinase (BTK), a member of the Tec family of non-transmembrane protein tyrosine kinases (PTKs)[10,11]. BTK phosphorylation after BCR ligation leads to the activation of canonical nuclear factor-κ light-chain enhancer of activated B (NF-κB) cell pathway, in addition to nuclear factor of activated T (NFAT) cells and extracellular signal-regulated kinase (ERK) pathways[12,13]. Crosslinking of the BAFF receptor activates the NF-κB pathway non-canonically via NF-κB-inducing kinase (NIK) and inhibitor of NF-κB, IκB kinase 1 (ref. 14). Although BAFF receptor signalling was first believed to be independent of BCR signalling, a recent report suggested that BAFF receptor signalling may also include the BCR signalling pathway components[15]. The NF-κB pathway substantially contributes to B-cell survival by inducing the expression of Bcl-2, Bcl-xL and Mcl-1 (ref. 13).

The carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), a member of the carcinoembryonic antigen and the immunoglobulin families, is engaged in intercellular binding interactions that affect various signal transduction pathways associated with cell proliferation and differentiation[16,17]. CEACAM1 usually acts via intercellular

adhesion through homophilic (CEACAM1–CEACAM1) or heterophilic (CEACAM1–CEACAM5, CEACAM1–CEACAM6 and CEACAM1–CEACAM8) interactions[17,18]. In mice, there are at least four CEACAM1 isoforms: CEACAM1-4L, CEACAM1-4S, CEACAM1-2L and CEACAM1-2S. The CEACAM1 ectodomain is composed of four (CEACAM1–4) or two (CEACAM1–2) highly glycosylated Ig-like domains, which are highly flexible and participate in anti-parallel (trans) and parallel (cis) homophilic binding[19]. The isoform with the short cytoplasmic tail (CEACAM1-S) can bind calmodulin, tropomyosin, actin, annexin II and PDIP38, and is phosphorylated on Ser449 through protein kinase C[20–22]. The isoform with the long cytoplasmic tail (CEACAM1-L) contains two additional immunoreceptor tyrosine-based inhibitory motif-carrying segments[17,23]. Within these immunoreceptor tyrosine-based inhibitory motif-carrying segments, phosphorylation at two tyrosines initiates signalling through CEACAM1-mediated signal transduction. Furthermore, trans-homophilic CEACAM1 binding induces cis-dimerization, and primarily dimeric but not monomeric CEACAM1-L recruits two specific Src homology region 2 domain-containing phosphatase-type phosphatases (PTPN6 and PTPN11)[19,24–26]. Crosslinking of CEACAM1 as monomers and dimers induces binding of the SRC family of PTK members to CEACAM1, which typically causes cell activation[19,26].

CEACAM1 is expressed by a broad range of cell types, including angiogenically activated endothelia/lymphendothelia, various leukocyte subpopulations, normal epithelial cells and tumour cells[16]. Although *Ceacam1*$^{-/-}$ mice do not exhibit this broad CEACAM1 expression, they develop normally and, in the absence of specific challenges, show no signs of disease[27]. CEACAM1 has been described primarily as a regulator of T cells in the gut[20,28–30]. Expression of CEACAM1-L inhibits T-cell proliferation and therefore prevents inflammatory bowel disease[30]. Expression of CEACAM1-S is essential for the development of follicular T helper cell-driven IgA production by gut B cells[20]. CEACAM1 also acts as a co-stimulatory molecule for T-cell receptor and BCR signalling[31–33]. The role of CEACAM1 in B-cell homeostasis and in antiviral B-cell responses *in vivo* remains unknown.

We report here that CEACAM1 is expressed on blood, bone marrow, lymph node, as well as splenic MZ and follicular zone (FO) B-cell subpopulations in mice. *In vitro* CEACAM1 expression induces the survival of proliferating B cells. In line with this finding, *Ceacam1*$^{-/-}$ mice carry reduced numbers of total B cells and virtually no MZ B cells. During viral infection, the absence of CEACAM1 on B cells leads to an insufficient antiviral B-cell

response, and *Ceacam1*$^{-/-}$ mice die early after infection with the cytopathic vesicular stomatitis virus (VSV).

3.3 *Results*

3.3.1 *CEACAM1 is expressed on B-cell subsets.*

We first analysed CEACAM1 expression on various cell populations in the peripheral blood of wild-type (WT) mice. Erythrocytes stained negative for CEACAM1 **(Supplementary Fig. 1)**. As previously reported[34–36], high levels of CEACAM1 expression were detected on blood granulocytes (Ly6G^{+}) and monocytes (CD115^{+}) with the anti-CEACAM1-specific monoclonal antibody (clone CC1, **Fig. 1a**). Cells from *Ceacam1*$^{-/-}$ mice stained negative for CEACAM1 (**Fig. 1a**). Next, we analysed CEACAM1 expression on lymphoid cells in the blood. CD90.2 cells, representing primarily T cells, showed weak CEACAM1 expression by individual cells (**Fig. 1b**), a finding suggesting that various T-cell subpopulations may differentially express CEACAM1. B cells in peripheral blood expressed CEACAM1 at high levels (**Fig. 1b**). Evaluation of precursor (B220^{+} CD43^{-} IgM^{+} IgD^{low}) and mature B cells (B220^{+} CD43^{-} IgD^{high}) in the bone marrow revealed strong CEACAM1 expression in the B-cell lineage (**Fig. 1c**). In line with this finding, lymph node B cells, follicular B cells (FO, B220^{+} CD19^{+} CD21/35^{low} CD23^{hi}) and MZ B cells (B220^{+} CD19^{+} CD21/35^{hi} CD23^{low}) in the spleen expressed CEACAM1 at high levels (**Fig. 1d,e**). Furthermore, quantitative RT–PCR analyses of expression of long and short isoforms of CEACAM1 showed that murine splenic B cells express more long than short isoforms (**Fig. 1f**). In conclusion, strong CEACAM1 expression was detected in all B-cell subpopulations tested, a finding implying that CEACAM1 is functionally relevant in B cells.

3.3.2 *CEACAM1 induces survival genes via Syk and Erk and NF-κB.*

Recently, expression of CEACAM1 in granulocytes was shown to lead to Syk phosphorylation[37]. In addition, expression of CEACAM1 participates in Btk phosphorylation[38]. In B cells, Syk and Btk are essential signalling molecules, which are phosphorylated after BCR or BAFFR crosslinkage and play an essential role in induction of survival signals[15,39]. We thus focused on the influence of CEACAM1 on Syk activity during CEACAM1 and BCR crosslinkage. Indeed, in B-cell lymphoma immunoprecipitation of CEACAM1 pulled down Syk (**Supplementary Fig. 2**), which confirms the recently published interaction of Syk and CEACAM1 (ref. 37). To get insights into the functional

relevance of CEACAM1 in B cells, we isolated splenocytes from WT and *Ceacam1*$^{-/-}$ mice and challenged them with anti-IgM, anti-CEACAM1 activating antibody (monoclonal antibody, CC1) or lipopolysaccharide (LPS). Ig-α, which associated with BCR, showed slight tyrosine-phosphorylation after IgM crosslinkage (**Fig. 2a**, left panel). Neither anti-CEACAM1 monoclonal antibody challenge nor LPS treatment induced phosphorylation of Ig-α (**Fig. 2a**, right panel). We subsequently analysed Syk tyrosine phosphorylation in WT and *Ceacam1*$^{-/-}$ mice. As expected BCR crosslinkage induced strong Syk phosphorylation. Absence of CEACAM1 strongly reduced Syk phosphorylation after BCR crosslinkage (**Fig. 2b**, left panel). Challenge with anti-CEACAM1 monoclonal antibody also triggered phosphorylation of Syk, which was stronger than LPS-induced Syk phosphorylation (**Fig. 2b**, right panel). Therefore, we concluded that CEACAM1 enhances Syk phosphorylation not only directly, but also after BCR crosslinkage. Next we analysed the downstream target of Syk, Btk, another member of the Tec family of non-transmembrane PTKs. Flow cytometric analysis showed that Btk is transiently phosphorylated in B cells derived from WT but not in *Ceacam1*$^{-/-}$ mice after challenge with anti-IgM (**Fig. 2c**, upper panel) antibody. Similarly, anti-CEACAM1 monoclonal antibody challenge induced Btk phosphorylation in WT B cells (**Fig. 2c**, lower panel). We therefore conclude that CEACAM1 directly influences the phosphorylation of Syk and Btk, after BCR and CEACAM1 crosslinkage. To gain insight into downstream targets of CEACAM1 activation, we analysed specific Syk substrates. We observed that the CEACAM1 signal phosphorylate Erk1 (Erk-42) but not Erk2 (Erk-44) and, to a lesser extent, p-38. LPS could phosphorylate both Erk1/2 and p-38 (**Fig. 2d,e**). Triggering with the anti-CEACAM1 monoclonal antibody induced degradation of IκBα and slight phosphorylation of p65, a finding suggesting that CEACAM1 signals activate the canonical NF-κB pathway (**Fig. 2f**).

Tonic BCR signalling followed by Syk phosphorylation induces several survival genes, which are essential for B cell survival[12]. To see whether lack of CEACAM1 influences expression of those genes, we cultured purified splenic B cells from WT and *Ceacam1*$^{-/-}$ mice for 24 h without further stimulation and measured mRNA expression of various NF-κB-regulated genes. Indeed, lack of CEACAM1 led to reduced expression of *Bcl6*, *Pax5*, *Bcl2a1* and *Xiap* (**Fig. 2g**). In line with the known positive feedback loop of NF-κB, we found reduced expression of *NF-κB* p65, *Rel-B*, *Nfatc1* and *Nfatc2* in *Ceacam1*$^{-/-}$ B cells (**Fig. 2h,i**). *c-Jun*, *c-Fos* and *Ap1* were not influenced by CEACAM1 expression (**Fig. 2j**). *Blimp1*, which is another key regulator of plasma cell differentiation, was not affected by

CEACAM1 expression (*Fig. 2k*). Taken together, we found that CEACAM1 contributed to the Syk phosphorylation during BCR and/or CEACAM1 crosslinkage, leading to the enhanced expression of NF-κB, NFAT and survival genes.

3.3.3 *CEACAM1 promotes survival of B cells in vitro.*

We found that expression of CEACAM1 enhanced BCR-dependent Syk phosphorylation. Therefore, absence of CEACAM1 reduced expression of survival genes, which are induced via the tonic BCR signal. To see how lack of CEACAM1 influenced proliferation and survival of B cells, we labelled WT and *Ceacam1*$^{-/-}$ B cells with carboxyfluorescein succinimidyl ester (CFSE) and challenged them with anti-CEACAM1 monoclonal antibody or the BCR-independent B-cell activator such as LPS, or CD40 ligand (CD40L) in combination with mouse interleukin (IL)-4. Challenge with anti-CEACAM1 (monoclonal antibody, CC1) induced some proliferation in WT but not in *Ceacam1*$^{-/-}$ B cells (**Fig. 3a**). Challenge with LPS (TLR4-ligand) or CD40/IL-4 induced proliferation that was comparable between WT and *Ceacam1*$^{-/-}$ B cells (**Fig. 3a**). Therefore, we conclude that the induction of proliferation via TLR4 or CD40 is not affected by CEACAM1.

Next, we analysed the impact of CEACAM1 on B-cell survival. First, we quantified the total number of living B cells with diamidino-2-phenylindole (DAPI) staining and fluorescenceactivated cell sorting (FACS) beads. Without any further challenge, WT B cells consistently died under our *in vitro* culture conditions (**Fig. 3b**), a finding that agrees with that of a previous study[40]. Lack of CEACAM1 enhanced death of B cells (**Fig. 3b**). When challenged with CD40/IL-4, the numbers of WT B cells were higher than those of non-activated B cells after 24 h (**Fig. 3b**). The numbers of *Ceacam1*$^{-/-}$ B cells 24 h after stimulation with CD40/IL-4 increased similarly to those of WT B cells, however, declined rapidly 24 h after challenge with CD40/IL-4 (**Fig. 3b**). This rapid decrease was probably due to apoptosis and cell death, because Annexin-V staining was significantly enhanced in none activated and activated DAPI^{-}, B cells (**Fig. 3c**). We postulate that defective survival of *Ceacam1*$^{-/-}$ B cells was due to limited Syk and Btk phosphorylation. To test this hypothesis, we treated WT and *Ceacam1*$^{-/-}$ B cells with the Btk inhibitor Ibrutinib[41]. We observed that inhibition of Btk in WT B cells led to the rapid death of B cells. This effect was less pronounced in *Ceacam1*$^{-/-}$ B cells (**Fig. 3d**). In conclusion, we found that lack of CEACAM1 signal limited survival of resting and activated B cells.

3.3.4 *CEACAM1 promotes B-cell differentiation and survival in vivo.*

We found that CEACAM1 is expressed on all B-cell subpopulations analysed and that it influences the survival of B cells, especially after activation. During maturation, several activating stimuli allow B cells to progress through various stages (**Fig. 4a**). To determine the influence of CEACAM1 on survival during B-cell proliferation, we analysed B-cell subsets in bone marrow, peripheral blood, lymph node and spleen of WT and *Ceacam1*$^{-/-}$ mice. We found that B-cell precursor subsets in the bone marrow are reduced only in stages B, C and C' (**Fig. 4b**). However, after stage C' they expand more rapidly; therefore, the numbers of newly formed B cells in stages D and E are similar in WT and *Ceacam1*$^{-/-}$ mice (**Fig. 4b**). This finding is concordant with the similar numbers of newly formed (immature, B220^{+} IgM^{+}) B cells in the peripheral blood (**Fig. 4c**). These findings are also in line with previously published experiments showing that there is no difference in B-cell numbers in the peripheral blood of *Ceacam1*$^{-/-}$ mice[27]. Therefore, we conclude that the generation of newly formed B cells is hardly affected by CEACAM1. B-cell analysis in the peritoneal cavity showed that the frequency of B2 (CD19^{+} B220^{+} CD43^{-}) B cells was slightly reduced. B1a (CD19^{+} B220^{+} CD43^{+} CD5^{+}) B-cell frequency was significantly reduced, whereas the B1b (CD19^{+} B220^{+} CD43^{+} CD5^{-}) B-cell population was higher in *Ceacam1*$^{-/-}$ mice (**Supplementary Fig. 4**).

Next, we examined whether CEACAM1 signals are essential for B cells in secondary lymphoid organs, where newly formed B cells undergo further proliferation and differentiation. In lymph nodes, the numbers of newly formed (CD19^{+} IgM^{+} IgD^{low}) B cells in WT and *Ceacam1*$^{-/-}$ mice were similar (**Fig. 4d**). In contrast, the numbers of matured (M, CD19^{+} IgD^{high}) B cells were significantly reduced in the lymph nodes of *Ceacam1*$^{-/-}$ mice (**Fig. 4d**). In the spleen, the T1, T2 and T3 populations of transitional B cells were reduced in the absence of CEACAM1 (**Fig. 4e**). The numbers of follicular B cells I (Fol I) and II (Fol II), MZ precursor cells and MZ B cells were dramatically reduced in the spleen of *Ceacam1*$^{-/-}$ mice (**Fig. 4e**). In line with this finding, the numbers of mature B cells (stage F, B220^{+} CD43^{-} IgD^{high}) in the bone marrow were reduced in *Ceacam1*$^{-/-}$ mice (**Fig. 4b**). Similarly, histologic analysis showed lower numbers of B cells in the spleen and lymph node (**Fig. 4f**) and the absence of MZ B cells as well as low numbers of metallophilic (CD169^{+}) macrophages in *Ceacam1*$^{-/-}$ mice (**Fig. 4g**). To strengthen our hypothesis that reduced Syk phosphorylation after B-cell-activating factor receptor (BAFFR) or BCR activation, but not TLR or Fcgr2b signalling, is responsible for reduced B-cell numbers in *Ceacam1*$^{-/-}$ mice, we

performed histology of *Jh*$^{-/-}$, *Baffr*$^{-/-}$, *Fcgr2b*$^{-/-}$ and *Myd88/Trif*$^{-/-}$ mice. Histological analysis showed that a lack of BCR and BAFFR signalling, but neither TLR nor Fcgr2b signalling, reproduces the B-cell deficiency seen in *Ceacam1*$^{-/-}$ mice (**Supplementary Fig. 5**).

Next, we determined whether defective intrinsic survival signals are responsible for reduced B-cell numbers in *Ceacam1*$^{-/-}$ B cells, we transferred the same number of B cells from WT or *Ceacam1*$^{-/-}$ mice into B-cell-deficient (*Jh*$^{-/-}$) mice. Adoptively transferred WT B cells survived in the *Jh*$^{-/-}$ mice and repopulated the spleen within 30 days (**Fig. 4h**). In contrast, *Ceacam1*$^{-/-}$ B cells were not detectable after 30 days (**Fig. 4h**), a finding suggesting that the absence of intrinsic CEACAM1 limits the survival of B cells after proliferation in *Jh*$^{-/-}$ mice. To gauge the direct influence of CEACAM1 expressed on B cells, we made use of mixed bone marrow chimeras, where the irradiation of WT (CD45.2) mice was followed by reconstitution with bone marrow from WT (CD45.1) and WT (CD45.2, mixed 1:1) or WT (CD45.1) and *Ceacam1*$^{-/-}$ (CD45.2, mixed 1:1) mice. Analysis of peripheral blood showed some, but not statistically significant difference in blood B-cell levels between WT and *Ceacam1*$^{-/-}$ cells (**Fig. 4i**). In contrast, splenic MZ B cells were only derived from WT (CD45.1) mice (**Fig. 4j**). FO B cells were also mainly from WT (CD45.1) origin (**Fig. 4j**). This suggests that CEACAM1 B-cell intrinsic signals are essential for B-cell survival after B cells home to the spleen. Next, we analysed how the lack of CEACAM1 contributes to antibody production in unchallenged mice. Analysis of various immunoglobulin (Ig) subtypes in the serum of naïve WT and *Ceacam1*$^{-/-}$ mice showed a reduction in IgM, IgG1, IgG2a, IgG2b, IgG3, IgA and IgE in *Ceacam1*$^{-/-}$ mice (**Supplementary Fig. 6**). In conclusion, CEACAM1 expression influences survival in mature B cells.

3.3.5 *CEACAM1 ensures mouse survival during VSV challenge.*

Next, we analysed the role of CEACAM1 during antigen challenge. We infected mice with VSV, a cytopathic pathogen for which systemic control strongly depends on the rapid induction of neutralizing antibodies[42]. We found strong expression of CEACAM1 in the MZ (**Fig. 5a**), a site at which VSV-specific B cells become activated[43,44]. Because CEACAM1 is a natural CEACAM1 ligand, this finding suggests that CEACAM1 may release an important survival signal during antigenic challenge. To determine the role of CEACAM1 during B-cell activation, we transferred same number of B cells from Vi10 mice (WT × Vi10), which express a VSV-specific BCR or from Vi10 mice crossed to *Ceacam1*$^{-/-}$ mice (*Ceacam1*$^{-/-}$ × Vi10), into WT mice and infected them with VSV. After 3 days, the number of WT × Vi10 B

cells but not *Ceacam1*$^{-/-}$ × Vi10 B cells was expanded (**Fig. 5b**). In the absence of stimulation, *Ceacam1*$^{-/-}$ × Vi10 B cells were reduced as compared to WT × Vi10 B cells (**Fig. 5b**), a finding indicating that CEACAM1 provides an important survival signal in antigen-activated and non-activated B cells. In line with these data, *Ceacam1*$^{-/-}$ mice displayed delayed induction of total VSV-neutralizing antibodies (**Fig. 5c**, left panel). In agreement with this finding, *Ceacam1*$^{-/-}$ mice failed to generate neutralizing IgG antibodies (**Fig. 5c**, right panel). ELISA confirmed that VSV-specific IgG levels were significantly low in *Ceacam1*$^{-/-}$ mice (**Supplementary Fig. 7**, right panel). Notably, anti-VSV IgM levels were significantly reduced in *Ceacam1*$^{-/-}$ mice (**Supplementary Fig. 7**, left panel). During challenge with non-replicating ultraviolet-inactivated VSV, *Ceacam1*$^{-/-}$ mice did not secrete neutralizing antibodies (**Fig. 5d**), a finding suggesting that *Ceacam1*$^{-/-}$ mice exhibit strongly impaired B-cell functions. To demonstrate that CEACAM1 expression by B cells is responsible for poor VSV neutralizing antibody responses, we adoptively transferred CEACAM1-competent VSV-specific B cells into WT and *Ceacam1*$^{-/-}$ mice. This adoptive transfer of B cells rescued VSV-neutralizing antibody responses (**Fig. 5e**). Detailed analysis showed that both MZ and FO B cells could rescue the VSV neutralizing antibody response in the *Ceacam1*$^{-/-}$ mice (**Supplementary Fig. 8**). Absence of early neutralizing antibodies can result in the spread of VSV to the nervous system[45]. To determine whether a reduction in the levels of neutralizing antibodies in *Ceacam1*$^{-/-}$ mice affected the immune response of virus control, we analysed organ virus titres 8 days after systemic VSV infection. Indeed, *Ceacam1*$^{-/-}$ mice exhibited VSV replication in the brain and spinal cord, whereas WT mice controlled VSV replication in all organs (**Fig. 5f**). VSV replication in *Ceacam1*$^{-/-}$ brains was associated with the death of mice (**Fig. 5g**). To determine whether a diminished B-cell response contributed to mortality, we performed survival experiments. *Ceacam1*$^{-/-}$ mice with adoptively transferred VSV-specific WT B cells survived VSV infection (**Fig. 5h**). Therefore, B-cell intrinsic expression of CEACAM1 is essential for initiating a protective antiviral immune response after exposure to VSV.

3.3.6 *CEACAM1 facilitates LCMV-dependent B-cell activation.*

Next, we infected WT and *Ceacam1*$^{-/-}$ mice with non-cytopathic lymphocytic choriomeningitis virus (LCMV) to confirm whether antigen-specific B-cell responses are impaired in *Ceacam1*$^{-/-}$ mice. Early control of an infection dose of 200 plaque-forming units (PFUs) of LCMV depends only on CD8^{+} T cells. However, at higher infection doses, B cells

are important for LCMV control[46]. WT mice produce higher titres of LCMV-specific antibodies than do *Ceacam1*$^{-/-}$ mice during LCMV infection (**Fig. 6a**). In line with this finding, LCMV replication in the liver of *Ceacam1*$^{-/-}$ mice was prolonged (**Fig. 6b**).

3.3.7 *Human B-cell subpopulations express CEACAM1.*

To determine whether CEACAM1 also plays a potential role in human B cells, we measured CEACAM1 expression by naïve B cells. Previous publications reported that most B cells isolated from peripheral blood express CEACAM1 (ref. 32). Also, we found that naive human B cells (IgD^{high} CD27^{-}) and memory B cells (CD27^{+}) express substantial levels of CEACAM1 (**Fig. 7a**). The expression of memory B cells is slightly higher than that of naïve B cells (**Fig. 7b**), a finding suggesting that the CEACAM1 signal in humans may be crucial for the survival of memory B cells rather than naïve B cells.

3.4 *Discussion*

The results of this study demonstrate that CEACAM1 expression is essential for the survival of murine B cells. The absence of CEACAM1 expression on murine B cells is associated with reduced numbers of B cells and a defective immune response after viral antigen challenge.

Greicius *et al.*[31] demonstrated that binding of CEACAM1 with an anti-CEACAM1 monoclonal antibody induces strong B-cell proliferation. Other studies showed that BCR activation in the presence of another anti-CEACAM1 monoclonal antibody (clone T84.1) limits B-cell proliferation[47,48]. In the current study, we demonstrate that CEACAM1 crosslinkage had some effect on proliferation. However, *in vitro* and *in vivo* we found a strong influence of CEACAM1 on the survival of activated B cells. Therefore, we consider that a prolonged signal via CEACAM1 ligation is essential for promoting survival signals and maintaining B-cell numbers, which could explain reduced B-cell numbers in *Ceacam1*$^{-/-}$ mice.

BCR crosslinking by auto-antigens has an essential role in the differentiation of transitional B cells into MZ B cells and follicular B cells[6]. In addition, this tonic BCR signal, together with constant signalling via the BAFF receptor, is the most important survival signal for naïve B cells. How this diverse process can be regulated by BCR signals remains unexplained. We suggest that CEACAM1 is another strong regulator of B-cell survival. This

is supported by previous findings that CEACAM1 regulates apoptosis in granulocytes[49]. Because CEACAM1 is expressed most strongly by B cells, and because CEACAM1 is its own ligand, we suggest that CEACAM1 induces positive survival signals primarily on B cells within the B-cell follicles and that CEACAM1 acts as a positive feedback loop once B cells reach the B-cell follicle. Therefore, in addition to chemokine gradients[50], CEACAM1 expression appears to contribute to the generation of B-cell follicles.

We found that Syk phosphorylation after BCR crosslinkage was dependent on expression of CEACAM1. In line with these results, monoclonal antibody activation of CEACAM1 led to Syk phosphorylation. These data suggest that CEACAM1 directly interacts with BCR signalling and therefore would influence the tonic BCR signal as well as antigen-dependent BCR activation. Tonic BCR signalling, but also BAFFR signalling, induces several survival genes via Syk. Therefore, lack of BCR and BAFFR signalling limits B-cell development and survival. Indeed, in line with data from *Jh*$^{-/-}$ or *Baffr*$^{-/-}$ mice, *Ceacam1*$^{-/-}$ mice showed strongly reduced B-cell numbers in naïve mice. Therefore, we concluded that CEACAM1, in addition to BAFFR, is another important membrane molecule influencing BCR signalling via Syk.

We found that CEACAM1 activation leads to Btk phosphorylation. This finding is related to the enhanced survival of B cells. Loss-of-function mutations in BTK lead to X-linked agammaglobulinemia because of a complete absence of mature B cells[51]. Mice deficient in CD19 or BCR exhibit a strongly reduced number of mature B cells[52]. This reduction in the numbers of MZ B cells in CEACAM1-deficient mice is also found in mice and humans lacking the Wiskott-Aldrich syndrome protein (WASp)[53]. WASp is also involved in BTK phosphorylation[54]. As we have shown for CEACAM1, the necessity for WASp in the generation of the MZ B-cell compartment is B-cell intrinsic[55]. Whether CEACAM1 affects WASP function must be investigated in future studies.

We found that CEACAM1 is expressed on human B cells. This could suggest that also in human antiviral immune response, CEACAM1 signalling may play a role in inducing sufficient antibody responses against different viruses. Therefore, we would suggest that lack of CEACAM1 could be another factor explaining B-cell deficiency in humans. If indeed CEACAM1 deficiency occurs in humans, remains to be fully elucidated.

Recently, we found that CD169^{+} macrophages enforce viral replication and therefore are essential for initiating an antiviral immune response[44,56]. B cells play an important role in recruiting CD169^{+} macrophages[3]. Therefore, B-cell deficiency leads to defects in enforced

virus replication (**Supplementary Fig. 9**) and a reduction in the innate immune response. Indeed, we found that in *Ceacam1*$^{-/-}$ mice the ability of CD169^{+} macrophages to enforce virus replication was impaired (**Supplementary Fig. 10**). Which could be an additional mechanism how CEACAM1 deficiency in B cells contributes to defective anti-VSV immune response.

In conclusion, we found that CEACAM1 expressed on murine B cells is an important regulator of B-cell homeostasis. During exposure with cytopathic virus, CEACAM1 was essential for inducing an efficient antiviral antibody response and thereby reducing mouse mortality.

3.5 *Methods*

3.5.1 *Mice*

All mice used in this study, including *Ceacam1*$^{-/-}$, *Jh*$^{-/-}$, *Tcrab*$^{-/-}$, *Aid*$^{-/-}$, *sIgM*$^{-/-}$, *Myd88/Trif*$^{-/-}$, *Baffr*$^{-/-}$ and *Fcgr2b*$^{-/-}$ mice, were maintained on the C57BL/6 genetic background (back-crossed at least 8 times and as many as 16 times) and were bred as homozygotes. Vi10/CD45.1 mice expressing VSV-specific BCR as a transgene were used for cell transfer studies, and mice expressing the CD45.1 transgene were used for reconstitution of bone marrow. Six to eight-week-old, age- and sex-matched mice were used for all the studies. All animals were housed in single ventilated cages. During survival experiments, the health status of the mice was checked twice daily. Animal experiments were authorized by the Nordrhein Westfalen Landesamt für Natur, Umwelt und Verbraucherschutz (Recklinghausen, Germany), and in accordance with the German law for animal protection or according to institutional guidelines at the Ontario Cancer Institute of the University Health Network and at McGill University. Animals exhibiting severe symptoms of sickness or paralysis or showing substantial weight loss during VSV infection were put to death and were considered dead for statistical analysis.

3.5.2 *Bone marrow chimeras*

For generation of bone marrow chimeras, C57BL/6 mice were irradiated twice for 7min each with a total of 1,050 rad. After 24 h, mice were reconstituted intravenously with 5×10^6 bone marrow cells from each donor for mixed bone marrow chimeras. Mice were analysed 40–45 days after reconstitution.

3.5.3 *Virus and plaque assays*

VSV, Indiana strain (VSV-IND, Mudd-Summers isolate), was originally obtained from Professor D. Kolakofsky (University of Geneva, Switzerland). Virus was propagated on BHK-21 cells at a multiplicity of infection of 0.01. VSV concentration was determined as described below, and was then plaque purified on Vero cells[57]. Mice were infected intravenously with VSV at the indicated doses. Virus titres were measured with a plaque-forming assay. For this assay, organs were smashed in Dulbecco's modified Eagle medium (DMEM) containing 2% fetal calf serum (FCS), titrated 1:3 over 12 steps, and plaqued on Vero cells. After a 2-h incubation at 37 ºC, overlay was added, and the virus preparation was again incubated at 37 ºC. Plaques were counted 24 h later by the use of crystal violet staining. The LCMV strain WE was originally obtained from F. Lehmann-Grube (Heinrich Pette Institute, Hamburg, Germany) and was propagated on L929 cells, MC57 cells or both. Mice were infected intravenously at the indicated dose. LCMV viral titres were detected by a plaque-forming assay on MC57 fibroblasts as previously described[58]. In short, smashed organs were plaqued with MC57 cells as described above and incubated at 37 ºC. After a 3-h incubation at 37 ºC, overlay was added, and the virus preparation was again incubated at 37 ºC. Plaques were counted 72 h later by LCMV NP staining. Cells were fixed (with 4% formaldehyde solution), permeabilized (with 1% Triton-X solution), blocked (with 10% FCS in phosphate-buffered saline) and stained with anti-LCMV NP antibody (made in house). ECL-conjugated anti-rabbit-IgG antibody was used as a secondary antibody. Plaques were detected by colour reaction (0.2M Na_2HPO_4 + 0.1M citric acid + 30% H_2O_2 + o-phenylenediamine dihydrochloride), all chemicals from Sigma-Aldrich.

3.5.4 *Neutralizing antibody assay*

Serum was prediluted (1:40). The complement system was inactivated at 56 ºC for 30 min. For analysis of IgG kinetics, diluted samples were treated with 2-mercaptoethanol (0.1 M) for removal of IgM. Serum was titrated 1:2 over 12 steps and was incubated with 1×10^3 PFU of VSV. After a 90-min incubation at 37 ºC, the virus–serum mixture was plaqued onto Vero cells. Overlay was added after 1 h, and the mixture was incubated again for 24 h at 37 ºC. Plaques were counted by crystal violet staining. Antibody titres are presented as two- or threefold dilution steps ($-\log_2$ and $-\log_3$) times the predilution (that is, × 40).

3.5.5 *B-cell culture*

Spleens retrieved from WT and *Ceacam1*$^{-/-}$ mice were homogenized in magnetic-activated cell sorting (MACS) buffer (1% FCS and 0.8% 0.5M EDTA). B220^{+} B cells were isolated by positive selection with CD45R-conjugated magnetic beads (MACS Miltenyi Biotech). Flow cytometry confirmed that the purity of B220^{+} cells was higher than 95%. For proliferation assays, cells were labelled with 5 mM carboxyfluorescein succinimidyl ester, and 2×10^5 cells per well were cultured in 96-well flat bottom plates in RPMI 1640 medium supplemented with 10% LPS-free FCS, 1% antibiotics and 0.1% 50mM 2-mercaptoethanol. They were then challenged with anti-CEACAM1 (20 μg ml^{-1}, clone CC1; a kind gift from Dr Kathryn V. Holmes, University of Colorado, Denver, CO) antibody or recombinant mouse CD40 ligand (1 μg ml^{-1}) in combination with mouse IL-4 (10 ng ml^{-1}; R&D Systems) or LPS (100 ng ml^{-1}; Sigma-Aldrich) for 48 h. Similarly, for inhibition experiments, purified B cells (as described above) were cultured in the medium described above with recombinant mouse CD40 ligand (1 μg ml^{-1}) in combination with mouse IL-4 (10 ng ml^{-1}; R&D Systems) and were treated with 10 mM Ibrutinib (Selleck). Control wells were supplemented with equal amounts of DMSO used to dissolve Ibrutinib. Cell death was measured by DAPI (Life Technologies) staining. For survival experiments, B cells were cultured as mentioned above, and cells were stained with Annexin-V (BD Biosciences) followed by DAPI. Proliferation and survival were determined by FACS at indicated time points. For cell signalling experiments, splenocytes were dissociated in VLE-DMEM supplemented with 10% LPS-free FCS and 1% antibiotics and were challenged with anti-CEACAM1 (clone CC1, 20 μg ml^{-1}; K. Holmes) and anti-IgM (10 μg ml^{-1}) antibody (Jackson ImmunoResearch Laboratories, Inc.) for various time periods at 37 ºC.

3.5.6 *Histology*

Histologic analyses of snap-frozen tissue were performed with a monoclonal antibody to VSV glycoprotein (Vi10; made in-house). Anti-CD45R (B220; RA3-6B2), anti-CD90.2 (53-2.1), anti-CD19 (1D3), anti-CD1d (1B1) and anti-CEACAM1 (CC1) monoclonal antibodies were purchased from eBioscience. Biotin-anti-CD169/SIGLEC1 (MOMA-1) was purchased from Acris. Sections were washed and stained with streptavidin (eBioscience). In short, sections were fixed with acetone, and nonspecific antigens were blocked in PBS containing 2% FCS for 15 min, followed by various stainings of antibodies, diluted 1:100 in

blocking solution for 45 min. Images of stained sections were acquired with a fluorescence microscope (KEYENCE BZ II analyzer).

3.5.7 *Flow cytometry*

Peripheral blood cells were stained with anti-Ly6G (RB6-8C5), anti-CD115 (AFS98), anti-CD45R (B220; RA3-6B2), anti-CD90.2 (30-H12), anti-CEACAM1 (CC1; with corresponding isotype control anti-IgG1 (M1-14D12)), anti-IgD (11-26c), anti-CD93 (AA4.1), anti-CD19 (1D3) (all from eBioscience) and anti-IgM (II/41; BD Biosciences) antibodies. Recovered bone marrow cells were resuspended in FACS buffer (0.5M EDTA, 0.1% sodium azide, 1% FCS in PBS) and incubated with anti-CD45R (B220; RA3-6B2), anti-IgD (11-26c), anti-IgM (II- 41), anti-CEACAM1 (CC1), anti CD24 (M1/69; all from eBioscience), anti-CD43 (1B-11), and anti-BP1 (6C3; from BioLegend) antibodies. Inguinal lymph nodes were disaggregated in FACS buffer, and cells were stained for anti-CD45R (B220; RA3-6B2), anti-CD19 (1D3), anti-IgD (11-26c; all from eBioscience), and anti-IgM (II/41; BD Biosciences) antibodies. Spleens were dissociated in FACS buffer, and splenocytes were incubated with anti-CD45R (B220; RA3-6B2), anti-CD19 (1D3), anti-CD93 (AA4.1), anti-CD21/35 (8D9), anti-CD23 (B3B4), anti-IgD (11-26c), anti-CEACAM1 (CC1), anti-CD45.1 (A20), anti-CD45.2 (104; all from eBioscience) and anti-IgM (II/41; BD) antibodies. Human peripheral blood samples were stained with anti-IgD (IA6-2) and anti-CD27 (M-T271) antibodies (both from BD Biosciences) and anti-CEACAM1 (monoclonal antibody, B3-17) antibody (from Dr Singer, Essen). Peritoneal B cells were stained for anti-CD19 (1D3), anti-CD45R (B220; RA3-6B2), anti-IgM (II/41; all from eBioscience), and anti-CD5 (53-7.3; BD Biosciences) and anti-CD43 (1B-11; from BioLegend) antibodies. Dead cells were discriminated by staining with propidium iodide (PI, eBioscience) and/or DAPI and were excluded from all analyses except for blood. For cell signalling experiments, cells were fixed and permeabilized according to the manufacturer's instructions (BD Phosflow, BD Biosciences). The cells were stained with anti-Btk (pY223)/Itk (pY180) (BD Phosflow) antibody. All antibodies were diluted 1:100 to their original concentration in FACS buffer. For quantification of total cell numbers, FACS beads were used (BD Biosciences). All stained cells were analysed on an LSRII or a FACS Fortessa (BD Biosciences) flow cytometer, and data were analysed with Flowjo software.

3.5.8 *Immunobloting*

Spleens were dissociated, and splenocytes were subjected to erythrocyte lysis buffer (MORPHISTO). Next, 10×10^6 cells were challenged with or without anti-CEACAM1 (clone CC1, 20 µg ml^{-1}) antibodies, anti-IgM (Jackson ImmunoResearch Laboratories, Inc., 10 µg ml^{-1}) antibodies, and LPS (Sigma-Aldrich, 100 ng ml^{-1}) treatment for indicated time points. Cells were lysed with boiling SDS buffer (1.1% SDS, 11% glycerol, 0.1M Tris; pH 6.8) with 10% 2-mercaptoethanol. Total cell extracts were examined by 10% SDS–PAGE gels and transferred onto Whatman nitrocellulose membrane (GE Healthcare) by standard techniques. Membranes were blocked for 1 h in 5% BSA (PAA Laboratories) in TBS supplemented with 1% Tween-20 and incubated with the following antibodies: antiphospho-Igα (Y182); anti-phospho-Syk/ZAP (Y352/Y319); anti-phospho-p44/42 (p-Erk1/2); anti-phopshpo-p38; anti-phospho-NF-κB p65; anti-Syk; anti-p44/42 (Erk1/2), anti-p38; anti-IκBα (all from Cell Signalling Technologies); anti-Igα (Thermo Scientific); and anti-NF-κB p65 (Santa Cruz). The secondary antibodies anti-actin (Cell Signalling Technologies) and anti-GAPDH (Meridian Life Science) were detected by horse radish peroxidase (HRP)-conjugated anti-mouse IgG (Bio-Rad) or anti-rabit IgG (GE Healthcare) antibodies, or both. Signals were detected with the BIO RAD ChemiDoc imaging system and analysed with the manufacturer's software. Blots were quantified with KODAK MI software. Images have been cropped for presentation purpose. Full-size images are presented in Supplementary Fig. 3.

3.5.9 *Immunoprecipitation*

Mouse B-lymphocyte H16-L10-4R5 cells were cultured in RPMI 1660 medium supplemented with 10% FCS and 1% antibiotics. 25×10^6 cells were lysed in dulbecco's phosphate-buffered saline (DPBS) supplemented with 1% Triton-X and protease inhibitor cocktail (both from Sigma-Aldrich). Lysates were incubated with anti-CEACAM1 antibody (CC1, Novus Biologicals, 5 µg ml^{-1}) overnight at 4º. CEACAM1 was immunoprecipitated with protein G dynabeads (Life technologies).

3.5.10 *RT–PCR*

Total RNA was extracted from MACS-sorted pure B cells with TRIZOL reagent (Ambion) according to the manufacturer's instructions. RNA was transcribed with a QuantiTect Reverse Transcription Kit (Qiagen). Quantitative real-time PCR amplification of

single genes was performed with SYBR Green quantitative PCR master mix in a Light Cycler 480 (Roche). QuantiTect Primer assays for Bcl-6, Pax-5, Rel-B, c-Jun, c-Fos, Ap1S1, CD5, NFATc1, NFATc2, NF-κB, BCL2A1, XIAP, and Blimp-1 (Qiagen) were used for quantification of mRNA expression of the respective genes. The following oligonucleotide primers that detect murine CEACAM1 isoforms were used for detection: mouse CEACAM1, a common sense primer that detects both long and short isoform FP-5'-GCCATGCAGCCTCTAACCCACC-3'; and two antisense primers that detect specific isoforms, mouse CEACAM1-L BP-5'-CTGGAGGTTGAGGGTTTGTGCTC-3' and mouse CEACAM1-S BP-5'-TCAGAAGGAGCCAGACCCGCC-3'. The product was analyzed on 3% agarose gels in Tris-borate-EDTA buffer and visualized by ethidium bromide staining. For analysis, expression levels of target genes were normalized to GAPDH, 18S r-RNA (Qiagen), or both as an internal control gene (ΔCt). Gene expression values were then calculated with the ΔΔCt method; the mean of 4 untreated WT B cells (0 hours after sorting) was used as a calibrator to which all other samples were compared. Relative quantities (RQ) were determined with the equation $RQ = 2^{-\Delta\Delta Ct}$.

3.5.11 *LCMV-glycoprotein GP1-specific IgG measurements*

The detection of LCMV glycoprotein GP-1-specific IgG by ELISA has been previously described[59]. In short, 96-well flat-bottom Nunc Immuno Plates (Thermo Scientific) were coated with anti-human IgG (Jackson ImmunoResearch Laboratories, Inc.) in coating buffer (0.1M Na_2CO_3 + 0.1M $NaHCO_3$; pH 9.6) overnight at 4 ºC. On the next day, plates were washed with washing buffer (PBS with 0.05% Tween-20), and unspecific binding was blocked with 2% FCS in PBS for 2 h. Plates were incubated with LCMV Gp-Fc supernatant (made in-house) for 3 h at room temperature. Plates were washed and titrated with pre-diluted (1: 20) serum over 12 wells with 1:3 dilutions in successive wells. After a 90-min incubation, plates were incubated with HRP-conjugated anti-mouse-IgG antibody (Sigma). After a 1-h incubation, plates were developed as described below.

3.5.12 *ELISA measurements*

For detection of VSV-specific anti-IgG and anti-IgM antibodies, 96-well flat-bottom Nunc Immuno Plates (Thermo Scientific) were coated with baculovirus VSV-GP[42] in coating buffer 0.1M $NaCO_3$ (0.1M Na_2CO_3 + 0.1M $NaHCO_3$; pH 9.6) overnight at 4 ºC. On the next day, plates were washed with washing buffer (PBS with 0.05% Tween-20), and unspecific

binding was blocked with 2% FCS in PBS for 1–2 h. Plates were washed once and titrated with prediluted (1:15) serum over 12 wells with 1:3 dilutions in successive wells. Plates were incubated at room temperature for 2 h. Plates were washed with washing buffer and incubated with HRP-conjugated anti-mouse-IgG (Sigma) or anti-mouse-IgM (Sigma) antibody for 30–60 min. Plates were washed and incubated with 1× TMB Substrate solution (eBioscience) in the dark, after which 10% H_2SO_4 solution was added to stop the colour reaction. Optical density was measured at 450 nm (FLUOstar Omega, BMG LABTECH). Various immunoglobulin isotypes and subtypes were measured in naïve serum of WT and *Ceacam1*$^{-/-}$ mice as described[55].

3.5.13 *Statistical analysis*

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 one-way analysis of variance (ANOVA) with Bonferroni or Dunnett post hoc tests. Survival was compared with log-rank (Mantel-Cox) tests. The level of statistical significance was set at $P < 0.05$.

3.6 *Acknowledgements*

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3.7 *Author contributions*

V.K. and V.D. designed, planned and performed the experiments, analysed data and wrote the paper. S.K.M. helped in experiments and was involved in data analysis. N.H. and

N.S. were involved in data discussion. A.P. helped in experiments. M.S. performed studies on human lymphocytes. V.P., H.C.X. and P.S. helped in experiments. F.B. and F.M. performed immunoglobulin isotype and subtype ELISAs. K.M. and E.L. helped in experiments. C.K., A.M.W., D.H., F.L., U.D., R.K., M.R., C.H. and I.S. were involved in the data discussion and in drafting the manuscript. N.B. provided the *Ceacam1*$^{-/-}$ mice and was involved in the data discussion and in drafting the manuscript. J.R.G. performed the B-cell analysis, provided the reagents, discussed the data and wrote the paper. B.B.S. initiated the study, provided reagents, organized and discussed the data and wrote the paper. P.A.L. discussed the data and wrote the paper. K.S.L. initiated, organized and designed the study, wrote the paper and completed the manuscript.

3.8 *Figure Legends*

3.8.1 *Figure 1: CEACAM1 is expressed on murine B-cell subsets.*

(a,b) Representative histogram showing CEACAM1 expression in leukocyte subpopulations isolated from wild-type (WT, black line) and *Ceacam1*$^{-/-}$ mice (red line). Isotype control antibody staining of leukocytes from WT mice is shown as a grey area. Peripheral blood leukocytes gated for Ly6G (granulocytes) and CD115 (monocytes; **a**) and CD90.2^{+} (T cells) or B220^{+} (B cells) cells **(b)**, as measured by flow cytometry (n = 6 per group). **(c–e)** Dot plot showing IgD and IgM expression of cells gated on B220, and histogram showing CEACAM1 expression (black line) versus isotype control antibody staining (grey area) in bone marrow **(c)**, lymph nodes **(d)** and spleens **(e)**, respectively, from WT mice, as measured by flow cytometry (n = 6 per group). **(f)** Representative expression levels of CEACAM1-S and CEACAM1-L isoforms in murine splenic B cells by real-time PCR (n = 6). $^{**}P<0.01$ (Student's t-test). Data are representative of two **(a–f)** experiments (mean±s.e.m., f).

3.8.2 *Figure 2: CEACAM1 in B cells induces survival genes via Syk and Erk and NF-κB.*

(**a,b**) Representative immunoblot probed with antibodies to phospho-Igα, Igα and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in wild-type (WT) and *Ceacam1*$^{-/-}$ splenocytes (**a**, left panel) or in WT Splenocytes (**a**, right panel) or probed with antibodies to phospho-Syk, Syk and GAPDH in WT and *Ceacam1*$^{-/-}$ splenocytes (**b**, left panel) or in WT splenocytes (**b**, right panel) after treatment with anti-IgM or anti-CEACAM1 monoclonal antibody or LPS for indicated time points (n = 4). (**c**) Representative flow cytometry

histogram of WT or *Ceacam1*$^{-/-}$ splenocytes gated on B cells showing p-Btk (pY223)/Itk (pY180) staining; splenocytes were left untreated (grey area) or were treated with anti-IgM or anti-CEACAM1 antibody for indicated time points at 37 ºC (n = 3). (**d,e**) Representative immunoblot probed with antibodies to Tyr-P-Erk (p-p44/42 MAPK), Erk (p44/42) and GAPDH (**d**, n = 4) or with antibodies to phospho-p38, p38 and GAPDH (**e**, n = 3) in WT splenocytes after treatment with anti-CEACAM1 antibody or LPS for indicated time points. (**f**) Representative immunoblot and quantification of specific bands after staining for p-NF-κB p65, NF-κB p65, IκBα and β-actin in WT or *Ceacam1*$^{-/-}$ B cells after challenge with anti-CEACAM1 antibody for indicated time points (n = 4). (**g–k**) RT–PCR analysis of representative transcription factors such as *Bcl-6*, *Pax-5* (**g**, n = 5), *Bcl2a1*, *Xiap* (**g**, n = 7), *NF- κB* p65, *Rel-B* (**h**, n = 5–7), *Nfatc1*, *Nfatc2* (**i**, n = 5) or *c-Jun*, *c-Fos*, and *Ap1s1* (**j**, n = 5) or *Blimp1* (**k**, n = 7) mRNA from WT and *Ceacam1*$^{-/-}$ B cells sorted from spleen and 24 h after without stimulation (values show fold change to expression in B cells 0 h after sorting). **P*<0.05; ***P*<0.01 and ****P*<0.001 (Student's t-test); NS = not significant. Data are representative of two (**g,h,i,j,k**), three (**c,e**) or four (**a,b,d,f**) experiments (mean±s.e.m., **f,g,h,i,j,k**). Immunoblot images have been cropped for presentation purpose. Full-size images are presented in Supplementary Fig. 3.

3.8.3 *Figure 3: CEACAM1 promotes survival of B cells in vitro*

(**a**) Representative flow cytometry histogram of proliferating B cells from wild-type (WT) or *Ceacam1*$^{-/-}$ mice that were left untreated (grey area) or were further challenged (red line) with anti-CEACAM1 antibody, with LPS, or with recombinant mouse CD40 ligand in combination with mouse IL-4 for 48 h, as determined by FACS analysis. Histograms show DAPI^{-} cells (n = 6). (**b**) Absolute numbers of living B cells (DAPI^{-}) for indicated time points (n = 3) sorted from spleen and after challenge with or without recombinant mouse CD40 ligand in combination with mouse IL-4 (n = 3) determined by FACS analysis. (**c**) Representative histogram and statistical analysis of Annexin-V^{+} B cells, which were stimulated with recombinant mouse CD40 ligand in combination with mouse IL-4 or were left unstimulated for 48 h (gated on DAPI^{-} B cells, n = 6). (**d**) Percentage of DAPI^{+} B cells sorted from spleen of WT and *Ceacam1*$^{-/-}$ mice after challenge with recombinant mouse CD40 ligand in combination with mouse IL-4 cultured in the presence or absence of Btk inhibitor Ibrutinib for 48 h determined by FACS analysis (n = 6). **P*<0.05; ***P*<0.01 and

***P<0.001 (Student's t-test). Data are representative of one of two (**b**) or two (**a,c,d**) experiments (mean±s.e.m. (**b–d**)).

3.8.4 *Figure 4: CEACAM1 promotes B-cell survival in vivo.*

(**a**) Scheme of developmental, maturation and migration stages of B cells in bone marrow, blood, lymph node and spleen. Arrows indicate most likely developmental pathway. Dotted arrow indicates still debated developmental pathway. Red arrow indicates differentiation after antigen challenge. (**b–e**) Representative dot blots, gating strategy and total numbers of B-cell subpopulations from wild-type (WT) and *Ceacam1*$^{-/-}$ mice as measured by flow cytometry in bone marrow (**b**, n = 6), in blood (**c**, n = 4), in lymph node (**d**, n = 4) and in spleen (**e**, n = 10). (**f,g**) Representative immunofluorescence of spleen and lymph node (**f**, n = 6) sections derived from naïve WT and *Ceacam1*$^{-/-}$ mice after staining for B cells (B220, blue) or T cells (CD90.2, green) and spleen sections stained for marginal zone B cells (CD1d, red), follicular B cells (CD19, green), marginal zone macrophages (CD169, red) or B cells (B220, blue; **g**, n = 6). (**h**) Representative immunofluorescence results of spleen sections from *Jh*$^{-/-}$ mice that were left untreated or were subjected to adoptive transference with 1 × 10^7 B cells derived from WT or *Ceacam1*$^{-/-}$ mice 30 days before analysis, stained for T cells (CD90.2, green) and B cells (CD19, blue; n = 3). (**i,j**) Representative dot blots, gating strategy and statistical analysis of B-cell subpopulations from murine bone marrow chimeras reconstituted with 1:1 composition of bone marrow from WT(CD45.1): WT(CD45.2) mice in one group and bone marrow from WT(CD45.1): *Ceacam1*$^{-/-}$ (CD45.2) mice in another group after 45 days of reconstitution (n = 5 per group) as measured by flow cytometry in blood (**i**) and spleen (**j**; only second group has been shown for FACS gating strategy). Scale bars, 300 mm. *P<0.05; **P<0.01 and ***P<0.001 (Student's t-test); NS = not significant. Data are representative of one (**h**) or two (**b–d,i,j**) or three (**e**) experiments (mean±s.e.m., **b–e**). One representative slide of three (**h**) or six (**f,g**) slides is shown.

3.8.5 *Figure 5: CEACAM1 ensures mouse survival during VSV challenge.*

(**a**) Immunofluorescence of spleen sections from naïve wild-type (WT) and *Ceacam1*$^{-/-}$ mice and WT mice 7 h after infection with 2 × 10^8 PFU of VSV (n = 6), stained for VSV glycoprotein (green) and CEACAM1 (red). Scale bars, 300 µm (main image) or 100 µm (insets). (**b**) Bar diagram showing percentage of WT × Vi10 and *Ceacam1*$^{-/-}$ × Vi10 B

cells that were adoptively transferred into WT mice (1×10^7 per mouse) on day −1 and which were infected with 2×10^6 PFU of VSV on day 0. The proliferation was analysed in spleen 3 days after infection by FACS (n = 3 per group). (**c,d**) Total VSV-neutralizing antibodies and neutralizing IgG antibodies generated in WT and *Ceacam1*$^{-/-}$ mice after intravenous infection with 2×10^6 PFU of VSV (**c**, n = 6–9 per group) and/or after intravenous infection with 2×10^8 PFU of ultraviolet (UV) light-inactivated VSV (**d**, n = 8–9 per group). (**e**) VSV-neutralizing antibody response and neutralizing IgG antibodies measured in sera of WT and *Ceacam1*$^{-/-}$ mice that also received 1×10^7 VSV-specific B cells (Vi10) on day −1 and were then intravenously infected with 2×10^6 PFU VSV on day 0 (n = 7–9 per group). (**f**) VSV titres in various organs of WT and *Ceacam1*$^{-/-}$ mice after intravenous infection with 2×10^6 PFU of VSV assessed 8 days after infection (n = 6 per group). (**g**) Survival of WT and *Ceacam1*$^{-/-}$ mice after intravenous infection with 2×10^6 PFU of VSV (n = 9–12 per group). (**h**) Survival of WT and *Ceacam1*$^{-/-}$ mice that were left untreated or were adoptively given 1×10^7 VSV-specific B cells (Vi10) on day −1 and then intravenously infected with 2×10^6 PFU of VSV on day 0 (n = 4–9). *P<0.05; **P<0.01 and ***P<0.001 (Student's t-test for **b,c,f**; Mentel-Cox survival test for **g** and **h**). Data are representative of one (**b**) or two (**a,f**) or three (**c–e**,**h**) or four (**g**) experiments (mean±s.e.m., **b**,**c**,**f**,**g**).

3.8.6 *Figure 6: CEACAM1 facilitates LCMV-dependent B-cell activation.*

(**a**)LCMV glycoprotein-specific IgG antibodies measured in sera of wild-type (WT) and *Ceacam1*$^{-/-}$ mice after intravenous infection with 200 PFU of LCMVWE (n = 3–6 per group). (**b**) Liver virus titres of WT and *Ceacam1*$^{-/-}$ mice 10 days after intravenous infection with 2×10^6 PFU of LCMV-WE (n = 3 per group). ***P<0.001 (Student's t-test). Data are representative of one (**b**) or two (**a**) experiments (mean±s.e.m., **a**,**b**).

3.8.7 *Figure 7: Human B-cell subpopulations express CEACAM1.*

(**a**) Representative FACS plot and histogram showing CEACAM1(monoclonal antibody, B3-17) expression in lymphocyte subpopulations in human peripheral blood on naive (IgD^+ $CD27^-$) and memory ($CD27^+$) B cells. Isotype control antibody staining of lymphocytes is shown as red line (n = 6). (**b**) Histogram plot showing differences in mean fluorescence intensity (MFI) levels between human naïve and memory B cells (n = 6). *P<0.05 (Student's t-test). Data are representative of independent staining from six donor samples (mean±s.e.m., **b**).

3.8.8 *Supplementary Figure 1: Erythrocytes stain negative for CEACAM1*

Representative dot plot and histogram showing CEACAM1 expression in peripheral blood erythrocytes from wild-type (WT, black line) and *Ceacam1*$^{-/-}$ mice (grey area) as measured by flow cytometry (n = 3−4 per group).

3.8.9 *Supplementary Figure 2: CEACAM1 interacts with Syk*

Immunoblot analysis of CEACAM1, Syk and GAPDH in mouse B-lymphocyte from whole cell lysate and after immunoprecipitation with or without anti-CEACAM1 antibody (mAb, CC1). Data are representative of three independent experiments.

3.8.10 *Supplementary Figure 3: Uncropped western blots shown in Figure 2.*

Proteins were loaded on 10% SDS-PAGE gels and transferred onto Whatman nitrocellulose membrane by standard techniques. Nitrocellulose membranes were subsequently developed from left to right.

3.8.11 *Supplementary Figure 4: CEACAM1 expression affects B1a B-cell proportion in peritoneum*

a: Representative dot blots, gating strategy, and percentage of B1 B-cell subpopulations from wild-type (WT) and *Ceacam1*$^{-/-}$ mice as measured by flow cytometry in peritoneal cavity (n = 5). **b:** Bar diagram showing average frequencies of indicated subsets calculated from the gates shown above. Numbers in plots represents the percentage of events as a function of indicated parent gate. B2 ($CD19^+B220^+CD43^-$), B1a ($CD19^+B220^+CD43^+CD5^+$), and B1b ($CD19^+B220^+CD43^+CD5^-$). $^{**}P < 0.01$; and $^{***}P < 0.001$ (Student's t-test); ns = not significant. Data are representative of two experiments (mean ± SEM).

3.8.12 *Supplementary Figure 5: BAFF receptor signaling resembles CEACAM1-mediated signaling*

Representative immunofluorescence of spleen sections derived from naïve wild-type (WT) (n = 6), *Myd88/Trif*$^{-/-}$ (n = 5), *Fcgr2b*$^{-/-}$ (n = 6), *Baffr*$^{-/-}$ (n = 6), and *Jh*$^{-/-}$ (n = 6) mice after staining for marginal zone B cells (CD1d, red), marginal zone macrophages (CD169, green), and B cells (B220, blue). Scale bars, 300µm.

3.8.13 *Supplementary Figure 6: CEACAM1 influences the levels of serum immunoglobulins*

Histogram showing levels of various serum immunoglobulin isotypes and subtypes in naïve wild-type (WT) and *Ceacam1*$^{-/-}$ mice (n = 8 per genotype). ***P* < 0.01; and ****P* < 0.001 (Student's t-test). Data are representative of two experiments (mean ± SEM).

3.8.14 *Supplementary Figure 7: CEACAM1 is essential for anti-VSV-specific Ig production*

Histogram showing anti-VSV–specific IgM and IgG antibodies generated in wild-type (WT) and *Ceacam1*$^{-/-}$ mice after intravenous infection with 2 × 10^6 PFU of VSV (n = 4–7 per group). **P* < 0.05; ***P* < 0.01; and ****P* < 0.001 (Student's t-test). Data are representative of two experiments (mean ± SEM).

3.8.15 *Supplementary Figure 8: MZ and FO B cells can rescue survival in Ceacam1*$^{-/-}$ *mice*

Histogram showing total VSV neutralizing antibody response (**a**) and neutralizing IgG antibodies (**b**) measured in sera of wild-type (WT) and *Ceacam1*$^{-/-}$ mice that also received 10 × 10^6 MZ and 1 × 10^6 FO VSV-specific B cells (Vi10) on day -1 and were then intravenously infected with 2 × 10^6 PFU VSV on day 0 (n = 3 per group).

3.8.16 *Supplementary Figure 9: B cells but not other cell types are important for the replication of vesicular stomatitis virus in the spleen and the activation of adaptive immunity.*

a: Spleen vesicular stomatitis virus (VSV) titers from wild-type (WT) or *Jh*$^{-/-}$ mice or from WT or *Tcrb*$^{-/-}$ mice (n = 6 per genotype) 7 h after intravenous infection with 2 × 10^6 PFU of VSV. **b:** Immunofluorescence assay of spleen sections from WT or *Jh*$^{-/-}$ mice or from WT or *Tcrb*$^{-/-}$ mice 7 h after intravenous infection with 2 × 10^8 PFU of VSV stained with VSV glycoprotein (green) and CD169 (red). One of six representative slides is shown. **c:** Spleen VSV titers from WT or *Aid*$^{-/-}$ mice or from WT or *sIgM*$^{-/-}$ mice (n = 6 per genotype) 7 h after intravenous infection with 2 × 10^6 PFU of VSV. Scale bars, 300 µm. ****P* < 0.001 (Student's t-test); ns = not significant. Data are representative of two (**a** & **c**) experiments. One representative slide of six (**b**) slides is shown (mean ± SEM, **a**, **c**).

3.8.17 *Supplementary Figure 10: Deficient marginal zone in Ceacam1$^{-/-}$ mice limits antiviral innate immune response*

a: Immunofluorescence of spleen sections from wild-type (WT) or *Ceacam1*$^{-/-}$ mice 7 h after intravenous infection with 2×10^8 PFU of vesicular stomatitis virus (VSV), stained for VSV glycoprotein (green), CD169 (red), and B220 (blue) (n = 6 per genotype). One of six representative slides is shown. Scale bar, 300 µm. **b:** Spleen VSV titers from WT or *Ceacam1*$^{-/-}$ mice 7 h after intravenous infection with 2×10^6 PFU of VSV (n = 6 per genotype). $^{**}P < 0.01$ (Student's t-test). Data are representative of two (**b**), or one of two (**a**) experiments (mean ± SEM, **b**).

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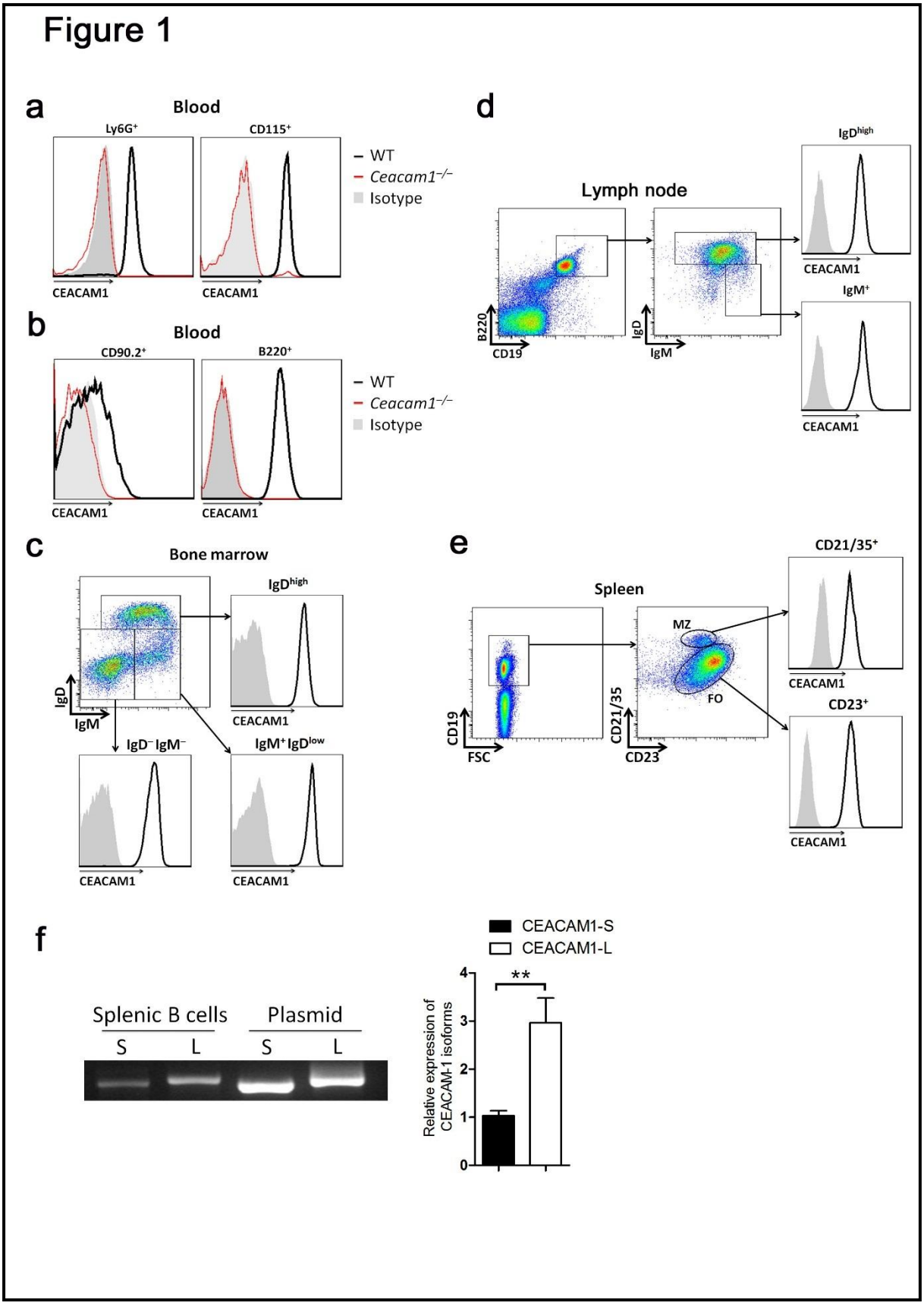


Figure 3-1: CEACAM1 is expressed on murine B-cell subsets.

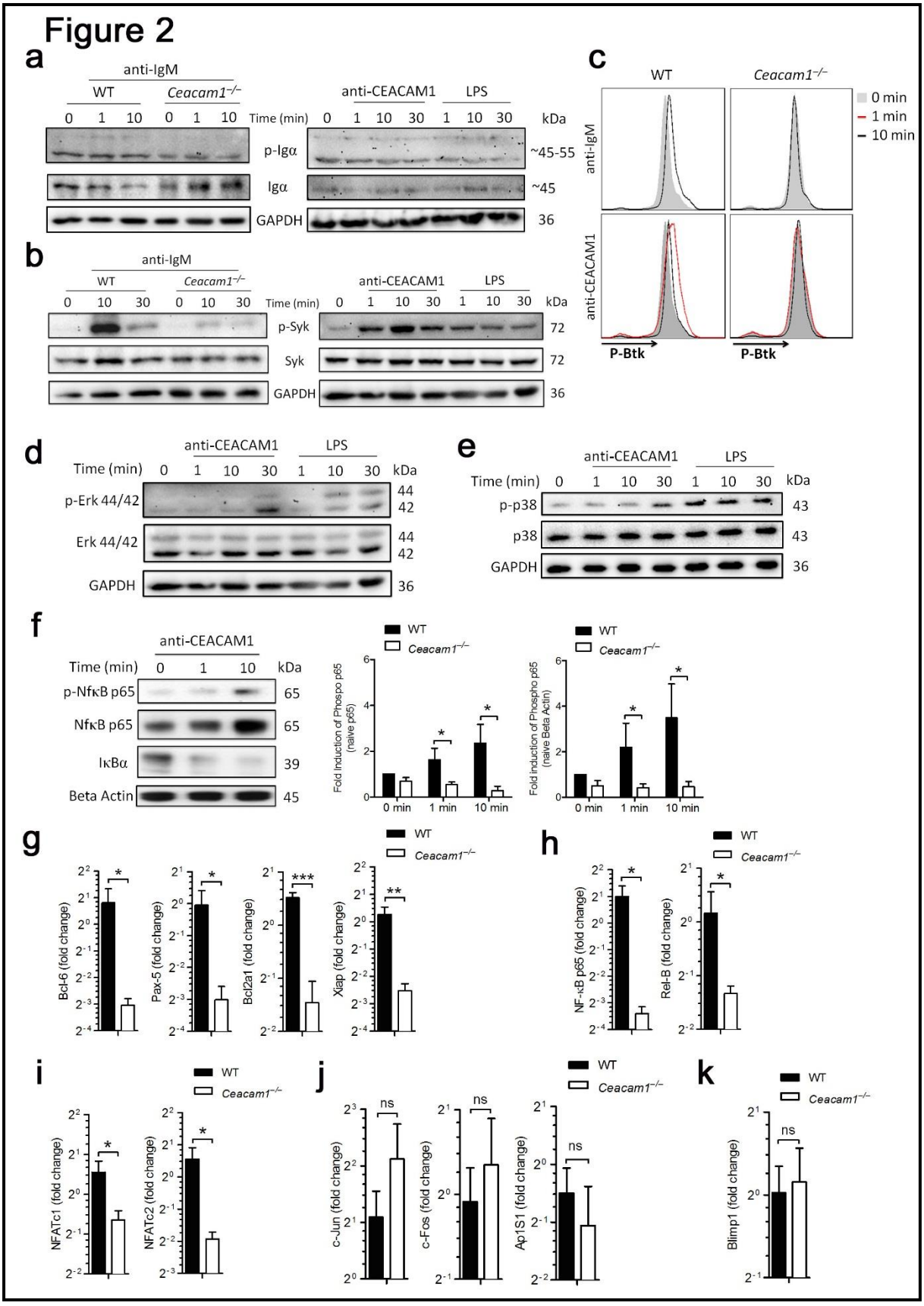


Figure 3-2: CEACAM1 in B cells induces survival genes via Syk and Erk and NF-κB.

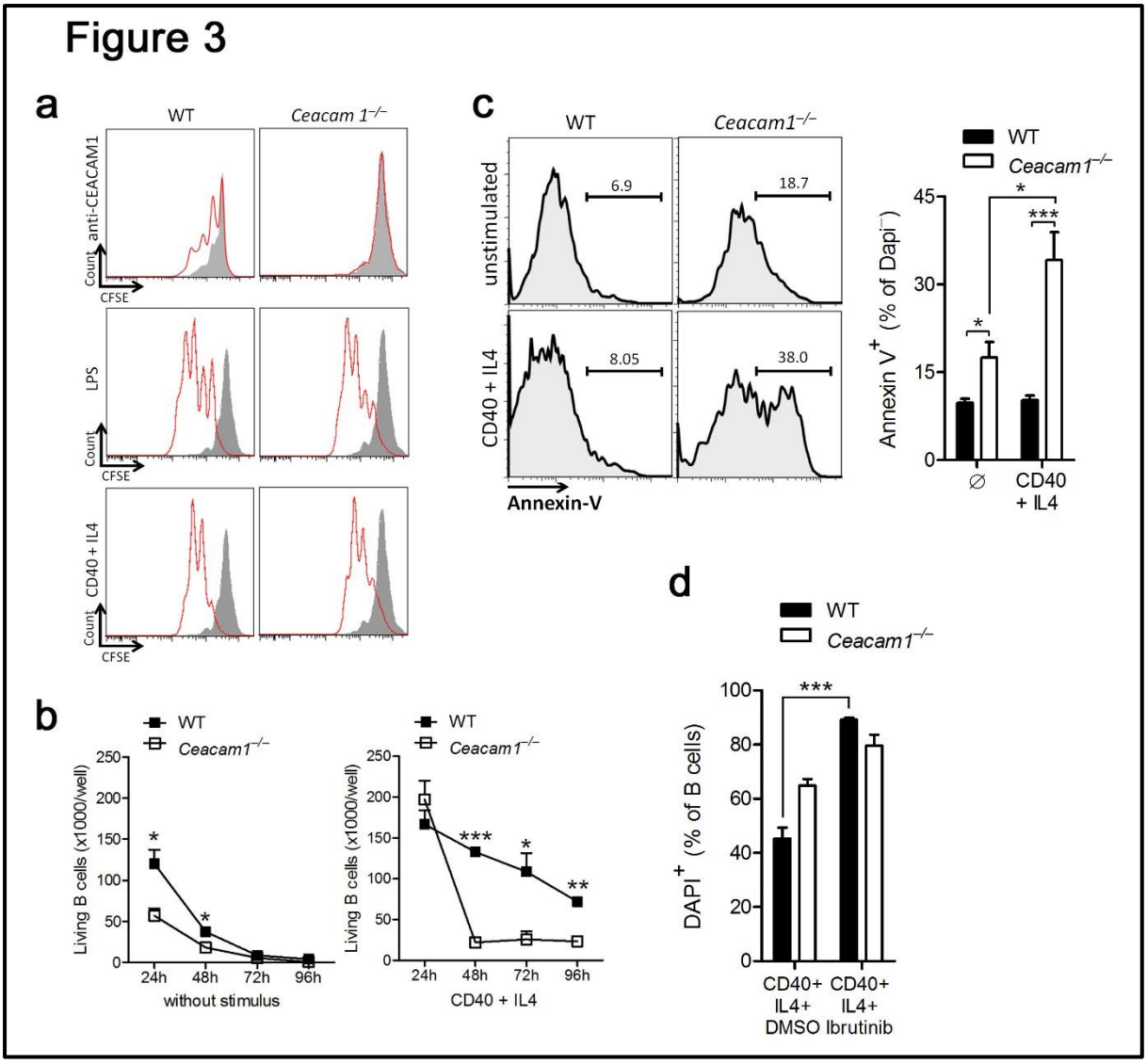


Figure 3-3: CEACAM1 promotes survival of B cells *in vitro*.

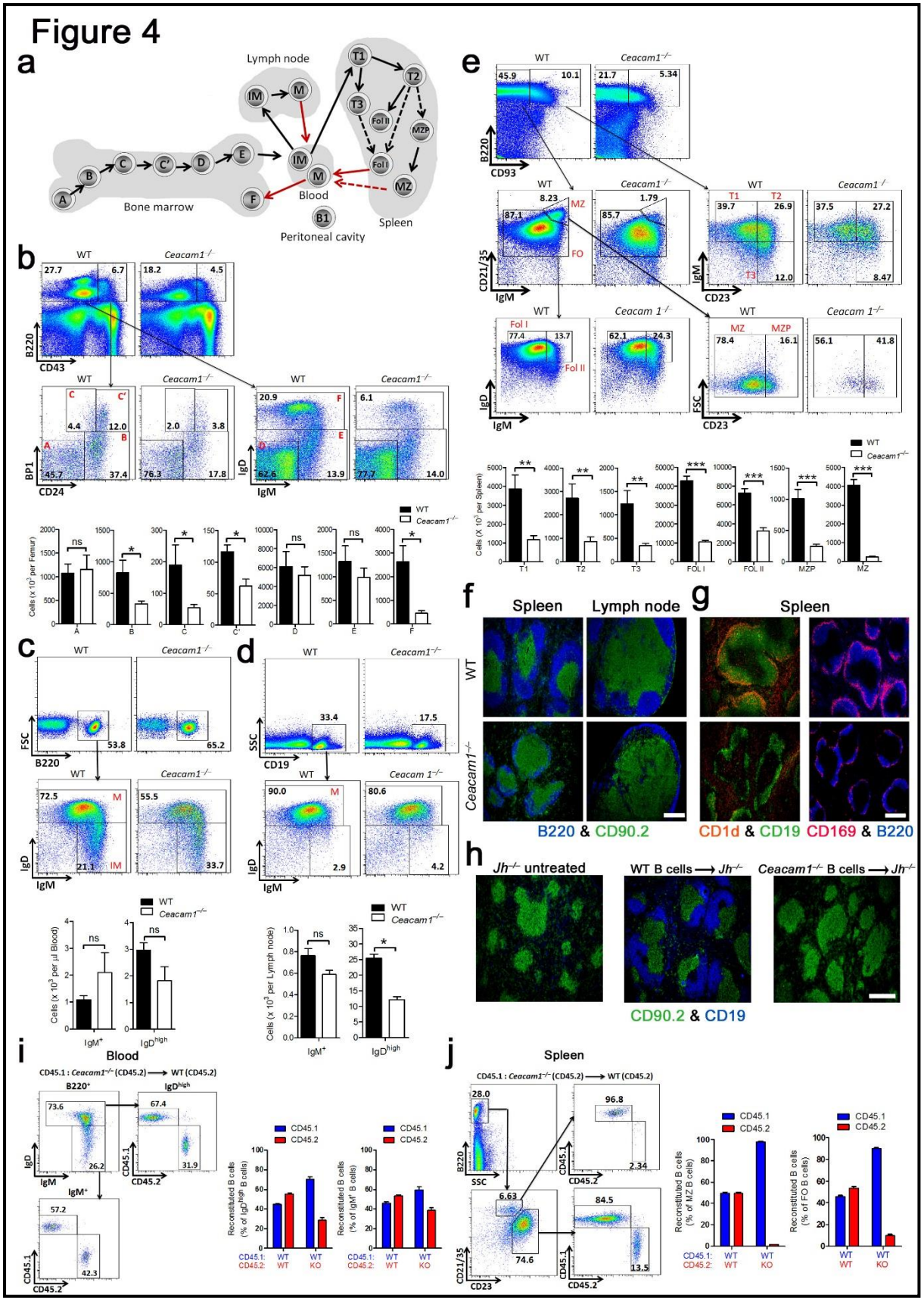


Figure 3-4: CEACAM1 promotes B-cell survival *in vivo*.

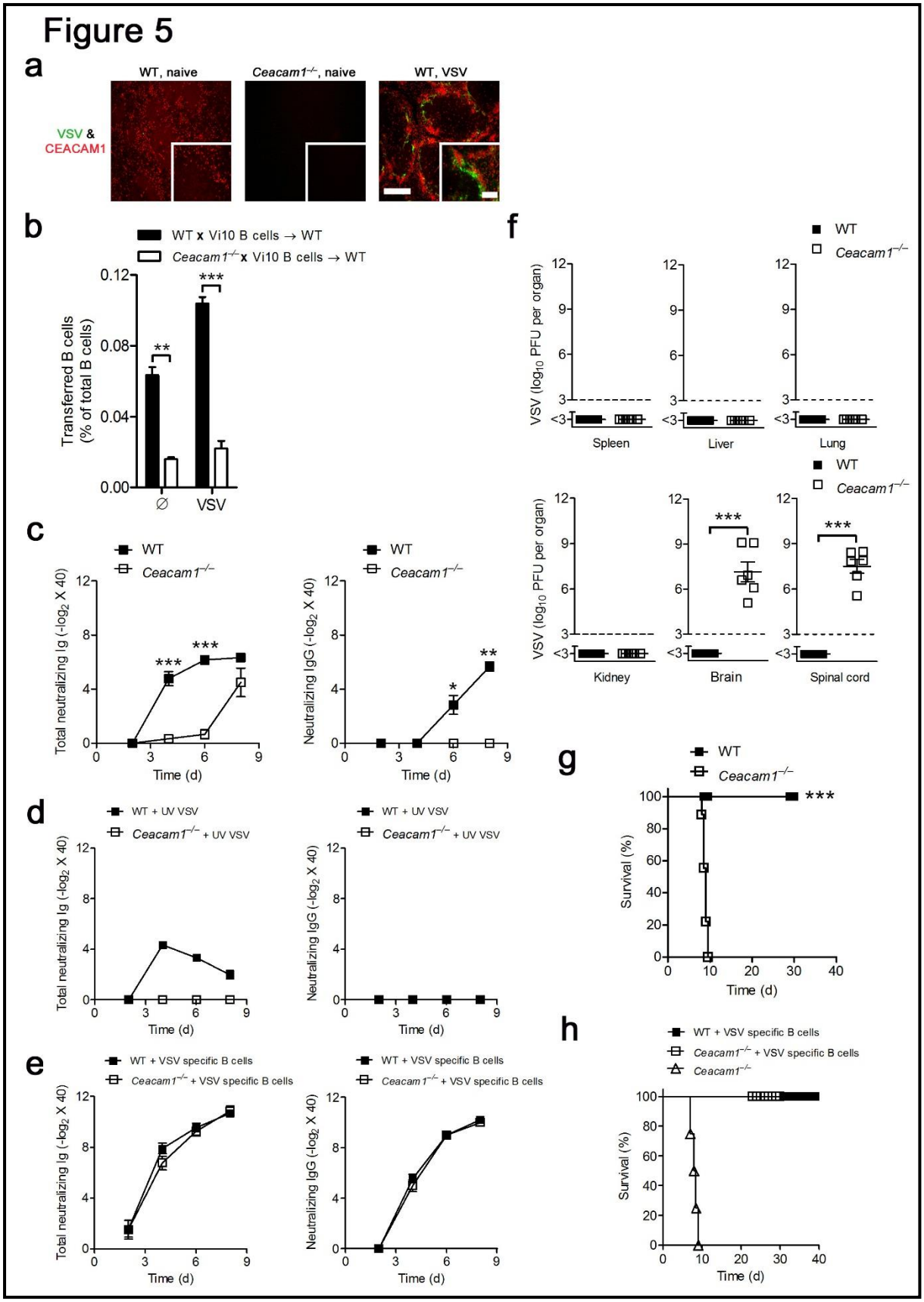


Figure 3-5: CEACAM1 ensures mouse survival during VSV challenge.

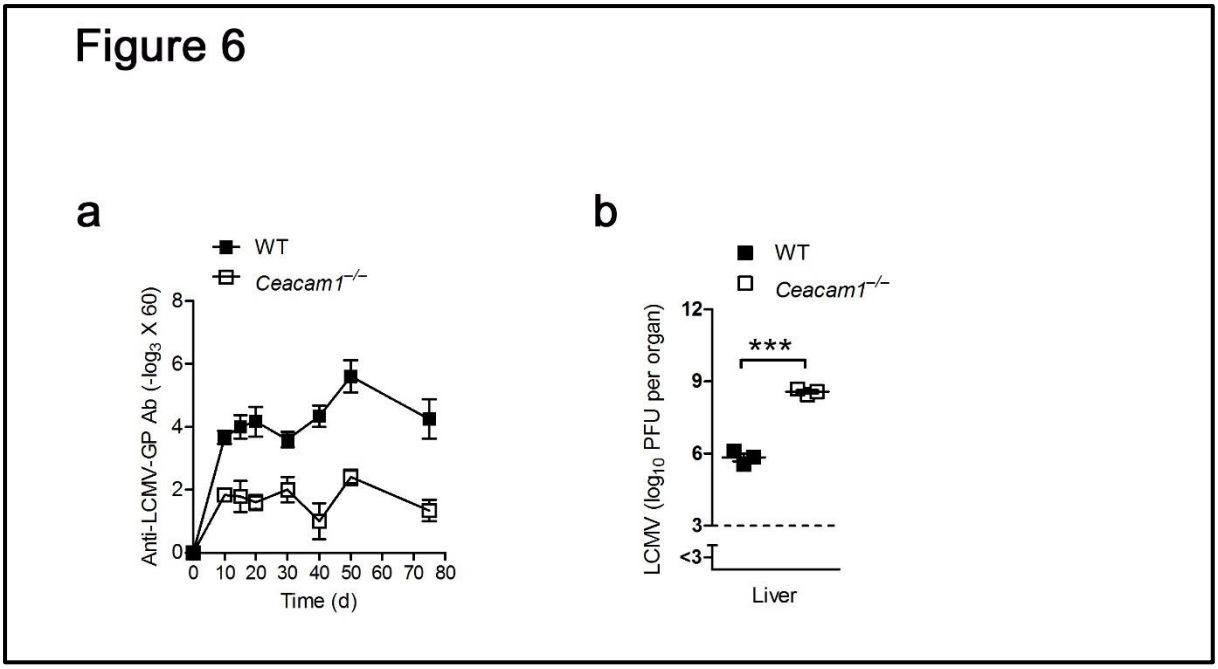


Figure 3-6: CEACAM1 facilitates LCMV-dependent B-cell activation.

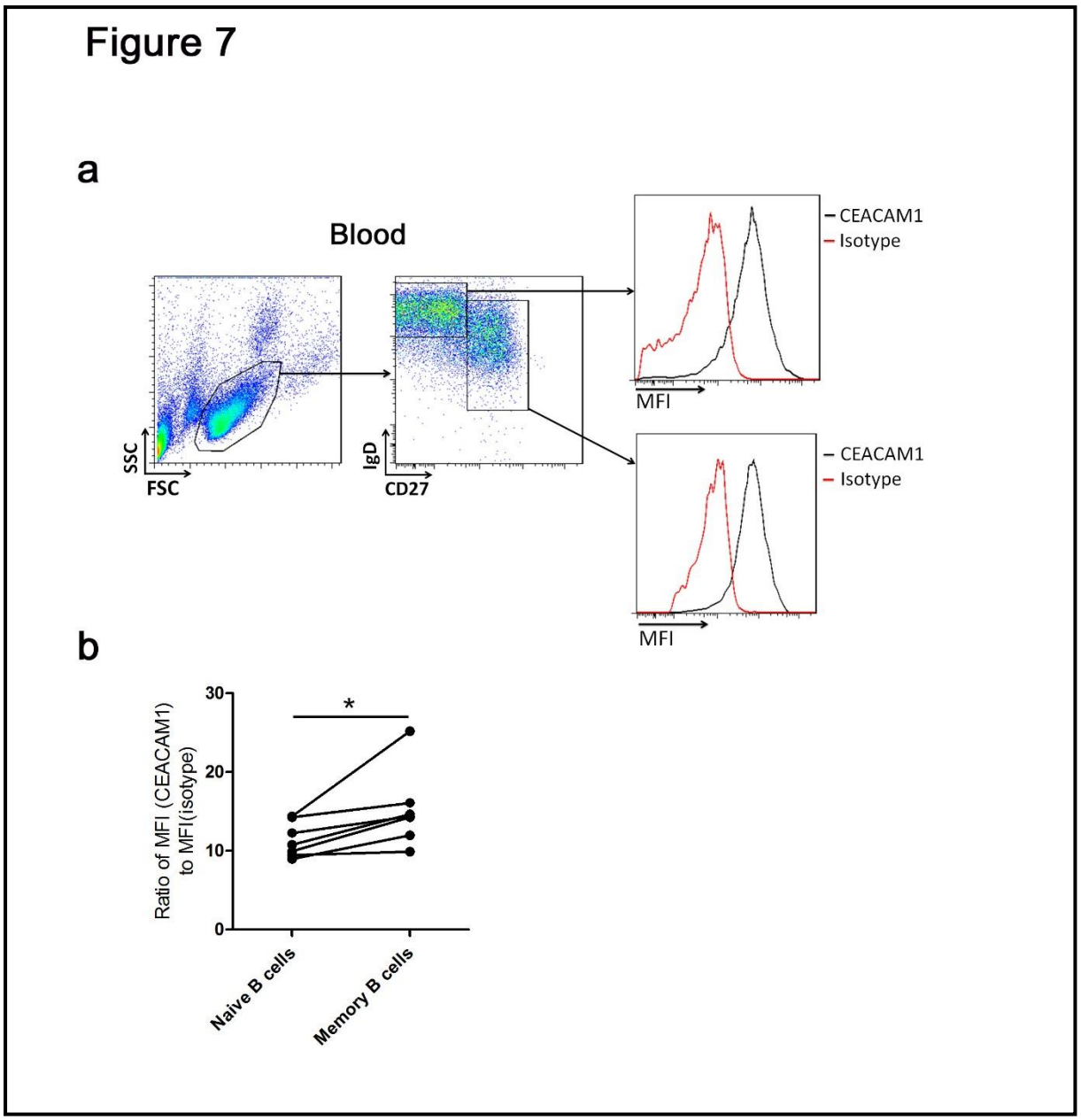
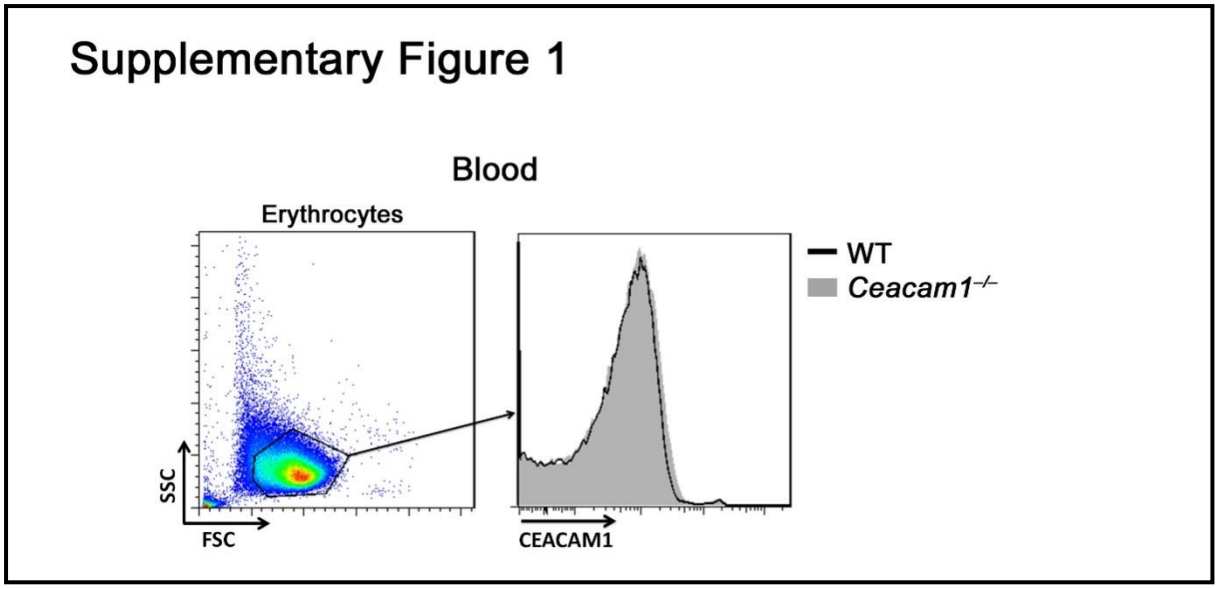
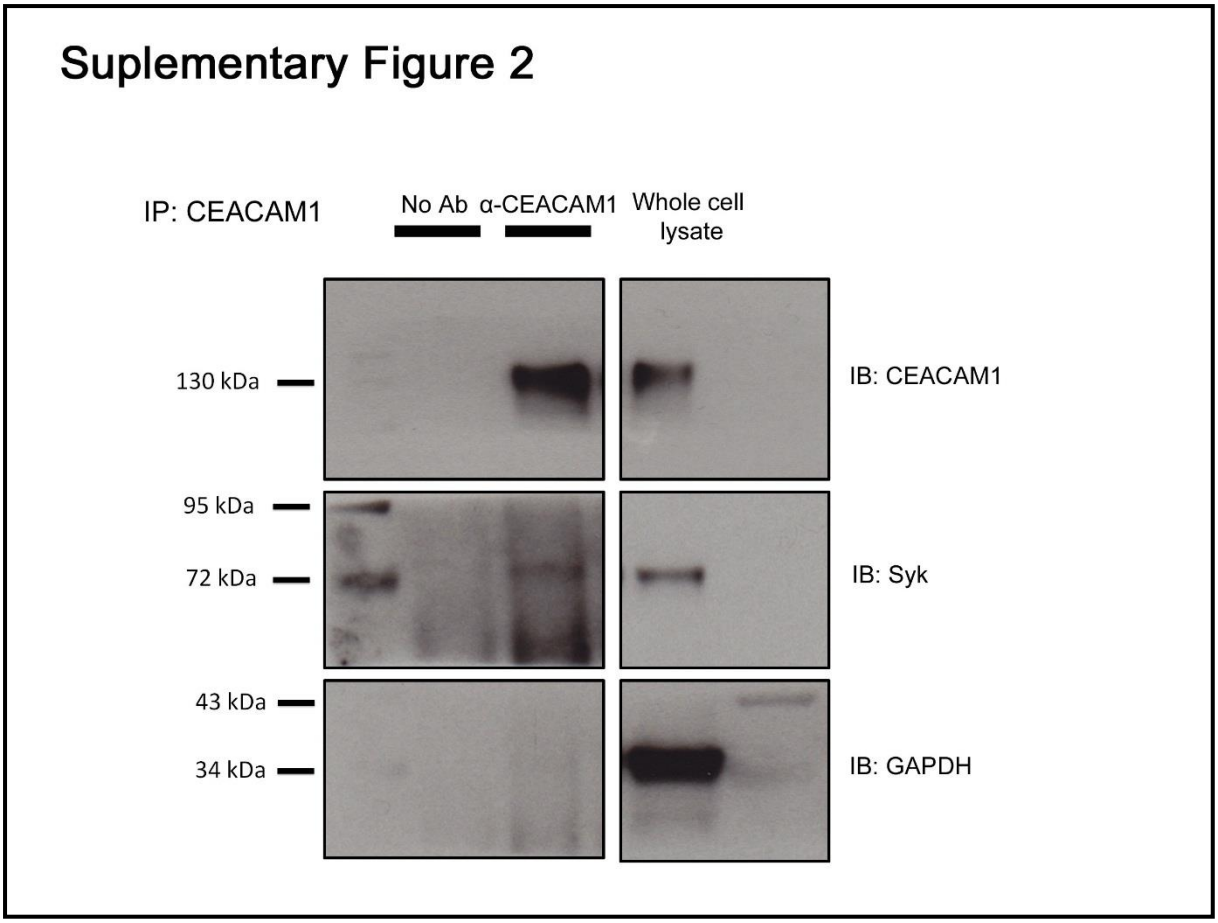


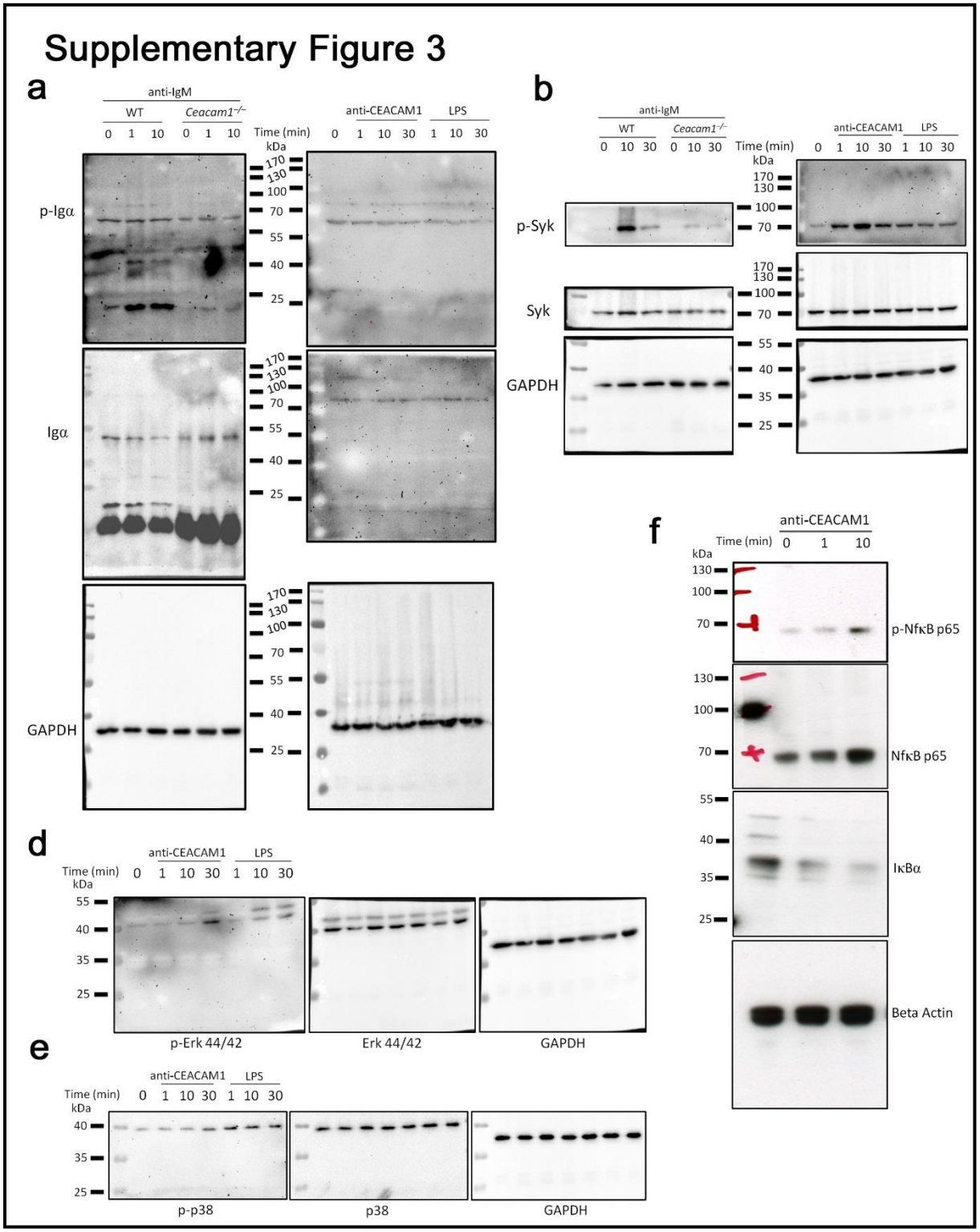
Figure 3-7: Human B-cell subpopulations express CEACAM1.



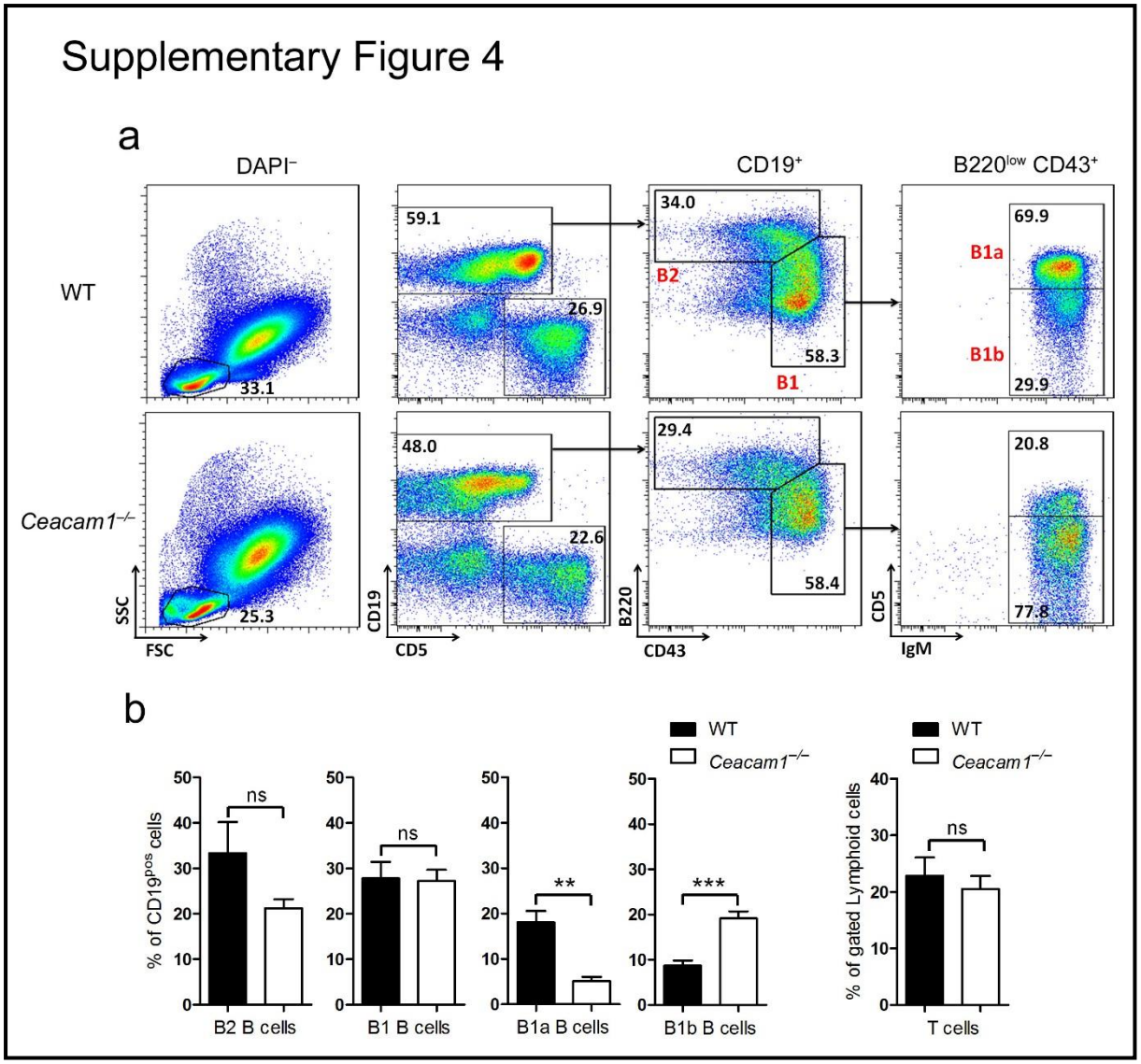
Supplementary Figure 3-1: Erythrocytes stain negative for CEACAM1



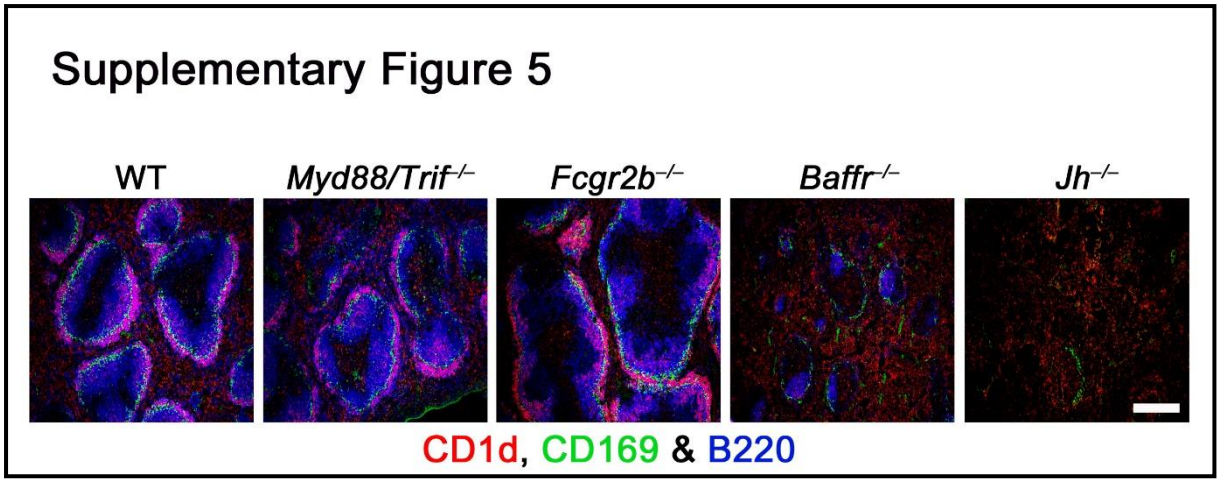
Supplementary Figure 3-2: CEACAM1 interacts with Syk



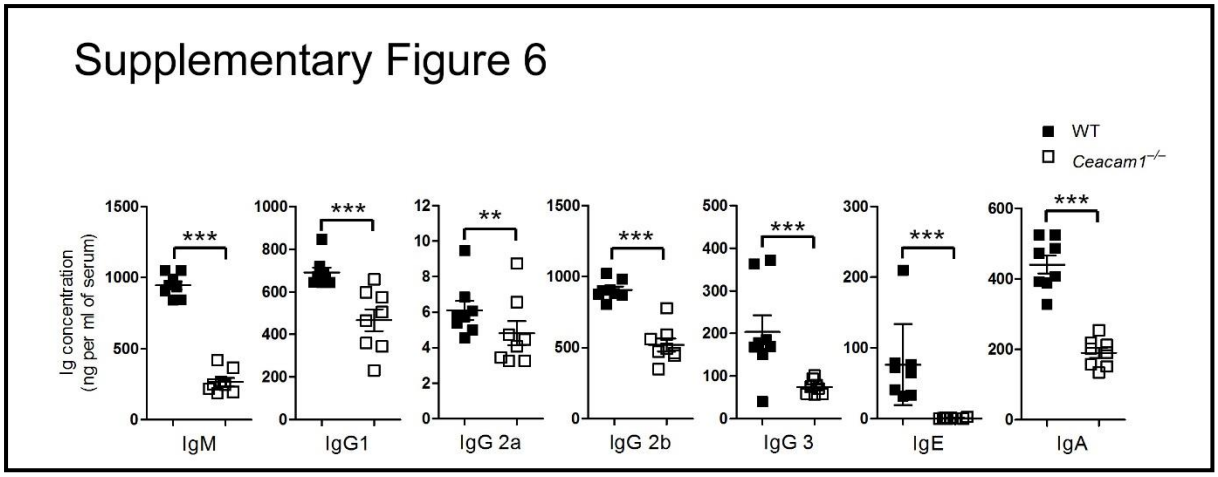
Supplementary Figure 3-3: Uncropped western blots shown in Figure 2.



Supplementary Figure 3-4: CEACAM1 expression affects B1a B-cell proportion in peritoneum

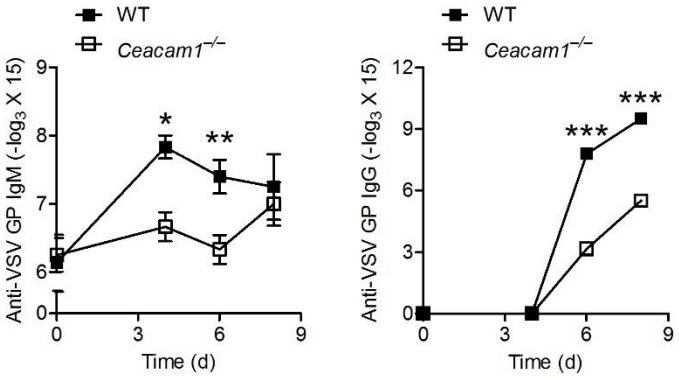


Supplementary Figure 3-5: BAFF receptor signaling resembles CEACAM1-mediated signalling

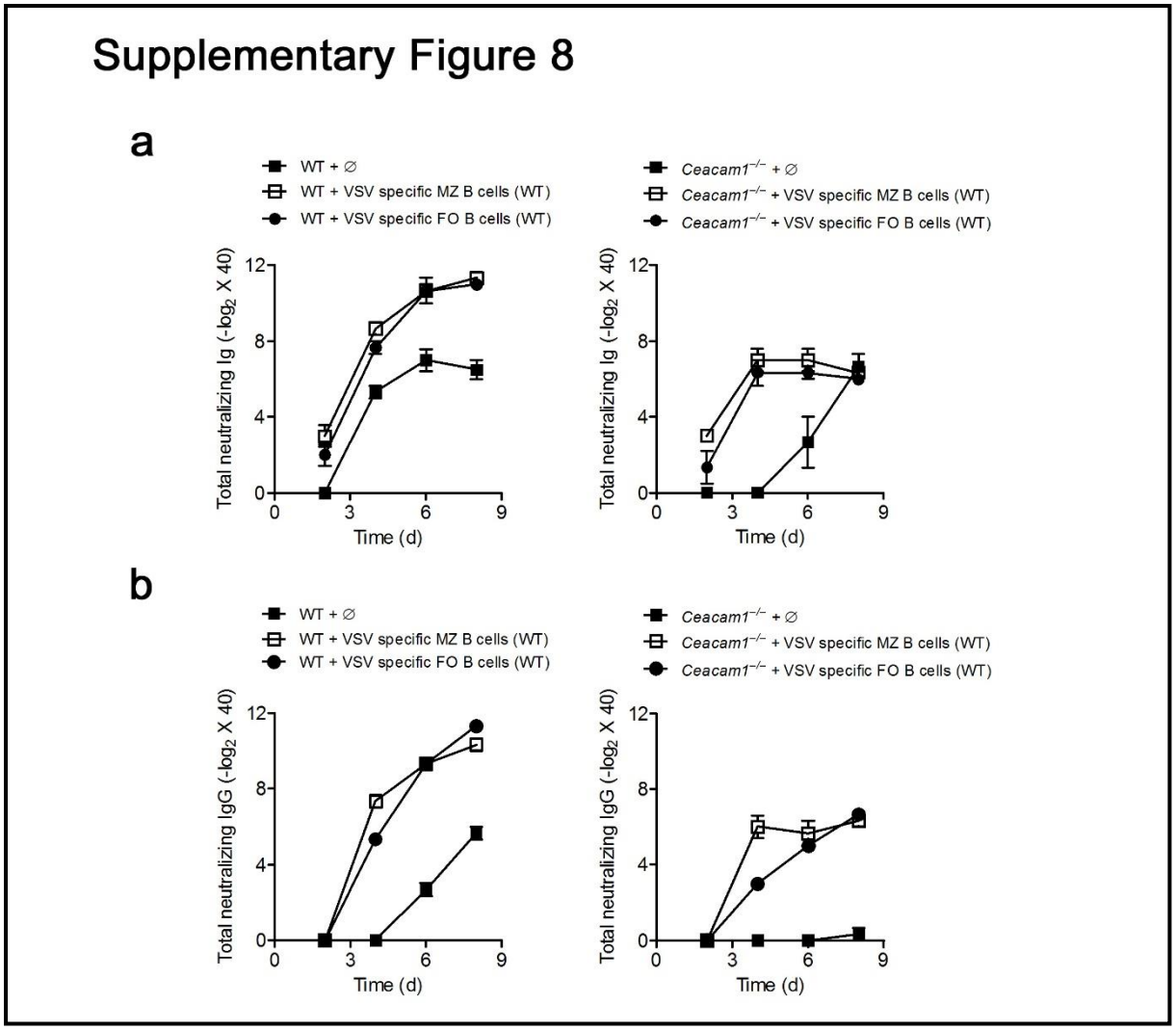


Supplementary Figure 3-6: CEACAM1 influences the levels of serum immunoglobulins

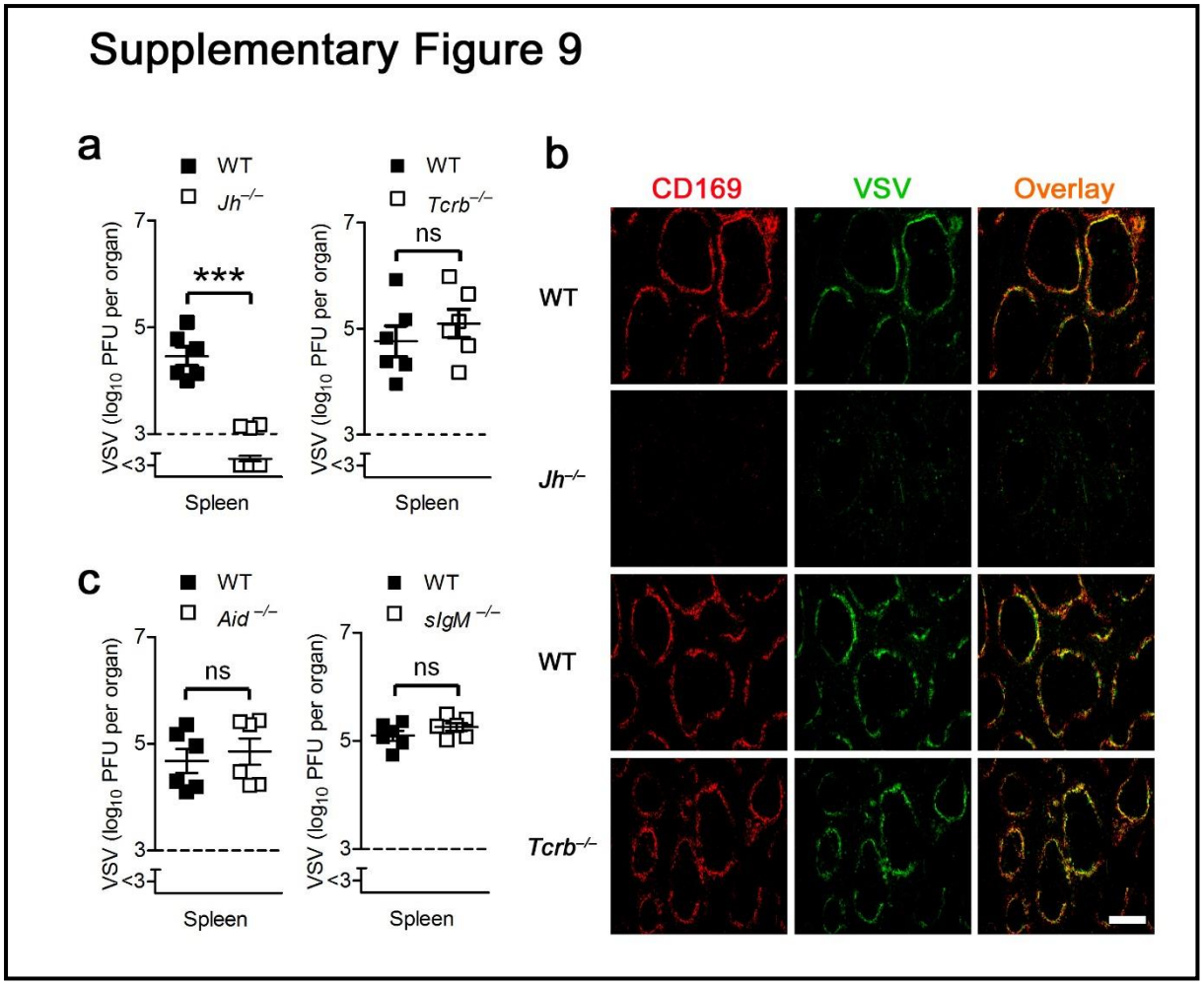
Supplementary Figure 7



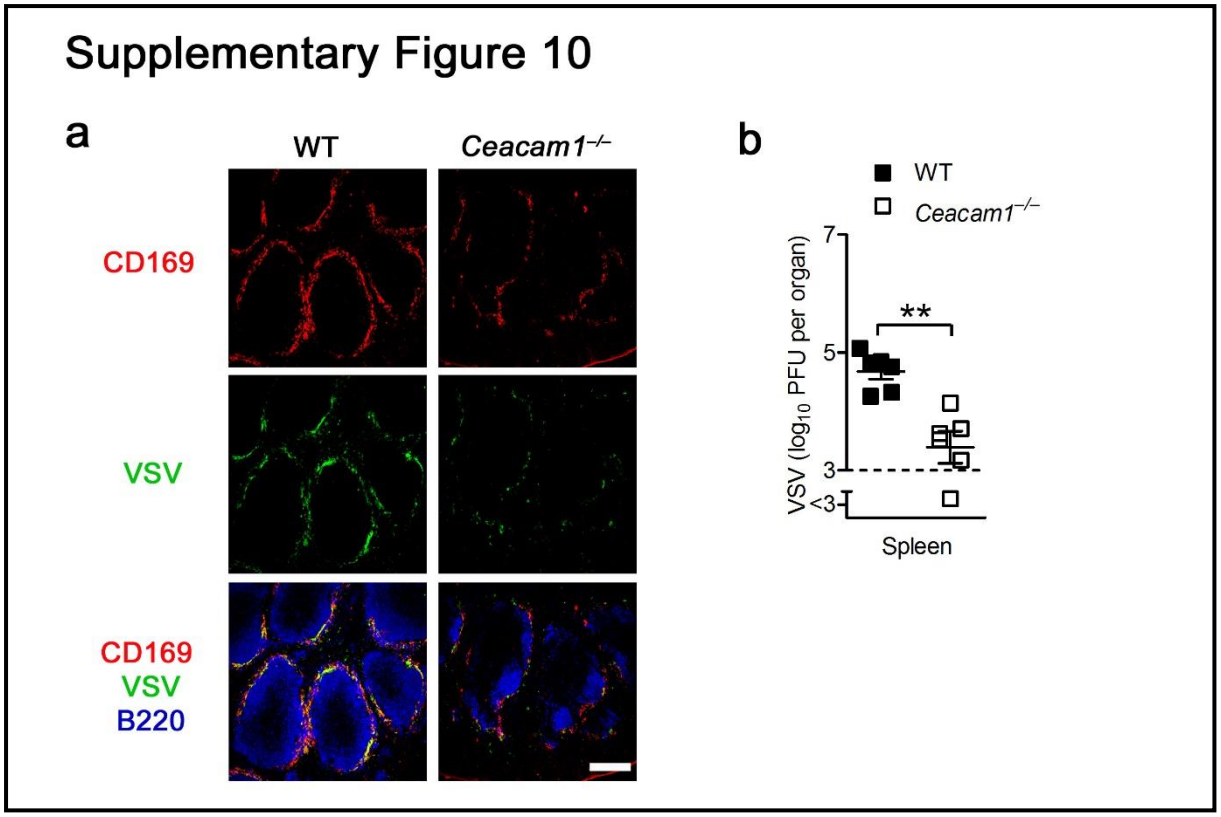
Supplementary Figure 3-7: CEACAM1 is essential for anti-VSV-specific Ig production



Supplementary Figure 3-8: MZ and FO B cells can rescue survival in *Ceacam1*$^{-/-}$ mice



Supplementary Figure 3-9: B cells but not other cell types are important for the replication of vesicular stomatitis virus in the spleen and the activation of adaptive immunity.



Supplementary Figure 3-10: Deficient marginal zone in *Ceacam1*$^{-/-}$ mice limits antiviral innate immune response

Article Statement:

1 Publication:

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- Writing and reviwing of the manuscript
  - Introduction: Part of the literature research and review
  - Material and Methods: All writing part of materials and methods with *Duhan V.*
  - Results: Planing of the experiments and in parts execution with *Duhan V, Manay SK, Pandyra A, Seifert M, Pozdeev V, Xu HC, Sharma P, Baldin F, Marquardsen F, Lang E, Göthert J.*

- Discussion: Writing part of discussion and critical reviewing with *Duhan V. Seifert M, Kirschning C, Westendorf AM, Häussinger D, Lang F, Dittmer U, Küppers R, Recher M, Hardt C, Scheffrahn I, Beauchemin N, Göthert JR, Singer BB, Lang PA, and Lang KS*

- Results
  - Fig. 1: FACS analysis of Blood samples and RT PCR for the experiments (with *Duhan V*).
  - Fig. 2: Western bloting (with *Pandyra A* and *Sharma P)*, FACS analysis of Blood samples (for 2c, with *Duhan V*) and RT PCR sample collection and analysis (with *Duhan V*).
  - Fig. 3: *In vitro* B cell proliferastion (Fig. 3a and 3b with *Manay SK* and *Pozdeev V*); FACS analysis and survival experiments with inhibitors.
  - Fig. 4: B cell FACS analysis (Fig. 4b, 4c, 4d, and 4e with *Göthert JR*). Histological analysis of spleen and lymph node samples (Fig 4f, 4g and 4h with *Duhan V*). FACS analysis of mice with mixed Bone marrow chimeras (Fig 4i, and 4j with *Xu HC*).
  - Fig 5: Histological analysis (Fig. 5a); *in vivo* B cell proliferation assay (Fig. 5b); Neutralizing antibody experiments (Fig. 5c, 5d, and 5e); plaque assay analysis and survival experiments (Fig. 5f, 5g, and 5h) with *Duhan V.*
  - Fig. 6: LCMV infection and ELISA from serum samples for measuring anti-LCMV-GP antibodies (Fig. 6a) and plaque assay analysis (Fig. 6b).
  - Fig. 7: FACS analysis for CEACAM1 expressin on different B cell sub-populations (Fig 7a and 7b) with *Seifert M.*
  - Supplementary Figures: Supp. Fig 1 (with *Lang L*); Supp. Fig. 2 (with *Manay SK*); Supp. Fig. 4 (with *Göthert JR*); Supp Fig. 5; Supp Fig. 6 (with *Baldin F, Marquardsen F*); Supp. Fig. 7; Supp Fig 8; Supp. Fig. 9 and Supp. Fig. 10 (with *Duhan V*)

- Revision and Proof reading
  - Discussion on the reviewer comments, data analysis and drafting the manuscript to its final online version. (with *Duhan V, Göthert JR, Singer BB, Lang PA, and Lang KS*)

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Vishal Khairnar **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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Vikas Duhan[1,*], Vishal Khairnar[1,*], Sarah-Kim Friedrich[1], Fan Zhou[1], Asmae Gassa[1,2], Nadine Honke[1], Namir Shaabani[1], Nicole Gailus[1], Lacramioara Botezatu[3], Cyrus Khandanpour[3], Ulf Dittmer[4], Dieter Häussinger[5], Mike Recher[6], Cornelia Hardt[1], Philipp A. Lang[5,7,*] & Karl S. Lang[1,5,*]

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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4. Chapter IV:

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

Vikas Duhan*, Vishal Khairnar*, Sarah-Kim Friedrich, Fan Zhou, Asmae Gassa, Nadine Honke, Namir Shaabani, Nicole Gailus, Lacramioara Botezatu, Cyrus Khandanpour, Ulf Dittmer, Dieter Häussinger, Mike Recher, Cornelia Hardt, Philipp A. Lang,* & Karl S. Lang*

4.1 *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.

4.2 *Introduction*

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 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[13].

Recently we found that antigen-presenting cells ($CD169^+$ macrophages and $CD11c^+$ dendritic cells) within the marginal zone specifically allow viral replication[14]. Enforced viral replication in the spleen is essential for activating the innate and adaptive immune systems[15]. 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.

4.3 *Results*

4.3.1 *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[15]. 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[16,17].

4.3.2 *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[18]. 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 and lymph node 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 **B**). 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.

4.3.3 *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.

4.3.4 *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.

4.3.5 *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.

4.3.6 *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[14,15]. 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[14,15]. 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.

4.4 *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[19]. Although specific antibodies mainly target infectious virus particles, virus-specific CD8$^+$ T cells can directly suppress viral replication in infected cells[20]. Currently available

vaccinations induce measurable antibodies against the pathogen but often fail to induce virus-specific $CD8^+$ T cells[21]. 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[22–24]. Indeed, in the Friend virus model, antibodies can enhance the virus-specific $CD8^+$ T-cell response[25]. 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[26]. In addition, complement may track virus into a separate compartment[27]. 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[28]. 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[29]. 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[30]. 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[31], and cellular immunity does not lead to a protective immune response upon challenge infection[32]. The fact that HIV induces marginal zone atrophy and marginal zone lymphoma[33,34] and the fact that $CD4^+$ T cells can be activated in the marginal zone and are the main target cells of HIV infection[14] 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[31], 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.

4.5 *Methods*

4.5.1 *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.

4.5.2 *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[35]. 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[36] 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.

4.5.3 *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.

4.5.4 *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).

4.5.5 *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).

4.5.6 *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.

4.5.7 *ALT and LDH measurement.*

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

4.5.8 *LCMV neutralization assay.*

The neutralizing capability of serum was measured with plaque-forming assays according to a previously published protocol[35].

4.5.9 *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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4.7 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.

4.8 Figure Legend

4.8.1 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.

4.8.2 *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.

4.8.3 *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.

4.8.4 *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).

4.8.5 *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).

4.8.6 *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).

4.8.7 *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).

4.8.8 *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).

4.8.9 *Supplementary 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 (CD45.1, 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).

4.8.10 *Supplementary 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.

4.8.11 *Supplementary 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).

4.8.12 *Supplementary 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.

4.8.13 *Supplementary 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

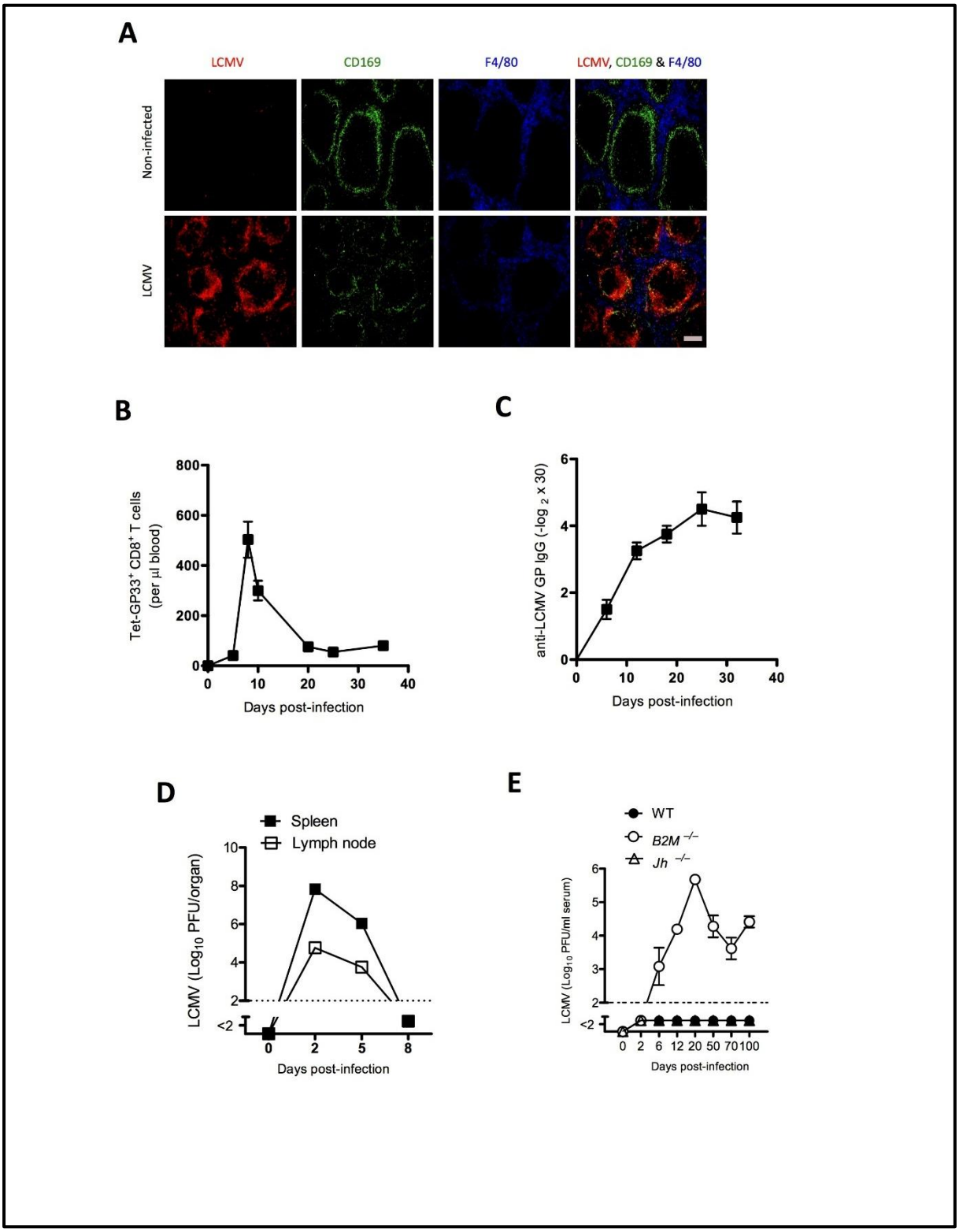


Figure 4-1: ***Replication of lymphocytic choriomeningitis virus (LCMV) in the marginal one is associated with immune activation and viral control.***

Figure 2

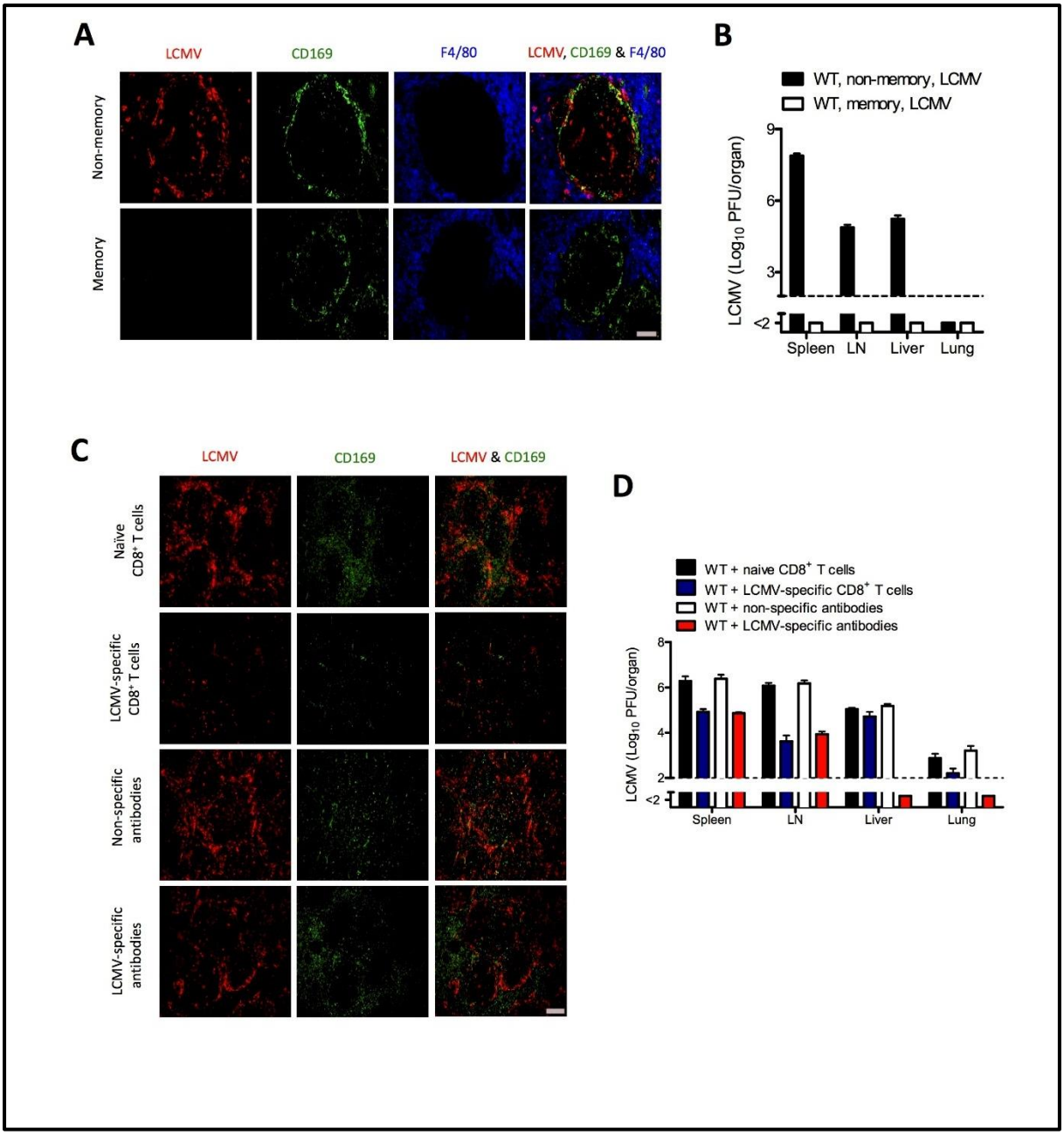


Figure 4-2: ***Virus-specific antibodies, but not virus-specific CD8$^+$ T cells, allow viral replication in the marginal zone.***

Figure 3

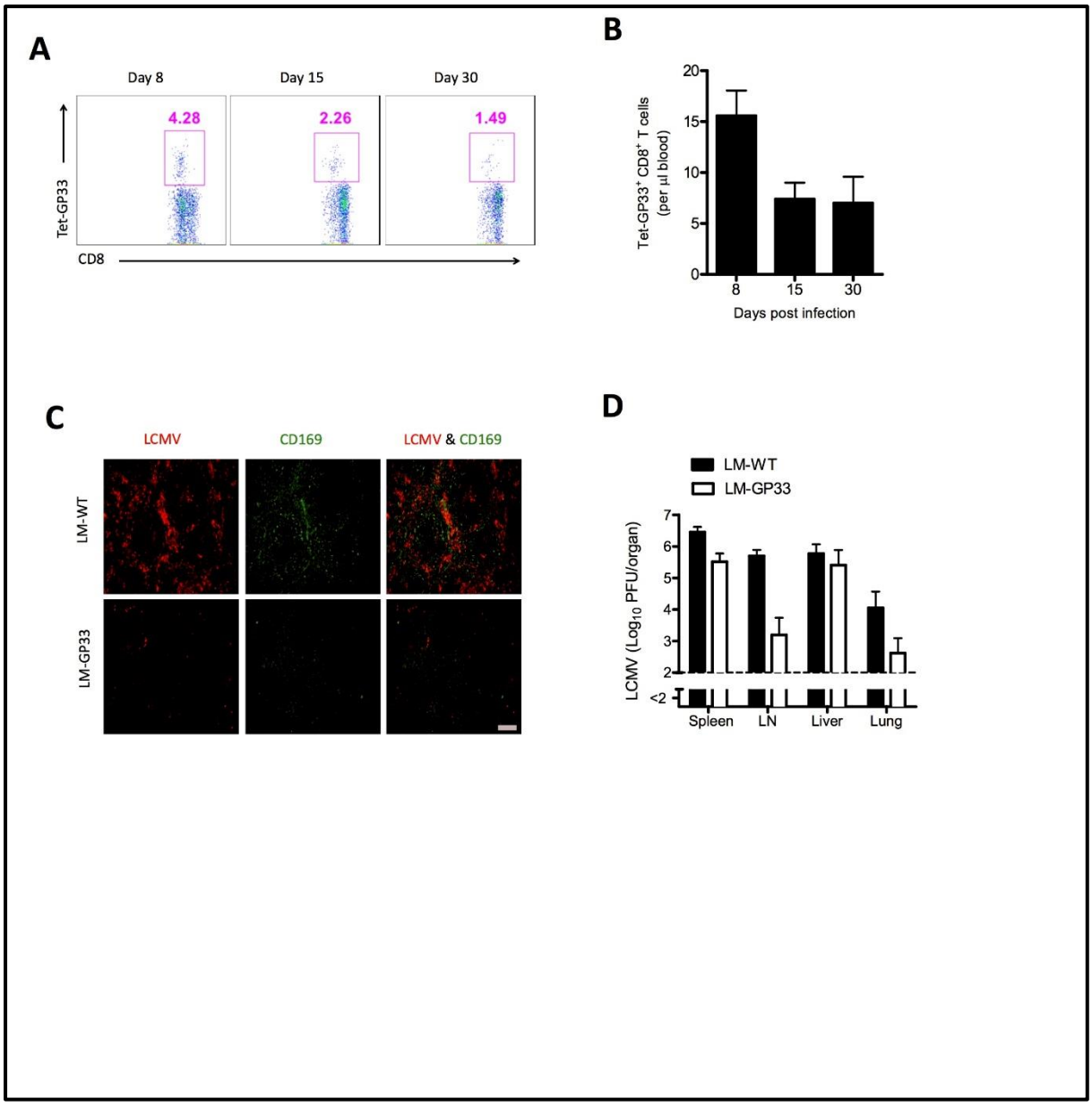


Figure 4-3: ***Inhibition of viral replication in splenic marginal zone of mice primed with recombinant Listeria monocytogenes expressing the glycoprotein of LCMV.***

Figure 4

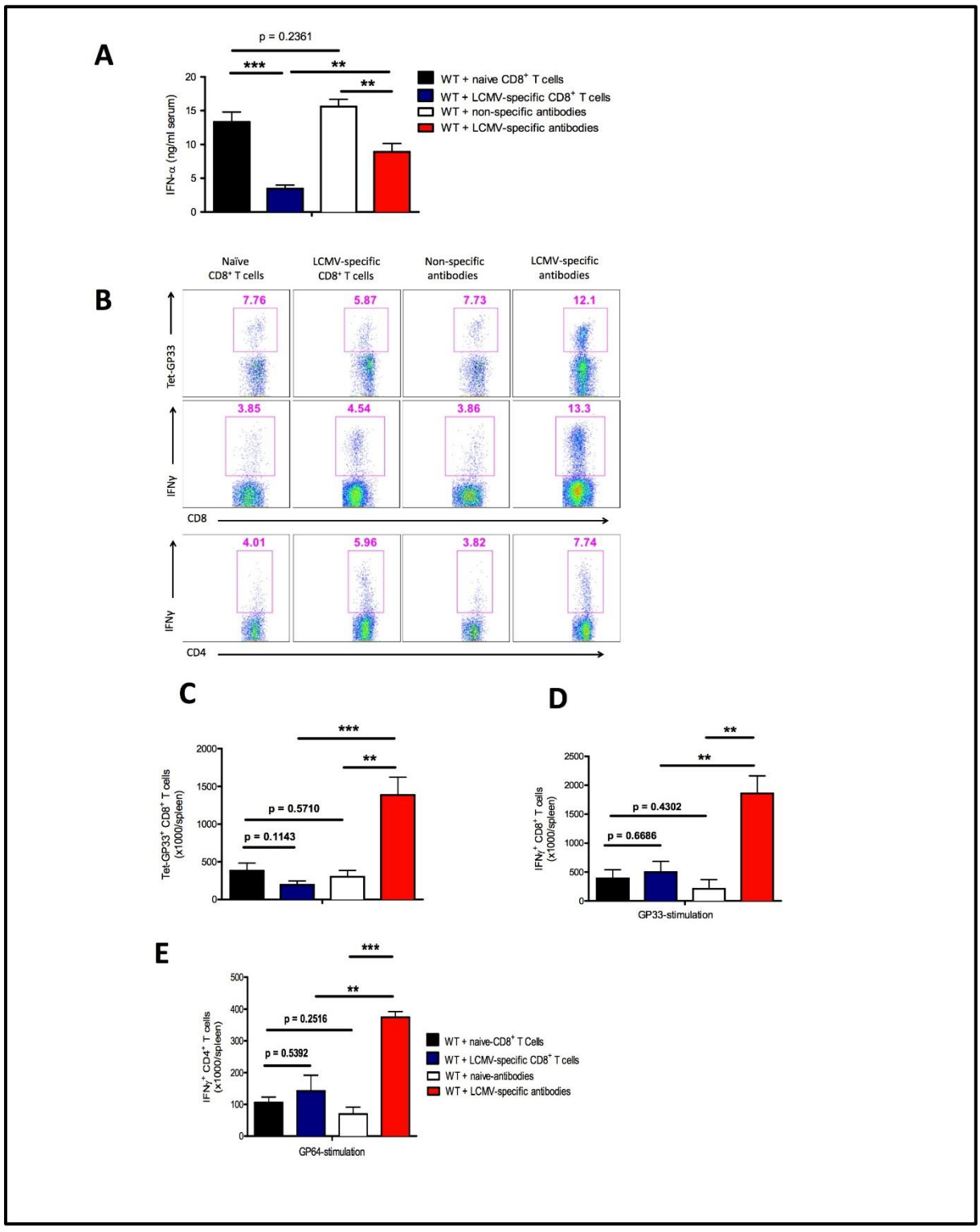


Figure 4-4. ***Virus-specific antibodies allow innate and adaptive immune activation.***

Figure 5

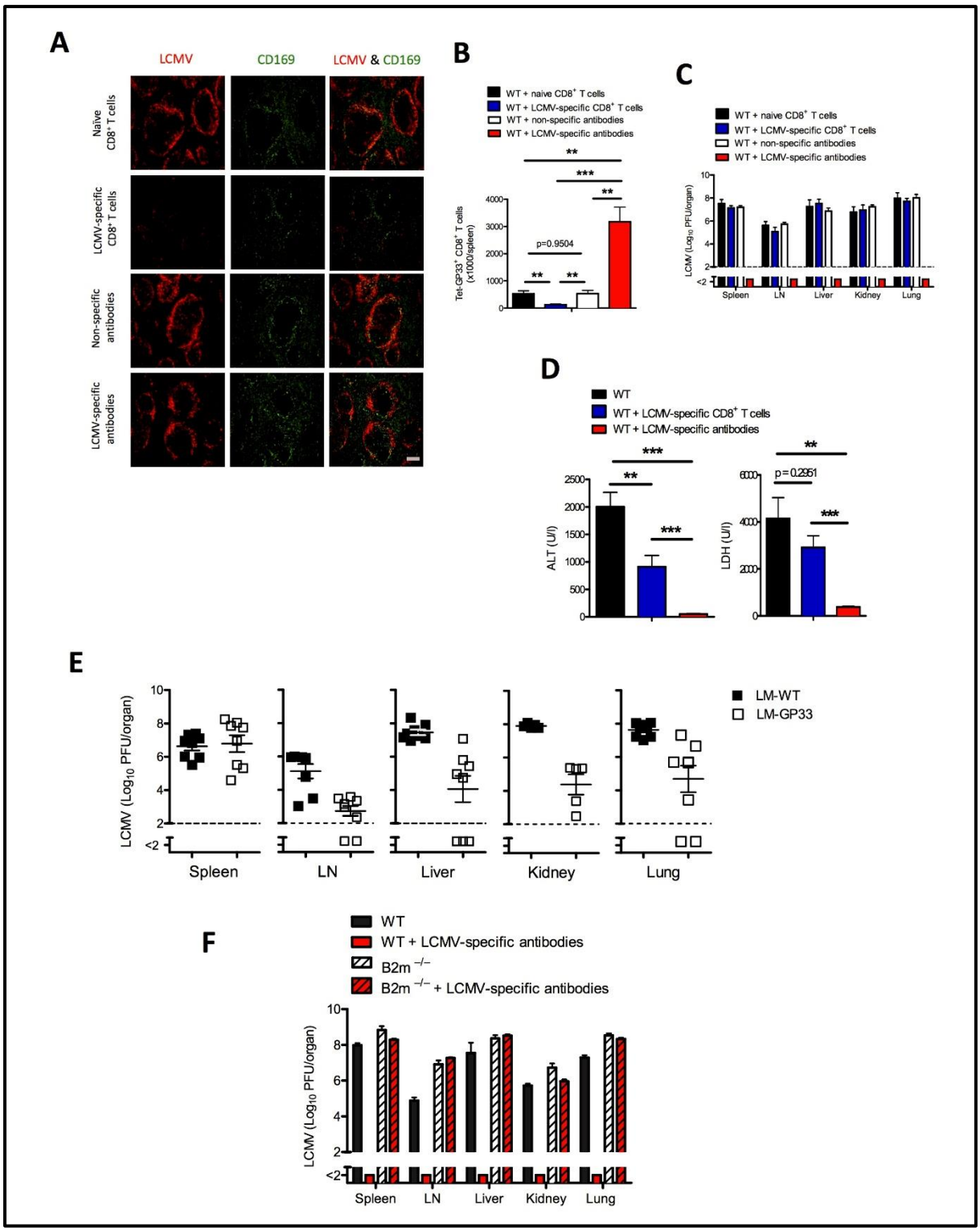


Figure 4-5: ***Virus-specific antibodies protect against immunopathology and lead to control of virus.***

Figure 6

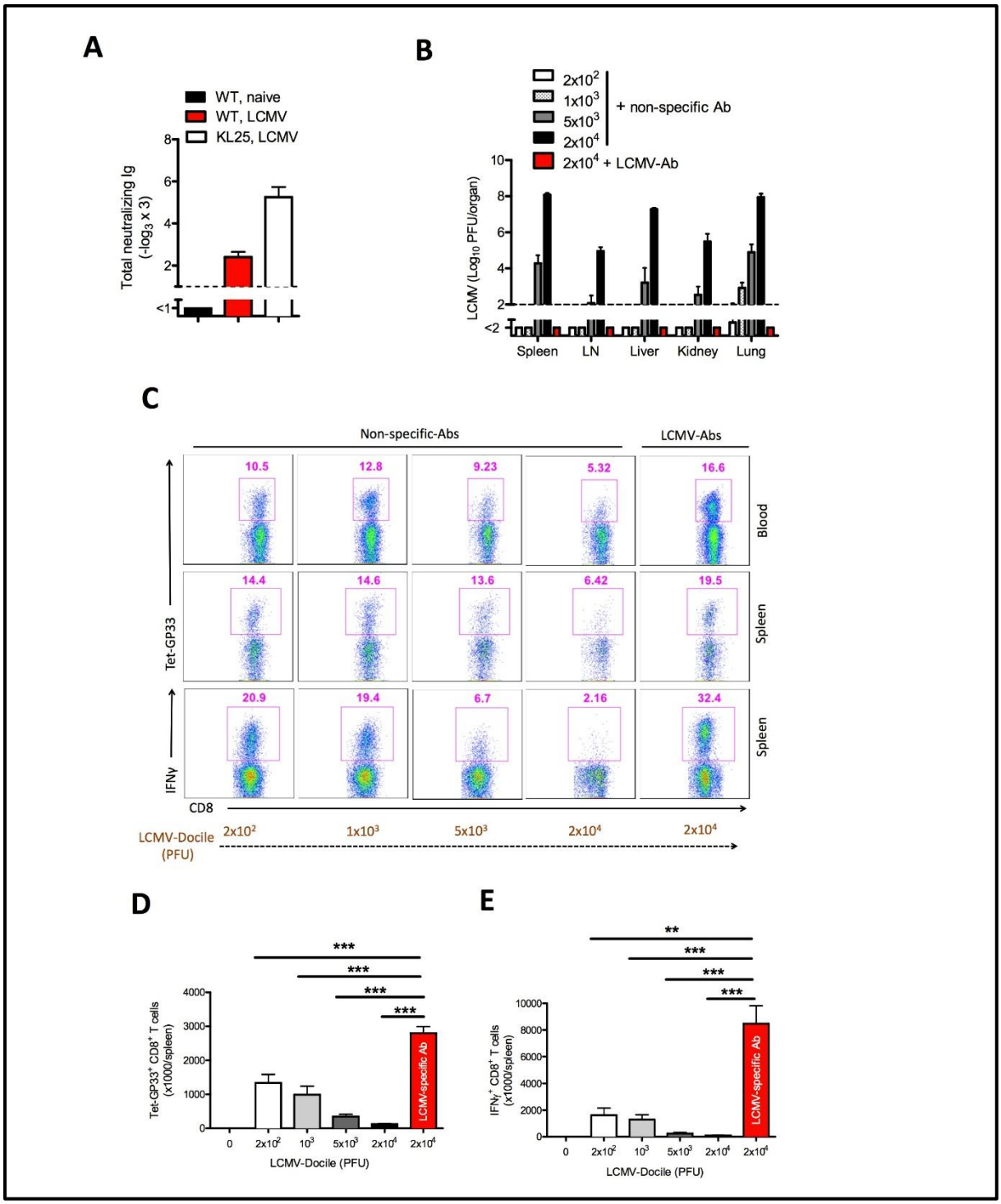


Figure 4-6: ***Virus-specific antibodies enhance priming and expansion of CD8⁺ T cells.***

Figure 7

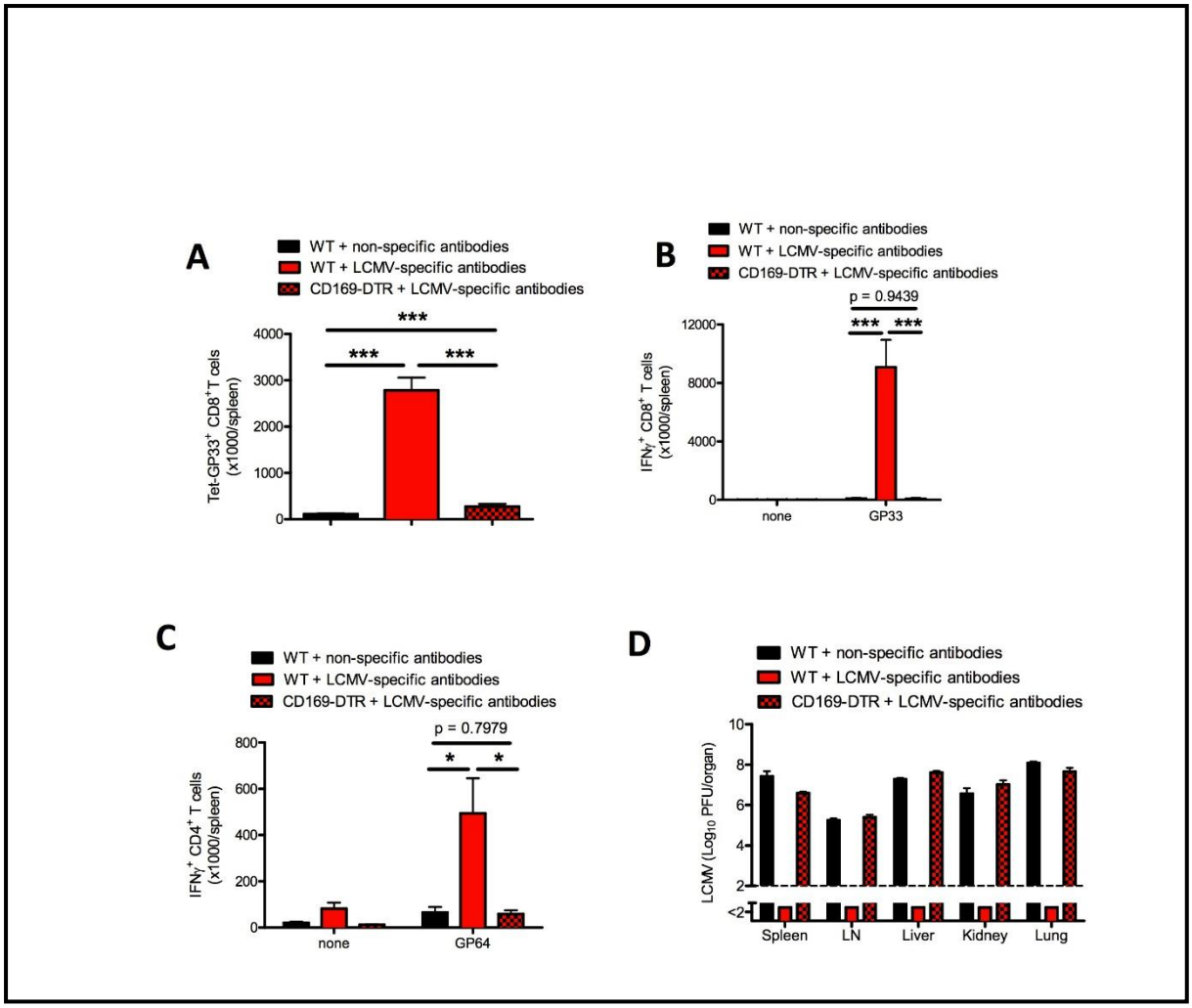


Figure 4-7: ***Immune activation in the presence of virus-specific antibodies is essential for controlling persistent viral infection.***

Figure 8

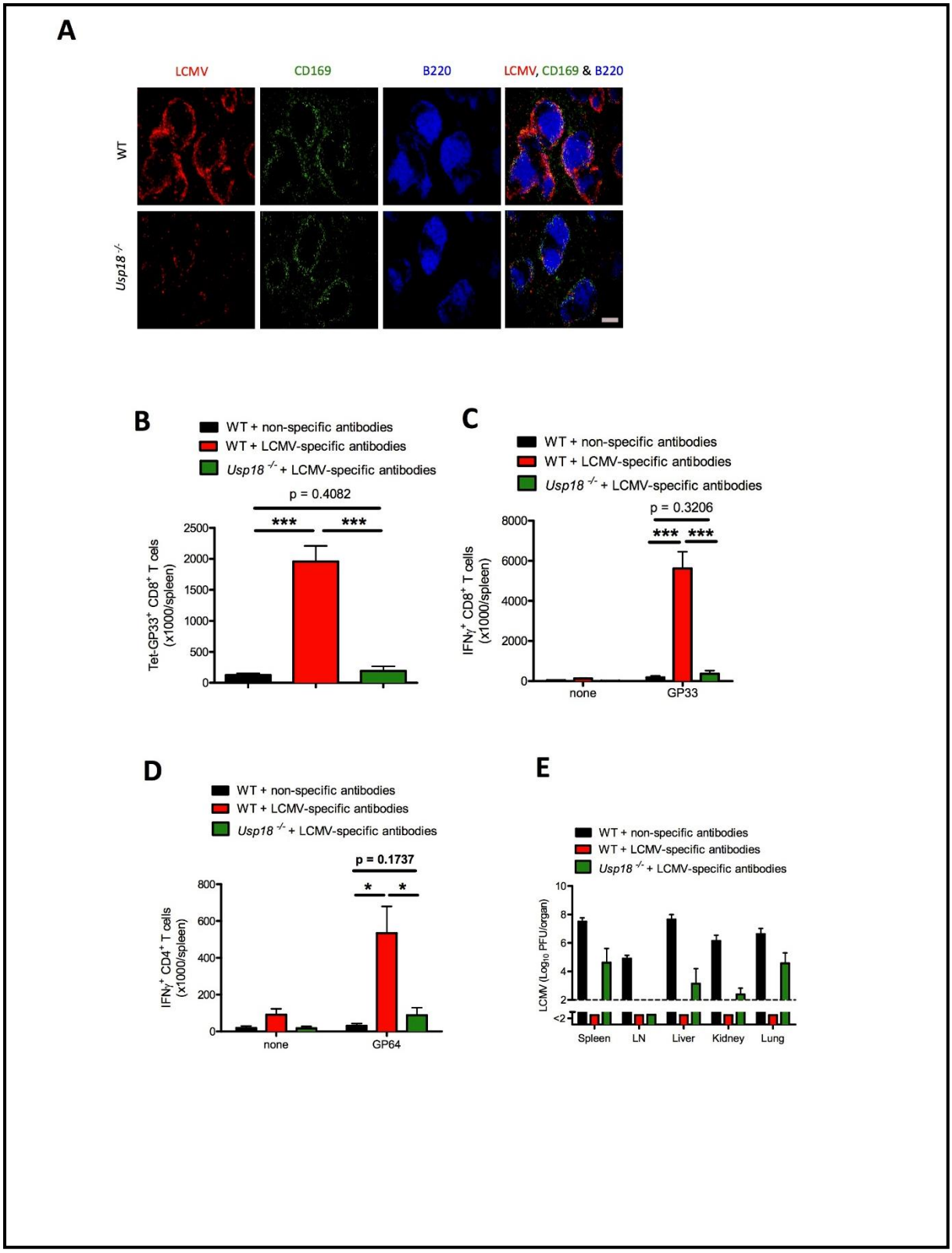
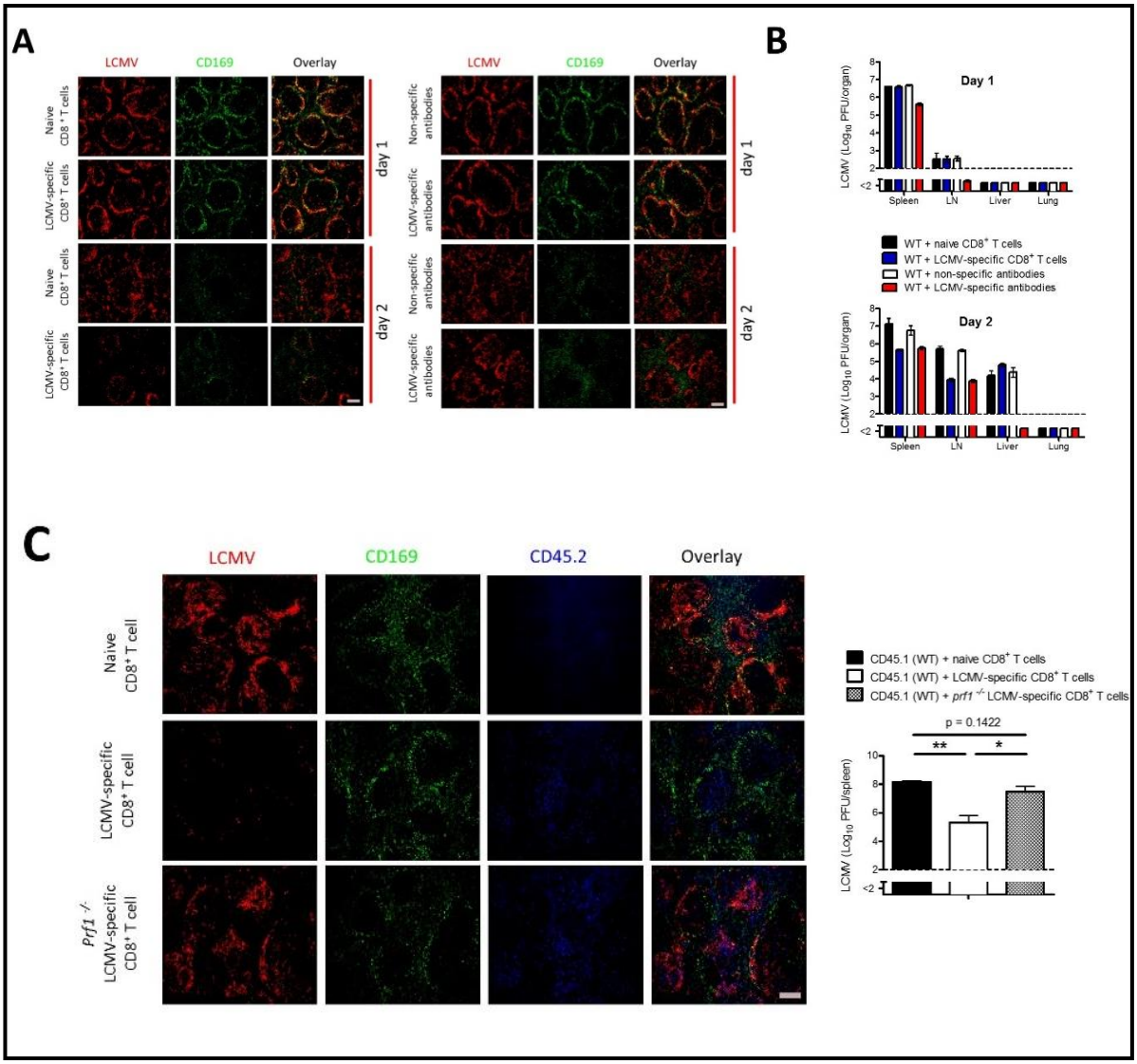


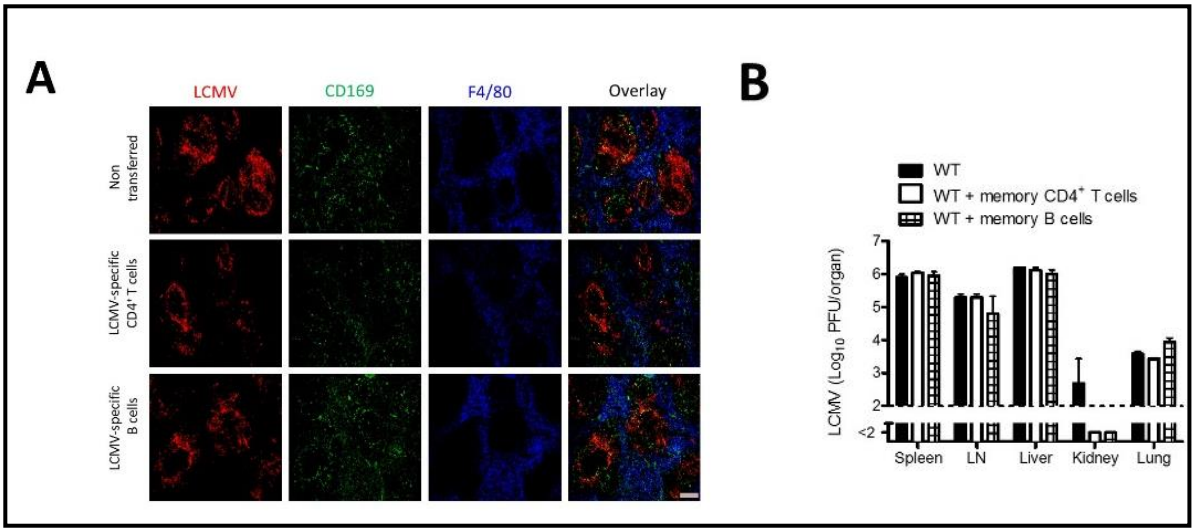
Figure 4-8: ***Immune activation in the presence of virus-specific antibodies is Usp18 dependent.***

Supplementary Figure 1



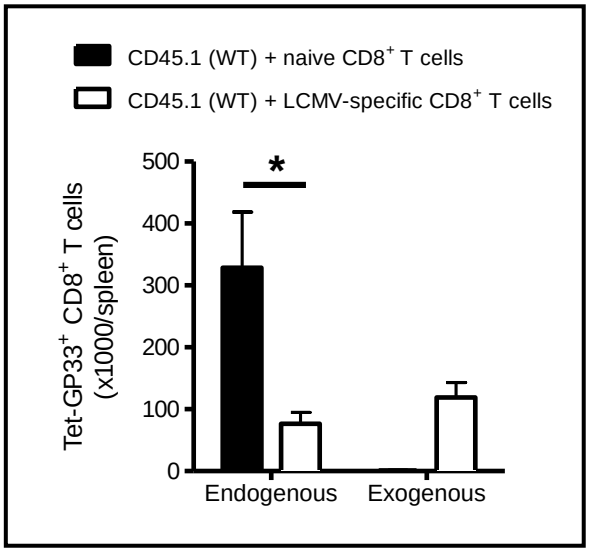
Supplementary Figure 4-1: ***Virus-specific antibodies, but not virus-specific CD8⁺ T cells, allow viral replication in the marginal zone.***

Supplementary Figure 2



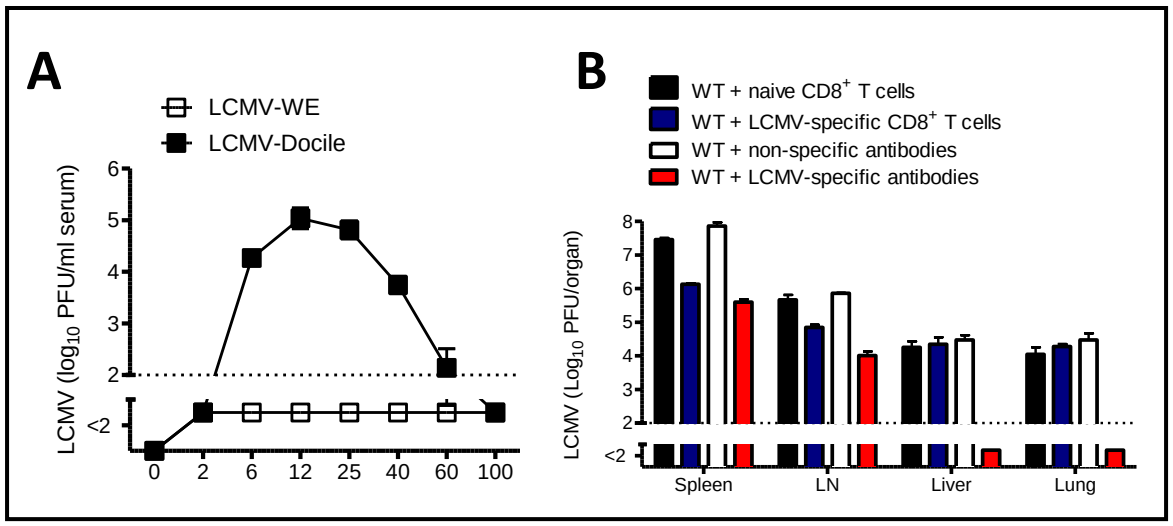
Supplementary Figure 4-2: ***Memory CD4$^+$ T cells and memory B cells has no effect on viral replication in the marginal zone.***

Supplementary Figure 3



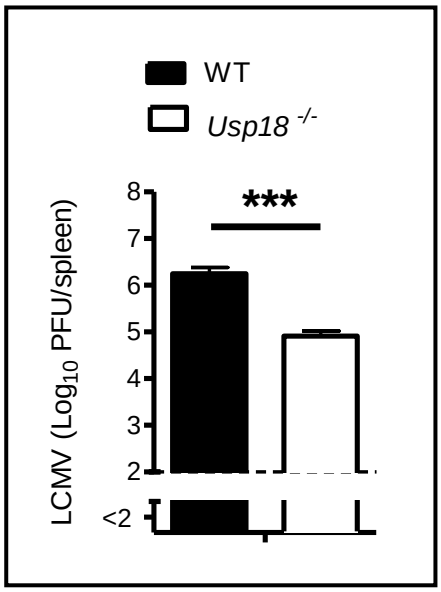
Supplementary Figure 4-3: ***Memory CD8⁺ T cells reduce the expansion of endogenous CD8⁺ T cells.***

Supplementary Figure 4



Supplementary Figure 4-4: ***Virus-specific antibodies inhibit persistent LCMV-Docile replication in peripheral organs.***

Supplementary Figure 5



Supplementary Figure 4-5: ***Usp18 promotes LCMV replication.***

4.9 References

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Article Statement:

1 Publication:

Vikas Duhan1, Vishal Khairnar*, Sarah-Kim Friedrich, Fan Zhou, Asmae Gassa, Nadine Honke, Namir Shaabani, Nicole Gailus, Lacramioara Botezatu, Cyrus Khandanpour, Ulf Dittmer, Dieter Häussinger, Mike Recher, Cornelia Hardt, Philipp A. Lang,* & Karl S. Lang,**

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

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Declatation:	*Mr. Vishal Khairnar planned and performed part of the experiments, analysed the data and wrote the manuscript.*

Contribution to the publication:

- Writing and reviwing of the manuscript
  - Introduction: Part of the literature research and critical review
  - Material and Methods: Writing part of materials and methods with *Duhan V.*
  - Results: Planing of the experiments and execution with *Duhan V, Friedrich SK, Zhou F, Gassa A, Honke N, Shaabani N, Gailus N, Botezatu L.*

- Discussion: Part of discussion writing and critical reviewing with *Duhan V. Khandanpour C, Dittmer U, Häussinger D, Recher M, Hardt C, Lang PA & Lang KS*.

➢ Results

- Fig. 1: Histology (Fig. 1A); FACS analysis of Blood samples (Fig. 1B); ELISA for anti-LCMV GP (Fig. 1C); and plaque assay from organ and Serum samples (Fig. 1D and 1E) with *Duhan V.*
- Fig. 2: Immunohistochemisrty analysis for Fig. 2A and 2C with *Duhan V.*
- Fig. 3: Immunohistochemistry for Fig. 3C with *Duhan* V.
- Fig. 5: Histological analysis for Fig. 5A with *Duhan V.*
- Fig 6: Tetramer analysis for Fig. 6C, Fig. 6D and Intercellular cytokine staining for Fig. 6E with *Duhan V.*
- Fig. 7: LCMV infection and plaue assay for Fig. 7D with *Duhan V.*
- Fig. 8: Immunohistology for Fig. 8A with *Duhan V.*
- Supplementary Figures: Immunhistochemistry for Supp Fig. 1A; Supp. Fig 2A with *Duhan V.*

➢ Revision and Proof reading

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

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Vishal Khairnar **Prof. Dr. Karl S. Lang**

5. Chapter V:
General Discussion and Conclusions

Using three different studies, we show in this thesis the role of lymphotoxin beta, CEACAM1 and virus specific antibodies in innate and adaptive immune activation.

Lymphotoxin plays a major role in a variety of immunological processes including lymph node development, generation and activation of DC's, B cell development, IFN-α production and $CD169^+$ macrophage function.[1, 2, 3] In the first part of this thesis we investigated different functions of *Usp18* and lymphotoxin beta during viral replication in spleen. In a recent study, it was shown that enforced viral replication in marginal zone ($CD169^+$) macrophages is essential for the generation of sufficient antigen particles to trigger enough IFN-α production which in turn activates the adaptive immune responses.[4] $CD169^+$ macrophages have higher *Usp18* expression and knockdown of *Usp18* or lack of CD169 macrophages leads to limited viral replication in spleen leading to impaired priming of adaptive immunity. Therefore, $Usp18^{-/-}$ mice lack neutralizing antibodies which in turn causes a higher mortality rate.[4] On the other hand, LTα1β2 produced from B cells is necessary to maintain the subcapsular sinus (SCS) macrophage phenotype within virus draining lymph nodes (LNs).[3, 5] Mice lacking LTα1β2 from B cells also fail to replicate VSV and lack sufficient type I interferon production which also leads to death of these mice.[3] Hence, expression of *Usp18* and secretion of LTα1β2 is integral for protection against fatal neuroinvasion.[4, 6]

In this study, we delineated distinct roles for these two molecules. *Usp18* and lymphotoxin beta both are required for viral replication in the spleen. *Usp18* influences viral replication whereas LT beta is involved in spreading the virus along the conduits of the marginal zone. *Usp18* is important for both systemic generation of type I interferon as well as CD8 T-cell priming. On the other hand, we found that LT beta is necessary only for IFN-α secretion but not for CD8 T-cell priming. Our results showed that after LCMV infection *Usp18* is important for cytokine secretion from CD8 T cells whereas, LT beta does not influence adaptive immune system activation. We determined that B cell-derived LT beta is an important contributor for the viral distribution along the marginal zone. Importance of viral distribution might be explained by large number of viral particles can activate pDC's. Also, a different composition of infected cell from adjacent non-infected cells are more likely to infect the pDC's leading in to activation of strong antiviral mechanisms.

Viral spread is an important hallmark of viral distribution in organs and T-cell exhaustion.[7] In our study, we suggest that drugs targeting the extracellular spread of virus can lead to better T-cell functions by preventing the exhaustion. Hence, formulation of drugs or

antibiotics which allow threshold viral replication can promote CD8 T cell function and can result in better viral control.

In conclusion, we have shown that viral replication in $CD169^{+}$ macrophages and viral spread along the conduits of the marginal zone are two separate mechanism of enforced viral replication. Viral spread primarily affect only systemic production of type I interferon and viral replication is absolute for activation of innate as well as adaptive immune function after antigen challenge. Other factors affecting lymphotoxin beta secretion or *Usp18* expression are still relatively unknown. The role of lymphotoxin beta in lymph node formation and B cell function hints to there being more factors affecting B cell survival, proliferation and antiviral functions. One of these molecules which we study in the second part of our study was CEACAM1.

In this portion of the thesis, we demonstrate that cell intrinsic signalling of CEACAM1 is essential for survival and proliferation of B cells. Lack of CEACAM1 leads to reduced numbers of B cells and defective immune responses after antigen challenge. Previous studies have demonstrated that CEACAM1 influences BCR-complex mediated activation.[8] CEACAM1 triggered strong proliferation of B cells when combined with surface IgM cross linking in B cells without affecting class switching and similar results were obtain with CEACAM1-expressing fibroblasts.[8] Mouse B cells express relatively high long isoform of CEACAM1 (CEACAM1-4L) over short isoform (CEACAM1-4s). Phosphorylation of ITIM's on long cytoplasmic tail give inhibitory signals in T cells by recruiting SHP-1 and SHP-2.[9] However, in current study we showed that CEACAM1 crosslinkage has some proliferative effects. Our *in vitro* and *in vivo* study suggest that CEACAM1 ligation induces proliferative signals and is important for survival of activated B cells. Like previous studies, we confirm lack of CEACAM1 induces apoptotic signals in B cells. This is in line with the findings which shows CEACAM1 induces apoptosis in granulocytes.[10] In another other study CEACAM1 was shown to act as a co-inhibitory receptor for granulocyte-colony stimulating factor receptor (G-CSFR) which regulate granulopoiesis.[11]

BCR is essential for survival of B-cells. In absence of BCR all B cells as well as peripheral B cells were lost. This suggests that BCR either provide constitutive or tonic survival signal or self-antigens recognize cognate BCR's on immature or transitional zone B cells.[12] As the BCR is responsible for cell survival, altering BCR signal strength should alter the B cell fate. Modulating Bruton's tyrosine kinase (Btk) pathway which is downstream of BCR revealed that antigen stimulation is necessary for survival of FO B cell and B-1 B cells.[13] Relative strong BCR crosslinking by auto antigens favours mature FO-B cell

development whereas weak BCR signal favours MZ B cell development.[12] In addition to BCR signal, constant signal via BAFF receptor (*Baffr*) is the most important signal for naïve B cell development.

In our study, we suggest that tonic signal via CEACAM1 strongly regulates B cell survival. Because CEACAM1 is its own ligand, we suggest that CEACAM1 induces positive survival signal on B cells and maintains the structure of the B-cell follicles. We found that CEACAM1 contributed to Syk phosphorylation after challenge with monoclonal anti-CEACAM1 antibody indicating that CEACAM1 is involved in the BCR complex. Unlike *Jh*$^{-/-}$, *Baffr*$^{-/-}$ mice, *Ceacam1*$^{-/-}$ mice showed similar reduction in B cell numbers particularly no MZ B-cell development in naïve mice. Similar to *Ceacam1*$^{-/-}$ mice, mice deficient in CD19 or BCR exhibit reduced number of B cells which can be linked to humans lacking the Wiskott-Aldrich syndrome protein (WASp)[14], which is also reported to involve in Btk phosphorylation[15] and MZ B-cell generation[16]. Reduced B cell numbers particularly MZ B-cells couldn't give early immune activation after VSV challenge as *Ceacam1*$^{-/-}$ mice lack enforced viral replication in CD169^{+} macrophages and failed to generate adaptive immune response in terms of neutralizing antibodies. Insufficient antibody generation led to a breach of the Blood-Brain barrier and hence significant mortality rate in *Ceacam1*$^{-/-}$ mice. We also showed CEACAM1 expression on human peripheral B cells suggesting that CEACAM1 could play major role in human antiviral responses.

Reduced number of B cells leads to deficiency in antiviral antibody production. Antibodies play an important role in generating adaptive immune responses. Hence, in the third study we checked the role of virus-specific antibodies and virus specific T-cells in CD8 T cell priming and virus control.

Vaccination ensures the induction of specific antibodies that can generate long lasting protection against pathogens. Natural viral infection causes B cell activation resulting in plasma cell differentiation and production of virus specific antibodies.[17] After vaccination or antigen activation B-1 cells differentiate into plasmablast generating polyspecific IgM antibodies which leads to the activation if CD8 and CD4 T cells. Because of CD4 T cell help naïve B cells become long lived plasma cell producing high number of neutralizing antibodies.[18]

In this study, we found that upon recall infection, virus specific antibodies limit the viral replication in peripheral organs but still would allow replication of viral antigen in splenic marginal zone. This threshold replication is beneficial for innate and adaptive immune activation as it induces strong CD8 T cell response, limiting the immunopathology

leading to faster clearance of the antigen. Virus specific antibodies can target infectious particles, virus-specific CD8 T cells can supress viral replication and eliminate the antigen.[19] The results from our study shows that antigen specific antibodies block the antigen in peripheral organs but still allow viral replication in splenic marginal zone. Differential expression of Fc receptors in different organs may contribute to organ specific antiviral capacities. To generate effective memory response virus must replicate in $CD169^{+}$ macrophages to generate functional CD8 T cell response (**Fig. 5-1**). Therefore, from our studies utilizing LCMV we can suggest that antibodies are more potent than CD8 T cells.

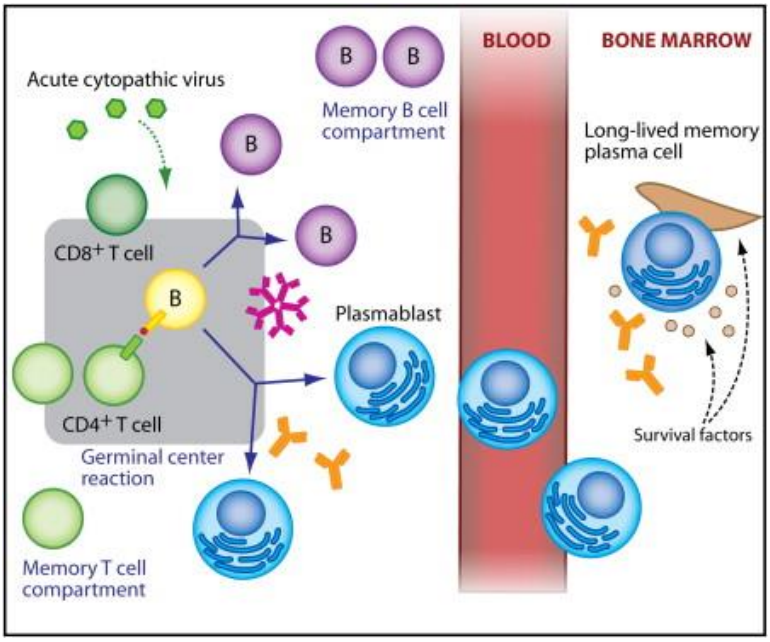

Fig: 5-1: Long term antiviral antibody induction. (Dörner T, and Radbruch A, *Immunity*, 2007)

From our study, we can suggest that for effective vaccine treatment, vaccine should allow minimal viral replication in certain secondary lymphoid organs in order to generate adaptive immune system but also to inhibit the spread of the virus to susceptible organs. This could be a better vaccine strategy for HCV infection because HCV E1 and E2 envelop protein specific antibodies lose their neutralizing capacity due to virus escape mutations.[20] However, for HIV infections replication in marginal zone could be not beneficial because the activated CD4 T cells are located mainly in marginal zone and would lead to direct elimination of virus and could lead to immunopathology however humanised mouse model studies could lead us draw a conclusion for antibodies, CD8 T cells and HIV infection.

In conclusion, with three different studies we checked the role of different parameters such as lymphotoxin beta, CEACAM1 and virus specific antibodies in viral infection and

their influence on enforced viral replication in activating innate and adaptive immune system. We found that viral replication and extracellular viral spread leads to distinct outcomes in disease. Intracellular viral replication is important for innate and adaptive immune activation particularly in generating CD8 T cell responses whereas, extracellular viral spread affect type I interferon production. Lack of lymphotoxin beta limits the spread of virus along the conduits resulting in limited IFN-α production but normal CD8 T cell response. Knowing the role of lymphotoxin in maintaining splenic architecture especially follicular DC's in B cell follicle, loss of CEACAM1 also lead to generate deficient B cell responses. CEACAM1 expression on murine B cells is an important regulator of B cell homeostasis and maintaining normal B- cell numbers. CEACAM1 is essential for protective antiviral antibody generation and the resulting reduction in mouse mortality against cytopathic viral infection. On the other hand, virus specific antibodies are more potent than virus specific CD8 T cells in chronic viral infection because they allow viral replication in splenic marginal zone and abolish the replication in peripheral organs resulting in generating effective adaptive immune responses. In contrast to specific antibodies, memory CD8 T cells inhibited viral replication in marginal zone but failed to protect mice from persistent viral infection.

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6. *Erklärungen*

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,‘‘Role of Lymphotoxin Beta and Cell Adhesion Molecule (CEACAM1) in Innate and Adaptive Immune Activation’’ zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von Vishal S. Khairnar befürworte.

Essen, den 08/02/2017_____ _____

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Erklärung:

Hiermit erkläre ich, gem. § 7 Abs. 2, d und f der Promotionsordnung der Fakultät für Biologie zur Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient habe und alle wörtlich oder inhaltlich übernommenen Stellen als solche gekennzeichnet habe.

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Hiermit erkläre ich, gem. § 7 Abs. 2, e und g der Promotionsordnung der Fakultät für Biologie zur Erlangung des Dr. rer. nat., dass ich keine anderen Promotionen bzw. Promotionsversuche in der Vergangenheit durchgeführt habe, dass diese Arbeit von keiner anderen Fakultät abgelehnt worden ist, und dass ich die Dissertation nur in diesem Verfahren einreiche.

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Finally, to all of you,

Thank you,

Namasthe.

8. *Curriculum Vitae*

The curriculum vitae is not included in the online version for reasons of data protection.