The role of coronin 1 during cell mediated immune responses

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Prof. Dr. Jörg Schibler

For Vera

and

Ani-Stella and Chloé my two beautiful nieces

Abstract

Coronin 1 is one of 7 mammalian isoforms member of the evolutionary conserved WD40-repeat proteins that are involved in a variety of activities such a cell migration and cytokinesis. Coronin 1 is predominantly expressed in cells of hematopoietic origin, and it is the most conserved coronin isoform. Analysis of mice lacking coronin 1 revealed coronin 1 as a crucial pro-survival factor for peripheral T lymphocytes. It was found that coronin 1 was essential for Ca^{2+} mobilization upon T cell receptor (TCR) triggering; in the absence of coronin 1, T cell signaling does not result in Ca^{2+} mobilization thereby causing a rapid clearance of the T cells through apoptosis. Nevertheless, coronin 1-deficient mice are capable to mount specific antibody responses after immunization, although somewhat delayed for T cell dependent antigens. Together these results suggest an important role for coronin 1 in T cell signaling and in naïve T cell homeostasis. Here, we investigate the cellular immune response to Murine cytomegalovirus (MCMV), Lympocytic choriomeningitis virus (LCMV) and Vesicular stomatitis virus (VSV), whose clearance and control are either dependent on $CD8^+$ T cells or on $CD4^+$ T cells, in wild type and coronin 1-deficient mice. Our results show surprisingly normal antiviral $CD8^+$ T cell responses concerning magnitude, kinetics and functionality of virus specific $CD8^+$ T cells. In contrast, virus specific $CD4^+$ T cell responses were significantly impaired leading to loss of viral control in VSV infection. These findings suggest a more important role of coronin 1 for $CD4^+$ T cell survival, activation and homeostatic proliferation in the periphery than for $CD8^+$ T cells.

In the second part of that thesis we investigate the Natural killer (NK) cell immune response in the absence of coronin 1. Delay in viral control can be due to impaired NK cell response in the absence of coronin 1, which is believed to interact with Phospholipase C γ (PLCγ) activity. Hence, NK cell receptor signaling share some similarities with TCR signaling. We analyzed the functionality of coronin 1-deficient NK cells after VSV infection, stimulation of NK cells with antibodies, YAC-1 tumor cell or Concanavalin A (Con A). We show that NK cell activation and functionality was not impaired in the absence of coronin 1. However, ConA treatment (*in vivo* and *ex vivo*) was associated with impaired Interferon γ (IFNγ) production and cytotoxicity against YAC-1 cells. We found that coronin 1-deficiency was associated with increased sensitivity of NK cells leading to increased apoptosis rather than impaired NK cell activation upon Con A treatment.

Overall, our results suggest that coronin 1 is crucial for peripheral $CD4^+$ T cell homeostasis and functionality but is largely dispensable for NK cell and $CD8^+$ T cell mediated antiviral immunity.

Table of Content

A. Index of Figures

1. Introduction

4. Results

5. Supplementary results

B. Index of Tables

1. Introduction

6. Supplementary results

C. Abbreviations

7-AAD	7-aminoactinomycin
ADCC	Antibody-dependent cell-mediated cytotoxicity
APC	Antigen presenting cell
BCR	B cell receptor
Ca	Calcium
CD	Cluster of Differentiation
CDR	Complementarity determining region
CLIP	Class II-associated invariant chain peptide
Cor 1	Coronin 1
CPM	Counts per minute
CRAC	Calcium release activating channel
cSMAC	Central supramolecular activation cluster
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen 4
DAG	Diacylglycerol
DC	Dendritc cell
DNA	Desoxyribo nucleic acid
ER	Endoplasmatic reticulum
FACS	Fluorescent Automated Cell Sorter
FCS	Fetal Calf Serum
i.v.	Intravenous
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IP_3	Inositol-1,4,5-trisphosphate
ITAM	Immunoreceptor-tyrosine-based-activating-motif
ITIM	Immunoreceptor-tyrosine-based-inhibitory-motif
ITSM	Immunoreceptor-tyrosine-based-switch-motif
kD	Kilo Dalton
KO	Knock-out
LAT	Linker for the activation of T cells
LCK	Lymphocyte specific protein tyrosine kinase
LCMV	Lymphocytic choriomeningitis virus
MCMV	Murine cytomegalovirus
MEM	Minimal Essential Medium

MHC	Major Histocompatibility Complex
NaCl	Natrium Chloride
NK	Natural killer
PBS	Phosphate Buffered Saline
PFU	Plaque forming unit
PI3K	Phosphatidyl-inositol-3-kinase
PIP_2	Phosphatidyl-inositol-4,5 -bisphosphate
PIP_3	Phosphatidyl-inositol-3,4,5-trisphosphate
PLC	Phospholipase C
pSMAC	Peripheral supramolecular activation cluster
RAG	Recombination activating gene
RNA	Ribonucleic acid
s.c.	Sub cutaneous
SAP	SLAM associated protein
SH	Src-homology
SLAM	Signaling lymphocytic activation molecule
TACO	Tryptophan Aspartate Containing Protein
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TLR	Toll like receptor
TNF	Tumor-necrosis-factor
VSV	Vesicular stomatitis virus
WD	Tryptophan - Aspartate
WT	Wild type
β-ME	β-Mercaptoethanol

1. Introduction

1. Introduction

1.1. The immune system

The Immune system is the most important feature of an organism to protect and defend itself against foreign pathogenic intruders such as bacteria, viruses, fungi and parasites. Over the last century, knowledge of the immune system has gained more and more importance due to vaccine development, autoimmune disease treatment and organ transplantation.

All organisms have developed their own way to protect from intruders. The immune system is capable to learn from passed infections and to evolve due to a constant selective pressure. In some species, such as in invertebrates, the immune system is well adapted to its ecological niches. Hence environmental changes, such as drop or increase in temperature, can seriously influence the functionality of the immune system. Moreover, introduction of an unknown pathogen into a population can lead to severe diseases and can have fatal consequences on the host population [1-4]. Vertebrates show common features with the primitive innate immune system of certain invertebrates. Additionally, vertebrates have a second line of defense. This is a more complex and evolved “specific” immune system termed as “adaptive immune system”.

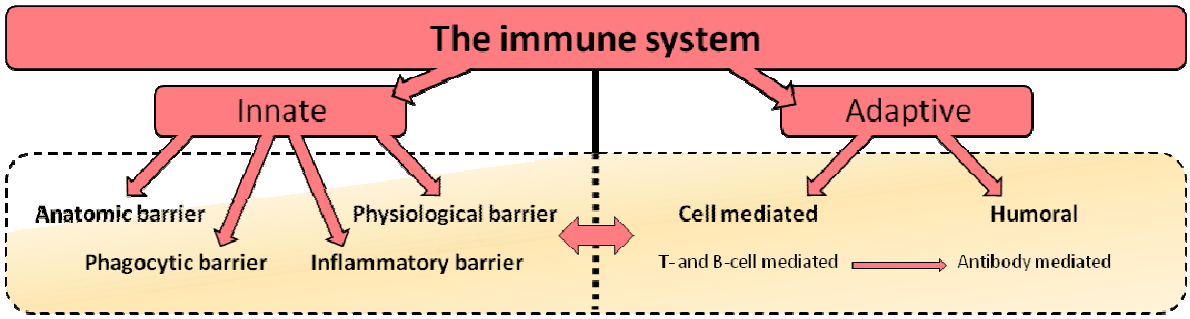


Figure 1. 1 The immune system divided in two major defense categories.

The innate (non-specific) and the adaptive (specific) immune system have to act tightly together to provide efficient protection against pathogenic microorganisms. The anatomic barrier is a mechanical barrier, which blocks the entry of pathogens. The physical, phagocytic and inflammatory barriers inhibit growth of pathogens and destroy them, thereby providing important activation signals for the adaptive immune system. On the other hand, antibodies can opsonize pathogens and activate the complement system.

The innate and the adaptive immune system have to act tightly together to provide efficient protection against pathogenic intruders (Figure 1. 1). Astonishingly, the immune system has the ability to distinguish between self and non-self molecules. However, in some circumstances, the immune system overreacts, such as in allergies, or it can become an aggressor toward the own host which is referred to autoimmunity. For instance, psoriasis, diabetes type I or multiple sclerosis are typical autoimmune diseases.

This introduction will give a brief overview of the innate immune system and will focus on the different aspect of the adaptive immune system and its role during viral infections. Additionally, the discovery and importance of the coronin 1 protein during immune response will be discussed.

1.1.1. **Haematopoiesis and the components of the immune system**

The immune system has an arsenal of different specialized cells. All blood cells are derived from haematopoietic stem cells located in the bone marrow. Haematopoiesis is the formation and development of red and white blood cells (leukocytes), and the latter give rise to two main distinct cell populations, the myeloid and the lymphoid progenitors (Figure 1. 2).

The lymphoid progenitors give rise to T cells, dendritic cells (DC), Natural killer (NK) cells and B cells which make up to 20-40% of all body leukocytes. On the other hand, myeloid progenitors give rise to granulocytes, monocytes, neutrophiles, eosinophiles, basophiles, dendritic cells, platelets and erythrocytes.

Haematopoiesis depends on the environment of the maturing cell and involves many factors. A complex regulation of different transcription factors and gene rearrangement, decides the fate of each cell and it is controlled by growth factors and cytokines [5, 6].

Myeloid progenitors develop in the bone marrow and further differentiate in the body's bloodstream (monocytes) and tissue. Lymphocytes mature in primary lymphoid tissues (bone marrow and thymus). Spleen, mucosal associated lymphoid tissue and lymph nodes are secondary lymphoid organs were antigens can be trapped and presented to naïve lymphocytes via antigen presenting cells (APC).

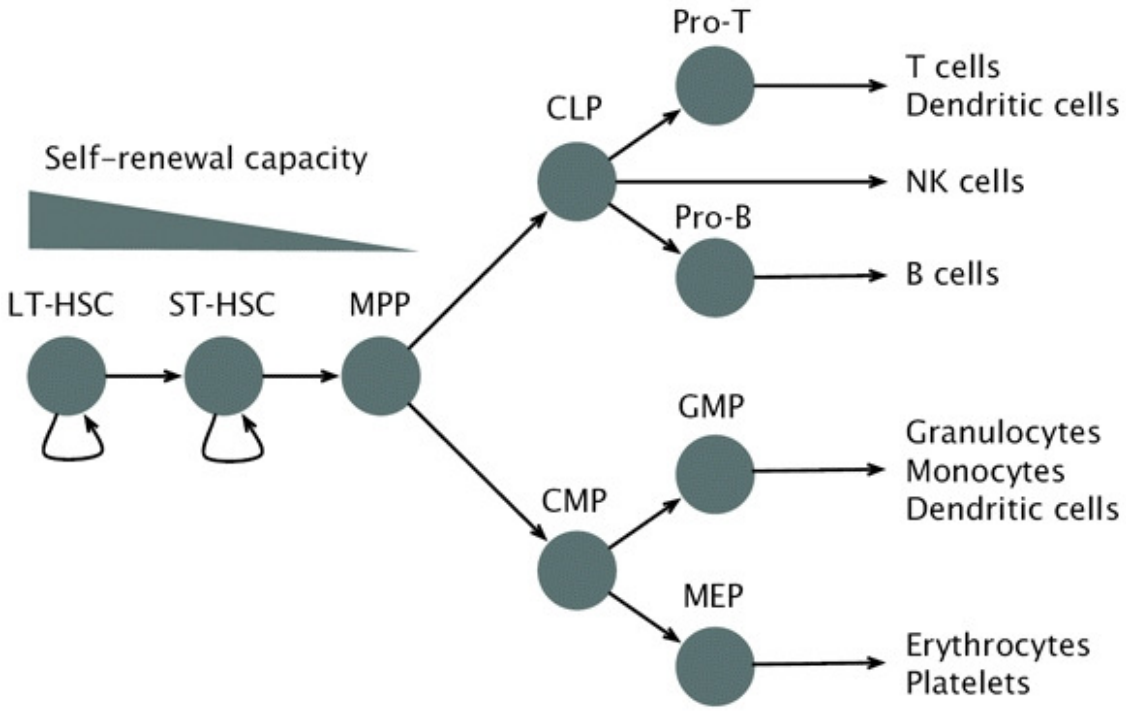


Figure 1. 2 Haematopoiesis.

Long lived and self renewing haematopoietic stem cell (LT-HSC) gives rise to a common lymphoid progenitor (CLP) and a common myeloid progenitor (CMP). All blood cells are derived from the LT-HSC [5].

The spleen is a specialized organ, which traps bloodstream derived antigens. It is a highly organized structure, which allows antigen to be efficiently trapped by APCs and presented to T cells. Lymph nodes resemble the spleen architecture and are connected via the lymphatic vessels, which form a network that collects interstitial fluid, the so called lymph. Lymph nodes filter toxins, antigens and waste products. The lymphatic system transports the interstitial fluid via the afferent lymph vessels into the lymph nodes were antigen is trapped and processed by APCs. The filtered fluid exits the lymph nodes via the efferent lymph vessel and re-enters the blood circulation through the thoracic duct [6, 7].

1.1.2. **Innate Immune system**

This chapter will give a brief overview of the different aspects of the innate immune system and will focus on the Natural killer (NK) cell immune response and NK cell receptor signaling.

The non-specific innate immune system is the first line of defense against pathogenic intruders. To prevent infection the innate immune system uses a combination of four

defensive barriers: Anatomic, physiologic, phagocytic and inflammatory barriers (Table 1. 1) [6].

1.1.2.1. Anatomic barriers

The anatomic barrier can be seen as a mechanical barrier which is provided by the skin (epidermis and dermis) and the mucosal membranes. They protect the organism by blocking the entrance of the invaders. In addition, commensal microbiota of skin and mucous membrane flora compete against invaders for attachment sites and nutrients. The low pH of the skin (pH 3-5) further limits the growth of bacteria and the mucous membranes entrap foreign microorganisms, where membrane-associated cilia propel them out of the body. However, pathogens can enter the body when skin integrity is damaged or through animal bites. In case of Influenza, the virus has evolved a mechanism to penetrate and infect the mucous membrane [6, 8].

1.1.2.2. Physiological barriers

As soon as a microorganism has invaded a host, normal body temperature or fever can inhibit its growth and if ingested most microorganism are destroyed by the acidic pH of the stomach. Different soluble chemical mediators such as lysozyme, collectin and complement help to kill invaders by either disrupting their cell walls, lysing the microorganism or enhancing phagocytosis [6].

1.1.2.3. Phagocytic and endocytic barriers

Foreign microorganisms and non-self macromolecules are recognized and get internalized by specialized cell types such as blood monocytes, macrophages and neutrophiles via a generalized process called endocytosis (Figure 1. 3). Phagocytosis is a type of endocytosis where the cell's plasma membrane wraps around the foreign particle.

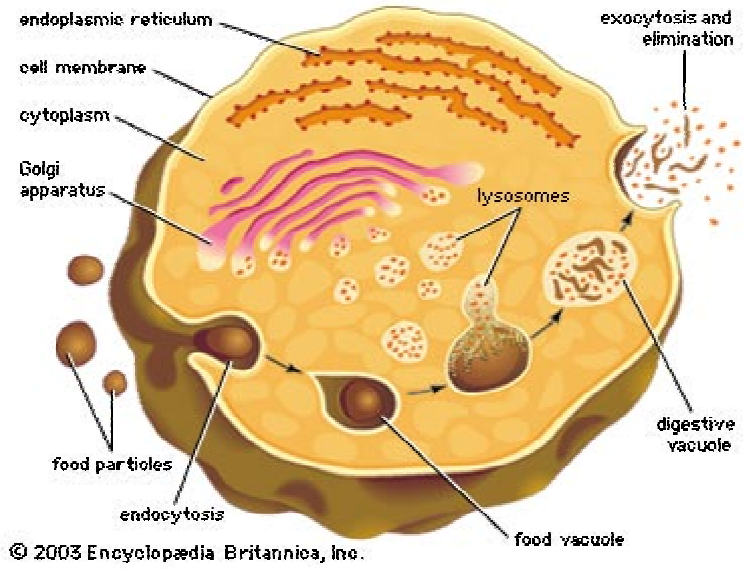


Figure 1. 3 Endocytic pathway of foreign particles recognized by specialized phagocytic cells [9].

The particle is then engulfed by the cell forming a phagosome. The phagosome then fuses with a lysosome, a vesicle containing hydrolytic enzymes, forming the phagolysosome [6, 9]. Some pathogens, like *Mycobacterium tuberculosis* or *Listeria monocytogenes* have evolved the ability to escape from intracellular digestion by either inhibiting phago-lysosome formation [10] or by lysing the phagosomal membrane, thereby entering the cytosol [11, 12].

1.1.2.4. Inflammatory barriers

Inflammatory molecules such as histamine or chemokines are rapidly produced after tissue damage and invading pathogenic microorganisms. Inflammatory responses can be divided in three steps:

1. Vasodilation
2. Increase in capillary permeability
3. Influx of phagocytes

First, vasodilation is characterized by an increase in the diameter of the blood vessels, which results in increase blood stream and temperature (erythema).

Table 1. 1 Overview of the innate immune system (adapted from [6])

Type	Mechanism
Anatomic barrier	
Skin	Mechanical barrier and acidic pH
Mucous membrane	Entraps foreign microorganism and propel them out the body with help of cilia
Physiological barrier	
Temperature	Normal body temperature and fever inhibits growth of some pathogens
Acidic stomach environment	Most ingested pathogens are killed by the low pH
Chemical mediators	Destroy cell walls (lysozyme, collectin), induce lysis and enhance phagocytosis (complement),
Phagocytic and endocytic barrier	Monocytes, Neutrophiles Macrophages kill microorganisms after phagocytes whole particles, where macromolecules are digested in specialized organelles containing reactive oxygen species.
Inflammatory barrier	Inflammatory molecules produced after infection or tissue damage induce influx of phagocytic cells into the affected area and production of proteins with antibacterial activity, transmit inflammatory signal upon pattern recognition (TLR) and induces antiviral state in nearby cells (interferon)

Second, increase in capillary permeability is important to facilitate influx of fluid containing high amounts inflammatory proteins (exudates). This influx leads to tissue swelling (edema). Third, phagocyte chemotaxis and extravasation to the site of inflammation is promoted by chemokines [6]. Moreover, the innate immune system has evolved receptors to recognize molecular patterns which are unique to prokaryotes and viruses and are usually not found among multicellular organism.

This type of pattern recognition is mainly mediated by Toll like receptors (TLR) or nucleotide oligomerization domain like receptors (NOD) [6]. In addition, interferons (Type I: IFNα/β;

Type II: IFNγ) are a class of proteins produced by virus-infected cells, thereby inducing a generalized antiviral state in nearby cells or by activating other immune cells [6].

1.1.2.5. Natural killer cells

Natural killer (NK) cells are cells derived from lymphoid progenitors and can make up to 15% of peripheral blood lymphocytes [13]. NK cells belong to the innate immune system and represent an early line of immune defense. NK cell activation is a complex mechanism, which needs to be tightly controlled. NK cells have an arsenal of activating as well as inhibitory receptors. Thus, for a proper function of NK cells, activation or inhibition of both types of receptors needs to be tightly coordinated [14, 15]. Besides, NK cells can be activated via soluble molecules such as IL2, IL12, IL15 and IFNα/β, which are released upon pathogenic infection [16]. Through direct cell-to-cell contact, NK cells sense their environment for missing inhibitory signals such as the MHC class I molecule, which is often downregulated in virus infected cells and tumors [16-20].

NK cells were shown, in human and mouse models, to play an important role in controlling early viral infection, especially Herpesvirus infection, such as Epstein-Barr virus (EBV) and Cytomegalovirus (CMV), and in tumor immunosurveillance [16]. Thus, lack of NK cells increases the susceptibility to viral infections. The antiviral effect of NK cells is based on their cytotoxicity against infected cells. Cytotoxicity is mediated by granule exocytosis which releases perforin and granzyme [21]. NK cells can also be activated directly via specialized receptors. Some NK cell receptors have evolved to recognize specific viral proteins. The mouse Ly49H NK cell receptor recognizes the M157 protein of MCMV on infected cells leading to NK cell activation [22]. Other mouse strains such as the Balb/c mouse do not express Ly49H, and are therefore more susceptible to MCMV infection. Recent findings provide evidence that NK cells play an active role in immune-regulation of the adaptive immune response after viral infection either by accelerating or limiting T cell responses [23, 24].

Table 1. 2 Important inhibitory and activating NK cell receptors (adapted and modified from [14, 25, 26]).

Gene	Other name	Species	Structure	Signaling	Ligand
Inhibitory NK receptors					
Klra1	Ly49a	Mouse	C-lectin homodimer	ITIM	H2-D^d, -D^k
Klra3	Ly49c	Mouse	C-lectin homodimer	ITIM	H2-K^b, -K^d, -D^d, -D^k
Klra5	Ly49e	Mouse	C-lectin homodimer	ITIM	?
Klra6	Ly49f	Mouse	C-lectin homodimer	ITIM	$H2^d$
Klra7	Ly49g	Mouse	C-lectin homodimer	ITIM	H2-D^d
Klra9	Ly49i	Mouse	C-lectin homodimer	ITIM	H2-D^k
Activating NK receptors					
Klra4	Ly49D	Mouse	C-lectin homodimer	DAP12	H2-D^d
Klra8	Ly49H	Mouse	C-lectin homodimer	DAP12	MCMV m157
Klrb1c	NKR-P1C, NK1.1	Mouse	C-lectin homodimer	FcεRIγ	?
KLRK1	NKG2D, CD314	Mouse Human	C-lectin Homodimer	Mouse DAP10 or DAP12 Human: DAP10 only	Mouse: Rae-1, H60, MULT1; Human: MICA, MICB, ULBP1-4
NCR1	NKp46, CD335	Mouse, human	Ig monomer	FcεRIγ, CD3ζ	Influenza hemagglutinins
CD244	2B4	Mouse, human	Ig monomer	ITSM, SAP	CD48
FCGR3	CD16	Mouse, human	Ig monomer	Mouse FcεRIγ; Human FcεRIγ or CD3ζ	IgG

1.1.2.6. NK cell signaling

NK cell activation is tightly controlled by a range of activating and inhibitory receptors. Activating signals are transmitted by small transmembrane-anchored adaptor proteins which contain immunoreceptor-tyrosine-based-activating-motif (ITAM), whereas inhibitory signals are transmitted by the receptor its cytoplasmatic domains, containing immunoreceptor-tyrosine-based-inhibitory-motif (ITIM) or immunoreceptor-tyrosine-switch-motif (ITSM, SAP; which can switch between activating and inhibitory motif) (Table 1. 2 and Figure 1. 4). The adaptor proteins FcεRIγ, CD3ζ and DAP10 (also known as HCST (hematopoietic cell signal transducer)) which signal through ITAMs are expressed as a disulfide-bonded homodimer or heterodimer. The adaptor protein DAP12 (also known as TYROBP (tyrosine kinase-binding protein)) is exclusively expressed as disulfide-bonded homodimer. The interaction between the receptors and the adaptor proteins predominantly takes place in their transmembrane region by oppositely charged amino acids forming salt bridges [14, 15, 27-29]. NK cell receptor triggering induces the phosphorylation of the ITAM, ITSM or ITIM by Src-family kinases, which provides docking sites for the SH2 (Src homology 2) domain for the family of intracellular kinases Syk and ZAP70 [27]. Syk and ZAP70 initiate a downstream phosphorlyation cascade after been recruited to the ITAM, which activates Phosphatidylinositol 3-kinase (PI3K). PI3K in turn phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP_2) to phosphatidylinositol-3,4,5-trisphosphate (PIP_3) which leads to a downstream signal promoting cell proliferation and survival and act as a docking station for PLCγ. In addition, PLCγ can cleave PIP_2 to inositol-1,4,5-trisphosphate (IP_3) and diacylglycerlol (DAG). IP_3 binds to the IP_3-receptor on the ER which then leads to Ca^{2+} efflux and to degranulation. On the other hand, DAG activates PKCΘ which leads to downstream phosphorylation of NF-κB. NF-κB translocates into the nucleus and controls the transcription of pro-inflammatory genes [14, 28, 30-32]. Importantly, NK cell receptor ITAM signaling through the Phospholipase C-γ (PLC-γ) isoforms (PLC-γ1 and PLC-γ2) may be redundant. Further, 2B4 is a member of the signaling lymphocyte activation molecule (SLAM) receptor family which can interact with the SLAM associated protein (SAP) adaptor protein and is an important modulator of NK- and T cell activity.

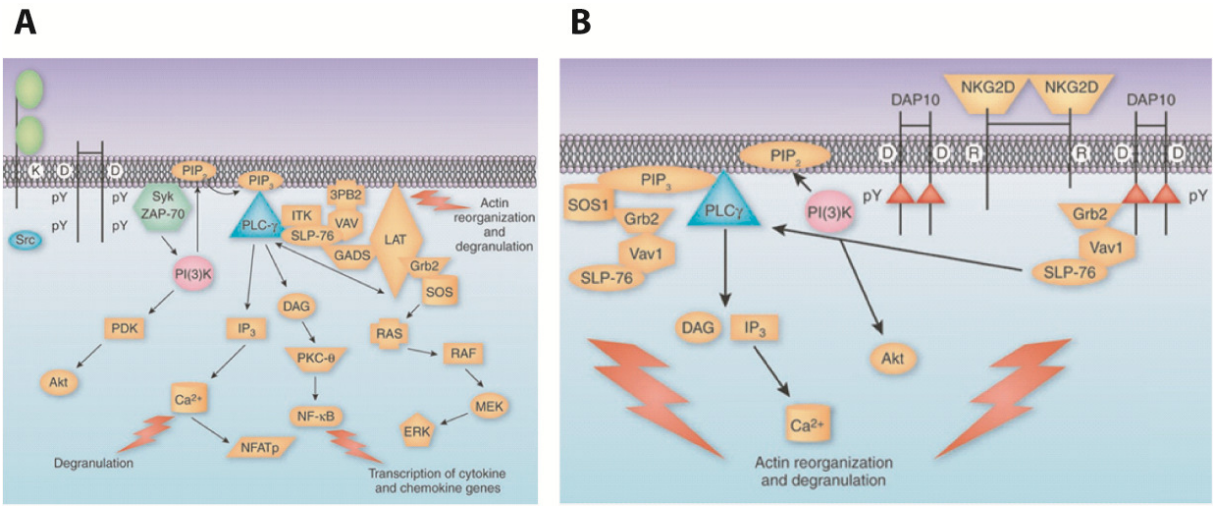


Figure 1. 4 NK cell signaling pathways.

A: After receptor activation ITAM- subunits are phosphorylated, by Src family kinases. Syk and/or ZAP-70 are recruited to the phosphorylated ITAMs, which initiate a phosphorylation cascade. B: DAP10-mediated signaling in NK cells. Cross-linking NKG2D causes NK cell activation that involves the recruitment of the p85 subunit of PI(3)K and recruitment of the Grb2-Vav1-Sos1 complex to the phosphorylated YINM motif in the cytoplasmic domain of DAP10. DAG, diacylglycerol; IP_3, inositol-1,4,5-trisphosphate; PIP_2, phosphatidylinositol-4,5-bisphosphate; PIP_3, phosphatidylinositol-3,4,5-trisphosphate; pY, phosphotyrosine; ITK, tyrosine kinase; GADS and 3BP2, adaptor proteins; NFATp and NF-κB, transcription factors; PDK, phosphoinositide-dependent protein kinase; PKC-θ, protein kinase C-θ; RAF, mitogen-activated protein (MAP) kinase kinase kinase; RAS, GTPase (adapted and modified from [14]).

2B4 can effectively co-stimulate the signals of other activating NK cell receptors and can enhance as well as inhibit the cytotoxic activity of antigen-specific T cells. 2B4 stimulates NK cell cytotoxicity and cytokine production and its activity is controlled by inhibitory receptors [28, 33, 34]. Upon activation, 2B4 recruits LAT on the ITSM to induce signaling cascade [35].

1.1.3. **The adaptive immune system**

The acquired immune system responds more slowly to microbial infections. The strength of the adaptive immune system lies in the specificity and the capacity to build a memory of encountered infection. For that, a network of diverse cell types has to act together, to provide recognition of foreign particles. This process is crucial to elicit an appropriate infection-specific immune response. The adaptive immune system is divided in two main arms, namely the “humoral immune response” and the “cellular immune response” (Figure 1. 5). The humoral immune response is mediated by B cells which produce antibodies. On the other hand, the cellular immune response is mediated by T cells.

1.1.3.1. B cells and the humoral immune response

B cells develop in the bone marrow and are derived from lymphoid progenitors. Bone marrow stromal cells create distinct microenvironments that provide support for haematopoiesis and B cell development [36]. B cells produce antibodies, which bind to pathogens, thereby preventing their entry into the host cells or opsonize them for destruction. Early B cell development depends on the expression of the Pax5 transcription factor, the E2A and early B cell factor (EBF) and IL7 [37-39]. B cell commitment is then followed by Ig gene rearrangements at the H chain locus. The construction of the H chain variable domain requires DNA recombination and ligation of the V_H, D_H and J_H elements to form a functional and productive pre-B cell receptor (BCR). A highly diverse BCR repertoire can be generated through junctional flexibility and nucleotide addition during DNA recombination. The formed pre-BCR on the immature B cells is then subjected to negative and positive selection. This occurs in the bone marrow where auto-reactive cells are deleted upon binding of self-antigens [6, 37, 39-42]. Naïve B cells expressing a functional BCR (mainly IgM and IgD) migrate then to secondary lymphoid tissues. Upon antigen encounter B cells become fully activated and are recruited to the germinal center where they become plasma and then memory B cells. In the germinal center the B cells undergo somatic hypermutation to edit the affinity of their BCR to the antigens. Depending on the nature of the antigen (organization, repetitive structure), the B cell activation can be independent (TI) or dependent (TD) on T cell help [6, 43]. For the latter, the interaction of the co-stimulatory B cell surface receptor CD40 and its ligand CD154 (expressed on $CD4^+$ T cells) are crucial for the proper activation of B cells and the production of antibody isotypes (IgA, IgE, IgG). Moreover, antibody class-switch depends on cytokine-signaling and on the activation-induced-cytidine-deaminase (AID). The different isotypes of antibodies possess distinct biological function. Hence, depending on the location and on the type of infection another antibody isotypes is favored [6, 37, 44, 45].

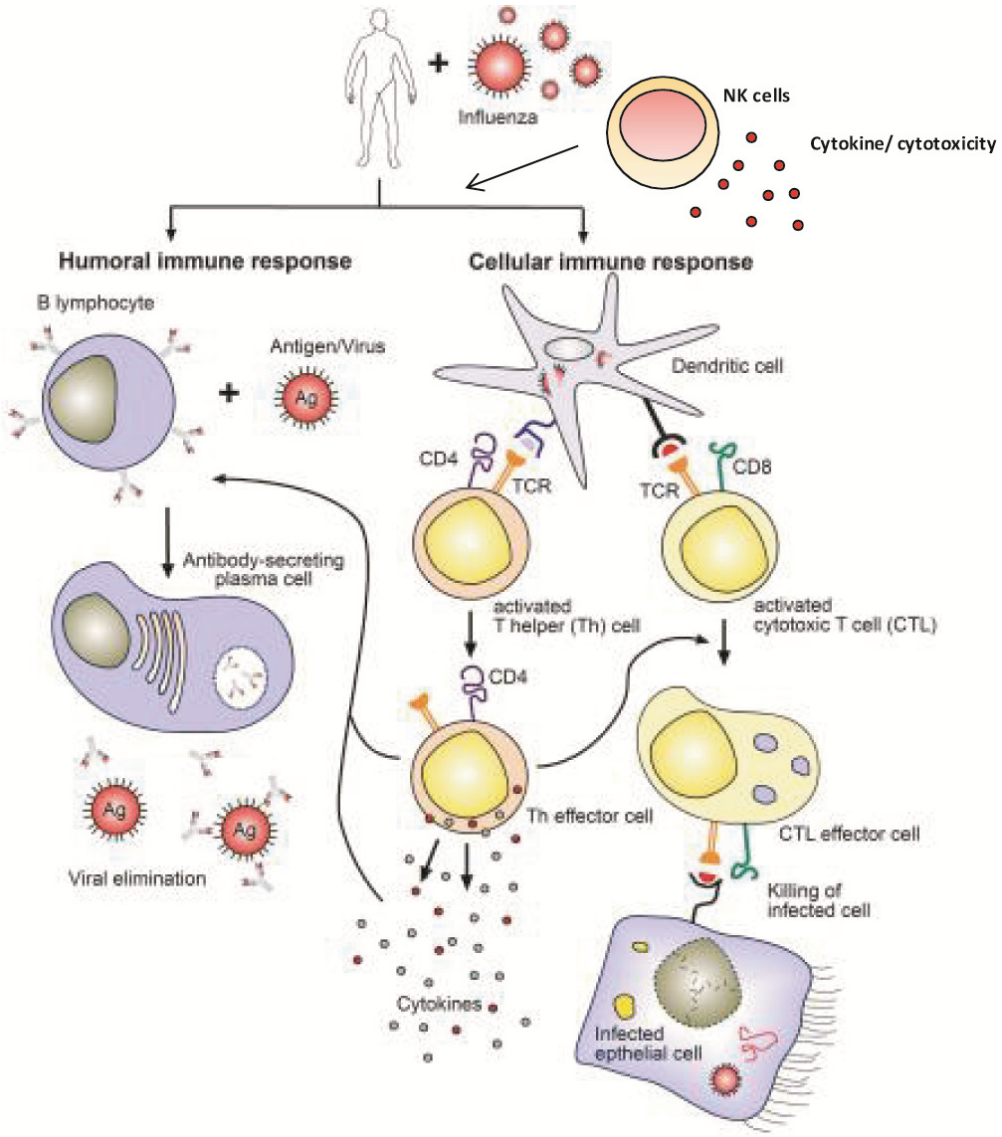


Figure 1. 5 The cell mediated and humoral immune response to a viral infections.

The humoral branch of the immune system comprises B lymphocytes *(left)*. After interaction with pathogens, B cells differentiate into antibody-secreting plasma cells. The cellular response *(right)* starts with the activation of NK cells and later on with antigen presentation via MHC I (black) and II (blue) molecules by dendritic cells. This leads to the activation, proliferation and differentiation of antigen-specific T cells (CD4 or CD8). These cells gain effector cell function to help B cell and cytotoxic T lymphocytes (CTL) response, release cytokine, or mediate cytotoxicity following recognition of antigen. Thus, the cellular and humoral immune responses have to act in concert to provide efficient protection against pathogens (adapted and modified from [46]).

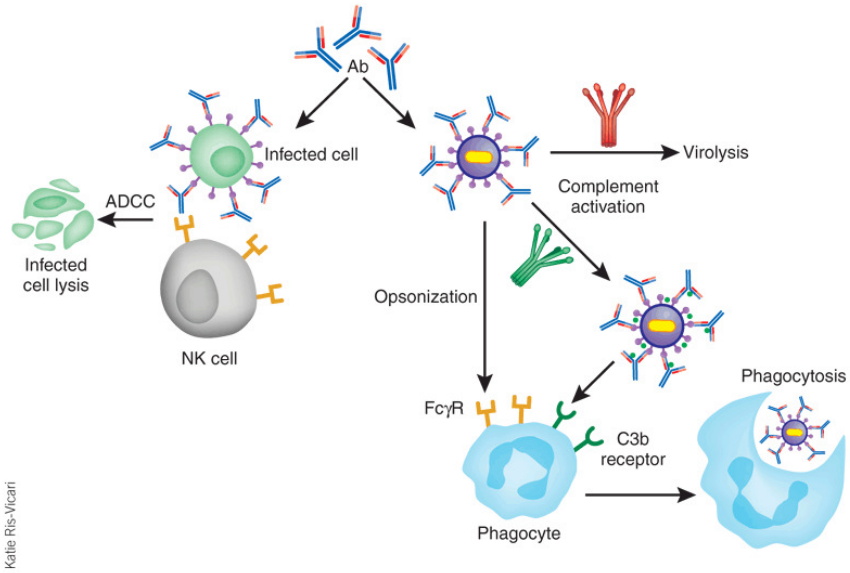


Figure 1. 6 Opsonization, antibody dependent cellular cytoxicity (ADCC) and complement activation [47].

An important feature of antibodies is to opsonize foreign antigens to facilitate their uptake by specialized Antigen presenting cells (APC) [48]. As soon as an antigen is recognized by specific antibodies on the surface of an infected cell, antibody dependent cellular cytotoxicity (ADCC) is promoted by NK cells which bear the Fc-receptor (CD16) on their cell surface [48, 49] (Figure 1. 6).

Most vaccines are based on the production of specific memory B cells and the production of specific neutralizing antibodies [50]. Neutralizing antibodies bind and block important molecules, on the surface of pathogens, which are required for infection. Impaired B cell responses, for instance by missing a $CD4^+$ T cell response (e.g. CD40-CD154 interaction), can have fatal consequences on the host survival after pathogenic infection. In case of Vesicular Stomatitis Virus (VSV) or Rabies virus infection, the virus invades the central nervous system (CNS) if no neutralizing IgG antibodies are produced, which results in lethal paralysis of the host [51-55]. On the other hand, some viruses such as the Dengue virus, have developed a mechanism to hitchhike the antibody pathway to enter cells via the Fc-receptor, a process called antibody-dependent enhancement [56]. Recent findings show that B cells also may act as immune-modulating cells during viral infection, questioning the importance of neutralizing antibodies [57, 58].

1.1.3.2. T cells and the cellular immune response

$CD8^+$ and $CD4^+$ T cells are derived from lymphoid progenitors and belong to the adaptive immune system. The T cell progenitors develop in the bone marrow and migrate to the thymus where the T cell receptor (TCR) is rearranged. This is comparable to the BCR rearrangement in the bone marrow. The T cell precursors pass different developing stages in the thymus cortex and subcapsular zone, which can be divided in DN1 (double negative for CD8 and CD4) ($CD117^{hi}$, $CD44^{hi}$, $CD25^-$, $CD24^{-/lo}$, $CD27^{hi}$, $CD3^-$), DN2 ($CD117^{hi}$, $CD44^{hi}$, $CD25^+$, $CD24^{hi}$, $CD27^{hi}$, $CD3^-$), DN3 ($CD117^{-/lo}$, $CD44^{-/lo}$, $CD25^+$, $CD24^{hi}$, $CD27^{-/lo}$, $CD3^{lo}$) and DN4 ($CD117^{-/lo}$, $CD44^{-/lo}$, $CD25^{-/lo}$, $CD24^{hi}$, $CD27^{hi}$, $CD3^{lo}$) [59-61]. At this point the DN4 precursor T cells start to express CD4 and CD8 and are therefore referred as double positive (DP) thymocytes. Depending on their interaction with the major histocompatibility complex (MHC) I or II expressed on cortical epithelial cells, precursor T cells develop into CD8 or CD4 single positive (SP) T cells, respectively. To ensure proper TCR recognition by peptide loaded MHC I/II (pMHC I/II) and to prevent auto-reactive T cells, CD8 and CD4 committed T cells are submitted to positive and negative selection. Self-peptides are presented on epithelial MHCs, which provide TCR stimulation. If the TCR signal provided by the self-pMHC is too strong or too low, then apoptosis is initiated (negative selection). Hence, if the signal is intermediate for the MHC and weak for the self-pMHC then T cells survive and proliferate and migrate to the periphery (positive selection) (Figure 1. 7) [61].

In the periphery, $CD8^+$ and $CD4^+$ T cells can exert their function after encountering foreign antigens. $CD8^+$ T cells, also referred to cytotoxic T lymphocytes (CTLs, killer cells), exert their killing function when they detect aberrant target cells, which present antigenic peptides on their MHC I molecules. $CD4^+$ T cells on the other hand (also referred as helper T cells), support $CD8^+$ T cell as well as B cell mediated immune response. $CD4^+$ T cells can be subdivided in three subsets: T_{H1} (IFNγ), T_{H2} (IL-4, IL-13) and T_{H17} (IL-17, IL-23). Whereas T_{H1} $CD4^+$ T cells mainly induce cell-mediated immunity and phagocyte-dependent inflammation, T_{H2} $CD4^+$ T cells support the CTLs and the humoral immune system. Next, T_{H17} $CD4^+$ T cells induce tissue inflammation and are associated with several autoimmune diseases [60, 62-64]. In the absence of $CD4^+$ T cells, various stages of the antigen specific $CD8^+$ T cell response are impaired, whereas the severity and the stage of interferences greatly depend on the challenging antigen.

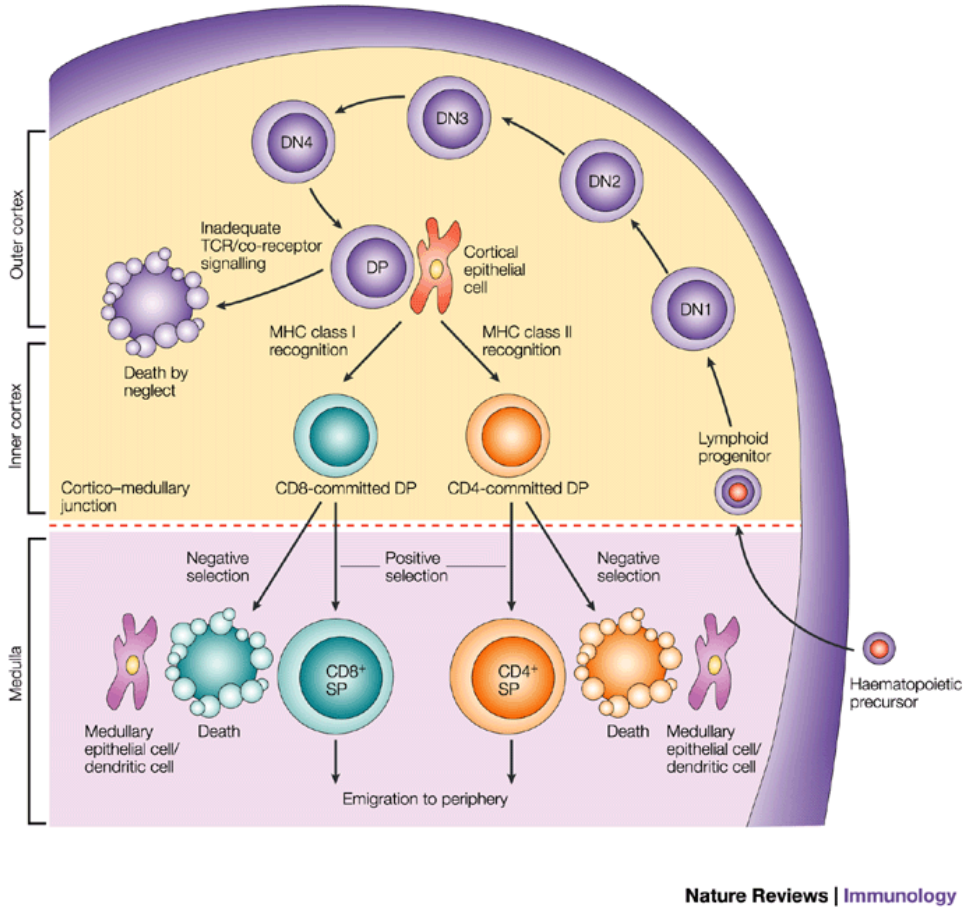


Figure 1. 7 T cell development in the thymus.

Early T cells progenitors lack expression of T-cell receptor (TCR), CD4 and CD8, and are termed double-negative (DN; no CD4 or CD8) thymocytes. DN thymocytes can be divided into four stages of differentiation DN1-4. Thymocytes start to express the pre-TCR as they progress to the DN4 stage. The rearranged αβ-TCR$^+$CD4$^+$CD8$^+$ (DP) thymocytes interact with the cortical epithelial cells that express a high density of MHC class I and class II molecules associated with self-peptides. Depending on the signaling that is mediated by the interaction of the TCR with the self-peptide–MHC ligands, T cells survive and proliferate (positive selection) or undergo apoptosis (too less or too much signal: negative selection). Thymocytes that express TCRs that bind self-peptide–MHC-class-I complexes become CD8$^+$ T cells, whereas those that express TCRs that bind self-peptide–MHC-class-II ligands become CD4$^+$ T cells; SP, single positive (adapted and modified from [60]).

The uptake, processing and presentation of foreign antigens to T cells by antigen presenting cells (APC) are the most important steps during the cell mediated immune response. This process is required to activate naïve CD8$^+$ and CD4$^+$ T cells.

Antigen is presented to T cells via the MHC molecules I and II to the TCR of CD8$^+$ and CD4$^+$ T cells, respectively. Endogenous antigens are primarily presented on MHC class I and exogenous on MHC class II molecules [65].

The CD8 and CD4 molecules along with other co-stimulatory molecules such as CD28, CD45 or CTLA-4 are mandatory to provide efficient T cell activation [66, 67]. Missing signals can result in anergic T cells which are unable to respond to further stimulation. Via the TCR and

co-stimulatory molecules, activation signals are transmitted into the cell, which in turn activate a different signaling cascade leading to production of inflammatory molecules such as cytokines. However, to become entirely activated in the periphery, T cells need a priming period of approximately three to four days [6]. The T cell response after infection can be divided into three stages: priming and expansion, contraction and the memory phase (Figure 1. 8A).

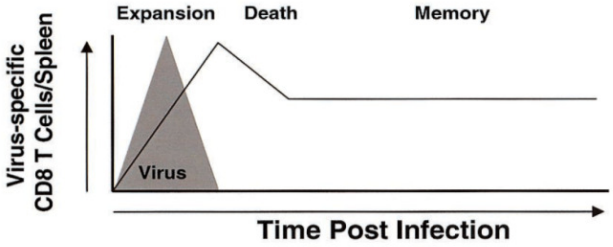
Primary antigen encounter induces specific T cells to become primed and to expand vigorously (4-6 hours per cell cycle; ~10'000 fold) [68]. These T cells start to express effector molecules and inflammatory cytokines such as IFNγ, Tumor necrosis factor α (TNFα) or IL2. Whereas IL2 promotes survival and proliferation of immune cells, IFNγ and TNFα limit viral dissemination by activating other immune cells and by increasing the antiviral resistance of neighbouring cells [69].

The T cell responses reach their peak approximately one week post infection and then contract to reach a low T cell number. During contraction (death) phase up to 95% of effector T cells undergo apoptosis. In the following memory phase, T cells form a stabile memory T cell population whose homeostatic turnover is independent of antigen (Figure 1. 8B). Memory T cells have different properties compared to naïve T cells. Upon a secondary challenge, memory T cells are less dependent on co-stimulatory molecules and have a rapid recall response resulting in a more effective pathogen clearance (Figure 1. 8C).

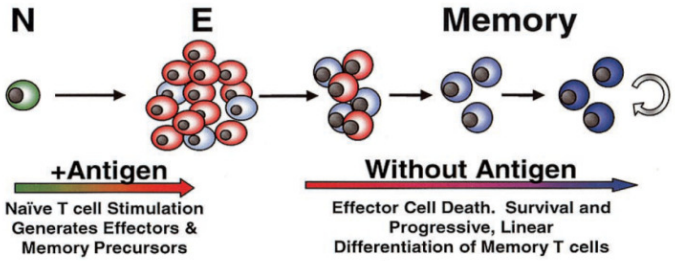
Memory T cell subsets can be classified according to their expression of surface molecules and receptors (Figure 1. 8D). For instance, memory T cells express high level of pro-survival molecules such as IL2 and IL7-receptors. Furthermore, naïve, memory and effector T cells can be classified according to their expression of adhesion (CD44) and lymph node homing molecules (CD62L) [60, 70].

Memory T and B cells activation is often bypassed by viruses. Pathogens, such as a virus, which are subjected to a selective pressure by the immune system, can introduce small mutations in their genome which results in changes in the epitopes recognized by the TCR. For instance, influenza, which infects 5-10% of the world population every year, and Human immunodeficiency virus (HIV) (2.7 Million new infections in 2008, WHO), introduce many of such mutations in their genome. On the other hand, vaccines which are based on the production of neutralizing antibodies directed against specific epitopes, cannot bind the target effectively, if the epitope mutated prior a secondary infection [71-73].

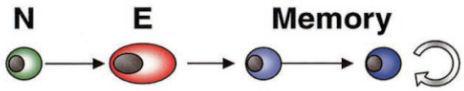
A.



B.

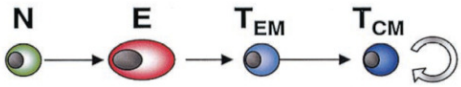


C.



		N	E	Memory	
Homing to:	2° Lymphoid Organs	++++	-/+	-/+	+++
	Peripheral Tissues	-/+	++++	++	+
Ex Vivo Effector Functions:	CTL	-	++++	++	-/+
Rapid Recall:	IFN-γ / TNF-α	-/+	++++	++++	++++
	IL-2	++	-/+	-/+	+++
Proliferation:	Antigen Driven	+++	+	++	++++
	Homeostatic	-	-	-/+	++++

D.



		N	E	T_{EM}	T_{CM}
Antigen Experienced T Cells					
CD44	Adhesion Molecule	Lo	Hi	Hi	Hi
CD11a	LFA1, α Integrin; Adhesion Molecule	Lo	Hi	Hi	Hi
Ly6C	GPI-linked; unknown function	Lo	Hi	Hi	Hi
CD122	IL-2/IL-15 receptor β chain	Lo	Hi	Hi	Hi
IL-15Rα	IL-15 receptor α chain	Lo	Hi	Hi	Hi
Associated with Effector T Cells					
CCR5	Chemokine Receptor	Lo	Hi		Lo
Perforin	Cytotoxic granule protein	Lo	Hi		Lo
Granzyme B	Cytotoxic granule protein	Lo	Hi	Int	Lo
Dynamically Regulated during Memory T Cell Development					
CD62L	Lymph node homing receptor	Hi	Lo	Lo	Hi
CCR7	Lymph node homing chemokine receptor	Hi	Lo	Lo	Hi
CD27	TNF receptor superfamily; costimulation	Hi	Lo/Int	Lo/Int	Hi
CD127	IL-7 receptor α chain	Hi	Lo	Lo/Int	Hi
Bcl-2	Anti-apoptotic/cell survival	Int	Lo	Int	Hi

Figure 1. 8 T cell response after acute infection.

A: The dynamics of a $CD8^+$ T cell response to acute infection. A $CD8^+$ T cell response to an acute viral infection undergoes an expansion phase, culminating in the generation of effector CD8+ T cells and viral clearance. The expansion phase is followed by a death phase. The surviving effector $CD8^+$ T cell pool further differentiates and generates a memory T-cell population that is maintained long-term in the absence of antigen. B: Memory $CD8^+$ T cell generation is linear and progressive. Antigenic stimulation causes naïve $CD8^+$ T cells to proliferate and acquire effector functions. Memory T cells continue to differentiate in the absence of antigen and acquire the ability to persist in the absence of antigen via homeostatic turnover. C: Memory $CD8^+$ T cell properties that change during the naïve → effector → memory transition are listed. D: Phenotypic changes that occur during the naïve → effector → memory transition are listed, including differences between the effector memory (T_{EM}) and central memory (T_{CM}) subsets of memory $CD8^+$ T cells. Int, intermediate. (adapted and modified from [70])

1.1.3.3. Antigen presentation and the Major Histocompatibility Complex (MHC) class I and class II molecules

Genes encoding for the major Histocompatibility Complex (MHC) are crucial for a proper functioning of the immune system. MHC molecules bind small peptides. A particular combination of peptide and MHC I or II is recognized by specific TCR on $CD8^+$ or $CD4^+$ T cells, respectively, leading to T cell activation. The mouse MHC is referred as the H2 complex. It is a tightly clustered set of genes. This cluster can be divided into four regions: K, I, S and D. On the basis of distinct structural and functional characteristics, the H2-genes are classified into class I, II and III [62, 65, 74-79].

The class I loci is located in the K and D region of the H2-complex, which encodes a single-chain protein of 44 kDa consisting of three external domains (α_{1-3}) and is expressed in all nucleated body cells. This α-chain is associated with a small 12 kDa protein (β_2-microglobulin) (Figure 1. 9A). Endogenous antigens, which are usually derived from intracellular viruses are proteolytically cleaved by the proteasome into 8-10 amino acid (AA) long peptides and subsequently are actively transported by the "transporter associated with antigen processing" (TAP) into the ER where it binds to the MHC class I molecule. Peptide loading of the MHC I is facilitated by the tapasin glycoprotein which mediates the interaction between the newly synthesized MHC I and TAP. The peptide MHC I complex is then transported via exocytosis to the cell membrane, where it is recognized by $CD8^+$ T cells harboring the appropriate TCR [62, 65, 76, 77, 79].

The class II loci is located in the I region of the mouse H2-complex. Two different types of chain (α and β) are separately encoded and are non-covalently associated on the cell surface (Figure 1. 9A). Unlike the MHC I molecule, MHC II is only expressed on a subset of immune cells that include B cells, macrophages, monocytes, dendritic cells and thymic epithelium, which are also referred as APCs [62]. Shortly after MHC II expression in the ER, MHC II is associated with the invariant chain (Ii), which blocks the binding groove of the MHC II to prevent intrinsic peptide binding. As the MHC II-Ii complex is transported through the Golgi to the endosome, Ii is partially degraded in such a way that the class II-associated invariant chain peptide (CLIP) remains in the binding groove. At this time point exogenous antigen is uptake through the endocytic pathway by the APCs and proteolytically digested in the lysosome into 13-30 AA long peptides [62, 80-86].

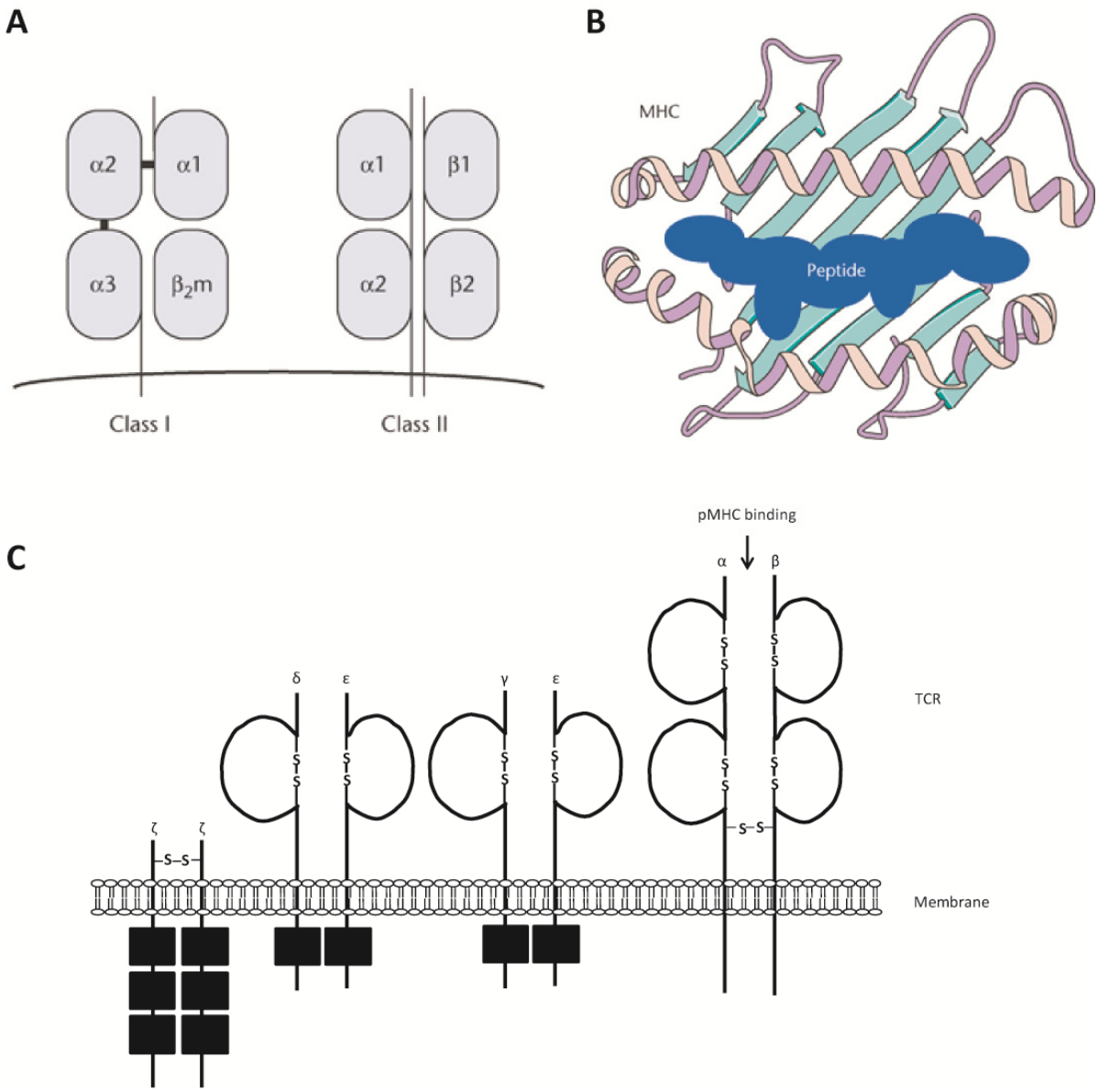


Figure 1. 9 Schematic structure of the MHC I/II and the T cell receptor

A: The MHC I molecule consist of three extracellular α domains and a β2 microglobulin domain. MHC I is expressed on every nucleated cell of the body and is recognized by the TCR of $CD8^+$ T cells. The MHC II molecule is composed of non-covalently associated α- and β-chain, which both are characterized by two extracellular domains. B: Antigen-derived peptides bind in the binding groove of the MHC molecule. MHC I peptide are usually 8-10 amino acid long and MHC II peptide can be up to 30 amino acids (adapted and modified from [62]). C: The αβ TCR cell surface expression is dependent on the association with CD3γε, δε, and ζζ signaling subunits. The black boxes represent the ITAMs [87].

Exchange of the CLIP fragment by the exogenous peptide is catalyzed by the MHC class II-related chaperone molecule H2-M (human: HLA-DM) at the endosomal/lysosomal site [82]. Upon formation of a stable peptide MHC II complex (pMHC), the pMHC II is transported by exocytosis to the cell surface, where it recognized by $CD4^+$ T cells expressing the restricted TCR [62, 83].

1.1.3.4. T cell receptor

The actual antigen specific TCR consists of an α and a β subunit. The α-chain contains multiple alleles for the V, J and C region, whereas the β-chain contains multiple allele for the V, D, J and C regions. These alleles are rearranged within each region in the thymus cortex using the recombinase activating genes (RAGs). The V gene segments encode for three hyper variable complementarity determining regions (CDRs). Through usage of different CDR1 and CDR2 sequences in the different V gene segments and a junctional variation in the CDR3, a high diversification of the TCR can be achieved. The naïve TCR repertoire is further increased by both, a lack of precision during V(D)J gene rearrangement and the addition of non-template encoded nucleotides at V(D)J junctions [88-91].

CD3 proteins exist as a series of Ig fold dimers including γε, δε and ζζ associated with a single αβ-TCR disulfide bonded heterodimer. This forms the αβ-TCR/CD3-complex (Figure 1. 9C). A small subset of T cells expresses γδ-TCRs composed of disulfide- bonded γ and δ chains. These bind directly to pathogen-derived glycoproteins or nonclassical MHC molecules [87]. The αβ TCR cell surface expression is dependent on the association with CD3γε, δε, and ζζ signaling subunits. The transmembrane domains of each CD3 chain and the αβ-TCR interact through highly conserved charged residues. The intracellular domains of each of the CD3 chains contain immunoreceptor-tyrosine-based-activation motifs (ITAMs) that serve as starting point for intracellular signal transduction upon TCR engagement. The CD3 δ, γ, and ε chains each contain one ITAM, and CD3ζ contains three ITAMs [88, 90, 91].

1.1.4. **T cell receptor signaling and T cell activation**

T cell activation is initiated at the contact side (also referred as immunological synapse) between a T cell and an antigen presenting cell (APC). At the immunological synapse, the TCR recognizes the peptide-MHC complex on the APC [92]. This interaction leads to clustering of additional co-stimulatory molecules such as CD28 and CTLA-4 [66, 93, 94]. The polarization of the co-stimulatory molecules to the T cell – APC interface is called central supramolecular activation cluster (cSMAC). The more distal part of the interaction where cell adhesion is taking place is referred as peripheral SMAC (pSMAC) [95].

The downstream activation signaling pathway strongly resembles the NK cell receptor signaling pathway mediated by the ITAM domains. TCR stimulation leads to phosphorylation of ITAM, which provides a docking site for other proteins (Figure 1. 10). The Syk kinase family member "ζ chain associated protein kinase" (ZAP-70, 70 kDa) is recruited to the phosphorylated ITAM and becomes phosphorylated by the CD4/CD8-associated src-family protein proto-oncogene tyrosine-protein kinase (Fyn) and lymphocyte-specific protein tyrosine kinase (Lck) [67, 96]. The Src homology 2 (SH2) domain-containing leukocyte phosphoprotein (SLP-76, 76kDa) forms a distinct microcluster with the membrane associated "linker for the activation of T cells" (LAT) at the proximal site of the cSMAC. This microcluster organizes the following effector molecules in a spatio-temporal manner. SLP-76 and LAT are phosphorylated by ZAP-70 [97, 98]. LAT phosphorylation induces the binding of the C-terminal SH2 domain of PLC-γ1 and the p85 subunit of PI3K [99]. SLP-76 interacts itself with the SH2 domain of Vav1 (a guanine nucleotide exchange factor) and Nck. This formation induces the phosphorylation of PLC- γ1, which hydrolyzes the membrane lipid PIP_2 to IP_3 and DAG [67, 95].

DAG activates two major pathways involving RAS (guanine nucleotide binding protein) and PKCΘ, which leads to a downstream activation of transcription factors, such as JNK and NF-κB translocation into the nucleus [67, 95, 100]. IP_3 binds to the Ca^{2+}-permeable ion channel receptor (IP_3R) on the ER, which leads to ER Ca^{2+} store release. Intracellular Ca^{2+} release leads to an opening of the Ca^{2+} release activated channels (CRAC) located at the plasma membrane via the "stromal interaction molecules" (STIM) and the "Calcium release-activated calcium channel protein" (ORAI), which promotes extracellular Ca^{2+} influx [101-103]. Ca^{2+} binds to calmodulin, which in turn activates calcineurin and Ca^{2+}-calmodulin-dependent-kinase (CaMK). Finally, these two pathways leads to the activation of two transcription factors NFAT and CREB, which translocate into the nucleus to induce important T cell activation genes such as IL2 [67, 95].

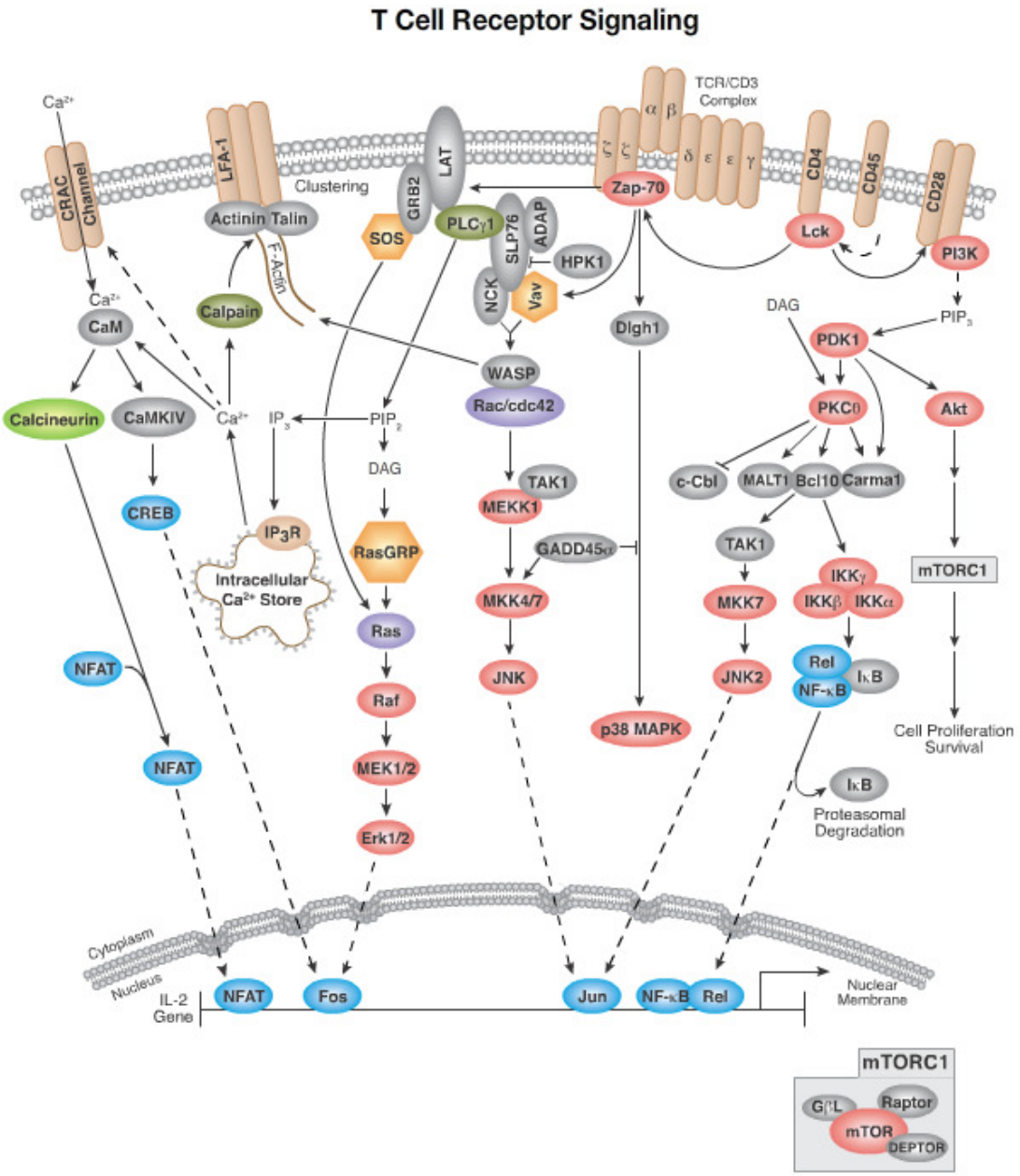


Figure 1. 10 T cell receptor signaling pathways.

TCR activation induces a signaling cascade that regulates cytokine production, cell survival, proliferation, and differentiation. Upon TCR activation ITAM becomes phosphorylated on the cytosolic side of the TCR/CD3 complex by lymphocyte protein-tyrosine kinase (Lck). The CD45 receptor tyrosine phosphatase modulates the phosphorylation and activation of Lck. ζ-chain associated Zap-70 is recruited to the TCR/CD3 complex, where it becomes activated, promoting recruitment and phosphorylation of downstream adaptor or scaffold proteins. Phosphorylation of SLP-76 by Zap-70 promotes recruitment of Vav the adaptor proteins NCK and GADS. Phosphorylation of PLCγ1 results in the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce the second messengers diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3). DAG activates PKCθ and the MAPK/Erk pathways, both promoting transcription factor NF-κB activation. IP_3 triggers the release of Ca^{2+} from the ER, which promotes the entry of extracellular Ca^{2+} into cells through CRAC. Calcium-bound calmodulin (Ca^{2+}/CaM) activates the phosphatase calcineurin, which promotes IL-2 gene transcription through the transcription factor NFAT. The incorporation of signals from additional cell surface receptors (such as CD28 or LFA-1) further regulates cellular response [104].

1.2. The cell mediated immune response to MCMV, LCMV and VSV

1.2.1. Murine cytomegalovirus (MCMV)

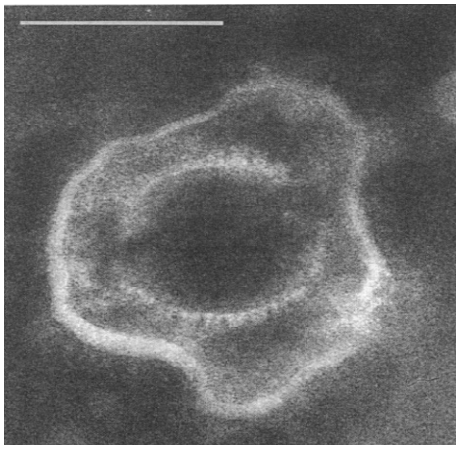


Figure 1. 11 The electron-microscopic appearance of CMV

The white bar represents 100 nm (adapted and modified from [105]

Cytomegalovirus (CMV) is a large, double-stranded DNA virus, which is classified in the β herpesvirus family (Figure 1. 11). Due to co-evolutionary adaptation, the virus is highly specific for its respective host species. CMVs have a large double-stranded linear genomic DNA of about 230 kbp, potentially encoding for more than 200 proteins. During viral replication, the viral gene expression is subdivided into three sequential stages called immediate early (IE), early (E) and late (L) phase.

After primary infection with CMV, the virus becomes latent in multiple organs and can later reactivate when the immune system is dysregulated [106]. Primary HCMV-infection of immune-competent individuals is usually asymptomatic. If symptoms occur, the disease is characterized by a self limiting febrile illness reminiscent of infectious mononucleosis (i.e. acute EBV-infection). In some rare cases, severe primary infection may cause hepatitis, pneumonitis or meningoencephalitis in immunocompetent host [105, 107-110]. Importantly, CMV is a common cause of mortality and morbidity in immunocompromised patients such as those with HIV/AIDS and transplant recipients on immunosuppressive therapy [106].

The murine CMV prominently replicates in salivary glands, liver, spleen, lung, kidney cortex and bone marrow stroma. CMV can infect many different cell types including endothelial and epithelial cells, bone marrow stromal cells, DCs and tissue macrophages, thereby establishing latency in macrophages, dendritic cells and endothelial cells [109, 111].

Despite a multi-layered immune response, CMV enters a latent stage leading to lifelong persistence in the infected host [112]. CMV has evolved diverse mechanisms to avoid immunosurveillance and establish persistence in host cells. CMV possesses a series of genes which are used to escape the immune system. Many of these gene-products interfere with antigen presentation in the infected cells, thereby down-regulating MHC I or II expression and escape cytotoxic $CD8^+$ or $CD4^+$ T cell recognition [106, 109, 113]. However, down-regulation of MHC class I expression renders the infected cells susceptible to lysis by NK cells. Hence, NK cell control is prevented by CMV gene products which mimic MHC molecules on the cell surface (e.g. the MCMV protein M157) [22, 114].

As for many viruses, NK cells play a major role in limiting viral replication, especially during acute MCMV infection. Moreover, $CD4^+$ T cells, $CD8^+$ T cells, and antibodies all play a role in controlling viral replication and spread. Fifty to sixty percent of all $CD8^+$ T cells during acute MCMV infection are virus specific, whereas about 30% of total $CD8^+$ T cells directed against MCMV-derived peptides remain in the circulation during latent MCMV infection phase [115, 116]. The high frequency of MCMV-specific $CD8^+$ T cells during viral latency is due to the long term accumulation of memory T cells, a phenomenon unique to CMV and referred as memory inflation [117-120]. Once latency is established, viral reactivation seems to be redundantly controlled by the combined efforts of NK cells, $CD4^+$, and $CD8^+$ T cells [109, 116, 121, 122]. In adoptive transfer experiments, primed B cells and T cells exerted antiviral effects and were able to reduce viral replication [110, 123]. Yet, CD4 T cell deficiency is associated with impaired lytic viral control in various organs, in particular in the salivary glands where shedding of infectious virus persists [110, 123, 124]. Thus, it is believed that $CD4^+$ T cells exert a T_{H1} phenotype by secreting TNFα and IFNγ, thereby inhibiting MCMV replication [110]. In addition, progressed HIV infection is characterized by a very low CD4-counts and a high risk for HCMV-reactivation. Overall, HCMV-specific $CD4^+$ T cells were shown to be important for long-term protection in transplant patients and immunocompetent children [125-127]. Together, these findings underline the importance of $CD4^+$ T cells in the long term control of HCMV-infection.

1.2.2. **Lymphocytic choriomeningitis virus (LCMV)**

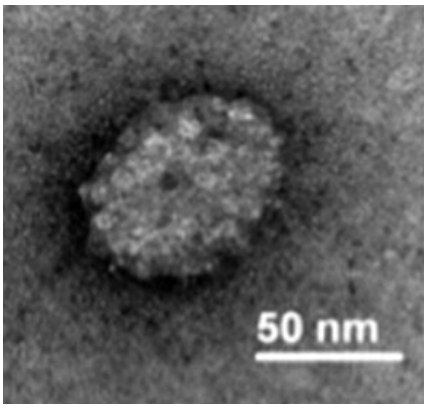


Figure 1. 12 Electron microscopic apparence of Lymphocytic choriomeningitis virus (LCMV) (adapted and modified from [128])

LCMV belongs to the arenavirus, which are enveloped viruses with a bi-segmented negative-strand RNA genome (Figure 1. 12). The genomic RNA segment, L (7.3 kb) and S (3.5 kb), uses an ambisense coding strategy to direct the synthesis of two polypeptides in opposite orientations. The S RNA encodes the viral glycoprotein precursor (GPC) and the nucleoprotein (NP). GPC is post-translationally cleaved by the cellular site 1 protease (S1P) to yield the two mature virion glycoproteins GP1 and GP2. GP1 and GP2 form the spikes on the virus surface and mediate receptor recognition and cell entry. The L RNA encodes the viral RNA (vRNA)-dependent RNA polymerase (RdRp, or L polymerase) and the small RING finger protein Z, which is the arenavirus counterpart of the M protein found in many other negative-strand RNA viruses [128, 129]. The natural host of LCMV is the mouse and other rodents and human infection with LCMV is very rare. However, in some area, 5% of the human population is seropositive for LCMV. LCMV can cause a variety of syndromes, from malaise to meningitis or encephalitis [130, 131].

Depending on the LCMV-isolate, virus dose and the immune-competence of the host, LCMV can cause a transient, acute or a persistent chronic infection [132]. For instance, infection of mice with the LCMV isolate Armstrong results in a rapid expansion of virus-specific effector $CD4^+$ and $CD8^+$ T cell responses that clear the infection within 8–10 days post-infection, whereas infection with LCMV Clone 13 leads to the emergence of functionally exhausted T

cells and decreased antibody responses that are ineffective at clearing the virus, resulting in a persistent infection. Exhausted T cells down-regulate TCR signaling molecules, increase the expression of inhibitory surface molecules, lose the ability to produce multiple immune-stimulatory and antiviral cytokines associated with reduced viral clearance during acute infection and have reduced proliferative capacity [133]. Hence, an exhausted T cell response to persistent LCMV may reflect T cell responses found in HIV infection [134].

Control of acute LCMV infection was shown to mainly dependent on LCMV-specific $CD8^+$ T cells. However, a strong and sustained LCMV-specific CTL response can cause severe and lethal immune pathology, which can be manifested by meningitis. During acute LCMV infection $CD8^+$ T cell numbers in the spleen increase five to ten fold. At the peak of the infection, 50-70% of these $CD8^+$ T cells appear to be specific for five LCMV derived epitopes (NP396, GP33, GP276, GP34 and NP205) [135, 136]. Notably, primary CTL responses against LCMV were shown to be normal in the absence of $CD4^+$ T cells. Whereas LCMV specific $CD8^+$ T cells are responsible for controlling acute LCMV infection, chronic LCMV infection is, however, dependent on $CD4^+$ T cell help, which support and maintain $CD8^+$ T cell and B cell responses. Hence, $CD4^+$ T cells are critical to control long-term LCMV infection. Moreover, impaired B cell responses, which result in low neutralizing antibody titers, were shown to be critical in controlling long term LCMV dissemination [137-141]. Additionally, NK cell depletion was shown to be dispensable for acute LCMV control [16, 142]. Nevertheless, NK cells were shown to modulate T cell responses and immunopathology upon LCMV infection [23, 143].

1.2.3. **Vesicular stomatitis virus (VSV)**

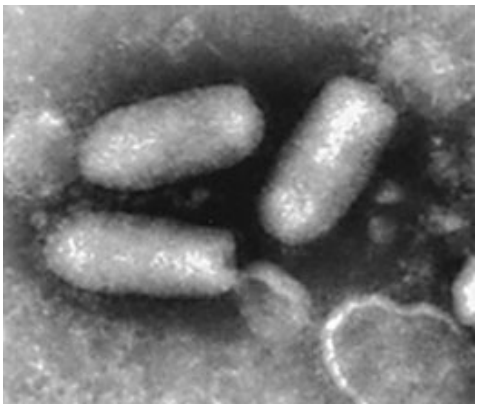


Figure 1. 13 Electron microscopic appereance of VSV (adapted and modified from [144])

Vesicular stomatitis virus (VSV) is an enveloped, bullet-shaped, non-segmented, negative-strand RNA virus belonging to the rhabdovirus family, which also includes the rabies virus (Figure 1. 13). The virion contains two nested, left-handed helices: an outer helix of matrix protein M and an inner helix of nucleoprotein N and RNA. M has a hub domain with four contact sites that link to neighboring M and N subunits, providing rigidity by clamping adjacent turns of the nucleocapsid. Side-by-side interactions between neighboring N subunits are critical for the nucleocapsid to form a bullet shape [145, 146]. The nucleocapsid forms a rigid and high repetitive structure which represents ideal activation conditions for B cells [43].

VSV is a highly cytopathic virus, which cause cytopathic effects as early as 1 to 2 h post-infection. However, VSV is highly sensitive to type I IFNs. Hence, VSV replication in somatic cells, including neurons, was shown to be inhibited by type I IFN at several stages of infection [57, 58, 147].

VSV replicates rapidly, resulting in the release of high numbers of progeny virus from infected cells. Intravenous infection of mice leads to little virus replication in the peripheral tissues. In the absence of a neutralizing antibody response within 6 to 8 days, the virus spread to the central nervous system (CNS) thereby inducing severe paralytic encephalitis. The clinical symptoms are similar to those of human rabies infection; death usually follows within a few days [51, 145].

Upon infection, VSV induces a short-lived helper-T-cell independent neutralizing immunoglobulin M (IgM) response around day 4 post infection. This response is followed by strictly $CD4^+$ T cell dependent neutralizing IgG response. All neutralizing antibodies are exclusively directed toward the glycoprotein (G) of VSV. Hence, mice depleted from either $CD4^+$ T cells or B cells are unable to mount a neutralizing IgG response and succumb to VSV infection. VSV also induces a vigorous CD8+ T cell response, which is mainly directed against the nucleoprotein (NP). However, $CD8^+$ T cells were shown to be dispensable for survival of mice upon VSV infection [51, 138, 148-153].

1.3. Coronin

1.3.1. **Coronin Protein family**

Coronins are proteins which so far, were described in all eukaryotes except plants. Coronins belong to the superfamily of WD40 repeat proteins and were characterized as proteins involved in several actin-dependent processes such as cytokinesis, cell motility, phagocytosis, transcription, vesicle trafficking and signaling [154-161].

The first member of the coronin family was described in 1991 in *Dictyostelium discoideum*, as a 57 kDa protein, which was named coronin, because of its association with crown-shaped cell surface projections of growth phase *D. discoideum* cells. Deletion of coronin in *D. discoideum* resulted in impaired cytokinesis, motility and phagocytosis. Moreover, coronin from *D. discoideum* co-precipitated with actin *in vitro*, which suggested that coronin may act as a cytoskeletal interactor protein [156, 157, 162, 163]. Notably, WD40 repeats, which are able to interact reversibly with multiple other proteins to form complexes, are found in other proteins such as the β subunit of G-proteins, which are transmembrane signal transducer proteins [156, 164-166]. Therefore, it is likely that coronins interact with other proteins, thereby playing a role during signal transduction events.

Coronin from yeast shows 57% similarity with *Dictyostelium* coronin. In addition, *yeast* coronin contains a proline rich central region of a 190 amino acid which is absent in *Dictyostelium* coronin. *In vitro* experiments showed that purified *yeast* coronin interacts with Arp2/3 thereby modulating F-actin polymerization. However, coronin-deficient yeast cells fail to reveal any defect cytoskeleton-related processes [167-169].

1.3.2. **Phylogeny, structure and implication of coronins**

Coronins can be categorized in 12 subfamilies (Table 1. 3). In mammals 7 coronin paralogs were found to be encoded by the genome [155, 161]. Members of the coronin gene family have two highly conserved domains of unknown function (DUF1899, C-terminal and DUF1900, N-terminal).

Table 1. 3 Nomenclatur and the species distribution for the mammalian coronin gene family (adapted and motified from [170, 171])

Protein Name	Synonyme	Species	Tissue expression
coronin 1	Coronin 1A, p57, TACO, ClipinA	vertebrates	Immune and nervous system
coronin 2	Coronin 1B, Coronin	vertebrates	Gastrointestinal system, lung liver, kidney
coronin 3	Coronin 1C, HCRNN4	vertebrates	Brain, liver, muscles, intestines, heart
coronin 4	Coronin 2A, IR10, ClipinB, WDR2	vertebrates	Immune system
coronin 5	Coronin 2B, ClipinC	vertebrates	Brain, ovary, heart
Coronin 6		vertebrates	
Coronin 7	P70, POD-1	Metazoa, fungi, Amoebae	Immune cells

These domains are interspaced by three canonical WD40 domains that form part of a 7-bladed β-propeller scaffold plus a variable "coiled coil domain" responsible for oligomerization and stabilization of the protein (Figure 1. 14A, B) [166, 170-174]. Mammalian coronins can be classified in two classes based on their amino acid sequence length. For instance, coronin 1-6 have three domains (N-terminal domain containing WD40 repeats, C-terminal extension and a unique region, C-terminal domain which forms a coiled coil structure), whereas coronin 7 and the POD-1 protein (a protein implicated in embryonic and neuronal development) from *C. elegans* and *D. melanogaster* contains two core WD-repeat domains rather than on and lack the coiled coil domains [166, 171, 172, 175-177]. Further, biophysical analysis of the C-terminal domain of coronin 1, which fold into a three-stranded coiled coil, showed that coronin 1 forms a trimeric structure [172, 174]. This generates cytoskeletal binding site at the positively charged residues at the linker region [173] (Figure 1. 14C). Additionally, modification of coronins by phosphorylation of serine and tyrosine residues seemed to be common for coronins. These results suggested that coronins link cytoskeleton remodeling and cell signaling [159, 175, 178].

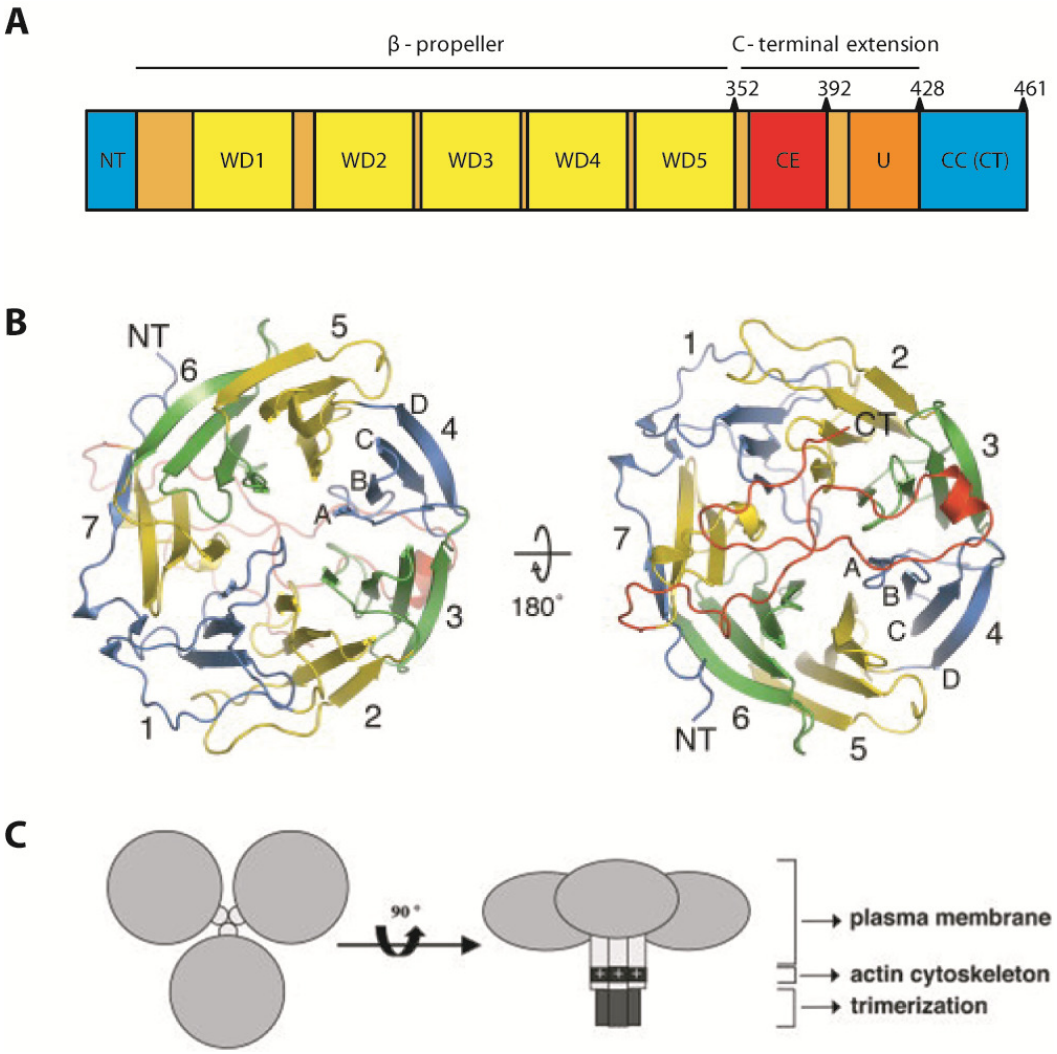


Figure 1. 14 Structure of the murine coronin 1.

A: Schematic domain structure of murine coronin 1. B: The structure can be divided into an N-terminal seven-bladed β propeller (residues 8–352; colored blue, yellow, and green) and a C-terminal extension (residues 353–402; colored red). The last ordered residues of the N and C termini are marked with an "NT" and "CT," respectively. The individual blades are numbered from one to seven; the strands in each blade are named A–D and are labeled in blade 4. C: Coronin 1 is a parallel homotrimeric protein consisting of three globular N-terminal β-propellers (light gray) assembled via the C-terminal coiled coil (dark gray). Association of coronin 1 with the cytoskeleton occurs via a stretch of positively charged residues in the linker region (light gray) and is dependent on trimerization. The F-actin–independent binding to the plasma membrane is mediated via the N-terminal globular β-propeller domain (adapted and modified from [166, 172, 173]).

1.3.3. **Coronin 1 in immunity**

Coronin 1 is the closest homolog to the *Dictyostelium* coronin of all seven mammalian coronin isoforms and is exclusively expressed in cells from haematopoietic origin and the nervous system [160, 179, 180]. The importance of coronin 1 during immunity was discovered when Ferrari et al. were analyzing molecules involved in mycobacterial survival inside the macrophage phagosome [160].

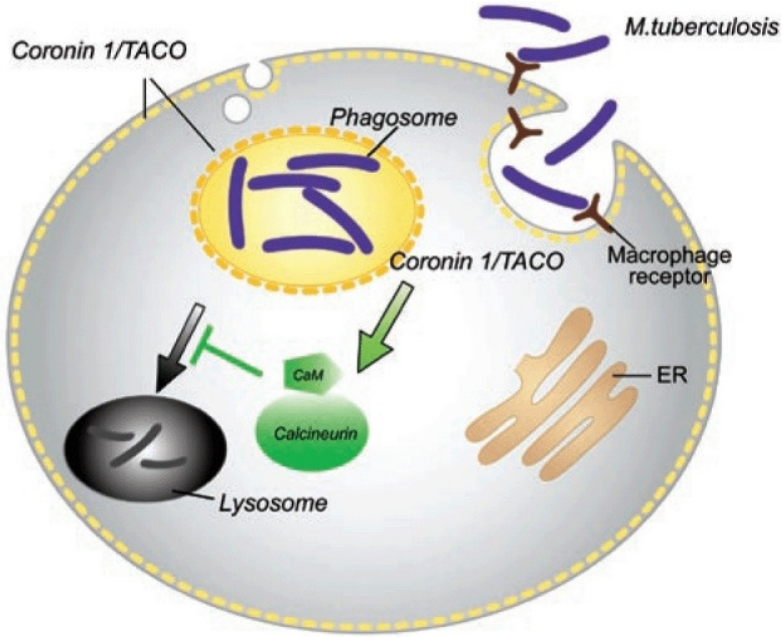


Figure 1. 15 Model for the activity of coronin 1 in macrophages.

In resting macrophages, coronin 1 is distributed in the cytoplasm as well as at the cell cortex. Upon pathogenic mycobacterial entry coronin 1 is actively recruited and retained at the phagosomal membrane, thereby ensuring the activation of calcineurin. Activation of calcineurin results in a block in the fusion of mycobacterial phagosomes with lysosomes. Thus, deletion of coronin 1 or inhibition of calcineurin activity results in the induction of phagosome lysosome fusion and subsequent mycobacterial killing (adapted and modified from [180]).

While most bacteria are internalized via endocytosis by macrophages for further degradation in the lysosome, *Mycobacterium tuberculosis* survives and replicates in the phagosome by preventing phagosomal fusion wth lysosomes [181]. It was found that mycobacteria actively recruit coronin 1 to the phagosomal membrane upon internalization, thereby blocking phago-lysosomal fusion [160]. Further investigations showed that coronin 1 elicits its function by activating the Ca^{2+}-dependent phosphatase calcineurin, leading to a block in lysosomal delivery of mycobacteria (Figure 1. 15). Notably, deletion of coronin 1 in mice had no impact on major cytoskeletal function as for instance phagocytosis, macropinocytosis, cell spreading, membrane, ruffling as well as cell migration [157, 162, 178, 180, 182-188]. Recently it was found that most immune cells such as neutrophils, B cells and Mast cells had no significant defect in the absence of coronin 1 [179, 182, 189, 190]. Interestingly, coronin 1-deficiency was associated with impaired Ca^{2+} mobilization upon TCR triggering with antibodies directed against the CD3 and CD28 molecules [183-185]. While T cells from coronin 1-deficient mice developed normal in the bone marrow and in the thymus, they were rapidly deleted through apoptosis in the periphery. These results

suggested that coronin 1 was important for modulating TCR signaling thereby providing pro-survival signals in naïve T cells. Importantly, coronin 1 was dispensable for T cell survival and chemotaxis in memory T cells. Thus, most T cells in the periphery of coronin 1-deficient mice show a central memory phenotype ($CD44^+$, $CD62L^+$) [183-185]. More recently, coronin 1-deficient mice were shown to be resistant to murine experimental autoimmune encephalomyelitis (EAE), despite normal mobilization of leukocyte subsets in the blood, as well as effector cytokine expression comparable with wild type T cells on polyclonal stimulation [191, 192]. In addition, antigen processing and presentation via the MHC I/II was shown to be independent of coronin 1 [193]. Thus far, the proper function of coronin 1 during immune response and in general still remains elusive.

2. Thesis Project

2. Thesis Project

Coronin 1 was shown to play an important role in peripheral T cell survival, which is dependent on TCR signaling. Hence, coronin 1-deficient mice show low numbers of peripheral T cells. It is proposed that coronin 1 acts downstream of the TCR signal by interacting with PLC-γ1 thereby mediating the generation of inositol-1,4,5-trisphosphate (IP_3). Yet, the precise role of coronin 1 has not been solved.

Viruses are obligate intracellular parasites. Resistance against viral infections is often mediated by T cell dependent mechanisms, either directly by CD8-dependent cytotoxicity, by secretion of antiviral cytokines or indirectly by promoting protective antibody responses. Therefore, we asked whether coronin 1-deficiency is associated with impaired viral control due to impaired TCR signaling.

This Thesis will be divided into two parts. In the first part, the above question will be addressed by investigating the $CD8^+$ and $CD4^+$ T cell response against three well established viral models, namely MCMV, LCMV and VSV.

Primary infection with MCMV is controlled by the concerted action of NK cells, $CD8^+$ T cells and $CD4^+$ T cells. After control of productive viral replication, maintenance of MCMV latency is also dependent on a functional immune system. Next, acute LCMV infection is largely controlled by a vigorous CTL response, whereas long-term control also requires $CD4^+$ T cells and B cells/antibodies. Finally, the immune response to VSV will be investgated. Control of VSV is mainly dependent on an early neutralizing IgG-response, which requires a functional B cell and $CD4^+$ T cells compartment. Hence, generation of neutralizing IgG against the viral surface glycoprotein is essential for mouse survival. In mice, in which IgG production is absent, such as in µMT (IgM heavy chain knockout), $CD40L^{-/-}$ and $MHCII^{-/-}$ mice, VSV invades the CNS leading to lethal paralysis.

In the second part, the functionality of NK cells in the absence of coronin 1 will be investigated. TCR signaling and NK cell receptor signaling is believed to have strong similarities. Therefore, it is important to evaluate the impact of coronin 1 during NK cell activation.

3. Material and Methods

3. Material and Methods

3.1. Animals

C57BL/6 mice and the previously generated transgenic coronin 1-deficient mice [194] were bred in-house and kept under specific pathogen free (SPF) conditions. All animal experiments were conducted according to the regulations of the Cantonal Veterinary Office (Zurich/Basel, Switzerland). Six-twelve weeks old mice were used for all the experiments, unless indicated otherwise.

3.2. Antibodies and Tetramers

Monoclonal antibodies for flow cytometry assays were purchased from Becton Dickinson (Switzerland) or BioLegend (Switzerland) if not indicated otherwise. The following antibodies were used: anti-CD8-Pacific Blue/PerCp/PE (clone 53-6.7), anti-CD4-Pacfic blue/PE/PerCp (clone RM4-5), anti-CD3ε-Pacific blue/PerCp/PE/APC (clone 145-2C11) anti-CD43-PE-Cy7 (clone 1B11), anti-CD44-PE-Cy7(clone IM7), anti-CD62L-PerCp (clone MEL-14), anti-CD27-PE (clone LG.3A10), anti-IFNγ-APC/PE-Cy7/PE (clone XMG1.2), anti-TNFα-PE (clone MP6-XT22), anti-CD107-APC/Brilliat-Violet 421 (clone 1D4B), anti-CD45.1-PE (Ly5.1) (clone A20), mouse V_β TCR screening panel (BD Pharmigen™ cat. No. 557004), anti-FITC-RPE (Polyclonal, AbD Serotec, Cat. No. 640009), PE Annexin V apoptosis detection kit (BD Pharmigen™, Cat. No. 559763). M38-/M45-specific (MCMV) and GP33 specific (LCMV) $CD8^+$ T cells were detected by MHC class I tetramer staining using APC- or PE-conjugated tetramers. **Tetramers:** Tetramers were produced as previously published (Altman et al., 1996). Briefly, inclusion bodies were purified from transformed *E. coli* expressing either the MHC heavy chain H-2Db or H-2Kb, or the β2 microglobulin (β2m). H-2Db or H-2Kb were refolded in the presence β2m and the peptide M45 (H-2Db), M38 (H-2Kb) or GP33 (H-2Db) in a refold buffer (100 mM Tris pH 8.1, 400 mM L-Arginine, 2 mM EDTA, 1 mM β-ME, 5.5 mM Cystamine) at 4°C for 24-72 hours. Protein were then concentrated using a Amicon stirred cell (400 ml, model 8400) and Amicon Ultra 15 ml-centrifugal filter devices (Millipore; Cat. No. UFC901096; 24pk;

regenerated cellulose 10,000MWCO). Protein was then equilibrated in BirA buffer (100 mM Tris pH 7.5, 200 mM NaCl, 5 mM $MgCl_2$) using PD10 desalting columns (GE Healthcare Europe GmbH; Cat. No. 17-0851-01). Next, Leupeptin (1 mg/ml; Sigma; Cat. No. L9783), Pepstatin (0.1 mg/ml; Sigma; Cat. No. P5318), ATP (100 mM; Sigma; Cat. No. A2383), d-Biotin (100 mM; Sigma; Cat. No. B4501) and BirA (0.6 mg/ml; Avidity LLC; Bulk Biotin Protein Ligase, Cat. No. BIRA) was added to biotinylate the refolded pMHC on a table shaker at RT over night. Monomers were then purified using a HiLoad 26/60 Superdex 75 pg column (GE Healthcare Europe GmbH; Cat. No. 17-1070-01) and a basic Aekta module (Pump Unit (P-920); UV-VIS Detector (UPC-900, incl. HG-optics and flow cell 5 mm); Conductivity Unit (pH/C-900); Mixing Unit (M-925); Fraction Collector (FRAC-950); 7-Port Injection valve (INV-907); Connecting Unit (CU-950 USB); GE Healthcare Europe GmbH; Unicorn software (version 5.11)). For further purification by anion exchange chromatography, monomers were equilibrated in a Ion exchange buffer A (20 mM Tris ph 8) and purified using a Mono Q 5/50 GL column (GE Healthcare Europe GmbH; Cat. No. 17-5166-01) and a basic Aekta module. 1 µg/ml Leupeptin and 1 µg/ml Pepstatin was then added to the purified fraction. Protein concentration was then determined in a biotin ELISA. Tetramers were formed by adding Streptavidin-Allophycocyanin conjugate (BD Biosciences; Cat. No. 554067) or Extravidin-R-Phycoerythrin conjugate (Sigma; Cat. No. E4011) in a 3.16:1 (monomer: conjugate) ratio to the monomers and incubated at 4°C over night.

Antibodies for stimulation and blocking were purchased from BioLegend (Switzerland): LEAF purified anti-Ly49D (Clone 4E5), LEAF purified anti-NKp46 (Clone 29A1.4), LEAF purified anti-NK1.1 (Clone PK136), LEAF purified anti-NKG2D (Clone A10), LEAF purified anti-CD16/32 (Clone 93)

3.3. Peptides

MCMV derived peptides $M45_{985\text{-}993}$ (HGIRNASFI, H2-D_b) and $M38_{316\text{-}323}$ (SSPPMFRV, H2-K_b), LCMV-derived peptide $GP_{33\text{-}41}$ (KAVYNFATC, H2-D_b), $NP396_{396\text{-}404}$ (FQPQNGQFI, H2-Db) and peptide $GP_{64\text{-}80}$ (GPDIYKGVYQFKSVEFD, I-Ab) and Vaccinia Virus derived peptide $B8R_{20\text{-}27}$ (TSYKFESV, H-2Kb) were purchased from EMC Microcollections (Tubingen, Germany).

3.4. Viruses

All viruses were provided by Prof A. Oxenius (ETH Zurich, Switzerland). Recombinant MCMV-ΔM157 [195] was propagated on mouse embryonic fibroblasts (MEF), VSV (serotype Indiana) on BHK-21 cells and LCMV strain WE was propagated on L929 fibroblast cells. Depending on the experiment, 200 PFU – 5 x 10^7 PFU of virus was used to infect mice. Mice were infected either intravenously (i.v.) into the tail or subcutaneously (s.c.) into the footpad.

3.4.1. **Virustiter determination**

3.4.1.1. Murine cytomegalovirus

MCMV-titres of virus stocks and organ homogenates were determined by MCMV plaque assay as described in [196]. Briefly, 10^5 MEFs/well were seeded into 24-well tissue culture plates. A 1:10 serial dilution of the sample was prepared in MEM with 2% FCS. After aspiration of the medium from the cells, 200 µl of pre-diluted sample were added to the cells. The plates were centrifuged at 2000 rpm for 30 min at 4°C and subsequently incubated for 1h at 37°C. Supernatant was removed and overlaid with 1 ml Avicel medium (3% Avicel, MEM, 10% FCS, 200 mM Glutamine, penicillin, streptomycin, 10 mM HEPES/$NaHCO_3$,) [197] and incubated for an additional four days at 37°C, 5% CO_2. The overlay was removed and plates were gently washed three times with PBS. Cells were stained and fixed with 1 ml of crystal violet solution (0.5% crystal violet, 1.85% formaldehyde, 50% ethanol, 0.8% NaCl dissolved in water) for 20 min at RT. Plates were washed three times with water and plaques were counted using an inverted microscope (Leitz Labovert FS; Objective: EF-4/0.12)

3.4.1.2. Lymphocytic choriomeningitis virus Focus Forming Assay

LCMV titer was determined as already described [198]. **Plaque assay**: All samples were frozen at -80°C in MEM 2% FCS and 100 U/ml Heparin (only for blood samples) after harvesting to disrupt intact cells. Organs were homogenized with a Qiagen tissue lyzer (45 – 60 sec, 25 Hz, 4°C) and centrifuged for 5 min at 500xg and 4°C to remove cell debris. For each organ or blood sample a 6-fold or a 4-fold 1:10 serial dilution, respectively, was performed in a 96-well plate. 1.6 x 10^5 MC57G cells in 200 µl MEM 2% FCS (8 x 10^5/ml) were

seeded into each well of a 24 well plate. 200 µl of each sample and dilution was added to the 24-well plate, mixed and incubated for 2-4 hours at 37°C, 5% CO_2. 200 µl of a mixture of 2x MEM and 2% methylcellulose were added to the wells and further incubated for 2 days.

Staining: The overlay was discarded and cells were fixed with 200 µl 4% Formalin-PBS for 30 min at RT. Cells were washed 2 x with PBS and permeabilized with 200 µl 1% Triton X – PBS solution for 20 min. Unspecific binding was blocked by incubating cells with 200 µl PBS containing 10% FCS for 60 min at RT. Next, cells were stained with the VL-4 rat anti-LCMV mAb for 60 min at RT. A 1:400 dilution in PBS-1% FCS of a secondary peroxidase – conjugated goat-anti-rat IgG (H^+L) (Jackson ImmunoResearch Laboratories, Inc; cat no. 112-035-003) was incubated for 60 min at RT. After washing, a color reaction was induced for 15-20 min by adding 500 µl OPD Buffer (0.025M $Na_2HPO_4 \cdot 2H_2O$, 0.025M citric acid, 0.4 mg/ml orhto-phenyldiamin, 30% H_2O_2).

3.4.1.3. Vesicular stomatitis virus neutralization assay

Neutralization assay was performed according to [54].Vero cells were resuspended in MEM 5% FCS at a concentration of 3-4 x 10^5 cells /ml. 100 µl (3-4 x 10^4 cells) of the cell suspension was added to each well of a 96-well flat bottom cell culture microtiter plate. Cells were incubated over night at 37°C, 5% CO_2. Mouse sera were collected at the indicated time points. For measuring IgG and IgM responses 10 µl serum was mixed with 10 µl PBS, and for measuring only neutralizing IgG, 10 µl serum was mixed with 10 µl of a 0.1M β-ME in 0.9% NaCl solution and incubated for 60 min at RT. Complement was inactivated by adding 380 µl MEM 5% FCS and incubating the samples for 30 min at 56°C. Next, a 12-fold 1:2 serial dilution of the samples was performed in a 96-well round-bottom microtiterplate. 100 µl of each dilution was subsequently mixed with 100 µl of a 500 PFU/ml stock VSV-Indiana and incubated 90 min at 37°C. The medium of the Vero cell monolayer was removed and 100 µl of the antibody-VSV mix was then transferred to the cells and incubated for 60 min at 37°C, 5% CO_2. To each well 100 µl of a RT pre-warmed 2 x MEM 10% FCS, 2% Methylcellulose was added and incubated for 1 day at 37°C, 5% CO_2. 100 µl of 4% Formalin was added to each well for 30 min to inactivate the virus. The medium was discarded and 150 µl 0.5% crystal violet solution was added to the wells and incubated for 45 min. The stain removed and the wells were washed 5 times under running tap water. The highest dilution of samples that

reduces the number of plaques by 50% was then recorded and expressed as neutralizing titre x –log2. Unreduced samples (IgG $^{+}$ IgM) are taken as IgM titer only if the corresponding reduced samples (IgG only) had at least 4-fold lower titer.

3.5. Buffers and Media

Buffers were prepared in endotxin free ddH_2O. The pH was adjusted by addition of 1 M NaOH or 0.5 M HCl.

PBS

NaCl (10 mM)	7.02 g
$HNa_2PO_4 \cdot 12H_2O$ (50mM)	17.9 g
ddH_2O	1 l
pH	8

EDTA stock

EDTA (0.5M)	186.12 g
ddH_2O	1 l
Ad ~20g NaOH and adjust to pH 8	
Autoclave	

MACS-buffer

PBS	980 ml
FCS (2%)	20 ml
From EDTA Stock (2mM)	4 ml
Filter sterilize	

ACK-buffer

NH_4Cl (155 mM)	8.29 g
$KHCO_2$ (10 mM) or (K_2CO_3)	1 g or (1.38 g)
From EDTA Stock (0.1 mM)	2 ml
ddH_2O	1 l
pH	7.4
Filter sterilize	

FACS-buffer

PBS	970 ml
FCS (2%)	20 ml
From EDTA stock (5mM)	10 ml
NaN_3 (0.05%)	0.5 g

4% Paraformaldehyde (50 ml)

PFA (Aldrich Cat. No. 15.812-7)	1.5 g
PBS	50 ml
$CaCl_2$ (0.1 mM)	5 µl
$MgCl_2$ (0.1 mM)	5 µl
pH	7.5
Filter sterilize	

0.5% Crystal violet

96% EtOH	500 ml
Crystal violet	5 g
NaCl	8 g
37% Formalin	50 ml
ddH_2O	450 ml

Carbonate buffer

Na_2CO_3 (29 mM)	0.303 g
$NaHCO_3$ (71 mM)	0.6 g
ddH_2O	100 ml
pH	9.5

Cell culture media

RPMI 1640 (Invitrogen AG; Cat. No. 31870-025)

MEM (Invitrogen AG; Cat. No. 21090)

10x DMEM (Sigma; Cat. No. D2429)

Red HBSS (Invitrogen AG; Cat. No. 24020-091)

HBSS without phenol red (homemade; NaCl (137 mM), KCl (5.4 mM), $Na_2HPO_4 \cdot 2H_2O$ (0.34 mM), KH_2PO_4 (0.44 mM), $MgCl_2 \cdot 6H_2O$ (0.5 mM), $MgS0_4.7H_2O$ (0.4 mM), $CaCL_2$ (1.3 mM), $NaHCO_3$ (4.2 mM), D(+)-Glucose Monohydrat (5 mM), pH 7.2)

3.6. Splenectomy

Splenectomy was modified from [199, 200]. One day before until 6 days after splenectomy mice received analgesia with buprenorphin (Temgesic®, Essex Chemie AG) into the drinking water. During the procedure, mice were anaesthetized with Isoflurane. After the surgical level of anesthesia was reached, the lower back of the mouse was shaved and disinfected with 70% ethanol. After a small transverse incision of the skin below the last rib, the peritoneal cavity was opened and the spleen was gently pulled out with sterile forceps. The gastro-splenic ligament was detached, the splenic artery and vein were ligated with a single knot and the spleen was entirely removed. The peritoneal cavity was sewed with a single stitch and the skin was closed with wound clips. During the following week, mice were checked daily for signs of pain or post-surgical complications. After 7-10 days of recovery mice entered the subsequent experiment.

3.7. Footpad swell measurements

The swell of the footpad was measured with a caliper. Footpad diameter was monitored starting at day 2 and up to day 17 post infection.

3.8. Cryosection and Hämalaun – Eosin Staining

Footpads were freezed in O.C.T^{TM} compound (Polyvinyl Alcohol <10%, Carbowax <5%, non-reactive ingredients >85%) at -80°C. 10-15 µm thin sections were cut at -20°C in a cryostat and stored on a glass slide at -80°C. Sections were fixed with 4% PFA in PBS for 60 min at room temperature. Slides were washed with water and stained 5 min in Mayers-Hämalaun solution (Merck; Cat. No. 109249). After rinsing with water slides were stained for 1 min with 0.5% Eosin. Slides were washed with water and rinsed with increasing concentration of EtOH. Sections were then washed 3 min with xylol and overlaid with Entellan neu® (Merck; Cat. No. 107961).

3.9. Cell isolation

3.9.1. Isolation of lymphocytes from lymphoid tissue

Thymus, spleen and lymph nodes were harvested in ice cold RPMI supplemented with 5% FCS and 200 mM glutamine, penicillin, streptomycin and smashed through a grid of stainless steel. Cell debris were removed by quick spin (300xg, 10 s). Depending on the experiment, the cell suspension was treated with 1 ml ACK buffer to lyse erythrocytes or left untreated to sort cells for T cells using an untouched T cell enrichment kit (Stemcell technologie, Easy sep). Cells were counted with a Neubauer counting chamber. Dead cells were excluded by using trypan blue.

3.9.2. Lymphocyte isolation from lung and liver

Mice were anaesthetized by injecting 250 µl (for a 20 g mouse) to 300 µl of a Xylazin (Bayer), Ketamin (Graeub), Acepromazin (Arovet AG) solution i.p. or euthanized with CO_2. After

perfusing the mice with 5-10 ml ice cold PBS via the right heart ventricle to remove all contaminating blood, the organs were harvested and cut in small pieces and digested with 3 ml of an enzyme cocktail containing 2.4 mg/ml Collagenase (Gibco, Cat. No. 17100-017) and 0.2 mg/ml DNase I (Roche, Cat. No. 1 284 932) dissolved in RPMI supplemented with 10% FCS for 30 min at 37°C, 5% CO_2. After tissue disruption, by pulling the sample through an 18G needle, the tissue was further digested for 20 min by adding 2 ml of fresh enzyme cocktail. Cell suspension was then homogenized through a 70 µm cell strainer (BD, Falcon) and washed twice with PBS. Lymphocytes were then resuspended in 90% Percol (0.15 M NaCl) and further isolated by a Percol (Amersham Biosciences Europe GmbH; Cat. No. 17-0891-02) gradient (overlaid by 37% Percol in Red HBSS followed by 30% in HBSS w/o Phenol red) centrifugation. Erythrocytes were then lysed by adding 1 ml ACK buffer for 3 min at 4°C. Cells were counted with a Neubauer counting chamber. Dead cells were excluded by using trypan blue.

3.10. FACS Staining

Blood (50-100 µl) or 0.5 – 2 x 10^6 cells isolated from organs were stained with fluorochrome conjugated monoclonal antibodies for 20 min at 4°C. Tetramer staining was performed for 20 min at 4°C or 37°C. Using BD FACS Lysing Solution or 2% PFA, cells were fixed for 10 min in the dark at RT. Cells were then washed and resuspended in 100 – 200 µl FACS buffer for analysis. Samples were measured with a 6-8 colour BD FACS Canto II Flow Cytometer using FACS Diva software. The data files were analyzed with Flowjo version 7.5.2.

3.11. Intracellular cytokine staining

5 x 10^5 – 2 x 10^6 lymphocytes were resuspended in 200 µl RPMI supplemented with 10% FCS. 200 µl RPMI, 10% FCS containing either Brefeldin A (20 µg/ml, Sigma; Cat. No. B7651) or Monensin (4 µM, Sigma; Cat. No. M5273) and 1 x 10^{-6} M peptide, if not indicated otherwise, was added to the cells. Depending on the experiment 1 µg of fluorescent dye conjugated anti-mouse CD107a antibody was added. Cells were incubated for 4.5 – 6 hours at 37°C, 5% CO_2. Cells were washed with FACS-Buffer and stained for surface markers for 20-30 min at

4°C. NK cells were treated prior to surface staining for 5 min at 4°C with a blocking anti-mouse CD16/32 antibody. Cells were then fixed and permeabilized with BD-FACS-lysing solution containing 0.05% Tween20 (for T cells) or 2% PFA in FACS buffer followed by permeabilization with 0.5% Saponin in FACS buffer (for NK cells) for 10 min at RT in the dark. After washing cells were stained intracellularly for IFNγ, TNFα or IL2, depending on the experiment for 20 – 30 min. After washing, samples were measured with a 6-8 colour BD FACS Canto II Flow Cytometer using FACS Diva software. The data files were analyzed with Flowjo version 7.5.2.

3.12. NK cell activation studies

3.12.1. **NK cell stimulation via antibody coated wells**

Maxi-sorp 96-well plates (Nunc; Cat. No. 439454) were coated at 4°C over night with anti-NK1.1 (5 µg/ml), anti-NKp46 (5 µg/ml), anti-Ly49D (5 µg/ml) or anti-NKG2D (10 µg/ml) antibodies in 200 µl carbonate buffer. The next day, plates were washed twice with PBS and 0.5-1 x 10^6 splenocytes in 200 µl RPMI, 10% FCS, 10 mM HEPES and 50 µM β-ME were added to the wells in the presence or absence (+ 1 µg anti-CD107a-Brilliant Violet 421) of Brefeldin A. Cell were cultured at 37°C for 4.5 hours prior staining for NK cell and IFNγ.

3.12.2. **^{51}Cr – cytotoxic assay**

Freshly isolated splenocytes or purified NK cells from spleen were either directly used or according to [201] treated for 20-22 hours with Con A (2.5 µg/ml), IL15 (10 ng/ml, Biolegend; Cat. No. 566302) or medium alone in a round bottom 96-well cell culture microtiterplate and used as a source of effector cells. YAC-1 cells were cultured in RPMI supplemented with 10% FCS and 200 mM L-Glutamine. 2 x 10^7 YAC-1 cells were loaded with 300 µCi $Na_2{}^{51}CrO_4$ (1 mCi/ml in PBS, Hartmann Analytic) for 90 min on a 37°C thermoshaker. YAC-1 cells were subsequently washed twice with PBS and resuspended at a concentration of 5 x 10^5 cells/ml RPMI, 10% FCS and used as target cells. 100 µl of the YAC-1 cell suspension (5 x 10^4 cells) was then added to the pre-titrated splenocytes to obtain the indicated splenocyte or effector : target ratio. Cell mixture was then incubated for 4.5 hours at 37°C, 5% CO_2. Plates were

centrifuged for 5 min at 500xg and 40 µl of supernatant was then transferred and mixed with 200 µl liquid scintillator. CPM was then counted in a Packard TopCount Microplate scintillation counter. Specific cell lysis was calculated as follow: (experimental release – spontaneous release) / (maximum release – spontaneous release).

3.13. NK cell survival

Freshly isolated splenocytes or purified NK cells were cultivated in the presence of Con A (2.5 µg/ml), IL15 (10 ng/ml) or medium alone (RPMI; 10% FCS, 200 mM L-Glutamine) at 37°C, 5% CO_2 for 4, 10 and 20 hours. At the indicated time points remaining cells were counted with a Neubauer counting chamber and subsequently stained for CD3, NK1.1, Annexin V and 7-AAD. Cells were then analyzed using a BD FACScanto II Flow Cytometer using FACS Diva software. The data files were analyzed with Flowjo version 7.5.2.

3.14. Statistical analysis

Non-parametric one-tailed or two-tailed Mann-Whitney t-test was used for group comparisons using Graph Pad Prism (GraphPad Software, La Jolla, CA). The p-values are indicated in the figure legends.

4. Results: Part one

T cell immune response in coronin 1-deficient mice after viral infection

Most of the results described in this chapter are part of the following paper:

Vincent S. Tchang, Andrea Mekker, K. Siegmund, Urs Karrer and Jean Pieters

Diverging role for coronin 1 in antiviral CD4$^+$ and CD8$^+$ T cell responses *(Mol. Immunology accepted)*

4. Results

4.1. Part one: T cell immune response in coronin 1-deficient mice after viral infection

4.1.1. Introduction

Coronin 1 is a member of the family of evolutionary conserved WD40-repeat proteins [154]. In the unicellular slime mold *Dictyostelium discoideum*, which expresses a short (coronin 12, corA) and a long coronin (coronin 7, corB) isoform, coronin was shown to be involved in a variety of activities such a cell migration and cytokinesis [157, 202]. In mammals, 7 coronin molecules are encoded by the genome [170]. Of these, coronin 1 is the most conserved coronin protein (also known as coro1a, TACO (tryptophan-aspartate containing coat protein) or p57). Coronin 1 is a 57 kD protein containing 5 WD repeats being part of a 7 bladed β-propeller at its N-terminal domain [172, 173]. Interestingly, coronin 1 is predominantly expressed in cells of hematopoietic origin [160, 203]. Coronin 1 was initially defined as a molecule that prevents the intracellular degradation of *Mycobacterium tuberculosis* within infected macrophages, by blocking phagosome lysosome fusion [160]. Recent work showed that coronin 1 exerts this activity by modulating Ca^{2+} mobilization upon mycobacterial entry within macrophages, thereby activating the Ca^{2+} dependent phosphatase calcineurin [194].

Recently, coronin 1-deficiency was associated with a defect in Ca^{2+} mobilization after T cell receptor (TCR) triggering and with a profound peripheral T cell deficiency due to a block in thymic T cell egress leading to severe T cell lymphopenia in blood and secondary lymphoid organs. Most remaining peripheral T cells express a central memory ($CD44^{hi}$ $CD62L^{lo}$) phenotype in naïve coronin 1-deficient mice [184]. Moreover, coronin 1 was shown to play an important role for naïve T cell survival and migration [183, 204]. Nevertheless, coronin 1-deficient mice are capable to mount specific antibody responses after immunization although somewhat delayed for T cell dependent antigens [189]. Together, these results suggest a profound defect in T cell mediated immunity in coronin 1-deficient mice.

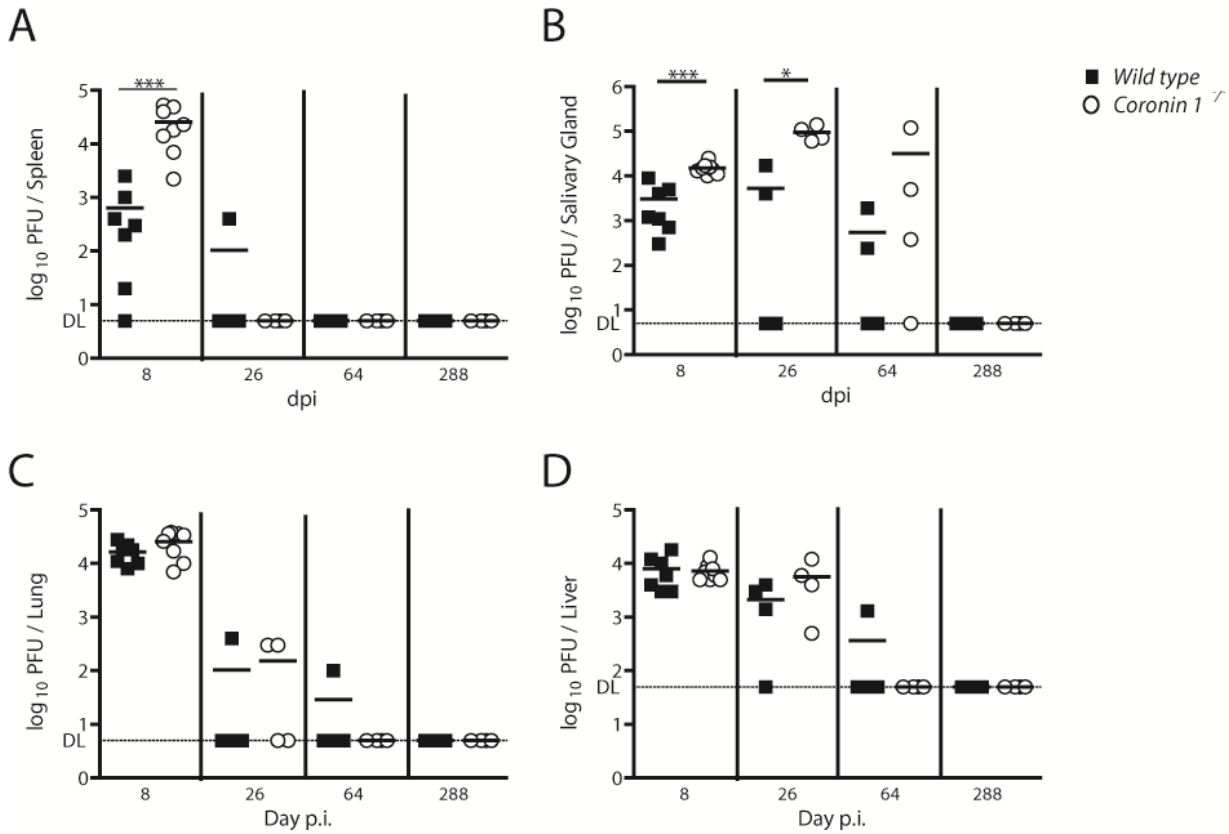


Figure 4. 1 Kinetics of MCMV clearance in wild type and coronin 1-deficient mice.

Wild type or coronin 1-deficient and wild type C57Bl/6 mice were infected with $2x10^6$ PFU MCMV-ΔM157 intravenously. Spleen (A), salivary gland (B), lung (C) and liver (D) were harvested at the indicated time points and viral titers were determined by plaque assay as described [196]. P values (***p<0.0007; *p<0.03) were calculated by using the two-tailed Mann-Whitney t-test. Circles and squares indicate individual mice; horizontal lines represent the mean of each group; DL: Detection limit.

Control of many viral infections is highly dependent on antiviral T cell immune responses. Until now, it is not known if coronin 1-deficiency is associated with impaired viral control. Therefore, we investigated the T cell immune response to viral infections in coronin 1-deficient mice with three well established murine viral models, namely Murine cytomegalovirus (MCMV), Lymphocytic choriomeningitis virus (LCMV) and Vesicular stomatitis virus (VSV). First, MCMV is a persistent herpes virus which induces specific memory $CD8^+$ T cell inflation [117]. Control of acute infection is mainly $CD8^+$ T cell dependent [117, 205]. However, $CD4^+$ T cell are required MCMV clearance in the salivary gland and help to prevent reactivation [110]. Second, acute LCMV induces a strong $CD8^+$ T cell dependent immune response which is required for viral control [135]. Also, infection with LCMV leads to a $CD4^+$ T cells and B cells dependent long term viral control [137, 138, 148]. Third, primary VSV infection is highly dependent on thymus dependent antibody class

switch. $CD4^+$ T cell or B cell deficiency leads to poor viral control which results in viral CNS invasion and paralysis [51, 148, 153].

Our results indicate that specific $CD8^+$ T cell responses in coronin 1-deficient mice were only marginally affected after MCMV and LCMV infection. Hence, the specific $CD8^+$ T cell response was comparable in coronin 1-deficient and wild type mice. Furthermore, peptide re-stimulation of MCMV and LCMV specific $CD8^+$ T cells were comparable between wild type and coronin 1-deficient animals, suggesting a coronin 1-independent mechanism for T cell activation via the TCR. Even more, in coronin 1-deficient mice $CD8^+$ T cell numbers increased to a number comparable to wild type $CD8^+$ T cells. However, $CD4^+$ T cells from coronin 1-deficient animals were not able to increase to a number comparable to wild type $CD4^+$ T cells. Moreover, acute $CD4^+$ T cell responses were strongly impaired during acute LCMV and VSV infection. Furthermore, coronin 1-deficiency was associated with high lethality after VSV infection. We conclude from our data that coronin 1 is more important for acute $CD4^+$ T cell immune responses than for $CD8^+$ T cell immune responses. Taken together, we showed that coronin 1 modulates T cell response in $CD8^+$ and $CD4^+$ T cell differently after viral infections suggesting a different requirement of intracellular components, especially coronin 1, during TCR signaling.

4.1.2. **Results**

4.1.2.1. Expansion of T cells after MCMV infection

Following infection with MCMV, acute virus control is dependent on NK cell and $CD8^+$ T cell responses [117, 206, 207], whereas $CD4^+$ T cells are required for long term viral control [110, 208]. Thus activation and clonal expansion of peripheral T cells is highly dependent on the interaction with MHC class I/II presenting antigen to the TCR [209]. Since TCR signaling depends on coronin 1 [184], we asked whether coronin 1-deficiency was associated with impaired viral control. For our experiments we used a recombinant MCMV lacking the gene M157, which encodes a stimulating ligand for the NK cell receptor Ly49H. Therefore, control of MCMV-ΔM157 is less dependent on NK cells and more on $CD8^+$ T cells [22, 110].

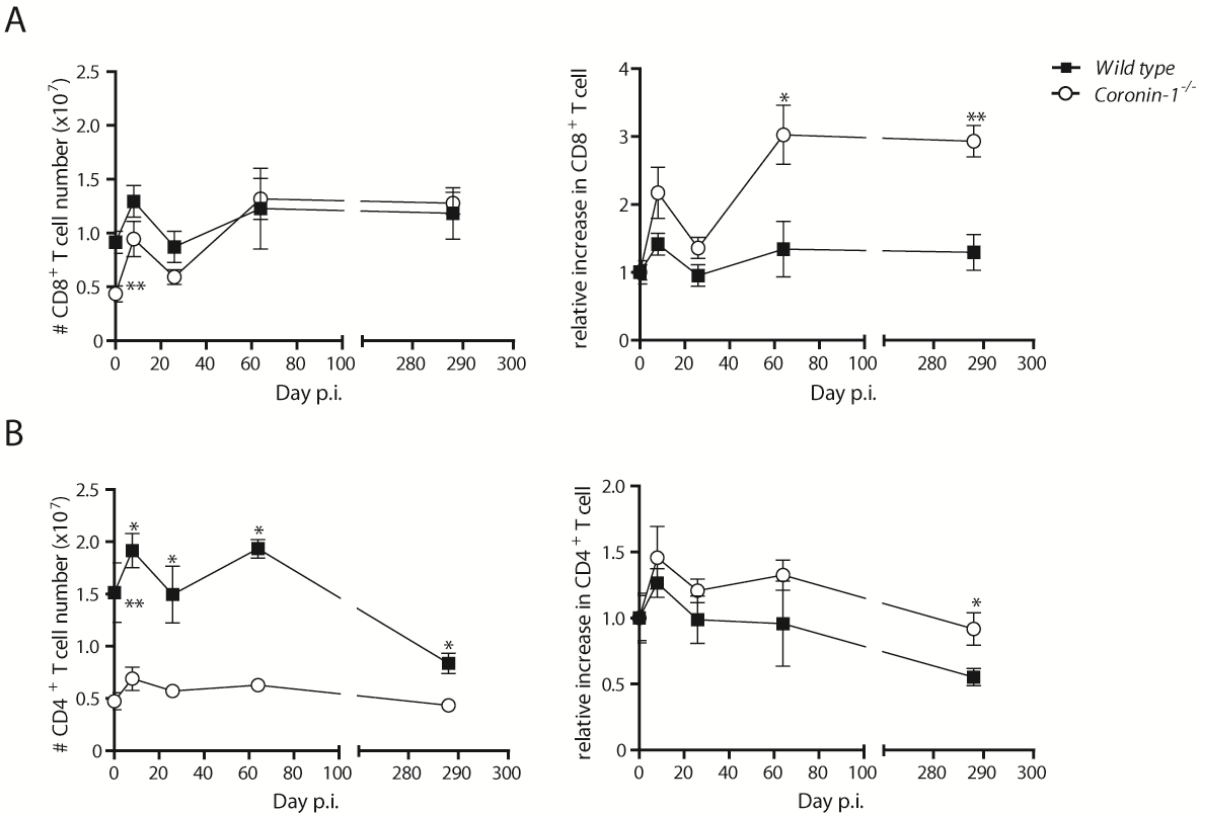


Figure 4. 2 Expansion of peripheral T cells in wild type and coronin 1-deficient mice after MCMV infection.

Splenocytes from MCMV infected mice were harvested at the indicated time points and counted and cells were stained for CD8 (A) or CD4 (B). Absolute numbers were calculated from the FACS data. P values (**p<0.008; *p<0.03) were calculated by using the one tailed (n=3-5). Error bars represent the SEM.

To this end, mice were intravenously infected with $2x10^6$ PFU MCMV-ΔM157. At the indicated time points, MCMV-titers were measured in different organs. As shown in Figure 4. 1, MCMV-titers were significantly higher in spleen and salivary gland on day 8 post infection. However, mice were able to control MCMV by day 26 in the spleen and day 288 in the salivary gland (Figure 4. 1A, B). While viral control was slightly delayed in the spleen and in the salivary gland in the absence of coronin 1, coronin 1-deficient mice were able to control MCMV by day 64 in the lung (Figure 4. 1C) and liver (Figure 4. 1D), which was comparable to wild type mice.

T cells are important for controlling primary viral infection and therefore we investigated the capacity of peripheral T cells to respond to the MCMV infection. For this, we infected coronin 1-deficient and wild type mice with $2x10^6$ PFU MCMV-ΔM157 and harvested the spleens at the indicated time points, and stained for CD8 and CD4, (Figure 4. 2). We show, in line with previous publications [183-185], that naïve coronin 1-deficient mice have

significantly lower $CD8^+$ and $CD4^+$ T cell number in the spleen. However, as shown in Figure 4. 2A, we found that the $CD8^+$ T cell subset expanded upon infection. Even more, $CD8^+$ T cell numbers from infected coronin 1-deficient mice increased to a number comparable to wild type mice. In contrast, determination of the $CD4^+$ T cell numbers from coronin 1-deficient mice showed only a slight increase upon infection (Figure 4. 2B) and these $CD4^+$ T cells did not expand to a number comparable to wild type $CD4^+$ T cells. Nevertheless, relative increase in $CD4^+$ T cell number was comparable in coronin 1-deficient and wild type animals. In addition, differences in expansion of $CD8^+$ and $CD4^+$ T cell were visualized by plotting the CD8:CD4 T cell ratio in the blood (Suppl. Figure 6. 4). However, overall the absence of coronin 1 resulted in a functional T cell response to ensure peripheral MCMV control.

4.1.2.2. Specific $CD8^+$ T cell response after MCMV infection

MCMV infection induces a strong $CD8^+$ T cell response which is directed against several viral proteins (e.g. M38, M45). The $CD8^+$ T cell response against the immuno-dominant epitope M45 exhibits a typical kinetic of rapid expansion peaking around day 8, followed by contraction and low level long term memory [118, 210]. On the other hand, the $CD8^+$ T cell response against M38 is subdominant during the acute infection phase, but then accumulates over time during a process called memory inflation leading to prominent effector memory T cell population late after MCMV infection. M38 specific memory T cell inflation was shown to be dependent on antigen presentation by non-hematopoietic cells [117, 211]. Moreover, it was shown that continuous re-stimulation of central memory $CD8^+$ T cells in the lymph nodes by infected non-hematopoietic cells ensured the maintenance of a functional effector $CD8^+$ T cell pool in the periphery, providing protection against viral reactivation events [211].

To analyze whether coronin 1-deficient mice were able to a MCMV specific T cell response and whether specific T cells were able to expand in peripheral tissue such as the lung, we infected wild type and coronin 1-deficient mice with $2x10^6$ PFU MCMV-ΔM157 and monitored the M38 and M45 specific T cell immune response in the blood, spleen and in the lung by tetramer staining over time (Figure 4. 3). In coronin 1-deficient mice the expansion of M45 specific T cell was delayed by 1 day compared to MCMV infected wild type mice.

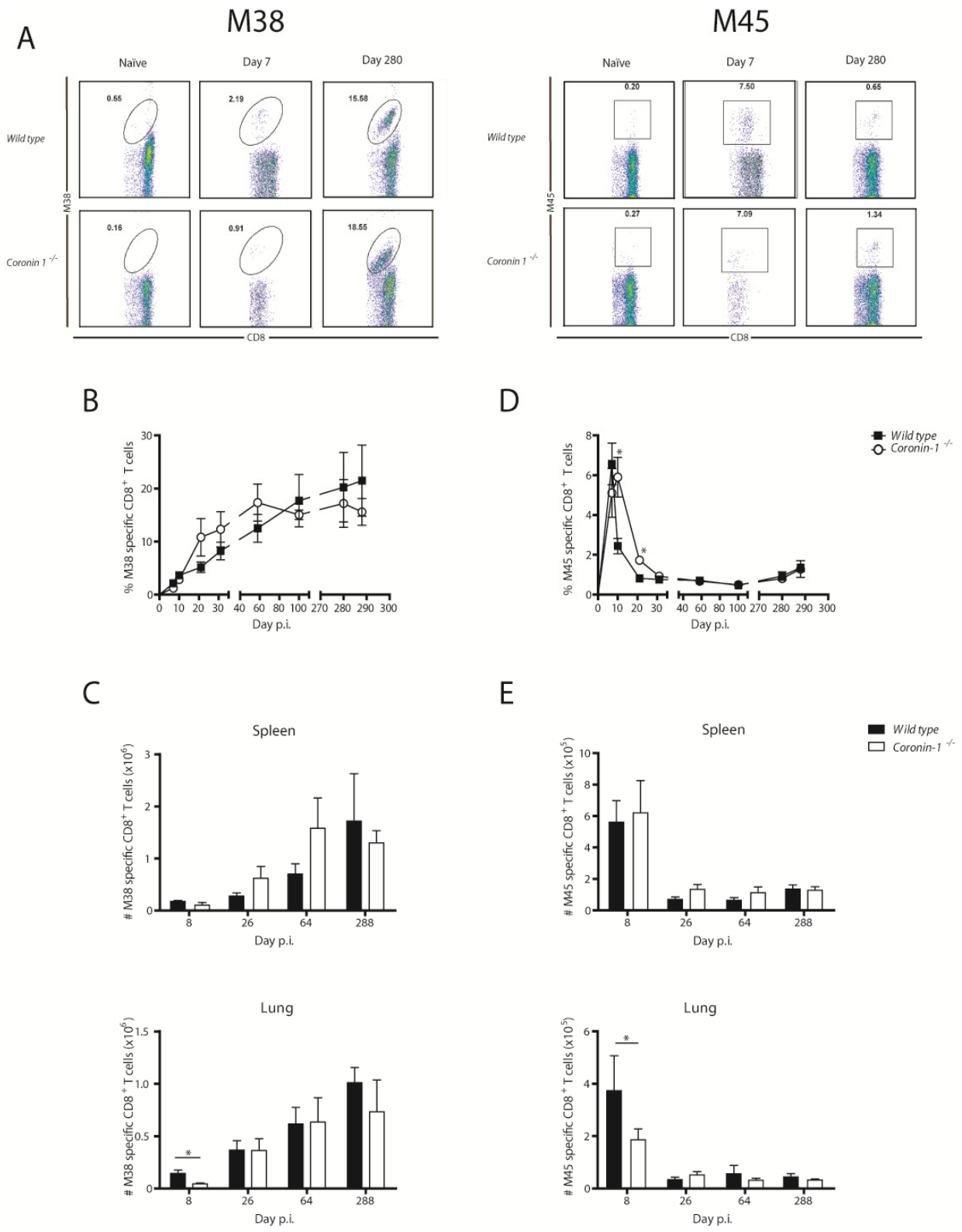


Figure 4. 3 Specific CD8$^+$ T cell response after MCMV infection.

Mice were infected intravenously with 2x10^6 PFU MCMV-ΔM157. Mice were bled or sacrificed at the indicated time points post infection. Organs were harvested and stained for CD8 and APC-conjugated tetramer M38 or M45. A: Representative FACS dot-plot from whole blood from coronin 1-deficient and wild type mice after infection with MCMV-ΔM157. B: Frequency of M38 specific CD8$^+$ T cells in the blood. C: Absolute M38-specific T cell numbers in the spleen lung after MCMV infection. D: Frequency of M45-specific CD8$^+$ T cells in the blood. E: Absolute M45-specific T cell numbers in the spleen lung after MCMV infection. Cells were counted and M38- or M45-specific CD8$^+$ T cell numbers were calculated from the FACS data. P values were calculated by using the two-tailed Mann-Whitney t-test. Error bars represent the SEM (n=4-5; *p<0.03).

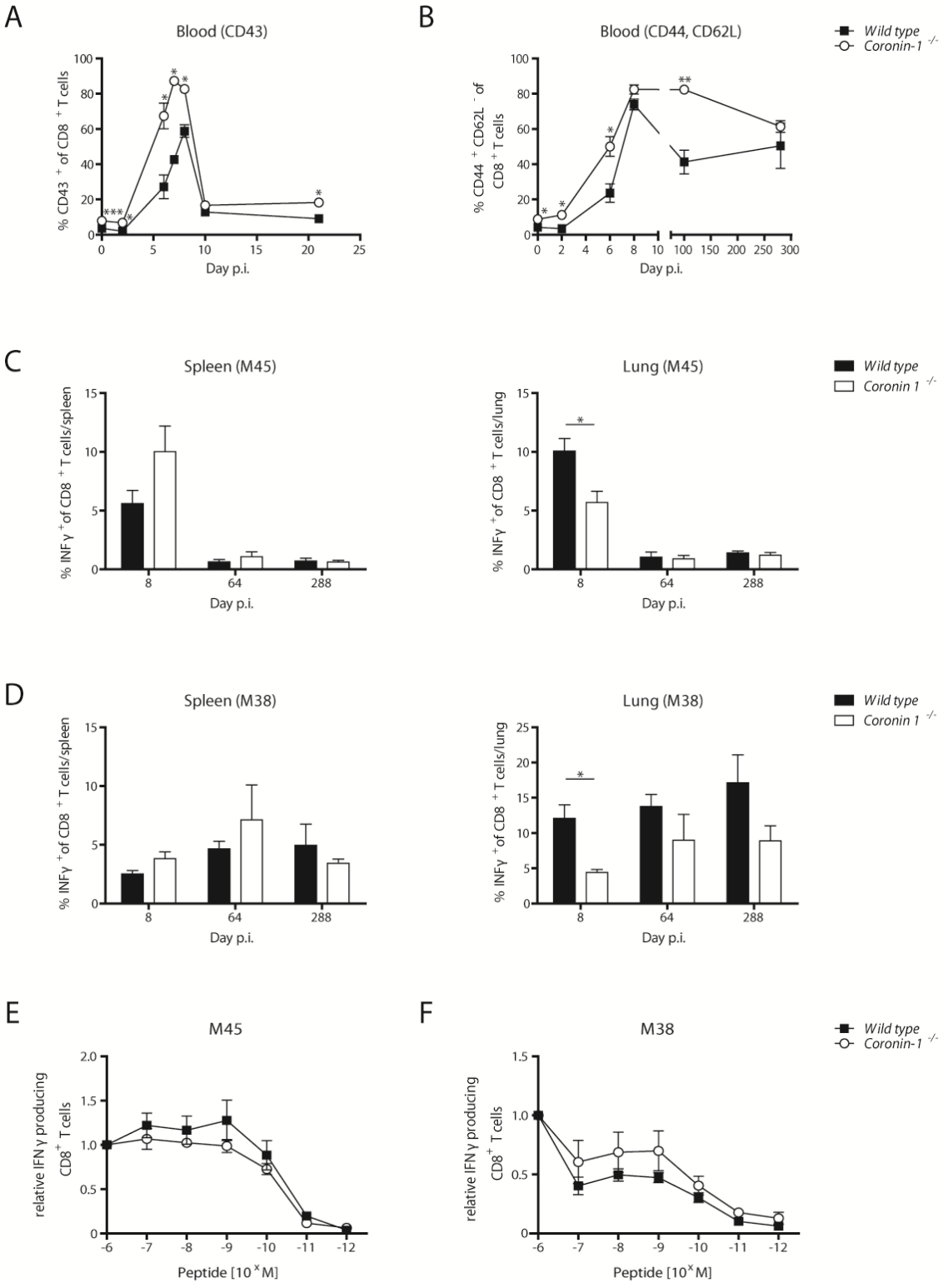


Figure 4. 4 Activation and functionality of T cells after MCMV infection

Coronin 1-deficient or wild type mice were infected with $2x10^6$ PFU MCMV-ΔM157 i.v. and sacrificed at the indicated time points. A, B: Blood were harvested and analyzed for CD43, CD44 and CD62L expression on $CD8^+$ T cells. A: Kinetic of CD43 expression on total $CD8^+$ T cells. B: Kinetic of $CD44^+$ $CD62L^-$ $CD8^+$ T cell on total $CD8^+$ T cells. C, D: Lymphocytes from spleen or lung were re-stimulated with the M45 (C) or M38 (D) peptide in the presence of Monensin for 6 hours and subsequently stained for CD8 and intracellularly for IFNγ. E, F: At day 8 p.i. splenocytes were isolated and stimulated with the indicated concentration of MCMV peptide M45 (E) and M38 (F) in the presence of Monensin for 6 hours. Cells were subsequently stained for CD8 and intracellularly for IFNγ. Results were normalized to the value of highest peptide concentration. P values were calculated by using the two-tailed Mann-Whitney t-test. Error bars represent the SEM (n=3-15; *p<0.03, **p<0.008, ***p<0.0001).

However, the contraction phase and formation of a M45 specific memory $CD8^+$ T cell pool was similar in wild type and coronin 1-deficient mice (Figure 4. 3A, D, E). On the other hand, the M38 specific $CD8^+$ T cell numbers were found to be reduced in the lung but not in the spleen at day 8 post infection (Figure 4. 3C). Nevertheless, M38 specific $CD8^+$ T cell numbers increased to the same extend in wild type and coronin 1-deficient mice. Overall, the kinetics with continuous accumulation of M38 specific $CD8^+$ T cells over time in the blood, spleen and lung was comparable between wild type and coronin 1-deficient mice (Figure 4. 3A, B, C).

Since numbers and frequencies of MCMV specific $CD8^+$ T cells were comparable between wild type and coronin 1-deficient mice after MCMV infection, we next evaluated whether the phenotype and functional characteristics of coronin 1-deficient T cells differed from wild type T cells. Therefore, we infected wild type and coronin 1-deficient mice with $2x10^6$ PFU MCMV-ΔM157 and harvested blood, spleen and lung to monitor activation and functionality of the T cells in the organs at the indicated time points post infection (Figure 4. 4). As we analyzed $CD8^+$ T cell activation in the blood, we found that naïve coronin 1–deficient mice had a slightly increased T cell frequency with an activated and effector memory phenotype, as measured by CD43 (Figure 4. 4A and Suppl. Figure 6. 3) or CD44, CD62L (Figure 4. 4B), compared to T cells from naïve wild type mice. Moreover, frequency of activated T cells increased up to 80-90% by day 8 post infection in the absence of coronin 1 (wild type 60-80%). Interestingly, whereas CD43 expression dropped down to basal level by day 21 in wild type and coronin 1-deficient $CD8^+$ T cells, we could measure increased frequency of effector memory ($CD44^+$, $CD62L^-$) $CD8^+$ T cells in the blood of coronin 1-deficient mice, which was maintained up to day 100 post infection (Figure 4. 4B). However, by day 288 frequencies of effector memory $CD8^+$ T cells were found to be similar in wild type and coronin 1-deficient mice.

Next, we investigated whether the T cells were functional in the absence of coronin 1. For this, we harvested and isolated lymphocytes from spleen and lung after MCMV infection. After re-stimulation of lymphocytes with the MCMV peptide M38 or M45 we measured the frequency of IFNγ producing $CD8^+$ T cell by intracellular cytokine staining (ICS) (Figure 4. 4C, D).

A

	LCMV positive mice (PFU/ml Blood)					
	Day 4	Day 8	Day 12	Day 41	Day 75	Day 103
Wild type	0/6	0/6	0/6	0/6	0/6	0/3
Coronin $1^{-/-}$	0/6	1/6 (4500)	1/6 (550)	0/6	0/6	0/4

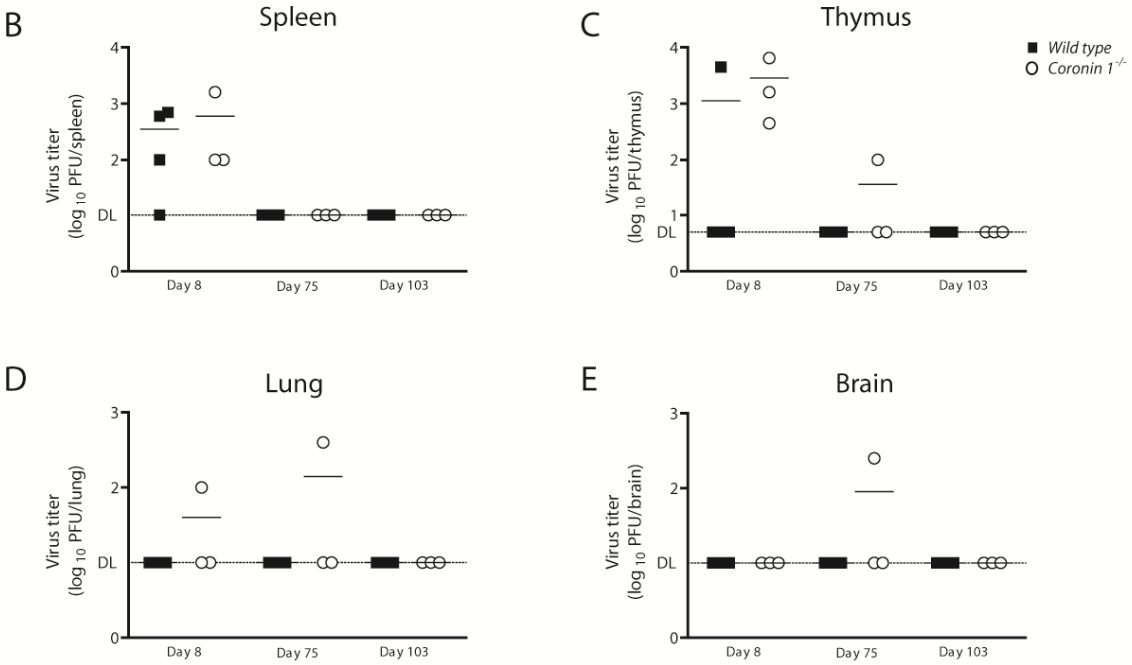


Figure 4. 5 Kinetics of LCMV clearance in wild type and coronin 1-deficient mice.

Wild type (■) and coronin 1-deficient (o) mice were infected with 200 PFU LCMV-WE i.v.. Blood (A), spleen (B), thymus (C), lung (D) and brain (E) were harvested at the indicated time and virus titer was determined by a LCMV focus forming assay as described by [198]. The mean of the viral titer is depicted for each experiment. DL, detection limit.

Our results show clearly that, despite a initial reduced frequency of IFNγ producing $CD8^+$ T cells, after *ex vivo* re-stimulation with M45 (Figure 4. 4C) or M38 (Figure 4. 4D), in the lung at day 8 post infection, the kinetic and functionality of coronin 1-deficient $CD8^+$ T cells were comparable to wild type $CD8^+$ T cells in the spleen and lung. Moreover, similar results were obtained by measuring CD107a upregulation, a marker for degranulation (not shown). In addition, functional avidity against M45 (Figure 4. 4E) and M38 (Figure 4. 4F) was found to be similar between wild type and coronin 1-deficent $CD8^+$ T cell at day 8 post infection.

Taken together, we conclude from these data that $CD8^+$ T cells can become activated and are functional upon peptide re-stimulation in the absence of coronin 1. These results imply that TCR signaling in MCMV specific $CD8^+$ T cells is taking place in a coronin 1-independent manner.

4.1.2.3. T cell responses and control of LCMV infection in coronin 1-deficient mice

Results obtained during the course of MCMV infection, suggest a functional but delayed T cell response in the absence of coronin 1. Interestingly, whereas coronin 1-deficient $CD8^+$ T cells reconstituted the periphery, $CD4^+$ T cells did not expand and stayed at low numbers after MCMV infection (Figure 4. 2). Since, MCMV infects mice latently and promotes T cell memory inflation [211], we suggested that T cell inflation contributes to enhanced T cell stimulation, which would bypass the need for coronin 1.

To investigate to which extend $CD8^+$ and $CD4^+$ T cells were affected by the lack of coronin 1 and to analyze whether the differences observed between $CD8^+$ and $CD4^+$ T cells after MCMV infection were also true for acute viral infections, we analyzed the T cell immune response to LCMV. Like for MCMV, the early control of LCMV is highly dependent on a robust $CD8^+$ T cell responses, but for the maintenance of long-term control $CD4^+$ T cells and antibodies are also required [135, 137, 138, 140, 141, 212, 213]. Therefore, we asked whether coronin 1-deficient mice were able to control LCMV infection. For this, we infected wild type and coronin 1-deficient animals with 200 PFU LCMV-WE and monitored the viral load in the blood as well as in spleen, thymus, lung and brain at several time points post infection (Figure 4. 5). LCMV control in the blood was similar between wild type and coronin 1-deficient mice. Out of 6 mice only 1, coronin 1-deficient mice was viremic at day 8 and day 12 after LCMV infection but cleared the virus thereafter (Figure 4. 5A). In all tested organs we did not find significant differences in LCMV-titers between wild type and coronin 1-deficient mice (Figure 4. 5B-E). However, one out of three coronin 1-deficient mice still harbored LCMV in the thymus, brain and the lung at day 75 (Figure 4. 5C-E). Notably, these results were similar to the results obtained during the MCMV infection experiments.

As the control of LCMV is dependent on T cell activation, we analyze whether coronin 1-deficient T cells were responding to the LCMV infection. We first analyzed total T cell numbers in the spleen and lung and T cell activation in the blood at the indicated time points after LCMV infection by staining for CD8, CD4 and the early activation marker CD43 (Figure 4. 6). Prior infection, naïve coronin 1-deficient mice had lower $CD8^+$ and $CD4^+$ T cell numbers in the spleen compared to wild type mice. However, we found similar numbers of $CD8^+$ T cells in the lung of naïve wild type and coronin 1-deficient mice. On the other hand, $CD4^+$ T cell number were also significantly reduced in the lung of naïve coronin 1-deficient mice

compared to wild type mice (Figure 4. 6A, B). After acute LCMV infection, we found that coronin 1-deficient $CD8^+$ T cells expanded in the spleen and lung similar to wild type $CD8^+$ T cells (Figure 4. 6A). However, at day 8 total $CD8^+$ T cell numbers were still reduced in the spleen but not in the lung of coronin 1-deficient mice. Nevertheless, coronin 1-deficient $CD8^+$ T cells expanded to numbers comparable to wild type $CD8^+$ T cell numbers in the spleen after long-term infection. On the contrary, $CD4^+$ T cells from coronin 1-deficient mice did not expand after LCMV infection in the spleen and lung and T cell numbers stayed significantly low compared to wild type mice, even after long-term infection (Figure 4. 6B). Furthermore, the kinetics of activated $CD43^+$ $CD8^+$ T cells was comparable between coronin 1-deficient and wild type animals (Figure 4. 6C). On the other hand, $CD4^+$ T cells from coronin 1-deficient mice did not become activated after acute infection compared to wild type $CD4^+$ T cells. However, $CD4^+$ T cells started to upregulated CD43 after long-term infection in the absence of coronin 1, whereas wild type $CD4^+$ T cells expressed basal level of CD43 (Figure 4. 6D).

We conclude from these data that coronin 1-deficient $CD8^+$ T cells can expand, become activated as well as migrate to peripheral tissue upon LCMV infection similar to wild type $CD8^+$ T cells. Surprisingly, whereas for $CD8^+$ T cells, expansion and activation was similar for wild type and coronin 1-deficient cells after LCMV infection, coronin 1-deficient $CD4^+$ T cell numbers, fail to expand upon infection in the spleen and lung (Figure 4. 6B). These findings were further visualized by plotting the CD8:CD4 T cell ratio (Suppl. Figure 6. 5). Consistent with this, we found comparable level of the activation marker $CD43^+$ on $CD8^+$ wild type and coronin 1-deficient and reduced CD43 upregulation on coronin 1-deficient $CD4^+$ T cells (Figure 4. 6C, D). However, CD43 was upregulated on coronin 1-deficient $CD4^+$ T cells at late stages of infection. These data suggests that coronin 1 is important for the activation of $CD4^+$ T cells during acute infection and but are dispensable during long-term infection.

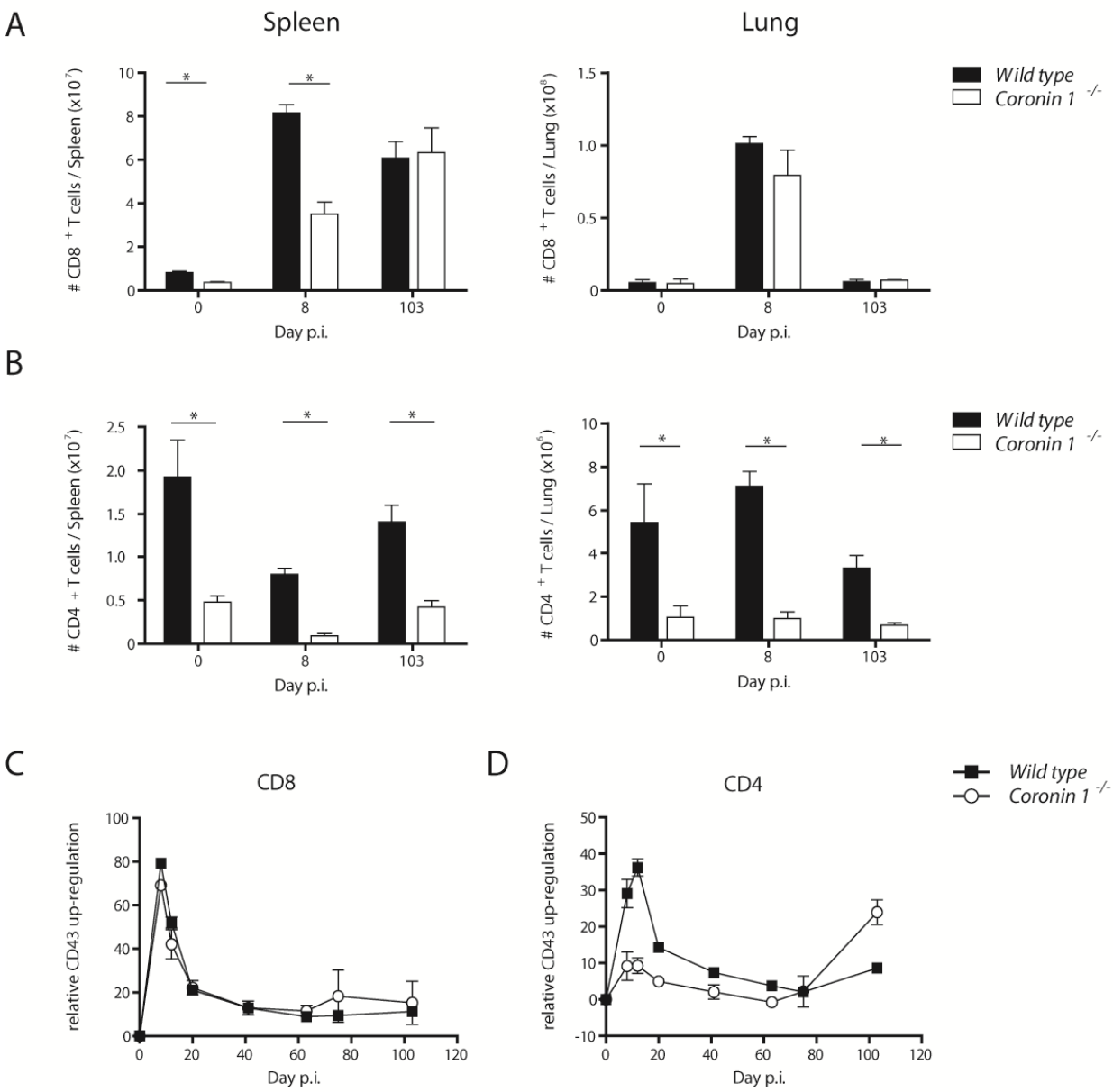


Figure 4. 6 Expansion and activation of T cells after low dose LCMV infection.

Coronin 1-deficient and C57Bl/6 wild type mice were infected with LCMV-WE i.v. A, B: Peripheral lymphocytes from spleen and lung were harvested and counted at the indicated time points and stained for for CD8 and CD4. Absolute $CD8^+$ (A) and $CD4^+$ (B) numbers in the spleen and lung after LCMV infection. C, D: Whole blood from LCMV infected mice was stained for CD8, CD4 and the early activation marker CD43 at the indicated time points. Relative upregulation of CD43 on $CD8^+$ (C) and $CD4^+$ (D) T cells in the peripheral blood. Relative CD43 up-regulation was calculated by subtracting basal CD43 expression level from the CD43 expression level of infected mice. T cell counts were calculated from the FACS data. P values (* $p<0.03$) were calculated by using the one tailed Mann-Whitney t-test (n=3-5). Error bars represents the SEM.

4.1.2.4. Specific T cell response after acute LCMV infection in coronin 1-deficient mice

Virus-specific $CD8^+$ T cells are crucial for controlling primary acute LCMV infection [135]. Therefore, we analyzed whether expanding $CD8^+$ T cells were virus-specific or not. For this, we monitored the two immune-dominant GP33- and NP396-specific $CD8^+$ T cell response at several time points after LCMV infection in the blood, spleen and lung by Tetramer staining (Figure 4. 7). We found that frequencies of GP33- and NP396-specific $CD8^+$ T cell were comparable between wild type and coronin 1-deficient mice in the blood, spleen and in the lung after acute infection (Figure 4. 7A-B). Yet, absolute virus-specific $CD8^+$ T cells were significantly lower in the absence of coronin 1 at day 8 post infection, which we conclude was due to the reduced total $CD8^+$ T cell numbers (Figure 4. 6A). However, GP33- and NP396-specifc $CD8^+$ T cell numbers were similar after long-term infection at day 103 post infection (Figure 4. 7C). Interestingly, at day 75 post infection we observed an increased frequency of NP396-specific $CD8^+$ T cell in coronin 1-deficient mice in the blood (Figure 4. 7A) and in the spleen and lung (not shown). In addition, while absolute numbers of NP396-specific $CD8^+$ T cells were increased in the lung, absolute NP396-specific $CD8^+$ T cell numbers were similar in the spleen of coronin 1-deficient mice compared to the NP396-specific $CD8^+$ T cell numbers in the organs of wild type mice at day 75 (not shown).

Overall, these results show that LCMV specific coronin 1-deficient $CD8^+$ T cells in the spleen and lung can expand to a number comparable to wild type specific $CD8^+$ T cells (Figure 4. 7C). Although expansion of virus-specific $CD8^+$ T cell numbers in the spleen was slightly delayed, we suggest that migration and clonal expansion of peripheral $CD8^+$ T cell to infected tissue is independent of coronin 1.

Control of LCMV infection is dependent on the functionality of cytotoxic T lymphocytes (CTL), including direct cytotoxicity by perforin and granzyme release and secretion of pro-inflammatory cytokines. Re-stimulation and activation of T cells is dependent on the TCR-MHC interaction and subsequently dependent on intracellular Ca^{2+} mobilization which initiates the downstream regulation of cytokine production [67]. Since coronin 1-deficiency is associated with a defect in Ca^{2+} mobilization downstream of TCR triggering [184, 185], we investigated if LCMV specific T cell were able to produce cytokines in the absence of coronin 1. To analyze cytokine production following LCMV infection, wild type and coronin 1-deficient mice were infected with low dose of LCMV-WE intravenously and the spleen and

peripheral lung lymphocytes were harvested at the indicated time points, followed by re-stimulation with the $CD8^+$ and $CD4^+$ T cell epitopes GP33 or GP64, respectively. Subsequently, IFNγ producing T cells were quantified by FACS (Figure 4. 8). At the early time points after infection (day=8), *ex vivo* peptide re-stimulation resulted in a similar frequency of IFNγ producing $CD8^+$ T cells in wild type and coronin 1-deficient mice following re-stimulation with the $CD8^+$-specific GP33 peptide (Figure 4. 8A, B). However, absolute IFNγ producing $CD8^+$ T cell numbers were still reduced after acute infection in coronin 1-deficient mice. Yet, frequency and numbers of IFNγ producing $CD8^+$ T cell numbers were increased in the spleen and lung after long-term infection (day=103). Similar results were obtained by re-stimulating $CD8^+$ T cell with the NP396 peptide (not shown).

In contrast, re-stimulation of $CD4^+$-specific with the GP64 peptide resulted in a significant (3-4 fold) reduction of IFNγ producing $CD4^+$ T cells (Figure 4. 8D-E). Hence, we suggest that re-stimulation of $CD8^+$ T cell via the TCR – MHC I complex after LCMV infection was independent of coronin 1. Interestingly, at late time points following LCMV infection (day=103), GP64 re-stimulation resulted in an increased percentage of IFNγ producing $CD4^+$ T cells. These results suggest an important need of coronin 1 for acute $CD4^+$ T cell responses, due to low virus specific pre-cursors frequencies, which can expand after long-term infection.

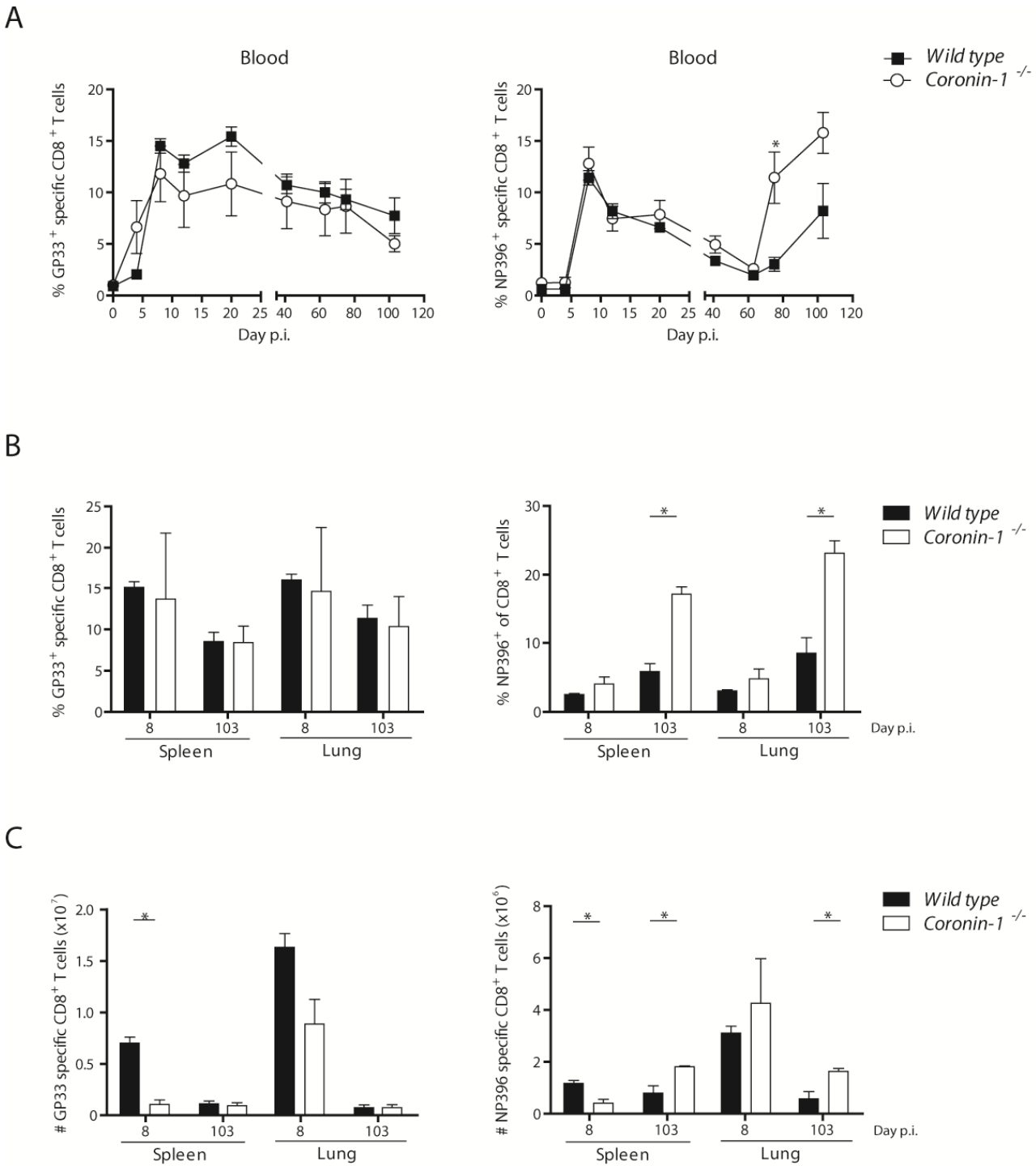


Figure 4. 7 Specific CD8$^+$ T cell immune response after LCMV infection.

Coronin 1-deficient and C57Bl/6 wild type mice were infected intravenously with LCMV-WE. Whole blood (A) as well as spleen and lung (B, C) lymphocytes were harvested and counted at day 8 and day 103 post infection and stained for CD8 and APC conjugated GP33- or NP396-specific tetramers. A, B: Frequencies of GP33- and NP396-specific CD8$^+$ T cells C: Absolute GP33- and NP396-specific T cell numbers. T cell counts were calculated from the FACS data. P values (* $p<0.03$) were calculated by using the one tailed Mann-Whitney t-test (n=3-5). Error bars represent the SEM.

4.1.2.5. $CD4^+$ T cell response is impaired during delayed type hypersensitivity in coronin 1-deficient mice

Delayed type hypersensitivity (DTH) can be used to monitor T cell mediated inflammation upon subcutaneous LCMV-WE infection [212]. Swelling of the footpad after local LCMV infection are mediated by sequential infiltration of inflammatory $CD8^+$ T cells around day 7-8 followed by $CD4^+$ T cells around day 11-13 in the infected tissue. To this end, we infected mice subcutaneously with 200 PFU LCMV-WE into the right and left footpad and monitored the swelling of the footpads (Figure 4. 9A). Footpads from wild type and coronin 1-deficient mice started to swell at day 6 and continue to swell up to day 9 post infection. Around day 11 footpad swell in coronin 1-deficient mice was mostly gone, whereas wild type mice still showed strong footpad swell (Figure 4. 9B). Histological analysis of footpad sections confirmed decreased inflammation in the footpad of coronin 1-deficient mice at day 13 (Suppl. Figure 6. 6). Along with this, the activation-kinetics (upregulation of CD43 and downregulation of CD62L on $CD44^+$ memory cells ($CD44^+$, $CD62L^-$)) of blood-derived T cells showed comparable $CD8^+$ T cell activation in coronin 1-deficient and wild type animals (Figure 4. 9D). Furthermore, CD43 expression on $CD8^+$ T cells in peripheral organs at day 17 was comparable between wild type and coronin 1-deficient animals (Suppl. Figure 6. 7A and Table 6. 1). In agreement with previous results, $CD4^+$ T cell activation could not be observed in coronin 1-deficient mice during acute infection (Figure 4. 9F and Suppl. Figure 6. 7B). At day 11, at the peak of the footpad inflammation, e*x vivo* re-stimulation of splenocytes with the LCMV peptide GP33 or GP64 induced cytokine production in coronin 1-deficient $CD8^+$ T cells but not in $CD4^+$ T cells, respectively (Figure 4. 9C, E).

Recently, a publication described a mutation in the coronin 1 locus which was associated with decreased T cell egress from the lymph node [188].

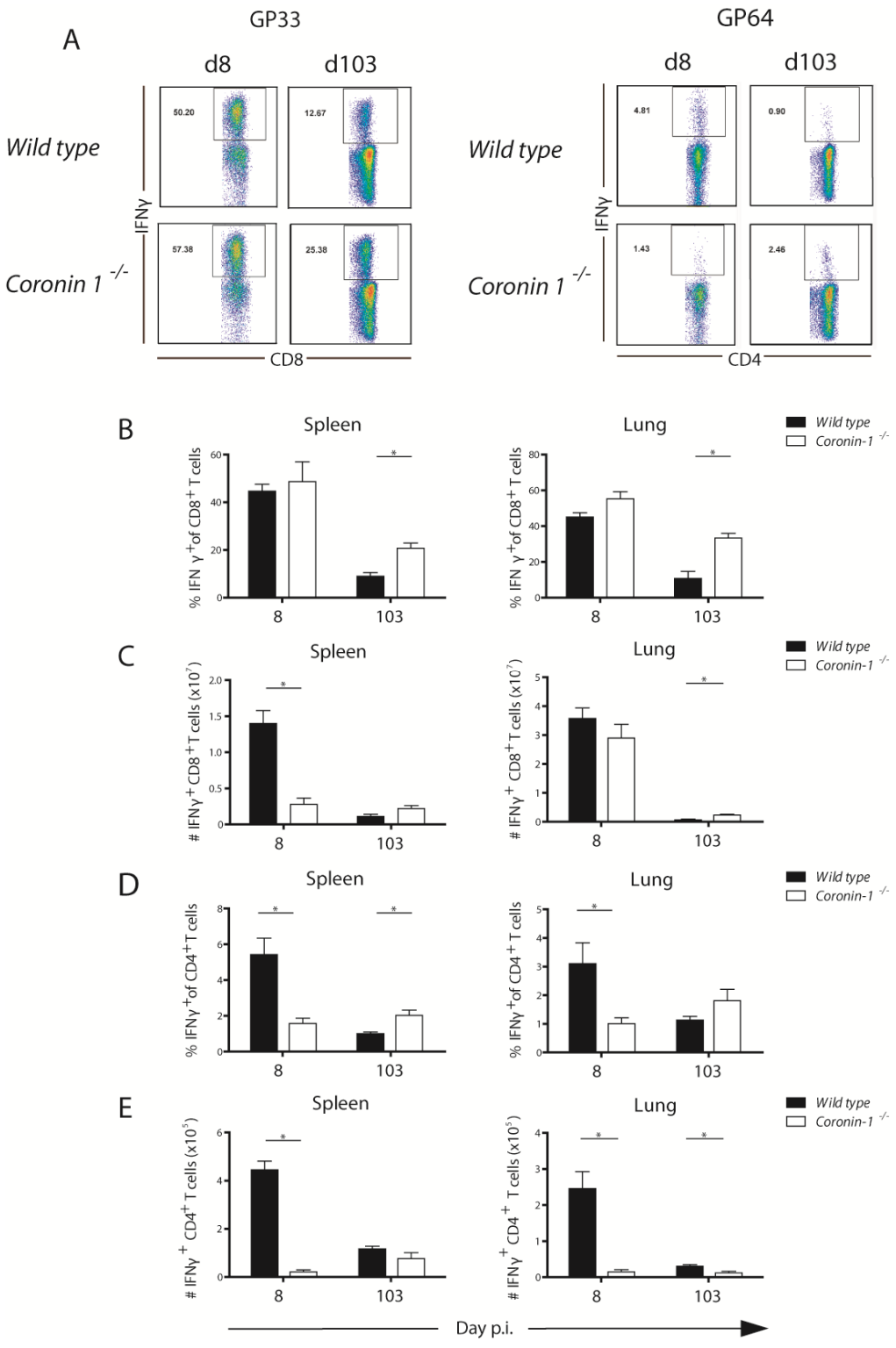


Figure 4. 8 Functionality of LCMV specific T cells.

At day 8 and 103 LCMV-WE infected coronin 1-deficient and wild type mice were sacrificed and splenocytes and lung-derived lymphocytes were harvested. Cells were re-stimulated *ex vivo* with the LCMV peptide GP33 or GP64 in the presence of Monensin at 37°C for 6 hours. Cells were subsequently stained for CD8 or CD4 followed by permeabilization with Tween 20 and stained intracellularly for IFNγ. A: Representative FACS dot-plot from splenocytes gated on either total $CD8^+$ T cells stimulated with GP33 or $CD4^+$ T cells stimulated with GP64. Frequency (B) and absolute numbers (C) of IFNγ producing $CD8^+$ T cells in the spleen and lung after GP33 re-stimulation. Frequency (D) and absolute numbers (E) of IFNγ producing $CD4^+$ T cells in the spleen and lung after GP64 re-stimulation. T cell numbers were calculated from the FACS data. P values (* $p<0.03$) were calculated by using the one tailed Mann-Whitney t-test. Error bars represent the SEM (n=3-4).

To confirm that footpad infiltrating T cells were derived from lymph nodes and not from spleen, we splenectomized mice prior to subcutaneous LCMV infection. Analysis of footpad swelling showed that primary swelling around day 8 post infection was comparable in wild type and coronin 1-deficient mice. Also in these experiments we found that the footpad swelling was mostly gone in the coronin 1-deficient mice around day 11 post LCMV infection (Suppl. Figure 6. 8A). Whereas the kinetics of activation marker (CD43, and $CD44^+$, $CD62L^-$) expression was similar in wild type and coronin 1-deficient $CD8^+$ T cells (Suppl. Figure 6. 8B), coronin 1-deficient $CD4^+$ T cells failed to upregulate CD43 and downregulate CD62L (Suppl. Figure 6. 8C). Importantly, results obtained after LCMV infection in splenectomized mice were comparable to results obtained during DTH of un-splenectomized mice. We conclude from these data, that in the absence of coronin 1 the $CD8^+$ T cell response is largely intact (lymph node egress and footpad migration) but slightly delayed due to the low T cell numbers in the absence of coronin 1. In contrast, the inflammatory $CD4^+$ T cell response was significantly impaired in the absence of coronin 1 following acute subcutaneous LCMV infection. Likewise, $CD8^+$ T cell upregulation of CD43 in peripheral organs was comparable in splenectomized coronin 1-deficient and wild type mice, whereas CD4 T cell activation was also impaired in the absence of coronin 1 (Suppl. Figure 6. 9 and Table 6. 2). These results suggest an important need of coronin 1 for $CD4^+$ T cell mediated immune response after acute LCMV infection.

4.1.2.6. Vesicular stomatitis virus (VSV) infection is associated with increased lethality in coronin 1 deficient mice

The above results suggest that coronin 1 is largely dispensable for antiviral $CD8^+$ T cell responses but required for CD4-dependent responses. To directly analyze the importance of coronin 1 for $CD4^+$ T cell immunity, we investigated the antiviral responses during an infection with vesicular stomatitis virus (VSV), which is controlled by neutralizing antibody responses and which is highly dependent on $CD4^+$ T cell help. VSV belongs to the Rhabdoviridae family and following an infection in mice, neutralizing IgG antibodies against the VSV glycoprotein are produced. However, missing IgG response results in invasion of the CNS by VSV, which results in limb paralysis and death, due to cytotoxic neuroinvasion [138, 214-216]. $CD4^+$ T cells provide a co-stimulation signal to promote antibody class switch in B

cells which produces neutralizing IgG against the VSV glycoprotein G in 6 to 8 days [214, 217]. Hence, $CD4^+$ T cells are essential for VSV control [51, 148, 150, 151, 153, 218, 219]. To analyze the immune response against VSV, wild type and coronin 1-deficient mice were infected intravenously with different doses of VSV-INDG. As shown in Figure 4. 10, while a low dose ($2x10^5$ PFU) of VSV-INDG had only minor effect on lethality in wild type mice, the same dose resulted in paralytic symptoms and death in the majority of the coronin 1-deficient mice (Figure 4. 10A). An infection of $5x10^7$ PFU VSV-INDG, resulted in up to 90% lethality in coronin 1-deficient mice, while only 40% of the wild type animals succumbed to the infection. These results suggest that the absence of coronin 1 is associated with a drastically increased lethality after VSV infection.

Increased lethality after intravenous VSV infection is related with impaired B cell response and neutralizing IgG antibody production. [152, 219-221]. To analyze whether defects in B cell function in the absence of coronin 1 may contribute to the observed differences in lethality upon VSV infection neutralizing IgG against VSV were measured. To that end, wild type and coronin 1-deficient mice were infected mice with $2x10^6$ PFU VSV-INDG intravenously and serum was harvested at the indicated time points post infection. We found that thymus independent neutralizing IgM production was comparable between wild type and coronin 1-deficient mice. However, thymus dependent neutralizing IgG (β-mercaptoethanol (β-ME) reduced serum) production was delayed in the absence of coronin 1 (Figure 4. 10B), which is consistent with earlier work by Combaluzier et al. [189].

We suggest from these results that the B cell response is not per se impaired but rather ineffective due to impaired $CD4^+$ T cell response, which is responsible for promoting antibody class switch via co-stimulatory molecules. As we analyzed T cell activation in coronin 1-deficient mice after VSV infection we found that relative upregulation of CD43 on $CD8^+$ T cells was only slightly reduced in coronin 1-deficient mice whereas relative upregulation of CD43 on coronin 1-deficient $CD4^+$ T cells was strongly reduced and delayed (Figure 4. 10C). Moreover, when we stimulated T cell *in vitro* with CD3/CD28 antibodies, coronin 1-deficient $CD4^+$ T cells were impaired in up-regulating CD40L, an important molecule to provide B cell help for antibody class switch [222] (Figure 4. 11). Importantly, we conclude from the MFI that the amount of CD40L expressed per $CD4^+$ T cell was similar between wild type and coronin 1-deficent cells (Figure 4. 10D).

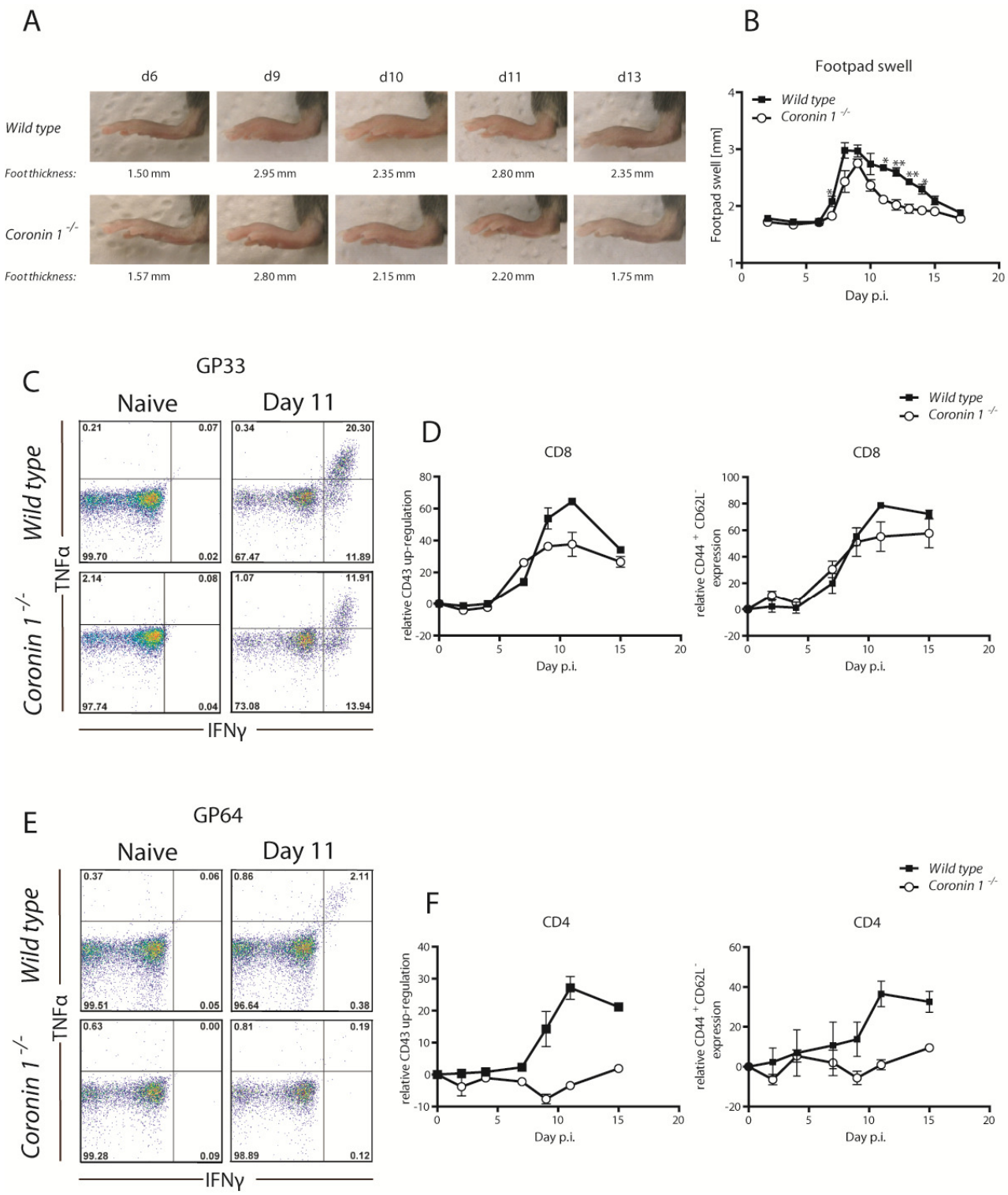


Figure 4. 9 Delayed type hypersensitivity after subcutaneous footpad LCMV infection.

Coronin 1-deficient and wild type C57Bl/6 mice were infected subcutaneously into the right and left footpad with LCMV-WE. Footpad swell was measured daily and whole blood was harvested at the indicated time points and stained for CD8, CD4, CD43, CD44 and CD62L. At day 11 spleen cells were harvested and restimulated with either GP33 or GP64 peptide and stained for CD8, CD4 and intracellularly for IFNγ and TNFα after Tween 20 permeablization. A, B: Footpad-swell was measured with a caliper at the indicated time points. C: Representative FACS dot-plot from splenocytes gated on total $CD8^+$ T cells stimulated with GP33. D: Relative activation of $CD8^+$ T cell after LCMV infection in whole blood. E: Representative FACS dot-plot from splenocytes gated on total $CD4^+$ T cells stimulated with GP64. F: Relative activation of $CD4^+$ T cell after LCMV infection in whole blood. Relative activation of T cells was calculated by subtracting the frequency of activated T cells from naïve mice from the frequency of activated T cells of infected mice. P values (* $p<0.03$, ** $p<0.008$) were calculated by using the two tailed Mann-Whitney t-test. Error bars represent the SEM (n=4-6).

Recently it was shown that B cells were required for maintaining subcapsular sinus (SCS) ($CD169^+$, $CD11b^+$) macrophages via LT α1β2 signaling, thereby preventing VSV dissemination to the CNS after subcutaneous infection. Moreover, it was shown that neutralizing antibodies were not required for VSV neutralization after subcutaneous VSV infections [57, 58]. To confirm that increased lethality was not due to lack of SCS macrophages in the absence of coronin 1, we analyzed the presence of SCS in the lymph nodes from wild type and coronin 1-deficient mice (Suppl. Figure 6. 10). We found that they were present in a similar frequency in the lymph nodes of both wild type and coronin 1-deficient animals. Moreover subcutaneous infection of VSV-INDG into the footpad did not lead to increase lethality of coronin 1-deficient mice (Suppl. Figure 6. 11).

Overall, these results showed that $CD8^+$ T cell responses were comparable between wild type and coronin 1-deficient mice after viral infection, whereas the $CD4^+$ T cell responses were strongly impaired, resulting in an increased mortality after acute VSV infection in the absence of coronin 1.

4.1.3. **Discussion**

Coronin 1-deficient mice have a strongly reduced peripheral T cell pool, and, naïve T cell are rapidly deleted through apoptosis in the periphery in the absence of coronin 1. Most of the remaining T cells express a central memory phenotype, which is probably due to lymphopenic induced proliferation (LIP) [183, 184]. In addition to LIP, partial TCR activation may lead directly to long live memory T cells [223]. Whether newly synthesized T cells are recruited to the periphery in adult mice in the absence of coronin 1, is not known.

Homeostatic proliferation in a lymphopenic environment usually results in a skewed T cell receptor repertoire [224]. Through, analysis of the TCR variable β-chain usage of coronin 1-deficient T cells, we found that V_β-chain usage was diverse in the absence of coronin 1 and that the distribution of their usage was comparable to those of wild type mice (Suppl. Figure 6. 1). From these data we conclude that thymocytes can exit the thymus, thereby contributing to a diverse T cell receptor repertoire in the periphery. However, why some naïve T cells survive in coronin 1-deficient mice and acquire a memory phenotype remains elusive.

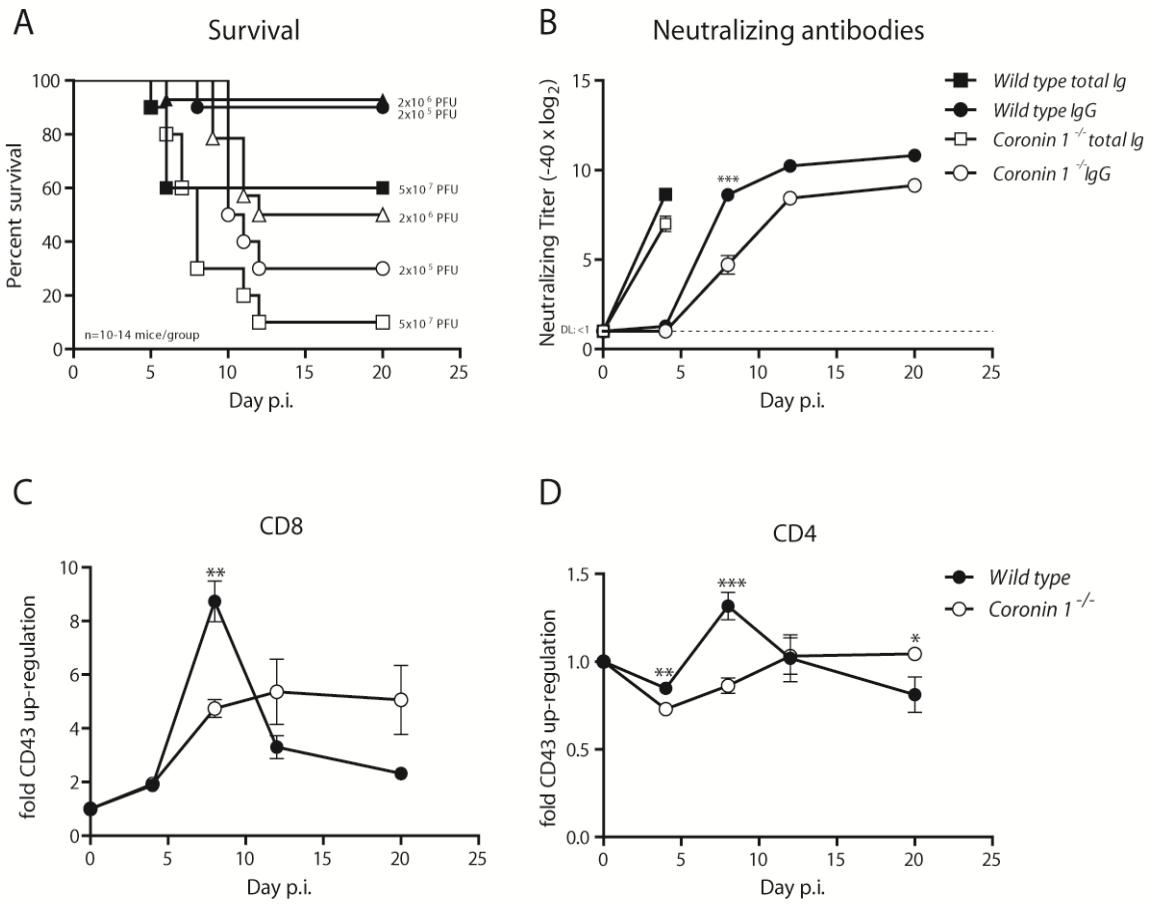


Figure 4. 10 Survival of wild type and coronin 1-deficient mice after VSV infection.

Wild type (black symbols) and coronin 1-deficient mice (open symbols) were infected with high dose ($5x10^7$ PFU, □), intermediate dose ($2x10^6$ PFU, Δ) or low dose ($2x10^5$ PFU, ○) of VSV-INDG. A: Survival of mice was monitored over 20 days. B: Serum was harvested at day 4, 8, 12 and 20 post infection from mice infected with $2x10^6$ PFU VSV-INDG and neutralizing IgG or total Ig titer against VSV was determined as described in the Methods. C, D: Blood from VSV-INDG ($2x10^5$ PFU) infected mice was harvested at the indicated time points and stained for CD8 (C), CD4 (D) and CD43. Relative activation was calculated by normalizing the frequency of $CD43^+$ T cells in infected mice to the frequency of $CD43^+$ T cell in uninfected mice. P values (*p<0.05;**p<0.006;***p <0.0004) were calculated by using the two-tailed Mann-Whitney t-test (n=10). Error bars represent the SEM.

We hypothesized, while most of the naïve T cells will undergo apoptosis in the absence of coronin 1, some naïve coronin 1-deficient T cells with a low TCR activation threshold might receive a partial activation signal resulting in differentiation into a memory T cell. Taken together, we suggest that coronin 1 act as modulator of the TCR signaling, thereby reducing the activation threshold of the TCR. Though, in the absence of coronin 1 only the T cells survive, which TCR activation threshold is low and signal is strong enough to bypass the need of coronin 1. As Mueller et al. showed [183], T cells can induce a very weak Ca^{2+} mobilization in the presence of extracellular Ca^{2+}. These results might suggest that weak Ca^{2+} signal,

induced by anti-CD3 and anti-CD28 antibodies, might be enough to promote T cell survival in T cells with low TCR activation threshold.

CD43 (leukosialin, sialophorin) is a glycosylated transmembrane protein expressed on a variety of hematopoietic cells, which is up-regulated after T cell activation [225-227]. It is found to provide important T cell activation signals as well as playing a role in negative T cell regulation [226, 228, 229]. Additionally, high level of CD43 expression was shown to inhibit T cell receptor/CD3-mediated apoptosis [225]. Interestingly, we showed that most $CD4^+$ T cells from uninfected coronin 1-deficient mice were activated compared to uninfected wild type mice, whereas expression of activation markers ($CD43^+$ and $CD62L^-$) on $CD8^+$ T cells from coronin 1-deficient and wild type mice were similar. Thus it seems that $CD4^+$ T cells are more affected by the absence of coronin 1. Correlating with this hypothesis we found that the CD8:CD4 T cell ratio in uninfected coronin 1-deficient mice was distorted in favor of $CD8^+$ T cells (Suppl. Figure 6. 2). These results suggested a more important role of coronin 1 for peripheral $CD4^+$ T cell survival than for $CD8^+$ T cells.

T cell immune response is required for controlling many microbial infections [51, 141, 230-232]. Upon infection, antigens are internalized and processed by antigen presenting cells (APC) and presented via their MHC molecule to the TCR [67, 233]. Activation and clonal expansion of T cells is dependent on the interaction of the TCR with the MHC I/II molecules that present antigenic peptides which leads to rapid intracellular Ca^{2+}-mobilization [67, 209]. T cell receptor signaling was shown to be impaired in the absence of coronin 1 [183-185]. Therefore, we asked whether coronin 1-deficient T cells could be activated upon viral infection and whether there is a difference in $CD8^+$ and $CD4^+$ T cell response. For this, we analyzed the T cell immune response upon MCMV and LCMV infection in coronin 1-deficient mice. Control of primary MCMV and LCMV infection is mainly due to CTL response, whereas $CD4^+$ help is mainly needed for long term protection and control of virus reactivation [110, 117, 135, 137, 140, 206-208, 212]. Infection of wild type and coronin 1-deficient mice with either MCMV or LCMV leads to no systemic infection and leads complete clearance of virus in all organs tested. However, clearance of virus was slightly delayed in coronin 1-deficient animals.

We conclude that delay in viral clearance was due to low T cell numbers as well as reduced $CD4^+$ T cell activity in coronin 1-deficient mice. Nevertheless, we suggested that T cell

immune response was taking place. Therefore, we analyzed T cell expansion and migration in the periphery following MCMV or LCMV infection. We showed that coronin 1-deficient $CD8^+$ T cell were expanding and were able to migrate to infected peripheral tissue, whereas coronin 1-deficient $CD4^+$ T cells did not. Moreover, activation kinetic of $CD8^+$ T cells in wild type and coronin 1-deficient mice were comparable after virus infection. Furthermore, MCMV and LCMV infection induced virus specific $CD8^+$ T cell expansion in the periphery in the absence of coronin 1. Interestingly, we observed an increase in frequencies and numbers of NP396-specifc $CD8^+$ T cells after long-term LCMV infection. NP presentation by APC was shown to be faster and immune-dominant during acute infection and LCMV challenge experiments [234]. In the light of the observed LCMV titer in the coronin 1-deficient mice at day 75, we suggest that LCMV can reactivate after long-term infection in the absence of coronin 1, thereby contributing to an expansion of NP396-specific $CD8^+$ T cells. Moreover, coronin 1-deficient virus specific $CD8^+$ T cells were fully functional upon peptide re-stimulation and functional avidity was comparable to wild type $CD8^+$ T cells. These findings demonstrate that activation, expansion, migration and functionality of specific $CD8^+$ T cells are not dependent on coronin 1 after virus infection.

However, as we analyzed $CD4^+$ T cell response in MCMV and LCMV acutely infected coronin 1-deficient mice, we found that coronin 1-deficient $CD4^+$ T cell did not expand could not get activated. In addition, we could hardly measure any IFNγ producing specific $CD4^+$ T cells upon peptide restimulation after acute LCMV infection. Interestingly, coronin 1-deficient $CD4^+$ T cells upregulated CD43 after long term LCMV infection and peptide re-stimulation leads to considerable IFNγ production in coronin 1-deficient $CD4^+$ T cells. These results suggested that coronin 1 is important for $CD4^+$ T cells activation during acute infection. On the other hand, virus-specific $CD4^+$ T cells might be so low in the absence of coronin 1 that they need more time to expand to a protective number. Another possibility would be, since MHC II is only expressed on restricted cells [62, 84], relative MHC II expression is lower compared to MHC I expression. This could influence and decrease the $CD4^+$ T cell stimulation and activation via TCR, resulting in a delayed $CD4^+$ T cell response. Importantly, coronin 1-deficiency has no influence on antigen processing, presentation and MHC I/II expression on dendritic cells [193].

Coronin 1 is required for peripheral survival, homeostatic proliferation and activation of naïve T cells through TCR signaling [183-186]. During viral infection, cytokines such as type I IFN are released by other immune cells or non-hematopoietic infected cells. We propose that these cytokines which re-activate T cells [235-237] renders the T cell activation independent of coronin 1 (Figure 4. 12). We suggest that delay in viral clearance was due to low $CD8^+$ T cell numbers prior infection rather than impaired CTLs. However, $CD4^+$ T cell seemed to be more affected by the lack of coronin 1 during both MCMV and LCMV infection. These results showed that coronin 1 was required for acute $CD4^+$ T cell immune term LCMV infection but was dispensable for CTL response.

Strong evidence of an impaired $CD4^+$ T cell response in the absence of coronin 1 prompted us to investigate the survival of coronin 1-deficient mice upon VSV infection. $CD4^+$ T cell deficiency is associated with poor VSV control leading to viral CNS entry and to paralysis [51, 151, 153]. As expected, upon intravenous VSV infection, coronin 1-deficiency was associated with increased lethality and delay in production of neutralizing anti-VSV IgG. Furthermore, we showed that CD40L upregulation $CD4^+$ T cell was significantly reduced on coronin 1-deficient compared to wild type $CD4^+$ T cells.

According to previous publications, coronin 1-deficient mice are able to mount thymus dependent antibody class switch, although with delayed kinetics [189]. Furthermore, it was shown that $CD4^+$ T cells provide important B cell help for promoting antibody class switch via the CD40L molecule [222]. Importantly, CD40L regulation is also dependent on TCR signaling and intracellular Ca^{2+} mobilization [238-240]. Yet, some coronin 1-deficient $CD4^+$ T cells were able to upregulate CD40L which indicate that TCR signaling is not completely abolished in the absence of coronin 1 but rather ineffective. These results suggest that T_{H2} dependent B cell help is taking place and would need further investigation. However, the delay in neutralizing IgG production might give enough time to VSV, to escape the immune system and enter the CNS, which would explain the increased lethality of coronin 1-deficient mice after VSV infection.

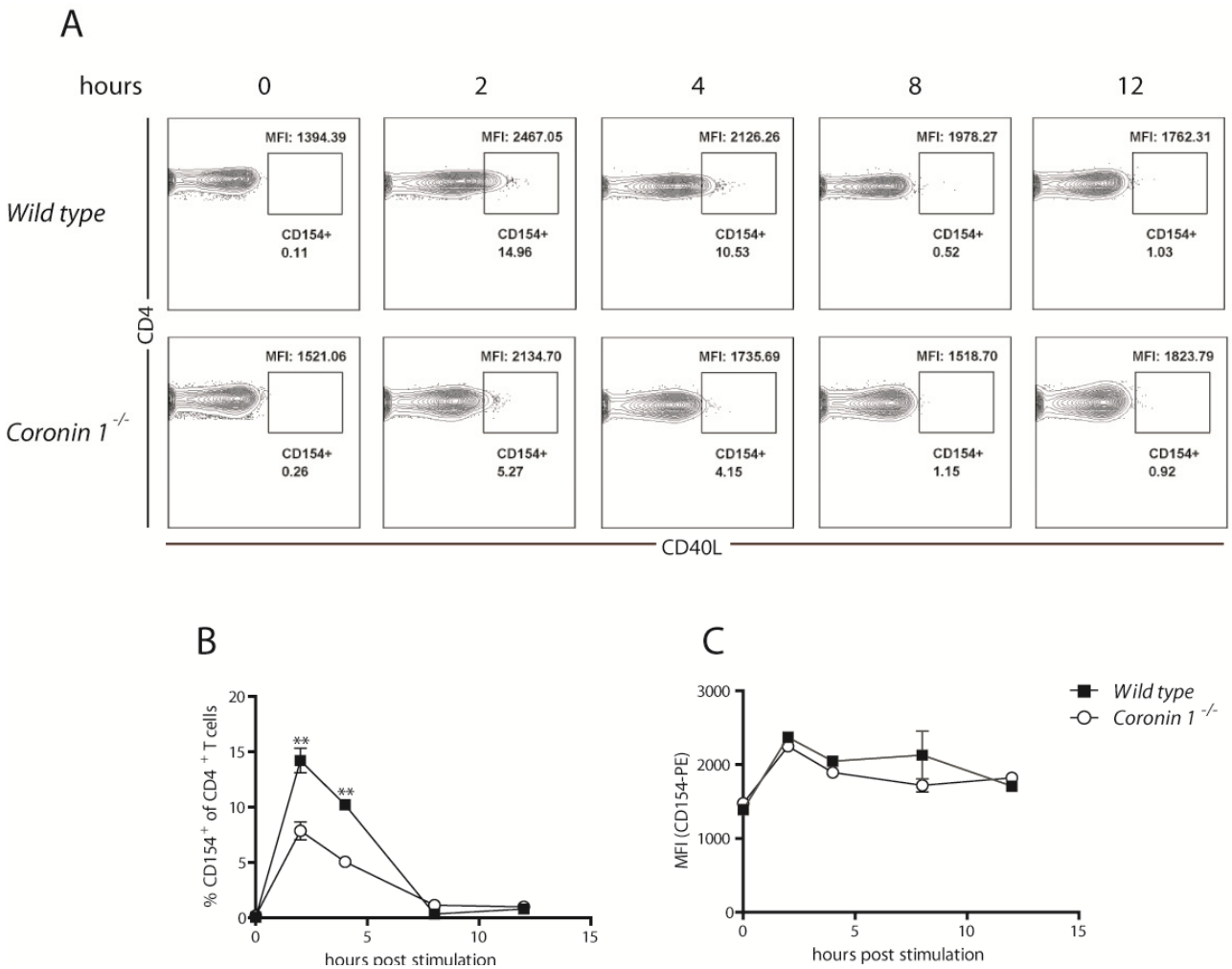


Figure 4. 11 Upregulation of CD40L on wild type and coronin 1-deficient CD4$^+$ T cell after TCR stimulation.

Freshly isolated splenocytes from coronin 1-deficient or wild type mice were stimulated for 12 hours with anti-mouse CD3 and anti-mouse CD28 monoclonal antibodies. Cells were harvested and stained for CD4 and CD154 (CD40L) at the indicated time points after stimulation. A: Representative FACS plots after TCR stimulation B: Frequency of CD40L expressing CD4$^+$ T cells. C: Mean fluorescence intensity of CD40L-PE. (n=4-6; significance was calculated using the two tailed Mann-Whitney t-test, **p<0.001). Error bars represent the SEM.

Taken together, our findings sustain the idea that in addition, low CD4$^+$ T cell numbers in the absence of coronin 1, CD4$^+$ T cell activation is impaired, resulting in a delayed antibody class switch. However, adoptive transfer experiments would dissect the importance of coronin 1 during T cell immune response in more detail, especially during viral infection. Overall, our results fits with previous finding that coronin 1-deficient mice are resistant to EAE which is mediated by CD4$^+$ T cells [191, 192]. Moreover, our results suggest a different requirement for coronin 1 in CD8$^+$ and CD4$^+$ T cell signaling. Several publications show that there are some differences between CD8$^+$ and CD4$^+$ during T cell activation. Especially, LCK was shown to have a greater impact on CD4$^+$ T cell signaling than for CD8$^+$ T cells [241-243].

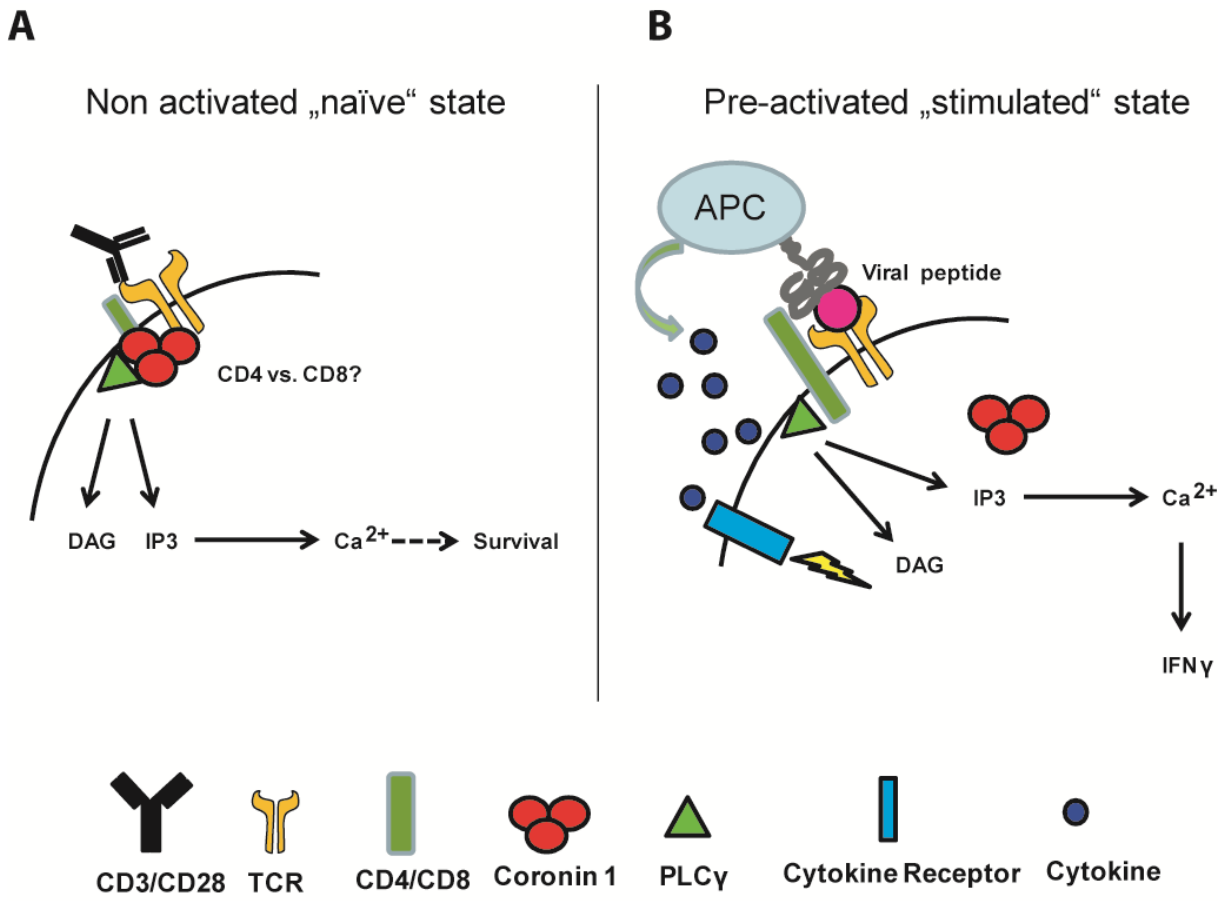


Figure 4. 12 Working model of coronin 1-independent T cell activation.

A: Stimulation of naïve T cells depends on the presence of coronin 1 for efficient T cell signaling. Our results show that coronin 1 may be more important for $CD4^+$ T cells than for $CD8^+$ T cells. Hence, it remains unclear what the contribution of coronin 1 in CD4 and CD8 T cells signaling, respectively, is. B: During viral infection, we hypothesize that effector cytokines are released by infected cells which induce re-localization of coronin 1. In effector T cells, coronin 1 seemed to be dispensable for T cell signaling.

Overall, we propose that coronin 1 is more important to maintain activation, survival and functionality of $CD4^+$ T cells than of $CD8^+$ T cells. Hence, coronin 1-deficient mice might be highly susceptible to pathogens which rely on functional $CD4^+$ T cells after acute infection. Besides, $CD4^+$ T cells are shown to play an important role in sustaining primary as well as secondary $CD8^+$ T cell response [110, 244, 245]. Pre-eliminary transfer experiments with P14 cells and subsequent challenging with Vaccinia virus expressing GP33 suggest that coronin 1-deficient $CD4^+$ T cells support $CD8^+$ T cell responses upon secondary infection (suppl. Figure 6. 12). These results, suggest that despite low $CD4^+$ T cell numbers in the absence of coronin 1, $CD4^+$ T cells from coronin 1-deficient mice are able to support primary as well as secondary $CD8^+$ T cell response. Yet, it remains unclear whether memory $CD8^+$ T cell and their responsiveness upon secondary challenge are maintained.

4. Results: Part two

The NK cell immune response in the absence of coronin 1

Most of the results described in this chapter are part of the following manuscript:

Vincent S. Tchang, K. Siegmund, Urs Karrer and Jean Pieters

Coronin 1 is important for NK cell maintenance but dispensable for NK cell immune responses

4.2. Second Part: The NK cell immune response in the absence of coronin 1

4.2.1. Introduction

During acute primary viral infection the innate immune system is the first line of defense. Natural killer (NK) cells play an important role for early control of viral infections as well as for anti-tumors defense. NK cell deficiency is associated with poor viral control such as EBV or increased occurrence of tumors [16, 20, 21, 246-248]. Upon infection, pro-inflammatory cytokines such as type I IFNs are released by infected and dendritic cells which leads to NK cell activation and proliferation [16]. Additionally, direct cell-to-cell contact is needed to promote efficient NK cell activation and cytotoxic function [14]. NK cells sense their environment for low MHC I expression, which are often down-regulated on virus infected-cells, to escape $CD8^+$ T cell immune control, and on tumor cells [17]. NK cell activation is tightly controlled by an arsenal of activating and inhibitory receptors [14, 15, 27]. NK cell activating receptors have similarities with B cell and T cell receptors but they miss the intracellular enzymatic activity. NK cell receptors are non-covalently associated with transmembrane molecules which transmit their signal via immunoreceptor tyrosine based activating motifs (ITAM) (CD3ζ, FcεRIγ, DAP10 and DAP12). ITAMs become phosphorylated by Src-familiy protein kinases which induce a downstream phosphorylation cascade resulting in intracellular Ca^{2+} mobilization and degranulation. On the other hand, inhibitory NK cell receptors contain immunoreceptor tyrosine based inhibitory motifs (ITIM), which recruit the tyrosine phosphatases SHP-1 and SHP-2 upon phosphorylation. SHP-1/2 abolish the phosphorylation cascade induced by ITAMs [15]. Hence, whether NK cell become activated depends on the balance and strength of the activating and inhibitory signals.

Coronin 1-deficiency is associated with impaired Ca^{2+} mobilization upon TCR signaling [183-185]. TCR and NK cell receptor signaling is believed to have strong similarities in terms of phosphorylation cascade resulting in intracellular Ca^{2+} mobilization [28, 249, 250]. Yet, a role for coronin 1, if any, in NK cell-mediated immune responses has not been analyzed. We have previously showed that acute MCMV and LCMV control is delayed and VSV control is highly impaired in the absence of coronin 1. We explained these results with an impaired $CD4^+$ T cell response in the absence of coronin 1. However, impaired NK cell-mediated immune

response in coronin 1-deficient mice may be another possibility to explain the delay in virus control. Therefore, we asked whether coronin 1-deficiency was associated with impaired NK cell activation. We analyzed NK cell activation upon VSV infection, after stimulation with antibodies directed against specific activating NK cell receptor and during Concanavalin A (Con A) induced hepatitis. Furthermore, we investigated the capacity of coronin 1-deficient NK cells to induce cytotoxicity in YAC-1 cells. We found that coronin 1-deficient NK cells were fully functional. VSV infection as well as antibody or YAC-1 stimulation was not associated with impaired NK cell activation in the absence of coronin 1. However, Con A treatment (*in vivo* and *ex vivo*) was associated with impaired IFNγ production and cytotoxicity against YAC-1 cells. These findings correlated with increased sensitivity to apoptosis of NK cells in the absence of coronin 1.

4.2.2. **Results**

4.2.2.1. NK cell immune response to VSV

NK cells are important for controlling viruses and tumors. Yet, the impact of coronin 1-deficiency on NK cells has not been analyzed thus far. Importantly, activating NK cell receptor signaling is similar to T cell receptor signaling, with both signaling cascades very dependent on PLCγ1 [14, 104, 251, 252], which was shown to interact with coronin 1 [184]. Thus, NK cell receptor signaling and NK cell activation may be impaired due to coronin 1-deficiency. Additionally, control of virus after acute infection is slightly delayed in the absence of coronin 1, which could be explained by an impaired NK cell functionality.

To investigate whether NK cell numbers were altered in the absence of coronin 1, we analyzed NK cell viability in coronin 1-deficient mice. For this, lymphocytes isolated from spleen or liver of wild type and coronin 1-deficient mice were stained for NK1.1 and CD3 to exclude NKT cells (Figure 4. 13). Interestingly, we found an increased NK cell frequency in spleen and liver of naïve coronin 1-deficient mice compared to wild type mice (Figure 4. 13A). However, absolute NK cell numbers were only marginally increased in the liver of coronin 1-deficient mice (Figure 4. 13B). We conclude from these data, that NK cell number are not reduced by the lack of coronin 1 but may be slightly increased to compensate for the periphery T cell lymphopenia.

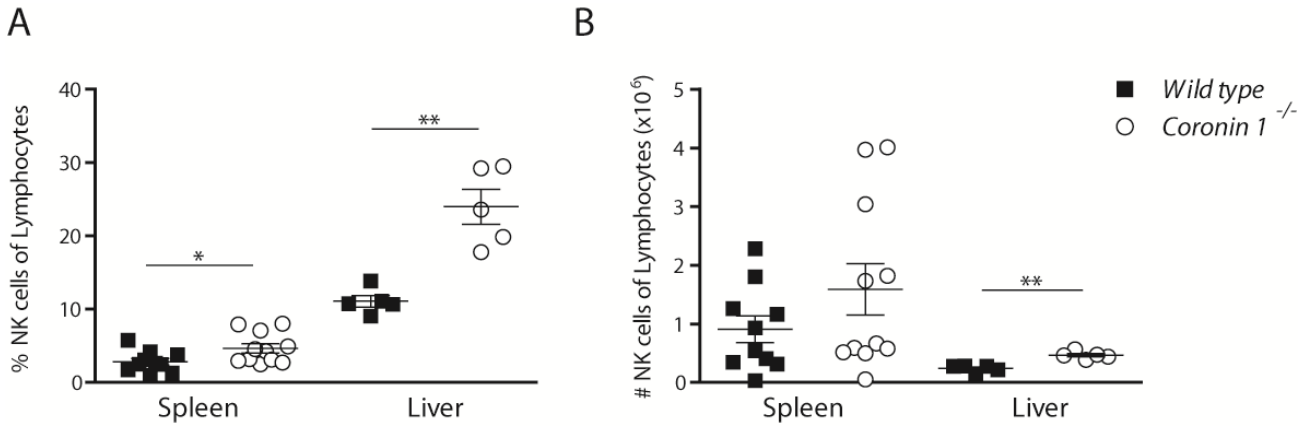


Figure 4. 13 NK cell frequencies and numbers in wild type and coronin 1-deficient mice.

Wild type and coronin 1-deficient mice were sacrificed and spleen and liver were harvested. Splenocytes were isolated by homogenizing the spleen through a metal grid. Liver tissues were digested enzymatically and intrahepatic liver lymphocytes (IHL) were isolated by centrifugation on a Percol gradient. Cells were counted and stained for NK1.1 and CD3. A: Frequency of NK cell in the spleen and liver. B: Absolute NK cell number in the spleen and liver. Absolute numbers were calculated from the FACS data. P values (** $p<0.008$; * $p<0.03$) were calculated by using the two tailed Mann-Whitney t-test. Error bars represent the SEM.

It was shown that NK cell activity may contribute to increased survival of mice after lethal VSV challenge [253]. To investigate a potential defect in antiviral NK cell responses in the absence of coronin 1 during VSV infection, we infected wild type and coronin 1-deficient animals with $5x10^6$ or $5x10^5$ PFU VSV-INDG i.v. and analyzed the capacity of NK cells to produce IFNγ (Figure 4. 14). 48 hours post infection we sacrificed the mice and harvested the liver and spleen. We cultured intrahepatic lymphocytes (IHL) and splenocytes for 4.5 hours in the presence of Brefeldin A to measure spontaneous IFNγ production by NK cells. While we observed a decrease in NK cell numbers in the spleen of infected mice, NK cell numbers in the liver remained constant when infected with high dose of VSV and were marginally increased when infected with low dose of VSV (Figure 4. 14B). We speculate that spleen derived NK cells were probably recruited to the infected area resulting in reduced NK cell numbers in the spleen. Nevertheless, coronin 1-deficient NK cells were able to produce IFNγ (Figure 4. 14C). Although, the frequency of IFNγ-producing NK cells seemed to be reduced in VSV infected coronin 1-deficient mice compared to wild type mice, their total number was similar (Figure 4. 14D).

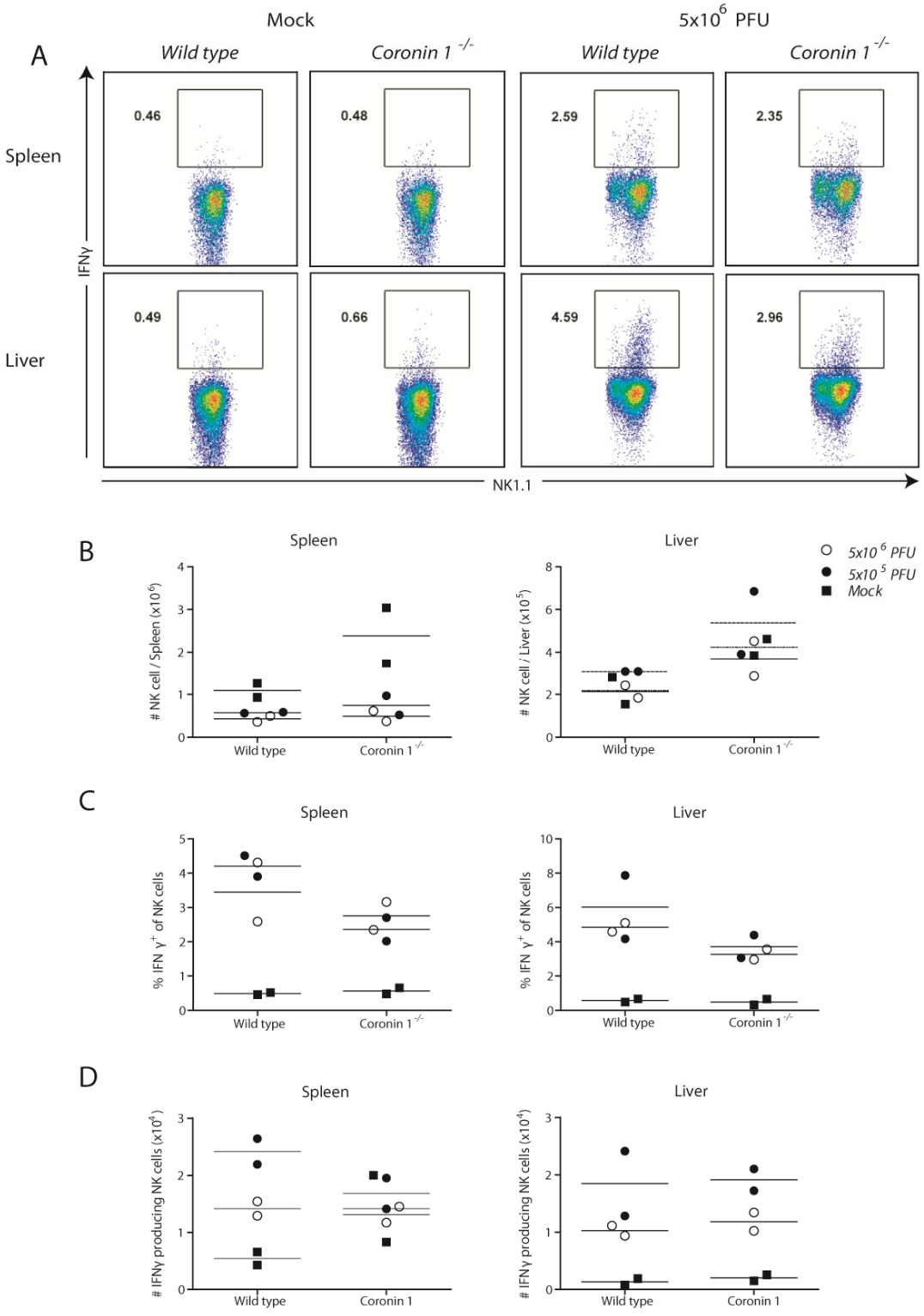


Figure 4. 14 NK cell activation after VSV infection in wild type and coronin 1-deficient mice.

Coronin 1-deficient and wild type mice were infected intravenously with $5x10^6$ or $5x10^5$ PFU VSV-INDG or infected with medium (Mock). Mice were sacrificed 48 hours post infection and spleen and liver were harvested. Splenocytes were isolated by homogenizing the spleen through a metal grid. Liver tissues were digested enzymatically and intrahepatic liver lymphocytes (IHL) were isolated by centrifugation on a Percol gradient. Cells were count and 0.5 - $2x10^6$ lymphocytes were then cultured for 4.5 hours at 37°C, 5% CO_2 in the presence of Brefeldin A. Cells were subsequently stained for NK1.1 and CD3. After permeabilzation of the cells with 0.5% saponin, cells were intracellular stained for IFNγ. Cells were then analyzed with a BD FACScanto II. A: Representative FACS dot-plot from splenocytes and IHL from mice infected with $5x10^6$ PFU VSV-INDG. B: Absolute NK cell numbers in the spleen and liver. C: Frequency of IFNγ producing NK cells in the spleen and liver. D: Absolute IFNγ producing NK cells after VSV infection. Absolute NK cell numbers were calculated from the FACS data.

These results showed that IFNγ production in NK cells is not impaired after VSV infection in the absence of coronin 1. Moreover, de-granulation of NK cells, measured by CD107a up-regulation was not affected by the lack of coronin 1 (not shown).

4.2.2.2. Stimulation of activating NK cell receptors induces activation of coronin 1-deficient NK cells

Virus infected cells secrete pro-inflammatory and pro-survival cytokines which sustain NK cell immune response [253-255]. As hypothesized earlier, cytokines may contribute to a pre-activation of T cells as well as NK cells which renders their response independent of coronin 1. To dissect whether ITAM signaling was impaired or intact in the absence of coronin 1 in uninfected mice, we stimulated NK cells from coronin 1-deficient or wild type spleen directly with plate bound specific antibodies against the activating receptors NK1.1, NKp46, Ly49D or NKG2D (Figure 4. 15). After stimulation with plate bound antibodies, both wild type and coronin 1-deficient NK cells were fully activated, leading to production of IFNγ (Figure 4. 15A) and to degranulation (Figure 4. 15C). We found increased frequencies of IFNγ producing NK cells in coronin 1-deficient mice after stimulating the Ly49D receptor (Figure 4. 15A). However, frequencies of degranulating NK cells were similar. NKG2D is a C-type lectin-like receptor which is up-regulated on NK cells upon infection, stimulation by IL2 or stress [14, 15, 256, 257]. Stimulation of freshly isolated splenocytes with anti-NKG2D antibody coated plate did not result in IFNγ production in both wild type and coronin 1-deficient NK cells (Figure 4. 15B and D). Therefore we cultured splenocytes in the presence of IL2 for 20 hours prior to stimulation with plate bound antibodies (Figure 4. 15B and D). Together with IL2 stimulation of the NKG2D receptor resulted in IFNγ production (Figure 4. 15B) and degranulation (Figure 4. 15D), with a slight decrease of the latter in coronin 1-deficient NK cells.

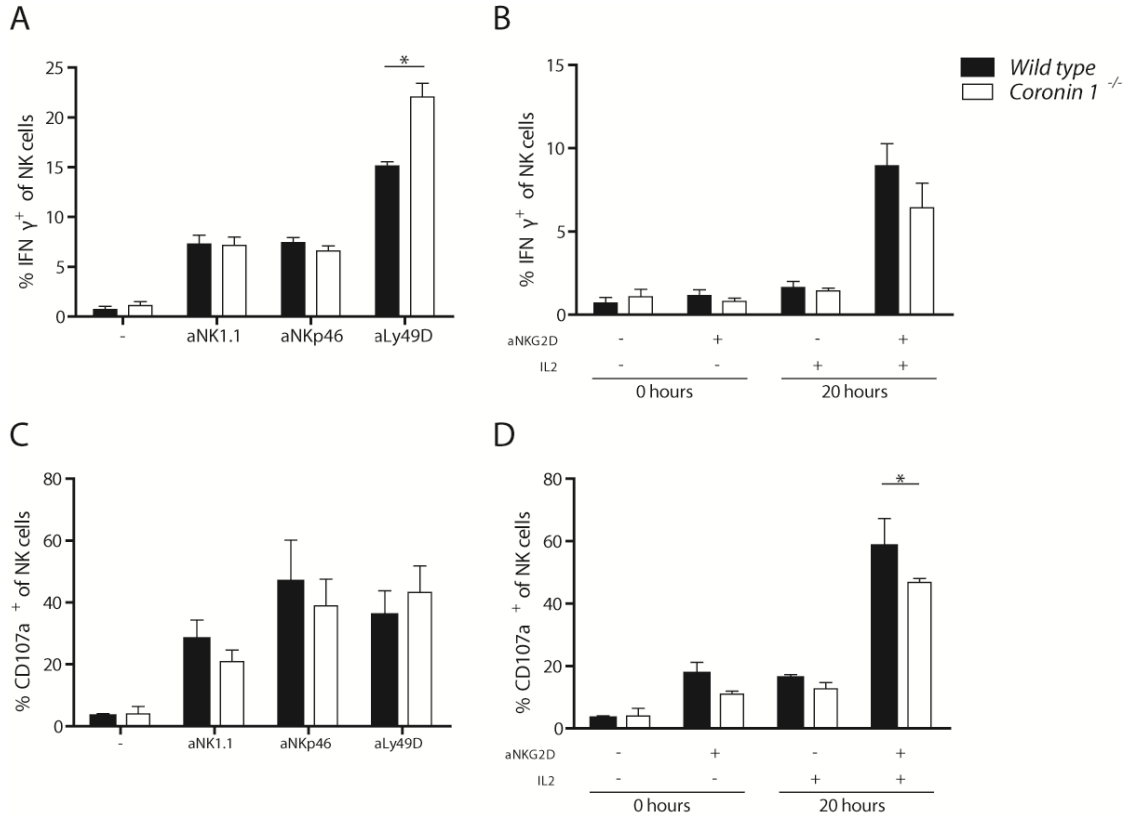


Figure 4. 15 Stimulation of NK cells by plate bound antibodies.

Wild type and coronin 1-deficient mice were sacrificed and spleen was harvested. 1x10^6 splenocytes were stimulated on antibody coated plates (no antibody, aNK1.1, aNKp46 and aLy49D, 5 µg/ml) for 4.5 hours at 37°C, 5% CO_2 or were cultured for 20 hours with 10 ng/ml IL2 prior to stimulation on aNKG2D (10 µg/ml) coated plates. A, B: Cells were cultured in the presence of Brefeldin A. Cells were harvested and stained for NK1.1 or NKp46 and CD3. After cell permeabilzation with 0.5% saponin cells were stained intracellular for IFNγ. C, D: Cells were cultured in the presence of Brilliant violet 421 conjugated anti-CD107a antibodies. Cells were then stained for NK1.1 or NKp46 and CD3 prior to FACS analysis. P values (* p<0.03) were calculated by using the two tailed Mann-Whitney t-test. Error bars represent the SEM.

4.2.2.3. NK cells induce cytotoxicity against YAC-1 cells in the absence of coronin 1

NK cells are important for controlling tumor growth [15, 19, 21]. Tumor cells are controlled by NK cells through perforin/granzyme-dependent necrosis of target cells which involves cell adhesion, NK cell receptor triggering, and granule release. On the other hand, NK cells induce apoptosis in tumor cells which is mediated by surface TNF ligand family members FasL, TNF-α and TRAIL, each of which interact with specific receptors on the target cell surface [248]. The ability of NK cells to produce IFNγ and to degranulate does not exclude an impaired cytotoxic function. Therefore, we investigated whether coronin 1-deficient NK cells were able to induce cytoxicity in the tumor cell line YAC-1 (Figure 4. 16). In a first experiment, we co-culture splenocytes from coronin 1-deficient or wild type mice with YAC-1 cells and measured the NK cell activation (Figure 4. 16A).

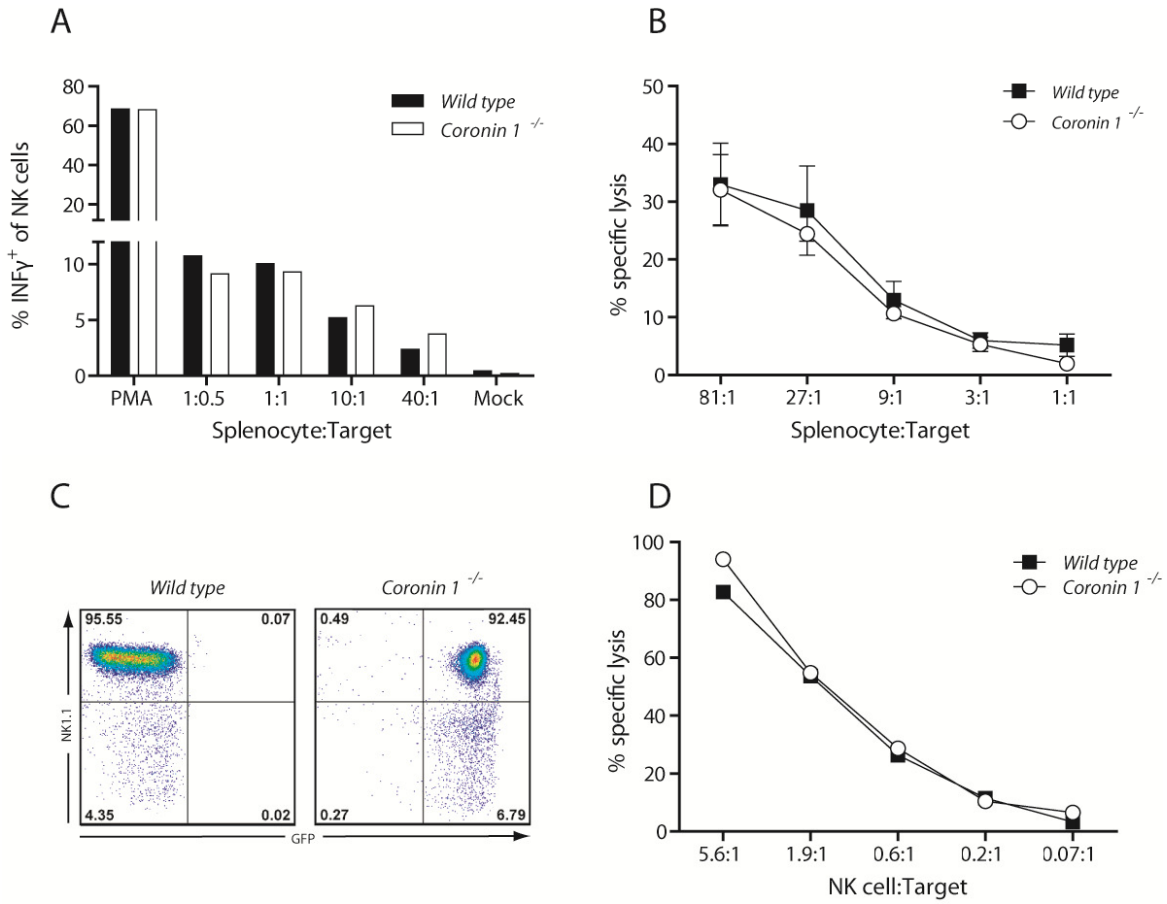


Figure 4. 16 Cytotoxic activity of wild type and coronin 1-deficient NK cells against YAC-1 cells.

Splenocytes were isolated from the spleen of wild type or coronin 1-deficient mice. A: Splenocytes were co-cultured at the indicated splenocyte:target ratio with YAC-1 cells for 4.5 hours. Cells were then harvested and stained for NK1.1, CD3 and intracellularly for IFNγ. B: Splenocytes were co-cultured at the indicated splenocytes:target ratio with $5x10^4$ Cr-51 radio-labeled YAC-1 cells and cytoxicity was measured in a 4.5 hours Cr-51-release assay (pooled data from four independent experiments). C: NK cells were purified and pooled from two spleens and stained for NK1.1 and CD3. FACS dot-plot of purified NK cells gated on total viable lymphocytes. D: Purified NK cells were used in a 4.5 hours cytoxtoxic Cr-51-release assay with $5x10^4$ Cr-51 radio-labeled YAC-1 cells. Error bars represent the SEM.

Ex vivo stimulation of splenocytes with YAC-1 cells induced IFNγ production in both wild type and coronin 1-deficient NK cells to the same extend. In addition, cytotoxicity of wild type and coronin 1-deficient NK cells was measured via a Cr-51-release assay. Also here both wild type and coronin 1-deficient NK cells were able to induce lysis of YAC-1 cells (Figure 4. 16B). To make sure that lysis of YAC-1 cells was mediated by NK cells and not by other immune cells; we purified NK cells from spleen of wild type and coronin 1-deficient (GFP^+) mice (Figure 4. 16C) and used these cells in a Cr-51-release assay (Figure 4. 16D). Also here, specific lysis of YAC-1 cells by wild type and coronin 1-deficient NK cells was similar. From these results we conclude that NK cell functionality is not impaired in the absence of coronin 1.

4.2.2.4. Increased reduction in NK cell functionality of coronin 1-deficient NK cells after Con A treatment

Concavalin A (Con A) from *Canavalia ensiformis* binds glycoproteins and glycolipids and is often used as a mitogen [258-263]. In vivo Con A administration into C57BL/6 mice induces strong hepatitis which is mediated by T, NKT and NK cells [264-266]. Siegmund et al. (unpublished) observed that intravenous Con A administration induces liver inflammation in wild type mice, which was reduced in coronin 1-deficient mice. Moreover, NK cells of coronin 1-deficient mice did not produce any IFNγ compared to wild type NK cells after Con A administration. Therefore, we investigated whether Con A treatment of coronin 1-deficient mice had any effect on NK cell cytotoxicity against YAC-1 cells. For this we treated wild type and coronin 1-deficient mice with 13 mg/kg Con A i.v. for 12 hours, isolated the splenocytes and co-cultured these cells with Cr-51 radio-labeled YAC-1 cells for 4.5 hours (Figure 4. 17A). We found that NK cells mediated cytotoxicity was strongly reduced in Con A treated coronin 1-deficient mice. To exclude any inhibitory effects from other immune cells, we purified NK cells from spleen of wild type and coronin 1-deficient Con A treated mice and analyzed NK cell functionality in Cr-51 release assay. Also in these experiments we found decreased cytotoxicity of coronin 1-deficient NK cells against YAC-1 cells compared to wild type NK cells (Figure 4. 17B). We conclude from these experiments that Con A treatment leads to a reduced NK cells activation and cytotoxicity against YAC-1 cells in the absence of coronin 1. We argue, that these observations might be due to an increased apoptosis or inhibition of coronin 1-deficient NK cells after Con A treatment [267, 268], since NK cell numbers in the spleen are comparable in Con A treated wild type and coronin 1-deficient mice (not shown).

To get a deeper insight into the mechanism of Con A induced reduction of NK cell functionality in the absence of coronin 1, we stimulated splenocytes from wild type or coronin 1-deficient mice for 20-22 hours *ex vivo* with 2.5 µg/ml Con A or IL15 (10 ng/ml), as pro-survival cytokine, prior to adding Cr-51 radio-labeled YAC-1 cells (Figure 4. 18). We found that only wild type splenocytes treated with Con A (Figure 4. 18A) or IL15 (Figure 4. 18B) maintained their functionality after 20 hours. However, coronin 1-defcient NK cells maintained their functionality upon stimulation with IL15.

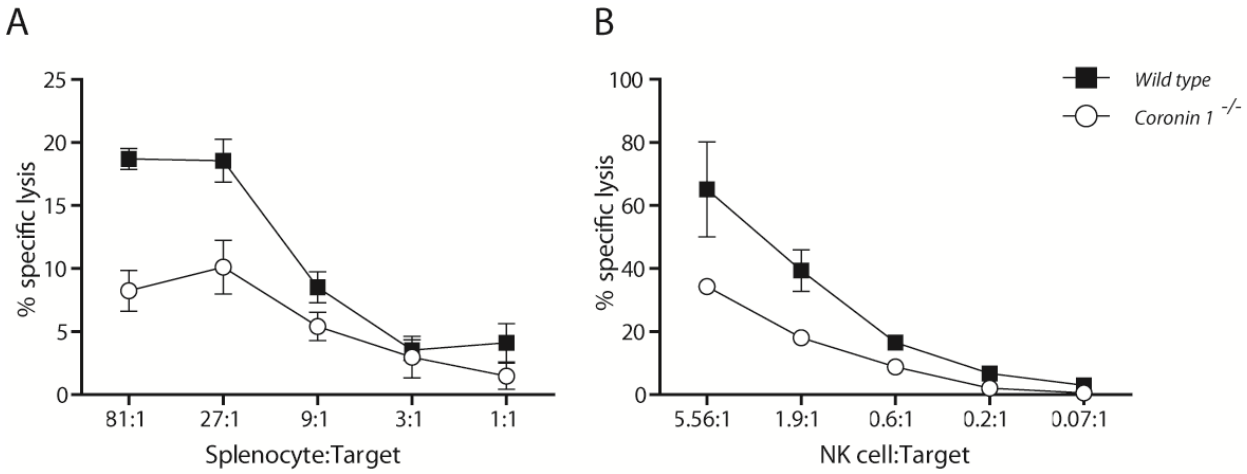


Figure 4. 17 Cytotoxicity of wild type and coronin 1-deficient NK cell against YAC-1 cells after Concanavalin A treatment *in vivo*.

Wild type and coronin 1-deficient mice were treated with 13 mg/kg Con A for 12 hours. A: Splenocytes were co-cultured at the indicated splenocytes:target ratio with $5x10^4$ Cr-51 radio-labeled YAC-1 cells and cytoxicity was measured in a 4.5 hours Cr-51-release assay (pooled data from four independent experiments). B: NK cells were purified from spleen and used in a 4.5 hours cytoxtoxic Cr-51-release assay with $5x10^4$ Cr-51 radio-labeled YAC-1 cells at the indicated effector:target ratio (pooled data from two independent experiments). Error bars represent the SEM.

Notably, stimulation of purified NK cells from wild type and coronin 1-deficient mice with Con A completely abolished cytotoxicity against YAC-1 cells after 20 hours stimulation (not shown). These results suggested that first, Con A stimulation can maintain NK cell functionality only in the presence of coronin 1 and second, whole splenocytes are needed to maintain functionality of NK cells in the presence of Con A.

4.2.2.5. Increased spontaneous apoptosis in coronin 1-deficient NK cells

We hypothesized that impaired NK cell functionality in the absence of coronin 1 is most likely because of a missing pro-survival signal secreted by spleen cell resulting in the deletion of NK cell by apoptosis. To investigate whether coronin 1-deficient NK cells functionality was impaired due to decreased cell viability and increased apoptosis, we stimulated whole splenocytes with or without Con A for 10 and 20 hours and analyzed cell viability and frequency of apoptotic cells (Figure 4. 19). Con A stimulation decreased NK cell recovery in the absence of coronin 1. On the other hand, wild type NK cell recovery was increased in the presence of Con A compared to NK cells cultured in medium alone (Figure 4. 19A).

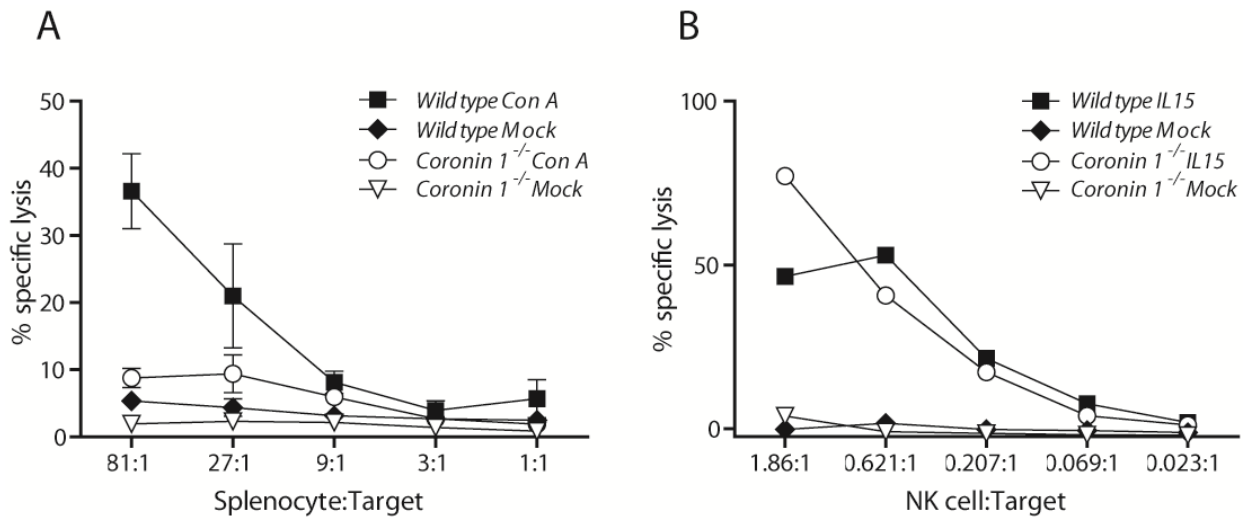


Figure 4. 18 Maintenance of NK cells functionality by Con A and IL15 stimulation *in vitro*.

Wild type and coronin 1-deficient mice were sacrificed and spleen cells were harvested and used for an *ex vivo* Cr-51 release assay. A: Splenocytes were cultured in a 96 round bottom wells with Con A (2.5 µg/ml) or medium (mock) for 20-22 hours. $5x10^4$ Cr-51 radio-labeled YAC-1 cells were then added to the splenocytes and cytotoxicity was measured in a 4.5 hours Cr-51-release assay (pooled data from three independent experiments). B: The percentage of NK cells in the spleen was measured by FACS analysis and the same NK cell number were distributed to the wells. Cells were stimulated with IL15 (10 ng/ml) or medium alone (mock) for 20-22 hours. $5x10^4$ Cr-51 radio-labeled YAC-1 cells were then added to the splenocytes and cytotoxicity was measured in a 4.5 hours Cr-51-release assay (pooled data from two independent experiments). Error bars represent the SEM.

By analyzing apoptotic cells by staining with Annexin V and 7-AAD, we found an increased frequency of apoptotic coronin 1-deficient NK cells compared to wild type NK cells with or without Con A (Figure 4. 19B). Even more, Con A treatment induced increased apoptosis in coronin 1-deficient NK cells (Figure 4. 19B). These results suggest that coronin 1-deficient NK cells were more prone to undergo spontaneous apoptosis and were more sensitive to Con A induced apoptosis.

4.2.3. **Discussion**

NK cells belong to the innate immune system, which are important for controlling acute viral infection and tumor cell growth [16, 19, 24, 246-248, 269-272]. Therefore we investigated the functionality of NK cells, which may be impaired in the absence of coronin 1. We infected mice with VSV and analyzed NK cell functionality after two days of infection. We found that NK cells were able to become activated by starting to produce IFNγ. These results showed that NK cells were functional upon VSV infection in the absence of coronin 1.

NK cell receptor signaling is very similar to TCR signaling [14, 28, 104, 252, 273]. On the other hand, NK cells use different activating receptors (e.g. NK1.1, NKp46, Ly49D and NKG2D) which transmit their signal via their specific ITAM containing adaptor molecules. One can differentiate between the adaptor molecules DAP12, CD3ζ and FcεRγ. These adaptor molecules recruit Src kinases (e.g. Syk, Fyn, LCK) and initiate a downstream phosphorylation cascade upon activation which results in intracellular Ca^{2+} release. Thus, the DAP10 adaptor molecule signaling is strictly dependent on PLCγ2 and PI3K activation [14, 15, 21, 257, 273]. Coronin 1 was shown to immunoprecipitate with PLCγ, which suggest a requirement of coronin 1 during TCR downstream signaling pathway [184]. To dissect whether one of the NK cell receptor signaling pathway was impaired in the absence of coronin 1, we stimulated NK cells specifically with plate bound antibodies directed against the NK cell activating receptors NK1.1 (DAP12), NKp46 (DAP12), Ly49D (CD3ζ and FcεRγ), NKG2D (DAP10). Stimulation of these receptors results in comparable IFNγ production and degranulation (measured by LAMP-1 (CD107a)) in coronin 1-deficient and wild type NK cells. These results showed that activating NK cell receptor signaling is not impaired in the absence of coronin 1. Similarly, it was shown that FcεRγ signaling was not impaired coronin 1-deficient mast cells [190]. In addition, NKG2D can signal through DAP10 or DAP12, whereas DAP10 signaling was shown to be highly dependent on PLC-γ2 activity [15]. Yet, activated NK cells (for instance by IL2 or stress) were shown to preferentially engage NKG2D signaling through the adaptor molecule DAP12 [14, 15, 256, 257]. Hence, it remains elusive whether DAP10 signaling is intact in the absence of coronin 1.

Next, we asked whether NK cells were able to become activated and lyse tumor cells. For this we performed Cr-51 release assay on radio-labeled YAC-1 cells. We found that NK cell from both wild type and coronin 1-deficient mice were able to produce IFNγ and to lyse YAC-1 cells to the same extend. Cytotoxicity against YAC-I cells was shown to be independent of Syk/Fyn signaling [27], which could explain the cytotoxicity against YAC-1 cells observed by coronin 1 deficient NK cells.

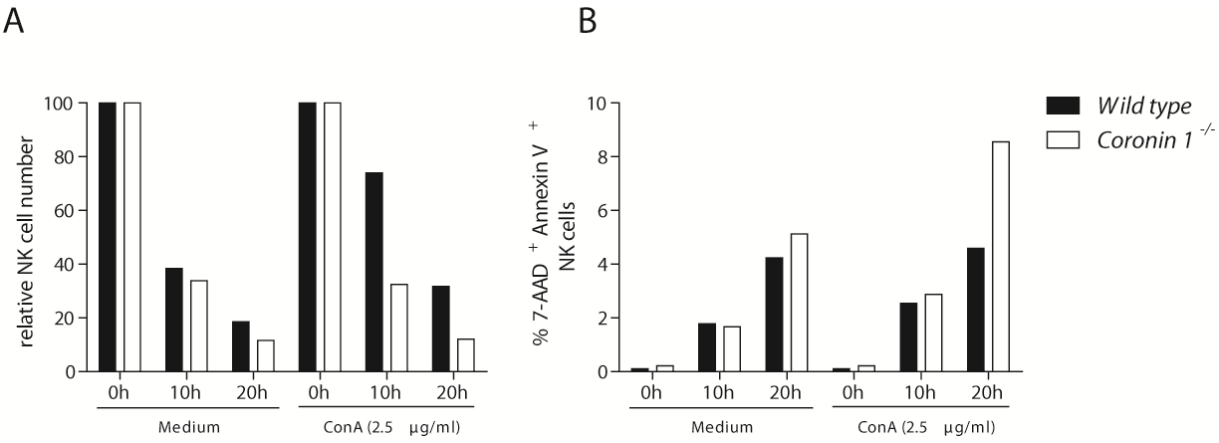


Figure 4. 19 Increased apoptosis of coronin 1-deficient NK cells.

$2x10^6$ splenocytes from wild type or coronin 1-deficient mice were treated *in vivo* with Con A (2.5 µg/ml) or medium alone for 10 or 20 hours. A: Cells were harvested and counted and stained for NK1.1 and CD3 and analyzed by FACS. NK cell numbers were normalized to the NK cell count at time point 0. B: Cells were harvested and stained for NK1.1, CD3, Annexin V and 7-AAD.

Together with the unpublished observation that coronin 1-deficient mice do not have increased tumor development compared to wild type mice and the comparable activation of NK cells, by a variety of activating NK cell receptors using different ITAM containing adaptor molecules, we conclude that NK cell receptor signaling and functionality is not impaired in the absence of coronin 1.

Siegmund et al. (unpublished) observed that Con A treatment of mice results in IFNγ production in wild type NK cells but not in coronin 1-deficient NK cells. Therefore, we investigated whether Con A treatment influences NK cell cytotoxicity against YAC-1 in the absence of coronin 1. Interestingly, we found a strong reduction in cytotoxicity of coronin 1-deficient NK cells against YAC-1, after *in vivo* and *in vitro* Con A treatment. Con A was shown to reduce cytotoxicity against P815 [267]. Moreover, Con A was shown to be toxic leading to apoptosis of lymphocytes [268, 274]. We hypothesize that Con A treatment inhibits NK cell functionality more efficiently in the absence of coronin 1 and leads to increased Con A mediated apoptosis. In addition, $CD4^+$ lymphocytes were shown to be important for Con A induced hepatits [266]. Since, coronin 1-deficient mice have reduced $CD4^+$ T cell numbers; it could explain the observed reduction in NK cell activation which would be dependent on $CD4^+$ and NKT cell [264]. Furthermore, *in vitro* Con A treatment of splenocytes abolished cytotoxicity of coronin 1-deficient but not of wild type NK cells. Moreover, we observed that incubation of purified NK cells with Con A abolished cytotoxicity against YAC-1 cells

completely, of both wild type and coronin 1-deficient NK cells, after 20 hours *in vitro* incubation. These results suggest that other spleen derived cells, such as $CD4^+$ T cells, are crucial for maintaining NK cell functionality upon Con A stimulation *ex vivo*. Notably, IL15 stimulation of splenocytes maintained NK cell cytotoxicity of both wild type and coronin 1-deficient NK cells. Interestingly, we found an increased rate of apoptosis of coronin 1-deficient NK cells cultured with or without Con A. According to these results, naive T cells were also shown to undergo increased apoptosis in the absence of coronin 1 [183]. Therefore it is possible that coronin 1 is an important pro-survival component in NK cells.

$CD4^+$ T cells are a source of pro-survival cytokines such as IL2 [244, 275]. Reduced $CD4^+$ T cell numbers and impaired activation could result in reduced secretion of pro-survival cytokines following Con A stimulation in coronin 1-deficient mice. Moreover, IL12 and IL15 are NK cell pro-survival cytokines, which are mainly produced by monocytes [276-279]. In addition to the low $CD4^+$ T cell numbers in coronin 1-deficient mice, production of IL12 and IL15 could be reduced upon Con A stimulation in the absence of coronin 1. This could explain the increased apoptosis and impaired cytotoxicty of NK cells in the absence of coronin 1.

Overall, we conclude that NK cell activation and functionality is not per se impaired in the absence of coronin 1, but that NK cells are more prone to undergo apoptosis due to a missing pro-survival factor, which may be $CD4^+$ T cell dependent. These results give an insight into the role of coronin 1 during NK cell activation and homeostasis, and suggest coronin 1 as pro-survival factor.

5. General discussion

5. General discussion

Coronin 1 belongs to the superfamily of WD40 protein and in mammals coronin 1 is predominantly expressed in cells from haematopietic origin and the nervous system [160, 162, 172, 173, 179, 180]. Coronin 1-deficiency was showed to be associated with impaired Ca^{2+} mobilization upon T cell receptor stimulation, resulting in rapid peripheral T cell deletion through apoptosis [183-185]. In the first part of that thesis, I have investigated the $CD8^+$ and $CD4^+$ T cell immune response to MCMV, LCMV and VSV, in the absence of coronin 1. We found that $CD8^+$ T cell responses to all three viruses were similar in wild type and coronin 1-deficient mice. On the other hand, $CD4^+$ T cell activation and expansion were impaired upon acute virus infection, in the absence of coronin 1. Hence, coronin 1-deficiency was associated with increased susceptibility to VSV infection. However, we propose that pro-inflammatory cytokines released from infected cells and dendritic cells after viral infection, pre-activate T cells, resulting in a coronin 1-independent T cell activation. Furthermore, coronin 1 might modulate the TCR activation threshold. Though, coronin 1-deficiency would lead to an increased deletion of T cell during negative and positive selection. Taken together, we suggest that coronin 1 is more important for $CD4^+$ T cell than for $CD8^+$ T cell response. Yet, the exact requirement of coronin 1 for $CD4^+$ T cell activation compared to $CD8^+$ T cell activation remains elusive.

Since Natural killer (NK) cell receptor signaling is believed to have strong similarities with the T cell receptor signaling pathway [14, 21, 28, 30, 32, 104, 242, 252], I analyzed, in the second part of that thesis, the NK cell mediated immune response in the absence of coronin 1. We found, that NK cell function is not impaired in the absence of coronin 1. Interestingly, Concanavalin A (Con A) treatment reduced coronin 1-deficient NK cell functionality significantly. We conclude that NK cells are highly sensitive to Con A induced apoptosis or inhibition. Moreover, we suggest that reduced NK cell functionality after Con A treatment is due to the low $CD4^+$ T cell numbers in the coronin 1-deficient mice, which were shown to indirectly activate NK cells after Con A treatment [264, 266]. Overall, we showed that coronin 1-deficiency was associated with decreased NK cell survival, proposing coronin 1 as a pro-survival factor for NK cells.

6. Supplementary results

6. Supplementary results

6.1. T cell repertoire of coronin 1-deficient mice

B cells, neutrophils and macrophages were shown not to have significant defect in the absence of coronin 1. Nonetheless, coronin 1-deficiency is associated with naïve T cell lymphopenia. Coronin 1-deficient T cell develop normally in the thymus and in the bone marrow, while naïve T cells get systematically deleted from the periphery [179, 182, 184, 189]. It was shown that coronin 1 is acting downstream of the TCR by interacting with PLCγ which cleaves PIP_3 to diacylglycerol and IP_3 which in turn leads to intracellular Ca^{2+} release from the ER. Therefore, coronin 1 was shown to provide pro-survival signal to naïve T cells by modulating Ca^{2+} mobilization. More recent coronin 1 was shown to be important in T cell migration and homeostasis [183, 204]. Interestingly, coronin 1-deficient T cells have mainly a central memory phenotype ($CD44^+$, $CD62L^+$).

Homeostasis and expansion of memory cells in a lymphopenic environment can result in a restricted T cell receptor repertoire [224]. Therefore, we were interested whether coronin 1-deficient mice had a limited T cell repertoire due to homeostatic proliferation in a lymphopenic environment. For this we isolated lymphocytes from thymus, spleen and lymph nodes and stained for $CD8^+$, $CD4^+$ and for the TCR variable β regions (Figure 6. 1). We found that usage of the TCR variable β chains on $CD8^+$ (Figure 6. 1A) as well as on $CD4^+$ (Figure 6. 1B) T cells was not restricted in the organs tested of coronin 1-deficient mice. We conclude from these results that newly developed T cell can reach the periphery, thereby contributing to a diverse peripheral T cell repertoire.

6.2. Distorted T cell ratio in coronin 1-deficient mice

We observed that the relative frequency of $CD4^+$ T cell was lower than the relative $CD8^+$ T cell frequency in the periphery of coronin 1-deficient mice compared to wild type mice. For this we have analyzed the CD8:CD4 T cell ratio in coronin 1-deficient and wild type mice (Figure 6. 2).

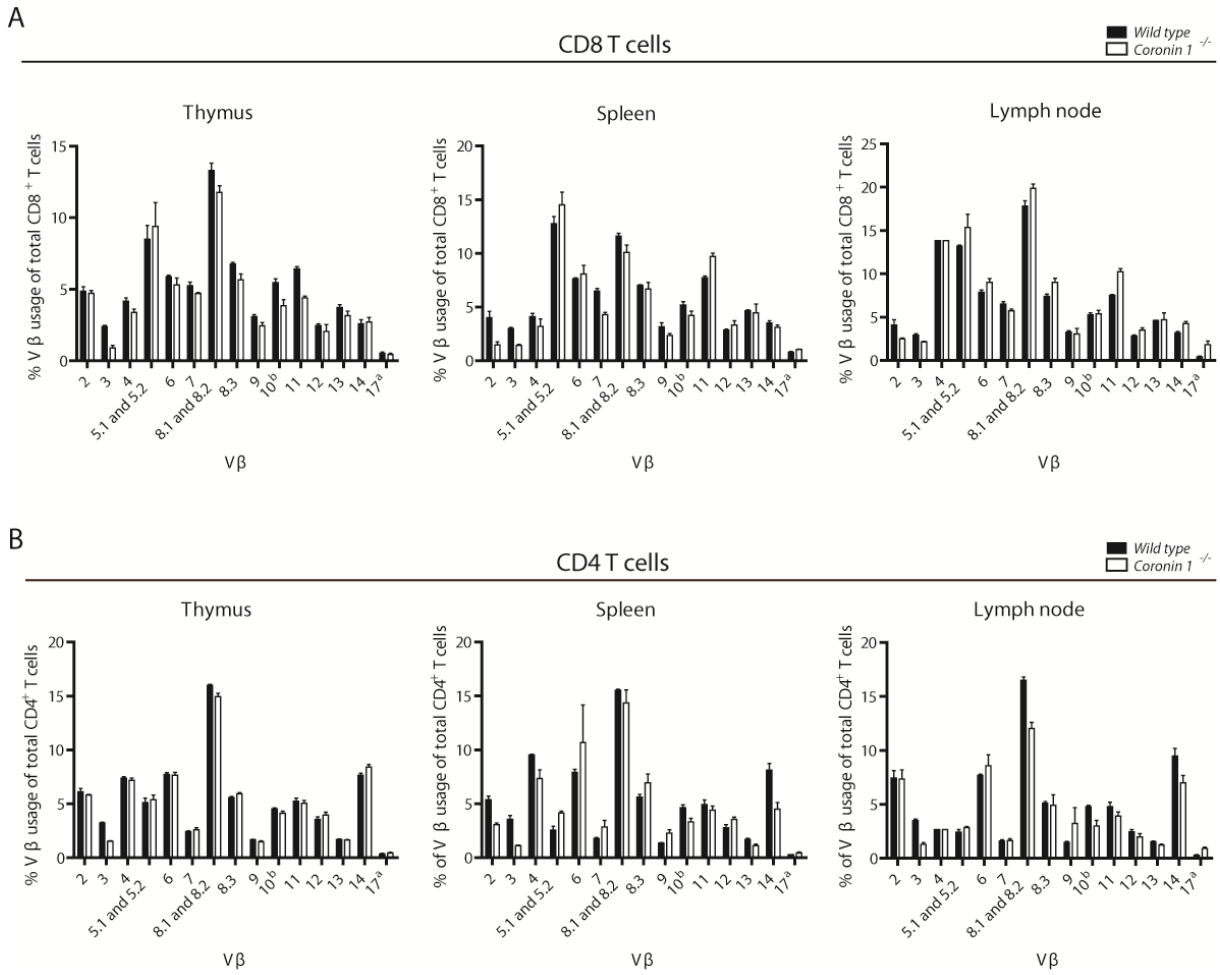


Figure 6. 1 TCR variable β chain usage of wild type and coronin 1-deficient CD8$^+$ and CD4$^+$ T cells.

Expression of the TCR variable β chain in CD8 (A) and CD4 T-cells (B). Lymphocytes from 5 month old wild type and 8 month old coronin 1-deficient mice were isolated from the thymus, spleen and lymph nodes and stained for CD8, CD4 and with the indicated anti-mouse Vβ-antibody. Cells were subsequently analyzed by FACS (n=3). Error bars represent the SEM.

We found that the CD8:CD4 T cell ratio was distorted in the blood, lung and spleen of coronin 1-deficient mice. However, CD8:CD4 T cell ratio in the lymph nodes of coronin 1-deficient mice was found to be normal compared to the T cell ratio from lymph nodes of wild type mice.

Survival of memory T cells was shown to be independent of pro-survival signal through TCR [280, 281]. Furthermore, expression of activation marker such as sialophorin (CD43) was shown to inhibit T cell receptor/CD3-mediated apoptosis [225]. Interestingly, we found that up to 40% of CD4$^+$ T cells from coronin 1-deficient mice (wild type mice ~5%) stained positive for CD43, whereas CD43 expression on CD8$^+$ T cells was comparable in coronin 1-deficient and wild type mice (Figure 6. 3).

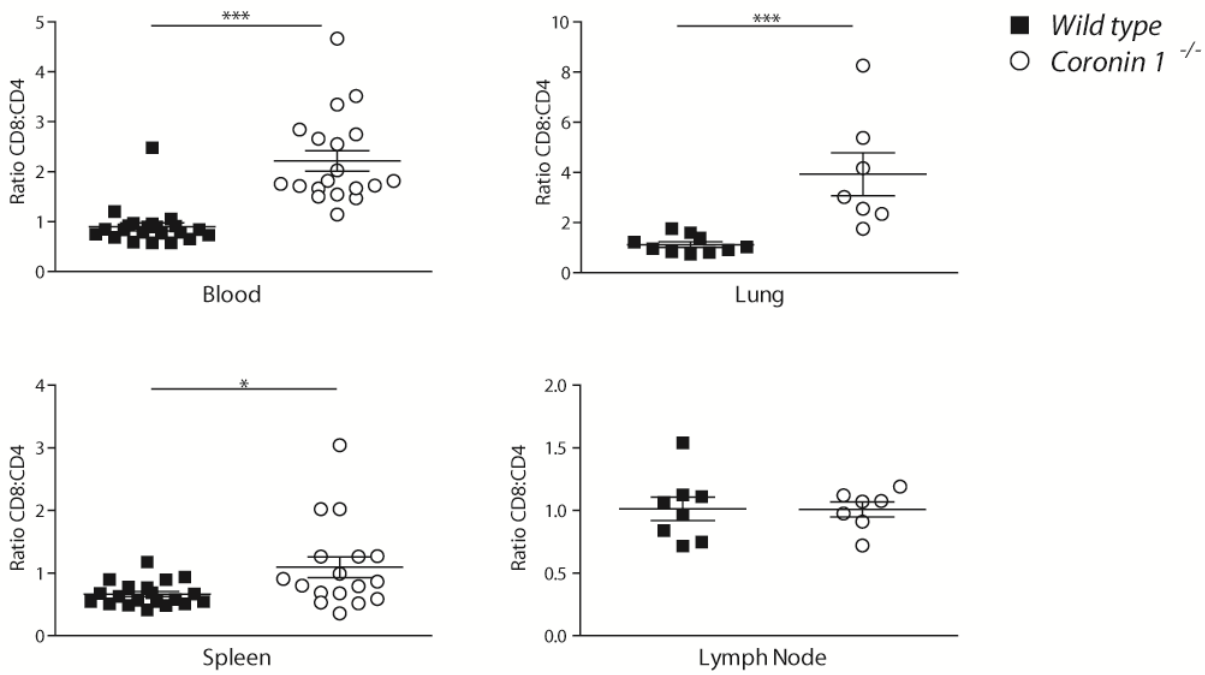


Figure 6. 2 Distorted T cell ratio in the periphery of wild type and coronin 1-deficient mice.

CD8:CD4 ratio in lymphoid and peripheral organs of naïve coronin 1-deficient and wild type C57Bl/6 mice. Lymphocytes were isolated from spleen, lung, whole blood and lymph nodes. Lymphocytes were stained for CD8 and CD4 and T cell ratio were analyzed by FACS. P values (* $p<0.015$; *** $p\leq0.0002$) were calculated by using the two-tailed Mann-Whitney t-test. Error bars represent the SEM.

Correlating with these results, coronin 1-deficient $CD4^+$ T cells were expressing CD62L to a lower frequency (not shown). These results, suggested that coronin 1 plays a differential role during homeostasis and activation of $CD8^+$ and $CD4^+$ T cells contributing to a balanced CD8:CD4 T cell ratio.

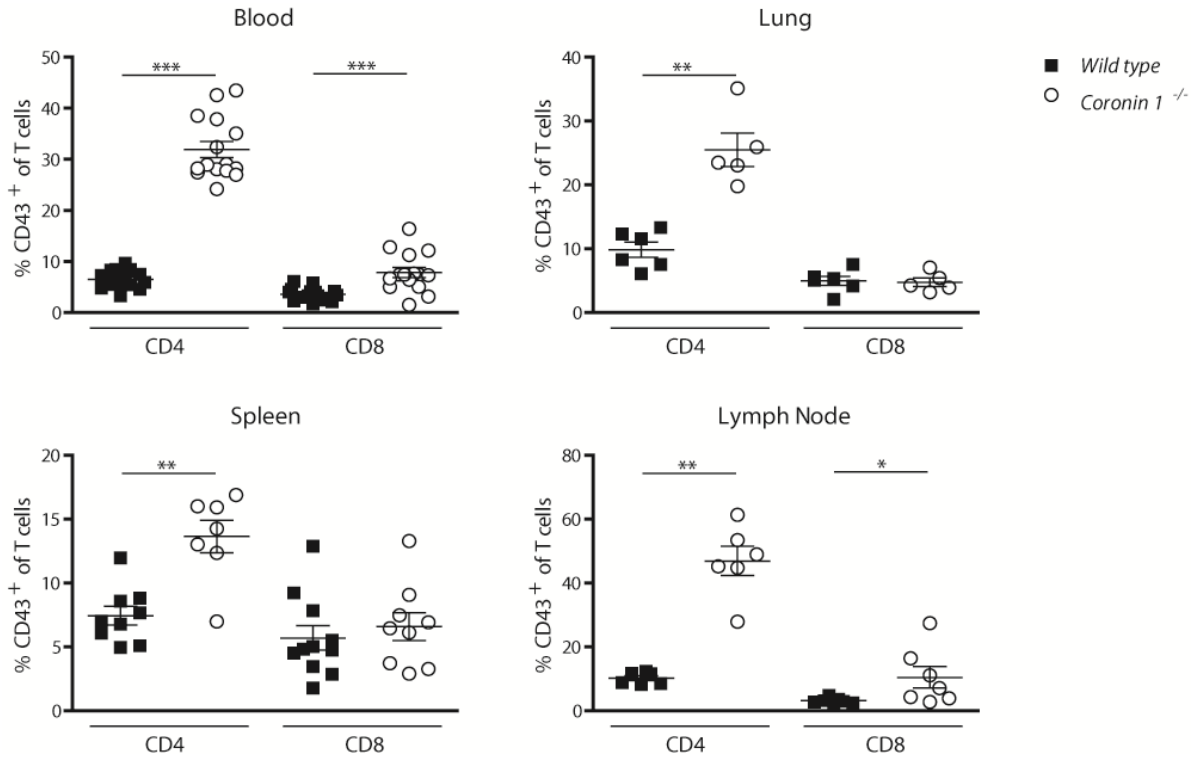


Figure 6. 3 CD43 expression on peripheral T cells from wild type and coronin 1-deficient mice.

Blood, lung, spleen and lymph node was harvested from uninfected coronin 1-deficient and wild type animals and stained for CD8, CD4 and the early activation marker CD43. P values (***p <0.0001; ** p<0.008; * p<0.03) were calculated by using the two-tailed Mann-Whitney t-test. Error bars represent the SEM.

6.3. T cell ratio after MCMV infection

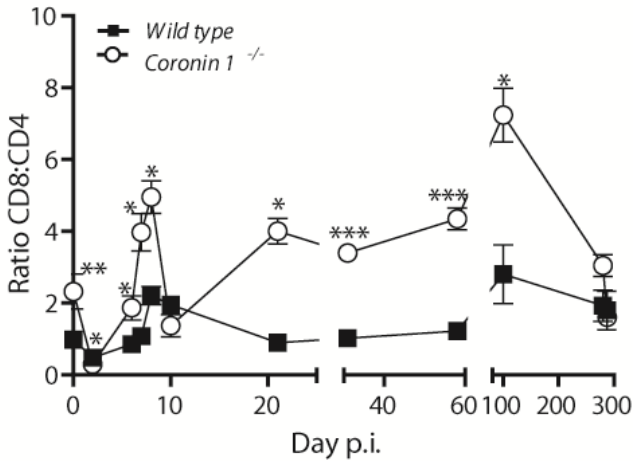


Figure 6. 4 T cell ratio after MCMV infection.

Wild type and coronin 1-deficient mice were infected with 2 x 10^6 PFU MCMV-ΔM157. Whole blood was harvested at the indicated time points and stained for CD8 and CD4. CD8:CD4 T cell ratio was calculated from the FACS data. P values (***p <0.0001; ** p<0.008; * p<0.03) were calculated by using the two-tailed Mann-Whitney t-test (n=3-5). Error bars represent the SEM.

6.4. T cell ratio after LCMV infection

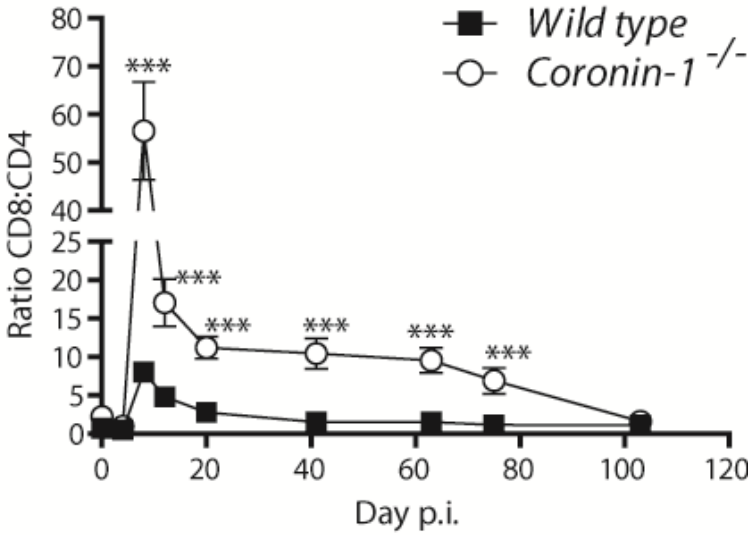


Figure 6. 5 T cell ratio after LCMV infection.

Wild type and coronin 1-deficient mice were infected with 200 PFU LCMV-WE. Whole blood was harvested at the indicated time points and stained for CD8 and CD4. CD8:CD4 T cell ratio was calculated from the FACS data. P values (***p <0.0008) were calculated by using the two-tailed Mann-Whitney t-test (n=3-6). Error bars represent the SEM.

6.5. Hämalaun – Eosin staining of LCMV infected footpads

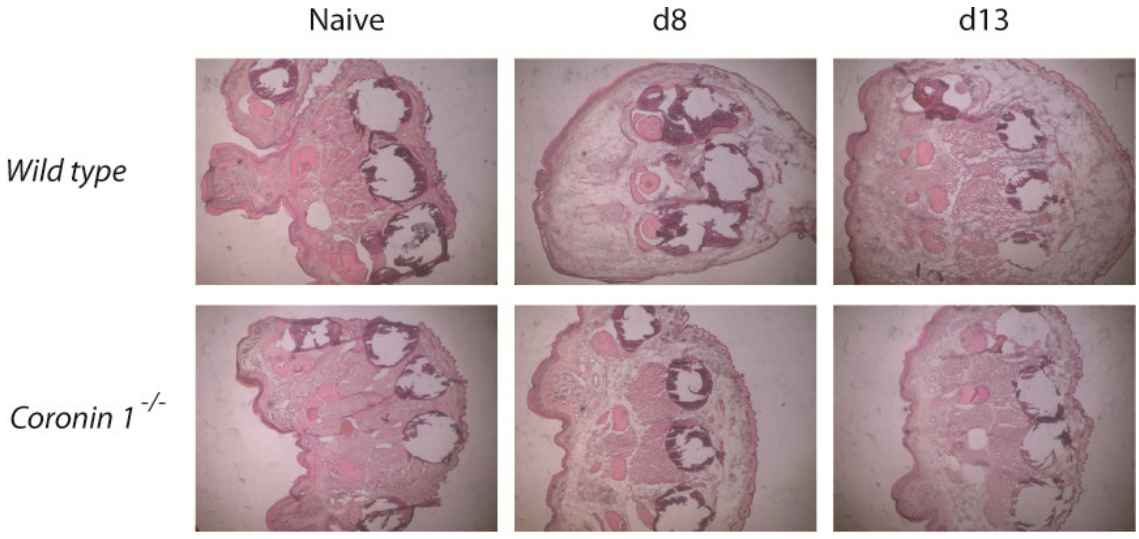


Figure 6. 6 Hämalaun – Eosin staining of LCMV infected footpads from wild type and coronin 1-deficient mice.

Wild type and coronin 1-deficient mice were subcutaneously infected into the right and left footpad with LCMV-WE. Mice were sacrificed at day 8 and 13 post infection and footpads were frozen in O.C.T compound at -80°C. 10 µm thin footpad sections were cut with a cryostat prior HE-staining.

6.6. CD43 expression on peripheral T cells after LCMV infection

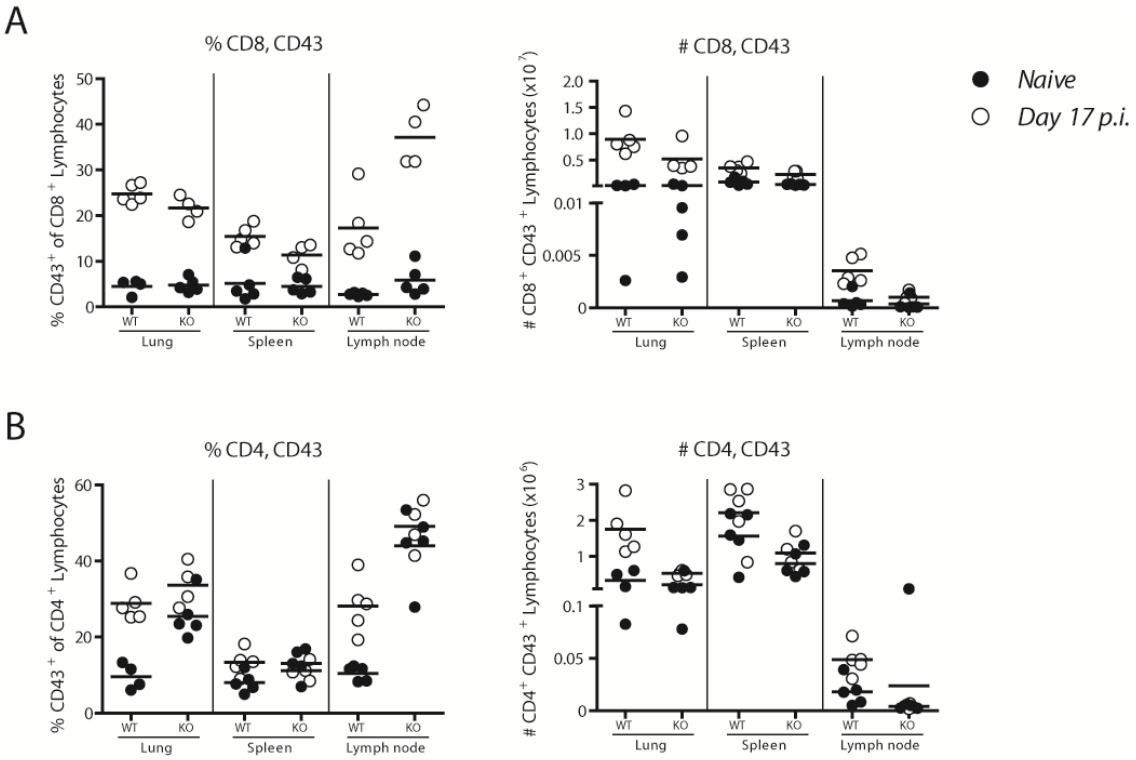


Figure 6. 7 CD43 expression on peripheral T cells after subcutaneous LCMV infection.

Mice were subcutaneously infected with LCMV-WE. At day 17 organs were harvested and lymphocytes were isolated. Cells were counted and subsequently stained for CD43, CD8 (A) and CD4 (B).

Table 6. 1 Relative increase in T cell frequency and upregulation of CD43 after subcutaneous footpad LCMV infection (average value compared to naïve mice)

Organ	Relative increase in T cell frequency				Relative increase in T cell activation (CD43)			
	CD8		CD4		CD8		CD4	
	Wild type	Coronin $1^{-/-}$	Wild type	Coronin $1^{-/-}$	Wild type	Coronin $1^{-/-}$	Wild type	Coronin $1^{-/-}$
Lung	3.86 ± 0.15	3.76 ± 0.12	0.73 ± 0.14	0.87 ± 0.1	5.48 ± 0.10	4.55 ± 0.09	2.99 ± 0.11	1.32 ± 0.06
Spleen	1.39 ± 0.34	1.66 ± 0.34	0.96 ± 0.14	1.8 ± 0.03	3 ± 0.61	2.53 ± 0.15	1.66 ± 0.13	0.85 ± 0.10
Lymph node	1.43 ± 0.07	4.84 ± 0.23	1.09 ± 0.05	1.57 ± 0.21	6.31 ± 0.15	6.36 ± 0.28	2.69 ± 0.08	1.12 ± 0.05

6.7. Delayed type hypersensitivity after LCMV infection in splenectomized mice

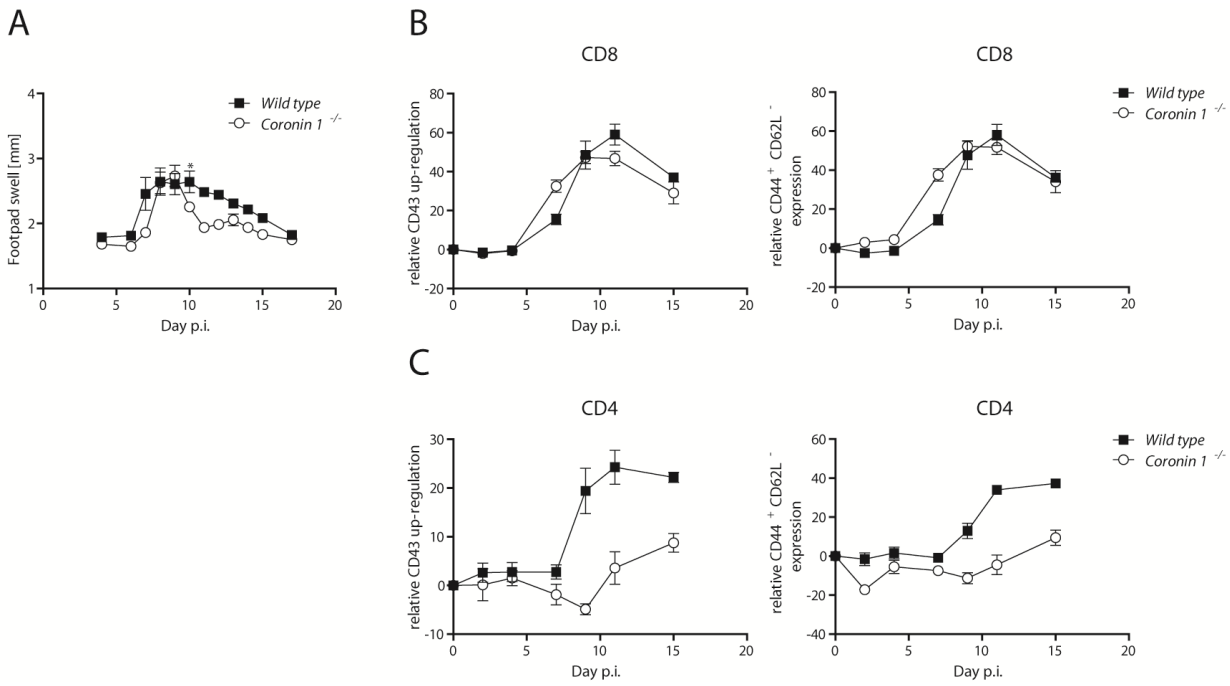


Figure 6. 8 Delayed type hypersensitivity after LCMV infection in splenectomized mice.

Wild type and coronin 1-deficient mice were splenectomized and left to recover from the surgery for one week. Mice were subcutaneously infected with LCMV-WE in the right and left footpad and inflammation was monitored over 17 days. A: Footpad swell measured with a caliper. B: Relative activation of blood derived $CD8^+$ T cell after LCMV infection. B: Relative activation of blood derived $CD4^+$ T cell after LCMV infection. Relative activation of T cells was calculated by subtracting the frequency of activated T cells from naïve mice from the frequency of activated T cells from infected mice. P values (* $p<0.03$) were calculated by using the two tailed Mann-Whitney t-test. Error bars represent the SEM (n=3-4)

6.8. CD43 expression on peripheral T cells in splenectomized mice after LCMV infection

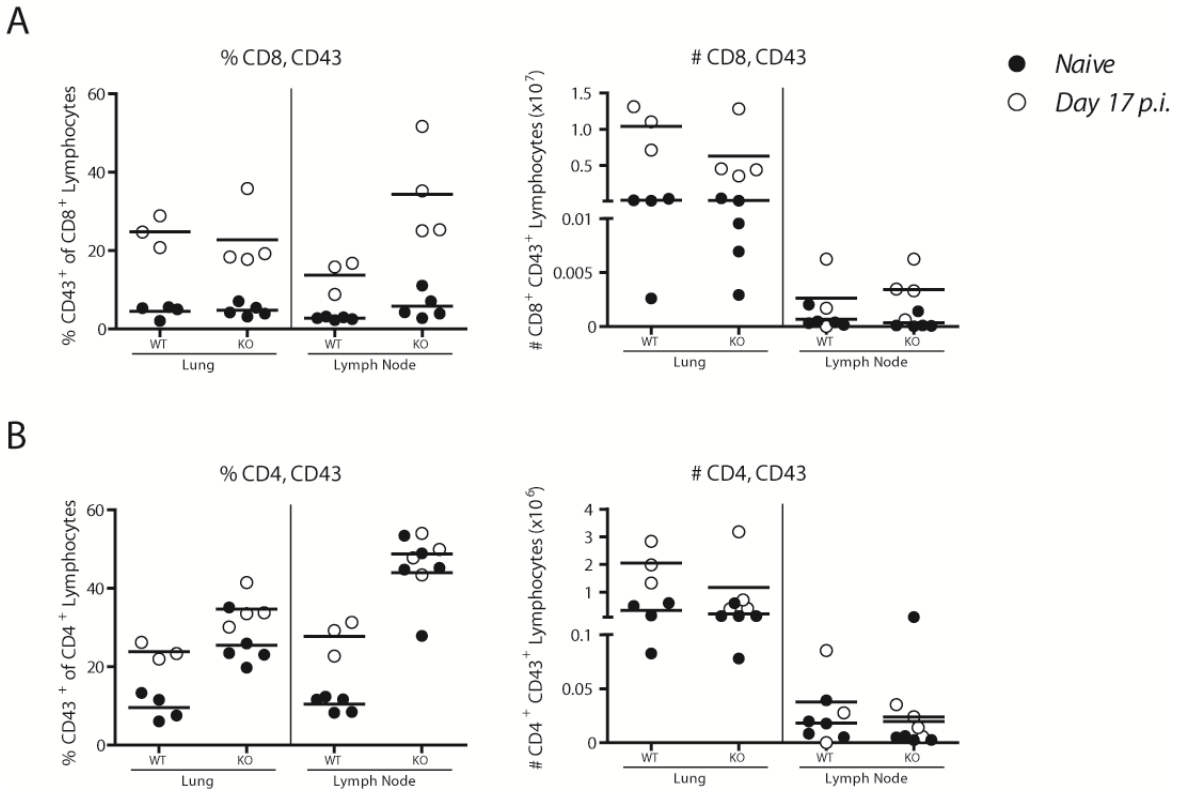


Figure 6. 9 CD43 expression on peripheral T cells in splenectomized wild type and coronin 1-deficient mice after subcutaneous LCMV infection.

Splenectomized mice were subcutaneously infected with LCMV-WE. At day 17 lung and lymph nodes were harvested and lymphocytes were isolated. Cells were counted and subsequently stained for CD43, CD8 (A) and CD4 (B).

Table 6. 2 Relative increase in T cell frequency and upregulation of CD43 after splenectomy and subcutaneous footpad LCMV infection (average value compared to naïve not splenectomized mice)

Organ	Relative increase in T cell frequency				Relative increase in T cell activation (CD43)			
	CD8		CD4		CD8		CD4	
	Wild type	Coronin $1^{-/-}$	Wild type	Coronin $1^{-/-}$	Wild type	Coronin $1^{-/-}$	Wild type	Coronin $1^{-/-}$
Lung	3.46 ± 0.17	3.70 ± 0.11	0.85 ± 0.07	1.27 ± 0.32	5.49 ± 0.11	4.79 ± 0.19	2.48 ± 0.10	1.36 ± 0.06
Lymph node	1.06 ± 0.08	2.24 ± 0.32	0.94 ± 0.04	1.01 ± 0.25	5.05 ± 0.08	5.88 ± 0.36	2.65 ± 0.05	1.11 ± 0.04

6.9. Subcapsular sinus macrophages in wild type and coronin 1-deficient mice

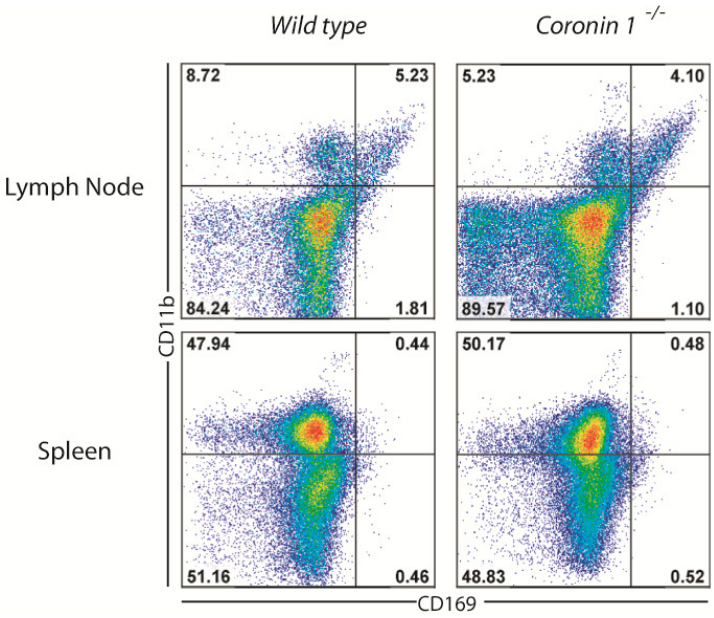


Figure 6. 10 Presence of subcapsular sinus macrophages in wild type and coronin 1-deficient mice.

Lymph nodes or splenocytes from wild type or coronin 1-deficient mice were isolated and stained for B220, CD11b and CD169. Representative FACS dot-plot gated on $B220^-$ lymphocytes.

6.10. Survival of wild type and coronin 1-deficient mice after subcutaneous VSV infection

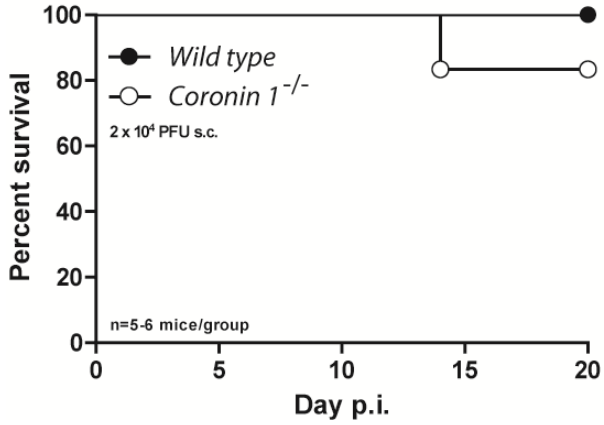
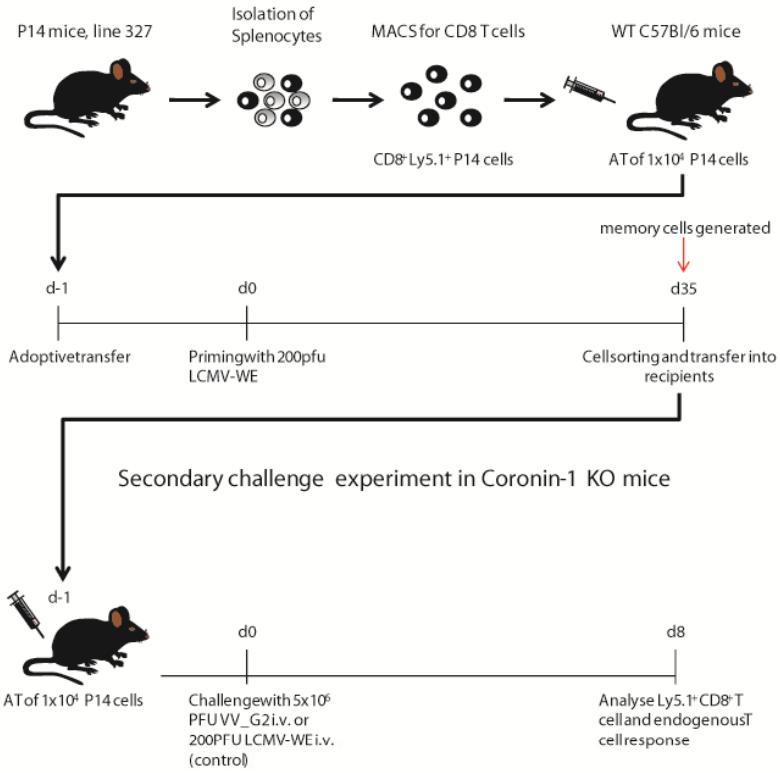


Figure 6. 11 Survival of wild type and coronin 1-deficient mice after subcutaneous VSV infection

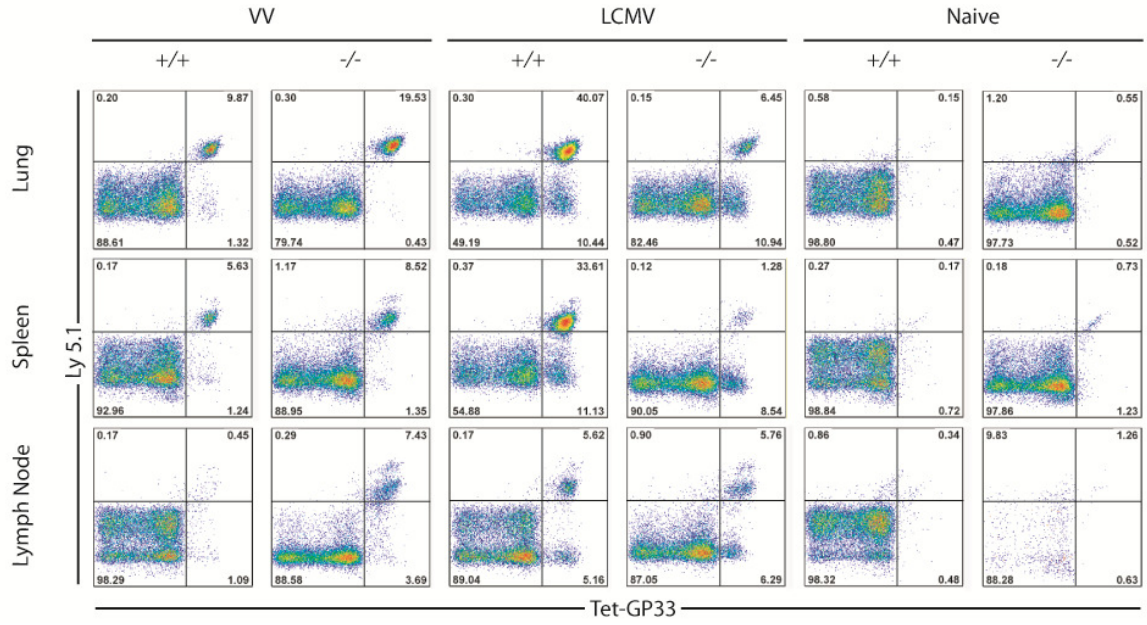
Wild type and coronin 1-deficient mice were infected subcutaneously with $2x10^4$ PFU VSV-INDG into the right hind footpad and survival of mice was monitored up to day 20 post infection.

6.11. CD4$^+$ T cell help upon secondary challenge in wild type and coronin 1-deficient mice

A



B



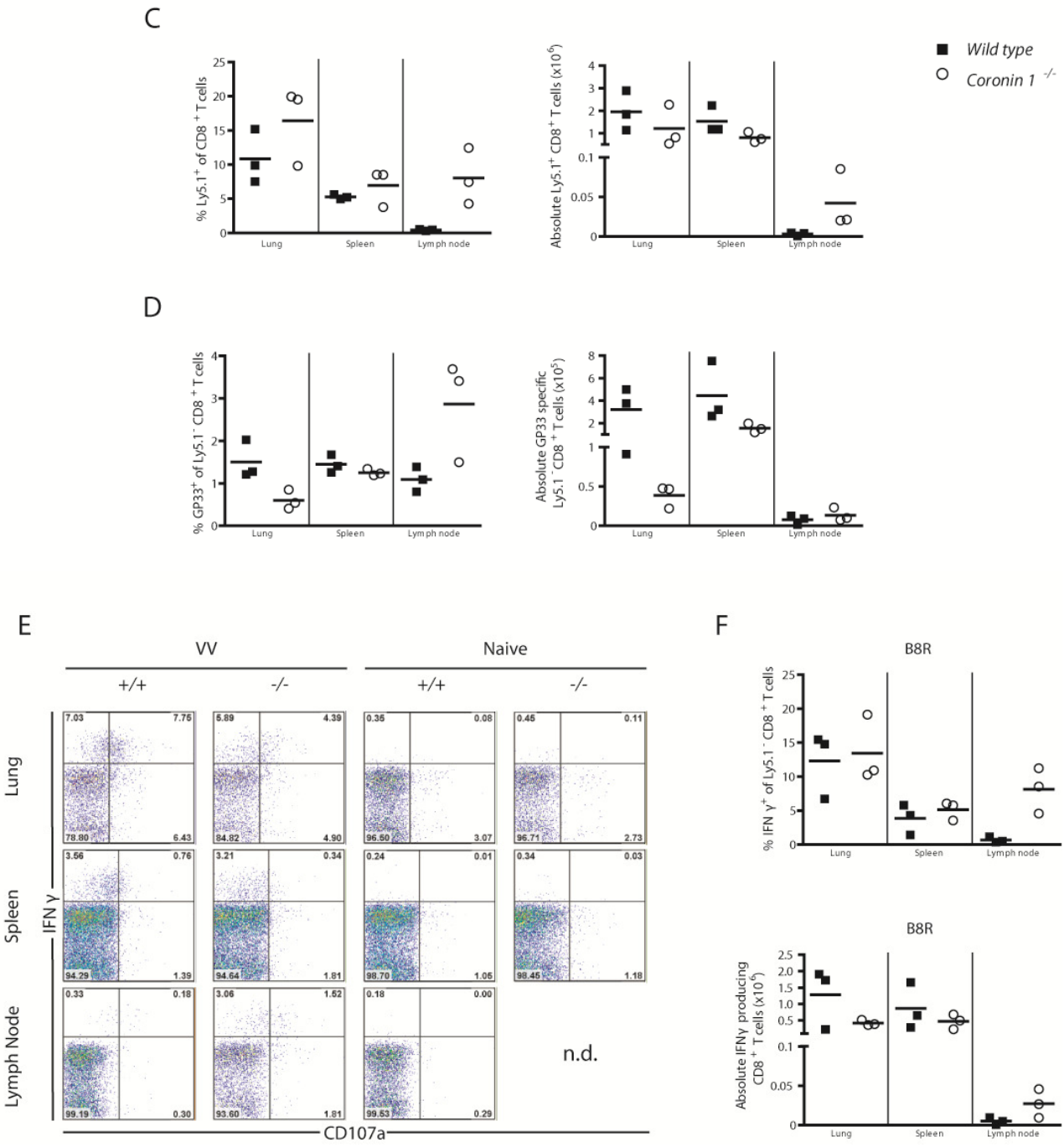


Figure 6. 12 CD4$^+$ T cell help upon secondary challenge in wild type and coronin 1-deficient mice.

A: Naïve Ly5.1 congenic P14 cells were adoptively transferred into C57Bl/6 mice, which were immunized one day later with low dose of LCMV expressing the OVA protein. At day 35 post infection, memory OT-I cells from these mice were FACS sorted and transferred into either naïve C57Bl/6 or coronin 1-deficient mice. One day later, mice were challenged with Vaccinia Virus expressing OVA or with LCMV-OVA as control. At day 8 post challenging, mice were sacrificed and organs were harvested and lymphocytes were isolated. B: Cells were stained for CD8, Ly5.1 and APC-conjugated GP33-tetramers. B: Representative FACS-dot plot gated on total CD8$^+$ T cells. Frequency and absolute numbers of Ly5.1$^+$ (C) and Ly5.1$^-$ (D) GP33$^+$ CD8$^+$ T cells. E: Representative FACS dot-plot gated on Ly5.1$^-$ CD8$^+$ T cells re-stimulated *ex vivo* with the specific CD8$^+$ T cells VV peptide B8R$_{20\text{-}27}$ in the presence of APC conjugated anti-CD107a and Monensin. Cell were then stained for CD8, Ly5.1 and intracellular for IFNγ. F: Frequencies and absolute numbers of Ly5.1$^-$ CD8$^+$ IFNγ producing T cells.

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9. Curriculum vitae

Education and Qualifications

2008-2012 **PhD thesis in immunology and infectious diseases at the University of Zurich and Basel**

Topic: The role of coronin 1 during cell mediated immune responses

Co-Supervisors: Prof. Dr. Jean Pieters and PD Dr. med. Urs Karrer

2007 **Master's degree in Microbiology, ETH Zurich**

Focus area: Immunology, Mycology, Food Microbiology, Medical Microbiology, Molecular and Genetic Microbiology

2005 – 2006 **Bachelor and Master's Thesis**

Institute of Food Science and Nutrition, ETH Zurich

Topic: Construction and functional analysis of recombinant *Listeria* phage endolysins

2003 – 2005 **Biology studies focused on chemistry**

Swiss Federal Institute of Technology Zurich (ETH Zurich)

Work Experience

2008 – 2012 **Teaching student courses in immunology and biochemistry**

University of Zurich and University of Basel

2007 (6 Weeks) **Coral conservation volunteer program**

NGO Blue Ventures, Madagascar

- Supporting projects in coral conservation, ecotourism and public health

2007 (4 weeks) **Institute of Experimental Immunology, University Hospital Zurich**

Research assistant in the group of Prof. MD D. Pinschewer

- Generation and screening of recombinant LCMV

2006 (8 weeks) **Institute of Microbiology, ETH Zurich**

Research assistant in the group of Prof. A. Oxenius

- Identification of protective B cell antigens of *L. pneumophila*

Certificates

2010 **Biosafety course for working in a BSL3 facility in Switzerland**
Institute of virology, University of Zurich

2008 **LTK Module 1E: Introductory Course in Laboratory Animal Science**
University of Zurich, Institute of Laboratory Animal Science Education and Training

1999/1998 **Federally certified Youth and Sports instructor (Jugend & Sport Leiter)**

Publications

- V. S. Tchang, A. Mekker, K. Siegmund, U. Karrer and Jean Pieters: Diverging role for coronin 1 in antiviral $CD4^+$ and $CD8^+$ T cell responses (*Molecular Immunology, article in press*)

- K. Westritschnig, S. BoseDasGupta, V. S. Tchang, K. Siegmund and Jean Pieters: Antigen processing and presentation by dendritic cells is independent of coronin 1 (*Molecular Immunology, 2012*)

- Mekker, V. S. Tchang, L. Haeberli, A.Trkola and U. Karrer: Relative Contributions of Age and Cytomegalovirus Infection (*PLOS Pathogens, 2012*)

- Stefan S. Weber, Nicole Joller, Anna Barbara Küntzel, Roman Spörri, Vincent Tchang, Elke Scandella, Christoph Rösli, Dario Neri, Burkhard Ludewig, Hubert Hilbi, and Annette Oxenius: Identification of protective B Cell Antigens of *Legionella pneumophila* (*Journal of Immunology, 2012*)

- Schmelcher M, Tchang VS, Loessner MJ.: Domain shuffling and module engineering of Listeria phage endolysins for enhanced lytic activity and binding affinity. (*Microb Biotechnol., 2011*)

- Schmelcher, M., Shabarova, T., Eugster, M.R., Eichenseher, F., Tchang, V.S., Banz, M., and Loessner, M.J.: Rapid multiplex detection and differentiation of *Listeria* cells using fluorescent phage endolysin cell wall binding domains (*Applied Environmental Microbiology, 2010*)

- U. Karrer, A. Mekker, K. Wanke, V. Tchang and Lea Haeberli: Cytomegalovirus and immune senescence: Culprit or innocent bystander? (*Experimental Gerontology, 2009*)

Languages

German, French: Native languages

English: Fluent in spoken and written language

Spanish: Basic communication

Poster and Oral Presentation

2012	Oral presentation, Biozentrum PhD reatreat, Bern
2011	Poster and 1st Posterprize, Biozentrum PhD retreat, Fribourg
2011	Poster, V World immune regulation meeting, Davos
2010/2011	Oral presentation, Wolfsberg meeting, Schloss Wolfsberg
2010	Poster, Biozentrum PhD retreat, Interlaken
2009	Poster, 2nd European Congress of Immunology, Berlin
2009	Oral Presentation, Joint Immunology meeting, Zürich

Prizes

Poster prize Biozentrum, PhD retreat 2011 in Fribourg May 26th / 27th