An MCMV mutant lacking the IFN antagonist pM27 elicits strong humoral immune responses and can serve as a live attenuated vaccine

Dissertation
for
the doctoral degree of
Dr. rer. nat.

from the Faculty of Biology
University of Duisburg-Essen
Germany

Submitted by

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Born in Drniš, Croatia

August, 2023

The experiments underlying the present work were conducted at the Institute of Virology, Universitätsklinikum Essen.

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Date of the oral examination: 23.11.2023

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DOI: 10.17185/duepublico/81304
URN: urn:nbn:de:hbz:465-20231130-132601-7

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„Der Zweifel ist der Beginn der Wissenschaft. Wer nichts anzweifelt, prüft nichts. Wer nichts prüft, entdeckt nichts. Wer nichts entdeckt, ist blind und bleibt blind.“

Teilhard de Chardin (1881-1955)

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Abbreviations

A/I	activating/inhibitory ratio (A/I)
Aa	amino acid
Ab	antibody
Act.D	Actinomycin D
ADCC	Antibody dependent cellular cytotoxicity
BAC	bacterial artificial chromosome
ChCMV	chimpanzee cytomegalovirus
cCMV	congenital cytomegalovirus
°C	degree Celsius
CD23	cluster of differentiation 23
CD4+ T cells	CD4 positive T lymphocyte cells
CD45R	CD45 receptor
CDR	complementary-determining region
CDS	coding sequence
CHX	cycloheximide
cm^2	square centimetre
CNS	central nervous system
CO_2	carbon dioxide
CTL	cytotoxic T- Lymphocytes
d.p.i	days post infection
DDB1	DNA damage binding protein 1
DMEM	Dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
E	early expression kinetics
dsDNA	double stranded DNA
EGFP	enhanced green fluorescent protein
ELISA	Enzyme-linked immunosorbent assay
ELIspot assay	Enzyme-linked immunospot assay
EtOH	Ethanol
Fab part	fragment antigen binding
Fc part	fragment crystallisable

FcR	Fc receptor
Fcγ	Fragment crystallisable from IgG
FcγR	Fc gamma receptor
G	Gauge
g	relative centrifugal force (rcf)
gB	glycoprotein B
h	Hours
H_2SO_4	sulfuric acid
HCMV	human cytomegalovirus
HSV-1	Herpes simplex virus type 1
i.p.	intraperitoneal
ICP	infected cell protein
ICs	immune complexes
IE	immediate early (expression kinetic)
IFN	Interferon
IFN-γ	interferon gamma
IgG	immunoglobulin G
IgK	immunoglobulin K
IgM	immunoglobulin M
IL-2	interleukin 2
IL-6	interleukine 6
IRS/TRS	internal/terminal repeat short
kDa	kilo-Dalton
L	late expression kinetics
LN	lymph node
MAbs	monoclonal antibodies
MBL	mannose-binding lectin
MCMV	mouse cytomegalovirus
MEC	primary mouse embryo cells
mg/ml	milligram per millilitre
MIE locus genes	major immediate early locus genes
min	minutes
ml	millilitre
mM	millimolar

MNC	mouse newborn cells
MOI	multiplicity of infection
nAbs	neutralizing antibodies
NK cells	natural killer cells
NKG2A, C, or D	natural-killer group 2, member A, C or D
nm	nanometer
NPC	nuclear pore complex
ORF	open reading frame
OVA	ovalbumin
p.a.	*pro analysi*
PAMP	pathogen-associated molecular pattern
PBS	phosphate-buffered saline
PFU	plaque forming unit
PFU/ml	plaque forming unit per millilitre
PRR	pattern recognition receptor
PVP	polyvinyl pyrrolidone
RhCMV	Rhesus Cytomegalovirus
rpm	revolutions per minute
RT	room temperature
s	second
SARS-CoV-2	Severe acute respiratory syndrome coronavirus type 2
SCID mouse	severe combined immuno-deficient mouse
SG	salivary gland (sublingual gland and submandibular gland)
SNP	single nucleotide polymorphism
STAT	signal transducer and activator of transcription
SV5-V	Simian virus 5 V protein
TMB	3,3',5,5'-tetramethylbenzidine
TNF-α	tumour necrosis factor alpha
TRL/IRL	terminal/internal repeat long
U/ml	units per millilitre
UL	unique long
US	unique short
v/v	volume per volume
VACV (VV)	Vaccinia virus

vFcγR	viral Fc gamma Receptor
VSB	virus standard buffer
w.p.i	weeks post infection
w/v	weight per volume
WHO	World Health Organization
μg/ml	microgram per millilitre
μl	microliter
μl/well	microliter per well

1 Introduction

1.1. Viruses

In 1892, a young Russian scientist, Dimitri Ivanovsky, reported that the tobacco mosaic disease was caused by a filterable infectious agent (Ivanovsky, 1892). Independently of Ivanovsky, Martinus Beijerinck determined that the infectious agent was able to multiply within living plants. He hypothesized that it was liquid, soluble, even circumvents the traditional filtering procedures used for trapping bacteria, and described it as a "*contagium vivum fluidum*" (contagious living fluid) (Mayer, 1886). Friedrich Loeffler and Paul Frosch in 1898 described and isolated the first filterable infectious agent from animals, the foot-and-mouth disease virus, and Walter Reed and his team in 1901 recognized the first human virus, the yellow fever virus. By the start of the twentieth century, the term of viruses was firmly established, derived from the Latin term poison (Lustig & Levine, 1992.).

Viruses are obligate intracellular parasites. Obligate intracellular parasites cannot reproduce outside their host cell, meaning that the parasite's reproduction is entirely reliant on intracellular resources. When infected, a host cell is often forced to rapidly produce thousands of copies of the original virus. When not inside an infected cell or in the process of infecting a cell, viruses exist in the form of cell-independent particles, or virions. Their genetic material consist of long molecules of DNA or RNA that encode the structure of the proteins; a protein coat, the capsid, which surrounds and protects the genetic material; and in some cases an outside envelope of lipids. The shapes of these virus particles range from simple helical and icosahedral forms to more complex structures. Double-stranded DNA viruses can express and duplicate their genetic material by processes, which are at least formally identical to those used by cells. Viruses with other types of genomes require special systems for replication and transcription. These processes make up the viral genetic system, and it is possible to group viruses according to general properties of their genetic systems (Baltimore, 1971).

1.1.1. Herpesvirus family

The family name is derived from the Greek word *herpein* meaning 'to creep' or serpent, referring to spreading cutaneous lesions, usually involving blisters (Beswick, 1962). Herpesviruses have a high prevalence worldwide. So far, nine human herpesviruses have been described:

- Herpes simplex virus type 1 and type 2 (HSV-1 and HSV-2) and varicella zoster virus (VZV or human herpesvirus 3 [HHV-3]) belong to the α-subfamily,
- Human cytomegalovirus (HCMV or [HHV-5]), human herpesvirus 6a and 6b ([HHV-6a] and [HHV-6b])) and human herpesvirus 7 ([(HHV-7[) belong to the β-subfamily,
- Kaposi´s sarcoma-associated herpesvirus (KSHV or HHV-8) and Epstein-Barr virus (EBV or [HHV-4[) belong to γ-subfamily.

All members of the *herpesviridae* have the same basic structure consisting of four layers. A core is composed of the virus dsDNA, and a sturdy represents icosahedral capsid composed of 162 capsomers that surrounds and protects the DNA. A thick layer of virus-encoded protein called the tegument lies between the capsid and the envelope membrane. A membrane derived from the host cell that contains virus-encoded glycoproteins is involved in entry and other functions. This whole particle is known as a virion (Figure 1).

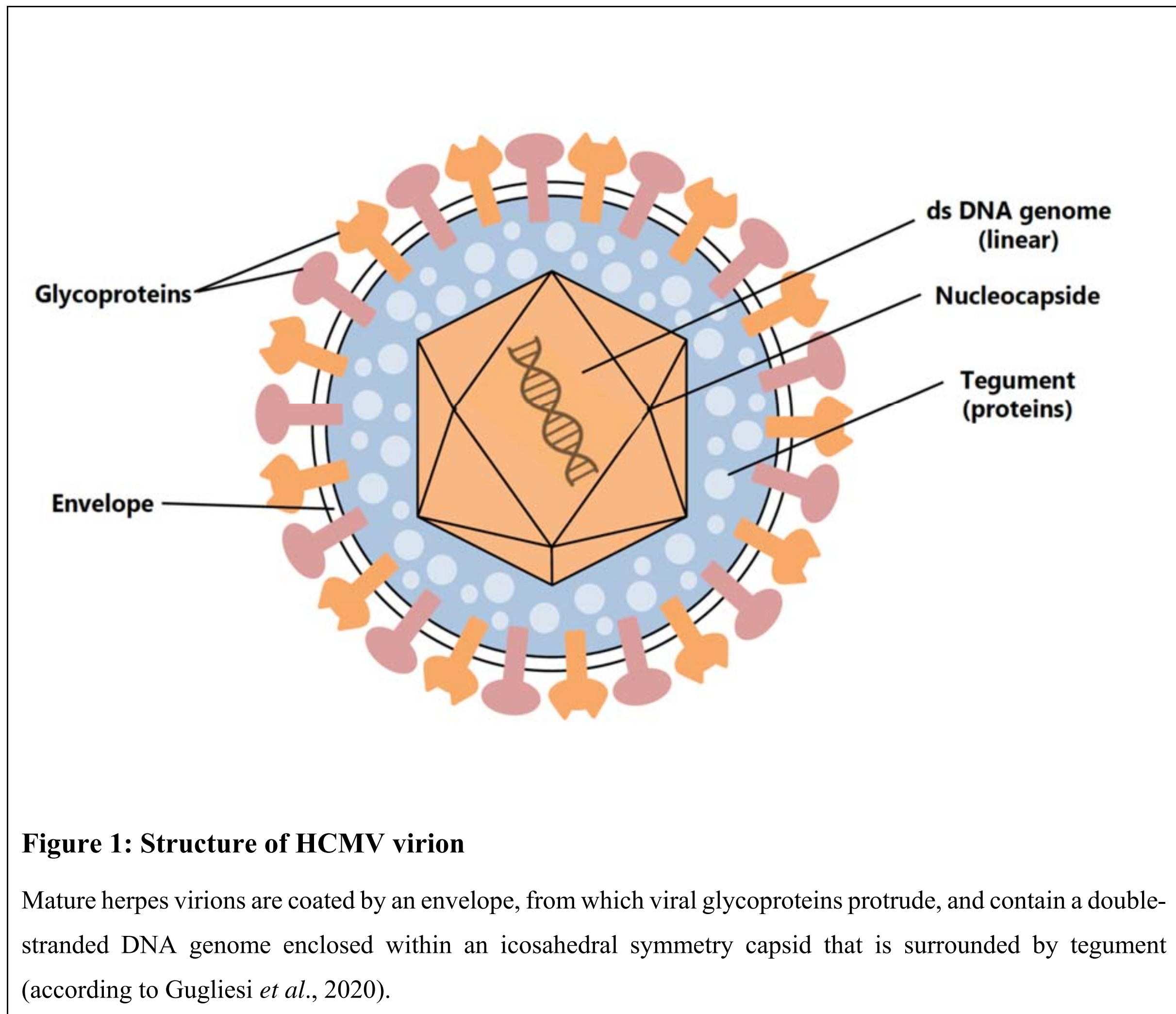


Figure 1: Structure of HCMV virion

Mature herpes virions are coated by an envelope, from which viral glycoproteins protrude, and contain a double-stranded DNA genome enclosed within an icosahedral symmetry capsid that is surrounded by tegument (according to Gugliesi *et al*., 2020).

All herpesviruses share some major characteristics:

1. The viral DNA is transcribed to mRNA within the infected cell's nucleus
2. Production of virions is accompanied by lytic infection, in most of cases
3. They encode a large array of enzymes involved in nucleic acid metabolism, DNA synthesis and processing of proteins
4. Herpesviruses can persist in the cell (and thus the host) establishing latency. While primary infection is often accompanied by a self-limited period of clinical illness, long-term latency is mostly symptom-free. Latent genomes retain the principle capacity to replicate and cause disease after reactivation.

Herpesviruses have different replicative strategies. One of them is latency with occasional re-emergence. A second option is the ‘hit-and-run’ approach. They display a rapid burst of replication but are subsequently cleared from the system. Depending on their hosts to shed large

quantities of viral particles, some find the way to novel individuals. The third option is the 'slow-and-low' tactic. These viruses replicate continuously, but at a low level, sufficiently not to induce an immune response resulting in their clearance from the system (Grinde, 2013). The target cells or tissues where every member of the herpesvirus family remains latent also differ between individual members of this family. For example, CMV remains latent in CD34+ blood progenitor cells and monocyte-derived macrophages (Jarvis & Nelson, 2002) and HSV-1 in sensory neurons (Cook *et al.*, 1991).

Despite biological divergence, human cytomegalovirus (HCMV, HHV-5) on the one hand, and the three human roseola viruses (HHV-6A, HHV-6B, HHV-7) on the other hand, share approximately 70 evolutionarily conserved and collinear genes (Mocarski, 2007). The high coding capacity of this virus family, with so much possible antigenic proteins to be targeted by the immune system, created a necessity to develop immune evasion mechanisms. This positions virus family as masters of immune evasion and molecular piracy (Hengel *et al.,* 1998). Immune evasion refers to virus-mediated mechanisms that counteract immune control. Molecular piracy means virus hijacked host genes and integrated it into its own genome. The immunocompetent host does not manage to eliminate the virus but maintains it under control and with viral immune evasion mechanisms allows the establishment of a lifelong latency with episodically reactivations (Sissons *et al.*, 2002).

1.1.2. Human cytomegalovirus

HCMV infection leads to clinical complications in immunocompromised patients such as AIDS and allograft transplantation patients or in patients with immature immune system as in foetuses or newborns. Infection in an immunocompetent host usually follows an asymptomatic or subclinical course of infection. The primary infection is usually effectively controlled by a competent immune system, but HCMV persists in the host for lifetime in a latent state with periodic reactivations. For HCMV transmission due to excretion of HCMV particles into saliva, genital fluids and breast milk, intimate contact is necessary. Vertical infection from mother to child by breastfeeding is a common mode of infection (Hamprecht *et al.*, 2001). HCMV shows natural transplacental transmission (Stagno *et al.*, 1986), and belongs to a TORCH complex (T-*Toxoplasma gondii*, other (*Listeria monocytogenes, Treponema pallidium*, parvovirus, HIV, varicella zoster virus, amongst others), Rubella, Cytomegalovirus (CMV), and Herpesviruses

(HSV) 1 and 2, and Zika virus (ZIKV)), together with other pathogens capable of creating the vertically transmitted infections (Arora *et al.*, 2017). The reported incidence of HCMV congenital infection is 0.5-2.0 % (Peckham, 1991) and is a leading cause of mental retardation and deafness in live-born infants. The dogma in research area of HCMV congenital infection was that maternal CMV seropositivity decreases the likelihood of congenital CMV transmission because the adaptive immunity confers certain protection (Ludwig & Hengel, 2009). However, in woman with nonprimary HCMV infections, the existing adaptive immunity to HCMV cannot prevent infection with a new strain of HCMV (Britt, 2017). Also, as HCMV sero-prevalence increases in maternal populations, the rate of congenital HCMV infection also increases suggesting that overall outcomes in congenitally infected infants are similar following both primary and nonprimary maternal HCMV infections (Britt, 2017). In Germany, seroprevalence in adults lies at approximately 60% depending on age, sex, and socio-economic circumstances (Enders *et al.,* 2003).

Primary cytomegalovirus infection is usually controlled by a combination of coordinated innate and adaptive immune responses. The first line of the immune response is innate immune response comprising interferons (IFNs) and activated natural killer cells (NK) cells. HCMV is a rather slowly replicating virus. NK cells are cytolytic effector cells which are able to recognize and lyse infected target cells before the formation of infectious virus. In addition, T and NK cells release specific cytokines, i.e., IFN-γ and TNF-α, which can efficiently inhibit late gene transcription and block CMV replication at the stage of nucleocapsid formation (Lučin *et al.*, 1994). The efficient protective adaptive response appears to be T-cell mediated (Reddehase *et al.*, 1987; Polić *et al.*, 1998). Antiviral antibodies (Abs), although not essential for the control of primary CMV infection and the establishment of latency, play a critical role in limiting the dissemination of recurrent virus (Jonjić *et al.*, 1994). Abs can modify the disease associated with HCMV infection in transplant recipients as well as congenital CMV infection in humans and experimental animal models (Nigro *et al.*, 2005; Nigro *et al.*, 2008; Bracher *et al.*, 1995; Cekinović *et al.*, 2008; Chatterjee *et al.*, 2001; Snydman *et al.*, 1987). Infection with HCMV is frequently associated with a number of transient humoral abnormalities - mixed cryoglobulins, cold agglutinins, rheumatoid factor, antinuclear antibodies, and anticomplementary activity (Klemola *et al.*, 1970) - that may be attributable to virus-induced polyclonal B cell activation (Hutt-Fletcher *et al.*, 1983). The appearance of auto antibodies in serum of mice infected with mouse cytomegalovirus (MCMV) may also be caused by widespread activation of B cells (Price *et al.*, 1993). Almost all human sero-positive sera contain antibodies against envelope glycoproteins gB, gH, the tegument protein pp150 and to a nonstructural DNA binding pp52

(Greijer *et al.*, 1999; Schoppel *et al.*, 1997). Other targets are matrix antigens like phosphoproteins pp71 and pp65. The role of antibodies is mainly to limit the severity of disease, since the antibody response is too late in comparison with the fast T cell response to control infection. However, the antibody response against CMV contributes to the control of reactivation and reinfection (Jonjić *et al.*, 1994). The transfer of memory B cells showed that, when present before an infection occurs, memory B cells do provide protection against MCMV. Also, memory B cells can be used therapeutically to protect from an ongoing CMV infection (Klenovšek *et al.*, 2007). CMV persists lifelong in its host, with phases of latent or non-productive and recurrent or productive infection, which expose the virus to the most part to an already primed immune system (Polić *et al.*, 1998). In the naive host, CMV replicates first in visceral organs, including the lungs, spleen, and liver. In a later phase of infection, when virus multiplication in these tissues is already ceasing due to clearance of infectious virus by $CD8^+$-dependent immune control functions, increasing titers are found in the salivary glands (Polić *et al.*, 1998).

In order to provide protection against HCMV and congenital CMV disease, the development of a vaccine is viewed as the most promising option. Considerable progress has been made over the last 30 years, but technical challenges make it uncertain when a vaccine will become available (Plotkin, 2002). Congenital CMV disease is most likely to occur following a primary infection in the mother. Primary infections occur in 1%-4% of sero-negative, pregnant women and lead to foetal infection in 40%-50% of these pregnancies. Maternal CMV reactivation or reinfection with a different CMV strain leads to foetal infection in about 1% of seropositive, pregnant women (Cannon & Davis, 2005). Approximately 10% of congenitally infected infants are symptomatic at birth, and of the 90% who are asymptomatic, 10%-15% will develop symptoms over months or even years (Stagno &Whitley, 1985). Permanent sequelae can result from CMV infection of the fetus during any trimester, but infection during early foetal development is likely to be especially damaging (Preece *et al.*, 1985; Stagno *et al.*, 1986). Since few newborns are screened for CMV, the true impact of congenital CMV infection is underappreciated (Cannon & Davis, 2005).

Currently, four antivirals are approved to be used in immunocompromised patients: Ganciclovir, Valganciclovir, Foscarnet, Cidofovir and Letermovir. Ganciclovir acts as nucleoside analogue. Its antiviral activity requires monophosphorylation by the HCMV protein kinase pUL97 (Sullivan *et al.*, 1992). The second drug, Cidofovir, is a nucleotide analogue which is already phosphorylated and thus active. Further phosphorylation to the triphosphate

form of Ganciclovir and Cidofovir is performed by cellular kinases. The nucleoside-triphosphate-analogues compete with the cellular nucleotide triphosphates at the viral polymerase pUL54 and are incorporated into the viral DNA. Foscarnet has a different mode of action. It directly inhibits polymerase function by blocking the pyrophosphate binding site of pUL54 (Tan, 2014). Finally, Letermovir binds to a components of CMV- terminase complex (UL51, UL56 and UL86) involved in the packaging of genome into preformed virus capsids (Marty *et al.*, 2017). Nevertheless, chronic administration of these antivirals can create resistant viruses. Therefore, patient treatment with hyper-immunoglobulin preparations can be an option. Hyper-immunoglobulin is an immunoglobulin G (IgG) containing a standardized number of antibodies to cytomegalovirus, so enriched pool of IgG from a large number HIV/HBV/HCV-negative donors. Alone or in combination with antiviral agent, hyper-immunoglobulin preparations may be used for the prophylaxis of cytomegalovirus disease associated with organ transplantation.

HCMV is the prototypical member of the β-subgroup of herpesviruses. The virion has a typical herpesvirus structure, but it is larger (200-300 nm in diameter). The 230-kbp double-stranded linear DNA genome is packaged within a 100-nm-diameter icosahedral capsid, which in turn is surrounded by a poorly characterized protein structure known as the tegument or matrix. A lipid envelope containing viral and host-derived glycoproteins further surrounds the tegument, rendering the diameter of the mature virion. In total, it is estimated that at least 30 different proteins, ranging in size from 11 to more than 200 kDa, form the complete infectious particle (Baldick & Shenk, 1996). The complex biology of HCMV begins with an initial interaction between the envelope of the infectious virion and the host cell. Understanding the initial events of infection requires an analysis of the glycoprotein components of the virion envelope as well as their expression in the membranes of the infected cell. The large genome of HCMV may encode for over 65 unique glycoproteins (Baldick & Shenk, 1996). Protein homologs of only four herpes simplex virus glycoproteins, gB, gH, gL and gM, have been identified, and potential functions have been postulated based on studies of specific glycoprotein null mutants of HSV and other herpesviruses. Several of the envelope glycoproteins elicit strong host immune responses, including the production of virus-neutralizing antibodies. Antigenic sites that elicit neutralizing antibodies are more heavily glycosylated than those that elicit non-neutralizing antibodies, suggesting that HCMV gB uses glycans to shield neutralizing epitopes while exposing non-neutralizing epitopes, thus helping HCMV to avoid clearance (Burke & Heldewein, 2015). Also, HCMV gB and gH/gL form stable, preformed complexes in extracellular virions independent of receptor binding (Vanarsdall *et al.*, 2016). These responses

are supposed to be a key component of host immunity and represent a goal of vaccine development (Britt & Mach, 1996).

HCMV has the largest genome of the human herpesviruses (Chee *et al.*, 1990). It is longer than all other human herpesviruses and one of the longest genomes of all human viruses in general. It has the characteristic herpesvirus class E genome architecture, consisting of two unique regions (unique long [UL] and unique short [US]), both flanked by a pair of inverted repeats (terminal/internal repeat long TRL/IRL and internal/terminal repeat short IRS/TRS) (Mocarski, 2007) which are present on viruses grown in cell culture (Trilling *et al.*, 2011). Instead of the IRL region, all clinical HCMV isolates have ULb´ region (Trilling *et al.*, 2011). Trilling and colleagues 2011. showed that AD169*var*L-infected cells exhibited TNF- induced IκBα degradation, whereas AD169*var*S-infected cells did not. An HCMV ULb´ gene product was identified, which selectively enhance TNFR1 surface density, namely pUL138 (Trilling *et al.*, 2011) (Figure 2).

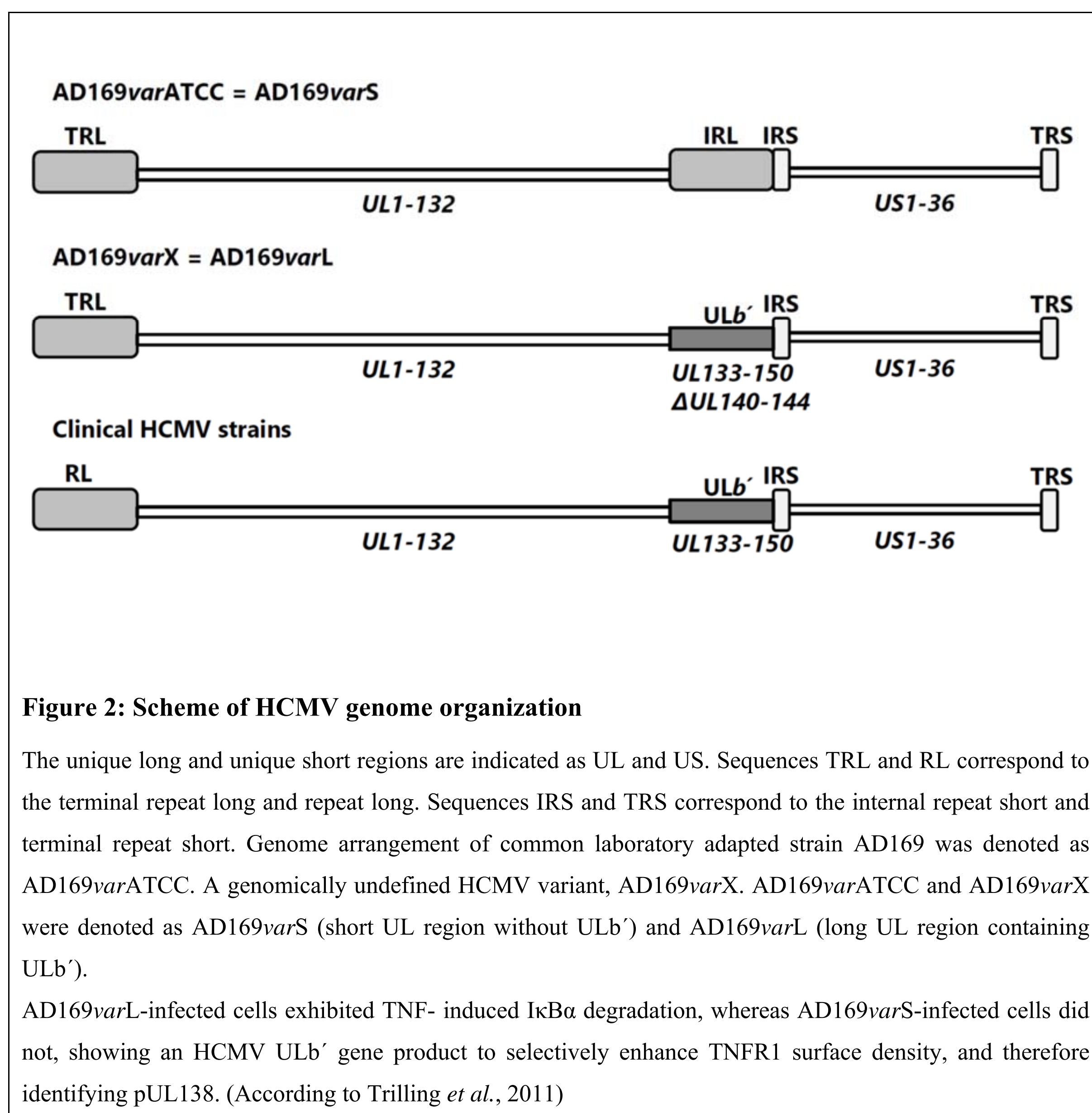


Figure 2: Scheme of HCMV genome organization

The unique long and unique short regions are indicated as UL and US. Sequences TRL and RL correspond to the terminal repeat long and repeat long. Sequences IRS and TRS correspond to the internal repeat short and terminal repeat short. Genome arrangement of common laboratory adapted strain AD169 was denoted as AD169*var*ATCC. A genomically undefined HCMV variant, AD169*var*X. AD169*var*ATCC and AD169*var*X were denoted as AD169*var*S (short UL region without ULb´) and AD169*var*L (long UL region containing ULb´).
AD169*var*L-infected cells exhibited TNF- induced IκBα degradation, whereas AD169*var*S-infected cells did not, showing an HCMV ULb´ gene product to selectively enhance TNFR1 surface density, and therefore identifying pUL138. (According to Trilling *et al.*, 2011)

The coding capacity of CMV estimation varies between 166 to more than 200 open reading frames (ORF) (Murphy *et al.*, 2003). Complete genome sequences have been determined for four bacterial artificial chromosome (BACmid) cloned strains like AD169 (Borst *et al.*, 1999), Towne and TB40E (Sinzger, 2008). This allows the genetic manipulation of the virus in *Escherichia coli* to create mutants lacking specific genes of CMV. Stern-Ginossar *et al.* identified 751 translated HCMV ORFs using ribosome profiling and transcript analysis (Stern-Ginossar *et al.*, 2012). In addition to the traditional immediate early (IE), early (E) and late (L) gene expression kinetics, there are also a newly defined groups of expression kinetics genes, known as early-late genes and late-translation-independent gene cluster (Rozman *et al.*, 2022)

Early-late genes (TC 4) show two waves of expression, one of which is dependent on *de novo* protein synthesis and a second, later wave of expression that depends on the onset of DNA replication. Late-translation-independent genes (TC 6) are expressed in the absence of *de novo* protein production, but their predominant expression occurs at late time points post infection and is highly dependent on viral DNA replication (Rozman *et al.*, 2022).

Replication cycle of HCMV starts by attachment of the virion at the cell of interest. Glycoproteins at membrane of virion (gB, gH, gL, gO and. pUL128, pUL130 as well as pUL131A) bind themselves with the receptor of the cell membrane (*attachment*). Identity of the cell membrane receptors is not clear, but candidates are membrane proteins called integrins, and nonintegrin receptors such as EGFR und PDGFRα (Feire *et al.*, 2004; Isaacson *et al.*, 2007; Soroceanu *et al.*, 2008; Wang *et al.*, 2005; Wang *et al.*, 2003). A gH/gL/gO trimeric complex is sufficient for viral attachment and entry into fibroblasts (Hahn *et al.*, 2004; Wang & Shenk, 2005), and a pentameric complex composed of gH, gL, and pUL128–131A, required for efficient entry into epithelial, endothelial, and dendritic cells as well as monocytes (Nogalski *et al.*, 2013). Although both the trimeric and pentameric gH/gL complexes are capable of binding β_1 and β_3 integrins, only HCMV possessing the pentameric complex can fully activate the integrin–c-Src signalling axis to promote efficient entry into monocytes (Nogalski *et al.*, 2013). Neuropilin-2 was identified as one of the membrane receptor involved in attachment of HCMV (Martinez-Martin *et al.*, 2018). These interactions trigger a fusion of the viral and the cellular membrane. Virus capsids get into the cytoplasm (*entry*) and then moves along the microtubules of the host cell to the cell nucleus. The capsid dissociates at the cell nucleus (*uncoating*) and the DNA enters the cell *via* the nuclear pore complex (NPC). In the cell nucleus, the viral DNA circularizes and allows DNA replication using so-called „rolling circle“mechanism (McVoy and Adler, 1994). The expression of viral genes occurs in a cascade-like manner and can be divided into different phases (Wathen & Stinski, 1982, Wathen *et al.*, 1981). The first, immediate early (IE) phase begins right after the virus enters the cell and circularizes the DNA in the nucleus (McVoy and Adler, 1994). For HSV-1 it has been shown that the circularization is not prerequisite for the expression of the IE genes (Umene & Nishimoto, 1996). Transcription of the genes expressed at this phase is induced by cellular or by viral transcription factors which have been brought by the virion. After transcription and translation, the IE proteins transactivate the early phase (E) of viral gene expression. The gene products of early genes (e.g. viral DNA polymerase) are responsible for viral genome replication. This is followed by the third, late phase (L). Some genes show so-called „real" late gene expression kinetics, which are only expressed after genome replication, and other exhibit

early/late gene (E/L) expression, which are expressed before viral genome replication but becomes upregulated once viral genome replication has started. The expression of genes of the „real" late genes is dependent on viral DNA replication. At the late phase mainly structural proteins of the next virus generation are expressed, as well as proteins that are responsible for the *assembly*, as well as for the exit of the virus particle from the cell (*egress*) (Landolfo *et al.*, 2003).

1.1.3. Mouse Cytomegalovirus

Mouse cytomegalovirus (MCMV, *Murid herpesvirus 1*) is a mouse-specific betaherpesvirus. MCMV infecting its murine host has been used extensively to model human disease with HCMV (Hudson *et al.*, 1978). MCMV is a natural pathogen of mice, being found in every wild *Mus musculus* population that has been investigated in Australia (Moro *et al.*, 1999; Smith *et al.*, 1993). There are many genetic similarities between HCMV and MCMV with multiple gene homologs, including structural and immune-evasion genes (Rawlinson *et al.*, 1996). However, there are important differences in the organization of the genetic information. For example, HCMV has unique long and unique short regions with terminal and internal repeat sequences (Van Damme *et al.*, 2014) compared with MCMV, which has a single unique sequence with short terminal direct repeats and several short internal repeats (Rawlinson *et al.*, 1996). Initially, an analysis of the complete 230-kb DNA sequence of the Smith strain of MCMV is predicted to contain 170 genes and was found to be essentially co-linear with the HCMV genome over the central 180 kb (Rawlinson *et al.*, 1996). The new annotation comprises 365 viral transcription start sites (TiSS) that give rise to 380 and 454 viral transcripts and ORFs, respectively (Lodha *et al.*, 2023). There are 24- and 27-kb regions at the left and right ends of the genome that encode genes currently only found in MCMV. Within these regions, families of tandemly arranged genes are predicted to encode membrane glycoproteins, a feature that resembles the RL11 and US6 families identified in HCMV. There are ORFs with homology to eukaryotic cellular genes (Rawlinson *et al.*, 1996).

MCMV is generally maintained either as salivary gland stock or as tissue culture-derived stock, usually prepared from the infection of embryonic mouse fibroblasts (Brizić *et al.*, 2018). This influences the nature of the infection that occurs *in vivo* (Osborn *et al.*, 1968). Salivary gland stock is generally composed of single capsid virions, derived from the cytoplasmic vacuoles within the serous acinar sinus cells of the salivary gland (Mims *et al.*, 1979). After MCMV

potassium tartrate density gradients purification, salivary gland stock consisted of single-capsid enveloped virus and free capsids. Successful separation was achieved by filtration through 220 and 450 nm Millipore membrane filters. It has been shown that naked capsids did not interfere with the action of (virulent) salivary gland virus in newborn mice (Chong *et al.*, 1981). Salivary gland stock produces acute infection and can be lethal when inoculated to juvenile (3 weeks old) mice. In contrast, virus stocks made from other organs (e.g., the liver and spleen) and also tissue culture-derived stock passaged in embryonic mouse fibroblasts are comprised of both single and multicapsid virions (Hudson *et al.*, 1976).

MCMV has been distinguished from other murine viruses on the basis of cytopathic effect, tissue tropism, and morphology (Osborn *et al.*, 1986). Both MCMV and HCMV have a highly restricted host range. The movement of MCMV between organs differs, depending on the route of infection (Brune *et al.*, 2001). There is a strong tropism for the salivary gland, regardless of the route of infection used. Dissemination from initial infection sites occurs via a leukocyte-associated viremia, seeding distal organs such as salivary glands (Collins *et al.*, 1993; Stoddart *et al.*, 1994). In many strains of mice, MCMV replication in the salivary gland continues for a prolonged period of time (e.g., several weeks to month). The tropism for the salivary gland is central to the biology and epidemiology of CMVs. Persistent and recurrent shedding from the salivary gland is believed to be the principal mean by which these viruses spread in the population (Manning *et al.*, 1992). As with all herpesviruses, MCMV infection has a latent phase (Pollock *et al.*, 1995).

The effect of MCMV has been studied in various mouse strains, which differ according to their MCMV resistance. Both H-2- and non-H-2-associated genes control this form of resistance, and were found to influence extent of viral replication during sublethal and severe infection (Allan *et al.*, 1984). In mice of the BALB/c background, the increase in resistance conferred by the *k* haplotype was about 8- to 10-fold that of the *d* and *b* haplotypes, which were equally susceptible. Non-H-2-associated genes also affect resistance to the virus, particularly in the C57BL/6 genetic background (Grundy *et al.*, 1981, Le-Trilling *et al.*, 2018). Other genetic factors involved in control of MCMV resistance include the Ly49H receptor encoded by the *cmv-1* gene (Brown *et al.*, 2001; Daniels *et al.*, 2001; Lee *et al.*, 2001). Welsh and colleagues observed that the subset of NK cells expressing the activating Ly49H receptor preferentially expressed IFN-γ after MCMV infection. Depletion of NK cells expressing Ly49H, but not other members of the Ly49 family, was required for host resistance to MCMV infection (Daniels *et al.*, 2001). Ly49H is present in strains demonstrating relative resistance to MCMV (e.g.,

C57BL/6), but is absent in susceptible strains (e.g. BALB/c, DBA/2). It was discovered that the MCMV-encoded major histocompatibility complex-like molecule m157 protein binds to Ly49H, confirming a direct interaction between the NK receptor and viral protein. The product of *m157* also binds to the 129/J strain allelic form of Ly49I, an inhibitory NK receptor. It has been proposed that *m157* might have originally evolved to serve as an immune evasion protein that delivers inhibitory signals to the NK cell subsets expressing Ly49I or Ly49I-like receptors (Arase *et al.*, 2002). Adler and colleagues sequenced the complete MCMV-BAC genome and identified a frameshift mutation within the ORF encoding MCMV chemokine MCK-2. This mutation reduced virus production in salivary glands (Jordan *et al.*, 2011). MCMV was used as a vaccine vector expressing the mouse ovarian glycoprotein zona pellucida 3 in studies investigating immune-mediated contraception, and binding the specific protein m157 to Ly49H was broadly associated with vaccine success (Lloyd *et al.*, 2007). This effect was abrogated using a different virus strain, G4, which does not have the same interaction with NK cell activation receptors.

MCMV has been used as a model for HCMV infection. The major reason for using MCMV as a model is the matching biological characteristics of these virus infections in their natural settings. Both MCMV and HCMV cause severe infections in the immunocompromised or immunologically immature host, resulting in similar clinical syndromes (Craighead *et al.*, 1992; Mutter *et al.*, 1988; Osborn *et al.*, 1986; Shellam *et al.*, 1985). In addition, both HCMV and MCMV are susceptible to the antiviral agent 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG or ganciclovir) (Shanley *et al.*, 1985). Many early CMV diseases were modelled using the intraperitoneal route of infection. The movement of a virus from the site of vaccination and the dissemination of a virus *via* peritoneal macrophages to visceral organs and, to the salivary gland (Hsu *et al.*, 2009). This route of inoculation does not correlate well with natural routes of infection, and other inoculation sites, such as the footpad, have also been used in some iterations of the MCMV disease model. By inoculation of a virus *via* the footpad, the infectious virus moves to the popliteal lymph node, infecting resident subcapsular sinus macrophages, although these cells do not allow lytic replication. The intranasal route of inoculation (simulating transmission from mother to offspring via grooming) has demonstrated that the visceral organs do not show the same level of viral replication as is seen with other routes of administration (Oduro *et al.*, 2016).

Innate immune responses determine the severity of MCMV infection, and NK cells play an important early role in defence. Presence of NK cell activating receptors and the antigen-

specific inflation of NK cell populations (Nabekura & Lanier, 2016) create the increased resistance of C57BL/6 mice to MCMV infection compared with BALB/c mice (Scalzo *et al.*, 2003). Cytokines produced as a result of MCMV infection are responsible for the tissue damage and suppression of pathology (Clement & Humpreys, 2019). Strong humoral and cell-mediated immunity is induced after primary infection with MCMV. $CD8^+$ T-cells are important for the control of viral clearance from many organs (Jonjić *et al.*, 1989) and also to inhibit MCMV-associated effects on haematopoiesis in bone marrow graft rejection (Renzaho *et al.*, 2020). $CD4^+$ T-cells produce granzyme B and may directly kill infected cells *in vivo* (Verma *et al.*, 2015). Antibodies were produced due to polyclonal B-cell responses (Price *et al.*, 1993). Recent data suggests that the administration of an antibody recognizing glycoprotein B, even where the antibody is unable to neutralize the virus, may provide protection from disease (Bootz *et al.*, 2017).

MCMV and HCMV have some biological differences, as transplacental transmission of MCMV has not been demonstrated and mouse models of foetal infection involve direct inoculation of MCMV into the central nervous system or uterus (Kashiwai *et al.*, 1992; Tsutsui *et al.*, 1993). One of the aspects of HCMV infection that has been attempted using manipulations of the basic MCMV/mouse model is congenital CMV infection (cCMV). Early studies of cCMV used relatively high titers of salivary gland virus and generally resulted in foetal loss (resorption) and growth restriction that was attributed to maternal illness or placental insufficiency (Johnson, 1969; Fitzgerald & Shellam, 1991), suggesting that fundamental differences between the mouse and human placentae may be responsible for this condition. Humans and mice placentae are both hemochorial (i.e., having a trophoblast surface that is in direct contact with maternal blood). The difference that probably results in reduced foetal infection, is that mice have three trophoblast layers-one is mononuclear and two are syncytial—separating the maternal and foetal blood, compared with the single syncytiotrophoblast layer found in human placentae (Georgiades *et al.*, 2002; Rossant & Cross, 2001). However, the reported congenital infection of severe combined immunodeficient (SCID) mouse pups leading to intrauterine growth restriction and microcephaly suggested that physical barrier may not be responsible for differences between mice and humans (Woolf *et al.*, 2007). The use of tissue culture virus rather than salivary gland virus has allowed some more subtle infections to be optimized, particularly involving infection of the placenta without significant resorption of foetuses. The groups of William J. Britt and Stipan Jonjić established a model of i.p. neonatal MCMV infection whose pathogenesis closely resembles congenital HCMV infection (Koontz *et al.*, 2008). A mouse model of an HCMV infection was introduced of the developing CNS

using peripheral inoculation of newborn mice with low titers of MCMV. Results indicated that virus replication in the liver and presumably the spleen was followed by spread to the brain (Koontz et al., 2008). To date, the most successful small animal model of congenital disease is the guinea pig model (Schleiss & McVoy, 2010).

Mouse embryonic stem cells have been reported to be refractory to MCMV infection, but neuronal stem progenitor cells are susceptible to infection (Tsutsui, 2009). Experiments using brain slice cultures have shown that the susceptibility of brain cells, particularly neural stem progenitor cells, to CMV infection may be associated with neurogenesis (Tsutsui *et al.*, 2008). Newborn pups were susceptible to brain infection after i.p. inoculation of MCMV, and has been noted that protection was afforded by $CD4^+$ T-cells (Brizić *et al.*, 2019), $CD8^+$ T-cells (Bantug *et al.*, 2008), and antibodies (Cekinović *et al.*, 2008). Virus-induced cochlear inflammation in newborn mice induces sensorineural hearing loss (Won Sung *et al.*, 2019).

1.2. Vaccines

The first vaccine was applied in 200 BC in China, where variolation was used against smallpox. Variolation referred to the subcutaneous instillation of smallpox virus into nonimmune individuals (Riedel, 2005). In the 18th century, during the smallpox epidemics in Europe, Edward Jenner inoculated a healthy individual using matter from fresh cowpox lesions, and six weeks later inocculated the individual with smallpox. No disease developed and Jenner concluded that protection was complete. As the Latin word for cow is *vacca*, and for cowpox is *vaccinia*; Jenner decided to call a new procedure vaccination (Willis, 1997).

There are several forms of vaccines that can induce a protective immune response. Live vaccines are capable of replication and usually induce both neutralizing antibodies and cytotoxic T-cell (T_C or CTL) responses, or helper T-cell (T_H) responses (Alarcon *et al.*, 1999). These vaccines include attenuated viruses or bacteria. The attenuated viruses carry mutations in the wild-type genome, or have been cultivated under conditions that reduce the pathogenicity of the virus, but the virus can still induce complex immune responses (Marthas *et al.*, 1992). Examples include the viral diseases yellow fever, measles, mumps, and rubella, and the bacterial disease typhoid. The live *Mycobacterium tuberculosis* vaccine developed by Calmette and Guérin is not made of a contagious strain but contains a virulently modified strain called "BCG" used to elicit an immune response (WHO, 2018). Although attenuated vaccines provoke durable immunological responses, they may not be safe for use in immune-compromised

individuals, and on rare occasions mutate to a virulent form and cause disease. For some viruses, such as the retrovirus HIV, the risk of reverse mutations is too high for the use of attenuated viruses (Whitney & Ruprecht, 2004).

Dead vaccines that can no longer multiply in the organism include dead viruses or bacteria, as well as protein (tetanus or diphtheria toxoid) and peptide vaccines (hepatitis B surface proteins). Protein and peptide vaccines usually only induce T_H- and neutralizing responses and no cytotoxic T-cell responses (Alarcon *et al.*, 1999). These vaccines have the disadvantage that they are only weakly immunogenic, and therefore have to be vaccinated very frequently in order to induce a proper immune response. However, they offer a very high level of safety because they only contain parts of the virus, such as the vaccine against hepatitis B (Beran, 2007). Adjuvants are the substances that enhance the immunogenicity of such protein or peptide vaccines, as well as other vaccines. Other additives are included to ensure that the antigen is released in a delayed manner, which leads to increased stimulation of the immune system. However, adjuvants sometimes trigger strong inflammatory reactions and tissue damage. In addition to classic adjuvants, genetic adjuvants such as cytokines or toll-like receptor ligands are also used (Nohria & Rubin, 1994; Thompson & Staats, 2011; Kalams et al., 2013; Peters *et al.*, 2013). The immunogenicity of a protein can also be increased by fusing it with a highly immunogenic molecule. This includes, for example, the enzyme thioredoxin, which can attract monocytes and T cells (Canali *et al.*, 2014), or the ligand CD154 (CD40L), which binds to CD40 on antigen-presenting cells and activates them (Auten *et al.*, 2012).

In addition to the dead and live vaccines, there are also viral vectors and DNA vaccines. Viral vectors usually use a virus to carry a transgene of pathogens for transient expression (Goff & Berg, 1976). The transgene usually replaces DNA sections of the viral genome that are required for their replication, so that the viral vector is replication-incompetent after cloning. An example of viral vectors are adenoviral vectors (Basset *et al.*, 2011). DNA vaccines are bacterial plasmids into which a transgene is introduced, usually under the control of a strong promoter (Liu *et al.*, 2016). The immunogenicity of DNA vaccines can be improved by choosing the right method of administration. The packaging in nanoparticles increases the immunogenicity of the applied DNA (Knuschke *et al.*, 2014). RNA vaccines are focused on mRNA, especially on libraries of gene-based constructs encoding various antigens (Pardi *et al.*, 2018).

1.2.1. CMV vaccine

The majority of the adult global population is latently infected with HCMV. Worldwide, the sero-prevalence of HCMV is 30-100%, depending on factors such as age, gender, and socio-economic conditions (Colugnati *et al.*, 2007; Lachmann *et al.*, 2018). Usually, the healthy adult immune system efficiently keeps HCMV under control. If the immune system is impaired, HCMV infections are not controlled, and can cause life-threatening disease. HCMV is transmitted from the pregnant mother through the placenta to the developing foetus (Pereira, 2018). In terms of the number of children with long-term sequelae, cCMV outnumbers several other well-known childhood disorders such as Down syndrome, Spina Bifida, and foetal alcohol syndrome (Cannon & Davis, 2005). However, despite relevant progress, an approved HCMV vaccine is not available (Schleiss *et al.*, 2017). Clinical HCMV isolates showing genetic heterogeneity are suggesting that HCMV super-infections can occur (Boppana *et al.*, 2001; Renzette *et al.*, 2011; Ross *et al.*, 2011; Pokalyuk *et al.*, 2017). Therefore, the question is can sterilizing immunity to CMV infection represent an achievable goal for a vaccine. However, it has been shown that vaccines with low efficacy can have substantial epidemiological and economic benefits (Sah *et al.*, 2018).

NAbs are crucial for protection against reinfection with viruses (Graham *et al.*, 1991; Ahmed *et al.*, 1996; Seiler *et al.*, 1998; Harada *et al.*, 2003). Protection by efficient vaccines that are in use today correlates closely with neutralizing antibody titres (Plotkin *et al.*, 2001, Zinkernagel, 2003). However, many epitopes exposed on viral or cellular surfaces are not involved in mediating virus entry and fusion and thus do not raise nAb responses (El-Bakkouri *et al.*, 2011; Laver *et al.*, 1990; Jegerlehner *et al.*, 2004; Carragher *et al.*, 2008; Ackerman *et al.*, 2013; van Zanten *et al.*, 1995). Adoptive transfer experiments provided a proof of principle for a role of non-neutralizing IgG in controlling primary and recurrent infections of particular viruses, including herpesviruses, MV, poxviruses, LCMV and influenza virus (Farell *et al.*, 1991; Partidos *et al.*, 1997; Klenovšek *et al.*, 2007; Carragher *et al.*, 2008; Wright *et al.*, 2009; Richter *et al.*, 2013; Straub *et al.*, 2013). Work from the last few years demonstrated the indispensable role of FcγR-mediated effector functions to confer IgG immune protection to various viruses *in vivo* including murine herpesvirus-68, HIV and influenza (Holl *et al.*, 2009; Wright *et al.*, 2009; Jegaskanda *et al.*, 2013; Bournazos *et al.*, 2014; DiLillo *et al.*, 2014; Bournazos *et al.*, 2015). Analysis of the Fc glycan composition of antigen-specific IgG elicited upon influenza hemagglutinin (HA) vaccination in humans revealed that specific sialylated and fucosylated Fc glycoforms are enriched at different stages after vaccination (Wang *et al.*, 2015). In a mouse

model, Hebeis and colleagues have shown that virus-specific B cells adoptively transferred into immune-deficient hosts can be stimulated to antibody production by antigen alone, without requiring T-cell help (Hebeis *et al.*, 2004). In addition, transfer of serum from MCMV-immune animals had a comparably protective effect against MCMV infection as transfer of memory B-cells, indicating that the production of IgG might represent the protective principle (Klenovšek *et al.*, 2007). Following the favourable outcome of the MCMV serum transfer, it has been shown that administration of CMV-hyper-immunoglobulin in pregnant women was associated with a significantly lower risk of cCMV infection (Nigro *et al.*, 2005). The same group reported that many symptoms of cCMV infection were caused by placental dysfunction, which was reduced in hyper-immunoglobulin treated women (Adler & Nigro; 2009; Maidji *et al.*, 2010). In further studies, hyper-immunoglobulin therapy caused *in utero* resolution of the signs of foetal disease and reduction of the severity of disabilities of already primary infected mothers (Nigro *et al.*, 2008 and 2012). The prevention of congenital infection was not confirmed in a controlled study (Revello *et al.*, 2014). However, this inactivity has been attributed to suboptimal administration schedules (Hamprecht *et al.*, 2014). Despite unclear results for hyper-immunoglobulin application, the benefit of anti-HCMV specific antibodies are evident. Pre-existing immunity reduces the intrauterine HCMV transmission from acquiring primary infection during pregnancy to about 1% in reinfection or recurrence of seropositive persons. Moreover, it is associated with less severe sequelae of congenital CMV infection than in primary infection (Fowler *et al.*, 1992 and 2003) and protection against transfusion-associated CMV infection in the immediate postnatal period (Syndman *et al.*, 1995).

Abs are necessary to prevent acquisition and spread of CMV by seronegative individuals, but T-cell responses are crucial to suppress reactivation of the virus in seropositive individuals. In the 1970, two vaccine strains were attenuated: AD169 and Towne (Elek &Stern, 1974; Plotkin *et al.*, 1975). The AD169 attenuated strain was soon abandoned, but the Towne attenuated strain went on to extensive testing. The investigational Towne strain vaccine could protect humans against a challenge with non-attenuated HCMV, but naturally acquired immunity protected against a higher dose challenge than did the vaccine (Plotkin *et al.*, 1989). In addition, the attenuated strain failed to prevent natural acquisition of HCMV by women exposed to children in day care (Alder *et al.*, 2016). The next important development was the purification of a surface protein of HCMV, gB, because of homology with a glycoprotein of other herpesviruses. When combined with the MF59 oil-in-water adjuvant, the levels of neutralizing antibodies were produced in humans after three injections over a six-month period (Pass *et al.*, 2009; Bernstein *et al.*, 2016). However, antibodies and efficacy faded quickly. In the year 2000, HCMV was

placed in its highest priority for vaccine development in the USA. In addition, it was discovered that a pentameric complex of proteins was present on the surface of CMV and that this structure, consisting of gH, gL, and the products of genes UL128, 130 and 131, elicited far more neutralizing antibodies than gB (Wang & Shenk, 2005). In parallel, a rapid response to the pentameric complex in pregnant women infected by HCMV was associated with protection against transmission to the foetus (Lilleri *et al.*, 2017). An attempt was made to increase the immunogenicity of the Towne attenuated virus by making recombinants with the Toledo low passage "wild" HCMV. One recombinant turned out to be suitably immunogenic (Alder *et al.*, 2016). However, another attractive approach that combines safety with immunogenicity is a replication-defective virus. V160 is a genetically engineered HCMV-AD169 strain expressing the pentameric entry complex, and harbouring two proteins rendered potentially unstable by chemical combination but stabilized by a synthetic compound called Shield-1 (Shld 1). V160 only replicates in the presence of the Shld-1. On injection into humans in the absence of Shld-1, the virus cannot form infectious particles but does express immunogenic proteins (Wang *et al.*, 2016). Due to its RL duplication, V160 lacks the ULb' region comprising *UL145*. Thus, replicating V160 should be unable to degrade STAT2. However, V160 is replication defective *in vivo*. In phase 1 of clinical trials, the replication-defective virus (NCT01986010 (Adler *et al.* 2019), and NCT03840174) gave significant immune responses (Fu *et al.*, 2014). However, V160 failed in outcome regarding the prevention of HCMV infection (NCT01986010). A peptides, DNA and mRNA vaccines also represent the significant candidates (Wloch *et al.*, 2008; La Rosa *et al.*, 2012; Geall *et al.*, 2012). For the moment, the cCMV vaccine is highly desirable. It was shown that maternal antibodies recognizing HSV-1 are transmitted to the offspring, and protect neonatal mice against HSV-1 neurological infection and death (Levington *et al.*, 2013; Jiang *et al.*, 2017; Patel *et al.*, 2019). Accordingly, live attenuated guinea pig CMV (GpCMV) vaccines improve the pregnancy outcome in the congenital GpCMV infection model (Levington *et al.*, 2013; Schleiss *et al.*, 2013; Schleiss *et al.*, 2015; Schleiss *et al.*, 2021), and passive immunization with GpCMV-specific antibodies decreased foetal infection, intrauterine growth retardation, and reduced pregnancy losses (Chartjee et al., 2005).

1.3. Innate immune system

The innate immune system is one of the two main immunity arms (the other being the adaptive immune system) in vertebrates. It is the dominant immune system response found in plants,

fungi, insects, and primitive multicellular organisms. The receptors used by the innate system are expressed broadly on a large number of cells, therefore this system acts rapidly and thus constitutes the initial host response (Chaplin, 2010). The innate immune system includes physical barriers, the secreted mucus layer that overlays the epithelium, soluble proteins and bioactive small molecules. Also, it includes membrane bound receptors and cytoplasmic proteins that bind molecular patterns expressed on the surfaces of invading microbes (Chaplin, 2010).

An invading pathogen or toxin is encountered by cells already present in all tissues, mainly resident macrophages, dendritic cells, histiocytes, Kupffer cells, and mast cells. These cells present receptors on the surface or within the cell, named pattern recognition receptors (PRRs), which recognize molecules that are broadly shared by pathogens but distinguishable from host molecules, referred to as pathogen-associated molecular patterns (PAMPs). At the onset of an infection, burn, or other injuries, these cells undergo activation (one of their PRRs recognizes a PAMP) and release inflammatory mediators, like cytokines and chemokines, which are responsible for the clinical signs of inflammation.

Mutations in elements of the innate immune response demonstrate that innate immune effectors are critical for effective host defence. Thus, the innate and adaptive arms of the immune response should be viewed as the complementary immune systems (Chaplin, 2010).

1.3.1. Interferon and JAK/STAT signalling pathway

Interferons (IFNs) are a group of proteins known as cytokines, made and released by host cells after recognition of PAMPs by PRRs on their surface. Interferons are named for their ability to "interfere" with viral replication (Parkin & Cohen, 2001) by protecting cells from virus infections. They are three IFN classes: type I IFN, type II IFN, and type III IFN (Figure 4). Type I interferons comprise all IFN-α subtypes (Cull *et al.*, 2003; Gerlach *et al.*, 2009; Gibbert & Dittmer, 2011) like IFN-β, IFN-ε, IFN-τ, IFN-κ, IFN-δ and IFN-ω (Pestka *et al.*, 2004; Liu *et al.*, 2005). Type I interferons have immunomodulatory functions, and are produced when the immune cells recognize an invading virus. The only known type II IFN is IFN-γ, which is induced by cytokines such as IL-12, and its expression is mostly restricted to immune cells such as T cells and NK cells. Although originally defined as an agent with predominantly immunomodulatory functions, the properties of IFN-γ include direct antiviral activity (Boehm *et al.*, 1997; Zimmermann *et al.*, 2005; Le *et al.*, 2008; Trilling *et al.*, 2009; Trilling *et al.*,

2011). IFN-λ forms the type III IFN family, which is composed of four members, IFN-λ1, IFN-λ2 and IFN-λ3, also named IL-29, IL-28A and IL-28B, respectively (Kotenko *et al.*, 2003; Sheppard *et al.*, 2003) and IFN-λ4 which has been described in humans (Prokunina-Olsson *et al.*, 2013). It contains a new transiently induced region that harbours a dinucleotide variant ss469415590 (TT or ΔG). ΔG is a frameshift variant that creates a novel gene, designated *IFNL4*, encoding the interferon-λ4 protein (IFNL4), which is moderately similar to *IFNL3* (Hamming *et al.*, 2013; Prokunina-Olsson *et al.*, 2013).

Type I IFNs bind to a specific cell surface receptor complex known as the IFN-α/β receptor (IFNAR) that consists of IFNAR1 and IFNAR2 chains. The IFNAR1 subunit is constitutively associated with tyrosine kinase 2 (TYK2), whereas IFNAR2 is associated with Janus kinase 1 (JAK1). After binding of type I IFN to its receptor, the receptor goes through conformational change including dimerization and autophosphorylation, which activates associated tyrosine kinases. The tyrosine kinases phosphorylate intracellular tyrosine residues of the receptor, which then act as a binding site for signal transducer and activator of transcription, STAT1 and STAT2. The highly conserved src homology 2 (SH2) domain of the STAT proteins are responsible for phosphorylation of STAT proteins at specific tyrosine residues (Y7101 at STAT1; Y690 at STAT2). The activated STAT proteins then form heterodimers and bind interferon regulatory factor 9 (IRF9). This heterotrimeric interferon-stimulated gene factor 3 (ISGF3) complex translocates into the nucleus and binds to specific DNA elements with a characteristic consensus sequence. This sequence is called interferon-stimulated response elements (ISRE) and the induction of the transcription of interferon-stimulated genes (ISGs) starts (Platanias 2005; Ivashkin & Donlin, 2014) (Figure 4). Evidence is accumulating for the existence of a STAT2/IRF9-dependent, STAT1-independent IFNα signalling pathway. This complex can induce antiviral ISRE-containing ISGs commonly up-regulated by STAT2/IRF9 and ISGF3 (Blaszczyk *et al.*, 2015). IFN-α (in addition to activating ISGF3) rapidly activates IFN-γ activated site (GAS) -binding factor, also known as the IFN-alpha activation factor (AAF) (Decker *et al.*, 1991).

Interferon type II (IFN-γ) binds to a receptor composed of the subunits IFNGR1 and IFNGR2 and linked to the tyrosine kinases Jak1 and Jak2. After binding of IFN-γ to its cognate receptor, signal transduction phosphorylate STAT1 molecules in the canonical pathway. STAT1 already exists in the non-phosphorylated state as a homodimer. These STAT1 dimers show an anti-parallel conformation (head-to-tail) and are not active. Only after activation do they undergo a transition into a parallel (head-to-head) conformation that is transcriptionally active (Venta *et*

al., 2008). Activated STAT1 homodimers translocate into the nucleus and bind to specific promoter regions with a characteristic consensus sequence (gamma-activated sequences [GAS]). IFN-γ can not only activate the transcription of different genes, but also suppress their transcription. These genes are called IFN-repressed genes (IRepGs) (Trilling *et al.*, 2013). Also, it has been shown that STAT2 plays an important role in IFN-γ signal transduction, where it is phosphorylated and forms ISGF-3 complex (Matsumoto *et al.*, 1999; Zimmerman *et al.*, 2005; Trilling *et al.*, 2013; Le-Trilling *et al.*, 2018).

Type III interferon acquires signals through a receptor complex consisting of IL10R2 (also called CRF2-4) and IFN28Ra (also called CRF2-12). The IFN-λ signalling pathway is activated *via* Jak1 and Tyk2 tyrosine kinases, which phosphorylate STAT1 and STAT2. It has been shown that IFN-λ has an antiviral effect against various viruses or fungi (Ank *et al.*, 2006; Pott *et al.*, 2011; Le-Trilling *et al.*, 2018).

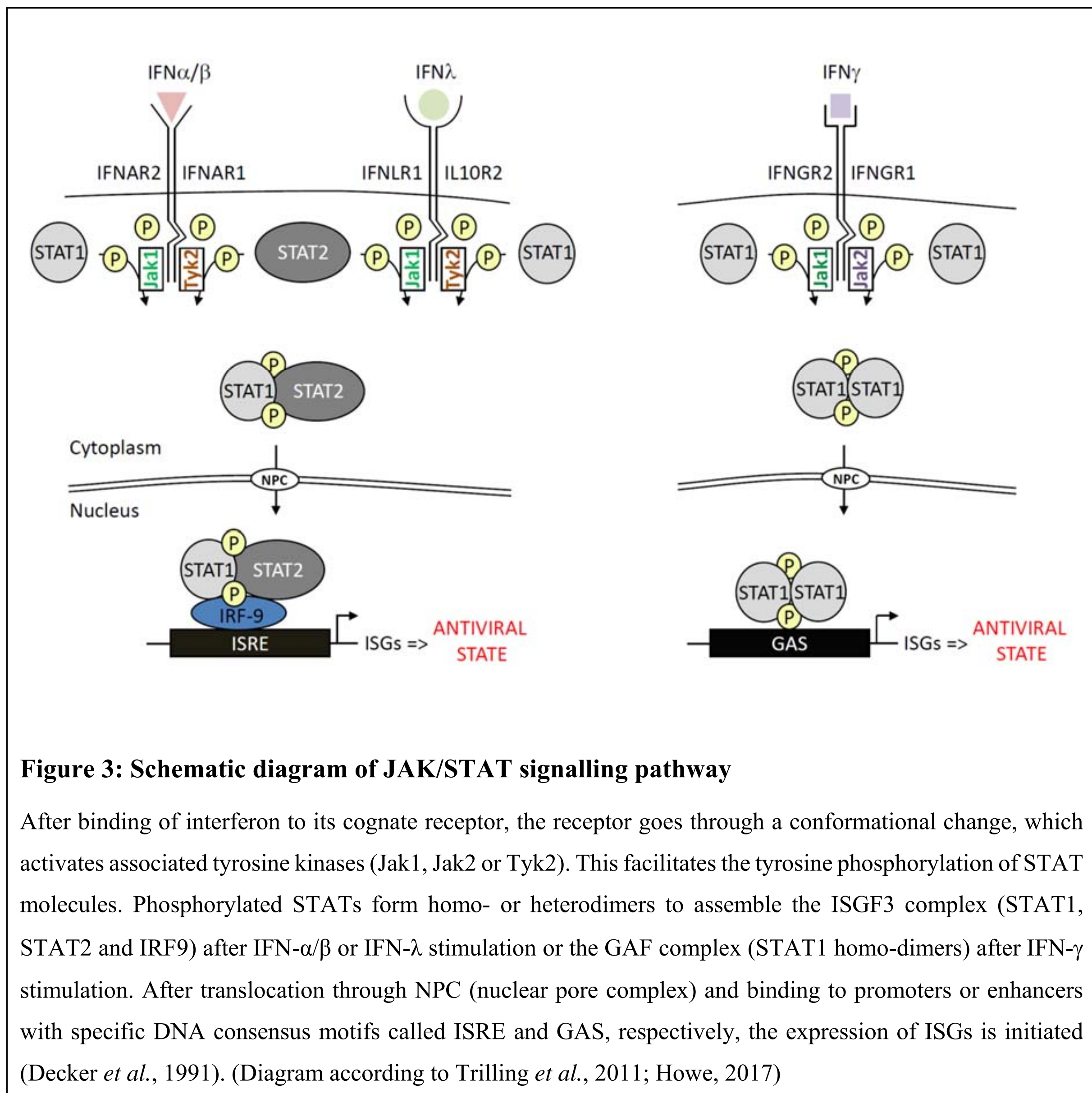


Figure 3: Schematic diagram of JAK/STAT signalling pathway

After binding of interferon to its cognate receptor, the receptor goes through a conformational change, which activates associated tyrosine kinases (Jak1, Jak2 or Tyk2). This facilitates the tyrosine phosphorylation of STAT molecules. Phosphorylated STATs form homo- or heterodimers to assemble the ISGF3 complex (STAT1, STAT2 and IRF9) after IFN-α/β or IFN-λ stimulation or the GAF complex (STAT1 homo-dimers) after IFN-γ stimulation. After translocation through NPC (nuclear pore complex) and binding to promoters or enhancers with specific DNA consensus motifs called ISRE and GAS, respectively, the expression of ISGs is initiated (Decker *et al.*, 1991). (Diagram according to Trilling *et al.*, 2011; Howe, 2017)

1.3.2. pM27

pM27 is an MCMV-encoded antagonist that blocks the JAK/STAT signalling pathway. An *M27* transposon insertion mutant with a disruption of open reading frame *M27* lead to the attenuation of viral virulence and deficient growth *in vivo* (Abenes *et al.*, 2001). *M27* is an early-late expressed gene and encodes a 79-kDa protein that selectively binds and downregulates STAT2, but it has no effect on STAT1 activation and signalling (Zimmermann *et al.*, 2005; Trilling *et al.*, 2014). Although it was not found in a systematic search for MCMV particle proteins (Kattenhorn *et al.*, 2004), pM27 appeared to be present in the virion since it could be detected in purified supernatant MCMV particle preparations. The pM27 recruits STAT2 and shuttles STAT2 to DNA-damage DNA-binding protein (DDB1), which is an adapter protein of the

Cul4A/B-Roc1 ubiquitin ligase complex. This is followed by polyubiquitination and proteasomal degradation of STAT2 (Trilling *et al.*, 2011) (Figure 5). Although encoded by MCMV, pM27 can also induce the proteasomal degradation of the human STAT2 (Trilling *et al.*, 2011; Landsberg *et al.*, 2018), probably by using STAT2 conserved regions recognized by pM27. Usually, interaction with DDB1 occur *via* WD40 domain with many interacting proteins. However, this sequence cannot be found in pM27 or paramyxoviral-encoded SV5-V protein, and therefore is not necessary for the interaction. SV5-V protein contains two zinc binding pockets critically required for DDB binding (Lin *et al.*, 1998), one of which with the sequence CxCxxC (aa$_{206-211}$) (Li *et al.*, 2006). A CxCxxC motif is conserved throughout cytomegalovirus evolution in *M27* homologs with the exception of HCMV and chimpanzee cytomegalovirus (ChCMV). CxCxxC motif is also present in pM27 (aa$_{274-279}$) suggesting that pM27 like pE27 is a Zn^{2+}-binding protein (Le-Trilling *et al.*, 2023). A mutation of this motif (C279A) resulted in a significant impairment of the interaction between pM27 and DDB1 (Trilling *et al.*, 2011). The pM27 co-precipitated DDB1 in human cells, consistent with the high degree of sequence conservation of DDB1 and the functional competence of pM27 in human cells (Trilling *et al.*, 2011). So, pM27-mediated inhibition of STAT2-dependent Jak/STAT signalling allows virus replication in the presence of type I and II interferons (Zimmermann *et al.*, 2005; Trilling *et al.*, 2011). Recently, it has been shown that pM27 also inhibits type III IFN-λ signalling (Le-Trilling *et al.*, 2018).

To monitor viral functions affecting IFN-inducible transcription throughout the CMV replication cycle, a reporter cell line expressing the luciferase gene under the control of an ISRE-dependent promoter was generated. After infection, the IFN-α–induced, ISRE-dependent luciferase activity declined and was abolished 36 h p.i. This effect required MCMV gene expression because UV-inactivated MCMV and the presence of the CMV polymerase inhibitor phosphonoacetic acid (PAA) was not capable of mediating this inhibition. To identify the MCMV gene products responsible for this inhibition, 3T3-ISRE-luc cells were infected with a large panel of reconstituted Tn*Max*16 containing MCMV BAC mutants and tested for an IFN-α–induced luciferase expression. Three independent mutants failed to suppress the IFN-α–stimulated luciferase expression. The Tn*Max*16 insertion site in all three mutants was mapped to the *M27* gene (Zimmermann *et al.*, 2005). While pM27 *in vitro* is not essential for viral replication in the absence of interferons, ΔM27-MCMV in the presence of IFN-α clearly shows reduced replication, and in the presence of IFN-γ almost no replication (Zimmerman *et al.*, 2005; Trilling *et al.*, 2011; Le-Trilling *et al.*, 2018). In STAT2-deficient cells, the ΔM27-MCMVs ability to replicate can be fully restored under IFN treatment (Le-Trilling *et al.*, 2018).

Upon infection, ΔM27-MCMV did not replicate in the liver and did not spread to the SGs. The deletion of *M27* resulted in a dramatic replication deficiency of MCMV *in vivo* (Abenes *et al.*, 2001; Le-Trilling *et al.*, 2018). Replication was largely restored in all tested organs of STAT2-deficient animals (e.g., >1,000-fold recovery in spleen and liver at 5 days p.i.). However, in contrast to the complete recovery of ΔM27-MCMV replication observed in IFN-conditioned cells *in vitro*, a small but statistically significant attenuation of ΔM27-MCMV in comparison to that of wt-MCMV remained in STAT2- deficient animals *in vivo*. This indicates that pM27 possesses at least one additional function and/or target beyond STAT2 antagonism, which is relevant *in vivo* but appears to be dispensable in fibroblasts *in vitro* (Le-Trilling *et al.*, 2018).

Like MCMV, HCMV expresses a protein that induces proteasomal STAT2 degradation (Le *et al.*, 2008a; Le *et al.*, 2008b; Weekes *et al.*, 2014). HCMV-encoded STAT2 degradation is not mediated by pUL27 - the HCMV-encoded position and sequence homolog of pM27 (Le *et al.*, 2008a; Trilling *et al.*, 2011). Instead, a short and a large protein isoform of the HCMV protein pUL145 induce STAT2 degradation (Le-Trilling *et al.*, 2020). Both, pM27 and pUL145 interact with STAT2 and exploit DDB1-containing Cul4A-Roc1 ubiquitin ligase to stimulate the poly-ubiquitination and subsequent proteasomal degradation of STAT2 (Trilling *et al.*, 2011; Becker *et al.*, 2019; Le-Trilling *et al.*, 2020).

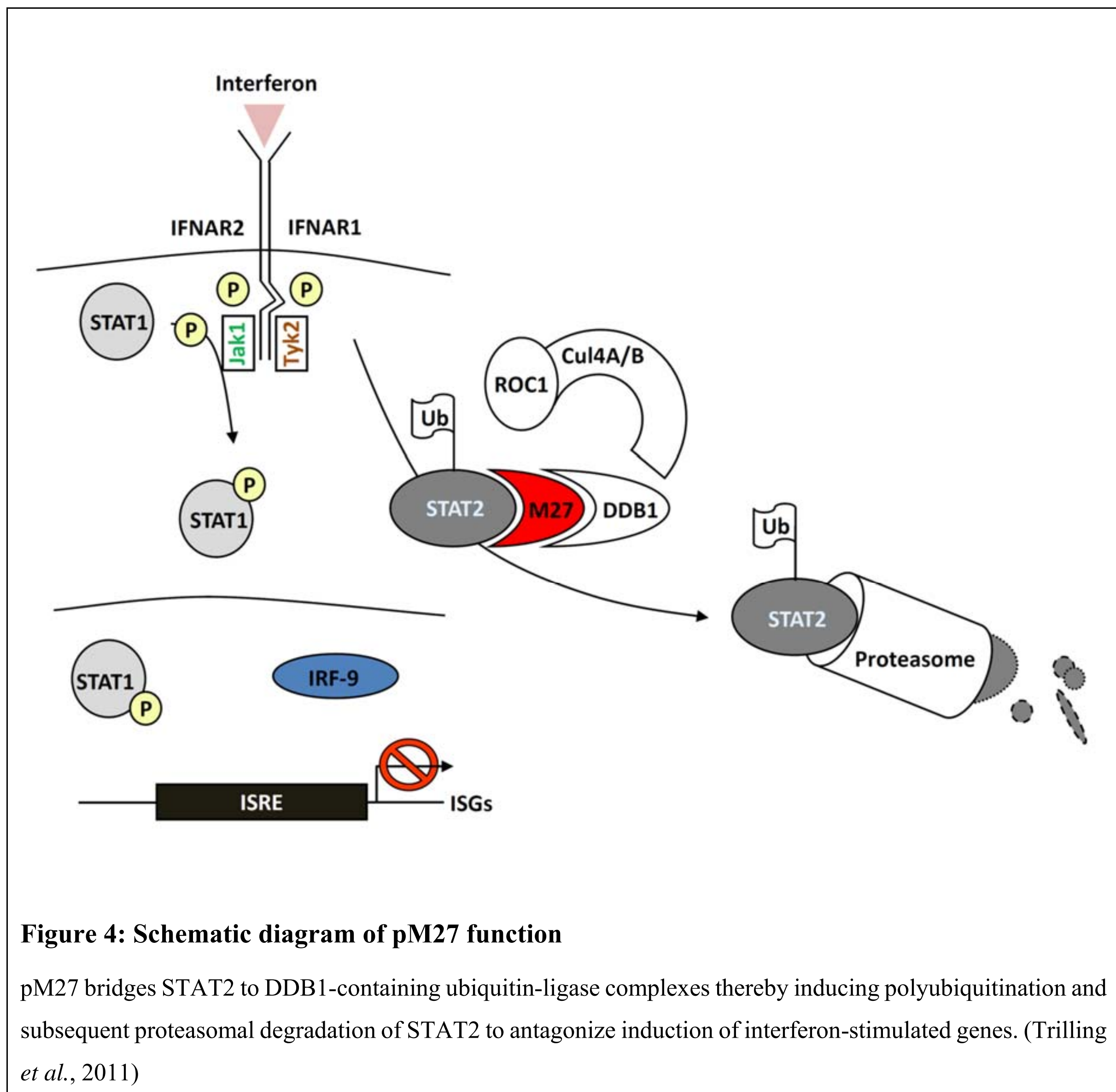


Figure 4: Schematic diagram of pM27 function

pM27 bridges STAT2 to DDB1-containing ubiquitin-ligase complexes thereby inducing polyubiquitination and subsequent proteasomal degradation of STAT2 to antagonize induction of interferon-stimulated genes. (Trilling *et al.*, 2011)

1.4. Immunoglobulin G (IgG)

Upon infection, the immune system tries to identify and neutralize foreign objects such as viruses and viral proteins. Therefore, the specific antibody responses against many viral proteins are produced. The earliest term of antibody came from von Behring, Kitasato, and Wernicke at 1890, when they discovered that serum from immunized animals was able to protected animals from diphtheria. The method was based on the principle of passive immunization (von Behring & Kitasato, 1991).

Antibodies are ~150 kDa glycoproteins belonging to the immunoglobulin superfamily. They have different structures, biological activities and distribution in the body. In humans and most mammals, an antibody unit consists of four polypeptide chains; two identical heavy chains and

two identical light chains connected by disulphide bonds and non-covalent interaction giving rise to the classical “Y”-shape (Woof and Burton, 2004) (Figure 6). Structurally, an antibody is also partitioned into the Fab part or fragment antigen binding that contains the paratope recognizing the epitopes expressed by pathogens/viruses; and the Fc part or fragment crystallisable which is important for mediating different effector functions against pathogens (Putnam *et al.*, 1979). The antibody chains are composed of repeated anti-parallel β-sheets, called Ig domain. The N-terminus of each chain is situated at the tip. The light chains possess two and the heavy chains four to five Ig domains. The paring of the variable domain of the light (V_L) and the heavy (V_H) chain creates the antigen-binding site (paratope). Each variable domain contains three hypervariable loops (CDR: complementary-determining region) in-between the β-sheet, which determine the antigen specificity (Al-Lazikani *et al.*,1997; North *et al.*, 2011; Nikoloudis *et al.*, 2014) . The variable domains (V_H and V_L) and the first constant Ig domains (C_H1 and C_L) form the Fab fragment, which is separated by an unstructured and flexible sequence, the hinge region, from the constant part (C_H2 - C_H3 or C_H2 - C_H4) (Putnam *et al.*, 1979).

Antibodies exist as five different classes: Immunoglobulin A (IgA), Immunoglobulin E (IgE), Immunoglobulin M (IgM), Immunoglobulin G (IgG), and Immunoglobulin D (IgD). Although the classes exhibit a similar structure, the obtained different protein sequences result in different numbers of Ig-like domains, numbers and positions of intra- /intermolecular disulphide bonds, N- and O-glycosylation sites, and the length of the hinge (Woof and Burton, 2004). IgM and IgA are found as pentamers and dimmers, respectively, in sera (Kenneth *et al.*, 2013).

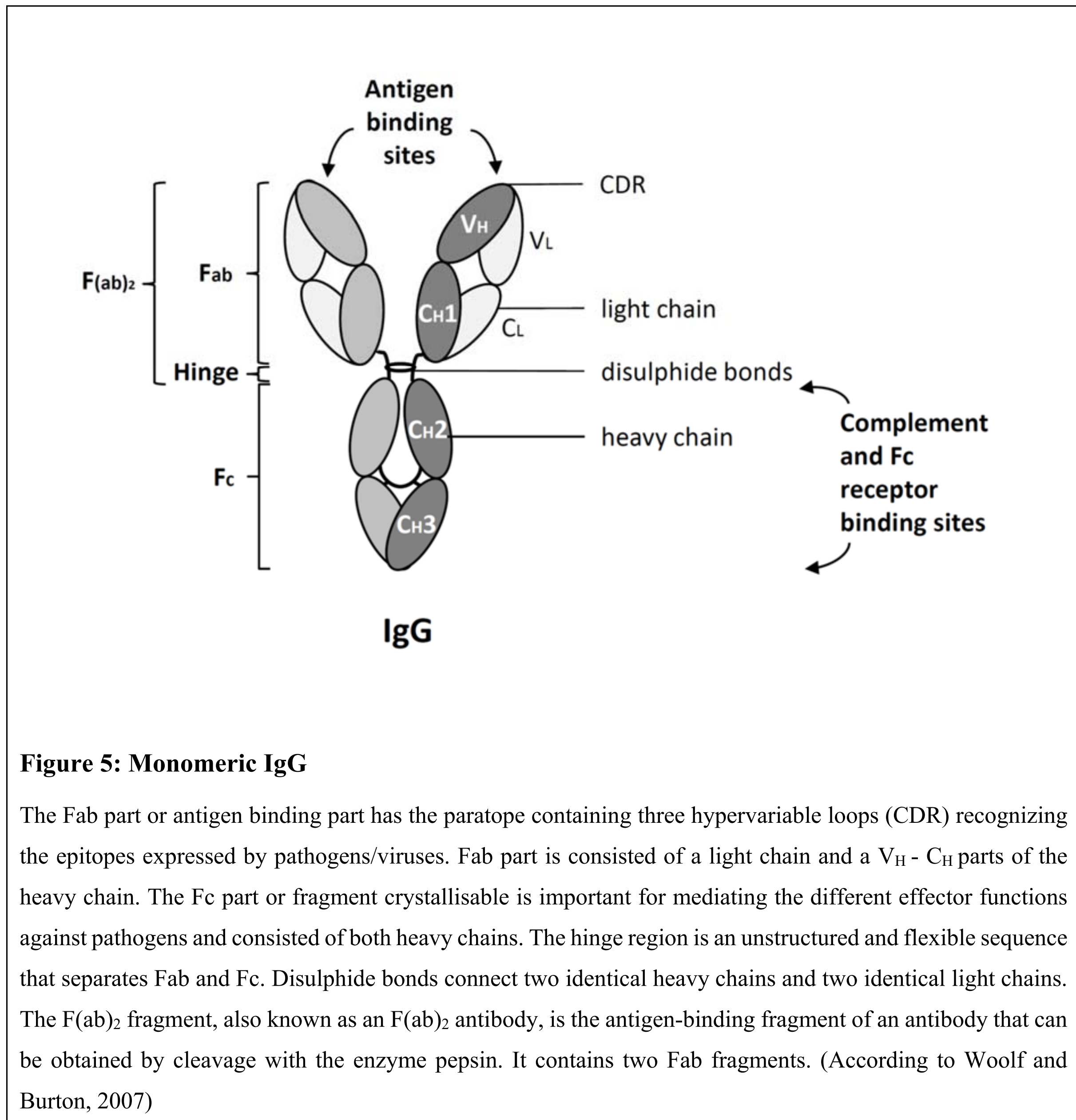


Figure 5: Monomeric IgG

The Fab part or antigen binding part has the paratope containing three hypervariable loops (CDR) recognizing the epitopes expressed by pathogens/viruses. Fab part is consisted of a light chain and a V_H - C_H parts of the heavy chain. The Fc part or fragment crystallisable is important for mediating the different effector functions against pathogens and consisted of both heavy chains. The hinge region is an unstructured and flexible sequence that separates Fab and Fc. Disulphide bonds connect two identical heavy chains and two identical light chains. The $F(ab)_2$ fragment, also known as an $F(ab)_2$ antibody, is the antigen-binding fragment of an antibody that can be obtained by cleavage with the enzyme pepsin. It contains two Fab fragments. (According to Woolf and Burton, 2007)

The antibody classes have the different serum abundance depended on age, sex, demographic factor, and the common habits. The different serum abundance typically depended on age: IgG >>> IgA >IgM > IgD > IgE (Gonzalez-Quintela *et al.*, 2008). IgM is the first Ig class produced in the primary immune response. It does not undergo affinity maturation resulting in a lower antigen affinity as other Ig classes. This is overcoming by the formation of pentamers, which increases the avidity of the molecule due to the higher number of antigen binding sides. IgM is found mainly in the blood stream, leading and a potent activator of the complement system (Kenneth *et al.*, 2013). The circulating immunoglobulin contains about 75% of IgG, making it the most abundant antibody class in serum. IgG has a crucial protective capacity against bacteria and viruses in a secondary immune response. IgG is present in the extracellular fluid (Kenneth

et al., 2013). The high abundance is also a consequence of its long half-life, which is regulated by the neonatal Fc receptor (FcRn) (Ghetie *et al.*, 1996; Ghetie *et al.*, 1997; Chaudhury *et al.*, 2003; Roopenian *et al.*, 2007; Ward *et al.*, 2009) (Figure 8). Additionally, the FcRn transports IgG across the placenta into the foetal blood stream (Story *et al.*, 1994; Firan *et al.*, 2001).

There are four subclasses: IgG1, IgG2, IgG3 and IgG4 in the human and IgG1, IgG2a, IgG2b and IgG3 in the mouse. Furthermore, IgG possesses a conserved N-glycosylation site at asparagine 297 in the C_H2 domain of the Fc part. The glycan consists of a heptasaccharide with variable additions of fucose, galactose, and/or sialic acid. The conformation of the Fc part is consisted of non-covalent interaction of the C_H3 domains of both heavy chains and the opening between the C_H2 domains. The Fc glycan modulates the conformation of the Fc part and differences in the glycan composition influence the effector function triggered by the IgG molecule (Bournazos *et al.*, 2015). Antibodies are crucial molecules that function as adaptors linking pathogens with appropriate pathogen elimination mechanisms (Nimmerjahn & Ravetch, 2010). Humoral immune response levels were assessed in sera from various mouse strains infected with MCMV. IgG levels rose by day 14, remained high until day 30 and then declined. IgM levels in sera from C57BL/10 (B10) and BALB/c mice increased 7-11 days after infection (Price et al., 1992). Increases in serum IgG were recorded in A/J and BALB.B mice infected with MCMV, which are more susceptible than BALB/c, and C3H/HeJ, and BALB.K mice, which are as resistant as CBA. Hence, Ig levels do not correlate with $H\text{-}2^k$-mediated resistance to MCMV, or with input virus dose (Price *et al.*, 1993). Furthermore, IgG subclasses are differentially induced. $CD4^+$ T cell differentiation is driven towards T helper cell type 1 or 2 (T_H1 or T_H2). Intracellular pathogens as viruses generally induce a T_H1 response resulting in IFN-γ as predominant cytokine and efficient class switching to IgG2a (Coutelier *et al.*, 1988). In contrast, T_H2 cells secret IL-4 and IL-5 and leading to a high level of IgG1 (and IgE) and TGF-β inducing IgG2b (and IgA) (Mosmann *et al.*, 1989). IgG1 represents the predominant subclass elicited by protein immunogens (Coutelier *et al.*, 1987).

The membrane-bound form of an antibody is called a surface immunoglobulin (sIg) or a membrane immunoglobulin (mIg). It is part of the B-cell receptor (BCR), which allows a B-cell to detect when a specific antigen is present in the body and triggers B-cell activation (Parker et al., 1993). The BCR is composed of surface-bound IgD or IgM antibodies and associated Ig-α and Ig-β heterodimers, which are capable of signal transduction (Wintrobe *et al.*, 2004). Binding of an antigen to the BCR leads to the internalization, processing, and presentation of peptides derived from the antigen by MHC-II molecules on the cell surface (Tollar *et al.*, 2008).

For the full activation of the B cell, the presented peptide has to be recognized by a $CD4^+$ T-cell. These $CD4^+$ T-cells are activated before by an antigen-presenting cell (APC), which had encountered the antigen as well. The $CD4^+$ T- and B cell recognize the same antigen but different epitopes of the antigen. The activated B cells expand clonally. Multiple rounds of expansion, somatic hyper-mutation of the variable regions, and subsequent selection of B cells with increased affinity to their antigen lead to the affinity maturation of the antibody (Goding *et al.*, 1978). An Ig class switch is induced resulting in the association of the variable regions with the constant region of another Ig subclass. These B cells terminally differentiate to plasma cells, which secrete soluble antibodies with the same paratope, or memory B cells that survive in the body to enable long-lasting immunity to the antigen (Borghesi & Milcarek, 2006). Because soluble antibodies are released into the blood and tissue fluids, traditionally known as humours, antibody-mediated immunity is considered a part of humoral immunity (Pier *et al.*, 2004). An alternative pathway to activate B cells can be triggered by certain microbial antigens, for example unmethylated CpG DNA or polysaccharides (Nutt *et al.*, 2015). Thereby, the activation signal is provided by the antigen. The signal provided by the antigen can be the induced by activation of PRRs or the extensive crosslinking of the BCR by repeating epitopes. This pathway is called thymus-independent (TI) in contrast to the B-cell activation with $CD4^+$ T-cell help, which is thymus-dependent (TD). TI antigens lead to short lived plasma cells with limited class switch, while TD antigens induce affinity maturation, class switch, and differentiation into long-lived memory cell or plasma cells (Bortnick *et al.*, 2012).

First, the variable region of the heavy chains is formed and paired with a germline encoded light chain surrogate. If the pre-BCR is successfully formed, the B-cell expands and the variable region of the light chain is rearranged. The mature B-cells possess BCRs of the type IgM and IgD. The exon for the variable region of the heavy chain is generated by the combination of three segments (V variable, D diverse, J joining) and exon of the light chain consists of two segments (V, D), whereby the V/D/J segments are randomly chosen from the respective gene segment cluster. These recombination processes is called V(D)J recombination (Nemazee, 2006). Each B cell expresses one specific antibody/BCR, which is generated by an irreversible rearrangement of genomic DNA segments, or high rate of a point mutation (somatic recombination) (Diaz *et al.*, 2002). As a consequence, any daughter B cells will acquire slight amino acid differences in the variable domains of their antibody chains. This serves to increase the diversity of the antibody pool and affects the antibody's antigen-binding affinity, causing B cells with low affinity die due to apoptosis (Honjo *et al.*, 1985). The process of generating antibodies with increased binding affinities is called affinity maturation (Neuberger *et al.*,

2000). Class switching allows different daughter cells from the same activated B cell to produce antibodies of different isotypes (IgA, IgE, or IgG). Only the constant region of the antibody heavy chain changes during class switching, and the variable regions, remain unchanged. Thus the progeny of a single B-cell can produce antibodies, all specific for the same antigen, but with the ability to produce the effector function appropriate for each antigenic challenge. Class switching is triggered by cytokines; the isotype generated depends on which cytokines are produced by follicular $CD4^+$ T-cells (Stavnezer & Amemiya, 2004). The DNA of a heavy chain exon is broken by the activity of enzymes at two conserved nucleotide motifs called switch (S) regions. The variable domain exon is the re-joined through a non-homologous end joining (NHEJ) (Durandy, 2003; Casali & Zan, 2004).

1.4.1. IgG-mediated effector functions

The IgG-mediated effector functions can be divided in two major groups (Parren and Burton, 2001):

1. One targeting directly the integrity of the virus particle:
    a) Neutralization: Inhibition of the virus binding to entry receptors and therefore, infection of the target cells (Figure 7 A).
    b) Complement-mediated virolysis: Activation of the classical pathway of the complement cascade which terminates in lysis of the envelope which surrounds the virion or capsid, therefore destroying the virus before infection (Figure 7 B).
    c) Virus aggregation: No binding to entry receptors or inability to lose the viral envelope (Figure 7 A2).
    d) Fc-mediated phagocytosis of immune complexes or pathogens: after binding of the antibody to the virion particle, host cells bearing FcγRs are recruited and phagocytosis occurs to improve antigen processing and presentation (Figure 7 D).
2. One targeting the virus-infected cells, thus impeding virus replication, spread and re-infection:

a) Complement mediated-cell lysis: activation of the classical pathway of the complement cascade resulting in lysis of the infected cell (Figure 7 B).

b) Antibody dependent cellular cytolysis (ADCC) upon binding of the cellular FcγR: degranulation of NK cells, macrophages, neutrophils or dendritic cells resulting of lysis of infected cells (Figure 7 C).

c) Induction of apoptosis of infected cells upon binding to host FcγRs: death receptors become activated and apoptosis mediators are released.

A minor fraction of antibodies is able to blunt infections directly by blocking essential mechanisms of attachment, entry or uncoating of intracellular pathogens like viruses (Burton *et al.*, 2002). These antibodies with direct antiviral capacity are referred to as neutralizing antibodies (nAbs). Neutralizing activity requires the Ab to be of relatively high affinity and/or avidity for exposed structures on the surface of the virus (Roost *et al.*, 1995; Bachmann *et al.*, 1997). Accordingly, they bind to structures that interfere with the interaction of the viral surface protein and its receptor by steric obstruction. Interference to the structures can occur at different stages of virion entry or egress (Reading *et al.*, 2007). Such nAbs can block sterically or prevent the conformational changes of the attachment or the entry of virion into the target cell, which are necessary to induce the fusion of the virus envelope with the plasma membrane (Figure 7 A2). Neutralization can also take place in endosomes, allowing the endocytosis, but inhibiting a viral escape into the cytoplasm by uncoating (Figure 7 A3). Also, nAbs can repress virion assembly and release from the plasma membrane (Klasse *et al.*, 2002) (Figure 7 A4). Moreover, they can inhibit cell-to-cell spread of viruses (Burioni *et al.*, 1994; Pantaleo *et al.*, 1995; Mannini-Palenzona *et al.*, 1998) (Figure 7 A5). Only a few human antibodies contact the receptor-binding site directly (Bizebard *et al.*, 1995; Smith *et al.*, 1996; Saphire *et al.*, 2001), and do so through long CDR3 regions (Saphire *et al.*, 2001). The vast majority of virus-specific antibodies have no neutralizing activity, they are called non-neutralizing antibodies (non-nAbs). Non-nAbs are often specific for particular proteins: internal proteins that are not accessible on intact virions or infected cells (viral nucleoproteins) (Battegay *et al.*, 1993; Leung *et al.*, 2004); proteins that have been denatured, degraded or incompletely translated or processed (in terms of cleavage or glycosylation) (Parren *et al.*, 1997; Sakurai *et al.*, 1999); or proteins that are not oligomerized (which is required for T-cell-independent activation of B-cells) (Sattenau & Moore, 1995; Sakurai *et al.*, 1999); proteins that are native surface antigens, but do not have neutralizing activity because they are directed against epitopes for which antibody binding does not interfere with viral attachment or entry (Lefrancois & Lyles, 1982).

Some virus species utilize non-nABs or insufficient amounts of neutralizing antibodies binding to virus particles to facilitate their uptake into host cells. This mechanism is known as antibody-dependent enhancement (ADE) (Tirado *et al.*, 2003). It has been observed for Dengue and Zika virus (Dejnirattisai *et al.*, 2016).

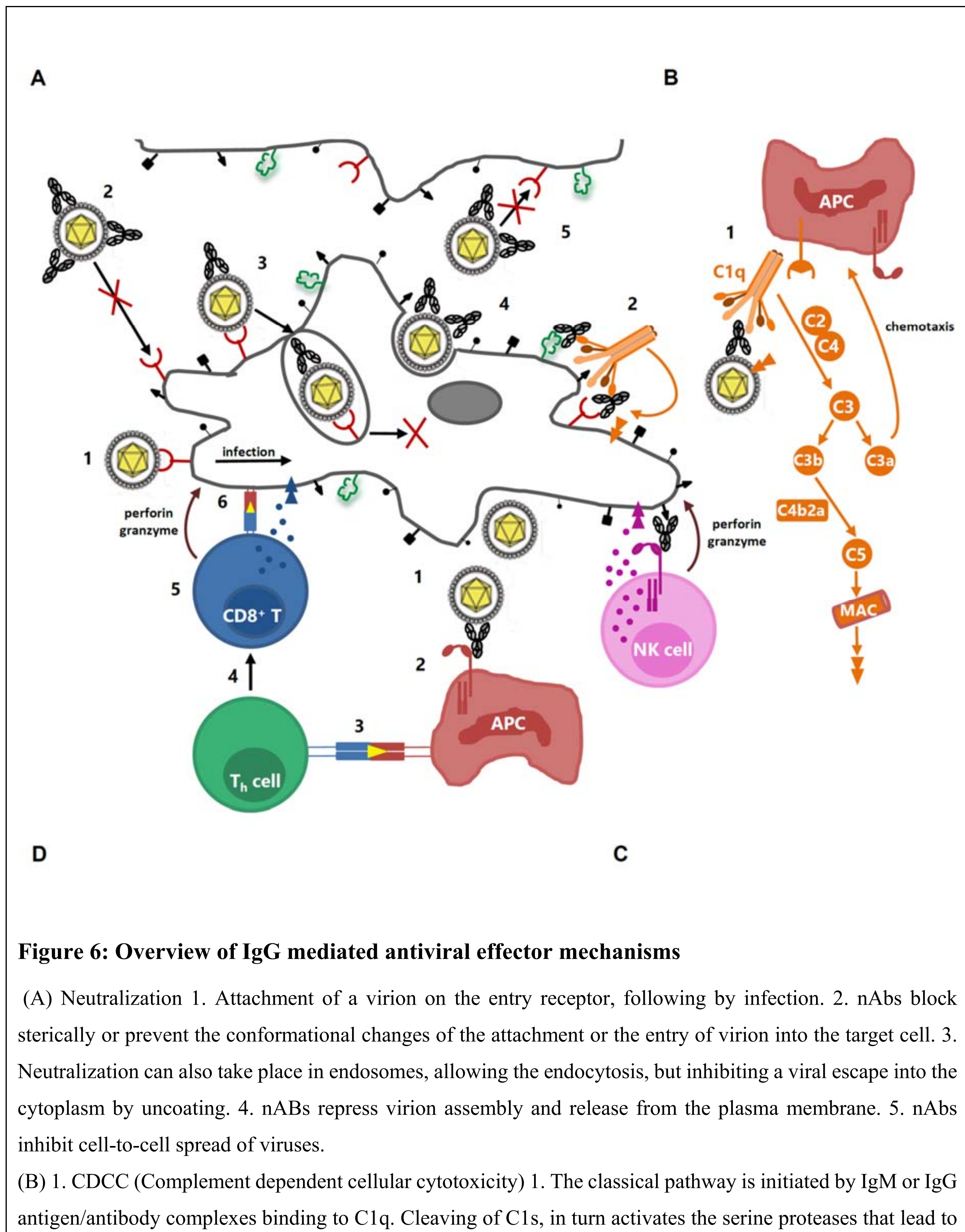


Figure 6: Overview of IgG mediated antiviral effector mechanisms

(A) Neutralization 1. Attachment of a virion on the entry receptor, following by infection. 2. nAbs block sterically or prevent the conformational changes of the attachment or the entry of virion into the target cell. 3. Neutralization can also take place in endosomes, allowing the endocytosis, but inhibiting a viral escape into the cytoplasm by uncoating. 4. nABs repress virion assembly and release from the plasma membrane. 5. nAbs inhibit cell-to-cell spread of viruses.

(B) 1. CDCC (Complement dependent cellular cytotoxicity) 1. The classical pathway is initiated by IgM or IgG antigen/antibody complexes binding to C1q. Cleaving of C1s, in turn activates the serine proteases that lead to

cleaving of C4 and C2, leading to formation of C3 convertase. This in turn cleaves C3 into C3a and C3b. C3a acts as a recruiter of inflammatory cells (anaphylatoxin), C3b binds to the C4b2a complex to form C5 convertase (C4b2a3b). The C5 convertase initiates the formation of the membrane attack complex (MAC) that inserts into membrane creating functional pores and leading to its lysis. 2. CDC (Complement dependent cytotoxicity) IgG or IgM are bound to surface antigen on target cell, the classical complement pathway is triggered, resulting in formation of a MAC and target cell lysis.
(C) ADCC (Antibody dependent cellular cytotoxicity) NK cells are expressing Fcγ receptor which binds to the Fc region of IgG Abs recognizing virus infected target cells This leads to the polarization of the cell mediating cell-to-cell contact and release of the preformed granules containing cytolytic substances in the direction of the target cell. Pores are formed in the target cell membrane by perforin and apoptosis is induced by proteolytic enzymes called granzymes.
(D) Presentation of antigenic peptides in the context of major histocompatibility (MHC) molecules to T-cells 1. Assembly of virions. 2. Uptake of virus by antigen presenting cell (APC). 3. Presentation of antigens, including epitopes, to T-cell receptor (TCR). 4. Activation of T helper (Th) cells and production of cytokines, that, recognized by 5. cytotoxic T-cells, 6. kill the virus. (According to Parren & Burton, 2001; Nimmerjahn, 2008)

NAbs against HCMV appear first approximately 13 weeks post primary infection (Eggers *et al.*, 1998; Eggers *et al.*, 2001). However, the inhibitory function of nAbs, particularly in HCMV infections differ in their blocking efficiency among susceptible cell types (Gerna *et al.*, 2008) due to neutralizeable protein complexes involved in virus entry (Wang *et al.*, 2005). The first neutralizing MCMV antibodies were detected late, 21 to 28 dpi, in contrast to all MCMV specific IgGs antibodies (Quinnan *et al.*, 1979; Hangartner *et al.*, 2006). During primary MCMV infection, there is no detectable physiological role for *de novo* generated antibodies regarding the organ clearance and the prevention of horizontal transmission. During recurrence, the presence of neutralizing antibody has a significant effect on virus spread, which proves the role of antibodies in limiting the spread of virus (Reddehase *et al.*, 1994). During recurrence after immunosuppression, however, antibodies are the only specific immune function that limits extracellular dissemination (Jonjić *et al.*, 1994). Nevertheless, nAbs are important in neutralizing the toxic effects of bacterial toxins. Example of nAb is diphtheria antitoxin, which can neutralize the biological effects of diphtheria toxin. Neutralizing Abs are not effective against extracellular bacteria, as the binding of antibodies does not prevent bacteria from replicating (Trefers, 2014).

Natural antibodies are low-affinity and poly-reactive antibodies that are present at low titres in the blood of naive individuals. In mice, natural antibodies are present under germ-free conditions (Haury *et al.*, 1997). The self-renewing $CD5^+$ B1 B-cell compartment secrete large

amounts of these antibodies (Haury *et al.*, 1997; Baumgarth *et al.*, 1999). The poly-reactive specificity of natural antibodies has been attributed to conformational flexibility within the CDR3 region of the immunoglobulin heavy chain (Ditzel *et al.*, 1996; Toran *et al.*, 1999). Natural antibodies provide a link between the innate and adaptive immune systems by restricting initial viral dissemination (Ochsenbein & Zinkernagel, 2000), and substantially contribute to the recruitment of viral antigens to secondary lymphoid organs, which is a prerequisite for the priming of adaptive immune responses (Ochsenbein *et al.*, 2000). Some natural antibodies can neutralize cytopathic viruses directly (Hangartner *et al.*, 2003). *In vitro* neutralization assays or *in vivo* protection assays are carried out to assess the biological function of antibodies. In the case of acutely cytopathic and intermittently cytolytic viruses, antibody kinetics detected by ELISA usually correlate with *in vitro* neutralization titres (Charan *et al.*, 1987; Murphy *et al.*, 1981; Usonis *et al.*, 2001; Giessauf *et al.*, 2004). However, correlation does not apply in case of the persistency prone, poorly cytopathic group of viruses, which induce ELISA-detectable antibodies early, and neutralizing antibodies weeks to months later (Cafruny *et al.*, 1986; Battegay *et al.*, 1993; Wei *et al.*, 2003; Richman *et al.*, 2003; Aasa-Chapman *et al.*, 2004).

Broadly neutralizing antibodies (bNAbs) can neutralize a wide range of virus strains by binding to conserved regions of the virus surface proteins that are unable to mutate because they are functionally essential for the virus replication. Most binding sites of bNAbs against HIV are on HIV's exposed surface antigen, the Env protein (a trimer composed of gp120 and gp41 subunits). These site include the CD4 binding site or the gp41-gp120 interface (Haynes *et al.*, 2019). BNAbs have been found in other viruses including influenza (Corti et al., 2017), hepatitis C (Colbert *et al.*, 2017) dengue (Durham *et al.*, 2019) and West Nile virus (Goo *et al.*, 2019).

Neutralizing and non-neutralizing antibodies can initiate Fc dependent responses. Besides Fc gamma receptors (FcγRs), the effector system involved in Fc dependent responses is the complement system (Janda *et al.*, 2016). The complement system consists of small inactive proteins participating in a cascade of subsequent proteolytic activation leading to clearance of foreign cells (Schifferli *et al.*, 1986). The outcomes include the induction of an inflammatory response, attraction of phagocytes by chemotaxis, opsonisation (facilitated phagocytosis), immune complex formation and removal, and the lysis of pathogens or infected cells (CDC: complement dependent cytolysis) (Nesargikar *et al.*, 2012) (Figure 7 B). The classical pathway is initiated by IgM or IgG antigen/antibody complexes binding to C1q (first protein of the cascade) leading to activation of C1r, which in turn cleaves C1s. This in turn activates the serine

proteases that lead to cleaving of C4 and C2, leading to formation of C4b2a (C3 convertase), which in turn cleaves C3 into C3a and C3b (Arumugam *et al.*, 2006). While C3a acts as a recruiter of inflammatory cells (anaphylatoxin), C3b binds to the C4b2a complex to form C5 convertase (C4b2a3b). The C5 convertase initiates the formation of the membrane attack complex (MAC) that inserts into membrane creating functional pores in bacterial membranes leading to its lysis (Morgan, 1999) (Figure 7 B2). Due to its oligomeric nature, IgM is the most potent Ig class for complement activation.

Since Behring, Kitasato, and Wernicke in 1890 discovered the principle of passive immunization, the method has been obtained till today, mostly because of its ability to be used for patients even if they do not have a healthy immune system (Salazar *et al.*, 2017). Today, the serum therapy is used as the first line of defence during an outbreak as it can relatively quickly be obtained (Kreil, 2015; Schmidt *et al.*, 2018). Serum therapy was shown to reduce mortality in patients during the 2009 swine flu pandemic and the Western African Ebola virus epidemic (Hung et al., 2011). For a more specific and robust treatment, purified polyclonal or monoclonal antibodies (mAb) can be used. Polyclonal antibodies are antibodies that target the same pathogen but bind to different epitopes (Bregenholt *et al.*, 2006). Polyclonal antibodies have been used as treatment for CMV, HBV, measles virus, and RSV. Monoclonal Abs, bind the same epitope with high specificity, resulting in recognition of different antigens (Casadevall *et al.*, 2004). The most common effector functions for therapeutic antibodies are the block or activation of a signalling cascade by binding the receptor or ligand, the recruitment of immune system inducing clearance / killing of the target (by FcγR and / or complement engagement), or a combination of mechanisms (Casadevall *et al.*, 2004). Antibody therapeutics are often combined with therapies like chemotherapy for cancer. For example, Rituximab, an anti-CD20 mAbs, induces deletion of B cells by apoptosis in addition to complement and FcγRs induced cytolysis (Maloney *et al.*, 2002). Furthermore, therapeutic mAbs can have a vaccination effect inducing long-term cellular immune responses due to FcγR engagement on APCs (DiLillo & Ravetch, 2015).

1.5. Host Fcγ receptors (FcγRs)

1.5.1. FcγR biology

Cellular receptors for the different immunoglobulin isotypes (IgA, IgE, IgM, and IgG), called Fc binding receptors (FcRs), are involved in regulating and executing antibody-mediated responses (Ravetch, 2003). They link the specificity of the adaptive immune system to the effector functions triggered by innate immune cells such as mast cells, neutrophils, monocytes, and macrophages (Nimmerjahn & Ravetch, 2007). One of the few hematopoietic cell types that do not show FcR expression are T cells (Hulett & Hogarth, 1994; Daëron, 1997; Ravetch, 2003). In general, there exist five different types of FcR, each for every immunoglobulin class (Van Vugt & Van den Winkel, 2001). The FcαRI (CD89) binds IgA and sIgA (soluble IgA) (Ottem & Edmond 2004). The FcεR (CD23) has a high affinity to IgE and plays an important role in mast cells as trigger for releasing immune mediators (Ochiai *et al.*, 1994). For the FcμR and FcδR, for IgM and IgD respectively, has been described that they help in the activation of B cells and in antibody production (Kubagawa *et al.*, 2014; Liu *et al.*, 2019).

Four different classes of FcγRs have been identified in rodents, which are called FcγRI, FcγRIIB, FcγRIII, and FcγRIV (Nimmerjahn & Ravetch, 2006; Nimmerjahn, 2005) (Figure 8). FcγRs are well conserved between different mammals and the corresponding human proteins are called FcγRIA (CD64), FcγRIIB (CD32B), FcγRIIA (CD32A), FcγRIIC, FcγRIIIA (CD16), and FcγRIIIB. FcRs as well as their ligands, the family of IgG molecules consisting of four members in mice (IgG1, IgG2a, IgG2b, and IgG3) and humans (IgG1–IgG4), belong to the large immunoglobulin superfamily. The majority of FcRs have two extracellular domains, while FcγRI has an additional third domain, which has been suggested to be important for the higher affinity of this receptor for monomeric IgG (Allen and Seed, 1989) (Figure 8). Only one of the two extracellular domains makes contact with the CH2 domain of the antibody Fc portion (Radaev & Sun, 2002), suggesting a 1:1 model of antibody–FcR interaction (Kato *et al.*, 2000; Zhang *et al.*, 2000) (Figure 8). This interaction site is different from other IgG-binding proteins such as protein A/G, mannose-binding lectin (MBL), or the FcRn (Jefferis & Lund, 2002) (Figure 8). Functionally, FcRs can be divided by two different ways: based on the affinity for their ligand and, based on the type of signalling pathway that is initiated on FcR cross-linking. FcγRIIB, FcγRIII, and FcγRIV as well as their corresponding human counterparts FcγRIIA/B/C and FcγRIIIA/B have a low affinity for the IgG Fc-portion (Dijstelbloem *et al.*, 2001;

Nimmerjahn & Ravetch, 2006; Nimmerjahn, 2005) enabling interaction with antibodies in the form of immune complexes (ICs). Only FcγRI displays a higher affinity for the IgG Fc-portion enabling significant binding to monomeric antibodies. Regarding the signalling pathways they initiate, there is one inhibitory receptor, FcγRIIB. All other FcRs with the exception of human FcγRIIIB, which has no signalling function, trigger activating signalling pathways (Ravetch, 2003).

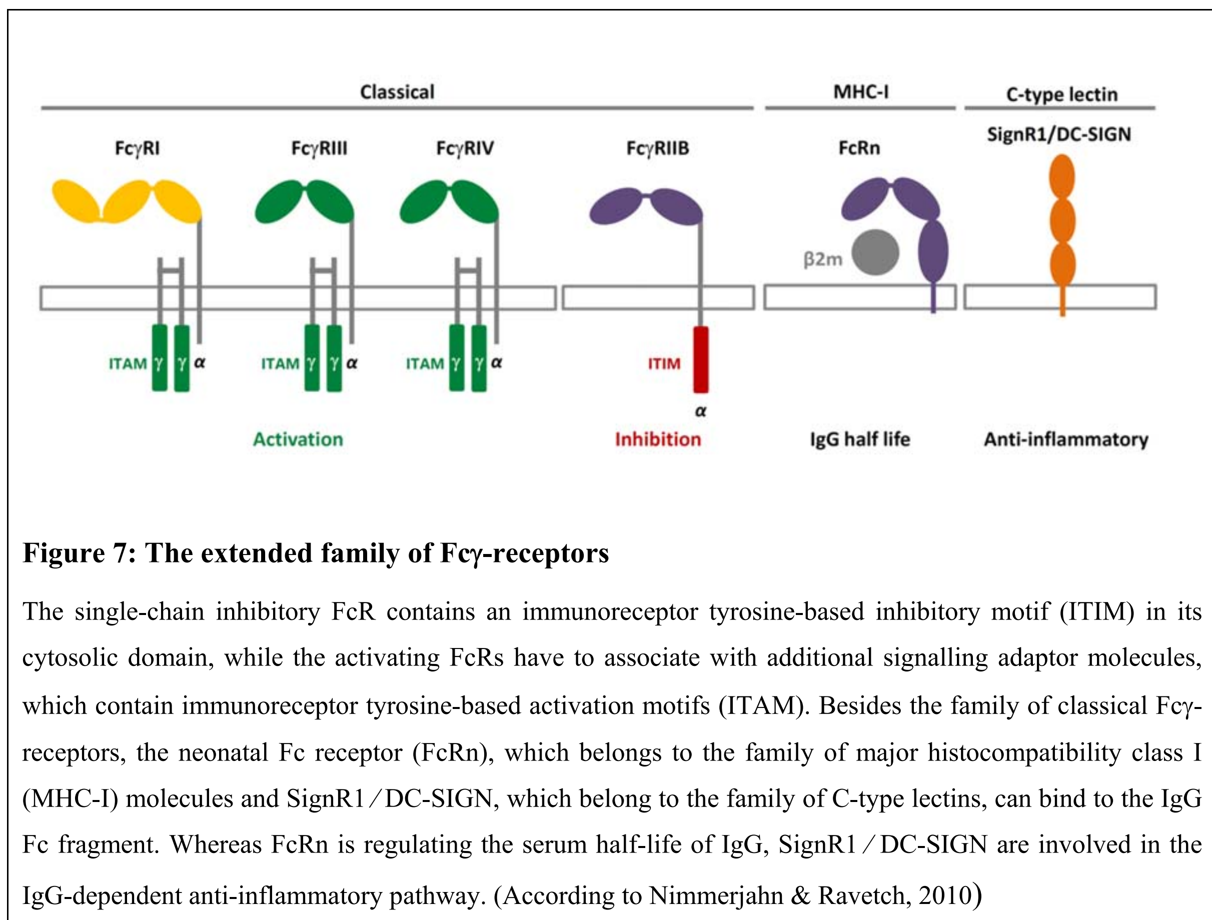


Figure 7: The extended family of Fcγ-receptors

The single-chain inhibitory FcR contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytosolic domain, while the activating FcRs have to associate with additional signalling adaptor molecules, which contain immunoreceptor tyrosine-based activation motifs (ITAM). Besides the family of classical Fcγ-receptors, the neonatal Fc receptor (FcRn), which belongs to the family of major histocompatibility class I (MHC-I) molecules and SignR1 ⁄ DC-SIGN, which belong to the family of C-type lectins, can bind to the IgG Fc fragment. Whereas FcRn is regulating the serum half-life of IgG, SignR1 ⁄ DC-SIGN are involved in the IgG-dependent anti-inflammatory pathway. (According to Nimmerjahn & Ravetch, 2010)

FcRs are co-expressed on the same cell (Ravetch, 2003). On innate immune effector cells such as mast cells, neutrophils, and macrophages, dual signals regulate a variety of downstream responses such as cell degranulation, phagocytosis, antibody-dependent cellular cytotoxicity (ADCC), and antigen presentation (Ravetch, 2003). On B cells that do not express activating FcRs, FcγRIIB regulates activating signalling pathways initiated by the B-cell receptor (BCR) (Bolland & Ravetch, 1999; Ravetch & Lanier, 2000). The single-chain inhibitory FcR contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytosolic domain (Figure 8). The activating FcRs, with the exception of human FcγRIIA/C, cannot signal autonomously

(Hulett & Hogart, 1994). They have to associate with additional signalling adaptor molecules, which contain immunoreceptor tyrosine-based activation motifs (ITAM) in their cytosolic portion (Figure 8). In NK cells, the ζ chain serves as an adaptor molecule. They become tyrosine-phosphorylated by members of the Src family of kinases, associated with the receptor in an inactive form on FcR cross-linking. On B cells, another ITIM- and SHIP-independent signalling pathway has been described that leads to apoptosis via an Abl-family kinase-dependent pathway. It is known for its selective cross-linking of FcγRIIB without concomitant triggering of the BCR (Pearse *et al.*, 1999; Tzeng *et al.*, 2005).

SignR1 represents a C-type lectin type of the Fc receptor recognizing a specific IgG glycovariant. Sialic acid-rich glycovariants have a reduced binding to the family of classical FcγRs (Kaneko *et al.*, 2006; Scallon *et al.*, 2007). The presence or absence of a single sugar residue leads to a change in receptor specificity. In contrast to the essential role of classical FcγRs for the pro-inflammatory activity, this type of receptors are essential for the anti-inflammatory activity. C-type lectins such as SignR1 can directly bind to bacterial and viral glycoproteins and additionally endogenous ligands (sialic acid-rich IgG) playing an important role during the steady state in the absence of microbial infections (Nimmerjahn & Ravetch, 2010).

The majority of innate immune effector cells express more than one activating FcR. For example, monocytes and macrophages express all activating FcγRs, followed by neutrophils that predominantly express FcγRIII and IV (Table 1). However, the individual activating FcRs have a differential affinity for different antibody isotypes (Nimmerjahn & Ravetch, 2005; Nimmerjahn *et al.*, 2005). While FcγRIII can bind to IgG1, IgG2a, and IgG2b subclasses *in vitro*, FcγRIV shows a more restricted specificity for IgG2a and IgG2b (Hirano *et al.*, 2007; Nimmerjahn *et al.*, 2005) (Table 1). Neither FcγRIV nor FcγRI binds to IgG1 antibody subclass (Nimmerjahn & Ravetch, 2006). FcγRI has high-affinity to bind to IgG2a, but the contribution of this FcR for mediating antibody activity is negligible, due to the saturation of this receptor with monomeric IgG2a serum antibodies in the steady state of peripheral tissues (Barnes *et al.*, 2002; Bevaart *et al.*, 2006; Ioan-Facsinay *et al.*, 2002). In humans, the same principles may apply for human FcγRIIIA that has a higher affinity for IgG1 compared to FcγRIIA. The level of negative regulation by the inhibitory FcR differs due to the differential affinity of various IgG subclasses (Table 1). IgG1 is strictly regulated due to the lower affinity of FcγRIII compared to FcγRIIB. In contrast, IgG2a and IgG2b are less regulated as FcγRIV has a much

higher affinity for these subclasses than FcγRIIB (Nimmerjahn and Ravetch, 2005). In FcγR activation assays, there was no correlation between FcγR-activating and neutralizing IgG responses. The pattern observed for the FcγRI-, FcγRIIA-, FcγRIIB- and FcγRIIIA-mediated responses differed substantially compared to neutralizing or ELISA IgG responses. The pattern for FcγR-activating and neutralizing IgG responses was individual (Corrales-Aquilar *et al.*, 2016).

Table 1: Mouse FcγRs

Name		FcγRI	FcγRIV	FcγRIII	FcγRII
		CD64	-	CD16	CD32
Function		activation			inhibition
Affinity to IgG		high	medium	low	
Binding of IgG subclasses				IgG1	IgG1
		IgG2a	IgG2a	IgG2a	IgG2a
			IgG2b	IgG2b	IgG2b
Expression	**Lymphoid**			NK, NKT	B cells
	Myeloid	Monocytes, Macrophages*, DC*	Monocytes, Macrophages*	Monocytes, Macrophages, DC	Monocytes, Macrophages, DC
	Granulocyte	Neutrophils#, Eosinophils#, Basophils#	Neutrophils	Neutrophils, Eosinophils#, Basophils, Mast cells	Neutrophils, Eosinophils, Basophils, Mast cells

*in subsets; # inducible expression (according to Kim *et al.*, 2006; Guillams *et al.*, 2014; Bruhn *et al.*, 2015; Ehrhardt, 2016; Bournazos & Ravetch, 2017; Bournazos *et al.*, 2020)

Activating FcR expression on innate immune cells can be increased by pro-inflammatory stimuli (such as LPS), T_H1 cytokines (such as IFN-γ), and the complement component C5a (Guyre *et al.*, 1983; Shushakova *et al.*, 2002; Nimmerjahn & Ravetch, 2006). In contrast, T_H2 cytokines such as IL-4, IL-10, or TGF-β down-regulate activating FcR expression and increase the level of FcγRIIB (Okayama *et al.*, 2000; Pricop *et al.*, 2001; Radeke *et al.*, 2002; Tridandapani *et al.*, 2003; Nimmerjahn *et al.*, 2005). Another factor that can have influence on antibody binding to FcRs is the heptameric core sugar structure consisting of N-acetylglucosamine (GlcNac) and mannose attached to all IgG subclasses at the asparagine residue 297 (N297) in the C_H2 region of the antibody constant region (Arnold *et al.*, 2007).

Genetic or biochemical deletion of this sugar side chain abrogates FcR binding but does not affect the interaction with other proteins such as the FcRn (Arnold *et al.*, 2007; Shields *et al.*, 2001). Core sugar structure contains variable amounts of branching and terminal sugar residues such as sialic acid, galactose, fucose, and GlcNac. Antibodies without fucose bind with up to 50-fold higher affinity to mouse activating FcγRIV and human FcγRIIIA (Nimmerjahn & Ravetch, 2005; Shields *et al.*, 2002; Shinkawa *et al.*, 2003). Dengue virus (DENV) infection induced a specific increase in IgG1 afucosylation, and the levels of afucosylated IgG1 were predictive of dengue disease severity (Bournazos *et al.*, 2021). It was shown that the anti-inflammatory activity of high doses of IVIG therapy can be potentiated by enriching IgG preparation for the sialic acid rich fraction (Kaneko *et al.*, 2006a; Nimmerjahn & Ravetch, 2007b). IVIG therapy is critically dependent on the presence of FcγRIIB as a negative regulator because sialylated IgG upregulates FcγRIIB expression on effector macrophages.

1.5.2. FcγR effector mechanisms

FcγRs are found on a number of cells in the immune system. They allow immune cells to bind Abs attached to the antigens expressed on the cell surface after infection with intracellular pathogens like viruses, especially during reinfection (Sulica *et al.*, 1995; Raghavan *et al.*, 1998). Typical effector functions are:

d) antibody dependent cytotoxicity (ADCC)

e) antibody dependent phagocytosis (ADCP)

f) secretion of cytokines, proinflammatory mediators, and reactive oxygen species (ROS)

ADCC is an immune mechanism that bridges the adaptive humoral and innate immune responses. ADCC has important applications in cancer treatment, via monoclonal antibody (mAb)-mediated ADCC killing of tumour cells (Scott *et al.*, 2012), and is also involved in host defence from viral infection and control of viremia, *via* ADCC-mediated killing of virus-infected cells (Schmaljohn, 2013). Abs bind pathogen antigens expressed on the membrane of infected cells, which can then recruit and activate FcγR-expressing cytotoxic effector cells to kill the infected cells (Roman *et al.*, 2013) (Figure 7 C). Classic ADCC is driven by natural killer (NK) cells expressing FcγRIIIa (CD16A), which binds to the Fc region of IgG Abs recognizing virus-infected target cells (Lu *et al.*, 2018) (Figure 7 C). This leads to the

polarization of the cell mediating extensive cell-to-cell contact and release of the preformed granules containing cytolytic substances in the direction of the target cell. Pores are formed in the target cell membrane by perforin and apoptosis is induced by proteolytic enzymes called granzymes (Kenneth, 2012). Removal of fucose from the IgG glycan can increase ADCC by enhancing antibody affinity for FcγRIIIa (Shields *et al.*, 2002; Zeitlin *et al.*, 2011). The addition of a GlcNAc prevents the addition of fucose and similarly enhances ADCC (Umana *et al.*, 1999). Other innate immune cells involved in ADCC that express FcγRIIIa, are macrophages and DCs (Biburger *et al.*, 2014; Weiskopf *et al.*, 2015; Bournazos *et al.*, 2016), and FcγRIIIb (also known as CD16b) on neutrophils. An increasing body of evidence suggests that ADCC contributes to protection against HIV-1 acquisition in pre-clinical studies (Gomez-Romanet *et al.*, 2005; Mabuka *et al.*, 2012; Fouts *et al.*, 2015; Bradley *et al.*, 2017). Assessing FcγRs activation upon ADCC lead to establishing a novel assays. Triggering of the chimeric FcγR–CD3ζ chain molecules by immune complexes formed on the surface of IgG-opsonized virus-infected target cells resulted in FcγR activation leading to IL-2 secretion by BW5147 hybridoma cells, easily measured by ELISA (Corrales-Aquilar *et al.*, 2013).

Phagocytosis of antibody-coated pathogens or ADCP involves the formation of endocytic vesicles that mature through fusion with different endosomal compartments. The crosslinking of FcRs results in signalling *via* ITAMs or ITIMs, which both impact the re-organization of microtubules to enable phagosome formation (Weber & Oxenius, 2013). ITAM recruitment leads to the rapid trafficking of pathogens to lysosomes for their degradation and directed antigen processing for presentation to T-cells (Boros *et al.*, 2014). ITIM signalling results in the retention of whole pathogen antigens for subsequent transfer to B-cells and the induction of humoral immunity (Bergtold *et al.*, 2005). ADCP activate distinct innate immune effector functions, depended on the innate cell type that has been recruited to clear the pathogen. This is mediated by mononuclear phagocytes (monocytes, macrophages and DCs), and granulocytes (neutrophils, eosinophils, basophils and mast cells) (Weber & Oxenius, 2013).

Phagocytic cells may integrate additional information *via* cooperative signals between FcRs and other pattern recognition receptors such as c-type lectin receptors (CLRs) and Toll-like receptors, which are found on the surface of the effector cell or within endocytic compartments (Rittirsch *et al.*, 2009; Anthony *et al.*, 2008; van Egmond *et al.*, 2015). The collaborative signalling lead to additional effector functions, which includes the release of proteases, defensins, cytokines, ROS and reactive nitrogen species that together recruit and additional arm of innate effector cells (Hoving *et al.*, 2014; van Egmond *et al.*, 2015).

1.6. Viral Fcγ receptors (vFcγRs)

Members of the α- and β-subfamily of the *Herpesviridae* encode transmembrane glycoproteins, which selectively bind IgG *via* its Fc domain. The Fc-binding proteins constitute viral Fcγ receptors (vFcγRs) expressed on the cell surface of infected cells. Moreover, vFcγRs are incorporated into the envelope of virions. Despite their molecular and structural heterogeneity, the vFcγRs interfere with IgG-mediated effector functions like ADCC, complement activation and neutralization (Budt *et al.*, 2004). The first vFcγR characterized was the HSV-1 encoded gE/gI. It inhibits neutralization, complement mediated virolysis and cytolysis, and host FcγR activation including ADCC (Frank *et al.*, 1989; Dubin *et al.*, 1991; Corrales-Aguilar *et al.*, 2014). For HCMV, four vFcγR have been identified: glycoprotein (gp) 34, gp68, gp95 (RL12), and RL13, with fast mutational rate by propagating the virus *in vitro* (Atalay *et al.*, 2002; Cortese *et al.*, 2012). For MCMV, *m138* was recognized so far (Thäle *et al.*, 1994). It codes for a highly glycosylated type I transmembrane protein, expressed in the early and late phases of MCMV replication. Comparison of the *m138* sequence with mouse host FcγR reveals a significant homology and predicts a composition of 3 IgSF-like domains (Budt *et al.*, 2004). The m138 protein is specific for IgG2a and IgG2b subclasses. In contrast to HSV-1, HCMV vFcγRs do not interfere with neutralization and complement mediated virolysis (Corrales-Aguilar *et al.*, 2014). Most of vFcγRs use a mechanism of antibody bipolar bridging, whereby the antibody binds the antigen with its Fab and simultaneously the Fc part is binds the vFcγR present on the same cells (Sprague *et al.*, 2006; Corrales-Aguilar *et al.*, 2014). HCMV gp34 and gp68 bind IgG simultaneously at topologically different Fcγ sites. While gp34 enhances immune complex internalization, gp68 acts as inhibitor of host FcγR binding to immune complexes. In doing so, gp68 induces Fcγ accessibility to gp34 and simultaneously limits host FcγR recognition (Kolb *et al.*, 2021).

1.7. Aim of the thesis

Since immunity afforded by natural primary HCMV infection may be inefficient at preventing reinfection, development of a vaccine require maximal induction of host immunity against multiple CMV antigens. While subunit, peptide, or DNA vaccines meet these criteria (Suhrbier *et al.*, 1995; Gonzales Armas *et al.*, 1996), we have begun to analyse vaccination against CMV by using a stably attenuated live vaccine lacking STAT2 antagonist, on the theory that effective

immunity may be easily and effectively induced by live attenuated vaccination (MacDonald *et al.*, 1998). We have evaluated the ability of the live-attenuated vaccine candidate to elicit humoral immune responses using ELISA on infected cell proteins and on virions. Specific IgG immune responses elicited by live attenuated vaccine were tested on their ability to generate neutralizing antibodies and to activate FcγRs (FcγRI [CD64], FcγRII [CD32], FcγIII [CD16] and FcγRIV), therefore connecting the innate and adaptive arm of the immune response. Live attenuated vaccine lacking STAT2 antagonist was examined in order to determine its capability to produce sufficient antigens and immune stimulation to mount protective immune responses. At the end, protection of neonatal mice from MCMV infection *via* maternal IgG was evaluated.

2 Materials and methods

2.1. Materials

2.1.1. Devices

-20 °C Refrigerator	Confort UG1211, Liebherr
	Medline, Liebherr
	KBS Kältetechnik
-80 °C Refrigerator	Ultra low temperature freezer, Sanyo
	Forma -86°C ULT Freezer, Thermo Scientific
	HeraFreeze, Heraeus
Vacuum aspiration system	Vacusafe, Integra
	2-9336, Neolab
	KNF, Neuberger
Sonicator	Branson Sonifier 450
Freezer boxes	CoolCell® FTS 30, biocision
Ice machine	AF 80, Scotsman
Precision balance	R 160 P, Sartorius Research
Homogenisator	Dounce Tissue Grinders, Wheaton
Incubators	CB150 E3, Binder
	Incubat, Melag
	Max Q6000, Thermo Scientific
Refridgerator	UK 1720, Liebherr
Magnetic stirrer	MR Hei-Standard, Heidolph
Microscope	CKX41, Olympus
	Primo Vert, Zeis
Microwave	HF12M240, Simens
Multimode Reader	Mithras2 LB 943, Berthold Technologies
pH meter	Lab 850, Schott Instruments
Pipette	8-5010, Neolab
	ErgoOne, Starlab
	Research, Eppendorf

Pipette controller	Pipetboy 2, Integra Biosciences
	Pipetman, Gilson
	Pipetus®, Hirschmann Laborgeräte
Ultrapure water system	Milli Q, Millipore
Nitrogen tank	1500 Series-190, MVE; MVE TEC 3000
Sterile bench	HERAsafe, Thermo Electron Corporation
Thermoblocks	Thermomixer Comfort, Eppendorf
	MBT 250, Kleinfeld Labortechnik
	ThermoStat plus, Eppendorf
Ultracentrifuge	Optima™ L-80 XP Ultracentrifuge, Beckman Coulter
Overhead shaker	Heidolph Reax 2
Vortexer	Vortexer™, Heathrow Scientific
	Vortex Genius 3, IKA Werke
Balance	EMB 1000-2, Kern
	572, Kern
UV-cross-linker	Stratagene
Water bath	GFL
Heavy duty shaker	3013, GFL
	Duomax 1030, Heidolph
Centrifuges	Centrifuge 5415D, Eppendorf
	Centrifuge 5417R, Eppendorf
	Centrifuge 5427R, Eppendorf
	Centrifuge 5424, Eppendorf
	Allegra® X-15R Centrifuge, Beckman Coulter
	Avanti® J-26 XP, Beckman Coulter
	Sigma 4-16K
Small Animal Anaesthesia Machine	UNO, Zevenaar

2.1.2. Consumables

Complete Protease Inhibitor Cocktail Tablets	Roche
Cryo.s™	Greiner Bio-One

LumiNunc F96 MicroWell Plates	Nunc
Multiwell Plates Cellstar (6-wells #657160, 12-wells #665180)	Greiner Bio-One
Multiwell Plates Cellstar (24-wells #662160, 48-wells #677180)	Greiner Bio-One
Multiwell Plates Cellstar (96-wells #353072)	Greiner Bio-One
Pipette tips TipOne	Starlab
Reaction vessels, 1,5 ml and 2 ml Safe Lock	Eppendorf
Saran wrap foil	Sarogold
Disposable injection needles	BD MicrolanceTM 3, BD biosciences
Disposable syringe 0,3 ml and 0,5 ml	BD biosciences
Capillary minicaps NA-HEP	Hirschmann® Laborgeräte
Serological pipettes 5 ml, 10 ml, 25 ml	Greiner Bio-One
Cell culture flasks Cellstar #690175, #658175, #660175	Greiner Bio-One
Cell scrapers M #99003	TTP
Centrifuge tubes (Falcontubes) 15 ml and 50 ml	Greiner Bio-One
96-well MaxiSorp Plates	Nunc
Cell strainers, 75 µm	Miltenyi
Ultracentrifuge Tubes	Beckman Coulter
Neubauer chamber	Merck

2.1.3. Kits

All kits were used according to the manufacturer's instructions.

1-Step™ Ultra TMB-ELISA Substrate Solution	Thermo Scientific
1 X GloLyse Buffer	Promega

2.1.4. Chemicals and antibiotics

96% Acetic acid	Roth

Bovines serum albumin (BSA) 30%	PAA Laboratories
Boric acid	Roth
Calcium chloride	Roth
Chloridic acid	Roth
Citric acid	Roth
Dinatriumhydrogenphosphate	Roth
Dimethylsulfoxide (DMSO)	Roth
Ethylenediaminetetraacetic acid (EDTA)	Roth
Ethanol p.a.	Roth
Ethanol, denatured 96%	Roth
Geneticin (G418)	Sigma-Aldrich
Glucose	Roth
Glycerol	Roth
Glycine	Roth
Hydrochloric acid	Roth
Hydrogen Peroxide 30%	Roth
Isopropanol	Roth
Isofluran	Abbott
Kaliumacetate	Roth
Kaliumchloride	Roth
Kaliumdihydrogenphosphate	Roth
Kaliumhydrogencarbonate	Roth
Kaliumhydroxide	Merck
L-Glutamin (200mM; 100x)	Invitrogen
Methanol	JT Baker
Magnesiumchloride	Roth
Maleic acid	Roth
Methylcellulose	Sigma-Aldrich
Phenol/Chloroform/Isoamyl alcohol	Roth
Saccharose	Roth
Skimmed milk powder	Sucofin
Sodium acetate	Roth
Sodium carbonate	Roth
Sodium chloride	Roth

Sodium hydroxide	Roth
Sodium citrate	Roth
Sodium phosphate	Roth
Sodium Pyruvate (100 mM)	Invitrogen
Sorbitol	Roth
Sulfuric acid	Roth
Tris-HCl	Roth
Trypan blue stain (0.4%)	Invitrogen
Trypsin 2.5%	Invitrogen
Tween-20	Sigma
Zeocin	Invitrogen

2.1.5. Cell culture media and additives

Penicillin (10.000 U/ml)/Streptomycin (10.000 µg/ml)	Gibco
Phosphate buffered saline (PBS)	Gibco
Trypsin	Gibco
Dulbecco's Modified Eagle Medium (DMEM)	Gibco
RPMI-1640 Medium	Gibco
10 X MEM	Thermo Fisher
FBS Superior (Fetal bovine serum)	Biochrom AG
FCS Superior (Fetal calb serum)	Biochrom AG

2.1.6. Solutions and buffers

10x PBS; pH 7,4	136 mM NaCl 2.6 mM KCl 1.8 mM Na_2HPO_4 x $2H_2O$ 1.5 mM KH_2PO_4
	15% Saccharose

MCMV-Saccharose-VSB (Virus Standard Buffer); pH 7.8	50 mM Tris /HCl 12 mM KCl 5 mM EDTA
Freezing medium	50% (v/v) DMEM or RPMI-1640 40% (v/v) FCS 10% (v/v) DMSO
10x TBST	0.1 M Tris/HCl; pH 8.0 1.5 M NaCl 5% (v/v) Tween-20
Methylcellulose	8,8 g Methylcellulose 360 ml H_2O
Methylcellulose medium	360 ml Methylcellulose Solution 40 ml 10 x MEM 20 ml FCS 5 ml Penicillin (10.000 U/ml)/Streptomycin (10.000 µg/ml) 5 ml 200 mM L-Glutamine 20 ml $NaHCO_3$ (55 g/l)
ELISA binding buffer	0.1 M Na_2HPO_4, adjust pH to 9.0
ELISA blocking buffer	PBS 10% (v/v) FCS
ELISA sample buffer	PBS 10% (v/v) FCS 0.1% (v/v) Tween-20
ELISA washing buffer	PBS 0.1% (v/v) Tween-20

ELISA Stop Solution	1M H_2SO_4

2.1.7. Cell lines

CIM	Crisis-immortalized MEF cells (Rattay *et al.*, 2015)
MEF	Primary murine fibroblasts (mouse embryonic fibroblasts) derived from C57BL/6 and BALB/c embryos (Le-Trilling & Trilling, 2017)
MNC	Primary murine fibroblasts (mouse embryonic fibroblasts) derived from C57BL/6 and BALB/c newborn mice (Le-Trilling & Trilling, 2017)
BW5147	mouse thymoma cells; ATCC TIB-47™
BW5147 m64	mouse BW5147 hybridoma cells stably expressing chimeric FcγR-CD3ζ chain molecules consisting of the extracellular domain of mouse FcγRI, fused to the transmembrane and intracellular domains of the mouse CD3ζ chain (Corrales-Aquilar *et al.*, 2013)
BW5147 m32	mouse BW5147 hybridoma cells stably expressing chimeric FcγR-CD3ζ chain molecules consisting of the extracellular domain of mouse FcγRII, fused to the transmembrane and intracellular domains of

	the mouse CD3ζ chain (Corrales-Aquilar *et al.*, 2013)
BW5147 m16	mouse BW5147 hybridoma cells stably expressing chimeric FcγR-CD3ζ chain molecules consisting of the extracellular domain of mouse FcγRIII, fused to the transmembrane and intracellular domains of the mouse CD3ζ chain (Corrales-Aquilar *et al.*, 2013)
BW5147 mIV	mouse BW5147 hybridoma cells stably expressing chimeric FcγR-CD3ζ chain molecules consisting of the extracellular domain of mouse FcγRIV, fused to the transmembrane and intracellular domains of the mouse CD3ζ chain (Corrales-Aquilar *et al.*, 2013)

2.1.8. Viruses

wt-MCMV (Mouse Cytomegalovirus MCMV-BAC MW97.01)	Wagner *et al.*, 1999; Jordan *et al.*, 2011
ΔM27-MCMV (Mouse Cytomegalovirus MCMV- pSM3fr-MCK-2fl derived)	recombinant MCMV with deletion of M27; derived from the wt Smith strain with repaired MCK-2 background (Jordan *et al.*, 2011: Le-Trilling *et al.*, 2018)

Δm157-MCMV:Luc	recombinant MCMV; derived from the wt Smith strain; expresses the firefly luciferase gene under the human major immediate early (MIEP) instead of m157 promoter (Trilling *et al.*, 2011)
Δm138-MCMV-FSH	recombinant MCMV with deletion of m138/fcr-1 ((ΔMC95.15; Crnković-Mertens *et al.*, 1998); available by Prof. Hartmut Hengel, Institute for Virology, Universitätsklinikum Freiburg
MCMV:eGFP	recombinant Mouse Cytomegalovirus expressing enhanced green fluorescent protein (eGFP); *eGFP* gene is placed under control of the native MCMV ie1/ie3 promoter (Henry *et al.*, 2000)

2.1.9. Mouse strains

Balb/c	Charles River Laboratories, Harlan Laboratories
C57BL/6	Charles River Laboratories, Harlan Laboratories

2.1.10. Antibodies

Table 2: Primary antibodies

Name (Species)	Firma/Producer	Catalogue number	Application	Dilution
Anti-mouse IL-2 JES6-5H4 (Rat)	BD Pharmingen	554426	ELISA Capture Ab	1:500

Anti-mouse IL-2 Biotinylated JES6-1A12 (Rat)	BD Pharmingen	554424	ELISA Detection Ab	1:500

Table 3: Secondary antibodies

Name (Species)	**Firma/Producer**	**Catalogue number**	**Application**	**Dilution**
Goat anti-mouse IgG, POD, Peroxidase Conjugated	Jackson ImmunoResearch	115-035-003	ELISA	1:5000
POD, Streptavidin Peroxidase Conjugated	Jackson ImmunoResearch	016-030-084	ELISA	1:1000

2.1.11. Database

National Center for Biotechnology Information	http://www.ncbi.nlm.nih.gov/

2.1.12. Software

End Note X8.2	Clarivate Analytics
Graph Pad Prism V6	Graph Pad Software, Inc.
MS Office (MS Word, MS Excel, MS PowerPoint)	Microsoft

2.2. Methods

2.2.1. Cell biological methods

2.2.1.1. Cultivation of eukaryotic cells

In order to maintain sterility, all cell biological work was carried out under a sterile bench. The cells were incubated at 37°C and 5% CO_2. Depending on their cell type, the cultivation of the cells was carried out in different nutrient media. Media were heat-inactivated (for 30 min at 56°C) and supplemented with 10% (v/v) FBS (foetal bovine serum), 100 U/ml penicillin, 100 µg/ml streptomycin and 2mM glutamine. When 90 - 100% confluence of adherent cells was reached, the medium was first discarded, cells were washed with warm (37°C) PBS, incubated with a trypsin solution (0.625% [w/v] in PBS) at 37°C for several minutes until all cells were detached. Thereafter, the cells were resuspended in fresh, warm (37°C) medium and in appropriate dilution transferred to a new cell culture flask.

Suspension cell lines were grown in RPMI 1640 medium containing 10% (v/v) FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM glutamine, and 1 mM of sodium pyruvate. These were centrifuged at 149 x g, washed with PBS and then taken up in fresh medium. The number of cells was counted using a Neubauer chamber and afterwards transferred to a new cell culture flask with fresh medium. Suspension transfectants were selected in RPMI medium containing 3 mg/ml G418 (Sigma-Aldrich) or 50 µg/ml Zeocin (Invitrogen).

2.2.1.2. Cryopreservation of eukaryotic cells

In order to frozen cells, a confluency should be reached of about 70-80%. The cells were maintained as described above (see 2.2.1.1. Cultivation of eukaryotic cells), first washed with PBS and detached with trypsin, and then the medium was added. After the centrifugation of the cells (335 g for 3 min), they were resusupended in a freezing medium and aliquoted into cryo tubes. Adherent cells were resuspended in 50% [v/v] FBS, 40% [v/v] medium and 10% [v/v] DMSO, whereas the suspension cells in 90% [v/v] FCS and 10% [v/v] DMSO. Freezing the cells was performed overnight at -80°C in Cryobox slowly (-1°C/min) before the cells were transplanted in liquid nitrogen tank (-196°C) were they were stored.

2.2.1.3. Cell counting

Adherent cells were collected as described in 2.2.1.1 and resuspended in 10 ml of medium. Suspension cells were centrifuged at 1000 x g for 5 min at RT and resuspended in 10 ml of medium. From each cell suspension a 5 µL was taken, mixed with 45 µL of Trypan Blue and put to a Neubauer chamber. To calculate the number of viable cells/mL the average cell count from each of the sets of 16 corner squares was taken multiplied by 10000 (chamber factor) and multiplied by a dilution factor.

2.2.1.4. Preparation of primary Mouse Embryonal Fibroblasts (MEF) and Mouse Newborn cells (MNC)

MEF and MNC were generated as described in Le-Trilling & Trilling, 2017. For MEF preparation, mouse embryos (day 16 to 17 post coitum) were dissected into 10 ml sterile PBS in a 100 mm tissue culture dish. Embryonic internal organs were removed from the abdominal cavity. For MNC preparation, 1 to 2 days old newborn mice were sterilized in 70% (v/v) ethanol and thoroughly washed in PBS before the head, limbs and visceral organs were removed. After the dissection, the remaining steps of both MEF and MNC preparation were the same. Liver and gut were removed, the remaining tissue was washed in PBS, and minced into small pieces. The tissue parts are resuspended by PBS ad 50 ml in a (or several) 50 ml tube(s), then sedimented by allowing the tube(s) to stand for a while and the PBS was removed. The washing was repeated until the supernatant was clear. To obtain a single cell suspension by enzymatic digestion of a connecting tissue, a cell culture grade trypsin solution (2.5% trypsin diluted 1:3 in sterile PBS; Gibco) was applied on the samples which were afterwards incubated at 37°C for 1-2 hours (in an incubator). A 5 ml of 2.5% trypsin solution and 100 µl of DNaseI solution (10 mg/ml; Roche) was added on samples and incubated 30 min at 37°C. 5 ml FCS was added to stop the trypsin reaction. In addition, the trypsin was removed by centrifugation for 5 min at 350 x g and subsequent removal of the supernatant trypsin solution. The pellet was resuspended in 25 ml DMEM supplemented with 10% (v/v) FCS. The pellet was finally intensely resuspended in 50 ml growth medium. The cell suspension was sedimented and the supernatant was transferred into tissue culture flask (175 cm^2) with a final volume of 60 - 90 ml medium per flask. Cells were grown in DMEM supplemented with 10% (v/v) FCS, 100 µg/ml streptomycin, 100 U/ml penicillin and 2 mM glutamine until confluency (2 to 4 days). The

medium was changed after the first day (passage 0), and cell density was monitored using an inverted microscope. Passage 0 cells were expanded 1:5 before passage 1 cells were frozen as described in 2.2.1.2. For all experiments, MEF and MNC were used in passage 3.

2.2.2. Virological methods

2.2.2.1. Infection of cells

A technique of centrifugation of inoculum was used to approach cells and the virus to each other (Osborn & Walker, 1968). For this purpose, the plates were centrifuged for 15 min at 900g and RT, then rotated by 180° and again centrifuged for 15 min at 900g and RT.

2.2.2.2. MCMV virus stock

MCMV-permissive cells were seeded in three 6-well plates and simultaneously in 20 175 cm^2 cell culture flasks. The next day, 70-80% confluent cells in the 6-well plates were infected with MCMV with a multiplicity of infection (MOI) between 0.1 and 0.3. For this purpose, the medium was replaced with 3 ml of virus dilution (DMEM with MCMV) and the plates were centrifuged as described in section 2.2.2.1. After CPE was observed (2-3 days), the cells and the medium from the 6-well plates were collected and transferred to one of the previously created 175cm^2 flasks. 6-9 days after infection (when CPE was pronounced), the cells were scraped and transferred to sterile centrifuge beakers with the medium. The cell suspension was centrifuged at 5000 x g and 10° C for 5 min (Beckman Coulter J2-21, Rotor JLA 10500). The supernatant was collected, the pellet resuspended in 5 ml and disrupted by a douncer. The disrupted cells and the supernatant were again transferred to beakers and centrifuged at 5,000 x g (Beckman J2-21, Rotor JLA 10500) and 10° C for 15 min. The supernatant was then transferred to a sterile centrifuge beaker and centrifuged at 20000 x g and 10° C for 4 h (Beckman Coulter J2-21, Rotor JLA 10500). Afterwards, the supernatant was discarded, 5 ml DMEM was added to the precipitate and incubated overnight on ice at 4° C, while the remaining supernatant loosened up the pellet. The next day, the precipitate was resuspended in the existing medium and homogenized in a douncer. The suspension was slowly placed on a VSB sucrose cushion (15% Saccharose/VSB) in an ultracentrifuge tube and ultracentrifuged for 70 min and 10°C at 100,000 x g (Beckman Coulter L-70K ZU, Rotor SW 32 Ti). The supernatant was then

removed and 1 ml of VSB-sucrose solution was added to each precipitate and dissolved on ice at 4° C overnight. The next day, the isolated virus was homogenized with a douncer, aliquoted and stored at -80°C.

2.2.2.3. MCMV virion stock

In order to obtain MCMV virion stock, MCMV permissive cells were seeded, propagated, and infected as in section 2.2.2.3. After CPE was observed (2-3 days), the cells and the medium from the 6-well plates were collected and transferred to one of the previously created 175 cm^2 flasks. At 6-9 days after infection, the media containing secreted MCMV virions was collected and spun at 5000 x g and 10° C for 5 min (Beckman Coulter J2-21, Rotor JLA 10500). The supernatants were transferred to beakers and again centrifuged at 5,000 x g (Beckman J2-21, Rotor JLA 10500) and 10° C for 15 min. This step was repeated but with centrifugation at 20000 x g and 10° C for 4 h (Beckman Coulter J2-21, Rotor JLA 10500). The pellet was resuspended in 5 ml of cold DMEM and incubated overnight at 4° C. A combined pellet suspension from all ultracentrifuge tubes were loaded on VSB sucrose cushion (15% Saccharose/VSB) made in PBS and spun at 100000 × g for 1 h at 4 °C (Beckman Coulter L-70K ZU, Rotor SW 32 Ti). The visible virion-containing band was collected into a new tube, mixed with 1 ml of 15% Saccharose/VSB made in PBS, and incubated on ice at 4° C overnight. The virions were resuspended in existing volume, aliquoted and stored at -80°C.

2.2.2.4. Plaque titration of a virus stocks

To determine the virus titer of a purified MCMV stock, MEFs were seeded up in 48-well plates one day before titration. The starting dilutions are prepared in a complete DMEM. A 1/10 dilution series (55 µl + 500 µl) was performed directly on the plate with eight dilution steps and each dilution was determined in triplicates. Virus suspension is infected by centrifugation (see 2.2.2.1.) and incubated for 2 h at 37 °C and 5% CO_2. The cells were then covered with medium containing methyl cellulose (approximately 500 µl/ well), and the plaques formed by cell-to-cell infection were counted 3 days after infection. The plaque forming units (PFU) per ml were calculated.

2.2.2.5. MCMV UV inactivation

For *in vivo* application, a fraction of the wt-MCMV virus used for immunization was inactivated by exposure to 1 kJ/cm^2 UV light with wavelength of 254 nm at a distance of 5 cm in a UV-cross-linker (Stratagene) at 4°C.

2.2.3. Immunological methods

2.2.3.1. MCMV vaccination

For vaccination with MCMV, C57Bl/6 and BALB/c mice were injected intraperitoneally with 2*10^5 PFU. The mouse was restrained in one hand fixing the neck with the angle of index finger and thumb, the tail with the little finger, and one leg with the ring finger. The needle of the syringe was inserted into the lower part of the abdomen in cranial direction. The volume was released and the syringe was pulled out after a short pause (Hedrich & Bullock, 2004). The immunization was carried out once or in a homologous prime-boost scheme at intervals of three weeks and twenty weeks.

2.2.3.2. Retroorbital blood sampling

Retroorbital blood sampling was performed according to Hedrich & Bullock, 2004. Under inhalation isoflurane anaesthesia, 6-8 drops of blood (about 200 µl) were taken retroorbitally from the mice. The skin above and below the eye was pulled outwards with the index finder and thump. The orbital sinus was incised with a thin glass capillary (Capillary minicaps NA-HEP, Hirschmann Laborgeräte) in the left or right corner of the eye and applying a short-time gentle pressure plus rotation. Releasing the pressure, the capillary was filled with blood by the capillary forces. The blood was collected, and the eyeball was pressed gently to stop the bleeding. A serum was prepared from collected blood as described in the section 2.2.3.4.

2.2.3.3. Total blood collection from the heart

Total blood collection from the heart was performed according to Hedrich & Bullock, 2004. The mouse was euthanized by cervical dislocation or by CO_2 inhalation. After washing with 70% EtOH, the mouse was fixed on its back, the skin was opened and pulled to the sites

exposing the thorax. The thorax was opened by cutting the sternum and extending the cut on both sites allowing the cut thoracic wall to be pulled upwards and revealing the beating heart while leaving the diaphragm intact. The venous blood was slowly collected from the right ventricle with a 26G needle and transferred into a tube. A serum was prepared from collected blood as described in the section 2.2.3.4.

2.2.3.4. Serum preparation

Collected blood was incubated for 30 min on RT. Serum was separated by centrifugation on 1000 x g for 10 min at 4°C. Supernatant was stored at -20°C.

2.2.3.5. Harvesting the mouse organs

Harvesting the mouse organs was performed according to Hedrich & Bullock, 2004. After washing with 70% (v/v) EtOH, the mouse was fixed on its back, the skin was opened and pulled to the sites exposing the thorax. First, the SG (sublingual gland and submandibular gland) were perpetrated. The thorax was opened to obtain the lung. The abdomen was opened and the spleen and the liver (a piece of the middle of the biggest lobe) were perpetrated. Every the organ was transferred into cryotubes and snap frozen in liquid nitrogen. The organs were stored at -80°C until the titration (2.2.2.5.).

2.2.3.6. Generation of organ homogenates

The organs were thawed on ice and homogenized by rinsing it through a 75 µm cell strainer. The cell strainer with organ is rinsed in a total volume of 2 ml cold 5% (v/v) FCS in PBS. The suspension was centrifuged at 100 x g for 10 min at 4°C, and the supernatant was transferred in a 15 ml tube, and centrifuged again at 1610 x g for 2 min at 4°C. The supernatant from this centrifugation (homogenate) was used for the titration.

2.2.3.7. Enzyme-linked Immunosorbent Assay (ELISA) Lysates

At the day before infection, permissive cells were seeded. The cells were virus infected with multiplicity of infection (MOI) = 3 - 5. Infected cells were collected with the cell scraper, centrifuged at 200 x g for 3 min at RT, and resuspended in 5 ml of PBS. This step was repeated. The pellets were resuspended in 200µl of mix of PBS-Protease Inhibitors (cOmplete protease inhibitors, Roche). The cells were lysed by sonication 3 x 10 sec (Branson Sonifier II 450). The cell debris was pellet at 26000 x g for 5 min at 4°C, and the supernatant was stored at -20°C. The volume used for coating of one ELISA plate was adjusted to number of seeded cells.

2.2.3.8. Enzyme-linked Immunosorbent Assay (ELISA)

For the quantification of MCMV-specific IgG present in mouse serum samples collected at different times after MCMV infection, an indirect ELISA was performed. The ELISA plate (96 well, Maxisorp, Nunc) was coated overnight at 4°C with MCMV-infected cell lysates (2.2.3.6.) or MCMV virions (2.2.2.4), diluted in PBS with 10% (v/v) FCS. Non-specific binding sites were blocked with 200 µl of 10% (v/v) FCS in PBS per well at RT for 1 hour. The wells were washed with 200 µl of PBS containing 0.1% (v/v) Tween 20 three times. Serum samples were serially diluted in a separate 96-well plate. In the first well, 220 µl of PBS with 10% (v/v) FCS were introduced, in each additional 100 µl. 2.2 µl of sera were added to the first well and the serial dilution continued stepwise with 50 µl from the previous well, resulting in six dilution steps. 100 µl of these dilutions were then transferred to the washed MaxiSorp plate and incubated at RT for 1.5 h. The ELISA sample buffer has been removed and the plate washed three times with 200 µl of PBS containing 0.1% (v/v) Tween 20. The second antibody was an HRP-coupled antibody Goat Anti-Mouse (GAM-POD, Jackson ImmunoResearch) diluted 1:5000 in PBS with 10% (v/v) FCS, added 100 µl and incubated for 1 h at RT. After another three washing steps, a development was performed by adding 50 µl of TMB substrate (3,3',5,5'-tetramethylbenzidine, 1-Step™ Ultra TMB-ELISA Substrate Solution, Thermo Scientific) per well until the samples turned blue. The reaction was stopped with 1 M H_2SO_4 before the absorbance was determined using a microplate multireader at 450nm (Mithras LB 943, Berthold). The antibody response was determined based on the subtraction the mean of absorbance and controls.

2.2.3.9. *In vitro* neutralization assay

To evaluate the neutralization effect of serum samples, an MCMV *in vitro* neutralization assay was conducted as described in Reinhard *et al*., 2011. First, the serum was incubated at 56°C for 30 min to inactivate the complement and afterwards diluted in DMEM supplemented with 10 % (v/v) FCS. Mixtures of Δm157-MCMV:Luc and sera were prepared by adding a serum dilution to an equal volume of an Δm157-MCMV:Luc suspension and incubated for 90 min at 37 °C. 100 µl of these mixtures were transferred into a 96 well cell-culture plate with a 90% confluent monolayer of permissive CIM cells. The plate was centrifuged for enhancement of infection as described in 2.2.2.1. The cells were incubated at 37°C in 5%CO_2 of atmosphere. At 24 h post-infection, the supernatant was aspirated and the cells were rinsed with PBS. By adding of LucLyse Buffer (Promega), cells were lysed and luciferase activity was measured according to the manufacturer's instructions using a microplate luminometer at 560nm (Mithras LB 943, Berthold). In order to obtain the dilution of IgG (sera) that resulted in 50% reduction of the infection, neutralizing capacity was calculated with untreated Δm157-MCMV:Luc as reference

2.2.3.10. IgG-dependent activation of the BW5147:FcγR-ζ reporter cells (*In vitro* FcγR activation assay)

The principle of this assay was established and described by Corrales-Aguilar *et al*., 2013 and 2016. The work of the IgG-dependent activation of the BW5147:FcγR-ζ reporter cells was performed at the Universitätsklinikum Freiburg. To quantify antiviral IgG antibodies able to trigger a specific FcγR, CIM cells were infected with Δm138-MCMV-FSH according to 2.2.2.1. CIM cells were seeded in 96 well cell-culture plate one day prior to infection. Afterwards, Δm138-MCMV-FSH-infected cells were incubated with serial dilutions of serum in DMEM containing 10% (v/v) FCS for 30 min at 37 °C in an atmosphere of 5% CO_2 and final volume of 100 µl per well. To remove non-immune IgG, cells were washed three times with 200µl of DMEM containing 10% (v/v) FCS before co-cultivation with BW5147:FcγR-ζ reporter cells was performed in RPMI containing 10% (v/v) FCS. The effector (BWFcγR-ζ transfectant) to target (virus-infected cell) E:T ratio was 20:1. After 16 h of co-cultivation, supernatants were diluted 1/2 in ELISA sample buffer (PBS with 10% [v/v] FCS and 0.1% [v/v] Tween-20) and the secreted murine IL-2 was measured in the mIL-2 ELISA (see below).

2.2.3.11. Interleukin-2 (IL-2) ELISA

Interleukin-2 (IL-2) ELISA was performed according to Corrales-Aguilar *et al.*, 2013. The rat-anti-mouse IL-2-Biotin capture antibody (clone JES6-1A12, BD Pharmingen) was diluted 1/500 (v/v) to obtain 1 µg/ml concentration in PBS with 10% (v/v) FCS, and 50 µl/well were added to the ELISA plate. The ELISA plate (Maxisorp, NUNC) was incubated overnight at 4°C. The plate was washed three times with 200 µl/well of PBS with 0.1% (v/v) Tween-20. The samples and mIL-2 standards were diluted 1:1 v/v in PBS with 10% (v/v) FCS and 0.1% (v/v) Tween-20 and incubated for 15 min at RT in order to allow a complete lysis of cells. The samples and standards were added 100 µl per well, and were incubated on the plate overnight at 4°C. The plate was washed three times with PBS containing 0.1% (v/v) Tween 20 and a biothinylated anti-IL-2 detection antibody (JES6-5H4, BD Pharmingen), diluted to 1 µg/ml (1 /500 [v/v]) in PBS with 10% (v/v) FCS and 0.1% (v/v) Tween-20 was added for 1 hour of incubation at RT. 50 µl per well was added. The plate was washed three times with PBS with 0.1% (v/v) Tween-20 and the POD-coupled Streptavidin (Jackson ImmunoResearch) diluted 1/1000 (v/v) in PBS with 10% (v/v) FCS and 0.1% (v/v) Tween-20; 50 µl per well was added. The plate was incubated exactly 30 minutes at RT, and then washed four times with PBS with 0.1% (v/v) Tween-20. Shortly before adding the substrate to the plate, 10 µl of 30% (v/v) H_2O_2 per 10 ml of a prepared TMB solution were added and the solution was rapidly and shortly vortexed. As quickly as possible, 50 µl per well of the substrate solution were added and let between 2-4 minutes to develop a blue colour. The reaction was stopped by adding 50 µl of 1M H_2SO_4 per well allowing a yellow colour to appear. The absorbance was determined using a microplate multireader at 450nm (Mithras LB 943, Berthold). A mIL-2 calibration curve with the standards was done and the respective concentration of mIL-2 in pg/ml of the samples was calculated.

2.3. Statistic analysis

Statistical analysis and graphs were created with Graph Pad Prism Version 6. Statistical significance was determined using unpaired t-test or Kruskal-Wallis test corrected for multiple comparisons as described in the figure legends.

3 Results

3.1. Humoral immune responses of ΔM27-MCMV

Acute phase of primary MCMV infection induces increases in the numbers of IgG-producing cells (Karupiah *et al.*, 1998). IgG-producing cells are affected in responding to MCMV antigens, suggesting an IFN-γ contribution upon MCMV infection (Karupiah *et al.*, 1998). Since pM27 is a STAT2 dependent IFN antagonist (Zimmermann *et al.*, 2005; Le-Trilling *et al.*, 2018), MCMV-specific IgG was quantified in the absence of pM27, using ΔM27-MCMV.

To examine humoral immune responses during MCMV infection a ΔM27-MCMV and wt-MCMV with repaired MCK-2 background, described in Le-Trilling *et al.*, 2018, susceptible and resistant mice from the BALB/c and C57BL/6 strain, respectively, were infected with ΔM27-MCMV and wt-MCMV intraperitoneally. Mice were terminated 3, 7, 14, and 21 day post infection and blood was collected from the heart.

3.1.1. ΔM27-MCMV elicits MCMV specific IgG responses on MCMV infected cell proteins (ICPs)

To test if ΔM27-MCMV is capable to elicit MCMV-specific IgG response in the serum of susceptible and resistant mice, an ELISA recognizing infected cell proteins (ICP) was applied (Materials and methods, 2.2.3.7). ICPs were created using mouse fibroblasts infected with viruses of interest and lysed by sonification (Materials and methods, 2.2.3.8), and afterwards immobilized on an ELISA plate. The collected samples of sera were diluted prior to the application. The MCMV-specific IgG response was determined by subtracting the mean of an absorbance of samples with control samples and plotting it against time. The number of cells and the multiplicity of infection (MOI) of viruses were constant for all shown ELISA experiments. To measure the specific IgG response in sera, a peroxidase-conjugated goat anti mouse (GAM) antibody that reacts with whole molecule of the mouse IgG, was used.

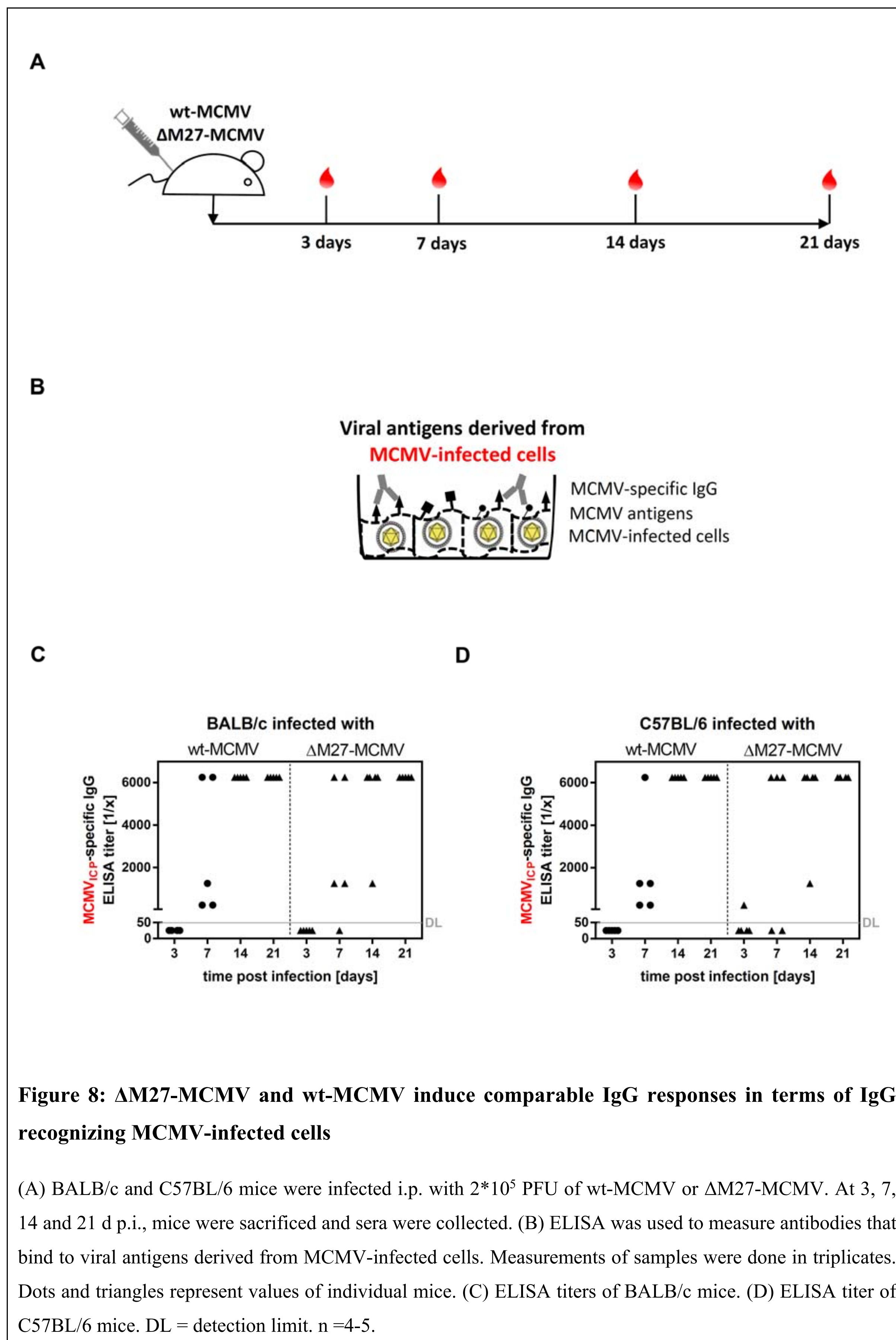


Figure 8: ΔM27-MCMV and wt-MCMV induce comparable IgG responses in terms of IgG recognizing MCMV-infected cells

(A) BALB/c and C57BL/6 mice were infected i.p. with $2*10^5$ PFU of wt-MCMV or ΔM27-MCMV. At 3, 7, 14 and 21 d p.i., mice were sacrificed and sera were collected. (B) ELISA was used to measure antibodies that bind to viral antigens derived from MCMV-infected cells. Measurements of samples were done in triplicates. Dots and triangles represent values of individual mice. (C) ELISA titers of BALB/c mice. (D) ELISA titer of C57BL/6 mice. DL = detection limit. n =4-5.

ΔM27-MCMV and wt-MCMV induced comparable IgG responses in terms of IgG recognizing MCMV-encoded proteins present in infected cells. The ΔM27-MCMV and wt-MCMV induced MCMV-specific IgG responses reaching their maximum 14 days post infection with high responses persisting further (21 days post infection) (Figure 11 C & D). All four mice infected with wt-MCMV induced a maximum MCMV-specific IgG responses at day 14 post infection, while a group of four mice infected with ΔM27-MCMV has accomplished the maximum MCMV-specific IgG responses at 21 day post infection (Figure 11 C & D). ΔM27-MCMV induces MCMV-specific IgG responses in 3 of C57BL/6 infected mice at day 7 post infection (Figure 11 D). Nevertheless, at the same day post infection, MCMV-specific IgG responses in infected BALB/c mice have reached the same level only in 2 mice.

3.1.2. ΔM27-MCMV elicits MCMV specific IgG responses on MCMV virions

With this experiment, our intention was to determine if ΔM27-MCMV and wt-MCMV stimulate a humoral immune response that can recognize virus-encoded proteins present in the MCMV particle. MCMV particles contain at least 58 virus-encoded proteins, from the capsid, tegument, glycoprotein, replication, and immunomodulatory protein families (Kattenhorn *et al.*, 2004). Since proteins associated with the viral particle were, among the others, proteins important for viral entry (Kattenhorn *et al.*, 2004), IgG recognizing MCMV virion proteins was determined by ELISA. Blood was collected 3, 7, 14, and 21 days post infection with ΔM27-MCMV or wt-MCMV from BALB/c and C57BL/6 mice.

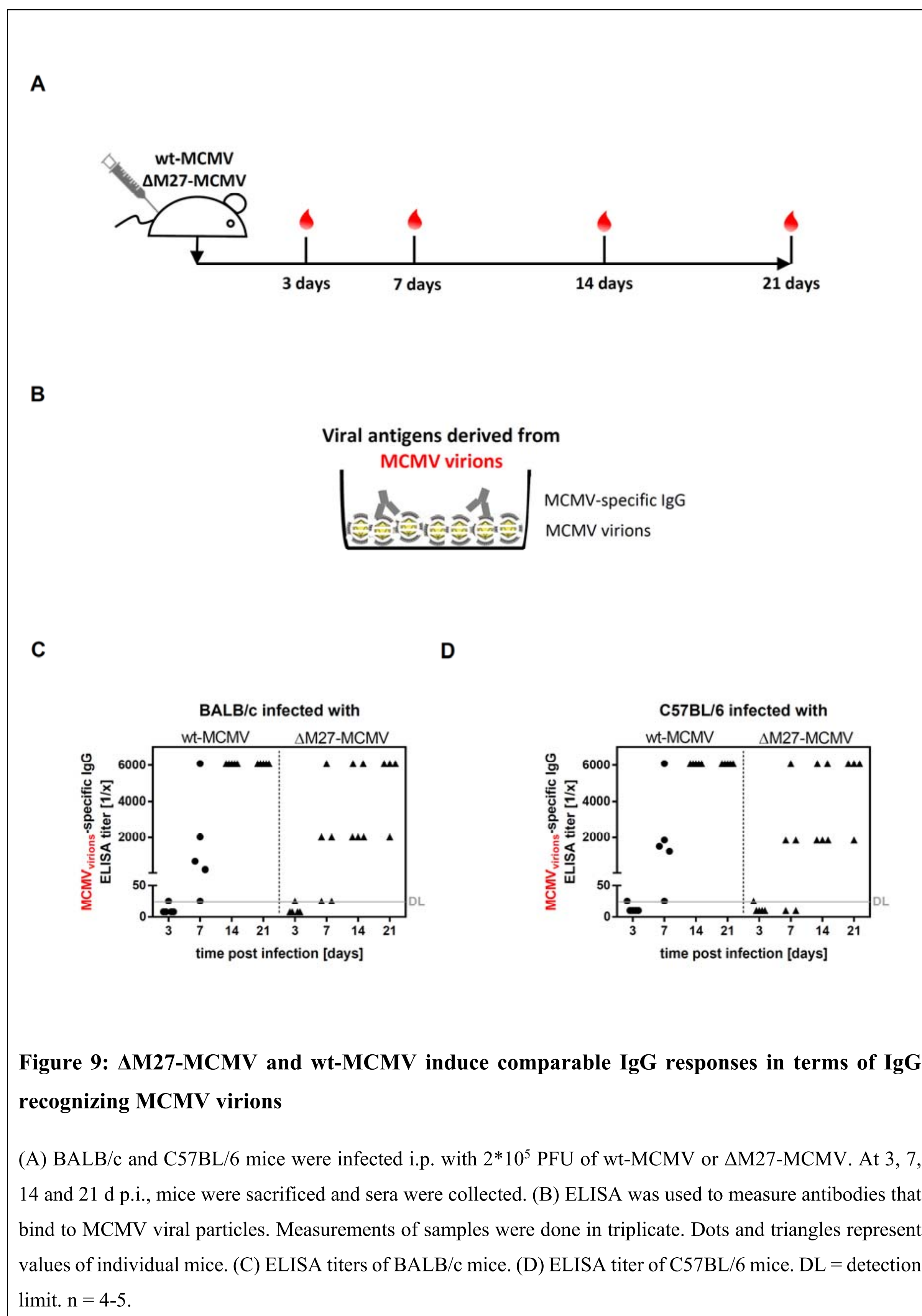


Figure 9: ΔM27-MCMV and wt-MCMV induce comparable IgG responses in terms of IgG recognizing MCMV virions

(A) BALB/c and C57BL/6 mice were infected i.p. with $2*10^5$ PFU of wt-MCMV or ΔM27-MCMV. At 3, 7, 14 and 21 d p.i., mice were sacrificed and sera were collected. (B) ELISA was used to measure antibodies that bind to MCMV viral particles. Measurements of samples were done in triplicate. Dots and triangles represent values of individual mice. (C) ELISA titers of BALB/c mice. (D) ELISA titer of C57BL/6 mice. DL = detection limit. n = 4-5.

The ΔM27-MCMV and wt-MCMV induced comparable IgG responses in terms of IgG recognizing MCMV virions. For ΔM27-MCMV and wt-MCMV, the maximum of MCMV virion-specific IgG responses were reached 21 days post infection (Figure 12 C & D). At day 14 post infection, 2 mice infected with ΔM27-MCMV have reached a maximum of IgG responses in terms of IgG recognizing virions, induced by infection of BALB/c and C57BL/6 mice with ΔM27-MCMV and wt-MCMV (Figure 12 C & D). For infection of BALB/c mice with wt-MCMV at same conditions, 14 days post infection, 5 infected mice have reached the maximum of IgG responses in terms of IgG recognizing virions (Figure 12 C). MCMV IgG response induced with ΔM27-MCMV and wt-MCMV show similar phenotype in terms of MCMV IgG response time points and regardless of infected mouse strain.

3.1.3. ΔM27-MCMV induces persistent immune responses

Infection of BALB/c mice with MCMV increased the proportions of spleen and lymph node cells expressing IgG (Price *et al.*, 1993). Although the number of antibody secreting cells decreased 15-21 days post infection, IgG levels rose by day 14, remained high until day 30 post infection, and then declined (Price *et al.*, 1993).

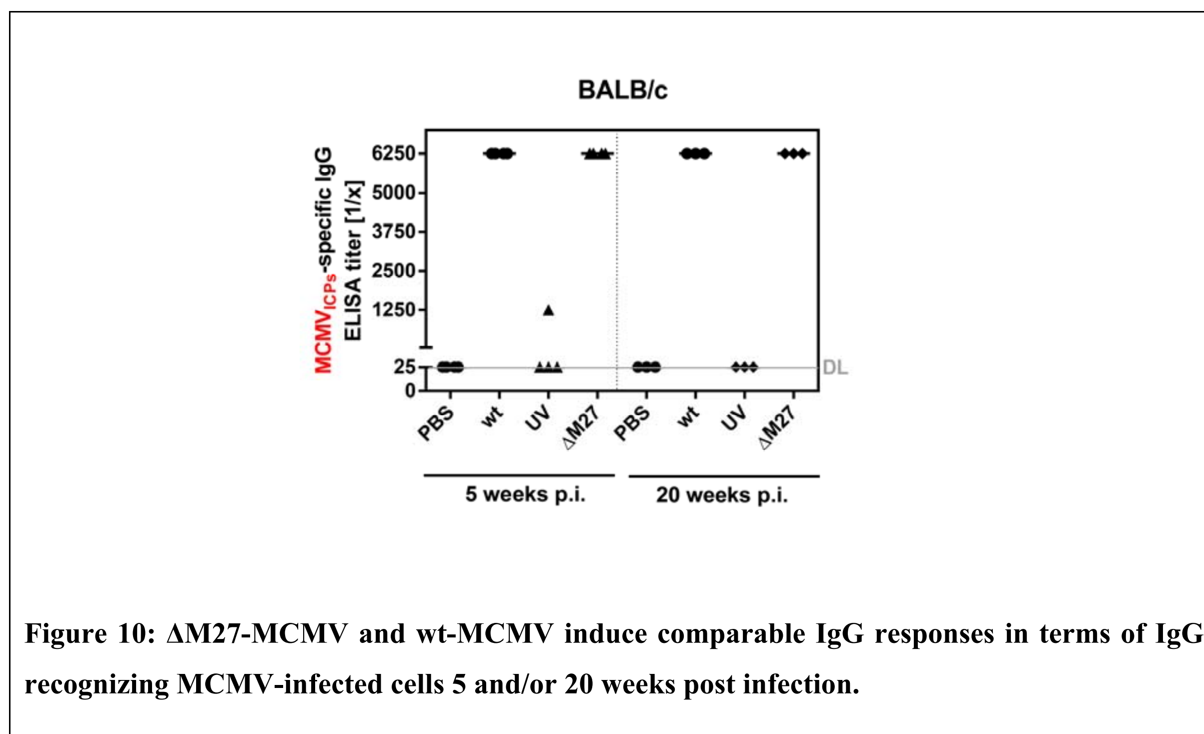


Figure 10: ΔM27-MCMV and wt-MCMV induce comparable IgG responses in terms of IgG recognizing MCMV-infected cells 5 and/or 20 weeks post infection.

BALB/c mice were infected i.p. with $2*10^5$ PFU of wt-MCMV or ΔM27-MCMV. At 5 and 20 weeks p.i., the sera were collected. ELISA was used to quantify antibodies, which bind to viral antigens derived from MCMV-infected cells. Measurements of samples were done in triplicates. Dots, diamonds, and triangles represent values of individual mice. DL = detection limit. n =3-5.

This prompted us to test if ΔM27-MCMV may be able to mount an adaptive humoral immune response for time periods longer than 21 days. We infected BALB/c mice with wt-MCMV or ΔM27-MCMV and blood was collected, sera isolated at 5 and 20 weeks post infection to quantify MCMV-specific IgG by ELISA on ICPs. Both viruses mounted very strong MCMV-specific ELISA-reactive IgG responses (Figure 13). Only in one infected BALB/c mouse UV-inactivated MCMV elicited minimal MCMV-specific ELISA-reactive IgG responses, showing that the virus must be infective in order to induce high MCMV-specific IgG responses.

3.2. ΔM27-MCMV induce immune responses capable of activating receptors recognizing the Fc part of IgG (FcγRs)

Interaction of antigen-bound IgG with activatory FcγRs results in phagocytosis of immune complexes and opsonized pathogens, antibody-dependent cell-mediated cytotoxicity (ADCC) by NK cells or macrophages, and the release of inflammatory cytokines, chemokines, or superoxide radicals (Nimmerjahn *et al.*, 2008). Therefore, we determined the FcγR-activating abilities of the MCMV-specific IgG raised upon immunization with ΔM27-MCMV.

3.2.1. An assay for measuring antiviral IgG antibodies triggering activation of individual host Fcγ receptors

Assessing FcγR activation *in vitro* is of fundamental importance, but technically difficult. Therefore, an assay for measuring antiviral IgG antibodies triggering activation of individual host Fcγ receptors has been established by Corrales-Aguilar *et al.* (Corrales-Aguilar *et al.*, 2013). The assay comprises the co-cultivation of virus-infected target cells with immune IgG antibodies and mouse BW5147 hybridoma cells stably expressing chimeric FcγR-CD3ζ chain molecules consisting of the extracellular domain of human FcγRIIIA, FcγRIIA or FcγRI fused to the transmembrane and intracellular domains of the mouse CD3ζ chain. Triggering of the

chimeric FcγR receptors by immune complexes formed on the surface of IgG-opsonized virus-infected target cells resulted in FcγR activation leading to IL-2 secretion by BW5147 cells, which was quantified as a surrogate marker in an ELISA (Corrales-Aguilar *et al.*, 2013). Also, mouse BW5147 hybridoma cells were available stably expressing chimeric FcγR-CD3ζ chain molecules consisting of the extracellular domain of mouse FcγRIV, FcγRIII, FcγRII or FcγRI.

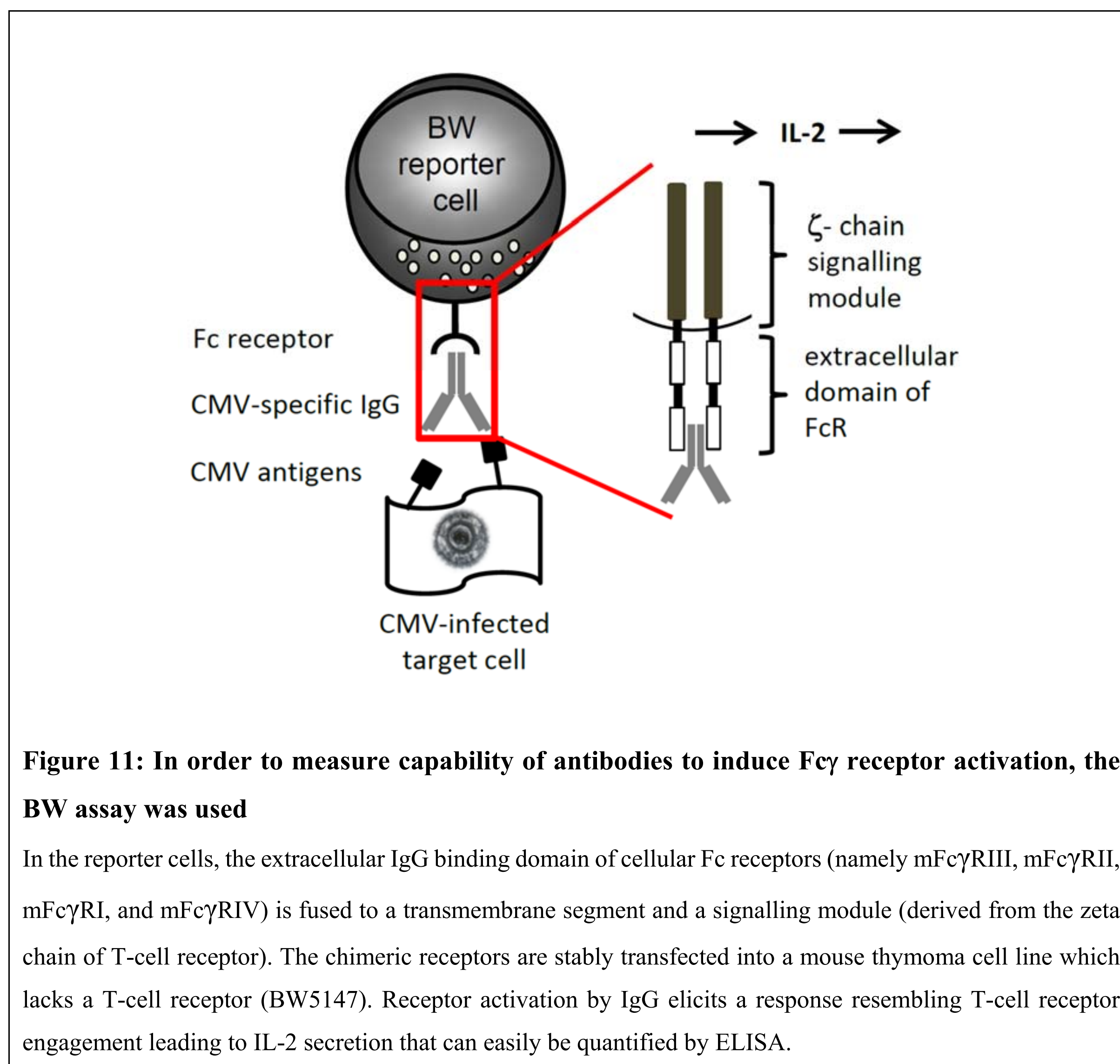


Figure 11: In order to measure capability of antibodies to induce Fcγ receptor activation, the BW assay was used

In the reporter cells, the extracellular IgG binding domain of cellular Fc receptors (namely mFcγRIII, mFcγRII, mFcγRI, and mFcγRIV) is fused to a transmembrane segment and a signalling module (derived from the zeta chain of T-cell receptor). The chimeric receptors are stably transfected into a mouse thymoma cell line which lacks a T-cell receptor (BW5147). Receptor activation by IgG elicits a response resembling T-cell receptor engagement leading to IL-2 secretion that can easily be quantified by ELISA.

3.2.2 ΔM27-MCMV and wt-MCMV infection, irrespective whether BALB/c or C57BL/6 mice were infected, induce antibodies with similar activation capacity of Fcγ receptors (FcγRIII, FcγRI, FcγRII, and FcγRIV)

To investigate capability of ΔM27-MCMV to induce antibodies that can also activate Fcγ receptors, sera collected 3, 7, 14 and 21 days from infected BALB/c and C57BL/6 mice were incubated with cells infected with Δm138-MCMV-FSH. The mouse thymoma cell line

(BW5147) stably expressing individual mFcγ receptor chimeras were added on fibroblasts infected with Δm138-MCMV-FSH. Upon activation, chimeric FcγR-CD3ζ chain molecules were capable of releasing mIL-2 quantified by ELISA.

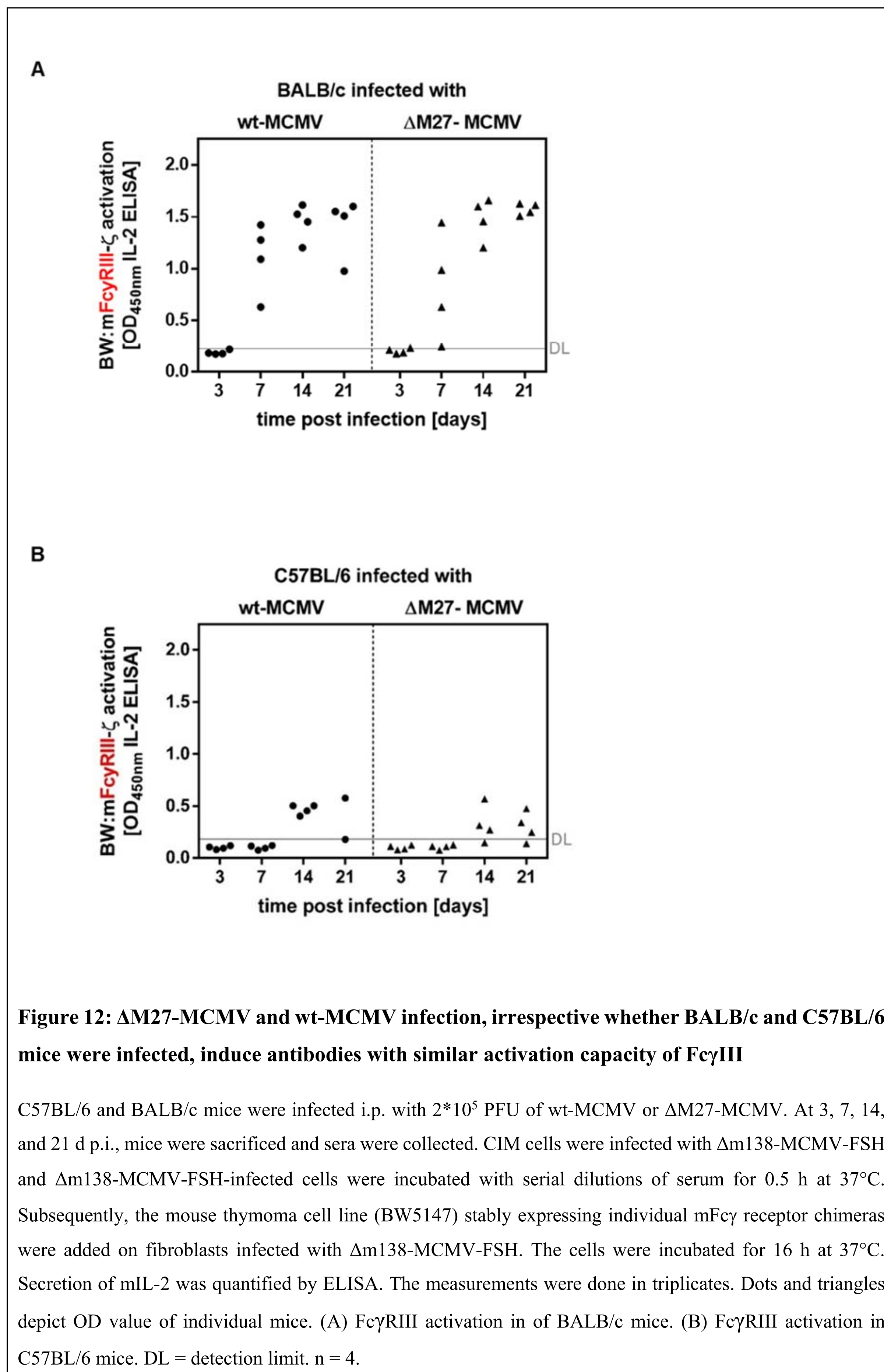


Figure 12: ΔM27-MCMV and wt-MCMV infection, irrespective whether BALB/c and C57BL/6 mice were infected, induce antibodies with similar activation capacity of FcγIII

C57BL/6 and BALB/c mice were infected i.p. with $2*10^5$ PFU of wt-MCMV or ΔM27-MCMV. At 3, 7, 14, and 21 d p.i., mice were sacrificed and sera were collected. CIM cells were infected with Δm138-MCMV-FSH and Δm138-MCMV-FSH-infected cells were incubated with serial dilutions of serum for 0.5 h at 37°C. Subsequently, the mouse thymoma cell line (BW5147) stably expressing individual mFcγ receptor chimeras were added on fibroblasts infected with Δm138-MCMV-FSH. The cells were incubated for 16 h at 37°C. Secretion of mIL-2 was quantified by ELISA. The measurements were done in triplicates. Dots and triangles depict OD value of individual mice. (A) FcγRIII activation in of BALB/c mice. (B) FcγRIII activation in C57BL/6 mice. DL = detection limit. n = 4.

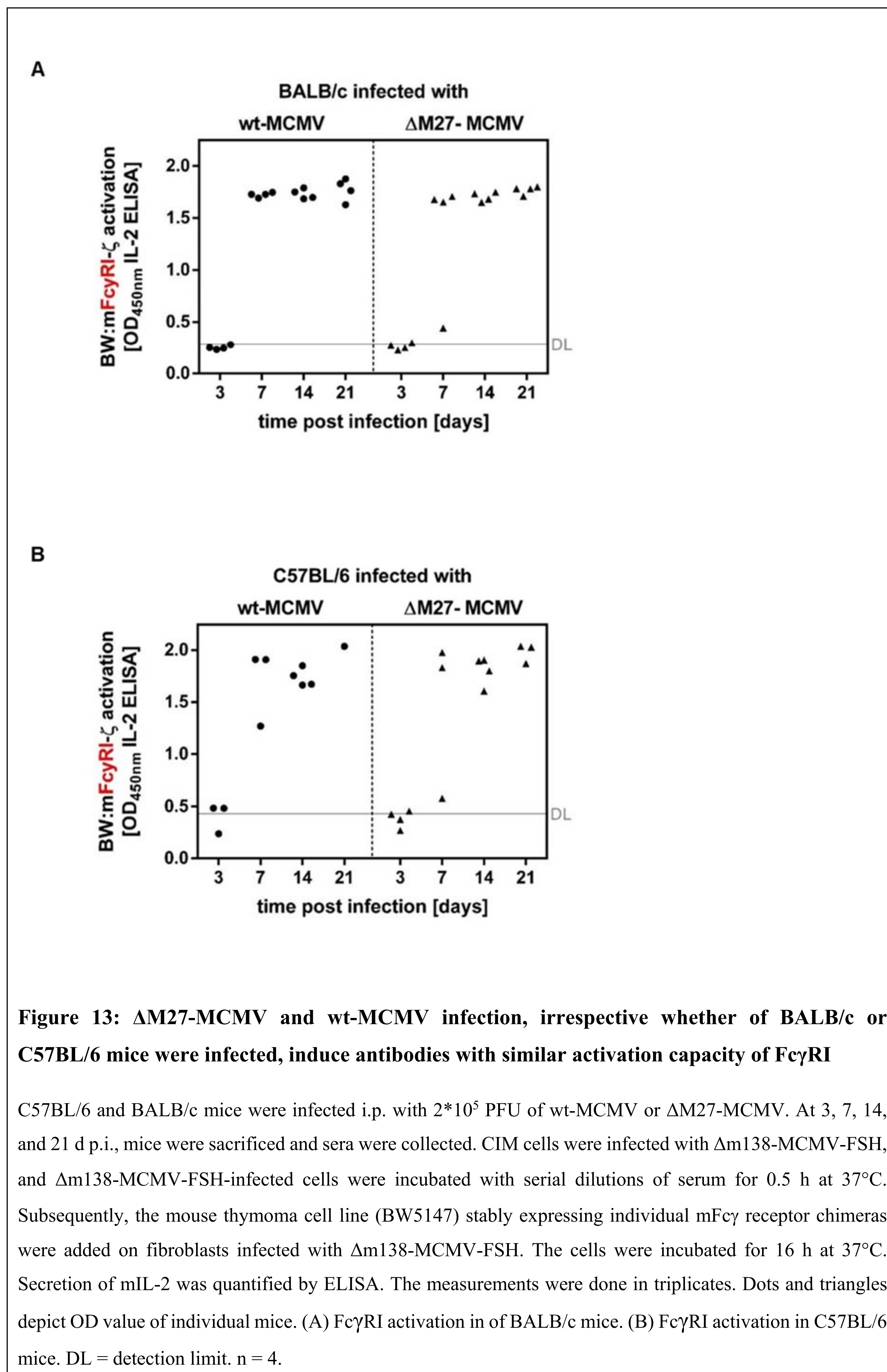


Figure 13: ΔM27-MCMV and wt-MCMV infection, irrespective whether of BALB/c or C57BL/6 mice were infected, induce antibodies with similar activation capacity of FcγRI

C57BL/6 and BALB/c mice were infected i.p. with $2*10^5$ PFU of wt-MCMV or ΔM27-MCMV. At 3, 7, 14, and 21 d p.i., mice were sacrificed and sera were collected. CIM cells were infected with Δm138-MCMV-FSH, and Δm138-MCMV-FSH-infected cells were incubated with serial dilutions of serum for 0.5 h at 37°C. Subsequently, the mouse thymoma cell line (BW5147) stably expressing individual mFcγ receptor chimeras were added on fibroblasts infected with Δm138-MCMV-FSH. The cells were incubated for 16 h at 37°C. Secretion of mIL-2 was quantified by ELISA. The measurements were done in triplicates. Dots and triangles depict OD value of individual mice. (A) FcγRI activation in of BALB/c mice. (B) FcγRI activation in C57BL/6 mice. DL = detection limit. n = 4.

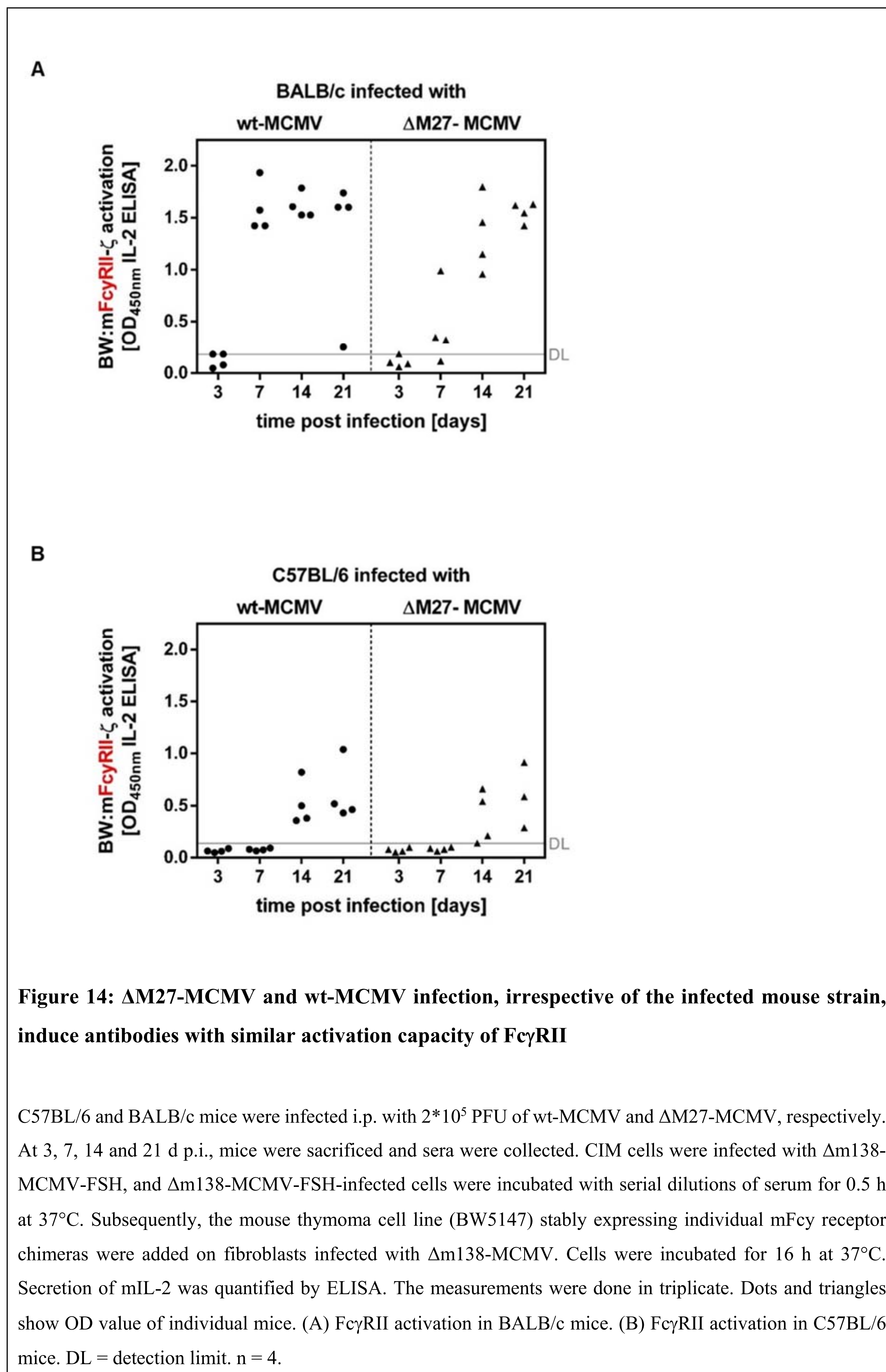


Figure 14: ΔM27-MCMV and wt-MCMV infection, irrespective of the infected mouse strain, induce antibodies with similar activation capacity of FcγRII

C57BL/6 and BALB/c mice were infected i.p. with $2*10^5$ PFU of wt-MCMV and ΔM27-MCMV, respectively. At 3, 7, 14 and 21 d p.i., mice were sacrificed and sera were collected. CIM cells were infected with Δm138-MCMV-FSH, and Δm138-MCMV-FSH-infected cells were incubated with serial dilutions of serum for 0.5 h at 37°C. Subsequently, the mouse thymoma cell line (BW5147) stably expressing individual mFcy receptor chimeras were added on fibroblasts infected with Δm138-MCMV. Cells were incubated for 16 h at 37°C. Secretion of mIL-2 was quantified by ELISA. The measurements were done in triplicate. Dots and triangles show OD value of individual mice. (A) FcγRII activation in BALB/c mice. (B) FcγRII activation in C57BL/6 mice. DL = detection limit. n = 4.

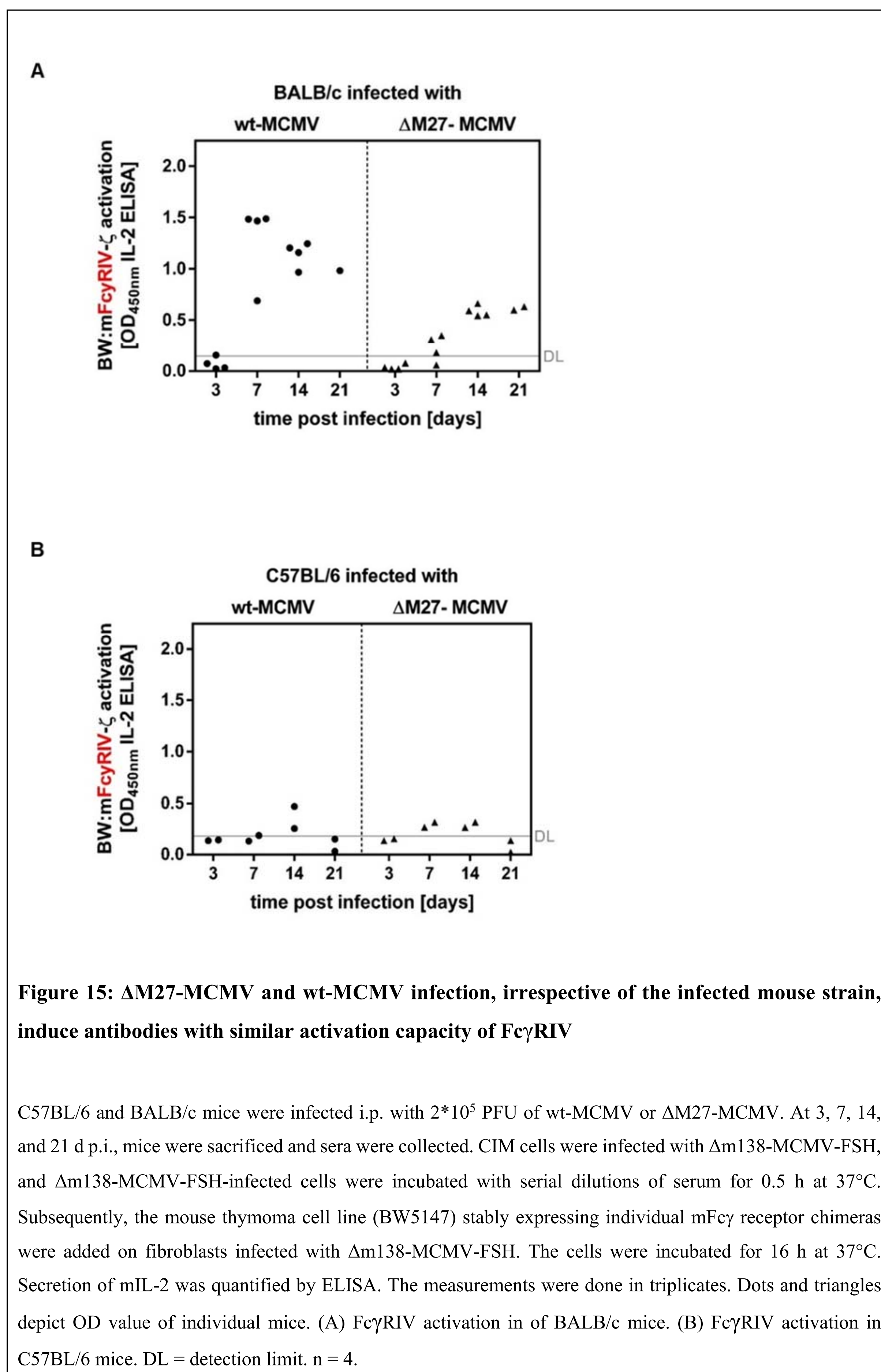


Figure 15: ΔM27-MCMV and wt-MCMV infection, irrespective of the infected mouse strain, induce antibodies with similar activation capacity of FcγRIV

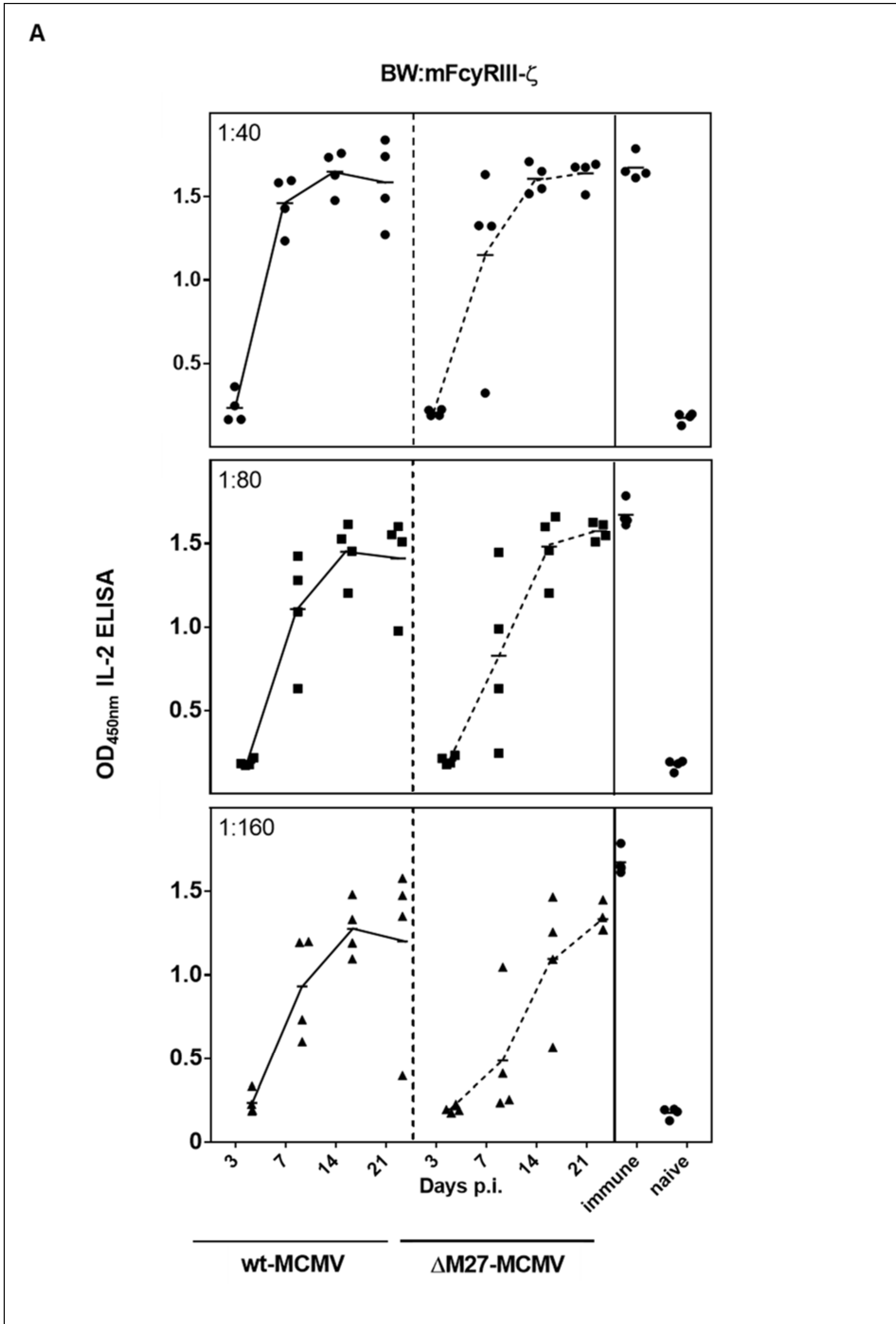
C57BL/6 and BALB/c mice were infected i.p. with $2*10^5$ PFU of wt-MCMV or ΔM27-MCMV. At 3, 7, 14, and 21 d p.i., mice were sacrificed and sera were collected. CIM cells were infected with Δm138-MCMV-FSH, and Δm138-MCMV-FSH-infected cells were incubated with serial dilutions of serum for 0.5 h at 37°C. Subsequently, the mouse thymoma cell line (BW5147) stably expressing individual mFcγ receptor chimeras were added on fibroblasts infected with Δm138-MCMV-FSH. The cells were incubated for 16 h at 37°C. Secretion of mIL-2 was quantified by ELISA. The measurements were done in triplicates. Dots and triangles depict OD value of individual mice. (A) FcγRIV activation in of BALB/c mice. (B) FcγRIV activation in C57BL/6 mice. DL = detection limit. n = 4.

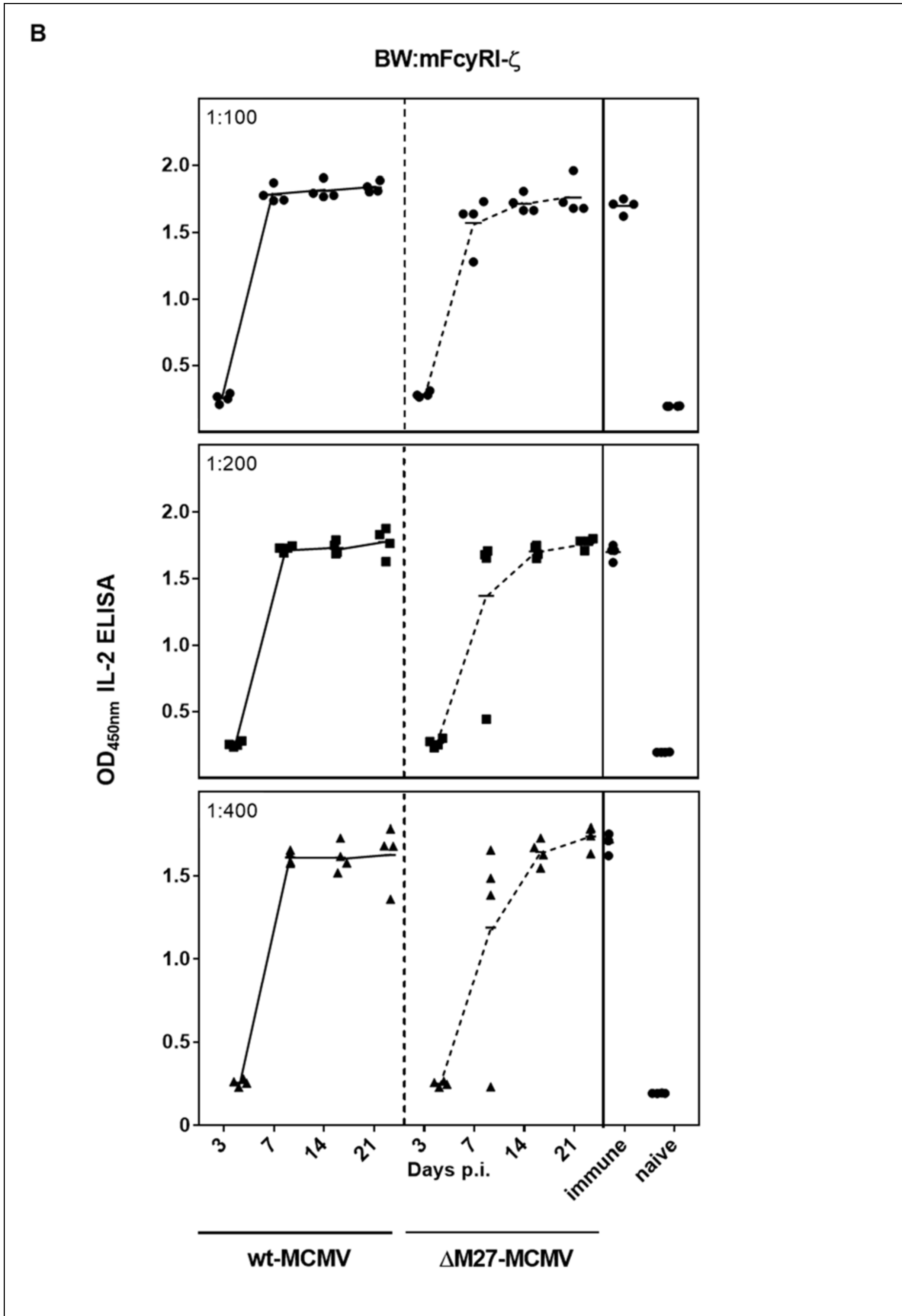
The wt- and ΔM27-MCMV raise similar FcγRIII [CD16], FcγRI [CD64] and FcγRII [CD32] activating IgG responses in BALB/c and C57BL/6 mice. FcγRI, FcγRII and FcγRIII have reached their maximum level of IgG activation upon infection with wt- and ΔM27-MCMV at 14 and 21 days post infection, respectively (Figure 15, 16, 17, and 18). The wt- and ΔM27-MCMV raise FcγRIV-activating IgG responses in BALB/c and C57BL/6 mice (Figure 18). All BALB/c and C57BL/6 mice infected with wt- and ΔM27-MCMV are capable of inducing IgG that activates FcγRI, FcγRII and FcγRIII responses 14 and 21 days post infection and also FcγRIV responses (Figure 15, 16, 17, and 18). C57BL/6 mice infected with wt- and ΔM27-MCMV are showing lower level of activation for FcγRIII, FcγRII and FcγRIV activating IgG responses compared to BALB/c mice (Figure 15, 17, and 18). Thus, an immunization with a live attenuated MCMV mutant lacking a STAT2 antagonist pM27 is sufficient to mount IgG responses that activate immune cells through Fcγ receptors.

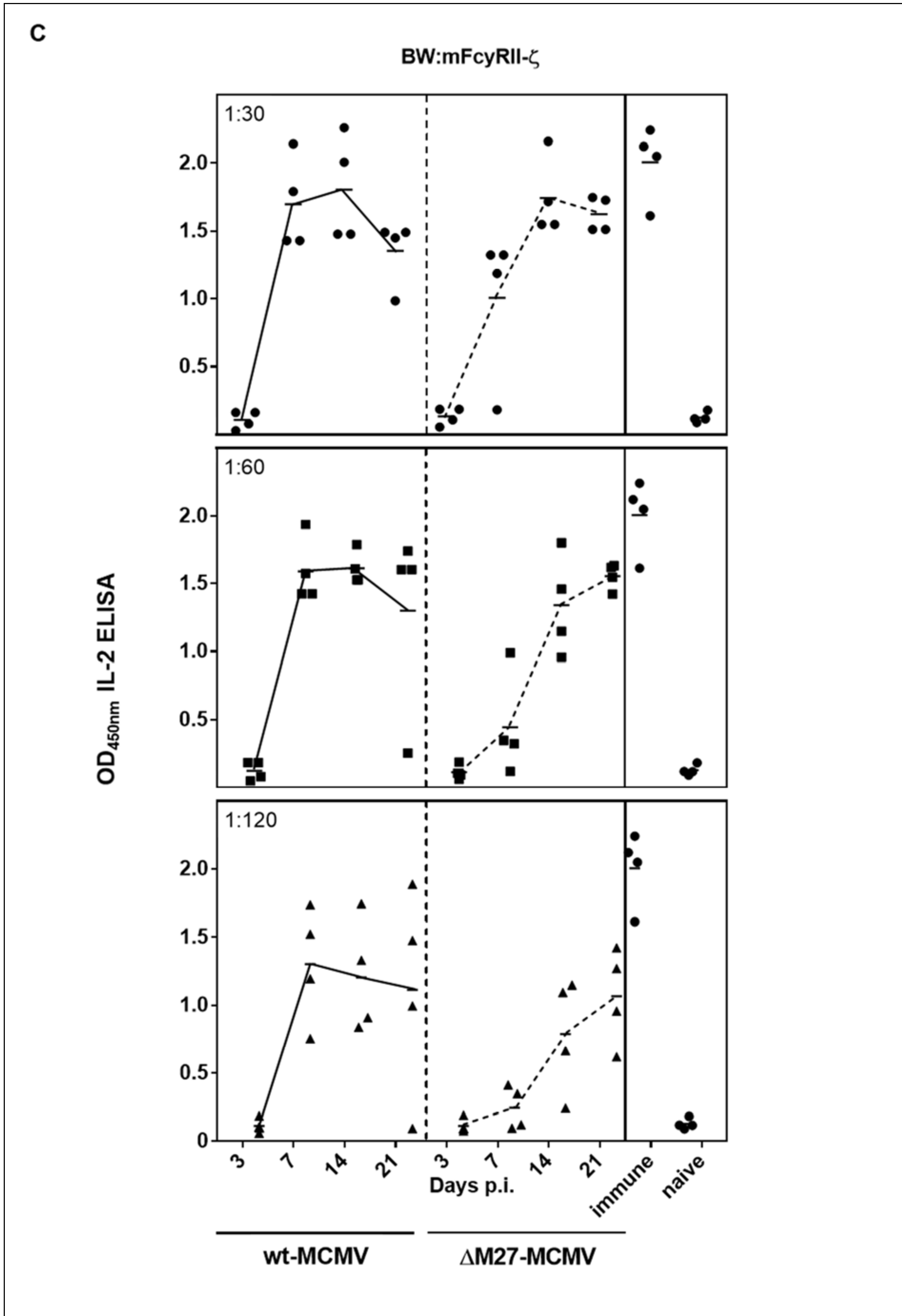
3.2.3. FcγR reporter cell have been activated in dose-dependent manner

Human IgG activates the human FcγR reporter cells in a dose-dependent manner indicating that the response is strictly IgG-dependent (Corrales-Aquilar *et al.*, 2016). The influence of IgG concentration on the activation of the BW:FcγR-ζ reporter cells is determined by using variable amounts of IgG. The same thing is also observed for mouse FcγR reporter cells. A clear dose-dependent activation by IgG creates mIL-2 production induced by the activation of the chimeric receptors correlates over a wide range with the amount of IgG used for FcγR activation (Corrales-Aquilar *et al.*, 2013).

The previous experiment showed that ΔM27-MCMV mounts sufficient IgG responses capable activate Fcγ receptors. Now, we wanted to test if graded serum concentrations induced different capability of chimeric FcγR-CD3ζ chain molecules responses upon ΔM27-MCMV infection. FcγR-CD3ζ responses were quantified per mIL-2 ELISA.


A
BW:mFcγRIII-ζ
1:40
1:80
1:160
OD_{450nm} IL-2 ELISA
0
0.5
1.0
1.5
3
7
14
21
3
7
14
21
immune
naive
Days p.i.
wt-MCMV
ΔM27-MCMV


B
BW:mFcγRI-ζ
1:100
1:200
1:400
OD450nm IL-2 ELISA
2.0
1.5
1.0
0.5
0
3
7
14
21
immune
naive
Days p.i.
wt-MCMV
ΔM27-MCMV


C
BW:mFcγRII-ζ
1:30
1:60
1:120
OD450nm IL-2 ELISA
2.0
1.5
1.0
0.5
0
3
7
14
21
Days p.i.
immune
naive
wt-MCMV
ΔM27-MCMV

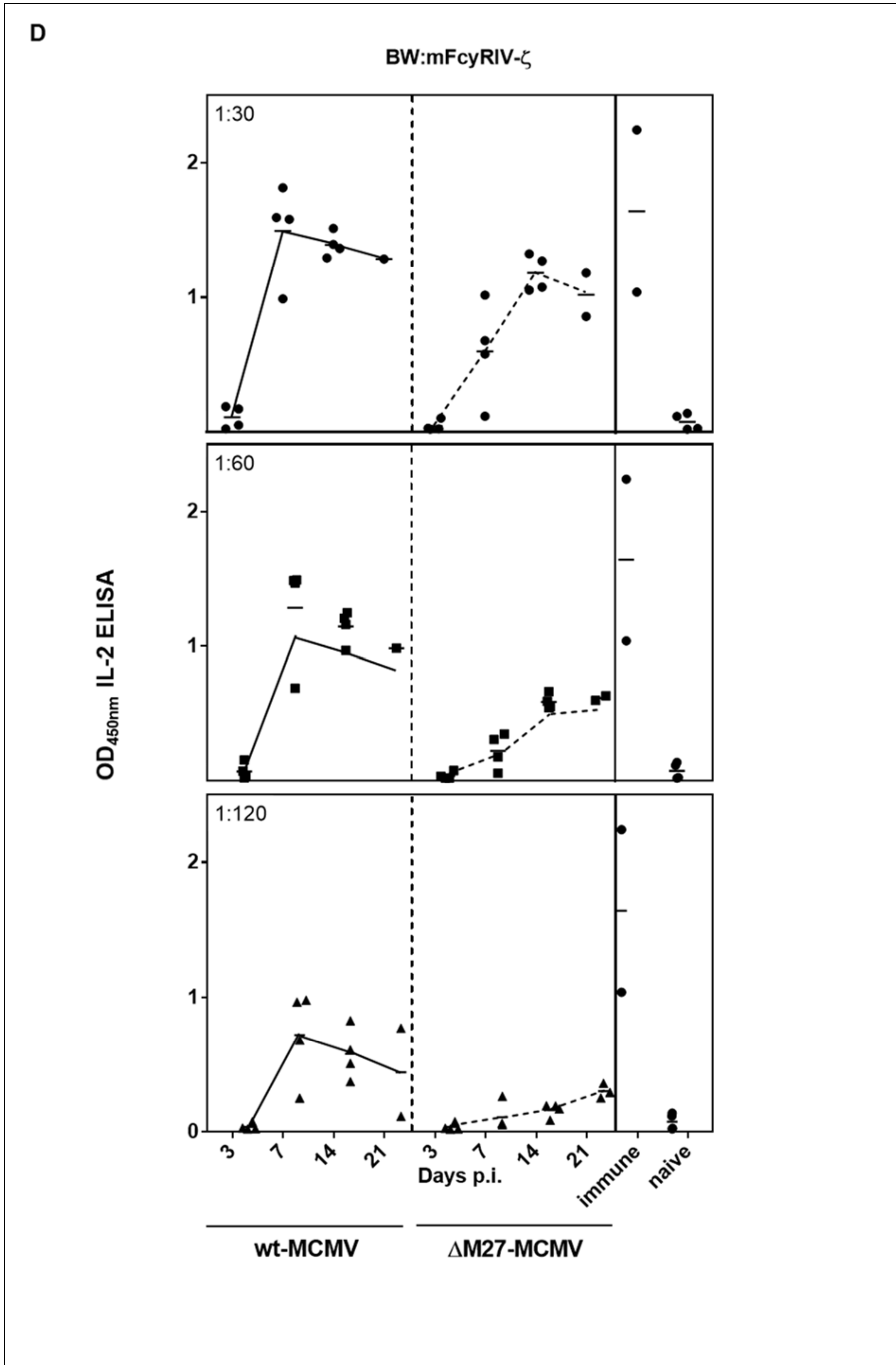
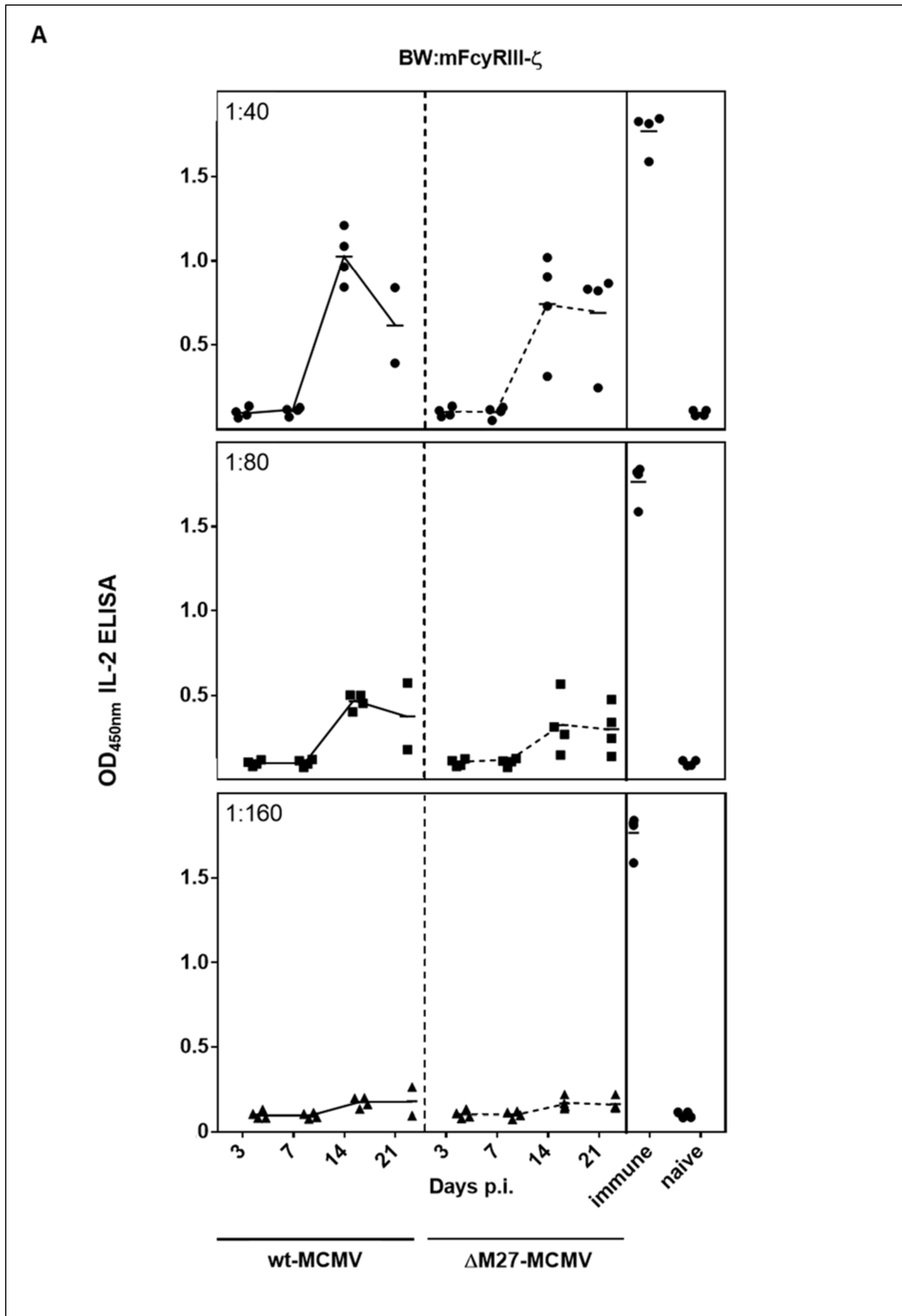
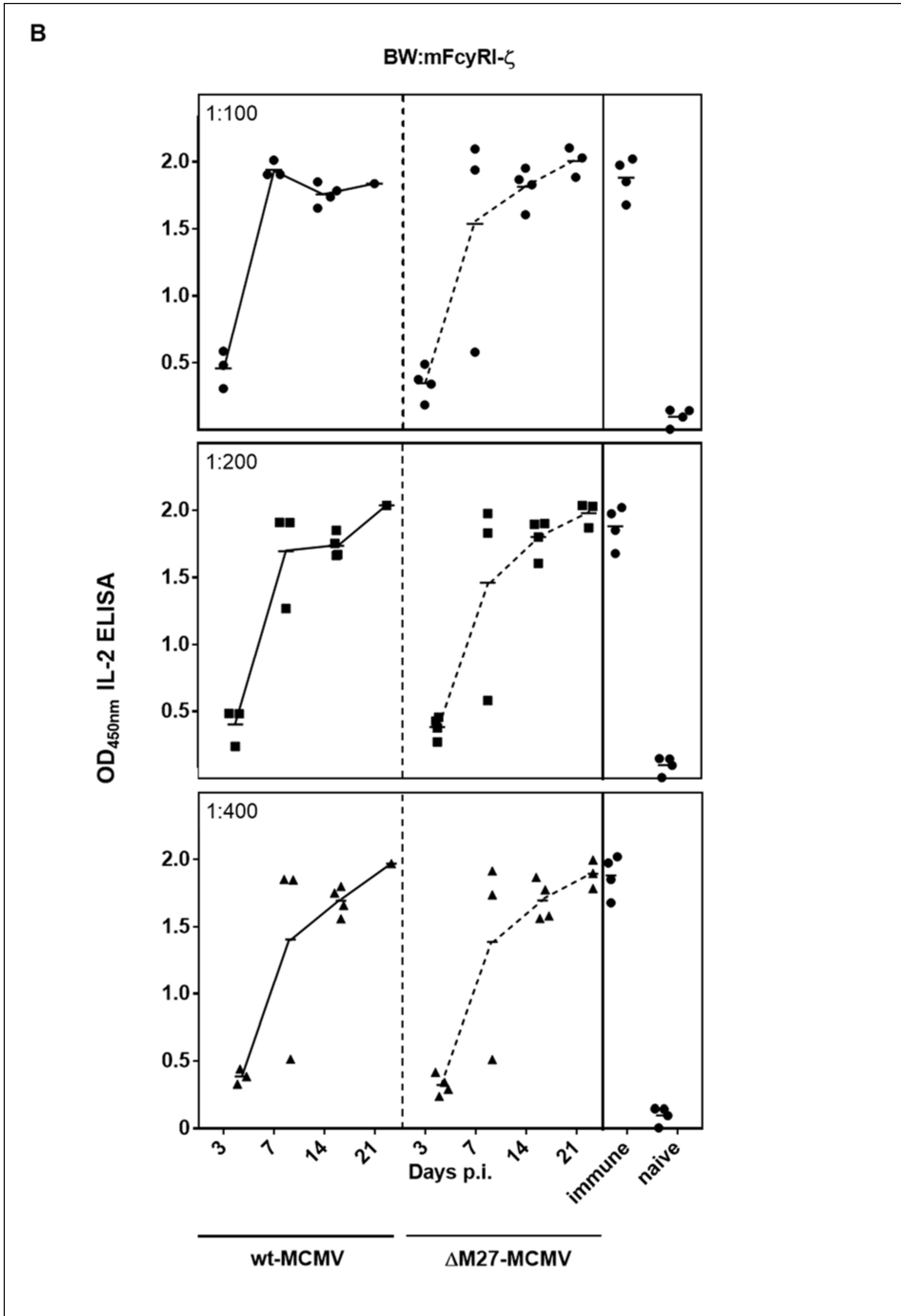

D
BW:mFcyRIV-ζ
1:30
1:60
1:120
OD_{450nm} IL-2 ELISA
0
1
2
3
7
14
21
Days p.i.
immune
naive
wt-MCMV
ΔM27-MCMV

Figure 16: Infection with ΔM27-MCMV elicits antibodies capable of dose-dependently activating FcγIII, FcγII, FcγI and FcγIV receptors in BALB/c mice

BALB/c mice were infected with $2*10^5$ PFU of wt-MCMV or ΔM27-MCMV. A sera was collected 3, 7, 14 and 21 days post infection. CIM cells were infected with Δm138-MCMV-FSH, and Δm138-MCMV-FSH-infected cells were incubated with serial dilutions of serum for 0.5 h at 37°CSubsequently, the mouse thymoma cell line (BW5147) stably expressing individual mFcγ receptor chimeras were added on fibroblasts infected Δm138-MCMV-FSH. The cells were incubated for 16 h at 37°C. Secretion of mIL-2 was quantified by ELISA. The measurements were done in triplicate. Dots and triangles show OD value of individual mice. (A) FcγRIII activation in of BALB/c mice. (B) FcγRII activation in of BALB/c mice. (C) FcγRI activation in of BALB/c mice. (D) FcγRIV activation in of BALB/c mice. All samples were measured in triplicate. Dots and triangles show OD value of individual mice. DL = detection limit. n = 4.


A
BW:mFcγRIII-ζ
1:40
1:80
1:160
OD_{450nm} IL-2 ELISA
0
0.5
1.0
1.5
3
7
14
21
3
7
14
21
immune
naive
Days p.i.
wt-MCMV
ΔM27-MCMV


B
BW:mFcyRI-ζ
1:100
1:200
1:400
2.0
1.5
1.0
0.5
0
OD450nm IL-2 ELISA
3
7
14
21
3
7
14
21
immune
naive
Days p.i.
wt-MCMV
ΔM27-MCMV

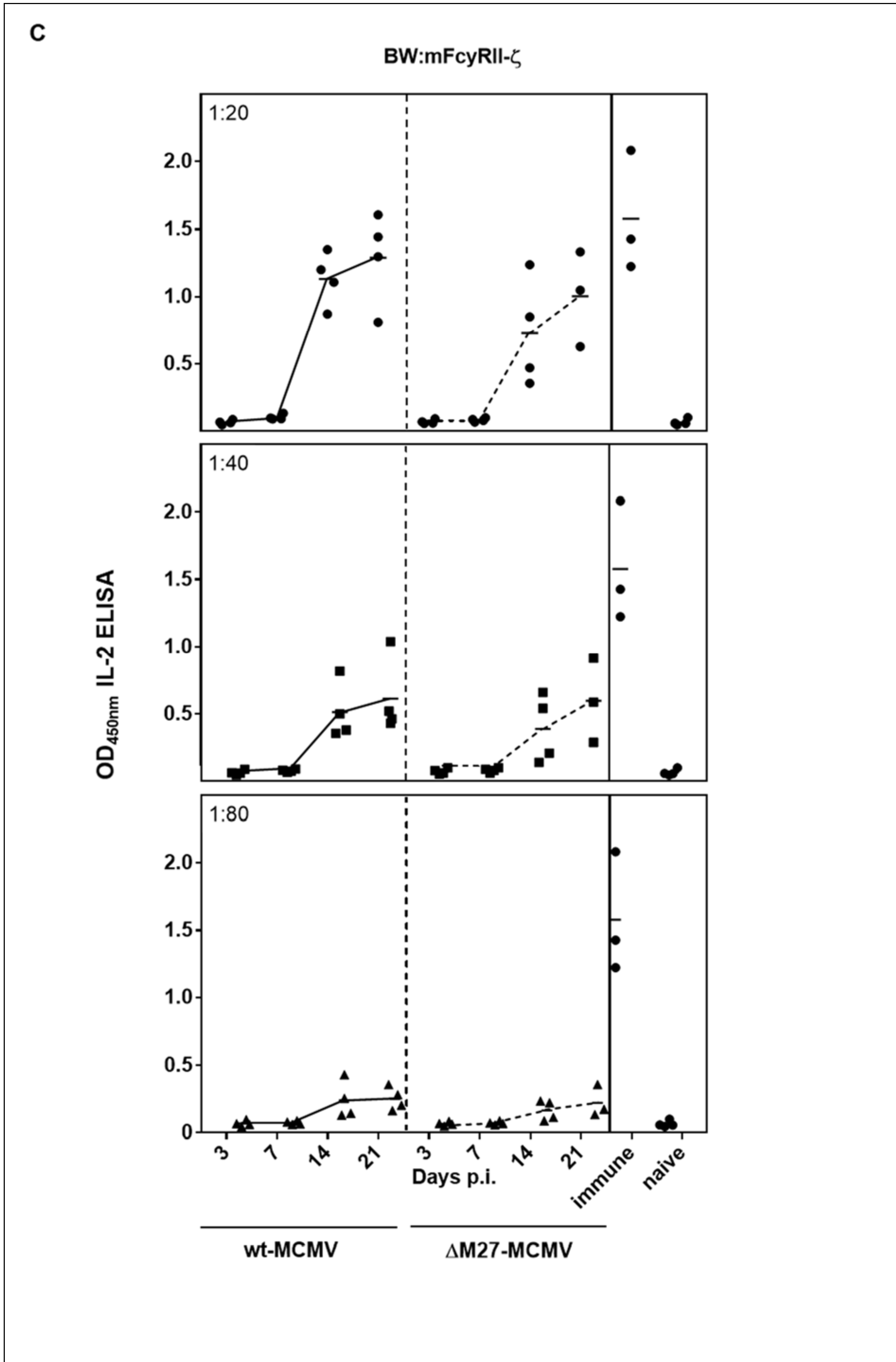

C
BW:mFcγRII-ζ
1:20
1:40
1:80
OD450nm IL-2 ELISA
2.0
1.5
1.0
0.5
0
3
7
14
21
immune
naive
Days p.i.
wt-MCMV
ΔM27-MCMV

Figure 17: Infection with ΔM27-MCMV elicits antibodies capable of dose-dependently activating FcγRIII, FcγII and FcγRI receptors in C57BL/6 mice

C57BL/6 mice were vaccinated with $2*10^5$ PFU of wt-MCMV or ΔM27-MCMV. A sera was collected 3, 7, 14 and 21 days post infection. CIM cells were infected with Δm138-MCMV-FSH, and Δm138-MCMV-FSH-infected cells were incubated with serial dilutions of serum for 0.5 h at 37°C. Subsequently, the mouse thymoma cell line (BW5147) stably expressing individual mFcy receptor chimeras were added on fibroblasts infected Δm138-MCMV. The cells were incubated for 16 h at 37°C. Secretion of mIL-2 was quantified by ELISA. The measurements were done in triplicate. Dots and triangles show OD value of individual mice. (A) FcyRIII activation in of C57BL/6 mice. (B) FcyRII activation in of C57BL/6 mice. (C) FcyRI activation in of C57BL/6 mice. All samples were measured in triplicate. Dots and triangles show OD value of individual mice. DL = detection limit. n = 4.

Grading serum concentrations induced FcγR-CD3ζ activation as indicated by mIL-2 production that was quantified by ELISA. For FcγRIII receptor a serum concentration of 1:40, 1:80, and 1:160 was used. For FcγRI receptor a serum concentration of 1:100, 1:200, and 1:400 was used. For FcγRII receptor a serum concentration of 1:20, 1:40, and 1:80 was used, respectively. The level of mIL-2 measured on time points when the sera were collected from infected BALB/c and C57BL/6 mice (3, 7, 14, and 21 days post infection) incline from 3 days post infection till 21 days post infection (Figure 19, and Figure 20). Also, the level of produced mIL-2 shows that the mIL-2 production path is the same for the same sera concentrations regardless of the virus (wt- and ΔM27-MCMV) and the mouse strain (BALB/c and C57BL/6) (Figure 19, and Figure 20). Therefore, the level of produced mIL-2 is the same for same sera concentrations, thereby confirming clear dose-dependent activation by IgG.

Thus, the wt- and ΔM27-MCMV raise similar FcγRIII [CD16], FcγRI [CD64], FcγRII [CD32] activating IgG responses in BALB/c and C57BL/6 mice. The wt- and ΔM27-MCMV raise FcγRIV activating IgG responses in BALB/c and C57BL/6 mice.

3.3. An MCMV mutant lacking the IFN antagonist pM27 induces MCMV-specific neutralization antibodies (nAbs)

The previous experiment confirmed that wt- and ΔM27-MCMV are capable to raise FcγR activating IgG immunoresponses in BALB/c and C57BL/6 mice. Besides of Fcγ- receptor activation, another IgG effector function is induction of neutralization antibodies. As IgG neutralizing antibodies are found 5 to 7 days post infection with MCMV and maximal IgG

neutralization titres are reached by 20 days p.i. (Lawson et al., 1988), we wanted to test if sera collected from BALB/c mice previously infected with wt- and ΔM27-MCMV can induce neutralization antibodies.

In order to test neutralization potential of sera collected from BALB/c mice 3, 7, 14, and 21 days post infection, a luciferase-expressing reporter MCMV (Δm157-MCMV:Luc) was used. Δm157-MCMV-Luc is a MCMV (reporter-) virus expressing the luciferase gene derived from the firefly *Photinus pyralis* under the control of the *m157* promoter-enhancer (Trilling *et al.*, 2011). The gene *m157* is expressed with early expression kinetics (Triphathy *et al.*, 2006).

Sera collected from BALB/c mice were incubated with Δm157-MCMV:Luc for 1.5 hours on 37°C and afterwards mouse fibroblasts were infected.

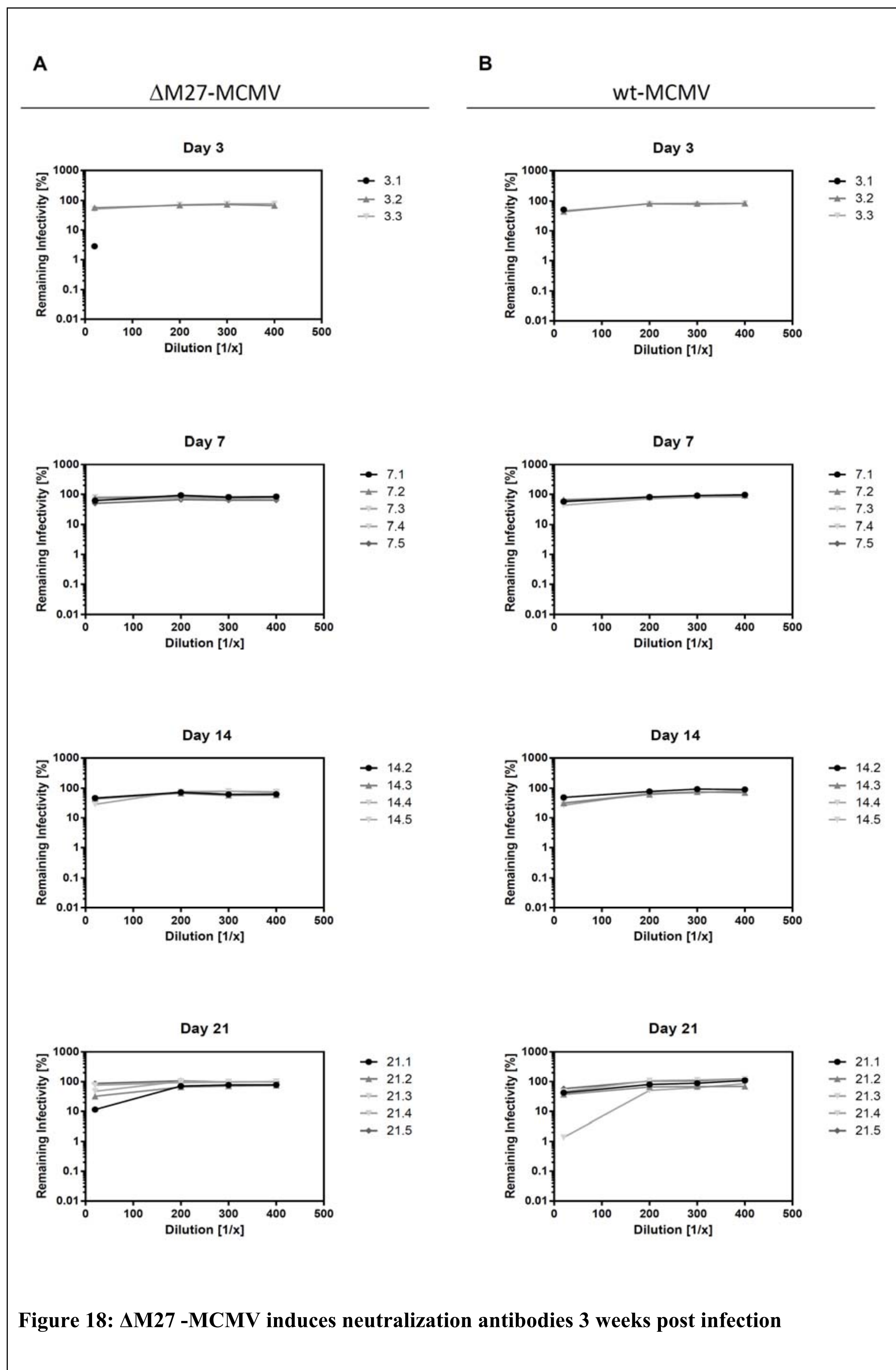


Figure 18: ΔM27 -MCMV induces neutralization antibodies 3 weeks post infection

BALB/c mice were infected i.p. with 2*10^5 PFU of MCMV:eGFP. At 3, 7, 14, and 21 days p.i., mice were sacrificed and sera was collected. A luciferase-expressing reporter MCMV (Δm157-MCMV:Luc) was incubated for 1.5 h at 37°C with serial dilutions of individual mouse sera (dilutions 1/20, 1/200, 1/300, and 1/400) prior to infection of fibroblasts. Infected cells were incubated for 1.5 days at 37°C. Lysates of infected cells were collected and luciferase activity was quantified.
All samples were measured in triplicate. Geometric shapes show neutralization titers of individual mice.

At day 3, 7, and 14 post infection, there are not significant differences in neutralization potential between different mice infected with ΔM27-MCMV and wt-MCMV (Figure 21 A & B). At the same timepoints, remaining infectivity was showed to be unchanged, presumably showing neutralization was not significant or not detectable. At 21 day post infection, BALB/c mice infected with ΔM27-MCMV are showing the lower remaining activity and neutralization if sera (collected from infected mice) together with Δm157-MCMV-Luc is applied on permissive fibroblasts (Figure 21 A). Only one individual mouse from groups infected with wt-MCMV has drastically diminished the remaining infectivity of Δm157-MCMV-Luc for more than 50%. At 21 days post infection with 1/20, 1/200, 1/300 and 1/400 sera dilutions, both ΔM27-MCMV and wt-MCMV induce specific antibodies capable of neutralization, however, induction of neutralization antibodies suggests they have not reached the neutralization maximum completely.

ΔM27-MCMV and wt-MCMV are capable to induce neutralization antibodies that diminished the remaining infectivity of Δm157-MCMV-Luc for more than 50% 21 day post infection (Figure 21 A & B). At this day post infection, the remaining activity was diminished for every individual BALB/c mouse per group not equally. Some of the BALB/c mouse infected with ΔM27-MCMV and wt-MCMV diminished its neutralization potential for 50% and some of them for less than 50% per group indicating individual pattern of producing neutralization antibodies. The previous experiment showed that BALB/c and C57BL/6 mice infected with wt- and ΔM27-MCMV are (individually per group) capable of inducing IgG that activates FcγRI, FcγRII, FcγRIII and FcγRIV responses 14 and 21 days post infection (Figure 15, 16, 17, and 18). If we compare this neutralization "pattern" with capability of ΔM27-MCMV and wt-MCMV to induce Fcγ receptor activation, a highly individual pattern can be noticed. Intriguingly, mice capable of inducing an IgG-specific activation of Fcγ receptors do not have to be capable inducing potent neutralization antibodies, indicating that the FcγR-mediated IgG responses cannot be extrapolated from ELISA.

3.4. Kinetics of MCMV:eGFP in resistant C57BL6 and susceptible BALB/c mice strain

In order to examine the course of MCMV infection and to compare the impact of viral infection on different genetic backgrounds, C57BL/6 and BALB/c mice were infected with enhanced green fluorescent protein (eGFP) expressing MCMV (MCMV:eGFP). The *eGFP* gene is placed under control of the native MCMV ie1/ie3 promoter as a means of visualizing directly sites of ie1 transcriptional activity (Henry *et al.*, 2000). This virus was initially described as derivative of the MCMV Smith strain deposited as VR-194 at the American Type Culture Collection (ATCC).

Le-Trilling and colleagues 2018 have shown that due to Ly49H receptor, MCMV replication in spleen and liver is largely impaired in BALB/c and C57BL/6 mice 3 days p.i., suggesting the the role of NK cells in control of MCMV replication at early time points (Le-Trilling *et al.*, 2018). To characterize the progress of acute MCMV infection after intraperitoneal (i. p.) inoculation, salivary glands of susceptible (BALB/c) and resistant (C57BL/6) strains of mice were collected at 3, 7, 14, and 21 days post infection. Salivary glands were snap frozen and viral titers from organ homogenates were determined by plaque titration as described in the Materials and methods, section 2.2.2.5.

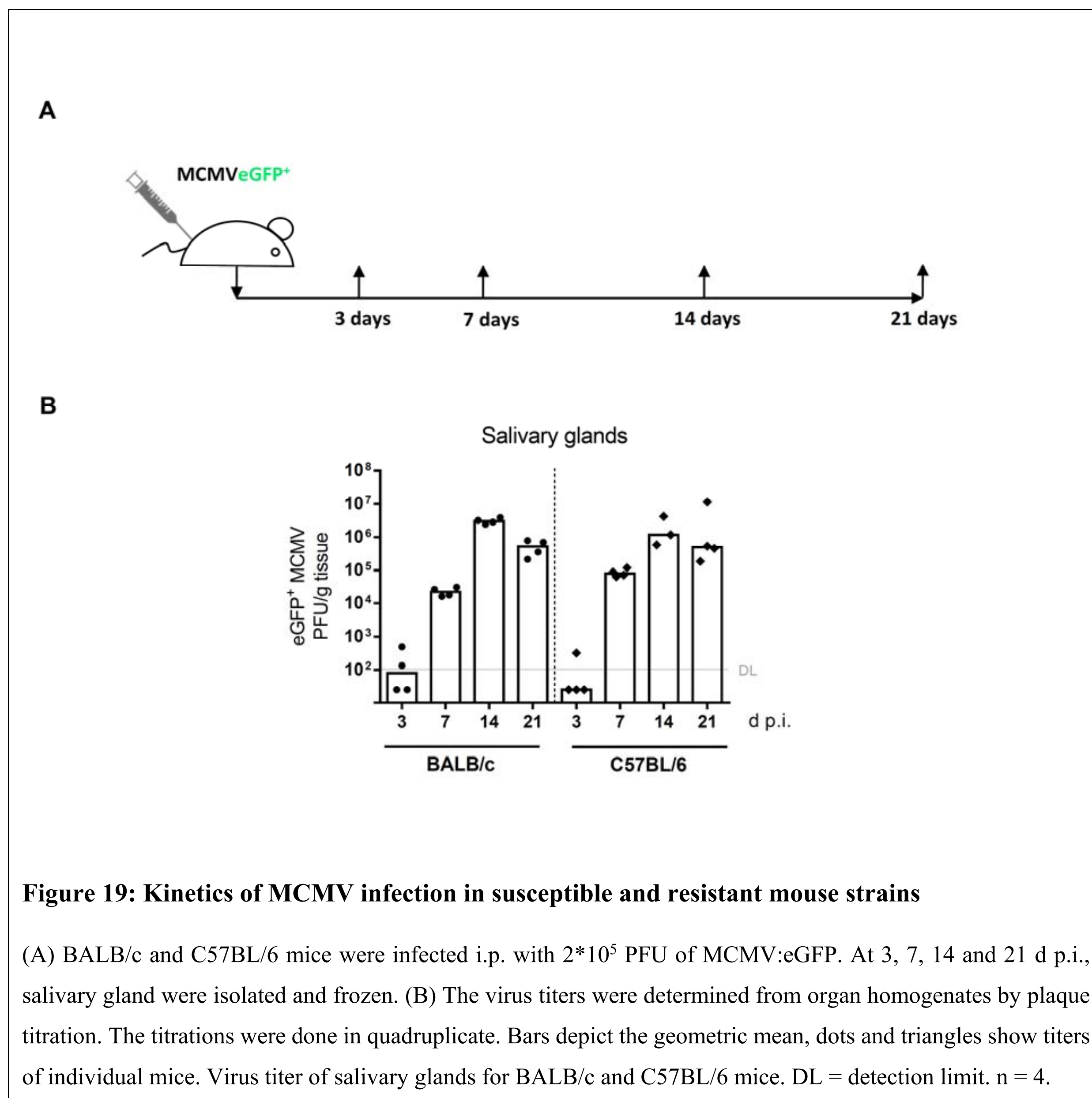


Figure 19: Kinetics of MCMV infection in susceptible and resistant mouse strains

(A) BALB/c and C57BL/6 mice were infected i.p. with $2*10^5$ PFU of MCMV:eGFP. At 3, 7, 14 and 21 d p.i., salivary gland were isolated and frozen. (B) The virus titers were determined from organ homogenates by plaque titration. The titrations were done in quadruplicate. Bars depict the geometric mean, dots and triangles show titers of individual mice. Virus titer of salivary glands for BALB/c and C57BL/6 mice. DL = detection limit. n = 4.

The kinetics of the infection in the resistant C57BL/6 mouse strain were similar to those observed in the susceptible BALB/c mouse strain (Figure 22). At day 3 and 14 post infection, the titer of the virus in salivary gland was 0.5-fold higher in the susceptible strain than in the resistant strain. Day 7 reveals 0.5 fold higher viral titer in mice of the resistant strain. Besides these slight differences probably caused by genetic differences in BALB/c and C57BL/6 strains, both strains showed a rather similar course of MCMV infection. This experiment confirmed possibility of using the susceptible BALB/c and the resistant C57BL/6 mouse strain in subsequent vaccination experiments. The highest viral titers have been achieved in C57BL/6 and BALB/c mice at 14 and 21 days p.i., still showing productive MCMV replication.

Therefore, the 21 day p.i. was defined as the optimal timepoint for salivary gland collection and titration in a subsequent vaccination experiments.

3.5. Vaccination with ΔM27-MCMV elicits immune responses that protect adult mice from challenge infections using another MCMV strain

MCMV mutants lacking the *M27* encoding gene are highly IFN susceptible *in vitro* (Zimmermann *et al.*, 2005, Trilling *et al.*, 2011, Le-Trilling *et al.*, 2018) and highly attenuated *in vivo* (Abenes *et al.*, 2001; Zimmermann *et al.*, 2005; Le-Trilling *et al.*, 2018). The IFN susceptibility and attenuation are direct consequences of the inability of ΔM27-MCMV to counteract STAT2-dependent IFN signalling. This is evident by experiments in cells and mice lacking STAT2, in which ΔM27-MCMV replication is restored and the attenuation reverted (Le-Trilling *et al.*, 2018). Despite the pronounced attenuation of ΔM27-MCMV, low but consistent virus replication is observed at 3 days post-infection in the spleen and the liver of C57BL/6, BALB/c, and 129 mice (Le-Trilling *et al.*, 2018). It was also shown that viral replication of a live attenuated MCMV vaccine is a prerequisite for the induction of protective immunity (Čicin-Šain *et al.*, 2007; Mohr *et al.*, 2008). So, we wished to address a question whether vaccination with an a live-attenuated MCMV mutant lacking a STAT2 antagonist (ΔM27-MCMV), results in establishment of systemic immune response by the attenuated virus under conditions which result in protective immunity.

To differentiate the primary and second virus inoculum and to challenge with a heterologous MCMV strain, we used the eGFP-expressing MCMV strain RVG102 generated by Henry and colleagues (Henry *et al.*, 2000). MCMV:eGFP represents a heterologous challenge virus suitable for experiments, in which mice were vaccinated with wt- and ΔM27-MCMV of Smith strain background.

To test whether the immune response induced by the ΔM27-MCMV infection is sufficient to protect the host from challenge infection, 6 - 8 weeks old BALB/c and C57BL/6 mice were injected (i. p.) with $2*10^5$ PFU of ΔM27-MCMV, UV-inactivated wt-MCMV or wt-MCMV 21 and 6 weeks prior to challenge with MCMV:eGFP. At 21 days post challenge infection, mice were sacrificed and salivary glands were collected. Viral titers in the tissue homogenates were determined by standard plaque assays on primary MEFs (Materials and methods, 2.2.2.5.).

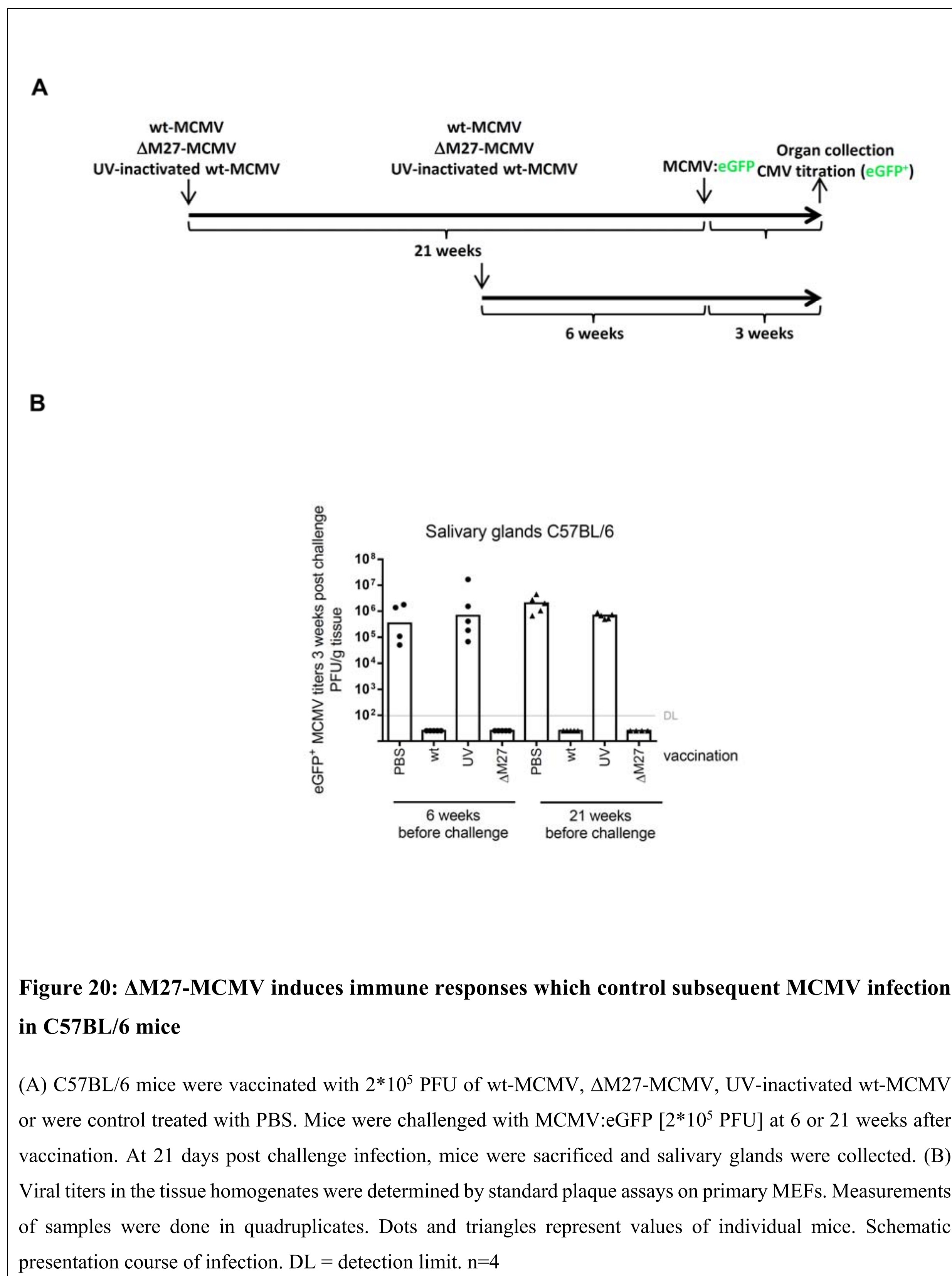


Figure 20: ΔM27-MCMV induces immune responses which control subsequent MCMV infection in C57BL/6 mice

(A) C57BL/6 mice were vaccinated with $2*10^5$ PFU of wt-MCMV, ΔM27-MCMV, UV-inactivated wt-MCMV or were control treated with PBS. Mice were challenged with MCMV:eGFP [$2*10^5$ PFU] at 6 or 21 weeks after vaccination. At 21 days post challenge infection, mice were sacrificed and salivary glands were collected. (B) Viral titers in the tissue homogenates were determined by standard plaque assays on primary MEFs. Measurements of samples were done in quadruplicates. Dots and triangles represent values of individual mice. Schematic presentation course of infection. DL = detection limit. n=4

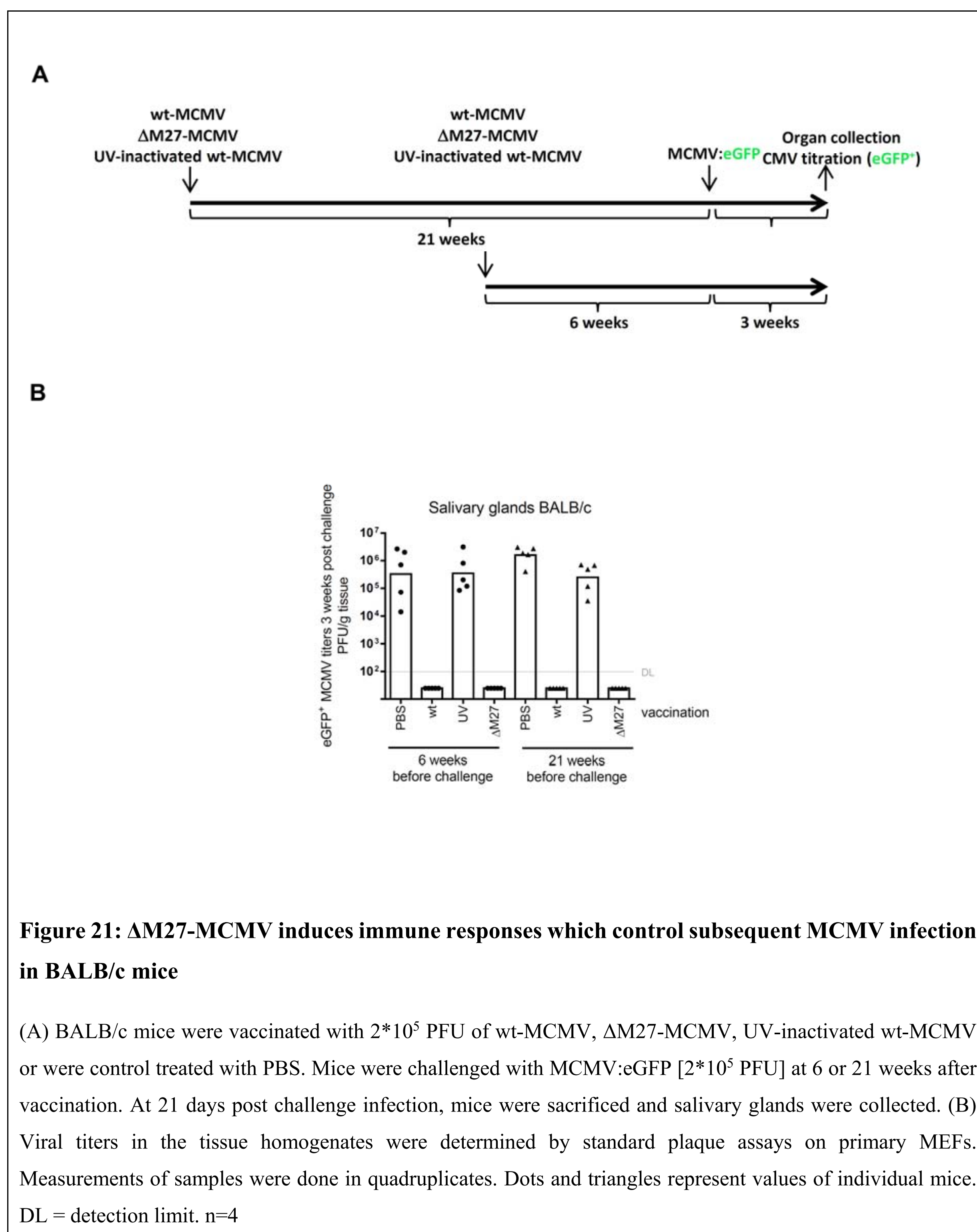


Figure 21: ΔM27-MCMV induces immune responses which control subsequent MCMV infection in BALB/c mice

(A) BALB/c mice were vaccinated with $2*10^5$ PFU of wt-MCMV, ΔM27-MCMV, UV-inactivated wt-MCMV or were control treated with PBS. Mice were challenged with MCMV:eGFP [$2*10^5$ PFU] at 6 or 21 weeks after vaccination. At 21 days post challenge infection, mice were sacrificed and salivary glands were collected. (B) Viral titers in the tissue homogenates were determined by standard plaque assays on primary MEFs. Measurements of samples were done in quadruplicates. Dots and triangles represent values of individual mice. DL = detection limit. n=4

While naive mice failed to control the infection, all of the mice immunized with ΔM27-MCMV, similar to the mice infected with wt-MCMV, showed capability for supressing viral titer of challenge infection with MCMV:eGFP (Figure 23 & 24). Notably, mice immunized with ΔM27-MCMV resisted the challenge infection with $2*10^5$ PFU of the MCMV:eGFP as wt-MCMV-infected mice, suggesting that the MCMV deletion mutant lacking interferon

antagonist (ΔM27-MCMV) provides an innate immune stimuli that influence the effectiveness of the adaptive immune response. UV-inactivated wt-MCMV showed an incapability to establish protection upon MCMV:eGFP challenge infection confirming that a low level of replication is necessary for establishing the protection from subsequent challenge infections. Ly49H-positive (C57BL/6) (Figure 24) and Ly49H-negative (BALB/c) (Figure 23) mouse strains infected with ΔM27-MCMV, and after 6 or 21 weeks challenged with MCMV:eGFP, established no difference in salivary gland viral titers showing no influence on protection of a soluble viral m157 protein binding to Ly49H. In addition, protection of ΔM27-MCMV has been successfully tested 21 weeks post primary infection, showing its capability to provide protection against infected mice. Taken together, the results indicate that immunization with ΔM27-MCMV induced an immune response that conferred protection against subsequent MCMV infection.

3.5.1. Analysis of humoral immune responses before and after challenge infection

3.5.1.1. Impact of nAbs on protection of ΔM27-MCMV against subsequent MCMV infections

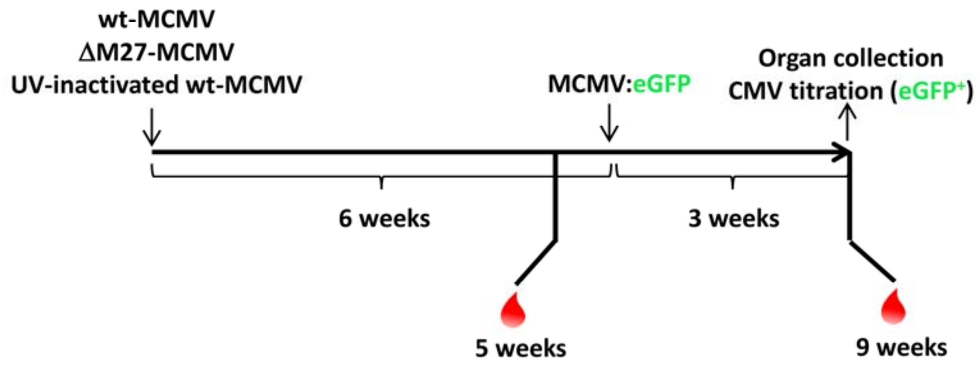
During primary MCMV infection, there is no detectable physiological role for generated antibodies regarding the organ clearance and the prevention of horizontal transmission. During recurrence, the presence of neutralizing antibody has a significant effect on virus spread, which proves the role of antibodies in limiting the spread of virus after focal recurrence (Reddehase *et al.*, 1994).

Neutralizing antibodies are crucial for protection against reinfection with viruses (Harada *et al.*, 2003; Seiler *et al.*, 1998, Graham *et al.*, 1991; Ahmed *et al.*, 1996). In a mouse model, Hebeis and colleagues have shown that virus-specific B cells, adoptively transferred into immunodeficient hosts can be stimulated to antibody production by antigen alone, without requiring T cell help (Hebeis *et al.*, 2004). Also, Klenovšek and colleagues 2007 have shown that transfer of serum from MCMV-immune animals has a comparably protective effect against MCMV infection as transfer of memory B cells, indicating that the production of IgG might represent the protective principle. Bongard and colleagues 2019 have shown that the protection conferred by a MCMV vector encoding F-MuLV envelope relies on the ability of mice to mount a neutralizing antibody response showed only upon prolonged challenge FV infection, and not

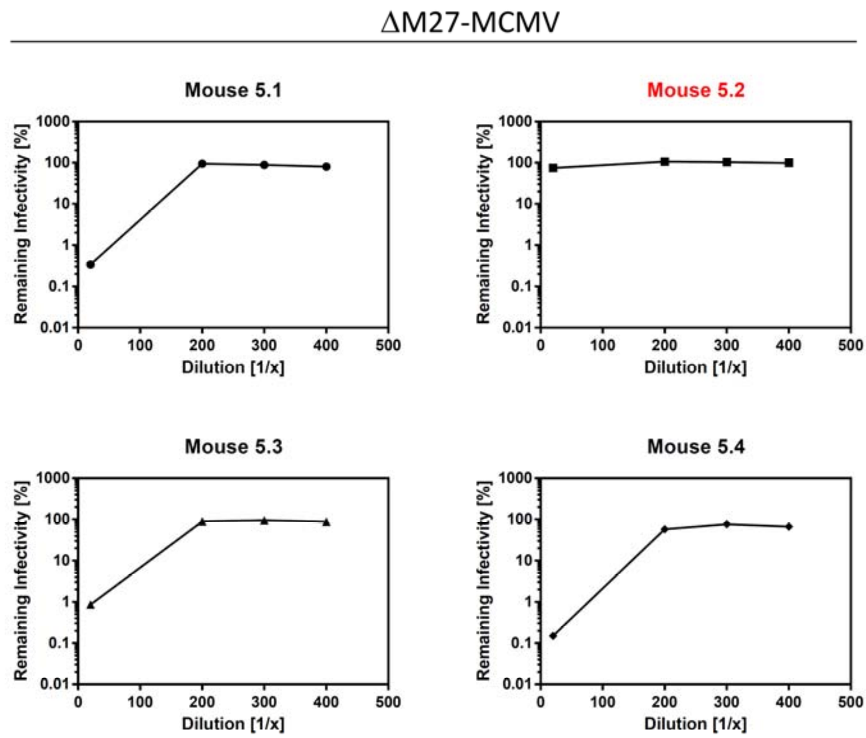
upon vaccination. These anamnestic antibody responses need a long time period for the maturation of the antibodies, and are crucial for the MCMV.env mediated protection (Bongard *et al.*, 2019).

To examine if antibodies induced by infection of BALB/c mice with ΔM27-MCMV represent the protective principle towards subsequent MCMV infection, a neutralization responses have been determined. BALB/c mice were vaccinated with $2*10^5$ PFU of wt-MCMV, ΔM27-MCMV, and UV-inactivated wt-MCMV or were control treated with PBS. Sera were collected 5 weeks post infection. Mice were challenged with MCMV:eGFP [$2*10^5$ PFU] 6 weeks after vaccination.

A



B



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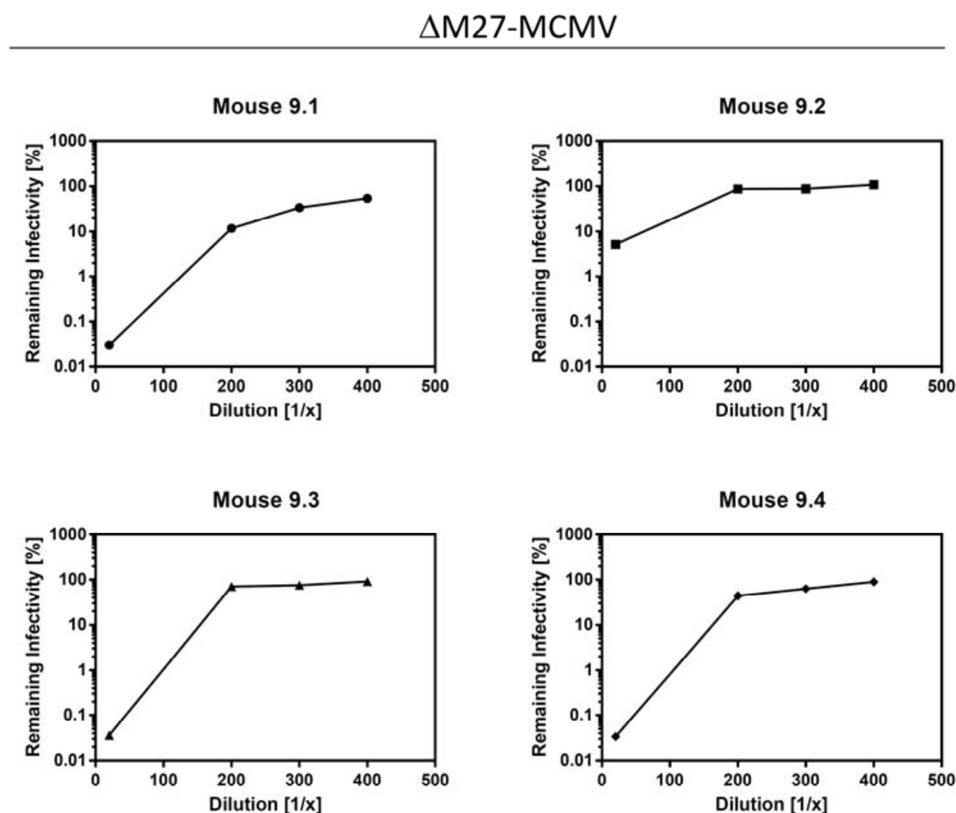

Figure 22: ΔM27-MCMV elicits neutralization antibodies comparable with wt-MCMV 5 weeks post vaccination and 3 weeks post challenge infection

BALB/c mice were vaccinated with $2*10^5$ PFU of wt-MCMV, ΔM27-MCMV, UV-inactivated wt-MCMV or were control treated with PBS. Sera were collected 5 weeks post infection. Mice were challenged with MCMV:eGFP ($2*10^5$ PFU) at 6 weeks after vaccination. At 21 days post challenge infection, mice were sacrificed and sera were collected. A luciferase-expressing reporter MCMV (Δm157-MCMV-luc) was incubated for 1.5 h at 37°C with serial dilutions of individual mouse sera (dilutions 1/20, 1/200, 1/300, 1/400). Subsequently, mouse fibroblasts were infected with virus-antibody mixture. Infected cells were incubated for 1.5 days at 37C. Lysates of infected cells were collected and luciferase activity was quantified. A) Schematic presentation blood collection at time points. (B) ΔM27-MCMV neutralization capacity 5 weeks post infection in BALB/c mice. (C) ΔM27-MCMV neutralization capacity 6 weeks post infection and 3 weeks post challenge infection in BALB/c mice. Experiments have been repeated for 3 times. All samples were measured in triplicate. Geometric shapes show neutralization titers of individual mice. DL = detection limit. n = 4.

We compared the neutralizing antibody responses to ΔM27-MCMV 5 weeks post immunization and 3 weeks post challenge infection (BALB/c mice were challenged 6 weeks

post immunization and blood was taken 3 weeks after challenge). Serial dilutions of sera were mixed with a luciferase-expressing Δm157-MCMV-Luc prior to infection of CIM cells. The reduction of the luciferase signal reflected the neutralizing capacity of the antisera. Immunization of BALB/c mice with ΔM27-MCMV induced amount of neutralizing antibodies comparable with wt-MCMV immunization (Figure 25) but in highly individual manner. Three of four BALB/c mice infected with ΔM27-MCMV were capable to induce neutralization antibodies, only one BALB/c mice was characterized as "weak" neutralizer. Three weeks upon challenge infection, the same BALB/c mouse vaccinated with ΔM27-MCMV was not capable to induce neutralization antibodies although it was protected from subsequent infection. Therefore, ΔM27-MCMV induces neutralization antibodies that might have influence on protection from subsequent infections; however, neutralization antibodies seem not to crucial in establishing long-term protection. Since all mice were fully protected in the very same experiment including the mice lacking detectable nAbs, our results suggest that protection can be achieved even if pre-existing nAbs are below the level of detection in *in vitro* assays. Immunization with UV-irradiated wt-MCMV abolished the induction of neutralizing antibodies (data not shown), confirming the virus must be fully infective in order to induce the protective immune responses.

3.5.1.2. ΔM27-MCMV elicits antibodies capable of activating FcγRIII and FcγRIV receptors

To determine levels of activation of Fcγ receptors induced by IgG upon infection of BALB/c mice with ΔM27-MCMV, blood was taken 5 weeks post immunization and 3 weeks post challenge infection (mice were challenged 6 weeks post immunization). The assay used for testing individual IgG-dependent activation of FcγRs is based on co-cultivation of antigen-bearing cells with BW5147 reporter cells stably expressing chimeric FcγR-ζ chain receptors, stimulating mouse IL-2 production in the presence of immune IgG, provided that the opsonizing IgG is able to activate the particular FcγR (Corrales-Aguilar *et al.*, 2013). The recent studies have demonstrated that improved ADCC capacity is related to the lack the core fucose of the IgG-Fc glycan which enhances FcγRIII and FcγRIV activity (Oosterhof *et al.*, 2022). The low affinity FcγRIII/CD16 is highly relevant for protective immune responses like ADCC (Lanier, 1998). In mice, FcγRIV is also crucial for several important IgG-mediated immune responses like in IgG2a- and IgG2b-dependent killing of B cells (Nimmerjahn *et al.*, 2010). Since

afucosylated IgG responses are important in preventing or improving the vaccination (Pereira, 2018), and CMV infection stimulates very strong afucosylated IgG responses (Larsen *et al.*, 2021), the capability of ΔM27-MCMV to activate Fcγ receptors induced by IgG before, and after the challenge infection was analysed. IgG-dependent activation of individual BW:FcγR-ζ reporter transfectants was performed by incubating mock and Δm138-MCMV-FSH-infected cells with serial twofold dilutions of mouse sera in DMEM 10 % (v/v) FCS for 30 min at 37 °C in an atmosphere of 5 % CO_2.

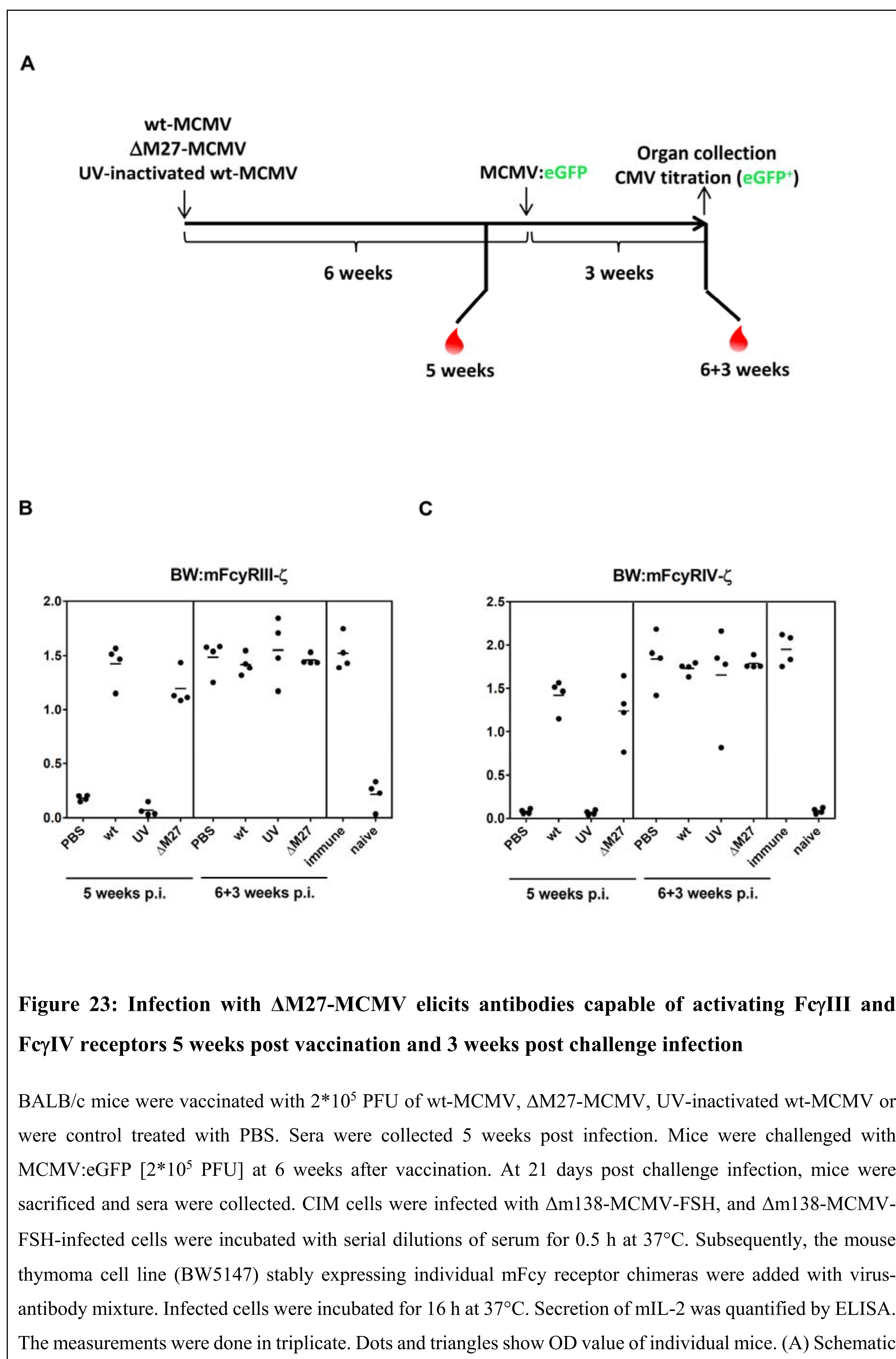

Figure 23: Infection with ΔM27-MCMV elicits antibodies capable of activating FcγIII and FcγIV receptors 5 weeks post vaccination and 3 weeks post challenge infection

BALB/c mice were vaccinated with $2*10^5$ PFU of wt-MCMV, ΔM27-MCMV, UV-inactivated wt-MCMV or were control treated with PBS. Sera were collected 5 weeks post infection. Mice were challenged with MCMV:eGFP [$2*10^5$ PFU] at 6 weeks after vaccination. At 21 days post challenge infection, mice were sacrificed and sera were collected. CIM cells were infected with Δm138-MCMV-FSH, and Δm138-MCMV-FSH-infected cells were incubated with serial dilutions of serum for 0.5 h at 37°C. Subsequently, the mouse thymoma cell line (BW5147) stably expressing individual mFcy receptor chimeras were added with virus-antibody mixture. Infected cells were incubated for 16 h at 37°C. Secretion of mIL-2 was quantified by ELISA. The measurements were done in triplicate. Dots and triangles show OD value of individual mice. (A) Schematic

presentation blood collection at timepoints. (B) FcγRIII activation in of BALB/c mice. (C) FcγRIV activation in BALB/c mice.
All samples were measured in triplicate. Dots and triangles show OD value of individual mice. DL = detection limit. n = 4.

Infection with ΔM27-MCMV elicits antibodies capable of activating FcγRIII and FcγRIV receptors 5 weeks post infection (Figure 26). As expected, IgG did not induce receptor activation (mIL-2 response) in the presence of PBS-infected mice 5 weeks post infection. Also, sera collected from BALB/c mice infected with wt-MCMV elicited efficient IgG response leading to FcγRIII and FcγRIV activation. While antigens of ΔM27-MCMV efficiently triggered FcγRIII and FcγRIV reporter cells, sera collected from BALB/c mice 5 weeks post infection infected with UV-inactivated wt-MCMV elicited very poor if any responses (Figure 26 B), confirming that lack of virus replication leads to reduction of MCMV-specific ELISA-reactive IgG responses and therefore FcγR activation. Thus, a challenge infection with MCMV:eGFP also established the conditions of vaccination to elicit immune IgG capable of inducing FcγRIII and FcγRIV immune receptor activation. FcγRIII and FcγRIV receptor showed comparable IgG immune responses upon vaccination with ΔM27-MCMV, confirming ΔM27-MCMV is sufficient to efficiently trigger adaptive immune response capable of establishing protection against subsequent viral infection.

3.6. Precursors that have influence of MCMV course of infection are dependent of dose of infection

3.6.1. ΔM27-MCMV vaccination is dose independent

MCMV infectious dose applied experimentally (10^2 - 10^6 PFU of MCMV) determines the total number of cytotoxic T- lymphocytes () generated, and is explained by the slow replication cycle of the virus (Reddehase *et al.*, 1994). The number of MCMV-specific IgG secreting long-lived plasma cells and consequently the magnitude of the antibody response are also dependent on the MCMV inoculum dose (Welten *et al.*, 2013), presumably having influence on vaccination responses.

Therefore, we wanted to examine do different doses of infection have an influence on ΔM27-MCMV vaccination.

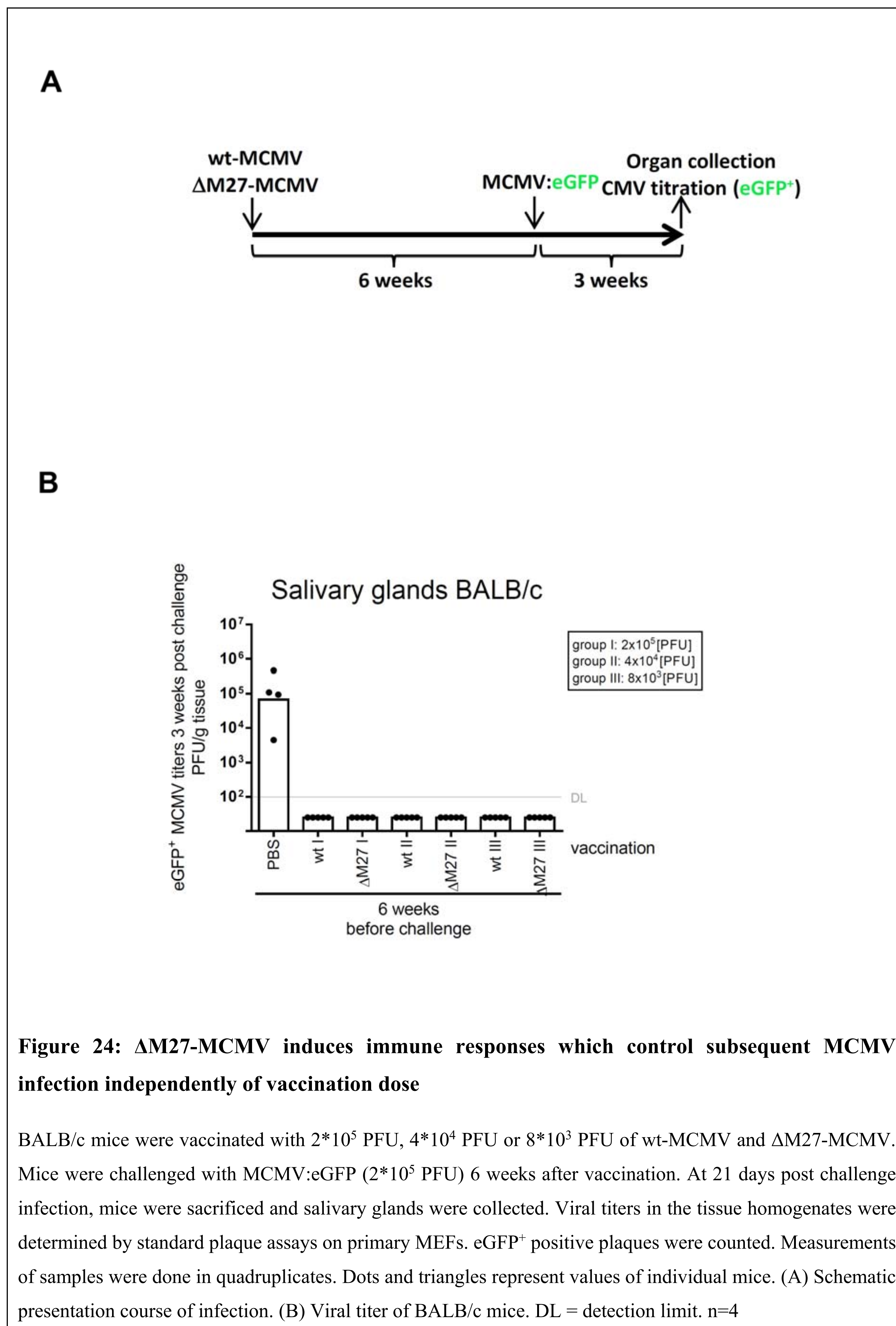

Figure 24: ΔM27-MCMV induces immune responses which control subsequent MCMV infection independently of vaccination dose

BALB/c mice were vaccinated with $2*10^5$ PFU, $4*10^4$ PFU or $8*10^3$ PFU of wt-MCMV and ΔM27-MCMV. Mice were challenged with MCMV:eGFP ($2*10^5$ PFU) 6 weeks after vaccination. At 21 days post challenge infection, mice were sacrificed and salivary glands were collected. Viral titers in the tissue homogenates were determined by standard plaque assays on primary MEFs. $eGFP^+$ positive plaques were counted. Measurements of samples were done in quadruplicates. Dots and triangles represent values of individual mice. (A) Schematic presentation course of infection. (B) Viral titer of BALB/c mice. DL = detection limit. n=4

ΔM27-MCMV vaccination is dose independent. Eventhough infection with dose of $2*10^5$ PFU of ΔM27-MCMV results in low ΔM27-MCMV replication in spleen and liver of BALB/c mice 3 days post infection, at 21 days post infection there is no detectable ΔM27-MCMV replication in organs (Le-Trilling *et al.*, 2018). This prompted us to use $2*10^5$ PFU of ΔM27-MCMV per vaccination. To determine if even lower doses of ΔM27-MCMV would be effective, we examined the effects of intraperitoneal vaccination with $4*10^4$ PFU or $8*10^3$ PFU. Six weeks after immunization, BALB/c mice were challenged with $2*10^5$ PFU of MCMV:eGFP, with the *eGFP* gene under control of the native MCMV ie1/ie3 promoter. Irrespective of a dose, salivary gland viral titers of all BALB/c mice immunized with ΔM27-MCMV have been below detection limit (detection limit was found to be 10^2 PFU). Thus, with vaccination with 100-fold less of ΔM27-MCMV, all immunized mice were protected (Figure 27).

In summary, ΔM27-MCMV was able to efficiently protect immunocompetent mice against challenge with MCMV:eGFP after vaccination with a single dose. Remarkably, vaccination with ΔM27-MCMV was as efficient as vaccination for long-term protection.

3.6.2. ΔM27-MCMV provided full protection against homotypic challenge

Safety is also a requirement for a useful vaccine vector (Redwood *et al.*, 2002). CMVs are typically non-pathogenic in immunocompetent hosts, making them potentially safer than other vectors such as lentiviruses. However, for use in humans, it may be important to produce vaccine vectors that are attenuated because of the number of immunocompromised individuals in the population (Redwood *et al.*, 2005).

Hence, we wanted to examine do ΔM27-MCMV itself indulges the safety criterion.

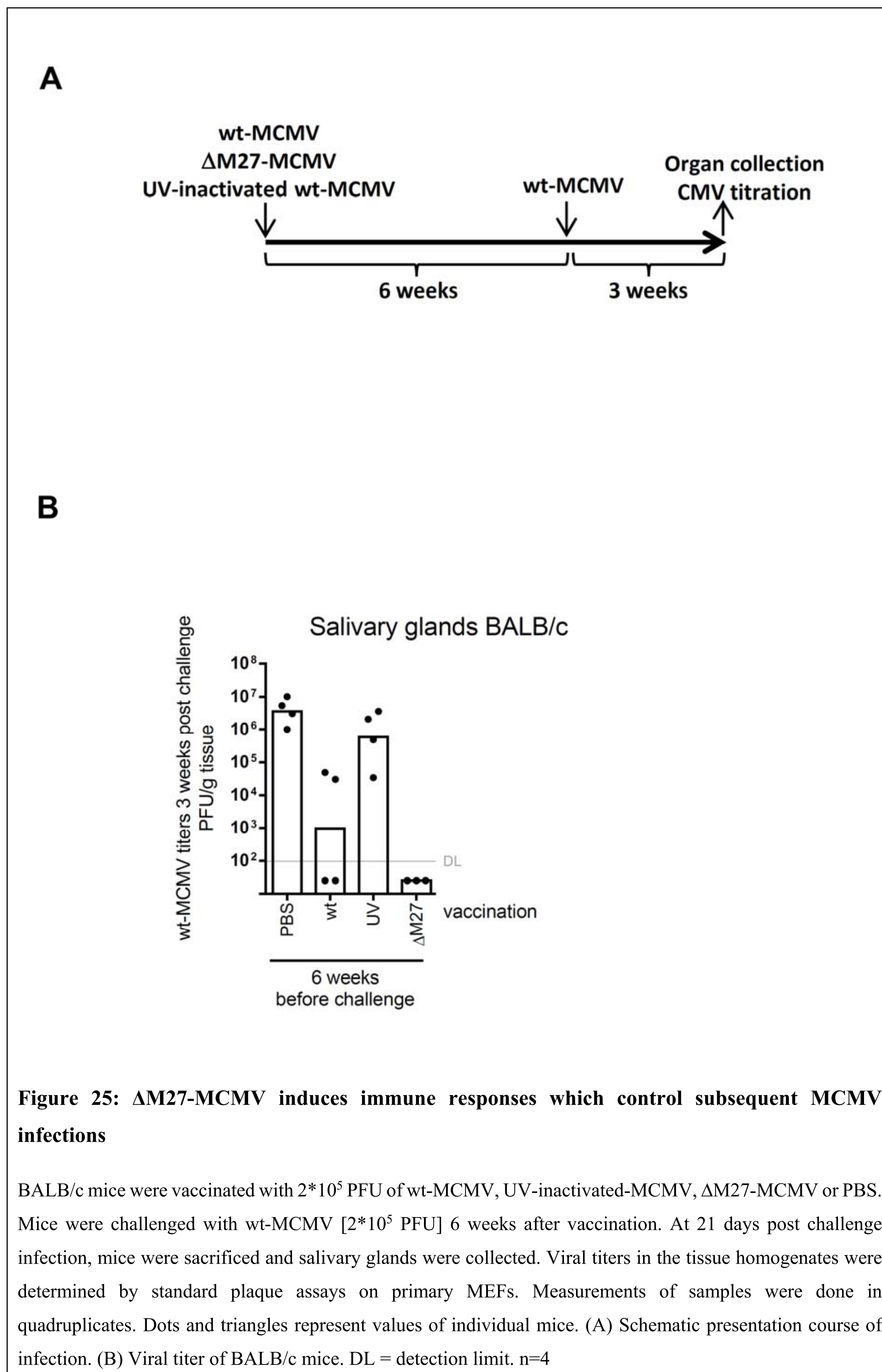

Figure 25: ΔM27-MCMV induces immune responses which control subsequent MCMV infections

BALB/c mice were vaccinated with $2*10^5$ PFU of wt-MCMV, UV-inactivated-MCMV, ΔM27-MCMV or PBS. Mice were challenged with wt-MCMV [$2*10^5$ PFU] 6 weeks after vaccination. At 21 days post challenge infection, mice were sacrificed and salivary glands were collected. Viral titers in the tissue homogenates were determined by standard plaque assays on primary MEFs. Measurements of samples were done in quadruplicates. Dots and triangles represent values of individual mice. (A) Schematic presentation course of infection. (B) Viral titer of BALB/c mice. DL = detection limit. n=4

As described above, all our vaccination experiments were performed using Smith/K181 MCMV:eGFP for the challenge. The *eGFP* gene was placed under control of the native MCMV ie1/ie3 promoter as a means of visualizing directly sites of ie1 transcriptional activity in acute infection (Henry *et al.*, 2000; Reddehase *et al.*, 2002). To exclude that this leads to exaggerated protection, e.g., due to an attenuation caused by introduction of the *eGFP* gene, the existence of foreign antigens, or the mosaic genome, we repeated the experiment by challenging with the autologous Smith strain-derived wt-MCMV (Messerle *et al.*, 1997; Wagner *et al.*, 1999). ΔM27-MCMV provided full protection against this homotypic challenge confirming that replacing the *ie2* by a reporter gene in Smith/K181 MCMV has no influence on result of vaccination.

4. Discussion

We analysed the potential of an MCMV mutant, ΔM27-MCMV, lacking its STAT2 antagonists to serve as live attenuated vaccine in mice. Infections with ΔM27-MCMV confirmed its capability for inducing ELISA IgG responses in terms of IgG recognizing MCMV-encoded proteins present in infected cells. Also, ΔM27-MCMV induced strong IgG responses in terms of IgG recognizing MCMV virions. The ΔM27-MCMV was capable to raise FcγRIII [CD16], FcγRI [CD64], FcγRII [CD32] and FcγRIV activating IgG responses in BALB/c and C57BL/6 mice. Infections with ΔM27-MCMV induced neutralizing antibodies.

BALB/c and C57BL/6 mice immunized with ΔM27-MCMV resisted the challenge infection with the MCMV:eGFP (21 and 6 weeks post infection), resulting in protection from subsequent challenge infections. The ΔM27-MCMV vaccination is repeated, challenged with wt-MCMV and observed full protection of ΔM27-MCMV-immunized mice against wt-MCMV challenge, showing that an attenuation is not caused by introduction of the *eGFP* gene. ΔM27-MCMV induces neutralization antibodies 5 weeks post immunization and 3 weeks post challenge infection, and antibodies that elicits ΔM27-MCMV are capable of activating FcγRIII and FcγRIV receptors 5 weeks post infection. Irrespective of the infection dose, all BALB/c mice immunized with ΔM27-MCMV were protected. Remarkably, ΔM27-MCMV was able to efficiently protect immunocompetent mice after vaccination with a single dose and vaccination was efficient for long-term protection.

4.1. Humoral immune responses induced by ΔM27-MCMV

It has been confirmed that during the acute phase of primary infection MCMV induces polyclonal B cell activation in mice. Flow cytometric analyses revealed that the surface expression of CD45R, IgM, and IgK by splenocytes from MCMV-infected mice was significantly reduced but the frequency of surface IgG-expressing cells increased. Also, ELISpot assays confirmed that the changes revealed by flow cytometry were paralleled by increases in the numbers of IgG-producing cells, especially those secreting IgG2a. Production of this IgG subclass is strongly stimulated by IFN-γ (Karupiah *et al.*, 1998). One of the MCMV-encoded IFN antagonists was previously identified as pM27 (Zimmermann *et al.*, 2005; Trilling

et al., 2011). We found that ΔM27-MCMV is capable of inducing IgG responses recognizing MCMV-encoded proteins present in infected cells. These responses were similar of those of wt-MCMV-induced IgG responses. Previously, MCMV virus titres were shown to influence the amount of IgG antibody detected by the ELISA as seen at day 10 p.i. with susceptible BALB/c mice (Lawson *et al.*, 1988). The ΔM27-MCMV and wt-MCMV-induced MCMV-specific IgG responses reaching their maximum 14 days post infection with high titers persisting further (21 days post infection) confirming previously observed results. In our hands UV-inactivated MCMV elicited antibody of the IgG classes but the titre was always less than that observed with ΔM27-MCMV and wt-MCMV salivary gland-derived viruses. Hence, the virus must be fully infectious in order to induce high antibody titres (Lawson *et al.*, 1988). In our experiments, the wt-MCMV reached a maximum of MCMV-specific IgG responses earlier than ΔM27-MCMV and ΔM27-MCMV induced MCMV-specific IgG responses in 3 of C57BL/6 infected mice at day 7 post infection. Although viral titer has an influence on producing MCMV-specific IgG responses, the genetic background of infected mice has an influence on the resistance of mice to MCMV We have shown that ΔM27-MCMV, inducing MCMV-specific IgG, faster reaches the maximum in C57BL/6 mice compared to BALB/c. Since, Lawson *et al.*, 1988 showed IgG levels of susceptible BALB. B and resistant B10.BR mice were found to correlate inversely with resistance status of mice till 20 days after infection, our finding suggest that ΔM27-MCMV induces MCMV-specific IgG differently in different mice strains presumably due to its difference in genetic background.

The *M27*-encoded, 79-kD protein blocked both IFN-α/β and IFN-γ responses via STAT2 and has a huge impact on viral fitness *in vivo*. The degradation of the STAT2 protein by pM27 is executed via the ubiquitin-proteasome pathway of protein degradation (Trilling *et al.*, 2011, Landsberg *et al.*, 2018). Although it was not found in a systematic search for MCMV particle proteins (Kattenhorn *et al.*, 2004), pM27 appeared to be present in the virion since it could be detected in purified supernatant MCMV particle preparations (Prof. Trilling, personal communication). In ELISAs using MCMV virions as antigen, we have shown that ΔM27-MCMV and wt-MCMV induced comparable IgG responses in terms of IgG recognizing MCMV virions. At the day 14 post infection, in our experiments, 2 mice infected with ΔM27-MCMV have stimulated maximum of IgG responses in terms of IgG recognizing virions (induced by infection of BALB/c and C57BL/6 mice with ΔM27-MCMV and wt-MCMV). Fourteen days post infection of BALB/c and C57BL/6 mice with wt-MCMV at same conditions, 5 infected mice have reached the maximum of IgG responses in terms of IgG recognizing virions. We have shown, for the first time, the MCMV-specific IgG responses

induced by ΔM27-MCMV and wt-MCMV show similar phenotypes regardless of the infected mouse strain.

UV-inactivated MCMV elicited MCMV-specific ELISA-reactive responses of the IgG classes, but less than with virulent salivary gland-derived wt-MCMV. Quinnan and colleagues have compared MCMV viral titers and IgG levels in MCMV-infected mice. MCMV titers in spleens reach a maximum within 4 days and then gradually declined within 3 weeks. Conversely, salivary gland virus titers continue to rise for 2-3 weeks after infection before they start to decrease (Quinnan *et al.*, 1979). Neutralizing antibodies in serum were first detected 21 days after infection. IgG levels rose by day 14, remained high until day 30 and then declined (Price *et al.*, 1992). For persistent infection, ΔM27-MCMV and wt-MCMV mounted very strong MCMV-specific ELISA-reactive IgG responses 5 and 20 weeks after infection of BALB/c mice. Hence, the virus must be fully infectious in order to induce high MCMV-specific IgG responses. The lower MCMV-specific ELISA-reactive IgG responses produced in mice immunized with UV-inactivated virus probably reflects a lack of virus replication and is consistent with results obtained with other viruses (Rosenberg & Notkins, 1974).

4.2. ΔM27-MCMV induces MCMV-specific IgG responses capable of activating Fcγ-receptors

First, antibody Fc-mediated effector systems can affect the antibody activity against free virus particles by activating the complement cascade. The activation of complement by antibodies that are bound to virus particles can lead directly to virolysis (Burton, 2002), and/or phagocytosis followed by inactivation of the virion (Burton, 2002). To exclude influence of the complement on our assays, we preheated the sera on 56°C. Second, Fc-mediated effector systems can lead to cell lysis or clearance by antibody-dependent cellular cytotoxicity (ADCC). The mediators of ADCC are Fcγ receptors which are cell surface receptors specific for the different antibody Fc fragments (Nimmerjahn & Ravetch, 2010). By applying a previously described test principle (Corrales-Aguilar *et al.*, 2016; Corrales-Aguilar *et al.*, 2013; Van den Hoecke *et al.*, 2017), we showed the ability to activate the murine Fcγ receptors with the MCMV-specific IgG raised upon immunization with ΔM27-MCMV. The wt- and ΔM27-MCMV raise similar FcγRIII [CD16], FcγRI [CD64], FcγRII [CD32]-activating IgG responses in BALB/c and C57BL/6 mice. FcγRI, FcγRII and FcγRIII have reached their maximum level

of IgG activation upon infection with wt- and ΔM27-MCMV at 14 and 21 days post infection, respectively. Also, the wt- and ΔM27-MCMV raise FcγRIV-activating IgG responses in BALB/c and C57BL/6 mice. Mice express two low-affinity activating FcγRs, FcγRIII and FcγRIV on myeloid cells and dendritic cells, as well as the low-affinity inhibitory FcγRIIB, which is widely expressed on mouse hematopoietic cells (DiLillo *et al.*, 2014). Therefore, we can presume uptake of myeloid and dendritic cells, as well as hematopoietic cells in immune response upon the infection of the BALB/c and C57BL/6 mice with wt- and ΔM27-MCMV. IgG2a antibodies are the most potently activating (with an activating/inhibitory [A/I] ratio of 69) (Nimmerjahn & Ravetch, 2005) and preferentially interact with the activating FcγRs, whereas IgG1 antibodies are the least activating ([A/I] = 0.1) and preferentially interact with inhibitory FcγRIIB. The balance between activating and inhibitory FcγRs determines the biological effect of circulating immune complexes or antibodies bound to pathogens or cells (Nimmerjahn & Ravetch, 2008). For the first time we have shown the ΔM27-MCMV-induced IgG responses were capable to produce sufficiently strong immune response to activate aforementioned activating and inhibiting FcγRs.

Graded serum concentrations induced different capabilities of chimeric FcγR-CD3ζ chain molecules to produce mIL-2, which can be easily quantified by ELISA. At 3, 7, 14, and 21 days post infection, the sera were collected from BALB/c and C57BL/6 mice (infected with wt-MCMV and ΔM27-MCMV) and the level of FcγR activation has been determined. Nevertheless, the level of produced mIL-2 is different for different sera concentrations, thereby confirming clear dose-dependent activation by IgG. It is likely that ΔM27-MCMV-induced IgG responses recognize receptors carrying viral antigen expressed on the surface of already infected cells, thereby activating FcγR-expressing monocytes, macrophages or NK cells to kill the infected cells through ADCC. The majority of vaccination-elicited antibodies apparently need the low serum concentrations of these antibodies which may necessitate FcγR interactions to be effective (Nimmerjahn *et al.*, 2010). In order to confirm the influence that FcγR interactions have on serum concentration or to effector functions that contribute to protection, interesting would be to perform experiments with FcγR-deficient mice.

4.3. ΔM27-MCMV induces MCMV-specific neutralization antibodies

On of the most marked antiviral activity of antibody and the activity important for antibody-mediated protection *in vivo* is the neutralization of free virus particles. Neutralization has been

defined as “the loss of infectivity which ensues when antibody molecule(s) bind to a virus particle (Burton et al. 2002). However, if antigen is present at a relatively low density on the surface of a virion, it could bind antibody without resulting in neutralization. Such non-neutralizing antibody could, nevertheless, trigger complement dependent virolysis or phagocytosis (Burton *et al.*, 2002). Antibodies can inhibit the release of viruses from infected cells (Gerhard, 2001) and the cell–cell transmission of viruses (Pantaleo *et al.*, 1995; Burioni *et al.*, 1994). Poor induction of neutralization antibodies that prevent viral entry is a problem of a HCMV infection (Landini *et al.*, 2001). The neutralizing efficacy of an antibody should be related to its affinity for antigen on the virion surface. So, a vaccine should aim to elicit antibodies of the highest affinity for virion surface antigen (Landini *et al.*, 2001). In order to test if ΔM27-MCMV is capable of inducing antibodies with high affinity for virion surface antigen, the sera collected from BALB/C mice at day 3, 7, 14 and 21 days post infection were incubated with Δm157-MCMV-Luc for 1.5 hours on 37°C and afterwards mouse fibroblasts were infected (Reinhard *et al.*, 2011). ΔM27-MCMV was capable of inducing luciferase expression with a promoter element due to a virus-dependent transactivation which requires viral gene expression of a viral transactivator proteins. This method required optimization of various parameters in order to find an adequate control.

At day 3, 7, and 14 post infection, there are not significant differences in neutralization potential between different mice infected with ΔM27-MCMV and wt-MCMV. So, the remaining infectivity was shown to be unchanged, presumably showing neutralization was not significant or not detectable. At 21 day post infection, BALB/c mice infected with ΔM27-MCMV are showing the lower remaining activity and, for the first time, the neutralization. Only one individual mouse from groups infected with wt-MCMV has drastically diminished the remaining infectivity of Δm157-MCMV-Luc for more than 50%. At 21 days post infection and with 1/20, 1/200, 1/300 and 1/400 sera dilutions, both ΔM27-MCMV and wt-MCMV induce specific antibodies capable of neutralization, however, induction of neutralization antibodies suggests they have not reached the neutralization maximum completely. Comparison of viral titers BALB/c mice infected with ΔM27-MCMV and wt-MCMV with neutralization potential of same groups suggested plateau titers were reached before the elimination of infectious virus (also confirmed in Jonjić *et al.*, 1994). To further dissect the timepoint when neutralization titers reach maximum, sera from BALB/c mice infected with ΔM27-MCMV and wt-MCMV should be collected after 21 days post infection.

Some of the BALB/c mice infected with ΔM27-MCMV or wt-MCMV diminished its neutralization potential for 50% and some of them for less than 50% per group indicating individual pattern of producing neutralization antibodies. Other experiments showed that BALB/c and C57BL/6 mice infected with wt- and ΔM27-MCMV are individually per group capable of inducing IgG that activates FcγRI [CD16], FcγRII [CD64], and FcγRIII [CD32], responses 14 and 21 days post infection and also FcγRIV responses. If we compare this neutralization "pattern" with the capability of ΔM27-MCMV and wt-MCMV to induce Fcγ receptor activation, a highly individual pattern can be noticed. Intriguingly, mice capable of inducing an IgG-specific activation of Fcγ receptors do not have to be capable inducing potent neutralization antibodies, indicating that the FcγR-mediated IgG responses cannot be extrapolated from ELISA. Since similar observations were previously confirmed and published for HCMV (Corrales-Aquilar *et al.*, 2016), our data, for the first time, suggested these conclusions can be applicable to a mice model.

4.4. Vaccination with ΔM27-MCMV elicits immune responses that protect adult mice from challenge infections using another MCMV strain

While subunit vaccines induce an immune response to selected viral proteins, the advantage of live vaccines is that they elicit an immune response that mimics natural immunity and provides broad protection (Slavuljica *et al.*, 2010). However, their use carries the risk of CMV disease caused by the vaccine strain or reactivation in the immunocompromised state, unless the vaccine virus is efficiently controlled by residual immunity (Slavuljica *et al.*, 2010). One approach to generating such an immunogenic, but safe live-attenuated vaccines is the deletion of viral genes that subvert the host immune response (Čičin-Šain *et al.*, 2007; Crumpler *et al.*, 2009) or essential genes (Mohr *et al.*, 2010). The other approach is the insertion of an immune-stimulatory ligand recognized by the activating receptor on immune cells into the CMV genome (Slavuljica *et al.*, 2010). Our candidate was an MCMV lacking the *M27* gene which results in an exaggerated IFN susceptibility *in vitro* (Zimmermann *et al.*, 2005; Trilling *et al.*, 2011; Le-Trilling *et al.*, 2018) and high attenuation *in vivo* (Abenes *et al.*, 2001; Zimmermann *et al.*, 2005, Le-Trilling *et al.*, 2018). The genetic factors involved in control of MCMV resistance include the Ly49H receptor, encoded by the *cmv-1* gene (Brown *et al.*, 2011; Daniels *et al.*, 2001; Lee *et al.*, 2001). Ly49H is present in strains demonstrating relative resistance to MCMV (e.g. C57BL/6), but is absent in susceptible strains (e.g. BALB/c, DBA/2).

Our experiments showed the kinetics of the infection in the resistant C57BL/6 strains were similar to those observed in the susceptible BALB/c strains.

To differentiate the primary and second virus inoculum and to challenge with a heterologous MCMV strain, we used the eGFP-expressing MCMV strain RVG102 generated by Henry and colleagues (Henry *et al.*, 2000). This virus was initially described as derivative of the MCMV Smith strain deposited as VR-194 at the American Type Culture Collection (ATCC). However, a recently published full genome sequence comparison found a high number of SNPs (Aquilar *et al.*, 2017). We reassessed these genetic differences using available reference sequence and other recently published MCMV genome sequences (Cheng *et al.*, 2010; Rawlinson *et al.*, 1996; Smith *et al.*, 2008). We found that RVG102 actually harbours a mosaic genome composition comprising alternating Smith strain-like (e.g., the region from M50 to the intergenic region preceding M70) and K181 strain-like gene regions (e.g., the region from m01 to m38). Thus, RVG102 ('MCMV:eGFP') most likely represents a heterologous challenge virus suitable for experiments, in which mice were vaccinated with wt- and ΔM27-MCMV of Smith strain background. At 2011. Jordan and colleagues provide evidence that pSM3fr-derived MCMV virus shows an attenuated growth phenotype in salivary glands due to a mutation in MCK-2, a CC(β) chemokine homologue. Le-Trilling and colleagues in 2018 have shown that irrespective of the MCK-2 functionality, ΔM27-MCMV was severely attenuated at 7 and 21 days post infection in the spleen, liver, and salivary glands of infected C57BL/6 mice.

BALB/c and C57BL/6 mice immunized with ΔM27-MCMV resisted the challenge infection with $2*10^5$ PFU of the MCMV:eGFP (21 and 6 weeks post infection) like wt-MCMV-infected mice, suggesting that the MCMV deletion mutant lacking interferon antagonist (ΔM27-MCMV) provides an innate immune stimuli that influence the effectiveness of the adaptive immune response. UV-inactivated wt-MCMV showed an incapability to establish protection upon MCMV:eGFP challenge infection confirming that a low level of replication is necessary for establishing the protection from subsequent challenge infections. The concept of 'vita-PAMPs' has been proposed (Sander *et al.*, 2011), which suggests that the innate immune system senses certain PAMPs that arise only during the active life cycle of the pathogen. Accordingly, replicating MCMV - but not UV-inactivated MCMV - induces the formation of dsRNA intermediates (Budt *et al.*, 2009).

The absence of detectable amounts of infectious virus in salivary glands of ΔM27-MCMV vaccinated mice 3 weeks after challenge implies that horizontal transmission to other mice *via saliva* might also be abrogated. It has been commonly accepted that a crucial difference between

MCMV (Klotman *et al.*, 1985) and HCMV is that only in the latter reinfections are common (Bale *et al.*, 1996; Boppana *et al.*, 2001; Coaquette *et al.*, 2004; Rasmussen *et al.*, 1997). However, experiments with Rhesus CMV (RhCMV) infected macaques confirmed this model as a crucial for testing the influence of pre-existing immunity (Bongard *et al.*, 2019) and as the only animal model that replicates the essential features of congenital CMV infection in humans (Gong *et al.*, 2022). Thus, it seems possible that a similar human vaccine will not be able to protect in all scenarios against reinfection. Nevertheless, it is possible that such an equivalent vaccine will protect against HCMV disease, similar to the protective effect of a pre-existing infection (Fowler *et al.*, 2003). This is supported by the observation that women who were exposed to HCMV were at lower risk of giving birth to children with symptomatic disease than non-infected women (Fowler *et al.*, 2003). The sero-positivity of the mother could not prevent infection of, but did prevent pathogenesis in the children. In addition, frequent exposure to different CMV strains could further increase immunity against reinfection (Adler *et al.*, 1995). It is therefore tempting to test an interferon antagonist pUL145-deficient HCMV (Le-Trilling *et al.*, 2020) vaccine for the induction of an immune response equal to natural infection, which might still protect against symptomatic HCMV infection without the risk for reactivation and pathogenesis.

Both innate and adaptive immune responses are important for the control of CMV infection (Arvin *et al.*, 2004; Krmpotić *et al.*, 2003; Koszinovski *et al.*, 1991; Reusser *et al.*, 1991; Einsele 2002; Peggs *et al.*, 2003). Innate immunity, in particular NK cells, plays a key role in limiting CMV infection at an early stage and in priming of the adaptive immune response (Robbins *et al.*, 2007; Rölle *et al.*, 2009). CD8+ T cells are the principal effectors required for resolution of productive infection and establishment of latency (Reddehase, 2002). Although CD8+ T cells play a dominant role, CD4+ T cells and NK cells contribute to the maintenance of latent CMV infection (Polić *et al.*, 1998). Antiviral Abs, although not essential for the control of primary CMV infection and the establishment of latency, play a critical role in limiting the dissemination of recurrent virus (Jonjić *et al.*, 1994). Abs can modify the disease associated with HCMV infection in transplant recipients as well as congenital CMV infection in humans and experimental animal models like mice and guinea pigs (Nigro *et al.*, 2005; Nigro *et al.*, 2008; Bracher *et al.*, 1995; Cekinović *et al.*, 2008; Chatterjee *et al.*, 2001; Snydman *et al.*, 1987). So far, for the first time, we have shown that an MCMV deletion mutant lacking interferon antagonist (ΔM27-MCMV) provides humoral immune response adequate to confer protection.

4.5. Analysis of humoral immune responses before and after challenge infection

4.5.1. ΔM27-MCMV induces neutralization antibodies involved, but not crucial for protection from a subsequent infection

The course of primary infection defines the overall load with latent CMV, and the copy number of latent viral genomes in organs correlate with the organ-specific risk of recurrence (Reddehase *et al.*, 1994). Also, the level of neutralizing antibodies that are induced during infection correlates with the degree of protection against disease for several viral vaccines (Whitton *et al.*, 2001). This does not necessarily mean that neutralizing antibodies are the agent of protection. In principle, they could simply be 'markers' of exposure to viral antigens as are antibodies to internal viral proteins. However, the protective activities that are described in passive-transfer studies indicate that it is unlikely that neutralizing antibodies are just markers and it is far more likely that they are actively involved in establishing control and resistance (Burton *et al.*, 2002).

In a mouse model, Hebeis and colleagues have shown that virus-specific B cells that are adoptively transferred into immune-deficient hosts can be stimulated to antibody production by antigen alone, without requiring T cell help (Hebeis *et al.*, 2004). Shanley and colleagues in 1981 showed that in normal animals, MCMV-immune serum administered six days after acute virus infection had been initiated did not influence the outcome. However, in immunosuppressed mice, virus dissemination during acute infection was substantially reduced by MCMV-specific antibodies (Shanley *et al.*, 1981; Farrell and Shellam, 1991). Also, Klenovšek and colleagues in 2007 showed that a transfer of serum from MCMV-immune animals had a protective effect against MCMV infection like a transfer of memory B cells, indicating that indeed the production of IgG might represent the protective principle.

A powerful vaccine would be the one that manages to minimize horizontal transmission of CMV to other individuals. Virus shedding from the epithelial cells of the serous acini in salivary glands is a major source for the horizontal transmission of cytomegalovirus where CD4+T lymphocytes are essential to terminate the productive infection (Jonjić *et al.*, 1989). With a mutant mice that do not produce antibodies because of a disrupted membrane exon of the immunoglobulin μ chain gene, Jonjić and colleagues proved that T-B cell cooperation and the production of antibodies are not required for this process. Therefore, antibodies are not essential for recovery from a primary MCMV infection. The adoptive serum transfer proved that

antibody is the limited factor that prevents virus dissemination in the immune-deficient host showing antibodies are important in limiting reactivation and dissemination of latent virus (Jonjić *et al.*, 1994).

We compared the neutralizing antibody responses to ΔM27-MCMV 5 weeks post immunization and 3 weeks post challenge infection. Immunization of BALB/c mice with ΔM27-MCMV induced amount of neutralizing antibodies comparable with wt-MCMV immunization but in highly individual manner. Three of four BALB/c mice infected with ΔM27-MCMV were capable to induce neutralization antibodies, only one BALB/c mouse was characterized as “poor” neutralizer. Three weeks upon challenge infection, the same BALB/c mouse vaccinated with ΔM27-MCMV was not capable to induce neutralization antibodies although it was protected from subsequent infection. Since this pattern has been noticed in only one BALB/c mouse infected with ΔM27-MCMV, hence, the number of infected mice per groups should be bigger. Nevertheless, ΔM27-MCMV induces neutralization antibodies that might have an influence on the protection from subsequent infections, however, neutralizing antibodies are not crucial in establishing long-term protection. Since all mice were fully protected in the very same experiment including the mice lacking detectable nAbs, our results suggest that protection can be achieved even if pre-existing nAbs are below the level of detection in *in vitro* assays. BALB/c mice infected with UV-irradiated wt-MCMV did not induce neutralizing antibodies. As expected, the virus must be fully infective in order to induce immune responses.

Therefore, it is unlikely that neutralizing antibodies that are present in the 1/20, 1/200, 1/300 and 1/400 serum dilutions of a vaccinated individual at the time of virus challenge are solely responsible for protection. One would predict that some virus-infected cells will escape elimination by antibody. Although ADCC-elicitng, in particular NK cells, play a key role in limiting CMV infection at an early stage (Robbins *et al.*, 2007; Rölle *et al.*, 2009), the obvious candidate for protection is viral-specific CD8+ T cells, and there is ample evidence for the ability of these cells to control the viral replication (Burton, 2002). Virus-specific CD8+ effector T cells could already be present at the challenge, be recruited from the vaccine-induced CD8+ T cell memory pool or be induced *de novo* (Figure 30 B). So, the kinetics of the virus-specific T cell response should be tested upon infection of mice with ΔM27-MCMV. Another possibility is that increased antibody concentration (as a result of the stimulation of memory B cells by viral antigen) contributes to protection (Burton, 2002). Also, the pre-existing antibody is in focus as the most powerful first line of defence against viral challenge. This antibody can

be maintained at relatively high levels for many years, probably produced by long-lived plasma cells (Slifka & Ahmed, 1998; Ochsenbein *et al.*, 2000) although this is not universally accepted (Janeway *et al.*, 1945). However, the second antibody memory component, memory B cells (Figure 30 B), might also be crucial for vaccine-mediated protection in some cases. Equally, the contact of memory B cells with viral antigen might be important to boost plasma-cell numbers and serum-antibody concentrations for the next encounter with the virus (Burton, 2002). The antibody mediated blunting, rather than ablation, of infection will facilitate this boost by increasing the amount of antigen that is available. Finally, other mechanisms, particularly innate immunity, might contribute to containing infection. During the acute phase of primary infection MCMV frequency of surface IgG-expressing cells increased, especially those secreting IgG2a. Production of this IgG subclass is strongly stimulated by IFN-γ (Karupiah *et al.*, 1998). Antigen-specific CD4+ T cells produce IL-2 and IL-12, which can lead to the production of cytokine-induced memory-like NK cells (CIML) (Figure 30 A1) and these cells are responsible for increasing levels of IFNγ (Forrest *et al.*, 2020). Natural CMV infection and some vaccine formulations may be able to induce adaptive NK cells via the induction of IL-12 (Forrest *et al.*, 2020). As-yet-undefined receptor-ligand interactions are also likely to be important for the induction of these cells (Figure 30 A3). According to this, it would be interesting to examine environmental factors as effective mediators of ADCC during the infection, induced by deletion mutant lacking interferon antagonist ΔM27-MCMV.

4.5.2. ΔM27-MCMV elicits antibodies capable of activating FcyRIII and FcyRIV receptors

Fc-FcγR interactions promote innate immune cell activation that lead to effector activities and the expression of pro-inflammatory and immunomodulatory mediators with an impact on cellular differentiation and survival (Bournazos & Ravetch, 2017). FcγR-mediated pathways control B-cell activation and selection, IgG affinity maturation, IgG production and plasma cell survival. Given the significant contribution of the FcγR-mediated immunomodulatory activities in the regulation of T- and B cell responses, manipulation of FcγR function could lead to the design of novel vaccination strategies with robust and sustained cellular and humoral immune responses (Bournazos & Ravetch, 2017).

Infection with ΔM27-MCMV elicits antibodies capable of activating FcγRIII and FcγRIV receptors 5 weeks post infection. While antigens of ΔM27-MCMV efficiently triggered FcγRIII reporter cells, sera collected from BALB/c mice 5 weeks post infection infected with UV-inactivated wt-MCMV elicited very poor if any responses (Figure 3.5.2 B) confirming that lack of virus replication leads to reduction of MCMV-specific ELISA-reactive IgG responses and therefore FcγR activation. The FcγRIII receptor compared to the FcγRIV receptor showed comparable IgG immune responses upon vaccination with ΔM27-MCMV, indicating that ΔM27-MCMV is sufficient to efficiently trigger adaptive immune responses capable of establishing protection against subsequent viral infection.

Van der Hoecke and colleagues in 2017 showed that the M2e-specific IgG2a antibody protected better against influenza A virus challenge than the M2e-specific IgG1 antibody, presumably because, as detected *in vitro*, MAb 65 (IgG2a) could engage all three activating Fcγ receptors, which are expressed on natural killer cells, neutrophils, monocytes, and macrophages (Bruhns, 2012; Nimmerjahn & Ravetch, 2011). Also, Kaugars and colleagues in 2021 demonstrated that the recombinant vaccine ΔgD-2:HAPR8 (a herpes simplex virus vaccine lacking the essential glycoprotein D [gD] gene), elicits protection against influenza with a high proportion of FcγRIV activating antibodies. By expressing the HA gene from influenza in the ΔgD-2 vector, they created a vaccine that effectively protects against otherwise lethal homologous PR8 influenza, HSV- 1, and HSV-2 challenge. Mice with high levels of humoral immunity against HSV-2 were protected against influenza challenge by ΔgD-2:HAPR8, showing advantage of using the ΔgD-2 vectors where preexisting immunity does not impede its ability to function as a vaccine (Kaugars *et al.*, 2021). Since HCMV can superinfect preimmune hosts (Mendez *et al.*, 2019; Meyer-Koenig *et al.*, 1998), CMV-based vaccines may be applicable regardless of preexisting immune responses raised by naturally acquired CMVs. Huang and colleagues 2021. explored the therapeutic potential of MCMV-based vaccines, using MCMV vectors expressing the small HBsAg and achieving the attenuation by deleting the IFN antagonist *M27*. The vaccination with the attenuated ΔM27-HBsAg provided protection to mice against HBV challenges (Huang *et al.*, 2021). Altogether, ΔM27-MCMV data corroborate the concept that CMV-based vectors are promising candidates for the development of live-attenuated vaccines.

What are the implications of our findings for the clinical development of live-attenuated vaccines based on interferon antagonist deletion mutants? ΔM27-MCMV immunity appears to operate in the presence of demonstrable virus neutralizing activity which engages Fc receptor-expressing myeloid cells. FcγRIV is a high-affinity receptor for mouse IgG2a and IgG2b, as it

binds monomers of both IgG subclasses (Mancardi *et al.*, 2008). FcγRIV has, however, no affinity for mouse IgG1 or IgG3. FcγRIII and FcγRIIB, the only inhibitory IgG receptor, are low-affinity receptors for mouse IgG1, IgG2a, and IgG2b (Daëron, 1997) but binding IgG1. Therefore, vaccine formulations that promote the induction of antigen specific IgG1, IgG2a and IgG2b should be used. Human IgG1 has the highest affinity for FcRI, which, as in mice, has a broad expression pattern (dendritic cells, monocytes, and macrophages) (Guillams *et al.*, 2014). The sequence of mouse FcγRIV suggests that it is related to human FcγRIIIA (expressed on natural killer cells, monocytes, and macrophages) (Nimmerjahn *et al.*, 2005, Mancardi *et al.*, 2008). Therefore, ΔM27-MCMV-specific IgG antibodies could possibly provide protection through multiple effector cells that are resident at or recruited to the site of infection.

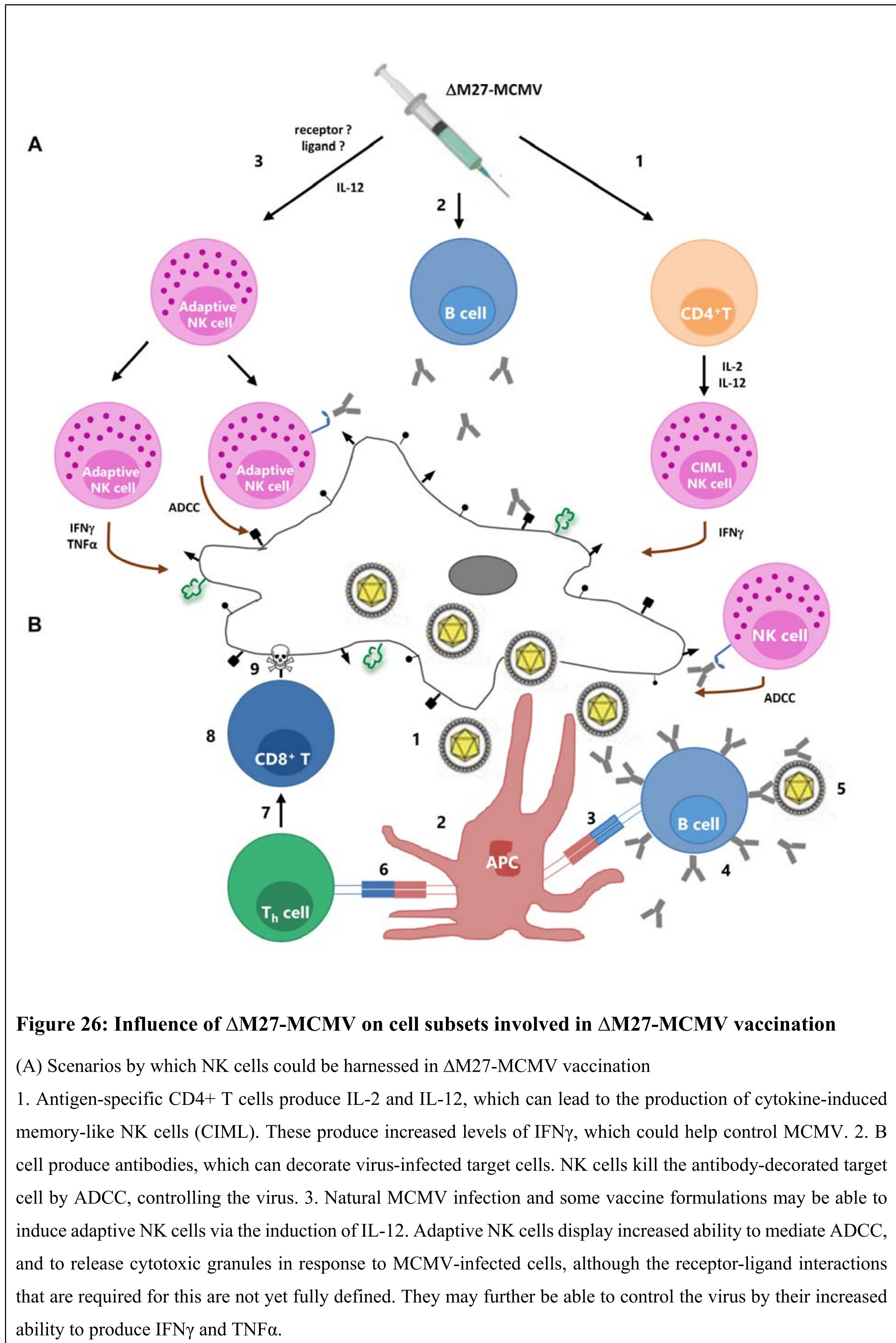

Figure 26: Influence of ΔM27-MCMV on cell subsets involved in ΔM27-MCMV vaccination

(A) Scenarios by which NK cells could be harnessed in ΔM27-MCMV vaccination

1. Antigen-specific CD4+ T cells produce IL-2 and IL-12, which can lead to the production of cytokine-induced memory-like NK cells (CIML). These produce increased levels of IFNγ, which could help control MCMV. 2. B cell produce antibodies, which can decorate virus-infected target cells. NK cells kill the antibody-decorated target cell by ADCC, controlling the virus. 3. Natural MCMV infection and some vaccine formulations may be able to induce adaptive NK cells via the induction of IL-12. Adaptive NK cells display increased ability to mediate ADCC, and to release cytotoxic granules in response to MCMV-infected cells, although the receptor-ligand interactions that are required for this are not yet fully defined. They may further be able to control the virus by their increased ability to produce IFNγ and TNFα.

(B) Cell subsets possible involved in ΔM27-MCMV protection from a subsequent challenge infection
1. Assembly of virions. 2. Uptake of virus by antigen-presenting cell (APC). 3. Presentation of antigens, including epitopes, to B-cell receptor (BCR). 4. Production of binding and neutralizing antibodies by B cells that, ideally, 5. neutralize the virus. 6. Presentation of antigens, including epitopes, to T-cell receptor (TCR). 7. Activation of T helper (T_h) cells and production of cytokines, that, recognized by 8. cytotoxic T-cells, 9. kill the virus. (according to Burton, 2002 and Forrest *et al.*, 2020)

4.6. ΔM27-MCMV vaccination presumes course of infection as independent of dose of infection

Reddehase and colleagues 1984. have shown that MCMV infectious dose applied experimentally (10^2 - 10^6 PFU of MCMV) determines the total number of generated MCMV-specific CD8+ T cells and is explained by the slow replication cycle of the virus (Reddehase *et al.*, 1984). Repetitive antigen exposure leads to memory MCMV-specific CD8+ T cell inflation (Seckert *et al.*, 2011), elicited by immediate early (IE) (e.g. IE3 in C57BL/6 and IE1 in BALB/c) and early antigens (E) (e.g. *M139* and pM38 in C57BL/6 mice and *M164* in BALB/c mice), albeit with different kinetics and magnitude (Welten *et al.*, 2013; Holtappels *et al.*, 2002). After low dose inoculum, the accumulation of inflationary MCMV-specific memory T cells was severely hampered and diminished antigen-driven T-cell proliferation (Redeker *et al.*, 2013). The memory phenotype of the IE3-specific cytotoxic T cells was mostly affected by the viral inoculum size as compared to the *M139*- and pM38-specific cytotoxic T cells, inducing a central memory phenotype of the inflationary T cells and improved capacity to expand after rechallenge (Redeker *et al.*, 2013). Also, the initial viral inoculum dose impacts the number of long-lived plasma cells and memory B cells maintained during chronic infection, thereby impacting the amount of IgG antibodies that are present in the serum. IgG antibody inflation, however, occurs despite differences in the initial viral inoculum (Welten *et al.*, 2016).

ΔM27-MCMV vaccination is dose independent. Initial experiments used $2*10^5$ PFU of ΔM27-MCMV per vaccination, since infection with identical dose [$2*10^5$ PFU] of ΔM27-MCMV results in no detectable ΔM27-MCMV replication in organs of BALB mice 21 days post infection (Le-Trilling *et al.*, 2018). We examined the effects of intraperitoneal vaccination with $4*10^4$ PFU or $8*10^3$ PFU. Six weeks after immunization, BALB/c mice were challenged with $2*10^5$ PFU of MCMV:eGFP. Irrespective of the vaccine dose, salivary gland viral titers of all BALB/c mice immunized with ΔM27-MCMV have been below the detection limit (detection

limit was 10^2 PFU). Thus, with vaccination with 100-fold less ΔM27-MCMV, all immunized mice were fully protected. This process was obviously not crucial for establishing conditions for ΔM27-MCMV protection on immunized mice. Our findings suggest that number of MCMV-specific CD8+ T cells that exerts lytic activity towards cells carrying viral structural proteins probably does not have the influence on ΔM27-MCMV in establishing the protection from subsequent challenge infections, and suggests further determination of MCMV specific CD8+ T cells upon ΔM27-MCMV infection.

In summary, ΔM27-MCMV was able to efficiently protect immunocompetent mice against challenge with MCMV:eGFP after vaccination with a single dose. Remarkably, vaccination with ΔM27-MCMV was as efficient as vaccination for long-term protection.

4.7. ΔM27-MCMV protects fully against the homotypic challenge

Safety is also a requirement for a useful vaccine vector (Redwood *et al.*, 2005). CMVs are typically non-pathogenic in immunocompetent hosts, making them potentially safer than other vectors such as lentivirus. However, for use in humans, it may be important to produce vaccine vectors that are attenuated because of the number of immunocompromised individuals in the population (Redwood *et al.*, 2005). This should be technically feasible, as vaccine strains of MCMV that replicate poorly *in vivo* yet protect from challenge with a virulent wt-MCMV have been produced (MacDonald *et al.*, 1998, Morley *et al.*, 2002).

All our vaccination experiments were performed using Smith/K181 MCMV:eGFP for the challenge. The *eGFP* gene was placed under control of the native MCMV ie1/ie3 promoter as a means of visualizing directly sites of ie1 transcriptional activity in acute and latent infection (Henry *et al.*, 2000). To exclude this leads to exaggerated protection, e.g., due to an attenuation caused by introduction of the *eGFP* gene, the existence of foreign antigens, or the mosaic genome, we repeated the experiment by challenging with the autologous Smith strain-derived wt-MCMV (Messerle *et al.*, 1997; Wagner *et al.*, 1999). ΔM27-MCMV provided full protection against this homotypic challenge confirming that placing visualization gene on behalf of *ie2* gene in Smith/K181 MCMV has no influence on result of vaccination.

5 Perspective

Despite huge efforts, no effective HCMV vaccine is currently available. Several features of HCMV make vaccine development extremely difficult. First, a large number of viral immunoevasion proteins subvert the host's immune responses (Hengel *et al.*, 1998). Second, immunity from naturally acquired infection is not completely protective against superinfection or CMV transmission from mother to foetus (Fowler *et al.*, 2003; Boppana *et al.*, 2001; Koontz *et al.*, 2008). Third, persistence of virus in the state of latency with the possibility of reactivation and disease in immunocompromised patients represents a safety concern (Redwood *et al.*, 2002). Still, a live, attenuated vaccine approach has several characteristics that finds it attractive. Unlike subunit vaccines, which induce cellular or humoral immune response to selected antigens only, live vaccines induce a much broader immunity that may mimic protection acquired following natural infection (Mohr *et al.*, 2010; Čičin-Šain *et al.*, 2007; Gill *et al.*, 2000; MacDonald *et al.*, 1998; Morello *et al.*, 1999; Snyder *et al.*, 2010). Cellular immunity against CMV follows unique kinetics characterized by maintenance or even expansion of the virus-specific CD8+ T cell response over time (Holtappels *et al.*, 2002; Karrer *et al.*, 2003). In addition, recombinant CMVs that expressed heterologous simian immunodeficiency virus, lymphocytic choriomeningitis virus, and influenza virus peptides have been shown to induce protective immunity against the respective viruses (Karrer *et al.*, 2004, Hansen *et al.*, 2009). Therefore, live, attenuated CMVs are attractive candidates for a CMV vaccine or a CMV-based vaccine vector provided that their pathogenicity is significantly attenuated but their immunogenicity is unaffected.

A better understanding of viral immunobiology and the introduction of BAC technology have made the CMV genome accessible to the design of rational mutants as CMV vaccine candidates (Messerle *et al.*, 1997, Wagner *et al.*, 2002). The vaccination potential of CMV mutants lacking nonessential viral genes has already been proven (Čičin-Šain *et al.*, 2007, Crumpler *et al.*, 2009). We show here that ΔM27-MCMV, a MCMV mutant lacking a specific inhibitor of IFN-induced STAT2 signalling, elicits strong humoral immune responses and confers full protection against subsequent challenge infections in adult mice.

The salivary glands are the organ for CMV replication in that productive infection continues long after innate and adaptive immune responses have cleared MCMV from other organs

(Reddehase *et al.*, 1994, Jonjić *et al.*, 1989). ΔM27-MCMV infection of mice led to the emergence of new, extremely favourable biological characteristics, including the lack of detectable infectious virus in salivary glands during primary or recurrent infection in BALB/c mice.

It is generally accepted that the innate immune system has a key role in determining the strength and quality of the adaptive immune response. Like MCMV, HCMV expresses a protein that induces proteasomal STAT2 degradation (Le *et al.*, 2008a; Le *et al.*, 2008b; Weeks *et al.*, 2014). HCMV mutant lacking its STAT2 antagonist pUL145 may constitute a very interesting candidate for the development of a live attenuated HCMV vaccine virus (Le-Trilling *et al.*, 2020). Based on the findings concerning the potential of cytomegaloviral vectors for vaccination against other infectious agents such as retroviruses (Hansen *et al.*, 2013; Hansen *et al.*, 2018) and the work in the mouse (Huang *et al.*, 2021) the herein described attenuation principle might also be applicable for the design of CMV-based vaccine vectors.

An important goal of CMV vaccine development is to develop a candidate vaccine that provides protection from disease associated with intrauterine CMV infection (Arvin *et al.*, 2004). Although mice have not been utilized as a model for congenital infection because of the restrictions of foetal infection secondary to the anatomy of the mouse placenta (Johnson *et al.*, 1969; Fitzgerald & Shellam, 1991), newborn animals have been used to model the CNS disease and sequelae associated with congenital CMV infection (Koontz *et al.*, 2008). Importantly, the newborn mouse is developmentally similar to the late-second-trimester human foetus, and infection delivered by an i.p. inoculation can lead to CNS infection and disease whose pathogenesis closely resembles that which is thought to occur in the infected human foetus (Slavuljica *et al.*, 2010). Passively acquired antiviral Abs are thought to modulate infection and disease in infected newborn mice in a fashion similar to transplacentally acquired maternal Abs in human foetuses infected *in utero* (Cekinović *et al.*, 2008). Infected neonates born to ΔM27-MCMV vaccinated mothers had diminished viral titers in all organs. Thus, this data provide evidence of protective immunity against neonatal infection and disease induced by maternal ΔM27-MCMV vaccination that is comparable to that induced by maternal wt-MCMV infection. Together, these findings demonstrate the efficacy of an attenuated MCMV vaccine lacking the interferon antagonist to generate a robust and persistent protective immune response.

Antibody Fc-mediated effector systems can affect antibody activity against free virus particles in several ways. First, the activation of complement by antibodies that are bound to virus particles (Burton, 2002). Second, complement activation can lead directly to virolysis (Burton,

2002). Third, Fc and complement receptors can bind antibody- and/or complement-coated virions, which leads to phagocytosis followed by inactivation of the virion in an intracellular compartment of the phagocyte (Burton, 2002). Significant contribution of the FcγR-mediated immunomodulatory activities has been shown in the regulation of T- and B cell responses and the manipulation of FcγR function could lead to the design of novel vaccination strategies with robust and sustained cellular and humoral immune responses (Bournazos & Ravetch, 2017). Vaccination with ΔM27-MCMV created IgG immune responses that activated activating FcγRIII and FcγRIV receptors, confirming ΔM27-MCMV is sufficient to efficiently trigger adaptive immune response capable of establishing protection against subsequent viral infection.

Multiple approaches have been proposed for attenuation of CMVs; however, since the biological target of attenuation is experimentally defined, optimal attenuation of this virus remains speculative (Slavuljica *et al.*, 2010). Optimal attenuation should result in a virus that can replicate sufficiently to induce adaptive immunity, thereby establishing immunological memory and an attenuated level of persistence at the same time (Slavuljica *et al.*, 2010). ΔM27-MCMV led to not only strong attenuation in immunocompetent host but also additional results beneficial for attenuated vaccine candidates, including

(a) enduring protective responses;

(b) attenuated phenotype even in immunologically immature or immunodeficient hosts;

(c) stability of deletion mutation despite of the strong selective pressure; and

(d) minimal risk of recurrence.

All benefits stated above emphasize the large potential of attenuated CMV viruses to serve as live vaccine vectors (Hansen *et al.*, 2009). In process of creating a safe and efficient vaccine, this approach - using live attenuated CMVs as vaccine candidates will hopefully gain more recognition in the near future.

6 Summary

Human cytomegalovirus (HCMV) frequently causes congenital infections, resulting in birth defects and developmental disorders. A vaccine is needed, but currently unavailable. We analysed the potential of an MCMV mutant, lacking its STAT2 antagonists to serve as live attenuated vaccine in mice. Infections with ΔM27-MCMV confirmed its capability for inducing ELISA IgG responses in terms of IgG recognizing MCMV-encoded proteins present in infected cells. For persistent infection, very strong MCMV-specific ELISA-reactive IgG responses were also mounted. After implementing ELISA on MCMV virions, ΔM27-MCMV induced strong IgG responses in terms of IgG recognizing MCMV virions. The ΔM27-MCMV was capable to raise FcγRIII [CD16], FcγRI [CD64], FcγRII [CD32] and FcγRIV activating IgG responses in BALB/c and C57BL/6 mice. FcγRI, FcγRII and FcγRIII have reached their maximum level of IgG activation upon infection with ΔM27-MCMV at 14 and 21 days post infection, respectively. It is likely that ΔM27-MCMV activating IgG responses recognize receptors expressed on the surface of already infected cells, thereby activating FcγR-expressing monocytes, macrophages or NK cells, to kill the infected cells through ADCC. Infections with ΔM27-MCMV induced neutralizing antibodies.

BALB/c and C57BL/6 mice immunized with ΔM27-MCMV resisted the challenge infection with the MCMV:eGFP (21 and 6 weeks post infection), suggesting that the MCMV deletion mutant lacking interferon antagonist (ΔM27-MCMV) provides an innate immune stimuli that influence the effectiveness of the adaptive immune response, resulting in protection from subsequent challenge infections. To exclude that this leads to exaggerated protection, e.g., due to an attenuation caused by introduction of the *eGFP* gene, the ΔM27-MCMV vaccination is repeated, challenged with wt-MCMV and observed full protection of ΔM27-MCMV-immunized mice against wt-MCMV challenge. We compared the neutralizing antibody responses to ΔM27-MCMV 5 weeks post immunization and 3 weeks post challenge infection, showing that ΔM27-MCMV induces neutralization antibodies that might have influence on protection from subsequent infections. Nevertheless, antibodies that elicits ΔM27-MCMV are capable of activating FcγRIII and FcγRIV receptors 5 weeks post infection. Irrespective of the infection dose, salivary gland viral titers of all BALB/c mice immunized with ΔM27-MCMV have been below detection limit (10^2 PFU), therefore suggesting that all immunized mice were protected.

ΔM27-MCMV was able to efficiently protect immunocompetent mice against challenge with MCMV:eGFP after vaccination with a single dose. Remarkably, vaccination with ΔM27-MCMV was as efficient as vaccination for long-term protection.

6 Zusammenfassung

Das humane Cytomegalovirus (HCMV) verursacht häufig kongenitale Infektionen, die zu Geburtsfehlern und Entwicklungsstörungen führen. Ein Impfstoff wird benötigt, ist aber derzeit nicht verfügbar. Wir untersuchten das Potenzial einer MCMV-Mutante, der ihr STAT2-Antagonist fehlt, als abgeschwächter Lebendimpfstoff in Mäusen zu dienen. Infektionen mit ΔM27-MCMV zeigten die Fähigkeit, ELISA-IgG-Reaktionen in Form von IgG zu induzieren, das MCMV-kodierte Proteine in infizierten Zellen erkennt. Bei persistierender Infektion wurden auch sehr starke MCMV-spezifische ELISA-reaktive IgG-Reaktionen beobachtet. Nach der Durchführung von ELISA auf MCMV-Virionen induzierte ΔM27-MCMV starke IgG-Reaktionen in Form von IgG, welches MCMV-Virionen erkennt. Das ΔM27-MCMV war in der Lage, FcγRIII [CD16], FcγRI [CD64], FcγRII [CD32] aktivierende IgG-Reaktionen in BALB/c und C57BL/6 Mäusen auszulösen. FcγRI, FcγRII und FcγRIII haben ihr Maximum an IgG-Aktivierung nach einer Infektion mit ΔM27-MCMV 14 bzw. 21 Tage nach der Infektion erreicht. Es ist wahrscheinlich, dass ΔM27-MCMV aktivierende IgG-Reaktionen Antigenrezeptoren erkennen, die auf der Oberfläche bereits infizierter Zellen exprimiert werden, und dadurch FcγR-exprimierende Monozyten, Makrophagen oder NK-Zellen aktivieren, um die infizierten Zellen durch ADCC abzutöten. Infektionen mit ΔM27-MCMV induzierten außerdem neutralisierende Antikörper.

BALB/c- und C57BL/6-Mäuse, die mit ΔM27-MCMV immunisiert wurden, widerstanden einer Testinfektion mit MCMV:eGFP (21 und 6 Wochen nach der Impfung), was darauf hindeutet, dass die MCMV-Deletionsmutante, der der Interferon-Antagonist fehlt (ΔM27-MCMV), einen Immunstimulus liefert, der die Wirksamkeit der adaptiven Immunantwort beeinflusst, was zu einem Schutz vor nachfolgenden Infektionen führt. Um auszuschließen, dass dies zu einem übertriebenen Schutz führt, z. B. aufgrund einer Abschwächung durch die Einführung des *eGFP*-Gens, wurde die ΔM27-MCMV-Impfung wiederholt, mit wt-MCMV herausgefordert und ein vollständiger Schutz von ΔM27-MCMV-immunisierten Mäusen gegen die Herausforderung durch wt-MCMV beobachtet. Wir verglichen die neutralisierenden Antikörperreaktionen auf ΔM27-MCMV 5 Wochen nach der Immunisierung und 3 Wochen nach der Challenge-Infektion und zeigten, dass ΔM27-MCMV neutralisierende Antikörper induziert, die einen Einfluss auf den Schutz vor nachfolgenden Infektionen haben könnten. Dennoch sind Antikörper, die ΔM27-MCMV auslösen, in der Lage, 5 Wochen nach der

Infektion die FcγRIII- und FcγRIV-Rezeptoren zu aktivieren. Unabhängig von der Infektionsdosis lagen die Virustiter in den Speicheldrüsen aller BALB/c-Mäuse, die mit ΔM27-MCMV immunisiert wurden, unter der Nachweisgrenze (10^2 PFU), was darauf schließen lässt, dass alle immunisierten Mäuse geschützt waren.

ΔM27-MCMV war in der Lage, immunkompetente Mäuse nach der Impfung mit einer Einzeldosis wirksam gegen eine Herausforderung mit MCMV:eGFP zu schützen. Bemerkenswerterweise war die Impfung mit ΔM27-MCMV ebenso effizient wie die Impfung zum Langzeitschutz.

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Acknowledgments

I would like to thank Prof. Dr. Mirko Trilling for giving me the opportunity to come in his lab and setting my interest on virology. Thank you for making me a researcher and stimulating my scientific curiosity. Thank you also, for helpful discussions and for giving, at the appropriate moment, the most-needed advice.

I would like to thank Dr. Vu Thuy Khanh Le-Trilling for scientific suggestions and support through laboratory work. Your remarks gave me the force for continuing and reaching my goals.

My special thanks goes to Prof. Dr. Astrid Westendorf for her willingness of being the Second Thesis Supervisor.

My thanks goes also to the entire working group (Benjamin, Christine, Fabienne, Kevin, Kerstin, Lejla, Lydia, Mareike, Meike, Sebastian, Tanja) for creating a pleasant working atmosphere, and nevertheless, for spending much great times discussing life and lab issues in company with a delightful food.

I am grateful for having the opportunity to meet and work with AG Hengel. I would like to thank Prof. Dr. Hartmut Hengel for allowing me to perform the important experiments in His laboratory. For professional support in establishing such experiments and always being available to answer my questions, I would like to thank to Dr. Katja Hoffmann. To the rest of the AG Hengel, especially to André, Carolin and Precious, thank you for the warm welcome.

I would like to cordially thank to my family who has always been there for me, even far away. You always believed in me and without you this would have never been possible.

„The last but not the least“, I thank all my friends who went with me through ups and downs, through good times and bad times. Thank you for giving me the needed impulse and strenght to proceed further.

Publications, presentations, posters

Publications

1. Le-Trilling VTK[#], **Jagnjic A[#]**, Brizic I[#], Eilbrecht M, Wohlgemuth K, Rozmanic C, Herdman A, Hoffmann K, Westendorf AM, Hengel H, Jonjic S, Trilling M. Maternal antibodies induced by a live attenuated vaccine protect neonatal mice from cytomegalovirus NPJ Vaccines. 2023 Feb 3;8(1):8 **(# contributed equally)**

2. Le-Trilling VTK, Wohlgemuth K, Rückborn MU, **Jagnjic A**, Maaßen F, Timmer L, Katschinski B, Trilling M. STAT2-dependent immune responses ensure host survival despite the presence of a potent viral antagonist J Virol. 2018 May, JVI.00296-18

Presentations

1. “Humoral immune responses raised against Cytomegalovirus mutants lacking interferon antagonists” Andreja Jagnjić, Kerstin Wohlgemuth, Katja Hoffmann, Hartmut Hengel, Vu Thuy Khanh Le-Trilling & Mirko Trilling
   Oral presentation at the **Betaherpesviruses Satellite Workshop of the 42nd International Herpesvirus Workshop (IHW)**, Ghent, Belgium, July 2017

2. “Humoral immune responses raised against Cytomegalovirus mutants lacking interferon antagonists” Andreja Jagnjić, Kerstin Wohlgemuth, Katja Hoffmann, Hartmut Hengel, Vu Thuy Khanh Le-Trilling & Mirko Trilling
   Oral presentation at the **16th GfV´s Workshop of the study group „Immunobiology of Viral Infections“**, Tauberbischofsheim, Germany, September 2017

Posters

1. “Humoral immune responses raised against Cytomegalovirus mutants lacking interferon antagonists” Andreja Jagnjić, Kerstin Wohlgemuth, Katja Hoffmann, Hartmut Hengel, Vu Thuy Khanh Le-Trilling & Mirko Trilling
   Betaherpesviruses Satellite Workshop of the 42nd International Herpesvirus Workshop (IHW), Ghent, Belgium, July 2017

2. "Vaccination with a live attenuated cytomegalovirus mutant lacking an interferon antagonist raises strong humoral immune responses and prevents subsequent challenge infections" Andreja Jagnjić, Ilija Brizić, Kerstin Wohlgemuth, Katja Hoffmann, Hartmut Hengel, Stipan Jonjić, Vu Thuy Khanh Le-Trilling & Mirko Trilling
28th Annual Meeting of the Society for Virology, Würzburg, Germany, March 2018

3. "Cytomegalovirus mutants lacking interferon antagonists as candidates for a live attenuated vaccine" Andreja Jagnjić, Kerstin Wohlgemuth, Wibke Bayer, Katja Hoffmann, Hartmut Hengel, Vu Thuy Khanh Le-Trilling & Mirko Trilling
27th Annual Meeting of the Society for Virology, Marburg, Germany, March 2017

4. "Towards the analysis of the role of STAT2 in MCMV infection *in vivo*" Andreja Jagnjić, Vu Thuy Khanh Le-Trilling, Mirko Trilling
25th Annual Meeting of the Society for Virology, Bochum, Germany, March 2015

5. "Vaccination with a live attenuated cytomegalovirus mutant lacking an interferon antagonist raises strong humoral immune responses and prevents subsequent challenge infection" Andreja Jagnjić, Ilija Brizić, Kerstin Wohlgemuth, Katja Hoffmann, Hartmut Hengel, Stipan Jonjić, Vu Thuy Khanh Le-Trilling & Mirko Trilling
16. Forschungstag der Medizinischer Fakultät, University Duisburg-Essen, University Hospital Essen, Essen, Germany, November 2017

6. „Cytomegalovirus mutants lacking interferon antagonists as candidates for a live attenuated vaccine" Andreja Jagnjić, Vu Thuy Khanh Le-Trilling, Wibke Bayer & Mirko Trilling
15. Forschungstag der Medizinischer Fakultät, University Duisburg-Essen, University Hospital Essen, Essen, Germany, November 2016

7. „The role of interferon-dependent JAK-STAT signalling for adaptive immune responses against cytomegaloviruses" Andreja Jagnjić, Vu Thuy Khanh Le, Mirko Trilling
13. Forschungstag der Medizinischer Fakultät, University Duisburg-Essen, University Hospital Essen, Essen, Germany, November 2014

Curriculum Vitae

The Curriculum Vitae is not included in the online version for data protection reasons.

Declarations

Declaration:

In accordance with § 6 (para. 2, clause g) of the Regulations Governing the Doctoral Proceedings of the Faculty of Biology for awarding the doctoral degree Dr. rer. nat., I hereby declare that I represent the field to which the topic "*An MCMV mutant lacking the IFN antagonist pM27 elicits strong humoral immune responses and can serve as a live attenuated vaccine"* is assigned in research and teaching and that I support the application of Andreja Jagnjic.

Essen, date ________ ______________________ ________________________________

Prof. Dr. Mirko Trilling

Declaration:

In accordance with § 7 (para. 2, clause d and f) of the Regulations Governing the Doctoral Proceedings of the Faculty of Biology for awarding the doctoral degree Dr. rer. nat., I hereby declare that I have written the herewith submitted dissertation independently using only the materials listed, and have cited all sources taken over verbatim or in content as such.

Essen, date _________________ ______________________________

Andreja Jagnjić

Declaration:

In accordance with § 7 (para. 2, clause e and g) of the Regulations Governing the Doctoral Proceedings of the Faculty of Biology for awarding the doctoral degree Dr. rer. nat., I hereby declare that I have undertaken no previous attempts to attain a doctoral degree, that the current work has not been rejected by any other faculty, and that I am submitting the dissertation only in this procedure.

Essen, date _________________ ______________________________

Andreja Jagnjić