Characterization of IgG Effector Responses to Mouse Cytomegalovirus in Susceptible and Resistant Mouse Strains

Inaugural-Dissertation

zur Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultät
der Heinrich-Heine-Universität Düsseldorf

vorgelegt von

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Düsseldorf, Juni 2012

aus dem Institut für Virologie
der Heinrich-Heine Universität Düsseldorf

Gedruckt mit der Genehmigung der
Mathematisch-Naturwissenschaftlichen Fakultät der
Heinrich-Heine-Universität Düsseldorf

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Tag der mündlichen Prüfung: 28.11.2012

To my parents

(Părinților mei)

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ABBREVIATIONS

A	Ampere
aa	Amino acid
Ab	Antibody
ADCC	Antibody dependent cellular cytotoxicity
ADVI	Antibody dependent virus inhibition
APS	Ammonium persulfate
ATCC	American type culture collection
ATP	Adenosintriphosphat
BAC	Bacterial artificial chromosome
bp	Base pairs
BMFZ	Biological Medical Research Center
BSA	Bovine serum albumin
°C	Celcius degree
CD	Cluster of differentiation
CDC	Complement dependent cytotoxicity
CMV	Cytomegalovirus
CMVIG	Cytomegalovirus hyperimmune globulin
CO_2	Carbon dioxid
CPE	Cytopathic Effect
Cys	Cystein
Da	Dalton
DC	Dendritic cell
DDB1	DNA-binding protein 1
DL	Detection limit
DMEM	Dulbeccos Modified Eagle Medium
DMSO	Dimethylsulfoxide
DNA	2-Desoxyribonucleic acid
d.p.i.	Days post infection
ds	Double strand
DTT	1,4- Dithiothreitol
E	Early
EBV	Epstein - Barr virus
EDTA	Ethylenediaminetetraacetic acid (+Na_2)

ELISA	Enzyme-Linked ImmunoSorbent Assay
Endo H	Endoglycosidase H
ERGIC	Endoplasmic reticulum Golgi intermediate compartment
Fab	Fragment antigen binding
FACS	Fluorescent activated cell sorting
Fc	Fragment crystallisable
Fcγ	Fragment crystallisable from IgG
FcR	Fc Receptor
FcRn	Fc Receptor neonatal
FcαR	Fc alpha Receptor
FcδR	Fc delta Receptor
FcεR	Fc epsilon Receptor
FcγR	Fc gamma Receptor
FcμR	Fc mu Receptor
FCS	Fetal Calf Serum
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
g	Gravity
G418	Geneticine G
GCR	G protein-coupled receptor
gp	Glycoprotein
gB	Glycoprotein B
gCIII	Glycoprotein complex III
gH	Glycoprotein H
gL	Glycoprotein L
gO	Glycoprotein O
GPI	Glycosylphosphatidylinositol
h	Hours
HA	Hemagglutinin from human influenza
HAT	Hypoxanthine-aminopterin-thimidine
HC	Heavy chain
HIV	Human immunodeficiency virus
H60	Minor histocompatibility antigen
HRP	Horseradish peroxidase

HSV	Herpes simplex virus
HCMV	Human Cytomegalovirus
HHU	Heinrich-Heine University
HHV	Human Herpesvirus
h.p.i.	Hours post infection
IE	Immediate early
IFN	Interferon
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IgSF	Immunoglobulin superfamily
IL	Interleukin
IP	Immunoprecipitation
i.p.	Intraperitoneal
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
k	Kilo (1000)
kbp	Kilobase pairs
K	Lysine
kDa	Kilo-Dalton
l	Liter
L	Late
LC	Light chain
LCMV	Lymphocytic choriomeningitis virus
Log	Logarithm
Luc	Luciferase gene
m	Milli (1/1000)
µ	Mikro ($1/10^6$)
mAb	Monoclonal Antibody
MCMV	Mouse Cytomegalovirus
Met	Methionine
MEF	Mouse embryonic fibroblast

MEM	Minimal Essential Medium
MHC	Major histocompatibility complex
MIEP	Major immediate early promoter
min	Minute
mg	Milligram
mM	Milli molar
MOI	Multiplicity of infection (pfu/cell)
MULT1	Mouse UL16-binding protein-like Transcript 1
mRNA	Messenger RNA
MWCO	Molecular weight cut off
µg	Microgram
NA	Natural antibodies
NCS	Newborn calf serum
n.s.	Not significant
NK	Natural Killer cells
NKG2D	Natural-killer group 2, member D
NN	Non-neutralizing
nm	Nanometer
ng	Nanogram
NP-40	Nonidet P-40 Detergent
n.t.	Not tested
NT	Neutralization
O.D.	Optical density
ORF	Open reading frame
PAGE	Polyacrylamide –Gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PI	Propidium iodide
pIGR	Poly IgA receptor
PFU	Plaque forming units
pH	*potentia hydrogenii (lat.)*
PMSF	Phenylmethan-Sulfonylflouride
pp	Phosphoprotein

R	Arginine
RAE-1	Retinoic acid early inducible 1
RAG	Recombination activating gene
RIPA	Radioimmunoprecipitation assay buffer
RLU	Relative light units
RNA	Ribonucleic acid
RSV	Respiratory Syncytial Virus
RT	Room temperature
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute (cell culture medium)
SD	Standard deviation
SDS	Sodium dodecylsulphate
sec	Second
SHIV	Simian human immunodeficiency virus
SN	Culture supernatant
SPF	Specific pathogen free
ss	Single Strand
STAT2	Signal Transducer and Activator of Transcription
SVG	Salivary gland virus
TBE	Buffer: Tris, Borat and EDTA in Water
TEMED	Tetramethylethylenediamine
TGFß	Transforming growth factor beta
TNF	Tumor necrosis factor
TRL	Terminal repeats long
TVA	Tierversuchanlage (animal facility)
UL	unique long
US7/8	unique shorts 7/8
ULBP	UL16-binding protein
US	United States of America
UV	Ultraviolet light
V	Volt
VACV	Vaccinia Virus
vFcγR	Viral Fc gamma Receptor
VSV	Vesicular stomatitis virus

VSB	Virus standard buffer
VZV	Varizella Zoster Virus
v/v	Volume/volume
Wt	Wild type
w/v	Weight/volume

SUMMARY

Despite the fact that IgG antibodies have been attributed a prominent role in limiting viral infection during the acute and memory phase, little is known how genetic factors of the host shape antibody effector responses to viruses. I have systematically characterized and compared two mouse strains that have distinctive susceptibilities to mouse cytomegalovirus (MCMV) infection, namely BALB/c (susceptible) and C57BL/6 (resistant), with focus on the total and virus-immune IgG, IgG subclass composition and IgG mediated effector functions. Besides virion neutralization, crucial IgG mediated immune control mechanisms include the activation of host FcγRs (FcγRI/CD64, FcγRII/CD32, FcγRIII/CD16, FcγRIV) through antibody-bound antigens which elicit important cell-mediated immune functions such as degranulation and killing of target cells by cytotoxic effector cells, cytokine secretion and phagocytosis. A novel *in vitro* method for detection of IgG Abs able to trigger host FcγRs was recently established in our group allowing to define the fraction of FcγRIII/CD16-activating IgG in BALB/c and C57BL/6 during all phases of MCMV infection, followed by a first identification of prototypic MCMV antigens able to trigger CD16 by monoclonal IgGs.

No major differences between BALB/c and C57BL/6 mice concerning the total amount of virus-specific IgG and IgM, their capacity to perform neutralization and to elicit FcγRIV-responses were observed, excluding a direct correlation with the resistant phenotype of C57BL/6 mice. Despite this, remarkable differences in the subclass composition of immune IgG (IgG2a > IgG2b > IgG3 > IgG1 in BALB/c versus IgG2b ≥ IgG2c > IgG3 > IgG1 in C57BL/6) were noted which might account for the differences in magnitude and effector quality of CD16-activating IgG responses. The much faster and stronger CD16-activation titres elicited in susceptible BALB/c compared with C57BL/6 argue for a less effective role of the IgG2c isotype in triggering CD16 when compared with IgG2a. Moreover, a strong increase in the overall concentration of IgG2a and IgG2c antibodies during and after MCMV-replication was noted in both strains of mice, leading to a long lasting IgG-subclass-selective hypergammaglobulinemia.

Screening of a large library of MCMV-specific mAbs generated from MCMV-infected mice by ELISA, FACS, immunoprecipitation, virion neutralization and CD16-activation assays revealed that a small fraction of IgG (4 out of 104 mAbs) is able to mediate CD16 activation in the presence of infected cells. Two of the mAbs with different IgG isotypes recognize MCMV gB, making this glycoprotein a major target not only for neutralizing, but also for CD16-activating Abs. IgG Abs eliciting CD16-effector functions represent new candidates for therapeutic intervention and should be further assessed for their *in vivo* protective capacities in MCMV-infected mice.

ZUSAMMENFASSUNG

Obwohl IgG-Antikörpern seit langem eine zentrale Rolle bei der Begrenzung von Virusinfektionen in der Akut- und Gedächtnisphase zugeschrieben wird, ist wenig darüber bekannt, inwieweit genetische Faktoren des Wirts Einfluss auf Antikörperantworten und antivirale Effektormechanismen nehmen. In der vorliegenden Arbeit wurden daher zwei Mausstämme mit unterschiedlicher Sensitivität gegenüber der Infektion mit dem murinen Cytomegalovirus (MCMV), nämlich BALB/c (anfällig) und C57BL/6 (resistent), systematisch im Hinblick auf Gesamt- und Immun-IgG, IgG-Subklassen-Zusammensetzung und IgG-vermittelte Effektorfunktionen verglichen.

Neben der direkten Virusneutralisation vermitteln IgG-Antikörper durch die antigenabhängige Aktivierung wirtseigener Fcγ-Rezeptoren (FcγRI/CD64, FcγRII/CD32, FcγRIII/CD16, FcγRIV) wichtige zellvermittelte Immunfunktionen wie die Aktivierung und Degranulation zytotoxischer Effektorzellen, Zytokinsekretion und Phagozytose. Ich habe eine durch unsere Gruppe etablierte neue *in vitro* Methode zum Nachweis FcγR-aktivierender IgG-Antikörper zur Messung des Anteils FcγRIII/CD16-aktivierender IgG-Antikörper in BALB/c und C57BL/6 im Infektionsverlauf verwendet und konnte erstmals prototypische MCMV-Antigene identifizieren, die die Bildung CD16-aktivierender IgG-Antikörper auslösen.

Die Messungen der Gesamtmenge an Virus-spezifischem IgG und IgM, der Neutralisationstiter und der FcγRIV-aktivierender Antikörper ergaben keine großen Unterschiede zwischen BALB/c und C57BL/6 Mäusen. Im Gegensatz dazu ergaben sich bemerkenswerte Unterschiede in der Zusammensetzung der IgG-Subklassen (IgG2a > IgG2b > IgG3 > IgG1 in BALB/c gegenüber IgG2b ≥ IgG2c > IgG3 > IgG1 in C57BL/6) und ein schneller ansteigender und auf Dauer höherer CD16-Aktivierungstiter in BALB/c im Vergleich zu C57BL/6-Mäusen. Dies deutet auf eine geringere Effektivität des IgG2c-Isotyps bei Auslösung von CD16-vermittelten Antworten im Vergleich zu IgG2a hin. Schließlich wurde eine starke Vermehrung der IgG2a bzw. IgG2c Antikörperkonzentration während und in Folge der MCMV Replikation in beiden Mausstämmen nachgewiesen, so dass eine langanhaltende Subklassenspezifische Hypergammaglobulinämie durch MCMV induziert wird.

Das Screening einer Bibliothek von MCMV-spezifischen monoklonalen Antikörper (mAK) in ELISA, Durchflußzytometrie, Immunpräzipitation, Virusneutralisationstest und CD16-Aktivierungsassays zeigte, dass nur ein kleiner Anteil dieser IgG (4 von 104 mAk) CD16-Aktivierung vermittelt. Zwei mAk unterschiedlichen IgG-Subtyps erkennen MCMV gB und identifizieren damit gB als ein wichtiges Zielantigen nicht nur für die Neutralisation, sondern auch für CD16-Aktivierung. IgG Ak mit CD16-Effektor-Funktionen stellen somit eine neue Option zur Herstellung therapeutischer Antikörper dar und sollten in Hinblick auf *in vivo* Schutzfunktion in MCMV-infizierten Mäusen untersucht werden.

I. INTRODUCTION

I.1 VIRUSES

The general accepted definition for a virus is referred to a submicroscopic infectious agent, obligatory intracellular parasite, not being able to replicate outside the living cells. Viruses do not have their own enzymatic equipment necessary for growing and multiplication and therefore require a host cell to make new viral particles. The word '*virus*' derives from Latin and it can be translated with '*poison*' or '*noxious substance'* (Douglas Harper, 2012).

The existence of viruses was first related with the ability of some pathogens to pass through Chamberland filters that otherwise stop bacteria. Studying the agent of tobacco mosaic in 1892, Dimitrii Ivanovsky proved the filterability of this agent, but he kept looking for a microbe or a filterable toxin produced by a small bacterium (Ivanovsky, 1882). In 1898 Martinus Willem Beijerinck confirmed the filterability of tobacco mosaic agent and published that infection of tobacco plants is not due to a microbe (a *contagium fixum*), but to a non-corpuscular entity which he named *contagium vivum fluidum* (Beijerinck, 1898). In the same year, Friedrich Loeffler and Paul Frosch reproduced the filterability concept on foot-and-mouth disease of cattle, concluding that the agent is not soluble but 'corpuscular' because it is retained by a fine-pored Kitasato filter. They included in the group of these '*minutest organisms'* agents of other infectious diseases of man and animals, such as smallpox, cowpox, scarlet fever, measles, thyphus and rinderpest (Loeffler and Frosch, 1898). In 1901 Walter Reed and his team discovered the first human virus, yellow fever virus (Reed et al., 1991).

By the start of the twentieth century, the initial term of a filterable agent, too small to be observed in the light microscope but able to cause disease by multiplying in living cells and tissue has rapidly evolved into what we know nowadays as '**virus**'.

Viruses display a wide diversity of morphologies, with a diameter between 10 and 300 nanometres. In general, their structures were determined using a transmission electron microscope. The genomic component of a virion consists of nucleic acid (either DNA or RNA) and is surrounded by a protective coat of proteins coded by the viral genome called *capsid* or *nucleocapsid*. Many viruses have also a lipid *envelope* derived from the host cell membrane (Caspar and Klug, 1962). After attachment and penetration into the

proper host cell, viral particles are able to replicate their genome and the newly formed virions are released from the host cell by lysis or budding (Collier et al., 1998).

I.1.1 Herpesviridae family

The *Herpesviridae* is a large family of DNA viruses that causes diseases in animals, including humans**.** Herpesvirus infections have been described as early as ancient Greek times. Hippocrates spoke about the cutaneous spreading of herpes simplex lesions and scholars of Greek civilization define the greek word *'herpes'* to mean *'to creep or crawl'* in reference the spreading nature of the herpetic skin lesions. In Roman times, the emperor Tiberius tried to stop an outbreak of mouth herpes by prohibiting kissing at public events and ceremonies.

Herpesviruses are highly disseminated in nature. There is at least one herpesvirus for almost each animal species, more than 200 herpesviruses being identified to date (Pellett and Roizman, 2007).

For humans, eight Herpesviruses have been characterized:

- *Herpes simplex virus 1* (HSV-1), *herpes simplex virus 2* (HSV-2) and *varicella-zoster virus* (VZV) belong to the *Alphaherpesvirinae* subfamily;
- *Human cytomegalovirus (HCMV), Human Herpesviruses 6* and *7* (HHV-6 and HHV-7) belong to the *Betaherpesvirinae* subfamily;
- *Epstein-Barr virus* (EBV) and *Human Herpesviruses 8* (HHV-8) belong to *Gammaherpesvirinae* subfamily.

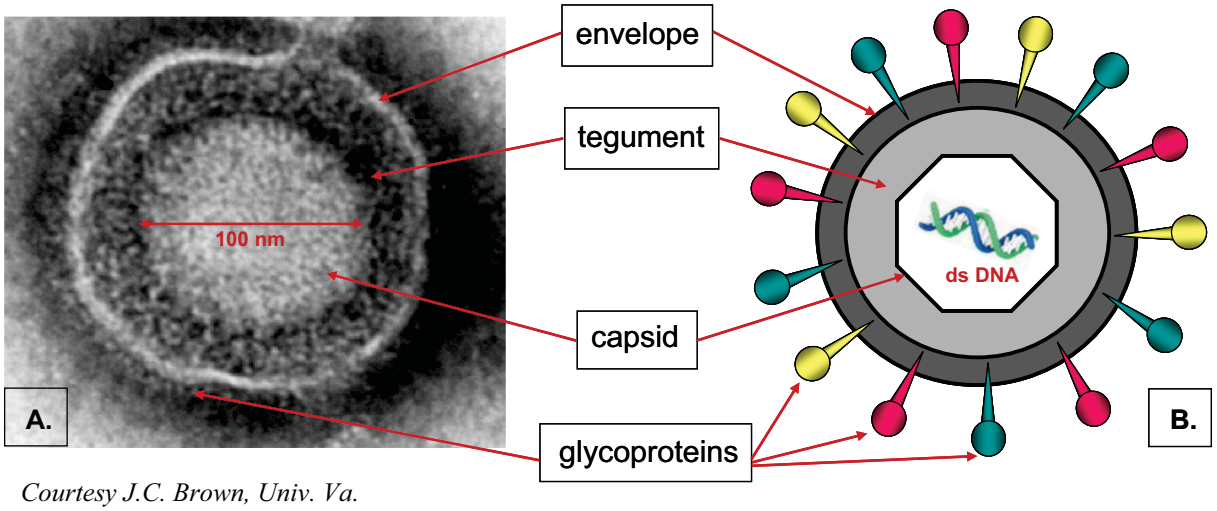
The classification of Herpesviridae family into the three subfamilies: *α-Herpesvirinae, β-Herpesvirinae* and *γ-Herpesvirinae* was realized in the late 1970s, on the basis of their biological properties such as host species specificity, growth kinetics, the ability to grow in cultured cells and clinical manifestations of disease they cause (Pellett and Roizman, 2007).

Despite these differences, all members of Herpesvirus family share common features based on the architecture of the virion.

A typical herpesvirion is composed of:

- a *core* containing a linear double-stranded DNA, with a variable length ranging from 124-230 kb (**Fig. I.1**);
- an icosahedral protein *capsid* approximately 125 nm in diameter and containing 162 capsomeres;
- an asymmetric protein layer called *tegument,* that surrounds the viral capsid;

- the *envelope*, a lipid bilayer membrane containing viral glycoproteins on its surface.



Courtesy J.C. Brown, Univ. Va.

Figure I.1 Herpesvirus virion architecture. The envelope, tegument, capsid and glycoproteins are indicated by red arrows. **A**. Electron microscopy image of HSV-1 structure kindly provided by Prof. Jay C. Brown, University of Virginia Cancer Center, Charlottesville, USA; **B**. Schematic representation; ds DNA, double-stranded DNA.

All members of the family *Herpesviridae* share four significant biological properties:

- they have a large number of enzymes with functions in nucleic acid metabolism, DNA synthesis and protein processing;
- viral DNA synthesis and capsid assembly take place in the nucleus;
- the lytic infection ends up with the formation of new virus particles and the destruction of the infected host cell;
- all described herpesviruses are able to remain latent in their natural hosts. In the latent phase viral genomes become closed circular molecules and only a reduced number of viral genes is expressed. During latency, herpesviruses do not replicate and infectious progeny is not produced. Reactivation from latency occurs differently for each member of this family via a mechanism that is not completely understood (Pellett and Roizman, 2007).

I.1.2 Cytomegaloviruses

Cytomegalovirus (CMV) is a prototypic member of the ß-subfamily of herpesviruses. It has a worldwide distribution and is characterized by salivary gland tropism, strict species specificity and slow replication in cultured cells (Mocarski et al., 2007).

In the past, cytomegaloviruses were also known as salivary gland viruses (SVG) (SMITH, 1956), until the actual name was proposed by Weller *et al*. based on the typical cytopathic effect on infected cells, involving characteristic nuclear and cytoplasmic inclusions and cell enlargement (Weller et al., 1960) (from Greek words *Kytos* = *Cell* and *megas* = large).

Human Cytomegalovirus (HCMV) is an important human pathogen that causes a very high disease burden in immunocompromised patients such as organ transplant recipients, HIV patients and congenitally infected newborns.

Primary infection in immunocompetent hosts usually follows an asymptomatic course and results in a latent infection. In contrast, primary infection or virus reactivation in immunocompromised hosts can cause severe or fatal diseases (Alford and Britt, 1993).

In Europe, congenital CMV infection represents a major cause of neurological disabilities in children, leading to mental retardation, hearing loss, neurodevelopmental delay and blindness (Ludwig and Hengel, 2009).

Due to its high morbidity and mortality in these risk groups, CMV is ranked as a top vaccine goal in the US, but no effective and safe vaccine is available to date. Although a number of CMV vaccines are currently being evaluated in clinical trials. These vaccines are based on different formats, including attenuated live virus, a recombinant protein in a fowl pox vector, a plasmid DNA vaccine and a recombinant protein given with a new adjuvant.

Food and Drug Administration (FDA) currently approved four antiviral agents shown to reduce or eliminate viremia or CMV shedding and to prevent and control CMV disease in immunocompromised patients. Their generic (trade) names are: Ganciclovir (Cytovene®), Valganciclovir (Valcyte®), Foscarnet (Foscavir®) and Cidofovir (Vistide®). Due to their significant toxicity, these agents have a reduced and limited utility for risk patients (Mocarski et al., 2007).

A safe and effective therapy for prevention of CMV disease in organ transplant recipients, premature newborns at risk and pregnant women could be represented by the

passive immunization with hyperimmune globulin preparations containing high levels of IgG antibody to CMV (CMVIG) (Mocarski et al., 2007; Nigro et al., 2005).
Ideally, immunizations for CMV would first be evaluated in animal models prior to human clinical trials. Unfortunately, the strict species specificity of CMVs does not permit infection of laboratory animals with human CMV (HCMV), reducing the possibilities to study the protective effects of a CMV therapy. As a result, several animal models for CMV immunity and pathogenesis research are described. Due to ethical difficulties and expensive costs of primates and big animals, small animal models such as guinea pigs and mice are preferred (Staczek, 1990).

I.1.3 Mouse Cytomegalovirus

Mouse Cytomegalovirus (MCMV) is the best characterized member of the animal CMVs. The natural host for MCMV is the house mouse *Mus musculus domesticus,* although MCMV is able to infect both laboratory and free-living wild mice.
Because the biological features of MCMV and HCMV infection are very similar, the mouse model has been extensively used for studies involving pathogenesis of acute, latent and recurrent virus infections (Krmpotic et al., 2003).
The virion of MCMV follows the structure of a typical herpesvirus, with a larger diameter of about 230 nM and with a more irregular envelope. The envelope contains several viral glycoproteins that are potential targets for antiviral antibodies directed against the virion (including neutralizing antibodies) or against infected cells. The major glycoprotein is the highly conserved glycoprotein B (gB), which is a dominant B-cell antigen in CMV-infected animals and humans. Glycoprotein B and the heterotrimeric complex gCIII composed of MCMV glycoproteins gH, gL and gO are essential for viral entry into the cells (Huber and Compton, 1998).
The core contains the dsDNA viral genome, which is packaged as a single linear molecule into the icosahedral protein capsid. The 235 kilobase pairs (kbp) MCMV genome was initially described as coding for 170 open reading frames (ORFs) (Rawlinson et al., 1996). Recently, further 34 ORFs have been predicted to exist in the MCMV genome (Brocchieri et al., 2005).
Expression of MCMV genes is tightly coordinated and occurs in a sequential manner with immediate early (IE), early (E) and late (L) kinetics. These genes are grouped in three gene families: α, β and γ, respectively.

IE transcription starts after the virus entry into the host cell and the viral genome is delivered to the nucleus. IE genes are under the control of major IE promoter (MIEP). A second phase of E gene expression follows, starting by 6 hours post infection (h.p.i.) and continues through 18 to 24 h.p.i. when viral DNA synthesis initiates. The third L phase is represented by genes which transcription depends on genome replication and maximum expression is seen after 24 h.p.i, mostly viral structural proteins.

There are several MCMV strains described to date, but only the Smith and the K181 strains have been widely used. The Smith strain of MCMV (ATCC VR-1399) was isolated by Margaret Smith in 1954 from the salivary gland tissue of infected laboratory mice (SMITH, 1954). The K181 strain of MCMV was isolated in 1982 by June Osborn and was considered as a more virulent Smith strain variant [cited in (Misra and Hudson, 1980)]. Since then, several low-passage isolates of MCMV were isolated from wild mice and their viral genomes were sequenced and mapped. It is well described that extensively passaged laboratory strains of HCMV differ significantly from clinical isolates. Regarding the MCMV, it was also found that between laboratory strains of MCMV and wild derived strains there are genotypic and phenotypic differences (Smith et al., 2008). Besides this, mouse inbred strains were found to have dramatic differences with regard to their susceptibility to MCMV infection (Schleiss, 2006). For example, BALB/c mice are considered to be susceptible to MCMV infection. In contrast, resistance of C57BL/6 mice strain to MCMV infection was associated with NK (natural killer) cell mediated protection (Scalzo et al., 1992). This protection is correlated with the presence of the NK cell activatory receptor Ly49H on the surface of NK cells, which interacts with the MCMV-encoded protein m157 on the surface of infected cells. This interaction activates NK cells, and as a result interferon-gamma and perforin are produced and infected cells are destroyed.

I.2 MCMV IMMUNE EVASION

I.2.1 Immune response to MCMV

In adult immunocompetent mice, a primary MCMV infection is controlled by a combination of coordinated innate and adaptive (acquired) immune responses. The cellular immunity and interferons play a central role in controlling primary MCMV

infection, establishment of latency and prevention of a productive virus reactivation (Lucin et al., 1994; Polic et al., 1998; Reddehase, 2002).

I.2.1.1 NK cell response

NK cells represent a component of the innate system, the first line of defence against a viral infection, along with macrophages. An effective NK cell response after MCMV infection is triggered in the resistant mouse strain C57BL/6 and is controlled by the *cmv-1* locus located inside the NK gene complex on mouse chromosome 6. This resistance is correlated with a reduction of MCMV replication in the spleen (Scalzo et al., 1990; Scalzo et al., 1992). $Ly49H^+$, the activator receptor encoded by the *cmv-1* gene locus and present on NK cells binds specifically to a protein encoded by the MCMV *m157* gene, a viral MHC I homolog.

In contrast, BALB/c mice, which are Ly49H negative, express another activator receptor on NK cells, called NKG2D. Their susceptibility to MCMV infection was explained by the ability of the virus to down-regulate the ligands of NKG2D receptor on infected cells (Krmpotic et al., 2002).

I.2.1.2 T cell immune response

$CD8^+$ T cells exert an essential role in virus clearance, demonstrated by adoptive cell transfer experiments. However, $CD8^+$ T cells deficient mice developed an efficient immune effector function and eliminated the virus from tissues with similar kinetics to normal mice (Polic et al., 1996). These results suggested that $CD4^+$ T cells were able to compensate the antiviral activity of $CD8^+$ T cells in animal hosts lacking the $CD8^+$ T cell subset. Additionally to $CD4^+$ T cells, macrophages are also required for limiting the viral spread (Selgrade and Osborn, 1974). An exception from this rule is represented by the fact that $CD8^+$ T cells were not able to eliminate the virus from salivary glands in mice depleted of $CD4^+$ T cells.

In conclusion, for an effective clearance of MCMV and prevention of horizontal transmission of infection, functions of both $CD8^+$ and $CD4^+$ T cells are required.

I.2.1.3 The role of IFNs and TNF-α during MCMV infection

IFN-α/β/γ and TNF-α are described to play an essential role in limiting virus replication during the early stage after infection. IFN-α/β is produced by a subset of dendritic cells and is important for the activation of NK cells, as well as specific $CD8^+$ T cells (Dalod et al., 2003). At a later stage of infection, IFN-γ and TNF-α preserve the presentation of peptide-loaded MHC class I molecules to $CD8^+$ T cells (Hengel et al., 1994; Hengel et al., 1995).

I.2.1.4 Antibody response to MCMV infection

Although during primary MCMV infection there is no prominent role for antibodies regarding organ clearance, antiviral antibodies are shown to prevent virus dissemination after reactivation from latency or reinfection (Jonjic et al., 1994; Polic et al., 1998). It is generally accepted that antiviral antibodies mediate their protective activity by direct virus neutralization or indirectly via complement activation and by promoting antibody-dependent cellular cytotoxicity (ADCC). Therefore, it is expected that antibodies may suppress CMV dissemination by direct virus neutralization and/or by cytotoxicity of cells carrying the virus. Moreover, adoptively transferred memory B cells from immune animals into T- and B-cell-deficient RAG-$1^{-/-}$ mice protected from MCMV infection by reduction of viral titres and DNA copies in several organs. The effect of memory B cells on the replication of MCMV is correlated with the production of protective antibodies. As well as memory B cells, transfer of serum from MCMV-infected animals into RAG-$1^{-/-}$ deficient mice had a comparable protective effect against MCMV infection.
In humans, administration of CMV hyperimmune globulin (HIG) to pregnant women with a primary CMV infection was effective regarding both endpoints i) the severity of HCMV disease and ii) prevention of foetal HCMV infection (Adler and Nigro, 2008; Adler and Nigro, 2009; Nigro et al., 2005; Nigro et al., 2008).

I.2.2 Immune evasion mechanisms of MCMV

Coevolution of CMVs with their hosts over millions of years has lead to sophisticated and highly complex strategies to escape recognition of immune effector cells. HCMV, as well as MCMV are described to code for a number of proteins that interfere with the

hosts NK/ T cells and antibody protection mechanisms. MCMV genes coding for these proteins and their immune-evasion functions are listed in **Table I.1**.

Table I.1 Immune evasion genes and their functions during immune response to MCMV

MCMV ORF	Function	References
m152/ gp40	-Inhibition of antigen presentation to $CD8^+$ T cells; -Retention of MHC class I complexes in the ERGIC/cis-Golgi -Down-modulation of RAE-1 family members	(Del Val et al., 1989; Del Val et al., 1992) (Ziegler et al., 1997) (Lodoen et al., 2003)
m04/ gp34	-Binds MHC class I and rescues its expression on the cell surface, possible NK cell decoy	(Kavanagh et al., 2001; Kleijnen et al., 1997)
m06/ gp48	Targeting of MHC I complexes into the endosome/lysosome for degradation	(Reusch et al., 1999)
m144	MHC I homologue: inhibition of NK function in vivo	(Cretney et al., 1999; Farrell et al., 1997; Kubota et al., 1999)
m131	ß chemokine homologue -proinflammatory effects: promotes dissemination to or replication of MCMV in salivary glands	(MacDonald et al., 1997) (Fleming et al., 1999)
M33	G-protein coupled receptor (GCR) homologue -promotes dissemination to or replication of MCMV in salivary glands -induces vascular smooth muscle cell migration	(Davis-Poynter et al., 1997) (Melnychuk et al., 2005; Waldhoer et al., 2002)
M78	Putative G-protein coupled receptor (GCR) homologue -facilitates accumulation of immediate-early mRNA	(Beisser et al., 1999; Oliveira and Shenk, 2001)
m138 /gp86/88/105	-Expression of a viral Fc receptor (fcr-1), binding of murine IgG; -down-modulates MULT-1 and H60; -interferes with clathrin-dependent endocytosis -down-modulates RAE-1ε	(Crnkovic-Mertens et al., 1998; Thale et al., 1994) (Lenac et al., 2006) (Arapovic et al., 2009)
m157	-Binding to Ly49H and activates NK cells (in C57BL/6 mouse strain) -Binding to Ly49I and inhibits NK cells (in MCMV-susceptible mouse strains)	(Smith et al., 2002) (Arase et al., 2002)
m155	Down-regulates the NKG2D ligand H60	(Hasan et al., 2005;

		Lodoen et al., 2004)
m145	Reduces expression of stress-induced MULT-1 molecule	(Krmpotic et al., 2005)
m147.5 (modB7-2)	-Encodes a spliced transcript -Down-regulates expression of the costimulatory molecules CD86 on the surface of antigen-presenting cells	(Loewendorf et al., 2004)
M44 /pp50	DNA polymerase accessory protein gene Role in MCMV replication cycle	(Loh et al., 1999; Loh et al., 2000)
unknown	Activates Ly49P on NK cells in context of H-2D^{k}	(Desrosiers et al., 2005) (Adam et al., 2006)
M27	-Viral determinant required for optimal MCMV growth and virulence in vivo; -encodes an inhibitor of STAT2 (Signal Transducer and Activator of Transcription) that disrupts IFN-gamma receptor signalling; -it forms complexes with DNA-damage DNA-binding protein 1 (DDB1)	(Abenes et al., 2001; Khan et al., 2004; Trilling et al., 2011; Zimmermann et al., 2005)

I.3 ANTIBODIES AND THEIR EFFECTOR FUNCTIONS FOR VIRUS CONTROL

Antibodies, also known as *Immunoglobulins (Ig)* are important components of the humoral immune response that mediate an efficient removal of pathogens from the organism. Immunoglobulins are glycoproteins synthesized and secreted by effector plasma cells that are derived from the B cells of the immune system.

The term of *antibody* or *antitoxin* was first mentioned in 1890 when Emil von Behring and Kitasato Shibasaburo studied diphtheria and tetanus toxins. They described the antibody activity and put the theory of *humoral immunity*, proposing that a mediator in serum could react with a foreign antigen (AGN, 1931). Seven years later, Paul Ehrlich proposed the *'side chain theory'* for antibody and antigen interaction. This theory described that receptors (referred here as *'side chains'*) on the surface of cells could bind specifically to toxins in a 'lock-and-key-interaction' that triggers the production of antibodies (Winau et al., 2004). Antibody opsonisation, as well as its protein structure were later on described and characterized.

Antibodies exist as five distinct classes or isotypes: IgA (Immunoglobulin A), IgE (Immunoglobulin E), IgM (Immunoglobulin M), IgG (Immunoglobulin G) and IgD (Immunoglobulin D). They have different structures, biological activities and

distributions in the body. The basic functional unit of each antibody is an immunoglobulin (Ig) *monomer*. Secreted antibodies can also be *dimeric*, with two Ig units, like IgA, or *pentameric* with five Ig units like IgM (Roux, 1999).

The general structure of an Ig monomer has a 'Y'-shape with two identical heavy chains (HC) and two identical light chains (LC), each folded into globular immunoglobulin domains and connected by disulphide bonds (Woof and Burton, 2004) (**Fig. I.2**).

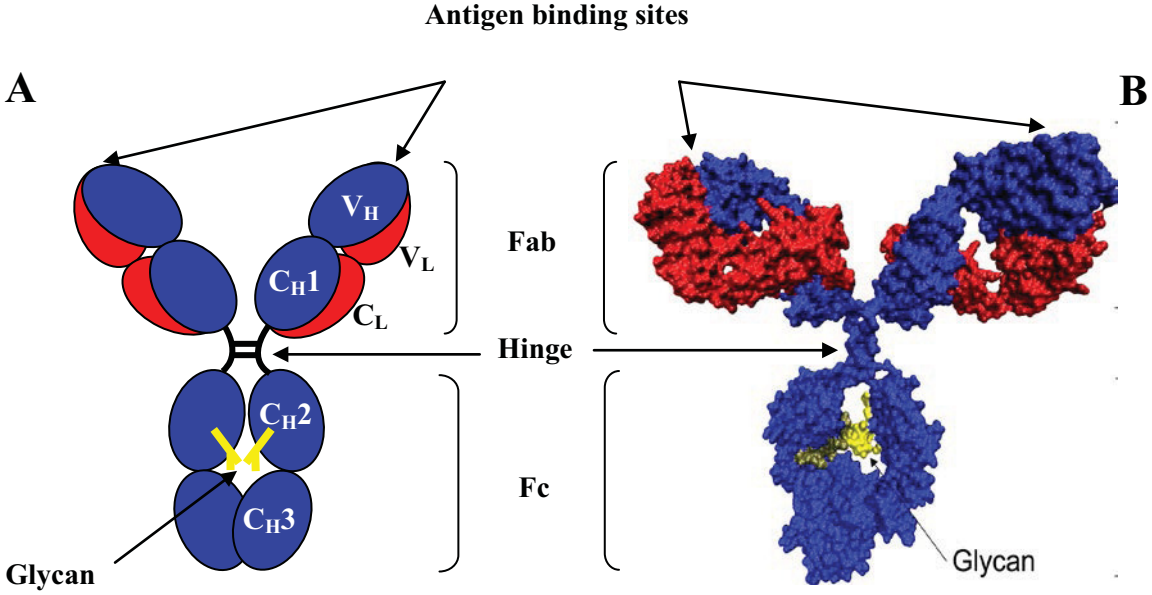


Figure I.2 The structure of monomeric IgG. A-schematic representation of monomeric IgG; heavy chains (HC) are coloured in blue and the light chains (LC) in red; brackets indicate the antigen-binding Fab' portion and the Fc portion of IgG; arrows indicate the antigen binding sites, the hinge region and the two conserved glycans (yellow); V_L, V_H - variable region of light and heavy chains respectively; C_L , C_H - constant region of light and heavy chains respectively; B- Crystal model of IgG generated by Mike Clark, Cambridge University, UK (Clark, 1997).

IgG represents 75% from all immunoglobulins in serum and provides the majority of antibody-based immunity against pathogens. IgG is the only antibody crossing the placenta conferring passive immunity to the foetus and has a crucial protective capacity against bacteria and viruses.

Based on small differences in the amino acid sequences in the constant region of the heavy chain and on differences in the hinge region, IgG can be divided into 4 subclasses: IgG1, IgG2, IgG3 and IgG4 in humans and IgG1, IgG2a/2c, IgG2b and IgG3 in mice.

The structural parts of the antibody have distinct and unique functions. The Fab region (*Fragment antigen-binding*) contains the sites that can bind two antigens. It is composed of one constant and one variable domain of each of the heavy and light chain (**Fig. I.2**). The two variable domains contain the *paratope (antigen-binding site)* that bind the *epitope* on their specific antigen.

The Fc (*Fragment crystallisable*) region represents the constant part of an antibody. It interacts with cell surface receptors called Fc receptors and some proteins of the complement system, triggering different effector functions, e.g. recognition of opsonised particles, lysis of cells and degranulation of mast cells, basophils and eosinophils.

The protective effects of antibodies can be divided into two groups (Burton, 2002; Crowe, Jr. et al., 2001):

1) One group targeting the free virus particles and preventing infection of host cells:
    a) Neutralization: blocking of virus entry into the cells upon binding of antibodies to the viral attachment proteins;
    b) Fusion inhibition: post-attachment neutralization by inhibiting virus replication after viral particles attach to the host cells;
    c) Virus aggregation: decreasing the number of infectious particles and preventing viral entry upon a multivalent antibody (e.g. IgM) aggregates the virion;
    d) Antibody-dependent virus inhibition (ADVI) and Complement-mediated virolysis are both neutralization mechanisms mediated by Ab-complement fixation and deposition of complement components on the virion surface, respectively;
    e) Fc-mediated opsonisation and subsequent elimination of the virus by phagocytic cells;

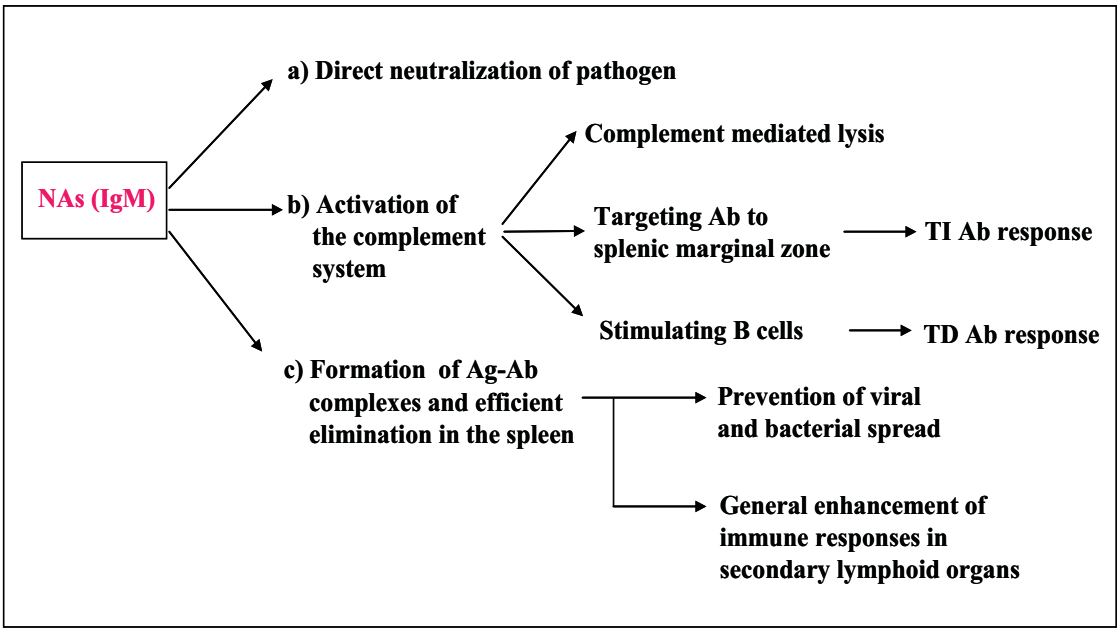
2) Another group is targeting the virus-infected cells, preventing virus replication and cell-cell transmission:
    a) Complement-dependent cytotoxicity (CDC): activation of the complement cascade that leads to lysis of infected cells;
    b) Antibody-dependent cell cytotoxicity (ADCC) is mediated through Fcγ receptors present on different effector cells (NK cells, macrophages,

neutrophils and dendritic cells); as a result, the effector cell is activated and degranulates, thus inducing a complete lysis of infected cell;

c) Apoptosis of infected cells induced upon binding to host Fcγ receptors and activation of death receptors.

I.3.1 Natural antibodies: first line of defence

Natural antibodies (NAs) represent an important link between innate and adaptive immunity and play a major role in the early protection of the host against an antigen reaching the blood and systemic distribution. NAs are present in low concentrations in serum of naïve humans before a viral infection. These antibodies have low-affinity (mostly IgM isotype) and are polyreactive with a variety of self or foreign antigens, including proteins, nucleotides, polysaccharides and lipids (Ochsenbein and Zinkernagel, 2000). Even though NAs were often excluded from immunological assays as 'background' (Ochsenbein et al., 1999), it was shown that they are able to limit dissemination of the pathogen present in the circulation by direct neutralization or indirectly by activation of the complement system and formation of immune complexes (Ochsenbein and Zinkernagel, 2000) (**Fig. I.3**.).



Modified from A.F. Ochsenbein and R.M. Zinkernagel, Immunology today, 2000

Figure I.3 Direct and indirect protection mechanisms of natural antibodies (NAs). NAs can activate complement system and enhance the specific Ab response, leading to a more efficient control of the

infection. These links to the adaptive immunity are highlighted in the scheme. Abbreviations: Ab, antibody; Ag, antigen; NA, natural antibody; TI, T-cell independent; TD, T-cell dependent.

The natural restriction of early viral dissemination and recruitment of viral antigens to secondary lymphoid organs represent important steps for priming of an adaptive immune response.

Spontaneous pre-immune neutralization titres of NAs can vary from relatively high for cytopathic viruses (e.g. 1:8 to 1:32 for vesicular stomatitis virus-VSV) (Ochsenbein et al., 1999) to low or below detection levels for poorly cytopathic viruses (e.g. < 1:2 against lymphocytic choriomeningitis virus-LCMV) (Hangartner et al., 2003). This difference could be due to the high avidity of the antibodies for the particular neutralizing determinants exhibited by the cytopathic viruses, thereby decreasing the lethality of the host and providing natural resistance.

I.3.2 Neutralizing versus non-neutralizing antibodies

Upon a viral infection, multiple antibody specificities against many viral proteins are produced. But only a small fraction of these antibodies exert direct antiviral effect *in vitro* and they are named neutralizing antibodies. The rapid kinetics of the neutralizing antibody production represents a decisive factor for host survival upon an acutely cytopathic virus infection (Lutz et al., 1998). Induction of early neutralizing IgM antibodies, independently of T-cell help, might overcome the delayed priming of $CD4^+$ T helper-cell responses (Hangartner et al., 2006).

A very important characteristic of the neutralization activity is the relatively high affinity and/or avidity of the antibody for the antigens expressed on the surface of the virion, early in the primary immune response (Bachmann et al., 1997; Roost et al., 1995). Higher affinity of an antibody correlated with increased antiviral activity. Therefore it is speculated that the important role of the somatic hypermutation (SHM) of antibody genes is not only to increase the diversity of epitopes recognized by the repertoire, but also to improve the affinity of individual B cell clones for their epitopes (Crowe, Jr. et al., 2001). IgM to IgG isotype class switching is crucial for the maintenance of protective high antibody titres that are necessary for the long-term control of viruses (Hangartner et al., 2006). Binding of neutralizing antibodies can induce conformational changes that abrogate the functionality of the viral surface protein and thus prevent virus entry into the host cell (Outlaw and Dimmock, 1993).

The majority of virus-specific antibodies, however, have no neutralizing activity. They are elicited by virion fragments or by viral proteins released from dying, infected cells (Battegay et al., 1993) and are directed to native proteins including surface antigens without any interference with viral attachment or entry (Lefrancois and Lyles, 1982). Non-neutralizing antibodies that bind to surface-accessible proteins were found to be protective against several viral infections by activating the complement system, enhancing phagocytosis and/or promoting ADCC. Most of these biological activities are mediated via interactions between the Fc part of the antibody and Fc receptors present on various immune effector cells.

ADCC and neutralizing antibodies differ from each other in that ADCC antibody is directed against infected cells and triggers cell death, whereas neutralizing antibody is directed against free virion and promotes virus inhibition.

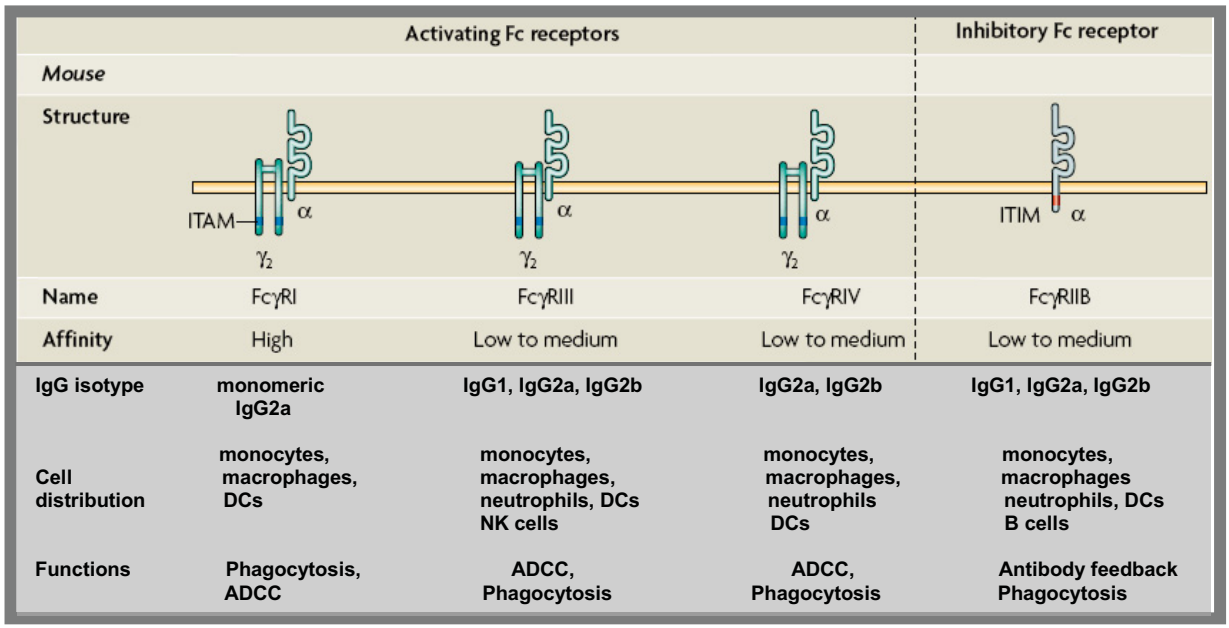
I.4 CELLULAR FCγ RECEPTORS (FCγR)

Fc receptors (FcRs) are type I transmembrane glycoproteins widely distributed on innate immune effector cells, such as macrophages, monocytes, neutrophils, dendritic cells (DC), NK cells and B cells and bind with different affinities the constant Fc part of an antibody. There are FcRs for each class of immunoglobulins and their names contain the Greek letter corresponding to the Latin letter describing the type of antibody they recognize. There are known FcRs for IgG (FcγRs), for IgA (FcαRs), for IgE (FcεRs), for IgM (FcμRs) and for IgD (FcδRs).

FcRs are considered to form a link between innate and adaptive immunity (Nimmerjahn et al., 2005), mediating varying effector functions (e.g. FcγRs, FcεRs, FcαR) or the transport of Ig across epithelial surfaces: poly IgA receptor (pIgR) and neonatal FcR (Ravetch, 1997).

Depending on the cell type, FcRs with immunoreceptor tyrosine-based activation motifs (ITAMs) trigger macrophage phagocytosis, NK cell ADCC, neutrophil activation, whereas those with immunoreceptor tyrosine-based inhibition motifs (ITIMs) negatively regulate cell activation (**Fig. I.4**). Due to their co-expression on the same effector cells, the balance between activating and inhibitory signals determines the outcome of an immune response and contributes to the maintenance of cell tolerance and prevention of autoimmune disorders. Based on genetic and structural analysis, four FcγRs subclasses in human and mice were characterized. Mouse FcγRI (CD64), FcγRII

(CD32), FcγRIII (CD16) and the newly described FcγRIV possess different affinities for the various IgG isotypes and each has distinct cell distribution and functions (**Fig. I.4**).



Modified with the permission of Prof. Nimmerjahn F.,University of Erlangen, Germany (from Nimmerjahn F. and Ravetch J.V., Nat.Rev.Immunol., 2008)

Figure I.4. Mouse cellular FcγRs: structure, affinity for Fc fragment of different IgG isotypes, cell distribution and functions. There are three activating FcγRs in mice: FcγRI (CD64), FcγRIII (CD16) and FcγRIV and one inhibitory receptor, FcγRIIB (CD32B). They differ in their affinity to the antibody Fc-fragment and in the signalling pathway they induce. FcγRI binds monomeric IgG2a with high affinity; the others have low to medium affinity for the Fc fragment: FcγRIII and FcγRIIB bind IgG1, IgG2a and IgG2b; FcγRIV has intermediate affinity for IgG2a and IgG2b-immune complexes. The inhibitory FcγRIIB contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic domain and negatively regulates cell activation. All the activating receptors contain immunoreceptor tyrosine-based activating motifs (ITAMs) in the signal-transducing γ-chain dimer and a α-chain with the ligand binding sites.

For a long time it was thought that the different IgG isotypes trigger their *in vivo* effector functions with the help of both complement and FcR system. However, recent studies have shown that the activity of most potent IgG isotypes was not modified in complement-deficient mice, but was completely abrogated in mice deficient in the γ-chain common for all activating FcγRs (Nimmerjahn and Ravetch, 2005; Nimmerjahn and Ravetch, 2006; Ravetch and Clynes, 1998; Sylvestre et al., 1996).

These data suggest that each IgG isotype mediates *in vivo* effector functions by triggering selective activating FcRs (Hamaguchi et al., 2006; Nimmerjahn and Ravetch, 2005).

In mice IgG2a and IgG2b are the most pro-inflammatory IgG molecules, showing a greater activity than IgG1 or IgG3 in clearing bacterial or viral infections, in killing tumor cells or in depleting platelets or red blood cells (Coutelier et al., 1987; Fossati-Jimack et al., 2000; Kaminski et al., 1986; Kipps et al., 1985).

Till recently it was considered that FcγRI and FcγRIII are the obvious candidates for the IgG2a and IgG2b effector functions *in vivo*, as FcγRI binds exclusively monomeric IgG2a with high affinity and FcγRIII binds IgG1, IgG2a and IgG2b with low affinity (Nimmerjahn and Ravetch, 2005).

Recent studies have shown that the IgG2a and IgG2b effects occur independently of these two receptors, but in a γ-chain dependent manner (Fossati-Jimack et al., 2000; Hazenbos et al., 1996; Meyer et al., 1998; Uchida et al., 2004). Only due to recent discovery of the mouse FcγRIV which binds IgG2a and IgG2b-immune complexes with 20-40 times higher affinity than FcγRIII (Nimmerjahn and Ravetch, 2006), it became clear that FcγRIV is important for the activity of IgG2a and IgG2b in models of ADCC and autoimmunity *in vivo* (Nimmerjahn et al., 2010). In contrast, deletion of the low-affinity receptor FcγRIII seriously impaired IgG1-mediated effector functions in models of arthritis, glomerulonephritis, IgG-dependent anaphylaxis, IgG-mediated haemolytic anemia and immunotrombocytopenia (Bruhns et al., 2003; Fossati-Jimack et al., 2000; Fujii et al., 2003; Hazenbos et al., 1996; Ji et al., 2002; Meyer et al., 1998), suggesting that FcγRIII effector functions in these disease models are triggered preferentially by IgG1 immune complexes.

I.5 VIRAL FCγRS

Herpesviruses have developed complex strategies to escape recognition of the immune system, due to a long history of immune selection and coevolution with their hosts. The immune evasion mechanisms target the host T-cell or NK cell response, as well as interferons and antibodies (see Chapter I.2.2 Immune evasion mechanisms of MCMV and **Table I.1**). CMVs, as well as HSV-1 and Coronaviruses (Oleszak and Leibowitz, 1990) encode FcγR-like proteins that limit the cellular FcγR-mediated effector

functions. These proteins bind the constant Fc part of the IgG molecule and interfere with the antibody-triggered effector functions, such as ADCC, complement-dependent neutralization and phagocytosis. The herpesviral-encoded FcγRs and their specific immune-evasion functions are listed shortly in **Table I.2**.

Table I.2 Herpesviral-encoded FcγRs and their immune-evasion mechanisms for viral protection

Herpesvirus	Viral FcγRs	Functions	References
HCMV	gp34(*TRL11*) gp68 (*UL119-118*) gp95 (*TRL12*)	-bind Fc part of human IgG	*(Atalay et al., 2002; Lilley et al., 2001)* *(Sprague et al., 2008)* *(Merce-Maldonado E.-manuscript in preparation)*
MCMV	fcr-1/m138 (*m138*)	-binds Fc part of mouse IgG -down-modulates MULT-1 and H60; -interferes with clathrin-dependent endocytosis -down-modulates RAE-1e	*(Thale et al., 1994; Crnkovic-Mertens et al., 1998)* *(Lenac et al., 2006)* *(Arapovic et al., 2009)*
HSV-1	gE (*US7*): gI (*US8*)	-protects HSV-infected cells from ADCC by 'antibody bipolar bridging'.	*(Dubin et al., 1991; Frank and Friedman, 1989; Johnson et al., 1988)*

II. AIM OF THE THESIS

Besides virus neutralization, a crucial IgG mediated immune control mechanism involves the activation of host FcγRs through immune complexes, i.e. antibody-bound antigens. As a result, several effector functions are elicited, such as degranulation and killing of target cells by NK cells, cytokine secretion, phagocytosis, enhancement of antigen presentation and modulation of dendritic cell functions. An *in vitro* method for detection of IgG Abs able to trigger host FcγRs (e.g. FcγRIII) was recently established by our group. Furthermore, this assay would allow us to define new antiviral IgG effector responses and also to identify the MCMV Ags expressed on the surface of infected cells that trigger immune responses via $Fc\gamma RIII^{+}$ immune cells.

Despite the fact that the general role of antibodies in limiting viral infection has been extensively studied in mouse models over the last years, there is little comprehensive knowledge on differences or similarities in Ab response to herpesviral infections in susceptible versus resistant strains of mice.

Therefore, a systematic comparison of two differentially susceptible strains upon primary MCMV infection with a focus on the total IgG, IgG subclass composition and various IgG mediated effector functions was pursued.

III. RESULTS

III.1 MCMV ANTIBODY RESPONSE IN GENETICALLY RESISTANT AND SUSCEPTIBLE MOUSE STRAINS

III.1.1 Kinetics of MCMV-specific IgM and IgG antibodies in susceptible BALB/c and resistant C57BL/6 mice

The total amount of IgM and the titres of IgG produced in BALB/c and C57BL/6 serum after infection with $4x10^6$ PFU MCMV (Smith strain) were calculated using ELISA measurements. ELISA plates were coated with lysates of mock- or MCMV-infected MEF. Serum samples representing a pool of sera collected from 5 mice / each time point and diluted 1/50 were incubated for 1 h at room temperature. IgM antibodies specific for MCMV antigens were detected by a goat anti-mouse IgM- peroxidase conjugated antibody, following the protocol described in experimental procedures. As shown in **Fig. III.1.A**, MCMV-specific IgM antibodies are produced early after infection in both mouse strains, a maximum amount being reached at day 7 p.i. After this time point, the amount of IgM was decreasing to a constant level around 1 µg/ml maintained till day 60 p.i.

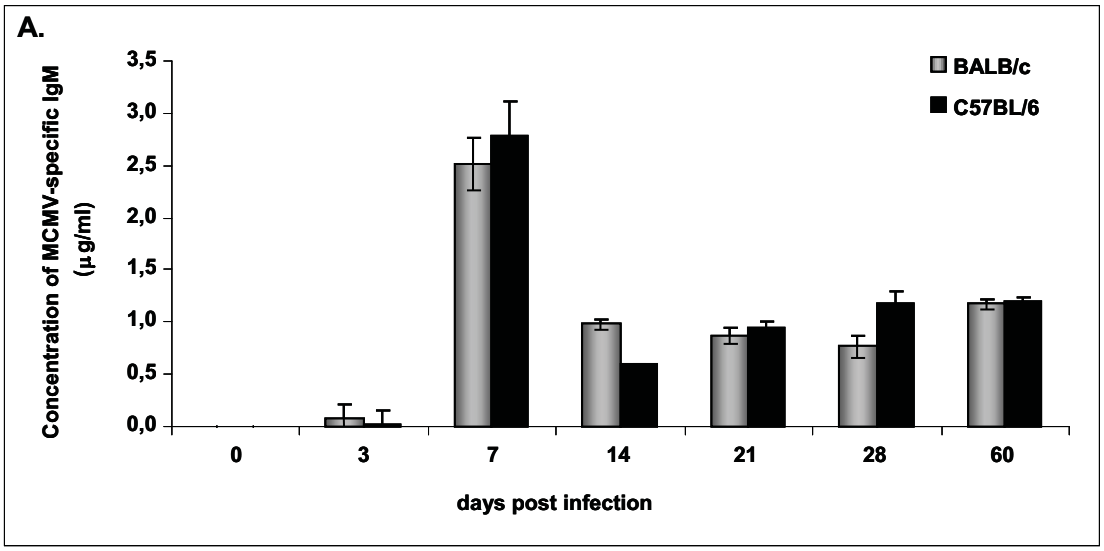


Figure III.1.A Concentration of MCMV-specific IgM (µg/ml) in immune sera of BALB/c and C57BL/6 mice. Sera were collected 0, 3, 7, 14, 21, 28 and 60 days after intraperitoneal (i.p.) infection with $4x10^6$ PFU (Plaque Forming Units) Smith MCMV and analyzed by ELISA. For each time point a pool of sera from 5 mice was tested. Lysates of mock and infected cells were coated on ELISA plates and

samples were added in a dilution of 1/50. Detection was made with goat anti-mouse IgM-peroxidase and optical density (O.D.) was measured at 450 nm. A standard curve with known concentrations of mouse purified IgM was simultaneously performed. Concentration of IgM from immune serum at different time points after infection was calculated using the linear regression equation given by the standard curves: $y=a+bx$, where y is the dependent variable (e.g. O.D. values measured at 450 nm for each sample), a is the intercept (in all cases zero), b is the slope of the regression line (a constant number that is specific for each linear standard curve) and x is the concentration value in ng/ml that has to be calculated. Further on, to calculate the final concentration in µg/ml, the obtained value has to be multiplied with the dilution factor of the sample (in this case 50) and divided by 1000. The final concentration values for each time point were calculated subtracting the concentration values of mock-infected cells, considered as background. Each sample was tested in triplicate and the indicated standard deviations are from two individual experiments.

Taken together, no significant differences at all time points between the two mouse strains were observed when comparing IgM production after MCMV infection.

In **Fig. III.1.B**, kinetics of 50% IgG ELISA titres to MCMV infection is indicated.

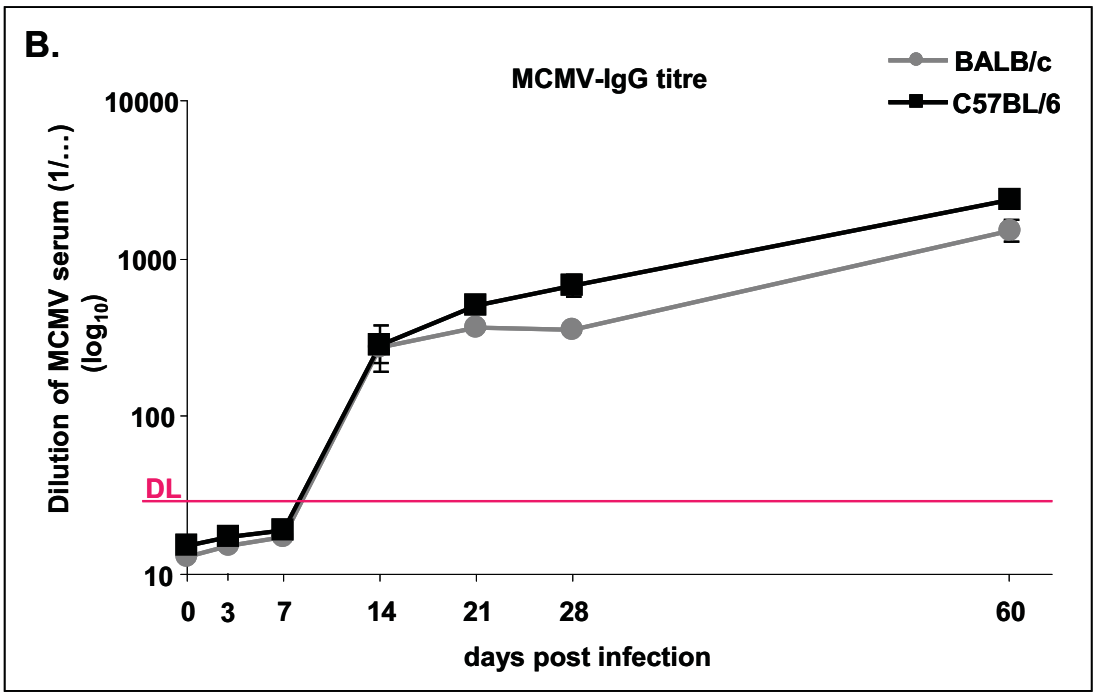


Figure III.1.B IgG antibody titres to MCMV produced by BALB/c (grey) and C57BL/6 (black) mice infected with 4x10^6 PFU of MCMV Smith strain by the i.p. route. The results are presented as the 50% ELISA titre and were calculated as the serum dilution having an O.D. value in the middle of the interval between the maximum O.D. and the cutoff. Maximum O.D. is given by the lowest dilution (1/25) of serum at day 60 p.i. Cutoff is represented by the O.D. value from the lowest dilution (1/25) of serum at day 0 p.i. (non-immune serum). Detection limit (DL) of the experiment is indicated by the red line and is defined by the lowest dilution (1/25) of serum that has MCMV-IgG titres below 50%. For days 0, 3 and 7

p.i. titres under the detection limit were obtained and therefore arbitrary units in the range 10-25 were attributed. The titres are indicated in a logarithmic scale (log_{10}) with standard deviations from two individual experiments.

IgG antibodies showed different kinetics in comparison with IgM, being produced later after infection in both mouse strains. 50% MCMV-IgG titres in ELISA rapidly increased between 7 and 14 days p.i. before slowly reaching a maximum after 60 and 90 days p.i.; before 7 d.p.i., IgG titres were very low (<1/25), similar with day 3 and with non-immune serum (day 0 p.i.), considered as background.

Moreover, IgG antibodies to MCMV were maintained at a relative constant level till later time points of infection (575 d.p.i., data not shown).
There was no major difference in the overall IgG ELISA titres to MCMV of BALB/c and C57BL/6 sera at all time points. As a negative control, serum from non-infected mice (day 0) was used in all tests. 50% IgG ELISA titres corresponding to days 0, 3 and 7 p.i. were situated below the detection limit and therefore arbitrary units in the range 10-25 were attributed.

Summary:

1) No major differences in the kinetics of virus-specific IgM and IgG levels after MCMV infection were observed between BALB/c and C57BL/6 mouse strains.
2) MCMV-specific IgM was detectable on day 3 p.i. in both mouse strains and reached the highest values at day 7 p.i.; afterwards virus IgM decreased to a constant level of about 1 µg/ml.
3) MCMV-specific IgG was detectable between 7-14 d.p.i. and the 50% IgG ELISA titres increased continuously till later time points of infection.

III.1.2 MCMV-specific IgG subclass composition in BALB/c and C57BL/6 sera

Previous studies showed that production of individual IgG subclasses is influenced by the type of cytokine triggered dependent on the Th1/Th2 balance of the CD4 T cell response. In mice, Th1 cell-mediated immunity is correlated with elevated IgG2a and/or IgG3 and IgG2b isotypes, induced by IFN-γ and TGF-ß, respectively (Snapper and Mond, 1993). Moreover, antibodies of the IgG2a subclass are the predominant isotype produced to viral infections (Coutelier et al., 1987).

For the characterization of IgG subclasses present in serum of susceptible and resistant mice infected with MCMV Smith strain, ELISA tests were established. To calculate the IgG concentration in µg/ml, separate standard curves for each IgG subclass were performed.

As shown in **Fig. III.2.A**, IgG2a and IgG2b are the predominant isotypes detected in BALB/c serum after MCMV infection (IgG2a > IgG2b > IgG3 > IgG1), indicating a Th1 response induced by MCMV infection.

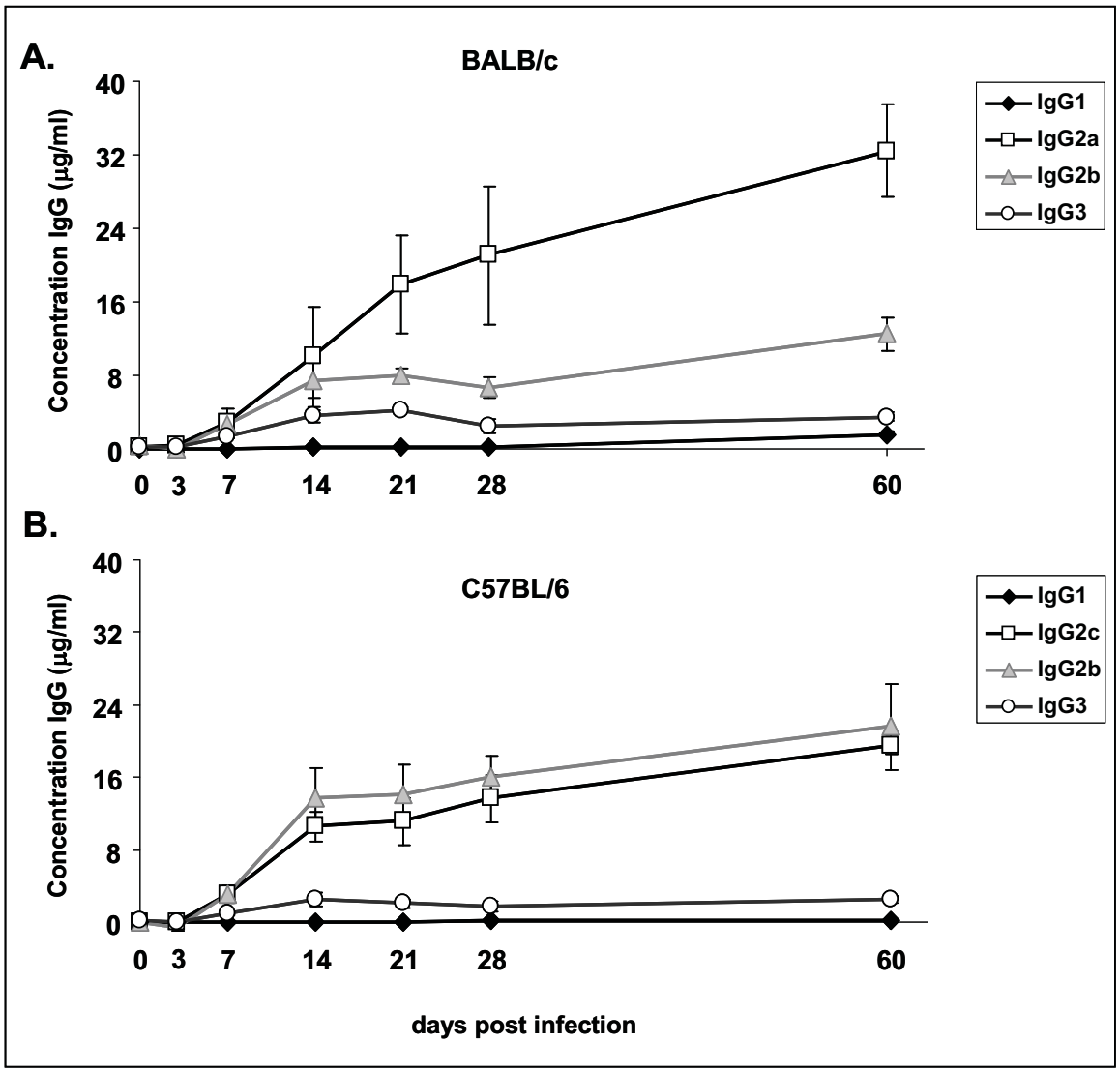


Figure III.2 MCMV-specific IgG isotypes in serum collected 0, 3, 7, 14, 21, 28 and 60 days post infection. Serum from BALB/c (A) and C57BL/6 (B) mice infected with $4x10^6$ Smith MCMV was assessed for MCMV-specific IgG subclass composition in a home-established ELISA test. Lysates of wild-type MCMV strain (C3X) -infected cells were performed as described in experimental procedures and coated on ELISA plates. Different dilutions of mouse serum were incubated with the lysates for 1 h at RT and detection was realized for the individual IgG isotype with the specified secondary antibodies: rat anti mouse IgG1-and IgG3-biotin conjugated (BD Pharmingen) and HRP-conjugated mouse IgG2a-, IgG2b-, IgG2c (Bethyl Laboratories). Standard curves obtained with known concentrations of purified

mouse IgG1, IgG3 and mouse IgG2a, IgG2b, IgG2c reference serum (two fold serial dilutions as indicated in the ELISA IgG subclass Quantitation sets, Bethyl) were performed. Concentration of each IgG subclass (in ng/ml) was calculated using the linear regression equation given by the standard curves: $y=a+bx$, where y is the dependent variable (e.g. O.D. values measured at 450 nm for each sample), a is the intercept (in all cases zero), b is the slope of the regression line (a constant number that is specific for each linear standard curve) and x is the concentration value in ng/ml that has to be calculated. Further on, to calculate the final concentration in µg/ml, the obtained value has to be multiplied with the dilution factor of the sample and divided by 1.000. As several dilutions of the same sample were used in more than 3 experiments, a chosen constant dilution was multiplied with the respective correction factor. The correction factor was necessary because the measured O.D. values did not reflect the dilution of serum used in the test. For example, when dilutions of serum 1/200 and 1/1.000 were used, O.D. of 1,075 and 0,514, respectively were obtained. Therefore, the correction factor in this case is 2,09 =1,075 /0,514. The final concentration (in µg/ml) when serum 1/1.000 dilution was used would be the value obtained from regression line equation * 200 (the constant chosen dilution) * 2,09 (the correction factor) / 1.000. Such calculations were done for all individual experiments and for each serum tested at all time points. For each time point a pool of 5 mice was tested in triplicate and the standard deviations from at least 3 independent experiments are indicated. Values from non-immune serum (day 0 p.i.) were set as background and subtracted from the values obtained at other time points after infection.

In C57BL/6 mice IgG2a could not be detected (data not shown); IgG2c isotype is present instead in this resistant strain (Jouvin-Marche et al., 1989; Martin et al., 1998; Morgado et al., 1989) and showed increased concentrations situated between 10 µg/ml at day 14 p.i. and 22 µg/ml at 60 d.p.i. (**Fig. III.2.B**). Levels of IgG2b were slightly greater or similar with those of IgG2c. Therefore, the viral IgG subclass profile specific for C57BL/6 is represented by IgG2b ≥ IgG2c > IgG3 > IgG1. Similarly, no IgG2c could be detected in BALB/c serum at either time points of MCMV infection (not shown).

IgG2a, IgG2b and IgG2c subclasses seemed to increase with the time of infection in both BALB/c and C57BL/6 serum as shown in **Fig. III.2.A and B**. These data revealed a time-dependent production of IgG subclasses that are considered important mediators of antiviral effector functions. In contrast, IgG3 and IgG1 only from C57BL/6 did not show an increase after infection and their concentrations were very low compared with the other predominant subclasses, suggesting little or no involvement in protection against MCMV. The time-dependent kinetics of MCMV-IgG3 was very similar between the tested mouse strains, with slightly higher concentrations of IgG3 antibodies detected in BALB/c between 7 and 28 d.p.i. Nevertheless, concentrations of MCMV-

IgG3 were maintained reduced (4 and 2 μg/ml in BALB/c and C57BL/6, respectively), being not affected by the course of viral infection.

Starting with 14 d.p.i., C57BL/6 mice develop two-fold more MCMV-IgG2b than it was detected in BALB/c serum, making IgG2b the major subclass in this resistant mouse strain after MCMV infection.

MCMV-IgG1 concentrations from BALB/c and C57BL/6 were shortly above the detection level as shown in **Fig. III.2.A and B** by the graphic scale of maximum 40 μg/ml. Considering the same values from previous figure but changing the scale to maximum 2 μg/ml, a clear increase in the MCMV-IgG1 concentrations in BALB/c but not C57BL/6 sera between 28 and 60 d.p.i. was observed (**Fig. III.3**).

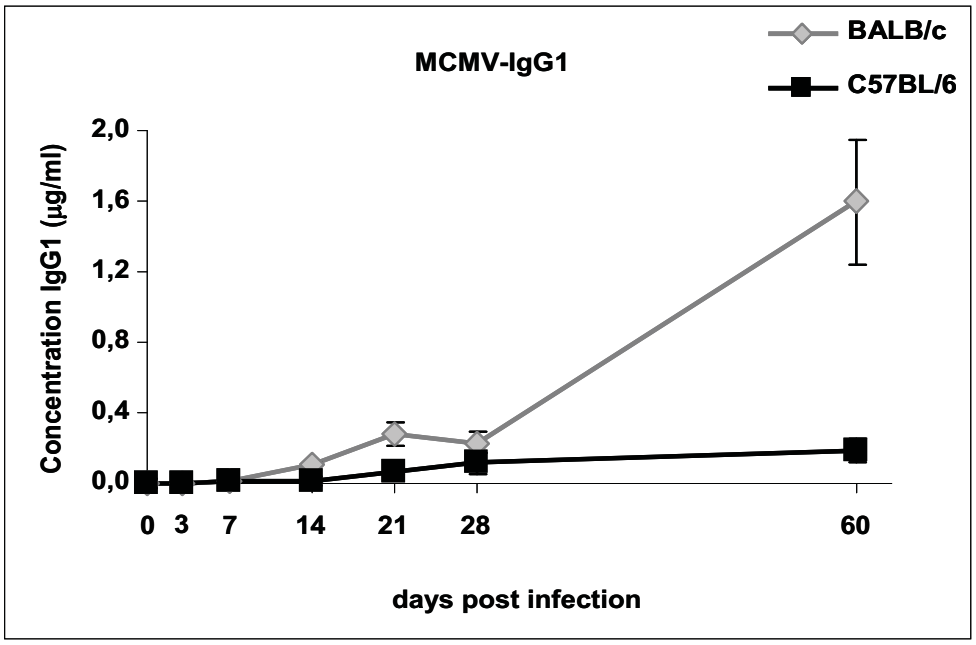


Figure III.3 Time-dependent production of MCMV-specific IgG1 in BALB/c and C57BL/6 mice. Sera collected from both mouse strains at days 0, 3, 7, 14, 21, 28 and 60 post Smith MCMV infection were analyzed in ELISA for the production of virus-specific IgG1, as described in experimental procedures and in the legend from Fig. III.2. Results are presented as concentration of IgG1 in μg/ml and the indicated standard deviations are from more than 3 independent experiments.

We have previously noted (**Fig. III.1.B**) that the total amount of IgG produced in response to MCMV infection does not differ significantly between both mouse strains. Moreover, this finding is consistent with the results obtained after summarizing individual concentration values of IgG1, IgG2a/IgG2c, IgG2b and IgG3, determined at each time point after infection, as shown in **Fig. III.4**. The overall amount of MCMV-IgG of all subclasses obtained in this way showed similar time kinetics for BALB/c and C57BL/6 serum, indicating that the susceptible and resistant mouse strain do not differ

in their total MCMV-specific IgG antibody concentration, but in the composition of IgG subclasses present in the serum of infected mice.

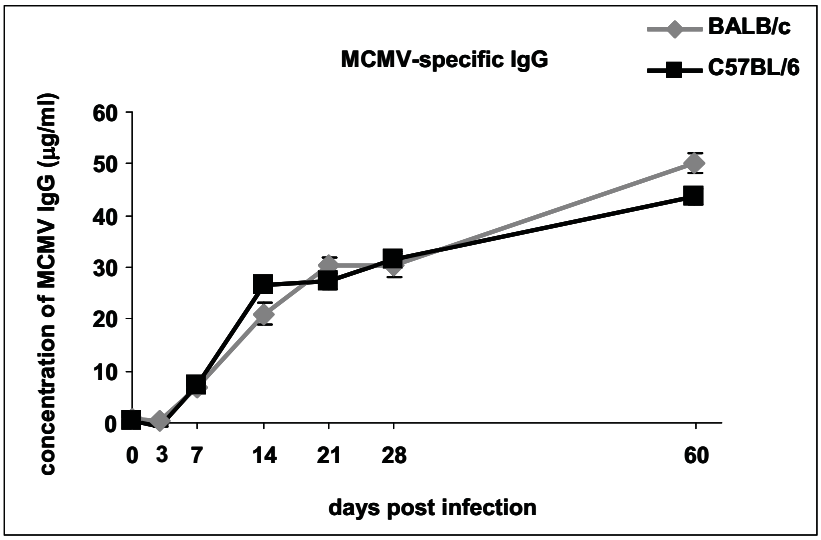


Figure III.4 Total MCMV-specific IgG in BALB/c and C57BL/6 mice. The indicated values represent the summarized concentrations of each MCMV-specific IgG subclass: IgG1, IgG2a/2c, IgG2b and IgG3 for BALB/c and C57BL/6, respectively and for each time point after infection. Concentrations in µg/ml are shown here in a linear scale.

Summary:

1) After MCMV infection the predominant IgG isotypes are IgG2a > IgG2b in BALB/c and IgG2b ≥ IgG2c in C57BL/6 serum, indicating a Th1 response induced by MCMV infection.
2) 2-fold more MCMV-IgG2b was produced in C57BL/6 serum in comparison to BALB/c at all time points after infection.
3) Though the amount of MCMV-IgG1 was very low in comparison with the other IgG subclasses, an increase of about 8-fold was observed in BALB/c but not C57BL/6 serum between 28 and 60 d.p.i., as detected by ELISA.
4) MCMV-specific IgG3 showed similar concentrations in BALB/c and C57BL/6 mice, without significant modifications over time.
5) When summarizing the amount of MCMV-immune IgG subclass antibodies (IgG1 + IgG2a + IgG2b + IgG3 in BALB/c and IgG1 + IgG2c + IgG2b + IgG3 in C57BL/6) at each time point after MCMV infection, the data obtained with the overall MCMV-IgG ELISA was confirmed. These data suggest that the difference observed between the susceptible and the resistant mouse strain is not the quantity of IgG antibodies produced against MCMV antigens, but rather the composition of IgG subclasses.

III.1.3 Total IgG subclass composition in BALB/c and C57BL/6 sera

The previous differences in MCMV-specific IgG subclass composition observed between the susceptible and resistant mouse strain (**Fig. III.2**) prompted us to measure the composition of total IgG subclasses in the particular sera of MCMV-infected BALB/c and C57BL/6 mice. Detection of IgG1 and IgG3 subclasses was determined using ELISA tests with mouse serum from indicated time points directly coated on plates. For total IgG2a, 2b and 2c detection, coating antibodies for each of the three subclasses as indicated in ELISA Quantitation sets (Bethyl) were used. In **Fig. III.5**, total IgG subclasses from BALB/c (A) and C57BL/6 (B) mice maintained under specific pathogen free (SPF) conditions but infected with MCMV Smith strain are indicated.

For BALB/c mice, IgG2a was again the predominant IgG subclass present in serum at all time points after MCMV infection and increased strongly between 7 and 21 d.p.i.; 12-fold more IgG2a was observed at day 21 p.i. when compared to non-infected serum from day 0 p.i. Afterwards the level of total IgG2a stayed relatively constant till later time points (approx. 650±63 µg/ml) (**Fig. III.5.A**). The strong increase of total IgG2a amounts in the early serum could be correlated with MCMV infection.
The other IgG subclasses did not considerably change with the time of infection. Their relative amounts fluctuated from 63 µg/ml for IgG1 up to 161 µg/ml of IgG3.
Amounts of total IgG2b in BALB/c varied slightly between 55 µg/ml in non-infected serum (day 0 p.i.) and 164 µg/ml (day 21 p.i.), showing only a 2,9-fold increase up to the indicated time point. The hierarchy of total IgG subclasses in BALB/c serum was represented by IgG2a >> IgG3 > IgG2b > IgG1.

Examination of C57BL/6 sera revealed a different picture, as shown in **Fig. III.5.B**. IgG2b seemed to be the predominant subclass present in C57BL/6 serum, independent of MCMV immune IgG2b, as indicated by the non-immune serum taken at day 0 p.i. A two-fold increase in the amount of total IgG2b in the early serum (7-14 d.p.i.) was also observed, but the total amount did not exceed 475±128 µg/ml.

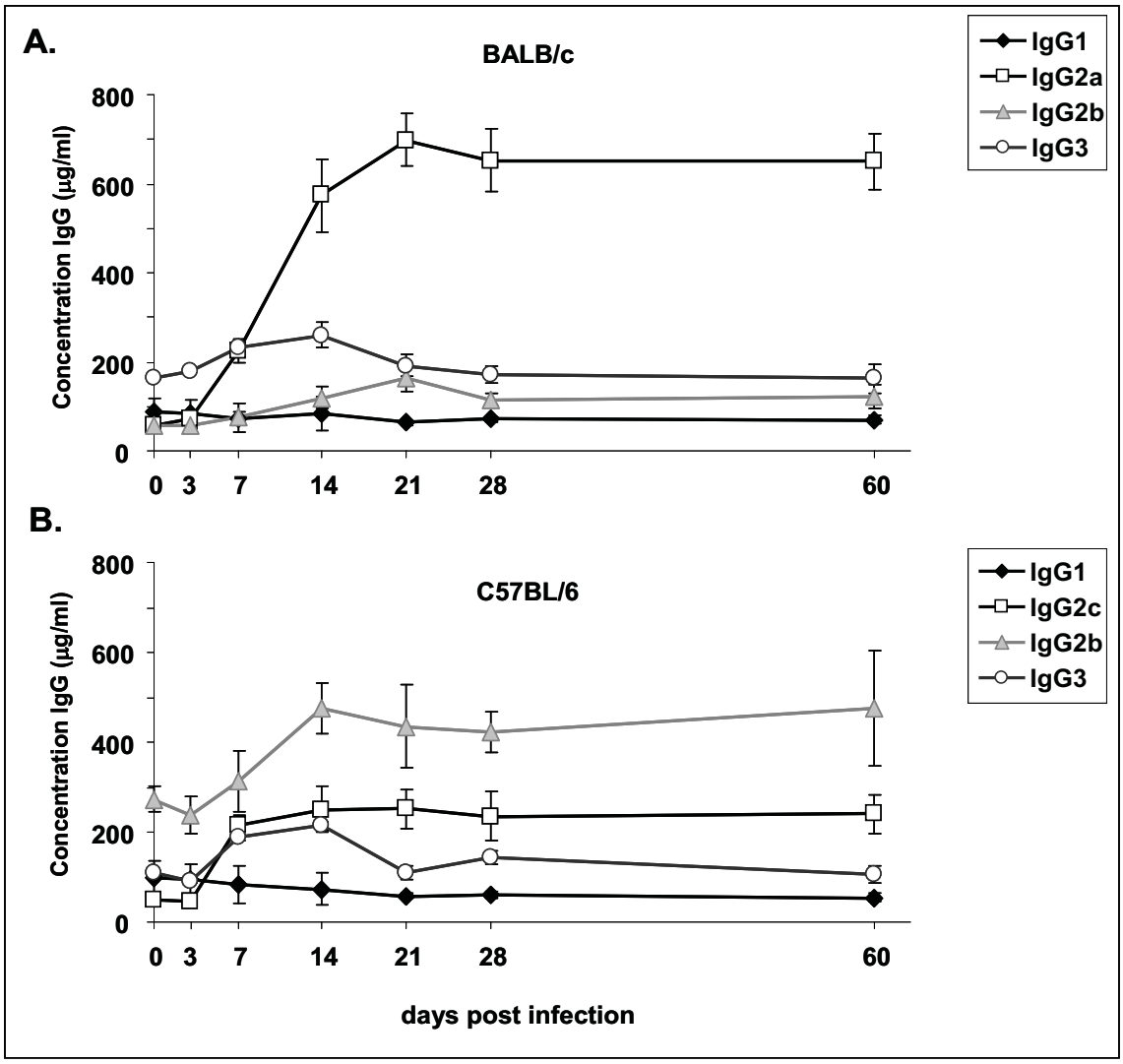


Figure III.5 Time-dependent composition of total IgG subclasses in BALB/c and C57BL/6 sera. Serum collected from the two mouse strains at 0, 3, 7, 14, 21, 28 and 60 days post Smith MCMV infection ($4x10^6$ PFU) was assessed for the overall concentration of IgG subclasses using ELISA tests. For total IgG1 and IgG3, ELISA plates were overnight coated with mouse serum diluted 1/5.000 and 1/2.000, respectively. Biotin rat anti-mouse IgG1 and IgG3 (BD Pharmingen) at a final concentration of 1 µg/ml were used for detection of the two IgG subclasses. For total IgG2a, IgG2c and IgG2b the protocols from mouse IgG2a/2c/2b ELISA Quantitation sets (Bethyl) were used. Serum was diluted 1/30.000, 1/20.000 and 1/1.500 for IgG2a, IgG2b and IgG2c, respectively. Dilutions of detection antibodies 1/17.000, 1/30.000 and 1/10.000 for IgG2a, IgG2b and IgG2c, respectively were established. Standard curves using known concentrations of purified mouse IgG1, IgG3 and mouse IgG2a, IgG2c, IgG2b reference serum (two fold serial dilutions as indicated in the Bethyl ELISA kits) were simultaneously performed. Concentration of each IgG subclass (in ng/ml) was calculated using the linear regression equation given by the standard curves: $y=a+bx$, where y is the dependent variable (e.g. O.D. values measured at 450 nm for each sample), a is the intercept (in all cases zero), b is the slope of the regression line (a constant number that is specific for each linear standard curve) and x is the concentration value in ng/ml that has to be calculated. Further on, to calculate the final concentration in µg/ml, the obtained

value has to be multiplied with the dilution factor of the sample and divided by 1.000. As several dilutions of the same sample were used in more than 3 experiments, a chosen constant dilution was multiplied with the respective correction factor. The correction factor was necessary because the measured O.D. values did not reflect the dilution of serum used in the test. For example, when dilutions of serum 1/1.500 and 1/3.000 were used, O.D. of 1,919 and 1,839, respectively were obtained. The correction factor is in this case 1,04 =1,919/1,839. Therefore, the final concentration (in µg/ml) when serum 1/3.000 dilution was used would be the value obtained from regression line equation multiplied with 1.500 (the constant chosen dilution) and multiplied with 1,04 (the correction factor), all divided by 1.000. Such calculations were done for all individual experiments and for each tested serum at all time points. For each time point a pool of 5 mice was tested in triplicate and the standard deviations from at least 3 independent experiments are indicated.

The total IgG2c found exclusively in C57BL/6 serum varied from 50 to 250 µg/ml, representing a 5-fold increase at 14 days post MCMV infection in comparison with the non-immune serum (0 d.p.i.); afterwards no further changes in the overall amount of IgG2c were detected.

Total IgG3 in C57BL/6 serum increased slightly from 108 µg/ml in non-infected serum (day 0 p.i.) to 213 µg/ml in serum collected at day 14 p.i. After this time point, the amount of IgG3 decreased till 105 µg/ml at day 60 p.i. (**Fig. III.5.B**). Total IgG1 showed no significant modification with the time of infection and the relative amounts were situated around 55 µg/ml. In C57BL/6 serum, the total IgG subclass profile was represented by IgG2b > IgG2c > IgG3 > IgG1.

Taken together, these results illustrate a significant increase of the total IgG2a in BALB/c and IgG2c in C57BL/6 (12- and 5-fold, respectively) during the primary phase of MCMV infection (till day 14 or 21 p.i.) that is induced by MCMV replication. The other IgG subclasses (IgG2b, IgG1 and IgG3) did not show a major modification over the course of MCMV infection.

Therefore, based on these findings it is tempting to speculate that MCMV infection induces a process that we designated as '*IgG subclass-selective hypergammaglobulinemia'*.

Additionally, when summarizing the concentrations in µg/ml of all four IgG subclasses previously obtained, we noticed a close resemblance between BALB/c and C57BL/6 mouse strains, with slightly lower levels of total IgG obtained for C57BL/6 mice at 21 d.p.i. (**Fig. III.6**). Once more, the differences we found are strictly related to the

composition of each IgG subclass in the mouse serum and not to the total IgG antibodies.

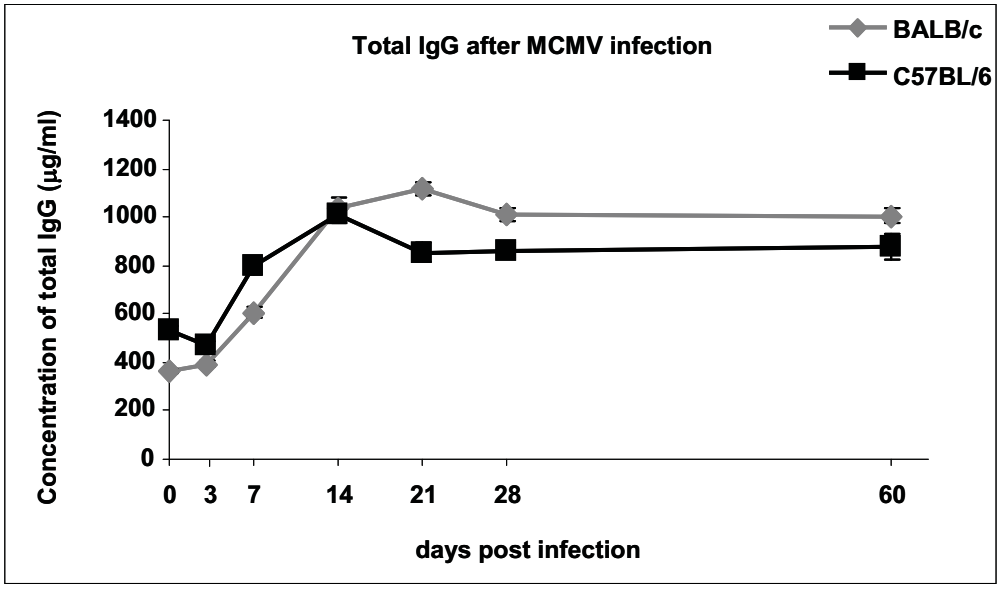


Figure III.6 Total IgG in mouse serum. The indicated values were obtained by summarizing concentrations of each IgG subclass from previous ELISA tests: IgG1, IgG2a, IgG2b, IgG3 for BALB/c; IgG1, IgG2c, IgG2b, IgG3 for C57BL/6. Results are presented in µg/ml in a linear scale. Sera from 0, 3, 7, 14, 21, 28 and 60 days post Smith MCMV infection are indicated in the graphic.

Summary:

1) The measurement of total IgG subclasses in serum collected from BALB/c and C57BL/6 mice infected with MCMV Smith strain indicated a 12- and 5-fold increase of the total IgG2a and IgG2c, respectively, early after MCMV infection (3-21 d.p.i.) and values stayed relatively constant during the subacute phase of infection (60 d.p.i.). These data suggest that MCMV infection is followed by an '*IgG subclass-selective hypergammaglobulinemia'* in these mouse strains.
2) Total IgG1 detected in serum was relatively stable at low levels for all tested time points and in both mouse strains, whereas total IgG3 showed a slow increase between 3 to 14 d.p.i., with no further alteration over time.
3) The total IgG subclass distribution reflected the subcomposition of IgG subclasses that are specific for MCMV antigens, where IgG2a is the predominant subclass in BALB/c and IgG2b in C57BL/6 serum.
4) Total IgG obtained by summarizing each IgG subclass concentration yielded similar values in the susceptible and resistant mouse strain, with slightly higher amounts at day 21 p.i. calculated for BALB/c.

III.1.4 Direct comparison of IgG subclasses

Previous findings showed that the IgG2a gene is absent in C57BL/6 mice, and instead the IgG2c gene is present, identified as a product of the *Igh-1b* allele (Jouvin-Marche et al., 1989; Martin et al., 1998; Morgado et al., 1989). When comparing the aminoacid sequence of IgG2a and IgG2c products of the two distinct isotypes *Igh-1a* and *Igh-1b*, respectively, it was found that 16% of the aminoacids are different, indicating a relative dissimilarity between both alleles (Martin et al., 1998). These data raised the question whether IgG2a and IgG2c might have also different immune functions in mice. Our ELISA results can partially answer this question by showing for the first time a clear difference between the concentrations of IgG2a from BALB/c and IgG2c from C57BL/6 serum. **Fig. III.7** points out that both MCMV-specific and total IgG2a antibodies are two and respectively three fold higher in BALB/c than IgG2c in C57BL/6 serum from 60 days p.i. Moreover, we calculated how much of the total IgG2a or IgG2c is directed against MCMV antigens, and the results revealed almost 5% of IgG2a and 8% of IgG2c (**Fig. III.7**). The percentage of IgG2a that is specific for MCMV antigens and calculated from the total amount of IgG2a present in mouse serum is also increasing over time, from 1,24% at 7 d.p.i. to 5% at day 60 p.i. In the same way, MCMV-specific IgG2c is increasing from 1,46% at day 7 p.i. up to 8% at day 60 p.i. (data not shown). These data indicate that only a small fraction of all detected IgG antibodies is directed against MCMV antigens; the rest of MCMV-induced non-immune IgGs could include autoantibodies triggered by MCMV infection.

Comparison of MCMV-immune IgG2b in the two mouse strains revealed that this isotype is the second major subclass after IgG2a in BALB/c serum, reaching 10% from the total IgG2b at day 60 post MCMV infection (**Fig. III.7.A**). In C57BL/6 mice, MCMV-specific and total IgG2b is two and respectively 4,5-fold higher than in BALB/c serum, demonstrating that IgG2b is the main subclass in this resistant mouse strain, followed by IgG2c isotype (**Fig. III.7.B**).

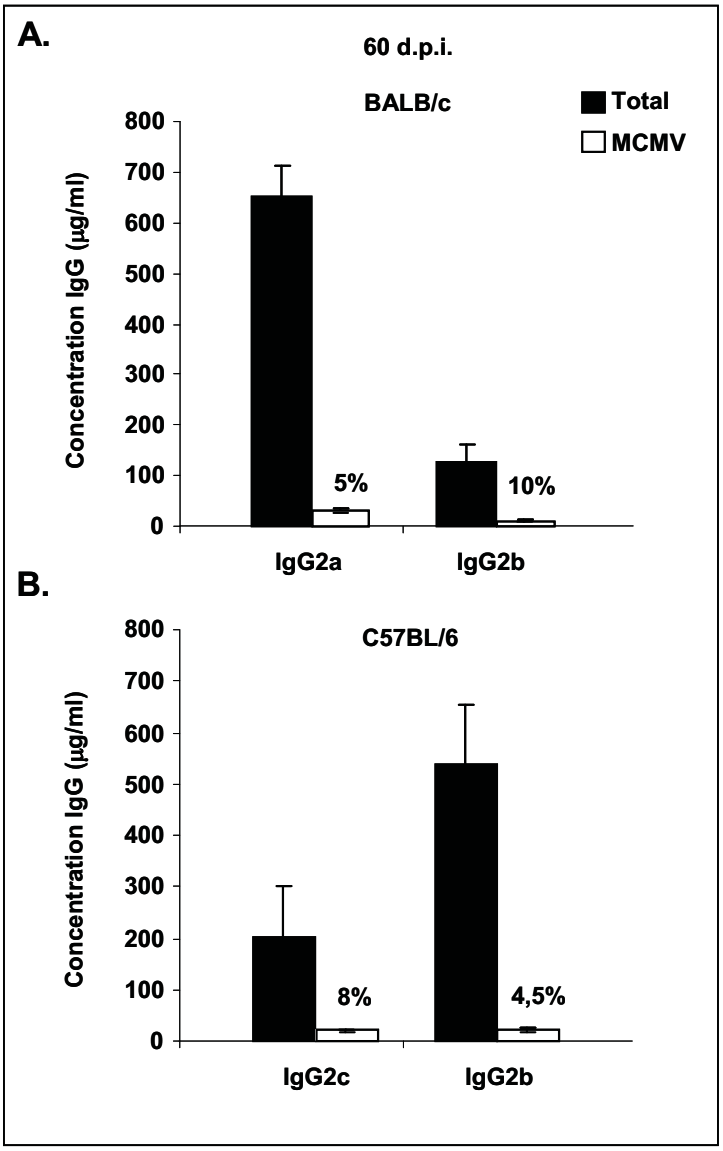


Figure III.7 Total versus MCMV-specific IgG subclasses in BALB/c (A) and C57BL/6 (B) serum collected 60 days post MCMV infection. This figure depicts another representation of the concentration values for IgG subclass composition from paragraphs III.1.2 and III.1.3. Protocols for MCMV-specific IgG subclasses and total IgG determined by ELISA tests are described in experimental procedures and in the legends from Fig.III.2 and Fig.III.5, respectively. Concentrations are calculated in µg/ml and standard deviations from at least three independent experiments are shown. Additionally, percentage (%) of MCMV-specific IgG2a, IgG2b and IgG2c is calculated from the total IgG2a, IgG2b and respectively IgG2c, and indicated above the white bars.

Summary:

1) Only a very small fraction of total IgG2a, IgG2b and IgG2c antibodies that are found in mouse serum are directed against MCMV antigens.
2) Direct comparison of IgG2a from BALB/c with IgG2c from C57BL/6 showed for the first time a disparity in the production of the two isotypes; IgG2a is produced in higher quantities in BALB/c serum, being the predominant IgG subclass in this susceptible mouse strain; on the other hand, IgG2c from C57BL/6 serum is produced in smaller amounts, representing only the second most abundand IgG subclass after IgG2b in the resistant strain of mice. These findings query whether the immune functions of IgG2a and IgG2c are similar.

III.1.5 MCMV-immune IgG mediated activation of FcγRIII and FcγRIV in BALB/c and C57BL/6 mice after MCMV infection

It is well described that host Fc gamma receptors (FcγRs) elicit multiple effector responses, like degranulation and killing of target cells by immune cells, cytokine secretion, phagocytosis, etc. Based on a recently developed FcγR activation method (Eugenia Corrales-Aguilar et al., 2012) we measured the ability of 'cytotoxic' IgG antibodies to activate host FcγRIII and FcγRIV after MCMV infection in susceptible BALB/c and resistant C57BL/6 mice, as described in experimental procedures, chapter VI.4.16. The read-out of the experiment represents the measurement of IL-2 released after activation of responder cells (BW5147 cells transfected with either FcγRIII-ζ or FcγRIV-ζ) (see chapter VI.4.17- IL-2 ELISA in experimental procedures).

Figure III.8.A shows FcγRIII-activation titres of IgG antibodies developed in BALB/c and C57BL/6 serum after MCMV infection in a time-dependent manner. The obtained data indicated a delay of about seven days in the production of FcγRIII-activating IgG in C57BL/6 comparing with BALB/c serum (day 21 and 14, respectively). Moreover, FcγRIII responses in BALB/c are stronger than those developed in C57BL/6, reaching a titre of 450 and respectively 99 at 60 days post MCMV infection. The difference in the FcγRIII titres may reflect the variability of MCMV-specific IgG subclasses observed in BALB/c and C57BL/6 mice and propose for the first time that IgG2a isotype triggers more efficiently the activation of this receptor than IgG2c from C57BL/6. For both mouse strains, FcγRIII-activation titres continue to increase till day 60 p.i., indicating a maturation of these responses with the time of antigen exposure.

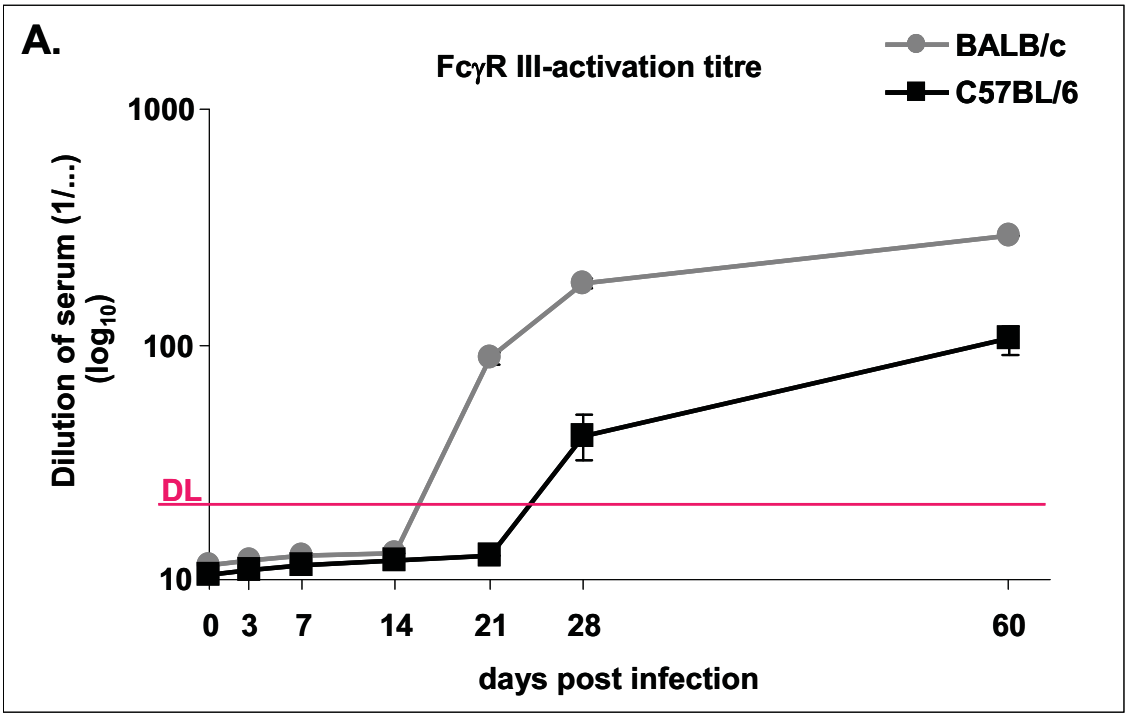


Figure III.8.A FcγRIII-activation titres of MCMV-specific IgG produced in BALB/c and C57BL/6 mice after MCMV Smith infection in a time dependent manner. BW5147 cells transfected with either FcγRIII-ζ (A) or FcγRIV-ζ (B) were incubated with MCMV-infected target cells (MEFs passage 3, 24 h.p.i.) and the secreted IL-2 was measured using an ELISA test; the O.D. at 450 nm is directly proportional to the activation of effector cells triggering ADCC responses via FcγRIII and FcγRIV, respectively. In Fig.III.8.A, results are expressed as FcγRIII-activation titres and represent the serum dilution that triggers 50% activation of the indicated Fc receptor. Titres were calculated using the O.D. at 450 nm of the 2-fold serial dilutions for serum collected 0, 3, 7, 14, 21, 28 and 60 days post infection (pooled serum). Detection limit (DL) showed by the red line represents the smallest serum dilution (1/25) that gives FcγRIII-activation titre below 50%. For serum collected at days 0, 3, 7, 14 and 21 p.i. (for C57BL/6) values in the range 10-25 were arbitrary attributed ,as their 1/25 dilutions were under the detection limit. The indicated standard deviations are calculated from two individual experiments.

In **Fig. III.8.B**, the time kinetics of FcγRIV-activating IgG in BALB/c versus C57BL/6 is indicated. Comparing the two mouse strains, we observed a similar generation of the MCMV-specific IgG antibodies able to activate FcγRIV-ζ transfectants over the time of infection. IgG antibodies with 50% FcγRIV-activating capabilities are generated at the same time for both mouse strains, starting with day 7 p.i. and strongly increasing till day 21 p.i.; afterwards, titres show a minor enhance till day 60 p.i.

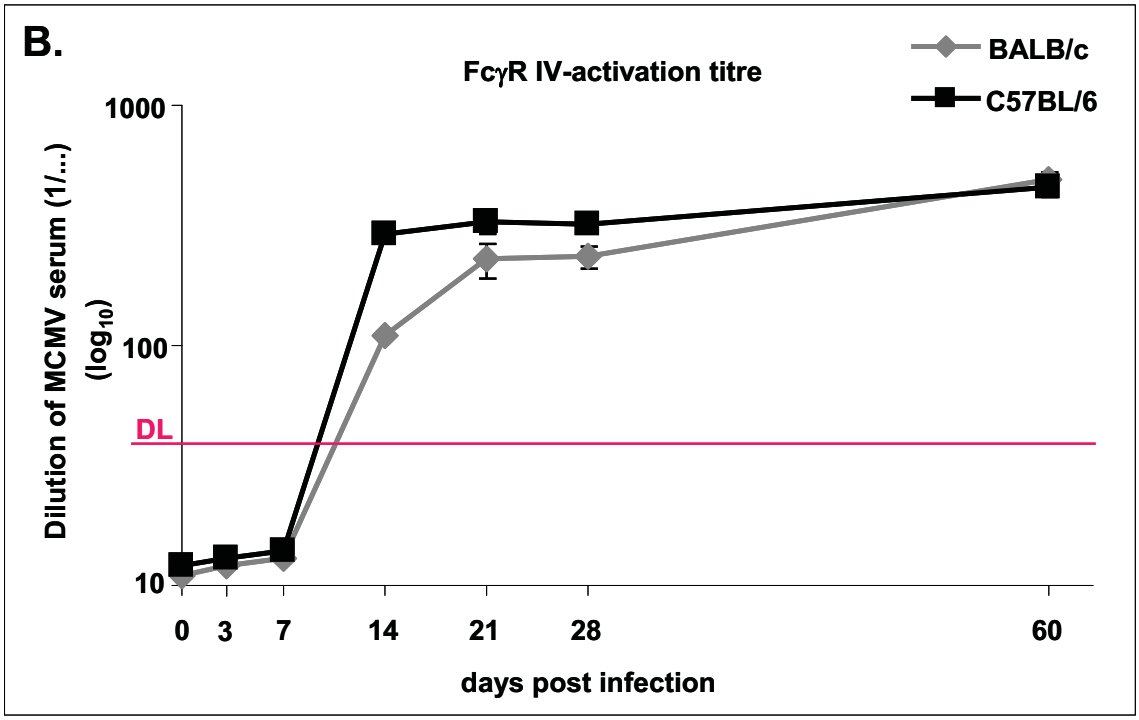


Figure III.8.B FcγRIV-activation titres of MCMV-specific IgG produced in BALB/c and C57BL/6 mice after Smith MCMV infection in a time dependent manner. The protocol for this test is described above in the legend of Fig.III.8.A. Results are expressed as FcγRIV-activation titres and represent the serum dilution that triggers 50% activation of the indicated receptor. Titres were calculated using the O.D. at 450 nm obtained for the 2-fold serial dilutions of pooled serum. The detection limit (DL), designated by the red line represents the smallest dilution (1/50) of serum from days 0, 3 and 7 p.i. that has a FcγRIV-activation titre below 50%. For sera collected at days 0, 3, 7 p.i. arbitrary units were attributed, as their 1/50 dilution was under the detection limit. Mean values from two individual experiments with their corresponding standard deviations are indicated.

To check if the difference in FcγRIV-activation titres observed at day 14 p.i. in BALB/c and C57BL/6 serum (**Fig. III.8.B**) is statistically significant, 2-fold serum dilutions from individual mice belonging to a group of 5 were additionally tested. For comparison, mathematical p values were afterwards calculated (**Fig. III.9**). For day 14 p.i., *p* values smaller than 0,01 show a moderate statistically significant difference (designated as **) between the two mouse strains, with higher FcγRIV-activation titres developed in C57BL/6 mice. In contrast, at day 21 p.i., *p* values of 0,068 clearly indicate no significant (n.s.) difference in the FcγRIV-activation titres between BALB/c and C57BL/6 mice.

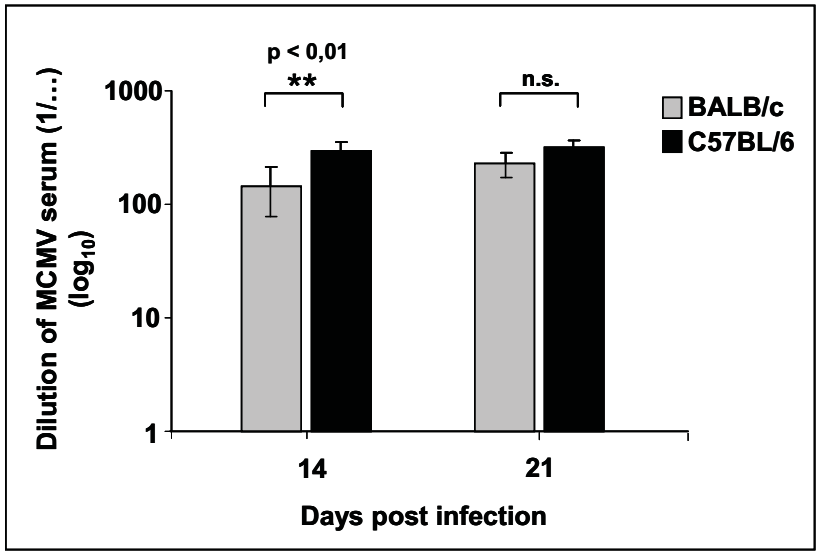


Figure III.9 FcγRIV-activation titres of BALB/c and C57BL/6 serum at 14 and 21 days post MCMV infection. Serum collected from 5 mice at day 14 and 5 mice at day 21 p.i. with Smith MCMV was individually tested in the FcγRIV-activation assay and 50% activation titres were calculated as described above (see legend from Fig. III.8.A). The mean values of five mice belonging to one time point and the corresponding standard deviations are indicated in a logarithmic scale (log_{10}). **, $p < 0,01$; n.s., not significant.

Summary:

1) The data indicate for the first time different responses of FcγRIII-activating IgG antibodies when comparing a MCMV-susceptible with a resistant mouse strain; the quality and magnitude of FcγRIII-responses is stronger in BALB/c mice at all time points tested.
2) There is a seven day delay in the production of FcγRIII-activating IgG in C57BL/6 mice, possibly due to the absence of the IgG2a isotype and to the less effective IgG2c in triggering CD16 responses.
3) There is a similar generation of FcγRIV-activating IgGs in the two mouse strains at all time points after infection; moreover, resistant C57BL/6 strain showed a slight increase in the magnitude of FcγRIV-activation titres at day 14 p.i. when compared to susceptible mice, possibly due to a higher production of IgG2b specific for MCMV antigens at this time point. These data suggest that the role of missing IgG2a isotype in C57BL/6 for activation of FcγRIV-bearing cells is overtaken by the IgG2b present in double amounts in resistant mice.

III.1.6 Time-dependent production of MCMV-specific neutralizing antibodies

Upon infection with MCMV, the immune system responds with producing neutralizing antibodies against epitopes present on the surface of the virion that block the entry and dissemination of the virus to neighboring cells. To compare susceptible and resistant mouse strains with respect to their neutralization capabilities and to find out whether neutralizing Abs may contribute to the resistance of C57BL/6 mice, a luciferase-based neutralization (NT) assay was performed. The luciferase NT method used in this work is based on the measurement of luciferase activity expressed as relative light units (RLU) in cell lysates prepared from cells infected with a Δm157-MCMV-*luciferase* mutant (Reinhard et al., 2011).

The measured RLUs are direct proportional with the amount of infectious viral particles. Construction of the deletion mutant Δm157-MCMV that has inserted the firefly luciferase (*luc*) gene in the *m157* gene was recently described (Trilling et al., 2011).

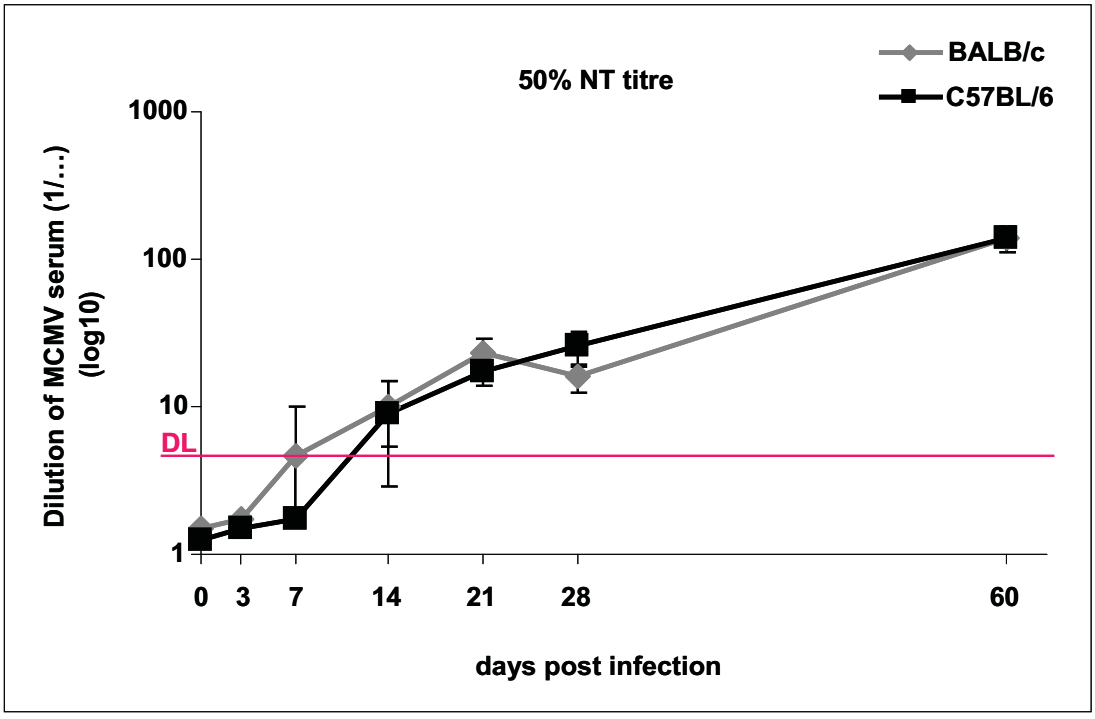


Figure III.10 Neutralization titres of antibodies in serum of BALB/c and C57BL/6 mice over the course of MCMV infection. Δm157-MCMV-*luc* was incubated with serial dilutions of pooled serum (1/5, 1/20, 1/80, 1/320) for 1,5 h at 37^0C and 5%CO_2 to allow the neutralization of virions take place. NIH 3T3 cells were afterwards infected with virus-Ab mix for 18 h; next day lysates of infected cells were performed as described in experimental procedures and the Relative Light Units (RLU) represented by the luciferase activity were measured. The 50% neutralization was first calculated using the measured

RLU with the following mathematical formula: [(1-$RLU_{sample}/RLU_{non\text{-}immune\ serum}$)*100]. Second, serum dilution that gives 50% reduction of the $1x10^3$ Δm157-MCMV-*luc* and designated as 50% NT titre is indicated in a logarithmic scale (log_{10}) for each time point. Detection limit (DL) is indicated by the red line and represents the minimum serum dilution (1/5) that has NT titres below 50%. Serum from days 0 and 3 p.i. (BALB/c) and 0, 3, 7 p.i. (C57BL/6) had NT titres situated below the detection limit and therefore arbitrary values were attributed. Mean values ± SD from three independent experiments are shown.

Figure III.10 shows that the capacity to neutralize MCMV virions was equally developed in susceptible and resistant mouse strains with similar antibody titres over the course of MCMV infection. 50% NT titres were acquired starting with 7 d.p.i. in BALB/c and 14 d.p.i. in C57BL/6. For days 0, 3 p.i. in BALB/c and 0, 3, 7 p.i. in C57BL/6 mice, the 50% NT titres were situated below the detection limit and arbitrary units in the range 1-5 were attributed. For both mouse strains the neutralization titres increased gradually with the time of infection, reaching a titre of 140 ± 28 in serum collected at 60 d.p.i.

These data suggest that the resistance of mice to MCMV is not correlated with the first detection or magnitude of NT Abs, as susceptible BALB/c seem to develop them earlier than resistant C57BL/6.

Summary:

1) BALB/c mice produced neutralizing antibodies early after infection, between 3-7 days p.i, whereas in C57BL/6 mice the neutralizing Abs are produced later, starting with day 7 p.i., and the 50% NT capacity of these Abs was reached only after 14 d.p.i. These data do not indicate any correlation with the genetically determined resistance to MCMV infection.
2) Neutralization titres increased with the time post infection in both mouse strains, suggesting a time-dependent maturation of the virus-specific antibodies, and reached similar maximum of NT IgG.

III.2 SCREENING A LIBRARY OF MCMV-SPECIFIC MONOCLONAL ANTIBODIES (MABS) (A) AND THE IDENTIFICATION OF ANTIGENS ELICITING FCγR ACTIVATING IGG RESPONSES (B)

Antiviral antibodies can mediate their effector functions either by directly neutralizing the virion or indirectly by targeting infected cells. The latter type of antibodies could be able to trigger Fcγ receptor-mediated responses (e.g. ADCC) and as a result several effector mechanisms are elicited, e.g. killing of target cells by NK cells, cytokine secretion, phagocytosis, etc.

In the case of MCMV, studies of passively transferred IgG from pre-immunized mice showed an efficient control of infection, although the neutralizing capacity of these antibodies was demonstrated to be very weak (Klenovsek et al., 2007). These data argue for a possible role in virus control of the MCMV-IgG antibodies that trigger FcγR-mediated immune responses. Moreover, studies from Hessell *et al*. with SHIV (Simian-Human Immunodeficiency Virus) highlighted that Fc-receptor-bearing effector cells but not neutralizing or the complement activating IgGs are important in reducing virus yield from infected cells (Hessell et al., 2007).

Based on this data and on our previous findings that showed a stronger CD16-activation by MCMV-immune serum of BALB/c than C57BL/6 mice, our aim was A) to define the subtype of MCMV-specific mAbs that are able to trigger CD16-responses in BALB/c mice and B) to identify their specific antigens in MCMV-infected cells. On a longer perspective, MCMV-mAbs should be further assessed for their protective capabilities (e.g. in immunocompromised mice) and the viral antigens that trigger ADCC-responses should be considered for possible vaccination studies. To achieve our goal, defined steps of testing were pursued (**Fig. III.11**).

First, a library of mAbs produced after immunization of BALB/c mice with wt MCMV should be established followed by a comprehensive investigation of their specificity, CD16-activation and neutralization capabilities (**Fig. III.11.A**). In the end, such defined mAbs are expected to recognize antigens expressed on the surface of virus-infected cells and should be able to activate CD16-bearing effector cells. The second step is represented by the characterization and identification of the antigens recognized by the CD16-activating mAbs using tandem mass spectrometry analysis (**Fig. III.11.B**).

All the steps in the screening procedure are described point by point in the next chapters of the experimental result section.

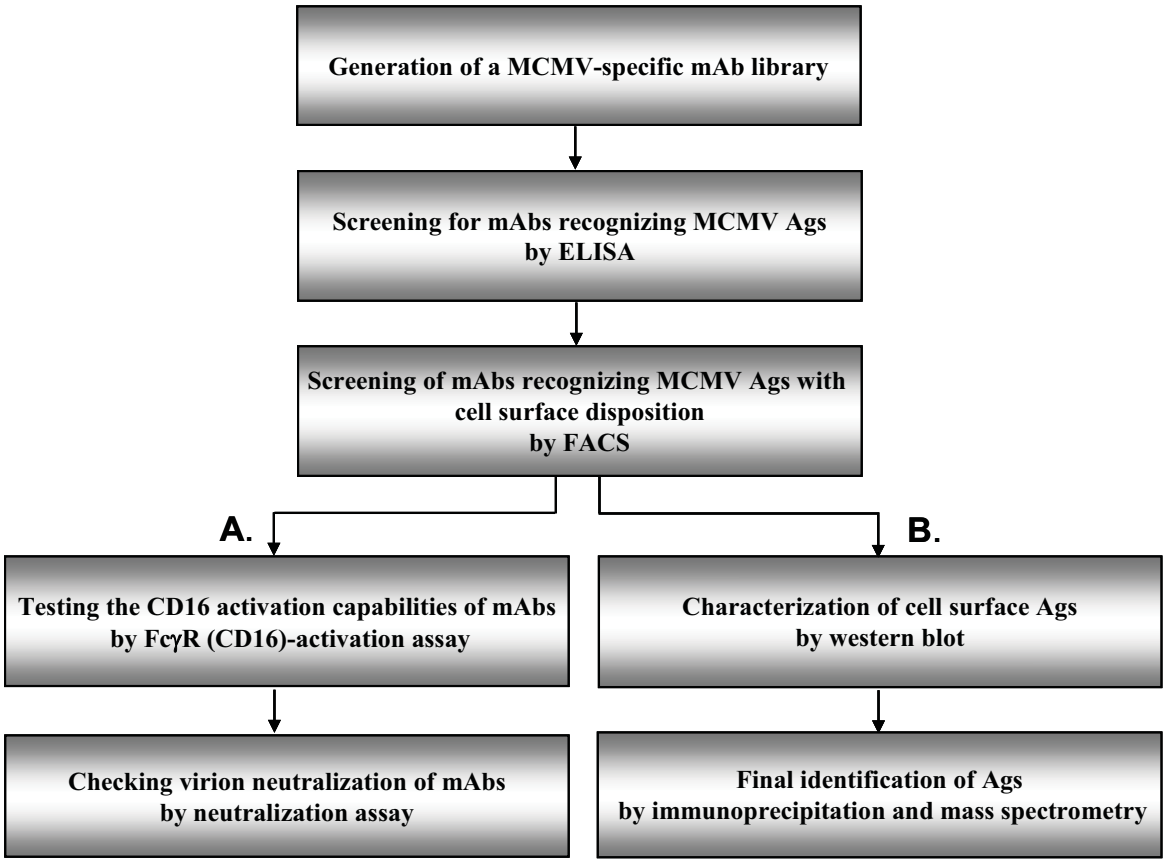


Figure III.11 Steps in screening the library of MCMV-specific mAbs.

Abbreviations: FACS, Fluorescent Activated Cell Sorting; mAbs, monoclonal antibodies; Ags, antigens.

III.2.1 Production of MCMV-specific mAbs library

The method used for generation of mAbs is based on the hybridoma technology developed by Cesar Milstein, Georges J.F. Köhler and Niels Kaj Jerne in 1975 and the protocol is described in detail in experimental procedures section VI.3.1. A hybridoma is a hybrid cell line formed after fusion between a specific antibody-producing B-cell (from spleen) with a myeloma cell. Before starting the fusion procedure, serum collected from five BALB/c females, previously intraperitoneally (i.p.) inoculated with wt MCMV and boosted with lysates of MCMV-infected MEFs had to be tested in ELISA for antibody production. In **Figure III.12**, immunized serum from five mice (serum 1-5) was 2-fold serially diluted (from 1/50 to 1/6.400) and incubated with lysates from MCMV-infected cells. IgG antibody titres to MCMV differ for each serum, being probably dependent on the age of mice by immunization. As seen in **Figure**

III.12, sera from mice 4 and 5 presented a relatively low antibody titre to MCMV, even at a low dilution of serum (1/50). Serum 3 showed intermediate to high O.D. values in ELISA, whereas mice 1 and 2 developed the highest MCMV-IgG titres as indicated by the increased O.D. values. Accordingly, spleens obtained from mouse 1 and 2 with the highest antibody response to MCMV were chosen for fusion and for mAbs production.

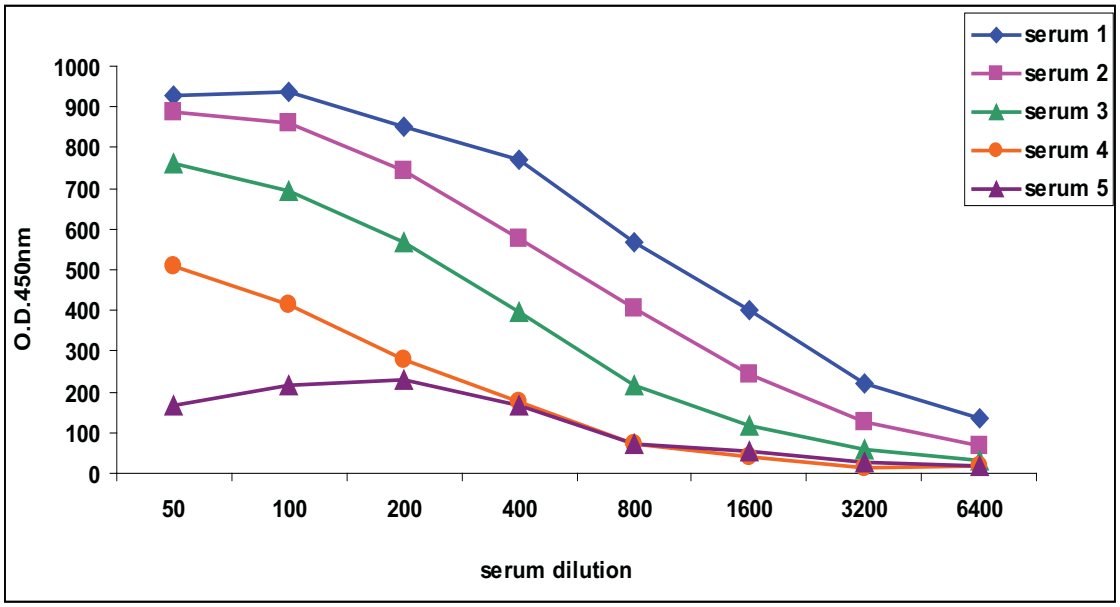


Figure III.12 MCMV antibody titres in immunized BALB/c mice. Serum collected at 90 days p.i. from five mice immunized with wt MCMV was tested in ELISA for the determination of their MCMV IgG titre. Two-fold serial dilutions of each immune serum were added to ELISA plates previously coated with lysates from MCMV-infected cells. Goat anti-mouse IgG-POD was used for detection and O.D. was measured at 450 nm.

After fusion of Sp2/O (ATCC CRL-1581^{TM}) myeloma cells with B cells from the spleens of the two immunized mice (mouse 1 and 2), a number of 50 hybridoma cells were selected. All cells were grown in HAT selection medium and only 42 out of 50 survived further selection and were cultured for production of mAbs. The protocol is described in experimental procedures, section VI.3.1.

Summary:

1) From five mice immunized with wt MCMV and boosted with lysates of infected cells, mouse 1 and mouse 2 that developed the highest MCMV-IgG titres were chosen for fusion and production of mAbs.
2) 42 out of 50 hybridoma cells obtained after fusion survived in the selection medium and were further cultured for mAb production.

III.2.2 Analysis of MCMV-specific IgG production in hybridoma culture supernatant

Culture supernatants (SN) from the 42 hybridoma cells that survived selection with HAT medium were afterwards tested using ELISA assay for the production of MCMV-specific antibodies (**Fig. III.13.A and B**).

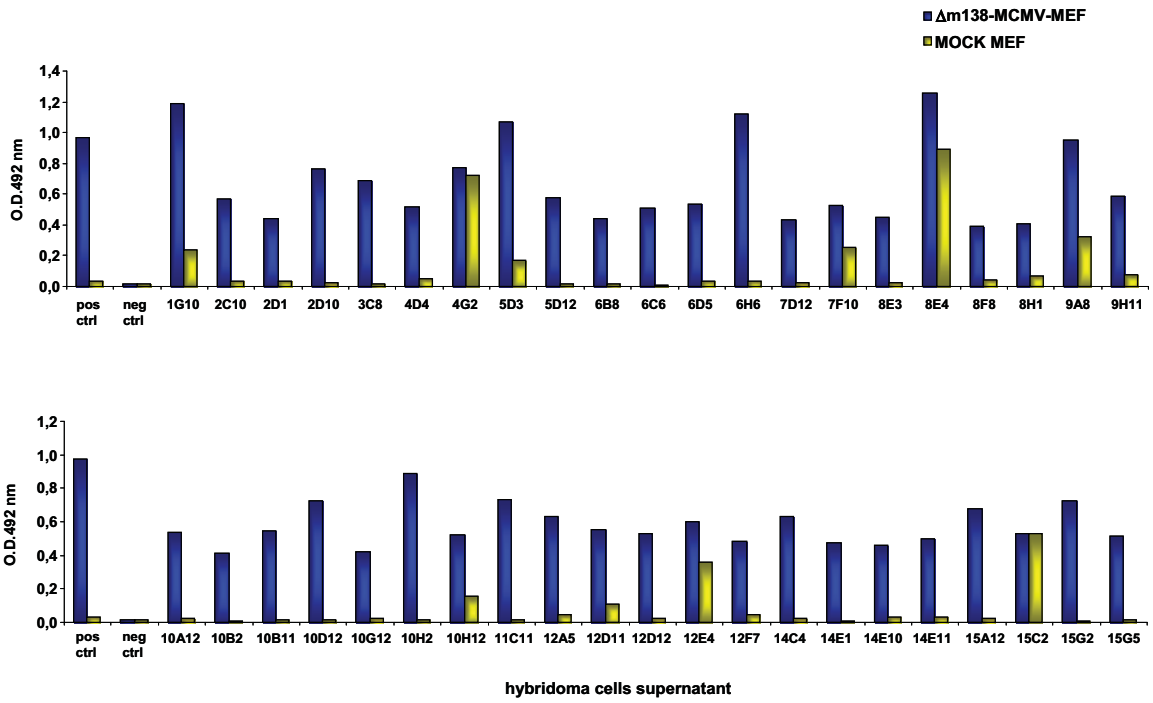


Figure III.13 ELISA test of hybridoma culture supernatant for the determination of MCMV reactivity of Abs. Culture supernatants of the 42 hybridoma cells were tested for the presence of IgG antibody in an MCMV-specific ELISA. A positive MCMV-specific signal was obtained for all supernatants (blue bars); additionally, for SN 1G10, 4G2, 5D3, 7F10, 8E4, 8H1, 9A8, 9H11, 10H12, 12A5 , 12D11, 12E4, 15C2 a significant reaction with non-infected cell lysates was detected (yellow bars). The name of each MCMV hybridoma is derived from the position of the cell culture in a 96-well format. The first number represents the plate number (1-15), the letter corresponds to the line (A-H) and the last number indicates the column of a 96 well-plate where a colony of hybridoma cells could be observed. For example, 1G10 means supernatant collected from hybridoma cell culture from the first 96 well-plate, line G, column 10. The optical density (O.D.) was measured at 492 nm and is direct proportional with the amount of Ab in the hybridoma culture SN. **A.** O.D. of the first 21 hybridoma culture SN, a positive and a negative control; **B**. O.D. of the following 21 hybridoma culture SN, the same positive and negative controls like in Fig.A. Abbreviations: Pos ctrl, positive control (immune serum); Neg ctrl, negative control (non-immune serum).

Previous findings showed that the viral fc receptor m138/fcr-1 (Thale et al., 1994) expressed on the surface of MCMV-infected cells is able to bind the Fc part of an IgG

and therefore could mediate antigen-independent antibody binding in different assays. Taking into account these facts and to exclude a false positive result, further experiments were performed using MCMV that has the ORF *m138* deleted (Δm138-MCMV) and no functional protein is expressed.

ELISA 96-well plates were coated with lysates from Δm138-MCMV- or MOCK-infected MEF and the 42 hybridoma SN, as well as a negative and a positive control serum (polyclonal mouse MCMV-immune serum) were used.

As presented in **Figure III.13.A** and **B**, all the 42 SNs tested in ELISA were strongly reactive with MCMV infected cell lysates, indicating the presence of Abs in the hybridoma culture SNs that are able to recognize viral antigens. Among them, 13 SNs (designated 1G10, 4G2, 5D3, 7F10, 8E4, 8H1, 9A8, 9H11, 10H12, 12A5, 12D11, 12E4 and 15C2) showed a low to high positive reaction also with the cellular antigens from uninfected MEFs (MOCK MEF). These data indicate that the 13 Abs appear to be multispecific for both virus and self-antigen

Summary:

1) All 42 hybridomas produced antibodies in the culture supernatant as shown by ELISA.
2) 29 out of 42 Abs (70%) were specific only for MCMV antigens, whereas 13 Abs (30%) reacted with both viral and self-antigens; these results support the previous publications that MCMV infection is associated with the production of autoantibodies to different tissues and organs.

III.2.3 Screening the surface reactivity of MCMV-specific IgGs

The next step in screening the library of MCMV-IgGs was to check their surface reactivity on MCMV-infected cells. This characteristic of a mAb is important to further define its CD16-activation capability, as the mechanism of ADCC is based on the interaction between the cellular FcγR (e.g. CD16) and a surface viral Ag bound by a specific mAb.

To do this, all 42 hybridoma culture SNs that were found positive in ELISA were further tested for their specificity in flow cytometry analysis (FACS). NIH 3T3 cells infected with Δm138-MCMV or MOCK-infected were stained 24 h.p.i. with each of the

indicated 42 mAbs and detection of the surface reactivity was done with a goat-anti mouse IgG-FITC conjugated antibody. A number of six hybridoma SN were found positive for the surface staining of MCMV-infected cells, i.e. 1G10, 2C10, 2D10, 4D4, 10B2, 15A12 (**Fig. III.14**).

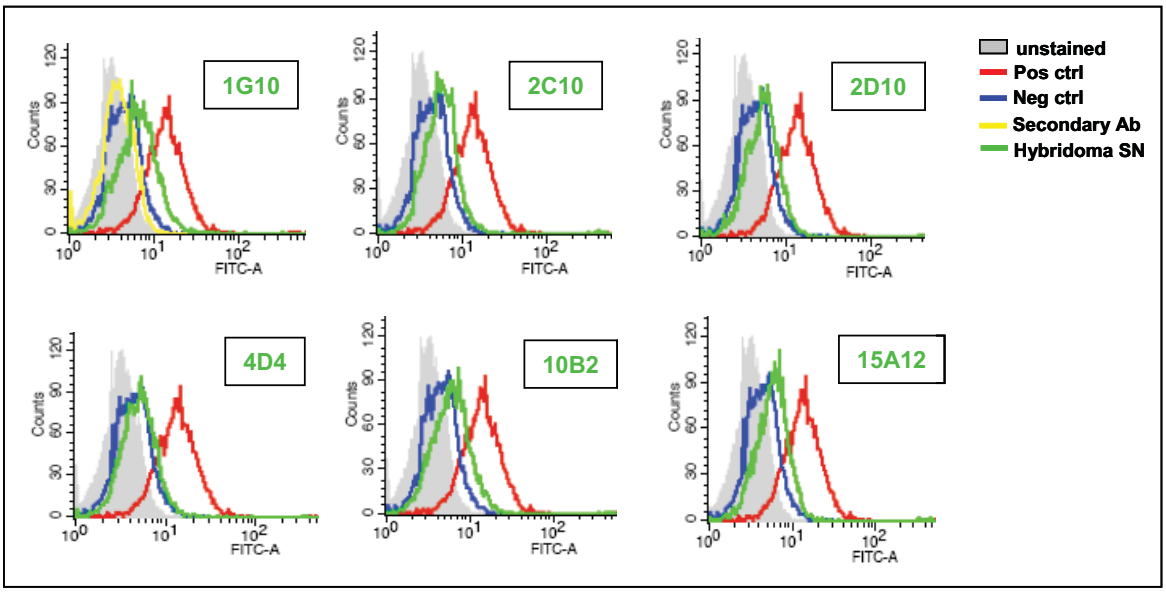


Figure III.14 FACS analysis for detection of surface reactivity of Abs selected by ELISA. Δm138-MCMV-infected NIH 3T3 cells were incubated 24 h.p.i. with each hybridoma SN before IgG binding was detected with goat anti-mouse IgG-FITC. The six hybridoma SN that positively stained the cell surface of MCMV-infected cells are represented in green. A staining with negative serum (blue), positive MCMV-immune serum (red) and only secondary antibody (yellow) is indicated. Unstained NIH 3T3 cells are represented by the filled grey colour. The histogram presented in this figure is one out of three independent experiments. Abbreviations: Pos ctrl, positive control (MCMV-immune serum); Neg ctrl, negative control (non-immune serum); Ab, antibody; SN, supernatant.

Some other hybridoma SN presented a very weak surface staining of the infected cells, i.e. 6B8, 6D5, 8F8, 12D11, 14C4, 14E11 (data not shown). In order to avoid elimination of some potential candidates from these weak positive Abs, they were further included for analysis.

Summary:

1) 6 out of 42 Abs showed a clear positive reactivity with surface viral antigens as indicated by FACS analysis.
2) 6 out of 42 Abs showed a very weak surface staining of infected cells.

III.2.4 Cloning by limiting dilution

All hybridoma cells producing IgGs targeting putative viral antigens on the surface of MCMV-infected cells were subsequently cloned by limiting dilution in order to obtain isolated clones derived from the same hybridoma cell. For each hybridoma mother well, one or more individual clones were obtained. These clones were further tested by FACS analysis for staining of the surface of MCMV-infected cells and the results are presented in **Table III.1**. Most of the clones (22 out of 27) appeared to bind to antigens on the surface of MCMV-infected cells, but not on MOCK cells. Surprisingly, clones derived from 2C10 mother well (2C10-C9 and 2C10-C12), those obtained from 8F8 mother well (8F8-B12 and 8F8-C10) and clone 15A12-E5 did not give any positive staining on infected cells, so they were excluded from further analysis.

Hybridoma SN	1G10		2D10		4D4				10B2				
Clone	B3	F5	A1	F9	A3	A6	C3	H6	B6	D3	D10	E1	E10
FACS	+	+	+	+	+	+	+	+	+	+	+	+	+

Hybridoma SN	2C10		15A12		6B8	6D5		8F8		12D11		14C4	14E11	
Clone	C9	C12	E5	H9	H10	F10	H8	B12	C10	D5	H8	A7	D4	E12
FACS	-	-	-	+	+	+	+	-	-	+	+	+	+	+

Table III.1 Cloning of the positive hybridoma SN by limiting dilution and testing of subclones for surface staining to cells by FACS. The clone names are derived from the original hybridoma SN (mother well) plus the coordinates of a 96-well plate where a single cell could be observed. For example, 1G10-B3 and 1G10-F5 are two individual clones obtained from the 1G10 mother well. Clones that positively stained viral surface antigens are shown here in red; the black colour represents clones found negative on infected cells in a surface FACS.

Summary:

1) Cloning of the 12 mother wells positive for surface MCMV-infected cells (6 strong and 6 weak positive) resulted in 22 subclones positive and 5 negative as indicated by extracellular FACS analysis.

III.2.5 Extending the library of MCMV-mAbs

The purpose to define mAbs with CD16-activating capabilities and later to identify their specific antigens involves screening of a large panel of mAbs. Our current library consists of 22 clones out of 27 that are positive for the surface viral antigens. To prevent that some Abs with the desired effector functions are excluded from the screening procedure, extension of the mAbs library is necessary. More SN collected from cloned hybridoma cells were received from Prof. Stipan Jonjic (Center for Proteomics, Rijeka, Croatia) and tested for their surface antigen reactivity using FACS analysis. Their names and the obtained FACS results are presented in **Table III.2**.

MCMV-mAbs	FACS	MCMV-mAbs	FACS	MCMV-mAbs	FACS	MCMV-mAbs	FACS	MCMV-mAbs	FACS
M37.01 . . M37.10	-	MCMV 1.26	+	MCMV 7.05	+	CROMA 69	-	CROMA 95	+
M151.01 . . M151.05	-	MCMV 1.28	+	MCMV 7.06	-	CROMA 70	-	CROMA 124	+
M153.01	-	MCMV 1.29	+	MCMV 97.3	+	CROMA 75 CROMA 76	-	CROMA 161	+
M153.05	-	MCMV 1.33	+	CROMA 5	+	CROMA 77	-	CROMA 212	-
M153.15	+	MCMV 1.34	+	CROMA 25	-	CROMA 78	-	CROMA 231	-
M153.16	+	MCMV 1.35	+	CROMA 26	+	CROMA 80	-	CROMA 262	-
MCMV 1.01 . . MCMV 1.22	+	MCMV 7.01 . . MCMV 7.03	-	CROMA 60	-	CROMA 88	+	CROMA 271	-
MCMV 1.23	-	MCMV 7.04	+	CROMA 67	-	CROMA 92	-	CROMA 373.10	-

Table III.2 New MCMV-specific mAbs and their surface specificity assessed by FACS. The new mAbs were tested for their surface antigen specificity in FACS analysis; with "+" and in red are indicated mAbs that stain the surface of MCMV-infected cells. The black coloured mAbs and indicated with "-" showed no detectable staining of the surface viral antigens.

Summary:

1) In order to extend the library of mAbs, additional 77 clones were tested in FACS analysis; 39 out of 77 mAbs showed a positive reaction with surface MCMV-infected cells.
2) In the end, the mAbs library consisted of 104 mAbs, 61 out of 104 being positive for staining the surface of MCMV-infected cells.

III.2.6 Assessment of CD16-activation capabilities of mAbs recognizing MCMV-antigens on the surface of infected cells

In order to facilitate a successful vaccine design or immunotherapy against MCMV infection, the antigenic targets recognized by the cytotoxic antibodies that elicit a high activation of the host FcγRs have to be identified. The method that offers us the possibility to screen for the IgG-mediated effector functions via CD16 (FcγRIII)-bearing cells is designated FcγR-ζ activation assay and was recently established by E. Corrales-Aguilar (Eugenia Corrales-Aguilar et al., 2012). The protocol is described in chapter VI.4.16 of experimental procedures.

Screening the large library of MCMV-mAbs by ELISA and FACS narrowed down the list of possible IgG candidates for the identification of the immunodominant MCMV antigens. The next step will provide us with the MCMV-mAbs with CD16-activation capacities.

Each of the 61 mAb was incubated with Δm138-MCMV-infected MEFs for 1 h and the BW5147-CD16-ζ transfected cells were added in a ratio of 20:1. Co-cultivation of target cells with effector cells resulted in the activation of the effector BW-CD16-ζ and IL-2 production. The IL-2 ELISA was performed as described in chapter VI.4.17 and the results are indicated as O.D. values in **Figure III.15**. The higher O.D. values correlate with a stronger activation of the effector cells triggered by CD16-bound antibodies.

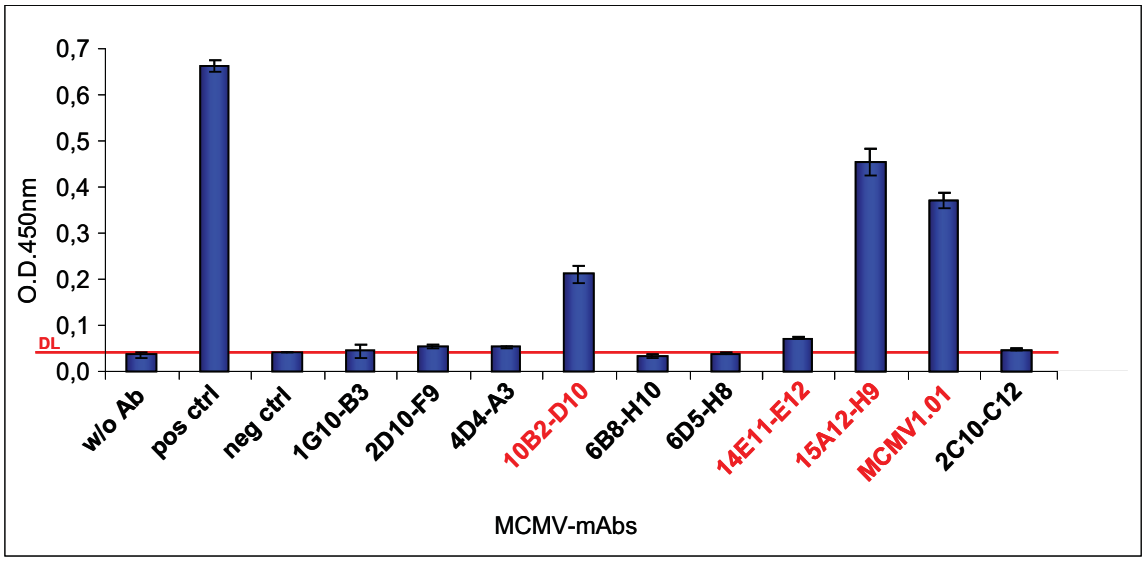


Figure III.15 CD16-activation assay for determination of effector functions of mAbs. 24 h after infection with Δm138-MCMV at a MOI of 1 MEF cells were incubated with indicated IgGs for 1 h at 37^0C and 5% CO_2. A co-cultivation of the target cells (infected MEF) with effector cells (BW5147-CD16-ζ) in a 1:20 ratio (T:E) was performed for 16 h at 37^0C and 5% CO_2. Next day mIL-2 secreted after activation of effector cells was measured in ELISA and O.D. at 450 nm was determined. A positive and a negative control serum (a pool of sera from MCMV infected and not infected mice, respectively), together with 10 out of 61 mAbs are indicated in the graphic. The red line is considered the detection limit of the experiment and delimitate the border between the negative O.D. (background) and the positive O.D. In red are indicated the mAbs that showed a clear positive CD16-activation capability. Each sample was tested in triplicate and the respective standard deviations are indicated. One representative out of 3 independent experiments is shown. Abbreviations: w/o Ab, without antibody; pos ctrl, positive control (immune serum); neg ctrl, negative control (non-immune serum); O.D., optical density; T, target cell; E, effector cell.

As shown in **Figure III.15**, from all 61 mAbs tested in CD16-activation assay, only **10B2-D10**, **15A12-H9** and **MCMV 1.01** showed a clear positive result, indicated by the relatively high O.D. values comparable with the positive control (MCMV-immune serum). Moreover, **14E11-E12** had a measured O.D. shortly above the detection limit. The other 57 mAbs were found negative in CD16-ζ-activation assay, indicating no detectable capacity of triggering CD16-dependent ADCC responses.

Taken together, these data indicate that the frequency of mAbs with CD16-activating capabilities is very low and represents only 3,8% from the total mAbs of the initial library (4 out of 104 mAbs).

Moreover, **10B2-D10**, **15A12-H9**, **MCMV1.01** and **14E11-E12** mAbs are further considered the final candidates for the identification of antigens triggering ADCC responses via CD16.

Summary:

1) The FcγR-ζ-activation assay revealed a number of only 4 mAbs (**10B2-D10**, **15A12-H9**, **MCMV 1.01** and **14E11-E12)** that were able to trigger CD16 immune effector functions *in vitro* by activating FcγRIII-bearing cells. These data suggest that CD16-activating Abs represent only a minor subspecies from the total IgGs produced after MCMV infection.

III.2.7 Testing the possible neutralization capabilities of mAbs triggering CD16-effector functions

Crowe Jr. *et al.* and Burton *et al.* (Burton, 2002; Crowe, Jr. et al., 2001) divided the protective effects of antibodies intro two groups: one group that targets the free virus particles and prevent infection of host cells by a process called neutralization; the other group targets virus-infected cells and prevents virus replication and cell-cell transmission. Therefore, due to their specialized functions, it is considered that Abs can either neutralize or trigger ADCC-responses via host FcγRs.

In order to test if our previously described IgG Abs that showed CD16-activation capabilities are also able to neutralize the virus, a conventional neutralization assay for the indicated mAbs on MEF cells was performed. The protocol is described in section VI.2.5 of experimental procedures.

The same amount of 10B2-D10, 14E11-E12, 15A12-H9 and MCMV 1.01 Abs was incubated separately with $1x10^5$ PFU Δm138-MCMV 1,5 h at 37^0C and 5% CO_2. After the neutralization process took place, the Ab-virus mixture was added over MEF cells and titrated. Four days later, primary plaques formed after virus infection were counted by microscopic inspection. Plaque number correlates with the number of viral particles infecting one cell (**Fig. III.16**).

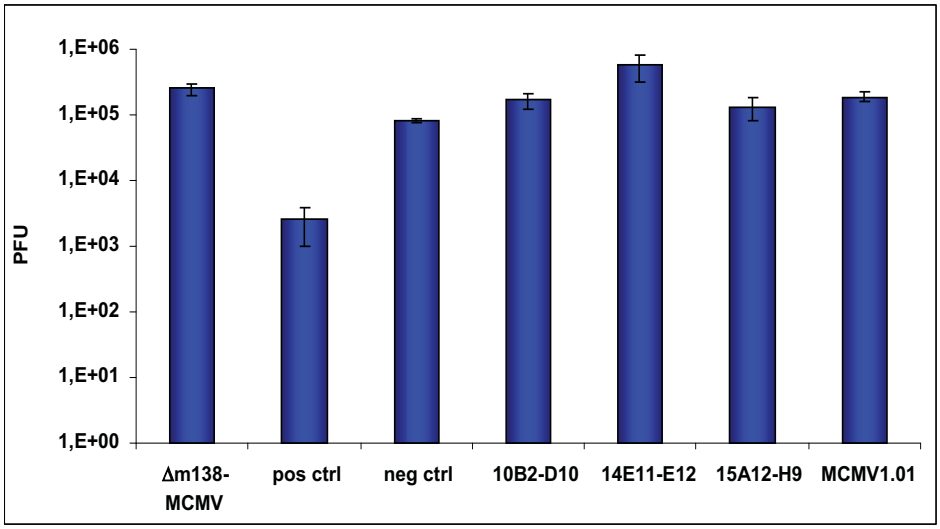


Figure III.16 Conventional neutralization test for the CD16-activating mAbs. A master mix of each indicated mAb and 1x10^5 PFU Δm138-MCMV were incubated 1,5 h at 37^{0}C and 5% CO_2. A positive and a negative control serum, as well as virus alone were also used in the experiment. The Ab-virus mixture was afterwards titrated over MEF cells, centrifuged for enhancement of viral infection and incubated for minimum 2 h at 37^{0}C and 5% CO_2. Later on, a semi-solid methylcellulose medium was added, to avoid spreading of virus particles from one cell to the other. Data are means ± standard deviations of three determinations of the same sample; Values as PFU (plaque forming units) in a logarithmic scale are indicated.

As shown in **Figure III.16**, the tested mAbs are not able to neutralize the Δm138-MCMV indicated by the number of counted plaques similar to those obtained for the negative control (non-immune serum); these data indicate that the tested MCMV-mAbs with CD16-activation capabilities have non-neutralizing effects when compared to the immune serum (positive control) that reduces infection of MEF cells with about 2 $\log_{10}$ steps.

Summary:

1) The conventional neutralization test confirms that MCMV1.01, 15A12-H9, 10B2-D10 and 14E11-E12 are not able to block viral entry by neutralizing the virion, so they have no neutralizing effect. These data confirm the hypothesis that after MCMV infection specialized Abs for either neutralization or ADCC are produced.

III.2.8 IgG subclass determination of mAbs

It was described that defined effector functions of antiviral Abs are restricted to only some IgG subclasses, indicating a natural selection of Abs that are more effective against a viral infection. In the mouse model, IgG2a and IgG2b are the most important isotypes in eliciting cytotoxic responses (Coutelier et al., 1987) via CD16 and FcγRIV. Our previous data (experimental results chapter III.1.2) also indicated a predominant production of IgG2a followed by IgG2b in BALB/c mice after MCMV infection. Therefore, a further characterization of the MCMV-mAbs that were found to trigger CD16-responses and having no direct neutralizing effect of the MCMV virion includes determination of IgG subclass assignment. For this purpose, Iso-Gold™ Rapid Mouse-Monoclonal Isotyping Kit (BioAssay) was used and the results are presented below (**Table III.3**). The protocol followed the instructions supplied with the manual's kit.

MCMV-mAbs	MCMV 1.01	10B2-D10	15A12-H9	14E11-E12
IgG subclass	IgG2b	IgG2a	IgG2a	IgG2a

Table III.3 IgG subclass of MCMV-mAbs able to trigger CD16 responses.

As shown in **Table III.3**, IgG subclass determination indicated the IgG2a isotype for the 10B2-D10, 15A12-H9 and 14E11-E12 antibodies and the IgG2b isotype for MCMV 1.01. The results are consistent with published data from Coutelier et al, 1987 where it was reported that mouse CD16 binds primarily antibodies of the IgG2a and IgG2b subclass (Coutelier et al., 1987).

Summary:

1) The CD16-activating Abs 10B2-D10, 15A12-H9 and 14E11-E12 belong to the IgG2a isotype, whereas MCMV 1.01 was found to be of IgG2b subclass.

III.2.9 Improving test results by concentrating the mAbs SN

Hybridoma cells differ in the quality and quantity of mAbs produced and secreted in the culture supernatant, therefore the concentration of mAbs in a SN may vary considerably. In order to compare the CD16-activation capabilities of the four mAbs produced by the hybridoma cells 10B2-D10, 15A12-H9, 4E11-E12 and MCMV 1.01, it was necessary to adjust the same concentration of the tested mAb. To achieve this goal, purification and concentration of these mAbs using Protein G columns was performed. The protocol was described in experimental procedures, paragraph VI.4.9.1. The concentration of the purified IgG from the culture SN was determined by the Bradford method using a BSA standard curve. For the purified mAbs, the following concentrations expressed in μg/ml were obtained (**Table III.4**).

MCMV-mAbs	MCMV 1.01	10B2-D10	15A12-H9	14E11-E12
Concentration (μg/ml)	759	240	85	262

Table III.4 Concentrations (μg/ml) of purified MCMV-mAbs from culture SN.

III.2.10 Concentration-dependent CD16-activation

The question whether activation of CD16 is dependent on the amount of tested Abs, as well as a comparison of the strength of CD16 responses using a defined concentration of IgG were further investigated. To do this, CD16-activation assay using defined concentrations of each of the four IgG candidates was performed. The protocol is described in the section VI.4.16 of experimental procedures. 5, 25, 50 μg/ml and the maximum concentration obtained after purification of either 10B2-D10 or 15A12-H9 Abs were tested; 5, 25, 150, 262 and 25, 150, 350, 759 μg/ml of 14E11-E12 and MCMV1.01, respectively were used. As shown in **Figure III.17**, CD16-activation triggered by 10B2-D10, 14E11-E12 and 15A12-H9 Abs in the presence of Δm138-MCMV infected cells was increasing dose-dependently. These results indicate that CD16 activation is dependent on the concentration of IgG and that an individual threshold amount of IgG is required for a minimal CD16 triggering: 25 μg/ml for 10B2-D10, 5 μg/ml of 14E11-E12, 50 μg/ml 15A12-H9 and 25 μg/ml of MCMV1.01.

Interestingly, when 240 µg/ml of 10B2-D10 or 262 µg/ml of 14E11-E12 Abs were used, a slight CD16-activation on MOCK cells was observed; these could be due to the formation of IgG aggregates that potentially trigger CD16 activation in MOCK cells.

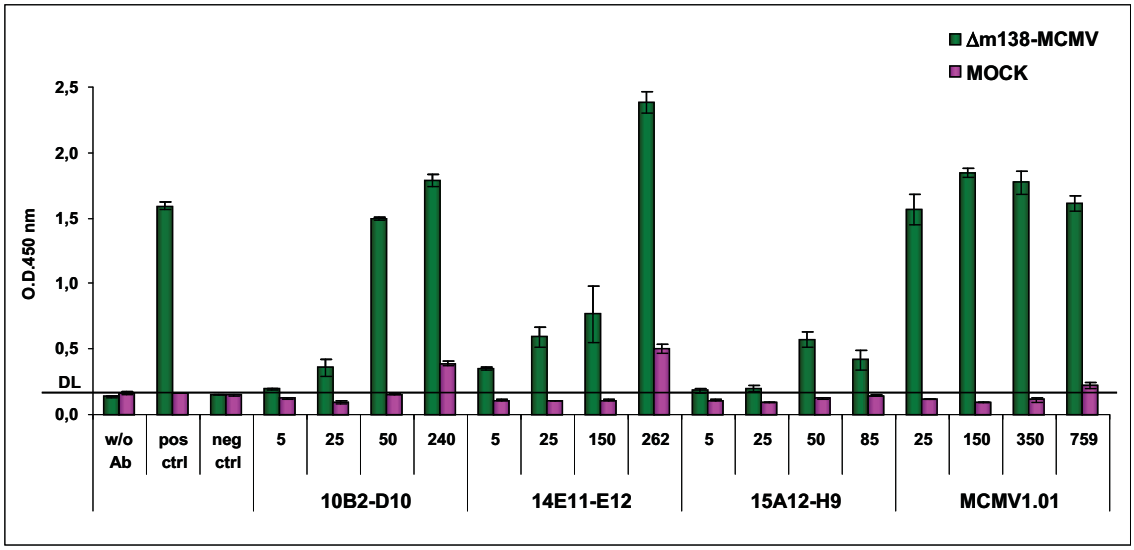


Figure III.17 Concentration-dependent CD16-activation profiles of the four MCMV-mAbs. MEF cells were either MOCK or Δm138-MCMV infected in triplicate with MOI 1. 24 h.p.i. different amounts (µg/ml) of indicated mAbs, as well as a positive and a negative control serum were incubated with the fibroblasts. After 1h, BW-CD16-ζ cells were added in a ratio of 20:1 (effector : target) and incubated for 16 h with Ab-bound target cells. Next day IL-2 secreted in the medium was analysed using mIL-2 ELISA and the O.D. at 450 nm was measured. In green and magenta, CD16-activation capabilities of indicated mAbs on virus-infected cells and MOCK cells, respectively are indicated. Error bars indicate standard deviations between triplicates. DL, detection limit defined by the O.D. of the negative control (non-immune serum).

MCMV1.01 did not show a significant modification in the CD16-activation profile at either low or high concentrations (**Fig. III.17**). The only slight increase in CD16-activation

triggered by MCMV1.01 Ab was observed between 25 and 150 µg/ml. Above 150 µg/ml, MCMV1.01 did not trigger a stronger CD16-activation, indicating that the saturation range is already reached.

Comparing the four mAbs at a chosen concentration of 25 µg/ml, the most potent CD16-activator is MCMV1.01 > 14E11-E12 > 10B2-D10 > 15A12-H9.

Summary:

1) Different concentrations of purified MCMV1.01, 10B2-D10, 15A12-H9 and 14E11-E12 Abs were determined of IgG preparations performed using protein G columns (**Table III.4**).
2) Comparison of the CD16-activation capability was done using the same amount of IgG (e.g. 25 µg/ml) and showed an activation profile of MCMV1.01 > 14E11-E12 > 10B2-D10 > 15A12-H9.
3) Activation of CD16-bearing BW5147 responder cells is dependent on the concentration of IgG present in the sample.

Summary of experimental results part A

As shown in **Figure S.1**, from a library of 104 MCMV-specific mAbs as revealed by ELISA that were further tested by FACS analysis for the detection of surface antigens, 61 mAbs (58,6%) were found positive for staining antigens on the surface of MCMV-infected cells. These IgGs were further tested in a FcγRIII-activation assay for determination of their individual activatory capabilities. Only 4 mAbs (approx. 3,8%) were eventually found to activate BWCD16-ζ transfected responder cells. The 4 MCMV-mAbs, designated MCMV1.01, 15A12-H9, 10B2-D10 and 14E11-E12 represent candidates for the identification of CD16-activating MCMV antigens; they were additionally tested in a conventional neutralization assay and showed no neutralizing effect, indicating that mAbs produced after MCMV infection may have selective functions, either neutralization or mediators of FcγR/ADCC-responses.

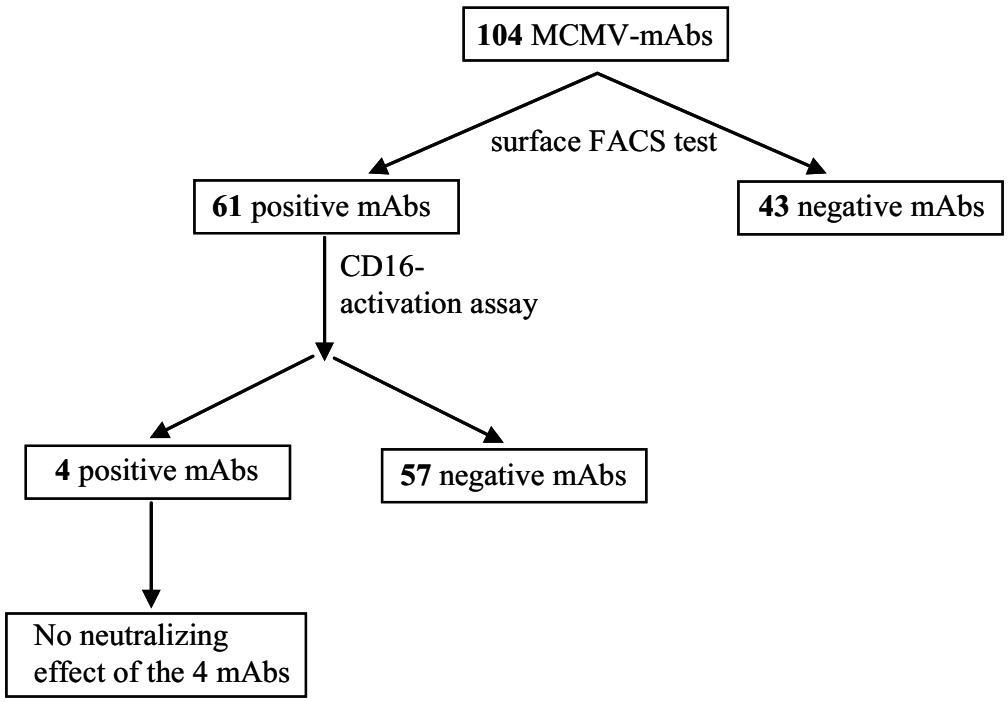


Figure S.1 Summary of experimental results A. Abbreviations: MCMV, Mouse Cytomegalovirus; mAbs, monoclonal antibodies.

III.2.11 Characterization of proteins precipitated with the CD16-activating mAbs (B)

After having identified four MCMV mAbs that are able to trigger CD16 activation in infected cells (15A12-H9, 14E11-E12, 10B2-D10 and MCMV1.01), the next challenge was to characterize and identify the viral proteins recognized by these mAbs. First, western blot analysis of lysates derived from infected and non-infected cells respectively, using each of the four mAb revealed clear protein bands of different molecular weights present only in infected cells (data not shown). Moreover, to be able to identify those viral proteins by mass spectrometry, immunoprecipitation (IP) from ^{35}S-metabolically labeled and non-labeled cells, followed by coomasie staining was performed.

Previously labelled ^{35}S-Met/Cys Δm138-infected or MOCK NIH 3T3 cells were lysed using the protocol described in section VI.4.2 of experimental procedures. 2 μg/ml of each purified mAb was added to the cell lysates and incubated overnight at 4^0C. Next day, a precipitation of immune complexes using protein G sepharose has been performed (see chapter VI.4.5 – experimental procedures). The samples containing precipitated proteins were loaded on a SDS-PAGE and separated overnight. To check whether the proteins are glycosylated, conditions with (+) and without (-) Endo H digestion were analysed (**Fig. III.18**).

As shown in **Figure III.18**, proteins of different molecular weight were precipitated from Δm138-MCMV infected NIH 3T3 and not in MOCK fibroblasts by MCMV1.01, 10B2-D10, 15A12-H9 and 14E11-E12 mAbs, suggesting viral origin of the recognized antigens.

MCMV1.01 and 15A12-H9 Abs (**Fig. III.18**, upper left and lower left, respectively) precipitated a viral protein of ca. 130 kDa. Both 130 kDa proteins are glycoproteins, as they are deglycosylated after EndoH digestion and their molecular weight is reduced to approx. 90 kDa.

Regarding 10B2-D10 Ab, two EndoH-resistant proteins at 63 and 49 kDa were precipitated only from virus-infected cells (upper panel right). There is no indication of glycans present in these two bands, as neither EndoH nor PNGase F digestion revealed any change in the molecular weight (data not shown). Additionally, 14E11-E12 Ab precipitated a diffuse fainted band of 120 kDa only from infected cells that is also EndoH-resistant (lower panel, right).

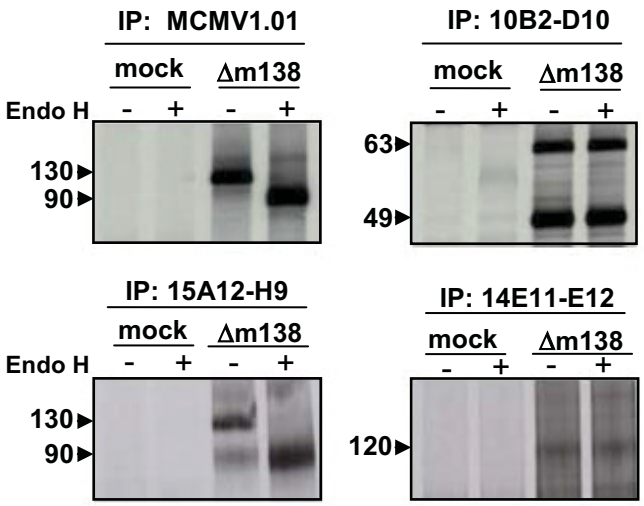


Figure III.18 IP of metabolically labeled MCMV-proteins. For each sample, MOCK and 24 h Δm138-MCMV-infected NIH 3T3 cells were labeled with ^{35}S-Met/Cys for 2-3 h at 37^{0}C, 5% CO_2 and then the lysates were prepared as described in experimental procedures. Cell lysates were overnight incubated with 2 μg/ml of the indicated mAb at 4^{0}C in an overhead tumbler; next day precipitation of immune complexes with protein G sepharose was performed. Samples without (-) and with (+) EndoH digestion for MOCK- and infected cells are indicated in the figure. Separation of samples was performed in a SDS-PAGE. The blot shown is one representative out of 3 independent experiments.

III.2.12 Mass spectrometric identification of proteins precipitated by MCMV-mAbs MCMV1.01, 10B2-D10, 15A12-H9 and 14E11-E12

The next step was aiming at the final identification of antigens recognized by MCMV-mAbs that trigger CD16 activation. Prior to the identification of bands by mass spectrometry, immune complexes recovered by each of the four MCMV-mAbs were analysed by coomassie staining, using the protocol described in section VI.4.7 of experimental procedures. The 130 kDa band precipitated with MCMV1.01 and 15A12-H9, as well as the 63/49 and 120 kDa bands precipitated by 10B2-D10 and 14E11-E12 Abs, respectively, were cut out from the coomassie stained gels and subjected to mass spectrometry analysis (**Fig. III.19**, red marked bands).

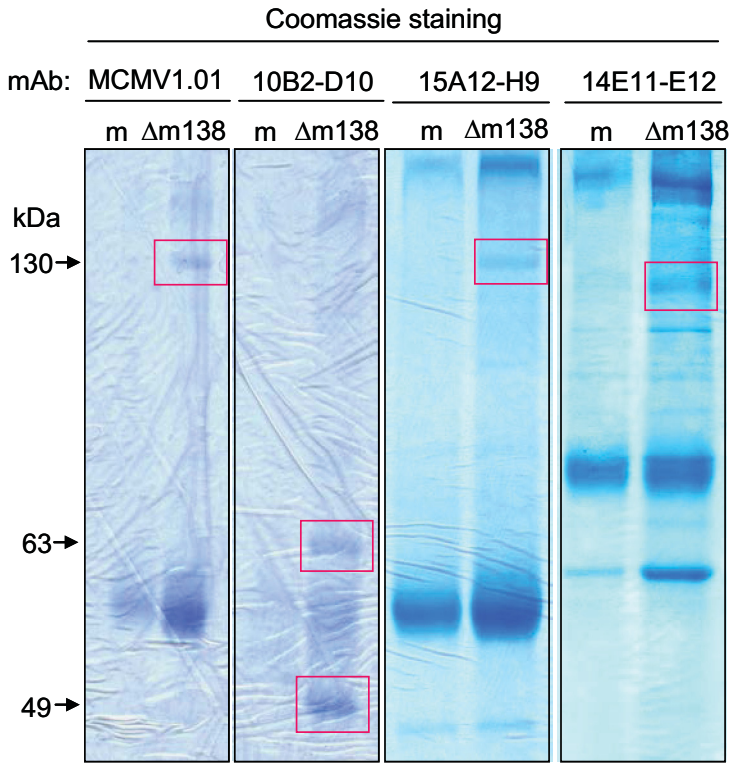


Figure III.19 Coomasie staining of proteins precipitated by MCMV-mAbs. Lysates of MOCK- (m) and Δm138-MCMV-infected NIH 3T3 cells were performed 24 h.p.i. as described in experimental procedures, section VI.4.2. Concentrations of 2 µg/ml of each indicated MCMV-mAb were used for the precipitation of viral proteins, following the protocol used in the case of non-metabolically labeled proteins. Separation of samples was carried out in big SDS-PAGE gels and afterwards the precipitated bands were stained with a solution of coomasie brilliant blue as described in section VI.4.7. The protein bands marked with red square were cut out from the gel and sent to mass spectrometric analysis.

Several peptides resulting from the tryptic digestion of the entire proteins were analysed by mass spectrometry for their molecular weight. Digestion was performed with trypsin (EC 3.4.21.4), a serine protease that cleaves the peptide chain after Lysine (K) or Arginine (R) residues. The obtained peptides were afterwards compared with those from the data bank and the results are presented in the table below:

Table III.5 Mass spectrometry results of the antigens precipitated by CD16-activating mAbs.

MCMV-mAb	IP profile	Peptides	Sequence similarity with data bank proteins
MCMV1.01	130 kDa	TYTEQDALLILR INPSNILSAIYERPVTAK TYFGELAAPK	MCMV-gB (Major Envelope Glycoprotein B)
15A12-H9	130 kDa	46 unique peptides	MCMV-gB (Major Envelope Glycoprotein B)
10B2-D10	63 kDa	SFFLTQTSER IGLLTVEFIYR VLCpTHVAAPPRPR	MCMV- m143 (member of US22 family homolog)
	49 kDa	GLVNFEPLYAER SADQVESFPGYPVWEMYGGAPAGR DVEVADALNDVDATFR TDAVFVM$_{ox}$SLSIDELAR	MCMV- m142 (member of US22 family homolog)
14E11-E12	120 kDa	52 peptides	Myosin IC isoform A (*Mus musculus*)

As indicated in **Table III.5**, the 130 kDa band precipitated by the MCMV1.01 mAb contained three peptides: TYTEQDALLILR, INPSNILSAIYERPVTAK and TYFGELAAPK, the sequences all of which indicated the **Major Envelope Glycoprotein B (gB)** of MCMV.

The protein recognized by the 15A12-H9 mAb was found to have 43,2% sequence coverage with MCMV **glycoprotein B** and a number of 46 unique peptides belonging to this protein were revealed by mass spectrometry and data bank analysis.

The 63 and 49 kDa bands precipitated by the 10B2-D10 mAb corresponded to **m143** and **m142,** respectively. Both proteins are described to be members of the *US22* gene family homolog of MCMV. For m143, three peptides were identified: SFFLTQTSER, IGLLTVEFIYR, VLCpTHVAAPPRPR, whereas for m142 a number of four viral peptides were indicated: GLVNFEPLYAER, SADQVESFPGYPVWEMYGGAPAGR, DVEVADALNDVDATFR and TDAVFVM$_{ox}$SLSIDELAR.

Surprisingly, the band precipitated at about 120 kDa by the 14E11-E12 Ab was found to yield to 52 peptides belonging to the isoform A of **myosin** from *mus musculus*.

Summary:

1) The western blot and IP analysis revealed one band at 130 kDa for MCMV1.01 and 15A12-H9 Abs, two bands precipitated at 49 and 63 kDa by 10B2-D10 Ab and a 120 kDa band for 14E11-E12 Ab.
2) MCMV1.01 and 15A12-H9 recognize both the MCMV glycoprotein B; 10B2-D10 binds to either m142 or m143 (both members of the US22 family homolog); and finally, the band precipitated by the 14E11-E12 Ab was identified as mouse myosin.

III.2.13 Neutralization and CD16-activation capabilities of anti-gB antibodies

Glycoprotein B is known to be a major neutralizing glycoprotein present in the envelope of the MCMV virion (Rapp et al., 1992) Our findings that the MCMV1.01 and 15A12-H9 mAbs are able to precipitate glycoprotein B of MCMV lead us to compare their neutralization capabilities with a known IgG2a neutralizing α-gB antibody namely MCMV 97.3 and kindly provided by Prof. Jonjic S., Croatia.

To this end, a luciferase-based neutralization test using Δm157-luc-MCMV and the three α-gB antibodies (undiluted SNs of MCMV1.01, 15A12-H9 and MCMV 97.3) was performed as described in experimental procedure (**Fig. III.20.A**).

As shown in **Figure III.20.A**, MCMV 97.3 mAb is able to neutralize almost 99% of the virus (almost 3 $\log_{10}$ scales), comparable to 78% neutralization induced by mouse immune serum (positive control); the measured relative light units for MCMV1.01 and 15A12-H9 SN are comparable with those measured with non-immune serum (negative control), suggesting a lack of neutralizing activity of these two α-gB antibodies.

In parallel, the three α-gB antibodies were compared with regard to their CD16-activation capabilities. To this end, FcγR-activation assay using BW5147-FcγRIII-ζ as effector cells was performed as previously described in experimental procedures.

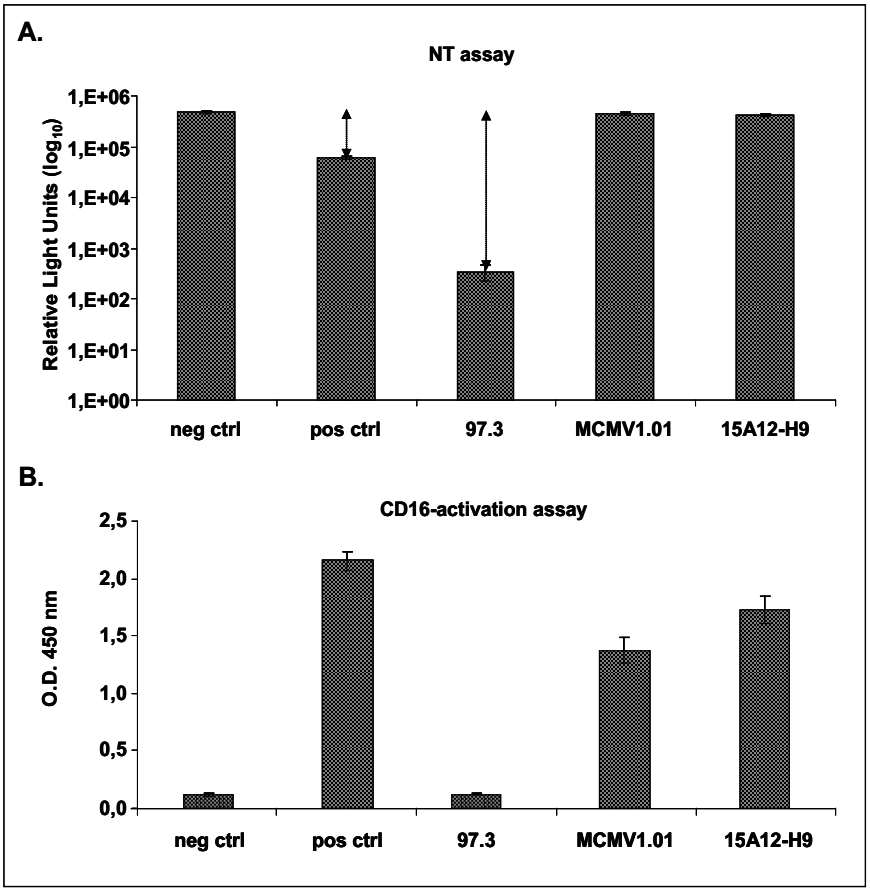


Figure III.20 Neutralization and CD16-activation capabilities of α-gB mAbs. Luciferase-based neutralization test (A) and FcγRIII-activation assay (B) with MCMV1.01, 15A12-H9 and MCMV 97.3 antibodies were performed as described in experimental procedures. In both assays, each mAb was used as undiluted culture supernatant. As controls, positive and negative (non-immune) polyclonal sera were used in experiments. Relative light units in a logarithmic scale (log_{10}) and optical densities measured at 450 nm are indicated in A respectively B.

As indicated in **Figure III.20.B**, MCMV1.01 and 15A12-H9 mAbs were seen to trigger CD16-responses comparable to immune serum (positive control); these results were previously confirmed by the experiment indicated in the **Fig. III.15**, where SNs of the indicated mAbs were tested. Conversely, the neutralizing 97.3 antibody did not elicit CD16-dependent effector functions. A negative control represented by serum collected from non-infected mice was used in these two experiments.

Summary:

1) Taken together, these data demonstrate for the first time the existence of antibodies directed against MCMV gB that have separate effector functions, being specialized either in neutralization or FcγRIII activation.

III.2.14 Identification of the putative antigen recognized by the 10B2-D10 Ab

The mass spectrometric analysis of the two bands precipitated by the 10B2-D10 Ab revealed two distinct proteins: one having a molecular weight of 49 kDa coded by the *m142* ORF of MCMV and the second of 63 kDa, identified as m143 MCMV protein.
As both m142 and m143 MCMV proteins are co-expressed at similar levels with the same subcellular distribution in infected cells (Hanson et al., 2005) and act in a heterodimeric complex (Child et al., 2006), one question arises from our findings: which of the both proteins is the direct target of the 10B2-D10 mAb? To answer to this question, an immuno-precipitation using 10B2-D10 Ab, MOCK- and Δm138-infected NIH 3T3 lysates, as well as lysates harvested from fibroblasts stably transfected with either m142-HA or m143-HA epitope tagged genes was performed, as described in sections VI.4.2 and VI.4.5 of experimental procedures (**Fig. III.21**).

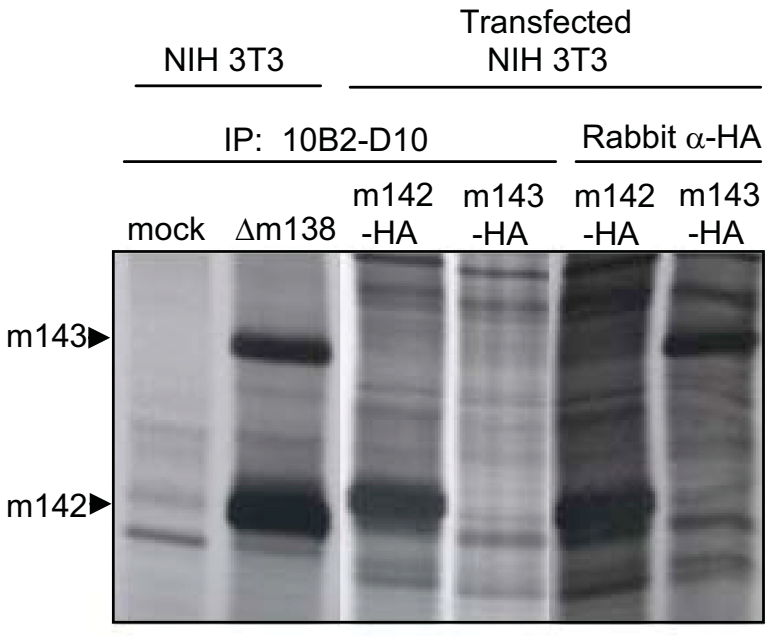


Figure III.21 IP of m142 and m143 in infected and transfected NIH 3T3 cells. NIH 3T3 cells were MOCK- or Δm138-MCMV infected with MOI 5. At 24 h.p.i., lysates of ^{35}S-metabolically labeled MOCK, Δm138-MCMV-infected NIH 3T3 cells, as well as of cells stably transfected either with m142-HA or m143-HA were harvested as described in experimental procedures. Immunoprecipitation with 10B2-D10 (2 μg/ml) or rabbit α-HA antibody (2 μg/ml) was performed. The samples were separated by SDS-PAGE using the protocol presented in section VI.4.5. The blot is one representative of two independent experiments.

As illustrated in **Figure III.21**, both m142 and m143 proteins were immuno-precipitated in Δm138-MCMV infected cells at 24 h.p.i., but not in MOCK cells (lanes

1 and 2), indicating the viral origin of these two bands. Moreover, 10B2-D10 Ab was able to precipitate m142-HA from m142-HA stably transfected fibroblasts (lane 3), but not when m143-HA transfected cells were used (lane 4). These results clearly show that MCMV m142 protein is the antigen recognized by 10B2-D10 Ab and that the m143 is co-precipitated, as both proteins are described to form heterodimers (Child et al., 2006; Valchanova et al., 2006). As control, a rabbit anti-HA antibody for detection of HA-tagged m142 or m143 in 3T3 NIH transfected cell lysates was used (**Fig. III.21**, lanes 5 and 6). The HA epitope tag is a synthetic peptide derived from human influenza hemagglutinin (HA) and contains 9 amino acids (YPYDVPDYA). It was used for fusion with MCMV m142 and m143 ORF, prior to transfection into NIH 3T3 fibroblasts. Therefore, the fusion protein expressed by transfected cells has a molecular weight consisting of the original protein (m142 or m143) plus the molecular weight of the HA tag (**Fig. III.21**, lanes 3, 5 and 6).

Summary:

1) Additional analysis showed that the m142 MCMV protein is the direct target of 10B2-D10 Ab and that the m143 is co-precipitated, as these two proteins form a complex.

III.2.15 Re-testing the surface reactivity of 10B2-D10 and 14E11-E12 Abs with IgG2a isotype controls

The identification of proteins precipitated by the MCMV-mAbs 10B2-D10 and 14E11-E12 i.e. m142/m143 and mouse myosin, raised a problem in that these proteins are not known to be exposed on the cell surface. Specifically, myosin was described to have a cytoplasmic localization and also to concentrate at the highly motile peripheral regions of most of the cells (Barylko et al., 1992; Drenckhahn and Dermietzel, 1988; Fath and Burgess, 1993; Wagner et al., 1992). M142/m143 were reported to form a heterodimer complex and a co-localization of both proteins predominantly in the cytoplasm, but also nucleus of infected cells was reported (Hanson et al., 2005).
There are several possibilities that could explain the obtained results:

a) The intracellular viral proteins could become targets for the antibodies with effector functions, following translocation of the antibody through pores into infected cells.
b) Primarily intracellular proteins might reach the cell surface and become exposed to antibodies, although not being transported along the secretory pathway of the cell (unconventional transport pathway to the cell surface, e.g. HCMV ICP22 (Mocarski et al., 1988; Romanowski and Shenk, 1997).
c) The 'molecular mimicry' phenomenon already described for some MCMV proteins that share common epitopes with mouse myosin (Lawson et al., 1992; O'Donoghue et al., 1990).
d) A further MCMV-encoded FcγR that is not yet identified and binds the Fc part of the antibody, leading to a 'false positive' staining in FACS analysis. Our immunoprecipitation assays come in accord to this hypothesis by showing the presence of an 'unidentified viral band' of ca. 50 kDa precipitated in the presence of MCMV-mAbs with IgG2a and IgG2b, but not with IgG1 subclass (data not shown).
e) 'Wrong bands' were excised for mass spectrometry analysis.

The hypothesis of another MCMV-encoded FcγR could be analyzed by re-testing the surface specificity of the 10B2-D10 and 14E11-E12 mAbs by FACS with the proper IgG2a isotype control (**Fig. III.22**).

In **Figure III.22**, for both 10B2-D10 and 14E11-E12 Abs the surface binding to infected cells was reproduced. The obtained results confirm the initial FACS data that suggested the presence of a viral antigen on the surface of infected cells, targeted by the two MCMV-specific mAbs. Mouse anti-V5 antibody, tested in the same concentration like 10B2-D10 and 14E11-E12 Abs (25 μg/ml) shows an intermediate surface staining of infected cells when compared to negative control (non-immune serum) and the two tested mAbs. Mouse α-V5 Ab is detecting recombinant proteins containing V5 epitope (Ausubel F.M. et al., 1994) and there is no target for the α-V5 Ab on mock or MCMV-infected NIH 3T3 cells.
Therefore, the use of a mouse monoclonal IgG2a Ab that is not MCMV specific but has the same IgG isotype like the MCMV-specific mAbs, could partially explain the

occurrence of the observed FACS staining with mAbs 14E11-E12 and 10B2-D10, respectively.

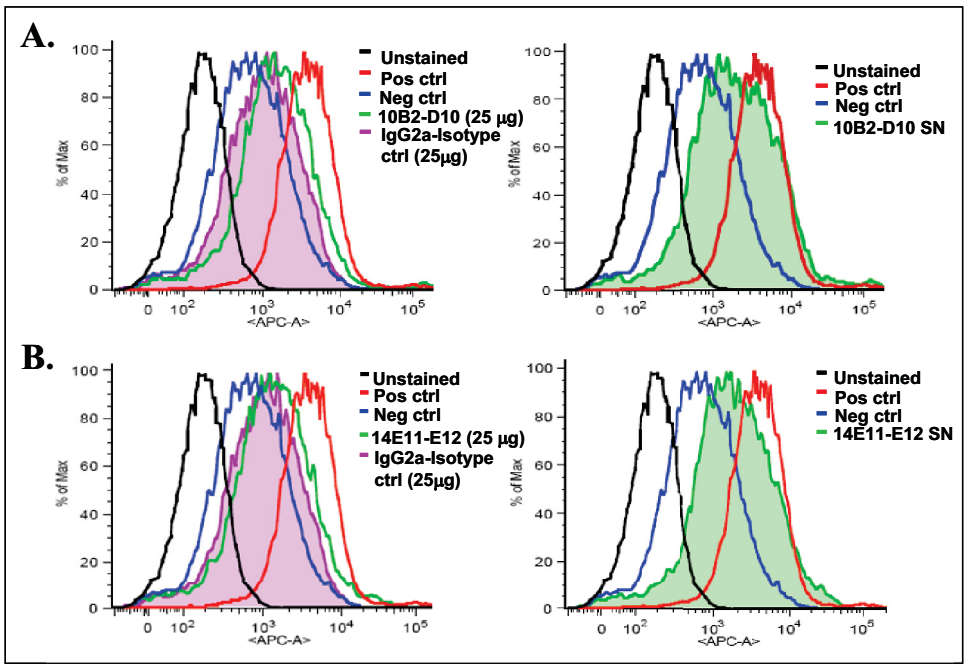


Figure III.22 Surface staining of MCMV-infected cells with 10B2-D10 and 14E11-E12 Abs assessed by FACS. NIH 3T3 cells were infected with Δm138-MCMV at a MOI of 2. 48 h.p.i cells were surface stained with either 10B2-D10 (A) or 14E11-E12 Abs (B) (represented in green). 25 µg/ml from the concentrated Abs (left panel) or undiluted culture supernatant (right panel) were used. A positive (red) and negative (blue) control serum (immune and non-immune serum, respectively), as well as mouse α-V5 Ab (IgG2a isotype control) (violet) were also included in the test. Primary antibodies were detected with goat anti-mouse IgG-Cy5 conjugated antibody and the fluorescence intensity was measured within the APC channel.

In order to control the surface viral specificity of generated MCMV-mAbs (including 14E11-E12 and 10B2-D10 mAbs), FACS analysis using not infected NIH 3T3 cells (MOCK) were simultaneously performed. As expected, no staining of the surface of MOCK cells was observed (data not shown).

Summary:

1) FACS re-tests confirmed the initial data showing 10B2-D10 and 14E11-E12 to stain the surface of MCMV-infected cells.
2) Moreover, a similar intensive staining of the surface of infected cells was observed with IgG2a isotype control Ab, suggesting the possibility of an unspecific Ab binding detected in FACS.

Summary of the experimental results part B

Characterization and the mass spectrometric identification of the antigens recognized by the four new MCMV-mAbs were performed. The band sizes precipitated by mAbs and their names as revealed by mass spectrometry are indicated in **Table S.2**. Moreover, a short characterization which implies FACS results and CD16-activation capabilities for each mAb are also showed in the table.

B.

MCMV-mAbs	IgG subclass	FACS	CD16-activation assay	Neutralization assay	IP major band	Mass Spectrometry protein identification
MCMV1.01	IgG2b	++++	++++	NN	130 KDa	α-gB
15A12-H9	IgG2a	++++	+	NN	130 KDa	α-gB
10B2-D10	IgG2a	++++	++	NN	63 KDa	α-m142 (m143 is co-precipitated)
14E11-E12	IgG2a	++++	+++	NN	120 KDa	α-mouse myosin

Table S.2 Complete characterization of the four MCMV mAbs that trigger CD16-responses and the names of the antigens they recognize (B). SN, Supernatant; NN, non-neutralizing; IP, immunoprecipitation; gB-glicoprotein B. The intensity of the test results (FACS and CD16-activation assay) is indicated by '+'.

IV. DISCUSSION

IV.1 CHARACTERIZATION OF THE MCMV-SPECIFIC ANTIBODY RESPONSE IN BALB/C VERSUS C57BL/6 MICE

The humoral response to MCMV infection was previously characterized by several investigators (Classen et al., 1987; Farrell and Shellam, 1989; Lawson et al., 1988; Selgrade et al., 1983), but the data were restricted to ELISA titres of IgM/IgG and/or neutralization capabilities of the immune serum. These data were extended in my studies by the determination of the total and MCMV-specific IgG subclass composition and their Fc-dependent effector functions, using the FcγR-activation assay recently described by our group (Eugenia Corrales-Aguilar et al., 2012).

As seen in **Fig. O**, a direct comparison of BALB/c and C57BL/6 antibody responses to MCMV infection over time was drawn. The summarizing graphic helps for a better understanding of the similarities and dissimilarities observed between the two mouse strains.

The assessment of the antibody response to MCMV infection revealed unexpected differences between the susceptible (BALB/c) and the resistant (C57BL/6) mouse strain in the capacity of antiviral IgG to trigger activation of FcγRIII- and partially FcγRIV-bearing cells (**Fig. O.A** and **B**).

Our hypothetical explanation for the observed variations includes qualitative differences in individual MCMV-IgG subclasses between the two strains. The earlier and stronger FcγRIII-activation in BALB/c can be attributed to the bulk of IgG2a elicited after MCMV infection in this susceptible strain, whereas the presence of IgG2c and the absence of IgG2a in C57BL/6 are correlated with later and lower FcγRIII-responses in this particular strain. Moreover, it is tempting to speculate that the more vigorous humoral immune response of BALB/c mice (partially) compensates the more pronounced genetic susceptibility of this host allowing a better control of MCMV replication.

No differences between BALB/c and C57BL/6 mice were observed when comparing the overall IgG and IgM triggered in response to MCMV infection and with regard to their neutralizing capacities (**Fig. O** and **Fig. III.1.A** in experimental results).

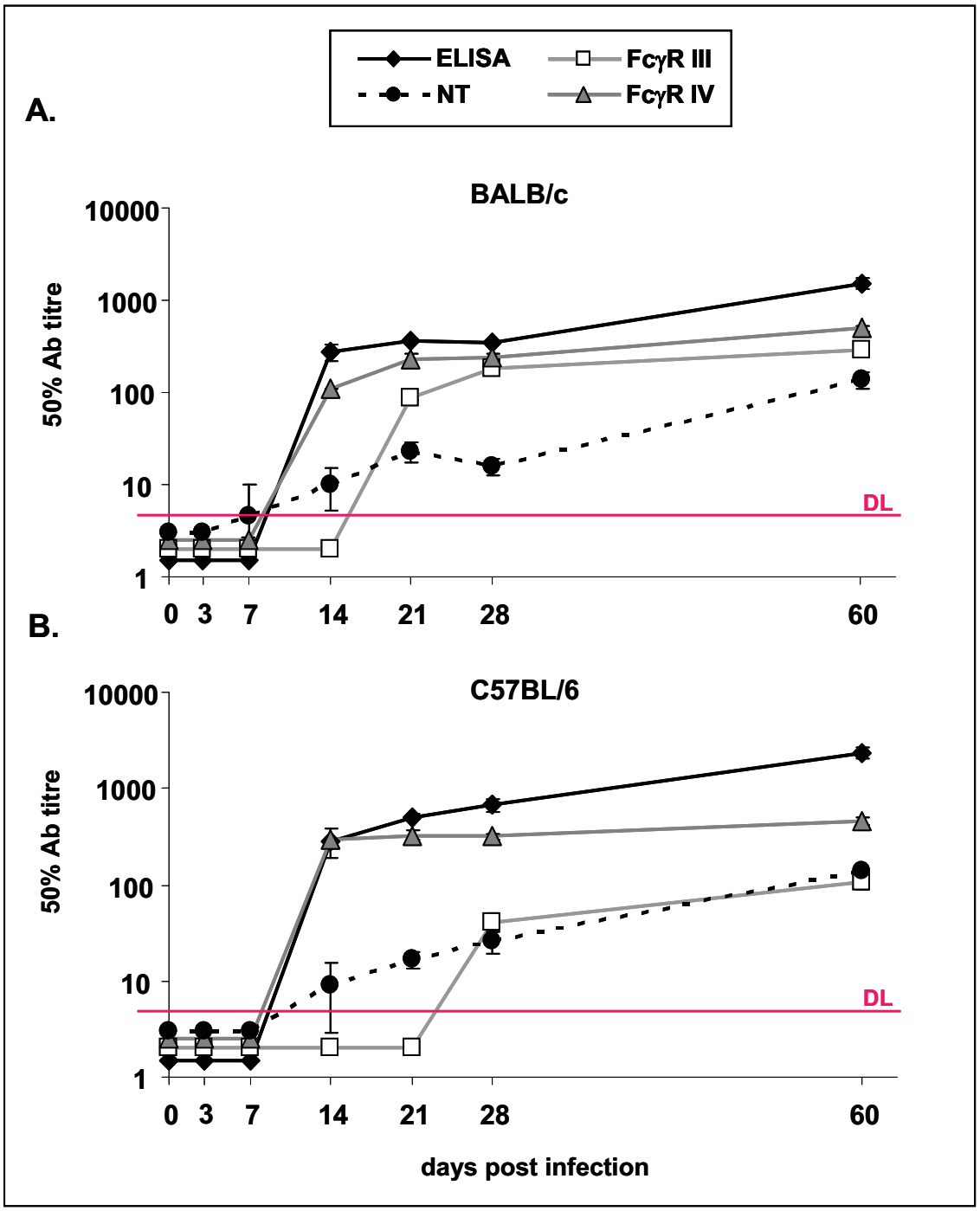


Figure O Antibody responses to MCMV infection in BALB/c and C57BL/6 mice. The BALB/c (A) and C57BL/6 (B) 50% titres in ELISA, FcγRIII- and FcγRIV-activation assay, as well as in neutralization (NT) test from the experimental results chapter are summarized here in one graphic. Detection limit (DL) indicated by the red line was settled to 1/5 in this figure, as for individual assays different detection limits were previously established: 1/25 for ELISA and FcγRIII-activation assay; 1/50 for FcγRIV-activation assay and 1/5 for neutralization test. Titres lower than 50% are arbitrary placed in the graphic below the detection limit. The values are presented in a logarithmic scale (log_{10}) with standard deviations from at least two independent experiments.

Specific aspects of the results obtained in this work are discussed in more detail in the following chapters. First, the specificity of IgG antibody response to MCMV infection is evaluated followed by the assessment of the IgG subclass profile and its Fc-dependent effector functions.

IV.1.1 Comparable MCMV-specific IgG and IgM titres in BALB/c and C57BL/6 mice

Studies with adoptively transferred anti-CMV antibodies have shown their protective role during acute infection (Araullo-Cruz et al., 1978; Farrell and Shellam, 1991; Lawson et al., 1988; Shanley et al., 1981), but failed to prevent the establishment of a latent infection (Shanley et al., 1981). In immunocompromised mice, the presence of adoptively transferred immune serum before viral infection prevents viral dissemination (Klenovsek et al., 2007) and protects from lethal primary infection. Although during the primary phase of infection there is no detectable role for antibodies in virus clearance in organs (as revealed in μMT/B-cell deficient mice), after reactivation from latency antibodies become a prominent immune function that limits virus dissemination and prevent MCMV disease (Jonjic et al., 1994; Polic et al., 1998).

Similarly with previous kinetics studies of MCMV specific IgG antibodies (Araullo-Cruz et al., 1978; Classen et al., 1987; Lawson et al., 1988; Selgrade et al., 1983), our data (**Fig. III.1.B** and **III.4**) show the development of MCMV-IgG between 3-7 d.p.i. with 50% ELISA IgG titres beginning after 7 d.p.i. with $4x10^6$ PFU Smith MCMV.

In addition to the published data, analysis of immune serum from aged infected BALB/c mice (90, 447 and 570 d.p.i.) showed a continuous increase in the IgG antibodies till 90 d.p.i., afterwards a slow and weak decline till 570 d.p.i. was observed; interestingly, the levels of MCMV-IgG were maintained relatively high up to 570 d.p.i., indicating a life-long persistence of anti-MCMV antibodies in mouse serum (data not shown).

Our ELISA measurements of MCMV-specific IgM antibodies (**Fig. III.1.A**) revealed an early production of this isotype after primary infection that reached a maximum at day 7 p.i, before decreasing gradually at later time points p.i.; additionally, the presence of MCMV-specific IgG starting with day 7 p.i. suggests that from this time point it is initiated the process of class switch recombination to IgG, performed by mature B cells, thereby enabling the selection of antibodies with increased affinity for the antigen.

Comparing susceptible BALB/c and resistant C57BL/6 mice with regard to their IgM and IgG response to MCMV infection, we found no significant discrepancy, suggesting

that the overall repertoire of IgG specificities is rather similar between both strains and is hardly influenced by the genetic background of the mice. These results are consistent with the previous (Farrell and Shellam, 1989; Lawson et al., 1988) and recent (Kuparinen et al., 2012) studies of MCMV and HCMV infection, respectively.

IV.1.2 Differences in the IgG subclass composition between BALB/c and C57BL/6

The assessment of the individual IgG subclasses developed in BALB/c and C57BL/6 serum upon infection with Smith MCMV revealed differences in the isotype composition and types of IgG subclasses expressed (**Fig. III.2.A** and **B**). In the case of BALB/c mice, the major production of the IgG2a subclass that we observed after MCMV infection is most likely due to the Th1 phenotype of $CD4^{+}$T cells that are activated in response to MCMV replication. In the Th1 response, cytokines like interferon-gamma (IFN−γ) are responsible for the class switch to the IgG2a isotype (Maloy et al., 2000; Selgrade et al., 1983; Snapper and Paul, 1987), whereas IL-4 selectively stimulates the production of murine IgG1 (Isakson et al., 1982) and IgE (Coffman and Carty, 1986), both members of the Th2-response. Similarly, TGF-β selectively stimulates production of IgG2b (McIntyre et al., 1993) in addition to IgA. The type of IgG subclasses raised is dependent on the context of antigen expression and the type of infection. Therefore, during infection with nematode parasites IgG1 and IgE are produced (Finkelman et al., 1986) a strong induction of IgG3 and IgG2a after bacterial infection was observed (Snapper and Mond, 1993).

Other mouse studies using a panel of RNA and DNA viruses representative of 11 genera (excluding MCMV) (Coutelier et al., 1987), as well as after immunization with Dengue virus (Smucny et al., 1995) clearly showed that the antiviral response is preponderant of the IgG2a subclass at all tested time points after infection. Coutelier *et al*. also suggested that the antibody response to these viruses is IgG2a independent of the strain of mice tested. Later publications contradict this notion by showing a complete absence of the IgG2a gene locus in mouse strains as C57BL/6, C57BL/10, SJL and NOD, but the presence of the *igh-1b* allele that encodes for the new isotype designated as IgG2c (Jouvin-Marche et al., 1989; Martin et al., 1998; Morgado et al., 1989). The genetic analysis of the variety of haplotypes present in laboratory and wild mice led to the interpretation of two distinct evolutionary models: on one hand IgG2a and IgG2c are considered two distinct alleles physically linked on the chromosome and

derived from separate genes (*igh-1a* and *igh-1b,* respectively) (Jouvin-Marche et al., 1989; Martin et al., 1998; Morgado et al., 1989); on the other hand, a recent model of IgG2a and IgG2c as allelic-single loci not physically linked on the chromosome was proposed (Zhang et al., 2012). Our data is consistent with the last findings and show that in C57BL/6 serum no IgG2a was measured at either time points after MCMV infection, IgG2c being detected instead (**Fig. III.2B**). Conversely, no IgG2c was detected in the serum of BALB/c–infected mice (**Fig. III.2.A**).

We were able to show for the first time that there is a difference in the time course of the individual IgG subclasses elicited in response to MCMV infection between the BALB/c and C57BL/6 mouse strains. In our ELISA assays, IgG2a reached almost 3-fold higher concentrations in BALB/c serum 60 days after MCMV infection, when compared to IgG2c in C57BL/6, drawing attention to an obvious difference between these isotypes (**Fig. III.2**). This finding and also the 16% aminoacid difference between IgG2a and IgG2c as reported by Martin *et al*. (Martin et al., 1998) arise the question whether the structural variation between IgG2a and IgG2c results in an yet unknown difference in their biological functions.

In C57BL/6 serum, similar amounts of IgG2b and IgG2c were observed after MCMV infection, making these two isotypes the predominant IgG subclasses triggered in response to MCMV infection. IgG1 and IgG3 responses to MCMV were found to be very low in both mouse strains (**Fig. III.2.A** and **B**). These results are in line with previous publications where IgG1 responses in mice were predominantly elicited by soluble proteins (Balkovic et al., 1987), whereas immunization with polysaccharides resulted in primarily IgG3 (Perlmutter et al., 1978). Furthermore, exposure of mice to live viruses usually elicits a predominant IgG2a response (Coutelier et al., 1987; Smucny et al., 1995), also confirmed by our data with MCMV in BALB/c mice, but not in C57BL/6 (**Fig. III.2.A** and **B**). In C57BL/6 serum a predominant IgG2b and IgG2c response was induced after infection with $4x10^6$ PFU Smith MCMV.

Taking a closer look at the MCMV-specific IgG1 concentrations obtained in ELISA measurements (**Fig. III.3**), we noticed another difference between the susceptible and the resistant mouse strain: BALB/c mice which have a Th2 bias developed higher amounts of this subclass than C57BL/6 from 28 up to 60 d.p.i. Similar results were found by Bickerstaff *et al*. when comparing serum from the two mouse strains by flow cytometry (Bickerstaff et al., 2007). Based only on IgG1 measurements and without

testing the other IgG subclasses, these authors concluded that C57BL/6 mice exhibit lower antibody responses than BALB/c. Our data obtained by summarizing each IgG subclass concentrations of BALB/c and C57BL/6 show very similar, almost identical concentrations for MCMV-specific IgG (**Fig. III.4**), indicating that the difference observed in the antibody response to MCMV between the susceptible and the resistant mouse strain is not in the total amount of IgG, but in the distribution of individual IgG subclasses present in the immune serum. Another observation that underlines our hypothesis is the 2-fold higher amount of MCMV-IgG2b elicited in C57BL/6 in comparison to BALB/c. In conclusion, smaller amounts of MCMV-IgG1 and the absence of IgG2a in C57BL/6 are compensated by the presence of IgG2c isotype and by the higher amounts of IgG2b, finally leading to a similar IgG response in both mouse strains.

The resistance of mouse strains to MCMV in the frame of an otherwise lethal primary infection was elegantly described as being dependent on the *cmv-1* (Scalzo et al., 1990; Scalzo et al., 1992) and more recently on the *cmv-4* locus (Adam et al., 2006) that results in a more effective NK cell response in the resistant strains. To date, no important role of the IgG antibodies in the resistance of mouse strains to MCMV infection was identified.

As mentioned above, B cell immunity was found to have no essential role in the immune control of primary MCMV replication as concluded from the analysis of μMT-chain deficient C57BL/6 mice (Jonjic et al., 1994). Therefore, the differences we show for the first time in the isotype distribution of MCMV-immune IgG antibodies between the tested mouse strains suggest no direct link with the susceptibility phenotype of MCMV primary infection and could be interpreted as a consequence of other host genetic factors.

IV.1.3 IgG2a/IgG2c subclass-selective hypergammaglobulinemia induced by MCMV

The ELISA determination of the total IgG subclasses in mouse serum collected from mice kept under pathogen free conditions after infection with MCMV showed interesting results, where IgG2a is present as the main subclass in BALB/c in much higher concentrations in comparison to other subclasses (**Fig. III.5.A**). We found that only a small fraction of the total IgG2a (5%) and 8% of the total IgG2b are directed against MCMV antigens. We hypothesize that the non-MCMV immune IgG2a and

IgG2b antibodies present in BALB/c serum could be directed against normal mouse tissues and organs (autoantibodies) (O'Donoghue et al., 1990; Price et al., 1993) or can have further specificities.

The total IgG subclasses reflect the pattern observed in the MCMV-IgG subclass profile, where IgG2b is the predominant subclass in C57BL/6 serum, followed by lower concentration of IgG2c. Again, no IgG2a was detected in serum from C57BL/6 mice, whereas no IgG2c was present in BALB/c serum. Surprisingly, when comparing the total IgG subclasses, we observed a rapid boost of the individual IgG2a/IgG2b and IgG2b/IgG2c (**Fig. III.5.A** and **B**) early between 3-14 d.p.i., most probably due to the MCMV infection that induces a strong polyclonal B cell activation during the acute phase of primary infection (Karupiah et al., 1998). Afterwards, the individual levels of IgG isotypes are maintained constantly in latently infected mice of both tested mouse strains.

Taken together, the most interesting result is represented by the strongest enhancement of total IgG2a antibodies (almost 12-fold), followed by IgG2c (5-fold) in comparison with other isotypes (**Fig. III.5.A and B**). This pattern is reminiscent with observations by Karrer *et al.* When studying the $CD8^+$T cell response after MCMV infection (Karrer et al., 2003), this group found a strong expansion, followed by a contraction phase and then again increase in the $CD8^+$T cells specific for the IE1/pp89 MCMV epitope, but not for other MCMV-derived epitopes. They designated the accumulation process of $CD8^+$T cells specific for few immunodominant epitopes as 'memory inflation'. By analogy with these studies, the accumulation of MCMV-immune IgG2a isotypes over the time of infection observed in our tests is due to a continuous expansion of only a B cell clone that produces the most effective IgG subclass for a better control of acute and latent MCMV infection.

A striking increase in the number of IgG2a-producing B cells after MCMV infection was also described by Karupiah *et al*., and it is most probably due to the early production of IFN-γ by IL12-activated NK cells (Karupiah et al., 1998). In these studies and also others (Price et al., 1993), the widespread activation of B cells may result in the appearance of autoantibodies in serum of mice infected with MCMV.

Our results showed for the first time a strong increase of the IgG2a/IgG2c subclasses (IgG2a >> IgG2c) in mouse serum after MCMV infection and therefore, we named this process '*IgG-subclass selective hypergammaglobulinemia*' induced by MCMV.

A similar pattern of an abnormal B-cell activation resulting in the elevation of serum IgG levels was noticed in patients after HIV infection (Amadori and Chieco-Bianchi, 1990; Martinez-Maza et al., 1987; Nagase et al., 2001).

IV.1.4 Strong activation of CD16 by BALB/c MCMV- immune serum

Using the FcγR-activation assay that was recently developed by our group (Eugenia Corrales-Aguilar et al., 2012) we further analyzed the capability of BALB/c and C57BL/6 immune serum to activate CD16 (FcγRIII)- and FcγRIV-bearing cells. Our data revealed unexpected results with an earlier and stronger activation of BW5147-CD16-ζ cells being triggered by BALB/c immune serum when compared to C57BL/6 (**Fig. III.8.A**). According to the 50% CD16-activation titres calculated for each time point after MCMV infection the appearance of this antiviral IgG fraction is observed as early as 14 d.p.i. in BALB/c mice, whereas in C57BL/6 they were first detected after 21 d.p.i. Additionally, the intensity of CD16-responses in BALB/c serum was significantly greater at all tested time points and increased gradually till 60 d.p.i., suggesting a functional maturation of CD16 responses with the time of antigen exposure. Surprisingly, these effector mechanisms seem to be developed later and at only very low levels in C57BL/6, suggesting no correlation between the MCMV resistant phenotype and the FcγRIII-activation capacity of immune IgG. One possible explanation for the observed discrepancies between the resistant and the susceptible mouse strain with regard to their CD16-activation titres is linked to the IgG subclass profile that clearly varies between these two mouse strains. It is obvious that IgG subclasses differ in their functional properties, including FcγR binding and activation. Among all four IgG subclasses in mice, IgG2a and IgG2b are considered the most important mediators of ADCC (Denkers et al., 1985) and have an increased efficiency in mediating protection to some, but not all viruses *in vivo* (Amadori and Chieco-Bianchi, 1990; Ishizaka et al., 1995; Markine-Goriaynoff and Coutelier, 2002; McKendall and Woo, 1988; Smucny et al., 1995). The protection mechanisms in response to viral infections include complement activation (Klaus et al., 1979), binding to Fc receptors (Heusser et al., 1977) and mediation of antibody-dependent cellular cytotoxicity (Kipps et al., 1985). Several previous studies analyzed the role of each FcγR *in vivo* and their binding affinities to the Fc part of individual IgG subclasses *in vitro*. Therefore, despite the *in vitro* high affinity binding of IgG2a to FcγRI,

intermediate affinity to FcγRIV and low affinity to FcγRIII (Nimmerjahn and Ravetch, 2005), the dominant effector function *in vivo* of this IgG isotype could result from engagement of one or more of these FcγRs, depending on the effector cell type involved. Similarly, IgG2b binds *in vitro* to FcγRIII and FcγRIV with intermediate affinity, but the IgG2b activity *in vivo* was shown to be dependent only on FcγRIV engagement (Nimmerjahn and Ravetch, 2005). Moreover, IgG1 was described to bind *in vitro* with 10x higher affinity to inhibitory FcγRIIB than to activating FcγRIII (Nimmerjahn et al., 2005), resulting in both negative and positive regulations of immune responses *in vivo.*

Based on these studies, it is tempting to speculate that the presence of high MCMV-specific IgG2a antibodies that we observed in BALB/c serum is responsible for the strong activation of FcγRIII-transfected cells. Therefore, we raise the hypothesis that this IgG subclass dominating the antiviral response could play a significant role in the differences regarding CD16 activation we observed. If this hypothesis is verified, the involvement of other CD16-binding IgG subclasses (IgG1 and IgG2b) should be further excluded. On one hand, it is very unlikely that IgG1 triggers FcγRIII-activation in BALB/c mice, as the production of this IgG subclass is restricted to Th2-bias and it is present only at low levels at all time points after MCMV infection (**Fig. III.2.A** and **B**). On the other hand, the two times more IgG2b measured in C57BL/6 serum when compared to BALB/c does not directly correlate with the reduced activation of CD16 in this resistant mouse strain.

In line with our hypothesis, the absence of IgG2a in C57BL/6 mice serum can be indirectly associated with the reduced CD16-triggering abilities detected in this mouse strain.

Taken together, these data suggest that MCMV-specific IgG2c present in C57BL/6 serum is less efficient in triggering CD16-activation as compared with IgG2a from BALB/c. In this way, a first important difference in the effector functions of these two isotypes is postulated.

One method to test this hypothesis is currently under investigation by Katrin Ehrhardt in our group. The project focuses on the generation of mouse monoclonal IgG antibodies that have the same epitope but exchanged constant heavy chains from each of the five mouse IgG subclasses: e.g. IgG1, IgG2a, IgG2c, IgG2b and IgG3. The epitope is directed against a model antigen with surface distribution on infected cells which was introduced into the MCMV genome by BACmid-based mutagenesis. In this way, the

efficiency of the individual IgG subclasses in triggering FcγR-activation can be assessed *in vitro* using the FcγR-activation assay recently described by our group (Eugenia Corrales-Aguilar et al., 2012). Therefore, a direct comparison of the CD16 or FcγRIV-activation triggered by IgG2a and IgG2c monoclonal antibodies should confirm our hypothesis. Further on, the *in vivo* protective capacities of the IgG subclasses will be determined by passive immunization followed by infection with the recombinant MCMV of the B- and T-cell-deficient mice.

In conclusion, the obtained data imply a possible role of the genetic background of the mice in the development of antiviral IgGs with regard to their FcγRIII-effector functions.

The lower CD16-activation triggered in C57BL/6 mice can be alternatively explained when taking into consideration the presence of virus-encoded FcγRs on the surface of infected cells with potential capacities of inhibiting ADCC responses. The MCMV-encoded viral FcγR fcr-1/m138 is a glycoprotein expressed on the surface of infected cells (Thale et al., 1994) with a series of immune evasion functions (Arapovic et al., 2009; Crnkovic-Mertens et al., 1998; Lenac et al., 2006; Thale et al., 1994). It was recently shown to act as antagonist or inhibitor of the host FcγRs (e.g. CD16) activation (Eugenia Corrales-Aguilar, 2008).

Moreover, the subclass specificity of fcr-1/m138 for the two most important cytotoxic antibodies in the mouse (e.g. IgG2a and IgG2b) (Matthias Budt, unpublished observations) arises the question whether IgG2c represents a likely candidate for the *in vitro* inhibition of its effector functions. Based on our CD16-activation results, one might speculate that fcr-1/m138 mediates a stronger inhibition of IgG2c than of IgG2a leading to a reduced activation of CD16 in strains with *igh-1b* haplotype.

IV.1.5 Similar FcγRIV-activation in the susceptible and the resistant mouse strain

The FcγRIV-ζ activation assay was used for the determination of that antibody fraction able to trigger FcγRIV-dependent effector functions. Similar 50% FcγRIV-activation titres were obtained for both BALB/c and C57BL/6 serum, with gradually production of this antibody fraction from 7 up to 60 days post MCMV infection (**Fig. III.8.B**). An intermediate significant difference ($p < 0,01$) between BALB/c and C57BL/6 mice was observed at day 14 p.i (**Fig. III.9**). Based on the previous studies showing FcγRIV

binding both IgG2a and IgG2b with intermediate affinity (Nimmerjahn et al., 2005; Nimmerjahn et al., 2010) the only possible explanation for the higher FcγRIV-activation titres in C57BL/6 can be associated with the 2x higher concentrations of IgG2b present in this mouse serum at day 14 p.i. (**Fig. III.3.A** and **B**), as comparable amounts of MCMV-specific IgG2a developed in BALB/c serum and IgG2c in C57BL/6 at 14 d.p.i. were detected. These data argue for a dominant role of IgG2b and to a lesser extent of IgG2a and IgG2c in triggering FcγRIV-responses after MCMV infection. In contrary to the possible differences in the efficiency of triggering CD16 activation suggested between IgG2a from BALB/c and IgG2c from C57BL/6, a similar capacity to elicit FcγRIV-responses of the two isotypes is here proposed. The method for testing the efficiency of the two isotypes in triggering FcγRIV-activation can be assessed using the model proposed above (the monoclonal IgG antibodies of different subclasses but a constant variable region and paratope).

The exponential increase of the FcγRIII- and FcγRIV-activating titres indicate that the IgG antibodies to MCMV are going through a process of affinity maturation, and as a result the effector functions of these antibodies are greatly improved over time. This hypothesis is also supported by unpublished data from our group where the avidity index of antibodies is increasing within 7 to 90 days of antigen encounter. Therefore, another confirmation that the antibodies to MCMV play important roles in the secondary immune response or reactivation from latency is here highlighted.

IV.1.6 Comparable neutralization titres of BALB/c and C57BL/6 immune serum

A systematic characterization of antibody responses with regard to their IgG profile and IgG-mediated effector functions over the course of MCMV infection was assessed. The last test that should offer us an indication about the antiviral activities of antibodies to MCMV is represented by virion neutralization. Comparing the susceptible and the resistant mouse strain with respect to their neutralizing capabilities, we were able to show a very similar pattern of neutralizing antibody development in both mouse sera over the course of infection. Based on our results of the IgM and IgG amount in the same mouse sera, we conclude that early after MCMV infection (3-7 d.p.i.) IgM antibodies with low neutralization activities (below 50%) are developed in BALB/c serum, as indicated in **Fig. III.10**. After 7 d.p.i., the B cell class switch is initiated and IgG antibodies with higher affinities for the virion envelope glycoproteins are produced.

The 50% neutralization titres were detected up to 14 d.p.i. for C57BL/6 mice in our sensitive luciferase neutralization assay, these results are consistent with previous studies (MEDEARIS, Jr., 1964; Quinnan, Jr. and Manischewitz, 1987), although others (Araullo-Cruz et al., 1978; Kim and Carp, 1973) have demonstrated neutralizing antibodies to MCMV by 3 d.p.i. using a sensitive complement-dependent neutralization assay, even though they showed very low titres by 50% plaque reduction. The same early serum have been shown to protect against MCMV infection when passively transferred (Araullo-Cruz et al., 1978). From our findings we can conclude that neutralizing antibodies do not correlate to the early resistance phenotype to MCMV infection, and argue for a role of minor importance of these antibodies in the primary infection in either BALB/c or C57BL/6 mouse strains. These conclusions are supported by earlier studies with susceptible and resistant mouse strains (Lawson et al., 1988; Grundy et al., 1982).

The protective capacity of passively transferred antibodies against MCMV infection was elegantly described by Klenovsek *et al*., but the neutralizing capacity of these antibodies was demonstrated to be very weak (Klenovsek et al., 2007). Therefore, the virus-IgG subfraction able to trigger FcγR-effector functions, but not neutralizing, may indeed correlate better with the protective capacity of MCMV immune sera. In accord with this idea, Hessell *et al.* reported that not neutralizing or complement activating IgG protect macaques against SHIV (simian-human immunodeficiency virus), but the Fc-receptor-bearing effector cells are important in reducing virus yield from infected cells (Hessell et al., 2007). Till lately there was little methodology to measure such IgG activities *in vitro*. Taking advantage of the recently described FcγR-ζ activation assay, we were able to define those fractions of IgG including cytotoxic antibodies able to trigger host FcγRs in both BALB/c and C57BL/6 serum. As indicated in **Fig. O.A** and **B**, from the total ELISA reactive-IgG, a relatively large fraction is represented by FcγRIV- and up from 14 and 21 d.p.i. by the FcγRIII- activation IgG in BALB/c and C57BL/6, respectively, whereas neutralizing Abs are present at relatively low titres. These data support an important role of the FcγR-activating IgG subfraction, and not of neutralizing antibodies in the control of MCMV infection. Moreover, in agreement with our results, a previous publication indicated that antibody titres obtained using the ADCC assay were about 10-fold higher than by neutralization (Manischewitz and Quinnan, Jr., 1980). These authors concluded that the ADCC response to MCMV is

likely to be highly efficient and may contribute significantly to control of both acute and later stages of infection. The role of NK cells in the early phases of a primary MCMV infection and also during reactivation from latency was elegantly described (Polic et al., 1998). Beside NK cells, neutrophils and macrophages were shown to be involved in the FcγR-mediated ADCC responses at later stages of infection (Kohl et al., 1979; Manischewitz and Quinnan, Jr., 1980).

However, our findings that FcγRIII-activation is triggered earlier and qualitatively better in BALB/c mice provoked us to further search for the MCMV-specific mAbs that are able to mediate such effector functions and to identify their respective viral antigens as potential therapeutic targets.

IV.2 BALB/C-DERIVED MABS WITH FCγRIII-ACTIVATION CAPABILITIES FOR POTENTIAL THERAPEUTIC USE

The use of monoclonal antibodies (mAbs) for immunotherapy has increased considerably after the hybridoma technique allowed their development in the mid 1970s (Kohler and Milstein, 2005). The main goal of vaccines developers is the elicitation of potent antibodies that prevent the morbidity and mortality caused by pathogens. In order to achieve this, an understanding of the molecular mechanisms by which antibodies mediate protection is very important. While in the past neutralization antibodies were considered the optimal drugs for the development of efficient Ab-based therapies, recent studies argue for the role of ADCC-mediating and not necessarily neutralizing Abs in the control of different viral infections (Hessell et al., 2007). These findings mark a new direction in the hunt for potential immunological targets.

Our results obtained when comparing a susceptible and a resistant mouse strain with regard to their antibody response to MCMV revealed a difference in the activation of FcγRIII-bearing cells (**Fig. III.8.A**). These data imply the possibility that in the susceptible BALB/c strain FcγRIII- effector functions are triggered more efficiently than in C57BL/6 resistant mice. In a first step to assess the protective capacity of mAbs, it was of particular interest to define the subfraction of antibodies with CD16-activation capabilities by testing a library of mAbs produced after immunization of BALB/c mice with wild type MCMV. Screening the large number of MCMV-specific mAbs by flow cytometry revealed that over 50% of mAbs are directed against surface viral antigens, whereas the other half was specific for intracellular viral proteins. Remarkably, only 4% of the total mAbs (4 out of 104) were able to elicit FcγRIII-effector functions as described in experimental results, **Fig. III.15**. These results emphasize that from the total anti-MCMV ELISA-reactive Abs only a small fraction may develop ADCC effector functions via FcγRIII, the rest being specialized for other or no Fcγ receptors or effector processes.

The IgG subclass determination of the four selected mAbs (chapter III.2.8 in experimental results) designated MCMV1.01, 15A12-H9, 10B2-D10 and 14E11-E12 revealed the same isotypes that were previously described to have binding affinities to FcγRIII: IgG2a and IgG2b (Nimmerjahn et al., 2005; Nimmerjahn and Ravetch, 2005), confirming once more the selectivity of the Fc receptors for the most efficient IgG

isotypes activating immune cells. However, the concentration dependency of mAb should be taken into account when comparing the CD16-responses of two or more mAbs *in vitro*, as we were able to show that the degree of FcγRIII-activation is directly proportional with the concentration of mAb present in a sample (**Fig. III.17**). Moreover, we showed that in the FcγRIII-activation assay a minimal concentration of mAb is required to elicit a response (**Fig. III.17**) and that for MCMV1.01 the threshold for mAb concentration is reached between 25 and 50 μg/ml. No increase in the activation pattern was detected when MCMV1.01 above 150 μg/ml was used. Thus, in order to check if there is any *in vivo* correlation in the protective ability of a mAb and its concentration, experiments with adoptively transferred amounts from the four CD16-activating mAbs into MCMV-infected immunodeficient hosts should be performed. Moreover, a threshold of the respective mAb avidity for its antigen should be also analyzed, as studies on VSV (vesicular stomatitis virus) showed that avidity maturation of a therapeutic mAb beyond a defined threshold may not improve its protective capacity (Bachmann et al., 1997).

Another characteristic of the CD16-activating Abs to be defined is related to their neutralizing capabilities. As previously described (Klenovsek et al., 2007), the neutralization capacity of protective polyclonal antibodies passively transferred into immunodeficient mice was demonstrated to be very weak. These data are highlighting the specialized functions of antiviral antibodies and the possible role of the ADCC-mediating Abs in virus control. Therefore, one would expect that antibodies with FcγR-activation properties may lack a neutralizing effect, a hypothesis that was also confirmed by our tests when MCMV1.01, 15A12-H9, 10B2-D10 and 14E11-E12 mAbs in a conventional neutralization assay were used (**Fig. III.16**).

IV.3 IDENTIFICATION OF ANTIGENS RECOGNIZED BY CD16 - ACTIVATING MABS IN BALB/C MICE

Defining the possible targets of therapeutic mAbs is also of great importance for the development of antiviral vaccines. Therefore, our next focus was the identification of the antigens recognized by the four mAbs with FcγRIII-activation capabilities: MCMV1.01, 15A12-H9, 10B2-D10 and 14E11-E12. Mass spectrometry analysis revealed following: MCMV glycoprotein B as target for MCMV1.01 and 15A12-H9;

m142 the direct target of 10B2-D10 mAb and mouse myosin recognized by 14E11-E12 mAb. The final results are summarized in **Table S.2** and are discussed below in more details.

IV.3.1 Glycoprotein B is a major target for both neutralizing and ADCC Abs

Glycoprotein B (gB), the major envelope glycoprotein is an important target for the humoral immune response against CMV due to its implication in the virus entry and virus-induced cell fusion. The CMV gB represents a predominant antigen for the induction of neutralizing antibodies and therefore a significant proportion of the neutralizing antibodies in sera from CMV infected hosts is directed against glycoprotein B (Britt et al., 1990; Gonczol et al., 1991; Rasmussen, 1990). The MCMV gB gene was identified as coding for a protein with a very high degree of homology with other herpesviruses (Loh et al., 1988; Loh, 1991; Rapp et al., 1992). Studies using this protein in different vectors, e.g. vaccinia virus (Rapp et al., 1992) or vesicular stomatitis virus (Wilson et al., 2008) showed protection against challenge with MCMV by induction of neutralizing antibodies and therefore provides a tool to test the usefulness of a gB vaccine in an animal model.

In our study, the 130 kDa band precipitated by MCMV1.01 and 15A12-H9 mAbs was identified by mass spectrometry and a number of three respectively 46 unique peptides to glycoprotein B from MCMV were identified. Since our goal was to define mAbs that have FcγRIII-activation capabilities, the finding that MCMV1.01 and 15A12-H9 are able to bind to gB expressed on MCMV-infected cells, the major neutralization target on virions was relatively surprising and unexpected. The additional characterization of MCMV-mAbs revealed that there is no neutralization effect when either MCMV1.01 or 15A12-H9 was incubated with the virus (**Fig. III.16**). Therefore, it is tempting to speculate that after MCMV infection mAbs against different epitopes of glycoprotein B are elicited and function as important mediators of the host immune response. In accord to our assumption, Loh *et al*. and Rapp *et al*. published that both neutralizing and non-neutralizing antibodies with specificity to MCMV gB have been produced (Rapp et al., 1992; Loh et al., 1988), but their specific roles in MCMV immunobiology were not described.

In addition to published data, our results show for the first time a potentially important immune function of antibodies against gB epitopes that are not neutralizing. Their involvement in the Fc-dependent functions, e.g. activation of FcγRIII- and FcγRIV-

transfected cells (data not shown) makes them crucial mediators of immunotherapeutic approaches. Additional experiments for determination of their immunologic role *in vivo* should be performed. Based on our results and on published data we consider a model by which one type of mAb against the BE2 region (residues 155-278) (Xu et al., 1996) of gB exerts its protection mechanisms *in vitro* and *in vivo* by directly neutralizing the virus and blocking the virus entry into neighbouring cells. A second type of α-gB Ab binds to other antigenic regions exposed by gB localized more probably on the surface of infected cells and is likely to mediate ADCC-responses via FcγRIII and FcγRIV expressed by immune effector cells. Supplementary data that also confirm our theory are presented in the **Fig. III.19.A** and **B** in experimental results. Using the neutralization and FcγRIII-activation assay we compared a neutralizing mAb against gB, namely MCMV 97.3 with MCMV1.01 and 15A12-H9 mAbs, which are also gB specific.

The strong neutralization capability of MCMV 97.3 mAb (**Fig. III.19.A**) and on the other hand the effective CD16-activation triggered by MCMV1.01 and 15A12-H9 mAbs (**Fig. III.19.B**), but not by 97.3 antibody sustain the theory of neutralizing and ADCC-mediating IgGs directed against different epitopes of the same viral protein.

However, the neutralization and FcγR-activation capabilities may not be in all cases separated, since our group found that a humanized therapeutic IgG1 mAb specific for the glycoprotein F of RSV (Respiratory Syncytial Virus), namely Palivizumab/ Synagis® (Saez-Llorens et al., 1998) is able to efficiently trigger activation of human FcγRs (CD16 > CD32 > CD64) (Corrales-Aguilar, E., unpublished observation), in addition to its well-described neutralization capacity.

The different isotypes detected for the three α-gB antibodies, namely MCMV1.01, 15A12-H9 and 97.3 mAbs, (**Table III.3**) and the clear separation in their immune functions offers us to further test for their *in vivo* protective capabilities and to clear distinguish between neutralization and Fc-dependent mechanisms.

IV.3.2 MCMV 14E11-E12 antibody precipitates mouse myosin

Another unexpected finding resulted from the identification of mouse myosin as the 120 kDa band precipitated by 14E11-E12 MCMV mAb. The mass spectrometry analysis revealed 52 unique peptides that offered 43,9% sequence coverage with the myosin IC isoform A from *mus musculus*. Several explanations of this finding are possible:

1) The fact that MCMV infection induces production of multiple autoantibodies in sera from BALB/c mice was elegantly described by Bartholomaeus *et al*. since 1988 (Bartholomaeus et al., 1988). Two years later, O'Donoghue *et al*. showed that autoantibodies to cardiac myosin in BALB/c are the most predominant and represent the major cause of MCMV-induced myocarditis (O'Donoghue et al., 1990). Therefore, one can speculate that in our experiment, after BALB/c mice were infected with MCMV and boosted with lysates of MCMV-infected cells, it is very likely that autoantibodies to different organs and tissues are elicited, the most predominant being myosin autoantibodies.
2) Likewise, a phenomenon called '*molecular mimicry'* was described also for MCMV antigens that share epitopes with mouse myosin (Lawson et al., 1992; O'Donoghue et al., 1990). Based on this information and on our experimental data, it is tempting to speculate that 14E11-E12 mAb binds to common epitopes shared by both myosin and a not yet defined viral protein. On one hand, FACS analysis revealed that 14E11-E12 Ab is able to stain the surface of MCMV- (**Fig. III.21**) and not of MOCK-infected cells (data not shown), indicating a viral specificity of this Ab. On the other hand, coomasie staining and western blot revealed that 14E11-E12 recognizes more bands in lysates from MCMV-infected cells: one at 120 kDa that was also found to be precipitated and identified as myosin; another one band of ca. 75-80 kDa detected only in coomasie staining (**Fig. III.19**) and western blot (not shown), and not after metabolic labeling and immunoprecipitation (**Fig. III.18**).
3) Based on the knowledge that the MCMV fcr-1/m138 is able to bind the Fc part of a mouse IgG antibody (Thale et al., 1994) and therefore can interfere with binding of antibodies in different assays (e.g. m138 is an efficient inhibitor of CD16 activation *in vitro* (Eugenia Corrales-Aguilar, 2008)), we chose Δm138-MCMV for all FACS, western blot and immunoprecipitation experiments previously depicted. In this way, an unspecific signal of the infected cells in the presence of 14E11-E12 in FACS analysis was partially excluded. However, what could not be prevented was the interference by an additional MCMV-encoded FcγR that is not yet identified and binds the Fc part of the mAb, leading to a 'false positive' staining in FACS analysis. In accord with this hypothesis are the FACS data that show a similar staining of the infected cells with both 14E11-E12 mAb and an IgG2a isotype control (anti-V5 antibody) (**Fig. III.22**).

Though, for the identification of the direct target of 14E11-E12 mAb, additional experiments properly controlled should be performed.

IV.3.3 Intracellular antigens as targets for ADCC antibodies

The last MCMV antibody that was found to trigger activation of CD16-ζ-BW transfected cells is 10B2-D10. Identification of its direct target revealed MCMV m142 protein, whereas m143 is co-precipitated, as they are described to function as a complex (Child et al., 2006). ORFs m142 and m143 are transcribed with immediate-early kinetics, but are abundantly expressed at early times (Hanson et al., 1999) in the cytoplasm of infected cells (Hanson et al., 2005).

The presumption that an intracellular MCMV antigen is targeted by an antibody with Fc-mediated effector functions deserves comment and can be explained by:

1) the antiviral antibody enters the cell (e.g. after destruction of the plasma membrane) and binds the intracellular antigen,
2) or the antigen is transported to the cell surface for a direct contact with the antibody.
3) the interference by a not yet described MCMV-encoded viral FcγR that is binding the Fc part of 10B2-D10 mAb, resulting in a 'false positive' signl. This theory may explain our FACS data when an IgG2a isotype control antibody and 10B2-D10 mAb were used (**Fig. III.22**); the α-V5 isotype control antibody was shown to stain the surface of Δm138-MCMV-infected cells almost as efficiently as 10B2-D10 mAb, in the absence of a specific target on infected cells.

In accord to the first supposition comes the biphasic model of antibody-FcγR control of viral infection built up by E. Corrales-Aguilar (Eugenia Corrales-Aguilar, 2008). In the first phase, antibodies detecting surface antigens activate FcγR-bearing cells, e.g. NK cells. Activation of NK cells leads to degranulation and release of perforin and granzymes that induce pores on the target cells. In this way, antibodies can enter the interior of the cell. The second phase consists of the activation and recruitment of NK cells upon binding of antibodies to intracellular antigens, followed by the induction of cytokines like IFN-γ. In our FcγR-activation assay a T cell line called BW5147 stably transfected with CD16 was used as effector cell instead of NK cells. Therefore, IL-2 is produced after activation of the effector cells by the antibody-bound viral antigens.

Taking into account the absence of perforin and granzymes in our *in vitro* ADCC assay, it is tempting to speculate that the target cell may lose its integrity due to the cytopathic effect of MCMV infection and that pores in the cell membrane are induced, facilitating antibody translocation in the lumen. In this way, antibodies with CD16-activation capabilities are not limited to surface resident antigens, but also to cellular lumen resident antigens, being the case also for m142 protein.

IV.4 FURTHER PERSPECTIVES

CMV is an important clinical pathogen and one of the major causes of fatal diseases in congenitally infected infants and patients with immunosuppression. Although the different attempts to prevent CMV disease in such patients are continuously developing, no effective and safe vaccine is available to date. Moreover, the high toxicity of the antiviral agents currently approved allows a reduced and limited utility in immunocompromised patients and not in pregnant women.

A safe and effective therapy for prevention of CMV disease in organ transplant recipients, premature newborns at risk and pregnant women could be represented by the passive immunization with CMV IgG antibodies. The use of mAbs for immunotherapy has increased considerably after the hybridoma technique allowed their development in the mid 1970s (Kohler and Milstein, 2005). The two most important molecular mechanisms by which antibodies can mediate protection is represented by neutralization of the virion and/or the killing of virus-infected cells via ADCC. Whereas the neutralizing mAbs were considered for a long time the optimal drugs for the development of an efficient Ab-based therapy, more recent studies mark a new direction in the hunt for potential immunological targets. The role of ADCC, rather than neutralizing antibodies in clearing viral infections was elegantly described for HIV (Hessell et al., 2007), as well as herpesvirus infections (Chu et al., 2008; Wright et al., 2009).

Since no previous data could help us understanding how the antibody responses are triggered in mouse strains with different susceptibilities to MCMV infection, the purpose of this work was extended from the systematic characterization of IgG development and IgG-effector mechanisms to the identification of those viral antigens recognized by CD16-activating mAbs.

For the development of a rational CMV Ab-based therapy, the following conditions should be fulfilled:

1) Targeted antigens should be abundantly localized on the surface of infected cells in order to facilitate the recognition by the therapeutic antibodies;
2) Antibodies should have an optimal IgG subclass activating effector responses;
3) Antibodies should be able to trigger efficient activation of the different host FcγRs (e.g. FcγRIII/CD16), thus promoting the antibody-dependent cell cytotoxicity;
4) Antibodies should be engineered to become resistant against the CMV-encoded FcγRs which block IgG-Fc functions;
5) The neutralization capacity of the antibody may be an additional advantage.

The passive immunization by several mAbs forming a combined therapy in a '*multi-pronged approach'* could be a safe and effective option for the prevention of CMV disease in immuno-compromised hosts and primary infected women.

V. MATERIALS

V.1 DEVICES

Microscope	Axiovert 25, Zeiss
	TS100, Nikon
	Axiovert 40 CFL, Zeiss
Incubators	BBD 6220, Heraeus
	G25 Incubator Shaker,
	New Brunswick Scientific
Nitrogen Tank	Biosafe MD, Cryotherm
Freezing container	Qualifreeze, Qualilab
Working sterile banks	Hera Safe, Heraeus
Centrifuges	5810R, Eppendorf
	5415D, Eppendorf
	5417R, Eppendorf
	3K30, Sigma
	J2-21, Beckman
	OptimaL-70K, Beckman
Thermoblocks	ThermoStat plus,
	Eppendorf
Thermomixer	Comfort,Eppendorf
Blot-shaker	3011, GFL
Vortexer	L46, Labinco
	VV3, VWR

SDS Electrophoresis

Equipment	Agagel mini, Biometra
	Agagel maxi, Biometra
	Minigel-Twin, Biometra
Power-Supplies	Power Pac 300, Biorad
	EPS 301, Amersham
Blotting-Equipment	Fastblot B64, Biometra

Sonicator	Branson Sonifier 450
ELISA Reader	Rainbow ELISA Reader, TECAN
FACS Equipment	FACSCalibur™and FACSCantoII™BD, Biosciences
FPLC ÄKTA™ Systems	ÄKTAbasic, GE Healthcare
Gel dryer	Gel Dryer 583, BioRad
Overheadshaker	REAX2, Heidolph
Amicon® Ultra-15 Centrifugal Filters	Millipore
Luminometer	Berthold

V.2 CHEMICALS AND BIOCHEMICALS

Ammonium Persulfate (APS)	Riedel-de Haen
Ampicilin	Roth
β-Mercaptoethanol	Roth
Bovine Serum albumin (BSA) 30%	PAA Laboratories
Bromphenol blue	Merck
Calcium Chloride	Roth
Ciprofloxacin Hydrochloride	ICN Biochemicals
Complete Protease Inhibitors	Roche
Chloridic acid	Roth
Citric acid	Roth
[^{14}C]-protein molecular weight marker	GE Healthcare
Dimethylsulfoxide (DMSO)	Roth
Dithiothreitol (DTT)	Merck
Dulbecco's Modified Eagle Medium (DMEM)	Invitrogen
EDTA	Merck
Ethanol	Merck
Endoglycosidase H	Roche
FCS (Fetal calf serum)	Invitrogen

Formaldehyde 37%	Merck
Glycerol	Roth
Glycin	Neolab
Geneticine (G418)	Sigma
Hydrogen Peroxide 30%	Roth
HiTrap Protein G HP columns (5 ml)	GE Healthcare
Isopropanol	Merck
Kaleidoscope Prestained Protein-Standard	Biorad
L-Glutamine (200 mM; 100x)	Invitrogen
Methanol	Merck
Minimal Essential Medium (MEM) 10x	Sigma
Nitrocellulose Transfer-Membrane (Protran)	Schleicher & Schuell
Nylonmembrane (Hybond NX)	GE Healthcare
NP40/Igepal	Sigma
OptiMEM	Invitrogen
Penicillin (10.000 U/ml)/Streptomycin (10.000 µg/ml)	Invitrogen
Pepstatin A	Sigma
Peptid-N-Glycosidase F (PNGase F)	Roche
Phenol/Chloroform/Isoamylalcohol	Roth
PMSF	Roth
Protein G-Sepharose 4 Fast Flow	GE Healthcare
PBS Tablets	OXOID
PBS solution	Invitrogen
Ponceau S solution	Sigma
Propidium Iodide	Sigma
Proteinase K	Roche
Redivue ProMix L-[^{35}S]Methionin	GE Healthcare
Rotiphorese-30-Acrylamid	Roth
RPMI 1640 Glutamax	Invitrogen
[^{35}S] Cell Labelling Mix (L-Methionine/L-Cysteine)	Amersham
Saccharose	Roth
Skim milk powder	Sucofin

SeaKem LE Agarose	BioWhittaker
Sodium acetate	Roth
Sodium chloride	Roth
Sodium citrate	Roth
Sodium dodecyl sulfate (SDS)	Roth
Sodium carbonate	Roth
Sodium hydroxide	Merck
Sodium phosphate	Merck
Sodium Vanadat	Alexis
Sodium Pyruvate (100 mM)	Invitrogen
Sulfuric acid	Roth
TEMED	Roth
3,3',5,5'-Tetramethylbenzidine (TMB) Tablets	Sigma
3,3',5,5'-Tetramethylbenzidine (TMB) solution	Thermo scientific
Tris-Base	Roth
Trypan blue stain (0.4%)	Invitrogen
Trypsin 2.5%	Invitrogen
Tween-20	Sigma

V.3 KITS

ECL Plus Western Blotting Detection System	Amersham
Hybridoma Subisotyping Kit, Mouse	Calbiochem
ImmunePure® Fab Preparation Kit	PIERCE
Iso-Gold™ Rapid Mouse-Monoclonal Isotyping Kit	BioAssay Works
Mouse Immunoglobulin Isotype Panel	Southern Biotech
Luciferase Reporter Gene assay high sensitivity kit	Roche
Mouse IgG2c/IgG2a/IgG2b ELISA Quantitation Set	Bethyl Laboratories

V.4 SOLUTIONS AND BUFFERS

Freezing medium

For adherent cells	50% (v/v) DMEM
	40% (v/v) FCS

	10% (v/v) DMSO
For suspension cells	90% (v/v) FCS
	10% (v/v) DMSO

Virus titration

MCMV-Saccharose-VSB	
(Virus Standard Buffer); pH 7.8	15% Saccharose
	50 mM Tris /HCl
	12 mM KCl
	5 mM EDTA

Methylcellulose semi-solid medium

8,8 g Methylcellulose
360 ml H_2O } autoclaved and mixed overnight at 4^0C

20 ml FCS
20 ml $NaHCO_3$ (55 g/l)
40 ml MEM 10x
5 ml Penicillin/Streptomycin
5 ml Glutamin

} mixed at RT for minimum 30 min

Native Lysis buffer pH 7.4	10 mM KCl
(Freshly prepared	200 mM NaCl
before use)	10 mM $MgCl_2$
	20 mM HEPES
	0,5% (v/v) NP40-Igepal
	100 mM EDTA
	10% Glycerol
	0,1 mM Sodium Vanadat
	0,1 mM PMSF
	1 mM DTT

	0,5 µM Pepstatin A
	Complete Protease Inhibitor
	Adjust to pH 7.4 with KOH 3 M

SDS-PAGE and Western Blot

1x RIPA Lysis Buffer	50 mM Tris-HCl pH 7.5
	150 mM NaCl
	1% NP40 (v/v)
	1% Sodium-deoxycholate (v/v)
	0,1% SDS (v/v)
	Complete Protease Inhibitor
5x SDS-Sample buffer	0,25 M Tris/HCl; pH 6.8
	25% (v/v) Glycerol
	20% (w/v) SDS
	0,5%(v/v)β-Mercaptoethanol
	Bromphenol blue
5x Endoglycosidase H Buffer	20mM Sodium phosphate pH 5.0
	100 mM EDTA
	0,1% SDS (v/v)
	1% β-Mercaptoethanol
Stacking gel	5% Acrylamide (v/v)
	65 mM Tris/HCl pH 6.8
	0,1% (w/v) SDS
	14% (w/v) Sucrose
	TEMED
	0,13% (w/v) APS
Separating gel	10% Acrylamide

	43 mM Tris/HCl, pH 8.8 0,1%SDS (w/v) TEMED 0,12% APS (w/v)
10x Lämmli-Electrophoresis Buffer	252 mM Tris 1,92 mM Glycin 1% (w/v) SDS
10x TBST	0,1 M Tris/HCl; pH 8.0 1,5 M NaCl 5% (v/v) Tween-20
Blotting Buffer	5,8 g Tris-Base 2,9 g Glycin 200 ml Methanol H_2O till 1000 ml

Immunoprecipitation

IP-Lysis Buffer (1% NP40 Lysis Buffer)	140 mM NaCl 5 mM $MgCl_2$ 20 mM Tris; pH 7.6 1% NP40 (v/v) 0,5-1 mM PMSF (before use)
IP-Wash Buffer B	150 mM NaCl 10 mM Tris; pH 7.6 2 mM EDTA 0,2% NP40 (v/v)
IP-Wash Buffer C	0,5 mM NaCl 10 mM Tris; pH 7.6 2 mM EDTA

	0,2% NP40 (v/v)
IP-Wash Buffer D	10 mM Tris; pH 8.0
Silver staining	
Fixation Buffer	50% Methanol 12% Acetic acid 38% H_2O
$Na_2S_2O_3$ working solution	2% $Na_2S_2O_3$ x $5H_2O$
Coomassie staining	
Coomasie staining solution Blue	0,02% Coomassie Brilliant G250 2% (w/v) Phosphoric acid 5% Aluminium sulphate 10% Ethanol
Fixation Buffer	30%Ethanol 2% (w/v) Phosphoric acid
FACS	
FACS Buffer	1x PBS 2,5% FCS 0,05% Sodium Azide
For intracellular FACS:	0,3% Saponin
ELISA	
Binding Buffer pH 9.0	0,1 M Na_2HPO_4, adjust to
Blocking Buffer	PBS 10% FCS
Sample Buffer	PBS

	10% FCS
	0,1% Tween-20
Washing Buffer	PBS
	0,1% Tween-20
Substrate Buffer	0,05 M phosphate-citrate buffer, pH 5.0
Solution A:	0,2 M Na_2HPO_4
Solution B:	0,1 M citric acid

Mix 25,7 ml of Sol. A with 24,3 ml of Sol. B and add 50 ml bi-distillate H_2O (pH 5.0)

Stop Solution	1 M H_2SO_4

Affinity chromatography

Elution Buffer	0,1 M Glycine-HCl, pH 2.7
Neutralization Buffer	1 M Tris-HCl, pH 9.0

Hybridoma technology

Lysis solution	0,02 M Tris
	0,14 M NH_4Cl , pH 7.2
HAT selection medium	RPMI 1640
	20% FCS
	0,5% Ciprofloxacin
	0,4% Fungizone (AmphotericinB)
	2%HAT-medium supplement 1x

V.5 CELL LINES

MEFs	mouse embryonic fibroblasts (home-made)
NIH 3T3	mouse fibroblast cell line (ATCC CRL-1658™)
NIH 3T3-m142-HA	
NIH 3T3-m143-HA	stably transfected with either m142-HA or m143-

	HA (provided by Dr. Le V. T., Institute for Virology, Düsseldorf)
BW5147	mouse thymoma cell line (ATCC TIB-47™)
BW5147-FcγRIII-ζ	stably transfected with mouse Fcγ receptor III-ζ (CD16-ζ) and provided by Dr. E. Corrales-Aguilar, Institute for Virology, Düsseldorf
BW5147-FcγRIV-ζ	infected with lentivirus expressing mouse FcγRIV-ζ and provided by K. Ehrhardt., Institute for Virology, Düsseldorf
Sp2/O	myeloma cell line (ATCC CRL-1581™)

V.6 VIRUSES

MCMV-C3X	BAC-derived wild-type recombinant MW97.01 (Wagner et al., 1999) and referred here as wild type (wt)
MCMV Smith	original isolate of wild-type MCMV (ATCC VR-194) (SMITH, 1954)
MCMV-Δm138	mutant derived from wt MCMV having a frame shift on *m138* gene locus (Lenac et al., 2006)
Δm157- MCMV-*luc*	MCMV-mutant having the *luciferase* reporter gene inserted instead of *m157* ORF (Trilling et al., 2011)

V.7 ANTIBODIES

Primary antibodies

Name	Firma/Producer	Catalogue number	Application
28-14-8s (α-mouse MHC I)	(Ozato and Sachs, 1981)	ATCC HB 27	FACS, BWCD16-z assay
Mouse anti-V5 (IgG2a)	Invitrogen	R960-25	FACS, BWCD16-z assay
Rat anti-mouse IL-2 JES6-5H4	BD Pharmingen	554426	ELISA Capture Ab
Rat Anti-mouse IL-2 Biotin Conjugated JES6-1A12	BD Pharmingen	554424	ELISA Detection Ab

Secondary Antibodies

Name	Firma/Producer	Catalogue number	Application
Goat anti-mouse IgG Peroxidase Conjugated	Dianova	115-035-003	Western Blot, ELISA
Goat anti-mouse IgG FITC Conjugated	Sigma-Aldrich	F2012	FACS
Goat anti-mouse IgG Cy5 Conjugated	Dianova (Jackson ImmunoResearch Laboratories)	115-175-062	FACS
Rat anti-mouse IgG1 Biotin Conjugated	BD Pharmingen™	550331	ELISA
Rat anti-mouse IgG3 Biotin Conjugated	BD Pharmingen™	553401	ELISA
Goat anti-mouse IgM-POD conjugated (mu chain)	Rockland	610-1307	ELISA
Rabbit anti-HA	Sigma	H6908-.5ML	IP

V.8 MICE

BALB/cJ (H-2^d)
C57BL/6J (H-2^b) } 8-12 weeks old females provided by the Animal Facility of Heinrich-Heine University of Düsseldorf, Germany

VI. EXPERIMENTAL PROCEDURES

VI.1 WORKING WITH EUKARYOTIC CELLS

VI.1.1 Cell culture

During the experimental procedures, culture of eukaryotic cells was performed under sterile conditions. MEF (mouse embryonic fibroblasts) cells were maintained in culture with DMEM (Invitrogen) containing 10% (v/v) heat-inactivated (30 min, 56^0C) FCS (Foetal Calf Serum), 1% Penicillin (10.000 U/ml), 1% Streptomycin (10 mg/ml). Usually MEFs were used in passage 2 or 3 for infection with Mouse Cytomegalovirus (MCMV). They were prepared in our laboratory as described by Brune *et al.* (Brune et al., 2001).

NIH 3T3 cells (ATCC CRL-1658^{TM}) were cultured using DMEM with 10% (v/v) heat-inactivated NCS (Newborn Calf Serum), 1% Penicillin and 1% Streptomycin.

Mouse BW5147 cells (ATCC TIB-47^{TM}) transfected with CD16-ζ were maintained in selection medium RPMI (Invitrogen) containing 10% FCS, 1% Penicillin, 1% Streptomycin and 4 mg/ml G418 (Sigma-Aldrich, Germany). For culturing the mouse BW-FcγRIV–ζ transfectants, the same medium as above was used, but without any selection marker.

Mouse hybridoma cells were cultured in RPMI medium supplemented with 5% (later 1% and then 0,5%) FCS, 1% Penicillin/Streptomycin and 0,1% 50 mM ß-Mercaptoethanol (Invitrogen).

For passaging the adherent cells, the cells were washed with sterile PBS (Invitrogen) and then detached using 0,6% Trypsin (v/v) diluted in PBS in the case of MEFs and 2 mM EDTA for NIH 3T3 respectively and then resuspended in fresh cell culture media.

When not used for experiments, cells were trypsinized, washed with PBS, centrifuged at 1.000 rpm for 5 min at room temperature (RT), resuspended in freezing medium and aliquoted in Cryo-Tubes (NUNC). The freezing medium is specially used in order to avoid formation of crystals during the freezing process that could destroy the cells. This medium contains 50% (v/v) DMEM, 40% (v/v) FCS and 10% (v/v) DMSO for adherent cells or 90% FCS and 10% DMSO for suspension cells. Cells were frozen slowly (-1^0C/1 min) to -80^0 C in a freezing container (Qualifreeze) and then stored into liquid N_2.

VI.2 WORKING WITH VIRUSES

VI.2.1 MCMV stock preparation

MCMV C3X (Wagner et al., 1999), Smith strain (ATCC VR-194) (SMITH, 1954) or Δm138-MCMV (Lenac et al., 2006) were propagated on MEF cells passage 2 or 3. For a stock preparation, a pre-stock of one T175 flask (175 cm^2) of MEF 90% confluence was infected for almost 4-5 days. Afterwards 30 T175 flasks with MEF passage 3 were infected with the supernatant of the pre-stock. Approximately 4-5 days after infection, when the cytopathic effect (CPE) was observed under the microscope and the cells started to detach, the supernatant was collected in 500 ml centrifuge containers and centrifuged at 5.000 g (6.000 rpm, J2-21, Beckman) for 30 min at 10^0C in order to pellet the cell debris. Afterwards the supernatant was transferred to new containers and centrifuged again for 3 h at 20.000 g (13.000 rpm, 4^0C, J2-21, Beckman) in order to pellet the virus. The virus pellet was incubated overnight at 4^0C in a volume of 5-7 ml supernatant. Next day the virus pellet was resuspended in the respective supernatant volume and homogenized with a Douncer (Wheaton) to make single viral particles. The viral suspension was pipetted carefully over a Saccharose (15% Saccharose/Virus Standard Buffer) cushion and ultra centrifuged 1 h at 60.000 g (20.000 rpm, 4^0C, OptimaL-70K, Beckman). After the ultracentrifugation, the supernatant was discarded and 1-2 ml Saccharose/VSB was added, dependent on the size of the virus pellet. The solution was left overnight at 4^0C to allow a better resuspension of the pellet. Next day the pellet was resuspended and homogenized with a Douncer. Aliquots of 20 µl were made and stored at -80^0 C.

VI.2.2 MCMV titration

For MCMV titration MEF passage 3 were platted in a 48-well culture plate (Greiner Bio One-Cellstar) until they reached 85-90% confluence. The virus stock was pre-diluted 1/100 and then 1/10 steps dilutions were added to the cells. Dilutions between 10^{-2} and ending with 10^{-10} µl stock/ml medium were used. The plate was centrifuged for enhancement of infection at 2.000 rpm for 15 min at RT, and then the plate was turned around and centrifuged in the other direction once again. After incubation for 2 h at 37^0C in 5% CO_2 atmosphere / 80% humidity, the medium was aspirated and a semi

solid medium with methylcellulose was added. After 3-4 days the primary plaques formed after viral infection can be counted. The titre will be given as PFU (plaque forming units)/ml.

VI.2.3 MCMV infection of cells

MEF passages 2 or 3 or NIH 3T3 cells were plated in an adequate culture dish one day prior to infection. The cells were infected in an adequate volume of medium (e.g. 100 µl for a well in a 96-wells plate, 2 ml for a well of a 6-wells plate) with a MOI (multiplicity of infection: number of PFU/cell) of 0,8-5 of MCMV. Enhancement of infection was always done by two steps centrifugation at 2.000 rpm (each time 15 min) at RT, changing the plate orientation between the two steps. After incubation for 24 or 48 h at 37^0C in 5% CO_2 atmosphere / 80% humidity, the different assays (e.g. lysates, FACS, BWFcγR-ζ assay, etc) were performed.

VI.2.4 MCMV infection of mice

Eight weeks old female BALB/c- (H-$2^{\mathbf{d}}$) and C57BL/6- (H-$2^{\mathbf{b}}$) mice obtained from the animal facility of Heinrich-Heine-University of Düsseldorf were intraperitoneally (i.p.) injected with $4x10^6$ PFU/mouse (virus titre calculated with centrifugation) of wild-type Smith MCMV in a total volume of 100 µl/mouse. Dilution of virus was performed in PBS. Mice were killed by cervical dislocation at different days after infection (0, 3, 7, 14, 21, 28 and 60) and blood was collected by cardiac puncture. Mice were maintained under selective pathogen-free conditions.

VI.2.5 MCMV plaque reduction neutralization

MEFs passage 3 were platted in 48-well culture plates (Greiner Bio One - Cellstar) till they reached 90% confluence. Separately, a mix of $1x10^5$ /well Smith MCMV and a 1/50 dilution of latent sera (or MCMV-specific mAbs) were incubated 1 h at 37^0C, to permit binding of the neutralizing antibodies to the viral antigens and blocking of the viral entrance into the cell. The total mix volume of 555 µl virus and sera/mAb was added per well in triplicate. Serial 1/10 dilutions of the mix per each well were

performed and then the plates were centrifuged 2 times 15 min at 2.000 rpm, with changing the plate orientation between the two steps. After incubation for 2 h at 37^0C in 5% CO_2 atmosphere / 80% humidity, the medium was aspirated and a semi-solid medium with methylcellulose was added. After 3-4 days the primary plaques formed after viral infection can be counted and the neutralization effect of sera/mAbs can be evaluated.

VI.2.6 Luciferase neutralization assay

Luciferase is a 61 kDa enzyme (EC 1.13.12.7) isolated from the firefly *Photinus pyralis.* Firefly luciferase is used in bioluminescence and catalyzes luciferin oxidation using ATP-Mg^{2+} as a co-substrate.

For determination of neutralization capabilities of MCMV immune serum, a luciferase neutralization assay was performed. The method is described elsewhere (Reinhard et al., 2011) and is based on the measurement of luciferase activity expressed as RLU (Relative Light Units) in cell lysates after Δm157-MCMV-*luciferase* infection. Δm157-MCMV-*luc* was kindly provided by Dr. Elke Bleifuß, HHU Düsseldorf, Germany and the construction of this virus was previously described (Trilling et al., 2011). The measured RLUs are direct proportional with the amount of infectious viral particles.

NIH 3T3 cells were plated one day before in a 24-wells format. The next day, 1/5 dilutions of BALB/c and C57BL/6 mouse sera, followed by four 4-fold dilutions were prepared. About 10^3 PFU of Δm157- MCMV-*luc* were incubated with different dilutions of sera at 37^0C for 90 min, in a final volume of 26,5 µl. The master mix represented by sera dilutions and virus particles was added over the NIH 3T3 cultured cells and a two steps centrifugation protocol (15 min in each direction, RT, 2.000 rpm) was performed. The plates were let in incubators overnight at 37^0C. Neutralization tests for all dilutions were run in duplicate.

Next day each well was washed with 1 ml PBS and then 100 µl/well of Lysis Buffer of the Luciferase Reporter Gene assay high sensitivity kit (Roche) was added. Plates were frozen at -80^0C for minimum 2 h. After the complete cell lysis was performed, plates were thawed at 4^0C, cells were scraped and the lysates from each well were individually transferred into an Eppendorf tube. Samples were centrifuged at 4^0C and 13.000 rpm for 5 min. 50 µl from each lysate was then transferred into a luminescence plate and the luciferase activity in cell lysates was measured using a luminometer (Berthold). The

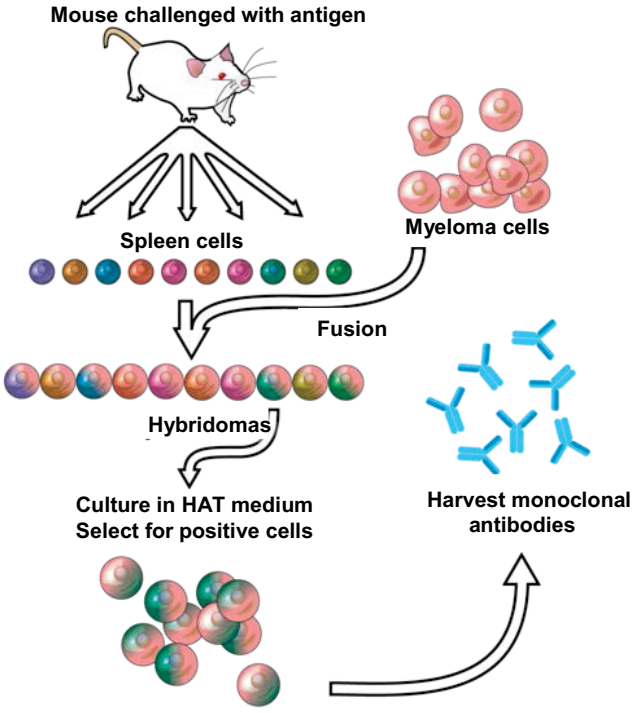
50% neutralization titres for each time point were afterwards calculated and represent the serum dilution neutralizing 50% of the virus.

VI.3 WORKING WITH MICE (IN VIVO)

VI.3.1 Production of MCMV specific monoclonal antibodies using hybridoma technology

Monoclonal antibodies (mAbs) can be produced in specialized cells through a technique known as hybridoma technology, invented by Cesar Milstein, Georges J.F. Köhler and Niels Kaj Jerne in 1975 (Kohler and Milstein, 2005). A hybridoma is a hybrid cell line formed after fusion between a specific antibody-producing B-cell with a myeloma (cancer) cell. The fused hybridoma has the antibody producing capability inherited from lymphocytes and has the ability to grow continuously like malignant cancer cells. Fused cells are selected in HAT medium (hypoxanthine-aminopterin-thymidine) for 10-14 days and then cloned by limiting dilution to obtain mAbs derived from only one hybridoma.

The basic steps in hybridoma technology are represented in the figure below:



Kuby Immunology (4th edition) *by Richard A. Goldsby, Thomas J. Kindt and Barbara A. Osborne. 2000. W. H. Freeman and Co., NY*

Figure VI.1 Production of monoclonal antibodies using hybridoma technology

Production of MCMV mAbs using hybridoma technology was done in collaboration with Karmela Miklic, Dr. Astrid Krmpotic and Prof. Stipan Jonjic from the University of Rijeka, Centre for Proteomics, Croatia.

Five females BALB/c mice were i.p. infected with wt MCMV and after 3 months mice were boosted i.p. with lysates of MCMV-infected MEFs 3 days prior to fusion. The boost induces a proliferation of MCMV-specific B cells already produced after the first immunization. The serum collected from all 5 mice was tested in ELISA for antibody response to MCMV infection and only two mice with the highest antibody titre were chosen for fusion. Spleens from these two mice were collected in tubes which contain RPMI medium (Invitrogen). Spleens were smashed through a 70 μm Cell Strainer (BD Falcon, Germany), taking care of dropping RPMI medium constantly over the spleen. In this way the spleen tissue was separated into single cells. The Falcon tube containing single cells from spleen in RPMI medium was centrifuged at 1.700 rpm and RT for 5 min. The supernatant was discarded and the pellet was resuspended in 5 ml lysis solution (0,02 M Tris, 0,14 M NH_4Cl, pH 7.2) for 5 min. Afterwards the Sp2/O (ATCC: CRL-1581^{TM}) myeloma cell line suspension was added over the spleen cells (approx. 6-8 x 10^7 myeloma cells/fusion). Tube was centrifuged at 1.700 rpm and RT for 5 min. Supernatant was discarded and 1 ml PEG (Polyethylene Glycol) was added drop by drop over the pellet, homogenizing it with the cells for 1 min. PEG is a polyether compound that has the property to open the cell membranes of both B cells and myeloma cells and as a result they fuse into one single hybridoma cell. After the fusion took place, the cell membranes start to close by adding RPMI medium drop by drop, till the total volume of 10 ml. The tube was centrifuged at 800 rpm and RT for 5 min. Supernatant was discarded and 30 ml of HAT medium was added strongly over the cell pellet, without resuspension. The total volume was diluted in HAT medium so that at the end the cell suspension was platted in 15 x 96 well plates, 200 μl/well. After they started to grow, hybridoma cells were fed everyday for 1 week with new HAT medium. In order to check if the obtained hybridomas were able to produce mAbs against MCMV, an ELISA test on lysates of MCMV-infected MEFs 10 days after the fusion was performed. The selected positive hybridomas were expanded and cloned by limiting dilution.

VI.3.2 Cloning by limiting dilution

Limiting dilution is a method to isolate individual hybridoma cells from each other and culture these isolated hybridomas to produce isolated clones. Once an antibody-secreting clone has been established, it can be expanded to allow for the production of large amounts of monoclonal antibody. The procedure of cloning by limiting dilution of the fused hybridoma cells previously described has been exclusively realized by the Centre for Proteomics of the University of Rijeka, Croatia, in the laboratory of Prof. Stipan Jonjic.

VI.3.3 Production of MCMV mAbs from hybridoma culture supernatants

Cloned hybridoma cells were cultured for several days in RPMI medium (Invitrogen) containing 5% heat-inactivated FCS, 1% Penicillin/Streptomycin and 0,1% 50 mM ß-Mercaptoethanol; during cultivation and expansion of cells, concentration of FCS was decreased to 1% and then in the end 0,5%. When the desired volume of culture supernatant was reached, cells were centrifuged for 1 h at 4.000 rpm and the supernatant which contains antibodies was collected in new tubes and stored at -20^{0}C.

VI.3.4 Production of MCMV immune serum

Blood collected from infected and non-infected control mice (5 mice per group) was allowed to clot at 4^{0}C for 30 min. A first centrifugation step at 8.000 rpm and 4^{0}C for 15 min was performed, followed by a second centrifugation at 13.000 rpm and 4^{0}C for 15 min. Sera present as supernatant was collected and stored at -20^{0}C till it was used for experiments.
Before performing the experiments, equal amounts of sera from all five mice of each group were pooled.

VI.4 IMMUNOLOGICAL/PROTEIN METHODS

VI.4.1 Generation of protein lysates for ELISA

72 h after MCMV infection, MEF passage 3 were scraped, pelleted and washed two times with PBS. The cell pellet was resuspended in a mix of 1 ml/tube PBS plus 30 µl Complete Protease Inhibitors Cocktail (Roche) and centrifuged for 1 min at 13.000 rpm and 4^0C. The cell pellet was again resuspended in PBS and sonicated 3 times 10 seconds (Branson Sonifier 450) for a complete cell lysis. Lysates were then frozen in 200 µl aliquots at -20^0C. There were approximately $1x10^6$ cells in 100 µl of lysates.

VI.4.2 Generation of protein lysates for western blot and immunoprecipitation

For the generation of protein lysates 1x RIPA Lysis Buffer for Western Blot or 1% NP40 Lysis Buffer for Immunoprecipitation was used. Approximately 10^6 NIH 3T3 cells per sample (MCMV- infected or mock cells) were scraped, pelleted and washed two times with cold PBS (centrifugation at 1.200 rpm, 3 min, 4^0C). Every step was done on ice. The cell pellet was resuspended in 300 µl 1x RIPA Lysis Buffer, followed by a 30 min incubation step on ice. Afterwards the tubes were centrifuged for 30 min at 4^0C and 13.000 rpm (5415D, Eppendorf). The SN was collected in new tubes and divided in two equal parts: in each part 1x Endoglycosidase H Buffer was added and all the samples were boiled for 5 min at 95^0C. The tubes were then cooled on ice and 2 µl Endoglycosidase H (Endo H) was added only in half of the samples. All the samples were then incubated overnight at 37^0C to allow the enzyme to deglycosylate the glycoproteins present in the cell lysates. Endoglycosidase H cleaves with highly specificity asparagine-linked mannose rich oligosaccharides from glycoproteins, but not highly processed complex oligosaccharides. Next day 1x SDS Sample Buffer was added and the tubes were stored at -20^0C.

VI.4.3 SDS-PAGE (Polyacrylamide - Gel electrophoresis)

For the reducing SDS-PAGE the Minigel Twin system from Biometra was used. Depending on the size of the proteins to be detected, a different percentage of acrylamide for the resolving gel was poured (**Table VI.1**). The separation of the

proteins was carried out at 20 mA per gel during 1-1,5 h, depending on the protein size. Before the samples were loaded to the SDS-PAGE, they were boiled for 5 min at 95^0C in 1x SDS sampler buffer.

Table VI.1 Components of the SDS-PAGE gel

	Resolving gel		**Stacking gel**
	8%	10%	5%
30% Acrylamide	3,2 ml	4 ml	1,5 ml
2 M Tris-HCl (pH 8.8)	2,5 ml		
0,5 M Tris-HCl (pH 6.8)	-		1,2 ml
60% Saccharose			2,1 ml
20% SDS	60 µl		45 µl
H_2O	6,1 ml	5,3 ml	4,2 ml
TEMED	24 µl		12 µl
10% APS	144 µl		120 µl

VI.4.4 Western blot

For the detection of proteins which were separated in the SDS-PAGE, specific antibodies (see materials) were used in western blot analysis, as previously described (Laemmli, 1970). In order to make the proteins accessible to antibody detection, they were transferred from SDS-PAGE gel onto a membrane made of nitrocellulose (Whatman PROTRAN). The blotting process was performed using a Semidry Blotting equipment (Biometra) and the protocol was as follows: at the anode (+) part there were 3 x 3 mm Whatman-Paper of the dimensions of the gel (9 cm x 8 cm), then comes the nitrocellulose membrane, the SDS-PAGE gel on top of it and another 3x Whatman-Paper at the cathode (-). All Whatman papers and the nitrocellulose membrane were previously inserted into the Blotting Buffer.

The transfer of the proteins was done for 1 h at 16V. Afterwards, the proteins transferred onto the membrane were stained with Ponceau red staining. After the removal of Ponceau staining, the membrane was incubated with a blocking solution of 5% (w/v) non-fat dry milk in TBST for at least 40 min, in order to block the non-specific binding between the membrane and the antibody used for detection of the target

protein. In the case of MCMV-mAbs, the membrane incubation with the primary antibody was performed overnight at 4^0C in a tumbler. The solution with the detection antibody, which is coupled with horseradish peroxidase (HRP), was let over the membrane for about 1 h at RT. After washing 5x with TBST (each time 5 min), the proteins were detected using the ECL Plus Western Blotting Detection Systems (GE Healthcare) and BioMax MR Films (Kodak).

VI.4.5 Immunoprecipitation (IP) of metabolically labeled proteins

For characterization of viral proteins recognized by MCMV-specific mAbs, radioactive immunoprecipitation was performed (Bjorck and Kronvall, 1984). MCMV-infected NIH 3T3 cells or cells stably transfected with m143-HA or m142-HA (platted in 6 well-plates) were washed 1x with PBS containing 3% FCS and incubated for 30 min in Met/Cys-free DMEM (starving medium) plus 5% FCS. Afterwards 10 µl/well of ^{35}S-Met/Cys (GE Healthcare) in a total volume of 1 ml starving medium/well was added. The cells were labeled for 2-3 h at 37^0C, 5% CO_2 atmosphere / 80% humidity. The generation of cell lysates was performed as described in the section VI.4.2 (Generation of protein lysates for western blot and immunoprecipitation).

After the cell lysis was performed, the MCMV-specific mAbs or rabbit α-HA antibody (Sigma) in a concentration of 2 µg/ml each were added per sample. Binding of antibodies to their targets was performed overnight in an overhead tumbler at 4^0C. A volume of 40 µl/sample of protein G Sepharose (previously washed 3 times with the IP buffer B and resuspended 1:1 in the same buffer) was added after the given incubation period. The sample tubes were rotated overhead at 4^0C for about 1 h. Afterwards a centrifugation step of 30 seconds at 16.000 g (13.000 rpm, 5417R, Eppendorf) was done. The sepharose pellet was washed 2x with IP wash buffer B, 2x with IP wash buffer C and 1x with IP wash buffer D. Between the washing steps, the sepharose was precipitated by centrifugation for 20 seconds at 13.000 rpm. After the last washing step, as much as possible of the buffer was removed from the sepharose pellet. The dry pellet was resuspended in 30 µl of 1x Endo H Buffer and cooked for 5 min at 95^0C. The samples were cooled on ice and centrifuged for 1 min at 13.000 rpm. In only half of the samples 2-3 µl of Endoglycosidase H was added and the digestion was carried out for 16 h at 37^0C. The next day samples were stored at -20^0C till further analysis. 40 µl of 5x

IP sample buffer was added to the samples before they were loaded on a SDS-PAGE big gel.

For the immunoprecipitation of non-metabolically labeled proteins, the same protocol was applied. The only difference was that cell lysates were performed directly, without a previous step of labeling the cells with ^{35}S-Met/Cys.

VI.4.6 Silver staining of proteins in SDS-polyacrylamide gels

A non-radioactive protein detection method used for the identification of MCMV-specific antigens able to trigger CD16-responses is the silver staining (Merril et al., 1981), which is 100-fold more sensitive than Coomassie Brilliant Blue staining. After the samples were loaded on an SDS-PAGE gel and run overnight, the following steps were performed for the silver staining of MCMV-specific proteins.

Table VI.2 Protocol for the staining of proteins in SDS-PAGE gels

Steps in silver staining protocol	Solutions	Time
Fixation	25 ml Fixation buffer + 12,5 µl 37% Formaldehyde	20 minutes
Washing	50% Ethanol	3x 5 min
Incubation	**Solution A**: 25 ml H_2O + 25 µl $Na_2S_2O_3$-working solution	1 minute
Washing	H_2O	3x 20 seconds
Incubation	**Solution B:** 0,04g $AgNO_3$ +18 µl 37% Formaldehyde in 25 ml H_2O	10-15 minutes
Washing	H_2O	2x 20 seconds
Developing	**Solution C:** 1g Na_2CO_3+6,25 ml $Na_2S_2O_3$-working solution+10 µl 37% Formaldehyde in 25 ml H_2O	From 10 minutes to 2 hours depending on your stained proteins concentration
Washing	H_2O	2x 10 seconds
Stopping	Fixation buffer	20 minutes

Afterwards the SDS-PAGE gel was washed with H_2O and dried for 2 h in a Gel Dryer (BioRad).

VI.4.7 Coomassie Brilliant Blue for staining of MCMV-specific proteins

For the identification of MCMV-antigens by mass spectrometry (MS), a Coomassie Brilliant Blue staining prior to the final identification was performed. The protocol used for staining was adapted from Kang *et al.,* Bull. Korean Chem. Soc. 2002, Vol.23, No.11 and is compatible with the MS method used by the Biological Medical Research Center (BMFZ) of the University of Düsseldorf.

The protein samples were loaded on a big SDS-PAGE gel and run overnight. Next day the gel was washed 3 times for 10 min with distillated H_2O. Proteins were then fixed on the gel with a fixation buffer composed of 30% Ethanol, 2% (w/v) Phosphoric acid for 30 min. The proteins present on the gel were stained overnight with a Coomassie Staining solution and the coloring process was stopped after washing the gel with water. Protein bands were cut from the gel with a clean knife and sent for identification by MS.

VI.4.8 Mass spectrometry analysis

Identification of MCMV-antigens able to trigger CD16-activation was performed in two different laboratories. Identification of Glycoprotein B (gB) and m142/m143 complex recognized by MCMV antibodies called MCMV1.01 and 10B2-D10 respectively, was performed in the Centre for Medical Biology Research (BMFZ) from University of Düsseldorf. Secondly, sequence identification of gB recognized by 15A12-H9 mAb, as well as of mouse Myosin were performed in the Centre for Molecular Medicine Cologne (ZMKK) from University of Köln.

VI.4.9 Affinity Chromatography

This method separates proteins on the basis of a reversible interaction between a protein (or a group of proteins) and a specific ligand coupled to a chromatography matrix. In this way the target protein is recovered by changing conditions to favor elution of the bound molecules. Elution is performed specifically using a competitive ligand or non-specifically by changing the pH, ionic strength or polarity. The target protein is collected in a purified, concentrated form.

VI.4.9.1 Purification of MCMV-specific IgGs from hybridoma culture supernatants using HiTrap Protein G columns

In order to purify and concentrate MCMV-specific IgGs from hybridoma culture supernatants, an affinity chromatography method based on HiTrap Protein G 5 ml columns and a liquid chromatography system such as ÄKTAdesign™ –prime plus system (GE Healthcare, Germany) has been performed. The basis for purification of IgG is the high affinity of Protein G for the Fc region of polyclonal and monoclonal IgG –type antibodies. In comparison to Protein A, Protein G (derived from groups C and G *Streptococci*) is able to bind to IgG Fc of most species, including rat and goat and recognizes most subclasses (including human IgG3 and mouse IgG1) but has a lower binding capacity. Therefore the HiTrap Protein G HP columns from GE Healthcare for the purification and isolation of mouse IgGs were preferred. The HiTrap Protein G HP 5 ml columns are prepacked with 5 ml of Protein G Sepharose™ High Performance, as an affinity matrix. The recombinant Protein G used in this process is produced in *E.coli* and contains two IgG binding regions. The albumin binding region of native Protein G has been genetically deleted, thereby avoiding undesirable cross-reactions with albumin. There are 3 major steps in performing the IgG purification using HiTrap Protein G columns as seen in the figure below:

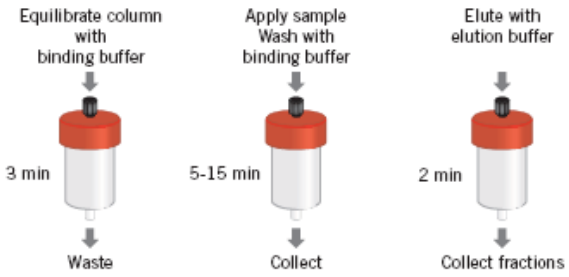


Figure VI.2 Affinity purification procedure using HiTrap Protein G prepacked columns (GE Healthcare)

1) Column equilibration

The HiTrap Protein G 5 ml columns were first washed with 5 column volumes of distillate H_2O and then equilibrated with the same volume of PBS (Binding Buffer). The pump flow rate was gradually increased from 1,5 ml/min to the maximum recommended of 5 ml/min for the given column.

2) Sample application

The mouse hybridoma culture supernatant was applied in a 50 ml superloop connected to the whole system through thin flexible tubes. The sample is slowly loaded into the system and reaches the HiTrap Protein G columns where the mouse IgG present in the supernatant is able to reversibly bind to the Protein G Sepharose matrix prepacked in the column. The total volume of 50 ml of supernatant is loaded in about 15-20 minutes at a relatively low flow rate, gradually increased from 1 to 5 ml/min, to ensure that the target protein binds strongly to the ligand. Afterwards the column was washed with 5 column volumes of PBS to remove all impurities and unbound material. This step is repeated until no protein is detected in the eluent (determined by UV absorbance at 280 nm). This will improve the purity of the eluted target protein.

3) Elution

Elution of IgG bound to the Protein G matrix is performed applying 2-5 column volumes of Elution buffer (0.1 M Glycine-HCl, pH 2.7). This change in pH values alters the degree of ionization of charged groups on the ligand and/or the bound protein. This change may affect the binding sites directly, reducing their affinities. The eluted IgG is collected as fractions into tubes and is represented in the graphic as a sharp peak (**Fig. VI.3**). Neutralization buffer (1 M Tris-HCl, pH 9.0) was added drop by drop with a pipette to return the fraction to a neutral pH. The column was also re-equilibrated with PBS to neutral pH.

The typical affinity chromatography profile could be seen in the figure below (**Fig. VI.3**):

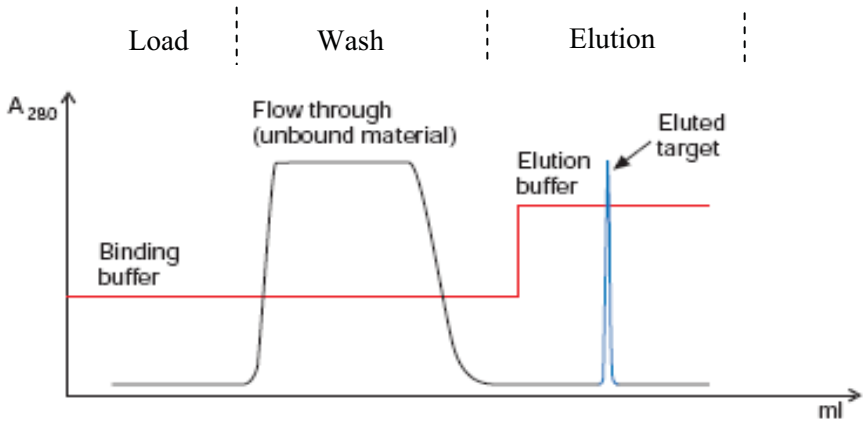


Figure VI.3 Affinity chromatography elution profile (GE Healthcare)

VI.4.10 Dialysis of the eluted IgG

Dialysis is a separation technique that facilitates the removal of small, unwanted compounds from macromolecules in solution by selective and passive diffusion through a semi-permeable membrane. For dialysis of eluted IgG, a Spectra/Por membrane (Spectrum Medical Industries, USA) with a molecular weight cut-off (MWCO) of 6.000-8.000 Da was used. The eluted IgG was filled in the tube formed by the membrane and placed in a 1 liter cylinder filled with PBS. The passive diffusion of molecules smaller than 6-8.000 Da was realized at 4^0C overnight.

VI.4.11 Determination of IgG concentration (Bradford Method)

After MCMV-specific IgG was purified from hybridoma culture supernatants using the previously described affinity chromatography method, concentration of eluted IgG was determined by Bradford method (Bradford, 1976). Measuring the O.D. of the samples at 595 nm and using a BSA standard curve, the final concentration of the eluted IgG was calculated and expressed as μg/ml.

VI.4.12 FACS (Fluorescence Activated Cell Sorting)

FACS was performed for the analysis of cell membrane protein expression or for staining of intracellular proteins. The basis of a FACS analysis is a labeled suspension of individual cells which passes a focused laser beam. Capillary forces cause the cells to pass the flow-cell, where the labels are stimulated by the laser light. The emitted fluorescent light from the fluorophores, which are coupled to the antibodies are detected by a photomultiplier tube or a light detector. By collecting the information from the light (scatter and fluorescence) and using the specific manufacture's software, the end results of the measurement are presented graphically.

NIH 3T3 cells were platted one day before infection in a 6 well-plate format, using approx. $1x10^6$ cells for 2 samples. Infected cells were washed carefully with PBS, detached from the bottom with 1 mM EDTA and transferred in 5 ml Falcon tubes for centrifugation. Non-infected (mock) cells were trypsinized for detachment. Both cell types were centrifuged at 1.300 rpm for 3 min at 4^0C, and the obtained pellet was resuspended in cold FACS Buffer (PBS + 3% FCS), 100 μl/sample. The cells were then

added into different wells of a 96-well V-bottom plate for analysis. For extracellular FACS, the cells were directly incubated with a proper dilution of the primary antibody (50 µl/well) for 30 min on ice. The plates were then centrifuged for 3 min at 1.300 rpm and 4^0C and the supernatant was carefully aspirated. The cells were washed two times with FACS buffer and incubated with the secondary antibody in a proper dilution (50 µl/well) for about 30 min in the dark. Incubation in the dark has been performed because the labeled antibodies are sensitive to light exposure. The plate was centrifuged at 1.300 rpm for 3 min and the supernatant aspirated. Cells were again washed two times with FACS buffer. After the last centrifugation, 100 µl/well of FACS buffer was added and the cells were resuspended and transferred into proper tubes for FACS measurement which contained 500 µl FACS buffer and 0,3 µl Propidium Iodide. Propidium Iodide (PI) intercalates into double-stranded nucleic acids. It is excluded by viable cells but can penetrate cell membranes of dying or dead cells. Approximately 10.000 cells per sample were read and a gate only on living cells was afterwards realized.

In order to screen for the extracellular specificity of the library of MCMV-specific mAbs, several FACS analysis were performed. Infected and mock NIH 3T3 cells were stained with each MCMV-specific mAb and the extracellular binding capability was detected using as secondary antibody, either Goat anti-Mouse IgG -FITC (Sigma-Aldrich, Germany) or Cy™5-conjugated AffiniPure Goat anti-Mouse IgG (H+L) (Jackson ImmunoResearch Laboratories, USA).

VI.4.13 MCMV specific IgG and IgM ELISA

For the detection of total amount of MCMV-specific IgG and IgM present in mouse serum collected at different times after MCMV infection, an indirect ELISA test was performed.

Microtitre plates (MaxiSorp Surface NUNC, Denmark) were coated with 50 µl/well lysates of mock- or C3X-infected MEFs in ELISA Binding Buffer (0,1 M Na_2HPO_4, pH 9.0). There were approximately $2x10^6$ lysed cells per one 96 wells plate. Elisa plates were left overnight at 4^0C; the nonspecific binding sites were blocked with a solution of 10% (v/v) FCS in PBS for 1 h at RT, 100 µl/well and then washed two times with ELISA Wash Buffer (PBS with 0,1% (v/v) Tween 20 (PBS-T)). Samples of pooled sera were 2-fold diluted in ELISA Sample Buffer (ELISA wash buffer with 10% FCS)

starting with dilution 1/25 till 1/1.600 in the case of IgG and 1/50 dilution of sera for IgM detection was used. 50 µl of each sera dilution was added per well and incubated at RT for 1,5 h. All the plates included a positive and a negative control serum. After two washing steps with PBS-T, peroxidase-conjugated AffiniPure goat anti-mouse IgG (Jackson ImmunoResearch Laboratory, West Grove, PA) or goat anti-mouse IgM (mu chain) Peroxidase Conjugated (Rockland -BioTrend) were used as detection antibodies at a dilution of 1/5.000 in ELISA Sample Buffer. A volume of 50 µl/well of conjugate dilution was incubated at RT for 1 h and the plates were then rewashed as described above. The colour reaction was developed with a solution of TMB substrate (3,3',5,5' tetramethylbenzidine, Thermo Scientific, Rockford, USA), 50 µl/well for 2-4 min. Reaction was stopped after adding a solution of 1 M H_2SO_4 (50 µl/well) and the O.D. was measured immediately at 450/620 nm using an automatic spectrophotometer (TECAN Sunrise, Germany). All the samples were tested in triplicate and the average of those three determinations was calculated. The results are presented as a 50% ELISA titre. The titre represents the serum dilution corresponding to the O.D. situated in the middle of the interval between the maximum (obtained for dilution 1/25 of serum from day 60 p.i.) and the minimum O.D. (cutoff) measured in ELISA.

For quantification of total IgM present in sera collected from infected animals, an additional IgM standard curve was performed, using different concentrations (from 125 ng/ml till 0,97 ng/ml) of purified mouse IgM (Southern Biotech) coated on ELISA plates. For detection, the same peroxidase-goat anti-mouse IgM (Rockland-BioTrend) antibody was used. Taken into account the measured O.D. at 450 nm, the standard curve equation and the serum dilution factor, concentrations of IgM (µg/ml) were calculated. The final concentration values for each time point were calculated subtracting the concentration values of mock-infected cells, considered as background.

VI.4.14 MCMV-specific IgG ELISA subclass

For quantification of MCMV-specific IgG subclass composition of sera from infected animals, an indirect ELISA test has been performed. The protocol is similar with the one described for whole IgG determination, with the difference that for each IgG subclass a specific detection antibody was used. For IgG1 and IgG3, rat anti-mouse IgG1- and rat anti-mouse IgG3-Biotin conjugated (BD Pharmingen) were used in a final

concentration of 1 µg/ml. In addition to the ELISA protocol described above, 50 µl/well of 1/1.000 dilution of peroxidase-conjugated streptavidin (Jackson ImmunoResearch) was added on ELISA plates for 30 min in the case of IgG1 and IgG3 detection. The colour reaction was developed as previously described. For the detection of MCMV-specific IgG2a, IgG2b and IgG2c in BALB/c and C57BL/6 serum, ELISA Quantitation Sets (Bethyl Laboratories) were utilized with the protocol described in the kits instruction manual. For each Bethyl detection antibody a titration was performed and dilutions of 1/17.000, 1/30.000, 1/10.000 for IgG2a, IgG2b and IgG2c, respectively were chosen.

After several titrations of pooled mouse serum, proper dilutions of 1/5.000, 1/1.000 and 1/500 for detection of IgG2a, IgG2b and IgG2c, respectively were applied. On the other hand, mouse serum was diluted 1/200 for both IgG1 and IgG3 detection.

Standard curves for IgG2a, IgG2b and IgG2c were realized as recommended by the kits protocols; instead, serial decreasing concentrations (from 125 ng/ml to 0.97 ng/ml) of purified IgG1 and IgG3 (Mouse Immunoglobulin Panel, Southern Biotech, USA) were performed.

Concentrations in µg/ml of each IgG subclass present in serum from MCMV-infected mice were calculated using the measured O.D., standard curves equation and the dilution factor.

VI.4.15 Total IgG subclass ELISA

In order to determine the total IgG subclass present in mouse serum at each time point after MCMV infection, another ELISA test was established. For IgG1 and IgG3, ELISA plates were overnight coated with mouse immune serum diluted 1/5.000 and respectively 1/2.000 in binding buffer. For total IgG2a, IgG2b and IgG2c detection, the protocols from mouse IgG2a/IgG2b/IgG2c ELISA Quantitation sets (Bethyl) were used. In this case, serum was diluted 1/30.000, 1/20.000 and 1/500 for IgG2a, IgG2b and IgG2c, respectively. Detection antibodies for each IgG subclass were diluted as previously described in the MCMV-IgG ELISA. The rest of the protocol, including standard curves and calculation of concentrations in µg/ml were done in the same manner as for MCMV-specific IgG subclasses.

VI.4.16 Fcγ Receptor-activation assay (BW-FcγR-ζ Assay)

To quantify antiviral IgG antibodies able to trigger CD16 (FcγRIII) and FcγRIV responses, target cells (MEFs p3) were infected with 0,5 PFU of MCMV Smith strain. Target cells (T) were normally seeded in 96-well culture plates one day prior to infection. 24 h post infection mock and virus-infected cells were incubated with 2-fold serial dilutions of sera (from 1/25 till 1/1.600) in complete D-MEM for 1 h at 37^0C in an atmosphere of 5% CO_2 / 80% humidity. Afterwards, target cells were co-cultivated with BW-CD16-ζ or BW-FcγRIV-ζ effector cells (E) for 16 h, in a ratio of 1:20 (T:E).
After co-cultivation, supernatants were diluted 1:1 v/v in ELISA sample buffer (PBS with 10% FCS and 0,1% Tween 20) and the secreted murine IL-2 was measured in the mIL-2 ELISA (see below). All tests were performed in triplicates.

VI.4.17 Interleukin-2 (IL-2) ELISA

Secreted IL-2 upon activation of BW-CD16-ζ or BW-FcγRIV-ζ transfectants was measured in an ELISA test using as capture antibody rat anti-mouse IL-2 (diluted in ELISA binding buffer at a final concentration of 1 μg/ml) and as detection antibody Biotin rat anti-mouse IL-2 (1 μg/ml) (BD Pharmingen™ , Belgium). The samples (secreted murine IL-2), previously diluted 1:1 (v/v) in sample buffer were added at 100 μl per well and incubated on the ELISA plates for minimum 2 h. All the subsequent steps were performed as in the ELISA protocol described above.

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VIII. ACKNOWLEDGEMENTS

My special acknowledgements are directed to Prof. Hartmut Hengel for giving me the opportunity to come closer to such a fascinating research field and for his permanent trust and encouragement during my PhD.
Prof. Johannes Hegemann is thankfully acknowledged for accepting being the thesis supervisor.

I am grateful to Prof. Stipan Jonjic and his team from the Centre of Proteomics in Rijeka, Croatia for a warm and exciting working environment during the two months training in his lab and for teaching me the technology of producing mouse monoclonal antibodies.

I want to thank Stefanie Jeschke for being the perfect example of 'an organized German person' and for a successful help with the animal experiments. I express my appreciation to PD. Albert Zimmerman for critically reading my thesis and for motivating scientific discussions.

Many thanks to Dr. Mihaela Gurgui from Bonn University for her lately scientific support, including critics and corrections of this manuscript and for being a true friend in bad and good times.

My family: parents, grandma, brother and his two lovely children Rares and Dragos fill a special place in my heart and I am totally grateful to them for the whole moral, spiritual and every now and then financial support. You encouraged me from far away to keep moving forward no matter what difficulties come across and to follow my goals with respect and dignity - *'Multumesc din suflet familiei: mama, tata, mamaia, Razvan, Alina, Rares si Dragos pentru intregul suport moral, spiritual si nu de putine ori finaciar. Voi m-ati incurajat permanent de la distanta sa imi urmez scopul cu respect si demnitate si sa merg mai departe indiferent ce greutati intalnesc in drumul vietii.'*

Last, but not least I want to thank the new and old members of the CMV group for a friendly working environment and critical scientific discussions.

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Cytomegalovirus-induced IgG-subclass selective hypergammaglobulinemia and emergence of antiviral IgG effector functions

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ERKLÄRUNG

Ich versichere an Eides Statt, dass die Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der "Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf" erstellt worden ist. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

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