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Pathogenesis of the highly passaged MCMV Smith strain and low passaged HaNa1 strain in Balb/c mice mimicking natural infection upon oronasal inoculation

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Promoter

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"It was the best of times, it was the worst of times, it was the age of wisdom, it was the age of foolishness, it was the epoch of belief, it was the epoch of incredulity, it was the season of light, it was the season of darkness, it was the spring of hope, it was the winter of despair"...

(Charles Dickens, A Tale of Two Cities)

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List of abbreviations

1E	ethmoid turbinate one
3E	ethmoid turbinate three
4E	ethmoid turbinate four
5E	ethmoid turbinate five
6E	ethmoid turbinate six
AIDS	acquired immune deficiency syndrome
AlHV-1	Alcelaphine herpesvirus 1
BAC	bacterial artificial chromosome
bp	base pair
$CaCl_2$	Calcium chloride
CCL	chemokine (C-C motif) ligand
CD4	cluster of differentiation 4
CD8	cluster of differentiation 8
cDCs	conventional DCs
ChHV-6	Chelonid herpesvirus 6
CMC	carboxymethylcellulose
CMV	cytomegalovirus
CPE	cytopathic effect
DABCO	1,4-diazabicyclo[2.2.2]octane
DCs	dendritic cells
DLM	dorsal lateral meatus
DM	dorsal medial meatus
DNA	deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
dpi	day(s) post inoculation
E	ethmoid turbinate
E genes	early genes
EDTA	Ethylenediaminetetraacetic acid
EHV-2	Equid herpesvirus 2
EHV-7	Equid herpesvirus 7
ElHV-1	Elephantid herpesvirus 1
ER	endoplasmic reticulum
ERGIC	ER-Golgi intermediate compartment
FCS	fetal calf serum
FITC	Fluorescein isothiocyanate
g	gravity
G+C	guanine+cytosine

GaHV-1	Gallid herpesvirus 1
GaHV-2	Gallid herpesvirus 2
gB	glycoprotein B
gH	glycoprotein H
gL	glycoprotein L
gM	glycoprotein M
gN	glycoprotein N
gO	glycoprotein O
H-2	histocompatibility-2
HCMV	Human cytomegalovirus
HEVs	high endothelial venules
HHV-1	Human herpesvirus 1
HHV-3	Human herpesvirus 3
HHV-4	Human herpesvirus 4
HHV-5	Human herpesvirus 5
HHV-6	Human herpesvirus 6
HHV-8	Human herpesvirus 8
HIV	human immunodeficiency virus
HP	hard palate
hpi	hour(s) post inoculation
i.f.	Intrafootpad
IE	Immediate-early genes
IFN	interferon
IL	interleukin
IP	intraperitoneally
IPMA	immunoperoxidase monolayer assays
IRL	long internal repeat
IRS	short internal repeat
kbp	Kilo-base pair
$KHCO_3$	potassium hydrogen carbonate
L genes	late genes
LM	lateral meatus
LN	lymph nodes
LNG	lateral nasal glands
LTα/β	Lymphotoxin α/β
LTβR	lymphotoxin β receptor
M	molar
M2-10B4	murine bone marrow stromal cell line

mAbs	Monoclonal antibodies
MCK-2	MCMV-encoded CC chemokine 2
MCMV	Murine cytomegalovirus
MCP	major capsid protein
MEFs	mouse embryonic fibroblasts
MEM	Minimum Essential Medium
mg	milligrams
$MgCl_2$	Magnesium chloride
MHC	major histocompatibility complex
MIE	major immediate-early genes
MIEP	major IE promoter
ml	millilitre
MM	middle medial meatus
mM	millimolar
mm	millimetre
mm^2	square millimeter
mRNA	messenger RNA
MS	maxillary sinus
MT	maxilloturbinate
MuHV1	Murid herpesvirus 1
MULT-1	murine UL-16 binding protein-like transcript −1
N	nasoturbinate
Na	naris
NA	Neutralizing assay
NALT	nasal associated lymphoid
ND	not determined
NEAA	non-essential amino acids
NH_4Cl	Ammonium chloride
NK	natural killer
NKG2D	natural-killer group 2 member D
nm	nanometre
NP	nasopharynx
NPM	nasopharyngeal meatus
OB	olfactory bulb
OE	olfactory epithelium
OMP	olfactory neuron marker
PAMP	pathogen-associated molecular pattern
PBLs	peripheral blood leukocytes

PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCR	polymerase chain reaction
pDCs	plasmocytoid DCs
PFU	plaque-forming unit
pα-MCMV Abs	biotinylated polyclonal anti-MCMV antibodies
qPCR	quantitative PCR
RAE-1	retinoic acid early inducible cDNA clone-1
RE	respiratory epithelium
RPMI	Roswell Park Memorial Institute
RT	room temperature
S	septum
SE	squamous epithelium
SG MCMV	salivary gland-derived MCMV
sgg 1	salivary gland gene 1
SuHV-2	Suid herpesvirus 2
TC MCMV	tissue culture MCMV
$TCID_{50}$	50% tissue culture infective dose
TE	transitional epithelium
TLR	toll-like receptor
TNF	Tumor necrosis factor
TRL	long terminal repeat
TRS	short terminal repeat
UV	ultraviolet
VM	ventral meatus
VO	vomeronasal organ
µl	microliter
µm	micrometre
µM	micromolar

Chapter 1

Introduction of Murine Cytomegalovirus

1.1 History and classification

Earlier researchers demonstrated that intranuclear inclusion bodies in the salivary glands of mice could be used to infect healthy mice [1]. The intranuclear inclusions were later identified as Murine cytomegalovirus (MCMV). MCMV, also known as Murid herpesvirus 1, was first isolated in mouse tissue culture in 1954 by Margaret Smith from the salivary gland of infected laboratory mice [2]. The term cytomegalovirus was introduced by Weller and colleagues [3].

According to their biological properties and genome content and organization, the *Herpesviridae* family (*Herpesvirales* order) is subdivided into three distinct subfamilies: the *Alphaherpesvirinae*, the *Betaherpesvirinae*, and the *Gammaherpesvirinae* (Table 1) [4, 5]. *Alphaherpesvirinae* are characterized by a variable host range, rapid spread in culture, relatively short replication cycle, efficient destruction of infected cells by cell lysis, and ability to establish latent infections typically in sensory neurons [4, 6]. This subfamily consists of four genera (*Simplexvirus*, *Varicellovirus*, *Mardivirus* and *Iltovirus*) and unassigned species. *Betaherpesvirinae* have a restricted host range and a long reproductive cycle that is reflected by the slow infection progress in cultures [4, 6]. Typically, infected cells enlarge (cytomegalia). Latent infection can be established in various distinct tissues/cell types such as secretory glands, kidneys, spleen, lymphoreticular cells. The subfamily *Betaherpesvirinae* consists of four genera (*Cytomegalovirus*, *Muromegalovirus*, *Roseolovirus* and *Proboscivirus*) and unassigned species. MCMV and Murid herpesvirus 2 (rat cytomegalovirus), together with the cytomegaloviruses of human, guinea pig and other species, belong to the *Betaherpesvirinae* [5]. *Gammaherpesvirinae* prefer to enter the latency program instead of leading to a productive replication [4, 6]. This subfamily consists of four genera (*Lymphocryptovirus, Rhadinovirus, Macavirus* and *Percavirus*) and unassigned species.

Despite their differences at the level of genome composition, host range and duration of the productive cycle, *Herpesviridae* share four significant biological properties: (1)

they intrinsically encode for a variety of enzymes involved in the process of nucleic acid metabolism, DNA synthesis and protein processing, (2) viral DNA synthesis and capsid assembly occur in the nucleus, whereas final processing of the virion takes place in the cytoplasm, (3) productive infection leads to cell destruction, (4) the herpesviruses can establish latency in their natural hosts [4].

Table 1. Taxonomy classification of the family *Herpesviridae* of the order *Herpesvirales.*

Subfamily	Genus	Representative virus	
		Formal name (acronyms)	Common name
Alphaherpesvirinae	Simplexvirus	*Human herpesvirus 1* (HHV-1)	Herpes simplex virus type 1
	Varicellovirus	*Human herpesvirus 3* (HHV-3)	Varicella-zoster virus
	Mardivious	*Gallid herpesvirus 2* (GaHV-2)	Marek's disease virus type 1
	Iltovirus	*Gallid herpesvirus 1* (GaHV-1)	Infectious laryngotracheitis virus
	Unassigned species	*Chelonid herpesvirus 6* (ChHV-6)	Lung-eye-trachea disease-associated virus
Betaherpesvirinae	Cytomegalovirus	*Human herpesvirus 5* (HHV5)	Human cytomegalovirus
	Muromegalovirus	*Murid herpesvirus 1* (MuHV1)	Mouse cytomegalovirus
	Roseolovirus	*Human herpesvirus 6* (HHV-6)	Human herpesvirus 6
	Proboscivirus	*Elephantid herpesvirus 1* (ElHV-1)	Elephant endotheliotropic herpesvirus
	Unassigned species	*Suid herpesvirus 2* (SuHV-2)	Pig cytomegalovirus
Gammaherpesvirinae	Lymphocryptovirus	*Human herpesvirus 4* (HHV-4)	Epstein-Barr virus
	Rhadinovirus	*Human herpesvirus 8* (HHV-8)	Kaposi's sarcoma-associated herpesvirus
	Macavirus	*Alcelaphine herpesvirus 1* (AlHV-1)	Malignant catarrhal fever virus
	Percavirus	*Equid herpesvirus 2* (EHV-2)	Equine herpesvirus 2
	Unassigned species	*Equid herpesvirus 7* (EHV-7)	Asinine herpesvirus 2

1.2 Virus characteristics

1.2.1 Structure of the cytomegalovirus

A mature MCMV virion consists of four morphologically distinct elements: the core, capsid, tegument, and envelope (Fig. 1). The core includes the double stranded DNA genome, which is packaged into the capsid. The tegument is a poorly defined layer of proteinaceous material between the capsid and envelope. The envelope is a lipid bilayer containing a number of different integral viral (glyco)proteins [5].

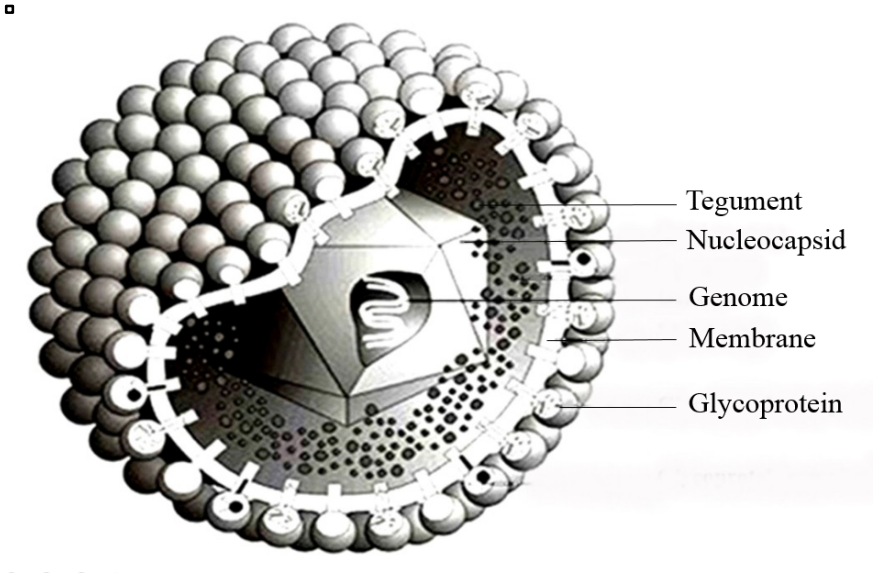

Figure 1. A cartoon depicting the structure of the cytomegalovirus virion. The double-stranded DNA is contained within a polyhedral viral capsid. The viral capsid is surrounded by the tegument. The membranous envelope surrounds the tegument. (Image adapted from (http://www.virology.net/big_virology/ bvdnaherpes.html website) with courtesy of Dr. Marko Reschke in Marburg, Germany).

1.2.2 Genome organization

Mature fully assembled MCMV is about 230 nm in size. The DNA molecule of the MCMV genome is a linear double strand of approximately 230 kbp. The genome of the Smith strain has an overall G+C content of 58.7% and consists of 230,278 bp [7], which is similar in size to the genomes of HCMV [8]. In total, the MCMV genome has 204 open reading frames based on comparative genomics and new predicting algorithms [7, 9]. Unlike HCMV, MCMV does not contain large internal repeats but is arranged as a single unique sequence bounded by short (31 bp) terminal direct

repeats not represented elsewhere in the genome (Fig. 2) [7].

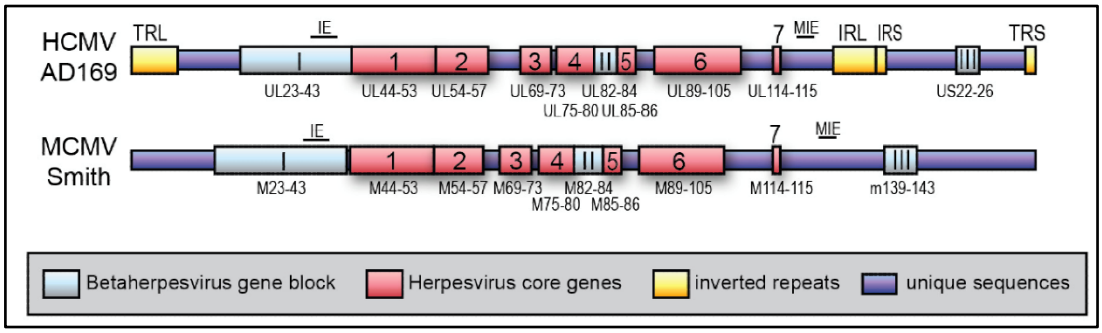

Figure 2. Comparison of HCMV and MCMV genome structures. MIE: major immediate-early genes; IE: Immediate-early genes; TRL: long terminal repeat; IRL: long internal repeat; IRS: short internal repeat; TRS: short terminal repeat. (Image obtained from Vanda Juranić Lisnić's Ph.D dissertation, 2013 [10])

1.2.3 Structural proteins

MCMV contains three kinds of structural proteins: capsid, tegument and envelope proteins. The capsid encloses the viral genome, consists of 162 capsomeres and exhibits icosahedral symmetry. Information on MCMV capsid proteins is mostly coming from their homology with the proteins of HCMV. The capsid of MCMV is composed of seven proteins: the major capsid protein (MCP) (encoded by M86), the minor capsid protein (encoded by M85), the minor capsid binding protein (encoded by M46), the smallest capsid protein (encoded by M48.5) and three distinct assembly-related proteins (encoded by M80, M80a and M80.5) [11]. The MCP is the most strongly conserved protein among herpesviruses [11].

The tegument of MCMV has an ordered structure, particularly proximal to the capsid as demonstrated by electron microscopy. Five genes encoding the tegument proteins of MCMV have homologues in HCMV: M32 (pp150), M48 (large tegument protein), M83 (pp65), M99, and M82 [11]. Nevertheless, the individual tegument proteins are conserved in the *Betaherpesvirinae* but are not shared with members of *Alpha*- and *Gammaherpesvirinae*.

The envelope of MCMV is a lipid bilayer derived from cellular membranes obtained during virion maturation and egress, and is spiked with virally encoded glycoproteins.

The major glycoprotein is the glycoprotein B (gB), which is one of the most highly conserved herpesvirus proteins and the target of many neutralizing antibodies [12]. MCMV gB has 45% similarity in amino acid sequence with HCMV gB [13]. Additionally, MCMV forms various gH/gL virion envelope glycoprotein complexes that serve as entry mediators and determinants of cell tropism *in vitro*. *In vivo*, the gH/gL/gO complex (encoded by M115, M75 and m74, respectively) is critical for establishing infection by efficient entry into diverse cell types, such as liver macrophages, endothelial cells and hepatocytes. Nonetheless, it is dispensable for intra-tissue spread [14]. The alternative complex gH/gL/MCK-2 (encoded by M115, M75 and m131-129, respectively) is required for intra-tissue virus spread only in the absence of the gH/gL/gO complex [14]. *In vivo* infection was abolished at all sites when both complexes were lacking gO and MCK-2 in double-knockout virus [14]. MCK-2 determines MCMV pathogenicity by involving a mechanism for virions to hijack CX3CR1^{hi} patrolling monocytes to serve as vehicles [15, 16].

1.3 Replication cycle

An overview of the CMV replication cycle is described in Figure 3.

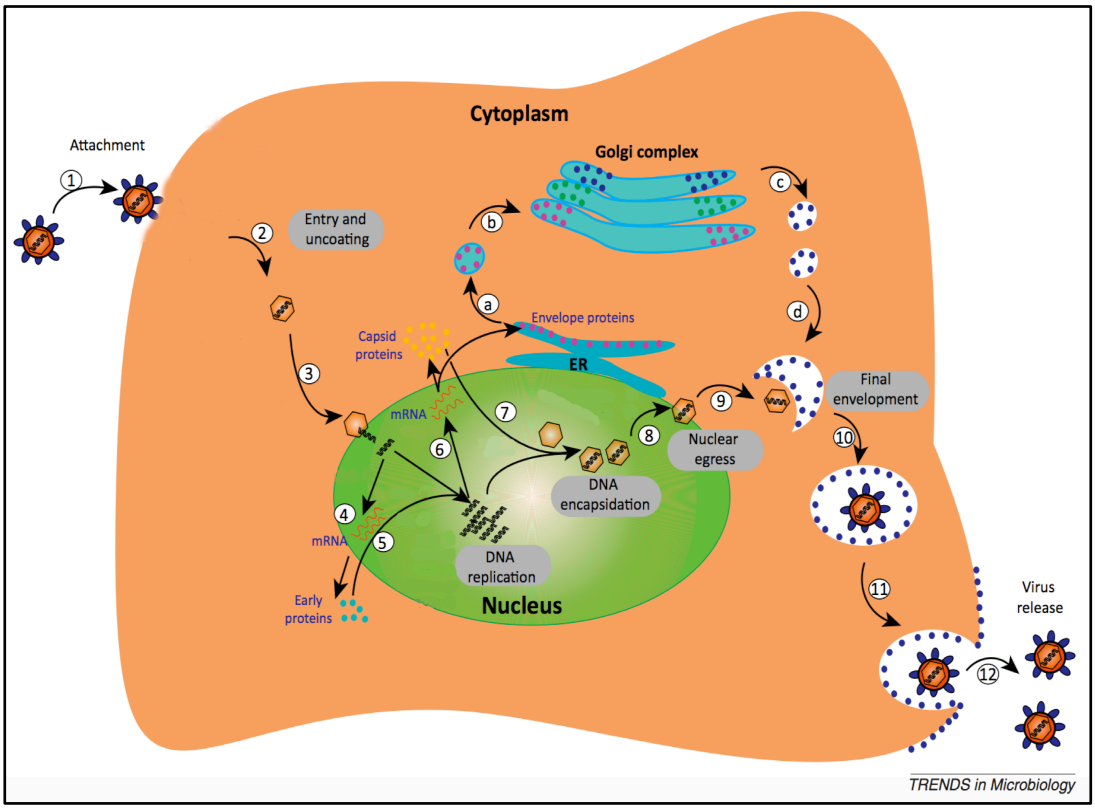

Figure 3. Productive replication cycle of cytomegalovirus. (1) Viral glycoproteins bind on the cell membrane of host cells. (2) The viral envelope fuses with the cell membrane and releases the nucleocapsid into the cytoplasm. (3) The nucleocapsid associates with cytoskeletal elements and translocates to the nucleus where the nucleocapsid interacts with nuclear pores and releases the viral genome into the nucleus. (4) The viral DNA is transcribed into early mRNAs which are then transported to the cytoplasm for early protein synthesis. (5) These early proteins are imported into the nucleus and promote viral DNA replication. (6) The viral DNA is then transcribed into the late mRNAs which are responsible for late viral protein synthesis (capsid and envelope proteins). (a–d) The viral envelope proteins (pink, green, and blue dots) are processed in the endoplasmic reticulum (ER) and Golgi complex. (7) The capsid proteins are imported into the nucleus and then encapsidate the newly replicated genomes. (8–10) These capsids egress from the nucleus and bud into the exocytotic vesicles. (11) The virion containing vesicles migrate to and fuse with the cell membrane. (12) Infectious viruses are then released from the cell. (Image adapted from reference [17]).

1.3.1 Entry

The replication cycle of MCMV is initiated with the binding of the viruses to cellular receptors on permissive cells. It is very well possible that more than one receptor is involved. Epidermal growth factor receptor (EGFR) was considered to be an HCMV receptor [18, 19], but later a study directly demonstrated no discernible role for EGFR in virus entry or signaling in a range of cell types containing fibroblasts, epithelial cells and endothelial cells [20]. Heparan sulfate proteoglycans contribute to MCMV binding and entry [21], and also to the binding of HCMV to susceptible cells [22]. In addition, MHC class I [23] and beta-2-microglobulin [24] have been shown to facilitate the binding of MCMV *in vitro*. After the process of membrane fusion or endocytosis and fusion, viral nucleocapsids enter the cytoplasm and DNA is transported through nuclear pores to the nucleus [25].

1.3.2 Replication and transcription

Once the genome enters the nucleus through the nuclear pore, it circularizes. Next, transcription is initiated and gene expression occurs in a cascade manner. All betaherpesviruses [26] including MCMV [27] have three gene families: α, β and γ, which are temporally regulated. The first genes that are transcribed are designated immediate early (IE) or α genes and are involved in the regulation of transcription and translation of early proteins. They do not require *de novo* protein synthesis but are controlled by the major IE promoter (MIEP) [28]. In MCMV, the MIEP controls expression of the IE1 (m123) and IE3 (M122) genes [29, 30]. MCMV IE1 (pp89) and IE3 are transcriptional activators [31]. The IE3 gene is essential for MCMV replication [32]. In contrast, the IE2 gene is not essential for MCMV replication either *in vitro* or *in vivo* [33]. After IE genes, early (E) or β genes are expressed, which requires the IE gene expression for regulation [34]. E genes are mostly involved in virus DNA replication [35]. Finally, last expressed are the late (L) or γ genes that encode proteins needed for virus assembly and egress, and their transcription requires proteins synthesized during the E phase of replication [11]. L phase genes mostly encode structural proteins.

1.3.3 Assembly and release

The formation of capsids and the packaging of viral DNA occurs in the nucleus of infected cells [36]. Capsid proteins that are produced in the cytoplasm are transported back to the nucleus across the nuclear membrane. Viral DNA is packaged into complete capsids and transported to the cytoplasm via the nuclear membrane, where the capsids acquire their primary envelope as they bud through the inner nuclear membrane. There are two herpesvirus genes (UL31 and UL34) that are involved in this process. Similar homologues (M53/p38 and M50/p35) are also identified in MCMV [37-39]. M50/M53 recruit cellular kinases like protein kinase C to degrade the inner nuclear membrane, especially the nuclear lamina that is a filamentous network that prevents budding [37]. Cellular kinases lead to phosphorylation and degradation of the lamina and increases egress of the virus from the nucleus.

The mechanism of egress of herpesviruses from infected cells remains incompletely understood. The envelope and tegument of perinuclear virions differ from those of mature virions [40, 41]. The major envelope glycoprotein complex (gp52/105/150) of MCMV have been characterized [42], which is encoded by the gB gene [13, 43]. A different ultrastructure has been observed between primary enveloped virions and mature virions, indicating a secondary envelopment. Nevertheless, the mechanism by which the virus loses the primary envelope and tegument and acquires the mature tegument and envelope is still not fully known. Recent studies with α- and β-herpesviruses suggest that the primary enveloped virus fuses with the outer nuclear membrane, there by losing its primary envelope and reordering the cytoplasm as a naked capsid [44]. Afterwards, the virus possibly migrates to the trans Golgi network, where the virions gain their tegument and final envelope [44, 45]. The mature enveloped virions are now present in secretory vesicles that are transported to the plasma membrane, where they are released into the extracellular space by exocytosis.

The formation of multicapsid virions, which appears to be unique to MCMV infection, is also not well understood [11]. It has been postulated that they are formed when capsids exit the nucleus via nuclear pores and form cytoplasmic aggregates of capsids

[46]. These capsids then receive an envelope via budding into extended cytoplasmic vacuoles derived from the Golgi apparatus. The multicapsid virions are also believed to be released from the cell by exocytosis [46].

1.4 Epizootiology

1.4.1 Natural history

In order to understand the genetics and the evolution of MCMV it is useful to have an understanding of the origins of the commonly used MCMV strains and the genetic backgrounds of the mice from which these strains were isolated. The natural host for MCMV is considered to be the *Mus musculus domesticus* (*musculus domesticus*), since to date the majority of low passage strains of MCMV were derived from these mice [47]. So far, there are 12 individual strains of MCMV, whose genomes have been completely sequenced (Table 2). Two laboratory strains of MCMV (Smith and K181) are in widespread use. The Smith strain was isolated in 1954 by Margaret Smith from the salivary glands of an infected laboratory mouse [2]. The K181 strain of MCMV was isolated in the 1970s by June Osborne [47]. Recently, comparing the Smith derived clone of MCMV, WT1 [48], with the sequence of Smith [7] and K181 [49] suggest that the early stocks of Smith were a mix of both Smith and K181 strains.

Table 2. Twelve fully sequenced MCMV strains

Strain	Location of isolation	Genome size (bp)	Genbank No.	Year	Ref.
Smith	St. Louis, USA	230,278	NC_004065	1954	[50]
K181	Wisconsin, USA	230,301	AM886412	1976	[51]
WT1[a]	USA	230,408	GU305914	2010	[48]
N1	Nannup, Australia	229,884	HE610454	1993	[52]
G4	Geraldton, Australia	230,227	EU579859	1993	[52]
C4A	Canberra, Australia	230,111	EU579861	2006	[53]
C4B	Canberra, Australia	230,154	HE610452	2006	[53]
C4C	Canberra, Australia	229,924	HE610453	2006	[53]
C4D	Canberra, Australia	229,935	HE610456	2006	[53]
WP15B	Walpeup, Australia	230,118	EU579860	2008	[54]
AA18D	Macquarie Island, Australia	229,543	HE610451	2013	[55]
N07	Beacon Island, Australia	229,452	HE610455	2013	[55]

[a] : WT1 is a variant of Smith strain MCMV, possibly a recombinant of Smith and K181 strains.

1.4.2 Host range

Cytomegaloviruses have a strict species-specificity. Indeed, MCMV only infects mice, rat CMV only infects rat, hamster CMV only infects hamster, HCMV only infects human [4, 56].

1.4.3 Modes of Transmission

Horizontal transmission - Salivary glands are believed to be the important sites for horizontal transmission, since the virus can be shed in saliva of both mice and human for a long time period [57, 58]. Three different salivary glands exist: submandibular glands, parotid glands and sublingual glands. Each gland has a single excretory duct terminating in the oral cavity. All three glands can be infected by MCMV. Nonetheless, virus infection appears to be more pronounced in the submandibular glands, followed by the sublingual and parotid glands [59]. The acinar epithelial cells are the major site of MCMV replication in the salivary gland, from which the virus is

shed into ducts leading to the mouth [60]. There are at least two viral genes m133 (sgg1) and m131/129 (MCK-2) determining the salivary gland tropism of MCMV. The sgg1 seems to specifically control the growth of MCMV in acinar cells [61]. In contrast, MCK-2 encodes a CC chemokine to facilitate the hijacking of patrolling monocytes to serve as vehicles transporting MCMV to salivary glands [16]. Additionally, M33 and M43 genes are also required for MCMV replication within the salivary glands [62, 63]. Besides saliva, infectious virus can be detected in tears, milk, urine and sperm [64-66], which are also sources for horizontal transmission.

Vertical transmission - Congenital HCMV infection affects 0.2-2.5% of the newborn infants [67]. While the majority of congenital HCMV infections are asymptomatic, it has been estimated that 5 to 10% of congenitally HCMV infected neonates exhibit symptomatic, generalized cytomegalic inclusion disease [68]. Typical clinical features of cytomegalic inclusion disease include petechiae, hepatosplenomegaly, jaundice and microcephaly [67]. Additional manifestations at birth are intrauterine growth retardation, prematurity, chorioretinitis, and central nervous system diseases such as intracranial calcifications, ventriculomegaly, lissencephaly, pachygyria, dysmyelination, paraventricular cysts and calcifications [69, 70]. Because CMV exhibits strict species-specificity, it has not been possible to use HCMV to create heterologous experimental animal models of congenital HCMV infection [71]. Vertical transmission of CMV can be induced in guinea pigs [72] and rhesus macaque monkey [73], however not yet in mice. This might be due to different anatomical features of the mouse placenta [74]. The anatomy of the mouse placenta differs in a number of aspects from that found in other mammalian orders. In particular, the structure of the trophoblastic layers of the murine chorioallantoic placenta is exceptional in that maternal blood is separated from fetal blood by three (hemotrichorial) trophoblast layers, a basement membrane, and a layer of fetal vascular endothelial cells. In contrast, there is only one trophoblast (monochorial) layer in other mammalian orders [74]. Possibly, the two extra layers of trophoblastic cells between the maternal and fetal blood in mice could provide a physical barrier

that may serve to limit the vertical transfer of MCMV from the mother to the conceptus. Other possible explanations may be the inoculation dose, the age of the embryo and the timing of infection. Overall, further studies will be needed to address the MCMV intrauterine infection in mice.

1.5 Pathogenesis

1.5.1 Genetic control of host resistance or susceptibility to MCMV

Inbred mouse strains have a different susceptibility to MCMV infection. In order to find the basis for susceptibility and resistance to MCMV infection, genes related to the control of MCMV replication were compared by Grundy *et al.* and Chalmer *et al.* [75, 76]. Their data revealed that both H-2 associated and non-H-2 associated genes determine resistance to the lethal effects of MCMV, as discussed below.

H-2 associated genes - First, CBA and C3H mice having H-2^k haplotype were found more resistant to lethal MCMV infection than BALB/c or C57BL mice after intraperitoneal inoculation with MCMV [75, 76]. Secondly, using BALB/c congenic strains carrying H-2^b, H-2^g, or H-2^k haplotypes rather than the H-2^d haplotype of BALB/C mice, resistance was found to be associated with H-2^k [76]. Finally, in a more detailed study using LD_{50} analysis, it was shown that the H-2^k haplotype rendered BALB/c mice 10.2 times more resistant to MCMV than H-2^d BALB/c [75]. Taken together, the H-2^k haplotype plays a critical role in host resistance to MCMV infection.

Non-H-2 associated genes - Comparison between two mice strains bearing the $H2^b$ haplotype (BALB.B and C57BL/10) showed that C57BL/10 mice were more resistant to MCMV than BALB.B mice, which showed that besides H-2 associated genes, non-H-2 associated genes were responsible for resistance in C57BL/10 mice [75]. Later, it was found that NK cells play important roles in the genetically controlled resistance, since the NK cell response was activating within 24 hours after MCMV infection in genetically resistant strains but not in susceptible strains [77]. In addition, MCMV titers were higher in beige mutant C57BL/6 and other strains in which NK

cell activity is abrogated [78, 79]. The ability of mouse strains to trigger an effective NK cell response is controlled by the *Cmv I* locus, positioned inside the NK gene complex on mouse chromosome 6. The alleles of the *Cmv I* locus can either confer susceptibility (*Cmv I*^{s}, a recessive allele) or resistance (*Cmv I*^{r}, a dominant allele) to MCMV [80]. The latter allelic form restricts early MCMV replication in the spleen of resistant strains [81, 82]. The Ly49h gene has been identified as the gene that mediates the *Cmv I* phenotype [83, 84]. Ly49h binds specifically to a protein encoded by the MCMV m157 gene, which has a structural homology to MHC class I. Recognition of viral m157 protein by NK-cell receptor Ly49H will lead to destruction of infected cells by release of NK cell perforin [80, 85, 86].

1.5.2 Effect of inoculation dose and route

Dose and route of inoculation significantly influence the outcome of MCMV infection. With a fourfold increase of the inoculum titer, the mortality increases from 0 to 100% in susceptible animals [87]. So far most researchers have used intraperitoneal or intravenous virus inoculation, which results in a rapid spread to the liver and spleen, after which the virus is disseminated to other organs such as submandibular glands [88, 89]. Intrafootpad (i.f.) inoculation leads to a lymph dissemination towards the draining lymph nodes [90]. The oronasal inoculation is considered as the best way to mimic natural infection. Unfortunately, there have only been a few studies on intranasal inoculation and all mice were under anesthesia/sedation during inoculation [91-95]. As the inoculum also reaches the lungs in anesthetized/sedated mice due to the absence of the swallowing/sneezing reflex, this inoculation cannot be accepted as a natural way of infection, and hence the primary replication site of the virus upon oronasal inoculation remains elusive.

1.5.3 Systemic dissemination of MCMV by patrolling monocyte-associated viremia

Cell free CMV has never been detected in blood of infected humans [96, 97] and mice [98], regardless of their immune status. The virus in blood is fully cell-associated, and

these infected peripheral blood leukocytes (PBLs) may cause transfusion-transmitted CMV infection both in humans [99, 100] and mouse [98, 101]. Mocarski and his team [16] recently demonstrated that patrolling monocytes (PM) carry MCMV during the dissemination stage of infection [16]. Afterwards, transmission occurs in a cell-associated way in between monocytic cells (direct transfer via viral synapses or fusion in internal lymphoid organs such as spleen), and then to tertiary shedding sites, such as the salivary glands [90]. Although the liver can also be infected, MCMV produced in the hepatocytes does neither contribute significantly to secondary viremia nor to the dissemination of the infection to other organs [102].

1.5.4 Virus distribution and replication in parenchymal organs

In models for systemic MCMV infection (i.e. after intraperitoneal inoculation), multiple organs have been reported to become infected, such as salivary glands, lungs, spleen, liver, kidneys and central nervous system.

Salivary glands - Virus replication is localized in the serous acinar cells of submandibular, parotid and sublingual glands [103, 104]. The intranuclear development of the virus is typical for herpesviruses; in contrast the cytoplasmic inclusions are different. They are composed of vesicles that sometimes contain more than 100 enveloped virions. These vesicles fuse with the plasma membrane and liberate the virions into the lumen, from which they are presumably transported along the ducts into the oral cavity as part of the salivary secretions [105]. The infected cells do not appear to be damaged. It looks like the virus simply utilizes the cell's normal secretory mechanism as a means of exit. The unusual cytoplasmic development of the virus explains why multicapsid virions are not present in these tissues, since each virion matures and leaves the cell as an individual entity, in contrast to the situation in other tissues [105]. Epithelial cells are identified as the main susceptible cell type in salivary glands [106].

Lungs - Pneumonitis is easily established after intranasal inoculation of anesthetized mice [107]. Afterwards, virus spreads to other organs via blood, while the virus in the

lungs becomes cleared [108]. Type 2 alveolar epithelial cells are highly susceptible to MCMV; additionally stromal cells such as fibroblasts can also be infected [107, 109, 110]. Alveolar macrophages can phagocytose virus or debris of infected cells, but they are not productively infected [110].

Spleen - The spleen is commonly involved in MCMV infection. In recent studies, the susceptible target cells were identified as macrophages and dendritic cells [103, 104, 111, 112], sinus-lining cells and reticular fibroblasts [88, 113], endothelial cells and hematopoietic cells [109, 114]. The reticular fibroblasts were identified as a reservoir for later reactivation [113].

Other lymphoid tissues - Several lymph nodes are also involved in the course of MCMV infection. It is interesting that various cells are found to be susceptible to the virus, such as subcapsular sinus macrophages in the mediastinal lymph nodes [88], medullary sinus macrophages and reticular fibroblasts in popliteal lymph nodes [90]. Subcapsular sinus macrophages take up the virus and are important in controlling its dissemination [90].

Liver - The liver is one of the first tissues infected after intraperitoneal inoculation. Hepatocytes [88, 102], hepatic endothelial cells [102, 109] and to some extent Kupffer cells [105] are permissive for virus replication. Nuclear inclusions represent the sites of assembly of nucleocapsids, while the cytoplasmic bodies are clusters of virions embedded in or associated with a densely staining matrix [105]. Nevertheless, the significance of the dense bodies is unknown, but their presence does not correlate with destruction of virus.

Kidneys - MCMV replicates in the kidneys during infection, resulting in shedding of MCMV in the urine [115]. In the kidneys, the endothelial cells [109], glomerular cells and renal tubules cells [103, 109] are susceptible cells.

Central nervous system - Most of the work reported on central nervous system involvement has used intracranial or intraorbital inoculation. A generalized encephalitis occurs with considerable necrosis and inflammatory response. Several

types of cells including neurons, macrophages and glial cells, can be infected and show typical nuclear and cytoplasmic inclusions [105].

1.5.5 Symptoms in immunocompetent, immunocompromised and immature mice

Symptoms in immunocompetent mice - Intraperitoneal or footpad injection of adult immunocompetent mice with 10^5 PFU tissue culture MCMV (TC MCMV) is usually asymptomatic and it is not associated with serious damage to organ systems [57, 90]. This is consistent with the fact that very little infectious virus can be detected in tissues of infected mice, with the exception of salivary glands, which support prolonged virus replication. On the other hand, injection of a similar dose of salivary gland-derived MCMV (SG MCMV) results in damage to multiple organs and tissues and high mortality even in fully immunocompetent hosts, which might in part be due to cytokines accounting for its virulent manifestations [57]. The primary features of an acute infection with SG MCMV include high levels of virus replication in the liver and spleen, with loss of liver function [116] and immunosuppression [117]. The extent of damage to the spleen and suppression of immune responses appears to be closely linked with the degree of liver dysfunction. This tissue damage ultimately leads to the death of infected mice [80].

Symptoms in immunocompromised mice - CMVs in general cause asymptomatic infections in immunocompetent hosts but are important opportunistic viruses affecting immunodeficient hosts. For example, HCMV infection is harmful to AIDS patients and causes variable clinical manifestations including pneumonitis, hepatitis, retinitis, cardiovascular disease and adrenalitis [11, 118]. Similarly, infection of immunocompromised mice with TC MCMV is associated with a high level of virus replication and multiple organ damage, resulting in high morbidity and mortality. Interstitial pneumonia can be established in γ-irradiated mice [119] or in adult Balb/c mice intranasally inoculated with $8x10^4$ PFU of MCMV, followed by a single dose of cyclophosphamide [93]. MCMV-induced hepatitis in mice has been widely used as a model of hepatitis induced by HCMV in immunocompromised patients. Several studies have shown that severe hepatitis is caused by lethal doses of MCMV in

susceptible strains of mice via intraperitoneal or intravenous inoculation. [116, 120]. Retinitis can only be induced when the virus is administered intraocularly via the supraciliary route [121]. MCMV can also be used as a model of HCMV infection in immunocompromised patients causing other disease, such as cardiovascular disease [122] and adrenalitis [123].

Symptoms in immature mice - The clinical and histological outcome of an MCMV infection of neonatal mice represents well that of a congenital HCMV infection [124]. In contrast to HCMV, MCMV cannot be transmitted transplacentally but can be transmitted to newborn mice during birth or afterwards by breast-feeding and saliva [125]. Unlike adult mice acutely infected with MCMV, in which productive infection is stopped within several weeks, newborn mice that survive an MCMV infection have a long-lasting persistence in salivary glands and shed the virus in saliva for several months before establishment of latency [126]. Antigen positive cells were found in a wide range of tissues and organs, such as salivary glands, lungs, liver and heart muscle [125]. MCMV infection in newborn mice is also characterized by virus dissemination within the central nervous system [127].

1.5.6 MCMV infection in mice as a model for HCMV infection in humans

Due to the strict species-specificity of CMV, it is not possible to study HCMV in experimental animals. Therefore, it is necessary to set up animal models for the study of HCMV. Up till now, a number of CMV infections in various animal species have been utilized for modeling HCMV infection in humans. Among the animal CMVs, pathogenesis studies have been reported for rhesus macaque CMV, porcine CMV, guinea pig CMV, rat CMV and murine CMV [80, 128-131]. MCMV infections in mice is the most commonly and widely used animal model for HCMV study due to the following reasons: (1) MCMV shares many features with HCMV [71], (2) the genomes of mice and MCMV are fully sequenced [7, 132] and (3) the small size, cheap price, short life span, ease of handling and high reproductive rate of mice make them most suitable.

MCMV has now been successfully used to investigate many pathological facets of CMV infection, including humoral/cellular immunity, host responses to congenital CMV infection and infection in immunosuppressed hosts. Additionally, various deletion mutants were constructed by application of bacterial artificial chromosome (BAC) mutagenesis [133] depending on the accuracy of genomic maps, which have been used to identify and characterize a multitude of immune evasion genes and functions of specific genes.

1.6 The immune response to MCMV infection

Immune responses in mice infected with MCMV involve both the innate and adaptive branches of the immune system. To establish persistent infection, MCMV has evolved mechanisms to modulate immune responses. In this section, a brief review on innate/adaptive immune responses and immune evasion are given.

1.6.1 Innate immunity

Innate immunity is the first and most ancient line of defense. It is immediate, but also non-specific. Indeed, innate effector cells, such as myeloid cells (dendritic cells (DCs) and monocytes/macrophages), and natural killer cells, recognize structurally conserved, self or non-self molecular patterns with the help of their germ-line encoded receptors.

Myeloid cells **-** Myeloid cells such as DCs and monocytes/macrophages are the primary sensors of MCMV infection via a number of innate immune receptors.

DCs are dispersed throughout the body and are mainly known for their professional antigen-presentation capacities [134]. DCs are distributed in lymphoid organs as well as peripheral tissues like skin, lungs, liver, kidneys, and intestinal tract. Immature DCs have a great capacity for endocytosis and antigen degradation, but express low levels of MHC-I, MHC-II, and co-stimulatory molecules [135]. Therefore, they are poor antigen-presenting cells. DC activation is mainly mediated by toll-like receptor (TLR) ligation and proinflammatory cytokines such as TNF-α and IL-1β [136]. These signals, together with the engagement of the CD40 receptor, stimulate DC maturation

and migration to secondary lymphoid organs. In contrast to the immature DCs, mature DCs are less endocytic, but become more potent in antigen presentation to T-lymphocytes. Besides that, mature DCs enables the initiation of adaptive immunity, and secretion of IFN-α/β [137]. DCs produce proinflammatory cytokines (IL-2, IL-12, IL-15, IL-18) needed for optimal NK-cell activity and for the regulation of adaptive immune responses [136]. CMVs can infect DCs and impair their functions such as antigen uptake and degradation, as well as maturation and migration [138, 139]. Infections also reduce the expression of MHC-I, co-stimulatory molecules and cytokine secretion [140].

Monocytes and tissue macrophages play a pivotal role in the pathogenesis of MCMV infection [141]. Both HCMV and MCMV use monocytes for their dissemination throughout the body [142-144]. Monocytes are recruited to sites of inflammation upon sensing a chemokine gradient (CCL2, CCL7 and CCL12), molecules whose expression are stimulated by infection. Once migrating inside tissues, monocytes differentiate into macrophages or DCs. Macrophages produce inflammatory cytokines, possess a great ability for pathogen recognition, and play a role in antigen presentation. They can identify foreign antigens via TLRs and other innate receptors. In addition, macrophages are also important mediators of innate and adaptive immune responses by their secretion of proinflammatory cytokine such as TNF-α, IL-1 and IL-12. When macrophages are infected with MCMV, their expression of MHC-II molecules decreases making them poor antigen-presenting cells [145].

The type I IFN response - Effective control of MCMV replication has long been associated with an increase in type I IFN secretion in the spleen, liver and serum during the first 48 hpi [146]. Type I IFNs can be produced by almost all nucleated cells; they belong to a large cytokine family, which includes type II IFN (IFN-γ) and type IIIs (IFN-λ1, IFN-λ2, IFN-λ3). Type I IFNs themselves include 16 members: IFN-β, IFN-ε, IFN-κ, IFN-ω and 12 subtypes of IFN-α. Thus far, virus-infected cells have been shown to preferentially produce IFN-α, IFN-β and IFN-ω [147]. Type I IFN secretion is biphasic in the infected animals. The first wave peaks at 8 hpi, while

the second does so at approximately 36 hpi. Each wave is associated with a particular cell type that recognizes the infection via specific receptors. The first wave of IFN-α/β secretion is initiated by lymphotoxin α and lymphotoxin β ligating with their cognate receptor, lymphotoxin β receptor (LTβR). Afterwards, the non-canonical NF-κB pathway is activated in the LTβR-bearing cell. Lymphotoxin α/β (LTα/β) expression is restricted to activated T-lymphocytes, NK cells, and a subset of follicular B-cells, whereas LTβR expression can be detected on a variety of cell types, including follicular DCs, high endothelial venules (HEVs), conventional DCs (cDCs), and macrophages [148]. Furthermore, it was demonstrated that the interaction between LTα/β$^+$ B-cells and LTβR$^+$ MCMV-infected stromal cells led to production of type I IFNs in the early hours following infection [149]. The second wave of IFN-α/β secretion mainly rests upon the recognition of viral pathogen-associated molecular pattern (PAMP) by TLRs expressed on plasmocytoid DCs (pDCs) [147]. These pDCs, also known as natural IFN-producing cells, are morphologically similar to plasma cells and secrete most of type I IFN following viral infection. The activated pDCs release IFN-α/β and IL-12. IFN-α/β increases the resistance of bystander cells to infection and stimulates NK-cell cytotoxicity, while IL-12 stimulates the secretion of IFN-γ by NK cells [147].

Natural killer cells - The crucial role of NK cells in controlling MCMV infection is best illustrated by the numerous evasion mechanisms devised by the virus to escape the NK-cell response (more details in chapter 1.6.3). Depletion of NK cell activity in adult mice using antibodies to asialo-GM1 or NK1.1 monoclonal antibodies rendered C5BL/6 mice susceptible to MCMV [81, 150, 151]. Infection of newborn mice with MCMV is usually lethal. However, the adoptive transfer of cloned NK cells or NK cell-enriched fractions from naive adults to neonatal mice prevented mortalities by reducing splenic viral titers [152-154]. Genetic susceptibility to infection by MCMV correlates with the inability to mount a sufficient NK cell response [77, 79]. NK cells mediate their function by the secretion of cytokine, the induction of target cell apoptosis via cell surface receptors, and the release of cytotoxic granules (granzymes

and perforin) [147].

1.6.2 Adaptive immunity

B cell-mediated immune responses - The primary roles of antibodies are to prevent infection and/or reduce the overall viral burden in the host. There are two types of antibodies detected during MCMV infection, namely neutralizing antibodies and non-neutralizing antibodies [155]. Serum IgM antibodies were detected in mice as early as 3-5 days, while IgG antibody was detected between 5-7 days, reaching peak levels at 20 days p.i. [156]. IgA antibodies were not detected in the sera of MCMV-infected mice [156]. Antibodies are not critical to control primary MCMV infection, since high antibody titers are detected with significant viral titers in organs [156]. However, they do play important roles in protection from infection. A number of early studies with MCMV using adoptive transfer of serum from infected mice showed obviously lower virus titers in organs following viral challenge [156, 157]. Later on, transfer of mAbs has been used to explore protection from subsequent infection. In 1990, several mAbs with highly divergent *in vitro* neutralizing activity have been used to demonstrate protection from a lethal challenge dose [158].

Antibodies are raised against a lot of viral proteins including structural and nonstructural components of the virion [159]. The major targets for induction of virus-neutralizing antibodies during natural infection have been identified: gB, gH, gL, gM, and gN [160].

T cell-mediated immune responses - Both $CD4^+$ and $CD8^+$ T cells play critical roles in the immune response to MCMV. The role of T cells in protective immunity was initially observed in T cell-deficient nude mice, which are very susceptible to MCMV [161, 162].

$CD4^+$ T lymphocytes are required for viral clearance in certain organs such as salivary glands [163, 164]. $CD4^+$ T cells can compensate for the absence of $CD8^+$ T cells. IFN-γ is required for $CD4^+$ T cell activity in the salivary glands [164]. The response to MCMV in the salivary glands is intriguing, it is likely that the cellular compartment

in salivary glands colonized by virus during early days post infection is under the control of $CD8^+$ T cells; nevertheless, the $CD4^+$ T cells become essential when the virus reaches the acinar glandular epithelial cells [165].

Adoptive transfer of the sensitized $CD8^+$ T cells plays pivotal roles in protecting mice against lethal disease, limiting MCMV dissemination and preventing tissue destruction [166, 167]. Cytotoxic T lymphocytes are crucial for the clearance of acute MCMV infection in Balb/c mice. However, $CD8^+$ T cell responses are not essential for the early control of MCMV replication in the presence of effective NK cell responses, such as those observed in genetically resistant C57BL/6 and CBA mice [168].

1.6.3 Immune evasion by MCMV

The most-studied immune evasion genes of MCMV are those that restrict NK cell responses. NK cell receptors may deliver either an activating or inhibitory signal into the cell. Several NK receptors bind MHC-I or MHC-like molecules (Fig. 4) [169, 170].

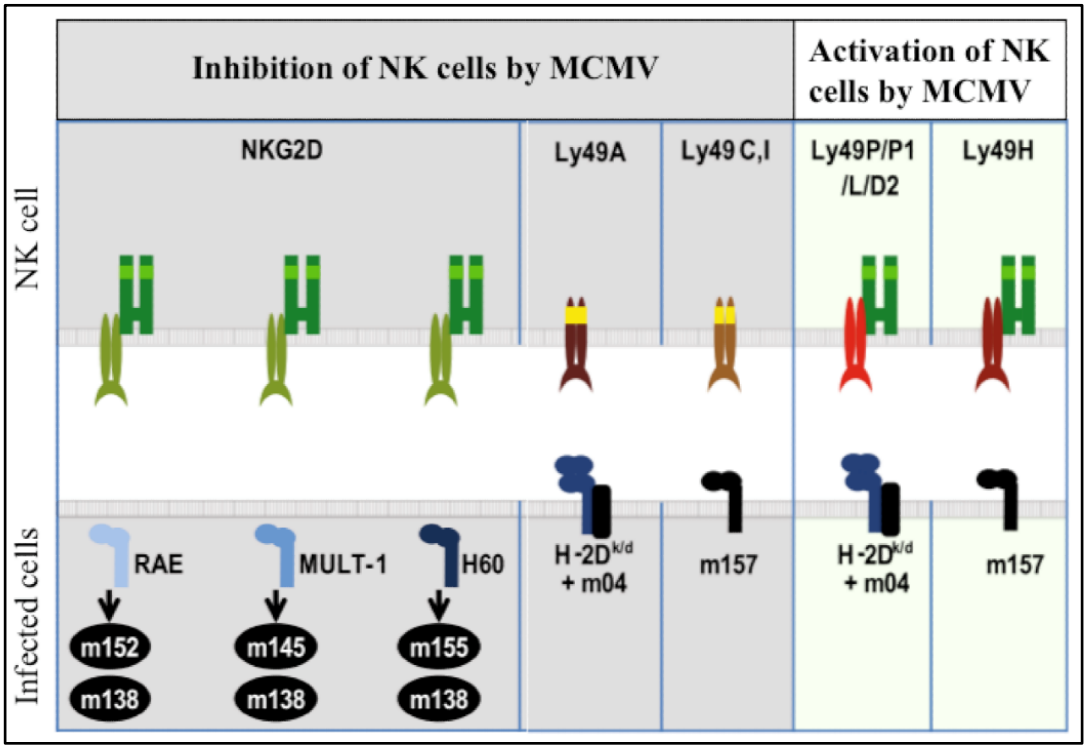

Figure 4. MCMV subverts NK cell receptors by different molecular mechanisms. Most of MCMV immune-evasion mechanisms are aimed to prevent surface expression of ligands for

activating NK cell receptors or include MHC I manipulation to avoid the 'missing self' recognition [165]. Activating receptor NKG2D is subverted by downregulation of ligands required for their engagement. In case of NKG2D, the downregulation is mediated by MCMV proteins that target either plasma membrane resident ligands (m138) or ligand maturation (m152). Inhibitory Ly49 receptors are subverted either by expression of MHC homologues (m157) or proteins mediating surface repopulation of MHC class I (m04). (Image adapted from reference [171])

Inhibitory Ly49 (A, C & I)-MHC-I axis - Inhibitory receptors bind MHC-I molecules expressed on virtually all cells, maintaining NK cells in the state of inhibition. The majority of NK cells express at least one of the inhibitory Ly49 receptors (Ly49A, Ly49C and Ly49I).

Modulation of MHC-I expression The virus can avoid cytotoxic cell detection by modulating the expression of MHC-I molecules on the surface of infected cells [172]. Nonetheless, the changes in MHC-I expression should be sensed by NK cells and in the case of down-regulation of MHC-I molecules should render the infected cells sensitive to NK-cell control via the 'missing self'-mediated mechanism, namely interactions between inhibitory Ly49 receptors and MHC-I molecules [173]. MCMV encodes three proteins (m04/gp34, m06/gp48 and m152/gp40) that tamper with the expression of MHC-I molecules. The m04/gp34 binding of MHC class I in the endoplasmic reticulum (ER) does not inhibit its expression on the cell surface, but escorts MHC-I molecules to the cell surface as a complex where gp34/MHC-I complexes may silence NK cells by serving as ligands for inhibitory Ly49 receptors [174]. The m06/gp48 protein interferes with the MHC class I pathway of antigen presentation by binding to complexes of MHC class I molecules and antigenic peptides, targeting them for degradation in the lysosome [175]. The reduced cell surface expression of these complexes results in reduced recognition and killing of these cells by $CD8^+$ T cells [174-176], but enhancing NK cells killing these cells via the missing-self mediated mechanism. The m152/gp40 prevents the transport of class I molecules to the cell surface, causing them to be retained in the ER-Golgi intermediate compartment (ERGIC) [175, 177]. Therefore, it leads to reduced recognition and killing of these cells by $CD8^+$T cells, but increasing the missing-self

mediated mechanism of NK cells to kill these infected cells.

Activating Ly49H/NKG2D receptor axis - Activating receptors such as NKG2D and Ly49H are well-studied. The other activating receptors are not discussed here.

Ly49H-m157 axis C57BL/6 mice have strong resistance to MCMV infection due to their expression of the activating receptor Ly49H on the surface of their NK cells [178, 179]. The product of the m157 gene is the exclusive ligand of Ly49H [180]. This direct ligation initiates an activating signaling cascade, resulting in clonal proliferation of $Ly49H^+$ NK cells, killing of infected cells, and secretion of cytokines (IFN-γ and TNF-α) [147]. Recently, m157 in particular has become the focus of much investigation. Sequence analysis of m157 derived from MCMV samples isolated from the wild showed that its sequence is highly variable. Intriguingly, the protein encoded by m157 has also been shown to serve as a ligand of the inhibitory Ly49I receptor.

Escape from NKG2D-mediated recognition The importance of NKG2D in the immune response against MCMV infection is best illustrated by the fact that MCMV has developed numerous immune evasion mechanisms against this receptor [181]. Indeed, four MCMV genes (m138, m152, m155, m145) encode proteins that downregulate the expression of ligands (MHC-I-like molecule RAE-1 [182, 183], MULT-1 [184] and H-60 [185]) of the NKG2D receptor [186].

The m138 protein has been shown to interfere with the recycling of the intracellular portion of MULT-1, resulting in its subsequent lysosomal degradation; H-60 surface expression is also downregulated by the gene product of m138 [187]. The m152 gene product gp40 inhibits NK cells by down-regulating the expression of the MHC-I-like molecule RAE-1. The m152/gp40 protein retains immature RAE-1 proteins in the endoplasmic reticulum-Golgi intermediate compartment *in vitro* [183, 188]. The products of m145 and m155 down-regulate the expression of MULT-1 and H-60, respectively, and deletion of these genes from MCMV enhances clearance of the virus due to NK cell-specific effects [183, 189-191].

In conclusion, understanding the role of immune evasion *in vivo* is complicated by a

number of factors. First, different inbred mouse strains have different Ly49 haplotypes, which leads to a high inter-mouse variation upon infection with the same virus strain. For example, the product of the m157 gene plays an important role in activating receptor Ly49H$^+$ on the surface of NK cells in C57BL/6 mice, but does not have the same effect in Balb/c mice since they are Ly49H$^-$. That is the reason why C57BL/6 mice are more resistant to MCMV infection than Balb/c mice. Second, certain viral genes exhibit dual functions. The m152/gp40 protein cannot only prevent the transport of MHC-I molecules to the cell surface but also downregulates the NK cell ligand RAE-I. Therefore, this viral gene has an inhibitory effect on both CD8$^+$ T cells and NK cells. Furthermore, new isolates of MCMV exhibit polymorphisms in a number of immune evasion genes. The MCMV G4 isolate encodes a variant of m157 that does not activate Ly49H [192]. Hence, Ly49H$^+$ mouse strains are as susceptible to G4 as the Ly49H$^-$ mouse strains. Given the complexity of MCMV immune evasion, knowing the genomes of both virus and mouse strain is of significant importance to understand the mechanism and impact of immune evasion by MCMV.

1.7 Anatomy of oral & nasal cavities and lymph nodes associated with alimentary & respiratory tracts in mice

In the present Ph.D thesis, oronasal inoculation without anesthesia will be used to mimic natural MCMV infection. In order to identify viral target cells in oral and nasal cavities, it is essential to have a full understanding of the anatomy of the oral and nasal cavities of mice. In addition, it is also necessary to know the anatomy of various lymph nodes associated with alimentary and respiratory tracts. They may be involved in virus transmission upon oronasal inoculation, since mucosal surface, mucosa-associated lymphoid tissues and mucosa-draining LN constitute the inductive sites for mucosal immunity against exogenous pathogens [193-195].

1.7.1 Anatomy of oral and nasal cavities of mice

The lips, teeth, oral mucosa, tongue and salivary glands consist of the basic components of the oral cavity, which all are engaged in the process of digestion (Fig.

5). The oral cavity contains the labial, palatine, buccal and gingival mucosa, which is lined by variably orthokeratinized squamous epithelium [196]. The thickness of the keratin layer varies with diet and frequency of ingestion. Remarkably, mice do not have tonsils in the oral cavity.

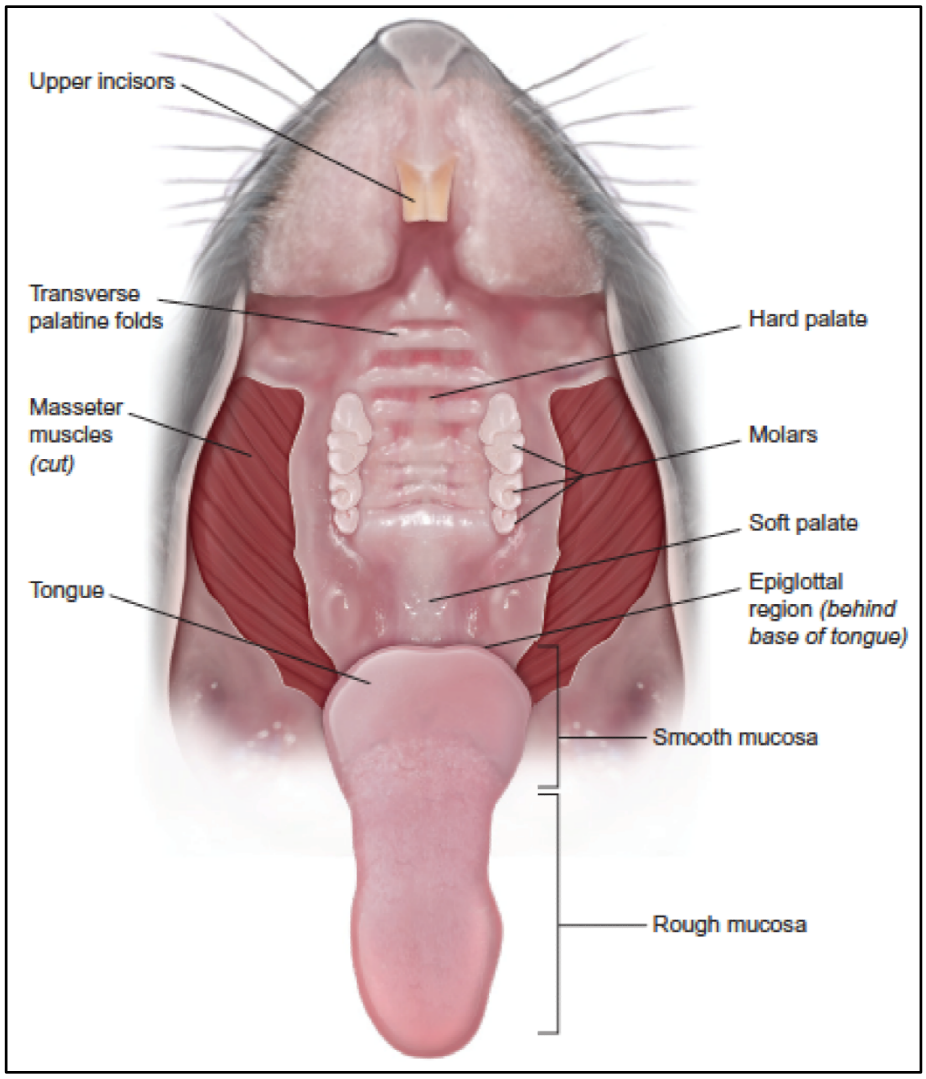

Figure 5. Mouse oral cavity. The hard palate extends from the incisors to the posterior of the third molars and has eight ridges. (Image obtained from reference [196])

The nose is the portal of entry for the respiratory system. In addition to serving as the sensory organ for smell (olfaction), the nose also functions as an air conditioner and a defender of the lower respiratory tract by humidifying, heating and filtering the inhaled air. Besides that, it protects the delicate gas-exchange regions of the lung by effectively absorbing water-soluble and reactive gases and vapors, trapping inhaled particles, and metabolizing airborne xenobiotics.

In mice, evolutionary pressure on olfactory function has defined the shape of the turbinate and the type and distribution of the cells lining these structures. In the proximal nasal airway, the complex naso- and maxilloturbinates of mice are thought to provide protection of the lower respiratory tract due to enhanced filtration, absorption, and disposal of airborne particles and gases. The highly complex shape of the ethmoid turbinate, lined predominantly by olfactory neuroepithelium, in the distal half of the nasal cavity of mice is suitably designed for acute olfaction [197]. The complexity of the gross turbinate structure throughout the nasal airway of the adult laboratory mouse is illustrated in Figure 6. There are four distinct nasal epithelium types in mice: 1) squamous epithelium (SE), which is primarily restricted to the nasal vestibule; 2) ciliated, pseudostratified, cuboidal/columnar epithelium, or respiratory epithelium (RE), in the main chamber and nasopharynx; 3) poorly ciliated cuboidal/columnar epithelium, often termed transitional epithelium (TE), lying between SE and RE in the proximal aspect of the main chamber; and (4) olfactory epithelium (OE), located in the dorsal or dorsoposterior aspect of the nasal cavity.

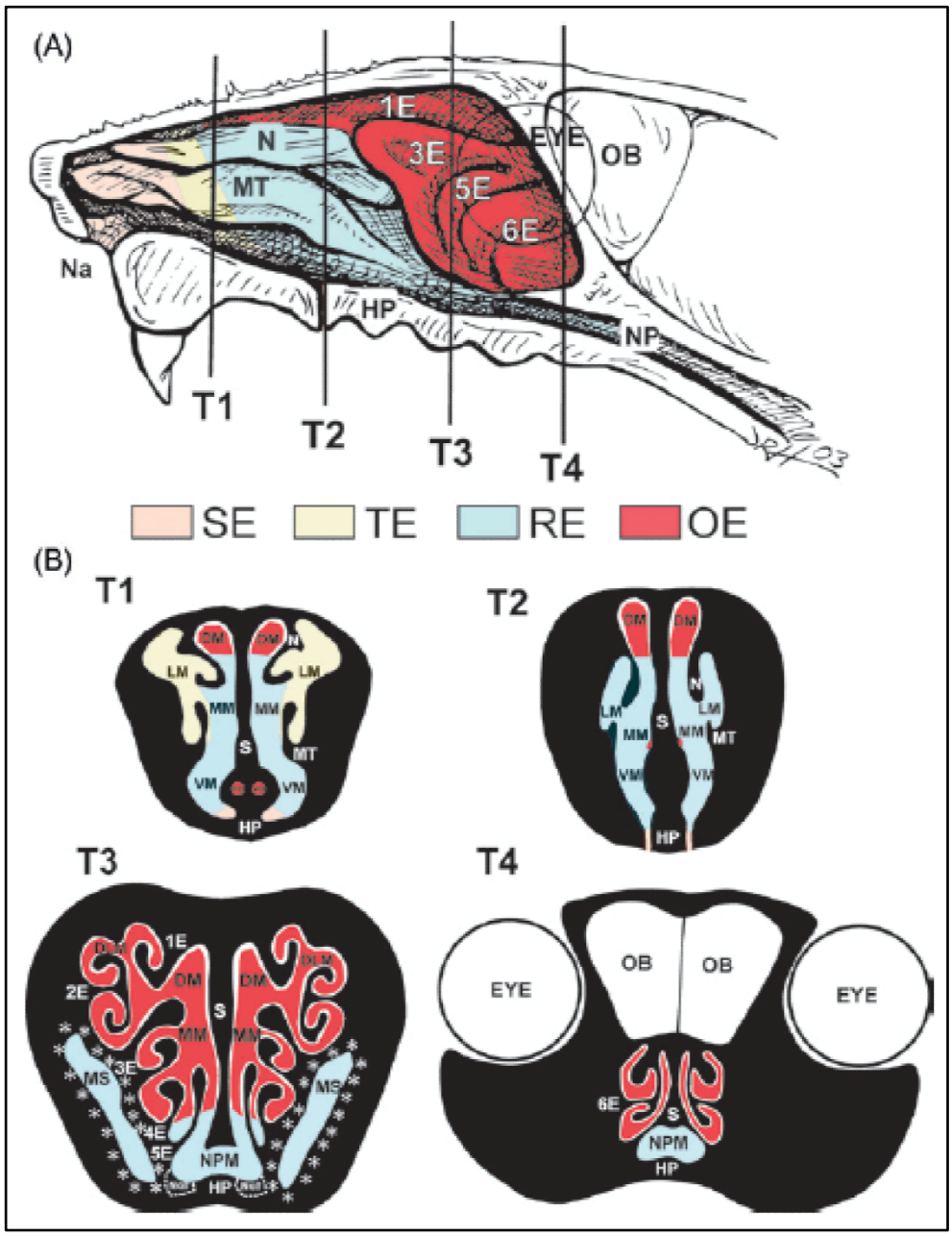

Figure 6. Location of nasal epithelia: (A) right nasal passage of the murine nose with septum removed, exposing nasoturbinate (N), maxilloturbinate (MT), and ethmoid turbinate (E1-6); vertical lines indicate anterior surfaces of transverse tissue blocks (T1-T4). SE, squamous epithelium; TE, transitional epithelium; RE, respiratory epithelium; OE, olfactory epithelium; Na, naris; NP, nasopharynx; OB, olfactory bulb; HP, hard palate. (B) Cross-sectional views of T1-4. DM, dorsal medial meatus; LM, lateral meatus; N, nasoturbinate; MM, middle medial meatus; MT, maxilloturbinate; S, septum; VO, vomeronasal organ; VM, ventral meatus; HP, hard palate; DLM, dorsal lateral meatus; 1E, ethmoid turbinate one; 2E, ethmoid turbinate two; 3E, ethmoid turbinate three; 4E, ethmoid turbinate four; 5E, ethmoid turbinate five; MS, maxillary sinus; NPM, nasopharyngeal meatus; 6E, ethmoid turbinate six; OB, olfactory bulb; Nalt, nasal associated lymphoid. Asterisks = anatomical location of the lateral nasal glands (LNG). (Image obtained from reference [198])

1.7.2 Anatomy of murine lymph nodes associated with alimentary and respiratory tracts

The various lymph nodes associated with alimentary and respiratory tracts are nasopharynx-associated lymphoid tissues (NALT, which is at the base of the nasal cavity and consists of bilateral strips of lymphoid tissues [199]), superficial parotid lymph nodes (LN), submandibular LN, deep cervical LN, mediastinal LN, mesenteric LN, Peyer's patches, thymus and spleen. Their locations are shown in Figure 7.

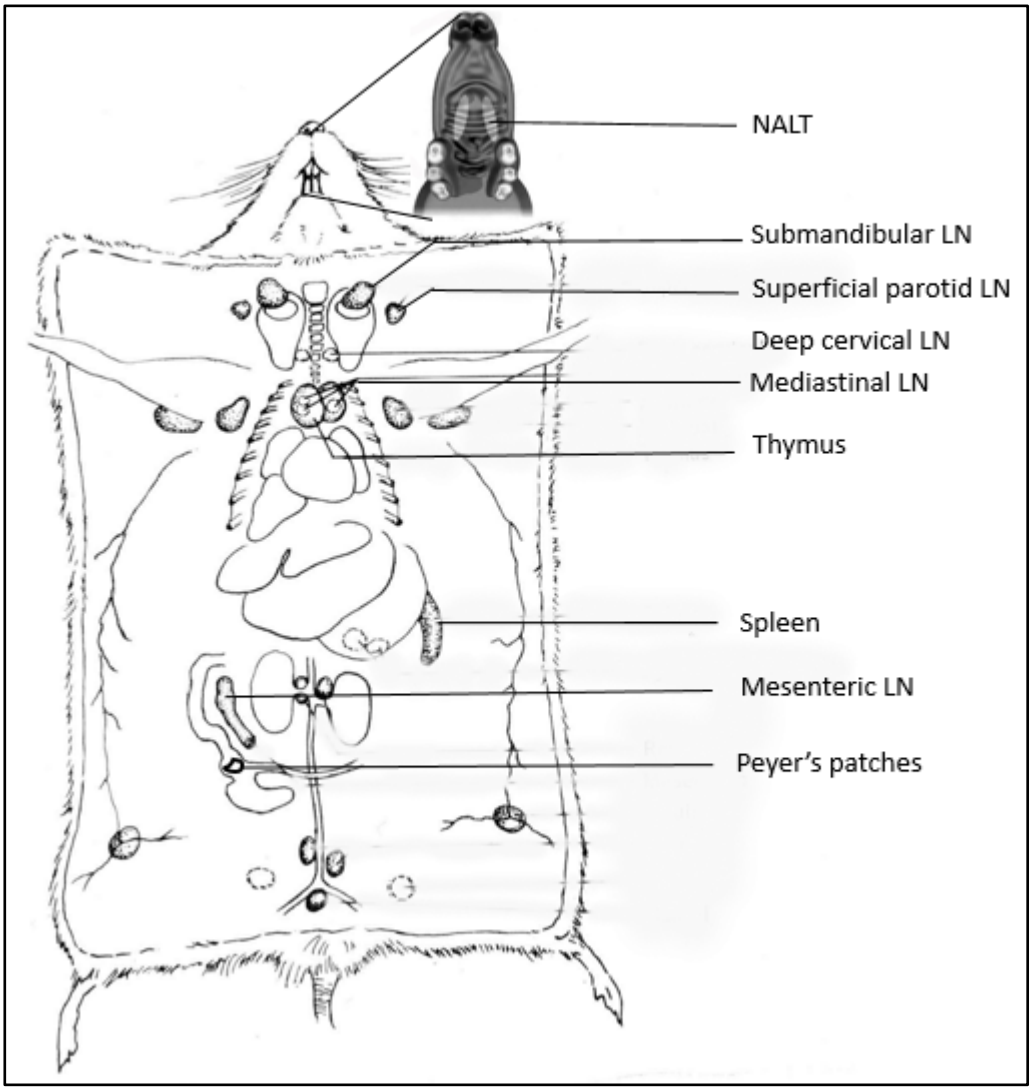

Figure 7. Scheme showing the localization of various lymph nodes associated with alimentary and respiratory tracts. The ventral view of the palate is shown at the top right corner, and the ellipsoid areas represent the position of the NALT in the nasal cavity.

1.8 Homologies and differences in genetic and biological characteristics between MCMV and HCMV

It is impossible to undertake experimental animal studies on the pathogenesis of HCMV infection due to its strict species specificity. Because MCMV infection in mice shares many features with HCMV infection in humans [11, 57], MCMV infection in mice has been widely used as an animal model for studies of HCMV infection. Here, an overview of homologies and differences between MCMV and HCMV is given.

Genome composition and structure - MCMV and HCMV are essentially co-linear over the central region, with significant amino acid sequence homology of the proteins encoded by the 78 genes [200]. Nonetheless, the two viruses have different genome structures. The genome of MCMV is arranged as a single unique sequence with short (31- bp) terminal direct repeats but does not contain large internal repeats [7]. In contrast, the genome structures of HCMV are consisting of long and short unique sequences flanked by inverted large terminal and internal repeats [201].

Biological characteristics - MCMV and HCMV infections are in general asymptomatic in the immunocompetent hosts with virus replication mainly detected in salivary glands, but are really harmful to the immunocompromised individuals such as fetus, AIDS patients and organ transplant recipients, with virus infection detected in salivary glands, lungs, spleen, liver, kidney, aortic wall, and adrenal glands [80, 202, 203]. MCMV infection in mice is a suitable animal model for studying HCMV-related diseases: retinitis [204, 205], interstitial pneumonitis [92, 93], hepatitis [206-208], myocarditis [209], atherosclerosis [210, 211] and adrenalitis [123]. Up till now, it cannot be used to study transplacental infection since transplacental spread of MCMV has not been demonstrated yet.

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Chapter 2

Aims

HCMV chronically infects more than 90% of the world's population. It is harmful for immunocompromised individuals, such as neonates, HIV patients, and recipients of organ transplants, but is in general asymptomatic in the immunocompetent hosts. The sporadic asymptomatic HCMV transmission makes early infection hard to study. MCMV infection in mice is a commonly used experimental animal model for studying HCMV infection. Therefore, MCMV may give more insight in how HCMV invades in its host under natural situation.

Natural MCMV entry routes remain ill-defined, although MCMV has been studied for more than 60 years. Most published studies on MCMV have utilized intraperitoneal inoculation, sometimes intracerebral, orbital or intravenous inoculation, but none of them can be considered as being natural. In general, only intranasal and oral inoculations are widely accepted as the route of natural infection of betaherpesviruses. Unfortunately, there is very limited information on how MCMV infection is established upon intranasal or peroral inoculation. Although a few previous published papers used intranasal inoculation, all inoculated mice were under anesthesia/sedation resulting in a lack of the sneezing reflex, and hence resulting in a direct delivery of virus into the lungs. This does not correlate with the natural infection. Transmission of MCMV by suckling virus contaminated breast milk is possible, likely due to the presence of milk and the neonatal Fc receptor, which may respectively provide additional buffering to avoid destruction by low gastric pH and capture virions bound by maternal antibodies. How MCMV invades the host by oral exposure is still unknown.

Besides the inoculation route, the virus strain should be taken into consideration during *in vivo* studies as well. Over the past decades, most published studies used the MCMV Smith or K181 that is derived from the early stocks of MCMV Smith. Both strains are highly passaged *in vivo* and *in vitro*, and it has become apparent that such strains have acquired genetic and biological differences during passaging. The same problem has been discussed for HCMV, where serially passaged laboratory strains, such as the commonly used HCMV AD169, exhibit significant biological differences

compared to the clinical isolates of HCMV. Therefore, using serially passaged strains of MCMV may not be suited to extrapolate results to the real life situation. It is of importance that recent isolates of MCMV, which are not undergone extensive cell culture passaging, should be tested to give more relevant information on the pathogenesis of MCMV.

Given the above observed problems, the general aim of the research carried out for this doctoral thesis was to obtain a good understanding of MCMV pathogenesis by mimicking the natural infection with a reliable and low passaged MCMV HaNa1 isolate.

The more specific aims were

1) To set up an animal model to mimic natural infection of MCMV and compare the pathogenesis of infection with two MCMV strains (the low passaged MCMV HaNa1 Belgian isolate and highly passaged MCMV Smith strain) in Balb/c female mice using the natural route of infection (oronasally) with a low (10^4TCID$_{50}$ per mouse) and high (10^6TCID$_{50}$ per mouse) inoculation dose without sedation/anesthesia (Chapter 3).

2) To elucidate the kinetics of virus dissemination throughout the body upon oronasal exposure through inoculation of BALB/c female mice with two MCMV strains (the highly passaged MCMV Smith strain and low passaged MCMV HaNa1 strain) (Chapter 4).

3) To demonstrate the role of the spleen in virus dissemination in the natural primary infection of MCMV by inoculating intact or splenectomized Balb/c mice with either MCMV Smith or MCMV HaNa1 (Chapter 5).

Chapter 3

Comparison of the pathogenesis of the highly passaged MCMV Smith strain with that of the low passaged MCMV HaNa1 isolate in BALB/c mice upon oronasal inoculation

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Summary

Murine cytomegalovirus (MCMV) Smith strain is widely used in mouse models to study HCMV infections. Due to highly serial passages, MCMV Smith has acquired genetic and biological changes. Therefore, a low passaged strain would be more relevant to develop mouse models. Here, the pathogenesis of an infection with MCMV Smith was compared with that of an infection with a low passaged Belgian MCMV isolate HaNa1 in BALB/c adult mice following oronasal inoculation with either a low (10^4 $TCID_{50}$/mouse) or high (10^6 $TCID_{50}$/mouse) inoculation dose. Both strains mainly replicated in nasal mucosa and submandibular glands for one to two months. In nasal mucosa, MCMV was detected earlier and longer (1-49dpi) and reached higher titers with the high inoculation dose compared to the low inoculation dose (14-35dpi). In submandibular glands, a similar finding was observed (high dose: 7-49dpi; low dose: 14-42dpi). In lungs, both strains showed a restricted replication. Only the Smith strain established a low level of productive infection in spleen, liver and kidneys. The infected cells were identified as olfactory neurons and sustentacular cells in olfactory epithelium, macrophages and dendritic cells in NALT, acinar cells in submandibular glands, and macrophages and epithelial cells in lungs for both strains. Antibody analysis demonstrated for both strains that IgG_{2a} was the main detectable antibody subclass. Overall, our results show that significant phenotypic differences exist between the two strains. MCMV HaNa1 has been shown to be interesting for use in mouse models in order to get better insights regarding HCMV infections in immunocompetent humans.

1 Introduction

Human cytomegalovirus (HCMV), also known as human herpesvirus 5 (HHV-5), is the prototype member of the *Betaherpesvirinae* within the family of the *Herpesviridae*. It is an ubiquitous virus with a worldwide distribution [1]. It is the main cause of congenital infections in the world, affecting 0.5-2.0% of live births [2, 3] and leading to central nervous damage, congenital deafness and mental retardation [4-7]. HCMV infection is also harmful for the immunocompromised individuals, such as HIV patients and recipients of organ transplants, but is in general asymptomatic in the immunocompetent host [8].

Due to the strict species-specificity of HCMV, it is not possible to study this virus in experimental animals. Therefore, it is necessary to set up animal models for the study of HCMV. Up till now, a number of CMV infections in various animal species have been utilized for modeling HCMV infection. Among the animal CMVs, pathogenesis has been reported for rhesus macaque CMV, porcine CMV, guinea pig CMV, rat CMV and murine CMV [9-13]. The mouse model with MCMV is the most commonly and widely used animal model for HCMV study, due to the following reasons: (1) MCMV shares many features with HCMV [4], (2) the genomes of mice and MCMV are fully sequenced [14, 15] and (3) the small size, short life span, ease of handling and high reproductive rate make them most suitable.

MCMV has been studied for more than 60 years. Most published studies used the MCMV Smith strain or MCMV K181 derived from the early stock of MCMV Smith strain [16], which were highly passaged *in vitro* or *in vivo*. It is now apparent that strains or variants of MCMV Smith that are in common use have acquired genetic and biological differences during passaging [17, 18]. The same problem has been discussed for HCMV, where serially passaged laboratory strains, such as the commonly used HCMV AD169, exhibit significant biological differences compared to the clinical isolates of HCMV [19, 20]. Therefore, using serially passaged strains of MCMV may not be able to reproduce the full range or extent of virus replication and clinical outcome that are associated with HCMV infections. It is important that

more emphasis is being placed on the use of recent isolates of MCMV and avoiding cell culture passaging of these isolates.

Besides the passage history, the inoculation route is an important factor during *in vivo* studies as well. The inoculation route should mimic the natural route of MCMV infection. Most of the published studies on MCMV have utilized the intraperitoneal inoculation, sometimes intracerebral, orbital or intravenous inoculation, none of which can be considered as being natural [21]. Although intramuscular or subcutaneous inoculation mimics natural infection upon biting, only intranasal and oral inoculations are widely accepted as the route of natural infection. Unfortunately, there is very limited information on natural infection upon oronasal inoculation, with only a few studies on viral kinetics, organ and tissue tropism, and host response [22-24].

In the present study, we have used two MCMV strains (low passaged MCMV HaNa1 isolate and highly passaged MCMV Smith strain) to set up mouse models using the natural route of infection (oronasally) with a low (10^4TCID$_{50}$ per mouse) and high (10^6TCID$_{50}$ per mouse) inoculation dose without sedation/anesthesia in order to compare the pathogenesis of a low passaged isolate HaNa1 and the well-studied Smith strain.

2 Materials and Methods

2.1 Ethics statement

All animal experiments (Case number 2013-47) were approved by the local Ethical Committee of the Faculty of Veterinary Medicine, Ghent University.

2.2 Cells and viruses

Primary BALB/c mouse embryonic fibroblasts (MEFs) at passage 2 were propagated at 37°C and 5% CO_2, in minimum essential medium with 10% fetal calf serum (FCS), 2% lactalbumin and a mixture of antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin and 50 µg/ml gentamicin). Viruses used in the present experiments

were the second passage of clone1 of the MCMV HaNa1 isolate, which was isolated by our laboratory from a domestic mouse, and the MCMV Smith strain at unknown passage. Up till now, six genes from MCMV HaNa1 have been sequenced and submitted to GenBank: m06 gene (accession No.: KR184668), m033 gene (accession No.: KR184669), mck-2 including exon1 (m131 gene) and exon2 (m129 gene) (accession No.: KR184670), m138 gene (accession No.: KR184671), m144 gene (accession No.: KR184672), m152 gene (accession No.: KR184673). The m157 gene has also been sequenced more recently and submitted to Genbank (accession No.: KT289520).

2.3 Multistep growth curves of two MCMV strains in MEFs

In order to have a better understanding of the *in vitro* viral replication kinetics of both MCMV strains, a growth curve analysis was performed. Monolayers of MEFs in 24-well plates were inoculated in triplicate with MCMV HaNa1 or MCMV Smith at 10^4 $TCID_{50}$/well. After inoculation for 1 hour at 37°C with 5% CO_2, the inoculum was removed, and cells were washed three times with 2ml PBS. Afterwards, 1ml of fresh culture medium was added per well. The supernatants (1ml) with the extracellular virus and the infected cells containing intracellular virus, which were resuspended in 1ml PBS, were collected at 1, 12, 24, 48 and 72 hpi. The virus inactivation curve was determined by keeping cell free virus in culture medium at 37°C with 5% CO_2. Samples were taken at different time points. The samples were stored at -70°C upon use at the end of the experiment. All samples were thawed and cleared of cellular debris, and then titrated to determine 50% tissue culture infectious dose ($TCID_{50}$) according to the Reed and Muench formula [25].

2.4 Animals and virus inoculation

A total of 135 specific pathogen-free 8-week-old BALB/c female mice were used. In both low dose groups (36 mice/group), each mouse was inoculated with 100µl PBS containing 10^4 $TCID_{50}$ MCMV HaNa1 or MCMV Smith via intranasal (25 µl) and peroral (75 µl) routes without sedation/anesthesia. For the intranasal inoculation, a

small amount of inoculum (5 µl) was repeatedly instilled in each nostril. Each application was done with several minutes interval. For the oral inoculation, 25 µl inoculum was given three times with a few minutes interval between each inoculation. Mice were kept in isolation and fed *ad libitum*. Three inoculated mice were euthanized at each time point (1, 3, 5, 7, 10, 14, 17, 21, 28, 35, 42 and 49 days post inoculation (dpi)). In both high dose groups (30 mice/group), each mouse was inoculated with 100µl PBS containing 10^6 $TCID_{50}$ MCMV HaNa1 or MCMV Smith via intranasal (25 µl) and peroral (75 µl) routes using the same methodology. Three infected mice were euthanized at each time point (1, 3, 5, 7, 10, 14, 17, 21, 35 and 49 dpi). Another 3 mice were mock inoculated with PBS and euthanized at the end of the experiment.

2.5 Collection of saliva, blood and tissues

Saliva was collected by swabs and stored in 0.3 ml of cold sterile PBS containing 1% fetal calf serum and a mixture of antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin and 50 µg/ml gentamicin). Upon anesthesia with 130 µl of 10 mg/ml sodium pentobarbital (KELA, Belgium) per mouse, 0.5 ml blood was collected from the orbital sinus with a heparinized pasteur pipet and kept in an eppendorf with 0.5 ml PBS containing 5 U/ml heparin (Leo Pharma, Zaventem, Belgium). Then, plasma was harvested through centrifugation (200g for 10 min) and stored at -70 °C for virus and antibody titration. Peripheral blood mononuclear cells (PBMC) were isolated on a Ficoll-Paque cushion according to manufacturer's protocol (GE Healthcare), washed three times, resuspended in 0.5 ml RPMI and counted with a hemocytometer. The fresh PBMC were used for co-culture studies. After blood collection, mouse was euthanized with 200 µl of 10 mg/ml sodium pentobarbital (KELA, Belgium). Various tissues were collected under aseptic conditions from the nerve system (olfactory bulb and brain), from the respiratory system (nasal mucosa, nasopharynx-associated lymphoid tissues (NALT), pharynx, trachea and lungs), from the alimentary system (submandibular glands, esophagus and small intestines), from the abdominal organs (liver and kidneys), from the reproductive system (uterus

and ovaries) and from the lymphoid organs (thymus and spleen). One part of an organ was stored at -70°C for virus titration. The other part was snap frozen with methocel and stored at -70°C for immunofluorescence staining.

2.6 Virus titration of tissues

A five percent homogenate was made of all collected tissues for virus titration. Briefly, tissues were thawed, weighed and homogenized by using a pestle, a small volume of sterile sand and DPBS with 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2x6H_2O$ and 0.002% phenol red, supplemented with 2% FCS and a mixture of antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin and 50 µg/ml gentamycin). Afterwards, the supernatants were collected after centrifugation (2400g, 10min). Virus titration was performed on the second passage of MEFs. After 7 days, the presence of a cytopathic effect (CPE) was assessed by light microscopy (Olympus Optical Co., Hamburg, Germany) and virus titer was calculated as 50% tissue culture infectious dose ($TCID_{50}$) according to the Reed and Muench formula [25].

2.7 Co-culture of PBMC

To examine the cell-associated viremia in PBMC, co-culture assays were performed. MEFs ($2x10^5$/well) were seeded in 24-well plates two days prior to co-culture. Freshly isolated PBMC were brought on the monolayer (PBMC from one mouse were equally divided into 2 wells in 24-well plate) and covered with 1ml carboxymethylcellulose (CMC) medium (1/4 2xMEM, 1/4 2xRPMI, 1/2 2xCMC supplemented with 5% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamicin, 0.1mM non-essential amino acids (NEAA) and 1mM Sodium pyruvate), and the plates were centrifuged 750 g for 10 min, afterwards cultivated at 37°C in the incubator for 8 days. Plaques were counted with a light microscopy (Olympus Optical Co., Hamburg, Germany).

2.8 Production of biotinylated polyclonal anti-MCMV antibodies (*pα-MCMV Abs*)

Anti-MCMV Smith hyperimmune sera were prepared as described before by Nigel

et al. with slight modification [26]. Briefly, MCMV Smith was grown in MEFs, the virus was released by sonication and virus suspension was clarified to remove cellular debris by centrifugation (4,000g for 20min). Mice were inoculated with 10^6 $TCID_{50}$ of clarified MCMV Smith intraperitoneally (IP), followed by two further IP inoculations at 2-week intervals. Afterwards, the plasma was collected at 7 days post last injection. IgG was isolated from plasma using Protein G Sepharose™ 4 Fast Flow (GE Healthcare), and protein concentration was determined by NanoDrop 2000 (Thermo Fisher Scientific). The purified antibodies were biotinylated with biotin reagents (EZ-Link® Sulfo-NHS-LC-Biotin, Thermo Fisher Scientific).

Pα-MCMV Abs were tested for their reactivity against viral immediate early proteins, early proteins or late proteins by a co-localization assay of *pα-MCMV Abs* and murine monoclonal antibodies against immediate early protein (mouse anti-m123/IE1, CROMA101, isotype IgG1 (Capri, Croatia)), early protein (mouse anti-M112-113/E1, isotype IgG1 (Capri, Croatia)) and late protein (mouse anti-M55/gB, isotype IgG2b (Capri, Croatia)). The co-localization assay showed that *pα-MCMV Abs* recognized the viral early and late proteins but not viral immediate early proteins.

2.9 Quantification of MCMV-infected cells in the nasal mucosa, lungs and submandibular glands

Immunofluorescence staining was used to quantify MCMV-infected cells in tissues (nasal mucosa, lungs and submandibular glands) that were MCMV HaNa1/MCMV Smith-positive after virus titration. The number of MCMV-infected cells in the nasal mucosa, submandibular glands and lungs of mice inoculated with the high dose (10^6 $TCID_{50}$/mouse) at 3, 7, 14 and 35 dpi was calculated. Forty consecutive cryosections (12µm) per organ were fixed in 4% paraformaldehyde at 4°C for 10 min and permeabilized with 0.1% Triton X-100 (Sigma) at room temperature (RT) for 10 min. Tissue sections were pretreated for 30 min with 10% negative goat serum and followed by incubating with *pα-MCMV Abs* (1:30) at 37°C for 1h. The cryosections were washed three times with PBS and incubated with the secondary antibodies:

streptavidin Alexa-fluor® 488 conjugate, 1:200 (Invitrogen) at 37°C for 1h. After three washings, cell nuclei were stained with 10µg/ml Hoechst 33342 (Invitrogen) at RT for 10min. Finally, cryosections were mounted with glycerin-DABCO (Acros Organics).

Infected cells within each cryosection were quantified with the Leica TCS SPE laser-scanning confocal microscopy (magnification 200x, Leica Microsystems, GmbH, Wetzlar, Germany) according to the quantification method of Beyer and colleagues [27]. Forty consecutive 12µm-sections were analyzed per organ. The size of the analyzed sections was determined by the number and size of the visual fields at a 200x magnification (diameter 1mm). Finally, the number of MCMV-infected cells was calculated as a value per $10mm^2$, independent of their localization and distribution within the cryosection.

2.10 Identification of MCMV-infected cells in the nasal mucosa, lungs and submandibular glands

Immunofluorescence double staining was used to identify MCMV-infected cells in the nasal mucosa, lungs and submandibular glands. Twelve µm viral antigen-positive cryosections of nasal mucosa, lungs and submandibular glands from 3, 7, 14 and 35dpi were prepared following the aforementioned protocol. Sections were incubated at 37°C for 1h with *pα-MCMV Abs* (1:30) and cell markers (rabbit polyclonal anti-cytokeratin-18 for epithelia, 1:150 (Abcam); goat anti-olfactory marker protein for olfactory neurons [28], 1:500 (Wako); rat anti-mouse CD68/FITC-rat-anti-mouse F4/80 for tissue macrophages, 1:50 (AbD Serotec); hamster anti-mouse CD11c for dendritic cells, 1:50 (eBioscience); rat anti-mouse B220 for pan-B cells, 1:50 (Biolegend) and Alexa Fluor® 488-hamster-anti-mouse CD3 for T cells). The cryosections were washed three times with PBS and incubated at 37°C for 1h with the corresponding secondary antibodies: streptavidin-Texas Red-X or FITC conjugate, 1:200 (Invitrogen); FITC-goat-anti-rabbit IgG, 1:200 (Invitrogen); Alexa Fluor® 594-rabbit-anti-goat IgG, 1:200 (Invitrogen); Alexa Fluor® 488-goat-anti-rat IgG, 1:200 (Invitrogen); Alexa Fluor®

488-goat-anti-hamster IgG, 1:200 (Jackson ImmunoResearch) and Alexa Fluor® 488-goat-anti-rat IgG, 1:200 (Invitrogen). After washing three times, cell nuclei were counterstained with 10µg/ml Hoechst 33342 (Invitrogen) at RT for 10min. Finally, cryosections were mounted with glycerin-DABCO (Acros Organics) and analyzed with the Leica TCS SPE laser-scanning confocal microscopy (Leica Microsystems, GmbH, Wetzlar, Germany).

2.11 Determination of total and isotype-specific anti-MCMV antibody titers

The titer of total and isotype specific anti-MCMV antibodies were determined in immunoperoxidase monolayer assays (IPMA) [29]. Briefly, monolayers of immortalized mouse embryo fibroblasts (MEFs) [30] in 96-well plates were inoculated with MCMV HaNa1 or MCMV Smith (10^3 $TCID_{50}$ per well), and cultivated for 3 days (37°C, 5% CO_2). Afterwards, the culture medium was removed, and cells were washed with PBS and dried at 37°C for 1h. The plates were covered with plastic covers and stored at -20°C until use. Plates were thawed at RT and cells were fixed with 4% paraformaldehyde for 10 min at RT. The paraformaldehyde was removed, and cells were washed twice with PBS. Afterwards, the cells were treated with 100% methanol supplemented with 1% H_2O_2 at RT for 5min. Plates were washed twice with PBS and serial twofold dilutions of plasma were added and incubated at 37°C for 1h. Plates were washed three times with PBS. To determine the virus-specific immunoglobulin classes and subclasses, 50µl biotinylated secondary antibody (rat anti-mouse IgA biotin, 1:100, (eBioscience); rat anti-mouse IgM biotin, 1:100, (eBioscience); sheep anti-mouse IgG biotin, 1:100, (GE healthcare); rat anti-mouse IgG_1 biotin, 1:100, (eBioscience); rat anti-mouse IgG_{2a} biotin, 1:100, (eBioscience); rat anti-mouse IgG_{2b} biotin, 1:100, (Biolegend); goat anti-mouse IgG_{2c} biotin, 1:100, (abcam); rat anti-mouse IgG_3 biotin, 1:100, (Biolegend)) were added respectively and incubated at 37°C for 1h. Afterwards, plates were washed three times and 50µl streptavidin-biotin horseradish peroxidase complex (1:200) was added per well and incubated at 37°C for 1h. Plates were washed three times and 50µl of a substrate solution of 3-amino-9-ethylcarbazole

(1/20) in 0.05 M acetate buffer, pH 5, with 0.024% H_2O_2 was added to each well and kept in RT for 30min. Finally, the reaction was stopped with sodium acetate and the IPMA titer was calculated as the reciprocal value of the highest serum dilution that induced visual staining of infected MEFs as determined by a light microscopy (Olympus Optical Co., Hamburg, Germany). All aforementioned biotinylated secondary antibodies have been validated with sera from mice inoculated with influenza A/New Caledonia/20/99 (NC) virus by IPMA. In addition, the specificity of biotinylated secondary IgG antibodies had also been assessed with a panel of murine anti-MCMV Smith specific IgG monoclonal antibodies with known subclasses (mouse anti-m112-113, CROMA 103, IgG1; mouse anti-M123, IE1.01, IgG2a; mouse anti-m55-MCMV, CROMA7, IgG2b; mouse anti-m04-MCMV, m04-KAC.10, IgG2c; all were purchased from Capri, Croatia). No apparent cross-reaction was found (data not shown).

2.12 Complement-dependent neutralization test

Hyperimmune sera to CMVs were found to mainly contain complement-requiring neutralizing antibodies. Assays to measure neutralizing antibody titers were performed as described before by Helen *et al.* and Lawson *et al.* with a few modifications [31, 32]. At day one, serial twofold dilutions of heat-inactivated (56°C, 30min) plasma in MEM (from 1:2 to 1:512) were incubated with an equal volume containing 500 $TCID_{50}$ MCMV Smith or 700 $TCID_{50}$ MCMV HaNa1 for 23h at 37°C. Meanwhile, MEFs were trypsinized and seeded in 96-well plates with 100µl per well at a concentration of $2.5x10^5$ cells/ml. After 23h, 25µl of guinea pig complement (0.5 µg/µl) was added to the virus/serum dilution mixtures for incubating another 1h at 37°C in a 5% CO_2 incubator. Then, the medium of cells was removed and the mixtures of serum/virus/complement were added to the confluent cell monolayers. The plates were kept for 7 days at 37°C. The neutralization titer of a plasma was expressed as the reciprocal of the highest serum dilution that was able to completely block MCMV infection in MEFs.

3 Results

3.1 Growth kinetics of MCMV strains in MEFs

In the first experiment, the *in vitro* viral growth characteristics of MCMV HaNa1 and MCMV Smith were compared in MEFs, as shown in Figure 1. The study demonstrated that HaNa1 grew to a ~10-fold lower yield in comparison with the Smith strain and that HaNa1 isolate was more cell-associated than the Smith strain. Therefore, it can be stated that the Smith strain replicated in MEFs much more easily than the HaNa1 isolate.

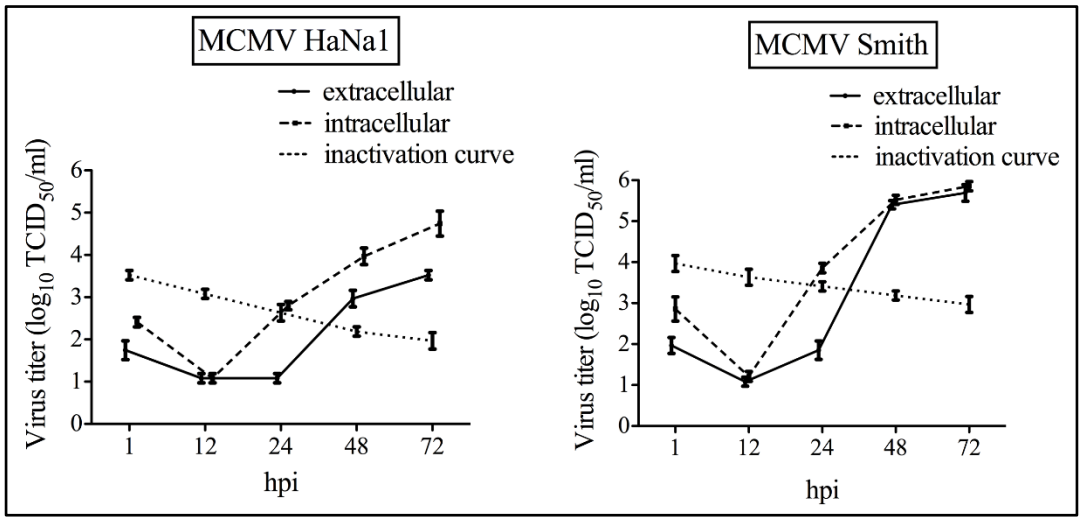

Figure 1. Growth kinetics of MCMV HaNa1 and MCMV Smith in MEFs. The virus titers generated in MEFs were determined, and growth curves of HaNa1 and Smith were plotted. The inactivation curve shows the drop of virus titers at 37 °C in culture medium due to inactivation events. The mean virus titer (log_{10} $TCID_{50}$/ml) and standard deviation (n=3) were shown in the diagram.

3.2 Virus titers in tissues

Low dose - After oronasal inoculation of 10^4 $TCID_{50}$ per mouse, MCMV HaNa1 was detected in the nasal mucosa from 14 till 35 dpi with the highest mean virus titer of $10^{3.53}$ $TCID_{50}$/g at 14 dpi, in submandibular glands from 14 till 35 dpi with the highest mean virus titer of $10^{4.93}$ $TCID_{50}$/g at 21 dpi (Fig. 2), and in lungs and saliva only at one time point (14 (n=1) and 28 (n=1) dpi, respectively). The other organs (olfactory bulb, brain, pharynx, trachea, esophagus, small intestines, liver, kidneys,

uterus, ovaries, thymus and spleen) remained all negative (under the detection limit).

MCMV Smith was detected in the nasal mucosa from 14 till 35dpi and in submandibular glands from 14 till 42dpi with the highest mean virus titer in the nasal mucosa ($10^{3.01}$ $TCID_{50}$/g) at 14 dpi and in the submandibular glands ($10^{3.72}$ $TCID_{50}$/g) at 17 dpi (Fig. 2). Lungs were positive only at 17dpi (n=2). Saliva and plasma were negative during the course of infection. MCMV Smith led to a productive infection with virus replication in the spleen at 17dpi (n=3) and 35dpi (n=1), in the liver at 14dpi (n=2) and 17dpi (n=3), and in the kidneys at 14dpi (n=1) and 17dpi (n=3) (Fig. 2). The other organs (olfactory bulb, brain, pharynx, trachea, esophagus, small intestines, uterus, ovaries and thymus) remained all negative (under the detection limit).

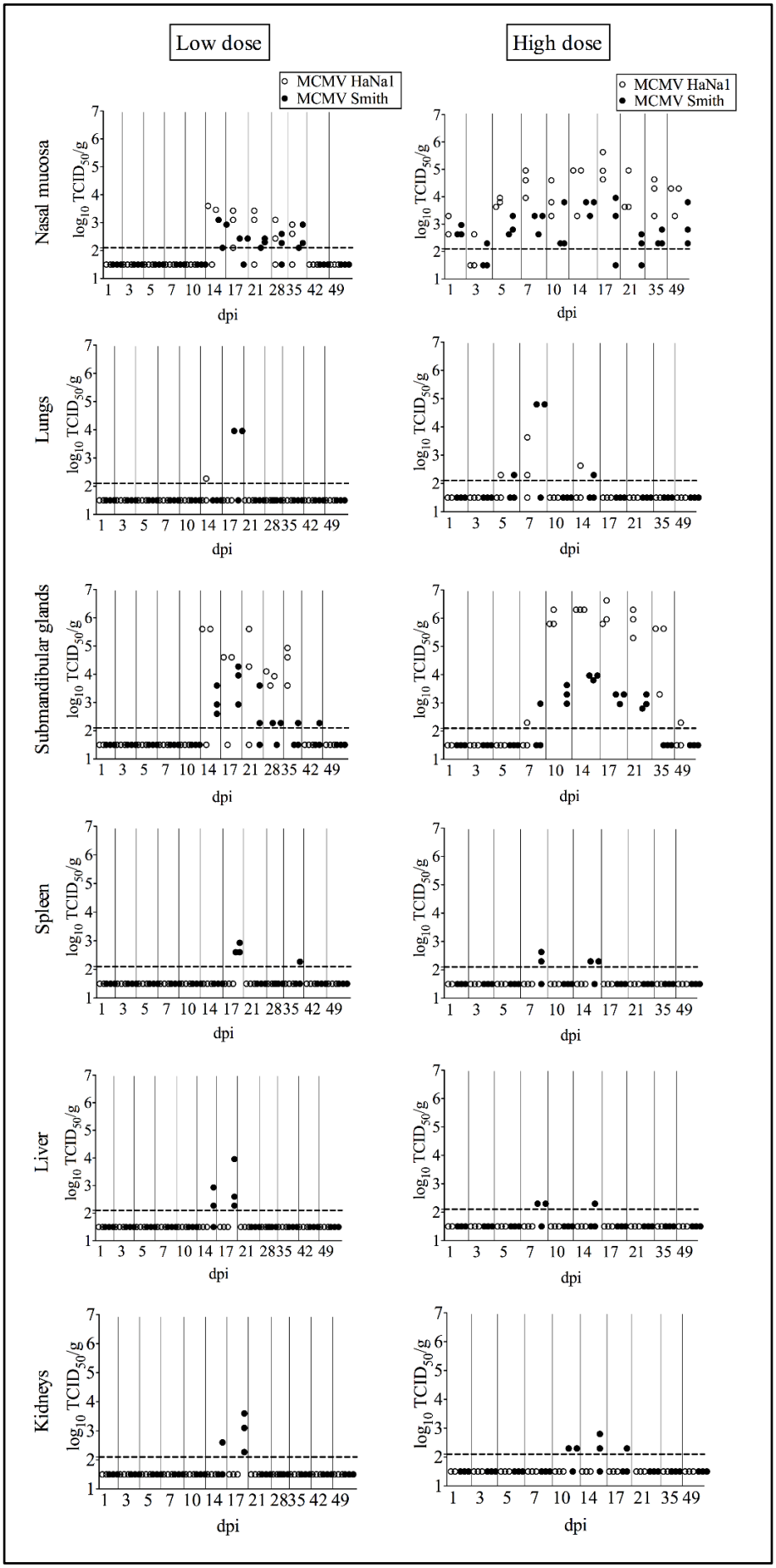
Low dose
High dose
MCMV HaNa1
MCMV Smith
Nasal mucosa
Lungs
Submandibular glands
Spleen
Liver
Kidneys
log10 TCID50/g
dpi
1 3 5 7 10 14 17 21 28 35 42 49
1 3 5 7 10 14 17 21 35 49

Figure 2. Virus titers in the nasal mucosa, lungs, submandibular glands, spleen, liver and kidneys. These tissues were collected from mice upon oronasal inoculation with either a low (10^4TCID$_{50}$/mouse, left column) or a high (10^6TCID$_{50}$/mouse, right column) inoculation dose. Virus titers ($\log_{10}$ TCID$_{50}$/g) were measured by titration. Open circles represent HaNa1-infected mice and closed circles represent Smith-infected mice. The detection limit for the titration ($10^{2.1}$ TCID$_{50}$/g) is shown by the horizontal dotted line.

High dose - After oronasal inoculation of 10^6 TCID$_{50}$ per mouse, MCMV HaNa1 was detected in the nasal mucosa from 1dpi till the end of the experiment (49dpi) with the highest mean virus titer of $10^{5.07}$ TCID$_{50}$/g at 17dpi, in submandibular glands from 7dpi till the end of the experiment 49dpi with the highest mean virus titer of $10^{6.3}$ TCID$_{50}$/g at 14dpi, and in lungs at 5, 7 and 14dpi with a low level of virus replication (Fig. 2). At none of the collected time points post inoculation, infectious virus was detected in saliva and plasma. The other organs (olfactory bulb, brain, pharynx, trachea, esophagus, small intestines, liver, kidneys, uterus, ovaries, thymus and spleen) remained negative throughout the experiment (under the detection limit).

MCMV Smith was detected in the nasal mucosa from 1dpi till the end of the experiment (49dpi) with the highest mean virus titer of $10^{3.63}$ TCID$_{50}$/g at 14 dpi, in submandibular glands from 7dpi till 35dpi with the highest mean virus titer of $10^{3.91}$ TCID$_{50}$/g at 14 dpi, and in lungs from 5dpi till 14dpi except 10 dpi with a low level of virus replication close to the virus detection limit except at 7dpi with a virus titer of $10^{4.8}$ TCID$_{50}$/g in 2 out of 3 mice (Fig. 2). MCMV Smith led to a productive infection with virus replication in the spleen at 7dpi (n=2) and 14dpi (n=2), in the liver at 7dpi (n=2) and 14dpi (n=1), and in the kidneys at 10dpi (n=2), 14dpi (n=2) and 17dpi (n=1) (Fig. 2). Infectious virus was not detected in saliva and plasma at any indicated time point. No virus was detected in the control mice. The other organs (olfactory bulb, brain, pharynx, trachea, esophagus, small intestines, uterus, ovaries and thymus) remained negative throughout the experiment (under the detection limit).

3.3 Co-culture of PBMC with MEFs

At none of the collected time points post inoculation, cell-associated virus was detected by co-culture for both strains at a low inoculation dose. At a high inoculation dose, cell-associated virus was detected in PBMC for both strains: at 7dpi (n=2) and 10dpi (n=1) in HaNa1-infected mice; at 7dpi (n=2) in Smith-infected mice.

3.4 Quantification of MCMV-infected cells in the nasal mucosa, lungs and submandibular glands

The nasal mucosa, lungs and submandibular glands from mice that received a high inoculation dose were collected at 3, 7, 14 and 35 dpi and the number of MCMV-infected cells in these organs was quantified. The same samples from mock-inoculated mice were negative for MCMV. Figure 3 shows the number of MCMV-infected cells in these organs.

For HaNa1-infected mice, infected cells in the nasal mucosa were first detected at 3dpi (0.09 cells per $10mm^2$), peaked at 7dpi (0.51 cells per $10mm^2$) and dropped sharply afterwards (at 35dpi: 0.09 cells per $10mm^2$). In lungs, they were first detected and also peaked at 7dpi (0.47 cells per $10mm^2$), decreased afterwards significantly and were undetectable at 35dpi. In submandibular glands, they were first noticed at 7dpi (0.13 cells per $10mm^2$), peaked at 14dpi (1.59 cells per $10mm^2$), and dropped sharply afterwards (0.80 cells per $10mm^2$ at 35dpi).

For Smith-infected mice, MCMV-infected cells in the nasal mucosa were first detected at 3dpi (0.07 cells per $10mm^2$), peaked at 7dpi (0.23 cells per $10mm^2$) and then decreased till 35dpi (0.08 cells per $10mm^2$). In lungs, they were first detected and also peaked at 7dpi (0.72 cells per $10mm^2$), afterwards fell dramatically and were undetectable at 35dpi. In submandibular glands, they were first detected at 7dpi (0.08 cells per $10mm^2$), reached a peak at 14dpi (0.49 cells per $10mm^2$) and decreased afterwards (0.05 cells per $10mm^2$ at 35dpi).

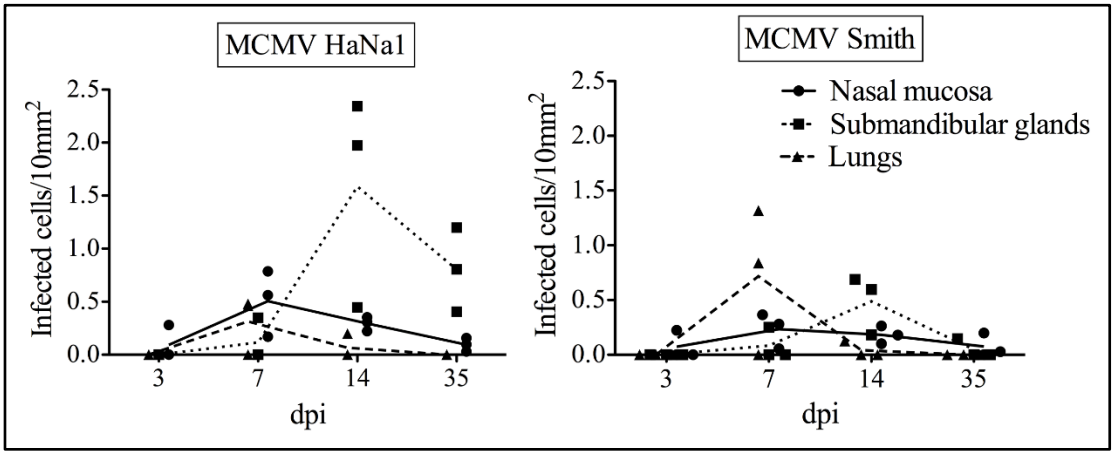

Figure 3. Quantification of MCMV-infected cells in the nasal mucosa, submandibular glands and lungs. Each time point has three individual animals. Forty consecutive cryosections per tissue were evaluated by immunofluorescence microscopy. The number of infected cells per 10 mm^2 is shown. The average values of infected cells at different time points were connected by lines.

3.5 Identification of MCMV-infected cells in the nasal mucosa, lungs and submandibular glands

Identification of the MCMV-infected cells in target tissues would help us to understand the cell tropism of MCMV. The nasal mucosa, lungs and submandibular glands from mice inoculated with a high dose were collected at 3, 7, 14 and 35 dpi to be stained for MCMV antigens and cellular markers simultaneously. The morphology of MCMV-positive cells consisted of a big round or oval unstained nucleus surrounded by a thick rim of positive cytoplasm (Fig. 4). Since both strains gave similar results, only staining of cryosections from HaNa1-infected mice were presented here. In the nasal mucosa, the infected cells were only found in the olfactory epithelium and nasopharynx associated lymphoid tissue (NALT) from 3dpi onwards. Based on our results, viral proteins were expressed in both sustentacular cells and neurons in the olfactory epithelium, and in CD68/CD11c positive cells (macrophages/dendritic cells) but not in B220/CD3 positive cells (B cells/T cells) in the NALT. In lungs, epithelial cells and macrophages were susceptible cell types at 7 and 14dpi. In submandibular glands, only epithelial cells were susceptible at 7, 14 and 35dpi.

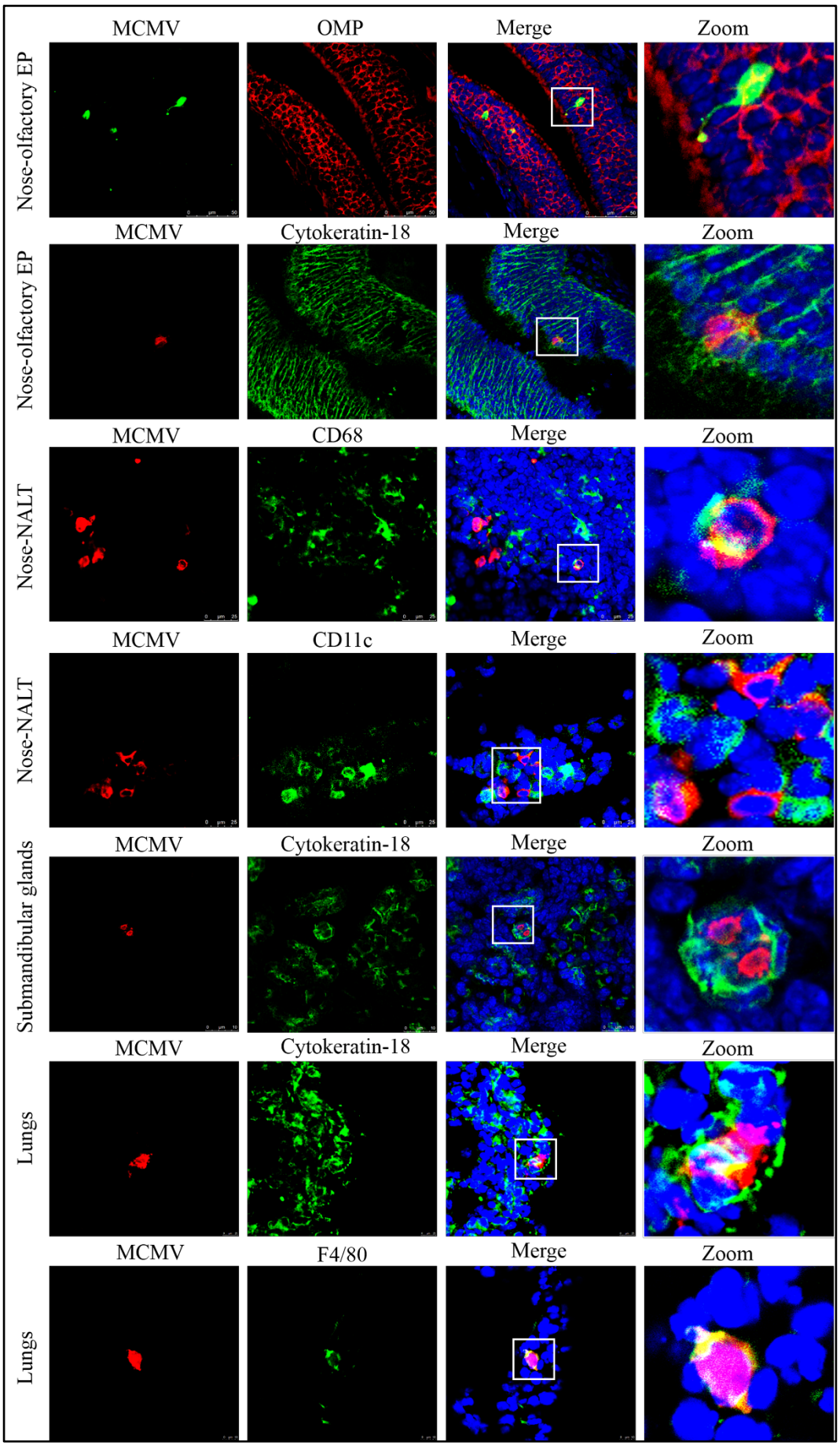
MCMV
OMP
Merge
Zoom
Nose-olfactory EP
MCMV
Cytokeratin-18
Merge
Zoom
Nose-olfactory EP
MCMV
CD68
Merge
Zoom
Nose-NALT
MCMV
CD11c
Merge
Zoom
Nose-NALT
MCMV
Cytokeratin-18
Merge
Zoom
Submandibular glands
MCMV
Cytokeratin-18
Merge
Zoom
Lungs
MCMV
F4/80
Merge
Zoom
Lungs

Figure 4. Identification of MCMV-infected cells in the nasal mucosa, lungs and submandibular glands. Nuclei were counterstained with Hoechst (Blue). Co-localization appears yellow in the merged images. The zoomed images show the boxed region of merged layers. Cryosections of the nasal mucosa were double-stained with antibodies against MCMV antigens (by *pα-MCMV Abs*) and cell markers: OMP (olfactory neuron marker), cytokeratin-18 (epithelial marker), CD68 (tissue macrophages) and CD11c (dendritic cells). Olfactory neurons and sustentacular cells of the olfactory epithelium (olfactory EP) in the nasal mucosa were susceptible for MCMV. Infected cells of NALT in the nasal mucosa were $CD68^+/CD11c^+$. Epithelial cells (cytokeratin-18^+) of submandibular glands were susceptible for MCMV. Epithelial cells (cytokeratin-18^+) and macrophages ($F4/80^+$) were positive for MCMV in lungs.

3.6 Serology

3.6.1 Viral-specific antibodies by IPMA

Low dose - MCMV HaNa1-specific antibodies were first detected at 10dpi. Afterwards, titers rose and reached a maximal level at 21dpi; MCMV Smith-specific antibodies showed a similar course with the exception that they appeared later (at 14dpi) (Fig. 5). ***High dose*** - The high dose (10^6 $TCID_{50}$/mouse) reduced the time of appearance of antibodies (HaNa1 at 7dpi; Smith at 10dpi). Both reached a maximal level at 17dpi.

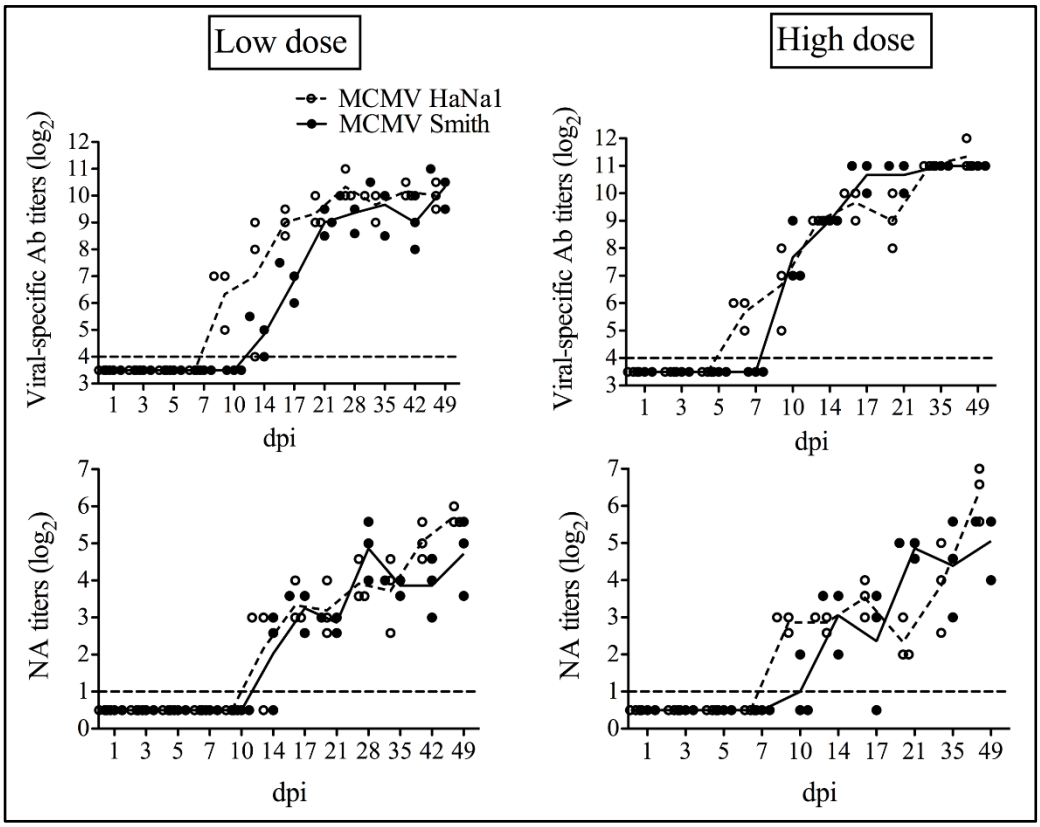

Figure 5. Viral-specific antibody titers by IPMA and neutralizing antibody (NA) titers by NA. They were determined in the Smith or HaNa1-infected mice that had been inoculated with either a low (10^4TCID$_{50}$/mouse) or a high (10^6TCID$_{50}$/mouse) inoculation dose. The average titers of 3 BALB/c mice at different time points were connected by lines. The detection limit was shown by the dotted line.

3.6.2 Complement-dependent neutralizing antibodies by NA test

Neutralizing antibodies without adding guinea pig complement were only detected at 35-49dpi with low NA titers (lower than or equal to 2) for both strains (data not shown). Complement-dependent neutralizing antibodies from mice inoculated with a low inoculation dose were first detected at 14dpi, afterwards increased till the end of experiment at 49dpi (Fig. 5). A similar pattern was found with a high inoculation dose, except that neutralizing antibodies appeared earlier at 10dpi.

3.6.3 Subclasses determination by IPMA

Low dose - The results of MCMV-specific Ig classes and IgG subclasses determination showed that only MCMV-specific IgG2a was detectable throughout the whole experiment, whereas the other (IgA, IgM and other IgG subclasses (IgG1, IgG2b, IgG2c and IgG3)) were not (Table 1). MCMV-specific IgG_{2a} subclass was detected earlier in HaNa1-infected mice at 14dpi than in Smith-infected mice at 17dpi. ***High dose*** - MCMV-specific IgG2a was the main antibody subclass (Table 2). MCMV-specific IgG1 and IgG2c antibodies were also detected for both strains but with lower titers (lower than or equal to 64) albeit at 35 and 49dpi.

Table 1. Course of isotype-specific anti-MCMV HaNa1 or anti-MCMV Smith antibody titers in BALB/c mice inoculated with 10^4TCID$_{50}$ per mouse

Strains	Classes/subclasses	Antibody titers											
		1d	3d	5d	7d	10d	14d	17d	21d	28d	35d	42d	49d
MCMV HaNa1	IgA	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-
	IgM	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-
	IgG_1	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-
	IgG_{2a}	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	32/-/-	32/32/16	64/32/64	128/256/128	128/64/128	128/128/128	128/128/128
	IgG_{2b}	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-
	IgG_{2c}	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-
	IgG_3	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-
MCMV Smith	IgA	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-
	IgM	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-
	IgG_1	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-
	IgG_{2a}	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	32/32/32	32/64/64	64/128/32	128/64/128	128/64/128	128/128/128
	IgG_{2b}	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-
	IgG_{2c}	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-
	IgG_3	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-

'-' represents that the antibody titer was under the detection limit (1/16)

Table 2. Course of isotype-specific anti-MCMV HaNa1 or anti-MCMV Smith antibody titers in BALB/c mice inoculated with 10^6TCID$_{50}$ per mouse

Strains	Classes/subclasses	Antibody titers									
		1d	3d	5d	7d	10d	14d	17d	21d	35d	49d
MCMV HaNa1	IgA	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-
	IgM	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-
	IgG_1	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	128/16/64	32/64/64
	IgG_{2a}	-/-/-	-/-/-	-/-/-	-/-/-	64/16/32	256/128/256	256/256/512	128/256/256	1024/1024/1024	256/1024/1024
	IgG_{2b}	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-
	IgG_{2c}	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	64/32/64
	IgG_3	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-
MCMV Smith	IgA	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-
	IgM	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-
	IgG_1	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/32/-	64/32/-
	IgG_{2a}	-/-/-	-/-/-	-/-/-	-/-/-	32/-/64	64/128/128	256/128/256	512/512/256	512/512/1024	1024/512/512
	IgG_{2b}	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-
	IgG_{2c}	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	16/32/16	64/16/64
	IgG_3	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-

'-' represents that the antibody titer was under the detection limit (1/16)

4 Discussion

To date, MCMV has been widely used in laboratory models for studying HCMV infections. Almost all the laboratory research on MCMV used either the Smith or the Smith-derived K181 strains, which are the two standard laboratory MCMV strains [18]. These strains have been serially passaged *in vitro* or *in vivo* for more than 60 years, which likely caused genetic and biological changes. Furthermore, non-natural inoculation routes (subcutaneously, intraperitoneally, or intravenously) have been utilized to inoculate animals in the previous studies, thus bypassing the mucosal sites of virus replication and the local immune response [4, 33]. Therefore, there is limited information on natural infections with low passaged MCMV. Here, an MCMV infection model has been established that mimics natural infection using a recently Belgian MCMV isolate HaNa1. We found that upon oronasal inoculation: 1) the nasal mucosa and submandibular glands were the main sites of productive MCMV replication; 2) only the Smith strain established a productive infection in spleen, liver and kidneys, whereas the HaNa1 isolate did not; 3) increasing the inoculation dose strongly elevated virus production in the nasal mucosa and submandibular glands, and also reduced the time of appearance of antibodies.

In the present study, it was examined whether HaNa1 and Smith differed in viral growth *in vitro* and *in vivo*. The growth kinetics *in vitro* demonstrated that the Smith strain replicated to a ~10-fold higher yield than the HaNa1 isolate. In contrast to the *in vitro* situation, in the nasal mucosa and submandibular glands, HaNa1 replicated to higher titers than Smith *in vivo*. This is consistent with their passage history. The MCMV with more passages in cells grows better *in vitro* but loses part of its replication ability *in vivo* [34, 35].

In vivo, both strains (Smith and HaNa1) were first detected in the nasal mucosa. Increasing the inoculation dose elevated virus production leading to early detection and higher virus titers. HaNa1 reached higher virus titers than Smith. In our study, the nasal mucosa was shown for the first time to be a susceptible organ for MCMV. Based on similar characteristics of human and murine CMVs, we hypothesize that

the nasal mucosa might also be a target organ for HCMV. In line with this, there have been several reports on the detection of HCMV in nasopharyngeal carcinomas, sinusitis and nasal polyposis [36-39]. In lungs, both strains showed a very restricted replication during the first three weeks after post inoculation, after which the infection was controlled. Our finding is consistent with a previous study [40]. The submandibular gland is another target organ for both strains. In contrast with the viral replication in the nasal mucosa, the viral replication in the submandibular glands always started after one week post inoculation and lasted longer than 49dpi post inoculation. HaNa1 reached much higher virus titers (>100 folds) than Smith. Similarly to the nasal mucosa, increasing the inoculation dose enhanced virus production in the submandibular glands. CMVs have been reported to mainly use salivary glands as target organ for virus persistence and shedding into saliva [33, 41, 42]. However, in our study, HaNa1 was detected in saliva only at one time point within one mouse. The low level of virus titers in saliva was quite surprising, as CMVs are thought to transmit orally via saliva. In future studies, these conflicting data will be further examined.

Cell-associated virus in PBMC was detected at 7-10dpi for both strains with a high inoculation dose. This shows that circulating PBMC are involved in the dissemination of MCMV, which corresponds with a previous study [43]. In our study, only the Smith strain was detectable by virus titration in spleen, liver and kidneys from the second week post inoculation onwards, providing evidence that only the Smith strain can establish a productive infection in internal organs of adult mice, whereas HaNa1 cannot. The latter is similar to the outcome of an HCMV primary infection in immunocompetent adults, during which it is only causing a limited virus-associated spread to the salivary glands but not to multiple internal organs [44].

Quantification of MCMV-infected cells in the nasal mucosa, lungs and submandibular glands revealed a good correlation between virus titers and infected cells. Identification of MCMV-infected cells demonstrated that in the nasal mucosa,

olfactory neurons as well as sustentacular cells in the olfactory neuroepithelium and CD68/CD11c positive cells (macrophages/dendritic cells) in NALT were the main susceptible cell types. Targeting the olfactory neurons raised the question if CMV may damage the smelling [45, 46]. This will be investigated in the future. According to the staining results, CD68/CD11c positive cells (macrophages/dendritic cells) in NALT were infected from 3dpi onwards, which indicates that NALT plays a very important role in MCMV infection. Therefore, we presume that the virus may be transmitted via lymphatic circulation to draining lymph nodes, ending up in the blood circulation. In lungs, both epithelial cells and macrophages are susceptible to both MCMV strains, which are the main cause of pneumonitis caused by MCMV [22, 40]. It is also a frequently observed manifestation of HCMV infection [47, 48]. In submandibular glands, epithelial cells are the main susceptible cell type for both MCMV strains, which is consistent with earlier published data [49]. However, up till now it is unclear how the viruses reach the submandibular glands and how the virus becomes transferred to the epithelial cells.

Serological analysis showed that IgG2a was the antibody subclass that was mainly produced except that low titers of IgG1 and IgG2c were also detected in mice inoculated with a high dose at 35-49dpi. IgM was not detected through the whole course of the experiments for both MCMV strains. This could be due to the low sensitivity of the MCMV-specific IPMA, which is consistent with our positive control (low sensitivity of the mice adapted influenza-specific IPMA), or the suppressive effect of MCMV on the production of IgM by T cell cytokines. T cell cytokines are responsible for the immunoglobulin class switching mechanism in mouse and human [50]. The strong induction of IgG2a is generally known to be mediated by interferon γ (IFNγ) [51, 52]. Because IFNγ as well as other T cell cytokines were not evaluated in this study, we could not assess the role of IFNγ in the orientation of the antibody isotype switch. The complement-dependent neutralization test demonstrated that complement plays a critical role in neutralizing MCMV since antibodies without complement/with inactivated complement did not

neutralize MCMV infection. Since IgG2a is the predominant viral-specific antibody, we can state that complement-dependent IgG2a-mediated inactivation of MCMV is an important anti-MCMV defense. This is consistent with the characteristics of antibody isotype IgG2a to fix complement in mice [53]. Neutralizing antibody titers were higher at 35 and 49dpi, which may explain in part the clearance of HaNa1 at 49dpi and Smith at 35 and 49dpi in submandibular glands in the high inoculation dose group. Similar patterns also occurred for both strains at 42 and 49dpi in the low inoculation dose groups. The control of virus infection at the end of the experiments is also most probably mediated by the cell-mediated immunity, which is generally considered to be the most important factor in controlling CMV infections [54, 55].

In summary, mouse models with MCMV mimicking natural infection were set up in our study. Through comparing the pathogenesis of two MCMV strains, we found that infections occurred via respiratory route and then local replication in tissues of the upper respiratory tract, after which the virus may spread via a cell-associated viremia to other target organs such as spleen, liver, kidneys and submandibular glands. Only Smith caused a productive infection in spleen, liver and kidneys, whereas the HaNa1 isolate did not. The latter is similar to the outcome of an HCMV primary infection in immunocompetent hosts; it is only causing a limited virus-associated spread to the salivary glands but not to multiple internal organs. Therefore, the newly isolated MCMV HaNa1 isolate is interesting to be used in mouse models in order to get better insights into HCMV natural infections in immunocompetent hosts via oronasal exposure. Increasing the inoculation dose strongly elevated virus production in the nasal mucosa and submandibular glands and cell-associated viremia during the early stage of infection, reduced the time of appearance of antibodies, and increased the level of antibodies. In this study, we predominantly focused on the kinetics of virus production in different organs. As known for a long time, CMVs spread in a strict cell-associated way. This was not investigated in depth in the present study. In the near future, we will focus on the kinetics of cell-associated motions of MCMV infected leukocytes in order to get a

full insight how the replications in the respiratory tract and submandibular glands are linked to each other. It is very important to understand how MCMV starts up replication in the submandibular glands and maintains its replication in this organ for a long period.

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Chapter 4

Pattern of circulation of MCMV mimicking natural infection upon oronasal inoculation

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Summary

Cytomegaloviruses may infect mammals via the oronasal route. However, up till now it remains unclear how this exposure leads to a general infection and shedding. To address this issue, BALB/c female mice were oronasally inoculated with either the highly passaged murine cytomegalovirus (MCMV) Smith or the low passaged MCMV HaNa1. Virus titration showed a productive virus replication of both strains in the nasal mucosa from 1 dpi until the end of the experiment (14 dpi), in lungs from 5 until 14 dpi, and in submandibular glands from 7 until 14 dpi. In contrast to MCMV HaNa1, MCMV Smith also established a low level productive infection in abdominal organs (spleen, liver and kidneys) from 5 dpi (spleen), 7 dpi (liver), and 10 dpi (kidneys) until the end of the experiment. Co-culture showed that for both strains, cell-associated virus was detected in a non-infectious form in nasopharynx-associated lymphoid tissues (NALT) from 1 until 14 dpi, in submandibular lymph nodes from 3 until 5 dpi, in deep cervical lymph nodes from 3 until 14 dpi, in mediastinal lymph nodes from 7 until 14 dpi, in spleen from 5 until at least 10 dpi and in the peripheral blood mononuclear cells (PBMC) at 7 and 10 dpi. The present study shows that upon oronasal exposure, MCMV first enters the nasal mucosa and NALT, from where the virus disseminates to the spleen possibly via the draining lymphatic system and blood; a subsequent cell-associated viremia transports MCMV to submandibular glands and for MCMV Smith also to liver and kidneys, where a second productive replication starts.

1 Introduction

Human cytomegalovirus (HCMV) is a ubiquitous pathogen that establishes life-long, mainly asymptomatic infections. However, these infections may be life-threatening in immunocompromised individuals, such as preterm neonates [1, 2], AIDS patients [3] and transplant recipients [4, 5]. Due to the strict species-specificity of HCMV, it is not possible to study its replication in experimental models. Because of high similarities in CMV's biology and disease spectrum, murine cytomegalovirus (MCMV) infections in mice have been most commonly and widely used as a model to understand the pathogenesis of HCMV infections in humans [6, 7].

Humans can be infected with HCMV by oral exposure, such as consuming HCMV-contaminated breast milk [8-12] and oral uptake of HCMV-contaminated saliva [13, 14]. HCMV was also recently detected in the nasal mucosa of healthy individuals [15], infants [16], transplant recipients [17], and AIDS patients [18-20]. This oronasal transmission may also be the main route for MCMV infections in mice, since mice have been successfully infected with MCMV by oral exposure to MCMV-contaminated breast milk [21], by oral inoculation [22, 23], and by nasal exposure [24, 25]. Therefore, the oral and nasal cavities are likely to be major primary replication sites of CMV and shedding [13, 15, 16, 18, 20]. In our previous work, an MCMV infection model has been established that mimics natural infection using a recent Belgian MCMV isolate HaNa1 [26]. However, it remains unclear how the virus enters and reaches other organs such as salivary glands after natural oronasal exposure.

As known for a long time, CMV spreads in a strict cell-associated way in mice and humans [27-29]. Different peripheral blood leukocyte (PBL) subsets have been involved in viremia and latency in both immune-compromised and immune-competent subjects [30-32]. Nonpermissive monocytes likely serve as latent reservoirs that support dissemination by maturing into permissive macrophages or dendritic cells that reactivate virus [33-35]. Besides blood circulation, the lymph drainage may be another important pathway for CMV dissemination as well, since

MCMV could be detected in draining lymph nodes [24].

In the oral and nasal cavities, mucosal surfaces, mucosa-associated lymphoid tissues and mucosa-draining lymph nodes (LN) constitute the inductive sites for mucosal immunity against exogenous pathogens [36-38]. Since mice have no tonsils, the nasopharynx-associated lymphoid tissues (NALT) are the most important tissues for the generation of mucosal immunity after inhalation of antigens [36, 39-42]. The NALT is found at the base of the nasal cavity and consists of bilateral strips of lymphoid tissue. In human, the functional homologue of rodent NALT is the Waldeyer's ring [43, 44]. As previous researchers have reported the detection of HCMV DNA in the Waldeyer's ring [45, 46], the exact role of these mucosa-associated lymphoid tissues in the dissemination of CMV requires further investigation.

In the present study, BALB/c mice were experimentally inoculated with two MCMV strains (the highly passaged MCMV Smith strain and the low passaged MCMV HaNa1 strain) to elucidate the kinetics of virus dissemination upon oronasal exposure. Blood, lymph nodes and other relevant organs were collected at different time points and used to detect infectious virus by virus titration and cell-associated virus by co-cultivation.

2 Materials and methods

2.1 Cells and viruses

Primary BALB/c mouse embryonic fibroblasts (MEFs) were cultivated at 37 °C with 5% CO_2, in minimum essential medium (MEM) with 10% fetal calf serum (FCS) and a mixture of antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin and 0.5 mg/ml gentamicin), and were used at the second passage. Viruses used in the present experiments were the second passage of clone1 of the MCMV HaNa1 strain, which was isolated from a Belgian domestic mouse in the laboratory of the authors [26], and the MCMV Smith strain from ATCC was at a high passage in the continuous murine bone marrow stromal cell line (M2-10B4). Seven genes from MCMV HaNa1 have been sequenced and submitted to GenBank: m06 gene (accession No.: KR184668), m033 gene (accession No.: KR184669), mck-2 including exon1 (m131 gene) and exon2 (m129 gene) (accession No.: KR184670), m138 gene (accession No.: KR184671), m144 gene (accession No.: KR184672), m152 gene (accession No.: KR184673) and m157 gene (accession No.: KT289520). The nucleic acid/deduced amino acid sequence alignments between the known HaNa1 genes and the corresponding Smith genes were done by MEGA5.21. The results are shown in Table 1.

Table 1. Nucleic acid/deduced amino acid sequence identity of genes/gene products of MCMV strains

Genes	Sequence identity MCMV HaNa1 & MCMV Smith	
	Nucleic acids	Deduced amino acids
m06	99.8%	100%
m33	99.7%	99.7%
m129	100%	100%
m131	100%	100%
m138	99.5%	99.1%
m144	95%	93.5%
m152	99.2%	98.4%
m157	95.7%	91.5%

2.2 Animals and virus inoculation

Specific pathogen-free 6 to 8-week-old BALB/c female mice were used in the present study. Eighteen mice were inoculated with 10^6 $TCID_{50}$ MCMV HaNa1 in 100 µl PBS via intranasal (25 µl) and peroral (75 µl) route without anesthesia. Since mice are obligate nose breathers [41] and intranasal administration of large volumes (>30µl) per time can result in direct delivery into lungs [47, 48], a small amount of inoculum (5 µl) was repeatedly instilled in each nostril. Each application was done with several minutes interval to avoid direct flow of the inoculum into the lungs. For the oral inoculation, 25 µl inoculum was given three times with a few minutes interval between each inoculation. Another 18 mice were inoculated with MCMV Smith using the same methodology. Mice were kept in isolation and fed *ad libitum*. For each MCMV strain, three infected mice were euthanized at 1, 3, 5, 7, 10 and 14 dpi. Another 3 mice were mock inoculated with PBS and euthanized at the end of the experiment.

2.3 Collection of blood and tissues

For blood collection, mice were anesthetized with 130 µl of 10 mg/ml sodium pentobarbital (KELA, Belgium). Afterwards, approximately 0.6 ml blood per mouse was taken from the orbital sinus with a heparinized Pasteur pipet and kept in an eppendorf with 0.5 ml PBS containing 5 U/ml heparin (Leo Pharma, Zaventem, Belgium). Plasma was collected by centrifugation (200 g for 10 min) and stored at -70 °C for virus titration. PBMC were isolated by density gradient centrifugation with Ficoll-Paque (GE Healthcare). After blood collection, mice were euthanized with another 200 µl of 10 mg/ml sodium pentobarbital (KELA, Belgium), and different tissues were collected under aseptic conditions. Tissues obtained from the respiratory system were nasal mucosa, pharynx, trachea, lungs, thymus, nasopharynx-associated lymphoid tissues (NALT) (collected as described in references [38, 49]), deep cervical LN and mediastinal LN. Tissues collected from the alimentary system were oral mucosa, submandibular glands, esophagus, small intestines, submandibular LN,

superficial parotid LN, Peyer's patches and mesenteric LN. Other abdominal organs that were collected were spleen, liver and kidneys. The lymph nodes, thymus, PBMC and half of the spleen were used for co-culture (see below). From the other half of the spleen and the other tissues, 10% homogenates were made in PBS and stored at -70 °C until virus titration was performed.

2.4 Virus titration

MEFs were inoculated with 50 µl of 10-fold serial dilutions of the 10% tissue homogenates. After 1 h incubation at 37 °C, 100 µl of medium were added and the cells were further incubated for 7 days. After 7 days, the presence of a cytopathic effect (CPE) was assessed by light microscopy (Olympus Optical Co., Hamburg, Germany) and the virus titer was determined as 50% tissue culture infectious dose ($TCID_{50}$) according to the method of Reed and Muench.

2.5 Preparation of single-cell suspensions for co-culture

As known for a long time, CMV spreads in a strict cell-associated way. In order to investigate the kinetics of cell-associated MCMV in different lymphoid organs, a co-culture assay was used in this study. For the preparation of single-cell suspensions from lymph nodes, two needles were used to shear lymph nodes into little pieces in 6-well plates with 150 µl RPMI 1640 medium supplemented with 2% FCS and a mixture of antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin and 50 µg/ml gentamycin). Afterwards, the cell suspensions were filtered using 100-µm-pore-size cell strainers (BD Biosciences, Bedford, MA) to obtain single-cell suspensions. Spleen and thymus were cut into small pieces and digested with trypsin for 5 min at 37 °C. Afterwards, the digested tissue was transferred to a 100-µm-pore-size cell strainer. The single-cell suspensions from the spleen were centrifuged at 200 g for 10 min. Pellets were resuspended in 3 ml of red blood cell lysis buffer (0.15 M NH_4Cl, 1 mM $KHCO_3$, 0.1 mM EDTA, pH 7.2) and incubated on ice for 5 min. The reaction was stopped by adding PBS. Cells were washed once with DPBS, centrifuged, and resuspended in RPMI 1640 medium. MEFs (2×10^5) were seeded in 24-well plates

two days before the co-culture assay was performed. All PBMC and single-cell suspensions from lymph nodes, thymus and spleen were brought onto the monolayer of MEFs in the 24-well plates and covered with 1 ml carboxymethylcellulose (CMC) medium (1/4 2 x MEM, 1/4 2 x RPMI and 1/2 2 x CMC supplemented with 5% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamycin, 0.1 mM NEAA and 1 mM sodium pyruvate). Afterwards, the plates were centrifuged at 750 g for 10 min. Subsequently, the cells were cultivated at 37 °C in an incubator for 8 days (lymph nodes and PBMC) or 12 days (spleen and thymus). The medium was partially changed every 4 days. Afterwards, the plaques were counted with a light microscope (Olympus Optical Co., Hamburg, Germany) to quantify the virus loads in various lymph nodes, spleen, thymus and PBMC.

2.6 Ethical statement

All animal experiments were approved by the local Ethical Committee of the Faculty of Veterinary Medicine, Ghent University (EC2013-47).

3 Results

3.1 Dissemination of the low passaged MCMV HaNa1 strain after oronasal inoculation

Table 2 represents the results of virus titration of various tissues at different time points after oronasal inoculation of BALB/c mice with MCMV HaNa1 and MCMV Smith. The nasal mucosa was the first site where infectious virus was found, with a mean virus titer of $10^{2.8}$ $TCID_{50}$/g at 1 dpi. From 5 dpi onwards, infectious virus was additionally detected in the lungs, and later on also in the submandibular glands (7 dpi). In all these tissues, virus replication persisted till the end of the experiment (14 dpi), and reached the highest mean virus titers in the nasal mucosa at 7 dpi ($10^{4.5}$ $TCID_{50}$/g) and submandibular glands at 14 dpi ($10^{6.3}$ $TCID_{50}$/g). Infectious virus was not detected in the oral mucosa, pharynx, trachea, esophagus, small intestines, spleen, liver, kidneys and plasma throughout the whole experiment.

Investigation of the cell-associated virus trafficking in lymphoid organs and blood by

a co-culture assay (Table 3) revealed that the cell-associated virus was detected in the NALT as early as 1 dpi, in the submandibular LN and deep cervical LN from 3 dpi, in the spleen from 5 dpi, in the PBMC and mediastinal LN from 7 dpi. The cell-associated virus persisted in the NALT from 1 dpi until the end of the experiment (14 dpi), in the submandibular LN from 3 dpi until 10 dpi (except for 7 dpi), in the deep cervical LN from 3 dpi until the end of the experiment (14 dpi), in the mediastinal LN at 7 and 14 dpi, in the spleen from 5 dpi until at least 10 dpi (co-culture of spleen was not done after 10 dpi), and in the PBMC from 7 dpi until 10 dpi. The other lymphoid tissues (superficial parotid LN, thymus, Peyer's patches and mesenteric LN) remained negative throughout the whole experiment. Overall, the cell-associated virus was most frequently detected in NALT, followed by the deep cervical LN.

Table 2[#]: MCMV HaNa1/MCMV Smith replication in different tissues collected at 1, 3, 5, 7, 10 and 14 dpi.

System	Samples	Virus titers (log_{10} $TCID_{50}$/g tissue or log_{10} $TCID_{50}$/ml plasma) (HaNa1/***Smith***)*					
		1 dpi	3 dpi	5 dpi	7 dpi	10 dpi	14 dpi
Respiratory system	Nasal mucosa	2.6/3.3/2.6	2.6/2.0/2.0	4.0/3.8/3.6	5.0/4.0/4.6	3.3/3.8/4.6	5.0/5.0/3.3
		2.6/2.6/3.0	***-/2.0/2.3***	***2.8/2.6/3.3***	***3.3/2.6/2.3***	***3.8/2.3/2.3***	***3.8/3.3/3.8***
	Pharynx	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-
		-/-/-	***-/-/-***	***-/-/-***	***-/-/-***	***-/-/-***	***-/-/-***
	Trachea (pool)	-	-	-	-	-	-
		-	***-***	***-***	***-***	***-***	***-***
	Lungs	-/-/-	-/-/-	2.3/-/-	2.3/-/3.6	-/-/-	-/-/2.6
		-/-/-	***-/-/-***	***-/2.3/-***	***4.8/4.8/-***	***-/2.0/-***	***-/-/2.3***
Alimentary system	Oral mucosa (pool)	-	-	-	-	-	-
		-	***-***	***-***	***-***	***-***	***-***
	Submandibular glands	-/-/-	-/-/-	-/-/-	2.3/-/-	5.8/6.3/5.8	6.3/6.3/6.3
		-/-/-	***-/-/-***	***-/-/-***	***-/-/3.0***	***3.6/3.3/3.0***	***4.0/3.8/4.0***
	Esophagus (pool)	-	-	-	-	-	-
		-	***-***	***-***	***-***	***-***	***-***
	Small intestines	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-
		-/-/-	***-/-/-***	***-/-/-***	***-/-/-***	***-/-/-***	***-/-/-***
Blood	Plasma	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-
		-/-/-	***-/-/-***	***-/-/-***	***-/-/-***	***-/-/-***	***-/-/-***
Abdominal organs	Spleen	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-
		-/-/-	***-/-/-***	***-/2.0/-***	***2.6/2.3/-***	***2.0/-/-***	***2.3/-/2.3***

Liver	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-
	-/-/-	***-/-/-***	***-/-/-***	***2.3/-/2.3***	***-/-/-***	***-/-/2.3***
Kidneys	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-
	-/-/-	***-/-/-***	***-/-/-***	***-/-/-***	***2.3/-/2.3***	***2.8/-/2.3***

[#] : The data of Table 2 were partially adapted from our previous published paper in Veterinary Research [26]. We have the right to reproduce our data for addressing a new unsolved question if the correct citation details are given according to the copyright policy of Veterinary Research (http://www.veterinaryresearch.org/about/faq/journal-copyright-policy).

* : data from MCMV HaNa1-infected mice were shown in regular text; data from MCMV Smith-infected mice were shown in bold/italics.

- : under detection limit ($10^{1.8}$ $TCID_{50}$/g tissue or $10^{0.8}$ $TCID_{50}$/ml plasma). At each time point, the data from three mice are given. Only trachea, oral mucosa and esophagus from three mice were pooled at each time point.

Table 3. Co-cultivation of single-cell suspensions of different lymphoid organs and PBMC from the MCMV HaNa1/MCMV Smith-infected mice with MEFs

Samples	Number of infected cells / all cells collected from the whole organ or all PBMC (HaNa1/***Smith***)*					
	1 dpi	3 dpi	5 dpi	7 dpi	10 dpi	14 dpi
NALT (pool)	19	26	34	75	53	60
	28	***22***	***24***	***41***	***33***	***35***
Submandibular LN	-/-/-	67/19/12	47/-/-	-/-/-	-/-/15	-/-/-
	-/-/-	***27/-/-***	***56/24/-***	***-/-/-***	***-/-/-***	***-/-/-***
Superficial parotid LN	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-
	-/-/-	***-/-/-***	***-/-/-***	***-/-/-***	***-/-/-***	***-/-/-***
Deep cervical LN	-/-/-	23/55/22	79/52/43	38/-/-	18/-/24	-/41/-
	-/-/-	***44/24/10***	***60/69/-***	***-/63/14***	***24/23/10***	***44/-/-***
Mediastinal LN (pool)	-	-	-	15	-	21
	-	***-***	***-***	***27***	***38***	***31***
Mesenteric LN	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-
	-/-/-	***-/-/-***	***-/-/-***	***-/-/-***	***-/-/-***	***-/-/-***
Peyer's patches (PP)	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-
	-/-/-	***-/-/-***	***-/-/-***	***-/-/-***	***-/-/-***	***-/-/-***
Thymus	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-
	-/-/-	***-/-/-***	***-/-/-***	***-/-/-***	***-/-/-***	***-/-/-***
Spleen	-/-/-	-/-/-	62/49/9	25/81/114	66/46/41	ND
	-/-/-	***-/-/-***	***113/160/82***	***215/290/133***	***201/110/125***	***ND***
PBMC	-/-/-	-/-/-	-/-/-	-/22/43	18/-/-	-/-/-
	-/-/-	***-/-/-***	***-/-/-***	***-/47/41***	***-/-/-***	***-/-/-***

* : data from MCMV HaNa1-infected mice are shown in regular text; data from MCMV Smith-infected mice are shown in bold/italics. ND : not determined.

3.2 Dissemination of the highly passaged MCMV Smith strain after oronasal inoculation

Dissemination of the highly passaged MCMV Smith strain was very similar to the replication pattern of the low passaged MCMV HaNa1 strain (Table 2, Bold/italic text). Infectious virus was first detected in the nasal mucosa (1 dpi), followed by lungs (5 dpi) and submandibular glands (7 dpi). Similarly to the HaNa1 strain, the Smith strain persisted in these tissues until the end of the experiment (14 dpi). Smith strain reached the highest mean virus titers at 14 dpi in the nasal mucosa ($10^{3.6}$ $TCID_{50}$/g) and submandibular glands ($10^{3.9}$ $TCID_{50}$/g). In comparison with the HaNa1 strain, a few clear differences were found. Only MCMV Smith caused a productive infection with a low level of virus replication in the spleen from 5 dpi until the end of experiment 14 dpi, in the liver at 7 dpi (n=2) and 14 dpi (n=1), and in the kidneys at 10 dpi (n=2) and 14 dpi (n=2). No infectious virus was detected in the oral mucosa, pharynx, trachea, esophagus, small intestines, and plasma throughout the whole experiment.

The results of the co-culture assay showed that dissemination of the Smith strain to the spleen and other lymphoid organs was comparable to the findings obtained with the HaNa1 strain, with the detection of cell-associated viruses in the NALT from 1 dpi until 14 dpi, in the submandibular LN from 3 dpi until 5 dpi, in the deep cervical LN from 3 dpi until 14 dpi, in the mediastinal LN from 7 dpi until 14 dpi, in the spleen from 5 dpi until at least 10 dpi (co-culture of spleen was not done after 10 dpi), and in the PBMC only at 7 dpi. The other lymphoid tissues (superficial parotid LN, thymus, Peyer's patches and mesenteric LN) remained negative throughout the whole experiment. Overall, the amount of cell-associated virus in the spleen of MCMV Smith-infected mice was remarkably higher compared to the HaNa1 strain. NALT was the most frequent site where the cell-associated virus was detected, followed by the deep cervical LN.

4 Discussion

CMV can certainly be transmitted from infected to susceptible hosts by oronasal route. However, it remains elusive how the virus spreads throughout the body upon oronasal exposure. In the present study, the viral dissemination was investigated. It was shown that a productive replication first occurred in the nasal mucosa (1 dpi), which coincided with the detection of cell-associated virus in the NALT. Subsequently, the virus was detected in the submandibular LN and deep cervical LN (3 dpi). Afterwards, the virus was found throughout the entire body, with detection of infectious and/or cell-associated viruses in the lungs (5 dpi) and spleen (5 dpi), and in PBMC, submandibular glands and mediastinal LN at 7 dpi. MCMV Smith, in contrast to MCMV HaNa1, was able to spread further to liver and kidneys with a productive replication.

Based on these results, a general hypothetical model for MCMV dissemination throughout the body can be proposed (Fig 1). Upon natural exposure to MCMV, the nasal mucosa and NALT serve as portal of entry for MCMV. From these primary replication sites, the virus is transported in non-productively infected leukocytes to the draining lymph nodes (submandibular LN and deep cervical LN) and finally to the spleen and lungs possibly via blood. The route of antigen uptake at the surface mucosae and drainage to the lymphoid tissues is consistent with previous reports using fluorescein isothiocyanate (FITC), radiochemical ^{131}I human serum albumin, horseradish peroxidase-gold complex particles and other viruses to study the patterns of drainage [36, 43, 50-54]. As the viremia could only be detected after the spleen became positive, we hypothesize that the spleen serves as a transfer hub from where a secondary replication is initiated in various other tissues such as submandibular glands (Smith and HaNa1), liver and kidneys (only Smith).

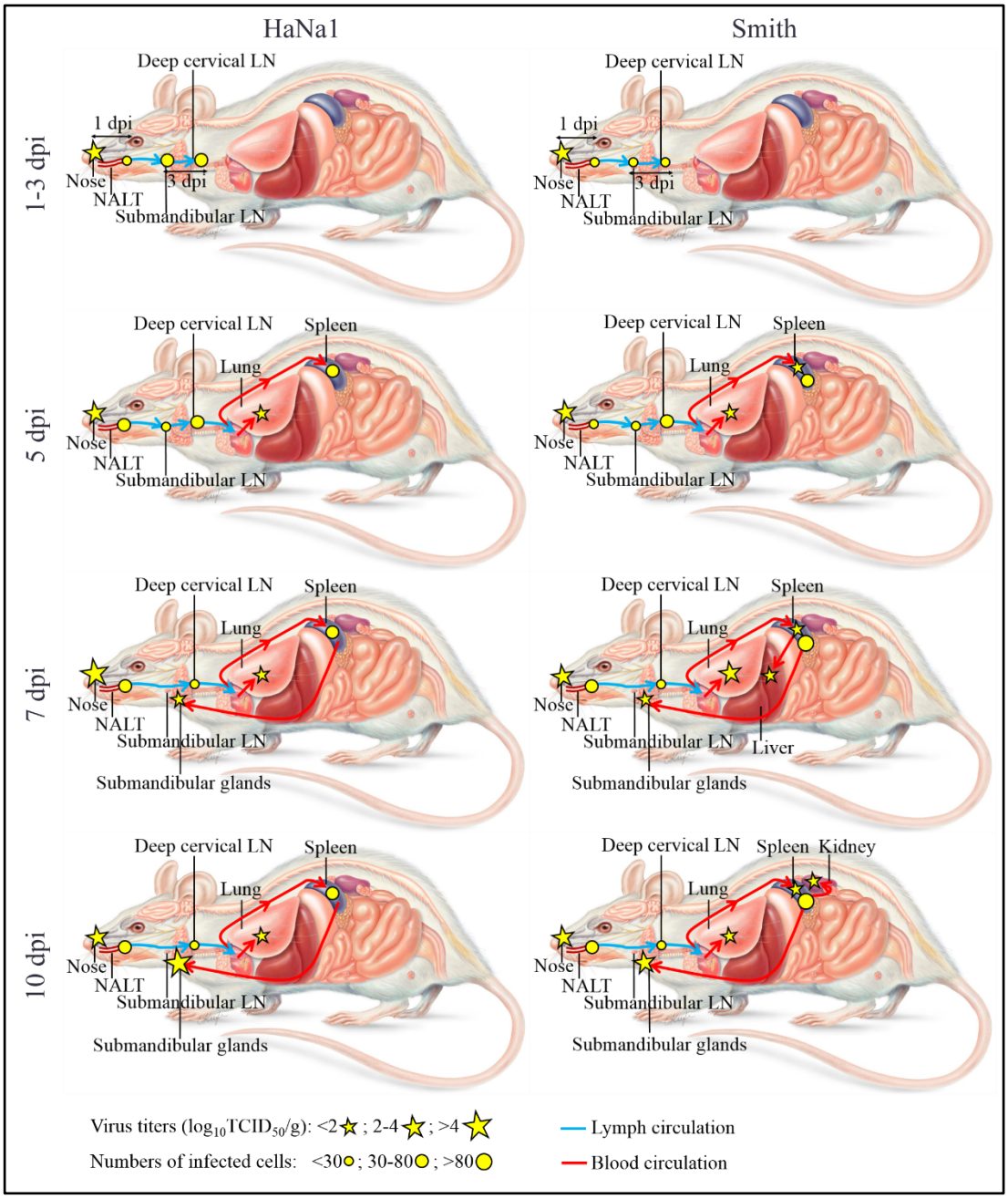

Figure 1. Illustration of MCMV HaNa1 and MCMV Smith dissemination upon natural oronasal inoculation. Stars represent infectious virus, and circles represent cell-associated virus. After natural oronasal inoculation, both strains first reach the nasal mucosa and NALT. Subsequently, both MCMV Smith and MCMV HaNa1 spread to the submandibular LN, deep cervical LN, lungs and spleen possibly via lymph and blood. A subsequent cell-associated viremia carries the virus from the spleen to various tissues such as submandibular glands (Smith and HaNa1), liver and kidneys (only Smith), where a secondary replication is started. Productive infections in abdominal organs such as spleen, liver and kidneys were only detected in MCMV Smith-infected mice. The mouse model pictures are derived from artist Laurie O'Keefe with her permission.

The present study provides information on the replication kinetics of MCMV in vivo. Findings in many but not all of the organs are consistent. In some organs, the variability might be an issue. However, when we put all our previous published studies [26, 55] and other unpublished data together, the aforementioned results are fully confirmed. They exclude that data from our study are coincidental findings due to limited animal numbers (n=3 per time point). Moreover, it is not appropriate to discuss and make overconclusions on individual animals in a kinetic pathogenesis study. For example, cell-associated virus (infected PBMC) was detected in only 4 out of 6 mice at 7 dpi in the present study. This is most probably due to the low sensitivity of co-cultivation. Indeed, in a more recent study, it was also observed that cell-associated virus in PBMC was only detected in 3 out of 6 inoculated mice at 7 dpi. However, by the use of an in-house qPCR, viral DNA could be detected in PBMC of all 6 inoculated mice (576-1670 copies/10^6 PBMC).

According to our results, viral trafficking likely occurs in a cell-associated manner upon oronasal inoculation. In blood, cell-associated virus (determined by co-culture of PBMC) but no cell-free virus (determined by virus titration of isolated plasma) was detected. Our data are in line with previous studies using intraperitoneal inoculation [29, 56]. In addition, in various lymph nodes, cell-associated virus (determined by co-culture of single cells) but no cell-free virus (determined by virus titration of lysed single cells; data not shown) was detected. In our study, the identification of the carrier cells was not investigated. Earlier studies demonstrated that MCMV-encoded CC chemokine, MCK-2, promotes recruitment of CX3CR1^{hi} patrolling monocytes to initial infection sites in the mouse, from where these cells become infected and traffic via the blood stream to distal sites [57, 58]. These studies indicate that CX3CR1^{hi} patrolling monocytes may be important carrier cells.

In the present study, NALT was identified as a portal of entry for MCMV upon intranasal inoculation. This finding is consistent with its anatomic and functional characteristics. In humans, the functional homologue of the rodent NALT is the Waldeyer's ring [44]. Previous researchers have reported the detection of HCMV

DNA in the Waldeyer's ring [45, 46], and hence, based on our results, it can be hypothesized that the Waldeyer's ring may be an important entry site for HCMV upon natural exposure.

The lungs are often affected by CMV. The pneumonia-causing ability of the virus has been studied by intranasal inoculation of anesthetized mice with MCMV. With this inoculation technique, it has been shown that the virus can directly flow into the lungs [48, 59]. Therefore, lungs are usually believed to be a primary target organ for MCMV via intranasal inoculation under anesthesia [25, 59]. However, it is very well possible that upon anesthesia, the inoculum directly flows into the lungs due to the absence of a sneezing reflex. Therefore, it remains to be elucidated how the virus reaches the lungs during a natural CMV infection. In the present study, infection of lungs was only detectable from 5 dpi, and no infectious virus was found in the trachea throughout the entire experiment. In addition, histopathological and immunohistochemical analysis of the lungs revealed a clear interstitial pneumonia and viral antigens in the interstitium (unpublished data). All these data suggest that infection of lungs in both MCMV Smith and HaNa1 infected mice result from the hematological spread of the virus, and not by inhalation as described by others for anesthetized mice [25, 59].

During the entire experiment, no infectious or cell-associated virus was detected in the pharynx, esophagus, small intestines, Peyer's patches and mesenteric LN. In consistence with a previous report [25], this illustrates that the digestive tract and associated lymphoid tissues are not an entrance gate for the virus. Potential explanations may be the absence of the Waldeyer's ring in the pharynx of mice and/or presence of a low pH and proteases in the stomach, detergents in the bile and proteases/lipases in the small intestines. The latter conditions are too harmful for this enveloped virus. In order to examine if MCMV can use the oral route for infecting mice, a small experiment was performed in which nine mice were inoculated with MCMV HaNa1 and nine mice were inoculated with MCMV Smith via oral route. Only 2 mice became infected with MCMV HaNa1, and 3 with Smith. The poor

infectivity of MCMV via oral route is quite surprising, as CMV is thought to spread horizontally via saliva. MCMV most possibly prefers spread via nasal secretions because nose-to-nose contacts and smelling are important activities in the social life of mice.

In contrast to MCMV HaNa1, MCMV Smith productively infects the spleen, liver and kidneys. Since the experimental hosts are identical, the degree of the viral replication (productive versus non-productive infection) is due to genetic differences between the two strains. One determinant of MCMV pathogenicity is MCMV-encoded chemokine 2 (MCK-2) [57, 60]. However, this chemokine is identical between HaNa1 strain and Smith strain (Table 1). In order to find the answer to this question, the sequencing of complete genome of HaNa1 is in progress and the full genome will be compared with that of the Smith strain. The results may pave the way to identify pathogenicity determinants.

In summary, we have shown that MCMV-infected leukocytes in blood and lymphoid organs contribute to virus dissemination within the host upon oronasal exposure. Considering that the shedding from salivary glands results from this systemic dissemination, further investigation of the critical role of blood, lymph nodes and spleen in CMV dissemination could lead to the identification of new targets to control viral transmission and disease. In the near future, we will focus on the role of spleen in MCMV dissemination in order to investigate whether MCMV exploits the spleen as a transfer hub for systemic dissemination upon oronasal inoculation.

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Chapter 5

MCMV exploits the spleen as a transfer hub for systemic dissemination upon oronasal inoculation

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Summary

Murine cytomegalovirus (MCMV) infection in mice is a commonly used animal model for studying human cytomegalovirus (HCMV) infections. In our previous studies, a mouse model based on an oronasal MCMV infection was set up for mimicking a natural infection, and the spleen was hypothesized to regulate viremia and virus dissemination to distal organs such as submandibular glands. Here, the role of the spleen during an MCMV infection was investigated by the comparison of intact and splenectomized Balb/c mice. Both highly passaged MCMV Smith and low passaged MCMV HaNa1 were used. Various samples were collected at 7, 14, and 21 days post inoculation (dpi) for analyses by virus isolation/titration, co-cultivation and qPCR. The results showed that for both virus strains, 1) cell-associated virus in PBMC (determined by co-cultivation) was detected in intact mice but not in splenectomized mice; 2) the mean viral DNA load in PBMC of splenectomized mice was 4.4-(HaNa1)/2.7-(Smith) fold lower at the peak viremia (7 dpi) compared with that of intact mice; and 3) infectious virus in the submandibular glands was detected later in splenectomized mice (14 dpi) than in intact mice (7 dpi). Moreover, the average virus titers in submandibular glands of splenectomized mice were 10-(HaNa1)/7.9-(Smith) fold lower at 14 dpi and 1.7-(HaNa1, $P = 0.45$)/2.1-(Smith, $P = 0.29$) fold lower at 21 dpi compared with that of intact mice. Upon inoculation with MCMV Smith, infectious virus was found in the kidneys and liver of intact mice, but not in splenectomized mice. Taken together, all these data clearly demonstrate that virus dissemination to distant organs is reduced in splenectomized mice, further confirming the importance of the spleen as a viremia booming site for a natural MCMV infection.

1 Introduction

Human cytomegalovirus (HCMV) is a significant viral pathogen, mainly affecting immunocompromised adults and developing fetuses [1]. HCMV infection in the immunocompetent host is usually asymptomatic or produces only mild symptoms, which makes early natural infection difficult to detect and study [2]. Splenomegaly is a common clinical finding in patients with symptomatic HCMV infection [3], but the exact role of the spleen in the pathogenesis of a natural HCMV infection is unknown. Due to the strict species specificity of cytomegaloviruses, it is difficult to study HCMV infection in experimental animals [1, 4]. Since HCMV shares a lot of similarities in pathogenesis with murine cytomegalovirus (MCMV), MCMV infection in mice has become a commonly used animal model for studying HCMV infection in humans [5, 6].

The systemic infection upon intraperitoneal inoculation of MCMV has been widely used to study the pathogenesis of a cytomegalovirus [5]. MCMV can infect various organs such as spleen, liver, kidneys and salivary glands in mice using intraperitoneal inoculation [4, 7-9]. Among all these organs, the spleen is a very important target organ during early infection [7, 9] and, together with the salivary glands, the main organ for viral latency [10, 11]. MCMV infection in mice following intraperitoneal inoculation is thought to occur by an initial dissemination of virus from the portal of entry to the spleen, followed by an abundant viral replication at this site [12, 13]. MCMV has a broad cell tropism in the spleen, with macrophages and dendritic cells [14-17], sinus-lining cells and reticular fibroblasts [9, 18], endothelial cells and hematopoietic cells [19, 20] as targets. Subsequent to the replication in the spleen during which a second viremia occurs, virus is disseminated via blood leukocytes, ultimately reaching additional sites of infection, such as salivary glands [9, 12, 13, 21]. All these previous studies indicate that the spleen plays an important role in the pathogenesis of a systemic MCMV infection. However, it remains elusive if the spleen also plays a central role in virus dissemination during a more natural infection via the oronasal route. In our previous studies, a mouse model based on an oronasal

MCMV infection was set up to mimic a natural CMV infection [21, 22]. We found that after oronasal infection, MCMV first replicates in the nasal mucosa and nasopharynx-associated lymphoid tissues (NALT), and then spreads to the submandibular lymph nodes, deep cervical lymph nodes and the spleen via lymph and blood. Subsequently, a cell-associated viremia carries the virus to distal organs such as submandibular glands, liver and kidneys. As the cell-associated viremia was not detectable until the spleen became positive, it was hypothesized that the spleen serves as a transfer hub for virus dissemination throughout the body during natural infection.

To investigate the role of the spleen during a natural MCMV infection, intact and splenectomized mice were inoculated oronasally with either the highly passaged MCMV Smith strain or the low passaged MCMV HaNa1 strain. Plasma and various organs were collected at different time points and used to detect infectious virus by virus titration, and the associated lymph nodes were collected and used to detect cell-associated virus by co-cultivation. In addition, the average MCMV DNA load in peripheral blood mononuclear cells (PBMC) of intact and splenectomized mice was determined by qPCR.

2 Materials and methods

2.1 Viruses, cells, and animals

Two MCMV strains were used in this study: the second passage of clone 1 of the MCMV HaNa1 strain and the MCMV Smith strain at a high passage in the continuous murine bone marrow stromal cell line (M2-10B4) [22]. Primary Balb/c mouse embryonic fibroblasts (MEFs) were cultivated in minimum essential medium (MEM) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 0.5 mg/ml gentamicin, 2% lactalbumin and 10% fetal calf serum (FCS). MEFs were used at the second passage. Virus titration was performed in 96-well plates and titers were expressed as the viral dilution infecting 50% of the cell cultures ($TCID_{50}$), following the methodology described by Reed and Muench [23].

2.2 Experimental design

According to our previous data [21], peak infection of blood leukocytes occurs at 7 dpi and virus can be detected in salivary glands, liver and kidneys at 14 dpi. Therefore, these time points were selected in the present study. Additionally, 21 dpi was selected to evaluate the effect of splenectomy on the course of the infection. Intact and splenectomized 6-week-old Balb/c female mice were used in this experiment. Nine intact and twelve splenectomized Balb/c mice were inoculated with 10^6 $TCID_{50}$ MCMV HaNa1 via intranasal (25 µl) and peroral (75 µl) route without anesthesia. Mice were kept in isolation and fed *ad libitum*. At each time point (7, 14, and 21 dpi), three intact and four splenectomized mice were euthanized. Another nine intact and nine splenectomized Balb/c mice were inoculated with MCMV Smith strain using the same methodology. Three intact and three splenectomized Balb/c mice were euthanized at 7, 14 and 21 dpi. Another three intact and three splenectomized Balb/c mice were mock inoculated with PBS and euthanized at the end of the experiment.

2.3 Collection of blood and tissues

For blood collection, each mouse was anesthetized by intraperitoneal administration with 130 µl of 10 mg/ml sodium pentobarbital (KELA, Belgium). According to a previously described method [22], 0.6 ml blood per mouse was taken from the orbital sinus with a heparinized Pasteur pipet and kept in an eppendorf with 0.5 ml PBS containing 5 U/ml heparin (Leo Pharma, Zaventem, Belgium). Plasma was collected through centrifugation (200xg for 10 min) and stored at -70 °C until virus titration. PBMC were isolated by density gradient centrifugation with Ficoll-Paque (GE Healthcare) and resuspended in 0.5 ml RPMI. After blood collection, mice were euthanized by intraperitoneal administration with another 200 µl of 10 mg/ml sodium pentobarbital (KELA, Belgium), and different tissues were collected under aseptic conditions. Tissues obtained from the respiratory system and draining lymphoid tissues were nasal mucosa, lungs, nasopharynx-associated lymphoid tissues (NALT), deep cervical lymph nodes (LN), and mediastinal LN. Tissues associated with the alimentary system that were collected were submandibular glands and submandibular

LN. Other abdominal organs that were sampled were spleen, liver, and kidneys. The different lymph nodes, 400 µl out of a total of 500 µl PBMC of each mouse, and half of the spleen were used for co-cultivation. From the other half of the spleen and the other tissues, 10% homogenates (w/v) were made in PBS and stored at -70 °C until virus titration was performed. Another 100 µl of PBMC were used to quantify the average MCMV DNA copies present in PBMC.

2.4 Virus titration

Fifty µl of tenfold serial dilutions of the supernatants of the homogenates were used to inoculate monolayers of MEFs. After incubation at 37 °C for 1 h, 100 µl of medium were added and the cells were further incubated for 7 days. After 7 days, the presence of cytopathic effect (CPE) was assessed by light microscopy (Olympus Optical Co., Hamburg, Germany) and the virus titer was determined as $TCID_{50}$ according to the method of Reed and Muench [23].

2.5 Preparation of single-cell suspensions for co-cultivation

As already known for a long time, CMV spreads in a strict cell-associated way [24-26]. In order to investigate the cell-associated virus in different lymphoid organs, co-cultivation was used in this study. For the preparation of single-cell suspensions from lymph nodes, two needles were used to tear the lymph nodes into pieces in 6-well plates with 150 µl RPMI 1640 medium supplemented with 2% FCS and a mixture of antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin and 50 µg/ml gentamycin) [21]. Afterwards, the cell suspensions were filtered using 100-µm-pore-size cell strainers (BD Falcon) to obtain single-cell suspensions. Splenocytes were isolated by cutting the spleen with scissors into small pieces, followed by trypsin digestion for 5 min at 37 °C. Afterwards, splenocytes were filtered through a 100-µm-pore-size cell strainer (BD Falcon), followed by lysis of red blood cells.

PBMC (400µl out of a total of 500 µl), single-cell suspensions from lymph nodes and half of the spleen per mouse were separately added to a monolayer of MEFs and were

later covered with 1 ml carboxymethylcellulose (CMC) overlay medium (1/4 2 x MEM, 1/4 2 x RPMI and 1/2 2 x CMC totally supplemented with 5% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamycin, 0.1 mM NEAA and 1 mM sodium pyruvate). Afterwards, the plates were centrifuged at 750xg for 10 min. Subsequently, the cells were cultivated at 37 °C in an incubator for 8 days (lymph nodes and PBMC) or 12 days (spleen). The medium was partially changed every 4 days. Afterwards, the numbers of plaques were counted using a light microscope (Olympus Optical Co., Hamburg, Germany) to quantify the viral loads in various lymph nodes, spleen and PBMC.

2.6 Quantification of MCMV DNA in PBMC using qPCR

2.6.1 Generation of standard DNA for absolute quantification of the viral load

DNA was extracted from MCMV HaNa1 stock viruses using the QIAamp DNA mini kit (Qiagen, California, USA). Real-time qPCR forward (5'-GTGCGTTCTTCGTGGAGC-3') & reverse (5'-CGCCTTTGTCTACGGTGT-3') primers were designed in a conserved region of the m152 gene, based on the nucleotide sequence of HaNa1 strain (accession No.: KR184673) using the Primer3Plus website. First, these primers were used to amplify the nucleotide sequence fragment of m152 gene in a 50 µl PCR reaction mixture containing 10 µl OneTaq Standard reaction buffer (New England Biolabs Inc., Massachusetts, USA), 1 µl dNTPmix, 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM), 0.25 µl OneTaq DNA-polymerase (New England Biolabs Inc.), 32.75 µl DNase/RNase free H_20, and 4 µl DNA. The PCR was performed by an initial incubation at 95 °C for 3 min to activate the enzyme with 35 amplification cycles consisting of denaturation at 95 °C for 20 s, annealing at 55 °C for 30 s and extension at 72 °C for 2 min. A final extension step was done for 10 min at 72 °C and stored at 12 °C until further processing. Amplification products were separated on a 1% agarose gel and identified with UV after incubation with ethidium bromide. Fragments with the correct length were excised and purified from the gel using the Nucleospin® Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany), and the nucleotide sequence was

characterised using Sanger Sequencing (performed by the GATC Biotech Company, Konstanz, Germany). The concentration of DNA was determined using spectrophotometry (Nanodrop 2000 system, Thermo Scientific). Ten-fold serial dilutions of the template DNA were made over a range of 6 log units (10^6-10^1) for the generation of the standard curve.

2.6.2 Real time qPCR

At each time point (7, 14, and 21 dpi), 100 µl of PBMC was used for DNA extraction, using the QIAamp DNA mini kit (Qiagen, California, USA) according to the manufacturer's instructions. Assays were prepared in 96-well optical reaction plates (Life technologies) in a total volume of 20 µl including 10 µl PrecisionPLUS 2x qPCR MasterMix with SYBR Green, ROX and inert blue dye (PrimerDesign Ltd., Southampton, UK), 50 nM forward primer 5'-GTGCGTTCTTCGTGGAGC-3', 50 nM reverse primer 5'-CGCCTTTGTCTACGGTGT-3', 6.8 µl LiChrosolv water, and 3 µl sample DNA or diluted DNA standard (see above). Thermal cycling started with an enzyme activation at 95 °C for 2 min, followed by 40 cycles of each 15 s at 95 °C and 60 s at 60 °C. A first-derivative melting curve analysis was performed by heating the mixture to 95 °C for 15 s, then cooling to 60 °C for 1 min, and heating back to 95 °C at 0.3 °C increments. Amplification and melting curve analysis were carried out in a Step One Plus™ real-time PCR system (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA). Each sample was measured twice and the average DNA copy number was used for quantification analysis.

2.7 Statistical analyses

Data from experimental groups were analyzed using the Mann-Whitney U test of GraphPad Prism v.5.0 software (GraphPad Software Inc., San Diego, CA, USA). P values ≤ 0.05 were considered as significant.

2.8 Ethical statement

All animal experiments were approved by the local Ethical Committee of the Faculty of Veterinary Medicine, Ghent University (EC2015-57).

3 Results

3.1 Organ specific differences in viral load between intact and splenectomized Balb/c mice inoculated with MCMV HaNa1 or MCMV Smith, as determined by virus titration

Figure 1 (top panel) represents infectious virus titers in various tissues at different time points after oronasal inoculation of intact and splenectomized Balb/c mice with MCMV HaNa1. Infectious virus was detected in the nasal mucosa at 7, 14, and 21 dpi for mice with and without spleen. In the intact mice, the average virus titers in the nasal mucosa were $10^{2.6}$ $TCID_{50}$/g at 7 dpi, 10^{3} $TCID_{50}$/g at 14 dpi, and $10^{3.7}$ $TCID_{50}$/g at 21 dpi. Compared with intact mice, the average virus titers in the nasal mucosa of splenectomized mice tended to be higher (10^{3} $TCID_{50}$/g at 7 dpi, 10^{4} $TCID_{50}$/g at 14 dpi, and $10^{4.5}$ $TCID_{50}$/g at 21 dpi), but this was not significantly different. In the submandibular glands, infectious virus was detected at 7 dpi (1 out of 3), 14 dpi (3 out of 3), and 21 dpi (3 out of 3) in intact mice, but only at 14 dpi (4 out of 4) and 21 dpi (4 out of 4) in splenectomized mice. The average virus titer was 10 times lower ($P = 0.0001$) at 14 dpi in splenectomized mice ($10^{5.7}$ $TCID_{50}$/g) compared to intact mice ($10^{6.7}$ $TCID_{50}$/g). Also at 21 dpi the virus titer was somewhat lower ($10^{5.5}$ $TCID_{50}$/g versus $10^{5.7}$ $TCID_{50}$/g) but not significantly different ($P = 0.45$). Infectious virus was not detected in the spleen (intact mice), liver, kidneys and plasma (both intact and splenectomized mice) throughout the whole experiment.

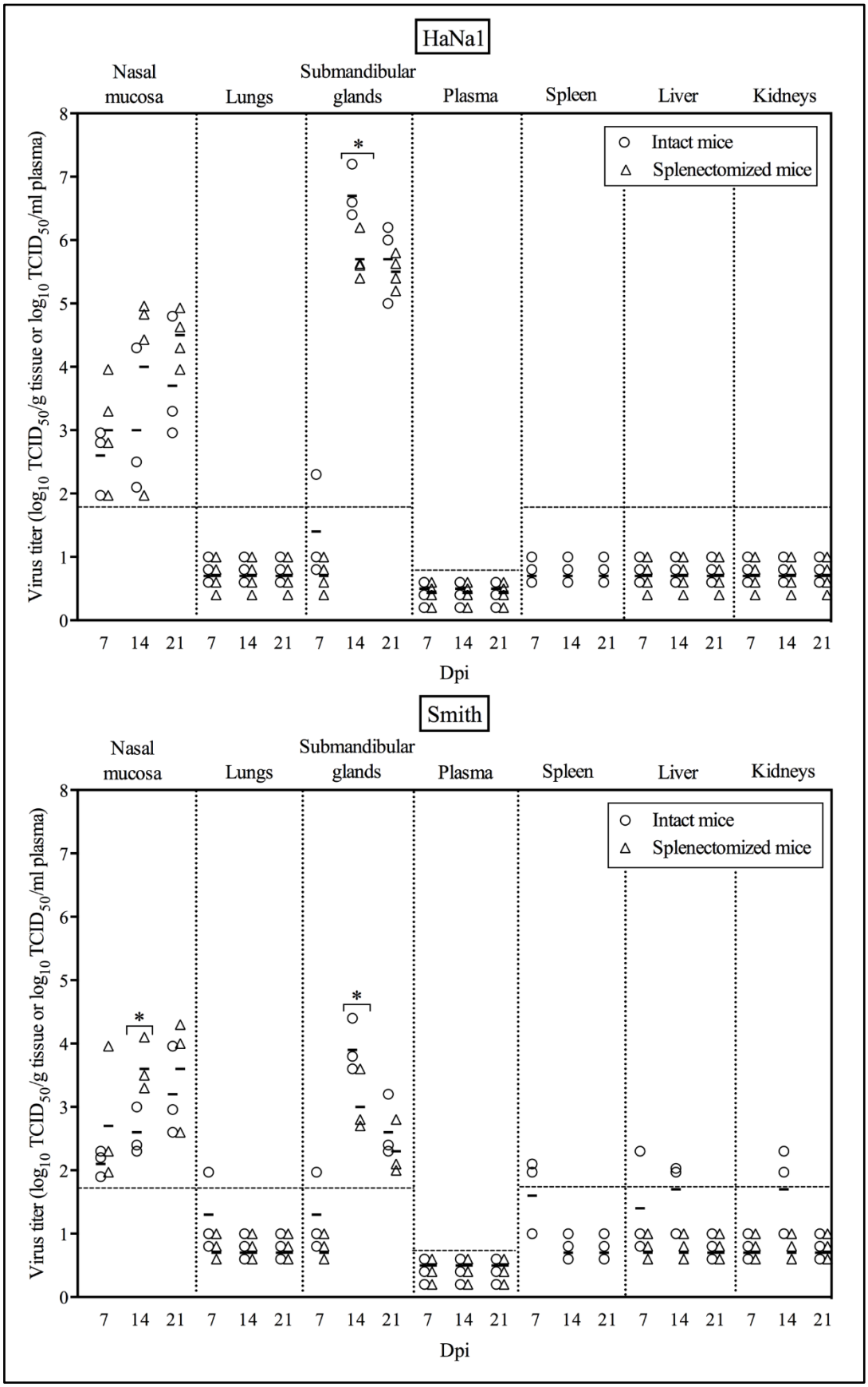

Figure 1. MCMV HaNa1 (top) and MCMV Smith (bottom) replication in various tissues from intact/splenectomized mice collected at 7, 14 and 21 dpi. At each time point, the data

from intact or splenectomized mice are given. Each open circle or triangle corresponds to one mouse. The center bar indicates the mean. The detection limit for the titration assay ($10^{1.8}$ $TCID_{50}$/g tissue or $10^{0.8}$ $TCID_{50}$/ml plasma) is shown by the horizontal dashed line. *, $P < 0.05$.

Figure 1 (bottom panel) represents the results of the virus titration of various tissues at different time points after oronasal inoculation of intact and splenectomized Balb/c mice with MCMV Smith. Infectious virus was detected in the nasal mucosa of both types of mice at 7, 14, and 21 dpi. In the intact mice, the average virus titers in the nasal mucosa were $10^{2.1}$ $TCID_{50}$/g at 7 dpi, $10^{2.6}$ $TCID_{50}$/g at 14 dpi, and $10^{3.2}$ $TCID_{50}$/g at 21 dpi. Compared with intact mice, the average virus titers in the nasal mucosa of the splenectomized mice were higher ($10^{2.7}$ $TCID_{50}$/g at 7 dpi, $10^{3.6}$ $TCID_{50}$/g at 14 dpi and $10^{3.6}$ $TCID_{50}$/g at 21 dpi). A significant difference was only reached at 14 dpi ($P = 0.0089$). In the submandibular glands, infectious virus was detected at 7 dpi (1 out of 3), 14 dpi (3 out of 3) and 21 dpi (3 out of 3) in intact mice, but only at 14 dpi (3 out of 3) and 21 dpi (3 out of 3) in splenectomized mice. In intact mice, the average virus titers in the submandibular glands were $10^{3.9}$ $TCID_{50}$/g at 14 dpi and $10^{2.6}$ $TCID_{50}$/g at 21 dpi. Compared with intact mice, the average virus titer in the submandibular glands of splenectomized mice was nearly 8 times lower at 14 dpi ($10^{3.0}$ $TCID_{50}$/g) ($P = 0.0048$) and 2 times lower at 21 dpi ($10^{2.3}$ $TCID_{50}$/g) ($P = 0.29$). In intact mice, infectious virus was also detected in other organs, such as lungs (1 out of 3 mice at 7 dpi), spleen (2 out of 3 at 7 dpi), liver (1 out of 3 at 7 dpi and 2 out of 3 at 14 dpi) and kidneys (2 out of 3 at 14 dpi). For splenectomized mice, no infectious virus was detected in lungs, liver and kidneys throughout the whole experiment. No virus was detected in any tissue of mock-inoculated mice.

3.2 Detection of cell-associated virus in lymph nodes and blood in intact and splenectomized Balb/c mice inoculated with MCMV HaNa1 or MCMV Smith

Figure 2 (top panel) shows the number of infected cells determined by co-cultivation of cells derived from lymph nodes and blood of intact and splenectomized Balb/c mice inoculated with MCMV HaNa1. Cell-associated virus was detected in pooled NALT for both types of mice at 7, 14, and 21 dpi, in submandibular LN at 14 (2/3)

dpi for intact mice and at 7 (3/3) dpi for splenectomized mice, in deep cervical LN at 7 (1/3), 14 (2/3) and 21 (2/3) dpi for intact mice and at 7 (3/4) and 14 (2/4) dpi for splenectomized mice, in the pooled mediastinal LN at 7 and 14 dpi for both types of mice, in the spleen at 7 (3/3) and 14 (3/3) dpi for intact mice, and in PBMC at 7 (2/3) dpi only for intact mice.

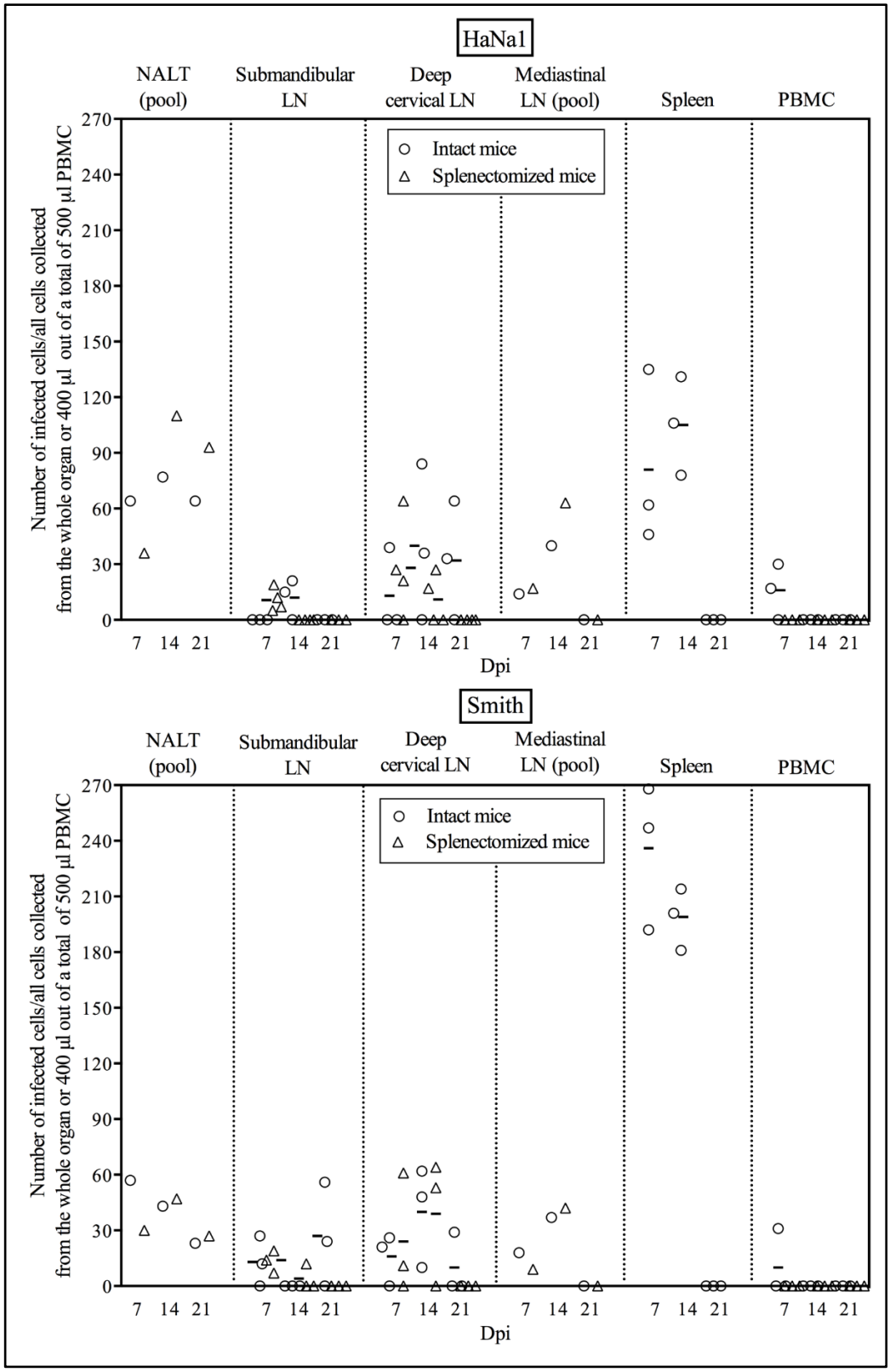

Figure 2. Co-cultivation of cell suspensions of different lymphoid organs and PBMC from MCMV HaNa1 (top) and Smith (bottom)-inoculated intact/splenectomized mice with MEFs. Each open circle or triangle corresponds to one mouse. The center bar indicates the mean. NALT and mediastinal LN are respectively pooled together for co-cultivation.

The results of the co-culture assay show that dissemination of the MCMV Smith strain to lymphoid organs in intact and splenectomized mice (Fig. 2, bottom panel) was similar to the findings obtained with the MCMV HaNa1 strain, with the detection of cell-associated virus in the pooled NALT at 7, 14, and 21 dpi, in the submandibular LN at 7 (2/3) and 21 (2/3) dpi for intact mice and at 7 (3/3) and 14 (1/3) dpi for splenectomized mice, in the deep cervical LN at 7 (2/3), 14 (3/3), and 21 (1/3) dpi for intact mice and at 7 (2/3) and 14 (2/3) dpi for splenectomized mice, in the pooled mediastinal LN at 7 and 14 dpi for both types of mice, in the spleen at 7 (3/3) and 14 (3/3) dpi for the intact mice, and in PBMC at 7 (1/3) dpi only for the intact mice. Overall, the amount of cell-associated virus in the spleen of MCMV Smith-inoculated intact mice was remarkably higher ($p < 0.5$) compared to that of the MCMV HaNa1-inoculated intact mice.

3.3 Quantification of MCMV DNA load in PBMC of intact and splenectomized Balb/c mice inoculated with MCMV HaNa1 or MCMV Smith, as determined by qPCR

Figure 3 shows the mean virus DNA load in PBMC of MCMV HaNa1 and MCMV Smith-inoculated intact and splenectomized mice. Ten-fold serial dilutions of the template DNA were made over a range of 6 log units (10^6-10^1) for the generation of the standard curve (efficiency: 105.4 %; R^2: 0.981). In the MCMV HaNa1-inoculated intact and splenectomized mice, the average MCMV DNA copy numbers were higher at 7 dpi compared to 14 and 21 dpi. The average DNA copies in PBMC of splenectomized mice (3.0 x 10^2 DNA copies/10^6 cells) were significantly lower (P = 0.022) compared with that of inoculated intact mice (1.3 x 10^3 DNA copies/10^6 cells), whereas no significant differences were found at 14 and 21 dpi.

Similar results were found in the MCMV Smith-inoculated intact/splenectomized mice. The average viral DNA copies were the highest at 7 dpi for both types of inoculated mice, but a significantly lower amount (P = 0.0468) was found in splenectomized mice (3.9 x 10^2 DNA copies/10^6 cells) compared to intact mice (1.0 x 10^3 DNA copies/10^6 cells). Only a very low load was found at 14 and 21 dpi, and no

significant difference were noticed between intact and splenectomized mice at these time points.

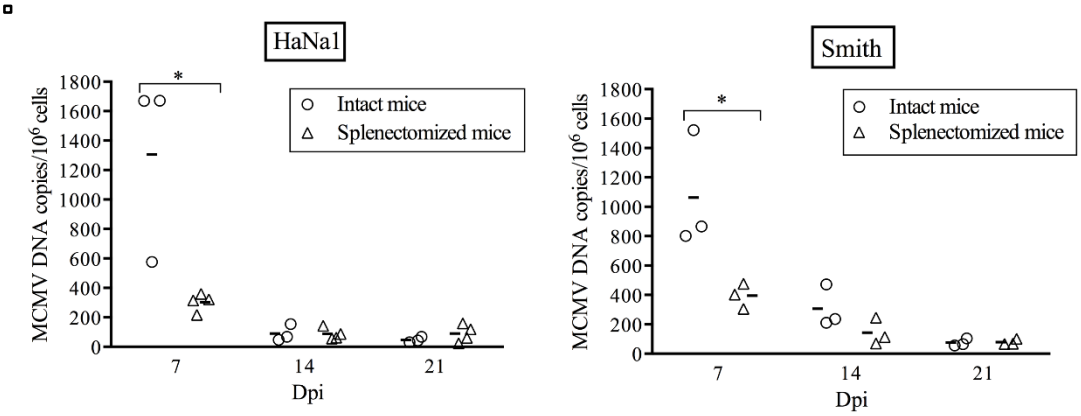

Figure 3. Quantification of MCMV DNA load in PBMC by qPCR from HaNa1-(left) and Smith-(right) inoculated intact/splenectomized mice were quantified. Each open circle or triangle corresponds to one mouse. The center bar indicates the mean. *, $P < 0.05$.

4 Discussion

Intraperitoneal inoculation of mice with MCMV has identified the spleen as an important target organ for early replication, systemic spread, and latency [7, 9-11]. Using oronasal inoculation (the natural infection route), our previous research also highlighted the spleen as a key determinant for virus dissemination during natural infections. Indeed, we showed that viremia and subsequent dissemination of the virus to distal organs (such as the liver, kidneys, and submandibular glands) was only detectable after replication had occurred in the spleen [21]. By comparing MCMV dissemination in intact and splenectomized mice, the present study confirmed the role of the spleen as transfer hub for virus dissemination during infection with both MCMV Smith and HaNa1. For both virus strains, a cell-associated viremia was detectable by co-cultivation in intact mice, but not in splenectomized mice. To exclude that this was a coincidental finding (since a cell-associated viremia was not detected in all intact mice and a rather limited number of animals were used), MCMV DNA load was determined in PBMC with qPCR. These data confirmed the restricted viremia in splenectomized mice.

For both virus strains, virus in the submandibular glands of splenectomized mice was

detected later and at lower titers compared with that of intact mice. For the Smith strain, infectious virus in liver and kidneys of splenectomized mice were not detected, whereas virus was frequently isolated in these organs of intact mice. For HaNa1, infectious virus in liver and kidneys was never detected in both intact and splenectomized mice, which is in line with our previous reports [21, 22], demonstrating that MCMV HaNa1 cannot lead to a productive infection in spleen, liver and kidneys in adult mice. Taken all data together, it can be concluded that the spleen can be considered as a key determinant for virus dissemination during natural infections. The precise mechanism for limiting virus dissemination over the body in splenectomized mice may be due to the reduction of numbers of MCMV infected leukocytes in blood by splenectomy. It is very well possible that these cells are patrolling monocytes. This hypothesis is supported by previous studies demonstrating that a larger number of patrolling monocytes are found in the spleen compared to blood [27, 28] and that these patrolling monocytes are hijacked by MCMV as immune-privileged vehicles for dissemination to salivary glands and other tissues [10]. Although patrolling monocytes can also be found in various lymph nodes [28, 29], the size of a spleen is remarkably larger compared with that of other lymphoid tissues, which leads to a higher number of infected patrolling monocytes in the spleen than in lymph nodes. This may be an additional explanation why the spleen is more important as a source of infected leukocytes in the blood and for virus dissemination.

For both virus strains, virus titers in the portal of entry (nasal mucosa) were higher in splenectomized mice compared with intact mice. This may be due to a reduced T cell-mediated immune response to MCMV infection after splenectomy, as the role of splenic effective T cells in providing protection against MCMV infection was demonstrated by several previous studies [30-33], and the numbers of effective T cells in lymph and blood circulation are reduced by splenectomy [31-34].

In order to detect and quantify cell-associated virus in various lymphoid tissues, a co-cultivation assay was used in the present study. Similar patterns were found for both virus strains except that the average number of cell-associated virus was lower in

NALT and deep cervical LN, but was remarkably higher in the spleen of Smith-inoculated mice compared with that of HaNa1-inoculated mice. The former finding may be due to the more extensive replication of HaNa1 in the nasal mucosa and the submandibular gland and the draining of these regions to NALT and deep cervical LN [35, 36]. The higher number of cell-associated virus in the spleen of Smith-inoculated intact mice may be explained by the finding that only the Smith strain causes a clear productive infection in the spleen and that both productively and latently infected cells are detected with the co-cultivation assay. In contrast to the higher number of virus-infected cells in the spleen of Smith-inoculated intact mice, cell-associated viremia and average viral DNA in PBMC were less pronounced for the Smith strain compared with the HaNa1 strain. Although hypothetical, this may be due to the fact that the Smith strain is causing a more productive infection in the spleen, leading to the death of the infected cell, whereas HaNa1 infection of the spleen may promote latently infected cells which are staying alive and may enter the blood circulation.

In conclusion, all these data suggest that the spleen serves as a viremia booming site for MCMV infections activating virus dissemination to distal organs such as salivary glands, liver and kidneys. These data identify the spleen as the target organ for a control of MCMV transmission.

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Chapter 6

General discussion

An HCMV infection is in general asymptomatic in immunocompetent hosts but harmful for immunocompromised individuals [1, 2]. The asymptomatic nature of HCMV infection makes the study of early infections hard to do. Further, due to its strict species-specificity, it is not possible to study the pathogenesis of infections with this virus in experimental animals. MCMV infection in mice is a widely used animal model for studying HCMV infection [3]. As such, MCMV models may provide insights in the way HCMV initiates replication in its host in a natural situation.

The routes of a natural MCMV entry remain illusive, despite the fact that MCMV has been investigated for several decades. In most previous published studies on MCMV pathogenesis, the intraperitoneal, intracerebral, orbital and intravenous inoculation routes have been utilized, but none of them can be recognized as a natural infection route [4]. In general, the routes of intranasal and peroral inoculation are nearly and widely accepted as the natural infection routes. Nonetheless, limited data on infection via intranasal and peroral inoculation are available so far. Even if intranasal inoculation was used in a few previous published papers, all mice were sedated or anesthetized [5, 6]. Concerning this, the aforementioned intranasal inoculation was not accepted as being natural. Oral inoculation of MCMV has also been investigated by suckling virus contaminated breast milk [7], but it was not demonstrated how infection was initiated in these animals.

Likewise, the virus strain is another factor, which should be taken into consideration during *in vivo* studies. Two laboratory strains of MCMV, Smith and K181, are in widespread use over the past decades [8]. Both strains have been extensively passaged *in vivo* or *in vitro* since their first isolation. Serial *in vivo* or *in vitro* passage is known to affect genome stability, thus leading to biological differences [9, 10]. The same issue has been addressed for HCMV [11, 12]. Hence, MCMV strains with serial passages may not generate closely correlated results in regard to the full range of virus replication and clinical outcome that are associated with CMV infections. It is critical that isolates of MCMV without undergoing extensive *in vivo/ in vitro* passages should be investigated to give more relevant data on the pathogenesis of CMV infections.

Therefore, in the present thesis, the pathogenesis of MCMV infection with the low passaged MCMV HaNa1 isolate was compared with that of highly passaged MCMV Smith, using oronasal inoculation without anesthesia to mimic natural infection.

This thesis provided detailed information on how MCMV disseminates throughout the body upon oronasal inoculation, and highlighted some important differences between the low passaged HaNa1 and highly passaged Smith strains. A hypothetical model depicting this dissemination is given in Figure 1 and discussed in detail below.

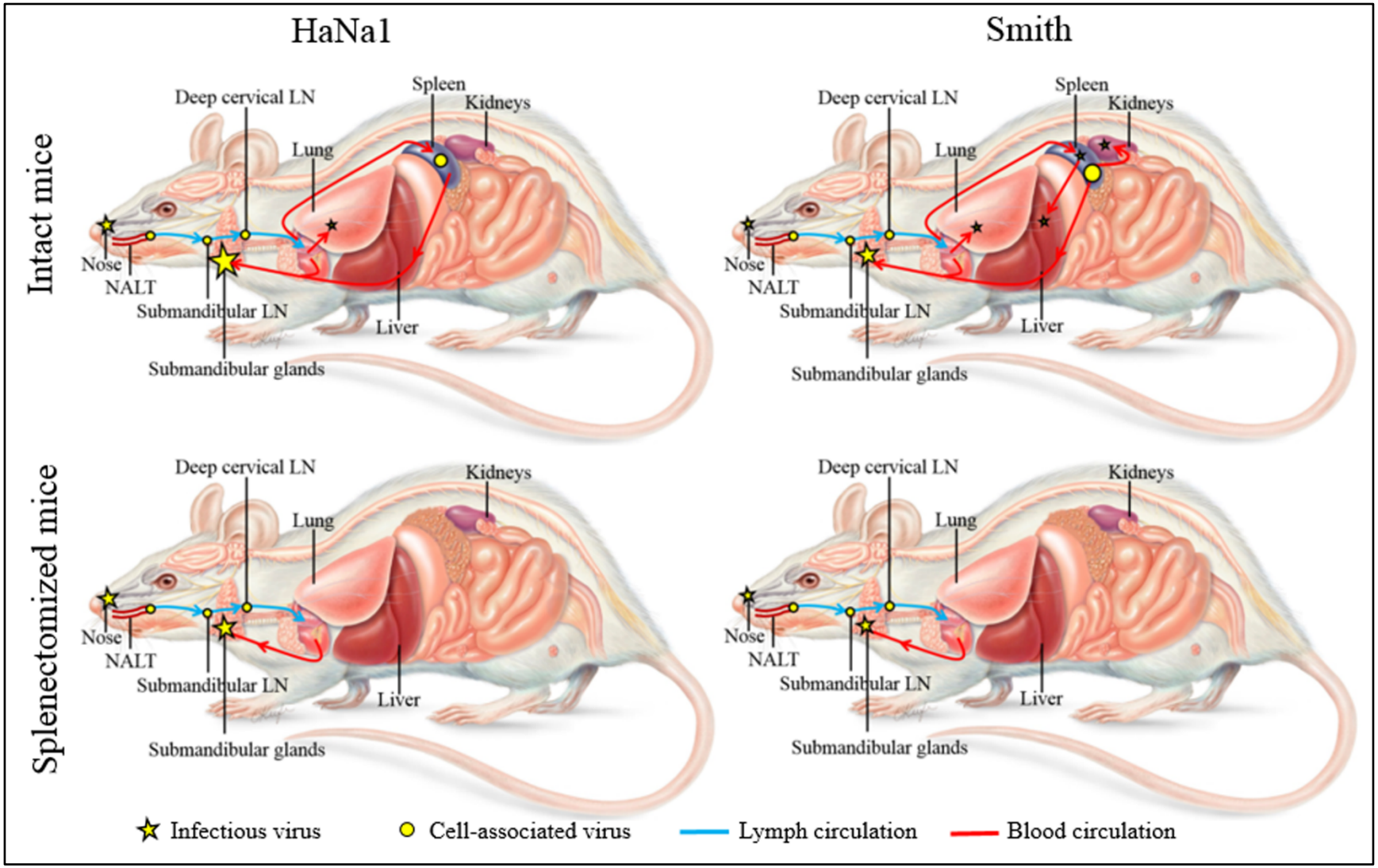
HaNa1
Smith
Intact mice
Splenectomized mice
Deep cervical LN
Spleen
Kidneys
Lung
Nose
NALT
Submandibular LN
Liver
Submandibular glands
Infectious virus
Cell-associated virus
Lymph circulation
Blood circulation

Figure 1. Illustration of MCMV HaNa1 and MCMV Smith dissemination upon natural oronasal inoculation and function of spleen in this process. The size of stars and enclosed circles respectively represent infectious virus and cell-associated virus production. For intact mice with natural oronasal inoculation, both virus strains (HaNa1 and Smith) first reach the nasal mucosa and NALT. Subsequently, both virus strains spread to the submandibular LN, deep cervical LN, lungs and spleen possibly via lymph and blood circulation. Afterwards, the spleen regulates a subsequent cell-associated viremia carrying the virus to various tissues such as submandibular glands (for both Smith and HaNa1), liver and kidneys (only for Smith), where a secondary replication is initiated. Productive infections in abdominal organs such as spleen, liver and kidneys were only detected in MCMV Smith-infected adult mice. For splenectomized mice inoculated with the same inoculation technique, splenectomy leads to an obviously less efficient virus dissemination manifested by a more restricted viremia detected at 7 dpi, significantly lower virus titers in the submandibular glands at 14 dpi for both virus strains, and additionally no infectious virus detected in the spleen, liver and kidneys at 7, 14 and 21 dpi for Smith. These data confirm that the spleen plays a vital role in regulating viremia and virus dissemination upon oronasal inoculation. The mouse model pictures are derived from artist Laurie O'Keefe with her permission.

1) The nose serves as portal of entry for MCMV

The nasal mucosa was demonstrated for the first time as a vital susceptible organ for MCMV. According to our *in vivo* results, upon oronasal inoculation, infectious and cell-associated virus was first detected in the nasal mucosa and NALT respectively from 1 dpi onwards for both virus strains with high inoculation dose. Manifestation of earlier detection and higher virus titers resulted from increasing the inoculation dose. HaNa1 reached to higher virus titers than Smith in the nasal mucosa. Cell-associated virus persisted in NALT until at least 14 dpi, which indicated that NALT plays a critical role in MCMV infection in general. We assume that the virus in NALT may be transmitted via lymphatic drainage to the draining lymph nodes, ending up in the blood circulation. NALT is identified as portal of entry for MCMV upon intranasal inoculation. This finding is in line with its anatomic and functional characteristics. The Waldeyer's ring in human is the equivalent of NALT in rodent [13]. In addition, HCMV DNA has been detected in the Waldeyer's ring [14, 15], and hence, the Waldeyer's ring can be hypothesized to be a vital entry site for HCMV upon natural exposure. For both virus strains, infected cells in the nose were identified as

macrophages and dendritic cells in the NALT, and as olfactory neurons and sustentacular cells in olfactory epithelium. Virus getting into the NALT is thought to occur via several routes: 1) transcytosis through microfold cells; 2) transport across the columnar epithelial cells, and 3) direct luminal sampling via DC dendrites [16]. The presence of heparan sulfate might be a potential explanation for virus infection in olfactory epithelium and absence of infection in respiratory epithelium. Heparan sulfate contributes to MCMV binding and entry, and virions unable to bind to heparan sulfate show poor host entry [17]. The respiratory epithelial cells only express heparan sulfate basolaterally [18], where it is inaccessible to virions from the lumen of the nose. The olfactory epithelium that contains olfactory neurons and sustentacular cells, unlike the nasal respiratory epithelium, expresses heparan sulfate both apically and basolaterally [18, 19]. Therefore, virions may easily bind to the heparan sulfate at the apical side and enter both cell types. This mechanism of virus infecting olfactory epithelium has also been reported for murid herpesvirus-4 [18, 19]. In addition, infecting the olfactory neurons raised the question whether CMV may damage the function of smell [20, 21]. This will be an interesting direction in the future investigation. The nasal mucosa is hypothesized to be a target organ for HCMV according to the similarities between human and murine CMV. Indeed, several reports on the detection of HCMV in nasopharyngeal carcinomas, sinusitis and nasal polyposis are consistent with this [22-25].

2) Virus dissemination occurs from the nose to various tissues/organs from 3 dpi via non-productively infected leukocytes

Cell-associated virus was first detected in the submandibular LN and deep cervical LN at 3 dpi. Subsequently, the virus was disseminated throughout the entire body, with detection of infectious and/or cell-associated virus in the lungs and spleen at 5 dpi, and in PBMC, mediastinal LN and submandibular glands at 7 dpi.

Both virus strains showed a very restricted replication in lungs since the infection was controlled in the first three weeks post inoculation. Our finding is in line with a previous study [26]. Moreover, cell-associated virus can only be detected in

mediastinal LN from 7 dpi onwards after the lungs become infected (5 dpi), which is in line with the drainage of mediastinal LN. CMV infected the lungs often leading to pneumonia, which has been demonstrated by intranasal inoculation of anesthetized mice with MCMV. It has been confirmed that the virus can directly flow into the lungs with this inoculation method [5, 27]. Hence, lungs are widely considered to be a primary target organ for MCMV via intranasal inoculation under anesthesia [5, 6]. In the present study, infectious virus was first detected in the lungs at 5 dpi, and not detected in the trachea throughout the entire experiment. Additionally, a clear interstitial pneumonia and viral antigens in the interstitium were examined in the lungs by histopathological and immunohistochemical analysis (unpublished data). Taken all these data together, the infection of lungs with both Smith and HaNa1 strains is associated with the hematological spread of the virus, and not due to inhalation as described by others for anesthetized mice [5, 6]. Viral antigen positive cells in lungs were identified as epithelial cells and macrophages for both MCMV strains [5, 26]. According to a more recent published study, it was precisely demonstrated that type 2 alveolar epithelial cells, but not type 1 alveolar epithelial cells, can be productively infected, and alveolar macrophages can phagocytose virus or debris of infected cells but are not productively infected [28].

Cell-associated virus was detected in PBMC at 7-10dpi for both strains with a high inoculation dose. This suggests that circulating PBMC are involved in the dissemination of MCMV, which is in line with a previous study [29]. Since the viremia could only be detected after the spleen became positive, the spleen is hypothesized to serve as a transfer hub for regulating viremia and virus dissemination over the body such as submandibular glands (for both HaNa1and Smith), liver and kidneys (only for Smith). This hypothesis was further confirmed by comparing MCMV dissemination in intact and splenectomized mice upon oronasal inoculation. Our data showed that for both virus strains, a cell-associated viremia was detectable by co-cultivation assay using PBMC of intact mice, but not of splenectomized mice. To exclude that this was a coincidental finding (since a cell-associated viremia was

not detected in all intact mice and a rather limited number of animals were used), the MCMV DNA load in PBMC was examined with qPCR. The restricted viremia was found in splenectomized mice. For both virus strains, virus in the submandibular glands of splenectomized mice was detected later and at lower titers compared with that of intact mice. For Smith, infectious virus in liver and kidneys of splenectomized mice was not detected, even though virus was frequently isolated from these organs of intact mice. For HaNa1, infectious virus in liver and kidneys was never detected in both intact and splenectomized mice, which is consistent with our previous reports [30, 31]. Taken all data together, it was concluded that the spleen can be regarded as a key determinant for regulating viremia and virus dissemination during natural infections. The precise mechanism for regulating viremia and limiting virus dissemination over the body in splenectomized mice may result from the reduction of numbers of MCMV infected leukocytes in blood by splenectomy. These cells possibly are patrolling monocytes. This hypothesis is supported by previous studies demonstrating that a larger number of patrolling monocytes are found in the spleen compared to blood [32, 33] and that these patrolling monocytes are hijacked by MCMV as immune-privileged vehicles for dissemination to salivary glands and other tissues [34]. Although patrolling monocytes can also be found in various lymph nodes [33, 35], the spleen is remarkably larger compared with that of other lymphoid tissues, which leads to a higher number of infected patrolling monocytes in the spleen than in lymph nodes. This may be an additional explanation why the spleen is more important as a source of infected leukocytes in the blood and for virus dissemination. Intriguingly, the spleen is also involved in the systemic transmission of murid herpesvirus-4 [36], but its exact role in virus dissemination may be different from MCMV. In the future, it is very interesting to compare the mechanisms involved in systemic spread by both MCMV and murid herpesvirus-4.

The submandibular gland is another main target organ for both strains. The viral replication in the submandibular glands always initiated one week later compared with that in the nasal mucosa and lasted longer than 49 days post inoculation. The

similar pattern in the nasal mucosa was also found in the submandibular glands. Namely, HaNa1 reached much higher virus titers than Smith, and increasing the inoculation dose enhanced virus production in the submandibular glands. The susceptible cell type in submandibular glands for both MCMV strains was identified as epithelial cells, which is in line with earlier published data [37]. CMVs have been reported to mainly use salivary glands as target organ for virus persistence and shedding into saliva for horizontal transmission [38-40]. Nevertheless, in our study, only HaNa1 was detected in saliva at only one time point within one mouse. The low level of virus titers in saliva was quite surprising, as CMVs are thought to be transmitted orally via saliva. These conflicting data will be further examined in future studies. Moreover, it is still unclear how the virus initiates replication in the submandibular glands.

MCMV Smith productively infects the spleen, liver and kidneys in adult mice compared with MCMV HaNa1. Since the experimental hosts are identical, the genetic differences between HaNa1 and Smith result in the different degree of viral replication (productive versus non-productive infection). MCMV-encoded chemokine 2 (MCK-2) has been identified as a determinant of MCMV pathogenicity [41, 42]. However, this chemokine is identical between HaNa1 and Smith and cannot explain the difference. In order to find an answer to this question on the strain difference, the full sequence of the MCMV HaNa1genome should be determined and compared with that of the Smith strain. The results may pave the way to identify pathogenicity determinants. The outcome of MCMV HaNa1 infection is more similar to that of an HCMV primary infection in immunocompetent adults, during which the virus is causing a limited virus replication in the salivary glands but not in multiple internal organs [43]. Therefore, MCMV HaNa1 is an interesting strain to be used in mouse models in order to get better insights into HCMV natural infections in immunocompetent hosts via oronasal exposure.

3) MCMV does not replicate in the digestive tract and associated lymphoid tissues

No infectious or cell-associated virus was detected in the pharynx, esophagus, small intestines, Peyer's patches and mesenteric LN throughout the entire experiment. Our date demonstrate that the digestive tract and associated lymphoid tissues are not efficient entrance sites for the virus, which is consistent with a previous published report [6]. Potential explanations may be the absence of the Waldeyer's ring in the pharynx of mice and/or presence of a low pH and proteases in the stomach, detergents in the bile and proteases/lipases in the small intestines. The latter conditions are too detrimental for this enveloped virus. In order to determine whether MCMV can effectively establish infections using peroral inoculation, a small experiment was carried out in which nine mice inoculated with MCMV HaNa1 and nine mice inoculated with MCMV Smith were via oral route. Only 2 mice were infected with MCMV HaNa1, and 3 with Smith. The poor infectivity of MCMV via oral route further confirmed that peroral inoculation is far less efficient to establish infections than intranasal inoculation. MCMV most possibly prefers spread via nasal secretions because nose-to-nose contacts and smelling are important activities in the social life of mice.

4) MCMV-infected mice specifically mount an IgG2a antibody response

Serological analysis revealed that IgG2a was the mainly produced antibody subclass throughout the entire experiment except that IgG1 and IgG2c were also detected with lower titers in mice inoculated with a high dose at 35-49dpi. IgM was not detected during the entire experiments for both MCMV strains. This could result from the low sensitivity of the MCMV-specific IPMA, or the suppressive effect of MCMV on the production of IgM by T cell cytokines. T cell cytokines determine the immunoglobulin class switching mechanism in mouse and human [44]. In general, interferon γ (IFNγ) plays a vital role in mediating the strong induction of IgG2a [45, 46]. The role of IFNγ in the orientation of the antibody isotype switch could not be assessed since IFNγ and other T cell cytokines were not determined in this study. The

complement-dependent neutralization assay demonstrated that complement is essential in neutralizing MCMV since antibodies without complement/with inactivated complement did not neutralize MCMV infection. The complement-dependent IgG2a-mediated inactivation of MCMV plays a vital role in anti-MCMV defense, as IgG2a is the predominant viral-specific antibody. This is consistent with the characteristics of antibody isotype IgG2a to fix complement in mice [47]. Neutralizing antibody titers were high at the end of the experiment, which may partially explain the clearance of both virus strains in the submandibular glands at the late stage of infection. Likewise, the cell-mediated immunity most probably mediate the control of virus infection at the end of the experiments, which is generally considered to be the most important factor in controlling CMV infections [48, 49].

In conclusion, our mouse models with two virus strains reveal that the nasal mucosa and NALT serve as portals of entry for MCMV, provides new insights into the dissemination of MCMV upon oronasal exposure, suggests that the Waldeyer's ring may be an important entry site for HCMV upon natural exposure because of its equivalent role with NALT in mouse, and also demonstrates that the spleen serves as a transfer hub for regulating viremia and virus transmission. These new insight can be valuable to control viral transmission and disease upon natural MCMV/HCMV infection.

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Summary-samenvatting

Summary

Human cytomegalovirus (HCMV) is a betaherpesvirus that causes subclinical infections in immunocompetent hosts but clinically significant diseases in immunocompromised patients. HCMV can certainly be transmitted from infected to susceptible hosts via the oronasal route. However, it remains elusive how the virus spreads throughout the body during a natural infection. Due to the strict species-specificity of HCMV, it is impossible to study the pathogenesis of HCMV infections in experimental animals. Therefore, animal CMV infection models are used. Murine cytomegalovirus (MCMV) infection in mice is a widely used animal model to study HCMV infection. However, knowledge about the invasion strategies of MCMV upon oronasal exposure was lacking.

In **Chapter 1**, an overview of the current knowledge on MCMV was given. First, an introduction was given on the history, the classification, the virus characteristics, and the replication cycle of MCMV. Further, the epizootiology, the pathogenesis of MCMV infection and the immune response during an infection of MCMV were reviewed. Finally, the anatomy of oral & nasal cavities and lymph nodes associated with alimentary & respiratory tracts in mice were briefly introduced.

In **Chapter 2**, the aims of the studies described in the thesis were presented. The first aim was to set up animal models for mimicking natural infection of MCMV in order to compare the pathogenesis of MCMV infection with two strains (the low passaged MCMV HaNa1 isolate and highly passaged MCMV Smith strain) in Balb/c female mice. The second aim was to elucidate the kinetics of virus dissemination throughout the body upon oronasal exposure. The third and last aim was to demonstrate the role of the spleen in virus dissemination during a natural primary MCMV infection.

In **Chapter 3**, the pathogenesis of an infection with the highly passaged MCMV Smith strain was compared with that of an infection with a low passaged Belgian MCMV isolate HaNa1 in BALB/c adult mice following oronasal inoculation with

either a low (10^4 $TCID_{50}$/mouse) or high (10^6 $TCID_{50}$/mouse) inoculation dose. Both strains were mainly replicating in nasal mucosa and submandibular glands for one to two months. In nasal mucosa, MCMV was detected earlier and longer (1-49dpi) and reached higher titers with the high inoculation dose compared to the low inoculation dose (14-35dpi). In submandibular glands, a similar finding was observed (high dose: 7-49dpi; low dose: 14-42dpi). In lungs, both strains showed a restricted replication. Only the Smith strain established a low level of productive infection in spleen, liver and kidneys. The infected cells were identified as olfactory neurons and sustentacular cells in olfactory epithelium, macrophages and dendritic cells in NALT, acinar cells in submandibular glands, and macrophages and epithelial cells in lungs for both strains. Antibody analysis demonstrated for both strains that IgG_{2a} was the main detectable antibody subclass. Overall, our results showed that significant phenotypic differences exist between the two virus strains. MCMV HaNa1 is a promising strain to use in mouse models in order to get better insights for HCMV infections in immunocompetent humans.

In **Chapter 4**, virus titration showed a productive virus replication of both HaNa1 and Smith in the nasal mucosa from 1 dpi until the end of the experiment (14 dpi), in lungs from 5 until 14 dpi, and in submandibular glands from 7 until 14 dpi. In contrast to MCMV HaNa1, MCMV Smith also established a low level productive infection in abdominal organs (spleen, liver and kidneys) from 5 dpi (spleen), 7 dpi (liver), and 10 dpi (kidneys) until the end of the experiment. Co-culture showed that for both strains, cell-associated virus was detected in a non-infectious form in nasopharynx-associated lymphoid tissues (NALT) from 1 until 14 dpi, in submandibular lymph nodes from 3 until 5 dpi, in deep cervical lymph nodes from 3 until 14 dpi, in mediastinal lymph nodes from 7 until 14 dpi, in spleen from 5 until at least 10 dpi and in the peripheral blood mononuclear cells (PBMC) at 7 and 10 dpi. This study showed that upon oronasal exposure, MCMV first enters the nasal mucosa and NALT, from where the virus disseminates to the spleen possibly via the draining lymphatic system and blood; a subsequent cell-associated viremia transports MCMV to submandibular glands and

for MCMV Smith also to liver and kidneys, where a second productive replication starts.

In **Chapter 5**, the role of the spleen during an MCMV infection was investigated by the comparison of intact and splenectomized Balb/c mice. Both highly passaged MCMV Smith and low passaged MCMV HaNa1 were used. Various samples were collected at 7, 14, and 21 days post inoculation (dpi) for analyses by virus isolation/titration, co-cultivation and qPCR. The results showed that for both virus strains, 1) cell-associated virus in PBMC (determined by co-cultivation) was detected in intact mice but not in splenectomized mice; 2) the mean viral DNA load in PBMC of splenectomized mice was 4.4-(HaNa1)/2.7-(Smith) fold lower at the peak viremia (7 dpi) in contrast to that of intact mice; and 3) infectious virus in the submandibular glands was detected later in splenectomized mice (14 dpi) than in intact mice (7 dpi). Moreover, the average virus titers in submandibular glands of splenectomized mice were 10-(HaNa1)/7.9-(Smith) fold lower at 14 dpi and 1.7-(HaNa1)/2.1-(Smith) fold lower at 21 dpi compared with that of intact mice. Upon inoculation with MCMV Smith, infectious virus was found in the kidneys and liver of intact mice, but not in splenectomized mice. Taken together, all these data clearly demonstrate that virus dissemination to distant organs is reduced in splenectomized mice, further confirming the importance of the spleen as a viremia booming site for a natural MCMV infection.

In **Chapter 6**, all data obtained in the present thesis were reviewed and discussed. A general hypothetical model for MCMV dissemination throughout the body was proposed: upon oronasal exposure to MCMV, the nasal mucosa and NALT serve as portal of entry for MCMV. From these primary replication sites, the virus is transported in non-productively infected leukocytes to the draining lymph nodes (submandibular LN and deep cervical LN) and finally to the spleen and lungs possibly via lymph and blood circulation. In the spleen, a subsequent cell-associated viremia is regulated, from where a second replication is initiated in various tissues such as submandibular glands (for both Smith and HaNa1), liver and kidneys (only for Smith).

Samenvatting

Human cytomegalovirus (HCMV) is een betaherpesvirus dat over het algemeen subklinische infecties veroorzaakt in immunocompetente individuen. In immunogecompromitteerde patiënten daarentegen kan het HCMV aanleiding geven tot klinisch significante ziekten. Het HCMV wordt overgedragen van geïnfecteerde gastheren op gevoelige gastheren via de oronasale route maar hoe het virus zich verspreidt doorheen het lichaam na blootstelling tijdens een natuurlijke infectie is nog niet geweten. De strikte gastheerspecificiteit van HCMV maakt het moeilijk om de pathogenese van humane cytomegalovirus infecties te bestuderen in experimentele diermodellen. Vandaar dat er momenteel gebruikt gemaakt wordt van infectiemodellen met dierlijke cytomegalovirussen. Infectie van muizen met het muriene cytomegalovirus (MCMV) is een goed diermodel voor de studie van HCMV infectie, maar de invasie strategie van het MCMV na oronasale trasmissie is eveneens ongekend.

Hoofdstuk 1 geeft een literatuuroverzicht van de huidige kennis van het MCMV waarbij eerst de historie, classificatie, virus eigenschappen en replicatie van het MCMV geïntroduceerd werden. Daarnaast werden ook de epizoötiologie en de pathogenese van MCMV infectie alsook de immune response tegen het MCMV gereviseerd . Tot slot, werd ook kort de anatomie van de orale en nasale caviteit en lymfeknopen geassocieerd aan het spijsverterings- en ademhalingsstelsel in de muis kort besproken.

In **hoofdstuk 2** werden de doelstellingen van deze thesis geformuleerd. Een eerste doelstelling was het ontwikkelen van een diermodel waarin een natuurlijke infectie met MCMV nagebootst word om zo de pathogenese van twee MCMV stammen te kunnen vergelijken, namelijk de “low-passaged” MCMV HaNA1 stam en de “highly-passaged” MCMV Smith stam, in vrouwelijke Balb/c muizen. Een tweede algemene doelstelling was om de kinetiek van virus disseminatie doorheen het lichaam na oronasale bloostelling te achterhalen. Een derde en laatste doelstelling was het ontrafelen van de rol die de milt speelt in virus disseminatie gedurende natuurlijke,

primaire MCMV infectie.

In **Hoofdstuk 3** werd de pathogenese van de hoge passage MCMV-Smith stam vergeleken met die van het lage passage Belgische HaNa1-isolaat. Hiervoor werden volwassen BALB/c-muizen oronasaal geïnoculeerd met ofwel een lage (10^4 $TCID_{50}$/muis) of hoge (10^6 $TCID_{50}$/muis) inoculatiedosis. Beide stammen repliceerden voornamelijk in de nasale mucosa en submandibulaire klieren gedurende één tot twee maanden. In de nasale mucosa werd MCMV vroeger en langer gedetecteerd (1-49 dpi) en bereikte hogere titers bij de hoge inoculatiedosis vergeleken met de lage inoculatiedosis (14-35 dpi). In de submandibulaire klieren werd een gelijkaardige bevinding gedaan (hoge dosis: 7-49 dpi; lage dosis: 14-42 dpi). In de longen, vertoonden beide stammen een beperkte replicatie. In de lever, milt en nieren kon alleen bij de Smith-stam een beperkte productieve infectie waargenomen worden. Voor beide stammen konden de geïnfecteerde cellen als volgt geïdentificeerd worden: de olfactorische neuronen en sustentaculaire cellen in het olfactorisch epitheel, de macrofagen en dendritische cellen van het neus-geassocieerd lymfoïd weefsel, de acinaire cellen in de submandibulaire klieren en in de longen macrofagen en epitheelcellen. Uit antistofanalyse bleek IgG2a voor beide stammen de belangrijkste detecteerbare antistofsubklasse te zijn. Onze resultaten tonen aan dat er significante fenotypische verschillen bestaan tussen beide virusstammen. Hier werd aangetoond dat MCMV-HaNa1-infectie in muizen een interessant model is om een beter inzicht te verwerven in HCMV-infecties bij immunocompetente mensen.

In **Hoofdstuk 4** toonde virustitratie een productieve virale replicatie aan van zowel HaNa1 als Smith in de nasale mucosa vanaf 1 dpi tot het einde van het experiment (14 dpi), in de longen vanaf 5 tot 14 dpi en in de submandibulaire klieren vanaf 7 tot 14 dpi. In tegenstelling tot MCMV-HaNa1, veroorzaakte MCMV-Smith wel een beperkte productieve infectie in abdominale organen (milt, lever en nieren) vanaf 5 dpi (milt), 7 dpi (lever) en 10 dpi (nieren) tot het einde van het experiment. Co-culturen toonden aan dat bij beide stammen cel-geassocieerd virus in een niet-infectieuze vorm aanwezig was in het neus-geassocieerd lymfoïd weefsel van dag

1 tot 14 post infectie, in de submandibulaire lymfeknopen van 3 tot 5 dpi, in de diepe cervicale lymfeknopen van 3 tot 14 dpi, in de mediastinale lymfeknopen van 7 tot 14 dpi, in de milt van 5 tot minstens 10 dpi en in de perifere bloed mononucleaire cellen (PBMC) vanaf 7 tot 10 dpi. Deze studie toont aan dat, na oronasale blootstelling, MCMV eerst de nasale mucosa en het neus-geassocieerd lymfoïd weefsel binnentreedt en van hieruit – mogelijks via de drainerende lymfe en het bloed - spreidt naar de milt; een hieruit volgende cel-geassocieerde viremie transporteert MCMV naar de submandibulaire klieren, alsook naar de lever en nieren bij MCMV-Smith, waar een secondaire productieve replicatie plaatsvindt.

In **Hoofdstuk 5** werd de rol van de milt tijdens een MCMV infectie onderzocht door gebruik te maken van intacte en gesplenectomeerde Balb/c muizen. De MCMV Smith stam, die al veel passages heeft doorgemaakt, en de MCMV HaNa1 stam, die slechts enkele passages heeft doorgemaakt, werden beiden gebruikt. Op 7, 14 en 21 dagen post inoculatie (dpi) werden verschillende stalen verzameld voor virusisolatie/-titratie, co-cultivatie en qPCR. De resultaten tonen aan dat voor beide stammen, 1) cel-geassocieerde viremie in PBMC (bepaald met co-cultivatie) werd gevonden bij intacte muizen maar niet in gesplenectomeerde muizen; 2) de gemiddelde virale DNA-load in PBMC van gesplenectomeerde muizen was 4.4-(HaNa1)/2.7-(Smith) keer lager tijdens de piek viremie (7dpi) dan in die van intacte muizen; en 3) infectieus virus werd later gevonden in de submandibulaire klieren bij gesplenectomeerde muizen (14dpi) dan in bij intacte muizen (7dpi). Bovendien waren de gemiddelde virus titers in de submandibulaire klieren van gesplenectomeerde muizen 10-(HaNa1)/7.9-(Smith) keer lager op 14dpi en 1.7-(HaNa1)/2.1-(Smith) keer lager op 21dpi dan bij intacte muizen. Na inoculatie met MCMV Smith stam werd er infectieus virus gevonden in de nieren en lever van intacte muizen, maar niet die van in gesplenectomeerde muizen. Samengevat tonen al deze data aan dat virus disseminatie naar perifere organen verminderd is in gesplenectomeerde muizen, wat het belang van de milt als viremie-booming-site tijdens een natuurlijke MCMV infectie bevestigt.

Hoofdstuk 6 geeft een overzicht van alle resultaten, en worden alle data nog eens besproken. Een algemeen hypothetisch model voor de spreiding van MCMV in het lichaam wordt weergegeven: na een oronasale blootstelling aan MCMV, dienen de nasale mucosa en NALT als intredepoort voor MCMV. Van deze primaire plaats van replicatie, wordt het virus getransporteerd in niet-productieve geinfecteerde leukocyten naar de drainerende lymfeknopen (submandibulaire LN en de diepe cervicale LN) om finaal de milt en longen te bereiken via de lymfe- en bloedcirculatie. In de milt ontstaat er een volgende cel-geassocieerde viremie, waardoor een secundaire replicatie kan optreden in verschillende weefsels zoals de submandibulaire klieren (voor beide Smith en HaNa1), lever en nieren (enkel voor Smith).

Curriculum Vitae

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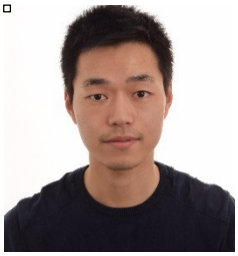

Shunchuan Zhang was born on 30th March 1986 in Sichuan, China.

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1. **Shunchuan Zhang**, Jun Xiang, Jan Van Doorsselaere, Hans J. Nauwynck. *Comparison of the pathogenesis of the highly passaged MCMV Smith strain with that of the low passaged MCMV HaNa1 isolate in BALB/c mice upon oronasal inoculation* (Veterinary Research, 2015; 46(1): 94.)
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2. **Shunchuan Zhang**, Jun Xiang, Jan Van Doorsselaere, Hans Nauwynck. *Comparison of the pathogenesis of the highly passaged MCMV Smith strain with that of the low passaged MCMV HaNa1 strain in BALB/c mice upon oronasal inoculation.* (BELVIR 2014, Belgium, oral presentation)
3. **Shunchuan Zhang**, Jun Xiang, Lowiese Desmarets, Hans Nauwynck. *Systemic dissemination of MCMV HaNa1 via non-productively infected leukocytes between nose, lymphoid organs and salivary glands upon oronasal exposure.* (International Herpesvirus workshop 2015, USA, poster)
4. **Shunchuan Zhang**, Jun Xiang, Sebastiaan Theuns, Lowiese M.B. Desmarets, Ivan Trus, Hans J. Nauwynck. MCMV exploits the spleen as a transfer hub for systemic dissemination upon oronasal inoculation. (BELVIR 2015, Belgium, oral presentation)

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