

Journal of General Virology

Murine cytomegalovirus infection via the intranasal route offers a robust model of immunity upon mucosal CMV infection --Manuscript Draft--

Manuscript Number:	JGV-D-15-00491R2
Full Title:	Murine cytomegalovirus infection via the intranasal route offers a robust model of immunity upon mucosal CMV infection
Short Title:	Memory inflation upon intranasal MCMV infection
Article Type:	Standard
Section/Category:	Animal - Large DNA Viruses
Corresponding Author:	Luka Čičin-Šain Helmholtz Centre for Infection Research Braunschweig, GERMANY
First Author:	Jennifer D. Oduro
Order of Authors:	Jennifer D. Oduro Anke Redeker Niels A.W. Lemmermann Linda Ebermann Thomas F. Marandu Iryna Dekhtiarenko Julia K. Holzki Dirk Busch Ramon Arens Luka Čičin-Šain
Abstract:	<p>Cytomegalovirus (CMV) is a ubiquitous virus, causing the most common congenital infection in humans, yet a vaccine against this virus is not available. The experimental study of immunity against CMV in animal models of infection, such as the infection of mice with the mouse CMV (MCMV), has relied on systemic intraperitoneal infection protocols, although the infection naturally transmits by mucosal routes via body fluids containing CMV. To characterize the biology of infections by mucosal routes, we have compared the kinetics of virus replication, the latent viral load, and CD8 T cell responses in lymphoid organs upon experimental intranasal and intragastric infection to intraperitoneal infection of two unrelated mouse strains. We have observed that intranasal infection induces robust and persistent virus replication in lungs and salivary glands, but a poor one in the spleen. CD8 T cell responses were somewhat weaker than upon intraperitoneal infection, but showed similar kinetic profiles and phenotypes of antigen-specific cells. On the other hand, intragastric infection resulted in abortive or poor virus replication in all tested organs, and poor T cell responses to the virus, especially at late times after infection. Consistent with the T cell kinetics, the MCMV latent load was high in the lungs, but low in the spleen of intranasally infected mice and lowest in all tested organs upon intragastric infection. In conclusion, we show here that intranasal, but not intragastric infection of mice with MCMV represents a robust model to study short and long-term biology of CMV infection by a mucosal route.</p>

1 **Murine cytomegalovirus infection via the intranasal route offers a robust model of**
2 **immunity upon mucosal CMV infection**

3
4 Running title: Memory inflation upon intranasal MCMV infection

5 Jennifer D. Oduro^a, Anke Redeker^b, Niels A.W. Lemmermann^c, Linda Ebermann^a,
6 Thomas F. Marandu^{ad}, Iryna Dekhtiarenko^a, Julia K. Holzki^a, Dirk H. Busch^{e,f,g}, Ramon
7 Arens^b, Luka Čičin-Šain^{a,g,h} #

8 Department for Vaccinology/Immune Aging and Chronic Infection, Helmholtz Centre for
9 Infection Research, Braunschweig, Germany^a; Department of Immunohematology and
10 Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands^b;
11 Institute for Virology and Research Center for Immunotherapy (FZI), University Medical
12 Center of the Johannes Gutenberg-University Mainz, Mainz, Germany^c; Dar es Salaam
13 University College of Education, Tanzania^d; Institute for Medical Microbiology,
14 Immunology and Hygiene, Technische Universität München, Munich, Germany^e; Focus
15 Group “Clinical Cell Processing and Purification,” Institute for Advanced Study,
16 Technische Universität München, Munich, Germany^f; German Center for Infection
17 Research (DZIF)^g; Department for Virology, Medical School Hannover, Hannover,
18 Germany^h

19 #Corresponding author: Luka.Cicin-Sain@helmholtz-hzi.de

20 0049-531-6181-4616

21 Keywords: cytomegalovirus, memory inflation, mucosal immunity, CD8 T cells

22 Subject category: 1b (Animal DNA viruses)

23 Word count: 5497 Word count (abstract): 250

24

25 **Abstract**

26 Cytomegalovirus (CMV) is a ubiquitous virus, causing the most common congenital
27 infection in humans, yet a vaccine against this virus is not available. The experimental
28 study of immunity against CMV in animal models of infection, such as the infection of
29 mice with the mouse CMV (MCMV), has mainly relied on parenteral infection protocols,
30 although the virus naturally transmits by mucosal routes via body fluids. To characterize
31 the biology of infections by mucosal routes, we have compared the kinetics of virus
32 replication, the latent viral load, and CD8 T cell responses in lymphoid organs upon
33 experimental intranasal (targeting the respiratory tract) and intragastric (targeting the
34 digestive tract) infection to systemic intraperitoneal infection of two unrelated mouse
35 strains. We have observed that intranasal infection induces robust and long-term virus
36 replication in lungs and salivary glands, but a limited one in the spleen. CD8 T cell
37 responses were somewhat weaker than upon intraperitoneal infection, but showed
38 similar kinetic profiles and phenotypes of antigen-specific cells. On the other hand,
39 intragastric infection resulted in abortive or poor virus replication in all tested organs, and
40 poor T cell responses to the virus, especially at late times after infection. Consistent with
41 the T cell kinetics, the MCMV latent load was high in the lungs, but low in the spleen of
42 intranasally infected mice and lowest in all tested organs upon intragastric infection. In
43 conclusion, we show here that intranasal, but not intragastric infection of mice with

44 MCMV represents a robust model to study short and long-term biology of CMV infection
45 by a mucosal route.

46

47 **Introduction**

48 Cytomegalovirus (CMV) infection is the most common congenital viral infection in
49 developed countries (Griffiths et al., 2015, Kenneson and Cannon, 2007). Congenital
50 HCMV infections occur upon infection or reinfection of adult women during pregnancy
51 (Boppana et al., 2001, Fowler et al., 2003). Infection can also occur via blood transfusion
52 and organ transplantation, which is especially problematic in immunosuppressed sero-
53 negative recipients, where such an infection may cause severe disease. The exact
54 natural route of transmission and entry into the body of most hosts infected with CMV
55 post-partum remains, however, uncertain. While it is reasonable to assume that infection
56 occurs via mucosal surfaces exposed to infectious secretions, the exact route(s) are
57 difficult to identify by epidemiological studies. HCMV can be shed for months, if not
58 years, upon infection by numerous body fluids (Cannon et al., 2011), arguing that CMV
59 may naturally transmit itself by more than one means of infection. Numerous studies
60 identified that HCMV shedding by breast milk (Diosi et al., 1967) may be a way of
61 transmission (Stagno et al., 1980, Dworsky et al., 1983), especially in pre-term infants
62 (Vochem et al., 1998).

63 Sexual activity may present a risk factor for HCMV infection in adult women (Fowler and
64 Pass, 1991) and CMV infection is common in day-care workers attending very young
65 children ([Adler, 1989](#)). However, multiple body fluids are likely to be simultaneously

66 exchanged during close contacts that are required for infection, and the lack of
67 pathognomonic symptoms complicates the epidemiological study of HCMV transmission
68 in adults.

69 Experimental studies of transmission in animal models, on the other hand, are limited to
70 HCMV-orthologue viruses, such as the rhesus CMV (RhCMV) or the murine CMV
71 (MCMV), due to the strict species specificity of cytomegaloviruses. Nevertheless, the
72 mouse model of CMV infection has provided deep insight in CMV pathogenesis and
73 immunity and reflects numerous aspects of HCMV biology (Redwood et al., 2013).
74 Surprisingly, little is known about the natural route of infection by MCMV. While
75 intranasal MCMV infection of adult mice has been occasionally used in previous reports
76 (Jordan, 1978, Shanley et al., 1997, Morello et al., 2005), the only effort to systematically
77 compare it to other routes of infection relied on low infection doses and was limited in its
78 interpretation because of wide variations of outcomes between cages (Doom and Hill,
79 2008). A study in the model of neonate mouse infection has reported that a transgenic
80 MCMV, expressing mCherry as a reporter gene may be found in the lungs of neonates
81 infected by the laryngopharyngeal route and adult mice infected intranasally (i.n.) (Stahl
82 et al., 2013). This study used a molecular clone of MCMV lacking the viral protein Mck-2
83 (Saederup et al., 2001), and a subsequent study by the same group showed that Mck-2
84 is a determinant of viral pathogenicity in lungs of neonate mice infected by the
85 laryngopharyngeal route (Stahl et al., 2015). Therefore, the replication and immune
86 response to mucosal infections by a wild-type MCMV remain unknown.

87 On the other hand, the well-studied model of adult mouse infection has relied on
88 systemic infection of mice by the intraperitoneal (i.p.) (Munks et al., 2006) or intravenous

89 (i.v.) (Karrer et al., 2003) route, or by injection of the virus into the footpad, assuming
90 that this may mimic a putative transmission by mouse bites (Podlech et al., 2002).
91 Parenteral infection results in MCMV dissemination to multiple organs during a primary
92 viremia phase, and a secondary viremia delivering the virus to the salivary glands
93 (Podlech et al., 2002). This also results in a remarkably robust CD8 T cell response
94 (Cicin-Sain et al., 2012), where virus specific CD8 T cells accumulate at the time of virus
95 latency (Holtappels et al., 2002), a phenomenon aptly named memory inflation (Karrer et
96 al., 2003, O'Hara et al., 2012). Inflationary responses were shown to consist
97 predominantly of CD62L⁻ effector memory cells (Holtappels et al., 2000, Karrer et al.,
98 2003), implying recent antigenic encounter, and similar types of responses were
99 observed in natural and experimental rhesus monkey infection with RhCMV (Hansen et
100 al., 2009, Cicin-Sain et al., 2011, Pitcher et al., 2002), or in people carrying latent HCMV
101 (Appay et al., 2002). However, it has remained unclear if mucosal infection would elicit
102 such responses.

103 To define the mucosal route reflecting the natural infection of an adult host, and whether
104 this would result in CD8 T cell responses matching those upon systemic infection, we
105 compared the kinetics of virus replication, the latent viral load, and CD8 T cell responses
106 in mice infected by the intranasal, the gastric lavage (gavage) and the intraperitoneal
107 route. We observed that i.n. infection results in robust virus replication during primary
108 infection and secondary dissemination to salivary glands, as well as elevated latent
109 loads in the lungs, whereas gavage infection was either abortive or resulted in poor virus
110 replication and very low latent loads in the tested organs. Inflationary responses were
111 less pronounced upon mucosal infection than upon the systemic one, but clearly
112 noticeable in all tested compartments upon i.n. infection. Therefore, the data presented

113 here suggest that intranasal MCMV infection may be a suitable model of infection of
114 adult mice, and potentially a model of natural mucosal CMV infection.

115

116 **Results**

117 ***Intranasal MCMV infection results in robust virus replication in the lungs, whereas*** 118 ***infection by gavage is inefficient***

119 Viral replication upon mucosal infection was compared to systemic infection via the i.p.
120 route by determining infectious virus titers in spleens and lungs of BALB/c mice at 5 and
121 14 days post infection (dpi), as well as in salivary glands at 14 dpi. In mice infected with
122 MCMV by gavage, infectious virus in spleen and lungs was almost undetectable. In half
123 of these mice, salivary gland MCMV titers were substantially lower than in i.p. infected
124 controls (Fig. 1a). In contrast, i.n. infection induced higher viral titers in the lungs than
125 i.p. injection. On the other hand, titers in spleens were reduced after i.n. infection.
126 Spread to the salivary glands had nearly the same efficiency in both infection routes
127 (Fig. 1a). Since high virus titers in the salivary glands are deemed important for
128 transmission to the next host, our results may suggest that transmission of CMV in mice
129 occurs via the respiratory rather than the gastrointestinal tract. Hence, we analysed
130 MCMV replication after infection via this route in detail.

131 Virus titers were determined for a week in spleen and lung samples harvested daily from
132 mice infected i.n. or i.p., and at 10 dpi. Virus was detected in lungs of i.n. infected mice
133 as soon as 1 dpi, and titers were increased several orders of magnitude above those in
134 mice infected by the i.p. route at all tested time points. It is important to note that i.n.

135 infected mice showed no obvious symptoms of disease at any time point, despite the
136 persistence of very high virus titers in their lungs. In a separate experiment, spleen, lung
137 and salivary glands were analysed for infectious virus titers at 7, 14 and 21 dpi. Elevated
138 titers in the lungs could be detected even by 21 dpi (Fig. 1c), and some infectious virus
139 could be detected in some mice as late as 42 dpi (not shown). In contrast, low titers of
140 infectious virus were detectable in the spleen only from 5 to 7 dpi after i.n. infection,
141 whereas i.p. infection induced substantially higher infectious titers which peaked by 4 dpi
142 and could still be detected by 21 dpi (Fig. 1b, 1c). Virus titers in salivary glands of i.n. or
143 i.p. infected mice were comparable at all tested time points (Fig. 1c).

144

145 ***IE1-specific CD8 T cells inflate in the blood after intranasal infection***

146 After systemic MCMV infection, CD8 T cells recognizing defined immunodominant
147 peptides have been shown to accumulate after clearance of lytic viral replication, a
148 phenomenon known as memory inflation (Karrer et al., 2003, Sierrro et al., 2005, Munks
149 et al., 2006, Holtappels et al., 2002, O'Hara et al., 2012). In order to test whether
150 memory inflation is induced upon mucosal MCMV infection, we compared CD8 T cell
151 kinetics after i.n. infection and infection by gavage to systemic infection by the i.p. route.
152 We monitored CD8 T cells for their response to MCMV infection in the peripheral blood
153 up to six months p.i.. Mice that showed no CD8 T cell response above background at
154 any tested time point were excluded from the CD8 T cell analysis, to censor false
155 negative events due to inefficient primary infection. One should note that these events
156 occurred only in gavage infection, consistent with data showing the absence of
157 infectious virus titers in organs (see Fig. 1a). The representative gating strategy for the
158 flow cytometry analysis is depicted in Fig. S1.

159 First, the percentage and absolute numbers of primed CD8 T cells (defined by
160 CD11^{high}/CD44^{high}) were monitored. Primed cells displayed a strong initial increase in
161 mice infected via the i.p. route by 1 week after infection, which slightly contracted by
162 week 4 and remained rather stable in relative and absolute terms later on. The initial
163 increase was delayed for a week after mucosal infection and the obtained values were
164 clearly lower than after i.p. infection. After a contraction around week 4, the frequency of
165 primed CD8 T cells remained rather stable in i.n. infected mice, but decreased to
166 background level in the gavage-infected group (Fig. 2a, left panel). The absolute
167 numbers of primed cells upon mucosal infection was hardly elevated over levels
168 observed in uninfected mice (Fig. 2a, right panel). While the difference was small, mice
169 infected via the i.n. route showed a higher frequency and number of primed CD8 T cells
170 than mice infected by gavage at all time points (Fig. 2a). Next, we analysed the CD8 T
171 cell response to an immunodominant MCMV-derived epitope; the IE1₁₆₈₋₁₇₆ epitope,
172 which induces CD8 T cells with an inflationary phenotype in mice with the H-2^d
173 haplotype (e.g. BALB/c) (Karrer et al., 2003, Sierro et al., 2005, Holtappels et al., 2002).
174 We used a MHC class I tetramer (IE1-tetramer) to stain IE1-specific CD8 T cells and
175 detect them via flow cytometry. In mice infected by the i.p. route, we observed the
176 previously reported inflationary kinetic profile of IE1-specific CD8 T cells, with highly
177 elevated relative and absolute counts throughout the time course of the experiment (Fig.
178 2b). Consistent with the kinetics of primed cells, the initial peak of IE1-specific responses
179 occurred later (in week 2 p.i.) upon both mucosal infection routes. Whereas the
180 frequency and counts of IE1-specific CD8 T cells declined in mice infected by gavage by
181 week 4 p.i. and remained low thereupon, in i.n. infected mice the percentage and
182 absolute number increased from week 4 to week 13 and stayed constant afterwards

183 (Fig. 2b). IE1-specific responses after i.n. infection were weaker than after i.p. infection
184 and this difference was more obvious for absolute (Fig. 2b, right panel) than for relative
185 numbers (Fig. 2b, left panel). These data suggest a hierarchy of CD8 T cell responses
186 upon the three infection routes tested: Responses are clearly strongest after i.p.
187 infection, followed by i.n. infection and only then by gavage inoculation.

188

189 ***IE1-specific CD8 T cells inflate in lymphoid organs after intranasal infection***

190 The robust T cell response in mice infected via the i.n. route prompted us to examine it
191 in more detail. Therefore, we investigated the virus-specific responses in several
192 lymphoid compartments in addition to the blood by determining the kinetics of primed
193 and MCMV-specific CD8 T cells in the spleen, the mediastinal lymph nodes (MLN) and
194 the inguinal lymph nodes (ILN) of BALB/c mice. The MLNs are the draining lymph nodes
195 of the lungs and have been shown to also drain substances administered via the i.p.
196 route (Kirby et al., 2009, Ple et al., 2010, Marco et al., 1992, Hsu et al., 2009), while the
197 inguinal lymph nodes were used as non-draining lymph nodes.

198 The kinetics of primed and IE1-specific CD8 T cells (Fig. 3a) in the blood was in essence
199 comparable to the previous experiment (Fig. 2), although the absolute numbers in i.p.
200 infected mice were lower in this experiment, resulting in a smaller difference to mice
201 infected via the i.n. route. In both infection routes, the kinetics of CD8 T cells in the
202 spleen (Fig. 3b) mimicked closely those in the blood (Fig. 3a). Both the percentages and
203 the numbers of primed and IE1-specific CD8 T cells were much lower in the lymph
204 nodes in general (Fig. 3c, 3d). Percentages of both primed and MCMV-specific CD8 T
205 cells increased in the non-draining ILNs only at late time points, but remained lower as in
206 the blood or spleen (Fig. 3c, first and third panel). Notably, the increase in the frequency

207 of primed CD8 T cells was also detected in uninfected controls, suggesting that it may
208 be a function of exposure to environmental antigens in aging mice, rather than caused
209 by MCMV infection. The numbers of primed CD8 T cells remained very low throughout
210 the experiment, with the exception of a transient increase in i.p. infected mice 1 week p.i.
211 (Fig. 3c, second panel), which was reflected as an early peak in the count of IE1-specific
212 cells as well (Fig. 3c, fourth panel). Furthermore, total counts of IE1-specific cells
213 increased in both infection groups at later time points, but again much less than in blood
214 or spleen (Fig. 3c, fourth panel). In the MLNs of i.n. infected mice, the absolute count of
215 primed and IE1-specific CD8 T cells exceeded those after i.p. infection early upon
216 infection (Fig. 3d, second and fourth panel), but this reversed for primed cells by 13
217 weeks p.i. (Fig. 3d, second panel). On the other hand, the percentage and number of
218 IE1-specific CD8 T cells was comparable in the MLN of i.p. and i.n. infected mice at later
219 time points (Fig. 3d, third and fourth panel).

220 In sum, these data suggest that i.n. infection initiates a robust MCMV-specific T cell
221 response in the draining lymph node and induces memory inflation, not only in the blood,
222 but also in the spleen and lymph nodes.

223

224 ***MCMV-specific CD8 T cells inflate after intranasal infection of 129/Sv mice***

225 To exclude that these results are restricted to the BALB/c mouse strain, we analysed the
226 kinetics of the CD8 T cell response in the blood upon mucosal MCMV infection in mice
227 of the 129/Sv strain. Like BALB/c mice, these mice do not express the resistant *Cmv1*
228 allele (Scalzo et al., 1995), and thus display similar levels of CMV control by NK cells.
229 We analysed the kinetics of primed CD8 T cells and CD8 T cells specific for an

230 inflationary K^b-restricted epitope corresponding to the amino acid position 316-323 of the
231 M38 protein of MCMV (Munks et al., 2006).

232 The largest discrepancy between BALB/c and 129/Sv mice was observed upon gavage
233 infection. Namely, 23 out of 35 129/Sv mice showed no MCMV-specific response to
234 infection at any time point, whereas this was the case in only 2 out of 20 BALB/c mice.
235 Among the mice showing responses, the kinetics of primed and M38-specific CD8 T
236 cells displayed the same hierarchy as observed for primed and IE1-specific cells in the
237 BALB/c model (Fig. 2). Systemic i.p. infection induced the strongest inflationary
238 response, followed by i.n. infection, which still induced inflating M38-specific CD8 T
239 cells, but at a lower level than i.p. infection. Infection by gavage resulted in the weakest
240 response and no increase of antigen-specific CD8 T cells after 4 weeks of infection
241 (Fig. 4a, 4b).

242

243 ***Most primed CD8 T cells display an effector memory phenotype upon intranasal***
244 ***infection***

245 Most inflationary CD8 T cells show an effector memory (EM) phenotype after systemic
246 i.p. infection, characterised by low expression of CD62L and high expression of KLRG1.
247 In contrast, non-inflationary cells usually assume a central memory (CM) phenotype
248 (Sierro et al., 2005, Snyder et al., 2008), defined here as CD62L^{hi} KLRG1^{lo}. We
249 analysed the phenotype of primed CD8 T cells upon MCMV infection in 129/Sv mice and
250 observed that most primed CD8 T cells of i.p. infected mice assumed an EM phenotype
251 (KLRG1⁺/CD62L⁻) throughout the infection. This percentage was a bit lower in mice
252 infected via the i.n. route, but the lowest percentage was observed upon gavage or in
253 uninfected mice (Fig. 4c). For cells displaying a CM phenotype, the hierarchy was

254 inverted (Fig. 4d). Kinetics of CM and EM phenotypes were similar in BALB/c mice, with
255 most EM cells upon systemic infection, the least in uninfected mice, and intermediate
256 phenotypes in the two mucosal infections (data not shown).

257 The phenotype of M38-specific CD8 T cells was also analysed in different compartments
258 (blood, spleen, ILN, MLN) at an early (week 2) and a late time point (week 26) after
259 infection. Early after infection, the phenotype was comparable between all infection
260 routes, with more KLRG1⁺/CD62L⁻ M38-specific cells in the blood and spleen (Fig. 4e,
261 upper panel) and with more KLRG1⁻/CD62L⁺ M38-specific cells in the lymph nodes (Fig.
262 4e, lower panel). Later after infection, this distribution remained the same, but mice
263 infected by gavage had clearly less EM cells and more CM cells in M38-specific CD8 T
264 cells of the blood and spleen than seen upon i.p. or i.n. infection (Fig. 4f).

265 In combination, our data argue that i.n. MCMV infection induces inflationary CD8 T cell
266 responses and that these inflationary cells retain the EM phenotype, although at slightly
267 lower frequencies than upon i.p. infection.

268

269 ***Latent virus is elevated in the lungs and low in the spleen after intranasal*** 270 ***infection***

271 It was recently suggested that the spleen might be an important site for the maintenance
272 of memory inflation (Smith et al., 2014, Redeker et al., 2014). In that case, the
273 attenuated memory inflation upon intranasal infection (Fig. 2-4) may be a reflection of
274 lower splenic viral genome load upon mucosal infection. Hence, we determined the
275 latent load in spleens of 129/Sv mice infected by the different infection routes at 26
276 weeks p.i.. I.p. infection resulted in the highest latent load in the spleen (Fig. 5a). This

277 pattern was not maintained in lungs, where i.n. infection led to the highest latent viral
278 genomes (Fig. 5b). The latent genome load was lowest after infection by gavage in both
279 organs (a more detailed comparison of latent loads is provided in Fig. S2). We repeated
280 this analysis in BALB/c mice with similar results (data not shown).

281 In sum, our results indicate that the latent MCMV load in an organ depends on the route
282 of infection. Considering the results shown in Fig. 1, we observed an association
283 between the magnitude of viral replication in an organ during primary infection, and the
284 latent genome load during latency.

285

286 **Discussion**

287 In this study we have systematically compared different routes of MCMV infection. We
288 show that the intranasal route results in robust and long-lasting virus replication in the
289 lungs and salivary glands, consistent with a previous report (Morello et al., 2005), and is
290 accompanied with memory CD8 T cell inflation, yet this is impaired upon intragastric
291 infection, even when we optimize pH conditions to avoid virus neutralization prior to its
292 entry into cells. This implies that the respiratory mucosa, rather than the gastrointestinal
293 one, may serve as a major site of viral entry into the host. Previous studies by the
294 Förster group showed similar results in newborn mice (Stahl et al., 2013), but they used
295 transgenic viruses containing three separate mutations, and it remained unclear if the
296 poor replication in the gut mucosa upon oral infection reflected the biology of the wild-
297 type MCMV infection. This is in stark contrast with data indicating that intragastric
298 infection of newborn mice with wild-type MCMV can result in viral dissemination (Wu et

299 al., 2011). However, that study was based on qualitative PCR of viral nucleic acids,
300 while we measured the replicating virus by a quantitative assay, allowing us not only to
301 identify its presence, but also to assess its abundance. Interestingly, the large majority of
302 mice infected by gavage had no detectable viral titers in the tested primary organs
303 (spleen, lungs), but a distinct number of those mice had detectable viral replication in the
304 salivary glands. As the salivary glands are generally thought to be targeted only upon
305 secondary viremia (Campbell et al., 2008), this may be either explained by virus
306 replication in a primary organ that was not tested by us, or by the possibility that virus
307 might disseminate without a major primary expansion.

308 Our results suggest that the wild-type MCMV infection by the intragastric route is
309 substantially less efficient than intranasal infection in adult mice, arguing that the
310 respiratory rather than the gastrointestinal mucosa, may be a more dominant natural site
311 of entry of this virus. One needs to note, however, that our study focused only on the
312 respiratory and gastrointestinal epithelium, whereas other mucosal surfaces, such as the
313 genitourinary mucosa or the epithelium of the oral cavity, need to be addressed in future
314 studies.

315 Surprisingly, the high viral titers observed upon i.n. infection resulted in no overt disease.
316 Since low pathology is also a feature of primary HCMV infections of adults, one may
317 speculate that i.n. infection reflects a natural infection condition. However, more studies
318 are required to validate this hypothesis.

319 Most evidence for memory T cell inflation has rested on systemic experimental infection
320 with a high dose of the virus (Karrer et al., 2003, Munks et al., 2006). Recently, it was
321 shown that low dose inoculum of MCMV results in a severely hampered inflation of

322 memory T cells (Redeker et al., 2014) suggesting that the amount of virus is also a
323 determinant of memory inflation. While it is generally accepted that systemic infection
324 occurs during congenital HCMV infection by transfer of the virus through placenta,
325 infections after birth most likely occur via mucosal routes. Therefore, it was not clear if
326 infection of adult hosts by a mucosal route would result in a sufficient level of acutely
327 replicating virus to elicit memory inflation. We showed here that inflation of MCMV-
328 specific T cells can occur upon i.n. infection, but is less pronounced than in mice
329 infected by the i.p. route (Fig. 3).

330 It has been proposed that intermittent low-level antigen expression during latent MCMV
331 infection induces inflationary CD8 T cell responses (Seckert et al., 2012), and it has
332 been recently reported that inflationary responses occur due to the proliferation of T cells
333 in contact with the blood circulation, rather than in organ parenchyma (Smith et al.,
334 2014), probably because endothelial cells lining blood vessels are a major site of MCMV
335 latency (Seckert et al., 2009). If this model is accurate, and considering that mice
336 infected by the i.n. route showed substantially higher latent loads in the lungs, but lower
337 ones in the spleen (Fig. 5), our data imply that latent viral load in the spleen, rather than
338 the lungs, predicts the magnitude of memory inflation, consistent with the recent
339 observation that CMV-specific T cells proliferate in the spleen of latently infected mice
340 upon i.p. infection (Redeker et al., 2014). We focused in our study on prototypical
341 inflationary epitopes. While it is likely that non-inflationary epitopes will exhibit a similar
342 hierarchy of responses upon various infection routes, this aspect needs to be formally
343 confirmed in future studies.

344 Taken together, our results argue that i.p. infection drives inflationary CD8 T cell
345 responses that are more pronounced than the responses observed upon i.n. infection,
346 but essentially both infection routes induce responses with similar kinetics and
347 phenotypes of responding T cells. Since CMV induced pneumonia is a major pathology
348 caused by this virus and mucosal infection is likely to reflect the natural infection better
349 than intraperitoneal injection, we propose that the i.n. infection route offers a robust
350 model for the study of CMV replication and latency and mucosal immune responses in a
351 highly relevant site of infection.

352

353 **Materials and methods**

354 ***Mice***

355 BALB/c mice were purchased from Janvier, 129/Sv mice were purchased from Charles
356 River. Animals were kept in SPF conditions at the animal facility of the HZI
357 Braunschweig. Housing and handling was performed in agreement with good animal
358 practice defined by Federation of Laboratory Animal Science Associations (FELASA). All
359 animal experiments were performed in accordance with the German animal protection
360 law (TierSchG BGBI S. 1105; 25.05.1998) and were approved by the responsible state
361 office (Lower Saxony State Office of Consumer Protection and Food Safety) under
362 permit number 33.9-42502-04-10/0109.

363

364 ***Cells***

365 The murine bone marrow stromal cell line M2-10B4 (CRL-1972) (Lemoine et al., 1988a,
366 Lemoine et al., 1988b) was purchased from ATCC and maintained in DMEM
367 supplemented with 10% FCS, 1% glutamine and 1% penicillin/streptomycin. C57BL/6
368 murine embryonic fibroblasts (MEFs) were generated and cultured as previously
369 described (Podlech et al., 2002).

370

371 ***Virus***

372 The BAC-derived mouse cytomegalovirus clone pSM3fr 3.3 (Jordan et al., 2011) was
373 used as MCMV^{wt}, it was propagated on M2-10B4 cells and titrated on MEFs as
374 previously described (Podlech et al., 2002).

375

376 ***Infection***

377 Six- to ten-weeks-old female BALB/c or 129/Sv mice were infected with 2×10^5 PFU
378 MCMV^{wt} using different infection routes. Virus diluted in 200 μ l PBS was i.p. injected.
379 20 μ l of virus solution (in PBS) were administered into both nostrils of mice anaesthetized
380 with 10 μ l/g bodyweight of ketamine (10 mg/ml) + xylazine (1 mg/ml) in 0.9% NaCl. We
381 used ketamine, because isoflurane anaesthesia resulted in less efficient infection
382 (detected by virus titers in the lungs on day 4 p.i. – Fig. S3). As a second mucosal route,
383 mice were infected by gavage. Mice were fasted for 5 hrs and stomach acid was
384 neutralized with 100 μ l of a 5% NaHCO₃ solution injected into the stomach by a feeder
385 needle prior to infection with virus in 200 μ l PBS by the same route.

386

387 ***Determination of infectious virus in organs***

388 Organs were sterilely harvested from infected mice and stored in DMEM at -70°C until
389 titration. Thawed organs were homogenised on 70 µm-pore-size cell strainers in 5 mL
390 DMEM (supplemented with 5% FBS, 1% glutamine and 1% penicillin/streptomycin) and
391 homogenates were titrated on MEFs with centrifugal enhancement as described
392 previously (Cicin-Sain et al., 2005).

393

394 ***Blood and organ collection, processing and counting***

395 Blood was collected from the retrobulbar venous plexus of isoflurane anaesthetized
396 mice. 50µl of blood was transferred into EDTA reaction tubes and used for lymphocyte
397 counting at the VetScan HM5 Hematocytometer (Abaxis). The remaining blood
398 (approximately 100µl) was mixed with 300µl of heparin in HBSS (2U/ml) to prevent
399 clotting. Erythrocytes were lysed and cell pellets were stained for flow-cytometric
400 analysis.

401 Mediastinal and inguinal lymph nodes as well as spleens were harvested in ice cold
402 RPMI (supplemented with 10% FBS, 1% glutamine, 1% penicillin/streptomycin, 1%
403 sodium pyruvate and 0.1% β-mercaptoethanol) before further processing. Organs were
404 homogenized on 70µm-pore-size cell strainers and pelleted by centrifugation. Lymph
405 node samples were resuspended in 300 µl RPMI; 60 µl were used for counting of
406 lymphocytes, 240 or 80 µl (detailed kinetic) were used for immunofluorescence staining.
407 Erythrocytes were removed from spleen samples by 1 min incubation in 5 ml ACK buffer
408 (155 mM NH₄Cl, 10 mM KHCO₃, 1 mM EDTA; pH = 7.3), which stopped by addition of
409 10 ml RPMI, centrifugation, a PBS wash and resuspension in 1 ml RPMI. 6 µl of each
410 sample was mixed with 54 µl RPMI (1:10 dilution) for counting. Additionally, one sample

411 of each group was counted using a Neubauer chamber and approximately
412 10^6 cells/sample were used for immunofluorescence staining.

413

414 ***Immunofluorescence staining and flow cytometry analysis***

415 Cells were stained for surface markers and multimers recognizing MCMV-specific CD8 T
416 cells. In this study, MHC-Streptamers recognizing T cells specific for M38₃₁₆₋₃₂₃
417 (SSPPMFRV) were used in case of samples from 129/Sv mice and a conventional
418 tetramer binding T cells specific for IE1/pp89₁₆₈₋₁₇₆ (YPHFMPTNL) was used for samples
419 from BALB/c mice. All multimers were generated in house as described previously
420 (MHC-Streptamer (Knabel et al., 2002); tetramer (Altman et al., 1996)).

421 MHC multimer staining was initiated for 15 min at 4°C; subsequently, cell surface marker
422 antibodies were added and staining was continued for 30 min at 4°C. IE1-specific
423 tetramer-APC was directly used with surface marker antibodies to stain cells for 30 min
424 at 4°C. The following surface marker antibodies were used: αCD3-APC-eFluor 780
425 (clone 17A2; eBioscience), αCD4-Pacific blue (clone GK1.5; Biolegend), αCD8-PerCp-
426 Cy5.5 (clone 53-6.7; BD Biosciences), αCD11a-PE-Cy7 (clone 2D7; BD Biosciences),
427 αCD44-Alexa Fluor 700 (clone IM7; Biolegend / BD Biosciences), αKLRG1-FITC (clone
428 2F1/KLRG1; Biolegend), αCD62L-eFluor605NC (clone MEL-14; eBioscience), α-
429 KLRG1-Biotin (clone 2F1/KLRG1; Biolegend). For the detection of KLRG1-Biotin a
430 secondary staining with streptavidin-Brilliant Violet 510 (Biolegend) was performed.

431 After washing cells twice with FACS buffer (2% FBS in 1x PBS), samples were acquired
432 using an LSR-Fortessa (BD Biosciences). Results were analysed using FlowJo software
433 version 9.7.6 (Treestar).

434

435 **Quantification of latent viral genomes**

436 For quantification of MCMV genomes, DNA was isolated from spleens or lungs using the
437 DNeasy Blood & Tissue Kit (QIAGEN) and quantitated by M55 (encoding gB)- specific
438 qPCR normalized to cell number by pthrp specific qPCR as previously described
439 (Lemmermann et al., 2010).

440

441 **Acknowledgments**

442 This work has been supported by grants by the Helmholtz Association (VH-NG-638) and
443 the European Research Council (StG 260934) to L.C.-S. JDO was supported in part by
444 the Hannover Biomedical Research School (HBRS) and the Center for Infection Biology
445 (ZIB).

446 We would like to thank Klaus Schugart and Nora Kühn (Department for Infection
447 Genetics, Helmholtz Centre for Infection Research, Braunschweig) for providing the
448 Vetscan HM5 Hematology System. We thank Lisa Borkner, Franziska Dag, Rosaely
449 Casalegno-Garduno, Bahram Kasmampour Seighalani, Zeeshan Chaudhry, Ilona Bretrag,
450 Jennifer Wolf, Ayse Barut, Inge Hollatz-Rangosch, Franziska Klann, Janine Schreiber,
451 Maximilian Seidel and Jan Manicke for technical assistance.

452

453 **References**

454

455 **Adler, S. P. (1989).** Cytomegalovirus and child day care. Evidence for an increased infection rate among
456 day-care workers. *N Engl J Med* **321**, 1290-6.

457 **Altman, J. D., Moss, P. A., Goulder, P. J., Barouch, D. H., Mcheyzer-Williams, M. G., Bell, J. I.,**
458 **McMichael, A. J. & Davis, M. M. (1996).** Phenotypic analysis of antigen-specific T lymphocytes.
459 *Science* **274**, 94-6.

460 Appay, V., Dunbar, P. R., Callan, M., Klenerman, P., Gillespie, G. M., Papagno, L., Ogg, G. S., King, A.,
461 Lechner, F. & 6 other authors (2002). Memory CD8+ T cells vary in differentiation phenotype in
462 different persistent virus infections. *Nat Med* **8**, 379-85.

463 Boppana, S. B., Rivera, L. B., Fowler, K. B., Mach, M. & Britt, W. J. (2001). Intrauterine transmission of
464 cytomegalovirus to infants of women with preconceptional immunity. *N Engl J Med* **344**, 1366-
465 71.

466 Campbell, A. E., Cavanaugh, V. J. & Slater, J. S. (2008). The salivary glands as a privileged site of
467 cytomegalovirus immune evasion and persistence. *Med Microbiol Immunol* **197**, 205-13.

468 Cannon, M. J., Hyde, T. B. & Schmid, D. S. (2011). Review of cytomegalovirus shedding in bodily fluids
469 and relevance to congenital cytomegalovirus infection. *Rev Med Virol* **21**, 240-55.

470 Cicin-Sain, L., Brien, J. D., Uhrlaub, J. L., Drabig, A., Marandu, T. F. & Nikolich-Zugich, J. (2012).
471 Cytomegalovirus infection impairs immune responses and accentuates T-cell pool changes
472 observed in mice with aging. *PLoS Pathog* **8**, e1002849.

473 Cicin-Sain, L., Podlech, J., Messerle, M., Reddehase, M. J. & Koszinowski, U. H. (2005). Frequent
474 coinfection of cells explains functional in vivo complementation between cytomegalovirus
475 variants in the multiply infected host. *J Virol* **79**, 9492-502.

476 Cicin-Sain, L., Sylwester, A. W., Hagen, S. I., Siess, D. C., Currier, N., Legasse, A. W., Fischer, M. B.,
477 Koudelka, C. W., Axthelm, M. K. & others authors (2011). Cytomegalovirus-specific T cell
478 immunity is maintained in immunosenescent rhesus macaques. *J Immunol* **187**, 1722-32.

479 Diosi, P., Babusceac, L., Nevinglovschi, O. & Kun-Stoicu, G. (1967). Cytomegalovirus infection associated
480 with pregnancy. *Lancet* **2**, 1063-6.

481 Doom, C. M. & Hill, A. B. (2008). MHC class I immune evasion in MCMV infection. *Med Microbiol*
482 *Immunol* **197**, 191-204.

483 Dworsky, M., Yow, M., Stagno, S., Pass, R. F. & Alford, C. (1983). Cytomegalovirus infection of breast
484 milk and transmission in infancy. *Pediatrics* **72**, 295-9.

485 Fowler, K. B. & Pass, R. F. (1991). Sexually transmitted diseases in mothers of neonates with congenital
486 cytomegalovirus infection. *J Infect Dis* **164**, 259-64.

487 Fowler, K. B., Stagno, S. & Pass, R. F. (2003). Maternal immunity and prevention of congenital
488 cytomegalovirus infection. *JAMA* **289**, 1008-11.

489 Griffiths, P., Baraniak, I. & Reeves, M. (2015). The pathogenesis of human cytomegalovirus. *J Pathol* **235**,
490 288-97.

491 Hansen, S. G., Vieville, C., Whizin, N., Coyne-Johnson, L., Siess, D. C., Drummond, D. D., Legasse, A. W.,
492 Axthelm, M. K., Oswald, K. & other authors (2009). Effector memory T cell responses are
493 associated with protection of rhesus monkeys from mucosal simian immunodeficiency virus
494 challenge. *Nat Med* **15**, 293-9.

495 Holtappels, R., Pahl-Seibert, M. F., Thomas, D. & Reddehase, M. J. (2000). Enrichment of immediate-
496 early 1 (m123/pp89) peptide-specific CD8 T cells in a pulmonary CD62L(lo) memory-effector cell
497 pool during latent murine cytomegalovirus infection of the lungs. *J Virol* **74**, 11495-503.

498 Holtappels, R., Thomas, D., Podlech, J. & Reddehase, M. J. (2002). Two antigenic peptides from genes
499 m123 and m164 of murine cytomegalovirus quantitatively dominate CD8 T-cell memory in the H-
500 2d haplotype. *J Virol* **76**, 151-64.

501 Hsu, K. M., Pratt, J. R., Akers, W. J., Achilefu, S. I. & Yokoyama, W. M. (2009). Murine cytomegalovirus
502 displays selective infection of cells within hours after systemic administration. *J Gen Virol* **90**, 33-
503 43.

504 Jordan, M. C. (1978). Interstitial pneumonia and subclinical infection after intranasal inoculation of
505 murine cytomegalovirus. *Infect Immun* **21**, 275-80.

506 Jordan, S., Krause, J., Prager, A., Mitrovic, M., Jonjic, S., Koszinowski, U. H. & Adler, B. (2011). Virus
507 progeny of murine cytomegalovirus bacterial artificial chromosome pSM3fr show reduced
508 growth in salivary Glands due to a fixed mutation of MCK-2. *J Virol* **85**, 10346-53.

509 **Karrer, U., Sierro, S., Wagner, M., Oxenius, A., Hengel, H., Koszinowski, U. H., Phillips, R. E. &**
510 **Klenerman, P. (2003).** Memory inflation: continuous accumulation of antiviral CD8+ T cells over
511 time. *J Immunol* **170**, 2022-9.

512 **Kenneson, A. & Cannon, M. J. (2007).** Review and meta-analysis of the epidemiology of congenital
513 cytomegalovirus (CMV) infection. *Rev Med Virol* **17**, 253-76.

514 **Kirby, A. C., Coles, M. C. & Kaye, P. M. (2009).** Alveolar macrophages transport pathogens to lung
515 draining lymph nodes. *J Immunol* **183**, 1983-9.

516 **Knabel, M., Franz, T. J., Schiemann, M., Wulf, A., Villmow, B., Schmidt, B., Bernhard, H., Wagner, H. &**
517 **Busch, D. H. (2002).** Reversible MHC multimer staining for functional isolation of T-cell
518 populations and effective adoptive transfer. *Nat Med* **8**, 631-7.

519 **Lemmermann, N. A. W., Podlech, J., Seckert, C. K., Kropp, K. A., Grzimek, N. K. A., Reddehase, M. J. &**
520 **Holtappels, R. (2010).** CD8 T-cell immunotherapy of cytomegalovirus disease in the murine
521 model. In *Methods in Microbiology*, 37, pp. 369-420. Edited by D. Kabelitz & S. H. E. Kaufmann.
522 Academic Press.

523 **Lemoine, F. M., Humphries, R. K., Abraham, S. D., Krystal, G. & Eaves, C. J. (1988a).** Partial
524 characterization of a novel stromal cell-derived pre-B-cell growth factor active on normal and
525 immortalized pre-B cells. *Exp Hematol* **16**, 718-26.

526 **Lemoine, F. M., Krystal, G., Humphries, R. K. & Eaves, C. J. (1988b).** Autocrine production of pre-B-cell
527 stimulating activity by a variety of transformed murine pre-B-cell lines. *Cancer Res* **48**, 6438-43.

528 **Marco, A. J., Domingo, M., Ruberte, J., Carretero, A., Briones, V. & Dominguez, L. (1992).** Lymphatic
529 drainage of *Listeria innocytogenes* and Indian ink inoculated in the peritoneal cavity of the
530 mouse. *Lab Anim* **26**, 200-5.

531 **Morello, C. S., Ye, M., Hung, S., Kelley, L. A. & Spector, D. H. (2005).** Systemic priming-boosting
532 immunization with a trivalent plasmid DNA and inactivated murine cytomegalovirus (MCMV)
533 vaccine provides long-term protection against viral replication following systemic or mucosal
534 MCMV challenge. *J Virol* **79**, 159-75.

535 **Munks, M. W., Cho, K. S., Pinto, A. K., Sierro, S., Klenerman, P. & Hill, A. B. (2006).** Four distinct
536 patterns of memory CD8 T cell responses to chronic murine cytomegalovirus infection. *J*
537 *Immunol* **177**, 450-8.

538 **O'Hara, G. A., Welten, S. P. M., Klenerman, P. & Arens, R. (2012).** Memory T cell inflation:
539 understanding cause and effect. *Trends Immunol* **33**, 84-90.

540 **Pitcher, C. J., Hagen, S. I., Walker, J. M., Lum, R., Mitchell, B. L., Maino, V. C., Axthelm, M. K. & Picker,**
541 **L. J. (2002).** Development and homeostasis of T cell memory in rhesus macaque. *J Immunol* **168**,
542 29-43.

543 **Ple, C., Barrier, M., Amniai, L., Marquillies, P., Bertout, J., Tscopoulos, A., Walzer, T., Lassalle, P. &**
544 **Duez, C. (2010).** Natural killer cells accumulate in lung-draining lymph nodes and regulate airway
545 eosinophilia in a murine model of asthma. *Scand J Immunol* **72**, 118-27.

546 **Podlech, J., Holtappels, R., Grzimek, N. K. & Reddehase, M. J. (2002).** Animal models: murine
547 cytomegalovirus. *Method Microbiol* **32**, 493-525.

548 **Redeker, A., Welten, S. P. & Arens, R. (2014).** Viral inoculum dose impacts memory T-cell inflation. *Eur J*
549 *Immunol* **44**, 1046-57.

550 **Redwood, A. J., Shellam, G. R. & Smith, L. M. (2013).** Molecular Evolution of Murine Cytomegalovirus
551 Genomes. In *Cytomegaloviruses: From Molecular Pathogenesis to Intervention*. Edited by M.J.
552 Reddehase. Caister Academic Press.

553 **Saederup, N., Aguirre, S. A., Sparer, T. E., Bouley, D. M. & Mocarski, E. S. (2001).** Murine
554 cytomegalovirus CC chemokine homolog MCK-2 (m131-129) is a determinant of dissemination
555 that increases inflammation at initial sites of infection. *J Virol* **75**, 9966-76.

556 **Scalzo, A. A., Lyons, P. A., Fitzgerald, N. A., Forbes, C. A., Yokoyama, W. M. & Shellam, G. R. (1995).**
557 Genetic mapping of Cmv1 in the region of mouse chromosome 6 encoding the NK gene complex-
558 associated loci Ly49 and musNKR-P1. *Genomics* **27**, 435-41.
559 **Seckert, C. K., Griessl, M., Buttner, J. K., Scheller, S., Simon, C. O., Kropp, K. A., Renzaho, A., Kuhnappel,**
560 **B., Grzimek, N. K. & Reddehase, M. J. (2012).** Viral latency drives 'memory inflation': a unifying
561 hypothesis linking two hallmarks of cytomegalovirus infection. *Med Microbiol Immunol* **201**, 551-
562 66.
563 **Seckert, C. K., Renzaho, A., Tervo, H. M., Krause, C., Deegen, P., Kuhnappel, B., Reddehase, M. J. &**
564 **Grzimek, N. K. (2009).** Liver sinusoidal endothelial cells are a site of murine cytomegalovirus
565 latency and reactivation. *J Virol* **83**, 8869-84.
566 **Shanley, J. D., Thrall, R. S. & Forman, S. J. (1997).** Murine cytomegalovirus replication in the lungs of
567 athymic BALB/c nude mice. *J Infect Dis* **175**, 309-15.
568 **Sierro, S., Rothkopf, R. & Klenerman, P. (2005).** Evolution of diverse antiviral CD8+ T cell populations
569 after murine cytomegalovirus infection. *Europ J Immunol* **35**, 1113-23.
570 **Smith, C. J., Turula, H. & Snyder, C. M. (2014).** Systemic hematogenous maintenance of memory
571 inflation by MCMV infection. *PLoS Pathog* **10**, e1004233.
572 **Snyder, C. M., Cho, K. S., Morrison, E. L., Dommelen, S. V., Shellam, G. R. & Hill, A. B. (2008).** Memory
573 inflation during chronic viral infection is maintained by continuous production of short-lived
574 functional T cells. *Immunity* **29**, 650-59.
575 **Stagno, S., Reynolds, D. W., Pass, R. F. & Alford, C. A. (1980).** Breast milk and the risk of cytomegalovirus
576 infection. *N Engl J Med* **302**, 1073-6.
577 **Stahl, F. R., Heller, K., Halle, S., Keyser, K. A., Busche, A., Marquardt, A., Wagner, K., Boelter, J.,**
578 **Bischoff, Y. & other authors (2013).** Nodular inflammatory foci are sites of T cell priming and
579 control of murine cytomegalovirus infection in the neonatal lung. *PLoS Pathog* **9**, e1003828.
580 **Stahl, F. R., Keyser, K. A., Heller, K., Bischoff, Y., Halle, S., Wagner, K., Messerle, M. & Forster, R. (2015).**
581 Mck2-dependent infection of alveolar macrophages promotes replication of MCMV in nodular
582 inflammatory foci of the neonatal lung. *Mucosal Immunol* **8**, 57-67.
583 **Vochem, M., Hamprecht, K., Jahn, G. & Speer, C. P. (1998).** Transmission of cytomegalovirus to preterm
584 infants through breast milk. *Pediatr Infect Dis J* **17**, 53-8.
585 **Wu, C. A., Paveglio, S. A., Lingenheld, E. G., Zhu, L., Lefrancois, L. & Puddington, L. (2011).** Transmission
586 of murine cytomegalovirus in breast milk: a model of natural infection in neonates. *J Virol* **85**,
587 5115-24.

588 **Figure legends**

589 **Fig. 1: Intranasal MCMV infection results in robust virus replication in the lungs**
590 **and efficient spread to the salivary glands.** BALB/c mice were infected with 2×10^5
591 PFU MCMV^{wt} using the indicated infection route. (a) Virus titers were determined in the
592 lungs and spleen 5 and 14 dpi and in the salivary glands 14 dpi by plaque assay. (b)
593 Lungs and spleens were harvested daily from 1 to 7 days p.i. and on day 10 p.i. Virus
594 titers were analysed in organ homogenates. The group means \pm SEM are displayed
595 (n=5), dashed lines show the detection limit. (c) Infectious virus was detected in lungs,

596 spleen and salivary glands on day 7, 14 and 21 p.i.. (a+c) Each symbol represents one
597 mouse, solid lines display the median and dashed lines show the detection limit. (a-c)
598 SG = salivary glands, DL = detection limit

599

600 **Fig. 2: IE1-specific CD8 T cells inflate in BALB/c mice upon intranasal MCMV**
601 **infection.** BALB/c mice were left untreated (MOCK) or were infected with 2×10^5 PFU
602 MCMV^{wt} using the indicated infection route. Each group consisted of at least 12 mice. At
603 least 5 mice per group were sacrificed and used for detailed analysis at 2 weeks p.i. The
604 rest (at least 7 mice per group) were analysed at indicated time points p.i. Blood from 5
605 mice was analysed prior to infection, to define the basis of the CD8 T cell fractions. For
606 the analysis of CD8 T cell subsets, blood leukocytes were stained with an IE1-tetramer
607 and with antibodies against CD3, CD4, CD8a, CD11a and CD44, and analysed by flow
608 cytometry. (a) Percentage and absolute counts of antigen experienced CD11a⁺ CD44⁺
609 CD8 T cells. (b) Percentage and absolute counts of CD11a⁺ IE1-tetramer⁺ CD8 T cells.
610 (a+b) Displayed are the group means \pm SEM ($n \geq 5$) at indicated time points.

611 **Fig. 3: Memory inflation in the spleen and lymph nodes upon systemic or**
612 **intranasal MCMV infection.** Two cohorts of BALB/c mice were infected with 2×10^5 PFU
613 MCMV^{wt} by the intraperitoneal or the intranasal route. 20 μ l PBS were administered via
614 the intranasal route to MOCK controls. The first cohort consisted of 25 mice per group,
615 sacrificed at week 1, 2, 4, 13 and 27, and the second cohort of 10 mice per group
616 analysed at week 1 or 27 only. Samples from 5 mice were collected prior to infection to
617 set the baseline of CD8 T cell subsets (time point 0). At indicated time points p.i., the
618 frequency and the absolute counts of primed (CD11a⁺CD44⁺) or IE1-tetramer⁺ CD8 T

619 cells were determined in (a) the blood, (b) the spleen, (c) the inguinal and (d) the
620 mediastinal lymph nodes. Leukocytes were stained with an IE1-tetramer and antibodies
621 against CD3, CD4, CD8a, CD11a and CD44 and analysed by flow cytometry. Displayed
622 are the group means \pm SEM ($n \geq 3$) at indicated time points; combined data from both
623 cohorts are shown at 1 and 27 weeks p.i. Absolute counts are shown per 100 μ l blood,
624 whole spleen or one lymph node. ILN = inguinal lymph nodes; MLN = mediastinal lymph
625 nodes

626

627 **Fig. 4: Intranasal MCMV infection of 129/Sv mice results in inflation of M38-**
628 **specific CD8 T cells with an effector memory phenotype.** Two cohorts of 129/Sv
629 mice were left untreated (MOCK) or were infected with 2×10^5 PFU MCMV^{wt} using the
630 indicated infection route. Each group in each cohort contained at least 5 mice. Blood
631 from 5 mice was analysed prior to infection to define the basis of the CD8 T cell
632 fractions. Infected mice were bled at indicated time points p.i., isolated leukocytes were
633 stained with an M38-multimer and with antibodies against CD3, CD4, CD8a, CD11a,
634 CD44, CD62L and KLRG1 and subsequently analysed by flow cytometry. (a)
635 Percentage and absolute counts of primed (CD11a⁺CD44⁺) cells in the CD8 T cell pool.
636 (b) Percentage and absolute counts of M38-specific (CD11a⁺M38-multimer⁺) in the CD8
637 T cell pool. (c) Percentage of EM (KLRG1⁺CD62L⁻) cells in the primed CD8 T cell pool.
638 (d) Percentage of CM (KLRG1⁻CD62L⁺) in the primed CD8 T cell pool. (a-d) Displayed
639 are the group means \pm SEM ($n \geq 3$) at indicated time points. (e) Frequencies of M38-
640 multimer⁺ CD8 T cells with EM (KLRG1⁺CD62L⁻) and CM (KLRG1⁻CD62L⁺) phenotypes
641 in indicated compartments at 2 weeks post systemic or mucosal infection. (f)

642 Phenotypes as in panel E at 26 weeks p.i. (e+f) Displayed are the group means + SEM
643 ($n \geq 5$) in indicated compartments.

644

645 **Fig. 5: Latent virus is elevated in the lungs of intranasally infected mice.** 129/Sv
646 mice were infected with 2×10^5 PFU MCMV^{wt} using the indicated infection route. Lungs
647 and spleens were harvested 26 weeks p.i. and latent viral genomes were determined in
648 these organs by real-time PCR. Latent viral genomes per million host cells in (a) spleen
649 and (b) lungs are shown. Each symbol represents one mouse, horizontal lines show the
650 medians.

651

652 **Fig. S1: Representative gating strategy.**

653

654 **Fig. S2: Latent virus in spleen and lungs is lowest after intragastric infection.**
655 129/Sv mice were infected with 2×10^5 PFU MCMV^{wt} using the indicated infection route.
656 Lungs and spleens were harvested 26 weeks p.i. and latent viral genomes were
657 determined in these organs by real-time PCR. Latent viral genomes per million host cells
658 in (a) spleen and (b) lungs are shown. To optimize presentation, the infection routes with
659 the highest latent viral genome loads are omitted. Each symbol represents one mouse,
660 horizontal lines show the medians.

661

662 **Fig. S3: Isoflurane anaesthesia results in inefficient intranasal MCMV infection.**
663 BALB/c mice were anaesthetized with either isoflurane or ketamine/xylazine (10mg/ml /
664 1mg/ml in 0.9% NaCl) and subsequently infected with 2×10^5 PFU MCMV^{wt} intranasally.
665 Infectious virus was determined in lungs 4 days p.i.. Each symbol represents one
666 mouse, solid lines display the median and the dashed line shows the detection limit
667 (DL). This analysis was performed once.

668

- 669 **ADLER, S. P. 1989. Cytomegalovirus and child day care. Evidence for an increased infection rate among**
670 **day-care workers. *N Engl J Med*, 321, 1290-6.**
- 671 **ALTMAN, J. D., MOSS, P. A., GOULDER, P. J., BAROUCH, D. H., MCHEYZER-WILLIAMS, M. G., BELL, J. I.,**
672 **MCMICHAEL, A. J. & DAVIS, M. M. 1996. Phenotypic analysis of antigen-specific T lymphocytes.**
673 ***Science*, 274, 94-6.**
- 674 **APPAY, V., DUNBAR, P. R., CALLAN, M., KLENERMAN, P., GILLESPIE, G. M., PAPAGNO, L., OGG, G. S.,**
675 **KING, A., LECHNER, F., SPINA, C. A., LITTLE, S., HAVLIR, D. V., RICHMAN, D. D., GRUENER, N.,**
676 **PAPE, G., WATERS, A., EASTERBROOK, P., SALIO, M., CERUNDOLO, V., MCMICHAEL, A. J. &**
677 **ROWLAND-JONES, S. L. 2002. Memory CD8+ T cells vary in differentiation phenotype in**
678 **different persistent virus infections. *Nat Med*, 8, 379-85.**
- 679 **BOPPANA, S. B., RIVERA, L. B., FOWLER, K. B., MACH, M. & BRITT, W. J. 2001. Intrauterine transmission**
680 **of cytomegalovirus to infants of women with preconceptional immunity. *N Engl J Med*, 344,**
681 **1366-71.**
- 682 **CAMPBELL, A. E., CAVANAUGH, V. J. & SLATER, J. S. 2008. The salivary glands as a privileged site of**
683 **cytomegalovirus immune evasion and persistence. *Med Microbiol Immunol*, 197, 205-13.**
- 684 **CANNON, M. J., HYDE, T. B. & SCHMID, D. S. 2011. Review of cytomegalovirus shedding in bodily fluids**
685 **and relevance to congenital cytomegalovirus infection. *Rev Med Virol*, 21, 240-55.**
- 686 **CICIN-SAIN, L., BRIEN, J. D., UHRLAUB, J. L., DRABIG, A., MARANDU, T. F. & NIKOLICH-ZUGICH, J. 2012.**
687 **Cytomegalovirus Infection Impairs Immune Responses and Accentuates T-cell Pool Changes**
688 **Observed in Mice with Aging. *PLoS Pathogens*, 8, e1002849.**
- 689 **CICIN-SAIN, L., PODLECH, J., MESSERLE, M., REDDEHASE, M. J. & KOSZINOWSKI, U. H. 2005. Frequent**
690 **coinfection of cells explains functional in vivo complementation between cytomegalovirus**
691 **variants in the multiply infected host. *J Virol*, 79, 9492-502.**
- 692 **CICIN-SAIN, L., SYLWESTER, A. W., HAGEN, S. I., SIESS, D. C., CURRIER, N., LEGASSE, A. W., FISCHER, M.**
693 **B., KOUDELKA, C. W., AXTHELM, M. K., NIKOLICH-ZUGICH, J. & PICKER, L. J. 2011.**
694 **Cytomegalovirus-specific T cell immunity is maintained in immunosenescent rhesus macaques.**
695 ***J Immunol*, 187, 1722-32.**
- 696 **DIOSI, P., BABUSCEAC, L., NEVINGLOVSCHI, O. & KUN-STOICU, G. 1967. Cytomegalovirus infection**
697 **associated with pregnancy. *Lancet*, 2, 1063-6.**
- 698 **DOOM, C. M. & HILL, A. B. 2008. MHC class I immune evasion in MCMV infection. *Medical***
699 ***Microbiology and Immunology*, 197, 191-204.**
- 700 **DWORSKY, M., YOW, M., STAGNO, S., PASS, R. F. & ALFORD, C. 1983. Cytomegalovirus infection of**
701 **breast milk and transmission in infancy. *Pediatrics*, 72, 295-9.**

702 FOWLER, K. B. & PASS, R. F. 1991. Sexually transmitted diseases in mothers of neonates with
703 congenital cytomegalovirus infection. *J Infect Dis*, 164, 259-64.

704 FOWLER, K. B., STAGNO, S. & PASS, R. F. 2003. MATernal immunity and prevention of congenital
705 cytomegalovirus infection. *JAMA*, 289, 1008-1011.

706 GRIFFITHS, P., BARANIAK, I. & REEVES, M. 2015. The pathogenesis of human cytomegalovirus. *J Pathol*,
707 235, 288-97.

708 HANSEN, S. G., VIEVILLE, C., WHIZIN, N., COYNE-JOHNSON, L., SIESS, D. C., DRUMMOND, D. D.,
709 LEGASSE, A. W., AXTHELM, M. K., OSWALD, K., TRUBEY, C. M., PIATAK, M., JR., LIFSON, J. D.,
710 NELSON, J. A., JARVIS, M. A. & PICKER, L. J. 2009. Effector memory T cell responses are
711 associated with protection of rhesus monkeys from mucosal simian immunodeficiency virus
712 challenge. *Nat Med*, 15, 293-9.

713 HOLTAPPELS, R., PAHL-SEIBERT, M. F., THOMAS, D. & REDDEHASE, M. J. 2000. Enrichment of
714 immediate-early 1 (m123/pp89) peptide-specific CD8 T cells in a pulmonary CD62L(lo)
715 memory-effector cell pool during latent murine cytomegalovirus infection of the lungs. *J Virol*,
716 74, 11495-503.

717 HOLTAPPELS, R., THOMAS, D., PODLECH, J. & REDDEHASE, M. J. 2002. Two Antigenic Peptides from
718 Genes m123 and m164 of Murine Cytomegalovirus Quantitatively Dominate CD8 T-Cell
719 Memory in the H-2d Haplotype. *Journal of Virology*, 76, 151-164.

720 HSU, K. M., PRATT, J. R., AKERS, W. J., ACHILEFU, S. I. & YOKOYAMA, W. M. 2009. Murine
721 cytomegalovirus displays selective infection of cells within hours after systemic administration.
722 *Journal of General Virology*, 90, 33-43.

723 JORDAN, M. C. 1978. Interstitial pneumonia and subclinical infection after intranasal inoculation of
724 murine cytomegalovirus. *Infection and Immunity*, 21, 275-280.

725 JORDAN, S., KRAUSE, J., PRAGER, A., MITROVIC, M., JONJIC, S., KOSZINOWSKI, U. H. & ADLER, B. 2011.
726 Virus progeny of murine cytomegalovirus bacterial artificial chromosome pSM3fr show
727 reduced growth in salivary Glands due to a fixed mutation of MCK-2. *J Virol*, 85, 10346-53.

728 KARRER, U., SIERRO, S., WAGNER, M., OXENIUS, A., HENGEL, H., KOSZINOWSKI, U. H., PHILLIPS, R. E. &
729 KLENERMAN, P. 2003. Memory Inflation: Continuous Accumulation of Antiviral CD8+ T Cells
730 Over Time. *The Journal of Immunology*, 170, 2022-2029.

731 KENNESON, A. & CANNON, M. J. 2007. Review and meta-analysis of the epidemiology of congenital
732 cytomegalovirus (CMV) infection. *Rev Med Virol*, 17, 253-76.

733 KIRBY, A. C., COLES, M. C. & KAYE, P. M. 2009. Alveolar Macrophages Transport Pathogens to Lung
734 Draining Lymph Nodes. *The Journal of Immunology*, 183, 1983-1989.

735 KNABEL, M., FRANZ, T. J., SCHIEMANN, M., WULF, A., VILLMOW, B., SCHMIDT, B., BERNHARD, H.,
736 WAGNER, H. & BUSCH, D. H. 2002. Reversible MHC multimer staining for functional isolation of
737 T-cell populations and effective adoptive transfer. *Nat Med*, 8, 631-7.

738 LEMMERMANN, N. A. W., PODLECH, J., SECKERT, C. K., KROPP, K. A., GRZIMEK, N. K. A., REDDEHASE,
739 M. J. & HOLTAPPELS, R. 2010. 16 - CD8 T-Cell Immunotherapy of Cytomegalovirus Disease in
740 the Murine Model. In: DIETER, K. & STEFAN, H. E. K. (eds.) *Methods in Microbiology*. Academic
741 Press.

742 LEMOINE, F. M., HUMPHRIES, R. K., ABRAHAM, S. D., KRYSTAL, G. & EAVES, C. J. 1988a. Partial
743 characterization of a novel stromal cell-derived pre-B-cell growth factor active on normal and
744 immortalized pre-B cells. *Exp Hematol*, 16, 718-26.

745 LEMOINE, F. M., KRYSTAL, G., HUMPHRIES, R. K. & EAVES, C. J. 1988b. Autocrine production of pre-B-
746 cell stimulating activity by a variety of transformed murine pre-B-cell lines. *Cancer Res*, 48,
747 6438-43.

748 MARCO, A. J., DOMINGO, M., RUBERTE, J., CARRETERO, A., BRIONES, V. & DOMINGUEZ, L. 1992.
749 Lymphatic drainage of *Listeria innocytogenes* and Indian ink inoculated in the peritoneal
750 cavity of the mouse. *Laboratory Animals*, 26, 200-205.

751 MORELLO, C. S., YE, M., HUNG, S., KELLEY, L. A. & SPECTOR, D. H. 2005. Systemic priming-boosting
752 immunization with a trivalent plasmid DNA and inactivated murine cytomegalovirus (MCMV)
753 vaccine provides long-term protection against viral replication following systemic or mucosal
754 MCMV challenge. *J Virol*, 79, 159-75.

755 MUNKS, M. W., CHO, K. S., PINTO, A. K., SIERRA, S., KLENERMAN, P. & HILL, A. B. 2006. Four Distinct
756 Patterns of Memory CD8 T Cell Responses to Chronic Murine Cytomegalovirus Infection. *The*
757 *Journal of Immunology*, 177, 450-458.

758 O'HARA, G. A., WELTEN, S. P. M., KLENERMAN, P. & ARENS, R. 2012. Memory T cell inflation:
759 understanding cause and effect. *Trends Immunol*, 33, 84-90.

760 PITCHER, C. J., HAGEN, S. I., WALKER, J. M., LUM, R., MITCHELL, B. L., MAINO, V. C., AXTHELM, M. K. &
761 PICKER, L. J. 2002. Development and homeostasis of T cell memory in rhesus macaque. *J*
762 *Immunol*, 168, 29-43.

763 PLE, C., BARRIER, M., AMNIAI, L., MARQUILLIES, P., BERTOUT, J., TSICOPOULOS, A., WALZER, T.,
764 LASSALLE, P. & DUEZ, C. 2010. Natural Killer Cells Accumulate in Lung-Draining Lymph Nodes
765 and Regulate Airway Eosinophilia in a Murine Model of Asthma. *Scandinavian Journal of*
766 *Immunology*, 72, 118-127.

767 PODLECH, J., HOLTAPPELS, R., GRZIMEK, N. K. & REDDEHASE, M. J. 2002. Animal models: murine
768 cytomegalovirus. *Methods in microbiology*, 32, 493-IN11.

769 REDEKER, A., WELTEN, S. P. & ARENS, R. 2014. Viral inoculum dose impacts memory T-cell inflation.
770 *Eur J Immunol*, 44, 1046-57.

771 REDWOOD, A. J., SHELLAM, G. R. & SMITH, L. M. 2013. Molecular Evolution of Murine Cytomegalovirus
772 Genomes. In: REDDEHASE, M. J. (ed.) *Cytomegaloviruses: From Molecular Pathogenesis to*
773 *Intervention*

774 Caister Academic Press.

775 SAEDERUP, N., AGUIRRE, S. A., SPARER, T. E., BOULEY, D. M. & MOCARSKI, E. S. 2001. Murine
776 cytomegalovirus CC chemokine homolog MCK-2 (m131-129) is a determinant of dissemination
777 that increases inflammation at initial sites of infection. *J Virol*, 75, 9966-76.

778 SCALZO, A. A., LYONS, P. A., FITZGERALD, N. A., FORBES, C. A., YOKOYAMA, W. M. & SHELLAM, G. R.
779 1995. Genetic mapping of Cmv1 in the region of mouse chromosome 6 encoding the NK gene
780 complex-associated loci Ly49 and musNKR-P1. *Genomics*, 27, 435-41.

781 SECKERT, C. K., GRIESSL, M., BUTTNER, J. K., SCHELLER, S., SIMON, C. O., KROPP, K. A., RENZAHO, A.,
782 KUHNAPFEL, B., GRZIMEK, N. K. & REDDEHASE, M. J. 2012. Viral latency drives 'memory
783 inflation': a unifying hypothesis linking two hallmarks of cytomegalovirus infection. *Med*
784 *Microbiol Immunol*, 201, 551-66.

785 SECKERT, C. K., RENZAHO, A., TERVO, H. M., KRAUSE, C., DEEGEN, P., KUHNAPFEL, B., REDDEHASE, M.
786 J. & GRZIMEK, N. K. 2009. Liver sinusoidal endothelial cells are a site of murine
787 cytomegalovirus latency and reactivation. *J Virol*, 83, 8869-84.

788 SHANLEY, J. D., THRALL, R. S. & FORMAN, S. J. 1997. Murine cytomegalovirus replication in the lungs of
789 athymic BALB/c nude mice. *J Infect Dis*, 175, 309-15.

790 SIERRA, S., ROTHKOPF, R. & KLENERMAN, P. 2005. Evolution of diverse antiviral CD8+ T cell
791 populations after murine cytomegalovirus infection. *European Journal of Immunology*, 35,
792 1113-1123.

793 SMITH, C. J., TURULA, H. & SNYDER, C. M. 2014. Systemic Hematogenous Maintenance of Memory
794 Inflation by MCMV Infection. *PLoS Pathogens*, 10, e1004233.

795 SNYDER, C. M., CHO, K. S., MORRISON, E. L., DOMMELEN, S. V., SHELLAM, G. R. & HILL, A. B. 2008.
796 Memory Inflation During Chronic Viral Infection is Maintained by Continuous Production of
797 Short-Lived Functional T Cells. *Immunity*, 29, 650-659.

798 STAGNO, S., REYNOLDS, D. W., PASS, R. F. & ALFORD, C. A. 1980. Breast milk and the risk of
799 cytomegalovirus infection. *N Engl J Med*, 302, 1073-6.

800 STAHL, F. R., HELLER, K., HALLE, S., KEYSER, K. A., BUSCHE, A., MARQUARDT, A., WAGNER, K., BOELTER,
801 J., BISCHOFF, Y., KREMMER, E., ARENS, R., MESSERLE, M. & FÖRSTER, R. 2013. Nodular
802 Inflammatory Foci Are Sites of T Cell Priming and Control of Murine Cytomegalovirus Infection
803 in the Neonatal Lung. *PLoS Pathog*, 9, e1003828.

804 STAHL, F. R., KEYSER, K. A., HELLER, K., BISCHOFF, Y., HALLE, S., WAGNER, K., MESSERLE, M. &
805 FORSTER, R. 2015. Mck2-dependent infection of alveolar macrophages promotes replication of
806 MCMV in nodular inflammatory foci of the neonatal lung. *Mucosal Immunol*, 8, 57-67.

807 VOCHER, M., HAMPRECHT, K., JAHN, G. & SPEER, C. P. 1998. Transmission of cytomegalovirus to
808 preterm infants through breast milk. *Pediatr Infect Dis J*, 17, 53-8.

809 WU, C. A., PAVEGLIO, S. A., LINGENHELD, E. G., ZHU, L., LEFRANÇOIS, L. & PUDDINGTON, L. 2011.
810 Transmission of Murine Cytomegalovirus in Breast Milk: a Model of Natural Infection in
811 Neonates. *Journal of Virology*, 85, 5115-5124.

812

813

Fig. 1

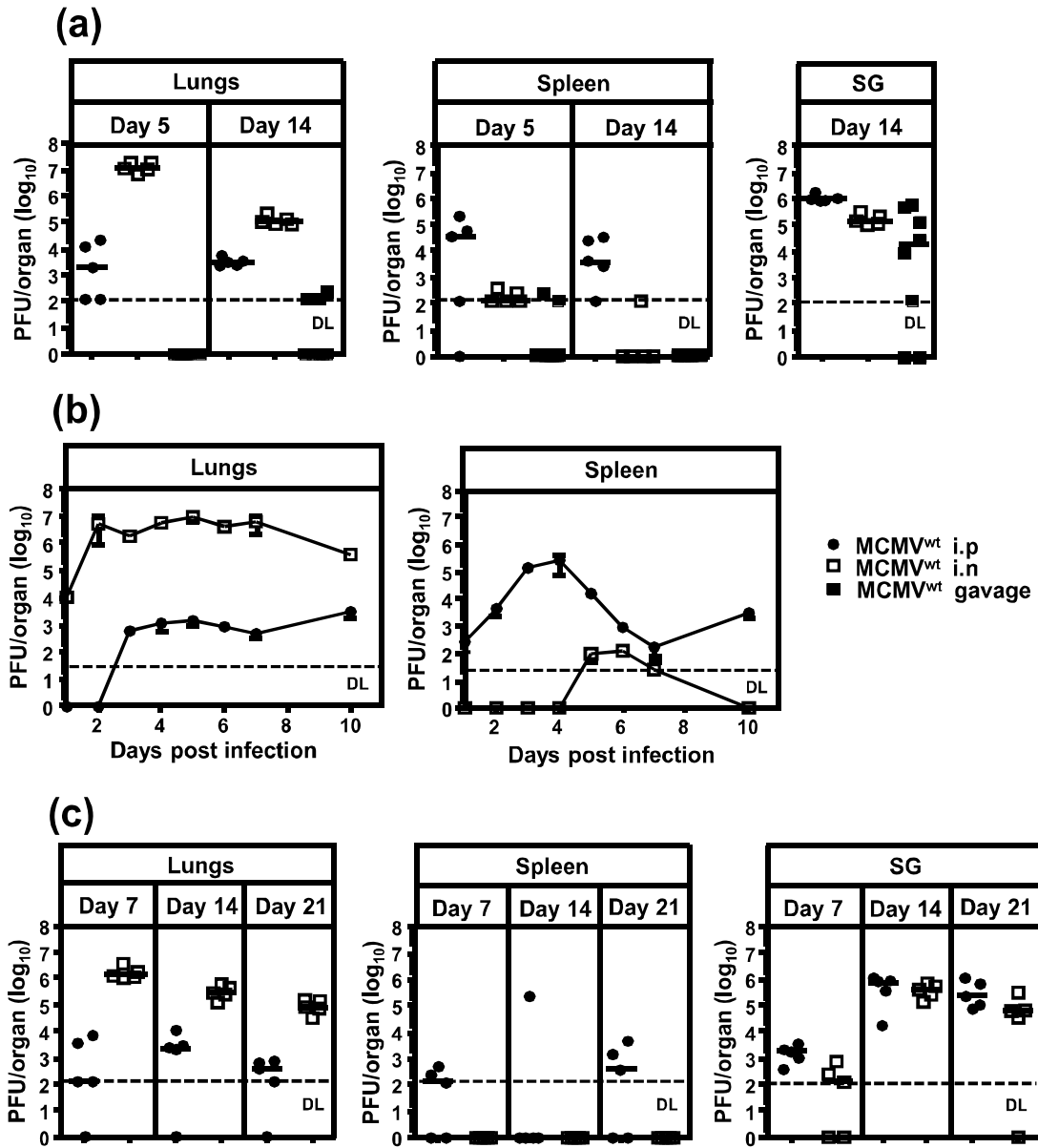


Fig. 2

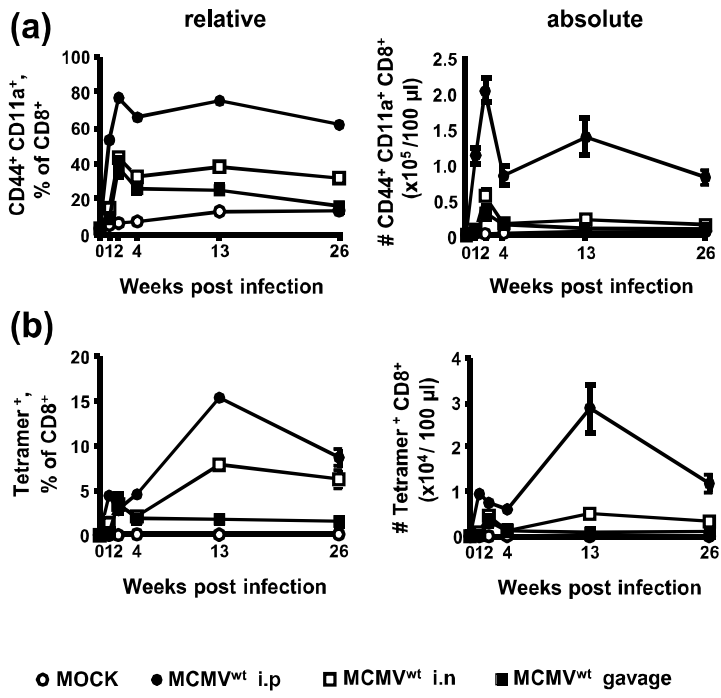


Fig. 4

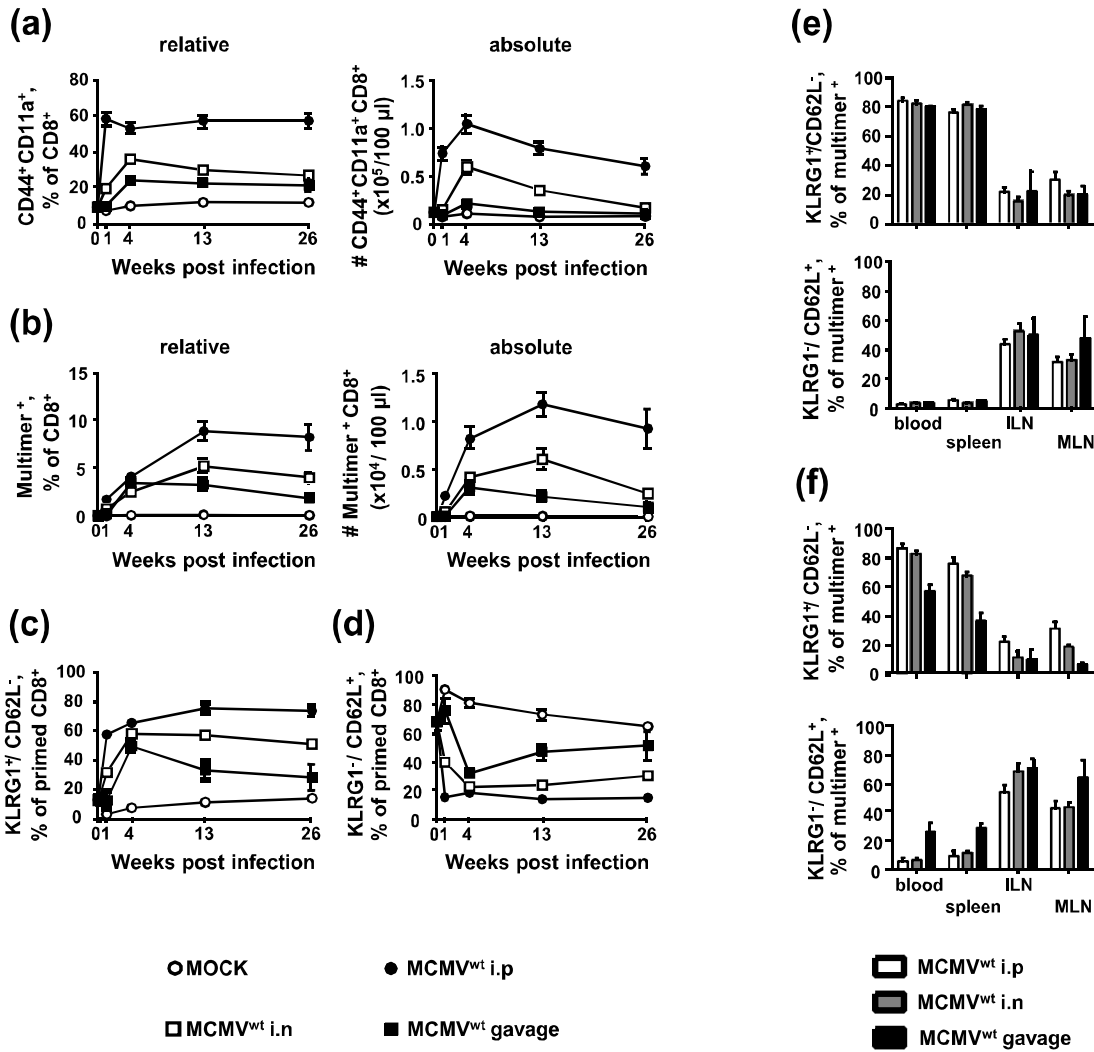


Fig. S1

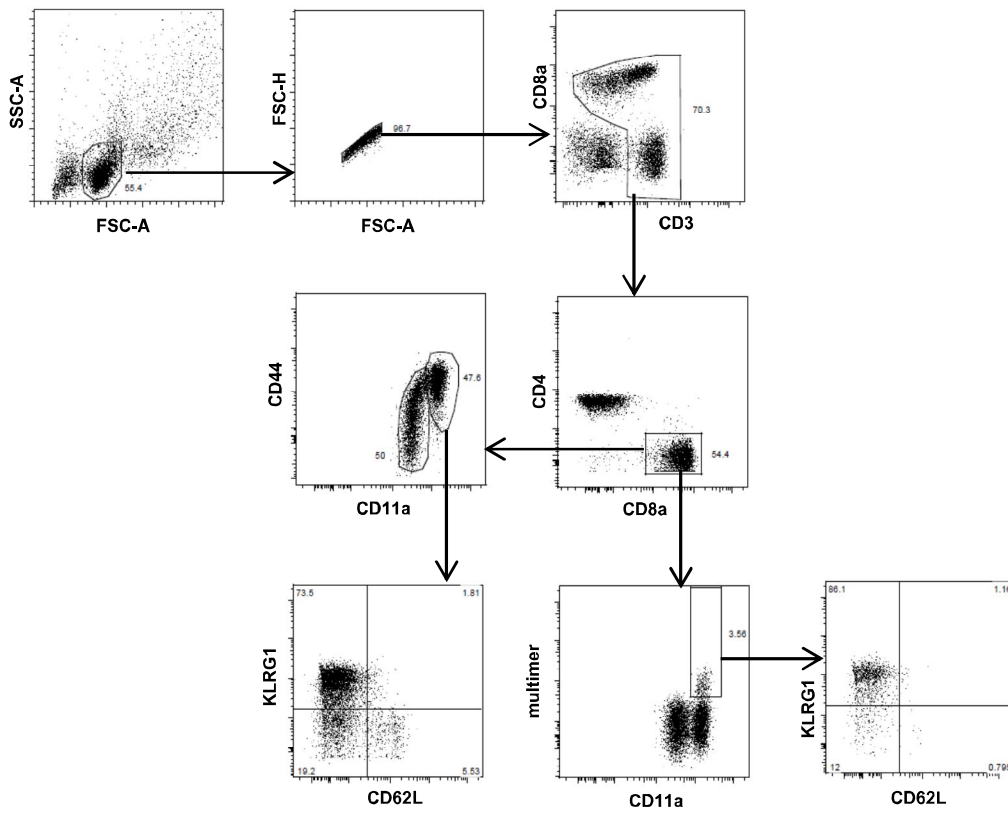


Fig. S1: Representative gating strategy.

Fig. S2

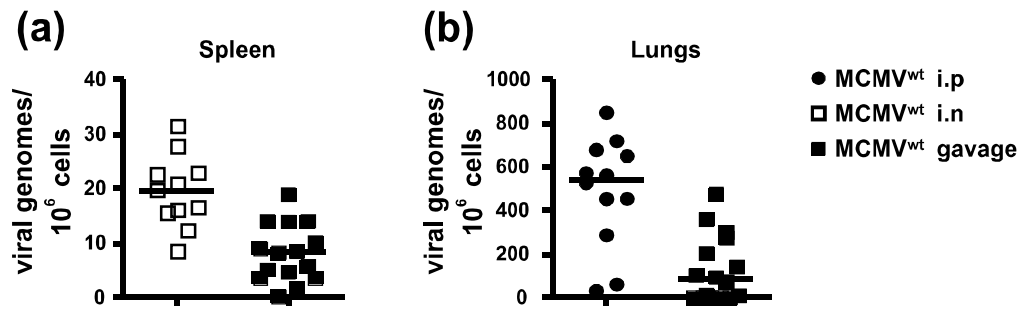


Fig. S2: Latent virus in spleen and lungs is lowest after intragastric infection.

129/Sv mice were infected with 2×10^5 PFU MCMV^{wt} using the indicated infection route. Lungs and spleens were harvested 26 weeks p.i. and latent viral genomes were determined in these organs by real-time PCR. Latent viral genomes per million host cells in (a) spleen and (b) lungs are shown. To optimize presentation, the infection routes with the highest latent viral genome loads are omitted. Each symbol represents one mouse, horizontal lines show the medians.

Fig. S3

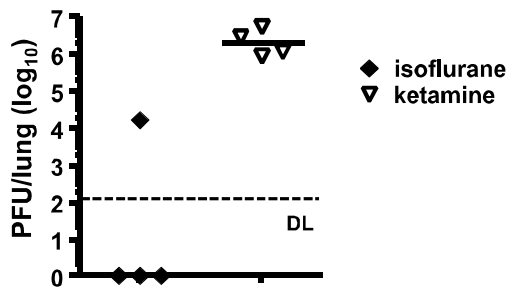


Fig. S3: Isoflurane anaesthesia results in inefficient intranasal MCMV infection.

BALB/c mice were anaesthetized with either isoflurane or ketamine/xylazine (10mg/ml / 1mg/ml in 0.9% NaCl) and subsequently infected with 2×10^5 PFU MCMV^{wt} intranasally. Infectious virus was determined in lungs 4 days p.i.. Each symbol represents one mouse, solid lines display the median and the dashed line shows the detection limit (DL).