Single cell detection of latent cytomegalovirus reactivation in host tissue

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The molecular mechanisms leading to reactivation of latent cytomegalovirus are not well understood. To study reactivation, the few cells in an organ tissue that give rise to reactivated virus need to be identified, ideally at the earliest possible time point in the process. To this end, mouse cytomegalovirus (MCMV) reporter mutants were designed to simultaneously express the red fluorescent protein mCherry and the secreted Gaussia luciferase (Gluc). Whereas Gluc can serve to assess infection at the level of individual mice by measuring luminescence in blood samples or by in vivo imaging, mCherry fluorescence offers the advantage of detection of infection at the single cell level. To visualize cells in which MCMV was being reactivated, precision-cut lung slices (PCLS) that preserve tissue microanatomy were prepared from the lungs of latently infected mice. By day 3 of cultivation of the PCLS, reactivation was revealed by Gluc expression, preceding the detection of infectious virus by approximately 4 days. Reactivation events in PCLS could be identified when they were still confined to single cells. Notably, using fractalkine receptor–GFP reporter mice, we never observed reactivation originating from CX3CR1+ monocytes or pulmonary dendritic cells derived therefrom. Furthermore, latent viral genome in the lungs was not enriched in sorted bone-marrow-derived cells expressing CD11b. Taken together, these complementary approaches suggest that CD11b+ and CX3CR1+ subsets of the myeloid differentiation lineage are not the main reservoirs and cellular sites of MCMV latency and reactivation in the lungs.

INTRODUCTION

Human cytomegalovirus (HCMV) infection is highly prevalent in the human population (Mocarski et al., 2007). Primary infection often occurs in early childhood, usually without symptoms or with mild symptoms only. The acute infection is cleared by the immune system, but viral genomes remain in specific cells of the organism in a latent state. Cells of the myeloid lineage such as monocytes, which are the progenitors of dendritic cells and macrophages, but also other cell types such as endothelial cells, are considered to form the reservoir for latent HCMV genomes (reviewed by Jarvis & Nelson, 2002; Sinclair, 2008). Latency is operationally defined by the absence of detectable infectious virus and the capacity of the latent viral genomes to give rise to recurrent infection (Roizman & Sears, 1987). Reactivation of viral gene expression may be a relatively frequent event if viewed over long periods of time, however, the immune system in healthy individuals can recognize and terminate reactivation events even before virion assembly (Simon et al., 2006; reviewed by Reddehase et al., 2008). In addition, the immune system limits the spread of already reactivated virus, thus preventing recurrent viraemia and cytomegalovirus (CMV) disease (Polić et al., 1998). Consequently, HCMV reactivation is primarily a health risk for immunocompromised individuals such as transplant patients.

The molecular events that lead to activation of the viral gene expression programme, resulting in lytic virus production...
in the immunocompromised host, are not completely understood. It is assumed that in latently infected cells CMV genomes reside in a kind of dormancy with very limited and restricted viral gene expression (Kurz et al., 1999; Grzimek et al., 2001). Signalling events, for instance induced by pro-inflammatory cytokines, as well as differentiation processes in the latently infected cells have been associated with reactivation (Koffron et al., 1999; Simon et al., 2005; Cook et al., 2006; reviewed by Stinski & Isomura, 2008). In the transplant setting, CMV reactivation was indeed observed in the context of immune reactions such as alloreactive tissue rejection or graft-versus-host disease (Mocarski et al., 2007). Owing to the host-species specificity of CMVs, HCMV infection cannot be studied in an animal model. Since mouse CMV (MCMV) and HCMV share many biological properties and display similarity in pathogenesis, MCMV has become a widely accepted model for the study of CMV latency and reactivation (Hummel & Abecassis, 2002; Reddehase et al., 2008). Experimentally, the molecular analysis of CMV reactivation is difficult to address even in the mouse model, owing to the low number of latently infected cells and the low frequency of reactivation events. To give an idea of the orders of magnitude involved, a recent report on MCMV latency in the liver has identified liver sinusoidal endothelial cells (LSECs) as the cellular site of latency in that specific organ, with only 1–2 of $10^4$ LSECs carrying $\sim$9 (range 4–17) copies of the latent viral genome, and the incidence of productive reactivation ex vivo was estimated to be 1 per 1000 latently infected LSECs (Seckert et al., 2009). For the lungs, a major organ site of MCMV latency (Balthesen et al., 1993), as few as 16 virus recurrences calculated for the whole lung were observed in explant cultures of solid tissue pieces on the basis of a latent virus load of $\sim$4000 copies per $10^6$ lung cells (Böhmann et al., 2009). Images obtained by in-situ PCR were indicative of MCMV genome colocalization with pulmonary macrophages during latency (Koffron et al., 1998). However, whether the amplified viral sequences represented reactivatable viral genomes or were engulfed remnants of productive infection could not be concluded. It thus remained open to question whether virus reactivation in the lungs originates from macrophages. Explant cultures of organ tissues have been widely used for studying MCMV reactivation (Cheung & Lang, 1977; Jordan & Mar, 1982; Presti et al., 1998), and more recently, results obtained with such cultures have suggested that reactivation involves opening of the latent viral chromatin-like structure (Hummel et al., 2007). However, we are far from a comprehensive understanding of the detailed mechanisms that lead to the switch from latent to lytic infection. The value of the reactivation system would be greatly increased by improved cultivation techniques and more sensitive and faster methods of detecting reactivation. In this study we report that, by the combined use of novel reporter viruses and precision-cut lung slices (PCLS) generated from the lungs of latently infected mice, reactivation events originating from single cells can reliably be detected as early as 3 days post-implantation. The results obtained with this advanced reactivation system did not support the assumption that virus is reactivated from CX3CR1$^+$ cells of the myeloid lineage.

### RESULTS

#### Construction and in vitro analysis of polyfunctional reporter mutants

As reactivation of CMV from latency in an organ is a rare event, potentially originating from one or a few cells only (Grzimek et al., 2001; Kurz et al., 1999; Kurz & Reddehase, 1999; Reddehase et al., 1994), the analysis of such reactivation events requires highly sensitive detection systems. Since even low activities of luciferase can be detected with high sensitivity (Tannous et al., 2005), we constructed MCMV reporter viruses that express the Gaussia luciferase (Gluc) under the control of the strong MCMV immediate–early promoter. Secretion of Gluc from cells offers the possibility of monitoring virus reactivation in explants by measuring luciferase activity in the culture supernatants over time, without destroying the tissue. In addition, we employed a previously established technique of preparing semi-thin slices of lung tissue (Held et al., 1999; Henjaković et al., 2008) for studying MCMV reactivation. Such PCLS maintain the microanatomy and important functional characteristics of lung tissue for several days and may thus allow detection of single MCMV-reactivating cells. To this end, we included the mCherry fluorescence marker in the expression cassette (Fig. 1a). To ensure simultaneous high-level expression, both markers were synthesized from a single ORF and the nascent proteins were separated by a picornaviral cotranslational cleavage site (Szymczak & Vignali, 2005). The expression cassette was inserted into the MCMV m157 ORF that encodes a ligand for the Ly49H receptor of NK cells (reviewed by Lenac et al., 2008). Accordingly, insertion of the expression cassette was not expected to result in virulence changes of the mutants in mice lacking Ly49H (e.g. BALB/c), whereas, in Ly49H-positive mouse strains (e.g. C57BL/6), m157 disruption causes enhanced viraemia during acute infection (Bubić et al., 2004). This is predicted to result in a higher load of latent MCMV genomes (Reddehase et al., 1994), a condition considered to be advantageous for studies on virus reactivation.

The reporter mutant MCMV-2D carries the mCherry–Gluc cassette in the genetic background of BAC-derived wild-type MCMV (MCMV-WT), whereas MCMV-3D and MCMV-3Δ are based on the viruses mCMV-SIINFEKL and mCMV-$\Delta m06m152$-SIINFEKL, respectively (Lemmermann et al., 2010). The latter two viruses encode the ovalbumin-derived T-cell epitope SIINFEKL, and MCMV-3Δ additionally lacks the ORFs for the immunoevasins m06/gp48 and m152/gp40. These features give the option of analysing the role of T cells and viral immunoevasins in the control of
reactivation by using SIINFEKL-specific T cells from OT-I mice (Hogquist et al., 1994).

Strong expression of mCherry was detected in cells infected with these reporter mutants by immunoblotting. The electrophoretic mobility of the mCherry protein was in accordance with its expected molecular mass of 34 kDa (Fig. 1b), indicating efficient separation from Gluc by the picornavirus P2A cleavage site. Luciferase activity could be measured in the supernatant of the infected cell cultures as early as 3 h post-infection (p.i.), and the signal increased steadily over the next 24 h, whereas the first mCherry expressing cells were detected at 4 h p.i. (Fig. 1c). Thus, both markers allow early detection of infection though serve different purposes: the luciferase allows for sensitive detection of infection in a cell population, while the mCherry marker is intended to detect single infected cells.

The growth kinetics of the reporter mutants in murine embryonic fibroblasts (MEF) was comparable to that of MCMV-WT (Fig. 2a). To determine how many infected cells are needed to detect a luciferase signal, reporter-virus-infected MEF were seeded into microtitre plates at a low number (one cell per well, statistically). From a total of 46 wells proven to contain just one cell, 43 wells (93 %) gave a signal in culture supernatants at least 1.5-fold higher than the baseline values, and thus could clearly be scored as being positive (Fig. 2b). Supernatants from cultures that contained two infected cells displayed a further twofold increase in signal. This result indicated that a luciferase signal originating from one cell can almost always be detected.

Next, we analysed the sensitivity of detection of the virally expressed Gluc over a time course by infecting fibroblasts with MCMV-3D at m.o.i. as low as 1 p.f.u. per 10, 100 and 1000 cells (Fig. 2c). At 24 h p.i., an m.o.i-dependent signal was detected, even in cultures infected with the lowest dose. The luciferase activity increased over time due to accumulation of secreted Gluc and viral spread to neighbouring cells. Comparison of Gluc activities and viral titres revealed a highly significant correlation between these parameters (Fig. 2d). We thus concluded that Gluc is a convenient surrogate marker for the infection of permissive cells, since it allows much earlier detection, namely at 3 h p.i. compared with 2 days p.i. for infectious virus (compare Figs 1c and 2a). Also, unlike the plaque assay, the Gluc assay is completed within minutes instead of 5 days.

**Fig. 1.** Construction and characterization of MCMV reporter mutants. (a) The expanded region below the HindIII map of the MCMV genome indicates the ORFs modified in the reporter viruses, m157 and m164. An expression cassette consisting of the MCMV major immediate–early promoter (mMIEP), and the mCherry and Gaussia luciferase (Gluc) ORFs separated by a picornavirus P2A cleavage site (P2A) was inserted into the m157 ORF. The sequence of m164 encoding an MCMV T-cell epitope was previously replaced with the sequence for the SIINFEKL peptide (Lemmermann et al., 2010). (b) Detection of mCherry expression. Lysates harvested at 48 h p.i. from MLE-12 lung epithelial cells (Wikenheiser et al., 1993), either mock infected or infected with the indicated reporter viruses at an m.o.i. of 1, were analysed by immunoblotting with an mCherry-specific antibody. Protein size markers are indicated to the left. (c) Time course of mCherry and Gluc expression. NIH3T3 cells were infected with MCMV-2D at an m.o.i. of 1 (with centrifugal enhancement), and at the indicated time points the cultures were analysed for mCherry and Gluc expression. Arrows mark the first detection of the respective reporter proteins.
Fig. 2. Growth kinetics of the reporter mutants in vitro and sensitivity of luciferase detection. (a) MEF were infected with the indicated viruses at an m.o.i. of 1; at the indicated time points culture supernatants were analysed for the presence of virus by plaque assay. Each data point represents the mean value of the titres from triplicate cultures (± SD). Dashed line marks the detection limit of the assay. (b) MCMV-2D-infected MEF were seeded into 96-well-plates at one cell per well. mCherry-positive cells per well were counted by three independent examiners, and Gluc activity in the supernatants was measured 4 days later. (c) NIH3T3 cells were infected with MCMV-3D at the indicated m.o.i. (with centrifugal enhancement), and Gluc activity (± SD) in the supernatants of triplicate cultures was determined at the time points shown. (d) Correlation of luciferase activity and viral titres. NIH3T3 cells were infected with centrifugal enhancement using graded dilutions (in log5 steps) of MCMV-3D, starting with an m.o.i. of 0.04. Viral titre and Gluc activity in the supernatants of the cultures were determined at 48 and again at 72 h p.i. For each time point the mean value of three cultures is shown.
Detection of the reporter mutants in acute *in vivo* infection

Efficient replication and dissemination of MCMV in the acute phase of host infection is a prerequisite for the establishment of latency with a high load of latent viral genomes in organs that is associated with a high probability of reactivation (Reddehase *et al.*, 1994). After infection of BALB/c mice, viral titres in spleen and liver, main target organs of acute infection, were within a comparable range for reporter mutants and MCMV-WT (Fig. 3a, b). Notably, distinct differences in viral titres were paralleled by differences in Gluc activity (Fig. 3c, d), thus demonstrating the adequacy of Gluc as a marker for *in vivo* studies of viral replication.

Next, we analysed whether mCherry can be used to detect infected cells in tissues. Following infection of mice by footpad inoculation, red fluorescent cells were observed in the subcapsular sinus area in the cortex of the draining popliteal lymph node (Fig. 4a), which is precisely the site of intranodal MCMV replication identified previously by immunohistology (Böhm *et al.*, 2008). Thus, mCherry is a strong marker for infected cells *in situ*. The Gluc marker offers the additional advantage of monitoring infection *in vivo* in a non-invasive manner. Specifically, infection of

![Graphs showing viral titres and luciferase activities](http://vir.sgmjournals.org/1283)

**Fig. 3.** *In vivo* growth of the MCMV mutants and luciferase activity in infected organs. BALB/c mice (4–6 weeks old) were infected intraperitoneally with 10^6 p.f.u. of the indicated viruses. At 3 days p.i., viral titres (a, b) and luciferase activities (c, d) were determined in spleen and liver. Horizontal bars mark median values and dashed lines indicate the detection limit of the centrifugal plaque assay.
mice with MCMV-2D resulted in a pronounced bioluminescence signal at the local site of infection (Fig. 4b), and Gluc activity could be detected in blood samples shortly after infection with the reporter mutants (Fig. 4c). Priming of MCMV-specific T cells is another sensitive indicator of infection, with memory cells testifying to past infection after the clearance of acute viral replication. In accordance with previous results (Lemmermann et al., 2010), CD8 T cells specific for the K\textsuperscript{b}-SIINFEKL complex were found in C57BL/6 mice infected with the SIINFEKL-encoding MCMV-3D (Fig. 4d). Taken together, the reporter mutants allow highly sensitive monitoring of infection in vivo with three different detection systems, all of which have specific applications.

Fig. 4. Reporter expression in vivo. (a) mCherry expression was assessed in a cryosection of the draining popliteal lymph node by epifluorescence microscopy 2 days after subcutaneous infection into the right hind footpad of C57BL/6 mice with $10^6$ p.f.u. of MCMV-2D. Nuclei were stained with DAPI. Bar, 100 μm. (b) Bioluminescence imaging of C3H/HeJ nude mice 5 days after infection into the right hind footpad with $10^6$ p.f.u. of MCMV-WT or MCMV-2D. Images were generated based on a 3 min data acquisition. The pseudocolour scale shows relative photon flux. (c) Luciferase detection in blood. Luciferase activity in whole blood was analysed 3 days after intraperitoneal infection of C57BL/6 mice with $10^6$ p.f.u. of the indicated viruses. Bars indicate median values. (d) Detection of specific CD8 T-cells in mice infected with the SIINFEKL-encoding MCMV-3D virus. Frequencies of peptide-specific (OVA, SIINFEKL; M38, STYTFVRT) CD8 T-cells in the blood were determined by IFN-γ-ELISpot assay (for three mice per group) 2 weeks after intraperitoneal infection of C57BL/6 mice with $10^6$ p.f.u. of MCMV-WT (●) and MCMV-3D (□). Horizontal bars mark median values.
Monitoring of reactivation events by use of the luciferase marker

Since the load of latent viral genomes in a tissue defines the probability of reactivation events occurring (Reddehase et al., 1994), we compared the capacity of the reporter mutants and MCMV-WT to establish latency. At 6 months after infection of C57BL/6 mice, a time point when latency was established, ~10,000 genome copies of each virus were found per 10^6 cells in the salivary glands (Fig. 5a), a major target organ of latent infection.

Reactivation of MCMV is often analysed in cultures of explanted organ tissues (see the Introduction) and the occurrence of reactivated virus is regularly monitored by plaque assay. We tested whether reactivation of the reporter mutants can alternatively be detected by luciferase activity in culture supernatants. At 3 weeks after explantation of small pieces of lung tissue, luciferase activity was found in a substantial fraction of the cultures (Fig. 5b). The observed variance in luminescence levels probably reflects time differences in the onset of reactivation. Reactivation of reporter mutants was also observed in other organs, including spleen, kidney and salivary glands (data not shown).

The following experiments to determine the onset of reactivation were performed with PCLS because tissue slices maintain cell viability, and thus tissue integrity, longer than solid lung pieces. First reactivation events were already detectable after 3 days of culture of PCLS (Fig. 6a). In other cultures, an increase in luciferase activity occurred later, which is in accordance with reactivation being a stochastic event (Böhm et al., 2009). Usually, luciferase activity in individual cultures increased over time, suggesting spreading of reactivated virus within the PCLS. In some cultures however, the luciferase activity vanished (labelled with arrows in Fig. 6a), possibly indicating unsuccessful reactivation events. Fig. 6(b) shows that first detection of Gluc activity preceded the detectability of virus by several days. In another set of experiments, Gluc activity in the culture supernatants was detected on average 4 days (median value; range, 0–12 days) earlier than infectious virus (Fig. 6c). These results document the advantage of the luciferase marker in early detection of reactivation events.

Monitoring of MCMV-reactivating cells in PCLS

To identify individual cells giving rise to reactivated virus, the integrity of explanted tissue as well as cell viability must be preserved and, moreover, the tissue must be accessible to microscopic examination. Since the lungs are a major site of latency and reactivation of MCMV (Balthesen et al., 1993), we reasoned that PCLS cultures (Fig. 7a) might be ideal for studying reactivation. Individual cultures were analysed by independent examiners for luciferase activity and for the appearance of red fluorescent cells. At 4 days post-explantation, those cultures with the highest luciferase activity scored positive for one or few mCherry-expressing cells (Fig. 7b). At 7 days of cultivation further elevated luciferase activities coincided with the formation of foci of mCherry expressing cells, indicating the spread of reactivated virus within the PCLS (Fig. 7c). Notably, reactivated viruses generally expressed both reporter proteins, indicating that there were no negative selection pressures on the reporter genes during latency or reactivation.
Search for the cell type in the lungs harbouring latent MCMV

Our data provide reasonable evidence to suggest that the dual reporter viruses, combined with the PCLS system, allow detection of reactivation events originating from single cells. We reasoned that this approach would enable us to characterize the cell type in the lungs that gives rise to reactivation of latent MCMV. In HCMV there is evidence that bone marrow (BM)-derived cells of the myeloid lineage (e.g. monocytes) carry latent viral genomes, and that virus reactivation can occur upon tissue immigration and differentiation, involving chromatin remodelling and stimulatory signalling (Söderberg-Nauclér et al., 1997; Söderberg-Nauclér et al., 2001; Reeves et al., 2005; Smith et al., 2010; reviewed by Sinclair, 2008). A similar scenario has been proposed for MCMV (Koffron et al., 1998).

To learn if the cell type harbouring the latent MCMV genome in the lungs is indeed of haematopoietic origin, we performed sex-mismatched BM transplantation with male BALB/c donors (carrying the Y-chromosome gene sry) and female BALB/c recipients, followed by establishing a latent MCMV infection in the recipients (Seckert et al., 2009). A cell suspension from latently infected lungs was found to be comprised of donor-derived sry+ and recipient-derived sry– cells, and contained latent viral genomes. Notably, whereas immunomagnetic enrichment of CD11b+ cells also enriched sry+ cells, latent viral genomes were lost and instead were localized to the CD11b– lung-cell subpopulation (Fig. 8a). These data gave a first hint towards the proposal that BM-derived CD11b+ cells of the myeloid lineage are not the main cellular site of MCMV latency in the lungs, although this experiment did not formally exclude a fraction of sry+ CD11b–/low lung-resident, resting macrophages (Vermaelen & Pauwels, 2004).

To visualize directly whether BM-derived myeloid cells give rise to reactivation, we made use of a mouse strain in which the GFP ORF is inserted into the fractalkine receptor (CX3CR1) gene locus (Jung et al., 2000). In the lungs of CX3CR1+GFP mice, GFP-expressing BM-derived monocytes and lung dendritic cells can easily be distinguished from other cell types (Srivastava et al., 2005). CX3CR1+GFP mice were infected with the MCMV-2D reporter virus and PCLS were generated from latently infected lungs. In 160 PCLS cultures examined, we found 29 slices with one single reactivation event. (b) Comparison of luciferase activity and virus production in an individual PCLS (cultivated without MEF monolayer). Samples of the supernatant were analysed in triplicate (with centrifugal enhancement being applied for the plaque assay). Arrows point to the earliest detection of reactivation by the respective assay. Dashed line marks the detection limit of the plaque assay. (c) Time difference between the first detection of luciferase activity and infectious virus. Supernatants of individual PCLS cultures (n=46) were monitored daily for luciferase activity and for the occurrence of virus.
cell displaying mCherry expression as an indicator of virus reactivation. Importantly, none of these mCherry-expressing cells was positive for GFP (Fig. 8b). Collectively, this set of experiments led us to conclude that neither CX3CR1+ monocytes nor pulmonary dendritic cells, nor other cell types expressing CX3CR1, represent prominent cellular sites of MCMV latency and reactivation in the lungs.

DISCUSSION

In this study we have introduced MCMV reporter mutants that allow an analysis of viral reactivation with unprecedented sensitivity and precision. In combination with the use of PCLS, we were able to literally shed light on the reactivation process in lung tissue explants of latently infected mice. The focal spread of reactivated virus in the lung slices (Fig. 7b, c) reproduces the characteristics of recurrent infection as it is proposed to occur in vivo. Gluc activity revealed reactivation events several days earlier than detection of virus in explant culture supernatants. Moreover, the luciferase assay can be completed in <1 h, whereas the plaque assay is more laborious and requires incubation for ~5 days before the results become available. As such, screening procedures aimed at the identification of physiological inducers of reactivation or – more importantly for antiviral therapies – of substances that block reactivation will therefore greatly benefit from the new approach. When tracking Gluc expression in PCLS cultures, we occasionally observed viral activity that did not lead to virus production (Fig. 6a). This is in accordance with the view that in vivo reactivation is controlled at multiple molecular checkpoints and with the finding that even in the absence of immunological control only a fraction of the reactivation events results in the recurrence of infectious virus (Kurz & Reddehase, 1999; reviewed by Reddehase et al., 2008).

The sensitivity of the Gluc assay enabled us to detect reactivation events that were still confined to single cells, which could then be identified microscopically in the PCLS with the aid of the mCherry marker. Macrophages have previously been proposed as candidates for harbouring MCMV DNA in the lungs of latently infected mice, based on similar microanatomical localization of PCR in situ hybridization signals and immunohistological staining (Koffron et al., 1998). This prompted us to revisit, here, the role of pulmonary myeloid cells as sites of MCMV latency with our advanced technologies. By employing...
CX3CR1+/GFP mice, however, we could not confirm that reactivation originates from cells of the myeloid lineage expressing CX3CR1. Furthermore, in a sex-mismatched BM chimera model, latent viral genomes were not found to be enriched in a pulmonary population of donor-derived cells expressing CD11b. One explanation for the discrepant results could be that we studied latency and reactivation at a later stage than in the above-mentioned study. Based on differential viral DNA clearance kinetics in BM, blood and various organ sites, it has been proposed that two types of latency exist, namely a ‘temporary latency’ in haematopoietic cell progeny and an ‘enduring latency’ in another cell type(s), which coexist only at an early stage of latency (reviewed by Reddehase et al., 2002). In organs other than the lungs, such as kidney, liver, spleen and heart, capillary endothelial cells are considered to be the cellular site of MCMV latency (Koffron et al., 1998). More precisely, CD31+CD146+ LSECs were recently identified as the cellular site harbouring the latent viral DNA in the liver, and adoptive transfer of these cells into naïve recipients verified replication competence by productive virus reactivation (Seckert et al., 2009). Lung endothelial cells are therefore one candidate cell type.

**Fig. 8.** Analysis of latently infected and reactivating cells in the lungs. (a) Cellular localization of latent viral genomes. At 11 months after sex-mismatched BM transplantation with male (sry+) donor and female recipient BALB/c mice followed by infection with MCMV-WT, lung cell suspensions (LCS) were prepared individually from five latently infected mice and separated by immunomagnetic cell sorting into CD11b+ (eluate) and CD11b− cells (flow through). Male (sry+) haematopoietic cells and latent viral DNA loads were determined by quantitative PCRs (qPCR) specific for genes sry and M55/gB, respectively. Each dot represents the median value of a qPCR run in triplicate. Short horizontal bars indicate the median values for the five latently infected mice tested. P values are indicated for group comparisons of interest. (b) 160 PCLS cultures generated from two CX3CR1+/GFP mice infected as 4-day-old pups with 10⁵ p.f.u. of MCMV-2D 14 months earlier were monitored daily for Gluc activity up to day 21. In 29 Gluc-positive cultures one single mCherry-expressing cell in the PCLS was found. Cells reactivating latent MCMV are visualized by mCherry (red) fluorescence and CX3CR1-positive cells are visualized by GFP (green) fluorescence. Left panel, fluorescence microscopic image of a PCLS. Right panel, 3D-reconstruction performed with the IMARIS program. Bars, 40 μm.
for harbouring latent MCMV genomes, besides others, such as possibly the long-living lung stem cell. A similar approach to that described here may help to solve this open question.

In this study, we employed the properties of the reporter virus primarily for the analysis of reactivation in vitro, but the reporter genes can also be used to follow other phases of MCMV infection in vivo. Since it is known that the course of the acute infection impacts the subsequent latent phase (Reddehase et al., 1994), it is convenient to examine MCMV infection in the acute phase via Gluc activity in blood or by bioluminescence in a minimally invasive manner without sacrificing the animals. The mCherry marker will also provide the opportunity to study the interaction of MCMV-infected cells with various immune cells in situ in the living animal or in explanted tissues. Compared with EGFP, detection of the red mCherry fluorescence is less influenced by the autofluorescence of tissues, which was an issue of concern when EGFP-reporter viruses (Henry et al., 2000) were used for monitoring MCMV infection (Benedict et al., 2006). Infection with the mutants that in addition to the reporter genes also express the well-characterized SIINFEKL T-cell epitope, and the availability of SIINFEKL-specific CD8 T cells from transgenic mice (Hogquist et al., 1994), will allow the study of the immune control of MCMV reactivation under defined conditions. So, for instance, an analysis can be done to find out if reactivation is contained by cytolytic or non-cytolytic immune effector mechanisms.

In conclusion, the newly developed sensitive detection system will enable further studies on the latently infected cell type(s), the molecular mechanisms of CMV reactivation and the control of CMV reactivation by immune cells in a well-defined quantitative manner.

**METHODS**

**MCMV infection of mice.** The MCMV strain referred to as MCMV-WT and the recombinant viruses were derived from the BAC-cloned strain MW97.01 (Wagner et al., 1999). Virus propagation and generation of virus stocks were performed as described by Brune et al. (1999). Mice housed in ventilated cages under specified pathogen-free conditions were infected by i.n. injection of 2 mg Ketamine and 80 gm l−1 in PBS) was included to control for interassay variation.

**Quantification of infectious virus.** Infection of cells in vitro was performed at the indicated m.o.i., followed by centrifugal enhancement (800 g for 30 min) if indicated. Organs were excised from sacrificed mice and homogenized with a MM200 tissue homogenizer (Retsch). Virus titres in organ homogenates or in culture supernatants were determined by plaque assay performed on MEF monolayers in triplicate (with the application of centrifugal enhancement if indicated).

**Generation of MCMV recombinants.** The Gluc ORF, amplified with primers 5′-GGCTGCTTAGGGCCGCGACGAGAAGAAACCGGGCCCATGGAATCCATCAAGGGGCGGAGG-3′ and 5′-TATGTGCCATGCGACGATGTAAGGTGAGGAGG-3′, was cloned into pmCMV-GluC (NEB) was cloned into pmCMV3 (Chatellard et al., 2007), resulting in pmMIEP-GluC. The mCherry ORF, amplified with primers 5′-GGGAGTTATGCTAGCCGGCCCATGTAAGGTGAGGAGG-3′ and 5′-GCGAAGCTTATGCATGCCGCCATGGTGAGCAAGGGCGAGG-3′, was cloned into pmCMV3 (Livet et al., 2007), was inserted into pmMIEP-GluC, resulting in pmMIEP-GluC. Finally, a kanamycin resistance marker flanked by FRT sites was inserted, yielding pmCherry2AGlucKanR. MCMV BACs were mutated by homologous recombination in *Escherichia coli* as described by Bot et al. (2007). Briefly, PCR fragments were generated using plasmid pmCherry2AGlucKanR and the primers 5′-AATCGTCTACGAGAAGGCTGAACTTAACGGCTGA-3′ and 5′-ACCCGGGCTCTTACGTTAGGACCTCGAAGGCTGTA-3′, containing BACs pSM3fr (Wagner et al., 1999), pSM3fr-m164_SIINFEKL or pSM3fr-D060m152_m164_SIINFEKL (Lemmermann et al., 2010). The kanamycin resistance marker was excised by FLP recombinase. Recombined BACs were characterized by restriction analysis.

**Generation of PCLS and ex vivo reactivation assay.** PCLS were generated as described (Henjakovic et al., 2008). In brief, mice were sacrificed, and the lungs were filled with 2% low-melting-point agarose (Sigma), excised and flooded with 20°C for 4 min. Then, the lungs were separated into lobes and cut into 150μm semi-thin slices using an oscillating tissue slicer (EMS 5000; Electron Microscopy Sciences). PCLS were incubated on MEF monolayers in 24-well plates, except when indicated otherwise. The Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FCS was renewed twice a week. Supernatants were analysed daily for Gluc activity and infectious virus (50 μl each).

**Reporter detection assays.** PCLS were analysed with an Observer Z1 microscope (Zeiss). Digitalized images were further processed using the AxioVision (Zeiss), or IMARIS programs (Bitplane, Scientific Software), or Photoshop (Adobe). Flow cytometry was performed using an LSRII (Becton Dickinson), and data were processed with FACS Diva software (Becton Dickinson). Luciferase activity was quantified with an Orion II Microplate Luminometer (Berthold Technologies) by adding equivalent volumes of the substrate solution (1 μg coelenterazine ml−1 in PBS) and of the sample to non-translucent 96-well plates. Tissue homogenates and blood samples were diluted 1 : 100 in DMEM prior to analysis. The measured values were normalized to the signals measured in samples obtained from MCMV-WT-infected or uninfected cells. A positive control with defined Gluc activity was included to control for interassay variation. For bioluminescence detection, mice were anaesthetized by i.p. injection of 2 mg Ketamine and 80 gm l−1 in PBS). Then, mice were injected intravenously. Photon counts were recorded for 3 min by using an IVIS Imaging System 200 and Living-Image software (Xenogen). Immunomagnetic cell separations as well as qPCRs for the quantification of viral genomes, based on the viral gene *M55/gB,* and in male cells, based on the *Y*-chromosomal gene *sry,* were described in greater detail previously (Seckert et al., 2009).

**ELISpot assay.** DC2.4 cells (Shen et al., 1997) were pulsed with synthetic peptides SIINFEKL or STYTVFRT (Eurogentec) at 10−8 M
for 2 h at 37 °C. Blood cells (10^7) isolated from infected C57BL/6 mice were co-incubated with 10^5 of the peptide-loaded DC2.4 cells for 20 h on a PVDF microplate coated with a mouse IFN-γ-specific antibody. Captured IFN-γ was visualized with a second IFN-γ-specific antibody and streptavidin-conjugated alkaline phosphatase (elbionics). Dots were counted with an automated ELSpot reader (Aelvis).

**Immunoblot analysis.** Proteins were separated by SDS-PAGE on a 12 % acrylamide gel and, after transfer to a nitrocellulose membrane, probed with a DsRed-specific antisemir (Clontech) and a HRP-conjugated goat anti-rabbit antibody, followed by enhanced chemiluminescence (ECL; Amersham) detection.

**Statistical analysis.** Correlation and statistical significance were calculated by the non-parametric, distribution-free Spearman test (Graph Pad Prism 5). The statistical significance of differences between two independent sets of data were evaluated by using the non-parametric, distribution-free Wilcoxon–Mann–Whitney (rank sum) statistics. Differences are regarded as significant if the P value (two-tailed test) is <0.05.

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