Systemic and Local Infection Routes Govern Different Cellular Dissemination Pathways during Gammaherpesvirus Infection *In Vivo*

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Human gammaherpesviruses cause morbidity and mortality associated with infection and transformation of lymphoid and endothelial cells. Knowledge of cell types involved in virus dissemination from primary virus entry to virus latency is fundamental for the understanding of gammaherpesvirus pathogenesis. However, the inability to directly trace cell types with respect to virus dissemination pathways has prevented definitive conclusions regarding the relative contribution of individual cell types. Here, we describe that the route of infection affects gammaherpesvirus dissemination pathways. We constructed a recombinant murine gammaherpesvirus 68 (MHV-68) variant harboring a cassette which switches fluorescent markers in a Cre-dependent manner. Since the recombinant virus which was constructed on the wild-type background was attenuated, in this study we used an M1-deleted version, which infected mice with normal kinetics. Infection of Cre-transgenic mice with this convertible virus was used to estimate the quantitative contribution of defined cell types to virus productivity and dissemination during the acute phase of MHV-68 infection. In systemic infection, we found splenic vascular endothelial cells (EC) among the first and main cells to produce virus. After local infection, the contribution of EC to splenic virus production did not represent such early kinetics. However, at later time points, B cell-derived viruses dominated splenic productivity independently of systemic or local infection. Systemic versus local infection also governed the cell types involved in loading peritoneal exudate cells, leading to latency in F4/80- and CD11b-positive target cells. Systemic infection supported EC-driven dissemination, whereas local infection supported B cell-driven dissemination.

Tumors caused by human gammaherpesviruses are of either lymphoid or endothelial cell (EC) origin. This raises the question of whether one of these cell types plays a key role in gammaherpesvirus pathogenesis during infection. Due to the strict species specificity of human gammaherpesviruses, these viruses cannot be studied in animals. Infection with murine gammaherpesvirus 68 (MHV-68) serves as an animal model to study gammaherpesvirus pathogenesis *in vivo* (1). MHV-68 is closely related to the two human gammaherpesviruses, Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV). All three viruses present an analogous genomic structure (2, 3), infect leukocytes and epithelial cells (4–8), and establish life-long latency in lymphocytes. Furthermore, mononucleosis is comparable in EBV and MHV-68 pathogenesis (7, 9), and all three viruses induce malignancies (10–12).

After intranasal (i.n.) inoculation of mice with MHV-68, the infection first locates in the respiratory tract for a few days. Infection of lung tissue by MHV-68 is typically followed by systemic virus dissemination associated with productive infection in multiple organs (7). After 2 to 3 weeks, the acute infection becomes controlled. During the last period of acute infection, infection of B cells, macrophages, and dendritic cells (DC) in lymphoid organs advances and the latent phase of MHV-68 infection is established (4–8). B cells represent the major reservoir of latent virus in the spleen, independently of the chosen route of infection (8, 13), while macrophages were described as the major reservoir of latency in the peritoneal cavity, at least after intraperitoneal infection (13). However, an efficient latency establishment in the peritoneal cavity of mice lacking B cells strongly suggests that this is also the case after intranasal infection (13, 14).

Until now, with the exception of lung epithelial cells (6), the role of specific cell types in MHV-68 infection was mainly inves-

tigated with respect to the establishment of latency. Since the productive infection in and dissemination from individual cell types could not be tested *in vivo*, it remained unclear how MHV-68 disseminates and eventually reaches the different sites of latency. Only in immune-deficient mice were two other cell types found to be infected with MHV-68, which have not been identified as sites of latency: in mice lacking the gamma interferon (IFN- γ) receptor, infected smooth-muscle cells were identified in aortic lesions, and more recently, endothelial cells (EC) in the lungs of CD8 knockout mice were found to be infected and to support persistent MHV-68 infection *in vitro* (15, 16). The extent of infection of these cell types, their productivity, and the relative contribution of their virus progeny to virus dissemination in the normal course of MHV-68 infection in immunocompetent mice, however, remained unknown.

The identification of the cell types supporting virus production in the immunocompetent host and their role for dissemination is basic for understanding viral pathogenesis in the immune-deficient setting. At best, *in vitro* infection of cell lines or primary cells

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and the detection of viral proteins and/or nucleic acids *in vivo* can only suggest a role of a specific cell type in the infection process *in vivo*.

To study specific biological features of defined cell types, the Cre-*loxP* system represents one of the most reliable genetic tools (17). Cre recombinase catalyzes the recombination of two directly repeated *loxP* sites, leading to the excision of an intervening DNA sequence and allowing the control of the expression of a gene or marker (18). The Cre-*loxP* system was applied to activate cell type-specific expression of viral genes *in vivo* (19), and our group introduced Cre-dependent marker conversion as an approach to quantify the contribution of cell types to productive virus infection and to trace the fate of viral progeny from defined cellular origins (20–23).

Recently, the contribution of dendritic cells (DCs) to MHV-68 infection was tested using a similar Cre-*loxP* system and mice expressing Cre in DCs (CD11c-cre) (24). This study indicated that DCs contribute to MHV-68 dissemination toward lymphoid organs and especially B cells. However, this contribution was never found to represent more than about 30% of the total virus load or infected cells. The authors advanced the hypothesis that a low degree of Cre expression in these mice could induce an underestimate of this contribution. However, the contribution of other cell types in MHV-68 dissemination could also explain these results, which is what we wished to test.

We modified the MHV-68 genome with bacterial artificial chromosome (BAC) technology and integrated a reporter for cell type-specific Cre recombination. This modified MHV-68 undergoes a genetic marker conversion upon infection of cells expressing Cre. Here, we studied, among other cell types, mainly B cells and EC as major sites of virus production during the initial phase of MHV-68 infection and describe, for the first time, their quantitative contribution to virus dissemination and to the establishment of latency.

MATERIALS AND METHODS

Ethical statement. All animal experiments were performed in compliance with the German animal protection law (TierSchG BGBl. S. 1105; 25.05.1998). The mice were housed and handled in accordance with good animal practice as defined by FELASA and the national animal welfare body GV-SOLAS. All animal experiments were approved by the responsible state office (Regierung von Oberbayern) under permit number 55.2-1-54-2531-03-10.

Cells and mice. BHK-21 and Ref-Cre cells as well as BALB/c-derived mouse embryo fibroblasts (MEF) were grown as described previously (25, 26). All animals were housed under specific-pathogen-free (SPF) conditions at the animal facility of the Max-von-Pettenkofer Institute. Transgenic Tek-cre (27), Tie2-cre (28) (kindly provided by B. Arnold, DKFZ, Heidelberg, Germany), Alb-cre (29), SM22-cre (30), CD11c-cre (31) (kindly provided by I. Förster, Life & Medical Sciences [LIMES] Institute, University of Bonn), knock-in CD19-cre (32), Mb-1-cre (33) (kindly provided by M. Reth, University of Freiburg, Germany), and F4/80-cre mice (34) were maintained on the C57BL/6J background. C57BL/6J mice were obtained from Janvier, France, and other mice (except Tie2-cre and Mb-1-cre) were from Jackson Laboratories. Experiments were performed with male and female mice at 2 to 14 months of age. Animal experiments were approved by the responsible state office.

Viruses and infection of mice. The recombinant MHV-68 variants described in this study were derived from the previously described MHV-68 bacterial artificial chromosome (BAC) (35). Viruses were reconstituted and propagated on BHK-21 cells and purified as described previously (26), except that at least 10 passages on BHK-21 were realized before

virus stock preparation to ensure BAC cassette removal. The removal of the BAC cassette from the viral genome was analyzed by southern blot analysis. To prepare Cre-recombined virus stocks for use in the studies, at least 4 passages on Ref-Cre cells were made before harvest. Virus quantification was done by standard plaque titration assay on MEF. Mice were infected intravenously (i.v.; into a tail vein) in a volume of 300 μ l or intranasally (i.n.) in a volume of 20 μ l (under ketamine/xylazine anesthesia).

Construction of recombinant viruses. The general strategy for the construction of the convertible MHV-68 recombinants was the following. First, the BAC cassette in the wild-type (wt) MHV-68 BAC (35) was first modified to permit the integration of a floxed color-switch cassette. The flanking *loxP* sites and the enhanced green fluorescent protein (EGFP) expression unit were removed from the BAC cassette. In order to allow the excision of the modified BAC cassette by homologous recombination during tissue culture passages of the reconstituted virus (36), the first 500 bp from the left end of the MHV-68 genome was inserted in front of the BAC cassette in a second copy, providing the necessary sequence duplication. A Cre-convertible color-switch cassette (see the construction details below) was inserted in the viral genome, either without deletion of viral sequences in front of the tRNA-2-like gene or by replacing a viral sequence of comparable size containing the M1 gene and tRNA-2-like to tRNA-6-like genes (see the construction details for BAC mutagenesis below). The insertion of the color-switch floxed cassette without viral deletion resulted in MHV-68-color-switch, and the replacement of M1 and tRNA-2-like to tRNA-6-like genes resulted in MHV-68-color-switch- Δ . The Cre-convertible color-switch cassette contained an mOrange expression unit in which the mOrange open reading frame (ORF) was flanked by *loxP* sites. In addition, after the mOrange ORF, an EGFP coding sequence was inserted. This cassette expressed mOrange by default, and EGFP expression was activated after Cre recombination.

Cre-convertible color-switch cassette. For the construction of the floxed convertible color-switch cassette, the cmc-tag sequence was cloned at the C-terminal end of the mOrange open reading frame in PJ51 (DNA2.0 Inc.). PCR was performed with primers morange-cmyc-EcoRIfor and morange-cmyc-XhoIrev (the sequences of the primers used in this study are listed in Table S1 in the supplemental material), and its product was inserted into PJ51-mOrange vector after digestion with XhoI/EcoRI. (All synthetic oligonucleotides used in this study are listed below in Table S1.) The poly(A) sequence of simian virus 40 (SV40) amplified from pHcRed-Tandem-C1 (Evrogen) was then inserted in the previous vector using an XbaI restriction site to obtain mO-cmyc-pA using primers SV40epAKXfor and SV40pAeKXrev. The mOrange-cmyc+polyA(SV40) fragment was then cloned using primers mOr-pASV40-KN-for and mOr-pASV40e-KN-rev into AccI/SacII sites of the previously described pCR3-floxstop-egfp-FRT-Kan-FRT (23) to obtain PCR3-mOrange-cmyc-in-egfp-floxout. The SV40 early promoter (pSV40) from pHcRed-tandem-C1 vector was cloned in the same vector in front of the HcRed-tandem gene using primers PSV40e-tHcRed-NheIa and PSV40e-tHcRed-AgeIb into NheI/AgeI sites. HSV-TK-PolyA, from pHcRed-tandem-C1 vector, was cloned into XhoI/EcoRI sites in the same vector after the HcRed-tandem gene using primers PolyAHSVTK-tHcRed-XhoIa and PolyAHSVTK-tHcRed-EcoRIb. The fragments containing pSV40, the HcRed-tandem gene, and HSV-TK-PolyA were then cloned into the DraIII site of pACYC177 vector (New England BioLabs) using primers ModifMHV68BAC-4a and ModifMHV68BAC-4b, resulting in pACYC177-HcRed.

BAC mutagenesis. The modifications performed on the MHV-68 BAC genome (35) were all done, if not otherwise stated, by markerless BAC mutagenesis as described previously (37, 38).

First, the 56 bp from the left extremity of the MHV-68 genome was inserted in the corresponding place on the MHV-68 BAC, whereas the *loxP* site at the 3' extremity of the BAC cassette and the already existing EGFP expression cassette were deleted. For that, a double-stranded DNA (dsDNA) fragment was synthesized with the single-stranded DNAs

(ssDNAs) corModifMHV68BAC-1a and corModifMHV68BAC-1b, digested with DraIII, and inserted into the DraIII site of pACYC177 vector. An I-SceI cassette then was amplified from the previous plasmid using primers corModifMHV68BAC-2a and ModifMHV68BAC-2b. MHV-68-BAC-332, MHV-68-BAC-382, and MHV-68-BAC-622 were created thusly. Second, we inserted a tandem-HcRed expression cassette at the end of the BAC cassette (fluorescence provided by this cassette was never observed). pACYC177-HcRed plasmid was digested with BamHI/BsaI, and an I-SceI cassette was created by PCR of the 4.8-kbp fragment using primers ModifMHV68BAC-5a and ModifMHV68BAC-5b, resulting in MHV-68-BAC-33266 and MHV-68-BAC-33268. In the third step, we deleted the *loxP* site at the 5' extremity of the BAC cassette and inserted in its place 500 bp identical to the left part of the MHV-68 genome: this 500 bp was amplified by PCR on MHV-68-BAC-332 with primers cormodifMHV68BAC-6a and ModifMHV68BAC-6b and were cloned into Aval/DrdI sites on pACYC184 vector (New England BioLabs). An I-SceI cassette was then created by PCR on this template with primers CorModifMHV68BAC-7a and CorModifMHV68BAC-7b. For this step, tetracycline was used in place of kanamycin. Successful recombination resulted in intermediate MHV-68 BAC clones, which were named 3326681 and 3326844. The last step, to insert the color-switch-floxed (DCF) cassette into 3326681 in front of or replacing tRNA-2 to tRNA-6 and the M1 viral gene, was realized by recombination with linear fragments as described previously (36). In order to construct the recombination fragment introducing the DCF cassette, sequences homologous to targeted regions of the MHV-68 genome were first cloned into PCR3-mOrange-cmyc-in-egfp-floxout plasmid. The upstream sequence was amplified by PCR on the 3326681 genome using primers Aa and Bb and inserted into the DraIII site of PCR3-mOrange-cmyc-in-egfp-floxout plasmid, resulting in the AB plasmid. The downstream sequences were amplified by PCR of the 3326681 genome using primers Ga and Hb and inserted into the closest Pci site in the AB plasmid, resulting in ABGH plasmid. The final linear fragments for recombination were obtained after digestion of ABGH plasmid with SmaI. After recombination and removal of the Kan^r marker, the final constructs were identified and named floxed color-switch MHV-68 (insertion) and floxed color-switch MHV-68-color-switch-floxed-Δ (ΔtRNA2-6 ΔM1). The new recombinant MHV-68 BACs were confirmed by restriction enzyme digestion and sequencing.

Analysis of the productive virus infection *in vivo*. The production of infectious virus particles after *in vivo* infection of mice was determined as described for murine cytomegalovirus (MCMV) by plaque assays (25). Briefly, the infected organs were harvested and homogenized, and subsequently MEFs were infected with serial dilutions of the organ homogenates. The load of nonrecombined or Cre-recombined MHV-68-color-switch-Δ virus was determined by counting plaques which were formed 5 days after infection of the MEFs using epifluorescence microscopy. The total virus load was calculated on the basis of the plaques, which were formed independently of the color of their fluorescence. In parallel, the titers of both the green and the red fluorescent plaques were also determined individually. The load of wt MHV-68 was also determined 5 days after infection of the MEFs by counting the induced plaques after staining with crystal violet solution. PFU were calculated per g of organs. Detection limits varied from one experiment to another depending on the dilutions chosen for titration and on the number of wells used per dilution.

Magnetic cell separation of B cells, DCs, and macrophages. To isolate splenocytes, spleens were harvested at the given days after infection into PBS, homogenized, and filtered through a 100-μm-pore nylon cell strainer (Becton, Dickinson). Erythrocytes were removed with BD Pharm Lyse buffer (BD biosciences). Fresh peritoneal exudate cells (PEC) were obtained by washing the peritoneal cavity with cold phosphate-buffered saline (PBS). B cells were enriched from splenocytes by positive isolation using anti-CD19 magnetic beads (Miltenyi Biotec) per the manufacturer's recommendations. DCs were then enriched the same way from the B cell-depleted splenocyte suspension using anti-CD11c magnetic beads (Miltenyi Biotec). Finally, monocytes-macrophages were enriched from B

cell- and DC-depleted splenocytes or from PEC using anti-CD11b magnetic beads (Miltenyi Biotec). Following separation, stained cell populations were analyzed by flow cytometry as described below.

Flow cytometry (FACS). Cells for fluorescence-activated cell sorter (FACS) analysis were resuspended in MACS buffer (Miltenyi Biotec) and were stained using a combination of the following antibodies: phycoerythrin (PE)-Cy5.5-conjugated antibodies to CD19 and CD11b, fluorescein isothiocyanate (FITC)-conjugated antibodies to B220, and PE-conjugated antibodies to CD11c. All antibodies were obtained from BD Pharmingen and used at a 1:400 dilution. Cells were incubated with antibodies for 30 min in the dark at 4°C before being washed in MACS buffer. Depending on the experiments, 10⁴ to 10⁶ events were collected on a BD FACS Canto (BD Biosciences). The data were analyzed using FlowJo software (Tree Star, Inc.).

Analysis of viral load of latently infected cells by infectious center assay. Both the *ex vivo* reactivation analysis and the infectious center assay were performed as previously described (39, 40), except that instead of staining with crystal violet solution, the epifluorescence of EGFP or mOrange was used to detect infectious centers. To control for the presence of preformed infectious virus particles, parallel to these assays, a proportion of each sample was subjected to 3 cycles of freezing-thawing. These cell lysates were plated onto MEF, and the productive virus infection was quantified as described above. Detection limits varied depending on the number of extracted and sorted cells used for each assay.

Statistical analysis. Comparison of percentage of recombined virus between time points and mouse lines was done with the two-tailed Wilcoxon rank-sum test using the website <http://elegans.som.vcu.edu/~leon/stats/utest.html>. *P* < 0.05 was considered a significant difference, and *P* > 0.1 was considered not significant.

RESULTS

Construction of an MHV-68 BAC expressing a color-switch cassette. To mark the murine gammaherpesvirus 68 (MHV-68) progeny in a Cre-dependent manner, a reporter cassette for switching between expression of two different fluorescent markers was constructed (floxed color-switch cassette) (Fig. 1B). The floxed color-switch cassette comprises the mOrange open reading frame (ORF) under the control of the human cytomegalovirus (HCMV) major immediate-early promoter. A translational and transcriptional stop sequence [poly(A)] immediately following the mOrange ORF prevented the expression of the downstream EGFP ORF. The mOrange ORF and the poly(A) site were flanked by two *loxP* sites (floxed). Cre recombination deleted the constitutively expressed mOrange ORF and the stop signals (Fig. 1B). Thus, the phenotype of this recombination should be the switch from mOrange expression to EGFP expression (Fig. 1B). More importantly, the constant presence of a positive marker identifies potential conditions of loss of fluorescence which are not due to Cre recombination (23, 41). The color-switch cassette was introduced into the recombinant MHV-68 by BAC mutagenesis (35, 42) (see Materials and Methods). The cassette was inserted directly after the tRNA-1 coding sequence either without deletion of the MHV-68 genome or with the replacement of the tRNA-2-like to tRNA-6-like genes and the M1 gene to prevent attenuation and genetic instability due to being too large (see Fig. 1A). The deletion of neither the tRNA-like sequences nor the M1 genes has been associated so far with effects on acute MHV-68 infection and on the establishment of latency (1, 43).

Cre expressed in specific cell types productively infected with the cassette-containing virus marks the progeny of the respective cell type (rec-MHV-68-switch-color). This allowed estimating the contribution of the cell type to the total virus production and

FIG 1 Construction of a color-switch virus permitting the tracing of the progeny from specific cell types by Cre-mediated recombination. (A) The BAC cassette (dashed box) of the wt MHV-68 BAC contained two *loxP* sites (black arrowheads) and an EGFP expression unit (green box). This cassette was inserted after the terminal repeats (TR) and the first tRNA-like locus (t.1-2). In the recombinant MHV-68 constructs used in this study, the *loxP* sites and EGFP expression unit were removed from the BAC cassette. Both features were reinserted as a part of the floxed color-switch cassette (underlined portion), which consisted of a constitutive CMV promoter, upstream *loxP* site, mOrange ORF, downstream *loxP* site, and the EGFP ORF flanked by a poly(A) site. The leftmost 500-nucleotide region of the MHV-68 genome (blue box) containing the first tRNA-like sequence was reinserted at the front of the BAC cassette, allowing the removal of the BAC backbone by homologous recombination. The floxed color-switch cassette was inserted into the MHV-68 genome by two ways. (i) The cassette was inserted between the first and the second tRNA-like loci (t.1-2 and t.3-4, respectively), resulting in the floxed color-switch MHV-68. (ii) The color-switch cassette replaced the second and third tRNA-like loci (t.3-4 and t.5-6, respectively) and the M1 gene, resulting in the floxed color-switch MHV-68-Δ. (B) A Cre-dependent expression cassette (floxed color-switch cassette) was inserted in the MHV-68 genome. In the absence of Cre recombination, only mOrange fluorescent protein is expressed. EGFP, under the control of the major immediate-early promoter of HCMV, is not expressed due to the presence of multiple translational stop signals (*) and the poly(A) sequence from SV40 (transcriptional stop sequence) between the HCMV promoter and EGFP gene. Cre-mediated removal of the sequence containing the mOrange gene and the stop sequence flanked by *loxP* sites (floxed) leads to expression of EGFP. (C and D) Our experimental system allows identification of productively infected cell types *in vivo* to quantify their progeny and to monitor virus spread to other cell types and organs. (C) This is achieved by cell type-specific Cre-mediated recombination of MHV-68 genomes. Infection of transgenic or knock-in mice expressing Cre specifically in certain cell types allows marking and tracing of MHV-68 originating from Cre-expressing cells. (D) Schematic setup of experiments and analysis. Transgenic or knock-in mice expressing Cre recombinase under the control of a cell type-specific promoter were infected with floxed MHV-68 color-switch virus. At different time points after infection, the PFU of nonrecombined and Cre-recombined MHV-68-color-switch in various organs was determined by plaque assay. The graph depicts total (nonrecombined plus recombined) virus load per gram of organ for individual mice as open circles that refer to the logarithmic scale on the right side. Horizontal bars mark mean values, and dotted lines indicate detection limits. Gray columns refer to the linear scale on the left side and show the mean percentage of EGFP⁺ plaques, with the standard deviations indicated by vertical bars.

FIG 2 Infection with MHV-68-color-switch- Δ is comparable to infection with wt MHV-68 during the productive phase of infection *in vivo*. (A) MEF cells were infected with wild-type MHV-68, nonrecombined color-switch MHV-68, or Cre-recombined color-switch MHV-68 (wt, floxed, and rec, respectively) (a) or wild-type MHV-68, nonrecombined-color-switch MHV-68- Δ , or Cre-recombined-color-switch MHV-68- Δ (b) at a multiplicity of infection (MOI) of 0.1. Cell supernatants were collected immediately (day 0, before washing the cells) or after 1, 2, 3, 4, 5, or 6 days postinfection (p.i.). The viral load of the supernatants was analyzed by plaque assay. Every time point of every infection was performed in triplicate. (B) C57BL/6 mice were infected i.n. with either 10^5 PFU of MHV-68-wt (wt), a mix (mix) containing 5×10^4 PFU floxed virus plus 5×10^4 PFU rec-virus, or a mix (mix Δ) containing 5×10^4 PFU floxed and 5×10^4 PFU rec-virus- Δ , and the viral load in lungs of 5 mice per time point was analyzed on days 6 and 10 p.i. For each infection and each time point, we represented the total viral load per gram of organ and the viral load in nonrecombined virus (mOrange⁺) and Cre-recombined virus (EGFP⁺). (C) C57BL/6 mice were infected i.n. with 10^5 PFU of wt or floxed virus- Δ , and the total viral load in lungs and spleen of 3 mice per time point was analyzed after 3, 6, 10, 14, 17, and 23 days p.i. Similar data were obtained in a second identical experiment with the time points 6, 10, 14, and 17 days p.i. (D and E) C57BL/6 mice were infected i.n. with 9×10^5 PFU of either wt, floxed, or rec virus- Δ , and the total viral load in lungs and spleen of 4 or 5 mice per time point was analyzed after 6, 13, and 39 days p.i. (D) At 39 days p.i., some living splenocytes were also extracted in order to perform an *ex vivo* reactivation assay (limiting-dilution analysis). (E) The graph on the left represents the percentage of wells positive for fluorescence (mOrange or EGFP), whereas the graph on the right represents the percentage of wells positive for lysis. A second independent experiment (D and E) was performed with similar results.

permitted tracing of the virus from Cre-positive cells to other cells (Fig. 1C). The number of EGFP⁺ plaques after the first round of virus replication reflects the relative contribution of the Cre-expressing cell type to total virus productivity in the tested organ. The comparison of different cell types which express Cre permits conclusions on the roles the different cell types play during infection (Fig. 1D).

In vitro, both floxed-MHV-68-switch-color- Δ and rec-MHV-68-switch-color- Δ replicated to titers similar to those of wt MHV-68 (Fig. 2A, b). However, floxed-MHV-68-switch-color was significantly attenuated compared to wt virus, while rec-MHV-68-switch-color seemed to replicate with higher titers than wt virus, creating an even bigger difference in replication between floxed and recombined viruses without deletion (Fig. 2A, a).

TABLE 1 Virus progeny derived from different cell types in various organs

Mouse type(s) and site of Cre expression	Route of infection ^a	% Cre recombination in virus load found in ^b :									
		Spleen		Lymph node		Lung		Heart		Liver	
		Min	Max	Min	Max	Min	Max	Min	Max	Min	Max
Tek-cre, Tie2-cre											
Endothelial cells	i.v.	25	100	0	45	14	64	73	98	8	30
Endothelial cells	i.n.	15	80	0	5	0	0				
CD19-cre, Mb-1-cre											
B cells	i.v.	0	100	0	100	0	5	0	0	0	100
B cells	i.n.	45	100	0	100	0	49				
F4/80-cre											
Macrophages	i.v.	0	2	0	2	0	1	0	0	0	1
SM22-cre											
Smooth-muscle cells	i.v.	0	0	1	3	0	3	0	5	0	1
Alb-cre											
Hepatocytes	i.v.	0	0	0	0	0	0	0	0	0	10
CD11c-cre											
Dendritic cells	i.v.	67	97	0	50	100	100	0	4	Not tested	Not tested
Dendritic cells	i.n.	44	66	10	95	5	10				

^a Mice were infected intravenously (i.v.) or intranasally (i.n.) with 2×10^6 PFU of floxed-MHV-68-color-switch- Δ virus.

^b Numeric values indicate the percent represented by Cre-recombined viruses compared to the total viral load (PFU/g) detected in the different organs. Four mice per time point were analyzed between days 2 and 10 p.i. for i.v. infections and days 3 and 13 p.i. for i.n. infections. Similar results were obtained in at least 2 independent experiments for each mouse line ($n = 2$ or more). Minimal (min) and maximal (max) values depend on the time point after infection. For some mice, after i.n. infection, no viral load above the detection limit could be measured in heart and liver.

These results were confirmed by the total viral load measured in organs from C57BL/6 mice intranasally (i.n.) infected with either wt MHV-68, a mix of floxed-MHV-68-switch-color and rec-MHV-68-switch-color viruses, or a mix of floxed-MHV-68-switch-color- Δ and rec-MHV-68-switch-color- Δ viruses (Fig. 2B). Both floxed-MHV-68-switch-color- Δ and rec-MHV-68-switch-color- Δ replicated comparably to wt MHV-68 in lungs and spleen from i.n.-infected mice during acute infection (Fig. 2B, C, and D), while floxed-MHV-68-switch-color was strongly and quickly attenuated compared to wt virus. These results (Fig. 2A, B, C, and D) supported our decision to use floxed-MHV-68-switch-color- Δ and rec-MHV-68-switch-color- Δ for all subsequent experiments.

Some attenuation of rec-MHV-68-switch-color- Δ , however, was also observed at later time points in the spleen, as measured by an *ex vivo* reactivation assay (39 days postinfection [p.i.]) (Fig. 2E). An explanation of this phenomenon given by the study of Braaten et al. showed that a strong CD8⁺ T cell response can limit long-term latent MHV-68 infection, especially if a gene with a presentable CD8⁺ T cell epitope is expressed by the virus under the control of the HCMV IE1 promoter, as this leads to an attenuation in viral persistence (44). Therefore, our results would be explained if EGFP possesses a presentable CD8⁺ T cell epitope while mOrange does not.

Consequently, we restricted our studies only to the initial phase of MHV-68 infection, namely, until 10 days after intravenous (i.v.) infection and 14 to 16 days after i.n. infection. This time restriction also aimed to reduce possible effects of M1 gene depletion in the MHV-68 genome, which have been associated with enhancement of reactivation from latency (43). Note that activa-

tion of EGFP expression was never observed after infection of mice with floxed-MHV-68-switch-color- Δ virus in the absence of Cre, and plaques always expressed either mOrange or EGFP stably and in a mutually exclusive fashion (data not shown). Based on these data, we found floxed-MHV-68-switch-color- Δ to be a suitable model for primary MHV-68 infection in subsequent studies.

The relative contribution of cell types to MHV-68 production is determined by the route of infection. Both human gammaherpesviruses, EBV and KSHV, are transmitted naturally by saliva (45, 46). In addition, systemic infections, namely, by transplantation or blood transfusion, are common for EBV and have also been described for KHSV (45–50). To test both major infection routes, we infected mice with floxed-MHV-68-switch-color- Δ either i.n. or i.v.

We used an array of different Cre-expressing mice to test the productivity of B cells (CD19-cre and Mb-1-cre) (32, 50), EC (Tek-cre and Tie2-cre) (27, 28, 51), macrophages (F4/80-cre) (34), smooth-muscle cells (SM22-cre) (30), hepatocytes (Alb-cre) (29), and dendritic cells (CD11c-cre) (31) upon MHV-68 infection *in vivo*.

The virus load of different organs and the recombination rates were determined by plaque assay 2, 4, 6, and 10 days after i.v. infection and 3, 7 (only for the spleen), 10, and 13 days after i.n. infection. Typical conversion rates in spleens, lymph nodes (superficial, deep cervical, brachial, and inguinal), lungs, heart, and liver are shown in Table 1. Very low levels or no recombined progeny was found in the different organs prepared from infected Alb-cre, F4/80-cre, and SM22-cre mice (Table 1), excluding an important role for hepatocytes, macrophages, and smooth-mus-

FIG 3 EC and B cells are infected early during MHV-68 infection and disseminate virus in different organs. (A) Tek-cre^{+/-} (a to e) and CD19-cre^{+/-} mice (f to j) were i.v. infected with 2×10^6 PFU of floxed-MHV-68-color-switch- Δ , and spleen (a and f), lymph nodes (b and g), lungs (c and h), heart (d and i), and liver (e and j) of 4 mice per time point were analyzed on days 2, 4, 6, and 10 (a to e) or 2, 4, 7, and 10 (f to j) p.i. ($n = 3$ for Tek-cre and $n = 2$ for CD19-cre). (B) Tek-cre^{+/-} (a to c) and CD19-cre^{+/-} mice (d to f) were infected with 2×10^6 PFU of floxed-MHV-68-color-switch- Δ i.n., and spleen (a and d), lymph nodes (b and e), and lungs (c and f) of 4 mice per time point were analyzed on days 3, 7 (for Tek-cre only for spleen), 10, and 13 p.i. For both panels, the PFU of nonrecombined and Cre-recombined MHV-68-color-switch in various organs was determined by plaque assay. The graph depicts total (nonrecombined plus recombined) virus load per gram of organ for individual mice as open circles that refer to the logarithmic scale on the right side. Horizontal bars mark mean values, and dotted lines indicate detection limits. The gray columns refer to the linear scale on the left side and show the mean percentage of EGFP⁺ plaques, with the standard deviations indicated by vertical bars ($n = 2$ for both mouse lines; the day 13 p.i. time point was performed 4 times, of which 2 were performed independently of the other time points).

cle cells, respectively, for virus production during primary infection. In contrast, substantial production of rec-MHV-68-switch-color- Δ virus was only found after infection of CD11c-cre, Tek-cre, Tie2-cre, CD19-cre, and Mb-1-cre mice. The results for CD11c-cre mice indicate a contribution of DC in virus production in spleen and lymph nodes after both i.v. and i.n. infection and in lungs after i.v. infection.

The results for CD19-cre and Tek-cre mice with respect to virus load and conversion rates are shown in Fig. 3. Similar results were obtained with Mb-1-cre and Tie2-cre mice, as well as with a lower dose of infection (data not shown).

B cells make a major contribution to the total viral load in the lymphoid organs of i.v.-infected mice (Fig. 3 and data not shown). The infection of B cells was expected, since they are the major cell type infected by MHV-68 in lymphoid organs, at least during latency. Notably, compared to total virus production in both spleen and lymph nodes, virus production by B cells was delayed: after i.v.

infection, less than 3 and 20% of viruses were derived from B cells after 2 and 4 days, respectively (Fig. 3). Although less prominent, this phenomenon was also detectable after i.n. infection. However, independently from the route of infection, at later times virus production became entirely dominated by B cells in spleen and lymph nodes. In contrast, almost no contribution of B cells was found in nonlymphoid organs, i.e., lungs (Fig. 3A, h, and B, f), heart (Fig. 3A, i), and liver (Fig. 3A, j).

EC-derived production of viruses appeared as prominent as that of B cells in the spleen after systemic infection but differed with respect to timing. EC-derived production represented almost the total splenic viral load at 2 days after i.v. infection (Fig. 3A, a). Hence, EC are an early, if not the first, cell type to produce virus in the spleen after i.v. infection. At later time points, EC progeny was still present in the spleen but represented only about 50% of the total viral load at a time when most virus was derived from B cells, as was seen in CD19-cre mice. In lymph nodes, however, EC-

derived virus was not produced earlier than B cell-derived virus after i.v. infection and never represented more than 50% of the total viral load (Fig. 3A,b). An early contribution of EC to virus production also could be observed after i.v. infection in nonlymphoid organs, such as lungs, heart, and liver.

After i.n. infection, however, the head start of virus production in splenic EC could not be observed (after 10 days p.i.) (Fig. 3B, a). After i.n. infection, the relative contribution of EC-derived virus was comparable to that observed after i.v. infection (45 to 50%), only at later time points (after 13 days p.i.) (Fig. 3B, a). In the lymph nodes, EC never contributed more than 5% to the infection after i.n. infection (Fig. 3B, b). Thus, after i.n. infection, cells other than EC or B cells must have produced the bulk of virus in the spleen and lymph nodes during the first week p.i. (Fig. 3B, a, b, d, and e).

Taken together, our data showed that the contribution of EC to virus productivity was dependent on the route of infection. The lack of contribution of vascular EC to the infection of lungs and lymph nodes after i.n. infection probably reflects the infection pathway, since the respiratory tract gets infected without the need for viral transport through blood vessels. Lymph nodes drain this tract, and viruses produced in respiratory tissues may circulate via lymph nodes without a major contribution of blood vessels and vascular EC. Indeed, among the tested lymph nodes, virus was mostly found in superficial lymph nodes after i.n. infection, and very little was found in brachial and inguinal ones (data not shown).

Not surprisingly, no virus production could be detected in the heart and the liver after i.n. infection of mice in the C57BL/6 background. In the literature, productive infection was detected in these organs only in BALB/c (very limited) or newborn mice (7, 52, 53). Moreover, it is possible that the early and short production of virus detected in these organs after i.v. infection, especially the part produced by EC, simply reflects viral replication within the vasculature without being specific to these organs. The same could also be true for virus production in lungs after i.v. infection, which is vastly different in timing and characteristics from lung infection after i.n. inoculation.

EC-derived MHV-68 disseminated to splenic B cells, DCs, and monocytes-macrophages. Since EC contribute early to virus productivity in the spleen, we wanted to investigate whether this cell type is a source of infection for other splenocytes. Therefore, we tested transmission of EC-derived virus to splenic B cells, DCs, and monocytes-macrophages after the first occurrence of virus replication as determined by infectious center assay with splenocytes (after 1 day for i.v. infection and 6 days after i.n. infection) (Fig. 4). In this assay, the virus reactivation is also measured, and therefore it is possible that the deletion of the M1 gene slightly influences the results in quantitative terms, but we believe that it is still appropriate to answer this question in qualitative terms.

The interval of only 24 h after i.v. infection was not sufficient for EC to begin to produce virus efficiently, and logically no transmission of EC derived virus to splenic B cells occurred so early (Fig. 4A, a). However, at this time point some splenic CD19⁺ cells were already nonproductively infected (Fig. 3A, f, and 4A, a). Similarly, 6 days after i.n. infection, less than 10% of infected B cells had received virus from EC (Fig. 4C). Therefore, the observed delay of virus production by B cells was not due to a delay of B cell infection in general. Some B cells were already infected before spread of EC virus progeny to B cells occurred. However, 2 to 8

days after i.v. infection, 40 to 60% of B cells were infected by EC-derived virus in the spleen (Fig. 4A and B), and 12 days after i.n. infection about 30% of B cells were infected by EC progeny (Fig. 4C). These results indicate that EC-derived virus is transmitted to B cells in the spleen, although some B cells are also infected by other sources, and infection of B cells is not directly followed by virus production. This delay in production probably explains the decrease in EC contribution to total splenic viral load over time after both i.v. and i.n. infection (Fig. 3A, a, and B, a), since at later time points, B cells which were infected earlier could contribute significantly to virus production.

Splenic DCs, representing 0.4 to 5% of all infected splenocytes, were infected during MHV-68 primary infection after both i.v. and i.n. infection (Table 2). The percentage of DCs infected with EC-derived virus after i.v. inoculation was comparable to that of B cells, indicating no selectivity in the spread of EC viral progeny to either cell type. The small number of infected DCs after i.n. infection was not sufficient to estimate the contribution of EC-derived virus (Fig. 4C and Table 1). Interestingly, while DCs produced virus at day 3 after i.v. infection, this production ceased after 7 days, although the number of infected DC still increased (Fig. 4B and Table 1). An early switch of infection to latency in DCs could explain this result.

CD11b⁺ splenocytes (monocytes and macrophages) were found infected at day 7 after i.v. infection, and the percentage of CD11b⁺ cells infected with EC-derived virus was comparable to that of B cells and DC. Also, at day 12 after i.n. infection, monocytes-macrophages were found to be infected (Fig. 4C), but the number of infected CD11b⁺ cells was not sufficient to measure the contribution of EC.

The infection of the CD11b⁺-enriched fraction at earlier time points was too limited to draw conclusions. No evidence of *in vivo* virus production was ever found in CD11b⁺ cells (Fig. 4B, b), at least not during the studied acute phase of MHV-68 infection. However, during this early splenic infection, the infected CD11b⁺ macrophages represented up to 1% of infected splenocytes (Table 2). An early switch to latency without productive infection would explain why Cre-recombined virus progeny derived from F4/80⁺ cells was not found in F4/80-cre^{+/-} mice (Table 1 and Fig. 5A), although macrophages are a major target for latent infection (4, 13).

Taken together, these results indicated that after i.v. infection, 35 to 50% of MHV-68-infected splenic B cells, DC, and macrophages received the virus from EC. Also, 12 days after i.n. infection, EC represented an important source for infection (31% of infected B cells). This viral transmission from EC to B cells is especially important, since it probably contributes significantly to the EC-derived virus population in the total viral load found in the spleen, especially at later time points. Indeed, since our color-tracing system identifies only the origin of the converted viruses and not the direct producer cell type at a given time point, the amount of viruses with recombined genome in an organ might be heavily influenced by virus spread between Cre-expressing cells and other highly productive cells. B cell infection with EC-derived virus could also explain the very slight (no more than 5%) contribution of EC to the infection of lymph nodes that appears late (at day 13) after i.n. infection. This, however, could also be the consequence of recirculation of infected B cells between spleen and lymph nodes (54).

Virus transmission from EC and B cells to peritoneal monocytes-macrophages. Latent infection of splenic macrophages is

FIG 4 Spread from EC-derived virus to B cells, DC, and monocytes-macrophages in the spleen after i.v. and i.n. infection. (A) Tek-cre^{+/-} mice were infected with 2×10^6 PFU of floxed-MHV-68-color-switch- Δ i.v., and splenic B cells (CD19⁺) were sorted after different time points p.i. An infectious center assay was performed (a and b), and a small part of the spleen was kept for titration (c and d). The 2 experiments (a and b) were performed independently. The results of the infectious center assay (i.e., the plaque assay with coculture of living splenocytes instead of organ homogenate) are presented in the same format as that described for Fig. 3. The total fraction represents every splenocyte without separation. (B) Tie2-cre^{+/-} mice were infected with 2×10^6 PFU of floxed-MHV-68-color-switch- Δ i.v., and splenic B cells, DC, and monocytes-macrophages (CD19⁺-enriched, CD19⁻ CD11c⁺-enriched, and CD19⁻ CD11c⁻ CD11b⁺-enriched fractions, respectively) were sorted after 3 and 7 days p.i. An infectious center assay was performed with each fraction (a), and the same assay was also performed after disruption of the cells in each fraction (b). In this experiment, 6 and 5 mice were used, respectively. Splenocytes from 2 mice were pooled before performing the cell separation ($\square$), except at 7 days p.i., when one mouse was used ($\circ$). (C) Tek-cre^{+/-} and Tie2-cre^{+/-} mice were infected with 2×10^6 PFU of floxed-MHV-68-color-switch- Δ i.n., and splenic B cells, DC, and monocytes-macrophages were sorted after 6 and 12 days p.i. as described for panel B. An infectious center assay was performed with each fraction (total, CD19⁺ enriched, CD19⁻ CD11c⁺ enriched, and CD19⁻ CD11c⁻ CD11b⁺ enriched). Six mice were used for each time point, and splenocytes from 2 mice were pooled before performing the cell separation. Tek-cre mice^{+/-} and Tie2-cre^{+/-} mice were used for the analysis 6 and 12 days p.i., respectively.

marginal, whereas this cell type is considered to represent a major cell reservoir for MHV-68 latency in the peritoneum (13). Accordingly, a minimal contribution of F4/80⁺ macrophages to MHV-68 production was found during the productive phase of infection in spleen, lymph nodes, and lungs of F4/80-cre^{+/-} mice (Table 1). Cell separation studies were performed with peritoneal exudate cells (PECs) to determine whether peritoneal macrophages become infected during primary infection and to measure the EC- and B cell-derived progeny with respect to dissemination to PEC (Fig. 5). Infectious center assays performed with CD11b⁺ cells isolated from PEC of i.v.- and i.n.-infected mice confirmed a major role for macrophages (Fig. 5B and C). Apparently, there is little or no virus production in the macrophage fraction during acute infection, although latent recombined virus can be very efficiently reactivated from F4/80-cre^{+/-} mice (Fig. 5A). Notably,

all virus-harboring cells among PEC are CD11b⁺ (Fig. 5B and C). Thus, there is either only a limited activity of Cre expression in F4/80⁺ mice that does not always suffice for recombination, or the F4/80 marker expressed on resident macrophages does not describe the whole MHV-68-susceptible population among CD11b⁺ cells.

The contribution of vascular EC (Tek-cre mice) and B cells (CD19-cre mice) to PEC infection during MHV-68 dissemination was studied by infectious center assays using total PEC or either CD11b⁺-enriched or -depleted PEC, respectively (Fig. 5B and C). After systemic (i.v.) infection, 35 to 40% of infected PEC or CD11b⁺ peritoneal macrophages were infected via EC, whereas less than 10% of them were infected via B cells (Fig. 5B, a, and C, a). In stark contrast, local (i.n.) infection led to the opposite result. Under these conditions, 60% of PEC or CD11b⁺ peritoneal mac-

TABLE 2 Evaluation of the total number of infected splenocytes from different cell types^a

Route of and no. of days after infection	Infected CD19 ⁺ (B220 ⁺) splenocytes (B cells)			Infected CD19 ⁺ CD11c ⁺ splenocytes (mostly DC)			Infected CD19 ⁺ CD11c ⁺ CD11b ⁺ splenocytes		
	Total no.	% EGFP	Production	Total no.	% EGFP	Production	Total no.	% EGFP	Production
i.v.									
3	3 × 10 ³	43	+	10 ²	46	+	6	34	+/-
7	6 × 10 ³	50	+	3 × 10 ²	36	+/-	6 × 10 ¹	39	+/-
i.n.									
6	7 × 10 ²	9	—	4	<50	—	5	<50	—
12	5 × 10 ³	31	—	2 × 10 ¹	3	—	10 ¹	4	—

^a Based on the experiments described in the legend to Fig. 4B and C, we calculated an estimation of the total number of different infected splenocytes (CD19⁺ [B cells], CD19⁺ CD11c⁺ [mostly DC], and CD19⁺ CD11c⁺ CD11b⁺ [mostly monocytes-macrophages]) based on the total number of splenocytes counted at each time point, the repartition of the different cell types in the total fraction (given by flow cytometry analysis at each time point), and the results of the infectious center assays. Total no., the number of infected cells in the spleen (order of magnitude). % EGFP, the percentage of cells infected with cre-recombined-MHV-68-color-switch-Δ. Production indicates our ability to detect infectious viral particles (PFU) in disrupted cells (virus production): +, virus production clearly above the detection limit; +/-, virus production below the detection limit for most mice; —, indicating no virus production above the detection limit.

rophages received their virus from B cells, whereas only a minority of the PEC virus represented EC-derived virus progeny (Fig. 5B, b, and C, b). In addition, and independently from the route of infection, a significant part of the PEC infection was derived from virus which was not recombined either in EC or B cells (about 40% of infected PEC carried nonrecombined virus after either i.v. or i. n. infection). These results suggested that a significant proportion of infected PEC received the virus, which might come from a third cell source. Interestingly, peritoneal macrophages, although infected during the early phase of infection, did not produce virus.

DISCUSSION

The infection of vascular EC in the context of viral dissemination is considered central, since they provide physical barriers between blood and parenchymal tissues. Consequently, during gamma-herpesvirus infection, EC are thought to play a particular role, and EC functions are modulated during virus infection. Here, we studied the productive infection of EC by MHV-68 *in vivo* and delineated to what extent EC infection contributes to virus dissemination. Since we used a recombinant virus lacking the M1 gene, which was comparable to wt virus in the acute phase of infection, we restricted our studies to the initial phase of MHV-68 infection. We showed that after i.v. infection, vascular EC are efficiently and productively infected by MHV-68 *in vivo* in normal immunocompetent mice. After i.n. infection, vascular EC also contributed significantly to virus production in the spleen of those mice. Moreover, EC seemed to play a major role in dissemination of the virus to splenic B cells, the major target of gammaherpesvirus latency. We also showed that both EC- and B cell-derived virus can load the compartment of latently infected peritoneal cells. The most salient new finding was that different routes of infection (local versus systemic) strongly affected the virus dissemination pathways.

So far, it cannot be avoided that the genetic modification of the viral genome has subtle effects on the fitness of the resulting viruses and/or on the expression of any inserted gene (5, 23, 24, 41). This problem was already observed in previous studies with MHV-68 using a fluorescent protein of the EGFP family (e.g., yellow fluorescent protein) to mark a virus *in vivo* (41). It is probably also due to the immunogenicity of these proteins (55, 56). Indeed, it has been shown that if a gene with a presentable CD8⁺ T cell epitope is expressed by MHV-68 under the control of the

HCMV IE1 promoter, like in our case, it leads to an attenuation in viral persistence (44). We bypassed this problem by strictly limiting our studies to acute infection, a period under which there were no significant differences observed between the recombined and the nonrecombined viruses.

The methodology we used in this study was adapted from the recently published Cre reporter system for MCMV infection (23). The general advantage of this system is the use of, with the exception of Cre expression, normal reporter mice. No additional systemic genetic or therapeutic manipulation of the recipients, which may change the infectious process, is needed in order to reveal virus infection and virus dissemination patterns.

However, the specificity of Cre expression in EC in the transgenic lines used in this study is crucial for the correct interpretation of the data we obtained. It was originally shown that a genomic reporter is converted in Tie-2-cre mice in EC and in most, if not all, hematopoietic cells. This was explained to be the expression of Cre in EC and also in some hematopoietic progenitor cells. It is important to note that Cre expression was not found in differentiated B and T cells (28, 51). We also confirmed this with the help of bone marrow chimeras in our previous study on MCMV dissemination. There, we documented that results from Tie2-Cre^{+/−} mice are not affected by contaminating Cre recombination derived from radiosensitive hematopoietic cells (23). Here, we also observed that in several organs and at time points, efficient recombination occurred in Tek-cre or Tie2-cre mice, while in the same organs and at the same time points no recombination was found in CD19-cre or Mb-1-cre mice (Fig. 3). This excluded the possibility that a significant ectopic Cre expression in B cells was responsible for the recombination rates we observed in Tie2-cre or Tek-cre mice. A similar conclusion is indicated regarding the more radioresistant macrophages by the data we obtained by infection of F4/80-cre and CD11c-cre mice (Table 1). Besides B cells, macrophages and DCs are the major known targets for MHV-68, therefore they represent the strongest threat for the interpretation of our results if some of these cells were supporting viral Cre recombination in Tek-cre or Tie2-cre mice. However, no virus production by macrophages was found in the tested organs of F4/80-cre mice, even if they were well infected and could recombine and reactivate the virus efficiently in F4/80-cre mice (Table 1 and Fig. 4 and 5). These characteristics also argue against

FIG 5 Monocytes-macrophages from peritoneal exudate cells (PEC) were partly infected with EC-derived virus after i.v. infection and with B cell-derived virus after i.n. infection. (A) F4/80-cre^{+/-} mice (a and b) were infected with 2×10^6 PFU of floxed-MHV-68-color-switch- Δ intravenously (i.v.), and PEC of 4 mice were analyzed 7 days p.i. by infectious center assay with living cells (a) or disrupted cells (b). (B and C) Tek-cre^{+/-} (B, graphs a and b) and CD19-cre^{+/-} (C, graphs a and b) mice were infected with 2×10^6 PFU of floxed-MHV-68-color-switch- Δ i.v. (B, graph a, and C, graph a) or i.n. (B, graph b, and C, graph b), and PEC of 4 mice were extracted after 7 (B, graph a, and C, graph a) or 14 (B, graph b, and C, graph b) days p.i., respectively. Then CD11b⁺ cells were separated by positive selection. An infectious center assay was performed with each fraction (B and C) as described in the legend to Fig. 3.

Cre recombination coming from macrophages in the viral production observed in Tek-cre and Tie2-cre mice. These results alone cannot exclude some contaminating activity coming from another unidentified, infected cell type that would express Cre in both Tek-cre and Tie2-cre mice; however, coupled with the results from Suarez et al. (15) showing productive infection of EC with MHV-68 *in vitro* and *ex vivo*, we were confident that EC are indeed productively infected with MHV-68 *in vivo*, and they contribute to virus dissemination.

Here, we also analyzed two independent strains of EC-specific Cre transgenic mice, which were characterized earlier in the context of infection with a reporter. These data showed that recombination of reporter viruses in Tie2-cre^{+/-} and Tek-cre^{+/-} mice resulted in comparable conversion rates. Reliability in the degree

and cell type specificity of Cre expression is crucial for quantitative data evaluation. The degree of Cre expression in these strains sufficed to cause recombined infection with MCMV in up to 100% of organs. Therefore, high or low conversion rates must reflect the nature of the infectious agent rather than a technical limitation of the system. However, knowledge on the strength of recombinase expression in reporter mice is mandatory for the interpretation of results (21). For most Cre-expressing mouse strains used in this study, we had no second strain to use as a control. The Alb-cre strain identified the hepatocytes as the dominant target for MCMV (23). That the hepatocyte represents a negligible target for MHV-68 is justified only based on the MCMV findings. For F4/80-cre^{+/-} or SM22-cre^{+/-} mice, the conversion rates were low, and there was no separate mouse strain and no other marker virus to confirm the quantitative aspects of the results. In CD11c-cre mice, on the other hand, the conversion rates were higher than expected, especially compared to the results from Gaspar et al. (24). Nevertheless, like in their study, very little contribution from DC was found for the very early infection of the respiratory tract after i.n. infection, and significantly more was found in lymphoid organs. However, the known Cre activity beyond the DC subset in these mice, a finding already observed during MCMV infection (57 and data not shown), coupled with a possible lack of efficiency in Cre recombination (24), makes it very difficult to draw definite conclusions, and that is why we did not make extensive use of that mouse strain in this study. Like Gaspar et al., we feel that dendritic cells contribute significantly to MHV-68 infection and dissemination. However, the relative importance of this cell type is difficult to evaluate.

While the low viral conversion rates observed in F4/80-cre^{+/-} mice could not be confirmed with a separate mouse strain to prove the quantitative aspects of the results, the results we obtained by infectious center assays after separation of macrophages mostly tend to confirm their pertinence. However, a slight contradiction appears in the results obtained from the peritoneal cavity with both methods. The 50% Cre recombination in infected PEC from F4/80-cre mice versus the 100% CD11b expression in infected PEC (Fig. 5) could be due to some deficiency in recombinase expression in this strain, as already explained. On the other hand, it is possible that in the CD11b⁺ PEC cell population there is an MHV-68-susceptible subset which does not express F4/80. B-1 cells, which express both CD11b and CD19 and represent about 80% of B cells in the peritoneal cavity, is a candidate for this possibility (58, 59). However, after i.v. infection, less than 10% of recombined virus was found in PEC in CD19-cre mice, indicating that less than 10% of infected PEC could be B-1 cells. Therefore, B-1 cells cannot be solely responsible for the difference of 50% described above, and they also cannot represent the major reservoir of infection in the peritoneal cavity after i.v. infection. Even after i.n. infection, they could not represent more than 60% of infected PEC at most if we considered that every PEC infected with recombined virus after i.n. infection was a B-1 cell, which is very unlikely. Also, the data obtained with PEC, while confirming the importance of the route of infection for dissemination leading to the peritoneal compartment, also underline that B cells are not essential for latency establishment in peritoneal macrophages (14).

In our study, we compared virus production by EC to virus production by B cells. One of the most striking findings was that the infection kinetics of EC and B cells are different. Splenic EC

infection is prominent during early time points after systemic infection and wanes over time (Fig. 3A, a). On the other hand, productive B cell infection is low at times when EC infection is highly productive, nevertheless the relative contribution of B cells to virus production in the spleen increases over time. Our experiments indicated that about 50% of productively infected B cells received their virus from EC. We concluded from these data that EC, if preferentially targeted by systemic infection, do disseminate their progeny to B cells. After i.n. infection, the contribution of EC to splenic virus production and to splenic B cell infection was also seen at later time points, but this was minimal at early time points after i.n. infection. In contrast, the kinetics of virus productivity by B cells was comparable between both infection routes. We therefore concluded that the increasing contribution to virus productivity by B cells is a general feature of MHV-68 infection and is independent from the infection route. It was recently shown that the efficiency of B cell infection *in vitro* was improved when MHV-68 was produced by myeloid cells, while virus produced by epithelial cells or fibroblasts poorly infected B cells (60). Our results suggest that virus produced by EC also infect B cells efficiently.

Infected EC were found in acutely infected lungs of immune-deficient mice receiving virus via the intranasal route (15). In immunocompetent mice, we could not detect any virus production derived from EC in lungs. Thus, immunological fitness defines whether or not a specific cell type gains a particular function in the infectious process. In fact, this was also shown for smooth-muscle cells in gamma interferon receptor knockout mice (16).

Imaging techniques provide a new dimension to resolve details of the infection process. Imaging of bioluminescent markers expressed by MHV-68 and other herpesviruses allows real-time whole-body monitoring of temporal and spatial distribution kinetics of the virus in organ sites (5, 22, 61). However, quantitative aspects can only be assumed because there is no clear connection between the degree of marker gene expression and virus production and release. Cell types involved in virus dissemination by productive infection therefore are not resolved by this approach. Conditional marker gene expression by the viral genome, as presented in this study, also provides the chance to describe cell type-specific events in quantitative terms.

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