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Review

Imaging cytomegalovirus infection and ensuing immune responses

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Cytomegaloviruses (CMVs) possess exquisite mechanisms enabling colonization, replication, and release allowing spread to new hosts. Moreover, they developed ways to escape the control of the host immune responses and hide latently within the host cells. Here, we outline studies that visualized individual CMV-infected cells using reporter viruses. These investigations provided crucial insights into all steps of CMV infection and mechanisms the host's immune response struggles to control it. Uncovering complex viral and cellular interactions and underlying molecular as well as immunological mechanisms are a prerequisite for the development of novel therapeutic interventions for successful treatment of CMV-related pathologies in neonates and transplant patients.

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Introduction

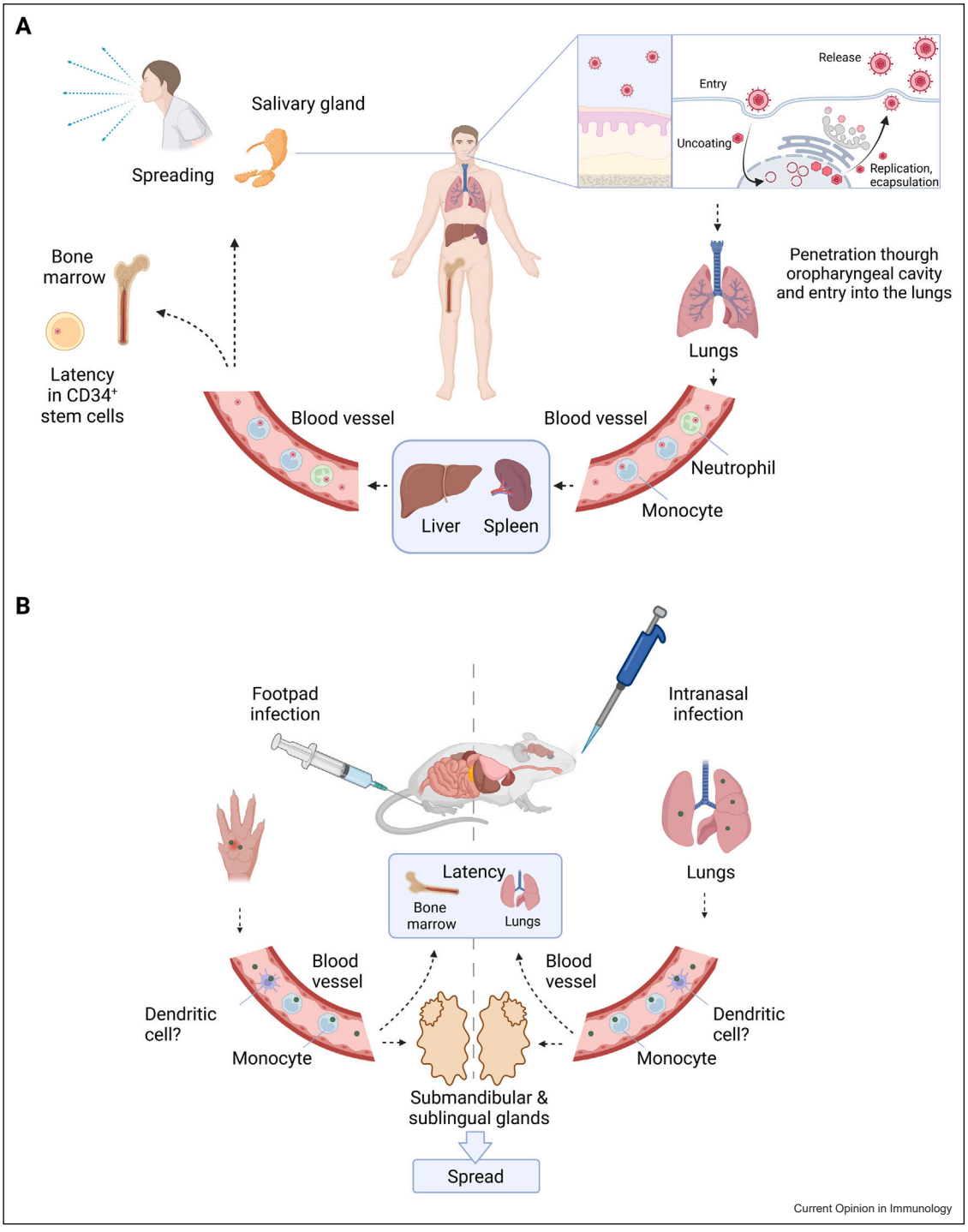
The cytomegaloviruses (CMVs), a genus of members of a beta herpesvirus family, adapted perfectly to their mammalian hosts during millions of years of coevolution. Human cytomegalovirus (HCMV; officially Human betaherpesvirus 5) became a ubiquitous human pathogen with seroprevalence ranging from 56% to 94% [1]. The virus developed multiple immune-modulatory strategies to escape mechanisms the immune system employs to fight HCMV infection [2–4]. Upon penetration into the organism predominantly through mucosal surfaces, HCMV manages to disseminate, infect various organs, and eventually establish a latency, hiding from the virus-specific T cells and antibodies (Figure 1a). The virus thus remains within the organism for the host's lifetime, requiring constant immunosurveillance to remain in the latent state [2]. HCMV is, therefore, a major cause of disease in immunosuppressed adults and vulnerable groups such as low birth-weight infants and congenitally infected unborn fetuses [5].

Understanding the mechanisms by which the immune system successfully controls, or fails to clear, a given viral pathogen benefits greatly from the visualization of infection location. The use of tailor-made, reporter CMV mutants enabled numerous new insights into mechanisms of viral penetration into the cells, immune system-mediated protection, viral immune evasion, and latency. Especially potent proved to be *in vivo* models using reporter mutants of murine cytomegaloviru (MCMV; Figure 1b), a natural mouse pathogen that serves as a reliable model for HCMV disease [6,7]. As discussed in this review, the combination of reporter CMV strains, various genetically modified mice, and advanced microscopy techniques, such as two-photon microscopy, proved to be essential for the understanding of all stages of CMV infection and protective immune response at the single-cell level.

Recombinant cytomegaloviruses that enable imaging

The use of originally passaged viruses provided snapshots of viral spread and underlying immune responses using methods such as *in situ* RNA hybridization, immunohistochemistry, or immunofluorescent microscopy. In combination with various immunocompromised mouse models, such assays contributed to our understanding of virus dissemination. To better understand the dynamic processes by which immune cells control CMV disease and the virus escapes them at the single cell and organ level *in vivo*, different MCMV and HCMV reporter viruses encoding fluorescent proteins

Figure 1



Schematic representation of the CMV life-cycle. Overview of **(a)** HCMV and **(b)** MCMV infection, replication, dissemination, spread to the new host, and establishment of latency. Created with BioRender.com.

were generated to facilitate imaging (Table 1 and Table 2). Additional expression of the Gaussia luciferase enables bioluminescent imaging of viral infection *in vivo*, semiquantitative estimation of virus amounts *ex vivo*, and ultrasensitive and fast infectious virus progeny detection upon reactivation *in vitro* [8–11]. To investigate T cell responses, some reporter viruses were equipped with peptides (e.g. SIINFEKL from ovalbumine (257-264) or

Table 1

Examples of reporter MCMV suited for imaging experiments.

Reporter virus designation	Fluorescent protein	Additional modifications	Application examples
Δm157-MCMV	GFP	Deletion of viral m157	Allows tracking of MCMV infection without control of NK cells in C57Bl/6 mice [13]
MCMV-Δm157-flox-egfp	GFP	Deletion of viral m157, truncated MCK2	In combination with Cre expressing mice enables identification of infected cell types and tracking of virus dissemination [47]
MCMV-Δm128-flox	GFP	Deletion of viral m128, truncated MCK2	In combination with Cre expressing mice enables identification of infected cell types and tracking of virus dissemination and the role of NK cells [47,68]
MCMV-Δm157-flox-egfp	GFP	Deletion of viral m157	In combination with Cre expressing mice enables identification of infected cell types and tracking of virus dissemination [49]
MCMV-IE1/3gfp	GFP	-	Subcellular localization and function of immediate early genes [69]
GLuc-MCMV	mCherry, Gaussia luciferase	-	*In vivo* and *ex vivo* imaging [8]
MCMV-2D	mCherry, Gaussia luciferase	deletion of viral m157, truncated MCK2	susceptibility of C57Bl/6 mice [9]
MCMV-3D	mCherry, Gaussia luciferase	deletion of viral m157, truncated MCK2, SIINFEKL	susceptibility of C57Bl/6 mice, role of specific T cells [9]
MCMV-3DΔ	mCherry, Gaussia luciferase	deletion of viral m157, truncated MCK2, SIINFEKL, deletion of m06 and m152	susceptibility of C57Bl/6 mice, role of specific T cells in context of missing MHCI down modulation [9]
MCMV-MIEP^{r}	YFP, tdTomato	-	Discrimination of the function of ie1 and ie2 [23]
MCMV-3DR	mCherry, Gaussia luciferase	deletion of viral m157, functional MCK2	susceptibility of C57Bl/6 mice, role of specific T cells, role of MCK2 for infectivity [10]
MCMV-GR	GFP, tdTomato	Deletion of viral m157	Identification of infected cell types in combination with Cre expressing mice; susceptibility of C57Bl/6 mice [29]
MCMV$^{IE2SL-MIEP}$	YFP, tdTomato	SSIEFARL peptide	Discrimination of the function of ie1 and ie2, role of specific T cells [12]
MCMV-3D-Ca	mCherry, GFP	Expression of Ca^{2+} sensor GCaMP6s, Deletion of viral m157, deletion of viral m157, truncated MCK2, SIINFEKL	Visualization of calcium fluxes during killing process, susceptibility of C57Bl/6 mice, role of specific T cells [39]
MCMV-3D-ΔvRAP-Ca	mCherry, GFP	Ultra-sensitive Ca2+ sensor GCaMP6s, Deletion of viral m157, deletion of viral m157, truncated MCK2, SIINFEKL, deletion of m06 and m152	Visualization of calcium fluxes during killing process, susceptibility of C57Bl/6 mice, role of specific T cells in context of missing MHCI down modulation [39]
MCMV-GFP-ie2ova	GFP	SIINFEKL	role of specific T cells [11]
MCMV-GFP-ie2ova.ΔM36	GFP	SIINFEKL, deletion of m36	role of specific T cells, impact of M36 in apoptosis [11]
MCMV-3D.ΔM36	mCherry Gaussia luciferase	deletion of viral m157, truncated MCK2, SIINFEKL, deletion of viral m36	susceptibility of C57Bl/6 mice, role of specific T cells, impact of M36 in apoptosis [11]
MCMV-3D.ΔvRAP.ΔM36	mCherry Gaussia luciferase	deletion of viral m157, truncated MCK2, SIINFEKL, deletion of m06, m152, m36	susceptibility of C57Bl/6 mice, role of specific T cells in context of missing MHCI down modulation, impact of M36 in apoptosis [11]
MCMV^{r}.ΔM36	YFP, tdTomato	Deletion of m36	Discrimination of the function of ie1 and ie2, impact of M36 in apoptosis [11]
rMCMV448	GFP	Deletion of viral m128	Influence of e1-promotor on pathogenicity [22]
rMCMV1373	GFP	Deletion of viral m128	Influence of e1-promotor on pathogenicity [22]
MCMV-GFP	GFP	GFP expression under m36 promotor, repaired MCK2	Studying early-late infection [26]
MCMV-IE3–142	GFP	contains four repeated targeting sites for the microRNA miR-142–3p in the 3' untranslated region of the essential viral gene IE3	Deficient replication in hematopoietic cells [50]
MCMV-IE3–015	GFP	-	control virus without miR-target sites [50]

Abbreviations: YFP, yellow fluorescent protein; MCK2, MCMV-encoded chemokine 2; MHCI, major histocompatibility complex I; NK cell, natural killer cell; SIINFEKL, peptide from chicken ovalbumine (257-264); SSIEFARL, peptide from Herpes symplex 1 glycoprotein B (498-505).

SSIEFARL from Herpes symplex 1 glycoprotein B (498-505)) recognized by T cells expressing a transgenic T cell receptor [9,11,12]. Finally, simultaneous deletion or insertion of additional viral genes facilitates the understanding of their function. For example, m157 or m129 were shown to be important for MCMV infectivity

Table 2

Examples of reporter HCMV used for visualization of infected cells.

Reporter virus designation	HCMV strain	Fluorescent protein(s)	Application examples
HCMV TB40 UL32-EGFP	TB40	EGFP	Tracking of a viral particle release [20], virus transfer from endothelial cells to monocytes [43], virus reactivation from latency [62], or screening for genes that promote HCMV entry [17]
TB40/E-IE2-EYFP virus	TB40	EYFP	Tracking of virus reactivation from latency [62]
HCMV TB40E-GATA2-mCherry	TB40	mCherry	Allows detection of latently infected cells based on mCherry expression [63]
HCMV TB40E-GFP	TB40	GFP under the control of an SV40 origin/promoter	Used for genome-wide CRISPR/Cas9 screen to detect cellular receptors [15], to determine dynamics of viral gene expression in lytic and latent infection [24,61], or to investigate T cell-mediated killing of latently infected monocytes upon virus reactivation [64]
HCMV-TB40-pp150-EGFP-gM-mCherry	TB40	EGFP and mCherry	Tracking of a viral particle release [24]
HCMV^{3F}	TB40	mNeonGreen, GFP, and mCherry	Monitoring of viral gene expression from different promotors and determination of antiherpesviral drug mechanisms [25]
NR-1 GFP	NR-1	GFP	Mechanisms of latency in hematopoietic progenitor cells [60]

Abbreviations: EGFP, enhanced green fluorescent protein; EYFP, enhanced yellow fluorescent protein.

[10,13], M36 for prevention of apoptosis [11], m06 and m152 for immune evasion from cytotoxic T cells [9], among others (Table 1).

Lessons from imaging of primary infected cells

The easy distinction of infected and uninfected cells using reporter CMVs (Figure 2a) enabled genetic screen experiments that extended the list of cellular molecules the virus uses to enter the cells. Recently, an olfactory receptor OR14I1 and the immunoglobulin family member CD147 were described to contribute to HCMV entry, besides platelet-derived growth factor receptor alpha, Neuropilin 2, and complement regulatory protein CD46 [14–17]. For MCMV, our data indicate that the major histocompatibility complex (MHC) class I molecules and CX3CR1 facilitate MCMV entry into macrophages [18]. On the other hand, the neuropilin 1 is a key molecule for the infection of fibroblasts and endothelial cells with this virus [19].

Within the infected cells, intracellular movements of viral particles and their egress pathways can be visualized by tracking fluorescently tagged viral proteins in combination with advanced imaging techniques [20,21]. Alternatively, cell-specific viral gene expression dynamics can be easily monitored by inserting fluorescent proteins under the control of different viral promotors [22–24]. These reporter viruses also allow investigating antiviral mechanisms of divergent molecules, such as interferon-gamma (IFN-γ) [8] and antiherpesviral drugs [25].

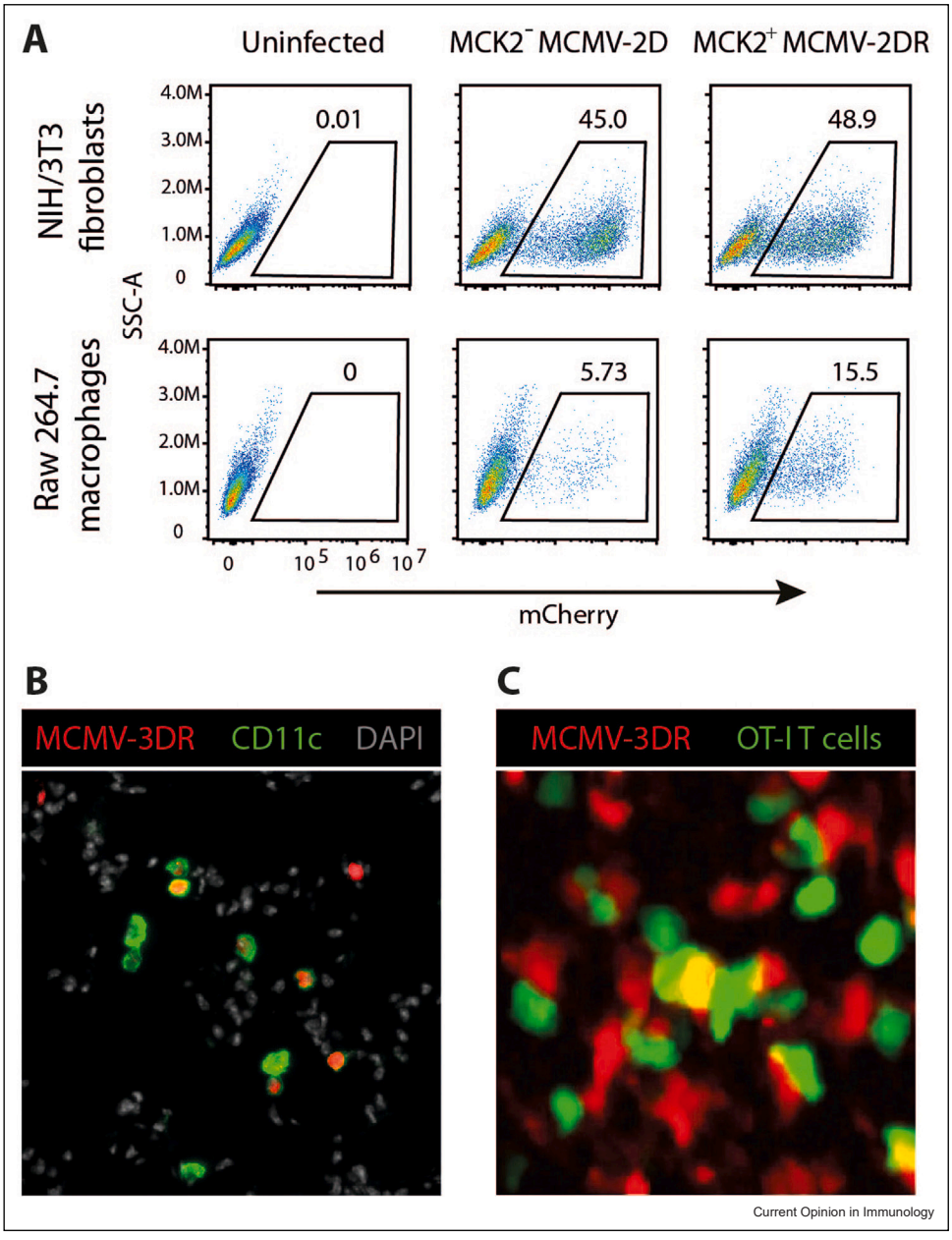
Furthermore, green fluorescent (GFP)-expressing MCMV enabled the detection of subversion mechanisms the virus uses to morphologically, metabolically, and immunophenotypically reprogram macrophages [26]. In infected cells, expression of classical macrophage markers such as CD64, F4/80, and CD11b and the antigen-presenting capacity are downregulated. At the same time, infected macrophages gain stem cell-like features and increase their motility. MCMV-induced macrophage reprogramming also affected their function *in vivo*. Alveolar macrophages promoted intercellular viral spread within the lungs and had impaired anti-inflammatory effects to secondary infection [26].

The location and type of infected cells depend on the route of MCMV inoculation. Intranasal MCMV infection primarily targets alveolar macrophages and type 2 alveolar epithelial cells, thus corresponding to the data obtained from CMV-infected human lungs (reviewed in [27]). In contrast, footpad inoculation, mimicking transmission by bites, leads to infection of CD169^{+} macrophages and fibroblasts in the subcapsular sinus of the draining popliteal lymph node [9,28]. Bioluminiscence imaging of MCMV strains encoding for Gaussia luciferase was crucial to establish that the respiratory but not the oral mucosa is a direct target for viral infection in neonatal mice [29,30]. In addition, fluorescently labeled rhesus cytomegalovirus enabled visualization of congenital infection in rhesus macaque [31]. Altogether, visualization of the primary infected cells *in vivo* is the cornerstone for understanding the mechanisms CMVs use to establish a foothold in the organism.

Insights in local immune response using imaging

Infection of first cells at the primary site of virus entry activates the immune system and immune cells swarm the site of infection. The detailed analysis of the ensuing immune response was strongly facilitated by the use of mouse models and depend on the route of virus inoculation. Here, we focus on the immune responses

Figure 2



Examples of the use of fluorescently labeled viruses. **(a)** Representative dot plots of 3T3/NIH fibroblasts and Raw 264.7 macrophages analyzed with flow cytometry without infection or at 17–24 h post infection (p.i.) with an mCherry expressing MCMV strains MCMV-2D (MCMV-encoded chemokine 2 (MCK2)-deficient) compared MCMV-2DR (MCK2-proficient). **(b)** Representative photomicrograph of a wild-type B6 mouse lung section stained with anti-CD11c antibody to label alveolar macrophages at 1 dpi with mCherry expressing MCK2-proficeint MCMV-3DR. **(c)** An example of two-photon image of mCherry-expressing MCMV-3DR infected cells surrounded with GFP+ OT-I CD8+ T cells recognizing the SIINFEKL peptide expressed by the virus.

induced by intranasal infection in adult mice or the laryngopharyngeal infection in neonatal mice, models that mimic the natural infection route [30,32–34]. The combination of fluorescent MCMV reporter viruses with immunofluorescence microscopy on tissue sections allows a better understanding of the location of infiltrating cells and their interaction with infected cells (Figure 2b).

During the first 3–8 days post infection (dpi), infiltrating immune cells form nodular inflammatory foci (NIF) surrounding infected cells. NIFs consist of different subsets of myeloid cells, such as macrophages or dendritic cells (DCs), and lymphocytes, including natural killer (NK) cells, CD8, or CD4 T cells [30,35]. Interestingly, B cells are only present at low numbers in NIF.

These NIF are crucial for eradication of infected cells, control of virus spread within the organ, and limiting of virus dissemination to other organs. *In vitro*, IFN-γ limits the cell-to-cell spread [30]. Hence, it is likely potential IFN-γ producers NK cells and macrophages present in NIF control virus spread by secreting IFN-γ [35]. In contrast to bronchus-associated lymphoid tissues (reviewed in [36]), NIF are nonstructured, dense infiltrations that dissolve once infected cells are killed.

In addition, virus-specific activated T cells can efficiently kill infected cells. T cells are present in NIF around 3 dpi. It is rather unlikely that, within this short period of time, T cells get primed in the draining lymph node and subsequently migrate to the site of infection. It is therefore likely that T cells get primed directly in the infected organ. Indeed, *ex vivo* imaging of lungs revealed long lasting contacts between CD8 T cells and $CD11b^+$ cells in NIF, identifying these structures a potential sites for CD8 T cell priming [30]. Efficient T cell priming is important to establish an inflationary $Tcf1^+$ $KLRG1^-$ CD8 T cell pool that controls virus reactivation in peripheral organs [37,38]. The additional presence of CD4 T cells improves the efficiency of CD8 T cells [35]. Whether CD4 T cells are only involved in CD8 T cell priming or also contribute to the control of virus spread by secreting IFN-γ, warrants investigation. Virus-specific activated CD8 T cells can kill infected cells in a contact-dependent manner *via* perforin secretion. Multiphoton imaging in lymph nodes revealed that the killing capacity of cytotoxic T cells *in vivo* differs considerably from results obtained by *in vitro* experiments [39] (Figure 2c). MCMV expresses immune-evading genes such as m06 and m152 encoding for viral regulators of antigen presentation (vRAP) [40]. The expression of these genes down-modulates MHC-I expression on the surface of infected cells and therefore interferes with antigen recognition by CD8 T cells. In the presence of vRAP the *in vivo* killing capacity of CD8 T cells is unexpectedly low, indicating that CD8 T cells mainly control MCMV infection in a contact-dependent manner. In the absence of vRAP, MHC-I molecules stay present on the surface of infected cells. Thus, the use of viruses deficient for vRAP genes allows to study the full capacity of T cell immunity and in combination with vRAP proficient viruses the impact of the immune evasion on virus control [9]. Results from these experiments revealed cytotoxic CD8 T cells rather form motile kinapses than static synapsis with virus-infected cells during the killing process [39]. Furthermore, it could be shown that the killing of infected cells becomes efficient if these cells get contacted by more than two CD8 T cells. Together, these studies revealed that a rather high amount of activated CD8 T cells is required to efficiently clear MCMV infection in lymph nodes and lungs [35,39].

Tracking of the virus dissemination

A long-standing Fenner's hypothesis based on mousepox viruses postulates that viruses initially disseminate *via* lymph to the draining lymph nodes. From there, the virus uses afferent lymphatics draining into the bloodstream to spread through the host body and infect various organs [41]. This hypothesis is believed to be also true for HCMV dissemination, with the difference that preferentially HCMV disseminates hidden in immune cells such as neutrophils and monocytes (reviewed in [42]; Figure 1a). *In vitro* experiments using reporter HCMV strains helped to establish the model that those leukocytes can be infected by passing through the layer of infected endothelial cells [43].

The analysis of HCMV dissemination is restricted to *in vitro* experiments and *ex vivo* sample analyses. Hence, CMV reporter viruses infecting animals were instrumental to study virus dissemination *in vivo*. A powerful combination represents the use of reporter MCMVs with genetically engineered mouse strains. These models indicated that intranasally or footpad-administered MCMV disseminates between days 5 and 7 directly to salivary glands, without the need to initially infect blood-filtering organs [10,29,30,35,44–46] (Figure 1b). Insertion of a Cre-inducible expression cassette into the m157 gene of MCMV permitted cell-type-specific labeling of virus progeny *in vivo* [47–49]. These MCMV strains established that hepatocytes do not spread the virus to other organs [47,49]. In contrast, endothelial-derived virus disseminated to various tissues, including spleen and lung [47,48]. Recently, an innovative recombinant MCMV reporter confirmed exclusive cell-mediated viral dissemination after local infection and highlighted the importance of cell-mediated immunity in its restriction [50]. In contrast, after systemic administration in immunocompetent mice or local administration in immunocompromised or neonatal animals, the virus efficiently spread to multiple organs, most likely *via* virion transport in the blood [10,30,35].

Which mononuclear cell type disseminates the virus after local infection is still debated. The literature offers evidence that MCMV travels within $CX3CR1^+$ patrolling monocytes [45], DCs [44,51], myeloid progenitor cells [52], or alveolar macrophages [26]. Unfortunately, currently available reporter viruses are not optimized to address this issue, as the majority of CMV genes are repressed in monocytes and myeloid progenitor cells. Nevertheless, recent data indicate that the expression of HCMV genes in monocytes is not completely switched-off [24,53]. Therefore, it seems possible to develop MCMV strains able to express fluorescent markers under

the control of genes expressed in mononuclear cells. Such reporters could help to characterize the rare cells transporting viruses within the bloodstream.

Besides the determination of cellular factors, reporter viruses enable also the evaluation of viral genes important for CMV dissemination. In the context of MCMV, regulation of MHC class I and NKG2D ligand cell surface levels by m04, m06, and m152 gene products is crucial for the escape of cells transporting the virus from NK cell-mediated depletion [51]. Another example is MCMV-encoded chemokine 2, which is crucial for the virus spread to the salivary glands [10,18,30,49,52–54].

How imaging contributed to the understanding of cytomegalovirus latency

Despite systemic virus spread, CMV infection in immunocompetent hosts is usually subclinical and the immune response efficiently eradicates lytically infected cells. However, CMV establishes latency in some cells, including $CD34^+$ hematopoietic progenitor cells and $CD14^+$ monocytes. In those cells, CMV exploits the cellular machinery to quiescent its gene expression and avoids the production of new viral particles. HCMV latency seems to involve a combination of viral G protein-coupled receptors, in particular the HCMV gene *US28*, cellular heterochromatin protein 1, transcription factors nuclear factor kappa B and Yin Yang 1 to suppress viral gene expression from the major immediate-early promoter/enhancer region (MIEP; reviewed in [55,56]). Hidden episomally in the nucleus, HCMV remains lifelong in the host, protected from detection and clearance by the immune system. However, the virus occasionally reactivates, thus requiring constant immune control. This makes HCMV especially dangerous for immunosuppressed or immunocompromised individuals, who often contract severe diseases after virus reactivation (reviewed in [57]).

Expression of only a few viral genes at a low level prevents the use of most reporter CMV strains for visualization of latently infected cells. Nevertheless, *in vitro* infection of monocytes using reporter HCMV strains lead to GFP expression that was detectable for 3–4 days, allowing comparison of gene expression programs in lytically and latently infected cells [24]. Together with RNA sequencing [58,59], these results established that latency is regulated by the unique repression of immediate-early genes rather than through the expression of a special, latency-specific viral gene set. Interestingly, it seems that relatively high expression of interferon-stimulated genes per cell is crucial for viral entry into the latency [24]. Moreover, latently infected monocytes seem to acquire an anergic-like state [60], characterized by lower expression of CD74 and response to IFN-γ [61]. Despite expressing viral genes at a low level, latently infected monocytes are still susceptible to NK cell [62] or neutrophil killing *via* antibody-mediated antigen-dependent cell cytotoxicity [63].

While visualization of latency is inefficient, reporter viruses represent an excellent tool to monitor elegantly CMV reactivation. In human monocytes, HCMV reactivation can be induced by their differentiation into DCs or macrophages as well as by coculturing with activated CD4 T cells [62]. This *in vitro* model of HCMV reactivation can also be used for testing drugs that could activate viral gene expression and predispose the latently infected cells to deletion by the cellular immune response, such as HDAC inhibitors [64]. In precision-cut lung slices prepared from the lungs of mice latently infected with MCMV, a combination of Gaussia luciferase measurements and mCherry fluorescence enabled the detection of reactivation events at the single-cell level [9]. Interestingly, these data indicated that $CD11b^+$ myeloid cells are not the main cellular sites of MCMV latency and reactivation within mouse lungs [9]. These data are aligned with multiple reports indicating that different types of endothelial cells are the sites of MCMV latency (reviewed in [65]). Whether endothelial cells represent a significant reservoir of latently HCMV-infected cells needs further investigation.

Potential pitfalls of recombinant cytomegaloviruses

Generation of recombinant herpesviruses encoding for fluorescent proteins requires the selection of single viral clones originating from CMV laboratory strains. However, such multipassaged CMV laboratory strains do not represent the full biological breadth of clinically isolated CMVs [66,67]. Hence, interpreting the results of studies conducted with those viruses requires special attention, as they do not mimic all biological properties of CMV clinical isolates. Additionally, fluorescent proteins might affect viral replication efficiency or alter the immunogenic properties of the virus by providing novel antigens to adaptive immune responses.

Conclusions

The integration of genes encoding fluorescent proteins is an excellent strategy to trace molecular and cellular processes crucial for divergent phases of the CMV's life cycle and ensuing immune responses. The combination of reporter CMVs with advanced imaging and single-cell sequencing methods allowed visualization of lytic and latent viral infections *in vitro* and in animal models. These results were crucial for understanding the complex interplay of CMV and the immune system. At the same time, some results revealed a need to redefine some of the current reporter viruses. For example, integration of the fluorescent reporter genes under the control of the US28 promotor might help to visualize

latently infected cells. Such experiments crucially contribute for the successful development of novel treatment options of CMV-induced pathologies.

Conflict of interest statement

None.

Data Availability

No data were used for the research described in the article.

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- •• of outstanding interest

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