Murine cytomegalovirus displays selective infection of cells within hours after systemic administration

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A distinctive feature of the cytomegaloviruses is their wide tissue tropism, demonstrated by the infection of many organs and cell types in an active infection. However, in experimental models of systemic infection, the earliest stages of infection are not well characterized, and it is unclear whether only certain cells are initially infected. Using a recombinant murine cytomegalovirus (MCMV) expressing green fluorescent protein (GFP), we tracked viral infection after systemic administration via intraperitoneal injection and showed that specific cells are infected within the first hours. We provide evidence that MCMV traffics as free virus from the peritoneal cavity into the mediastinal lymphatics, providing access to the bloodstream. We demonstrate that MCMV productively infected CD169+ subcapsular sinus macrophages in the mediastinal lymph nodes, ER-TR7+ CD29+ reticular fibroblasts in the spleen and hepatocytes. Infection in the spleen followed a distinctive pattern, beginning in the marginal zone at 6 h and spreading into the red pulp by 17 h. By 48 h after infection, there was widespread infection in the spleen and liver with degeneration of infected cells. In addition, infected dendritic cells appeared in the white pulp of the spleen at 48 h post-infection. On the other hand, cowpox virus showed a different pattern of infectivity in the spleen and liver. Thus, early MCMV infection produces a distinct pattern of infection of selective cells.

INTRODUCTION

Human cytomegalovirus (HCMV) is a betaherpesvirus that causes clinically significant disease mostly in immunocompromised patients. Infection can affect nearly every organ, resulting in hepatitis, pneumonitis, retinitis and colitis, among others (Mocarski et al., 2007; Sinzger et al., 1995). Inasmuch as there is species tropism, murine cytomegalovirus (MCMV) has become a good animal model for systemic HCMV infections, with similarities in pathogenesis. Like HCMV, MCMV can infect many different organ and cell types (Jordan & Takagi, 1983; Shellam et al., 2006). MCMV is commonly administered via intraperitoneal (i.p.) injection in experimental models of systemic infection, where it can cause significant damage to the host. High levels of viral replication are seen in the spleen and liver, and lethal infection is often associated with destruction of the liver (Katzenstein et al., 1983; Shanley et al., 1993). Replicating virus is found in most visceral organs within the first week. Acute infection is often studied in the spleen 2–4 days post-infection (p.i.) after systemic administration. However, there are conflicting data over whether sinus-lining cells and endothelial cells or macrophages are the primary cell type infected (Henry et al., 2000; Benedict et al., 2006; Mercer et al., 1988; Stoddart et al., 1994). In addition, early infection of dendritic cells (DCs) is thought to contribute to an effective innate response to MCMV by natural killer (NK) cells (Andoniou et al., 2005). Thus, multiple cell types are implicated in an acute infection. However, a time course for the appearance of these infected cells before 2 days p.i. has not been detailed.

Additionally, despite the ubiquitous use of the i.p. route of infection, the mechanism by which this causes systemic infection has not been well studied. It is unclear whether inoculation of MCMV into the peritoneal cavity leads to infection of peritoneal cells, or whether virions traffic from the peritoneal cavity to initially infect cells elsewhere. Stoddart et al. (1994) suggested that mononuclear phagocytes in the peripheral blood disseminate MCMV after i.p. infection, and that these infected cells are then found in infected organs. However, the trafficking mechanism from the peritoneal cavity was not described and infected mononuclear cells were observed 5 days p.i., after the peak of acute infection. Thus, further elucidation...
of MCMV trafficking at earlier time points is warranted for understanding the progression of early MCMV infection.

Here, we use a recombinant MCMV that expresses enhanced GFP (MCMV–GFP) under an intermediate-early promoter to visualize the time course of infection as early as 6 h p.i. We show that MCMV injected into the peritoneal cavity appears to traffic as free virus to infect specific cells in the lymph nodes (LNs), spleen and liver within the first hours after infection. These studies were aided by use of inert beads that fluoresce in the near-infrared (NIR) range, which allowed us to visualize the trafficking pattern from the peritoneal cavity. Thus, MCMV displays specificity within the first day of infection, before dissemination to other organs and cell types as the infection progresses.

METHODS

Mice and viruses. Wild-type C57BL/6 mice were obtained from either NCI (Frederick, MD, USA) or Jackson Laboratories (Bar Harbor, ME, USA) and maintained under specific-pathogen-free conditions. Mice were used at 12–16 weeks. Smith strain MCMV–GFP was a generous gift from S.C. Henry and J. Hamilton (Duke University, Durham, NC, USA) and propagated in salivary glands of BALB/c mice. The titre was determined by plaque assay using 3T12 cells (ATCC). Mice were infected with $10^5$ p.f.u. MCMV–GFP either by i.p. or tail vein injection. CPXVA203–GFP was created in our laboratory and propagated in Vero cells in vitro, then purified using a sucrose cushion as described by Byun et al. (2007). Although CPXVA203–GFP is a deletion virus, its only known difference from the wild-type virus is a partial decrease of the major histocompatibility complex (MHC) class I downregulation in infected cells. As we were mostly interested in the cells that were initially infected by CPXV, this deletion should not affect our results. Mice were infected with $5.5 \times 10^6$ p.f.u. by i.p. infection.

NIR fluorescent microbeads. Carboxyl fluorescent ‘Aqua Green’ particles were purchased (Spherotech) in 0.3, 1 and 5 μm sizes. Maximum excitation and emission for these beads are 775 and 789 nm, respectively. Beads were treated with 0.05 M NaOH for approximately 1 day to remove any endotoxins and resuspended in sterile water. Beads were confirmed to be endotoxin-free by the Limulus Amebocyte Lysate (LAL) test (Associates of Cape Cod). Prior to use, beads were maintained with inhaled isoflurane (2 %, v/v) for 1 day to remove any endotoxins and resuspended in sterile water. Beads were treated with 0.05 M NaOH for approximately 1 h. Approximately $8 \times 10^7$ 5 μm beads were injected into mice per day. Beads were adsorbed overnight with 10% mouse serum prepared from C57BL/6 mice. To separate aggregates, beads were vortexed and sonicated adsorbed overnight with 10% mouse serum prepared from C57BL/6 C57BL/6 mice with salivary gland preparations of MCMV–GFP under an intermediate-early promoter (K. Hsu et al., 2008) and propagated in Vero cells in vitro, then purified using a sucrose cushion as described by Byun et al. (2007). Although CPXVA203–GFP is a deletion virus, its only known difference from the wild-type virus is a partial decrease of the major histocompatibility complex (MHC) class I downregulation in infected cells. As we were mostly interested in the cells that were initially infected by CPXV, this deletion should not affect our results. Mice were infected with $5.5 \times 10^6$ p.f.u. by i.p. injection.

In vivo imaging. To facilitate imaging, hair was removed from the ventral side of mice prior to injection by using Sally Hansen Brush-On Hair Remover (Del Laboratories). Mice were anaesthetized and maintained with inhaled isoflurane (2 %, v/v) for in vivo imaging. Images from the mice were captured using the Kodak IS4000MM multimodal imaging system (Eastman Kodak Company) at various time points between 30 min and 4 days after injection of fluorescent beads. Fluorescence images were acquired by 60 s exposure using excitation and emission collection centred at 755 and 830 nm, respectively. Digital X-ray images were subsequently acquired for anatomical orientation of the NIR images (Backer et al., 2007). Images were analysed with Kodak Molecular Imaging Software (Eastman Kodak Company).

Immunofluorescence. Spleens, livers and LNs were harvested from mice and fixed overnight in PLP fixative (0.05 M lysine phosphate buffer, pH 7.4, 0.05 % glutaraldehyde, 0.002 mg NaIO 4 ml $^{-1}$, 0.5 % paraformaldehyde) with 0.1 % Triton X-100. Tissues were then dehydrated in 30 % sucrose for approximately 1 day and frozen in Tissue-Tek (Sakura Finetek) using cooled 2-methylbutane over dry ice and stored at $-80 \degree$ C. Cryostat sections were cut 9 μm thick and rehydrated in PBS. Sections were blocked with 10% normal goat serum in 0.5 % Triton X-100 in PBS for 1 h. Sections were then incubated with the primary antibody for 2 h, washed with 0.5 % Triton X-100/PBS, and incubated with the secondary antibody for 1 h. Primary antibodies used included anti-GFP (Abcam), anti-MADCAM-1 (BD Pharmingen), anti-CD169 (AbD Serotec), anti-CD29 (BD Pharmingen) and anti-ER-TR7 (Abcam). Secondary antibodies used included Alexa Fluor 660-conjugated goat anti-rabbit IgG and Alexa Fluor 568-conjugated goat anti-mouse IgG, both from Molecular Probes. Directly conjugated antibodies were incubated on tissue for 1 h after blocking. F4/80-phycocerythrin (PE), CD11c-PE, CD3e-allophycocyanin and CD11b-PE were obtained from eBioscience; B220-PE, NK1.1-PE and MADCAM-PE were obtained from BD Pharmingen; MARCO-PE was obtained from AbD Serotec; CD146-PE was obtained from Chemicon International. Images were acquired using a Nikon Eclipse 80i microscope and processed using MetaMorph (Molecular Devices).

Quantification of the percentage of infected cells co-localizing with a particular marker was performed by an experimenter blinded to the identity of the marker. Infected cells from several different sections and locations were analysed at $\times 60$, and approximately 150 GFP$^+$-infected cells were counted and among those, the number of cells with a given marker was determined.

RESULTS

MCMV infects splenic marginal zone (MZ) and red pulp cells within the first hours after infection

To study the in vivo pathogenesis of MCMV, we infected C57BL/6 mice with salivary gland preparations of MCMV–GFP, allowing for the detection of infected cells by 4 h after infection by GFP expression. Using MADCAM-1, a marker of MZ sinus-lining cells (Mebius & Kraal, 2005), to delineate the MZ of the spleen, we saw infected cells appear in the MZ by 6 h (Fig. 1). Infected cells clearly surrounded the MZ by 8 h, with increased infection of cells in the red pulp by 17 h. By 48 h, infected cells were found throughout the red pulp and many appeared to be enlarged and degenerating. There was disruption of the MZ and marked loss of cellularity in the red pulp at this stage as noted by 4,6-diamidino-2-phenylindole (DAPI) nuclear counterstain (data not shown). Additionally, the appearance of occasional infected cells in the white pulp was first noted at 48 h, suggesting that this was a later consequence of MCMV infection. Thus, MCMV infects cells in the MZ of the spleen by 6 h and then preferentially infects cells in the red pulp instead of the white pulp by 17 h, leading to significant destruction of the red pulp by 48 h.

In addition to the spleen, GFP$^+$ cells were also detected in the liver in the first hours after i.p. infection (Supplementary Fig. S1 available in JGV Online). Infected cells in the liver were large, polygonal and often...
binucleate, indicating that they were most likely hepatocytes. By 48 h p.i., increased infection of hepatocytes was accompanied by focal necrosis of hepatocytes. Further staining revealed an influx of macrophages around these infected cells (data not shown). No GFP\(^+\) cells were found in the kidneys, lung, heart or intestines up to 48 h p.i. (data not shown). Thus, the spleen and liver appear to be the two primary organs for early viral infection and replication, and both sustain significant damage by 48 h.

**MCMV directly infects spleen and liver cells as free virus**

The early infection of MZ cells in the spleen suggested haematogenous spread of the virus, either as virus-infected cells or as free virus, because blood passes from the splenic arterioles through the MZ into the red pulp. To determine whether injection of virus directly into the bloodstream would result in a similar pattern of infection, we infected mice with MCMV–GFP intravenously (i.v.) via tail vein injection. The distribution and pattern of infection were essentially identical – the spleen and liver were infected early, with cells infected in the MZ and red pulp of the spleen (Fig. 2) and infected hepatocytes in the liver (Supplementary Fig. S1). Infected cells had similar morphologies regardless of the route of infection. Moreover, the infected cells in both i.p. and i.v. inoculations expressed the same cell surface markers by immunohistochemistry (data not shown) as discussed below. Thus, MCMV appears to spread via haematogenous route upon i.p. inoculation.

It was possible that peritoneal cells were directly infected by MCMV and then traffic to the spleen. However, neither *in vivo* nor *ex vivo* experiments supported this hypothesis (data not shown). Collection of peritoneal exudate cells by peritoneal lavage at serial time points (4–48 h) after i.p.
inoculation produced <1% GFP+ cells. In an ex vivo experiment, fresh peritoneal exudate cells were removed from naïve mice by peritoneal lavage and infected with an m.o.i. ranging from 0.1 to 2. Again, <1% of the cells were infected at 48 h. Given the large number of infected cells seen in the spleen at 48 h, it appears unlikely that infected cells traffic from the peritoneal cavity to the spleen.

**MCMV follows the normal trafficking mechanism from the peritoneal cavity to mediastinal LNs**

To explore the possibility that free virus was trafficking from the peritoneal cavity to directly infect resident spleen and liver cells following an i.p. injection, we first mapped out the default trafficking pathway using inert beads. Beads fluorescing in the NIR range were injected i.p. in varying sizes (0.3, 1 and 5 μm) and whole mouse in vivo imaging was used to detect bead movement. The 0.3 μm beads approximated the size of an MCMV virion, while 5 μm beads were closer to the size of a small leukocyte (Shellam et al., 2006; Junqueira et al., 1998). After 2 h, 0.3 and 1 μm beads were detected in the mediastinum and remained there until the mice were sacrificed, at most, 4 days after the initial injection (Fig. 3a). In contrast, the 5 μm beads never appeared in the mediastinum, even after 4 days. Thus, beads smaller than 5 μm were capable of trafficking to the mediastinum, while 5 μm beads were not, consistent with the possibility that free virus could directly traffic from the peritoneal cavity to the spleen.

The location of the beads was more precisely determined by removing the diaphragm and selected mediastinal and abdominal LNs for fluorescent microscopy. A schematic diagram of LNs sampled is shown in Fig. 3b. The cranial mediastinal (CM) LNs are paired and lateral to the heart, while the tracheobronchial (TB) LN is located at the tracheal bifurcation (Van den Broeck et al., 2006). Abdominal LNs sampled include mesenteric, pancreaticoduodenal and para-

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**Fig. 3.** Lymphatic distribution of inert 0.3 μm beads and MCMV–GFP from the peritoneal cavity were similar. (a) Representative digital X-ray images overlaid with NIR fluorescence intensities from a living mouse 1 min (left) and 6 h (right) after i.p. injection of 0.3 μm NIR fluorescent beads are shown. NIR fluorescence was detected from the mediastinum beginning 2 h after injection. (b) Schematic diagram of LNs that were sampled for both bead and MCMV–GFP experiments. (c) Removal of LNs demonstrated that beads were contained within mediastinal LNs, particularly the top CM and TB LNs. Other LNs appeared relatively dark due to proportionately lesser number of beads. The diaphragm also contained many beads, in contrast to the control diaphragm which did not. (d) Mice injected with MCMV–GFP i.p. had a distribution of GFP+ MCMV-infected cells similar to the distribution of 0.3 μm beads. GFP+ cells were noted as bright dots in the mediastinal LNs and diaphragm, whereas only background autofluorescence was seen in the abdominal LNs and control diaphragm. LNs and diaphragm shown here were taken from mice sacrificed 24 h p.i. CM, Cranial mediastinal LN; RT, right top; RB, right bottom; LT, left top; LB, left bottom; TB, tracheobronchial LN; MES, mesenteric LN; PD, pancreaticoduodenal LN; PA, para-aortic LN.
aortic LNs. The smaller, 0.3 and 1 μm, beads were readily found in the mediastinal LNs (Fig. 3c). Specifically, the top CM LNs had the greatest amount of bead accumulation, with markedly less in the corresponding bottom CM LNs. Beads were also found in the TB LN. Presumably, one route by which the beads reached the mediastinum was through the diaphragmatic lymphatics, as streaks of beads were observed there. In contrast, few 5 μm beads were found in the mediastinal LNs or diaphragm, with more 5 μm beads in the mesenteric LNs (data not shown). Thus, trafficking of beads from the peritoneal cavity occurs through a size-dependent mechanism through the lymphatics, with smaller beads travelling through the diaphragmatic lymphatics to the mediastinal LNs.

To compare the trafficking of inert beads to MCMV, we injected mice with MCMV–GFP i.p. and sampled mediastinal and abdominal LNs as before (Fig. 3d). The distribution of GFP+ cells was similar to that of the smaller beads (0.3 and 1 μm). Infected cells were found in the lymphatics of the diaphragm and in the mediastinal LNs, with few GFP+ cells in the abdominal LNs. These data suggest that free MCMV virions traffic from the peritoneal cavity into the mediastinal LNs and then into the thoracic duct, thereby accessing the bloodstream to eventually infect the spleen and liver.

Early preferential MCMV infection of subcapsular sinus macrophages in mediastinal LNs

Sectioning of LNs 8 h after injection showed peripheral accumulation of beads, suggesting that most of the lymph is not filtered through the parenchyma of the LN, but instead preferentially flows around the parenchyma through the subcapsular sinus (Fig. 4a). The same pattern was observed in the MCMV–GFP-infected LNs, suggesting that the virus was being carried in the lymph, and that it also inefficiently penetrates into the parenchyma (Fig. 4b). Furthermore, approximately 80% of MCMV–GFP-infected cells in the subcapsular sinus were CD169+, suggesting that they were macrophages (Fig. 4c). Infected cells were often next to, but did not co-localize with, Lyve-1+ lymphatic endothelial cells of the subcapsular sinus (data not shown). These data strongly suggest that subcapsular sinus macrophages are selectively infected early in the LNs.

Early MCMV infection of stromal cells in the spleen

To determine whether macrophages were also being infected in the spleen, the identity of the GFP+ -infected cells in the spleen after 8 h was further explored by immunofluorescence. The data presented below were consistent in both i.p. and i.v. inoculations. Although the initial MZ distribution of the GFP+ -infected cells presented the possibility that MCMV may be infecting MZ macrophages, GFP+ cells did not co-localize with the MZ macrophage marker MARCO or the MZ metallophilic macrophage marker CD169 (Fig. 5a). Further staining suggested that these early infected cells were not leukocytes, as they did not co-localize with CD45, CD11c, CD11b, NK1.1, CD3 or B220 (Fig. 6 and data not shown).

Because at 8 h p.i. GFP+ -infected cells in the spleen did not appear to be leukocytes, we explored the possibility that MCMV was infecting other major non-haematopoietic...
populations in the MZ and red pulp, including sinus-lining cells that line the MZ, endothelial cells that line the splenic sinuses and reticular fibroblasts that form the splenic cords. While 28% of infected cells near the MZ displayed MADCAM-1 positivity, suggesting some sinus-lining cells were infected, the majority of infected cells did not appear to be these cells (Fig. 7). GFP+ cells did not co-localize with CD146, which marked endothelial cells of splenic sinuses in the red pulp (Supplementary Fig. S2 available in JGV Online), although some infected cells appeared to extend around the CD146+ cells. Instead, 66% of GFP+ cells co-localized with reticular fibroblast marker ER-TR7 (Fig. 5a), found in the red but not the white pulp of the spleen, and many displayed a morphology characteristic of these cells with multiple thin cytoplasmic extensions. This is also consistent with their proximity to endothelial cells, as reticular fibres are known to envelope sinusoids across the transverse axis of the sinusoid (Mebius & Kraal, 2005). GFP+ cells (94%) also co-localized with a less specific stromal cell-associated marker, CD29, which stained both sinus and cord structures in the red pulp (Fig. 7). At 17 h p.i., infected cells spread into the red pulp still co-localized with ER-TR7 and CD29 (Fig. 7). Given that large numbers of cells were infected throughout the MZ and red pulp, and that many of these cells co-localized with fibroblastic marker ER-TR7 and showed a reticular morphology, it is probable that many of the early infected cells are the reticular fibroblasts that give structure to the MZ and red pulp.

As previously discussed, significant changes have occurred in the spleen by 48 h p.i. Occasional infected cells began to appear in the white pulp at this time, and the distribution of these cells within the white pulp was similar to that of DCs in the white pulp. Indeed, GFP+ cells in the white pulp co-localized with the DC marker CD11c, while most of the infected cells in the MZ and red pulp did not (Fig. 5b). This is consistent with previous observations that DCs are infected in the first few days after infection (Andoniou et al., 2005; Andrews et al., 2001). In addition, by 48 h p.i., changes were seen in other splenic populations, including the disorganization of B- and T-cell regions, CD11b+ cell infiltration into the red pulp, disappearance of NK cells from the red pulp and loss of ER-TR7 positivity on infected cells (Fig. 6). In contrast, at 8 h, these populations still appeared normal. These data indicate the evolution of infection in both haematopoietic and stromal compartments of the spleen by 48 h p.i.
Cowpox virus (CPXV) produces a distinct pattern of infection

To determine whether the pattern of infection seen with MCMV–GFP was due to selectivity of anatomical structures or specific to the virus, we infected C57BL/6 mice with CPXV for comparison. Like MCMV, CPXV is also a large DNA virus of approximately the same size as MCMV, is endemic in natural rodent reservoirs, and is also known to have a wide tissue tropism (Buller & Palumbo, 1991). However, the pattern of CPXV infection was distinct from MCMV infection in both the spleen and liver. In the spleen, infected cells were also found around the MZ, but extended into the white pulp instead of the red pulp (Fig. 8). Many of the infected cells were found around the MZ, but extended into the white pulp instead of the red pulp (Fig. 8). Many of the infected cells were found inside of the ER-TR7 staining. A proportion of these cells co-localized with the MZ metallophilic macrophage marker CD169, and a proportion co-localized with the MZ macrophage marker MARCO, suggesting that several macrophage populations have been infected. In the liver, infected cells had a dendritic morphology and co-localized with the macrophage marker F4/80, suggesting that these were Kupffer cells (Supplementary Fig. S3a available in JGV Online). Thus, CPXV appears to have a specificity for macrophages in the spleen and liver in a manner that is distinct from MCMV.

In contrast, in the mediastinal LNs, CPXV-infected cells appeared around the edges of the LN and co-localized with CD169, similar to MCMV (Supplementary Fig. S3b available in JGV Online). These data suggest that despite specificity differences in the spleen and liver, CPXV infection in the LN is similar to MCMV infection.

DISCUSSION

Our data strongly suggest that the mechanism of viral spread in the i.p. MCMV infection model involves free virus trafficking to the mediastinal LNs before entering the systemic circulation to reach the spleen and liver. We significantly extend both our preliminary work with soluble dyes [methylene blue (data not shown)] and other studies using India ink (Abu-Hijleh et al., 1995; Shibata et al., 2007) by using endotoxin-free beads of different sizes to demonstrate that particles smaller than 5 μm, such as viruses, are much more efficient at employing this pathway. It has been suggested that the diaphragm acts as a sieve, allowing the passage of particles that can pass through the stomata and lymphatic vessels (Abu-Hijleh et al., 1995; Shinohara, 1997). As both stomata and lymphatic vessels are generally less than several micrometres wide, it is likely that the larger beads could not traffic to the mediastinum due to these diaphragmatic barriers. MCMV as free virus, however, was readily able to travel through the diaphragm and to the mediastinal LNs but surprisingly, not the mesenteric LNs.

MCMV can infect many different organs, and, in vitro, many different cell lines (Jordan & Takagi, 1983; Shellam et al., 2006; Krmpotic et al., 2003). Despite this broad
tropism and its dissemination as free-viral particles, MCMV displayed a selective infection within the first hours after infection. Following i.p. inoculation, MCMV virions infected subcapsular sinus macrophages in the mediastinal LNs. Haematogenous spread of the virus resulted in infection of stromal cells in the spleen, particularly reticular fibroblasts, beginning in the MZ at 6 h p.i. and spreading into the red pulp. Hepatocytes were targeted in the liver, but we did not see infected cells elsewhere. MCMV–host interactions appear to be different.
in the LNs and the spleen, as MCMV-infected cells in the LNs were CD169+ macrophages, and this population was not initially infected in the spleen. Thus, selected cell populations were infected in selective sites after MCMV infection, raising the possibility that MCMV is handled in an organ-specific manner.

Recent studies have suggested that subcapsular sinus CD169+ macrophages can capture fluorescently labelled vesicular stomatitis virus, vaccinia virus and adenovirus in the draining popliteal LN after footpad injection (Junt et al., 2007). While we add MCMV and CPXV to this list, our study differs in four major aspects: (i) we show productive infection of subcapsular sinus macrophages by MCMV and CPXV, as evidenced by GFP expression under the control of a viral promoter. (ii) We show infection of these cells in the mediastinal LNs after i.p. injection. (iii) Within a few hours after injection, we visualized productive infection of only a few medullary cells, whereas labelled viruses were readily seen in the medulla. (iv) We infected mice with sublethal doses of MCMV and CPXV, while labelled viruses were injected in quantities 1–2 logs above the standard p.f.u. required. Nevertheless, these studies when taken together suggest that subcapsular sinus macrophages have a generic host mechanism for pathogen capture that is not specific to the virus. As lymph appears to flow preferentially through the subcapsular sinus, sentinel cells in that location would then be advantageous for the early detection of a pathogen and initiation of an immune response. Moreover, subcapsular sinus macrophages can also bind both antigen and immune complexes (Carrasco & Batista, 2007; Phan et al., 2007), suggesting that they possess mechanisms to sample the lymph broadly.

Although early capture of fluorescently labelled viral particles by subcapsular sinus macrophages can lead to B-cell stimulation (Junt et al., 2007), the role of the initially infected cells in the spleen in the progression or suppression of infection is largely unknown. The selective infection of non-immune cells in the spleen opens up the possibility that epithelial and stromal cells, although themselves not classic effector cells in the immune system, may play important roles in alerting the immune system to viral infection. In particular, reticular fibroblasts closely interdigitate with leukocytes in the red pulp, and would be in an ideal position to signal neighbouring immune cells to infection. Consistent with this possibility, stromal cells in the spleen may be the initial producers of the type I interferons after MCMV infection (Schneider et al., 2008), which are known to have a role in limiting early MCMV replication (Orange & Biron, 1996). Moreover, while this manuscript was being prepared, endothelial cells were shown to be the apparent source of virions for secondary viraemic spread (Sacher et al., 2008). Although we found that most infected cells in the spleen were initially reticular fibroblasts, there was also a population of infected MADCAM-1+ sinus-lining cells, consistent with this observation. In either case, here we show directly the initial cells infected with MCMV after systemic administration.

**Fig. 8.** CPXV produced a different pattern of infection in the spleen as compared with MCMV infection. C57BL/6 mice were infected with 5.5×10⁶ p.f.u. CPXV–GFP and sacrificed at 8 h p.i. GFP+ cells were found in the MZ and the white pulp of the spleen, with few in the red pulp. Unlike MCMV-infected cells, CPXV-infected cells were inside of ER-TR7 staining and there was co-localization with both MARCO and CD169, suggesting that macrophages were infected in the spleen.
As NK cells are known to be important in controlling early MCMV infection (Brown et al., 2001), this then leads to the question of whether the initially infected cells in the LNs or alternatively the selectively infected cells in the spleen or the liver may be the direct targets of NK cells. Interestingly, however, the particular strain of MCMV–GFP we used here had a point mutation in the m157 open reading frame (data not shown). m157 is a ligand for the activation receptor Ly49H involved in the genetic control of MCMV infection within the first 3 days (Smith et al., 2002; Arase et al., 2002). Although C57BL/6 mice are genetically resistant to MCMV because they have Ly49h (Cmv1+) and most other laboratory strains of mice lack Ly49h and are thus genetically susceptible (Scalzo et al., 1990; Lee et al., 2001; Daniels et al., 2001), our experiments here with C57BL/6 mice may be more similar to that of genetically susceptible strains of mice than resistant strains.

The initial interactions between MCMV and host progresses from infection of the selective cell types to a more widespread infection that is observed several days after infection. Previous in vivo studies detailing acute MCMV infection have observed both disruption of splenic architecture and a variety of cell types that are initially infected in the spleen, but these studies were conducted 2–3 days p.i. (Benedict et al., 2006; Mercer et al., 1988; Stoddart et al., 1994). This appears to be too late to observe the actual initially infected cells, as our studies indicate that by 48 h, the homeostasis of the host has been dramatically changed by the virus and host response, and it is likely that at least one cycle of lytic viral replication has occurred by this point. In the stromal population, infected cells were found from the MZ to the red pulp within 24 h, leading to disruption and degeneration of these areas by 48 h. We found ER-TR7 to be a good marker for infected cells only in the first hours after infection. It is possible that infected cells have downregulated ER-TR7 or undergone apoptosis at later time points, leading to a decreased signal at 2–3 days p.i. The leukocyte population in the spleen was not initially infected by MCMV; however, the infection evolved by 48 h p.i. In particular, DCs are thought to be efficiently infected in early acute MCMV infection (Andoniou et al., 2005; Andrews et al., 2001), but our experiments showed that infected CD11c+ DCs appear relatively late, at 48 h, mostly in the white pulp of the spleen. It is possible that DCs were infected elsewhere, and traffic to the white pulp within 48 h. However, we have not noted infected DCs in any other organs that we examined within the first day of infection (data not shown), and our results suggest that the vast majority of infected cells in the spleen were stromal cells, not DCs. Alternatively, it is possible that the GFP+ DCs had simply engulfed other infected cells, without becoming productively infected. In any case, GFP+ DCs were not noticeable in the infection until 48 h p.i., after the infection of other cells. Additionally, movement of lymphocytes, macrophages, and NK cells at 48 h p.i. suggests that by this time, these cells have been alerted to the infection and were involved in the host response.

Finally, we showed that CPXV infected subcapsular sinus macrophages in the LNs similar to MCMV. Inasmuch as salivary gland MCMV and sucrose cushion-purified CPXV initially infected the same cell in the proximal draining LN, it is unlikely that the salivary gland preparation itself affected initial MCMV localization. CPXV also infected cells in the MZ of the spleen, suggesting similar haematogenous spread as in MCMV. However, cells infected in the spleen and liver by CPXV were macrophages that were morphologically different from MCMV-infected cells, and the spread of infection in the spleen was into the white pulp instead of the red pulp. Like MCMV, CPXV is also known to infect many different cell types (Buller & Palumbo, 1991), but this does not appear to be the case early in infection. Thus, our data suggest differences in the initial host response to these two viruses, as a result of selective early infection of different cells.

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