B-cell-independent lymphoid tissue infection by a B-cell-tropic rhadinovirus

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Lymphocytes provide gammaherpesviruses with a self-renewing substrate for persistent infection and with transport to mucosal sites for host exit. Their role in the initial colonization of new hosts is less clear. Murid herpesvirus 4 (MuHV-4), an experimentally accessible, B-cell-tropic rhadinovirus (gamma-2 herpesvirus), persistently infects both immunocompetent and B-cell-deficient mice. A lack of B-cells did not compromise MuHV-4 entry into lymphoid tissue, which involved myeloid cell infection. However, it impaired infection amplification and MuHV-4 exit from lymphoid tissue, which involved myeloid to B-cell transfer.

Murid herpesvirus 4 (MuHV-4) is B-cell-tropic rhadinovirus related to the Kaposi’s sarcoma-associated herpesvirus (human herpesvirus 8) and more distantly to Epstein–Barr virus (EBV; human herpesvirus 4) (Efstathiou et al., 1990). It persists in laboratory mice and like its human counterparts establishes a largely latent infection of B-cells (Sunil-Chandra et al., 1992). It persists also in B-cell-deficient mice, infecting myeloid (Weck et al., 1996) and epithelial cells (Stewart et al., 1998). EBV, a lymphocryptovirus (gamma-1 herpesvirus), does not seem to persist in B-cell-deficient humans (Faulkner et al., 1999).

However limitations on human analysis make infection hard to exclude without serology: it is certain only that without B-cells, long-term EBV infection is hard to detect. The idea that EBV infects just B-cells (Thorley-Lawson et al., 1996) has an appealing simplicity, but does not explain the diversity of EBV-infected cancers (George et al., 2012). KSHV also causes non-B-cell cancers. Therefore, it is important to understand what non-B-cell infections contribute to gammaherpesvirus host colonization.

With MuHV-4 we can ask what different cell types contribute to rhadinovirus host colonization. B-cells are not a target for host entry: MuHV-4 enters the upper respiratory tract via olfactory neurons and sustentacular cells (Milho et al., 2012), and the lower respiratory tract via alveolar macrophages (Lawler et al., 2015). Its main defect in colonizing B-cell-deficient hosts via the lower respiratory tract is a lack of spread to the spleen (Usherwood et al., 1996). Intraperitoneally inoculated MuHV-4 directly infects marginal zone (MZ) macrophages (Frederico et al., 2014) and colonizes the spleens of B-cell-deficient mice (Weck et al., 1996). Therefore, a lack of B-cells seems to impair MuHV-4 propagation from the lungs to the spleen.

Infected B-cells amplify the MuHV-4 latent load through proliferation in germinal centres (Simas & Efstathiou, 1998). They also transport systemic infection back to mucosal sites (Frederico et al., 2012), providing a basis for transmission. Their role in infection spread in new hosts is less clear. Viruses, being immotile, must spread via host pathways; peripheral-to-lymphoid antigen transport is a myeloid rather than a B-cell function, and MuHV-4 accordingly enters lymph nodes (LNs) via myeloid cells (Gaspar et al., 2011). Indeed B-cell tropism requires passage through a myeloid cell (Frederico et al., 2012). However B-cells influence myeloid function (You et al., 2011; Moseman et al., 2012); B-cell-derived IgM promotes early antigen capture (Heyman, 2014), and B-cells transport antigens from myeloid cells into LN and splenic follicles (Phan et al., 2007; Cinamon et al., 2008). Therefore, B-cell deficiency could compromise MuHV-4 spread indirectly by compromising myeloid infection. It could also impair subsequent steps directly. To understand where B-cells contribute to host colonization, we tracked LN and spleen infections in B-cell-deficient mice.

We infected immunocompetent C57BL/6 and B-cell-deficient µMT mice in the upper respiratory tract by allowing them to inhale spontaneously 10⁴ p.f.u. MuHV-4 as a 5 µl droplet (Tan et al., 2014). We titrated infectious virus in noses by plaque assay, and total virus in superficial cervical LNs (SCLNs) and spleens by infectious centre assay.
(de Lima et al., 2004) (Fig. 1a). Early nose titres were similar in C57BL/6 and μMT mice, emphasizing that B-cells are not required for host entry. C57BL/6 mice then cleared infectious virus from noses more efficiently than did μMT mice. Initiating SCLNs infection did not require B-cells, as day 9 SCLN titres were significantly higher in μMT mice at day 9 post-inoculation (P<0.02) and significantly lower thereafter (P<0.01). Spleen titres were significantly higher in C57BL/6 mice from day 9 post-inoculation (P<0.02). (b) C57BL/6 and μMT mice were infected intranasally as in (a), but with MHV-GFP. At day 7 post-inoculation, SCLNs were stained for viral EGFP, B220 (B-cells), F4/80 (medullary sinus macrophages) and CD169 (subcapsular and medullary sinus macrophages). Arrows show examples of co-localization between viral EGFP and cellular markers. Bar, 30 μm. Across 10 sections from three mice per group, EGFP+ cells in C57BL/6 SCLNs were (mean±SEM) 35.6±10.2 % B220+, 17.4±9.8 % CD169+ and 6.2±3.5 % F4/80+. EGFP+ cells in μMT SCLNs were 63.3 %±13.8 % CD169+ and 32.6±12.0 % F4/80+; none was B220+.

Subsequently, consistent with infected B-cell proliferation amplifying latent viral loads.

Splenic infection was detectable from day 9 post-inoculation. C57BL/6 spleens contained consistently more virus than μMT. Nonetheless, most μMT spleens had detectable infection, so B-cells were non-essential for SCLN-to-spleen virus transport. The failure of Usherwood et al. (1996) to detect splenic infection possibly reflected lower assay sensitivity,
as their peak C57BL/6 infectious centres in spleens were ‘>5000’, whereas we detected up to 100 000. Their lower respiratory tract inoculation would not have compromised virus spread, as such inoculations also reach the upper respiratory tract (Tan et al., 2014). Infected B-cell proliferation presumably increased splenic viral loads both directly and indirectly by amplifying SCLN viral loads. B-cells also seemed to promote virus release from SCLNs, as day 9 post-inoculation splenic viral loads were higher in C57BL/6 mice despite higher SCLN loads in μMT mice.

We used EGFP expression from an EF1α promoter (MHV-GFP; May & Stevenson, 2010) to visualize LN infection directly and without a need for ongoing lytic gene expression. Immunostaining SCLN sections for EGFP (Frederico et al., 2014) at day 7 post-inoculation showed follicular B-cell infection in C57BL/6 mice, whereas μMT

![Fig. 2. PLN colonization by replication-competent and replication-deficient MuHV-4 in μMT mice. We infected μMT mice by intra-footpad inoculation of replication competent (ORF50⁺) or replication-deficient (ORF50⁻) MHV-GFP (10⁵ p.f.u.). At day 3 post-inoculation, PLN sections were stained for EGFP, F4/80 and CD169. The ‘overview’ image shows the region of PLNs sampled. Bar, 250 μm. The ‘zoom’ images show the boxed region at higher magnification. Bar, 50 μm. Arrows show examples of co-localization between viral EGFP and cellular markers. Across nine sections from three mice per group, the cells infected by ORF50⁺ MHV-GFP were (mean ± SEM) 74.6 ± 14.9 % CD68⁺ and 48.7 ± 10.5 % CD169⁺. Those infected by ORF50⁻ MHV-GFP were 69.9 ± 11.8 % CD68⁺ and 56.5 ± 12.1 % CD169⁺.](image-url)
Fig. 3. Splenic colonization by intraperitoneal MuHV-4 in C57BL/6 and µMT mice. (a) C57BL/6 and µMT mice were infected intraperitoneally with MHV-GFP (10⁵ p.f.u.). At day 4 or 8 post-inoculation, spleens were titrated for total recoverable virus by infectious centre assay and for pre-formed infectious virus by plaque assay. Circles show individual mice; horizontal bars show means. Total virus titres were significantly higher in µMT than in C57BL/6 mice at day 4 post-inoculation and significantly lower at day 8 post-inoculation (P<0.02 by Student’s unpaired two-tailed t-test). In both C57BL/6 and µMT mice, infectious virus titres were not significantly different from total virus titres at day 4 post-inoculation (P>0.5), but significantly lower at day 8 (P<0.001). ND, Not detected. (b) Spleens of C57BL/6 and µMT mice infected as in (a) were analysed for infection distribution by immunostaining for EGFP and cellular markers. Arrows show examples of co-localization. Bar, 50 μm. Across 10 sections from three mice per group, EGFP⁺ cells in C57BL/6 spleens at day 4 post-inoculation were (mean±SEM) 60.2±5.0 % CD169⁺, 31.5±11.2 % F4/80⁺ and 6.7±2.6 % B220⁺; and at day 8 post-inoculation were 19.3±5.2 % CD169⁺, 21.2±7.2 % F4/80⁺ and 70.7±16.3 % B220⁺. EGFP⁺ cells in µMT spleens at day 4 post-inoculation were 65.4±11.3 % CD169⁺ and 44.1±9.2 % F4/80⁺; and at day 8 post-inoculation were 39.5±6.0 % CD169⁺ and 52.7±11.1 % F4/80⁺.
infection was confined to F4/80+ and CD169+ cells around the SCLN margin (Fig. 1b). LN subcapsular and medullary sinus macrophages express CD169, but only the latter express F4/80 (Gray & Cyster, 2012). Therefore MuHV-4 reached the subcapsular and medullary sinuses independently of B-cells, but poorly colonized other LN areas.

MuHV-4 inhalation provides a realistic way to study host entry. However SCLN infection is asynchronous, making early events hard to capture. This reflects the fact that mucosal penetration requires viral replication: a replication-deficient mutant (ORF50-MHV-GFP; Milho et al., 2009) gave no EGFP+ cells in SCLNs (data not shown). Therefore, we also tracked popliteal LN (PLN) infection after MuHV-4 inoculation into footpads (105 p.f.u. in 50 μl), from where it can enter lymphatics directly. Immunostaining μMT PLNs 3 days after intra-footpad MHV-GFP showed CD68+ and CD169+ EGFP+ cells (Fig. 2). Replication-deficient ORF50-MHV-GFP also infected CD68+ and CD169+ cells. Therefore, intra-footpad inoculated virions reached directly the sinus-lining macrophages of μMT LNs. The PLN cells infected by ORF50-MHV-GFP clustered more consistently around the subcapsular sinus than those infected by ORF50-MHV-GFP, so MuHV-4 may spread serially between sinus macrophages. However, total EGFP+ PLN cell numbers were similar at day 3 between ORF50-MHV-GFP (mean±SEM for nine sections from three mice=16.4±1.7 EGFP+ cells per section) and ORF50-MHV-GFP (13.4±1.4 EGFP+ cells per section), so any such spread was inefficient.

B-cells receive antigen from macrophages around the subcapsular sinus (Carrasco & Batista, 2007) and from dendritic cells around high endothelial venules (Braun et al., 2011). They proliferate in germinal centres and then migrate to efferent lymphatics, differentiating into plasma cells in LN medullary cords or rejoining the circulation as memory cells (MacLennan et al., 1992). Plasma cell differentiation triggers gammaherpesvirus reactivation (Laichalk & Thorley-Lawson, 2005; Liang et al., 2009). MuHV-4 reactivates in splenic plasma cells after intraperitoneal infection (Liang et al., 2009) and SCLN B-cells express viral lytic antigens after intranasal infection (Frederico et al., 2014). Memory B-cells recirculate through the splenic MZ, but by analogy with antigen-stimulated B-cells should become plasma cells in submucosal lymphoid tissue or bone marrow, rather than in the spleen (MacLennan et al., 1992). Thus, we hypothesize that MuHV-4 normally reaches the splenic MZ via virion release from medullary cord plasma cells into the efferent lymph.

MuHV-4 given intraperitoneally to C57BL/6 mice passes from MZ macrophages to MZ B-cells, so macrophage infection is productive (Frederico et al., 2014). However, intraperitoneally inoculated μMT mice are reported to show only latent splenic infection (Weck et al., 1996). To address this discrepancy and examine B-cell-independent splenic infection directly, we gave MHV-GFP intraperitoneally to C57BL/6 and μMT mice (105 p.f.u.), and then compared virus titres with EGFP expression 4 and 8 days later (Fig. 3). At day 4 post-inoculation, plaque assays (pre-formed infectious virus) and infectious centre assays (infectious plus latent virus) gave comparable titres (Fig. 3a), so most infection was lytic. The μMT mice showed no deficit in this phase of infection. By day 8 post-inoculation, infectious centre titres had increased in C57BL/6 spleens and decreased in μMT. Neither retained pre-formed infectious virus. This result was consistent with B-cell infection being more tightly latent than macrophage infection and comprising the main viral latency reservoir. MuHV-4 lytic spread in spleens was likely greater after intraperitoneal than intranasal inoculation because intraperitoneal virus reaches the spleen directly, before T cell immunity is established. The conclusion of Weck et al. (1996) that intraperitoneally inoculated MuHV-4 does not replicate lytically in B-cell-deficient spleens reflected their analysis starting at day 9 post-inoculation, by which time lytic infection is largely cleared.

Immunostaining C57BL/6 spleens 4 days after intraperitoneal inoculation showed viral EGFP expression commonly in CD169+ (MZ) and F4/80+ (red pulp) macrophages, and rarely in B220−B-cells (Fig. 3b). By contrast, at day 8 post-inoculation, most infected cells were in B-cell follicles and B220+. Day 4 post-inoculation μMT spleens also contained CD169+ and F4/80+ EGFP+ cells, but this pattern was maintained at day 8 post-inoculation. Therefore, as in LNs, splenic infection started in myeloid cells, independently of B-cells, then either passed to B-cells or was arrested in myeloid cells, consistent with virus spread via serial myeloid cell/B-cell exchange (Frederico et al., 2014). Although intraperitoneal MuHV-4 efficiently infected B-cell-deficient spleens and intranasal MuHV-4 efficiently infected B-cell-deficient SCLNs, without B-cells to provide transit through LNs, acute splenic infection by intranasal MuHV-4 was poor.

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