Arch. Virol. July 12, 2012. DOI: 10.1007/s00705-012-1397-5

Experimental infection of laboratory-bred bank voles (*Myodes glareolus*) with Murid Herpesvirus 4

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Abstract

MuHV-4 is a natural pathogen of rodents of the *Apodemus* genus (e.g. wood mice, yellow-necked mice) and *Myodes glareolus* (bank voles). We report experimental MuHV-4 infection of bank voles in comparison with infection of *A. sylvaticus* (wood mice) and BALB/c mice. Like in wood mice, the level of productive replication in the lungs of bank voles was significantly lower than in BALB/c mice. In contrast to other hosts, however, the level of latent infection in the lung and spleen of bank voles was extremely low. These findings together previous studies suggest that bank voles are an occasional and inefficient host for MuHV-4.

Human gammaherpesviruses are clinically significant pathogens associated with various malignancies. However, due to their narrow host range, studying their pathogenesis is difficult. To overcome this issue, murid herpesvirus 4 (MuHV-4) infection of in-bred strains of laboratory mice (*Mus musculus*) is now a widely utilised model for studying gammaherpesvirus pathogenesis [11]. MuHV-4 was originally isolated from a bank vole (*Myodes glareolus*) [2]. However, a molecular-based study of free-living rodents captured in the U.K. and Germany demonstrated that *Apodemus spp.* (including *A. flavicollis*, *A. agrarius* and *A. sylvaticus*) are natural hosts of MuHV-4 [4]. Due to the host specificity of this group of viruses and the impact this is likely to have on their pathogenesis, we and others have begun the important work of investigating infection of the various identified murid hosts of MuHV-4. As the usual hosts are clearly *Apodemus* spp., a likely explanation of why bank voles are infected with MuHV-4 is due to their sympatric relationship with wood mice.

Given that gammaherpesviruses have intimately evolved with their hosts [10], we recently completed an evaluation of experimental infection of laboratory-bred wood mice (*A. sylvaticus*) and compared this to infection of BALB/c mice, a widely used laboratory mouse strain for studying MuHV-4 pathogenesis [8]. Strikingly, we observed numerous differences (see below) [8], some of which we were able to attribute to the actions of the MuHV-4-encoded chemokine binding protein, M3 [9]. Taking a similar tack, Francois et al. have recently compared MuHV-4 infection of bank voles and laboratory mice (BALB/c and CD1 mice; both *M. musculus*). In that study, significant differences were also observed between the two species, further highlighting the importance of host selection when interpreting pathogenesis-related observations. To complete the picture, it will be important to compare infection of the other species of *Apodemus* that MuHV-4 infects.

Although the recent study by Francois and colleagues was noteworthy, we thought it important to directly compare infection of bank voles *and* wood mice with the commonly used laboratory host, BALB/c mice . Furthermore, we have investigated latent infection in the lungs of bank voles – this is particularly important given the central role latency plays in the lungs of wood mice [7, 8]. The bank voles used for this study were obtained from an out-bred colony established at the University of Liverpool, and were negative by serology and PCR for MuHV-4 and other major infections associated with laboratory animals. They were also negative for the recently-discovered *Myodes glareolus* rhadinovirus 1 [4]. Infections were performed under UK Home Office Project Licence number 40/2483 and Personal Licence number 60/6501.

Cohorts of animals (bank voles, wood mice and BALB/c mice) were infected intranasally with 4 x 10^5 PFU of the MHV-68 isolate of MuHV-4 (strain g2.4) or sterile PBS [11, 13]. The titre of virus at d7 p.i. was measured as described elsewhere [13]. Approximately 10^2 PFU per lung were detected in wood mice, and ca. 10^5 PFU per lung were recovered from infected BALB/c mice ($P < 0.01$, Student's *t*-test). In agreement with a recent report, the titres of virus recovered from bank vole lungs were ca. 10^2 PFU per lung and similar to those observed for wood mice (Fig. 1A); again these were significantly lower than those seen in BALB/c mice ($P < 0.01$, Student's *t*-test.)

We have reported that despite the levels of productive virus measured from the lungs of wood mice were significantly lower than in BALB/c mice, viral DNA loads were equivalent. Further work showed that this was due to high numbers of latently infected cells present within inducible bronchus associated lymphatic tissue (iBALT). Indeed, infection of wood mice with a mutant virus unable to initiate iBALT resulted in significantly fewer latently infected cells. Therefore, we next measured the viral DNA load in the three infected species by qPCR [12]. As expected, the results (Fig. 1B) revealed roughly equivalent levels of viral DNA in the lungs of wood and BALB/c mice.

However, significantly lower levels of viral DNA were detected in the lungs of bank voles ($P < 0.05$). Hence, the low levels of infectious virus recovered from bank vole lungs were likely due to relatively poor establishment of infection in this species, either productive or latent.

After intranasal infection of common laboratory (e.g., BALB/c) and wood mice with MuHV-4 and replication in the lungs, the virus traffics to the spleen, a major site of virus latency [5, 13, 15]. Previous qPCR analysis revealed that viral DNA can be detected in the spleens of wood and BALB/c mice at 7 days p.i. [8]. Our analysis here (Fig. 1C) showed that viral DNA copy numbers in bank vole spleens were equivalent to those of wood and BALB/c mice at 7 days p.i. (300 – 1000 copies per 200 ng spleen DNA in the three species tested). However, by 14 days p.i., viral DNA copy number had not significantly increased but actually decreased in the bank vole spleens, in contrast to a significant increase observed in the wood and BALB/c mice ($P < 0.05$) (Fig. 1C). Although Francoise and colleagues used a different assay to determine the levels of latency in the spleens of bank voles (infectious centre assay), a similar result was observed. Indeed, infective centres could only be detected in 50% of infected bank voles after 14 days, and by 21 days, only one out of ten bank vole spleens was latently infected (albeit, very low); this was in stark contrast to the robust levels of latency found in BALB/c and CD1 mice. These data demonstrate that, while virus traffics to the spleen within infected bank voles, in this host there is inferior amplification of virus latency, despite the artificially high titre of virus used for these types of studies. This is surprising given that this organ is the primary site of viral latency in a natural host such as the wood mouse (including in the wild) [1, 8], as well as in laboratory mouse strains.

We next investigated the host responses in these species by histological analysis (immunohistology for viral antigen and RNA-*in situ* hybridisation [RNA-ISH] for the detection of viral *tRNA*-like transcripts [*vtRNAs*] using established protocols [8]) of tissue

harvested at 7 days p.i. The results revealed similar changes in both species. Lungs had mildly increased interstitial cellularity represented by increased numbers of lymphocytes, and a B cell-dominated perivascular and peribronchial lymphocyte-dominated infiltration. This was much less intense in bank voles than in wood mice (Fig. 2A, B, [8]). In addition, necrotic alveolar epithelial cells were observed in both species (Fig. 2C, D). In bank voles, these were observed in association with a few focal aggregates of macrophages and fewer lymphocytes and neutrophils (granulomatous infiltrates; Fig. 2D). In wood mice, the necrotic cells were seen disseminated and as small aggregates and the granulomatous infiltrates were more numerous (Fig. 2C and (9)). In both species, virus antigen (detected by a rabbit polyclonal antibody to virus structural antigens [8]) was detected in alveolar epithelial cells and in macrophages within granulomatous infiltrates, but to a far lower degree in bank voles than we have observed in wood mice (Fig. 2E, F and (9)). Viral *tRNA* transcripts indicative of virus latency [3] were detected in small numbers of lymphocytes within the perivascular/peribronchial accumulations and occasionally within veins in the bank voles (Fig. 2G), whereas they were only found in a single alveolar epithelial cell in one wood mouse (data not shown). Furthermore, regional (mediastinal/bronchial) lymph nodes of bank voles exhibited ill-defined follicles without evidence of germinal centre formation, and mild sinus histiocytosis (presence of macrophages in the sinuses). Here, scattered macrophages expressing viral antigen (Fig. 2H) and scattered lymphocytes expressing *vtRNA* (Fig. 2I) were seen. In contrast, we found that the mediastinal/bronchial lymph nodes of wood mice exhibited large secondary follicles and mild to moderate sinus histiocytosis, while again only scattered lymphocytes expressed *vtRNA*, Virus antigen-positive macrophages were more numerous than in bank voles.

Within the spleen at 7 days p.i., neither species showed any changes in comparison to uninfected animals.

At day 14 p.i. the type and distribution of changes in the lungs were similar in bank voles and wood mice. A mild diffuse increased interstitial cellularity was seen and there were still small aggregates of mononuclear cells (macrophages, lymphocytes; granulomatous infiltrates). Moderate (bank voles) to intense (wood mice) diffuse perivascular and peribronchial lymphocyte infiltration was observed. Immunohistology identified T and B cells in approximately equal amounts in these infiltrates in both species. In addition, focal lymphocyte accumulations were seen in a perivascular/peribronchial distribution. In wood mice, these were often large B-cell dominated follicles with germinal centres that exhibited tingible body macrophages and some degree of cell turnover as identified by the presence of mitotic cells and apoptotic cells, representing iBALT (Fig.3A, B). In bank voles, they represented smaller B cell accumulations that contained or were surrounded by some T cells and, although reminiscent of follicles, did not exhibit germinal centres (Fig.3C-E). Viral antigen was not detected and, in contrast to wood mice where *vtRNA* expression within lymphocytes in perivascular/peribronchial infiltrations is relatively intense (9), *vtRNA* expression in bank voles was restricted to scattered lymphocytes in perivascular and peribronchial infiltrates (data not shown); results that support our qPCR analysis (Fig. 1B).

Finally, we found that the spleens of bank voles and wood mice exhibited similar features at 14 days p.i. The white pulp was comprised of moderately sized, well delineated secondary follicles with well defined germinal centres, and relatively small T cell zones. However, while wood mouse spleens had viral antigen in variable numbers of macrophages in the red pulp and occasional follicular tingible body macrophages, there was no evidence of viral antigen expression in bank vole spleens (data not shown). In wood mice, *vtRNA* was detected in variable numbers of lymphocytes/lymphoblasts within follicle centers and occasional lymphocytes and macrophages in the red pulp (9),

while in bank voles it was only seen in scattered lymphocytes within follicles in one of the three tested animals (data not shown), consistent with an inefficient establishment of latency.

Here we report experimental MuHV-4 infection of bank voles, an apparent natural but relatively rare host of MuHV-4 in the wild. Like in wood mice, the level of productive replication in the lungs of bank voles was significantly lower than in BALB/c mice. However, in the absence of high titres of infectious virus, a high degree of latency is still observed in wood mice lungs, presumably resulting from virus-driven iBALT [8]. This differs in bank voles, where very few cells harboured latent virus. Similarly, MuHV-4 was unable to establish a robust latent infection in the spleens of bank voles. Interestingly, although the virus did infect the spleen, there was no apparent amplification of latently infected cells in bank voles, as seen in BALB/c and wood mice. While in preparation, Francois et al [6] published a similar study comparing infection of bank voles with laboratory mice (BALB/c and CD1) showing results similar to ours, except importantly they did not compare infection with the more usual *Apodemus* host or characterise the level of MuHV-4 persistence in the lung, which is a prominent feature of infection in wood mice. Furthermore, in that study the authors downplay the large quantitative differences between the two host species they studied and state that *M. musculus* represents a suitable host for studying gammaherpesvirus pathogenesis with MuHV-4. We entirely agree with the latter view and have never attempted to diminish the importance of this model system – indeed, we continue to use it. Nevertheless, it is imperative that we understand the differences and limitations of all model systems if we are to make conclusions regarding gammaherpesvirus pathogenesis especially since it is universally agreed that this group of viruses have intimately evolved with their hosts.

Two separate studies [1, 14] have reported higher seroprevalence of MuHV-4 in wood mouse populations compared to bank vole populations. It is possible that these

studies may have been detecting WMHV [7] (a recently discovered virus highly similar to MuHV-4) or a combination of MuHV-4 and WMHV. However, a molecular study did not find any evidence of MuHV-4 in Bank voles [4]. These findings together with the present study suggest that bank voles are an occasional and inefficient host for MuHV-4.

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Figure legends

Fig. 1. Reduced establishment of MUHV-4 infection in bank voles. Bank voles, wood and BALB/c mice were inoculated intranasally with 4 X 10^5 PFU MUHV-4, and lung and spleen tissue from three animals per species was harvested at the indicated time points for virological analysis. (A) Productive virus replication in the lungs of each species 7 days p.i. measured by plaque assay from lung lysates (after three freeze-thaw cycles). Represented are the mean numbers of plaques counted. (B) Mean copies of MUHV-4 DNA in the lungs of infected animals 7 days p.i. using a previously reported qPCR protocol [12]. The amount of input MUHV-4 DNA (measured by amplifying the viral gene *gp150*) was normalised by concurrently quantifying copies of the cellular gene *rpl8*. (C) Mean copies of MUHV-4 DNA in the spleens of each species, comparing days 7 and 14 p.i. DNA was quantified as in (B). Note the lack of virus amplification in bank voles between days 7 and 14. Error bars represent the standard deviation from the mean and statistically-significant differences between species were determined by two-way ANOVA with Bonferroni post-tests.

Fig. 2. Host response in the lungs of wood mice and bank voles at day 7 p.i. (A) Wood mouse. Several vessels exhibit a moderate lymphocyte-dominated perivascular inflammatory infiltrate (arrows). HE stain. (B) Bank vole. Occasional vessels exhibit a mild lymphocyte-dominated perivascular inflammatory infiltrate (arrow). HE stain. (C) Wood mice exhibit extensive multifocal macrophage-dominated mononuclear (granulomatous) infiltrates with necrotic cells (arrow). (D) In bank voles, the focal granulomatous infiltrates are far less numerous and contain only scattered necrotic cells (arrow).. A-D: HE stains. (E) Wood mouse: Viral antigen is extensively expressed in macrophages in granulomatous infiltrates (arrows) and occasionally seen in pneumocytes (arrowhead). Bar = 20µm. (F) In bank voles, viral antigen is expressed less abundantly, in macrophages in granulomatous infiltrates (arrow) and in scattered alveolar epithelial cells (arrowhead). E, F: Peroxidase anti-peroxidase method, Papanicolaou's haematoxylin counterstain. (G) In bank voles, scattered lymphocytes in the perivascular and peribronchiolar infiltrates exhibit *vtRNA* expression (arrows). Inset: Intravascular lymphocyte showing *vtRNA* expression (arrow). RNA-in situ hybridisation, Papanicolaou's haematoxylin counterstain. (H, I) Mediastinal lymph node, bank vole. (H) Several sinus macrophages express viral antigen (arrows). Peroxidase anti-peroxidase method, Papanicolaou's haematoxylin counterstain. (I) Scattered lymphocytes express *vtRNA* (arrow). RNA-in situ hybridisation, Papanicolaou's haematoxylin counterstain.

Fig. 3. Host response in the lungs of wood mice and bank voles at day 14 PI. (A, B) Lung, wood mouse. (A) Peribronchial follicle-like lymphocyte aggregates with germinal centre formation (iBALT; arrows). Inset: Mitotic (arrow) and apoptotic (arrowhead) cells in the follicles indicate cell turnover. HE stain. (B) Follicles are comprised of B cells (CD45R-positive). Avidin biotin peroxidase complex method, Papanicolaou's haematoxylin counterstain. (C-E) Lung, bank vole. (C) Peribronchial lymphocyte aggregates (arrows). HE stain. (D) Peribronchial lymphocyte aggregates are dominated by B cells (CD79a-positive), avidin biotin peroxidase complex method, Papanicolaou's haematoxylin counterstain. (E) T cells (CD3-positive) are in the minority in the peribronchial lymphocyte aggregates. Peroxidase anti-peroxidase method, Papanicolaou's haematoxylin counterstain. A, artery; B, bronchiole; V, vein

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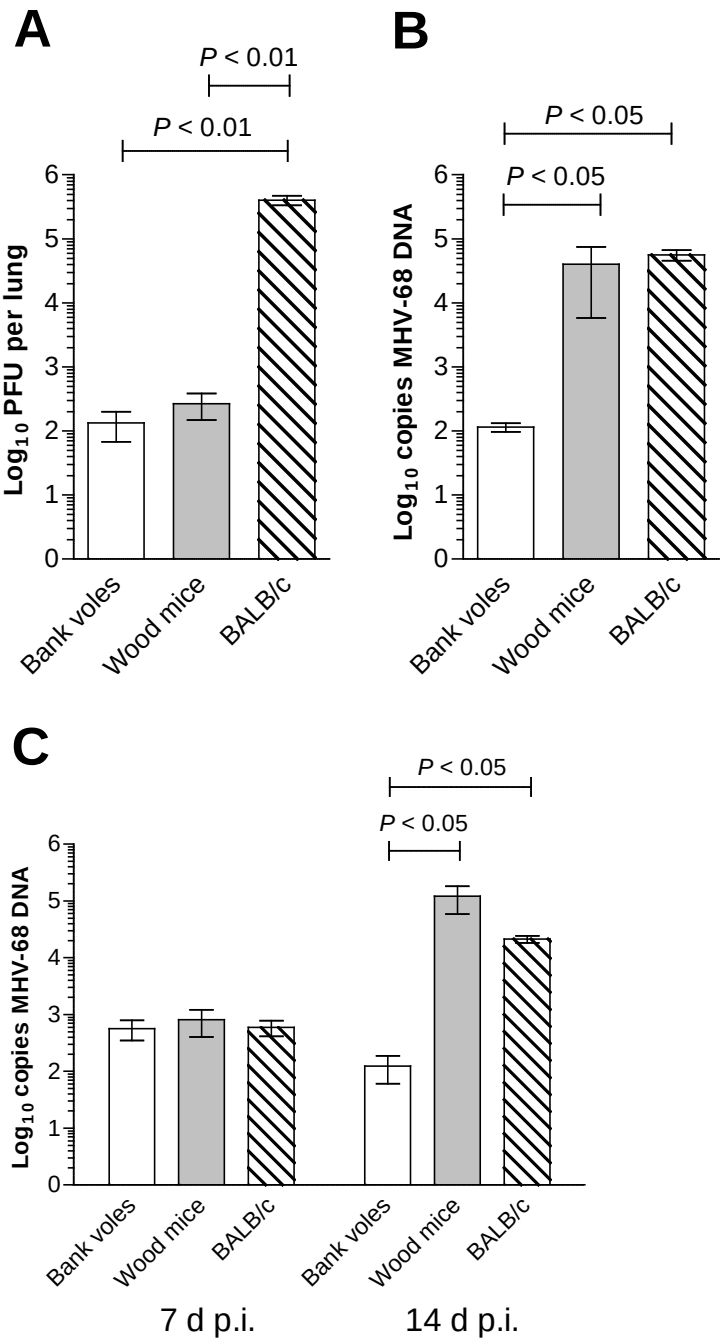
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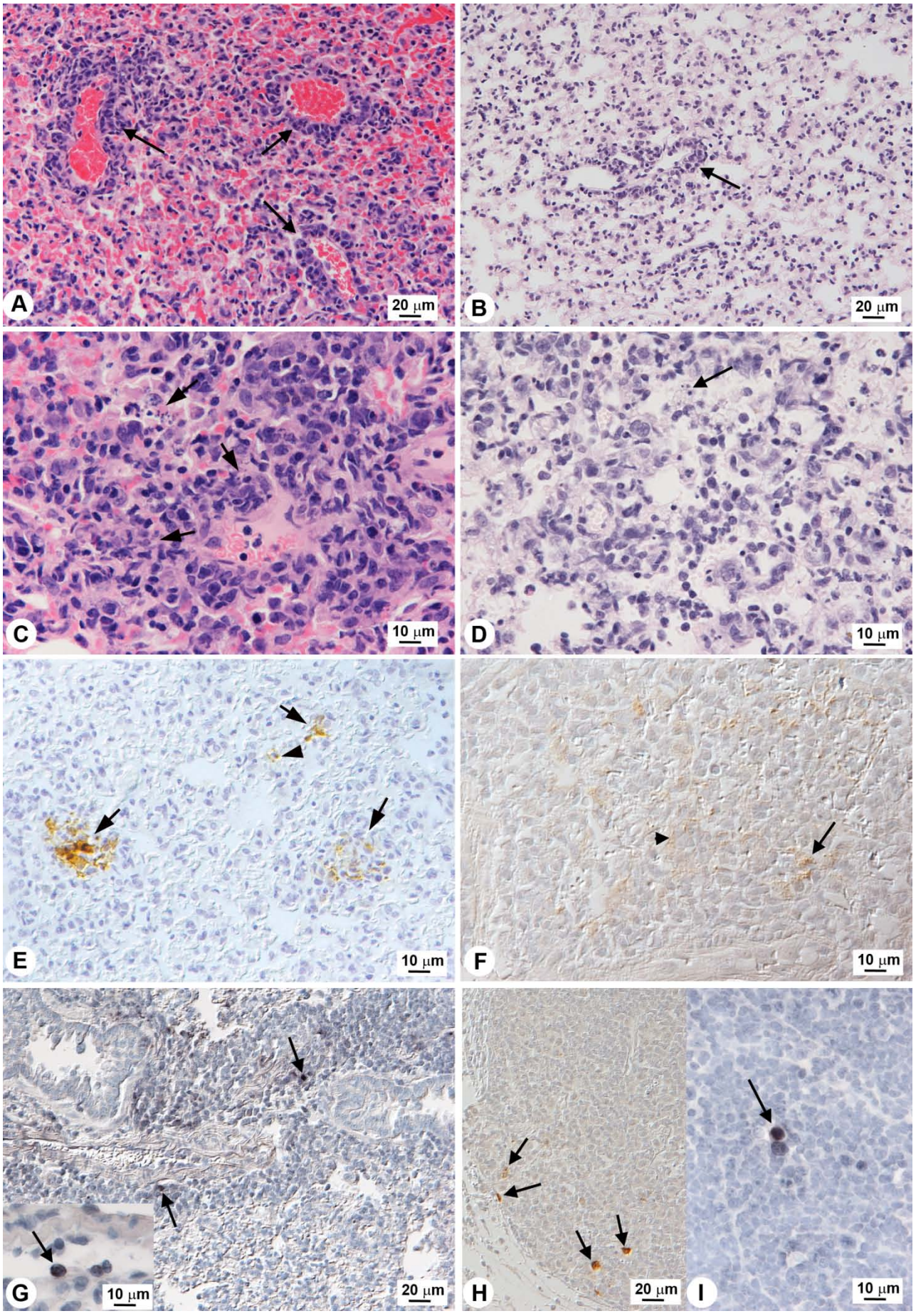
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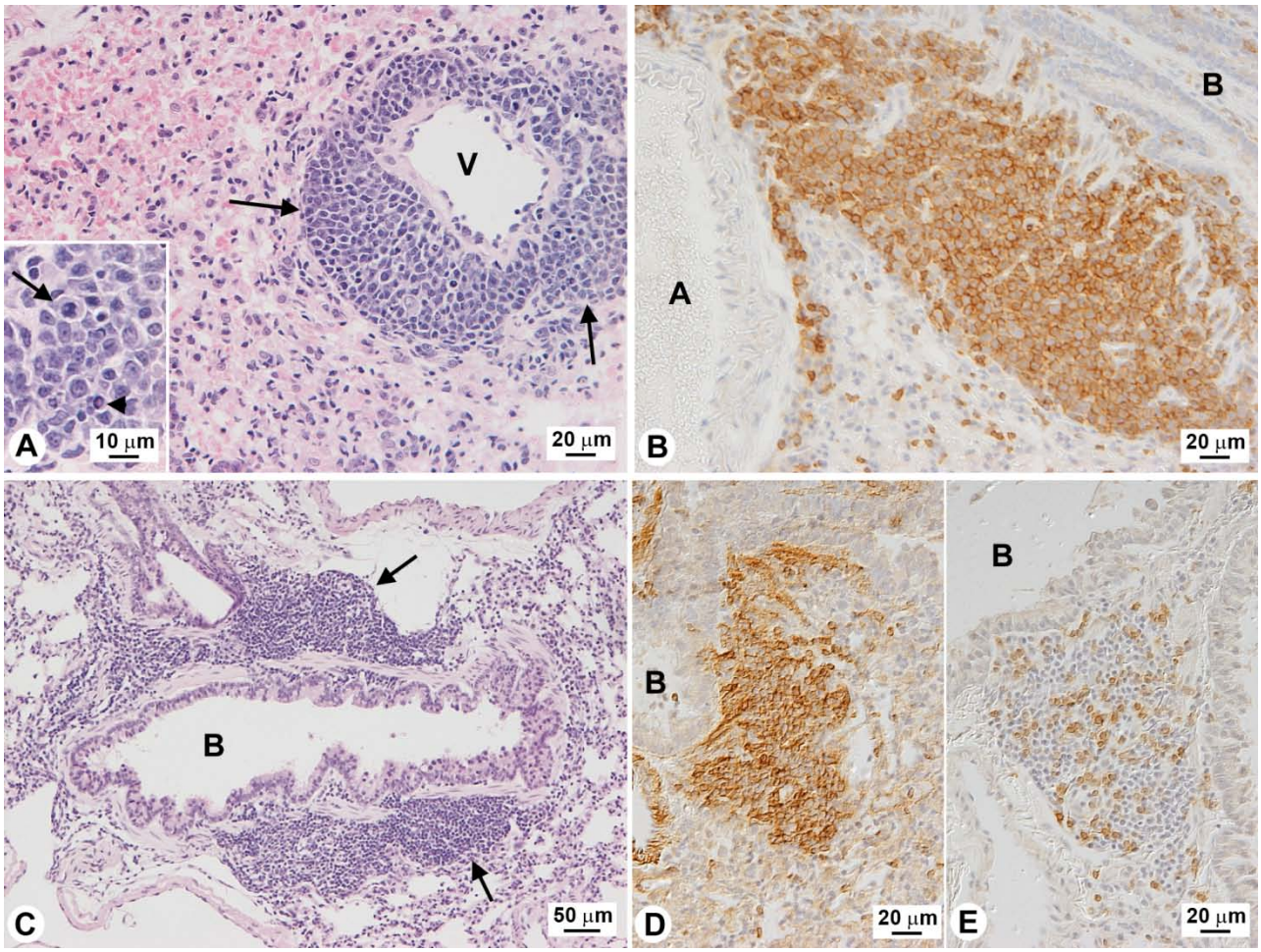
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