Altered Host Response to Murine Gammaherpesvirus 68 infection in mice lacking the tachykinin 1 gene and the receptor for substance P.

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ABSTRACT

The tachykinins are implicated in neurogenic inflammation and the neuropeptide substance P in particular has been shown to be a proinflammatory mediator. A role for the tachykinins in host response to viral infection has been previously demonstrated using either TAC1- or NK1 receptor-deficient transgenic mice. However, due to redundancy in the peptide-receptor complexes we wished determine whether a deficiency in TAC1 and $NK1^R$ in combination exhibited an enhanced phenotype. TAC1 and $NK1^R$-deficient mice were therefore crossed to generate transgenic mice in both ($NK1^{-/-}$ x $TAC1^{-/-}$). As expected, after infection with the respiratory pathogen murine gammaherpesvirus (MHV-68), TAC1 and $NK1^R$-deficient mice were more susceptible to infection than wild-type C57BL/6 controls. However, unexpectedly, $NK1^{-/-}$ x $TAC1^{-/-}$ mice were more resistant to infection arguing for a lack of feedback inhibition through alternative receptors in these mice. Histopathological examination did not show any great differences in the inflammatory responses between groups of infected animals, except for the presence of focal perivascular B cell accumulations in lungs of all the knockout mice. These were most pronounced in the $NK1^{-/-}$ x $TAC1^{-/-}$ mice. These results confirm an important role for TAC1 and $NK1^R$ in the control of viral infection but reinforce the complex nature of the peptide-receptor interactions

1. INTRODUCTION

The tachykinin family of neurotransmitters are not only involved in central and peripheral nervous system function but also have a role in inflammation (termed neurogenic inflammation) and adaptive immunity (Goetzl & Sreedharan, 1992; Maggi, 1997). The most characterized member of the family is substance P (SP). This is encoded by the preprotachykinin A (*PPT-A*) or *TAC1* locus. Alternative splicing of *TAC1* RNA and processing of the propeptide yields, in addition to SP, neurokinin A (NKA) and neuropeptides K and γ. Although local nerves have been believed to be the major source of tachykinins in the peripheral tissues, *TAC1* has been shown to be induced and expressed in other cell types such as monocytes, macrophages, pancreatic islet cells and various tumour cell types (Germonpre et al., 1999; Ho et al., 1997; Lambrecht et al., 1999; McGregor et al., 1995; Singh et al., 2000). This has led to the hypothesis that SP not only acts as a mediator of the neuroimmune system but is also involved in direct interaction between immune cells in a paracrine and/or autocrine fashion independent of sensory nerves (Ho et al., 1997; Lai et al., 1998). The tachykinins can modulate the immune response and SP has been shown to regulate production of a number of cytokines including IL-1, IL-6, IL-8 and TNFα to mediate inflammatory and cell proliferative responses (Lotz et al., 1988; Palma & Manzini, 1998).

We have previously addressed the actions of the *TAC1* gene expression and function in virus infection in the lung and in particular early function of neuropeptides released from non neuronal cells in the lung. The tachykinins have been extensively implicated in the initiation and progression of lung disease processes such as bronchitis and asthma (D'Agostino et al., 2002; Joos et al., 2000; Kamijo et al., 2001; Noveral & Grunstein, 1995), although it was presumed that it was the release from sensory ganglia that was the major effector. A number of studies have shown that viruses (e.g. respiratory syncytial virus and murine gammaherpesvirus, MHV-68) can induce SP and neurogenic inflammation, particularly in lungs in the context of a respiratory challenge (Payne et al., 2001; Piedimonte et al., 1999; Tripp et al., 2000). Consistent with this NK1 is upregulated in lymphocytes (especially T cells) in the lung in response to viral infection in the lung (Tripp et al., 2002)

and antibody to SP decreases inflammatory responses to pulmonary viral infection (Haynes et al., 2002). This indicates a primary role for SP in the lung in the response to respiratory viral infection. More recently we have demonstrated in a transgenic model in which the *TAC1* gene is co-expressed with the *LacZ* marker gene (MacKenzie et al., 2000; MacKenzie & Quinn, 2002) that non-neuronal *TAC1* expression early after infection may have important clinical implications for the progression or management of lung disease or infection aside from the well characterised later involvement of the tachykinins during the inflammatory response (Stewart et al., 2008). The action of MHV-68 is similar to that of RSV in the lung as regards tachykinin expression and function in which SP is increased (Dakhama et al., 2005) and altered $NK1^R$ expression is observed (Piedimonte, 2003). This alteration of tachykinin function and expression is observed in other challenges in the lung (Hegde et al., 2007; Ng et al., 2008; Puneet et al., 2006; Zhang et al., 2007). We therefore view the tachykinin pathway as a key major modulator of the immune response to lung challenge.

Experiments are usually done with either knock outs of the neuropeptide gene (*TAC1*) or the high affinity receptor ($NK1^R$). These knockouts have been generated on a variety of strains and strain differences have been demonstrated to have a major affect on biological importance. Further, in addition to the classical peptides SP and NKA, the recently discovered peptides such as hemokinin 1 and the endokinins have been found in non-neuronal cells/tissues such as pulmonary and cardiovascular tissue, articular cartilage and cells of the immune system (Zhang et al., 2000; Zhang & Paige, 2003) and they signal through affinity SP receptors, $NK\text{-}1^R$ (Morteau et al., 2001) adding a further layer of complexity to the tachykinin function in host defence. Given the redundancy and complexity in tachykinin peptides and receptors, we wished to generate a more stringent knockout that defined tachykinin function better. We surmised that a mouse lacking tachykinin peptides and high affinity receptor would address the problems of redundancy. We therefore generated a double knockout of *TAC1* and $NK1^R$ on the C57BL/6 genetic and studied their

response to respiratory infection with murine gammaherpesvirus 68 – a commonly used model pathogen of laboratory mice.

2. MATERIALS AND METHODS

2.1 Cell culture and virus stocks.

Murid herpesvirus 4 strain 68 (MHV-68) was originally isolated during field studies from the bank vole *Myodes glareolus* (Blaskovic et al., 1980) and was subsequently plaque purified on BHK-21 cells to obtain clone g2.4 as described previously (Efstathiou et al., 1990). Viruses were propagated and titrated using BHK-21 cells as described previously (Sunil-Chandra et al., 1992).

2.2 Generation of transgenic Tac1$^{-/-}$ x NK1$^{-/-}$ mice.

All animal work was performed under UK Home Office Project Licence number 40/2483. Transgenic NK1$^{-/-}$ (De Felipe et al., 1998) and TAC1$^{-/-}$ (Zimmer et al., 1998) mice both on a C57BL/6 background had been derived as described previously. Control C57BL/6 mice for use as controls were bred in house mice or purchased from Bantin and Kingman (Hull, UK). The NK1$^{-/-}$ and Tac1$^{-/-}$ mouse lines were cross bred together to produce an F1 progeny of mice that where heterozygous for the null deletion at both the *NK1* and *Tac1* allele locus (NK1$^{-/+}$, Tac1$^{-/+}$). The F1 offspring where then backcrossed at sexual maturity to a null NK1$^{-/-}$ to produce an F2 generation of which 1 in 4 were null at the *NK1* locus and heterozygous at the *Tac1* locus (NK1$^{-/-}$, Tac1$^{-/+}$). The F2 (NK1$^{-/-}$, Tac1$^{-/+}$) mice were then crossed together to produce an F3 generation in which 50% of the offspring were null mutants at both the *NK1* and *Tac1* loci. These mice were breed and the double knockout line was maintained with sister x brother mating. This breeding strategy was chosen to adhere to the 3Rs (reduction, replacement, refinement) with regards to reduction of mice with unwanted genotype and to maximise double knockout survival chances (refinement). Males and females were separated at weaning (19-21 days after birth) and housed in conventional cages. They were grouped up to six animals per cage for maintenance under standard conditions (12h light/dark cycle, 22 +/- 1 ^{o}C ambient temperature, 60% relative humidity, food and water provided *ad libitum*).

2.3 Infection of animals.

Animals 5 – 7 weeks of age were anesthetized with halothane and inoculated with 4 x 10^5 plaque forming units (PFU) in 40 μl of sterile phosphate buffered saline (PBS). At various times between day 3 and 40 p.i., animals were euthanized and tissues were harvested.

2.4 Real-time quantitative PCR analysis.

To determine the viral DNA (vDNA) load, DNA was extracted from splenic and pulmonary tissue using DNeasy kits (Qiagen) and quantified by UV spectrophotometry. Quantification of vDNA copy number was performed using an Opticon Monitor 2 real-time PCR machine (MJ Research) using primers specific for the MHV-68 gp150 gene as described previously (Hughes et al., 2010). Copy numbers were normalized relative to the murine ribosomal protein L8 (*rpl8*) gene Mean viral genome copy numbers were determined from four individual animals. Statistical analysis was performed using student's T-test with a confidence limit of 95%.

2.5 Histology, immunohistology and RNA-in situ hybridization.

Lungs from all animals were fixed in 4% buffered paraformaldehyde (PFA; pH 7.4) and embedded into paraffin wax. Sections (3-5 μm) were cut and stained with haematoxylin and eosin, or used for immunohistology and RNA-*in situ* hybridization (RNA-ISH).

Immunohistology was performed on lungs, to detect viral antigen, to identify infiltrating leukocytes (T cells, B cells and macrophages), using both the peroxidase anti-peroxidase (PAP) method and the avidin biotin peroxidase complex (ABC) method as described previously (Kipar et al., 2001). For the detection of MHV-68 antigen, a polyclonal rabbit antiserum was used that had been generated in rabbits injected with purified MHV-68 particles. T cells were detected by a cross-reacting rabbit anti-human CD3 antibody (DAKOCytomation), and B cells were identified using rat anti-mouse CD45R (clone RA3-6B2; SouthernBiotech). Macrophages were identified using rat anti-mouse F4/80 antigen

(clone Cl:A3-1; Serotec) and a cross-reacting rabbit antibody against human lysozyme (DAKOCytomation).

RNA-ISH followed previously published protocols (Hughes et al., 2010) and used digoxigenin (DIG; Roche) labeled sense and antisense transcripts to the MHV-68 tRNA molecules 1-4, which were made from plasmid pEH1.4 as described previously (Bowden et al., 1997).

3. RESULTS

3.1 TAC1$^{-/-}$ x NK1$^{-/-}$ mice are more resistant to MHV-68 infection.

Groups of mice (n = 4) from each genetic background (C57BL/6, NK1$^{-/-}$, TAC1$^{-/-}$, NK1$^{-/-}$ x TAC1$^{-/-}$) were infected with MHV-68 via the intra-nasal route. At 7 days post-infection (p.i.), the mice were euthanized and lungs analyzed for viral load by quantitative PCR (q-PCR). Two lobes were separately processed for histopathologcial analysis (see below). In the first experiment, infection of wild-type C57BL/6 was compared with NK1$^{-/-}$ and TAC1$^{-/-}$ mice. The results (Fig. 1A) showed that viral loads in TAC1$^{-/-}$ and NK1$^{-/-}$ mice were approximately 3 fold higher than in wild-type mice ($P < 0.01$). In contrast, in a comparable experiment (Fig 1B) it was found that the viral load at 7 days p.i. in NK1$^{-/-}$ x TAC1$^{-/-}$ x mice was approximately 15 times lower than in wild-type mice. Thus, while mice lacking either the peptides produced by the TAC1 locus or their receptors are more sensitive to MHV-68 infection, those lacking both peptides and receptors are more resistant to infection.

3.2 Uninfected transgenic mice show no abnormalities at the histopathological level

Uninfected control animals (n = 3) from each group of transgenic mice (C57BL/6, NK1$^{-/-}$, TAC1$^{-/-}$, NK1$^{-/-}$ x TAC1$^{-/-}$ were examined histologically for phenotypic changes. Brain, lungs, small and large intestine, as well as lymphatic tissues (spleen, thymus, mediastinal lymph nodes) were assessed and did not provide any evidence of pathological changes. In particular the lymphatic tissues were similar in all groups with regard to activity and cellular composition, as identified by immunohistology for T cells, B cells and macrophages.

3.3 NK1$^{-/-}$, TAC1$^{-/-}$ and NK1$^{-/-}$ x TAC1$^{-/-}$ mice all develop focal perivascular B cell aggregates at 7 days p.i.

At 7 days p.i., C57BL/6 mice showed the typical lung changes previously described in laboratory mice (Hughes et al., 2010). This comprised a moderate to marked multifocal infiltration by T cells with fewer macrophages and B cells in association with variable numbers of necrotic cells, a proportion of which showed evidence of viral antigen

expression. Viral antigen was also observed in alveolar epithelial cells (type I and II pneumocytes). There was evidence of leukocyte recruitment (T and B cell rolling) and vasculitis (mainly phlebitis (Fig. 2A), occasionally arteritis). Mild to moderate T cell dominated perivascular lymphocyte infiltration was also observed (Fig. 2B, C). There was a general increase in the number of interstitial (lysozyme-positive) macrophages and alveolar lumina often contained individual desquamed alveolar macrophages.

In $NK^{-/-}$ mice, the changes were very similar to those observed in WT mice. Vasculitis was present, but generally less intense than in WT mice. All animals showed distinct T and to a lesser extent B cell recruitment from vessels, mild to moderate phlebitis and perivascular T and B cell infiltration (Fig. 2D, E). However, in contrast with infected WT mice, focal perivascular B cell aggregates were observed (Fig. 2F).

In infected $TAC^{-/-}$ mice, changes very similar to those observed in $NK^{-/-}$ mice were seen. There was evidence of an increase in the proportion of B cells in the perivascular lymphocyte infiltrates together with the presence perivascular B cell aggregates (Fig. G-I).

In infected $NK^{-/-}$ x $TAC^{-/-}$ mice, changes were in general similar to those seen in the other groups (Fig. J, K). However, the perivascular focal B cell aggregates were larger than in $NK^{-/-}$ or $TAC^{-/-}$ (Fig. 2L). The inflammatory changes in blood vessels, in particular the phlebitis, appeared more intense than in the $NK^{-/-}$ x $TAC^{-/-}$ mice and were similar to WT mice (Fig. 2J).

4. DISCUSSION

In this study, using genetically manipulated mice, we have confirmed previous results that deletion of the *TAC1* gene and consequent lack of both SP and NKA leads to an increased susceptibility to murine gammaherpesvirus 68 infection. NK1 receptor deficiency mirrored the lack of *TAC1* peptides. Interestingly, the lack of both *TAC1* and *NK1*^{R} led to an increased resistance to murine gammaherpesvirus 68 infection. The pattern of histopathological changes after infection was generally similar in all animals, except for the presence of focal perivascular B cell accumulations in all the knockout mice. These were most pronounced in the NK1$^{-/-}$ x TAC1$^{-/-}$ mice.

Tachykinins either released locally from nerves, epithelial cells or macrophages in the lung affect inflammation by acting at receptors localized on peripheral nerve terminals, vascular endothelial, bronchial epithelial and inflammatory cells. We and others have shown that mice deficient in either TAC1 peptides or NK1^{R} are more susceptible to virus infection likely due to a decreased inflammatory response and specific cytotoxic T cell response (Elsawa et al., 2003; Payne et al., 2001; Svensson et al., 2005). The increased resistance of NK1$^{-/-}$ x TAC1$^{-/-}$ to infection is therefore surprising and suggests that while the interaction of TAC1 peptides with NK-1^{R} is clearly important for the host response to viruses, there are complex regulatory mechanisms in play. Thus, in the absence of TAC1, although TAC1 peptide-NK1^{R} interactions are absent, NK1^{R} interactions with other tachykinins e.g hemokinin 1 would still be possible. Likewise, in the absence of NK1, while TAC1 peptide-NK1^{R} and hemokinin-NK1^{R} responses are absent, TAC1 peptides could still interact with other receptors, e.g. NK2. An increased resistance in the absence of both TAC1 peptides and NK1^{R} may suggest a lack of inhibitory signaling on components of the host response mediated by these peptide-receptor combinations.

The major difference in pathology between the groups of mice was the presence of perivascular B cell accumulations in all 3 groups of knockout animals, which were not observed in wild type mice. The differences in viral load observed did not correlate with the presence or absence of these B cell accumulations. Thus, they did not appear to be

contributing the antiviral response in the lung. However, the focal B cell aggregates were associated with a lack of TAC1 peptide-NK1 interactions and may suggest an inhibitory action of $NK1^R$ on B cell recruitment and accumulation during viral infection. Focal accumulations of B cells in the lung and the induction of bronchus-associated lymphoid tissue after viral infection are chemokine-driven and not generally seen in rodents, unlike humans and other large mammals. Indeed, this has only been observed in genetically altered mice deficient in secondary lymphoid organs (Moyron-Quiroz et al., 2004). Thus, the observation of focal B cell accumulations after infection is intriguing and suggests an important role for $NK1^R$ and cognate peptides in the regulation of their formation. These mice might also be important tools for studying the generation of B cells in the lung in response to infection.

The results presented here emphasize the important role that TAC1 peptides and NK1 receptor have in the response to murine gammaherpesvirus 68 infection. However, further work is required to dissect out the complex interactions involved.

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FIGURE LEGENDS

Figure 1. Virological Analysis of Infection in the Lung. Real-time quantitative fluorescent PCR analysis of the amount of viral DNA (vDNA) present in the lungs mice. Data are presented as $\log_{10}$ copies of vDNA relative to the amount of cellular *rpl 8* gene DNA present in 200 ng of lung DNA sample analyzed. The data were compiled from analyses of four individual mice at each time point.

FIGURE 2. Histopathological Analysis of Lungs at Day 7 p. i. A-C. C57BL/6 mouse. A. Large vein showing lymphocyte recruitment (arrowhead), lymphocyte infiltration of the wall (phlebitis) and mild perivascular lymphocyte infiltration. Inset: Small artery with leukocyte infiltration (arteritis; arrow). T cells (B) dominate the perivascular infiltrates while B cells (C) represent a minority. D-F. $NK^{-}/^{-}$ mouse. Moderate circular perivascular lymphocyte infiltration

with T cells (E) and B cells (F), the latter also as small focal aggregates (arrow). G-I. $TAC^{-/-}$ mouse. Moderate circular perivascular lymphocyte infiltration. T cells (H) are numerous in the infiltrate, but B cells (I) form focal aggregates (arrow). J-L. $NK^{-/-}$ x $TAC^{-/-}$ mouse. J. Moderate circular perivascular lymphocyte infiltration with lymphocyte recruitment and infiltration of the vessel wall (phlebitis; arrows). The perivascular infiltrates are comprised of T cells (K) and B cells (L). The latter also form larger focal aggregates. A, D, G and J: Haematoxylin eosin stain. B, E, H and K: Staining for CD3 antigen, Peroxidase anti-peroxidase method, Papanicolaou's haematoxylin counterstain. C, F, I and L: Avidin biotin peroxidase complex method, Papanicolaou's haematoxylin counterstain. V: vein; A: artery.

Figure(s)

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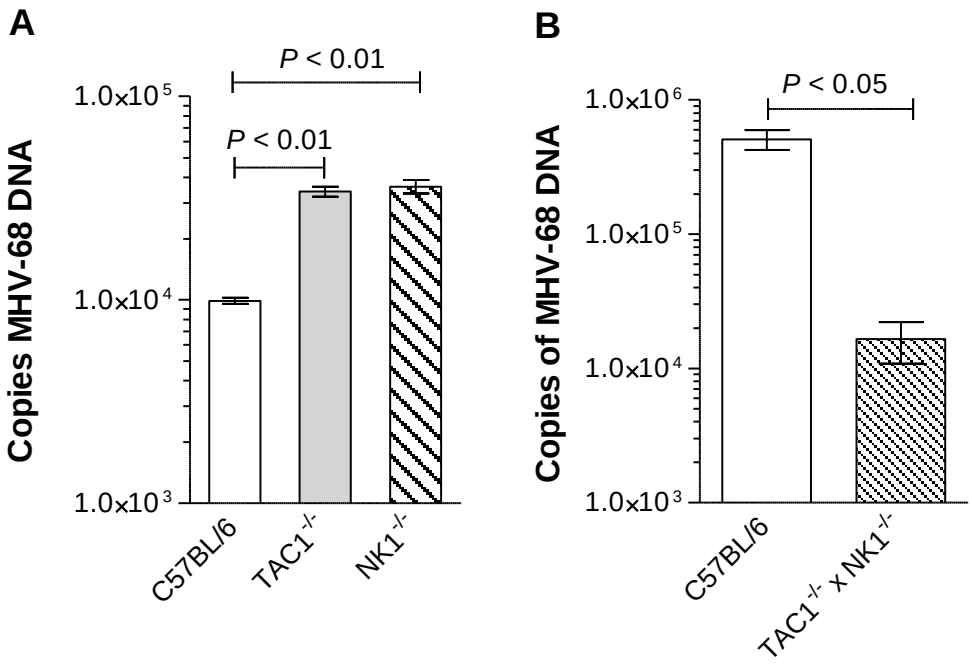

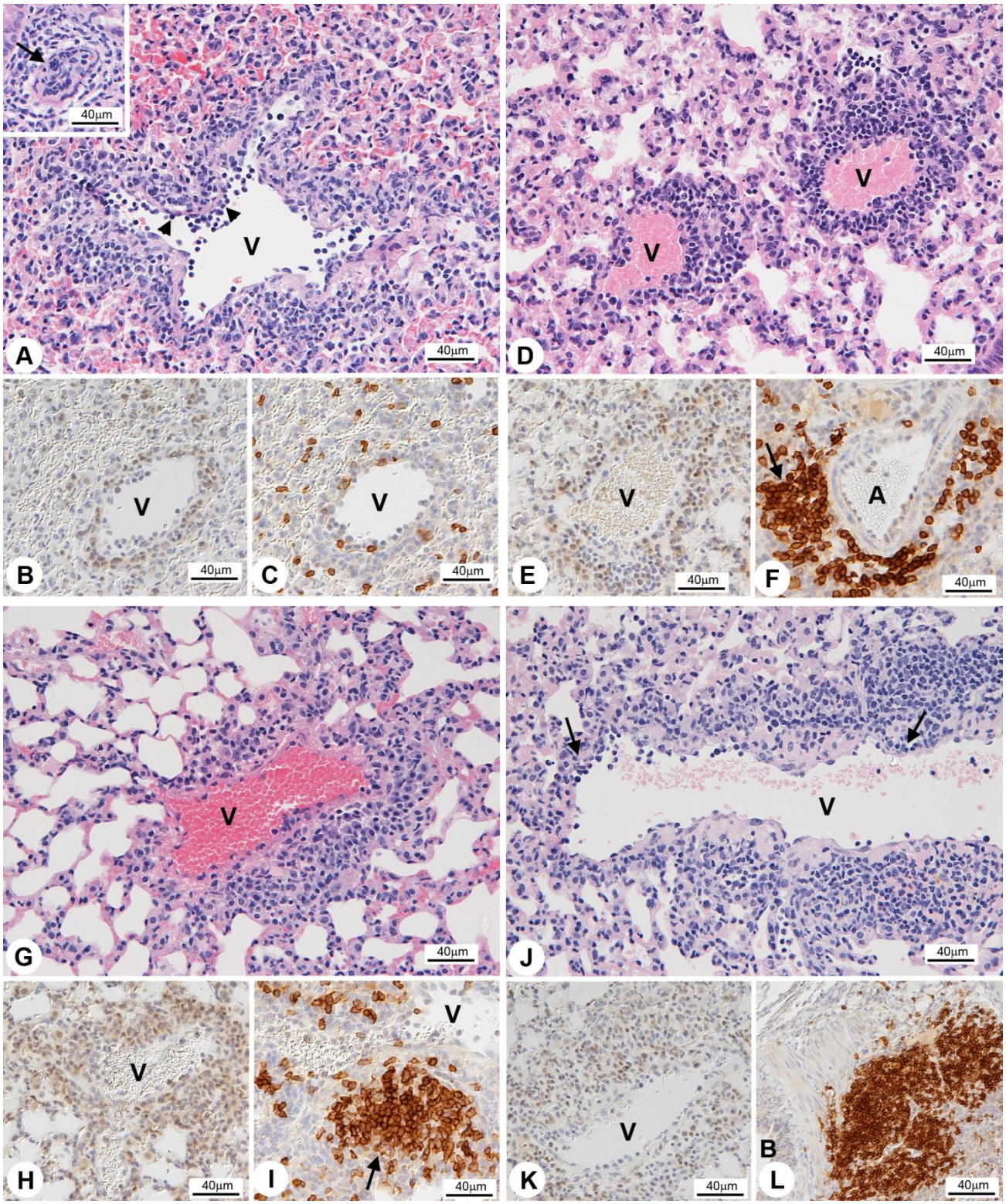
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