Alveolar macrophages are the main target cells in feline calicivirus-associated pneumonia

Monné Rodriguez, J M ; Soare, T ; Malbon, A ; Blundell, R ; Papoula-Pereira, R ; Leeming, G ; Köhler, K ; Kipar, A

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Original Article

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Abstract

Feline calicivirus (FCV) is a pathogen of felids and one of the most common causative agents of feline upper respiratory disease (URD). Reports of natural FCV pneumonia in the course of respiratory tract infections are sparse. Therefore, knowledge on the pathogenesis of FCV-induced lung lesions comes only from experimental studies. The aim of the present study was to assess the type and extent of pulmonary involvement in natural respiratory FCV infections of domestic cats and to identify the viral target cells in the lung. For this purpose, histology, immunohistochemistry and RNA-in situ hybridisation for FCV and relevant cell markers were employed in diagnostic post-mortem specimens collected after fatal URD, virulent systemic FCV or other conditions. All groups of cats exhibited similar acute pathological changes, dominated by multifocal desquamation of activated alveolar macrophages (AM) and occasional type II pneumocytes with fibrin exudation, consistent with diffuse alveolar damage (DAD). In fatal cases, this was generally seen without evidence of epithelial regeneration. In cats without clinical respiratory signs, type II pneumocyte hyperplasia was present alongside the other changes, consistent with the post-damage proliferative phase of DAD. FCV infected and replicate in AM and, to a lesser extent, type II pneumocytes. This study shows that lung involvement is an infrequent but important feature of FCV-induced URD. AM are the main viral target cell and pulmonary replication site, and their infection is associated with desquamation and activation, as well as death via apoptosis.

Keywords: Feline calicivirus; Pulmonary infection; Alveolar macrophages; Immunohistochemistry; RNA-in situ hybridisation

Introduction

Feline calicivirus (FCV) is one of the most common causative infectious agents of feline upper respiratory disease (URD; Bannasch and Foley, 2005; Di Martino et al., 2007; Gaskell et al., 2012). URD is widespread and common in cats and presents with a variety of clinical signs, including conjunctivitis, rhinitis, oral ulcers and, occasionally, pneumonia (Hurley and Sykes, 2003; Gaskell et al., 2006; Radford et al., 2007; Pesavento et al., 2008; Radford et al., 2009). The mortality rate is usually low, but occasionally kittens develop fatal pneumonia (Love and Baker, 1972; Turnquist and Ostlund, 1997).

FCV is a member of the Caliciviridae, which possess a non-enveloped, positive-sense, single-stranded RNA genome of approximately 7.5 kb (Clarke and Lambden, 1997). The virus exhibits high genetic variability and there are a variety of natural strains (Radford et al., 2003) with variable cell tropism, pathogenesis and virulence, resulting in different clinical manifestations, such as self limiting URD, lameness due to acute synovitis (Dawson et al., 1994) and systemic disease caused by highly virulent strains, so-called virulent systemic (VS)-FCV (Hurley and Sykes, 2003; Radford et al., 2007; Pesavento et al., 2008). The genetic diversity of FCV is a consequence of changes in the hypervariable C and E regions that encode part of the capsid proteins involved in the generation of neutralising antibodies (Radford et al., 1999; Geissler et al., 2002). Animals develop a protective immune response against FCV (Kahn et al., 1975; Kahn and Hoover, 1976; Scott, 1977); however, continual genomic changes in the virus can lead to reinfections with different strains or closely related variants of the same strain, despite previous infection episodes (Johnson, 1992; Radford et al., 2003; Coyne et al., 2006b, 2007). Due to the genetic instability of FCV, effective disease prophylaxis has remained challenging (Radford et al., 1997) and, after years of vaccination programmes, infection is still widespread, leading to the search for new heterologous vaccines

that can protect against more than one strain (Poulet et al., 2005; Radford et al., 2006). Moreover, in 1998, new highly virulent VS-FCV strains emerged. The first VS-FCV cases were observed in California (Pedersen et al., 2000), but further outbreaks have subsequently been reported from the USA (Schorr-Evans et al., 2003; Hurley et al., 2004; Pesavento et al., 2004) and, more recently, Europe (Coyne et al., 2006a; Schulz et al., 2011; Battilani et al., 2013; Velasco et al., 2013). The most frequent clinical signs are cutaneous oedema (mainly on the head and limbs), ulceration of the skin and mucosa, predominantly affecting the oral cavity, nares, pinnae and footpads, and clinical signs due to multi-organ necrosis, most commonly affecting the liver, but occasionally also the spleen, pancreas or lungs (Pedersen et al., 2000; Hurley and Sykes, 2003; Coyne et al., 2006a; Radford et al., 2007; Pesavento et al., 2008; Radford et al., 2009).

Reports of natural FCV pneumonia are sparse and our understanding of the pathogenesis of FCV-induced lung lesions is from experimental studies only. These used high doses (up to 2×10^4 median tissue culture infective dose) of virus propagated in tissue culture in an aerosol for the intranasal infection of kittens and young cats, which often resulted in pulmonary involvement (Holzinger and Kahn, 1970; Kahn and Gillespie, 1971; Hoover and Kahn, 1973, 1975; Love, 1975; Langloss et al., 1978a). The development and type of lung lesions was generally similar; after an initial phase of pneumocyte injury with exudation and neutrophil infiltration, proliferation of type II pneumocytes and desquamation of alveolar macrophages (AM) into the alveolar lumen was observed. Virus was detected by immunofluorescence in pneumocytes and AM (Holzinger and Kahn, 1970; Kahn and Gillespie, 1971). Some authors suggested a correlation between the type of virus inoculum and the clinical signs (Love, 1975; Ormerod et al., 1979), and it has since been accepted that the experimental studies overemphasise the relevance of FCV-associated pneumonia, since

natural infection mainly occurs via the oronasal route (Radford et al., 2007; Gaskell et al., 2012). However, other authors have suggested that severe pneumonia might not be rare in naturally infected cats (Pesavento et al., 2008).

Definitive reports of pneumonia as a complication of severe, naturally acquired FCV-associated respiratory disease are very rare (Love and Baker, 1972; Turnquist and Ostlund, 1997). Furthermore, attempts to demonstrate the virus in the lungs to assess whether FCV or bacteria, such as *Bordetella bronchiseptica*, were the relevant pulmonary pathogen, have not been made (Turnquist and Ostlund, 1997). This study, which was initiated after acute pneumonia and FCV infection had been diagnosed post-mortem in a number of cats with URD, aimed to assess the type and extent of pulmonary involvement in natural respiratory FCV infections of domestic cats, and to identify the viral target cells in the lung. For this purpose, histopathology, immunohistochemistry (IH) and RNA-in situ hybridisation were employed on post-mortem specimens from cats with fatal URD, VS-FCV or other conditions.

Materials and methods

Animals and tissues

The study was performed on specimens from cats from Germany, the UK, Finland, Italy and Spain that had undergone full diagnostic post-mortem examinations. Tissue specimens had been collected for histological examination and, in some cases, for virological and bacteriological examinations (Tables 1-4). Four groups were included. Group 1 comprised eight cats with clinical histories of URD and pneumonia. FCV was isolated from the lungs by virus culture and involvement of FCV in the pneumonia was confirmed by IH. Group 2 comprised five cats with URD and pneumonia. Virus culture was not performed, but FCV involvement was confirmed by IH. Group 3 comprised two cats without URD. These had been euthanased because of feline parvovirus infection, but exhibited histopathological

findings consistent with FCV pneumonia, confirmed by FCV IH. Group 4 comprised four cats with VS-FCV; pulmonary specimens from these cats were examined by IH for the presence and distribution of FCV. Most of the cats in group 4 have since been reported as confirmed cases of VS-FCV in the UK (Coyne et al., 2006a), Italy (Battilani et al., 2013) and Spain (Velasco et al., 2013).

All lung tissue specimens were fixed in 10% non-buffered formalin for 24-72 h, followed by trimming and routine paraffin wax embedding. Specimens of tongue, larynx or nose were also examined when they showed gross lesions associated with URD. Other tissues/organs (spleen, liver, kidney, intestine, brain) were processed for histological examination, to identify or exclude any concurrent disease.

Histology

Sections (3-5 µm thick) were prepared and stained with haematoxylin and eosin for histopathological examination. Consecutive sections were prepared and mounted on polysine slides (VWR International Eurolab S.L) for IH and RNA-in situ hybridisation.

Immunohistochemistry

IH was used to demonstrate viral antigen (FCV and, in most specimens from groups 1 and 2, FHV, to exclude FHV infection of the lung and to confirm the involvement of FHV in one case of ulcerative glossitis,), type II pneumocytes (surfactant protein, SP-C, positive), AM (CD18 positive), apoptotic cells (cleaved caspase-3 positive) and the functional marker matrix metalloproteinase (MMP)-9 in the lungs. FCV antigen expression was also assessed in other lesions consistent with URD to confirm aetiology and in other organ specimens from cats with VS-FCV (Coyne et al., 2006a; Battilani et al., 2013).

The peroxidase anti-peroxidase (PAP), avidin biotin complex peroxidase (ABC) and horseradish peroxidase (HRP) methods were applied, as previously described (Kipar et al., 2005; Coyne et al., 2006a; Leeming et al., 2006). All antibodies, antigen retrieval and detection methods are listed in Table 5. The two antibodies against FCV were both used on consecutive sections of the same case (cats in group 1; with similar staining results), or were used alternatively.

A formalin-fixed and paraffin-embedded cell pellet prepared from an FCV infected tissue culture (see below), and sections prepared from a case of glossitis with strong expression of FCV antigen, a similarly prepared FHV-infected cell pellet and a specimen from a cat with necrotising rhinitis with strong FHV antigen expression served as positive controls for the virus IH; a specimen with granulomatous dermatitis was used as control for the functional markers. Negative controls were represented by consecutive sections in which the primary antibody was omitted or replaced by a non-reacting polyclonal antibody against *Toxoplasma gondii* or a mouse monoclonal antibody against canine distemper virus.

RNA-in situ hybridisation

Virus cultivation, RNA extraction and cDNA preparation - The FCV strain F9 (Carter et al., 1992) was cultured in feline embryo A (FEA) cells using standard protocols (Povey and Johnson, 1971; Carter et al., 1992) and a multiplicity of infection of 0.001 plaque forming units/cell in a volume of 5 mL growth medium. RNA was extracted and reverse transcribed into cDNA, as previously described (Coyne et al., 2007).

174 *Preparation of riboprobes* - Nine full genome FCV nucleotide sequences were retrieved
175 from GenBank[1] (accession numbers AY560115, AY560113, M86379, D31836, AF479590,
176 L40021, AF109465, DQ424892 and AY560117) and aligned using CLUSTAL W alignment
177 editor MEGA version 4 (Tamura et al., 2007). A pair of primers, forward and reverse, were
178 designed to amplify a relatively conserved region of ORF1 from residues 2420 to 2550 of the
179 FCV genome (FCV 2420-fw: 5'-GAACTACCCGCCAATCAACATGTGGTAAC-3' and
180 FCV 2550-rev: 3'-GGGACAGTTAGCACRTCRTATGCGGC-5'), to produce an amplicon of
181 130 base pairs (A.D. Radford, personal communication). The PCR product amplified from the
182 viral cDNA was cloned into a plasmid vector (pCRII, Invitrogen, Life Technologies), using a
183 commercially available kit (TOPO TA cloning kit, Invitrogen), and a digoxigenin (DIG)-
184 labelled probe was generated by in vitro transcription, as previously described (Hughes et al.,
185 2011), using a DIG RNA labelling kit (Roche Diagnostics) to generate sense and anti-sense
186 riboprobes. The correct concentration of the probe for use on tissue sections was determined
187 empirically using dot blots and RNA-ISH on formalin-fixed, paraffin-embedded pelleted cells
188 from FCV-infected cell cultures.
189
190 *RNA-in situ hybridisation* - RNA-ISH was performed according to a previously
191 published protocol (Kipar et al., 2005). After deparaffinisation, sections were digested with
192 proteinase K (1 µg/mL, Roche Diagnostics) for 25 min to expose the target RNA, followed by
193 post-fixation, acetylation and prehybridisation steps. Hybridisation was performed by
194 incubation of slides at 37 ºC for 15-18 h; the hybridisation mix contained either the anti-sense
195 probe (for the detection of positive strand viral RNA) or the sense probe (for the detection of
196 negative strand, replicative intermediate stage viral RNA), respectively. After post-
197 hybridisation washing, probe binding was visualised with alkaline phosphatase-coupled anti-

[1] See: https://www.ncbi.nlm.nih.gov/genbank/.

198 DIG antibody (anti-DIG-AP FAb fragments; Roche Diagnostics) and nitroblue tetrazolium
199 chloride (NBT)/5-bromo-4-chloro-3-indolylphosphate (BCIP; SigmaFast BCIP/NBT; Sigma-
200 Aldrich). Sections were counterstained for 10 s with Papanicolaou's haematoxylin (Merck
201 KGaA). Sections that were incubated with the hybridisation mix alone served as negative
202 controls.
203
204 **Results**
205 *Histopathology associated with feline calicivirus infection of the lungs*
206 Pulmonary specimens from cats with URD and histological evidence of pneumonia,
207 from which FCV was isolated in culture (group 1), served to identify the type of pathological
208 changes induced in the lungs after natural FCV infection. Subsequent examination of the
209 lungs of all other groups (group 2: URD with pneumonia, confirmed by FCV IH alone; group
210 3: no URD, but pulmonary changes similar to groups 1 and 2; group 4: VS-FCV) confirmed a
211 range of key histopathological features that were consistently observed in all cats.
212
213 The main finding was multifocal desquamation of large, round, often vacuolated
214 mononuclear cells into the alveolar lumen, generally in association with fibrin exudation (Fig.
215 1). The extent of this feature varied, both with regard to the number and distribution of
216 affected alveoli and the number of desquamated cells in individual alveoli. The desquamated
217 cells were mostly AM, confirmed by their consistent, strong CD18 expression (Fig. 2A).
218 Among these were occasional type II pneumocytes, which were identified by their expression
219 of SP-C (Fig. 2B). The morphology of type II pneumocytes (large, cuboidal cells) often
220 suggested an activated state, which was also confirmed by IH, since both these cells and AM
221 expressed MMP-9 (Fig. 2C). A variable proportion of the desquamated cells were undergoing
222 apoptosis, exemplified by the typical morphology and the expression of cleaved caspase-3

(Fig. 2D). In areas with extensive alveolar fibrin exudation, fibrin was also found on the pleura. Occasionally, erythrocyte extravasation into the alveolar lumen was also observed (Fig. 1B). In rare cases (groups 2 and 3, $n = 1$ each), mild to focally moderate type II pneumocyte hyperplasia was observed (Fig. 1C). FCV antigen was expressed by variable numbers of desquamated AM, which were often apoptotic (Fig. 3). Occasional type II pneumocytes also expressed viral antigen (Fig. 3B, 3C inset).

In all eight cats in group 1, pulmonary changes were acute and locally extensive to diffuse, without evidence of alveolar epithelial cell regeneration or substantial interstitial leucocyte infiltration. Apart from one case (cat 1.5), all cats had alveolar fibrin exudation along with desquamation of AM and type II pneumocytes in the lungs (Table 1). When tested, FCV antigen was also demonstrated in URD lesions, i.e. ulcerative glossitis (cats 1.4 and 2.3) and rhinitis (cat 2.6). Interestingly, cat 1.5 exhibited FCV and FHV co-infection in the ulcerative glossitis, but only FCV was detected in the lung by both virus isolation and IH.

Bacteriology had been performed on all cats in group 1. Pulmonary bacterial infection with *Pasteurella multocida* was identified in two cats (cats 1.4 and 1.7); in one case (cat 1.4) there was a co-infection with γ-haemolytic *Streptococcus* sp. Septicaemia was diagnosed in six cats, either with a mixed population of bacterial isolates (cats 1.3-1.6) or haemolytic *Escherichia coli* (cats 1.1 and 1.8). One cat (cat 1.4) also had feline parvovirus enteritis, confirmed by IH.

Group 2 comprised six cats with URD and pulmonary changes that were identical to those in group 1. In these cases, FCV involvement was confirmed only by IH for FCV antigen. In most cases, IH for FHV had been performed concurrently to confirm that FHV

248 was not involved (Table 2). In group 2, all but one case (cat 2.4) had alveolar fibrin exudation.
249 In one case (cat 2.5), the presence of mild focal type II pneumocyte hyperplasia and moderate
250 interstitial macrophage infiltration indicated a slightly longer course of disease. FCV antigen
251 was also present in URD lesions, i.e. laryngitis (cat 2.2), glossitis (cat 2.3), and rhinitis (cat
252 2.6). In one case (cat 2.1), an unidentified *Mycoplasma* sp. was isolated from the lungs. In the
253 remaining cases, bacteriological examination either was not performed or did not yield any
254 predominant bacterial isolates.
255
256 Group 3 was represented by two cats that had been euthanased due to feline parvovirus
257 enteritis, without clinical or gross pathological signs of URD or pneumonia. Both exhibited
258 histopathological changes in the lung that were similar to those observed in groups 1 and 2,
259 the only difference being that they were generally only mild and patchy in the group 3 cats.
260 However, the presence of moderate multifocal type II pneumocyte hyperplasia (Fig. 1C)
261 indicated a longer course of disease than in most cats from groups 1 and 2 (Table 3).
262
263 In all cats in group 4, VS-FCV was confirmed by the presence of typical necrotising
264 organ lesions and, with the exception of cat 4.3, the isolation of FCV from oropharyngeal
265 swabs or organs, PCR and sequencing (Coyne et al., 2006a; Battilani et al., 2013; Velasco et
266 al., 2013). The lungs were always involved and showed typical multifocal desquamation of
267 AM and occasional type II pneumocytes (Table 4). One cat (cat 4.3) had marked alveolar
268 fibrin exudation and intense desquamation of cells into the alveolar lumina. A few
269 bronchioles had focal loss of the respiratory epithelium and apoptosis of sloughing epithelial
270 cells (Fig. 1D). These cells expressed FCV antigen, similar to the desquamated cells in the
271 alveolar lumina (Fig. 3D).
272

Feline calicivirus replication in the lung

Alongside the demonstration of viral antigen by IH, FCV was demonstrated by RNA-ISH (positive strand viral RNA; binding to anti-sense riboprobe) within desquamated AM (Fig. 4A, B). RNA-ISH using the sense probe yielded a positive signal in a small proportion of these cells, confirming that the virus was not only taken up by the cells, but replicated in the infected cells (Fig. 4A). Viral replication was also demonstrated in activated and hyperplastic type II pneumocytes (Fig. 4B, C). In the VS-FCV case with infection of bronchiolar respiratory epithelial cells, viral replication was also demonstrated in these cells (Fig. 4D).

Discussion

This retrospective study aimed to provide insight into the relevance and effect of FCV in the lungs of naturally infected cats. For this purpose, we initially examined a group of cats with URD that had been euthanased, underwent post-mortem examination and had histological evidence of acute pneumonia in which FCV was suggested to play a role, since the virus was isolated from the lungs. The key histological feature in these lungs was severe acute diffuse alveolar damage (DAD), represented by extensive multifocal to diffuse desquamation of AM (CD18 positive) and occasional type II pneumocytes (SP-C positive; Mulugeta and Beers, 2006) that accumulated in the alveolar lumen, together with variable amounts of fibrin and occasional erythrocytes. IH demonstrated FCV antigen in the desquamated cells, providing strong evidence that the virus played a pathogenic role in the lungs. The apparent lack of alveolar epithelial regeneration and/or inflammatory cell recruitment suggests that the cats died or were euthanased due to virus-induced acute DAD, as a fatal complication of severe FCV-induced URD.

297 We also included cats that had presented with URD and identical acute pulmonary
298 changes in which a virological examination had not been undertaken. Based on the results of
299 the first group, we considered IH demonstration of FCV in the desquamated cells to be
300 diagnostic for acute FCV-associated pneumonia. Subsequent application of the histological
301 criteria identified another two cases of suspected FCV-induced lung lesions, in which the
302 involvement of FCV was confirmed by IH. Interestingly, both cats also had feline parvovirus
303 enteritis. Considering that infection with feline parvovirus not only induces enteritis, but also
304 marked leucopenia and immunosuppression (Truyen et al., 2009), it is possible that the
305 observed, albeit mild, pneumonia, was a consequence of viral recrudescence from sites of
306 persistence, such as the tonsils, due to immunosuppression (Dick et al., 1989; Radford et al.,
307 2007; Gaskell et al., 2012). Also, in both cats, there was evidence of a slightly longer time
308 course of disease, since both exhibited multifocal type II pneumocyte hyperplasia, a feature
309 generally observed 6-7 days after pneumocyte damage (Myers et al., 1993; Caswell and
310 Williams, 2007). This has also been observed 7-14 days after experimental infection of cats
311 with FCV by the aerosol route (Hoover and Kahn, 1973, 1975; Ormerod et al., 1979).
312
313 The desquamated cells found in the alveolar lumen were mainly AM, admixed with a
314 few type II pneumocytes. These cells, both detached and, less frequently, attached to the
315 alveolar wall, were activated, as evidenced by their expression of MMP-9, a gelatinase (92
316 kDa gelatinase B) that digests collagen IV and thereby the basement membrane, and is
317 assumed to facilitate the inflammatory cell recruitment (Pardo et al., 1999; Kim et al., 2009).
318 In most cases with more extensive changes (i.e. severe URD and pneumonia), this cellular
319 reaction was associated with alveolar fibrin exudation, indicating that not only alveolar cells,
320 but also vascular endothelial cells were injured, leading to increased vascular permeability.
321 These vascular effects are typical of acute DAD and result from damage to the extracellular

matrix induced by, among other factors, MMP-9; consequently, DAD can be mediated by AM alone (Gibbs et al., 1999; Gushima et al., 2001).

FCV pneumonia shares many histopathological features with the lungs of fatal human avian influenza cases; both exhibit DAD, with an acute exudative phase and, later, a proliferative phase (Nakajima et al., 2013), whereby type II pneumocyte hyperplasia is a consequence of the initial epithelial and endothelial cell damage (Langloss et al., 1978a; Myers et al., 1993).

Pulmonary FCV infection was either associated with URD or appeared to be the only virally induced lesion in the respiratory tract, indicating that FCV reaches the lung by inhalation of aerosols after natural infection, confirming that the experimental approach of older studies was appropriate (Holzinger and Kahn, 1970; Kahn and Gillespie, 1971; Hoover and Kahn, 1973, 1975; Love, 1975; Langloss et al., 1978a; Ormerod et al., 1979). The pulmonary changes observed in the present study are similar to those described in experimentally infected cats, apart from the initial neutrophil recruitment in the latter, which might be due to the high viral dose administered (Holzinger and Kahn, 1970; Kahn and Gillespie, 1971; Hoover and Kahn, 1973, 1975; Love, 1975; Langloss et al., 1978a). However, in the lungs of human beings infected with avian influenza, substantial neutrophil infiltration has been documented (Nakajima et al., 2013).

The lung lesions in the four VS-FCV cases (group 4) were histopathologically similar to those in the other three groups. VS-FCV strains are highly virulent and have both a wider target cell spectrum and more intense cytopathic effect than other FCV isolates (Radford et al., 2007; Pesavento et al., 2008). In the lungs, this can result in infection and death of

347 respiratory epithelial cells, as indicated by the focal FCV infection and apoptotic loss of
348 bronchial epithelium observed in one cat in the present study.
349
350 In FCV pneumonia, the virus was mainly identified within AM. RNA-ISH to
351 demonstrate both positive and negative strand viral RNA showed that it is not only
352 phagocytosed by the AM, as suggested by previous in vitro studies (Langloss et al., 1978b),
353 but it also replicates in these cells. Similarly, we found that FCV infected both activated and
354 hyperplastic type II pneumocytes in the acute lesions and also replicated in those cells.
355 Hyperplastic type II pneumocytes could be a site for viral persistence in the lungs. Feline
356 junctional adhesion molecule A (fJAM-A), an immunoglobulin-like molecule that is
357 expressed in a wide range of feline cells, including epithelial and endothelial cells and
358 leucocytes (Makino et al., 2006; Ossiboff and Parker, 2007; Pesavento et al., 2011), mediates
359 FCV attachment and entry into cells. The selected cell tropism of most FCV strains, however,
360 is likely mediated by α2,6-sialic acids, which are also responsible for the macrophage tropism
361 of murine noroviruses, which are also members of the Caliciviridae (Makino et al., 2006;
362 Ossiboff and Parker, 2007; Stuart and Brown, 2007). In contrast, VS-FCV might have an
363 altered receptor usage, which allows it to infect a wider range of cell types.
364
365 Our study provides strong evidence that FCV induces apoptosis in infected
366 macrophages and respiratory epithelial cells in vivo. This supports in vitro studies, which
367 demonstrated that FCV infection induces caspase activation, including caspase-3, which we
368 used to confirm cell death via apoptosis using IH (Sosnovtsev et al., 2003; Natoni et al.,
369 2006).
370

In a few cases, we found co-infection of FCV with FHV-1 or bacteria. FCV and FHV-1 co-infection of the upper respiratory tract is a known feature of URD and was recently found in 9% of cats that tested positive for either virus in a cat population in Brazil (Henzel et al., 2012). The presence of a concomitant bacterial infection with *Pasteurella multocida* or *Mycoplasma* spp., both opportunistic pathogens that are part of the normal nasopharyngeal bacterial population (Gaskell et al., 2012), are secondary and are likely be established after DAD.

Conclusions

In this study, lung involvement was an infrequent, but relevant, feature of FCV-induced URD. Acute FCV-induced lung lesions were consistent with DAD. AM were the main viral target cells and the site of viral replication in the lung, and their infection was associated with desquamation, activation and apoptosis.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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Table 1

Relevant gross and histological findings for group 1: Cats with upper respiratory disease (URD) and pneumonia, involvement of feline calicivirus (FCV) confirmed by isolation of FCV from the lungs and immunohistochemistry (IH) for FCV antigen.

Case	Breed	Age	Sex	Lung histopathology	Demonstration of FCV
1.1	ESH	11 years	F	Multifocal mild to moderate fibrin exudation and low numbers of AM/type II pneumocytes [a] in AL	IH: Scattered cells in AW (type II pneumocytes) and in AL (AM)
1.2	DSH	8 weeks	NK	Multifocal moderate to marked fibrin exudation and moderate numbers of often apoptotic [b] AM/type II pneumocytes with a few neutrophils in AL; bronchiolar lumen with similar content; moderate hyperaemia and perivascular oedema	IH: Moderate numbers of cells in AL, occasional cells in AW ISH (S, AS): Moderate number of cells in AL (IH, FHV: Negative)
1.3	DSH	12 weeks	F	Multifocal marked fibrin exudation and abundant AM/ type II pneumocytes in AL; bronchiolar lumen with similar content; diffuse fibrin exudation on pleura	IH: Low numbers of cells in AL ISH (S, AS): Low number of cells in AL
1.4	DSH	8 weeks	F	Multifocal moderate to marked fibrin exudation and abundant, occasionally apoptotic AM/type II pneumocytes in AL; moderate numbers of extravasated erythrocytes and erythrophagocytosis in AL; bronchioles with similar content and with focal loss and degeneration of epithelial cells and presence of macrophages	IH: Low to moderate numbers of cells in AL, some macrophages/degenerate epithelial cells in bronchiolar wall. ISH (S, AS): Low number of cells in AL
1.5	ESH	6 years	MN	Multifocally, low to moderate numbers of AM/type II pneumocytes in AL; marked alveolar oedema and occasional extravasated erythrocytes in AL	IH: Low numbers of cells in AL (IH, FHV: Positive, tongue; negative, lung)
1.6	MC	1 week	M	Multifocal mild to moderate alveolar fibrin exudation and desquamation of abundant, often apoptotic AM and type II pneumocytes; bronchioles with similar content	IH: Moderate numbers of desquamated cells in alveoli ISH (S, AS): Scattered cells in alveolar lumen
1.7	MC	12 weeks	F	Multifocal moderate fibrin exudation and abundant, often apoptotic AM and type II pneumocytes in AL, in focal area with extensive fibrin exudation and necrosis, and neutrophil infiltration	IH: Moderate numbers of cells in AL; cell free viral antigen in area with necrosis (IH, FHV: Negative)
1.8	DSH	4 weeks	M	Multifocal extensive fibrin exudation and abundant, often apoptotic AM/type II pneumocytes in AL, mild erythrocyte extravasation in AL.	IH: Scattered desquamated cells in AL

ESH, European shorthair; DSH, Domestic shorthair; BSH, British shorthair; MC, Maine Coon; F, female; M, male; N, neutered; NK, not known; AL, alveolar lumina; AM, alveolar macrophages; AS, anti-sense probe; AW, alveolar wall; FHV, feline herpesvirus type 1; ISH, RNA-in situ hybridisation; S, sense probe.

[a] AM/type II pneumocytes, numerous CD18 positive AM and scattered individual SP-C positive type II pneumocytes.

[b] Apoptosis/apoptotic: confirmed by IH (cleaved caspase-3 positive).

644 **Table 2**

645 Relevant gross and histological findings for group 2: Cats with upper respiratory disease (URD) and pneumonia,
646 involvement of feline calicivirus (FCV) confirmed by immunohistochemistry (IH) for FCV antigen.

647

Case	Breed	Age	Sex	Lung histopathology	Demonstration of FCV
2.1	DSH	12 weeks	M	Multifocal moderate fibrin exudation and very abundant AM/type II pneumocytes in AL; bronchioles with similar content	IH: Low number of cells in AL and in bronchiolar lumina
2.2	DSH	4 years	F	Multifocal mild fibrin exudation and moderate numbers of AM/type II pneumocytes in AL	IH: Scattered cells in AL (IH, FHV: negative)
2.3	DSH	8 weeks	F	Multifocal extensive fibrin exudation and moderate to large numbers of AM/type II pneumocytes in AL	IH: Several cells in AL ISH (S, AS): Several cells in AL (IH, FHV: Negative)
2.4	Oriental	12 weeks	F	Mutifocal low to moderate numbers of AM/type II pneumocytes in AL	IH: Several cells in AL
2.5	DSH	3.5 years	MN	Multifocal moderate fibrin exudation and abundant, often apoptotic AM/type II pneumocytes in AL; mild focal type II pneumocyte hyperplasia; moderate interstitial macrophage infiltration	IH: Moderate to high number of cells in AW (type II pneumocytes). (IH, FHV: Negative)
2.6	DSH	11 weeks	F	Multifocal moderate fibrin exudation and moderate numbers of occasionally apoptotic AM/type II pneumocytes in AL; moderate amount of lymphocytes and plasma cells in interstitium	IH: Moderate number of cells in AL (IH, FHV: Negative)

648

649 DSH, Domestic Shorthair; F, female; M, male; N, neutered; AM, alveolar macrophages; AW, alveolar wall; AL, alveolar lumina; ISH, RNA-
650 in situ hybridisation; S, sense probe; AS, anti-sense probe; FHV, Feline herpesvirus type 1.

651 **Table 3**

652 Relevant gross and histological findings for group 3: Cats without upper respiratory disease (URD) and
653 histological findings consistent with feline calicivirus (FCV) pneumonia, involvement of FCV confirmed by
654 immunohistochemistry (IH) for FCV antigen.

655

Case	Breed	Age	Sex	Primary disease	Lung histopathology	Demonstration of FCV
3.1	DSH	8 weeks	M	Feline parvovirus enteritis	Multifocal fibrin exudation and abundant, partly apoptotic AM/type II pneumocytes; mild to moderate multifocal type II pneumocyte hyperplasia; moderate interstitial macrophage infiltration	IH: Moderate number of cells in AL ISH (S, AS): Several cells in AL
3.2	BSH	1 year	MN	Feline parvovirus enteritis	Multifocally, low numbers of partly apoptotic AM/type II pneumocytes in AL	IH: Scattered cells in AW (type II pneumocytes?) or in AL

656

657 DSH, Domestic shorthair; BSH, British shorthair; M, male; N, neutered; AM, alveolar macrophages; AW, alveolar wall; AL, alveolar
658 lumina; ISH, RNA-in situ hybridisation; S, sense probe; AS, anti-sense probe.

659 **Table 4**

660 Relevant gross and histological findings for group 4: Cats with virulent systemic feline calicivirus (FCV) and

661 demonstration of FCV antigen in the lungs by immunohistochemistry (IH)

662

Case	Breed	Age	Sex	Lung histopathology	Demonstration of FCV
4.1 [a]	DSH	3 years	FN	Multifocal desquamation of low numbers of AM/type II pneumocytes	Isolation and PCR (oropharyngeal swab); PCR IH: Several cells in AL
4.2 [b]	DSH	10 years	MN	Multifocally, low numbers of AM/type II pneumocytes in AL	FCV (oropharyngeal swab, tissues); PCR IH: Several cells in AL
4.3	DSH	6 weeks	NK	Multifocal marked fibrin exudation and moderate numbers of partly apoptotic AM/type II pneumocytes in AL; focal extensive apoptosis and loss of bronchiolar epithelial cells	IH: Moderate number of intact and apoptotic cells and cell free in AL ISH (S, AS): Moderate number of (apoptotic) cells in AL and focally numerous (apoptotic) bronchiolar epithelial cells
4.4 [c]	DSH	Adult	M	Multifocally, low numbers of AM/type II pneumocytes in AL	Isolation (spleen); PCR; IH: A few cells in AL

663

664 DSH, Domestic shorthair; F, female; M, male; N, neutered; NK, not known; AM, alveolar macrophages; AL, alveolar lumina; ISH, RNA-in

665 situ hybridisation; S, sense probe; AS, anti-sense probe

666 [a] Coyne et al. (2006).

667 [b] Battilani et al. (2013).

668 [c] Velasco et al. (2013).

669 **Table 5**

670 Antibodies, detection method and antigen retrieval for immunohistochemistry.

671

Antibody	Source	Antigen retrieval	Detection method
Rabbit anti-FCV capsid	University of Liverpool (Coyne et al., 2006)	CB pH 6.0, 96 °C	PAP
Mouse anti-FCV (clone FCV2-16)	Custom Monoclonals International	CB pH 6.0, 96 °C	PAP
Mouse anti-FHV (clone FHV5)	Custom Monoclonals International	None	PAP
Mouse anti-SP-C (clone FL-197)	Santa Cruz Biotechnology	None	PAP
Rabbit anti-cleaved caspase 3	Cell Signaling	CB pH 6.0, 96 °C	PAP
Mouse anti-feline CD18 (clone FE3.9F2)	Leukocyte Antigen Biology Laboratory (University of California)	Bacterial protease	HP (Envision; Dako)
Mouse anti-human MMP-9 (clone IIA5)	NeoMarkers	CB pH 6.0, 96 °C	PAP

672

673 FCV, feline calicivirus; FHV, feline herpesvirus; SC, surfactant protein; MMP, matrix metalloproteinase; CB, citrate buffer; PAP, peroxidase
674 anti-peroxidase method; HP, horseradish peroxidase method.

675

676 **Figure legends**

677

678 Fig.1. Histological features of feline calicivirus (FCV)-associated lung lesions. (A) Cat with
679 upper respiratory disease (URD) and isolation of FCV from the lung (cat 1.2). Variable
680 numbers of desquamated alveolar macrophages (arrows) and scattered neutrophils and small
681 amounts of fibrin (arrowhead) are present in alveolar lumina. Bar = 20 µm. (B) Cat with URD
682 and immunohistochemical confirmation of FCV infection (cat 2.5). Alveoli contain variable
683 numbers of desquamated alveolar macrophages (arrows) and a few extravasated erythrocytes.
684 Type II pneumocytes (arrowheads) often appear activated. Bar = 20 µm. (C) Cat without
685 URD, but with immunohistochemical confirmation of FCV infection (cat 3.1). Apart from the
686 presence of fibrin and desquamated cells in alveolar lumina (arrowhead), moderate type II
687 pneumocyte hyperplasia (arrows) is seen in several alveoli. Bar = 20µm. (D) Cat with virulent
688 systemic FCV (cat 4.3). Alveoli contain abundant fibrin and several apoptotic alveolar
689 macrophages (arrowheads). The bronchiole exhibits focal apoptotic loss of respiratory
690 epithelial cells (arrow). Bar = 20 µm. Haematoxylin and eosin staining.

691

692 Fig. 2. Immunohistochemical characterisation of feline calicivirus (FCV)-associated lung
693 lesions. (A) Cat with upper respiratory disease (URD) and isolation of FCV from the lung (cat
694 1.2). Expression of CD18 is seen in the vast majority of desquamated cells, which can be
695 identified as alveolar macrophages. Horseradish peroxidase method. Bar = 20µm. (B, C) Cat
696 with URD and immunohistochemical confirmation of FCV infection (cat 2.5). (B) Staining
697 for surfactant protein-C identifies type II pneumocytes (arrowheads), either attached to the
698 alveolar wall or, occasionally, desquamated. Peroxidase anti-peroxidase method. Bar = 20
699 µm. (C) MMP-9 expression is seen in both desquamated alveolar macrophages (arrows) and
700 activated type II pneumocytes (arrowhead). Peroxidase anti-peroxidase method. Bar = 10 µm.

(D) Cat without URD and immunohistochemical confirmation of FCV infection (cat 3.1). Staining for cleaved caspase-3 confirms that numerous desquamated cells in alveolar lumina undergo apoptosis (arrows). Peroxidase anti-peroxidase method. Bar = 20 µm. Papanicolaou's haematoxylin counterstain.

Fig.3. Expression of feline calicivirus (FCV) antigen in FCV-associated lung lesions. (A) Cat with upper respiratory disease (URD) and isolation of FCV from the lung (cat 1.2). Numerous desquamated alveolar macrophages (arrows) and scattered type II pneumocytes in the alveolar wall express viral antigen. Bar = 20 µm. (B) Cat with URD and immunohistochemical confirmation of FCV infection (cat 2.5). Apart from desquamated alveolar macrophages (arrows), a few type II pneumocytes attached to the alveolar wall (arrowheads) express viral antigen. Bar = 10 µm. (C) Cat without URD and immunohistochemical confirmation of FCV infection (cat 3.1). Numerous, often apoptotic cells in alveolar lumina (arrows) express viral antigen. Bar = 20 µm. Inset: FCV antigen expression in an activated type II pneumocyte. Bar = 10 µm. (D) Cat with VS-FCV (cat 4.3). Alveoli contain numerous cells, often apoptotic (arrows), that express viral antigen. A bronchiole exhibits focal loss and apoptosis of respiratory epithelial cells that exhibit strong viral antigen expression (arrow heads). Numerous FCV antigen positive degenerate cells are also found in the bronchiolar lumen (BL). Bar = 20 µm. Peroxidase anti-peroxidase method. Papanicolaou's haematoxylin counterstain.

Fig. 4. Demonstration of feline calicivirus (FCV) genome and replication in FCV-associated lung lesions. (A) Cat with upper respiratory disease (URD) and isolation of FCV from the lung (cat 1.2). Several, partly apoptotic desquamated cells in the alveolar lumen show a positive signal (arrows) for positive strand viral RNA (top); a few also express negative

726 strand, replicative intermediate stage viral RNA (bottom). Bars = 20 µm. (B, C) Cat with
727 URD and immunohistochemical confirmation of FCV infection (cat 2.5). (B) Desquamated
728 cells in the alveolar lumen show a positive signal (arrows) for positive strand viral RNA (top).
729 Activated type II pneumocytes exhibit a signal for negative strand, replicative intermediate
730 stage viral RNA (bottom); V, vessel. Bars = 10 µm. (C) Focal areas with type II pneumocyte
731 hyperplasia, with abundant signal for negative strand, replicative viral RNA (binding of sense
732 probe) in type II cells (arrows). Bar = 20 µm. (D) Cat without URD or immunohistochemical
733 confirmation of FCV infection (cat 3.1). Alveoli contain numerous cells, often apoptotic
734 (arrows), that express negative strand, replicative viral RNA (arrows), detected by binding of
735 the sense probe. A bronchiole exhibits focal loss and apoptosis of respiratory epithelial cells
736 that exhibit strong signals for negative strand, replicative viral RNA (arrowheads). BL,
737 bronchiolar lumen. Bar = 10 µm. RNA-in situ hybridisation; NBT/BCIP. Papanicolaou's
738 haematoxylin counterstain.

Figure 1

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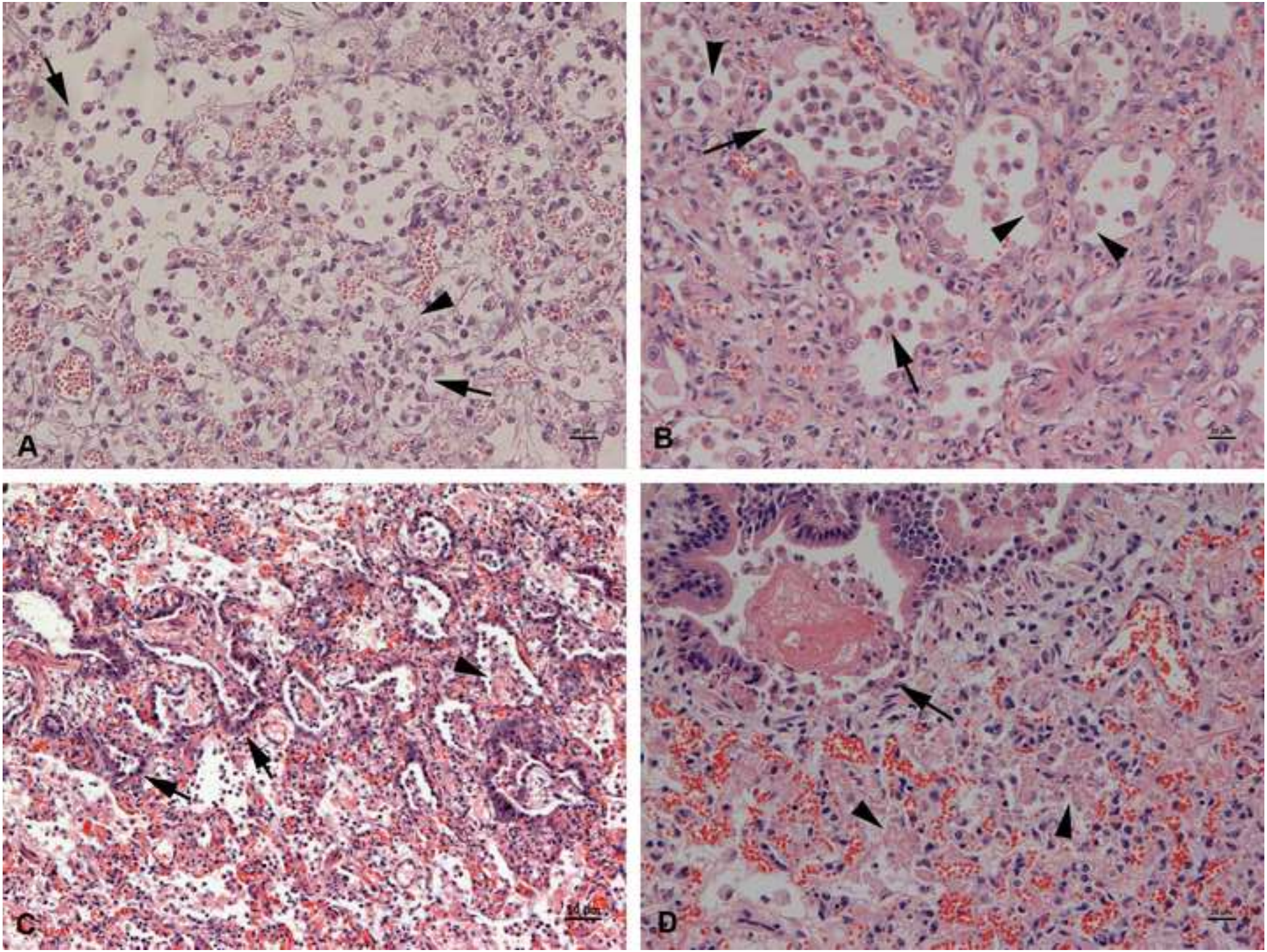

Figure 2

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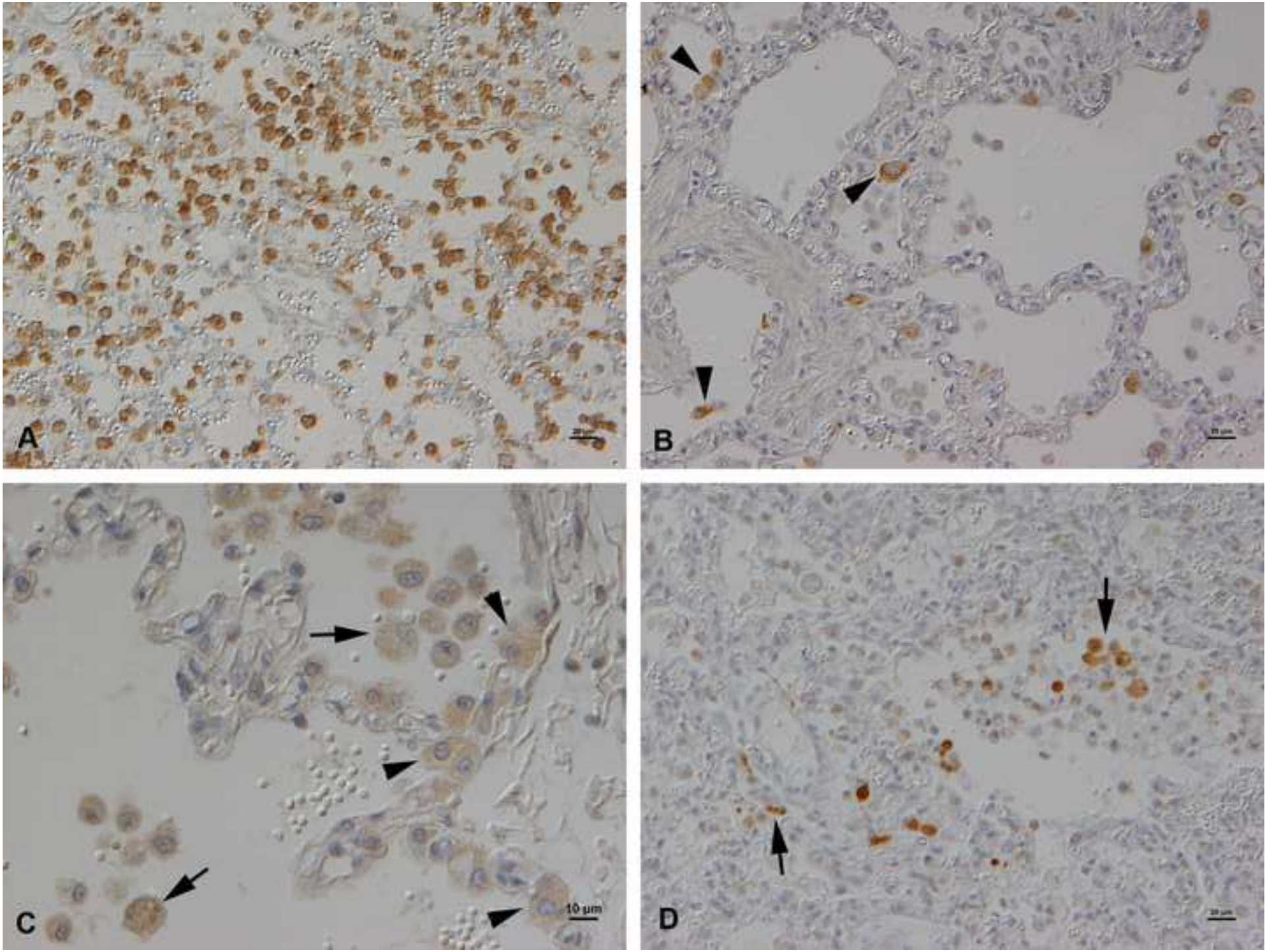

Figure 3

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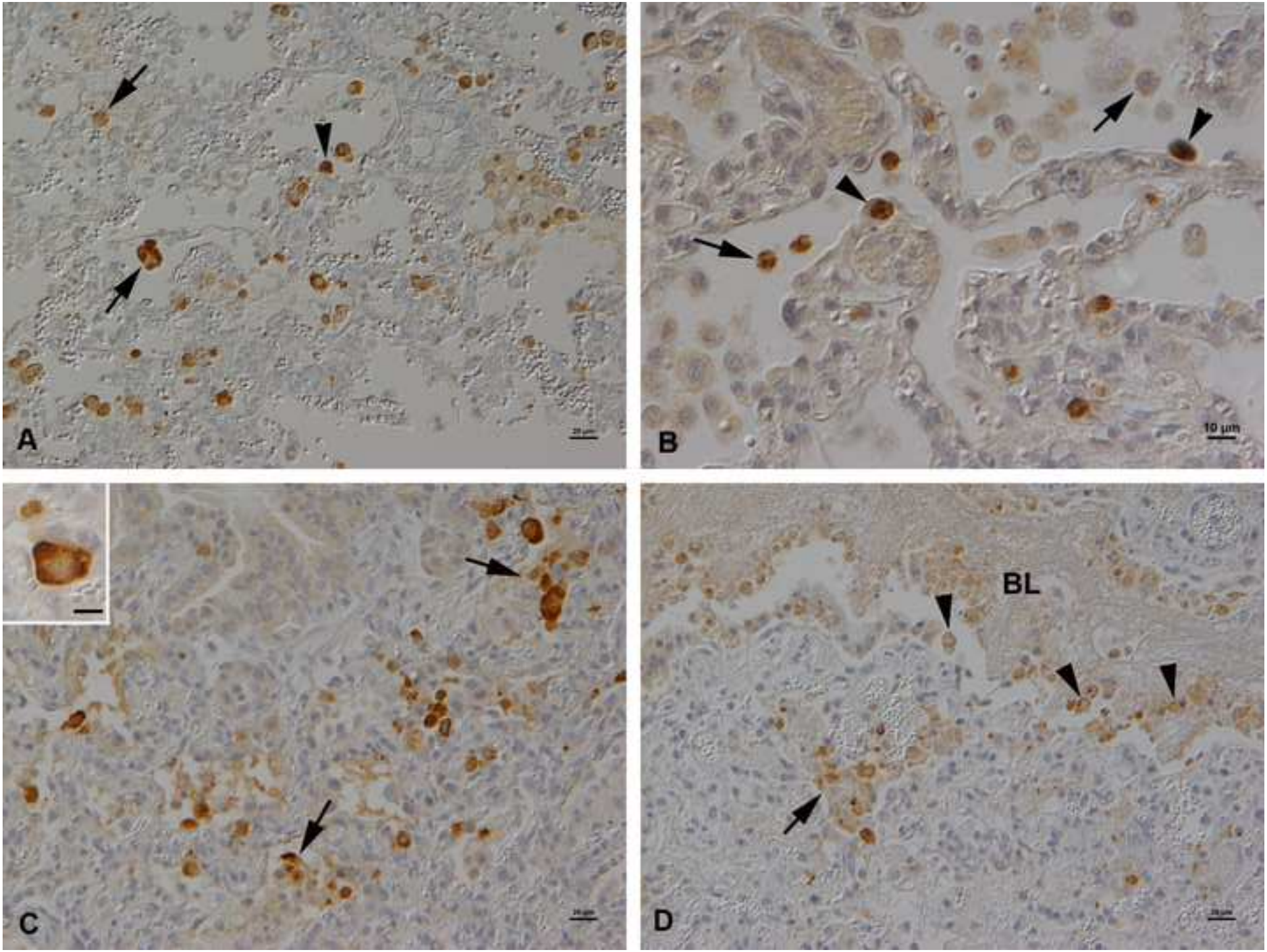
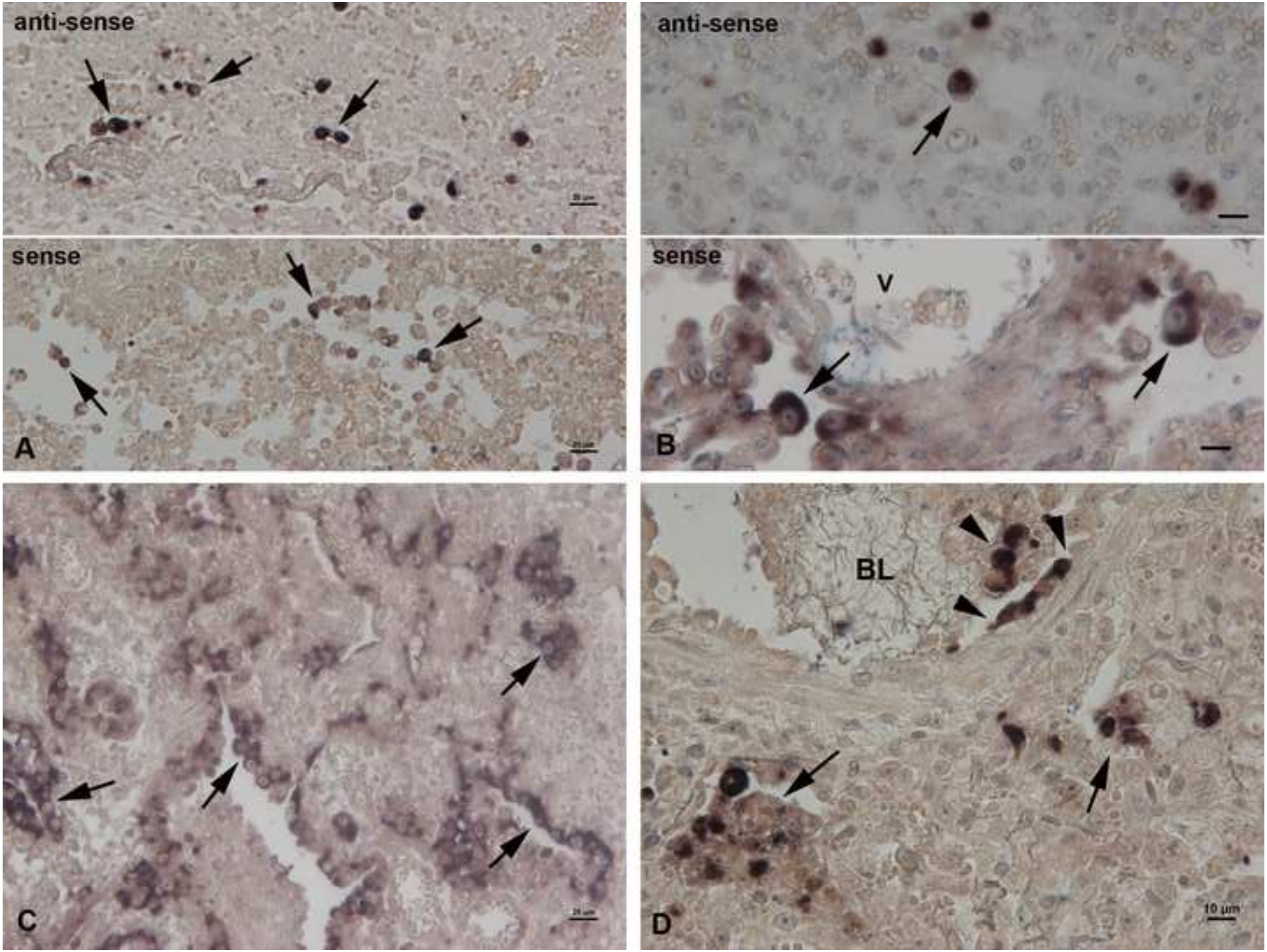


Figure 4

Click here to download high resolution image



Revision Note

We thank the editor and reviewers for their feedback on our manuscript and have carefully attended all their comments.

Editor's comment:

We thank the editor for editing our manuscript. From the editor's feedback, the main issue was the following:

Your manuscript is currently 4134 words long. Can you please try to revise the word count down so that it is closer to the 3000 word limit for Original Articles in TVJ?

Response: We have carefully gone through the manuscript again and have made some changes to shorten the text. However, the reviewers requested a few changes that then added further words to the manuscript. At present, it has a length (introduction to conclusions) of 3,902 words. We feel that further cuts would reduce the value of the manuscript but are happy to undertake another attempt if the editor wishes us to and is happy with a less thorough report and discussion.

Reviewer #1:
Thsi is a well conducted useful study which sheds new light on the role of FCV in the lungs of naturally infected cats.

Response: We thank the reviewer for the positive comment.

line 4 - can the authors's justify the use of the word "mediate" in the title

Response: We agree with the reviewer that "mediate" might not be the best term to describe the role of alveolar macrophages in FCV-associated pneumonia and have therefore changed the title to "Alveolar macrophages are the main target cells in feline calicivirus-associated pneumonia" in the hope that the reviewer will agree with this title.

Line 28 - there are some reports of FCV-like viruses in dogs (Martella V, Pratelli A, Gentile M, Buonavoglia D, Decaro N, Fiorente P, Buonavoglia C (2002). Analysis of the capsid protein gene of a feline-like calicivirus isoalted from a dog. Veterinary Microbiology, 85:315-322.)

Response: In order to avoid any discussion about the host specificity, in particular in light of the publication mentioned by the reviewer, we have taken out the words "host specific".

Line 36 - VS in full Line 64 - don't need both "Caliciviridae" and "family"

Response: The text has been shortened and this text passage been deleted.

Line 68 - UDR

Response: The text has been changed accordingly.

Line 72 - C and E do not encode proteins, rather part of a protein

Response: We are grateful to the reviewer for pointing this out and have changed the text accordingly.

Line 188 - relatively conserved

Response: The text has been changed accordingly.

Line 735 - don't understand the "B, C" in the middle of this line.

Response: The “B, C.” refers to the subsequent text, meaning that Fig. 4B and C are from a “Cat with URD and immunohistological confirmation of FCV infection (case No. 2.5).” We believe that this is clear enough.

Line 308 - this conclusion seems unproven. Yes you have lesions in a dead cat. But that does not mean the lesions were associated with the cause of death. Apologies if I am missing something.

Response: The text has been changed to make the conclusion more understandable. For this purpose, we have again mentioned the severity of the pulmonary lesions (acute alveolar damage) which can alone be responsible for the animals’ death (lines 302 - 304 in the revised manuscript).

Line 372 - don't need to repeat the (Langloss et al., 1978b),

Response: The text has been changed accordingly.

Line 374 - persistence. This got me thinking. I would be interested in the authors' thoughts on what they would see if they were able to carry out their analyses on a recovered cat. Perhaps some of the staining they see represents "normal" lung. Perhaps the authors should comment on the need to analyse such cases in subsequent studies, as an additional control.

Response: This is an interesting point. The presence of hyperplastic tyoe II pneumocytes in some animals indicates recovery of the lungs from initial FCV induced alveolar epithelial cell damage. The fact that viral RNA was found in occasional hyperplastic type II cells indicates that the virus can persist in these cells. However, with further recovery, these cells would further differentiate, mainly into functional type I cells, to ensure effective gas exchange. It is unlikely that these fully differentiated cells would still carry FCV, and if they did, in amounts high enough to be detected by RNA-ISH. The more sensitive PCR would not help much, since it would not allow identification of infected cells. Therefore, this question could only be addressed in a large experimental study, which would be extremely difficult to realise, at least in Europe.

Line 382/3 - don't need both family and Coronviridae

Response: The word “family” has been deleted.

Line 387 - have you formally confirmed? You have shown apoptosis. You have shown FCV infection. But you can't prove a link between the two. The figure 2d legend has it about right. Or perhaps I have missed the logic. I think you can say you have provided further evidence, this time in vivo, for …..

Response: The staining of consecutive sections demonstrates FCV and the apoptosis marker in the same lesion and thereby group of cells, which we consider as sufficient to use the term “confirm”. However, since we have not performed double staining, we of course did not show both features in the same cell. Therefore, the statement has been toned down (line 376 in the revised manuscript).

Line 393-398 - Possibly delete this last paragraph.

Response: We have decided to keep the text.

Line 405 - again, possibly delete the sentence relating to immunosuppressed cats.

Response: The sentence has been deleted.

Figure 2 legend may be made easier to follow by starting each section with the name of the antigen target. This reviewer found it hard to decipher.

Response: The text has been amended to provide information on the antigens that are detected at the beginning of each sentence on the relevant photo.

Reviewer #2:
In this study the involvement of FCV infection in pulmonary lesions in cats with natural respiratory disease was determined. So far mainly data from experimental infections are known which may not represent the type and extent of lesions found in naturally infected cats. Therefore, this study adds to our knowledge of the pathogenesis of FCV infection in domestic cats.
The study was done with a selected number of cats in which pulmonary involvement was already evidenced by virus isolation or virus antigen detection by immunohistochemical staining. Several genetic and immunological tools were used to identify the type of cells and the presence of viral antigen and/or viral replication. These tools and techniques provide sufficient information to draw the conclusions about the type and extent of lung lesions. There are some ambiguities regarding materials and methods that need to be addressed. Also some of the results need to be clarified.

Comments
- Four different groups of cats were included in the study. The second group consists of cats that had URD and pneumonia in which FCV involvement was evidenced only by immunohistology. From the M&M paragraph it is not clear if virus isolation was not performed or was performed but unsuccessful. In the results section authors state that FCV involvement was confirmed by immunohistology alone, which does not exclude that virus isolation was attempted. Only when reading the discussion it becomes clear that virological examination had not been undertaken.

Response: We agree with the reviewer; the text in M&M was ambiguous. We have amended it to clarify that virus culture was only undertaken in animals of group 1 (lines 130 and 131 in the revised manuscript).

- Besides staining for FCV antigen some samples were stained for FHV as well. It is not clear why only a selected number of cases were stained for FHV and which criteria were used to decide whether staining was performed or not. This information should be included.

Response: The immunohistology for FHV was done to exclude that FHV played a role in the lung lesions. It was therefore mainly done in group 2, where virus culture was not performed, which itself ruled out FHV infection of the lung when negative (Group 1). We have provided information on this in the M&M section (lines 151-153 in the revised manuscript).

- In cats from group 1, one case (No. 1.5) was shown to exhibit co-infection with FHV and FCV. However FHV staining was only performed in 3 out of 8 cats. The relevance of this finding should be discussed knowing that not all cats were screened for the presence of FHV (see also previous comment). Also the conclusion that co-infection was found in a few cases (discussion, last paragraph) seems of little value if not all samples were tested.

Response: As mentioned above, immunohistology for FHV was performed in the lungs to confirm that only FCV was involved. We consider this as relevant in the cats with FCV pneumonia in which virus culture had not been performed (group 2). We have tried to make this clearer in the text (results sections: lines 254/255).

- Staining for FCoV was only performed in 2 cases (based on the information in Table 1). The reason for performing this staining only in these 2 cases is not discussed. This cannot be concluded based on the histological information given in table 1.

Response: We did undertake FCoV immunohistologie in just a few cases, as a diagnostic panel that had been decided upon at the time of necropsy. However, we agree with the reviewer that this does not add anything to the manuscript and have therefore deleted all mentioning of the FCoV immunohistology, since FIP was not a differential diagnosis in any of these cases.

- Negative controls for the in situ hybridization are important to proof the specificity of the reactions. Information on the negative controls used in the in situ hybridization is lacking. These should be included and discussed.

Response: Information on the negative controls for ISH is now provided (lines 209/201 in the revised manuscript).

- In the results section authors refer to the occasional extravasation of erythrocytes into the alveolar lumen (fig 1B,C). This is not mentioned in the legend of the figure and also not indicated within the figure.

Response: Erythrocyte extravasation into the alveolar lumen is obvious in particular in Fig. 1B. A reference to this has now been included into the figure legend and the text been changed accordingly.

A few typos .
Introduction Line 68: self limiting UDR; should be: URD

Response: The text has been changed accordingly.

Introduction line 100 "into the aleolar lumen": change to: alveolar

Response: The text has been changed accordingly.

Discussion, line 383; Coronviridae: change to Coronaviridae

Response: The text has been changed accordingly.

We hope that the revised manuscript will now be acceptable for publication in The Veterinary Journal.

Sincerely,

Anja Kipar