Original Article

Coronavirus Infection in Ferrets: Antigen Distribution and Inflammatory Response

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Abstract

Multisystemic granulomatous lesions are the most common finding in ferrets infected by ferret systemic coronavirus (FRSCV). To characterize the inflammatory response developed against this virus, lesions from 4 naturally infected ferrets were examined. Lesions were classified into the 4 known types of granulomas (granulomas without necrosis [G], granulomas with necrosis [G-N], granulomas with neutrophils [G-NL], and diffuse granulomatous inflammation [DG]). The cellular composition of the lesions was characterized on the basis of cellular morphology and immunohistochemistry using markers for T and B-lymphocytes, plasma cells, macrophages, and neutrophils. The extent and distribution of viral antigen expression was also assessed. In G lesions, macrophages were mainly located in the center of the granuloma, with a moderate number of T-lymphocytes scattered among the macrophages, plasma cells, and B-lymphocytes. G-N lesions exhibited a necrotic center surrounded by abundant macrophages, some T-lymphocytes, plasma cells, and a few B-lymphocytes. In G-NL lesions, there was a central area dominated by neutrophils with low numbers of macrophages, plasma cells, and lymphocytes. DG presented similar cell proportions, but distributed evenly throughout the lesions. FRSCV was expressed in G, G-NL, G-N, and DG, with decreasing numbers of immunoreactive cells. This study reveals the important role of macrophages in the inflammatory response of ferrets against the virus and the variable proportions of leukocytes among different types of lesions, indicating their variable age. The results also confirm the similarities of the disease in ferrets to feline infectious peritonitis.

Ferret systemic coronavirosis is an emerging fatal disease of ferrets caused by ferret systemic coronavirus (FRSCV) that shares clinical-pathological characteristics with the dry form of feline infectious peritonitis (FIP) in cats.[3,4,10,17] The severity of the disease and the increased popularity of ferrets as exotic pets have generated a growing interest in ferret systemic coronavirosis.[4] FRSCV was described as causing multisystemic granulomatous lesions.[11] However, other diseases in ferrets are also associated with granulomatous inflammatory lesions, such as mycobacterial infections,[15] pyogranulomatous pleuropneumonia associated with *Pseudomonas luteola* infection,[9] and endogenous lipid pneumonia. Moreover, ferrets were also used in the past as an animal model for Crohn's disease, where artificially induced intestinal infarction developed into different lesions including chronic transmural inflammation, ulceration and granuloma formation.[13] Hence, a granulomatous reaction in ferrets appears to be a common inflammatory response to different agents.

The clinical signs and lesions described with ferret systemic coronavirosis are similar to those seen in cats with FIP. The pathogenesis of FIP has been extensively studied. It is believed that the activation of monocytes and macrophages leads to the pathological features of FIP, including vasculitis, body cavity effusions, and fibrinous and granulomatous inflammatory lesions.[6,7] However, the pathogenesis of the disease has not been studied in ferrets. As a first step toward the understanding of its pathogenesis, the present study was established. Its purpose was to characterize the cellular composition and distribution of the inflammatory cells involved in the granulomatous inflammatory reaction in ferrets naturally infected with FRSCV and compare

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Table 1. Immunohistochemical Quantification of the FRSCV Viral Antigen and Inflammatory Cells Involved in Different Types of Granulomatous Lesions. For Each Immunohistochemical Marker, 12 Granulomas Corresponding to Each Type of Lesion Were Evaluated.

	Immunohistochemistry				
Type of lesion	FRSCV	Lysozyme Macrophages/Neutrophils	CD3	CD20	Lambda Light Chain
G	30%	+++/+	++	+	++
G-N	15%	+++/+	++	+	+
G-NL	20%	++/+++	++	+	+
DG	11%	+++/+	+	+	++

Abbreviations: FRSCV, Ferret Systemic Coronavirus; data are expressed as mean. G, granuloma without necrosis; G-N, granuloma with necrosis; G-NL, granuloma with neutrophils; DG, diffuse granulomatous inflammation.
Note. + = Mild, ++ = Moderate, +++ = Abundant.

the results with those described in cats. We hypothesize that the morphological features and cellular composition of the inflammatory response in ferrets with ferret systemic coronavirosis are similar to those described for cats with FIP.

Material and Methods

Samples

The study was performed on paraffin-embedded tissues with characteristic lesions obtained from necropsies of 4 ferrets with ferret systemic coronavirosis, between 2004 and 2010. These tissues belonged to the archive of the Veterinary Pathology Diagnostic Service (SDPV), Facultat de Veterinària, Universitat Autònoma de Barcelona. Inclusion criteria were the presence of the characteristic granulomatous lesions described for ferret systemic coronavirosis in ferrets and the detection of coronavirus antigen by immunohistochemistry.[11] The samples were taken from 2 females of 11 months and 1.5 years, and 2 males of 5 months and 1 year of age, who either died of or were euthanized as a result of the disease. The following tissues were examined: spleen (n = 2), kidney (4), liver (3), brain (2), lung (1), lymph node (1), intestine (2), and pancreas (1). Peritoneal serosa was also studied in detail.

Histology and Immunohistochemistry Techniques

Formalin-fixed, paraffin-embedded tissues were sectioned at 3 μm, and stained with hematoxylin and eosin (HE). Immunohistochemistry (IHC) was performed using the PT-Link automatic System (Dako Glostrup, Denmark) for deparaffinization, rehydration and epitope retrieval. Immunostaining was performed on a Dako Autostainer Plus, using procedures, buffers and solutions provided by the manufacturer. Primary antibodies and dilutions are shown in Supplemental Table 1. The Rabbit/Mouse EnVision Detection System (Dako Ref.: K5007) was used at the dilution recommended by the manufacturer. After washing, slides were incubated for 5 min. in DAB-Chromogen-hydrogen peroxide (Dako Ref.: K3468) to reveal binding. After washing, slides were counterstained in Mayer's haematoxylin for 10 seconds, washed in running tap water, and then automatically dehydrated, cleared, and

mounted. FRSCV antigen was demonstrated using a mouse monoclonal antibody against feline coronavirus (clone FCV3-70; Custom Monoclonals International, West Sacramento, CA, USA) previously shown to cross react with FRSCV.[11]

To immunohistochemically characterize the different types of granulomatous inflammation observed in each animal, lesions were divided into 4 types: granulomas without necrosis (G), granulomas with necrosis (G-N), granulomas with neutrophils (G-NL), and diffuse granulomatous inflammation (DG). The criteria used for this classification, and to determine the cellular composition and the expression of the viral antigen, were those previously described.[11] This classification was based on the type and distribution of the cells within the lesion.

To assess the cellular composition and the amount of FRSCV antigen present within the 4 types of granulomatous lesions, a panel of primary antibodies were used following the protocols described previously.[16] Antibodies were used for the detection of cells based on the expression of the following antigens: lysozyme (rabbit antihuman lysozyme; Dako, Glostrup, Denmark) for macrophages and neutrophils, lambda light chain (rabbit antihuman lambda light chains; Dako, Glostrup, Denmark) for plasma cells, CD3 (rabbit antihuman CD3; Dako, Glostrup, Denmark) for T-lymphocytes, and CD20 (rabbit antimouse CD20; Thermo Scientific, San Ramon, CA) for B-lymphocytes (Supplemental Table 1). Consecutive tissue sections were incubated with PBS instead of the primary antibodies to be used as negative controls. As a positive control, tissue from a ferret kidney immunoreactive for FRSCV antigen was included.

Immunohistochemical Scoring

For each granulomatous lesion type, 3 tissue sections were evaluated regardless of the animal and the organ from which they came. Within each section, 4 granulomatous lesions were evaluated, thus, 12 granulomas in each category were investigated. Six different areas from every granuloma were randomly evaluated at high power field (40×). Expression of all markers was evaluated in each granuloma using a semiquantitative approach based on the percentage of immunoreactive cells for each of the antibodies. A final average was calculated for each type of lesion. Results were expressed as: + (less than 20%), ++ (between 20 and 40%), and +++ (more than 40%)
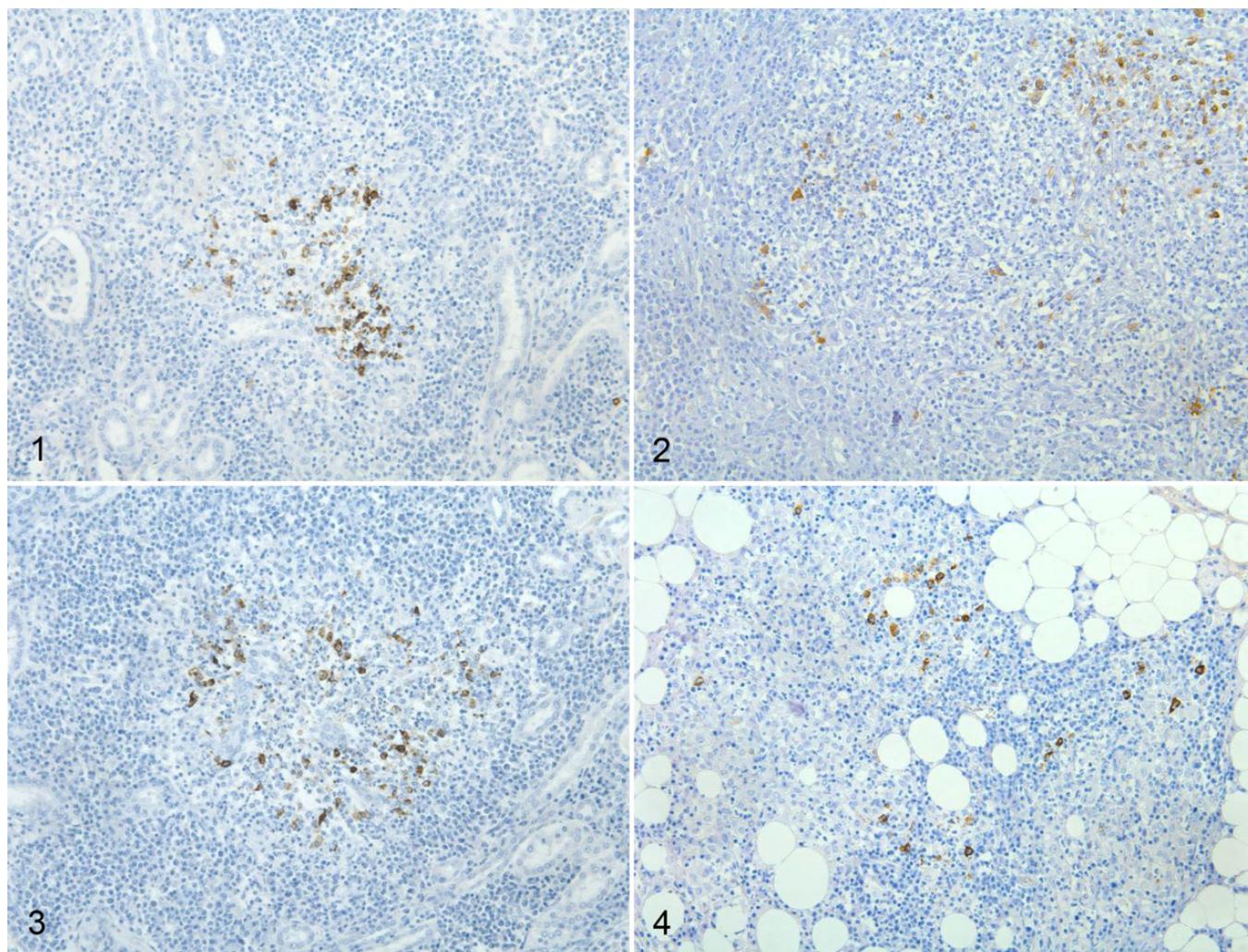
Figure 1. Granuloma without necrosis, kidney, ferret. Expression of abundant ferret systemic coronavirus (FRSCV) antigen within macrophages localized in the center of the granuloma. IHC for FRSCV. **Figure 2.** Granuloma with necrosis, kidney, ferret. Expression of FRSCV antigen within macrophages in the periphery of the granuloma. IHC for FRSCV. **Figure 3.** Granuloma with neutrophils, liver, ferret. Expression of FRSCV antigen within macrophages. IHC for FRSCV. **Figure 4.** Diffuse granulomatous inflammation, intestinal serosa, ferret. Isolated macrophages in the diffuse granulomatous inflammation with strong immunoreactive in the cytoplasm for FRSCV antigen. IHC for FRSCV.

(Table 1). Cells immunoreactive for lysozyme were separated into neutrophils and macrophages based on their morphology.

Statistical Analysis

For FRSCV antigen immunostaining, the approximate percentage of immunoreactive cells was assessed from the total number of macrophages in each granuloma. For each category of lesion, the average percentage of FRSCV labeled cells from the 12 granulomas was calculated (Table 1). To compare the percentage of FRSCV immunoreactive cells between the 4 types of lesions, results were statistically analyzed by a 1-way non-parametric analysis of variance (ANOVA), followed, when necessary, by a Student-Newman-Keuls multiple comparisons test. Data were considered statistically significant when $P < .05$.

Expression of all markers was recorded using a semiquantitative grading scheme based on the percentage of immunoreactive cells for each of the antibodies.

Results

The different types of lesions were observed in the same animal and in the same organ (Supplemental Table 2). Five organs showed a predominance of G (spleen, kidney, pancreas, liver, lymph node), 3 organs exhibited G-N (liver, lung, spleen), 3 organs G-NL (2 kidneys, liver), and 5 organs DG (2 brains, 2 intestines, kidney). In 2 animals, lesions were also observed in the peritoneal serosa. Immunohistochemistry was performed on sections of granulomas that were thick enough to ensure the lesion was present on all serial sections.
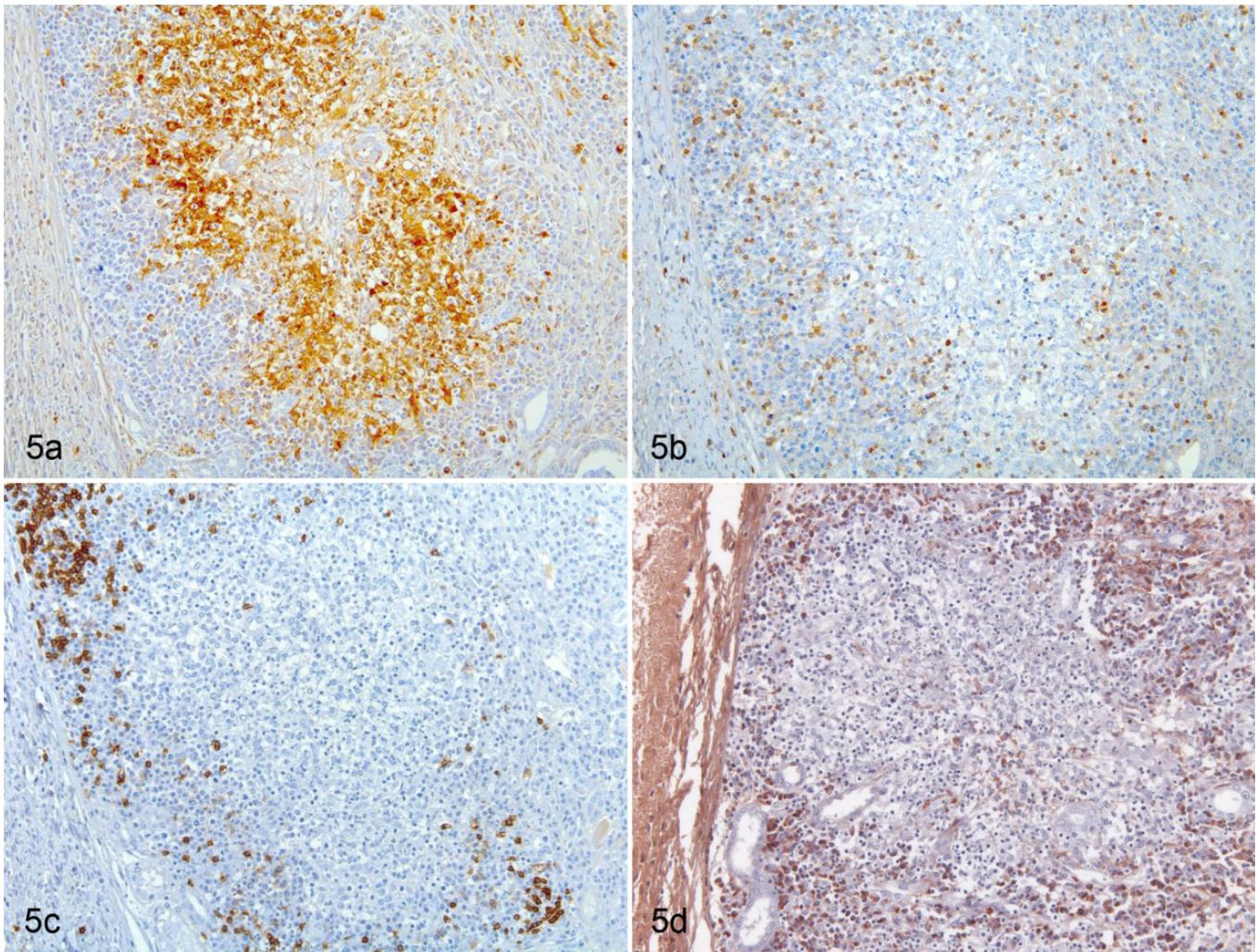
Figure 5. Granuloma without necrosis, kidney, ferret. Figure 5a. IHC for lysozyme shows large amount of macrophages and scant neutrophils in the center of the lesion. Figure 5b. IHC for CD3 shows presence of many T-lymphocytes diffusely distributed among macrophages and in the periphery of the granuloma. Figure 5c. IHC for CD20 shows presence of B-lymphocytes located at the periphery of the granuloma. Figure 5d. IHC for lambda light chain shows presence of many plasma cells diffusely distributed in the periphery of the granuloma.

G were studied in detail in the spleen, kidney and pancreas (Supplemental Table 2). In this type of lesion, there were numerous lysozyme immunoreactive cells and moderate numbers of T-lymphocytes. There were large number of centrally located macrophages and low numbers of neutrophils. Moderate numbers of T-lymphocytes were detected diffusely throughout the granuloma. The remaining cell population was classified as plasma cells and B-lymphocytes and were located at the periphery of the lesion. Approximately 30% of inflammatory cells within the granulomas were macrophages containing detectable FRSCV antigen (Table 1) (Figs. 1, 5, 6)

G-N lesions were evaluated in liver, spleen, and lung (Supplemental Table 2). The extent of the necrosis varied. The majority of the inflammatory cells were macrophages with no neutrophils present. In the larger necrotic areas, the macrophages were located at the periphery, while within the smaller necrotic areas, the macrophages were randomly scattered through the necrosis. There were a moderate numbers of

T-lymphocytes and low numbers of plasma cells distributed at the periphery of the granuloma. Low numbers of B-lymphocytes were present along the border of the granuloma. The percentage of macrophages with FRSCV viral antigen was about 19% (Figs. 2, 6).

G-NL were observed in 1 liver and 2 kidney specimens (Supplemental Table 2). A center of the granuloma contained large numbers of neutrophils and low number of macrophages. Low numbers of T-lymphocytes, plasma cells, and B-lymphocytes were present along the periphery of the granuloma. The percentage of macrophages with FRSCV viral antigen was 15%. FRSCV antigen was not detected in the granulomas with the largest amount of neutrophils (Figs. 3, 6).

DG infiltration was found in the meninges and choroid plexus of the brain (Supplemental Table 2). There was also diffuse granulomatous inflammation in the intestinal serosa of 2 animals. The majority of cells present were macrophages and they, along with moderate number of plasma
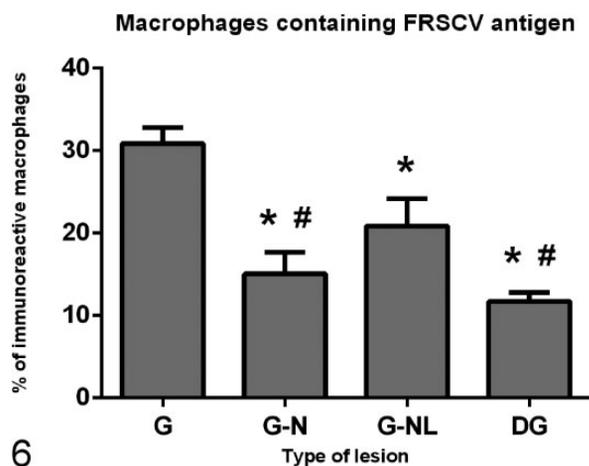
Macrophages containing FRSCV antigen

Figure 6. Percentage of macrophages with immunoreactive ferret systemic coronavirus antigen for each granuloma category. *Significant differences between the percentage of immunoreactive cells in granulomas without necrosis (G) and granulomas with necrosis (G-N), granulomas with neutrophils (G-NL) and diffuse granulomatous inflammation (DG). #Significant differences between G-NL and G-N and DG. Data are expressed as mean. Data were analyzed by a 1-way nonparametric analysis of variance (ANOVA), followed, when necessary, by a Student-Newman-Keuls multiple comparisons test. Data were considered statistically significant when $P < .05$.

cells, were diffusely detected throughout the inflammatory infiltrate. Low number of T and B lymphocytes were detected randomly scattered throughout the inflammatory lesion. Viral antigen expression was detected in 11% of the macrophages (Figs. 4, 6).

In 1 animal, focal phlebitis of medium-sized veins was seen in the meninges. The inflammatory pattern was consistent with DG and was composed of abundant macrophages and lymphocytes and few neutrophils. Some of the neutrophils were attached to endothelium and infiltrating the vascular walls. Some of the perivascular macrophages were strongly immunoreactive for FRSCV antigen.

None of the studied organs had evidence of fibrosis.

Statistical analysis revealed higher number of macrophages containing FRSCV antigen in G compared to the other lesions. Moreover, G-NL had a significantly higher number of the immunoreactive macrophages than G-N and DG (Fig. 6). There was no cell-free viral antigen in the lesions.

Discussion

This study was carried out on tissues from 4 ferrets naturally infected with FRSCV. The purpose was to study the inflammatory reaction triggered by FRSCV, and compare the morphological features and inflammatory cellular composition with those described in FIP.

The morphology and inflammatory cell composition of the granulomas were similar to those described in FIP.[5] FIP granulomas were described as being initially dominated by macrophages which were progressively replaced by B-lymphocytes

and plasma cells. T-lymphocytes comprised a minority of cells; neutrophils were rarely seen, and plasma-cells were present in all types of granulomas, and some were immunoreactive for coronavirus-specific antibodies.[5,7] In FRSCV-infected ferrets, the cellular composition was heterogeneous as well, depending on the type of lesion. About half of the cells were macrophages in each of the different types of granulomas. The remaining cells were T-lymphocytes, plasma cells, and B-lymphocytes, in order of prevalence. In DG lesions, plasma cells were more abundant than T-lymphocytes. A similar finding has been reported in cats, where typical serosal FIP lesions exhibit an underlying predominance of B-lymphocytes and plasma cells, some of them containing coronavirus-specific antibodies.[7] These authors also described that macrophages were progressively replaced by B-lymphocytes and plasma cells, and suggested that humoral response can limit disease progression, at least to some extent or for a limited period of time. In the present study, the presence of FRSCV-specific antibodies was not investigated. However DG lesions in the mesentery suggest a similar pathogenic response to that described in cats.

In FIP, a complex involvement of the immune system with concurrent detection of humoral and cellular findings is mentioned in previous studies, and type III and type IV hypersensitivity are suspected to contribute in the pathogenesis of the disease.[7,14] The morphology of the lesions in ferret systemic coronavirosis is similar to those in FIP with regard to the distribution of the immune cells and virus in the lesions. Thus both type III and IV hypersensitivities could be involved in the pathogenesis of the lesions. In this way, to better understand the pathogenic mechanism and the immune system involvement in ferret systemic coronavirosis, data on hematology, circulating leukocyte subsets, antibody titers, and interleukin and serum proteins profiles of the infected animals would be needed. This would provide more information about the dynamic immune response of FRSCV-infected ferrets.

FRSCV viral antigen was detected in the cytoplasm of the macrophages. Viral antigen expression was variable among the different types of granulomas and was expressed in G, G-NL, G-N, and DG, with decreasing numbers of immunoreactive cells. This could be a consequence of the number of macrophages present in each lesions as well as the size of the focal lesion. G lesions were extensive and had the highest number of macrophages, and G-NL had a larger amount of neutrophils than macrophages, reflected in the lower percentage of FRSCV immunoreactive cells compared to G. In contrast, G-N presented a large amount of necrotic cells, including macrophages, so that the viral antigen expression was lower. In DG, although the proportion of macrophages was similar to G, the extension of the lesion was limited, and there were very few viral antigen-immunoreactive cells. In FIP, diffuse granulomatous lesions were also shown to occasionally contain only rare FCoV-immunoreactive cells. However, granulomas with areas of necrosis had higher viral expression than granulomas without necrosis (where the number of macrophages and viral expression was low).[7] The chronological evolution of the different granulomatous lesions observed in these ferrets is difficult to

ascertain, as they originate from natural cases in which the clinical history was not available. Moreover, different types of granulomatous inflammation were observed in the same ferret and in the same organ. This could be a consequence of different episodes of viremia, as described in cats with FIP.[6] This suggests some similarities in the pathogenesis of FIP and ferret systemic coronavirosis. In cats, based on the variable pathological changes and the apparent multiphasic nature of the disease, authors proposed that the fulminant monocyte activation, which is essential for the development of FIP vasculitis, only occurs as brief bouts, followed by a phase in which self-sustained granulomatous lesions develop.[5] This could also explain why viral antigen was more abundant in G lesions (where macrophages were more abundant) in ferrets, than in the rest of the granulomas. As inflammation progressed and neutrophils and necrosis appeared, or the number of plasma cells increased, the number of virus antigen-immunoreactive macrophages decreased.

Vasculitis has been described to be developed as a consequence of the activation of monocytes in cats with FIP,[5] and is one of the most characteristic lesions of the disease. The morphological features are of granulomatous phlebitis and periphlebitis that developed through direct interaction between monocytes and activated endothelial cells.[5] This phlebitis was dominated by activated virus-infected monocytes, which lacked the features of immune complex vasculitis, such as the involvement of arteries and the dominance of neutrophils.[6] However, there was also some evidence that a type III hypersensitivity reaction contributed to the pathogenesis of this lesion, at least in some cases.[5] In the present study, vasculitis (in the form of granulomatous phlebitis) was only observed in the meningeal veins of 1 animal. There are some possible explanations for this observation. It is possible that vascular lesions may be an initial event in the pathogenesis of ferret systemic coronavirosis, but these could be obscured by the subsequent granulomatous reaction. Another possibility would be that the disease progresses more slowly in ferrets than FIP in cats. Accordingly, the vasculitis could be replaced by the other granulomatous lesions by the time the animals become clinically ill.

When comparing FRSCV lesions in ferrets with other infectious diseases that induce granulomatous reactions, there are some histopathological characteristics in common. Experimental infection of ferrets with *Mycobacterium bovis* produced granulomatous lesions with extensive tissue necrosis and macrophage infiltration.[2] Other reports described sporadic mycobacteriosis in ferrets with infiltration of macrophages, epithelioid cells and a small number of lymphocytes and neutrophils.[12,15] *Pseudomonas luteola* was reported to produce multifocal necrotizing pyogranulomatous pleuropneumonia and lymphadenitis in naturally infected ferrets, with absence of multinucleated giant cells.[9] In ferrets infected with FRSCV, multinucleated giant cells within the granulomatous reaction were rarely described.[3] In our study, necrosis was observed in some of the lesions (G-N), however neither fibrosis nor multinucleated giant cells were observed. When comparing the morphological features of all these diseases in ferrets, it is

surprising that multinucleated giant cells and fibrosis were scant or nonexisting. In general, differences in the granulomatous reaction among various diseases depends on the type of the inciting agent, the host immune response, and the interplay of cytokines produced by cells within the chronic inflammatory response.[1] In this way, 2 types of macrophage response against the agent have been described: 1 with strong microbicidal activity (M1) and 1 leading to tissue repair and fibrosis (M2).[8] In ferrets, and particularly in those infected by FRSCV, the findings suggest that the M1 macrophage response predominates.

In conclusion, this study characterized the inflammatory cell composition and distribution in the granulomatous inflammatory reaction in naturally occurring cases of ferret systemic coronavirosis. This work revealed the important role of macrophages in the ferret inflammatory response to the virus and suggests that the disease has similarities to FIP in its pathogenesis. However, several questions remain to be answered with regard to the pathogenesis of ferret systemic coronavirosis, such as the role of the humoral and cellular immunity on disease onset, the role of vasculitis as the initial event in lesion development, and the type of granulomatous inflammation produced against the virus. The use of experimentally infected animals may help to define different stages of infection and further the understanding of the pathogenesis of this emerging disease of ferrets.

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