

# Systemic immune responses in *Cytauxzoon felis*-infected domestic cats

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**Objective**—To characterize systemic immune responses in *Cytauxzoon felis*-infected cats.

**Sample**—Blood and lung samples obtained from 27 cats.

**Procedures**—Cats were allocated into 4 groups: cats that died of cytauxzoonosis, acutely ill *C felis*-infected cats, healthy survivors of *C felis* infection, and healthy uninfected cats. Serum concentrations of tumor necrosis factor- $\alpha$  and interleukin-1 $\beta$  were measured and serum proteins characterized. Blood smears were stained immunocytochemically and used to assess immunoglobulin deposition. Immunohistochemical expression of CD18 and tumor necrosis factor- $\alpha$  were compared in lung tissues obtained from cats that died and healthy uninfected cats. A real-time reverse-transcription PCR assay for CD18 expression was performed on selected blood samples from all groups.

**Results**—Concentrations of both cytokines were greater and serum albumin concentrations were significantly lower in cats that died of cytauxzoonosis, compared with results for all other groups. Erythrocytes from acutely ill cats and survivors of *C felis* infection had staining for plasmalemmal IgM, whereas erythrocytes from the other groups did not. Increased staining of *C felis*-infected monocytes and interstitial neutrophils for CD18 was detected. The real-time reverse-transcription PCR assay confirmed a relative increase in CD18 expression in cats that died of cytauxzoonosis and acutely ill cats, compared with expression in other groups. Immunostaining for TNF- $\alpha$  in lung samples confirmed a local proinflammatory response.

**Conclusions and Clinical Relevance**—Results indicated immunopathologic responses were greater in cats that died of *C felis* infection than in cats that survived *C felis* infection. (*Am J Vet Res* 2013;74:901–909)

Cytauxzoonosis is a fatal disease of domestic cats in the Midwestern, mid-Atlantic, southeastern, and south-central United States caused by *Cytauxzoon felis*, a tick-borne parasite in the order Piroplasmida, family Theileriidae.<sup>1,2</sup> Infected ticks transmit *C felis* during feeding, which is followed by schizogony of the parasite in monocytes throughout the body.<sup>3</sup> Although *C felis* infection can be subclinical, clinical signs in infected cats include anorexia, signs of depression, lethargy, dehydration, pyrexia, dyspnea, icterus, and dark urine as well as the less common clinical signs of pallor, anemia-

## ABBREVIATIONS

BHQ1	Black hole quencher-1
Ct	Cycle threshold
FAM	Fluorescein amidite
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
IL	Interleukin
LLD	Lower limit of detection
qRT	Quantitative real-time reverse transcription
SPE	Serum protein electrophoresis
TNF	Tumor necrosis factor

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induced heart murmur, and increased capillary refill time. Hematologic findings may include normocytic, normochromic, nonregenerative anemia with pancytopenia or moderate neutrophilia.<sup>2,4</sup>

Since the discovery and description of *C felis* infections in the 1970s, little has been published regarding the immune response of cats to this disease. Investigators in 1 report<sup>5</sup> described the formation of antibodies against the nonpathogenic, erythrocytic stage of *C felis*. In 2 studies,<sup>6,7</sup> investigators confirmed that monocytes were the infected cells in the leukocytic stage of the disease. Interstitial pneumonia commonly evident in cats

that die of *C felis* infection has been described and categorized, and the author of that report<sup>8</sup> suggested that this inflammation is likely caused by release of proinflammatory cytokines and chemokines by the infected monocytes. One of the main histopathologic characteristics of cytauxzoonosis is the presence of giant, infected, intravascular monocytes, many of which are adhered to the vascular endothelium and that possibly have involvement with and cause activation of CD18.

The CD18 integrin is present on most leukocytes, especially when they are activated,<sup>9–19</sup> and this integrin plays a role in the adherence of leukocytes to the endothelium during inflammation. Different molecules, such as chemokines, fibrinogen, selectins, and cytokines such as TNF- $\alpha$ , can activate integrins. Chemokines are among the most important and common activating molecules for integrins.<sup>15,16,18–21</sup>

The objective of the study reported here was to characterize the systemic immune response of cats to *C felis* infection. We hypothesized that uncontrolled proinflammatory immune responses, caused in part by increased CD18 expression, contribute to the pathogenesis of cytauxzoonosis.

## Materials and Methods

**Cats**—Blood samples were obtained from cats at participating veterinary clinics that were confirmed by diagnostic laboratories or participating veterinarians as infected with *C felis* on the basis of results of blood smear analysis. Practitioners at veterinary clinics in Georgia and Arkansas were contacted via telephone, e-mail, and facsimile during March 2009 through September 2012, informed about the study, and asked to participate. Serum samples also were obtained from 5 healthy uninfected cats that were blood donors owned by the investigators or part of the blood donor colony at the University of Georgia College of Veterinary Medicine. Owner consent was obtained for use of investigator-owned blood donors, and the study was conducted in compliance with the University of Georgia College of Veterinary Medicine Clinical Research Committee.

**Clinical history of *C felis*-infected cats**—Copies of the medical records or brief summaries of the clinical history were solicited from participating veterinarians for all cats infected with *C felis*. Information extracted from these records included the date of diagnosis, number of days the cat had been ill before *C felis* infection was diagnosed, date the blood sample was collected, treatment history (including any hematologic analyses, if performed), and case resolution (survival to discharge or death). None of the cats in the group that died were euthanized (ie, all of them died of *C felis* infection). On the basis of that information, the cats were allocated into 3 groups: *C felis*-infected cats that died of the disease, acutely ill *C felis*-infected cats that survived the infection until discharge, and cats that survived *C felis* infection and subsequently were healthy.

**Collection of blood samples**—Blood samples (1 to 5 mL/sample) were collected into EDTA-containing tubes and tubes without any anticoagulant (clot tubes) from the 3 groups of cats with *C felis* infection (cats

that died of the disease, acutely ill *C felis*-infected cats, and healthy survivor cats). Blood samples were also collected from some cats into an RNA tube<sup>a</sup> to prevent RNA degradation. For the 3 groups, these blood samples were collected 0 to 2 days after initial evaluation of these cats at participating veterinary clinics, which was approximately 1 or 2 days after owners noticed clinical signs. These samples were used to confirm infection with *C felis*.

Serum samples were also used for measurement of cytokine concentrations for all groups. For the healthy survivor group (which included 2 of the acutely ill cats that subsequently recovered from the disease), convalescent blood samples were collected at least 2 weeks after resolution of the disease. Duration of the disease was approximately 3 to 5 days after initial examination by a participating veterinarian. Resolution of disease was determined by participating veterinarians on the basis of improvement in clinical signs and an increase in appetite. The interval between acute and convalescent blood samples for the healthy survivors was approximately 1 month.

Blood samples were collected from 5 healthy uninfected control cats; these samples were collected during the same time period as the submission of blood samples from participating veterinarians.

**Processing of blood samples**—After samples arrived at our laboratory, blood smears were prepared from EDTA-anticoagulated blood samples, which were used to confirm the previous diagnosis of cytauxzoonosis in affected cats and to provide slides for immunocytochemical analysis. Serum was immediately separated from blood samples shipped in clot tubes; serum was stored at  $-20^{\circ}\text{C}$  for 7 to 30 days until a sufficient number of samples was amassed for cytokine quantitation. Serum samples obtained from the healthy uninfected cats were handled in the same manner as samples from clinically affected cats; these serum samples were stored at  $-20^{\circ}\text{C}$  at least overnight and were assayed along with the clinical samples.

**ELISA**—A sandwich ELISA was performed with reagents optimized for detection of feline TNF- $\alpha$  and IL-1 $\beta$ .<sup>b</sup> All assays were conducted in accordance with the protocol provided by the manufacturer. All serum samples (control and clinically affected cats) were assayed in duplicate or triplicate, depending on sample volume. Assays were conducted in several batches that coincided with acquisition of a sufficient number of samples. Flat-bottom, 96-well microplates<sup>c</sup> were used for the assay. A 7-point standard curve was generated with each batch; the standards were created via 2-fold dilution in 1% bovine serum albumin in PBS solution. The high standard was 1,000 pg/mL for TNF- $\alpha$  and 2,000 pg/mL for IL-1 $\beta$ , and the LLD was 15.6 pg/mL for TNF- $\alpha$  and 31.25 pg/mL for IL-1 $\beta$ .

Evaluation of results of preliminary experiments with the assays indicated that cytokine concentrations were too low to allow for dilution in 10% fetal bovine serum and PBS solution as recommended by the manufacturer; therefore, the samples were routinely not diluted prior to assay. Spike-and-recovery experiments were performed to validate the use of undiluted sam-

ples; results indicated recovery of 80% to 100%. Optical density was determined with a plate reader and associated software<sup>d</sup>; absorbance was set at 450 nm.

**SPE**—Serum samples remaining after the ELISA was performed were sent to another diagnostic laboratory<sup>c</sup> for SPE. Results were compared with established reference intervals and interpreted by 2 board-certified veterinary clinical pathologists.

**IgM immunocytochemical analysis**—Blood smears made at the time of receipt at our laboratory were stained immunocytochemically<sup>f</sup> with mouse anti-feline IgM monoclonal antibody and isotype control antibody.<sup>8</sup> Before starting the procedure, the blood smear on each slide was divided in half with a hydrophobic pen. Blood smears were fixed by incubation in acetone for 3 minutes at room temperature (approx 25°C). Each half of the slide was incubated at room temperature for 1 hour with mouse anti-feline IgM (1:50) or an isotype control antibody (1:50). Slides then were counterstained with Gill hematoxylin, air-dried, mounted, and subjectively examined via light microscopy by one of the investigators. Results for slides were considered negative when cells had no staining for IgM and positive when most of the erythrocytes had staining for IgM.

**CD18 and TNF- $\alpha$  immunohistochemical analysis**—Samples of lung tissues were obtained from cadavers of cats that had died of *C felis* infection and from healthy uninfected control cats. Tissues were fixed in neutral-buffered 10% formalin, embedded in paraffin, cut at a thickness of 4  $\mu$ m, and placed on glass slides. Antigen retrieval was performed in accordance with the manufacturer's protocol.<sup>h</sup> Antibodies used included mouse anti-feline CD18 monoclonal antibody,<sup>i</sup> goat anti-mouse TNF- $\alpha$  polyclonal antibody<sup>j</sup> (which has been found to cross-react with feline TNF- $\alpha$ <sup>22</sup>), and isotype control antibodies.<sup>8</sup> A commercial kit<sup>f</sup> was used for stain development. Each slide was incubated with primary antibody (dilution of 1:50 for CD18 and 1:100 for TNF- $\alpha$ ) at room temperature for 30 minutes. Slides were counterstained with Gill hematoxylin stain and rinsed with deionized water, and a coverslip was then added.

Staining distribution was subjectively compared via light microscopy by a board-certified veterinary pathologist. The CD18 and TNF- $\alpha$  immunoreactivity was characterized as low, moderate, or high. For CD18, low staining was defined as staining of only the cell membrane of a few alveolar macrophages. Moderate staining was defined as staining of cells other than alveolar macrophages in addition to staining of alveolar macrophages, but less than half of all inflammatory cells in the section. High staining was defined as staining of more than half of the inflammatory cells. For TNF- $\alpha$ , low staining was defined as extremely light, diffuse, background staining (but more staining than negative control samples) and no cytoplasmic staining in the inflammatory cells. Moderate staining was defined as diffuse TNF- $\alpha$  immunoreactivity evident in the cytoplasm of less than half of the inflammatory (mostly infected) cells. High staining was defined as staining evident in more than half of the inflammatory cells.

**RNA isolation and cDNA synthesis**—Blood samples collected in RNA tubes were stored at 5°C until

use. The RNA was extracted in accordance with the manufacturer's protocol<sup>k</sup> but with a slight modification. The resulting eluate was incubated for 5 minutes at 65°C before storage at -80°C until needed.

The cDNA was obtained via a procedure performed in accordance with the manufacturer's instructions.<sup>l</sup> Before cDNA synthesis, the concentration of RNA obtained from each blood sample was measured with a spectrophotometer.<sup>m</sup> For this experiment, 40 ng of RNA from each sample were used. Samples were stored at -20°C.

**Primer and probe design**—Primers and probes<sup>n</sup> were designed with available software.<sup>o</sup> As the specific feline CD18 sequence has not been reported in GenBank, a consensus of various other mammalian CD18 sequences was used to design the primers and probes. The selected forward primer was 5'-CACAAAGTC-CATACCCAGGATCT-3', and the reverse primer was 5'-GGAGATCACATGACTTAAAAGGAC-3'. To perform real-time PCR analysis, a linear fluorescent-labeled probe was designed to anneal within the targeted CD18 amplicon. The probe sequence was 5'-AGGGCCTCCTGTGGGATTCTGG-3' and was labeled with 5'-FAM fluorescence and 3'-BHQ1 quencher. The GAPDH primers and probes were also designed with available software.<sup>o</sup> The selected forward primer was 5'-CATCTTCCAGGAGCGAGAT-3', and the selected reverse primer was 5'-CCACAACATACTCAGCACCA-3'. The sequence for the fluorescent-labeled probe designed to anneal with the GAPDH amplicon was 5'-CGCCAACATCAAATGGGGTG-3', with 5'-FAM fluorescence and 3'-BHQ1 quencher.

**qRT-PCR assay**—All qRT-PCR assays were performed with an automated system.<sup>p</sup> The concentrations for all the primers and probes were determined after performing optimization experiments. Reactions for GAPDH and CD18 were conducted simultaneously via separate tubes for each sample. The thermal cycler program was as follows: 10 minutes at 95°C, which was followed by 40 cycles of 15 seconds at 95°C, 30 seconds at 52°C, and 30 seconds at 72°C. The Ct values were obtained from the PCR graphs, and the relative expression of CD18 was calculated via the comparative Ct method ( $2^{-\Delta\Delta Ct}$ ).<sup>23</sup>

**Statistical analysis**—Mean and SD values were obtained, and error bars were used to represent SEMs.<sup>q</sup> For cytokine results, differences between SDs were large so the means were logarithmically transformed; an ANOVA, followed by the Tukey-Kramer multiple comparisons test, was used to detect significant differences among groups. Differences were considered significant at a value of  $P < 0.05$ .<sup>r</sup> Protein concentrations obtained via SPE were analyzed in a similar manner, although these data did not require logarithmic transformation prior to statistical analysis.

For immunocytochemical analysis, samples were categorized and analyzed by group. The number of positive slides per subgroup, as determined subjectively by one of the investigators, was divided by the total number of slides in that subgroup, and those values and the percentage were reported. A Fisher

Table 1—Number of samples that were obtained from acutely ill *Cytauxzoon felis*-infected cats that subsequently died of the disease, acutely ill *C felis*-infected cats that survived until discharge, cats that survived *C felis* infection and subsequently were healthy, and healthy uninfected (control) cats and used in each of several assays.

Group	IgM		CD18 qRT-PCR assay	CD18		TNF- $\alpha$ immunohistochemical analysis
	ELISA	SPE		immunohistochemical analysis	immunohistochemical analysis	
Died (n = 8)	8	4	5	3	7	7
Acutely ill (n = 7)	7	3	5	4	0	0
Healthy survivor (n = 8)	8*	6†	7	7	0	0
Healthy uninfected (n = 5)	5	4	4	5	2	2

Samples were obtained from cats that died of cytauxzoonosis and acutely ill cats during the illness at 1 or 2 days after cats became ill, from healthy survivor cats at least 2 weeks after cats recovered from cytauxzoonosis, and from healthy uninfected control cats during the same period as the submission of blood samples from participating veterinarians for the other 3 groups. Samples from each cat were not used in all assays because of limitations in sample type or volume of samples obtained.

\*Includes 2 cats that were part of the acutely ill group. †Includes 1 cat that was part of the acutely ill group.

exact test was used to determine significant differences between groups.

For immunohistochemical analysis, only samples of lung tissues obtained from cats that died of *C felis* infection and healthy uninfected control cats were compared subjectively by one of the investigators. A Fisher exact test was performed to determine significant differences between these 2 groups in terms of expression of CD18 and TNF- $\alpha$ .

For the qRT-PCR assay, values obtained with the software were imported to a spreadsheet program,<sup>9</sup> and calculations based on published methods<sup>24</sup> were used to obtain fold changes and SDs. Samples were categorized as described previously, and an ANOVA followed by the Tukey-Kramer multiple comparisons test was used to analyze differences.

## Results

**Samples**—Blood and serum samples were obtained from 27 cats and analyzed via various assays (Table 1). Of the 7 acutely ill cats that had samples analyzed via ELISA, 2 were subsequently included in the healthy survivor group for analysis via ELISA because they were available for collection of convalescent samples after clinical recovery. Of the 3 acutely ill cats that had samples analyzed via SPE, 1 was subsequently included in the healthy survivor group because the cat was available for collection of convalescent samples after clinical recovery.

For 4 cats (1 in the group that died, 2 in the group of acutely ill cats, and 1 in the healthy survivor group), there was insufficient sample volume to allow the ELISA to be performed in triplicate. For the TNF- $\alpha$  assay, 2 acutely ill cats, 3 healthy survivor cats, and 4 healthy uninfected cats had concentrations below the LLD. For the IL-1 $\beta$  assay, 3 cats that died, 2 acutely ill cats, 5 healthy survivor cats, and 5 healthy uninfected cats had concentrations below the LLD.

Complete hematologic analysis was performed on a subset of the 3 groups of *C felis*-infected cats. The main abnormalities detected were anemia, thrombocytopenia, and leukopenia; however, there were no significant differences in these abnormalities among the 3 groups of *C felis*-infected cats.

**Proinflammatory cytokines**—Concentrations of the major proinflammatory cytokines (TNF- $\alpha$  and IL-1 $\beta$ ) were higher for cats that died of the disease,

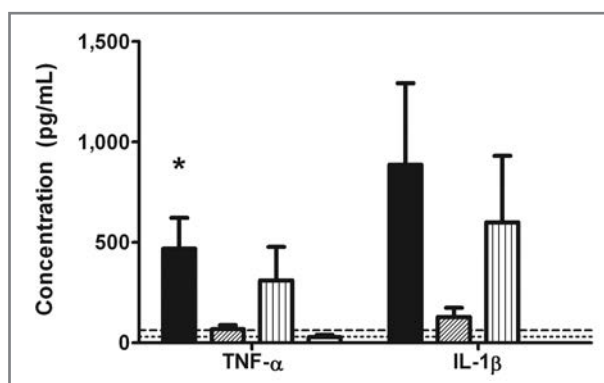


Figure 1—Mean  $\pm$  SEM serum TNF- $\alpha$  and IL-1 $\beta$  concentrations, as determined via an ELISA, for acutely ill *Cytauxzoon felis*-infected cats that subsequently died of the disease (n = 8; black bars), acutely ill *C felis*-infected cats (7; diagonal-striped bars), healthy cats that survived *C felis* infection (8; vertical-striped bars), and healthy uninfected control cats (5; gray bars). Blood samples were collected 1 or 2 days after onset of disease for the cats that died of the disease and the acutely ill cats and were collected at least 2 weeks after complete clinical recovery for the healthy survivor cats; 2 of the healthy survivor cats were also included in the group of acutely ill cats. Notice the LLD for TNF- $\alpha$  (31.25 pg/mL [dotted line]) and IL-1 $\beta$  (61.5 pg/mL [dashed line]). \*Within a cytokine, the value for this group of cats differs significantly ( $P < 0.05$ ) from the value for the group of healthy uninfected control cats.

compared with concentrations for the other 3 groups, although results were only significantly different for TNF- $\alpha$  concentrations between the group of cats that died and the group of healthy uninfected cats (Figure 1). Although healthy survivor cats had higher concentrations of these cytokines, compared with concentrations for the acutely infected cats and healthy uninfected cats, the concentrations of TNF- $\alpha$  and IL-1 $\beta$  did not differ significantly.

Differences in cytokine concentrations among the groups were not considered attributable to a delay before serum separation and harvest. Paired serum samples (serum was separated immediately after clotting or after samples were allowed to sit for 24 hours at 4°C) were obtained from a control cat with naturally high concentrations of TNF- $\alpha$  and IL-1 $\beta$ . There were no significant differences in TNF- $\alpha$  or IL-1 $\beta$  concentrations in these paired serum samples (data not shown).

All lung tissues obtained from cats that died of *C felis* infection had staining for TNF- $\alpha$  via immunohistochemical analysis. The tissues were characterized

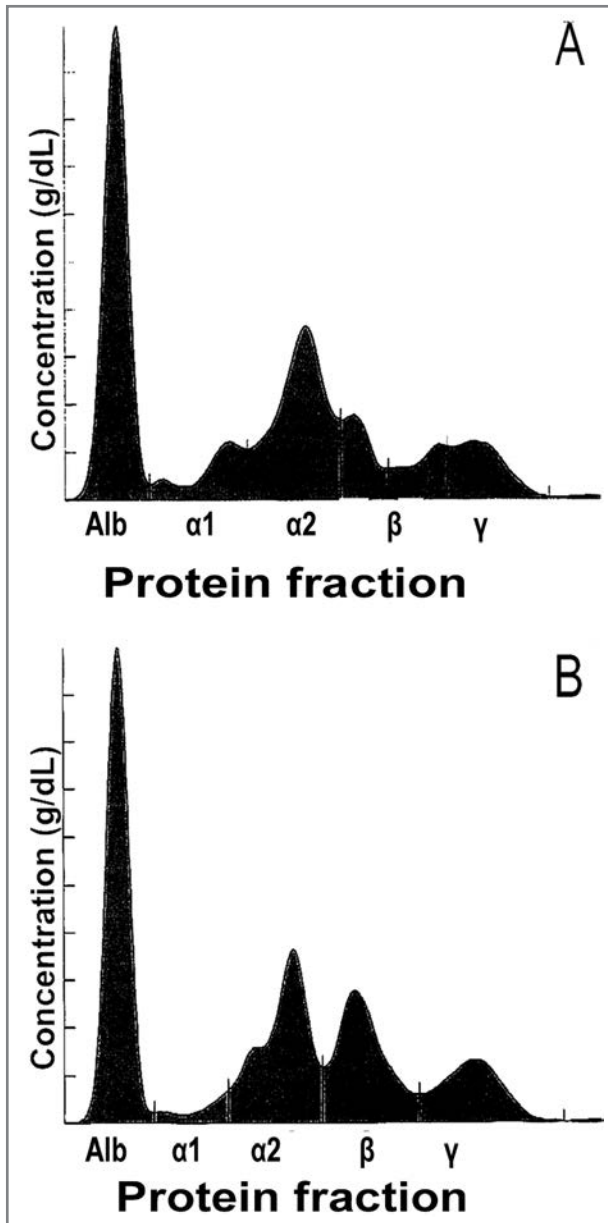


Figure 2—Serum protein electrophoretograms of representative cats with cytauxzoonosis that had increased  $\alpha$ -globulin concentrations (A) and increased  $\beta$ -globulin concentrations (B). Alb = Albumin.

as having high staining, particularly in the cytoplasm of infected monocytes that lined vessels and other inflammatory cells within the pulmonary interstitium and alveoli (data not shown).

**Serum protein**—All groups contained at least 2 cats (4 cats that died of the disease, 2 acutely infected cats, 3 healthy survivor cats, and 2 healthy uninfected cats) for which the results were interpreted as a non-specific, acute-phase inflammatory response, which was primarily attributed to increases in  $\alpha_2$ -globulin concentrations (Figure 2). Four cats (1 acutely ill cat, 2 healthy survivor cats, and 1 healthy uninfected cat) had results that were deemed not clinically relevant. Three cats (1 acutely ill cat and 2 healthy survivor cats) had mild to marked increases in total  $\beta$ -globulin concen-

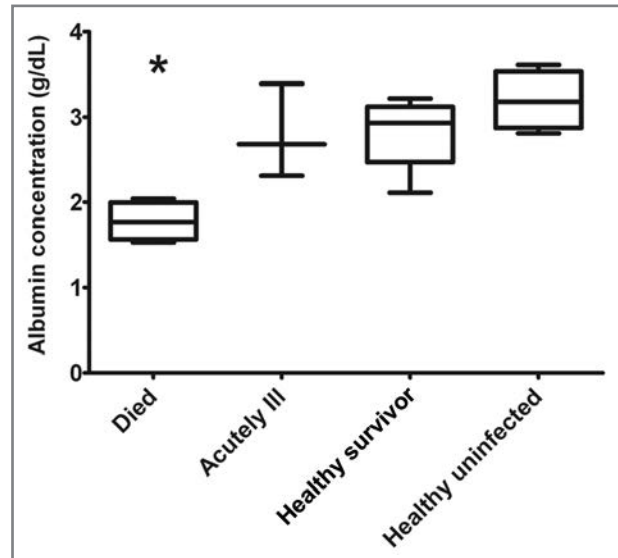


Figure 3—Box-and-whisker plot of serum albumin concentrations as determined via SPE for cats that died of cytauxzoonosis ( $n = 4$ ), cats acutely ill with cytauxzoonosis (3), healthy cats that survived *C felis* infection (6), and healthy uninfected control cats (4). One of the healthy survivor cats was also included in the acutely ill group. For each group, the upper and lower boundaries of the box represent the range, the horizontal bar in each box represents the median, and the whiskers represent the SEM. \*Median values for this group differ significantly ( $P < 0.05$ ) from the values for all other groups.

trations that were attributed to hemolysis of the blood samples. One cat in the group of cats that died of the disease had results consistent with restricted oligoclonal gammopathy.

Comparison of mean specific protein concentrations revealed several differences among the groups. Total protein concentration was significantly ( $P = 0.01$ ) lower in the group of cats that died of *C felis* infection (5 mg/dL), compared with concentrations for the other 3 groups. Similarly, the mean albumin concentration for the group of cats that died (1.78 g/dL) was significantly lower, compared with concentrations for the other groups (Figure 3). The mean albumin concentration for the group of cats that died of *C felis* infection was significantly ( $P = 0.01$ ) lower, compared with concentrations for the healthy survivor cats and the healthy uninfected cats.

**IgM immunocytochemical analysis**—The blood smears of 5 cats that died of the disease were tested; one of these had positive results for IgM as indicated by staining of erythrocytes and neutrophils. Three of 5 blood smears for acutely ill cats and 3 of 7 blood smears for healthy survivor cats also had staining for IgM. None of the blood smears of the 4 healthy uninfected cats had staining for IgM deposition (Figure 4). Staining differed among groups, but not significantly, as determined with the Fisher exact test.

**CD18 expression**—Immunohistochemical staining for CD18 revealed that expression was extremely low in all healthy uninfected cats and was restricted to plasmalemmal staining of a few scattered alveolar macrophages. Cats that died of *C felis* infection had moderate to high CD18 expression, as indicated by plasma-

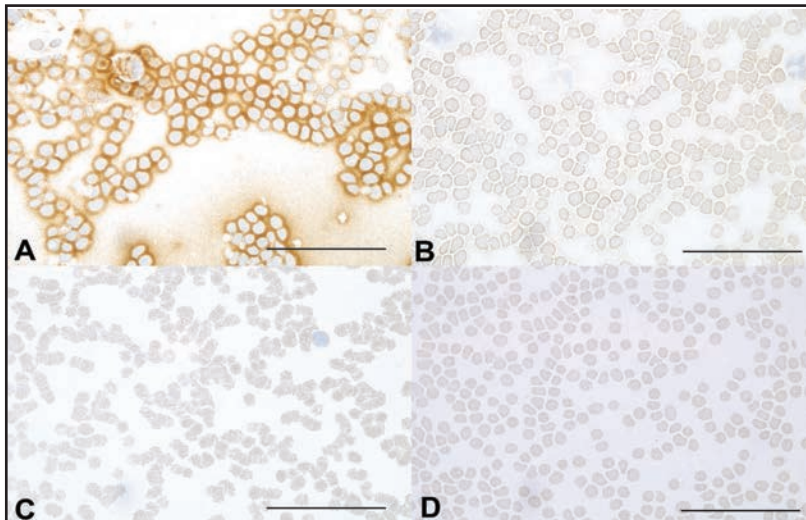


Figure 4—Photomicrographs of results of IgM immunocytochemical analysis of blood samples obtained from a *C felis*-infected cat (A and B) and a healthy uninfected cat (C and D). An anti-IgM antibody was used in panels A and C, and an isotype control antibody was used in panels B and D. In panel A, notice the intense staining of the erythrocytes. 3,3'-diaminobenzidine chromogen with hematoxylin counterstain; bar = 50  $\mu$ m.

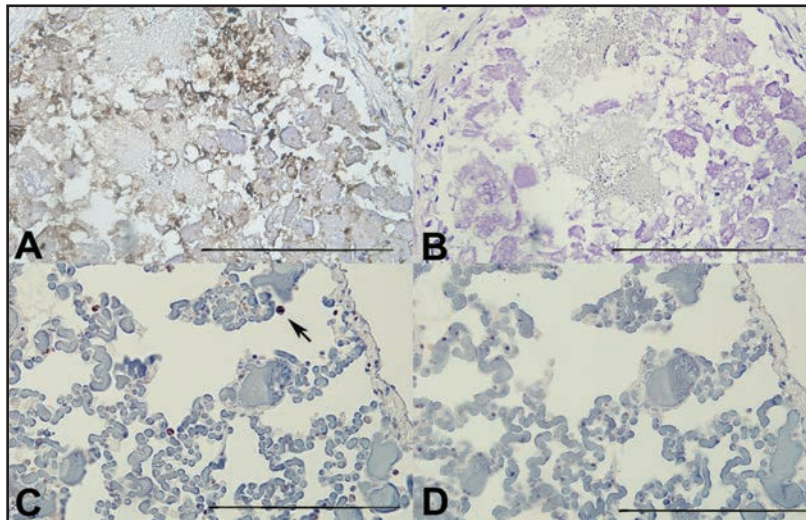


Figure 5—Photomicrographs of results of CD18 immunohistochemical analysis of lung sections obtained from a *C felis*-infected cat (A and B) and a healthy uninfected control cat (C and D). An anti-CD18 antibody was used in panels A and C, whereas an isotype control antibody was used in panels B and D. In panel A, notice the plasmalemmal staining of infected monocytes within a blood vessel. In panel C, notice the CD18 staining of an alveolar macrophage (arrow). 3,3'-diaminobenzidine chromogen with hematoxylin counterstain; bar = 200  $\mu$ m.

lemmal staining for CD18 (Figure 5). A significant difference in staining for CD18 was observed between the cats that died of the disease and the healthy uninfected cats. To confirm upregulation of CD18 at the transcriptional level, mRNA concentrations of CD18 in peripheral blood leukocytes were compared among the 4 groups. Compared with CD18 expression (normalized on the basis of GAPDH expression) for healthy uninfected cats, cats that died of the disease had an 11.6-fold increase in CD18 expression, whereas acutely ill cats had a 9.0-fold increase and healthy survivor cats had a 1.3-fold increase (Figure 6). There was a significant difference in CD18 expression between cats that died of the disease and healthy survivor cats, cats that died

of the disease and healthy uninfected cats, acutely ill cats and healthy survivor cats, and acutely ill cats and healthy uninfected cats.

## Discussion

Many studies of cytauxzoonosis have focused on the cause and transmission of the disease, but 1 study<sup>25</sup> focused on immunologic aspects, host response, and pathogenesis of the disease. The authors of that study<sup>25</sup> described an indirect fluorescent antibody test for the detection of the leukocytic stage of *C felis*; they also described attempts to maintain the parasite in vitro, serologic assays, and antibody responses and reported that infected cats did not develop detectable antibody titers or had an extremely low response late in the disease that was insufficient to enable affected cats to recover from the disease. The combined immunologic, histologic, and molecular findings of the present study of cats naturally infected with *C felis* provide additional information on the pathogenesis of cytauxzoonosis. The findings also highlight the need for further studies to enable a better understanding of the feline immune response to this parasite and means to better combat this disease with pharmaceuticals.

Although there were higher concentrations of the proinflammatory cytokines TNF- $\alpha$  and IL-1 $\beta$  in cats that died of the disease than in cats of all other groups, the concentrations were only significantly higher for TNF- $\alpha$  concentrations in cats that died of the disease, compared with concentrations in the healthy uninfected cats. The large variations were likely attributable to differences in the time of collection of a single sample during the course of the disease, with the potential that the peak cytokine concentration could have been missed. In addition, there may have been differences attributable to genetics among breeds of cats. The release of proinflammatory cytokines can cause lesions that are evident histologically<sup>8</sup> and may be part of an exaggerated systemic immune response, as was suggested in that study.<sup>8</sup> This was also supported by the marked staining for TNF- $\alpha$  detected during immunohistochemical analysis.

Analysis of serum protein profiles for most of the samples from the infected cats revealed nonspecific acute inflammatory responses; these findings were not unexpected, given the other results in the present study. Surprisingly, similar patterns were commonly seen in the healthy uninfected control cats, and the underlying causes for these responses in those animals were not clear. These findings highlight a major limitation

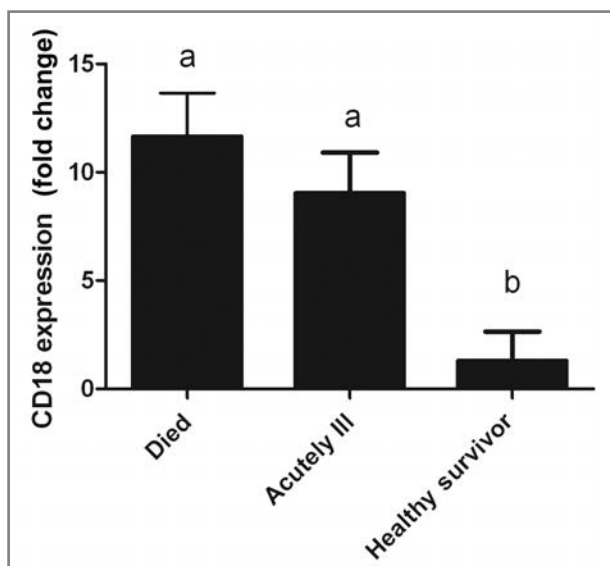


Figure 6—Mean  $\pm$  SEM fold change in CD18 mRNA expression (normalized on the basis of GAPDH expression) for cats that died of cytauxzoonosis, cats acutely ill with cytauxzoonosis, and healthy cats that survived *C felis* infection, compared with results for healthy uninfected control cats. <sup>a,b</sup>Values with different letters differ significantly ( $P < 0.05$ ).

for SPE interpretation in this study in that electrophoretograms are typically not indicative of a particular etiologic agent. As a result, cats infected with *C felis* or cats with unrelated conditions, some of which may be subclinical, could have similar electrophoretograms. This was further supported by the healthy survivor cats that had recovered from cytauxzoonosis but still had SPE patterns consistent with inflammation. This may have suggested there was a lingering systemic effect of the disease, perhaps related to the persistent erythrocytic infection<sup>26</sup>; however, it cannot be excluded that those cats had a new, unrelated reason for an acute inflammatory response. In 1 cat in the healthy survivor group, the inflammatory profile was evident in the acute sample and persisted in the convalescent sample, although it was less evident in the convalescent sample. This suggested that in some cats with *C felis* infection, serial monitoring of SPE may be more helpful than single evaluations, but further investigation is necessary to confirm this assumption.

Most of the samples submitted for SPE had evidence of a nonspecific, acute-phase response, regardless of *C felis* status of the cats from which they were obtained; however, the albumin concentration was significantly different among groups. Albumin is considered a negative acute-phase protein, the concentration of which decreases during an acute inflammatory response.<sup>27–31</sup> This decrease is caused in part by an increase in proinflammatory cytokines, such as TNF- $\alpha$  and IL-1 $\beta$ , during acute inflammation. These high concentrations of cytokines have inhibitory effects on the hepatic synthesis of albumin.<sup>29</sup> The decrease in albumin concentration was more noticeable in cats that died of *C felis* infection, which suggested an increase in the overall inflammatory response. Although inflammation was likely at least partially responsible for changes detected among the groups, the magnitude of the decrease

in the albumin concentration suggested 1 or more concurrent processes, such as vasculitis, liver dysfunction, renal loss, or even protein-losing enteropathies, were also contributing to the hypoalbuminemia. Alternative explanations for hypoalbuminemia were not investigated because of study limitations.

Blood smears from acutely ill cats and healthy survivor cats were more likely to have staining for IgM than were blood smears from cats that died of *C felis* infection or healthy uninfected control cats. Interestingly, most of the acutely ill cats with staining for IgM also had increases in the amount of total  $\beta$ -globulins (the band in which IgM can migrate during gel electrophoresis). Although antibody responses to *C felis* have been described,<sup>25</sup> they were not found to be protective in that study<sup>25</sup> and may perhaps play a role in extravascular hemolysis (via deposition on erythrocytes as found in the present study) and icterus commonly observed in animals with cytauxzoonosis. Similarly, IgM-positive slides, for the most part, were not associated with higher concentrations of  $\gamma$ -globulins in the electrophoretogram, and 1 cat with staining for IgM eventually died of cytauxzoonosis. On the other hand, cats that died of cytauxzoonosis were less likely to have IgM deposition detected during immunocytochemical analysis than were acutely infected cats, despite the fact that both groups of cats were likely to be at a similar stage of the disease course at the time blood samples were collected (ie, the day the cats were first examined by a referring veterinarian), which suggested that antibodies against the erythrocytic phase of the parasite may have some protective effect. However, it should be mentioned that accuracy of the duration of clinical signs was unknown, considering that the onset of disease was reported by the owners. Therefore, it is possible that samples with negative results for IgM were obtained before an antibody response could be mounted.

Immunohistochemical analysis of *C felis*-infected tissue samples revealed a qualitative increase in the surface expression of CD18 (an important leukocyte adhesion molecule upregulated during inflammation), compared with results for uninfected tissues. We detected CD18 only in alveolar macrophages and a few neutrophils within the lungs of the healthy uninfected control cats, which is consistent with their sentinel role. A marked increase in CD18 staining was evident in *C felis*-infected cats, mainly on infected or activated monocytes within blood vessels as well as on interstitial neutrophils. It was suggested in 1 study<sup>32</sup> that CD18 contributes to an increase in the expression of proinflammatory cytokines, including TNF- $\alpha$  and IL-1 $\beta$ .

Although immunohistochemical analysis yields qualitative information regarding CD18 expression, qRT-PCR assay provides quantitative information regarding CD18 mRNA upregulation. In the present study, expression of CD18 was increased in cats that died of cytauxzoonosis, acutely ill cats, and healthy survivor cats, compared with results for healthy uninfected control cats. Cat that died of cytauxzoonosis and acutely ill cats had the highest expression of CD18, which was expected and supported (at least for the cats that died of cytauxzoonosis) by the immunohistochemical results. The group of acutely ill cats had lower expression of

CD18 than did cats that died of cytauxzoonosis, which could possibly have been related to lower expression of the proinflammatory cytokines. Increases in CD18 expression are relevant because they relate to leukocyte activation,<sup>9-19</sup> and interaction of CD18 with its ligands can in turn upregulate proinflammatory cytokines that can cause vascular leakage or damage.<sup>33</sup> Upregulation of CD18 and adhesion was also consistent with the common finding of leukopenia in that adhered leukocytes would not be collected during venipuncture.

Tests that are more specialized for the determination of the exact concentration of specific proteins would be helpful in further characterizing the inflammatory response. In cats, these would include tests to measure concentrations of serum amyloid A as well as  $\alpha$ -1-glycoprotein and haptoglobin, the major and moderate acute-phase proteins in cats, respectively.<sup>26,29,34</sup> Alternatively, capillary electrophoresis (which is a more sensitive method of electrophoresis) could be used to help detect subtle changes in serum proteins and better separate protein fractions.<sup>35</sup> As described in a recent case report,<sup>36</sup> clinicians detected biclonal gammopathy in a cat with a plasma cell neoplasm by use of a capillary electrophoresis method; that gammopathy had not been detected with regular SPE. A direct Coombs' test would also be valuable for confirming antibody deposition on erythrocytes as a mechanism for hemolysis during cytauxzoonosis. Unfortunately, because of limited sample volume and a recent reduction in the number of samples submitted to the University of Georgia during the years of this project, these methods of analysis were beyond the scope of the present study.

Results of a combination of immunologic, histologic, and molecular techniques for the present study support the hypothesis that cytauxzoonosis causes a robust systemic proinflammatory response characterized by increases in proinflammatory cytokines, including a local inflammatory response, an acute-phase response, IgM deposition on erythrocytes, and upregulation and expression of CD18 on leukocytes. This response is more severe in cats that die of *C felis* infection, compared with the response in cats that survive the disease, which suggests that the immune response is important in the pathogenesis of this disease.

- a. PAXgene blood tube, Qiagen, Valencia, Calif.
- b. DuoSet, RnD Systems, Minneapolis, Minn.
- c. Nunc-Immuno, Sigma-Aldrich, St Louis, Mo.
- d. KC4, BioTek, Winooski, Vt.
- e. Animal Health Diagnostic Center, Cornell University, Ithaca, NY.
- f. Dako Envision system, Dako, Carpinteria, Calif.
- g. Clone AA124, AbD Serotec, Raleigh, NC.
- h. L.A.B. solution, Polysciences, Warrington, Pa.
- i. Clone Fe3.9F2, provided by Dr. Peter Moore, Department of Pathology, Microbiology, and Immunology, School of Veterinary Medicine, University of California-Davis, Davis, Calif.
- j. Clone M-18, Santa Cruz Biotechnology, Santa Cruz, Calif.
- k. PAXgene blood RNA kit, Qiagen, Valencia, Calif.
- l. ThermoScript RT-PCR system, Invitrogen, Grand Island, NY.
- m. NanoDrop 2000, Thermo Scientific, Waltham, Wash.
- n. Integrated DNA Technologies, Coralville, Iowa.
- o. Primer3, Broad Institute, Cambridge, Mass. Available at: [www.genome.wi.mit.edu/genome\\_software/other/primer3.html](http://www.genome.wi.mit.edu/genome_software/other/primer3.html). Accessed Mar 1, 2009.
- p. iCycler IQ system, Bio-Rad, Hercules, Calif.

- q. Excel 2007, Microsoft Corp, Redmond, Wash.
- r. InStat, GraphPad Software Inc, La Jolla, Calif.

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