FELINE IMMUNE RESPONSE TO INFECTION WITH CYTAUXZOOM FELIS AND THE ROLE OF CD18 IN THE PATHOGENESIS OF CYTAUXZOOMOSIS

by

KARELMA FRONTERA-ACEVEDO

(Under the Direction of Kaori Sakamoto)

ABSTRACT

Cytauxzoonosis is a highly fatal, hemoproteozal disease of cats in the Mid-Western, Mid-Atlantic, and Southeastern United States, caused by *Cytauxzoon felis*. Although the causative agent has been recognized since 1976, no study has profiled the immune response of infected cats, there is no definitive cure, and *C. felis* has not been successfully maintained in cell cultures *in vitro*, thwarting research efforts. One of the main histopathologic characteristics of this disease is the presence of giant, infected, intravascular macrophages, many of which are adhered to the vascular endothelium. The main goals of this project are: 1) to characterize the feline immune response to *C. felis*; 2) to develop a cell culture system in order to study *C. felis in vitro*; and 3) to determine whether CD18 plays a role in the pathogenesis of cytauxzoonosis.

INDEX WORDS: Cat, *Cytauxzoon felis*, pathogenesis, protozoal disease, veterinary pathology
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by

KARELMA FRONTERA-ACEVEDO

BS, University of Florida, 2004
DVM, Louisiana State University, 2008

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial
Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2013
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by

KARELMA FRONTERA-ACEVEDO

Major Professor: Kaori Sakamoto
Committee: Corrie Brown
Bridget Garner
David S Peterson
Wendy Watford

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
May 2013
DEDICATION

This work is dedicated to the author’s grandparents, José Guillermo Frontera Reichard and Ramón Isaac Acevedo Irizarry, who always supported her curiosity and encouraged learning. May they rest in peace.
ACKNOWLEDGEMENTS

The author would like to thank everyone who has helped her with this document and in her academic career. These include all her committee members, the faculty and her colleagues at the Departments of Pathology and Infectious Diseases, and the technical personnel and support staff in all the laboratories for the help and advice offered. Also, she thanks the Athens and Tifton Georgia Diagnostic Laboratories, the Arkansas Diagnostic Laboratory, and all of the other private practitioners in Georgia and Arkansas for submitting case samples.
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Cytauxzoonosis is a fatal hemoprotezoal disease of cats (*Felis domesticus*) in the Mid-Western, Mid-Atlantic, South-central, and Southeastern United States caused by *Cytauxzoon felis*. This apicomplexan parasite is classified in the order Piroplasmida, family Theileriidae. Protozoa in this family have both an erythrocytic and a leukocytic or tissue phase. It was first reported in domestic cats in 1976 in southwestern Missouri, and it is now considered an emerging infectious disease. In the life cycle of *C. felis*, the schizonts develop in intravascular monocytes, developing into numerous merozoites that eventually fill the entire cytoplasm and greatly expand the infected cell. This stage is also what separates it from the closely-related *Theileria* spp, in which the schizonts tend to occur within lymphocytes, although the tissue phase has also been found in dendritic cells and macrophages in *Theileria annulata*, which infects cattle from North Africa to China, and causes a disease called tropical theileriosis. Eventually the *C. felis*-infected cell ruptures, releasing merozoites into the blood. These then go on to invade erythrocytes and produce a late-stage parasitemia. In these cells, *C. felis* reproduces asexually by binary fission. This stage is detected in routine blood smears of both acutely ill and survivor cats.

The prepatent period of *Cytauxzoon felis* is between 2 to 3 weeks, and most of the clinical signs develop during the schizogony phase and rapid asexual multiplication of merozoites,
perhaps as a result of mechanical obstruction of blood flow,\textsuperscript{1,10,12} or by activation of infected monocytes. By-products of the parasites are also thought to be toxic, pyrogenic, and vasoactive.\textsuperscript{10,11} Infected cats die in a shock-like state or terminal hemolytic crisis.\textsuperscript{10,11} The hemolytic crisis is likely secondary to destruction of infected erythrocytes by macrophages. The shock-like state may be a result of vascular occlusion affecting several organs, such as the lungs. Another possibility is that the shock is secondary to a release of pro-inflammatory cytokines by neutrophils and activated monocytes in circulation.\textsuperscript{10,13}

Clinical signs in cats include anorexia, dyspnea, lethargy, dark urine, dehydration, depression, icterus, pallor, anemic heart murmur, increased capillary refill time, and pyrexia. Hematologic findings include pancytopenia with mild to moderate normocytic, normochromic, nonregenerative anemia.\textsuperscript{3,5,10,14} Infected erythrocytes are present late in the disease, and although erythroparasitemia can cause erythrocyte destruction and erythrophagocytosis, without the tissue phase it is of little clinical significance.\textsuperscript{5,10,11,15}

Clinical diagnosis is commonly made by finding the erythrocytic phase in blood smears stained with Romanowsky stains.\textsuperscript{5,10,11,14} The organism can look similar to other blood parasites, such as \textit{Mycoplasma haemofelis} and \textit{Babesia felis},\textsuperscript{5} the latter not being found in the United States. It can also look similar to Howell-Jolly bodies, stain precipitates, and water artifact, and a thin layer of blood smear is necessary so that erythrocytes can spread out and examination can more easily be made.\textsuperscript{5} The signet ring-shaped organisms, usually one or two per cell, are the most characteristic finding. However, other forms include tetrad (multiple organisms) or safety-pin configurations.\textsuperscript{10} Parasitemia is usually low; and this is a biological limitation, since the clinical signs can start before development of the erythroparasitemia.\textsuperscript{5,14} Monocytes infected with the tissue phase are considered to be rarely observed in peripheral blood smears, and are
more common in smears prepared from heart blood or impression smears of tissues such as spleen, liver, and lung.\textsuperscript{5,10,11}

Care of infected cats consists mostly of supportive care (fluid therapy and anticoagulant administration, for example),\textsuperscript{14} and antiprotozoal therapy has had limited success.\textsuperscript{16} One of the most commonly used drugs, imidocarb, has potential parasympathetic side effects,\textsuperscript{5,10} while a more recently published treatment involving atovaquone and azithromycin was found to be more effective.\textsuperscript{16} An earlier study that treated cytauxzoonosis with diminazene diaceturate, a product not available for animal use in the United States, found it highly effective in treating acutely ill cats, but not in eliminating the carrier state.\textsuperscript{17} Similarly, treatment with two of the drugs used to treat theileriosis in cattle, parvaquone and buparvaquone, was unsuccessful in the treatment of experimentally infected cats.\textsuperscript{18} Even with this new atovaquone treatment, though, the disease remains highly fatal, with a third of the patients dying despite aggressive treatment.\textsuperscript{16} Unlike related \textit{Theileria} spp.,\textsuperscript{7,19,20} no vaccine is currently available for \textit{C. felis}.\textsuperscript{5,14} Therefore, diligent application of tick-controlling products and limiting outdoor exposure are the two most effective ways to prevent this disease in cats.

Gross findings in dead cats include dehydration, pallor, icterus, hydropericardium, hydrothorax, enlarged, edematous, and hemorrhagic lymph nodes, an accentuated hepatic lobular pattern, congested veins, splenomegaly, and petechial and ecchymotic hemorrhages on the serosal surfaces of abdominal organs and throughout the lungs.\textsuperscript{10} The main histologic lesion consistent with feline cytauxzoonosis is the accumulation of numerous, markedly enlarged, monocytes parasitized with merozoites in various stages of development within blood vessels in multiple organs.\textsuperscript{5,10,21} This is in contrast to related organisms like \textit{Theileria} spp. or other protozoa like \textit{Toxoplasma gondii}, where the infected cells proliferate and are able to cross tissue
barriers, and do not appear to be attached to the endothelium.\textsuperscript{22} It is similar, though, to \textit{Plasmodium falciparum} and \textit{Babesia bovis} were in those cases infected erythrocytes can be found adhered to the vessel wall.\textsuperscript{23-26} Other changes include erythrophagocytosis,\textsuperscript{12} hemorrhage, edema, and vascular damage. Inflammation can be variable, but a recent article\textsuperscript{13} describes moderate interstitial pneumonia and moderate to severe vascular occlusion in lungs of infected cats. These pulmonary changes were first documented in a report by Meier and Moore.\textsuperscript{27} In this case, radiographs demonstrated severe bronchointerstitial pulmonary changes in a cat that eventually developed respiratory distress and died of cytauxzoonosis.

Electron microscopy studies are few,\textsuperscript{2,3} and study the different stages of \textit{C. felis} development within the macrophage and infection of erythrocytes. Depending on the stage, organelles, including the nucleus, mitochondria, and rhoptries, can be identified, but in other stages, the parasite does not show distinguishing features. Both studies concluded that the tissue phase of \textit{Cytauxzoon felis} was similar to other species previously studied, such as \textit{Theileria}. The report by Kier was also the only study that used scanning electron microscopy to study the parasite and observed infected mononuclear phagocytes attached to the endothelium of the splenic vein endothelium.

Because of the severe illness and usually fatal outcome, the domestic cat is considered an incidental dead-end host,\textsuperscript{10} with a definitive host present in the wildlife population.\textsuperscript{11} The bobcat (\textit{Lynx rufus}) is considered the natural reservoir for the parasite.\textsuperscript{1,5,10-12,28-30} In one study, one subspecies of bobcat (\textit{Lynx rufus floridanus}) developed the disease and died, while another subspecies (\textit{Lynx rufus rufus}) was parasitemic but remained asymptomatic.\textsuperscript{31} Curiously, in the same study, sheep developed a low persistent parasitemia but were also asymptomatic. Other studies have found that bobcats can get infected and die (at a much lower incidence), but that the
schizogony period is short and survived by most bobcats, which then become persistently parasitemic and act as reservoirs.\textsuperscript{5,32,33} Most recently, captive tigers (\textit{Panthera tigris}) without clinical signs tested positive for \textit{C. felis}, both by PCR and blood smears,\textsuperscript{34} and in other cases at least a few have died.\textsuperscript{5,35} Reasons for the subclinical infections could be that the tigers were able to overcome the tissue phase of \textit{C. felis}, or that they were only inoculated with the erythrocytic phase of \textit{C. felis}, if they attacked and killed a carrier cat or bobcat that wandered into the enclosure. \textit{Cytauxzoon felis} has also been documented, both by blood smears and by PCR, in wild Florida pumas (\textit{Puma concolor coryi}),\textsuperscript{36-38} but at a much lower incidence than bobcats.\textsuperscript{39} Little is known about the disease in this species, but based on the findings in that paper; it is possible that there are various strains of \textit{C. felis} circulating in the wildlife population.

The geographical distribution of this parasite corresponds most likely with the distribution of the Lone Star tick, \textit{Amblyomma americanum}, which has a more limited range than \textit{Dermacentor variabilis}, similar to that of reported cases of cytauxzoonosis.\textsuperscript{1} The first reports and compiled studies on transmission of \textit{Cytauxzoon felis} considered \textit{Dermacentor variabilis} to be the main tick vector,\textsuperscript{2,3,10,14,27} and this tick was used in early transmission experiments. With \textit{Amblyomma americanum}, it was initially unclear whether ticks were infected before biting the cat or whether they were infected during feeding. A recent article\textsuperscript{40} confirms that \textit{Amblyomma americanum} is capable of transmitting \textit{Cytauxzoon felis} among cats. This has been confirmed by the publication of more recent articles relating to the epidemiology of \textit{C. felis} in both cats and wild felids, mostly bobcats.\textsuperscript{30,41,42} Because the distribution of the tick vector is more widespread than the distribution of the currently reported cases, it has been suggested that \textit{C. felis} has the potential to spread and infect a larger geographical area than was previously thought.\textsuperscript{30,42} The schizogony phase of the life cycle occurs in natural infections only after tick transmission of the
organism, and perinatal transmission of the hemoparasite has not been documented nor was it found in a recent experiment. Diagnosis of *Cytauxzoon felis* appears to follow a bimodal pattern with two annual peaks. The larger peak occurs between April and June, while the second peak occurs during August and September. The majority of cases are reported during May. Most of the cases are found in low density residential areas around urban edges, and associated with wooded natural or unmanaged areas.

*Cytauxzoon felis* is not the only member of the genus that can at least partially infect domestic cats. *Cytauxzoon manul* is a related, recently described parasite that has been found in Pallas’ cats (*Otocolobus manul*), originally from Mongolia. In a recent study, a group of domestic cats was first challenged by blood from parasitemic Pallas’ cats, and they all developed a persistent parasitemia, without evidence of schizogony. This intraerythrocytic parasitemia did not protect against a second challenge with a spleen from a naturally *C. felis*-infected cat. This is similar to other earlier reports with *C. felis*, which found that although giving *C. felis*-infected blood from persistently parasitemic bobcats or domestic cats to healthy uninfected cats made them parasitemic, it neither gave them clinical disease nor protected them from later challenge with tissue homogenates. These studies once again demonstrate that the tissue phase of *Cytauxzoon felis* is the disease-producing stage, and do not rule out the possibility that *C. manul* could also cause disease in cats, if they are infected with blood or tissues containing *C. manul* schizont-laden monocytes. Another *Cytauxzoon* spp. has been detected in various, highly endangered Iberian lynxes (*Lynx pardinus*), but very little is known about whether it can cause disease in lynxes or domestic cats. Other studies have reported *Cytauxzoon* spp. in domestic cats in Spain; however, these cases have not been fully characterized or identified.
The cytauxzoonosis mortality rate has historically been thought to approach 100%, yet recent clinical cases have been described in which cats survived with and without treatment.\textsuperscript{1,14,51} These cats may have the capacity to serve as reservoirs instead of just dead-end hosts.\textsuperscript{29,52} Some of these cats had very low parasitemias, but tested positive for \textit{C. felis} in repeated PCR testing. Recent studies have shown differences in the ribosomal DNA genotype of \textit{C. felis}.\textsuperscript{51,52} These different strains correspond to different geographic areas, but are not necessarily associated with a better prognosis.\textsuperscript{51,52} Although it is possible that infection with less pathogenic parasites increases survival, this study did not look at the host response or the disease pathogenesis. In the related \textit{Theileria} spp.,\textsuperscript{4,7,19,20,53,54} various cattle breeds have been found to be more susceptible or resistant to infection, and cell lines derived from different animals show differential expression of immunological mediators, such as major histocompatibility molecule class II, IL-1\(\alpha\), IL-6, IL-10, IL-4, IFN-\(\gamma\), and TNF-\(\alpha\).\textsuperscript{55} Currently, no studies have been done with cats to demonstrate whether the mortality due to \textit{C. felis} infection may be due to genetic variability amongst infected cats.

The cell infected during the tissue phase and schizogony of \textit{C. felis} has been described since its discovery as being part of the reticuloendothelial system.\textsuperscript{2,56-58} This term is no longer used today, and instead has been replaced by the mononuclear phagocyte system (MPS),\textsuperscript{54,59-64} which includes monocytes and macrophages as part of the cells involved in the innate immune response,\textsuperscript{22,25} both in the acute and chronic phases of inflammation.\textsuperscript{57} Another recent study\textsuperscript{13} suggested that the cells are likely activated, but did not have immunohistochemical, molecular, or serological data to confirm it. Monocytes are considered the first mature but not completely differentiated member of the MPS to leave the bone marrow and circulate in the vasculature.\textsuperscript{59,61} Monocytes have limited recirculation and replication capabilities.\textsuperscript{61,65} Although the
classifications are still being studied, and may vary by species, two common types of blood monocytes have been found: one that eventually becomes activated macrophages and localizes to sites of inflammation, and another resting pool that is responsible for repopulating resident tissue macrophages.\textsuperscript{61,63,65} Activated macrophages are also divided into two main groups, those that are pro-inflammatory and histotoxic (M1), and those that are broadly anti-inflammatory and involved in tissue repair (M2).\textsuperscript{58,62,63} Pro-inflammatory (M1) macrophages produce inflammatory mediators, such as inducible nitric oxide synthetase (iNOS),\textsuperscript{59} and express class II major histocompatibility complex (MHC II) molecules.\textsuperscript{58,60,61,66-68} They also secrete large amounts of pro-inflammatory cytokines such as TNF-\(\alpha\), IL-1\(\beta\), and IL-6, which help modulate the immune response,\textsuperscript{57} and other molecules involved in tissue injury (reactive oxygen species, proteases) and tissue repair (growth factors, angiogenic factors).\textsuperscript{57}

Studies on the host response and pathogenesis of cytauxzoonosis are few. At first, many of the studies focused on characterizing the clinical disease and studying its transmission. These include an early study by Wagner,\textsuperscript{47} which was the first to describe the parasite and focused on experimentally infecting healthy cats with \textit{Cytauxzoon felis} and keeping a continuous passage of parasites from tissues of moribund cats to healthy cats parenterally. Another early experiment studying parasite transmission was described by Kier, Wagner, and Morehouse.\textsuperscript{28} Using blood and tissue obtained from an acutely ill bobcat, they were able to cause fatal disease in inoculated cats. Cats inoculated with blood and tissues of asymptptomatically parasitemic bobcats and domestic cats, however, did not develop cytauxzoonosis, suggesting the parasite is infective to other monocytes during the tissue phase, and that once it develops to the erythrocytic stage, it loses its pathogenicity to other monocytes within the host.
One of the earlier studies, focusing more on immunology, host response, and pathogenesis, by Shindel et al., described an indirect fluorescent antibody test for the detection of the leukocytic stage of *Cytauxzoon felis*. This study also mentioned one of the earliest attempts at maintaining the parasite *in vitro*, and was one of the first to perform serology and study antibody responses. Their results demonstrated that infected cats either do not develop an antibody response, or developed a very low response late in the disease that was insufficient for recovery. A later paper described the development of anti-*Cytauxzoon* antibodies in a group of healthy, experimentally infected cats. These cats were injected with parasitized blood from yet another experimentally-infected cat. At this time, it was already known that transmission of the intraerythrocytic stage from one cat (or bobcat) to another would only induce parasitemia without concurrent clinical disease. A crude mixture of parasitemic blood was used as antigen to detect possible anti-*C. felis* antibody in the serum of the experimentally-infected cats. It is possible then, that the antibodies detected in these cats corresponded to an immune response against the intraerythrocytic stage or perhaps against the foreign red blood cells, and not a response to the disease-causing, tissue stage of *C. felis*. A similar conclusion was reached in a study by Uilenberg et al., in which, although they were able to obtain antibodies against the intraerythrocytic stage, these not protective when cats were challenged with homogenized organs containing *C. felis* schizonts. Motzel and Wagner also reported that, although inoculation with blood from carrier cats did not cause disease in naïve cats, neither did it protect them against challenge with subcutaneously-injected, spleen inoculum from an infected cat that died from this disease. The same study did find that challenging survivor cats with the same inoculum did not result in clinical disease, suggesting that immunity against the tissue phase does develop in cats that survive the infection.
Leukocytes, including monocytes and macrophages, contain different surface markers that regulate cell signaling and movement from blood to tissues. These molecules include the family of integrins, which are composed of two different transcytoplasmic chains, and are classified according to the structure of these chains. Integrins can be classified according to which cells express them and to what ligands they bind, and one class of integrins are specifically involved in firm adhesion of leukocytes to endothelial cells. Although these molecules can normally be expressed in leukocytes, they are expressed at low levels and in a low-affinity state. Different molecules can activate integrins, such as chemokines, cytokines like TNF-α, fibrinogen, and selectins. Of these, chemokines are among the most important and common activating molecules for integrins. Chemokines released during an inflammatory response bind to chemokine receptors in leukocytes, resulting in their activation, which is called “inside-out” signaling, and involves multiple kinases and GTPases. Integrins can also be activated and undergo functional changes following ligand binding, and this is known as “outside-in” signaling. Activated leukocytes express more clustered integrins in a higher affinity and higher avidity state, increasing the adhesion of circulating leukocytes to endothelial cells. Therefore, in integrin function, conformation is as important as expression, as low affinity conformation can lead to reduced outside-in signaling, for example. In turn, these activated leukocytes secrete pro-inflammatory cytokines that induce endothelial cells to express more ligands for the integrin molecules. A major component of many integrins, the β2-integrin also known as CD18, is expressed by most white blood cells, especially when they are activated.

Once the integrins are bound to their ligand, they are able to deliver stimulatory signals to their cells by different pathways, and the end results vary with cell type. In some leukocytes,
these integrin-mediated signals can lead to increased phagocytic capacity and intercellular communication, cell migration, generation of reactive oxygen species, inflammatory gene expression, regulation of cytokine secretion, and apoptosis.\textsuperscript{66,71,73,76,80,84,85,92,97,98,100-107} An older study\textsuperscript{97} reported increases in cytosolic calcium concentrations with the binding of anti-CD18 antibodies to CD18, but this increase varied with antibody type, and in some cases, there is no significant increase, or only increases in certain subsets of leukocytes. This was determined to be due to the recognition of different epitopes by the anti-CD18 antibodies. Other studies implicate several different kinases, which include mitogen-activated protein (MAP) kinases, tyrosine kinases, such as Syk, and protein kinase C, as part of CD18 signaling in eosinophils, neutrophils, and monocytes.\textsuperscript{76,77,83-85,87,98,101,103,105} These kinases and pathways eventually activate the major pro-inflammatory regulatory molecule, nuclear factor-kappaB (NF-\kappaB).\textsuperscript{106-108} The short cytoplasmic tails of integrins are capable of propagating signals by coupling to cytoplasmic proteins, such as kindlin and talin, and forming large complexes with different signaling enzymes.\textsuperscript{75,76,92,100,103,109} The end products of integrin-mediated gene expression can, in turn, increase the inflammatory response and signal up-regulation of pro-inflammatory products, strengthening the response and supporting leukocyte emigration to the sites of inflammation.\textsuperscript{100} Integrins also form type 3 and type 4 complement receptors, binding a fragment of C3b, and are thus involved in phagocytosis. \textsuperscript{66,71,77,92,94,98,104,110,111}

Neutralization of CD18 has been shown to inhibit adhesion and cytotoxicity of activated monocytes, macrophages, and neutrophils on endothelial cells,\textsuperscript{89,107,112-115} inhibit local neutrophil accumulation and emigration,\textsuperscript{81,89,92,114} suppress eosinophil adhesion,\textsuperscript{84} and reduce cerebral edema, aortic aneurysm expansion, organ failure, and mortality in various animal models of infectious disease.\textsuperscript{71,81,96,115-117} In \textit{Clostridium difficile} infection, for example, inhibition of CD18
reduces the inflammation and fluid loss into the lumen. Another study reports that bovine leukocytes stimulated by leukotoxin (LKT) and lipopolysaccharide (LPS), toxins secreted by *Mannheimia hemolytica*, cause increased expression of LFA-1, which is in part formed by CD18. In this study, LKT caused direct cytotoxicity after binding to LFA-1, which had increased expression, affinity, and avidity due to prior exposure to both LKT and LPS. An earlier study suggested that the increased expression of CD18 contributed to the increased expression of pro-inflammatory cytokines, including TNFα, IL-1β, and IL-6. Neutralization of CD18 on pulmonary alveolar macrophages before administering an exotoxin significantly reduced the expression of IL-1β and TNF-α. The inhibition of leukocyte adhesion and aggregation also prevents vascular leakage and direct cytotoxic damage in some studies, which could prove to be useful in the treatment of cytauxzoonosis, where interstitial pneumonia with edema and vascular damage have been found to be one of the major histopathologic findings. It has been suggested in the recent literature that the infected macrophages are responsible for some of the histopathologic findings, and neutralization of these infected cells could also ameliorate the pro-inflammatory response. Alternatively, neutralization of CD18 in neutrophils could also decrease pulmonary lesions and improve survival. As mentioned previously, cats infected with *C. felis* develop severe lung lesions, which may include interstitial pneumonia, and die in what is considered a shock-like state. Some of these effects may also be mediated by activated neutrophils, and as has been suggested in another study, these cells contribute to acute respiratory distress syndrome, which is present during sepsis, by causing tissue damage. CD18 expression is increased in animals and humans during these conditions.

Integrins, and CD18 in particular, bind various molecules, including many cell-surface receptors. Amongst these, a group called intercellular adhesion molecules (ICAMs) are the most
important. ICAM-1 (also known as CD54) is the specific receptor for CD18, and this molecule, although constitutively expressed, is upregulated by inflammatory stimuli, such as CD18, IL-6, IL-1β, and TNF-α, which can in turn be upregulated by CD18 activation. ICAM-1 molecule is expressed on many cells, such as B and T lymphocytes, dendritic cells, macrophages, and endothelial cells. The interaction of ICAM-1 and CD18 is important in the formation of the tight adhesion that is part of the transmigration of leukocytes from the vessels to the tissues. The expression of ICAM-1 is elevated in various inflammatory conditions, such as hepatitis, dermatitis, and graft rejections, and its presence correlated with the expression of CD18. Upregulation of ICAM-1 has also been found after infection in humans with another hemoprotozoa, Plasmodium falciparum, which causes malaria, and in particular, the upregulation is noted in the more severe form of cerebral malaria. Similar to CD18, administration of anti-ICAM-1 antibody has decreased inflammatory response and graft rejection by minimizing micro-vascular damage.

Objectives and Structure

As mentioned in the previous section, Cytauxzoon felis causes a nearly fatal disease in cats, and few studies have been done to investigate its pathogenesis, host response, and possible mechanisms by which it causes disease. The main hypothesis proposed in this project is that C. felis infected-macrophages adhere to host feline blood vessels because of up-regulation of adhesion molecules, such as CD18 and ICAM-1, and then damage the endothelium by local excessive production of pro-inflammatory cytokines or by direct cytotoxic effects between leukocytes (mostly macrophages but also neutrophils) and endothelial cells. CD18, as mentioned
previously, has been shown to play an important role in the pathogenesis of various inflammatory and infectious diseases in both animals and humans. Neutralizing this molecule with anti-CD18 antibodies should then decrease the secretion of pro-inflammatory cytokines and cell-mediated cytotoxicity that occurs during this disease.

Very little is known about the pathogenesis and host immune response to this parasite. It is possible that cytauxzoonosis survivors have a different immune response compared to *C. felis*-infected cats that die, and this could be helpful in developing prognostic indicators for cytauxzoonosis. Based on preliminary results, it is proposed that cats that died from the infection develop an uncontrolled immunopathologic response compared to a more moderate response in those cats that survive infection with *C. felis*.

*Cytauxzoon felis*, unlike the related *Theileria* spp. has not been established and studied *in vitro*.\(^7-9,19,20,127\) This limits the study of the organism to samples obtained from blood and tissues collected from naturally- or experimentally- infected cats. In the case of naturally-infected cats, it is difficult to study the pathogenesis and host response of *C. felis in vivo* because the exact disease timeline is not known, and cats may die or be euthanized at different times during the disease, which could affect data interpretation. In experimentally-infected cats, the timing of inoculation is known, most variables are controlled, and study animals either die or can be euthanized at known time points. Still, *C. felis* is a high morbidity, high mortality disease, and an *in vitro* system would enable one to study the parasite without relying solely on an animal model. It is proposed that *C. felis*, if isolated, will only infect cells that have been immunohistochemically confirmed to be of monocyte-macrophage lineage. As part of this investigation, an attempt was made to isolate the parasite and use it to infect feline cell lines. Cell culture techniques were used to infect established feline cell lines, as well as to establish
newly-developed, feline cell lines that will be capable of propagating the parasite *in vitro*, similar to the way other *Theileria* spp. are kept in established cell lines and used for immunological studies and vaccine development.\(^7,8,19,20\) The establishment of such a cell line for *C. felis* would help further characterize the disease by allowing the study of the molecules activated and secreted at various time points of infection, without depending on either naturally infected cats or infecting healthy cats with a fatal disease.

As an adhesion molecule, CD18 may play a role in the attachment of *C. felis*-infected macrophages to the endothelium that is observed histologically and by scanning electron microscopy. Also, since CD18 has been found to increase the pro-inflammatory response, activation of this molecule could be one of the causes of the increase in pro-inflammatory cytokines and lesions noted histologically. Again, based on preliminary data and what was discussed regarding integrins, it is proposed that CD18 is upregulated in *C. felis*-infected cats compared to healthy, uninfected cats. It is also proposed that differential upregulation of CD18 is present between cats that die versus those that survive cytauxzoonosis. Finally, because CD18 and the other pro-inflammatory cytokines can upregulate the major CD18 ligand, ICAM-1, it is proposed that an upregulated CD18 expression correlates with upregulated ICAM-1 expression, and that the presence of both in activated states is what causes the binding between infected macrophages and endothelial cells.

The following chapters are each written and organized in a manuscript format, starting with the second chapter, which has been accepted for publication by the American Journal of Veterinary Research. Any alterations made to the submitted and approved manuscript are only those that would conform to the guidelines established by the University of Georgia Graduate School style. The other chapters closely mirror this second chapter in organization, in an effort
to remain consistent throughout the text. The last chapter is an overall conclusion, bringing all of the findings together.
CHAPTER 2

SYSTEMIC IMMUNE RESPONSES IN *CYTAUXZOOON FELIS*-INFECTED DOMESTIC CATS†

† Karelma Frontera-Acevedo; Nicole Balsone; Melissa Dugan; Cheryl Makemson; Llewelyn B. Sellers; Holly Moore Brown; David S. Peterson; Kate Creevy; Bridget Garner; Kaori Sakamoto

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Abstract

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

Samples--Blood and lung samples from 29 cats.

Procedures--Cats were divided into four groups:  A) cats that died from cytauxzoonosis (n = 8); B) acutely ill, *C. felis*-infected cats (n = 7); C) healthy survivors of *C. felis* infection (n = 8); and D) healthy, uninfected cats (n = 5). Serum concentrations of tumor necrosis factor-α, and interleukin-1β were measured by enzyme-linked immunosorbent assay, and serum proteins were characterized by serum protein electrophoresis. Blood smears were also stained immunocytochemically for immunoglobulin deposition. Immunohistochemical expression of CD18 and tumor necrosis factor-α were compared between Groups A (dead) and D (healthy). Real-time, reverse transcription polymerase chain reaction for CD18 expression was performed on selected blood samples from all four clinical groups.

Results--Concentrations of all three cytokines were greater overall, and serum albumin concentrations were significantly lower in Group A compared to all other groups. Erythrocytes from acutely ill and convalescent cats stained positively for plasmalemmal immunoglobulin M, as compared to Groups A and D. Increased staining of *C. felis*-infected macrophages and interstitial neutrophils for CD18 was detected. Real-time, reverse transcription polymerase chain reaction confirmed a relative increase in CD18 expression in Groups A and B compared to other groups. Positive immunostaining for TNF-α in the lung confirmed a local pro-inflammatory response.

Conclusions and Clinical Relevance--Data suggest that immunopathologic responses are greater in cats that die from *C. felis* infection compared to survivors.
**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BHQ1</td>
<td>Black hole quencher-1</td>
</tr>
<tr>
<td>Ct</td>
<td>Cycle threshold</td>
</tr>
<tr>
<td>FAM</td>
<td>Fluorescein amidite</td>
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<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>LLD</td>
<td>Lower limit of detection</td>
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<tr>
<td>qRT</td>
<td>Quantitative real-time reverse transcription</td>
</tr>
<tr>
<td>SPE</td>
<td>Serum protein electrophoresis</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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Introduction

Cytauxzoonosis is a fatal disease of domestic cats in the Midwestern, Mid-Atlantic, Southeastern and Southcentral United States, caused by *Cytauxzoon felis*, a tick-borne parasite in the order Piroplasmida, family Theileriidae.\textsuperscript{10,11} An infected tick transmits *C. felis* while feeding, followed by schizogeny of the parasite in monocytes throughout the body.\textsuperscript{128} While this infection can be asymptomatic, clinical signs in infected cats include anorexia, depression, lethargy, dehydration, pyrexia, dyspnea, icterus, dark urine, and less commonly, pallor, anemic heart murmur, and increased capillary refill time. Hematologic findings may include normocytic, normochromic, nonregenerative anemia, with pancytopenia or moderate neutrophilia.\textsuperscript{10,40}

Since its discovery and description in the 1970s, very little has been published regarding the feline immune response to this disease. A previous study reported the formation of antibodies against the non-pathogenic, erythrocytic stage of *C. felis*.\textsuperscript{70} Kier *et al.*\textsuperscript{3} and Susta *et al.*\textsuperscript{21} confirmed the macrophage identity of the infected cells in the leukocytic stage of the disease. Snider *et al.*\textsuperscript{13} described and categorized the interstitial pneumonia commonly present in cats that died from *C. felis* infection, and suggested that this inflammation is likely caused by release of pro-inflammatory cytokines and chemokines by the infected macrophages. One of the main histopathologic characteristics of cytauxzoonosis is the presence of giant, infected, intravascular macrophages, many of which are adhered to the vascular endothelium, possibly with involvement and activation of CD18.
The CD18 integrin is present on most leukocytes, especially when they are activated, and plays a role in their adherence to the endothelium during inflammation. Different molecules can activate integrins, such as chemokines, fibrinogen, selectins, and cytokines such as TNF-α. Of these, chemokines are among the most important and common activating molecules for integrins.

The overall objective of the current study was to characterize the systemic immune response to *C. felis* infection, with the hypothesis that uncontrolled pro-inflammatory 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 *C felis*–infected on the basis of results of blood smear analysis. Practitioners at veterinary clinics in Georgia and Arkansas were contacted via telephone, email, and facsimile during March 2009 through September 2012, informed about the study, and asked to participate. Serum samples were also obtained from 5 healthy uninfected cats that were volunteer blood donors owned by the co-authors or part of the blood donor colony at the University of Georgia College of Veterinary Medicine. Owner consent was obtained for volunteer 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 later survived the infection, and cats that had survived *C. felis* infection and subsequently were healthy.

Collection of blood samples—Blood samples (1-5 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. Blood samples were also collected from some cats into an RNA tube to prevent RNA degradation. Cats that died of the disease and acutely ill *C. felis*–infected cats, and healthy survivor cats were acutely ill at the time of sample collection. 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*.

These samples were also used for measurement of cytokine concentrations for the acutely ill cats and cats that subsequently died. For the healthy survivor group (which included some of the acutely ill cats that subsequently recovered from the disease), convalescent blood samples were collected two weeks after resolution of the disease. Duration of the disease was approximately 3-5 weeks. Resolution of disease was determined by the referring clinician on the
basis of improved clinical signs and increased appetite. The interval between acute and convalescent blood samples for the healthy survivors was approximately one 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°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°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-α and IL-1β. 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 as samples were acquired. Flat-bottom, 96-well microplates were used for the assay. A 7-point standard curve was always created with each batch and used with 2-fold dilution in 1% bovine serum albumin in PBS solution. The high standard was 1,000 pg/mL for TNF-α and 2,000 pg/mL for IL-1β and the LLD was 15.6 pg/mL for TNF-α and 31.25 pg/mL for IL-1β.

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 (Appendix A) to validate the use of undiluted samples; results indicated recovery of 80% to 100%. Optical density was determined with a plate reader and associated software; absorbance was set at 450 nm.

SPE—Serum samples remaining after the ELISA was performed were sent to another diagnostic laboratory 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 then stained immunocytochemically with mouse anti-feline IgM monoclonal antibody and isotype control antibody. 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 were then counterstained with Gill’s hematoxylin, air dried, mounted, and subjectively examined via light microscopy by one of the investigators. Results for slides were considered negative when no staining for IgM was detected around cells and positive when most of the erythrocytes had staining for IgM.

CD18 and TNF-α immunohistochemical analysis—Samples of lung tissues were obtained from cadavers of cats that had died of C. felis infection and from the healthy, uninfected, control cats, which were part of the feline blood donor program. Tissues were fixed in neutral-buffered 10% formalin, embedded in paraffin, cut at a thickness of 4 µm, and placed on glass slides. Antigen retrieval was performed in accordance with the manufacturer’s protocol. Antibodies used included mouse anti-feline CD18 monoclonal antibody, goat anti-mouse TNF-α polyclonal antibody (which has been found to cross react with cats), and isotype
control antibodies. A commercial kit was used for stain development. Each slide was incubated with primary antibody (dilution of 1:50 for CD18 and 1:100 for TNF-α) at room temperature for 30 minutes. Slides were counterstained with Gill’s 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-α 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 the inflammatory cells were stained. High staining was defined as staining of more than half of the inflammatory cells. For TNF-α, low staining was defined as extremely light, diffuse, background staining (but more staining than for negative control samples) and no cytoplasmic staining in the inflammatory cells. Moderate staining was defined as diffuse TNF-α 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. The resulting eluate was incubated for 5 minutes at 65° before storage at −80°C until needed.

The cDNA was obtained via a procedure performed in accordance with the manufacturer’s instructions. Before cDNA synthesis, the concentration of RNA obtained from each blood sample was measured with a spectrophotometer. 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 were designed with available software. 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’-CACAAGTCCATACCCAGGATCT-3’ and the reverse primer was 5’-GGAGATCACATGACTTTAAAAGGAC-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. The selected forward primer was 5’-CATCTTCAGGAGCGGAGAT-3’, and the selected reverse primer was 5’-CCACAACATCTCAGCACA-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. 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 \(2^{-\Delta\Delta C_t}}\).129 130

Statistical analysis—Mean and SD values were obtained, and error bars were used to represent SEMs. 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 \( P < 0.05 \). Protein concentrations obtained by 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 the values and the percentage were reported. A Fischer 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 Fischer 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, and calculations based on published methods 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**

**Sample**—Blood and serum samples were obtained from 28 cats and analyzed via various assays (Table 2.1). Of the 7 acutely ill cats that had samples analyzed via ELISA, 2 were subsequently included in the healthy survivor group for analysis via the ELISA, as they were available for repeat sampling after clinical recovery. Of the 3 acutely ill cats that had samples
analyzed via SPE, 1 was subsequently included in the healthy survivor group, as the cat was available for repeat sampling 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-α assay, 2 acutely ill cats, 3 healthy survivor cats, and 4 healthy uninfected cats had concentrations below the LLD. For the IL-1β 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-α and IL-1β) were higher for cats that died of the disease, compared with concentrations for the other 3 groups, although results were only significantly different for TNF-α concentrations between the group of cats that died and the group of healthy uninfected cats (Figure 2.1). 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-α and IL-1β 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 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-α and IL-1β. There were no
significant differences in TNF-α or IL-1β concentrations in these paired serum samples (Appendix A).

All lung tissues obtained from cats that died of C. felis stained positively TNF-α by immunohistochemistry (Figure 2.2). The tissues were characterized as having high staining, particularly in the cytoplasm of infected macrophages that lined vessels and other inflammatory cells within the pulmonary interstitium and alveoli.

**Serum protein**—All groups contained at least 1 cat (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 nonspecific, acute-phase inflammatory response, which was primarily attributed to increases in α-2 globulin concentrations (Figure 2.3). 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 β-globulins 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, a term used for a polyclonal gammopathy that has a restricted immunoglobulin migration pattern.

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 2.4). 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 plasmalemmal 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 2.5). Staining differed among groups, but not significantly, as determined with the Fischer exact test.

**CD18 expression**—Immunohistochemistry 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 plasmalemmal staining for CD18 (Figure 2.6). 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 to GAPDH expression) for healthy uninfected cats, cats that died of the disease had 11.6-fold increases in CD18 expression, whereas acutely ill cats had a 9.0-fold increase and healthy survivor cats had a 1.3-fold increase (Figure 2.6). 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 focused on immunologic aspects, host response, and pathogenesis of the disease. The authors of that study 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 important information on the pathogenesis of cytauxzoonosis. The findings also highlight the need for further studies to enable a better understanding of the feline immune system response to this parasite and the means to better combat this disease with pharmaceuticals.

Although there were higher concentrations of the proinflammatory cytokines TNF-α and IL-1β in cats that died of the disease than in cats of all other groups, the concentrations were only significantly higher for TNF-α concentrations in cats that died of the disease, compared with concentrations in the healthy uninfected cats. The TNF-α concentrations may have been statistically higher than others perhaps because not only it is one of the first ones produced, this cytokine is in this disease the most important pro-inflammatory cytokine involved compared to others. 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\textsuperscript{13} and may be part of an exaggerated systemic immune response.\textsuperscript{13} This was also supported by the marked staining for TNF-\(\alpha\) detected by immunohistochemistry.

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 for SPE interpretation in this study, in that electrophoretochromograms are typically not indicative of a particular etiologic agent. As a result, cats infected with \textit{C. felis} or cats with unrelated conditions, some of which may be subclinical, could have similar electrophoretochromograms. 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;\textsuperscript{52} 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 \textit{C. felis} infection, serial monitoring of SPE may be more helpful than single evaluations, but further investigation is necessary to confirm this theory.

Most of the samples submitted for SPE had evidence of a nonspecific, acute-phase response, regardless of the \textit{C. felis} infection 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. This decrease is caused in part by an increase in proinflammatory cytokines, such as TNF-α and IL-1β, during acute inflammation. These high concentrations of cytokines have inhibitory effects on the hepatic synthesis of albumin. The decrease in albumin concentration was more noticeable in cats that later 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 that one 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 stain positively for IgM than 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 β-globulins (the band in which IgM can migrate during gel electrophoresis), but other than those cases, expression of IgM by immunocytochemistry was not associated with increase in the total amount of β-globulins. Although antibody responses to C. felis have been described, they were not found to be protective in that study and may perhaps play a role in extravascular hemolysis, via deposition on erythrocytes as found in the present study, and the icterus commonly observed in animals with cyauxzoonosis. On the other hand, cats that died of cyauxzoonosis were less likely to have IgM deposition detected by immunocytochemistry than were acutely infected cats, despite the fact that both groups of cats were likely to be at a similar stage of the disease at the time of blood samples collection (the day cats were examined by a referring veterinarian). This suggests that antibodies against the
erythrocytic phase of the parasite may have some protective effect. However, it should be mentioned that the accuracy of the duration of clinical signs provided was uncertain, 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 plasmalemmal expression was evident in *C. felis*–infected cats, mainly on infected or activated macrophages within blood vessels, as well as on interstitial neutrophils. It was suggested in 1 study\textsuperscript{104} 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. Cats 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 immunohistochemistry. The group of acutely ill cats had lower expression of CD18 than did the 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,\textsuperscript{66,71,72,82,84,95-99} and interaction of CD18 with its
ligands can in turn upregulate proinflammatory cytokines that can cause vascular leakage or damage.\textsuperscript{135} 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 α-1-glycoprotein and haptoglobin, the major and moderate acute-phase proteins in cats, respectively.\textsuperscript{52,133,136} 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.\textsuperscript{137} In a recent case report,\textsuperscript{138} clinicians detected a biclonal gammopathy in a cat with a plasma cell neoplasm by use of a capillary electrophoresis method; that gammopathy had not been detected by SPE. A direct Coombs test would also be valuable to pursue 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 \textit{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.
Footnotes

a. PAXgene blood tube, Qiagen, Valencia, CA
b. DuoSet, RnD Systems, Minneapolis, MN
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, CA
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, CA
j. Clone M-18, Santa Cruz Biotechnology, Santa Cruz, CA
k. PAXgene blood RNA kit, Qiagen, Valencia, CA
l. ThermoScript RT-PCR system, Invitrogen, Grand Island, NY
m. NanoDrop 2000, Thermo Scientific, Waltham, WA
n. Integrated DNA Technologies, Coralville, IA
p. iCycler IQ system, Bio-Rad, Hercules, CA
q. Excel 2007, Microsoft Corp, Redmond, WA

r. InStat, Graphpad Software, La Jolla, CA
Figure 2.1—Mean ± SEM of serum TNF-α and IL-1β concentrations, as determined by ELISA, for acutely ill *Cyttauxzoon felis*–infected cats that subsequently died of the disease (n = 8; black bars), acutely ill *C. felis*–infected cats (7; diagonal stripes), healthy cats that survived *C. felis* infection (9; 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 two 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-α (15.625 pg/mL [dotted line]) and IL-1β (31.25 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.*
Figure 2.2—Photomicrographs of TNF-α immunohistochemistry of lung samples obtained from a *C. felis*–infected cat (A and B) and a healthy uninfected control cat (C and D). An anti-TNF-α antibody was used in panels A and C, whereas an isotype control antibody was used in panels B and D. 3,3′-diaminobenzidine chromogen with hematoxylin counterstain; bar = 200 μm
Figure 2.3—Serum protein electrophoretograms of representative cats with cytauxzoonosis that had increased α-globulin concentrations (A) and increased β-globulin concentrations (B). Alb = Albumin.
Figure 2.4—Box-and-whisker plots of serum albumin concentrations as determined via SPE for cats that died of cytauxzoonosis (n = 8), cats acutely ill with cytauxzoonosis (7), healthy cats that survived *C. felis* infection (8), and healthy uninfected control cats (5). 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 standard error of the means. *Median values for this group differ significantly (P < 0.05) from the values for all other groups.*
**Figure 2.5**—Representative photomicrographs of IgM immunocytochemistry 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, note the intense staining around the erythrocytes. 3,3’-diaminobenzidine chromogen with hematoxylin counterstain; bar = 50 μm.
Figure 2.6—Photomicrographs of CD18 immunohistochemistry of lung samples 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, note the plasmalemmal staining of infected macrophages. In panel C, note the CD18 staining of an alveolar macrophage (arrow). 3,3′-diaminobenzidine chromogen with hematoxylin counterstain; bar = 200 μm.
Figure 2.6—Mean ± SEM fold changes in CD18 mRNA expression (normalized on the basis of GAPDH expression) for cats that died of cytauxzoonosis, acutely ill cats, and healthy cats that survived *C. felis* infection, compared with results for healthy uninfected control cats. \( ^{\text{a,b}} \) Values with different letters differ significantly \( (P < 0.05) \).
Table 2.1—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 later recovered, healthy cats that had survived *C felis* infection, and healthy uninfected (control) cats, and used in each of several assays.

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 two weeks after cats recovered from cytauxzoonosis, and from the 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.

<table>
<thead>
<tr>
<th>Group</th>
<th>ELISA</th>
<th>SPE</th>
<th>IgM immuno-histochemical analysis</th>
<th>CD18 qRT-PCR assay</th>
<th>CD18 immunohistochemical analysis</th>
<th>TNF-α immunohistochemical analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Died (n = 8)</td>
<td>8</td>
<td>4</td>
<td>5</td>
<td>3</td>
<td>7</td>
<td>7</td>
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<tr>
<td>Acutely ill (n = 7)</td>
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<td>3</td>
<td>5</td>
<td>4</td>
<td>0</td>
<td>0</td>
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<tr>
<td>Healthy survivor (n = 8)</td>
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<td>6†</td>
<td>7</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Healthy uninfected (n = 5)</td>
<td>5</td>
<td>4</td>
<td>4</td>
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CHAPTER 3
LOCAL IMMUNE RESPONSES IN CYTAUXZOOM FELIS-INFECTED DOMESTIC CATS

Introduction

Cytauxzoonosis is a fatal disease of domestic cats in the Midwestern, Mid-Atlantic, Southeastern and Southcentral United States, caused by *Cytauxzoon felis*, a tick-borne parasite in the order Piroplasmida, family Theileriidae.\(^{10,11}\) An infected tick transmits *C. felis* while feeding, followed by schizogeny of the parasite in macrophages throughout the body.\(^{128}\) While this infection can be asymptomatic, clinical signs in infected cats include anorexia, depression, lethargy, dehydration, pyrexia, dyspnea, icterus, dark urine, and less commonly, pallor, anemic heart murmur, and increased capillary refill time. Hematologic findings may include normocytic, normochromic, nonregenerative anemia, with pancytopenia or moderate neutrophilia.\(^{10,40}\)

Since its discovery and description in the 1970s, very little has been published regarding the feline immune response to this disease. A previous study reported the formation of antibodies against the non-pathogenic, erythrocytic stage of *C. felis*.\(^{70}\) Kier *et al.*\(^3\) confirmed the macrophage identity of the infected cells in the leukocytic stage of the disease. Snider *et al.*\(^{13}\) described and categorized the interstitial pneumonia commonly present in cats that died from *C. felis* infection, and suggested that this inflammation is likely caused by release of pro-inflammatory cytokines and chemokines by the infected macrophages. One of the main histopathologic characteristics of cytauxzoonosis is the presence of giant, infected, intravascular
macrophages, many of which are adhered to the vascular endothelium, possibly with involvement and activation of CD18.

The CD18 integrin is present on most leukocytes, especially when they are activated, and plays a role in their adherence to the endothelium during inflammation. Different molecules can activate integrins, such as chemokines, fibrinogen, selectins, and cytokines. Of these, chemokines are among the most important and common activating molecules for integrins. The CD18 integrin is also responsible for upregulating many cytokines and pro-inflammatory molecules, including TNF-α, IL-1β, and IL-6. It also upregulates some of its ligands, such as ICAM-1.

The ICAM-1 molecule is expressed on many cells, such as B and T lymphocytes, dendritic cells, macrophages, and endothelial cells. The interaction of ICAM-1 and CD18 is important in the formation of the tight adhesion that is part of the transmigration of leukocytes from the vessels to the tissues. The expression of ICAM-1 is elevated in various inflammatory conditions, such as hepatitis, dermatitis, and graft rejections, and its presence correlated with the expression of CD18. Upregulation of ICAM-1 has also been found after infection in humans with another hemoprotozoa, Plasmodium falciparum, which causes malaria, and in particular, the upregulation is noted in the more severe form of cerebral malaria.

Interleukin-1β and IL-6 are considered two important pro-inflammatory cytokines in the acute and innate immune response. Major sources of IL-1β include activated macrophages, endothelial cells, and to a lesser extent, neutrophils. Some of its major effects include further activation of endothelial cells, fever, and synthesis of acute phase proteins. These effects are concentration-dependent, as lower concentrations exert more local effects, such as increase in the expression of adhesion molecules on the endothelium, while larger quantities cause
systemic effects. Unlike TNF-α, IL-1β by itself does not cause the pathologic changes of septic shock.\textsuperscript{139} Interleukin-6 can function in both innate and immune responses, and it is produced by various cells, including macrophages, endothelial cells, and fibroblasts.\textsuperscript{120,139} In innate immunity, IL-6 contributes to the synthesis of acute phase response proteins in the liver, along with IL-1β and TNF-α.\textsuperscript{59,120,139} Although IL-6 was not discussed in the previous chapter, and IL-1β was not found to be statistically different in the overall serum concentration between the various cat groups, both are still pursued in this chapter because their expression at the local level can have effects that may not be reflected by their total systemic serum concentration.

Nitric oxide (NO) is produced by activated macrophages (amongst many other cell types), via inducible nitric oxide synthase (iNOS), and combines with other reactive molecules in the phagolysosome to kill microbes.\textsuperscript{57,59,140} Besides iNOS, two other similar enzymes, neuronal NOS (nNOS) and endothelial NOS (eNOS) are involved in NO production\textsuperscript{57,140-142} but usually regulate NO during physiological conditions, and unlike the other two enzymes, once iNOS is activated, it is constantly active and not regulated by intracellular calcium.\textsuperscript{143} Although NO can have anti-inflammatory properties, excessive release of NO can cause damage to the surrounding host tissue\textsuperscript{59,141,143} by various mechanisms, including injuring vascular cells and increasing vascular permeability,\textsuperscript{57,140,144} activating endothelial cells, and increasing expression of adhesion molecules, and enhance cytokine expression.\textsuperscript{57,140,141}

Major histocompatibility complex (MHC) molecules present peptide fragments to specific T lymphocytes, thus activating the adaptive immune response.\textsuperscript{60} Based on where they are present and what type of peptide fragments they present to specific T lymphocytes, MHC molecules can be classified into two main classes, both of which are highly polymorphic.\textsuperscript{60,66} Class II MHC (MHCII) molecules present peptide fragments obtained from digested
extracellular molecules. This class of MHC is thus mainly expressed on antigen presenting cells such as macrophages (particularly activated macrophages), lymphocytes, and dendritic cells.\textsuperscript{60,61}

Studies done on parasites related to \textit{Cyttauxzoon felis}, like \textit{Theileria parva}, have found a concomitant increase in parasite load and pro-inflammatory cytokine upregulation, and this may be involved in the severity of clinical signs observed in East Coast Fever, caused by \textit{T. parva}.\textsuperscript{4} Another study regarding surface markers in \textit{T. annulata}-infected macrophages shows that this infection downregulates surface markers and the secretion of some immune mediators, such as NO.\textsuperscript{8}

The previous chapter characterized the systemic immune response to \textit{C. felis} infection, and supported the hypothesis that cytauxzoonosis causes a robust systemic pro-inflammatory response characterized by increases in pro-inflammatory cytokines, an acute-phase response, IgM deposition on erythrocytes, and upregulation and expression of CD18 on leukocytes. This response more severe in cats that died of \textit{C. felis} infection, compared with the response in cats that survived the disease, which suggests that the immune response is important in the pathogenesis of this disease. The previous observation that increases in inflammatory cytokines played a role in the severity of disease was also noted in diseases caused by related parasites, such as \textit{Theileria parva}.\textsuperscript{4}

The overall objective of work presented in the current chapter is to characterize further the local pulmonary immune response to \textit{C. felis} infection, with the hypothesis that uncontrolled local pro-inflammatory immune responses (caused in part by increased CD18 expression) contribute to the pathogenesis of cytauxzoonosis. This increased CD18 upregulation in turn causes an upregulation of ICAM-1, its ligand present in the endothelial cells. The combined upregulation of CD18 and ICAM-1 then causes the attachment of the infected macrophages that
is noted histologically. Immunohistochemical detection of the pro-inflammatory cytokines IL-1β and IL-6, and of the immunoregulatory enzyme iNOS, which produces NO, were used to characterize the local inflammation present in the lungs, which has been one of the most studied organs in cytauxzoonosis and one with the most severe histologic changes. This complements the findings in the previous chapter that included immunohistochemistry of TNF-α and CD18. Lastly, real-time, reverse transcription polymerase chain reaction (qRT-PCR) was performed on RNA extracted from formalin-fixed, paraffin-embedded tissues by laser capture microdissection. In particular, the relative expression of the CD18 molecules in infected macrophages and ICAM-1 on endothelial cells was compared to tissue macrophages and endothelium obtained from healthy, uninfected cats.

**Materials and Methods**

**Case selection**—Nineteen samples of lung tissues from cats that died of cytauxzoonosis and were submitted for necropsy at the Athens Diagnostic Laboratory between the years 2005-2013 were selected for further immunohistochemical studies. A subset of three cases from these 19 was further used in laser capture microdissection and gene expression studies. For all the cases selected, the original hematoxylin and eosin slides containing lung tissue were examined by a board-certified veterinary pathologist.

For negative controls, one archived research case from a healthy control cat used in another experiment was used. Two cats that died of conditions unrelated to cytauxzoonosis or pulmonary disease (hemolytic anemia and cholangiohepatitis) were also used as controls.
Tissues from three healthy uninfected control cats used in other research studies were used for laser capture microdissection.

**IL-1β, IL-6, iNOS, and MHCII immunohistochemical analysis**—Tissues were fixed in neutral-buffered 10% formalin, embedded in paraffin, cut at a thickness of 4 μm, and placed on glass slides. If antigen retrieval was performed, a 10 mM sodium citrate solution (pH 6.0) was used. Antibodies used included mouse anti-feline IL-6 monoclonal antibody, a goat anti-feline IL-1β polyclonal antibody, b mouse anti-human HLA-DR, α-chain monoclonal antibody, c and rabbit anti-mouse iNOS polyclonal antibody d (the latter two have been found to cross-react in cats 145-147) and their appropriate isotype control antibodies. e-h Two different kits were used for stain development, i,j based on published protocols and manufacturer’s recommendations. Each slide was incubated with primary antibody either overnight at 4°C (IL-1β, IL-6, iNOS), or at room temperature for 3 hours (MHCII). Slides were counterstained with Gill’s hematoxylin stain and rinsed with deionized water, and a coverslip was then added. More details about these procedures are presented in Table 3.1.

Staining distribution was subjectively compared via light microscopy by a board-certified veterinary pathologist. The IL-1β, IL-6, and iNOS immunoreactivity was characterized as low or high. For both cytokines and iNOS, low staining was defined as extremely light, diffuse, background staining (but more staining than for negative control samples) and scattered intracytoplasmic staining in few scattered alveolar macrophages. High staining was characterized as more diffuse intracytoplasmic staining present in at least half of the various cell types examined. The MHC II immunoreactivity was characterized as low, moderate, or high. For MHCII, low staining was defined as staining of only the cell membrane of a few alveolar macrophages. Moderate staining was defined as either staining of cells besides alveolar
macrophages, or plasmalemmal staining of the endothelium, but not both present at the same time. High staining was defined as staining both leukocytes and the endothelium.

**Laser capture microdissection and cDNA synthesis**—The selected paraffin-embedded tissues were sectioned to 4 μm thickness, placed on special membrane slides, and stained with specific nuclease-free reagents before placing them in the selected laser capture microdissection system. Special isolation cap tubes were used to collect the selected cells. Two tubes were used per slide, one for macrophages and the other for endothelium. Approximately 500-1000 cells were collected for each tube. The RNA was extracted in accordance with the manufacturer’s protocol. The resulting eluate was stored at –80°C until needed. The cDNA was obtained via a procedure performed in accordance with the manufacturer’s instructions. Before cDNA synthesis, the concentration of RNA obtained from each blood sample was measured with a spectrophotometer. Samples were stored at –20°C.

**Primer and probe design**—Primers and probes were designed with available software. The same CD18 and GAPDH primers and probes used in the previous chapter were used for the work presented in this chapter. The selected forward CD18 primer was 5’-CACAAGTCCATACCCAGGATCT-3’ and the CD18 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’. The selected GAPDH forward primer was 5’-CATCTTCCAGAGGAGGAGAT-3’, and the selected GAPDH reverse primer was 5’-CCACAACATGACTCAGCCTCAA-3’. The sequence for the fluorescent-labeled probe designed to anneal with the GAPDH amplicon was 5’-CGCCAACATCAAATGGGGTG-3’. The selected ICAM-1 forward primer was 5’-GGCAAGGACCTCACCCTGCG-3’, and the selected ICAM-1
reverse primer was 5’-GTGAACGTGACCTCGGCCGGG-3’. The sequence for the fluorescent labeled probe designed to anneal with the ICAM-1 amplicon was 5’-TGCTGCTCCGTGGGGAGGAG-3’. All probes were labeled with 5’-FAM fluorescence and 3’-BHQ1 quencher.

**qRT-PCR assay**—All qRT-PCR assays were performed with an automated system. The concentrations for all primers and probes were determined after performing optimization experiments. Reactions for GAPDH and CD18 were conducted simultaneously via separate tubes for each sample of collected macrophages. Reactions for GAPDH and ICAM-1 were conducted simultaneously via separate tubes for each sample of collected endothelial cells. 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 expressions of CD18 and ICAM-1 were calculated via the comparative Ct method (2^−ΔΔCt).\textsuperscript{129,130}

**Statistical analysis**—For immunohistochemical analysis, a Fischer exact test was performed to determine significant differences between infected and noninfected groups in terms of expression of IL-1β, IL-6, iNOS, and MHC II.

For the qRT-PCR assay, values obtained with the software were imported to a spreadsheet program, and calculations based on published methods\textsuperscript{129,130} were used to obtain fold changes and SDs. Samples, both from the endothelial cells and from the macrophages, were categorized between uninfected and infected cats, and a one-sided, unpaired student’s t-test with Welch correction was used to analyze differences.\textsuperscript{v}

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Results

**Histopathological results**—The major pulmonary histopathological findings are summarized in Table 3.2. Of the nineteen cases examined, four lungs were diagnosed by the anatomic pathologist who performed the necropsy as pneumonia (3 interstitial pneumonias, one bronchopneumonia), and three were diagnosed with vasculitis, not just limited to the lung lesions. Figure 3.1 exemplifies all of the major findings present.

**Pro-inflammatory cytokines and iNOS immunohistochemistry**—Of the 19 lung tissues from cats that died of *C felis*, 16 had moderate staining for IL-1β by immunohistochemistry, particularly in the cytoplasm of infected cells that line vessels and other inflammatory cells within the pulmonary interstitium and alveoli (Figure 3.2). Occasional staining was also noted in the cytoplasm of endothelial cells, surrounding alveolar macrophages, and intra and extravascular lymphocytes. The lung tissues from the three uninfected cats had low staining for IL-1β, and in some cases, complete absence of staining. A significant difference in staining for IL-1β was observed between cats that died of cytauxzoonosis and the uninfected cats.

Of the 19 lung tissues from infected cats, 16 had moderate staining for IL-6 by immunohistochemistry, particularly in the cytoplasm of infected cells and other noninfected monocytes, alveolar macrophages, lymphocytes, and neutrophils, with rare intracytoplasmic staining of the vascular endothelium (Figure 3.3). Only one case from an infected cat had low immunoreactivity for both IL-1β and IL-6; another two cases that had low immunoreactivity for IL-6 did have high immunoreactivity for IL-1β. The lung tissues from the three uninfected cats
also had low or absent staining for IL-6. A statistical difference in staining for IL-6 was observed between cats that died of cytauxzoonosis and the uninfected cats.

Fourteen lung tissues from infected cats had moderate immunoreactivity for iNOS, while only 5 lung tissues from infected cats had low immunoreactivity. All tissues from uninfected cats had low immunoreactivity to iNOS. The staining was characterized by cytoplasmic staining in the infected cells, and also in the surrounding monocytes and alveolar macrophages (Figure 3.4). One case that had low staining for IL-6 also had low staining for iNOS, but the other 4 cases that had low staining for iNOS had moderate staining for both IL-1β and IL-6. A significant difference in staining for iNOS was observed between C. felis-infected cats and uninfected cats.

**MHCII immunohistochemical expression**—Immunohistochemical staining for MHCII revealed that expression was extremely low in all uninfected cats and was restricted to plasmalemmal staining of a few scattered alveolar macrophages. Cats that died of C. felis infection had moderate (3) to high (16) MHCII expression, as indicated by plasmalemmal staining for MHCII in various cells (Figure 3.5), including in the endothelium (Figure 3.6). Only three lung tissues from cats that died of cytauxzoonosis did not demonstrate staining of infected cells, but had MHCII plasmalemmal immunoreactivity in the endothelium. A significant difference in staining for MHCII was observed between the cats that died of the disease and the uninfected cats.

**CD18 expression**—Compared with CD18 expression (normalized on the basis of GAPDH expression) for healthy uninfected cats, cats that died of the disease had an 30-fold increase in CD18 expression, (Figure 3.7). There was a significant difference in CD18 expression between cats that died of the disease and healthy survivor cats.
ICAM-1 expression—Compared with ICAM-1 expression (normalized on the basis of GAPDH expression) for healthy uninfected cats, cats that died of the disease had a 1.7-fold increase in ICAM-1 expression (Figure 3.8). Despite this trend of increased ICAM-1 mRNA upregulation in the endothelium of cats that died of the disease compared to healthy cats, this finding was not statistically significant.

Discussion

While the previous chapter was concerned mainly with characterizing the systemic immune response to C. felis, and only briefly mentioned some local inflammatory responses, this chapter focuses on the local pulmonary responses, and in the upregulation of CD18 and ICAM-1. This upregulation would result in the firm attachment of infected cells to the endothelium, one of the main histologic characteristics of this disease. The combined histologic and molecular findings of the present study of cats naturally infected with C. felis provide important information on the pathogenesis of cytauxzoonosis.

Although a complete detailed analysis of histopathologic findings was not performed as described by Snider,13 a combination of the main significant findings he observed was present in all of our cases (Table 3.2, Figure 3.1). The lesions in three of the cases were interpreted by the pathologists who performed the necropsy as interstitial pneumonia, while in another three cases; the lesions were interpreted as vasculitis. Characteristics of interstitial pneumonia include edema, thickening of the interstitium by edema and neutrophilic exudate, and alveolar neutrophilic infiltrate, among others.148 At least two of these were found in all lung sections examined; confirming that infection with C. felis causes an interstitial pneumonia. There were
also three cases that were interpreted to have vasculitis, an inflammatory lesion that can be a component of a systemic disease\textsuperscript{61}, such as \textit{C. felis} infection. Characteristics of vasculitis include the presence of leukocytes within and around the affected vessels, as well as damage to the vessel walls, usually observed by necrosis or fibrin deposits.\textsuperscript{149} In the cases observed, although necrosis was not noted and fibrin was rare, the common presence of alveolar hemorrhages implies that there has been damage to the vessel wall. In addition, in many cases, neutrophils were observed surrounding the small- to medium-caliber vessels and the alveolar capillaries. It does not appear, based on what was found in this and the previous chapter, that the vascular lesions found in cytauxzoonosis would correspond to a type III hypersensitivity reaction, caused by deposition of antigen-antibody complexes.\textsuperscript{61,149} The antibody response in this disease has been found to be limited, compared to a potential large excess of available \textit{C. felis}-derived antigens present in the blood, which would indicate that the concentrations are not adequate to create large amounts of immune complex deposits capable of activating the complement system and neutrophils.

Immunohistochemistry of \textit{C. felis}–infected tissue samples revealed a widespread qualitative increase in the expression of both the pro-inflammatory cytokines IL-1\textbeta and IL-6 and iNOS, compared with results from uninfected tissues. Detection of these molecules in uninfected control cats was either completely absent or very limited and scattered. A study characterizing the morphological features of feline infectious peritonitis (FIP)\textsuperscript{150} described similar increases in the pro-inflammatory cytokines TNF-\alpha and IL-1\textbeta in the tissues of FIP-infected cats, and related this expression to upregulation by CD18, something that has also been suggested by other studies.\textsuperscript{104,119,150} The previous chapter found not only a qualitative immunohistochemical increase in CD18, but also a relative increase in the concentration of
CD18 mRNA in cats that died of *C. felis* infection compared to uninfected cats; therefore, the qualitative increase in the pro-inflammatory cytokines observed can be related back to an increase in the expression and regulation of CD18.

The occasional immunoreactivity for pro-inflammatory cytokines noted on the endothelium was also described in the vessels of FIP-infected cats, and this was explained in that article as a local paracrine effect of the infected macrophages on the endothelium, instead of a systemic response. Unlike the other two cytokines, IL-6 was found to be less disseminated, perhaps because IL-6, although still part of the innate immune response, is produced slightly later than the other two cytokines, by the time the cat may have already been suffering systemic effects and close to its death. The upregulation of both of these cytokines was also present in a study correlating *Theileria parva* parasitic load and pro-inflammatory cytokines, and they have also suggested that a protozoal-induced cytokine dysregulation contributes to the disease. Similarly, some cells infected with *T. annulata* were reported to produce IL-1β, IL-6, and TNF-α. A previous study on *C. felis* had already addressed the possibility of pro-inflammatory cytokines being involved in the pathogenesis of the pulmonary lesions, and the present immunohistochemical study confirms the extensive presence of the pro-inflammatory cytokines, IL-1β and IL-6.

The strong iNOS immunoreactivity present in the infected cases demonstrates that the infected macrophages are producing reactive nitrogen intermediates, which is absent in resting macrophages and monocytes, but induced in response to activation. This combined expression of pro-inflammatory cytokines and iNOS in infected macrophages, demonstrates they are activated, as was suggested in a previous study. This local production of pro-inflammatory mediators contributes to the histological lesions, morbidity, and mortality noted, as has been
suggested previously and discussed in the previous chapter. In contrast, a study on *T. annulata*-infected macrophages showed a decrease in production of NO, likely because of reduced expression of iNOS.

The expression of pro-inflammatory cytokines (TNF-α, IL-1β, IL6) and iNOS is an immunohistochemical feature also found in what is considered acute pulmonary distress syndrome (ARDS), both in humans and animals, and Snider had suggested that cats that died may be affected with this, based on the histopathologic findings. Acute respiratory distress syndrome is a clinical diagnosis that, in veterinary medicine, has been defined as a condition meeting four of the following five criteria: 1) acute onset of tachypnea and difficulty breathing; 2) presence of risk factors (which includes inflammation, sepsis, or infection); 3) evidence of pulmonary capillary leak without increased pulmonary capillary hydrostatic pressure; 4) inefficient gas exchange; and 5) evidence of diffuse pulmonary inflammation. One of the main features of ARDS is the increase in alveolar septal and vascular permeability, something that was noted in both Snider’s study and ours. One histopathologic feature, though, that is commonly found in human cases of ARDS, but was rarely found by Snider or the current study, is the presence of hyaline membranes covering the alveolar epithelium. Of note, cats have resident pulmonary intravascular macrophages (PIM), unlike other species such as dogs and humans. Studies performed in sheep, which also have PIM, have suggested that the lung lesions are caused primarily by these cells, in contrast to species that do not have them, making them more susceptible to ARDS. Although ARDS is usually caused in humans by sepsis, it is also on occasion caused by a parasitic infection, most notably malaria, caused by various *Plasmodium* spp. In those cases, parasite sequestration in the vascular endothelium causes release of pro-inflammatory mediators (TNF-α, IL-1β, IL-6, NO) and tissue damage.
The immunohistochemical surface expression of MHCII was also increased in *C. felis*-infected lung samples compared to uninfected lungs. This is not only due to the marked increase of antigen-presenting cells in the infected tissue, but also in the expression of MHCII in the endothelium. Although originally, MHCII immunohistochemistry was used in this study as an additional marker to identify the infected cells as macrophages, the expression of MHCII in the endothelium was a striking finding. Endothelial cells are not cell types associated with constitutive MHCII expression, but MHCII expression can be activated and induced by immune mediators, such as IFN-γ or TNF-α,\textsuperscript{66,157,158} in various species. In one study, the other pro-inflammatory cytokines studied failed to induce MHCII expression in porcine endothelial cells.\textsuperscript{157} From the previous chapter, a marked increase in TNF-α concentration and expression were noted serologically and immunohistochemically, respectively, and it is possible that this cytokine was responsible for the induction of MHCII expression in endothelial cells. Unlike endothelial expression of pro-inflammatory cytokines and molecules, endothelial expression of MHCII happened independently of the presence of nearby infected (and immunoreactive) macrophages. In three cases, the infected macrophages failed to immunoreact with MHCII, but had marked endothelial MHCII immunoreactivity, suggesting that there was an underlying systemic immune response that activated the endothelium. This endothelial MHCII expression was considered a systemic response by another study,\textsuperscript{150} unlike the endothelial expression of the pro-inflammatory cytokines. Activated endothelium can also modulate inflammatory responses, and can contract,\textsuperscript{140} allowing fluid to leak into the extravascular space and causing some of the edema that was noted in the lungs histologically in this study and others.\textsuperscript{13} They can also secrete NO, which although usually a vasodilator, can increase inflammation and damage the surrounding tissues in large amounts.\textsuperscript{59,140} As noted by immunohistochemistry, the endothelial
cells in this study did occasionally express iNOS, and the overexpression of NO by vascular walls is known to contribute to certain shock symptoms, for example hypotension and microvascular damage. These effects can then cause congestion and edema, which are part of the common gross findings noted in *C. felis*-infected cats.  

Regulation of MHCII is observed in various cow subspecies infected with a parasite closely related to *C. felis, Theileria annulata*. In those cases, regulation of MHCII varied according to breed, and the types of MHCII receptors were regulated differently. It was also suggested that some alterations were host responses to combat the *T. annulata* infection and propagation. Not enough is currently known regarding feline MHCII molecules to know the specific subtypes and how they are expressed in various cat breeds. The lack of MHCII immunoreactivity in infected macrophages in two of the cases in this study could either be a host response in an attempt to control the infection, or perhaps *C. felis* intervened and regulated the MHCII expression in order to evade detection while reproducing in an antigen-presenting cell like the macrophage. It is also possible that, even if there is strong MHCII immunoreactivity noted, this MHCII could be functionally inactivated or dysregulated. Degradation and inactivation of MHCII molecules is a known method of evasion in some species of *Leishmania*.

A big limitation of these immunohistochemical studies is that, while they helped to characterize the local immune response and confirm the presence of pro-inflammatory cytokines in the affected tissues, they were not a quantitative assay that could tell the concentration of cytokines produced, or the relative increase in cytokine mRNA expression. The previous chapter was able to demonstrate a marked increase in the serum concentration of TNF-α and IL-1β in cats that died of the disease compared to cats that survive, but it also had the limitation that the
disease course was not necessarily uniform throughout the study because it was based on naturally-infected cats. The early studies that characterized cytauxzoonosis in cats by experimental infection described the gross and histological characteristics of the disease, but at that time did not concern themselves with upregulation of genes and cytokine concentrations. The lack of a *C. felis in vitro* culture system also limits the study of this disease and the immune response while avoiding the infection of live cats.

The previous chapter had already demonstrated a significant difference in the upregulation of CD18 mRNA expression in cats that died of the disease compared to acutely ill cats that survive, healthy survivors, and healthy uninfected cats. The studies described in this chapter specifically target the CD18 mRNA expression in infected macrophages compared with healthy circulating monocytes and tissue macrophages found in healthy, uninfected cats. The results from the qRT-PCR assay provide quantitative information that indicates an even higher CD18 mRNA upregulation than that which was found in the blood. Increases in CD18 expression are relevant because they relate to leukocyte activation, and interaction of CD18 with its ligands can in turn upregulate pro-inflammatory cytokines that can cause vascular leakage or damage. This increase in CD18 and the related increase in pro-inflammatory cytokines are also important in the activation of one of the major ligands of CD18, ICAM-1. Regarding the upregulation of ICAM-1 mRNA, although there was a slight increase in ICAM-1 mRNA expression, unfortunately, this was not statistically significant. Considering that there are multiple other molecules involved with leukocyte adhesion and diapedesis, such as molecules from the PECAM, VCAM, and JAM families, it is possible these other molecules are involved and/or affected. Alternatively, the adhesion of infected macrophages to the vessel walls and inability to migrate across the vessel walls may be
due to other dysregulated molecules. Susta et al. mentioned the lack of MAC387 expression in infected macrophages, compared to other monocytes present in the same tissues and control tissues from uninfected cats.\textsuperscript{21}

Overall, the findings in this chapter and the previous one confirm previous suggestions\textsuperscript{13} that the \textit{C. felis}-infected macrophages are activated in a classical or M1 fashion, secreting and/or expressing various pro-inflammatory mediators. Although typically an M2 macrophage response is expected during parasite infections,\textsuperscript{68} it is possible that \textit{C. felis}, like its related \textit{Theileria} parasites, can somehow alter and modulate the response in the macrophage, to ensure survival and replication.

**Footnotes**

a. Clone 341031, RnD Systems, Minneapolis, MN
b. Catalog Number AF1796, RnD Systems, Minneapolis, MN
c. Clone TAL 1B5, Santa Cruz Biotechnology, Santa Cruz, CA
d. Catalog Number PA3-030A, Thermo Scientific, Rockford, IL
e. Clone 73009, RnD Systems, Minneapolis, MN
f. Polyclonal goat IgG, RnD Systems, Minneapolis, MN
g. Mouse IgG\textsubscript{1} negative control, AbD Serotec, Raleigh, NC
h. Polyclonal rabbit IgG, RnD Systems, Minneapolis, MN
i. Dako Envision system, Dako, Carpinteria, CA
j. Cell and Tissue Staining kit, RnD Systems, Minneapolis, MN
k. MMI Membrane Slides, RNase free, Molecular Machine & Industries, Haslett, MI
l. MMI H&E Staining Kit Plus, Molecular Machine & Industries, Haslett, MI
m. MMI CellCut Plus, Molecular Machine & Industries, Haslett, MI
n. MMI Isolation Diffuser Caps, Molecular Machine & Industries, Haslett, MI
o. QuickExtract FFPE RNA Extraction Kit, Epicentre Biotechnologies, Madison, WI
p. ThermoScript RT-PCR system, Invitrogen, Grand Island, NY.
q. Take3 Multi-volume plate, Biotek Instruments Inc., Winooski, VT.
r. Integrated DNA Technologies, Coralville, IA
s. Primer3, Broad Institute, Cambridge, Mass. Available at:
   www.genome.wi.mit.edu/genome_software/other/primer3.html.
t. Geneious software, Biomatters, Auckland, New Zealand
u. iCycler IQ system, Bio-Rad, Hercules, CA
v. InStat, Graphpad Software, La Jolla, CA
w. Excel 2007, Microsoft Corp, Redmond, WA
**Figure 3.1**—Photomicrograph of a section of severely affected lung from a *C. felis*–infected cat.

Note the near total occlusion of the vessel by macrophages infected by *C. felis* merozoites (1), adjacent alveolar edema (2), and small multifocal hemorrhages (3). Also note the random foci of neutrophilic infiltration of the vessel wall (4), and multiple alveolar macrophages phagocytozing erythrocytes (5). Finally, within the vessel, notice multifocal fibrin deposits (6). Hematoxylin and eosin; bar = 50 µm
**Figure 3.2**—Photomicrographs of results of IL-1β immunohistochemistry of pulmonary vasculature obtained from a *C felis*–infected cat (A and B) and an uninfected control cat (C and D). An anti-IL-1β antibody was used in panels A and C, whereas an isotype control antibody was used in panels B and D. In panel A, note the intracytoplasmic staining in infected cells and other inflammatory cells, and diffuse extracellular staining. 3,3′-diaminobenzidine chromogen with hematoxylin counterstain; bar = 20 µm.
Figure 3.3—Photomicrographs of results of IL-6 immunohistochemistry of pulmonary vasculature obtained from a *C. felis*–infected cat (A and B) and an uninfected control cat (C and D). An anti-IL-6 antibody was used in panels A and C, whereas an isotype control antibody was used in panels B and D. In panel A, note the intracytoplasmic staining of infected cells and other inflammatory cells. 3,3′-diaminobenzidine chromogen with hematoxylin counterstain; bar = 20 µm.
Figure 3.4—Photomicrographs of results of iNOS immunohistochemistry of pulmonary vasculature obtained from a *C. felis*–infected cat (A and B) and a healthy uninfected control cat (C and D). An anti-iNOS antibody was used in panels A and C, whereas an isotype control antibody was used in panels B and D. In panel A, note the intracytoplasmic staining of infected cells and other inflammatory cells. In panel C, the inflammatory cells are not stained. 3,3′-diaminobenzidine chromogen with hematoxylin counterstain; bar = 20 µm.
Figure 3.5—Photomicrographs of results of MHCII immunohistochemistry of pulmonary vasculature obtained from a *C. felis*–infected cat (A and B) and a healthy uninfected control cat (C and D). An anti-MHCII antibody was used in panels A and C, whereas an isotype control antibody was used in panels B and D. In panel A, note the cytoplasmic staining of infected cells and other inflammatory cells. In panel C, rare scattered alveolar macrophages and lymphocytes stain for MHCII. 3,3′-diaminobenzidine chromogen with hematoxylin counterstain; bar = 20 µm.
Figure 3.6—Photomicrograph of MHCII intracytoplasmic staining in the endothelium of a *C. felis*-infected cat (A). This was not present in control cats (B), and only noticed in some of the infected cats. 3,3′-diaminobenzidine chromogen with hematoxylin counterstain.
Figure 3.7—Mean ± SEM fold changes in CD18 mRNA expression (normalized on the basis of GAPDH expression) in infected macrophages of cats that died of cytauxzoonosis, compared with results from tissue macrophages of healthy, uninfected, control, cats. * Values differ significantly ($P < 0.05$).
Figure 3.8—Mean ± SEM fold changes in ICAM-1 mRNA expression (normalized on the basis of GAPDH expression) in the endothelial cells of cats that died of cytauxzoonosis, compared with results for endothelial cells of healthy, uninfected, control cats.
Table 3.1—Primary antibodies, dilutions, and antigen retrieval methods used. N/A - No antigen retrieval used, ABC - Avidin-biotin complex kit, Polymer - Polymer/streptavidin kit

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Dilution</th>
<th>Final concentration (µg/mL)</th>
<th>Antigen retrieval method</th>
<th>Detection method</th>
</tr>
</thead>
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<tr>
<td>IL-1β</td>
<td>1:10</td>
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<td>Citrate</td>
<td>ABC</td>
</tr>
<tr>
<td>IL-6</td>
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<td>50</td>
<td>N/A</td>
<td>ABC</td>
</tr>
<tr>
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<td>1:100</td>
<td>N/A</td>
<td>Citrate</td>
<td>ABC</td>
</tr>
<tr>
<td>MHCII</td>
<td>1:50</td>
<td>4</td>
<td>Citrate</td>
<td>Polymer</td>
</tr>
</tbody>
</table>
Table 3.2—Distribution of major pulmonary histopathologic findings in *C. felis*-infected cats.

The numbers are out of the nineteen *C. felis*-infected cases that were selected for immunohistochemical studies.

<table>
<thead>
<tr>
<th>Major pulmonary histopathological findings</th>
<th>Edema</th>
<th>Alveolar hemorrhage</th>
<th>Thickened septa</th>
<th>Neutrophils within septa</th>
<th>Alveolar macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>19</td>
<td>12</td>
<td>11</td>
<td>9</td>
<td>16</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER 4

ISOLATION OF CYTAUXZOON FELIS FROM BLOOD AND TISSUES

Introduction

Cytauxzoonosis is a fatal disease of domestic cats in the Midwestern, Mid-Atlantic, Southeastern, and Southcentral United States, caused by *Cytauxzoon felis*, a tick-borne parasite in the order Piroplasmida, family Theileriidae. An infected tick transmits *C. felis* while feeding, followed by schizogeny of the parasite in macrophages throughout the body. Numerous studies have been conducted regarding the epidemiology of cytauxzoonosis, and a few have also tried to further characterize the infection based on formalin-fixed, paraffin-embedded tissues. A major obstacle in further characterizing the pathogenesis is the inability to isolate and propagate the parasite in vitro, as has been done with the related *Theileria* species that affect cattle and wild ruminants. Our current characterization of the disease is based on the study of cats naturally infected with *C. felis* and on cats experimentally infected with homogenized tissues or blood obtained from *C. felis*-infected cats. No currently established cell line exists that can propagate *C. felis in vitro*, and no attempts at isolating the parasite from infected macrophages have been published to date.

These early characterization studies include one by Wagner, who was also the first to describe the parasite, which focused on experimentally infecting healthy cats with *Cytauxzoon felis* and keeping a continuous passage of parasites from tissues of moribund cats to healthy cats.
parenterally. Another early experiment studying parasite transmission was described by Kier, Wagner, and Morehouse[^28]. Using blood and tissue obtained from an acutely ill bobcat, they were able to cause fatal disease in inoculated cats. Cats inoculated with blood and tissues of asymptptomatically parasitemic bobcats and domestic cats, however, did not develop cytauxzoonosis, suggesting that the parasite is infective to other monocytes only during the tissue phase, and that once it develops to the erythrocytic stage, it loses its pathogenicity within the host. More recently, a group of domestic cats that had been infected with the erythrocytic stage of the related parasite *Cytauxzoon manul* was challenged by a splenic homogenate from a *C. felis*-infected cat that died of the disease, and all the cats in the group developed classical clinical signs of feline cytauxzoonosis.[^44]

Electron microscopy studies of *Cytauxzoon felis* are few[^2][^3] and they study the different stages of *C. felis* development within the macrophage and erythrocytes. Depending on the stage, organelles, including the nucleus, mitochondria, and rhoptries, can be identified, but in other stages, the parasite does not show distinguishing features. These studies[^2][^3] concluded that the tissue phase of *Cytauxzoon felis* was similar to other species previously studied, such as *Theileria*.

This chapter describes a method for isolating the *C. felis* parasites from the blood and tissues of cats naturally infected with *C. felis*, and an attempt at infecting peripheral blood monocytes (fPBMC) with the parasites. Early studies were able to infect healthy cats with cytauxzoonosis by administering tissue infected with the infected macrophages.[^28][^31][^47] Based on the published protocols, which included the use of fresh or previously frozen homogenized splenic samples from *C. felis*-infected dead cats, it appears that the parasite was able to survive extreme freezing temperatures (-80 °C to -120 °C) and freezing methods (use of DMSO).
Therefore, isolating the parasite, freezing it for later use, and infecting fPBMCs should be possible. Cytology and electron microscopy were used to identify the parasite and confirm that the sample was not contaminated with other cells or organisms. If the infection is successful, and if the parasite behaves like the related Theileria, infected cells may become established and continue proliferating by clonal expansion.\textsuperscript{7,8} Cytology was used to identify infected cells, and concentrations of the pro-inflammatory cytokines TNF-\(\alpha\), IL-1\(\beta\) and IL-6 were measured by ELISA.

\textit{Materials and Methods}

\textbf{Cats}—Blood and tissue samples were obtained from cats at participating veterinary clinics that were confirmed by diagnostic laboratories or participating veterinarians as \(C\ \text{felis}\)–infected on the basis of blood smear analysis, and that later died of the infection. Tissues from these cats had also been used on the previous chapters to characterize the local and systemic immune response to \(C.\ \text{felis}\). The whole blood used to establish a feline mononuclear cell culture was obtained from healthy uninfected cats that were part of the blood donor colony at the University of Georgia College of Veterinary Medicine. The study was conducted in compliance with the University of Georgia, College of Veterinary Medicine Clinical Research Committee and Institutional Animal Care and Use Committee.

\textbf{Collection of heart blood and tissue samples}—Heart blood samples (5-7 mL/sample), which are known to contain more infected macrophages than what is noted in samples taken from peripheral blood,\textsuperscript{27} were collected into EDTA-containing tubes from \(C.\ \text{felis}\)-infected cats.
during necropsy. Similarly, samples of lung, liver, and spleen from these cats were collected and placed in cryogenic vials, then stored at -80°C until needed.

**Processing of heart blood samples**—Once the samples arrived at our laboratory, blood smears were prepared from the EDTA-anticoagulated blood samples, which were used to confirm the previous diagnosis of cytauxzoonosis in affected cats. The blood components were separated using a density gradient, commercially available product, and following the manufacturer’s instructions.

The pellet obtained was mixed with freezing medium containing 90% heat inactivated, fetal bovine serum and 10% DMSO. The cells and medium was then stored in liquid nitrogen until needed.

**Processing of tissue samples**—The following protocols were adapted from previously published material, mostly involving extraction of *Leishmania* organisms from tissues, as this parasite also infects tissue macrophages. Approximately 1 to 3 grams of tissue (either lung, liver, or spleen), were thawed in a 37°C water bath, cut into approximately 5 mm sections, and added to 25 mL, 0.25% trypsin/EDTA solution. The mixture was warmed in a 37°C water bath for 4 hours, with occasional vortexing. The solution was filtered using a 70 µm cell strainer, and the large remaining pieces were separated and discarded. The remaining solution was centrifuged for 8 minutes, at room temperature and 2400 g. The supernatant was discarded, and 5 mL of RPMI 1641 solution were added, and the centrifugation step was repeated.

**Isolating *C. felis* merozoites from infected macrophages**—The monolayer obtained by density gradient separation using the Histopaque system was thawed, mixed with 5 mL of RPMI 1640, and centrifuged once at 2400 g for 8 minutes at room temperature. At this point, both
heart blood-derived and tissue-derived infected macrophages were at the same stage of processing, and the rest of the methodology applies to both types of parasite extraction.

The supernatant was discarded, and 10 mL of RPMI 1640 mixed with 0.08% SDS were added, followed by vortexing. The solution remained at room temperature for 5 minutes, when 5 mL of cell culture medium (Appendix B) was added and the solution was then centrifuged for 8 minutes at room temperature and 2400 g. The supernatant was discarded and the pellet was retained.

The pellet was mixed with 5 mL of cell culture medium and centrifuged for 8 minutes at room temperature and 70 g. The supernatant was carefully removed and saved, and 5 mL more of cell culture medium were added to the pellet. The centrifugation process was repeated two more times, each time collecting the supernatant.

The approximately 15 mL of supernatant collected from the previous steps were then centrifuged once more for 8 minutes at room temperature and 2400 g. This time, the pellet was kept and resuspended in 1 mL cell culture medium. Approximately 100 µL were added to a gluteraldehyde fixative solution for electron microscopy studies, 3-5 µL were used to make a cytologic smear to confirm the presence of the parasite, and the rest was kept for immediate use in infecting fPBMCs. The remaining suspension after inoculating cell wells, if any, was centrifuged again for 8 minutes, at room temperature and 2400 g, and the pellet mixed with freezing medium as described above and stored in liquid nitrogen until later use.

**Staining and cytologic examination for C. felis parasites**—Approximately 3-5 µL of the final resuspended pellet were used to make a smear on a clean, sterile slide, which was then allowed to air-dry. The slide was fixed with methanol for 2 minutes at room temperature and again allowed to air-dry. The slide was covered by a filtered Wright’s solution and stained for
10 minutes. Gentle washing with deionized water removed the excessive stain, and the slide was allowed to completely dry before being examined microscopically by a board certified veterinary pathologist.

**Processing of blood samples**—Once the blood from healthy uninfected cats was obtained, the same density gradient solution and process was used, following the manufacturer’s instructions. The mononuclear layer obtained was then washed as described by the manufacturer, and finally resuspended in 1 mL of phosphate buffer solution. A cell count using a hematocytometer and trypan blue dye was used to quantitate the total number of mononuclear cells collected.

**Culture of white blood cells, C. felis infection, LPS stimulation** — Depending on the number of leukocytes collected, a range of approximately $1 \times 10^5$ to $1 \times 10^6$ cells per mL were added to each well (at least 3 wells for control cells, 3 wells for infection with *C. felis*, and 3 wells for LPS stimulation). The cell medium (1 mL/well) used was the same as described above. Before adding the cells, and for later immunocytochemical and cytologic studies, round, glass cover slips were placed on the bottom of each well. The cells were allowed to acclimate in the incubator at 37°C and 5% CO$_2$ overnight. The next day, half of the medium was removed from all the wells. In the control wells, the amount removed was replaced by the same amount of fresh culture medium (500 µL). In the LPS-stimulated wells, the medium removed was replaced by 200 ng/mL of LPS in culture medium, for a final concentration of 100 ng/mL of LPS per well. For the *C. felis*-infected wells, the medium removed was replaced by thawed or freshly obtained parasites mixed with fresh culture medium. The cells were then incubated overnight under the same conditions. The next day, the supernatant was removed and stored in -20°C until needed.
Throughout the study, the wells were examined daily for evidence of bacterial or fungal contamination. Only data from wells that were never contaminated were included in this study.

**ELISA**—A sandwich ELISA was performed with reagents optimized for detection of feline TNF-α, IL-1β, and IL-6. All assays were conducted in accordance with the protocol provided by the manufacturer. All supernatant samples were assayed in triplicate. Flat-bottom, 96-well microplates were used for the assay. A 7-point standard curve was always created for each batch and used with a 2-fold dilution in 1% fetal calf serum in PBS. The high standard was 1,000 pg/mL for TNF-α and 2,000 pg/mL for IL-1β and IL-6, and the lower limit of detection (LLD) was 15.6 pg/mL for TNF-α and 31.25 pg/mL for IL-1β and IL-6. Optical density was determined with a plate reader and associated software; absorbance was set at 450 nm.

**Cytologic examination**—First, one round cover slip from each group was fixed with methanol and then stained with filtered Wright’s stain. The lid was then air dried and mounted on a glass slide before being examined by light microscopy by a board certified veterinary pathologist.

**Statistical analysis**—Mean and SD values were obtained, and error bars were used to represent SEMs. For cytokine results, an ANOVA, followed by the Tukey-Kramer multiple comparisons test, was used to detect significant differences among groups (control, LPS-stimulated, and *C. felis*-infected cells). Differences were considered significant at a value of $P < 0.05$.

**Results**

**Isolation of *C. felis* parasites**—Immediately after the final pellet was obtained and resuspended, cytologic examination was performed and compared to broken *C. felis*-infected
cells (Figure 4.1). A typical smear was sparsely cellular, and contained multiple, 1-3 µm in
diameter, irregularly shaped organisms that contained wispy pale blue cytoplasm, and a single,
dark purple eccentric round nucleus.

Ultrastructural findings (Figure 4.2) include a double membrane surrounding the
organisms, a nucleus, and occasional food vacuoles. Many organisms noted did not have intact
cytoplasmic membranes.

**Proinflammatory cytokines**— Concentrations of the three major pro-inflammatory
cytokines studied (TNF-α, IL-1β, and IL-6) were significantly higher in the supernatant of LPS-
stimulated fPBMC cells, compared to all other wells. Only in one attempt at culture, was there
any secretion of pro-inflammatory cytokines by the *C. felis*-infected cells (Appendix E).

**Discussion**

Unlike the related *Theileria* protozoa,\(^7\)\(^-\)\(^9\),\(^19\),\(^54\),\(^167\) isolation of *Cytauxzoon felis* and infection
of a naïve population of peripheral blood monocytes has not been described in the literature.
One early article\(^69\) does mention an attempt at infection of Vero cells, which are derived from
kidney cells of African green monkeys, but no other mention of this has been made, and no
functional test was mentioned in the article to confirm the presence of the parasite in the infected
cells. It appears *C. felis* is relatively species-specific, so far, infecting only various domestic and
wild felids,\(^31\),\(^34\),\(^168\)-\(^174\)-\(^176\) and also very cell-specific, infecting only monocytes and
erthrocytes,\(^2\),\(^13\),\(^21\) it is highly unlikely that the parasite infected the Vero cells. It is possible,
however, that what the authors were describing as infected cells were part of the original
inoculum of infected cells, that remained alive, and perhaps even replicating, for some passages.
Unfortunately, no more information has been published regarding this finding, and a cell line of immortalized, \textit{C. felis}-infected cells is not available, as it is for related parasites. Alternatively, it is possible that \textit{C. felis} behaves like other members of the \textit{Theileria} genus, which only have a transient tissue phase and are mainly known as intraerythrocytic parasites.\(^{177}\) This would conform to the findings that the tissue phase is very rarely found in the natural reservoir, the bobcat. The domestic cats, as aberrant hosts, may somehow be unable to survive the tissue phase.

\textit{Cytauxzoon felis} is an intracellular parasite, and the parasite is usually described, even ultrastructurally, within the infected macrophages, except for rare mentions of merozoites invading erythrocytes.\(^{2,3}\) Descriptions of free parasites are scarce, and only in cases where the infected macrophages are ruptured due to processing, such as a cytologic smear, are free parasites observed. A ruptured infected macrophage was used for comparison by light microscopy, and the results demonstrate that the product obtained after all centrifugation steps is similar to what is described for \textit{C. felis} (\textbf{Figure 4.1}). Bacterial contamination was not noted and very little to any cells or cell fragments were observed in most of the isolation attempts. Ultrastructural examination revealed large numbers of ruptured cytoplasmic membranes and scattered parasites (\textbf{Figure 4.2}). Unfortunately, many parasites also had ruptured cytoplasmic membranes. This may indicate that either the current method of parasite isolation or the preparation and processing for electron microscopy cause damage to the cellular membranes that could affect the infectivity.

The closest to a mononuclear phagocyte cell line available is the \textit{Felis catus} whole fetus (FCWF-4) cell line, which has been primarily used to study FIP \emph{in vitro}.\(^{178-180}\) Although according to its source,\(^{181}\) this cell line has some macrophage characteristics, such as phagocytic
activity and Fc receptors, neither the cell line description nor the published articles mention the expression of other characteristics, such as response to LPS and/or expression of surface markers. Based on experiments performed, this cell line does not respond to LPS stimulation (Appendix B), and does not express the surface marker CD18 (Appendix C). As has been discussed in previous chapters, C. felis infection causes an increase in pro-inflammatory mediators, likely regulated by infected and activated macrophages. Because this established cell line did not appear to respond to LPS, which is known to cause robust release of pro-inflammatory mediators, it was not used for the experimental infection of C. felis. Instead, temporary cell culture of feline peripheral blood mononuclear cells was used to test whether the isolated parasite was able to infect circulating monocytes and promote the release of pro-inflammatory mediators, such as TNF-α, IL-1β, and IL-6. One disadvantage of this is that, although the number of cells on each well was consistent within an experiment, the number of fPBMCs collected varied depending on the number of cats and the total amount of blood available.

Despite numerous attempts, only once was a temporary infection with C. felis obtained. Optimization of in vitro infection with C. felis will require the creation of a more permanent cell line of C. felis-infected macrophages, similar to what has been done with some of the related Theileria spp.,7,8,19,20,127,182 where the infected cells can continuously propagate, as long as they are infected with the parasite.182 In the one case in which the addition of C. felis merozoites resulted in an infection, it was observed that C. felis could infect peripheral blood monocytes, and that infected cells secreted pro-inflammatory cytokines, particularly TNF-α and IL-1β. This confirmed findings in the previous two chapters, where expression and secretion of pro-inflammatory cytokines were noted in C. felis-infected cats, particularly those that did not
survive infection, compared to healthy uninfected cats. The control cells expressed little to no pro-inflammatory cytokines, particularly TNF-α. The higher concentrations of pro-inflammatory cytokines in LPS-stimulated cells compared to *C. felis*-infected cells could be due to various reasons. First, LPS is a potent stimulator and activator of macrophages, and the dose (100 ng/mL) given is considered a high dose in the immunology literature. Unfortunately, these results could not be replicated, perhaps because of the large variability in obtaining both feline cat blood cells and infective, viable, *C. felis* parasites. The administration of a higher number of viable parasites could have resulted in a higher concentration of pro-inflammatory cytokines, but at this time, it is not possible to calculate the dose of *C. felis* parasites added to each well, nor the infective dose of *C. felis*.

**Footnotes**

a. Histopaque 1077, Sigma-Aldrich, St. Louis, MO
b. DuoSet, RnD Systems, Minneapolis, MN
c. Nunc-Immuno, Sigma-Aldrich, St Louis, MO
d. Gen 5, Biotek Instruments Inc., Winooski, VT
e. Excel 2007, Microsoft Corp, Redmond, WA
f. InStat, Graphpad Software, La Jolla, CA
Figure 4.1—Photomicrographs comparing a broken *C. felis*-infected macrophage surrounded by free merozoites (A) and the pellet contents at the end of the isolation process (B). Wright’s stain, bar = 10 µm.
Figure 4.2—Electron photomicrographs comparing two different ultrastructural images of *C. felis*. Both have a double membrane (arrow), nucleus (N), and (A) has a food vacuole (v). Uranyl acetate and lead citrate, bar = 500 nm.
CHAPTER 5

DEVELOPMENT OF A FELINE CELL LINE FOR IN VITRO STUDIES

Introduction

There are very few feline cell lines available to study infectious disease pathogenesis or cancer biology \textit{in vitro}, without depending on infecting live animals or using natural-occurring cases. Currently, there is only one commercially-available, feline cell line\textsuperscript{181}, the \textit{Felis catus} whole fetus (FCWF-4) cell line, that has been described in various articles as macrophage-like and primarily used to study feline infectious peritonitis (FIP) \textit{in vitro}.\textsuperscript{178,179} According to these articles and to the information provided by the cell culture bank, this cell line possesses Fc receptors, phagocytic activity, and nonspecific esterase production.

As mentioned previously, \textit{Cytauxzoon felis} has not been cultured conclusively \textit{in vitro}. One early article\textsuperscript{69} does mention an attempt at the infection of Vero cells, which are derived from kidney cells of African green monkeys, but no other mention of this has been made, and no functional test was mentioned in the article to confirm the presence of the parasite in the infected cells. Since it appears \textit{C. felis} is relatively species-specific, infecting mostly wild and domestic felids, and very cell-specific, infecting only monocytes and erythrocytes,\textsuperscript{2,13,21} it is highly unlikely that the parasite infected the Vero cells. This limits the study of the organism to samples obtained from blood and tissues collected from naturally- or experimentally- infected cats. In the case of naturally-infected cats, it is difficult to study the pathogenesis and host response of \textit{C. felis in vivo} because the exact disease timeline is not known, and cats may die or
be euthanized at different times during the disease, which could affect data interpretation. In experimentally-infected cats, the timing of inoculation is known, most variables are controlled, and study animals either die or can be euthanized at known time points. Still, *C. felis* is a high morbidity, high mortality disease, and an *in vitro* system would enable one to study the parasite without relying solely on an animal model. It is proposed that *C. felis* will only infect cells that have been immunohistochemically confirmed to be of monocyte-macrophage lineage.

The original plan was to use the FCWF-4 cell line to attempt the culture of the schizogony phase of *C. felis in vitro*. Preliminary studies ([Appendices C and D](#)) demonstrated that the FCWF-4 cells did not produce cytokines in response to LPS stimulation and did not express the leukocytic cell surface marker CD18, a $\beta_2$-integrin, which is present on all leukocytes.\(^{66,71,72,77,82,84,95-99}\) Due to these findings, an attempt was made to create a macrophage-like feline cell line that could be used to study *C. felis in vitro*.

*Materials and Methods*

**Case**—A 2.5 year-old, male, neutered cat presented to the University of Georgia Veterinary Teaching Hospital with a 5-month history of progressive right hind limb lameness. The laboratory clinical data was unremarkable. Based on the cytologic findings, the main differentials at that time included histiocytic sarcoma and an undifferentiated sarcoma. The clinical pathologists communicated to us their findings and of the owner’s decision to amputate the leg, and we obtained consent from the owner and clinician to collect some fresh tissue samples at the same time that the anatomic pathologists collected tissue for histopathologic examination.
**Processing of tissue samples**—The following protocols were adapted from previously published material.\textsuperscript{171-173} Approximately 3 grams of tumor tissue were cut into approximately 5 mm sections, and added to 25 mL, 0.25\% trypsin/EDTA solution. The mixture was warmed in a 37$^\circ$C water bath for 4 hours, with occasional vortexing. The solution was filtered using a 70 $\mu$m cell strainer, and the large remaining pieces were separated and discarded. The remaining solution was allowed to cool down at room temperature, and gradient separation using Histopaque\textsuperscript{a} was used, following manufacturer’s protocol.

**Establishment of the feline cell line**—The cells from the monolayer observed were removed, mixed with modified cell culture medium (\textbf{Appendix B}) containing 20\% heat-inactivated feline cat serum, and transferred to a T25 flask and incubated at 37$^\circ$ in 5\% CO$_2$. The flask was examined every day using an inverted microscope for signs of cellular adhesion and replication. Carefully and slowly, approximately 5 mL of medium were removed every three days, and replaced with 5 mL of fresh medium.

After one week, round to spindle-shaped cells were noted to be attached to the bottom of the flask. Eventually, the cells divided and became confluent, making necessary a transfer to a T75 flask.

**Splitting and preserving cells**—The culture medium was removed from the flask and the flask was washed a couple of times with 5 mL of sterile, phosphate buffer solution. Approximately 5 mL of 0.25\% trypsin/EDTA solution were added to the flask, and the flask was placed in a 37$^\circ$C, 5\% CO$_2$ incubator for approximately 3 minutes. The flask was then examined under the microscope, and once the cells were observed to be dislodged from the flask, the trypsin was neutralized with 5 mL of cell culture medium, and the cells were centrifuged at 1000
revolutions per minute (rpm) for 5 minutes at room temperature. The supernatant was discarded and the pellet of cells was mixed with cell culture medium and passed to a T75 flask.

Once the cells became confluent in the T75 flask, the same procedure for splitting the cells was repeated, only this time 6 mL of fresh cell culture medium were added after the supernatant was removed, and only 2 mL of that mixture were transferred to the next T75 flask. The remaining cells were either discarded or frozen.

To freeze the cells, the mixture was again centrifuged at 1000 rpm, for 5 minutes at room temperature. The supernatant was discarded, and 3 mL freezing medium (cell culture medium + 5% DMSO) was added and mixed with the pellet. The medium was aliquoted into three, 1 mL cryogenic vials, and placed immediately into liquid nitrogen. To thaw cells, the frozen cells were thawed in a 37°C waterbath, mixed with cell culture medium, and centrifuged at 1000 rpm for 5 minutes at room temperature before placing them in a T25 flask and restarting the cell culture. This was done days, weeks, and months after the initial cell line was established, and the culture was able to replicate each and every time, with a similar morphology as the original cells observed.

After five passages, the feline serum in the cell culture medium was replaced by fetal bovine serum, and no difference in growth and replication was observed. The cell line had become established and was able to continue propagating without using feline serum.

**LPS stimulation**—In order to collect cells for cytologic examination, sterilized round coverslips were added to the wells prior to adding the cells. After 10 passages, the cells were counted using a hematocytometer, and approximately 5 x 10^5 cells were added to six wells in a 24-well plate. At the same time, feline peripheral blood mononuclear cells were collected under aseptic conditions using Histopaque®, and the cells were equally split between six wells. At least
three wells of the cell culture line were used for control, and at least three wells were selected to be stimulated with a concentration of 100 ng/mL of LPS. Three wells were reserved for control, nonstimulated fPBMCs and three wells were reserved for LPS-stimulated fPBMCs. The total amount of cell culture medium used in each cell was 1 mL/well. The cells were allowed to acclimate in the incubator at 37°C and 5% CO₂ overnight. The next day, half the medium (500 µL) was removed from all wells. In the control tumor cell culture and fPBMC wells, the amount removed was replaced by the same amount of fresh culture medium. In the LPS-stimulated tumor cell culture and fPBMC wells, the medium removed was replaced by 200 ng/mL of LPS in culture medium, for a total of 100 ng/mL of LPS per well. The cells were then incubated overnight under the same conditions. The next day, the supernatant was removed and stored in -20°C until needed. Throughout the study, the wells were examined daily for evidence of bacterial or fungal contamination. Only data from wells which were never contaminated were included in this study. The round coverslips with cells attached were preserved for cytologic studies.

**ELISA**—A sandwich ELISA was performed with reagents optimized for detection of feline TNF-α, IL-1β, and IL-6. All assays were conducted in accordance with the protocol provided by the manufacturer. All supernatant samples were assayed in triplicate. Flat-bottom, 96-well microplates were used for the assay. A 7-point standard curve was always created with each batch and used with a 2-fold dilution in 1% fetal calf serum in PBS solution. The high standard was 1,000 pg/mL for TNF-α and 2,000 pg/mL for IL-1β and IL-6, and the lower limit of detection (LLD) was 15.6 pg/mL for TNF-α and 31.25 pg/mL for IL-1β and IL-6. Optical density was determined with a plate reader and associated software; absorbance was set at 450 nm.
Cytologic and CD18 immunocytochemical analysis—Cells grown over sterile round glass coverslips were first stained with filtered Wright’s stain and then mounted on glass slides for cytology. The remaining two coverslips from each group were also stained immunocytochemically with mouse anti-feline CD18 monoclonal antibody and isotype control antibody. Before starting the procedure, one of the two coverslips picked from each group was selected as isotype control. The round coverslips were fixed by incubation in acetone for 3 minutes at room temperature (approx. 25°C). Each coverslip was incubated at room temperature for 1 hour with mouse anti-feline CD18 (1:20) or an isotype control antibody (1:20). The coverslips were then counterstained with Gill’s hematoxylin stain, air-dried, mounted, and subjectively examined via light microscopy by a board-certified veterinary pathologist. Results for slides were considered negative when no plasmalemmal staining for CD18 was detected around cells and positive when CD18 was detected.

Electron microscopy—At the 8th to 10th passage, the cells that were not split to a new flask were centrifuged again for 5 minutes at 1000 rpm in room temperature. Feline tumor cells were immediately fixed in 2% (para)formaldehyde, 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3, overnight. After washing several times in 0.1 M phosphate buffer, the samples were placed in microfuge tubes and suspended in 3% noble agar at 58 - 60°C. They were then quickly spun at 13,000 rpm in a microfuge for 10 minutes to condense the cells into a pellet. The agar-embedded cells were allowed to cool completely before removing the pellets. The agar-pellets of cells were introduced again into phosphate buffer and then post-fixed in 1% osmium tetroxide in buffer for 1 hour. The cells were washed several times in deionized water and then placed in 0.5% aqueous uranyl acetate for 1 hour. After several more washes in deionized water, the samples were dehydrated in an ethanol series and cleared in two changes of acetone and two
changes of propylene oxide. The samples were infiltrated with a 1:1 mixture of propylene oxide and plastic mixture for four hours, and then two changes of the plastic for at least one hour each before embedding the samples in fresh plastic using flatbed molds. The embedded samples were allowed to polymerize in a 75°C oven overnight. Using an ultramicrotome, thin sections of approximately 50-55 nm were obtained and placed on 200-mesh copper grids. The sections were post-stained with 5% methanolic uranyl acetate and Reynold’s lead citrate. The grids were viewed using an electron microscope.

**Statistical analysis**—Mean and SD values were obtained, and error bars were used to represent SEMs. For cytokine results, an ANOVA, followed by the Tukey-Kramer multiple comparisons test, was used to detect significant differences among groups (control, LPS-stimulated, and *C. felis*-infected cells). Differences were considered significant at a value of *P* < 0.05.

**Results**

**Cell culture morphology**—The tumor cells in this culture ranged in size from 10 to 50 µm, and were mainly stellate to spindle-shaped, with rare round cells. Anisocytosis, anisokaryosis, and multinucleation were common in all passages examined. Similarly, each nuclei contained at least one, commonly multiple, large nucleoli, and mitotic figures were common (**Figure 5.1**).

Ultrastructurally, the cells appeared plump and round to spindle-shaped, with numerous cytoplasmic processes. The cytoplasm contained large numbers of ribosomes, rough endoplasmic reticulum, and abundant mitochondria. Only one large nucleus was observed in
most of the cells examined, with a prominent nucleolus (Figure 5.2). Closer examination also showed occasional coated vesicles.

**CD18 immunocytochemical results**—The tumor cell line did not express the CD18 cell surface marker, compared to the fPBMCs, which did demonstrate CD18 plasmalemmal staining (Figure 5.3).

**Pro-inflammatory cytokines**—Concentrations of the major proinflammatory cytokines (TNF-α, IL-1β, and IL-6) were significantly higher for LPS-stimulated fPBMCs than all other groups (Figure 5.4). LPS-stimulated tumor cells failed to secrete any detectable concentration of pro-inflammatory cytokines.

**Discussion**

Unfortunately, by the time the cell line became established, but before we could finish characterizing it, further immunohistochemical studies had been made on the formalin-fixed tissue removed from the same mass, and a final diagnosis of either synovial sarcoma or malignant fibrous histiocytoma was given, based on immunohistochemistry (Table 5.1).

Based on the ultrastructural study, the tumor cell line is more compatible with published descriptions of synoviocytes and synovial sarcoma cell lines obtained from various species. More specifically, the morphology is similar to the type B synoviocyte that has been described in rats, horses, and humans, among other species. These cells are characterized ultrastructurally by rough endoplasmic reticulum and dendritic processes. Functionally, they are considered to secrete collagens, fibronectin, and other glycosaminoglycans into the joint cavity. Immunohistochemical markers for this specific cell type are not completely known, but it has
been suggested that protein gene product (PGP) 9.5 is a reliable marker for this cell type, at least in horses. Between these ultrastructural images (Figure 5.2) and the immunohistochemical findings of the formalin-fixed tissues, (Table 5.1), these cells are classified as neoplastic synoviocytes, and the original tumor was most likely a synovial cell sarcoma.

The tumor cells failed to express CD18, and were not able to secrete pro-inflammatory cytokines when stimulated by LPS, and therefore could not be used in in vitro studies for *Cytauxzoon felis* infection. Nevertheless, an established feline synovial sarcoma cell line has not been previously described or published. To identify this cell line more conclusively as synovial in origin, immunocytochemistry against vimentin and cytokeratin should be performed. Once cell origin is established, further functional studies can be performed to study possible pharmacological treatments and cellular pathways involved in the pathogenesis of synovial cell sarcoma in cats.

**Footnotes**

a. Histopaque 1077, Sigma-Aldrich, St. Louis, MO
b. DuoSet, RnD Systems, Minneapolis, MN
c. Nunc-Immuno, Sigma-Aldrich, St Louis, MO
d. Gen 5, Biotek Instruments Inc., Winooski, VT
e. Dako Envision system, Dako, Carpinteria, CA
f. Clone Fe3.9F2, provided by Dr. Peter Moore, Department of Pathology, Microbiology, and Immunology, School of Veterinary Medicine, University of California-Davis, Davis, CA
g. Mouse IgG1 negative control, AbD Serotec, Raleigh, NC
h. JEM-1210 Transmission Electron Microscope, JEOL USA, Inc., Peabody, MA
i. Excel 2007, Microsoft Corp, Redmond, WA
j. InStat, Graphpad Software, La Jolla, CA
**Figure 5.1**—Photomicrograph depicting the morphology of the tumor cell line. Note the spindle to stellate-shaped cells, anisokaryosis and anisocytosis, multiple nucleoli, binucleation (*), and mitotic activity (mf). Wright’s stain, bar = 10 µm.
Figure 5.2—Photomicrograph depicting the ultrastructural morphology of the tumor cell line. Notice the multiple nucleoli (A), the numerous mitochondria (*), the rough endoplasmic reticulum (rer), and the free cytoplasmic ribosomes. Uracyl acetate and lead citrate, bar = 2 µm.
Figure 5.3—Photomicrographs of CD18 immunocytochemistry of tumor cell line samples (A and B) and fPBMCs from a healthy, uninfected cat (C and D). An anti-CD18 antibody was used in panels A and C, and an isotype control antibody was used in panels B and D. 3,3'-diaminobenzidine chromogen with hematoxylin counterstain; bar = 10 μm.
Figure 5.4—Mean ± SEM of supernatant TNF-α, IL-1β, and IL-6 concentrations, as determined via an ELISA, for LPS-stimulated fPBMCs (n = 6, black bars), and LPS-stimulated tumor cells (n = 9; white bars). Concentrations were obtained from culture supernatant collected 24 hours after stimulation and incubation in a 37°C, 5% CO₂ environment. The LLD for IL-6 and IL-1β are 31.25 pg/mL (single line), and the LLD for TNF-α is 15.625 pg/mL (dashed line). * Within a cytokine, the value for this group of cats differs significantly (P < 0.05) from the other group.
Table 5.1—Immunohistochemical stains performed on the formalin-fixed tissue obtained from the same mass as the tumor cell line.

The following includes the immunohistochemistry performed to better characterize the neoplastic mass from which the tumor cell line was obtained. Most of these tests and results were performed after the cell line had already been established. Note that the mass was only confirmed positive for vimentin and cytokeratin (AE1/AE3 and Lu-5).

<table>
<thead>
<tr>
<th><strong>Immunohistochemistry</strong></th>
<th><strong>Results</strong></th>
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<tr>
<td>Vimentin</td>
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<td>Cytokeratin</td>
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<td>CD18</td>
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CHAPTER 6
CONCLUSIONS

The previous chapters have all dealt with the three main goals of this project, namely the characterization of the feline immune response to infection by *Cytauxzoon felis*, the role of the integrin CD18 in the pathogenesis of this disease, and the establishment of protocols to study cytauxzoonosis *in vitro*. Chapters 2 and 3 dealt mostly with the first and second goals, while chapters 4 and 5 attempted to accomplish the third goal. In summary, the pulmonary and systemic immune responses to cytauxzoonosis involve the secretion of pro-inflammatory cytokines, and at least for one cytokine (TNF-α), a significant difference in the systemic concentration was present between the cats that eventually die of the disease and the cats that survived. At a local level, it was also found that the endothelium was activated, based on its expression of some immune mediators like IL-1β, IL-6, iNOS, and MHCII. This correlates with previous histopathologic findings and suggestions that the pulmonary lesions are caused by the release of large amounts of pro-inflammatory cytokines, causing a shock-like state that impairs organ function and can lead to death. Regarding the role of CD18, this was found to be both qualitatively and quantitatively upregulated in cats that died of the disease compared to cats that survived or healthy uninfected cats. This difference was more striking when the expression of CD18 by infected macrophages was compared to the expression by other tissue macrophages present in healthy uninfected cats. This increase in CD18 could be due to dysregulation caused by the parasite itself, or secondary to paracrine and autocrine stimulation by the release of pro-
inflammatory cytokines. Although it is possible that this increase does not correlate with a functional increase, as CD18 activation also depends on conformational changes, not just gene expression, the findings do suggest that the reason the infected macrophages appear adhered to the endothelium is because they are actually attached by CD18-dependent mechanisms.

Achieving the second goal has been more difficult, and only partially successful. A method to isolate *C. felis* from tissues has been developed and optimized, and although it seems that a relatively pure sample of *C. felis* parasites can be obtained, at the same time, infecting feline peripheral blood monocytes (fPBMCs) with these isolates has yielded mixed results. This can be due to various reasons, from the inability to obtain a proper number of fPBMCs needed to establish an infection that can produce a detectable level of cytokines, to the possibility that the isolating method causes the death and/or destruction of most of the parasites, and this decreases the amount of viable parasites available for monocyte infection. It is also possible that, since the isolated parasites were intracytoplasmic, they had not yet expressed the genes needed to survive extracellularly and/or infect other monocytes and continue the schizogony phase of the life cycle. Of all of the attempts made at infecting fPBMCs with isolated *C. felis*, only one attempt was successful, at least based on the secretion of detectable numbers of the pro-inflammatory cytokines, IL-1β and TNF-α.

The use and establishment of a feline macrophage-like cell line has also been difficult. First, the reported and published feline macrophage-like cell line failed multiple times to secrete pro-inflammatory cytokines, even at LPS concentrations that caused regular fPBMCs to secrete high levels of the same cytokines. The cell line also did not express CD18, which is the main molecule of interest in our study and one that should be present on all leukocytes, either constitutively or after LPS stimulation. Because of these drawbacks, another feline cell line
needs to be found and/or established. Despite multiple attempts to create a long-lasting feline monocytic culture that would survive multiple attempts at splitting and replating, as well as freezing and thawing, we were not successful. Despite these drawbacks, a successful culture from another feline tumor, later characterized as a synovial sarcoma, was able to be established and partially characterized. Unfortunately, this tumor cell line behaved similarly to FCWF-4, and thus it was not usable for the study of \textit{C. felis in vitro}. Still, this new cell line has not been studied before, and could be useful for research involving oncogenesis and tumor pathology in cats.

One of the main challenges in studying this disease remains the lack of an effective \textit{in vitro} culture system, which would facilitate further immunologic and parasitologic studies. One approach could be to isolate infected macrophages and grow them directly in culture. If \textit{C. felis} behaves similarly to \textit{Theileria}, it would be possible to establish a cell line. Alternatively, establishment of a more stable feline primary cell line could be pursued by inducing replication of the fPBMC or bone marrow hematopoietic precursors with the addition of colony-stimulating factors, as is done in other species. Lastly, it is possible that \textit{C. felis} might infect a commercially available macrophage cell line from a different species, although this is unlikely, given the host-specificity.

One of the main findings of this work is the role of various immune mediators in the pathogenesis of cytauxzoonosis and as the cause of morbidity and death in infected cats. Further investigation into the role of other immune mediators, for example the anti-inflammatory cytokine, IL-10, could provide more insights into the immunopathogenesis of this disease. Information obtained from such a study could then direct the development of alternative treatments that could be used for \textit{C. felis}-infected cats to improve survival rates.
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APPENDIX A
SPIKE AND RECOVERY RESULTS

Spike and recovery experiments were run following the protocols outlined, recommended, and provided by RnD Systems. The results are presented below. For TNF-α and IL-1β, the percentage of recovery for undiluted samples was between the 80-120% range recommended by the manufacturer to indicate confidence in using this kit for unvalidated samples.

For TNF-α undiluted sample:

\[
\% \text{ Recovery} = \left( \frac{\text{Observed} - \text{Neat}}{\text{Expected}} \right) \times 100
\]

\[
\% \text{ Recovery} = \left( \frac{1315.445 - 570.337}{916.372} \right) \times 100
\]

\[
\% \text{ Recovery} = \left( \frac{745.108}{916.372} \right) \times 100
\]

\% \text{ Recovery} = 81.310

Observed = Spiked sample value = 1315.445 pg / mL

Neat = Unspiked sample value = 570.337 pg / mL

Expected = Amount spiked into sample = 916.372 pg / mL

% Recovery is between 80 – 120%, which (per RnD supplied protocol), increases confidence in using for undiluted samples.
For IL-1β undiluted sample:

\[
\% \ Recovery = \frac{791.580 - 280.927}{575.476} \times 100
\]

\[
\% \ Recovery = 88.736
\]

Observed = 791.580 pg / mL

Neat = 280.927 pg / mL

Expected = 575.476 pg / mL

% Recovery is between 80 – 120 %, demonstrating confidence of using the kit for the other undiluted samples.
APPENDIX B

COMPARISON BETWEEN NON-SEPARATED SERUM KEPT OVERNIGHT AT 4°C AND IMMEDIATELY SEPARATED AND STORED SERUM AT -20°C

The white bars represent the cytokine concentrations, as determined by ELISA, of serum that was not separated from the other blood components and kept overnight in 4°C, while the black bars represent serum that was separated immediately and stored in -20°C. No significant differences were observed between the two methods of sample handling.
APPENDIX C

CELL CULTURE MEDIUM

Cell culture medium used for peripheral blood monocytes:

- 20% heat-inactivated fetal bovine serum
- 1% non-essential amino acids
- 1% L-glutamine
- 1% penicillin-streptomycin (100 IU/mL of penicillin, 100 µg/mL of streptomycin)
- <1% nystatin solution (50 µg/mL)

Base medium is Dulbecco’s Modified Eagle’s Medium

This was also the same cell culture medium used for the establishment of the feline cell culture in Chapter 5, with the following modification for the first 5 passages of the cell line:

- 20% heat-inactivated feline serum
APPENDIX D

LPS STIMULATION ON FCWF-4 VS. FPBMC

Mean ± SEM of cell culture supernatant TNF-α, IL-1β, and IL-6 concentrations, as determined via an ELISA, for LPS-stimulated feline peripheral blood mononuclear cells (n = 3; black bars) and FCWF-4 cells (n=66). The supernatants were collected after 24 hours of incubation with a 100 ng/mL of LPS. Note the LLD for IL-1β and IL-6 (31.25 pg/mL [single line]), and the LLD for TNF-α (15.625 pg/mL [dashed line]). *Within a cytokine, the value for this group of cells differs significantly (P < 0.05) from the value for the FCWF-4 cells.
APPENDIX E

CD18 IMMUNOCYTOCHEMICAL EXPRESSION IN FCWF-4 VS FPBM C

Photomicrographs of results of CD18 immunocytochemistry of FCWF-4 cells (A-C) and feline peripheral blood monocytes (D-E). Panel A demonstrates the morphology of FCWF-4. An anti-CD18 antibody was used in panels B and D, whereas an isotype control antibody was used in panels C and E. Wright’s stain (A) 3,3’-diaminobenzidine chromogen with hematoxylin counterstain (B-E); bar = 10 µm
APPENDIX F

PRO-INFLAMMATORY CYTOKINE EXPRESSION IN ONE CASE OF EXPERIMENTAL IN VITRO C. FELIS INFECTION

Mean ± SEM of serum TNF-α, IL-1β, and IL-6 concentrations, as determined via an ELISA, for control, uninfected fPBMCs (n = 6; white bars), *Cytaxzoon felis*-infected fPBMCs (n = 9; grey bars), and LPS-stimulated fPBMCs (n = 3, black bars). Concentrations were obtained from culture supernatant collected 24 hours after infection and incubation at 37°C, 5% CO₂. Note the LLD for IL-6 and IL-1β (31.25 pg/mL [dashed line]). Also note the LLD for TNF-α (15.625 pg/mL [solid line]). a,b Within a cytokine, the value for this group of cats differs significantly (P < 0.05) from the value for the group of control, uninfected cells.