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Ex vivo coccidioidal antigen chitinase-1 stimulation increases production of interleukin-1β in healthy cats

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Objective
To evaluate coccidioidal antigen–stimulated production of cytokines in healthy cats and determine optimum testing conditions (ie, incubation time and antigen concentration).

Methods
6 client-owned, healthy cats that were seronegative for *Coccidioides* spp antibodies were included in this prospective experimental study. Whole blood cultures were performed with exposure to PBS (vehicle control) or a coccidioidal antigen (recombinant coccidioidal antigen chitinase-1 [rCTS1]$_{105–310}$) at 5 final well concentrations (1 µg/mL, 10 µg/mL, 25 µg/mL, 50 µg/mL, and 75 µg/mL) with incubation times of 12 hours and 24 hours. A validated feline-specific, multiplex bead–based assay was used to measure 10 cytokines in cell culture supernatant.

Results
Supernatant concentrations of IL-1β increased when stimulated with rCTS1$_{105–310}$ for 12 hours at 50 µg/mL (274.4 pg/mL; *P* = .02) and 75 µg/mL (298.4 pg/mL; *P* = .003) compared to control (PBS; 102.9 pg/mL). Similarly, IL-1β concentrations increased after exposure to rCTS1$_{105–310}$ for 24 hours at 50 µg/mL (340.3 pg/mL) and 75 µg/mL (364.8 pg/mL) compared to control (110.5 pg/mL; *P* = .04). Production of IL-1β was greater when whole blood was stimulated with rCTS1$_{105–310}$ at 1 µg/mL for 24 hours (237.0 pg/mL) compared with 12 hours (116.8 pg/mL; *P* = .04). No other differences in cytokine concentrations were identified at either incubation time or rCTS1$_{105–310}$ concentration compared to control.

Conclusions
Exposure of whole blood to rCTS1$_{105–310}$ increases production of IL1β in healthy cats. Incubation time and rCTS1$_{105–310}$ concentration did not seem to impact production of most cytokines in this study.

Clinical Relevance
These results provide information on rCTS1$_{105–310}$-stimulated cytokine concentrations in healthy cats.

Keywords: valley fever, cytokine, cytokine signature, coccidioidomycosis, immune

Coccidioidomycosis is an endemic disease in the southwestern US caused by the dimorphic fungi *Coccidioides immitis* and *Coccidioides posadasii.*[1,2] Infection is usually initiated through the inhalation of arthrospores present in the environment. After arthrospores transform into the spherule phase, a spectrum of disease, from subclinical infection to severe respiratory tract infection and/or disseminated disease, can occur.[1,2] Approximately 60% of *Coccidioides* spp infections are subclinical in humans, and approximately 70% are suspected to be subclinical in dogs.[3–5]

Information regarding coccidioidomycosis in cats is sparse compared to that for dogs; however, cats have an increased frequency of disseminated disease (50% to 60%) compared to dogs (20% to 42%).[6–10] Additionally, dermatologic lesions are a common manifestation of disseminated coccidioidomycosis

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in cats (43% to 56%), whereas the most common sites of dissemination in dogs include bones, joints, and lymph nodes.[1,6–9] It is unknown whether the increased frequency of disseminated disease is associated with cats being diagnosed later in the stage of disease or whether there are differences in the host immune response to infection compared to dogs.[6] Like humans and dogs, serologic tests to detect anti–*Coccidioides* spp immunoglobulin (Ig)-M and IgG antibodies remain a focal point in establishing a clinical diagnosis of coccidioidomycosis in cats. Regardless of species, coccidioidal antigen chitinase-1 (CTS1), also referred to as complement fixation antigens, is used to detect IgG reactivity.[11]

Stimulation of early immune response that manifests following exposure to *Coccidioides* spp has shown to be predictive of disease progression in humans.[12–14] A cell-mediated response with a release of T-helper (Th)-1 cytokines, including interferon-γ (IFN-γ) and IL-2, has been associated with disease resolution and a good outcome.[15,16] Neutralization of *Coccidioides* spp endospores relies on the release of the proinflammatory cytokines IFN-γ and tumor necrosis factor-α (TNF-α), and secretion of these cytokines by peripheral blood mononuclear cells has been shown to occur following in vitro whole-blood coccidioidal antigen stimulation.[17–20] More recent studies[14,21–23] in mice substantiate a role of Th17 cells and the release of IL-17 in long-term immunity. Conversely, a Th2 response has been associated with chronic disease.[12,24] While the evaluation of immune response to *Coccidioides* spp has been explored in humans and, to a much lesser extent, in dogs, it has, to the authors' knowledge, not been evaluated in cats. Understanding the early immune response to *Coccidioides* spp in cats may provide insight into the various clinical manifestations and progression of disease in this species.

The focus of the present study was to evaluate the pattern of cytokine expression in whole blood from healthy cats incubated with recombinant CTS1 ($rCTS1_{105–310}$). Coccidioidal antigen chitinase-1 is a chitinase involved in spherule growth and endospore release, is immunogenic, and is used in serodiagnostic testing.[25,26] The objectives of this study were therefore to (1) describe the ex vivo $rCTS1_{105–310}$-stimulated cytokine profile in healthy cats and (2) determine the $rCTS1_{105–310}$ concentration and incubation time that optimized cytokine concentrations. We hypothesized that $rCTS1_{105–310}$ would stimulate Th1-related and proinflammatory cytokine production in the whole blood of healthy cats.

Methods

Animals

Six healthy, adult cats were enrolled in this study. All participants were privately owned cats of employees or students at Midwestern University. Cats lived entirely inside the owners' residence. Inclusion criteria included healthy status based on unremarkable physical examination and review of a CBC, serum biochemistry profile, and, when possible, a urinalysis by a board-certified small-animal internist (JAJ; Antech Diagnostics). Included cats were also required to have negative anti–*Coccidioides* spp antibody (IgM and IgG) titer results measured with agar gel immunodiffusion at the time of enrollment (Antech Diagnostics). Exclusion criteria included prior medical illnesses or current administration of medications outside of standard monthly parasite preventatives within 60 days of enrollment. This study was approved by the IACUC at Midwestern University (IACUC #AZ-4009), and informed consent was obtained from all owners.

Sample collection and processing

Medical records were reviewed for each cat enrolled. The age, sex, weight, and breed were recorded. Blood samples were collected into blood tubes with lithium heparin as an anticoagulant, and study experiments were started within 1 hour of sample collection.

Coccidioidal antigen chitinase-1 preparation

Recombinant CTS1 is a truncated polypeptide encompassing the protein segment that is immunodominant in human serum. Recombinant CTS1 was expressed and purified as previously described.[27] Briefly, the aa105–310 truncation of rCTS1 was cloned into a ligation-independent cloning vector pMCSG7 that contained a T7 promoter and N-terminal 6X His-tag.[28] The plasmid was grown in BL21 Gold (Agilent) cells in Terrific Broth (Millipore) to optical density $(OD)_{600}$ 0.6 and induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (Thermo Fisher). Cells were collected and resuspended in 20 mM Tris (pH 8), 200 mM NaCl, 25 mM imidazole, and 5% glycerol with protease inhibitors and deoxyribonuclease. Cells were sonicated, and the soluble fraction was run on an immobilized nickel-affinity chromatography (HisTrap FF; GE Healthcare). Proteins were further purified by size-exclusion chromatography (Superdex 200; GE HealthCare) using a buffer of 20 mM Tris (pH 8), 200 mM NaCl, and 5% glycerol. Protein was concentrated if necessary, and concentration ion was determined by absorbance at 280 nm using molecular weight and the extinction coefficient. Samples at > 10 µM were aliquoted into thin-walled PCR tubes and flash frozen using liquid nitrogen.

Whole-blood cytokine stimulation and measurement

Heparinized whole blood was diluted 1:2 with RPMI culture medium (Thermo Fisher Scientific) containing 200 U of penicillin/mL and 200 mg of streptomycin/mL. The blood-RPMI mixture from each cat was then transferred to 96-well plates and incubated with $rCTS1_{105–310}$ at 5 final well concentrations (1 µg/mL, 10 µg/mL, 25 µg/mL, 50 µg/mL, and 75 µg/mL) or PBS as a vehicle control. Twelve wells were used per cat to reflect the 6 exposure conditions (ie, $rCTS1_{105–310}$ or PBS) and 2 incubation times. Plates were incubated for 12 hours and 24 hours at 37°C in 5% CO_2 in the dark. Following incubation at both time points, plates were centrifuged (400 X *g* for 7 minutes) at 21°C. The supernatant was collected and stored at −80°C for batch analysis. For analysis, samples were thawed, and then TNF-α, IL-6, IL-12, IL-2, IL-4,

IL-1β, IL-8, granulocyte macrophage CSF (GM-CSF), monocyte chemoattractant protein-1, and IFN-γ were measured with a feline cytokine–specific, multiplex bead–based assay (Milliplex MAP; EMD Millipore Corp) as described elsewhere.[29] The median fluorescence intensity and cytokine concentration in each sample were measured in duplicate with appropriate manufacturer controls and associated data analysis software (Milliplex Analyst, version 5.1; EMD Millipore Corp). The lower limit of detection (LLD) for TNF-α, IL-2, IL-8, IL-12, and GM-CSF was 41.15 pg/mL. The LLD for IFN-γ, IL-4, and IL-6 was 205.76 pg/mL. The LLD for IL-1β and monocyte chemoattractant protein-1 was 102.88 l pg/mL and 1,235 pg/mL, respectively.

Statistical analysis

Statistical analyses were performed using statistical software (R, version 4.1; R Foundation for Statistical Computing). Descriptive continuous data, including age and weight, were normally distributed as determined by Shapiro-Wilk tests and presented as mean and SD. Descriptive data from the CBC were non-normally distributed and presented as median and IQR. When the measured cytokine concentration fell below the LLD, data were recorded at the LLD for statistical purposes. The statistical analysis used to investigate the effect of $rCTS1_{105–310}$ concentration on cytokine by time (an analysis for 12 hours and 24 hours) was the Kruskal-Wallis test. For any statistically significant results between groups overall, the Dunn test for multiple comparisons with a *P* value adjustment using the Bonferroni method was conducted. The analysis for the effect of time on cytokine concentration by $rCTS1_{105–310}$ concentration (an analysis for each $rCTS1_{105–310}$ concentration of 1, 10, 25, 50, and 75 µg/mL + control) was the Wilcoxon signed-rank test with Bonferroni correction. The significance level was set at $\alpha < 0.05$.

Results

Animals

Six healthy, adult domestic long- or shorthair cats (4 neutered males, 2 spayed females) ranging from 1 to 8 years of age (mean ± SD age, 3.3 ± 2.4 years) and weighing 4.4 to 8.8 kg (mean ± SD weight, 5.8 ± 1.8 kg) were enrolled in this study. Descriptive data from the CBC were WBCs (median, 8.8 X 10^3/µL; IQR, 7.0 to 12.7), neutrophils (median, 4,588/µL; IQR, 3,286.3 to 5,558.0), monocytes (median, 193.5/µL; IQR, 138.0 to 335.0), lymphocytes (median, 3,624.0/µL; IQR, 3,021.0 to 6,284.0), and eosinophils (median, 390/µL; IQR, 116.3 to 678.8).

Whole-blood cytokine stimulation and measurement

Concentrations of GM-CSF and IFN-γ were below the LLD in all samples at all timepoints, including the unstimulated controls and all concentrations of $rCTS1_{105–310}$ at both the 12-hour and 24-hour incubation periods. Therefore, these 2 analytes were excluded from subsequent analyses.

For the analysis of $rCTS1_{105–310}$ concentration by time on cytokine concentration, Kruskal-Wallis tests revealed a significant difference for IL-1β at both the 12-hour (*P* = .003) and 24-hour (*P* = .02) incubation times compared with control. Post hoc comparisons using the Dunn test with Bonferroni adjustment showed that the incubation of whole blood with $rCTS1_{105–310}$ for 12 hours for IL-1β at 50 µg/mL (mean, 274.4 pg/mL; SD, 110.2; *P* = .02) and 75 µg/mL (mean, 298.4 pg/mL; SD, 110.7; *P* = .003) resulted in increased supernatant concentrations of IL-1β compared to control (mean, 102.9 pg/mL; SD, 0.0; **Table 1**). Similarly, results from the Dunn tests revealed that exposure with $rCTS1_{105–310}$ for 24 hours for IL-1β at 50 µg/mL (mean, 340.3 pg/mL;

Table 1—Supernatant cytokine concentrations (picograms per milliliter) in 6 healthy cats following whole-blood incubation for 12 hours with various concentrations of recombinant coccidioidal antigen chitinase-1 $(rCTS1)_{105–310}$ antigen or PBS control.

	1 µg/mL (n = 6)	10 µg/mL (n = 6)	25 µg/mL (n = 6)	50 µg/mL (n = 6)	75 µg/mL (n = 6)	PBS control (n = 6)	*P* value
IL-1β							.003
Mean (SD)	116.847 (14.770)	213.250 (93.156)	252.915 (114.996)	274.352 (110.167)	298.442 (110.706)	102.880 (0.000)	
Range	102.880–137.080	102.880–325.550	102.880–422.970	102.880–395.220	121.010–441.020	102.880 – 102.880	
IL-2							1.00
Mean (SD)	49.492 (20.433)	45.428 (10.480)	47.822 (16.342)	46.958 (14.227)	44.525 (8.267)	47.107 (14.591)	
Range	41.150–91.200	41.150–66.820	41.150–81.180	41.150–76.000	41.150–61.400	41.150–76.890	
IL-12							.83
Mean (SD)	132.490 (59.299)	129.395 (55.122)	126.250 (50.343)	138.413 (58.382)	133.603 (59.718)	118.260 (57.096)	
Range	41.150–226.780	44.340–216.380	41.630–196.080	41.150–221.410	44.170–230.430	41.150–216.540	
IL-4							1.00
Mean (SD)	379.423 (378.187)	289.358 (180.307)	322.325 (249.463)	349.815 (300.416)	311.955 (204.564)	296.843 (213.658)	
Range	205.760–1,147.000	205.760–655.810	205.760–829.200	205.760–959.540	205.760–722.870	205.760–732.860	
IL-6							.99
Mean (SD)	268.568 (153.648)	244.838 (68.586)	251.913 (102.294)	257.193 (101.986)	238.866 (67.295)	239.005 (81.433)	
Range	205.760–582.200	205.760–373.960	205.760–459.890	205.760–460.710	205.760–373.966	205.760–405.230	
IL-8							.40
Mean (SD)	923.180 (544.502)	1,086.988 (785.533)	996.940 (641.445)	1,005.037 (641.713)	1,084.312 (710.446)	442.323 (637.907)	
Range	408.270–1,734.000	467.690–2,449.000	434.030–2,095.000	418.680–2,043.000	498.790–2,169.000	41.150–1,647.000	
MCP-1							.97
Mean (SD)	2,531.167 (1,301.730)	2,324.500 (899.517)	2,429.667 (1,096.709)	2,577.833 (1,138.479)	2,332.167 (731.251)	2,277.333 (1,053.958)	
Range	1,235.000–4,969.000	1,235.000–3,906.000	1,235.000–4,443.000	1,235.000–4,672.000	1,235.000–3,402.000	1,235.000–4,237.000	
TNF-α							.35
Mean (SD)	186.772 (150.228)	201.650 (178.887)	198.215 (133.989)	244.010 (173.882)	234.782 (155.734)	107.218 (76.532)	
Range	43.940–433.120	48.140–538.450	49.400–409.810	63.200–516.950	65.700–518.610	41.150–246.690	

Data were analyzed using Kruskal-Wallis tests.
MCP = Monocyte chemoattractant protein. TNF = Tumor necrosis factor.

SD, 129.7; *P* = .02) and 75 µg/mL (mean, 364.8 pg/mL; SD, 151.9; *P* = .01) increased IL-1β concentrations compared with control (mean, 110.5 pg/mL; SD, 18.7; **Table 2**). There were no other significant differences in cytokine concentrations for any of the other $rCTS1_{105–310}$ concentrations at either the 12-hour or 24-hour incubation times (all *P* > .05).

Wilcoxon signed-rank tests were performed to investigate the effect of time on cytokine concentration by $rCTS1_{105–310}$ concentration and revealed that for the 1-µg/mL $rCTS1_{105–310}$ concentration, IL-1β concentrations were greater after incubation for 24 hours (mean, 237.0 pg/mL; SD, 117.4) than 12 hours (mean, 116.8 pg/mL; SD, 14.8; *P* = .04; **Table 3**). There were no other significant differences in cytokine concentrations for any other $rCTS1_{105–310}$ concentration between 12 hours and 24 hours (all *P* > .05; **Supplementary Tables S1–S5**). Regardless of incubation time, the exposure of cells to PBS resulted in IL-1β concentrations below the LLD in most cats.

Table 2—Supernatant cytokine concentrations (picograms per milliliter) in 6 healthy cats following whole-blood incubation for 24 hours with various concentrations of $rCTS1_{105–310}$ antigen or PBS control.

	1 µg/mL (n = 6)	10 µg/mL (n = 6)	25 µg/mL (n = 6)	50 µg/mL (n = 6)	75 µg/mL (n = 6)	PBS control (n = 6)	*P* value
IL-1β							.02
Mean (SD)	237.035 (117.427)	332.020 (177.406)	319.135 (152.939)	340.307 (129.719)	364.805 (151.901)	110.500 (18.665)	
Range	102.880–435.370	102.880–538.420	102.880–507.020	132.740–466.590	173.250–585.350	102.880–148.600	
IL-2							1.00
Mean (SD)	47.775 (16.228)	49.145 (19.584)	49.030 (19.302)	47.107 (14.591)	44.550 (8.328)	51.313 (24.895)	
Range	41.150–80.900	41.150–89.120	41.150–88.430	41.150–76.890	41.150–61.550	41.150–102.130	
IL-12							.93
Mean (SD)	139.510 (60.774)	149.613 (67.362)	138.307 (63.016)	141.717 (55.966)	140.813 (55.945)	119.398 (44.697)	
Range	42.700–233.000	41.150–242.300	41.150–239.640	48.590–222.400	53.070–223.400	41.150–165.810	
IL-4							1.00
Mean (SD)	326.487 (234.693)	373.575 (322.174)	361.263 (303.530)	328.442 (231.857)	298.950 (168.694)	352.292 (299.073)	
Range	205.760–800.280	205.760–1,024.000	205.760–974.920	205.760–795.070	205.760–635.680	205.760–959.420	
IL-6							.36
Mean (SD)	315.530 (96.031)	322.163 (127.382)	277.592 (122.626)	258.953 (92.035)	237.458 (52.479)	269.592 (135.541)	
Range	205.760–454.190	205.760–545.810	205.760–515.480	205.760–432.930	205.760–330.120	205.760–543.820	
IL-8							.69
Mean (SD)	1,606.440 (1,068.187)	1,554.570 (1,223.085)	1,704.215 (1,286.326)	1,681.827 (1,188.246)	1,661.225 (1,126.172)	913.337 (1,027.100)	
Range	539.430–3,233.000	487.850–3,725.000	488.650–3,338.000	628.040–3,453.000	617.020–3,488.000	90.600–2,773.000	
MCP-1							.98
Mean (SD)	2,402.500 (1,056.953)	2,519.333 (909.334)	2,484.000 (862.447)	2,546.500 (1,034.421)	2,446.167 (852.354)	2,438.833 (1,237.086)	
Range	1,235.000–4,309.000	1,235.000–4,002.000	1,235.000–3,886.000	1,235.000–4,384.000	1,235.000–3,866.000	1,235.000–4,760.000	
TNF-α							.90
Mean (SD)	140.848 (84.060)	170.932 (139.397)	157.148 (112.497)	153.375 (105.793)	144.680 (94.065)	112.988 (106.986)	
Range	43.940–261.370	52.330–379.780	53.170–307.640	59.030–303.300	61.540–303.950	41.150–322.920	

Data were analyzed using Kruskal-Wallis tests.

Table 3—Supernatant cytokine concentrations (picograms per milliliter) in 6 healthy cats following whole-blood incubation of $rCTS1_{105–310}$ antigen at 1 µg/mL for 12 hours versus 24 hours.

	12 hours (n = 6)	24 hours (n = 6)	*P* value
IL-1β			
Mean (SD)	116.847 (14.770)	237.035 (117.427)	.04
Range	102.880–137.080	102.880–435.370	
IL-2			
Mean (SD)	49.492 (20.433)	47.775 (16.228)	1.00
Range	41.150–91.200	41.150–80.900	
IL-12			
Mean (SD)	132.490 (59.299)	139.510 (60.774)	.48
Range	41.150–226.780	42.700–233.000	
IL-4			
Mean (SD)	379.423 (378.187)	326.487 (234.693)	.93
Range	205.760–1,147.000	205.760–800.280	
IL-6			
Mean (SD)	268.568 (153.648)	315.530 (96.031)	.16
Range	205.760–582.200	205.760–454.190	
IL-8			
Mean (SD)	923.180 (544.502)	1,606.440 (1,068.187)	.24
Range	408.270–1,734.000	539.430–3,233.000	
MCP-1			
Mean (SD)	2,531.167 (1,301.730)	2,402.500 (1,056.953)	1.00
Range	1,235.000–4,969.000	1,235.000–4,309.000	
TNF-α			
Mean (SD)	186.772 (150.228)	140.848 (84.060)	.52
Range	43.940–433.120	43.940–261.370	

Data were analyzed using Wilcoxon signed rank tests.

Only 1 cat had measurable IL-1β (148.6 pg/mL) when cells were incubated with PBS.

Discussion

Whole-blood stimulation with immunogenic antigens is a straightforward and rapid method to evaluate the response to various stimuli, which can aid in characterizing the associated innate and adaptive immune responses.[30–34] Stimulation with pathogen-associated molecular patterns and subsequent evaluation of cytokines and other inflammatory markers has been performed previously in cats to characterize the cytokine signature.[29,30] These studies have identified cytokine signatures in response to whole-blood stimulation with bacterial pathogen-associated molecular patterns, including lipopolysaccharide, lipoteichoic acid, and peptidoglycan; however, to the authors' knowledge, this represents the first report of whole-blood stimulation with a single recombinant coccidioidal antigen in cats.[29,30]

Incubation of whole blood with various concentrations of $rCTS1_{105–310}$ for 12 and 24 hours was performed to determine the minimum concentration and incubation time to stimulate cytokine production. A significant difference in cytokine stimulation between the 12-hour and 24-hour incubation periods was not observed, except for IL-1β, where a significantly increased concentration was noted following the 24-hour incubation only when stimulated with $rCTS1_{105–310}$ at a concentration of 1 µg/mL. These findings suggest that while a 12-hour incubation period may be sufficient, 24 hours may be ideal. The concentration of $rCTS1_{105–310}$ used for antigenic stimulation was only significant for IL-1β production. Specifically, $rCTS1_{105–310}$ concentrations of 50 µg/mL and 75 µg/mL for 12 hours increased IL-1β production compared to control. Similarly, an $rCTS1_{105–310}$ concentration of 75 µg/mL for 24 hours also increased supernatant concentrations of IL-1β. Taken together, our results suggest that the exposure of cells to $rCTS1_{105–310}$ at 75 µg/mL for 24 hours is likely optimal for the production of IL-1β in cats. The small sample population of this study was an important limitation that may have impeded the identification of true significant differences (a type 2 error). As with most exploratory studies, obtaining estimates of SD to plan future studies in this area of research was still valuable despite very likely being underpowered.

Changes in ex vivo coccidioidal antigen–stimulated cytokine concentrations in human patients and dogs with and without coccidioidomycosis are variable; however, an increase in TNF-α, GM-CSF, and IFN-γ seem to be consistently increased in both humans and dogs. In a study conducted by Ampel et al,[20] whole-blood stimulation with the coccidioidal antigen T27K was performed on blood samples from humans with a recent diagnosis of coccidioidomycosis and 2 control groups of patients without a clinical diagnosis of coccidioidomycosis, including an immune and nonimmune control group. Increased concentrations of GM-CSF, IL-1β, IL-2, IFN-γ, IL-13, and TNF-α were observed following whole-blood stimulation in patients recently diagnosed with pulmonary coccidioidomycosis and immune control subjects when compared to healthy nonimmune subjects.[20] The nonimmune human subjects failed to exhibit a significant increase in proinflammatory cytokine concentrations. A recent study conducted by Jaffey et al[34] in dogs evaluated ex vivo changes in cytokine concentrations following whole-blood stimulation with $rCTS1_{105–310}$ in cases newly diagnosed with coccidioidomycosis and healthy controls. Increased supernatant concentrations of TNF-α, IL-6, GM-CSF, IL-8, and IL-10 were noted in control dogs and those with coccidioidomycosis.

Our study had several limitations that must be considered. As mentioned earlier, the small sample population with experiments under various different conditions led to limited observations and likely being underpowered to detect differences in all scenarios. Data obtained from this study will be instrumental in sample size calculation and study design in future studies investigating $rCTS1_{105–310}$ as a coccidioidal antigen in cats. There remains a void in definitively differentiating between immune and nonimmune in client-owned domestic pets (ie, cats and dogs), which was a limitation of our study and the recently published Jaffey et al[34] study that focused on dogs. Control pets in both studies were healthy and seronegative for *Coccidioides* spp antibodies, but this does not exclude the possibility of a previous subclinical infection. Utilization of healthy cats from a nonendemic area or specific pathogen-free cats would provide more clarity on whether immune status for *Coccidioides* spp affects ex vivo cytokine production following antigen stimulation.

The inclusion of only healthy cats was purposefully strict in this exploratory study as to avoid the inclusion of confounding variables that may have affected cytokine production, such as comorbid disorders or medication administration. Whole-blood cultures, rather than isolated immune cell types, were used to investigate cytokine production because this microenvironment allowed for cellular reactions to occur in a more physiologic milieu, possibly improving the clinical relevance of these ex vivo results. Many of the cytokines evaluated in our study were below the LLD regardless of whether samples were stimulated or not. This may be a true representation of cytokine production in cats with $rCTS1_{105–310}$ stimulation or a potential limitation of the feline-specific, magnetic bead–based multiplex immunoassay.[29] Most cytokines were below the LLD in a similarly designed ex vivo study in healthy cats using other antigenic stimulants (ie, lipopolysaccharide, lipoteichoic acid, peptidoglycan) and the same multiplex immunoassay.[29] Future studies that focus on evaluating the cytokine immune profile in coccidioidal antigen–stimulated immune cells from cats may consider using PCR to detect cytokine mRNA transcripts or flow cytometry for the identification of intracellular cytokine concentration. Recombinant $CTS1_{105–310}$ is a truncated polypeptide similar in structure to chitinase-1, a protein utilized by *Coccidioides* spp organisms to weaken the spherule cell wall before endospore release. Coccidioidal antigen chitinase-1

antigen has long been integral in the serodiagnostics of coccidioidomycosis in various species; however, the magnitude of its immunogenicity and specific signaling pathways remain unknown. Therefore, it is unclear whether the cytokine signature identified in this study was the result of a yet-to-be-determined signaling pathway or nonspecific antigenic stimulation. Additional research is needed to further classify this response. Lastly, the antigen used for stimulation experiments was a single recombinant polypeptide with no carbohydrate (sugar) moieties. Cytokine results may have been more similar to mouse and human patient studies if a more complex antigen were used to stimulate the cells from cats. Undoubtedly, additional work is needed to optimize this assay in domestic pets, including the antigens used, incubation time, and cytokines measured. The overarching goal with data from these future studies will be to better understand the immune response to *Coccidioides* spp in cats that may fill gaps in our comprehension of circumstances that surround infection susceptibility, severity, and progression to disseminated disease.

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Disclosures

The authors have nothing to disclose. No AI-assisted technologies were used in the composition of this manuscript.

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Supplementary Materials

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