



Ex Vivo Cytokine Release, Determined by a Multiplex Cytokine Assay, in Response to Coccidioidal Antigen Stimulation of Whole Blood among Subjects with Recently Diagnosed Primary Pulmonary Coccidioidomycosis

Neil M. Ampel,^a Ian Robey,^a Chinh T. Nguyen,^{a,b} Brentin Roller,^{a,b} Jessica August,^b Kenneth S. Knox,^c Demosthenes Pappagianis^d

^aSouthern Arizona Veterans Affairs Health Care System, Tucson, Arizona, USA

^bDivision of Infectious Diseases, University of Arizona College of Medicine, Tucson, Arizona, USA

^cDivision of Pulmonary Medicine, University of Arizona College of Medicine, Tucson, Arizona, USA

^dDepartment of Microbiology and Immunology, University of California at Davis, Davis, California, USA

ABSTRACT The elements of the cellular immune response in human coccidioidomycosis remain undefined. We examined the *ex vivo* release of an array of inflammatory proteins in response to incubation with a coccidioidal antigen preparation to ascertain which of these might be associated with diagnosis and outcome. Patients with a recent diagnosis of primary pulmonary coccidioidomycosis and a control group of healthy subjects were studied. Blood samples were incubated for 18 h with T27K, a soluble coccidioidal preparation containing multiple glycosylated antigens, and the supernatant was assayed for inflammatory proteins using the multiplex Luminex system. The presentation and course of illness were compared to the levels of the inflammatory proteins. Among the 31 subjects studied, the median time from diagnosis to assay was 15 days. Of the 30 inflammatory proteins measured, the levels of only 7 proteins, granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-1 receptor alpha (IL-1RA), interleukin-1 β (IL-1 β), interferon gamma (IFN- γ), IL-2, IL-13, and tumor necrosis factor alpha (TNF- α), were more than 10-fold above the levels seen without antigen stimulation. The levels of IFN- γ and IL-2 were significantly elevated in those subjects not receiving triazole antifungal therapy compared to those who were receiving triazole antifungal therapy. While the levels of IL-1RA were nonspecifically elevated, elevated levels of IL-13 were seen only in those with active pulmonary coccidioidomycosis. Only six cytokines were specifically increased in subjects with recently diagnosed primary pulmonary coccidioidomycosis. While IFN- γ , IL-2, and TNF- α have been previously noted, the finding of elevated levels of the innate cytokines GM-CSF and IL-1 β could suggest that these, as well as IL-13, are early and specific markers for pulmonary coccidioidomycosis.

IMPORTANCE Coccidioidomycosis, commonly known as Valley fever, is a common pneumonia in the southwestern United States. In this paper, we examined the release of 30 inflammatory proteins in whole-blood samples obtained from persons with coccidioidal pneumonia after the blood samples were incubated with a preparation made from the causative fungus, *Coccidioides*. We found that six of these proteins, all cytokines, were specifically released in high concentrations in these patients. Three of the cytokines were seen very early in disease, and an assay for all six might serve as a marker for the early diagnosis of Valley fever.

KEYWORDS cellular immunity, coccidioidomycosis, cytokines

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Address correspondence to Neil M. Ampel, nampel@email.arizona.edu.

Coccidioidomycosis is a fungal disease that is endemic to large portions of the southwestern United States as well as northern Mexico and portions of Central and South America (1). Cellular immunity has long been recognized to play a critical role in protection against coccidioidal infection (2). While measurement of cellular immunity in coccidioidomycosis was pioneered by Smith et al. using the skin test (3), *ex vivo* release of cytokines by peripheral blood mononuclear cells (4) or even whole blood (5) after coccidioidal antigen incubation also appears to predict the development of an appropriate cellular immune response and allows a more detailed assessment of the immunological response than the skin test.

We have previously shown that the coccidioidal antigen preparation T27K induces a specific *ex vivo* cellular immune response among humans with coccidioidomycosis that correlates with delayed type hypersensitivity to coccidioidin skin testing (6). These studies have focused principally on the T-helper type 1 cytokines interferon gamma (IFN- γ), interleukin-2 (IL-2), and tumor necrosis factor alpha (TNF- α). In the present study, we have broadened our approach and attempted to determine what other cytokines, chemokines, and growth factors are released *ex vivo* in response to incubation of whole-blood samples with T27K, studying subjects with recently diagnosed primary pulmonary coccidioidomycosis. In addition, we have tried to ascertain which of these correlate with initial clinical expression of illness and which correlate with the eventual outcome. We used a magnetic multiplex system that measures a total of 30 inflammatory proteins.

(This work was presented in part at the 7th International Coccidioidomycosis Symposium held 10 to 13 August 2017 at Stanford University.)

RESULTS

Description of the cohort. A total of 31 subjects were enrolled in the study. Their characteristics are displayed in Table 1. The median age was 64 years, and 29 of the subjects were male. Twenty-one were white, non-Hispanic. There was a median of 15 days from the time of diagnosis to the time of assay, and all but two subjects were tested within 1 month of diagnosis. Two patients were tested beyond this point; one at 43 days and the other at 98 days. In both cases, this occurred because of a delay in referring the patients to the coccidioidomycosis clinic. An underlying disease was present in 18 subjects, with diabetes being the most common. Twenty-six subjects had pulmonary coccidioidomycosis, followed by five who presented with a positive serology only. Among those with pulmonary disease, 13 had primary pneumonia, and 13 had sequelae from this, with seven subjects presenting with a pulmonary nodule and six with a pulmonary cavity. The complement fixation (CF) titer was positive in 24 subjects. Among those with a positive CF, the median titer was 1:4 with a range from <1:2 to 1:64. Five subjects were on the antifungal fluconazole at the time of the assay.

Overall cytokine results. Table 2 displays the 30 inflammatory proteins released in response to T27K antigen stimulation based on their mean fold level above the unstimulated control level. As can be seen, 19 proteins were <5-fold above the control level, 4 were 5- to 10-fold above the control level, and 7 were >10-fold above the control level. The latter were all cytokines: granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-2 receptor alpha (IL-2RA), interleukin-1 β (IL-1 β), interferon gamma (IFN- γ), IL-2, IL-13, and tumor necrosis factor alpha (TNF- α). The levels of IL-2 were more than 100-fold above the control level.

Among the 31 subjects, there were no differences between released concentrations of GM-CSF, IL-1RA, IL-1 β , IFN- γ , IL-2, and IL-13 whether the patient had a detectable anticoccidioidal complement fixation (CF) antibody titer or not ($P > 0.05$ for all; data not shown). The median concentrations of TNF- α released were 3,342 pg/ml in those with positive CF titers compared to 205 pg/ml in those with a negative CF titer ($P = 0.016$).

In this same group, when the 26 subjects who were not on antifungal therapy were compared to the five subjects receiving such therapy, there were no differences in the released concentrations of GM-CSF, IL-1RA, IL-1 β , or TNF- α ($P > 0.05$ for all; data not

TABLE 1 Description of the 31 subjects in the study

Demographic or clinical characteristic	No. of patients with the characteristic or value(s) for the characteristic
Age (yr), median (range)	64 (27–80)
Sex	
Male	29
Female	2
Race	
White, non-Hispanic	23
African-American	4
White, Hispanic	3
Asian	1
Time of assay from diagnosis (days), median (range)	15 (6–98)
Underlying disease	
Diabetes	11
Chronic lung disease	8
Cancer	6
Heart disease	4
Rheumatologic disease	2
Immunosuppressive therapy	2
Type of coccidioidomycosis	
Serologically positive only	5
Pulmonary nodule or cavity	13
Acute pneumonia	13
Complement-fixing serology titer, median (range)	1:4 (negative to 1:64)
Antifungal therapy	
Yes	5
No	26

shown). The median concentrations of IFN- γ and IL-2 were significantly higher in those not on antifungal therapy, 550 and 889 pg/ml, respectively, compared to 5 and 20 pg/ml, respectively, in those on antifungal therapy ($P = 0.014$ and 0.016 , respectively). While it did not reach statistical significance, median concentrations of IL-13

TABLE 2 Cytokines released in response to stimulation with the coccidioidal antigen preparation T27K based on their average fold level above the unstimulated control samples

Cytokine showing the following level above the control level ^a :		
<5-fold ($n = 19$)	5- to 10-fold ($n = 4$)	≥ 10 -fold ($n = 7$) ^b
EGF	IL-5	GM-CSF
Eotaxin	MIG	IL-1RA
FGF	MIP-1 α	IL-1 β
IFN- α	VEGF	IFN- γ
G-CSF		IL-2
HGF		IL-13
IL-2R		TNF- α
IL-4		
IL-6		
IL-7		
IL-8		
IL-10		
IL-12		
IL-15		
IL-17		
IP-10		
MCP-1		
MIP-1 β		

^a n shows the number of subjects in each group.

^bThe cytokine in boldface type was released >100 -fold.

TABLE 3 Comparison of *ex vivo* cytokine release from blood samples incubated with T27K from subjects with active coccidioidomycosis compared to results from 15 control subjects

Cytokine	Median cytokine concn (pg/ml) (range) in samples from:			P value ^a
	Subjects with coccidioidomycosis (n = 31)	Immune controls (n = 6)	Nonimmune controls (n = 9)	
GM-CSF	204 (0–1,744)	44 (5–221)	0 (0–59)	<0.001
IL-1RA	2,461 (0–692,208)	4,638 (1,551–36,004)	1,977 (0–5,441)	0.114
IL-1 β	309 (0–3,998)	36 (4–2,255)	4 (0–309)	0.011
IFN- γ	381 (0–1,854)	155 (90–455)	0 (0–12)	<0.001
IL-2	741 (1–2,058)	202 (137–310)	0 (0–4)	<0.001
IL-13	44 ^b (0–854)	0 (0–11)	0 (0–1)	<0.001
TNF- α	2,584 (1–29,374)	543 (23–11,784)	8 (0–4,198)	0.046

^aThe P values were determined using the nonparametric Kruskal-Wallis test comparing the three groups. The P values in boldface type indicate statistical significance ($P \leq 0.05$).

^b $P = 0.0032$ comparing subjects to immune controls only.

were 44 pg/ml in those on no therapy compared to 0 pg/ml for those on antifungal therapy ($P = 0.0959$).

Comparing subjects with active coccidioidomycosis to controls. Focusing on the seven cytokines whose *ex vivo* concentrations after T27K stimulation were >10-fold above those of unstimulated controls, Table 3 compares the results from subjects to results from 15 healthy controls without a clinical diagnosis of coccidioidomycosis. The controls were divided into two groups that included six immune controls, based on the *ex vivo* release of their whole blood of ≥ 100 pg/ml IL-2 above the unstimulated control sample in response to T27K, and nine nonimmune controls, whose blood samples released less than that in response to incubation with T27K. As can be seen, six of the seven cytokines were specifically and significantly increased in both the subjects with active coccidioidomycosis and the immune controls compared to the nonimmune samples. *Ex vivo* release of IL-1RA appeared to occur in all subjects, suggesting a nonspecific response and therefore was not subsequently analyzed. In addition, although there was a trend for higher concentrations of GM-CSF and IL-1 β in subjects with active coccidioidomycosis compared to immune controls, only the concentrations of IL-13 were significantly higher ($P = 0.0032$).

Comparing acute pulmonary disease with other forms of active coccidioidomycosis. Table 4 compares the 13 subjects with active coccidioidomycosis presenting with acute pneumonia to the 18 subjects presenting either with a pulmonary nodule or cavity or with positive serology only. This categorization was made because it is likely that the latter group represents subjects with a longer period of infection prior to diagnosis. There were no differences in age, time from diagnosis to assay, or coccidioid CF titer between the subjects with acute pneumonia and those with pulmonary nodules or cavities or positive serology only (data not shown). However, those with nodules, cavities, or who were seropositive only were more likely to be white, non-

TABLE 4 Comparison of *ex vivo* cytokine release from samples incubated with T27K from 13 subjects with acute pulmonary coccidioidomycosis to those from 18 subjects with either pulmonary nodules or cavities or with a positive serology only

Cytokine	Median cytokine concn (pg/ml) (range) in subjects with:		P value ^a
	Acute pneumonia (n = 13)	Nodule, cavity, or serology only (n = 18)	
GM-CSF	324 (5–1,745)	132 (0–1,478)	0.045
IL-1 β	610 (6–3,998)	114 (0–3,067)	0.037
IFN- γ	514 (19–1,845)	338 (0–1,729)	0.215
IL-2	999 (14–2,013)	679 (1–2,058)	0.379
IL-13	76 (2–854)	23 (0–301)	0.109
TNF- α	6,615 (27–29,374)	874 (1–14,206)	0.078

^aThe P values in boldface type indicate statistical significance.

TABLE 5 Relation of *ex vivo* cytokine concentrations to follow-up of subjects

Result or parameter at follow-up	<i>Ex vivo</i> cytokine concn (pg/ml) (range) or the <i>P</i> value ^a					
	GM-CSF	IL-1 β	IFN- γ	IL-2	IL-13	TNF- α
Outcome						
Improved (<i>n</i> = 18)	244 (0–1,744)	465 (0–3,998)	303 (0–1,854)	562 (1–2,013)	42 (0–854)	6,689 (1–29,374)
Not improved (<i>n</i> = 8)	182 (3–1,478)	385 (3–3,067)	859 (12–1,847)	771 (11–2,058)	48 (3–122)	465 (3–6,692)
<i>P</i> value	<i>0.868</i>	<i>1.00</i>	<i>0.317</i>	<i>0.657</i>	<i>0.781</i>	<i>0.085</i>
IDCF titer						
Positive (<i>n</i> = 12)	360 (5–1,478)	364 (16–3,998)	560 (23–1,847)	679 (28–2,058)	60 (5–301)	6,662 (57–29,374)
Negative (<i>n</i> = 14)	170 (0–1,744)	470 (0–2,084)	211 (0–1,854)	571 (1–2,013)	26 (0–854)	809 (1–14,206)
<i>P</i> value	<i>0.304</i>	<i>0.441</i>	<i>0.136</i>	<i>0.472</i>	<i>0.382</i>	<i>0.165</i>
Chest imaging						
Improved (<i>n</i> = 15)	324 (5–1,744)	455 (0–3,453)	311 (12–1,854)	1,072 (14–2,058)	52 (2–854)	3,785 (57–24,253)
Not improved (<i>n</i> = 10)	126 (0–1,478)	369 (1–3,998)	448 (0–1,180)	499 (1–2,013)	36 (0–104)	905 (1–29,374)
<i>P</i> value	<i>0.120</i>	<i>0.912</i>	<i>0.471</i>	<i>0.183</i>	<i>0.222</i>	<i>0.346</i>
Antifungal therapy						
Yes (<i>n</i> = 12)	232 (0–1,478)	456 (1–3,998)	211 (0–1,687)	166 (1–1,913)	36 (0–158)	2,206 (1–29,374)
No (<i>n</i> = 14)	225 (3–1,744)	435 (0–1,697)	595 (12–1,854)	1,053 (11–2,058)	67 (2–854)	5,200 (3–8,908)
<i>P</i> value	<i>0.797</i>	<i>0.440</i>	<i>0.181</i>	<i>0.031</i>	<i>0.304</i>	<i>0.877</i>

^aThe *P* values are shown in italic type in the table. The italic boldface *P* value indicates statistical significance.

Hispanic (*P* = 0.018) and to have an underlying disease (*P* = 0.009) compared to the acute pneumonia group. All concentrations of *ex vivo*-released cytokines were higher in those with primary pneumonia compared to the nodule, cavity, and serology group. Notably, GM-CSF and IL-1 β were significantly higher with a trend toward significance for TNF- α .

Outcome of coccidioidomycosis related to cytokine results. Of the 31 subjects, 26 had a follow-up of more than 30 days. Median follow-up for these subjects was 313 days with a minimum of 36 days and a maximum of 806 days. We examined the outcomes based on the last anticoccidioidal CF titer, whether the patient was treated with antifungal therapy, whether their chest radiographs had improved, and whether they were judged to be clinically improved at their last follow-up. These outcomes were then compared to the *ex vivo* release concentration of the six cytokines upon entry into the study. These data are displayed in Table 5. The only significant finding was that patients who did not require subsequent antifungal therapy had blood samples that expressed significantly higher levels of IL-2.

DISCUSSION

Six inflammatory proteins, GM-CSF, IL-1 β , IL-2, IFN- γ , IL-13, and TNF- α , were found to be specifically released *ex vivo* when whole-blood samples from subjects with recently diagnosed primary pulmonary coccidioidomycosis were incubated with the coccidioidal antigen preparation T27K. While we have previously shown that the T-helper type 1 cytokines IL-2, IFN- γ , and TNF- α are released *ex vivo* when exposed to coccidioidal antigen (5, 7, 8), the findings for the other cytokines are new and unexpected. We did not find evidence for increased release of IL-6 or IL-17A, as we have previously observed (7). However, that earlier work did not examine *ex vivo* cytokine responses early in diagnosis of pulmonary coccidioidomycosis, as was done here. Moreover, a different assay for cytokines was used in these two studies, and these studies may not be strictly comparable (9).

Given that the levels of released GM-CSF and IL-1 β were higher in those with acute pneumonia compared to those with pulmonary nodules or cavities or positive serology only on presentation, these two cytokines might serve as sentinel markers for early coccidioidomycosis. GM-CSF is a multipotential cytokine that has been shown to be critical for T cell immunity (10) and appears to play an important part in fungal immunity. In murine models, GM-CSF was protective during early infection with *Histoplasma capsulatum* (11) and infection with *Cryptococcus neoformans* (12) and may play

a role in human *Cryptococcus gattii* infection (13). Similarly, it might be expected that IL-1 β could be part of an early and innate response in coccidioidal immunity given its pivotal position as an early mediator of inflammation (14). With regard to fungal infection, IL-1 β has been shown to have a place in a protective murine vaccine against *Coccidioides posadasii* (15) as well as in the response to infection with *Paracoccidioides brasiliensis* (16) and *Candida albicans* (17).

While all released cytokine concentrations were higher in those with active coccidioidomycosis compared to those with evidence of immunity but without disease activity, only IL-13 was significantly higher in the active disease group. IL-13 may serve to distinguish new disease from established infection. IL-13 has been implicated in allergic airway inflammation and eosinophilia (18), the latter being a frequently observed phenomenon during primary pulmonary coccidioidomycosis (19). Additionally, IL-13, in concert with IL-33, has been associated with increased resistance to murine infection with *Candida albicans* (20).

We found few associations between the *ex vivo* release of the six inflammatory cytokines and clinical disease or outcome among the donors with coccidioidomycosis. Higher levels of IFN- γ and IL-2 were associated with subjects who were not started on antifungal therapy, and elevated IL-2 levels on entry were associated with subjects not requiring subsequent antifungal therapy, suggesting that in particular, IL-2 could be a predictor of an appropriate and controlling immune response.

While this study has several strengths, including performing the assay close to the time of diagnosis and examining only those with primary pulmonary coccidioidomycosis, it also has weaknesses. These weaknesses include the predominantly descriptive nature of the study, the relatively small cohort consisting predominantly of white males, the lack of control over clinical management, and the inability to obtain *ex vivo* cytokine levels later in the course of illness. Moreover, while many of the subjects with active coccidioidomycosis had underlying diseases, none of those in the control group did. In addition, we were not able to compare our results of subjects with known coccidioidal pneumonia with a group of subjects who had pneumonia due to other causes. Such a comparison would likely have strengthened our conclusions and will be considered for future studies. Despite these concerns, the results suggest that assessing the *ex vivo*-released antigen-induced levels of six cytokines using a multiplex assay technique might provide important diagnostic and prognostic information in human coccidioidomycosis.

MATERIALS AND METHODS

Subjects. Patients attending the Southern Arizona Veterans Affairs Health Care System (SAVAHCS) for nondisseminated coccidioidomycosis at their initial visit after the diagnosis were eligible for study. After informed consent was obtained, clinical data were collected, including the time from diagnosis to assay, the type of coccidioidomycosis, and the results of the serologic coccidioidal complement fixation titer by immunodiffusion (IDCF titer). IDCF titration was performed by the clinical laboratory of SAVAHCS as previously described (21). The medical records of each subject were reexamined at the end of the study to determine the clinical outcome. In addition, 15 healthy subjects were recruited as controls. The study was approved by the Human Subjects Protection Committee of SAVAHCS.

Whole-blood cytokine assay. As previously described (7), approximately 5 ml of blood was drawn by venipuncture from each subject and placed into a sterile tube containing sodium heparin. Aliquots were dispensed and incubated at 37°C in 5% CO₂ and 95% air with 20 μ g/ml of the coccidioidal antigen preparation T27K or without T27K to serve as an unstimulated control. After 18 h of incubation, the supernatant plasma sample was aspirated and frozen at -80°C until assayed for cytokine concentrations. T27K is the water-soluble supernatant of disrupted, mature, endospore-forming spherules of the Silveira strain of *Coccidioides posadasii* that were killed with thimerosal and subsequently centrifuged at 27,000 \times g (22).

Cytokine concentrations in each sample were determined using the Invitrogen Luminex cytokine human magnetic 30-plex panel assay according to the manufacturer's directions (Thermo Fisher Scientific, Waltham, MA). This technology allows simultaneous assay for 30 human proteins for each sample. Results are expressed as picograms per milliliter.

The 30 inflammatory proteins assayed included 19 cytokines, 7 chemokines, and 4 growth factors. The 19 cytokines were granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon alpha (IFN- α), IFN- γ , interleukin-1 β (IL-1 β), interleukin-1 receptor alpha (IL-1RA), IL-2, interleukin-2 receptor (IL-2R), interleukin-4 (IL-4), IL-5, IL-6, IL-7, IL-8, IL-10, both the p40 and p70 subunits of interleukin-12 (IL-12), interleukin-13 (IL-13), IL-15, IL-17, and tumor necrosis

factor alpha (TNF- α). The seven chemokines were eotaxin, IFN- γ -inducible protein (IP-10), monocyte chemoattractant protein 1 (MCP-1), monokine induced by IFN- γ (MIG), macrophage inhibitory protein 1 α (MIP-1 α), macrophage inhibitory protein 1 β (MIP-1 β), and RANTES (regulated on activation, normal T cell expressed and secreted). The four growth factors were epidermal growth factor (EGF), fibroblast growth factor-basic (FGF), hepatocyte growth factor (HGF), and vascular endothelial growth factor (VEGF).

Statistical analysis. Medians with ranges were used to express summarized data. The nonparametric Mann-Whitney rank sum test was used for two-group analysis of continuous variables, and the Kruskal-Wallis test was used for analysis of continuous variables that contained more than two groups. Categorical variables were assessed using the χ^2 test. A *P* value of ≤ 0.05 was considered to be statistically significant. The average fold level of the antigen-stimulated sample above control was obtained by dividing the mean cytokine concentration of the T27K-stimulated samples by the mean unstimulated controls. All data were analyzed using Stata version 14 (Stata Corp., College Station, TX).

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