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Fluconazole treatment of intrauterine *Candida albicans* infection in fetal sheep

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BACKGROUND: Intrauterine *Candida albicans* infection causes severe fetal inflammatory responses and fetal injury in an ovine model. We hypothesized that intra-amniotic antifungal therapy with fluconazole would decrease the adverse fetal effects of intra-amniotic *C. albicans* in sheep.

METHODS: Sheep received an intra-amniotic injection of 10^7 colony-forming units *C. albicans*. After 2 d, animals were then randomized to: (i) intra-amniotic and fetal intraperitoneal saline with delivery after 24 h (3 d *C. albicans* group); (ii) intra-amniotic and fetal intraperitoneal injections of fluconazole with delivery after either 24 h (3 d *C. albicans* plus 1 d fluconazole group) or 72 h (5 d *C. albicans* plus 3 d fluconazole group). Controls received intra-amniotic injections of saline followed by intra-amniotic and fetal intraperitoneal fluconazole injections.

RESULTS: Intra-amniotic *C. albicans* caused severe fetal inflammatory responses characterized by decreases in lymphocytes and platelets, an increase in posterior mediastinal lymph node weight and proinflammatory mRNA responses in the fetal lung, liver, and spleen. Fluconazole treatment temporarily decreased the pulmonary and chorioamnion inflammatory responses.

CONCLUSION: The severe fetal inflammatory responses caused by intra-amniotic *C. albicans* infection were transiently decreased with fluconazole. A timely fetal delivery of antimicrobial agents may prevent fetal injury associated with intrauterine infection.

Preventing preterm birth (delivery before 37 wk completed gestation) is a major challenge in perinatal medicine with the potential to prevent ~1 million perinatal deaths each year. Although the causes of preterm birth are multifactorial, intrauterine infection is frequently implicated (1). Many intrauterine infections associated with preterm labor are indolent, not clinically apparent and polymicrobial in nature (2,3). Although the most common microorganisms associated with preterm delivery that are identified by culture and molecular-based analyses are bacteria such as the *Fusobacterium*, *Ureaplasma*, *Mycoplasma*, *Streptococcus*, *Bacteroides*, and *Prevotella* spp., up to 40% of pregnant woman may also have vaginal colonization with the yeast, *Candida* spp. (2,4).

The detection of Candida *spp.* is twofold higher than for nonpregnant women (5), and *Candida* spp. have been isolated from the amniotic fluid of women with spontaneous preterm birth (6–8). Although congenital candidiasis is an uncommon clinical finding, recent molecular data suggest that *C. albicans* may colonize the amniotic cavity more frequently than initially indicated by culture-based analyses (7). Indwelling contraceptives and cervical cerclage are also associated with funisitis and chorioamnionitis caused by *Candida* spp. (2,9). Intra-amniotic *Candida* spp. infection can cause fetal death or fetal candidiasis with impaired neurodevelopmental outcomes (10,11). Again, although vaginal *Candida* spp. colonization is not frequently associated with increased risk of preterm delivery or low birth weight (12), there is some evidence that eradication of *Candida* spp. in pregnancy may reduce the risk of late miscarriage and preterm birth. A large retrospective cohort study demonstrated that the use of clotrimazole was associated with a significant reduction in preterm births and an increase in mean gestational age at birth (13). A recent study also reported that nearly 20% of women had asymptomatic vaginal candidiasis and there was a tendency toward reduction in spontaneous preterm birth among women who were treated with clotrimazole (14).

We previously described models of the fetal inflammatory response syndrome in sheep using the proinflammatory mediators *Escherichia coli* lipopolysaccharides (15), interleukin (IL)-1 (16), and live *Ureaplasma parvum* (17). In contrast to the low-grade fetal inflammation caused by these agonists, we recently reported that intra-amniotic *C. albicans* caused a severe, progressive fetal inflammation within 2 d, with the severity of skin, lung inflammatory, and systemic responses threatening fetal viability (18).

Recent studies have described cases of intra-amniotic *C. albicans* infection that were successfully treated with

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intra-amniotic fluconazole (19). Fluconazole is a trizaole antifungal agent that is commonly used to treat vaginal candidiasis. Although there are some concerns regarding the use of fluconazole in pregnancy, its efficacy against *Candida* spp., long half-life and gastrointestinal uptake make it a potentially useful agent to treat intrauterine infections caused by *C. albicans* (20–22). Therefore, we hypothesized that timely antifungal treatment with fluconazole would decrease the adverse fetal effects of intra-amniotic *C. albicans* in a pregnant sheep model.

RESULTS

Outcomes at Delivery and Cells in Cord Blood

The fetal mortality rate was 14% (6/40 animals) (**Table 1**). One saline-treated control fetus died prior to delivery. 25% of the 3 d *C. albicans*-plus 1 d fluconazole and 37% of the 5 d *C. albicans* plus 3 d fluconazole-exposed animals died. We did not include a 5 d *C. albicans* only group as we anticipated 100% mortality based on the significant degree of *C. albicans* infection-associated pathology identified in our earlier work with this model system (18). There were no differences in birth weights between saline-only and fluconazole-only control treatment animals, and these were combined as a single control group. Lymphocyte counts for the 3 d *C. albicans* group significantly decreased relative to control ($P < 0.05$). In contrast, there were no significant changes in neutrophil and monocyte counts between groups. Platelets significantly decreased in the 3 d *C. albicans* group and 3 d *C. albicans* plus 1 d fluconazole group relative to control ($P < 0.05$). Thus, intra-amniotic *C. albicans* infection caused a systemic fetal inflammatory response as indicated by lymphopenia and thrombocytopenia in fetal blood counts.

Detection of *C. albicans*

All animals in the 3 d *C. albicans* group had positive cultures for *C. albicans* in amniotic fluid (**Table 1**). All of *C. albicans*-exposed animals that were treated with fluconazole also had positive cultures for *C. albicans* in the amniotic fluid. The majority of the *C. albicans*-exposed fetuses also had positive blood cultures, irrespective of fluconazole treatment 1 or 3 d prior to delivery. qPCR analysis demonstrated significantly increased *C. albicans* RNA in the fetal lung in all groups relative to control ($P < 0.05$). The amount of *C. albicans* RNA trended to be less for the 3 d *C. albicans* plus 1 d fluconazole group than for 3 d *C. albicans* group ($P = 0.06$). No *C. albicans* RNA was detected in the fetal liver in any of the groups (**Table 1**).

Inflammation in Fetal Lungs

Pulmonary inflammation was evaluated by measuring mRNA expression for proinflammatory cytokines (**Figure 1**). mRNA for IL-1β, IL-8, MCP-1, and TNF-α in the fetal lung greatly increased in all *C. albicans*-exposed groups relative to control. mRNA expression for all cytokines significantly increased in the 3 d *C. albicans* group and 5 d *C. albicans* plus 3 d fluconazole group ($P < 0.05$), except for IL-1α, which only significantly increased in the 5 d *C. albicans* plus 3 d fluconazole group ($P < 0.05$). Interestingly, mRNA expression for IL-6 and MCP-1 in the lung significantly decreased in the 3 d *C. albicans* plus 1 d fluconazole group relative to 3 d *C. albicans* group ($P = 0.002$ and $P = 0.008$, respectively).

To better demonstrate the cellular inflammatory response and activation in the lung, inflammatory cell counts were performed for cells expressing CD3 (T-cell), PU.1 (a maturation marker for myeloid and lymphoid cells, found in high levels in

Table 1. Birth weight, and total and differential white blood cell counts

	Control	3 d *C. albicans*	3 d *C. albicans* + 1 d fluconazole	5 d *C. albicans* + 3 d fluconazole
Fetal death	1	0	2	3
Alive at birth	15	7	6	5
Birth weight, kg	2.8 ± 0.3	2.5 ± 0.2	2.8 ± 0.4	2.6 ± 0.3
Arterial blood pH	7.2 ± 0.07	7.2 ± 0.09	7.3 ± 0.07	7.05 ± 0.07**
$PaCO_2$	70.7 ± 9.7	77.0 ± 12.3	65.2 ± 11.0	96.6 ± 8.2**
PaO_2	5.6 ± 1.7	5.8 ± 3.9	6.5 ± 2.1	5.5 ± 1.3
Blood counts				
White blood cells, 10^9/l	2.7 ± 1.1	1.4 ± 0.8	2.2 ± 0.9	2.3 ± 1.2
Neutrophil, 10^9/l	0.2 ± 0.1	0.1 ± 0.3	0.2 ± 0.3	0.4 ± 0.4
Lymphocyte, 10^9/l	1.7 ± 0.8	0.7 ± 0.4*	1.2 ± 0.5	1.2 ± 0.9
Monocyte, 10^9/l	0.1 ± 0.06	0.1 ± 0.1	0.08 ± 0.06	0.2 ± 0.2
Platelet, 10^9/l	606 ± 157	297 ± 108*	387 ± 84*	408 ± 105
% Culture of *C. albicans*				
Amniotic fluid	0	100%	100%	100%
Blood	0	71%	83%	60%
C. albcans RNA, μg RNA/μl, lung	0	17.4 ± 35.5	1.6 ± 1.3	10.4 ± 11.1

Values are mean ± SD.
*$P < 0.05$ vs. control. **$P < 0.05$ vs. all groups.

mature macrophages) and myeloperoxidase (MPO) (a marker for activated neutrophils and monocytes). CD3-positive cell counts significantly increased in the 3 d *C. albicans* group and 5 d *C. albicans* plus 3 d fluconazole group relative to control ($P < 0.05$) (**Figure 2a**) and counts for MPO-positive cells significantly increased in the 3 d *C. albicans* group relative to control ($P < 0.05$) (**Figure 2b**). PU.1-positive cell counts also significantly increased in the 3 d *C. albicans* group ($P < 0.05$) (**Figure 2c**). Consistent with the mRNA data, CD3- and PU.1-positive cell counts significantly decreased in the 3 d *C. albicans* plus 1 d fluconazole group compared with the 3 d *C. albicans* group ($P = 0.004$ and $P = 0.008$, respectively). Immunohistochemical analysis of lung sections also demonstrated that there was a significant decrease in PU.1-positive cells in the 3 d *C. albicans* plus 1 d fluconazole group compared with the 3 d *C. albicans* group (**Figure 2d**).

Lung gas volume measured at 5, 10, 15, 20, and 40 cmH_2O significantly decreased in the 5 d *C. albicans* plus 3 d fluconazole group relative to control. In the 3 d *C. albicans* group, the lung gas volume also significantly decreased at 5, 10, 20, and 40 cmH_2O relative to control. The lung gas volume significantly increased in the 3 d *C. albicans* plus 1 d fluconazole group at 10, 20, and 40 cmH_2O compared with the 3 d *C. albicans* group. (**Figure 3a**) The increase in SP-A, SP-B, and SP-D mRNA levels indicates a lung maturation signal (**Figures 3b–d**). SP-A mRNA levels were significantly increased in the 3 d *C. albicans* group and 5 d *C. albicans* plus 3 d fluconazole group relative to control ($P < 0.05$). SP-B mRNA levels were significantly increased only in the 3 d *C. albicans* group and SP-D mRNA levels trended to increase in all groups relative to control.

Inflammation in Other Organs

Inflammation of the chorioamnion was assessed by measuring expression of proinflammatory cytokines (**Figure 4a–c**). mRNA expression of IL-6 significantly increased in the 5 d *C. albicans* plus 3 d fluconazole group relative to control ($P < 0.05$). In the 3 d *C. albicans* plus 1 d fluconazole group, mRNA expression for IL-1β, IL-6, and MCP-1 significantly decreased relative to the 3 d *C. albicans* group ($P = 0.03$, $P = 0.001$, and $P = 0.03$, respectively). No changes in IL-1α, IL-8, and TNF-α mRNA expression were detected (data not shown). Histology evaluated with hematoxylin and eosin staining demonstrated minimal inflammatory cells in the chorioamnion (data not shown). The membranes were clear except for large white plaques (**Figure 4d**).

We evaluated the weight of the posterior of mediastinal lymph node as this node receives lymphatic flow from the lung and gastrointestinal tract (23). The posterior of mediastinal lymph node to body weight ratio significantly increased in the 3 d *C. albicans* group and 5 d *C. albicans* plus 3 d fluconazole group relative to control ($P < 0.05$) (**Figure 5a**). The counts for MPO-positive cells in posterior of mediastinal lymph node

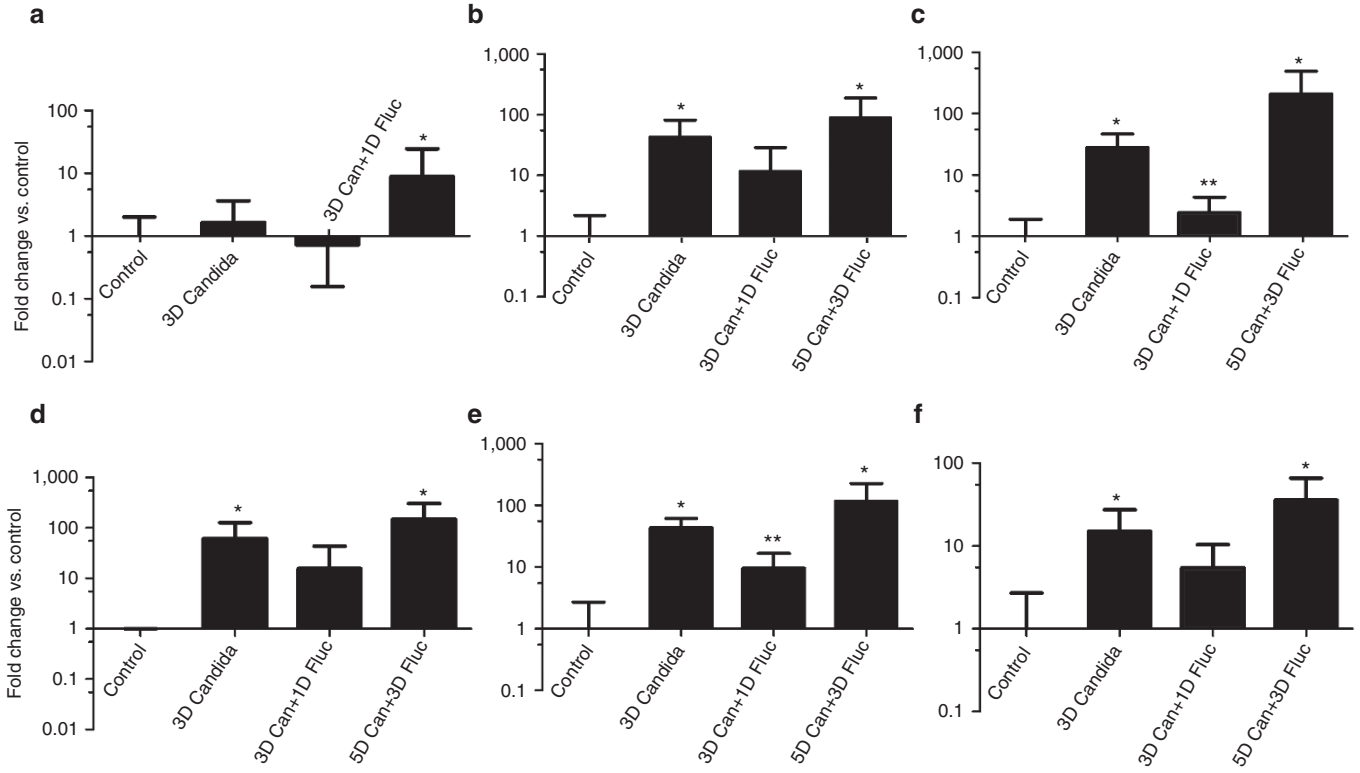

Figure 1. Lung cytokine expression following intra-amniotic *C. albicans* infection and fluconazole treatment. (**a–f**) Quantification of mRNAs for IL-1α, IL-1β, IL-6, IL-8, MCP-1, and TNF-α, respectively, was performed using real-time PCR with ovine-specific primers. Levels for each group were expressed as fold increase relative to control. mRNA expression for IL-1β, IL-6, IL-8, MCP-1, and TNF-α was significantly increased in the 3 d *C. albicans* group and 5 d *C. albicans* plus 3 d fluconazole group relative to control. mRNA expression for IL-6 and MCP-1 was significantly decreased for the 3 d *C. albicans* plus 1 d fluconazole group relative to the 3 d *C. albicans* group (*$P < 0.05$ vs. control, **$P < 0.05$ vs. the 3 d *C. albicans* group).

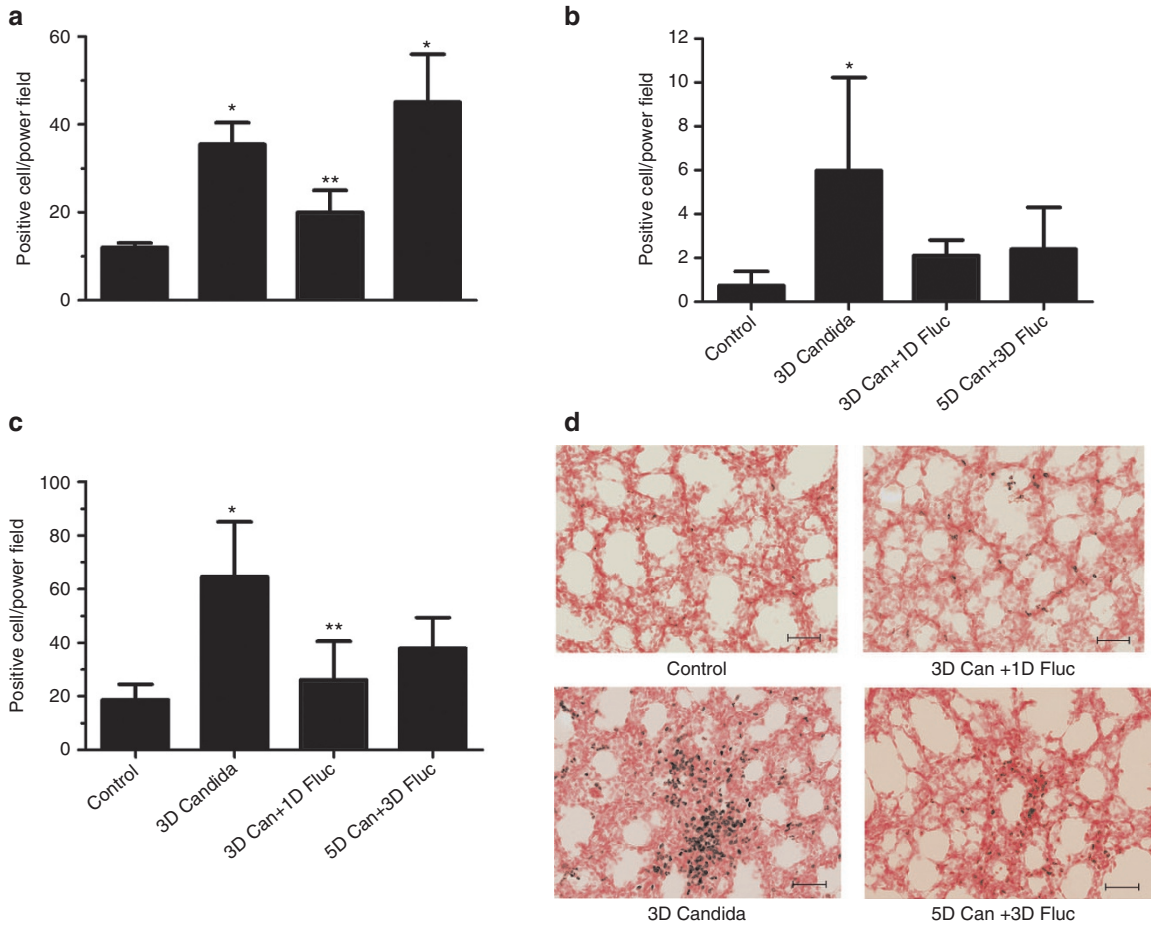

Figure 2. CD3, myeloperoxidase (MPO), and PU.1 expression in the lung following intrauterine *C. albicans* infection and fluconazole treatment. (**a**) CD3-positive cell counts significantly increased in the 3 d *C. albicans* group and 5 d *C. albicans* plus 3 d fluconazole group relative to control. (**b**) Counts for MPO-positive cells significantly increased in the 3 d *C. albicans* group relative to control. (**c**) PU.1-positive cell counts significantly increased in the 3 d *C. albicans* group relative to control and significantly decreased in the 3 d *C. albicans* plus 1 d fluconazole group relative to the 3 d *C. albicans* group. (**d**) Representative photomicrographs are shown for PU.1 immunostaining. All images 40× magnification and the scale bar showed 50 mm. The immunostained inflammatory cells (dark brown) were significantly increased in the 3 d *C. albicans* group (*$P < 0.05$ vs. control, **$P < 0.05$ vs. the 3 d *C. albicans* group).

tended to increase in the 3 d *C. albicans* group but decreased in both 3 and 5 d *C. albicans* and fluconazole-exposed groups relative to control (data not shown). Hepatic inflammation was assessed by measuring mRNA expression for the acute phase response proteins, SAA3 and C-reactive protein (CRP). SAA3 and CRP mRNA expression was significantly increased in all groups relative to control ($P < 0.05$) (**Figure 5b,c**). In the fetal spleen, mRNA for IL-6 significantly increased in the 3 d *C. albicans* group and 5 d *C. albicans* plus 3 d fluconazole group relative to control ($P < 0.05$) (**Figure 5d**).

DISCUSSION

Systemic fungal and yeast infections are a significant problem for infants requiring intensive care, and the major yeast pathogen in preterm infants is *C. albicans* (10,24). In healthy pregnant women, *Candida* spp. are common vaginal organisms that rarely infect the normal fetus. However, *Candida* spp. can cause ascending infection in association with preterm labor or preterm rupture of membranes (25). *Candida* spp. have been cultured from amniotic fluid of women with spontaneous preterm birth (7,26). In humans and animal models, there is limited information about the inflammatory responses of the fetus to *C. albicans*. We previously described the multiorgan fetal inflammatory responses to intra-amniotic *C. albicans* in an ovine model of pregnancy (19). In this current study, we replicate this inflammatory response and demonstrate that a single dose of the fungal-static drug fluconazole initially suppresses the fetal infection and associated fetal inflammatory response but is unlikely to be effective in a single-dose regimen for the treatment of intra-amniotic *C. albicans* infection.

The characteristics of the fetal inflammatory response caused by *C. albicans* colonization of the amniotic cavity should be interpreted within the context of other sheep models of chorioamnionitis and fetal inflammatory response syndrome. An intra-amniotic injection of lipopolysaccharides causes acute chorioamnionitis, striking lung inflammation, lung maturation, and a mild systemic inflammation in multiple organs including the gut, liver, skin, and the immune system (spleen, thymus, lymph node) (15,27). In contrast, *U. parvum* colonizes the amniotic fluid, fetal membranes, and lung but with

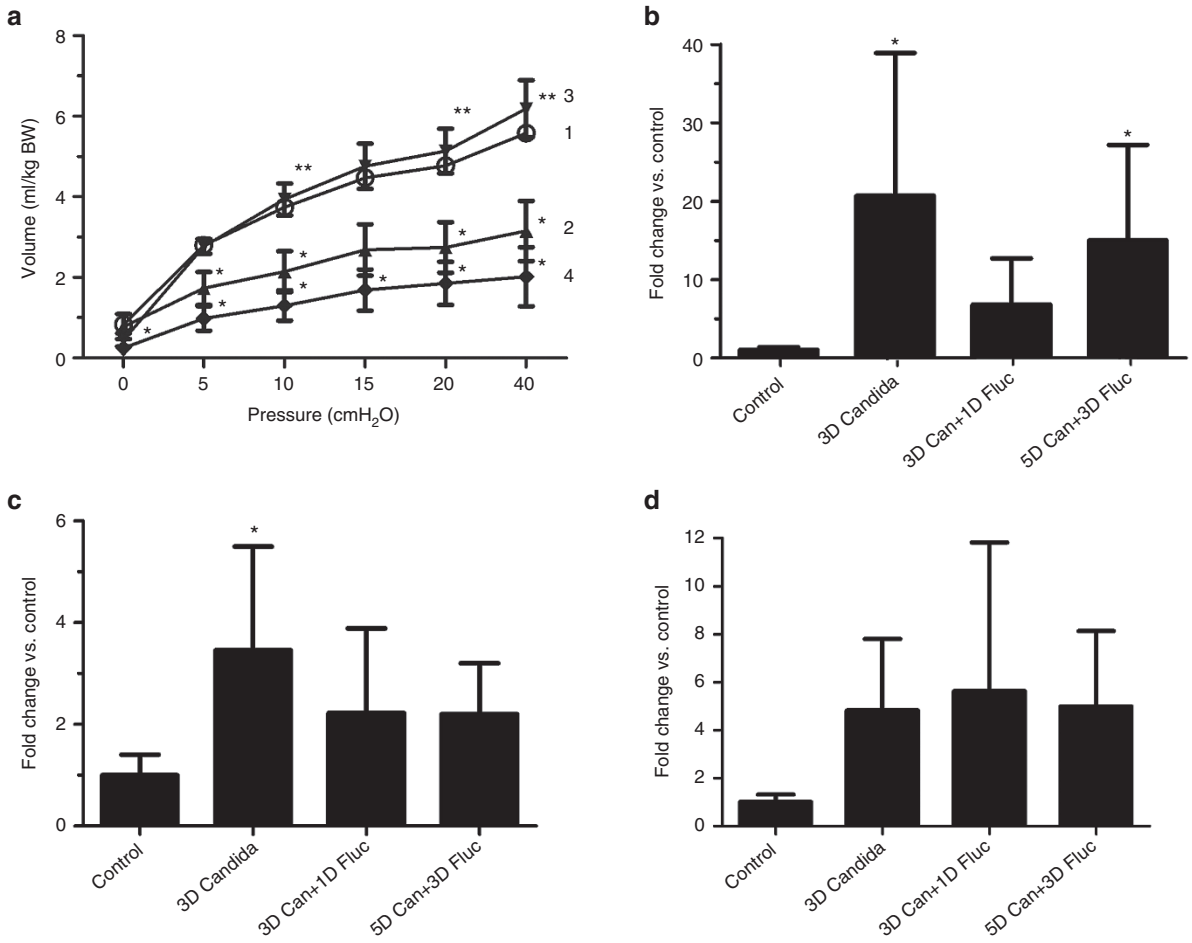

Figure 3. Lung gas volume and surfactant protein mRNA. (**a**) Pressure–volume curves demonstrated a significantly decreased lung volume in the 3 d *C. albicans* group and the 5 d *C. albicans* plus 3 d fluconazole group relative to control. The lung gas volumes significantly increased in the 3 d *C. albicans* plus 1 d fluconazole group at 10, 20, and 40 cmH_2O relative to the 3 d *C. albicans* group. (1, control group;, 2, 3 d *C. albicans* group;, 3, 3 d *C. albicans* plus 1 d fluconazole group;, 4, 5 d *C. albicans* plus 3 d fluconazole group). (**b–d**) Quantification of mRNAs for SP-A, SP-B, and SP-D, respectively, was performed using real-time PCR with ovine-specific primers. SP-A mRNA levels were significantly increased in the 3 d *C. albicans* group and 5 d *C. albicans* plus 3 d fluconazole group relative to control. SP-B levels were significantly increased in the 3 d *C. albicans* group relative to control (*$P < 0.05$ vs. control, **$P < 0.05$ vs. the 3 d *C. albicans* group).

less systemic inflammation and inconsistent lung maturation (17). Intra-amniotic *C. albicans* causes a substantially different inflammatory response, characterized by severe inflammation in the fetal lung, inflammation and edema of the fetal skin (18), and fetal death beyond 3 d of exposure. Interestingly, despite a robust intrauterine response, there were no overt effects on the well-being of the ewe, further underscoring the potential for significant fetal involvement to adopt a subclinical course. There is less histological chorioamnionitis observed with *C. albicans*, but the multiorgan fetal effects are far more severe. At 3 d postfetal exposure, lung cytokine levels are very high, and inflammatory cells are activated in the lungs. However, there is lung maturation response characterized by increased SP-A, SP-B, and SP-D mRNA levels. There is no lung maturation as assessed by a pressure volume curve, probably due to the progressive pneumonia caused by *C. albicans*. Of the *C. albicans*-exposed animals, 5 of 18 fetuses died, and 72% of the survivors had positive blood cultures at delivery. This demonstrates the ability of *C. albicans* to cause acute sepsis in these animals.

Intrauterine fluconazole treatment decreased the adverse effects of intra-amniotic *C. albicans* infection. mRNA expression of IL-6 MCP-1, CD3, and PU.1-positive cell counts in the fetal lung significantly decreased in the 3 d *C. albicans* plus 1 d fluconazole group relative to the 3 d *C. albicans* group. In addition, the lung gas volume in the 3 d *C. albicans* plus 1 d fluconazole group significantly increased compared with the 3 d *C. albicans* group. This is consistent with a reduction of lung inflammation in the 3 d *C. albicans* plus 1 d fluconazole group. In the chorioamnion, mRNA expression of IL-1β, IL-6, and MCP-1 significantly decreased in the 3 d *C. albicans* plus 1 d fluconazole group relative to the 3 d *C. albicans* group. Although the culture results demonstrated that most of the *C. albicans*-exposed animals had positive cultures, irrespective of the fluconazole treatment, the amount of *Candida* RNA in the fetal lung was decreased by 1 d of fluconazole ($P = 0.06$) (**Table 1**). Fluconazole is a fungal-static drug and would not be expected to clear the infection without effective innate immune responses, which may be minimal in these naive

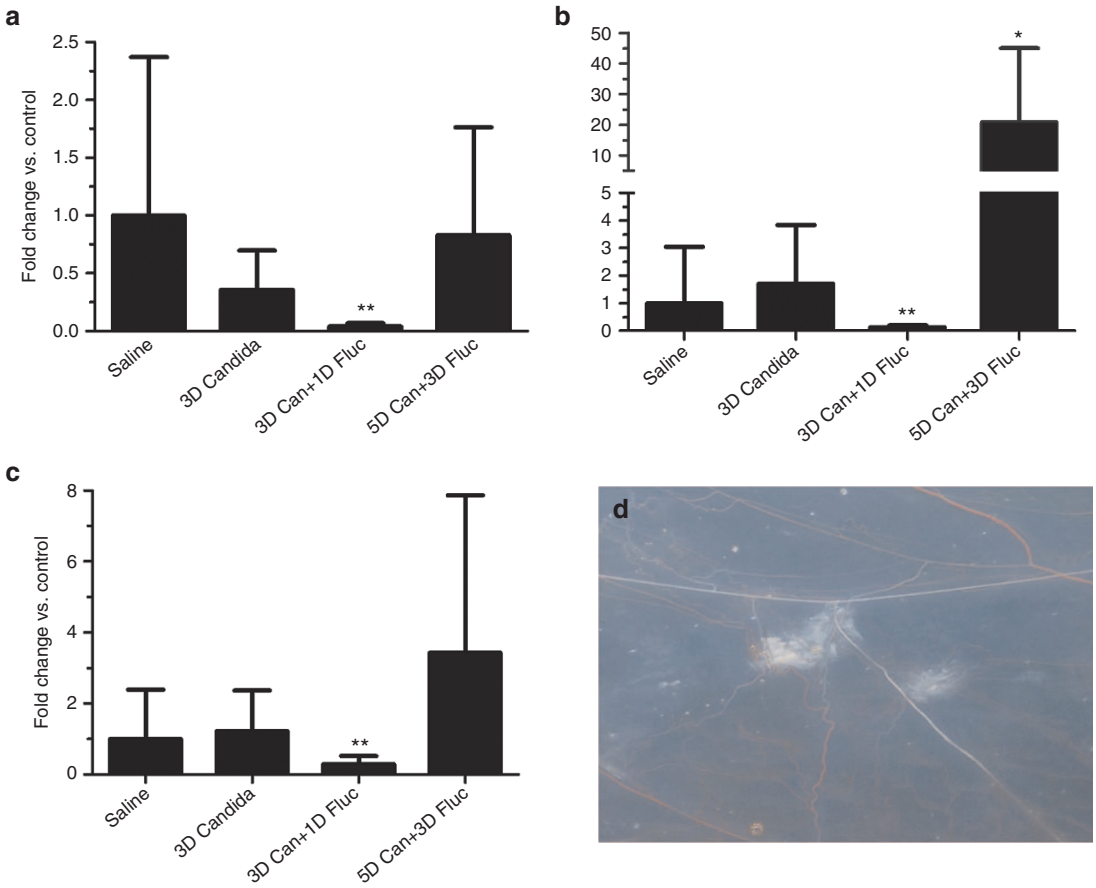

Figure 4. Cytokine expression in the chorioamnion following intra-amniotic *C. albicans* infection and fluconazole treatment. (**a–c**) Quantification of mRNA for IL-1β, IL-6, and MCP-1 respectively was performed using real-time PCR with ovine-specific primers. Levels for each group were expressed as fold increase relative to control. Only mRNA expression of IL-6 significantly increased in the 5 d *C. albicans* plus 3 d fluconazole group relative to control. In the 3 d *C. albicans* plus 1 d fluconazole group, mRNA expression of IL-1β, IL-6, and MCP-1 significantly decreased compared with the 3 d *C. albicans* group. (**d**) Representative photograph shows large white plaques on the chorioamnion membrane (*$P < 0.05$ vs. control, **$P < 0.05$ vs. the 3 d *C. albicans* group).

premature fetuses. Nevertheless, a single intra-amniotic and fetal intraperitoneal dose resulted in survival for five of eight animals exposed to *C. albicans* for greater than 4 d despite a significantly increased inflammatory response relative to control animals. However: the increase in inflammatory markers in multiple organs; the presence of viable *C. albicans* in the amniotic fluid; a ~7-fold increase in mean *C. albicans* RNA in the fetal lung; increases in both CD3-positive cell counts and PU.1-immunostained inflammatory cells; decreases in lung volume as indicated by pressure-volume curves and increases in the weight of the posterior mediastinal lymph node; in 5 d-treated compared to 3 d-treated animals, all indicate continued infection. The initial benefit of fluconazole treatment in the 3-d group was lost 24–72 h post-treatment. Based on our previous studies, it is likely all fetuses would have died beyond 5 d as a result of a re-established *C. albicans* infection (18).

Although there have been many previous attempts to reduce the rate of preterm birth with the use of antibiotic therapy, all with varying results (28), very few studies have demonstrated the effects of intrauterine antifungal therapy in pregnant women with *Candida* spp. infection. In one report, a pregnant woman with premature rupture of membranes and intra-amniotic infection with *C. albicans* was treated with transcervical amniofusion of amphotericin B and the infant was delivered without complications (29). Recently, Bean *et al.* (19) reported the resolution of two cases of intra-amniotic *C. albicans* infection with maternal and intra-amniotic fluconazole treatment. Thus, treatment of intra-amniotic *C. albicans* infection with intrauterine antifungal therapy may be helpful but further studies are needed to evaluate the effectiveness and possible toxicity of the therapeutic agent.

A limitation of this study is the absence of measurements of fluconazole levels within the fetal circulation, making it difficult to determine whether the five fetal deaths that occurred among the 16 animals comprising the fluconazole-treated groups are due, at least in part, to fetotoxic drug effects. The use of a combined intra-amniotic and fetal intraperitoneal dosing strategy was designed to ensure drug delivery to both the fetus and amniotic fluid. A drawback to this approach is that we are unable to determine differences or additive effects in therapeutic efficacy or fetotoxicity conveyed by these two delivery strategies. There are concerns relating to the use of fluconazole in pregnancy; it has been shown to have teratogenic effects in several animal model studies and recent *in vivo* work in a mouse model suggests that fluconazole perturbs CYP 26, an enzyme involved in retinoic acid homeostasis (30).

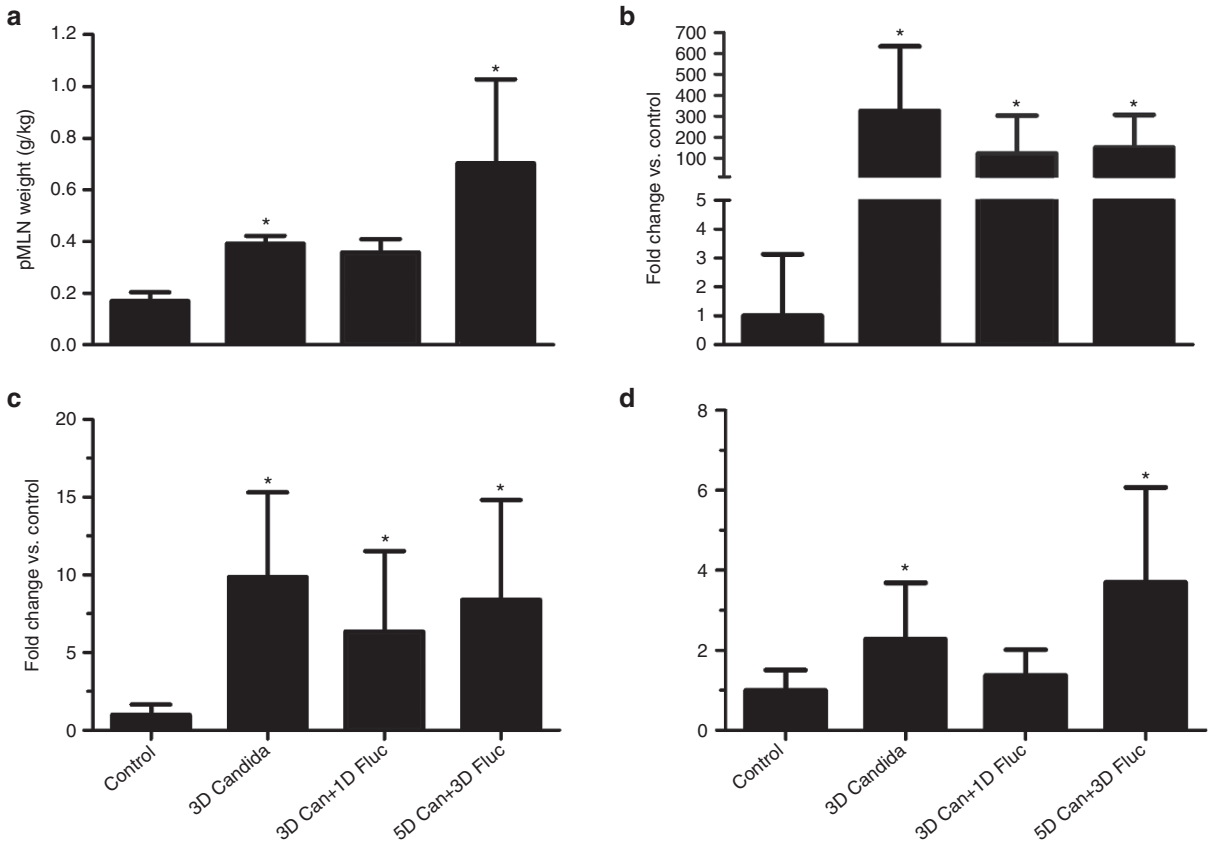

Figure 5. Systemic responses to *C. albicans*. (**a**) The weight of the posterior mediastinal lymph node significantly increased in the 3 d *C. albicans* group and 5 d *C. albicans* plus 3 d fluconazole group relative to control. (**b,c**) Acute phase protein gene expression in the liver. Quantification of mRNAs for SAA3 and C-reactive protein (CRP), respectively, was performed using real-time PCR with ovine-specific primers and probes. SAA3 and CRP significantly increased in all groups relative to control. (**d**) Quantification of mRNA for IL-6 in the spleen significantly increased in the 3 d *C. albicans* group and 5 d *C. albicans* plus 3 d fluconazole group relative to control (*$P < 0.05$ vs. control).

Interestingly, fluconazole treatment was well tolerated in the control group, suggesting that if fluconazole does have fetotoxic effects it may be more pronounced in conjunction with a pre-existing pathology or fetal stress, including intrauterine infection. Perhaps reassuringly, a number of retrospective cohort studies have failed to demonstrate an increased risk of congenital malformation in association with fluconazole use in pregnancy (22,31,32). Irrespective, further studies are warranted in order to determine the potential adverse effects deriving from exposing the fetus to high levels of fluconazole. A further limitation of this study was our decision to deliver two of the animals in the 5 d *C. albicans* plus 3 d fluconazole group 24 h early (equivalent to 4 d *C. albicans* and 2 d fluconazole exposure) due to concerns over fetal well-being. Although we are able to demonstrate that treatment efficacy was similarly reduced in these animals, data from this group does need to be interpreted with this difference in exposure length in mind.

In the current study, we administered a high dose by intra-amniotic and fetal intraperitoneal injections but failed to clear *C. albicans* from the amniotic fluid within 24 h. The efficacy of drugs may be altered in pregnant women due to physiologic changes and some drugs require higher dosing as a result of changes in fluid volumes. Administration of medication by amniocentesis facilitates the direct treatment of the fetus (19). Fluconazole is absorbed orally and thus could achieve continuous but decreasing fetal exposures with fetal swallowing. Our novel data demonstrate that although intra-amniotic fluconazole therapy for intrauterine *C. albicans* infection initially decreases adverse fetal effects, as indicated by a reduction in the lung and chorioamnion inflammation in a single-dose regimen, it is unlikely to prolong fetal survival beyond 5 d. A better understanding of the host immune response to *C. albicans* infection and as well as responses to multiple dose antifungal treatment regimens will help in the design of new therapies in perinatal medicine.

METHODS

Animals

All procedures involving animals were conducted at The University of Western Australia (Perth, WA) following review and approval by the animal care and use committees of The University of Western Australia and Cincinnati Children's Hospital (Cincinnati, OH). Date-mated Merino ewes with singleton pregnancies were randomized to one of the following exposure groups: (i) Control: intra-amniotic injection of 2 ml saline (n = 11) or intra-amniotic and fetal intraperitoneal injections of fluconazole (Claris Lifesciences Limited, Ahmedabad, India) (n = 5); or (ii) *C. albicans* at day 3 prior to delivery: intra-amniotic injection of 10^7 colony-forming unit (CFU) *C. albicans* in 2 ml saline 3 d prior to delivery (n = 8; one animal was found to be uninfected at postmortem and was eliminated from subsequent analyses, yielding a group n of 7); or (iii) *C. albicans* at day 3 prior

to delivery and fluconazole at day 1 prior to delivery: intra-amniotic injection of 10^7 CFU *C. albicans* in 2 ml saline 3 d prior to delivery, followed by intra-amniotic and fetal intraperitoneal injections of fluconazole 1 d prior to delivery, yielding 3 d of antenatal *C. albicans* exposure and 1 d of antenatal fluconazole exposure ($n = 8$); or (iv) *C. albicans* at day 5 prior to delivery and fluconazole at day 3 prior to delivery: intra-amniotic injection of 10^7 CFU *C. albicans* 5 d prior to delivery followed by intra-amniotic and fetal intraperitoneal injections of fluconazole 3 d prior to delivery, yielding 5 d of antenatal *C. albicans* exposure and 3 d of antenatal fluconazole exposure ($n = 8$).

The ultrasound guided dose of fluconazole was 30 mg per injection (12 mg/kg based on an estimated fetal weight of 2.5 kg) and was divided and given in equal 15 mg doses by intra-amniotic and fetal intraperitoneal injections of 7.5 ml each. Fluconazole was selected on the expectation that its prior successful use in treating intra-amniotic candidiasis, long half-life and the potential for uptake from AF swallowed by the fetal gut would allow us to deliver a lasting therapeutic dose from a single intra-amniotic and fetal intraperitoneal administration (20,21). Successful placement of intra-amniotic injections were confirmed with electrolyte (Cl-) analysis of amniotic fluid using a Siemens Rapidlab 1265 Analyser (Siemens, Munich, Germany). Successful placement of fetal intraperitoneal injections were confirmed by ultrasound visualization of fluid after injection. Combined intra-amniotic and fetal intraperitoneal injections were administered in an attempt to promote high fetal uptake of fluconazole as data on the bioavailability of this drug within sheep are not known.

All fetuses were surgically delivered at 122 ± 1 day GA, and euthanized with intravenous pentobarbital (100 mg/kg). Each fetus was weighed and fetal cord blood was collected for plasma, cell counts (VetPath, Perth, Western Australia), blood gas, and pH measurements. Fetal lung fluid and tissues for protein and mRNA expression analyses were collected at autopsy and snap frozen in liquid nitrogen. For determination of pulmonary compliance, a deflation air pressure–volume curve was measured from a static inflation of 40 cmH_20 airway pressure with the chest open (33).

C. albicans Culture and Detection

A Western Australian clinical isolate of *C. albicans* (19) was cultured on Difco Sabaraud-Dextrose agar (Becton Dickinson, Franklin Lakes, NJ) at 37 °C for 48 h and single colonies were inoculated into sterile phosphate-buffered saline (PBS) (Sigma-Aldrich, St. Loius, MO) (18). *C. albicans* colonial morphology was confirmed by growth on Brilliance Candida Agar (Oxoid, Adelaide, Australia). Inoculums were quantified using a plate dilution series as per standard microbiological methods and recorded as CFU/ml. Quantified inoculums (10^7 CFU in 2 ml PBS) were stored at −80 °C prior to use. For amniotic fluid culture, 100 µl of fresh amniotic fluid was inoculated onto Difco Sabaraud-Dextrose agar and evenly distributed across the plate with a sterile spreader. Incubation conditions were as described above. For blood culture, 2 ml fetal blood samples were inoculated into BACTEC Peds Plus culture vials (Becton Dickinson, Franklin Lakes, NJ) and incubated aerobically at 37 °C for 72 h. Every 24 h, a 1 ml sample was aseptically removed and 100 µl of this was subcultured on 5% sheep blood agar (Pathwest Laboratories, Perth, Western Australia) at 37 °C for 48 h. For both amniotic fluid and blood cultures, *C. albicans* colonial morphology was confirmed as described above. RNA extracted from fetal lung was screened using a real-time PCR assay targeting the RNase P RNA gene of *C. albicans* (19).

Quantification of mRNA Expression

Total RNA was extracted from fetal lung, chorioamnion, liver, and spleen tissues and homogenized in TRIzol (Life Technologies, Carlsbad, CA) as per manufacturer's instructions, and mRNA quantitation was performed by real-time PCR (27). mRNA was reverse transcribed to yield double-stranded cDNA (Verso cDNA kit, Thermo Scientific, Waltham, MA), which was used as template in real-time PCR assays with ovine-specific primers/Taqman probes (Applied Biosystems, Carlsbad CA) (34). Gene expression was measured for cytokines: IL-1α, IL-1β, IL-6, IL-8, TNF-α, monocyte chemotactant protein-1 (MCP-1), serum amyloid protein A3 (SAA3), and CRP. The values for each mRNA target were normalized to internal 18S rRNA values. Final expression data are represented as fold increase relative to control values.

Histology

Five-micrometer-thick sections from formalin-fixed lung and chorioamnion tissues embedded in paraffin blocks were stained with hematoxylin and eosin. Sections for immunohistochemical analysis were deparaffinized and rehydrated before microwave-assisted antigen retrieval in citric acid buffer at pH 6.0. Endogenous peroxidase activity was blocked with CH_3OH/H_2O_2. Sections were blocked with 2% goat serum in PBS. Sections were incubated for 16 h at 4 °C with primary antibodies specific for either PU.1 (Sc-352, Santa Cruz Biotechnology, CA, 1:400 dilution), CD3 (A0452, Dako, Glostrup, Denmark, 1:50) or MPO (catalogue #CMC028 Cell Marque, Rocklin, CA, 1:50) diluted in 2% goat serum in PBS. Sections were washed repeatedly in PBS before being incubated with an appropriate species-specific secondary antibody (1:200) for 30 min at room temperature. Slides were repeatedly washed in PBS before antigen:antibody complexes were visualized with a Vectastain ABC peroxidase Elite kit (Vector Laboratories, Burlingame, CA). Antigen detection was enhanced with nickel-diaminobenzidine, followed by incubation with TRIS-cobalt to give a black precipitate. Nuclei were counterstained with Nuclear Fast Red for photo-microscopy. Blind scoring of tissues was done by counting PU.1-, CD-3-, or MPO-positive cells in 10 comparable non-overlapping high power fields (×40 objective).

Statistical Analyses

All values are expressed as mean ± SD. Initial comparisons were made using Kruskal–Wallis one-way ANOVA across the four study groups. Selective comparisons between groups were made with two-tailed unpaired *t*-tests. Nonparametric data were tested for significance with Mann–Whitney tests. Statistical analysis was performed by GraphPad Prism v5.0. Significance was accepted at the level of $P < 0.05$.

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