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ORIGINAL ARTICLE

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Alteration of the mare's immune system by the synthetic progestin, altrenogest

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Abstract

Problem: Progestins are immunomodulatory in a variety of species. In the horse, the most commonly administered synthetic progestin is altrenogest (ALT), but its effect on the immune system of the non-pregnant mare is unknown.

Methods: Peripheral blood mononuclear cells (PBMCs) from diestrous mares were incubated with varying concentrations of progesterone (P4) or ALT to assess intracellular production of IFNγ and the expression of select cytokines. Additionally, ten mares received either ALT or VEH daily utilizing a switchback design beginning on the day of ovulation and continuing for 7 days. Circulating PBMCs and endometrial biopsies were obtained to assess the production and expression of the same cytokines.

Results: In vitro, both P4 and ALT caused a dose-dependent decrease in intracellular IFNγ in PBMCs. P4 caused a dose-dependent decrease in the expression of *IFNγ*, *IL-10* and *IL-4*, while ALT caused an increase in the expression of *IL-6* and *IL-1β* in PBMCs. In vivo, ALT suppressed the intracellular levels of IFNγ in PBMCs on d6. While control mares experienced a decrease in *IL-1β* expression from d0 to d6, ALT-treated mares did not. In the endometrium, ALT increased the expression of *IL-1RN* and *IFNγ* in comparison with VEH-treated mares.

Conclusion: P4 and ALT appear to alter the immune system of the non-pregnant mare both systemically in addition to locally within the endometrium. Further research is necessary to determine the pathways through which this synthetic progestin functions on the immune system of the horse, and the consequences it may have.

KEYWORDS

altrenogest, cytokine, endometrium, equine, glucocorticoid receptor, peripheral blood mononuclear cell, progesterone

1 | INTRODUCTION

The hormonal milieu of mammals is in constant fluctuation throughout the life cycle, with progesterone (P4) and its metabolites serving as a primary constituent. In many species, P4 is believed to be highly anti-inflammatory, while a variety of synthetic progestins have been found to alter the immune response in comparison with that of progesterone.[1] Medroxyprogesterone acetate (MPA), levonorgestrel (LNG), noresthisterone acetate, and d-norgestrel have been shown to bind selectively to either the progesterone (PR) or glucocorticoid receptor (GR) on a variety of immune cell types, including macrophages, dendritic cells, natural killer cells, and eosinophils

where they alter the production of select cytokines.[2-6] This can lead to an alteration of both the innate and adaptive immune responses. Women placed on these synthetic progestin-based contraceptives may be at a higher risk for a variety of reproductive diseases, including human immunodeficiency virus (HIV),[7] herpes simplex virus-2 (HSV-2),[8] chlamydia,[9] and gonorrhea.[10]

In the horse, synthetic progestins are commonly administered to mares for a variety of reasons, including therapeutic, estrous cycle control, and behavioral modifications.[11-13] The most commonly administered progestin is altrenogest (ALT), which has been found to maintain pregnancies in ovariectomized mares without the production of luteal progesterone, and is a more potent agonist of the progesterone receptor than P4 itself.[14,15] ALT gained popularity in the equine industry in the early 1980s for its use in estrous synchronization and in its ability to hasten the first ovulation in transitional mares.[11,16] Since then, ALT has been shown to maintain pregnancies in the absence of luteal progesterone.[17] Other synthetic progestins have been found not effective in preventing pregnancy loss in the absence of luteal progesterone, including medroxyprogesterone acetate (MPA), hydroxyprogesterone hexanoate (OHPC), norgestomet, and megestrol acetate.[18] ALT is thought to function in pregnancy maintenance by modifying myometrial contraction patterns in addition to the secretory function of the endometrium, while also increasing cervical tone and length in the absence of luteal P4.[19,20] While safety studies were performed on ALT and its effect on maternal and fetal viability, fertility, and reproductive development of offspring,[12,21,22] concerns have been noted in the literature following chronic administration of ALT. These include shortened gestational length, an imbalanced lymphocyte/neutrophil ratio as well as decreased respiratory function in foals born to mares receiving ALT, and an increased number of neutrophils in the endometrium of mares postpartum, indicating that it may have negative effects in both the pregnant mare and fetus.[23,24]

Altrenogest is a promiscuous activator of various steroid receptors, binding to the bovine PR with ten times the affinity of P4, and to the human androgen receptor (AR) at 75% the affinity of dihydrotestosterone (DHT).[25] In other species, various synthetic progestins have been found to bind to both the PR in addition to the GR, whereas others bind preferentially to just the PR, such as LNG. The receptor binding profile is thought to be controlled by the structure of the individual progestin, with those structurally similar to progesterone able to activate both the PR and GR, while those which mimic the 19-nortestosterone structure being unable to bind to the GR In addition, progestins appear to bind selectively in a species-specific manner, and the functionality of this binding is also species specific. This has been described in the horse, where MPA was unable to suppress estrus, which is in contrast to its ability to disrupt the menstrual cycle in women.[18] The binding capacity or activation of the equine GR by ALT has not been determined to our knowledge.

It is unknown if ALT alters the immune system of the non-pregnant mare, although research in other species supports that this may be the case.[1] In addition, while ALT is believed to be progestagenic by nature, it is structurally similar to an alkyl-substituted 19-nortestosterone and therefore may be unable to activate the equine GR, although no work has been performed to determine this. Thus, we hypothesize that ALT will alter the expression and production of cytokines through activation of the PR, which is located throughout the body on both immune and epithelial cells. Therefore, the objectives of this study were (a) to compare the effect of ALT to progesterone on lymphocyte function and expression of selected cytokines in vitro, (b) to assess if ALT alters both the systemic and local immune systems of the reproductive tract in the horse in vivo, and (c) to evaluate the ability of ALT to activate the equine GR Given the common use of ALT in horses, further understanding the effect of this synthetic progestin is imperative to the health and well-being of the animal.

Highlights

- The synthetic progestin altrenogest altered the immune system of the non-pregnant mare.
- This alteration occurred both systemically (in PBMC populations) and locally to the reproductive tract (endometrium)
- The alteration of cytokine production occurred without activating the glucocorticoid receptor.

2 | MATERIALS AND METHODS

2.1 | Animal usage

Twenty mares of mixed breeds, age, and weight (5-20 y/450-600 kg) were used over 2 years for the study. Mares were kept on grass pasture with grain supplementation and access to water and minerals ad libitum at the University of Kentucky's Maine Chance Farm in Lexington, KY, USA. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Kentucky (protocol #2017-2626). During the duration of the study (May-August), mares were examined daily via transrectal palpation and ultrasonography of their reproductive tract for follicular development, endometrial edema, as well as uterine and cervical tone. When the presence of a preovulatory follicle was noted (>35 mm) combined with reduced uterine tone, increased endometrial edema, and a relaxed cervix, mares received 3000 IU of human chorionic gonadotropin (hCG; Intervet International BV) intravenously to induce ovulation. Ovulation (d0) was confirmed by transrectal ultrasonography.

2.1.1 | In vitro progestin treatment animal usage

Ten non-pregnant mares were examined via rectal palpation and ultrasonography as previously described. On day 6 post-ovulation, blood was obtained aseptically via jugular venipuncture for the isolation of peripheral blood mononuclear cells (PBMCs). In brief, three

red top tubes were retrieved from each mare, and 100 USP heparin sodium injection (Procine; Henry Schein Animal Health) added to each as an anticoagulant for retrieval of plasma. Blood was taken to the laboratory at ambient temperature for further processing and analysis.

2.1.2 | In vivo progestin treatment animal usage

Ten additional non-pregnant mares were examined daily as previously described. Beginning on the day of ovulation and continuing daily for 7 days, mares received one of two treatments (a) 10 mL 0.044 mg/kg ALT PO as recommended (Regu-Mate; Merck Animal Health), or (b) 10 mL pure vegetable oil (Wesson®; vehicle control) with an estrous cycle in between to serve as washout. The converse treatment was administered after the washout cycle was passed, and the initial order of treatment was randomized. Blood was obtained on days 0 (ovulation) and 6 for the isolation of PBMCs as described below. On day 7 following ovulation, the perineum was washed with 2% chlorhexidine scrub and allowed to dry. A sterile alligator jaw biopsy forceps was inserted through the vulva and cervix and an endometrial biopsy obtained from the bifurcation of the right uterine horn. Tissue was immediately placed in RNALater® (Applied Biosystems) for preservation, initially at 4°C for 24 hours before being stored in −20°C for future processing for qPCR as described below. All mares received 7.5 mg of prostaglandin $F_2\alpha$ ($PGF_2\alpha$; Lutalyse, Pfizer) administered intramuscularly following the cessation of treatments.

2.2 | Laboratory work

2.2.1 | Peripheral blood mononuclear cells

In vitro stimulation of PBMCs with progestins

Peripheral blood mononuclear cells (PBMCs) were isolated from non-pregnant mares as previously described by Adams et al[26] on day 6 post-ovulation. Heparinized blood was used to isolate PBMCs by Ficoll-Paque PlusTM (Amersham Biosciences) gradient centrifugation. For in vitro stimulation, PBMCs were incubated in 1.0 mL c-RPMI (RPMI-1640; Gibco), supplemented with 2.5% heat-inactivated fetal equine serum (FES; Sigma-Aldrich), 100-U/mL penicillin/streptomycin (Sigma), and 55-mmol/L 2-mercaptoethanol (Gibco) media. Cells were plated at a concentration of 4×10^6 cells/well on a 24-well plate. Then, wells were treated with individual concentrations of progesterone (P4; 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} mol/L), altrenogest (ALT; 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} mol/L), dexamethasone (DEX; 10^{-4} mol/L), or vehicle control (<2%v/v ethanol) and incubated at 37°C in 5% CO_2 for 2 hours. Following the treatment, all cells were stimulated with Brefeldin A (10 μg/mL; Sigma) and select wells with the positive control phorbol 12-myristate 13-acetate (PMA; 25 ng/mL; Sigma) and ionomycin (1 mmol/L; Sigma) for 4 h. Then, 2×10^6 cells were separated for the determination of cytokine gene expression as described by Elzinga et al[27] using Trizol (Invitrogen) to isolate RNA, and additional cells were set aside for further analysis via flow cytometry.

Peripheral blood mononuclear cell isolation and in vivo progestin stimulation

Peripheral blood mononuclear cells (PBMCs) were isolated on days 0 (ovulation) and 6 of diestrus immediately prior to in vivo ALT treatment as previously described. Cells were plated at a concentration of 4×10^6 cells/well on a 24-well plate. All wells were stimulated with Brefeldin A (10 μg/mL; Sigma) and select wells with the positive control phorbol 12-myristate 13-acetate (PMA; 25 ng/mL; Sigma) and ionomycin (1 mmol/L; Sigma) for 4 hours. Following this, 2×10^6 cells were separated for the determination of cytokine gene expression as described by Elzinga et al[27] using Trizol (Invitrogen) to isolate RNA, and additional cells were set aside for further analysis via flow cytometry.

2.2.2 | Flow cytometry

To determine the intracellular production of IFNγ, 1×10^6 cells were assayed by flow cytometry as previously described by Siard et al.[28] In brief, aliquots (200 μL each) of cells from each well were placed into duplicate 96-well V-bottom plates. Plates were centrifuged, resuspended in 2% paraformaldehyde (Sigma), and stored at 4°C overnight. Cells were then washed in saponin buffer [PBS supplemented with 1% fetal bovine serum (FBS), 0.1% saponin, and 0.1% sodium azide (Sigma)], and IFNγ FITC mouse anti-bovine antibody (AbD Serotec, Raleigh, NC; 0.1 mg) was added at a 1:100 dilution in saponin buffer. After overnight storage, IFNγ staining was carried out as previously described by Adams et al.[29] In brief, after the cells were stained, aliquots of cells were resuspended in 1X PBS on a 96-well round-bottom plate for flow cytometric analysis. Using a FACS Calibur flow cytometer (Becton Dickinson) and Cell Quest®software (Becton Dickinson), samples were analyzed, and lymphocyte populations were gated on cell size and granularity to determine percent IFNγ-positive lymphocytes as well as mean fluorescence intensities (MFI) of lymphocytes.

2.2.3 | Quantitative polymerase chain reaction analysis

To assess the expression of targets on tissues, total RNA from PBMCs and endometrial biopsies was extracted using TRIzol® Reagent (Invitrogen) as described by the manufacturer. Total RNA was precipitated using sodium acetate and isopropanol, resuspended in ddH_2O and DNAse treated (DNA-free™, Applied Biosystems), and then analyzed for quantity and quality via a NanoDrop® spectrophotometer (Thermo Scientific) and only samples with a 260/280 ratio >2.0 were assessed. RNA was reverse transcribed, and qPCR was performed as previously described by Fedorka et al.[30] Briefly, 1.5 μg of RNA in 41.5 μL ddH_20 was reverse transcribed using Promega reagents; 0.5 μL AMV Reverse Transcriptase, 16 μL 5x RT Buffer, 1 μL RNAsin®, 16 μL MgCl, 4 μL dNTP, and 1 μL Oligo(dT) Primer (Promega). Samples were incubated at 42°C for 60 minutes followed by 95°C for 5 minutes. cDNA was diluted 1:1 with ddH_20, and qPCR was performed using 4.5 μL of cDNA, 5 μL of Sensimix™ II (Bioline), and 0.5 μL of a custom primer/probe set from Applied Biosystems. Transcripts of interest were chosen based on previous

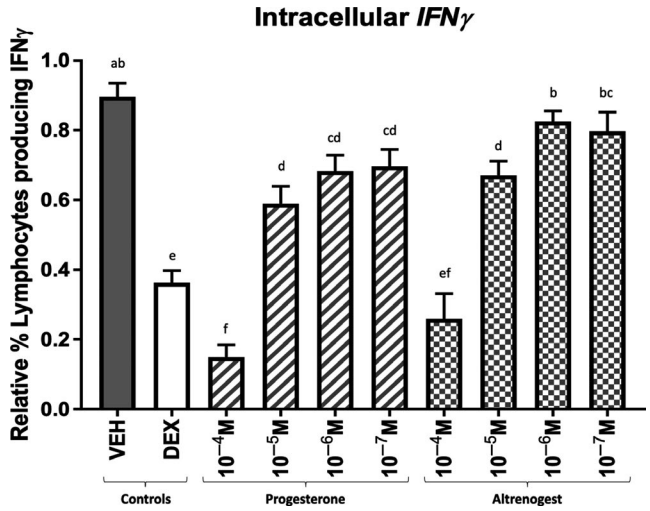

FIGURE 1 Effect of in vitro treatment of progesterone or ALT on production of intracellular *IFNγ* by equine lymphocytes. In vitro treatment with either P4 or ALT caused a dose-dependent suppression of the intracellular production of *IFNγ* by lymphocytes. All data are displayed as the relative production of treated cells producing *IFNγ* in comparison with the untreated cells producing *IFNγ* ± SEM. ^{a,b,c,d,e}values with different subscripts differ; $P < 0.05$

data regarding the effect of synthetic progestins on the immune system[1] and included the pro-inflammatory cytokines *IL-1β*, *IL-8*, *IFNγ*, *TNF*, the modulating cytokine *IL-6*, and the anti-inflammatory cytokines *IL-4* and *IL-10*. Primer sequences were designed using the TaqMan® Gene Expression System (Thermo Fischer) as previously described.[30,31] Reactions were performed in duplicate with the geometric mean of GAPDH and ACTB as the reference gene[32] for samples using the ViiA 7 Real-Time PCR System (Applied Biosystems). Samples were incubated at 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds. PCR efficiencies were calculated using LinRegPCR (version 2013.0). Results were expressed as the mean relative quantification (RQ), following the method described by Livak and Schmittgen.[33] RQ relates the PCR signal of the target transcript in a treatment group to that of an untreated control. In brief, the change in comparative threshold (Ct) between the gene of interest at the geometric mean of the reference genes was assessed and deemed the change in Ct (ΔCt). This was then standardized to the average of the negative controls (calibrator), and this was deemed the change of the ΔCt (ΔΔCt). For PBMCs, the calibrator was the average output of the cells stimulated by BFA alone. For endometrium, the calibrator was the average of negative control samples. Finally, the relative quantification (RQ) was determined through quantifying $2^{-\Delta\Delta Ct}$.

2.3 | Equine glucocorticoid receptor luciferase reporter assay

Equine Derm NBL-6 cell line was purchased from ATCC (Manassas, VA) and maintained in monolayer culture in Eagle's minimum essential medium (Mediatech, Manassas, VA) containing 10% (charcoal-stripped) fetal bovine serum (JR Scientific) and 1% penicillin/streptomycin (Gibco, Life Technologies). Replicates of cells (n = 4) were plated at 2.5×10^4 cells per well in a 96-well plate. After 24 hours, the cells were transiently transfected as recommended by the manufacturer (Lipofectamine 2000, Invitrogen) with a plasmid containing the steroid-responsive mouse mammary tumor virus promoter linked to a construct encoding luciferase (MMTV-luciferase; 0.25 μg plasmid DNA per well). Cells were grown for 24 hours and then incubated with increasing concentrations (0-300 nmol/L) of cortisol (Steraloids) P4 or ALT. Luciferase expression was measured 48 hours later on a Berthold Detection Systems luminometer (Oak Ridge, TN). Data were collected in relative light units (RLU) and expressed as a percentage increase over baseline (0 ng/mL steroid).

2.4 | Statistics

For the in vitro data reduction, the effect of treatment was normalized to the positive control of PMA/ionomycin and is therefore shown as relative expression. Comparisons were made between individual treatments. The in vivo data are shown without this transformation and made utilizing relative quantification (RQ) and is shown as relative expression. Comparisons were made between treatment groups and day. Data were analyzed using SAS 9.4 (SAS Institute Inc). Data were assessed for normality using a Shapiro-Wilkes test and equal variances with Bartlett's test. For the assessment of endometrial cytokine expression, data were analyzed with a general linear model (*proc glm*), with treatment as a fixed effect and mare as a random effect. When evaluating the effect of day and treatment on cytokine and receptor expression/production in PBMCs and endometrium, a repeated-measures analysis of variance was performed using a general linear model (*proc glm*) with treatment and day as fixed effects and mare as a random effect. For the in vitro data, the relative expression following treatment was assessed using a proc glm with treatment and concentration as a fixed effect. Only significant interactions were spliced for further analysis to investigate individual treatment groups, cytokines, and dosages. For receptor activation, a repeated-measures analysis of variance was performed with concentration and treatment as fixed effects. Comparisons were made between treatments using the mean ± the standard error with post hoc analysis performed using Tukey's test with significance set to $P < 0.05$ and trends $P \leq 0.1$. Data are presented as the mean ± the standard error of the mean.

3 | RESULTS

3.1 | In vitro

There was a significant interaction between the variable of treatment and intracellular production of *IFNγ*, and when this interaction was spliced, it was found that both P4 and ALT caused a dose-dependent decrease in the intracellular production of *IFNγ in* PBMCs

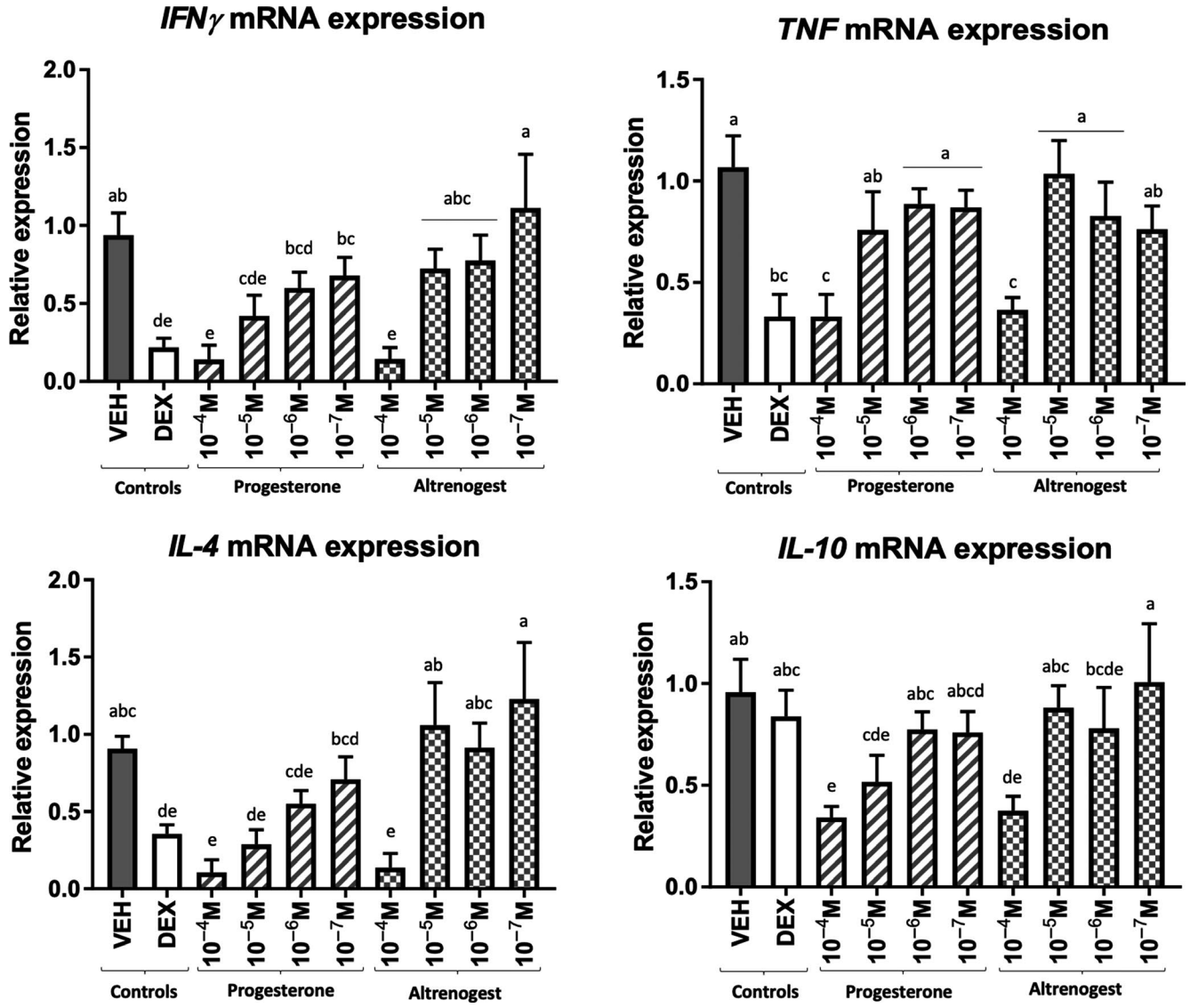

FIGURE 2 P4-induced suppression of mRNA expression of cytokines by equine lymphocytes following in vitro treatment. Increasing concentrations of P4 caused a dose-dependent decrease in the mRNA expression of pro-inflammatory cytokine *IFNγ*, in addition to the anti-inflammatory cytokines *IL-4* and *IL-10*. In contrast, ALT did not cause a dose-dependent decrease in these cytokines. All gene expressions are normalized to the geometric mean of ACTB and GAPDH and are displayed as a relative expression which describes relative quantification of treated cells in comparison with the relative quantification of the untreated cells ± SEM. ^{a,b,c,d,e,f}values with different superscripts differ; $P < 0.05$

($P < 0.0001$; Figure 1). When mRNA expression was assessed via qPCR, alterations in response were noted in the comparison between P4 and ALT. P4 caused a dose-dependent decrease in the mRNA expression of the pro-inflammatory cytokine interferon gamma (*IFNγ*; $P < 0.0001$) in addition to the anti-inflammatory cytokines interleukin-4 (*IL-4*; $P < 0.0001$) and interleukin-10 (*IL-10*; $P < 0.0001$; Figure 2). While the highest concentration of 10^{-4} mol/L ALT caused a suppression of these same cytokines, lower concentrations (10^{-5} mol/L/10^{-6} mol/L/10^{-7} mol/L) did not suppress the expression of these cytokines in comparison with negative controls. Although there was an overall effect of progestin treatment on *TNF* expression in PBMCs ($P < 0.001$), only the highest concentrations of P4 and ALT (10^{-4} mol/L) and the positive control of dexamethasone showed a significant suppression on expression in comparison with negative controls when spliced for individual treatment (Figure 2).

In contrast, ALT caused a dose-dependent increase in the mRNA expression of the pro-inflammatory cytokines *IL-1β* ($P = 0.014$) and the inflammatory-modulating cytokine *IL-6* ($P = 0.0012$; Figure 3); however, this increase was not noted in the P4-treated PBMCs. Altrenogest caused an increase in expression of *IL-6*, which reached a level of significance in comparison with that of both positive control cells (DEX) in addition to P4 at both 10^{-4} and 10^{-5} mol/L. A similar trend was noted for *IL-1β*, where ALT-treated PBMCs saw an increase in expression, but this only reached significance in comparison with

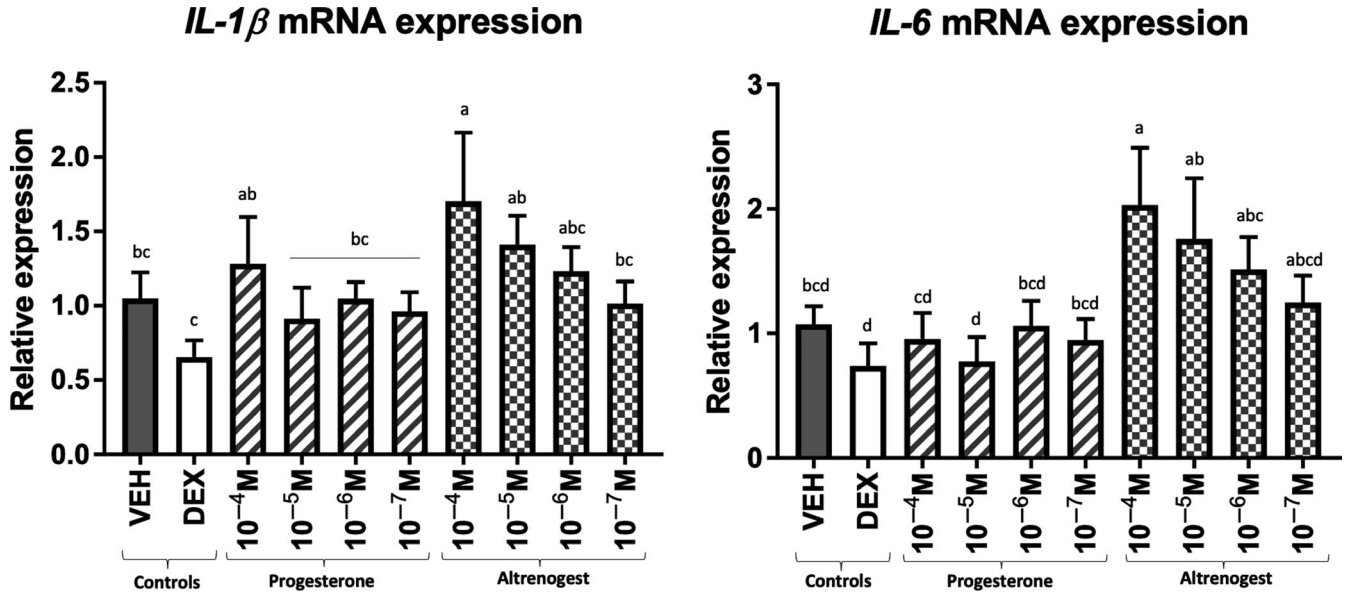

FIGURE 3 ALT-induced increase in the mRNA expression of cytokines by equine lymphocytes following in vitro treatment. Incubation of lymphocytes with increasing concentrations of ALT in vitro caused a dose-dependent increase in the expression of the pro-inflammatory cytokine *IL-1β* and the inflammatory-modulating cytokine *IL-6*. In contrast, no significant effect was noted following P4 treatment. All gene expression data are normalized to the geometric mean of ACTB and GAPDH and displayed as a relative expression which describes relative quantification of treated cells in comparison with the relative quantification of the untreated cells ± SEM with media as the calibrator. ^{a,b,c,d,e}values with different superscripts differ; $P < 0.05$

controls at the highest concentration of 10^{-4} mol/L. In regard to the assessment of both cytokines, the inflammatory-suppressive control of dexamethasone still functioned as anti-inflammatory, and this was in contrast to the effect of ALT. The treatment of P4 and ALT had no overall effect on the mRNA expression of the pro-inflammatory cytokine *IL-8* ($P = 0.13$), nor the anti-inflammatory cytokine *IL-1RN* ($P = 0.08$).

3.2 | In vivo

Systemically, both ALT treatment and control mares saw a decreased production of intracellular protein levels of *IFNγ* by PBMCs by day 6 of diestrus ($P = 0.037$; Figure 4). The mRNA expression of *IFNγ* in PBMCs also decreased by day 6 of diestrus in both ALT ($P = 0.025$) and control mares (CON; $P = 0.019$) in comparison with pre-treatment (day of ovulation; Figure 5). By day 6 of treatment, the CON group showed a suppression in the mRNA expression of *IL-1β* in PBMCs compared to that of day 0 ($P = 0.043$), in addition to a trend toward a decrease in the mRNA expression of *IL-8* ($P = 0.084$), but this decrease was not seen in the ALT-treated mares (Figure 5), indicating an ALT-induced increase in these two cytokines (*IL-1β* and *IL-8*) by day 6 of diestrus in comparison with CON.

There was a significant interaction between the treatment of ALT and mRNA expression of cytokines in endometrial biopsies ($P = 0.049$). When the interaction was spliced for specific cytokines, it was found that the treatment of ALT significantly increased the mRNA expression of the anti-inflammatory cytokine *IL-1RN* in comparison with vehicle control ($P = 0.018$). Altrenogest also increased

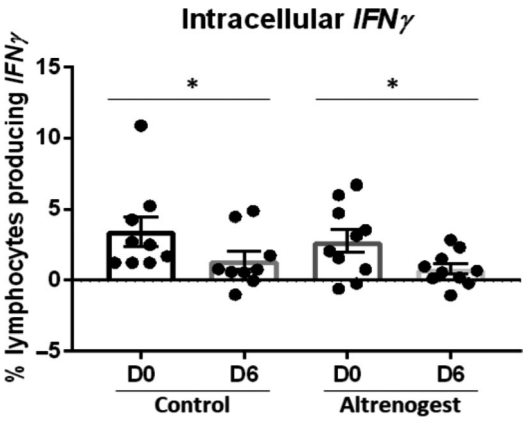

FIGURE 4 Effect of in vivo treatment of ALT on production of intracellular *IFNγ* by equine lymphocytes. In vivo treatment with ALT caused a suppression of the intracellular production of *IFNγ* by lymphocytes when comparing day 6 to day 0, and this decrease in *IFNγ* was also noted in control animals. There were no differences between day 6 treated and control animals. All data are displayed as the % cells producing *IFNγ* ± SEM with media as the calibrator. *; $P < 0.05$

the mRNA expression of the pro-inflammatory cytokine *IFNγ* within the endometrium at 7 days after the onset of treatment ($P = 0.012$). There was a trend toward an increase in the mRNA expression of the anti-inflammatory cytokine *IL-10* ($P < 0.1$; Figure 6). No changes were noted following ALT treatment on the endometrial mRNA expression of *TNF*, *iNOS*, or *IL-1β*, and expression levels of *IL-6* and *IL-8* were consistently below the limit of detection and therefore was not assessed (data not shown).

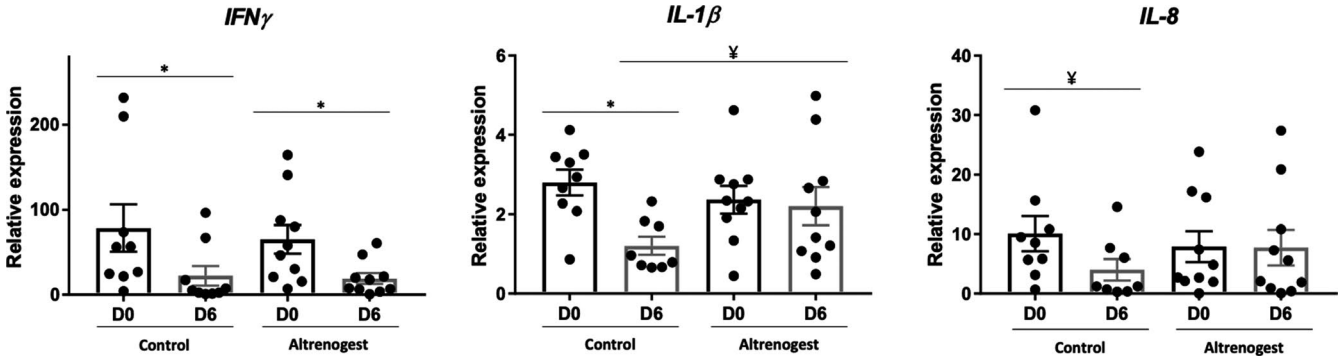

FIGURE 5 mRNA expression of cytokines in equine peripheral blood mononuclear cells (PBMCs) after in vivo ALT treatment. ALT caused a decrease in the PBMC expression of *IFNγ* by day 6 in comparison with day 0, and this decrease was by day 6 also seen in controls. There was no effect of ALT treatment on the mRNA expression of *IL-1β*. In contrast, the control group had a decrease in *IL-1β* mRNA expression from day 0 to day 6. When comparing day 6 of control animals to day 6 of ALT-treated animals, a trend toward an increase in *IL-1β* mRNA expression was noted in the treated animals ($P = 0.08$). A similar trend toward a decrease was noted in control animal mRNA expression of *IL-8* by day 6 ($P < 0.1$), and this again was not seen in ALT-treated animals. There were no differences in expression of any of the other cytokines examined, including *IL-10*, *IL-1RN*, *IL-6*, or *TNF* (data not shown). All gene expressions are normalized to the geometric mean of ACTB and GAPDH and displayed as the relative expression values with the average of control samples serving as the calibrator following the relative quantification method ± SEM with media as the calibrator. *$P < 0.05$ ¥$P < 0.01$

As determined by the luciferase reporter assay, the highest concentration of ALT or P4 tested did not activate the equine glucocorticoid responsive cell line, although the positive control of cortisol did so, and this reached significance at the 30 ng/mL concentration (Figure 7). Based on qPCR, mRNA for both glucocorticoid and progesterone receptors was expressed in the peripheral blood mononuclear cells and endometrium in diestrus (Figure 8).

4 | DISCUSSION

In this study, the synthetic progestin ALT was found to alter aspects of both the innate and adaptive immune system in non-pregnant mares in vivo and in vitro, and this was at times in contrast to both endogenous and exogenous P4. Administration of ALT was shown to have a significant effect on the immune system both systemically on circulating PBMCs and locally in the endometrium. To our knowledge, this is the first report on the alteration of the immune system of the non-pregnant mare by a synthetic progestin, and this finding deserves further investigation into its consequences.

The pharmacokinetics of ALT indicate that the standard dose of 0.044 mg/kg administered once a day results in a plasma concentration of 23-75 ng/mL, which is comparable to 10^{-6} mol/L/10^{-7} mol/L in vitro.[34] Additionally, concentrations of circulating P4 range from <1 ng/mL in early diestrus to an average of 11 ng/mL by 30 days of gestation[35] and are therefore more comparable to 10^{-7} mol/L. In this study, minimal alterations within the immune system were noted at these lower concentrations in vitro, while considerable alteration in response was seen at the in vitro concentrations of 10^{-4} mol/L/10^{-5} mol/L in addition to in vivo. This may

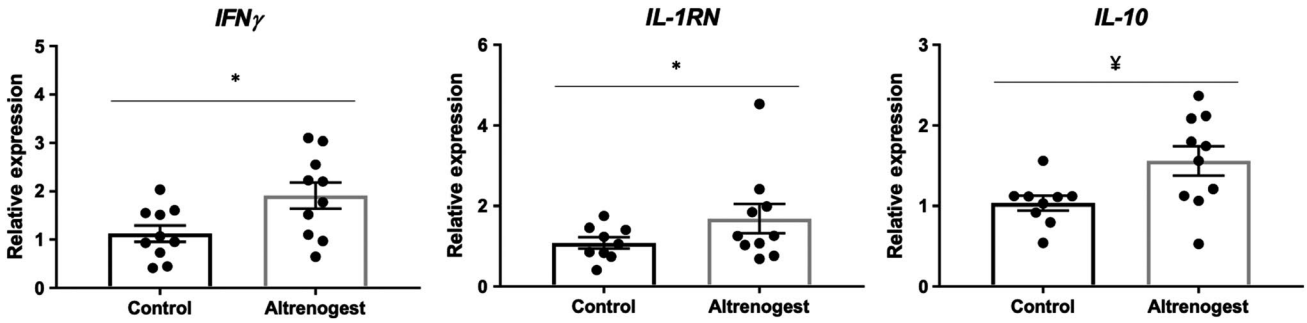

FIGURE 6 The effect of ALT administration on equine endometrial cytokine expression. ALT increased ($P < 0.05$) the mRNA expression of *IFNg*, *IL-1RN*, and tended to increase *IL-10* ($P < 0.1$) when compared to controls on day 7 of diestrus. No significant differences were noted in expression of *IL-1β*, *TNF*, or *iNOS*, and expression levels of *IL-6* and *IL-8* were undetectable in over half of the samples; therefore, analysis was not performed (data not shown). Gene expression data are normalized to the geometric means of ACTB and GAPDH and displayed as relative expression values with the average of control samples serving as the calibrator following the relative quantification method. Data are presented as dot plot with the middle horizontal line represented the mean while error bars represent the standard error of the mean (SEM). Asterisks indicate differences *$P < 0.05$ ¥$P < 0.01$

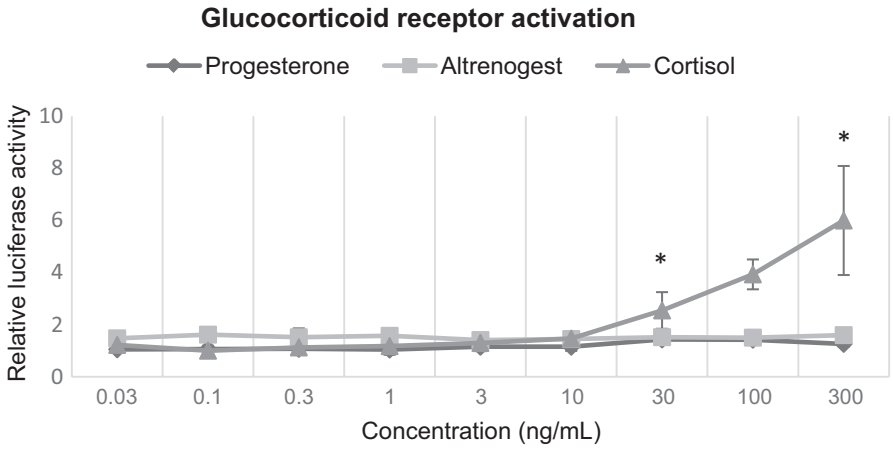

FIGURE 7 Relative equine glucocorticoid receptor (GR) luciferase activity following ALT and P4 stimulation. Neither ALT nor P4 stimulated the production of luciferase following incubation with an equine dermal cell that was cortisol responsive. The positive control of cortisol increased luciferase production. Data are presented in relative light units (RLU) and expressed as the mean of the percentage increase over baseline (0 ng/mL steroid) while error bars represent the standard error of the mean (SEM). Asterisks indicate differences $^{*}P < 0.05$

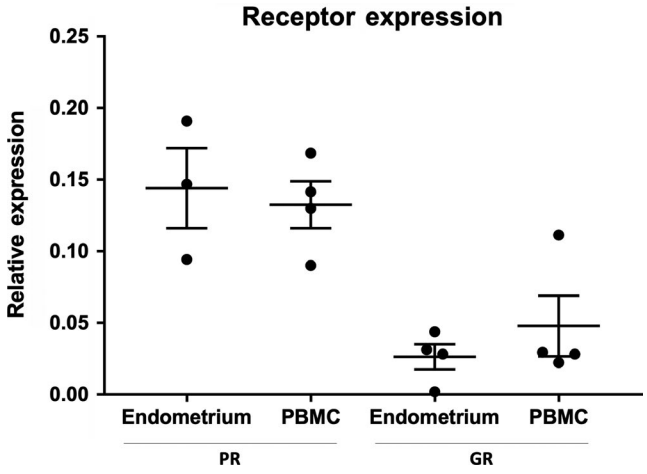

FIGURE 8 Relative expression of the equine progesterone receptor (PR) and glucocorticoid receptor (GR) in endometrium and peripheral blood mononuclear cells (PBMC). Both endometrium and PBMCs had notable levels of mRNA expression for PR and GR The gene expressions were normalized to the geometric mean of ACTB and GAPDH and displayed as relative expression values with the average of control samples serving as the calibrator following the relative quantification method. Data are presented as dot plot with the middle horizontal line represented the mean while error bars represent the standard error of the mean (SEM)

be due to increased physiological levels of these hormones within tissue in comparison with circulation, which has been noted for P4, and various endogenous progestins, including 5α-pregnane, 3,20-dione (5α-DHP), and 20α-hydroxy-5α-pregnan-3-one (20α-5P). This has been determined for both the endometrium (unpublished data) and chorioallantois [36] during pregnancy, in addition to fetal tissues such as adrenal and gonad, indicating a more local effect within the tissue. To our knowledge, no one has assessed the concentration of altrenogest present in the tissue of the non-pregnant mare following administration of standard doses, but justifies the range selected of 10^{-4} to 10^{-7} mol/L, and changes found at higher concentrations should be considered as causative of local effects.

In this study, ALT significantly altered numerous pro-inflammatory cytokines, including *IL-1β*, *IL-6*, *IL-8*, and *IFNγ*. In vitro, ALT increased the mRNA expression of *IL-1β* and *IL-6*, and this increase in pro-inflammatory cytokine expression in the endometrium was also noted after in vivo administration. By day 6 of diestrus, control mares had a decreased expression of both *IL-1β* and *IL-8* in comparison with day 0, although no decrease was noted in either cytokine in ALT-treated mares. It is believed that normal decrease in *IL-1β* and *IL-8* in control animals may be due to increased levels of endogenous P4 during the luteal phase of the cycle, and this decrease was inhibited following ALT treatment. This increase in pro-inflammatory cytokine expression in the ALT-treated mares is intriguing but has been documented in other species. Deese et al. compared the cytokine protein concentrations of endocervical swabs in women receiving varying progestin-based contraceptives and found the effect to be progestin specific. Depot medroxyprogesterone acetate (DMPA) was found to significantly increase the concentration of *MIP-1α*, *MIP-1β*, *IL-6*, *IL-8*, and *RANTES* while norethisterone oenanthate only increased *IL-6*, *IL-8*, and *RANTES* protein concentrations in comparison with untreated women.[3] This increase in the pro-inflammatory cytokines was hypothesized to predispose women to HIV infection by activating NFκβ, which binds to the terminal repeat on HIV and promotes viral replication. Potentially more comparable to ALT in structure, the 19-nortestosterone levonorgestrel (LNG) has been found to alter the immune function of women without activating the glucocorticoid receptor (GR) and does so by increasing in the expression and production of pro-inflammatory cytokines such as *IL-1β* and *IL-8* in the reproductive tract.[37,38] This alteration of the reproductive tracts cytokine profile under the influence of LNG has been shown to increase the risk of Chlamydia trachomatis[38] and herpes simplex

virus-2.[39] Under the confines of this study, it is unknown if the ALT-induced increase in *IL-1β*, *IL-6*, or *IL-8* expression predisposes mares to diseases of the reproductive tract, but our data suggest that further work in this area is justified.

One of the cytokines most notably altered by the progestins studied in the current experiment was the pro-inflammatory cytokine *IFNγ*. Systemically, both treated and control animals experienced a decreased production of this cytokine by day 6 of diestrus compared to day 0. This was further confirmed in vitro, where it was noted that both ALT and P4 have a dose-dependent suppressive effect on lymphocyte production of *IFNγ*, with the highest concentrations (10^{-4} mol/L) causing a comparable suppression to that of the anti-inflammatory dexamethasone at the same concentration. Luteal P4 is crucial for the switch from the pro-inflammatory events surrounding fertilization to the regulated inflammation involved with embryo migration, fixation, and implantation. In addition, *IFNγ* plays a role in the relationship between the innate and adaptive immune responses as it is involved in the cell-mediated response to intracellular pathogens,[40] and an alteration in *IFNγ* expression or production may increase risk of disease.[41] Miyaura and Iwata found that P4 suppressed *IFNγ* production in a dose-dependent manner in the T cells of mice.[42] A decrease in *IFNγ* production in the control mares by day 6 can therefore be explained by the increase in endogenous P4 at this time, which may be innately leading to the shift in a predominantly Th2 system for pregnancy recognition and acceptance.

In vitro, P4 caused a dose-dependent suppression of the cytokines *IFNγ*, *IL-4*, and *IL-10* while ALT did not. In comparison with P4 at similar concentrations, in vitro ALT increased the production and expression of the pro-inflammatory cytokine *IFNγ*, the anti-inflammatory cytokine *IL-4*, in addition to a trend toward an increase in the expression of the anti-inflammatory cytokine *IL-10*. In vivo, *IFNγ* expression was increased within the endometrium following ALT treatment, and again, there was a trend toward an increase in *IL-10* within the same tissue. This increase in *IFNγ* expression following ALT treatment could impact fertility, as any increase in *IFNγ* within the endometrium during pregnancy has been found to be detrimental to embryo survival.[43] The increase in endometrial expression of *IL-10* has been noted in other species, specifically following treatment with LNG in the human.[44] In the same study, an increase in *IFNγ* and *IL-1β* expression within the cervix was noted in addition to the increase in *IL-10* in women utilizing the LNG-IUD in comparison with controls.[44] The increase in *IL-10* within the endocervical region of the reproductive tract is associated with the presence and risk of sexually related diseases. It is therefore interesting that a similar increase was noted following ALT administration, a progestin that is structurally similar to that of LNG, and this deserves further investigation into any involvement with equine diseases involving the genital tract.

In this study, ALT treatment altered the immune response when compared to P4, and this may be explained by the preferential binding to the various receptors that each progestin activates. It has been documented that both MPA and megestrol acetate are able to bind to both the human PR and GR, while the 19-nortestosterone-related progestins, including LNG, norethisterone, and d-norgestrel, are devoid of binding capacity to the GR[45] In the current study, ALT did not activate the cortisol-responsive equine cells at concentrations well above those expected to be achieved systemically.[34] The PR and GR were found to be expressed within both the endometrium and PBMC cell population, insinuating that ALT may enact its immune alterations either selectively through the PR, which it binds to with a 13-fold increase in progesterone itself,[15] or through an entirely alternate pathway which may include the nuclear receptors due to their ease of diffusion across cell membranes. Additionally, P4 did not increase luciferase output within the GR assay, consistent with studies in other species including the human,[46] and this is in contrast to the response noted with MPA.[47] Investigations into this phenomena on other steroid nuclear receptors have determined that the species-specific interactions within various steroids, both endogenous and synthetic, are largely caused by the differences in transcriptional activation by progestins of full-length and truncated receptors and their interactions with the N-terminal domain and C-binding ligand binding domain.[48] It is unknown if this is true in the horse but may explain the differences between both species-specificity and progestin-specific binding preference.

This study demonstrates an alteration of the immune system of the non-pregnant mare by ALT, which is potentially executed through the PR located within a variety of tissues and cell types, including lymphocytes. The relationship between the endocrine and immune systems of the non-pregnant mare is poorly understood, although this field is developing in other species. It is believed that the endocrine changes of both the estrous cycle in addition to pregnancy have a significant effect on the effector functions of the immune system, and that this occurs for crucial events to occur, including ovulation, implantation of the early pregnancy, and the responses to vaccines required for herd immunity. In this study, ALT altered the effects of P4 on numerous endpoints, including in an increase in the expression and production of many pro-inflammatory cytokines. In other species, this increase in pro-inflammatory cytokine production activates the NFκβ pathway and increases pathogen load to the reproductive tract, worsening disease outcome. Therefore, further investigation into the consequences of the ALT-induced alteration of the immune system is warranted and may lead to improved management of the non-pregnant mare.

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CONFLICT OF INTEREST

None.

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