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STANDARD ARTICLE

Can levamisole upregulate the equine cell-mediated macrophage (M1) dendritic cell (DC1) T-helper 1 (CD4 Th1) T-cytotoxic (CD8) immune response in vitro?

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Background: Equine protozoal myeloencephalitis (EPM) is a common and devastating neurologic disease of horses in the United States. Because some EPM-affected horses have decreased immune responses, immunomodulators such as levamisole have been proposed as supplemental treatments. However, little is known about levamisole's effects or its mechanism of action in horses.

Objective: Levamisole in combination with another mitogen will stimulate a macrophage 1 (M1), dendritic cell 1 (DC1), T-helper 1 (CD4 Th1), and T-cytotoxic (CD8) immune response in equine peripheral blood mononuclear cells (PBMCs) in vitro as compared to mitogen alone.

Animals: Ten neurologically normal adult horses serologically negative for *Sarcocystis neurona*.

Methods: Prospective study. Optimal conditions for levamisole were determined based on cellular proliferation. Peripheral blood mononuclear cells were then cultured using optimal conditions of mitogen and levamisole to identify the immune phenotype, based on subset-specific activation markers, intracellular cytokine production, and cytokine concentrations in cell supernatants. Subset-specific proliferation was determined using a vital stain.

Results: Concanavalin A (conA) with levamisole, but not levamisole alone, resulted in a significant decrease ($P < .05$) in PBMC proliferation compared to conA alone. Levamisole alone did not elicit a specific immune phenotype different than that induced by conA.

Conclusion and Clinical Importance: Levamisole co-cultured with conA significantly attenuated the PBMC proliferative response as compared with conA. If the mechanisms by which levamisole modulates the immune phenotype can be further defined, levamisole may have potential use in the treatment of inflammatory diseases.

KEYWORDS

EPM, immunomodulators, immunology

1 | INTRODUCTION

Equine protozoal myeloencephalitis (EPM) is a common and costly neurologic disease of horses in the United States. Despite a high seroprevalence nationally (>50%), it is unclear why only a small percentage (0.5%-1%) of horses develop clinical disease. Performance and shipping have been shown to be risk factors for EPM.[1] Other studies have demonstrated that some EPM-affected horses have decreased immune responses, including decreased antigen-specific proliferation, as well as increased interleukin-4 (IL-4) and decreased interferon-gamma (IFN-γ) expression, supporting a cluster of differentiation 4 (CD4) T helper 2 (Th2) response.[2–7] Additional studies in mice in have supported a role for CD4 Th1 CD8 IFN-γ in protection because immunocompetent mice develop CD4 and CD8 memory responses, whereas IFN-γ knockout (KO) and CD8 KO mice develop

Abbreviations: ANOVA, analysis of variance; BrdU, bromodeoxyuridine; CD, cluster of differentiation; conA, concanavalin A; DC, dendritic cell; EPM, equine protozoal myeloencephalitis; FACS, fluorescent activated cell sorting; FBS, fetal bovine serum; I, ionomycin; IFN, interferon; Ig, immunoglobulin; IL, interleukin; KO, knockout; M, macrophage; MHC, major histocompatibility complex; NK, natural killer; PBMC, peripheral blood mononuclear cell; PMA, phorbol myristate acetate; RT, room temperature; Th, T helper.

TABLE 1 Levamisole nonenzymatic breakdown products

	Storage condition	Chemical name	Product	Effect on conA stimulated proliferation
Levamisole	37°C, pH 7.0-7.5	3-(2-mercaptoethyl)-5-phenyl imidazolidine-2-one	Product 1	Inhibited lymphocyte proliferation
Levamisole	4 °C, pH 7.5-8.0	6-phenyl-2,3-dihydroimidazo(2,1-b) thiazole	Product 2	Increased lymphocyte proliferation
Levamisole	37°C, pH 7-7.5	Bis (3-(2-oxo-5-phenylimidazolidin-1-yl) ethyl) disulfide	Product 3	No effect on proliferation

Abbreviation: conA, concanavalin A

disease.[8–10] Therefore, if efficacious immunomodulators could be found, they could be used as supplemental treatments for EPM.

Previously, levamisole has been proposed as an immunomodulatory treatment for EPM.[11] Levmamisole is an imidothiazole derivative with several actions that include antihelminthic, anti-inflammatory, antioxidant, antineoplastic, and immunomodulatory effects.[12] Experimental studies have demonstrated that levamisole can upregulate or downregulate, tolerize, or have no detectable effect on the immune response.[13,14] Some of the variables in levamisole function appear to be related to the immune competence or phenotype of the individual animal and levamisole storage conditions (eg, temperature, pH, and concentration). Levamisole breaks down into 3 different metabolites (Table 1).[15] Metabolite 1 after storage at 37°C or freshly prepared limits proliferation. Levamisole stored at 4°C generates metabolite 2, which is stimulatory. Metabolite 3 is generated from storage at 37°C but did not affect lymphocyte function. The nature of the metabolite(s) present may explain some of the differences in reports on levamisole function. Most of the studies that have already been performed, regardless of species, have only identified how levamisole affects specific aspects of the immune system. These studies have not identified which levamisole metabolites are present. Without knowing which metabolites are present, it is unknown if other factors are present (eg, unknown levamisole metabolites, other cells that levamisole may affect, species differences in how levamisole affects immune responses) that influence how levamisole affects immune function.[15,16]

Levamisole administered to pregnant mares increased immunoglobulin G3 (IgG3) concentration in colostrum.[17] Additionally, neutrophils from the foals of treated mares had enhanced phagocytic activity. Few additional studies have explored levamisole's effects on the equine immune response. Our goal was to evaluate the immunomodulatory effects of levamisole on the equine immune response and determine its potential use as a supplementary treatment for EPM. The predicted protective immune response against *Sarcocystis neurona* is a cell-mediated immune response. Because proliferation of cells involved in cell-mediated immunity is 1 of the best methods of assessing immune function, our first objective was to define optimal in vitro conditions for detecting an effect of levamisole on the mitogenic response of stimulated equine peripheral mononuclear cells (PBMCs). Based on previous studies,[14,15] we predicted that levamisole alone may have a minimal effect on the ability of cells to respond in vitro. We predicted levamisole would need to be combined with a mitogen to determine how levamisole affects proliferation of equine PBMCs. Therefore, to identify the predicted maximal response, we measured the change in levamisole effect with a mitogen to the effect of levamisole alone. We predicted the combination of levamisole with a mitogen would lead to the largest change in proliferation, which is a critical measure of immune function as opposed to activation only of cells. This system

then was used to examine changes in PBMC phenotype associated with levamisole co-culture.

2 | MATERIAL AND METHODS

Equine PBMCs were isolated from 10 healthy neurologically normal adult horses and used to identify the optimal (ie, conditions that stimulated the largest change in proliferation between levamisole alone versus levamisole with a mitogen) conditions for levamisole in vitro based on cell proliferation. We predicted that this approach would allow us to identify the greatest potential for levamisole to affect the immune response. Equine PBMCs then were cultured using optimized conditions of levamisole to identify the immune phenotype based on proliferation of specific subsets of cells and cytokine production using flow cytometry and ELISAs. This study was approved by Institutional Animal Care and Use Committee (VT14-097).

2.1 | Horses

Peripheral blood mononuclear cells were isolated from 10 adult horses ranging in age from 2 to 24 years. Horse breeds included 4 Arabians, 2 Warmbloods, 2 Standardbreds, 1 Thoroughbred, and 1 Quarter horse. There were 7 geldings and 3 mares. Horses were determined to be healthy based on normal physical and neurologic examination findings. Horses were current on vaccinations and Coggins status, and had not been vaccinated within 2 weeks of the study. They were negative for *Sarcocystis neurona* based on a negative serum surface antigen 1, 5, 6 peptide ELISA (Pathogenes, Inc.).

2.2 | Collection of PBMCs

Blood samples were aseptically collected into lithium heparinized tubes by jugular venipuncture from each horse.[18] Peripheral blood mononuclear cells were isolated as previously described.[6,18] Briefly, diluted blood was layered over an isosmotic density gradient material (Lymphoprep 1.077 g/mL; Nycomed (Zurich, Switzerland)). Samples were centrifuged, and the buffy coat isolated and washed 3 times. Cells were counted and resuspended in Roswell Park Memorial Institute Media (RPMI) 1640 complete media (10% heat inactivated fetal bovine serum [FBS], L-glutamine, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid [Thomas Scientific] Sweedsboro, NJ, and penicillin/streptomycin [Cellgro] Sweedsboro, NJ) at a concentration of 2×10^6 cells/mL.[6,18]

2.3 | Treatment conditions

Cells were treated according to conditions predicted to produce maximal stimulation and inhibition of leukocyte subsets in mice.[15,16]

Aliquots of cells (2×10^5 cells/well in 100 μL of complete media) from each horse were plated in triplicate in round bottom 96-well plates with 1 of the following treatments and a final concentration per well as follows: media only (negative control); concanavalin A (conA; 5 μg/mL; Sigma; positive control); fresh levamisole (Sigma; 1 μg/mL); fresh levamisole (10 μg/mL); levamisole 4°C (1 μg/mL); levamisole 4°C (10 μg/mL); levamisole fresh (1 μg/mL) and conA (5 μg/mL); levamisole fresh (10 μg/mL) and conA (5 μg/mL); levamisole 4°C (1 μg/mL) and conA (5 μg/mL); levamisole 4°C (10 μg/mL) and conA (5 μg/mL). All the same treatments were also used with phorbol myristate acetate (20 μg/mL) and ionomycin (10 pg/mL; PMA/I) with and without levamisole.[18] Fresh levamisole was prepared immediately before use, whereas levamisole 4°C was stored 2 weeks before at 4°C, pH 7.5 before (levamisole 4°C)[15,16] to replicate conditions for different levamisole metabolites. Levamisole prepared immediately before use was predicted to generate levamisole metabolite 1. Levamisole stored at 4°C for 2 weeks as described previously was predicted to generate levamisole metabolite 2 (Table 1).[15] Cells were stimulated for 72 hours. These studies were performed sequentially, and new preparations of levamisole were made for each study.

2.4 | Determination of proliferation using bromodeoxyuridine assay

After incubation of cultures for 48 hours, 20 μL of bromodeoxyuridine (BrdU) solution (Roche Life Sciences 11647229001) was added to each well. After 12 hours of incubation (72 hours total for cells), plates were harvested. Supernatants were collected and frozen at −80°C for cytokine analysis. The plates were centrifuged at 300g at 23°C for 10 minutes. Supernatants were removed, and FixDenat (200 μL/well) was added without resuspending the cells. The cells were incubated for 30 minutes at room temperature (RT), and the FixDenat was removed. Anti-BrdU-peroxidase (POD) (100 μL/well) working solution was added, and the plates were incubated for 90 minutes at RT. Plates were wash 3 times with 200 μL/well washing solution, after which substrate solution (100 μL/well) was added. Plates were incubated at RT to allow color development. Plates were read at 370 nm with a reference wavelength of 492 nm. Both proliferation as well as change in proliferation between levamisole and a mitogen were determined.

2.5 | Experimental overview and treatment conditions for study 2

The PBMCs were isolated as described for study 1. Cell Trace was added to the cells from each horse before stimulation. Optimal conditions identified from study 1 indicated that fresh levamisole at 1 μg/mL with conA significantly decreased PBMC proliferation as compared to conA stimulation alone. Therefore, treatment conditions that were identified to determine how levamisole altered the immune phenotype were 5 μg/mL conA and 1 μg/mL fresh levamisole. For comparison, cells were cultured in media only (unstimulated), conA (5 μg/mL) only, and levamisole (1 μg/mL made fresh) only. Cells were stimulated for 72 hours before harvesting for flow cytometry.

2.6 | Proliferation based on CellTrace Violet staining

Cells were stained with CellTrace Violet Cell Proliferation Assay (Life Technologies) before plating. Briefly, 0.2 μL of the CellTrace solution per milliliter of cells (for a final working solution of 1 μM CellTrace) was added to the cells, which then were mixed gently. The cells were incubated at 37°C for 20 minutes. A solution of RPMI with 1% FBS was added to quench the samples for 5 minutes. The cells were centrifuged at 1300 rpm × 5 minutes. Cells were resuspended in prewarmed complete media and counted. Samples were cultured with media only (unstimulated), with mitogen (5 μg/mL conA) only, with mitogen (5 μg/mL conA) and levamisole (1 μg/mL made fresh), and with levamisole alone (1 μg/mL made fresh).

2.7 | Cell surface, activation marker, and intracellular cytokine staining

Cells were plated and stimulated at 5×10^6/mL 100 μL/well for a total of 72 hours in complete media. Intracellular cytokine staining was performed using a commercially available kit, Cytofix/Cytoperm (BD Biosciences), according to the manufacturer's instructions. After 48 hours of stimulation, brefeldin A (1 μL/mL) was added to each sample. Cells were resuspended and stained with optimized concentrations of cell surface antibodies CD21 (BD557327), CD4 (Abd Serotec MCA1078F), CD8 (Abd Serotec MCA1080PE), CD14 (Wagner laboratory clone 105), CD172a (Kingfisher Biotech WS0567B-100) and activation markers major histocompatibility complex (MHC) class II (Abd Serotec MCA1085F), CD86 (BD Biosciences 555 665), and FoxP3 (ebioscience 53-4776) were added to the samples in different combinations (Table 2) to allow determination of subset-specific activation and cytokine production and were incubated at 4°C for 20 minutes. The samples were then washed twice, fixed, and permeabilized with a commercially prepared solution of formaldehyde and saponin (Cytofix/Cytoperm reagent). Samples were incubated at 4°C for 30 minutes, and then cells again were washed with a commercially available solution of FBS and saponin from the Cytofix/cytoperm kit. Antibodies against intracellular cytokines (IFN-γ; AbD Serotec MCA1783A647), and the remainder of antibodies from the Wagner laboratory: IL-4 (clone 13G7), IL-10 (clone 165-2) were diluted in FBS/saponin solution and added to each well. As appropriate based on primary antibody (CD14, DH59B), the secondary antibody was added as needed: (ebioscience 46-4015) Peridinin Chlorophyll Protein Complex (PerCP-ef710). Samples were incubated for 30 minutes and subsequently washed with the FBS/saponin solution and resuspended in phosphate buffered saline. Samples were stored overnight at 4°C and analyzed the next day using a fluorescent activated cell sorting (FACS) Aria flow cytometer.

2.8 | Flow cytometric analysis using the FACS Aria

The percentages of each cell subset (CD4, CD8, CD21, CD14, and CD172a) as well as the percentages of each cell subtype producing IFN-γ, IL-4, and IL-10 were determined. The percentage of CD14 and CD172a positive cells expressing activation markers MHC class II and CD86 were determined. The percentage of CD4 cells expressing FoxP3 was determined. Changes in the percentages of each cell subtype, cytokine production, and activation marker expression with each

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TABLE 2 Staining combinations used for immune phenotype analysis

Cell surface marker	Cytokine	Subset detection
1. CD21		B cell
2. CD4	IFN-γ	Th1/Tc1
CD8		
3. CD4	IL-4	Th2
CD8		
4. CD4	IL-10	Treg
FoxP3		
5. CD8	IL-10	
6. CD14	IFN-γ	DC activation and function (DC1)
CD86		
MHC class II		
7. CD14	IL-4	DC activation and function (DC2)
CD86		
MHC class II		
8. CD14	IL-10	DC activation and function for Treg pathway
CD86		
MHC class II		
9. CD172a	IFN-γ	M1 activation and function
CD86		
MHC class II		
10. CD172a	IL-4	M2 activation and function
CD86		
MHC class II		
11. CD172a	IL-10	M activation and function for Treg pathway
CD86		
MHC class II		

Abbreviations: CD, cluster of differentiation; conA, concanavalin A; DC, dendritic cell; IFN, interferon; IL, interleukin; MHC, major histocompatibility complex; Th, T helper; Treg, T regulatory.

treatment were calculated and analyzed for statistical significance. Percentages of dividing (proliferating) cells were determined based on CellTrace.

2.8.1 | IL-6 cytokine production on cell supernatants (Genorise Scientific)

One hundred microliters of each sample or standard were added to the plate following the template and incubated 2 hours at RT. Samples were removed by aspiration and 1X wash buffer was used for 3 washes. A 100 μL working dilution detection antibody was added to all wells, and the plate incubated for 2 hours at RT. The aspiration and wash were repeated for 3 washes. One hundred microliters of the working dilution of conjugate were added to all wells and incubated for 20 minutes at RT. The aspiration and wash were repeated for 3 washes. One hundred microliters of the substrate solution were added to each well and incubated for 15 minutes. Fifty microliters of the stop solution were added to each well. The optimal density was determined at 450 nm.

2.8.2 | Cytokine production by stimulated supernatants

Samples also were analyzed by the Equine 5 plex assay for IFN-α, IL-4, IL-10, IFN-γ, and IL-17.

2.9 | Statistical analysis

2.9.1 | BrdU data

A mixed model analysis of variance (ANOVA) was used to analyze the proliferation data for statistical significance comparing treated samples to untreated samples. A Freidman Chi Square was used to examine the difference between each levamisole treatment compared to untreated cells, and between mitogen with each levamisole treatment compared to mitogen stimulation alone. Statistical significance was set at $P < .05$.

2.9.2 | Flow cytometry data

A mixed model ANOVA was used to analyze the flow cytometry data for statistical significance comparing treated cells to untreated cells, and comparing levamisole treatment with mitogen to mitogen treatment alone. Statistical significance was set at $P < .05$.

3 | RESULTS

3.1 | Brdu proliferation assay

Concanavalin A and PMA/I with or without levamisole resulted in statistically significant greater differences compared to unstimulated cells. Levamisole alone did not induce any significant changes in proliferation (Figure 1). Concanavalin A with fresh levamisole at 1 μg/mL, predicted to induce levamisole metabolite product 1, significantly decreased proliferation compared to conA alone (Figure 2).[16]

3.2 | Immune phenotyping based on subset-specific activation

Limited changes were observed potentially because of the effect of CellTrace on viability, activation, and proliferation. Concanavalin A with or without levamisole induced significant increases in CD172/CD86 double positive cells compared to unstimulated cells alone. Both conA and conA with levamisole induced significant increases in CD14+ and CD172a+ MHC class II/CD86 double positive cells compared to unstimulated cells.

Concanavalin A with levamisole induced a significant increase in CD4. Concanavalin A with or without levamisole induced a significant increase in CD4 FoxP3 expression compared to unstimulated cells.

3.3 | Immune phenotype based on subset-specific cytokine production

Concanavalin A with or without levamisole induced a significant increase in CD4 IFN-γ, CD8 IFN-γ, CD14 IFN-γ, and CD172a IFN-γ production compared to unstimulated cells (Figure 3). Concanavalin A with or without levamisole induced a significant increase in CD4 IL-4, CD14 IL-4, CD14 IL-4, and CD172a IL-4 compared to unstimulated cells. Concanavalin A with or without levamisole induced a significant increase in CD4 IL-10, CD8 IL-10, CD14 IL-10, and CD172a IL-10 compared to unstimulated cells. No significant differences in cytokine production were observed between conA with levamisole and conA alone.
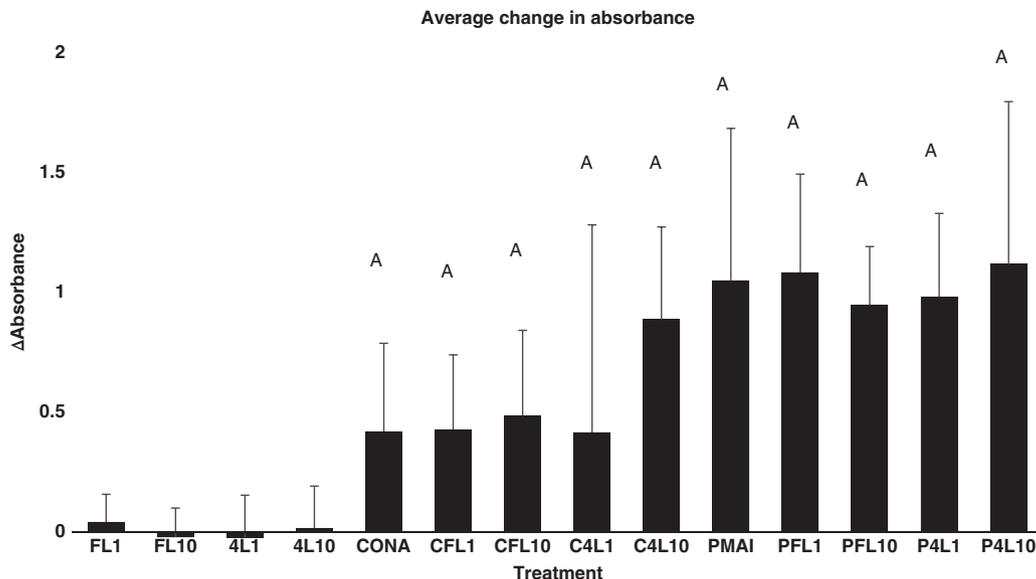
FIGURE 1 Average change in absorbance of stimulated cells compared to unstimulated cells. Isolated PBMCs from each horse were plated in triplicate at 2×10^5 cells/well in complete media at 37°C. The following treatment conditions were used: No mitogen, FL1 = 1 μg/mL levamisole freshly made, FL10 = 10 μg/mL levamisole freshly made, 4 L1 = 1 μg/mL levamisole stored at 4°C for 2 weeks, 4 L10 = 10 μg/mL levamisole stored at 4°C for 2 weeks, conA = Concanavalin A 5 μg/mL alone, CFL1 = conA and 1 μg/mL levamisole freshly made, CFL10 = conA and 10 μg/mL levamisole freshly made, C4L1 = conA and 1 μg/mL levamisole stored at 4°C for 2 weeks, C4L10 = conA and 10 μg/mL levamisole stored at 4°C for 2 weeks, PMAI = Phorbol myristate acetate 20 μg/mL and ionomycin 10 pg/mL (PMA/I), PFL1 = PMA/I and 1 μg/mL levamisole freshly made, PFL10 = PMA/I and 10 μg/mL levamisole freshly made, P4L1 = PMA/I and 1 μg/mL levamisole stored at 4°C for 2 weeks, P4L10 = PMA/I and 10 μg/mL levamisole stored at 4°C for 2 weeks. After 48 hours of stimulation, 20 μL of BrdU solution (Roche Life Sciences 11647229001) was added to each well. After 12 hours of incubation (72 hours total for cells), plates were harvested and proliferation was determined (Roche Life Sciences 11647229001). Statistically significant differences compared to unstimulated cells are marked with an "A" $P < .05$. The error bars represent SD.
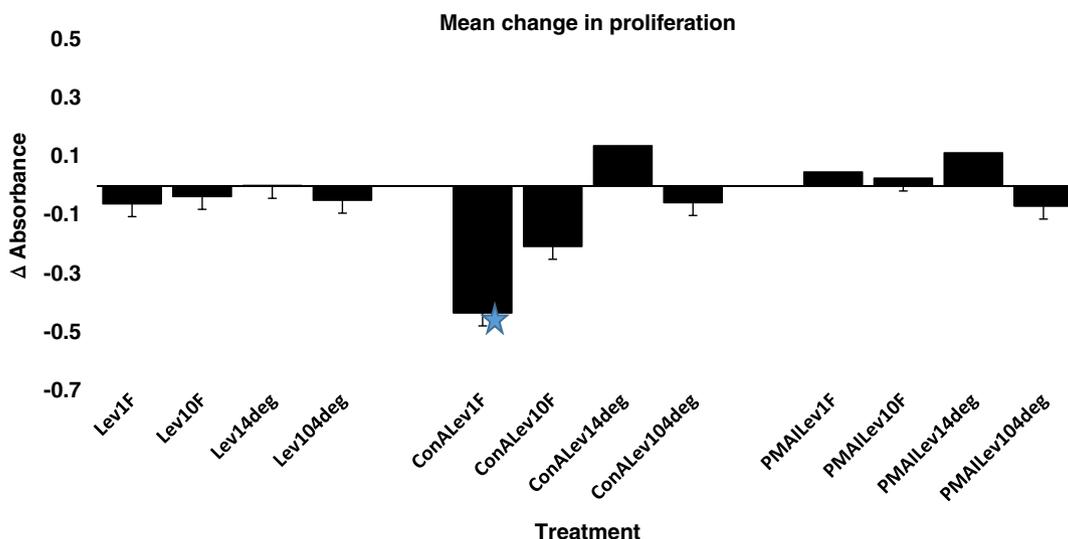

FIGURE 2 Mean change in proliferation with levamisole treatment compared to unstimulated cells, conA (5 μg/mL) stimulated cells, or PMA/I (20 μg/mL/10 pg/mL) stimulated cells. Isolated PBMCs from each horse were plated in triplicate at 2×10^5 cells/ well in complete media at 37°C. the following treatment conditions were used: No mitogen, FL1 = 1 μg/mL levamisole freshly made, FL10 = 10 μg/mL levamisole freshly made, 4 L1 = 1 μg/mL levamisole stored at 4°C for 2 weeks, 4 L10 = 10 μg/mL levamisole stored at 4°C for 2 weeks, conA = Concanavalin A 5 μg/mL alone, CFL1 = conA and 1 μg/mL levamisole freshly made, CFL10 = conA and 10 μg/mL levamisole freshly made, C4L1 = conA and 1 μg/mL levamisole stored at 4°C for 2 weeks, C4L10 = conA and 10 μg/mL levamisole stored at 4°C for 2 weeks, PMAI = Phorbol myristate acetate 20 μg/mL and ionomycin 10 pg/mL (PMA/I), PFL1 = PMA/I and 1 μg/mL levamisole freshly made, PFL10 = PMA/I and 10 μg/mL levamisole freshly made, P4L1 = PMA/I and 1 μg/mL levamisole stored at 4°C for 2 weeks, P4L10 = PMA/I and 10 μg/mL levamisole stored at 4°C for 2 weeks. After 48 hours of stimulation, 20 μL of BrdU solution (Roche Life Sciences 11647229001) was added to each well. After 12 hours of incubation (72 hours total for cells), plates were harvested and proliferation was determined (Roche Life Sciences 11647229001). Change in proliferation between a mitogen with levamisole minus levamisole alone were determined. Statistically significant differences compared to unstimulated cells are marked with a star, $P < .05$. The error bars represent SD.
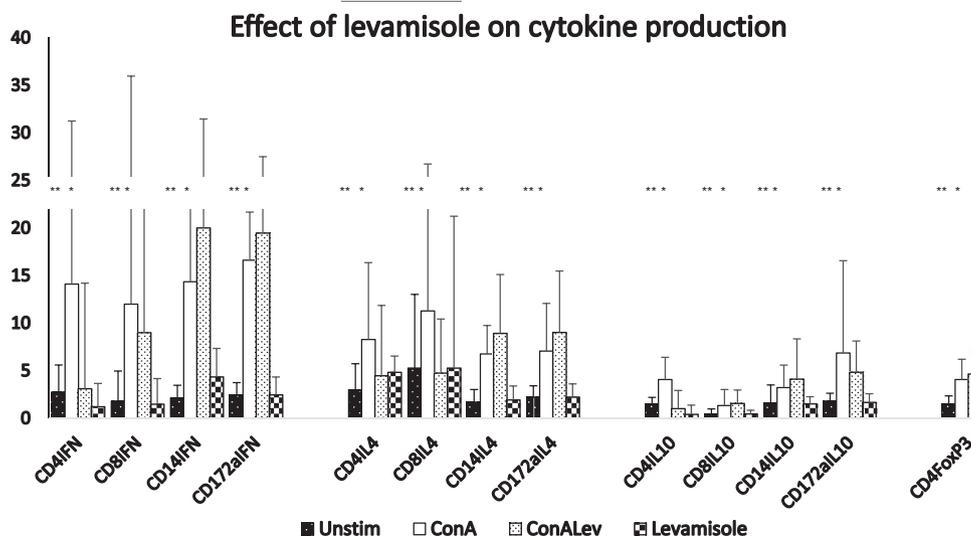
FIGURE 3 Mean percentage of cells producing cytokines compared in each group of cells. Isolated PBMCs from each horse were stained with CellTrace and then plated at 5×10^5 cells/well in complete media at 37°C. cells were stimulated with the following treatment conditions for a total of 72 hours: No stimulation, FL1 = 1 μg/mL levamisole freshly made, conALev = conA and 1 μg/mL levamisole freshly made, Con A 5 μg/mL alone, levamisole = 1 μg/mL levamisole freshly made. Cell surface staining as well as cytokine determination was performed as fully described in the methods. Percentages of cells producing IFN-γ is indicated in the first group, percentages of cells producing IL-4 is indicated in the second group, and percentages of cells producing IL-10 is indicating in the third group. Percentages of cells expressing FoxP3 is indicated in the last group. The black hashed is unstimulated cells, the clear box represents conA stimulated cells, the white hashed is conA and levamisole stimulated cells, and the black and white hatched represents cells stimulated with levamisole only. Statistically significant differences compared to unstimulated cells are marked with an ** versus *; $P < .05$. The error bars represent SD.

3.4 | Immune phenotype based on cell supernatant cytokine production by multiplex or ELISA

The concentrations of IFN-α, IL-4, IFN-γ, IL-17, and IL-10, all were significantly increased by the addition of conA or conA with levamisole as compared to unstimulated cells. However, there were no significant differences between conA and conA with levamisole. No significant differences were observed for IL-6 (Table 3).

3.5 | Determination of proliferation using CellTrace

Although we followed the protocol for Cell Trace and optimized it for our laboratory, after additional review of our data, we determined that CellTrace induced some degree of toxicity in our cells. Because we believe this toxicity impacted our proliferation results as detected by CellTrace, these data are not presented. Although the CellTrace may have influenced our remaining data, the results still are significant and valid under the conditions reported.

4 | DISCUSSION

Our data from study 1 supports that fresh levamisole at 1 μg/mL with conA induced a significant decrease in proliferation compared to conA only. Therefore, these conditions were used for study 2 to identify the immune phenotype associated with levamisole. Our data indicate that conA with levamisole and conA alone induced a significantly different phenotype compared to unstimulated cells, but conA with levamisole did not stimulate a significantly different phenotype than conA alone. With conA as well as conA with levamisole, based on cytokine production, we did see increased numbers of CD4 Th1 IFN-γ CD8 IFN-γ-producing cells compared to other cytokine production by those cells (Figure 3). Table 2 suggests, based on cell supernatants, that both conA and conA with levamisole stimulate a mixed innate and CD4 and CD8 response based on significantly increased innate factors (IL-6, IFN-α) as well as IFN-γ, IL-4, IL-10, and IL-17.

TABLE 3 Cytokine production from supernatants based on median ± SE

	IL-6	IFN-α	IL-4	IFN-γ	IL-17	IL-10
Unstimulated	0 ± 0	0 ± 0**	1075 ± 314.90**	107 ± 156.78**	400 ± 250.97**	1454.5 ± 253.41**
conA	40 ± 23.61	6 ± 6.68*	13 463 ± 4789*	385.5 ± 193.97*	3380 ± 687.8*	2594 ± 337.1*
conALev	40.5 ± 22.1	6.5 ± 17.84*	19 047 ± 4433.75*	559 ± 194.18*	4275.5 ± 745.79*	2963.5 ± 381.4*
Levamisole	0 ± 0	0 ± 0.13	827 ± 342.01	128 ± 148.4	372.5 ± 162.89	1193.5 ± 262.91

$P < 0.05$; ** versus *.
Abbreviations: conA, concanavalin A; IFN, interferon; IL, interleukin.

Our initial results from study 1 were unanticipated. We predicted that levamisole with a mitogen would increase proliferation, likely because of the formation of levamisole product 2. Concanavalin A or PMA/I with levamisole at 10 μg/mL significantly (Figure 1) increased proliferation as compared to cells alone. However, when we calculated the change in proliferation between mitogen with levamisole minus mitogen alone, the conA with freshly made levamisole at 1 μg/mL significantly inhibited PBMC proliferation. Although we predicted, based on decreased proliferation that levamisole product 1 was present and had decreased proliferation, limited resources prevented identification of the levamisole metabolites present. In future studies, the levamisole products should be determined.

Our results also were unexpected that levamisole with conA did not induce a significantly different immune phenotype than did conA alone. One factor that may have affected these results is individual animal variability, which may have limited our ability to identify a significant difference. Another possibility is that conA with levamisole induced a different immune phenotype beyond what we measured. Although we measured traditional markers for immune phenotypes, conA with levamisole may have induced other changes in macrophage, dendritic cell (DC), CD4, CD8 activation and function including CD80, TNF-α, IL-12, CD69, CD44, or other markers. Some of these other markers could be analyzed in future studies.

Additionally, Cell Trace may have affected our cell viability, cell activation, cytokine function, or all, which could have limited our ability to detect a difference in the immune phenotypes. Although we optimized Cell Trace conditions, when we later reviewed the data, it was apparent that it still affected the viability of the cells. Although the results and differences were significant and meaningful, all the data from study 2 was collected using Cell Trace treated cells, and therefore our flow cytometry cytokine results may have been affected. Our cytokine results, using the cell supernatants from study 1 with untreated CellTrace treated cells, had similar results, in that differences found between conA alone as compared to conA with levamisole as compared to media but not conA with levamisole as compared to conA alone. These results support the validity of our findings. However, in future studies, another vital dye should be used to determine proliferation.

If levamisole product 1 is present, this potentially could decrease CD4 and CD8 cytokine production. Future studies should determine whether levamisole differentially activates macrophage and DC as compared to CD4 and CD8 cells.

In conclusion, we determined that conA with fresh levamisole at 1 μg/mL significantly decreased conA-induced proliferation. Levamisole did not alter the immune phenotype induced by conA based on the end points we measured. Concanavalin A alone and conA with levamisole induced a mixed IL-6, IFN-α, IFN-γ, IL-4, IL-10, and IL-17 response. Total supernatants overall had increased cytokine concentrations indicating that levamisole had an effect on inhibiting some cytokines but not others. Based on these results, some of the cell types that levamisole could be affecting based on cytokine production include macrophages and DC which can produce any of the following: IL-6, IFN-γ, IL-4, IL-10, and IL-17. T cells also could be affected based on IFN-γ, IFN-α, IL-4, IL-10, and IL-17 production. Natural killer (NK) cells could be involved based on IFN-γ production, and IFN-α

also is produced by macrophages, NK cells, and B and T cells. Further studies are warranted to elucidate the precise mechanisms and signal transduction pathway by which levamisole operates. Levmisole potentially could be used in clinical cases to decrease the overall immune response in some inflammatory diseases. Based on our results, levamisole may benefit horses with EPM by modulating an active immune response, but it has little measureable effect on immune function in the absence of stimulation. These findings indicate that levamisole is most effective in modulating an existing active infection or immune response. However, the possible effect of levamisole on immune preparedness (ie, the rate at which specific subsets of stimulated lymphocytes undergo phenotypic differentiation and initiate cytokine production) was not evaluated in our study. Additonal studies are warranted to elucidate these mechanisms.

CONFLICT OF INTEREST DECLARATION

Dr. Ellison owns Pathogenes and sells levamisole alone or in combination with decoquinate as treatment for equine protozoal myeloencephalitis. Pathogenes performed the IL-6 ELISA. Other than providing the IL-6 data, Pathogenes did not have access to the data until it was presented in the manuscript.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off label use of microbials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Manuscript had IACUC approval, VT14-097.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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