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ORIGINAL ARTICLE

Analysis of the immunomodulatory properties of mycobacterium cell wall fraction on the cytokine production of peripheral blood mononuclear cells of healthy dogs

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Abstract

Background: Mycobacterium cell wall fraction (MCWF) is derived from nonpathogenic *Mycobacterium phlei* and is used as an immunomodulatory compound in clinical practice, yet its mode-of-action requires further research.

Objective: To evaluate the host response to MCWF in canine peripheral blood mononuclear cells (PBMCs) by using enzyme-linked immunosorbent assays (ELISA) and quantitative reverse transcription (qRT)-PCR for assessment of cytokines.

Animals: Eight healthy Labrador retrievers.

Materials and Methods: PBMCs were isolated from whole blood using density centrifugation. The cells were cultured with different concentrations of MCWF or a potent stimulator of cytokine production, phorbol 12-myristate 13-acetate/ionomycin, or left in cell culture medium for 24, 48 and 72 h. Cytokines were measured by ELISA for interleukin (IL)-4, IL-10 and interferon-gamma (IFN-γ), and by qRT-PCR for IL-4, IL-10, IL-13, IFN-γ, tumour necrosis factor alpha (TNF-α) and transforming growth factor-beta.

Results: A significant increase of IL-10 messenger ribonucleic acid (mRNA) was detected at all time points for all concentrations of MCWF ($p<0.05$). Protein analysis reflected this finding, with a maximum IL-10 concentration of 300.6±38.3 μg/mL. Compared to the negative control, post-stimulation elevation of IFN-γ mRNA was noted at 24 h with all concentrations of MCWF ($p<0.01$), and TNF-α mRNA was increased for 0.5 μg/dL MCWF only at 72 h ($p<0.05$).

Conclusions and Clinical Relevance: MCWF stimulation of PBMCs results in the elevation of both proinflammatory and regulatory cytokine mRNA. Further research into the role of MCWF as a systemically administered regulatory immunomodulator or adjuvant to allergen-specific immunotherapy should be considered.

KEYWORDS
IFN-γ, IL-10, immunocidin, mycobacterium cell wall fraction, peripheral blood mononuclear cells

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INTRODUCTION

The Mycobacteriaceae are known to have immunomodulatory and antitumour activity in both human[1] and veterinary medicine.[2] Original investigations into the use of mycobacterial species as an immunostimulant used Bacillus Calmette–Guerin (BCG) derived from attenuated *Mycobacterium bovis*,[3] while modern mycobacterium cell wall fraction (MCWF) products (Immunocidin; Novavive) use the purified fragments of *Mycobacterium phlei* DNA and cell wall components adjuvanted in 2% squalene to improve the safety profile of these products.[4,5] MCWF also has substantially higher immunostimulatory activity than whole mycobacterial extracts or DNA alone.[6] This formulation consists of high concentrations of muramyl dipeptides (MDP), trehalose dimycolate (TDM), lipid mycolic acid (MA) and glycolipid lipoarabinomannan (LAM).

In vitro, immunomodulatory mechanisms of MCWF have been attributed to interleukin (IL)-12 synthesis by macrophages,[7] induction of tumour necrosis factor alpha (TNF-α) and IL-1β gene transcription[8] and enhanced lymphocyte proliferation.[9] One in vitro study has been performed in dogs, which showed that MCWF inhibited the proliferation of bladder cancer cells via caspase-3-mediated apoptosis.[10]

In vivo, MCWF has been used primarily in a series of open, uncontrolled veterinary pilot studies to treat proliferative and neoplastic disorders.[11–13] However, one longitudinal study combined MCWF as an immunomodulator with *Culicoides* allergen to successfully treat horses with *Culicoides* hypersensitivity,[14] while other attempts at hyposensitisation with crude *Culicoides* extract failed.[15,16]

In order to evaluate the potential role of MCWF as an immunomodulator during immunotherapy in dogs, further understanding of its interaction with the canine immune system must be established. The objective of this study was to characterise the immune response of MCWF in an in vitro model. The messenger ribonucleic acid (mRNA) and protein levels of cytokines in peripheral blood mononuclear cells (PBMCs) were measured following stimulation by MCWF at three different time points. The use of PBMCs as a model is an inexpensive and practical way of studying the host response with minimal impact on animal welfare. To the best of the authors' knowledge, no previous studies have investigated the effect of MCWF on canine PBMCs.

It was hypothesised that MCWF would induce a T-helper (Th)-1-mediated response such as that expected of a mycobacterial infection.[17]

MATERIALS AND METHODS

Ethics

All procedures were in accordance with the guidelines defined by the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care, 1993) and the Animal Care Ethics Committee of the University of Guelph Ontario Veterinary College, University of Guelph. Informed consent was obtained from the owner of the dogs used in this experiment.

Study population and sample collection

Eight intact, female, Labrador cross dogs ranging in age from 1.5 to 5.2 years of age (mean 3.7 ± 0.47 years) and weighing between 22.5 and 29.6 kg (mean 25.8 ± 0.89 years) were enrolled in the study. All dogs were judged to be healthy based on normal clinical history and physical examination. The dogs did not receive any medication for at least three months before enrolment or vaccinations within a month of blood collection. At least 10 mL of blood were obtained from the cephalic veins and immediately placed into ethylenediaminetetraacetic acid (EDTA)-coated Vacutainer tubes (Becton Dickinson). The tubes were kept on ice, and PBMCs were isolated within two hours of collection.

PBMC isolation and stimulation

Cells were isolated by density gradient centrifugation using Histopaque 1077 (Sigma-Aldrich) according to methods reported previously.[18] Briefly, whole blood from each dog was diluted 1:1 with phosphate-buffered saline (DPBS; Wisent Bio) and layered onto 4 mL of Histopaque (Sigma-Aldrich) with a density of 1.077 g/mL in separate 15 mL conical centrifuge tubes (ThermoFisher Scientific). After centrifugation without braking (21 min, 740 ***g***, 4°C), the concentrated PBMCs at the interphase were removed by pipetting. The cells were washed twice in a PBS-PenStrep solution (Gibco by Life Technologies; ThermoFisher Scientific) at 4°C and resuspended thereafter in 5 mL of complete cell culture medium (445 mL of RMPI-2640 [Gibco by Life Technologies], 50 mL of 10% heat-inactivated fetal bovine serum [Gibco by Life Technologies] and 5 mL of PenStrep). Cells were kept on ice from this point onwards. PBMC viability and number were determined by 0.02% trypan blue staining (ThermoFisher Scientific) and manually counted using a haemocytometer. Dogs that had an insufficient PBMC yield ($<7.2 \times 10^6$ viable cells) were excluded from the study. Cell density was adjusted to 5×10^5 cells/mL.

The PBMCs were isolated and incubated with a potent stimulator of cytokine production, phorbol 12-myristate 13-acetate 50 ng/mL and Ca^{2+} ionomycin 1 μg/mL (PMA/ionomycin; ThermoFisher Scientific) (positive control), the cell culture medium (negative control) or 0.5, 2.5, 12.5 or 62.5 μg/mL of MCWF. The plates were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 24, 48 and 72 h. A total of 2×10^5 PBMCs were cultured with MCWF in 24-well flat-bottom culture plates (Corning Inc.) in a total volume of 800 μL of complete cell culture medium. Duplicate cultures were stimulated for each of the

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six treatment groups per dog. Following stimulation, the plates were centrifuged at 400 ***g*** for five minutes, and then 600 μL of supernatant were collected into Eppendorf tubes (ThermoFisher Scientific) and frozen at –80°C until further analysis. Any early adherent cells were washed from the base of the culture plates using gentle pipetting to avoid loss of macrophages owing to plastic adherence. The cells were lysed in 500 μL of TRIzol reagent (ThermoFisher Scientific) and stored separately at –80°C.

RNA extraction and quantitative reverse transcription (qRT)-PCR

After stimulation, RNA was extracted from the PBMCs by using TRIzol, as described previously.[19] RNA was re-suspended in 12.5 μL of ultrapure, nuclease-free water (DEPC-dH2O). Residual genomic DNA was digested by DNase I (ThermoFisher Scientific). RNA quality and yield were measured using nanodrop spectrophotometry (ThermoFisher Scientific). Samples had a final concentration of 20.3–308.1 ng/μL. RNA was standardised to 50 ng per reaction pending complementary DNA (cDNA) synthesis. The recovered RNA was stored at –80°C until cDNA was synthesised using a modified Superscript II protocol.[20] Quantification of mRNA concentration was performed by qRT-PCR using SYBR green technology (QuantiTect SYBR Green PCR Kit; Qiagen). Primers were grouped based on similar annealing temperatures. The plates were transferred to the LightCycler 480 II (Roche Diagnostics) and pre-incubated at 95°C for five minutes, followed by 50 cycles at 95°C for 20 s, and 55–65°C (primer-specific annealing temperatures) for 15 s, in addition to 10 s elongations at 72°C. The melting curve was done by 10 s incubation at 95°C. The reaction was then cooled down to 65°C for one minute, followed by heating to 95°C. Canine β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes were used as the reference genes to calculate the relative expression of all selected cytokines in this study. Primers were designed for Th1-related genes (interferon-gamma [IFN-γ] and TNF-α), Th2-related genes (IL-4 and IL-13) and genes associated with regulatory function (IL-10, transforming growth factor [TGF]-β and forkhead box P3 [FOXP3]) based on the sequences described in other canine studies using the Primer-Blast designing tool (Table 1). The primers were sourced from Invitrogen Custom Primers (ThermoFisher Scientific).

Enzyme-linked immunosorbent assay

The supernatant concentrations of the cytokines IFN-γ, IL-10 and IL-4 were determined using DuoSet enzyme-linked immunosorbent assays (ELISA) Development Systems (R&D Systems, Inc.) with detection limits of 31.3–2000, 31.3–2000 and 93.8–6000 pg/mL, respectively. All samples were analysed in duplicate, in accordance with the manufacturer's instructions. Absorbance values were read at 450 nm (reference

TABLE 1 Primer sequences for quantitative reverse transcription PCR.

Target gene	Forward primer sequence	Reverse primer sequence	Annealing temperature (°C)	Accession number/reference sequence	Reference
TNF-α	TCATCTTCTCGAACCCCAAG	ACCCATCTGACGGCACTATC	60	NM_001003244.4	23
IL-10	TCTGTTGCTGCCTGGTCCT	TGATGTCTGGGTCGTGGTT	54.3	NM_001003077.1	24
FOXP3	CAAATGGTGTCTGCAAGTGG	GTGCTCTGCCCTTCTCATCT	59	NM_001168461.1	25
IL-4	CCAAAGAACACAAGCGATAAGGAA	GTTTGCCATGCTGCTGAGGTT	61	AF091132.1	25
IL-13	GAGGAGCTGGTCAACATCA	TGCAGTCGGAGACATTGA	59	AF244915.1	25
TGF-β	CAAGGATCTGGGCTGGAAGTGGA	CCAGGACCTTGCTGTACTGCGTGT	65	NM_001003309.1	25
IFN-γ	AGCGCAAGGCGATAAATG	GCGGCCTCGAAACAGATT	55.8	NM_001003174.1	25
β-Actin	CCGCCTATTCCAGGATTCTCT	GGACCTTCCCAACCCTGTTAG		AF021873	26
GAPDH	AAGGCTGAGAACGGGAAACT	TACTCAGCACCAGCATCACC		AB038240.1	27

Abbreviations: FOX, forkhead box; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFN, interferon; IL, interleukin; TGF, transforming growth factor; TNF, tumour necrosis factor.

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wavelength) using an automatic microELISA reader (Bio-Tek Instruments). Cytokine concentrations were calculated by interpolation from a standard curve obtained with the controls provided in the kits.

Statistical methods

Raw ELISA optical density values were converted to cytokine concentrations based on the standard curve using GainData (Arigo Biolaboratories; https://www.arigobio.com/elisa-analysis). Specific cytokine concentrations were extrapolated using the four-parameter logistic model, as recommended by the manufacturer of the ELISA kits.

A Shapiro–Wilk test was performed to assess normality of data. Outliers were identified and excluded from analysis if the data was not normally distributed. A total of 13 outliers were identified and excluded if there were more than three data points available. Following this, a one-way ANOVA with Tukey's pairwise comparison using PRISM 10 (GraphPad, Inc.) software was performed on normal data to establish which specific treatments and time points shared grouping. Concentrations of mRNA in the treatment groups were determined using the LightCycler 480 software (Roche Diagnosticsand) relative to the reference genes β-actin and GAPDH. Logarithmic transformations were performed when the error deviations did not have homogenous variance across treatment groups. Analysed data are presented with means plus standard deviation (SD). PRISM 10 (GraphPad, Inc.) was used for graphical representations. A *p*-value of <0.05 was considered statistically significant, and asterisks were used to indicate the level of significance on the figures.

RESULTS

Three of the eight dogs (Dogs 1, 4 and 8) were excluded from the study as a consequence of suboptimal PBMC yield. For the other samples, the percentage of viable cells ranged from 98% to 100%. However, the expression of IL-13 (160 of 180) and FOXP3 (139 of 180) was not detectable in most of the samples, so these results were excluded from the analysis.

The qRT-PCR results revealed that IL-10 mRNA was elevated for MCWF at 0.5 and 2.5 μg/dL ($p<0.001$), as well as 12.5 and 62.5 μg/dL ($p<0.01$) compared to the negative control at 24 h (Figure 1). Interestingly, at 24 h, there seemed to be an inverse concentration-dependent increase of IL-10, with the highest gene expression induced by 0.05 μg/dL of MCWF. Likewise, elevations of IL-10 mRNA in cells stimulated by all concentrations of MCWF were found at 48 ($p<0.01$) and 72 h ($p<0.05$) compared to the positive and negative controls (Figure 1). PMA ionomycin did not cause any increase of IL-10 mRNA compared to the negative control at any time point.

IFN-γ mRNA was increased for all concentrations of MCWF compared to the negative control at 24 h ($p<0.01$) and not at 48 and 72 h (Figure 2). IFN-γ mRNA was increased by PMA/ionomycin stimulation at all time points compared to the negative control. An increase of TNF-α mRNA was detected in cells stimulated with PMA/ionomycin at 48 ($p<0.05$) and 72 hours

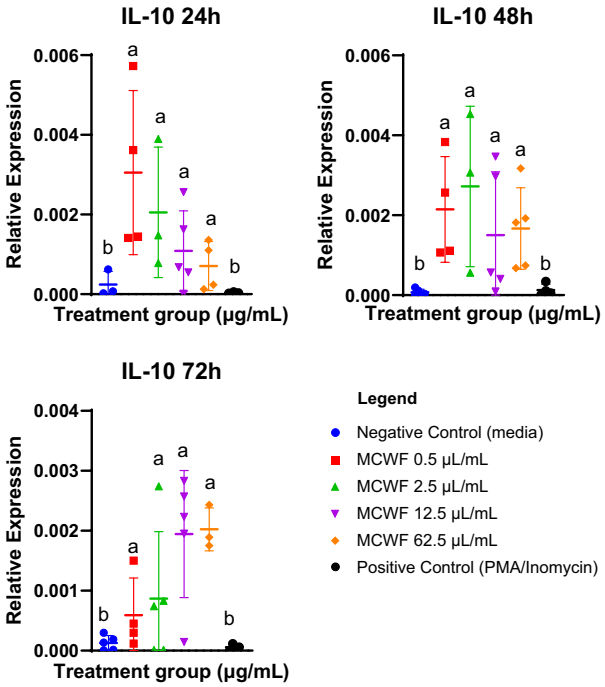

FIGURE 1 Interleukin (IL)-10 messenger (m)RNA at 24, 48 and 72 h postincubation of peripheral blood mononuclear cells (PBMCs) with mycobacterium cell wall fraction (MCWF). Group means that share the same letter do not differ significantly ($p<0.05$). Mean is represented with the straight line and the standard deviation is represented with bars.

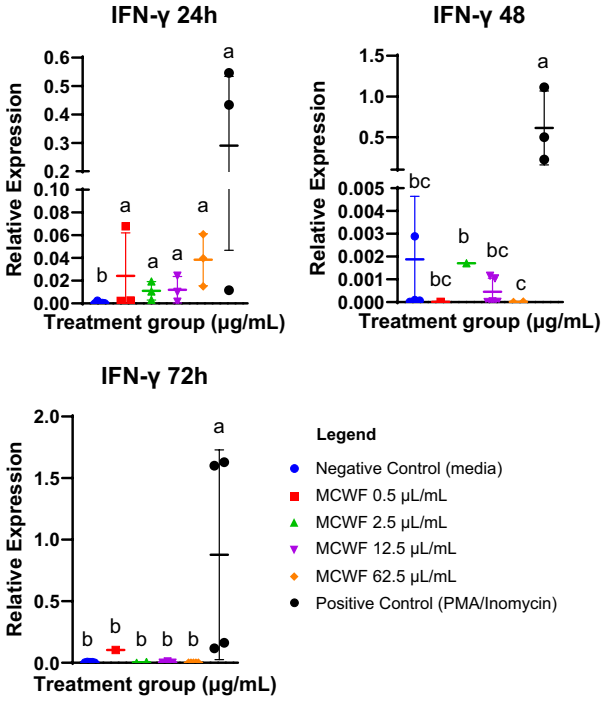

FIGURE 2 Interferon (IFN)-γ messenger (m)RNA at 24, 48 and 72 h postincubation of peripheral blood mononuclear cells (PBMCs) with mycobacterium cell wall fraction (MCWF). Group means that share the same letter do not differ significantly ($p<0.05$). Mean is represented with the straight line and the standard deviation is represented with bars.

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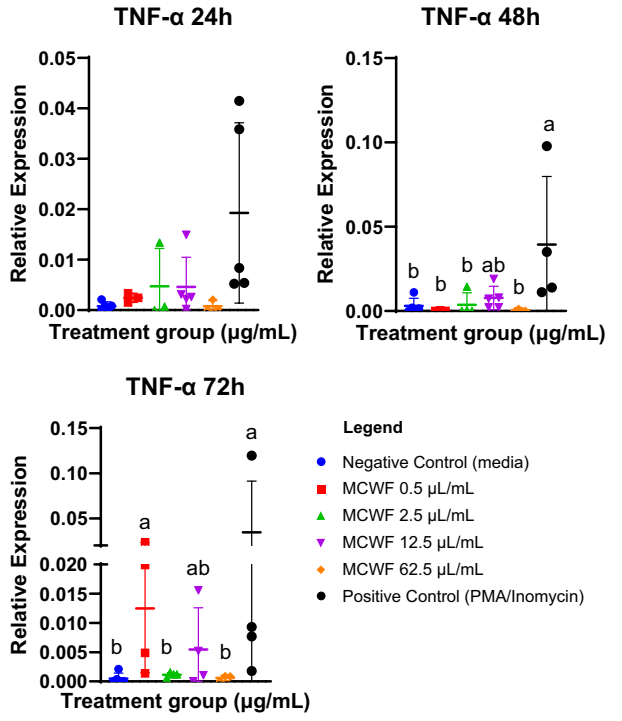

FIGURE 3 Tumour necrosis factor (TNF)-α messenger (m)RNA at 24, 48 and 72 h postincubation of peripheral blood mononuclear cells (PBMCs) with mycobacterium cell wall fraction (MCWF). Group means that share the same letter do not differ significantly ($p<0.05$). Mean is represented with the straight line and the standard deviation is represented with bars.

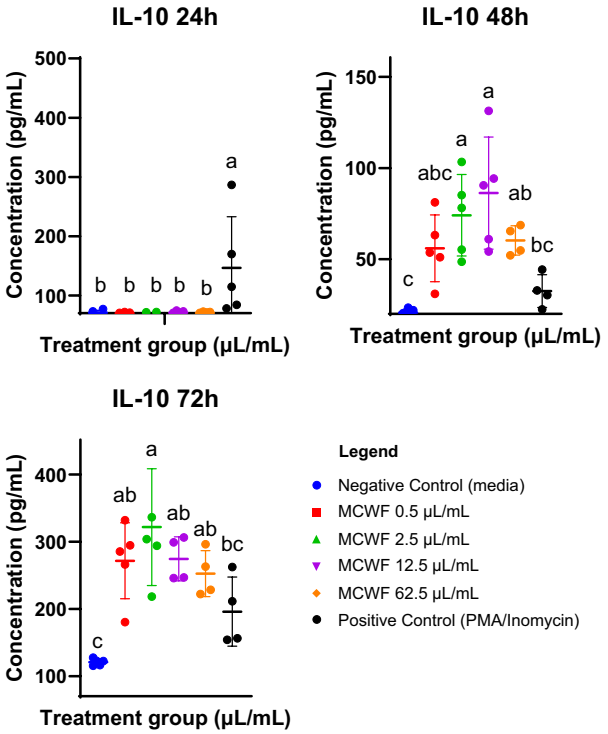

FIGURE 4 Interleukin (IL)-10 protein concentration measurement by sandwich ELISA at 48 and 72 h postincubation of peripheral blood mononuclear cells (PBMCs) with mycobacterium cell wall fraction (MCWF). Group means that share the same letter do not differ significantly ($p<0.05$). Mean is represented with the straight line and the standard deviation is represented with bars.

($p<0.001$) compared to the negative control. At 72 h, a significant difference in the relative expression of TNF-α was noted between the 0.5 μg/dL MCWF treatment group and the negative control ($p<0.05$), and at no other concentrations of MCWF (Figure 3). There were no significant changes in the relative expression of IL-4 or TFG-β following incubation with MCWF.

IL-10, IFN-γ and IL-4 production were measured by ELISA from supernatants of cell cultures following stimulation with MCWF at 0.5, 2.5, 12.5 and 62.5 μg/dL, along with the positive and negative controls at 24, 48 and 72 h postculture. Stimulation resulted in significantly higher concentrations of IL-10 by all MCWF treatment groups when compared to negative controls at both 48 and 72 h ($p<0.01$) and not at 24 h (Figures 4). At 48 h, the concentration of IL-10 was highest in the 12.5 μg/dL MCWF group reaching a peak of 86.28 pg/mL. At 72 h, the greatest IL-10 concentration was induced by the 2.5 μg/mL group achieving 300.6 pg/mL. No detectable IL-4 or IFN-γ was produced at any time point by any concentration of MCWF. Significant elevation of IFN-γ mRNA was detected at all time points following stimulation with PMA/ionomycin.

DISCUSSION

This is the first study examining the effect of MCWF on the ex vivo cytokine production of canine PBMCs. Cytokine mRNA and protein produced by PBMCs following stimulation at three different time points were investigated. Relative expression to constitutively expressed genes (e.g. GAPDH) can help identify changes in the expression of cytokines and, thus, by extrapolation, the immune response. These 'housekeeping genes' can be considered a control to which other genes can be compared.

The first major finding was a significant elevation of IL-10 mRNA among all PBMCs that were stimulated by MCWF. The inverse concentration-dependent increase of IL-10 at 24 h is surprising and may have been to the result of persistent outliers, as reflected in the large SD, particularly given that this was not the case at 48 and 72 h. It cannot be entirely excluded that squalene may have had an immunoregulatory effect[21]; however, it would be expected that this would appear at the highest concentrations of MCWF as well owing to the serial dilutions of the compound. A similar increase of translated IL-10 was observed at 48 and 72 h. IL-10 is a Janus kinase (JAK)-1-dependent cytokine, which is produced mainly by Th cells, T-regulatory (T_{reg}) cells, B-regulatory (B_{reg}) cells and a small fraction of natural killer (NK) cells, neutrophils, macrophages and dendritic cells.

This increase of IL-10 mRNA and protein is consistent with observations from other toll-like receptor (TLR) agonists, such as lipopolysaccharide, which can induce IL-10 as part of a negative feedback mechanism,[22] yet contrasted with our initial hypothesis that there would be a predominantly pro-inflammatory response. A pro-inflammatory response was expected based on its licensed application in neoplastic conditions and raises concerns about the anti-inflammatory effect of MCWF in this context. IL-10 has been shown

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to enhance tumour cell survival, proliferation and metastasis.[23] However, when used locally for neoplastic conditions, a far greater, cytotoxic concentration of MCWF is used.

More specifically, within MCWF, both MDP[10] and glycolipid LAM[24] have been shown to trigger the production of IL-10. IL-10 production during chronic *Mycobacterium tuberculosis* infections promotes disease exacerbation and bacterial growth.[25] Blockade of IL-10 receptor signalling can enhance T-cell recruitment, leading to more prolonged survival associated with reduced bacillary load, which could be part of the pathogenesis of certain strains of mycobacteria.[26] This may explain the increase of IL-10 produced by cells stimulated with MCWF found in this study. Recent studies have found that both innate (monocytes) and adaptive ($CD4^+$ T cells) immune systems express and produce IL-10 during an in vitro mycobacterial infection[27] by both $CD8^+$ T cells[28] and $CD4^+$ cells[29] in murine models. Mycobacteria can also promote C–C motif chemokine receptor type 5 (CCR5) expression in macrophages, leading to increased IL-10 production.[30] Any of these mechanisms could have led to the elevations of IL-10 observed in the present study. Further investigation is warranted into which specific cells within PBMCs are stimulated to produce IL-10 in dogs.

The induction of IL-10 by MCWF could have positive therapeutic potential. Impaired T_{reg}-mediated suppression is considered to be involved in the pathogenesis of atopic dermatitis, and IL-10 increases have been measured following initiation of allergy immunotherapy.[31] IL-10 directly downregulates major histocompatibility complex (MHC) class II and costimulatory molecules on the surface of macrophages and monocytes.[32] Furthermore, IL-10 inhibits the expression of many chemokines, chemokine receptors and proinflammatory cytokines such as TNF-α and IFN-γ.[33]

The second major finding of this study was the increased IFN-γ mRNA that was detected at 24 h following stimulation with MCWF. IFN-γ is a Type II interferon produced by Th1 cells, cytotoxic T cells, B cells, NK cells and macrophages to a lesser extent. It is induced following *M. tuberculosis* infection by NK cells through activation of TLR2, TLR4 and the mannose receptor.[34] A subsequent study showed that MCWF is a potent stimulus for the production of IFN-γ by murine T cells; however, the exact mechanism by which it stimulated the production could not be identified.[35] Within MCWF, lipid MA, MDP and phosphatidylinositol mannosides could all have induced the secretion of IFN-γ by PBMCs.[10,36]

The present study showed that IFN-γ protein was not detected at any of the time points following stimulation of PBMCs with MCWF despite gene transcription increases at 24 h. Increased IFN-γ concentrations were detected on Day (D)4, D7 and D10 in an endometrial equine model following stimulation with MCWF.[37] Therefore, discrepancies in findings could be a consequence of the timing of sample collections or because of the in vitro nature of the experiment. Alternatively, as IL-10 was produced following stimulation of the PBMCs, it may be that, despite early detected IFN-γ mRNA, its subsequent expression was inhibited by the immunomodulatory effect of IL-10. It has been shown previously that IL-10 can inhibit the expression of IFN-γ.[38]

The third significant finding was the elevation of TNF-α mRNA in PBMC cultures stimulated with 0.5 µg/dL of MCWF at 72 h. Several other concentrations of MCWF seemed to induce an increase of TNF-α at all time points; however, despite increased mean C_t values in these groups, there was no significant difference compared to the negative control.

Along with IFN-γ, TNF-α is a critical part of the immune system's anti-mycobacterial response.[39] The main TNF-α-producing cells are macrophages, T cells and dendritic cells. IFN-γ and TNF-α have been found to act synergistically to activate macrophages and produce reactive nitrogen intermediates during tuberculosis infections.[40] Mycobacterial cell wall components induce macrophages to secrete TNF-α through a TLR2-mediated pathway.[41] However, there is evidence that mycobacteria can decrease the production of TNF-α in human PBMCs,[33] which may suppress inflammation and protect these bacteria from rapid destruction. Additionally, the lipid components of mycobacterial cell walls have been shown to dampen the expression of TNF-α from human PBMCs.[28]

A similar immunomodulatory effect was frequently noted between the different concentrations of MCWF at all of the measured time points. This finding is consistent with other in vitro studies, upon which the concentrations of MCWF were based.[42] It would have been interesting to assess MCWF concentrations outside those investigated herein to establish efficacy and toxicity thresholds, yet the limited yield of PBMCs prohibited this.

There are a few limitations to the current study. A small number of healthy, female dogs were included, so care must be taken when extrapolating the effect of MCWF in a larger population and in diseased animals. The dogs were deemed healthy based on a history and clinical examination, yet no haematological analysis was performed to confirm the absence of subclinical disease. Another limitation is that there may be phenotypic differences between the cells found in circulation compared to affected cutaneous tissue, as well as the lack of environmental stimuli to which these cells would be exposed in vivo. For logistical reasons, a limited number of cytokines and concentrations were selected for analysis following stimulation based on anticipated responses. The use of multiplex assays, genome-wide analysis or flow cytometry could have identified changes in gene transcription and protein translation beyond the narrow spectrum of cytokines investigated in this study. Finally, rather than using media alone, the control group should have included the vehicle of the commercial product without the MCWF component, as squalene can modulate the immune response.[21]

In conclusion, the results presented here show that in vitro stimulation of PBMCs with MCWF is associated with an increase of both IL-10 mRNA and protein.

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In addition, IFN-γ and TNF-α mRNA were elevated in some treatment groups. Further studies should focus on further elucidating the underlying intracellular mechanism by which these cytokines are influenced, as well as animal safety and toxicity studies.

AUTHOR CONTRIBUTIONS

Robert Ward: Conceptualization; methodology; software; data curation; investigation; formal analysis; validation; funding acquisition; writing – original draft; writing – review and editing; project administration; visualization. **Geoffrey A. Wood:** Methodology; writing – original draft; conceptualization; supervision. **Charlotte Pye:** Conceptualization; writing – original draft; writing – review and editing; methodology; supervision. **Khalil Karimi:** Supervision; methodology; data curation; investigation; validation. **Anthony Yu:** Funding acquisition; conceptualization; methodology; writing – original draft; writing – review and editing; resources; supervision; visualization. **Myles St-Denis:** Methodology; investigation; data curation; software. **Katherine Blake:** Validation; investigation; methodology. **Sugandha Raj:** Methodology; investigation; writing – original draft. **Samson Oladokun:** Software; data curation; investigation; supervision. **Shayan Sharif:** Conceptualization; methodology; resources; supervision; funding acquisition; writing – original draft; project administration.

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CONFLICT OF INTEREST STATEMENT

Complimentary Immunocidin vials provided by Novavive.

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Zusammenfassung

Hintergrund: Mycobakterieller Zellwandbruch (MCWF) stammt von nicht pathogenem *Mycobacterium phlei* und wird als immunmodulatorischer Bestandteil in der klinischen Praxis eingesetzt, wobei seine Wirkungsweise weiterer Aufklärung bedarf.

Ziel: Eine Evaluierung der Wirtantwort auf MCWF in caninen peripheren Blut Mononuklearzellen (PBMCs) mittels Enzyme-linked Immunosorbent Assays (ELISA) und einem quantitativen real-time (qRT)-PCR zur Bestimmung von Zytokinen.

Tiere: Acht gesunde Labrador Retriever.

Materialien und Methoden: PMBCs wurden mittels Dichtegradienten-Zentrifugation aus Vollblut isoliert. Die Zellen wurden mit verschiedenen Konzentrationen von MCWF oder mit einem potenten Stimulator der Zytokinproduktion, nämlich Phorbol 12-Myristate 13-Acetate/Ionomycin, kultiviert oder für 24, 48 und 72 h im Kulturmedium belassen. Die Zytokine Interleukin (IL)-4, IL-10 und Interferon-Gamma (IFN-γ) wurden mittels ELISA bestimmt und IL-4, IL-10, IL-13, IFN-γ, Tumornekrosefaktor-alpha (TNF-α) und Transforming Growth Factor-beta mittels qRT-PCR.

Ergebnisse: Eine signifikante Zunahme der IL-10 Messenger (m)RNA wurde zu allen Zeitpunkten für alle MCWF-Konzentrationen festgestellt ($p<0.05$). Die Proteinanalyse bestätigte dieses Ergebnis bei einer maximalen IL-10 Konzentration von 300.6±38.3 μg/mL, was auf die Fähigkeit einer regulatorischen Wirkung hinweist. Im Vergleich zur Negativkontrolle wurde nach der Stimulation eine Erhöhung der IFN-γ mRNA nach 24 h bei allen MCWF-Konzentrationen gefunden ($p<0.01$) während festgestellt wurde, dass TNF-α mRNA nur bei 0.5 μg/dL MCWF nach 72 h erhöht war ($p<0.05$).

Schlussfolgerungen und klinische Bedeutung: Die Stimulation von PBMCs mittels Mycobakteriellem Zellwandbruch resultiert in einer Erhöhung sowohl der proinflammatorischen wie auch der regulatorischen Zytokin mRNA. Weitere Studien über die Rolle der MCWF als systemisch verabreichter regulatorischer Immunmodulator oder als Adjuvans bei Allergen-spezifischer Immuntherapie sollte bedacht werden.

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摘要
背景: 分枝杆菌细胞壁成分 (MCWF) 源自无致病性的草分枝杆菌,在临床中用作免疫调节化合物,但其作用方式需要进一步研究。
目的: 使用酶联免疫吸附试验 (ELISA) 和定量实时 (qRT)-PCR 评估细胞因子,评估犬外周血单核细胞 (PBMC) 中宿主对 MCWF 的反应。
动物: 八只健康的拉布拉多猎犬
材料和方法: 使用密度离心法从全血中分离 PBMC。将细胞与不同浓度的 MCWF 或强效的细胞因子产生刺激剂佛波醇 12-肉豆蔻酸酯 13-乙酸酯/离子霉素一起培养,或留在细胞培养基中 24、48 和 72 小时。通过 ELISA 测量细胞因子的白细胞介素 (IL)-4、IL-10 和干扰素-γ (IFN-γ),并通过 qRT-PCR 测量 IL-4、IL-10、IL-13、IFN-γ、肿瘤坏死因子 α (TNF-α) 和转化生长因子-β。
结果: 在所有时间点,在所有浓度的 MCWF 中均检测到 IL-10 信使 (m)RNA 显著增加 ($p<0.05$)。蛋白质分析反映了这一发现,最大 IL-10 浓度为 300.6±38.3μg/mL,表明具有调节作用的潜力。与阴性对照相比,在 24 小时后,所有浓度的 MCWF 均观察到 IFN-γ mRNA 刺激后升高($p<0.01$),而 0.5μg/dL MCWF 仅在 72 小时后 TNF-α mRNA 升高 ($p<0.05$)。
结论和临床意义: PBMCs 的分枝杆菌细胞壁成分刺激会导致促炎和调节性细胞因子 mRNA 升高。应考虑进一步研究 MCWF 作为全身给药调节性免疫调节剂或过敏原特异性免疫疗法佐剂的作用。

Résumé
Contexte: La fraction de paroi cellulaire de Mycobacterium (MCWF) est dérivée de *Mycobacterium phlei* non pathogène et est utilisée comme composé immunomodulateur en pratique clinique, mais son mode d'action nécessite des recherches supplémentaires.
Objectif: Évaluer la réponse de l'hôte à la MCWF dans les cellules mononucléaires du sang périphérique canin (PBMC) en utilisant des tests immuno-enzymatiques (ELISA) et la PCR quantitative en temps réel (qRT) pour l'évaluation des cytokines.
Animaux: Huit Labrador retrievers en bonne santé
Matériels et méthodes: Les PBMC ont été isolés à partir du sang total par centrifugation de densité. Les cellules ont été mises en culture avec différentes concentrations de MCWF ou avec un puissant stimulateur de la production de cytokines, le phorbol 12-myristate 13-acétate/ionomycine, ou laissées dans un milieu de culture cellulaire pendant 24, 48 et 72 heures. Les cytokines ont été mesurées par ELISA pour l'interleukine (IL)-4, l'IL-10 et l'interféron-gamma (IFN-γ), et par qRT-PCR pour l'IL-4, l'IL-10, l'IL-13, l'IFN-γ, le facteur de nécrose tumorale alpha (TNF-α) et le facteur de croissance transformant-bêta.
Résultats: Une augmentation significative de l'ARN (m) messager de l'IL-10 a été détectée à tous les points dans le temps pour toutes les concentrations de MCWF ($p<0.05$). L'analyse des protéines a reflété cette découverte, avec une concentration maximale d'IL-10 de 300.6±38.3μg/mL, indiquant la possibilité d'un effet régulateur. Par rapport au contrôle négatif, l'élévation post-stimulation de l'ARNm de l'IFN-γ a été notée à 24h avec toutes les concentrations de MCWF ($p<0.01$), et l'ARNm du TNF-α a augmenté pour le MCWF de 0.5μg/dL seulement à 72h ($p<0.05$).
Conclusions et pertinence clinique: La stimulation des PBMC par la fraction de paroi cellulaire de *Mycobacterium* entraîne une augmentation de l'ARNm des cytokines pro-inflammatoires et régulatrices. Il convient d'envisager des recherches supplémentaires sur le rôle de la MCWF en tant qu'immunomodulateur régulateur administré par voie systémique ou en tant qu'adjuvant de l'immunothérapie spécifique à l'allergène.

要約
背景: マイコバクテリウム細胞壁画分(MCWF)は、非病原性マイコバクテリウム(*Mycobacterium phlei*)由来の画分であり、臨床において免疫調節化合物として使用されているが、その作用機序についてはさらなる研究が必要である。
目的: 本研究の目的は、酵素結合免疫吸着測定法(ELISA)および定量的リアルタイム(qRT)-PCRを用いたサイトカインの評価により、イヌ末梢血単核球(PBMC)におけるMCWFに対する宿主応答を評価することであった。
供試動物: 健常ラブラドール・レトリバー8頭
材料と方法: PBMCを密度遠心法により全血から分離した。この細胞を、異なる濃度のMCWF、またはサイトカイン産生の強力な刺激因子であるフォルボール12-ミリスチン酸13-アセテート/イオノマイシンとともに、あるいは細胞培養液中に24、48、72時間放置して培養した。サイトカインは、インターロイキン(IL)-4、IL-10、インターフェロン-γ(IFN-γ)についてはELISA法で、IL-4、IL-10、IL-13、IFN-γ、腫瘍壊死因子α(TNF-α)、トランスフォーミング増殖因子βについてはqRT-PCR法で測定した。
結果: IL-10メッセンジャー(m)RNAの有意な増加が、すべてのMCWF濃度においてすべての時点で検出された($p<0.05$)。タンパク質分析もこの所見を反映し、IL-10の最大濃度は300.6±38.3μg/mLであり、調節作用の可能性を示した。陰性対照と比較して、IFN-γ mRNAの刺激後の上昇は、すべての濃度のMCWFで24時間後に認められ($p<0.01$)、TNF-α mRNAは0.5μg/dLのMCWFでのみ72時間後に上昇した($p<0.05$)。
結論と臨床的意義: PBMCのマイコバクテリウム細胞壁画分刺激は、炎症性サイトカインおよび制御性サイトカインのmRNAの上昇をもたらした。全身投与される調節性免疫調節因子またはアレルゲン特異的免疫療法のアジュバントとしてのMCWFの役割について、さらなる研究を検討すべきであった。

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Resumo

Contexto: A fração da parede celular de *Mycobacterium* (MCWF) é derivada de *Mycobacterium phlei* não patogênico e é usada como um composto imunomodulador na prática clínica, mas seu modo de ação requer mais pesquisas.

Objetivo: Avaliar a resposta do hospedeiro ao MCWF em células mononucleares do sangue periférico canino (PBMCs) usando ensaios imunoenzimáticos (ELISA) e PCR quantitativa em tempo real (qRT) para avaliação de citocinas.

Animais: Oito labrador retrievers saudáveis.

Materiais e Métodos: PBMCs foram isoladas do sangue total usando centrifugação de densidade. As células foram cultivadas com diferentes concentrações de MCWF ou um potente estimulador da produção de citocinas, forbol 12-miristato 13-acetato/ionomicina, ou armazenadas em meio de cultura celular por 24, 48 e 72 h. As citocinas interleucina (IL)-4, IL-10 e interferon-gama (IFN-γ) foram medidas por ELISA, e IL-4, IL-10, IL-13, IFN-γ, fator de necrose tumoral alfa (TNF-α) e fator de crescimento transformador-beta por qRT-PCR.

Resultados: Um aumento significativo do mRNA da IL-10 foi detectado em todos os pontos de tempo experimental para todas as concentrações de MCWF ($p<0.05$). A análise de proteínas refletiu essa descoberta, com uma concentração máxima de IL-10 de 300.6±38.3 μg/mL, indicando o potencial para um efeito regulatório. Comparado ao controle negativo, a elevação pós-estimulação do mRNA de IFN-γ foi observada em 24 h com todas as concentrações de MCWF ($p<0.01$), e o mRNA de TNF-α foi aumentado para 0.5 μg/dL de MCWF apenas em 72 h ($p<0.05$).

Conclusões e Relevância Clínica: A estimulação de PCMBs pela fração da parede celular de *Mycobacterium* de PBMCs resultou na elevação do mRNA de citocinas pró-inflamatórias e reguladoras. Mais pesquisas sobre a função do MCWF como um imunomodulador regulador administrado sistemicamente ou adjuvante à imunoterapia específica para alérgenos devem ser consideradas.

Resumen

Introducción: La fracción de pared celular de Mycobacterium (MCWF) se deriva de *Mycobacterium phlei* no patógeno y se utiliza como un compuesto inmunomodulador en la práctica clínica, aunque su modo de acción requiere más investigación.

Objetivo: Evaluar la respuesta del huésped a MCWF en células mononucleares de sangre periférica canina (PBMC) mediante ensayos inmunoabsorbentes ligados a enzimas (ELISA) y PCR cuantitativa en tiempo real (qRT) para la evaluación de citoquinas.

Animales: Ocho perros labradores retriever sanos

Materiales y métodos: Las PBMC se aislaron de sangre completa mediante centrifugación de densidad. Las células se cultivaron con diferentes concentraciones de MCWF o un potente estimulador de la producción de citoquinas, forbol 12-miristato 13-acetato/ionomicina, o se dejaron en un medio de cultivo celular durante 24, 48 y 72 h. Las citoquinas se midieron mediante ELISA para interleuquina (IL)-4, IL-10 e interferón-gamma (IFN-γ), y mediante qRT-PCR para IL-4, IL-10, IL-13, IFN-γ, factor de necrosis tumoral alfa (TNF-α) y factor de crecimiento transformante beta.

Resultados: Se detectó un aumento significativo del ARN mensajero (m) de IL-10 en todos los puntos temporales para todas las concentraciones de MCWF ($p<0.05$). El análisis de proteínas reflejó este hallazgo, con una concentración máxima de IL-10 de 300.6±38.3 μg/mL, lo que indica el potencial de un efecto regulador. En comparación con el control negativo, se observó una elevación del ARNm de IFN-γ después de la estimulación a las 24 h con todas las concentraciones de MCWF ($p<0.01$), y el ARNm de TNF-α aumentó para 0.5 μg/dL de MCWF solo a las 72 h ($p<0.05$).

Conclusiones y relevancia clínica: La estimulación de PBMCs con la fracción de la pared celular de Mycobacterium da como resultado la elevación del ARNm de citoquinas proinflamatorias y reguladoras. Se debe considerar la realización de más investigaciones sobre el papel del MCWF como inmunomodulador regulador o adyuvante de la inmunoterapia de alérgenos administrado sistémicamente.

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