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In vitro effects of monophosphoryl lipid A and Poly I:C combination on equine cells

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ABSTRACT

Background: Toll-like receptor (TLR) agonists have been used as adjuvants to modulate immune responses in both animals and humans.
Objectives: The objective of this study was to evaluate the combined effects of the TLR 4 agonist monophosphoryl lipid A (MPL) and the TLR 3 agonist polyinosinic:polycytidylic acid (Poly I:C) on equine peripheral blood mononuclear cells (PBMCs), monocyte-derived dendritic cells (MoDCs), and bone marrow-derived mesenchymal stromal cells (BM-MSCs).
Methods: The PBMCs, MoDCs, and BM-MSCs collected from three mixed breed horses were treated with MPL, Poly I:C, and their combination. The mRNA expression of interferon gamma (IFN-γ), interleukin (IL)-1β, IL-4, IL-6, IL-8, IL-12p40, tumor necrosis factor alpha (TNF-α), vascular endothelial growth factor (VEGF), and monocyte chemoattractant protein-1 (MCP-1) was determined using real-time polymerase chain reaction.
Results: The combination of MPL and Poly I:C significantly upregulated immunomodulatory responses in equine cells/ without cytotoxicity. The combination induced greater mRNA expression of pro-inflammatory cytokines IFN-γ and IL-6 than MPL or Poly I:C stimulation alone in PBMCs. In addition, the combination induced significantly higher mRNA expression of IL-1β, IL-6, and IL-12p40 in MoDCs, and IL-8, MCP-1, and VEGF in BM-MSCs compared to stimulation with a single TLR agonist.
Conclusions: The combination of MPL and Poly I:C can be used as a potential adjuvant candidate for vaccines to aid in preventing infectious diseases in horses.

Keywords: Toll-Like Receptors; peripheral blood mononuclear cell; dendritic cells; bone marrow stromal cell; immunomodulation

INTRODUCTION

In animal vaccines, an immunostimulatory material known as 'adjuvant' is included in the vaccine to improve its immunogenicity and enhance immune response. Various types of adjuvants are available in vaccine systems, such as pathogen-associated molecular patterns (PAMPs) that target pattern recognition receptors (PRRs). Among PAMPs, Toll-like receptor (TLR) agonists have often been used as adjuvants because of their ability to modulate innate immune response and increase the magnitude of protective adaptive immunity [1,2].

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Conflict of Interest

The authors declare no conflicts of interest.

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TLR agonists are categorized into two types based on the location of target receptors: receptors expressed on the cell surface that respond to the extracellular components of pathogens (TLR 1, 2, 4, 5, and 6) and endosomal receptors that recognize intracellular components of the pathogens (TLR 3, 7, 8, and 9) [3]. To date, more than five types of TLR agonists have been tested in vaccines, and these agonists have elicited enhanced immune responses in *in vitro* and *in vivo* experiments [1,3-6]. Considering the improved immune response in mouse models, experiments using different animal models have been conducted to verify the effectiveness of various TLR agonists.

In contrast to mouse models that have genetically identical traits and result in statistically significant data, *in vivo* experiments using livestock have limitations because of their genetic differences and laborious factors. To overcome these shortcomings, alternative *in vitro* experimental methods have been applied using animal cells obtained from blood or bone marrow.

In previous research, a variety of TLR agonists have been tested in many equine cells, including cytosine-phosphate-guanosine oligodeoxynucleotides and monophosphoryl lipid A (MPL) in peripheral blood mononuclear cells (PBMCs), polyinosinic-polycytidylic acid (Poly I:C), and lipopolysaccharide (LPS) in bone marrow-derived mesenchymal stromal cells (BM-MSCs) [7-11]. However, little information is available on the simultaneous use of multiple TLR agonists in equine cells. Recently, a strategy to apply multiple adjuvant components to mouse immune cells was examined to evaluate the possibility that combined adjuvants can induce synergistic effects on the immune environment, such as improved recruitment of immune cells, enhancement of antigen presentation and uptake by antigen-presenting cells (APC), or increased expression of costimulatory cytokines that stimulate T cell proliferation and differentiation [12-14]. However, the effects of combined adjuvants on immune cells of different species, including equine models, are rarely evaluated. Therefore, in this study, we used the TLR 4 agonist MPL and TLR 3 agonist Poly I:C simultaneously in equine PBMCs, monocyte-derived dendritic cells (MoDCs), and BM-MSCs to verify their combined effects in horse *in vitro* models and compared the immune response to those treated with MPL or Poly I:C alone.

MATERIALS AND METHODS

Animals

Five mares of different breeds (mixed breed, n = 3; Shetland pony, n = 1; Thoroughbred, n = 1) and one stallion (Selle Francais, n = 1) were used in this study. The horses were between 5 and 24 years old. PBMCs, MoDCs, and BM-MSCs were collected from three mixed-breed horses. Blood from the Thoroughbred, Shetland pony, and Selle Francais were used for the allogenic mixed lymphocyte reaction. Before conducting the experiment, the physical condition of the horses was clinically examined. All the experiments were performed according to the guidelines of Jeju National University (JNU) and approved by the Institutional Animal Care and Use Committee (IACUC) protocol (protocol number 2021-0035).

Reagents

Poly I:C and MPL were purchased from InvivoGen (USA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), sodium dodecyl sulfate (SDS), fluorescein isothiocyanate (FITC)-dextran, and 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE) were obtained from Sigma-Aldrich (USA). All the reagents were prepared according to the manufacturer's instructions.

Preparation, culture, and stimulation of PBMCs

Fifty milliliter of blood samples were collected from the jugular veins of three mixed breed horses using heparinized tubes. PBMCs were isolated by density centrifugation (400 g, 30 min, 4°C) using Ficoll Histopaque-1077 (Sigma-Aldrich) and washed twice in sterile phosphate-buffered saline (PBS). PBMCs (5×10^6 cells/well) from each horse were seeded into a 6-well plate and cultured in complete media consisting of Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich) supplemented with 10% complement-inactivated fetal bovine serum (FBS) and 1× antibiotic-antimycotic (Gibco BRL; Thermo Fisher Scientific, USA). PBMCs were stimulated with MPL (0.25 μg/mL), Poly I:C (2.5 μg/mL), or both for 18 h at 37°C. PBMCs incubated with complete media alone were used as negative controls. After incubation, all the cells were harvested using a cell scraper and transferred to a 1.7 mL tube for RNA extraction.

Cell viability assessment

An MTT assay was performed to evaluate the viability of PBMCs stimulated with TLR agonists. The assay was performed according to a previously described protocol [15]. Briefly, PBMCs from three mixed breed horses were seeded in a 96-well plate (4×10^5 cells/well) with 200 μL of complete medium. The cells were incubated with Poly I:C at concentrations of 0.625, 1.25, 2.5, 5, and 10 μg/mL and MPL at concentrations of 0.0625, 0.125, 0.25, 0.5, and 1 μg/mL. All the cells were incubated for 2 days at 37°C. After incubation, 10 μL of 10 mg/mL MTT solution was added to each well and incubated for 4 h. Next, 100 μL of 10% SDS solution was added to each well and incubated for 2 h. The optical density of each well was measured using a microplate reader at 570 nm.

Preparation, culture, and stimulation of MoDCs

MoDCs were generated according to a previously described protocol with minor modifications [16]. After seeding PBMCs into a 6-well plate, as described above, the cells were incubated for 2 h at 37°C. Non-adherent cells and culture medium were removed after incubation. Fresh complete medium containing 10 ng/mL of recombinant equine interleukin-4 (eq IL-4) (Abcam, UK) and 50 ng/mL of recombinant equine granulocyte-macrophage colony stimulating factor (eq GM-CSF) (Abcam) was added to the adherent cells, which were incubated at 37°C for 6 days to stimulate MoDC generation. Floating cells were removed, and fresh complete media with eq IL-4 and eq GM-CSF was refilled every 2 days. On day 6, the generated MoDCs were stimulated with TLR agonists and incubated at 37°C for 18 h. After incubation, all the cells were harvested and prepared for RNA extraction.

Evaluation of antigen uptake capacity of MoDCs

An endocytosis assay using Fluorescein isothiocyanate (FITC)-dextran was performed to evaluate the antigen uptake capacity of MoDCs stimulated with TLR agonists. The assay was performed as described previously [17]. Briefly, MoDCs were treated with TLR agonists for 18 h and harvested using cell scrapers. The cells were resuspended in 1 mL of fresh complete medium at a density of 5×10^5 cells/sample. Ten microliters of FITC-dextran solution was added to each sample and incubated at 4°C or 37°C for 1 h. After incubation, all the cells were washed twice with ice-cold PBS and resuspended in 400 μL flow cytometry staining (FACS) solution containing 0.1% sodium azide, 5% FBS, and 1% paraformaldehyde, before analysis. After staining, the cells were analyzed using the BD LSR Fortessa and BD FACS Diva program (BD Biosciences). Data analysis was performed using FlowJo software (FlowJo, LLC, USA).

Allogeneic mixed lymphocyte reaction assay

To evaluate the ability of MoDCs to induce non-specific T cell proliferation, an allogeneic mixed lymphocyte reaction assay was conducted using three different horse species (Thoroughbred, Shetland Pony, and Selle Francais). Allogenic naive lymphocytes were collected from PBMCs and stained with 2 μm of carboxyfluorescein succinimidyl ester (CFSE) (Sigma-Aldrich) at 37°C for 10 min. Subsequently, CFSE-labeled lymphocytes were washed with complete media, diluted to a concentration of 2×10^6 cells/mL, and co-cultured with control or TLR agonist pretreated MoDCs in 96 U-bottom well plates at 37°C for 5 days. The ratio of MoDCs to lymphocyte was 1:20. After 5 days of co-culture, all the cells were collected and resuspended in 400 μL FACS buffer consisting of PBS and 2% FBS. To exclude dead cells, live/dead AmCyan (LIVE/DEAD Fixable Aqua Dead Cell Stain Kit; Thermo Fisher Scientific) was added to each sample. The stained cells were analyzed with flow cytometry, and the results were analyzed using FlowJo software.

Preparation, culture, and stimulation of bone marrow-derived mesenchymal stromal cells

BM-MSCs were generated according to a previously described protocol [18]. Briefly, BM-MSCs were isolated from the bone marrow blood samples collected from the sternum of three mixed breed horses and cryopreserved at passage 1. Before stimulation with TLR agonists, the cells were thawed and cultured at the end of passage 2. BM-MSCs (5×10^6 cells/well) from each horse were seeded into a 6-well plate and cultured in complete media consisting of Dulbecco's Modified Eagle Medium (DMEM)-1640 medium (Sigma-Aldrich) supplemented with 10% FBS and 100 IU penicillin + streptomycin (Gibco BRL; Thermo Fisher Scientific). BM-MSCs were stimulated with TLR agonists and incubated for 18 h at 37°C. BM-MSCs incubated with complete media alone were used as negative controls. After incubation, the BM-MSCs were harvested and used for RNA extraction.

RNA extraction and complementary DNA preparation

Total RNA was prepared from cultured PBMCs, MoDCs, and BM-MSCs using an RNA extraction kit (iNtRON Biotechnology Inc., Korea) according to a previously described protocol [19]. The concentration and purity of each RNA sample was determined using a DS-11 spectrophotometer (DeNovix Inc., Wilmington, DE, USA). For complementary DNA (cDNA) preparation, 1 μg of total RNA was used to synthesize cDNA using a cDNA synthesis kit (iNtRON Biotechnology Inc.) following the manufacturer's protocol. The concentration and quality of the synthesized cDNA were evaluated as described above and diluted to an appropriate concentration for subsequent polymerase chain reaction (PCR). After synthesis, all the cDNA samples were stored at −80°C until use.

Quantification of cytokine gene expression using real-time PCR

Real-time PCR was used to measure changes in the mRNA expression of interferon gamma (IFN-γ), IL-1β, IL-4, IL-6, IL-8, IL-12p40 tumor necrosis factor alpha (TNF-α), vascular endothelial growth factor (VEGF), and monocyte chemoattractant protein-1 (MCP-1). Glyceraldehyde 3-phosphate dehydrogenase was used as an endogenous control. Primer information was obtained from previously published data [20,21]. **Table 1** lists the primer sequences. The PCR reaction had a final volume of 20 μL and consisted of 10 μL real-time PCR master mix reagents (iNtRON Biotechnology Inc.), 1 μL of 10 μM forward and reverse primers, 1 μL cDNA, and 7 μL nuclease-free water. PCR was performed using a Thermal Cycler Dice Real-Time System II (Takara Bio Inc., Japan). All the samples were measured in triplicate. The thermal profile consisted of an initial hold at 95°C for 10 min, followed by

Table 1. Oligonucleotide primer sequence of target genes

Target gene	Oligo	Primer sequence
GAPDH	Forward	GGTGAAGGTCGGAGTAAACG
	Reverse	AATGAAGGGGTCATTGATGG
IFN-γ	Forward	CTATTACTGCCAGGCCGCGTT
	Reverse	TCCTCTTCCGCTTCCTCAGGTT
IL-1β	Forward	ACCATAAATCCCTGGTGCTG
	Reverse	CGTCCCACAAGACAGGTACA
IL-4	Forward	CCGAAGAACACAGATGGAAAGGA
	Reverse	TCACAGTACAGCAGGTCCCGTTT
IL-6	Forward	AGCAAGGAGGTACTGGCAGA
	Reverse	CCTTTTCACCCTTGAACTCG
IL-8	Forward	CGCACTCCAAACCTTTCAAT
	Reverse	TCAAAAACGCCTGCACAATA
IL-12p40	Forward	TGCTGTTCACAAGCTCAAGTATGA
	Reverse	GGGTGGGTCTGGTTTGATGA
TNF-α	Forward	GCCCAGACACTCAGATCATCTTC
	Reverse	CATTTGCACGCCCACTCA
VEGF	Forward	CAACGACGAGGGCCTAGAGT
	Reverse	CATCTCTCCTATGTGTGGCTTTG
MCP-1	Forward	ATTGGCCAAGGAGATCTGTG
	Reverse	ATATCAGGGGGCATTTAGGG

GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; IFN-γ, interferon gamma; IL, interleukin; TNF-α, tumor necrosis factor alpha; VEGF, vascular endothelial growth factor; MCP-1, monocyte chemoattractant protein-1.

40 cycles of denaturation at 95°C for 15 s, annealing at 54.5–61°C for 30 s, and extension at 70°C for 30 s, followed by a melting curve (60–95°C). The ΔΔCt method was used to quantify relative mRNA expression [22].

Statistical analysis

All the results are presented as mean ± SEM. Statistical significance was determined using one-way ANOVA followed by Tukey's multiple comparison test. Statistical significance was set at $p < 0.05$. All data were analyzed using the Prism software (GraphPad Software, USA).

RESULTS

The combination treatment of TLR agonists did not affect the cell viability of equine PBMCs

Equine PBMCs were treated with MPL, Poly I:C, and a combination of MPL and Poly I:C, to evaluate the cytotoxicity of TLR agonists and determine the treatment dose. MPL did not exhibit cytotoxicity to equine PBMCs at concentrations between 0 and 1 μg/mL, compared to the control (**Fig. 1A**). Poly I:C did not show significant cytotoxicity to equine PBMCs at concentrations between 0 and 10 μg/mL (**Fig. 1B**). According to single treatment results, the MPL + Poly I:C ratio was 1:10. Cytotoxicity was not observed in the combination treatment, and highest cell viability was observed with the combination treatment of 0.25 μg/mL of MPL and 2.5 μg/mL of Poly I:C (**Fig. 1C**).

Pro-inflammatory cytokine and chemokine mRNA expression of equine PBMCs was induced by a combination of MPL and Poly I:C

To investigate the effects of TLR agonists on pro-inflammatory cytokine and chemokine production in PBMCs, equine PBMCs from three mixed breed horses were stimulated with MPL, Poly I:C, or their combination, followed by expression analysis of eight immunomodulatory genes using real-time PCR (**Fig. 2**). MPL treatment increased IFN-γ,

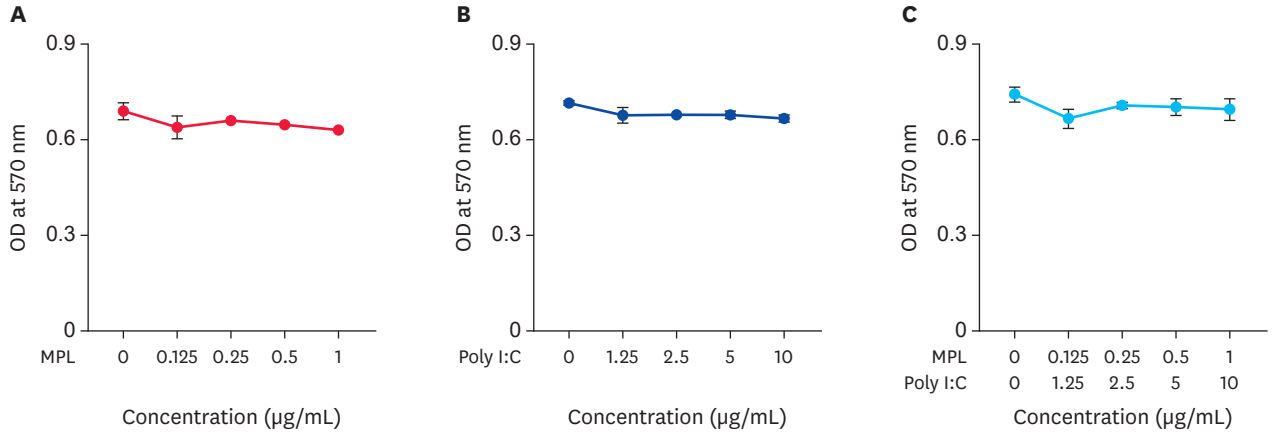

Fig. 1. *In vitro* effects of TLR agonists on the viability of equine PBMCs (n=3). Equine PBMCs were obtained from mixed breed horses and cultured with TLR agonists of different concentrations. Cell viability of PBMCs treated with MPL (A), Poly I:C (B) or MPL + Poly I:C (C). Cell viability was determined with an MTT assay after 2 days of culture with TLR agonist.
OD, optical density; MPL, monophosphoryl lipid A; Poly I:C, polyinosinic-polycytidylic acid; TLR, Toll-like receptor; PBMC, peripheral blood mononuclear cell.

IL-6, and MCP-1 expressions, whereas treatment with Poly I:C alone could not induce any cytokine or chemokine mRNA expression. However, the MPL and Poly I:C combination induced significant mRNA expression of IFN-γ ($p < 0.01$) and IL-6 ($p < 0.05$) (**Fig. 2A and B**). The mean fold-changes in IL-4 and MCP-1 expression stimulated with MPL + Poly I:C were higher than those in the other groups, but there was no significant difference between the groups (**Fig. 2C and D**). MPL + Poly I:C did not induce the expression of genes encoding IL-1β, IL-8, IL-12p40, and TNF-α (data not shown).

MoDCs were efficiently stimulated by a combination of MPL and Poly I:C to express pro-inflammatory cytokines

Dendritic cell (DCs) are the most potent APCs that stimulate both innate and adaptive immune responses and can be a good target for vaccine adjuvants [16,21]. To evaluate the effects of MPL and Poly I:C on cytokine production in MoDCs, MoDCs were stimulated with each TLR agonist or their combination, followed by expression analysis of four pro-inflammatory genes using real-time PCR. The combination of MPL and Poly I:C significantly upregulated mRNA expression of IL-1β compared to MoDCs stimulated with Poly I:C alone ($p < 0.05$) or MPL alone ($p < 0.05$) (**Fig. 2E**). Likewise, the combination significantly induced mRNA expression of IL-6 compared to MoDCs stimulated with each TLR agonist ($p < 0.001$) (**Fig. 2F**). In addition, the combination induced a higher mRNA expression of IL-12p40 than MoDCs stimulated with Poly I:C alone ($p < 0.01$) or MPL ($p < 0.05$) alone (**Fig. 2G**). The gene encoding TNF-α was significantly upregulated only in MoDCs stimulated with a combination of MPL and Poly I:C ($p < 0.05$) (**Fig. 2H**).

MPL + Poly I:C significantly enhanced antigen uptake of MoDCs

To evaluate the effect of TLR agonists on the antigen uptake capacity of MoDCs, an FITC-dextran endocytosis assay was performed (**Fig. 3**). MoDCs incubated at 4°C were used as a negative control for antigen uptake. MoDCs stimulated with all adjuvants displayed an upregulated endocytic profile. Treatment with a combination of MPL and Poly I:C significantly enhanced the FITC-dextran uptake of MoDCs compared to MoDCs without stimulation ($p < 0.05$). Non-stimulated MoDCs and MoDCs stimulated with single TLR agonist endocytosed similar amounts of FITC-dextran at 37°C.

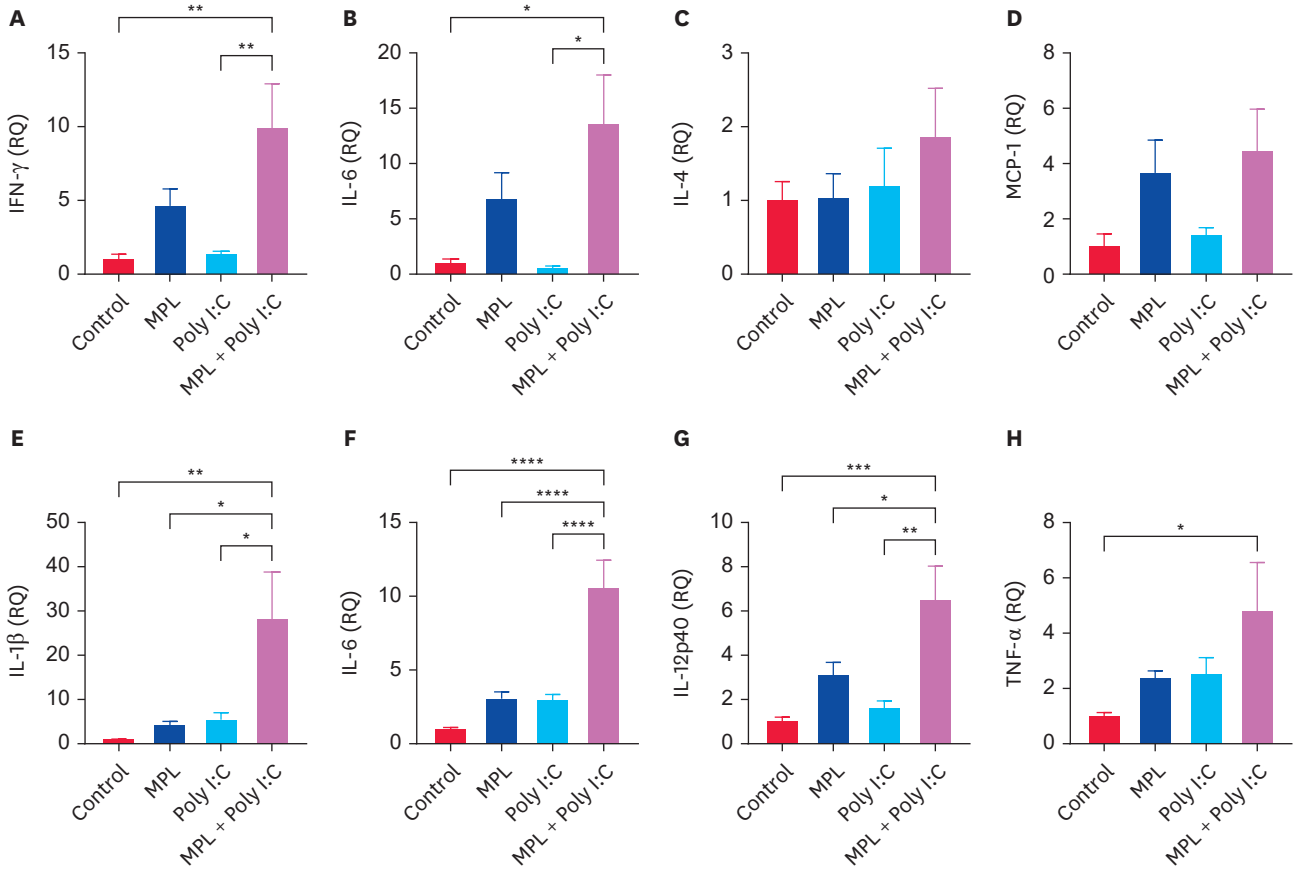

Fig. 2. Relative expression of the genes encoding IFN-γ (A), IL-6 (B), IL-4 (C), and MCP-1 (D) in equine PBMCs (n = 3) and IL-1β (E), IL-6 (F), IL-12p40 (G), and TNF-α (H) in equine MoDCs (n = 3) stimulated with Toll-like receptor agonists. Equine MoDCs were generated from PBMCs using equine granulocyte macrophage colony stimulating factor and IL-4. The PBMCs and MoDCs were cultured in plain growth medium or cultured with MPL (0.25 μg/mL), Poly I:C (2.5 μg/mL), and MPL (0.25 μg/mL) + Poly I:C (2.5 μg/mL). The cytokine gene expression was normalized to the reference gene and calibrated to that in the medium control. All data are shown as mean ± SEM. Statistical analysis between the groups were performed using one-way ANOVA and Tukey's multiple comparison test.
IFN-γ, interferon gamma; MPL, monophosphoryl lipid A; Poly I:C, polyinosinic-polycytidylic acid; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; TNF-α, tumor necrosis factor-alpha; PBMCs, peripheral blood mononuclear cells; MoDCs, monocyte derived dendritic cells.
$^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.005$; $^{****}p < 0.001$.

Pretreatment with a combination of MPL and Poly I:C stimulates T cell proliferation induced by MoDCs

After functional MoDC activation, the cells acquire the ability to induce T cell proliferation and activation. To investigate the T cell stimulating capacity of MoDCs treated with TLR agonists, we evaluated allogeneic T cell proliferation after the co-culture of naïve T cells with MoDCs pretreated with TLR agonist (**Fig. 4**). MoDCs pretreated with MPL alone or MPL + Poly I:C, induced significantly higher levels of T cell proliferation than unstimulated MoDCs, suggesting that a combination of MPL and Poly I:C effectively enhanced the activation of functional equine DC.

MPL + Poly I:C also induced BM-MSCs to express pro-inflammatory cytokine and chemokine genes

To evaluate the effects of MPL and Poly I:C on cytokine expression by BM-MSCs, BM-MSCs were stimulated with each TLR agonist or their combination, followed by expression analysis of four pro-inflammatory cytokine genes using real-time PCR. Only the combination of MPL and Poly I:C induced significant mRNA expression of IL-6 ($p < 0.05$), MCP-1 ($p < 0.01$),

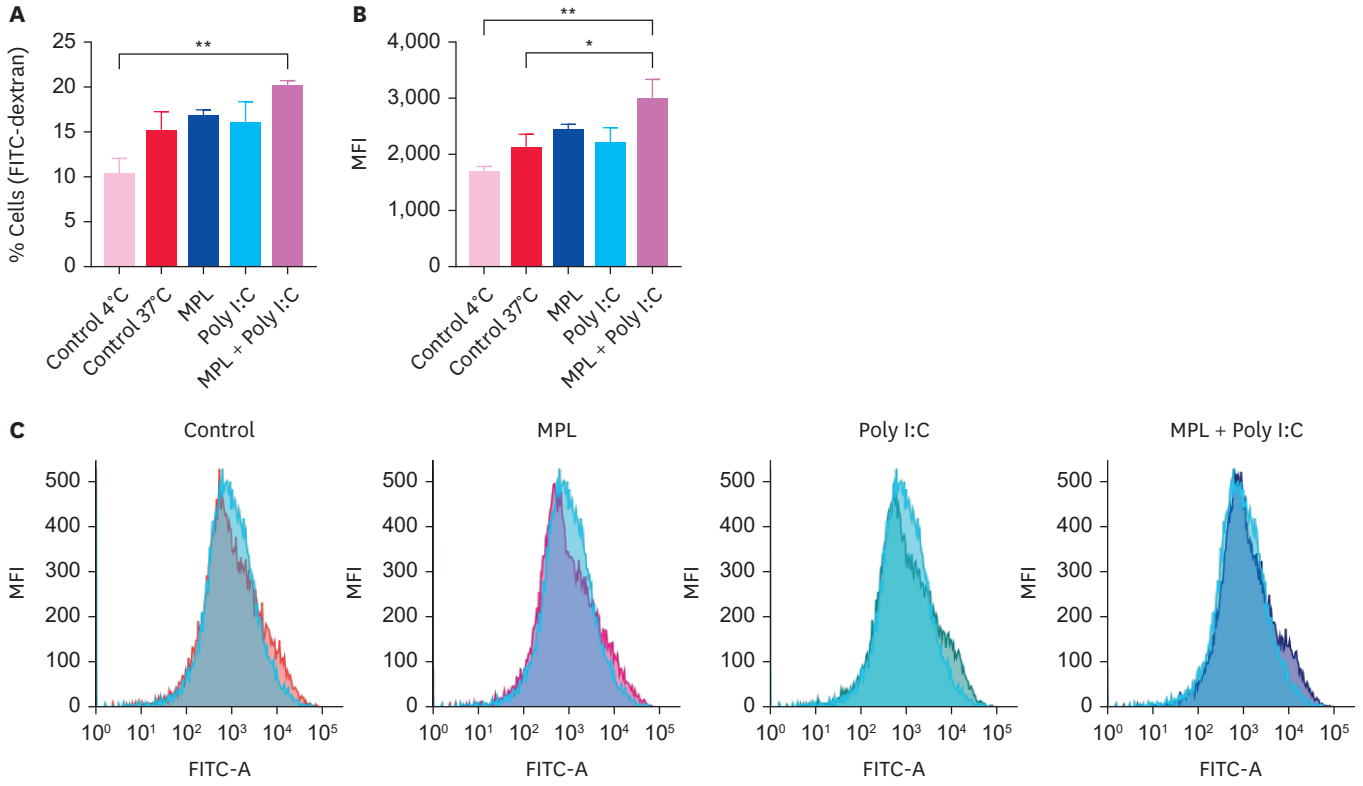

Fig. 3. Equine MoDCs (n = 3) stimulated with Toll-like receptor agonists acquired antigen uptake ability. FITC-dextran was used as a soluble antigen, which was added to equine MoDCs cultured for 6 days. MoDCs were incubated for 1 h at 37°C or 4°C. Cells were analyzed with flow cytometry and data analysis was performed using FlowJo software program. Percentage (A) and mean fluorescent intensity (B) of MoDCs taking up FITC-dextran was determined after gating on the cell population. The overlayed histograms (C) depict the uptake of FITC-dextran of MoDCs incubated at 37°C and 4°C (skyblue histogram). All data are shown as mean ± SEM. Statistical analysis between the groups were performed using one-way ANOVA and Tukey's multiple comparison test.
FITC, fluorescein isothiocyanate; MPL, monophosphoryl lipid A; Poly I:C, polyinosinic-polycytidylic acid; MFI, mean fluorescence intensity; MoDCs, monocyte derived dendritic cells.
$^*p < 0.05$; $^{**}p < 0.01$.

and VEGF ($p < 0.01$) relative to that in unstimulated BM-MSCs (**Fig. 5A-C**). TLR agonists, especially the combination of MPL and Poly I:C, also increased IL-8 mRNA expression, but the difference was not statistically significant (**Fig. 5D**).

DISCUSSION

The present study was conducted to evaluate the immune response of various equine cell lines treated with either MPL and Poly I:C alone or their combination. Despite inter-species variations, stimulation with TLR agonists results in distinct cytokine and chemokine responses and immune reactions. Thus, it can be assumed that *in vitro* experiments using horses could provide statistically significant data and clues for the basis of *in vivo* experiments.

In this study, we evaluated the effect of different concentration of Poly I:C (1.25–10 μg/mL) and MPL (0.125–1 μg/mL) on equine PBMCs and applied 2.5 μg/mL of Poly I:C and 0.25 μg/mL of MPL to induce cytokine expression. The concentration of MPL used was much lower than that used in previous studies where 5 μg/mL of MPL was applied to stimulate equine PBMCs [7,9]. Although the results of the MTT assay indicated that the cell viability of PBMCs stimulated with high concentration MPL (> 0.25 μg/mL) was not significantly

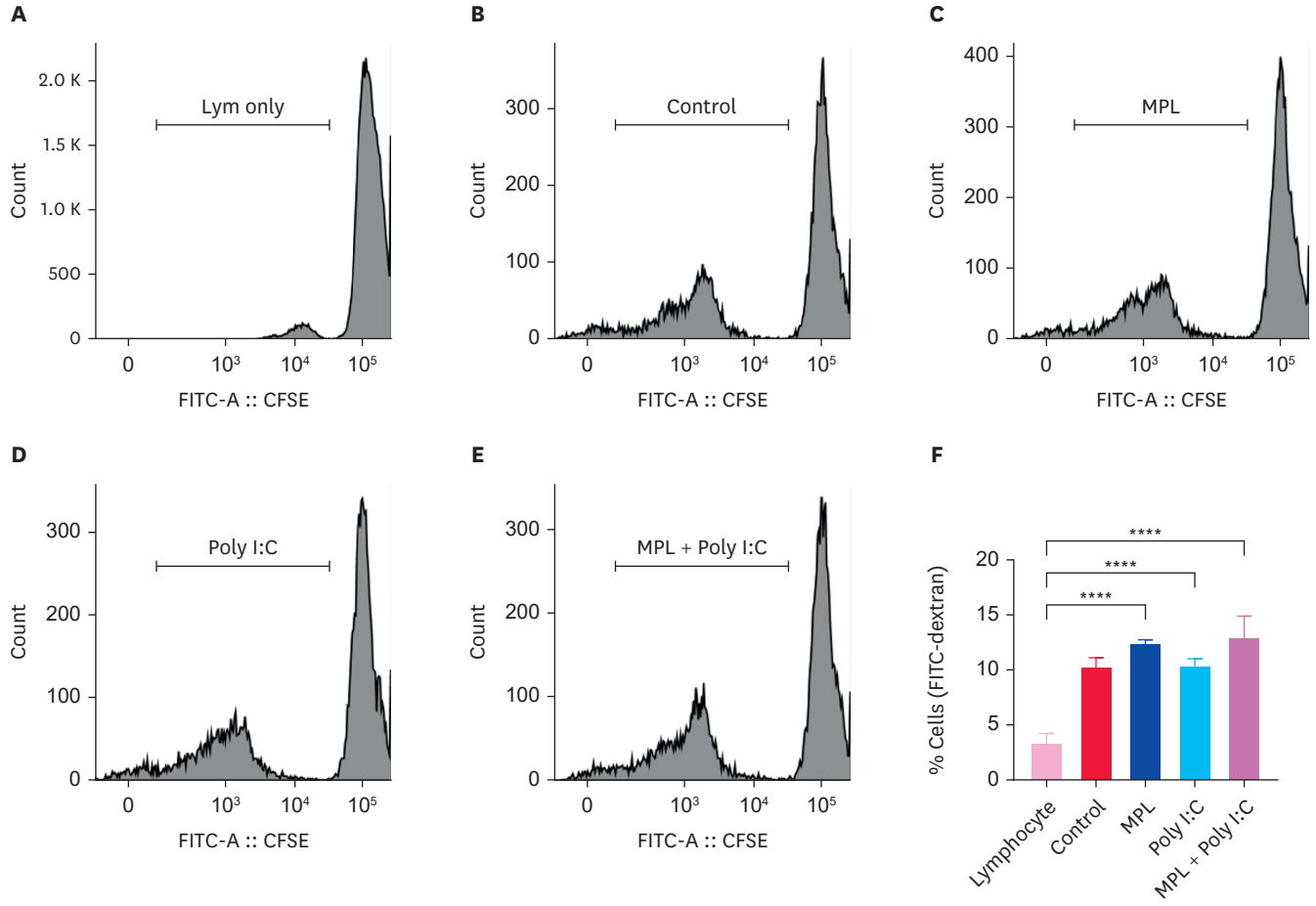

Fig. 4. Allogenic T cell proliferation after co-culture with TLR agonists pretreated MoDCs. Immature MoDCs obtained from mixed breed horses (n = 3) were stimulated with TLR agonists for 2 days. Allogenic Lyms were harvested from Thoroughbred, Shetland pony, and Selle Francais horse. CFSE-labeled Lyms were cultured alone (A) or co-cultured with nontreated MoDC (B), or MPL (C), Poly I:C (D), and MPL + Poly I:C (E) pretreated MoDCs for 5 days. T cell proliferation was determined with flow cytometry (F). All data are shown as mean ± SEM. Statistical analysis between the groups were performed using one-way ANOVA and Tukey's multiple comparison test.
FITC, fluorescein isothiocyanate; CFSE, 5(6)-carboxyfluorescein diacetate N-succinimidyl ester; MPL, monophosphoryl lipid A; Poly I:C, polyinosinic-polycytidylic acid; TLR, Toll-like receptor; MoDCs, monocyte derived dendritic cells; Lym, lymphocyte.
$^{*}p < 0.05$; $^{**}p < 0.01$; $^{****}p < 0.001$.

different, we decided to use a low concentration based on the previous results where 5 μg/mL of MPL suppressed IFN-γ production in equine PBMCs, and 1 μg/mL of MPL had a lower IFN-γ induction than that of 0.1 ug/ml of MPL in murine macrophages [7,23]. These data indicate that high concentration adjuvants do not always cause strong cytokine induction, which means that applying a new adjuvant necessitates dose-response analysis. Also, in the preliminary study, we used 1 μg/mL of Poly I:C and 0.1 μg/mL of MPL and evaluated cytokine expression by equine PBMCs. However, there was no significant induction of cytokine mRNA expression in the 1 μg/mL Poly I:C and 0.1 μg/mL of MPL adjuvanted groups compared to that in the non-stimulated group (data not shown). In summary, the concentration used in this study was appropriate for inducing cytokine expression.

The results obtained from this study indicate that the MPL and Poly I:C combination can effectively induce mRNA expression of IL-6 in equine PBMCs. Our finding is consistent with previous results, where the combination of MPL and Poly I:C induced significantly higher IL-6 production compared to control or single-adjuvanted groups in mice [14]. During infection,

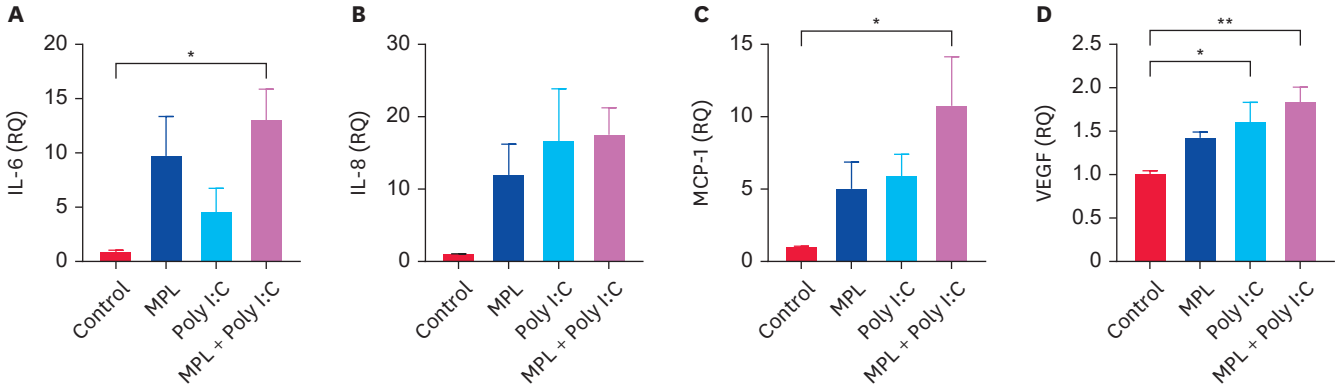

Fig. 5. Relative expression of the genes encoding IL-6 (A), IL-8 (B), MCP-1 (C), and VEGF (D) in equine BM-MSCs (n = 3) stimulated with Toll-like receptor agonists. Equine BM-MSCs were isolated from bone marrow of mixed breed horses. The BM-MSCs were cultured in plain growth medium or cultured with MPL (0.25 μg/mL), Poly I:C (2.5 μg/mL), and MPL (0.25 μg/mL) + Poly I:C (2.5 μg/mL). The cytokine gene expression was normalized to the reference gene and calibrated to that in the medium control. All data are shown as mean ± SEM. Statistical analysis between the groups were performed using One-way ANOVA and Tukey's multiple comparison test.
IL, interleukin; RQ, relative quantification; MPL, monophosphoryl lipid A; Poly I:C, polyinosinic-polycytidylic acid; MCP-1, monocyte chemoattractant protein-1; VEGF, vascular endothelial growth factor; BM-MSC, bone marrow derived mesenchymal stromal cell.
$^{*}p < 0.05$; $^{**}p < 0.01$.

IL-6 production by immune cells is known to promote antiviral activity by suppressing viral replication in a dose-dependent manner, and is also required for protection against intracellular bacterial infections such as *Rhodococcus equi* and *Listeria monocytogenes* [8,24,25]. Thus, the strong induction of IL-6 by equine PBMCs cultured with a combination of MPL and Poly I:C might provide effective protection against bacterial and viral pathogens in horses.

IFN-γ is important in Th1 differentiation by upregulating IL-12 production in macrophages and DCs, which are activated by encounters with intracellular bacteria or bacterial products such as LPS [26]. As IFN-γ-mediated activity is critical for the immune environment to prevent infectious diseases, strategies using a variety of adjuvant components to improve IFN-γ induction have been examined [8,14,27]. In this study, the increase of IFN-γ mRNA expression in response to MPL combined with Poly I:C was more than additive, indicating synergy. While stimulation with MPL only moderately increased IFN-γ mRNA expression, stimulation with Poly I: C alone resulted in a relatively low induction of IFN-γ. These results suggest that MPL is more closely related to Th1 cytokine profile and that the combination of MPL and Poly I:C could be an effective candidate to protect against microbial diseases by upregulating IFN-γ responses.

In contrast to our results, where there was no enhancement of genes encoding IL-1β, IL-8, IL-12p40, and TNF-α, previous *in vitro* studies reported an increased expression of TNF-α, IL-1, and IL-8 in equine PBMCs and monocytes after exposure to LPS stimulation, which may be attributed to the differences between LPS and MPL or the stimulation period and concentration [28,29].

The capacity to secrete pro-inflammatory cytokines is a feature of DC maturation. Exposure to PAMP activates the PRR of DCs, which leads to the secretion of pro-inflammatory cytokines. Recently, stimulation with more than one PRR has been used to improve cytokine production, and several studies have reported synergistic activation of immune responses by applying multiple TLR agonists simultaneously in a vaccine [13,30]. In this study, we tested whether stimulation with a combination of TLR agonists could improve the capacity of

equine MoDCs to secrete pro-inflammatory cytokines. Evidence suggests that DCs cultured with either Poly I:C or LPS (a TLR 4 agonist) induce IL-1β, IL-6, IL-12p40, and TNF-α in horses and other species [31-33]. Likewise, Poly I:C or MPL alone also induced IL-1β, IL-6, and IL-12p40 mRNA expression in equine MoDCs, which was significantly increased by the combination of Poly I:C and MPL. The induction of TNF-α was significantly higher in the combination group than in the control group ($p < 0.05$).

IL-12p70 (IL-12), secreted by DCs, is a heterodimer composed of p35 and p40 units. In DCs, IL-12 modulates and activates $CD4^+$ T cells and induces their proliferation, which promotes the development of acquired immunity. In a previous study, equine IL-12p35 and IL-12p40 mRNA were both induced after exposure to LPS for 24 h in equine MoDCs [32]. However, in this study, while both MPL and Poly I:C induced IL-12p40 mRNA expression, there was little or no induction of IL-12p35 mRNA expression compared to the control group (data not shown). This result is in agreement with that of a previous study, which showed that the p40 and p35 subunits were independently regulated in horses [8].

In the current study, we evaluated the antigen uptake capacity of equine MoDCs after stimulation with MPL or Poly I:C. Normally, mature DCs show downregulated endocytic activity. However, in this study, all groups stimulated with TLR agonists showed upregulated endocytic uptake of FITC-dextran compared to the unstimulated MoDCs. These results support those of previous studies that showed that the endocytic activity of DCs does not always correlate with the expression of maturation or that MoDCs may be blocked at an intermediate state of maturation [32,34].

In this study, we used TLR agonist-pretreated MoDCs from three mixed breed horses and CFSE-labeled allogenic lymphocytes from different species of horses to perform mixed lymphocyte reactions. Although the results showed that pretreatment with MPL alone, in MoDCs, significantly improved T cell proliferation ($p < 0.05$), the induction was considerably more effective when combined with Poly I:C ($p < 0.01$). Our results differed from previous data where murine DCs pretreated with Poly I:C alone were more effective in inducing both CD4 and CD8 T cell proliferation than murine DCs pretreated with MPL alone [14]. However, since this study only applied flow cytometry using forward and side scatter properties, cell viability dyes, and CFSE, and did not use fluorescent-labeled anti-horse antibodies such as anti-CD3, CD4, and CD8, which are used to identify T cell subsets, further studies using fluorescent conjugated antibodies are required to confirm our data.

Stimulation with TLR or nucleotide binding oligomerization domain-like receptor (NLR) agonists in MSC has been shown to increase their immunomodulatory properties [10,11,35]. In this study, we investigated the effect of TLR agonist combinations on cytokine induction by equine BM-MSCs. A previous study has shown that TLR 3 stimulation with Poly I:C or TLR 4 stimulation with LPS modulated the immune response of MSCs by increasing the expression of IL-6, C-C motif chemokine ligand 2 (CCL2), and C-X-C motif chemokine ligand 10 (CXCL10) [10]. Likewise, stimulation with MPL (TLR 4) also improved the immunomodulatory capacity of MSCs, which was significantly enhanced by combination with Poly I:C, except for IL-8. This is consistent with our results concerning PBMCs and MoDCs, which highlight the potential use of combined TLR agonists of different equine cells.

MCP-1 is the main paracrine cytokine of MSC and is involved in the recruitment of macrophages and monocytes. Increased MCP-1 levels are associated with paracrine

recruitment of innate immune cells and phagocytosis of bacteria by neutrophils, which could help eradicate infectious diseases [11]. In addition, MCP-1 exhibits angiogenic properties by inducing endothelial cell proliferation and migration through chemokine receptor 2 [36]. In a previous study, MCP-1 deficient MSC showed reduced therapeutic efficacy in an animal wound healing model, which indicates that MCP-1 is required for wound healing [37]. Therefore, upregulation of MCP-1 mRNA expression through MPL and Poly I:C stimulation in MSC could be used to enhance wound healing function as well as immune stimulation.

MSCs can modulate immune responses of other immune cells by regulating cytokine and growth factor production. In addition to the immune-modulating roles, it is well-known that BM-MSCs play a critical role in homeostasis of hematopoietic stem cells and tissue repair by secreting a variety of growth factors, including VEGF, hepatocytes, and insulin-like growth factors [38]. VEGF is an important effector molecule because it promotes tissue repair, regulates differentiation, and enables MSCs to enhance the expression of osteogenic marker genes [38]. Therefore, the induction of increased VEGF mRNA expression through the combination of MPL and Poly I:C could influence therapeutic potentials by promoting tissue repair and modulating immune responses.

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