Suppressive modulation of host immune responses by *Dermanyssus gallinae* infestation

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ABSTRACT The poultry red mite (*Dermanyssus gallinae*, **PRM**) is a blood-sucking ectoparasite in chickens and is one of the most serious threats to poultry farms. Mass infestation with PRMs causes various health problems in chickens, resulting in significant productivity reduction in the poultry industry. Infestation with hematophagous ectoparasites, such as ticks, induces host inflammatory and hemostatic reactions. On the other hand, several studies have reported that hematophagous ectoparasites secrete various immunosuppressants from their saliva to suppress host immune responses to maintain blood sucking. Here, we examined the expression of cytokines in peripheral blood cells to investigate whether PRM infestation affects immunological states in chickens. In PRM-infested chickens, anti-inflammatory cytokines, *IL-10* and *TGF-β1*, and immune checkpoint molecules, *CTLA-4* and *PD-1*, were highly expressed compared to noninfested chickens. PRM-derived soluble mite extracts (**SME**) upregulated the gene expression of *IL-10* in peripheral blood cells and HD-11 chicken macrophages. In addition, SME suppressed the expression of interferons and inflammatory cytokines in HD-11 chicken macrophages. Moreover, SME induces the polarization of macrophages into anti-inflammatory phenotypes. Collectively, PRM infestation could affect host immune responses, especially suppress the inflammatory responses. Further studies are warranted to fully understand the influence of PRM infestation on host immunity.

Key words: *Dermanyssus gallinae*, poultry red mite, chicken, immunity, immunosuppression

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INTRODUCTION

The poultry red mite (*Dermanyssus gallinae*, **PRM**), a hematophagous ectoparasite of chickens, is one of the most devastating agents in the poultry industry (Sparagano et al., 2014; George et al., 2015). Chickens infested by PRMs exhibit various health problems, including anemia, hyposthenia, and lower feed conversion, which results in reduced egg production and significant economic losses (van Emous, 2005; Mul et al., 2009; Sparagano et al., 2009). Moreover, PRM is suggested to be a vector of pathogens such as the avian influenza virus, *Salmonella* Gallinarum, and *Erysipelothrix rhusiopathiae* (Eriksson et al., 2009; Sommer et al., 2016; Pugliese et al., 2019; Schiavone et al., 2022). Control of PRMs with chemical acaricides is usually insufficient because PRMs can develop resistance to acaricides (Beugnet et al., 1979; Zeman and Zelezný, 1985; Marangi et al., 2009). Thus, prevention of PRM infestation is crucial for poultry farming.

Several studies have demonstrated that hematophagous ectoparasites secrete effector molecules in their saliva to modulate their host's innate and adaptive immunity to maintain an environment conducive for blood sucking (Barriga, 1999). Salivary gland extracts (**SGE**) of *Ixodes ricinus* reduce superoxide and nitric oxide production, phagocytosis, and the production of interferon gamma (**IFN-γ**) and tumor necrosis factor alpha (**TNF-α**) by macrophages (Kuthejlová et al., 2001; Kýcková and Kopecký, 2006). In addition, SGE of *Rhipicephalus appendiculatus, Dermacentor andersoni*, and saliva of *I. ricinus* reduce lymphocyte proliferation (Wikel, 1982; Ramachandra and Wikel, 1992; Rolníková et al., 2003). Salivary factors derived from mosquitoes are also known to modulate host immune responses; for instance, SGE from *Aedes aegypti* suppresses the production of Th1 cytokines and IFNs (Cross et al., 1994;

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Schneider et al., 2004), and these immunosuppressive effects are associated with arbovirus transmission (Schneider et al., 2004; Schneider and Higgs, 2008; Guerrero et al., 2020).

In PRMs, a previous study demonstrated that chronic stress by PRM infestation changes protein composition in the plasma of the affected chickens, suggesting that it contributes to decreased humoral immunity (Kowalski and Sokół, 2009). In addition, PRM infestation negatively affected the levels of antibody titers in chickens immunized with vaccines against several avian infectious agents (Kaoud and El-Dahshan, 2010). Moreover, the percentages of B cells and T helper cells in chickens that were raised in PRM-contaminated cages were negatively correlated with the number of PRMs, while there was a positive correlation between the percentage of cytotoxic lymphocytes and the number of PRMs (Koziatek-Sadłowska and Sokół, 2020). These observations suggest that PRM infestation could affect host immunity, and impair antibody production after vaccination (Kowalski and Sokół, 2009; Kaoud and El-Dahshan, 2010; Koziatek-Sadłowska and Sokół, 2020). Thus, blood-sucking by PRMs has the potential to modulate host immune responses, including the expression of cytokines in immune cells of chickens. However, chicken immune reactions, such as cytokine profiles, against infestation by PRMs are yet to be fully described. In this study, we examined whether PRM infestation affect the expression of pro-inflammatory and anti-inflammatory cytokines in the peripheral blood cells of infested chickens. We compared the gene expression of several cytokines and their products in plasma between PRM-infested and noninfested chickens. In addition, to investigate the potential of PRM-derived molecules to modulate the function of immune cells, chicken peripheral blood cells, and HD-11 chicken macrophage-like cells were cultured with soluble proteins extracted from PRMs, and the gene expression of cytokines was examined.

MATERIALS AND METHODS

Ethics Statement

The chicken blood samples used in this study were obtained with informed consent from the farm owners. Sample collection was performed according to the procedures and guidelines of the Ethics Committee of the Faculty of Veterinary Medicine, Hokkaido University. All animal experiments were approved by the Institutional Animal Care and Use Committee, Hokkaido University (Approval number: 22−0095).

Plasma and PBMC Samples of Chickens

Heparinized fresh blood samples were collected from 13 noninfested healthy chickens maintained at the Field Science Center for Northern Biosphere, Hokkaido University (NIE) and 6 chickens from a commercial egg-laying farm that is free from PRMs in Japan (noninfested commercial chickens, **NIC**), or 16 chickens from a PRM-eroded egg laying farm in Japan ("Infested" chickens). In addition, blood samples were collected from 8 noninfested chicks raised at a PRM-free coop (before exposure, **b.e.**) and 8 infested chickens that were transferred from a PRM-free coop to a PRM-contaminated coop (post exposure, **p.e.**). The presence of PRMs in the poultry houses were confirmed by visual observation or by monitoring PRMs that invaded cardboard boards placed in the poultry houses. Samples of b.e. and p.e. were collected from the same flock. The age of b.e. chickens was 106 or 114 days old and that of p.e. chickens was 364 or 471 days old, while NIC, NIE, and Infested samples were collected from adult chickens of mixed ages. NIE chickens were not vaccinated, whereas chickens from other groups were vaccinated to prevent several infectious diseases (such as Marek's disease, avian infectious bronchitis, Newcastle disease, mycoplasmosis, and fowl pox, among others). All the samples were collected from commercial egg-layers originating from Rhode Island Red or White Leghorn. For each sampling, chickens were randomly selected in each flock, and approximately 500 μL of blood was collected from the wing vein. Samples were transferred to the laboratory in cool condition. Plasma was isolated from the blood by centrifugation at 2,000 $\times$ g for 10 min, and peripheral blood mononuclear cells (**PBMC**) were isolated by density gradient centrifugation in Percoll solution (GE Healthcare, Chicago, IL) within 48 h of blood sample collection.

RNA Isolation and cDNA Synthesis

Total RNA of isolated PBMC was extracted using TRI reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's instructions. Remnant DNA was removed from the RNA extracted from PBMC with DNase I (amplification grade; Invitrogen, Carlsbad, CA) treatment for 10 min at 65°C. The RNA quality and quantity were analyzed using NanoDrop 8000 (Thermo Fisher Scientific, Waltham, MA), and then cDNA was synthesized from 1 μg of RNA using PrimeScript Reverse Transcriptase (Takara Bio Inc., Shiga, Japan) with 200 pmol of oligo (dT) 18 primer (Hokkaido System Science, Hokkaido, Japan).

Preparation of Soluble Mite Extract

PRMs of mixed developmental stages and sexes were collected from a laying hen's farm in Japan, where Infested samples described above were collected. PRMs were maintained at 25°C in 70 % humidity for one week, transferred to 5°C with 70% humidity and maintained for a 2-wk period. PRMs were then collected in 1,200 mL extra-long filter tips (WATSON Bio Lab, Tokyo, Japan) and stored at −80°C until use.

Approximately 500 mg of PRMs were suspended in 5 mL of phosphate-buffered saline (**PBS**) with 200 U/mL penicillin and 200 μg/mL streptomycin (Invitrogen) and incubated at 37°C for 1 h. After washing twice with 10 mL of cold PBS, PRMs were suspended in

1 mL of cold PBS and homogenized by sonication four times for 15 s on ice using an Ultrasonic homogenizer UX-050 (Mitsui Electric, Chiba, Japan). The homogenate was centrifuged at 20,000 × *g* for 20 min at 4°C. The supernatant (soluble mite extract, **SME**) was collected and filtered using a 0.2 mm filter (Nihon Pall Ltd., Tokyo, Japan). SME was stored at −80°C until use.

Cell Cultures and Stimulation

The chicken HD-11 macrophage-like cell line transformed by a myc-containing MC29 retrovirus (Beug et al., 1979) was kindly provided by Dr. Furusawa (Hiroshima University, Japan). Chicken PBMC isolated from NIE chickens as described above and the HD-11 cells were suspended in RPMI1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (Cell Culture Technologies LLC, Gravesano, Switzerland), 0.01% L-glutamine, 200 U/mL penicillin, and 200 μg/mL streptomycin (Invitrogen). PBMC or HD-11 cells (2.0×10^6 cells) were cultured at 39°C and 5% CO_2 for 24 h in the presence of 1% SME or the same volume of sterilized PBS. The supernatants were removed, and the cells were suspended with TRI reagent (Molecular Research Center, Inc.). RNA extraction and cDNA synthesis were performed as described previously.

Real-Time Quantitative RT-PCR

Specific primer sets for the chicken cytokine, chemokine, and *β-actin* genes were designed using Primer3Plus (Untergasser et al., 2007), and all the primers were searched in BLAST against chicken DNA (*Gallus gallus*, NCBI:txid9031) to verify the amplification specificity (Supplementary Table 1). Real-time quantitative RT-PCR (**qRT-PCR**) was performed using cDNA samples from chicken PBMC or HD-11 cells with LightCycler480 System II (Roche Diagnostics, Mannheim, Germany) using TB Green Premix DimerEraser (TaKaRa Bio Inc.) according to the manufacturer's instructions. The cycling conditions consisted of initial denaturation at 95°C for 30 s, followed by 45 cycles of 95°C for 5 s, each annealing temperature shown in Supplementary table 1 for 30 s, and 72°C for 30 s. To evaluate primer pairs for their specificities, a final melting curve analysis was performed from 65°C to 95°C at a rate of 0.1°C/s. To generate the standard curves for quantification, serial dilutions of T-vector pMD20 (TaKaRa Bio Inc.) or pGEM-T Easy Vector (Promega Corporation, Madison, WI) inserted with each amplicon were used. The mRNA expression of each target molecule was presented as the ratio obtained by dividing the concentration of the target mRNA by that of *β-actin* mRNA.

Enzyme-Linked Immunosorbent Assay

The concentrations of Interleukin-1β (**IL-1β**), transforming growth factor β1 (**TGF-β1**), and IL-10 in the plasma samples were determined by ELISA using the Chicken Interleukin 1b ELISA kit (CUSABIO TECHNOLOGY LLC, Wuhan, China), chicken transforming growth factor β1 ELISA kit (CUSABIO TECHNOLOGY LLC), and chicken IL-10 Do-It-Yourself ELISA (Kingfisher Biotech Inc., Saint Paul, MN), respectively, according to the manufacturer's instructions. The plasma samples were diluted 2 × or 5 × with PBS for this assay.

Statistics

Differences were analyzed using the Mann-Whitney *U* test for gene expression and ELISA analyses using blood samples from infested and noninfested chickens. For multiple comparisons, the Steel-Dwass' test was performed. Wilcoxon signed rank test and Student's *t* test were conducted to analyze the difference in qRT-PCR using PBMC and HD-11 cells, respectively. Statistical significance was set at $P < 0.05$ and < 0.01.

RESULTS

Anti-Inflammatory Cytokines Were Highly Expressed in Chickens From PRM-Eroded Farms

To analyze the immunological status of chickens chronically infested with PRMs, we first examined the gene expression of pro-inflammatory cytokines, Th1 cytokines, and type I interferon in the PBMC of PRM-infested chickens raised at a PRM-eroded commercial egg-laying farm and noninfested chickens maintained at a PRM-free experimental farm (NIE sample). While the expression of *IL-1β* gene was significantly higher in PRM-infested chickens than in NIE chickens, there was no difference in the expression levels of other pro-inflammatory cytokines, *IL-6* and *TNF-α* (Figure 1A). In addition, no significant difference in the expression levels of Th1 cytokines, *IL-2, IL-12*, and *IFN-γ*, and a type I interferon, *IFN-α*, was observed (Figures 1B and 1C). Thus, the expression of pro-inflammatory cytokines, except for *IL-1β*, Th1 cytokines, and interferons in infested chickens was not significantly higher than that in NIE chickens.

We next examined the expression of anti-inflammatory cytokines, *IL-10, TGF-β1, TGF-β4*, and other molecules that contribute to immune suppression. The expression levels of *IL-10* and *TGF-β1* in infested chickens were significantly higher than those in NIE chickens, whereas there was no difference in the expression levels of *TGF-β4* between infested and NIE chickens (Figure 2A). Additionally, we analyzed the gene expression of a marker for regulatory T (Treg)-like cells in chickens, *CD25* (Shanmugasundaram and Selvaraj, 2011), immune checkpoint molecules, cytotoxic T-lymphocyte antigen 4 (*CTLA-4*), and programmed death-1 (***PD-1***) and PD-ligand 1 (***PD-L1***). The expression levels of *CD25, CTLA-4*, and *PD-1* were significantly higher in infested chickens than in NIE chickens, whereas no difference in the expression of *PD-L1* was observed (Figure 2B). Taken together, the expression levels of several indicators of

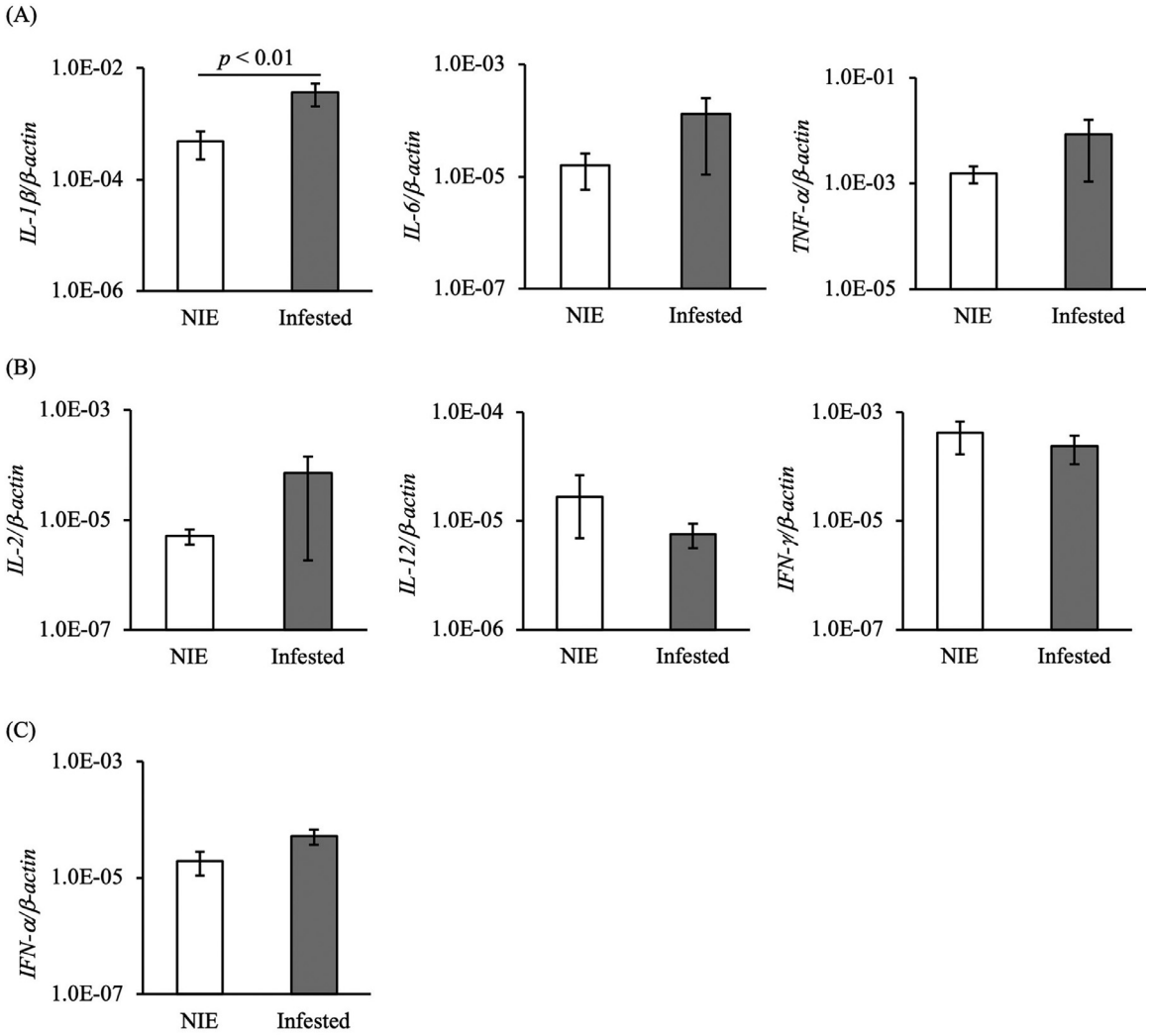

Figure 1. Gene expressions of inflammatory cytokines, Th1 cytokines, and a type I interferon in non-infested and infested chickens. The mRNA of (A) inflammatory, (B) Th1 cytokines, and (C) a type I interferon in PBMC collected from noninfested experimental (NIE) chickens raised at an experimental farm without contamination with poultry red mites (PRMs) ($n = 11$) and infected chickens from a PRM-eroded commercial laying hen farm ($n = 16$) were examined by qRT-PCR. The mRNA expression levels of each target were presented as ratios obtained by dividing the concentrations of target mRNA by those of *β-actin* mRNA. Data are presented as mean ± standard error of the mean. Statistical significance was determined using the Mann-Whitney *U* test. Statistical significance was set at $P < 0.05$ and < 0.01.

immune suppression were increased in chickens chronically infested with PRMs.

Plasma Concentration of Suppressive Cytokines Was Increased in Chickens From PRM-Eroded Farms

To confirm protein levels of molecules whose gene expression was upregulated in infested chickens, we compared the concentrations of *IL-1β*, *IL-10*, and *TGF-β1* in plasma samples collected from infested and NIE chickens. In addition, considering that other extrinsic factors such as vaccination may affect immunological state, plasma from noninfested chickens raised at a PRM-uncontaminated commercial laying hen's farm (NIC) were also tested. No difference in *IL-1β* concentration between the NIE and infested groups was observed, whereas *IL-1β* concentration in NIC chickens was significantly higher than that in NIE chickens and infested chickens (Figure 3). Although *IL-10* was not detected in both NIE and NIC chickens, it was detectable in 8 out of 11 infested chickens (Figure 3), suggesting that the plasma concentration of *IL-10* in PRM-infested chickens was high, consistent with the kinetics of gene expression observed in PBMC from PRM-infested chickens. The concentration of *TGF-β1* in the plasma from infested chickens tended to be higher than those in NIE chickens, although NIC chickens tended to have higher concentrations of *TGF-β1* than NIE chickens (Figure 3). However, statistical differences were not determined among the 3 groups, because NIE and NIC chickens contained a sample that was below the quantitation limit. Collectively, these results suggest that chickens chronically infested with PRMs are in an immunosuppressive state.

PRM-Infestation Potentially Upregulates the Expressions of Anti-inflammatory Cytokines

To further examine whether PRM infestation modulates cytokine expression, we assessed the concentrations

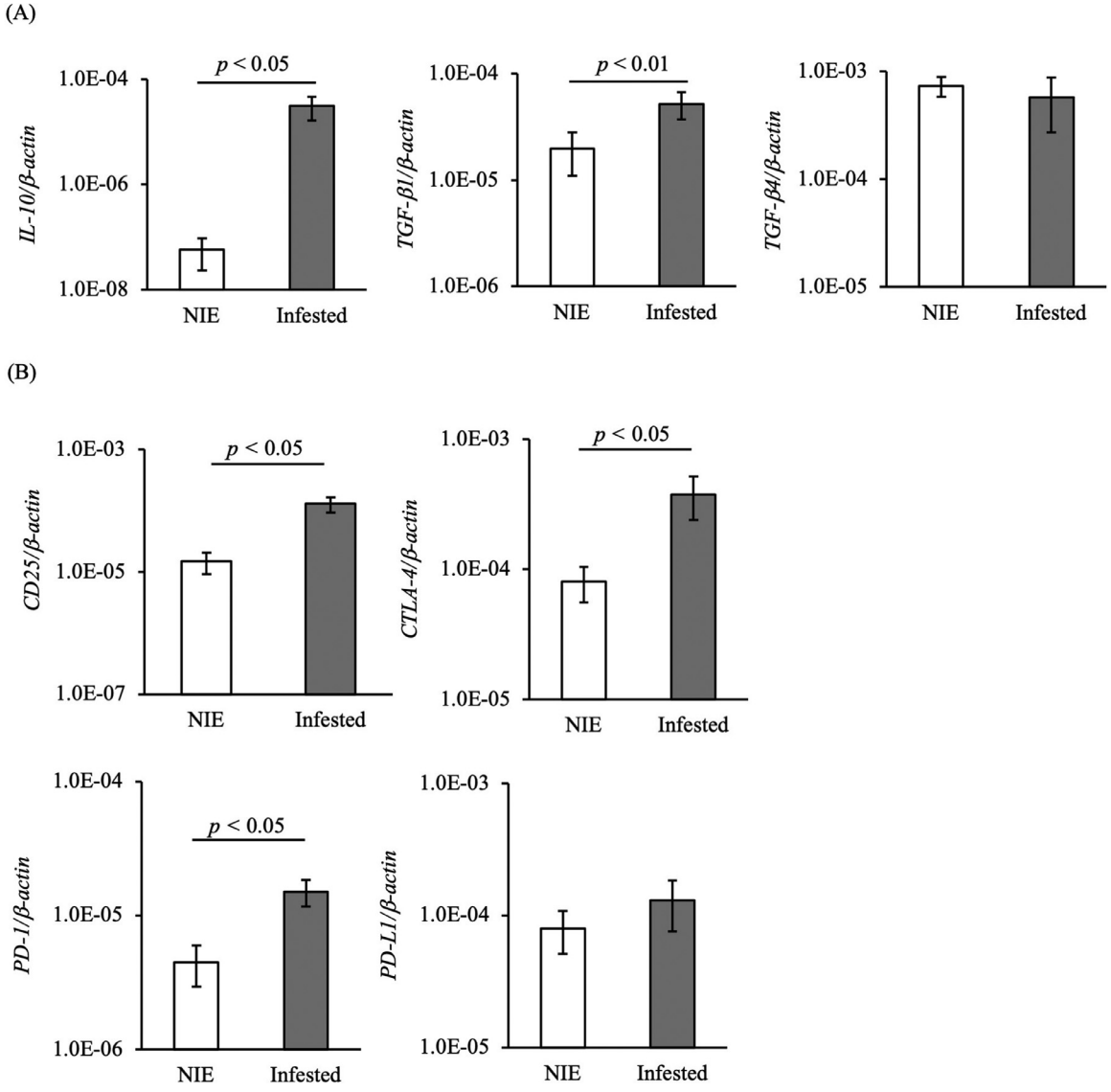

Figure 2. Gene expressions of anti-inflammatory cytokines and the molecules related to the immunosuppression in noninfested and infested chickens. The mRNA levels of (A) anti-inflammatory cytokines and (B) *CD25,* a marker for regulatory T-like cells in chickens, and immune checkpoint molecules in PBMC collected from NIE chickens raised at a PRM-free experimental farm ($n = 11$) and infested chickens from a PRM-eroded commercial laying hen's farm ($n = 16$) were examined by qRT-PCR. The mRNA expression levels of each target were presented as ratios obtained by dividing the concentrations of target mRNA by those of *β-actin* mRNA. Data are presented as mean ± standard error of the mean. Statistical significance was determined using the Mann-Whitney *U* test. *Statistical significance was set at* $P < 0.05$ and < 0.01 were considered statistically significant.

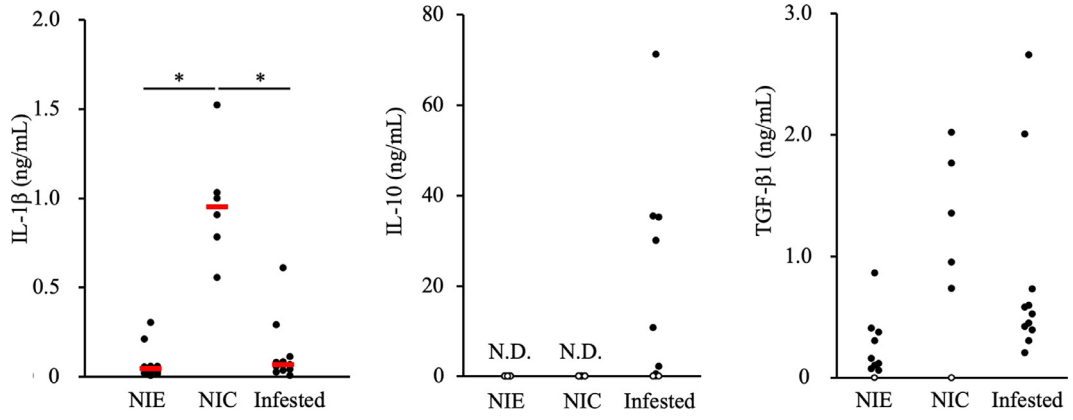

Figure 3. Plasma concentrations of IL-1β, IL-10, and TGF-b1 in noninfested and infested chickens. The concentrations of IL-1β, IL-10, and TGF-β1 in the plasma samples collected from noninfested chickens raised at a PRM-free experimental farm, noninfested commercial (NIC) chickens raised at a PRM-free laying hen's farm, and infested chickens from a PRM-eroded commercial laying hen farm were determined by ELISA. IL-1β, NIE: $n = 13$, NIC: $n = 6$, Infested: $n = 12$; TGF-β1, NIE: $n = 12$, NIC: $n = 6$, Infested: $n = 12$; IL-10, NIE: $n = 10$, NIC: $n = 6$, Infested: $n = 11$. Statistical differences were determined using the Steel-Dwass test. White circles indicate samples that were less than the quantitation limit. $^*P < 0.05$. N.D.: not detected.

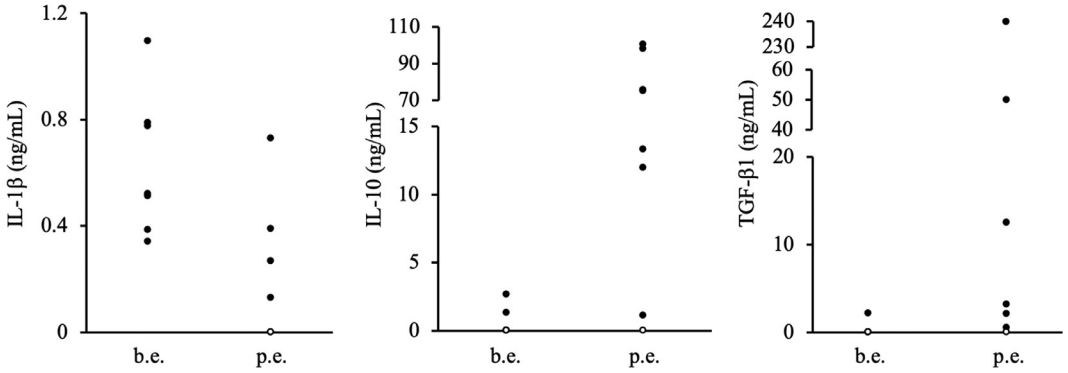

Figure 4. Changes in plasma concentrations of cytokines postexposure to PRMs. The concentrations of IL-1β, IL-10, and TGF-β1 in the plasma samples collected from noninfested chickens raised at a PRM-free coop (before exposure, b.e.) and infested chickens that were transferred from a PRM-free coop to PRM-contaminated coop (postexposure, p.e.) were determined by ELISA. IL-1β and TGF-β1, b.e.: $n = 7$, p.e.: $n = 7$; IL-10, b.e.: $n = 8$, p.e.: $n = 8$. White circles indicate samples that were less than the quantitation limit.

of *IL-1β, IL-10,* and *TGF-β1* in the plasmas collected from the same flock before (b.e.) and after exposure (p.e.) to PRMs. Statistical analyses were not performed because samples that were below the quantitation limit were included in each analysis. *IL-1β* concentrations in p.e. chickens were lower than those in b.e. chickens, and 3 out of 8 p.e. samples showed values less than the quantitation limit (Figure 4). On the other hand, the concentrations of *IL-10* and *TGF-β1* were higher in p.e. chickens than in b.e. chickens as expected. Six out of 8 b.e. samples and one out of 8 p.e. samples in the assay for *IL-10,* and 6 out of 7 b.e. samples and one out of 7 p.e. samples in the assay for *TGF-β1* indicated values less than the quantitation limit (Figure 4). Thus, following exposure to PRMs, the production of anti-inflammatory cytokines, *IL-10* and *TGF-β1*, was increased, whereas the plasma concentration of *IL-1β* was reduced.

PRM-Derived Soluble Extracts Modulate the Gene Expression of Cytokines in Chicken PBMC

To investigate the direct effects of PRM-derived molecules on chicken immune cells, PBMC from NIE chickens were cultured with soluble mite extracts (SME). Remarkably, mRNA expression of IFN-α, a representative cytokine that contributes to the innate immune response, was significantly decreased in the presence of SME (Figure 5A). In contrast to the results observed in Figures 1A and 1B, the gene expression of several pro-inflammatory and Th1 cytokines was significantly upregulated in SME-stimulated PBMC compared with those in the unstimulated PBMC (*IL-1β, IL-2,* and *IFN-γ*) (Figure 5A). In terms of anti-inflammatory cytokines, the treatment with SME drastically upregulated the expression of *IL-10* gene, while it did not affect the expression level of *TGF-β1* (Figure 5B). As we observed higher expression of other molecules that contribute to immune suppression, *CD25, CTLA-4,* and *PD-1*, in PRM-infested chickens (Figure 2B), we next examined whether SME promoted the expression of these molecules. The mRNA expression of *CD25* and *CTLA-4* was significantly decreased by SME stimulation (Figure 5C). In contrast, SME stimulation significantly upregulated the mRNA expression of *PD-1* and *PD-L1* (Figure 5C). Thus, PRM-derived SME activated chicken immune cells, perhaps mainly T cells, and promoted the expression of pro-inflammatory and Th1 cytokines, whereas PRM-derived molecules decreased *IFN-α* expression and upregulated the expression of *IL-10* and immune checkpoint molecules, *PD-1* and *PD-L1*. These results suggest that PRM-derived molecules have the potential to suppress host immunological states, although the inflammatory response could be activated by exposure to PRMs.

PRM-Derived SME Polarized Macrophages to the M2-Like Subtype

In chickens from commercial farms chronically infested with PRMs, the expression levels of *CD25*, a marker for Treg-like cells in chickens, and immune checkpoint molecules, *CTLA-4* and *PD-1*, were increased (Figure 2B), whereas stimulation with SME did not upregulate the expression levels of *CD25* and *CTLA-4* in chicken PBMC (Figure 5C). These results suggest that the suppressor cells, which are different from Treg-like cells, may produce suppressive cytokines during the initial phase of PRM-infestation. Therefore, we next focused on the suppressor macrophages, and gene expression of cytokines and related molecules were examined in HD-11, a chicken macrophage cell line, cultured in the presence of SME. SME exposure significantly downregulated the expression of *TNF-α, IFN-α*, and *IFN-γ* in HD-11 cells, while *IL-1β* expression was upregulated (Figure 6A). On the other hand, mRNA expression of *IL-10* and *PD-L1* increased in response to treatment with SME (Figure 6B). Finally, to investigate the functional phenotypes of HD-11 cells treated with SME, we analyzed the expression levels of interferon regulatory factor-5 (***IRF5***), which regulates inflammatory M1 macrophage polarization (Krausgruber et al., 2011), and *IRF4* and Arginase 2 (***Arg2***), which are considered markers of M2 macrophages (Satoh et al., 2010; Hardbower et al., 2016). The expression level of *IRF5*

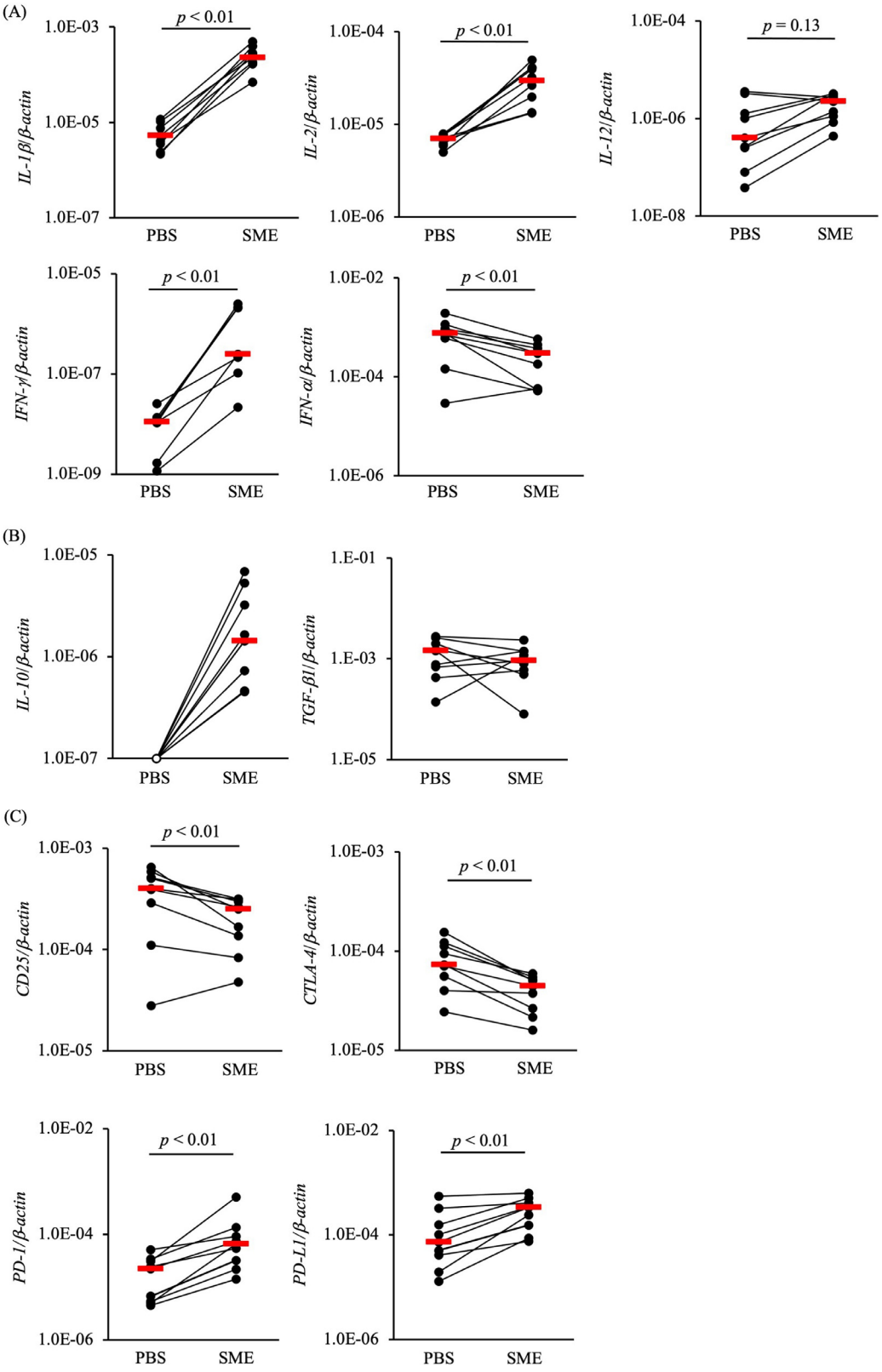

Figure 5. Gene expressions of cytokines and the molecules related to immunosuppression in SME-stimulated chicken PBMC. PBMC collected from NIE chickens raised on a PRM-free experimental farm were cultured with soluble mite extracts (SME) or PBS for 24 h ($n = 9$). Gene expression of (A) pro-inflammatory cytokines, Th1 cytokines, and a type I interferon, (B) anti-inflammatory cytokines, and (C) *CD25*, a marker for regulatory T-like cells in chickens, and the immune checkpoint molecules were examined by qRT-PCR. The extent of the expression of target genes was calculated by dividing the copy number of each target by that of *β-actin*. Statistical significance was determined using the Wilcoxon signed-rank test. White circles indicate samples that were less than the quantitation limit. Statistical significance was set at $P < 0.05$ and < 0.01. Abbreviations: NIE, noninfested experimental; PBMC, peripheral blood mononuclear cells.

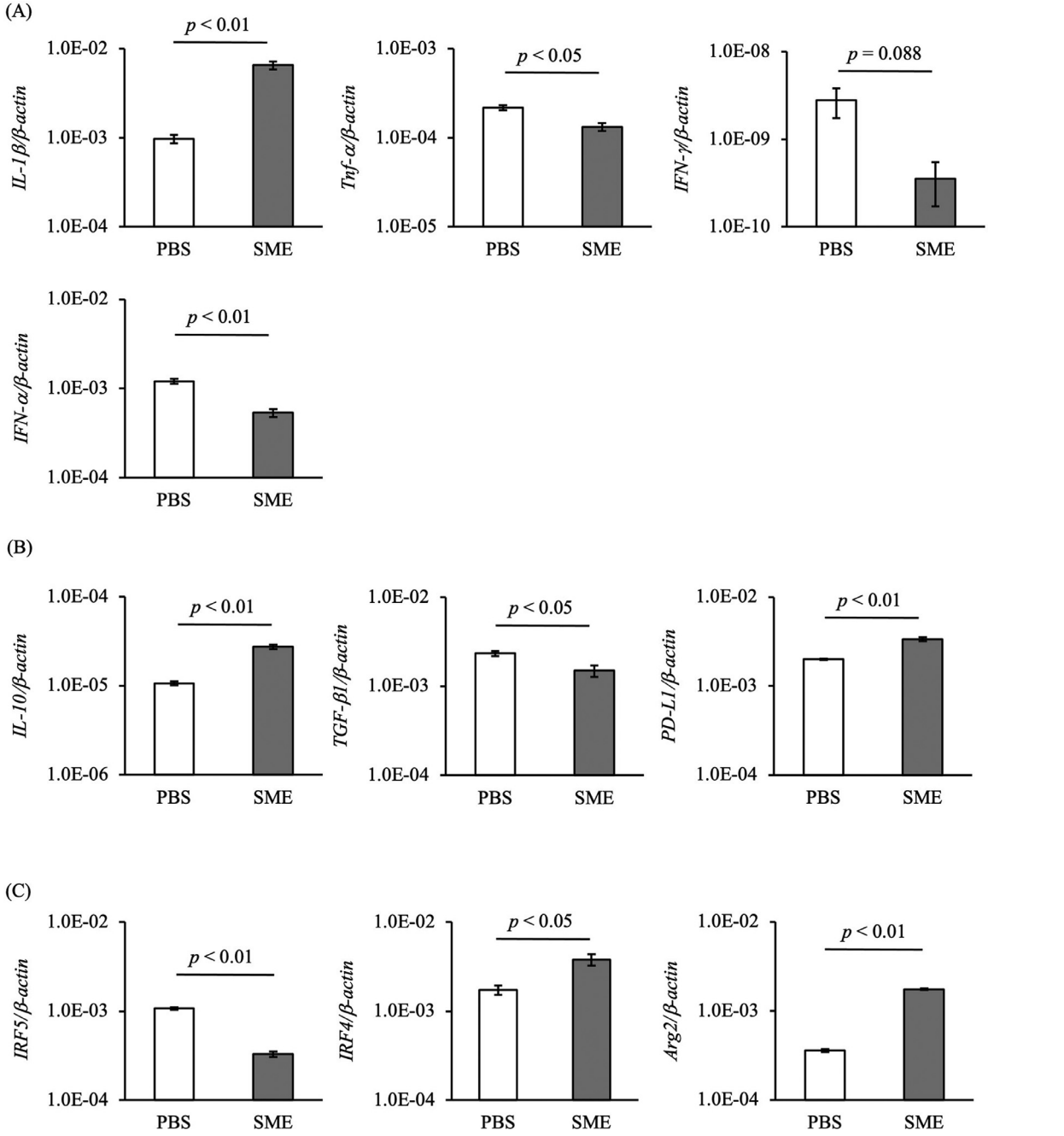

Figure 6. Changes in gene expressions of chicken macrophages in response to SME exposure. HD-11 chicken macrophages were cultured with SME or PBS for 24 h ($n = 5$). Gene expressions of (A) *IL-1β*, *IFN-γ*, *TNF-α*, and *IFN-α*, (B) anti-inflammatory cytokines and an immune checkpoint molecule, *PD-L1*, (C) the molecules related to the polarization of macrophages to M1 or M2 phenotypes, were examined by qRT-PCR. The extent of the expression of target genes was calculated by dividing the copy number of each target by that of *β-actin*. Statistical significance was determined using Student's *t* test. Statistical significance was set at $P < 0.05$ and < 0.01.

was significantly reduced in SME-treated HD-11 cells, whereas *IRF4* and *Arg2* were highly expressed following treatment with SME (Figure 6C). Taken together, PRM-derived molecules seem to be partially involved in the suppression of inflammatory responses in chickens by facilitating the polarization of macrophages into the M2-like anti-inflammatory phenotype.

DISCUSSION

Several studies have reported that various effector molecules secreted from their saliva modulate host immunological states during infestation (Cross et al., 1994; Mejri et al., 2002; Schneider et al., 2004; Brake and Pérez de León, 2012; Kotál et al., 2015). This not only contributes to establishing a suitable environment for undisturbed blood sucking but also prompts pathogen transmission (Schneider et al., 2004; Šimo et al., 2017; Nuttall, 2019; Guerrero et al., 2020; Schneider and Higgs, 2008). Thus, understanding host responses against infestation by ectoparasites is important for establishing strategies to control infectious diseases as well as parasitical burdens. Here, we demonstrated that chickens chronically infested with PRMs are in immunosuppressive states and that PRM-

derived soluble molecules possibly induce the polarization of macrophages into the M2-like phenotype, resulting in immunosuppression. This is the first report exploring detailed immune reactions, including the expression of cytokines against PRM infestation.

The increase in mRNA expression of inflammatory cytokines, except for *IL-1β*, and Th1 cytokines was not observed, although it was hypothesized that inflammatory responses would be induced in the peripheral blood by PRM-infestation (Wikel, 1982; Šimo et al., 2017). Throughout this study, the plasma concentration of IL-1β in PRM-infested chickens tended to be lower than that in noninfested chickens, although an opposite tendency was observed in the gene expression of *IL-1β*. IL-1β is synthesized as an inactive pro-IL-1β in the cytoplasm and processed by the cysteine protease caspase-1, which is secreted from the cells as a mature IL-1β (Li et al., 1995; Qu et al., 2007). Several studies have reported the presence of cysteine protease inhibitors in tick saliva (Kotsyfakis et al., 2006; Sajiki et al., 2020), cysteine protease inhibitors contained in PRM saliva may inhibit the processing of cytoplasmic pro-IL-1β and reduce the plasma concentration of mature IL-1β.

Mounting studies have reported that tick saliva suppresses the production of pro-inflammatory cytokines, such as TNF-α and IFN-γ, and upregulates the expression of immune checkpoint molecules (Chen et al., 2012; Poole et al., 2013; Sajiki et al., 2021). Consistently, PRM-derived SME suppressed the expression of interferons and inflammatory cytokines from HD-11 chicken macrophages, and notably, we discovered that SME exposure triggered the polarization of macrophages into the anti-inflammatory M2-like phenotype. In contrast, certain subsets of immune cells, probably T cells, seemed to be activated by treatment with SME, as indicated by the upregulation of Th1 cytokines, *IL-2* and *IFN-γ*, in SME-stimulated PBMC. As an explanation for this contradictory phenomenon, IL-10 may be a possible regulator of cell type-selective modulation. IL-10, which is a pleiotropic cytokine, exerts anti-inflammatory effects on macrophages, whereas it elicits inflammatory functions of $CD8^+$ T cells, depending on the expression level of its low-affinity receptor IL-10Rβ (Saxton et al., 2021). In the present study, the expression of IL-10 was significantly increased in PRM-infested chickens and SME-stimulated PBMC and HD-11 cells, suggesting that IL-10 may mediate PRM-driven immunomodulation as a critical regulator. Further experiments, including the blockade of IL-10, are required to elucidate the molecular mechanisms of host immune responses mediated by PRM-derived molecules.

Chickens raised at farms continuously contaminated with PRMs are chronically exposed to PRMs. In the PBMC samples from chickens chronically infested with PRMs, the expression levels of immune checkpoint molecules, *CTLA-4* and *PD-1*, were highly expressed, suggesting that chronic or severe infestation by PRMs would result in T cell exhaustion (Wherry and Kurachi, 2015; Buchbinder and Desai, 2016). Interestingly, *PD-L1* expression in PBMC and HD-11 macrophages was increased by treatment with SME; therefore, its continuous expression may contribute to the development of T-cell exhaustion. As another possibility that indicates immunosuppressive state, the expression of *CD25*, in addition to the immune checkpoint molecules, was upregulated in PBMC from chickens chronically infested with PRMs, implying an increase in Treg-like cells. In addition, the higher expression of *TGF-β1* observed in chronically infested chickens seems to indicate an increase in Treg-like subpopulations (Vignali et al., 2008), because M2-like HD-11 macrophages polarized by stimulation with SME did not exhibit increased expression of *TGF-β1*. In contrast, the expression levels of *TGF-β1, CD25*, and *CTLA4* were not increased in SME-treated PBMC in this study, suggesting that transient stimulation with PRM-derived molecules could not promote the development of Treg-like subpopulations. Although further experiments including flow cytometric analyses are warranted to elucidate the association with Treg-like cells in a state of immunosuppression in chickens chronically infested with PRMs, T cell responses seem to be suppressed in affected chickens.

We demonstrated that PRM infestation potentially modulates host immune responses; however, there are still some limitations. In this study, we analyzed samples collected from chickens that were randomly selected on farms uncontaminated and contaminated with PRMs. Therefore, it should be noted that the samples were obtained from chickens of mixed ages and breeds. A recent study has demonstrated that the age of chickens could affect the biology of PRMs, as represented by the decrease in egg laying in PRMs that infested aged chickens (Nunn et al., 2020). Thus, the biology of the PRMs could be affected by a variety of factors in chickens, such as ages and breeds. This may affect the immunological states of infested chickens. Therefore, further investigation using age-matched and breed-matched controls is required to precisely clarify the immunomodulatory effects of PRM infestation on chickens. In addition, in this study, contamination with PRM on poultry farms was confirmed visually or with traps; however, evaluating the degree of PRM contamination on each farm is difficult, and the comparison cannot be performed using samples from farms with similar levels of PRM contamination. As another limitation of this study, in cell culture experiments, we used SME that contained not only salivary proteins but also secretory proteins from other tissues, such as the midgut and hemosome. Techniques to collect saliva or salivary glands have yet to be developed due to the small sizes of PRMs. Furthermore, in the present study, the possibility that lipopolysaccharide (**LPS**) contained in SME influenced the cytokine expression could not be excluded, although it is less likely since the mRNA expression of *TNF-α*, the representative cytokine induced by LPS stimulation (Beutler and Rietschel, 2003), was not upregulated in HD-11 cells. Considering these limitations, analyses using samples from chickens experimentally infested with PRMs are required to corroborate the immunomodulatory effects of PRM infestation.

In the present study, we propose that PRM infestation or PRM-derived soluble molecules can suppress the host immune response. In addition, the expression of *IFN-a*, which contributes to the innate immune response, was decreased in SME-treated PBMC and HD-11 macrophages. Therefore, immunosuppression by infestation with PRMs may contribute to pathogen transmission, as described in tick studies (Kotál et al., 2015). Further investigations such as experimental infections in PRM-infested chickens are needed to examine the impact of PRM infestation on the poultry industry.

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DISCLOSURES

TS, EO, and AT are employed by Vaxxinova Japan K.K, Tokyo, Japan. The other authors have no financial conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2023.102532.

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