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J Vet Res 64, 33-38, 2020
DOI:10.2478/jvetres-2020-0009

Potential role of macrophage migration inhibitory factor in the pathogenesis of Marek's disease

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Received: July 9, 2019 *Accepted: January 20, 2020*

Abstract

Introduction: Marek's disease virus (MDV) can cause malignant T-cell lymphomas and immunosuppression in chickens. Macrophage migration inhibitory factor (MIF) not only plays a critical role in inhibiting T-cell responses, but also contributes to multiple aspects of tumour progression. The aim of this study was to reveal the potential role of MIF in the pathogenesis of MDV infection. **Material and Methods:** MIF gene expression levels were measured by using real-time PCR. Expression was assayed at different times in chicken embryo fibroblast (CEF) cells and tissue samples of SPF chickens infected with different MDV strains and fold change was calculated by the $2^{-\triangle\triangle CT}$ method. **Results:** The expression of MIF was significantly downregulated ($p < 0.05$ and $FC > 2$) in CEF cells infected with the very virulent MDV RB1B strain at 48 h post infection (hpi) and in the skin and spleen at 14 days post infection (dpi). The reduction of MIF expression was also found in CEF cells infected by reticuloendotheliosis virus (REV), avian leukosis virus subgroup J (ALV-J), and MDV vaccine strain CVI988 or in HD11 cells stimulated with TLR2, 3, 4, and 7 ligands. Interestingly, MIF expression decreased continuously from 7 to 28 dpi in the thymus after RB1B virus infection while it increased after CVI988 virus infection. Upregulated expression of MIF was found in CEF infected with RB1B at 96 hpi and in the spleen and skin at 21 and 28 dpi. **Conclusion:** The present study revealed the different expression pattern of MIF in response to MDV infection and indicated that MIF level may be associated with MDV pathogenesis.

Keywords: Marek's disease virus, macrophage migration inhibitory factor, chicken embryo fibroblasts, pathogenesis.

Introduction

Marek's disease (MD) is a lymphoproliferative disease of birds caused by a highly oncogenic, cell-associated α-herpesvirus termed Marek's disease virus (MDV) (3). This virus can cause malignant T-cell lymphomas and immunosuppression in chickens. The primary target cells for virus infection in the chicken are B lymphocytes. The virus destroys the cells in a few days after infection and then enters a latent phase. During latent infection of activated T cells, expressed genes are low in abundance, but the virus can be obtained from the lymphocytes (21). These latently infected T lymphocytes are the means of virus dissemination to the skin and feather follicle epithelial cells. Toll-like receptors (TLRs) have key roles in the recognition of pathogens and the initiation of the innate immune response that subsequently primes the specific adaptive immune response during infection. In addition, the activation of TLRs not only has implications for antiviral defence but also contributes to tumour suppression. Increased expression of TLR2, TLR3, TLR4, and TLR7 was found in MDV-infected chicken tissues (10).

Macrophage migration inhibitory factor (MIF) is a classic pro-inflammatory cytokine secreted by several cell types, including activated T lymphocytes and macrophages, and plays a central role in the control of the host inflammatory and immune response (4). MIF was initially described as a soluble mediator secreted by activated T cells that inhibits the migration of macrophages. MIF antibody treatment has been shown to elicit a significant increase in cytotoxic T lymphocyte (CTL) response, as well as increased

levels of interferon gamma (IFN-γ) expression (1). MIF not only plays a critical role in inhibiting T-cell responses but also contributes to multiple aspects of tumour progression through modulating several important biological mechanisms and processes (19). In addition, mounting evidence suggests that inflammation is closely associated with many types of cancer and MIF is a potent molecular link between inflammation and cancer (16). Moreover, MIF antibody treatment effectively suppressed tumour growth and tumour-associated angiogenesis (23). Taken together, these actions of MIF define it as important for the development and progression of cancer and render it exploitable as a marker for tumour detection.

In the previous study, MIF was identified as a differentially expressed protein in chicken thymus infected with the very virulent MDV RB1B strain, suggesting this protein might be involved in the pathogenesis of Marek's disease in poultry (9). Functional characterisation of avian MIF demonstrated the inhibition of macrophage migration, similarly to mammalian MIF, and the mediation of inflammatory responses during antigenic stimulation (12). However, there was no further investigation of its role in the pathogenesis of MDV infection or tumour progression in birds, and little is known about whether MIF is associated with the TLR-mediated immune response. In this study, we explore the potential role of MIF in the pathogenesis of MDV and make an attempt to identify the areas where knowledge is lacking in this field.

Material and Methods

Animals, cells and viruses. All chickens used in this study were one-day-old specific-pathogen-free (SPF) white Leghorns obtained from Merial Vital (Laboratory Animal Technology Co., Beijing, China). The chickens were housed in an isolation facility at the College of Marine and Biological Engineering, Yancheng Teachers' University. The RB1B (passage 15) strain of very virulent MDV and MDV vaccine strain CVI988 were maintained in the cell engineering laboratory in College of Marine and Biological Engineering, Yancheng Teachers' University.

Primary chicken embryo fibroblast (CEF) cells were prepared by standard methods from ten-day-old SPF embryos. The cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% foetal bovine serum (FBS) and antibiotics (100 U/mL of penicillin and 100 U/mL of streptomycin, Gibco) and were incubated at 37°C in 5% CO_2 for 24 h. After incubation, secondary CEF was used for virus infection. HD11, an avian macrophage cell line, was cultured in RPMI 1640 medium (Gibco) supplemented with 10% FBS, 10% tryptose phosphate broth (Sigma-Aldrich, St. Louis, MO, USA) and antibiotics (100 U/mL of penicillin and 100 U/mL of streptomycin, Gibco) at 41°C, 5% CO_2 and 95% humidity.

Infection of CEF cells with MDV, reticuloendotheliosis virus (REV), or avian leukosis virus subgroup (ALV-J). MDV infections of CEF cells were conducted as previously reported (14). Secondary CEF cells were seeded on 6-well plates in DMEM with 5% FBS at 37°C, 5% CO_2, and 95% humidity. After 24 h incubation, the cells were infected with two MDV strains (RB1B and CVI988) separately at 0.1 multiplicity of infection (MOI) and each virus infected three wells on six-well plates. Then the cells were collected at 24, 48, 72 and 96 h post infection (hpi). REV and ALV-J infections of CEF cells were conducted by the same method, and the infected cells were collected at 24, 72, 120, and 168 hpi.

TLR stimulation experiments. HD11 cells were seeded on six-well plates and incubated for 4–16 h in medium containing selected TLR ligands (all from InvivoGen, Hong Kong, China) used at a unified concentration of 10 μg/mL. The stimulants were *E. coli* 0111:B4 ultrapure TLR2 ligand peptidoglycan (PGN-EB), dsRNA poly analogue synthetic TLR3 ligand (I:C) of a high molecular weight, *E. coli* 0111:B4 ultrapure TR4 ligand lipopolysaccharide (LPS-EB), and small synthetic antiviral molecule Imiquimod TLR7 ligand (R837). After incubation, HD11 cells were collected at 4, 8, 12, and 16 h.

Experimental animals. This experimental work was performed as reported previously (9). Briefly, 72 one-day-old chickens were randomly divided into three equal groups (an RB1B-infected group, a CVI988-infected group, and a control group). The chickens were kept in separate units under similar environmental conditions. The chickens in infected groups received an intraperitoneal inoculation of 0.5 ml of RB1B or CVI988 virus solution at a dose of 2,000 plaque-forming units. At 7, 14, 21, and 28 dpi, nine chickens (three RB1B-infected, three CVI988-infected, and three uninfected control birds) were sacrificed and samples of the thymus, spleen, bursa, and skin were excised rapidly, rinsed with ice cold PBS to remove blood contaminants and immediately stored in liquid nitrogen until the real-time PCR analyses.

RNA isolation and quantitative real-time PCR. These procedures were conducted by methods previously reported (8). Briefly, total RNA was extracted from each tissue (0.1 g) or cell sample (2.5×10^6 per well) using an AxyPrep Multisource Total RNA Miniprep Kit (Axygen, Union City, CA, USA), and each RNA sample (1 μg) was reversed into first-strand cDNA using the PrimeScript RT Master Mix (TaKaRa Biomedical Technology, Beijing, China) following the manufacturer's instructions. Then the cDNA was diluted with nuclease-free water to 1:10, and 1 μL of the diluted sample was used for the real-time PCR with 400 nM of primers and 10 μL of SYBR Green Master Mix (TaKaRa) in a final volume of 20 μL. The primer sequences for MIF, GAPDH (12), and 18S rRNA (15)

have been previously reported and are given in Table 1. The expression level of MIF was determined using real-time PCR (7500 Real-Time PCR System, Applied Biosystems, Foster City, CA, USA) with amplification conditions of 95°C for 30 s, 40 cycles of 95°C for 5 s, and 60°C for 34 s. The expression level of MIF was normalised against the expression of chicken GAPDH in tissues or 18S mRNA in CEF cells and fold change in gene expression was calculated by the $2^{-\Delta\Delta CT}$ method.

Statistical analysis. Statistical analysis was conducted with the Statistical Package for the Social Sciences in version 16.0 (IBM, Armonk, NY, USA). Student's *t*-test was used to determine significant difference between fold change values of control and infected or treated MIF genes. Fold changes (FC) that had both $P < 0.05$ and greater than two-fold difference in expression levels were considered significantly different. Standard error was calculated using the FC values of three replicates for each gene measured.

Results

Differential expression pattern of MIF in chicken fibroblasts (CEF). To analyse the potential role in the pathogenesis of MDV infection, we first detected the expression of MIF in CEF cells infected with two MDV strains (RB1B and CVI988). We found that the expression of MIF was obviously upregulated at 24 hpi and significantly down regulated ($P < 0.001$ and $FC > 2$) at 48 hpi in CEF cells infected with the RB1B strain. After 48 hpi, the expression trend of MIF was gradually rising and it was significantly upregulated ($P < 0.01$ and $FC > 2$) at 96 hpi. Unlike in RB1B infection, MIF expression sharply reduced in CVI988 strain-infected-CEF cells at 24 hpi and then significantly augmented ($P < 0.01$ and $FC > 2$) at 72 and 96 hpi (Fig. 1A). To further confirm whether MIF induction is a unique characteristic for MDV infection in three avian viruses which induced immunosuppressive and tumour diseases in poultry (MDV, REV, and ALV), we next detected the expression of MIF in CEF cells infected with REV or ALV-J. Interestingly, MIF was not induced by REV or ALV-J and both reduced MIF expression after infection (Fig. 1B). MIF was actually significantly decreased by REV at 72 ($P < 0.001$ and $FC > 2$) and 120 hpi ($P < 0.01$ and $FC > 2$).

Differential expression pattern of MIF in chicken avian macrophage cell line (HD11). To investigate whether MIF is associated with the expressions of TLRs, we detected the expression levels of MIF in HD11 cells treated with TLR ligands. We found that TLR2 and TLR4 stimulation elicits a continuous and gradual reduction of MIF expression from 4 to 16 h while there is a 4 h delay for TLR3 and TLR7 stimulation (Fig. 1C and D).

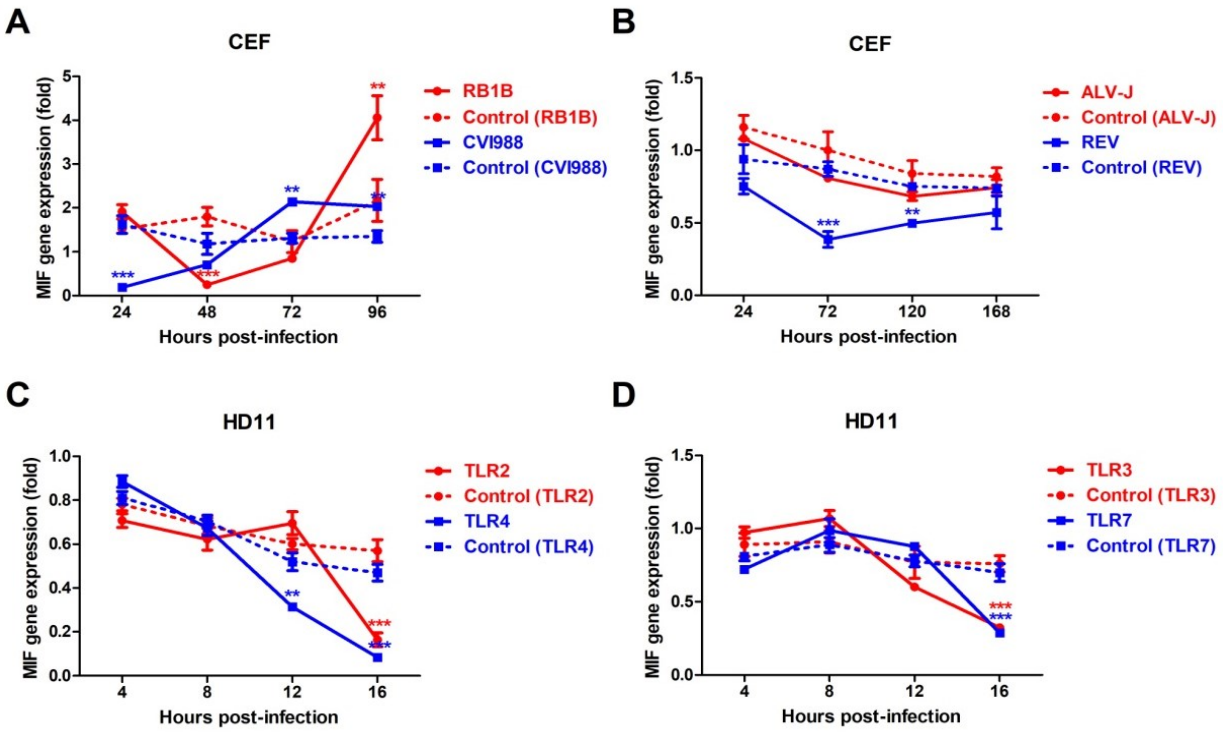

Fig. 1. MIF expression in CEF and HD11 cells. A – expression of MIF in CEF cells infected with RB1B or CVI988 strain; B – expression of MIF in chicken fibroblasts infected with REV or ALV-J; C – expression of MIF in HD11 in response to TLR2 and four stimulations; D – expression of MIF in CEF in response to TLR3 and TLR7 stimulations. The different number of asterisks (*) indicates statistically significant difference for the comparison of control (uninfected or untreated) and infected (or stimulated) transcripts at the same time point as determined by Student's *t*-test. ** – $P < 0.01$, *** – $P < 0.001$. Error bars represent standard error

Table 1. Primers used for real-time PCR

Gene	Primer Sequence (5′–3′)	Product size (bp)	Accession number
MIF	F: GCCCGCGCAGTACATAGC	57	XM42_5824
	R: CCCCCGAAGGACATCATCT		
GAPDH	F: AGGGTGGTGCTAAGCGTGTTA	78	NM_204305
	R: TCTCATGGTTGACACCCATCA		
18S rRNA	F: TCAGATACCGTCGTAGTTCC	154	AF173612
	R: TTCCGTCAATTCCTTTAAGTT		

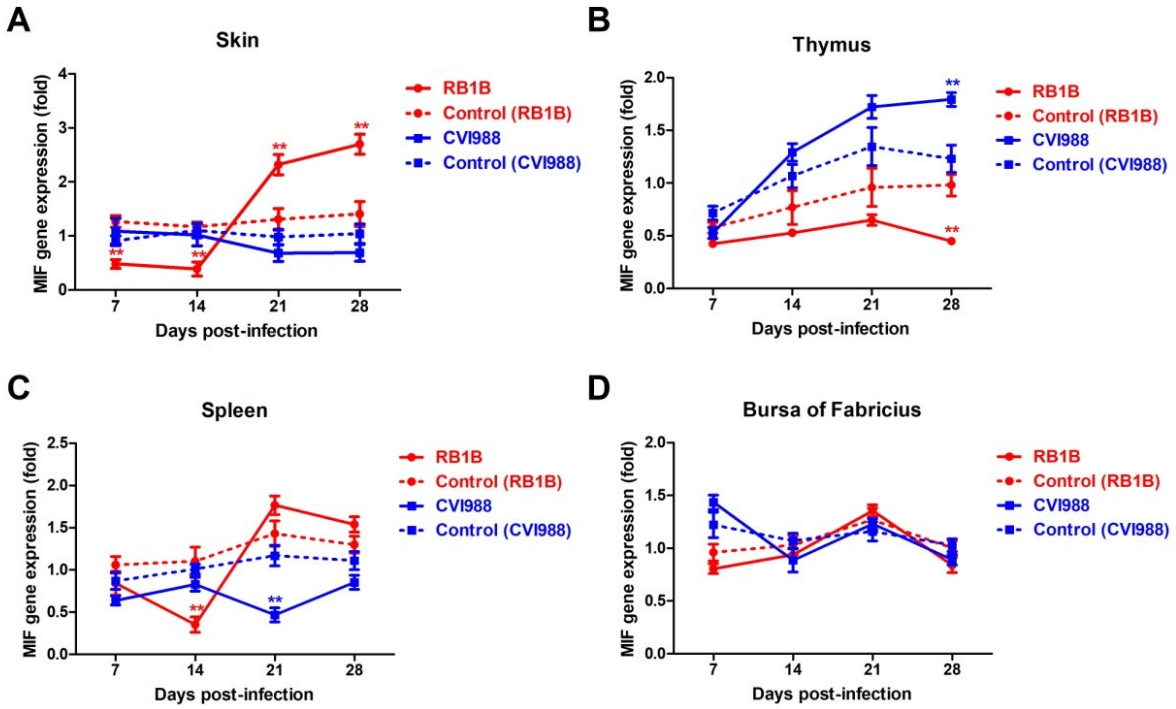

Fig. 2. MIF expression in chicken skin infected with RB1B or CVI988 strain. The different number of asterisks (*) indicates statistically significant difference for the comparison of control (uninfected) and infected transcripts at the same time point as determined by Student's *t*-test. ** – $P < 0.01$. Error bars represent standard error

Differential expression pattern of MIF in chicken tissues. MIF expression was significantly diminished ($P < 0.01$ and $FC > 2$) in RB1B-infected chicken skin at 7 and 14 dpi and elevated at 21 and 28 dpi, whereas it did not show any significant change in CVI988-infected chicken skin at the same time points (Fig. 2A). Reduced expression of MIF was also observed in RB1B-infected chicken thymus at four time points while the MIF expression trend was persistently increased from 7 to 28 dpi in CVI988-infected chicken thymus (Fig. 2B). In the spleen, MIF expression was slightly reduced by CVI988 infection and significantly downregulated ($P < 0.01$ and $FC > 2$) at 21 dpi. MIF expression level was also significantly decreased ($P < 0.01$ and $FC > 2$) by RB1B infection at 14 dpi while obviously upregulated at 21 and 28 dpi (Fig. 2C). In addition, we did not find any significant change of MIF expression in chicken bursa of Fabricius infected with RB1B or CVI988 strains (Fig. 2D).

Discussion

Despite our knowledge of molecular and cellular mechanisms of immunity against MD, we still have a limited understanding of the process and dynamics of T-cell mediated responses to the virus. Moreover, significant information on critical aspects of virus latency in lymphoid cells and the virus-host interaction in MDV-induced lymphoma is lacking. Importantly, there is little known about the molecular determinants of the host which govern T-lymphocyte immune response and transformation in latent MDV infection. T-lymphocytes are of key importance to the immune system and are at the core of adaptive immunity, thus the virus is not sufficient by itself for induction of T-cell lymphomas and the regulatory mechanisms of T-cell immunity could be employed by MDV. Reports showed that MDV influences the expression of genes associated with T lymphocyte responses during MDV infection (13). MIF plays a critical role in inhibiting T-cell responses, and has assumed a centrally important

mediatory function for innate immunity. In this study, we have further revealed the different expression pattern of chicken MIF in response to MDV infection and discussed in detail the potential role of this factor in the course of MDV infection.

MIF has emerged as a pivotal mediator of innate immunity (4). This protein modulates not only macrophage but also T cell functions (4), and especially exerts significant effects on regulation of anti-tumour and antigen-specific cytotoxic T-lymphocyte responses. The downregulation of the MIF gene in avian cells reflects the host immune response to virus infection or TLR stimulations. In fact, MIF expression was also decreased during early MDV infection, as MIF showed reduction in CEF at 24 hpi or 48 hpi and in chicken tissues at 7 dpi or 14 dpi. In addition, we also observed a continuous and gradual reduction of MIF expression in the avian HD11 macrophage cell line after a cell response was elicited by TLR stimulation, indicating that MIF in immune cells can be affected by TLR status. Studies *in vivo* showed that neutralisation of MIF can promote cytotoxic T-lymphocyte activity, increase expression levels of IFN-γ, and increase T lymphocyte homing to sites of tumour invasion (1), while expression of MIF leads to the inhibition of antitumour T lymphocyte reactivity (25) and T lymphocyte activation (24). A possible theory is that high MIF levels cause activation-induced T-cell death through an IFN-γ pathway and may eliminate activated T cells from the tumour microenvironment and thus facilitate the tumour's evasion of immune surveillance (24). Interestingly, the very virulent and oncogenic RB1B strain causes reduced expression of MIF in an infected chicken thymus while the non-oncogenic CVI988 vaccine strain causes increased expression. At present, there is little reported research into the function of chicken MIF, and we deduced that this protein plays either a protective or deleterious role in the immune response to different pathogens. Increased expression of MIF after latent MDV infection could inhibit T-cell responses.

Increased expression of MIF in the spleen at 21 and 28 dpi and in CEF at 96 hpi could be relevant to MDV RB1B strain infection and replication, while in skin at 21 and 28 dpi it might be associated with the production of MDV virus particles. Firstly, we observed that the expression trend of MIF was gradually rising along with the replication of MDV after 48 hpi for RB1B or 24 hpi for CVI988 and was significantly up regulated at 72 and/or 96 hpi. The results suggested that MIF expression was influenced by MDV during the different stages of pathogenesis. However, MIF was not induced by the other two of the three avian viruses (ALV-J and REV) which induced immunosuppressive and tumourigenic diseases in poultry in infected CEF cells. This may indicate a direct role for MIF in MDV replication or pathogenesis. Induction of MIF expression was also found in herpes simplex virus type 1 (HSV-1) (18), human cytomegalovirus (HCMV), (6) and dengue virus (5). HCMV paralyses macrophage motility through release of MIF (6), and MIF promotes HIV-1 replication through the activation of HIV-1 long terminal repeats (LTR) (22).

However, the reduction of MIF in skin, spleen, and thymus during early and latent infection may promote MDV spread. This is because strong macrophage migration activity when MIF is reduced will not only enhance random migration of macrophages but elicit T lymphocyte activation, and this could offer an opportunity for macrophages that carry MDV to spread the virus to T lymphocytes, and then latently infected lymphocytes can disseminate the virus to different sites. Indeed, infection of macrophages *in vivo* by MDV has been reported (2). Some MDV strains can replicate in macrophages, which leads to increased macrophage death (2) and a heavy infiltration of macrophages occurs around blood vessels at 8–10 days post MDV infection (7). In addition, macrophages play an essential role in the sensing and elimination of invasive microorganisms and this also offers the better option for virus contact with other immune cells. Thus, macrophages are excellent candidates for transporting MDV to primary lymphoid organs during the earliest stages of pathogenesis, and the reduction of MIF enhanced macrophage migration might be a potential mechanism employed by MDV to increase virus transport.

In the chicken spleen and skin infected with the very virulent and oncogenic strain RB1B, we observed the increased expression of MIF gene at 21 and 28 dpi. However, MIF expression was reduced in the non-oncogenic vaccine strain CVI988-infected spleen and in skin its expression did not show any significant change at the same time points. These findings suggested that MIF might be employed by MDV to induce lymphoma occurrence. Firstly, MIF sustains macrophage survival and function by suppressing p53-dependent apoptosis (17) and this is important for MDV-infected macrophage to spread the virus. Secondly, MIF exerts significant pro-tumour effects by regulation of anti-tumour T-lymphocyte responses. Host T-lymphocyte immunity is the biggest obstacle for MDV infection and the virus is not sufficient by itself for induction of T-cell lymphomas. MIF plays a dual role in both inhibiting T-cell responses and promoting tumour cell growth, and thus this regulatory mechanism could be employed by MDV to promote lymphoma occurrence. More importantly, MIF has been shown to mediate several important biological mechanisms and processes by which tumours thrive and spread. One of these mechanisms is the negative regulation of the important p53 tumour suppressor pathway (11), and the other is the modulation of hypoxic adaptation within the tumour microenvironment through the direct promotion of hypoxia-induced stabilisation of HIF-1α (20). However, the contributions of MIF to MDV-specific T-cell immunity and the mechanism of MD lymphoma occurrence need further investigation.

In summary, the present results provide the different expression pattern of MIF gene in response to the very virulent RB1B strain and CVI988 vaccine strain infections and might reveal a potential role of MIF in the pathogenesis of MDV infection. MIF might be a mechanism employed by MDV to increase virus replication and transport and promote MD lymphoma occurrence and evolution.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

Financial Disclosure Statement: This research was supported by the Guiding Plan for Agricultural Science and Technology of Yancheng City (YKN2013014).

Animal Rights Statement: The animal experiments were conducted in accordance with the guidelines provided by the Chinese Council on Animal Care. All experiments complied with institutional animal care guidelines and were approved by the Animal Ethics Committee of Yancheng Teachers' University.

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