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Unmasking the dynamics of *Mycoplasma gallisepticum*: deciphering HD11 macrophage polarization for innovative infection control strategies

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ABSTRACT *Mycoplasma gallisepticum* (**MG**) is a highly contagious avian respiratory pathogen characterized by rapid spread, widespread distribution, and long-term persistence of infection. Previous studies have shown that chicken macrophage HD11 cells play a critical role in the replication and immunomodulation of MG. Macrophages are multifunctional immunomodulatory cells that polarize into different functions and morphologies in response to exogenous stimuli. However, the effect of MG infection on HD11 polarization is not well understood. In this study, we observed a time-dependent increase in both the expression of the MG-related virulence protein pMGA1.2 and the copy number of MG upon MG infection. Polarization studies revealed an upregulation of M1-type marker genes in MG-infected HD11 cells, suggesting that MG mainly induces HD11 macrophages towards M1-type polarization. Furthermore, MG activated the inflammatory vesicle NLRP3 signaling pathway, and NLRP3 inhibitors affected the expression of M1 and M2 marker genes, indicating the crucial regulatory role of the NLRP3 signaling pathway in MG-induced polarization of HD11 macrophages. Our findings reveal a novel mechanism of MG infection, namely the polarization of MG-infected HD11 macrophages. This discovery suggests that altering the macrophage phenotype to inhibit MG infection may be an effective control strategy. These findings provide new perspectives on the pathogenic mechanism and control measures of MG.

Key words: *mycoplasma gallisepticum* (MG), macrophage polarization, NLRP3, chicken

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INTRODUCTION

Chronic respiratory disease (**CRD**) caused by *Mycoplasma gallisepticum* (**MG**) is the most serious economic infectious disease in poultry industry (Bao, et al., 2020; Tian, et al., 2016). *Mycoplasma gallisepticum* infection causes reduced productivity, compromised immune function in poultry, and increased susceptibility to co-infection with pathogens such as *E. coli* and avian influenza (Stipkovits, et al., 2012; Wu, et al., 2020). A highly pathogenic strain of MG, MG-HS, isolated in China, attacks the host immune system by binding its surface adhesion protein pMGA1.2 to the surface lipid protein ApoA-I of host respiratory epithelial cells, triggering an inflammatory response (Hu, et al., 2016). Due to its lack of cell wall and rapid mutation biology, MG exhibits an immune evasion capability that permits persistent survival and proliferation in the host (Pflaum, et al., 2018). Currently, MG has been reported globally and causes substantial economic losses in the poultry industry each year, leading to its listing as a priority concern by the World Organization for Animal Health. However, its specific pathogenic mechanisms remain largely unexplored (Mugunthan et al., 2023).

Macrophages are the first line of host immune defense and play pivotal roles in inflammatory response and antigen presentation (Lugg et al., 2022). These highly heterogeneous immune cells polarize into various phenotypes in response to exogenous stimuli, a process crucial for immune regulation (Shapouri-Moghaddam et al., 2018). Macrophage polarization, based on their function and characteristics, primarily falls into M1-type and M2-type classifications (Wen et al., 2022). M1-type macrophages exhibit classical polarization, mediating pathogen resistance while promoting the expression of pro-inflammatory factors such as tumor necrosis factor-α (**TNF-α**) and inducible nitric oxide synthase (**iNOS**), thereby contributing to the inflammatory response (Mohd Yasin, et al., 2022; Rao et al., 2022). Conversely, M2-type macrophages, also known as alternatively activated macrophages, are characterized by high

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expression of interleukin-10 (**IL-10**) and arginase-1 (**ARG1**) and exhibit anti-inflammatory functions (Lescoat et al., 2020). Macrophage polarization is an important physiological process that regulates the inflammatory response. The activation of NLRP3 inflammasome plays a crucial role in the pathogenesis of various inflammatory diseases. Liu et al. (2021) found that ubiquitin-specific protease19 (**USP19**) promotes M2 polarization in macrophages, converting the pro-inflammatory function of NOD-like receptor thermal protein domain associated protein 3 (**NLRP3**) into an anti-inflammatory function. Recently, there has been growing interest in the relationship between macrophage polarization and NLRP3 on disease processes, with most studies focusing on viruses such as human immunodeficiency virus (**HIV**) and Porcine reproductive and respiratory syndrome virus (**PRRSV**) (Alfano, et al., 2013; Wang, et al., 2017).

Avian macrophages have been demonstrated to be activated and regulate the host immune response by a wide range of avian pathogens, such as Newcastle disease virus, and avian pathogenic Escherichia coli. (Cui et al., 2021; Peng et al., 2018). In this study, we investigated the polarization phenotype of chicken macrophage HD11 cells after MG infection and explored the relationship between inflammatory vesicles NLRP3 and HD11 macrophage polarization, laying the foundation for MG treatment.

MATERIALS AND METHODS

Reagents

cDNA Synthesis SuperMix was purchased from TaKaRa (Tokyo, Japan). The Hieff UNICON qPCR SYBR Green Master Mix and TRITC Phalloidin (40734ES75) were purchased from Yeasen Biotechnology Co., Ltd. (Shanghai, China). The DNA primer sequences presented in Table 1 were synthesized by Tsingke Biotechnology Co., Ltd. (Wuhan, China). The BeyoClick EdU kit (C0071S) was purchased from Beyotime Biotechnology Co.,Ltd. (Jiangsu, China). The Cell Genetic DNA Extraction Kit (YDP304) was purchased from TIANGEN Biotechnology Co.,Ltd (Beijing, China). The cell culture dishes were purchased from SORFA Biotechnology Co.,Ltd (Beijing, China). The NLRP3 inhibitor (MCC950) was purchased from MCE Biotechnology Co., Ltd. (Shanghai, China). All other chemical reagents were of reagent grade.

Table 1. Sequences of gene primer.

Gene name	Primer sequence (5′-3′)
iNOS-F	ATCCTGGAGGTCCTGGAAGAGT
iNOS-R	CCTGGGTTTCAGAAGTGGCA
TNF-α-F	CTATGCCAACAAGTACACC
TNF-α-R	AAGAGGCCACCACACGACA
IL10-F	GCTGCGCTTCTACACAGATG
IL10-R	TCCCGTTCTCATCCATCTTC
PPARγ-F	GGGCGATCTTGACAGGAA
PPARγ-R	GCCTCCACAGAGCGAAAC
GAPDH-F	CCTCTCTGGCAAAGTCCAAG
GAPDH-R	TTGATGTTGCTGGGGTCACG

Preparation of MG and Cell Culture

The highly pathogenic virulent strain MG-HS is stored in the Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University. According to the previously reported method (Wang et al., 2022), the MG virulence of 1×10^{13} CCU/mL was determined using the color-changing unit (**CCU**) method.

HD11 cells were provided by Professor Zhuang Ding from the Key Laboratory of Zoonosis Research, Ministry of Education, Jilin University in Changchun, China. HD11 cells cultured in high-glucose medium containing 10% fetal bovine serum at 37 ± 0.5 °C with 5% CO_2.

RNA Isolation and Quantitative Real-Time PCR

Cells were inoculated into 6-well cell culture dishes and subsequently infected with MG (100 uL/well) at the appropriate density. No treatment was administered to the control group. RNA from HD11 cells at different time points post-MG infection was extracted using the TRNzol Universal reagent kit (TIANGEN, Beijing, China) and then reverse transcribed to cDNA according to the instructions provided with the reverse transcription PCR (**RT-PCR**) kit (TaKaRa, Tokyo, Japan). Quantitative real-time polymerase chain reaction (**qRT-PCR**) was performed with Hieff UNICON® qPCR SYBR Green Master Mix (Vazyme, Nangjing, China).

DNA Isolation and MG Quantitative

Cells were inoculated into 6-well cell culture dishes and subsequently infected with MG (100 uL/well) at the appropriate density. No treatment was administered to the control group. DNA from HD11 cells at different time points post-MG infection was extracted using the Cell Genetic DNA Extraction Kit from TIANGEN. The primers MG-F: AGCTAATCTGTAAAGTTGGTC and MG-R: CGCTTCCTTGCGGTT AGCAAC were identify DNA copies of MG.

Immunofluorescence Detection

Cells were inoculated into 24-well cell culture dishes with coverslips and subsequently infected with MG (30 uL/well) at the appropriate density. MG-infected HD11 cell samples were fixed in paraformaldehyde for 30 min followed by permeabilization with 0.5% Triton100 for 20 min, and then incubated in goat serum for 2 h at room temperature. Subsequently, all the samples were incubated separately with iNOS or IL-10 separately at 4°C for 12 h. After that, well diluted CY3 and FITC (1:300; Abclonal Co. Ltd, Wuhan, China) secondary antibodies were incubated separately, and DAPI was used for cell nuclear labelling. Photographs were observed under a fluorescence inverted microscope (Nikon TE2000).

5-Ethynyl-2′-deoxyuridine Assay

Cells were inoculated into 24-well cell culture dishes with coverslips and then subsequently infected with MG (30 uL/well) for 3, 6, 12, 24, 36, and 48 h at the appropriate density. HD11 cell were incubated with 10 mM EdU medium diluent (Beyotime, Jiangsu, China) for 2 h. The cells were then fixed and permeabilized with 4% paraformaldehyde and 0.3% Triton100, respectively, and subsequently labelled with EdU according to the instructions. DAPI was used to label the nuclei. After, EdU-positive cells were observed and photographed with a fluorescence inverted microscope (Nikon TE2000) and analyzed with ImageJ software (Bethesda, MD).

Western Blotting

Cells were inoculated into 6-well cell culture dishes and then infected with MG (100 uL/well) for 12 h at the appropriate density. No treatment was administered to the control group. In brief, the extracted cell proteins were quantified using the bicinchoninic acid protein assay reagent kit (Transgene, Shanghai, China). Subsequently, electrophoresis was performed using the PAGE Gel Fast Preparation Kit (Vazyme, Nanjing, China), followed by blocking with 5% BSA in BSA for 1 h after membrane transfer. The membrane was then incubated overnight at 4°C with the following primary antibodies: anti-pMGA1.2 (Polyclonal antibody, kept by ourselves), TNF-α (Proteintech, 60291-1-Ig), iNOS (Proteintech, 22226-1-AP), IL-10 (Abmart, TD6894), PPARγ (Wanlei, WL01800), IL-1β (Abmart MK56352), Caspase 1 (Abmart, MU167952), ASC (Abclonal, A1170), NLRP3 (Abmart, TD7438), and β-actin (Abmart, T40104). Then, the membranes were incubated with secondary antibody for 2 h at room temperature. Finally, the protein membranes were visualized in the imager using enhanced chemiluminescence (ECL).

Statistical Analysis

The fluorescence statistics were determined by image J. Anova analysis was performed on all data using spss software and differences were considered significant at $p \leq 0.05$, bar charts were produced by GraphPad prism and presented as means ± standard error (**SE**).

RESULTS

MG Proliferation at Different Infection Times

Various pathogens proliferate by invading host cells. In this study, we performed infection of HD11 cells with MG and detected the expression of MG virulence-related genes pMGA1.2 and copy numbers using qRT-PCR. At 3 hours post-infection, the morphology of HD11 cells changed compared with the normal group (Figure 1A). The results showed that MG replication increased in a time-dependent manner, as evidenced by the expression of MG virulence-related genes pMGA1.2 and the copy numbers (Figures 1B and 1C).

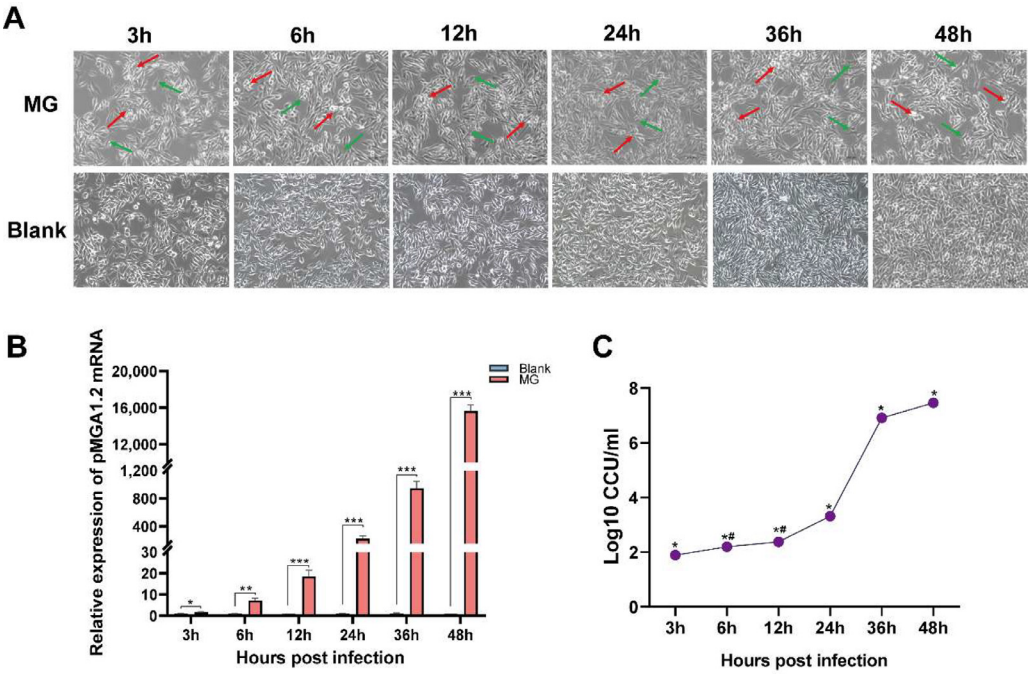

Figure 1. MG infection in HD11 macrophages. (A) Morphology of HD11 cells at different times of MG infection. HD11 cells morphology were recorded at 3, 6, 12, 24, 36, and 48 h in MG infected or non-infected. (B) MG virulence-related pMGA1.2 gene expression. MG virulence-related gene pMGA1.2 mRNA expression at 3, 6, 12, 24, 36, and 48 h of MG-infected HD11 cells. (C) MG copy numbers. MG copy numbers at 3, 6, 12, 24, 36, and 48 h of MG-infected HD11 cells. MG mRNA copies were calculated by the formula: y = -3.0391x + 39.833 (R^2 = 0.9856). Bar graph represents mean results ± SE (n = 3). Red arrows represent cell death and green arrows show cells with pseudopods. * means the significance of between MG-infected cells with Blank cells. *means $p < 0.05$, ** means $p < 0.01$, and *** means $p < 0.001$, *# means no difference (p>0.05).

MG Infection Inhibited HD11 Cell Proliferation

Pathogens propagate self-proliferation by regulating cell proliferation is an important characteristic in its pathogenic mechanism (Zhang et al., 2022). In this study, cell proliferation was detected in MG -infected HD11 cells in EdU progression (Figure 2A). The results showed that HD11 cell proliferation increased between 0-12 h of MG infection and peaked at the 12 h, while decreased as the infection progressed (Figure 2B). This finding indicates that during the initial stage of MG infection, HD11 cell proliferation remains unaffected. However, with the prolongation of infection time, MG significantly inhibits HD11 cell proliferation.

Effect of MG Infection on the Polarization Phenotype of HD11 Macrophages

Similar to MG infection of chicken embryo fibroblasts (DF1 cells), MG is able to infect HD11 cells to infect HD11 cells. Additionally, we observed a significant decrease in HD11 cell proliferation and a corresponding increase in MG copy numbers at 12 h post-infection (Figure 2). Therefore, we suggest that the 12 h time point post-infection is optimal for investigating cell function. To investigate the pathogenic mechanism of MG infection, we conducted cytoskeletal immunofluorescence observations on MG-infected HD11 cells (Figure 3A). The results revealed the detection of MG around the nucleus of HD11 cells, indicating that the invasion of MG into HD11 cells triggers the pathogenic mechanism. Subsequently, we analyzed the polarization marker genes of MG-infected HD11 macrophages. The results of the immunofluorescence test indicate that the M1-type polarization marker iNOS was significantly elevated in HD11 macrophages after 12 h of MG infection (Figures 3B and 3C). Conversely, the expression of the M2-type polarization marker IL-10 was significantly suppressed after MG infection (Figures 3D and 3F). qPCR and WB were used to further validate the effect of MG infection on polarization marker expression in HD11 macrophages. Consistent with the immunofluorescence results, MG increased the expression of the M1-type polarization genes iNOS and TNF-α protein levels, while decreasing the protein expression of the M2-type polarization genes IL-10 and PPARγ (Figure 4).

MG Stimulated NLRP3 Pathway-Related Gene Expression in HD11 Macrophages

In the MG-infected HD11 cell model, the expression of the MG virulence gene pMGA1.2 is the most direct

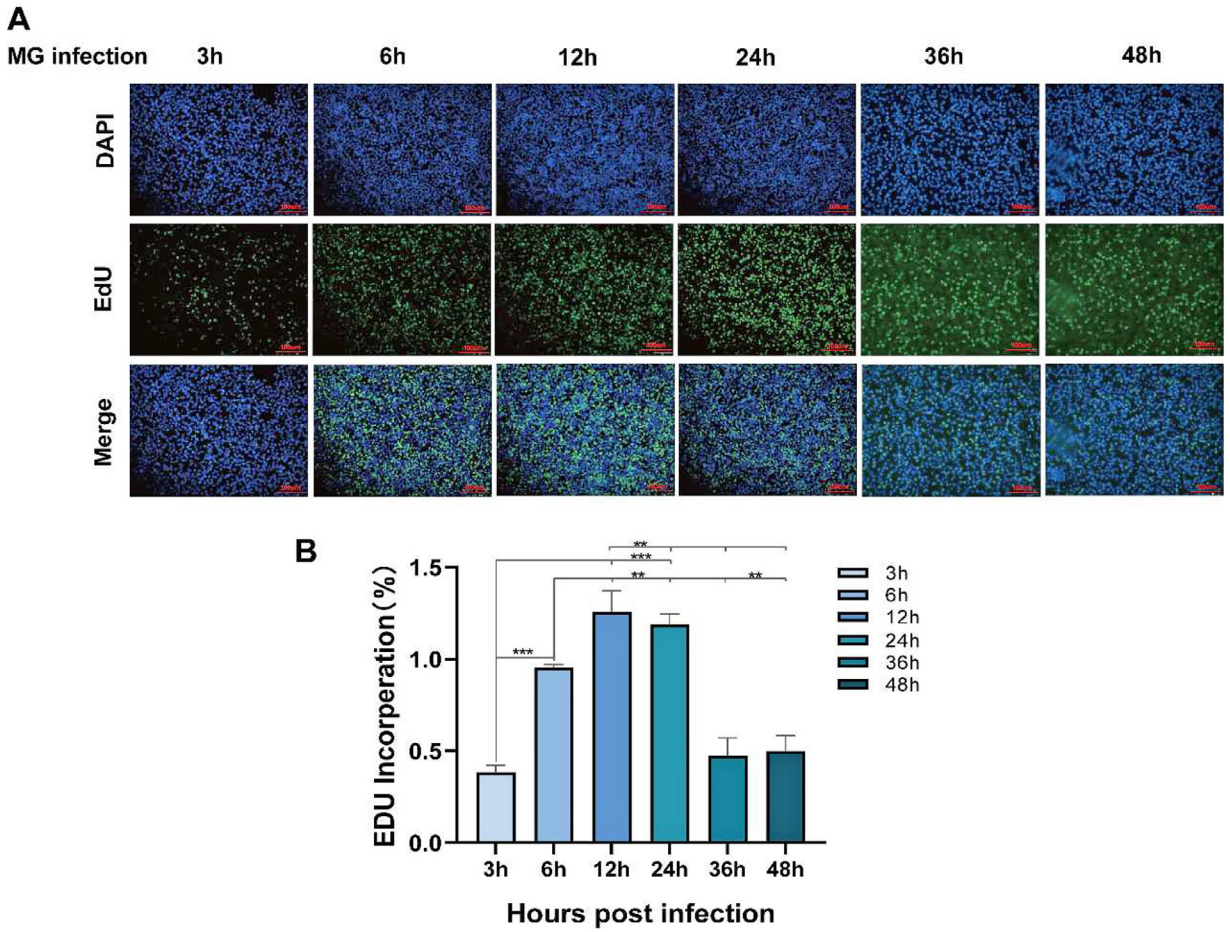

Figure 2. Immunofluorescence staining of EdU for MG-infected HD11 cells. (A) EdU immunofluorescence staining of MG-infected HD11 cells at 3, 6, 12, 24, 36, and 48 h. (B) Quantification of the progression of EdU-labelled cells in MG-infected HD11 cells at different times. Bar graph represents mean results ± SE (n = 3). *means $p < 0.05$, ** means $p < 0.01$, *** means $p < 0.001$.

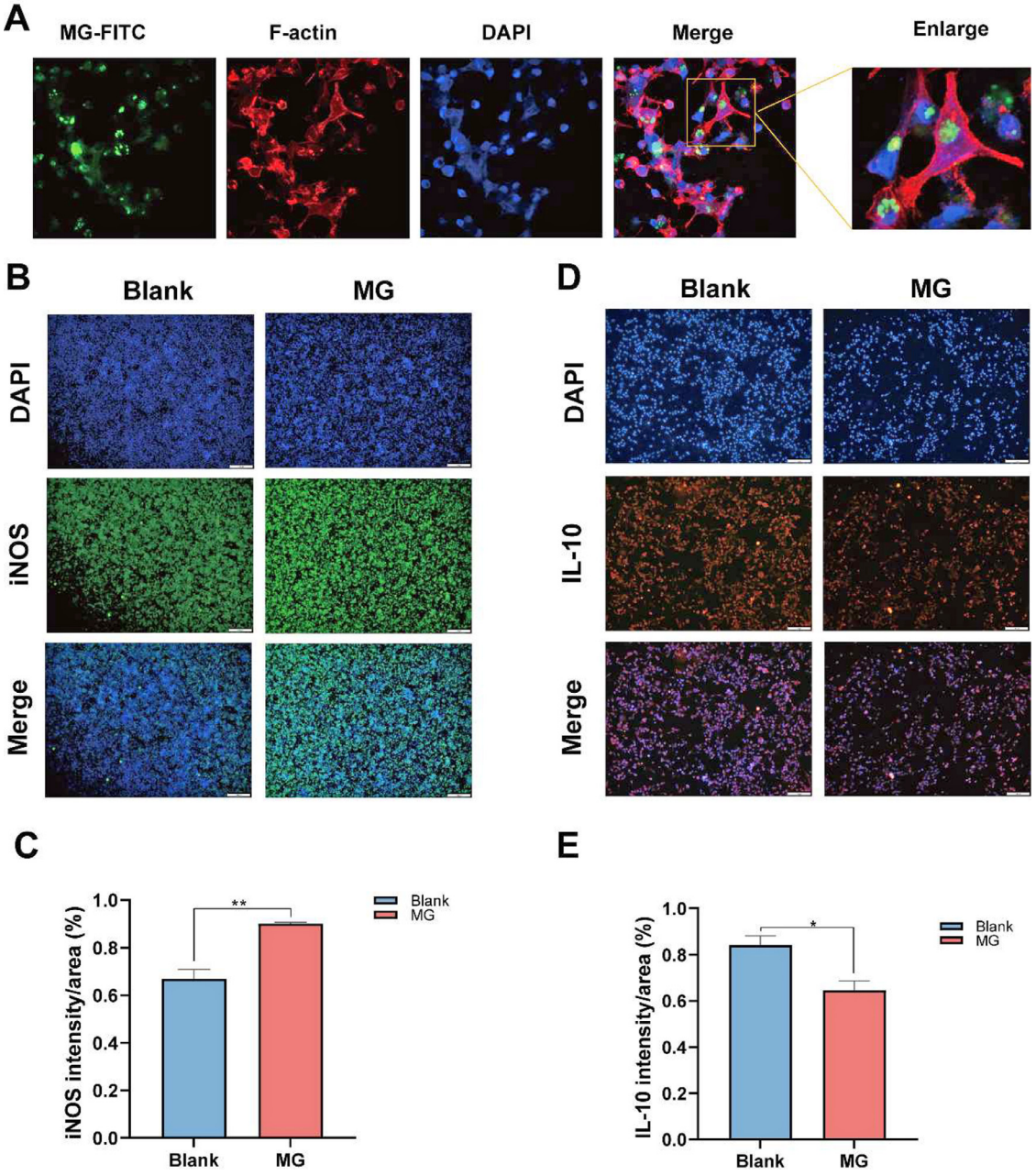

Figure 3. Immunofluorescence for MG-infected HD11 cells. (A) Colocalisation detection of MG localisation in HD11 cells. MG was labelled with FITC, F-actin was used to label the cytoskeleton and DAPI labelled the nucleus. (B and D) Immunofluorescence detection of iNOS and IL-10 in MG-infected and uninfected HD11 cells. (C and E) Quantification of the progression of iNOS and IL-10 positive cells in MG-infected HD11 cells. Scale: 100 um. Bar graph represents mean results ± SE (n = 3). * means the significance of between MG-infected cells with Blank cells. *means $p < 0.05$, ** means $p < 0.01$, *** means $p < 0.001$.

evidence of MG proliferation. The results demonstrate that MG significantly stimulated the expression of the pMGA1.2 protein compared to the blank group (Figures 5A and 5B), indicating successful MG infection. MG-induced M1 polarization of HD11 macrophages is typical of an inflammatory response process characterized by high expression of pro-inflammatory cytokines. The inflammatory response is often accompanied by aberrant expression of the inflammatory vesicle NLRP3 (Xu and Nunez, 2023). In this study, NLRP3 pathway-related genes expression were determined by WB (Figure 5). The WB analysis revealed that NLRP3, caspase-1, ASC, and IL-1β were significantly upregulated in HD11 cells after MG infection compared with the blank group (Figures 5A, 5C, 5D, 5E, and 5F). These results suggested that MG triggers an inflammatory response associated with the NLRP3 pathway.

MCC950 Inhibited MG-Induced M1 Polarization in HD11 Macrophages

MCC950, a specific inhibitor of the NLRP3 inflammasome, is commonly used to prevent NLRP3 activation (Corcoran, et al., 2021). Here, we utilized MCC950 (MCE) to investigate the impact of NLRP3 on MG-induced HD11 macrophages polarization (Figure 6). Immunofluorescence results indicated that MCC950 (10

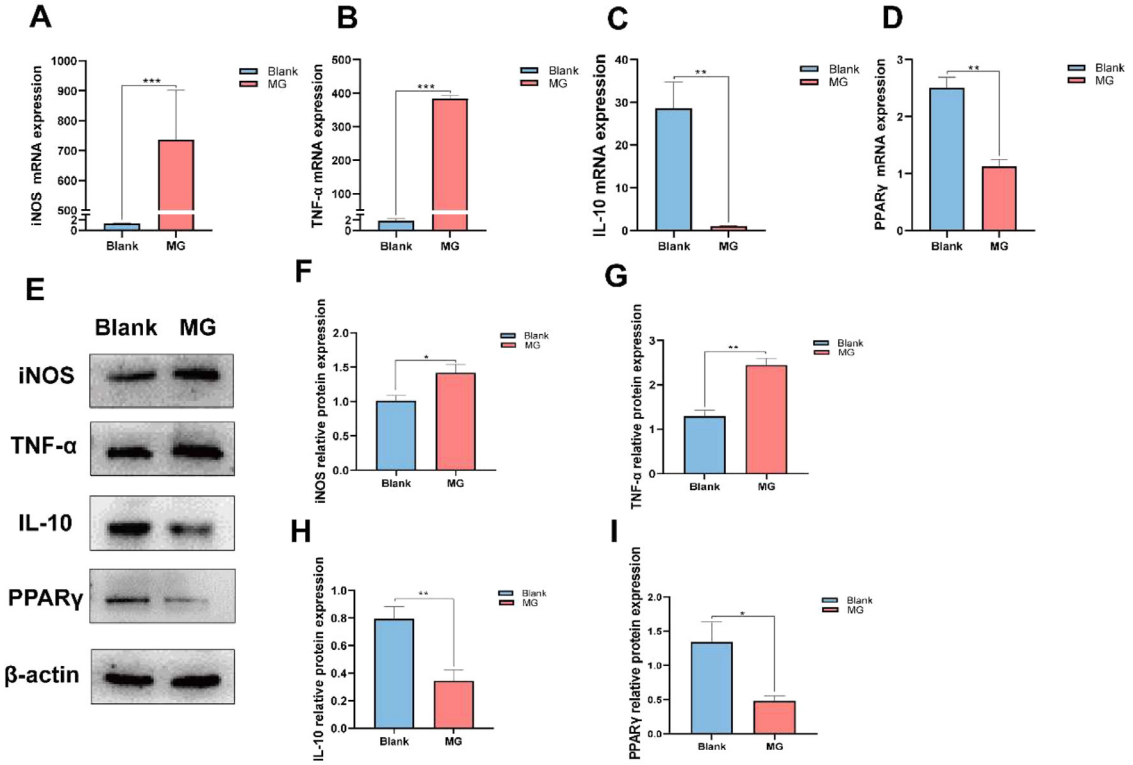

Figure 4. Effect of MG on protein level expression of polarization genes in HD11 macrophages. HD11 cells were infected by MG for 12 h, and the uninfected blank group was used as control. (A-D) qPCR detected the mRNA expression of iNOS, TNF-α, IL-10, and PPARγ. (E) WB showing changes in iNOS, TNF-α, IL-10, and PPARγ protein expression in MG-infected HD11 macrophages. (F, G, H, and I) Quantification of iNOS, TNF-α, IL-10, and PPARγ protein expression in MG-infected HD11 macrophages. Bar graph represents mean results ± SE (n = 3). * means the significance of between MG-infected cells with Blank cells. *means $p < 0.05$, ** means $p < 0.01$, *** means $p < 0.001$.

μM) significantly inhibited the expression of the MG-induced M1 polarization gene iNOS in HD11 macrophages (Figures 6A and 6C), while significantly promoting the expression of the M2 polarization gene IL-10 (Figures 6B and 6D). In line with the immunofluorescence findings, the qPCR (Figures 6E and 6F) and WB (Figures 6G, 6H, and 6I) results demonstrated that MCC950 (10 μM) down-regulated the protein expression of the MG-induced M1-polarized gene iNOS and up-regulated the protein expression of the M2-polarized gene IL-10.

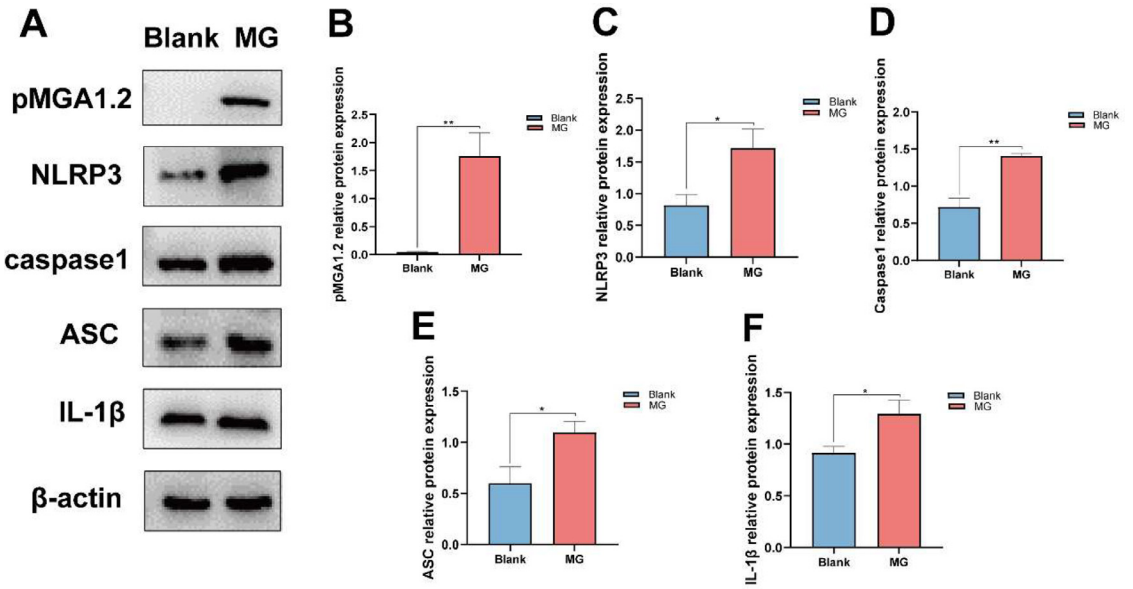

Figure 5. Effect of MG on protein level expression of NLRP3 pathway-related gene in HD11 macrophages. HD11 cells were infected by MG for 12 h, and the uninfected blank group was used as control. (A) WB showing changes in pMGA1.2, NLRP3, caspase-1, ASC, and IL-1β protein expression in MG-infected HD11 macrophages. (B, C, D, E, and F) Quantification of pMGA1.2, NLRP3, caspase-1, ASC, and IL-1β protein expression in MG-infected HD11 macrophages. Bar graph represents mean results ± SE (n = 3). * means the significance of between MG-infected cells with Blank cells. *means $p < 0.05$, ** means $p < 0.01$, *** means $p < 0.001$.

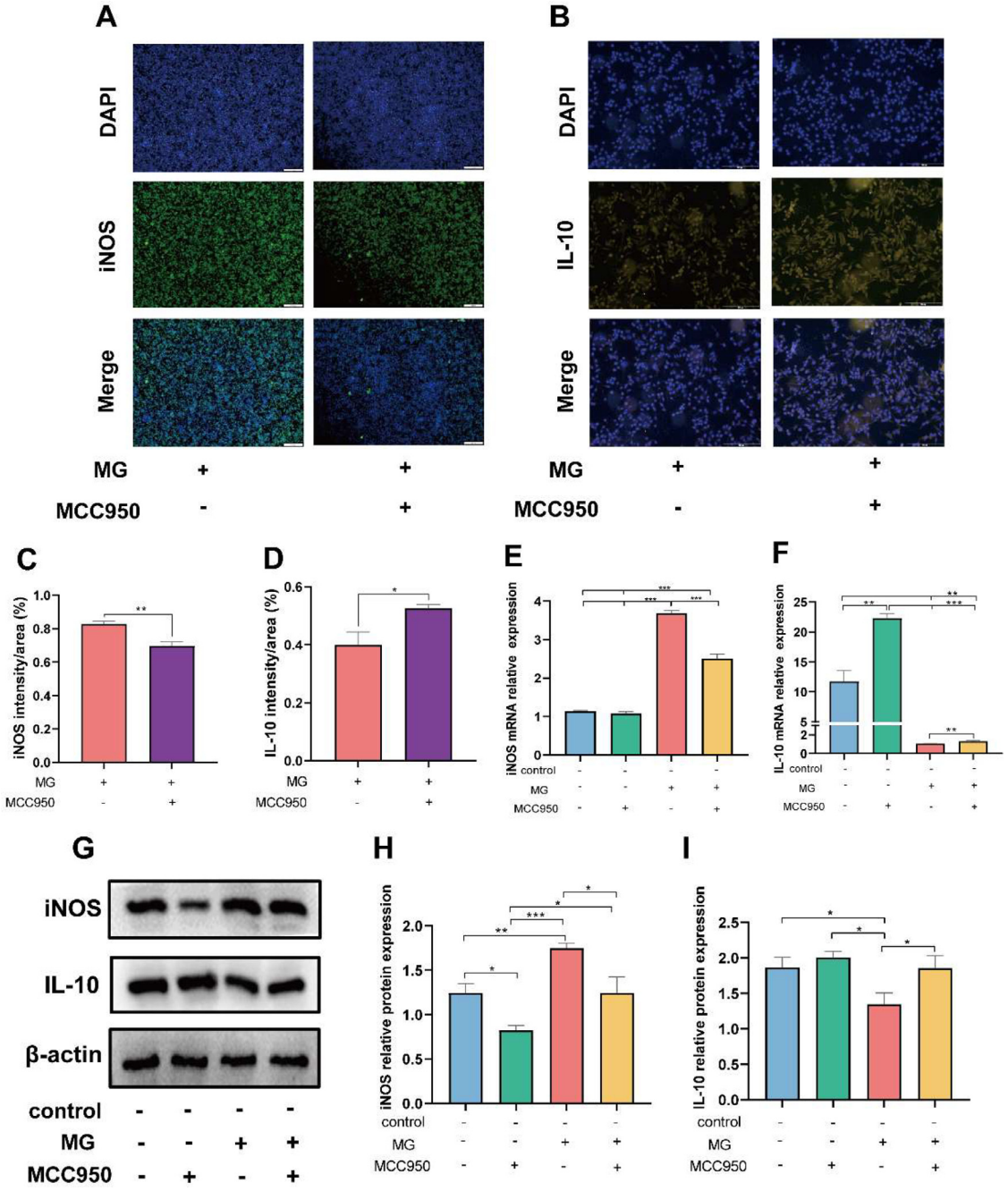

Figure 6. Effect of MCC950 on MG-induced polarization of HD11 macrophages. (A and B) Immunofluorescence detection of iNOS and IL-10 in MG-infected and uninfected HD11 cells. (C and D) Quantification of the progression of iNOS and IL-10 positive cells in MG-infected HD11 cells. (E and F) qPCR detected the mRNA expression of iNOS and IL-10 in MG-infected HD11 macrophages. (G) WB shown changes in iNOS and IL-10 protein expression in MG-infected HD11 macrophages. (H and I) Quantification of iNOS and IL-10 protein expression in MG-infected HD11 macrophages. Bar graph represents mean results ± SE (n = 3). * means the significance of between MG-infected cells with Blank cells. *means $p < 0.05$, ** means $p < 0.01$, *** means $p < 0.001$.

These results indicated that MCC950 may regulate MG-induced polarization type in HD11 macrophages.

MCC950 Inhibited MG-Induced NLRP3 Inflammatory Pathway-Related Gene Expression in HD11 Macrophage

To further investigate whether MCC950 affects NLRP3 inflammatory pathway-related gene expression, WB was performed to detect the protein expression of NLRP3 pathway-related genes. The results showed that MCC950 significantly inhibited the protein expression of MG virulence gene pMGA1.2 (Figures 7A and 7B). Furthermore, in the presence of MCC950, the protein expression of NLRP3, caspase-1, ASC, and IL-1β was significantly reduced in uninfected cells. Additionally, the inhibition of MG-induced protein expression of NLRP3, caspase-1, ASC, and IL-1β was statistically significant (Figures 7A, 7C, 7D, 7E, and 7F). These findings indicated that MG regulates HD11 polarization through the NLRP3 signaling pathway.

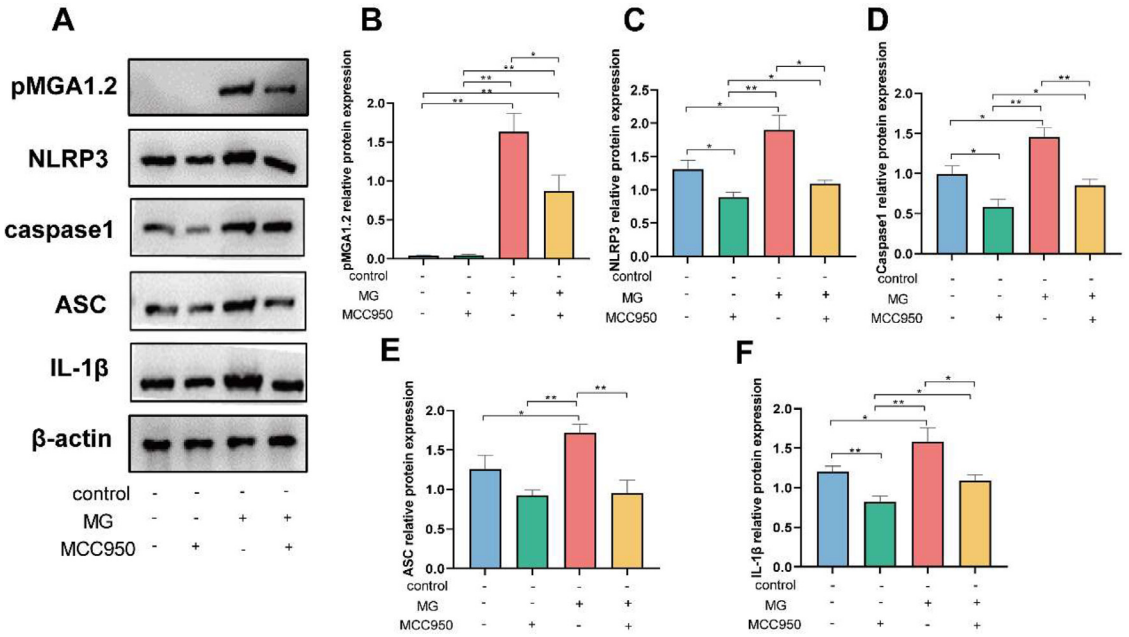

Figure 7. Effect of MCC950 on protein level expression of NLRP3 pathway-related gene in HD11 macrophages. (A) WB showing changes in pMGA1.2, NLRP3, caspase-1, ASC, and IL-1β protein expression in MG-infected HD11 macrophages. (B, C, D, E, and F) Quantification of pMGA1.2, NLRP3, caspase-1, ASC, and IL-1β protein expression in MG-infected HD11 macrophages. Bar graph represents mean results ± SE (n = 3). * means the significance of between MG-infected cells with Blank cells. *means $p < 0.05$, ** means $p < 0.01$.

DISCUSSION

MG infection has been found to reduce chick survival and decrease egg production in laying hens, which is the most pathogenic economic mycoplasma infection in poultry (Levisohn and Kleven, 2000; Machado, et al., 2017). Previous studies have shown that HD11 cells are one of the targets of MG and that IL-8 and IL-6 gene expression is higher in HD11 cells after MG infection than in other cells (Lam, 2004). IL-8 and IL-6, secreted by macrophages, are known to cause neutrophil aggregation (An et al., 2019). HD11 macrophages, similar to other macrophages, play crucial roles in host defense, maintaining the internal homeostasis, and initiating inflammatory responses as essential components of host immunity (Peng et al., 2020). Macrophages are known to polarize into different phenotypes in response to various stimuli and secrete different types of cytokines as part of the immune response (Yunna et al., 2020). However, the effects of MG on HD11 macrophage polarization remain poorly understood. A comprehensive understanding of MG's role in HD11 macrophage polarization is vital for elucidating MG pathogenesis and developing treatment and control strategies to address MG infections.

Macrophages are recognized for the highly tolerant to viral proliferation. However, as viruses proliferate in large numbers during the middle and late stages of infection, the physiological state of macrophages undergoes dramatically changes (De Simone et al., 2021). In our morphological observation of HD11 cells, we noted the appearance of tentacles in the periphery of the cells after 3 h of MG infection compared to the control group. This finding is consistent with previous reports by Cui et al. (2021), which demonstrated that *Avian Tembusu virus* (**TMUV**) infection induced alterations in HD11 cell morphology. In the *tracheal organ culture* (**TOC**) model, the expression of MG virulence genes gapA/crmA increased with infection time in the presence of different MG virulence level (Ruger et al., 2022). Here, we observed that as the duration of MG infection increased, the morphology of MG-infected HD11 cells changed more significantly compared to the control group, accompanied by a gradual increase in MG virulence gene pMGA1.2 expression and copy numbers (Figure 1). Cell proliferation capacity serves as an intuitive indicator of cell physiological status (Ma et al., 2020). Using EdU detection, we observed a linear increase in HD11 cell proliferation from 0 to 12 h after MG infection, peaking at 12 h, followed by a significant decrease (Figure 2). These changes in morphology and decreased proliferative capacity of HD11 cells may be attributed to the massive proliferation of MG. However, the precise mechanisms by which MG attacks HD11 cells remain unclear. Macrophage defense against pathogen invasion is an important component of host innate immunity (Hikosaka et al., 2022). Currently, it is not clear in what way MG attacks HD11 cells. It has been shown that viruses can invade cells by autophagy for immune evasion to facilitate self-replication (Zhang et al., 2023). Our findings from labelled MG infection revealed the presence of MG surrounding the cell nucleus (Figure 3A), suggesting that MG can invade HD11 cells for survival and proliferation. This invasion mechanism may contribute to MG evasion of immune defenses and the establishment of persistent infection.

Cell polarization characterizes an important regulatory reaction of macrophages in response to pathogen invasion. Macrophages are classified into typical M1-type polarization and M2-type alternative activation

based on the expression of surface markers following macrophage polarization (Murray et al., 2014). Macrophage polarization is not solely about M1-type and M2-type phenotypes, which are dynamic processes that exhibit different phenotypes in different physiological and pathological states (Wang et al., 2010). In this study, we observed that MG induced HD11 macrophages to polarize towards the M1 type, as evidenced by the increased expression of macrophage polarization markers M1 (iNOS) and M2 (IL-10) following MG infection (Figures 3B-3E). The polarized phenotype may be influenced by Th1-type cytokines (such as LPS, TNF-α) stimulating pro-inflammatory macrophages (M1) and Th2 cytokines (such as IL-4, IL-10) promoting polarization towards the M2 phenotype in the microenvironment upon pathogen stimulation (Bai et al., 2021). To further verify the type of MG-induced HD11 macrophage polarization, we employed qPCR and WB analysis to detect the expression of M1-type markers (iNOS, TNF-α) and M2-type markers (IL-10, PPARγ) (Figure 4). Consistent with the immunofluorescence results, the protein expression levels of M1-type and M2-type polarization markers indicated that MG-induced HD11 macrophages predominantly exhibited an M1-type polarization phenotype. Previous studies have reported the involvement of the NLRP3 signaling pathway in the regulation of macrophage polarization (Hou et al., 2022).

The NLRP3 inflammasome, composed of NLRP3, ASC, and caspase-1, is extensively studied and plays a crucial role in the host immune response to pathogen infection (Mangan et al., 2018; Zhuang et al., 2021). It regulates Th cell differentiation by cleaving the inactive precursor form of pro-IL-1β into mature IL-1β via caspase-1(Barker et al., 2011). Recent studies have increasingly confirmed the involvement of NLRP3 inflammatory vesicles in macrophage polarization. Treponema pallidum induces macrophage M1 polarization by increasing IL-1β secretion through activation of NLRP3 inflammatory vesicles (Lin et al., 2018). Similarly, activation of NLRP3 inflammatory vesicles in stimulated human periodontal fibroblasts induces M1 polarization in macrophages, leading to increased IL-1β release and stimulation of root resorption (Zhang et al., 2020). Our results align with these findings, as we observed MG-induced M1-type polarization of HD11 macrophages accompanied by increased protein expression levels of NLRP3, ASC, caspase1, and IL-1β in NLRP3 inflammatory vesicles (Figure 5). Furthermore, treatment with the NLRP3 specific inhibitor, MCC950, reduced the level of macrophage M1-type polarization (Figure 6) and NLRP3 inflammasome activation (Figure 7). These findings suggest that NLRP3 inflammasome may influence the immune activation function of MG-induced HD11 macrophages towards the M1 type, while inhibition of NLRP3-mediated inflammasome activation alleviates MG-induced HD11 macrophage M1 type overpolarization.

In conclusion, MG can invade into HD11 macrophages, inducing M1-type polarization and activating the NLRP3 inflammasome, thereby contributing to specific diseases associated MG. Our study provides a basis for investigating immune evasion and pathogenic mechanisms of MG through the exploration of MG-induced HD11 macrophage polarization and biological functions. Targeted treatments for polarization and NLPR3 signaling may contribute to the control of MG.

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Authors Contributions: TW performed experiments, wrote the manuscript and analyzed the data. YX and WZ performed experiments. SL helped to write, revise the manuscript. YW and QG provided advice for the study. XP conceived and designed the study and helped to revise the manuscript. All authors read and approved the final manuscript.

DISCLOSURES

The authors report no declarations of interest in the present study.

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