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RESEARCH ARTICLE

The CIITA/MHC-II/NF-κB Pathway Mediates Full Activation of TLR4-Driven Innate Immunity in RAW264.7 Macrophages Challenged with *Giardia duodenalis*

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ABSTRACT

Giardia duodenalis is a protozoan parasite responsible for waterborne diarrheal diseases in humans and animals worldwide. TLR4 is a key pattern recognition receptor of the innate immune system, and MHC-II bridges innate and adaptive responses, while role of these receptors in the activation of macrophages by *G. duodenalis* still needs further investigation. This study confirmed knockdown efficiency using RT-qPCR and western blotting then employed multiple molecular and cellular assays to investigate the functions of TLR4 and MHC-II in RAW264.7 macrophages during defense against *G. duodenalis*. Results showed that the significant upregulation of proinflammatory cytokines (e.g., 5.18-fold increase in IL-6 expression) and marked activation of NF-κB pathways (marked enhancement of p65 fluorescence in the cell nucleus) in macrophages following *G. duodenalis* infection were dependent on TLR4, as demonstrated by their attenuation after TLR4 knockdown. Interestingly, we also observed that the knockdown of MHC-II produced similar results, and the elevated expression of MHC-II in macrophages induced by *G. duodenalis* was inhibited by the interference of class II transactivating factor (CIITA). Overall, CIITA/MHC-II/NF-κB pathway is critical for full activation of TLR4-mediated innate immune response in macrophage initiated by *G. duodenalis*, which elaborated the innate immune network in the interaction between *G. duodenalis* and macrophages, holds significant implications for the immunotherapeutic strategies targeting Giardiasis.

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INTRODUCTION

G. duodenalis is a zoonotic protozoan parasite that colonizes the small intestine and causes giardiasis in humans and animals worldwide (Midlej *et al.*, 2024). In most immunocompetent hosts, infection is self-limiting, whereas immunocompromised individuals often develop severe diarrhea, dehydration, and malabsorption (Ihara *et al*., 2024; Kalavani *et al*., 2024a; Kalavani *et al*., 2024b; Klimczak *et al*., 2024). Innate immune responses encompass various immune cells (e.g., neutrophils, macrophages, eosinophils) and immune factors (e.g., chemokines, cytokines, defensins), which constitute the primary biological defence mechanism against diverse pathogens (Carpenter and O'Neill, 2024; Kaur and Secord, 2021). Research indicates that *G. duodenalis* infection releases protease-1 and cysteine proteases, which impair the intestinal epithelial barrier, and this breach triggers the recruitment of macrophages from the lamina propria and initiates broader innate immune responses (Zhao *et al*., 2022; Yu e*t al*., 2024). Given the central role of macrophages in the early host response to *G. duodenalis*, delving into the intricate immune interaction network between host macrophages and *G. duodenalis* may help us identify novel targets for the treatment of Giardiasis.

As key pattern recognition receptors, TLRs are essential for macrophages to detect pathogen-associated molecular patterns and launch the innate immune response (Wang *et al*., 2024). The activation of TLR4 on macrophages by pathogenic ligands triggers NF-κB activation and the production of IL-6, IL-12 and TNF-α, and these cytokines are critical for the effective clearance

of microbial infections (Saez *et al*., 2023). Studies suggest a key role for the TLR4/NF-κB signaling pathway in mediating innate immunity against a broad spectrum of intestinal protozoans (Lu *et al*., 2021; Marongiu *et al*., 2019; Muñoz-Caro *et al*., 2021). For example, *Toxoplasma gondii*, *Cryptosporidium parvum* and *Entamoeba histolytica* have been shown to activate the TLR4/NF-κB pathway in macrophages, thereby promoting the upregulation of proinflammatory cytokine secretion (Zhou *et al.*, 2024; Maldonado-Bernal *et al*., 2005; Mead, 2023). The activation of TLR4 has been demonstrated in *G. duodenalis* infection, yet the corresponding mechanism remains inadequately documented (Fernández-Lainez *et al*., 2022; Liu *et al*., 2021).

MHC-II expression is restricted to antigen-presenting cells, such as macrophages, and its expression is tightly regulated and almost exclusively controlled by a single main regulatory factor, which is termed as class II transactivating factor (CIITA) (Han *et al*., 2023; Han *et al*., 2023). MHC-II primarily functions to present processed exogenous antigens to $CD4^+$ T cells, a process that requires co-stimulatory molecules like CD80/CD86 (Buxadé *et al*., 2018; MacNabb *et al*., 2023). MHC-II contributes in a comparable way during parasite infection. Reduced MHC-II expression on mouse macrophages impairs antigen presentation, thereby weakening the immune defense against *Toxoplasma gondii* and resulting in more severe infection (Debierre-Grockiego *et al*., 2009; Jafari *et al.*, 2023). MHC-$II^{-/-}$ mice showed an increased susceptibility to *Strongyloides venezuelensis* infection (Rodrigues *et al*., 2013). Furthermore, MHC-II has been shown to regulate cytokine production (Dong *et al*., 2011). The lack of expression or low level of MHC-II resulted in decreased secretion of proinflammatory cytokines in macrophages after LPS stimulation (Liu *et al*., 2011). However, in the context of *G. duodenalis* infection, the involvement of MHC-II in the regulatory mechanism of proinflammatory cytokine in macrophages remains poorly understood.

Consequently, this study aimed to elucidate the roles of TLR4 and MHC-II in macrophage responses to *G. duodenalis*, thereby identifying potential immunotherapeutic targets for giardiasis.

MATERIALS AND METHODS

Cells and parasites: RAW264.7 macrophages were donated by professor Hongying Zhang's lab at Henan Agricultural University. The cells were cultured in RPMI 1640 medium (GIBCO, New York, USA) at 37°C with 5% CO_2. The culture medium was supplemented with 10% fetal bovine serum (BI, Beit-Haemek, Israel), 100U/ml penicillin and 0.1mg/ml streptomycin liquid (Solarbio, Beijing, China) at 37°C with 5% CO_2. The trophozoites of *G. duodenalis* which belongs to assemblage A of *G. duodenalis* were kindly obtained from Jilin University were cultured in modified TYI-S-33 medium solution at 37°C with 5% CO_2. RAW264.7 macrophages were inoculated on cell culture plate (12-well plate, 1.0×10^6 cells/well; 6-well plate, 2.0×10^6 cells/well). When reached 80% confluence, the *G. duodenalis* trophozoites were added to RAW264.7 macrophages for dedicated period of time. The ratio of *G. duodenalis* trophozoites to RAW264.7 macrophage was 10:1.

RT-qPCR assay: The cells were seeded in 12-well plate (1.0×10^6 cells/well) and were exposed to *G. duodenalis* trophozoites (1.0×10^7 trophozoites/well) for 3, 6, 9 and 12h. Total RNA was isolated from cells with RNA-easy Isolation Reagent (Vazyme, Nanjing, China), the HiScript III All-in-one RT SuperMix Perfect for qPCR (Vazyme, Nanjing, China) was used for cDNA synthesis and 1μg cDNA was added to ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China), amplified on Quantitative Real-Time PCR instrument (Analytik jena, Jena, Germany) and the reaction conditions were as follow, 95℃ for 30s; 95℃ 10s→60℃ 30s, 40 cycles; 95℃ 15s→60℃ 60s→95℃ 15s. Relative gene expression of *TLR4*, *NF-κB*, *IL-6*, *IL-12*, *TNF-α*, *CIITA*, *MHC-II*, *CD80*, *CD86* and *β-actin* were detected. Relative gene expression was analyzed by normalizing to β-actin as an internal control and applying the $2^{-\Delta\Delta Ct}$ calculation (Liu *et al*., 2021). All primers (Table 1) were designed with NCBI's Primer-BLAST tool and commercially synthesized by Sangon Biotech (Shanghai, China).

Table 1: List of primers used in RT-qPCR

Gene Name	Primer Type	Primer Sequences
TLR4	Forward	TTTCACCTCTGCCTTCACTACA
	Reverse	AGATACACCAACGGCTCTGAAT
NF-κB	Forward	AGCAACCAAAACAGAGGGGAT
	Reverse	TTGCAAATTTTGACCTGTGGGT
IL-6	Forward	CACATGTTCTCTGGGAAATCG
	Reverse	TTGTATCTCTGGAAGTTTCAGATTGTT
IL-12	Forward	TACAAGGTTCAGGTGCGAGC
	Reverse	ATGTATCCGAGACTGCCCAC
TNF-α	Forward	CAGGCGGTGCCTATGTCTC
	Reverse	CGATCACCCCGAAGTTCAGTAG
CIITA	Forward	TGTTTTGGATGCTGCAAGGC
	Reverse	AAGGCACAGTGGTATTCCCG
MHC-II	Forward	GCCCCACACCCTTATCTGCTTTG
	Reverse	GCTGGTCTCATAAACGCCGTCTG
CD80	Forward	GCTGAACAGACCGTCTTCCT
	Reverse	TTTGCAGAGCCAGGGTAGTG
CD86	Forward	TTGTGTGTGTTCTGGAAACGGAG
	Reverse	AACTTAGAGGCTGTGTTGCTGGG
β-actin	Forward	AGTGTGACGTTGACATCCG
	Reverse	GCAGCTCAGTAACAGTCCGC

Western blotting: The cells were seeded in 6-well plate (2.0×10^6 cells/well) and the cells were exposed to *G. duodenalis* trophozoites (2.0×10^7 trophozoites/well) for 3, 6, 9 and 12h. 200μl of RIPA lysate (Epizyme, Shanghai, China) containing 2μl protlytic protease and phosphatase inhibitor cocktail (NCM Biotech, Suzhou, China) was used to isolate the protein following the instructions. Concentration of the protein was measured by Omni-Easy™ Ready-to-use BCA protein Quantification Kit (Epizyme, Shanghai, China). The protein sample was mixed with protein loading buffer (Epizyme, Shanghai, China) and boiled for 5min. Protein samples (30μg/well) were separated with 10% sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) gel by electrophoresis in Tris-Glycine-SDS buffer (80V/30min and120V/30min), and the protein sample was transferred to polyvinylidene difluoride (PVDF) membrane via a rapid electrophoresis (400mA/22min). The membrane was blocked in 5% skim milk (PBST buffer) at 37°C for 2h and incubated with primary antibodies rabbit anti-MHC-II (Thermo Fisher Scientific, Shanghai, China), rabbit anti-CIITA (Thermo Fisher Scientific, Shanghai, China), rabbit anti-CD86 (Wanleibio, Shenyang, China), rabbit anti-CD80

(Wanleibio, Shenyang, China), rabbit anti-TLR4 (Wanleibio, Shenyang, China), rabbit anti-p65 (Abways Techology, Shanghai, China), rabbit anti-p-p65 (Abways Techology, Shanghai, China), rabbit anti-IκBα (Abways Techology, Shanghai, China), rabbit anti-p-IκBα (Abways Techology, Shanghai, China) and rabbit anti-β-actin (Abways Techology, Shanghai, China) at 4°C. Subsequently, the PVDF membranes incubated for 2h at 37°C with an HRP-conjugated goat anti-rabbit IgG (Abbkine, Wuhan, China). After three additional 5min washings with 1× PBST, the membrane was subjected to detection with ECL basic supersensitive luminescent solution (Kermey, Zhengzhou, China). The imprints were captured on a Tanon 5200 automated imaging system (Tanon, Shanghai, China) with subsequent analysis performed using ImageJ (NIH, Bethesda, USA). β-actin was used as an internal reference to quantify the relative protein expression by density analysis.

Enzyme linked immunosorbent assay: After the macrophages were exposed to *G. duodenalis* for 3, 6, 9 and 12h, the cell supernatant was collected and the secretion of IL-6, IL-12 and TNF-α in macrophages was detected with commercial enzyme-linked immunosorbent assay kit (MEIMAN, Jiangsu, China) following the manufacturer's instruction. Coefficient of variation within batches < 0.1, coefficient of variation between batches <0.12.

Immunofluorescence assay: RAW264.7 macrophages were exposed to *G. duodenalis* trophozoites for 3, 6, 9 and 12h and siMHC-II RNA interfered-macrophages exposed to *G. duodenalis* trophozoites for 9h. After the cells inoculated on a 12-well plate were exposed to *G. duodenalis* trophozoites for a specified time. The nuclear translocation of NF-κB p65 in macrophages was assessed via immunofluorescence, as described by Pu *et al*. (2021).

Inhibitor and RNA-Mediated Knockdown assays: To monitor the roles of activated TLR4 in response to *G. duodenalis* in RAW264.7 macrophages, cells were pretreated with 10μM TAK-242 (an inhibitor of TLR4, by binding TLR4 intracellular regions, Selleck, Shanghai) for 6h before stimulation.

The MHC-II-specific small-interfering RNA (siMHC-II RNA), CIITA-specific small-interfering RNA (siCIITA RNA) and negative control siRNA (siNC RNA) were transfected into the cells via Lipofectamine 2000 (Thermo Fisher Scicentific, Shanghai, China). Briefly, Lipofectamine 2000 and siRNAs were separately diluted in antibiotic-free Opti-MEM medium (Thermo Fisher Scientific, Shanghai, China) and incubated at 37°C for 5min. The solutions were then combined, mixed, and incubated for 15min to form complexes. Finally, the transfection mixture was applied to the cells and cultured for 8h at 37°C with 5% CO_2. Following discarding of the transfection medium, the cells were cultured under normal conditions for 24h, and the subjected to different treatments and detection of interference efficiency using RT-qPCR and Western blotting. All siRNA sequences (Table 2) were commercially synthesized by Sangon Biotech (Shanghai, China) and all subsequent procedures were performed under aseptic conditions.

Table 2: siRNA sequences used in knockdown assays

Gene Name	Type	Sequences
MHC-II siRNA-181	sense	GCAUUUCGUGUACCAGUUCAUTT
	antisense	AUGAACUGGUACACGAAAUGCTT
MHC-II siRNA-228	sense	CGCAGCGCAUACGAUAUGUGATT
	antisense	UCACAUAUCGUAUGCGCUGCGTT
MHC-II siRNA-526	sense	GACAGAUUUCUACCCAGCCAATT
	antisense	UUGGCUGGGUAGAAAUCUGUCTT
CIITA siRNA-2036	sense	CCCAGCUACCUUGUACACUUATT
	antisense	UAAGUGUACAAGGUAGCUGGGTT
CIITA siRNA-2875	sense	CAAGACUUACAUGAGGCACUATT
	antisense	UAGUGCCUCAUGUAAGUCUUGTT
CIITA siRNA-1866	sense	GCAGAGGAGAAGUUCACCAUUTT
	antisense	AAUGGUGAACUUCUCCUCUGCTT
Negative Control (NC)	sense	UUCUCCGAACGUGUCACGUTT
	antisense	ACGUGACACGUUCGGAGAATT

CCK-8 assays: Seed 1×10^4 RAW264.7 macrophages into a 96-well plate. When cell density reaches 80%, divide the cells into the following groups: Blank group (culture medium only), Control group (cells+culture medium), TAK-242 group (cells+culture medium+TAK-242 inhibitor), siMHC-II-228 group (cells+medium+siMHC-II-228 RNA) and siCIITA-1866 group (cells+medium+siCIITA-1866 RNA) with three biological replicates per group. After 8hrs of incubation, 10μl CCK-8 reagent was added, mixed thoroughly, and incubated in the dark area for 30min. Subsequently, the absorbance at 450nm was measured using a microplate reader. Cell viability was calculated using the formula: Cell viability (%) = [OD (inhibited group) - OD (blank group)]/[OD (control group) - OD (blank group)].

Statistical analysis: Statistical analyses were conducted with SPSS 20.0 (IBM, New York, USA). Unpaired Student's t-test was employed to compare the *G. duodenalis*-treated group with the control (CON) group, while one-way ANOVA was used for multi-group comparisons in RNA-mediated knockdown experiments. Post-hoc multiple comparisons were performed using Tukey's test. Data are expressed as mean ± SEM from three independent experiments and each experiment was performed in triplicate (n=3 biological replicates). GraphPad Prism 7.0 (GraphPad Software, Franklin, USA) was used to generate the graphical data. Differences were statistically significant, when $P<0.05$.

RESULTS

***G. duodenalis* induced morphological changes of macrophages:** Firstly, morphological changes of RAW264.7 macrophages were observed at 3, 6, 9 and 12h after *G. duodenalis*-stimulation. As shown in Fig. 1, the morphology of macrophages undergoes significant changes, specified by increased formations of membrane protrusions, and the protrusions of lamellipodia and filopodia changed the cell shape from rounded and upright to flattened after stimulated with *G. duodenalis* for 3, 6, 9 and 12h compared with the cells without *G. duodenalis* stimulation.

***G. duodenalis* infection was associated with increased TLR4 and NF-κB activity:** To assess whether *G. duodenalis* elicits a TLR4-mediated proinflammatory immune response in RAW264.7 macrophages, we examined the expression of pertinent genes and proteins in

macrophages after stimulating with *G. duodenalis* for 3, 6, 9 and 12h. Results showed that mRNA and protein levels of *TLR4* and *NF-κB* in macrophages were significantly higher than those in the CON group after stimulating with *G. duodenalis* for 3, 6, 9 and 12h, ($P<0.05$, Fig. 2A, B), and levels reached the highest after 9 h-stimulation, exhibiting a pattern of initial increase followed by decline (Fig. 2A, B). Compared with the CON group, the phosphorylated p65 and IκBα (Fig. 2B), the nuclear translocation of NF-κB p65 (Fig. 2C) and mRNA levels and protein levels of IL-6, IL-12 and TNF-α (Fig. 2D, E) in macrophages exhibited trends similar to those of TLR4 and NF-κB throughout the process. These results suggest that the production of proinflammatory cytokines in macrophages induced by *G. duodenalis* is correlated with the TLR4/NF-κB pathway.

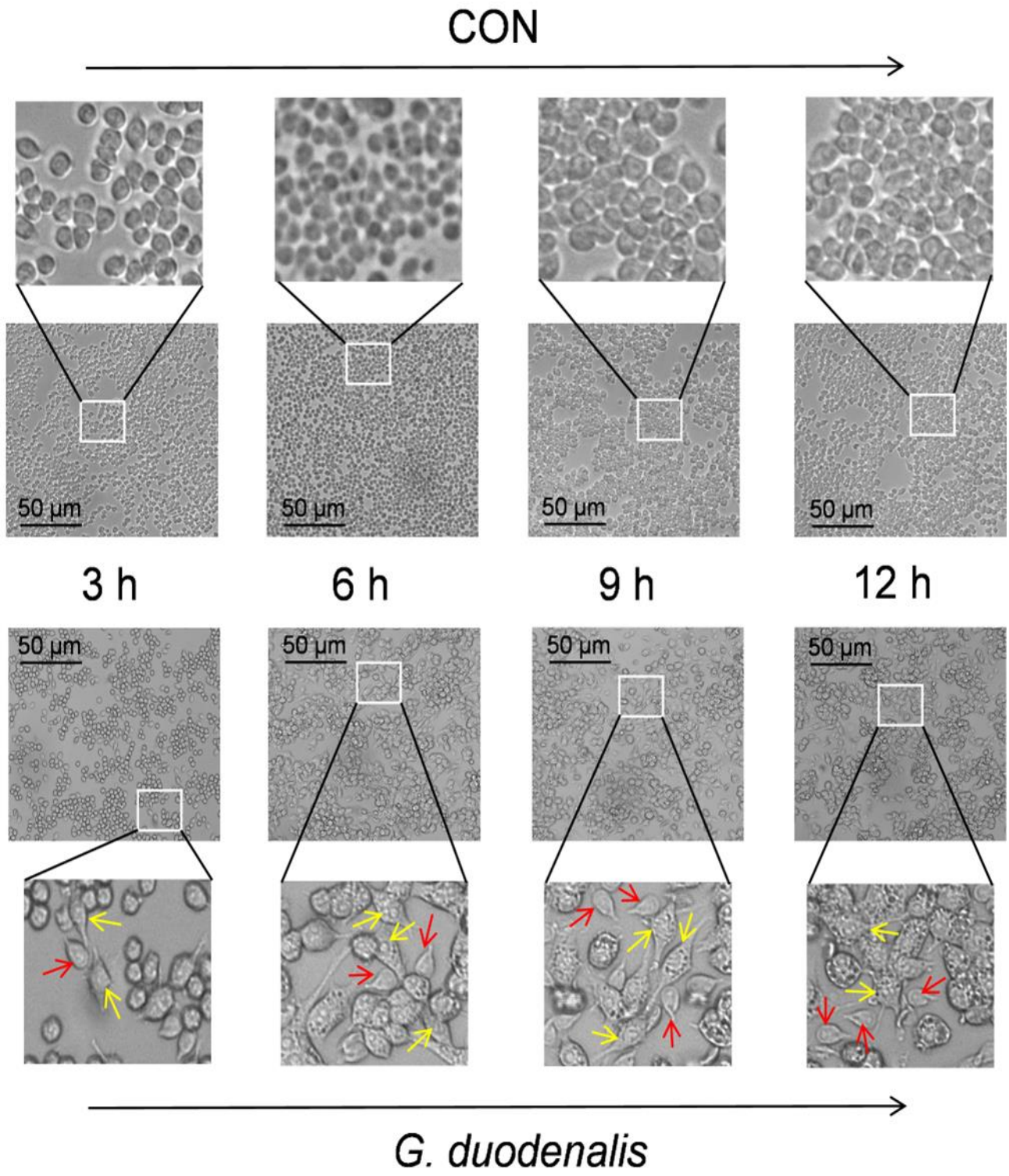

Fig. 1: Morphological changes of RAW264.7 macrophages in different groups. The morphology of RAW264.7 macrophages was observed by microscope at different time points after *G. duodenalis* stimulation. CON represent macrophages untreated by *G. duodenalis*; 3 h, 6 h, 9 h, and 12 h represent macrophages treated by *G. duodenalis* for 3, 6, 9, and 12h. Red arrows indicate *G. duodenalis*, yellow arrows indicate polarized macrophages. Scale bars = 50 μm.

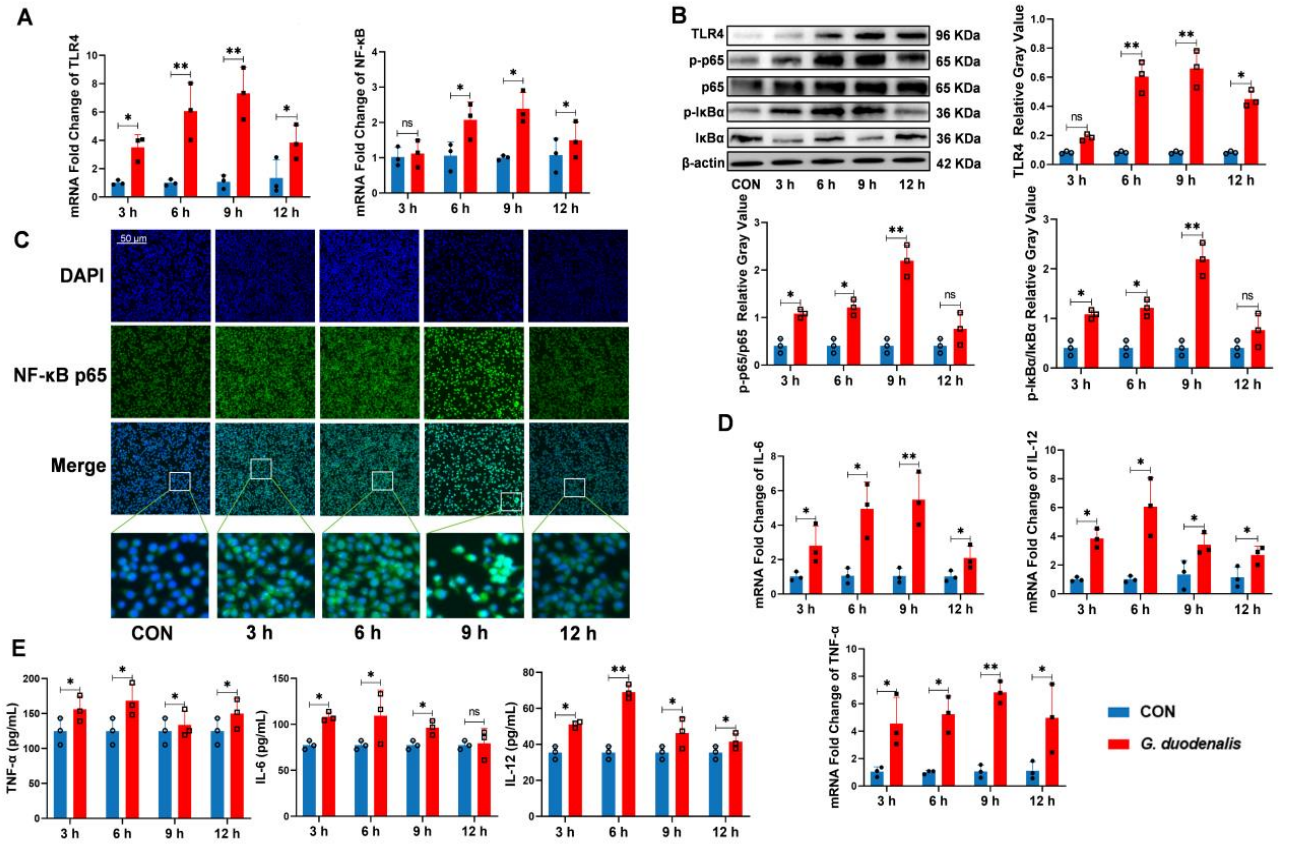

Fig. 2: *G. duodenalis* activated the macrophages TLR4/NF-κB signaling pathway to promote the generation of proinflammatory cytokines. (A) The mRNA levels of *TLR4* and *NF-κB* in macrophages after *G. duodenalis*-stimulation for 3, 6, 9 and 12h, detected with RT-qPCR. (B) The representative western blotting bands and the quantitative analysis of TLR4, p65, p-p65, IκBα and p-IκBα in macrophages after *G. duodenalis*-stimulation for 3, 6, 9 and 12h, detected with western blotting. (C) Nuclear translocation of macrophage NF-κB p65 in different groups, detected with immunofluorescence, which was evaluated with confocal microscopy. The nucleus was stained with DAPI (blue); NF-κB p65 was stained with sheep anti rabbit immunoglobulin labeled with Alexa Fluor488 (green); Scale bars=50μm. (D) The mRNA levels of *IL-6*, *IL-12* and *TNF-α* in macrophages after *G. duodenalis*-stimulation for 3, 6, 9 and 12h, detected with RT-qPCR. (E) The secretion of IL-6, IL-12 and TNF-α in macrophages after *G. duodenalis*-stimulation for 3, 6, 9 and 12h, detected with ELISA. ns, no significant difference; *P<0.05, **P<0.01, significant difference.

Inhibition of TLR4 attenuated the activation of NF-κB and the expression of proinflammatory cytokines in RAW264.7 macrophages induced by *G. duodenalis*: To determine whether the mechanism of *G. duodenalis*-induced proinflammatory cytokine elevation involves the TLR4/NF-κB pathway, RAW264.7 macrophages were pretreated with or without the TLR4 inhibitor TAK-242 for 6h at 37°C (TAK-242 had no significant effect on cell viability, P>0.05, Fig. S2). Results displayed that mRNA levels of macrophages NF-κB in *G. duodenalis*+TAK-242 group were significantly lower than those in the *G. duodenalis* group (P<0.05, Fig. 3B). Protein levels of the phosphorylated p65 and IκBα in macrophage in *G. duodenalis*+TAK-242 group were significantly lower than those in the *G. duodenalis* group (P<0.05, Fig. 3C). Additionally, the nuclear translocation of NF-κB p65 of macrophages in *G. duodenalis*+TAK-242 group were significantly lower than those in the *G. duodenalis* group (Fig. 3D). mRNA levels (Fig. 3E) and secretion levels (Fig. 3F) of IL-6, IL-12 and TNF-α in macrophages in *G. duodenalis*+TAK-242 group were significantly lower than those in the *G. duodenalis* group (P<0.05, Fig. 3E-F). These results indicated that TLR4/NF-κB signaling pathway played crucial role in the enhanced expression of proinflammatory cytokine induced by *G. duodenalis* in RAW264.7 macrophages.

***G. duodenalis* stimulation upregulated the expression of MHC-II , CD80 and CD86 in RAW264.7 macrophages:** To investigate the changes of MHC-II, CD80 and CD86 in RAW264.7 macrophages after *G. duodenalis* stimulation, the gene and protein changes of MHC-II, CD80 and CD86 were detected at 3, 6, 9 and 12h after stimulation. Results showed that mRNA levels of *MHC-II*, *CD80* and *CD86* in macrophages were significantly higher than those in the CON group after stimulating with *G. duodenalis* for 3, 6, 9 and 12h (P<0.05, Fig. 4A), and levels reached the highest after 6 h or 9h-stimulation (Fig. 4A). Moreover, protein expression of MHC-II, CD80 and CD86 in macrophages were significantly higher than those of the CON group after stimulating with *G. duodenalis* for 3, 6, 9 and 12h (P<0.05, Fig. 4B), and levels reached the highest after 6 or 9h-stimulation (Fig. 4B). Results shown that *G. duodenalis* stimulation upregulated MHC-II and co-stimulatory molecules CD80/CD86 expression, suggesting a potential activation toward an antigen-presenting phenotype.

MHC-II knockdown suppresses inflammatory signaling in *Giardia*-stimulated macrophages: The above results confirmed that *G. duodenalis* could induce TLR4-mediated proinflammatory immune response and upregulate the expression of MHC-II in macrophages. So,

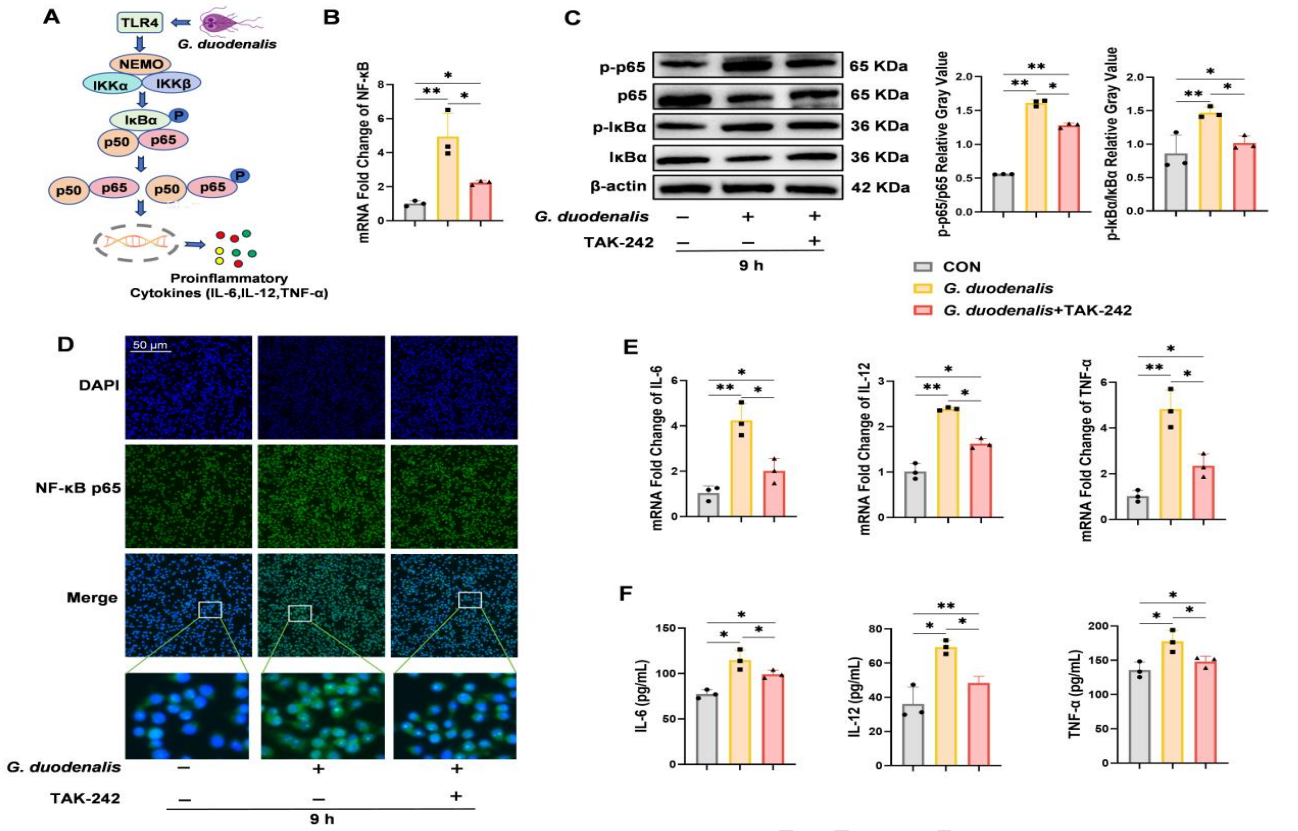

Fig. 3: *G. duodenalis* promotes TLR4 and NF-κB activation and proinflammatory cytokine production in macrophages. (A) A diagram of *G. duodenalis* inducing macrophages to produce proinflammatory cytokines via TLR4/NF-κB mediated signaling pathways. (B) Changes in mRNA levels of *NF-κB* in different groups of macrophages, detected with RT-qPCR. (C) The representative western blotting bands and the quantification analysis of p-p65, p65, p-IκBα and IκBα in different groups of macrophages, detected with western blotting. (D) Effect of TAK-242 on nuclear translocation of NF-κB p65 in *G. duodenalis*-treated macrophages, detected with immunofluorescence, which was evaluated with confocal microscopy. The nucleus was stained with DAPI (blue); NF-κB p65 was stained with sheep anti-rabbit immunoglobulin labeled with Alexa Fluor488 (green); Scale bars=50 μm. (E) Changes in mRNA levels of *IL-6*, *IL-12* and *TNF-α* in different groups of macrophages, detected with RT-qPCR. (F) Changes in secretion levels of IL-6, IL-12 and TNF-α in different groups of macrophages, detected with ELISA. ns, no significant difference; *P<0.05, **P<0.01, significant difference.

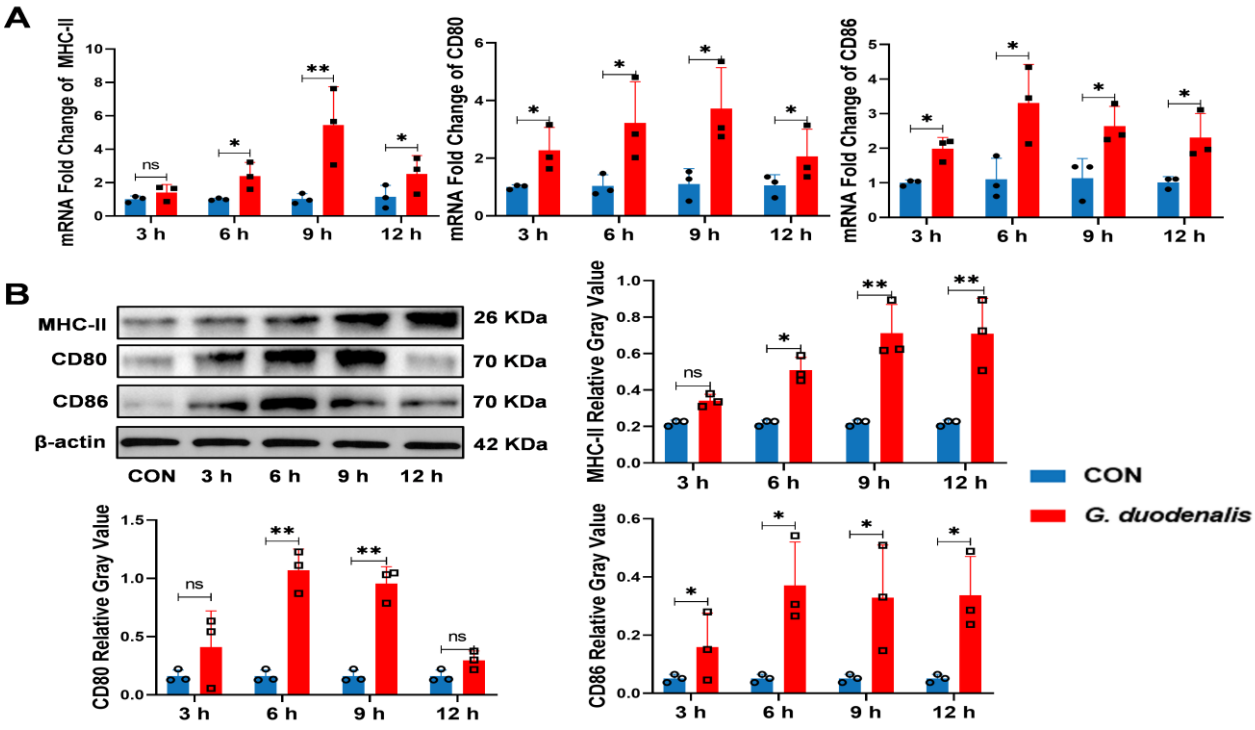

Fig. 4: The changes of MHC-II, CD86 and CD80 in macrophages after *G. duodenalis*-stimulation. (A) Changes in mRNA levels of *MHC-II*, *CD86* and *CD80* in macrophages after *G. duodenalis*-stimulation for 3, 6, 9 and 12h, detected with RT-qPCR. (B) The representative western blotting bands and the quantification analysis of CIITA, MHC-II, CD80 and CD86 in macrophages treated with *G. duodenalis* for 3, 6, 9 and 12h, detected with western blotting. ns, no significant difference; *P<0.05, **P<0.01, significant difference.

to further investigate whether the up-regulation of macrophage proinflammatory cytokines induced by *G. duodenalis* is related to the up-regulation of MHC-II. The siMHC-II RNA was used to knock down the expression of MHC-II in macrophages. Among the tested siRNAs, siMHC-II-228 exhibited the most potent interfering effect, significantly reducing MHC-II mRNA and protein levels compared to the control (CON) group (P<0.01, Fig. S1A, C), and siMHC-II-228 had no significant effect on cell viability (P>0.05, Fig. S2). Consequently, siMHC-II-228 was selected for subsequent experiments.

The mRNA level (Fig. 5B) and the protein expression (Fig. 5C) of MHC-II in macrophages of *G. duodenalis*+siMHC-II-228 group were significantly lower than those in *G. duodenalis* and *G. duodenalis*+siNC group (P<0.05). Additionally, the mRNA level of NF-κB (Fig. 5B), the phosphorylated p65 and IκBα (Fig. 5C) in macrophages of *G. duodenalis*+siMHC-II-228 group were significantly lower than those in *G. duodenalis* and *G. duodenalis*+siNC group (P<0.05). Nuclear translocation of NF-κB p65 (Fig. 5D) in macrophages of *G. duodenalis*+siMHC-II-228 group was significantly attenuated compared with that in *G. duodenalis* group and *G. duodenalis*+siNC group. Similarly, mRNA levels (Fig. 5E) and secretion levels (Fig. 5F) of IL-6, IL-12 and TNF-α in macrophages of *G. duodenalis*+siMHC-II-228 group were significantly lower relative to those in *G. duodenalis* group and *G. duodenalis*+siNC group (P<0.05).

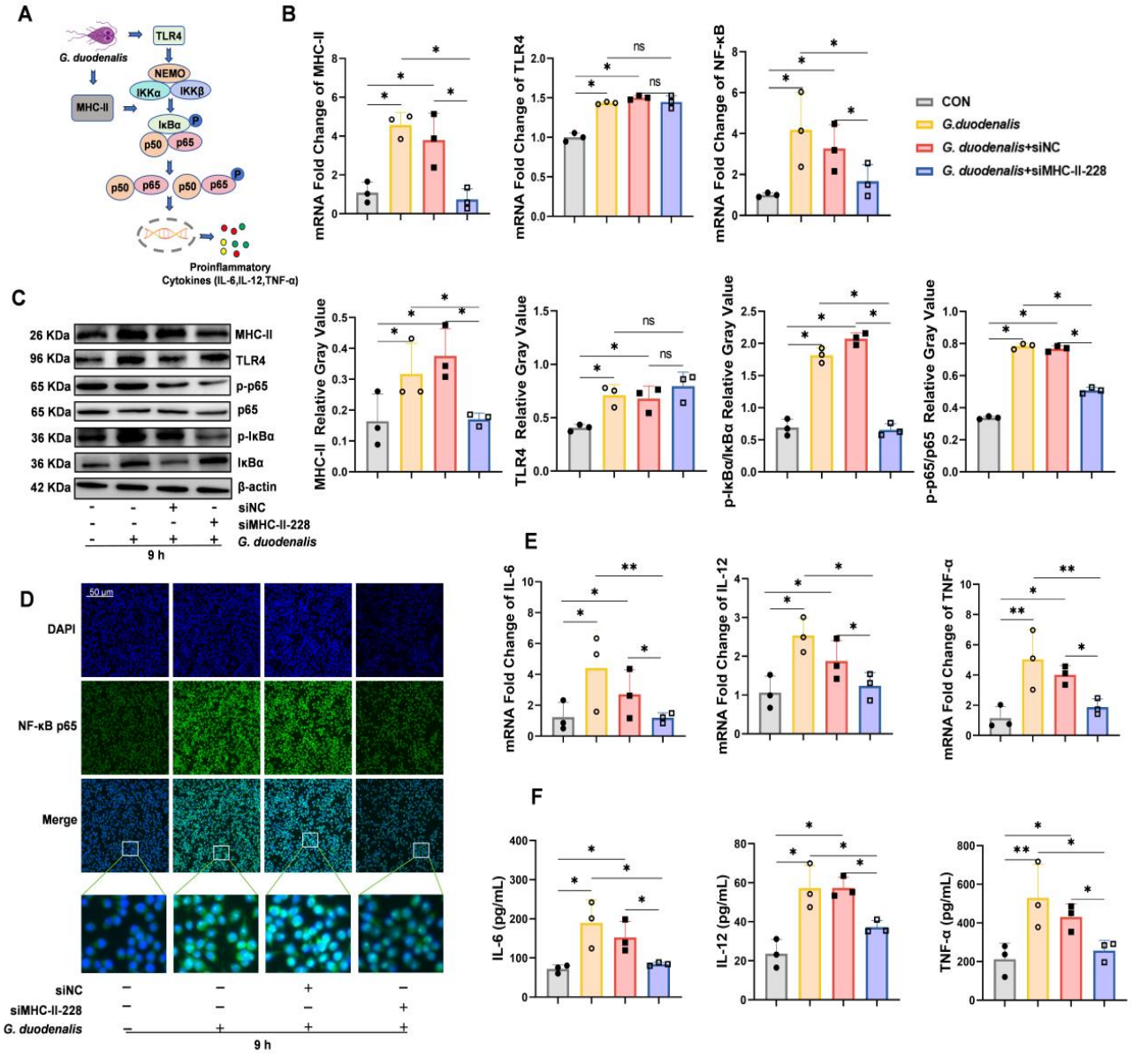

Fig. 5: Effect of siMHC-II RNA on proinflammatory cytokines via TLR4/NF-κB pathway in *G. duodenalis*-treated macrophages. (A) A diagram of *G. duodenalis* promoting macrophage proinflammatory cytokine production via MHC-II/NF-κB pathway. (B) Changes in mRNA levels of *MHC-II*, *TLR4* and *NF-κB* in different groups of macrophages, detected with RT-qPCR. (C) The representative western blotting bands and the quantification analysis of MHC-II, TLR4, p-p65, p65, p-IκBα and IκBα in different groups of macrophages, detected with western blotting. (D) Effect of siMHC-II RNA on nuclear translocation of NF-κB p65 in *G. duodenalis*-treated macrophages, detected with immunofluorescence, which was evaluated with confocal microscopy. The nucleus was stained with DAPI (blue); NF-κB p65 was stained with sheep anti-rabbit immunoglobulin labeled with Alexa Fluor488 (green); Scale bars=50μm. (E) Changes in mRNA levels of *IL-6*, *IL-12* and *TNF-α* in different groups of macrophages, detected with RT-qPCR. (F) Changes in secretion levels of IL-6, IL-12 and TNF-α in different groups of macrophages, detected with ELISA. ns, no significant difference; *P<0.05, **P<0.01, significant difference.

Knock-down of CIITA attenuated the expression of MHC-II and proinflammatory cytokines in RAW264.7 macrophages triggered by *G. duodenalis*: To investigate whether CIITA plays a role in the upregulation of MHC-II in macrophages treated with *G. duodenalis*, the CIITA gene in macrophages was knocked down by siCIITA RNA. Among the tested siRNAs, siCIITA-1866 exhibited the most potent interfering effect, significantly reducing CIITA mRNA and protein levels compared to the control (CON) group (P<0.01, Fig. S1B, D), and siCIITA-1866 had no significant effect on cell viability (P>0.05, Fig. S2). Consequently, siCIITA-1866 was selected for subsequent experiments.

Knockdown of CIITA with siCIITA-1866 significantly suppressed the mRNA (Fig. 6B) and protein (Fig. 6C) expression of both CIITA and MHC-II in macrophages treated with *G. duodenalis*, compared to the *G. duodenalis* groups and *G. duodenalis*+siNC groups (P<0.01). Consistent with this, the mRNA (Fig. 6D) and protein (Fig. 6E) levels of the proinflammatory cytokines IL-6, IL-12, and TNF-α were also markedly lower in the *G. duodenalis*+siCIITA-1866 group (P<0.05). These results suggest that CIITA mediates *G. duodenalis*-induced cytokine expression through regulation of MHC-II.

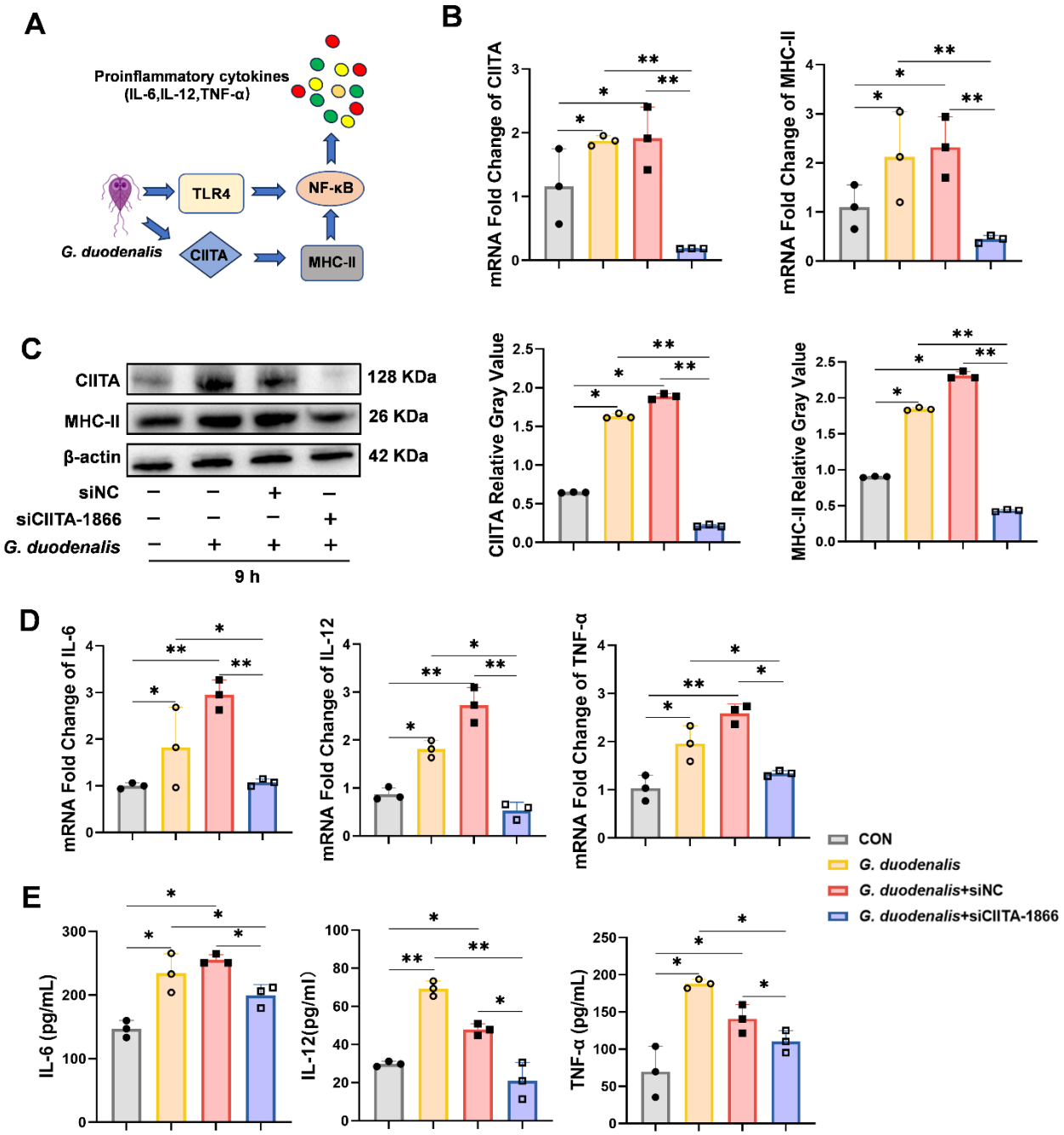

Fig. 6: Effect of CIITA on the expression of MHC-II and inflammatory cytokines in macrophages of different groups. (A) A diagram of *G. duodenalis* promoting macrophage proinflammatory cytokine production via CIITA/MHC-II/NF-κB pathway. (B) Changes in mRNA levels of CIITA and MHC-II in different groups of macrophages, detected with RT-qPCR. (C) The representative western blotting bands and the quantification analysis of CIITA and MHC-II in different groups of macrophages, detected with western blotting. (D) Changes in mRNA levels of IL-6, IL-12 and TNF-α in different groups of macrophages, detected with RT-qPCR. (E) Secretion levels of IL-6, IL-12 and TNF-α in different groups of macrophages, detected with ELISA. ns, no significant difference; *P<0.05, **P<0.01, significant difference.

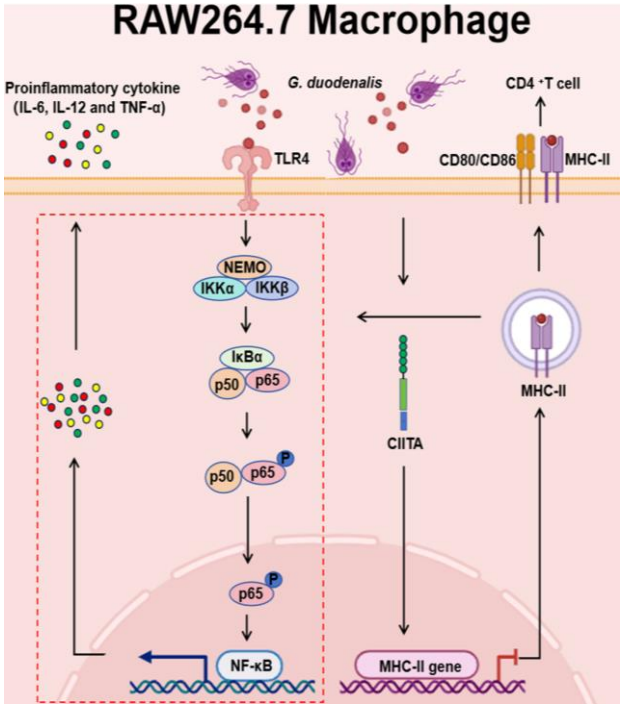

Fig. 7: Model for the role of MHC-II in the full activation of the macrophage TLR4-mediated innate immune response triggered by *G. duodenalis*. In the present study, RT-qPCR, Western blotting, ELISA, immunofluorescence, inhibitor assay and siRNA were utilized to probe the role of TLR4 and MHC-II in RAW264.7 macrophages-mediated defenses against *G. duodenalis*. Moreover, it was also found that MHC-II plays an important role in full activation of TLR4-mediated innate immune response in macrophage initiated by *G. duodenalis*.

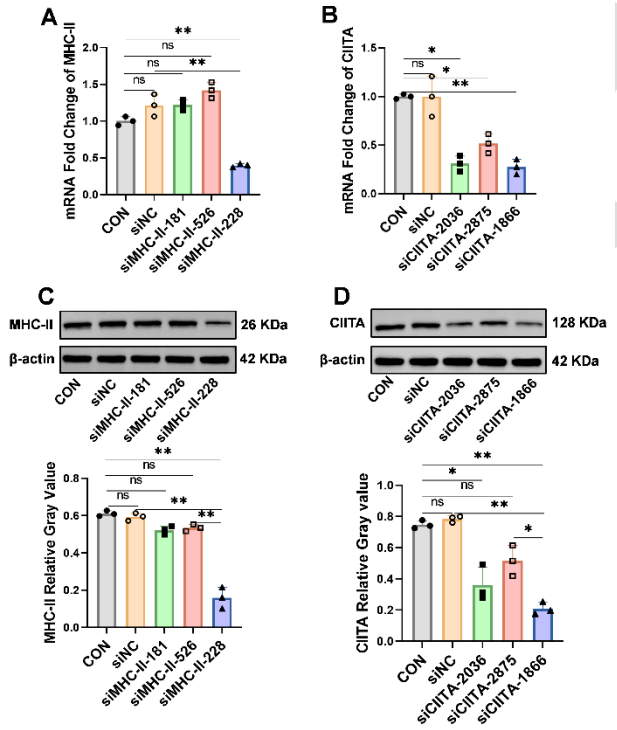

Fig. S1: The interference efficiency of siRNA was assessed using RT-qPCR. (A) Changes in mRNA levels of MHC-II in different siMHC-II RNA groups of macrophages, detected with RT-qPCR. (B) Changes in mRNA levels of CIITA in different siCIITA RNA groups of macrophages, detected with RT-qPCR. (C-D) The representative western blotting bands and the quantification analysis of MHC-II and CIITA in different groups of macrophages, detected with western blotting. ns, no significant difference; *P<0.05, **P<0.01, significant difference.

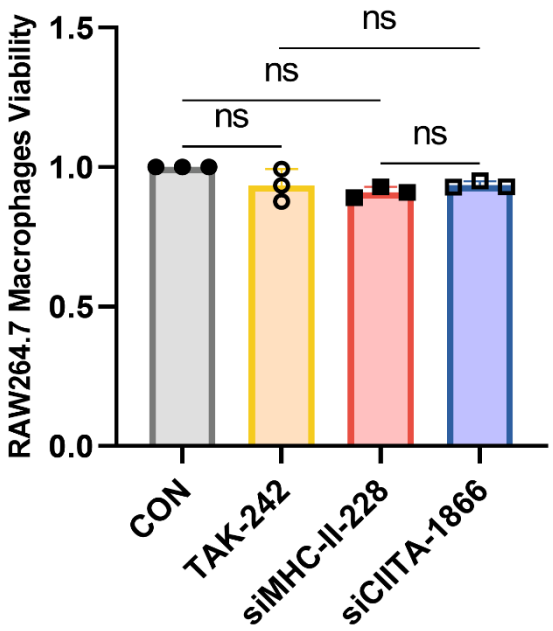

Fig. S2: Cell viability was assessed using the CCK-8 assay. ns, no significant difference, P>0.05.

DISCUSSION

Macrophages play a pivotal role throughout the course of *G. duodenalis* infection (Fink *et al*., 2019). In this study, our results show that *G. duodenalis* induced an increase in formations of membrane protrusions of RAW264.7 macrophages, and the formation of lamellipodia and filopodia altered macrophage morphology toward a flattened, activated phenotype, which preliminarily verified that the immune control of *G. duodenalis* infection is highly dependent on macrophage activity. As pattern recognition receptors, TLRs localize to macrophage membranes or endosomes, and they play an essential role in mediating macrophage immune responses (Alhamdan *et al*., 2024). TLR4 signaling includes the recruitment of different adaptor molecules that activate various transcription factors, such as NF-kB, IRF3/7 and MAP kinases, to induce the production of proinflammatory cytokines, which critically involved in the host's immune response to many protozoan parasites (Dos-Santos *et al*., 2016; Muñoz-Caro *et al*., 2021; Shen *et al*., 2023). For instance, the TLR4/NF-κB pathway is also critical for initiating proinflammatory responses against Leishmania, controlling parasite replication and directing adaptive immunity (Dos-Santos *et al*., 2016). Similarly, infection with *Entamoeba histolytica* triggers NF-κB activation in macrophages and epithelial cells, leading to the production of cytokines like TNF-α and IL-8 (Chadha et *al*., 2021). In this study, the results shown that the mRNA levels and secretion of IL-6, IL-12 and TNF-α were increased, the mRNA levels and protein expression of TLR4 was upregulated in response to *G. duodenalis* stimulation in macrophages. Similarly, the increased nuclear translocation of p65 and the enhanced phosphorylation of p65 and IκBα protein was also observed in response to *G. duodenalis* stimulation in macrophages. Following TLR4 inhibition, these proinflammatory responses were

markedly attenuated, though not entirely eliminated (e.g., 1.2-fold remained increase in IL-6 expression), indicating that the TLR4/NF-κB pathway plays a central but not exclusive role in macrophage activation. These observations align with patterns reported in other protozoan infections and suggest that giardiasis involves additional, yet-to-be-characterized signaling mechanisms warranting further investigation. Furthermore, following TLR4 inhibition, macrophage MHC-II may exhibit similar changes, though this requires further validation through in-depth experiments, particularly regarding the interaction between *G. duodenalis* and macrophages. Although our data using TAK-242 indicate TLR4 as the key mediator, the presence of minor interference from other TLRs cannot be ruled out due to its selective inhibition of TLR4. Future studies employing genetic approaches such as TLR4 gene knockout will be valuable for confirming this relationship.

MHC-II and its associated costimulatory molecules (CD80/86) orchestrate $CD4^+$ T cell activation by presenting processed antigens, thereby triggering the adaptive immune response (Buxadé *et al*., 2018). Research shows that malarial hemozoin secreted by malaria parasite upregulate the expression of MHC-II, CD86 and CD80 in mouse macrophages to enhance immunity against malaria parasites (Waseem *et al*., 2016). In this study, we found that MHC-II, CD80 and CD86 were also significantly increased in macrophages treated with *G. duodenalis*. A similar result was also observed in previous study in which they demonstrated that expression of MHC-II was increased after low concentrations of *G. duodenalis* stimulation in bovine DC (Grit *et al*., 2014), and the upregulation of MHC-II may enhance the antigen-presenting ability of macrophages, which could explain why most *G. duodenalis* infections are self-limiting. CD86 is a surface marker for M1-type macrophages (Liu *et al*., 2022), and the upregulation of macrophages CD86 in this study indicated that *G. duodenalis* stimulation promotes the shift of macrophages toward M1-like polarization, which was confirmed by the upregulation of proinflammatory cytokines in macrophages stimulated with *G. duodenalis* in the present study.

Apart from its primary antigen-presenting function, MHC-II triggers intracellular signals upon binding to ligands in a variety of cell types, including the regulation of cell adhesion, cytokine production as well as the induction of apoptosis, proliferation, and differentiation. (Dong *et al*., 2011; Mourad *et al*., 1990; Spertini *et al*., 1992). For example, insufficient or low levels of MHC-II in mice resulted in reduced secretion of proinflammatory cytokines after LPS stimulation (Piani *et al*., 2000). The levels of TNF-α, IL-6, IL-10 and IL-12 from peritoneal macrophages of MHC-II knockout mice was diminished following LPS treatment compared to that of wild-type mice (Frei *et al*., 2010). In this study, MHC-II knockdown reduced *G. duodenalis*-induced IL-6, IL-12 and TNF-α expression, suggesting MHC-II facilitates cytokine production in macrophages. NF-κB, a key transcriptional regulator of proinflammatory cytokines, has previously been linked to MHC-II-mediated immune responses. For example, knockdown of MHC-II attenuated LPS-stimulated NF-κB activation, leading to a decrease in proinflammatory cytokines (Guo *et al*., 2024; Liu *et al*., 2017). Consistent with this, we found that *G. duodenalis*-triggered NF-κB activation was suppressed upon MHC-II interference in macrophages, which inidcated that beyond its classical role in antigen presentation, MHC-II appears to modulate TLR4/NF-κB-mediated inflammatory signaling during macrophag*e-G. duodenalis* interactions. Research has revealed that intracellular MHC-II molecules interact with the tyrosine kinase Btk via the co-stimulatory molecule CD40, maintaining its activated state. Btk then interacts with the adapter molecules MyD88 and TRIF, promoting NF-κB activation and thereby enhancing TLR signaling (Liu *et al.*, 2011). In this study, MHC-II silencing downregulates NF-κB mRNA expression, which may be related to this mechanism. Future studies should aim to identify specific *G. duodenalis* antigens capable of modulating macrophage MHC-II expression, which could inform new therapeutic approaches. Although our data demonstrates a strong correlation between MHC-II downregulation, suppression of NF-κB pathway activity, and reduced cytokine secretion, the precise causal relationship requires further validation.

Expression of MHC-II is strictly regulated by complex mechanisms and CIITA is a master regulator that governs nearly all qualitative and quantitative aspects of MHC-II expression (Boss, 1997; Reith *et al*., 2005). This study also demonstrates that gene and protein expression of MHC-II were decreased in siCIITA RNA-interfered macrophages treated with *G. duodenalis* and similar trend was also observed in the expression of IL-6, IL-12 and TNF-α. These findings suggest that G. duodenalis modulates CIITA to regulate MHC-II expression, indirectly affecting NF-κB-driven cytokine output. Overall, MHC-II plays a role in promoting *G. duodenalis* to induce macrophages to produce proinflammatory cytokines. This finding contributes to our understanding that, although *G. duodenalis* infection typically remains non-inflammatory (Solaymani-Mohammadi, 2022), chronic macrophage activation through MHC-II–NF-κB crosstalk may contribute to localized intestinal inflammation during prolonged infection.

Conclusions: Overall, this study elucidates the critical roles of TLR4 and MHC-II in mediating the innate immune response of macrophages against *G. duodenalis*. Our findings demonstrate that *G. duodenalis* activates macrophage proinflammatory responses through modulation of the TLR4-mediated pathway, primarily via the CIITA/MHC-II/NF-κB signaling axis. This integrated pathway amplifies cytokine production and macrophage activation, revealing a previously underappreciated layer of innate immune regulation during *G. duodenalis* infection. As summarized in Fig. 7, these insights not only expand our understanding of host-parasite interactions but also highlight potential immunomodulatory targets for future therapeutic strategies against giardiasis.

Author contributions: FL and HyD conceived and designed the project. LxZ, HjD and JqL was responsible for supervision, writing review and funding. PtS performed the experiments, conducted data analysis and article writing. LlL, JkD, SjZ, YyW, HyD and FL checked the manuscript for grammar and spelling. All the authors critically read and approved the manuscript.

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Data availability statement: All data were available in this manuscript and the supporting information.

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