Original Article

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Entamoeba moshkovskii as a potential model organism for Gal/GalNAc lectin intermediate subunit exhibition and functional identification

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SUMMARY In humans, *Entamoeba histolytica* is the main pathogen causing various amoebiases, while *E. moshkovskii* falls between being a pathogen and non-pathogen. The two species have similar behavior patterns but differ significantly in pathogenicity, with previous studies and clinical data indicating that *E. moshkovskii* has a low level of pathogenicity. Meaningfully, the biological characteristics of *E. moshkovskii* make it a potential model organism and a protein display platform for studying the functions of important *Entamoeba* proteins. Here, an Amoeba-pcDNA3.1 vector capable of overexpressing *E. histolytica*-sourced Igl-C protein was constructed and successfully transfected into *E. moshkovskii*. High levels of expression of the *Igl-C*, *EGFP*, and *NeoR* genes were identified in Igl-C-transfected trophozoites using qRT-PCR, and they were subsequently confirmed using immunoblotting. Transfection of Igl-C protein improved the adherence and phagocytosis of *E. moshkovskii*, demonstrating that *E. histolytica* Igl mediated amoebic adhesion. Moreover, as a manifestation of protein virulence, the ability of post-transfected trophozoites to induce inflammation in host macrophages was also enhanced. In conclusion, this study utilizing the characteristics of *E. moshkovskii* confirmed its potential to serve as a model organism. *E. moshkovskii* could replace *E. histolytica* as the target of gene editing, allowing more efficient study of amoebic pathogenicity.

Keywords *Entamoeba moshkovskii*, *Entamoeba histolytica*, Gal/GalNAc lectin, model organism, transfection

1. Introduction

Entamoeba spp. are a group of facultative anaerobic parasites that primarily live in the intestinal tract of their host (*1-3*). In humans, *E. histolytica* is the main pathogen responsible for various amoebiases, such as amoebic colitis and amoebic liver abscess, resulting in 100,000 deaths annually (*4,5*). *E. moshkovskii*, also known as an *E. histolytica*-like amoeba, is morphologically similar to *E. histolytica*, and their cysts are almost identical (*6*). However, previous studies have shown that the two *Entamoeba* species share no serum cross-reactivity and have different isoenzyme profiles (*2*). Diarrhea caused by *E. moshkovskii* is limited to infants and immunocompromised populations, so only a few relevant cases have been reported in Bangladesh, eastern India, and Australia in recent years (*7-14*).

In the early stages of amoebiasis, *E. histolytica* trophozoites need to penetrate the mucus layer of the host intestine and invade the intestinal wall to induce tissue destruction and even severe ulceration. During this process, galactose (Gal)- and *N*-acetyl-D-galactosamine (GalNAc)-inhibitable lectins play an indispensable role by facilitating adherence of the parasite to mucins, gut microbiota, and host cells (*15,16*). The Gal/GalNAc lectins consist of a 260 kDa heterodimer and a non-covalently associated 150 kDa intermediate subunit (Igl), in which Igl is a specific cysteine-rich protein contributing to adherence and cytotoxicity (*17,18*). Previous studies have found that Igl, and especially its C-terminal segment (Igl-C), has the potential to serve as a potential vaccine against amoebiasis, but its complex function in pathogenesis still needs to be elucidated further (*19*).

E. histolytica is a polyploid organism, which hampers the full elucidation of the regulatory mechanism of its gene expression and the effectiveness of gene editing (*20,21*). At present, CRISPR technology is not yet mature enough for use in this genus, and other gene editing methods can only knock down a certain gene's expression in *E. histolytica*, being unable to reach the level of knocking out a gene in mammalian cells (*22*). For many years, this has limited the study of the

pathogenic mechanisms of *E. histolytica*, indicating the importance of developing new models.

E. moshkovskii and *E. histolytica* are closely related in terms of genetic distance and have similar biological characteristics, so the former has the potential to be a model organism for studying the function of *Entamoeba*'s virulence proteins. By modifying the appropriate vector and overexpressing *E. histolytica*-sourced Igl fragments, the aim of the current study was to investigate the potential of *E. moshkovskii* to serve as a model organism and a protein display platform. The overexpressed Igl fragment in *E. moshkovskii* was confirmed as correct and then sent for functional detection and comparison. To the extent known, this is the first study to successfully transfect an *E. histolytica* virulence protein in neighboring species, providing a novel method for the study of *Entamoeba* pathogenicity.

2. Methods

2.1. Amoeba and cell cultures

Trophozoites of *E. histolytica* HM-1:IMSS or *E. moshkovskii* were grown axenically in YIMDHA-S medium containing 10% (v/v) heat-inactivated adult bovine serum (Sigma-Aldrich, USA) at 36.5°C or 30°C, respectively (*17*). Jurkat Clone E6-1 and RAW264.7 cells were separately cultured in RPMI-1640 medium and Dulbecco's modified Eagle's medium (Corning, USA) supplemented with 10% fetal bovine serum (HyClone Laboratories, USA). Cells were grown in an incubator maintained at 37°C with 5% CO_2.

2.2. Vector construction

For the overexpression of certain genes in the trophozoite, this study constructed an *Entamoeba* transfection vector from HA-FLAG-pcDNA3.1 (Addgene, USA). The promoter sequence of the actin gene and the terminator sequence of the cysteine synthase gene were amplified, and then the original promoter of the NeoR resistance region and the original terminator of the recombinant protein expression region on the vector were replaced. Igl signal peptide gene sequence, Kozak-Signal-HA sequence, and P2A-EGFP sequence fragments were all synthesized by overlapping PCR to replace the 5' to 3' regions on the vector. After cDNA synthesis, the *E. histolytica* Igl-C sequence was amplified and inserted between the HA and P2A fragments. The primers used are listed in Table 1.

Table 1. Primers used in the construction of recombinant vectors

Name	Primer sequence (5' - 3')
Ac5'-F-SexAI	GCACCAGGTAAATGATGCTATATTTTG
Ac5'-R-SmaI	GCCCCGGGTGAATGTTCAATTCAGTT
Ac3'-F-BstBI	GCTTCGAATAATTTACTTTCTCATTTG
Ac3'-R-BstI17I	GCGTATACTCTCCATGTTCTTCATGA
CS5'-F-MluI	GCGACGCGTACACTTAATTAAGTAATT
CS5'-R-NheI	GCGGCTAGCTGAATCTTGTGTAACAAC
CS3'-F-KpnI	CGGGGTACCTTTGAATTGAACTCTTCT
CS3'-R-HindIII	GCGAAGCTTCATTAATTCCAAAAACTG
Igl-C-F-XhoI	CCCTCGAGGAAGGACCAAATGCAGAAG
Igl-C-R-BamHI	CGGGATCCGAACATAAATGCTAACAT
P2A-BamHI	CGGGATCCGCTACTAATTTCTCCTTGCTT AAGCAAGCTGGTGATGTTGAAGAAAATC CTGGTCCT
LINK	TTGAAGAAAATCCTGGTCCTATGGTGAG CAAGGGCGAGGA
EGFP-R-KpnI	GGGGTACCCTTGTACAGCTCGTCCAT
Signal-HA-F-NheI	CCGCTAGCCACCATGTTTATTCTTCTTTT ATTCATATCAATTTCACTTGGTGATTACC CA
Signal-HA-R-XhoI	CCCCTCGAGTCTAGAACCTCCACCTCCA CCAGCGTAATCTGGAACATCGTATGGGT AATCA

All primers were synthesized by Invitrogen.

2.3. Liposome transfection

E. moshkovskii trophozoites were harvested in the late logarithmic growth phase and then washed, resuspended, and spread in 48-well cell culture plates at a density of 3×10^5 per well. After they were fixed, a liposome transfection reagent containing 2 μg of plasmid was added to trophozoites. The reagent was aspirated and replaced with fresh RPIM 1640 medium after incubation for 5 h, and trophozoites were then transferred to glass tubes for incubation for 48 h. G418 screening was selectively performed in half of the trophozoites to obtain a stable transfected amoeba strain (*21*).

2.4. Quantitative real-time RT-PCR

E. moshkovskii cDNA was synthesized through a reverse transcriptase polymerase chain reaction (RT-PCR) with total RNA extracted from transfected trophozoites. On an ABI 7500 real-time PCR system (ABI, USA), transcript levels of three genes, *Igl-C*, *EGFP*, and *NeoR*, were detected in a final reaction volume of 20 μL in accordance with the manufacturer's recommendations (*23,24*). RAW264.7 cells were incubated with transfected *E. moshkovskii* trophozoites (ratio 2:1) at 37°C for 1 h to detect the expression of the *Tnf*, *Il1b*, *Il6*, *Nos2*, and *Actb* genes. Reactions were performed in 96-well plates with a SYBR Premix Ex Taq (TaKaRa, Japan) under the following amplification cycling conditions: 30 s at 95°C; 40 cycles of 5 s at 95°C and 35 s at 60°C. For gene expression of each cytokine, qRT-PCR was performed in the logarithmic phase of product accumulation, during which the threshold cycle (Ct) values were linearly correlated with relative DNA copy numbers. The primers used are listed in Table 2.

2.5. Dot blotting

For dot blotting experiments, *E. moshkovskii*

Table 2. Primers used in quantitative real-time RT-PCR

Amplified gene	Primer sequence (5' - 3')
Entamoeba moshkovskii (amoeba)	
Igl-C	
F	GGTTCACAGGTTGGTGCTTG
R	AGTACATGGCTTTTCTCCGGT
EGFP	
F	CCCGACAACCACTACCTGAG
R	GTCCATGCCGAGAGTGATCC
NeoR	
F	CAGACAATCGGCTGCTCTGA
R	CCTTCCCGCTTCAGTGACAA
Actin	
F	CGCACGACTTCAAAGGGACT
R	TCCTTATGACCTGGCGGAGT
Mus musculus (mouse)	
Tnf	
F	GTCGTAGCAAACCACCAA
R	GGCAGCCTTGTCCCTTGA
Il1b	
F	ACATCAGCACCTCACAAGCAG
R	TTAGAAACAGTCCAGCCCATAC
Il6	
F	TGCCTTCTTGGGACTGAT
R	TTGCCATTGCACAACTCTTT
Nos2	
F	TCCTGGAGGAAGTGGGCCGAAG
R	CCTCCACGGGCCCGGTACTC
Actb	
F	CACTGTCGAGTCGCGTCC
R	TCATCCATGGCGAACTGGTG

All primers were synthesized by Invitrogen.

trophozoites were blotted on a nitrocellulose membrane using the Vacuum Blotter. Filter strips were blocked with 5% bovine serum albumin (BSA) in PBS and allowed to react with diluted anti-HA antibody or EH3077 monoclonal antibody for 60 min. HRP-labeled goat anti-mouse IgG antibody (MP Biomedicals, USA) was used as the second antibody. The strips were then developed with an Enhanced HRP-DAB Chromogenic Substrate Kit (Tiangen Biotech, China).

2.6. Fluorescence imaging of trophozoites

Transfected trophozoites were used in confocal microscopy (*25*). Smears of post-transfected trophozoites in the logarithmic growth phase were prepared with a Cytospin 4 Cytocentrifuge (Thermo Scientific, USA) and then fixed with 4% paraformaldehyde for 30 min. After blocking with 5% BSA-PBS, trophozoites on the smears were incubated with an anti-HA antibody or EH3077 monoclonal antibody for 1 h, followed by a fluorescently labeled secondary antibody. DAPI was used for chromosome staining. Cytoslides were finally sealed and observed under a Leica TCS SP8 microscope (Leica, USA). Fluorescently stained trophozoites and cells in amoebic adherence and phagocytosis assays were also imaged using fluorescence microscopy.

2.7. Amoebic adherence assay

Erythrocytes of human blood group O were from healthy adult volunteers (*26*). In brief, *E. moshkovskii* trophozoites and erythrocytes were separately adjusted to 5×10^6 / mL and 2.5×10^7 /mL, and then 100 µL of trophozoites and 100 µL of erythrocytes were co-incubated at 4°C for 30 min. After fixation with 2.5% glutaraldehyde for 30 min, samples were washed with PBS, and erythrocytes were stained with a 3,3-diaminobenzidine (Sigma-Aldrich, USA) solution containing 0.2% H_2O_2. The rate of adherence was determined by examining how many of 300 trophozoites had adhered to at least three erythrocytes.

2.8. Amoebic phagocytosis assay

Both Jurkat Clone E6-1 cells and erythrocytes were used in phagocytosis assays. After obtaining erythrocytes of human blood group O from healthy adult volunteers, untransfected amoebas or post-transfected *E. moshkovskii* trophozoites were incubated with carboxyfluorescein succinimidyl ester (CFSE) at room temperature for 10 min, while Jurkat Clone E6-1 cells or erythrocytes were separately incubated with DiD at 55°C or room temperature for 20 min. Density: trophozoites were adjusted to 5×10^6 /mL, Jurkat cells to 1×10^7 /mL, and erythrocytes to 2.5×10^7 /mL. After incubating 100 µL of trophozoites with 100 µL of Jurkat cells or 100 µL of erythrocytes at 30°C for 30 min, samples were fixed for 30 min with 4% paraformaldehyde and subjected to flow cytometry or detection with a microplate reader.

2.9. Preparation of recombinant proteins

The amino acid sequence of *E. histolytica* Igl was used as a template to search for Igl-like protein sequences of *E. moshkovskii* using the NCBI Blast tool. After amplifying and sequencing the corresponding nucleic acid sequences, recombinant *E. histolytica* Igl-C (603-1086 aa) and *E. moshkovskii* Igl-C (591-1086 aa) proteins were prepared as previously described (*19*). In brief, pET-19b vectors were transformed into *Escherichia coli* BL21 Star(DE3)pLysS competent cells (Novagen, USA), followed by culturing in Luria-Bertani medium containing 100 µg/mL ampicillin and induction with isopropyl-β-d-thiogalactopyranoside (Amresco, USA) at a final concentration of 1 mM. Since proteins exist in the form of inclusion bodies, refolding was conducted using a Protein Refolding Kit (Novagen, USA) in accordance with the manufacturer's instructions.

2.10. Transcriptome sequencing

RAW264.7 cells were stimulated with the two recombinant *Entamoeba* Igl-C proteins whose total RNA was extracted at 12 h and 24 h with the RNeasy Plus Mini Kit (QIAGEN, Germany). They were then sent for transcriptome sequencing. After constructing cDNA

libraries, transcriptome sequencing was performed on the Illumina NovaSeq 6000 platform (Illumina, USA) in accordance with the manufacturer's procedure (*27*).

2.11. Statistical analysis

All statistical analyses were performed using SPSS, version 20 (IBM, USA). Amoebic adherence, amoebic phagocytosis, and qPCR results were analyzed using a two-tailed Student's *t*-test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Construction and transfection of amoeba overexpression vectors

As a powerful method for studying gene function and regulation, DNA transfection systems have been developed in *E. histolytica* in recent years. The actin and lectin gene promoters have already been reported to be successfully used to drive the expression of certain genes in transfected *E. histolytica*. Our parallel work, presented here, also focused on developing a system for successful transfection and expression of an important reporter gene in *E. moshkovskii*. Verified with PCR and sequencing, an eukaryotic expression vector, Amoeba-pcDNA3.1, suitable for *Entamoeba* spp. was successfully constructed in this study. The replacement genes involved were identical to those in the *E. histolytica* HM-1:IMSS gene database in GenBank (Figure 1A).

Based on the detected fluorescence signal intensity, the level of erythrocytic phagocytosis by *E. histolytica*

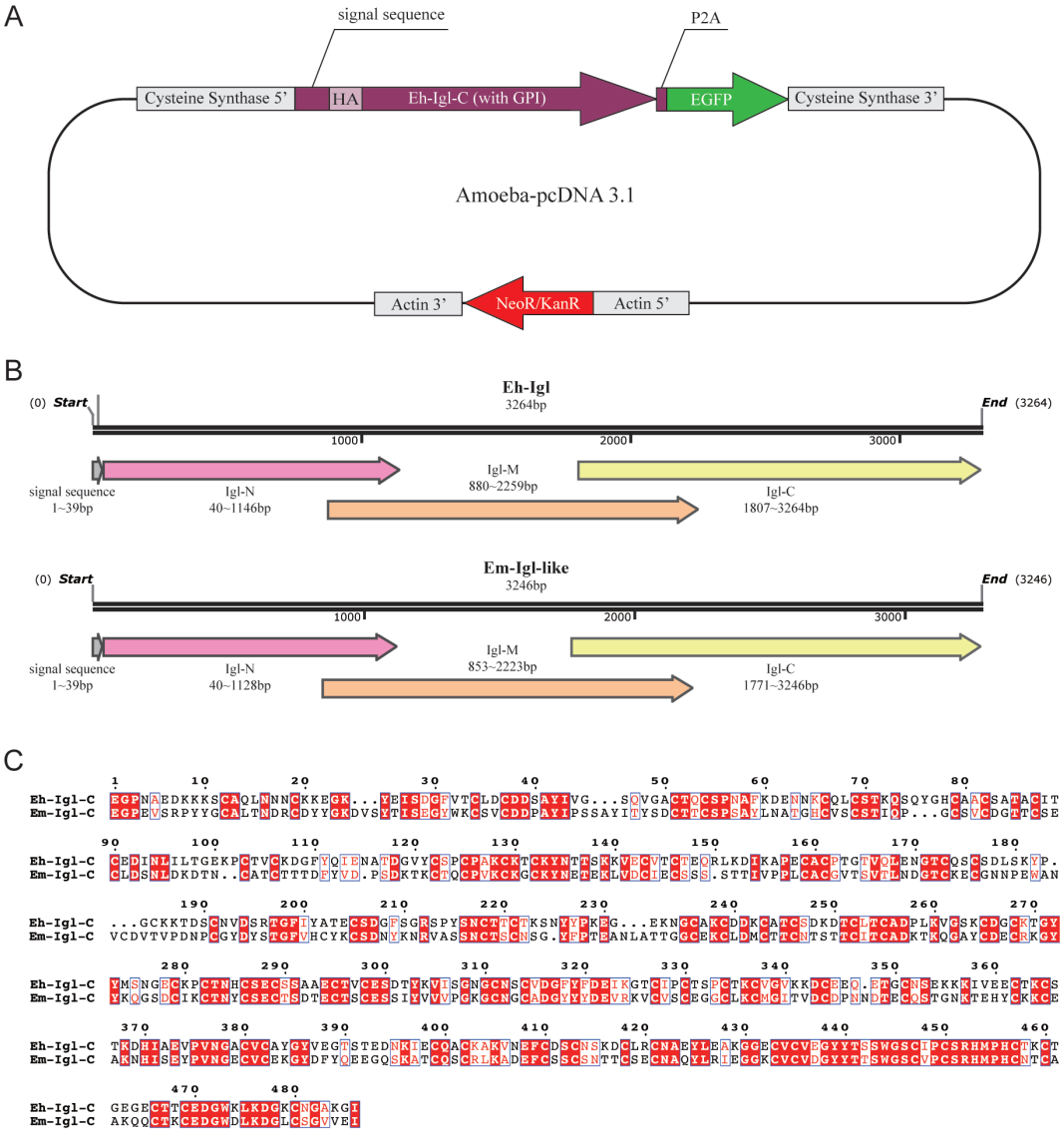

Figure 1. Construction of Amoeba-pcDNA3.1 vectors. (A) Schematic diagram of a recombinant vector. The actin gene promoter and cysteine synthase gene terminator sequences of *E. histolytica* were used to replace the original promoter of the NeoR resistance region and the original terminator of the recombinant protein expression region on the vector, respectively. Igl signal peptide gene, Kozak-Signal-HA, and P2A-EGFP sequences were replaced in the 5' to 3' regions on the vector, and then the *E. histolytica* Igl-C gene sequence was selectively inserted between the HA and P2A fragments. **(B)** *E. moshkovskii* Igl division in accordance with the segmentation study of *E. histolytica* Igls. **(C)** Amino acid sequence alignment between Igl-C proteins of *E. histolytica* and *E. moshkovskii*.

was about 30% higher than that by *E. moshkovskii* (Figures S1A and S1B, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=201*), indicating the latter had a relatively low level of pathogenicity. The *E. histolytica* Igl-C gene sequence was transfected with Amoeba-pcDNA3.1, in which HA was a fusion expression tag. After G418 screening, more stable transfection strains of the Igl-C vector group and empty vector group were selected for downstream experiments.

3.2. Validation of *E. histolytica* Igl-C's transcription and translation

First, qRT-PCR was used to confirm gene transcription in the recombinant vectors (Figures 2A to 2C). High levels of expression of the *Igl-C*, *EGFP*, and *NeoR* genes were identified in the Igl-C vector group, while high levels of expression of *EGFP* and *NeoR* genes were observed in the empty vector group in comparison to the untransfected controls. *Igl-C* was only highly expressed in the Igl-C vector group, indicating successful transfection. Levels of *EGFP* and *NeoR* expression in the empty vector group were higher than those in the Igl-C vector group, suggesting the influence of *Igl-C* overexpression. In summary, qRT-PCR indicated significant expression of vector genes after transfection. For verification at the translation level, *E. moshkovskii* was further blotted on a nitrocellulose membrane at a concentration of about 5×10^4 trophozoites per dot. In dot blotting experiments, trophozoites in the Igl-C vector group were reactive to both HA and EH3077 antibodies, and trophozoites in the empty vector group were only reactive to HA antibody, indicating different expression of Igl-C and HA proteins (Figures 2D and 2E). The results were consistent with those in qRT-PCR experiments.

The fluorescence intensity of EGFP in transfected *E. moshkovskii* trophozoites was determined using a confocal microscope (Figure 2F). EGFP protein expression in both the Igl-C vector group and empty vector group was observed 72 h after transfection. The trophozoite with weak fluorescence intensity was an untransfected control. In an immunofluorescence assay, trophozoites in the Igl-C vector group were reactive to

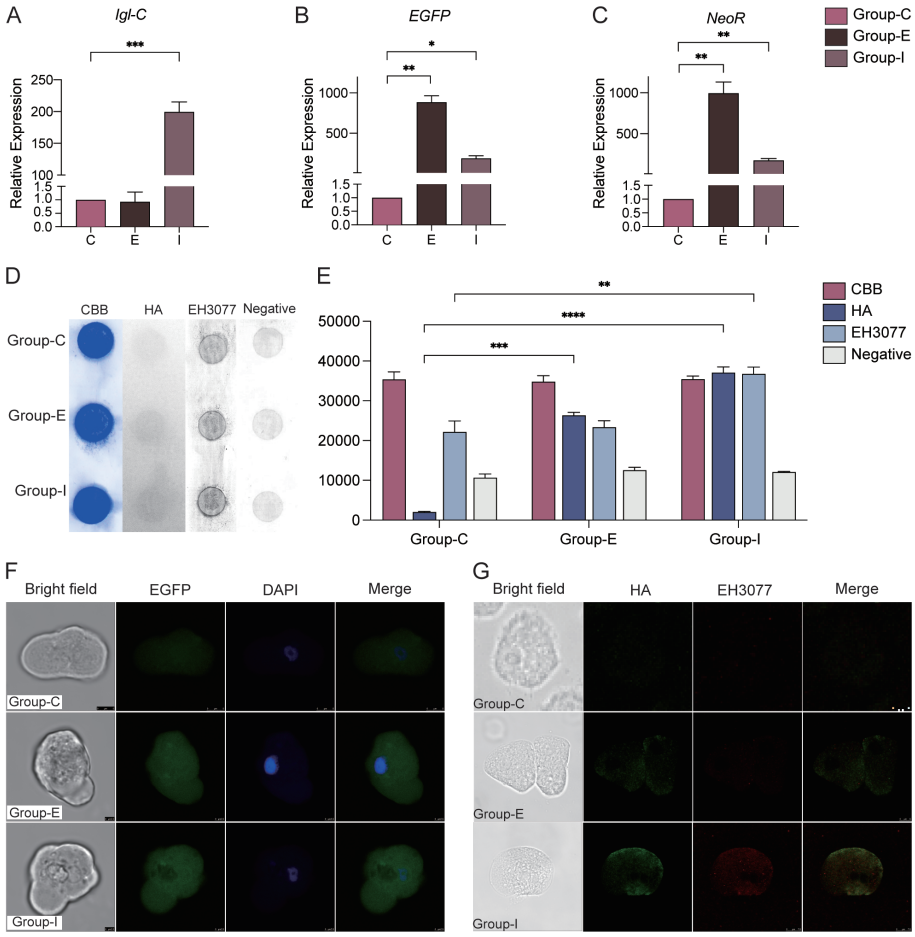

Figure 2. Verification of target gene expression after transfection. Transcription of target genes *Igl-C* **(A)**, *EGFP* **(B)**, and resistance gene *NeoR* **(C)** in the untransfected control group (Group-C), empty vector group (Group-E), and Igl-C vector group (Group-I) was detected using qRT-PCR. Protein expression in the three groups was verified with dot blotting **(D)**, while the gray values were analyzed using ImageJ to draw a histogram **(E)**. **(F)** Detection of EGFP protein expression under a confocal microscope, with the chromosome stained with DAPI. **(G)** The position of the target protein in trophozoites was verified with immunofluorescence assays. Trophozoites were stained with anti-HA antibody (green) and EH3077 monoclonal antibody (red). ****$P < 0.001$, ***$P < 0.005$, **$P < 0.01$, *$P < 0.05$.

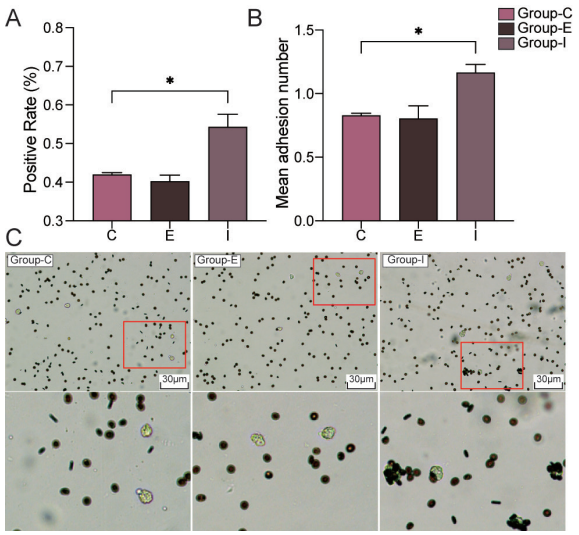

Figure 3. Amoebic adherence assay. The rate of adherence was determined by examining how many of 300 trophozoites had adhered to at least three erythrocytes. The positive rate of adhesion **(A)** and the average number of adhering erythrocytes per trophozoite **(B)** in the untransfected control group (Group-C), empty vector group (Group-E), and Igl-C vector group (Group-I) were determined. **(C)** Images of amoebic adhesion in the three groups observed under a microscope. The red box in the second row is an enlarged view. *$P < 0.05$.

both HA and EH3077 antibodies, while trophozoites in the empty vector group were only reactive to HA antibody (Figure 2G). These results revealed increased expression of the *Igl-C*, *EGFP*, *NeoR*, and *HA* genes 72 h after transfection, indicating the initial success of using the Amoeba-pcDNA3.1 vector in *Entamoeba* species. Transfection of the recombinant vector could be an effective method for inducing Igl-C overexpression in cultured *E. moshkovskii*.

3.3. Amoebic adherence and phagocytosis

Transfected *E. moshkovskii* has been confirmed to have the ability to express Igl-C on its cell membrane, but whether the protein can function normally still needs to be investigated further. Here, adherence and phagocytosis assays were used to evaluate the changes in adhesion of *E. moshkovskii* trophozoites after Igl-C transfection. After incubation with erythrocytes, the rate of adhesion (Figure 3A) and the average number of trophozoites adhering to erythrocytes (Figure 3B) in the Igl-C vector group both increased significantly in comparison to the untransfected controls. The rate of adherence was determined by examining how many of 300 trophozoites had adhered to at least three erythrocytes, indicating that *E. histolytica* Igl-C significantly enhanced the binding of *E. moshkovskii* trophozoites to erythrocytes (Figure 3C).

The ability of transfected *E. moshkovskii* trophozoites to phagocytose Jurkat Clone E6-1 cells and erythrocytes was also evaluated. The rate of erythrocyte ingestion by trophozoites in the Igl-C vector group was more than 88%, while that of trophozoites in the empty vector group and the untransfected control group was about 84%, indicating a significant difference in phagocytosis (Figures 4A to 4D). After labeling *E. moshkovskii* trophozoites with CFSE and Jurkat cells with DiD, phagocytosis was assessed in the same manner (Figures 4E to 4H). After Igl-C transfection, the proportion of trophozoites phagocytosing Jurkat cells increased to 31%, and that proportion in the untransfected controls was only 14%. These results indicated that the ability of *E. moshkovskii* to adhere to and phagocytose cells increased after *E. histolytica* Igl-C transfection. Igl, as an important surface lectin of *E. histolytica*, mediates the adhesion of *Entamoeba* trophozoites.

3.4. Effects of amoebic virulence

E. histolytica Igl is a highly immunogenic and virulent protein, so the virulence of transfected *E. moshkovskii* trophozoites on host cells was finally verified. In the *E. moshkovskii* (taxid: 41668) whole-genome shotgun contigs database, an Igl-like nucleic acid sequence (*E. moshkovskii* Igl) was obtained with NCBI "tblastn," and it was 3,264 bp in length and had a 39% similarity to the *Igl-1* gene of *E. histolytica*. According to the segmentation study of *E. histolytica* Igls, *E. moshkovskii* Igl was also divided into N, M, and C segments (Figures 1B and 1C). Both *E. moshkovskii* Igl-C and *E. histolytica* Igl-C were successfully expressed, with SDS-PAGE indicated that they could be used in subsequent experiments (Figure S2A, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=201*).

RAW264.7 cells were first stimulated with prokaryotic *E. moshkovskii* Igl-C and *E. histolytica* Igl-C, respectively, and the transcriptomic data suggested that the protein virulence of these two species differed substantially (Figure S2B, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=201*). Volcano plots revealed that approximately 2,000 genes were up- or down-regulated in the *E. histolytica* Igl-C stimulation group, while fewer than 500 genes were altered in the *E. moshkovskii* Igl-C stimulation group (Figures S2C to S2H, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=201*). This finding indicated that *E. histolytica* Igl-C stimulated host cells more potently, inducing more changes in gene expression. Interestingly, *E. moshkovskii* trophozoites transfected with *E. histolytica* Igl-C induced a more potent inflammatory response in RAW264.7 cells (Figure 5A). In the transcriptomic data, related genes labeled as Amoebiasis in the KEGG-Pathway database were further screened, and differences in the levels of expression in different groups were analyzed. At 12 h and 24 h, 27 and 26 genes in the *E. histolytica* Igl-C stimulation group changed significantly (Figures 5B and 5C), respectively, while only 4 and 5 genes changed significantly in the *E. moshkovskii* Igl-C stimulation group (Figures 5D and 5E). In comparison, the most obvious changes were in inflammatory genes, such as *Tnf*, *Il1b*, *Il6*, and *Nos2*, and their changes were consistent with those in the cells

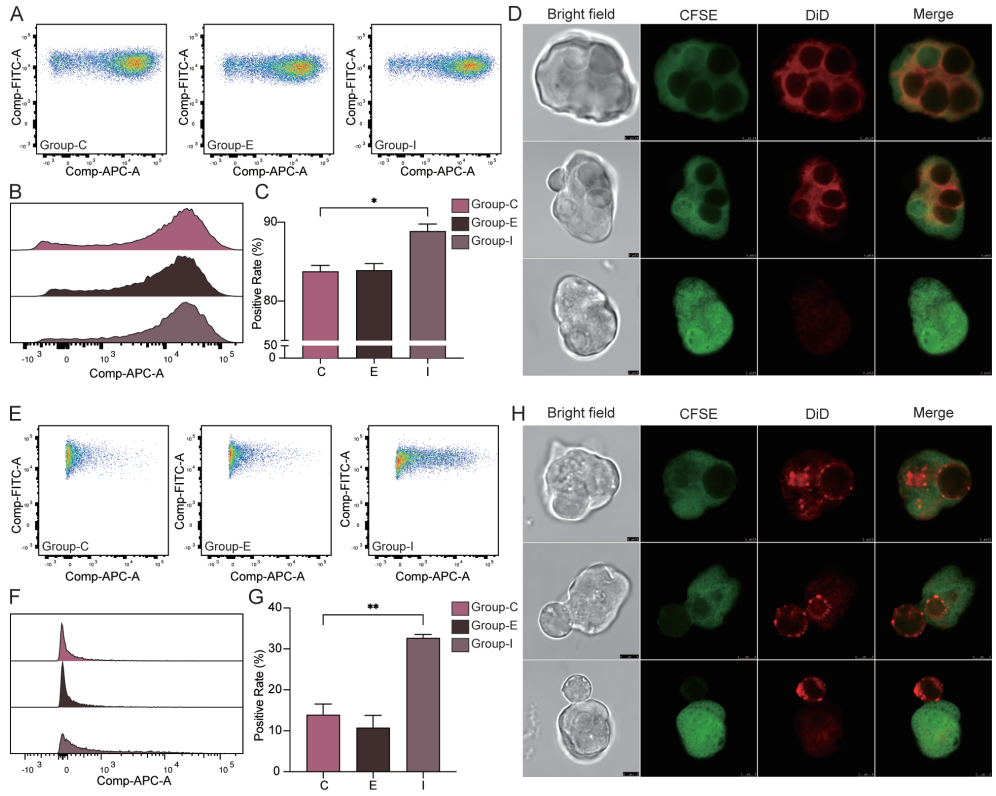

Figure 4. Amoebic phagocytosis assay. (A-D) Assay of erythrocyte phagocytosis. **(A)** Panels obtained from flow cytometry of the untransfected control group (Group-C), empty vector group (Group-E), and Igl-C vector group (Group-I), with the horizontal axis representing a red fluorescence channel and the vertical axis representing a green fluorescence channel. Red fluorescence frequency plots **(B)** and their statistical graphs **(C)** of erythrocyte phagocytosis in the three groups. Of the FITC+ trophozoites, the proportion of those that were APC+ (due to phagocytosis) was calculated. **(D)** Images of erythrocyte phagocytosis taken under a confocal microscope. Trophozoites were stained with CFSE (green) and erythrocytes were stained with DiD (red). **(E-H)** Jurkat cell phagocytosis assay. **(E)** Panels obtained from flow cytometry of the three groups. Red fluorescence frequency plots **(F)** and their statistical graphs **(G)** of Jurkat cell phagocytosis in the three groups. **(H)** Images of Jurkat cell phagocytosis taken under a confocal microscope. $^{**}P < 0.01$, $^{*}P < 0.05$.

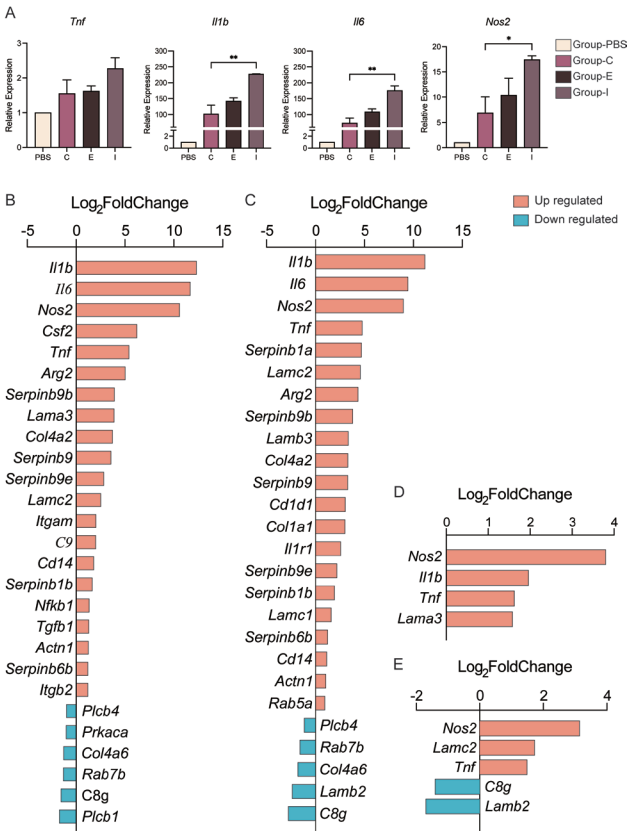

Figure 5. Comparison of the virulence of transfected trophozoites. (A) Expression of *Tnf*, *Il1b*, *Il6*, and *Nos2* in RAW264.7 cells detected with qRT-PCR after co-incubation with transfected *E. moshkovskii*. **(B-E)** Changes in expression of amebiasis-related genes in the KEGG database among transcriptomic data on RAW264.7 cells stimulated by two *Entamoeba* Igl-C proteins. The group added PBS was used as a blank control. Results of stimulation at 12 h **(B)** or 24 h **(C)** with *E. histolytica* Igl-C and stimulation at 12 h **(D)** or 24 h **(E)** with *E. moshkovskii* Igl-C are shown, respectively. $^{**}P < 0.01$, $^{*}P < 0.05$.

stimulated by transfected *E. moshkovskii* trophozoites. The results demonstrated that *E. moshkovskii* could be effectively used as a potential model organism.

4. Discussion

As a pathogenic species, *E. histolytica* pathogenic proteins had significantly more activity, variety, and quantities than those of other non-pathogenic *Entamoeba* proteins. Classic pathogenic proteins of *E. histolytica*, such as surface Gal/GalNAc lectins, cysteine proteases, and amoebapores, all have several to dozens of subtypes, with their functions frequently overlapping each other (*28-30*). When gene editing was performed in this species using conventional methods, convincing results were usually seldom obtained due to low silencing efficiency and the presence of other protein subtypes with similar functions (*20-22*). Thus, expressing the proteins in another type of cell to study their functions is a promising option (*31-34*). Our laboratory used to successfully express *E. histolytica* Igl fragments in mammalian cells, but the results indicated obvious differences in the survival mode and cell behavior pattern between the two species, making the detection of transfected proteins' related functions quite difficult. If there was thus a similar species whose own expression of disease-related proteins was maintained at a low level, which means low background, low interference, and similar behavioral patterns, it could probably serve as an excellent model organism. Among *Entamoeba* spp., *E. nuttalli*, and *E. dispar*, the closest relatives of *E. histolytica*, are also pathogenic or difficult to culture, making them unsuitable as alternatives. Thus, *E. moshkovskii* appears to be a practical choice as a potential pathogen.

As an amoeba in the digestive tract, the pathogenicity of *E. moshkovskii* has always been a concern. *E. moshkovskii* had been detected in epidemiological surveys in many regions, but only a few studies indicated the higher incidence of diarrhea in populations positive for it, suggesting that this species is a conditionally pathogenic parasite (*7-14*). The morphological similarities between *E. moshkovskii* and *E. histolytica* showed that differential diagnosis of individuals infected with the two parasites may be difficult in conventional clinical testing (*10*). *E. moshkovskii*'s behavior pattern is almost the same as that of *E. histolytica*, which is contact-dependent, but it has significantly lower pathogenicity. In the current study, *E. moshkovskii* trophozoites had significantly weaker adherence and phagocytosis, and its Igl protein only induced a mild inflammatory response in host macrophages. Moreover, *E. moshkovskii* does not have very strict nutritional requirements and is relatively easy to culture, so it has certain advantages in scientific research on amoebas (*6*). These characteristics all suggested that *E. moshkovskii* could become an outstanding model organism for studying *Entamoeba* spp. pathogenic proteins. By transfecting and expressing pathogenic *Entamoeba* proteins, their functions could be studied against a relatively clean biological background by detecting changes in the behavior patterns of the parasite. As a surface lectin of *E. histolytica*, Igl protein not only mediates the adhesion of amoeba itself but has potent immunogenicity and virulence (*17,19*). After transfection with *E. histolytica* Igl-C protein in the current study, the ability of *E. moshkovskii* to adhere to cells improved significantly. Due to increased adhesion, the transfected trophozoites had more opportunities to ingest Jurkat cells and erythrocytes, thereby further increasing their phagocytosis. As a manifestation of protein virulence, *E. moshkovskii* also had greater ability to induce inflammation after *E. histolytica* Igl-C transfection.

In this study, a modified vector with the actin gene promoter and cysteine synthase gene terminator of *E. histolytica* was used to transfect and express the Igl-C protein in *E. moshkovskii*. *E. moshkovskii* and *E. histolytica* are closely related, but directly using this modified vector in the former is a challenge, which means expressing heterogeneous proteins in *E. moshkovskii* through the promoters and terminators of *E. histolytica* genes. Results revealed a very high level of transcription and relatively lower levels of protein expression, indicating the availability of the vector in *E. moshkovskii*. Due to the minuscule differences between species, the transcriptional efficiency of the *E. histolytica* promoter may decrease slightly. The level of protein expression was not high, but Igl-C was successfully expressed and localized on the cell membrane of *E. moshkovskii* trophozoites, and its adhesion and virulence were also verified. As a heterogeneous protein, *E. histolytica* Igl-C is unlikely to play a synergistic or regulatory role in downstream signaling pathways of *E. moshkovskii*, so the increase in adherence was directly related to Igl-C, while the increase in phagocytosis might be a feedback regulatory effect when adhering to more cells. At present, the use of *E. moshkovskii* as a model organism to express a certain pathogenic *Entamoeba* protein only allows the study of the direct functions of the protein itself, and research on complicated mechanisms such as protein-protein interactions or protein regulations is still not feasible. In the future, we hope to study interactions among multiple proteins through cotransfection or other methods in this protein display platform, so *E. moshkovskii* may be used more widely as a model organism.

The aim of the current study was to use *E. moshkovskii*, which falls between a pathogen and non-pathogen, in basic research. Infection with the parasite is fairly common, but it is quite rarely pathogenic in humans. Utilizing its characteristics, this study attempted to demonstrate the potential of *E. moshkovskii* to serve as a model organism and a protein display platform to study the functions of pathogenic *Entamoeba* proteins. As a close relative of *E. histolytica*, *E. moshkovskii* can

replace it as the target of gene editing, allowing more efficient study of *E. histolytica*'s pathogenic mechanisms.

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