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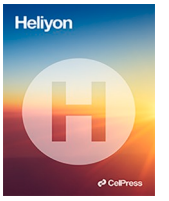

Helicobacter pylori infection contributes to the expression of Alzheimer's disease-associated risk factors and neuroinflammation

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ABSTRACT

Over time, mounting evidence has demonstrated extra-gastric manifestations of *Helicobacter pylori* infection. As such, a number of studies demonstrated the potential contribution of *H. pylori* infection to the incidence and progression of Alzheimer's disease (AD). Considering unanswered questions regarding the effect of *H. pylori* infection on brain activity, we sought to investigate the impact of *H. pylori* infection on the expression of AD-associated risk factors. We used two *H. pylori* clinical strains obtained from two patients with peptic ulcer and evaluated their influence on the expression level of AD-associated genes (*APP*, *ApoE2*, *ApoE4*, *ABCA7*, *BIN1*, *Clu*, *CD33*) and genes for inflammatory markers (TLR-4, IL-8, TNF-α) by RT-qPCR in human glioblastoma (U87MG) and astrocyte (1321N1) cell lines. The expression of inflammatory cytokines was further assessed by ELISA assay. The exposure of U97MG and 1321N1 cells to *H. pylori* strains resulted in a significant enhancement in the expression level of the risk allele *ApoE4*, while reducing the expression of the protective allele *ApoE2*. *H. pylori* infection remarkably increased the expression level of main AD-associated risk genes, and also pro-inflammatory cytokines. Furthermore, we noticed a substantial elevation in the mRNA expression level of transmembrane receptor TLR-4 following *H. pylori* infection. Our findings presented the potential for *H. pylori* to stimulate the expression of AD-associated risk genes and trigger neuroinflammation in the brain tissue. This, in principle, leads to the recommendation that AD patients should perhaps test for *H. pylori* infection and receive treatments upon positive detection.

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1. Introduction

Helicobacter pylori (*H. pylori*) is a Gram-negative bacterium classed as a human carcinogen that is strongly correlated with the development of gastric disorders. *H. pylori* specifically colonizes the gastric epithelium and usually persists for decades, developing a dynamic and persistent equilibrium with its human host [1]. *H. pylori* pathogenesis largely depends on the deleterious impact of virulence factors VacA (vacuolating cytotoxin A) and oncoprotein CagA (cytotoxin-associated gene A) [2]. The presence of *H. pylori* virulence factors in the gastric epithelium may stimulate various inflammatory and tumorigenic signaling pathways [3]. Most certainly, the presence of various gastrointestinal disorders is coupled with *H. pylori* infection, including chronic gastritis, peptic ulcer disease (PUD), chronic atrophic gastritis, and gastric adenocarcinoma. However, epidemiological studies further revealed the influence of *H. pylori* colonization on the pathogenicity of extra-gastrointestinal diseases including those in hematologic, cardiopulmonary, metabolic, neurologic, and dermatologic systems [4–7]. Several mechanisms have been suggested through which *H. pylori* could affect the progression of disorders localized outside the stomach, such as low-grade systemic inflammation, molecular mimicry mechanisms, and alterations in the gut microbial composition [8,9]. In this regard, neurodegenerative diseases appeared as a field of particular interest, especially Alzheimer's disease (AD), due to its notable public health burden.

AD is a progressive age-related neurodegenerative disorder characterized by cognitive dysfunction and memory decline. Being the most common cause of dementia, AD develops due to the accumulation of amyloid and neurofibrillary tangles in the brain and within neurons, respectively [10]. Major risk factors for the development and progression of AD include the presence of risk loci, apolipoprotein E (ApoE) polymorphic alleles, and neuroinflammation [11–13]. Considering *H. pylori*-AD interrelationship, this bacterium and its metabolites can reach the central nervous system (CNS) through the oral-nasal olfactory pathway, retrograde gastrointestinal tract neural pathway, infection of circulating monocytes, and disrupted blood-brain barrier [14]. However, meticulous interconnections between *H. pylori* infection and the risk of AD development remain elusive. Thus, this study aimed to investigate the effect of *H. pylori* clinical strains on the expression levels of *ApoE* polymorphic alleles (*ApoE2* and *ApoE4*), risk genes involved in neurodegenerative disorders, pro-inflammatory cytokines (IL-8/interleukin-8 and TNF-α/tumor necrosis factor-α), and inflammatory receptor (TLR-4/toll-like receptor-4), presenting the potential for *H. pylori* to induce neurodegenerative disorders.

2. Material and methods

2.1. H. pylori strains and growth condition

Two *H. pylori* clinical strains of PUD patients including *H. pylori* HC168 ($CagA^+$/VacA s1m2/$BabA2^+$/$SabA^+$) and *H. pylori* OC824 ($CagA^+$/VacA s1m2/$BabA2^-$/$SabA^-$) were obtained from the Helicobacter Research Laboratory collection of the Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran [15]. Briefly, the strains were cultured on Brucella agar plates (Merck, Darmstadt, Germany) supplemented with 7% (v/v) horse blood, 10% fetal calf serum (FCS), Campylobacter-selective supplement (vancomycin 2.0 mg/L, polymyxin 0.05 mg/L, trimethoprim 1.0 mg/L), and amphotericin B (2.5 mg/L). The cultured plates were incubated at 37 °C under a microaerophilic atmosphere (5% O_2, 10% CO_2, and 85% N_2) in a CO_2 incubator for 3–7 days.

2.2. Cell culture

Human glioblastoma U-87MG (ATCC HTB-14) and astrocyte 1321-N1 (ATCC HTB-16) cell lines were obtained from the Tarbiat Modares University, Tehran, Iran. The U87MG and 1321N1 cells were routinely grown in high-glucose Dulbecco's Modified Eagle Medium (Gibco-Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (Gibco-Invitrogen, Carlsbad, CA), 2 mM of L-glutamine, 100 U/mL of penicillin, and 100 μg/mL of streptomycin and were incubated in a 5% CO_2 humidified atmosphere at 37 °C.

2.3. Cell viability assay

U87MG and 1321N1 cells were seeded in 96-well plates at a density of 1×10^5 cells/well, and treated with *H. pylori* strains at multiplicity of infection (MOI) 50 and 100, when reached ~80% confluence for 24 h. The treated groups and untreated controls were then incubated with MTT solution (Sigma Aldrich, St. Louis, MO, USA) at a concentration of 5 mg/L for 4 h and consequently dissolved in 100 μl of DMSO. The cell viability was assessed by a microplate reader (Eon, BioTek Instruments, USA) with an absorbance of 570 nm and a reference wavelength of 630 nm.

2.4. Cell culture treatment

The U87MG and 1321N1 cells were counted and seeded in 24-well tissue culture plates at a density of 1×10^5 cells/well and grown in a CO_2 incubator for 24 h. Prior to treatment, the 80–90% confluent monolayers were washed three times with PBS (pH 7.2) (Gibco-Invitrogen, Carlsbad, CA), and the media were replaced with antibiotic- and serum-free complete DMEM overnight. Then, the cells were infected for 3 h, 6 h, 12 h, and 24 h at 37 °C with an MOI of 100 for each *H. pylori* strain. The untreated cells were harvested as the control group. The experiments were performed in duplicate and repeated at least three times.

2.5. ELISA measurement for pro-inflammatory cytokines

Cell culture supernatants were collected from *H. pylori*-treated and untreated control cells and subsequently centrifuged at 4 °C and 1000×*g* for 10 min. The concentration of pro-inflammatory cytokines IL-8 and TNF-α were assessed using enzyme-linked immunosorbent assay (ELISA) kits (Thermo Scientific, MA, USA) according to the manufacturer's instructions. A nonlinear regression model was used to determine the concentration of each cytokine with GraphPad Prism software version 8 (Inc., San Diego, CA, USA).

2.6. RNA extraction and cDNA synthesis

Total RNA from 1321N1 and U87MG cells was extracted using RNeasy Plus Mini Kit (Qiagen, GmbH, Germany) following the manufacturer's instructions. RNA concentration and purity were assessed using a NanoDrop spectrophotometer (ND-1000, Thermo Scientific, MA, USA) by the A260/280 ratio, and ribosomal RNA (rRNA) was assessed by electrophoresis. The RNA samples were frozen at −80 °C until used for cDNA synthesis. The RNA was reverse-transcribed to cDNA using the PrimeScript™ RT Reagent Kit (Takara, Kyoto, Japan) according to the manufacturer's protocol. All cDNA preparations were frozen at −20 °C until further use.

2.7. Quantitative real-time PCR (RT-qPCR)

The RT-qPCR was performed by the Rotor-Gene® Q real-time PCR system (Qiagen, GmbH, Germany) using BioFACT™ 2X Real-Time PCR Master Mix (BIOFACT CO., Ltd. Daejeon, South Korea). The oligonucleotide sequences used for gene expression analysis are listed in Table 1. To confirm amplification specificity, a melting analysis and subsequent agarose gel electrophoresis were performed after each run. All reactions were run in triplicate and the results of fold change in mRNA expression were given relative to the control samples using the comparative Ct formula "$2^{-\Delta\Delta CT}$", and the RNA input was normalized against the housekeeping gene *β-actin* [16].

2.8. Statistical analysis

Statistical analysis was carried out with GraphPad Prism software version 8 (Inc., San Diego, CA, USA). One-way analysis of variance (ANOVA) and Tukey's post hoc test were used to determine the statistical significance between the groups. The data were presented as the averages of at least three independent experiments; error bars represent the standard deviations (SD). Differences were considered statistically significant when $P < 0.05$; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$.

3. Results

3.1. Cell viability

For evaluating the toxicity of *H. pylori* strains on U87MG and 1321N1 cells, the MTT assay was performed to measure the viability of U87MG and 1321N1 cells treated with *H. pylori* (MOI 50 and 100) bacteria. MTT results presented a non-significant reduction in the

Table 1
Specific primers used for RT-qPCR.

Primer	Primer sequence (5′–3′)	Size of amplicon (bp)	Reference
APP	F: GCCATCATCGGACTCATGGT R: ATCCGTTCTGCTGCATCTTG	169	[29]
ApoE2	F: CGGACATGGAGGACGTGT R: CTGGTACACTGCCAGGCA	173	[49]
ApoE4	F: CGGACATGGAGGACGTGC R: CTGGTACACTGCCAGGCG	173	[49]
Bin1	F: CCTGCTGTGGATGGATTACC R: GCTTTCTCAAGCAGCGAGAC	219	[50]
ABCA7	F: TCCTTTGGAACAGCCTTTTG R: CTGCCCTTGAGATGTTGC	157	[51]
CD33	F: GATCTTCTCCTGGTTGTCAG R: CTGTGGAACATAGGTGACGTTG	184	This study
Clu	F: ACATTTGGTGCCCAGAAGTC R: CTGTGGTCCAGGGAAAGGTA	190	[52]
TLR4	F: CGAGGAAGAGAAGACACCAGT R: CATCATCCTCACTGCTTCTGT	106	[53]
TNF-α	F: CCCAGGGACCTCTCTCTAATC R: ATGGGCTACAGGCTTGTCACT	84	[54]
IL-8	F: CTCTTGGCAGCCTTCCTGATT R: ACTCTCAATCACTCTCAGTTCT	147	[54]
β-actin	F: CTGGAACGGTGAAGGTGACA R: AAGGGACTTCCTGTAACAATGCA	140	[55]

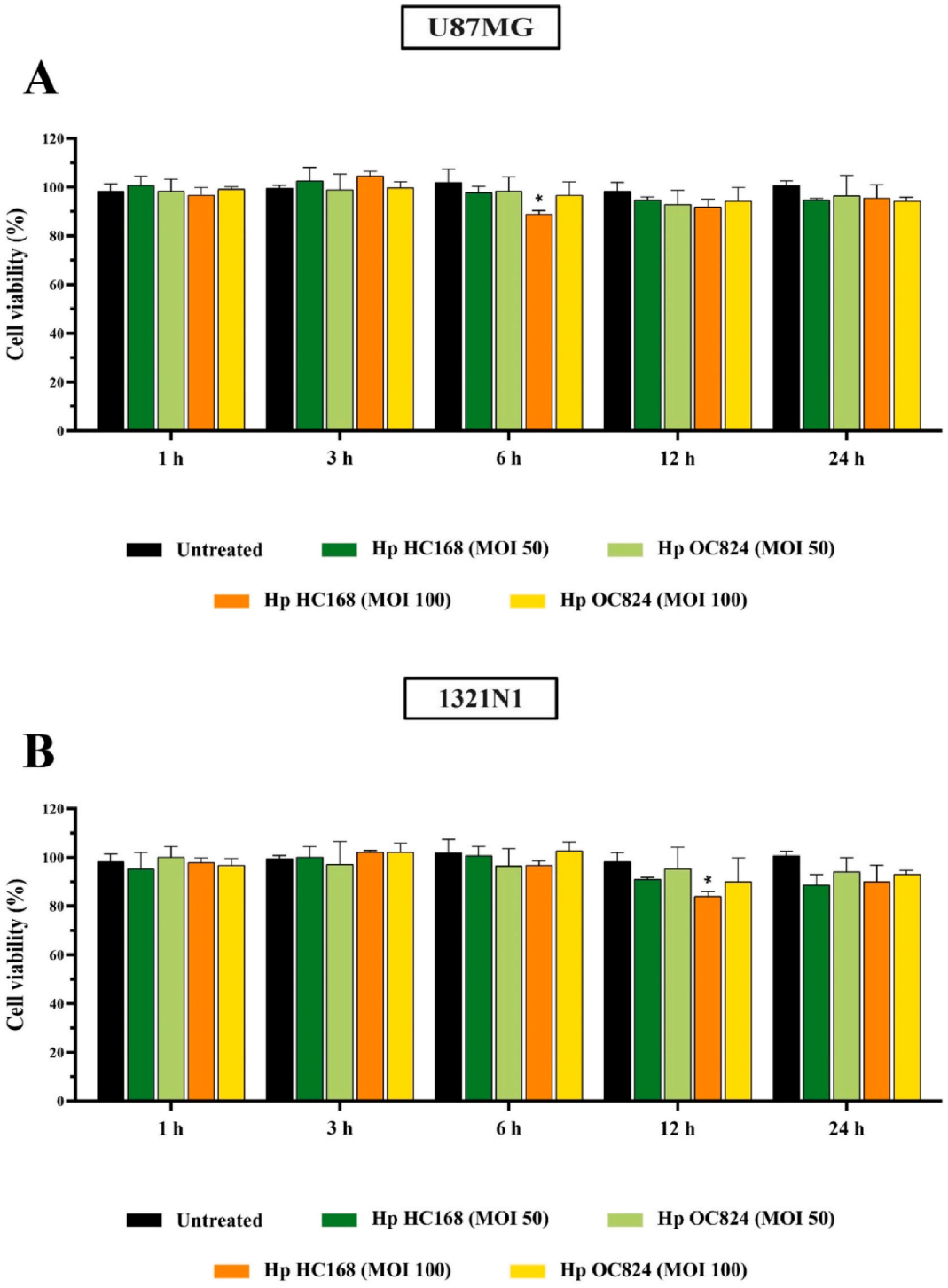

Fig. 1. Cell viability was determined using MTT assay for (A) U87MG and (B) 1321N1 cell lines treated with *H. pylori* (MOI 50 and 100) for 24 h (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$).

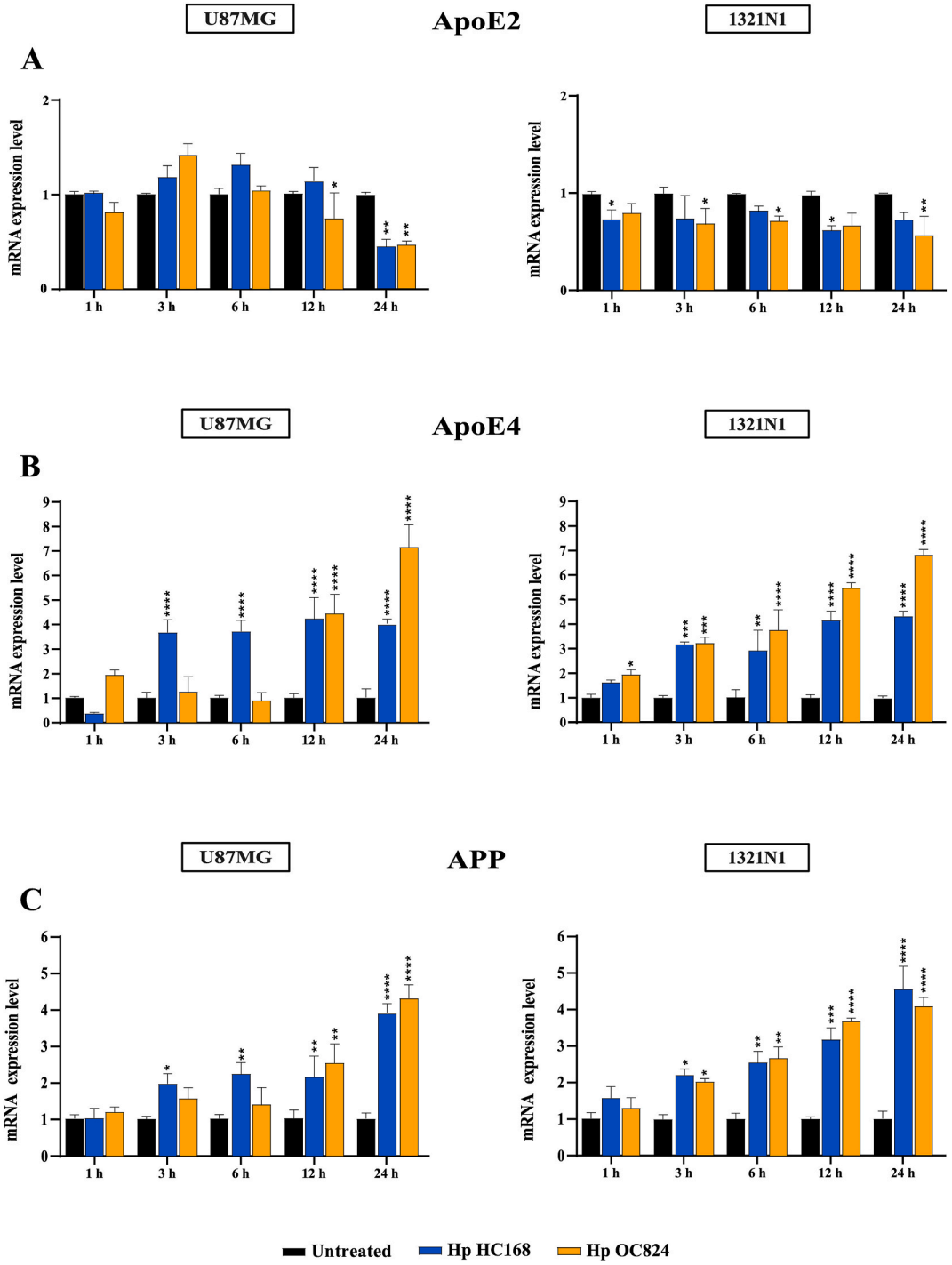

Fig. 2. The mRNA expression level of (A) ApoE2 and (B) ApoE4 in U87MG and 1321N1 cell lines upon exposure to *H. pylori* (Hp) HC168 and Hp OC824 strains at 1 h, 3 h, 6 h, 12 h, and 24 h (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$).

viability of both cell lines particularly after 24 h of infection, compared to the untreated control (Fig. 1A and B). Considering the cell viability results, *H. pylori* at MOI 100 was selected for further cell culture experiments.

3.2. Effects of H. pylori on the gene expression level of ApoE and APP in brain cells

To evaluate the effects of *H. pylori* on the gene expression level of the apolipoprotein isoforms *ApoE2* and *ApoE4*, U87MG and

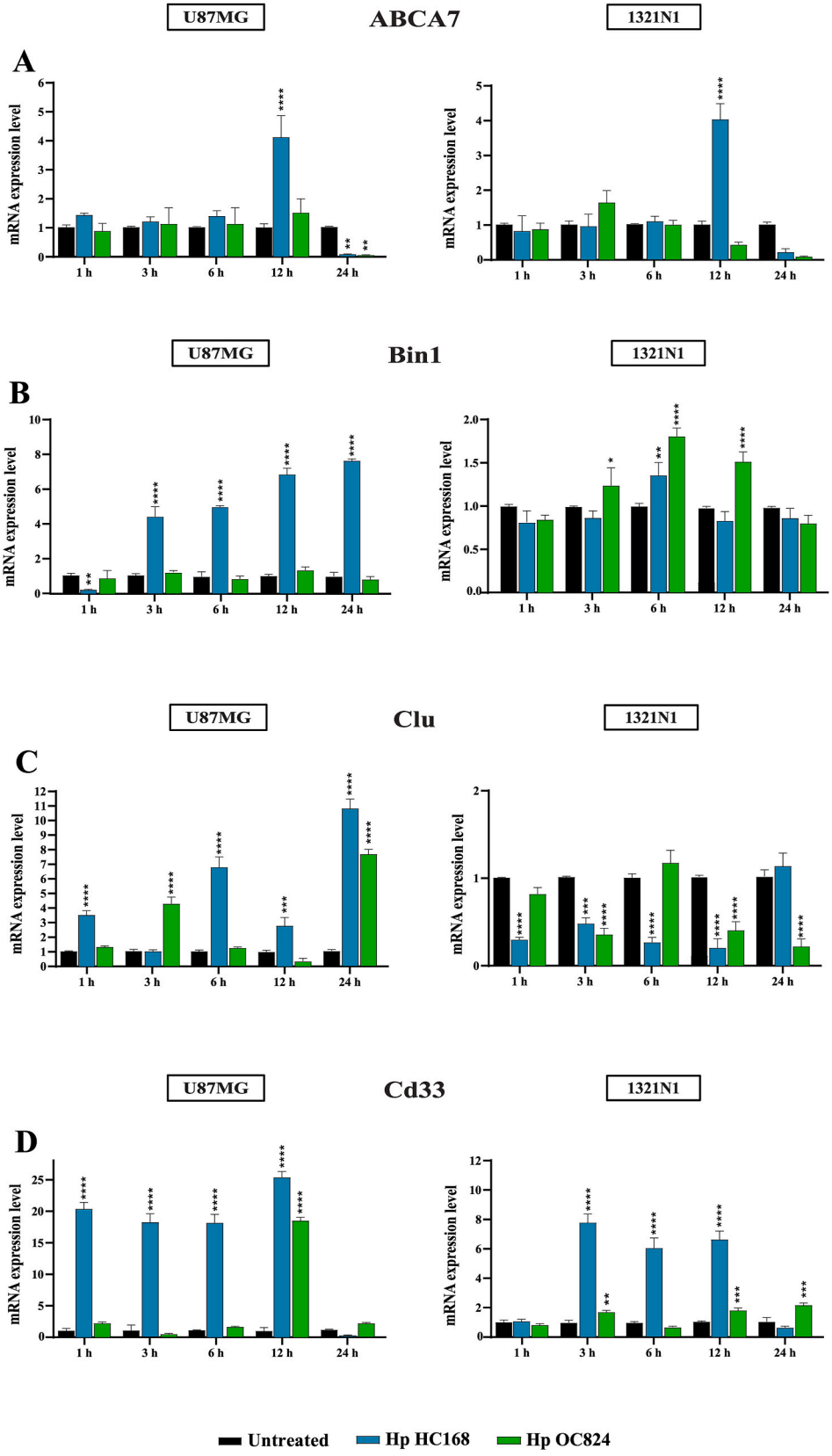

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Fig. 3. The mRNA expression level of (A) ABCA7, (B) Bin1, (C) Clu, and (D) Cd33 in U87MG and 1321N1 cell lines upon exposure to *H. pylori* (Hp) HC168 and Hp OC824 strains at 1 h, 3 h, 6 h, 12 h, and 24 h (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$).

1321N1 cells were infected with *H. pylori* bacteria for different time points. In U87MG cells, *ApoE2* expression was reduced by *H. pylori* HC168 after 24 h ($P < 0.01$) and by *H. pylori* OC824 after 12 h ($P < 0.05$) and 24 h ($P < 0.01$). The exposure of 1321N1 cells to *H. pylori* HC168, however, demonstrated a significant reduction in the expression level of *ApoE2* isoform after 1 h ($P < 0.05$) and 12 h ($P < 0.05$) of infection while *H. pylori* OC824 substantially reduced *ApoE2* expression after 3 h ($P < 0.05$), 6 h ($P < 0.05$), and 24 h ($P < 0.01$) (Fig. 2A). On the contrary, *H. pylori* infection significantly increased *ApoE4* gene expression in both the U87MG cells and 1321N1 cells (Fig. 2B). Furthermore, our results presented a remarkable time-dependent overexpression of *APP* gene in both cell lines after *H. pylori* infection (Fig. 2C).

3.3. Effects of H. pylori on the expression level of genes related to AD in brain cells

In this study, the effects of *H. pylori* HC168 and OC824 on gene expression levels of ATP binding cassette subfamily A member 7 (*ABCA7*), bridging integrator 1 (*BIN1*), clusterin (*Clu*), and *CD33* were investigated in U87MG and 1321N1 brain cells during different time points of infection. As shown in Fig. 3A, a significant increase was found for *ABCA7* gene expression level in both U87MG ($P < 0.0001$) and 1321N1 ($P < 0.0001$) cells infected with *H. pylori* HC168 after 12 h ($P < 0.0001$). However, a notable reduction in the expression level of the *ABCA7* gene was observed for *H. pylori* HC168 and OC824 in both U87MG ($P < 0.0001$) and 1321N1 ($P < 0.0001$) cells after 24 h of infection, in comparison with untreated cells.

We found a notable time-dependent enhancement in the gene expression level of *BIN1* in U87MG cells treated with *H. pylori* HC168. Except for the remarkable reduction at 1 h ($P < 0.01$), this strain could significantly increase *BIN1* expression in U87MG cells and only after 6 h ($P < 0.01$) of infection in 1321N1 cell, compared with the control group. Whilst, *H. pylori* OC824 could induce a significant upregulation in the expression level of the *BIN1* gene in 1321N1 cells after 3 h ($P < 0.05$), 6 h ($P < 0.0001$), and 12 h ($P < 0.0001$) of infection (Fig. 3B).

As depicted in Fig. 3C, *H. pylori* HC168 could markedly increase the expression level of the *Clu* gene in U87MG cells, whereas significantly reduced its expression in 1321N1 cells. Moreover, the *Clu* gene expression was substantially induced by *H. pylori* OC824 infection in U87MG cells only after 3 h ($P < 0.0001$) and 24 h ($P < 0.0001$) of infection, whilst *H. pylori* OC824 significantly reduced *Clu* gene expression after 3 h ($P < 0.0001$), 12 h ($P < 0.0001$), and 24 h ($P < 0.0001$) of infection in 1321N1 cells.

As shown in Fig. 3D, the gene expression level of *CD33* was markedly upregulated in U87MG cells after 1 h ($P < 0.0001$), 3 h ($P < 0.0001$), 6 h ($P < 0.0001$), and 12 h ($P < 0.0001$) and also in 1321N1 cells after 3 h ($P < 0.0001$), 6 h ($P < 0.0001$), and 12 h ($P < 0.0001$) of infection with *H. pylori* HC168, in comparison with control cells. *H. pylori* OC824 could induce the gene expression level of *CD33* in U87MG cells only at 12 h ($P < 0.0001$) and in 1321N1 cells at 3 h ($P < 0.01$), 12 h ($P < 0.001$), and 24 h ($P < 0.001$), in comparison with untreated control cells.

3.4. H. pylori induced TLR-4 gene expression in brain cells

We sought to examine whether *H. pylori* strains could upregulate the TLR-4 gene expression in U87MG and 1321N1 cells. As shown in Fig. 4, both *H. pylori* strains increased the expression levels of TLR-4 in infected cells when compared to the untreated cells. *H. pylori* HC168 significantly increased TLR-4 gene expression in U87MG cells at 3 h ($P < 0.0001$), 6 h ($P < 0.05$), 12 h ($P < 0.05$), and 24 h ($P < 0.0001$) and in 1321N1 cells at 3 h ($P < 0.0001$), 12 h ($P < 0.01$), and 24 h ($P < 0.01$), compared to the untreated control. Considering

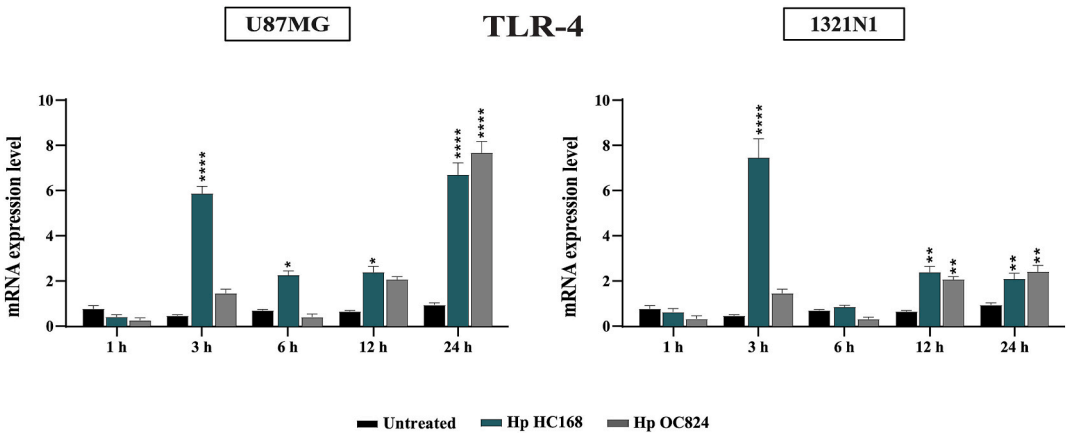

Fig. 4. The mRNA expression level of TLR-4 in U87MG and 1321N1 cell lines upon exposure to *H. pylori* (Hp) HC168 and Hp OC824 strains at 1 h, 3 h, 6 h, 12 h, and 24 h (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$).

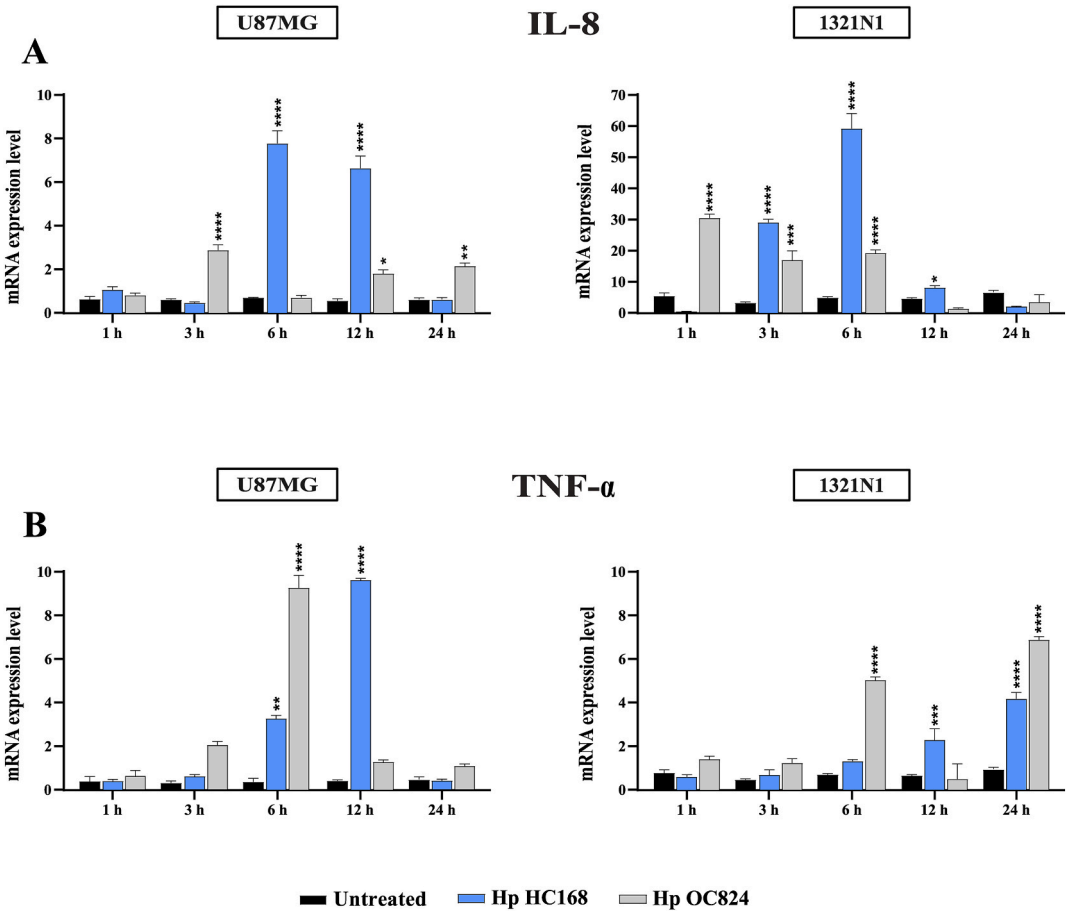

Fig. 5. The mRNA expression level of (A) IL-8 and (B) TNF-α in U87MG and 1321N1 cell lines upon exposure to *H. pylori* (Hp) HC168 and Hp OC824 strains at 1 h, 3 h, 6 h, 12 h, and 24 h (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$).

H. pylori OC824, however, TLR-4 expression level was substantially upregulated after 24 h ($P < 0.0001$) of infection in U87MG cells and following 12 h ($P < 0.01$) and 24 h ($P < 0.001$) of infection in 1321N1 cells.

3.5. H. pylori upregulated the expression and production of IL-8 and TNF-α in brain cells

We examined the effect of *H. pylori* on the gene expression level of pro-inflammatory cytokines in U87MG and 1321N1 cells. As expected, both *H. pylori* strains enhanced the expression of pro-inflammatory cytokines IL-8 and TNF-α (Fig. 5). *H. pylori* HC168 significantly induced IL-8 expression at 6 h ($P < 0.0001$) and 12 h ($P < 0.0001$) in U87MG cells and also at 3 h ($P < 0.0001$), 6 h ($P < 0.0001$), and 12 h ($P < 0.05$) in 1321N1 cells. Whilst, *H. pylori* OC824 notably increased the mRNA expression level of IL-8 at 3 h ($P < 0.0001$), 12 h ($P < 0.05$), and 24 h ($P < 0.01$) in U87MG cells and also at 1 h ($P < 0.0001$), 3 h ($P < 0.001$), and 6 h ($P < 0.001$) in 1321N1 cells (Fig. 5A). The gene expression of TNF-α was increased following the treatment of U87MG cells with *H. pylori* HC168 at 6 h ($P < 0.01$) and 12 h ($P < 0.0001$) and also in 1321N1 cells at 12 h ($P < 0.001$) and 24 h ($P < 0.0001$), in comparison to control cells. Moreover, *H. pylori* OC824 significantly induced the expression of TNF-α at 6 h ($P < 0.0001$) in U87MG cells and at 6 h ($P < 0.0001$) and 24 h ($P < 0.0001$) in 1321N1 cells (Fig. 5B). Consistent with the RT-qPCR results, ELISA assay presented noticeable increase in the production of IL-8 and TNF-α pro-inflammatory cytokines after *H. pylori* treatment in both the U87MG and 1321N1 cells (Fig. 6A and B).

4. Discussion

The gut microbiota-brain axis is the biological network of bidirectional interactions between the gut microbiota and the brain, preserving homeostasis of the gastrointestinal, nervous, and microbial systems in humans. Considering the involvement of multiple biological systems, these communication networks are orchestrated through various mechanisms and pathways that signal by chemical transmitters, multi-neuronal networks, and the immune system [17]. Several cross-sectional studies have demonstrated the alteration of the gut microbiota composition in patients with neurological disorders, indicating the contribution of specific microbiota

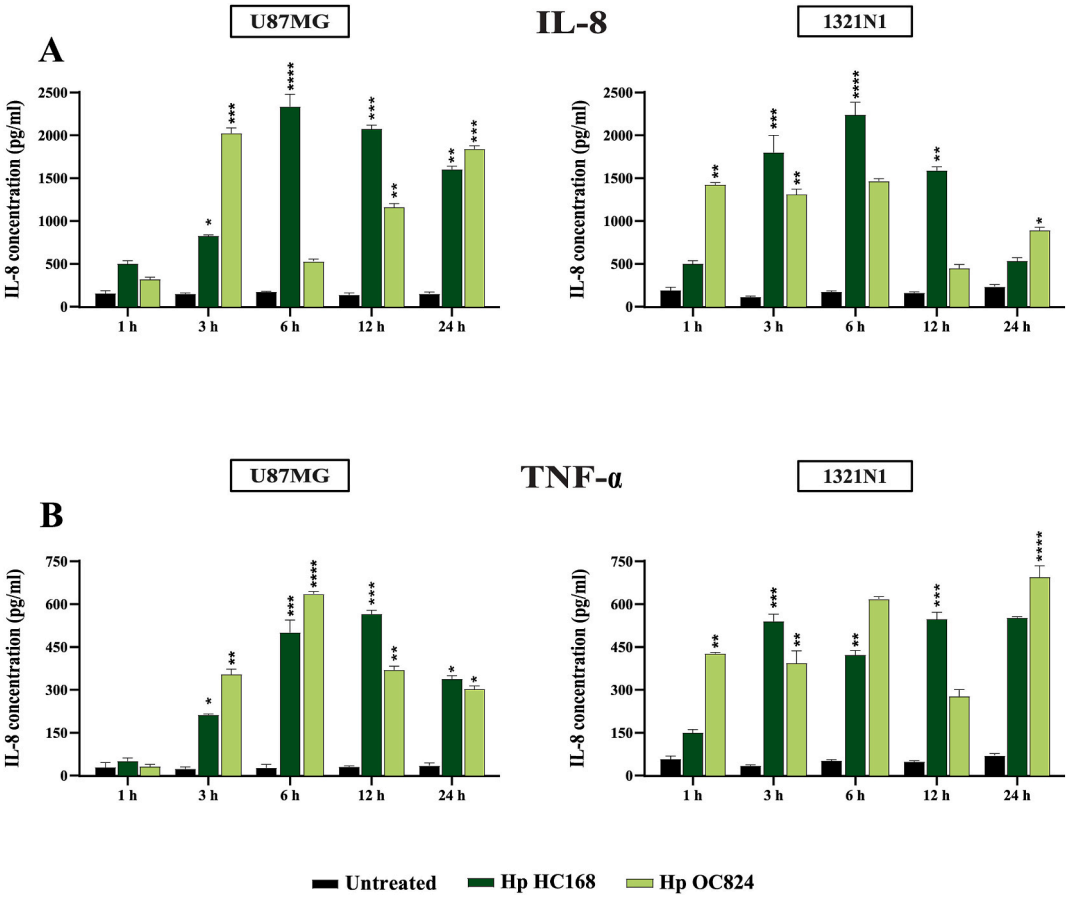

Fig. 6. The production of (A) IL-8 and (B) TNF-α in U87MG and 1321N1 cell lines upon exposure to *H. pylori* (Hp) HC168 and Hp OC824 strains at 1 h, 3 h, 6 h, 12 h, and 24 h (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$).

to brain pathology [18–20]. Accumulating evidence supports the potential influence of *H. pylori* on brain function and AD development. Clinical studies have reported significantly higher serum *anti-H. pylori* IgG and IgA titer in patients with AD or mild cognitive impairment [21–23]. Furthermore, *H. pylori* eradication improved the cognitive and functional status of AD patients and was associated with a significantly higher survival rate [24,25]. Studying activated genes and dysregulated pathways of human gastric MNK-45 cell line by a RNA-seq approach following treatment with *H. pylori* peptide Hp(2–20), Contaldi et al. [26] demonstrated alterations in the expression level of 77 genes, some of which (*APP*, *ApoE*, *PSEN1*, and *PSEN2*) contributing to AD development. Furthermore, similar dysregulated pathways during AD were also presented in the gastric cell line, emphasizing the biological possibility of the connection between *H. pylori* infection and predisposition to AD incidence. There are, however, inconsistencies about the potential influence of *H. pylori* on brain function, and different hypotheses have been presented so far (Fig. 7). It has been proposed that *H. pylori* might translocate to the brain through the oral-nasal-olfactory pathway or by circulating monocytes through the disrupted blood-brain barrier. *H. pylori* can also reach the brain tissue via the gastrointestinal retrograde neural pathways [27]. Moreover, *H. pylori* metabolites and by-products, such as outer membrane vesicles (OMVs), could cross biological barriers and ultimately access the brain [28]. To investigate the impact of *H. pylori* infection on the brain tissue, we treated U87MG and 1321N1 cell lines with two *H. pylori* strains (HC168, OC824) obtained from patients with PUD and evaluated the expression of pro-inflammatory cytokines and AD-associated risk genes.

Amyloid accumulation is a major characteristic of AD development, which is the consequence of proteolytic processing of APP transmembrane protein. As the first identified protein with a major contribution to AD, APP physiological activity has been vastly studied [29]. High expression levels of APP have been reported in AD and Down syndrome patients, which cause amyloid generation and neuritic plaque formation [30]. Therefore, we evaluated the impact of *H. pylori* infection on *APP* gene expression and demonstrated a marked time-dependent increase in its mRNA expression level in both cell lines. It could be interpreted from our results that *H. pylori* infection might contribute to the development of AD through the overexpression of APP and subsequent accumulation of amyloid in the brain.

A major cholesterol transporter in the brain, ApoE consists of three main allelic variants ε2, ε3, ε4, of which ApoE4 contributes to a higher risk while ApoE2 contributes to a lower risk of AD, compared to the common ApoE3 phenotype [31]. We noticed a significant elevation in the expression level of ApoE4 in both cell lines upon infection with either *H. pylori* strain. On the contrary, the exposure of

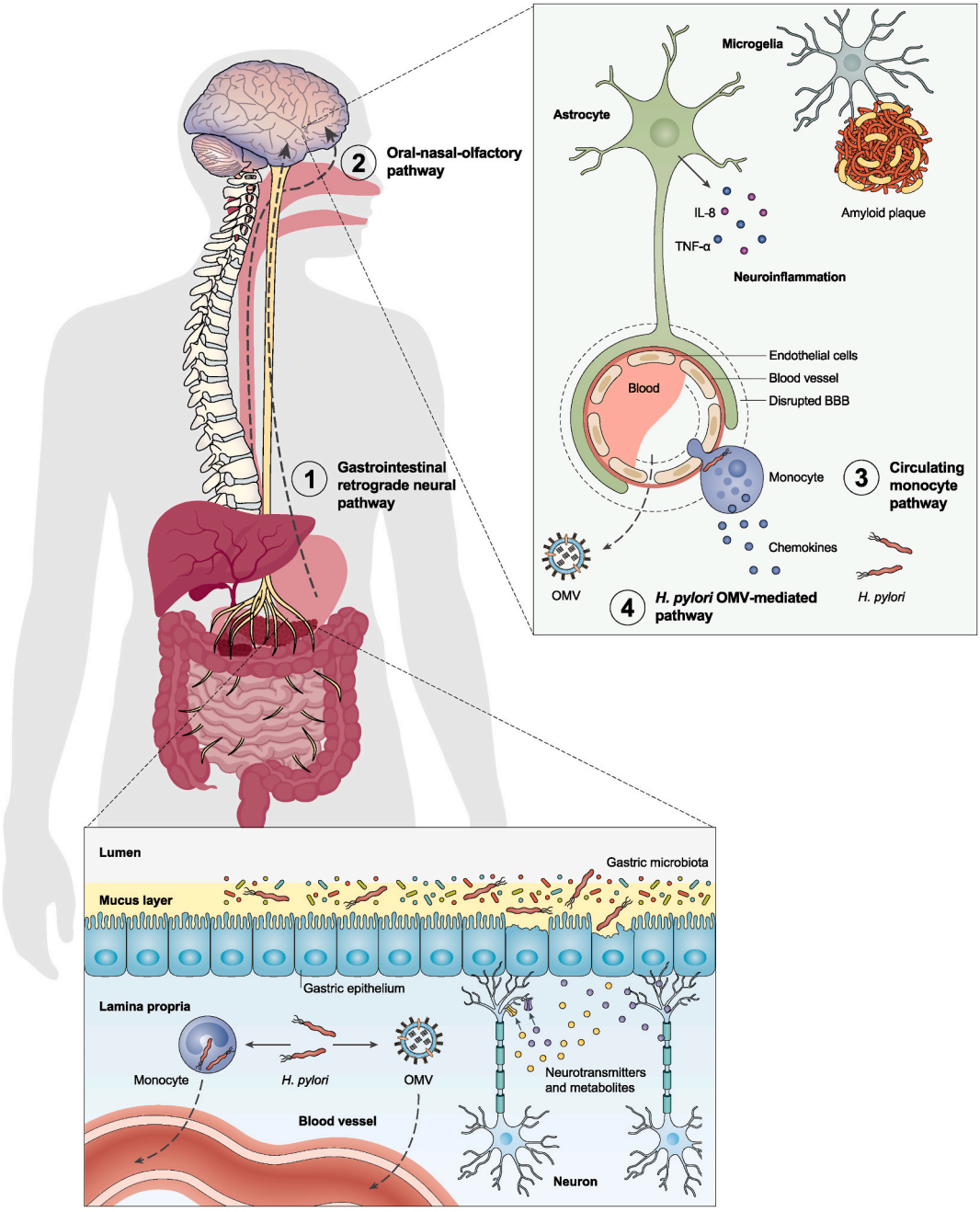

Fig. 7. Potential mechanisms through which *H. pylori* might access and affect brain function. *H. pylori* can penetrate the gastric epithelial barrier and reach the lamina propria. Thereafter, a disrupted blood-brain barrier (BBB) might allow *H. pylori* to reach the brain through (1) the gastrointestinal retrograde neural pathway, (2) the oral-nasal-olfactory pathway, or (3) circulating monocyte pathway. Furthermore, (4) *H. pylori*-derived OMVs could also cross biological barriers and eventually reach the brain (*H. pylori* OMV-mediated pathway). Subsequently, *H. pylori* induces neuroinflammation (e.g. IL-8 and TNF-α production) and increases the accumulation of amyloid plaque, ultimately leading to behavior and brain disorders.

U87MG and 1321N1 cells to *H. pylori* notably reduced *ApoE2* expression level. Although *H. pylori* strains demonstrated these substantial changes at different time points in the cell lines, *H. pylori* infection presented potential modification in the expression of AD-associated risk factor ApoE. By changing the *ApoE* expression level, *H. pylori* could affect neurodegeneration [32], microglial homeostasis [33], synaptic integrity [34], lipid transport [35], and glucose metabolism [36]. Furthermore, a recent study presented the influence of the gut microbiota and metabolome alteration on neurodegeneration in an ApoE-dependent manner in a mouse model

[37]. Therefore, *H. pylori*-mediated changes in the structure of the gut microbiome and metabolome might contribute to the pathogenicity of AD.

Following *ApoE*, *BIN1* is considered the second most important genetic susceptibility locus for AD [38]. Furthermore, genome-wide association studies (GWASs) have identified several other risk genes for AD development such as *Clu*, *ABCA7*, and *CD33* [39–41]. Except for a significant reduction in the expression level of *ABCA7* at 24 h and *BIN1* at 1 h in the U87MG cell line, as well as reduced expression of *Clu* at different time points in the 1321N1 cell line, both *H. pylori* strains presented a remarkable enhancement in the expression levels of AD-associated risk genes. Overall, we noticed a more substantial elevation in the expression of risk genes following *H. pylori* HC168 infection, compared to the *H. pylori* OC824 infection. This might be probably due to the absence of *H. pylori* adhesion proteins BabA and SabA in the *H. pylori* OC824 strain, hindering its ability to properly attach to glioblastoma and astrocyte cells [42]. The attachment of *H. pylori* to the host cell critically contributes to its pathogenicity and intracellular translocation of *H. pylori* virulence factors CagA and VacA [43]. The presence of different variants of *H. pylori* virulence factors such as variants of CagA EPIYA motifs could be another ration for distinctive pathogenicity of these strains [44]. Also, different cellular characteristics of glioblastoma and astrocyte cells including their morphological differences could be the underlying reason for their similar yet unique responses to the same *H. pylori* strain. Nevertheless, our findings demonstrated the capacity of *H. pylori* to simulate the expression of AD-associated risk genes.

Solid evidence suggests the involvement of TLR-4-mediated signaling in the pathogenesis of AD [45]. The detection of danger/damage-associated molecular patterns (DAMPs), such as high mobility group box 1 (HMGB1), or pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS), by TLR-4 can cause neuroinflammation and neuronal damage contributing to AD incidence [46]. *H. pylori* LPS, which is considered as an essential pathogenic virulence factor, is well known to induce inflammatory reactions through TLR-4 activation. Furthermore, neuroinflammation, which is an inflammatory response within the CNS featured by the excessive production of pro-inflammatory cytokines, has been demonstrated as a key risk factor for AD [13]. Therefore, we evaluated the impact of *H. pylori* infection on the expression level of TLR-4 and exhibited a substantial elevation in the expression of this receptor by both *H. pylori* strains in both cell lines. To further evaluate the potential capacity for *H. pylori* to induce neuroinflammation, we assessed the expression level of pro-inflammatory cytokines IL-8 and TNF-α. Our findings showed significant enhancement in the expression of these cytokines by both *H. pylori* strains, particularly IL-8 in the 1321N1 cell line. In this regard, *H. pylori* LPS and *H. pylori*-induced expression of HMGB1 can stimulate TLR-4 and subsequent NFKB/NF-κB signaling [47,48], eventually inducing pro-inflammatory cytokine production and neuroinflammation.

5. Conclusion

In a nutshell, we demonstrated the increased expression of AD-associated risk genes in U87MG and 1321N1 cell lines following *H. pylori* infection at different time points. Our findings further exhibited the potential capacity of *H. pylori* to stimulate the expression of pro-inflammatory cytokines in neural cells and induce neuroinflammation. Therefore, it is plausible that *H. pylori* infection predisposes AD incidence and progression, which is yet to be further validated by preclinical and clinical studies. Limitations to our study include the absence of in vivo models or human subjects to validate the results of this study. The results of the present study should also be evaluated by protein expression assays. Further mechanism-oriented studies and clinical trials are required to elucidate *H. pylori* mechanistic action of inducing AD development and progression.

Ethics statement

The study protocol conformed to the ethical guidelines of the Institutional Ethical Review Committee of Research Institute for Gastroenterology and Liver Diseases at Shahid Beheshti University of Medical Sciences, Tehran, Iran (RIGLD 1050, Project No. IR.SBMU.RIGLD.REC.1398.023).

Author contribution statement

Maryam Noori: Performed the experiments; Analyzed and interpreted the data; Wrote the paper. Ramina Mahboobi; Shaghayegh Jamshidizadeh; Farzaneh Fakharian: Performed the experiments. Ali Nabavi-Rad: Analyzed and interpreted the data; Wrote the paper. Abbas Yadegar: Conceived and designed the experiments; Analyzed and interpreted the data. Mohammad Reza Zali: Contributed reagents, materials, analysis tools or data.

Data availability statement

Data included in article/supp. Material/referenced in article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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