Effective targeting of CagA and autophagy-related Atg12 as a potential therapeutic approach for *Helicobacter Pylori* infection

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ABSTRACT

Helicopter pylori (H. Pylori) begins its long-term infection in the stomach, where the immune system responds with local neutrophil, lymphocyte, and macrophage infiltration. *H pylori* infection is known to cause stomach ulcers, but scientists are still largely in the dark about this critical area of health. We transfected HeLa cells with specific siRNA antagonist bacterial CagA gene or siRNA antagonist autophagy-related Atg12, and then *H. Pylori* reproduction was monitored. We also looked at proinflammatory cytokine secretion from treated cells to see if there was a link between bacterial CagA, host autophagy, and inflammatory events like tumor necrosis factor-alpha (TNF-) and interleukin-6 (IL-6). When compared to control-transfected cells, our findings revealed previously unnoticed changes in cell morphology, number of survived cells, and lactate dehydrogenase (LDH) production after transfection with siRNA targeting CagA or Atg12. Furthermore, targeting CagA effector and Atg12 successfully disrupts *H. pylori* reproduction in infected cells, as measured by quantitative real-time PCR of bacterial 16S ribosomal RNA (16S rRNA) (q-RT-PCR). Furthermore, CagA downregulation causes an obvious decrease in Raf-1 gene expression, which is an indicator of Megaton-Activated Protein Kinase (MAPK) signaling in infected cells. Finally, IL-6 and TNF- production was mediated in siRNA-transfected cells that attacked either CagA or Atg12 effectors. In conclusion, these findings suggest that

targeting the bacterial CagA effector and/or autophagy-related Atg12 as a potential therapeutic strategy for _H. pylori_ infection could present a novel mechanism to disrupt bacterial replication.

Keywords: _H. pylori, CagA effector, autophagy-related Atg12, TNF-α, IL-6, HeLa cell_

INTRODUCTION

Helicobacter pylori (H. pylori) strains are Gram-negative bacteria that cause an inflammatory response in the stomach and are the most common infectious cause of ulcers worldwide (Kusters et al., 2006). _H. pylori_ cause chronic inflammation (gastritis) (Vac-A) by attacking the interior layers of the stomach and releasing the bacterial cytotoxin effector known as vacuolating cytotoxin A. As a result, peptic ulcers can develop (Palframan et al., 2012). Although many _H. pylori_ patients have no symptoms, others may experience frequent belching, bloating, nausea, and vomiting. The more serious infection causes stomach pain, fatigue, diarrhoea, and peptic ulcers, whereas the less serious illness does not (Kusters et al., 2006). _H. Pylori_ infection causes a variety of cellular signalings that either prevent or promote intracellular reproduction. MAPK signaling, mechanical autophagy, the phosphatidylinositol 3-phosphate kinase signaling pathway, and proinflammatory cytokine overexpression are important cellular signaling pathways that are upregulated during bacterial infection. (Pachathundikandi et al., 2013). It is worth noting that chronic infection with _Helicobacter pylori_ CagA-positive strains is the most common cause of gastric cancer. CagA protein is produced by the cagA gene and is transported to stomach

epithelial cells via the type-4 bacterial secretion system. CagA is phosphorylated on tyrosine at the sequence "Glu-Pro-Ile-Tyr-Ala" (EPIYA) and interacts with host molecules such as the pro-oncogenic phosphatase SHP2 and the polarity-regulating kinase PAR1/MARK to modulate cellular signaling pathways (Hatakeyama, 2017). After cellular immunity or host defence detects an intracellular pathogen, mechanical autophagy aggregates in host cells to maintain cellular homeostasis. The host defence, particularly autophagy, is critical in the face of _H. pylori_ infection. Autophagy was discovered to be a resource-saving process that involves transferring superfluous cytoplasmic components and misfiled proteins to lysosomes for recycling (Ma et al., 2013). Hunger, viral infection, and bacterial infection, in general, promote the formation of autophagy maturation vacuoles. Autophagy proteins Atg5-Atg12, Atg6-Atg9, and Atg16L are acquired by pre-autophagosomal vesicles. The unconjugated or unlipidated isoform of autophagy-related Atg8 (LC3-I) is then recruited to vesicles and converted to LC3-II, a membrane or lipidated isoform (Abd El Maksoud et al., 2020). Importantly, knocking out one or more autophagy genes with specific siRNA shuts down the entire autophagy process. Notably, _H. pylori_-induced autophagy does not persist

during infection; thus, understanding the relationship between *H. pylori* infection and autophagy in the right model should aid in the identification of autophagy-associated treatment targets for *H. pylori* infection (Zhang et al., 2019). We planned to target the expression of CagA effector and the autophagy-related Agt12 with specific siRNA in infected cervical cancer cells, the HeLa cell line, to evaluate the efficacy of down-regulation of both components in addressing *H.pylori*.

Materials and Methods

Cell line

The HeLa cell line was obtained from VACSERA (Giza, Egypt) and cultured *in vitro* in Roswell Park Memorial Institute (RPMI1640) medium (Invitrogen, Waltham, MA, USA) supplemented with 3 mM L-glutamine, 3 mM sodium pyruvate, 25 mM HEPS, and 7% fetal bovine serum (FBS). In a CO_2 incubator, the cells were kept at 37°C. (Vision Scientific, Daejeon, South Korea) and subcultured by using Trypsin/EDTA solution (Sigma-Aldrich) (Khalil et al., 2017b, 2018).

Transfection protocol

In a 6-well plate, HeLa cells were plated at a density of $2x10^5$ cells per well and incubated overnight in a CO_2 incubator.

The cells were then transfected with CagA, Atg12, or luciferase siRNA antagonists at a concentration of 100 pmol/well.

The cells were then transfected with siRNAs targeting the autophagy-related gene Atg12, the *H. pylori* CagA gene, or anti-Luciferase (human-unrelated siRNA). As a result, 20 ul HiPerFect Transfection Reagent (Qiagen, USA) contains cationic and neutral lipids that promote siRNA uptake and release inside cells was gently mixed with 200 ng of the corresponding siRNA against Atg12, transcript variant 5, (5-CUUGCUACAUGAAAUGGAUU-3), or against the coding area of CagA, (5-AGCUUGCCUGUUAUCCCUAUCAUUC-3), or Luciferase (5-AACUUACGCUGAGUACUUCGA-3). The optimum media (recommended media for transfection) was used to suspend the transfection reagents, which were introduced to HeLa cells for 6 hours before being cultured in full RPMI 1640 Media for 2 days before being infected with *H. pylori* (Abd El Maksoud et al., 2020).

Bacterial strain and infection protocol

H. pylori strain P12 (Kindly provided by Wixner Medical Center, Ohio State University, USA) was grown on agar plates containing 10% horse serum for five days under microaerophilic conditions at 37°C and 10% CO_2. The Brucella broth liquid medium supplemented with 10% heat-inactivated FBS was then used to preserve and cultivate *H. pylori*, as previously stated (Blanchard and Nedrud, 2006). Several aliquots of the growing *H. pylori* were maintained in PBS contains 10% serum, 1%DMSO, and 15%

glycerol and kept at -80°C. Bacteria were collected from a culture in phosphate-buffered saline (PBS) and measured using a spectrophotometer at an optical density of 600 nm, where "each 1.0 value" equals 1108 (CFU/ mL). (Jung et al., 2015). At a multiplicity of infection of one (MOI = 1), the bacterial stock was used to infect Hela cells for 48 hours. The changes in cell morphology after infection were observed using an inverted microscope (Schneider et al., 2011).

Cell viability and cytotoxic effects

Researchers used several survived cells and representative photos of cell shape taken with an inverted microscope to achieve variations in cell morphology and cell viability rate following transfection in infected cells. The generation of Lactate dehydrogenase (LDH) in the infectious medium of treated cells was examined in a 96-well plate using an Lactate Dehydrogenase (LDH) Activity Assay Kit (Abcam, ab102526). The same volume of sample and LDH buffer containing the primary antibody were incubated for 2 hours before being treated for one hour with LDH substrate containing the secondary antibody, as directed by the manufacturer. Using ELISA plate reader (IndiaMART, Delhi, India) 470 nm absorbance readings, the relative activity of LDH was calculated. Cells treated with Triton X-100 were utilized as a positive control for cytotoxicity (Khalil et al., 2018). The relative LDH production was calculated by dividing the mean absorbance values of infected

cells on the mock (clean media). The cytotoxic potential of the indicated agents was further enhanced by using an inverted microscope to examine cell morphology and accounting for the number of survived cells. The number of cells that survived treatment was manually counted by removing the old media from the treated cells, washing the cells with PBS, trypsinizing the attached cells, and finally counting the number of cells with a hemocytometer.

Total RNA isolation and cDNA synthesis

Transfected and infected cells were collected from cell culture plates in clean, RNase-free tubes. Total RNAs were isolated using TRIzol reagent (Invitrogen, Waltham, MA, USA). By resuspending the isolated RNA in DNase and RNase-free water, the concentration of all samples was adjusted to 100 ng /ul. Then, 10 ul of purified total RNA was used to make cDNA with a cDNA synthesis kit (Qiagen, USA). According to the manufacturer's instructions, prepared RNA was incubated with Oligo(dT) 20 primer and M-MLV Reverse Transcriptase (Invitrogen, USA) at 55°C for 2 hours, followed by 5 minutes at 75°C to inactivate the Rtase enzyme. The cDNA was then kept at -20°C until needed (Farghaly et al., 2018).

Agarose gel electrophoresis

Purified RNA was loaded into a 1.5 percent agarose gel prepared in a clean cylinder by dissolving 1 gram agarose (UltraPure™ Agarose, Invitrogen, USA) in 75 ml TAE (1X).

The flask was microwaved, covered, until completely melted. The warm gel was loaded with 10 ul of ethidium bromide 10 mg/mL and placed in the DNA electrophoresis apparatus, which was kept cool. After placing the samples on the gel, electrophoresis at 50 volts was performed for one hour (Dahb Hassen *et al.*, 2017).

Quantitative real-time PCR (Q-RT-PCR) assay

Q-RT-PCR was used in transfected and infected HeLa cells to detect the expression profile of *H.pylori*-16S ribosomal RNA (16S rRNA), CagA, and Atg12 genes to evaluate both bacterial replication and cellular immune response. The relative gene expression of the indicated genes was measured using the QuantiTect SYBR Green PCR Kit (Qiagen, USA) and oligonucleotides specific for each gene, CagA-For-5´-ATGACTAATGAAACCATTGATCAAA-3´, CagA-Rev-5´-AAAGCTCTCTACAGAAGGAATCTAC-3´, Atg12-For-5´-CACGAACCATCCAAGGACTCA-3´, Atg12-Rev-5´-TTTGTGGTTCATCCCCACG-3 (Khalil et al., 2019). For 16S rRNA the following oligonucleotides was used F-5'-TCGGAATCACTGGGCGTAA-3', R-5'-TTCTATGGTTAAGCCATAGGATTTCAC-3'. Level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was improved using specific oligonucleotides, gapdh-for-5'-TGGCATTGTGGAAGGGCTCA-3' and gapdh-rev-5'-TGGATGCAGGGATGATGTTCT-3' (Abd El Maksoud *et al.*, 2020). The RT-PCR procedure used 96°C for 9 minutes, 40 rounds (96°C for 30 seconds, 59°C for 30 seconds, and 72°C for 30 seconds), and 4°C for stopping the reaction and holding samples. The indicated Ct values were investigated using Ct equations (Farghaly *et al.*, 2018).

Enzyme-linked immunosorbent (ELISA)

Human ELISA kits were used to measure the levels of tumour necrosis factor (TNF-) and interleukin 6 (IL-6) in treated cells (Abcam, 181421 and Abcam, 46028, respectively). In a 96-well plate, cells were seeded, then transfected with the corresponding siRNAs and infected with H. pylori at a MOI of 1. On day 1, day 2, and day 3, the media was collected and the level of TNF- was measured in a time-course experiment. 100 µl of lysed cells were placed into an ELISA plate reader at each time point and treated for 3 h at room temperature (RT) with the same volume of control solution and 50 µl 1× biotinylated antibody. After cleaning, the wells were filled with 100 µl of 1× streptavidin–horseradish peroxidase (HRP) solution, which was incubated for 30 minutes away from the light. Finally, each well received 100 µl of the chromogen TMB substrate solution, which was followed by a 15-minutes incubation period at room temperature in the dark. 100 µl stop solution was added to each well to halt the

reaction, and the absorbance of each well was measured at 450 nm. The final concentration of indicated cytokines was achived as previously described (Khalil *et al*., 2017).

Statistical Analysis:

Microsoft Excel was used to create graphs and histograms. For statistical analysis, the Student's two-tailed t-test was used, with p-values 0.05 considered significant and p-values 0.01 considered highly significant. Using the equations: delta–delta Ct analysis was used to calculate the foldchanges of the quantitative mRNA assessed by q-RT-PCR:

(1) delta Ct = Ct for gene − Ct for GAPDH, (2) delta–delta Ct = delta Ct for experimental − delta Ct for control), and (3) expression fold change = $(2^{-\Delta\Delta ct})$ (Khalil et al., 2016).

Results and Discussion

Transfection of siRNA-mediated CagA and Atg12 has no cytotoxic effects on HeLa cells

To evaluate if siRNA transfection was toxic to H. Pylori-infected Hela cells, the cell shape and number of surviving cells were analyzed. LDH formation by transfected cells was also examined in order to determine the transfection's potential toxicity. LDH production outside of cells indicates a disruption in cell proliferation and the activation of programmed cell death (Urbaska and Orzechowski, 2019). Transfection of the suggested siRNAs had little negative effects on transfected cells when compared to ant-

luciferase siRNA transfection, as measured by cellular shape and the number of living cells (Figure 1A and B). Furthermore, LDH synthesis was increased up to 5 times in both control-transfected and infected cells, indicating that *H. Pylori* infection was cytotoxic. Meanwhile, the level of LDH produced in transfected cells with siRNA targeting CagA and Atg12 was comparable, indicating that, on the one hand, the transfection's toxic effect was overlooked, and, on the other hand, transfected cells' ability to prevent *H. Pylori* infection cytotoxicity was overlooked (Figure 1C). These findings indicate that transfecting HeLa cells with siRNAs targeting CagA and Atg12 has no negative effects on cell growth and can prevent cytotoxicity caused by *H. Pylori* infection.
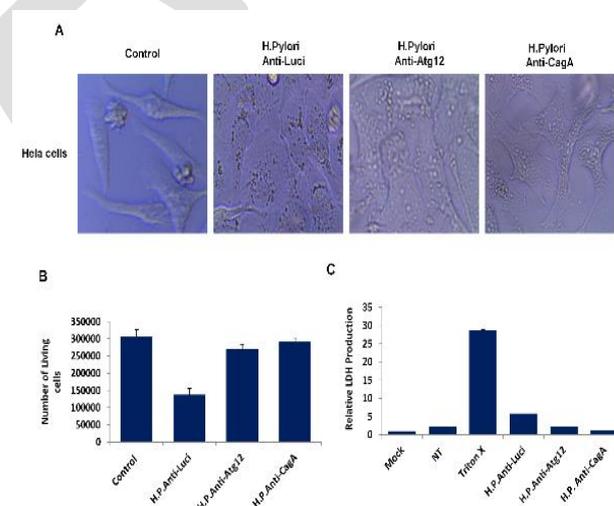
Figure 1: Cytotoxic effects of siRNA transfection:
 A) A comparison of HeLa cells transfected with anti-Atg12 and Anti-cagA siRNAs to anti-luciferase siRNA transfected cells, with representative cell photos indicating cell shape.
B) The number of viable cells after transfection was manually determined.
(C) LDH production in transfected cells compared to Triton X-100-treated cells and non-treated cells (NT). The error bars show the standard deviation (SD) of two independent experiments.

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Down-regulation of CagA and Atg12 inhibits *H. Pylori* replication in HeLa cells

CagA is one of the most studied pathogenicity effectors of *Helicobacter pylori*, with the ability to inject into host cells via the type four secretion system. CagA is an oncogenic protein that has been linked to gastric cancer (Jiménez-Soto and Haas, 2016). Alternatively, Atg12 has been identified as a mitochondrial apoptosis mediator that regulates the apoptotic signaling pathway during autophagosome formation (Rubinstein *et al.*, 2011). To see if targeting CagA and Atg12 can disrupt *H. Pylori* replication, bacterial replication in treated HeLa cells was quantified using q-RT-PCR of steady-state mRNA of bacterial 16S RNA (Figure 2B). In contrast to transfected cells, our findings revealed that the bacterial-16S rRNA expression profile was significantly (p-values =0.02) disrupted in transfected cells using siRNA against either CagA or Atg12 (more than 70 percent reduction). As a result, Agarose gel electrophoresis of pure RNA 24 hours after infection 16S rRNA revealed distinction between all infected samples and the control, demonstrating that *H. Pylori* can replicate intracellularly in Hela cells (Figure 2A). However, its expression was significantly reduced 48 hours after infection in transfected cells with anti CagA and anti Atg12, as measured by q-RT-PCR. (Shanjana, A. and Archana, A., 2003). This finding suggests that

suppressing CagA and Atg12 may be effective in preventing *H. Pylori* replication.
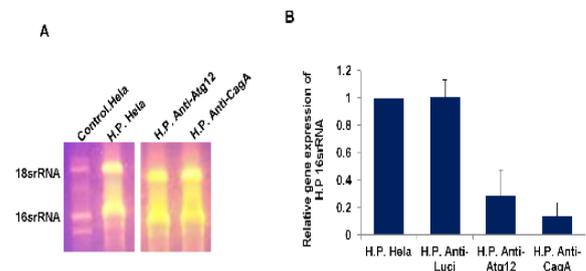
Figure 2: Expression profile of *H. Pylori* 16S rRNA in transfected HeLa cells
(A) Total RNAs were isolated and purified from transfected and infected Hela cells using 1.5 % agarose gel electrophoresis.
(B) In comparison to control-transfected cells, relative gene expression of 16S
(C) Expression fold change = (2−ΔΔct) rRNA demonstrates *H. Pylori* dominance in HeLa cells transfected with siRNA antagonist CagA and Atg12. The error bars represent the standard deviation (SD) of two independent experiments.

Inhibition of CagA and Atg12 expression by targeting in transfected HeLa cells

During *H. pylori* infection, several external and intracellular signaling pathways have been identified, with protein kinase C (PKC) playing a key role in several of them. PKC signalling activates several downstream signaling pathways, including mitogen-activated protein kinase (MAPK), which activates the RAS/RAF/MEK/ERK signalling pathways (Griner and Kazanietz, 2007). Only cells transfected with anti-Atg12 siRNA had lower levels of autophagy-related Atg12 gene expression (Figure 3A). Down-regulation of bacterial CagA was found to have a competitive

inhibitory effect on the prevalence of *H. Pylori* in HeLa cells, as well as a significant reduction in CagA gene expression. As a result of targeting the bacterial effector CagA and autophagy signaling was disrupted, resulting in lower bacterial particle replication levels in HeLa cells. Also, siRNA nanosomes were formulated by packaging lipid nanoparticles with multiple siRNAs that target the highly conserved 5′-untranslated region of the hepatitis C virus (HCV) genome. In mice, systemic treatment with siRNA nanosomes significantly inhibited HCV replication (Yang et al., 2019).
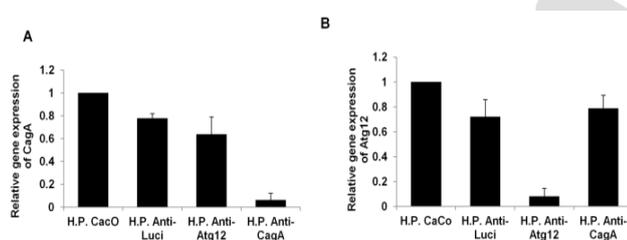
Figure 3: CagA and Atg12 expression in transfected and infected cells
(A) Comparison of CagA expression profiles in HeLa cells transfected with CagA or Atg12 specific siRNA to other transfected and infected Hela cells, normalized to GAPDH gene expression.
(B) Autophagy is indicated by the expression of Atg12 in transfected and *H. pylori* infected cells. The error bars depict the standard deviation of two separate experiments.

Down regulation of CagA and Atg12 regulates TNF-α and IL-6 in infected cells

Several studies have found that a lack of MAPK signal is associated with the production of pro-inflammatory cytokines such as tumour necrosis factor-alpha (TNF-) and interleukin-6 (IL-6) as well as anti-inflammatory cytokines such as IL-10 via p38 and c-Jun N-terminal kinase (JNK) (Park *et al*., 2010). Furthermore, the RAF-1

signalling pathway promotes microbial replication, including *H. Pylori*, hepatitis C virus, and influenza A virus replication (Khalil, 2017). Both IL-6 and TNF- concentrations were significantly (p-values = 0.03) reduced in Atg12-transfected cells over time. According to these findings, disrupting MAPK signaling causes HeLa cells to modify their pro-inflammatory cytokine release in response to *H. pylori* infection.
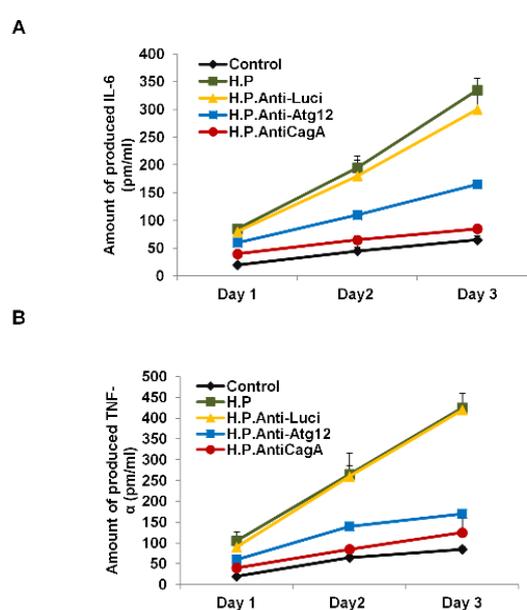
Figure 4: Levels of released IL-6 and TNF-α in transfected and infected Hela cells
(A) IL-6 concentration (pm/ml) in the fluid medium of *H. Pylori* infected HeLa cells transfected with specific siRNAs at the indicated time points, compared to transfected cells.
(B) TNF concentration (pm/ml) in the fluid medium of infected HeLa cells transfected with anti-Luci, anti-Atg 12, and anti-CagA siRNAs versus transfected cells at the indicated time points.

In this study, we examined the effect of anti-Atg12 and anti-CagA medicine on the relative gene expression and cytokine production associated with *Helicobacter pylori* infection.

Surprisingly, when HeLa cells were infected with *H. pylori*, the expression profile of survival genes such as CagA and Atg12, as well as inflammatory factors such as IL-6 and TNF-α, increased dramatically. These findings highlight the importance of *H. pylori* in preventing apoptosis and facilitating bacterial vacuolation after infection by preserving cell viability. Targeting the bacterial CagA effector, for example, was able to successfully limit IL-6, and TNF- α expression, as well as block bacterial reproduction in HeLa cells. Accordingly, The level of IL-8 induced by CagA positive strain H. pylori NCTC 11637 was higher than that produced by infection with the CagA negative strain NCTC 11639. Treatment with SiRNA resulted in a decrease in IL-8 production of more than 50% and a corresponding decrease in CagA mRNA (Yan et al., 2007). Similarly, the autophagy-related protein Atg12 is downregulated, causing bacterial replication to be disrupted and regulating proinflammatory cytokine release. We developed a precise and sensitive design of CagA gene and Atg12 gene siRNA cassettes that is effective in reducing *H. pylori* infection and its associated inflammatory progression. *Helicobacter pylori* cytotoxin-mediated gene A (CagA) protein has been linked to increased virulence and the risk of gastric cancer (Kontizas *et al*., 2020). The CagA gene produces the effector CagA protein, which changes cell shape, improves cell mobility, avoids junctional

activity, and promotes epithelial type transition to a mesenchymal-like phenotype (Buti *et al*., 2011). Furthermore, CagA inhibits the tumor suppressor role of p53 apoptosis-stimulating protein 2 (ASPP2) by causing its degradation, which reduces its activity. When CagA enters the host tissue, it activates ASPP2. Proinflammatory cytokines, particularly IL-6 and TNF- α, have been linked to increased apoptosis in the presence of *Helicobacter pylori*, independently of the bacterial effectors VacA and CagA (Kivrak Salim et al., 2016) According to previous research, *H. pylori* infection promotes cell survival signaling after infection to allow intracellular replication while avoiding apoptotic signaling with a low cytotoxic response (Thalmaier *et al*., 2002). TNF- is a proinflammatory cytokine that becomes active after binding to receptors 1 and 2. (TNFR1 and TNFR2). According to new research, TNF- plays a role in DEP-induced pulmonary inflammation, and TNFR2 is the most important receptor in mediating these inflammatory processes (Kumar *et al*., 2017). Following infection of HeLa cells, the proinflammatory cytokines TNF- were likely significantly reduced in a time-dependent manner in response to CagA and Atg12 targeting.

Conclusions

Our findings revealed that Transfecting HeLa cells with siRNAs targeting CagA and Atg12 has no negative effects on cell growth and can prevent cytotoxicity caused by *H. Pylori*

inoculation, bacterial-16S rRNA expression profile was significantly disrupted in transfected cells using siRNA against either CagA or Atg12. Targeting CagA effector and Atg12 successfully disrupts *H. pylori* reproduction in infected cells. Targeting the bacterial CagA effector, for example, was able to successfully limit IL-6, and TNF- expression. These findings suggest that targeting the bacterial CagA effector and/or autophagy-related Atg12 as a potential therapeutic strategy for *H. pylori* infection could present a novel mechanism to disrupt bactrial replication.

Conflicts of Interest:

The authors declare no conflict of interest.

Data Availability:

The data that support the findings of this study are available from the corresponding author, (Hany Kh.), upon reasonable request.

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