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RESEARCH ARTICLE

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Helicobacter pylori cholesterol glucosylation modulates autophagy for increasing intracellular survival in macrophages

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Abstract

Cholesterol-α-glucosyltransferase (CGT) encoded by the type 1 capsular polysaccharide biosynthesis protein J (*capJ*) gene of *Helicobacter pylori* converts cellular cholesterol into cholesteryl glucosides. *H. pylori* infection induces autophagy that may increase bacterial survival in epithelial cells. However, the role of *H. pylori* CGT that exploits lipid rafts in interfering with autophagy for bacterial survival in macrophages has not been investigated. Here, we show that wild-type *H. pylori* carrying CGT modulates cholesterol to trigger autophagy and restrain autophagosome fusion with lysosomes, permitting a significantly higher bacterial burden in macrophages than that in a *capJ*-knockout (ΔCapJ) mutant. Knockdown of *autophagy-related protein 12* impairs autophagosome maturation and decreases the survival of internalised *H. pylori* in macrophages. These results demonstrate that CGT plays a crucial role in the manipulation of the autophagy process to impair macrophage clearance of *H. pylori*.

KEYWORDS

autophagy, cholesterol glucosylation, *Helicobacter pylori*, lipid rafts, macrophages

1 | INTRODUCTION

Helicobacter pylori is a spiral-shaped Gram-negative bacterium that infects human stomachs in approximately half of the worldwide population (Marshall, 2002). Infection with *H. pylori* that colonise human gastric epithelium causes gastrointestinal illness with variable severity (Wroblewski, Peek Jr., & Wilson, 2010). Although several types of immune cells are recruited to the infected regions, the host immune response fails to completely eradicate *H. pylori* in a large proportion of infected subjects (Gobert et al., 2001; Monack, Mueller, & Falkow, 2004).

Lipid rafts serve as entry portals for several pathogens including *H. pylori* (Lai et al., 2008, Lu et al., 2012, Wang & Hajishengallis, 2008). *H. pylori* hijacks lipid rafts to inject virulence factors into the host cells via type IV secretion system (Lai et al., 2006; Lai, Hsu, Wang, & Wang, 2013). Notably, this peculiar bacterium possesses unique ability to manipulate cellular cholesterol, which is an important component of lipid rafts in cell membranes to protect itself from immune attack; cholesterol glucosylation by *H. pylori* plays a major role in evading immune surveillance (Brown & London, 1998, Wunder et al., 2006). It is thus of interest to understand how *H. pylori* exploits lipid rafts for its internalisation and multiplication in immune cells in the context of cholesterol glucosylation.

The human immune system has developed several mechanisms to protect the host from bacterial infection. One such means is through autophagy, which forms autophagosomes and then fuse with lysosomes of which contents are subsequently lysed by degradative enzymes (Greenfield & Jones, 2013, Kang, Zeh, Lotze, & Tang, 2011). In the context of degrading intracellular pathogens, such as *H. pylori*, autophagy contributes to immune defence to lessen *H. pylori* burden (Liao et al., 2017b, Mizushima, Levine, Cuervo, & Klionsky, 2008, Yang & Chien, 2009, Zhao et al., 2008). Interestingly, an earlier report revealed that glucosylation of *H. pylori* enhanced its survival in infected macrophages because of its sustained arrest in autophagosomes, implicating that these autophagic vesicles could provide a unique niche for *H. pylori* multiplication (Deen, Gong, Naderer, Devenish, & Kwok, 2015; Wang, Wu, & Lei, 2009a). However, the detailed mechanisms by which *H. pylori* regulates autophagy machinery to impair the efficiency of bacterial clearance remain unclear.

Helicobacter pylori absorbs host cholesterol, which is subsequently assimilated into glycosylated/lipidated forms as structural components of the bacterial cell wall. Notably, cholesteryl glucosides account for ~25% of cell wall glycolipids and promote immune evasion (Lebrun et al., 2006; Wunder et al., 2006). The key cholesterol-modifying enzyme has been identified as cholesterol-α-glucosyltransferase (CGT) encoded by the *HP0421* gene, type 1 capsular polysaccharide biosynthesis protein J (hence also known as CapJ) (Lebrun et al., 2006). *H. pylori* CGT is an important enzyme that catalyses the conversion of cholesterol into cholesterol-α-glucosides (CGs). Glycosylated cholesterol is then subjected to further modification, yielding a complex of CGs, including cholesteryl-α-D-glucopyranoside, cholesteryl-6′-α-*O*-tetradecanoyl-α-D-glucopyranoside, and cholesteryl-6′-α-*O*-phosphatidyl-α-D-glucopyranoside (Hirai et al., 1995). Loss of CGT activity reduces the ability of *H. pylori* to evade phagocytosis by macrophages and the T cell-mediated immune response (Beigier-Bompadre et al., 2011; Wunder et al., 2006). Our previous study demonstrated that CGs partition into cholesterol-rich microdomains around host–pathogen contact sites and contribute to membrane remodelling in bacteria-induced pathogenesis (Wang, Cheng, Cheng, Lai, & Wang, 2012). However, the association of *H. pylori*-synthesised CGs with the autophagy process, as well as the likely interference from innate immune defences during such interactions, has not been explored.

In the present study, J774A.1, which is frequently utilised as an *H. pylori*-infected macrophage model, was employed to study the involvement of cholesterol in the induction of autophagy activation in innate immune cells during *H. pylori* infection. The role of *H. pylori* CGT in the enhanced bacterial burden in macrophages was investigated, including whether this process is mediated through the exploitation of lipid rafts and manipulation of the autophagy pathway. Our results provide insights into the mechanisms underlying the involvement of cholesterol glucosylation in contributing to autophagy regulation and inhibition of lysosome fusion in the impairment of macrophage clearance of *H. pylori*.

2 | RESULTS

2.1 | *Helicobacter pylori* CGT increases bacterial survival in macrophages

Because cholesterol glucosylation of *H. pylori* can delay bacterial internalisation and interrupt phagosome maturation (Du et al., 2016), we hypothesised that this mechanism might be due to the manipulation of the autophagy process by *H. pylori* CGT. First, we examined whether CGT was involved in the increased *H. pylori* survival in macrophages. The macrophage cell line J774A.1 and a gentamicin protection assay were employed to analyse the intracellular survival of *H. pylori*. As shown in Figure 1a, the survival of the *capJ*-knockout (ΔCapJ) mutant (black bars) was much lower at all infection time points in comparison with that of the wild-type (WT) strain (open bars). We next evaluated whether restoration of CapJ expression could compensate the intracellular survival by using a *capJ* complementary (ΔCapJ-in) strain (grey bars) introduced with an intact *capJ*. The results showed a significantly rescued degree of the intracellular survival of bacteria in macrophages infected by ΔCapJ-in as compared with that of ΔCapJ-infected cells. Furthermore, the highest level of bacterial survival was found at the 6-hr post-infection for the WT-infected cells. Therefore, this time point was selected for the following experiments. To exclude the possibility that the difference in bacterial burden was due to the initial entry of the bacteria in the cells, J774A.1 cells were infected with WT or ΔCapJ mutant for 2 or 6 hr, followed by the measurement of the total bacteria in the cells (Figure S1). The level of bacterial load of the WT strain was comparable with that of ΔCapJ at 2- or 6-hr post-infection point, indicating that the higher intracellular survival was attributed to the WT strain possessing CGT.

We next evaluated whether CGT-dependent *H. pylori* survival in macrophages was related to dysregulated autophagy. The expression of proteins involved in autophagy was first characterised. Treatment of cells with rapamycin induced the conversion of microtubule-associated protein light chain 3 (LC3) from LC3-I to LC3-II, which is

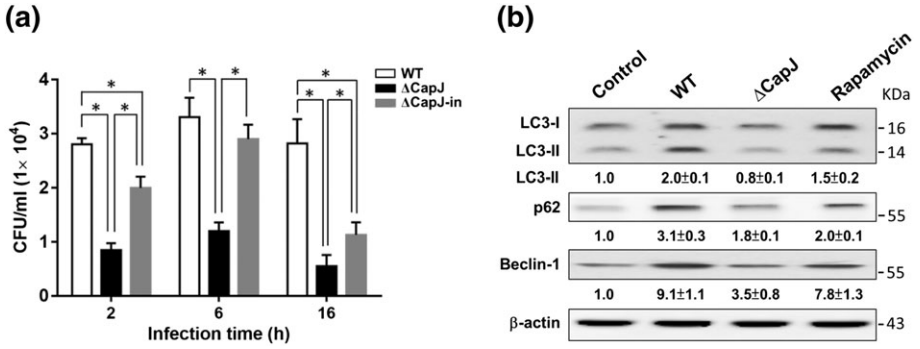

FIGURE 1 *Helicobacter pylori* cholesterol-α-glucosyltransferase enhances bacterial survival and induces autophagy-related protein expression in macrophages. (a) J774A.1 cells were infected with wild-type (WT), isogenic mutant *capJ*-knockout (ΔCapJ), or *capJ* complementary (ΔCapJ-in) *H. pylori* at a multiplicity of infection of 100 for 2, 6, and 16 hr. The infected cells were then treated with gentamicin (100 μg/ml), and the viable bacteria were determined using a gentamicin protection assay. $^{*}P < 0.05$ as compared to cells infected with ΔCapJ *H. pylori* using a Student's *t* test. (b) J774A.1 cells were treated with rapamycin (100 nM) or infected with WT and ΔCapJ strains for 6 hr. The expression levels of LC3-II, p62, and Beclin-1 were assessed by western blot analysis. β-Actin was used as a loading control. The results are the means and standard deviations of three independent experiments and are indicated at the bottom of each lane

considered a marker of autophagy (Klionsky et al., 2012). The data showed that infection of macrophages with WT *H. pylori* increased LC3-II expression, whereas the ΔCapJ variant did not. The expression levels of the autophagy-related proteins, SQSTM1/p62 and Beclin-1, that participate in the initiation of autophagy with LC3-II were assessed (Kang et al., 2011; Levine, Mizushima, & Virgin, 2011). The data showed that infection with the WT strain and rapamycin treatment increased the expression of p62 and Beclin-1 as compared with that in either the uninfected control or ΔCapJ-infected groups (Figure 1b). We then tested whether pretreatment with purified recombinant CGT (rCGT) could restore the level of LC3-II that is reduced in ΔCapJ-infected macrophages. As shown in Figure S2, ΔCapJ-infected cells had a lower level of LC3-II than WT-infected cells. After treatment of cells with rCGT (10 μg/ml), ΔCapJ-infected cells had a greatly increased LC3-II signal, comparable with that in WT-infected cells. These results suggest that *H. pylori* containing CGT interfered with autophagy-associated protein expression and increased bacterial survival in macrophages.

2.2 | *Helicobacter pylori* CGT inhibits lysosome formation

To further explore whether *H. pylori* survival in macrophages is dependent upon CGT dysregulated autophagy, cells were treated with 3-methyladenine (3-MA) to inhibit initial autophagosome formation. The 3-MA treatment dramatically increased WT *H. pylori* survival in macrophages (Figure 2a). Notably, the level of intracellular survival was much lower in ΔCapJ than in the WT strain regardless of 3-MA treatment. We then investigated whether *H. pylori* CGT promoted the formation of autophagosomes but subsequently reduced the fusion with lysosomes. Infection with WT *H. pylori* elicited the expression of early endosome antigen 1 and autophagy-related protein 12 (Atg12), specific markers for early endosome and autophagosome formation, respectively, but decreased lysosome-associated membrane protein-1 expression compared with ΔCapJ-infected cells (Figure 2b), indicating that ΔCapJ lost resistance to lysosomal degradation. Direct observations were further used to establish if *H. pylori* CGT dysregulated

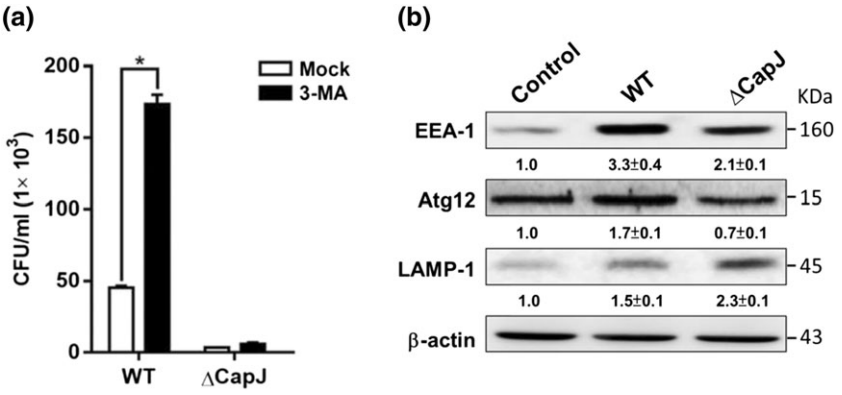

FIGURE 2 *Helicobacter pylori* cholesterol-α-glucosyltransferase increases bacterial growth in macrophages. (a) J774A.1 cells were treated with 5 mM 3-methyladenine (3-MA) for 24 hr, followed by infection with wild-type (WT) or *capJ*-knockout (ΔCapJ) *H. pylori* at a multiplicity of infection of 100 for 6 hr. The viable bacteria were determined using a gentamicin protection assay, and the results were expressed as the colony-forming unit (CFU). Statistical significance was evaluated using a Student's *t* test. $^{*}P < 0.05$. (b) J774A.1 cells were infected with WT or ΔCapJ strains for 6 hr, and the expression levels of EEA-1, Atg12, and LAMP1 were examined by western blot analysis. β-Actin was used as a loading control. The results are the means and standard deviations of three independent experiments and indicated at the bottom of each lane

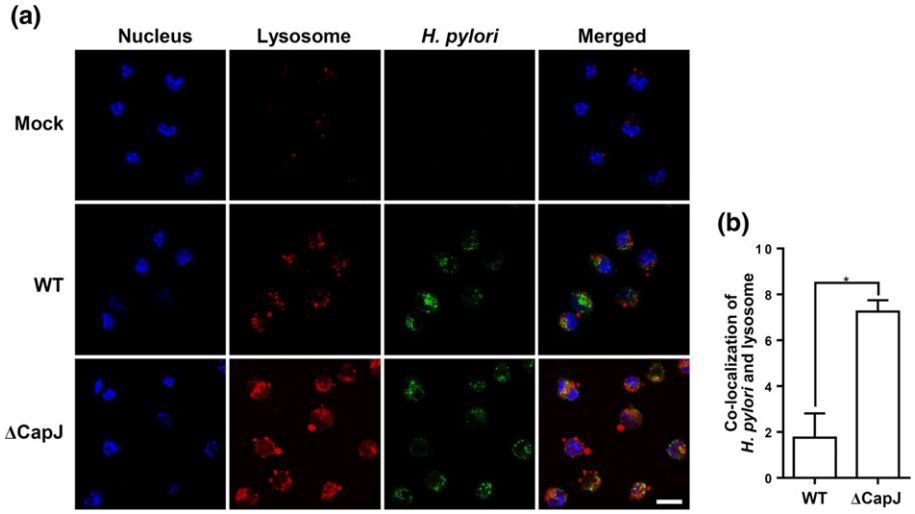

FIGURE 3 *Helicobacter pylori* cholesterol-α-glucosyltransferase decreases lysosome formation in macrophages. (a) J774A.1 cells were infected with wild-type (WT) or *capJ*-knockout (ΔCapJ) *H. pylori* at a multiplicity of infection of 100 for 6 hr. Cells were fixed and stained for nuclei (Hoechst33342, blue), lysosomes (red), and *H. pylori* (green), and then analysed by confocal microscopy. (b) Quantification represented the number of colocalised *H. pylori* and lysosomes in each cell (100 cells were counted per sample). $^{*}P < 0.05$ as determined by Student's *t* test. Scale bar: 10 μm

autophagy and thereby delayed fusion with the lysosomes. Analysis of the colocalisation of *H. pylori* and lysosomes showed that the WT-infected macrophages possessed significantly fewer bacteria–lysosome fusion events than did ΔCapJ-infected macrophages (Figure 3). These findings together suggest that *H. pylori* CGT might dysregulate the autophagy process to restrain lysosome fusion, revealing a key role of CGT in evading innate immune defence by macrophages.

2.3 | Impairment of autophagosome formation reduces *Helicobacter pylori* survival in macrophages

To examine whether CGT induces the expression of Atg12 that is important for the formation of autophagosome, J774A.1 cells were treated with shRNA as a control (shVector) or silencing *atg12* (shatg12) and then infected with WT and ΔCapJ strains, respectively. A significant increase in *atg12* mRNA levels was seen in WT-infected cells than in uninfected or ΔCapJ-infected cells (Figure 4a). Western blot analysis showed that WT *H. pylori*-infected cells significantly induced the expression of Atg12 and LC3-II, which was not seen in ΔCapJ-infected cells (Figure 4b). The WT bacterial survival was dramatically reduced in *atg12*-knockdown cells as compared with that in shVector controls (Figure 4c). Similar results were obtained for ΔCapJ-infected cells, also showing reduced intracellular survival following *atg12* knockdown. Taken together, the above results indicate that infection with *H. pylori*-induced autophagosome maturation is CGT-dependent and is essential for bacterial survival in macrophages.

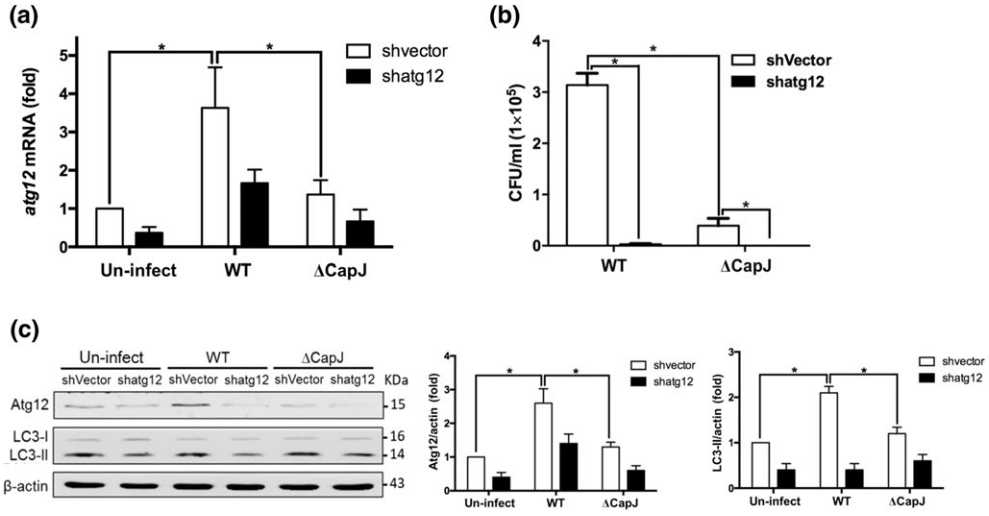

FIGURE 4 Knockdown of *atg12* impairs autophagosome formation and decreases *Helicobacter pylori* survival in macrophages. (a) J774A.1 cells were treated with shRNA as a control (shVector) or silencing *atg12* (shatg12) for 48 hr. Total RNA was isolated, and *atg12* mRNA levels were assessed by quantitative real-time PCR. (b) Cells were treated with shatg12 for 48 hr, followed by infection with WT or *capJ*-knockout (ΔCapJ) *H. pylori* for 6 hr. The protein expression levels of Atg12, LC3-II, and β-actin were analysed by western blot. The protein levels of Atg12 and LC-3-II normalised to β-actin are shown at right panels. (c) The intracellular survival of *H. pylori* was determined using a gentamicin protection assay. The statistical significance was evaluated using a Student's *t* test. $^{*}P < 0.05$. WT: wild type

2.4 | Involvement of lipid rafts in *Helicobacter pylori* CGT-interfered autophagy

Lipid rafts (also called cholesterol-rich microdomains) are required for *H. pylori* invasion of cells (Du et al., 2016; Wunder et al., 2006). However, the involvement of lipid rafts in CGT dysregulated autophagy that enhances bacterial intracellular survival remains poorly understood. Therefore, CGT's role in recruiting cholesterol and its requirement in *H. pylori*-induced autophagosome formation was investigated. Infection with WT *H. pylori* elicited formation of abundant autophagosome puncta (Cyto-ID) colocalised with lipid rafts (cholera toxin subunit B [CTX-B]; Figure 5). In contrast, minimal Cyto-ID colocalisation was observed with CTX-B in ΔCapJ-infected cells, similar to the results observed in uninfected controls. Methyl-β-cyclodextrin (MβCD) was then used to deplete cholesterol from membrane lipid rafts. The level of cellular cholesterol was reduced with MβCD treatment (Figure 6a), whereas bacterial entry was barely affected by MβCD treatment in both WT- and ΔCapJ-infected cells (Figure 6b). Disruption of lipid raft formation by using MβCD significantly reduced WT *H. pylori* survival in macrophages, with a less effect observed in ΔCapJ-infected cells (Figure 6c). Infection with ΔCapJ-in possessing an intact *capJ* significantly restored bacterial survival in macrophages, and that was similarly attenuated following MβCD treatment.

To further assess the impact of *H. pylori* CGT-exploited cholesterol on autophagosome formation, time-lapse fluorescence analysis was performed. WT *H. pylori* were found to cluster at the CTX-B-staining region (red) of the cell membrane (Figure 7a). During the course of the infection time (420–660 s), CTX-B was largely recruited by the bacteria, colocalised with autophagosomes (Cyto-ID, green) in

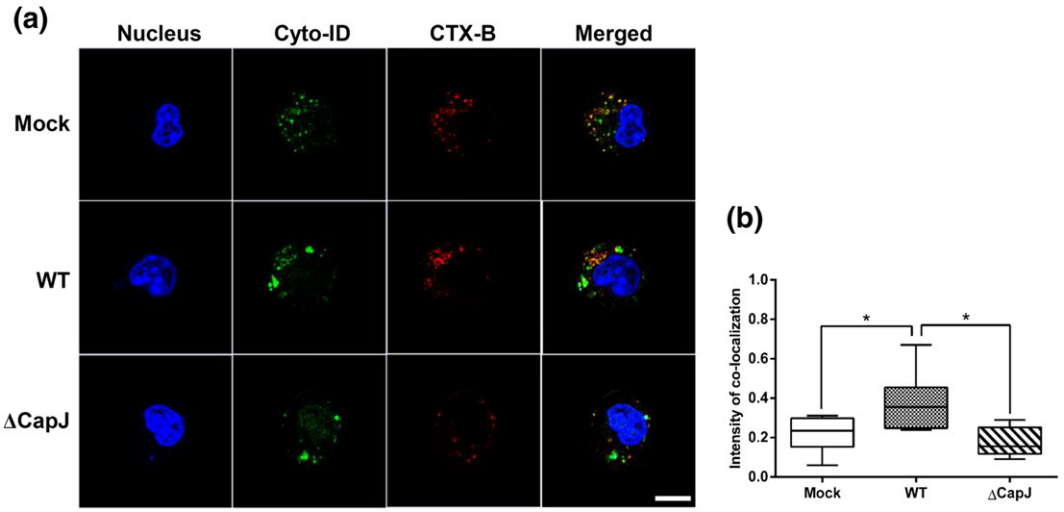

FIGURE 5 Involvement of lipid rafts in *Helicobacter pylori* cholesterol-α-glucosyltransferase-induced autophagosome formation in macrophages. (a) J774A.1 cells were infected with wild-type (WT) or *capJ*-knockout (ΔCapJ) *H. pylori* at a multiplicity of infection of 100 for 6 hr, and the cells were stained for nuclei (Hoechst33342, blue), autophagosomes (Cyto-ID, green), and lipid rafts (cholera toxin subunit B [CTX-B], red), respectively. The stained samples were analysed by confocal microscopy. (b) Box plots summarise the pixel of colocalisation signals of Cyto-ID puncta and CTX-B, which were analysed by Axiovision 4.7.1 software (Carl Zeiss). $^{*}P < 0.05$ as determined by a Student's *t* test. Scale bar: 10 μm

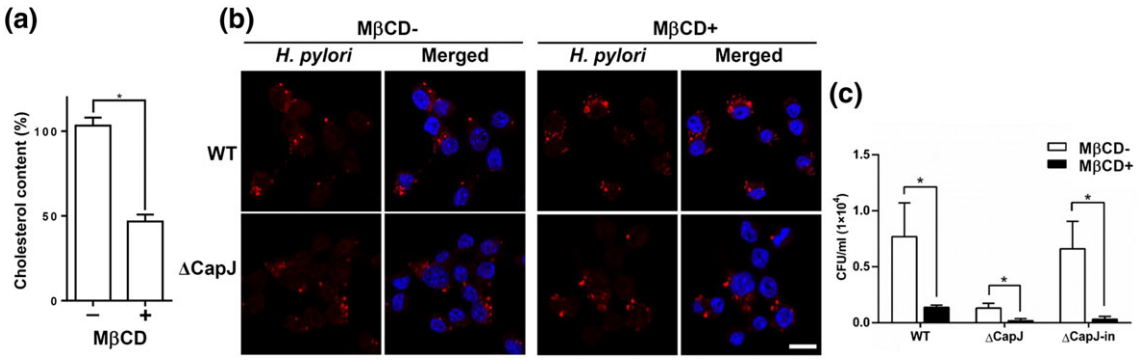

FIGURE 6 Sufficient cholesterol is essential for *Helicobacter pylori* intracellular survival in macrophages. (a) J774A.1 cells were untreated or treated with 10 mM methyl-β-cyclodextrin (MβCD) at 37°C for 30 min and harvested for cholesterol level analysis. (b) Cells were pretreated with 10 mM MβCD at 37°C for 30 min and infected with wild-type (WT) or *capJ*-knockout (ΔCapJ) *H. pylori* at a multiplicity of infection of 100 for 2 hr. Cells were stained for *H. pylori* (red) and nuclei (Hoechst33342, blue) and then analysed by confocal microscopy. (c) Cells were pretreated with 10 mM MβCD at 37°C for 30 min and infected with WT, ΔCapJ, or *capJ* complementary (ΔCapJ-in) *H. pylori* for an additional 6 hr. Viable bacteria were determined using a gentamicin protection assay, and the statistical significance was evaluated using a Student's *t* test. $^{*}P < 0.05$ as compared with the results for each MβCD-untreated group. Scale bar: 10 μm

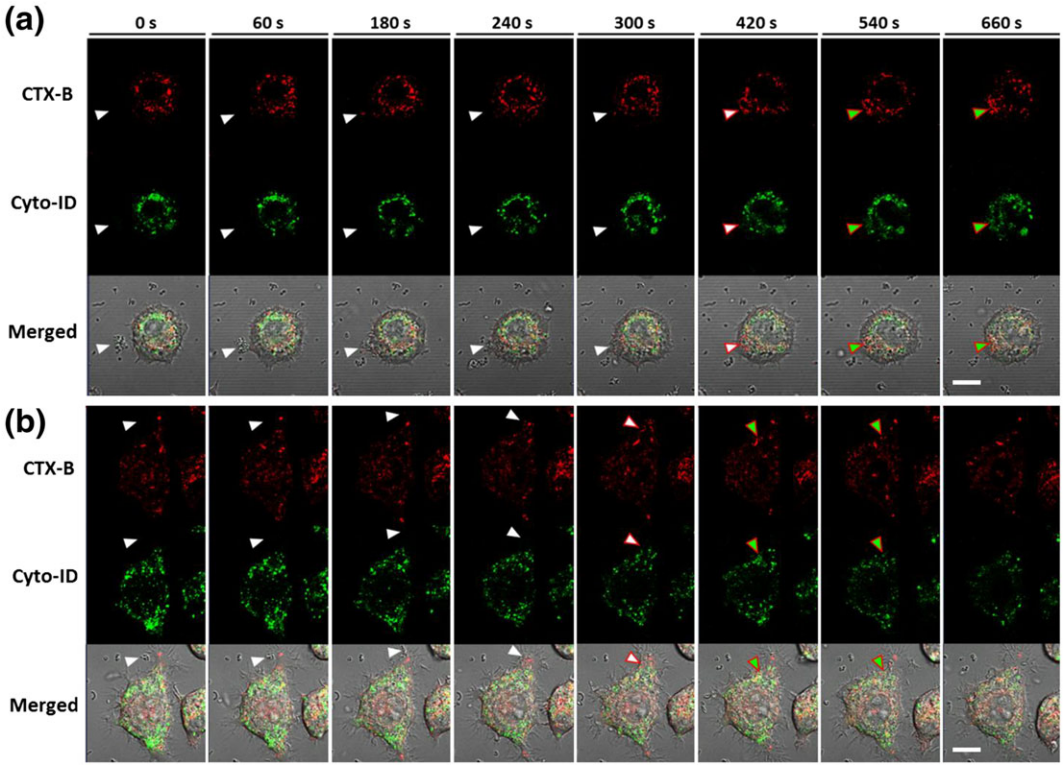

FIGURE 7 *Helicobacter pylori* cholesterol-α-glucosyltransferase recruitment of autophagosomes is mediated by lipid rafts. J774A.1 cells were stained for lipid rafts (cholera toxin subunit B [CTX-B], red) and autophagosomes (Cyto-ID, green), followed by infection with (a) wild-type or (b) *capJ*-knockout (ΔCapJ) *H. pylori* for the indicated times. Time-lapse images were collected using a Zeiss Axiovert Z1 inverted fluorescence microscope as described in the Experimental Procedures section. Arrows indicate *H. pylori* interactions with lipid rafts and autophagosomes. Scale bar: 10 μm

WT *H. pylori*-infected cells. However, in ΔCapJ-infected cells, minimal and scattered distribution of CTX-B to the sites of bacterial infection was observed (Figure 7B). Moreover, ΔCapJ-accumulated autophagosomes in the cells were transient (300–420 s) and disappeared over time. These results demonstrate that *H. pylori* CGT-promoted autophagosome maturation is cholesterol-dependent and further reduces autophagosome–lysosome fusion, thereby facilitating bacterial survival in macrophages.

3 | DISCUSSION

Autophagy induced by microbial infection acts as an innate immune defence to eliminate intracellular pathogens (Birmingham, Smith, Bakowski, Yoshimori, & Brumell, 2006; Nakagawa et al., 2004). Certain bacteria have thus evolved multipronged strategies to dysregulate autophagy machinery, thereby enhancing intracellular bacterial survival (Deretic & Levine, 2009, Dortet et al., 2011, Ouimet et al., 2016). However, the precise mechanisms by which *H. pylori* manipulates the autophagy process to sustain intracellular survival remain unclear (Deen, Huang, Gong, Kwok, & Devenish, 2013). In the present investigation, we show that *H. pylori* CGT encoded by *capJ* interferes with autophagy progression so as to reduce the lysosome fusion (Figure S3). Knockdown of *atg12* impaired the formation of autophagosome, which is crucial for *H. pylori* survival in infected macrophages. Furthermore, cholesterol depletion inhibited WT *H. pylori* intracellular survival, suggesting that *H. pylori* evasion of innate defence is cholesterol-dependent through CGT's action. The findings elucidate the mechanisms of the interactions between autophagy and *H. pylori* through the cholesterol glucosylation.

Atg12 plays a crucial role in phagophore elongation and autophagosome maturation (Fukuda & Itoh, 2008). In the Atg12-conjugation system, Atg12 can be activated by Atg7 and transferred to Atg10; the complex is then conjugated to Atg5 to form an Atg12–Atg5 complex (Geng & Klionsky, 2008). This complex non-covalently associates with Atg16L1 to constitute a ubiquitin protein-ligase-like enzyme that is critical for regulating autophagy and performing protein-lipid-conjugation reactions, implying that Atg12 is an important component for LC3 lipidation and autophagosome formation (Hanada et al., 2007). In this study, *atg12* knockdown-compromised autophagy and subverted autophagosome formation significantly reduced *H. pylori* survival in macrophages. The findings are consistent with previous reports that *H. pylori* infection induces autophagic processes that sequester bacteria within autophagosomes and inhibits compartmental fusion with lysosomes, resulting in bacterial survival in macrophages (Wang, Wu, & Lei, 2009b).

Vacuolating cytotoxin A (VacA) is an important player in inducing *H. pylori* autophagy for its persistent colonisation in this unique ecological niche. Yahiro et al. (2012) reported that VacA binds to the surface receptor LRP1 and induces the formation of LC3-II, an important marker of autophagy. Additionally, knockdown of Atg5 (Yahiro et al., 2012) and Atg12 (Terebiznik et al., 2009) led to a reduced level of VacA-induced autophagy (Terebiznik et al., 2009). The *ATG16L1T300A* variant in Crohn's disease results in impaired autophagic responses to VacA treatment, and those carrying the risk polymorphism have increased susceptibility to *H. pylori* infection (Raju

et al., 2012). Together, these results suggest that VacA-triggered autophagy proceed through the autophagy elongation complex (Atg5–Atg12–Atg16L1) after binding to LRP1. Conversely, RPTPα and RPTPβ, which are other VacA receptors, did not affect VacA-dependent autophagy, suggesting that VacA mediates distinct intracellular trafficking through binding to different surface receptors. We have previously shown that VacA is distributed among the detergent-resistant membranes that are rich in cholesterol, the depletion of which greatly reduced VacA intoxication and *H. pylori* internalisation activity (Kuo & Wang, 2003, Lai et al., 2008). Interestingly, *H. pylori* CGT extracts and modifies host cholesterol so as to produce lipidated forms, which partition in detergent-resistant membranes and reorganise the membrane architecture (Wang et al., 2012). It is likely that the CGs-containing raft membrane environment contributes to the VacA-induced autophagy. Nevertheless, the detailed mechanisms underlying how cholesterol or CGs affect VacA-induced autophagy need to be further investigated.

Helicobacter pylori cag-pathogenicity island, which encodes the type IV secretion system (TFSS) and CagA, is important for CagA translocation and signalling into cells. On the basis of the presence of CagA and VacA, *H. pylori* clinical isolates are classified into two major types: type I strains possess VacA and CagA, whereas type II bacteria lack VacA and CagA (Xiang et al., 1995). Most importantly, type I *H. pylori* delays the engulfment by macrophages due to the formation of megasomes, enhancing bacterial survival in the cells (Allen, Schlesinger, & Kang, 2000). Further investigation indicated that *H. pylori* inhibits phagocytosis by immune cells through the TFSS apparatus (Ramarao, Gray-Owen, Backert, & Meyer, 2000). The major antiphagocytic activity mediated by TFSS was characterised as VirB7, a key component of T pilus filament, and the VirB11 ATPase. However, neither CagA nor TFSS are essential for *H. pylori*-induced autophagy (Ricci, 2016; Terebiznik et al., 2009). Perhaps, CagA and VacA hijack host cells via independent strategies towards succeeding enduring gastric inhabitation.

Recent studies have shown that bacterial infections may participate in atherosclerosis by inducing inflammation response. For instance, the important role for *Chlamydophila pneumoniae* phospholipase D in the pathogenesis of atherosclerosis has been demonstrated (Benagiano et al., 2012). The results revealed that *C. pneumoniae* phospholipase D is able to drive the expression of pro-inflammatory cytokines to activate a Th17 immune response that plays a crucial role in the development of atherosclerosis. In addition, the cinaedi atherosclerosis inflammatory protein from *Helicobacter cinaedi* has been found to promote the differentiation of the pro-inflammatory profile of macrophages, triggering the formation of foam cells, which are important for the genesis of atherosclerotic plaques (D'Elios et al., 2017). Of note, *H. pylori* DNA was detected in atherosclerotic plaques, suggesting the association of *H. pylori* with the pathogenesis of atherogenesis (Kaplan et al., 2006). Although *H. pylori* CGT can catalyse the conversion of cellular cholesterol to evade phagocytosis by macrophages and attenuate the T cell-mediated immune response, the contribution to the bacteria-induced atherosclerosis is required for further investigations.

Although *H. pylori* is generally considered as an extracellular pathogen, an increasing number of studies indicate that this bacterium can invade and survive in both phagocytic and non-phagocytic cells, suggesting it as a facultative intracellular pathogen (Dubois & Boren, 2007; Lai et al., 2006; Petersen & Krogfelt, 2003; Wang et al., 2009b). Moreover, several investigations demonstrate that the sustained colonisation of *H. pylori* in this unique ecological niche is because this peculiar bacterium prevents itself from lysosomal degradation in immune cells (Wang et al., 2009a; Wang, Gorvel, Chu, Wu, & Lei, 2010). A recent study using macrophage lines indicated that a small subpopulation of intracellular *H. pylori* are hidden in autophagosomes, providing a potential refuge for the phagocytosed *H. pylori* (Deen et al., 2015). Although autophagosomes are reported to be intracellular niches for *H. pylori*, the underlying mechanisms by which *H. pylori* triggers the bacteria-associated autophagy machinery were poorly understood (Terebiznik et al., 2009; Wang et al., 2009a). The present study demonstrates that CGT plays a crucial role in *H. pylori*-induced autophagosome maturation and inhibition of autolysosome formation, both of which are cholesterol-sensitive scenarios. Our findings suggested that CGT contributed to two beneficial pathways for *H. pylori* survival in macrophages. First, CGT triggers autophagy to capture *H. pylori*, thereby recruiting lipid rafts to develop intracellular sanctuaries. In this case, *H. pylori* can be efficiently transferred into autophagosomes similar to *Listeria monocytogenes* and uropathogenic *Escherichia coli* (Amer, Byrne, & Swanson, 2005). In both pathogens, there is a rapid transfer from lipid raft vacuoles to autophagosomes. Second, *H. pylori* CGT interferes with autophagosome–lysosome fusion, thereby inhibiting bacterial degradation in cells. Similar mechanisms were observed with *Anaplasma phagocytophilium* (Niu, Yamaguchi, & Rikihisa, 2008) and *Staphylococcus aureus* (Schnaith et al., 2007), both of which are capable of multiplying in autophagosomes. Accordingly, CGT enables the exploitation of autophagic machinery to promote *H. pylori* survival in macrophages, which may be important for protecting bacteria from antibiotic treatment and avoiding elimination by host immune defences (Lai et al., 2013).

In summary, our findings demonstrate that cholesterol glucosylation is crucial for *H. pylori* exploitation of lipid rafts to control the autophagic process. Furthermore, the results demonstrated that the formation of autophagosomes provided a niche for CGT-containing *H. pylori* survival in macrophages. Cholesterol glucosylation is likely to contribute to the interference with autophagosome–lysosome fusion, thereby promoting evasion of innate immune defences. These results suggest that inhibition of lipid raft-mediated autophagic processes might be an interesting target for the development of novel drugs for the treatment of *H. pylori*-related diseases.

4 | EXPERIMENTAL PROCEDURES

4.1 | Antibodies and reagents

The LC3 monoclonal antibody was purchased from Cell Signaling Technology (Beverly, MA). Antibodies against SQSTM1/p62, Beclin-1, and LAMP-1 were purchased from Abgent (San Diego, CA). Antibody specific to Atg-12 was purchased from GeneTex (Irvine, CA). Rabbit anti-early endosome antigen 1 antibody was purchased from

Abcam (Cambridge, UK). Rabbit anti-*H. pylori* antibody was purchased from Novus Biologicals (Littleton, CO). Mouse anti-caveolin-1 and anti-transferrin receptor antibodies were purchased from BD Pharmingen (San Jose, CA). Fluorescein isothiocyanate-conjugated CTX-B was purchased from Molecular Probes (Carlsbad, CA). Cyto-ID™ autophagy detection kit was purchased from Enzo Life Sciences (Villeurbanne, France). Rapamycin, 3-MA, and MβCD were purchased from Sigma-Aldrich (St. Louis, MO). Hoechst33342 was purchased from AAT Bioquest (Sunnyvale, CA).

4.2 | Construction of ΔCapJ and ΔCapJ-in *Helicobacter pylori*

Helicobacter pylori 26695 (ATCC 700392) were cultured on blood agar plates containing 10% sheep erythrocytes and incubated in a microaerophilic environment (10% CO_2, 5% O_2, and 85% N_2) at 37°C. A 1,170 bp genomic fragment containing HP0421 (*capJ*) was amplified by PCR with forward and reverse primers: pcapJBamHI-F (GCGGGATCCATGGTTATTGTTTTAGTCGTGGATAG) and pcapJPstI-R (GCGCGATCGTTAATGCTGATAAGGTTTTA AAGAGATG), respectively. The amplified *capJ* was cloned into the BamHI and PstI sites of pGEM-T (Promega). The isogenic mutant *H. pylori* (ΔCapJ) was generated by insertion of the chloramphenicol resistance cassette (*cat*) from pUOA20 into the *capJ* of *H. pylori* through allelic replacement and selection of chloramphenicol-resistant clones (Wang, Roos, & Taylor, 1993). A ΔCapJ-in strain was prepared based on Δ*capJ H. pylori*. A full-length *capJ* along with kanamycin resistance gene cassette (Km^r) was inserted into position of HP0421 in ΔCapJ (Wang et al., 2012). The mutation of *capJ* was verified by DNA sequencing. Correct integration of the antibiotic resistance cassettes into the target genes of the chromosome were verified by PCR (Figure S4). All experimental bacterial strains including WT, ΔCapJ, and ΔCapJ-in *H. pylori* were verified by western blot analysis (Figure S5). WT and mutant *H. pylori* were incubated for 24–48 hr to achieve optimum microbial activity prior to infection of macrophages as described previously (Hung et al., 2015). To prepare the rCGT, *capJ* was sub-cloned and expressed in *E. coli* as described previously (Wang et al., 2012). The activity and concentration of rCGT were assessed by thin-layer chromatography analysis.

4.3 | Cell culture

Macrophage cell line J774A.1 (ATCC TIB67) cells were cultured in Dulbecco's Modified Eagle's Medium, supplemented with 10% endotoxin-free fetal bovine serum (HyClone, Logan, UT) in a humid atmosphere containing 5% CO_2. The medium used in the bacterial infectious system contained without antimicrobial agents.

4.4 | Bacterial internalisation assay

A gentamycin protection assay was employed in this study as described previously (Lai et al., 2008). Briefly, J774A.1 cells (1×10^5) were seeded in 24-well plates and incubated for 16 hr at 37°C. Cells were infected with WT, ΔCapJ, and ΔCapJ-in *H. pylori* at a multiplicity of infection (MOI) of 100 for 2, 6, and 16 hr, respectively. Gentamycin (100 μg/ml) was added into each well and incubated for 1.5 hr. The cells were washed with phosphate-buffered saline and then lysed with sterilised H_2O. The diluted cell lysates were applied to sheep blood agar plates. *H. pylori* colonies were counted after 3- to 5-day incubation. The number of colonies were calculated and represented in colony-forming units.

4.5 | Western blot analysis

J774A.1 cells (2×10^6) were seeded in 6-well plates prior to infection with *H. pylori* at an MOI of 100 for 6 hr. *H. pylori*-infected cells were washed three times with phosphate-buffered saline and then boiled in SDS-PAGE sample buffer for 5 min. The samples were resolved by 12% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA). The membranes were incubated with antibodies followed by exposed to horseradish peroxidase-conjugated secondary antibody (Invitrogen, Carlsbad, CA). The proteins of interest were detected using the ECL Western Blotting Detection Reagents (GE Healthcare, Piscataway, NJ).

4.6 | Immunofluorescence labelling and confocal microscopic analysis

J774A.1 cells (2×10^5) were seeded on cover glass in 6-well plates at 37°C for 16 hr. The cells were infected with WT or ΔCapJ *H. pylori* at an MOI of 100 for 6 hr and fixed with 3.7% paraformaldehyde for 30 min. The cells were then permeabilised with 0.1% Triton X-100 for 30 min and probed with Hoechst 33342, Cyto-ID, CTX-B, and anti-*H. pylori* antibody to observe nuclei, autophagy, lipid rafts, and *H. pylori*, respectively (Liao et al., 2017a). The lysosome formation was stained by using a Cell Navigator Lysosome Staining Kit (AAT Bioquest, Sunnyvale, CA). The stained cells were then analysed using a confocal laser scanning microscope (LSM 780, Carl Zeiss, Göttingen, Germany) with a 100× objective as described previously (Lin et al., 2016).

4.7 | Lentiviral vector production and short hairpin RNA transfection

Short hairpin (sh) RNA lentiviral particles and shRNA were purchased from National RNAi Core Facility (Academia Sinica, Taipei, Taiwan). The targeting sequence for *atg12* shRNA (TRCN0000257708) was TGGTAAACTGGTCCTGCATTA. Sequence for negative control shRNA (TRCN0000072224) was CGCGATCGTAATCACCCGAGT. The lentiviral vectors were constructed using standard molecular cloning procedures as described previously (Lai et al., 2014). Lentivirus was packaged in HEK293FT cells. J774A.1 cells were then infected with recombinant lentiviruses, and stable cell lines were selected with 3 μg/ml puromycin for 3 days.

4.8 | Quantitative real-time reverse transcription PCR

Total RNA was isolated from cells using TRIzol reagent (Invitrogen) and then reverse transcribed into cDNA using the oligo (dT) primer as described previously (Lu et al., 2012). Quantitative real-time PCR using SYBR Green I Master Mix and a model 7900 Sequence Detector System was conducted according to the manufacturer's instructions

(Applied Biosystems, Foster, CA). The oligonucleotide primers used corresponded to *atg12* (forward 5′-GGCCTCGGAACAGTTGTTTA-3′ and reverse 5′-CAGCACCGAAATGTCTCTGA-3′) and glyceraldehyde-3-phosphate dehydrogenase (forward 5′-AACGGATTTGGTCG TATTGGG-3′ and reverse 5′-CAGGGGTGCTAAGCAGTTGG-3′). After pre-incubation at 50°C for 2 min and 95°C for 10 min, PCR was performed with 40 cycles of 95°C for 15 s and 60°C for 1 min. The threshold was set above the non-template control background and within the linear phase of target gene amplification in order to calculate the cycle number at which the transcript was detected (denoted as C_T).

4.9 | Time-lapse image analysis

J774A.1 cells were stained with Alexa 555-conjugated CTX-B for labelling GM1 and Cyto-ID for labelling autophagosomes and incubated for 30 min. The treated cells were infected with *H. pylori* (MOI = 10). Infected live cells were inspected using a Zeiss Axiovert Z1 inverted fluorescence microscope at 20-s intervals for 10 min. The time-lapse sequence images were analysed by Axiovision 4.7.1 software (Carl Zeiss) as described in our previous study (Wang et al., 2012).

4.10 | Statistical analysis

Differences in results between two groups were evaluated using Student's *t* tests. For analyses of variance, one-way analysis of variance (one-way analysis of variance with post hoc Tukey Honestly Significant Difference test) was utilised. The data were displayed as mean ± standard error of the mean. Statistical analyses were performed using SPSS program (version 11.0, SPSS Inc., Chicago, IL). The difference was considered significant when $P < 0.05$.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTION

C. H. L. and W. C. W. contributed to the conception or design of this work and wrote the manuscript. J. C. H., H. H. C., H. Y. H., M. Z. H., Y. A. C., C. Y. H., Y. C. P., Y. T. C., W. Y. S., J. S. L. conducted the experiments. Y. F. W., M. C. W., T. J. C., Y. F. C., and H. J. W. analysed and interpreted the data. All authors gave final approval of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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