RESEARCH ARTICLE

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Insufficient Acidification of Autophagosomes Facilitates Group A Streptococcus Survival and Growth in Endothelial Cells

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ABSTRACT **Group A streptococcus (GAS) is an important human pathogen, and its invasion via blood vessels is critically important in serious events such as bacteremia or multiorgan failure. Although GAS was identified as an extracellular bacterium, the internalization of GAS into nonphagocytic cells may provide a strategy to escape from immune surveillance and antibiotic killing. However, GAS has also been reported to induce autophagy and is efficiently killed within lysosome-fused autophagosomes in epithelial cells. In this study, we show that GAS can replicate in endothelial cells and that streptolysin O is required for GAS growth. Bacterial replication can be suppressed by altering GAS gene expression in an acidic medium before internalization into endothelial cells. The inhibitory effect on GAS replication can be reversed by treatment with bafilomycin A1, a specific inhibitor of vacuolar-type H^+-ATPase. Compared with epithelial cells in which acidification causes autophagy-mediated clearance of GAS, there was a defect in acidification of GAS-containing vesicles in endothelial cells. Consequently, endothelial cells fail to maintain low pH in GAS-containing autophagosomes, thereby permitting GAS replication inside LAMP-1- and LC3-positive vesicles. Furthermore, treatment of epithelial cells with bafilomycin A1 resulted in defective GAS clearance by autophagy, with subsequent bacterial growth intracellularly. Therefore, low pH is a key factor for autophagy-mediated suppression of GAS growth inside epithelial cells, while defective acidification of GAS-containing vesicles results in bacterial growth in endothelial cells.**

IMPORTANCE **Previous reports showed that GAS can induce autophagy and is efficiently killed within lysosome-fused autophagosomes in epithelial cells. In endothelial cells, in contrast, induction of autophagy is not sufficient for GAS killing. In this study, we provide the first evidence that low pH is required to prevent intracellular growth of GAS in epithelial cells and that this mechanism is defective in endothelial cells. Treatment of GAS with low pH altered GAS growth rate and gene expression of virulence factors and resulted in enhanced susceptibility of GAS to intracellular lysosomal killing. Our findings reveal the existence of different mechanisms of host defense against GAS invasion between epithelial and endothelial cells.**

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Group A streptococcus (GAS) (*Streptococcus pyogenes*) is a common human pathogen and causes a wide spectrum of human diseases. Despite the availability of effective antimicrobial agents, there has been a worldwide increase in the incidence of serious invasive GAS infection in recent years (1–5). Although GAS is considered an extracellular pathogen, internalization of GAS by nonimmune cells may provide a way for bacteria to escape from phagocytosis and antibiotic killing (6–9). Small numbers of GAS may hide inside cells during a carrier stage in asymptomatic hosts (10–13).

Autophagy is an intracellular degradative process that is important for balancing sources of energy at critical times in development and in response to nutrient stress. An autophagosome is established at the interaction site of the endoplasmic reticulum and mitochondria (14) and is formed by an elongating double-membrane structure, called an isolation membrane, that surrounds cargo and then closes to form a large vacuole-like structure. Long-lived proteins or damaged organelles, such as endosomes, lysosomes, or mitochondria, are targeted by an isolation membrane and degraded in the resultant autophagolysosome (15–18). In addition to intracellular components, autophagy is also important for defense against invading pathogens (19–22), although it is not always beneficial to host cells (23, 24). Similar to endosomes and phagosomes, autophagosomes also rely on lysosome function to degrade cargo. The lysosome contains digestive hydrolytic enzymes and a low-pH microenvironment. This envi-

ronment is maintained by proton pumps and chloride ion channels and is necessary for enzyme activity (25). However, in addition to its necessity for enzyme function, this acidity in itself may be able to quickly suppress pathogen growth in late-endosomes/phagosomes upon fusion with lysosomes (26).

It has been previously shown that following entry into epithelial cells, GAS escapes from the endosome into the cytoplasm and is trapped in autophagosome-like compartments, which in turn fuse with lysosomes, resulting in GAS death (20, 22, 27–29). Nevertheless, a globally disseminated serotype, M1T1, of GAS can evade autophagy and replicate efficiently in the cytosol of infected epithelial cells (30). Compared with epithelial cells, both M1 and M3 GAS have been found to invade endothelial cells (31–33). In addition to invasion, M1 GAS can also survive in endothelial cells (33). However, the reasons as to why and how GAS grows inside endothelial cells remain unclear. In the present study, we show that GAS can replicate in endothelial cells irrespective of autophagy induction. Not only the M1 serotype but also other serotypes tested, including M4, M6, M12, and M49, can grow inside endothelial cells. Compared to epithelial cells in which acidification causes autophagy-mediated clearance of GAS, low pH is not maintained in GAS-containing autophagosomes in endothelial cells. In this study, we show that low pH is important for suppressing GAS survival and that deacidification in endothelial cells inhibits lysosomal bacterial killing.

RESULTS

Replication of GAS with different M serotypes in endothelial cells. A previous study showed clearance of GAS by autophagy in epithelial cells (22). In contrast, M1 GAS invades and grows in endothelial cells (33). However, the machinery of GAS replication inside endothelial cells remains unclear. In this study, we checked the fate of GAS inside endothelial cells. We infected HMEC-1 cells (human microvascular endothelial cell line 1) with different strains of GAS for 1 h and used gentamicin to kill extracellular bacteria. Results showed that intracellular bacterial numbers of GAS strains A20 (M1 serotype), 125 (M4 serotype), 712 (M6 serotype), 733 (M12 serotype), and NZ131 (M49 serotype) showed a time-dependent increase postinfection in HMEC-1 cells (Fig. 1A). In contrast to the growth in HMEC-1 cells, these GAS strains did not grow in A549 epithelial cells (see Fig. S1 in the supplemental material). To confirm that the GAS particles existed within the cell and were not associated with the plasma membrane, NZ131-infected HMEC-1 cells were stained with 4′,6′-diamidino-2-phenylindole (DAPI) and observed under confocal microscopy. The GAS particles in cytoplasm, as detected by DNA staining of DAPI, increased in a time-dependent manner (Fig. 1B). GAS replication inside HMEC-1 cells was further confirmed using polyclonal anti-GAS antibodies followed by flow cytometric analysis (Fig. 1C and D).

A previous report showed that streptolysin O (SLO) is required for damage of the endosomal membrane and further contributes to the escape of GAS from the endosome to the cytoplasm of the epithelial cell (22). In addition, recent reports showed that SLO cooperates with NAD-glycohydrolase against lysosomal killing and prolongs the intracellular survival of GAS in keratinocytes and macrophages (34, 35). To determine whether SLO is also required for GAS growth in endothelial cells, we examined bacterial growth of wild-type and SLO-deleted GAS. The results showed that the SLO-deleted A20 strain could not grow in endothelial cells (see Fig. S2A in the supplemental material). We also tested wild-type JRS4 (M6 serotype) and SLO-deleted mutant strains, which were previously examined in epithelial cells (22). The results showed that wild-type JRS4 grew in endothelial cells, whereas the SLO mutant strain could not even survive in the cells (Fig. S2B). Therefore, SLO is required for GAS growth in endothelial cells.

Low pH affects GAS growth in endothelial cells. The consumption of nutrients and the accumulation of metabolic byproducts are sensed by bacteria which alter gene expression and growth phase (36–38). Previous reports showed that pH was decreased in GAS long-term culture medium and that a modified medium with adjusted pH could alter GAS gene expression and survival (39–43). We confirmed the pH change in the broth medium during growth, and the results were consistent with previous findings that pH gradually decreased with GAS growth over time and that the lowest pH level (pH ~5.5) was reached when bacterial growth reached stationary phase (Fig. 2A). We therefore compared the growth rate in endothelial cells between stationary-phase GAS and GAS collected in log phase. There was no significant difference in the internalization efficiency of GAS between log and stationary phases (see Fig. S3A in the supplemental material). GAS collected in the stationary phase grew much more slowly inside endothelial cells than did GAS collected in log phase (Fig. 2B). To investigate whether low pH affects GAS growth inside endothelial cells, we infected cells with acid-pretreated GAS. The results indicated that with no difference in the internalization efficiency (Fig. S3B), acid-pretreated GAS had a decreased ability to grow and survive in endothelial cells (Fig. 2C).

We hypothesized that pH change is sensed by the bacteria which then attenuate growth to adapt to the environmental change. To confirm that pH was a cause of decreased bacterial growth, we adjusted the pH levels of fresh broth medium and measured GAS growth. Our results showed that an acidic environment suppressed GAS growth (Fig. 2D). When GAS was first incubated in neutral (pH 7) or acidic (pH 5.5) medium and then shifted into fresh neutral medium, results showed that restoring pH from pH 5.5 to neutral pH rescued the growth rate to levels seen for non-acid-treated GAS (Fig. 2E). Furthermore, treatment of endothelial cells with bafilomycin A1 (Baf A), a specific inhibitor of vacuolar-type H^+-ATPase, rescued growth of bacteria pretreated with low pH (Fig. 2F). We next separated GAS-infected cells into two types, one we called GAS low-growth type (L type; Fig. 3Aa and b) and the other GAS high-growth type (H type; Fig. 3Ac and d). The results showed that the distribution of acid-pretreated GAS in endothelial cells was limited to the L-type pattern, while nonacidified GAS showed an H-type distribution (Fig. 3Ae and B). We also confirmed that acid-pretreated GAS was restricted to LAMP-1-positive vacuoles, a late endosomal or lysosomal marker (Fig. 3C). These data indicate that suppression of GAS growth requires a consistent low pH in bacterial culture medium as well as in the intracellular bacterium-containing vacuolar environment.

Low pH alters GAS virulence gene expression. In addition to affecting GAS growth, a simple 1-h acid treatment could directly alter GAS virulence gene expression, including that of *spn* (*nga*), *slo*, *sagB*, and *speB* (Fig. 4A). The *slo* and *sagB* genes are responsible for producing hemolytic proteins (SLO and SLS), which damage cell membrane structure and help GAS escape from endosomes (22). Expression of *spn* (*nga*) gene produces a NAD-glycohydrolase, which is important for the virulence of GAS (44)

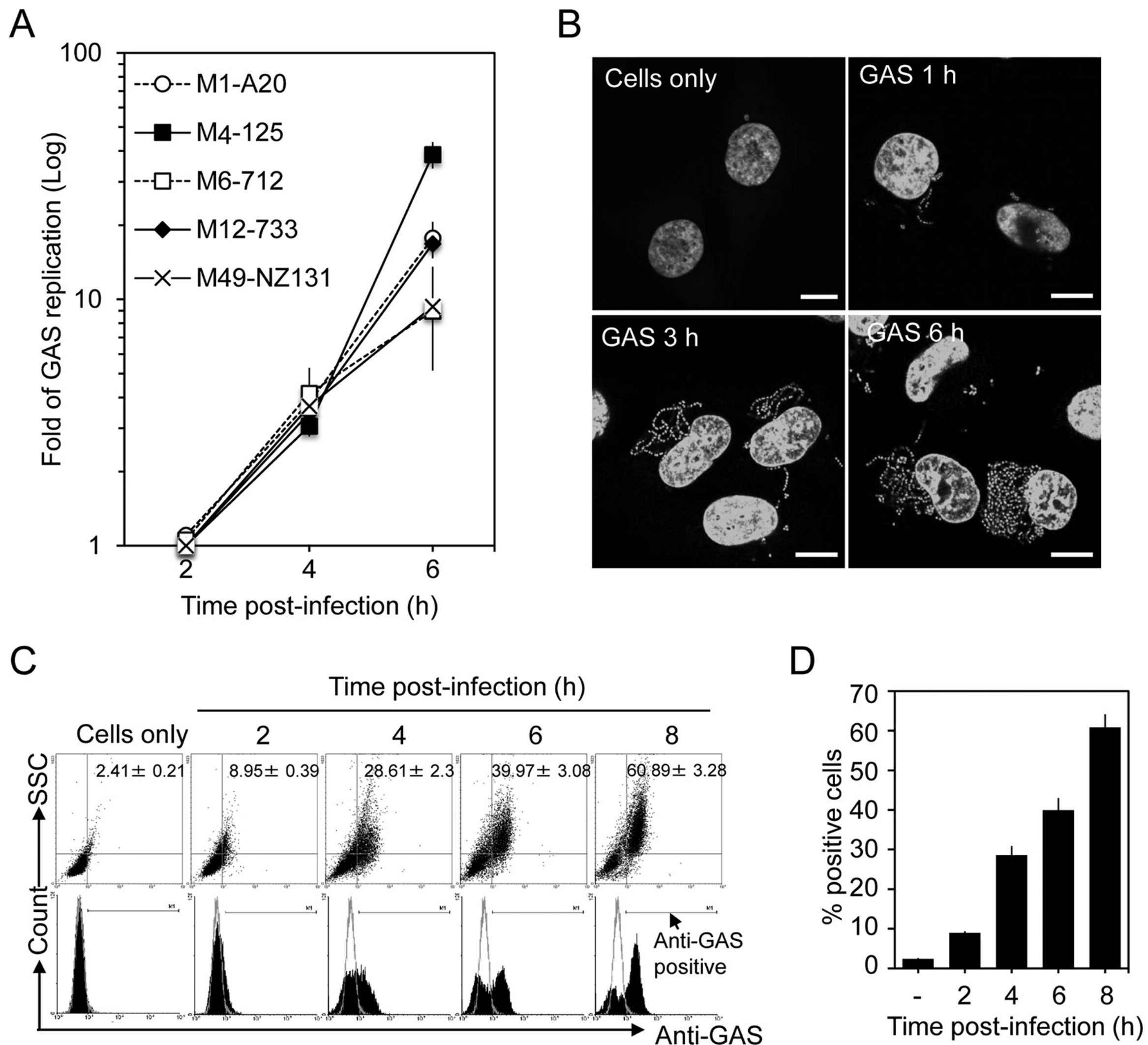

FIG 1 Replication of GAS in endothelial cells. (A) HMEC-1 cells were infected with serotype M1 (A20 strain), M4, M6, M12 (clinically isolated invasive strains 125, 712, and 733 [strain numbers assigned serially] of serotypes M4, M6, and M12, respectively) and M49 (NZ131 strain) at an MOI of 1 for 1 h. Extracellular bacteria were killed by gentamicin treatment. The numbers of intracellular bacteria were determined via a colony-forming assay at various time points postinfection. The fold GAS replication was calculated and normalized to the number of internalized bacteria at 2 h postinfection. Data represent the means ± standard deviations (SD) (error bars) from three independent experiments. (B) NZ131-infected HMEC-1 cells were stained with DAPI and observed by confocal microscopy at 1, 3, and 6 h. Bars, 10 μm. (C) HMEC-1 cells were infected with strain NZ131 at an MOI of 25 for 1 h and then treated with gentamicin. At various time points postinfection, cells were stained with anti-GAS polyclonal antibody and analyzed by flow cytometry. (C and D) One set of histograms is shown (C), and the means plus SD from three independent experiments are shown (D). SSC, side scatter, is represented as cell granularity or internal complexity.

and for avoidance of phagolysosome killing (34, 35). Our results indicated that following low-pH treatment, GAS showed reduced intracellular survival. Without the ability to rupture the endosomal membrane and resist antilysosomal digestion, the acid-pretreated GAS could be easily cleared by endosomal degradation in endothelial cells.

Instead of suppressing gene expression, acid treatment strongly increased *speB* gene expression (Fig. 4A), and this is consistent with our previous finding that low pH stimulates SpeB protein production (43). SpeB, streptococcal pyrogenic exotoxin, is a cysteine protease which allows GAS to digest host tissue and immune mediators (45). A recent report showed that in addition to a role for GAS dissemination among host tissues, SpeB also provides protease activity to cleave autophagy receptors and enhance GAS intracellular survival in epithelial cells (30). However, our results showed that low-pH treatment induced *speB* expression but did not enhance GAS intracellular growth in endothelial cells. Moreover, the growth activity of the *speB*-deleted GAS strain was similar to that of the wild-type GAS (Fig. 4B). However, both wild-type and *speB*-deleted strains were suppressed in epithelial cells (Fig. 4C). Taken together, these results indicate that acid pretreatment suppresses expression of various virulence genes, but not *speB*, and that acid pretreatment abolishes the growth of GAS in endothelial cells.

Acidification is defective in GAS-containing vesicles in endothelial cells. In general, vesicular pH begins to drop immediately after bacteria are internalized into endosomes or autophagosomes, and this is expedited by subsequent fusion with lysosomes, which possess a more acidic microenvironment. To investigate whether lysosome function is deficient and results in bacterial

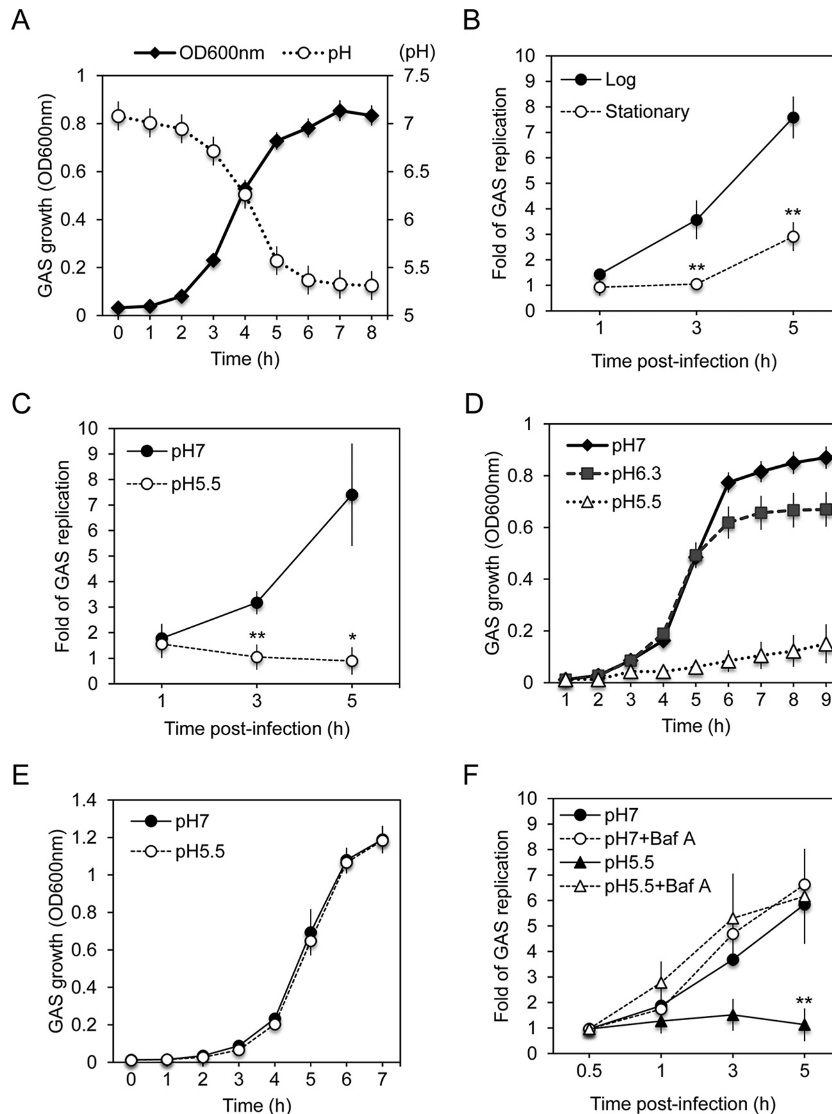

FIG 2 Low pH affects GAS growth in endothelial cells and in TSBY broth. After overnight culture, GAS (NZ131 strain) bacteria were transferred to fresh broth and cultured at 37°C. (A) Bacterial growth was determined at OD_{600} at various time points. Bacterial culture supernatants were collected by centrifugation and filtration at the time points indicated, and pH was determined in the supernatants. (B) Bacteria cultured overnight were used as "stationary-phase" GAS, and bacteria treated with fresh medium for 3 h (refreshed GAS) were used as "early-log-phase" GAS. Early-log-phase GAS depicted here was used as the standard culture for the rest of experiments. (C) GAS bacteria refreshed for 3 h were further incubated in fresh neutral or low-pH broth for 1 h, depicted as acid pretreatment. HMEC-1 cells were infected with indicated GAS (B and C) at an MOI of 5 for 30 min, and then gentamicin was added to kill extracellular bacteria. Bacteria were collected at the indicated time points after gentamicin treatment, and the fold GAS replication was calculated and normalized to the number of internalized bacteria (see Fig. S3 in the supplemental material). (D) After overnight culture, GAS bacteria were transferred to fresh or pH-adjusted TSBY broth (pH ± 0.2). (E) Acid-pretreated GAS bacteria were further transferred into fresh neutral broth. Bacterial growth in the broth was measured at OD_{600} at various time points. (F) HMEC-1 cells were pretreated with bafilomycin A1 (Baf A) (100 nM) for 1 h and infected with indicated GAS for 30 min. The colony-forming assay as in panels B and C was then performed. Data represent the means ± SD from three independent experiments. *, $P < 0.05$; **, $P < 0.01$.

growth in endothelial cells but not in epithelial cells, we first used LAMP-1 as a lysosome marker to observe the locations of lysosomes and autophagosomes. The results showed that LAMP-1 colocalized with LC3, an autophagosome marker, in both cell types after GAS infection (Fig. 5). However, LC3 and LAMP-1 double-positive vesicles were enlarged in endothelial cells compared with epithelial cells at 6 h postinfection. Although GAS-induced autophagy is known for its large autophagosome size compared to canonical autophagy, this large autophagolysosome did not suppress bacterial growth inside endothelial cells. Ultimately, the physical force of GAS replication was apparently sufficient to rupture the damaged vesicle (Fig. 5A, white arrows). Furthermore, bacterial growth in endothelial cells was also observed outside LC3-positive vesicles as shown by DAPI staining (Fig. 5A). In contrast, GAS-containing LC3-positive vesicles gradually disappeared in epithelial cells, indicating that autophagic flux proceeded to completion and that GAS growth was suppressed (Fig. 5B, white arrowheads). These data indicate that the recruitment of LAMP-1 and LC3 does not provide the same suppressive activity in endothelial cells as in epithelial cells.

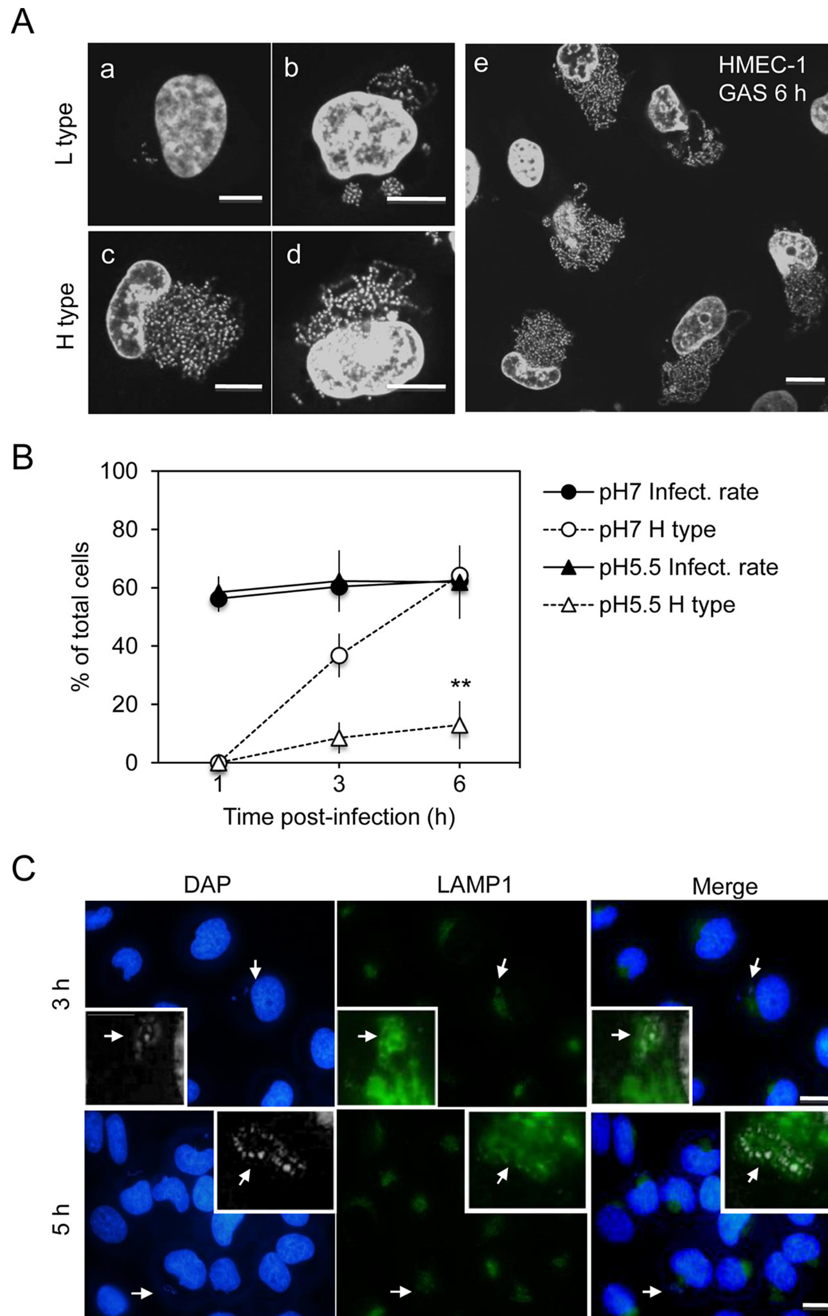

FIG 3 Low pH is required for suppression of GAS replication in endothelial cells. (A) The intracellular GAS (NZ131 strain) as stained by DAPI was classified into two groups, low growth (L type) (a and b) and high growth (H type) (c and d). After 6-h infection, most of the infected HMEC-1 cells have an H-type pattern (e). Bars, 10 μm. (B) HMEC-1 cells infected with GAS (with or without acid pretreatment) were stained with DAPI, and the percentages of cells with GAS infection and cells with H-type pattern were calculated by confocal microscopy. More than 100 cells in each sample were observed, and the means ± SD from three independent experiments are shown. **, $P < 0.01$. (C) HMEC-1 cells were infected with acid-pretreated GAS and then fixed and stained with anti-LAMP-1 antibody and DAPI at 3 and 5 h postinfection. Images were observed under confocal microscopy. Bars, 10 μm.

Since lysosome fusion can induce a low-pH environment in endosomes or autophagosomes and contribute to suppression of bacterial growth, one outstanding question is why lysosomal function is insufficient in endothelial cells to clear GAS. We therefore measured the pH of the GAS-containing, LC3 and LAMP-1 double-positive compartment using lysotracker as a low-pH indicator (pH < 5.5). LC3- and LAMP-1-positive vesicles were highly decorated with lysotracker dye in epithelial cells but not in endothelial cells (Fig. 6A, white arrows and areas surrounded by a dotted line). The quantitative results showed that GAS-containing autophagosomes (LC3 positive) were highly colocalized with lysosomes (LAMP-1) and that the efficiency of recruitment was not different between the two cell types. However, the percentage of lysotracker positivity with LC3 and LAMP-1 double-positive GAS

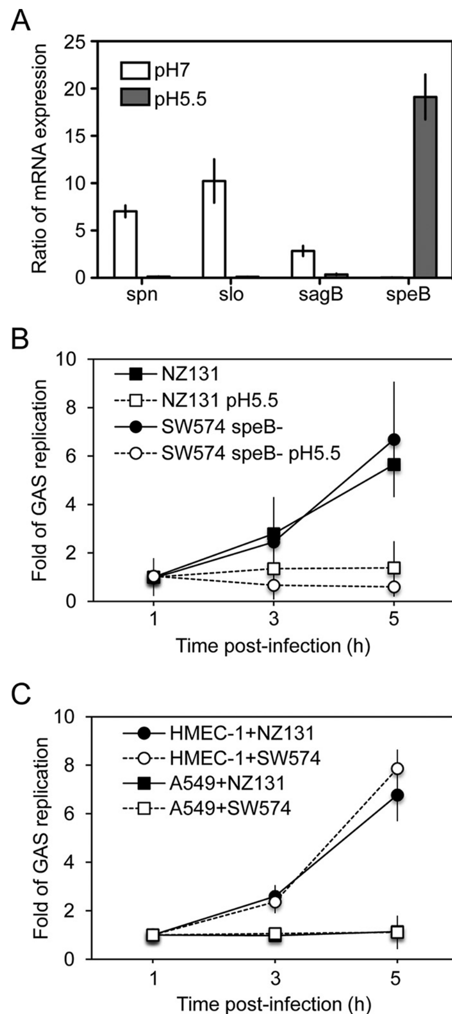

FIG 4 Low pH affects GAS virulence factor gene expression. (A) Refreshed GAS (NZ131 strain) bacteria were transferred to neutral or acidic TSBY for 1-h incubation. GAS mRNAs were collected and detected by real-time PCR using KAPA SYBR fast PCR kits. Data represent the means ± SD from three independent experiments. (B and C) HMEC-1 (B and C) and A549 cells (C) were infected with wild-type and *speB*-deleted NZ131 with or without acid pretreatment. Gentamicin was added to kill extracellular bacteria. The colony-forming assay was performed, and the fold GAS replication was calculated and normalized to the number of internalized bacteria at 1 h postinfection. Data represent the means ± SD from three independent experiments.

was much lower in endothelial cells than in epithelial cells (Fig. 6B). Restricted GAS growth within acidic autophagosomes was also confirmed in normal rat kidney epithelial cells (NRK) (see Fig. S4 in the supplemental material). These data indicate that low pH is important for epithelial cells to suppress GAS growth via autophagy, while a loss of low-pH preservation in LC3- and LAMP-1-positive vesicles occurs in endothelial cells.

Low pH is required for suppression of GAS intracellular growth. In order to further confirm whether acidic pH is a key factor affecting intracellular GAS growth, we used Baf A to block vesicle acidification (see Fig. S5A in the supplemental material) and confirmed its nontoxic effect on GAS growth (Fig. S5B). We found that autophagy-suppressed GAS can grow in Baf A-treated epithelial cells, indicating that even autophagosomes require low pH to kill bacteria (Fig. 7A). Furthermore, the distribution of GAS in epithelial cells was limited to the L-type pattern with very few H types, while GAS-infected endothelial cells showed an H-type distribution (Fig. 7B). Taken together, these data indicate that maintaining a low-pH environment in vesicles is important for intracellular suppression of GAS growth.

DISCUSSION

In the present study, low pH is identified as a major factor in reducing GAS replication in the intracellular environment. The process of vesicular acidification is strictly regulated in cells, and its compartmentalization in vesicles contributes to intracellular bacterial clearance. In epithelial cells, autophagosomes engulf GAS to provide an intact compartment for maintenance of low pH and lysosome function. Bacterial growth could be suppressed through endosomal degradation when GAS was pretreated with low-pH medium before internalization. In endothelial cells, however, acidification is not maintained inside GAS-containing vesicles, even though LC3 and LAMP-1 markers are readily recruited to them. Our results provide evidence for the requirement of low pH to suppress intracellular GAS growth in epithelial cells by autophagy, while defective acidification permits GAS replication in endothelial cells (Fig. 8).

After GAS-containing endosomes mature into late endosomes or GAS is surrounded by an autophagosome, the pH inside starts to decrease in either case and facilitates fusion with lysosomes (26, 46). Although our results indicate that LAMP-1 tagged LC3-positive vesicles, we cannot be sure whether lysosomes, specifically, had already fused with them or not because LAMP-1 is a marker for both lysosomes and late endosomes. However, irrespective of which stage of maturation these vesicles are, (i.e., late endosomal, autophagolysosomal, or lysosomal), low pH is required for normal processing. Most strikingly, a low-pH compartment was not maintained in endothelial cells, and this resulted in a lack of suppression of GAS growth.

Loss of low pH could possibly be due to a defect in proton transport from the cytoplasm into vesicles or due to protons leaking out from pores made by bacterial virulence factors. Although we treated cells with Baf A, which specifically blocks proton transfer from the cytoplasm into vesicles and abolishes the inhibitory activity of GAS replication in epithelial cells, the exact mechanism of proton loss from vesicles in GAS-infected cells remains unclear. Previous reports showed that loss of acidification abrogated phagosome maturation and that treatment with Baf A could affect lysosome fusion with autophagosomes (47). However, we found that LAMP-1 was localized on neutral-pH autophagosomes in endothelial cells, indicating that these events can be uncoupled.

Modification of pH has been reported to affect transcription of virulence factor genes, including *slo*, *sagB*, and *mga*, of GAS in stationary phase and might also relate to bacterial growth phase (40). Expression of the *mga* gene regulates GAS surface proteins, controls GAS colonization, and impairs phagosome acidification in cells of the vascular system (48, 49). We also demonstrated that a simple treatment with low-pH medium could reduce GAS virulence factor gene expression, and these were important for GAS intracellular survival. This implies that early acidification could be a protective mechanism by the host to reduce *slo*, *sagB*, *spn* (*nga*), and *mga* gene expression and decrease their virulent effects on membrane rupture and phagosome acidification. This suggests there may be competition between GAS virulence factor activation and host vesicle acidification. A recent report showed that

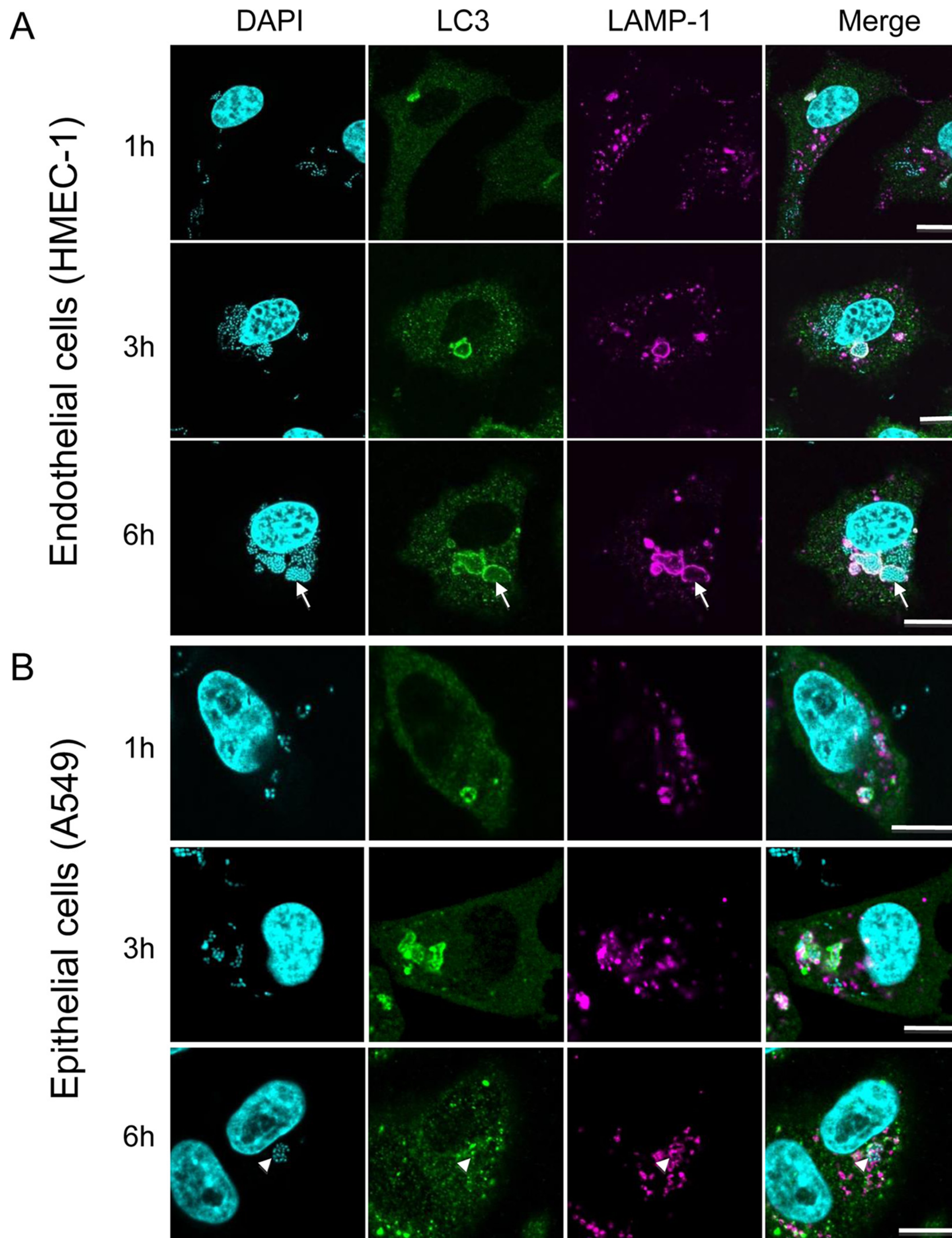

FIG 5 Lysosomes fuse with LC3-positive autophagosomes. (A and B) HMEC-1 (A) and A549 (B) cells were infected with GAS (NZ131 strain) for 30 min, and then gentamicin was added to kill extracellular bacteria. Cells were fixed and stained with anti-LC3 and anti-LAMP-1 antibodies at the indicated time points and then observed by confocal microscopy. Bars, 10 μm.

SLO and NAD-glycohydrolase prevented phagolysosome acidification and promoted GAS survival in macrophages (35). Therefore, there are complex interplays between bacterial virulence factors and autophagy components (50). Consequently, epithelial cells respond quickly to endosomal damage by enlisting the autophagic machinery to capture GAS directly or even the GAS-containing endosome within an autophagosome prior to the formation of a large enough rupture in the endosomal membrane that would allow GAS or proton escape. This process is highly effective in suppressing GAS growth during the very early stages of infection, and it also highlights the importance of maintaining an acidic compartment as a vital first step in the fight against invading bacteria that can damage membranes.

However, a previous report showed that SpeB cleaves autophagy receptors and contributes to M1T1 GAS escape from autophagy in epithelial cells (30). In that study, the growth of intra-

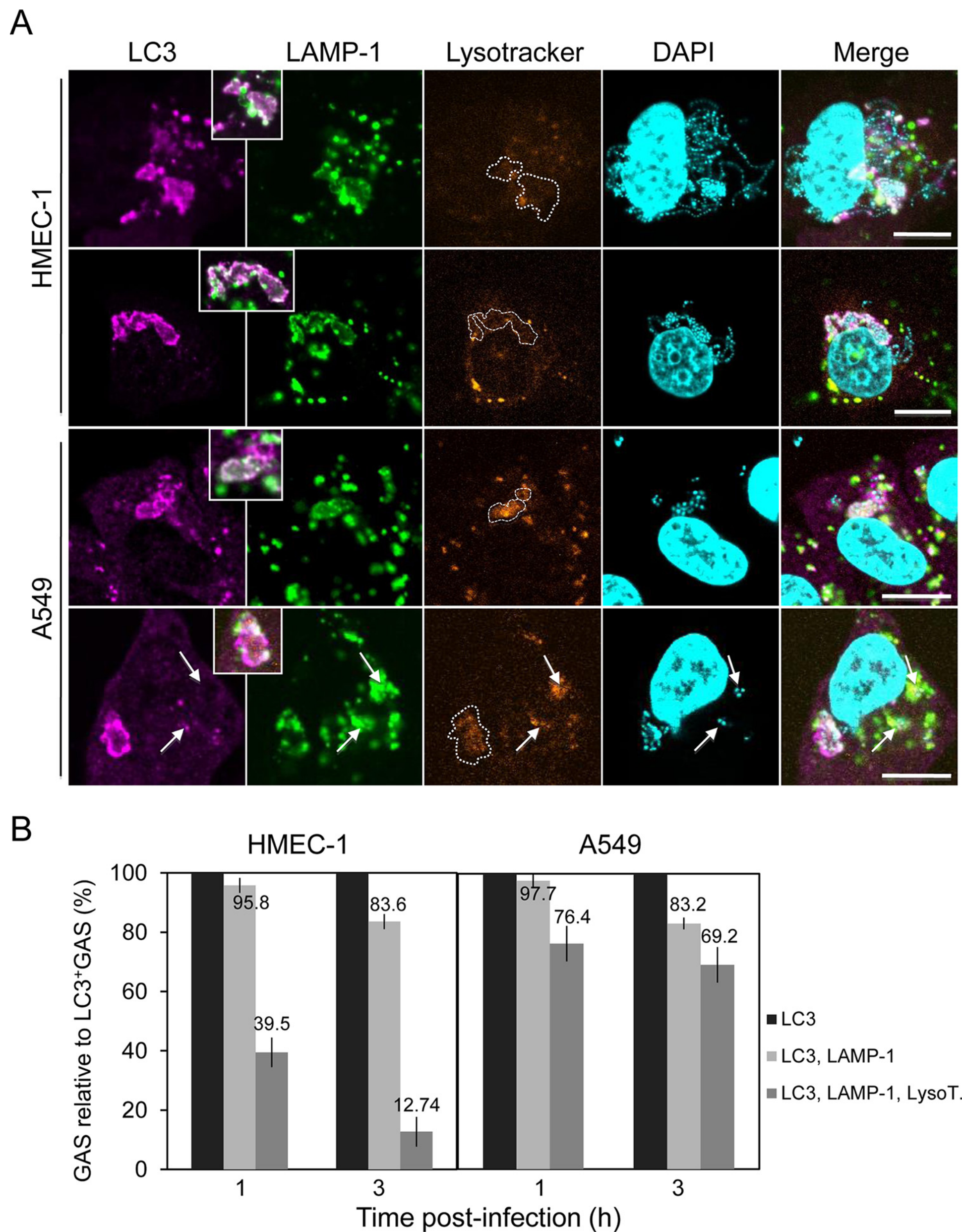

FIG 6 Lysosome-fused vesicles in endothelial cells do not maintain an acidic environment. After lysotracker (75 nM) prestaining for 1 h, the cells were infected with GAS (NZ131 strain) for 30 min, and then gentamicin was added to kill extracellular bacteria. Cells were fixed and stained with anti-LC3 and anti-LAMP-1 antibodies at 1 and 3 h postinfection. Samples collected at 3 h postinfection were imaged by confocal microscopy (A). Lysotracker (LysoT.) stains strongly in LC3- and LAMP-1-positive vesicles in A549 cells, but not in HMEC-1 cells (white arrows and areas surrounded by a dotted line). More than 100 cells in each sample were observed, and the means ± SD from three independent experiments are shown (B). Bars, 10 μm.

cellular GAS was only about twice as high in the wild type as in *speB*-deleted M1T1 GAS. Possible reasons for the observed differences in the two studies may include differences in the time points of bacterial assay (2, 4, and 6 h versus 8 h postinfection). Our results do not exclude a possible role of SpeB in intracellular protection from autophagic killing in epithelial cells but indicate that such a role may be relatively minor.

One of the GAS strains we used, A20, is also an M1T1 serotype strain, which was isolated from blood from a sepsis patient and with a highly SpeB-expressing phenotype. However, we found that strain A20 could not survive and grow in epithelial cells (see Fig. S1 in the supplemental material). In contrast, A20 could replicate in endothelial cells, and the bacterial number was 10 times higher at 6 h postinfection than the internalized bacterial number (Fig. 1A). We further showed that there was no difference in bacterial numbers between wild-type NZ131 and *speB*-deleted SW574 in endothelial cells (Fig. 4C). Furthermore, acid pretreatment, which increased *speB* expression (Fig. 4A), did not promote GAS intracellular survival (Fig. 4B). In contrast, SLO is required for intracellular survival of GAS, while acid-pretreated GAS and *slo*-deleted GAS decreased the ability of GAS growth in endothelial cells. Our results indicate that the growth of GAS is a cell type-

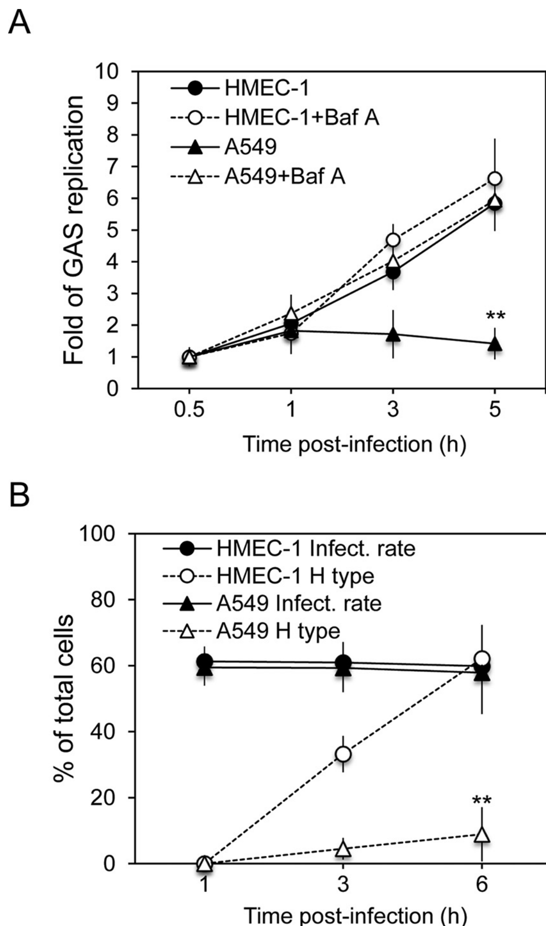

FIG 7 Low pH in epithelial cells is required for suppression of GAS replication. (A) HMEC-1 and A549 cells with or without 1-h pretreatment of bafilomycin A1 (100 nM) were infected with GAS (NZ131 strain) for 30 min and then treated with gentamicin to kill extracellular bacteria. Bacteria were collected at the indicated time points after gentamicin treatment, and the fold GAS replication was calculated and normalized to the number of internalized GAS at 0.5 h postinfection. Data represent the means ± SD from three independent experiments. (B) The H-type GAS pattern, as described in the legend to Fig. 3, was measured in infected HMEC-1 and A549 cells. The percentages of cells with GAS infection and cells with H-type pattern were calculated by confocal microscopy. More than 100 cells in each sample were observed, and the means ± SD from three independent experiments are shown. **, $P < 0.01$.

dependent phenotype and that various virulence factors, but not SpeB, are involved in the intracellular bacterial growth.

Acid pretreatment and a stationary-phase environment may be considered similar to the situation found in the epidermis where colonizing GAS encounter limited nutrients and a gradually decreasing environmental pH and accumulating metabolic products. Taking advantage of the decrease in virulence gene expression upon pH sensing by GAS, keratinocytes and epithelial cells can easily control and clear internalized GAS, especially compared to endothelial cells that encounter GAS in a neutral-pH setting (28, 34). Once GAS spreads into the blood circulation system in sepsis, the neutral pH and energy richness of serum induce GAS to revert to a more virulent phase of gene expression, one that could possibly cause more injury to blood vessels. Using an air pouch infection model established previously (51), our preliminary results indicate that mice are more resistant to stationary-phase GAS than to early-log-phase GAS (data not shown).

Epithelial cells and endothelial cells possess different physiological functions. Epithelial cells generally line externally exposed surfaces which are designed to interact with foreign materials and to provide defense against pathogens. In contrast, endothelial cells generally occupy an internal, sterile, and nutrient-rich environment, which may require less-stringent autophagic responses. A strong autophagic response (e.g., in epithelial cells) is able to suppress intracellular GAS growth, whereas a defective autophagic response such as that seen in *atg7* knockout epithelial cells leads to poor GAS killing and allows for GAS replication (see Fig. S6A and B in the supplemental material). This indicates that besides differences in physiological functions between epithelial and endothelial cells, autophagy is a key step to maintaining low pH in lysosomal/autophagosomal compartments and to suppress intracellular GAS growth. In addition, we confirmed that, standardized to cell size, the original average pH levels of cells are similar between these two cell types (Fig. S7). However, inefficient autophagy in endothelial cells causes the loss of ability to preserve low pH in GAS-containing autophagosomes which results in GAS replication. Most importantly, we showed that both the endothelial cell line (HMEC-1) and also primary endothelial cells (human umbilical vein endothelial cells [HUVECs]; Fig. S8) are unable to clear intracellular GAS due to defective acidification during the autophagy process. It will be of interest in the future to examine other types of endothelial cells for their responses to GAS infection.

Low pH not only influences bacterial virulence directly, it also influences the host response as low pH is vitally important for the activity of degradative enzymes in lysosomes (25). The mechanism as to how a cell maintains an acidic environment in a specific location, such as late endosomes, autophagosomes, and lysosomes, is an important question in cell biology. Here, we show that low pH is maintained through use of the autophagic machinery, which allows cells to defend against intracellular GAS. The detailed molecular mechanisms regarding how GAS can replicate in an uncontrolled manner in endothelial cells requires further investigation. Recent reports showed that phagosomal association with LC3, in the absence of isolation membrane, was required for phagosomal maturation and the subsequent decrease in pH and recruitment of acidic hydrolases. LC3-associated phagosomes fuse with lysosomes and inactivate invading pathogens (52). It has not been reported whether or not LC3 association is required for successful bacterial killing in nonphagocytes. Knocking out core autophagic proteins, including FIP200 and Atg9, in mouse embryonic fibroblast cells led to a lack of isolation membrane formation and bacterial growth suppression, even though LC3 was recruited to *Salmonella*-containing endosomes (53). We speculate that the absence of isolation membrane in endothelial cells, even though LC3 was still found on GAS-containing vesicles, may result in GAS replication inside the ruptured membrane and subsequent growth in the cytoplasm. This hypothesis remains to be confirmed.

In conclusion, low pH directly affects GAS growth in culture medium and is required for autophagy suppression of GAS replication inside cells. The findings obtained from this study may help to gain better understanding and provide potential therapeutic strategies in GAS diseases.

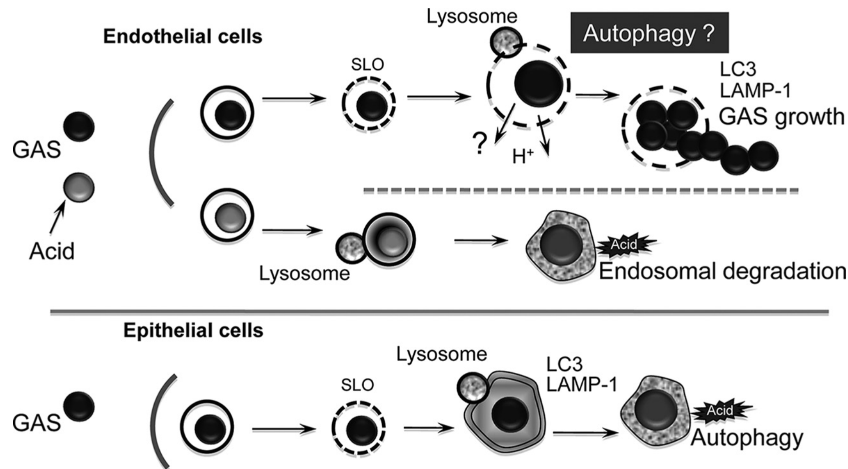

FIG 8 Influence of pH on GAS growth in endothelial and epithelial cells. In endothelial cells, GAS is internalized into cells through endocytosis and escape from endosomes by secreting virulence factors, such as SLO, which rupture membrane structure. Autophagy partially decorates GAS with LC3 and LAMP-1 markers, but this does not preserve a low-pH environment, and GAS replicates inside endothelial cells. Autophagy is inefficient in endothelial cells. Acid pretreatment decreases GAS virulence and growth before infection, and after internalization, endosomal acidification may suppress GAS replication through endosomal degradation. In epithelial cells, on the other hand, autophagy provides better preservation of low pH and efficiently restricts and kills bacteria.

MATERIALS AND METHODS

Cell culture. Human microvascular endothelial cell line 1 (HMEC-1 cells), obtained from the Centers for Disease Control and Prevention, USA, were grown in culture plates containing endothelial cell growth medium M200 (Cascade Biologics) composed of 10% fetal bovine serum (FBS), 1 μg/ml hydrocortisone, 10 ng/ml epidermal growth factor, 3 ng/ml basic fibroblast growth factor, and 10 μg/ml heparin. HMEC-1 cells retain the morphological, phenotypic, and functional characteristics of normal human microvascular endothelial cells (54). Human lung carcinoma epithelial A549 cells and normal rat kidney epithelial cells (NRK) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. Cells were cultured at 37°C in 5% CO_2 and detached with 1,000 U/ml trypsin and 0.5 mM EDTA for passage. Once cell confluence reached 80%, the cells were detached with trypsin-EDTA and seeded at 1×10^6 cells in 10-cm dishes for maintenance, 2×10^5 cells in 6-well plates for flow cytometry analysis, 8×10^4 cells in 24-well plates for colony-forming assay, and 6×10^4 cells in 24-well plates with cover glass for fluorescence microscope observation. For acidification inhibition, cells were pretreated with bafilomycin A1 (catalog no. B1793; Sigma) at a concentration of 100 nM for 1 h and then infected with group A streptococcus (GAS) as described in the infection protocol.

Bacteria. GAS strain NZ131 (M49 serotype) was a gift from D. R. Martin (New Zealand Communicable Disease Center, Porirua), and *speB*-deleted strain SW574 was described previously (55). A20 (M1T1 serotype), SW555 (*slo*-deleted A20 strain), JRS4 (M6 serotype), and *slo*-deleted JRS4 strains were from J. J. Wu (Department of Medical Laboratory Science and Biotechnology, National Cheng Kung University Medical College, Tainan, Taiwan), and other clinically isolated strains were obtained from C. C. Liu (Department of Pediatrics, National Cheng Kung University Medical College, Tainan, Taiwan). All of these GAS strains were isolated from patients with GAS-infected invasive diseases, such as sepsis, streptococcal toxic shock syndrome (STSS), glomerulonephritis, and necrotizing fasciitis. GAS grew at 37°C in tryptic soy broth containing 0.5% yeast extract (TSBY) overnight and was transferred to fresh broth at 1:50 dilution for 3 h (refreshed GAS). The refreshed GAS, early-log GAS, was used as the standard culture for the experiments. The bacteria were harvested by centrifugation (3,500 rpm, 10 min, 4°C), resuspended in phosphate-buffered saline (PBS), and the concentration was determined using an optical density at 600 nm (OD_{600}) of 0.2 as 1×10^8 CFU/ml and confirmed by viable-colony counting.

pH detection. After GAS growth for various times, bacterial culture supernatant was collected by centrifugation and then put through a 0.22-μm filter. The pH values were measured with a pH meter (F-52; Horiba). For acid inhibition experiments, fresh broth pH was adjusted with hydrochloric acid (HCl) and added with GAS to culture for the indicated time periods.

Infection model. Cells at 80% confluence were plated in 24-well plates or 6-well plates and incubated overnight. The prepared bacteria with or without acidic medium pretreatment for 1 h were directly added into wells at various multiplicities of infection (MOI). In order to ensure simultaneous infection of cells, the plates were centrifuged at $500 \times g$ for 5 min at 4°C. After 30-min or 1-h incubation, the cell culture was washed three times with PBS, and fresh medium containing 100 μg/ml gentamicin was added to kill extracellular bacteria. The cells were collected at various periods of time as indicated in each experiment.

Flow cytometry analysis for intracellular GAS staining. Cells were seeded at 2×10^5 in 6-well plates for overnight culture and infected with GAS at an MOI of 25 for 1 h. Gentamicin was added to kill extracellular bacteria. Cells were collected by trypsinization and fixed with 4% paraformaldehyde, permeabilized by 0.1% saponin in PBS containing 2% FBS, and stained with 1:10,000 diluted rabbit polyclonal anti-GAS antisera for 1 h, which were generated in J. J. Wu's laboratory. After Alexa Fluor 488-conjugated secondary antibody staining, samples were analyzed by flow cytometry (FACSCalibur; BD Biosciences). Staining with secondary antibody alone was used as the background control.

Real-time PCR. GAS RNA was isolated as described previously (56). After reverse transcriptase PCR, cDNA was measured by real-time PCR, according to KAPA SYBR fast PCR kits (KAPA Biosystems, Woburn, MA). The oligonucleotide sequences of primers were as follows: 5′-TCC TGCGGATGTGTTTGATA-3′ and 5′-TGCACTAAAGGCCGCTTC-3′ for *slo*, 5′-CTGTGCTTCAGGTGGAGGTT-3′ and 5′-ATAGCACCGTA TTCCGCAAA-3′ for *sagB*, 5′-TGTTGCTATTGCTTTGGCTG-3′ and 5′-TTGAGCCGTCTAATGTGTGC-3′ for *spn* (*nga*), and 5′-AATTGATGG CTGATGTTGGTAT-3′ and 5′-GCTTCCCAATCTTGTTTGGCT-3′ for *speB*. LightCycler 3.0 software (version 3.0; Roche Diagnostics, Indianapolis, IN) was used for analysis of the crossing point (CP). For other parameters, GAS gyrase subunit A (*gyrA*) was set as a reference, the gene of interest was set as target, and then products collected from acid-treated or nontreated GAS were set as sample or control. The relative levels of gene

expression were calculated by the formula: ratio = $2^\wedge[\Delta CP_{target}$ (control − sample) − $\Delta CP_{reference}$ (control − sample)] (57).

Immunofluorescence staining. Cells were seeded at 6×10^4 in 24-well plates with cover glass for overnight culture and infected with GAS for 30 min. For acid indicator stain, lysotracker (red DND-99; Invitrogen) was added at a final concentration of 75 nM for 1-h incubation before GAS infection. Extracellular bacteria were killed by 100 μg/ml gentamicin. At various time points postinfection, the cells were fixed with 4% paraformaldehyde, permeabilized with 50 μg/ml digitonin, and stained with anti-LC3 (pM036; MBL) and anti-LAMP-1 (H4A3; Santa Cruz) antibodies at room temperature for 1 h. After the cells were washed with PBS, they were stained with Alexa Fluor-conjugated secondary antibodies and DAPI for 40 min, and the samples were then analyzed by confocal microscopy (FV1000; Olympus).

Statistical analysis. Statistical analysis was performed using the paired *t* test. Significant differences were set at *P* values of <0.05 (GraphPad Prism software version 5).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01435-15/-/DCSupplemental.

Figure S1, TIF file, 0.2 MB.
Figure S2, TIF file, 0.1 MB.
Figure S3, TIF file, 0.2 MB.
Figure S4, TIF file, 1.2 MB.
Figure S5, TIF file, 0.9 MB.
Figure S6, TIF file, 0.3 MB.
Figure S7, TIF file, 0.1 MB.
Figure S8, TIF file, 0.1 MB.

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