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Differential Modulation of Intracellular Survival of Cytosolic and Vacuolar Pathogens by Curcumin

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Curcumin, a principal component of turmeric, acts as an immunomodulator regulating the host defenses in response to a diseased condition. The role of curcumin in controlling certain infectious diseases is highly controversial. It is known to alleviate symptoms of *Helicobacter pylori* infection and exacerbate that of *Leishmania* infection. We have evaluated the role of curcumin in modulating the fate of various intracellular bacterial pathogens. We show that pretreatment of macrophages with curcumin attenuates the infections caused by *Shigella flexneri* (clinical isolates) and *Listeria monocytogenes* and aggravates those caused by *Salmonella enterica* serovar Typhi CT18 (a clinical isolate), *Salmonella enterica* serovar Typhimurium, *Staphylococcus aureus*, and *Yersinia enterocolitica*. Thus, the antimicrobial nature of curcumin is not a general phenomenon. It modulated the intracellular survival of cytosolic (*S. flexneri* and *L. monocytogenes*) and vacuolar (*Salmonella* spp., *Y. enterocolitica*, and *S. aureus*) bacteria in distinct ways. Through colocalization experiments, we demonstrated that curcumin prevented the active phagosomal escape of cytosolic pathogens and enhanced the active inhibition of lysosomal fusion by vacuolar pathogens. A chloroquine resistance assay confirmed that curcumin retarded the escape of the cytosolic pathogens, thus reducing their inter- and intracellular spread. We have demonstrated that the membrane-stabilizing activity of curcumin is crucial for its differential effect on the virulence of the bacteria.

Curcumin, a pigment from turmeric, is known to have a vast array of therapeutic potential, ranging from anti-inflammatory to anticancer effects. It has also been shown to exhibit antimicrobial effects. However, its role as an antimicrobial agent remains controversial. Curcumin shows its antimicrobial effect against *Helicobacter pylori* (14, 19), *Bacillus subtilis* (38), *Plasmodium falciparum* (11, 34), etc. On the other hand, the role of curcumin as a promicrobial has been demonstrated in *Leishmania* (1) and *Salmonella* spp. (30). Curcumin is known to suppress the type 1 immune response (1, 24), which is important for the clearance of intracellular pathogens. In the following study, we sought to assess the effect of curcumin on the virulence of a few medically important intracellular foodborne pathogens.

The constant battle between the pathogen and host highlights the crux of host-pathogen interactions. The host has a repertoire of combative cells to keep the infection at bay (15, 28, 40). Similarly, pathogens employ different strategies to hijack the host immune system (15, 28, 40). Once the pathogen is sensed by the host immune system, it initiates an inflammatory response, recruiting different phagocytic cells to the site of infection. Macrophages, the key players in eliminating pathogens (32, 46), phagocytose the bacteria and use various tools to clear pathogens (27, 32, 43). The foremost tool is lysosomal degradation of the invading pathogen. During the process of phagocytosis, the bacteria or any foreign material passes through the endophagocytic pathway (18), where the engulfed material is present in the vacuole, which matures by sequentially fusing with the early endosome, the late endosome, and subsequently, the lysosome, where it gets degraded. However, pathogens have adopted various strategies to avoid degradation by lysosomal enzymes (18, 25). A few enteric pathogens, such as *Salmonella* spp., *Yersinia* spp., and *Staphylococcus* spp., have acquired machinery that inhibits the fusion of the pathogen-containing vacuole (PCV) with lysosomes (18, 25, 37), whereas other pathogens, such as *Shigella* spp. and *Listeria* spp., escape into the cytosol, thereby preventing lysosomal degradation (18, 25, 36). *Salmonella* spp., *Staphylococcus* spp., and *Yersinia* spp. multiply within the vacuole (25, 37), while *Shigella* spp. and *Listeria* spp. multiply in the cytosol (25, 36). On the basis of their intracellular localization, these pathogens can be classified into two groups, namely, vacuolar (salmonellae, *Yersinia* spp., staphylococci, etc.) and cytosolic (*Shigella* spp. and *Listeria* spp.). Here we show that curcumin differentially modulated the pathogenicity of cytosolic and vacuolar bacteria/pathogens. This differential effect was attributed to the membrane-stabilizing ability of curcumin.

MATERIALS AND METHODS

Eukaryotic cell lines and growth conditions. RAW 264.7 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% fetal calf serum (Sigma). Peritoneal macrophages were isolated from BALB/c mice and grown in RPMI medium (Sigma) supplemented with 10% fetal calf serum (Sigma). All these cells were maintained at 37°C in 5% carbon dioxide.

Bacterial strains and growth condition. The strains used in the study are listed in Table 1. *Salmonella enterica* serovar Typhi CT18, *Salmonella enterica* serovar Typhimurium, *Staphylococcus aureus*, *Salmonella enterica* serovar Typhimurium Δ*aroA*, *Shigella flexneri*, and *Yersinia enterocolitica* were grown in Luria broth (LB; Himedia), and *Listeria monocytogenes* was grown in brain heart infusion (BHI) medium (Himedia) at 37°C.

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M.S. and I.D. made equal contributions to this article.

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TABLE 1 Bacterial strains used in this study

Bacterial species	Strain	Source
Salmonella enterica serovar Typhimurium	NCTC 12023	M. Hensel, Institut fűr Klinische Mikrobiologie, Germany
Salmonella enterica serovar Typhimurium Δ*aroA*	Background strain, NCTC 12023	M. Hensel, Institut fűr Klinische Mikrobiologie, Germany
Salmonella enterica serovar Typhi CT18		Postgraduate Institute of Medical Education and Research, Chandigarh, India
Shigella flexneri clinical isolate 1		S. Mahadevan, Indian Institute of Science, Bangalore, India
Shigella flexneri Δ*aroA*	Background strain, *S. flexneri*, clinical isolate 1	Generated in this study
S. flexneri clinical isolate 2		H. Srinivasa, St. John's Medical College, Bangalore, India
S. flexneri	MTCC 1457	IMTECH, Chandigarh, India
Staphylococcus aureus (methicillin resistant)	COL	Gayathri Arakere, Sir Dorabji Tata Center for Tropical Diseases, Bangalore, India
S. aureus (methicillin susceptible)	ATCC 25923	Diagnostic Lab, Institute of Clinical Microbiology, Immunology and Hygiene, Erlangen, Germany
Listeria monocytogenes	MTCC 839	IMTECH, Chandigarh, India
L. monocytogenes	ATCC 19112	Devi Kalyan Kumar, Defense Food Research Laboratory, Mysore, India
Yersinia enterocolitica	MTCC 840	IMTECH, Chandigarh, India

Construction of *aroA* deletion mutant in *S. flexneri*. The one-step inactivation method described by Datsenko and Wanner (13) was used to generate the Δ*aroA* strain of *S. flexneri*. Briefly, *S. flexneri* (clinical isolate 1) was electrotransformed with plasmid pKD46. Electrocompetent cells of this strain were then transformed with the PCR product that was obtained using primers Δ*aroA* forward (GGGGTTTTTATTTCTGTTGTAGAGAGTTGAGTTCATGGAATCGTGTAGGCTGGAGCTGCTTC) and Δ*aroaA* reverse (GGCCGTGCATTTGGGATCAAGAATCGTCACTGGTGTATCTGCATATGAATATCCTCCTTA) (16) on the template, plasmid pKD4. Kanamycin-resistant clones were confirmed by PCR using confirmatory primers Δ*aroA* confirm forward (CTGTTGTAGAGAGTTGAGTTC) and Δ*aroA* confirm reverse (ATGTTTTGGCCGTGCATTTGG).

Intracellular survival assay. The intracellular survival assay was performed as described previously (30). Briefly, RAW 264.7 cells were seeded into a 24-well plate and were either mock treated or treated with gamma interferon (IFN-γ; 20 U/ml, 12 h), curcumin (10 μM, 2 h), or erythromycin (100 μg/ml, 1 h). These cells were then infected with the respective pathogens, and 100 μg/ml gentamicin was added to the cell culture medium to get rid of extracellular bacteria. Cells were maintained in 25 μg/ml of gentamicin to prevent the growth of extracellular bacteria. After specific incubation periods, cells were lysed with 0.1% (vol/vol) Triton X-100 (Sigma). The infected cells were lysed at an early (2 h) and a later (16 to 20 h) time point to determine the fold proliferation of the pathogen. However, cells infected with *S. flexneri* were lysed at 1 h and 3 h, as *S. flexneri* induces apoptosis at 3 to 4 h postinfection (5, 48), and those infected with *S. aureus* were lysed at 2 h and 6 h, as the intracellular bacteria start declining in number after 8 h (unpublished data). The lysates were plated onto LB agar plates with appropriate antibiotics (*Salmonella enterica* serovars Typhi and Typhimurium, *Shigella flexneri*, and *Yersinia enterocolitica*), Vogel-Johnson agar (*Staphylococcus aureus*), or BHI agar (*Listeria monocytogenes*), and the colonies were counted to determine the number of CFU.

Survival assay against reactive oxygen and nitrogen species. The survival assay was performed as described previously with some modifications (6, 9). Briefly, overnight cultures of methicillin-resistant *S. aureus* (MRSA; strain COL) were diluted to 10^5 CFU in Mueller-Hinton medium. $NaNO_2$ and H_2O_2 were freshly prepared at 200 mM, 1 mM, and 2 mM. Fifty microliters of the diluted culture was mixed with 50 μl of each of the above concentrations of $NaNO_2$ and H_2O_2 to get final concentrations of 100 mM, 500 mM, and 1 mM, respectively. The plates were incubated at 37°C under shaking conditions. After 2 h and 6 h of exposure, serial dilutions were made and plated onto Vogel-Johnson agar plates for the enumeration of surviving bacteria. The percent survival was calculated as follows: (number of CFU in the presence of stress × 100)/number of CFU in the absence of stress.

Confocal microscopy. RAW 264.7 cells were grown on the coverslips placed in a 24-well plate and were infected as described for the intracellular survival assay (30). Cells were fixed with 3.5% (wt/vol) paraformaldehyde at different time points. Lysosomes were either marked by pulse-chasing with Texas Red-conjugated ovalbumin 90 min prior to initiation of experiment or stained with antibody against LAMP1. Samples were observed with a confocal microscope (510 LSM Meta; Carl Zeiss) and analyzed for colocalization of bacteria with lysosome. The samples were scanned using appropriate lasers, and the *Z* stacks (optical sections) of the image were obtained. The colocalization was measured in maximum-intensity projections with *Z* stacks. The percent colocalization was calculated by using the colocalization coefficient *m*1, which was determined by the built-in software in the Carl Zeiss Meta 510 microscope. The colocalization coefficient values were multiplied by 100 to get the percent colocalization and plotted.

To label the lysosomes with LAMP1, the cells were stained with primary antibody against LAMP1 (DSHB) and then with secondary antibody conjugated to Cy5 (Dianova). To visualize bacteria, the bacteria (*S.* Typhimurium and *S. flexneri*) were either engineered to harbor green fluorescent protein or stained with specific primary polyclonal antibody (*S. aureus*, *Y. enterocolitica*, and *L. monocytogenes*), followed by staining with fluorescence (Cy2 or fluorescein isothiocyanate)-conjugated secondary antibody (Dianova). The antibody against *Listeria* was purchased as antisera from Difco, whereas the antibodies against *S. aureus* and *Y. enterocolitica* were generated in-house by immunizing rabbits with heat-killed bacteria, and antiserum was obtained after three immunizations given at 10-day intervals. The dilution factor for all the antibodies was 1:100.

Chloroquine resistance assay. The chloroquine resistance assay was performed as described previously (28). RAW 264.7 cells, treated or untreated with curcumin, were infected with *S. flexneri* (clinical isolates 1 and 2) or *L. monocytogenes* (ATCC 19112) at a multiplicity of infection of 50, as described for the intracellular survival assay. Chloroquine (50 μg/ml) was added at the desired time points (3 h for *S. flexneri* and 5 h for *L. monocytogenes*) postinfection, and the cells were incubated for an additional 1 h to allow the killing of bacteria inside the vacuole by chloroquine. The cells were washed and lysed with 0.1% Triton X-100, and the cell lysates were diluted and plated on agar plates to calculate the number of CFU.

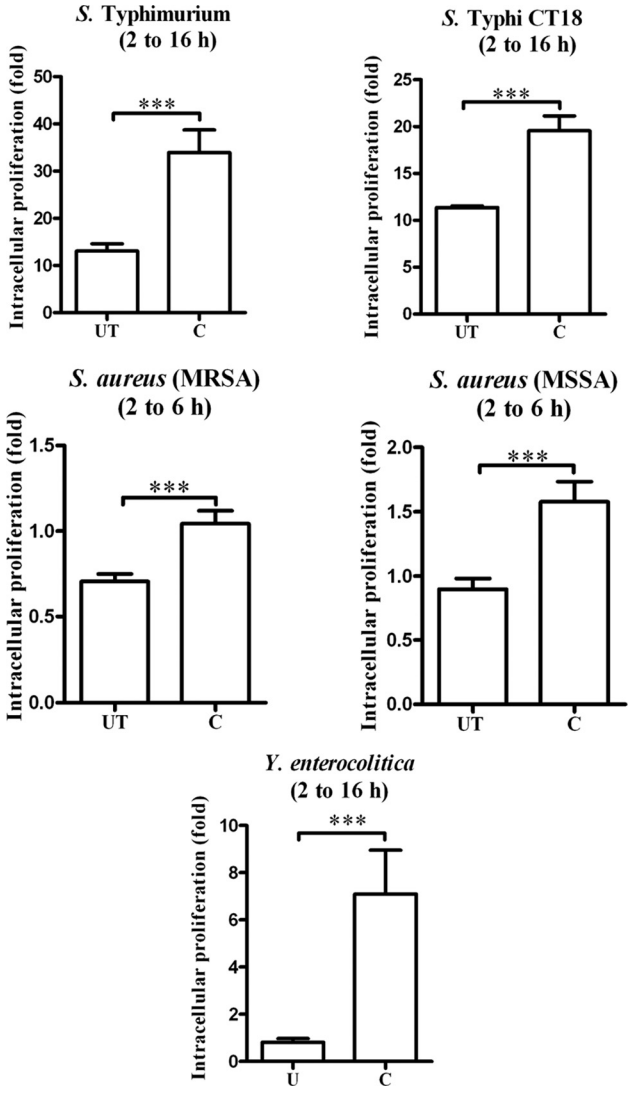

FIG 1 Curcumin increased the fold proliferation of vacuolar pathogens. Untreated and curcumin-treated (10 μM, 2 h) RAW 264.7 cells were infected with the respective vacuolar pathogens. The infected cells were lysed at different time points postinfection, and the fold replication of the bacteria was calculated. UT or U, untreated cells; C, curcumin-pretreated cells; MSSA, methicillin-susceptible *S. aureus*. Student's *t* test was used for testing the statistical significance of the data. ***, $P < 0.001$.

Membrane stability assay. The membrane stability assay was performed as described previously (47). The cells were collected from a healthy donor (rabbit) in tubes containing an anticoagulant, heparin (20 U/ml of blood). The cells were washed three times with 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (200 mM; NaCl, 110 mM), pH 5, by centrifugation at 200 × *g* for 5 min at 4°C, followed by redispersion. Erythrocytes (RBCs) were diluted (1:100) in MES buffer. The RBCs were treated with curcumin (10 μM, 2 h) or erythromycin (100 μg/ml, 1 h). The cells were washed twice with MES buffer, and hemolysis assay was performed. RBCs suspended in MES buffer were mixed with *L. monocytogenes* (10^7), and the mixture was incubated at 37°C for 20 min. The cells were pelleted at 5,000 × *g* for 10 min, and the supernatant was read at 540 nm in an enzyme-linked immunosorbent assay (ELISA) microplate reader (ELx 800 MS). As a positive control for 100% lysis, RBCs were resuspended in distilled water and incubated at 37°C. The suspension was then centrifuged at 5,000 × *g*, and the supernatant was read at 540 nm in the ELISA microplate reader.

Statistical analysis. All the experiments were performed in triplicate. The results are represented as means ± standard errors. All the analyses were done using GraphPad Prism (version 5) software. Wherever applicable, a *P* value of ≤0.05 was considered statistically significant.

RESULTS

Regulation of intracellular replication of different pathogens in macrophages by curcumin. Food-borne diseases are one of the leading causes of morbidity and mortality throughout the world (45). *Salmonella* spp. are among the major causative agents of food-borne diseases. In our study, we have tested the ability of curcumin, a pigment from turmeric, to regulate the pathogenesis of *Salmonella* spp. The macrophages were pretreated with curcumin (10 μM) for 2 h and infected with *Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar Typhi CT18 (a clinical isolate), after which their intracellular proliferation was evaluated. We found that in curcumin-treated cells, these pathogens showed increased intracellular fold proliferation (Fig. 1). To understand whether curcumin would exhibit a similar effect on other intracellular pathogens, we also analyzed the intracellular proliferation of other food-borne pathogens, *Y. enterocolitica* and *S. aureus*, in curcumin-treated cells. We found that curcumin increased the fold proliferation of these pathogens as well (Fig. 1). As reactive oxygen species (ROS) and nitrogen intermediates (RNIs) are the key players in the clearance of intracellular pathogens (in macrophages), we hypothesized that the antioxidant and anti-inflammatory property of curcumin might be responsible for the

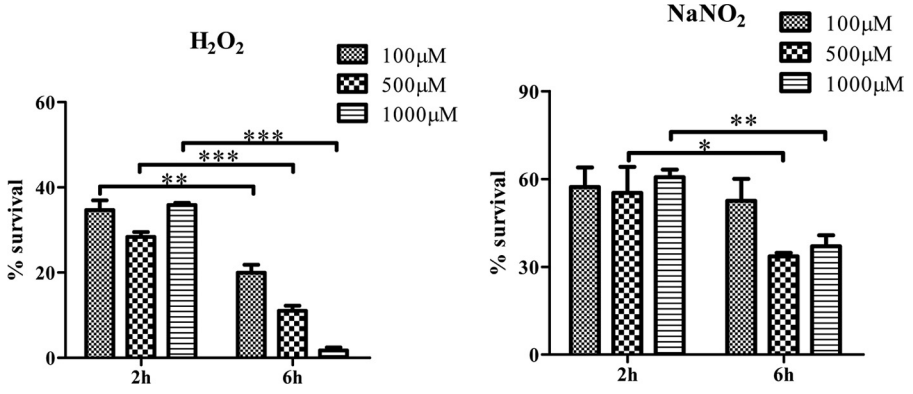

FIG 2 Sensitivity of MRSA to ROS and RNIs. The bacteria were incubated in the presence of either H_2O_2 or $NaNO_2$ in Mueller-Hinton broth, pH 5, and survival was assessed. Student's *t* test was used for testing the statistical significance of the data. ***, $P < 0.001$; **, $0.001 \leq P < 0.01$; *, $0.01 \leq P < 0.05$.

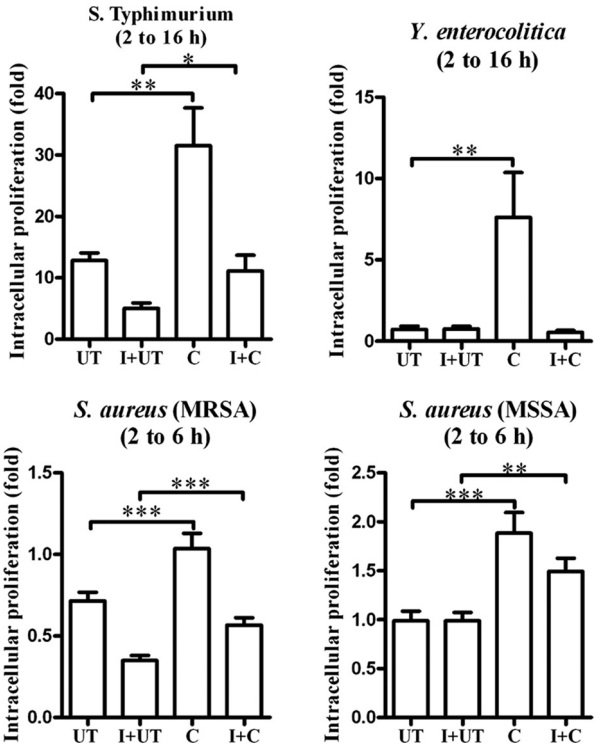

FIG 3 The antioxidant and anti-inflammatory property of curcumin is not responsible for the protection of intracellular pathogen. RAW 264.7 cells were primed with IFN-γ (20 U/ml, 12 h) and then treated with curcumin (10 μM, 2 h). These cells were infected with the respective vacuolar pathogens. The infected cells were lysed at different time points postinfection, and the fold replication of the bacteria was calculated. UT, untreated cells; C, curcumin-pretreated cells; I, IFN-γ-primed cells. Student's *t* test was used for testing the statistical significance of the data. ***, $P < 0.001$; **, $0.001 \leq P < 0.01$; *, $0.01 \leq P < 0.05$.

increased intracellular survival of the pathogens tested. To validate our hypothesis, the macrophage cells (RAW 264.7 cells) were pretreated with IFN-γ to induce the generation of ROS and RNIs in the cells (39), and the survival of the pathogens was analyzed in these cells with and without curcumin treatment. It was observed that *S.* Typhimurium and *S. aureus*, both susceptible to oxidative and nitrosative stress (9, 30, 42) (Fig. 2), showed increased proliferation in the IFN-γ-primed cells treated with curcumin compared to that in curcumin-untreated but IFN-γ-primed cells (Fig. 3). This indicates that the antioxidant and anti-inflammatory property of curcumin might be responsible for protecting these pathogens from the IFN-γ-induced ROS and RNIs. However, contrary to this observation, we found that curcumin did not confer any protection to *Y. enterocolitica* in IFN-γ-primed cells, indicating that a property distinct from the antioxidant and anti-inflammatory effect of curcumin might have a role in increasing the intracellular survival of the pathogens tested. To further validate this, the survival of an ROS- and RNI-resistant strain of *S. aureus* (methicillin-sensitive strain ATCC 25923; unpublished data) was tested in IFN-γ-primed cells treated or untreated with curcumin. *S. aureus* (ATCC 25923) did not show decreased survival in cells primed with IFN-γ; nevertheless, this pathogen proliferated almost 2-fold more in cells (primed or unprimed with IFN-γ) treated with curcumin. This indicates that the anti-inflammatory property of curcumin does not have a crucial role in improving the survival of *Y. enterocolitica* and *S. aureus* (ATCC 25923) in macrophages.

The effect of curcumin on the intracellular survival of a few other medically important food-borne pathogens, *Shigella flexneri* and *Listeria monocytogenes*, was tested. The intracellular survival of these pathogens was analyzed in macrophages either primed or

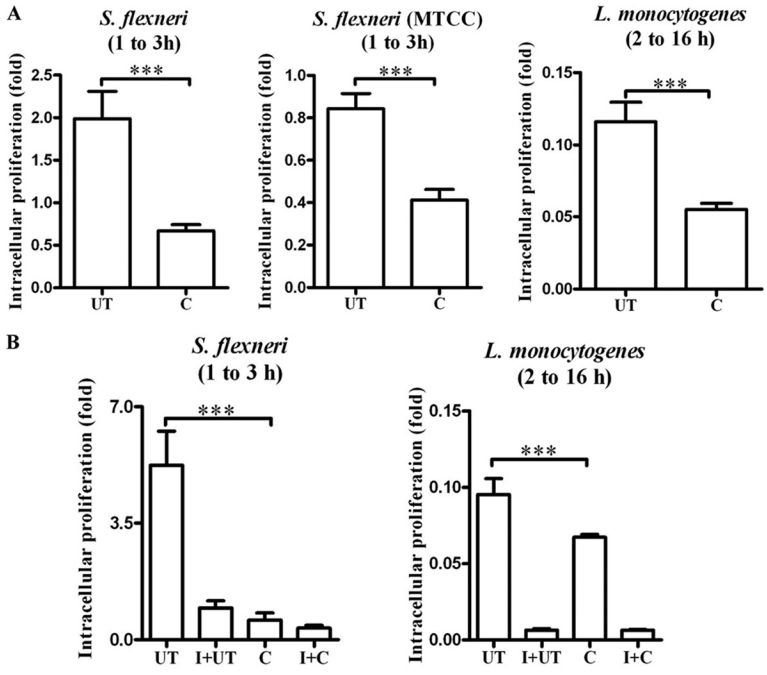

FIG 4 Curcumin decreased the fold proliferation of cytosolic pathogens. Untreated and curcumin-treated (10 μM, 2 h) RAW 264.7 cells either unprimed (A) or primed (B) with IFN-γ (20 U/ml, 12 h) were infected with the respective cytosolic pathogens. The infected cells were lysed at different time points postinfection, and the fold replication of the bacteria was calculated. UT, untreated cells; C, curcumin-pretreated cells; I, IFN-γ-primed cells. Student's *t* test was used for testing the statistical significance of the data. ***, $P < 0.001$.

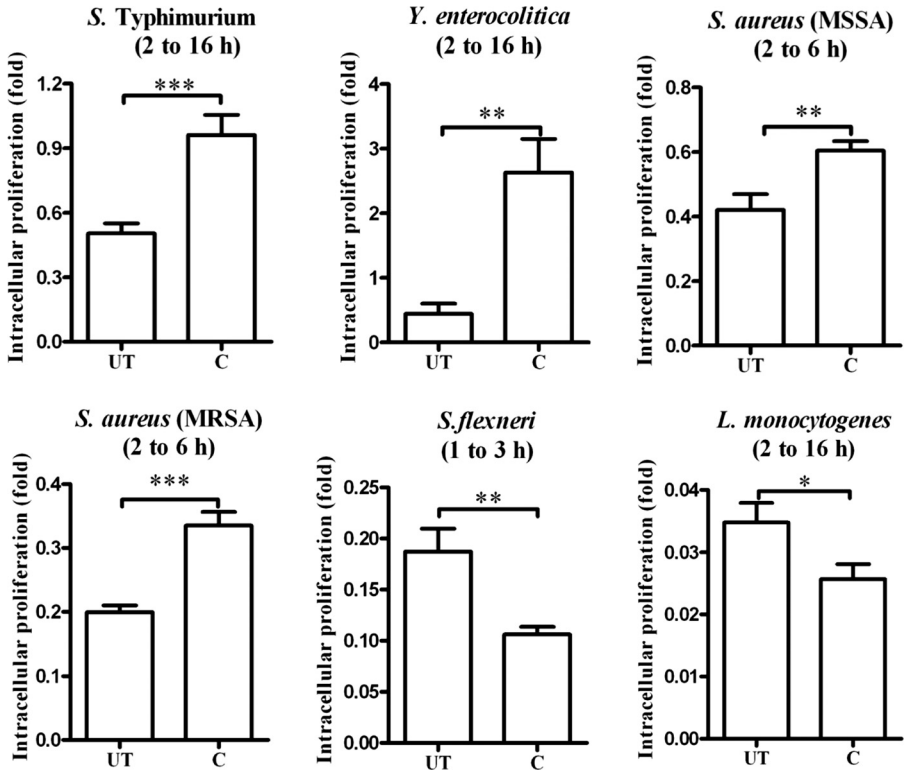

FIG 5 Differential modulation of fold proliferation of vacuolar and cytosolic pathogens in primary cells. The untreated and curcumin-treated (10 μM, 2 h) peritoneal macrophages were infected with the respective pathogens. The infected cells were lysed at different time points postinfection, and the fold replication of the bacteria was calculated. UT, untreated cells; C, curcumin-pretreated cells. Student's *t* test was used for testing the statistical significance of the data. ***, $P < 0.001$; **, $0.001 \leq P < 0.01$; *, $0.01 \leq P < 0.05$.

unprimed with IFN-γ and treated or untreated with curcumin. To our surprise, we found that curcumin decreased the survival of both *S. flexneri* and *L. monocytogenes* in IFN-γ-unprimed macrophages (Fig. 4A). Also, IFN-γ treatment led to similar growth inhibition of these pathogens in both curcumin-treated and -untreated cells (Fig. 4B), again confirming that the anti-inflammatory property of curcumin (or the type 1 suppression caused by curcumin) might not have a role in modulating the pathogenesis of intracellular bacteria.

Thus, taken together, curcumin positively regulated the pathogenesis of *S.* Typhimurium, *S. aureus*, and *Y. enterocolitica* and negatively regulated that of *S. flexneri* and *L. monocytogenes*. This differential regulation of pathogenesis by curcumin was also tested in primary cells, peritoneal macrophages, from mice. Similar to the results obtained in RAW 264.7 cells, pretreatment of peritoneal macrophages with curcumin increased the intracellular survival of *S.* Typhimurium, *S. aureus*, and *Y. enterocolitica* and decreased that of *S. flexneri* and *L. monocytogenes* (Fig. 5).

Curcumin increased the colocalization of cytosolic pathogens and decreased that of vacuolar pathogens with the lysosome. The other defensive strategy of macrophages against infection is to kill/destroy any foreign particle via phagocytosis. Almost all intracellular pathogens have evolved strategies to escape phagocytosis (18, 25). This is achieved either by inhibiting phagocytosis, by arresting phagosomal maturation at an early stage, whereby the pathogens survive and multiply in the vacuolar compartment, or by escaping to the cytosol. The cytosolic pathogens hijack the cytoskeletal machinery for their intracellular and intercellular dispersal (18, 25). Vacuolar bacteria, on the other hand, have an inherent mechanism to prevent the fusion of the pathogen-containing vacuole (PCV) with the lysosome (15, 46). However, few bacteria (10 to 30%) are unable to maintain the vacuolar integrity and can fuse with the lysosome (15, 46). Our results suggest that pretreatment of macrophages with curcumin leads to increased proliferation of vacuolar pathogens (*S.* Typhimurium, *S. aureus*, and *Y. enterocolitica*) and decreased proliferation of cytosolic pathogens (*S. flexneri* and *L. monocytogenes*). We hypothesized that curcumin might inhibit the escape of the cytosolic pathogen into the cytosol, thereby limiting its intracellular spread and increasing its fusion with the lysosomes. The stabilization of the vacuolar membrane would aid the inherent mechanism of the vacuolar pathogen to prevent the fusion of PCV with lysosome. In this context, we analyzed the colocalization of the pathogens with the lysosomal compartment in the curcumin-treated and -untreated cells.

The lysosomal compartments were stained red with either a fluid-phase marker, Texas Red-conjugated ovalbumin, or antibody against LAMP1. The colocalization of different pathogens with the lysosomal compartment was analyzed using confocal microscopy. We found that curcumin increased the colocalization of cytosolic pathogens but not that of vacuolar pathogens with lysosomal compartments (Fig. 6A), as indicated by the differential colocalization with Texas Red. However, as the vacuolar pathogens (*S.* Typhimurium, *S. aureus*) recruit LAMP1 to PCV

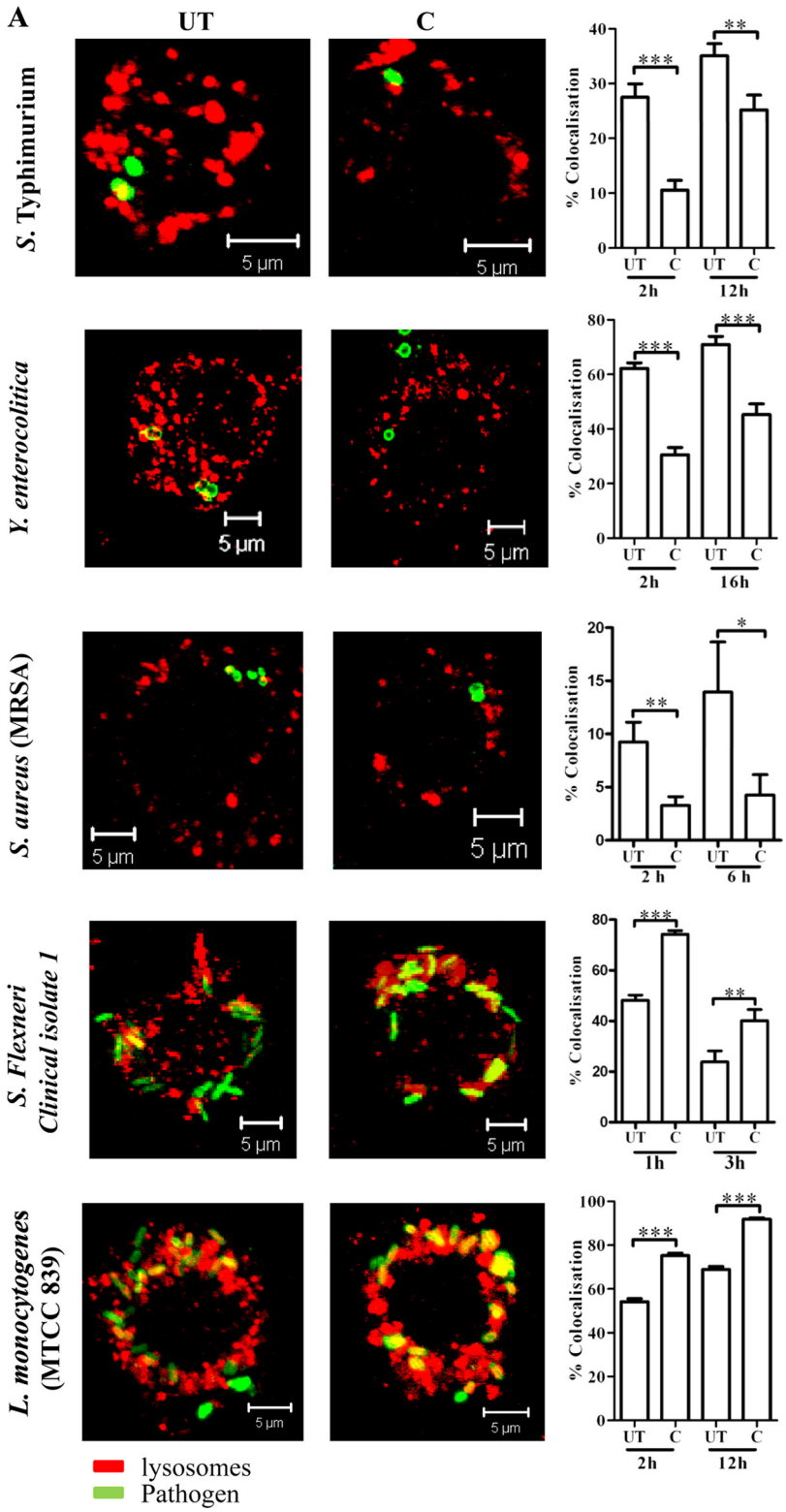

FIG 6 (A) Curcumin treatment leads to differential colocalization of cytosolic and vacuolar pathogens with the lysosome. RAW 264.7 cells were pulse-chased with Texas Red-conjugated ovalbumin and left untreated or were treated with curcumin (10 μM, 2 h). The cells were infected with the respective pathogens and processed for confocal microscopy. (B) Curcumin treatment leads to containment of vacuolar pathogens into the secluded vacuole. RAW 264.7 cells were untreated or treated with curcumin (10 μM, 2 h). The cells were infected with the respective pathogens and processed for confocal microscopy. The cells were stained for the LAMP1 marker and respective pathogen. UT, untreated cells; C, curcumin-pretreated cells. Student's *t* test was used for testing the statistical significance of the data. ***, $P < 0.001$; **, $0.001 \leq P < 0.01$; *, $0.01 \leq P < 0.05$.

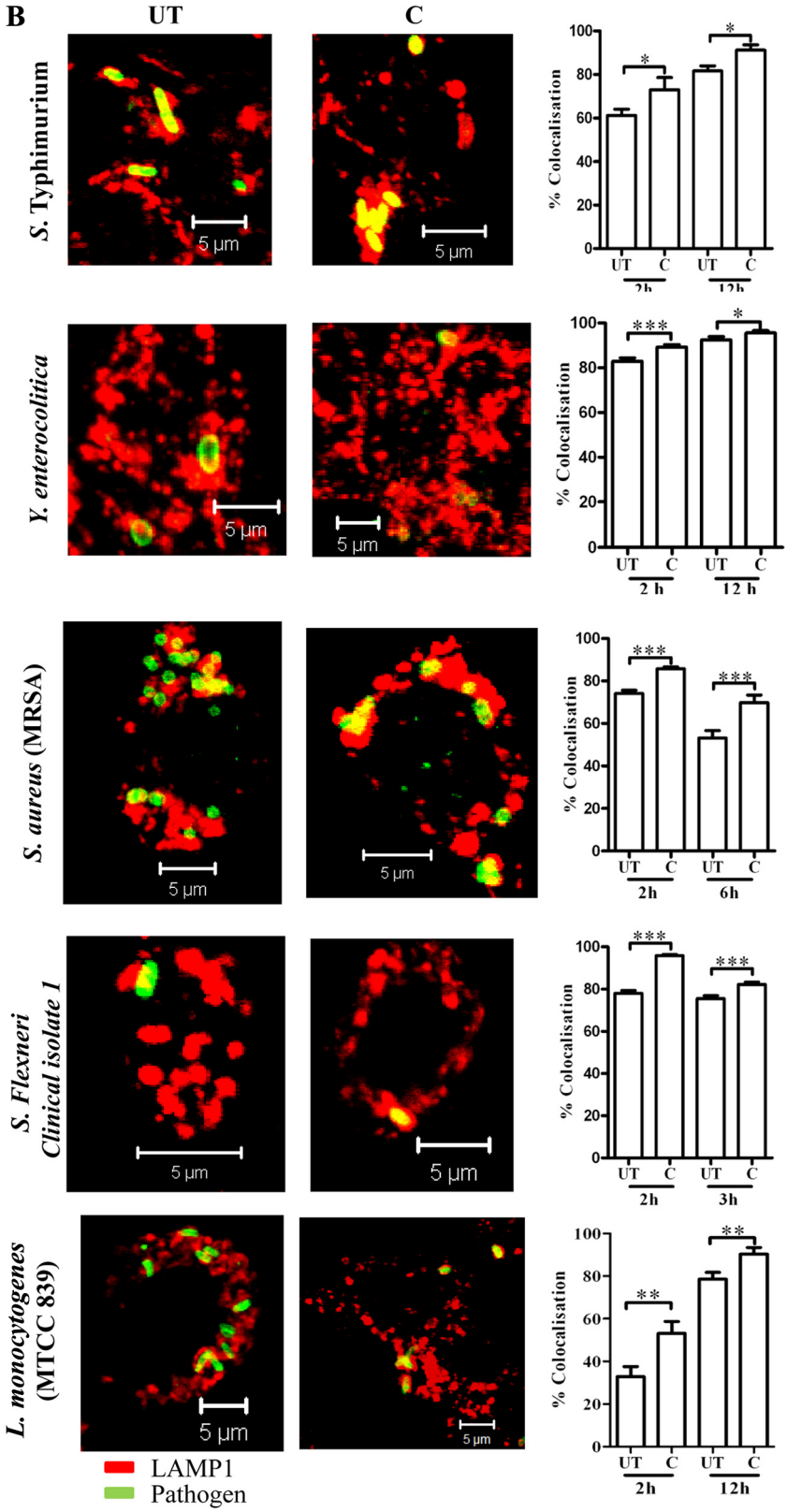

FIG 6 continued

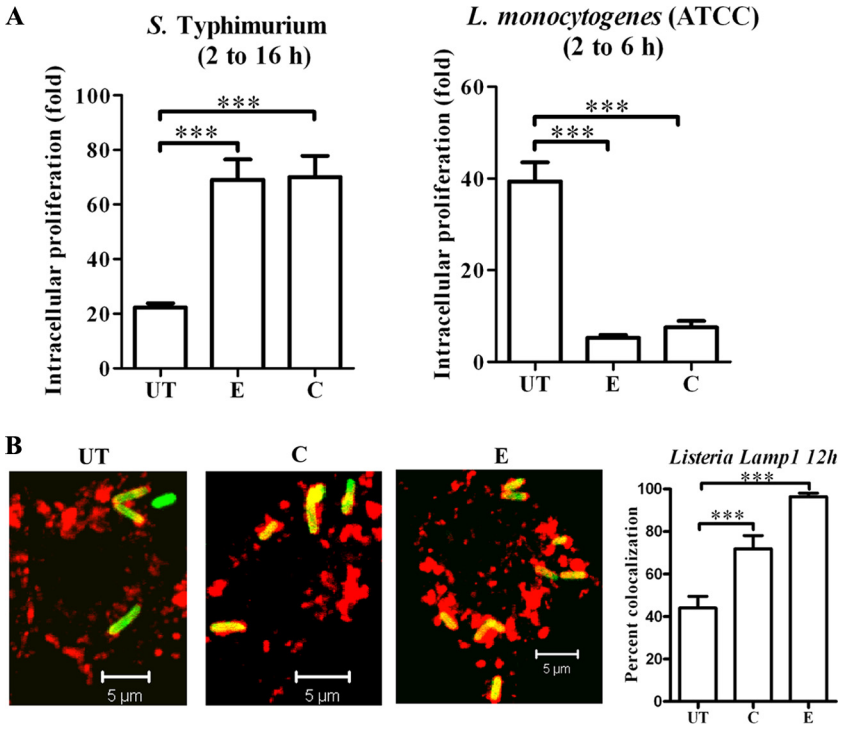

FIG 7 (A) Curcumin and erythromycin increased the fold proliferation of *S.* Typhimurium and decreased that of *L. monocytogenes* (ATCC 19112). RAW 264.7 cells treated or untreated with curcumin (10 μM, 2 h) and erythromycin (100 μg/ml, 1 h) were infected with the respective pathogens. The infected cells were lysed at different time points postinfection, and the fold replication of the bacteria was calculated. (B) Curcumin and erythromycin increased the colocalization of *L. monocytogenes* (ATCC 19112) with lysosomes. RAW 264.7 cells were untreated or treated with curcumin (10 μM, 2 h) and erythromycin (100 μg/ml, 1 h). The cells were infected with *L. monocytogenes* and processed for confocal microscopy. The lysosomes and *L. monocytogenes* were stained red (Cy5, with LAMP1-specific antibody) and green (Cy2, with *L. monocytogenes*-specific antibody), respectively. UT, untreated cells; C, curcumin-pretreated cells; E, erythromycin-pretreated cells. One-way analysis of variance followed by Tukey's multiple-comparison test was used for testing the statistical significance of the data. ***, $P < 0.001$.

(2, 23, 31), the differential colocalization of cytosolic and vacuolar pathogens with LAMP1, a lysosomal marker, was not observed in curcumin-treated cells (Fig. 6B). In fact, both vacuolar and cytosolic pathogens showed increased colocalization with LAMP1 in curcumin-treated cells (Fig. 6B). This indicates that curcumin is facilitating the ability of vacuolar pathogens to stay secluded in the PCV, thus avoiding fusion with lysosomes, and, at the same time, increasing the fusion of cytosolic pathogens with lysosomes (Fig. 6).

Thus, we can conclude that curcumin is either blocking (cytosolic pathogens) or aiding (vacuolar pathogens) the active mechanism of the pathogens. If so, the differential colocalization seen in curcumin-treated cells should not be observed for dead pathogens or an inert particle like latex beads. When analyzed for the colocalization of the dead pathogens and latex beads with either the fluid-phase molecular marker or LAMP1, the differential colocalization seen on curcumin treatment was abrogated (data not shown). This suggests that curcumin prevents the active escape of cytosolic pathogens into the cytosol, leading to their enhanced clearance via lysosomal degradation. However, it aids vacuolar bacteria by helping the bacteria to remain secluded in the vacuole. This indicates that curcumin might stabilize the vacuolar membrane to exert differential effects on the intracellular survival of cytosolic and vacuolar bacteria.

Curcumin stabilizes the vacuolar membrane to prevent the escape of cytosolic pathogens from the vacuole/endosome. With the indication that the membrane stabilization caused by curcumin might be responsible for the differential regulation of the intracellular survival of different pathogens, we tested whether a similar effect is exerted by erythromycin, a known membrane stabilizer/membrane-stabilizing agent (4). An intracellular survival assay was performed in cells treated or untreated with erythromycin and curcumin. It was found that both curcumin and erythromycin pretreatment increased proliferation of *S.* Typhimurium, a vacuolar pathogen, and decreased that of *L. monocytogenes*, a cytosolic pathogen (Fig. 7A). When analyzed for the localization of these pathogens with the lysosome, we observed that, similar to curcumin, erythromycin also increased the colocalization of a cytosolic pathogen (*L. monocytogenes*) with the lysosomes (Fig. 7B). Further, the effect of curcumin on the ability of *L. monocytogenes* and *S. flexneri* to escape the phagocytic vacuole was tested using a chloroquine resistance assay.

Upon exposure of the cells to chloroquine, it gets concentrated in the endosomes (41). Within endosomes, the acidic pH of the vacuole leads to its protonation and the protonated form of chloroquine gets trapped within endosomes (41). This concentrated chloroquine can kill bacteria within the phagocytic vacuole without any effect on cytosolic bacteria (7, 10, 26, 35). We have shown that curcumin treatment prevents the escape of cytosolic bacteria, *Shigella flexneri* and *Listeria monocytogenes*, probably by stabilizing the endosomal membrane. If it is true, then chloroquine

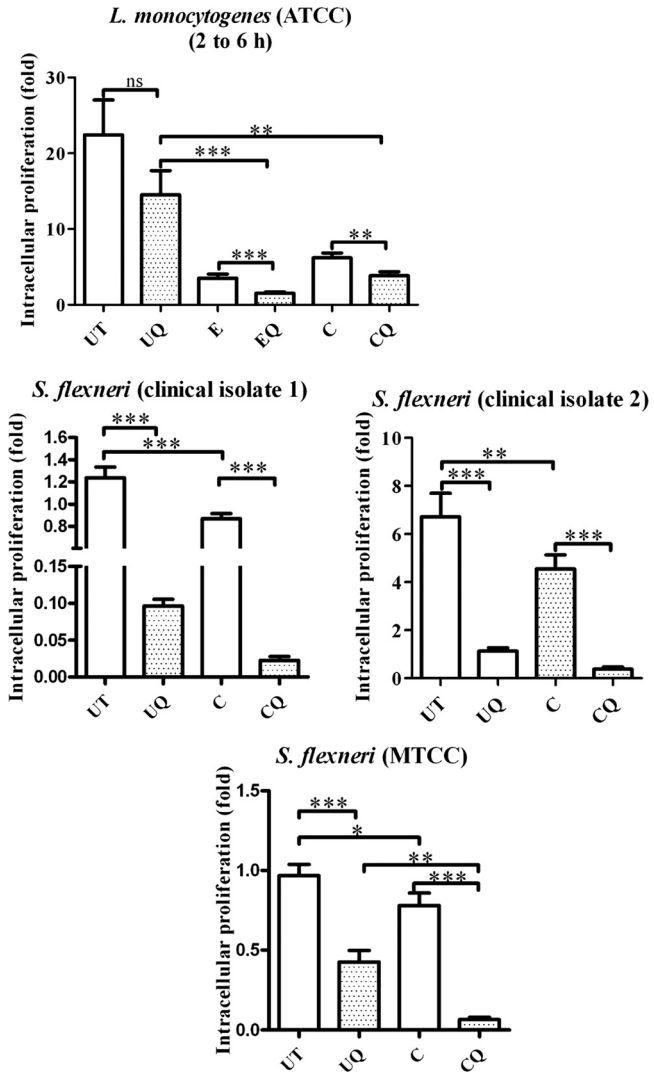

FIG 8 Curcumin and erythromycin prevented the escape of the cytosolic pathogens, leading to their enhanced killing by chloroquine. RAW 264.7 cells treated or untreated with curcumin (10 μM, 2 h) and erythromycin (100 μg/ml, 1 h) were infected with the respective pathogens. One set of infected cells was lysed at 1 h, and lysate was plated to determine the number of CFU. After 3 h (*S. flexneri*) and 5 h (*L. monocytogenes*), another set of infected cells was either treated (stippled bars) or untreated with chloroquine for 1 h and then lysed to determine the number of CFU. Fold replication (1 to 4 h, *S. flexneri*; 1 to 6 h, *L. monocytogenes*) of the bacteria was calculated. UT or U, untreated cells; C, curcumin-pretreated cells; E, erythromycin-pretreated cells; Q, chloroquine-exposed cells. One-way analysis of variance followed by Tukey's multiple-comparison test was used for testing the statistical significance of the data. ***, $P < 0.001$; **, $0.001 \leq P < 0.01$; *, $0.01 \leq P < 0.05$; ns, not significant.

should kill more bacteria in curcumin-pretreated cells. We proceeded with the intracellular survival assay in cells pretreated with curcumin in the presence and absence of chloroquine. Chloroquine was added 1 h before the lysis of the infected cells at the desired time point. The intracellular fold replication of *S. flexneri* and *L. monocytogenes* was reduced in the presence of chloroquine and was further decreased in curcumin- and erythromycin-treated cells (Fig. 8), suggesting that the bacteria trapped within endosomes due to the stabilizing effect of curcumin are effectively killed by chloroquine.

If our speculation is true, curcumin should exhibit similar effects on the pathogenic strains deficient in intracellular survival. Δ*aroA* strains of *S.* Typhimurium and *S. flexneri* are defective in intracellular survival due to their inability to synthesize aromatic amino acids (8, 16, 20, 21). An intracellular survival assay and confocal microscopic analysis were performed with Δ*aroA* strains of *S.* Typhimurium and *S. flexneri*. We indeed observed that curcumin differentially modulated the intracellular survival of these strains and their localization to the lysosomes in RAW 264.7 cells (Fig. 9A). *S.* Typhimurium Δ*aroA* showed increased intracellular proliferation and decreased localization to lysosomes (Fig. 9B), whereas *S. flexneri* Δ*aroA* showed decreased intracellular proliferation and increased localization to lysosomes (Fig. 9B).

To further confirm the membrane-stabilizing ability of curcumin, a hemolytic assay was performed. Erythrocytes, which are devoid of organelles, have been used as a model for the endosomal membrane (33, 47). Hence, we used them to analyze the membrane-stabilizing ability of curcumin. Saponin and a hemolytic strain of *L. monocytogenes* were used as membrane-destabilizing agents. Curcumin-pretreated RBCs showed less hemolysis upon saponin (0.01%) addition or on infection with *L. monocytogenes* than curcumin-untreated erythrocytes (Fig. 10). These results corroborate the membrane-stabilizing property of curcumin.

DISCUSSION

Curcumin, with its multifaceted roles, has proven to be a potent therapeutic for a variety of diseases (3, 17, 29). It is known to possess antibacterial activity. However, the antibacterial effect has mostly been demonstrated under *in vitro* conditions (12, 38) or against extracellular pathogens (14, 19). Its effect against intracellular pathogens remains less explored. Curcumin alleviates symptoms of various disorders, especially type 1-mediated diseases (1, 24, 29). The type 1 immune response is important against various intracellular pathogens. Thus, suppression of the type 1 immune response might pave the way for these pathogens to successfully establish an infection (1). In our study, we have evaluated the role of curcumin, a suppressor of the type 1 immune response, in modulating the pathogenesis of the different intracellular pathogens. We hypothesized that curcumin, being a suppressor of the type 1 immune response, should increase the intracellular survival/proliferation of all the intracellular pathogens. To test our hypothesis, we chose bacteria belonging to both the Gram-positive and Gram-negative groups of bacteria, cocci, coccobacilli, and bacilli (Table 1). The pathogens chosen in this study are known to breach the epithelial barrier and survive in macrophages. All survival experiments were carried out in macrophages, as they are responsible for the systemic dissemination of pathogens. Contradictory to our hypothesis, we found that curcumin did not increase the intracellular proliferation of all these pathogens. Instead, it differentially regulated the intracellular proliferation of these bacteria. Treatment of macrophages with curcumin increased the fold proliferation of vacuolar pathogens and decreased that of cytosolic pathogens. This implicates that a distinct role of curcumin is responsible for this differential regulation.

Pathogens have adopted various strategies to escape host defenses and establish an infection (25). One of them is the ability to evade the phagocytic response (18, 25). Either they inhibit phagocytosis or the fusion of PCV with lysosome or they escape into the cytosol (18, 25). The vacuolar pathogens, with the help of their

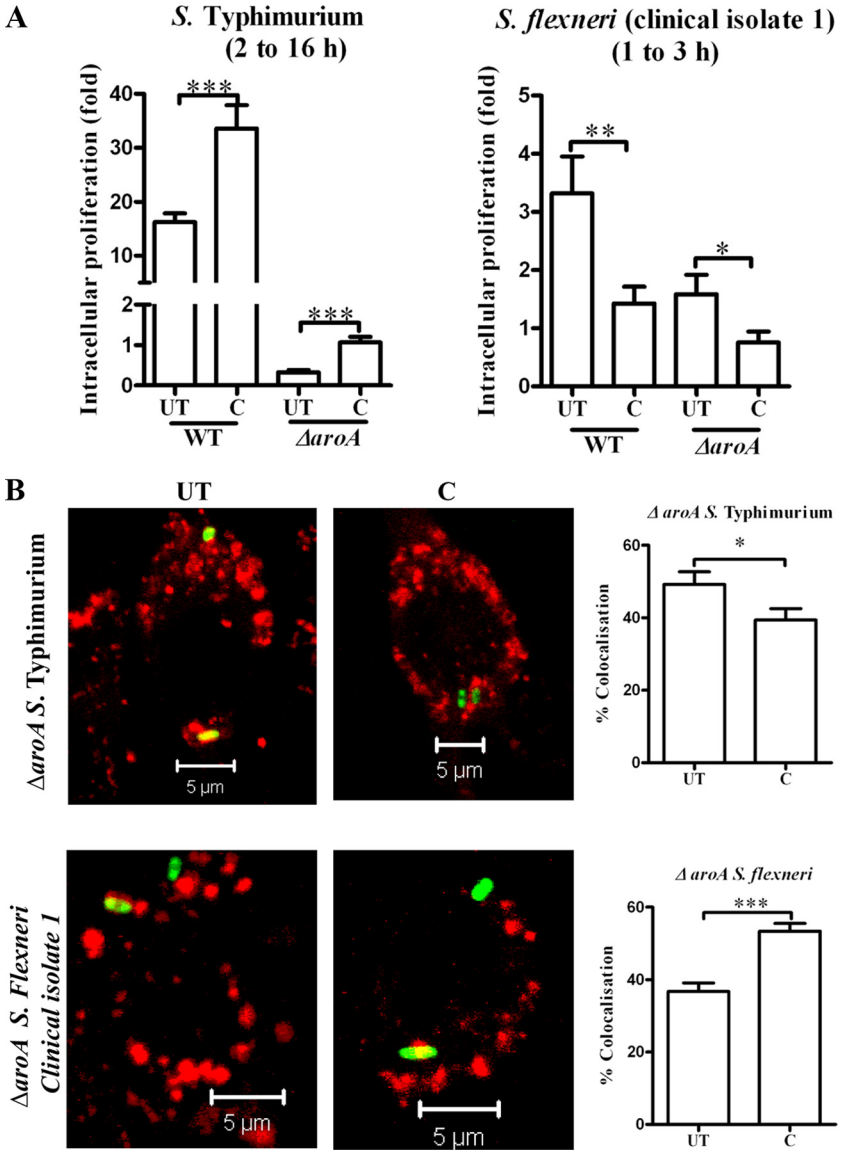

FIG 9 (A) Curcumin increased the fold proliferation of a Δ*aroA* strain of *S.* Typhimurium and decreased that of a Δ*aroA* strain of *S. flexneri.* RAW 264.7 cells treated or untreated with curcumin (10 μM, 2 h) were infected with the respective pathogens. The infected cells were lysed at different time points, and the number of CFU was determined. The fold replication of the bacteria was calculated. WT, wild type. (B) Curcumin decreased the colocalization of *S.* Typhimurium and increased that of *S. flexneri* with lysosomes. RAW 264.7 cells were untreated or treated with curcumin (10 μM, 2 h). The cells were infected with the respective pathogens (engineered to contain green fluorescent protein) and processed for confocal microscopy. The lysosomes were stained red by pulse-chasing the macrophages with Texas Red-conjugated ovalbumin. Student's *t* test was used for testing the statistical significance of the data. ***, $P < 0.001$; **, $0.001 \leq P < 0.01$; *, $0.01 \leq P < 0.05$.

virulence factors, stay secluded in their vacuolar niche without fusing with the lysosome (18, 25). However, the cytosolic pathogens (*viz.*, *L. monocytogenes*, *S. flexneri*, and *T. cruzi*) have tools to destabilize the vacuolar membrane and escape into the cytosol (18, 22, 25). The exact mechanism of membrane destabilization is not yet understood completely. However, it is known that these pathogens carry pore-forming proteins, such as listeriolysin O (LLO) and Ipa, that get incorporated into the vacuolar membrane, thereby leading to the degradation of the vacuole (22). To elucidate the mechanism of the differential regulation of pathogenesis of various pathogens by curcumin, we hypothesized that curcumin might stabilize the vacuolar membrane, thereby preventing the escape of cytosolic pathogens, thus leading to their fusion with lysosomes and increased killing. However, the stabilization of the vacuolar membrane might aid the ability of vacuolar pathogens to remain cloistered in the vacuole, avoiding fusion and degradation by the lysosome. Our hypothesis gains significance considering the fact that curcumin is known to possess a membrane-stabilizing ability (44). Confocal experiments demonstrated that curcumin increased the colocalization of cytosolic pathogens with the lysosomal compartment and decreased that of vacuolar pathogens. This suggests that curcumin indeed stabilizes the vacuolar membrane and prevents the escape of cytosolic pathogens, consequently leading to their increased fusion with lysosomes. The

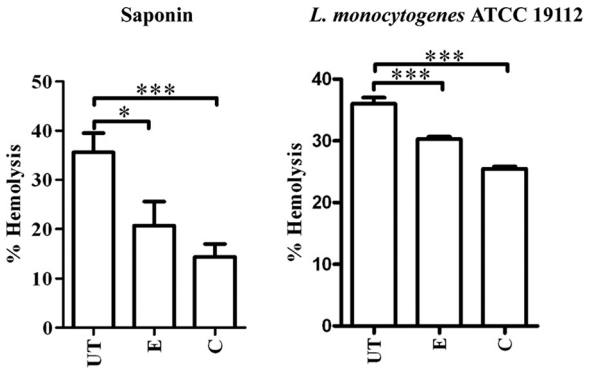

FIG 10 Curcumin and erythromycin retarded the lysis of erythrocytes by saponin and *L. monocytogenes*. The erythrocytes were treated with curcumin (10 μM, 2 h) and erythromycin (100 μg/ml, 1 h) and exposed to either saponin (0.001%, 5 min) or *L. monocytogenes* (10 to 15 min). The supernatant was assayed for the amount of hemolysis at 540 nm. The percent hemolysis was calculated by considering the sample incubated with H_2O as 100% lysis. UT, untreated cells; C, curcumin-pretreated cells; E, erythromycin-pretreated cells. Student's *t* test was used for testing the statistical significance of the data. ***, $P < 0.001$; *, $0.01 \leq P < 0.05$.

pathogens residing in vacuoles have an inherent potential for preventing the fusion of PCV with lysosomes. The stabilization of the vacuolar membrane by curcumin would aid this process, thus decreasing their fusion with lysosomes. The proof for membrane stabilization by curcumin was obtained through its ability to prevent lysis of RBCs by either detergent, saponin, or a hemolytic strain of *L. monocytogenes*. Effects similar to those of curcumin were also seen with erythromycin, a known membrane stabilizer. Thus, the differential modulation of the pathogenicity of cytosolic and vacuolar pathogens by curcumin can be attributed to its membrane-stabilizing ability that differentially regulated the fusion of pathogens with lysosomes (Fig. 11). Thus, even if curcumin acts as a suppressor of the type 1 immune response, as shown in previous studies, it does not appear to exacerbate infection/intracellular survival of all intracellular pathogens.

Our study mainly involves enteric pathogens; because curcumin is a dietary compound, it might hold promise in the regulation of the pathogenesis of other clinically important enteric pathogens as well.

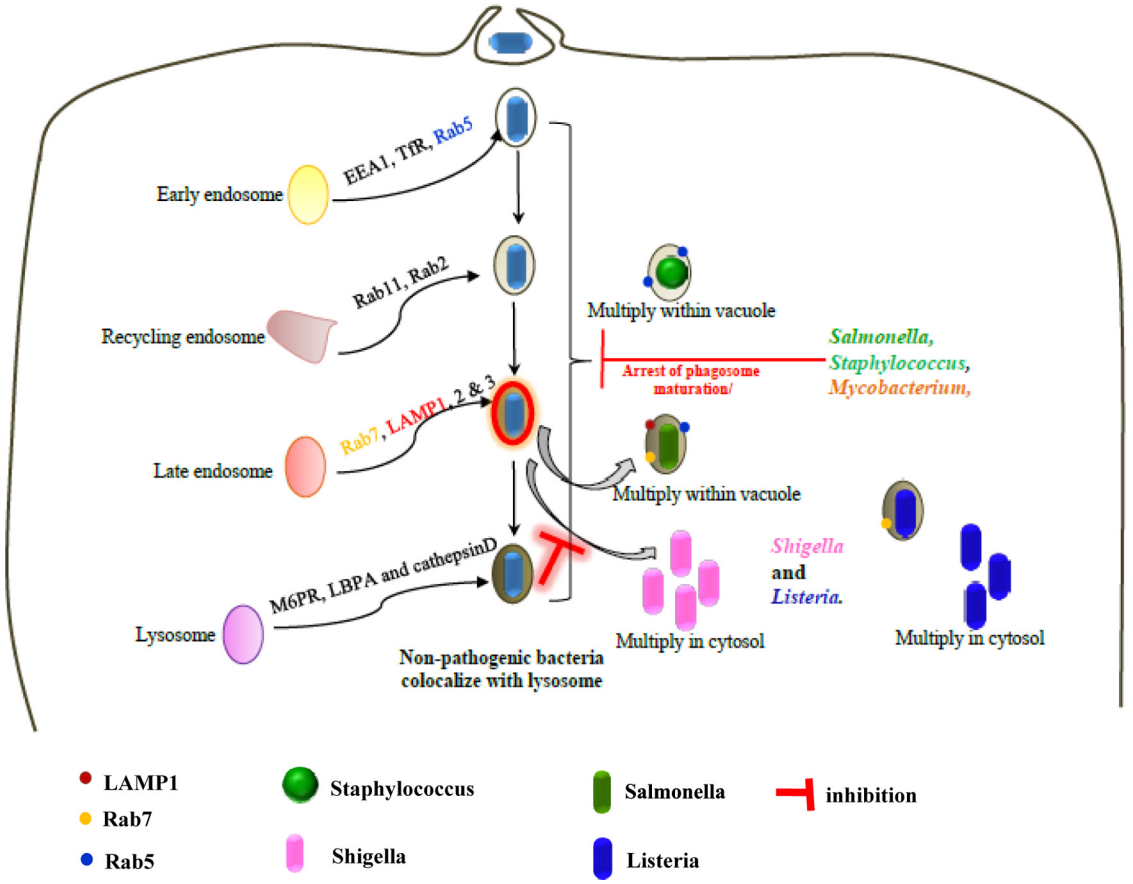

FIG 11 Schematic representation of mode of action of curcumin in differentially regulating the pathogenicity of different bacteria. The macrophages phagocytose the microorganism, which sequentially fuses with early endosomes, late endosomes, and ultimately, the lysosome, wherein it gets degraded. A few enteric pathogens, such as *Salmonella*, *Mycobacterium*, and *Staphylococcus*, have acquired machinery that inhibits the fusion of the pathogen-containing vacuole with lysosomes, whereas other pathogens, such as *Shigella* and *Listeria*, escape into the cytosol, thereby preventing lysosomal degradation. Membrane stabilization by curcumin (vacuole highlighted with a red outline) interferes with this mechanism by enhancing the ability of vacuolar bacteria to avoid fusion with lysosome, whereas it inhibits the escape of the cytosolic pathogen into the cytosol.

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