Original Article

Autophagy counters LPS-mediated suppression of lysozyme

Sudha B Singh[1,2] and Henry C Lin[1,2]

Abstract

Impaired Paneth cell expression of antimicrobial protein (AMP) lysozyme is found in patients with Crohn's disease with the autophagy gene *ATG16L1* risk allele, in mice with mutations in autophagy genes *Atg16L1*, *Atg5* and *Atg7*, and in *Irgm1* knockout mice. Defective autophagy is also associated with expansion of resident Gram-negative bacteria in the intestinal lumen. These findings suggest that autophagy may control extracellular resident microbes by governing expression of lysozyme. To test the hypothesis that autophagy may have a defensive role in host response to resident extracellular microbes, we investigated the relationship between gut microbes, autophagy, and lysozyme. RAW 264.7 macrophages were treated with fecal slurry (FS), representing the resident microbial community; lipopolysaccharide (LPS); or butyrate, representing microbial products; or a representative resident Gram-negative bacterium *Desulfovibrio vulgaris* (DSV). FS, LPS, and DSV inhibited lysozyme expression, whereas butyrate had no effect. Induction of autophagy by rapamycin countered this inhibition, whereas silencing of the autophagy gene *Irgm1* exacerbated the inhibitory effects of LPS on lysozyme expression. LPS also inhibited lysozyme activity against DSV and autophagy reversed this effect. Our results provide a novel insight into an interaction between gut bacteria, autophagy and AMP whereby autophagy may defend the host by countering the suppression of antimicrobial protein by Gram-negative bacteria.

Introduction

Autophagy has been well characterized for its function in eliminating intracellular pathogens. However, its potential role in controlling extracellular, non-pathogenic resident gut microbes remains unexplored. A recent study provided clues suggesting this function of autophagy. Crohn's disease (CD) patients homozygous for the *ATG16L1 T300A* risk allele were found to have an increased number of Gram-negative resident gut microbes in the intestinal lumen belonging to the families Enterobacteriaceae, Bacteroidaceae and Fusobacteriaceae.[1] The underlying mechanism of this association with the mutated autophagy gene remains unknown. Another study on CD patients homozygous for the *ATG16L1 T300A* risk allele found that there was an abnormal distribution of antimicrobial protein lysozyme in intestinal Paneth cells.[2] This finding was further validated in mouse models of defective components of autophagy machinery, such at Atg5, Atg16l1, Atg7 and Irgm1.[3,4]

Antimicrobial peptides/proteins (AMPs) are ancient immune defense mechanisms that are found in all organisms. AMPs have potent microbial killing properties against wide spectrum of organisms, including bacteria, viruses, fungi, and protozoa. AMPs are expressed both broadly and in specialized cells and occur as inducible or constitutively expressed molecules. Some examples of AMPs include lysozyme, phospholipase A2, cathelicidins, α-defensins (e.g. HD5 and HD6), and β-defensins (e.g. HBD-1 and HBD-2). AMPs not only protect the host against invading pathogens, but also shape the composition of resident

[1]Section of Gastroenterology, Medicine Service, New Mexico VA Health Care System, Albuquerque, NM, USA
[2]Division of Gastroenterology and Hepatology, Department of Medicine, the University of New Mexico, Albuquerque, NM, USA

Corresponding author:
Henry C Lin, New Mexico VA Health Care System 1501 San Pedro SE, Albuquerque, NM 87108, USA.
Email: helin@salud.unm.edu

commensal gut microbes.[5] Defective AMP production was found to be associated with alteration of gut microbial community.[6]

These findings suggest that autophagy may also have a role beyond the defense against intracellular pathogens. Could autophagy also control the extracellular resident gut microbial population via secretion of AMPs such as lysozyme? In this study, we tested the hypothesis that autophagy may have a defensive role in the host response to resident extracellular microbes by testing the relationship between gut microbes, autophagy, and lysozyme *in vitro* in macrophages.

Materials and methods

Cell culture and treatments

RAW264.7 cells were purchased from and grown according to ATCC methods, in DMEM supplemented with 10% FBS. No antibiotics or any other antimicrobial reagents were added to the culture medium, as they were found to interfere with the LPS inhibitory effects on lysozyme expression. Cells were treated for 3 h with filtered fecal slurry (in PBS) obtained from euthanized mice, or with LPS (L4391; Sigma, St. Louis, MO, USA), sodium butyrate (B5887; Sigma) or *Desulfovibrio vulgaris* (DSV) for 24 h. Polymyxin (P4932; Sigma) was added to a final concentration of 0.1 mg/ml 1 h before the LPS challenge. Rapamycin (R8781; Sigma) at 100 nM was added to the cells 1 h prior to challenge with test reagents. DMSO-treated cells (volume equivalent to what was used for 100 nM rapamycin) were used as controls. Rapamycin was also added to the cells 24 h after LPS challenge. In this case, rapamycin was added for 24 h in the continued presence of LPS. Cells were harvested and processed for preparation of protein and RNA samples.

DSV culture

The sulfate-reducing bacterium, DSV Hildenborough NCBI 8303, was grown on a lactate–sulfate medium according to previously published methods.[7]

siRNA transfection

siRNA against mouse *Irgm1* was purchased from Dharmacon (Lafayette, CO, USA). Thirty-five nM siRNA was used for transfection of RAW cells. Transfection was carried out with Lipofectamine 2000 (52758; Thermo Fisher Scientific, Waltham, MA, USA) using manufacturer's protocol. At 24 h post-transfection, cells were trypsinized and plated into six-well plates. After an additional 24 h, cells were treated with the test reagents. *Irgm1* knock down was confirmed with Western blotting using *Irgm1* Ab (sc11075; Santa Cruz, CA, USA).

Western blot

RAW cells were lysed in Lysis buffer (87787; Thermo Fisher Scientific) containing protease and phosphatase inhibitors (1861281; Thermo Fisher Scientific). Fifty μg protein samples were run on SDS-PAGE and transferred onto a nitrocellulose membrane. Membranes were blocked in 5% milk for 30 min followed by overnight (~18 h) incubation in lysozyme Ab (ab108508; Abcam, Cambridge, UK), actin Ab (4970; Cell Signaling, Danvers, MA, USA) or LC3 Ab (27755; Cell Signaling). Actin and LC3 Abs were diluted according to the manufacturer (1:1000 in 5% BSA). Lysozyme Ab was diluted 1:5000 in 5% milk. All the dilutions were made in wash buffer PBS–0.1% Tween 20. Blots were incubated with secondary Abs (7074; Cell Signaling) at room temperature (~20°C) for 1 h (dilution of 1:2000). Blots were developed using enhanced chemiluminescence (32106; Thermo Fisher Scientific).

Quantitative PCR

RNA (RNeasy Mini Kit, 74106; Qiagen, Hilden, Germany) was extracted from cells and cDNA synthesis was carried out using cDNA kit (18080-051; Thermo Fisher Scientific). Real-time quantitative PCR (qPCR) was performed using SYBR green (204145; Qiagen). The primer sequences were as follows: 18s F: GTAACCCGTTGAACCCCAT; 18s R: CCATCC AATCGGTAGTAGCG; Lyz F: GGATCAATTGCA GTGCTCTG; Lyz R: CAGTTCCGAATATACTGG GAC. qPCR conditions were as follows: 95°C for 15 min for 40 cycles; then 30 s at 95°C, 30 s at 51°C and 30 s at 72°C; and for the melting curve: 95°C for 15 s, 60°C for 30 s, 95°C for 15 s

Filtered mouse fecal slurry

The luminal contents of the cecum and colon were collected from mice and re-suspended in PBS. The slurry was filtered through glass wool followed by filtration through 0.2-μm filters. The filtrate was then added to RAW cells for 3 h.

Lysozyme activity assay

The protocol for lysozyme activity of the cells against *Micrococcus lysodeikticus* (M3770; Sigma) was adopted from a published protocol.[8] Enzyme activity against DVS in the culture supernatant of RAW cells was analyzed as described.[9]

For M. lysodeikticus. Briefly, cells were lysed and protein concentration was normalized to 1 mg/ml. *Micrococcus lysodeikticus* was prepared (2 mg/ml in PBS, pH 7.0) and further diluted to obtain ~0.7 OD_{450}. Two hundred μl of this solution was mixed with 100 μl protein sample

in a 96-well plate in triplicates and OD_{450} was analyzed at 90 min.

For DSV. DSV culture was centrifuged and re-suspended in 0.1 M EDTA in 0.05 M Tris pH 9.0 and incubated in ice for 10 min. Bacteria were washed in 0.05 M Tris pH 7.0 and re-suspended in the same buffer. The bacterial solution was then diluted to the OD_{600} of ~ 0.8. One ml of this solution was added to 100 μl of the culture supernatant (prepared by concentrating 2 ml of culture supernatant using Amicon filters to a volume of 100 μl). OD_{600} was analyzed at 60 min.

Lactate Dehydrogenase (LDH) cytotoxicity assay

LDH activity was determined using a commercially available kit (8895; Thermo Fisher Scientific) based on the manufacturer's protocol.

Lysozyme detection in the culture supernatant

Culture supernatants (for extracellular lysozyme), as well as the cell lysates (for cellular lysozyme), were precipitated with nine volumes of ice-cold ethanol and stored at –20° C for 1 h. The samples were centrifuged at 13,000 g for 15 min at 4°C and the supernatant discarded. The pellets were air dried and re-suspended in PBS. Protein concentration was measured with Bradford reagent and 50 μg protein from the supernatant or the lysate was analyzed by Western blotting to detect lysozyme.

Statistical analysis

All graphs were generated using Graph Pad Prism 5 (GraphPad, La Jolla, CA, USA). For qPCR data analysis, we compared differences between the treatments groups using a two-tailed *t*-test. *P*-Values < 0.05 were considered significant.

Results

Resident gut microbes, LPS and resident gut bacterium DSV inhibited lysozyme expression

To test directly the relationship between gut microbial products, autophagy and AMP, we carried out *in vitro* studies using RAW 264.7 macrophages. In the gut, lysozyme is produced mainly by macrophages and Paneth cells. While Paneth cells are found primarily in the ileum, macrophages are present throughout the length of the gut.[10]

We first tested the effect of filtered mouse fecal slurry (FS), comprised of metabolites and other gut microbial products, as a surrogate for gut microbes on lysozyme expression in RAW cells. We found that FS had an inhibitory effect on lysozyme expression (Figure 1a).

We then tested the effect of bacterial endotoxin LPS, one of the most abundant bacterial products found in feces.[11] We found that purified LPS also inhibited lysozyme expression, an effect similar to FS (Figure 1a). Quantification of Western blot revealed a significant difference in lysozyme expression in control (1.13 ± 0.05) vs. FS $(0.32 \pm 0.14; P < 0.01)$ and in control (0.99 ± 0.04) vs. LPS-treated cells $(0.10 \pm 0.03; P < 0.001)$. This is consistent with previously published studies on the inhibition of lysozyme expression by LPS in macrophages.[12,13] FS and LPS also inhibited lysozyme expression at the mRNA level (Figure 1b). Compared with control cells (1.01 ± 0.10), a significant decrease was observed in lysozyme expression in FS-treated $(0.62 \pm 0.02; P < 0.05)$ and LPS-treated $(0.43 \pm 0.14; P < 0.05)$ cells. LPS induced an increase in IL-1β mRNA level in our experiments, confirming its expected pro-inflammatory role (data not shown).

To test whether or not the inhibitory effect of LPS is specific we next tested the effect of butyrate, a Gram-positive bacterial product, on lysozyme expression. Butyrate, a microbe-derived short-chain fatty acid, is a nutrient vital for the host and has anti-inflammatory properties.[14] We found that treatment with sodium butyrate (NaBu) had no effect on lysozyme production (Figure 1c). Moreover, pre-incubation of cells with butyrate did not confer protection against inhibitory effect of LPS on lysozyme. Thus, inhibition of lysozyme may be specific to certain bacterial products such as LPS produced by Gram-negative bacteria.

We further tested the effects of LPS on lysozyme expression in the presence of polymyxin B, a LPS neutralizing agent. We found that in the presence of polymyxin B there was no inhibition of lysozyme by LPS (Figure 1d).

Autophagy counters the inhibitory effect of LPS and DSV on lysozyme

We next investigated the effect of autophagy on the inhibition of lysozyme by LPS. Using siRNA, we silenced the autophagy gene for the protein Irgm1.[15] Suppression of Irgm1 exacerbated the inhibitory effect of LPS on lysozyme (Figure 2a). A significant difference was observed in protein levels of lysozyme in the presence of LPS in scrambled (scr) (0.55 ± 0.11) vs. Irgm1 knockdown cells (siIrgm1) $(0.19 \pm 0.01; P < 0.05)$ (Figure 2b). Conversely, induction of autophagy with rapamycin protected against the effects of LPS on lysozyme when cells were treated with rapamycin prior to challenge with LPS (Figure 2c, d). While a significant difference was observed in lysozyme expression in control cells (1.0 ± 0.19) compared with LPS-treated cells (LPS) $(0.27 \pm 0.10; P < 0.05)$, no difference was observed between control (1.0 ± 0.19) and rapamycin plus LPS-treated cells (Rapa + LPS) $(0.65 \pm 0.21; P > 0.05)$. This was also true at the mRNA level
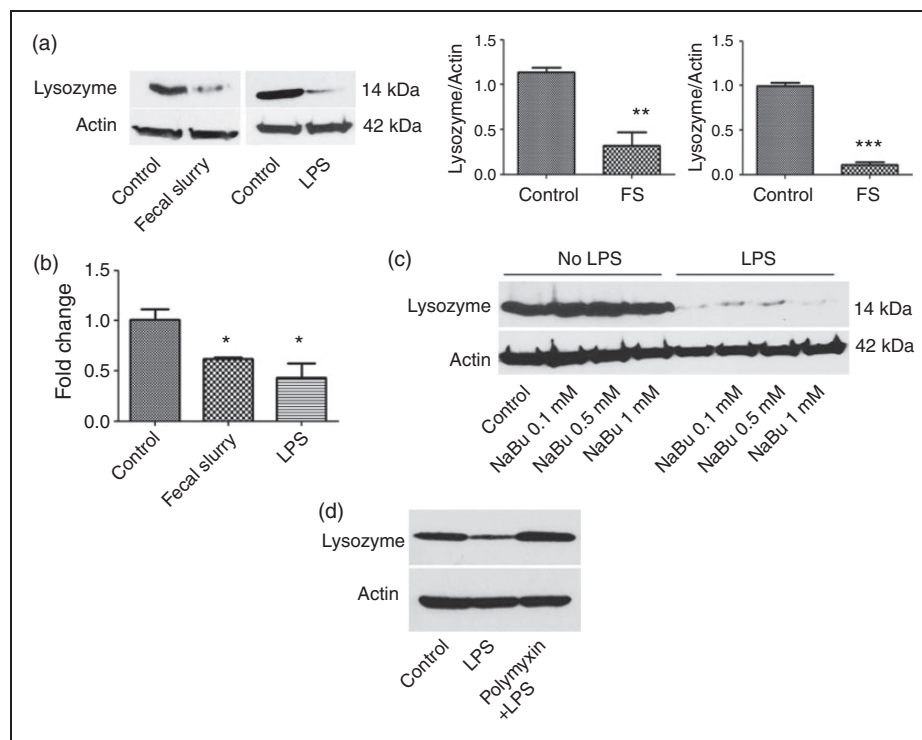
Figure 1. FS and LPS down-regulated lysozyme (a) RAW 264.7 cells grown on a six-well plate were treated with freshly prepared FS (200 µl) for 3 h and LPS (100 ng/ml) for 24 h and cells were processed for immunoblotting with lysozyme antibody. The ratio of lysozyme to actin was quantified using ImageJ software (NIH, Bethesda, MD, USA). Graph represents mean ± SEM ($n = 3$); **$P < 0.05$, ***$P < 0.01$ (two-tailed t-test). (b) RNA was isolated from the control or the FS- or LPS-treated cells and lysozyme transcription levels were analyzed with qPCR. Results are shown as mean ± SEM; *$P < 0.05$ (two-tailed t-test). (c) Cells were treated with different concentrations of NaBu for 24 h. Cells were further incubated in the presence or absence of LPS (100 ng/ml) for 24 h and analyzed by Western blotting for lysozyme expression (d). Cells were treated with polymyxin B (0.1 mg/ml) for 1 h prior to LPS challenge for 24 h and processed for Western blotting.

where a significant difference was observed between LPS-treated (0.24 ± 0.07) without pre-treatment vs. cells pre-treated with rapamycin (1.13 ± 0.19; $P < 0.05$) (Figure 2e). Activation of autophagy by rapamycin was confirmed by showing an increase in expression of the autophagic marker LC3II. We observed a slight increase in autophagy by LPS, which is in line with the other studies (Figure 2c).[16,17] However, a mild induction of autophagy by LPS alone was not sufficient to overcome the inhibitory effects of LPS on lysozyme.

We next investigated the effect on lysozyme expression when rapamycin was added after LPS treatment. Cells were first treated with LPS (100 ng/ml) for 24 h and subsequently treated with various doses of rapamycin for 24 h in the continued presence of LPS. We found that rapamycin reversed the inhibitory effects of LPS on lysozyme expression (Figure 2f). Thus, rapamycin not only conferred protection against subsequent challenge with LPS, but also reversed the inhibitory effects on lysozyme in cells previously treated with LPS.

To test the direct effect of a Gram-negative resident microbe, we also tested the effect of DSV on lysozyme expression in RAW cells. DSV is an LPS-producing

Gram-negative bacterium that belongs to a group of sulfate-reducing bacteria which are found in healthy subjects but may be increased in number in patients with inflammatory bowel diseases such as ulcerative colitis.[18,19] DSV inhibited lysozyme protein expression comparable with LPS (Figure 3a), and induction of autophagy by rapamycin (Rapa + DSV) overcame this inhibitory effect. We found the DSV also inhibited the lysozyme gene expression (control 1 ± 0 vs. DSV 0.30 ± 0.07; $P < 0.05$) in RAW cells and these effects could be reversed by rapamycin (DSV 0.30 ± 0.07 vs. Rapa + DSV 1.04 ± 0.17; $P < 0.05$) (Figure 3b).

We next tested the effect of LPS and rapamycin treatment on cell viability using lactate dehydrogenase activity assay (LDH) (Figure 4a). LDH is released into the culture medium when the plasma membrane is damaged. The amount of the enzyme released is measured by it enzymatic activity. The culture supernatants were collected and analyzed for LDH activity. The supernatant:pellet ratio of LDH activity was calculated and values were plotted as fold change difference vs. control. We observed a small increase in LDH activity with LPS treatment (1.38 ± 0.04) vs. control (1.00 ± 0.09; $P < 0.05$). Prior treatment with rapamycin
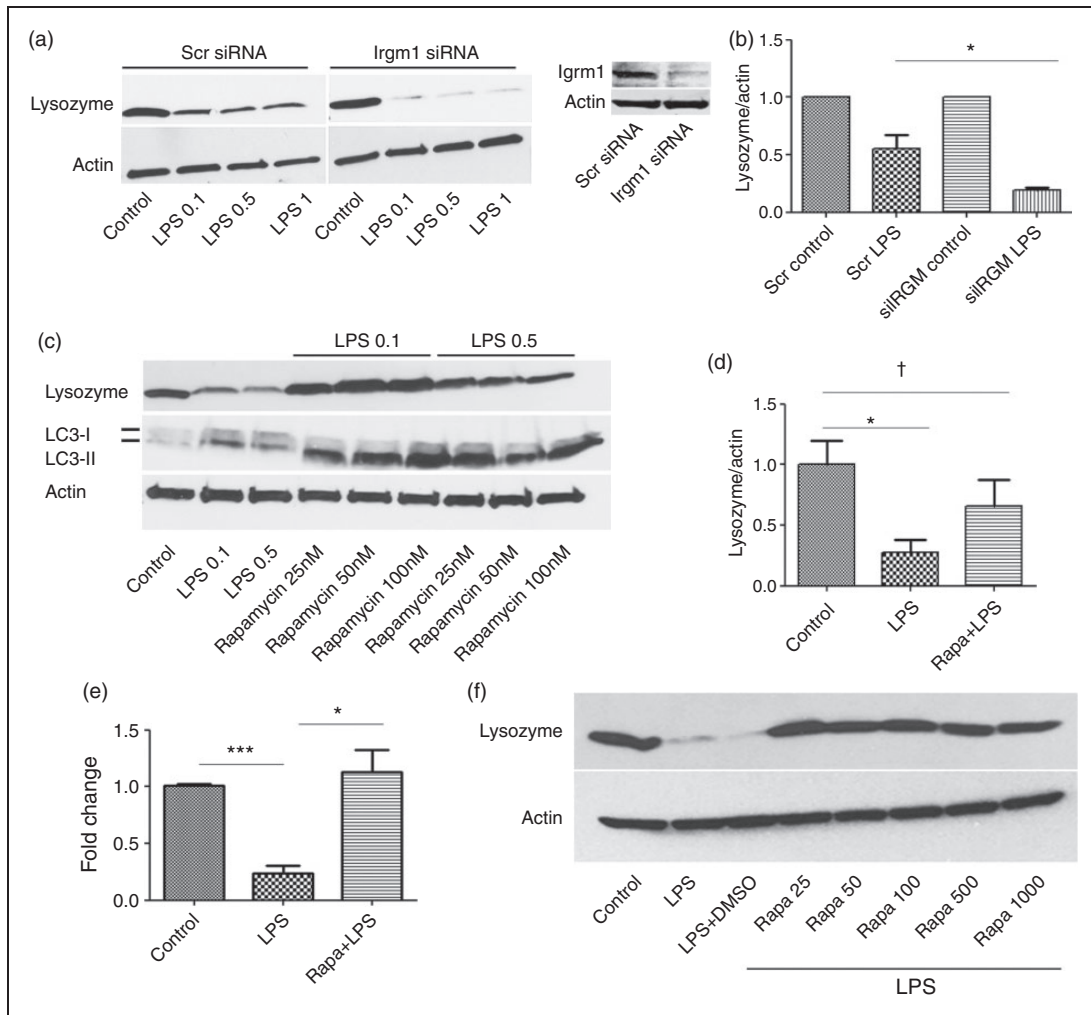
Figure 2. Autophagy overcomes inhibitory effect of LPS on lysozyme. (a) Cells were transfected with control or *Irgm1* siRNA for 48 h and then incubated with different LPS concentrations for 24 h and analyzed for lysozyme by immunoblotting. Knockdown was confirmed with Irgm1 Ab. (b) Quantification of lysozyme protein in scrambled vs. silrgm1 knockdowns in the absence of presence of LPS. Graph represents mean ± SEM ($n = 3$); *$P < 0.05$ (two-tailed t-test). (c) Cells were incubated with or without rapamycin at different concentrations for 1 h and challenged with LPS (0.1 or 0.5 µg/ml) for 24 h in the presence or absence of the drug. (d) RAW cells were pre-incubated with or without rapamycin before LPS treatment. Lysozyme/actin ratio was quantified using ImageJ. Data represents mean ± SEM ($n = 3$); *$P < 0.05$, †$P > 0.05$ (two-tailed t-test). Fold change differences compared with control were reported for all quantifications. Lysozyme was assessed at mRNA level. (e) RAW cells were pre-incubated with or without rapamycin and further incubated with LPS. Lysozyme was assessed at mRNA level. Data represent mean ± SEM ($n = 3$); *$P < 0.05$, ***$P > 0.05$ (two-tailed t-test). (f) Cells were treated with LPS (100 ng/ml) for 24 h followed by treatment with different doses of rapamycin (nM) in the presence of LPS for another 24 h. Cells were then processed for Western blotting.

caused a reduction in LDH activity (1.19 ± 0.041) vs. LPS (1.38 ± 0.04; $P < 0.05$) (Figure 4a).

To check whether the observed inhibition of lysozyme by LPS was due to increased secretion of lysozyme, we analyzed the levels of extracellular lysozyme in the culture supernatant following treatments (Figure 4b). While the levels of lysozyme in cell lysates yielded expected results (Figure 4c), the levels of secreted lysozyme appeared comparable in control, LPS- and rapamycin + LPS-treated cells (Figure 4d). These results argue away from the possibility that the observed decrease in cellular level of lysozyme in LPS treated cells was due to increased secretion of lysozyme.

Given our findings, we further tested if decreased expression of lysozyme in response to LPS corresponded to decreased biological activity of lysozyme and if autophagy protected against this effect. We found that LPS treatment caused a decrease in the antimicrobial activity of lysozyme against the standard target *M. lysodeikticus* (control 13263.7 ± 3527 vs. LPS 5505 ± 1297) and pre-treatment with rapamycin countered this effect (Rapa + LPS 11343 ± 2751 vs. LPS 5505 ± 1297; $P < 0.05$) (Figure 4e).

The lytic effect of pure lysozyme on DSV has been previously documented.[9] When we tested the lytic activity of culture supernatants of RAW cells on DSV,
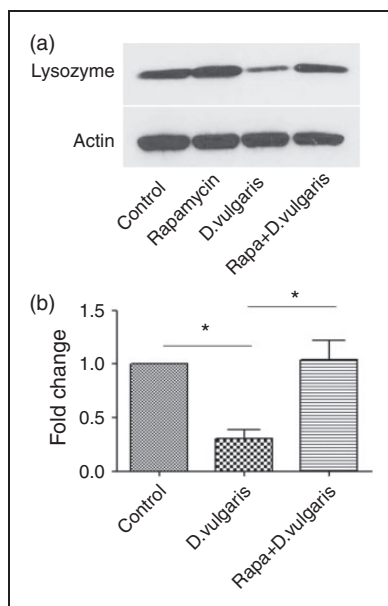
Figure 3. Autophagy overcomes inhibitory effect DSV on lysozyme. (a) Cells were treated with DMSO or rapamycin (100 nm) 1 h prior to adding DSV for 24 h. (b) Lysozyme mRNA levels were assessed in the absence or presence of rapamycin prior to treatment with DSV. Data represent mean ± SEM ($n = 3$); *$P < 0.05$, †$P > 0.05$ (two-tailed t-test).

a significant inhibitory effect in enzyme activity was observed between the control (6135.4 ± 239.2) compared with LPS-treated supernatant (4442.19 ± 182.7; $P < 0.01$) (Figure 4f). Pre-incubation of cells with rapamycin prior to LPS challenge recovered lytic activity in the LPS-treated supernatant (6649.83 ± 332.1; $P < 0.01$).

Discussion

In this study, we investigated the relationship between gut microbial products, autophagy and lysozyme to test the hypothesis that autophagy may have a role in the host response to resident extracellular microbes. For this purpose, we selected RAW 264.7 macrophages. Macrophages play a key role in the host innate immune response.[20] These cells are located in the strategic boundary between the luminal content and the host and contribute to innate immunity via phagocytosis and secretion of lysozyme and anti- and pro-inflammatory cytokines.[21] Macrophages in the gut protect against invading pathogens while maintaining tolerance to the resident commensal microbes. In the intestine, lysozyme is also secreted by Paneth cells. However, while Paneth cells are found primarily in the ileum, macrophages are present throughout the length of the gut.[10] These properties and functions of macrophage make them suitable candidates to study host response to microbes and microbial products.

We found that FS suppressed lysozyme expression suggesting that the predominant effect of resident gut microbes is inhibition of host lysozyme expression. To further break down the components in FS, we tested the effect of LPS, a Gram-negative bacterial product and butyrate, a Gram-positive product on lysozyme expression. We found that LPS but not butyrate inhibited lysozyme expression. This is consistent with previous studies showing inhibition of lysozyme by LPS in mouse macrophages,[12,13] but divergent from LPS obtained from *Salmonella enterica* sv. Typhimurium, which stimulated lysozyme production in chicken myelomonocytic cells and in Atlantic salmon macrophages.[22,23] LPS derived from *Escherichia coli* 0127:B8 did not have any effect on lysozyme activity in RAW 264.7 cells.[8] Thus, LPS has been shown to be both inhibitory and stimulatory for lysozyme production with specific effect of LPS depending on the source, concentrations and the length of treatment and the targeted cells. The inhibitory effect of LPS on lysozyme in our study was not universal to bacterial products as we did not observe the same phenomena with butyrate, another gut microbial metabolite.

We tested the effect of a representative LPS-producing Gram-negative resident gut bacterium, DSV, which is not an intracellular pathogen. We found that DSV inhibited lysozyme expression, possibly owing to its LPS effects. This result demonstrated an inhibitory effect of an extracellular, non-pathogenic resident gut microbe.

To test the role of autophagy in LPS-mediated inhibition of lysozyme, we tested the effects of rapamycin, an inducer of autophagy. Rapamycin was successful in countering the inhibitory effects of LPS on lysozyme production, demonstrating that autophagy is a positive regulator of lysozyme expression.

LPS may induce autophagy in RAW cells via a TLR4-dependent pathway.[16] We also found a moderate induction of autophagy by LPS in our study which is consistent with the previous findings (Figure 2c). However, this increase from LPS alone was insufficient to counter the inhibition of lysozyme by LPS in the absence of the stronger inducer of autophagy. One possible explanation for this is that LPS also induces NF-κB and NF-κB signaling can inhibit autophagy.[24,25] Since rapamycin has been shown to inhibit NF-κB activation,[26–28] this may explain the successful overcoming of inhibitory effects of LPS by rapamycin-induced autophagy in our study.

Triggering of autophagy alone, however, is not enough to affect lysozyme since we did not observe any effect on lysozyme expression when cells were treated with rapamycin alone (Figure 3a). LPS and autophagy are intimately linked in such a way that the full defensive role of autophagy is only revealed in the presence of the inhibitory effects of LPS. Our findings are in agreement with a published study which showed that
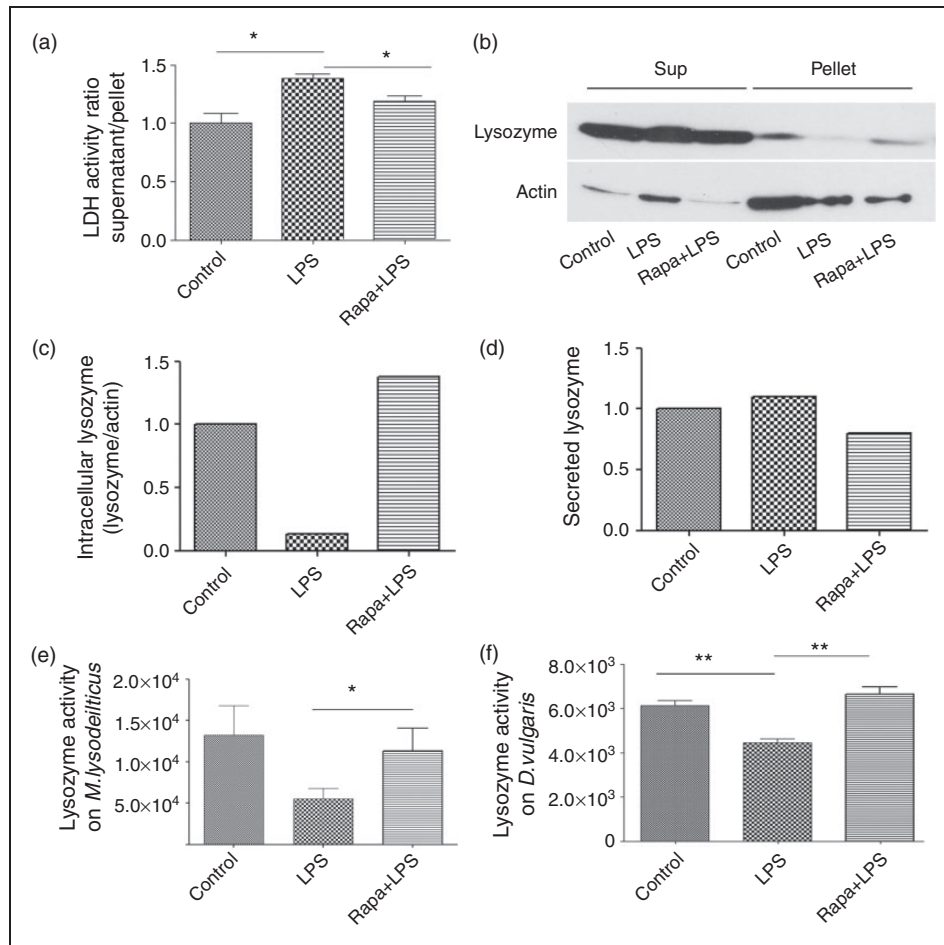
Figure 4. Effect of rapamycin and LPS on lysozyme secretion and activity. (a) RAW 264.7 cells were tested for viability with LDH cytotoxicity assay kit in response to treatment with LPS in the absence of presence of rapamycin. Values are represented as the ratio of supernatant to pellet activity and are normalized against control. (b) Proteins in culture supernatants and cell lysates were precipitated using ethanol as described. Fifty μg protein was loaded on SDS-PAGE for Western blot analysis for lysozyme detection. (c, d) Densitometric analysis of Western blot using ImageJ for (c) cellular lysozyme, as a ratio of lysozyme to actin and (d) extracellular lysozyme. Values were normalized against controls in both cases. (e) Cells were pre-treated with DMSO or rapamycin and further incubated with LPS (100 ng/ml) for 24 h. Cells were lysed and lysozyme activity was measured using *M. lysodeikticus* as substrate. (f) Cells were treated the same as before, but the culture supernatant was collected after treatment of the cells and checked for the lytic activity of lysozyme on DSV. Results represented as mean $\pm$ SEM. Data are from three different experiments. *$P < 0.05$; **$P < 0.01$ (two-tailed t-test).

adiponectin treatment counteracted LPS-induced TNF-α expression via autophagy induction.[29] In another published study, treatment of human monocytes with vitamin D and LPS, but not LPS alone, caused upregulation in genes of autophagy and antimicrobial peptide pathways.[30] These findings support the idea that induction of autophagy can overcome LPS-meditated effects and this is independent of autophagy triggered by LPS treatment alone.

Suppression of lysozyme expression by LPS and countering this effect by autophagy is clinically significant. One important example is that of a life-threatening Gram-negative sepsis, a major cause of mortality in hospitalized patients. The lethality of this condition may be understood in terms of a failure of autophagy on LPS-mediated inhibition of host defense.

Since lysozyme decreases the toxicity of LPS and has an anti-septic effect,[31–34] inhibition of lysozyme by LPS would favor the bacterial infection. We found not only that the prior treatment with rapamycin conferred protection against LPS-induced inhibition of lysozyme, but also reversed the effects of LPS on lysozyme expression when rapamycin was added after LPS treatment. Our findings suggest the therapeutic potential of both preventing and treating sepsis via induction of autophagy. Thus, our findings provide an explanation for the reported protective role of autophagy in sepsis.[35,36] This protective effect of autophagy may be the result of its successful over-ride of endotoxin-mediated suppression of host production of AMPs. In contrast, lethal sepsis occurs when autophagic response is impaired. Our findings would explain why

polymorphism in autophagy genes *IRGM* and *ATG16L1* have been associated with susceptibility to sepsis.[37,38]

The loss of intracellular lysozyme is not due to increased secretion as confirmed by our finding that levels of lysozyme in the culture supernatant treated with LPS was comparable with control and in cells treated with rapamycin prior to LPS challenge. While our data show that lysozyme is inhibited at transcription and translation levels, it remains to be determined whether lysozyme is degraded by intracellular pathways in the presence of LPS.

Our data showed lytic activity of the culture supernatant against DSV, which was reduced by LPS in association with a significant decrease in lysozyme protein expression. We found that induction of autophagy by rapamycin protected against this effect. The observed lytic activity of lysozyme against DSV suggests that lysozyme activation downstream of autophagy may function to control the load of these extracellular resident gut bacteria and that deficiency in autophagic machinery may lead to decrease in lysozyme, which, in turn, may allow overgrowth of DSV in disease conditions.

The mechanism by which changes in the gut bacteria signal autophagic machinery and subsequent AMP production may also hold the key to potential treatment options for disorders linked to dysbiosis. In conclusion, our findings suggest that the role of autophagy in controlling microbes may be larger than our current understanding of its role as antimicrobial defense against intracellular pathogens.

Declaration of Conflicting Interests

Funding

References

1. Sadaghian Sadabad M, Regeling A, de Goffau MC, et al. The ATG16L1-T300A allele impairs clearance of pathosymbionts in the inflamed ileal mucosa of Crohn's disease patients. *Gut* 2015; 64: 1546–1552.

2. Cadwell K, Liu JY, Brown SL, et al. A key role for autophagy and the autophagy gene *Atg16l1* in mouse and human intestinal Paneth cells. *Nature* 2008; 456: 259–263.

3. Cadwell K, Patel KK, Komatsu M, et al. A common role for Atg16L1, Atg5 and Atg7 in small intestinal Paneth cells and Crohn disease. *Autophagy* 2009; 5: 250–252.

4. Liu B, Gulati AS, Cantillana V, et al. Irgm1-deficient mice exhibit Paneth cell abnormalities and increased susceptibility to acute intestinal inflammation. *Am J Physiol Gastrointest Liver Physiol* 2013; 305: G573–G584.

5. Ostaff MJ, Stange EF and Wehkamp J. Antimicrobial peptides and gut microbiota in homeostasis and pathology. *EMBO Mol Med* 2013; 5: 1465–1483.

6. Salzman NH. Paneth cell defensins and the regulation of the microbiome: détente at mucosal surfaces. *Gut Microbes* 2010; 1: 401–406.

7. Biswas KC, Woodards NA, Xu H, et al. Reduction of molybdate by sulfate-reducing bacteria. *Biometals* 2009; 22: 131–139.

8. Babcock AS, Anderson AL and Rice CD. Indirubin-3′-(2,3 dihydroxypropyl)-oximether (E804) is a potent modulator of LPS-stimulated macrophage functions. *Toxicol Appl Pharmacol* 2013; 266: 157–166.

9. Findley JE and Akagi JM. Lysis of *Desulfovibrio vulgaris* by ethylenediaminetetraacetic acid lysozyme. *J Bacteriol* 1968; 96: 1427–1428.

10. Smythies LE, Sellers M, Clements RH, et al. Human intestinal macrophages display profound inflammatory anergy despite avid phagocytic and bacteriocidal activity. *J Clin Invest* 2005; 115: 66–75.

11. Saene JJM Van, Stoutenbeek CP and Van Saene HKF. Faecal endotoxin in human volunteers: normal values. *Microb Ecol Health Dis*, http://www.tandfonline.com/doi/abs/10.3109/08910609209141584 (2009, accessed 2 December 2015).

12. Warfel AH and Zucker-Franklin D. Down-regulation of macrophage lysozyme by lipopolysaccharide and interferon. *J Immunol* 1986; 137: 651–655.

13. Schoeniger A, Adolph S, Fuhrmann H, et al. The impact of membrane lipid composition on macrophage activation in the immune defense against *Rhodococcus equi* and *Pseudomonas aeruginosa*. *Int J Mol Sci* 2011; 12: 7510–7528.

14. Säemann MD, Böhmig GA, Osterreicher CH, et al. Anti-inflammatory effects of sodium butyrate on human monocytes: potent inhibition of IL-12 and up-regulation of IL-10 production. *FASEB J* 2000; 14: 2380–2382.

15. Singh SB, Davis AS, Taylor GA, et al. Human IRGM induces autophagy to eliminate intracellular mycobacteria. *Science* 2006; 313: 1438–1441.

16. Xu Y, Jagannath C, Liu X-D, et al. Toll-like receptor 4 is a sensor for autophagy associated with innate immunity. *Immunity* 2007; 27: 135–144.

17. Delgado MA, Elmaoued RA, Davis AS, et al. Toll-like receptors control autophagy. *EMBO J* 2008; 27: 1110–1121.

18. Loubinoux J, Bronowicki J-P, Pereira IAC, et al. Sulfate-reducing bacteria in human feces and their association with inflammatory bowel diseases. *FEMS Microbiol Ecol* 2002; 40: 107–112.

19. Gibson GR, Cummings JH and Macfarlane GT. Growth and activities of sulphate-reducing bacteria in gut contents of healthy subjects and patients with ulcerative colitis. *FEMS Microbiol Ecol* 1991; 9: 103–111.

20. Cipriani G, Gibbons SJ, Kashyap PC, et al. Intrinsic gastrointestinal macrophages: their phenotype and role in gastrointestinal motility. *C Cell Mol Gastroenterol Hepatol* 2016; 2: 120–130.e1.

21. Nagashima R, Maeda K, Imai Y, et al. Lamina propria macrophages in the human gastrointestinal mucosa: their distribution, immunohistological phenotype, and function. *J Histochem Cytochem* 1996; 44: 721–731.

22. Goethe R and Loc PV. The far upstream chicken lysozyme enhancer at -6.1 kilobase, by interacting with NF-M, mediates lipopolysaccharide-induced expression of the chicken lysozyme gene in chicken myelomonocytic cells. *J Biol Chem* 1994; 269: 31302–31309.

23. Paulsen SM, Engstad RE and Robertsen B. Enhanced lysozyme production in Atlantic salmon (*Salmo salar* L.) macrophages treated with yeast beta-glucan and bacterial lipopolysaccharide. *Fish Shellfish Immunol* 2001; 11: 23–37.

24. Trocoli A and Djavaheri-Mergny M. The complex interplay between autophagy and NF-κB signaling pathways in cancer cells. *Am J Cancer Res* 2011; 1: 629–649.

25. Schlottmann S, Buback F, Stahl B, et al. Prolonged classical NF-κB activation prevents autophagy upon *E. coli* stimulation in vitro: a potential resolving mechanism of inflammation. *Mediators Inflamm* 2008; 2008: 1–15.

26. Lai J and Tan T. CD28 signaling causes a sustained down-regulation of I kappa B alpha which can be prevented by the immunosuppressant rapamycin. *J Biol Chem* 1994; 269: 30077–30080.

27. Romano MF, Avellino R, Petrella A, et al. Rapamycin inhibits doxorubicin-induced NF-κB/Rel nuclear activity and enhances the apoptosis of melanoma cells. *Eur J Cancer* 2004; 40: 2829–2836.

28. Giordano A, Avellino R, Ferraro P, et al. Rapamycin antagonizes NF-kappaB nuclear translocation activated by TNF-alpha in primary vascular smooth muscle cells and enhances apoptosis. *Am J Physiol Heart Circ Physiol* 2006; 290: H2459–H2465.

29. Pun NT, Subedi A, Kim MJ, et al. Globular adiponectin causes tolerance to LPS-induced TNF-α expression via autophagy induction in RAW 264.7 macrophages: involvement of SIRT1/FoxO3A axis. *PLOS ONE* 2015; 10: e0124636.

30. Kariuki SN, Blischak JD, Nakagome S, et al. Patterns of transcriptional response to 1,25-dihydroxyvitamin D3 and bacterial lipopolysaccharide in primary human monocytes. *G3 (Bethesda)* 2016; 6: 1345–1355.

31. Takada K, Ohno N and Yadomae T. Detoxification of lipopolysaccharide (LPS) by egg white lysozyme. *FEMS Immunol Med Microbiol* 1994; 9: 255–263.

32. Kurasawa T, Takada K, Ohno N, et al. Effects of murine lysozyme on lipopolysaccharide-induced biological activities. *FEMS Immunol Med Microbiol* 1996; 13: 293–301.

33. Lee W, Ku S-K, Na DH, et al. Anti-inflammatory effects of lysozyme against HMGB1 in human endothelial cells and in mice. *Inflammation* 2015; 38: 1911–1924.

34. Bu HF, Wang X, Zhu YQ, et al. Lysozyme-modified probiotic components protect rats against polymicrobial sepsis: role of macrophages and cathelicidin-related innate immunity. *J Immunol* 2006; 177: 8767–8776.

35. Takahashi W, Watanabe E, Fujimura L, et al. Kinetics and protective role of autophagy in a mouse cecal ligation and puncture-induced sepsis. *Crit Care* 2013; 17: R160.

36. Yen Y-T, Yang H-R, Lo H-C, et al. Enhancing autophagy with activated protein C and rapamycin protects against sepsis-induced acute lung injury. *Surgery* 2013; 153: 689–698.

37. Kimura T, Watanabe E, Sakamoto T, et al. Autophagy-related IRGM polymorphism is associated with mortality of patients with severe sepsis. *PLOS ONE* 2014; 9: e91522.

38. Savva A, Plantinga TS, Kotanidou A, et al. Association of autophagy-related 16-like 1 (ATG16L1) gene polymorphism with sepsis severity in patients with sepsis and ventilator-associated pneumonia. *Eur J Clin Microbiol Infect Dis* 2014; 33: 1609–1614.