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Helminth Infection Impairs Autophagy-Mediated Killing of Bacterial Enteropathogens by Macrophages

Chien-wen Su,*† Yue Cao,*† Mei Zhang,* Jess Kaplan,* Libo Su,* Ying Fu,* W. Allan Walker,* Ramnik Xavier, † Bobby J. Cherayil,* and Hai Ning Shi*

Autophagy is an important mechanism used by macrophages to kill intracellular pathogens. The results reported in this study demonstrate that autophagy is also involved in the macrophage killing of the extracellular enteropathogen Citrobacter rodentium after phagocytosis. The process was significantly impaired in macrophages isolated from mice chronically infected with the helminth parasite Heligmosomoides polygyrus. The H. polygyrus-mediated inhibition of autophagy was Th2 dependent because it was not observed in macrophages isolated from helminth-infected STAT6-deficient mice. Moreover, autophagy of Citrobacter was inhibited by treating macrophages with IL-4 and IL-13. The effect of H. polygyrus on autophagy was associated with decreased expression and processing of L chain protein 3 (LC3), a key component of the autophagic machinery. The helminth-induced inhibition of LC3 expression and processing was STAT6 dependent and could be recapitulated by treatment of macrophages with IL-4 and IL-13. Knockdown of LC3 significantly inhibited autophagic killing of Citrobacter, attesting to the functional importance of the H. polygyrus-mediated downregulation of this process. These observations reveal a new aspect of the immunosuppressive effects of helminth infection and provide mechanistic insights into our earlier finding that H. polygyrus significantly worsens the in vivo course of Citrobacter infection. The Journal of Immunology, 2012, 189: 1459–1466.

Helminths are estimated to infect 3 billion people worldwide (1, 2). The distribution of several helminth pathogens coincides geographically with many devastating microbial diseases including HIV, malaria, and tuberculosis, and it is possible that the strong immunomodulatory effects of helminths on host responses may have a significant impact on such coincident infections (3, 4). Indeed, we have found that infection of mice with the intestinal helminth parasite Heligmosomoides polygyrus exacerbates the colitis caused by concurrent infection with Citrobacter rodentium, a Gram-negative organism used as a model of noninvasive, effacing bacterial enteropathogens such as enteropathogenic Escherichia coli and enterohemorrhagic E. coli (5). The severity of the colitis in the coinfected mice was correlated with high Citrobacter loads in the gut, translocation of the bacteria into mucosal and systemic immune compartments, and uncontrolled bacterial growth (5, 6). Subsequent investigations from our laboratory showed that the increased bacterial translocation and replication were associated with a significantly impaired ability of macrophages from helminth-infected animals to kill phagocytosed C. rodentium, an abnormality that was dependent on intact Th2 responses (7). The mechanisms responsible for the helminth-induced impairment of bacterial killing by macrophages are yet to be elucidated.

Macrophages can contribute significantly to the effector phase of the host antimicrobial response, that is, elimination of bacteria. Recent experiments indicate that one of the important aspects of this process is autophagy. Autophagy is a fundamental cellular homeostatic mechanism that has long been known to be involved in degrading and recycling proteins and organelles. Studies carried out over the last few years have shown that it is also involved in destroying several types of intracellular pathogens, including Mycobacterium tuberculosis, Shigella flexneri, Salmonella typhimurium, and Toxoplasma gondii (8–11). During autophagy, cytosolic proteins and organelles are sequestered by a double membrane. The resulting vacuoles (i.e., autophagosomes) go through a series of maturation steps and eventually fuse with lysosomes for degradation. By a similar process, autophagy can capture and eliminate intracellular pathogens, acting as an important innate immune effector mechanism. Autophagy can be monitored by changes in the microtubule-associated L chain protein 3 (LC3/Atg8), which is converted from the 16-kDa LC3 I form to the lipidated 14-kDa LC3 II form and is recruited to the autophagosomal membrane (12, 13). In addition to its involvement in the removal and destruction of intracellular microbial pathogens, autophagy also plays a role in Ag presentation (the delivery of microbial peptides to endosomes or MHC class II loading compartments) and, thus, in the activation of adaptive immunity (reviewed in Ref. 14). Recent genome-wide association studies have provided evidence linking autophagy and Crohn’s disease (15–17). It has been speculated that mutations in the autophagy pathway may alter the normal gut response to intestinal commensals or pathogens (15–17).

In this study, we show that macrophages normally eliminate phagocytosed Citrobacter by autophagy, and that this process is inhibited, in a Th2-dependent fashion, in animals infected with H. polygyrus. The underlying mechanism involves helminth-induced downregulation of LC3 expression and processing. These results

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Abbreviations used in this article: Arg1, arginase 1; IBD, inflammatory bowel disease; KO, knockout; LB, Luria broth; LC3, L chain protein 3; siRNA, small interfering RNA.
reveal a novel immunosuppressive consequence of helminth infection.

Materials and Methods

Mice

Six- to 8-wk-old female BALB/cByl and STAT6 knockout (KO) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). They were fed autoclaved food and water, and were housed in a specific pathogen-free facility at Massachusetts General Hospital. All animal experiments were approved by the Institutional Subcommittee on Research Animal Care.

H. polygyrus infection

H. polygyrus was propagated as previously described and stored at 4°C until use (2). Mice were inoculated orally with 200 third-stage larvae (L3).

C. rodentium infection

Mice were orally inoculated with C. rodentium (strain DBS100; American Type Culture Collection). Bacteria were grown overnight in Luria broth (LB) and resuspended in PBS before infecting the mice (0.5 ml/mouse; 5 x 10^8 colony-forming units (CFU) C. rodentium). GFP-expressing C. rodentium (GFP-C. rodentium; provided by Dr. L. Bry, Brigham and Women’s Hospital, Boston, MA) were grown overnight in LB containing carbenicillin (100 μg/ml) and resuspended in PBS before in vitro infection assays.

Real-time quantitative RT-PCR

Total RNA was prepared from thioglycollate-elicited peritoneal macrophages using TRIZol reagent (Invitrogen Life Technologies) following the manufacturer’s recommendations. cDNA was synthesized using 2 μg total RNA (Ready-to-Go kit; GE Healthcare). The cDNA samples were then tested for the expression of FIZZ1, Ym1, and arginase 1 (Arg1) by real-time RT-PCR. Real-time RT-PCR was performed using SYBR Green PCR Master Mix for 38 cycles on an Opticon II DNA engine (MJ Research). GAPDH was used as an internal control. Negative controls included the amplification of samples without prior reverse transcription. LightCycler relative quantification software was used to normalize data to the same GAPDH mRNA level. Samples were run in triplicate. The sequences for the sense and antisense primers used to quantify mRNA were: GAPDH, 5'-TGAGATCCTTGGCATCCATGAAAC-3' and 5'-TAAAAACGACGTCAGTAACGTCG-3'; Arg1, 5'-CAGAACGAGTAAGGACATGTCG-3' and 5'-CATGATGCGGAGGTACC-3'; Ym1, 5'-TACAGGTGCAATCTTCTTG-3' and 5'-TTGGTCTTCTTGGGCTTCTTC-3'; Fizz1, 5'-TCACAGATCAGTGAAAC-3' and 5'-CCTACTGAGATCC-CAAAAGGAA-3'; and mLC3, 5'-GACCCTGTTAATGAA-3' and 5'-CTTGACCA-ACT-3'.

Gentamicin protection assay

The peritoneal macrophages were collected from mice infected with helminths (3 wk after H. polygyrus infection) or uninfected control mice. After infection, cells were collected by complete DMEM for 2 h, nonadherent cells were removed by washing and the cells were cultured overnight. The adherent cells were incubated in complete DMEM at 37°C overnight and then infected for 1 h with 10^7 C. rodentium (multiplicity of infection of 10:1) in antibiotic-free medium. After completion of the infection period, the cells were washed with cold PBS (3×) and incubated with gentamicin-containing medium (100 μg/ml) for 2 h, which kills the extracellular bacteria. Because gentamicin is not cell permeable, intracellular bacteria are not killed by this antibiotic. The cells were then washed (3×) with sterile PBS and then lysed immediately in 0.2 ml sterile 1% Triton X-100 in water or after a further 4 h in medium containing 10 μg/ml gentamicin. The lysates were mixed with 0.8 ml PBS, and serial dilutions were made before plating 100 μl of the appropriate dilutions on LB agar. Colonies were counted after overnight incubation at 37°C, and the number of bacteria present inside the cells at each time point was calculated.

Immunofluorescence microscopy and LC3 detection

Peritoneal macrophages were collected from normal and helminth-infected mice (days 7–14 postinfection), grown on coverslips, and infected with GFP-expressing C. rodentium for 1 h, followed by incubation with gentamicin-containing medium for 2 h (as described earlier). After fixation, the cells were stained with rabbit anti-mouse LC3B primary Ab (Cell Signaling) followed by incubation with anti-rabbit IgG-Cy3 (Cedarlane Laboratories). Sections were analyzed by immunofluorescence microscopy (Nikon ECLIPSE 80i).

Small interfering RNA-mediated silencing of LC3

LC3-specific and irrelevant small interfering RNAs (siRNAs) were purchased from Dharmacon. siRNAs were transfected using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s protocol. Analysis of LC3 expression and bacterial killing was performed 2 d after transfection.

Western blot analysis of LC3

CD11b+ peritoneal macrophages were isolated from normal and H. polygyrus-infected BALB/c or STAT6 KO mice and the cells were pretreated with Th1 (IFN-γ) or Th2 (IL-4, IL-13) cytokines overnight. The cells were then exposed to C. rodentium for 1 h. Cellular lysates were prepared and protein content was determined using BCA protein assay (Bio-Rad Laboratories). Proteins were separated by SDS-PAGE for Western blot analysis. LC3 protein expression was determined by immunoblotting with polyclonal rabbit anti-mouse LC3B primary Ab (1:2000; Cell Signaling), followed by a goat anti-rabbit Ab conjugated to HRP (1:5000 dilution; Biosource). Each blot was analyzed for GAPDH protein expression as an internal loading control using a specific rabbit anti-mouse GAPDH Ab (1:5000).

Statistical analysis

All results were expressed as the mean ± SD. N refers to the number of mice used. Statistical differences were determined using one-way ANOVA test (Tukey’s multiple comparison test) with GraphPad Prism. A p value <0.05 was considered significant.

Results

Autophagy is involved in macrophage killing of Citrobacter and is enhanced by IFN-γ

Although C. rodentium typically colonizes the intestinal epithelial cell surface, bacterial translocation occurs in hosts with a dysregulated or compromised immune system (5, 7). Therefore, tissue resident macrophages can be involved in the elimination of the translocated bacteria. We carried out experiments to determine whether autophagy plays a role in this process. We isolated peritoneal macrophages from normal BALB/c mice and incubated the cells in the absence or presence of the PI3K inhibitor wortmannin, a pharmacologic strategy that is often used to evaluate the involvement of autophagy (18). After overnight incubation, cells were infected with C. rodentium (or GFP-expressing C. rodentium) for 1 h, followed by a gentamicin protection assay (7). Our results show that macrophages from normal mice are able to effectively control internalized C. rodentium as evidenced by a time-dependent reduction in intracellular bacterial numbers (Fig. 1A). This reduction was abolished in the wortmannin-treated cells (Fig. 1A). The results, therefore, suggest a potential role for autophagy in the control of C. rodentium by macrophages. Similar results were reported recently for another noninvasive bacterial enteropathogen, Helicobacter pylori (19). Autophagy involves the formation of a double-membrane structure called the autophagosome (20). Accordingly, we used electron microscopy to analyze Citrobacter-infected macrophages and found characteristic autophagic double-membrane structures partially enclosing the internalized bacteria (Fig. 1B). This observation further supports the idea that autophagy is involved in killing phagocytosed C. rodentium.

Autophagy is commonly characterized by the redistribution of the LC3 protein onto autophagosomes (12, 13, 21, 22). Immunostaining for LC3, therefore, is a relatively simple method to reveal a novel immunosuppressive consequence of helminth infection.
autophagy (23), whereas it was inhibited by wortmannin, an autophagy inhibitor (Fig. 2A–C, 2G). Moreover, the changes in colocalization correlated with intracellular bacterial numbers (Fig. 2H). These results provide further evidence that autophagy is involved in the control of phagocytosed *C. rodentium* by macrophages. The observations expand our current understanding of the mechanisms by which macrophages limit the replication of internalized bacterial enteropathogens.

**FIGURE 1.** Autophagy-mediated bacterial killing is impaired in macrophages from helminth-infected mice. (A) Treatment of macrophages with wortmannin, an autophagy inhibitor, resulted in impaired bacterial killing. Peritoneal macrophages were isolated from normal BALB/c mice and exposed to *C. rodentium* (10 bacteria/cell) for 1 h. At different time points (2, 6 h) postinfection, the number of viable intracellular bacteria was determined by plating the cell lysates onto LB plates. Data shown represent the mean ± SD of triplicate cultures and are from one of three experiments showing similar results. Additional cultures of macrophages without infection by *C. rodentium* showed no detection of viable bacteria (data not shown). (B) Autophagic double-membrane structures are present in macrophages with phagocytosed *C. rodentium*. Peritoneal macrophages were isolated from normal BALB/c mice, pretreated with IFN-γ (100 U/ml) overnight, and exposed to *C. rodentium* for 1 h. The ultrastructure of the cells was examined by transmission electron microscopy (original magnification ×60,000). Arrows (and the inset) indicate the presence of double-membrane structures surrounding the internalized bacteria. Scale bar, 500 nm.

**FIGURE 2.** Helminth infection resulted in reduced colocalization of LC3 and *C. rodentium* in macrophages. (A–F) Immunofluorescence microscopy data show the detection of LC3, an autophagy marker, internalized GFP-expressing *C. rodentium*, and colocalization of LC3 and GFP-*C. rodentium* in peritoneal macrophages (stained with anti-LC3 and Cy3: red) from normal (A–C) and *H. polygyrus*-infected (D–F) mice at 6 h after *C. rodentium*-GFP infection. Wortmannin-treated cells displayed reduced LC3-GFP bacterial colocalization (C, F). IFN-γ treatment resulted in an increase in LC3 expression and colocalization of LC3 with GFP-*C. rodentium* (B, E). Scale bar, 10 μm. (G) Frequency of LC3-GFP-*C. rodentium* colocalization within macrophages isolated from normal and helminth-infected BALB/c mice with and without wortmannin and IFN-γ. Data shown are from one of three experiments showing similar results. Arrows indicate the presence of colocalization of LC3 and GFP-expressing *C. rodentium*. *p < 0.05 for a comparison of two groups. (H) IFN-γ treatment of the cells results in enhanced bacterial killing. Peritoneal macrophages were isolated from normal BALB/c mice, then exposed to *C. rodentium* for 1 h with or without overnight IFN-γ pretreatment. The number of viable intracellular bacteria recovered from the macrophages was determined by plating the cell lysates onto LB plates 6 h after gentamicin treatment. Data represent the mean ± SD of triplicate cultures. *p < 0.05 for a comparison of two groups.
Macrophages from H. polygyrus-infected mice have compromised autophagic killing of Citrobacter

Intestinal helminth infections have been shown to have a significant impact on the occurrence and course of a number of other illnesses. This may be particularly important in the developing world where chronic helminth infections coexist commonly with other pathogens. Helminths are classical Th2 inducers, and helminth infection has been shown to dampen Th1 responses to other infections (24–27). The impairment of the bacterial killing capacity of macrophages has been attributed to the alternative activation of these cells in the mucosal immune compartment by helminth infection (7). Based on this information and on the results described earlier, we carried out experiments to determine whether an ongoing helminth infection might influence autophagic killing of bacteria by macrophages. First, we examined phenotypic alterations of peritoneal macrophages induced by helminth infection. Our real-time RT-PCR analysis showed that peritoneal macrophages isolated from helminth-infected mice displayed an alternatively activated phenotype as indicated by the upregulation of Ym1, Arg1, and Fizz1 genes that are characteristic of this phenotype (28, 29) (Fig. 3A). This finding is consistent with our earlier observation that macrophages isolated from the GALT of H. polygyrus-infected mice were alternatively activated (7).

We went on to evaluate the impact of helminth infection on the ability of peritoneal macrophages to kill phagocytosed Citrobacter. Macrophages from control and helminth-infected mice were exposed to C. rodentium (~10 bacteria/cell), and a gentamicin protection assay was performed to enumerate intracellular bacteria surviving at different times postinfection (7). In contrast with macrophages from normal mice, which showed an autophagy-mediated killing of bacteria (Fig. 1A), macrophages that were isolated from helminth-infected hosts had impaired killing of internalized C. rodentium, as indicated by unchanged levels of intracellular bacteria at 2 time points (2, 6 h; Fig. 3B). Furthermore, the colocalization of LC3 with GFP-expressing Citrobacter was significantly reduced in macrophages from H. polygyrus-infected mice, even when the cells were treated with IFN-γ (Fig. 2D, 2E, 2G). Colocalization was further reduced when the cells were treated with wortmannin (Fig. 2F, 2G). Transmission electron microscopic analysis failed to detect the characteristic autophagic double-membrane structure surrounding intracellular bacteria in cells isolated from helminth-infected mice (data not shown). Macrophage infection with Salmonella has been used as one of the models for studying the protective role of autophagy in immune defense against intracellular pathogens (10). To further evaluate the impact of helminth infection on autophagy, we examined colocalization of LC3 with GFP-expressing Salmonella. We observed clear colocalization of LC3 and GFP-Salmonella in macrophages from control mice, whereas colocalization in macrophages from helminth-infected mice was extremely infrequent (Fig. 3C).

Taken together, our results indicate clearly that helminth infection impairs the ability of macrophages to kill phagocytosed bacteria by autophagy.

Effect of H. polygyrus on the autophagy of C. rodentium is mediated by a Th2 response

The enteric pathogens involved in this study induce distinct immune responses, with H. polygyrus and C. rodentium inducing strong Th2 and Th1 responses, respectively (5). Th1/Th2 cytokine responses play a regulatory role in the autophagy process (30). The Th1 cytokine IFN-γ, through mechanisms that are not yet fully understood, induces or augments autophagy, and thus contributes to the elimination of intracellular bacteria (Fig. 2B, 2G) (9). Impaired Th1 immunity observed in mice that were coinfected with Th2-inducing H. polygyrus has been suggested to contribute to their enhanced susceptibility to C. rodentium and intestinal injury (7). The Th2 cytokines IL-4 and IL-13 have been shown to abrogate autophagy and autophagy-mediated killing of intracellular bacteria in macrophages (31). Recently, we have shown that helminths impair host defense against enteric bacterial infection through a Th2-dependent mechanism (7). Therefore, we carried out experiments to determine whether the effects of H. polygyrus infection on autophagy are dependent on Th2 responses.

Accordingly, we treated peritoneal macrophages in vitro with Th2 cytokines (IL-4/IL-13), or left them untreated (as controls) and infected the cells with GFP-C. rodentium. Our immunofluorescence analysis showed, similar to the results presented in Fig. 2, that colocalization of LC3 and GFP bacteria was detected in cells isolated from normal mice (Fig. 4A, 4D). In contrast, colocalization of LC3 and GFP bacteria was markedly reduced in cells that were pretreated with Th2 cytokines (IL-4/IL-13), similar to what was seen in cells isolated from H. polygyrus-infected mice (Fig. 4B, 4C, 4E). These observations raise the possibility that the helminth-induced impairment of autophagic bacterial killing may be mediated by the effects of the Th2 response in worm-infected animals. To test this hypothesis, we infected STAT6 KO mice with H. polygyrus, isolated macrophages from both helminth-infected and uninfected hosts, and infected with GFP-Citrobacter (Fig. 4F, 4G). The immunofluorescence analysis revealed that colocalization of LC3 and GFP-Citrobacter is restored to normal in macrophages from helminth-infected STAT6 KO mice (Fig. 4H).
Effect of \( H. \) polygyrus on autophagy of \( C. \) rodentium is associated with a decrease in LC3 expression and processing

During autophagy, the cytosolic form of LC3 (LC3I) is conjugated to phosphatidylethanolamine to form LC3II, which is recruited to autophagosomal membranes. In the next set of experiments, we determined whether the ongoing helminth infection influences the expression of LC3I and LC3II in vivo. We isolated macrophages from helminth-infected and control mice, and cultured the cells in vitro with and without IFN-\( \gamma \) or starvation treatment, both of which can induce autophagy (16). Immunoblotting with an anti-LC3 Ab revealed two species of LC3, as shown in Fig. 5A: a 16-kDa band corresponding to unmodified LC3I and a lower band corresponding to LC3II. Our results show that treatment of macrophages isolated from uninfected control mice with IFN-\( \gamma \) or starvation resulted in decreased detection of the unmodified LC3I (Fig. 5A). Strikingly, total LC3 levels, as well as processing to LC3 II (LC3II/GAPDH; in response to IFN-\( \gamma \) treatment or starvation), were significantly reduced in cells isolated from helminth-infected mice as compared with cells isolated from control animals (Fig. 5B, 5C). These results suggest that the induction of autophagy in macrophages can be negatively regulated by an ongoing helminth infection.

It has been shown previously that the effects of Th2 cytokines on starvation-induced autophagy is dependent on signaling via the Akt pathway, whereas the effect of Th2 cytokines on IFN-\( \gamma \)-induced autophagy is Akt independent and STAT6 dependent (30).

To directly demonstrate the influence of the helminth-induced Th2 cytokine response on LC3 during \( C. \) rodentium infection, in our next set of experiments, we used peritoneal macrophages isolated from helminth-infected or noninfected Th2-deficient, STAT6 KO mice (BALB/c mice used as control) and examined the influence of ongoing helminth infection on LC3 expression. In contrast with what was observed in WT mice (Fig. 5B), helminth-induced downregulation of LC3 levels was not seen in cells isolated from STAT6 KO mice (Fig. 5D). These results suggest that the helminth-induced Th2 response inhibits autophagic killing of phagocytosed bacteria by reducing LC3 expression. Our findings are consistent with a previous report showing that STAT6 is required for the suppression of IFN-\( \gamma \)-induced autophagy (31).

To further elucidate the mechanism responsible for the reduction in LC3 protein expression in peritoneal macrophages from helminth-infected mice, we next examined LC3 mRNA levels in macrophages that were isolated from STAT6 KO and WT mice with and without \( H. \) polygyrus infection. The real-time RT-PCR analysis showed a marked reduction of LC3 mRNA in macrophages from helminth-infected WT mice, an effect that was not seen in helminth-infected STAT6 KO mice (Fig. 6). IFN-\( \gamma \) up-regulated LC3 mRNA expression in macrophages from uninfected mice, whereas this effect was blunted in macrophages from helminth-infected animals. In contrast with IFN-\( \gamma \), IL-4/IL-13 treatment of macrophages downregulated LC3 mRNA. These results indicate a marked influence of Th1 and Th2 cytokines on...
autophagy and also support the notion that the effects of helminth infection on autophagy are mediated by the worm-induced Th2 response.

To determine whether the observed inhibition of LC3 expression by helminth infection can influence autophagy, which, in turn, resulted in reduced bactericidal activity of macrophages, we analyzed the effects of siRNA-mediated silencing of LC3 in the RAW264.7 macrophage cell line. Immunofluorescence microscopy indicated that the LC3-specific siRNA used in our experiments effectively downregulated LC3 expression (Fig. 7A). Digital quantification of LC3 fluorescence intensity confirmed that LC3 expression was significantly reduced in the cells transfected with the LC3-specific siRNA (Fig. 7B). The results from a gentamicin protection assay showed that siRNA-mediated knockdown of LC3 in macrophages was associated with impaired bacterial killing, as indicated by an increase in intracellular *Citrobacter* numbers at 6 h postinfection in IFN-γ–treated cells (Fig. 7C). We also examined the effects of LC3 silencing in peritoneal macrophages isolated from normal and *H. polygyrus*-infected STAT6 KO mice.

**FIGURE 5.** *H. polygyrus* infection reduces LC3 expression and LC3 II conversion in macrophages via a STAT6-dependent mechanism. (A) Peritoneal macrophages were isolated from normal and *H. polygyrus*-infected BALB/c mice (2 wk postinfection). Autophagy was induced in macrophages by incubating the cells overnight in the presence and absence of recombinant IFN-γ (100 U/ml) or by amino acid and serum starvation at 37˚C for 3 h. Cell lysates were collected for detection of LC3 (LC3I and LC3II) by Western blotting. GAPDH was used as the loading control. Each lane represents the sample from an individual mouse. (B and C) The bar graphs show the total LC3 density (LC3I+LC3II), LC3I, LC3II density, and the LC3II/LC3I ratio (mean density + SD from three experiments) based on the Western blotting analysis of LC3 expression (in A). *p < 0.05 for a comparison of two groups. (D) Western blotting analysis of LC3 expression in peritoneal macrophages isolated from normal and *H. polygyrus*-infected STAT6 KO mice.

**FIGURE 6.** *H. polygyrus* infection downregulates LC3 gene expression in wild-type mice but not in STAT6 KO mice. Peritoneal macrophages were isolated from normal or *H. polygyrus*-infected wild-type and STAT6 KO BALB/c mice. After overnight incubation in vitro in the presence or absence of Th1 or Th2 cytokines, total RNA was isolated. LC3 expression was determined using real-time RT-PCR. Values are the fold decrease or increase compared with baseline obtained from uninfected control mice. The data shown are from one of two experiments showing similar results.

siRNA-mediated suppression of LC3 in RAW264.7 cells and primary macrophages results in reduced autophagic killing of bacteria

To determine whether the observed inhibition of LC3 expression by helminth infection can influence autophagy, which, in turn, resulted in reduced bactericidal activity of macrophages, we analyzed the effects of siRNA-mediated silencing of LC3 in the RAW264.7 macrophage cell line. Immunofluorescence microscopy indicated that the LC3-specific siRNA used in our experiments effectively downregulated LC3 expression (Fig. 7A). Digital quantification of LC3 fluorescence intensity confirmed that LC3 expression was significantly reduced in the cells transfected with the LC3-specific siRNA (Fig. 7B). The results from a gentamicin protection assay showed that siRNA-mediated knockdown of LC3 in macrophages was associated with impaired bacterial killing, as indicated by an increase in intracellular *Citrobacter* numbers at 6 h postinfection in IFN-γ–treated cells (Fig. 7C). We also examined the effects of LC3 silencing in peritoneal macrophages. We collected macrophages from normal BALB/c mice, pretreated the cells with IFN-γ, and transfected the cells with siRNA before GFP-*Citrobacter* exposure. Similar to the results in the RAW264.7 macrophage cell line, a marked reduction in LC3 intensity was observed in the
siRNA-treated cells (Fig. 7D), which correlated with an increased frequency of GFP-Citrobacter in the cells (Fig. 7C). These observations support the idea that reduced LC3 expression is responsible for the impairment of autophagy-mediated bacterial killing in macrophages from helminth-infected mice.

**Discussion**

Helminth infection has been known to alter, and in some cases to compromise, the immune response to other pathogens (5, 24, 25, 27). Our own work in a mouse coinfection model has demonstrated that infection with the helminth *H. polygyrus* significantly impairs the ability of the host to eliminate the bacterial enteropathogen *C. rodentium* (5, 6). One aspect of this impairment is the helminth-induced, Th2-dependent alternative activation of macrophages, which inhibits the ability of these cells to kill *Citrobacter* (7). The results of this study extend these observations to shed light on one of the macrophage antimicrobial mechanisms that is affected by helminth infection. Our observations indicate that autophagy plays an important role in macrophage killing of phagocytosed *Citrobacter*, an observation that is consistent with increasing evidence that this process mediates disposal of a wide variety of pathogens (8–11, 27, 32, 33). They are also in keeping with a recent study showing that in vivo treatment of mice with wortmannin impaired clearance of *Citrobacter*, although this effect was attributed to alterations in PI3K- and Akt-mediated intestinal epithelial proliferation (34). Interestingly, we found that autophagy-mediated elimination of *Citrobacter* was significantly compromised in macrophages isolated from helminth-infected mice. The underlying mechanism appears to involve decreased expression and processing of LC3, a key component of the autophagy machinery (35). These effects were dependent on the helminth-induced Th2 response, as indicated by their absence in macrophages from STAT6-deficient mice and by their induction by the Th2 cytokines IL-4 and IL-13. The molecular details of how IL-4 and IL-13 downregulate LC3 expression are yet to be elucidated and are the subject of ongoing studies in our laboratory.

To our knowledge, the results reported in this article represent the first demonstration that helminth infection impairs autophagy. They reveal a novel aspect of the immunomodulatory effects of helminth infection and provide an in vivo, clinically relevant context to earlier observations showing that in vitro exposure of macrophages to IL-4 and IL-13 inhibited autophagy-mediated killing of mycobacteria (31). They also provide a mechanistic explanation for our previously reported finding that *H. polygyrus* significantly worsens the course of *Citrobacter* infection (5). Because autophagy is involved in defense against many different pathogens, our results have implications for a number of infectious diseases, particularly those that occur in areas of the world where chronic helminth infections are endemic. They could help to explain, for example, the increased incidence of tuberculosis in individuals chronically infected with helminth parasites (36). Defects in autophagy have also been implicated, on the basis of experimental models as well as genome-wide association studies in humans, in the pathogenesis of inflammatory bowel disease (IBD) (15–17). Although deliberate infection with helminths has demonstrated some success as a treatment for IBD (37), the data presented in this article raise the possibility that this approach may actually worsen the disease by impairing autophagy. Indeed, we have evidence suggesting that *H. polygyrus* infection can enhance the severity of intestinal inflammation in mouse models of IBD (M. Zhang, C.-w. Su, and H.N. Shi, unpublished data).

In summary, the results reported in this article have added autophagy to the list of immunological processes that are affected by helminth infection. Determining the contribution of altered autophagy to the diverse immunomodulatory effects of helminths will be an interesting area for future investigation.

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**Disclosures**

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