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Helminth infection impairs autophagy-mediated killing of bacterial enteropathogens by macrophages[1]

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Abstract

Autophagy is an important mechanism used by macrophages to kill intracellular pathogens. The results reported here demonstrate that autophagy is also involved in the macrophage killing of the extracellular enteropathogen *Citrobacter rodentium* following 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 since 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 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. Knock-down of LC3 significantly inhibited autophagic killing of *Citrobacter*, attesting to the functional importance of the *H. polygyrus*-mediated down-regulation 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.

Keywords

Parasitic helminth; alternatively activated macrophage; autophagy

Introduction

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 non-invasive, attaching-effacing bacterial enteropathogens such as enteropathogenic *E.*

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coli (EPEC) and enterohemorrhagic *E. coli* (EHEC) (5). The severity of the colitis in the co-infected 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 anti-microbial response, i.e. 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 light 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 antigen presentation (the delivery of microbial peptides to endosomes or MHC II loading compartments) and, thus, in the activation of adaptive immunity (reviewed in (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 the present work, 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 down-regulation of LC3 expression and processing. These results reveal a novel immunosuppressive consequence of helminth infection.

Materials and Methods

Mice

Six to 8 week old female BALB/c ByJ and STAT6 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 (5). Mice were inoculated orally with 200 third-stage larvae (L_3). Seven days following parasitic infection, a subset of the *H. polygyrus*-infected mice was fed *C. rodentium*.

C. rodentium infection

Mice were orally inoculated with *C. rodentium* (strain DBS100 from American Type Culture Collection). Bacteria were grown overnight in Luria broth (LB) and resuspended in PBS before infecting the mice (0.5 ml/mouse; 5×10^8 CFU of *C. rodentium*). GFP-expressing *C. rodentium* (GFP-*C. rodentium*) (provided by Dr. L. Bry at 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 of 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'-TGGAATCCTGTGGCATCCATGAAAC-3' and 5'-TAAAACGCAGCTCAGTAACAGTCCG-3'; Arg1, 5'-CAGAAGAATGGAAGAGTCAG-3' and 5'-CAGATATGCAGGGAGTCACC-3'; Ym1, 5'-TCACAGGTCTGGCAATTCTTCTG-3' and 5'-TTTGTCCTTAGGAGGGCTTCCTC-3'; Fizz1, 5'-TCCCAGATACTGATGAGA-3' and 5'-CCACTCTGGATCTCCCAAGA-3'; and iNOS2, 5'-CTGGAGGAGCTCCTGCCTCATG-3' and 5'-GCAGCATC CCCTCTGATGGTG-3'; and mLC3, 5'-GACCGCTGTAA-3' and 5'-CTTGACCAACT-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 incubation in complete DMEM for 2 h, non-adherent 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 of 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 of 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 (day 7 to 14 post-infection), 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 above). After fixation, the cells were stained with rabbit anti-mouse LC3B primary antibody (Cell Signaling), followed by incubation with anti-rabbit IgG-Cy3 (Cedarlane Laboratories). Sections were analyzed by immunofluorescence microscopy (Nikon ECLIPSE 80i).

siRNA-mediated silencing of LC3

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

Western blot analysis of LC3

$CD11b^+$ peritoneal macrophages were isolated from normal and *H. polygyrus* infected BALB/c or STAT 6 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 antibody (1:2000, Cell Signaling), followed by a goat anti-rabbit antibody conjugated to horseradish peroxidase (1: 5000 dilution, Biosource). Each blot was analyzed for GAPDH protein expression as an internal loading control using a specific rabbit anti-mouse GAPDH antibody (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 analysis of variance test (Tukey's multiple comparison test) with GraphPad Prism. A value for $p < 0.05$ 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 phosphatidylinositol (PI) 3-kinase 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 (Figure 1 A). This reduction was abolished in the wortmannin treated cells (Figure 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 non-invasive 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 (Figure 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 detect autophagy (23). To further assess the role of autophagy in the control of phagocytosed *C. rodentium*, we examined LC3 distribution and co-localization with GFP-expressing bacteria in macrophages. The results showed that co-localization of LC3 with the GFP-*Citrobacter* was readily detectable in macrophages isolated from normal mice (Figure 2

A & G). In support of the idea that this co-localization represented the formation of autophagosomes, we found that it was enhanced by IFN-γ, a known inducer of autophagy (23), whereas it was inhibited by wortmannin, an autophagy inhibitor (Figure 2 A, B, C and G). Moreover, the changes in co-localization correlated with intracellular bacterial numbers (Figure 2 H). 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.

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 above, we carried out experiments to determine whether an on-going 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 up-regulation of Ym1, Arg1, and Fizz1, genes that are characteristic of this phenotype (28, 29) (Figure 3A). This finding is consistent with our earlier observation that macrophages isolated from the gut-associated lymphoid tissue 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* (about 10 bacteria/cell) and a gentamicin protection assay was performed to enumerate intracellular bacteria surviving at different times after infection (7). In contrast to macrophages from normal mice, which showed an autophagy-mediated killing of bacteria (Figure 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 h and 6 h) (Figure 3 B). Furthermore, the co-localization of LC3 with GFP-expressing *Citrobacter* was significantly reduced in macrophages from *H. polygyrus*-infected mice, even when the cells were treated with IFNγ (Figure 2 D, E, G). Co-localization was further reduced when the cells were treated with wortmannin (Figure 2 F, G). 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 co-localization of LC3 with GFP-expressing *Salmonella*. We observed clear co-localization of LC3 and GFP-*Salmonella* in macrophages from control mice, whereas co-localization in macrophages from helminth-infected mice was extremely infrequent (Figure 3 C). Taken together, our results indicate clearly that helminth infection impairs the ability of macrophages to kill phagocytosed bacteria by autophagy.

The 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 (Figure 2B and 2G) (9). Impaired Th1 immunity observed in mice that were co-infected 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 dependent mechanism (7). Therefore, we carried out experiments to determine if 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 Figure 2, that co-localization of LC3 and GFP-bacteria was detected in cells isolated from normal mice (Figure 4 A & D). In contrast, co-localization of LC3 and GFP-bacteria was markedly reduced in cells that were pre-treated with Th2 cytokines (IL-4/IL-13), similar to what was seen in cells isolated from *H. polygygrus* infected mice (Figure 4 B, C & E). 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 un-infected hosts and infected with GFP-*Citrobacter* (Figure 4 F & G). The immunofluorescence analysis revealed that co-localization of LC3 and GFP-*Citrobacter* is restored to normal in macrophages from helminth-infected STAT6 KO mice (Figure 4 H).

The 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 IFNγ or starvation treatment, both of which can induce autophagy (16). Immunoblotting with an anti-LC3 antibody revealed two species of LC3, as can be seen in Figure 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 un-infected control mice with IFN-γ or starvation resulted in decreased detection of the unmodified LC3I (Figure 5 A). Strikingly, total LC3 levels as well as processing to LC3 II (LC3II/GAPDH) (in response to IFN-γ treatment or starvation) were significantly reduced in cells isolated from helminth-infected mice as compared to cells isolated from control animals (Figure 5B & C). 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 IFNγ-induced autophagy is Akt-independent and STAT 6 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 utilized peritoneal macrophages isolated from helminth-infected or non-infected Th2-deficient, STAT6 KO mice (BALB/c mice used as control) and examined the influence of ongoing helminth-infection on LC3 expression. In contrast to what was observed in WT mice (Figure 5B), helminth-induced down-regulation of LC3 levels was not seen in cells isolated from STAT6 KO mice (Figure 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 IFNγ-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 STAT 6 KO mice (Figure 6). IFNγ up-regulated LC3 mRNA expression in macrophages from uninfected mice, whereas this effect was blunted in macrophages from helminth-infected animals. In contrast to IFNγ, IL-4/IL-13 treatment of macrophages down-regulated 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.

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, the effects of siRNA-mediated silencing of LC3 in the RAW264.7 macrophage cell line were analyzed. Immunofluorescence microscopy indicated that the LC3-specific siRNA used in our experiments effectively down-regulated LC3 expression (Figure 7A). Digital quantification of LC3 fluorescence intensity confirmed that LC3 expression was significantly reduced in the cells transfected with the LC3-specific siRNA (Figure 7B). The results from a gentamicin protection assay showed that siRNA-mediated knock-down of LC3 in macrophages was associated with impaired bacterial killing, as indicated by an increase in intracellular *Citrobacter* numbers at 6 hours post-infection in IFNγ-treated cells (Figure 7C). We also examined the effects of LC3 silencing in peritoneal macrophages. We collected macrophages from normal BALB/c mice, pre-treated the cells with IFNγ 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 (Figure 7D), which correlated with an increased frequency of GFP-*Citrobacter* in the cells (Figure 7 C). 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, 27, 32). Our own work in a mouse co-infection 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 the present study extend these observations to shed light on one of the macrophage anti-

microbial 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, 33, 34). 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 phosphatidylinositol-3-kinase- and Akt-mediated intestinal epithelial proliferation (35). 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 (36). 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 down-regulate LC3 expression are yet to be elucidated and are the subject of on-going studies in our laboratory.

To our knowledge, the results reported here 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). Since autophagy is involved in defense against many different types of 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 (37). 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 (38), the data presented here 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 (unpublished data).

In summary, the results reported in this manuscript 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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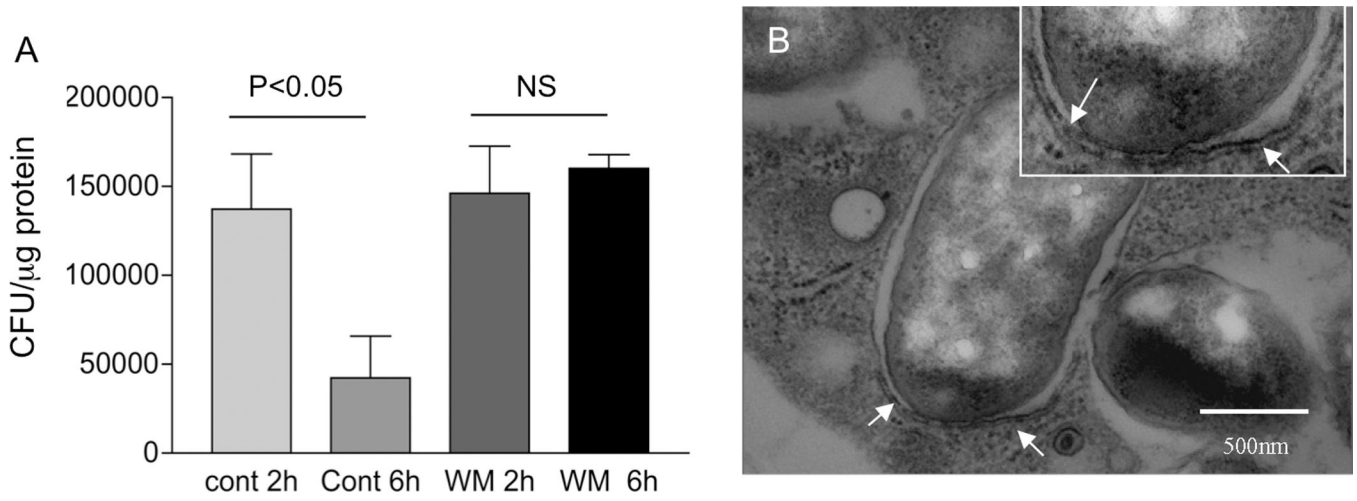

Figure 1.

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 and 6 h) after infection, the number of viable intracellular bacteria was determined by plating the cell lysates onto LB plates. The 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 show). **B:** Autophagic double-membrane structures are present in macrophages with phagocytosed *C. rodentium*. Peritoneal macrophages were isolated from normal BALB/c mice, pre-treated with IFN**γ** (100 U/ml) overnight and exposed to *C. rodentium* for 1 h. The ultra-structure of the cells was examined by transmission electron microscopy (images shown are 60,000×). Arrows (and the inset) indicate the presence of double-membrane structures surrounding the internalized bacteria. Scale bar represents 500 nm.

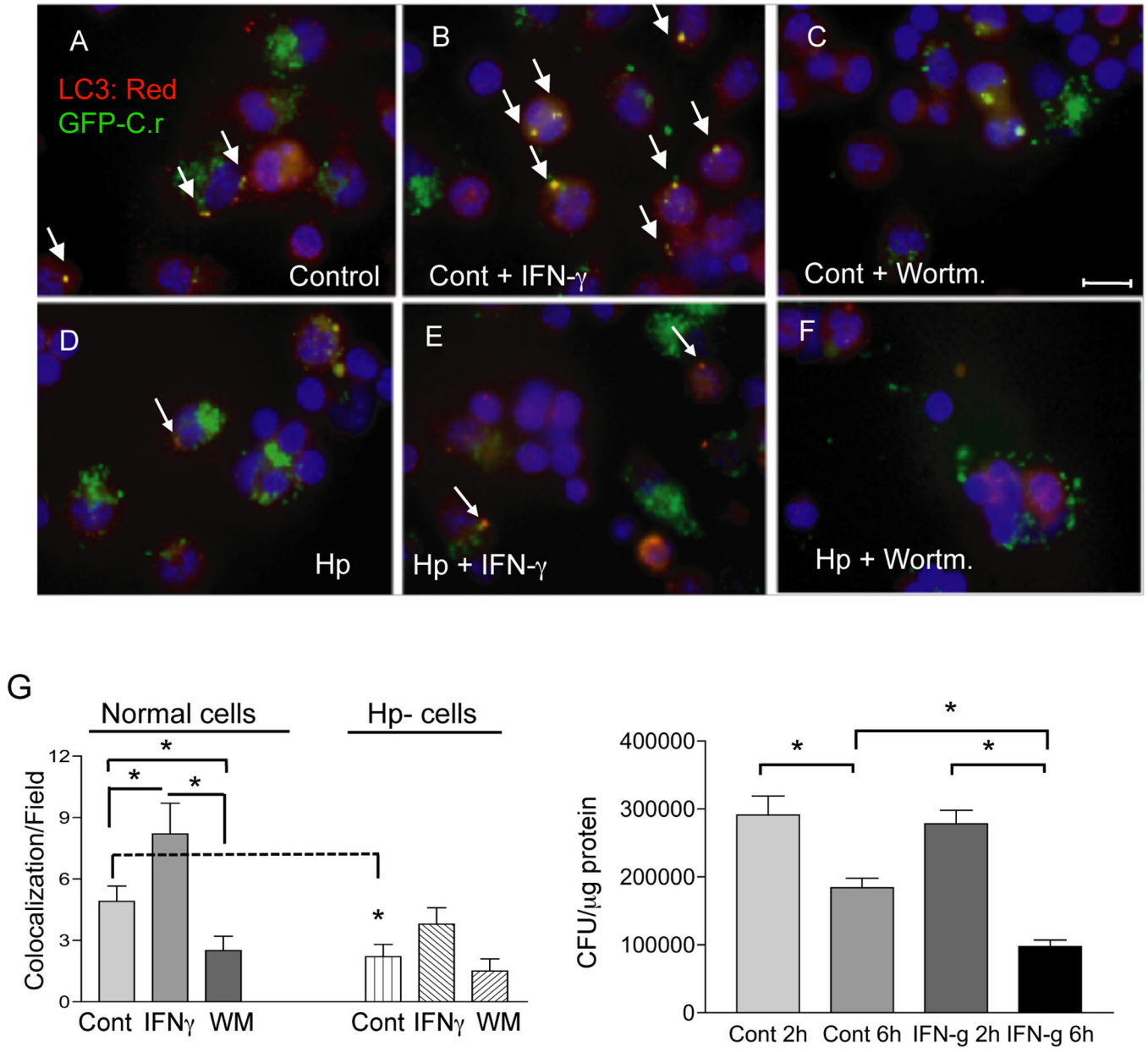

Figure 2.

Helminth-infection resulted in reduced co-localization 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, B and C**) and *H. polygyrus*-infected (**D, E and F**) mice at 6 h after *C. rodentium*-GFP infection. Wortmannin-treated cells displayed reduced LC3-GFP bacterial colocalization (**C and F**). IFNγ treatment resulted in an increase in LC3 expression and colocalization of LC3 with GFP-C. *rodentium* (**B and E**). Scale bar represents 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 IFNγ. The data shown are from one of three experiments showing similar results. Arrows indicate the presence of co-localization of LC3 and GFP-expressing *C. rodentium*. *p < 0.05 for a

comparison of two groups. **H:** IFNγ treatment of the cells results in enhanced bacterial killing. Peritoneal macrophages were isolated from normal BALB/c mice, and then exposed to *C. rodentium* for 1 h with or without overnight IFNγ 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. The data represent the mean ± SD of triplicate cultures. *$p < 0.05$ for a comparison of two groups.

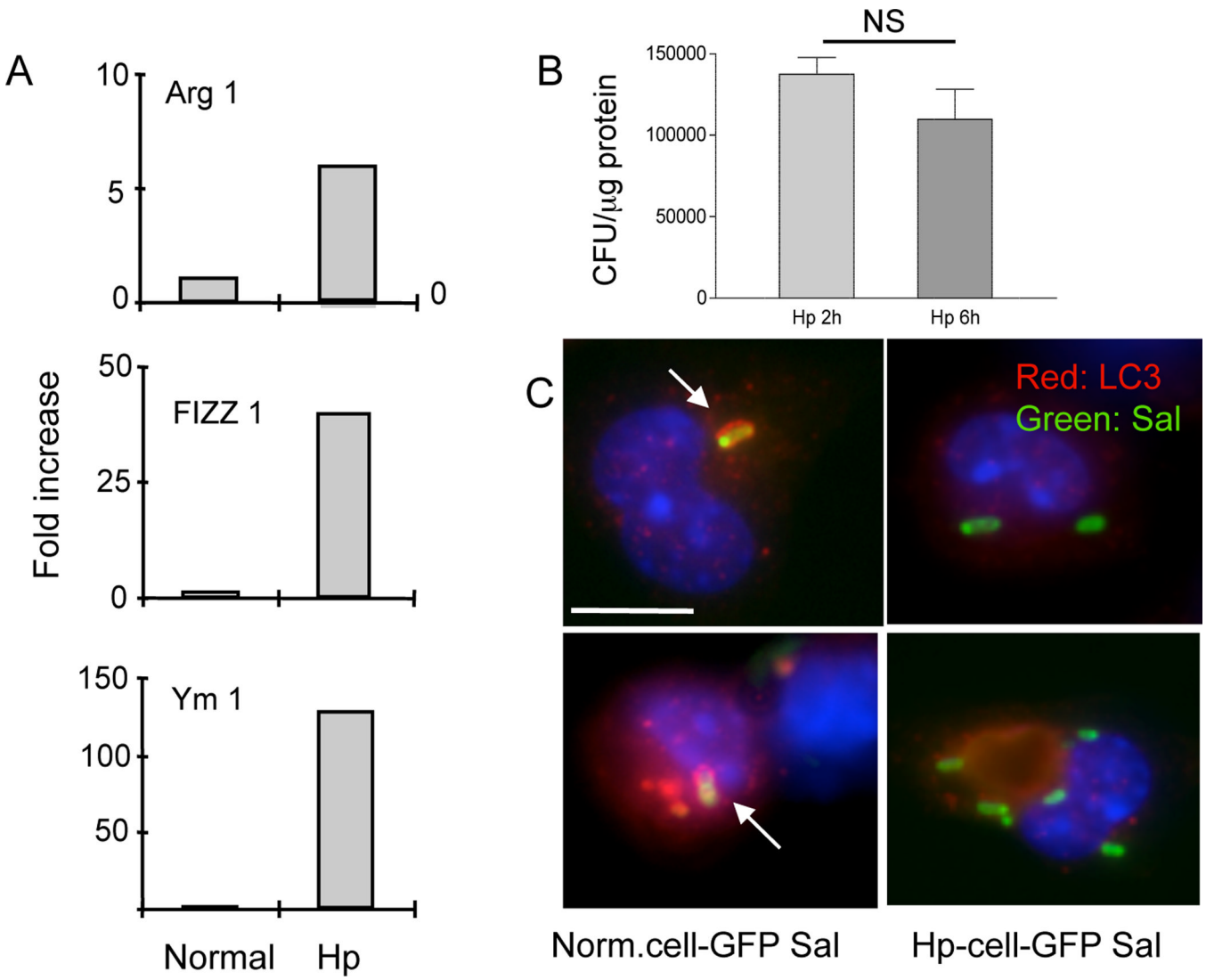

Figure 3.

A. *H. polygyrus* infection up-regulates Ym1, Arg1 and Fizz1 expression in peritoneal macrophages and impairs bacterial killing by macrophages. Peritoneal macrophages were isolated from normal and helminth-infected BALB/c mice at 7 days post infection. Total RNA was isolated from the cells. Ym1, Arg1 and Fizz1 expression was determined using real-time RT-PCR. Values are the fold increase compared with baseline obtained from uninfected control mice. **B**. *H. polygyrus* infection impairs bacterial killing by macrophages. Peritoneal macrophages were isolated from *H. polygyrus* infected mice and used for gentamicin protection assay. The results show similar numbers of viable internalized bacteria recovered in macrophages isolated from heminth-infected mice at 2 and 6 h post bacterial infection *in vitro*. $P > 0.05$. **C.** Immunofluorescence microscopy shows LC3-*Samonella* co-localization in macrophages from control and helminth-infected mice. Scale bar indicates 10 μm. The data shown are from one of three experiments with similar results.

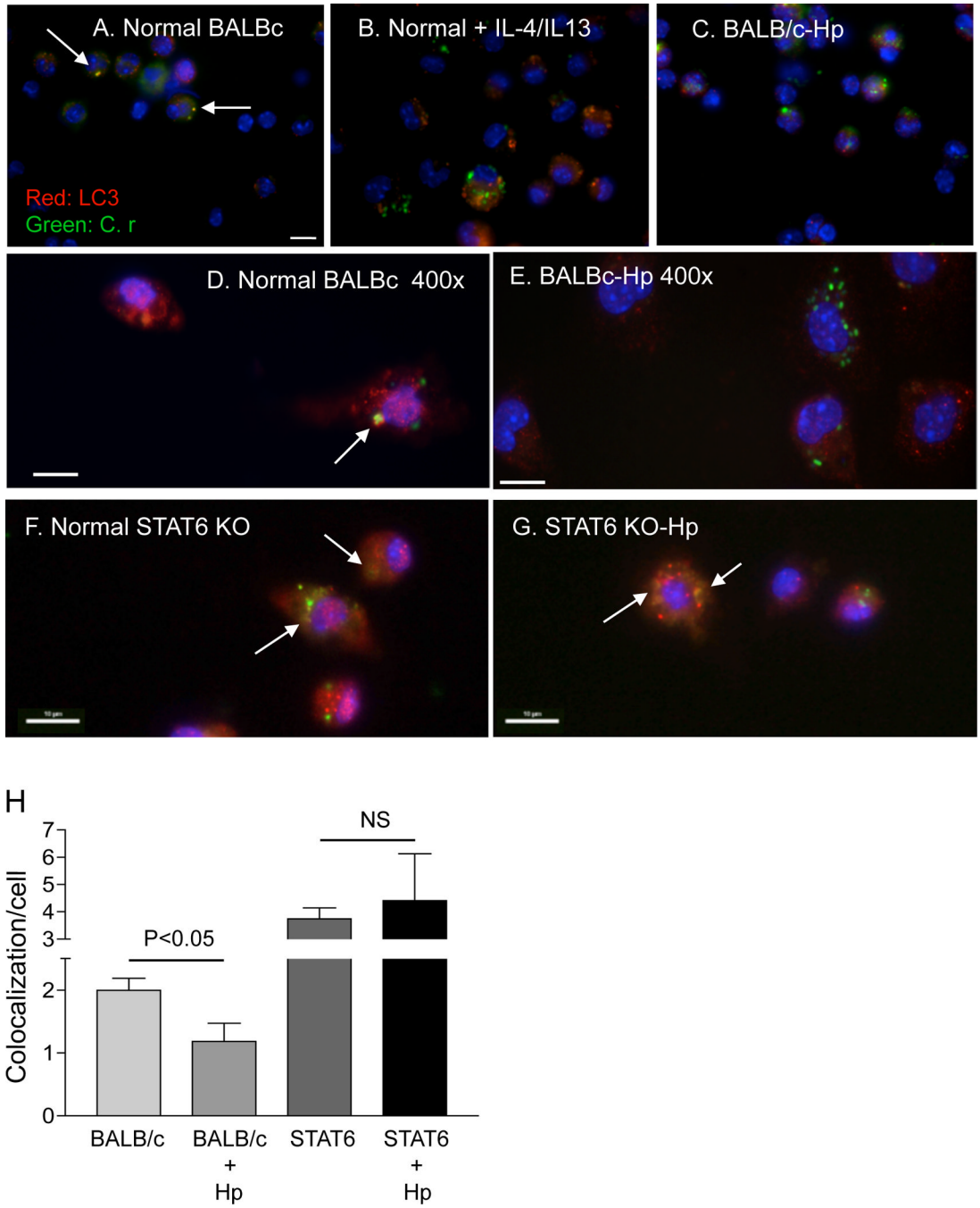

Figure 4.

The effect of *H. polygyrus* on autophagy of *C. rodentium* is mediated by a Th2 dependent mechanism. **A–G**: 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 isolated from normal BALB/c (**A** & **D**), *H. polygyrus*-infected BALB/c (**C** & **E**), normal STAT6 KO (**F**) or *H. polygyrus*-infected STAT6 KO mice (**G**). **B**: Peritoneal macrophages were isolated from normal BALB/c mice, pre-treated with IL-4/IL-13 (3 ng/ml) overnight and then exposed to GFP-*C. rodentium* for 1 h. Cells were collected 2 h after infection, fixed and stained for LC3. Scale bar represents 10

μm. For a detailed description, see Figure 1 **C–H**. H. Frequency of LC3-GFP-*C. rodentium* colocalization within macrophages isolated from normal and helminth-infected BALB/c and STAT6 KO mice. The data represent the mean (co-localization of LC3 and GFP-*C. rodentium*/cell) ± SD obtained from 30–50 cells per slide (1 slide from each of the two experiments was examined).

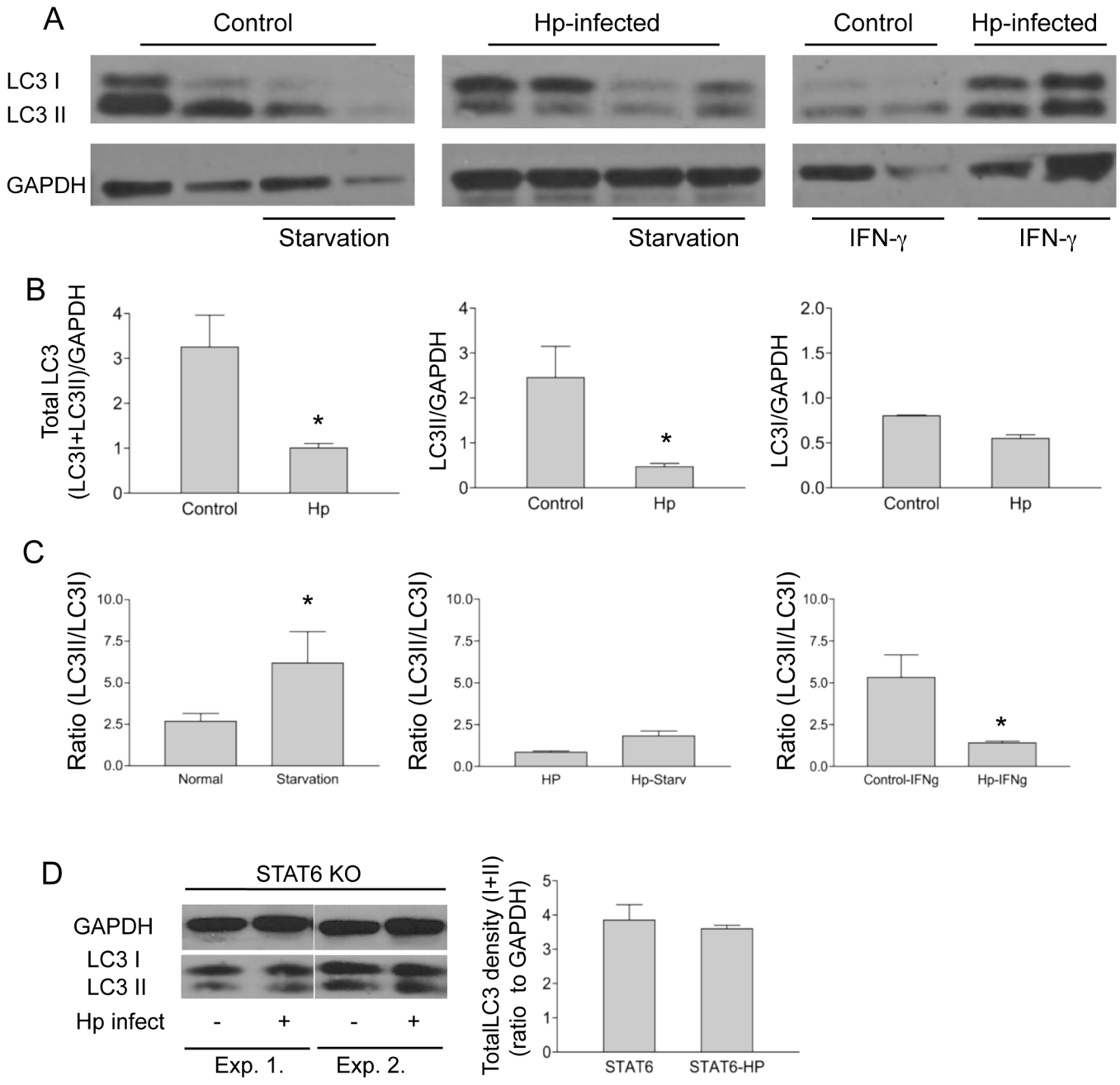

Figure 5.

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 weeks after infection). Autophagy was induced in macrophages by incubating the cells overnight in the presence and absence of recombinant IFNγ (100u/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 3 experiments) based on the western blotting

analysis of LC3 expression (in A). **D.** Western blotting analysis of LC3 expression in peritoneal macrophages isolated from normal and *H. polygyrus* infected STAT 6 KO mice.

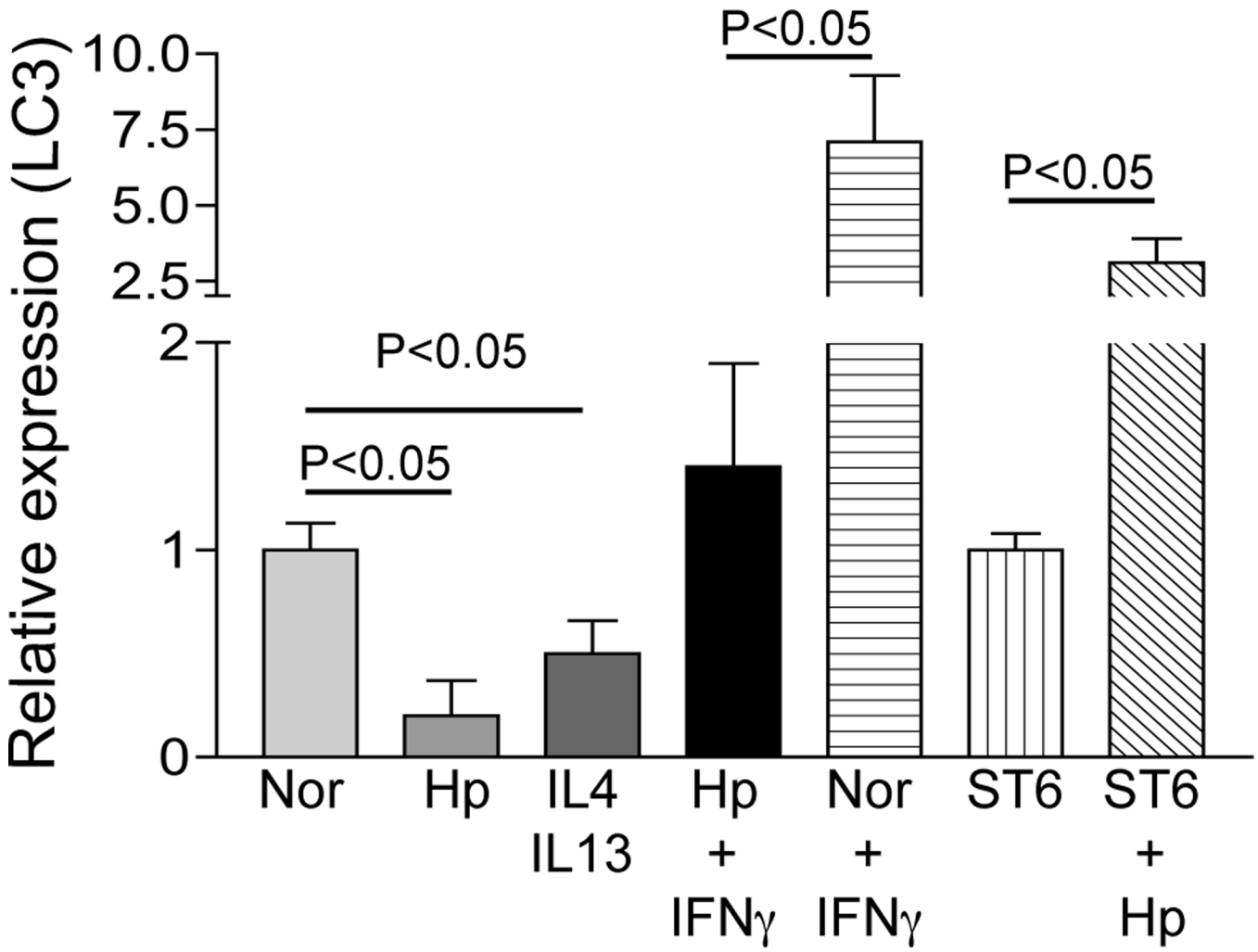

Figure 6.

H. polygyrus infection down-regulates 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.

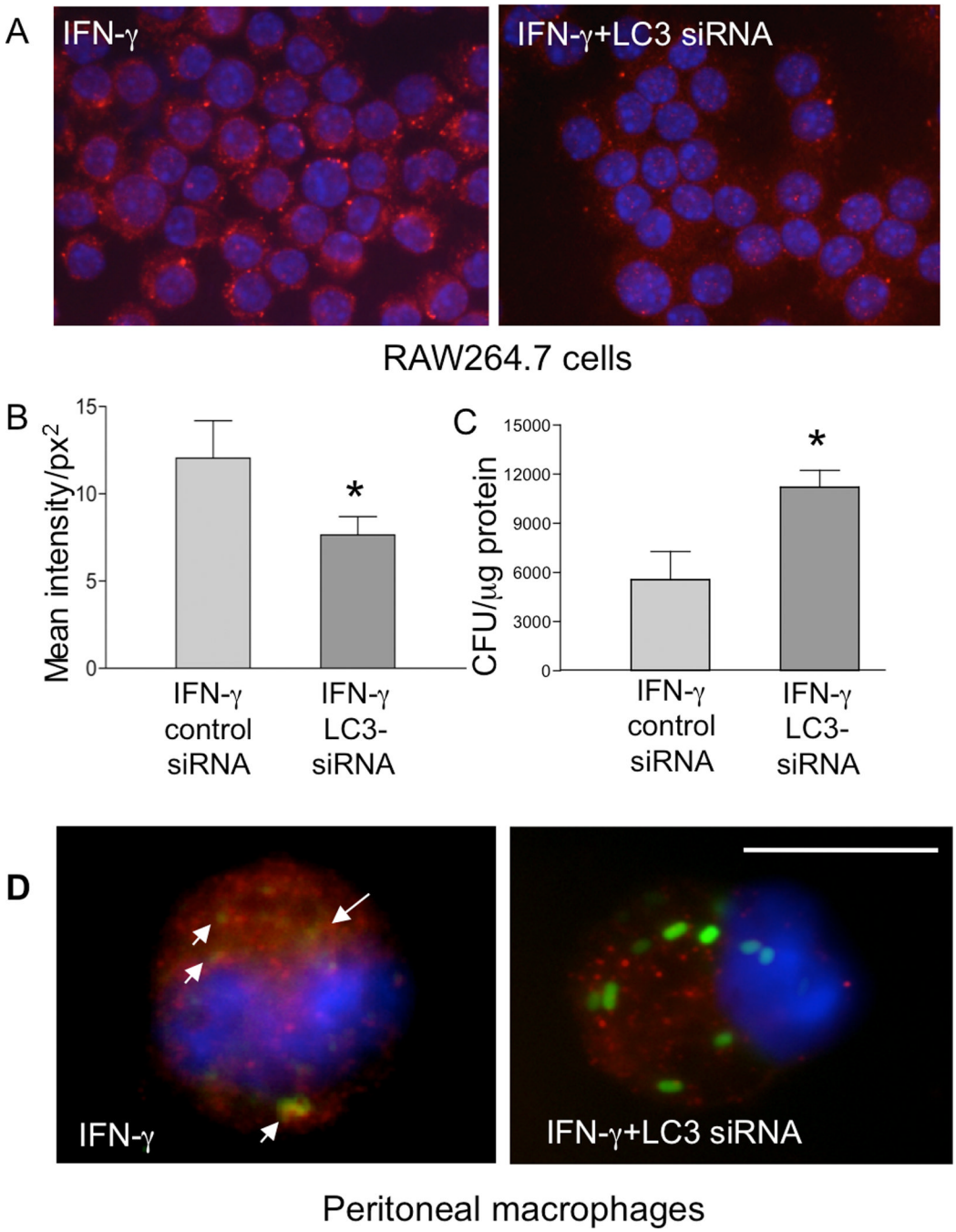

Figure 7.

siRNA-mediated knock-down of LC3 leads to impaired bacterial killing in RAW264.7 cells and peritoneal macrophages *in vitro*. **A**. RAW264.7 cells, with or without overnight IFN**γ** pretreatment, were transfected with or without an siRNA specific for LC3. The cells were then stained with anti-LC3 antibody as described in Figure 1. Results show that IFN**γ**-induced LC3 expression was effectively decreased by the siRNA (400 ×). **B**. Quantification of LC3 fluorescence intensity in RAW264.7 cells transfected with either a control or LC3-specific siRNA. The LC3 fluorescence intensity per cell was measured digitally using the Graph digitizing software (Nikon Imaging Software Elements). The data shown represent

the mean values of fluorescence intensity obtained from 50–60 cells per slide (1 slide from each of the two experiments was examined). *$p < 0.05$ for a comparison of two groups. **C**. After knocking down LC3 expression with the siRNA, the RAW264.7 cells were used for gentamicin protection assay. The data shown represent the mean ± SD of triplicate cultures. *$p < 0.05$ for a comparison of two groups. D: Peritoneal macrophages were isolated from normal BALB/c mice and treated with IFNγ overnight *in vitro*. The cells were transfected with siRNA specific for LC3 and infected 48 h later with C. rodentium for 1 h. LC3, GFP-*Citrobacter* and colocalization of LC3 and bacteria were examined (1000 ×). Scale bar represents 10 μm.