Autophagy Induction by a Small Molecule Inhibits *Salmonella* Survival in Macrophages and Mice.

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Running head: Novel molecule induces autophagy to kill *Salmonella*

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Abstract

*Salmonella enterica* are natural bacterial pathogens of humans and animals that cause systemic infection or gastroenteritis. During systemic infection, *Salmonella* generally reside within professional phagocytes, typically macrophages, whereas gastroenteritis is caused by infection of epithelial cells. We are only beginning to understand which host pathways contribute to *Salmonella* survival in particular cell types. We therefore sought to identify compounds that perturb *Salmonella*-host interactions using a chemical genetics approach. We found one small molecule, D61, that reduces *Salmonella* load in cell-line and primary macrophages but has no effect on *Salmonella* growth in epithelial cells or rich medium. We determined that in macrophages D61 induces LC3II, a marker of the autophagy pathway, and promotes aggregation of LC3II near *Salmonella*. We found that D61 antibacterial activity depends on the VPS34 complex and on ATG5. D61 also reduced *Salmonella* load in the spleens and livers of infected mice. Lastly, we demonstrate that D61 antibacterial activity in macrophages is synergistic with the antibiotic chloramphenicol, but that this synergy is largely independent of the known autophagy-stimulating activity of chloramphenicol. Thus, a small molecule has antibacterial activity specifically in macrophages and mice based on the promotion of bacterial degradation by autophagy.

Importance

Autophagy is a conserved cellular response to metabolic stress and to invading pathogens. For many pathogens, including *Salmonella*, autophagy can play a detrimental or beneficial role during infection depending on the cellular context. We
combined chemical genetics with single cell analyses and murine infection to dissect host-pathogen interactions. We identified a small molecule that reduces bacterial load in macrophages by increasing autophagic flux. This compound also reduces bacterial colonization of tissues in infected mice. These observations demonstrate the potential therapeutic utility of stimulating autophagy in cells and animals to curb infection.

INTRODUCTION

Persistent, clinically relevant human pathogens that live within macrophages include species of *Salmonella, Mycobacterium, Brucella* and also fungi such as *Cryptococcus* and *Histoplasma*. Elucidating the precise molecular interactions between a pathogen and the host is crucial to understanding infection processes and to developing therapeutics. *Salmonella enterica* serovar Typhimurium (*Salmonella*) is an excellent model Gram-negative pathogen because it is easy to work with and causes natural infections of humans and mice. *Salmonella* causes gastroenteritis in humans by colonizing epithelial cells in the gut (1). In contrast, prior to systemic infection of mice, *Salmonella* traverses the gut epithelial cell layer and is phagocytosed by cells of the monocyte lineaging, including macrophages, which deliver the pathogen to the spleen and liver (2). Although *Salmonella* has a hyper-replicative phase within the cytosol of epithelial cells, the bacteria are contained in all encountered cell types for at least some time within a specialized vesicular compartment termed the *Salmonella*-containing vacuole (SCV) (3, 4).
To identify small molecules that interfere with *Salmonella* survival or replication in macrophages, we recently screened the 14,400-compound Maybridge HitFinder™ v11 library in a high-content screen for Anti-infectives using Fluorescence microscopy of IntracellaR Enterobacteriaceae (SAFIRE) (5). Infected macrophage-like cells were treated with compound from 2 to 18 hours post-infection and then the cellular load of GFP-expressing *Salmonella* was quantified with automated imaging. A secondary screen in which lysed macrophages were plated for colony-forming units (CFU) identified 58 small molecules that reduce *Salmonella* growth and/or survival in macrophages. Unlike traditional antibiotics, these hit compounds lack antibacterial activity in standard microbiological medium (5). Here we focused on one, D61, which was particularly effective at reducing bacterial load and survival in macrophages.

**RESULTS**

A compound that reduces *Salmonella* load in macrophages.

D61 is a small aromatic molecule not previously described as having biological activity (Fig 1A). In the SAFIRE assay, D61 (25 μM) reduced bacterial load (GFP-signal in RAW 264.7 macrophages) by approximately 20-fold, with a half-maximum inhibitory concentration (IC$_{50}$) of 1.3 μM (Fig 1 B - D). Live imaging of RAW 264.7 cells treated with D61 (10 μM) two hours after infection revealed reduced bacterial GFP-signal compared to control within four hours of compound treatment, suggesting D61 acts rapidly to curb infection (Fig 1 E, Movie S1 (DMSO) and Movie S2 (D61)). Treatment with D61 (25 μM) also reduced the number of recoverable bacteria in macrophages by 20-fold, as monitored by plating for colony-forming units. Thus, D61 does not simply
prevent GFP production but indeed leads to bacterial death within macrophages (Fig 1F). The antibacterial activity of D61 appears to be specific for intracellular bacteria, as the mean inhibitory concentration (MIC) of this compound in broth is greater than 200 μM. Finally, D61 does not appear to have synergistic activity in broth with gentamicin, the antibiotic used in the cell culture infection experiments to limit extracellular bacterial replication (Fig 1G). These data indicate that treatment with D61 specifically inhibits *Salmonella* replication and/or survival within cell culture macrophages.

**D61 has antibacterial activity in primary mouse and human macrophages.**

The RAW 264.7 cell line used in the SAFIRE and colony-forming unit assays is a proxy for macrophages and is considerably more permissive for *Salmonella* than resting primary mouse macrophages, due in part to differential production of reactive oxygen and nitrogen species (6–8). RAW 264.7 cells also have homozygous loss-of-function mutations in Nramp1, which encodes an iron transporter that restricts the replication of intra-vesicular pathogens, including *Salmonella* (8). We therefore examined whether D61 prevents *Salmonella* replication in bone marrow-derived mouse macrophages (BMDMs) from Sv129S6/SvEvTac (Sv129) mice, which are Nramp1+/−. Treatment with D61 (25 μM) reduced recovery of bacterial colony-forming units from primary mouse macrophages by 17-fold (Fig 2A), and the compound has an IC₅₀ of 7.9 μM (Fig 2B). To examine whether D61 is antibacterial in animal species other than mice, we infected primary human bone marrow macrophages with wild-type *Salmonella* and treated with D61 (25 μM). Treatment reduced recovery of bacterial colony-forming units by 23-fold at 18 hours post infection (Fig 2C). These data demonstrate that D61 is antibacterial in...
mouse and human primary macrophages, indicating the compound may have in vivo relevance.

D61 is not antibacterial in HeLa cells and does not impact host cell survival. While *Salmonella* appears to reside in myeloid cells such as macrophages during systemic infection, gastrointestinal distress is associated with colonization of epithelial cells (9). We therefore examined the effect of D61 on *Salmonella* in HeLa cells, which are derived from the human epithelium. Neither quantification of bacterial load in the SAFIRE assay nor plating lysed HeLa cells for colony-forming units demonstrated that treatment with D61 (25 μM) reduced bacterial load within 18 hours of infection (Fig 2 D, E). These experiments, and the macrophage experiments above, were carried out with *Salmonella* grown to stationary-phase, which prevent T3SS-1-mediated killing of macrophages by repressing T3SS-1 (10–12). We next infected HeLa cells with *Salmonella* grown to late-log phase, which express T3SS-1, but did not observe D61 antibacterial activity (Fig S1). Thus, while D61 has antibacterial activity in human macrophages, it does not appear to be similarly active in HeLa cells.

Since host cell death over the course of infection has the potential to confound experimental interpretation of bacterial load (4), we established whether D61 treatment differentially kills RAW 264.7 and/or HeLa cells. First, examination of micrographs of D61 (25 μM) versus DMSO-treated macrophages at 18 hours after infection did not suggest differences in cell death (Fig S2A). We then monitored the escape of lactate dehydrogenase (LDH) from uninfected and infected cells with and without D61
treatment. Infection alone enhanced LDH release, as expected. However, treatment with D61 (25 μM) did not significantly increase LDH release compared to treatment with DMSO in either cell type (Fig S2 B, C). We also monitored LDH release across a dosage range of D61 in human HepG2 hepatocytes, which are typically used in toxicity studies. D61 had a half-maximal cytotoxic concentration (CC\textsubscript{50}) of 113 +/- 18 μM, approximately 100-fold higher than the IC\textsubscript{50} for reducing bacterial load in macrophages (Fig S2 D). Together, these data suggest that differences in D61 activity between macrophages and HeLa cells do not reflect differences in host cell killing.

**The in-cell antibacterial activity of D61 is independent of NOS.**

In the SAFIRE protocol, compound is added to host cells two hours after infection. Four hours later, *Salmonella* numbers in RAW 264.7 cells diverge from DMSO-treatment controls (Fig 1E). Therefore, D61 may amplify or extend early macrophage defenses, including the production of reactive nitrogen species (NOS) (7, 13, 14). We measured supernatant NOS using the Greiss assay. Treatment with D61 had no or modest effects on NOS accumulation whether cells were infected or not (Fig S3 A). As expected, treatment with the iNOS inhibitor L-nil reduced nitric oxide accumulation (Fig S3 A). However, neither L-nil, or a scavenger of H2O2-derived oxidants (acetovanillone) altered the antibacterial activity of D61 (Fig S3 B). Thus, D61 antibacterial activity may be independent of NOS.

*Salmonella is suppressed by D61 over the course of macrophage infection.*
Macrophages produce more and varied kinds of antimicrobial defenses as infection progresses and depend upon transcription and translation to do so. To establish when and for how long D61 must be present to inhibit *Salmonella* in macrophages, we treated cells from 2-6, 6-18 or 2-18 hours after infection and monitored bacterial load at 6 or 18 hours. The micrographs show, as expected, a decline in the density of macrophages observed by DAPI staining at 6 versus 18 hours in all samples, confirming that infection reduces cell number (Fig 3A). Quantification of GFP signal per macrophage area revealed that levels of bacterial replication at 6 hours post-infection were consistent with time-lapse microscopy data, with or without D61 (Fig 1E, Fig 3B). All three D61 treatment timeframes yielded similar, low levels of GFP signal at 18 hours post-infection, including when treatment occurs well after SCVs are established (6 hours post-infection). These results suggest that D61 exerts an antibacterial effect within hours and continues to suppress bacterial replication and/or survival over the entire 16-hour incubation period. D61 may therefore interfere with a complex and/or sustained macrophage defense mechanism.

**LC3 and LC3II accumulate in macrophages in response to D61 treatment.**

Autophagy is a host cell process that can clear bacteria, including *Salmonella*, *Shigella*, *Brucella*, *Mycobacterium tuberculosis*, and Group A *Streptococcus* (15, 16). To establish whether D61 may boost autophagy and could thereby cause the killing of wild-type *Salmonella* in macrophages, we infected RAW 264.7 cells with stationary-phase *Salmonella*, treated with D61 at two hours post-infection, and after 18 hours monitored the levels of LC3, a protein that contributes to, and is a marker of, autophagosome
Fluorescent microscopy revealed accumulation of total LC3 (I and II) in macrophages upon infection (Fig 4 A, B), consistent with previous observations of macrophages exposed to other Gram-negative bacteria (20, 21). Treatment of uninfected macrophages with D61 also robustly increased LC3 levels, compared to vehicle alone (Fig 4 A, B). We also compared the dosages at which D61 reduced bacterial load and increased LC3. The IC50 values were similar (Fig 4 C), suggesting that the ability of D61 to increase LC3 levels may be related to D61-mediated killing of Salmonella.

The anti-LC3 antibody we used for microscopy recognizes both cytosolic LC3I, and LC3II, the lipidated form, which attaches to the double membrane of the developing autophagosome and has differential mobility on SDS-PAGE gels. Immunoblot analysis of samples from uninfected and infected macrophages demonstrated an increase in LC3II in response to D61 treatment, while LC3I was not significantly affected (Fig S4 A, B). These observations together suggest that D61 promotes the accumulation of LC3II in macrophages, and that LC3II accumulation correlates with Salmonella killing by D61.

D61 induces autophagic flux through to acidification of the autophagosome. LC3II and autophagosome accumulation may suggest that D61 either blocks acidification/degradation of the autophagolysosome or stimulates autophagy through to acidification of the autophagosome. To distinguish between a block in autophagy or full flux, which includes acidification, we used a dual approach. First, we employed a tandem GFP-mCherry-LC3 sensor to establish the fate of LC3 in individual cells treated...
with D61. With this sensor GFP is quenched upon acidification, while mCherry expression remains intact (22–24). Macrophages stably expressing the tandem sensor construct were treated with vehicle (DMSO), D61 (25 μM), or rapamycin (500 nM) for six hours (Fig S4C). Treatment with bafilomycin A1 (200 nM) for the last four hours of the experiment, which is commonly used to inhibit autophagic flux by blocking acidification (24–26), served as a negative control. Macrophages were fixed, mCherry-positive puncta were identified to mark autophagosomes, and GFP signal in the identified puncta was quantified using MATLAB analysis. We plotted the number of non-acidified autophagosomes (GFP+, mCherry+) and total autophagosomes across all images. While treatment with bafilomycin A1 increased the total number of autophagosomes (red bars), nearly all of them remained non-acidified (blue bars), indicating autophagic flux was indeed inhibited. In contrast, rapamycin, a known activator of autophagic flux (27, 28), or D61 increased the total number of autophagosomes, with only a small fraction remaining non-acidified (Fig S4D). Live imaging of macrophages stably expressing the tandem sensor treated with D61 revealed that autophagosomes indeed acidify and degrade GFP, and change from yellow to red (Fig S4E). Examination of the fraction of acidified autophagosomes within the total autophagosome population revealed that treatment with bafilomycin A1 blocked GFP quenching, and thus acidification of the autophagosomes. Rapamycin increased the frequency of acidified autophagosomes, compared to DMSO (Fig 4D) and D61 treatment yielded a similar but less marked result. These population and single cell-based data suggest that D61 does not block acidification of the autophagosome but instead, similar to rapamycin, promotes the induction of autophagy, which includes acidification by the lysosome.
If D61 promotes autophagic flux, then co-treatment of cells with both D61 and bafilomycin A1 would be expected to increase LC3 protein levels above that of either treatment alone (17, 24). We therefore quantified total LC3 protein levels in the presence or absence of D61 and/or bafilomycin A1 by immunoblotting (17, 24). We treated uninfected macrophages with D61 (25μM) for 14 hours and/or with bafilomycin A1 (200 μM) for an additional four hours. As expected, bafilomycin A1 or D61 alone increased LC3II by approximately 2.2-fold or 1.8-fold, respectively (Fig 4E). Co-treatment with bafilomycin A1 and D61 increased LC3II up to 3-fold compared to untreated samples. Together with the tandem sensor data, these results demonstrate that upon D61 treatment of macrophages, more autophagosomes form and proceed through to acidification by the lysosome at all time points examined. Therefore, D61 induces the autophagic pathway and does not block trafficking to the lysosome and subsequent acidification.

**D61 induces LC3 aggregation near Salmonella in macrophages.**

Prior to microbial engulfment by an autophagosome, LC3 is recruited to the vicinity of the microbe (20, 29, 30). To establish whether D61 treatment affects *Salmonella* association with LC3 in macrophages, we determined the amount of LC3 localized around individual wild-type *Salmonella*. Infected cells were fixed, stained with an anti-LC3 antibody, and monitored by immunofluorescence (Fig 5A). Bacteria expressing RFP were identified as a region of interest using MATLAB scripting, and total LC3 signal intensity within a 40-pixel radius of the bacterium was quantified. D61 increased LC3
signal intensity around wild-type bacteria (Fig 5B, S5A). Examination of the percentage of the 40-pixel radius surrounding the bacterium that was positive for LC3 signal indicated that LC3 was more frequently found around those bacteria in macrophages treated with D61 (Fig 5C). An average of 19.3% +/- 9% of the identified bacteria were at least one half surrounded with LC3 upon DMSO treatment, whereas upon D61 treatment, an average of 38.9% +/- 5% of the identified bacteria were at least one half surrounded with LC3 (Fig 5 C). Manual counts of bacteria that were fully encircled by or decorated with aggregated LC3 at their poles, which is presumably forming autophagosomes (31), revealed that with D61 treatment, 13.3% +/- 6.1% of bacteria were associated with these patterns of LC3, while only 4.6% +/- 1.6% of bacteria were associated with these patterns of LC3 during DMSO treatment. These data corroborate and expand the immunoblotting and bacterial load results (Fig 4) by demonstrating that, within individual macrophages, D61 treatment correlates with LC3 recruitment to phagosomes and with bacterial killing.

The antibacterial activity of D61 in macrophages requires early steps in the autophagy pathway.

To establish where in the autophagy pathway D61 may act, we asked whether known chemical inhibitors of early autophagy steps reduce the antibacterial activity of D61. PI3K and VPS34 both play roles in the pathway prior to the recruitment of LC3II. PI3K functions at a step upstream of VPS34, a phosphatidylinositol 3-kinase that forms the initiation complex of the pathway. To blunt autophagy, we pre-treated RAW 264.7 macrophages with an inhibitor of VPS34 (VPS34i; SAR405 (32, 33)), or a more
generalized inhibitor of PI3K that also inhibits VPS34 ((PI3Ki; LY294002) (34, 35)). Two hours after infection we treated with D61 (25 μM), and 18 hours post-infection we quantified bacterial load based on GFP signal. Macrophage pre-treatment with VPS34i or PI3Ki decreased D61 antibacterial activity by approximately 40% (Fig 6 A, B). These results indicate that D61 relies upon early-stage autophagy to kill *Salmonella* in macrophages.

To genetically interrogate the role of autophagy in D61 antibacterial activity, we examined the effect of reducing the levels of ATG5 on D61-mediated killing of *Salmonella* in RAW 264.7 macrophages. ATG5 associates with ATG12 and is crucial for the conjugation of LC3 to autophagosomes (36). Transfection of RAW 264.7 cells with a pool of ATG5-directed siRNA led to a 20-60% reduction in ATG5 levels compared to macrophages transfected with non-targeting siRNA (Fig 6 C). Quantification of bacterial load based on GFP signal revealed D61 antibacterial activity was reduced to only 30% in ATG5 knockdown cells, while it remained above 90% in control macrophages (Fig 6 D). These results confirm that D61 curbs *Salmonella* infection by inducing autophagic flux.

**D61 reduces *Salmonella* load in infected mice.**

Our *in vitro* data suggests D61 is a highly potent antimicrobial agent, so we next tested whether D61 is effective in mice by comparing bacterial burden in the tissues of infected and treated mice. C57Bl/6 mice were intraperitoneally inoculated with 1 x 10<sup>4</sup> wild-type *Salmonella* and treated with 10-20 mg/kg of D61 at 20 minutes and 24 hours post-
infection. Spleen and liver were harvested at 48 hours post-infection and processed for
enumeration of CFU. Treatment with D61 significantly reduced Salmonella CFU in the
spleens and livers of infected mice indicating that this compound in particular and the
promotion of autophagy in general may have therapeutic value (Fig 7).

Chloramphenicol and D61 synergize to reduce bacterial load in macrophages.
The antibiotic chloramphenicol inhibits the bacterial 50s ribosomal subunit and also
induces autophagy in eukaryotic cells (37–41). We therefore determined whether D61
may act in synergy with chloramphenicol against Salmonella in primary macrophages.
Bone marrow-derived macrophages were infected with Salmonella, treated with single
or dual therapy, and lysed at 18 hours to enumerate colony-forming units, as displayed
in a checkerboard format (Fig 8A). Treatment with only D61 significantly reduced
bacterial load from 6.25 μM - 25 μM, and treatment with only chloramphenicol
significantly reduced bacterial load at all concentrations tested. However, D61
potentiates 12.5 μg/ml chloramphenicol treatment from 3.13 μM - 25 μM and
potentiates 6.3 μg/mL chloramphenicol treatment from 12.5 μM - 25 μM (Fig 8B). To
determine whether synergy relied upon chloramphenicol anti-ribosomal activity or
autophagy induction, we examined a Salmonella strain resistant to chloramphenicol
(Salmonella + pYAC184(CmR)). Chloramphenicol and D61 were synergistic only at
the highest concentration of D61 (25μM) suggesting synergy relies primarily upon
chloramphenicol inhibition of bacterial protein synthesis (Fig 8 C, D). These results
underscore the potential and complexity of dual treatment to increase the efficacy of
antibiotic therapy.
**DISCUSSION**

Omic studies have revealed many host cell changes that occur in the context of *Salmonella* infection, but it is not clear how these alterations contribute to controlling bacterial replication, nor are their roles in different cell types understood. The use of chemical genetics to identify small molecules of unknown function that alter the host-pathogen interaction is an alternative, not widely utilized approach to understanding disease and potential approaches to therapy (42, 43). Our current work describes a small molecule, D61, that is antibacterial in human and murine macrophages but not epithelial cells. We found that D61 induces autophagy to kill bacteria in macrophages.

Autophagy is a conserved cellular response to metabolic stress and to invading pathogens that all cell types examined can activate. Damaged organelles or intracellular bacteria are recognized by proteins within the autophagy pathway (e.g., p62, LC3), and engulfed by a double membrane autophagosome, which then fuses with the lysosome for degradation of the vesicle contents. In macrophages, autophagy appears to eliminate *Salmonella* that are attenuated for virulence, including strains that cannot deploy T3SS2 (16, 44). T3SS2 is needed by *Salmonella* to block AMPK activation and thereby protect the bacteria from autophagy during the first four hours of infection in Nramp1<sup>−/−</sup> BMDMs (45). In fibroblasts autophagy captures and removes some wild-type *Salmonella* and allows others to escape (46), but what determines the fate of an individual bacterium is not clear. Our results show that D61 curbs wild-type *Salmonella* infection in macrophages beginning within four hours of treatment and sustains and/or increases in potency over the course of 16 hours of infection. These results are similar...
to another small molecule, AR-12, which also reduces Salmonella load in macrophages and mice by inducing autophagy (47). A chemical genetics screen for compounds that prevent mycobacterial intracellular replication likewise identified an activator of autophagy, nortriptyline (42), consistent with reports that autophagy reduces mycobacterial colonization of host cells (48). These results illustrate the utility of empirical screens for dissecting host pathways that defend against intracellular pathogens.

Our observation that D61 does not interfere with Salmonella replication in HeLa cells supports previous reports that the autophagy pathway does not promote the killing of Salmonella and instead may benefit bacteria in this cell type. For example, the autophagy pathway has been implicated in the repair of SCV membranes damaged by T3SS1 and subsequent bacterial replication (49). TS331-damaged SCVs are vulnerable to recognition by LC3 and subsequent targeting for bacterial destruction early during infection of HeLa cells (50). On the other hand, it has been reported that recruitment of LC3 to the Salmonella SCV may facilitate bacterial access to nutrients in a manner dependent on the T3SS1 effector SopB (51). It was also recently reported that in the absence of the T3SS2 effectors SseF and SseG, autophagy kills Salmonella in HeLa cells (52). Our data, taken with existing studies, highlight the idea that autophagy can benefit or destroy Salmonella depending on the microenvironment, yielding different outcomes for different bacteria within a given cell. Additional studies at the single cell level will be needed to solve this riddle.
We also found that co-treatment with low doses of D61 and the antibiotic chloramphenicol more effectively reduced bacterial load in macrophages than either single therapy. Most of the impact of chloramphenicol was mediated by the effect of this antibiotic on the bacterial ribosome as opposed to its demonstrated ability to stimulate autophagy. We speculate that *Salmonella* exposed to chloramphenicol does not respond properly to the environment of the SCV due to failure to translate virulence factors such as T3SS2 (44), and is therefore more susceptible to D61-mediated autophagy than chloramphenicol-resistant bacteria. The original indication of chloramphenicol was for typhoid fever (53), but due in part to *Salmonella* resistance, chloramphenicol is now used for treatment of ocular infections, bacterial meningitis, and *Staphylococcus* brain abscesses, and is being considered as a treatment for vancomycin-resistant *Enterococcus* (54–57). However, because side effects of chloramphenicol may be severe (58), the pursuit of combination therapies has appeal. For instance, it may be possible to co-treat with a drug that promotes autophagy and thereby lower the effective dose of a toxic antibiotic.

Our studies on D61 extend previous observations regarding the cell-type-specific roles of autophagy and its effects on *Salmonella* survival and replication in macrophages versus epithelial cells. We note that single-cell quantitative microscopy was essential for establishing that D61 is a driver of autophagy, demonstrating that stimulating autophagy is sufficient to contain *Salmonella* within these cells. Why D61 treatment alone did not kill *Salmonella* in HeLa cells remains unclear. In addition, we do not know the molecular target of D61 nor whether D61 may have off-target effects, as do many small molecules
Advanced characterization of D61, such as Drug Affinity Responsive Target Stability (DARTS) (60), RNAi screening or proteomics analysis of macrophages treated with D61 may lead to a more refined understanding of the mode of action and determine whether control of the bacteria is linked directly to sequestration of *Salmonella* in autophagosomes and subsequent destruction, or indirectly via effects on autophagy induction and autophagosome formation elsewhere in infected cells. Nevertheless, by establishing how compounds such as D61 modulate the host to reduce bacterial load at the pathway level, we uncover molecular and even cell-type specific interactions between the host and pathogen that may ultimately be targeted to curb infection.

**MATERIALS AND METHODS**

**Bacterial strains**

For imaging experiments, macrophages were infected with *Salmonella enterica* serovar Typhimurium strain SL1344 (*sifB::gfp*) (61) and epithelial cells were infected with strain SL1344 SM022 (*rpsM::gfp*) (62). For chloramphenicol synergy experiments, SL1344 harbored *gfp* on pACYC184(*Cm*). For all other experiments, strain SL1344 was used for infection. Bacteria used for stationary-phase infections were grown in Luria-Bertani Broth (LB) with 30 µg/ml streptomycin (and 30 µg/ml kanamycin for GFP+ strains) for 18 hours at 37 °C with aeration. For infection with late-log phase bacteria, overnight cultures were diluted 1:33 in LB and grown for four hours at 37 °C with aeration.

**Cell culture**
Murine macrophage-like RAW 264.7, BMDMs, and HeLa human epithelial cells were grown in DMEM high glucose (Sigma) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, and 50 μM β-mercaptoethanol. Cells were maintained in a 5% CO₂ humidified atmosphere at 37 °C. Experiments using RAW 264.7 or HeLa cells were performed with cultures between passages four and ten. BMDMs were isolated as described (63). Briefly, bone marrow was flushed from femurs and tibias of 4-10 week old wild-type Sv129S6/SvEvTac (SV129) mice (Taconic). Mononuclear cells were separated using Histopaque-1083 (Sigma), washed, and directly seeded into assay plates at 1 x 10⁵ cells/mL in complete medium supplemented with 35% conditioned media from 3T3 cells expressing MCSF (64). Media were refreshed three days later. After one week, media were replaced with 200 µL fresh media and cells were infected.

Human monocyte-derived macrophages were derived from primary bone marrow mononuclear cells (ATCC). Monocytes were grown in DMEM high glucose (Sigma) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, and 50 μM β-mercaptoethanol containing 100 ng/mL mCSF. Cells were maintained in a 5% CO₂ humidified atmosphere at 37 °C. At day 7, macrophages were scraped and seeded to 96-well tissue culture-coated plates for further application.

**Bacterial infections for SAFIRE, CFU plating and protein extraction**

SAFIRE – Infections were performed as described previously (5). Briefly, RAW 264.7 macrophages (5 x 10⁴ macrophages in 100 µL) were seeded in 96-well black-walled
glass-bottomed plates (Brooks Automation). Twenty-four hours post-seeding, bacteria in
50 µL PBS were added to a final concentration of 1 x 10⁷ CFU/mL, which is
approximately a multiplicity of infection of 30 bacteria to one cell. Forty-five minutes
after bacterial addition, 50 µL gentamicin was added to a final concentration of 40
µg/mL, which did not affect intracellular infection but inhibited replication of extracellular
bacteria. At two hours post-infection, 1µL of compound (D61; SEW06622; MolPort) or
vehicle control was added to yield a final concentration of 25 µM. At 17.5 hours post-
infection, PBS containing MitoTracker Red CMXRos (Life Technologies) was added to a
final concentration of 100 nM. Thirty minutes later, 16% paraformaldehyde was added
to a final concentration of 1% and incubated at room temperature for 15 minutes. Wells
were washed, stained with 1 µM DAPI and stored in 90% glycerol in PBS until imaging.
Image analysis was performed with MATLAB R2018a (University of Colorado at Boulder
License Number: 361635), as described (5).

HeLa cells - Infections with Salmonella were performed as above except that 1 x 10⁴
cells were seeded (approximately at a multiplicity of infection of 150 bacteria to one
cell). Cells were infected with Salmonella constitutively expressing GFP from the rpsM
locus (SL1344 (rpsM::gfp)). Plates were centrifuged for five minutes at 500 x g after
addition of bacteria to enhance infection.

CFU - Infections were performed as described above, except cells were seeded in 96-
well tissue culture coated plates (Greiner). At 18 hours post-infection, wells were
washed three times in PBS, lysed with 30 µL 0.1% Triton X-100, diluted and plated to determine CFU.

Immunoblotting- 1 x 10⁶ macrophages were seeded in 6-well tissue culture-coated plates in 1 mL of medium. Twenty-four hours post-seeding, bacteria in 500 µL PBS were added to a final concentration of 1 x 10⁷ CFU/mL, which is approximately a multiplicity of infection of 15 bacteria to one macrophage. Forty-five minutes after bacterial addition, 500 µL gentamicin was added to a final concentration of 40 µg/mL, which did not affect intracellular infection but inhibited replication of extracellular bacteria. At two hours post-infection, 2.5 uL of compound or vehicle control was added to yield a final concentration of 25 µM. At 18 hours post-infection, wells were washed twice with PBS and scraped in 300 µL ice-cold RIPA buffer (10mM Tris-HCl (pH 8.0), 1mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl, 1mM PMSF, 10 mM vanadate). Lysate was incubated on ice for 15 minutes to extract protein. Cells were centrifuged at maximum speed in a microfuge. Supernatants were collected and 5x loading buffer including 0.1% β-mercaptoethanol was added, followed by boiling at 100 °C for five minutes, and storage at -20 °C until use. Equal protein was separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene difluoride membrane (PVDF, Sigma). Protein levels were assessed by Western blotting using anti-LC3I/II (1:1000, Cell Signaling Technology) or anti-actin (1:3000, Sigma) antibodies. Primary antibodies were detected using goat anti-mouse (1:3000) or goat anti-rabbit (1:3000) horseradish peroxidase-conjugated secondary
antibodies and visualized by Supersignal West Pico Chemiluminescence Substrate (Thermo) according to the manufacturer’s instructions using X-ray film.

**Broth antibacterial activity assays**

Overnight *Salmonella* cultures were washed three times in PBS and diluted to an OD of 0.01 in M9 minimal media supplemented with 100 mM Tris pH 7.4, 0.35% glycerol, 0.002% histidine, 10 mM MgCl$_2$, and 0.1% casamino acids. Where indicated, media was supplemented with 5 µg/mL polymyxin B. Compound was added using a pin tool (CyBio) or manually, yielding a final concentration of no more than 1% DMSO. Plates were grown with shaking at 37 °C and OD$_{600}$ was monitored using a BioTek Eon incubator shaker microplate absorbance reader.

Checkerboard assays used D61 diluted two-fold into DMSO and stored at room temperature. MHB (30 µL) was distributed into 96-well flat-bottom plates and gentamicin was serially diluted 1.5-fold down the plate. D61 was added followed by 270 µL of *Salmonella* from overnight cultures that were washed three times with PBS and diluted in MHB to an OD of 0.01. Plates were incubated at 37 °C for 18 hours and read at OD$_{600}$ on a plate reader (BioTek Eon or Synergy H1).

**LC3B immunofluorescence and determination of localization**

Macrophages were plated as described for SAFIRE imaging. After fixation, plates were rinsed with PBS and incubated with 1.5% BSA/PBS containing 0.1% Triton X-100 for one hour at room temperature. Plates were incubated with rabbit polyclonal anti-LC3
antibody (Cell Signaling Technology) at 1:200 in 1.5% BSA/PBS containing 0.1% Triton X-100 overnight at 4 °C. Washed slides were incubated with goat anti-rabbit AlexaFluor 488-conjugated antibody (Invitrogen) at 1:1000 for one hour at room temperature. Nuclei were stained with DAPI and wells were stored with 100 μL 90% glycerol. Images were acquired using the Yokogawa CellVoyager™ CV1000 Confocal Scanner System with a 100x/1.40NA OIL WD 0.13 (mm) objective and a Hamamatsu Photonics ImagEM X2 EM-CCD Camera C9100-14 High Resolution Format (1024 x 1024 pixels). Briefly, all images acquired included three channels, 23 Z-slices and a Z-range of 15 μm. Images were acquired as large montages (4496 x 2761 pixels; defined as a field of view), converted to Maximum Intensity Projections and stitched using the CV1000 viewer software version 1.06.06 and ImageJ 1.47n. Images were imported as multidimensional (3D) tiff stacks and processed using MATLAB. This script is available at MATLAB Central and titled LC3B ROI Quantification. Briefly, the LC3B ROI Quantification script analyzes high resolution monochrome images of cells infected with bacteria within a region of interest (ROI). The code auto defines thresholds for all three channels, performs watershed segmentation for the first and third channels, and quantifies the number of bacteria and nuclei present across the entire image. Next, ROIs with a radius of 40 pixels are defined with the centers of identified bacteria acting as the centroid. Finally, the integrated density of the identified objects surrounding the bacterium is quantified for each ROI.
Inhibitors used to determine autophagic flux

To determine whether D61 antibacterial activity was dependent on the autophagic pathway, macrophages were pretreated with SAR405 (100 nM) or LY294002 (50 μM) one hour prior to infection.

Transient transfection with small interfering RNA (siRNA)

RAW 264.7 cells (2.5x10⁴) in 48- or 96-well plates were transiently transfected using Accell siRNA technology (Dharmacon), in accordance with the manufacturer’s instructions. Briefly, cells were incubated with 1 μM Accell non-targeting or SMARTPool ATG5 siRNA using Accell delivery media for 96 hours. Medium was then replaced with complete DMEM and used for SAFIRE, Western blot, or CFU assays.

GFP-mCherry-LC3 Tandem sensor

Briefly, to generate tandem sensor-expressing macrophages, RAW 264.7 cells were transfected using lipofectamine 2000 and the Addgene pBABE-puro-mCherry-EGFP-LC3B-AMP (80432) plasmid. After selection, cells were flow-sorted to isolate a subset of cells with stable and consistent expression for mCherry. Sterile #1.5 High Tolerance MatTek coverslips were placed into 6 well plates and were seeded at 1 x 10⁶ cells/well in 2 mL of complete DMEM. Cells were then allowed to expand in an incubator for 24 hours at 37 °C/5% CO₂. Medium was exchanged with fresh DMEM 23.5 hours after seeding. Thirty minutes later, compounds (DMSO (0.125%), D61 (25 μM), or rapamycin (500 nm)) were added for six hours. Bafilomycin A1 (200 nM) was added for the last
four hours of the experiment. At six hours post-compound addition, cells were washed twice with 1X PBS/5 μM HEPES buffered at a pH of 7.4 and then fixed in 3.7 % PFA for 20 mins in the dark. Cells were then washed twice with 1X PBS/5 μM HEPES buffered at a pH of 7.4, stained with 1 μM DAPI, washed twice with 1X PBS/5 μM HEPES buffered at a pH of 7.4 and fixed using 18 μL of Invitrogen Prolong Glass Antifade Mounting Medium. Samples were then placed on a flat surface in the dark for two days to allow the medium to reach peak refractive index. Samples were imaged using the Yokogawa CellVoyager™ CV1000 Confocal Scanner System with a 100x/1.40NA OIL WD 0.13 (mm) objective and a Hamamatsu Photonics | ImagEM X2 EM-CCD Camera C9100-14 High Resolution Format (1024 x 1024 pixels). Images were acquired as volumes with the Z dimensions range set to 12 μM and a step size of 300 nm. All volumes contained a DAPI, EGFP, and mCherry channel. Finally, all RAW 264.7 cells expressing the pBABE-puro-mCherry-EGFP-LC3B-AMP plasmid were analyzed via MATLAB. This script is available at MATLAB Central and titled LC3 Tandem Puncta Quantification.

GFP-mCherry-LC3 tandem sensor MATLAB script-
The LC3 Tandem Puncta Quantification script is designed to quantify puncta in high resolution volumes on the single cell level. The code reads in 16-bit images in the form of multidimensional 4D Tiff stacks. Briefly, the code takes each volume independently, splits the channels into sub volumes and finally undergoes a 3D controlled watershed to obtain single cell volumes which are isolated for analysis. Each single cell volume is analyzed for the number of EGFP and mCherry puncta (note the user must set a
threshold for EGFP puncta only and the code will adapt this threshold to accommodate differences between EGFP and mCherry quantum yields. This threshold should be adjusted for each cell line using the negative controls average endogenous EGFP puncta signal. The results for each image are exported as a dot structure with the original volumes file path, filename, and the analyzed single cell volume results for the EGFP and mCherry puncta counts for each cell. Acidified autophagosomes were defined as GFP+, mCherry+ LC3 and non-acidified autophagosomes were defined as GFP+, mCherry+ LC3.

Infection and treatment of mice

This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (65), and all protocols were approved by the University of Colorado Institutional Committees for Biosafety and for Animal Care and Use. 7-8 week-old C57Bl/6 mice were intraperitoneally (IP) inoculated with 1 x 10^4 CFU and the infectious dose was verified by plating for CFU. Mice were IP-treated with vehicle (70% DMSO) or 10-20 mg/kg of D61 at 20 minutes and 24 hours post-infection. At 48 hours post-inoculation, infected animals were euthanized by CO2 asphyxiation, followed by cervical dislocation. Spleen and liver were collected, homogenized in 1 ml PBS and then serially diluted for plating to enumerate CFU. Dosages were determined based on LDH toxicity and SAFIRE IC₅₀ assays in primary macrophages, according to approved IACUC protocols.

Statistics
Each data set was analyzed for outliers and Gaussian distribution. Statistical tests were applied as indicated in figure legends using GraphPad/Prism or MATLAB. For experiments where fewer than three biological replicates were performed, or technical replicates are shown, each experiment is represented as a single dot in a scatter plot with a bar graph overlay. Bar graphs with mean and standard error of the mean are shown for all experiments with three or greater biological replicates.

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FIGURE LEGENDS

Figure 1. D61 is a potent antibacterial compound in cell culture macrophages. A) Structure of D61. B-F) RAW264.7 macrophage-like cells were infected with stationary phase Salmonella harboring a chromosomal sifB::GFP insertion. Cells were treated at two hours post-infection with vehicle (DMSO), D61, or as indicated. Cells were, at 18 hours post-infection, (B-D) fixed and imaged, or (F) lysed and plated for enumeration of CFU. Alternatively, (E) cells were live imaged. B) Representative micrographs from cells treated with DMSO (left) or D61 (25 μM, right). C) GFP+ Macrophage Area quantified from micrographs of cells treated with DMSO, D61 (25 μM) or rifampicin (Rif, (10 μg/mL)). GFP+ Macrophage Area is defined as the number of GFP+ pixels per macrophage divided by the total number of pixels per macrophage, averaged across all macrophages. ** P ≤ 0.005, *** P ≤ 0.0005 compared to infected DMSO by one-way ANOVA with Dunnett’s multiple comparison test. D) GFP+ Macrophage Area dose-response using two-fold dilutions of D61 from 25 μM. E) Live imaging of macrophages over 13 h with DMSO (gray) or D61 ((10 μM), blue). Integrated density of GFP+ Macrophage Area across four fields. F) CFU of cells treated with DMSO or D61 (25 μM). Mean CFU/mL DMSO = 8.1 x 10^6. Mean CFU/mL D61 = 3.6 x 10^5. Mean and SEM of three biological replicates. *** P ≤ 0.0005 vs. DMSO by unpaired t test. G) Wild-type stationary phase Salmonella were grown in MHB with a dose range of D61 and gentamicin. Mean and SEM of two independent biological replicates.

Figure 2. D61 is effective in primary mouse and human macrophages but not in HeLa cells. A, B) Mouse primary Sv129 BMDMs or C) human primary BMDMs were
infected with wild-type stationary phase *Salmonella*. Cells were treated at two hours post-infection with DMSO or D61 (25 μM), and at 18 hours post-infection were lysed and plated for enumeration of CFU. Mean CFU/mL DMSO in mouse primary macrophages = 5.0 x 10^8. Mean CFU/mL D61 in mouse primary macrophages = 2.4 x 10^5. Mean CFU/mL DMSO in human primary macrophages = 1.3 x 10^3. Mean CFU/mL D61 in human primary macrophages = 8.9 x 10^1. B) Dose response of BMDMs using two-fold dilutions of D61 from 25 μM. D) HeLa cells were infected with wild-type stationary phase *Salmonella*. Cells were treated at two hours post-infection with D61 (25 μM) and at 18 hours post-infection were plated for enumeration of CFU. Mean CFU/mL DMSO in HeLas = 1.2 x 10^6. Mean CFU/mL D61 in HeLas = 1.1 x 10^6. E) HeLa cells were infected with wild-type stationary phase *Salmonella* harboring prpsM::GFP. Cells were treated at two hours post-infection with D61 (25 μM) and at 18 hours were processed according to the SAFIRE protocol. Data are mean and SEM of four (C,D), three (A,B), or two (E) independent biological replicates. *** P ≤ 0.0005 relative to DMSO, unpaired t test; ns, not significant.

Figure 3: D61 suppresses *Salmonella* over the course of macrophage infection. A) RAW264.7 macrophage-like cells were infected with stationary phase *Salmonella* harboring a chromosomal *sifB::GFP* insertion. Cells were treated with vehicle control or D61 (25 μM) at indicated time points. B) At 6 or 18 hours, cells were processed according to the SAFIRE protocol. Data are mean and SEM from four independent biological replicates. * P ≤ 0.05, ** P ≤ 0.005, unpaired t test; ns, not significant.
Figure 4. D61 induces autophagic flux through to acidification of the autophagolysosome. A) Representative images of RAW264.7 macrophage-like cells that were mock-infected or infected with stationary phase *Salmonella* and were treated at two hours post-infection with vehicle or D61 (25 μM). Cells were stained with DAPI (blue) and immunostained for LC3 (green). B) Images from (A) were quantified for the intensity of LC3 signal using MATLAB SAFIRE scripts. **P ≤ 0.005 compared to uninfected, DMSO-treated cells by one-way ANOVA with Dunnett’s multiple comparison test. C) Macrophages treated with two-fold dilutions of D61 from 25 μM for 18 hours and were processed and quantified as in A and B (green line) and plotted against the data from Fig 1 D (blue line, GFP+ Macrophage Area). D) Macrophages stably expressing an LC3-GFP, mCherry tandem sensor were treated with DMSO, D61 (25 μM) or rapamycin (500 nM) (positive control) for six hours; as a negative control, macrophages were treated with bafilomycin A1 (200 nM) for the last four hours of the experiment. Cells were fixed, imaged and quantified using MATLAB. Acidified autophagosomes were identified as GFP-, mCherry+. Data are shown as histograms of acidified versus total autophagosomes derived from macrophages treated with DMSO (n = 247), D61 (n = 257), rapamycin (n = 279) or bafilomycin A (n = 326). E) Macrophages were treated with vehicle or D61 (25 μM). At 14 hours post-treatment, vehicle or bafilomycin A1 (200 nM) was added. At 18 hours post-treatment, protein was extracted, resolved on SDS-PAGE and immunoblotted for LC3 and actin. Data are mean and SEM of three independent biological replicates. *P ≤ 0.05 compared to treatment with bafilomycin A1 alone, unpaired t test.
**Figure 5. LC3 intensity increases with D61 treatment in macrophages**

**A**) Representative images of RAW 264.7 macrophages infected with wild-type stationary phase *Salmonella* expressing pRFP and treated at two hours post-infection with DMSO or D61 (25 μM). At four hours post-infection cells were fixed and stained with DAPI (blue) and for LC3 (green). **B,C**) Using MATLAB, macrophages within four large fields of view (4496 x 2761 pixels each) across four independent biological replicates were first identified. Total macrophages analyzed: WT DMSO (296), WT D61 (318). Then bacteria were defined as regions of interest, and the LC3 intensity within a 40 pixel radius of each bacterium (red) was measured. Total bacteria analyzed: WT DMSO (207), WT D61 (128). **B** The average LC3 intensity per bacterium was calculated for each biological replicate. Mean and SEM are shown. * P ≤ 0.05 compared to WT, DMSO-treated cells by students t-test. **C** Frequency distribution of the percentage of the bacterium that is positive for LC3. DMSO-treated, black; D61-treated-blue.

**Figure 6. The VPS34 complex and ATG5 contribute to D61 antibacterial activity.**

RAW 264.7 macrophages were pretreated with a **A** VPS34 inhibitor (SAR 405) or **B** PI3K inhibitor (LY294002) for one hour. Cells were then infected with *Salmonella* harboring a chromosomal sifB::GFP insertion. **C, D** Macrophages harboring non-targeting or ATG5 siRNA were infected with *Salmonella sifB::GFP*. For each treatment group, average fractional GFP area (GFP+ macrophage area) was determined using...
SAFIRE and used to calculate antibacterial activity. Mean and SEM from three to five independent biological replicates. * \( P \leq 0.05 \), ** \( P \leq 0.005 \); unpaired t test.

**Figure 7.** D61 reduces *Salmonella* tissue load in C57Bl/6 mice. Mice were intraperitoneally inoculated with \( 1 \times 10^4 \) wild-type CFU. At 20 minutes and 24 hours post-infection, mice were dosed with 10-20 mg/kg of D61 by intraperitoneal injection. At 48 hours post-infection, mice were sacrificed, and the spleen and liver were immediately homogenized and plated for enumeration of CFU. * \( P \leq 0.05 \), Mann-Whitney.

**Figure 8.** D61 potentiates the activity of chloramphenicol in primary macrophages

BMDMs were infected with stationary phase A,B) wild-type or C,D) wild-type *Salmonella* harboring a plasmid conferring chloramphenicol resistance. At two hours post-infection, BMDMs were treated with single or combinatorial therapy of chloramphenicol and D61. At 18 hours post-infection cells were lysed and plated for enumeration of CFU. Results from four independent experiments were A, C) plotted on a heat map or B, D) presented as CFU vs. concentration of D61.
**Figure 1**

A. D61 molecule structure.

B. Images showing GFP-expressing Salmonella, MitoTracker, and nuclei.

C. Bar graph showing GFP-positive macrophage area (%).

D. Graph showing D61 concentration vs. macrophage area (normalized to DMSO). IC50 is 1.3 µM.

E. Graph showing integrated density vs. time post-infection.

F. Graph showing CFU/mL normalized to DMSO.

G. Heatmap showing Gentamicin concentration vs. OD600.

Legend: DMSO, D61, Rif

**Table:**

| Gentamicin (µM) | OD600 = 0.003 | OD600 = 1.124 |
|-----------------|---------------|---------------|
| 0.60            | 4.18          | 1.84          |
| 1.21            | 2.78          | 1.51          |
| 1.84            | 1.21          | 0.92          |
| 2.78            | 0.60          | 0.31          |
| 4.18            | 0.00          | 0.00          |
Figure 2.

A

***

B

D

E

***

ns ns C

IC_{50} 7.39 \mu M

CFU/mL (normalized to DMSO)

DMSO

D61

0.0

0.5

1.0

CFU/mL

(Normalized to DMSO)

DMSO

D61

0.0

0.5

1.0

GFP^+ Macrophage Area (%)
Figure 3

![Image of GFP+ Macrophage Area (%)](attachment:image.png)

| Treatment (hrs) | Timepoint (hpi) |
|----------------|-----------------|
| 2-6            | 6               |
| 6-18           | 18              |
| 2-6            | 18              |
| 2-18           | 18              |

DMSO

D61

* ns

** ns
Figure 4

A

Infected Mock-infected

DMSO D61

B

Ave area intensity

GFP+ pixels

C

GFP+ Macrophage Area

Normalized to DMSO

D

% of cells

DMSO D61

Rapamycin Bafilomycin A1

E

Fold over DMSO

D61 BafA1

LC3I LC3II Actin

on March 5, 2020 by guest
Downloaded from http://aac.asm.org/
Figure 6

A

* * 
**

B C D

ATG5
Actin

0
20
40
60
80
100

D61 Antibacterial Activity (%)

DMSO
VPS34i

0
20
40
60
80
100

D61 Antibacterial Activity (%)

DMSO PI3Ki

0
20
40
60
80
100

D61 Antibacterial Activity (%)

C

Non-targeting
ATG5
siRNA

Actin

ATG5

D

Non-targeting
ATG5
siRNA

* *
Figure 7

![Graph showing CFU/g log10 for Spleen and Liver at D61 with negative and positive signs indicating statistical significance.](image-url)
