# ATG16L1 deficiency in macrophages drives clearance of uropathogenic *E. coli* in an IL-1β-dependent manner

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Urinary tract infections (UTIs) are frequent, commonly recurrent, and costly. Deficiency in a key autophagy protein, ATG16L1, protects mice from infection with the predominant bacterial cause of UTIs, Uropathogenic *E. coli* (UPEC). Here, we report that loss of ATG16L1 in macrophages accounts for this protective phenotype. Compared with wild-type macrophages, macrophages deficient in ATG16L1 exhibit increased uptake of UPEC and enhanced secretion of interleukin-1 $\beta$  (IL-1 $\beta$ ). The increased IL-1 $\beta$  production is dependent upon activation of the NLRP3 inflammasome and caspase-1. IL-1 $\beta$  secretion was also enhanced during UPEC infection of ATG16L1-deficient mice *in vivo*, and inhibition of IL-1 $\beta$  signaling abrogates the ATG16L1-dependent protection from UTIs. Our results argue that ATG16L1 normally suppresses a host-protective IL-1 $\beta$  response to UPEC by macrophages.

### INTRODUCTION

Half of all women will suffer from a urinary tract infection (UTI) at some point in their lives. Moreover, in the United States, UTIs account for 8 million visits to emergency departments and clinics every year and 15% of all outpatient antibiotic prescriptions.<sup>1</sup> Despite effective clearance of the acute infection, as many as 10% of those who suffer from a UTI will go on to have recurrent infections that cannot be cured with antibiotics.<sup>2</sup> Thus, development of effective therapeutics will require an understanding of the ways in which bacteria infect the urinary tract and the defense mechanisms used by the host to combat the infection.

Uropathogenic *E. coli* (UPEC) is the causative agent in 75% of all UTIs.<sup>2</sup> Mouse models of UTI have revealed that clearance of UPEC from the bladder mucosa depends on the early recruitment of innate immune cells.<sup>3,4</sup> On the other hand, UPEC have evolved several mechanisms to actively subvert recruitment and response of host immune cells.<sup>5,6</sup> For example, UPEC suppresses production of pro-inflammatory cytokines such as interleukin-6 (IL-6)<sup>7</sup> and attenuates the ability of

neutrophils to migrate toward the sites of infection.<sup>8</sup> However, the exact molecular mechanisms underlying the recruitment and functionality of innate immune cells, especially those of the monocyte/macrophages lineage, in response to UPEC infection remain unclear.

Host mucosal defense against pathogens requires coordination of multiple signaling pathways within innate immune cells. One such pathway is autophagy, a cellular recycling pathway responsible for lysosomal degradation of cytosolic components, including damaged organelles, protein aggregates, and intracellular pathogens.<sup>9,10</sup> The autophagy pathway in general, and the autophagy gene/protein ATG16L1 in particular, has an important role in innate immune responses to infection.<sup>11</sup> A common polymorphism in *ATG16L1* (T300A) remains prevalent within the Caucasian population and is associated with Crohn's disease,<sup>12,13</sup> a form of inflammatory bowel disease. An open question remains as how this 'bad' risk allele is prevalent in the population.

Recent studies have revealed that autophagy and ATG16L1 can dampen activation of the innate immune response to

Received 3 July 2014; accepted 11 December 2014; published online 11 February 2015. doi:10.1038/mi.2015.7

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infection as well as activation of inflammasomes,<sup>10,11</sup> key signaling complexes that detect pathogenic microorganisms and which in turn activate the highly pro-inflammatory cytokine IL-1 $\beta$ .<sup>14</sup> In particular, ATG16L1 and autophagy have been suggested to have a role in the production of IL-1 $\beta$ <sup>15,16</sup> as well as another pro-inflammatory cytokine tumor necrosis factor alpha (TNF $\alpha$ ).<sup>17,18</sup> However, the mechanisms by which autophagy and ATG16L1 regulates the inflammasome in the context of infection are still uncertain.

We recently showed that mice deficient in ATG16L1 (Atg16L1<sup>HM</sup>,<sup>19,20</sup> carrying a hypomorphic allele that reduces *Atg16l1* expression) cleared UPEC infection more rapidly and thoroughly than controls.<sup>21</sup> We further demonstrated that ATG16L1 deficiency in the hematopoietic compartment was the primary driver of increased clearance of UPEC, although the adaptive immune compartment did not contribute to the protective phenotype.<sup>21</sup> These findings argue that loss of ATG16L1 in the innate immune system is responsible for resistance to UTIs, yet the underlying mechanism for this resistance remains to be examined.

In this report, we demonstrate that macrophages are essential for the control and clearance of UPEC from the bladders of ATG16L1-deficient mice. Mechanistic studies using primary macrophages revealed that loss of ATG16L1 increases bacterial uptake and enhances release of the cytokine IL-1β, but not TNF $\alpha$ , in response to UPEC. The increased IL-1 $\beta$  production is independent of NOD2 or gross lysosomal damage, but is dependent on caspase-1 and the NLRP3 inflammasome. Finally, we show that augmented IL-1 $\beta$  signaling is the primary mechanism responsible for enhanced clearance of UPEC from the urinary tract in ATG16L1-deficient mice. Together, our findings show that ATG16L1 deficiency makes macrophages better able to control UPEC infection in vivo during a UTI by regulating levels of IL-1β. Our findings have implications for elucidating how UPEC is able to evade host innate defenses to cause a UTI and suggest that polymorphisms in ATG16L1 may be maintained in the population because of protective effects from a common infection.

### RESULTS

### Macrophages are required for UPEC clearance in Atg16L1<sup>HM</sup> mice

We previously found that monocyte recruitment was enhanced in the bladders of infected ATG16L1-deficient (Atg16L1<sup>HM</sup>) mice compared with those of infected wild-type (WT) animals.<sup>21</sup> Furthermore, Atg16L1<sup>HM</sup> mice cleared their UTIs more rapidly than WT mice. To determine whether monocytes and macrophages were necessary for this effect, we depleted monocytes and macrophages from Atg16L1<sup>HM</sup> mice by treating them with clodronate-containing liposomes (clodrolip)<sup>22,23</sup> followed by transurethral infection with a well-characterized clinical cystitis strain of UPEC (UTI89). Clodrolip treatment decreased the number of systemic macrophages and monocytes in the spleens of Atg16L1<sup>HM</sup> mice by greater than 60% (**Supplementary Figure 1**), reduced the recruitment and number of macrophages in the bladder mucosa after infection



**Figure 1** Macrophages and monocytes are essential for bacterial control in Atg16L1<sup>HM</sup> mice. (**a**, **b**) Colony forming unit (CFU) counts of bacteriuria (**a**) or 3 dpi bladder homogenates (**b**) from Atg16L1<sup>HM</sup> mice treated with clodrolip or control liposomes as displayed as mean Log<sup>10</sup>CFU per ml and s.e.m. For **a**, n = 3 independent experiments, for a total of 12 clodrolip-treated mice and 13 control liposome-treated mice. For **b**, n = 2 independent experiments, for a total of 9 clodrolip-treated mice and 9 control liposome-treated mice. Two-way ANOVA with matching by animal, \*\*\**P*<0.001, with Bonferroni post-test at individual time points (**a**), and unpaired *T*-test (**b**), \**P*<0.05. (**c**) ATG16L1 levels in bone marrow-derived macrophages (BMDMs) from wild-type (WT) and Atg16L1<sup>HM</sup> mice as measured by western blot. (**d**) Western blot of p62 in samples of WT and ATG16L1-deficient BMDMs. Starvation (Starv) was used to induce autophagy. GAPDH, glyceraldehyde 3-phosphate dehydrogenase, used as a loading control in (**c**) and (**d**). Data are representative of three independent experiments (**c** and **d**).

(**Supplementary Figure 2**) and significantly attenuated their enhanced ability to clear the infection (**Figures 1a** and **b**). Furthermore, clodrolip treatment resulted in higher urine and bladder titers at 3 days post infection in Atg16L1<sup>HM</sup> mice relative to WT mice (**Figures 1a** and **b**). Clodrolip treatment can also deplete CD11c + dendritic cells; however, it has been previously shown that dendritic cells are dispensable for UPEC pathogenesis.<sup>24</sup> Thus, we argue that macrophages or monocytes are essential for enhanced clearance of UTI89 in Atg16L1<sup>HM</sup> mice.

### Altered UTI89 uptake and processing by ATG16L1-deficient macrophages

Macrophages have direct bactericidal functions and also secrete pro-inflammatory cytokines to orchestrate the host response to pathogens. Given the evidence that ATG16L1 deficiency in macrophages was crucial for the observed enhanced clearing of UTI89 *in vivo*, we sought to elucidate the molecular mechanisms underlying how ATG16L1 deficiency altered the response of macrophages to UTI89. To address this question, we isolated bone marrow-derived macrophages (BMDMs) from WT and Atg16L1<sup>HM</sup> mice and challenged them with UTI89. As expected, BMDMs from Atg16L1<sup>HM</sup> mice displayed a 90% reduction of ATG16L1 protein (**Figure 1c**). Consistent with a reduced rate of autophagic flux, they also accumulated the adaptor protein p62 (**Figure 1d**).

To assess the direct effect of ATG16L1 deficiency on bacterial clearance, we determined intracellular bacterial load at multiple time points postinfection and found that Atg16L1<sup>HM</sup> BMDMs contained significantly more bacteria than WT BMDMs at 1 h post infection (h.p.i.) (Figure 2a). Likewise, immunofluorescence staining showed that more  $\mathrm{Atg16L1}^{\mathrm{HM}}$  BMDMs contained intracellular bacteria than WT macrophages (Figure 2b; Supplementary Figure 3a). To determine whether ATG16L1 deficiency affects degradation of intracellular bacteria, we quantified the intracellular bacterial load in BMDMs over time as compared with levels at 2 h.p.i. We observed a similar pattern of decrease in bacterial counts between WT and ATG16L1deficient macrophages, arguing that the dynamics of bacterial killing over time were not altered by ATG16L1 deficiency (Figure 2c). Together, these findings indicate that ATG16L1 deficiency enhances killing of UTI89 by increasing bacterial uptake at early stages of infection.

To examine whether ATG16L1 deficiency affected intracellular trafficking of UTI89, we performed immunofluorescence and observed co-localization of UPEC with the lysosomal marker LAMP1 in both WT and ATG16L1-deficient macrophages (Supplementary Figure 3b). To further evaluate the intracellular localization of UTI89 within macrophages, we used transmission electron microscopy (TEM). At 1 and 3 h.p.i, consistent with the immunofluorescence results, UTI89 was consistently found in single-membrane compartments in both WT and ATG16L1-deficient macrophages (Figure 2d). We counted the numbers of bacteria in electron-lucent or highly electron-dense compartments. At 1 h.p.i, the majority of bacteria appeared to be in highly electron-dense lysosomal compartments in both WT and ATG16L1-deficient macrophages (Figure 2e). However, by 3 h.p.i, whereas fewer bacteria were within highly electron-dense compartments in WT macrophages, the majority of bacteria still remained in these compartments in ATG16L1-deficient macrophages (Figure 2e). This suggests that UTI89 alters its phagocytic compartment in macrophages over time but does so more slowly in ATG16L1deficient cells. Thus, ATG16L1-deficient macrophages may sense and respond to intracellular UTI89 differently than WT macrophages do.

To determine whether the increased uptake by ATG16L1deficient macrophages was specific to a UPEC strain or would be elicited by any intracellular E. coli, we challenged the macrophages with a commensal *E. coli* (MG1655).<sup>25</sup> At 1 h.p.i, macrophages of both genotypes took up the commensal bacteria more efficiently than UTI89 (compare Figure 3a with Figure 2a). However, no significant differences in uptake (Figure 3a) or survival rates of MG1655 (Figure 3b) were observed between WT and ATG16L1-deficient macrophages. TEM analysis revealed that, at 1 and 3 h.p.i, MG1655 was in single-membrane compartments, the majority of which were not highly electron dense (Figure 3c). Moreover, the distribution of types of compartments containing MG1655 did not differ between WT and ATG16L1-deficient macrophages (Figure 3d). We conclude that loss of ATG16L1 affects intracellular trafficking of UTI89 but not of a commensal strain of E. coli.



**Figure 2** ATG16L1-deficient macrophages take up more bacteria than wild-type (WT) macrophages. (a) Intracellular bacterial load of WT and ATG16L1-deficient macrophages at different times after UTI89 challenge. (b) Percent of macrophages that contained intracellular bacteria by immunofluorescence. (c) Intracellular bacterial survival displayed as percentage of intracellular colony forming unit (CFU) load at 2 h. (d) Transmission electron microscopy (TEM) images of UTI89 within singlemembrane vacuoles of different electron densities at different time points (scale bars = 500 nm), and (e) quantification of the distribution of vacuole types within which uropathogenic *E. coli* (UPEC) was found in WT and ATG16L1-deficient macrophages. \*\*P < 0.01, \*P < 0.05. Paired *t*-test (b) or two-way ANOVA with Bonferroni post-tests (a, c). Data are from three independent experiments, with three replicates per experiment (a, c) or four fields per experiment (b) presented as mean and s.e.m.



ATG16L1-deficient macrophages secrete more IL-1 $\beta$  in response to UPEC challenge

Given the important link between autophagy and inflammation, we reasoned that the cytokine response elicited by UTI89 may be altered in macrophages deficient in ATG16L1. To test this hypothesis, we challenged WT and ATG16L1-deficient macrophages with UTI89, MG1655, or lipopolysaccharide (LPS). In response to these stimuli, WT and ATG16L1deficient macrophages secreted similar amounts of TNF $\alpha$  at 24 h.p.i (**Figure 4a**). In contrast, IL-1 $\beta$  release was significantly higher in UPEC-infected ATG16L1-deficient macrophages compared with control macrophages at 24 h.p.i (**Figure 4b**) and this response could be detected as early as 8 h.p.i (**Figure 4c**). No IL-1 $\beta$  was detected in WT and ATG16L1-deficient macrophages at 2 h.p.i suggesting that increased IL-1 $\beta$  did



Figure 3 ATG16L1-deficient macrophages take up MG1655, an avirulent *E. coli* strain differently than the uropathogenic *E. coli* (UPEC) strain. (a) Intracellular bacterial load of wild-type (WT) and ATG16L1-deficient macrophages at 1 and 2 h after MG1655 challenge. (b) Intracellular bacterial survival displayed as percentage of intracellular colony forming unit (CFU) load at 2 h. (c) Transmission electron microscopy (TEM) images of MG1655 within single-membrane vesicles of different electron densities at different time points, and (d) quantification of the distribution of vesicle types within which MG1655 was found in WT and ATG16L1-deficient macrophages. Not significant by two-way ANOVA with Bonferroni post-tests (a, b). Data are from three independent experiments, with three replicates per experiment (a, b) presented as mean and s.e.m.

**Figure 4** ATG16L1-deficient macrophages produce more interleukin-1 $\beta$  (IL-1 $\beta$ ) than wild-type (WT) macrophages. Levels of (**a**) tumor necrosis factor alpha (TNF $\alpha$ ) and (**b**) IL-1 $\beta$  in the supernatants of WT and ATG16L1-deficient cells 24 h after challenge with UTI89, MG1655, or lipopolysaccharide (LPS). (**c**) Levels of IL-1 $\beta$  in the supernatant after 8 h of challenge with UTI89, MG1655, or LPS as determined by ELISA. \*\*\*P<0.001. Two-way ANOVA with Bonferroni post-tests. Data are from three (**a**, **c**) and four (**b**) independent experiments presented as mean and s.e.m.

### ARTICLES

not directly enhance UPEC uptake (**Supplementary Figure 4a**). Interestingly, IL-1 $\beta$  release only occurred following infection with the UPEC strains (UTI89 and CFT073,<sup>26</sup> a pyelonephritis strain, **Supplementary Figure 4b**), not in response to commensal *E. coli* or LPS (**Figures 4b** and **c**). Further, dose-dependent increases in IL-1 $\beta$  production were observed in WT macrophages treated with varying concentrations of 3-MA, an autophagy inhibitor that targets VPS34, suggesting that the increased IL-1 $\beta$  production by ATG16L1-deficient macrophages in response to UTI89 may be the result of their autophagy deficiency (**Supplementary Figure 5**).

# Increased IL-1 $\beta$ production by ATG16L1-deficient macrophages is independent of NOD2 activity and enhanced transcription of pro-IL-1 $\beta$

IL-1 $\beta$  release is tightly controlled through the combination of two distinct triggers. First, activation of Toll-like receptor (TLR) or Nod-like receptors (NLRs), such as by LPS, induces transcription of pro-IL-1 $\beta$ .<sup>27,28</sup> In a second step, pro-IL-1 $\beta$ protein is cleaved by caspase-1, a process that is regulated by inflammasomes, which are defined by their NLR component, which can include AIM2, NLRC4, NLRP1, and NLRP3.<sup>28</sup> Thus, we wanted to know whether ATG16L1 deficiency affected pro-IL-1 $\beta$  generation (signal 1) or assembly of the inflammasome complex (signal 2).

No significant differences were observed in level of pro-IL-1 $\beta$  mRNA (**Figure 5a**) or protein (**Figure 5b**) between ATG16L1deficient and WT macrophages challenged with UTI89, even though increased cleaved IL-1 $\beta$  was observed in the supernatants by western blot. Additionally, caspase-11 and NLRP3 protein levels increased upon infection, but there was no significant difference between WT and ATG16L1-deficient macrophages (**Supplementary Figure 6**). Furthermore, when we pretreated cells with LPS to activate transcription of pro-IL-1 $\beta$  before activation by UTI89 ATG16L1-deficient macrophages still produced more IL-1 $\beta$  in response to UTI89 than WT cells did (**Figure 5c**). These findings argue enhanced IL-1 $\beta$ release in ATG16L1-deficient macrophages does not occur via modulation of signal 1.

Recent studies have suggested that ATG16L1 is important for controlling secretion of IL-1 $\beta$  and TNF $\alpha$  in response to agonists of NOD2, an NLR that recognizes cytosolic bacterial components.<sup>17,29,30</sup> We thus asked whether NOD2 was required for the increased IL-1 $\beta$  secretion of UTI89-challenged ATG16L1-deficient macrophages. We found that macrophages from Nod2<sup>-/-</sup> and WT mice produced equivalent levels of IL-1 $\beta$  (**Figure 5d**). Likewise, upon challenge with UTI89, macrophages from Nod2<sup>-/-</sup>; Atg16L1<sup>HM</sup> mice produced similar amounts of IL-1 $\beta$  as macrophages from Atg16L1<sup>HM</sup> mice (**Figure 5d**), suggesting that NOD2 does not contribute to ATG16L1 deficiency-mediated increased secretion of IL-1 $\beta$ upon UPEC infection.

### UTI89 activates NLRP3 inflammasomes in macrophages

As our findings suggested that ATG16L1 deficiency likely impacts signal 2, we sought to define that mechanism by which



**Figure 5** Increased interleukin-1 $\beta$  (IL-1 $\beta$ ) secretion by ATG16L1-deficient macrophages is not due to enhanced signal 1 or NOD2. (a) Levels of pro-IL-1 $\beta$  mRNA determined by quantitative PCR from macrophages at baseline or after 3 h of challenge with UTI89 or MG1655. (b) Representative western blot of cleaved IL-1 $\beta$  in the supernatant at 24 h and pro-IL-1 $\beta$  and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels at 3 h from macrophages treated with phosphate-buffered saline (PBS) or challenged with UTI89 or MG1655. (c) IL-1 $\beta$  level in the 24-h supernatant of bone macrow-derived macrophages (BMDMs) pretreated with lipopolysaccharide (LPS) or PBS for 4 h before challenge with UTI89 or MG1655. (d) Levels of IL-1 $\beta$  in the supernatants of BMDMs from wild-type (WT), ATG16L1<sup>HM</sup>, Nod2<sup>-/-</sup>, and Nod2<sup>-/-</sup>; Atg16L1<sup>HM</sup> mice 24 h after challenge with UTI89. \*\*\**P*<0.001. Paired *i*-test (c) or two-way ANOVA with Bonferroni post-tests (a, d). Data are from two (c) or three (a, d) independent experiments with three replicates per experiment presented as mean and s.e.m.



**Figure 6** TLR4 and NLRP3 activation by uropathogenic *E. coli* (UPEC) is important for interleukin-1 $\beta$  (IL-1 $\beta$ ) production. (**a**) Amount of IL-1 $\beta$  secreted by wild-type (WT) and Tlr4<sup>-/-</sup> macrophages in response to UTI89 and MG1655. (**b**) Amount of IL-1 $\beta$  secreted by WT, caspase1/11 double knockouts, or caspse11<sup>-/-</sup> macrophages in response to UTI89 and MG1655. (**c**) Levels of NIrp3 and NIrc4 mRNA (determined by quantitative PCR) in macrophages after challenge with UTI89 or MG1655 for 3 h, presented relative to the 0-h time point. (**d**) Secreted IL-1 $\beta$  detected by ELISA from macrophages from WT, NIrp3<sup>-/-</sup>, NIrc4<sup>-/-</sup>, and Aim2<sup>-/-</sup> mice in response to UTI89, MG1655, and lipopolysaccharide (LPS) challenge. (**e**) Measurement of cytotoxicity as lactate dehydrogenase (LDH) release by bone marrow-derived macrophage (BMDM) exposed to UTI89 for 3 or 24 h (2h exposure to extracellular bacteria followed by incubation for 22 h in gentamicin) as a percentage of total LDH release upon Triton-X treatment. \**P*<0.05, \*\*\**P*<0.001. Two-way ANOVA and Bonferroni post-test for each pair. Data are from two (**a**, **b**, and **d**) or three (**c** and **e**) independent experiments with three individual replicates per experiment. TLR, Toll-like receptor.

UTI89 activates the inflammasome. Importantly, inflammasome activation in macrophages in response to UPEC had not been previously examined. Thus, we first confirmed that UTI89-induced IL-1ß production was dependent on TLR4 activation; as expected, BMDMs from TLR4<sup>-/-</sup> mice produced significantly decreased levels of IL-1 $\beta$  (Figure 6a). Next, we determined whether both caspase-1 and caspase-11 were essential for IL-1 $\beta$  secretion in response to UTI89. BMDMs isolated from caspase-1/caspase-11 double knockouts had minimal secretion of IL-1 $\beta$  in response to UTI89 and MG1655, but caspase-11-deficient mice had no defect in IL-1β secretion in response to UTI89 (Figure 6b). This suggests that UTI89 activates the canonical inflammasome rather than the noncanonical caspase-11 activated inflammasome.<sup>31</sup> To further define the key inflammasome proteins activated upon UPEC challenge, we performed q-RTPCR analysis of genes encoding two inflammasome proteins that sense and respond to bacteria (NLRC4 and NLRP3)<sup>27,32,33</sup> and found that whereas Nlrc4 transcription was minimally induced, Nlrp3 transcription was highly upregulated in response to UTI89 infection of both WT and ATG16L1-deficient macrophages (Figure 6c). We then examined IL-1 $\beta$  production by macrophages from mice lacking the inflammasome proteins NLRP3, NLRC4, or AIM2. Macrophages from  $Nlrp3^{-/-}$  mice secreted significantly less IL-1 $\beta$  than WT macrophages (Figure 6d). In contrast,

macrophages from Nlrc4<sup>-/-</sup> and Aim2<sup>-/-</sup> mice produced roughly the same amount of IL-1 $\beta$  as WT macrophages. Together, these findings demonstrate that most IL-1 $\beta$  secretion in response to UTI89 requires TLR4 and NLRP3 inflammasome activation.

In response to infection, macrophages can kill intracellular pathogens by undergoing a form of cell death called pyroptosis, which is associated with IL-1 $\beta$  release and caspase-1 activation.<sup>34</sup> It is not known whether UPEC induces pyroptosis in macrophages. Thus, we investigated the effect of ATG16L1 deficiency on pyroptosis in response to UTI89 infection by using the CytoTox 96 assay, which measures the levels of release of the cytoplasmic enzyme lactate dehydrogenase into the supernatant. We detected no difference in total cell death between WT and ATG16L1-deficient macrophages challenged with UTI89 or MG1655 (**Figure 6e**), suggesting that pyroptosis is not a mechanism of fast clearance of UTI89 in Atg16L1<sup>HM</sup> mice.

## ATG16L1 deficiency enhances IL-1β secretion in response to UTI89 and other NLRP3 activators due to increased caspase-1 activation

Given that pro-IL-1 $\beta$  levels were similar between WT and ATG16L1-deficient macrophages, we hypothesized that the increased IL-1 $\beta$  secretion by ATG16L1-deficient macrophages

was due to increased activation of the NLRP3 inflammasome and caspase-1. To determine whether ATG16L1 deficiency enhanced NLRP3 activation, we treated WT and ATG16L1deficient macrophages with four known NLRP3 activators: Streptolysin-O, ATP, Alum, and Silica.35,36 Each caused secretion of IL-1 $\beta$  by both types of macrophages, but in each case, ATG16L1-deficient macrophages produced more IL-1ß than WT macrophages did (Figure 7a). Given that lysosome damage can trigger NLRP3 activation,<sup>28,37,38</sup> in conjunction with our data indicating that UPEC traffics to lysosomes in macrophages, we reasoned that UPEC might activate the NLRP3 inflammasome by impairing lysosome integrity and inducing release of cathepsin B and other vacuolar contents into the cytosol. To test this, we treated UTI89 infected WT and ATG16L1-deficient macrophages with the cathepsin B inhibitor CA-074Me.<sup>38</sup> As expected, CA-074Me decreased the secretion of IL-1 $\beta$  in response to Alum, a known inducer of lysosome damage.  $^{36}$  In contrast, the release of IL-1  $\beta$  in response to UPEC was unaffected by cathepsin B inhibition (Figure 7b).

Thus, inflammasome activation by UTI89 occurs without major lysosomal injury and cathepsin B release. This is consistent with our TEM observation that UTI89 was found within intact vacuoles in both WT and ATG16L1-deficient macrophages. These findings suggest that activation of the NLRP3 inflammasome occurs independently of gross lysosomal damage by UTI89.

We then determined the effect of ATG16L1 deficiency on caspase-1 activation. The levels of pro-caspase-1 in uninfected ATG16L1-deficient macrophages were higher than those observed in WT macrophages (**Figures 7c** and **d**). Upon challenge with UPEC, ATG16L1-deficient macrophages released more caspase-1 into the supernatant than WT macrophages, consistent with enhanced activation of this protease (**Figure 7e; Supplementary Figure 6**). To determine whether the difference observed in IL-1 $\beta$  secretion by WT and ATG16L1-deficient macrophages was due to changes in caspase-1 activity, we applied a caspase-1 inhibitor during infection. Caspase-1 inhibition, using 10  $\mu$ M Z-WEHD-FMK,



**Figure 7** ATG16L1-deficient macrophages produce more interleukin-1 $\beta$  (IL-1 $\beta$ ) due to increased NLRP3 and caspase-1 activity. (**a**) Levels of IL-1 $\beta$  in the supernatants of wild-type (WT) and ATG16L1-deficient macrophages after pretreatment with lipopolysaccharide (LPS) followed by exposure to Streptolysin O (with 10 mm DTT), Alum, Silica, or ATP. (**b**) Levels of IL-1 $\beta$  in the supernatants of WT and ATG16L1-deficient macrophages challenged with UTI89 for 24 h or the NLRP3 activator Alum for 8 h with or without the Cathepsin B inhibitor CA-074Me. (**c**) Representative western blot of pro-caspase-1 protein levels normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) values from macrophages at baseline and after 3 h of UTI89 challenge, quantification (**d**) displayed normalized to baseline WT levels. AU, arbitrary units. (**e**) Level of caspase-1 in the supernatants of macrophages challenged with UTI89 for 24 h. (**f**) Level of IL-1 $\beta$  in the supernatants of macrophages challenged with UTI89 for 24 h with or without a caspase-1 linhibitor during the entire experiment. (**g**) Level of IL-1 $\beta$  in the supernatants of macrophages challenged with UTI89 for 24 h with increasing concentrations of glyburide, an NLRP3 inhibitor. \**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.001. Paired *t*-test (**a**) or two-way ANOVA and Bonferroni post-test at for each condition (**b**, **d**–**g**). Data are from three independent experiments with three replicates per experiment (**d**) presented as mean with s.e.m.

eliminated the difference in secretion of IL-1 $\beta$  between WT and ATG16L1-deficient BMDMs (**Figure 7f**). Additionally, inhibiting NLRP3 with glyburide was able to inhibit IL-1 $\beta$  production in both WT and ATG16L1-deficient BMDMs in a dose-dependent manner (**Figure 7g**). We conclude that NLRP3 and caspase-1 activation is enhanced by ATG16L1 deficiency, and this pathway mediates the enhanced release of IL-1 $\beta$  by ATG16L1-deficient macrophages in response to UTI89 challenge.

### Blocking IL-1 signaling in Atg16L1<sup>HM</sup> mice reduces clearance of UPEC during a UTI

Our data suggest that Atg16L1<sup>HM</sup> mice are better able to clear a UPEC infection than WT mice for two reasons. First, as previously shown, more macrophages are recruited to the bladder mucosa.<sup>21</sup> Second, ATG16L1-deficient macrophages produce more IL-1 $\beta$  *ex vivo* in response to UPEC challenge due to increased caspase-1 activation (**Figure 4b**). In agreement with our mechanistic *ex vivo* studies, we found significantly more IL-1 $\beta$  in urines from Atg16L1<sup>HM</sup> mice than in urines from WT mice at 6 h.p.i (**Figure 8a**). To determine the physiological importance of IL-1 $\beta$  *in vivo*, we treated both WT and Atg16L1<sup>HM</sup> mice with Anakinra (a recombinant form of the endogenous IL-1 receptor antagonist, IL-1Ra, which blocks both IL-1 $\alpha$  and IL-1 $\beta$  signaling through IL-1 receptor (IL-1R)) to inhibit IL-1 signaling during infection.<sup>39</sup> Anakinra reduced bacterial clearance in Atg16L1<sup>HM</sup>

mice compared with vehicle-treated controls at 1dpi, whereas bacteriuric titers were not significantly affected in WT mice given vehicle (phosphate-buffered saline, PBS) or Anakinra at 1 dpi (**Figure 8b**). In fact, the infection phenotype of Anakinra-treated Atg16L1<sup>HM</sup> mice was similar to WT animals. In addition, Anakinra-treated Atg16L1<sup>HM</sup> mice had a significantly worse course of infection than vehicle-treated Atg16L1<sup>HM</sup> mice (**Figure 8c**, right), whereas Anakinra treatment did not significantly change the course of infection in WT animals (**Figure 8c**, left). Thus, our data argue that in the context of WT macrophages, subverting IL-1 $\beta$  production is not a part of the UPEC arsenal of host defense evasion. However, our results demonstrate that the augmented IL-1 $\beta$  release from macrophages in Atg16L1<sup>HM</sup> mice is the primary driver of enhanced bacterial clearance in those mice.

### DISCUSSION

Autophagy and its proteins are important for combating intracellular pathogens and have generally been considered to be anti-pathogenic.<sup>11</sup> However, emerging evidence suggests that autophagy proteins can also be pro-pathogenic.<sup>21,40,41</sup> In previous work, we found that ATG16L1-deficient mice display reduced levels of bacteriuria and bladder titers early in infection.<sup>21</sup> Our work suggested that hematopoieitic cells were important for ATG16L1 deficiency-mediated protection, but did not reveal the mechanism by which this occurred.



**Figure 8** Interleukin-1 $\beta$  (IL-1 $\beta$ ) production is enhanced in Atg16L1<sup>HM</sup> mice and is important for faster clearance of uropathogenic *E. coli* (UPEC). (a) Urine levels of IL-1 $\beta$  at baseline and 6 h post infection (h.p.i.) as measured by ELISA (b) colony forming unit (CFU) counts of day 1 bacteriuria from wild-type (WT) and Atg16L1<sup>HM</sup> mice given intraperitoneal (IP) phosphate-buffered saline (PBS) or Anakinra. (c) Bacteriuria over time from WT (left) or Atg16L1<sup>HM</sup> (right) mice treated with Anakinra or PBS displayed as mean Log<sup>10</sup>CFU per ml. \**P*<0.05, Two-way ANOVA with matching by animal and Bonferroni post-test at each time point (**a**, **c**) and Mann–Whitney test (**b**). Data are from (**a**) 2 independent experiments with a total of 7 WT and 6 Atg16L1<sup>HM</sup> mice examined, and from (**b**, **c**) four independent experiments with 13 PBS treated Atg16L1<sup>HM</sup> mice, 11 Anakinra-treated Atg16L1<sup>HM</sup> mice, 8 PBS-treated WT mice, and 7 Anakinra-treated WT mice, all are presented as mean with s.e.m.

In this report, we showed that ATG16L1 deficiency-mediated protection from UTIs is dependent on the macrophage/monocyte lineage *in vivo*. ATG16L1 deficiency in the macrophage/monocyte lineage promoted a more robust IL-1 $\beta$  response to UPEC challenge, and blockade of IL-1 signaling *in vivo* abolished the protective phenotype mediated by ATG16L1 deficiency. Additionally, our findings show for the first time that the NLRP3 inflammasome is the principal inflammasome activated by UPEC during a UTI, with minimal IL-1 $\beta$  production as a result of activation of other inflammasomes including, NLRC4 or AIM2. Furthermore, our work has revealed that the IL-1 $\beta$  response mediated by NLRP3 was dependent on caspase-1 activation. Altogether, our results indicate that loss of ATG16L1 promotes a beneficial innate immune response during a UTI.

UTIs are not the only type of infection in which loss of ATG16L1 can be beneficial. Deficiency of this gene was recently shown to provide significant protection from Citrobacter rodentium infection of the intestine.<sup>40</sup> Moreover, ATG16L1deficient macrophages and monocytes were found to be essential for the protection against both infections in these distinct mucosae. However, the underlying mechanisms differ in two ways. First, whereas an ATG16L1-deficient hematopoietic compartment is the main driver for the protective phenotype in UTI,<sup>21</sup> deficiency in the non-hematopoietic compartment also contributes to reduced Citrobacter titers and increased survival.<sup>40</sup> Second, although NOD2 has an essential role in ATG16L1 deficiency-induced resistance to Citrobacter, we found that NOD2 was dispensable for ATG16L1 deficiencyinduced resistance to UPEC42 and now show that NOD2 does not affect ATG16L1 deficiency-induced enhanced IL-1ß secretion by BMDMs in response to UPEC. These differences suggest that ATG16L1 has distinct roles in the pathogenesis of different infections.

Nonetheless, these studies indicate that ATG16L1 deficiency may be protective against a broader range of pathogens, particularly those that are not obligate intracellular pathogens and do not thrive within macrophages. For such pathogens, alterations in immune signaling and immune cell recruitment may outweigh any potential defects in controlling intracellular bacteria. Additionally, because UPEC do not thrive within macrophages and are primarily degraded,<sup>43</sup> we suggest that increased uptake of UPEC or similar pathogens by ATG16L1deficient macrophages could itself be protective to the host. In contrast, increased uptake of intracellular pathogens that do thrive within macrophages could be detrimental.

IL-1 $\beta$  is a pro-inflammatory cytokine that has a key role in the host response to infection and injury. This cytokine is tightly controlled at the transcriptional and post-translational level by two distinct signals, which prevent inappropriate activation and release.<sup>44</sup> An additional level of control exists through production of an endogenous IL-1Ra that competes with IL-1 $\beta$  for binding to the IL-1R. Our work adds to evidence that ATG16L1 and autophagy can modulate the production and release of active IL-1 $\beta$ . Saitoh *et al.*<sup>15</sup> were the first to show this connection when they discovered that both ATG16L 1-deficient fetal liver-derived macrophages and peritoneal macrophages in which basal autophagy was inhibited produced more IL-1ß in response to LPS and commensal bacteria but not Salmonella. Later, Harris et al.<sup>16</sup> showed that autophagy induction promotes the degradation of pro-IL-1ß in response to LPS. Most recently, Lee et al.45 demonstrated that ATG16L1 regulates IL-1ß signaling by controlling p62 levels by autophagosomal and proteosomal degradation. Our results suggest that increases in IL-1 $\beta$  in response to UPEC in our ATG16L1deficient macrophages are primarily a result of altered caspase-1 activation rather than upregulation of transcription as was reported in studies of the T300A variant of ATG16L1.46 Interestingly, in contrast to what was observed previously,<sup>15</sup> we found that LPS stimulation of Atg16L1<sup>HM</sup> macrophages was not sufficient to induce robust IL-1ß secretion; this may be related to the degree of ATG16L1 deficiency. Nevertheless, our work provides the first evidence that, instead of harming the host, increased IL-1 $\beta$  secretion due to ATG16L1 deficiency can lead to a more effective response to infection.

The finding that elevated IL-1 signaling was important for the ability of Atg16L1<sup>HM</sup> mice to control UPEC suggests that ATG16L1 has a role in dampening the bladder mucosal production of IL-1 $\beta$  in response to UPEC infection. Such dampening may be helpful in preventing excessive inflammation in mucosae that cannot resolve the inflammation. For example, elevated levels of IL-1ß due to ATG16L1 deficiency are detrimental in the context of chemically induced colitis.<sup>15</sup> Inhibition of autophagy in cells infected with Borrelia burgdorferi has been shown to lead to increased IL-1β, which is associated with worsened disease progression.<sup>47</sup> By contrast, increased macrophage-driven inflammation may be helpful in the bladder mucosa,<sup>48</sup> which is not permeable or absorptivelike the gut mucosa and is usually sterile. Thus, the normal balance maintained by ATG16L1 may prevent infections by obligate intracellular pathogens in most cells but may lead to insufficient responses to other pathogens, such as UPEC. Furthermore, the enhanced secretion of IL-1 $\beta$  due to loss of ATG16L1 appears to uncover a vulnerability in the ability of UPEC to evade early innate immune defenses and colonize and persist in the urinary tract. Enhanced secretion of IL-1 $\beta$  is protective in the Atg16L1<sup>HM</sup> mice with inhibition of IL-1 signaling leading to bacteriuria levels similar to those of the WT mice at 1 dpi, yet inhibition of IL-1 signaling in WT mice did not make the infection worse, suggesting that the level and timing of IL-1 $\beta$  secretion may be important. Given the high numbers of UTIs and the increasing antibiotic resistance among UPEC isolates, the potential of this new knowledge to contribute to development of new treatment regimens to elicit an effective immune response to UPEC could be vital.

Our data and that of others suggest that the hyperinflammatory responses observed by ATG16L1 deficiency<sup>17,18</sup> can be damaging or protective in different mucosal environments in response to different stimuli.<sup>15,49</sup> This may explain why the T300A polymorphism in *ATG16L1* is highly prevalent in the Caucasian population<sup>12</sup> even though it increases susceptibility to Crohn's disease.<sup>12,13</sup> Recent studies have shown that the

proteins produced by the T300A (human) or T316A (mouse) variants of ATG16L1 are more easily degraded by caspase-3, resulting in reduced expression of ATG16L1.49,50 ATG16L1 deficiency in the gastrointestinal tract is thought to cause intestinal dysbiosis because of an aberrant pro-inflammatory response to commensal gut flora.<sup>19,20,51</sup> Our work suggests that aberrant pro-inflammatory responses to commensals in the gut may occur as a trade-off for productive responses to pathogens in other tissues. The improved function of macrophages and increased production of IL-1 $\beta$  in response to UPEC by the hypomorphic ATG16L1 suggests that humans with the T300A variant may be more protected against pathogens such as UPEC where increased IL-1 $\beta$  would help clear the infection. Future genomic studies to investigate the possibility of associations between the T300A mutation and common infections such as UTI will help to reveal the selective pressure on maintenance of polymorphisms in ATG16L1 in the human population.

### METHODS

Mice, treatments, and infections. All protocols were approved by the animal studies committee of the Washington University School of Medicine (Animal Welfare Assurance #A-3381-01). Mice were maintained in a barrier facility under pathogen-free conditions and a strict 12-h light/dark cycle. Atg16L1^{HM} and WT mice on a C57BL/6 background were used at 7–9 weeks of age for infection (females) or to obtain BMDMs (males). Atg16L1<sup>HM</sup> mice were given two intraperitoneal injections of 100 mg kg<sup>-1</sup> of clodronate-containing liposomes (clodrolip) or the equivalent amount of control liposomes: 2 days and again 30 minutes before infection. Mice were given two intraperitoneal injections of 1 mg of Anakinra (Kineret, Swedish Orphan Biovitrum, Stockholm, Sweden) diluted in Dulbecco's phosphate-buffered saline (DPBS): 16 h and again 30 min before infection. For infections, the UPEC strain UTI89 was grown statically for 17 h in Luria-Bertani broth at 37 C; anesthetized mice were transurethrally inoculated with  $10^7$  colony forming units (CFUs) of UTI89 in 50 µl of PBS as previously described.<sup>52</sup> Urines from infected mice were collected at multiple time points, serially diluted in PBS, and plated on LB plates.<sup>52</sup> Three days post infection, bladders were aseptically removed, homogenized in 0.1% Triton-X, serially diluted in PBS, and plated on LB plates.<sup>21</sup>

**Flow-cytometry analysis**. Single-cell suspensions from spleens were stained with the following antibodies (eBioscience, San Diego, CA) or fluorescent dyes at 1:100 dilutions unless stated: anti-mouse GR1 FITC, anti-mouse F4/80 PE, anti-mouse CD11b APC, anti-mouse CD45 Pacific Blue, and 7AAD (1:500, AnaSpec, Fremont, CA). Cells were fixed with 1% paraformaldehyde (PFA) for 20 min with Actino-mycin (ACROS Organics, Geel, Belgium). Stained cells were analyzed on a BD FACSCantoII using BD FACS DIVA software (BD Bioscience, San Jose, CA).

**Isolation, differentiation, and challenge of BMDMs.** Bone marrow was aseptically isolated from Atg16L1<sup>HM</sup>, Tlr4<sup>-/-</sup>, Nlrp3<sup>-/-</sup>, Nlrc4<sup>-/-</sup>, Aim2<sup>-/-</sup>, Nod2<sup>-/-</sup>, Nod2<sup>-/-</sup>; Atg16L1<sup>HM</sup>, 12986 (caspase-11<sup>-/-</sup>), Caspase-1/Caspase-11 double knockout, and WT mice and differentiated on non-tissue-culture-treated petri dishes for 7 days at 37 °C with 5% ambient CO<sub>2</sub> in DMEM with 15% FBS, 30% L929 conditioned media, 1% Glutamax, 1% Na-Pyruvate, and 1% Penicillin and Streptomycin. Then on day 7 post isolation, non-attached cells were aspirated, and the media was changed to DMEM with 10% FBS, 1% Glutamax, and 1% Na-Pyruvate. On day 8, cells were incubated with ice-cold DPBS, removed with a cell scraper, counted, and plated at  $5 \times 10^5$  cells per ml. On day 9, BMDMs were challenged with UTI89 or

**Intracellular bacteria quantification**. Intracellular CFU were determined by serially diluting and plating cell lysates from BMDMs that had been challenged with bacteria for 1 or 2 h and then treated with 100  $\mu$ g ml<sup>-1</sup> gentamicin for 15 min. For bacterial survival time course, BMDMs were challenged with UPEC for 2 h, treated with 100  $\mu$ g ml<sup>-1</sup> gentamicin for 1 h, and then 10  $\mu$ g ml<sup>-1</sup> gentamicin for the remaining time stated. For imaging analysis, cells were stained with rabbit anti-*E. coli* (1:500, US Biologicals, Swampscott, MA)<sup>21</sup> and rat anti-LAMP1 (1:50, clone ID4B; Developmental Studies Hybridoma Bank, Iowa City, IA),<sup>21</sup> and antigen–antibody complexes were detected with anti-rabbit Alexa Fluor-488-conjugated secondary antibody and anti-rat Alexa-594-conjugated secondary antibody (both at 1:500, Invitrogen; Carlsbad, CA). Images were obtained with a Zeiss Apotome microscope (Zeiss, Jena, Germany).

MG1655 (ref. 25) grown as described above for mouse infections at an

ELISAS. BMDMs were incubated with UTI89, CFT073, or MG1655 for 2 h, then treated with  $100 \,\mu g \,m l^{-1}$  gentamicin containing medium for 1 h and finally incubated with  $10 \,\mu g \,ml^{-1}$  gentamicin containing medium for 5 h (8-h samples) or 21 h (24-h samples) at which point the supernatant was collected for ELISAs. IL-1ß DuoSet ELISA kit (R&D Systems, Minneapolis, MN), TNFa DuoSet ELISA kit (R&D Systems), and Caspase-1 (mouse) matched pair detection set (Adipogen, San Diego, CA) were used according to the manufacturer's instructions. Where indicated, the following inhibitors were used throughout the experiment: the Cathepsin B inhibitor CA-074 Me (Enzo Life Sciences, Farmingdale, NY) was used at 10 µM, the caspase-1 inhibitor Z-WEHD-FMK (R&D Systems) was used at 10 µm, and the VPS34 inhibitor 3-MA (Calbiochem, Millipore, Billerica, MA) was used at 2, 5, and 10 µm. Alum- and Silica-treated samples were pretreated with 100 ng ml<sup>-1</sup> LPS(*E. coli* strain 055:B5, Sigma, St. Louis, MO) for 2 h, and then treated with 200  $\mu$ g ml<sup>-1</sup> of Inject Alum (Thermo Scientific, Rockford, IL) or 100  $\mu$ g ml<sup>-1</sup> of Silica (Thermo Scientific) in fresh media for 6 h. Streptolysin O (SLO) (Sigma) was used at 10  $\mu$ g ml<sup>-1</sup> with 10 mM DTT for 5 min, and then the medium was changed and collected 6 h later.

**qPCR**. TRIzol (Invitrogen) was used to isolate RNA from  $4 \times 10^6$  BMDMs that had been challenged with bacteria or LPS for 2 h and then treated with gentamicin for 1 h. RNA was treated with DNase1 (Ambion, Austin, TX), and Superscript II RNase H reverse transcriptase (Invitrogen) was used to synthesize cDNA from 1 µg of total RNA. Gene expression was determined by calculating  $\Delta\Delta$  CT with values normalized to 36B4 RNA levels and then to values from PBS-treated samples.

**Western blots.** Protein was isolated from cells as described.<sup>21</sup> Briefly, cell lysates were electrophoresed on 4–20% Pierce Precise Protein Gels (Thermo Scientific) and transferred onto nitrocellulose membranes. Membranes were probed with the following primary antibodies: rabbit-anti-ATG16L1 (1:500, Sigma) guineapiganti-p62 (1:1,000, Progen Biotechnik, Heidelberg, Germany), rabbit anti-Caspase-1 (1:5,000, Dr Gabriel Nuñez), rat anti-Caspase-11 (1:250, Sigma), rat anti-NLRP3 (1:250, R&D Systems), and rabbitanti-GAPDH (1:1,000, Cell Signaling Technology, Danvers, MA). Goat-anti-rabbit IgG-HRP, goat-anti-guinea pig IgG-HRP, and goat-anti-rat IgG-HRP secondaries (Santa Cruz Biotechnology, Dallas, TX) were used and detected by SuperSignal West Dura (Thermo Scientific).

**Statistical analyses**. For time-course experiments, two-way ANO-VAs with matching were used with Bonferroni post-tests for individual time points. To determine significance between two samples, unpaired t-tests, paired *t*-tests, or non-parametric Mann–Whitney *U* tests were performed by using the Graph Prism software (GraphPad Software, Inc., San Diego, CA). A value of P < 0.05 was used as the cutoff for statistical significance.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/mi

### ACKNOWLEDGMENTS

We thank Dr Herbert 'Skip' Virgin for Atg16L1<sup>HM</sup> mice, Drs Deborah J Frank, Jason C Mills, and Ken Cadwell for comments, Kassandra Weber for technical support, Dr Kyle Bauckman for assistance with western blotting, Dr Robyn Klein for the Caspase1/11 double-knockout mice, and Dr Wandy Beatty, Director of Imaging Facility, Department of Molecular Microbiology, for TEM assistance. This work was funded in part by R01Al063331 and R01DK091191 (to GN), NIH KO8 HL09837305 (to JDS), T32 Al 007172-35 (to NOB), K99/R00 DK080643; P30 DK052574; and R01 DK100644 (to IUM).

### AUTHOR CONTRIBUTIONS

J.W.S., C.W., and I.U.M. designed the research; J.W.S. did the research; J.T. did the T.E.M. enumeration; N.O.B. did the immunostaining experiments; R.S. provided the clodronate-containing liposomes; G.N. provided NIrp3, Aim2, and NIrc4 knockout mouse bones; J.D.S. provided technical support and conceptual advice; J.W.S. and I.U.M. analyzed data; and J.W.S., C.W., and I.U.M. wrote the manuscript.

#### DISCLOSURE

The authors declared no conflict of interest.

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