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The *Drosophila* Deubiquitinating Enzyme dUSP36 Acts in the Hemocytes for Tolerance to *Listeria monocytogenes* Infections

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Key Words
Autophagy · Deubiquitinating enzymes · Intracellular pathogens · Ubiquitin-specific protease · USP36

Abstract
Listeria monocytogenes is a facultative intracellular pathogen which can infect *Drosophila melanogaster*. Upon infection, *Drosophila* mounts an immune response including antimicrobial peptide production and autophagy activation. A set of previously published results prompted us to study the role of the deubiquitinating enzyme dUSP36 in response to *L. monocytogenes* infections. We show in this report that flies with *dUsp36*-specific inactivation in hemocytes are susceptible to *L. monocytogenes* infections (as are flies with autophagy-deficient hemocytes) but are still able to control bacterial growth. Interestingly, flies with *dUsp36*-depleted hemocytes are not sensitized to infection by other pathogens. We conclude that *dUsp36* plays a major role in hemocytes for tolerance to *L. monocytogenes*.

Introduction

Listeria monocytogenes, a Gram-positive, facultative intracellular bacterium, is responsible for severe foodborne infections that primarily affect immunocompromised individuals and pregnant women [1]. It has been widely used as a model pathogen to study the molecular and cellular aspects of intracellular pathogenesis. *L. monocytogenes* is taken up by the host cell through pathogen-induced endocytosis and enclosed in a vacuole, from which the bacteria can evade by expressing a set of toxins [2, 3]. Following escape from the vacuole, the bacteria grow and divide in the host cytosol [4, 5]. Using host actin to form comet-like tails, *L. monocytogenes* propels itself through the cytosol and into neighboring cells [6, 7].

The fruit fly *Drosophila melanogaster* has been established as a model host for *L. monocytogenes* infections [8]. The immune system of *Drosophila* relies on two conserved nuclear factor-κB-like signaling pathways, *Toll* and *imd (immune deficiency)*, which are induced upon infection [9, 10]. The *Toll* pathway is activated by Lys-type peptidoglycans and results in the activation of a set of specific antimicrobial peptide genes [11–13] whereas the *imd* pathway is activated by diaminopimelic acid (DAP)-containing-peptidoglycans and results in the activation of another set of antibacterial peptide genes [14–17]. Both *Toll* and *imd* pathways are required for *Drosophila* survival to *L. monocytogenes* infections [8]. Moreover, *Drosophila* survival after *L. monocytogenes* infections also relies on autophagy [18].

Autophagy is the major lysosomal degradation pathway in cells. It is a highly conserved cellular mechanism in which cytoplasmic components are sequestered into

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double-membrane structures called autophagosomes and are eventually degraded in lysosomes [19]. Autophagy is involved in diverse functions, including the removal of damaged organelles, protein turnover, supply of nutrients in nutrient-deprived conditions, and cell survival and death. In addition to *L. monocytogenes,* autophagy is also involved in innate immune defenses against other invading pathogens such as group A streptococci, *Shigella flexneri*, *Mycobacterium tuberculosis* and *Toxoplasma gondii*, as shown in human cells cultures [20].

Besides these resistance mechanisms (responsible for the control of pathogen growth), tolerance plays also a major role in the survival of infected organisms. Tolerance is defined as the set of physiological mechanisms that keep organisms healthy during infections or that help enduring infections [21, 22]. Although most of the work on *Drosophila* immunity has been focused on resistance mechanisms, a number of studies now points out the role of tolerance in particular during *L. monocytogenes* infections [23, 24].

We have previously demonstrated that the dUSP36 (syn. *Scrawny* or *Emperor's thumb*) deubiquitinating enzyme acts as a negative regulator of the *imd* pathway by deubiquitinating the IMD protein [25]. We have also shown that *dUsp36* controls cell growth and selective autophagy activation by ubiquitinated proteins [26]. Interestingly, a genome-wide RNAi-based screen conducted to identify host processes that contribute to *L. monocytogenes* pathogenesis identified *dUsp36* (referred to as *CG5505*) as part of a group of genes whose knockdown led to enhanced *L. monocytogenes* intracellular growth [27]. These results place *dUsp36* at the crossroads between immune signaling, autophagy and *L. monocytogenes* intracellular growth containment.

In this report, we show that *dUsp36* function is required in vivo in adult hemocytes for survival to *L. monocytogenes* infections. We also confirm the role of autophagy as a protective mechanism during *L. monocytogenes* infections. Interestingly, flies with *dUsp36*-depleted hemocytes, although being sensitive to *L. monocytogenes* infections, do not display increased bacterial loads, which indicates defects in tolerance mechanisms. These results are a first step towards the understanding of the role of the dUSP36 deubiquitinating enzyme during infections by the intracellular pathogen *L. monocytogenes*.

Materials and Methods

Fly Strains and Culture

The UAS-*dUsp36-IR* transgenic line was obtained from the Vienna *Drosophila* RNAi Center and the UAS-*Atg5-IR* transgenic line from Dr. Thomas P. Neufeld (University of Minnesota). The Bloomington stock center provided the *hml-*, *hmlΔ-* and *Hemese*-GAL4 lines (stock Nos. 6395, 30139 and 8699, respectively) and the *Tub-GAL80^{ts}* line for the TARGET system (stock No. 7019).

Flies were kept in standard fly vials or bottles containing dextrose medium and raised under a 12-hour light-dark cycle at 18, 25 or 29°C prior to infections.

Bacterial Strains and Culture

L. monocytogenes strain 10403s was grown standing overnight in BHI medium at 37°C and injected at an OD_{600} of 0.01, 0.001 and 0.0001 for 1,000, 100 and 10 colony forming units (CFUs), respectively. *Streptococcus pneumoniae* was grown standing at 37°C in a 5% CO_2 incubator and injected at an OD_{600} of 0.2. *Salmonella typhimurium* was grown standing in LB medium at 37°C and injected at an OD_{600} of 0.1.

Infection

Five- to 7-day-old males were used for injection. Flies were anesthetized with CO_2 and injected with 50 nl of diluted culture using a Picospritzer (Parker Hannifin) and pulled glass needles. Flies were then placed in vials containing dextrose medium in groups of 20 and incubated at 29°C for *L. monocytogenes* and *S. typhimurium* infections or at 25°C for *S. pneumoniae* infections under a 12-hour light-dark cycle.

Survival Curves

After infection, the number of dead flies was counted daily. Using the GraphPad Prism software, Kaplan-Meier survival curves were generated and statistical analysis was performed using log-rank analysis. Survival was tested at least two times on more than 60 flies and gave similar results for each trial.

CFU Determination and Gentamicin Chase

Infected flies were homogenized in PBS. Appropriate dilutions of the homogenates were plated on LB agar plates using a spiral plater (QCL) and incubated overnight. The data were plotted using the GraphPad Prism software and the p values were determined according to an unpaired two-tailed t test. For the gentamicin chase experiments, flies were injected with 50 nl of 1 mg/ml gentamicin 3 h prior to homogenizing and plating.

Results

dUsp36 Is Required in Adult Hemocytes for Survival to L. monocytogenes Infections

A former RNAi screen showed that *L. monocytogenes* intracellular growth containment in the *Drosophila* hemocytic-like S2 cells requires *dUsp36* function [27]. To determine whether *dUsp36* is also required in the hemocytic lineage for fighting *L. monocytogenes* infections in vivo, we have first assessed the survival of flies with *dUsp36*-depleted hemocytes infected with wild-type *L. monocytogenes* (fig. 1). *dUsp36* hemocyte-specific knockdown was achieved by expressing a double-strand-

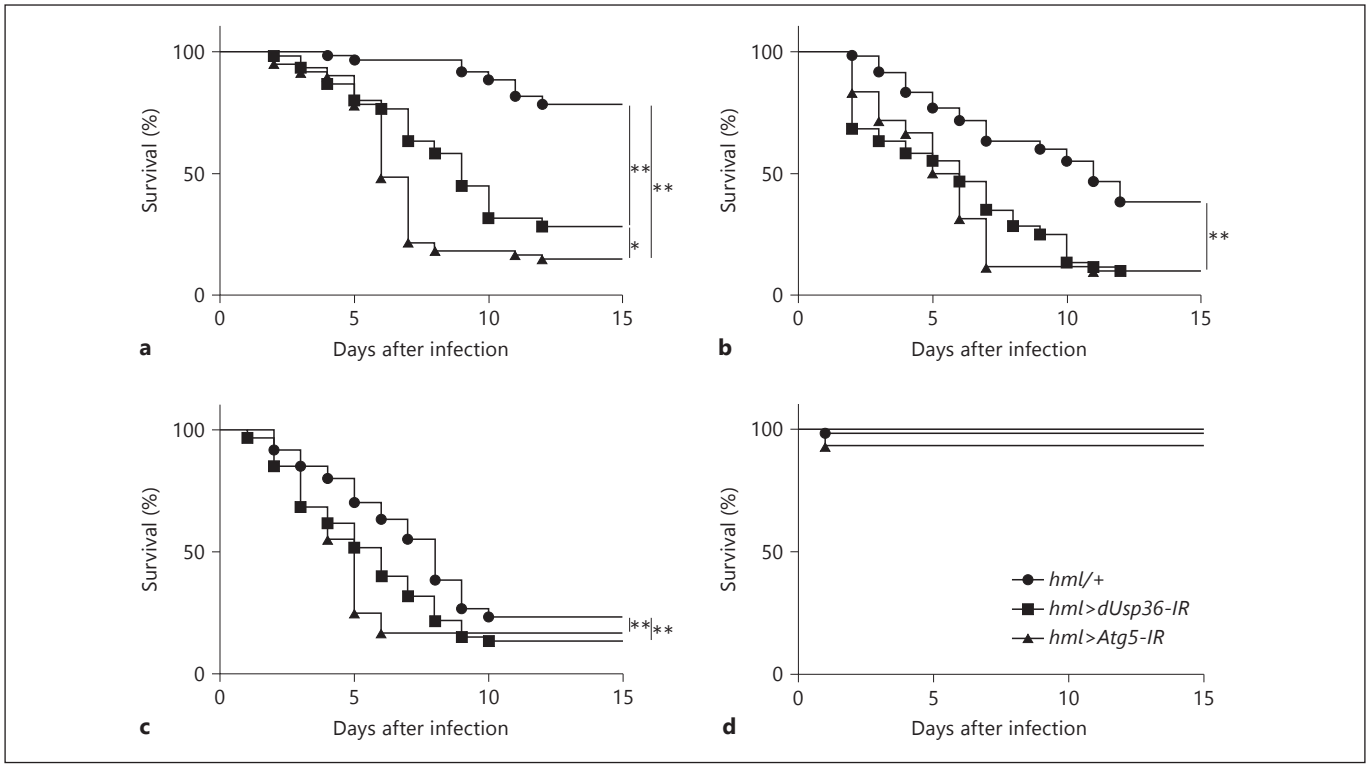

Fig. 1. *dUsp36* inactivation in hemocytes sensitizes flies to *L. monocytogenes* infections. Flies were injected with 10 (**a**), 100 (**b**) or 1,000 (**c**) CFUs of wild-type *L. monocytogenes* and PBS (**d**), and monitored for survival. Significance was determined by log-rank analysis of the survival curves (* $p < 0.05$, ** $p < 0.001$).

ed RNA (dsRNA) targeting *dUsp36* in hemocytes using the driver line *hml-Gal4* [28]. The efficiency and specificity of the dsRNA construct used in this study have already been thoroughly characterized [26]. We observed that, with the three different doses of *L. monocytogenes* used for infection (10, 100 or 1,000 CFUs), flies with *dUsp36*-depleted hemocytes (*hml>dUsp36-IR*) died significantly faster than control flies (*hml/+*; fig. 1a–c) whereas survival of PBS-injected flies was not affected (fig. 1d). Moreover, a second dsRNA transgene targeting a different sequence in *dUsp36* [25] also induced a significant sensitivity to *L. monocytogenes* infections (online suppl. fig. 1; see www.karger.com/doi/10.1159/000360293) indicating that the observed phenotype is not due to putative off-target effects but is actually the consequence of *dUsp36* loss of function.

Autophagy is required for *Drosophila* survival after *L. monocytogenes* infections [18]. The susceptibility of *hml>dUsp36-IR* individuals was thus compared to that of flies with autophagy-deficient hemocytes. First, we confirmed that flies with a hemocyte-targeted inactivation of the *Atg5* gene, which encodes an obligatory component of the autophagy machinery, are indeed more sensitive to *L. monocytogenes* infections than control flies (fig. 1). Even if, at the 10-CFU dose, flies with autophagy-deficient hemocytes died significantly faster than flies with *dUsp36*-depleted hemocytes (fig. 1a), similar survival kinetics between autophagy-deficient and *dUsp36*-depleted flies were observed when flies were infected with 100 and 1,000 CFUs (fig. 1b, c). This indicates that flies with autophagy-deficient hemocytes might be only slightly more susceptible to *L. monocytogenes* infections than flies with *dUsp36*-depleted hemocytes.

We also used two additional transgenic lines specifically expressing the GAL4 protein in hemocytes: *hmlΔ-Gal4* [29] and *Hemese-Gal4* [30]. We found that, using these Gal4 lines to express the dsRNA targeting *dUsp36*, flies with *dUsp36*-depleted hemocytes were systematically and significantly more sensitive to *L. monocytogenes* infections than the control flies (fig. 2a), which indicates that this sensitivity is actually the result of *dUsp36* loss of function in hemocytes.

We have previously shown that *dUsp36* controls cell growth during larval development [26] and the three Gal4

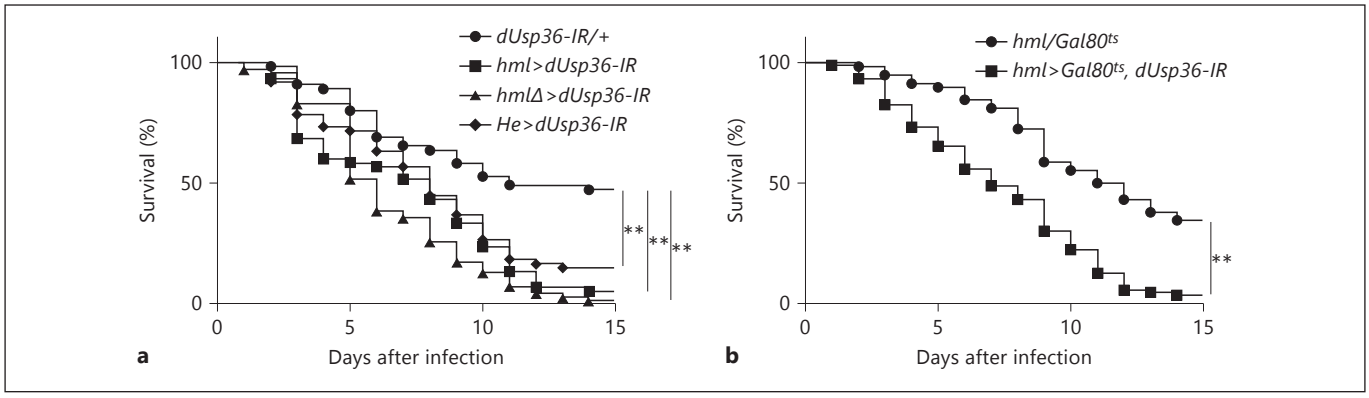

Fig. 2. *dUsp36* function is required in adult hemocytes for survival to *L. monocytogenes* infections. Flies were injected with 100 CFUs of wild-type *L. monocytogenes* and monitored for survival. *dUsp36* hemocyte-specific inactivation was carried out either throughout life time with different hemocyte-specific driver lines (**a**) or only during the adult stage (**b**). Significance was determined by log-rank analysis of the survival curves (** $p < 0.001$).

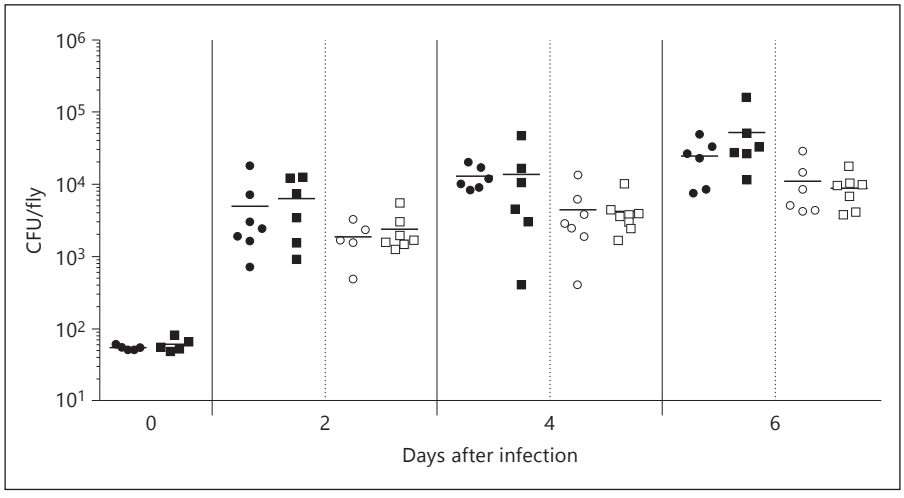

Fig. 3. *dUsp36* inactivation in hemocytes does not increase bacterial loads. Flies were injected with 100 CFUs of wild-type *L. monocytogenes* and bacterial loads were determined by plating at different time points after infection. Significance was determined by Student's t test. ● = *hml/+* total bacterial load; ■ = *hml>dUsp36-IR* total bacterial load; ○ = *hml/+* intracellular bacterial load; □ = *hml>dUsp36-IR* intracellular bacterial load.

lines used in this study are expressed in the hemocytic lineage during larval development [28–30]. This raises the possibility that the sensitivity of flies with *dUsp36*-depleted hemocytes to *L. monocytogenes* infections may be a consequence of developmental defects rather than a consequence of an actual role of *dUsp36* in adult hemocyte functions. To investigate this question, we used the TARGET system [31] to temporally control *dUsp36* inactivation. Development and the first 5 days of the adult life were achieved at restrictive temperature (18°C, no expression of the dsRNA targeting *dUsp36*). Five-day-old flies were then shifted to permissive temperature (29°C) 2 days before infection allowing for the expression of the dsRNA targeting *dUsp36*. We observed that flies with the adult-specific inactivation of *dUsp36* in hemocytes are still more sensitive to *L. monocytogenes* infections than control flies (fig. 2b), which indicates that this sensitivity is not the result of developmental defects.

Taken altogether, these results show unambiguously that *dUsp36* function is required in the adult hemocytes for survival to *L. monocytogenes* infections.

dUsp36 Inactivation in Hemocytes Does Not Increase L. monocytogenes Load

We have then quantified CFUs obtained from control and *hml>dUsp36-IR* flies at different time points after *L. monocytogenes* infection (0, 2, 4 and 6 days after infection) and, using a gentamicin chase to specifically eliminate the extracellular bacteria, we have also quantified the intracellular *L. monocytogenes* load (fig. 3). We observed

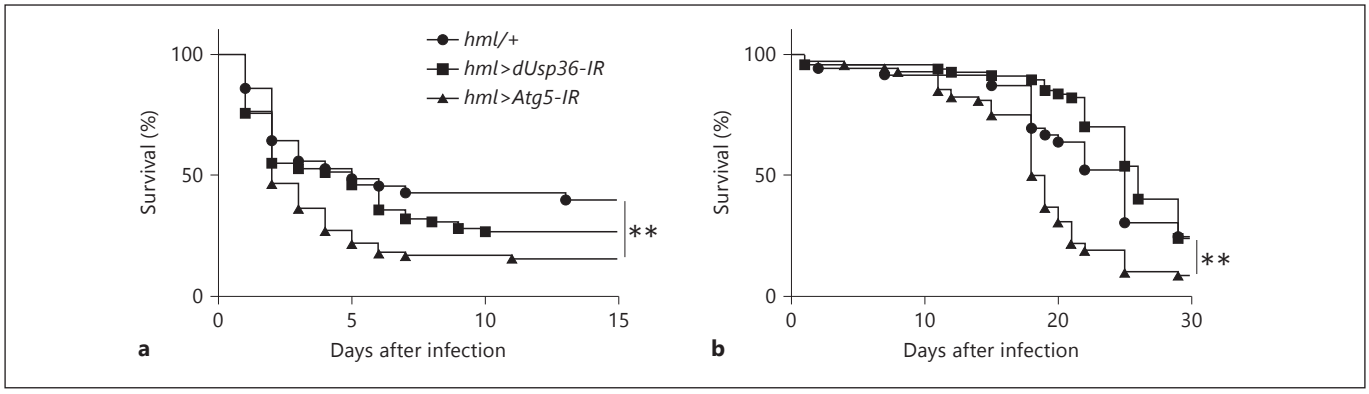

Fig. 4. *dUsp36* inactivation in hemocytes does not sensitize flies to *S. pneumoniae* and *S. typhimurium* infections. Flies were injected with *S. pneumoniae* (**a**) or *S. typhimurium* (**b**) and monitored for survival. Significance was determined by log-rank analysis of the survival curves (** $p < 0.001$).

that the total and intracellular bacterial loads increase with time, which indicates bacterial growth. However, comparison of the CFUs of control and *hml>dUsp36-IR* at the same time point reveals no significant differences. This indicates that the total and intracellular growth rates of *L. monocytogenes* are not different in *dUsp36*-deficient and wild-type hemocytes. These results further show that the sensitivity of flies with *dUsp36*-depleted hemocytes to *L. monocytogenes* infections is not accompanied by an increase in the total or intracellular bacterial load.

dUsp36 Inactivation in Hemocytes Does Not Compromise Survival to Other Infections

We have next infected flies with the hemocyte-specific inactivation of *dUsp36* with a variety of bacteria: a DAP-type extracellular microbe such as *S. pneumoniae* (fig. 4a) and a Lys-type intracellular pathogen such as *S. typhimurium* (fig. 4b). We observed that survival rates of control and *hml>dUsp36-IR* flies are not significantly different when infected with these pathogens. We also observed that flies with autophagy-deficient hemocytes are more susceptible to these infections than control flies. This indicates that hemocyte-specific inactivation of *dUsp36* does not result in a general sensitivity towards DAP-type or intracellular bacteria but rather in a host-pathogen interaction defect with *L. monocytogenes*.

Discussion

We have shown that *dUsp36* inactivation in hemocytes is sufficient to sensitize adult flies to *L. monocytogenes* infections. This is not a consequence of a putative *dUsp36* requirement during hemocyte development that would reduce their number or alter their functions since *dUsp36* adult-specific inactivation has the same effects. Moreover, we have observed that flies with *dUsp36*-depleted hemocytes are not sensitized to *S. pneumoniae* and *S. typhimurium* infections. This indicates that *dUsp36* inactivation in hemocytes does not impair major immune or cellular functions. From these experiments, we conclude that *dUsp36* plays an important role in hemocytes during *L. monocytogenes* infections.

To get further insight into the nature of this role, we have measured the total and intracellular bacterial loads of *L. monocytogenes*-infected flies and observed no difference between flies with *dUsp36*-depleted hemocytes and control flies. This observation is surprising for two reasons. First, *dUsp36* had been identified in a genome-wide RNAi-based screen performed in the hemocytic-like S2 cell line as part of a group of genes whose knockdown led to enhanced *L. monocytogenes* intracellular growth [27]. If this function also takes place in the adult hemocytes, an increase in the intracellular bacterial load should have been observed. This discrepancy is probably due to a difference between S2 cells and adult hemocytes. S2 cells are derived from embryos, are not fully mature and are closely related to larval circulating hemocytes. Published results demonstrating substantial differences between larval and adult hemocytes support this hypothesis [32–34].

Second, as stated earlier, no significant difference in the total bacterial load has been observed in flies with *dUsp36*-depleted hemocytes compared to control flies, which, combined with the fact that they are not sensitive to the other pathogens tested, indicates that their immune system is functional. This raises the question

as to why flies with *dUsp36*-depleted hemocytes succumb faster whereas they are still able to control *L. monocytogenes* infections. Decreased survival with no change in the associated pathogen load is the hallmark of a tolerance defect [21], which indicates that *dUsp36* is required in the adult hemocyte for tolerance to *L. monocytogenes* infections. The mechanisms involved in this process are still unclear and may imply a role of the dUSP36 deubiquitinating enzyme in some specific aspects of stress resistance or metabolism [35] or in the degradation of specific toxins during *L. monocytogenes* infections.

In conclusion, we have shown that *dUsp36* is required in vivo in the adult hemocyte for survival to *L. monocytogenes* infections by acting on tolerance mechanisms. We have also confirmed the previously demonstrated role of autophagy during *L. monocytogenes* infections. These results are a first step towards the understanding of the role of the dUSP36 deubiquitinating enzyme during *L. monocytogenes* infections in vivo.

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