Inventory of Supplemental Information

Figure S1 (related to Figure 2)

WT and Atg16L1^{HM} murine embryonic fibroblasts and peritoneal macrophages display similar intracellular bacterial burden following *in vitro S. aureus* infection.

Figure S2 (related to Figure 4)

Atg16L1^{HM} mice are more resistant to USA300 *Δhla* infection compared with WT mice.

Figure S3 (related to Figure 5)

The process of generating bone marrow chimeras alters disease course following *S. aureus* infection.

Figure S4 (related to Figure 6)

Atg16L1^{HM} mice have increased E-cadherin cleavage upon USA300 infection and α-toxin treatment.

Figure S5 (related to Figure 7)

Atg16L1^{HM} peritoneal macrophages are not more susceptible to α-toxin compared to WT.

Supplemental Experimental Procedures

Supplemental References

Figure S1, related to Figure 2

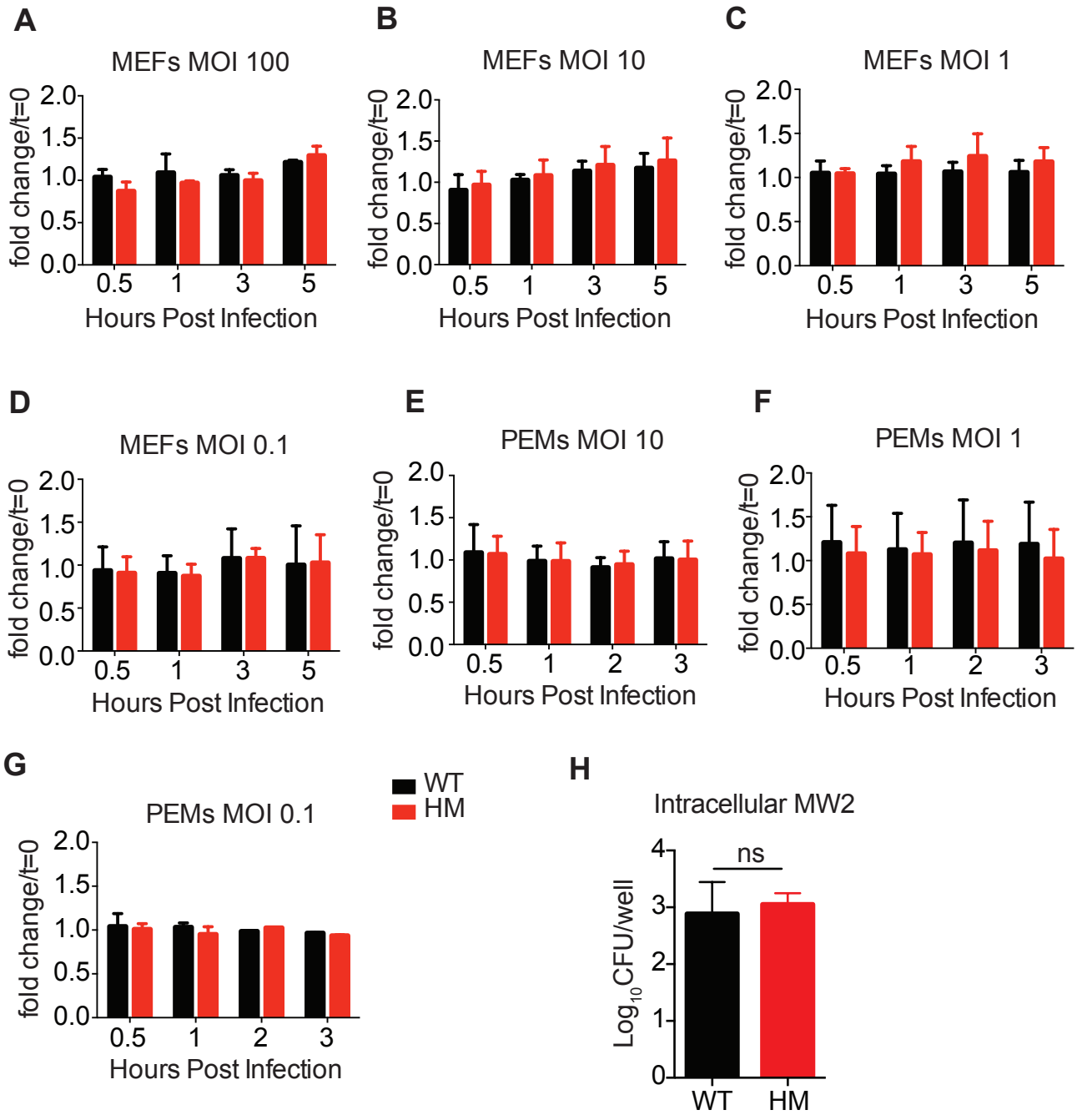


Figure S1, related to Figure 2. WT and Atg16L1^{HM} murine embryonic fibroblasts and peritoneal macrophages display similar intracellular bacterial burden following *in vitro S. aureus* infection.

(A-G) WT and Atg16L1^{HM} (HM) murine embryonic fibroblasts (MEFs) (A-D) or peritoneal macrophages (E-G) were infected with the indicated multiplicities of infection (MOI) of USA300 for 15 minutes. Cells were washed (time 0), and intracellular bacteria was quantified after culturing for the indicated amounts of time. Values represent fold change normalized to time 0. Bars represent the mean and SEM of 3 independent experiments.

(H) Quantification of intracellular bacterial burden of WT and Atg16L1^{HM} MEFs infected with USA400 (MW2).

Bars represent the mean and SEM of log transformed bacteria from 2 independent experiments.

Figure S2, related to Figure 4

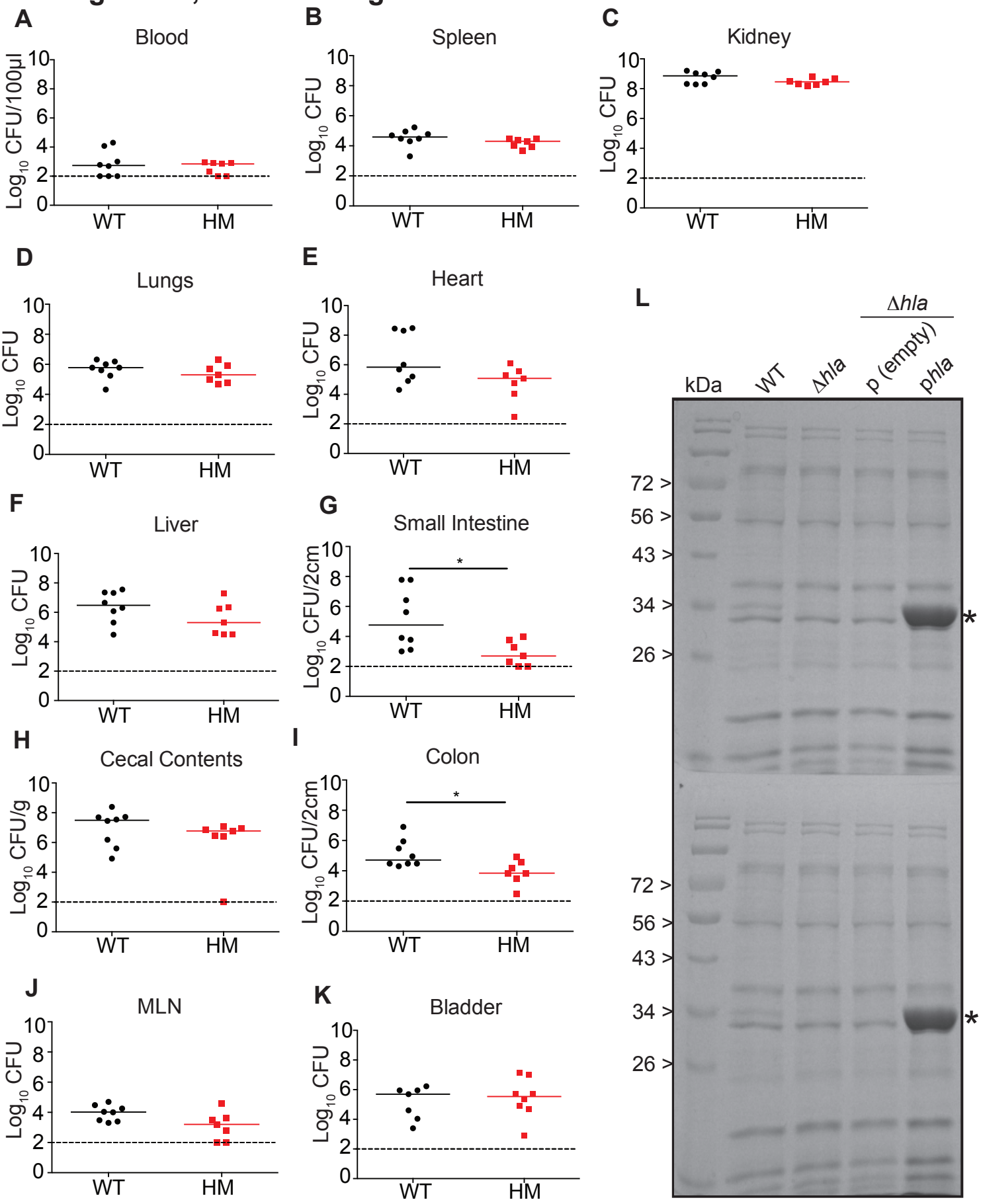


Figure S2, related to Figure 4. Atg16L1^{HM} mice are more resistant to USA300 *Δhla* infection compared with WT mice.

(A-K) Bacterial burden was quantified in the indicated organs and blood harvested from WT and Atg16L1^{HM} mice injected i.v. with 5x10^{7} cfu USA300 *Δhla* on day 3 post infection. Data points represent bacterial burden in individual mice, bars represent median, and dashed line represents limit of detection. *n*=7-8 mice/group from two independent experiments.

(L) Exoprotein profile of 2 independent colonies each of USA300 WT, USA300 *Δhla*, USA300 *Δhla* + EV (empty vector), and USA300 *Δhla* + p*hla*. Asterisk indicates α-toxin band.

Figure S3 related to Figure 5

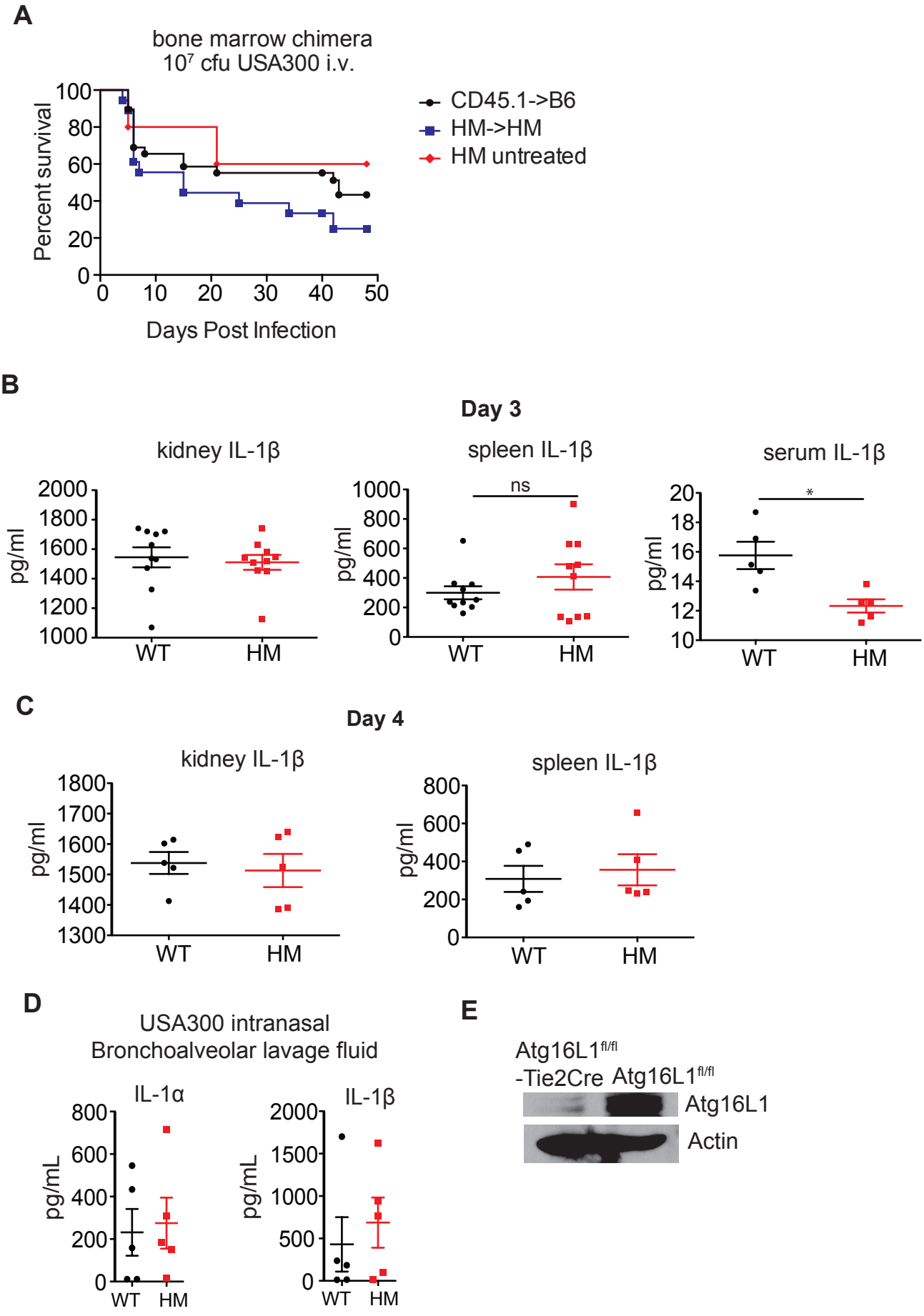


Figure S3, related to Figure 5. The process of generating bone marrow chimeras alters disease course following *S. aureus* infection.

(A) Bone marrow chimeras are often used to differentiate the effect of a mutation in the hematopoietic and non-hematopoietic compartments. To determine if this approach can be used to investigate the effect of Atg16L1 mutation on survival following *S. aureus* infection, we generated controls in which irradiated WT mice were reconstituted with WT bone marrow (WT→WT) and Atg16L1^{HM} mice were reconstituted with Atg16L1^{HM} bone marrow (HM→HM) as described previously (Marchiando et al., 2013). Animals were infected with 10^7 cfu USA300 i.v. 10 weeks following bone marrow transplantation. Survival curve indicates that this approach cannot be utilized for this model for two reasons. First, HM→HM were more susceptible to lethality than Atg16L1^{HM} mice, indicating that the procedure increases susceptibility. Second, we could not achieve significant separation between WT→WT and HM→HM. Thus, infecting chimeric mice (WT→HM or HM→WT) would yield un-interpretable results. Note that CD45.1 congenic mice were used as WT donors, which allowed us to confirm successful reconstitution. *n*=20 mice each for WT→WT and HM→HM in 2 independent experiments. *n*=5 Atg16L1^{HM} were included in the second experiment.

(B-C) IL-1β at day 3 and 4 after i.v. injection of 10^7 cfu USA300 and measured by ELISA in the indicated organs. *n*=5-10 mice/group in 2 independent experiments.

(D) IL-1α and IL-1β in bronchoalveolar lavage fluid following intranasal infection with USA300 measured by cytokine bead array analysis. *n*=5 mice/group

(E) Representative Western blots for Atg16L1 and actin in endothelial cells harvested from Atg16L1$^{fl/fl}$-Tie2Cre mice and Atg16L1$^{fl/fl}$ control mice.

Figure S4, related to Figure 6

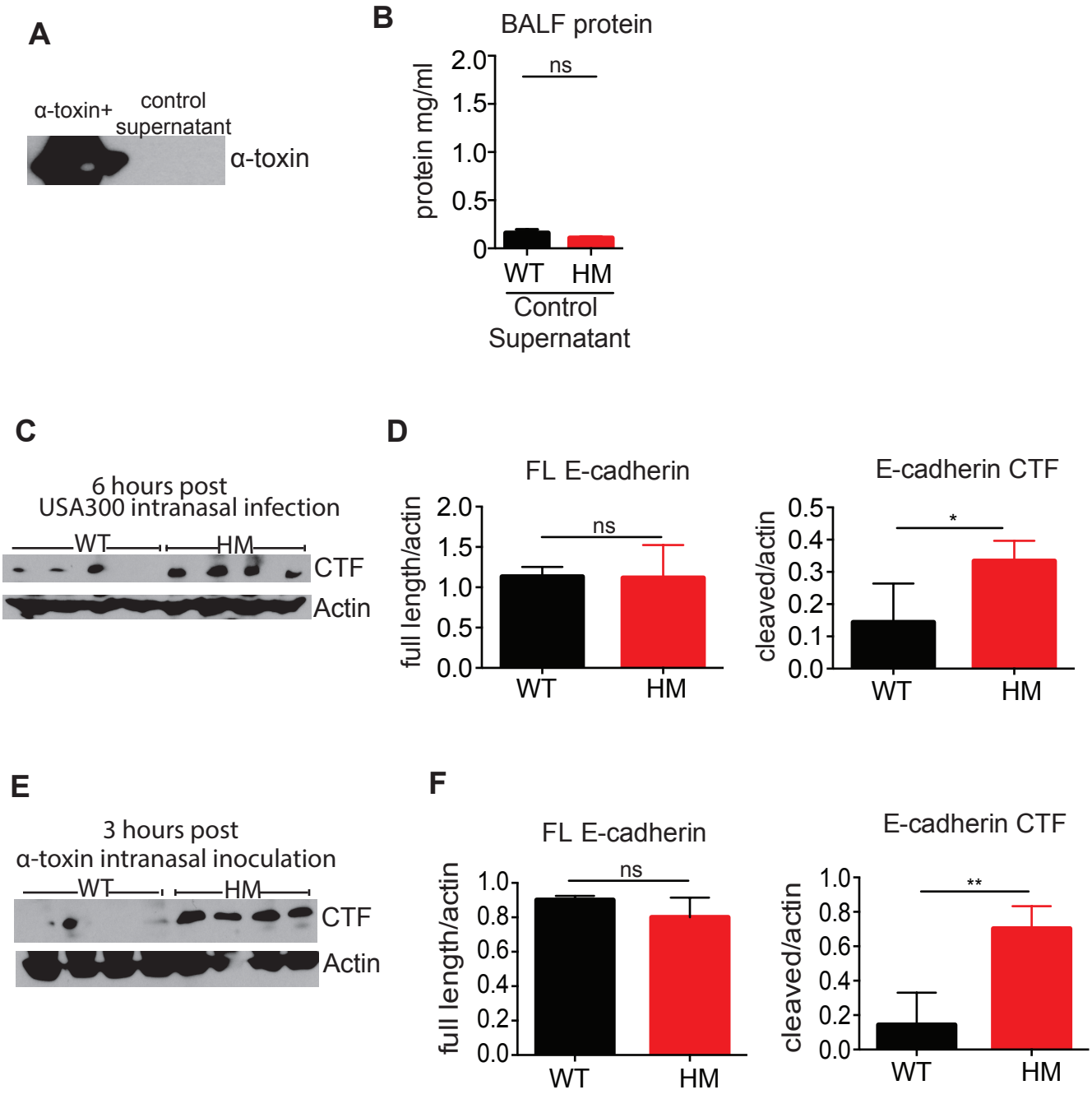


Figure S4, related to Figure 6. Atg16L1^{HM} mice have increased E-cadherin cleavage upon USA300 infection and α-toxin treatment.

(A) Western blot analysis of supernatant harvested from the engineered toxin-deficient *S. aureus* strain overproducing α-toxin or control supernatant from the parental strain.

(C) Bronchoalveolar lavage protein content of WT and Atg16L1^{HM} mice 3 hours post-intranasal inoculation with toxin-deficient control supernatant. *n*=4-5mice/group.

(D) Western blot for the cleaved C-terminal fragment (CTF) of E-cadherin and actin in lungs of WT and Atg16L1^{HM} mice following 6 hrs post intranasal infection with 4x10^{8} cfu USA300. Each lane represents an individual mouse.

(E) Quantification of full length (FL) and CTF E-cadherin from (D).

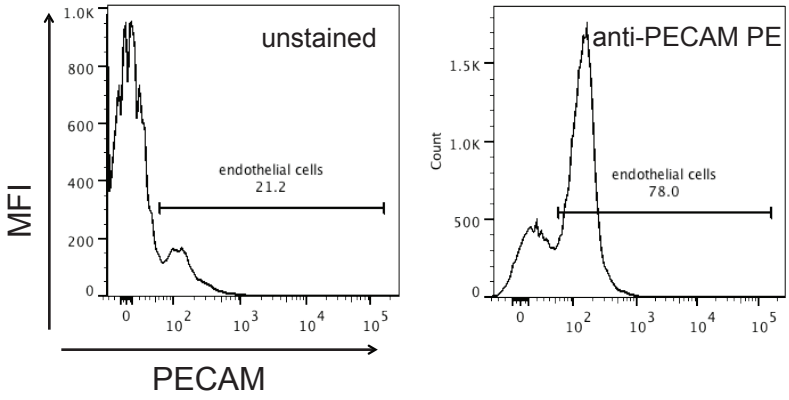
(F) Western blot for the CTF of E-cadherin and actin in lungs of WT and Atg16L1^{HM} mice following 3 hrs post inoculation with 20µl α-toxin. Each lane represents an individual mouse.

(G) Quantification of full length (FL) and CTF E-cadherin from (D).

*p<0.05, **p<0.01.

Figure S5, related to Figure 7

A



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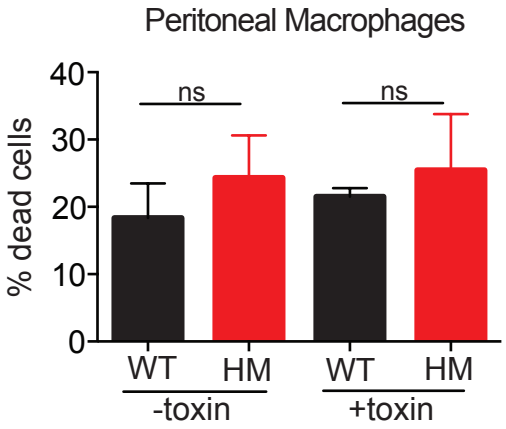

Figure S5, related to Figure 7. Atg16L1^{HM} peritoneal macrophages are not more susceptible to α-toxin compared to WT.

(A) PE-CAM expression in cultured endothelial cells from WT and Atg16L1^{HM} mice confirms the majority of cells in the population are positive for endothelial cell marker PE-CAM, compared to unstained control (left panel). Representative histogram from 2 mice/group.

(B) Percent cell death measured by the lactate dehydrogenase (LDH) assay in cultured peritoneal macrophages harvested from WT and Atg16L1^{HM} mice inoculated with α-toxin or control suspension. Data represents 3 independent experiments, each using cells pooled from 2 WT or Atg16L1^{HM} mice.

Data represent mean and SEM.

Supplemental Experimental Procedures

Strain List

Strain	VJT#	Reference
USA300 (LAC)	VJT 12.61	(Diep et al., 2006)
USA400 (MW2)	VJT 4.79	(Baba et al., 2002)
USA500 (BK2395)	VJT 27.36	(Benson et al., 2014)
USA300-LAC (AH1263)	VJT 15.77	(Boles et al., 2010)
USA300-LAC Δ*agr* (AH1292)	VJT 36.34	(Benson et al., 2011)
USA300-LAC *hla::erm*	VJT 36.63	This study
Newman quadruple KO	VJT 31.58	(Reyes-Robles et al., 2013)
Newman quadruple KO / pDU1212	VJT 32.57	This study
USA300-LAC *hla::erm* / pOS1-p_{hrt}	VJT 44.39	This study
USA300-LAC *hla::erm* / pDU1212 (P_{hla}-*hla*)	VJT 44.41	This study

Quantification of bacterial burden and disease

Bacterial burden in blood and organs was quantified by homogenizing organs and plating serial dilutions on Mannitol Salt Agar (MSA) plates, which preferentially grow *Staphylococci* and on which *S. aureus* can be distinguished by presence of yellow colonies. It has been previously shown that no colonies grow from stool homogenates from uninfected WT animals (Kernbauer et al., 2015). Colon and small intestine were washed in PBS to remove non-adherent bacteria to distinguish tissue-associated from lumenal bacteria. Activity assessment score was evaluated as described previously (Inoshima et al., 2011). Mice were assessed and scores were assigned as follows: 1 – mice were bright, alert, active, normal food and water intake; 2 – alert, less active, animals move spontaneously upon visual inspection of cage; 3 – animals were less alert but not huddled, fur not ruffled, only move when cage is disturbed; 4 – animals were huddled with ruffled fur, only move when touched, intact “righting reflex”; 5 – animals were hunched with ruffled fur, did not move when touched, absent “righting reflex”; 6 – recumbent ; 7 – dead. Sections of lungs were prepared by excising lungs and inflating alveoli with formalin. For pathology analysis, sections were stained with Hematoxylin and eosin (H&E) and analyzed blindly by a pathologist (J.D.). Analysis was converted to a numerical score by assigning one

point for each of the following criteria: presence of visible cocci, hemorrhage, fibrin thrombi in vessels, necrotic foci, lack of a robust neutrophil response. Half a point was assigned if any of the preceding criteria was classified as “rare”. TUNEL staining was performed by the NYU IHC Core. For bronchoalveolar lavage fluid (BALF) protein content, mice were inoculated intranasally with 20µl supernatant containing α-toxin or control suspension and euthanized at 3 hours post inoculation. BALF was collected by injecting 500µl PBS + protease inhibitors into the lungs via the trachea and subsequently aspirating the fluid with the same syringe. Protein concentration was determined by Bradford Assay.

Cell culture and *in vitro* assays

Peritoneal macrophages were harvested as described previously (Marchiando et al., 2013). Peritoneal macrophages and previously described WT and Atg16L1^{HM} murine embryonic fibroblasts (MEFs) (Cadwell et al., 2008) were plated at a density of 1x10^4 cells/well in 48 well plates and infected with USA300 or USA400 as indicated in triplicate at the indicated multiplicity of infection (MOI) and synchronized by centrifugation at 1000rpm for 3 minutes. After a 15 minute incubation, cells were washed in PBS to remove extracellular bacteria; this timepoint was t=0. Cells were lysed using 1% saponin, and intracellular bacterial load was quantified by serial dilutions on TSB plates. Additional timepoints were treated similarly, and data was expressed as fold change in cfu over t=0 for USA300 infection and as total cfu/well for USA400 infection. For LDH assays, cells were plated at in 48 well plates, and grown for 5 days or until confluent. At least 3 wells per condition were analyzed per experiment. 20µl of α-toxin containing supernatant or toxin-less supernatant was added per well. 2% triton-100x was added to positive control wells. A media only well was used for background measurement. At 6 hours post intoxication, 50µl of supernatant was taken from each well and LDH release was measured using the CytoTox-One Homogeneous Membrane Integrity Assay (Promega) according to the manufacturer’s instructions.

Autophagy modulation in endothelial cells

For each experiment, endothelial cells were harvested as described below and pooled from 2 WT mice and plated in 24 well plates. 2-4 wells per experiment were treated with the indicated autophagy inducing or inhibiting conditions. For serum starvation, wells were washed once with PBS and DMEM with or without serum was added to wells for 6 hours. For epirubicin, NH_4Cl, and Lys05 treatment, stock solutions were made in PBS, and small volumes were added to wells with fresh complete media to reach desired concentrations. An equal volume of PBS was added to negative control wells. Cells were treated for 6 hours with 100μM epirubicin, 24 hours with 20mM NH_4Cl, or 24 hours with 5 μM Lys05. At the indicated timepoint, cells were lysed with RIPA buffer (1% Triton-X 100, 1% sodium deoxycholate, 0.1%SDS, 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2).

Western blotting, ELISA, and qPCR

Western blot analysis of whole cell lysates prepared from animal tissue resuspended in RIPA buffer was performed. α-toxin production by *S. aureus* strains was determined by analyzing filter-sterilized supernatants harvested from bacteria cultured in RPMI/CAS for 5 hours after diluting an overnight culture 1:100. After a 30 min incubation on ice, 150 μl of 100% trichloroacetic acid (TCA) were added to 1.3 ml of filtered culture supernatants to precipitate proteins, incubated overnight at 4°C, and centrifuged for 15 minutes at 15,000 RPM, 4°C. The resulting protein pellets were washed on 100% ethanol for 1 hr at 4°C, then centrifuged for 15 minutes at 15,000 RPM, 4°C, and the pellets allowed to dry overnight. The dried pellets were resuspended in 30 μl of 1x TCA-SDS sample buffer and boiled for 10 minutes. 5 μl were run on a 15% SDS-PAGE gel. Membranes were incubated with the following primary antibodies: Atg16L1 (Sigma, A7356), α-toxin (Sigma, S7531), Adam10 (Millipore, AB19026), E-Cadherin C-

terminus (BD, Clone 36) and Actin (Abcam). Quantification of bands was performed using ImageJ or BioRad ImageLab software. Analysis of IL-1β in serum, spleen, and kidney homogenate was performed using Quantikine IL-1β ELISA kit (R&D systems). Cytokine bead array analysis for IL-1α and IL-1β (BD) was used to analyze cytokine content in bronchoalveolar lavage fluid. qPCR was performed as described previously using the following primers: Adam10: Forward- GGGAGGCACATGGGAGCCATA Reverse- CCTGGGTTGGAGCACCTTCAG GAPDH: Forward- TGCCCCCATGTTTGTGATG Reverse- TGTGGTCATGAGCCCTTCC

Microscopy

Endothelial cells were plated on gelatin/collagen-coated glass coverslips in 6 well plates. For Adam10 immuno-fluoresence cells were blocked with 1% BSA in PBS, then stained with a rabbit anti-Adam10 primary antibody (Millipore, AB19026) overnight at 4°C, followed by anti-rabbit Alexa488 secondary antibody. For analysis of pore formation, EtBr was added to the wells at a final concentration of 1:2000 6 hrs after incubation with 20µl of α-toxin containing supernatant or toxin-less supernatant. The cell monolayer was visualized at 488nm in the FITC channel using the 20x objective of a Zeiss Axioplan microscope for quantification and images were taken by confocal microscopy using a the 63x objective of a Zeiss 710 microscope.

Endothelial cell isolation

Endothelial cells were isolated by adapting a previously described protocol (Fehrenbach et al., 2009). 6 week old mice were anesthetized with ketamine-xylazine. The thoracic cavity was exposed, and blood was flushed from the lungs by perfusion via the right ventricle with 5ml PBS. 1ml of collagenase VIII (0.5mg/ml) was instilled into the lung through the trachea, and the trachea was quickly tied off. Thymus and heart were removed, and lungs were excised and incubated for 30 minutes in a 37°C water bath in 5ml collagenase VIII. The tube was inverted

every 5-8 minutes during incubation. Following incubation, 25ml PBS was added to the tube, and lungs were dissociated by vigorous shaking for 30 seconds, then filtered through a 70μm cell strainer. Cells were centrifuged at 1200rpm for 5 minutes and resuspended in complete media (DMEM+10%FCS+1%penicillin/streptomycin+1%L-glutamine). Endothelial cells were selected by magnetic sorting using Dynabeads coated with rat anti mouse CD31 antibody, washed 3x, resuspended in complete media, and added to plates coated with 0.5% gelatin and 50μg/ml collagen. Media was exchanged on day 3 post-harvest and cells were used for experimentation on day 5. For lactate dehydrogenase (LDH) release assays, endothelial cells were pooled from 2-3 mice per group for each experiment.

Flow cytometry

Lungs were perfused and digested by injecting 1ml of 0.5mg/ml collagenase VIII through the trachea and incubated for 30 minutes in 3ml 0.5mg/ml collagenase. Single cell suspensions were centrifuged at 1200 rpm for 5 minutes and resuspended in 1ml PBS. 100μl of cell suspension was transferred to a 96-well plate and resuspended in 100μl of Fc block (1:400, BioLegend) in FACS buffer (1% BSA in PBS) for 15 minutes at room temperature. Cells were washed and resuspended in surface staining solution containing anti-CD45 APCCy7 (1:100, BioLegend), anti-EpCAM/CD326 PECy7 (1:100, BioLegend), anti-ICAM-1/CD54 Pacific Blue (1:100, BioLegend), and anti-PECAM/CD31 APC (1:100, BioLegend). After a 15 minute incubation at 4 °C, cells were washed twice and resuspended in 200 μl FACS buffer. Propidium iodide (BioLegend) was added at 1:400 15 minutes prior to analysis. Cells were analyzed by LSR II flow cytometer (BD).

Generation of *hla* mutant strains

To generate *hla* mutant strains, ermS LAC *hla::erm* was transformed with empty plasmid or plasmid overexpressing *hla*. To evaluate the exoprotein profiles of these strains, overnight

cultures of two independent colonies were subcultured 1:100 in RPMI (Gibco) supplemented with 1% casamino acids (RPMI/CAS) and grown for 5 hours at 37°C, 180 RPM. The cultures were centrifuged for 15 minutes at 15,000 RPM, 4°C, and the supernatants filter-sterilized using a 0.22 µm filter, followed by incubation on ice for 30 minutes. Trichloroacetic acid (TCA) was added to the filtered culture supernatants at a final concentration of 10% (v/v) to precipitate proteins and incubated overnight at 4°C. The TCA-treated supernatants were centrifuged for 15 minutes at 15,000 RPM, 4°C. The resulting protein pellets were washed on 100% ethanol for 1 hr at 4°C, then centrifuged for 15 minutes at 15,000 RPM, 4°C, and the pellets allowed to dry for 2 hours. The dried pellets were resuspended in 30 µl of 1x TCA-SDS sample buffer and boiled for 10 minutes. 20 µl were run on a 12% SDS-PAGE gel at 80 V for 3 hours, then stained with Coomassie blue. EZ-Run Prestained Recombinant Protein Ladder (Fisher BioReagents) was used as a molecular weight marker.

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