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Supplemental Information

The Ubiquitin Ligase Smurf1 Functions in Selective Autophagy of *Mycobacterium tuberculosis* and Anti-tuberculous Host Defense

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Figure S1. Related to Figure 1

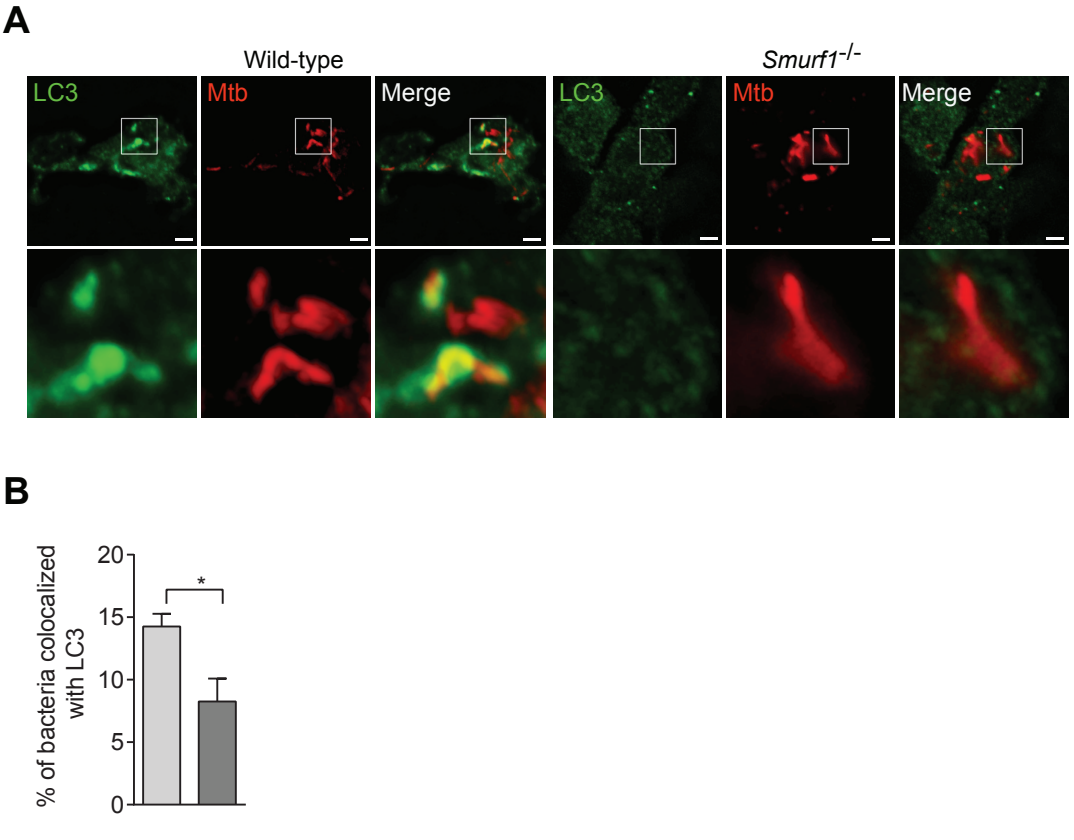


Figure S2. Related to Figure 4

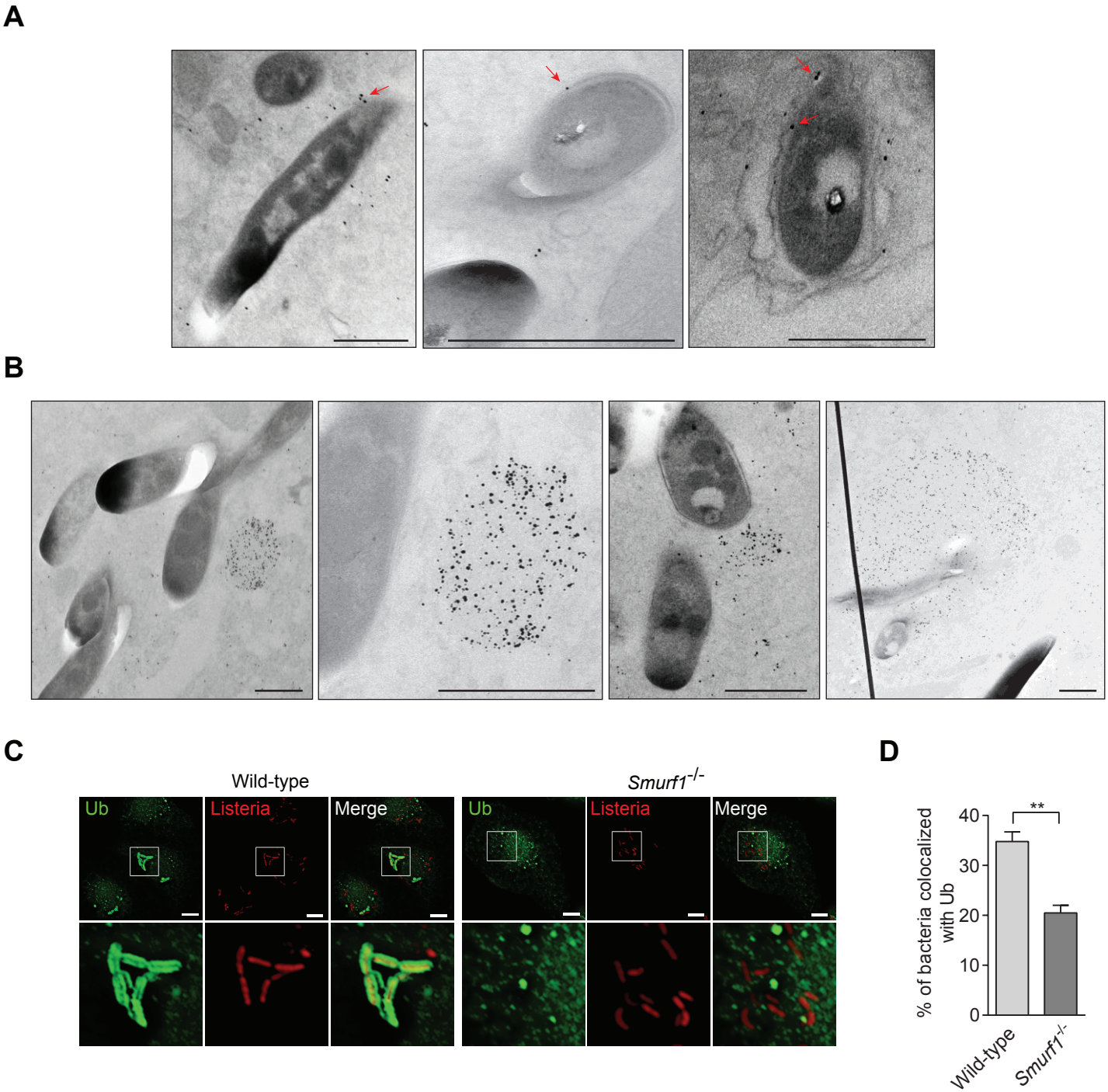


Figure S3. Related to Figure 5

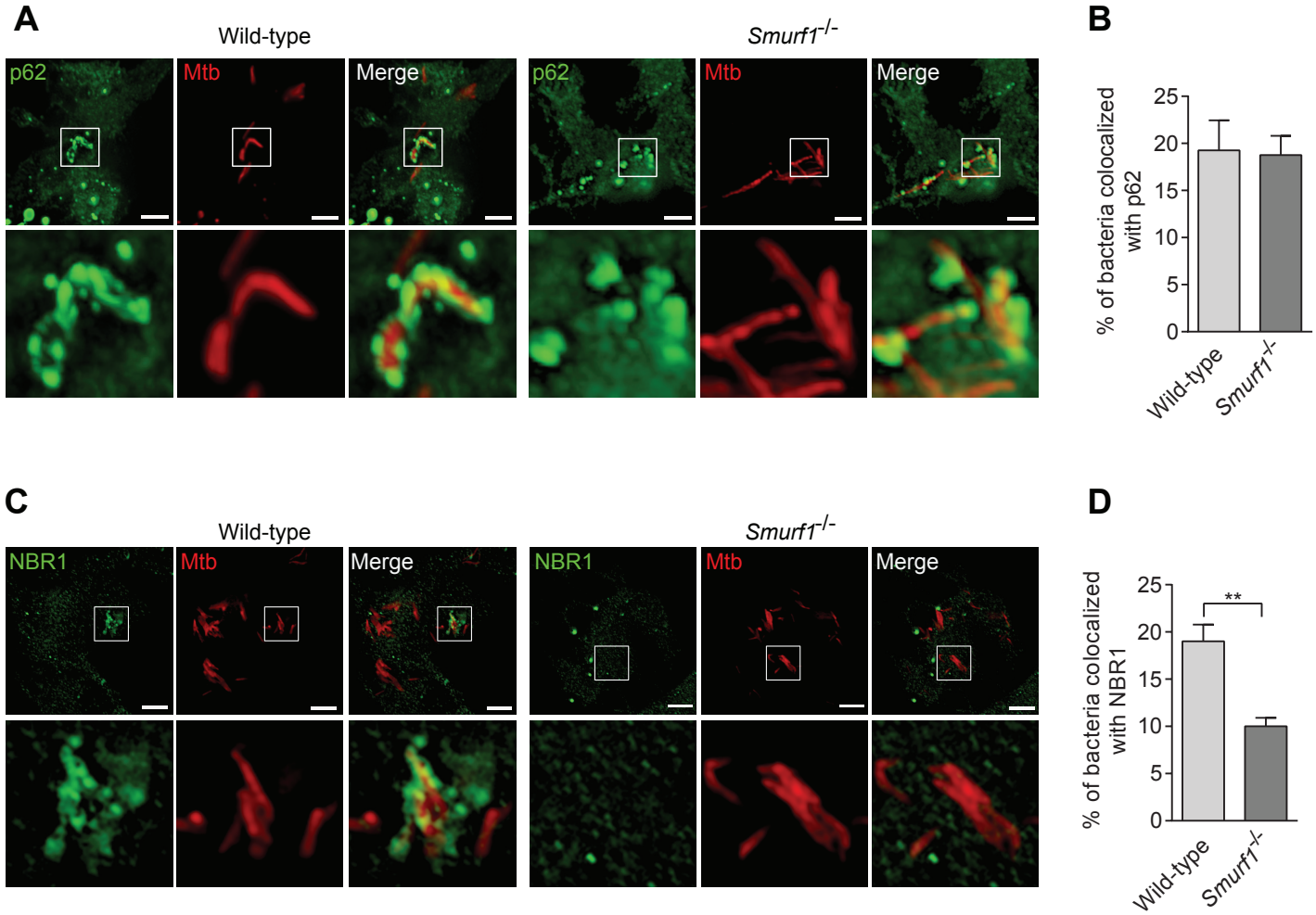


Figure S4. Related to Figure 6

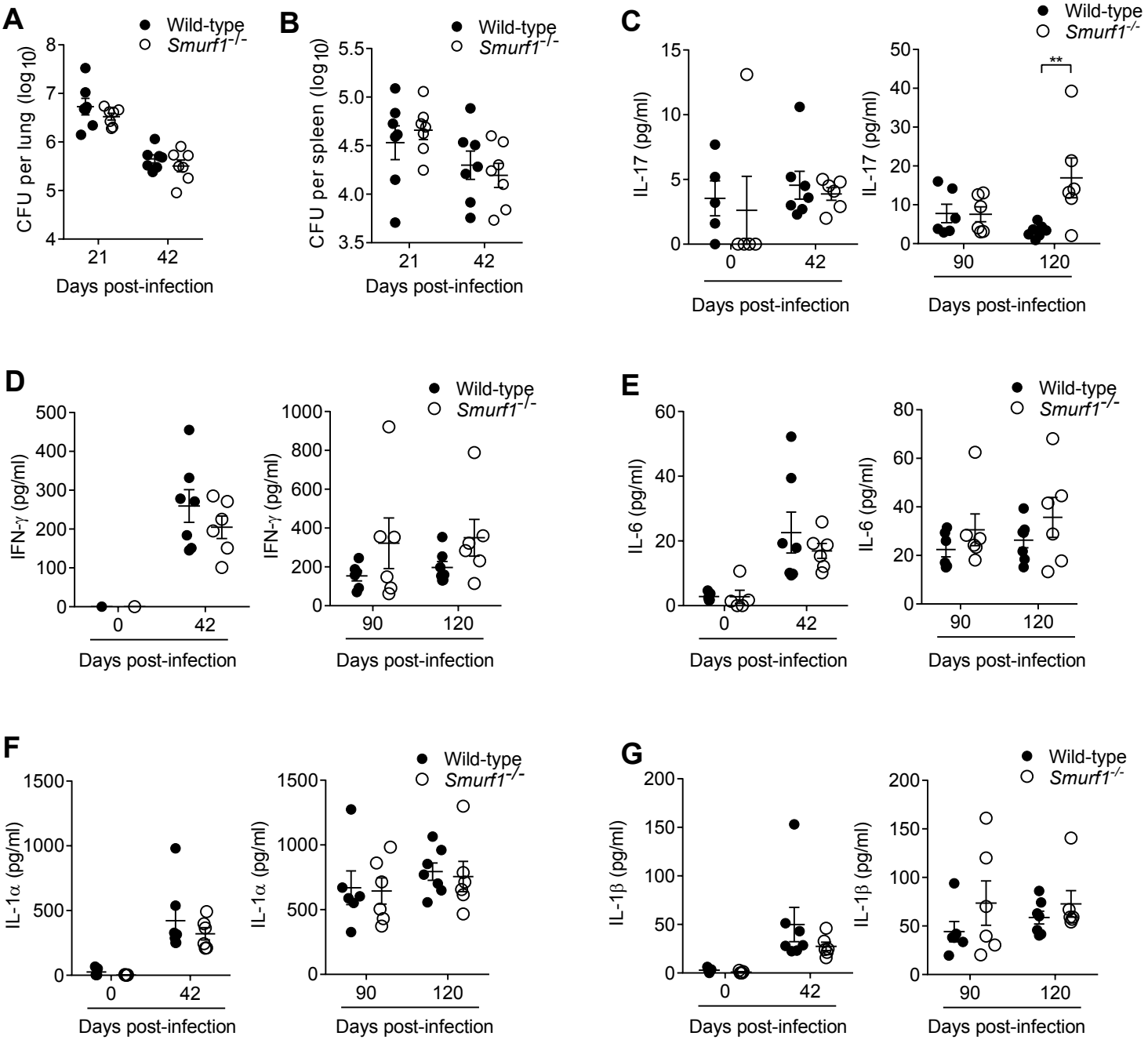
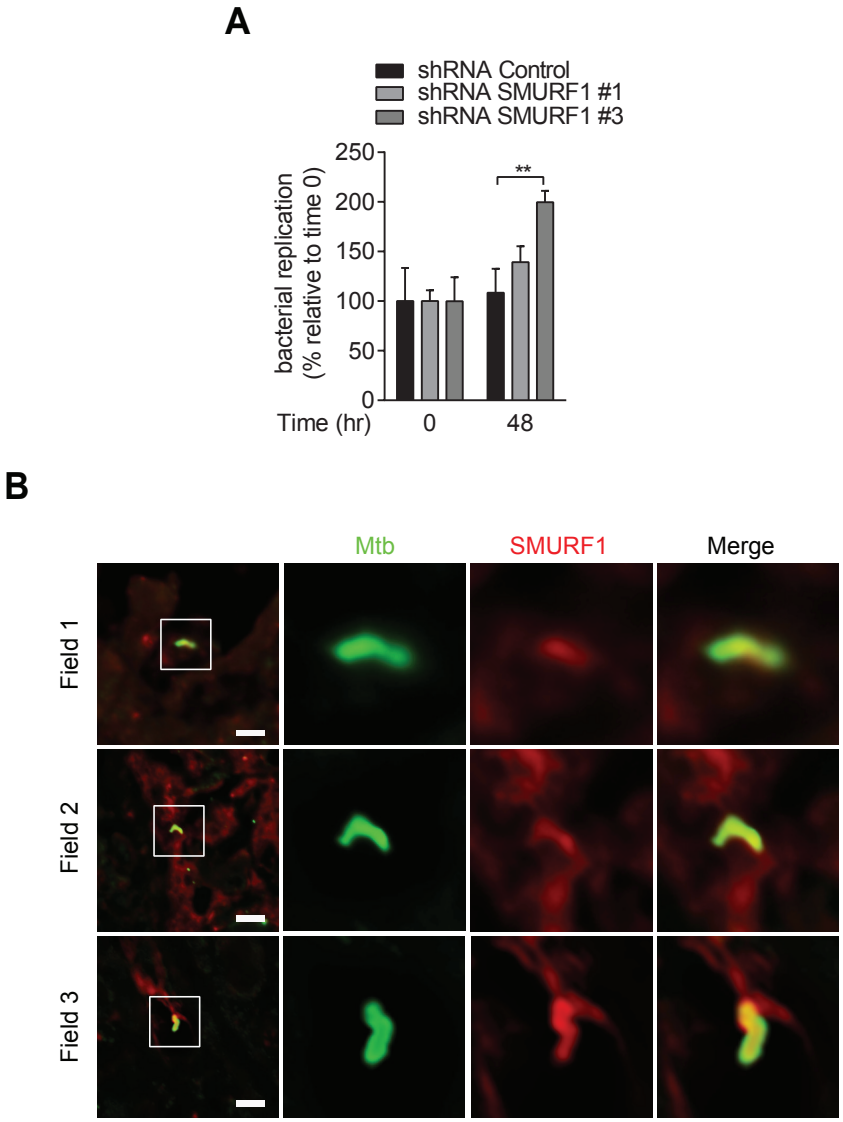


Figure S5. Related to Figure 7



SUPPLEMENTAL FIGURE LEGENDS

Figure S1, Related to Figure 1. Colocalization of Endogenous LC3 and *M. tuberculosis* is Impaired in *Smurf1*$^{-/-}$ BMDMs.

(A and B) Photomicrographs (**A**) and quantitation (**B**) of the colocalization of mCherry-Mtb and endogenous LC3 in wild-type or *Smurf1*$^{-/-}$ BMDMs 15 hr after infection. Insets (lower panels) in (**A**) show representative mycobacteria that would be considered colocalized with LC3 in wild-type BMDMs or not colocalized with LC3 in *Smurf1*$^{-/-}$ BMDMs. Bars are mean ± SEM of quadruplicate samples (100 bacteria evaluated per sample per genotype) from a representative experiment. Similar results were observed in at least 3 independent experiments. **P*<0.05, *t*-test. Scale bars, 4 μm.

Figure S2, Related to Figure 4. K48-Ubiquitin Association with Mtb-Associated Structures Visualized by Immunogold Labeling and Association of Polyubiquitin With *L. monocytogenes*

(**A and B**) Immunogold labeling of K48 in BMDMs infected with Mtb 15 hr after infection. In (**A**) K48 immunogold is found in close proximity to Mtb (left panel); in apparent direct contact with the Mtb bacterial membrane (middle panel) or associated with membranous structures surrounding Mtb that may be phagolyososomes or autolysosomes (right panel). Red arrows denote representative K48 immunogold particles near Mtb. In (**B**) K48 immunogold is found in aggregates near Mtb, either with a distinct spherical structure (left panels) or more dispersely located near tuberculous bacilli (right panels). Scale bars, 500 nm.

(C and D) Photomicrographs (**C**) and quantitation (**D**) of the colocalization of RFP-expressing *L. monocytogenes* ΔActA mutant and polyubiquitin in wild-type or *Smurf1*$^{-/-}$ BMDMs 2 hr after infection. Insets (lower panels) in (**C**) show representative bacteria that would be considered colocalized with polyubiquitin in wild-type BMDMs or not colocalized with polyubiquitin in *Smurf1*$^{-/-}$ BMDMs. Bars are mean ± SEM of quadruplicate samples (150 bacteria evaluated per sample per genotype) from a representative experiment. Similar results were observed in at least three independent experiments. **P*<0.05, *t*-test. Scale bars, 5 μm.

Figure S3, Related to Figure 5. Role of Smurf1 in Recruitment of p62 and NBR1 to *M. tuberculosis*-Associated Structures.

(A and B) Photomicrographs (**A**) and quantitation (**B**) of the colocalization of mCherry-Mtb and p62 in wild-type or *Smurf1*$^{-/-}$ BMDMs 15 hr after infection. Insets (lower panels) in (**A**) show representative mycobacteria that would be considered colocalized with p62 in wild-type or *Smurf1*$^{-/-}$ BMDMs. Scale bars, 4 μm.

(C and D) Photomicrographs (**C**) and quantitation (**D**) of the colocalization of mCherry-Mtb and NBR1 in wild-type or *Smurf1*$^{-/-}$ BMDMs 15 hr after infection. Insets (lower panels) in (**C**) show representative mycobacteria that would be considered colocalized with NBR1 in wild-type BMDMs or not colocalized with NBR1 in *Smurf1*$^{-/-}$ BMDMs. Scale bars, 5 μm.

For **B** and **D**, bars are mean ± SEM of quadruplicate samples (100 bacteria evaluated per sample per genotype) from a representative experiment. Similar results were observed in at least 3 independent experiments. ***P*<0.01, for indicated comparison; *t*-test.

Figure S4, Related to Figure 6. Bacterial CFUs and Production of Cytokines in Lungs of *M. tuberculosis*-infected Mice.

(A and B) Bacterial CFUs in lungs (**A**) and spleens (**B**) of wild-type or *Smurf1*$^{-/-}$ mice at indicated time-point after aerosol infection with Mtb. Results are mean ± SEM of 5 animals/group.
(C-G) Cytokine production in lungs homogenates of uninfected (day 0) or Mtb-infected mice at the indicated time-points. Results are mean ± SEM for n=5-7 animals/group. $^{**}P<0.01$; two-way ANOVA.

Figure S5, Related to Figure 7. SMURF1 Colocalizes With *M. tuberculosis* in Lung of Patient With Active Pulmonary Tuberculosis and Controls Replication of *M. tuberculosis* in vitro.
(A) Mycobacterial growth in primary human monocyte-derived macrophages transduced with control scrambled shRNA or shRNA targeting *SMURF1* and infected with Mtb. Infected cells were lysed at the indicated time-points and mycobacterial growth was determined by counting CFUs. Bars are mean ± SEM of quadruplicate samples; each sample was normalized to day 0. Similar results were observed in two independent experiments. $^{**}P<0.05$; two-way ANOVA.
(B) Immunofluorescence staining using anti-Mtb (green) and anti-SMURF1 (red) antibodies of a lung biopsy from a patient with active pulmonary tuberculosis. Insets show representative mycobacteria that would be considered colocalized with SMURF1 in three different fields that were imaged. Scale bars, 5 μm.

Table S1, Related to Figure 6. H & E-stained lung sections from mice at 120 days after infection with *M. tuberculosis.**

Genotype	Polymorphonuclear Cell Infiltration	Mononuclear Cell Infiltration	Bronchitis/ Bronchiolitis
Wild-type (1)	+1	+2	+1
Wild-type (2)	+1	+2	+1
Wild-type (3)	+1	+1	+1
Wild-type (4)	+1	+2	+1
Wild-type (5)	+3	+1	+1
Wild-type (6)	+2	+2	+1
Wild-type (7)	+2	+2	+1
Smurf1$^{-/-}$ (1)	+1	+3	+1
Smurf1$^{-/-}$ (2)	+1	+3	+2
Smurf1$^{-/-}$ (3)	+1	+3	+2
Smurf1$^{-/-}$ (4)	+1	+2	+2
Smurf1$^{-/-}$ (5)	+1	+2	+1
Smurf1$^{-/-}$ (6)	+1	+2	+1

*Lung sections were analyzed by a pathologist blinded to the genotype of each mouse. Analysis was performed using standard criteria for scoring mouse lungs with the following semiquantitative scale: 0: negative; +1: mild; +2: moderate; +3: severe.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cells and Cell Culture

Bone marrow-derived macrophages (BMDMs) were generated from mice by culturing bone marrow cells from mouse femurs and tibia in DMEM supplemented with 20% FBS, 30% L929 cell-conditioned media (Shiloh et al., 2008) and 1% sodium pyruvate (Sigma). After 7 days in culture, differentiated BMDMs were harvested and cultured in DMEM supplemented with 10% FBS and 5% L929 cell-conditioned media. The RAW 264.7 murine macrophage cell line was cultured in DMEM supplemented with 10% FBS. Primary human macrophages were obtained from buffy coats from anonymous donors provided by a local blood bank. Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Paque gradient (GE Healthcare) and adherent monocytes were cultured in RPMI 1640 supplemented with 10% human AB serum (Corning) and 50 ng/mL recombinant human M-CSF (R&D Systems) for 7 days. Cells were not exposed to antibiotics during processing or differentiation.

M. tuberculosis and *L. monocytogenes* Strains

Mtb Erdman was used as the wild-type strain throughout. The EccD1 mutant and mCherry-expressing strain were as described previously (Collins et al., 2015). Wild-type Mtb was grown in 7H9 medium supplemented with 0.5% glycerol, 0.05% Tween-80 and 10% Middlebrook OADC (BD Biosciences). Mtb expressing mCherry (mCherry-Mtb) and Mtb carrying a transposon mutation in EccD1 (Mtb EccD1::*tn*) (Stanley et al., 2003) were grown in the same conditions as the wild-type strain with addition of kanamycin (50 µg/mL). *L. monocytogenes* was obtained from D. Portnoy (UC Berkeley). For listeria experiments, we used *L. monocytogenes* 10403s ΔActA stably expressing RFP under the control of actA promoter (ΔActA-PACTA-RFP). *L. monocytogenes* ΔActA-PACTA-RFP was grown overnight to stationary phase in brain heart infusion broth media (BHI) for subsequent infection of BMDMs.

Macrophage Infections and Immunofluorescence Analyses

Cells were seeded on glass coverslips, incubated overnight and infected as follows. For Mtb infection, bacterial cultures were washed twice with PBS, bacterial aggregates were removed by low-speed centrifugation (300 x *g*), and the mycobacteria in suspension were sonicated to generate a single cell suspension. Bacteria were resuspended in DMEM supplemented with 10% horse serum for the infection (Shiloh et al., 2008; Stanley et al., 2003). Prior to infection, cells were washed with PBS, inoculated with the Mtb suspension [multiplicity of infection (MOI) ~1.0 colony-forming-units/cell] and centrifuged at 1500 rpm for 10 min and incubated at 37°C for 1 hr to allow BMDM phagocytosis of Mtb. BMDMs were then washed twice to remove extracellular bacteria, fresh macrophage media was added, and the cells were incubated at 37°C, 5% CO_2 for 15 hr. Where indicated, BMDMs were treated with the active autophagy-inducing Tat-Beclin 1-L11 peptide (sequence: YGRKKRRQRRR-GG-VWNATFHIWHD) or F270S inactive control peptide (which has a substitution from phenylalanine to serine at position 2 of the Beclin 1-derived C'terminal 11-mer) (sequence: YGRKKRRQRRR-GG-V**S**NATFHIWHD). These peptides have been previously published (Pietrocola et al., 2016) and were derived from longer peptide sequences described in (Shoji-Kawata et al., 2013). All peptides were synthesized by the University of Texas Southwestern Medical Center (UTSW) Protein Chemistry Technology Core and purified to >95% by HPLC (confirmed by mass spectrometry) and Tat-Beclin 1-L11 is commercially available (Biotechne). For *L. monocytogenes* infection, bacteria were grown overnight at 37°C in BHI media, washed two times with PBS, diluted in DMEM 10% FBS and BMDMs were infected at an MOI of 5. Plates were centrifuged 3500 rpm for 5 min and incubated at 37°C for 30 min to allow bacterial phagocytosis. Cells were washed twice and incubated at 37°C for 2 hr prior to fixation.

For LC3 immunofluorescence, infected cells were fixed for 10 min with methanol at -20°C. For all the other proteins labelled in this study, cells were washed in PBS, fixed with 4% paraformaldehyde and permeabilized with Triton X-100 0.3% for 10 min. Cells were incubated with primary antibodies (overnight for LC3 or Flag or 1 hr at 37°C for all the other proteins) and visualized with fluorescent secondary antibodies. The primary antibodies used were: anti-LC3 (Sigma L7543); anti-LAMP1 (Santa Cruz SC-8098); anti-polyubiquitin (Enzo FK1), humanized anti-K63 and anti-K48 (Matsumoto et al., 2012; Newton et al., 2012; Newton et al., 2008) (Genentech), anti-proteasome 20S β2i subunit (Enzo BML-PW8150), anti-Flag M1 (Sigma), anti-p62 (Abnova H00008878-M01), anti-NBR1 (Abcam ab55474). For GFP-LC3 puncta assays, uninfected GFP-LC3 BMDMs were seeded in glass coverslips and treated for 3 hr with Tat-Beclin 1-L11 or control peptides diluted in DMEM 10% FBS. Peptide-containing media was then washed out and replaced with fresh media and cells were cultured for an additional 3 hr. Bafilomycin A1 (Baf A1) (100 nM) was or was not added during the last 3 hr of culture. Coverslips were mounted

with Prolong Gold antifade with DAPI (Invitrogen). Z-stacks were acquired with a Zeiss AxioImager M2 microscope equipped with a Photometrics CoolSnap HQ2 camera and then deconvolved with AutoDeBlur (Bitplane). Imaris (7.4.0 version; Bitplane) was used for analysis. A minimum of 100 bacteria was analyzed per sample and quadruplicate samples were analyzed for each condition, or 100 cells were analysed per sample in GFP-LC3 puncta assays.

Measurement of Bacterial Growth in Macrophages

Mtb single cell suspensions were prepared as described above. Human macrophages or BMDMs were seeded in 48-well plates and infected at a multiplicity of infection (MOI) of 1 colony-forming unit (CFU)/cell in quadruplicate. Where indicated, infected BMDMs were treated with the active autophagy-inducing Tat-Beclin 1-L11 peptide or F270S inactive control peptide as described above. Cells were lysed at the indicated time-points with 0.5% Triton X-100 (Sigma) in water and serial dilutions were plated on 7H11 plates. CFUs were counted after 20-25 days of incubation. For *L. monocytogenes* CFU experiments, bacterial suspensions were prepared as described above. BMDMs were seeded in 96-well plates and infected at an MOI of 1. Plates were incubated at 37º C for 30 min to allow bacterial phagocytosis, followed by two washes with DMEM 10% FBS to remove extracellular bacteria. Infected macrophages were washed every 2 hr to remove extracellular bacteria. Cells were lysed at the indicated time-points with 0.5% Triton X-100 in water and serial dilutions were plated on brain heart infusion (BHI) media. CFU were counted after 24 hr of incubation at 37ºC.

Lentiviral Transduction For Transgene Expression or Knockdown

For transgenic expression of SMURF1, full-length human SMURF1, SMURF1ΔC2 (deleted of amino acids 1-119) or SMURF1 C699A (Zhu et al., 1999) were cloned into the entry vector pENTR1A containing the 3xFlag sequence, followed by subcloning into the lentiviral GATEWAY destination vector pLENTI CMV-Puro using LR recombinase (Invitrogen). All plasmids were sequenced to confirm the correct gene sequence. For *SMURF1* knockdown, shRNA specific for human *SMURF1* was obtained from Sigma (MISSION shRNA; TRCN0000003471 and TRCN000003473). A scrambled sequence was used as a control. Lentiviral particles were obtained by transfecting HEK293T cells with lentiviral vectors and the packaging plasmids pCMVΔ8.91 and pMDG for transgenic expression in mouse cells or psPAX2 and pMD2.G for gene knockdown. For *SMURF1* transgene expression in BMDMs, bone marrow cells were harvested and incubated for 3 days with DMEM supplemented with 20% FBS and 30% L929 cells conditioned media. Lentivirus-containing supernatant was added to cultures at days 3 and 4 with incubation at 37º C in 5% CO_2. The supernatant was replaced by fresh media and cells were incubated for 48 hr in the presence of 3 µg/mL of puromycin for selection prior to infection with Mtb. For *SMURF1* transgene expression in RAW 264.7 cells, virus-containing supernatant was incubated with cells for 48 hr, followed by selection with puromycin (0.5 µg/mL) for at least 10 days. The expression of 3xFlag-SMURF1 was evaluated by immunobloting as described below. For *SMURF1* knockdown in primary human macrophages, adherent monocytes isolated from PBMC were cultured with 50 ng/mL recombinant human M-CSF (R&D Systems) for 3 days and adherent cells were detached and seeded in 48-wells plates. Lentivirus-containing supernatants were added at days 3 and 4. At day 5, supernatant was replaced by fresh media and cells were incubated for 48 hr in the presence of puromycin for selection. *SMURF1* mRNA levels were evaluated by RT-qPCR using KiCqStart pre-designed primers from Sigma. The sequences of the primers used were: forward: GAGACGTTCGATGAAGAAAG; reverse: GAATGTCGATCCGGTTAAAG.

Western Blot Analyses

To evaluate LC3 lipidation and p62 expression by immunoblotting, BMDMs were treated for 2 hr with the active autophagy-inducing Tat-Beclin 1-L11 peptide or F270S inactive control peptide (Pietrocola et al., 2016) diluted in OPTI-MEM (Gibco) acidified with 0.15% (v/v) 6N HCl (Shoji-Kawata et al., 2013). Cells were lysed in a buffer containing 100 mM NaCl, 20 mM Tris (pH 7.6), 10 mM EDTA (pH 8), 0.5% SDS, 1% Triton X-100, Halt phosphatase inhibitor cocktail (Thermo) and protease inhibitor cocktail (Roche). Laemmli sample buffer was added to lysates and samples were boiled for 5 min. Proteins were separated by SDS-PAGE in 4-20% polyacrylamide resolving gel (Bio-Rad). After electrophoresis, proteins were transferred to nitrocellulose membranes. To detect expression of SMURF1 in *Smurf1*$^{-/-}$ BMDMs transduced with 3xFlag-SMURF1 wild-type, SMURF1 C699A or SMURF1ΔC2, cells were lysed and proteins were separated by SDS-PAGE as described above. The following antibodies were used: anti-LC3 (Novus NB600-1384), anti-p62 (Abnova H00008878); anti-actin-HRP (Santa Cruz sc-47778), anti-Flag-HRP (Sigma A8592).

Immune Electron Microscopy:

To detect K48 ubiquitin at the ultrastructural level, BMDMs were infected with Mtb for 15 hr. Cells were fixed in 4% paraformaldehyde and 7.5% sucrose in 0.1 M sodium phosphate buffer (pH 7.4) for 30 min, followed by permeabilization and blocking with 3% BSA and 0.1% saponin for 40 min. Primary anti-K48 antibody (Genentech) diluted in blocking buffer (1:1000) was incubated with samples for 1 hr at 37ºC. Cells were washed and incubated with the secondary 1.4 nm gold-conjugated Fluoronanogold anti-human AlexaFluor 488 antibody (Nanoprobes 7253) diluted in blocking buffer (1:100) for 1 hr at room temperature. Cells were washed and incubated with 1% glutaraldehyde for 10 min, followed by incubation with 50 mM glycine for 5 min. After washing in water, immunogold-labeled samples were silver-enhanced for 2 min using an HQ Silver Enhancement kit (Nanoprobes), followed by washing in 1% thiosulfate. Samples were incubated overnight with 2.5% glutaraldehyde in 0.1 M cacodylate buffer. The following day, samples were washed in 0.1 M cacodylate buffer and incubated with 1% osmium, 0.8% K3[Fe(CN)6] in 0.1 M cacodylate buffer for 1 hr. Samples were dehydrated in graded ethanol and embedded in resin. Thin sections were stained with uranyl acetate and lead citrate before imaging using a Tecnai Spirit Biotwin (FEI) electron microscope.

Aerosol Infection

Mtb aerosol infection was performed as described (Collins et al., 2015). Briefly, cultures were washed twice with PBS, sonicated to disperse clumps and resuspended in PBS at an O.D. of 0.1. Bacterial suspension was introduced into the nebulizer of a GlasCol aerosol chamber to infect mice with ~200 bacteria per animal. On day zero of infection, lungs were collected from 5 mice and the entire lung homogenate was plated on 7H11 plates to determine the initial inoculum. Subsequently, left lungs, livers and spleens were collected, homogenized and plated on 7H11 plates for quantitation of CFUs at the indicated time points. For survival studies, infected mice were sacrificed when they had lost 15% of their maximal body weight (Collins et al., 2015).

Cytokine Quantitation

Cytokines were measured in lungs homogenates from mice using the MILLIPLEX Map Kit (Millipore) according to the manufacturer's instructions.

Histopathology

Lungs from infected mice were collected and treated with 10% buffered formalin for 24 hr, transferred to PBS, paraffin embedded, and histological sections were stained with haematoxylin and eosin (H&E). Slides containing lungs sections were scanned and quantitation of the inflammation area in each lung was performed with ImageJ software (NIH). Histopathological analysis of bronchitis, monocytic inflammation, and neutrophil inflammation was performed by an experienced pathologist blinded to the genotype of each mouse lung. Each lung was analyzed using standard criteria for scoring mouse lungs with the following semiquantitative scale: 0: negative; +1: mild; +2: moderate; +3: severe. After unblinding, the average score from 6-7 mice per genotype was calculated.

Immunohistochemistry and Immunofluorescence in Human Specimens

For immunohistochemical staining of archived human lung tissue, paraffin-embedded tissue biopsy samples were deparaffinized in xylene; antigen retrieval was performed in heated citric acid 0.01 M (pH 6.0) and blocked in 2% goat serum. SMURF1 was stained with anti-SMURF1 produced in mouse (1:100; Sigma WH0057154M1) and secondary biotinylated anti-mouse 1:50. Staining was performed with AB reagent (Vectastain) and detected with DAB reagent (Thermo Scientific). For immunofluorescence, paraffin-embedded human specimens were processed as described above and Mtb was detected using anti-Mtb (1:100; antiserum guinea pig, BEI Resources, NR-13818) and secondary donkey anti-guinea pig conjugated to Alexa-Fluor 488 (1:500; Life Technologies). SMURF1 was detected using mouse anti-SMURF1 (Sigma) and secondary bovine anti-mouse conjugated to HRP (1:500; Santa Cruz) followed by amplification with tyramide (1:100; PerkinElmer) according to the manufacturer's instructions. Prolong Gold antifade with DAPI (Invitrogen) was used to visualize the nuclei and to mount the slides. Z-stacks were acquired with a Zeiss AxioImager M2 microscope equipped with a Photometrics CoolSnap HQ2 camera and then deconvolved with AutoDeBlur (Bitplane). Imaris (7.4.0 version; Bitplane) was used for analysis. This study was approved by the Institutional Review Board of the University of Texas Southwestern Medical Center.

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