Supporting Information

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SI Materials and Methods

FACS Analysis. MH–S cells were resuspended in PBS-EDTA 2 mM, fixed with 3% paraformaldehyde (PFA) for 30 min, and then were permeabilized or not with 0.1% Triton X-100 at 4 °C. After FcR blockage with anti-mouse CD16/CD32 antibody (Ab) (Pharmingen), cells were labeled with phycoerythrin-conjugated mouse IgG2a anti-TLR5 (Imgenex) or isotype-control (MOPC-173; eBioscience) antibodies. FACS analysis was performed using the FACScan flow cytometer instrument with CellQuest software (Fig. S1).

RNA Extraction and Quantitative RT-PCR. Total lung RNA was extracted with the RNeasy kit (Qiagen). Purified RNA (1 μg) was reverse-transcribed using MMLV reverse transcriptase (Promega) and a random primer p(dN)6 (Roche Diagnostics) according to the protocol of the manufacturer. The TLR5 and 18s mRNA levels were determined by real-time quantitative RT-PCR using 75000 Real-time PCR Systems (Applied Biosystems) and were performed in quadruplicate. QuantiTect SYBR Green PCR kit (Qiagen) was used according to the instructions of the manufacturer. Relative gene expression was calculated by comparing the number of thermal cycles that were necessary to generate threshold amounts of product (CT). CT was calculated for the TLR5 gene and for the housekeeping gene 18s. For each cDNA sample, the 18s CT was subtracted from the CT for TLR5 to yield ΔCT, thus normalizing the initial amount of RNA used. The amount of TLR5 mRNA was calculated as $2^{-\Delta\Delta CT}$, where ΔΔCT is the difference between the ΔCT of the two cDNA samples to be compared. Sequences of murine TLR5 primers were as follows: mTLR5-F, GCATAGCCTGAGCCTGTTTC; mTLR5-R: AAGTTCCGGGGAATCTGTTT) (Fig. S1).

Bacterial Binding Assays. MH–S cells (1.5×10^5 cells) were fixed in 3% PFA. After treatment with different amounts (0.5–2 μg) of purified *P. aeruginosa* flagellin, cells were incubated with WT PAK labeled with FITC (as described in the protocol of the phagocytosis assay, see *Materials and Methods*) at an MOI of 100 for 1 h. Then, nonadherent bacteria were removed by washing cells with PBS. The fluorescence arbitrary units, corresponding to bacterial binding, were measured on a FluoStar Optima (BMG Labtech) (Fig. S2).

Western Blot. Primary AMs (5×10^5) were infected with bacteria (MOI: 1) for 2–4 h and then were lysed in buffer [5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 50 mM Tris HCl (pH 7.4)] with anti-proteases (Roche Diagnostics). Protein lysates were run in a 12% SDS/PAGE, transferred onto PVDF membranes, and probed with Ab against murine pro-IL-1β, caspase-1 p10 (Santa Cruz Biotechnology), AEP (R&D Systems), or actin (Sigma) (Fig. 3 and Fig. S3).

Luminescence Activity. Cell supernatants of PAK-infected MH–S cells (MOI: 10; 4 h) were collected and centrifuged to eliminate bacteria and incubated with a culture of luminescent bacteria (PAKLux) to mid-log phase. PAKLux growth was evaluated by quantification of photon emission expressed as relative luciferase units (RLUs), using an EGNG Berthold luminometer. As a positive control of bactericidal activity, PAKLux was cultured with tobramycin (40 μg/mL), and the resultant growth was assayed as above (data not shown) (Table S1).

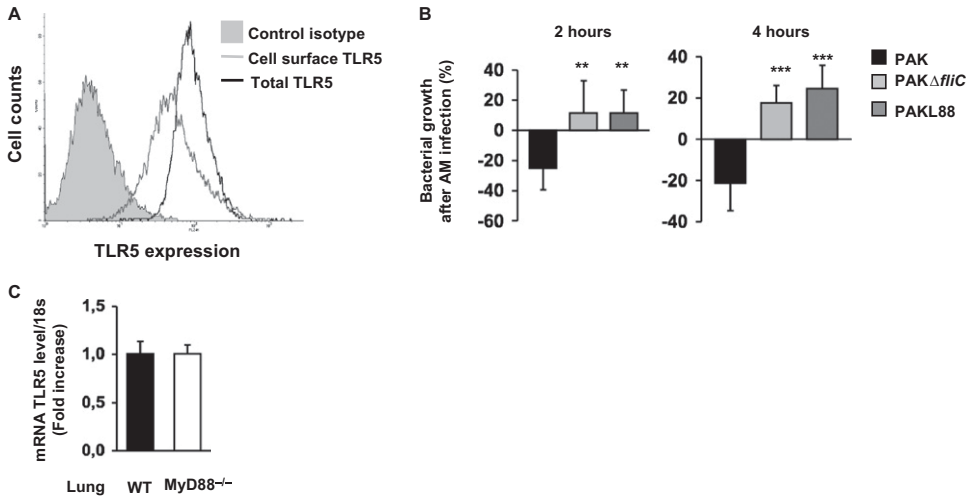

Fig. S1. Unflagellated *P. aeruginosa* or mutants expressing a flagellin mutated into TLR5-site recognition are not killed by AMs. (*A*) Surface (gray line) or total (black line) TLR5 expression of MH–S cells was analyzed by FACS. (*B*) MH–S cells were infected with WT PAK, PAKD*fliC* (deficient for flagellin expression), or PAKL88 (expressing a flagellin mutated into TLR5-recognition site) mutants at an MOI of 0.1 during 2 h or 4 h. CFUs were quantified in cell supernatants pooled with cell lysates after 0.1% Triton X-100 treatment. Results are means ± SD of three experiments (n = 3/group for each experiment) and are expressed as in Fig. 1. (**$P < 0.01$; ***$P < 0.001$.) (*C*) TLR5 mRNA expression in lung of WT or MyD88$^{-/-}$ mice. TLR5 mRNA was analyzed by quantitative RT-PCR and normalized with 18s levels. Results are expressed as fold increase over WT mice.

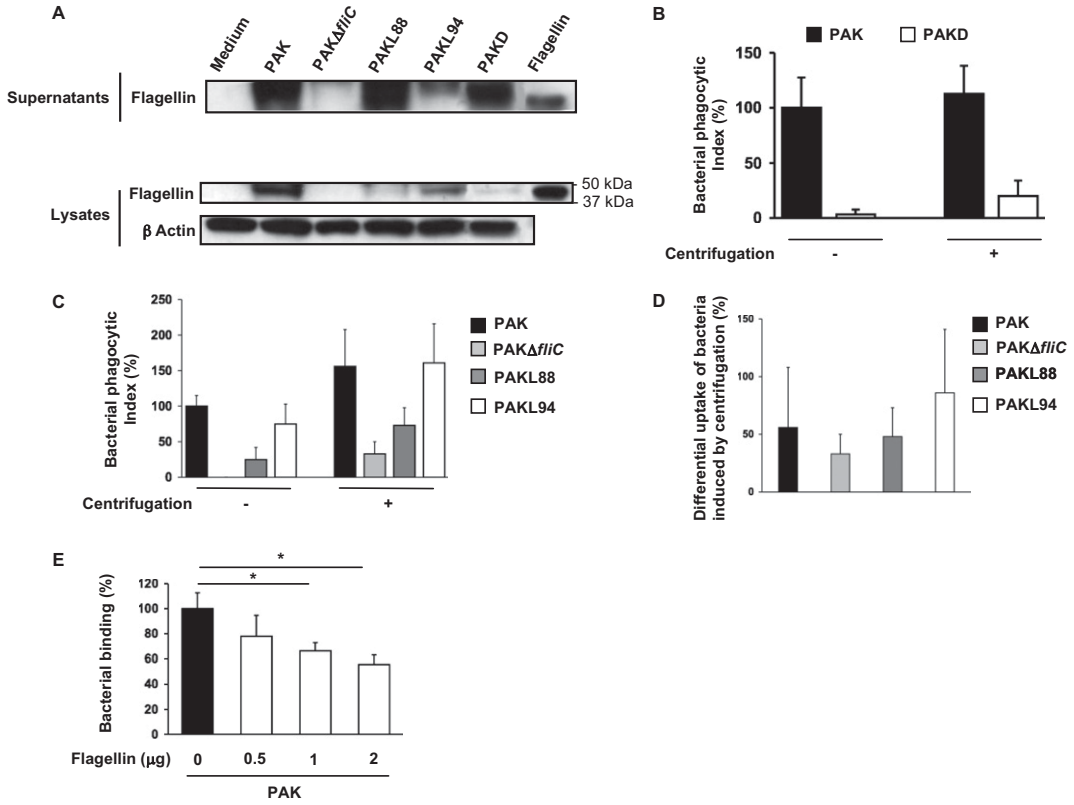

Fig. S2. The specific flagellum/TLR5 interaction plays a major role in *P. aeruginosa* binding to macrophages and in *P. aeruginosa* internalization. (*A*) Western blot analysis of flagellin in supernatants or in lysates of MH–S cells (5×10^5 cells) infected with either WT PAK, PAKΔ*fliC*, PAKL88, PAKL94, or PAKD mutants at an MOI of 10 for 1 h. Recombinant *P. aeruginosa* flagellin, purified from *E. coli* strain BL21 expressing WT flagellin was used as a positive control for migration. Flagellin was revealed with a specific anti-flagellin antibody (1, 2). Equal loading was controlled for by β-actin detection. (*B* and *C*) MH–S cells were infected with either WT PAK or PAKD, PAKD*fliC*, PAKL88, or PAKL94 mutants at an MOI of 10 for 1 h with or without centrifugation. Then, cells were washed and treated with 40 μg/mL tobramycin for 30 min, and the number of ingested bacteria was determined by counting CFUs on LB agar plate in cellular lysates after 0.1% Triton X-100 treatment. (*B*) Results are means ± SD of two experiments (n = 3/group for each experiment) and are expressed as in Fig. 2. (*C*) Data are means ± SD (n = 3/group for each group) and are expressed as in Fig. 2. Results are representative of three independent experiments. (*D*) Centrifugation enhances bacterial strains uptake by MH–S cells independently of the TLR5-interaction. The centrifugation-induced bacterial uptake was quantified from data presented in *C* as follows: CFU counts in cells centrifuged − CFU counts in cells not centrifuged. Data are means (n = 3/group). Results are representative of three independent experiments. (*E*) Binding of WT PAK-FITC in presence of purified *P. aeruginosa* flagellin. MH–S cells were fixed in 3% PFA, washed and treated with purified *P. aeruginosa* flagellin for 1 h, and then infected with WT PAK-FITC. Results are means ± SD of two experiments (n = 4–6/group; $^*P < 0.05$) and are expressed as percentage of bacterial binding = [fluorescence arbitrary units (AU) in cells treated with flagellin and WT PAK-FITC/fluorescence AU in cells treated with WT PAK-FITC alone] × 100.

1. Franchi L, et al. (2007) Critical role for Ipaf in Pseudomonas aeruginosa-induced caspase-1 activation. *Eur J Immunol* 37:3030–3039.
2. Lightfield KL, et al. (2011) Differential requirements for NAIP5 in activation of the NLRC4 inflammasome. *Infect Immun* 79:1606–1614.

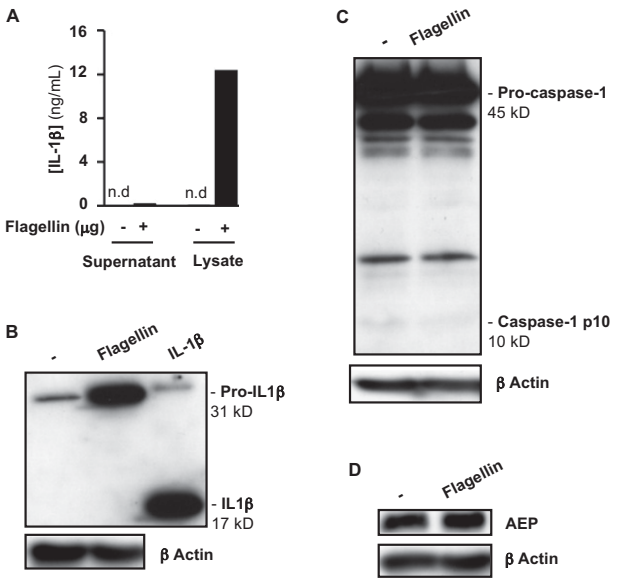

Fig. S3. IL-1β processing and production and AEP expression following purified flagellin stimulation of primary AMs. Primary AMs (5×10^5 cells) were stimulated with recombinant purified flagellin (1 μg/mL). After 4 h, levels of IL-1β were measured by ELISA in supernatants or AM lysates (*A*). n.d, not detected. IL-1β (*B*), caspase-1 (*C*), and AEP (*D*) expression was analyzed by Western blot in AM lysates. Equal loading was controlled for by β-actin detection. Results are representative of two independent experiments.

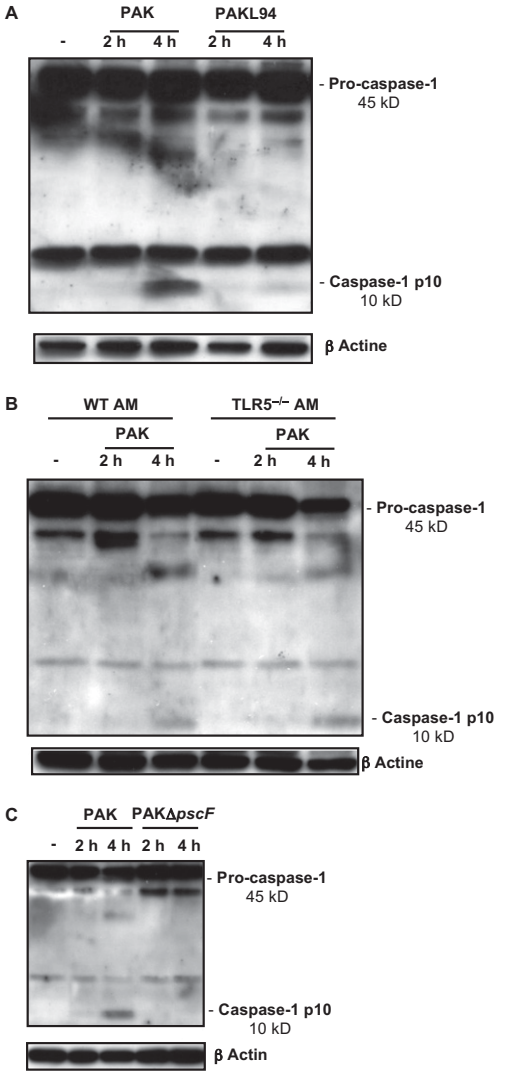

Fig. S4. Caspase-1 activation following bacterial infection of primary AMs. Primary WT AMs (5×10^5 cells) were infected with either WT PAK, PAKL94 (*A*), or PAKΔ*pscF* (*C*) mutant (MOI: 1). After 2 or 4 h, caspase-1 processing was analyzed in cell lysates by Western blot. (*B*) In similar experiments, primary WT or TLR5$^{-/-}$ AMs were infected with WT PAK (MOI: 1) and caspase-1 activation was analyzed as above. Equal loading was controlled for by β-actin detection. Results are representative of three independent experiments.

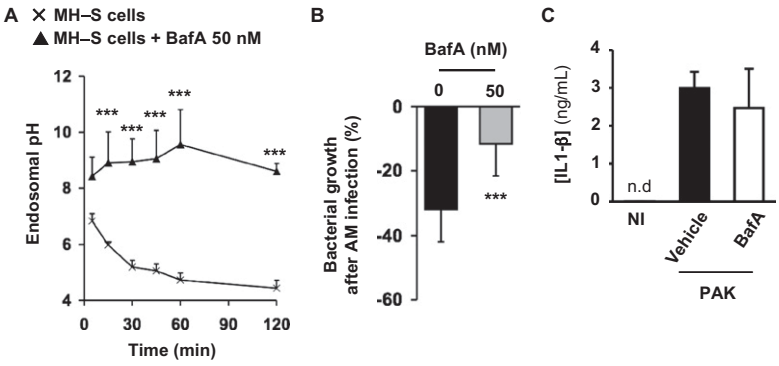

Fig. S5. Inhibition of endosomal acidification reduces *P. aeruginosa* clearance by AMs but has no effect on IL-1β secretion. (*A*) Kinetics of endolysosomal pH in MH–S cells was measured in the presence or not of BafA (50 nM; Sigma). Results are means ± SD of three experiments (n = 3/group for each experiment). Unpaired t test was used to assess statistical significance between same time points (***$P < 0.0001$). (*B*) MH–S cells were incubated with or without BafA (50 nM) for 1 h, infected with WT PAK (MOI: 0.1) during 4 h and CFU were quantified as in Fig. 1. Results are means ± SD of three experiments (n = 3/group for each experiment) and are expressed as in Fig. 1. (***$P < 0.001$.) (*C*) Primary WT AMs (10^5) were incubated with or without BafA (50 nM) for 1 h and then infected with WT PAK (MOI: 1) for 4 h. IL-1β was then measured in culture supernatants by ELISA. Results are means ± SD of three experiments (n = 3/group for each experiment). NI, noninfected cells; n.d, not detected.

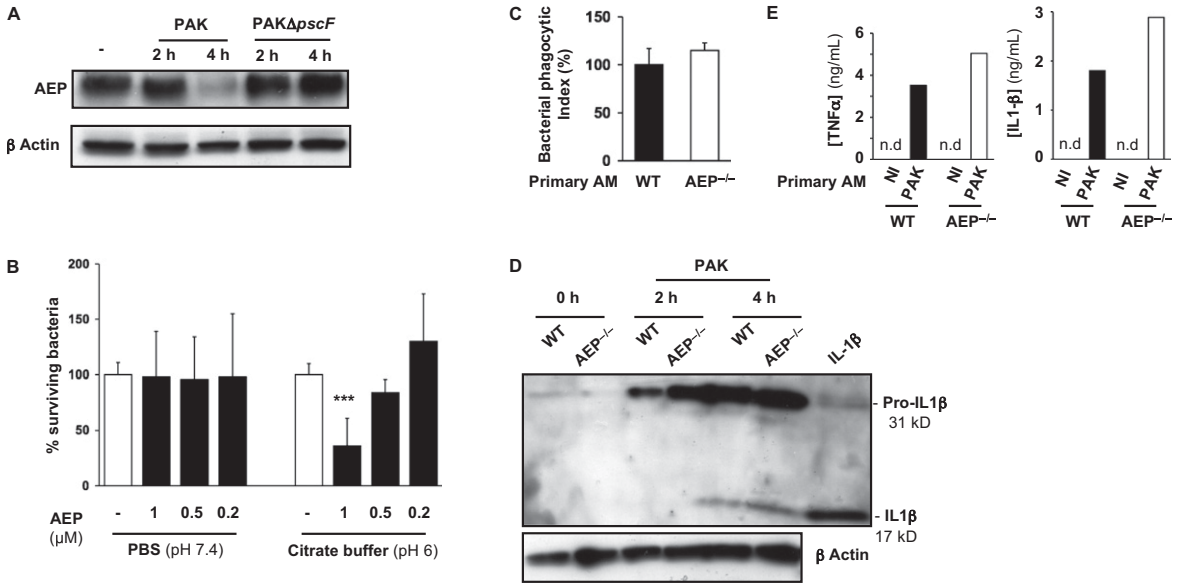

Fig. S6. AEP participates in AM-mediated *P. aeruginosa* killing without modifying bacterial uptake nor cytokine production. (*A*) AEP expression was analyzed by Western blot in lysates from primary WT AMs (5×10^5 cells) infected with WT PAK or PAKΔ*pscF* mutant (MOI: 1). Equal loading was controlled for by β-actin detection. Results are representative of three independent experiments. (*B*) Bacteriolytic assays were performed at 37 °C in either PBS (pH 7.4) or 50 mM citrate buffer (pH 6) by incubating during 2 h WT PAK (5×10^5 CFU) with 0.2, 0.5, or 1 μM of recombinant AEP protease (kindly given by Dr. C. Watts, College of life Sciences, University of Dundee, Dundee, UK). Surviving bacteria numbers were determined by counting CFU on LB agar plates. (*C*) Primary WT or AEP$^{-/-}$ AMs (5×10^5 cells) were infected with WT PAK (MOI: 10) during 1 h. After infection, cells were washed and treated with 40 μg/mL tobramycin 30 min, and the number of ingested bacteria was determined by counting CFU on LB agar plate. Results are representative of three experiments (n = 3/group for each experiment) and are expressed as in Fig. 2. (*D*) Lysates from primary WT or AEP$^{-/-}$ AMs (5×10^5 cells) infected with WT PAK (MOI: 1) were assessed for pro-IL-1β processing. Equal loading was controlled for by β-actin detection. (*E*) Primary WT or AEP$^{-/-}$ AMs (1×10^5 cells) were infected with WT PAK (MOI: 1). After 4 h, TNFα and IL-1β were measured in culture supernatants by ELISA. Results are means (n = 3/group for each experiment) and are representative of two independent experiments. NI, noninfected cells; n.d, not detected.

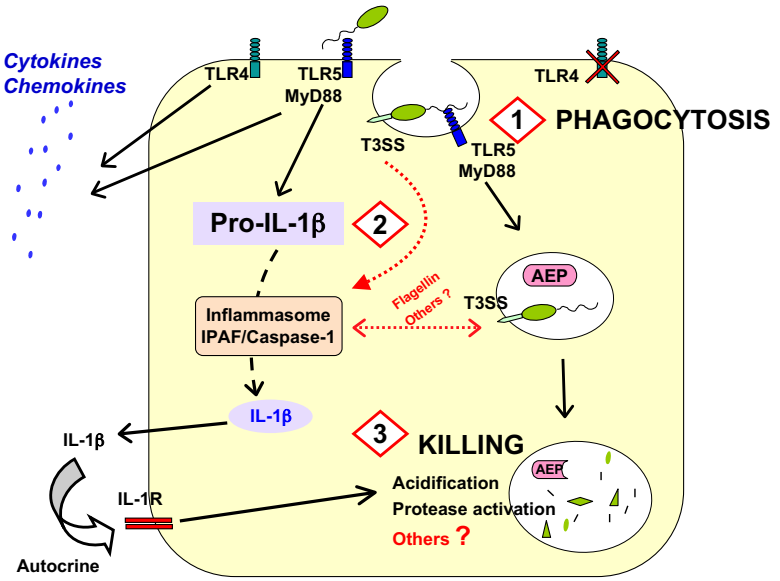

Fig. S7. Phagocytosis and bacterial clearance of *P. aeruginosa* by AMs. During an infection of AMs with flagellated *Pseudomonas aeruginosa*, bacteria are phagocytosed following a TLR5-MyD88 interaction, without the involvement of TLR4 (1). The flagellin/flagellum interaction with TLR5 is crucial for signaling and pro-IL-1β production (2). Furthermore, the presence of an intact T3SS, secreted through the syringe, either before or after engulfment into the phagolysosome, is necessary for inflammasome engagement, activation of caspase-1, and maturation of pro-IL-1β into mature IL-1β. The latter is key in the induction of phagolysosome acidification (3). Finally, acidification triggers the activation of the cysteine lysosomal protease AEP, which is shown to be a key factor in the killing of phagocytosed bacteria (3).

Table S1. Strains of *P. aeruginosa*

Strain	Relevant characteristics	Flagellin	Motile or nonmotile	Ref.
PAK	Wild-type laboratory strain expressing different virulent factors (pili, flagella, the type II and III secreted enzymes)	Wild-type	Motile	1
PAKΔ*fliC*	Nonflagellated PAK mutant with a deletion of the flagellin gene (*fliC*, the primary flagellar unit)	—	Nonmotile	1
PAKL88	Flagellated PAK mutant with an L88A amino acid substitution into TLR5-binding site on flagellin, defective in stimulating an inflammatory response	TLR5-binding site mutant L88	Motile	1
PAKL94	Flagellated PAK mutant with an L94A amino acid substitution into TLR5-binding site on flagellin, defective in stimulating an inflammatory response	TLR5-binding site mutant L94	Motile	1
PAKD	Nonflagellated PAK mutant with gentamicin resistance gene cassette inserted into flagellar cap gene, production of unpolymerized flagellin monomer	Wild-type	Nonmotile	2
PAKΔ*pscF*	Flagellated PAK mutant with a deletion of *pscF* gene (a major needle protein of the T3SS), defective in T3SS	Wild-type	Motile	3
PAKLux	Luminescent PAK by inserting *LuxAB* gene into the neutral *att* site of PAK chromosome	Wild-type	Motile	2

PAKLux growth was determined by quantification of photon emission expressed as relative luciferase units (RLUs). Results were expressed using the formula: (RLU after 1 h incubation with supernatants/RLU at t_0) × 100. Data are representative of two independent experiments.

1. Verma A, Arora SK, Kuravi SK, Ramphal R (2005) Roles of specific amino acids in the N terminus of Pseudomonas aeruginosa flagellin and of flagellin glycosylation in the innate immune response. *Infect Immun* 73:8237–8246.
2. Balloy V, et al. (2007) The role of flagellin versus motility in acute lung disease caused by Pseudomonas aeruginosa. *J Infect Dis* 196:289–296.
3. Jyot J, et al. (2011) Type II secretion system of Pseudomonas aeruginosa: *In vivo* evidence of a significant role in death due to lung infection. *J Infect Dis* 203:1369–1377.

Table S2. Supernatants of AMs infected with bacteria have no bacteriolytic activity on PAKLux

Supernatant of infected MH–S cells	PAKLux growth (% of PAKLux-inoculum)
PAK	171% ± 18%
PAKΔ*fliC*	169% ± 23%
PAKL88	212% ± 24%
PAKL94	139% ± 5%