Supplemental Materials

Supplemental Figures

Figure S1. Phospho-tyrosine Western blot profiles from Figure 2 analyzed by GeneTools (Syngene) Software, related to Figure 2 A. Translocated $TEM^{pos}Ly6G^{+}$ PMNs or non-translocated $TEM^{neg}Ly6G^{+}$ PMNs isolated from spleens of mice infected with WT-ETEM or Δ*yopH*-ETEM for 5 days. (B) Magnification of (A). (C) BMPMNs infected with WT, Δ*yopH* or uninfected at MOI 10:1 for 30 min. Software scans each individual lane (higher to lower molecular weight) and assigns Rf values to each detected band and represents them as histogram plots (Profile heights vs Rf values). Each peak represents a band and the level of phosphorylation is represented as the area under that peak. Lanes can be compared by overlapping them.

Figure S2. Model of SLP-76 mediated immuno-receptor and integrin signaling, related to Figure 3. (1) Immuno-receptor ligation initiates activation of Src family kinases (SFK) that phosphorylates ITAMs present on the receptor chains. (2) Syk is recruited, activated, and phosphorylates LAT and SLP-76. Phosphorylated LAT associates with phospho lipase gamma (PLCγ) and Gads and in doing so, recruits SLP-76 to the membrane. (3) Phosphorylated SLP-76 associates with Vav1, Nck, and Itk, which together activate PLCγ and initiate Ca^{+2} flux and actin polymerization. (4) Association of SLP-76 with the ADAP/SKAP55/RIAM complex promotes inside-out signaling by localizing active Rap1. (5) Integrin-mediated signaling events that occur downstream of ligand binding requires structural changes and activation of Src family kinases that phosphorylaties of a variety of downstream molecules. These include

ITAM-containing adapters that, when phosphorylated, lead to the recruitment and activation of Syk kinase. (6) Syk in turn phosphorylates various substrates, including SLP76 and Vav. (7) SFKs can also activate FAK (focal adhesion kinases) such as paxillin. Figure S2 related to Figures 3, 4 and 5.

Figure S3. Levels of known YopH targets in different cell lines, related to Figure 4. J774 macrophages, BMPMNs, HEp2 (human epithelium cells), Jurkat T cells and mouse embryonic fibroblast (MEF) cells were lysed and 10µg of protein/lane were loaded into a SDS-PAGE gel and analyzed by Western blot for the presence of some of the known YopH targets.

Figure S4. Calcium flux inhibition prevents IL-10 production in neutrophils and IL-10 production is inhibited by YopH from extracellular *Yptb,* related to Figure 6. (A-B) Groups of five mice were infected IV with either WT-ETEM or *ΔyopH*-ETEM. Five days p.i, spleens were collected and TEM^{pos} $Gr1^{+}CD11b^{+}$ and TEM^{neg} $Gr1^{+}CD11b^{+}$ cells (both neutrophils and iMo) were sorted and lysed. IL-10 (A) and TNF-α (B) were isolated from clarified supernatants using the BD™ Cytometric Bead Array following manufacturer's instructions and analyzed by FACS. The average and STD from 5 experiments are shown. Statistical significance was calculated by one-way ANOVA followed by Tukey's multiple comparison post-test. (C-D) Single cell suspensions of spleens from mice intravenously infected for 4 days with 1,000 CFUs of WT-ETEM, 10,000 CFUs of *ΔyopH*-ETEM or 5,000 CFUs of *ΔyopE*-ETEM were assayed for the presence of intracellular bacteria using a gentamicin protection assay – spleen

cells were assayed for total CFU (C) and with gentamicin treatment (D). (E-F) Single cell suspensions of spleens from mice intravenously infected for 5 days with 1,000 CFUs of WT-ETEM were plated for total CFU (E). Splenocytes were then incubated with 1µg/ml CCF4-AM (Invitrogen), 1.5mM probenecid (Sigma) and 100 µg/ml gentamicin. TEM^{pos} and TEM^{neg} cells were sorted, collected, lysed and plated for CFUs and the number of gentamicin-resistant bacteria per 100,000 TEM^{pos} or TEM^{neg} cells was determined (F). (G-H) BMPMNs ($8x10^4$ cells/well) were treated with 50µM APB, or 5µM EGTA for 30 min before BMPMNs were infected with Δ*5*+pBAD, Δ*5*+pYopH or Δ*5*+pYopE at MOI 10:1 for 4hours, then 100µg/ml gentamicin was added and supernatant collected12 hours later. Supernatants were collected and IL-10 concentrations (G) and LDH released (H) were determined. Three independent experiments were performed in triplicate. The average +/- STD of one representative experiment is shown. Statistical significance was calculated using one-way ANOVA followed by Tukey's multiple comparison post test.

Supplemental Table 1*. *Yersinia* strains used in this study related to Figures 1-6.

Strains		
Y. pseudotuberculosis	IP2666c $pIBI^+$ *yopH*(NdeI)	(Ivanov et al., 2005)
IP2666 ETEM	Amino acids 1-100 of YopE+TEM1	(Harmon et al., 2010)
IP2666 Δ*yopH*-ETEM	Deletion of *yopH* +Amino acids 1-100 of YopE+TEM1	This work
IP2666 Δ*yopE*-ETEM	Deletion of *yopE* +Amino acids 1-100 of YopE+TEM1	Cynthia Castillo
IP2666 Kan	Wild type *Yptb* IP2666c $pIBI^+$ *yopH*(NdeI): Kan^R	(Fisher et al., 2007)
IP2666 Δ*yopH*	Deletion of *yopH*	(Fisher et al., 2007)
IP2666 Δ5	Deletion of *yopH*, *yopE*, *yopJ*, *yopM*, *yopO*	(Auerbuch et al., 2009)
IP2666 Δ5 + pBAD33	Deletion of *yopH*, *yopE*, *yopJ*, *yopM*, *yopO* + pBAD33	This work
IP2666 Δ5 + pYopH	Deletion of *yopH*, *yopE*, *yopJ*, *yopM*, *yopO* + pYopH	This work
IP2666 Δ5 + pYopE	Deletion of *yopH*, *yopE*, *yopJ*, *yopM*, *yopO* + pYopE	This work
Plasmids		
pSR475-ETEM	YopE-TEM1; Kan^R	(Harmon et al., 2010)
pBAD33	Arabinose-inducible expression vector; Cm^R	(Guzman et al., 1995)
pYopH	Expressed *yopH* from pBAD33; Cm^R	Lauren Logsdon
pYopE	Expressed *yopE* from pBAD33; Cm^R	Lauren Logsdon

*Table S1 related to Figures 1-6

Supplemental Experimental Methods

Cytokine detection in PMNs isolated from spleens of infected mice

Groups of five mice were infected IV with either WT-ETEM or *ΔyopH*-ETEM. Five days p.i, spleens were collected and $TEM^{pos}Gr1^{+}CD11b^{+}$ and TEM^{neg} $Gr1^{+}CD11b^{+}$ cells (both neutrophils and iMo) were isolated as described above with the addition of 3.0 µg/ml brefeldin A to enhance detection of intracellular cytokines. $TEM^{pos}Gr1^{+}CD11b^{+}$ and TEM^{neg} $Gr1^{+}CD11b^{+}$ cells were lysed with a eukaryotic lysis buffer (Durand et al., 2010) for 30 minutes at $4^{o}C$ in the presence of protease inhibitor cocktail (Sigma P8340), 100µM sodium orthovanadate and 20mM sodium fluoride. Cytokines were isolated from clarified supernatants using the BD™ Cytometric Bead Array following manufacturer's instructions and analyzed in a FACS LSR II. Statistical significance was calculated by one-way ANOVA followed by Tukey's multiple comparison post test.

Gentamicin protection assay from total splenocytes

Single cell suspensions of spleens from animals intravenously infected for 4 days with 1,000 CFUs of WT-ETEM, 10,000 CFUs of *ΔyopH*-ETEM or 5,000 CFUs of *ΔyopE*-ETEM were prepared by gently dissociating the tissue through 70 µm nylon mesh. An aliquot was plated for total CFUs before 100 µg/ml of gentamicin was added. After 2 hour at 37°C in 5% CO_2, the cells were washed three times with PBS, lysed with 100 µl of 1% Triton-X-100 for 15 min followed by the addition of 900 µl PBS, then lysates diluted and plated for CFUs to determine the number of total and gentamicin-protected bacteria. The percentage of gentamicin-protected bacteria was determined by dividing the number of

gentamicin-protected CFU by the total number of CFU and multiplying that by 100.

Gentamicin protection assay of sorted TEM^{pos} and TEM^{neg} splenocytes

Single-cell suspensions of spleens from mice intravenously infected for 5 days with 1,000 CFUs of WT-ETEM were prepared by gently dissociating the tissue through 70 µm nylon mesh. Samples were assayed for CFUs before 100 µg/ml of gentamicin was added. Splenocytes were labeled for 30 minutes in the dark at room temperature in media containing 1µg/ml CCF4-AM (Invitrogen), 1.5mM probenecid (Sigma) and 100 µg/ml gentamicin. 1x10^5 TEM^{pos} and TEM^{neg} cells were collected by FACS, washed, and lysed with 100 µl of 1% Triton-X-100 for 15 min followed by the addition of 900 µl PBS, and then the lysates were plated for CFUs to determine the number of gentamicin-protected bacteria.

References

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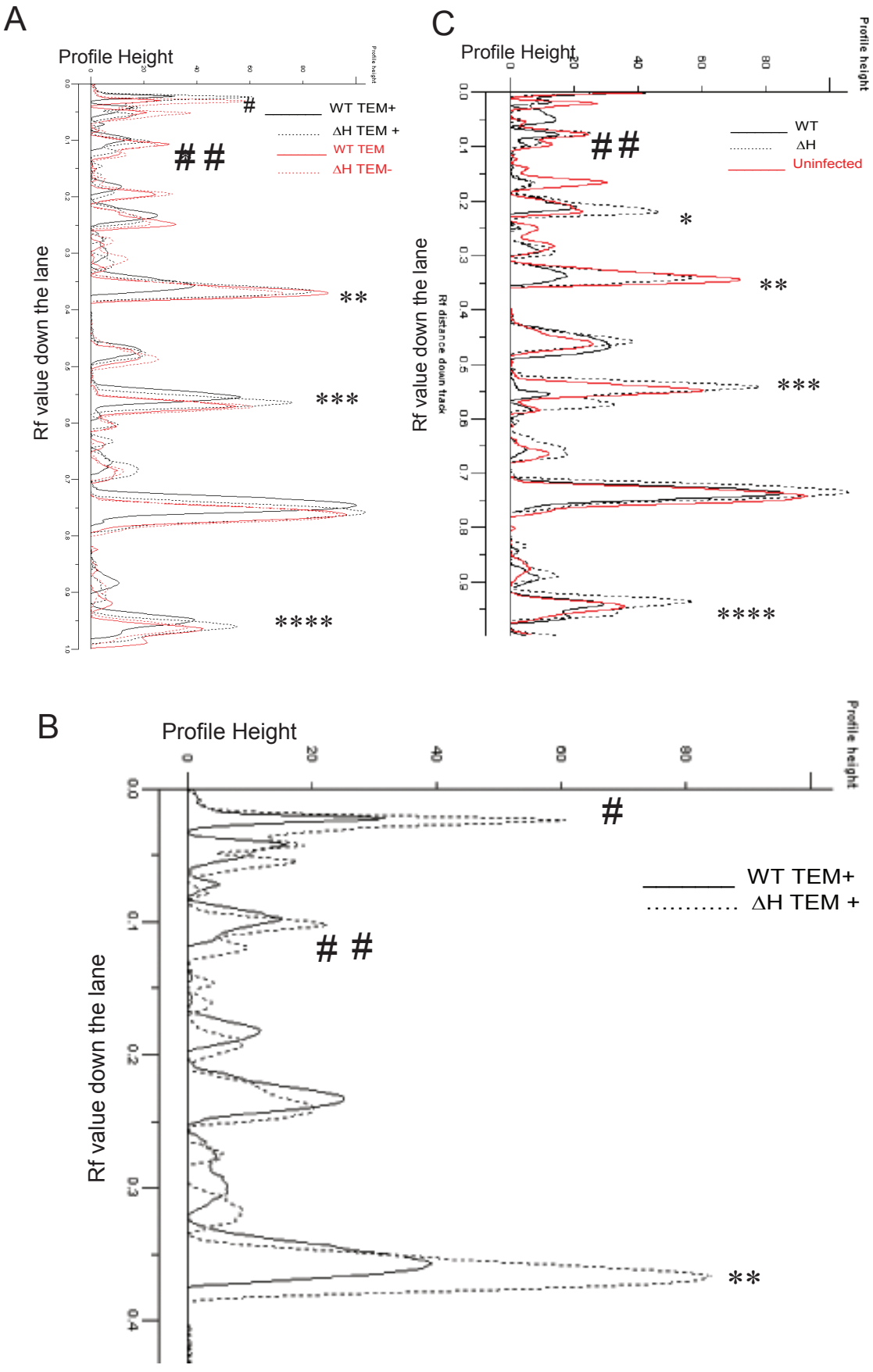
Figure S1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WT TEM+
ΔH TEM +
WT TEM
ΔH TEM-
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C
Profile Height
Rf value down the lane
Rf distance down track
Profile height
WT
ΔH
Uninfected
##
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B
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Rf value down the lane
Profile height
#
# #
WT TEM+
ΔH TEM +
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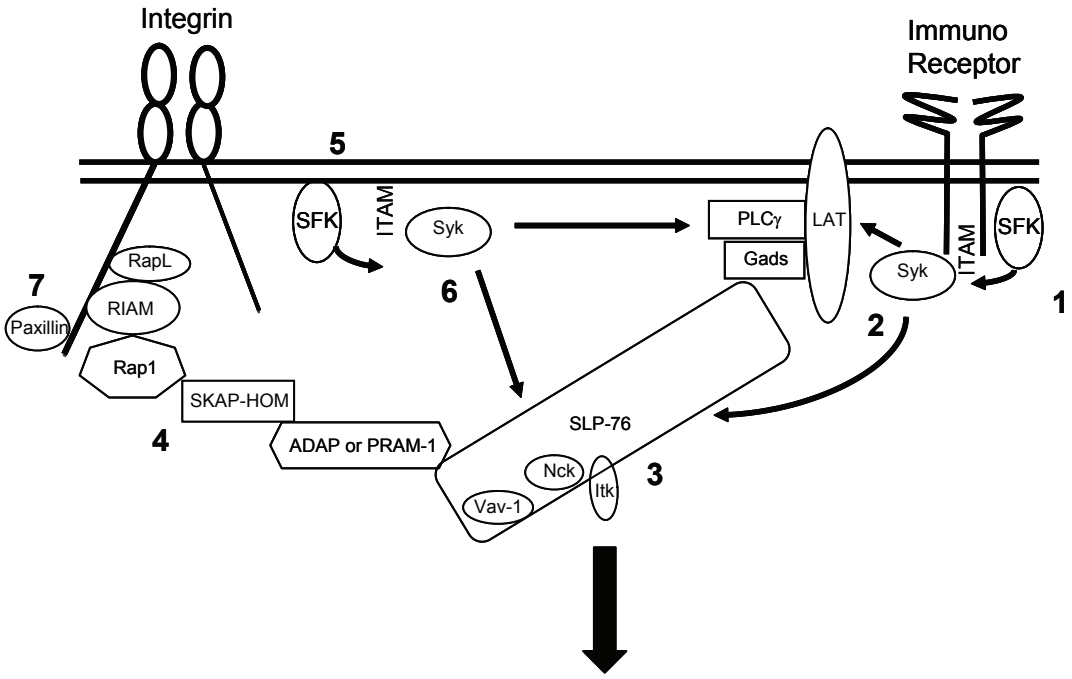
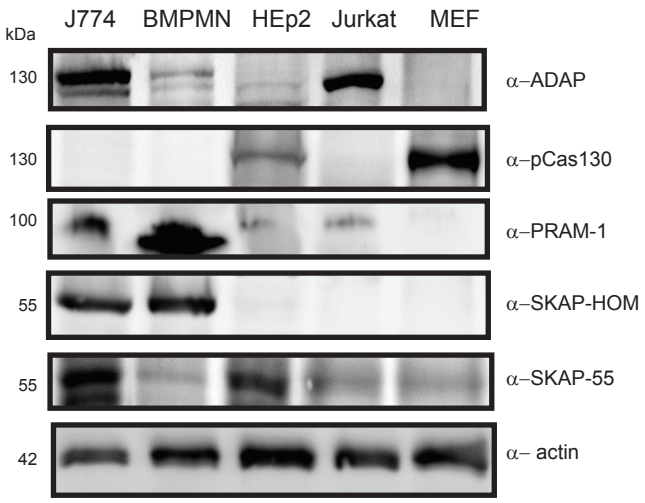
Figure S2



Ca^{+2} Flux ROS Erk activation Rap1 Activation

Adhesion Actin polymerization

Figure S3


J774
BMPMN
HEp2
Jurkat
MEF
kDa
130
α–ADAP
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α–PRAM-1
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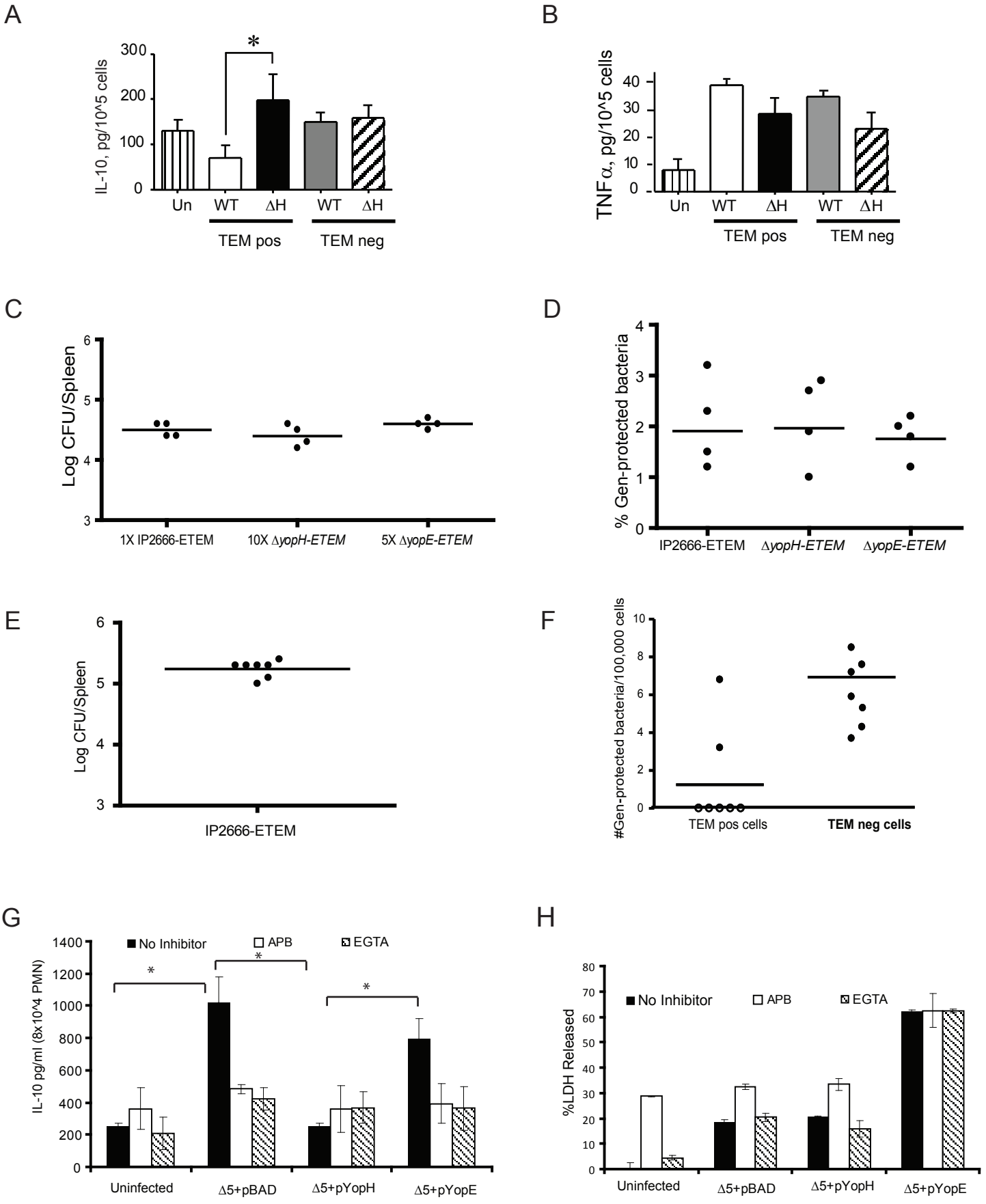
α–SKAP-HOM
55
α–SKAP-55
42
α– actin

Figure S4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200
100
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*
Un
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ΔH
WT
ΔH
TEM pos
TEM neg
B
TNFα, pg/10^5 cells
40
30
20
10
0
Un
WT
ΔH
WT
ΔH
TEM pos
TEM neg
C
Log CFU/Spleen
6
5
4
3
1X IP2666-ETEM
10X ΔyopH-ETEM
5X ΔyopE-ETEM
D
% Gen-protected bacteria
4
3
2
1
0
IP2666-ETEM
ΔyopH-ETEM
ΔyopE-ETEM
E
Log CFU/Spleen
6
5
4
3
IP2666-ETEM
F
#Gen-protected bacteria/100,000 cells
10
8
6
4
2
0
TEM pos cells
TEM neg cells
G
IL-10 pg/ml (8x10^4 PMN)
1400
1200
1000
800
600
400
200
0
No Inhibitor
APB
EGTA
*
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Uninfected
Δ5+pBAD
Δ5+pYopH
Δ5+pYopE
H
%LDH Released
80
70
60
50
40
30
20
10
0
No Inhibitor
APB
EGTA
Uninfected
Δ5+pBAD
Δ5+pYopH
Δ5+pYopE