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Supporting Online Material for

Host Resistance to Lung Infection Mediated by Major Vault Protein in Epithelial Cells

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Supporting Online Material

Materials and Methods

Cell lines. Human lung epithelial cells (CFT1) from a ΔF508 homozygous CF patient were immortalized and stable transfection with a vector encoding either WT-CFTR (CFT1-LCFSN) or β-galactosidase (CFT1-LC3) established the cell lines used in these studies (*S1*). For immunofluorescence studies, IBS3-1 cells from a CF patient heterozygous for 2 mutant alleles of CFTR (ΔF508 and W1282X) were transfected with a WT-GFP-CFTR-encoding plasmid carrying a geneticin resistance marker. Clones were derived by single-cell propagation in a geneticin-containing medium. Unaltered GFP-CFTR post-translational modification, subcellular localization and cAMP-inducible chloride transport were confirmed (unpublished data).

Bacterial strains. In addition to the *P. aeruginosa* strain PAS01-V, LPS smooth, non-mucoid CF clinical isolates of *P. aeruginosa* obtained from two CF patients early in the course of infection (N6, N8) were used, as was a corneal isolate (6294). Mutant *P. aeruginosa* strains deleted for either the *algC* (*S2*) or *galU* (*S3*) genes, resulting in an LPS with a truncated core and no O side chains, were also used. These bacteria have been shown to be deficient in CFTR-mediated epithelial cell uptake (*S4*). *P. aeruginosa* strain PA01-V tagged with enhanced cyan-fluorescent protein (CFP) in a mini-Tn7 construct was prepared as described (*S5*). Bacteria were grown and used for assays as previously described (*S6*).

Proteomic analysis by LC-MS/MS. MALDI-TOF analysis was performed as previously

described (*S7*).

Discontinuous sucrose gradients/Western blotting/vRNA detection. For analysis of proteins recruited to lipid rafts, cells were infected with ~1X10^9 bacteria at 37°C for 15 min in the presence or absence of 5 mM methyl-β-cyclodextrin as previously described (*S6*). Cells were lysed in 2 ml of 1% Triton X-100 in MBS (25 mM MES, pH 6.5, 150 mM NaCl) at 4°C for 20 min in the presence of Complete Mini Protease Inhibitor Cocktail (Roche Applied Science, Indianapolis, IN) and passed five times through a syringe and 21-gauge needle. In one experiment, the lysis buffer was supplemented with octyl-D-glucoside to 60 mM to solubilize rafts to show specificity of the detection system for raft proteins. The 2-ml lysate was added to 2 ml of 85% sucrose in MBS (42.5% final concentration of sucrose), placed in an ultracentrifuge tube and overlaid with 6 ml of 35% sucrose followed by an additional 2 ml of 5% sucrose. Samples were spun overnight at 39,000 rpm in a SW41 rotor (Beckman, Palo Alto, CA), and 1-ml fractions were removed from the top of the gradient, yielding fractions 1 through 12. The Triton-insoluble lipid rafts float to the interface of the 5% and 35% sucrose layers and were found to be in fractions 2-5. Fractions 2 and 3 were combined and used as the raft fractions for the proteomic screen and additional experiments.

Raft fractions were precipitated on ice for 1 hour by combining 900 µl with 900 µl of 40% trichloroacetic acid (TCA). The precipitate was pelleted at maximum speed in a microcentrifuge for 15 min, and the pellet was washed twice with acetone to remove residual TCA. Pellets were dissolved in sample buffer and analyzed by SDS-PAGE and/or Western blots as described previously (*S6*). For detection of MVP, anti-LRP mAb

(Transduction Laboratories/BD Biosciences, San Jose, CA) was diluted 1:2000 in 5% skim milk in PBS + 0.05% Tween-20 (PBS/Tween). vPARP was detected using anti-vPARP mAb 193.4 (Axxora, San Diego, CA) according to the recommendations of the manufacturer. An affinity-purified rabbit anti-TEP1 polyclonal antiserum was a generous gift from Dr. L. Harrington (University of Toronto, Canada) and used at a 1:2000 dilution in PBS/Tween supplemented with 5% skim milk. HRP-conjugated goat anti-mouse IgG (Southern Biotech, Birmingham, AL) and HRP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) were used at a 1:10,000 – 1:50,000 dilutions for detection. NIH Image software was used to determine relative band intensities. The presence of vRNAs in lipid raft fractions was determined by RT-PCR essentially as described (*S9*) with the exception that RNA was isolated from lipid raft fractions using RNA-Bee (Campro Scientific, Veenendaal, The Netherlands).

Immunofluorescence analysis of cultured cells and infected lungs. GFP-CFTR-expressing cells were infected with CFP-expressing PA01-V (*S10*) at 37°C for 90 min at a ratio of 10 bacteria per cell. Cells were fixed in 2% formaldehyde for 30 min at room temperature (RT) and washed twice with PBS, and the remaining formaldehyde was quenched by incubation with NH_4Cl for 10 min at RT. Cells were washed twice with PBS and permeabilized with 0.0075% saponin in blocking buffer (BB = 0.2% bovine serum albumin and 2% normal goat serum in PBS) for 10 min at RT, and non-specific binding was further blocked by pre-incubation with BB for 30 min at RT. Cells were labeled overnight at 4°C with monoclonal antibody directed against MVP (LRP-56, Axxora), diluted 1:40 in BB. Cells were then washed with PBS and incubated for 1 hour

at RT with Alexa Fluor 555-tagged goat anti-mouse IgG polyclonal antibody (Invitrogen, Carlsbad, CA), diluted 1:1000 in BB. Labeled cells were visualized using a Zeiss 510 LSM META confocal microscope in multitracking mode, which eliminated potential crosstalk between CFP (shown as red color) and GFP (shown as green color) or between GFP and Alexa Fluor 555 (shown as blue color).

Sections of lungs from WT and MVP$^{-/-}$ mice infected with PA01-V (see below) were examined by immunofluorescence microscopy after sectioning and removal of the paraffin embedding. Macrophages were stained with a mAb to the surface glycoprotein F4/80 (Clone BM8, diluted 1:50, Accurate Chemicals, Westbury, NY), PMN were detected with a mAb to the Ly6G antigen (mAb 1A8, diluted 1:50, BD Pharmingen, San Diego, CA), and epithelial cells were stained with mAb 1095 directed against the EGF receptor (diluted 1:50, R&D Systems, Minneapolis, MN). *P. aeruginosa* PA01-V was stained with a polyclonal rabbit antibody (diluted 1:500) raised to a live, attenuated vaccine version of this strain (*S3*). Each section was incubated with the primary antibody in a humidified chamber for 90 min. After three washings with PBS, Alexa Fluor 555-conjugated goat-anti-mouse IgG and Alexa Fluor 488-conjugated goat-anti-rabbit IgG were added (both 1:500 dilution, Invitrogen) and slides were incubated for 90 min in the humidified chamber. Control sections received either no primary antibody or no secondary antibody. After three additional PBS washes, the slides were covered with 0.1M *N*-propyl gallate to reduce photobleaching, sealed and examined at 100X magnification by confocal microscopy.

siRNA treatment. 24 hours after plating, cells were treated for 5 hours with 100 nM of

either anti-MVP pre-designed siRNA (Ambion, Austin, TX) or a scrambled sequence in Opti-MEM media (Invitrogen) containing a 1:500 dilution of Oligofectamine Reagent (Invitrogen). The following day, the cells were trypsinized, replated, and treated with siRNA again. This process was repeated again, for a total of three consecutive siRNA treatments. 24 hours after the third treatment, cells were plated in 6-well plates and used in assays the following day.

Bacterial ingestion by epithelial cells and NF-κB activation. Gentamicin-exclusion assays to determine bacterial uptake by epithelial cells and ELISAs to detect NF-κB released from the IκB inhibitor were performed as described previously (*S6*), with the exception that for the NF-κB experiments the cells were infected with a multiplicity of infection (MOI) of 500 for 60 min, and whole cell lysates were assayed rather than nuclear extracts.

IL-8 release. Cells were infected for 15 min with a MOI of 100, and then incubated in 500 μg tobramycin /ml for 3 h. The media were collected, filtered through a 0.22-μm cellulose acetate filter to remove bacteria and assayed for IL-8 using a Quantikine human IL-8 ELISA kit (R & D Systems, Minneapolis, MN) as per the manufacturer's instructions.

Apoptosis. Cells were infected with a MOI of 3 for 2-4 hours and then incubated overnight in media containing 150 μg neomycin /ml. Cells were lysed with sample buffer and 4 μg of the lysates were analyzed by Western blot for the 85 kDa caspase-cleaved form of poly-ADP ribose polymerase (PARP) as a measure of apoptosis. Anti-PARP (214/215) antibody (Sigma, St. Louis, MO), was used at a dilution of 1:2,000,

followed by a 1:10,000 dilution of HRP-conjugated goat anti-rabbit IgG (Southern Biotech) for detection.

Mouse infection studies. MVP$^{-/-}$ mice and littermate controls (C57Bl/6 background) were produced as described (*S8*), and were age- and sex-matched for infection experiments. To infect the mice for determinations of bacterial internalization and measurements of lung counts of *P. aeruginosa*, animals were anesthetized with ketamine hydrochloride (65 mg/kg) and xylazine (13 mg/kg) and infected intranasally with ~1.5 X 10^7 bacteria as previously described (*S11*). After 6 hours the mice were sacrificed using CO_2 inhalation and the lungs were removed and weighed. Single-cell suspensions of the lungs were obtained by using a syringe to pass the tissue through a fine mesh screen into F12 media (Invitrogen) with 10% FBS. Half of this cell suspension was pelleted and lysed in 1 ml of F12 + FBS with 0.5% Triton X-100, and serially diluted and plated on *Pseudomonas*-selective cetrimide agar to measure the total number of bacteria in the lungs. The remaining half was incubated at 37°C for 45 min in 2 ml of media containing 300 μg gentamicin /ml to kill external bacteria. After two washes to remove the antibiotic, the cells were lysed, and plated as above. Percent internalization was calculated as (internalized bacteria/total bacteria) X 100. For survival studies, mice were infected as above with ~4 X 10^6 cfu of *P. aeruginosa* and followed for up to 96 hours for lethality. Moribund mice were sacrificed and counted as dead for purposes of these experiments.

Statistical analysis. One-sample two-tailed t test was used to analyze *in vitro* bacterial uptake. Two-tailed unpaired t tests were used to analyze NF-κB activation and IL-8

release data as well as the cfu internalized and total bacterial burdens in the mouse infection experiments. These statistical analyses along with survival curve comparisons were performed using the GraphPad Prism3 Software Program for Macintosh.

SOM References

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Table S1. Proteins specifically recruited to lipid rafts by *P. aeruginosa* infection of cells expressing WT-CFTR; these proteins were not present in rafts of uninfected cells.

major vault protein
clathrin heavy chain 1
Ahnak
78 kDa gastrin-binding protein
integrin alpha-2
inosine monophosphate dehydrogenase 2
filamin a
reticulon 4
exportin 1
cleft lip and palate associated transmembrane protein 1
amine oxidase a
adenylate kinase isoenzyme 2
beta filamin
vacuolar atp synthase subunit c
rab11b
synaptic glycoprotein sc2
7 dehydrocholesterol reductase
similar to yeast translation activator GCN1
flotillin 2
hnrnp f
hypothetical protein LOC25923
nuclear chloride ion channel 27
nucleoside diphosphate kinase a
myosin regulatory light chain 2
ADP ribosylation factor 1
cathepsin d
L-lactate dehydrogenase
ADP-ribosylation factor 4
Golgi autoantigen, golgin subfamily a, 7
glutamate dehydrogenase 1
peroxiredoxin 1
emopamil binding protein
beta spectrin
rap1a
Moesin
malate dehydrogenase
prohibitin 2
CD81
vesicle associated membrane protein 2
peptidylprolyl isomerase F
KIAA1271
casein kinase I
importin beta 1
hepatitis b virus x interacting protein
AHNAK-related protein
hla class I histocompatibility antigen
late endosomal/lysosomal mp1 interacting protein
homologue of yeast Tim50
homologue of mouse immediate early response 3 interacting protein
rab18
vacuolar atp synthase subunit f
bifunctional aminoacyl-trna synthase
6-phosphofructokinase
ubiquinol cytochrome c reductase complex
phosphoprotein associated with glycosphingolipid-enriched microdomains
microsomal glutathione s-transferase 3
transmembrane protein 4
Hypothetical protein PRO1855
fructose bisphosphate aldolase c
hypothetical protein LOC493856
microsomal signal peptidase 12kDa subunit
tropomyosin 3
low-density lipoprotein receptor
rhoa
TRAP-1
myosin heavy chain, nonmuscle, differentially spliced
atp synthase b chain
microsomal glutathione S-transferase 1
aspartyl/asparaginyl beta-hydroxylase
40s ribosomal protein s17
fatty acid synthase
pantophysin
rab35
hypothetical protein LOC283635
presenilin 1
rab5c
HSPC121
succinate dehydrogenase
calgranulin B
lamin a
protein disulfide isomerase a4

tumor protein d54
nuclear pore complex protein nup155
transmembrane protein 32
db83
PNAS-117
inter-alpha (globulin) inhibitor H5-like
histidine triad nucleotide binding protein 1
superoxide dismutase
CD3e-associated protein
atp synthase f chain
mitochondrial import receptor subunit
dnase I-like
transmembrane protein tmp21
protein disulfide isomerase-associated 6
microsomal signal peptide 25 kDa subunit
DNAj homologue subfamily c member 5
ras GTPase-activating-like protein IQGAP1
glucosidase II alpha-subunit
poliovirus receptor related protein 1
caspase recruitment domain protein 11
ring finger protein 153
NADPH-ubiquinone oxidoreductase 30 kDa subunit
barrier to autointegration factor 1
antioxidant protein 2
uncharacterized hematopoietic stem/progenitor cells protein MDS029
angiotensin II receptor-associated protein
hsc70-interacting protein
SLC39A10 protein
macrophage migration inhibitory factor
h-ras-1
putative atp-dependent clp protease proteolytic subunit
cytochrome p450 51
nucleoside diphosphate kinase b
rap1B
atp synthase a chain
rab33b
lanosterol synthase
cytochrome c
golgi apparatus protein 1
ras-related C3 botulinum toxin substrate 1 isoform Rac1c
Ras-related protein ral-a
C19orf10 protein
Cadherin 13
r-ras
serotransferrin
peroxisomal membrane protein 20
LOC51234 protein
ubiquitin-specific protease otubain 1
heterogeneous nuclear ribonucleoprotein H2
minor histocompatibility antigen h13
Rer1
nuclear protein hcc-1
importin beta 3
elongation factor tu
potential membrane protein C14orf1
cytochrome c oxidase subunit Va
lamin b receptor
transferrin receptor protein 1
cytokeratin 15

Table S2. Proteins specifically recruited to lipid rafts by *P. aeruginosa* infection of cells expressing WT-MVP; these proteins were not present in rafts of infected MVP-knockdown cells.

filamin b
caveolin 1
NDK8 (putative nucleoside diphosphate kinase)
ras-related protein rab-7
guanine nucleotide-binding protein g(i)/g(s)/g(t) beta subunit 2
s100 calcium-binding protein a2
stomatin-like protein 2
14-3-3 protein gamma
ribophorin ii
annexin a3
guanine nucleotide-binding protein g(i)/g(s)/g(o) gamma-12 subunit
beta-tubulin
synaptosomal-associated protein 23
guanine nucleotide-binding protein g(k), alpha subunit
vacuolar atp synthase 16 kda proteolipid subunit
calm3
atp synthase d chain, mitochondrial
annexin a8
heat shock protein 90bb
brain acid soluble protein 1
chromosome segregation 1-like protein
mannose-6-phosphate receptor-binding protein 1

Table S3. Proteins present in lipid rafts of *P. aeruginosa*-infected MVP-knockdown cells that were not present in rafts of infected cells expressing WT-MVP

myosin 1
myosin 13
myosin 4
myosin 2
myosin 6
tropomyosin alpha 3

Supplementary Figures and Figure Legends

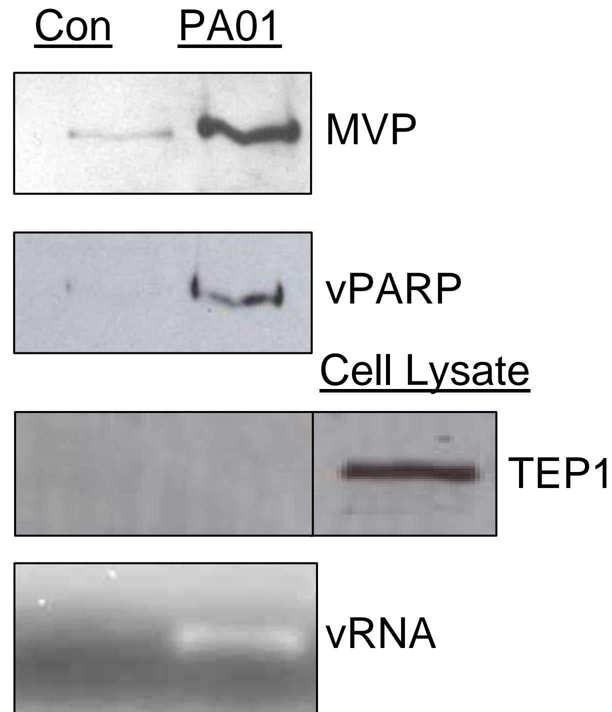

Fig. S1. Vault components found in raft fractions formed during 15 min of *P. aeruginosa* infection. Control (uninfected) and *P. aeruginosa* strain PA01-V (PA01)-infected human airway epithelial cells expressing WT-CFTR were lysed in 1% Triton X-100 and separated on discontinuous sucrose gradients. Proteins in raft fractions at the 5% to 35% interface were precipitated and subjected to electrophoresis and immunoblot analysis using antibodies directed against human MVP, vPARP and TEP1. To detect vRNA, RT-PCR was performed on RNA isolated from the raft fractions. PCR products were separated by agarose gel electrophoresis and products visualized by ethidium bromide staining. Whole cell lysates of WT-CFTR expressing cells were analyzed for TEP1 by Western blot to confirm expression.

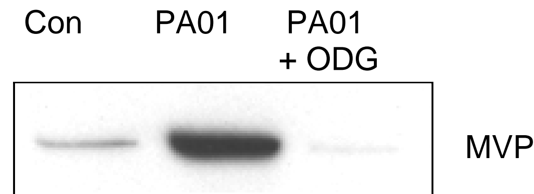

Fig. S2. MVP recruitment to lipid rafts formed during 15 min of *P. aeruginosa* infection is lost after treatment of CFT1-LCFSN cells with octyl-D-glucoside (ODG). Control (uninfected) and *P. aeruginosa* strain PA01-V-infected human airway epithelial cells expressing WT-CFTR were lysed in 1% Triton X-100 or in the presence of 60 mM ODG and separated on discontinuous sucrose gradients. Proteins in raft fractions at the 5% to 35% interface were precipitated and subjected to electrophoresis and immunoblot analysis using antibodies directed against human MVP.

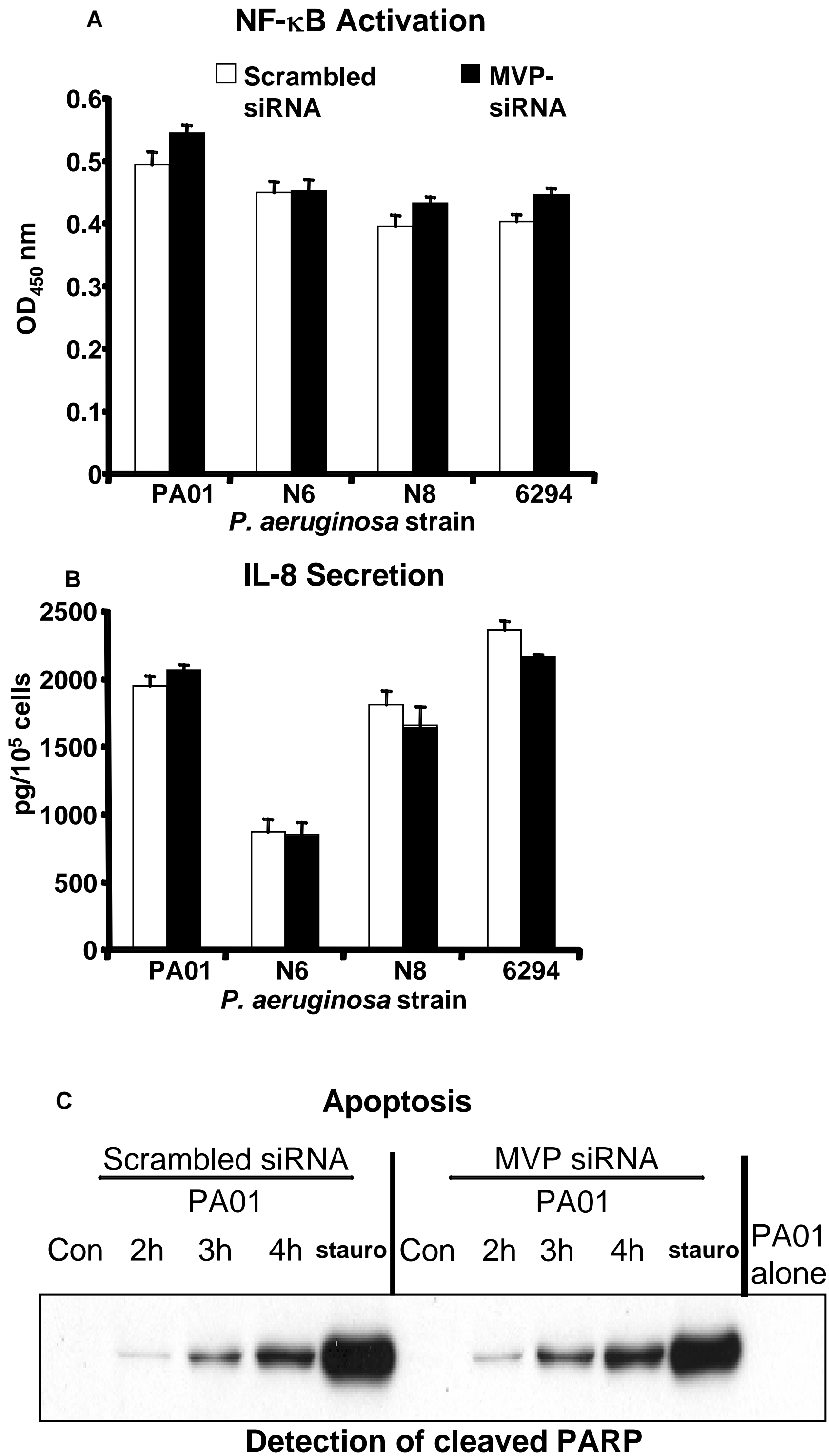

Fig. S3. MVP is not required for *P. aeruginosa*-induced NF-κB activation, IL-8 secretion, or

apoptosis in cells with WT-CFTR (CFT1-LCFSN cells). **(A)** Scrambled or MVP-specific siRNA-treated WT-CFTR cells were incubated with the indicated *P. aeruginosa* strain for 60 min and the level of NF-κB activation was determined. OD_{450}, reflecting the results of an ELISA specific for active NF-κB, is plotted. Error bars represent standard error of the mean. $p > 0.05$ by unpaired two-tailed t test between scrambled and MVP siRNA-treated cells. **(B)** Scrambled or MVP-specific siRNA-treated WT-CFTR cells were incubated with *P. aeruginosa* strains for 15 min, then 500 μg tobramycin /ml media was added and, after an additional 3 hours the level of IL-8 secretion was determined. An IL-8 ELISA was used to plot pg IL-8 released/1 X 10^5 cells. Error bars represent standard error of the mean. $p > 0.05$ by unpaired two-tailed t test between scrambled and MVP siRNA-treated cells. **(C)** Scrambled or MVP-specific siRNA-treated WT-CFTR cells were either left uninfected or infected with *P. aeruginosa* strain PA01-V (MOI of 3) for 2-4 hours then incubated overnight in media containing 150 μg neomycin/ml. To detect apoptosis, lysates were subjected to immunoblot analysis using Poly ADP-Ribose Polymerase (PARP) 214/215-specific antibodies that bind to the PARP cleavage site exposed by apoptosis. Con (control): no bacteria added. Staurosporine (stauro) was used as a positive control for apoptosis induction. PA01 alone: bacteria without epithelial cells.

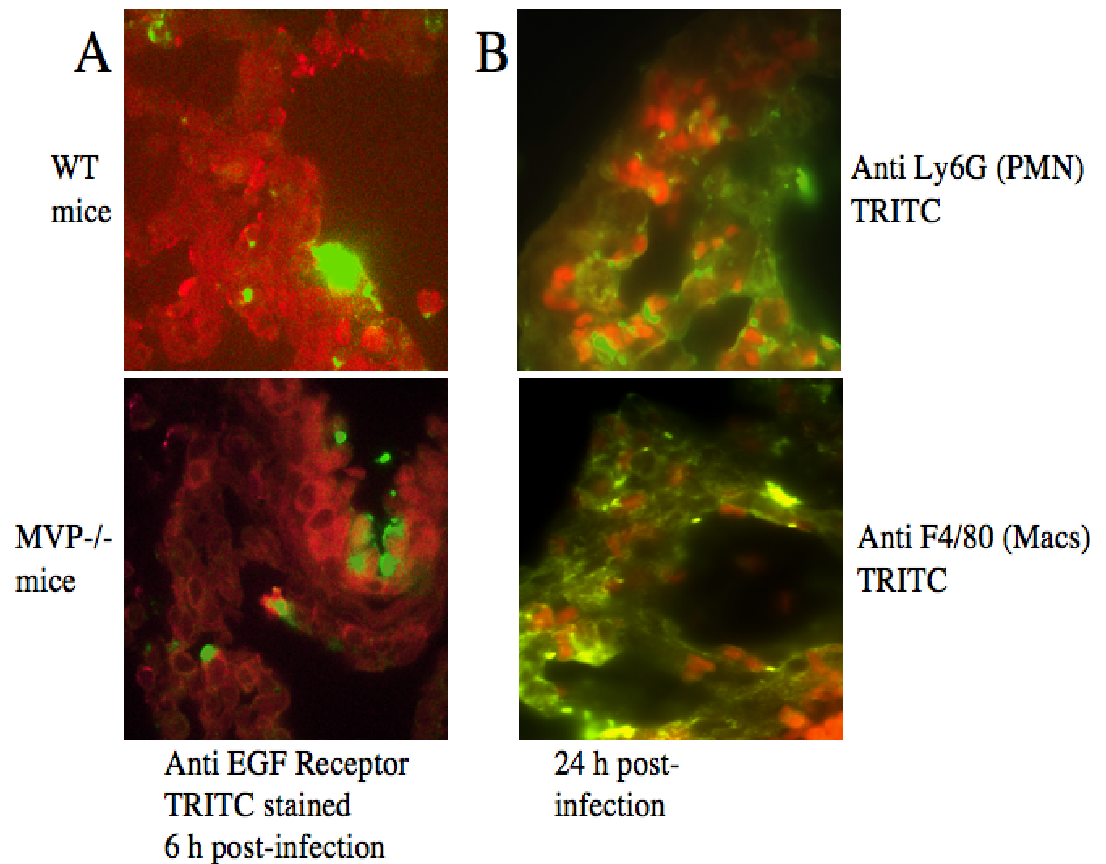

Fig. S4. *P. aeruginosa* cells or aggregates are associated with epidermal growth factor (EGF)-positive epithelial cells in WT or MVP$^{-/-}$ mice 6 hours post-infection. **(A)** Section of lung from WT or MVP$^{-/-}$ mice 6 hours after intranasal infection with *P. aeruginosa* PA01-V showing green bacterial cells or aggregates associated with epithelial cells (red). No bacterial cells were associated with PMN or macrophages at 6 hours post-infection. **(B)** As a control, lungs from WT mice 24 hours post-infection showed interstitial PMN and macrophages (red) associated with *P. aeruginosa* cells (green). Slides prepared without primary or secondary antibodies had no visible fluorescence. Sections X 100.