SUPPLEMENTAL MATERIAL

SUPPLEMENTAL METHODS

Mice

8–12 week-old C57Bl/6 (JANVIER), CD18$^{-/-}$ [1], *Sell*$^{-/-}$ [2], ADAM17$^{-/-}$ [5] and L(E) mutant mice[3] were used throughout this study. CD18$^{-/-}$ mice were a kind gift of Prof. Tobias Goerge, University of Muenster (Germany). Animals were maintained in a special pathogen–free facility at the University of Muenster. The Animal Care and Use Committee of the University of Muenster (Germany) approved all animal experiments.

Bacterial Strains and Growth Conditions

Overnight cultures (37 °C) of *K. pneumoniae* (ATCC strain 13883) were grown in Tryptic Soy medium, washed and resuspended in sterile saline (0.9%).

Lung infection with E. coli

Overnight cultures (37 °C) of *E. coli* (ATCC strain 25922) were grown in Tryptic Soy medium, washed and resuspended in sterile saline (0.9%). Mice were anaesthetized by intraperitoneal injection of ketamine (125 µg g^{-1} body weight; Pfizer, New York, USA) and xylazine (12.5 µg g^{-1} body weight; Bayer, Leverkusen, Germany). Subsequently, the trachea was exposed, and a 50 µl inoculum of bacterial suspension was administered via a 30-gauge needle. Animals were challenged with 5×10^6 viable *E. coli /* mouse. At this inoculation dose, all mice survived the 24 h observation period. After 24 h, the mice were sacrificed and the lungs were lavaged four times with 0.7 ml physiologic saline solution. The number of neutrophils in the BAL was counted using kimura staining. Neutrophils were counted and further discriminated by flow cytometry. Colony-forming units in the BAL, lung, blood and spleen were counted by serial plating on Tryptic Soy agar plates.

L-selectin TAT-fusion mutants

Briefly, isolated murine bone-marrow neutrophils were incubated with TAT-fusion mutants (GenScript, 2 μM, 37ºC, 30 min). Cells were incubated either with an L-selectin Tat-peptide, containing the intracellular tail of L-selectin plus a TAT-sequence (YGRKKRRQRRRGRRLKKGKKSKRSMNDPY), or a scrambled control peptide (YGRKKRRQRRRGPRMGRKRKKLSYNKKSD). The uptake and the specificity of the peptides have already been published [4].

Oxidative burst

Isolated murine bone marrow neutrophils were left untreated or were preincubated with antibodies, Tat-peptides or inhibitors as indicated and subsequently applied to uncoated, pRGD (Sigma-Aldrich, 20 μg/ml, 3 h, RT), rmICAM-1 (R&D Systems, 1 μg/ml, 3 h, RT), or fibrinogen (from bovine plasma, Sigma-Aldrich, 150 μg/ml, 3 h, RT)-precoated 96-well plates (Immunolon-4 HBX; Thermo Fisher Scientific) with 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1 mM cytochrome c (Sigma-Aldrich), and 50 ng/ml rmTNF-α, where indicated. Absorbance at 550 and 490 nm was recorded every 2 min for 90 min at 37°C in a plate reader. For calculation, each wavelength value was corrected by its superoxide dismutase control value.

Isolated human neutrophils from whole blood were pretreated with DMSO or GM6001 as indicated and subsequently applied to fibrinogen (from bovine plasma, Sigma-Aldrich, 150 μg/ml, 3 h, RT)-precoated black 96-well plates (Greiner Bio-one) with 1 mM $CaCl_2$, 1 mM $MgCl_2$, 20 μg/ml horseradish peroxidase (Sigma-Aldrich), 35 μg/ml luminol (Sigma-Aldrich) and 50 ng/ml rhTNF-α, where indicated. Luminescence was recorded every 2 min for 90 min at 37°C in a plate reader.

Biochemical experiments

For biochemical assays, bone-marrow derived murine neutrophils were isolated and suspended in 1x or 0.65x PBS (containing 1 mM each $CaCl_2$ and $MgCl_2$). Subsequently the cells were incubated for 10 minutes on uncoated or pRGD (20 µg/ml) coated coverslips in multiwell plates. Cells were lysed in RIPA buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100 buffer, 1% sodium deoxycholate, 0.1% SDS, 1 mM Na3VO4, 50 mM NaF, 2 mM EDTA, 1 mM Pefabloc, 10 µg/ml of leupeptin, 2 µg/ml of aprotinin, 1 mM dithiothreitol, 1 µg/ml of pepstatin and 1 mM di-isopropyl fluorophosphate) and lysates were boiled with sample buffer (10 min, 95°C) or incubated with Sepharose A/G beads (Santa Cruz Biotechnology, Inc., Dallas, USA) in combination with anti-Syk antibody (polyclonal rabbit IgG, Santa Cruz Biotechnology) or anti-IRhom2 antibody (polyclonal rabbit IgG, Thermo Fisher) for 4 h at 4°C. Beads were washed four times, and bound proteins were eluted by adding boiling sample buffer. Cell lysates and immunoprecipitates were run on 10% SDS-PAGE and immunoblotted using antibodies against phosphotyrosine (clone 4G10; Millipore), IRhom2 (polyclonal rabbit IgG, Thermo Fisher), Phospho-(Ser) 14-3-3 Binding Motif mouse mAb (mouse IgG1, 4E2, Cell Signaling), phospho-Syk (Tyr525/526, clone C87C1, Thermo Fisher), Akt (clone C67E7), phospho-Akt (Ser473, clone 193H12), PLCγ2 (polyclonal rabbit IgG) or phospho-PLCγ2 (Tyr1217, polyclonal rabbit IgG) (all from Cell Signaling Technology, Danvers, USA). Immunoblots were developed using an ECL system (GE Healthcare). Densitometric quantification was performed using ImageJ software.

In vitro Mac-1 clustering

Isolated bone marrow neutrophils were suspended in PBS containing 1 mM $MgCl_2$ and $CaCl_2$. Cells were either left unstimulated or were stimulated on µ-Slide 8 wells (Ibidi) precoated with 20 µg/ml pRGD (Sigma-Aldrich, 20 µg/ml, 3 h, RT) for 10 min at RT. Then, cells were fixed with 2% paraformaldehyde at room temperature and subsequently incubated with Alexa Fluor

488–conjugated anti–Mac-1 antibody (clone M17/4; BioLegend) or an IgG control at 4°C, 30 min. Clustering was analyzed using ImageJ.

Phagocytosis

K. pneumoniae bacteria were labeled with pHrodo-dye by using pHrodo™ Red Phagocytosis Particle Labeling Kit for Flow Cytometry (Thermo Fisher). pHrodo-labeled *E. coli* (Thermo Fisher) or *K. pneumoniae* bioparticles were opsonized with 50% mouse serum at 37°C for 30 min. Isolated bone marrow neutrophils were left untreated or were preincubated with LFA-1- (selfmade, clone M17/4) or Mac-1-blocking (selfmade, clone M1/70) antibodies for 10 min, where indicated and subsequently incubated with opsonized bioparticles at 37°C for 1 h at a ratio of 1:10 (neutrophils/bioparticles). Negative controls were kept 1 h on ice. Cells were washed, fixed, stained with FITC-conjugated anti–Ly-6B.2, and analyzed by flow cytometry.

Sell ELISA

Bone-marrow derived murine neutrophils were isolated and suspended in 1x or 0.65x PBS (containing 1 mM each $CaCl_2$ and $MgCl_2$). Cells were left untreated or were preincubated with antibodies, Tat-peptides or inhibitors as indicated and subsequently incubated for 10 minutes on uncoated or pRGD (Sigma-Aldrich, 20 µg/ml, 3 h, RT) coated coverslips in multiwell plates. Following centrifugation (5 min, 400 g), supernatants were collected and mouse Sell ELISA (Sigma Aldrich) was performed according to the manufacturer's instruction.

ADAM17 activity

Bone-marrow derived murine neutrophils were isolated and suspended in PBS (containing 1 mM each $CaCl_2$ and $MgCl_{2)}$. Cells were left untreated or were preincubated with R18 inhibitor as indicated and subsequently incubated for 10 minutes on uncoated or pRGD (Sigma-Aldrich, 20 µg/ml, 3 h, RT)) coated coverslips in multiwell plates. Following centrifugation (5 min, 400

g), cell pellets were lysed in assay buffer and SensoLyte 520 TACE (α-Secretase) Activity Assay (AnaSpec) was performed according to the manufacturer's instruction.

Statistics

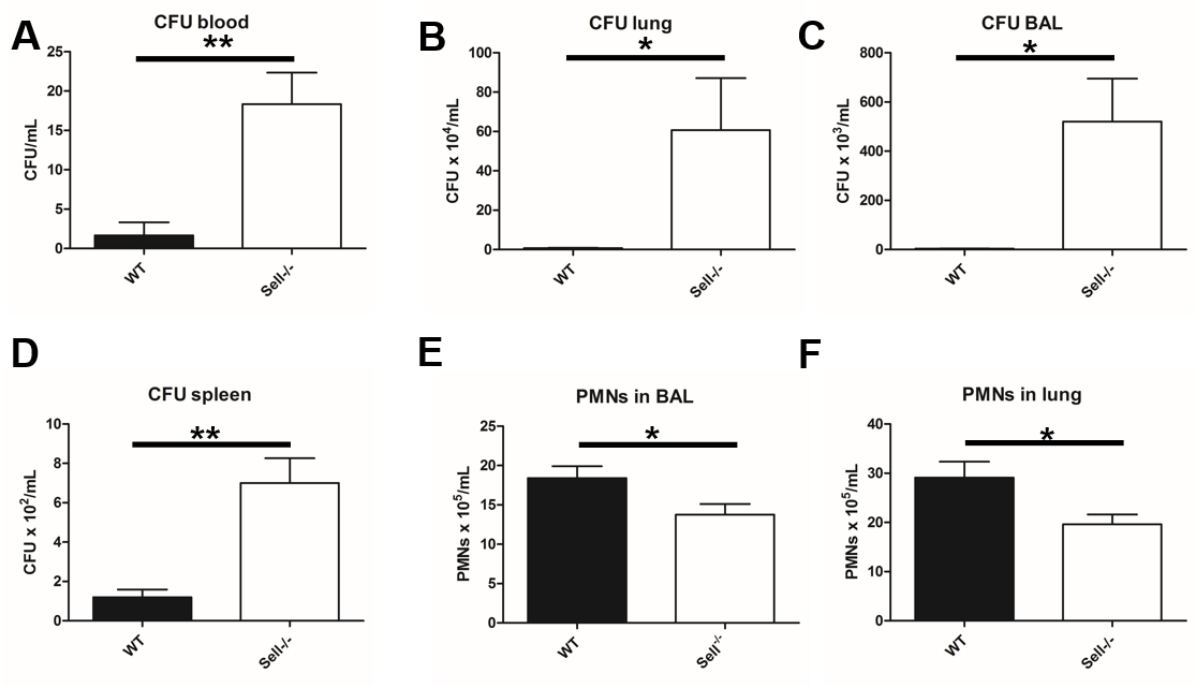
The number of experimental repeats is specified in the corresponding figure legend. Where appropriate, the total number of individual mice used per experimental group is also indicated. Statistical analysis was performed with SigmaPlot and GraphPad Prism 5. Differences between the groups were evaluated by one-way or two-way analysis of variance (ANOVA), Student-Newman-Keuls test, Rank Sum test or t-test where appropriate. Data are presented as mean ±SEM, and $p < 0.05$ was considered statistically significant.

SUPPLEMENTAL REFERENCES

1. Wilson RW, Ballantyne CM, Smith CW, et al. Gene targeting yields a CD18-mutant mouse for study of inflammation. *J Immunol*. 1993;151(3):1571-1578.
2. Arbones ML, Ord DC, Ley K, et al. Lymphocyte homing and leukocyte rolling and migration are impaired in L-selectin-deficient mice. *Immunity*. 1994;1(4):247-260.
3. Venturi GM, Tu L, Kadono T, et al. Leukocyte migration is regulated by L-selectin endoproteolytic release. *Immunity*. 2003;19(5):713-724.
4. Stadtmann A, Germena G, Block H, et al. The PSGL-1-L-selectin signaling complex regulates neutrophil adhesion under flow. *J Exp Med*. 2013;210(11):2171-2180.
5. Horiuchi K, Kimura H, Miyamoto T, et al. Cutting edge: TNF-a-converting Enzyme (TACE/ADAM17) Inactivation in Mouse Myeloid Cells Prevents Lethality from endotoxin Shock. J Immunol. 2007; 179:2686-2689

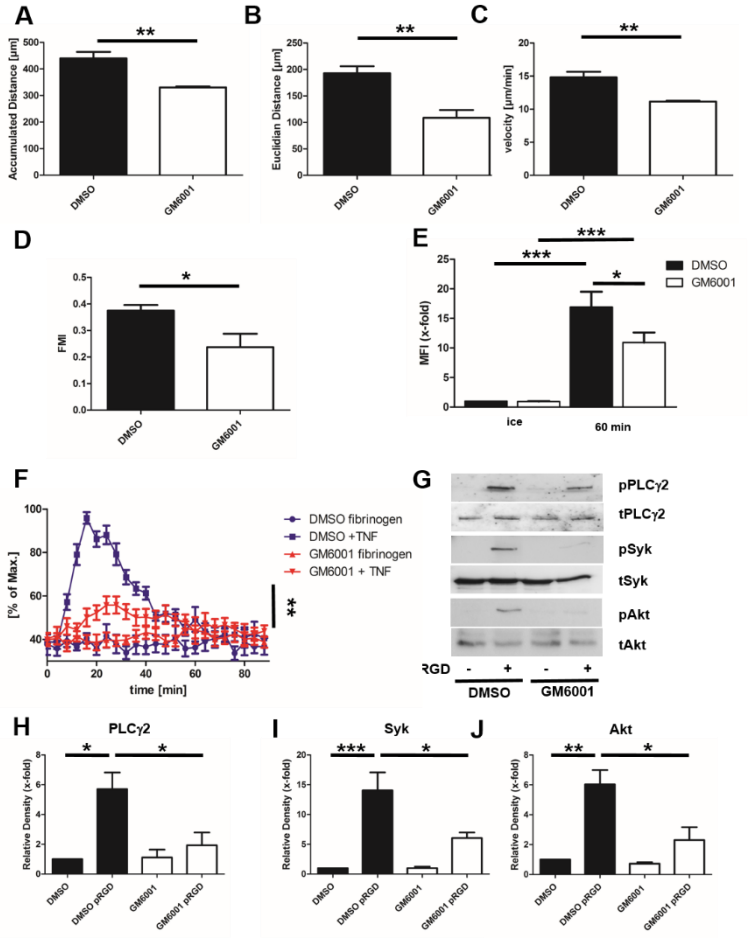
SUPPLEMENTAL FIGURES

SUPPLEMENTAL FIGURE 1



Supplemental Figure 1: L-selectin is required for host defense during *E. coli*-induced pneumonia. (A-F) WT and *Sell*$^{-/-}$ mice were subjected to *E. coli* intratracheal injection. (A-D) Bacterial burden in terms of CFUs in blood (A), lung (B), BAL (C) and spleen (D) as well as neutrophil recruitment into the alveoli (E) and lung tissue (F) were determined 24h after *E. coli* injection. n=6 mice/genotype, mean ± SEM, t-test *p=0.05

SUPPLEMENTAL FIGURE 2



Supplemental Figure 2: Shedding via metalloproteinases plays a pivotal role for neutrophil effector functions of human neutrophils. (A-D) Chemotaxis of DMSO and GM6001 pretreated isolated human neutrophils on fibronectin in response to a soluble IL-8 gradient *in vitro*. Accumulated (A) and euclidian distance (B), migration velocity (C) and forward migration index (D) of chemotaxing neutrophils are shown. 40 cells/experiment were analyzed. Phagocytosis of pHrodo *K. pneumoniae* particles (E) by DMSO or GM6001 pretreated human neutrophils. (F) Adhesion-dependent oxidative burst of DMSO or GM6001 pretreated human neutrophils plated on fibrinogen alone or in the presence of TNFα. (G-J) DMSO or GM6001 pretreated human neutrophils were plated on pRGD for 10 min, and lysates were immunoblotted with (G+H) anti–p-PLCγ2 and anti-PLCγ2, (G+I) anti-phospho Syk and total Syk and (G+J) anti–p-Akt and anti-Akt. n=3. Mean ± SEM, *p=0,05 one-way or two-way ANOVA.

SUPPLEMENTAL VIDEOS

Supplemental Video 1: Confocal microscopy of WT viable *ex vivo* lung sections following *K. pneumoniae* infection

Supplemental Video 2: Confocal microscopy of *Sell*$^{-/-}$ viable *ex vivo* lung sections following *K. pneumoniae* infection