Supplemental Files for

The GPCR Adaptor Protein Norbin Suppresses the Neutrophil-Mediated Immunity of Mice to Pulmonary Pneumococcal Infection

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Supplemental Methods

Materials: Rabbit polyclonal Norbin C1 antibody was a kind gift from Dr Kei Maruyama, Tokyo Institute of Psychiatry, Japan [1]. Kwick-Diff stain (9990700) was from Thermo Scientific Shandon. Antibodies for the identification of neutrophils and of leukocytes in broncho-alveolar lavages and perfused lung homogenates by flow cytometry are listed in Supplemental Table 1. Ly6G antibody clone 1A8 (BE0075-1, IgG2a) used to deplete neutrophils *in vivo*, and isotype control clone 2A3 (BE0089, IgG2a), were both from BioXCell. FITC-CD24 antibody clone M1/69 (101805) from Biolegend was used to test for the efficiency of neutrophil depletion. eF780-Fixable Viability Die (FVD) (65086514) was from eBiosciences. Fc block (553141) was from BD Biosciences. Antibodies to test the levels of GPCRs and adhesion molecules on the neutrophil surface by flow cytometry are listed in Supplemental Table1. Antibodies used for western blotting are listed in Supplemental Table 2.

Mice: We generated the $Ncdn^{\Delta mye}$ mouse strain, which is Norbin deficient in the myeloid cell lineage, by crossing $Ncdn^{fl/fl}$ mice [2] to $LysM^{Cre}$ [3]. We generated $Ncdn^{\Delta mye}Prex1/2^{-/-}$ mice with combined Norbin and Prex deficiency by crossing $Ncdn^{\Delta mye}$ mice with $Prex1/2^{-/-}$, a strain globally deficient in *Prex1* and *Prex2* [4]. This constitutes effectively a Norbin/Prex1 double deficiency in myeloid cells, as Prex2 is not expressed in myeloid cells [5]. All mouse strains were on C57BL/6 genetic background, either from inception or by backcrossing to C57BL/6 at least 7 times. $Prex1/2^{-/-}$ and $Prex1/2^{+/+}$ ($Prex1^{+/+}Prex2^{+/+}$) control mice were intercrossed at least every two years to minimise genetic drift, as were $Ncdn^{\Delta mye}$, $Ncdn^{fl/fl}$ and $LysM^{Cre}$ strains. Mice were group-housed (up to 5) in individually ventilated cages in the Babraham Instiute Small Animal Unit that uses 12 h light/dark cycles with dusk and dawn settings, and were fed chow diet and water *ad libitum*. Mice from both sexes were used for experiments with isolated neutrophlis, at young-

adult age (between 8 and 14 weeks), and were sex-and age-matched between genotypes within experiments. Males in that same age-range were used for *in vivo* experiments, again age-matching between genotypes within experiments. *Ncdn*$^{fl/fl}$, *LysM*^{Cre} and *Prex*$^{+/+}$ mice were used as control strains, as appropriate. For infection with *S. pneumoniae and E. coli*, animals were housed in individually ventilated isocages in the Babraham Institute biosafety level 2 containment facility. Animal breeding and experiments were carried out with approval from the local Animal Welfare Ethical Review Body under the British Home Office Animal Scientific Procedures Act 1986.

Neutrophil purification: Mature primary neutrophils were freshly isolated each day by Percoll^{PLUS} gradient at 4°C from mouse bone marrow using endotoxin-free reagents throughout, essentially as previously described [6], except that two consecutive Percoll^{PLUS} gradients were used for increased purity. Mouse bone-marrow was flushed from femurs, tibias and pelvic bones with ice-cold Hank's Balanced Salt Solution without Ca^{2+} or Mg^{2+} (HBSS^{--}, Sigma H6648) supplemented with 15 mM HEPES, pH 7.4 (RT) and 0.25% endotoxin-free and fatty acid-free (FAF) BSA (Sigma A8806) (HBSS^{--++}), triturated and filtered through a 40 µm cell strainer. 58% isotonic Percoll^{PLUS} (GE Healthcare, 17544501) in HBSS^{--++} was underlayed and samples spun at 1620 × g without brake for 30 min at 4°C. The lower 5 ml were collected and subjected to another Percoll^{PLUS} gradient. The lower 5 ml of the second gradient were resuspended in 40 ml HBSS^{--++} and centrifuged at 326 × g for 10 min at 4°C. Erythrocytes were lysed in Geye's solution (130 mM NH_4Cl, 5 mM KCl, 780 µM Na_2HPO_4, 176 µM KH_2PO_4, 5.5 mM glucose, 1 mM $MgCl_2$, 280 µM $MgSO_4$, 1.54 mM $CaCl_2$, 13.4 mM $NaHCO_3$) for 3 min at RT. 10 volumes of ice-cold HBSS^{--++} were added and cells sedimented again. Neutrophils were resuspended in ice-cold Dulbecco's Phosphate Buffered Saline with Ca^{2+} and Mg^{2+} (DPBS^{++}, Thermo Fisher Scientific, 14040117) supplemented with 0.1% glucose and 4 mM $NaHCO_3$ (DPBS^{++++}) and kept on ice while aliquots were counted by hemocytometer and purity assessed by

DiffKwik-staining of cytospins. Preparations were >90% pure, usually 95-98%. Neutrophils were sedimented again and resuspended in the buffer appropriate for the subsequent assay.

Norbin expression: To evaluate the deletion of Norbin from peritoneal leukocytes, total lysates were prepared of leukocytes recovered by peritoneal lavage from mice treated with LPS to induce peritonitis, as described below, and of resident peritoneal leukocytes recovered from mice mock-treated with saline. To evaluate the deletion of Norbin from neutrophils, total lysates were prepared from mature neutrophils isolated from bone marrow as described above, after treatment with the cell-permeable serine protease inhibitor diisopropyl-fluorophosphate (DFP, 7 mM, Sigma, D0879) for 10 min at RT and a wash in $DPBS^{++++}$. To prepare total lysates, cells were sedimented, resuspended in boiling 1.3 x SDS-PAGE sample buffer, and boiled for 5 min with repeated trituration through a 25 x G needle. Norbin levels were analyzed by western blotting with Norbin C1 antibody as previously described [7].

Western blotting: Proteins were denatured in in boiling SDS-PAGE sample buffer, separated by SDS-PAGE, transferred onto PVDF, blocked and probed with the appropriate antibodies listed in Supplemental Table 2 and detected using ECL or ECL Prime (GE Healthcare). Where required, membranes were stripped in 25 mM glycine, pH 2.0, 1% SDS for 5 min at RT and reprobed with different antibodies. Protein loading was assessed by coommassie staining. Densitometric analysis of western blots was done using ImageJ.

Pulmonary Streptococcus pneumoniae infection: *S. pneumoniae* (TIGR4 serotype 4) was maintained by *in vivo* passage every 6–12 months as described [8]. Bacteria were grown in a biosafety level 2 facility to mid-log phase (OD_{500} = 0.5–0.7) in Todd-Hewitt broth (Oxoid) with 0.5% yeast extract (Oxoid) at 37°C, 5% CO_2, pelleted, resuspended in PBS/20% glycerol, and aliquots snap-frozen and stored at −80°C. Titer, viability

and purity were assessed after culture on LB agar containing 5% defibrinated sheep blood (Oxoid) (blood agar) for up to 24 h, by CFU count and by the characteristic α-hemolytic zone around colonies. Prior to infection, bacterial stocks were thawed, washed twice in DPBS without Ca^{2+} and Mg^{2+} ($DPBS^{--}$, Invitrogen 14190-094), resuspended at 4×10^7 bacteria/ml in ice-cold $DPBS^{--}$, kept on ice and used within 2 hours. The infection dose was routinely confirmed by plating serial dilutions of the inoculum on blood agar.

Mice were lightly anaesthetized by inhalation of 3% isoflurane and infected intranasally with 50 µl *S. pneumoniae* suspension (2×10^6 bacterial CFU per animal), or were mock-treated with 50 µl $DPBS^{--}$. Upon inhalation of the inoculum and full recovery from the anesthesia, mice were returned to their home cages with food and water *ad libitum*. At specified times following infection, mice were euthanized by CO_2 inhalation, followed by severing of the femoral artery to confirm death. Bronchoalveolar lavage (BAL) was performed by three repetitions of slow injection and aspiration of 1 ml $DPBS^{--}$ using a Venflon cannula (1.1 x 32 mm, BD 391452) inserted through a small incision in the trachea. The BAL was stored on ice until further use. Lungs were perfused with 10 ml $DPBS^{--}$ through the right heart ventricle, removed using aseptic surgical technique, minced using razor blades, and placed into GentleMACS C tubes (Miltenyi Biotec, 130093237) containing 2 ml $DPBS^{--}$ and Miltenyi Mouse Lung Dissociation kit enzymes (130095927) according to the manufacturer's instructions, and samples were processed in a GentleMACS tissue homogenizer (setting 2.1, lung) for 40 s, followed by incubation at 37°C for 20-45 min and a second round in the homogenizer. The perfused lung homogenate was washed in 10 ml ice-cold $DPBS^{--}$ by centrifugation at 500 x g for 5 min at 4°C.

For the analysis of bacterial burden, aliquots of the BAL and perfused lung homogenate were serially diluted in ice-cold $DPBS^{--}$, plated onto blood agar, incubated for up to 24 h at 37°C, and CFU enumerated on plates of comparable colony density (≈20-200 CFU/plate).

Leukocytes in BAL and perfused lung homogenate were analyzed by flow cytometry. Cells were incubated with FVD (see 'Materials') for 15 min, washed in $DPBS^{--}$ by centrifugation at 500 x g for 5 min, stained with the antibody cocktail detailed in 'Materials' for 30-40 min on ice in the dark, washed again, resuspended in fixation buffer (Biolegend, 420801), incubated for 20 min at RT, and washed again. Spherotech ACBP-50-10 standard beads (5.0-5.9 μm) were added to enable the quantification of cells, and samples were analyzed using a BD Biosciences Fortessa flow cytometer. Data were analyzed using FlowJo (Tree Star). Among single, live $CD45^{+}$ leukocytes, neutrophils were identified by $CD11b^{hi}$, $Ly6G^{hi}$ staining; alveolar macrophages as $CD11c^{+}$, $SiglecF^{+}$ and inflammatory monocytes as $CD11b^{hi}$, $Ly6C^{hi}$ cells. Total monocyte/macrophages were defined as the sum of alveolar macrophages, inflammatory monocytes and other $CD11b^{+}$ macrophage-like leukocytes. For BAL samples, the volume of BAL recovered was taken into account in the quantification.

Macrophage and neutrophil depletion: To deplete macrophages, mice were treated with Clodronate liposomes [9] (Liposoma), by 100 μl *i.v.* injection and 50 μl *i.n.* inhalation, under general anaesthesia with recovery by 3% isofluorane inhalation at 72 h prior to infection with *S. pneumoniae*. Control mice were mock-treated with control liposomes (Liposoma). Animals were observed to ascertain inhalation of the full dose of liposomes and recovery from the anaesthesia before being returned to their home cages with food and water *ad libitum*. To deplete neutrophils, mice were injected *i.p.* with 25 mg/kg 1A8 monoclonal anti-Ly6G neutrophil depletion antibody (see 'Materials') [10] in 200 μl PBS, at 24 h and 0 h prior to infection with *S. pneumoniae*. Control mice were mock-treated with 25 mg/kg 2A3 isotype control antibody. Macrophage- or neutrophil-depleted and control mice were infected with *S. pneumoniae,* or were mock-infected, euthanized after 6 h, BAL prepared and CFU enumerated as described here-above. Leukocytes were analyzed by flow cytometry as described above except that CD24 antibody was used instead of Ly6G in neutrophil-depleted samples, because 1A8-mediated depletion targets the Ly6G epitope. To monitor

neutrophil depletion in peripheral blood, 50 µl tail blood samples were taken prior to each antibody injection, collected into EDTA-coated Microvette capillary tubes, centrifuged at 600 × g for 6 min at RT, and the supernatant discarded. Erythrocytes were lysed in 500 µl Geye's A solution (130 mM NH_4Cl, 5 mM KCl, 780 µM Na_2HPO_4, 176 µM KH_2PO_4, 5.5 mM glucose) for 5 min at RT prior to addition of 1.5 ml $DPBS^{++++}$. Samples were centrifuged again and leukocytes analyzed by flow cytometry as described above.

Peritonitis: Pathogenic *E. coli* O18:K1 bacteria [11] were grown in a biosafety level 2 facility to mid-log phase in LB at 37°C, 5% CO_2, pelleted, snap-frozen in PBS/20%glycerol and stored in aliquots at −80°C. Titers were determined by CFU count on blood agar plates. Prior to infection, a fresh aliquot of bacterial stock was thawed, washed in ice-cold $DPBS^{--}$ by centrifugation at 10,000 × g for 2 min at 4°C, resuspended at 5 × 10^5 bacteria/ml in ice-cold $DPBS^{--}$, kept on ice and used within 1.5 h. To induce septic peritonitis, mice in a biosafety level 2 containment facility were injected *i.p.* with 200 µl of the *E. coli* suspension (1 × 10^5 bacteria per animal), or were mock-treated with $DPBS^{--}$, before being returned to their home cages with food and water *ad libitum*. 3 h later, mice were euthanized by CO_2 asphyxiation, death confirmed by pithing, and peritoneal lavages performed by *i.p.* injection and aspiration of 8 mL $DPBS^{--}$, 5 mM EDTA. A second lavage was performed, pooled with the first, and samples stored on ice. An aliquot of the lavage fluid was taken for enumeration of bacteria, serially diluted in ice-cold $DPBS^{--}$, plated onto blood agar plates, cultured overnight at 37°C, 5% CO_2, and CFU counted on plates of comparable density (20-200 bacteria). The remaining lavage cells were pelleted at 450 x g for 10 min at 4°C, erythrocytes lysed by resuspending cells in 1 ml Geye's solution and incubated at RT for 150 s, prior to the addition of 10 ml of $DPBS^{++++}$. Leukocytes were centrifuged again and resuspended 1 ml fixation buffer (Biolegend, 420801), incubated for 20 min at RT, washed again, and resuspended in 1 ml $DPBS^{--++}$. Aliquots of fixed cells were counted by hemocytometer and the rest cytospun onto glass coverslips, stained with FITC-Ly6G antibody (BioLegend, #127605) and Hoechst 33342 DNA stain (Thermo Fisher, 62249) for 30 min in the dark,

washed twice, mounted, and imaged on a Nikon Eclipse Ti-E widefield system using the 20x objective and 3x3 image-stitching function. $Ly6G^+$ neutrophils were quantified by ImageJ analysis, taking into account the lavage volume recovered.

Aseptic peritonitis experiments were done essentially as previously described [12]. Mice were injected *i.p.* with 0.25 ml sterile 3% thioglycollate (TGC, Sigma, T9032) in H_2O, or were mock-treated with H_2O. 3 h later, mice were euthanized and peritoneal lavages performed and erythrocytes lysed as described above. Leukocytes were resuspended in 1.25 ml $DPBS^{++++}$. 1 ml of the sample was processed for flow cytometry by staining leukocytes with AF647-Cd11b and FITC-Gr1 antibodies in $DPBS^{++++}$ with Fc block for 20 min on ice in the dark, washing in $DPBS^{++++}$, 5 mM EDTA and resuspension in 500 μl $DPBS^{++++}$ containing 1 μg/ml DAPI and 1.25×10^5 Spherotech ACBP-50-10 standard beads/ml (5.0-5.9 μm). Flow cytometry was carried out in a BD Biosciences LSRII flow cytometer, and FlowJo was used for data analysis. Neutrophils were identified by $Cd11b^{hi}$, $Gr1^{hi}$ staining and enumerated by taking into account the lavage volume recovered. The remaining lavage leukocytes were counted by hemocytometer and analyzed by Kwick-Diff staining of cytospins as an alternative method of identification. To induce peritonitis with LPS, mice were challenged *i.p.* with 25 μg/kg LPS (Sigma) in sterile saline, or were mock-treated with saline, and euthanized by CO_2 asphyxiation after 4 h. Peritoneal lavages were performed and lavage leukocytes analyzed by flow cytometry and Kwick-Diff staining of cytospins as described above.

Histology: Mice were infected with *S. pneumoniae*, or mock-infected, and euthanized 6 h later as described above. Lungs were inflated with 0.5 ml 10% neutral-buffered formalin (Sigma) using a Venflon cannula (1.1 x 32 mm, BD 391452) inserted through a small incision in the trachea. Lungs were excised, and fixed in formalin. Samples were processed by Abbey Veterinary Services (Newton Abbot) for paraffin embedding, preparation of 5 μm sections and H&E staining. Slides were analyzed essentially as previously

described [13]. 3 sections/mouse were imaged on a Zeiss Microbeam microscope using the 20x objective and 3x3 image-stitching function, which generated images of 1.247 mm x 0.935 mm areas, and 6 such areas distributed throughout each lung were captured. For image analysis, 10 grids of 40,000 μm^2 were placed within each of those areas, such that they encompassed vascular, interstitial and epithelial tissue where possible. Neutrophils within these grids were identified by their characteristic nuclear morphology and quantified according to their localization within 10 μm of the vasculature, bronchiolar or alveolar epithelium, or within the interstitium. 60 such grids from 6 areas of lung were analyzed per mouse.

In vivo chemokine/cytokine production: Mice were infected with *S. pneumoniae*, or mock-infected, euthanized 3 h later, and BAL performed as described above, and the BAL fluid was stored at -80°C prior to analysis. The levels of mouse TNFα, CXCL1 and IL6 in the BAL fluid were analyzed by ELISA as previously described [13], using DuoSet ELISA kits DY410, DY453 and DY406 from R&D Systems according to the manufacturer's instructions.

Killing of bacteria by isolated neutrophils: *Staphylococcus aureus* (Wood 46) was stored at -80°C as glycerol stocks. Prior to experiments, bacteria were subcultured in LB at 37°C to logarithmic growth, sedimented, washed in $DPBS^{++++}$, opsonised with 10% mouse serum in $DPBS^{++++}$ for 15 min at 37°C, washed and resuspended in $DPBS^{++++}$ at 0.5 or 1 x 10^8/ml. Purified bone marrow-derived neutrophils at 2.5 x 10^7/ml in $DPBS^{++++}$ were primed with 20 ng/ml murine TNFα (R&D Systems, 410-MT-010) and 50 ng/ml GM-CSF (Peprotech, 315-03) for 45 min at 37°C. 50 μl of serum-opsonised *S. aureus* were incubated with 200 μl primed neutrophils for 15 min at 37°C, at the indicated ratios of bacteria to neutrophils. In some experiments, the ROS inhibitor diphenyleneiodonium (DPI, Sigma D2926) was added during the incubation at the indicated concentrations. After the incubation, 50 μl aliquots of each sample were added

to 950 μl ice-cold LB, 0.05% saponin, sonicated in a Misonix 3000 Probe Sonicator (output 1.5, 10 s), and serial dilutions were plated onto LB-agar and incubated overnight at 37°C to enumerate bacterial colonies. Samples containing bacteria but not neutrophils, with or without DPI, were processed in parallel as controls. Killing assays *with S. pneumoniae* were carried out in the same manner in a biosafety level 2 containment facility, except that the bacteria were opsonized straight after thawing, a bath sonicator was used to avoid aerosols, and bacteria were plated on blood agar.

ROS: ROS production was measured by luminol chemiluminescence assay in a Berthold MicroLumat Plus luminometer (Berthold Technologies), essentially as previously described [14]. Purified bone-marrow derived neutrophils were resuspended at 5×10^6 cells/ml in ice-cold $DPBS^{++++}$ and primed with 5 ng/ml murine TNFα, 100 ng/ml GM-CSF for 45 min at 37°C, or with 1 μg/ml LPS (E. coli LPS, Sigma, L3024) for 90 min at 37°C, with occasional flicking to prevent settling, or they were mock-primed in $DPBS^{++++}$ under the same conditions. Unprimed neutrophils were kept on ice and prewarmed to 37°C for 3 min prior to the assay. Stimuli (fMLP, C5a, PMA, bacteria, fungal particles) were prepared as 2.5x stocks in $DPBS^{++++}$. Heat-killed *S. pneumoniae* were prepared by growing bacteria to mid-log phase as described above, washing twice in phosphate buffered saline (PBS) and inactivating at 60˚C for 1 h prior to storage at −80°C. The efficacy of heat-inactivation was verified by culture on blood agar before removing bacteria from biosafety level 2 containment. 2.5x stocks were prepared by washing thawed aliquots of heat-inactivated *S. pneumoniae* in $DPBS^{++++}$, opsonisation with 10% mouse serum in $DPBS^{++++}$ for 15 min at 37°C, washing again and resuspending in $DPBS^{++++}$ at 7.5×10^8/ml. 2.5x stocks of *S. aureus* Wood 46 were prepared from logarithmically growing cultures, by washing in $DPBS^{++++}$, opsonisation with 10% mouse serum in $DPBS^{++++}$ for 15 min at 37°C, washing again and resuspending in $DPBS^{++++}$ at 1.875×10^7/ml. 2.5x stocks of zymosan A (*S. cerevisiae* yeast particles, Thermo Fisher, Z2849) were prepared by washing particles twice in $DPBS^{++++}$ and resuspending in $DPBS^{++++}$ at 1.125×10^7/ml. Prior to the assay, an equal volume of prewarmed

Detect buffer ($DPBS^{++++}$ containing 16 units/ml horseradish peroxidase (HRP, Sigma, P8375) and 120 μM luminol (Sigma-Aldrich, 123072)) was added to the prewarmed (primed, mock-primed or unprimed) neutrophils. The neutrophils/Detect mix was incubated for 3 min at 37°C, before 150 μl/well were dispensed into a prewarmed 96-well luminometer plate. 100 μl of prewarmed 2.5x stimulus in $DPBS^{++++}$, or $DPBS^{++++}$ control, was added either by automatic injection port (fMLP, C5a) or manually (bacteria, zymosan, PMA), and real-time ROS production was recorded at 37°C. Final assay concentrations were 1.5×10^6 neutrophils/ml and 3 μM fMLP, 25 nM C5a, 500 nM PMA, $3x10^8$ heat-killed *S. pneumoniae*/ml, $7.5x10^6$ *S. aureus*/ml, or $4.5x10^6$ zymosan A particles/ml. ROS production was quantified by integrating the area under the curve (AUC) of the ROS response over 2 min for fMLP and C5a, over 10 min for PMA and bacteria, and over 60 min for zymosan. In some assays, neutrophils were incubated with the indicated concentrations of the Rac inhibitor EHT 1864 (Tocris 3872; 100 mM stock in water) or the Erk1/2 inhibitor SCH772984 (Cayman Chemicals, 19166; 10 mM stock in DMSO) during priming with TNFα and GM-CSF for 1 h prior to stimulation of ROS production with fMLP as described above. The DMSO concentration during priming in the presence of SCH772984 was adjusted to 1% in all samples.

Degranulation: Gelatinase (MMP9) degranulation was measured by in-gel zymography essentially as described [15]. Purified bone marrow-derived neutrophils were resuspended at 5×10^6/ml in $DPBS^{++++}$ and either left unprimed on ice, or primed with 20 ng/ml murine TNFα and 50 ng/ml GM-CSF for 45 min at 37°C. Neutrophils (80 μl/well) were pipetted into a 96-well plate (Nunc; pre-blocked with 10% heat-inactivated FBS) containing 20 μl 5x fMLP and cytochalasin B in $DPBS^{++++}$, or $DPBS^{++++}$ alone, and incubated for 30 min at 37°C in 5% CO_2, followed by centrifugation at 300 × g for 10 min at 4°C. 40 μl of the conditioned supernatant was mixed with 20 μl 3x non-reducing SDS-PAGE sample buffer (160 mM Tris, pH 6.8, 8% SDS, 50% glycerol, bromophenol blue) at RT. 5 μl aliquots were separated by SDS-PAGE gel containing 0.067% gelatine B. Gels were equilibrated in 2.5% Triton X-100 for 30 min and in developing

buffer (50 mM Tris, pH 7.5, 200 mM NaCl, 5 mM $CaCl_2$, 0.02% Triton X-100) overnight at RT, allowing the gelatinase to digest the gelatine in the gel. Gels were coomassie-stained and gelatinase activity analyzed by densitometry using ImageJ.

Phagocytosis: Zymosan particles (Molecular Probes, Z2849) were opsonized using the manufacturer's opsonizing reagent (Molecular Probes, Z2850) according to their instructions and stored in $DPBS^{++++}$ at 4°C. Purified bone marrow-derived neutrophils were resuspended at 1×10^7/ml in $DPBS^{++++}$, and 200 μl cells/sample were allowed to adhere to glass coverslips in a 12-well plate for 15 min at 37°C, 5% CO_2, prior to the addition of 100 μl opsonised zymosan particles at a ratio of 5 particles/neutrophil. Samples were incubated for 30 min at 37°C, 5% CO_2, fixed in 4% paraformaldehyde (PFA)/PBS for 15 min at RT, washed twice in PBS, permeabilised in PBS/0.1% Triton X-100 for 10 min at RT, washed again twice, stained with FTIC-Gr1 antibody and DAPI in PBS, Fc block for 40 min at RT, washed three times and mounted. Slides were imaged using a ZEISS Axio Imager D2 widefield system and images analyzed using ImageJ for the number of zymosan particles per neutrophil.

NETs: *S. aureus*-induced NET production was assayed essentially as described [16]. *S. aureus* were cultured to logarithmic growth as described above, sedimented for 2 min at 12,000 x g, washed in Dulbecco's Modified Eagle's Medium (DMEM), 10 mM HEPES, pH 7.4, and opsonised with 10% mouse serum in DMEM, 10 mM HEPES for 30 minutes at 37°C, washed twice and resuspended at 5×10^7 bacteria/ml in DMEM, 10 mM HEPES. Purified bone marrow-derived neutrophils were resuspended at 8×10^5 cells/ml in DMEM, 10 mM HEPES, and 250 μl were seeded into each well of an 8-well glass bottom slide (ibidi 80827) and allowed to adhere for 30 min at 37°C, 5% CO_2, prior to the addition of 40 μl opsonised *S. aureus* (ratio of 10 bacteria per neutrophil), or were mock-treated with DMEM, 10 mM HEPES, for the indicated periods

of time. 15 minutes before the end of incubation, 0.1 µM Sytox Green (ThermoFisher, S7020; non-cell permeable) and 1.25 µg/ml Hoechst Hoechst 33342 (cell permeable) DNA stains were added and samples were live-imaged on the Nikon eclipse Ti-E widefield system using the 20x objective. 9 FOVs were captured per image and Sytox Green^{+} cells with NETs were counted manually. Phase contrast was used to quantify the total cell number, as not all cells were stained efficiently with Hoechst under these conditions.

Chemokine/cytokine release by neutrophils: Purified bone marrow-derived neutrophils were resuspended at 1 x 10^7 cells/ml in DPBS^{++++} and 100 µl added to each well of a 96-well plate (Thermo Fisher, Nunc 167008), which had been pre-blocked with 10 % heat-inactivated FBS for 1 h at RT. Cells were stimulated with zymosan particles (see above) in DPBS^{++++} at a ratio of 5 particles/neutrophil, or were mock-stimulated, for 4 or overnight (16 h) at 37°C, 5% CO_2. For overnight incubations, 10% heat inactivated FBS was added to the medium. Cells were sedimented for 10 min at 300 x g and supernatants collected and stored at -20°C until the analysis. The release of mouse TNFα, CXCL-1 and IL-6 by neutrophils into the supernatant was quantified by DuoSet ELISA kit as described above.

Adhesion, spreading and polarization: Purified bone marrow-derived neutrophils were resuspended at 2 × 10^6/ml in DPBS^{++++}, kept unprimed on ice, or were primed with 20 ng/ml murine TNFα, 50 ng/ml GM-CSF for 45 min at 37°C. 500 µl of cells were added onto 13 mm glass coverslips in each well of a 24-well plate (Thermo Fisher, Nunc 142475) containing 500 µl of 3 µM fMLP in DPBS^{++++}, or DPBS^{++++} alone, and were incubated for 10 or 25 min at 37°C at 5% CO_2, before fixing in 4% PFA, DPBS^{++++} for 15 min at RT. Cells were washed twice in PBS and stained with FTIC-Gr1 antibody and Hoechst DNA stain in PBS, Fc block, for 30 min at RT. Coverslips were washed three times in PBS, rinsed in H_2O and mounted onto slides with ProLong Gold Antifade (Life Technologies, P36934). Imaging was done using the 'large image capture'

function of a Nikon Eclipse Ti-E widefield system, taking 27 (3 x 9) fields-of-view (FOV) per coverslip at 100x magnification, and duplicate coverslips per condition. Image analysis was done using ImageJ. To quantify neutrophil adhesion, neutrophils/FOV were counted. Spreading was quantified by drawing a mask for each neutrophil and determining the area of the mask. To evaluate polarization, each neutrophil was assigned manually to one of four morphology categories (round, round/spread, polar, polar/spread).

Cell surface levels of receptors and adhesion molecules: Bone marrow cells were flushed from bones with ice-cold $HBSS^{--++}$, filtered through 40 µm cell strainers, counted by hemocytometer, pelleted at 326 × g for 10 min at 4°C, and resuspended in ice-cold $DPBS^{++++}$ at 4×10^7/ml. Cells were either kept on ice to minimize receptor trafficking, or were incubated for 30-45 min at 37°C to allow constitutive trafficking, or were primed with 20 ng/ml murine TNFα, 50 ng/ml GM-CSF for 45 min at 37°C to induce maximal receptor upregulation to the plasma membrane. In some instances, cells were stimulated for 10 or 30 min with 100 nM C5a to elicit agonist-induced receptor internalization. Cells were then centrifuged at 10,000 × g for 30 s at 4°C, resuspended in an ice-cold cocktail containing Fc block and antibodies for neutrophil markers, as well as PE- or AF647-labelled antibodies for GPCRs (C5aR1, CXCR1, CXCR2, CXCR4) or adhesion molecules (L-selectin, PSGL-1, LFA-1, Mac-1) in $DPBS^{++++}$, and incubated for 20 min on ice. Cells were sedimented at 10,000 × g for 30 s, resuspended in 1 ml ice-cold $HBSS^{--++}$, 1 mM EDTA and kept on ice. Flow cytometry was performed using a BD Biosciences LSR-Fortessa or Fortessa 5 flow cytometer, and data were analyzed using FlowJo. Neutrophils were identified by $Gr1^{hi}$ or $CD11b^{hi}$, $Ly6G^{hi}$ staining. GPCR and adhesion molecule levels on the neutrophil surface were quantified as the mean PE- or AF647-fluorescence signals from the relevant antibodies after subtraction of isotype control antibody background.

Integrin avidity: β2-integrin clustering was used as a measure of avidity essentially as previously described [17]. Purified bone marrow-derived neutrophils were resuspended at 3 x 10^6/ml in $DPBS^{++++}$ and primed with 20 ng/ml TNFα, 50 ng/ml GM-CSF for 45 min at 37°C. 200 µl aliquots were stimulated with 1.5 µM fMLP and AF594 anti-mouse CD18 antibody (BioLegend, 101416), or with AF594 anti-mouse CD18 antibody alone, for 3 min in a thermomixer at 37°C, 300 rpm. 3 mM Mn^{2+} was added to some samples as a positive control. To stop the stimulation, 1 ml $DPBS^{--}$ was added and cells centrifuged at 9,184 x g for 30 s at 4°C. Cells were washed in ice-cold $DPBS^{--}$, fixed in 4% PFA at 4°C, washed twice in $DPBS^{--}$ by centrifugation at 367 x g for 4 min, resuspended in 200 µl $DPBS^{--}$ and allowed to settle onto electrostatically charged slides (Superfrost Plus, VWR) for 30 min at RT. Slides were washed in H_2O and mounted in AquaPolymount using glass coverslips. Samples were imaged with a Nikon confocal AR1 microscope using the 60x objective. 30-40 cells pre coverslip were analyzed by ImageJ, R and Cell Profiler softwares for the presence of integrin clusters. Clusters were defined as areas of integrin pixel signal of at least 1.25 x the median intensity value of the cell. The number, size and localization of integrin clusters was determined. Clusters centered within 1/4 of the cell periphery around the largest cluster in the cell were defined as polarized.

Rac activity: Rac activity was assessed by Pak-CRIB pull down, essentially as described [18]. GST-Pak-CRIB bait was purified from bacterial culture as described [19] and stored in GST-FISH buffer (10% glycerol, 50 mM Tris pH 7.4, 100 mM NaCl, 1% NP-40, 2 mM $MgCl_2$, 2 mM DTT, 100 µM PMSF, and 10 µg/ml each of leupeptin, pepstatin A, aprotinin and antipain) at 4°C for up to one week. Purified bone marrow-derived neutrophils were resuspended at 1 × 10^7/ml in $DPBS^{++++}$ and pre-warmed for 3 min at 37°C. 200 µl aliquots were stimulated with 10 x fMLP (Sigma, F3506) at the indicated concentrations in $DPBS^{++}$ for 10 s, or were mock-stimulated. The reaction was stopped by the addition of 1 ml of ice-cold GST-FISH buffer containing 1.2% NP-40 (to give a final concentration of 1% NP-40), and cells were lysed by incubation on ice for 2 min with frequent vortexing. Samples were centrifuged at 12,000 x g for 3 min at 2°C to sediment debris, the

supernatant transferred into fresh precooled tubes, and 2% kept as a total lysate control. The remaining sample was incubated with GST-Pak-CRIB beads by end-over-end rotation for 15 min on ice. Samples were washed 5 times in GST-FISH buffer before adding boiling 1.3x SDS-PAGE sample buffer and boiling the samples for 5 min. To process total lysate samples, boiling 4× SDS-PAGE sample buffer was added to final 1.3x, and samples boiled for 5 min. GTP-Rac and total Rac were quantified by western blotting with Rac1 and Rac2 antibodies and densitometry using ImageJ.

Other GPCR signaling pathways: To determine the activities of $p38^{MAPK}$, Erk1/2, Jnk and Akt, purified bone marrow-derived neutrophils were resuspended at 1 × 10^7 cells/ml in $DPBS^{++++}$, primed with 20 ng/ml murine TNFα, 50 ng/ml GM-CSF for 45 min at 37°C, and 150 µl aliquots were stimulated for various time points with 50 µl 4 x fMLP at various concentrations, or mock-stimulated, as indicated. The reaction was stopped by the addition of 1 ml ice-cold $DPBS^{++}$. Cells were sedimented at 12,000 x g for 30 s at 2°C and lysed with 150 µl ice-cold RIPA buffer (30 mM Hepes pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 5 mM EGTA, 4 mM EDTA), supplemented with 1 mM DTT, 1 mM PMSF, protease inhibitors (10 µg/ml each of leupeptin, pepstatin A, aprotinin, antipain) and phosphatase inhibitors (50 mM NaF, 10 mM β-glycerophosphate, 10 mM $Na_2P_2O_7$, 1.5 mM Na_3VO_4) by incubation on ice for 5 min and vortexing. Debris was sedimented at 12,000 x g for 30 s at 2°C, the supernatant transferred to fresh pre-cooled tubes, boiling 4 x SDS-PAGE sample buffer added to final 1.3 x, and samples boiled for 5 min. Vav activity was determined in a similar manner, except that neutrophils were primed at 4 × 10^7 cells/ml, and 50 µl aliquots were stimulated with 1 µM fLMP, or mock-stimulated, for 10 s. The reaction was stopped by the addition of 150 µl RIPA buffer with the same supplements as described here-above except that leupeptin, pepstatin A, aprotinin, antipain were used at 25 µg/ml, Na_3VO_4 at 5 mM, 1x PhosSTOP (Roche, 04 906 845 001) was added, and the lysate was incubated on ice for 3 min. Samples were western blotted for total $p38^{MAPK}$,

Erk1/2, Jnk, Akt and Vav levels and for activating phosphorylations of these proteins using the antibodies detailed in Supplemental Table 2. Activities were quantified by densitometry of blots using ImageJ.

Data collection and statistical analysis: Sample size was determined using power calculations to yield 80% power, based on results of pilot experiments and on previously published data as referenced in 'Methods'. *In vivo* experiments were performed at least twice with independent cohorts of mice in order to determine inter-experimental variability as well as inter-individual variability. *In vitro* experiments were performed at least three times. Sample size and numbers of independent experiments are detailed in Figure Legends and Supplemental Figure Legends. Norbin-deficient and control mice were age- and sex-matched for experiments. Within those parameters, mice were selected for cohorts at random by the staff of the Biological Support Unit. Image analysis was performed double-blind by one person marking slides with random IDs and another person performing ImageJ analysis. Excel 2016 and GraphPad Prism 8.0 were used for tabulation, statistical analysis and plotting graphs.

Data were tested for normality of distribution using Shapiro-Wilk test to determine if parametric or non-parametric statistical analysis was required. Where warranted by variance between groups, data were log-transformed or square root-transformed prior to statistical analysis. Statistical outliers were identified using Tukey's test and removed from datasets. Other samples were only excluded when there was a known technical problem affecting the analysis. Comparisons between single independent measurements from two groups were made by two-tailed unpaired Student's t-test or Mann-Whitney U-test, as appropriate. Depending on experimental design, single or repeated measures one-way analysis of variance (ANOVA) or two-way ANOVA was used to test for effects of interventions. Group sizes (n) are listed in figure legends. Effect size and variance are reported as group mean ± standard error. P-values reported are from t-tests, or from multiplicity-adjusted Dunnett's, Tukey's, Sidak's or Holm-Sidak's post-

hoc comparisons, as selected to be most appropriate by the GraphPad Prism software. The threshold for statistical significance was set at $P<0.05$.

References for Supplemental Methods

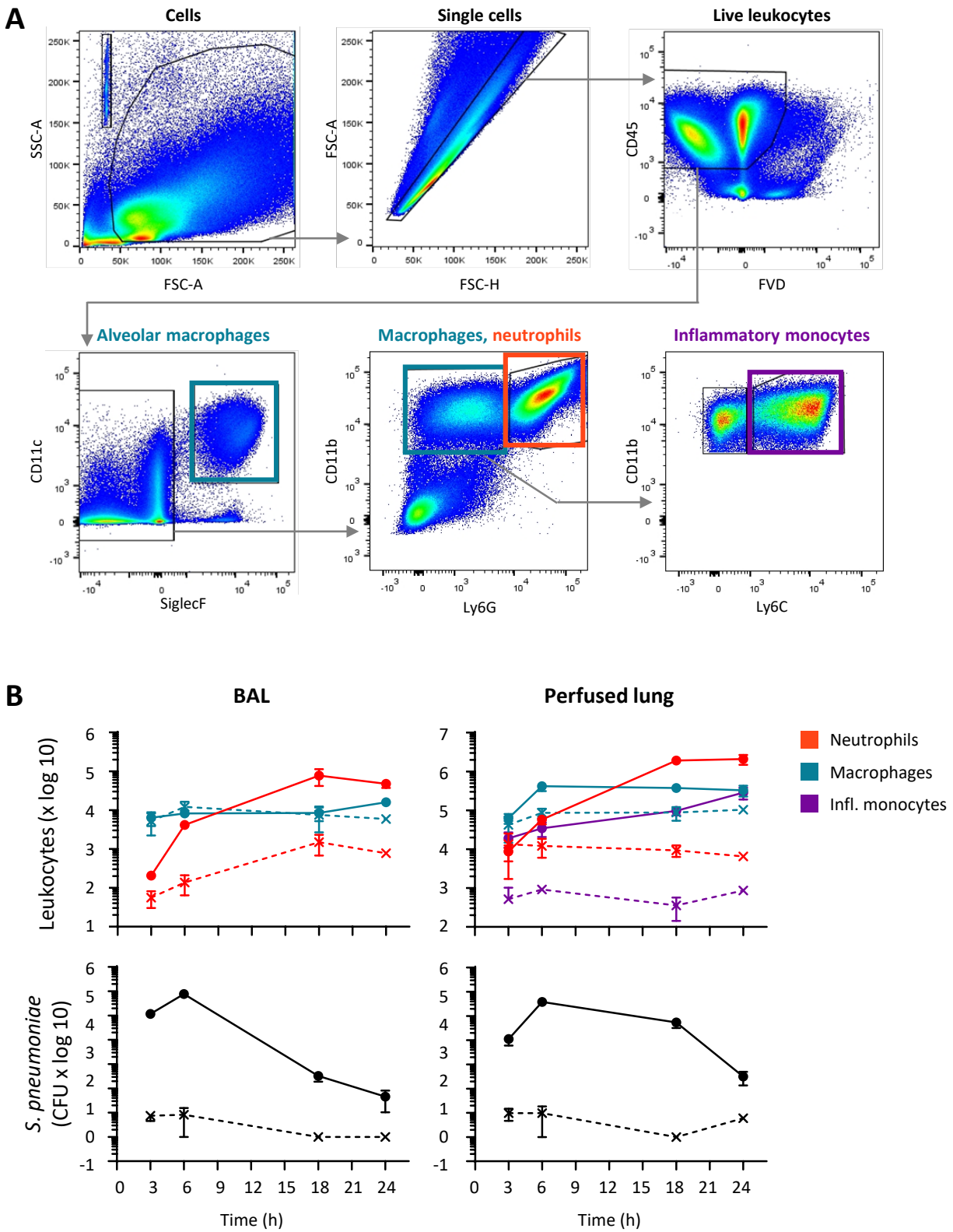
1. Shinozaki K, Maruyama K, Kume H, Kuzume H, Obata K. A novel brain gene, Norbin, induced by treatment of tetraethylammonium in rat hippocampal slice and accompanied with neurite-outgrowth in Neuro 2a cells. *Biochem Biophys Res Commun*. 1997;240(3):766-771.
2. Dateki M, Horii T, Kasuya Y, et al. Neurochondrin negatively regulates CaMKII phosphorylation, and nervous system-specific gene disruption results in epileptic seizure. *J Biol Chem*. 2005;280(21):20503-20508.
3. Clausen BE, Burkhardt C, Reith W, Renkawitz R, Forster I. Conditional gene targeting in macrophages and granulocytes using LysMcre mice. *Transgenic Res*. 1999;8(4):265-277.
4. Donald S, Humby T, Fyfe I, et al. P-Rex2 regulates Purkinje cell dendrite morphology and motor coordination. *Proc Natl Acad Sci U S A*. 2008;105(11):4483-4488.
5. Welch HC. Regulation and function of P-Rex family Rac-GEFs. *Small GTPases*. 2015;6(2):1-11.
6. Damoulakis G, Gambardella L, Rossman KL, et al. P-Rex1 directly activates RhoG to regulate GPCR-driven Rac signalling and actin polarity in neutrophils. *J Cell Sci*. 2014; 127(11):2589-2600.
7. Pan D, Barber MA, Hornigold K, et al. Norbin stimulates the catalytic activity and plasma membrane localization of the guanine-nucleotide exchange factor P-Rex1. *J Biol Chem*. 2016;291(12):6359-6375.
8. Stark AK, Chandra A, Chakraborty K, et al. PI3Kdelta hyper-activation promotes development of B cells that exacerbate Streptococcus pneumoniae infection in an antibody-independent manner. *Nat Commun*. 2018;9(1):3174.
9. Van Rooijen N, Sanders A. 1994. *Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications.* J. Immunol. Methods. 174 (1-2):83-93.
10. Daley JM, Thomay AA, Connolly MD, Reichner JS, Albina JE. Use of Ly6G-specific monoclonal antibody to deplete neutrophils in mice. *J Leukoc Biol*. 2008;83(1):64-70.
11. van 't Veer C, van den Pangaart PS, Kruijswijk D, et al. Delineation of the role of Toll-like receptor signaling during peritonitis by a gradually growing pathogenic Escherichia coli. *J Biol Chem.* 2011; 286(42): 36603–36618.
12. Welch HC, Condliffe AM, Milne LJ, et al. P-Rex1 regulates neutrophil function. *Curr Biol*. 2005;15(20):1867-1873.
13. Pan D, Amison RT, Riffo-Vasquez Y, et al. P-Rex and Vav Rac-GEFs in platelets control leukocyte recruitment to sites of inflammation. *Blood*. 2015;125(7):1146-1158.
14. Lawson C, Donald S, Anderson K, Patton D, Welch H. P-Rex1 and Vav1 cooperate in the regulation of fMLF-dependent neutrophil responses *J Immunol*. 2011;186(3):1467-1476.
15. Mocsai A, L. AC, Jakus Z, Hu Y, Lanier LL, Lowell CA. Integrin signaling in neutrophils and macrophages uses adaptors containing immunoreceptors tyrosine-based activation motifs. *Nat Immunol*. 2006;7(12):1326-1333.
16. van der Linden M, Westerlaken GHA, van der Vlist M, van Montfrans J, Linde Meyaard L. Differential Signalling and Kinetics of Neutrophil Extracellular Trap Release Revealed by Quantitative Live Imaging. *Sci Rep.* 2017;7: 6529.
17. Boras M, Volmering S, Arne Bokemeyer A, et al. Skap2 is required for β2 integrin–mediated neutrophil recruitment and functions. *J Exp Med.* 2017 Mar 6; 214(3): 851–874.
18. Johnsson AE, Dai Y, Nobis M, et al. The Rac-FRET mouse reveals tight spatiotemporal control of Rac activity in primary cells and tissues. *Cell Rep*. 2014;6(6):1153-1164.
19. Sander EE, van Delft S, ten Klooster JP, et al. Matrix-dependent Tiam1/Rac signaling in epithelial cells promotes either cell-cell adhesion or cell migration and is regulated by Phosphatidylinositol 3-Kinase. *J Cell Biol*. 1998;143(5):1385-1398.

Laser	Fluorochrome	Antibody Target	Clone Provider (catalogue number)	Dilution
(1) Identification of neutrophils				
405 nm 525/50	BV510	Ly6G	1A8 BioLegend (127633)	1:500
640 nm 670/14	AF647	CD11b	M1/70 BD Biosciences (557686)	1:1000
Or, where indicated				
488 nm 530/30	FITC	Ly6G/Ly6C (Gr1)	RB6-8C5 BD Biosciences (553126)	1:800
640 nm 670/14	AF647	CD11b	M1/70 BD Biosciences (557686)	1:1000
(2) Identification of leukocytes in BAL and perfused lung homogenate				
355 nm 379/28	BUV395	CD45	30-F11 BD Biosciences (564279)	1:300
405 nm 525/50	BV510	Ly6G	1A8 BioLegend (127633)	1:500
640 nm 670/14	AF647	CD11b	M1/70 BD Biosciences (557686)	1:1000
405 nm 450/50	BV421	Ly6C	AL-21 BD Biosciences (562727)	1:500
405 nm 780/60	BV785	CD11c	N418 BioLegend (117335)	1:500
561 nm 585/15	PE	SiglecF	E50-2440 BD Biosciences (552126)	1:200
(3) Analysis of neutrophil depletion efficiency: as (2), except replacing anti-Ly6G with				
488 nm 530/30	FITC	CD24	M1/69 BioLegend (101805)	1:400
(4) Analysis of macrophage depletion efficiency: as (2), except replacing anti-CD11c with				
488 nm 530/30	AF488	CD11c	N418 eBioscience (53-0114-82)	1:250
(5) Analysis of neutrophil GPCR surface levels: as (1), plus one of these				
561 nm 585/15	PE	C5aR1	20/70 Abcam (53434)	1:60
561 nm 585/15	PE	CXCR1	U45-632 BD Pharmingen (566383)	1:60
561 nm 585/15	PE	CXCR2	SA0044G4 BioLegend (149303)	1:60
561 nm 585/15	PE	CXCR4	2B11 Invitrogen (12-9991-81)	1:60
(6) Analysis of neutrophil adhesion molecule surface levels: as (1), plus one of these				
640 nm 670/14	AF647	CD11b (Mac-1)	M1/70 BD Biosciences (557686)	1:1000
640 nm 670/14	AF647	CD11a (LFA-1)	M17/4 BD Biosciences (563668)	1:400
561 nm 585/15	PE	CD62L (L-selectin)	MEL-14 BD Biosciences (553151)	1:100
561 nm 585/15	PE	CD162 (PSGL-1)	2PH1 BD Biosciences (555306)	1:200

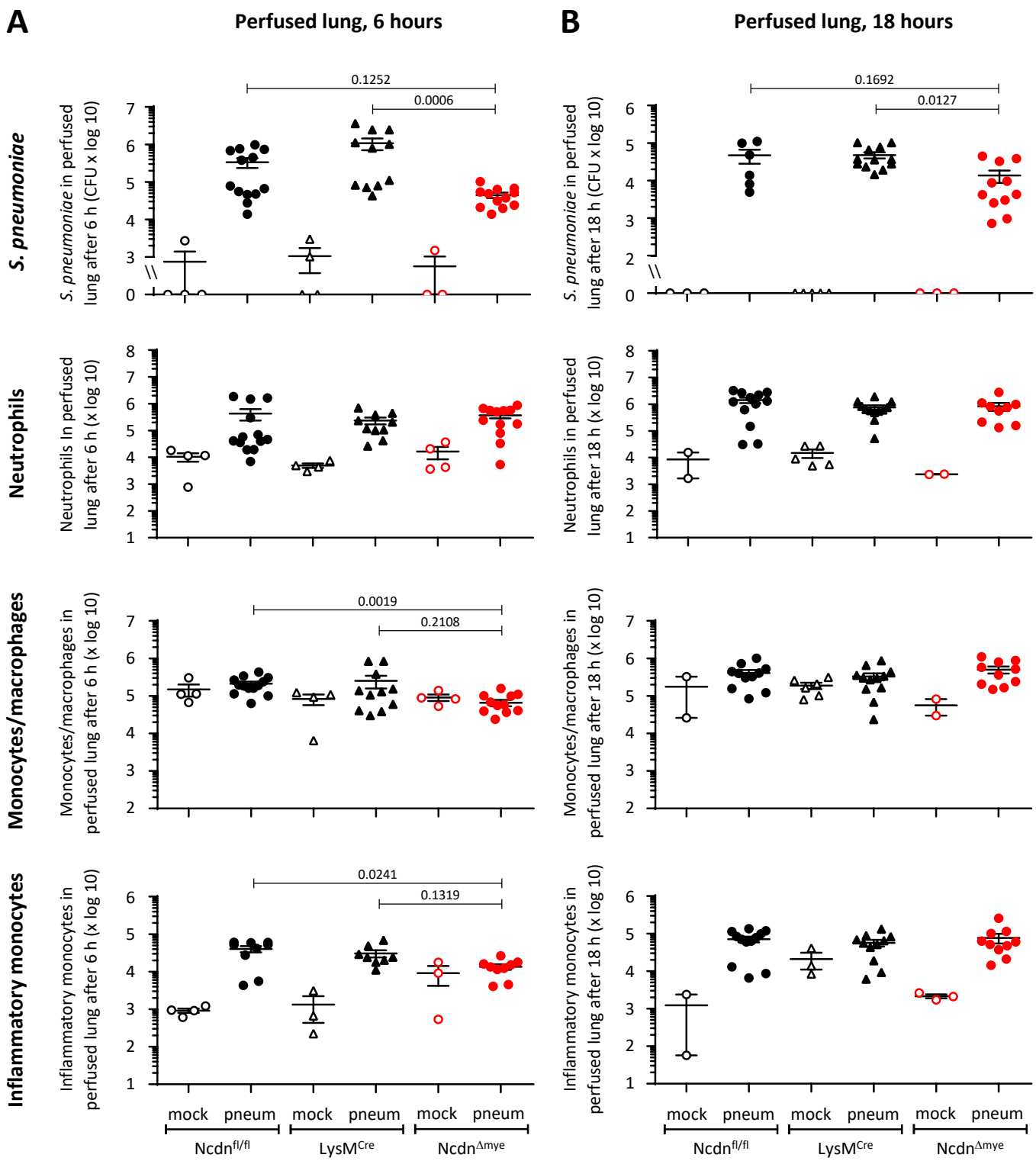
Supplemental Table 1: Antibodies used in flow cytometry

Antibody target	Antibody type	Provider	Dilution
Norbin	Rabbit polyclonal (C1)	Dr Kei Maruyama, Tokyo Institute of Psychiatry, Japan	1:1000
Rac1	Mouse monoclonal	Millipore (05-389)	1:3000
Rac2	Goat monoclonal	Millipore (07-604)	1:5000
Phospho-p38 MAPK (Thr180/Tyr182)	Rabbit polyclonal	Cell Signaling (9211)	1:1000
p38 MAPK	Rabbit polyclonal	Cell Signaling (9212)	1:500
Phospho-Vav (Tyr173)	Rabbit polyclonal	ProSci (79-457)	1:1000
Vav	Rabbit polyclonal	Dr Martin Turner, Babraham Institute	1:1000
Phospho-p44/42 MAPK (Thr202/Tyr204)	Mouse polyclonal	Cell Signaling (9106)	1:1000
p44/42 MAPK (Erk1/2)	Rabbit polyclonal	Cell Signaling (9102)	1:1000
Phospho-SAPK/JNK (Thr183/Tyr185)	Rabbit polyclonal	Cell Signaling (9251)	1:200
SAPK/JNK	Rabbit polyclonal	Cell Signaling (9252)	1:200
Phospho-AKT (Thr308)	Rabbit polyclonal	Cell Signaling (9275)	1:200
AKT	Rabbit polyclonal	Cell Signaling (9272)	1:200

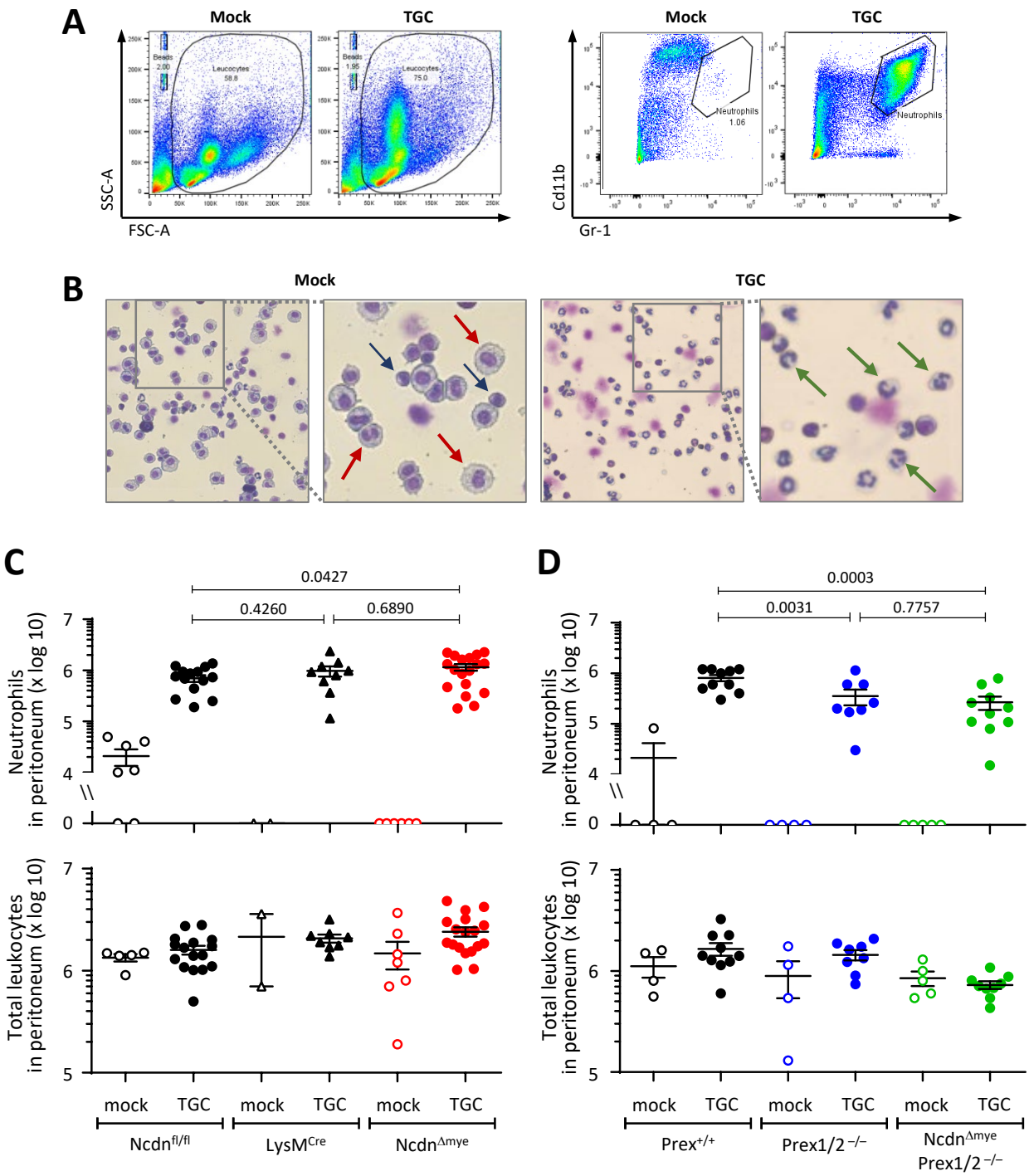
Supplemental Table 2: Antibodies used for western blotting



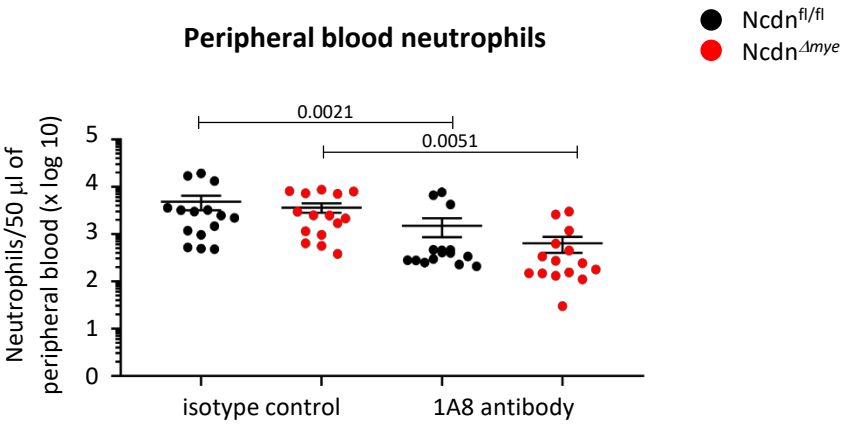
Supplemental Figure 1: Gating strategy for flow cytometry and time-course of neutrophil recruitment and clearance of bacteria in Ncdn$^{fl/fl}$ control mice during pulmonary *S. pneumoniae* infection



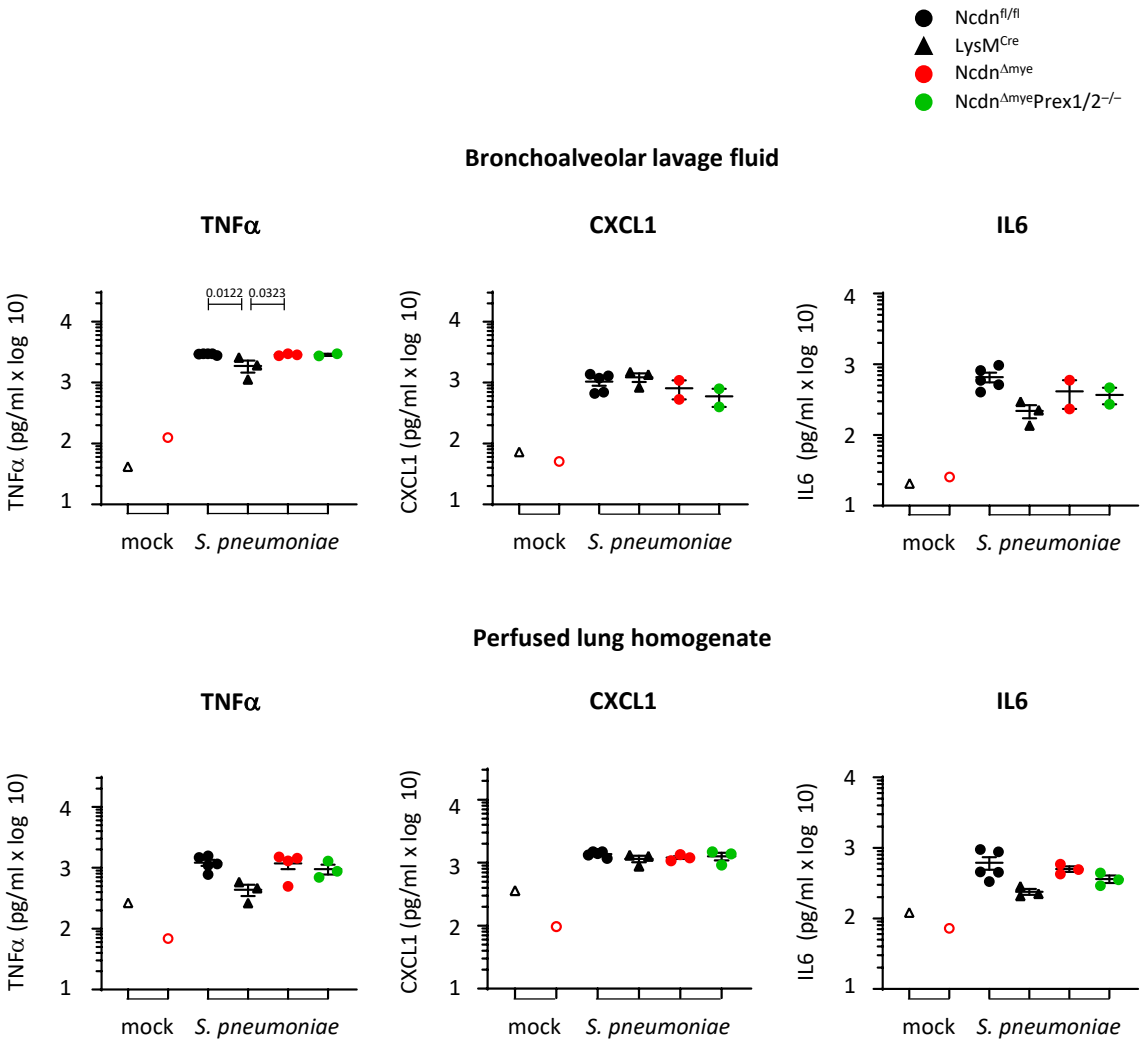
Supplemental Figure 2: Improved clearance of *S. pneumoniae* from the lung parenchyma of Ncdn$^{\Delta mye}$ mice



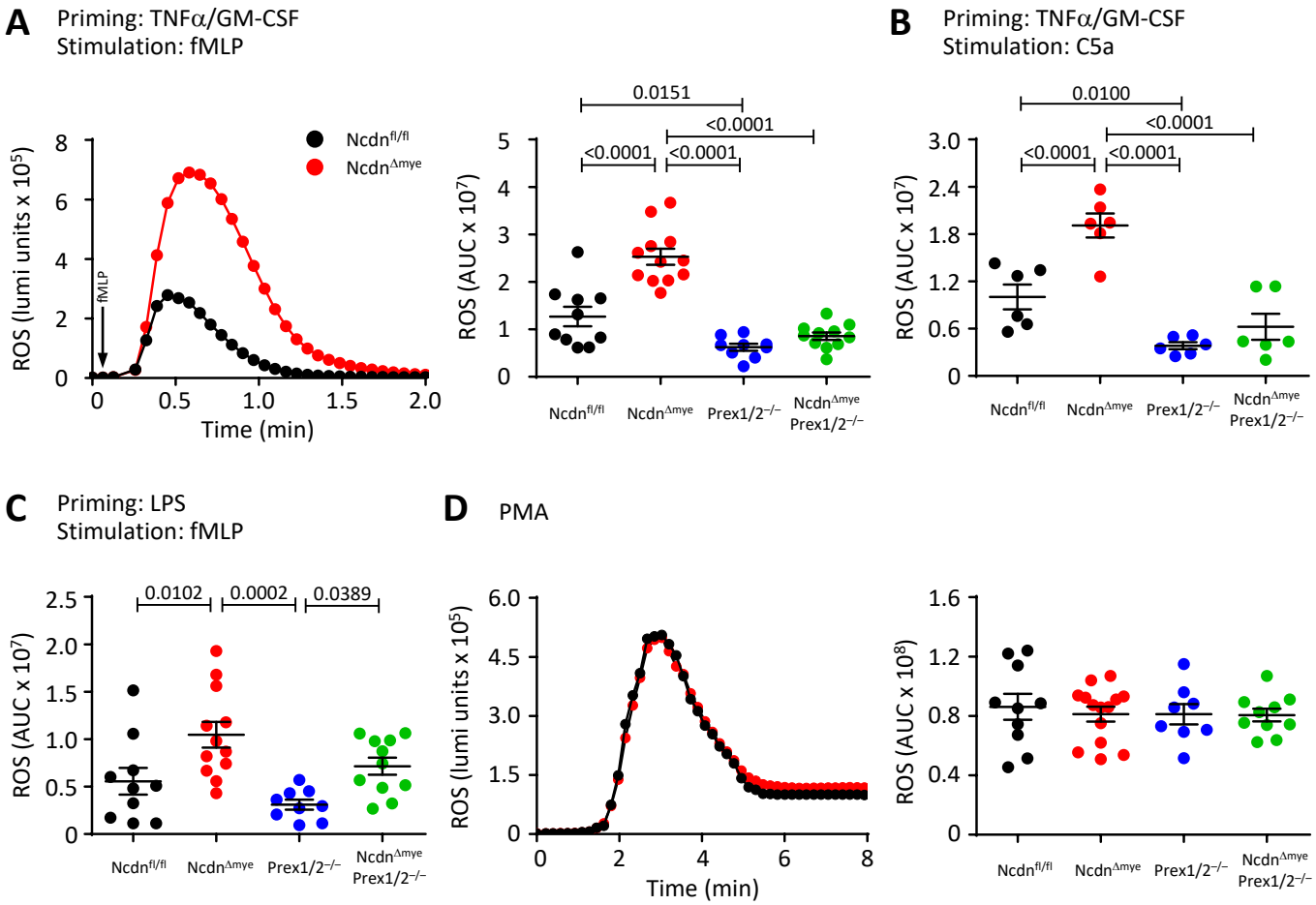
Supplemental Figure 3: Norbin deficiency does not affect Prex-dependent neutrophil recruitment during aseptic peritonitis.



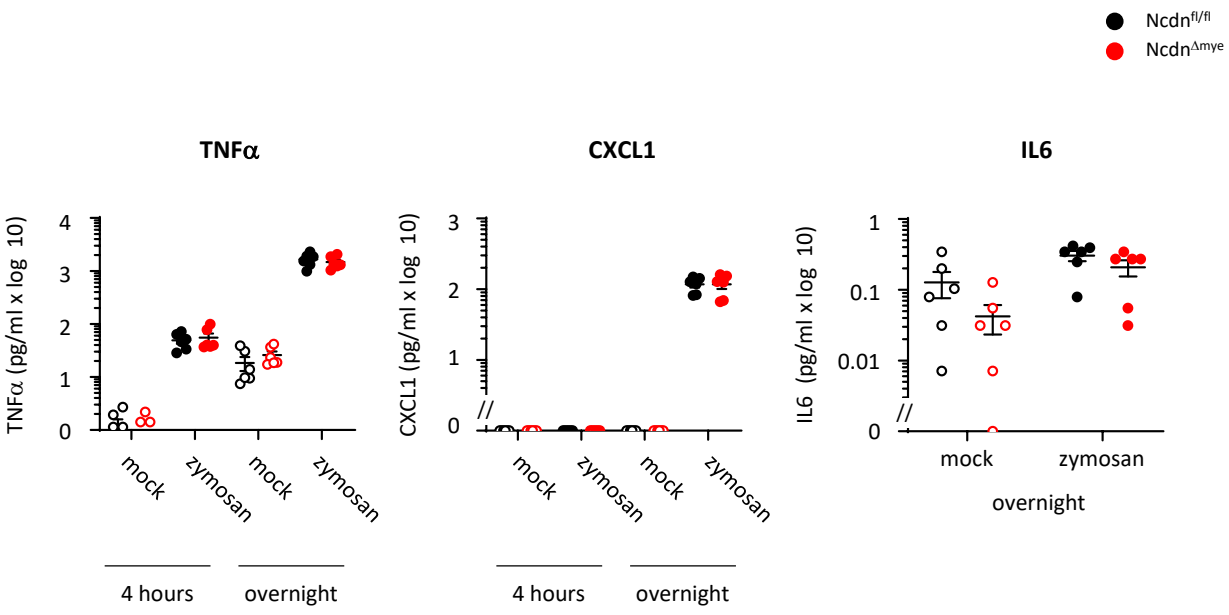
Supplemental Figure 4: Efficacy of 1A8 antibody in depleting neutrophils from peripheral blood



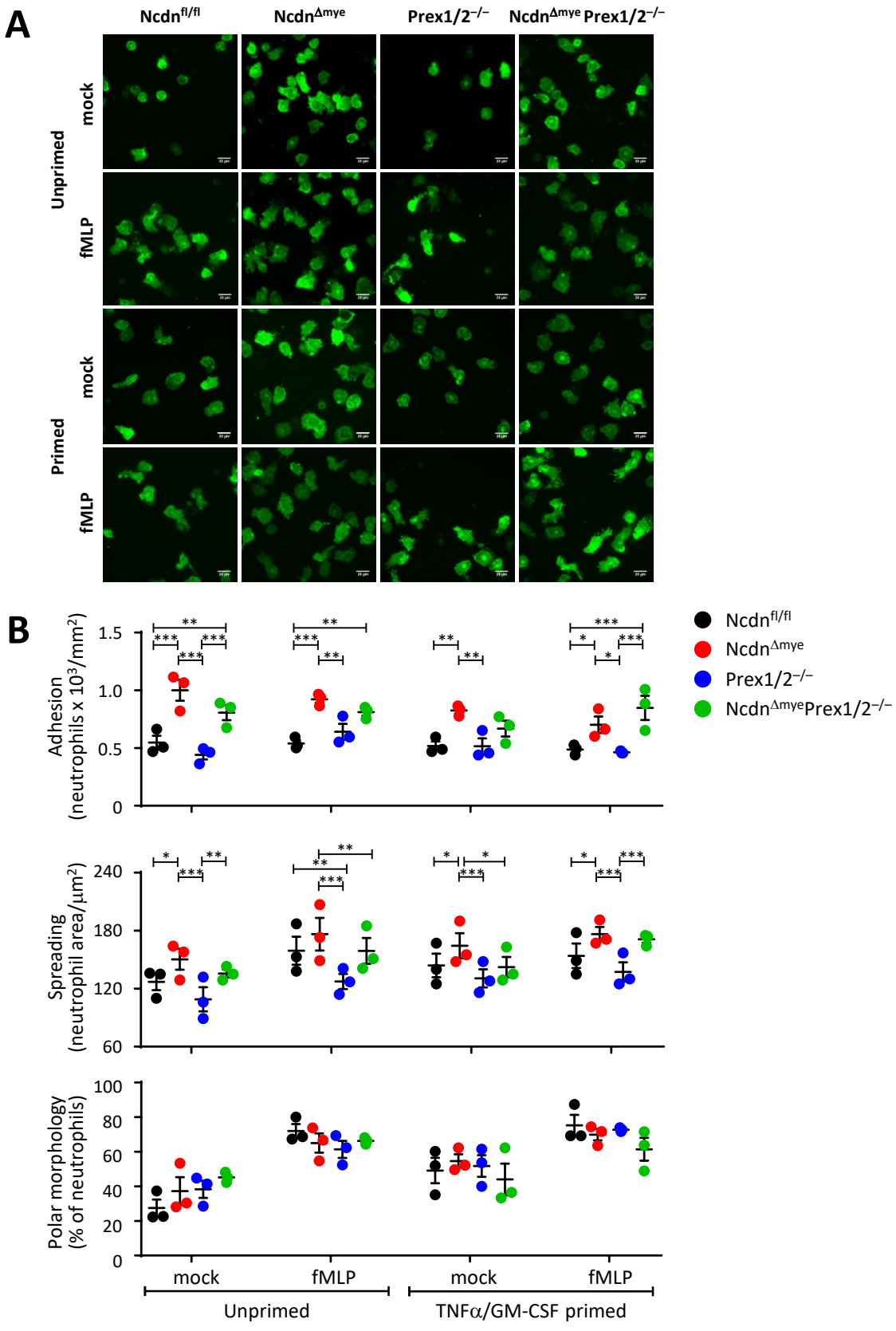
Supplemental Figure 5: Myeloid Norbin deficiency does not affect the production of TNFα, CXCL1 and IL6 during pneumococcal infection



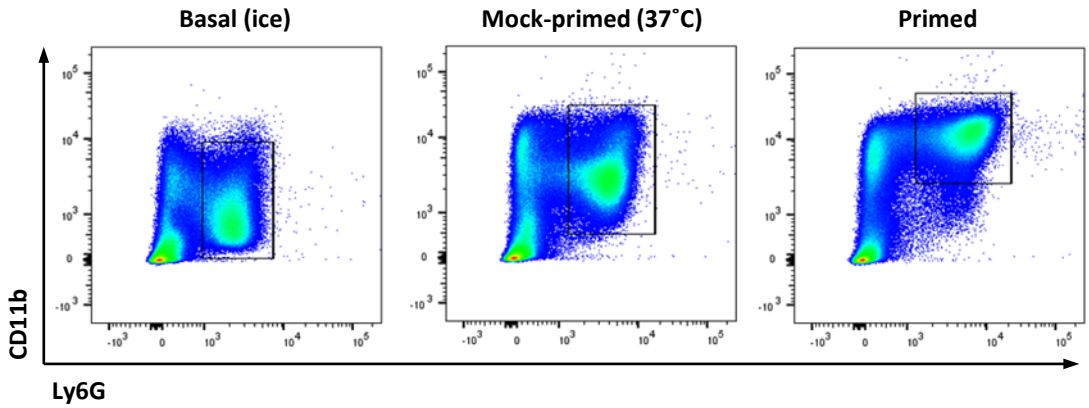
Supplemental Figure 6: Ncdn$^{\Delta mye}$ neutrophils have increased ROS production in response to GPCR stimulation



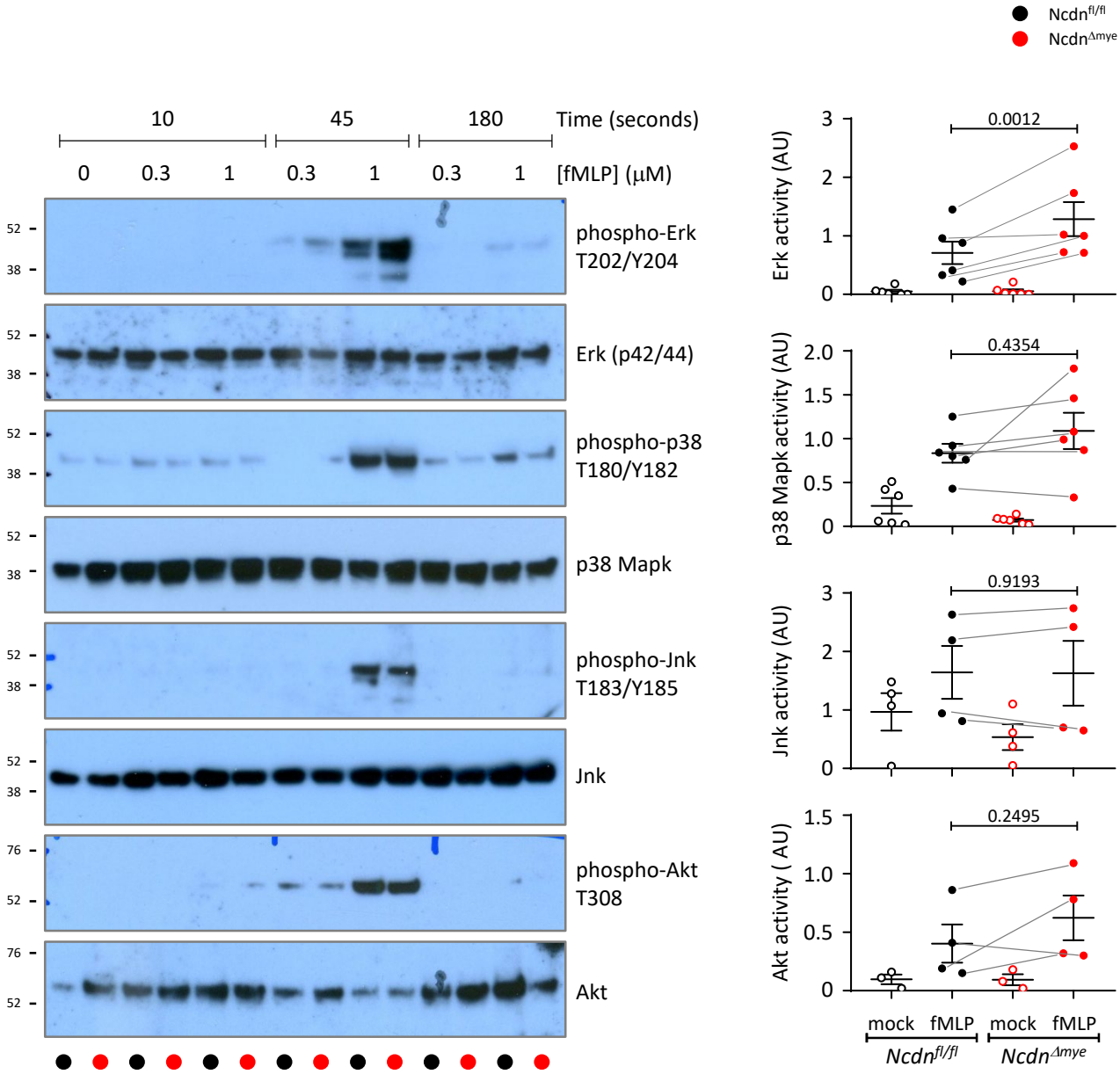
Supplemental Figure 7: Norbin-deficient neutrophils produce normal levels of TNFα, CXCL1 and IL6 upon zymosan stimulation



Supplemental Figure 8: Ncdn$^{\Delta mye}$ neutrophils show increased adhesion and spreading



Supplemental Figure 9: Mock-priming and priming increase the surface level of CD11b in Ncdn$^{fl/fl}$ neutrophils



Supplemental Figure 10: Ncdn$^{\Delta mye}$ neutrophils have increased fMLP stimulated Erk activity, whereas other signalling pathways seem unaffected

Supplemental Figure Legends

Supplemental Figure 1. Time course of neutrophil recruitment and bacterial clearance during pneumococcal infection

(A) Gating strategy to identify leukocyte populations by flow cytometry, here shown for the perfused lung homogenate of *Ncdn*$^{fl/fl}$ mice infected *i.n.* with 2 x 10^6 *S. pneumoniae* for 18 h. Single-cell suspensions were prepared, live leukocytes identified by viability dye (FVD) and CD45 expression. Among these, alveolar and other macrophages (blue boxes) were identified as CD11c$^+$, SiglecF$^+$ cells and CD11b$^+$, Ly6G^{lo} cells, respectively; neutrophils (red box) as CD11b^{hi}, Ly6G^{hi} cells; and inflammatory monocytes (purple box) as CD11b$^+$, Ly6C$^+$ cells. Where we use the term 'macrophages', we mean the combined cell populations in both blue boxes under conditions where inflammatory monocyte numbers are negligible. Where we use the term monocytes/macrophage, we mean the combined cell populations in the blue and purple boxes. **(B)** Time course of infection. *Ncdn*$^{fl/fl}$ mice were infected *i.n.* with 2 x 10^6 *S. pneumoniae* (solid lines), or were mock-infected (stippled lines), and culled at the indicated times after infection. BAL (left) and perfused lung homogenates (right) were prepared and analyzed for numbers of neutrophils (red), macrophages (blue) and inflammatory monocytes (purple) by flow cytometry (top panels), and for bacterial burden by quantification of CFU (bottom panels). Alveolar macrophages were the only monocyte/macrophage population in BAL after 6 h. Data are mean ± SEM of 1-2 mock-infected and 2-3 *S. pneumoniae*-infected mice per time point and experiment, pooled from 4 independent experiments, except at 24 h, which is 1 mock-infected and 7 *S. pneumoniae*-infected mice from 1 experiment.

Supplemental Figure 2. Improved clearance of *S. pneumoniae* from the lung parenchyma of *Ncdn*$^{\Delta mye}$ mice

Ncdn$^{fl/fl}$, *LysM*^{Cre} and *Ncdn*$^{\Delta mye}$ mice were infected *i.n.* with 2x10^6 *S. pneumoniae* (filled symbols), or were mock-infected (open symbols), and culled after 6 h **(A)** or 18 h **(B)**, prior to preparation of perfused lung homogenates, which were assessed for bacterial burden by quantifying CFU (top panels) and for the numbers of neutrophils, monocytes/macrophages and inflammatory monocytes by flow cytometry, as indicated. Perfused lung homogenate samples in (A) are from the same mice as the BAL samples shown in Figure 2A. Data are mean ± SEM, pooled from 4 independent experiments for (A) on 5 for (B), typically 1 mock-infected and 2-3 infected mice/genotype/experiment; dots represent individual mice. Statistics are Kruskal-Wallis analysis with Dunn's multiple comparisons test.

Supplemental Figure 3. Norbin deficiency does not affect Prex-dependent neutrophil recruitment during aseptic peritonitis

Mice of the indicated genotypes were injected *i.p.* with 0.25 mL 3% thioglycollate (TGC; filled symbols), or were mock-treated (open symbols), culled 3 h later, peritoneal lavages performed, and the number of neutrophils and total leukocytes in the lavages assessed by hemocytometer and cytospin microscopy, as well as by flow cytometry. **(A)** Representative forward (FSC) and side scatter (SSC) flow cytometry plots and neutrophil gating (Cd11b^{hi}, Gr1^{hi}) from mock- and TGC-treated *Ncdn*$^{fl/fl}$ mice. **(B)** Representative Kwick-Diff stained cytospin images of peritoneal lavage cells from mock- and TGC-treated *Ncdn*$^{fl/fl}$ mice. Red arrows denote resident peritoneal macrophages, blue arrows resident peritoneal lymphocytes and green arrows recruited neutrophils, identified by their characteristic nuclear morphology. **(C, D)** Quantification of neutrophil recruitment (top panels) and total leukocyte numbers (bottom panel) in the peritoneum of mice from the indicated genotypes, quantified by hemocytometer and cytospin. Data are mean ± SEM, pooled from 7 independent experiments in (C), and 4 in (D), typically 1 mock-treated and 2-

3 TGC-treated mice/genotype/experiment; dots represent individual mice. Statistics are two-way ANOVA with Tukey's multiple comparisons test.

Supplemental Figure 4. Efficacy of 1A8 antibody in depleting neutrophils from peripheral blood

Ncdn$^{fl/fl}$ and *Ncdn*$^{-/-}$ mice were treated with 1A8 monoclonal Ly6G antibody or isotype control IgG at -24 h and 0 h prior to *i.n.* infection with 2 x 10^6 *S. pneumoniae* and a 50 μl blood sample was taken at 0 h, and peripheral blood neutrophils (CD11b^{hi}, CD24^{hi}) were quantified by flow cytometry. Data are mean ± SEM pooled from 5 independent experiments; each dot represents one mouse. These are the same mice analysed further in Figure 3B. Statistical significance was assessed using two-way ANOVA with Sidak's multiple comparisons test.

Supplemental Figure 5. Norbin-deficient and Norbin/Prex-deficient mice produce normal levels of TNFα, CXCL1 and IL6 during pneumococcal infection

Mice of the indicated genotypes were infected *i.n.* with 2x10^6 *S. pneumoniae* (filled symbols), or were mock-infected (open symbols), and culled after 3 h. Broncho-alveolar lavage (BAL) was performed and BAP fluid assessed for levels of TNFα, CXCL1 and IL6 by ELISA. Data are mean ± SEM, pooled from 2 independent experiments with 1 mock-infected mouse, where indicated, and 1-3 infected mice/genotype/experiment; dots represent individual mice. Statistics were done for infected mice only and are one-way ANOVA with Tukey's multiple comparisons test.

Supplemental Figure 6. Norbin-deficient neutrophils have increased ROS production in response to stimulation of GPCRs

ROS production was measured in isolated neutrophils from mice of the indicated genotypes by real-time chemiluminescence assay in the presence of luminol and HRP to assess extra-and intracellular ROS. **(A)** ROS production primed with 5 ng/ml TNFα, 100 ng/ml GM-CSF and stimulated with 3 μM fMLP (arrow denotes time of fMLP injection). Representative traces (left-hand panel) and quantification (AUC, right-hand panel). **(B)** ROS production primed as in (A) and stimulated with 25 nM C5a. **(C)** ROS production primed with 1 μg/ml LPS and stimulated with 3 μM fMLP. **(D)** ROS production stimulated with 500 nM PMA. Representative traces (left-hand panel) and quantification as AUC (right-hand panel). Mock-stimulated ROS production was negligible (A-D) and is not shown for clarity. Data are mean ± SEM of the indicated numbers of independent experiments; each dot is the mean AUC from one experiment. Statistics are one-way Anova with Tukey's multiple comparisons test.

Supplemental Figure 7. Norbin-deficient neutrophils produce normal levels of TNFα, CXCL1 and IL6 in response to zymosan

Isolated *Ncdn*$^{fl/fl}$ and *Ncdn*$^{\Delta mye}$ neutrophils were stimulated with zymosan (5 particles/neutrophil; filled symbols), or were mock-stimulated (open symbols), for 4 h or overnight (16 h), as indicated, and the levels of TNFα, CXCL1 and IL6 released into the medium measured by ELISA. Data are mean ± SEM of 6 independent experiments; dots represents one experiment. Statistics are two-way ANOVA with Sidak's multiple comparisons test.

Supplemental Figure 8. Norbin-deficient neutrophils show increased adhesion and spreading

Neutrophils isolated from mice of the indicated genotypes were kept on ice (unprimed) or were primed with 20 ng/ml TNFα and 50 ng/ml GM-CSF for 45 min at 37°C, plated onto glass coverslips and stimulated with 1.5 μM fMLP for 10 min, or were mock-stimulated, fixed with PFA, and stained with FITC-Gr1 antibody

and Hoechst strain. **(A)** Representative images (FITC signal) from one field-of-view of 27 (3 x 9) per coverslip and duplicate coverslips per condition for each experiment. **(B)** Adhesion, quantified as mean number of neutrophils per mm^2. **(C)** Spreading, quantified by drawing a mask for each neutrophil using ImageJ and calculating the mean surface area of the mask. **(D)** Polarization, assessed by assigning each neutrophil manually to either polar or non-polar morphology categories. Data (B-D) are mean ± SEM of 3 independent experiments. Statistical significance was assessed using two-way Anova with Holm-Sidak's multiple comparisons test.

Supplemental Figure 9. Neutrophil priming upregulates CD11b to the cell surface

Ncdn$^{fl/fl}$ bone marrow cells were kept on ice (basal), incubated for 45 min at 37°C (mock-primed) or primed with 20 ng/ml murine TNFα and 50 ng/ml GM-CSF for 45 min at 37°C before transfer onto ice. Neutrophils were stained with CD11b and Ly6G antibodies, and were analyzed by flow cytometry. Representative flow cytometry plots from one of 6 experiments are shown.

Supplemental Figure 10. Norbin-deficient neutrophils have increased fMLP-stimulated Erk activity, whereas several other GPCR signaling pathways seem unaffected

Ncdn$^{fl/fl}$ and *Ncdn*$^{\Delta mye}$ neutrophils were primed with 20 ng/ml TNFα, 50 ng/ml GM-CSF for 45 min at 37°C, stimulated with the doses of fMLP and for the times indicated, lysed and lysates western blotted as indicated. Left-hand panels show representative western blots. Right-hand panels show the quantification of signaling protein activity at the conditions of 1 μM fMLP, 45 s stimulation (filled symbols) and mock-stimulation (open symbols), done by densitometry and expressed as band intensity of phospho-protein over total protein. Data are mean ± SEM of 3-6 independent experiments; each dot represents one

experiment. Statistical significance was assessed on log-transformed raw data using two-way Anova with Sidak's multiple comparisons test.