Supplementary Materials

Title: Lung endothelial ADAM17 regulates the acute inflammatory response to lipopolysaccharide

Authors: Daniela Dreymueller, Christian Martin, Tanja Kogel, Jessica Pruessmeyer, Franz Martin Hess, Keisuke Horiuchi, Stefan Uhlig, Andreas Ludwig

Table of content:

Results:

Characterization of Tie2-adam17$^{-/-}$ mice

We confirmed the knockout of ADAM17 in isolated endothelial cells, which has already been reported for this mice strain (1), by differential CD45- and CD31-sorting of digested lung cells. The CD31^{+}-fraction showed a 50% reduction of ADAM17 mRNA expression in Tie2-*adam17*$^{-/-}$ mice, whereas no differences to control mice were observed for the CD45^{+}-fraction (supplemental figure 5A,B). Additionally, we showed that the blood of control and Tie2-*adam17*$^{-/-}$ mice (both without inflammatory stimulus) did not differ with respect to the total leukocyte, neutrophil, or monocyte counts (supplemental figure 5C-E) nor in their ADAM17 mRNA expression level in these cells (supplemental figure 5F,G). Moreover, the ADAM17-dependent TNFα-release upon LPS stimulation of primary alveolar macrophages did not differ between control and Tie2-*adam17*$^{-/-}$ mice (data not shown). Thus, Tie2-*adam17*$^{-/-}$ mice showed the expected knockout of ADAM17 in endothelial cells and had normal numbers of peripheral blood leukocytes with normal ADAM17 mRNA expression and normal capacity of TNFα-release by alveolar macrophages.

Materials and Methods

Antibodies, cytokines and inhibitors

Mouse monoclonal antibodies against human ADAM10 and ADAM17, respectively, mouse IgG2b and IgG1 isotype controls were from R&D System (Wiesbaden, Germany). The APC-conjugated and the HRP-conjugated goat anti-mouse secondary antibodies were from Jackson ImmunoResearch Laboratories, Inc (West Grove, USA). The Alexa555-conjugated goat anti-rabbit secondary antibody was from Invitrogen (Frankfurt, Germany). Rat monoclonal antibody against murine CD31 (PECAM) (PE-labeled and unlabeled) was from BD Pharmingen (Heidelberg, Germany) and anti-rat magnetic microbeads from Miltenyi (Bergisch Gladbach, Germany). The rat allophycocyanin (APC)-labeled monoclonal antibody against murine Ly6G (APC-anti-mLy6G) and the rat monoclonal VioBlue-labeled anti-mCD45R were from Miltenyi. Rat monoclonal antibodies APC-labeled anti-mCD4 and PacificBlue-labeled anti-mCD8a were from BD Pharmingen. Fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-labeled rat monoclonal antibody against murine F4/80 was from AbD Serotec (Düsseldorf, Germany). Cy5-conjugated PE-labeled (PeCy5) armenian hamster monoclonal antibody against murine CD3e was from eBiosciences (NatuTec, Frankfurt, Germany). Cy™7-conjugated PE-labeled (PE-Cy™7) monoclonal rat antibodies against murine NK1.1, CD4, and CD11b were from BD Pharmingen. Cy™5-conjugated PE-labeled antibodies against murine CD8 and CD19 were obtained from BD Pharmingen. CD16/32 Fc-Block was obtained from eBiosciences. Rabbit polyclonal antibody against β-catenin was from Santa Cruz Biotechnology, Inc (Delaware, USA). The mouse monoclonal antibody against human JAM-A was from BD Pharmingen. Lipopolysaccharide (LPS) from E. coli 0127:B8 was from Sigma-Aldrich (Munich, Germany), human and murine TNFα and IFNγ were from R&D Systems. Human LBP was from Peprotech (Rocky Hill, USA). The metalloproteinase inhibitor GW280264 was synthesized and assayed for inhibition of human and mouse ADAM17 and ADAM10 as described (2). The Complete Protease Inhibitor was from Roche (Munich, Germany).

Primary cells and cell culture

Lung human microvascular endothelial cells (HMVEC-L) (Lonza, Belgium) were cultured in EGM-2 MV medium (Lonza) and subcultured following the manufacturer's protocol. Cells were used in passage 4 for viral transduction and passage 5 to 6 for all assays. Recombinant lentiviruses were produced by transient transfection of 293T packaging cells according to standard protocols (3) as described earlier (4). The targeting sequences were: 5'-

ACAGTGCAGTCCAAGTCAA-3'annealing from bp 1947 to bp 1965 of ADAM10 mRNA (LV-anti10) and 5'-AGGAAAGCCCTGTACAGTA-3'annealing from bp 2061 to bp 2079 of ADAM17 mRNA (LV-anti17). A sequence of 5'-CCGTCACATCAATTGCCGT-3' served as scramble control (LV-scramble). After 48 to 72 h, transduction was examined by fluorescence microscopy. Alveolar macrophages were isolated out of BAL fluid and seeded for 2 hours (5). Before stimulation with 1 μg/ml LPS, non-adherent cells were removed, and after 4 hours the TNFα release to the cell culture supernatant was measured by ELISA.

Immunohistochemistry

HMVEC-L were grown on 48-well tissue culture plates to complete monolayers. Cells were washed with PBS and fixed for 15 min in 4% PFA, followed by 10 min incubation with 50 mM NH_4Cl and permeabilization in 1% Triton X-100 in PBS for 4 min. After washing, cells were blocked in PBS/5% FBS for 30 min, followed by incubation with rat anti-m-β-catenin antibody for 30 min (6). Cells were washed, incubated with Alexa555-coupled anti-rat antibody for 30 min, DAPI-stained and at least covered with Immu-Mount (ThermoScientific, USA). Fluorescence microscopy was performed using the Leica DMI14000B (Leica Microsystems, Germany) with an image analysis package (DISKUS, Technisches Büro Carl H. Hilgers, Königswinter, Germany).

RT-qPCR analysis

The mRNA levels of ADAM10 and ADAM17 in murine lung tissue and BAL fluid cells as well as the mRNA levels of ADAM9, 10, 15, and 17, and MMP7 in HMVEC-L were quantified by RT-qPCR analysis and normalized to the mRNA level of murine RPS29 or human GAPDH. RNA was extracted using RNeasy Kit (Qiagen, Hilden, Germany) and quantified by spectrophotometry (NanoDrop, Peqlab, Germany). RNA (equal amounts within each data set) was reverse transcribed using RevertAid First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's protocol. PCR reactions were performed using LightCycler®480 SYBR Green I Master Mix (Roche) according to the manufacturer's protocol. Following primers were used with the specific primer annealing time given in brackets: madam10 forward, agcaacatctggggacaaac, madam10 reverse, tggccagattcaacaaaac (57°C); madam17 forward, aaaccagaacagacccaacg, madam17 reverse, gtacgtcgatgcagagcaaa (57°C); mRps29 forward, gagcagacgcggcaa, mRps29 reverse, cctttctcctcgttgggc (61°C); hadam17 forward, gaagtgccaggaggcgatta, hadam17 reverse, cgggcactcactgctattacc (55°C); hadam10 forward, ggattgtggctcattggtgggca, hadam10 reverse,

actctctcggggccgctgac (61°C); hmmp7 forward, caaagtggtcacctacaggatcgta, hmmp7 reverse, tcccaggcgcaaaggcatga (62°C); hadam9 forward, ggtgacagatttggcaattgtg, hadam9 reverse, ttgtgccttcgttaaccatcc (58°C); hadam15 forward, ccgacgggccctggagaaag, hadam15 reverse, gctgggcataggaggcacaac (63°C); hGAPDH forward, cggggctctccagaacatcatcc hGAPDH reverse, ccagccccagcgtcaaaggtg (66°C). For hadam15, 2mM $MgCl_2$ were additionally added to the reaction mix. All PCR reactions were run on a LightCycler® 480 System (Roche) with the following protocols. 40 cycles of 10 s denaturation at 95°C, followed by 10 s annealing at the indicated temperature and 15 s amplification at 72°C. Standard curves were determined by a serial dilution of a defined cDNA standard within each data set. Data were obtained as cycle CP values and calculated as delta CP values using the LightCycler®480 software and used for statistic analysis.

Peptide cleavage assay

Cell lysates were prepared in extraction buffer (0.15 M sodium citrate, 1% Tween 80, pH 8.0). After removal of cell debris by centrifugation at 16000 g and 4°C for 10 min, lysates were corrected for same protein amount (determined by BC assay kit), diluted 1:2 in reaction buffer (25 mM Tris-HCl, 0.01% Triton-X 100, pH 8.0) with or without GW280264 (10 μM) for monitoring ADAM-dependent activity. After incubation for 20 min, a fluorogenic peptide-based substrate mimicking the α-cleavage site of amyloid-precursor protein (APP, Ac-RE(EDANS-VHHQKLVF-K(DABCYL)-R-OH, 5 μM, Calbiochem) was added. Complete inhibition of general protease activity was obtained by using an inhibitor mix (Complete, Roche). The generation of the fluorescent cleavage product was monitored at 37°C over time in a spectral fluorimeter (Fluostar Optima, BMGLabtech, Offenburg, Germany) at 355 nm excitation and 510 nm emission wavelength. Linearity was determined by a serial dilution of a defined standard.

Transmigration assay

Neutrophils were isolated as described before (4). HMVEC-L were seeded on collagen-coated polycarbonate transwell filters with 5 μm-pores (Costar, Lowell, USA) and grown to confluence. 24-well culture plates were filled with 600 μl/well DMEM containing 0.2% BSA with or without 10 ng/ml IL-8 (R&D Systems). The transwells were transferred into the wells and filled with 100 μl of neutrophil suspension ($2x10^5$ neutrophils per well). After incubation for 30 min at 37°C and 5% CO_2, transwells were carefully removed and transmigrated cells were quantified by measurement of endogenous glucuronidase activity as describe before (7).

For inhibition of ADAM10 and ADAM17, GW280264 (10 μM) was added to HMVEC-L layers 1.5 h prior to the assay.

Permeability assay

HMVEC-L were seeded on collagen-coated polycarbonate transwell filters with 5 μm-pores (Costar) and grown to confluence. For lentivirally-transduced HMVEC-L expressing green fluorescent protein (GFP), confluence was controlled by fluorescence microscopy. After 24 h stimulation with LPS (1 μg/ml) or/and GW280264 (10 μM), the cell culture medium in the upper chamber was replaced by 70 kDa TRITC-dextran suspension (Sigma-Aldrich, 1 mg/ml in PBS with 0.2% BSA,). After 90 min incubation at 37°C and 5% CO_2, permeability was analyzed by measurement of the fluorescence intensity in the lower chamber at 535 nm excitation and 595 nM emission wavelength using XFluor4 Genios (Tecan, Grödig, Austria).

JAM-A cleavage assay

HMVEC-L were seeded on 6-wells and grown to confluence. JAM-A release to cell culture supernatant was performed as previously described (8).

LPS and TNF model of acute lung inflammtion

Mice were anesthesized by intraperitoneal injection of ketamin (100 mg/kg) und xylazin (5 mg/kg). LPS (400 μg/kg in PBS) or PBS as control was intranasally instilled in the presence of GW280264 (40 μg/kg, solved in DMSO, end concentration 0.06%) or vehicle control (0.06% DMSO). Mice were housed for 4 or 24 h. TNFα (200 μg/kg in PBS) was intranasally instilled in the presence of 0.06% DMSO, and mice were housed for 24 hours.

For collection of BAL fluid and lung tissue, mice were sacrificed by injection of ketamin (500 mg/kg) and xylazin (50 mg/kg). Lungs were lavaged intratracheally with 1 ml ice-cold PBS and 4 times aspiration with avoidance of air bubbles. The BAL fluid was kept at 4°C for the whole procedure. BAL fluid was centrifuged at 400 g and 4°C for 10 min and the cell sediment was resolved in flow cytometry assay buffer or fixed in PBS/1% PFA. The supernatant was again centrifuged at 16100 g and 4°C for 10 min, stored at –80°C and used for determination of cytokine content via ELISA and the determination of protein content using a commercial BC assay kit (Interchim, Montiucon, France).

After collection of BAL fluid, animals were perfused with ice-cold PBS. The lung were excised, cut in pieces and digested in 0.1 % collagenase type 2 (Worthington, Lakewood, USA) for 2 h. A single cell suspension was prepared by pipetting through a 27-gauche needle

and using 70 μm cell strainers (BD Falcon, Germany). After centrifugation for 8 min at 400 g, the pellet was resolved in erythrocyte lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA in H_2O) and incubated at 37°C for 5 min. Cell lysis was stopped by adding a 10-fold volume of ice-cold PBS. After sedimentation for 8 min at 400 g, cells were resolved in flow cytometry assay buffer or fixed in PBS/1% PFA. For determination of wet-dry-ratio, tissue samples were taken from both lobes of 24 h-challenged animals. The weight of the wet tissue was expressed in relation to the weight of the dry tissue determined after 2 and 3 days of drying at 60°C.

The BAL fluid content of TNFα, IL-6, JAM-A, KC, VE-cadherin and CX3CL1 was measured using commercially available ELISA Kits (R&D Systems and USCN Life Science). Values were standardized on equal volumes of BAL recovery.

Determination of protein content

The protein content of BAL fluid, tissue lysates and cell lysates (each in equal volumes of recovery) was determined using a commercial BC assay kit (Interchim) following the manufacturer's protocol. Linearity was determined using a bovine serum albumin (0 to 3000 ng/ml) standard in the aquivalent probe solution.

Flow cytometry

PBS supplemented with 1% FCS and 5 mM EDTA (sterile filtered) was used as assay buffer, and all steps of the staining process were performed at 4°C. L-HMVEC cells were detached from culture dishes using accutase (PAA, Cölbe, Germany) and analyzed for expression of ADAM10 and ADAM17 by incubation with mouse monoclonal antibodies against ADAM10 and ADAM17 (1 μg/ml and 5 μg/ml resp.) followed by incubation with a APC-conjugated anti-mouse antibody (1:100). Isotype controls for mouse IgG1 and mouse IgG2a, respectively, were used in parallel. Single cell suspensions of lung tissue and BAL fluid cells were Fc-blocked (ebioscience) according to the manufacturer's protocol. For determination of neutrophil and macrophage gates, cells were stained for CD3, CD4, CD8e, CD45, Ly6G, F4/80 and NK1.1 and cross-checked with the appropiate isotype controls following the manufacturer's protocols. Additionally, autofluorescence was used to further distinguish the dendritic cell and macrophage gate (9). Heparin blood was collected from *vena cava* and two times subjected to erythrocyte lysis. The total number of leukocytic cells within BAL fluid and blood samples was determined using CountBright™ absolute counting beads (Invitrogen, Germany) following the manufacturer's protocol. The fluorescence signal was detected by

flow cytometry (FACS Canto, BD Biosciences) and analyzed with FlowJo 8.7.3 software (Tree Star, Inc., Ashland, USA). Blood samples were sorted for lymphocytes, neutrophils and PBMCs by CD19, CD4, CD8, Ly6G and CD11b surface staining. Endothelial cells were sorted by CD45-negative and CD31-positive selection. Samples were sorted using the FACS Aria (BD Bioscience).

Histology

For histological examination, lungs were fixed by intratracheal instillation of Roti-Fix® (Roth, Germany) followed by bronchial ligation after 5 min. After 48 hours of fixation, the tissue was dehydrated, embedded in paraffin and cut in 3 μm slices. Hematoxylin-eosin staining was performed using standard protocols. Images were taken with a Zeiss microscope (AxioLab.A1, Carl Zeiss MicroImaging GmbH, Germany) and analyzed for thickness of alveolar septa and influx of polymorphnuclear cells using the AxioVision software (Carl Zeiss MicroImaging GmbH).

Statistics

Quantitative data are shown as mean and SEM calculated from three independent experiments/cell isolates/animals if not indicated otherwise within figure legends. Written fold-changes are rounded off and down, respectively, to the first dezimale. Percentages were arc sin-transformed for statistical analysis. Data were statistically analyzed as indicated in figure legends by either one-way ANOVA followed by Newman-Keuls post hoc analysis corrected for multiple comparison, two-way ANOVA followed by Bonferroni's post hoc analysis corrected for multiple comparison or unpaired t-test using GRAPH PAD PRISM 5.0 program (GraphPad Software, La Jollla, CA). Statistically significant differences ($p < 0.05$) are indicated as asterisks.

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Figure legends

Supplemental figure 1: Effect of LPS-challenge on ADAM9 and ADAM15 mRNA expression

HMVEC-L were incubated with and without 1 μg/ml LPS in the presence of 100 ng/ml LBP in the absence of serum for the indicated time. ADAM9 (**A**) and ADAM15 (**B**) mRNA expression was examined by RT-qPCR analysis. Data are expressed as change of expression compared to control cells. **A-C:** Data represent means ± SEM (n=3 per group). Significance was calculated using one-way ANOVA followed by the Newman-Keuls post-test and is indicated by asterisks (***p<0.001). Asterisks without line indicate significance to the appropriate controls.

Supplemental figure 2: Effect of ADAM inhibition on intercellular gap formation of cultured HMVEC-L.

HMVEC-L were grown to full confluence and stimulated for 24 h with LPS (1 μg/ml) in the presence of GW280264 (10 μM) or vehicle control (0.1% DMSO). Intercellular gap formation was visualized by reduced membrane-associated β-catenin-staining. Representative pictures were performed with 20x magnification. Data are representative for three independent experiments.

Supplemental figure 3: Flow cytometric analysis of lung tissue cells

Lung tissue of PBS-treated (**A**) and LPS-treated mice (**B**) was enzymatically disintegrated and passed through a cell strainer. Isolated lung cells were examined for forward and side scatter (FSC/SSC) as well as surface marker expression by flow cytometry. Gate (R1) contained $CD45^+$ cells (**A/B**). Gate R2 contained neutrophils (A/B), identified by $Ly6G^+$ surface expression (**C**). The FSC/SSC parameters were hold constant throughout all experiments, and 20000 cells were counted in R1 and analyzed for the percentage of neutrophils. Due to lung wet-dry-ratio changes upon LPS-treatment, absolute counting of lung tissue cells was not feasible as standardization on same tissue amounts was not possible.

Supplemental figure 4: *Adam*$17^{flox/flox}$ are not different from wild-type animals.

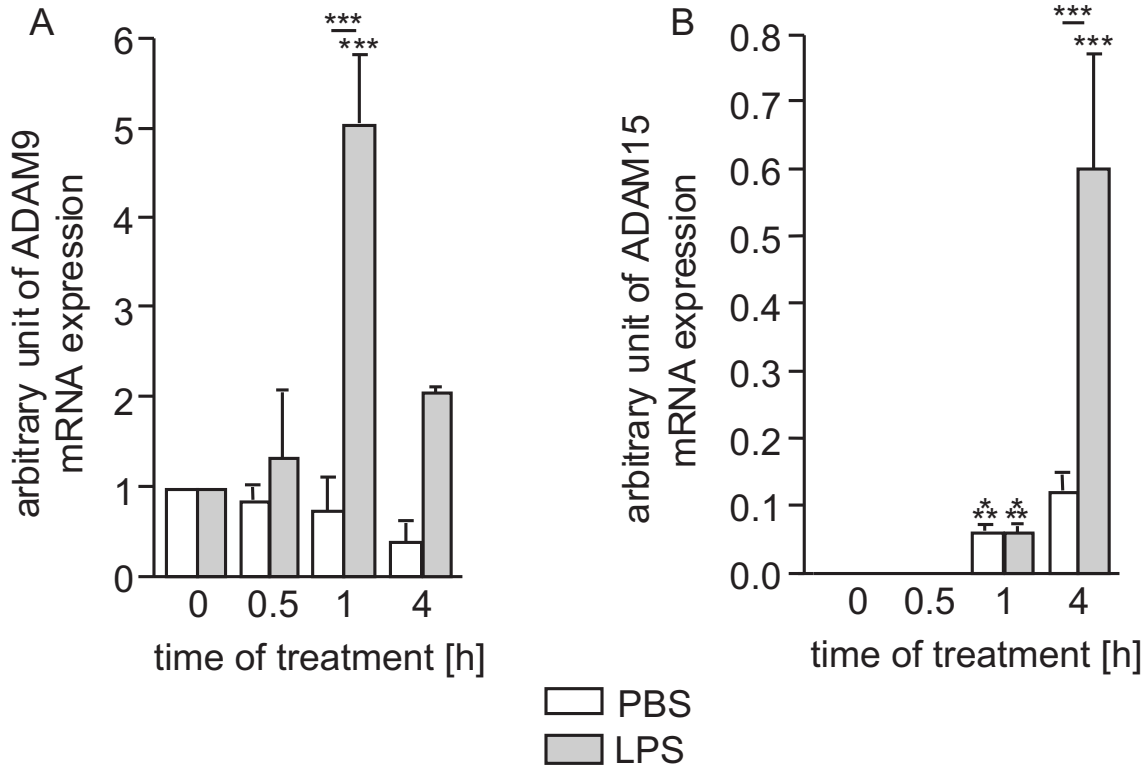
Appropriate littermate animals (*adam*$17^{flox/flox}$) were compared to wild-type animals (control animals) for LPS-induced lung inflammation. **A:** Lung wet-dry-ratio was determined 24 h after intranasal application. **B/C:** Release of IL-6 and TNFα was tested by ELISA measurement of BAL fluid 4 h after intranasal application. **D/E**: The total leukocyte cell

number (**D**) as well as the amount of neutrophils (**E**) in BAL fluid were determined by flow cytometry. **A-D:** Data represent means ± SEM (n=3 per group). Significance was calculated using one-way ANOVA followed by the Newman-Keuls post-test and is indicated by asterisks (*p<0.05, **p<0.01, ***p<0.001). Asterisks without line indicate significance to the appropriate PBS-treated controls.

Supplemental figure 5: Effects of endothelial *adam17*-knockout on *adam17* mRNA expression in lung tissue cells and basal peripheral blood leukocyte composition
Control and Tie2-*adam*17$^{-/-}$ mice were compared for blood cell composition and mRNA expression in various lung tissue and blood cells. **A/B:** Lung tissue cells were sorted for endothelial-enriched cells ($CD31^+CD45^-$) and leukocytes ($CD31^+CD45^-$) and analyzed for ADAM17 mRNA expression by RT-qPCR. **C-E:** Venous blood was collected from non-challenged animals and analyzed for leukocyte composition by flow cytometry. Results were expressed as cell number per ml blood. **F/G**: Blood leukocytes were sorted for PBMC ($Ly6G^-$) and neutrophils ($Ly6G^+$) by flow cytometry. B and T lymphocytes were separated by surface staining with CD19, NK1.1, CD4, and CD8. Negative sorted cells were further subgrouped by forward and sideward scatter and sorted for $FSC^{mid}SSC^{mid}Ly6G^+$ and $FSC^{high}SSC^{high}Ly6G^-$ cells to separate PMBC and neutrophils. The seperated cell populations were analyzed for ADAM17 mRNA expression by RT-qPCR. **A-G:** Data represent means ± SEM (n=3 per group in A, B, F, G; n=6 in C, D, E). Significance was calculated using Student's t test and is indicated by asterisks (***p<0.001). Asterisks without line indicate significance to the appropriate control.

Supplemental figure 6: Role of ADAM10 and ADAM17 for LPS-induced JAM-A release
HMVEC-L were transduced with lentivirus encoding shRNA (LV-scramble, LV-anti10 or LV-anti17). HMVEC-L were stimulated for 24 h with LPS (1 μg/ml) or left unstimulated (PBS) in the presence of 100 ng/ml LBP and in the absence of serum. Conditioned media were analyzed by western blotting followed by densiometric quantification.

Supplemental figure 1



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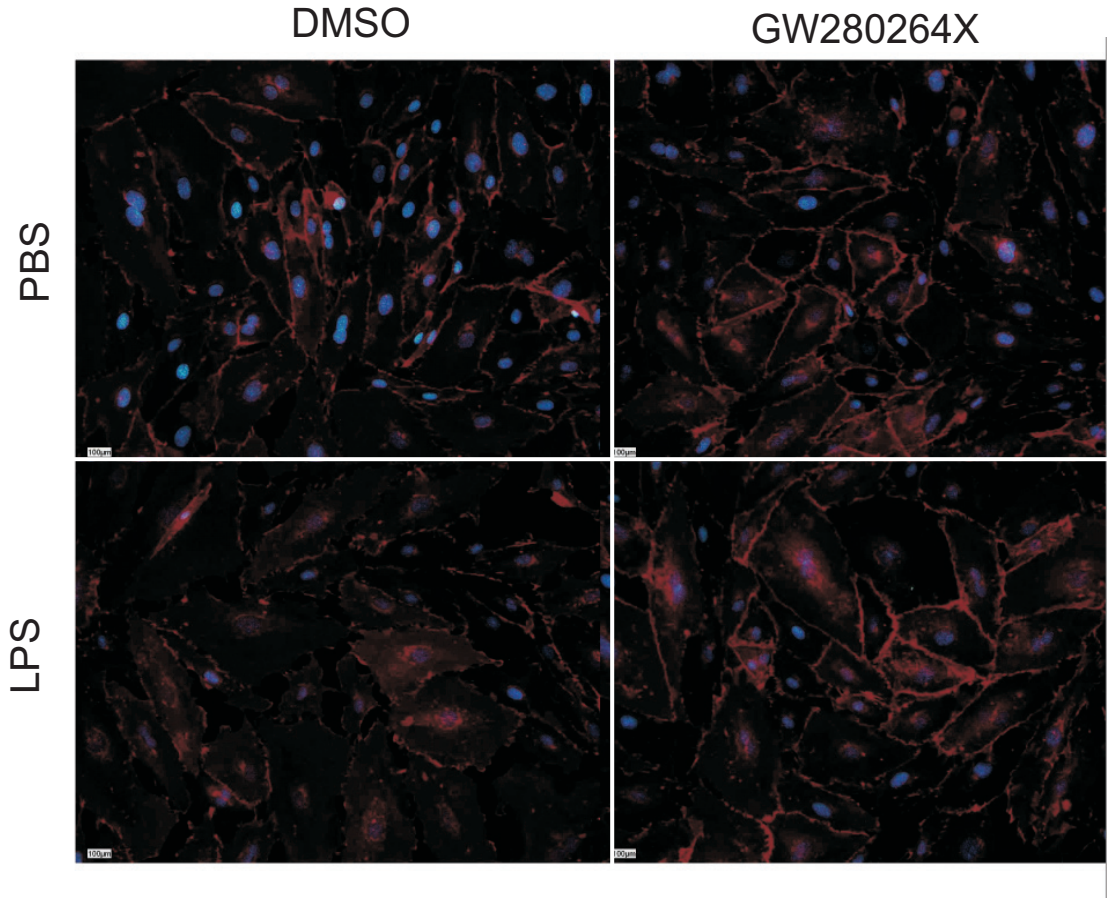

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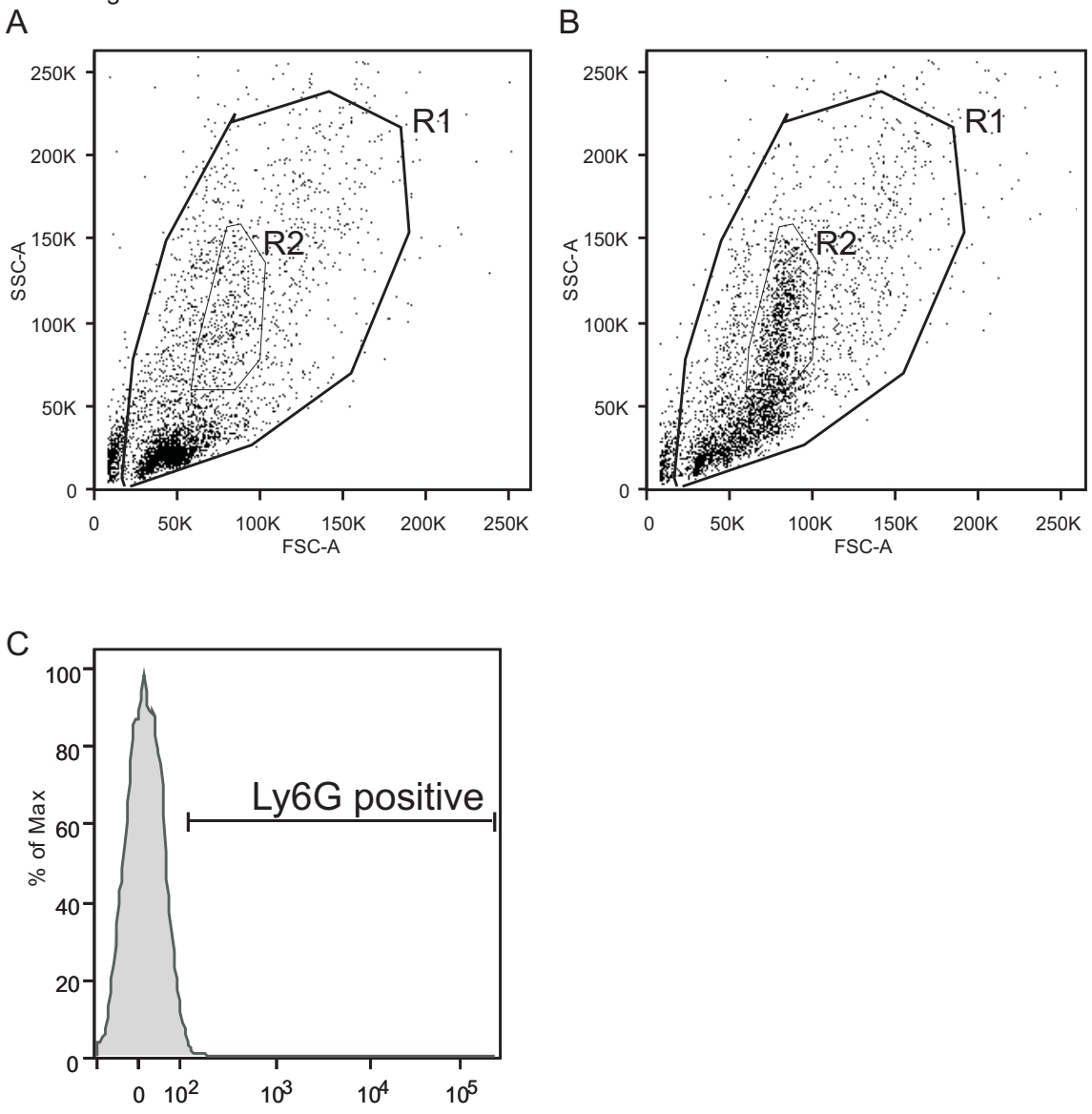

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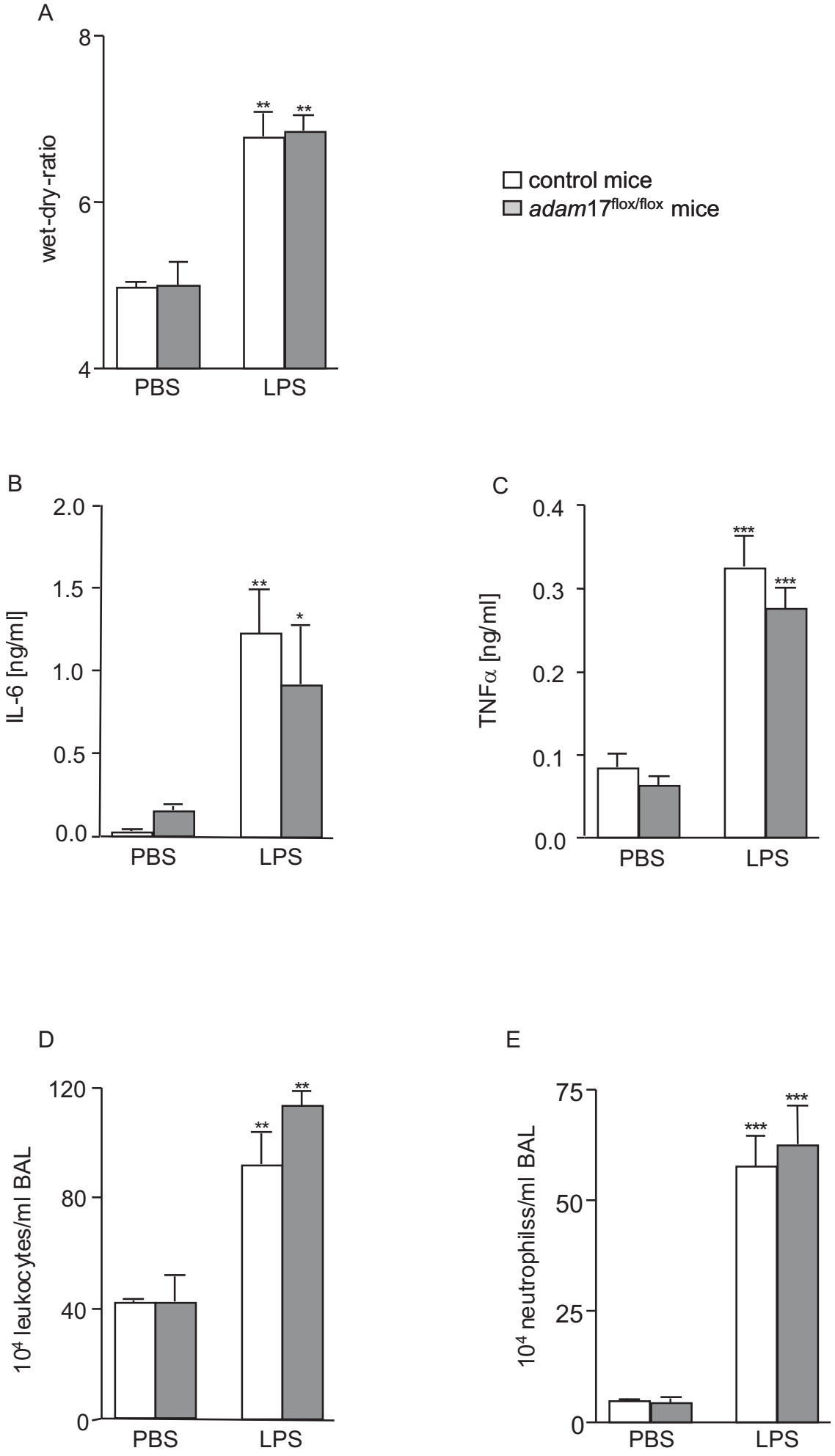

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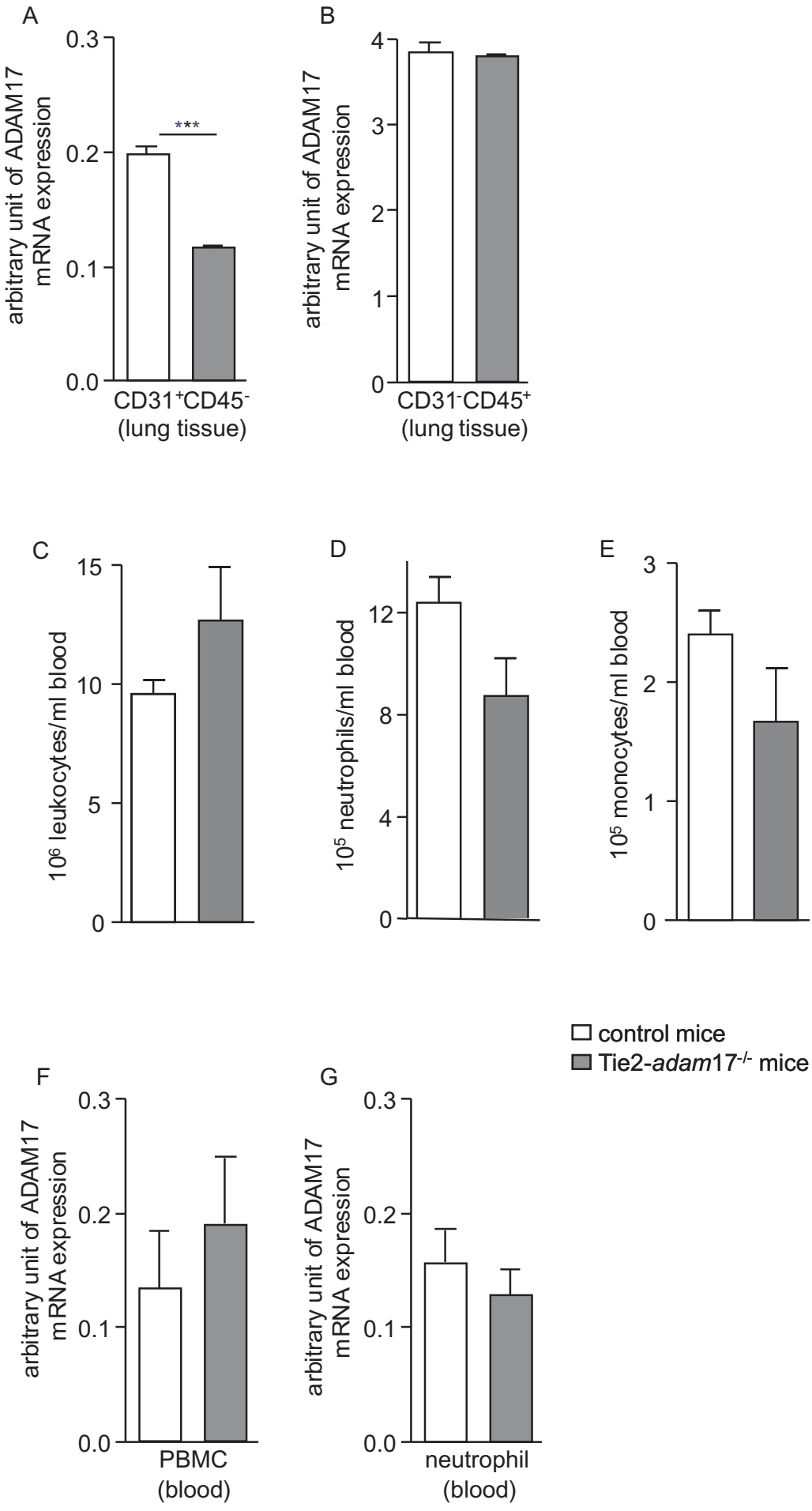

Supplemental figure 6