Lung endothelial ADAM17 regulates the acute inflammatory response to lipopolysaccharide

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Acute lung injury (ALI) is associated with increased vascular permeability, leukocyte recruitment, and pro-inflammatory mediator release. We investigated the role of the metalloproteinase ADAM17 in endotoxin-induced ALI with focus on endothelial ADAM17. *In vitro*, endotoxin-mediated induction of endothelial permeability and IL-8-induced transmigration of neutrophils through human microvascular endothelial cells required ADAM17 as shown by inhibition with GW280264X or shRNA-mediated knockdown. *In vivo*, ALI was induced by intra-nasal endotoxin-challenge combined with GW280264X treatment or endothelial *adam17*-knockout. Endotoxin-triggered upregulation of ADAM17 mRNA in the lung was abrogated in knockout mice and associated with reduced ectodomain shedding of the junctional adhesion molecule JAM-A and the transmembrane chemokine CX3CL1. Induced vascular permeability, oedema formation, release of TNF-α and IL-6 and pulmonary leukocyte recruitment were all markedly reduced by GW280264X or endothelial *adam17*-knockout. Intranasal application of TNF-α could not restore leukocyte recruitment and oedema formation in endothelial *adam17*-knockout animals. Thus, activation of endothelial ADAM17 promotes acute pulmonary inflammation in response to endotoxin by multiple endothelial shedding events most likely independently of endothelial TNF-α release leading to enhanced vascular permeability and leukocyte recruitment.

INTRODUCTION

Excessive endothelial leakage is a hallmark of acute lung injury (ALI), followed by the accumulation of leukocytes in the alveolar space (Alm et al, 2010; Reiss et al, 2012). Resident and recruited leukocytes fulfil important functions in the clearance of pathogens, but may also damage the endothelial and epithelial cell layer (Gungor et al, 2010; O'Grady et al, 2001; Strieter & Kunkel, 1994). The lung inflammatory response is regulated by a number of soluble and surface-expressed molecules originating from endothelial cells, smooth muscle cells, epithelial cells and leukocytes (Strieter & Kunkel, 1994; Zhang et al, 2000). Many of these molecules exist as transmembrane molecules that become released from the cell surface by limited proteolysis close to the cell membrane. This process is termed ectodomain shedding and represents a critical regulatory mechanism for the release of cytokines (*e.g.* TNF-α), cytokine receptors (*e.g.* TNFR1 and IL-6R) (Canault et al, 2006), chemokines (*e.g.* CXCL16 and CX3CL1; Abel et al, 2004; Hundhausen et al, 2003; Schwarz et al, 2010), adhesion molecules (*e.g.* ʟ-selectin) (Walcheck et al, 2006) and surface proteoglycans (*e.g.* CD44 and syndecans; Haczku et al, 2000; Pruessmeyer et al, 2009). Members of the ADAM (a disintegrin

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and a metalloproteinase) family, in particular ADAM17, are involved in the ectodomain shedding of these surface molecules (Dreymueller et al, 2011; Pruessmeyer & Ludwig, 2009), and evidence is accumulating that ADAM17 is an important regulator of the acute inflammatory response (Cesaro et al, 2009). In vitro, enhanced ADAM17 expression and activity have been demonstrated in endothelial cells, leukocytes, smooth muscle cells, and epithelial cells upon stimulation with proinflammatory factors such as nitric oxides or cytokines like TNF-α and IFN-γ (Bzowska et al, 2004; Canault et al, 2006; Cesaro et al, 2009; Dijkstra et al, 2009). In contrast, in a model of Escherichia coli-induced peritonitis, mice with a targeted deletion of adam17 in monocytic cells showed improved survival, probably because they were able to recruit more neutrophils to the peritoneal cavity whilst systemic TNF-α, IL-1 and IL-6 levels were reduced (Long et al, 2009).

ADAM17 is widely expressed throughout lung tissue (Dijkstra et al, 2009), and its expression is upregulated in asthma and chronic obstructive pulmonary disease (Dijkstra et al, 2009; Paulissen et al, 2009). The regulation and relevance of lung tissue ADAM17 in ALI is only poorly understood. Additionally, the wide range of functions of ADAM17, evidenced by the fact that ADAM17-deficient mice die shortly after birth (Peschon et al, 1998), necessitates to define its role in specific cells.

Since endothelial barrier dysfunction is critical for the development of ALI, the present study was designed to investigate the relevance of endothelial ADAM17 activity in acute lung inflammation induced by intranasal instillation of E. coli lipopolysaccharide (LPS). Inhibition and gene silencing experiments with LPS-stimulated lung microvascular endothelial cells as well as pharmacological inhibition experiments with LPS-challenged mice suggested a critical role of endothelial ADAM17 for barrier dysfunction and transmigration of leukocytes. This was studied in more detail in mice with targeted deletion of adam17 in endothelial cells. These animals showed diminished cellular infiltration, cytokine secretion and oedema formation upon intranasal LPS-challenge. Similar observations were made when ALI was induced by TNF-α, indicating that the beneficial effects of ADAM17 deficiency were not only related to endothelial cell ectodomain shedding of this cytokine, but of other ADAM17 substrates including junction molecules. Our findings show that endothelial ADAM17 activity is critical for the development of ALI.

RESULTS

LPS induces peptide cleavage activity and gene expression of ADAM10 and 17 in microvascular endothelial cells

The role of ectodomain shedding for barrier functions of cultured human microvascular endothelial cells (HMVEC-L) was analysed by pharmacological inhibition with GW280264X blocking both ADAM10 and ADAM17. The particular contribution of ADAM10 and ADAM17 was addressed by specific lentiviral knockdown of their mRNA expression using shRNA. The knockdown was verified by surface staining of the transmembrane proteases (Fig 1A and B). Treatment of HMVEC-L with LPS resulted in increased peptide cleavage activity as measured by the proteolysis of a fluorogenic peptide substrate for ADAM10 and ADAM17 (Fig 1C). Addition of the inhibitor GW280264X reduced the cleavage activity by ~50% in both unstimulated and stimulated HMVEC-L. The same degree of cleavage inhibition was observed after knockdown of either ADAM10 or ADAM17 (Fig 1C).

ADAM10 and ADAM17 can be regulated by altering either enzyme activity or gene transcription. By RT-qPCR analysis we confirmed that mRNA expression of both proteases is induced already 1 h after stimulation (Fig 1D and E), as also observed for ADAM9 (Supporting Information Fig S1A). After 4 h of stimulation, ADAM17 mRNA expression remained high, whereas the mRNA expression of ADAM10 slightly decreased and that of ADAM9 almost returned to baseline levels. ADAM15 showed delayed induction of mRNA expression after 4 h of LPS challenge (Supporting Information Fig S1B). Analysis of matrix metalloproteinase-7 (MMP7) mRNA expression revealed an upregulation upon 4 h of stimulation (unpublished observation).

Thus, LPS increases both sheddase activity and mRNA expression of ADAM10 and ADAM17 in microvascular endothelial cells.

Microvascular permeability and transendothelial migration are modulated by the activity of ADAM10 and ADAM17 in vitro

Lipopolysaccharide induces paracellular gap formation by shedding of junction proteins, including JAM-A and cadherins, which are intracellularly linked to β-catenin (Nonas et al, 2006). Silencing of ADAM17 results in reduced ectodomain shedding of JAM-A from endothelial and epithelial cells as we have previously described (Koenen et al, 2009). Following LPS treatment (24 h), we observed enhanced permeability measured by TRITC-dextran diffusion, which was completely suppressed by GW280264X-treatment (Fig 2A). Silencing of either ADAM10 or ADAM17 suppressed the permeability change to a similar degree as GW280264X (Fig 2A) suggesting that both proteases are required for the control of microvascular permeability under inflammatory conditions. Additionally, immunohistochemistry of β-catenin as an indicator of established cell junctions showed that LPS treatment (24 h) led to paracellular gap formation, also inhibited by GW280264X-treatment, without influencing the basal cell morphology (Supporting Information Fig 2).

To investigate whether the LPS-induced increase in permeability was linked to ADAM10- and ADAM17-mediated shedding of junction molecules, we investigated the release of soluble JAM-A by LPS-challenged HMVEC-L. Release of soluble JAM-A was 1.3-fold enhanced by 4 h of LPS stimulation, further increasing to 2.3-fold by stimulation for 24 h (Fig 2B) and was completely inhibited by GW280264X-treatment. The knockdown of ADAM10 or ADAM17 by shRNA indicated the involvement of ADAM17 and to a lesser extent of ADAM10 in JAM-A release (Supporting Information Fig 6).

We further examined whether ADAM10/17 activity might influence transendothelial migration of neutrophils. The inhibitor GW280264X reduced transmigration in response to the neutrophil-attracting chemokine IL-8 by 70% (Fig 2C).
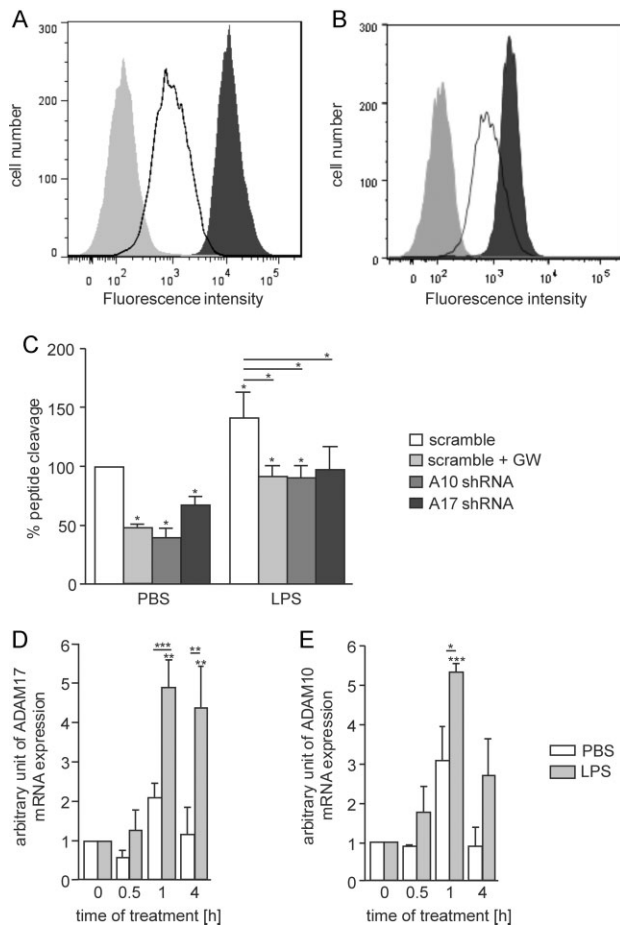
Figure 1. Influence of LPS challenge on ADAM10 and ADAM17 activity and mRNA expression *in vitro*. Data represent means ± SEM ($n = 3$ per group, **C–E**). Significance was calculated using one-way ANOVA followed by the Newman–Keuls post-test (in **D,E**) or by two-way ANOVA followed by the Bonferroni post-test for double-treated cells (in **C**) and is indicated by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Asterisks without line indicate significance to the appropriate control.

A-B. HMVEC-L were transduced with lentivirus encoding shRNA (LV-scramble, LV-antiA10 or LV-antiA17). Downregulation of ADAM10 (**A**) or ADAM17 (**B**) (black line) was analysed by surface staining with antibodies to ADAM10 or ADAM17 compared to isotype controls (light grey tinted) and surface stained scramble transduced cells (black tinted) followed by flow cytometry. Representative histograms of three independent experiments are shown.

C. HMVEC-L were stimulated for 24 h with LPS (1 μg/ml) or vehicle control (PBS). Cell lysates were analysed for sheddase activity using a fluorogenic peptide cleavage assay. Results were expressed as percentage of sheddase activity in relation to the unstimulated control receiving the vehicle DMSO only (= 100%).

D-E. HMVEC-L were stimulated for 0–4 h with LPS (1 μg/ml) or vehicle control (PBS) in the presence of 100 ng/ml LBP and in the absence of serum. ADAM17 (**D**) and ADAM10 (**E**) mRNA expression were examined by RT-qPCR analysis. Data are expressed as change of expression rate compared to control cells.
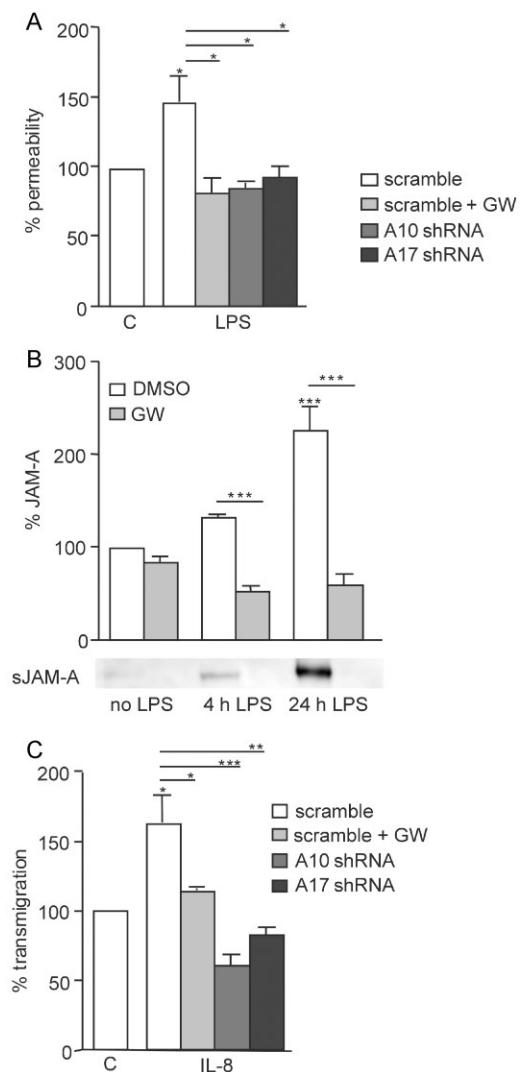
Figure 2. Role of ADAM10 and ADAM17 in lung microvascular endothelial cells *in vitro*. HMVEC-L were transduced with lentivirus encoding shRNA (LV-scramble, LV-antiA10 or LV-antiA17). Data represent means ± SEM ($n = 3$). Significance was calculated using one-way ANOVA followed by the Newman-Keuls post-test (in **B** and **C**) or by two-way ANOVA followed by the Bonferroni post-test for double-treated cells (in **A**) and is indicated by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Asterisks without line indicate significance to the appropriate control.

A. Transduced HMVEC-L were grown in transwell inserts and stimulated for 24 h with LPS (1 μg/ml) or were left unstimulated (PBS) in the presence or absence of GW280264X (10 μM). Permeability was measured by TRITC-dextran diffusion. Data are shown as percentage of TRITC-dextran permeability in relation to the unstimulated DMSO-treated control (= 100%).

B. HMVEC-L were stimulated for 4 or 24 h with LPS (1 μg/ml) or were left unstimulated (PBS) in the presence of 100 ng/ml LBP and in the absence of serum. Cells were treated either with GW280264X (10 μM) or with vehicle control (0.1% DMSO). Conditioned media were analysed by immuno-blotting followed by densitometric quantification. A representative immunoblot of three independent experiments is shown below the graph.

C. Transduced HMVEC-L were pre-treated with or without GW280264X (10 μM) for 1.5 h and examined for IL-8-induced (10 ng/ml) transmigration of neutrophils. Experiments were performed with neutrophils from three different donors. Results were expressed as percentage of transmigration in relation to the LV-scramble-transduced control (C) receiving no IL-8.

Silencing of either ADAM10 or ADAM17 alone was sufficient to abrogate transmigration in response to IL-8 (Fig 2D). Thus, endothelial ADAM10 and ADAM17 are both required for microvascular permeability and for IL-8-mediated transmigration of neutrophils *in vitro*.

Dual ADAM10/17 inhibition attenuates lung oedema formation and cytokine secretion in response to intranasal LPS

Based on the critical role of ADAM10 and ADAM17 for microvascular permeability and transmigration *in vitro*, we continued to examine their significance in an ALI model *in vivo*. Intranasal application of LPS strongly increased protein influx into the bronchalveolar lavage (BAL) fluid already 4 h after LPS treatment (Fig 3A, 1.9-fold), and also increased the tissue wet–dry-ratio, which was prominent after 24 h (Fig 3B, 1.4-fold). Both effects, indicating increased vascular permeability and oedema formation, were abrogated by treatment with GW280264X to simultaneously block ADAM10 and 17 (Fig 3A and B).

We next investigated the release of the chemokine CX3CL1 (fractalkine), which is shed from the cell surface by the activity of ADAM10 and ADAM17 after stimulation with pro-inflammatory stimuli (Hundhausen et al, 2003). CX3CL1 release was 1.9-fold increased within the first 4 h of LPS challenge (Fig 3A). This increase was inhibited by intranasal treatment with GW280264X, whereas basal levels in PBS-challenged controls did not differ.

Release of the pro-inflammatory cytokine TNF-α was prominent 4 h after challenge (66-fold increase, Fig 3D) and was declining after 24 h (unpublished observation). This release was markedly inhibited by intranasal treatment with GW280264X, whereas basal levels in PBS-challenged controls were not affected. The observation that inhibition of TNF-α release was not complete may be explained by the fact that TNF-α is produced at many sites and by many cell types, which may be difficult to access by the inhibitor. Finally, LPS stimulation also induced the release of IL-6 (28-fold increase, Fig 3E), which is not an ADAM17 substrate *per se*. Nevertheless, IL-6 release was considerably reduced by treatment with the inhibitor, which may reflect the fact that IL-6 is produced in response to other inflammatory stimuli such as TNF-α.

These inhibition experiments suggest that either ADAM10 or ADAM17 or both are involved in the regulation of vascular permeability and oedema formation as well as the release of inflammatory mediators in the course of LPS-induced ALI.

ADAM10 and 17 attenuate leukocyte recruitment in response to LPS challenge

The recruitment of leukocytes to the alveolar space (BAL fluid) and the lung tissue was studied by flow cytometry. LPS challenge increased the total leukocyte number and the number of neutrophils recovered from BAL fluid (Fig 4A and C), which was detectable after 4 h (unpublished observation) and more prominent after 24 h of LPS challenge (11.5-fold increase of neutrophil BAL count). LPS-induced neutrophil recruitment was reduced to about 50% by GW280264X-treatment (Fig 4C). The levels in PBS-challenged controls remained unaffected by the inhibitor treatment.
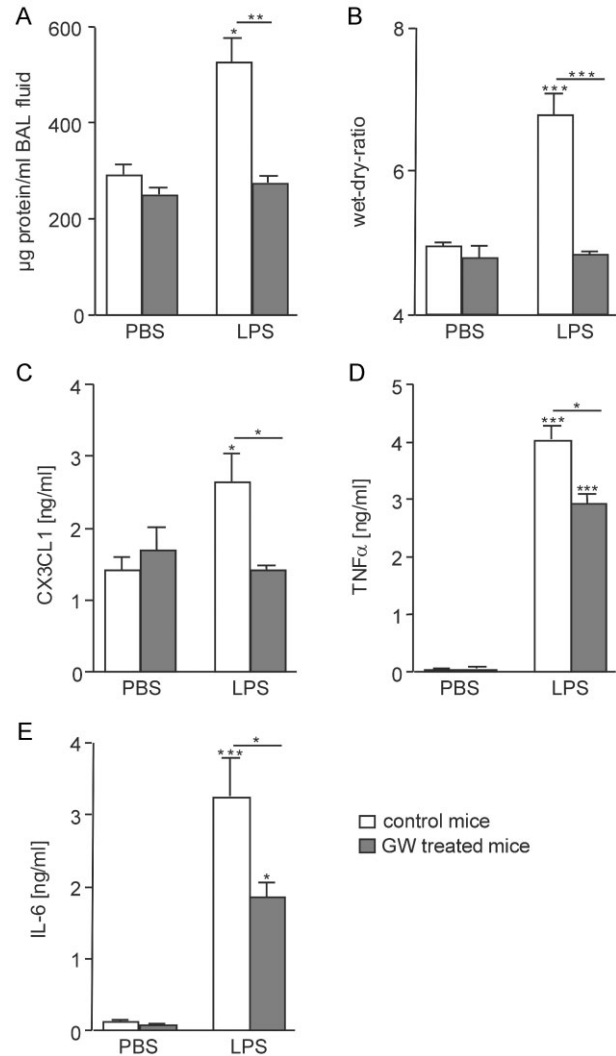

Figure 3. Effect of ADAM10 and ADAM17 inhibition on permeability, oedema formation and cytokine secretion in LPS-induced lung inflammation. Mice were intranasally treated with LPS (400 μg/kg) or vehicle control (PBS) with or without the metalloproteinase inhibitor GW280264X (40 μg/kg). Data represent means $\pm$ SEM ($n = 3$ per group). Significance was calculated using one-way ANOVA followed by the Newman–Keuls post-test and indicated by asterisks (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Asterisks without line indicate significance to the appropriate control.

A. Whole protein content of BAL fluid was determined 4 h after LPS application.

B. Lung wet–dry-ratio was determined 24 h after intranasal LPS application.

C-E. Release of CX3CL1 (**C**), TNF-α (**D**) and IL-6 (**E**) into BAL fluid was determined by ELISA 4 h after intranasal application.

No additional macrophages were recruited upon LPS challenge (24 h). Instead, their recovery from the BAL fluid of LPS-challenged animals was reduced compared to PBS-challenged controls (Fig 4B). However, in both PBS- and LPS-challenged animals, the number of macrophages in the BAL fluid was significantly reduced upon application of GW280264X.
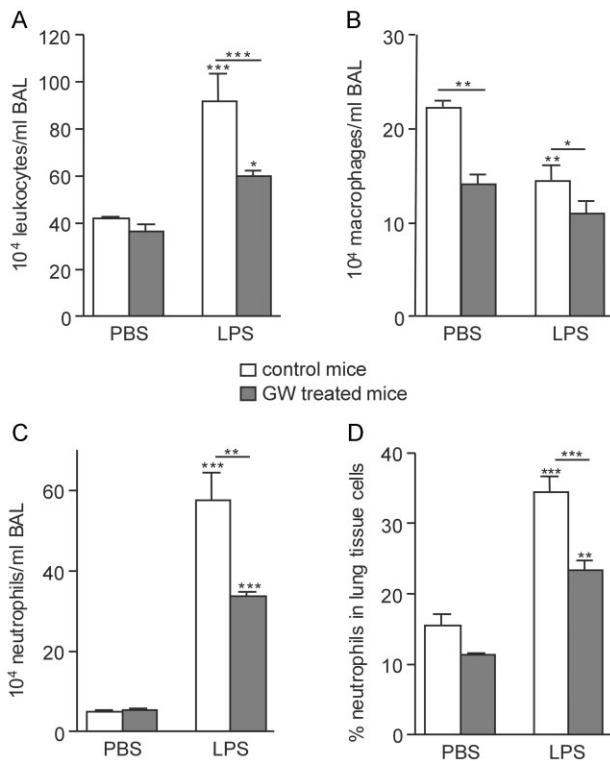
Figure 4. Influence of ADAM10 and ADAM17 inhibition on leukocyte recruitment in LPS-induced lung inflammation. Mice were intranasally treated with LPS or vehicle control (PBS) with or without the metalloproteinase inhibitor GW280264X. After 24 h, lungs were lavaged and the lung tissue was enzymatically disintegrated. 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.

A-D. The total leukocyte cell number (**A**) as well as the number of macrophages (**B**) and neutrophils (**C**) in BAL fluid and the amount of neutrophils in lung tissue suspension (**D**) were determined by flow cytometry. Results were expressed as cell number per ml BAL fluid (**A–C**) or as percentage of the total number of lung tissue cells examined (**D**).

For analysis of leukocyte recruitment into interstitial spaces, the lung tissue was disintegrated by enzymatic treatment and analysed for neutrophil content by flow cytometry (see Supporting Information Fig 3 for determination of percentages). Twenty-four hours after LPS challenge, the number of neutrophils in the lung tissue was 2.2-fold enhanced (Fig 4D). This response was almost completely suppressed by GW280264X treatment, whereas the levels in PBS-challenged animals were not significantly affected. These pharmacological studies suggest that either ADAM10 or ADAM17 or both proteases facilitate leukocyte recruitment to the acutely inflamed lung.

Reduced shedding in endothelial cell-specific adam17^{−/−} mice

We found that LPS challenge increased ADAM17 mRNA in lung tissue (perfused free of blood) of control mice (Fig 5A), whereas ADAM10 expression was not affected. We therefore hypothe-
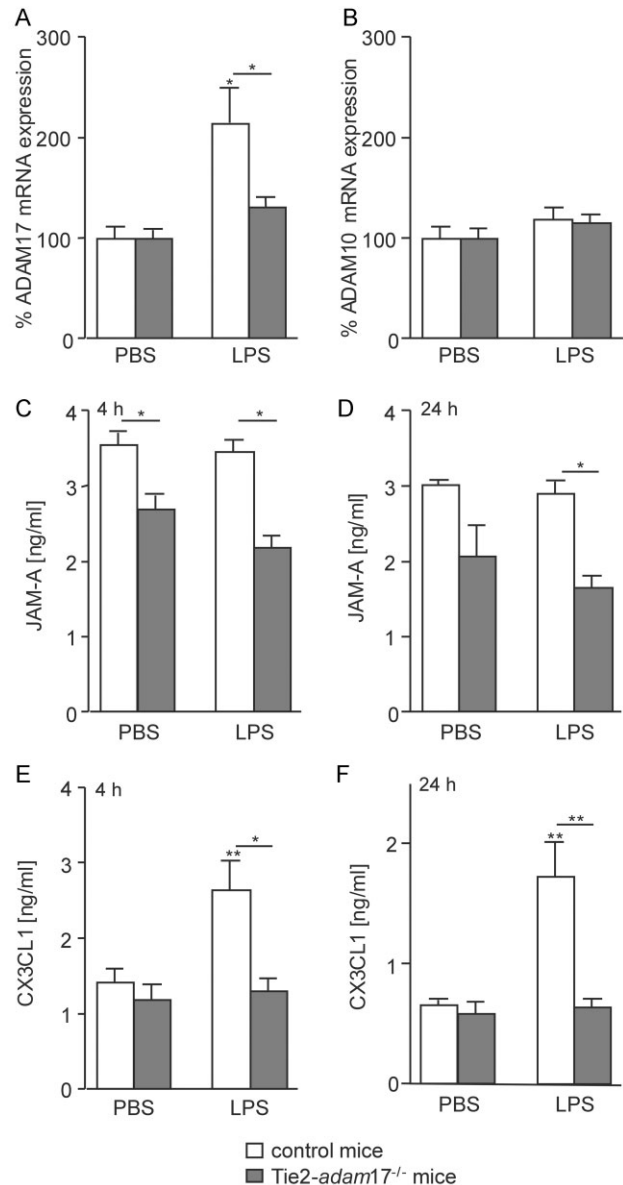

Figure 5. Role of endothelial ADAM17 on shedding processes in LPS-induced lung inflammation. Control and Tie2-adam17^{−/−} mice were intranasally treated with LPS or vehicle control (PBS). Data represent means ± SEM ($n = 3$ per group). Significance was calculated using Student's t-test (in **A** and **B**) or using one-way ANOVA followed by the Newman–Keuls post-test (in **C–F**) and is indicated by asterisks ($^*p < 0.05$, $^{**}p < 0.01$). Asterisks without line indicate significance to the appropriate PBS-treated controls.

A-B. After challenge for 24 h, BAL and vascular perfusion, ADAM17 (**A**) and ADAM10 (**B**) mRNA expression in lung tissue were examined by RT-qPCR analysis. Data are expressed as percentage of expression in relation to PBS-treated controls.

C-D. Release of soluble JAM-A into BAL fluid was measured by ELISA 4 and 24 h after intranasal application.

E-F. Release of soluble CX3CL1 into BAL fluid was determined by ELISA 4 and 24 h after intranasal application.

sized that ADAM17 is more relevant as a regulatory protease in LPS-induced responses.

Based on our *in vitro* observation with HMVEC-L that ADAM17 critically controls the induction of endothelial permeability and transmigration, we decided to specifically address the role of endothelial ADAM17 in ALI utilizing *adam17*$^{-/-}$ mice with Cre recombinase-mediated deficiency of the protease in endothelial cells (Tie2-*adam17*$^{-/-}$). These mice lack an obvious phenotype, and the absence of ADAM17 mRNA expression in heart and lung endothelial cells was demonstrated (Weskamp et al, 2010). We confirmed the endothelial specificity of ADAM17 knockout in those mice by differential cell sorting of lung tissue and blood cells followed by RT-qPCR analysis of mRNA expression. Additionally, control mice and Tie2-*adam17*$^{-/-}$ mice did not differ with regard to their blood cell composition and the capacity of TNF-α-release by alveolar macrophages (for detailed characterization of the mice see Supporting Information Fig 5 and Supporting Information Results).

We then continued to use Tie2-*adam17*$^{-/-}$ mice in our model of LPS-induced lung injury. In these mice, LPS failed to stimulate ADAM17 mRNA expression in lung tissue compared to control animals (Fig 5A), whereas ADAM10 expression did not vary (Fig 5B). Since ADAM17 is involved in the constitutive and induced ectodomain shedding of endothelial JAM-A, we next determined the level of released soluble JAM-A in the BAL fluid 4 and 24 h after LPS challenge. In PBS-treated mice, the release of JAM-A was generally reduced in Tie2-*adam17*$^{-/-}$ mice in comparison to control animals and this difference was more pronounced (up to 30%) when the animals were treated with LPS (Fig 5C and D). In addition, we investigated the level of soluble CX3CL1, which is generated from its transmembrane form via ADAM17-independent constitutive cleavage and ADAM17-mediated induced shedding upon cell stimulation (Schwarz et al, 2010). LPS challenge for 4 h increased the release of CX3CL1 into the BAL fluid (Fig 5E, 1.9-fold) in control animals. As expected, induced CX3CL1 release was abrogated in Tie2-*adam17*$^{-/-}$ mice (Fig 5E). CX3CL1 release was generally lower after 24 h of challenge, but was also suppressed in Tie2-*adam17*$^{-/-}$ mice (Fig 5F).

Lung endothelial ADAM17 mediates lung oedema formation and cytokine secretion in response to LPS challenge

Because of the observed deficit of Tie2-*adam17*$^{-/-}$ mice in ectodomain shedding of JAM-A, which is an important component of endothelial tight junctions, we next studied these mice for oedema formation in LPS-induced ALI. The knockout did not alter the basal level of BAL fluid protein content, but abrogated protein influx in response to LPS (Fig 6A). Similar to the experiments with GW280264X (Fig 3), LPS-induced oedema formation was completely inhibited in Tie2-*adam17*$^{-/-}$ mice (Fig 6B). This was confirmed by histological analysis of the lung tissue. In control mice, LPS treatment increased the thickness of interalveolar septa, whereas Tie2-*adam17*$^{-/-}$ mice were protected (Fig 6C and D). Additionally, only LPS-challenged control mice showed strong tissue damage with loss of parenchymal architecture, whereas the lung tissue of Tie2-*adam17*$^{-/-}$ mice stayed intact (Fig 6C).
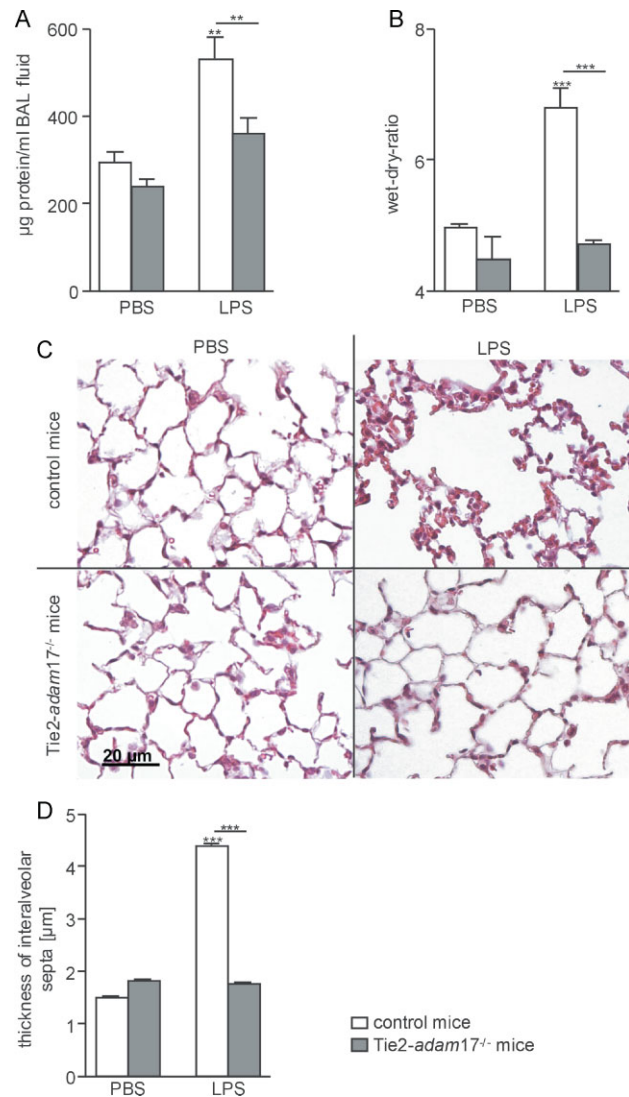

Figure 6. Role of endothelial ADAM17 for permeability changes and tissue damage in LPS-induced lung inflammation. Control and Tie2-*adam17*$^{-/-}$ mice were intranasally treated with LPS or vehicle control (PBS). Data represent means ± SEM ($n = 3$ per group for **A** and **B** and $n = 10$ per group for **D**). Significance was calculated using one-way ANOVA followed by the Newman–Keuls post-test and is indicated by asterisks (**$p < 0.01$, ***$p < 0.001$). Asterisks without line indicate significance to the appropriate PBS-treated controls.

A. Whole protein content of BAL fluid was determined 4 h after application.
B. Lung wet–dry-ratio was examined 24 h after application.
C-D. After 24 h, 3 μm-sections of formalin-fixed and paraffin-embedded lung tissue were stained with hematoxylin–eosin. Representative images are shown in panel (**C**). Ten images per animal were analysed for the thickness of interalveolar septa using AixoVision software (**D**).

Cytokine determination within BAL fluid revealed that LPS-challenged Tie2-*adam17*$^{-/-}$ mice showed reduced release of IL-6 (Fig 7A, 3.9-fold) and TNF-α (Fig 7B, 1.6-fold), whereas no effect was observed in PBS-challenged mice. Interestingly, the 37-fold increased release of the neutrophil attracting chemokine KC (Fig 7C, 36.5-fold) in LPS-challenged mice did not differ between the two groups.
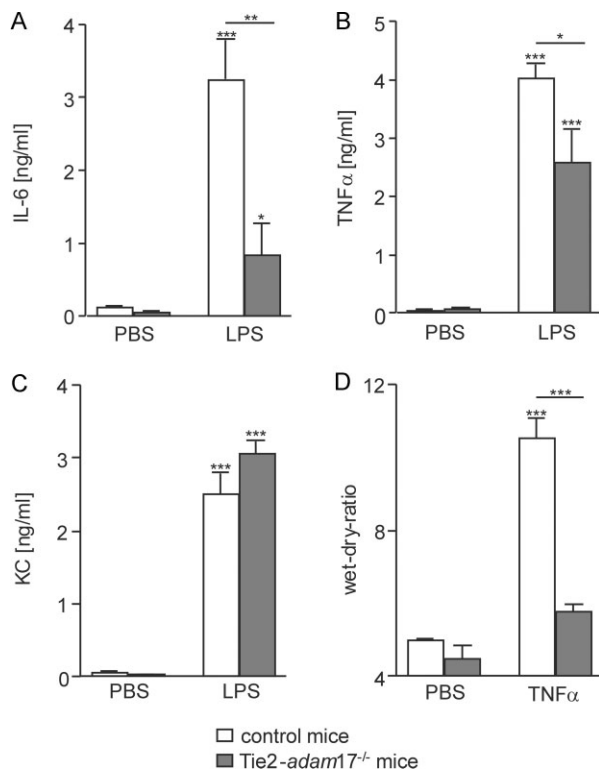
Figure 7. Role of endothelial ADAM17 for cytokine secretion in LPS-induced lung inflammation. Control and Tie2-$adam17^{-/-}$ mice were intranasally treated with LPS or vehicle control (PBS). Data represent means ± SEM (n = 3 per group). Significance was calculated using one-way ANOVA followed by the 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.

A-C. After 4 h of challenge, release of IL-6 (**A**), TNF-α (**B**) and KC (**C**) into BAL fluid was determined by ELISA measurement.

D. Control and Tie2-$adam17^{-/-}$ mice were compared for TNF-α-induced lung inflammation. The wet-dry-ratio of lung tissue was determined after 24 h of intranasal TNF-α-challenge.

Soluble TNF-α is generated by ADAM17 and this cytokine is a mediator of ALI (Mazzon & Cuzzocrea, 2007; Smith et al, 1998; Song et al, 2001). Since soluble TNF-α levels were only moderately reduced in the Tie2-$adam17^{-/-}$ mice, we questioned whether the protection of vascular leakage and tissue damage, which was seen in the endothelial $adam17$-deficient mice, could be explained by the reduced release of TNF-α. Control and Tie2-$adam17^{-/-}$ mice were studied for oedema formation after intranasal TNF-α challenge. The TNF-α-induced increase in lung tissue wet–dry-ratio was almost completely abrogated (Fig 7D) in Tie2-$adam17^{-/-}$ mice. Therefore, even in the presence of surplus soluble TNF-α, mice lacking endothelial ADAM17 are still protected from ALI.

Lung endothelial ADAM17 facilitates leukocyte recruitment in response to LPS challenge

Given the fact that LPS-induced TNF-α release was only moderately reduced and production and release of KC was not

affected in mice with endothelial ADAM17 deficiency, we asked whether the almost completely restored vascular permeability could still be associated with altered recruitment of leukocytes in the lungs of these mice. Both the total leukocyte and neutrophil number in BAL fluid 24 h after LPS challenge were significantly reduced in Tie2-$adam17^{-/-}$ mice (Fig 8A and B), whereas the cell number in BAL fluid upon PBS challenge did not differ. Neutrophils in the lung tissue of LPS-treated mice were strongly reduced in Tie2-$adam17^{-/-}$ mice (Fig 8D), similar to mice treated with GW280264X (Fig 4). This was confirmed by neutrophil counts in histological sections (Fig 8E).

Since Tie2-$adam17^{-/-}$ animals showed reduced ectodomain shedding of the transmembrane chemokine CX3CL1, which mediates transendothelial recruitment of monocytic cells, we further investigated the number of macrophages in the BAL fluid 24 h after LPS challenge. Remarkably, in both PBS- and LPS-treated groups, the number of macrophages was reduced in Tie2-$adam17^{-/-}$ animals (Fig 8C), suggesting that endothelial ADAM17 may regulate the number of macrophages in the alveolar space.

Notably, also in TNF-α-treated Tie2-$adam17^{-/-}$ mice, neutrophil recruitment to BAL fluid was reduced up to 50% (Fig 8F) in comparison to TNF-α-treated control mice. Therefore, LPS-induced lung injury does not appear to particularly depend on endothelial TNF-α release by ADAM17. Instead, endothelial ADAM17 regulates the inflammatory response at multiple levels, including the shedding of junctional adhesion molecules, and thereby affecting endothelial permeability and the generation and release of various inflammatory cytokines and chemokines such as TNF-α, IL-6 and CX3CL1 that promote leukocyte recruitment.

DISCUSSION

Endothelial barrier dysfunction is a hallmark of ALI. Here, we provide *in vitro* and *in vivo* evidence that endothelial ADAM17 plays a critical role in this process. To this end, we used human microvascular endothelial cells of the lung (HMVEC-L) in culture and endothelium-specific ADAM17 knockout mice (Tie2-$adam17^{-/-}$) to demonstrate that endothelial ADAM17 is critically involved in endothelial barrier dysfunction, oedema formation, cytokine release as well as leukocyte transmigration and recruitment in response to LPS. As shown by successful treatment with GW280264X, ADAM17 may be a worthwhile therapeutic target for the treatment of ALI.

Lipopolysaccharide treatment *in vitro* and *in vivo* increased the ADAM17 mRNA expression in cultured lung endothelial cells and in the lungs of mice, respectively. The upregulation was associated with an increase in ADAM17 activity and cleavage of ADAM17 substrates within a similar time frame. Dysregulation of ADAM17 on the level of gene transcription has been reported for other inflammatory diseases including atherosclerosis (Canault et al, 2006). In fact, the protease was found to play a critical pathogenic role in various models of inflammation (Canault et al, 2010; Chalaris et al, 2010; Horiuchi et al, 2007; Li et al, 2006; Long et al, 2009). Moreover, ALI was
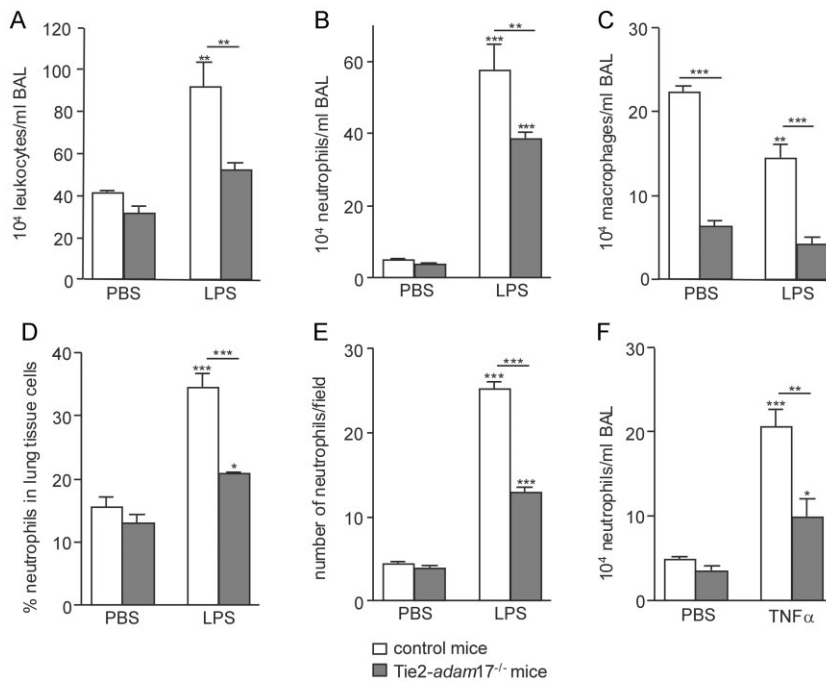
Figure 8. Role of endothelial ADAM17 for leukocyte recruitment in LPS-induced lung inflammation. Control and Tie2-adam17$^{-/-}$ mice were intranasally treated with LPS or vehicle control (PBS). Data represent means ± SEM ($n = 3$ per group for **A–D** and **F** and n = 10 per group for **E**). Significance 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.

A-D. Twenty-four hours after application, the number of total leukocytes (**A**), neutrophils (**B**) and macrophages (**C**) in BAL fluid and the amount of neutrophils in lung tissue suspension (**D**) were determined by flow cytometry. Results were expressed as cell number per ml BAL fluid (**A–C**) or as percentage of the total number of lung tissue cells investigated (**D**).

E. Ten images of hematoxylin–eosin stained 3 μm-sections per animal were analysed for invading neutrophils using the AixoVision software.

F. Control and Tie2-adam17$^{-/-}$ mice were compared for TNF-α-induced lung inflammation. The amount of neutrophils in BAL fluid was determined by flow cytometry after 24 h of intranasal TNF-α-challenge.

reduced by metalloproteinase inhibitors (PKF242-484, PKF241-466, Y-41654; Shimizu et al, 2009; Trifilieff et al, 2002). These inhibitors could not distinguish between ADAM17 and matrix metalloproteinases or other ADAM proteases, and did not include early and late time point measurements. Therefore, these pharmacological studies provided no conclusive evidence for a critical role of ADAM17 in ALI and could not address a cell-type specific contribution of the protease. Genetic studies in this area are complicated by the fact that adam17 total knockout leads to rapid postnatal lethality (Horiuchi et al, 2007; Peschon et al, 1998). As vascular permeability caused by endothelial barrier changes is central for the development of ALI, we used Tie2-adam17$^{-/-}$ mice that lack ADAM17 in their endothelial cells only (Weskamp et al, 2010) to investigate the contribution of ADAM17 in more detail.

The Tie2-adam17$^{-/-}$ mice have been characterized thoroughly for the lack of adam17 in endothelial cells (Weskamp et al, 2010). It has been argued that the Tie2-promoter might be also active in monocytes and neutrophils (Pucci et al, 2009) but we ruled this possibility out by showing normal ADAM17 mRNA expression in peripheral blood cells of Tie2-adam17$^{-/-}$ mice as well as normal TNF-α production in alveolar macrophages isolated from Tie2-adam17$^{-/-}$ mice. Taken together, we conclude that Tie2-adam17$^{-/-}$ mice lack ADAM17 in endothelial cells only.

Acute lung injury was studied in a model of LPS-induced pulmonary inflammation that resembles the reaction of the human lung to endotoxin exposure (Matute-Bello et al, 2008; Nonas et al, 2006). In this model, animals treated with the dual ADAM10/17 inhibitor GW280264X, but also Tie2-adam17$^{-/-}$ mice were protected on many levels: cytokine release, permeability, oedema formation and neutrophil recruitment.

These results indicate a critical role of endothelial ADAM17 in the development of ALI.

There are several ways how ADAM17 substrates could contribute to barrier dysfunction, cytokine release and cell recruitment in ALI. Increased vascular permeability is caused by paracellular gaps that are formed by subsequent reorganization of the cytoskeleton and redistribution of junctional proteins including JAM-A, which is involved in the regulation of brain endothelial and intestinal epithelial permeability (Haarmann et al, 2010; Laukoetter et al, 2007). ADAM17 also regulates leukocyte rolling along activated endothelium by shedding L-selectin (Ponnuchamy & Khalil, 2008) and leukocyte transmigration by cleaving CX3CL1 (Schwarz et al, 2010), ICAM-1 and VCAM-1 (Pruessmeyer & Ludwig, 2009; Singh et al, 2005) as well as JAM-A (Koenen et al, 2009). Inhibition of these shedding events results in reduced or abrogated leukocyte transmigration. Indeed, we observed reduced release of soluble JAM-A from LPS-stimulated HMVEC-L upon GW280264X treatment or shRNA-mediated knockdown of ADAM17 as well as reduced release of JAM-A and CX3CL1 in Tie2-adam17$^{-/-}$ mice, exemplifying the importance of ADAM17 for endothelial barrier dysfunction and leukocyte recruitment. Thus, multiple cleavage events within the lung and here particularly in endothelial cells contribute to the regulation of vascular permeability and neutrophil transmigration in vitro and in vivo, and therefore no single ADAM17 substrate can be held responsible for this process. Moreover, the pro-migratory function of endothelial ADAM17 might not be limited to neutrophils, but might also account for macrophages even in the absence of inflammation as indicated by the finding that the basal number of macrophages in the lungs was reduced in Tie2-adam17$^{-/-}$ mice.

The paper explained

PROBLEM:

Pulmonary or systemic inflammation can lead to ALI and in severe cases to respiratory distress syndrome and death (25–58%). oedema formation and leukocyte recruitment within the diseased lung tissue are early hallmarks of the inflammatory process. These responses are mediated by the action of cytokines and chemokines, adhesion molecules and junction molecules. Many of these pro-inflammatory mediator molecules require regulation by limited proteolysis at the cell surface, and in most of the cases, the disintegrins and metalloproteinases ADAM10 and 17 have been made responsible for this shedding process. Therefore, these proteases might represent potential targets for the protein- and site-specific treatment of lung disease. We investigated the influence of ADAM17 on ALI induced by endotoxin exposure with special focus on endothelial ADAM17.

RESULTS:

In vitro, endotoxin exposure enhanced expression and activity of ADAM10 and ADAM17, which was associated with increased endothelial permeability and release of the junctional adhesion molecule JAM-A. Regulation of permeability as well as IL-8-induced transmigration of neutrophils through human microvascular endothelial cells required both ADAM10 and 17, as shown by pharmacological inhibition or specific shRNA-mediated knockdown of the proteases. *In vivo*, ALI was induced by intranasal endotoxin challenge in combination with metalloproteinase inhibitor treatment or *adam17* knockout in endothelial cells. Endotoxin triggered the upregulation of

ADAM17 gene expression in the lung, which was abrogated in knockout mice. Induced vascular permeability, oedema formation, release of TNF-α and IL-6 and pulmonary leukocyte recruitment were all markedly reduced by inhibitor treatment to block ADAM10 and ADAM17 or endothelial *adam17* knockout. This was associated with reduced shedding of JAM-A and the transmembrane chemokine CX3CL1. Intranasal application of TNF-α could not restore leukocyte recruitment and oedema formation in knockout animals indicating that these responses are independent of TNF-α-release by endothelial cells. Taken together, our *in vitro* and *in vivo* experiments show that ADAM17, particular in endothelial cells, acts as a central pro-inflammatory regulator of pulmonary inflammation. This action appears on different levels, by increase of endothelial permeability involving shedding of junction molecules, enhanced release of inflammatory mediators and facilitation of leukocyte recruitment.

IMPACT:

Metalloproteinases of the ADAM family have been recognized as potential therapeutic targets in several diseases. However, due to their broad activity, systemic application of inhibitors may not represent an appropriate treatment strategy. Here, we present first evidence for a critical role of lung endothelial ADAM17 in the development of ALI. Further research is warranted to demonstrate that local ADAM17 inhibition avoiding systemic side effects can represent a promising approach for the treatment of this disease.

While we believe that the regulation of endothelial barrier function accounts for the majority of the protection by ADAM17 inhibition against LPS-induced lung injury, we also observed reduced levels of TNF-α and IL-6 in both mice with pharmacological inhibition of ADAM10/17 or genetic ablation of *adam17* in endothelial cells. Among these, in particular TNF-α, a well-known substrate of ADAM17, is known for its role in endotoxin-induced lung injury and other forms of ALI (Mazzon & Cuzzocrea, 2007; Schnyder-Candrian et al, 2005; Smith et al, 1998; Song et al, 2001). However, in our study, the reduction in TNF-α levels by pharmacological inhibition of ADAM10/17 or genetic ablation of *adam17* in endothelial cells was less pronounced compared to studies using rather unspecific inhibitors, where the LPS-induced TNF-α levels were almost abolished (Trifilieff et al, 2002). This can be explained by the fact that TNF-α is predominantly derived from epithelial cells and alveolar macrophages rather than endothelial cells (Ward, 2003) and that other proteases potentially contribute to TNF-α cleavage (Armstrong et al, 2006). ADAM9, ADAM10 and ADAM19 are capable of cleaving TNF *in vitro* and also MMP7 as well as proteinase-3 can shed TNF *in vivo*. Besides, recruited neutrophils further secrete proinflammatory cytokines and tissue damaging substances (Yoshikawa et al, 2004). Therefore,

reduced leukocyte recruitment in mice with pharmacological inhibition of ADAM10/17 or genetic ablation of *adam17* in endothelial cells is a likely explanation for the observed decrease in cytokine production such as TNF-α and IL-6 (Ward, 2003). Notably, we observed that endothelial hyperpermeability and leukocyte recruitment were reduced in Tie2-*adam17*$^{-/-}$ mice regardless of whether ALI was induced by LPS or TNF-α, indicating that this protection seen in Tie2-*adam17*$^{-/-}$ mice is independent of TNF-α-release by endothelial ADAM17.

Taken together, ADAM17 mediates a number of shedding events that influence several components of acute lung inflammation including vascular leakage, leukocyte recruitment and cytokine release. Particularly in endothelial cells, ADAM17 appears to act as a central regulator in pulmonary inflammation. We conclude that inhibition of ADAM17, possibly locally to reduce systemic side affects, may be a promising approach for the treatment of ALI.

MATERIALS AND METHODS

Antibodies, cytokines, inhibitors, flow cytometry
Antibodies for flow cytometry and immunohistochemistry as well as cytokines and commercially available inhibitors are described in the

supplement. The metalloproteinase inhibitor GW280264X was synthesized and assayed for inhibition of human and mouse ADAM17 and ADAM10 as described (Ludwig et al, 2005; Rabinowitz et al, 2001).

Cell culture, lentiviral knockdown of adam10 and 17 and immunohistological staining

Culture of HMVEC-L (Lonza, Belgium) and alveolar macrophages are described in the supplements. Vectors for lentiviral knockdown of *adam10* and *17* have been previously described (Schwarz et al, 2010). Lentiviral transduction and immunohistological examination of HMVEC-L for β-catenin are detailed in the supplements.

RT-qPCR analysis

The mRNA levels were quantified by RT-qPCR analysis. RNA was extracted using RNeasy Kit (Qiagen, Hilden, Germany), reverse transcribed using RevertAid First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany) and PCR reactions performed using LightCycler®480 SYBR Green I Master (Roche, Munich Germany) according to the manufacturer's protocols. Primers and amplification protocols are included in supplements.

Peptide cleavage assay

ADAM activity in cell lysates was measured as cleavage of a fluorogenic peptide-based substrate mimicking the α-cleavage site of amyloid-precursor protein. Changes in ADAM10/17 activity were also measured in terms of JAM-A release. For details see Supporting Information.

Transmigration assay

Neutrophils were isolated and analysed for transmigration through HMVEC-L as described (Schwarz et al, 2010). Transmigrated cells were quantified by measurement of endogenous glucuronidase activity as detailed in Supporting Information.

Permeability assay

Permeability was determined by TRITC-dextran diffusion as described before and within Supporting Information (Koenen et al, 2009).

Animals

Animal experiments were approved by the local authorities and performed with 6–8 week old Tie2-*adam17*$^{-/-}$ mice or wild type C57BL/6 mice (Janvier, Le Genest sur l'Isle, France), respectively. Tie2-*adam17*$^{-/-}$ mice for targeted disruption of *adam17* in endothelial cells were generated and characterized in the laboratory of Carl Blobel (Cornell University, New York, USA) and were kindly provided for this study (Weskamp et al, 2010).

LPS and TNF model of acute lung inflammation

Mice were anesthesized by intraperitoneal injection of ketamin (100 mg/kg) and xylazin (5 mg/kg). LPS (400 μg/kg in PBS) or PBS as control were intranasally instilled in the presence or absence of GW280264X (40 μg/kg, dissolved in DMSO, end concentration 0.06%) or vehicle control (0.06% DMSO). After 4 or 24 h, BAL fluid was collected for determination of protein influx, ELISA measurements of

cytokine release and flow cytometric analysis of cell recruitment. Lung tissue was used for determination of wet–dry-ratio and disintegrated for analysis of leukocyte infiltration. TNF-α (200 μg/kg in PBS) was intranasally instilled in the presence of 0.06% DMSO, and lung inflammation examined after 24 h. For detailed description see Supporting Information.

Statistics

Quantitative data are shown as mean ± SEM from three independent experiments/cell isolates/animals unless indicated otherwise. Statistical analysis was performed using GraphPad PRISM 5.0 (GraphPad Software, La Jollla, CA). For details see figure legends and the Supporting Information.

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