Leukocytes require the metalloproteinase ADAM10 but not ADAM17 for cell migration and for inflammatory leukocyte recruitment into the alveolar space

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Left running head: Pruessmeyer et al.
Right running head: ADAM10 is essential for leukocyte migration

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Key points

1. ADAM10 but not ADAM17 on leukocytes is essential for chemokine-induced signaling, adhesion, cytoskeletal rearrangement, and migration.

2. Leukocyte-expressed ADAM10 promotes leukocyte recruitment and edema formation in a murine model of acute pulmonary inflammation.

Abstract

Inflammation is a key process in various diseases, characterized by leukocyte recruitment to the inflammatory site. The present study investigates the role of a disintegrin and a metallopeptinase 10 (ADAM10) and ADAM17 for leukocyte migration \textit{in vitro} and in a murine model of acute pulmonary inflammation. Inhibition experiments or RNA knockdown indicated that monocytic THP-1 cells and primary human neutrophils require ADAM10 but not ADAM17 for efficient chemokine-induced cell migration. Signaling and adhesion events that are linked to cell migration such as p38 and Rho GTPase-family activation, F-actin polymerization, adhesion to fibronectin, and upregulation of $\alpha_5$-integrin were also dependent on ADAM10 but not ADAM17. This was confirmed with leukocytes isolated from mice lacking either ADAM10 or ADAM17 in all hematopoietic cells (Vav-Adam10$^{+/+}$ or Vav-Adam17$^{+/+}$ mice). In LPS-induced acute pulmonary inflammation alveolar recruitment of neutrophils and monocytes was transiently increased in Vav-Adam17$^{+/+}$ but steadily reduced in Vav-Adam10$^{+/+}$ mice. This deficit in alveolar leukocyte recruitment was also observed in LysM-Adam10$^{+/+}$ mice lacking ADAM10 in myeloid cells and correlated with protection against edema formation. Thus, with regard to leukocyte migration, leukocyte-expressed ADAM10 but not ADAM17 displays proinflammatory activities and may therefore serve as a target to limit inflammatory cell recruitment.
Introduction

Leukocyte recruitment and tissue infiltration is an early key event in acute inflammation. The recruitment is a multistep process involving rolling, capture, firm adhesion, transendothelial migration and chemotaxis towards the inflammatory site. This sequence of steps is regulated by an interplay of several soluble and surface expressed mediators. Adhesion molecules promote the adhesive interaction of leukocytes and endothelial cells. Cytokine-induced chemokine production is critical for mediating arrest of the leukocytes and directional migration leading to transmigration of the endothelial cell layer. Subsequently, leukocytes cross the basement membrane and follow a chemotactic gradient towards the site of inflammation. Prototype chemokines are CC-chemokine ligand 2 (CCL2/MCP-1) that recruits monocytic cells and CXC chemokine ligand 8 (CXCL8/IL-8) or murine CXCL1/KC that attracts neutrophils.

Cell recruitment by classical cytokines, chemokines and adhesion molecules is further regulated by several other molecules present on the cell surface, including members of the ADAM family. While protease independent activities of ADAMs are often carried out by their disintegrin domain and contribute to adhesive cell interactions, the proteolytic activity is mediated by the metalloproteinase domain and mediates the shedding of other surface molecules from the cell membrane. Especially ADAM10 and ADAM17 have been related to shedding of inflammatory substrate molecules by leukocytes or endothelial cells. Some substrates are shed by ADAM17 (e.g. TNF and L-selectin), others by ADAM10 (e.g. most cadherins), and some by both proteases (e.g. IL6 receptor/IL6R, CX3C-chemokine ligand 1/CX3CL1). The fact that ADAM10- or ADAM17-deficient mice die early in development or shortly after birth, respectively, necessitates to study conditional and/or cell-specific knockout of the proteases. On leukocytes TNF is predominantly shed by ADAM17 and this cleavage is critically involved in LPS-induced septic shock. Also L-selectin on neutrophils is shed by ADAM17, which process downregulates neutrophil adhesiveness and recruitment to inflammatory sites. At present no information exists on leukocyte-specific knockout of ADAM10 in models of acute pulmonary inflammation.

This study compares the contribution of leukocyte-expressed ADAM10 and ADAM17 to the migration and inflammatory recruitment of leukocytes in vitro and in acute pulmonary inflammation in vivo. Pharmacological inhibition and transcriptional silencing demonstrate that ADAM10 but not ADAM17 is essential for chemokine-induced migration of human and murine monocytic cells and neutrophils. This associates with the involvement of ADAM10 in
chemokine-induced signaling, adhesion, cytoskeletal rearrangement, and integrin-upregulation. Mice with selective protease deficiency in hematopoietic or myeloid cells were analyzed in a model of LPS-induced acute pulmonary inflammation. The results indicate that ADAM10 but not ADAM17 on hematopoietic cells promotes alveolar recruitment of tissue infiltrating leukocytes and development of pulmonary edema.
Materials and Methods

Antibodies, chemokines, inhibitors

LPS was from *E. coli* strain 0127:B8 (Sigma-Aldrich, Munich, Germany), other sources are listed in the supplements.

Mice

Vav-Adam10⁻/⁻ mice and Vav-Adam17⁻/⁻ mice expressed Cre recombinase under control of the Vav-promotor and were homozygous for floxed Adam10 or floxed Adam17, respectively. ⁸⁻¹¹ LysM-Adam10⁻/⁻ mice expressed Cre recombinase via the LysM-promotor and were homozygous for floxed Adam10. Littermates (“litter control”) of the same background expressed Cre recombinase but no floxed protease genes (see also supplements). Animal experiments were approved by the local authorities and performed with 6 to 8 week old female mice on C57BL/6 background (82-02.04.2011.A335 and 87-51.04.2010.A026, LANUV NRW, Germany).

Cell culture and cell preparation

THP-1 cells, lung human microvascular endothelial cells (HMVEC-L), human embryonic kidney cells (HEK293) and human epithelial bladder carcinoma cells (ECV304) were cultured as described.¹²⁻¹⁴ For isolation and culture of human and murine blood cells see supplements.

Lentiviral transduction

Short hairpin RNA (shRNA) targeting ADAM10 and ADAM17 was inserted into the lentiviral expression vector pLVTHM (Addgene plasmid 12247), lentiviral particles were prepared and used for transduction as described.¹⁵ The shRNA sequences are detailed in the supplements.

RT-qPCR

mRNA levels for indicated genes in murine BMDM as well as human THP-1 cells were quantified by RT-qPCR analysis and normalized to the mRNA level of murine RPS29 or human GAPDH as described.¹² For primer sequences see supplements.

Flow cytometric analysis

Cells were analyzed for surface expression of ADAM10 and ADAM17 as described.¹⁵ The same procedure was used to study integrin expression with antibodies against either total or
active integrin subunits. For analysis of murine BAL fluid, blood samples, neutrophil, macrophage and lymphocyte gates were defined by staining for CD11b, CD4, CD8e, Ly6G, and F4/80 and cross-checked with the appropriate isotype controls (see supplements).12

**Chemotaxis, transmigration, and adhesion assay**

Assays were performed and quantified as described (see supplements).13;16

**Phalloidin stain**

F-actin polymerization in THP-1 cells and BMDM was measured by phalloidin stain and is detailed in the supplements.

**ERK1/2, p38 activation and Rho GTPase activation**

Assays were performed with commercial antibodies and kits according to the manufacturer. See supplements for details.

**LPS-induced acute pulmonary inflammation**

The LPS-induced model of acute pulmonary inflammation has been described and is detailed in the supplements.12

**Statistics**

Quantitative data are shown as mean ± SEM calculated from at least three independent experiments and cell isolates. Animal numbers per group are indicated within the figure legends. Percentage data were arc sin-transformed. Statistics were calculated using PRISM5.0 (GraphPad Software, La Jolla, CA). Where applicable, data were analyzed by one-way ANOVA followed by Bonferroni correction, and differences were indicated by crosses (***p<0.0001, **p<0.01, *p<0.05). To test for difference to a single hypothetical value (e.g. 100%) the one-sample t-test was used, and differences were indicated by asterisks (**p<0.0001, *p<0.01, *p<0.05).

This study was conducted in accordance with the Declaration of Helsinki.
Results

ADAM10 is required for monocytic cell migration

The contribution of ADAM10 and ADAM17 to leukocyte migration was studied by chemotaxis experiments with THP-1 cells using the CC-chemokine CCL2 as chemoattractant. Pretreatment of cells with the metalloproteinase inhibitor GI254023X (GI) blocking ADAM10 with 100-fold more potency than ADAM17\(^ {17}\) reduced CCL2-induced chemotaxis in a concentration dependent manner reaching half maximal inhibition at approximately 1µM \((\text{Figure 1A})\) corresponding to the reported IC\(_{50}\) value for inhibition of cell-expressed ADAM10.\(^ {18,19}\) The inhibitor TAPI-1 predominantly blocks ADAM17,\(^ {15}\) but also inhibits ADAM10 at higher concentrations. This inhibitor had a less pronounced effect on cell migration \((\text{Figure 1B})\). There also was an effect of GI254023X on random migration in the absence of chemotactic stimulus, but compared to the inhibition of chemokine-induced migration the effect was rather small \((\text{suppl. Figure 1A})\). When migration experiments were extended to longer time periods, the migratory response to CCL2 reached a maximum at 4h, whereas cells treated with GI254023X showed a delayed response still increasing over a period up to 6h \((\text{suppl. Figure 1B})\).

Effective inhibition by GI254023X was achieved by a short preincubation time (15 to 30min). When cells were washed directly before the migration assay to remove free inhibitor, cell migration was still suppressed \((\text{Figure 1C})\). However, when inhibitor-treated cells were further incubated for 60min in the absence of inhibitor and then assayed for chemotaxis, the response recovered. This excludes the possibility that the effect of GI254023X is mediated by induction of long-term adaptations or by general effects on cell viability.

The inhibition experiments suggested that ADAM10 could be critically involved in cell migration but did not rule out an additional contribution of ADAM17. Therefore, ADAM10 and ADAM17 in THP-1 cells were downregulated on the transcriptional level using lentiviral vectors coding for specific shRNA (two different shRNA vectors for each ADAM protease or a scramble control shRNA vector). Effective downregulation of ADAM10 and ADAM17 expression was shown by RT-qPCR and flow cytometry \((\text{suppl. Figure 1C/D})\). The knockdown of ADAM10 reduced migration in response to CCL2 \((\text{Figure 1D})\) while the knockdown of ADAM17 had no effect. To clarify whether a similar effect could be seen for transendothelial migration of THP-1 cells, human microvascular endothelial cells of the lung (HMVEC-L) were cultured on transwell filters. CCL2-induced transendothelial migration of
THP-1 cells was also suppressed by ADAM10 silencing but not by ADAM17 silencing (suppl. Figure 1E).

Next, we investigated which step of migration is affected by the ADAM10 inhibitor GI254023X. CCL2 increased migration through the pores of the chemotaxis membranes, and cells that were still in the process of transmigrating through the pores could be visualized by staining (Figure 1E). Of note, GI254023X increased the number of cells that remained within the pores. Again, a very similar observation was made upon silencing of ADAM10, while silencing of ADAM17 had no effect (suppl. Figure 1F). Thus, leukocytes require ADAM10 to effectively migrate through the pores and finally detach from the filter. This was further analyzed in a transcellular migration setup in which THP-1 cells transmigrated through a layer of ECV-304 cells. These cells do not represent endothelial cells but they can be grown as a monolayer that is thick enough for confocal Z-stack analysis to visualize migrating leukocytes on, within or below the ECV304 cell layer. In the presence of GI254023X, THP-1 cells were able to migrate into the ECV304 cell layer (pos.1) but were unable to migrate further to the lower side of the membrane (pos.2, Figure 1F).

**ADAM10 promotes chemokine-induced signaling, adhesion to fibronectin and α5-integrin surface expression**

We next investigated possible effects of ADAM10 on CCL2-induced intracellular signals involved in the regulation of adhesion and chemotaxis. Assessment of intracellular calcium transients in response to CCL2 did not indicate any influence of GI254023X or knockdown of ADAM10 or 17 (suppl. Figure 2A-D). We then studied the influence of CCL2-induced phosphorylation of extracellular regulated kinases ERK1/2 and p38 kinase. Inhibition by U0126 and SB203580 confirmed that ERK1/2 and p38 signaling is important for CCL2-mediated THP-1 cell migration (suppl. Figure 2E). In the presence of GI254023X, phosphorylation of ERK1/2 in response to CCL2 was slightly reduced at early time points of stimulation (5min), but this difference vanished at later time points (Figure 2A). Phosphorylation of p38, however, was more efficiently suppressed over a longer time period. This was confirmed by shRNA-mediated silencing of ADAM10 leading to inhibition of early ERK1/2 phosphorylation and p38 phosphorylation in response to CCL2 (Figure 2B). Since ERK and p38 activation might arise from stimulation by EGFR that can be activated by metalloproteinase dependent shedding of growth factors,20 we next probed the EGFR
inhibitors PD168393 and cetuximab for their effect on CCL2-induced chemotaxis. In fact, cell migration was reduced by both inhibitors (suppl. Figure 2F).

Next, we studied whether ADAM10, ERK1/2, p38 or EGFR inhibition would affect THP-1 cell adhesion to coated integrin ligands, e.g. fibronectin or ICAM-1. Stimulation with CCL2 enhanced binding to fibronectin and this interaction was reduced by ERK1/2 or p38 inhibition (suppl. Figure 2G). By contrast, EGFR inhibition had no effect on CCL2-induced adhesion to fibronectin suggesting that EGFR is not relevant for this process (suppl. Figure 2H). Nevertheless, GI254023X completely suppressed adhesion to fibronectin (Figure 2C) indicating that ADAM10 is a key molecule for this type of cell adhesion. The effect of the ADAM10 inhibitor was less pronounced for ICAM-1-mediated adhesion and did not reach significance (suppl. Figure 2I).

Our findings suggested that fibronectin binding by α5β1-integrin and ICAM-1 binding by αMβ2-integrin, which are both expressed by THP-1 cells, could be differentially regulated by ADAM10. Analysis of integrin surface expression by flow cytometry indicated an upregulation of α2-, αL- and αM-integrins by CCL2, which was not sensitive to treatment with GI254023X (suppl. Figure 2J-L). There was no effect on the surface level of activated β1-integrin (suppl. Figure 2M). By contrast, CCL2-induced upregulation of α5-integrin was suppressed by the ADAM10 inhibitor (Figure 2D,E). Similar to the adhesion experiments, upregulation of α5-integrin was also blocked by inhibition of ERK1/2 and p38 but not by EGFR inhibition (suppl. Figure 2N, O). Since integrin-mediated signals in cell migration involve activation of the small GTPase family Rho21 we also studied the effect of ADAM10 inhibition on this pathway. Again we observed profound inhibition of CCL2-mediated Rho family activation by GI254023X (Figure 2F). Rho activation is a critical event in chemokine-induced F-actin polymerization and, indeed, treatment with GI254023X or ADAM10 silencing but not ADAM17 silencing abrogated F-actin polymerization in response to CCL2 (Figure 2G).

ADAM10 is required for migration and adhesion of primary human and murine neutrophils and monocytic cells

To clarify whether the observed inhibition of cell migration would also account for other cell types and for primary cells, human neutrophils were assayed for chemotaxis induced by the human neutrophil attracting chemokine CXCL8/IL-8. As seen for CCL2-induced migration of THP-1 cells, CXCL8-induced neutrophil migration was considerably decreased in the
presence of GI254023X (Figure 3A and suppl. Figure 3A) and this was associated with an accumulation of cells within the pores of the membrane (suppl. Figure 3B). Inhibition was also observed with TAPI-1 (Figure 3A and suppl. Figure 3C). Adhesion experiments demonstrated that GI254023X reduces CXCL8-induced neutrophil adhesion to fibronectin (Figure 3B) but not to ICAM-1 (suppl. Figure 3D). As seen for THP-1 cells, the inhibitor prevented chemokine-induced upregulation of $\alpha_5$-integrin (Figure 3C).

We further analyzed bone-marrow-derived macrophages (BMDM) for migration in response to murine CCL2. Treatment with GI254023X again suppressed the migratory response to CCL2 and showed almost no effect on random BMDM migration in the absence of CCL2 (Figure 3D). For the further study of leukocyte-expressed ADAM10 or ADAM17 in mice, animals with floxed Adam10 or Adam17 gene were crossed with Vav-cre mice for conditional knockout in hematopoietic cells. BMDM were generated from these mice and investigated by flow cytometry for F4/80 expression to confirm purity (> 95%, suppl. Figure 3E). The selective knockout was demonstrated by flow cytometry for ADAM10 and RT-qPCR for ADAM10 and ADAM17 (suppl. Figure 3F/G).

ADAM10-deficiency in BMDM reduced CCL2-induced cell migration to a similar extent as the ADAM10 inhibitor (Figure 3E). By contrast, ADAM17-deficiency in these cells did not affect cell migration. As seen for THP-1 cells and neutrophils, GI254023X reduced chemokine-induced BMDM adhesion to fibronectin (Figure 3F) but not to ICAM-1 (suppl. Figure 3H). Moreover, ADAM10-deficiency reduced adhesion to fibronectin to a similar extent as the ADAM10 inhibitor while ADAM17-deficiency had no effect (Figure 3F). Adhesion to ICAM-1 was again not affected by ADAM10- or ADAM17-deficiency (suppl. Figure 3H). Overexpression of ADAM10 in ADAM10-deficient BMDM reconstituted cell adhesion to fibronectin, which was prevented by GI254023X (Figure 3G). Finally, as seen for THP-1 cells, ADAM10-deficiency in BMDM also prevented chemokine-induced activation of p38 (Figure 3H), upregulation of $\alpha_5$-integrin surface expression (Figure 3I), F-actin polymerization (Figure 3J) and Rho GTPase activation (suppl. Figure 3I).

Subsequently, migration of murine blood leukocytes in response to non-heat-inactivated FBS as chemotactic stimulus was analyzed. As detected by flow cytometry analysis and gating of the migrated cells (suppl. Figure 3K), GI254023X or ADAM10-deficiency but not ADAM17-deficiency reduced the CCL-2-induced migration but not the random migration of blood neutrophils (suppl. Figure 3J).
Leukocyte expressed ADAM10 and ADAM17 differentially contribute to alveolar leukocyte recruitment in LPS-induced acute pulmonary inflammation

To investigate the *in vivo* role of ADAM10 or ADAM17 for leukocyte migration, a model of acute pulmonary inflammation was used. LPS was applied intranasally leading to increased leukocyte infiltration into the lung after 24h, which was quantified by flow cytometric analysis of the BAL fluid cells (Figure 4A). In line with previous studies, BAL fluid from LPS-treated litter controls contained more neutrophils and monocytes than PBS-treated control mice (Figure 4B-C). At the same time, monocyte and granulocyte counts but not lymphocyte counts in blood increased as a consequence of LPS treatment (suppl. Figure 4A and B). Compared to the litter controls, neither Vav-Adam10/+ nor Vav-Adam17/+ mice showed any change in blood leukocyte composition within each treatment group (PBS or LPS). Basal leukocyte numbers in BAL of PBS-treated animals was low and did not differ among the genotypes. However, in LPS-treated animals the influx of neutrophil and monocyte recruitment into the airspaces was reduced in Vav-Adam10-/- mice compared to the litter controls (Figure 4B/C). By contrast, LPS-treated Vav-Adam17-/- mice showed a tendency to enhanced leukocyte recruitment although this did not quite reach significance (p=0.06 using Student’s t-test).

ADAM10 or ADAM17 in hematopoietic cells differentially contribute to early leukocyte tissue infiltration, edema formation and cytokine release

To study whether differences in alveolar leukocyte recruitment would be related to differences in leukocyte tissue infiltration, lungs were perfused to remove vessel-attached leukocytes, histological analysis of lung sections was performed (Figure 5A/B), and tissue resident neutrophils and monocytes were counted. As expected, LPS-treatment of Adam10 and Adam17 litter control mice profoundly increased the number of neutrophils and monocytes within the tissue (Figure 5C/D). Of note, LPS-treated Vav-Adam10+/- mice contained even more neutrophils and monocytes in the lung tissue. Taken into account that leukocytes of Vav-Adam10+/- mice did not further migrate into the BAL fluid as shown by reduced BAL cell numbers, this indicates that cells accumulate in the tissue when crossing the endothelium. Lungs of LPS-treated Vav-Adam17+/- mice also contained more leukocytes within the lung tissue than LPS-treated litter control mice.

In order to assess edema formation, the alveolar influx of protein in response to LPS and the lung wet-to-dry ratio were measured (Figure 5E/F). After 24h of LPS exposure, this response
was, however, not affected by the Vav-Adam10<sup>−/−</sup> genotype. Thus, leukocyte arrest in the tissue and decreased alveolar leukocyte recruitment was not associated with a reduction in tissue damage. By contrast, in Vav-Adam17<sup>−/−</sup> mice increased edema formation and protein influx were observed, correlating with the increased tissue infiltration and alveolar recruitment of leukocytes in these mice.

Vav-Adam10<sup>−/−</sup> and Vav-Adam17<sup>−/−</sup> mice were also analyzed for protein levels of pro-inflammatory mediators in the BAL fluid. As expected, LPS stimulation increased the release of TNF, CCL2, CXCL1, and sIL-6R in the BAL of the litter controls (suppl. Figure 5A-D). ADAM10-deficiency reduced LPS-induced CXCL1 and IL-6R release, whereas ADAM17-deficiency showed a tendency to enhance CCL2 release although this did not reach significance (p=0.06). Of note, ADAM10- or ADAM17-deficiency in leukocytes did not affect TNF release into BAL suggesting that cell types other than leukocytes are more relevant for the release of soluble TNF into the alveolar lumen.

**Deficiency of ADAM10 reduces edema formation at a later stage**

To investigate a potential long term effect of leukocyte expressed ADAM10 and ADAM17 on lung tissue inflammation we extended the time of LPS treatment to 72h. At this time point numbers of neutrophils and monocytes in the blood were not increased in response to LPS treatment any more. Again we could not observe a difference in blood leukocyte composition among the genotypes (suppl. Figure 6A-B). At 72h after LPS exposure, monocyte and granulocyte recruitment was less compared to the 24h time point, but still there was a reduction in Vav-Adam10<sup>−/−</sup> mice (Figure 6A/B) but not in Vav-Adam17<sup>−/−</sup> mice (Figure 6D/E) compared to the litter controls. Importantly, at this later stage of the inflammatory response, ADAM10-deficiency was associated with a protection against edema formation (Figure 6C), whereas ADAM17-deficiency had no influence (Figure 6F). To further support the role of ADAM10 in a later stage of pulmonary inflammation, we extended the study to mice with LysM-driven deficiency of ADAM10 in myeloic cells. Deficiency of ADAM10 in these cells was confirmed by analysis of BMDM for ADAM10 mRNA and protein surface expression (suppl. Figure 6C, D). Compared to litter controls, LysM-Adam10<sup>−/−</sup> mice did not show a difference in blood leukocyte composition (suppl. Figure 6E). Again, 72h after LPS treatment we observed a reduced neutrophil and monocyte recruitment into the alveolar lumen (Figure 6G/H), which was associated with a protection against edema formation (Figure 6I).
Discussion

The present study provides *in vivo* and *in vitro* evidence that leukocytes need ADAM10 but not ADAM17 for their migration. Pharmacological inhibition, gene silencing and gene knockout experiments consistently demonstrate that ADAM10 is necessary for efficient migration of human and murine monocytic cells and neutrophils in response to the chemokines CCL2 and CXCL8, respectively. Moreover, ADAM10 on leukocytes is vital for p38 signaling, Rho activation, cell adhesion, upregulation of α5-integrin and F-actin polymerization in response to chemokines. *In vivo* experiments with Vav-driven Adam10-deficient mice show that the protease on hematopoietic cells is required for effective alveolar leukocyte recruitment during the early (24h) and later phase (72h) of LPS-induced acute pulmonary inflammation. At the later phase ADAM10-deficiency correlates with decreased edema formation in response to LPS challenge. This was also observed in mice with LysM-driven Adam10-deficiency in myeloic cells. By contrast, ADAM17 is not required for leukocyte migration *in vitro* and *in vivo*. ADAM17 on leukocytes rather seems to mitigate migration, alveolar leukocyte accumulation, edema formation and mediator release. However, these effects seem to be limited to the early phase of acute inflammation (24h) as ADAM17-deficiency had no effect on leukocyte recruitment and edema formation at a later stage of the inflammatory event.

This study demonstrates that pharmacological or genetic targeting of leukocyte expressed ADAM10 reduces migration of human and murine monocytes and neutrophils *in vitro* and *in vivo*. This effect was correlated on the one hand with a reduction in adhesive properties to fibronectin and deficient upregulation of α5-integrin. The upregulation of α5-integrin by cytokines had been previously shown for TNF stimulation of tumor vascular leukocytes. This upregulation may be brought about via vesicular transport by microtubule motors such as KIF1C or other yet undefined mechanisms. The α5-integrin is part of the fibronectin receptor and required for adhesion and chemotaxis of monocytes as well as for neutrophil and monocyte recruitment in LPS-induced pulmonary inflammation. In fact, α5β1-integrin blockage using RGDS-peptides prevents lung damage induced by LPS. On the other hand targeting of ADAM10 led to incomplete cell migration *in vitro* and *in vivo* as seen by an arrest of migrating cells in the chemotaxis membranes and within the lung tissue. Combining these two observations it can be speculated that the reduced adhesion to matrix components such as fibronectin hinders cell migration and thereby prolongs the migration time through the chemotaxis membranes, through the endothelium and through interstitial lung tissue, similar to the observed inhibition by RGDS-peptides. Therefore, fewer cells would have completed
migration while the majority would still be in the progress of migration. Thus, ADAM10-deficiency leads to accumulation of slow-migrating cells in the chemotaxis membranes and interstitial accumulation within the lung. Interestingly, Mx-driven ADAM10 deficiency in mice leads to leukocyte accumulation in the spleen \(^{27,28}\) and this effect could be in part due to the inability of leukocytes to pass through the spleen filter. Additionally, B cell specific ADAM10-deficiency results in impaired marginal zone development and asthmatic responses \(^{29,30}\), which might also depend on ADAM10-mediated B cell migration.

In acute pulmonary inflammation, ADAM10-deficiency in all hematopoietic cells or preferentially in myeloic cells reduced recruitment of neutrophils and monocytes to the alveolar lumen. In line with the \textit{in vitro} findings, the reduced ability of the cells to complete cell migration was associated with an accumulation of the infiltrating neutrophils and monocytes within the tissue. This may explain the observation why Vav-driven deficiency of ADAM10 does not protect against early changes in lung edema formation. Once activated the trapped cells in the lung tissue including many neutrophils could cause destruction of the surrounding tissue leading to increased permeability and edema formation. The fact, that lung tissue damage was not further enhanced could be due to reduced release of the neutrophil attracting chemokine CXCL1 and sIL-6R in the BAL fluid, which would also limit further cell recruitment and tissue damage. At a later stage, however Vav-driven or LysM-driven deficiency of ADAM10 conferred protection against LPS-induced lung damage. This may indicate that ADAM10-dependent long-lasting leukocyte recruitment contributes to maintain lung inflammation over a longer time period.

There are several possibilities, how ADAM10 can affect cell adhesion and migration. First of all this seems to be a rapid and reversible mechanism, suggesting that potential effects of ADAM10 on cell differentiation do not play a role. This was confirmed with BMDM in which ADAM10 did not affect the expression of the classical differentiation markers iNOS, CD206 and ICAM-1 or members of the Notch-signaling pathway. One possible pathway may be indirect activation of ERK1/2 and p38 via ADAM10. We here show that monocytic cell migration, adhesion to fibronectin, and upregulation of \(\alpha_5\)-integrin depend on both signals, and at least p38 activation requires the presence of ADAM10. It is known that ADAM10 and ADAM17 can induce shedding of EGFR ligands resulting in autocrine and paracrine EGFR transactivation \(^{20}\) followed by ERK1/2 and p38 phosphorylation. This EGFR pathway enhances migration of endothelial and endometriotic cells. \(^{31,32}\) Importantly, we found that
CCL2-induced α5-integrin upregulation and adhesion to fibronectin are not affected by EGFR inhibition. While there may be a general effect of EGFR on cell migration, our data suggest that this is not the key molecule by which ADAM10 contributes to leukocyte recruitment and cell adhesion. Even though the responsible cleavage event by ADAM10 still needs to be determined there obviously is an immediate effect of ADAM10 on the regulation of α5-integrin surface expression. Outside in signaling by integrins is well known to mediate Rho GTPase activation and actin polymerization.21 In fact, we found that both responses are dependent on ADAM10. The hindrance of cytoskeletal rearrangement may then explain our observation that ADAM10-deficient leukocytes cannot efficiently transmigrate through the pores of the chemotaxis membranes in vitro and cannot efficiently transmigrate through the lung tissue in vivo.

In comparison to ADAM10, the closely related protease ADAM17 seems to hold a different role in cell migration. By conditional knockout of ADAM17 in hematopoietic cells or adoptive transfer of ADAM17-deficient bone marrow we and others consistently found increased neutrophil recruitment in the early phase of the inflammatory response when leukocytes lack ADAM17.9;33;34 This increased presence of leukocytes would lead to enhanced tissue damage and may thus explain the increased edema formation observed in mice lacking leukocyte ADAM17. ADAM17 but not ADAM10 mediates L-selectin shedding, and loss of L-selectin on the surface of neutrophils would lead to enhanced rolling and adhesion to the endothelium,35 which would then promote inflammatory recruitment of neutrophils.9;36 In fact, loss of L-selectin shedding by ADAM17 has been correlated with reduced pulmonary recruitment of neutrophils.31 A recent study reported a crucial role of ADAM17 in monocyte transendothelial migration but not in endothelial adhesion. This was associated with surface regulation of MAC-1 (αMβ2-integrin) which was ADAM17- but not ADAM10-dependent.36 We here confirm that ADAM10 has no influence on chemokine-induced upregulation of the MAC-1 components β2-integrin and αM-integrin, and we also did not observe an effect on the LFA-1 (αLβ2-integrin). However, we found prominent effects of ADAM10 on chemokine-induced upregulation of α5-integrin in both neutrophils and monocytes. While MAC-1 and LFA-1 are involved in leukocyte interactions with endothelium, α5-integrin is part of the fibronectin receptor (α5β1) and therefore important for cell matrix interaction.37 Thus, ADAM17 and ADAM10 could play different roles in the interaction of leukocytes with endothelial cells and extracellular matrix components.

There is increasing evidence that metalloproteinase inhibitors can limit hallmarks of acute pulmonary inflammation.12;38 This is most likely a result of distinct metalloproteinase
activities on multiple cell types. For ADAM17, we have recently reported that the protease on endothelial cells acts proinflammatory in a murine model of acute pulmonary inflammation.\textsuperscript{12} However, this activity may be counteracted at least in part by ADAM17 on leukocytes limiting neutrophil migration as reported in the present study and by others.\textsuperscript{9,33,34} The role of ADAM10 is apparently different because its proinflammatory activities on endothelial cells may synergize with its proinflammatory activities on leukocytes that have been identified in the present study.\textsuperscript{12,13,39} Thus, local treatment with selective ADAM10 inhibitors may represent a novel strategy to limit pulmonary inflammation.
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Contribution: JP, FMH, HA, EG, TP, SN, JK, EV, and DD performed experiments and analyzed data; AL, DD, JP and FMH wrote manuscript and designed study; CM, SU, MD, and PS provided vital expertise, animal model or transgenic mice.

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Figure legends

Figure 1: Effect of metalloproteinase inhibitors and ADAM10/ADAM17 knockdown on THP-1 cell migration.

(A/B) THP-1 cells were preincubated with varying concentrations of the inhibitors GI254023X (GI) in A or TAPI-1 in B or appropriate dilutions of vehicle (DMSO) for 15min and then assayed for cell migration in response to 3nM CCL2. The number of migrated cells in the lower compartment was determined by measurement of endogenous glucuronidase activity and results are expressed in relation to cells receiving no chemoattractant and no inhibitor. Migration of cells receiving no chemoattractant is indicated by dotted lines. (C) THP-1 cells were pretreated with GI254023X (10µM) or DMSO (0.1%) for 30min. Subsequently, cells were either directly assayed for CCL2-induced cell migration or washed to remove free inhibitor. Washed cells were either directly investigated for CCL2-induced chemotaxis or further incubated for 60min (recovery) before the assay. Results are expressed in relation to the untreated control receiving no chemoattractant (dotted line). (D) THP-1 cells were transduced with different lentivirus encoding scramble-shRNA (scr), ADAM10-shRNA (A10-650 and A10-1947) or ADAM17-shRNA (A17-2061 and A17-2646) and assayed for CCL2-induced cell migration. Results are expressed in relation to the scramble control receiving no chemoattractant (dotted line). (E) Following CCL2-stimulation in presence of 10µM GI254023X or 0.1% DMSO (15min pretreatment) cells were removed from the top of the chemotaxis membrane and remaining cells were stained in the chemotaxis membrane. Representative images (left) and quantitative data (right) are shown. (F) GFP-expressing THP-1 cells were pretreated with 10µM GI254023X or 0.1% DMSO and subsequently investigated for transmigration through dsRed-expressing ECV304 cells grown on transwell filters. THP-1 cells were visualized by confocal microscopy and Z-stack analysis (left) and quantified within (pos.1) and below (pos.2) the ECV304 layer (right). Quantitative data represent means ± SEM of three independent experiments. Significance was calculated using one-way ANOVA and Bonferroni post-test and is indicated by crosses. Asterisks without line indicate significant differences to the non-treated control analyzed by one-sample t-test.
Figure 2: Effect of ADAM10 inhibition or knockdown on CCL2-induced signaling, adhesion and F-actin polymerization in THP-1 cells.

(A/B) THP-1 cells were treated with 10µM GI254023X or DMSO for 15min in A or transduced with lentivirus encoding shRNA targeting ADAM10 or scramble shRNA in B. Subsequently, cells were assayed for CCL2-induced phosphorylation of ERK1/2 and p38 at the indicated time points by Western blotting. Samples treated with DMSO and GI254023X were run on the same gel. Total ERK1/2 and p38 levels were determined in parallel (separate blots had to be used for pp38). Signals were quantified by densitometry, normalized to the expression of total kinase and expressed in relation to the phosphorylation level of the unstimulated control (0min) for DMSO-treated THP-1 cells in A and separately for each transduced THP-1 cell type in B. (C-F) THP-1 cells were pretreated with 10µM GI254023X or 0.1% DMSO for 15min and stimulated with CCL2 (3nM) or left unstimulated. Subsequently, cells were assayed for binding to coated fibronectin in C, upregulation of α5-integrin surface expression in D/E (D: representative histograms, E: quantification) and Rho GTPase activation in F. Rho activation was quantified as active Rho protein in relation to total Rho protein as determined by Western blotting. (G) THP-1 cells were treated with 10µM GI254023X or transduced with lentivirus to downregulate ADAM10. DMSO and scramble shRNA were used as controls. Subsequently, cells were assayed for polymerization of F-actin by flow cytometry using fluorophore-labeled phalloidin. Results were expressed as percentage of the controls (DMSO or scramble). Quantitative data represent means ± SEM of three independent experiments. Crosses indicate significance among treated cells calculated using one-way ANOVA and Bonferroni post-test. Asterisks without line indicate significant differences to the non-treated control analyzed by one-sample t-test.

Figure 3: ADAM10 promotes migration and fibronectin binding of human neutrophils and murine bone-marrow-derived-macrophages (BMDM).

(A-C) Human neutrophils were pretreated with 10µM GI254023X or 0.1% DMSO or left untreated for 15min and were subsequently assayed for cell migration in A, adhesion to fibronectin in B and α5-integrin surface expression in C, induced by CXCL8 (10 nM). (D) Murine BMDM were generated from wild type mice treated with GI254023X (10µM) or DMSO (0.1%) and investigated for chemotaxis induced by various concentrations of CCL2. (E/F) Murine BMDM from Vav-Adam10−/− and Vav-Adam17−/− mice and litter controls were pretreated with 10µM GI254023X or vehicle (DMSO) and investigated for CCL2-induced cell migration in E and adhesion to fibronectin in F. (G) ADAM10-deficient BMDM were
transfected with bovine ADAM10, treated with 10µM GI254023X or 0.1% DMSO and investigated for CCL2-induced adhesion to fibronectin after 15min. (H) BMDM from Vav-Adam10± and litter control mice were stimulated with murine CCL2 (3nM) for the indicated time periods and investigated for phosphorylation of p38 by Western blotting. Samples were run on the same gel. (I) BMDM from Vav-Adam10± and litter controls were treated for 15min with CCL2 and investigated for upregulation of α5-integrin surface expression. (J) BMDM from Vav-Adam10± and litter controls were treated with 3nM CCL2 for the indicated time periods and examined for polymerization of F-actin by flow cytometry. Quantitative data represent means ± SEM of three independent experiments and cell preparations. Crosses indicate significance among treated cells calculated using one-way ANOVA and Bonferroni post-test. Asterisks without line indicate significant differences to the non-treated control analyzed by one-sample t-test.

Figure 4: Deficiency of ADAM10 abrogates alveolar leukocyte recruitment in LPS-induced acute pulmonary inflammation.
(A-C) Vav-Adam10±, Vav-Adam17± mice and respective litter controls were intranasally treated with 400µg/kg LPS or vehicle (PBS). After 24h, lungs were lavaged, and BAL fluid was investigated for content of leukocytes in A, neutrophils in B, and monocytes in C by flow cytometry. Results were expressed as cell number per ml BAL fluid. Data represent means ± SEM (n=5). Significance was calculated using one-way ANOVA and Bonferroni post-test and is indicated by crosses. Crosses without line indicate significance to the PBS-treated control.

Figure 5: Influence of ADAM10- or ADAM17-deficiency on cell recruitment to lung tissue and on edema formation.
(A-F) Vav-Adam10±, Vav-Adam17± mice and respective litter controls were intranasally treated with 400µg/kg LPS or vehicle (PBS). 3µm-sections of formalin-fixed and paraffin-embedded lung tissue were stained with hematoxylin-eosin to differentiate monocytic and polymorphnuclear cells. Representative overview images are shown for Vav-Adam10± mice in A and Vav-Adam17± mice in B (upper panel). Alveolar lumen (Al), neutrophils (filled arrowheads) and monocytic cells (open arrowheads) were marked in the high magnifications (lower panel). Ten images per animal were analyzed for the recruitment of neutrophils in C and monocytes in D using AixoVision software. 24h after application, alveolar protein influx was determined in E and lung wet-dry-ratio in F, respectively. Quantitative data represent means ± SEM (n=5). Significance was calculated using one-way ANOVA and Bonferroni
post-test and is indicated by crosses. Crosses without line indicate significance to the PBS-treated control. Bar legends are the same for C/E and D/F, respectively.

**Figure 6: ADAM10-deficiency attenuates LPS-induced acute pulmonary inflammation.** (A-I) Vav-Adam10<sup>-/-</sup> (A-C), Vav-Adam17<sup>-/-</sup> (D-F), LysM-Adam10<sup>-/-</sup> (G-I) and respective litter control mice were intranasally treated with 400µg/kg LPS or vehicle (PBS). After 72h, lungs were lavaged, and BAL fluid was analyzed for content of neutrophils (A, D, G) and monocytes (B, E, H) by flow cytometry. Results were expressed as cell number per ml BAL fluid. After 72h, lung wet-dry-ratio was determined (C, F, I). Data represent means ± SEM (n=5). Significance was calculated using one-way ANOVA and Bonferroni post-test and is indicated by crosses. Crosses without line indicate significance to the PBS-treated control.
Figure 4

A

Leukocytes in BAL [10^6/ml]

PBS  LPS

B

Neutrophils in BAL [10^6/ml]

PBS  LPS

C

Monocytes in BAL [10^6/ml]

PBS  LPS

litter control  Vav-Adam10^+/+
Vav-Adam17^+/+
Leukocytes require the metalloproteinase ADAM10 but not ADAM17 for cell migration and for inflammatory leukocyte recruitment into the alveolar space

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