

Piezo1-ADAM axis promotes inflammatory alveolar-capillary crosstalk resulting in transendothelial leukocyte migration

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Abstract

Mechanical forces continuously maintain pulmonary homeostasis during respiration. However, excessive mechanical stress can promote inflammatory lung injury characterized by alveolar-capillary barrier dysfunction and leukocyte infiltration. Although the mechanosensitive ion channel Piezo1 has emerged as an important regulator of pulmonary mechanotransduction, the molecular mechanisms linking epithelial Piezo1 activation to a pro-inflammatory endothelium and leukocyte recruitment remain poorly understood. Here, we provide new insights into a mechanism by which Piezo1-activated lung epithelial cells promote inflammatory epithelial-endothelial crosstalk through release of epithelial surface proteins via proteases of the ADAM family, thereby initiating an endothelial signaling that ultimately enhances leukocyte infiltration. The dissection of this signaling cascade was resolved using a stepwise experimental approach, sequentially analyzing the activation of lung epithelial cells (H441/hAELVi) with the Piezo1 agonist Yoda1 in the first step, resulting in the release of a broad pro-inflammatory secretome, which composition was considerably modulated by a dual ADAM10/17 inhibition. Subsequently, the resulting conditioned medium triggered endothelial signaling pathway activation and inflammatory transcriptional reprogramming with enhanced expression of leukocyte recruitment-associated genes. This inflammatory response triggered increased leukocyte transmigration across the endothelial barrier, which was reduced by epithelial and endothelial treatment with the ADAM10/17 inhibitor or suppression of selected endothelial signaling pathways. Mechanistically, our findings identify epithelial ADAM proteases as central inflammatory switches coupling epithelial Piezo1 activation to paracrine endothelial activation and leukocyte recruitment. Collectively, this study provides a molecular link between mechanical stress and inflammatory activation of the alveolar-capillary barrier, emphasizing Piezo1 and ADAM proteases as valuable targets for local therapy to limit pulmonary injury.

Introduction

The respiratory system is subject to persistent mechanical forces caused by ventilation, hemodynamic forces, airway constriction, leukocyte trafficking and alterations in extracellular matrix stiffness. As a result, mechanotransduction evoked by physiological forces plays a fundamental role in maintaining lung homeostasis by coordinating essential cellular processes including tissue remodeling, maintenance of alveolar-capillary barrier integrity and inflammatory signaling. Mechanotransduction in response to stronger, pathological forces, however, can contribute to the pathogenesis of respiratory diseases correlated with an accumulation of red blood cells, macrophages and neutrophils in the alveoli [1]. This process typically relies on a cellular crosstalk from the epithelium exposed to mechanical forces, towards the endothelium which then becomes activated to promote the recruitment of inflammatory cells. Excessive or persistent leukocyte infiltration can itself become a major driver of lung injury by disrupting epithelial and endothelial barrier integrity through the release of reactive oxygen species, proteases and neutrophil extracellular traps, thereby amplifying pulmonary inflammation [2]. This recruitment process involves three major steps: The sequential upregulation of adhesion molecules and chemokines promoting leukocyte rolling, the firm adhesion and the ultimately transendothelial migration across the interstitial space into the alveolar compartment [3].

Despite the recognized contribution of mechanical stress to pulmonary inflammation, the molecular mechanisms linking mechanical stimulation of the alveolar epithelium to endothelial activation and subsequent immune cell recruitment remain poorly understood. Elucidating the mechanosensors and downstream signaling pathways governing this epithelial-endothelial communication therefore represents an important step toward understanding inflammatory lung pathologies. Among other mechanosensors, the mechanosensitive ion channel Piezo1 has attracted increasing attention due to its unique propeller-shaped structure enabling the direct conversion of membrane tension changes into rapid Ca^{2+} influx [4]. Consistent with its central role in mechanotransduction, the channel has been implicated in a wide range of pulmonary pathologies, including impaired bacterial clearance, apoptosis of pneumocytes, hypoxic vasoconstriction, endothelial barrier dysfunction and remodeling of alveolar epithelial cell junctions [5–9]. The diverse biological functions of Piezo1 are dominantly mediated through activation of Ca^{2+} -dependent downstream signaling pathways including cell type-dependent regulation of cell survival, inflammatory responses, cytoskeletal remodeling, intercellular junction integrity and vascular permeability [10–13]. Several of these processes rely on the proteolytic regulation of membrane-bound signaling molecules which is the key function of members of the a disintegrin and metalloproteinase (ADAM) family.

By mediating proteolytic ectodomain cleavage, also called shedding, ADAM10 as well as ADAM17 control the release of membrane-bound cytokines like tumor necrosis factor α (TNF α), cytokine receptors including interleukin 6 receptor (IL-6R), growth factors like heparin-binding EGF-like growth factor (HB-EGF) as well as adhesion molecules controlling barrier integrity and leukocyte recruitment like junctional adhesion molecule A (JAM-A), vascular endothelial (VE)-cadherin, intercellular adhesion molecule 1 (ICAM1) and vascular cell adhesion molecule 1 (VCAM1) [14]. Shedding of these effector molecules generates soluble forms which can act in an autocrine or paracrine manner to amplify inflammatory signaling. Recent findings indicate that these shedding events can be triggered by Piezo1-dependent signaling in response to mechanical forces through activation of ADAM10 [15] and ADAM17 [9, 16]. Therefore, within the alveolar-capillary barrier, ADAM-dependent generation of soluble inflammatory mediators is thought to provide an important mechanism for epithelial-endothelial communication. As a consequence, endothelial activation leads to the expression of inflammatory cytokines, chemokines and adhesion molecules, resulting in increased vascular permeability and transendothelial leukocyte migration to sites of pulmonary inflammation [17]. Despite increasing evidence linking Piezo1 and ADAM10/17 activation to inflammatory signaling and barrier regulation, it remains poorly understood whether this axis mediates epithelial-endothelial communication and subsequent processes like leukocyte transmigration across the alveolar-capillary barrier.

In the present study, we uncovered step by step the role of the lung epithelial Piezo1-ADAM signaling axis in regulating inflammatory epithelial-endothelial communication in a pharmacologically activated alveolar epithelial cell model. To address this, we studied how the epithelial secretome is affected by Piezo1-mediated cell activation and ADAM protease-dependent shedding. With medium transfer experiments we then examined its capacity to activate downstream signaling pathways driving transcriptional regulation of inflammatory mediators in endothelial cells. Finally, we assessed the

functional contribution of this axis and the identified signaling pathways in transendothelial leukocyte migration assays. The presented data demonstrate that Piezo1-induced epithelial activation induces ADAM protease-dependent shedding which then promotes paracrine endothelial activation and finally results in leukocyte recruitment.

Methods

Materials

Dimethylsulfoxide (DMSO) was purchased from Roth (Karlsruhe, DE). Yoda1 and GW280264X (GW) were obtained from Tocris (Minneapolis, USA). BAY11-7082 (BAY) and Baricitinib were obtained from Selleckchem (Houston, USA), LY294002 (LY), SCH772984 (SCH) and SR11302 (SR) were purchased from MedChemExpress (Monmouth Junction, USA). Afatinib and Dacomitinib were obtained from Biomol (Cay11492 and TGM-T2483, Hamburg, DE). For the antibodies used in this study, see the respective method section.

Isolation and cultivation of HUVECs

Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords of cesarean sections, covered by the ethics approval EK241/18 from the local ethics committee. The isolation was performed as described previously in [16]. For the cultivation process, 1% collagen type 1 coating (Sigma-Aldrich, St. Louis, USA) in PBS and Endopan3 medium (PAN-Biotech, Aidenbach, DE) including all supplements were used. Experiments were performed in Endopan3 medium lacking Heparin and Hydrocortisone. For each experiment, cells from at least three donors were used to rule out donor-specific effects. The cells were used until passage 7. At a confluence of 90%, HUVECs were detached by using Accutase (PAN-Biotech) and seeded 24 h before starting the experiment. Cell concentrations and plate formats were adjusted for the corresponding method and described in the respective method section. A pre-inhibition for 1 h was performed prior to every inhibitor treatment. The inhibitors were also present during the entire stimulation period.

Isolation of polymorphonuclear neutrophils from human blood

Human granulocytes, further referred to as leukocytes, were isolated from citrated peripheral blood of healthy volunteers, covered by the ethics approval EK241/18 from the local ethics committee. The blood was diluted 1:1 with PBS followed by a density gradient centrifugation step using Pancoll (400g, 30 min without break). The granulocyte phase, located on the erythrocyte pellet, was subsequently incubated in red blood cell lysis buffer (155 mM NH_4Cl , 10 mM NaHCO_3 , 5 mM ethylenediaminetetraacetic acid, pH 7.4) for 5 min. After two PBS washing and centrifugation steps, cells were counted and diluted to 2×10^6 cells/ml in Endopan3 (without heparin and hydrocortisone) right before starting the transmigration experiment.

Cultivation of epithelial cell lines

The human adenocarcinoma-derived epithelial lung cell line NCI-H441 (ATCC [HTB-174™]) was cultured in RPMI medium (PAN-Biotech) with 10% FCS (PAN-Biotech) and 1% Penicillin/Streptomycin (Sigma-Aldrich). Human alveolar epithelial lentivirus immortalized cells (Ci-hAELVi) (InSCREENex GmbH, Braunschweig, DE) were cultured in huAEC Medium (InSCREENex GmbH) on recommended coating (InSCREENex GmbH). Cells were seeded 48 h prior experiment start. For inhibitor treatments, the cells were pre-incubated for 1 h with the inhibitor, followed by the main stimulation period, in which the inhibitors were also present.

Preparation and transfer of conditioned medium

Epithelial cells (H441 or hAELVi) were seeded in 6-well plates at a density of 600 000 cells/well (H441) or 374 000 cells/well (hAELVi) in 2 ml RPMI or huAEC medium per well 48 h before stimulation to reach 100% confluence. Cells were washed once with PBS, followed by a 5 min 10 μ M Yoda1 (Yoda1 pulse) or DMSO stimulation in endothelial Endopan3 medium lacking heparin and hydrocortisone. To prevent transfer of Yoda1 to HUVECs, the cells were washed 3 times with PBS (5 min, 3 min, 1 min) and fresh Endopan3 medium was added subsequently. After 24 h, the medium was harvested, centrifuged and used as conditioned medium (CM) to stimulate endothelial cells.

HUVECs were seeded 24 h before starting the stimulation with the CM. If inhibitors were used, the pre-incubation was performed in Endopan3 medium without heparin and hydrocortisone. After pre-inhibition, if not already containing, the inhibitors were added to the CM for the stimulation period. The stimulation periods can be found in the respective method section.

Enzyme-linked immunosorbent assay (ELISA)

H441 cells or hAELVi cells were seeded in 6-well plates at a density of 600 000 cells/well (H441) or 374 000 cells/well (hAELVi) for 48 h until 100% confluence. Following treatment with Yoda1 and inhibitors (as described in the previous method section), the supernatant was collected after 24 h and cleared from cell debris by centrifugation. The concentration of released IL-8 (human IL-8 DuoSet ELISA Kit, R&D Systems, Minneapolis, USA), IL-6 (human IL-6 DuoSet ELISA Kit, R&D Systems), HB-EGF (human HB-EGF DuoSet ELISA Kit, R&D Systems) and IL-6R (human IL-6R DuoSet ELISA Kit, R&D Systems) were quantified according to the manufacturer's protocol. For the reaction, the chromogenic reaction substrate BM Blue POD substrate (Roche, Basel, Switzerland) was used and stopped by 5% H_2SO_4 . Subsequently, the measurement was performed by using a SpectraMAX iD3 multimode microplate reader (Molecular Devices, San Jose, USA).

Calcium assay

To measure intracellular calcium levels the fluorescent calcium-binding probe Cal-590 AM (AAT Bioquest, Pleasanton, USA) was used. HUVECs were seeded in black, clear bottom 96 well plates at a density of 10.000 cells/well and cultured for two more days. After 100% confluence was reached, and washed once with HEPES buffer, the cells were stained for 2.5 h at 37°C with the fluorescent dye, washed three times with HEPES buffer, followed by a resting time in fresh Endopan3 medium for 30 min. Subsequently before the injection of the conditioned medium from epithelial cells or Yoda1-containing medium, a baseline of 12.5 s was measured with a SpectraMAX iD3 multimode microplate reader (Molecular Devices, San Jose, USA). For measuring the excitation and emission the wavelengths Ex./Em.= 540/590 nm were used, and the fluorescence was recorded every 0.5 s. After injection, the fluorescence was measured for 60 s as an indicator of the intracellular calcium level.

Western blot

For signaling pathway analysis, HUVECs were seeded in a density of 300 000 cells/well in 6-well plates and were cultured until 100% confluence was reached. Before adding, the conditioned medium, the cells were starved with M199 medium (PAN-Biotech) containing no supplements for 1 h with additional inhibitors for pre-inhibition, if used. After indicated incubation time, the medium was replaced by the conditioned medium and incubated 20–45 min depending on the experimental design and readout. For cell lysis, the cells were washed once with PBS and immediately lysed with 350 µl lysis buffer (50 mM Tris; 137 mM NaCl; 2 mM EDTA; 10 mM 1,10-Phenanthroline; pH 7.5) supplemented with 1x cOmplete™ protease inhibitor (Sigma-Aldrich) and 1 mM Na₃VO₄, 10 mM β-Glycerophosphate, 30 mM NaF) followed by a clearance step using centrifugation (5 min, 16 000 g, 4°C). For denaturation, reducing loading buffer (0.1 M Tris-HCl (pH 6.8), 3% (w/v) SDS, 16% glycerol, 8% β-mercaptoethanol, and 0.01% (w/v) bromophenol blue) was added and samples were heated 20 min at 60°C. SDS-PAGE was used to separate proteins according to their size and transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-FL, Sigma-Aldrich). The membranes were blocked using 3% (w/v) bovine serum albumin (BSA, AppliChem GmbH, Darmstadt, DE) in TBST buffer (50 mM Tris; 150 mM NaCl; 0.1% Tween; pH 7.4) for 20 min at room temperature. Primary antibodies diluted in TBST containing 1% (w/v) BSA were incubated overnight at 4°C. Afterwards, the membranes were washed three times with TBST, followed by the incubation with the secondary antibody for 1 h at room temperature. At least, membranes were washed with TBST and TBS and proteins were detected using the Odyssey 9120 imager system (LI-COR, Lincoln, USA). The band intensities were calculated with the Image studio Lite software version 5.2 (LI-COR, Lincoln, USA) and the biological replicates were normalized by dividing the band intensity of each sample by the total of sample values from the same membrane sum of replicate method [18]. Used antibodies with indicated dilutions: apAKT (1:1000, 4060, Cell Signaling), αAKT pan (1:2000, 2920, Cell Signaling), apERK1/2 (1:1000, AF1018, R&D), αERK1 (1:2000, sc-271269, Santa Cruz), apSTAT3 (1:1000, 9131, Cell Signaling), αSTAT3 (1:1000, 9139, Cell Signaling), apNFκB p65 (1:1000, 3033, Cell Signaling), αNFκB (1:1000, 6956, Cell Signaling), αGAPDH (1:2000, MA5-15738, ThermoFisher), DyLight-680-conjugated mouse (1:10000, 35519, Thermo Scientific), DyLight-800-conjugated rabbit (1:10000, 35571, Thermo Scientific).

qPCR

HUVECs were seeded in 12-well plates at a density of 150 000 cells/well. After 24 h, the cells were stimulated with 500 µl conditioned medium for 1, 2, 4 or 24 h followed by a lysis step and the RNA extraction by the RNeasy Kit (Qiagen, Hilden, DE). The RNA concentration was quantified photometrically using the NanoDrop (Peqlab, Erlangen, DE). 300 ng RNA was reverse transcribed into cDNA by the PrimeScript™ RT Reagent Kit (Takara Bio Europe, St-Germain-en-Laye, FR) according to the manufacturers' protocol. To quantify the mRNA level by qPCR, a 10 µl volume containing 5 µl iTaq Universal SybrGreen Supermix (Bio-Rad, Hercules, USA), 2.9 µl nuclease-free H₂O, 1 µl cDNA template and 0.55 µl forward and reverse primer (500 nM final concentration) was used. The quantified mRNA level was normalized to the mRNA level of the reference gene RPL13A (quantified by reference gene analysis via CFX Maestro Software 2.3 (Bio-Rad)). A list of the used primers and corresponding temperatures can be found in the Suppl. Table 1. The qPCR running protocol included a 5 min denaturation step at 95°C, followed by 41 cycles of 10 s denaturation at 95°C, 10s annealing at the primer-dependent temperature and 15 s amplification at 72°C. The LinRegPCR version 2020.0 software was used, to determine the efficiency of the uncorrected RFU values. For the final quantification served the CFX Maestro Software 2.3 (Bio-Rad).

Leucocyte transmigration

HUVECs were seeded in collagen-coated transwells (6.5 mm insert, 5 µm polycarbonate membrane, Corning, New York, USA) 48 h before starting the stimulation. After 24 h, medium was exchanged. After 48 h, medium in the transwells and the lower compartments was replaced by conditioned medium, or by Endopan3 medium without heparin and hydrocortisone for the negative and positive (IL-8) control, for the following 24 h. Subsequently, the medium in the lower compartments of the controls were replaced by medium containing DMSO or IL-8 (10 ng/ml), and a PMN suspension was added to the transwells. The transmigration process was performed for 1 h and afterwards migrated cells in the lower compartments were counted with a Neubauer chamber.

Statistics

Quantitative data are expressed as mean ± standard deviation (SD) and are based on at least three independent experiments. Statistical analyses were conducted using generalized linear mixed models (PROC GLIMMIX, SAS version 9.4, SAS Institute Inc., Cary, NC, USA), assuming a normal, beta, or lognormal distribution as appropriate. The number of experimental replicates or HUVEC donors was included as a random effect to account for variability in treatment responses across experiments. Model assumptions were evaluated by inspection of residual plots and by the Shapiro–Wilk test to assess homoscedasticity and normality. Degrees of freedom were estimated using the Kenward–Roger approximation. If the assumptions of homoscedasticity were not met, a nonparametric Kruskal–Wallis test was applied. To correct for multiple testing, p values were adjusted using the false discovery rate (FDR) method. A minimum of three donors was included in each endothelial experiment, and p values < 0.05 were considered statistically significant.

Results

ADAM10/17 activity is required to mount a full pro-inflammatory mediator release from lung epithelial cells in response to Piezo1 activation

Piezo1 contributes to the maintenance of pulmonary homeostasis by regulating cellular integrity and coordinating injury induced repair processes [6, 7, 9, 12, 19–22]. Conversely, aberrant Piezo1 activation has been associated with various pulmonary diseases characterized by acute inflammatory responses and the release of pro-inflammatory mediators, including cytokines, chemokines, growth factors and soluble receptors [23]. Many of these mediators are released through ectodomain shedding mediated by the metalloproteases ADAM10 and ADAM17 [14]. In a previous work, we could already link stretch-induced Piezo1-activation in lung epithelial cells to ADAM10 and ADAM17 activation resulting in an increased shedding of the adhesion molecule JAM-A and the growth factors AREG and TGF α [9].

To investigate the specific effects of epithelial Piezo1 activation on the epithelial-capillary interface, we first characterized the secretory response of human alveolar epithelial (hAELVi) cells following pharmacological Piezo1 activation using the selective small-molecule agonist Yoda1. To determine the optimal time point for conditioned medium (CM) collection, hAELVi cells were stimulated with 10 μ M Yoda1 for 5 min, after which the supernatants were harvested after another 2, 4, 8, and 24 h (Fig. 1A).

The concentrations of the pro-inflammatory mediators in the supernatant were quantified by ELISA. We focused on heparin-binding epidermal growth factor-like growth factor (HB-EGF) and soluble interleukin-6 receptor (sIL-6R) since both molecules are highly relevant in mechanically induced epithelial responses and are released from the cell surface via ADAM protease-dependent shedding. We also included interleukin-8 (IL-8) and interleukin-6 (IL-6), as key soluble mediators released without shedding.

sIL-6R, IL-8 and IL-6 were detectable in supernatants but displayed distinct release kinetics. Yoda1-induced sIL-6R release was first significant after 8 h and peaked after 24 h compared with the respective control (Fig. 1B). In contrast, IL-8 secretion significantly increased as early as 2 h after Yoda1 stimulation and continued to rise, reaching maximal levels after 24 h (Fig. 1C). IL-6 exhibited a comparable temporal profile, although significant increases started after 4 h but with lower basal variability, and likewise reached its highest concentration at 24 h (Fig. 1D). Based on these kinetics, CM collected 24 h after stimulation was used for all subsequent experiments.

To determine whether ADAM10 and ADAM17 contribute to Piezo1-induced mediator release, cells were pretreated for 1 h with the dual ADAM10/17 inhibitor GW280264X (GW), followed by a 5-min Yoda1 pulse and a subsequent 24-h incubation with fresh medium in the continuous presence of the inhibitor. As expected, shedding of the established ADAM17 substrates sIL-6R and HB-EGF were almost completely abolished by GW treatment (Fig. 1E, F). Pharmacological inhibition of ADAM10/17 had no effect on Yoda1-induced IL-8 secretion (Fig. 1G). In contrast, Yoda1-induced IL-6 release was also reduced (Fig. 1H), although IL-6 is a soluble mediator and not a direct ADAM10/17 substrate. Thus, ADAM10 and

ADAM17 may contribute to a full inflammatory response not only through direct shedding of membrane-bound substrates but also through indirect regulation of soluble mediators such as IL-6.

To assess whether these observations were cell line-specific, the experiments were repeated in the human lung adenocarcinoma epithelial cell line H441. In these cells, basal IL-8 and sIL-6R levels were already comparable to the Yoda1-induced levels observed in hAELVi cells and no further increase following Piezo1 activation could be detected. Nevertheless, Yoda1 significantly enhanced IL-6 release in H441 cells, and both IL-6 and sIL-6R secretion remained sensitive to ADAM10/17 inhibition (Supplementary Fig. 1A–C), indicating that ADAM-dependent regulation of these mediators is conserved despite differences in basal secretory activity among the two cell lines.

To obtain a broader overview of the epithelial secretome following Piezo1 activation, supernatants from hAELVi cells stimulated with Yoda1 in the presence or absence of GW were analyzed using a Proteome Profiler Array (Fig. 1I). Piezo1 activation markedly increased the abundance of numerous proteins implicated in inflammatory and immune responses. In addition to IL-6 and IL-8, elevated levels of granulocyte-macrophage colony-stimulating factor (GM-CSF), monocyte chemoattractant protein-1 (MCP-1/CCL2), extracellular matrix metalloproteinase inducer (EMMPRIN/CD147/Basigin), ST2 (IL1RL1), IL-17A, macrophage migration inhibitory factor (MIF), endoglin (CD105), and transforming growth factor α (TGF α) were detected (Fig. 1I, J; Supplementary Fig. 1D). Several of these mediators have previously been implicated in acute inflammatory responses, tissue injury and pulmonary endothelial activation and dysfunction [24–31]. Furthermore, Piezo1 activation increased the release of multiple mediators involved in immune cell recruitment and endothelial barrier regulation, including growth-regulated oncogene- α (GRO α /CXCL1), macrophage inflammatory protein-3 α (MIP-3 α /CCL20), and RANTES (regulated and normal T cell expressed and secreted/CCL5) and urokinase plasminogen activator receptor (uPAR) [32–35] (Fig. 1I, J; Supplementary Fig. 1D). The dependence on ADAM10/17 varied substantially among individual mediators. As expected, the Yoda1-induced release of described transmembrane ADAM substrates as EGF, TGF α , CD30, transferrin receptor (TfR) or EMMPRIN was markedly reduced by GW treatment [14, 36, 37]. Moreover, also the secretion of soluble mediators such as IL-6, ST2, IL-17A, GRO α , MIP-3 α , and MIF was likewise reduced indicating a major indirect contribution of ADAM proteases to the Piezo1-driven inflammatory secretory response (Fig. 1I, J; Supplementary Fig. 1D, E). Collectively, these findings demonstrate that activation of Piezo1 elicits a broad pro-inflammatory epithelial secretome that is partially dependent on ADAM10/17 activity and exhibits a strong paracrine signaling capacity, with the potential to modulate neighboring endothelial cells and promote inflammatory responses within the lung microenvironment.

Epithelial-derived factors released upon Piezo1 activation induce the expression of leukocyte transmigration-associated genes in endothelial cells

To investigate the paracrine signaling potential of epithelial Piezo1 activation toward the vascular endothelium, CM derived from Yoda1-stimulated lung epithelial cells was transferred to cultured human umbilical vein endothelial cells (HUVECs). Since several epithelial mediators identified in the previous

experiments (Fig. 1) exhibited ADAM-dependent release, we further examined whether these mediators lead to a response in the endothelium and if the investigated proteases contribute to the transmission of Piezo1-induced signals from epithelial to endothelial cells.

To exclude the possibility that residual Yoda1 contained within the CM directly activates endothelial Piezo1, epithelial cells were subjected to a 5-min Yoda1 pulse followed by extensive washing before fresh medium was added for the subsequent 24-h incubating period (Fig. 2A). The absence of residual Piezo1 agonist in the CM was verified using intracellular calcium measurements in HUVECs directly after stimulation with CM. Medium containing Yoda1 and fresh culture medium served as positive and negative controls, respectively. Conditioned medium derived from Yoda1-treated H441 cells (Fig. 2B, C) as well as hAELVi cells (Fig. 2D, E) failed to induce intracellular calcium influx compared with direct Yoda1 stimulation, indicating that the CM itself does not activate Piezo1 in endothelial cells. Notably, even Yoda1 concentrations 100-fold lower than those used for epithelial stimulation elicited substantially stronger calcium responses than the conditioned media (Fig. 2B, C), making it highly unlikely that the observed endothelial effects result from residual agonist.

Having excluded direct Piezo1 activation, CM was subsequently used to assess paracrine transcriptional responses in endothelial cells. To determine whether ADAM-dependent epithelial mediator release contributes to endothelial activation, both epithelial cells and HUVECs were pretreated with the dual ADAM10/17 inhibitor GW prior to stimulation. Following the Yoda1 pulse, CM was generated in the continuous presence of the inhibitor, which was likewise maintained throughout endothelial stimulation to ensure persistent ADAM10/17 inhibition during both phases of the experimental setup (Fig. 2A). The endothelial response was assessed by quantifying the mRNA expression for proteins involved in leukocyte recruitment and inflammatory activation by qPCR. These included the adhesion molecules intercellular adhesion molecule 1 (ICAM1), vascular cell adhesion molecule 1 (VCAM1), and E-selectin (SELE), as well as MCP1, all of which play central roles in cytokine-induced leukocyte adhesion and transmigration. In addition, the inflammatory cytokines IL-6, IL-8, and IL-1 β were analyzed as established markers of inflammatory activation [38–41]. Conditioned medium derived from Piezo1-activated H441 cells markedly increased the expression of endothelial adhesion molecules as well as cytokine and chemokine genes (Fig. 2F–L). A comparable transcriptional response was observed following stimulation with CM from the more physiologically relevant hAELVi cell line (Fig. 2M–S), demonstrating that epithelial Piezo1 activation induces robust paracrine communication with neighboring endothelial cells across both epithelial models. Despite the similar qualitative response, substantial quantitative differences were observed between the two epithelial cell lines. CM from H441 cells induced markedly higher basal expression levels of all analyzed genes (Fig. 2F–L) than CM derived from hAELVi cells (Fig. 2M–S), consistent with the intrinsically more inflammatory phenotype of the adenocarcinoma-derived epithelial cell line.

Interestingly, the contribution of ADAM-dependent signaling differed considerably between both epithelial models. In H441-derived CM, ADAM10/17 inhibition selectively prevented the induction of *VCAM1* and *IL1B* (Fig. 2G, K), whereas the expression of the other tested genes was largely unaffected

(Fig. 2F–L). In contrast, inhibition of ADAM10/17 during generation of hAELVi-derived CM almost completely abolished the induction of all analyzed adhesion molecules and *MCP1*, while leaving the transcriptional regulation of *IL6*, *CXCL8* (IL-8), and *IL1B* largely unchanged (Fig. 2M–S). These findings suggest that epithelial Piezo1 activation induces both ADAM-dependent and ADAM-independent paracrine signaling pathways and further indicate that the composition of the epithelial secretome, as well as the downstream signaling mechanisms activated in endothelial cells, differs between transformed H441 cells and the more physiological hAELVi model.

Piezo1-activated epithelial cells release mediators inducing inflammatory signaling pathways in endothelial cells

The pronounced transcriptional response of endothelial cells following stimulation with CM from Piezo1-activated epithelial cells suggests that soluble epithelial-derived mediators activate intracellular signaling pathways in the endothelium. Numerous pro-inflammatory cytokines, chemokines, and growth factors, including IL-6, IL-1 β , TNF α , IL-8, MCP1, VEGF, and EGF, are known to activate signaling cascades such as PI3K/AKT, MAPK/ERK and JAK/STAT3 through their respective receptors [23, 42, 43]. We therefore investigated whether these pathways are activated in endothelial cells in response to the Piezo1-induced epithelial secretome.

Stimulation of HUVECs with CM derived from Yoda1-treated H441 cells (CM(Y)) (Fig. 2A) resulted in a pronounced increase in AKT phosphorylation compared with control CM (CM(Ctrl)) (Fig. 3A, B). Inhibition of ADAM10/17 during CM generation only moderately attenuated AKT phosphorylation, indicating that AKT signaling is only partially dependent on ADAM-mediated epithelial factor release. ERK phosphorylation was likewise increased following exposure to CM from Piezo1-activated H441 cells but was markedly reduced in the presence of the ADAM10/17 inhibitor GW, suggesting a stronger contribution of ADAM-dependent mediators to ERK phosphorylation (Supplementary Fig. 2A–C). STAT3 signaling pathway activation displayed a distinct response pattern. Even CM derived from untreated H441 cells induced robust STAT3 phosphorylation, reaching levels comparable to those observed following stimulation with hyper-IL-6 (hIL6), a fusion protein consisting of IL-6 and soluble IL-6R that potently activates gp130-dependent JAK/STAT3 signaling [44, 45]. CM from Yoda1-treated epithelial cells further enhanced STAT3 phosphorylation by nearly two-fold. Importantly, inhibition of ADAM10/17 almost completely prevented this additional Piezo1-induced STAT3 pathway activation, demonstrating that ADAM activity in H441 cells is a major determinant for crosstalk towards endothelial STAT3 signaling (Fig. 3C, D).

To compare these findings with the more physiological hAELVi model, phosphorylation kinetics of AKT, ERK, and STAT3 were initially analyzed following stimulation with CM derived from control- and Yoda1-treated hAELVi cells. Based on these experiments, 45 min was selected as the common endpoint for all subsequent analyses (Supplementary Fig. 2D–J). Conditioned medium derived from hAELVi cells

induced a signaling profile closely resembling that observed with H441-derived CM, although differences in signaling intensity and ADAM dependency became apparent. Phosphorylation of AKT, ERK, and STAT3 was consistently increased following stimulation with CM from Piezo1-activated hAELVi cells (Fig. 3E–I). While ERK phosphorylation was quantitatively more pronounced than in H441-CM stimulated endothelial cells, its inhibition by GW was comparatively less effective, indicating a lower dependence of the ERK signaling cascade on ADAM10/17-mediated mediator release in this epithelial model.

Since NFκB represents another central signaling pathway controlling inflammatory gene expression, its activation was analyzed in parallel. Indeed, CM derived from Piezo1-activated hAELVi cells significantly increased NFκB phosphorylation in HUVECs. Similar to STAT3 pathway activation, this response was markedly attenuated by ADAM10/17 inhibition, indicating that ADAM-dependent released epithelial mediators substantially contribute to NFκB activation in endothelial cells (Fig. 3J, K).

Collectively, these findings demonstrate that Piezo1 activation in lung epithelial cells induces the release of soluble mediators capable of activating multiple pro-inflammatory signaling pathways in neighboring endothelial cells. Activation of the PI3K/AKT, ERK, STAT3, and NFκB pathways is partially mediated by ADAM-dependent epithelial factor release, identifying these signaling cascades as potential drivers of the transcriptional endothelial response observed before.

PI3K, JAK, and NFκB signaling mediate the endothelial transcriptional response induced by epithelial Piezo1 activation

To identify the signaling pathways responsible for the transcriptional activation of endothelial cells induced by CM from Piezo1-activated epithelial cells, pharmacological inhibitors targeting PI3K (LY294002 (LY)), JAK1/2 (Baricitinib), ERK1/2 (SCH772984 (SCH)), NFκB (BAY11-7082 (BAY)), and AP-1 (SR11302 (SR)) were employed (Fig. 4A). To confirm inhibitor specificity, PI3K/AKT and JAK/STAT3 pathway activation was first assessed by Western blot analysis following stimulation of HUVECs with CM derived from H441 cells. Inhibition of PI3K completely abolished AKT phosphorylation without affecting either STAT3 or ERK phosphorylation (Supplementary Fig. 3A–E). Conversely, JAK inhibition selectively prevented STAT3 phosphorylation while leaving AKT and ERK phosphorylation unchanged, confirming effective and pathway-specific inhibition under the experimental conditions (Supplementary Fig. 3A–E).

To determine the functional contribution of these signaling pathways to endothelial activation, the mRNA expression of inflammatory and leukocyte transmigration-associated genes was analyzed following pathway inhibition. PI3K inhibition did not significantly affect *ICAM1* expression (Fig. 4B) but markedly reduced the induction of *VCAM1*, *SELE*, *CXCL8*, *IL1B*, and *MCP1* following stimulation with H441-derived CM (Fig. 4C–G). Notably, LY also reduced basal expression levels of several genes, indicating that PI3K signaling contributes to transcriptional activity induced by both constitutive and Piezo1-induced epithelial-derived mediators. Combined inhibition of PI3K and JAK produced the strongest suppression

of *ICAM1* and *CXCL8* expression (Fig. 4B, E), suggesting cooperative regulation through both signaling pathways. Among all genes analyzed, *MCP1* displayed the most pronounced dependence on JAK/STAT3 signaling, as Baricitinib significantly reduced its expression in addition to the effects of PI3K inhibition (Fig. 4G). These findings identify especially *MCP1* as a downstream target integrating signals from both PI3K/AKT and JAK/STAT3 pathways. To determine whether these observations extend to the more physiological epithelial model, selected target genes were analyzed following stimulation with CM derived from hAELVi cells. Expression of *SELE*, *IL1B*, and *MCP1* exhibited a response pattern comparable to that observed with H441-derived CM (Fig. 4H–J). All three genes were strongly dependent on PI3K signaling, whereas inhibition of JAK signaling predominantly affected *SELE* and *MCP1* expression, indicating that both pathways contribute to the endothelial response elicited by Piezo1-activated alveolar epithelial cells. Since ERK phosphorylation was consistently induced by epithelial CM (Fig. 3F, I, J; Supplementary Fig. 2A–C), we next investigated whether ERK signaling contributes to the observed transcriptional responses. Surprisingly, pharmacological inhibition of ERK had little or no inhibitory effect on gene expression and, in several cases, even resulted in a tendency toward increased transcript levels (Fig. 4K–Q), suggesting that ERK activation is largely dispensable for the induction of these inflammatory genes.

In contrast, inhibition of NFκB and AP-1 with BAY and SR substantially altered the endothelial transcriptional response. Both pathways contributed predominantly to the induction of the adhesion molecules *ICAM1* and *VCAM1* (Fig. 4K, L), whereas NFκB inhibition additionally markedly reduced the expression of *SELE*, *IL6*, and *MCP1* (Fig. 4M, N, Q). Conversely, *IL1B* expression remained largely unaffected by both inhibitors, while *CXCL8* expression was even unexpectedly increased following NFκB inhibition (Fig. 4O, P), suggesting differential regulatory mechanisms governing individual inflammatory genes.

Given the appearance of growth factors within the epithelial secretome (Fig. 1I, J), we further examined the contribution of ErbB receptor signaling using the pan-ErbB inhibitors Afatinib (Afa) and Dacomitinib (Daco). Both inhibitors substantially reduced AKT phosphorylation, whereas ERK activation was only minimally affected (Supplementary Fig. 3F–H), indicating that activation of PI3K/AKT is at least partially mediated through EGFR/HER family receptors. Although the inhibition of ErbB signaling had effects on phosphorylation patterns, downstream gene expression was only modestly affected. While *SELE*, *CXCL8*, and *IL6* displayed a tendency toward reduced expression, the induction of *ICAM1*, *VCAM1*, *IL1B*, and *MCP1* was largely unaffected or showed inconsistent responses (Supplementary Fig. 3I–O).

Collectively, these findings identify PI3K/AKT, JAK/STAT3, and NFκB as the principal signaling pathways mediating the endothelial transcriptional response to epithelial Piezo1 activation. In contrast, ERK signaling appears to play only a minor role in regulating inflammatory gene expression despite its robust phosphorylation, whereas growth factor receptor signaling contributes primarily to PI3K/AKT activation rather than directly controlling downstream transcriptional responses.

Epithelial-derived mediators promote transendothelial leukocyte migration

The induction of pro-inflammatory signaling pathways together with the increased endothelial expression of adhesion molecules, cytokines, and chemokines suggested that epithelial Piezo1 activation functionally modulates endothelial cells in a manner that may facilitate leukocyte recruitment and transendothelial migration. To directly investigate this hypothesis, leukocyte transmigration was analyzed using a transwell co-culture system, allowing the separate assessment of endothelial activation and leukocyte migration across an endothelial monolayer (Fig. 5A, B).

To initially assess the functional consequences of the epithelial secretome, CM derived from H441 cells was applied to confluent HUVEC monolayers for 24 h prior to the transmigration start. Freshly isolated human polymorphonuclear neutrophils (PMNs), further referred to as leukocytes, were subsequently allowed to migrate across the endothelial layer toward the CM for 1 h. Fresh culture medium and recombinant IL-8 served as negative and positive controls, respectively. As expected, recombinant IL-8 significantly increased leukocyte migration, confirming the functionality of the experimental system (Fig. 5C). Since IL-8 primarily acts as a potent chemoattractant, this positive control mainly reflects directed leukocyte chemotaxis rather than endothelial activation. Interestingly, CM derived from untreated H441 cells (CM(Ctrl)) already increased leukocyte transmigration to levels comparable to those observed with recombinant IL-8, indicating that constitutively released epithelial factors possess substantial pro-migratory activity. Conditioned medium from Yoda1-treatment further enhanced leukocyte transmigration, consistent with the increased endothelial activation observed before and the transcriptional induction of multiple adhesion molecules and inflammatory mediators known to facilitate leukocyte extravasation.

To further dissect the underlying mechanisms, CM derived from hAELVi cells was used to distinguish between the contributions of epithelial chemoattractants and endothelial activation (Fig. 5D). Continuous stimulation of endothelial cells with Yoda1-derived CM throughout the entire experiment (24 h preincubation followed by 1 h during transmigration (25 h CM(Y))) resulted in a marked increase in leukocyte migration compared with control CM. Replacing the CM immediately before the migration phase with the same but unused CM from Yoda1-treatment (24 h + 1 h CM(Y)) slightly reduced transmigration, suggesting that endothelial-derived mediators released during the preincubation period further contribute to obtain optimal leukocyte recruitment. Conversely, when endothelial cells were maintained in fresh medium during the initial 24-h incubation and exposed to CM only during the 1-h migration period (24 h Med. + 1 h CM(Y)), leukocyte transmigration was markedly reduced despite the continued presence of epithelial chemoattractants. Under these conditions, migration was comparable to that observed with control CM, indicating that short-term exposure to epithelial factors is insufficient to induce a fully activated endothelial phenotype. In contrast, after endothelial pre-activation with CM(Y) for 24 h and subsequent replacement of the CM with fresh medium during the 1-h migration phase (24 h CM(Y) + 1 h Med.) leukocyte transmigration was abolished, despite endothelial stimulation (Fig. 5D).

Together, these findings demonstrate that efficient leukocyte transmigration requires both sustained endothelial activation and the continuous presence of epithelial-derived chemoattractants. While epithelial mediators appear to provide the dominant chemotactic gradient directing leukocyte migration, endothelial activation is essential for establishing a permissive endothelial barrier and optimal transmigration.

To determine whether ADAM-dependent epithelial signaling contributes to this functional response, the dual ADAM10/17 inhibitor GW was added to epithelial cells during generation of conditioned media and remained present during the experiment. Inhibition of ADAM10/17 almost completely abolished the Piezo1-induced increase in leukocyte transmigration, reducing migration to levels comparable to the control condition (Fig. 5E). These findings identify ADAM activity as a central regulator of the epithelial-endothelial crosstalk that promotes leukocyte recruitment.

Finally, the signaling pathways identified in previous experiments (Fig. 4) were examined for their contribution to the functional transmigration response. Surprisingly, despite its pronounced role in regulating inflammatory gene expression, treatment of endothelial cells with the PI3K inhibitor LY294002 (LY) had no detectable effect on leukocyte transmigration (Fig. 5F), indicating that PI3K-dependent transcription alone is insufficient to drive this complex functional process. In contrast, inhibition of JAK1/2 with Baricitinib significantly reduced leukocyte migration under both basal and Piezo1-stimulated conditions (Fig. 5F), demonstrating an essential role for JAK/STAT signaling in endothelial activation. Likewise, inhibition of NFκB with BAY11-7082 (BAY) significantly attenuated transmigration, reducing leukocyte passage across the endothelial monolayer by approximately one-third compared with fully activated endothelial cells (Fig. 5G). Together, these findings identify ADAM10/17-dependent epithelial signaling and downstream JAK/STAT and NFκB activation as key regulators of Piezo1-induced leukocyte transmigration across the endothelial barrier.

Collectively, our data indicate that epithelial Piezo1 functions as a central upstream regulator of inflammatory epithelial-endothelial crosstalk. Through the ADAM10/17-dependent release of soluble mediators, Piezo1 activation orchestrates endothelial signaling and inflammatory gene expression, ultimately establishing a microenvironment that promotes leukocyte recruitment and transendothelial migration.

Discussion

In the present study, we provide a stepwise mechanistic characterization of a signaling cascade linking epithelial Piezo1 activation to inflammatory endothelial responses in the lung. Specifically, we demonstrate that Piezo1 activation induces ADAM-dependent mediator release, which promotes endothelial signaling pathway activation, transcriptional reprogramming and ultimately enhanced leukocyte transmigration across the endothelial barrier. These findings identify a previously unrecognized epithelial-endothelial signaling axis that may play a critical role in driving inflammatory processes during mechanically induced pulmonary injury.

Previous studies have predominantly investigated Piezo1 either in isolated cell types or within complex *in vivo* models, making it difficult to resolve sequential signaling events in the pulmonary tissue. To dissect the individual steps of this signaling cascade, we chose a stepwise experimental approach in which the use of conditioned medium allowed us to separately investigate epithelial and endothelial events. By first characterizing the epithelial secretome, followed by analyses of endothelial signaling and gene expression prior to assessment of leukocyte transmigration, we were able to temporally dissect the sequence of events linking epithelial Piezo1 activation to endothelial dysfunction. Such mechanistic resolution would have been considerably more challenging in animal experiments or direct co-culture systems, where the cellular origin of detected mediators and signaling events is often difficult to distinguish.

A similar mechanistic strategy was applied to the activation of Piezo1 itself. Rather than using mechanical stimulation, we employed the selective Piezo1 agonist Yoda1 to specifically activate the channel. Although multiple mechanosensors as epithelial sodium channels (ENaC), transient receptor potential vanilloid (TRPV4) or two-pore domain potassium (K2P) channels contribute to pulmonary mechanotransduction [19, 46–48], Piezo1 has emerged as one of the major mechanosensitive ion channels regulating cellular responses to mechanical stress. Pharmacological activation therefore allowed us to specifically investigate Piezo1-dependent signaling while minimizing confounding effects arising from the simultaneous activation of numerous mechanosensitive pathways, cytoskeletal changes and membrane remodeling during mechanical stimulation.

Among the downstream effectors proposed to mediate Piezo1 signaling, ADAM10 and 17 have recently gained considerable attention. Although the precise molecular mechanisms linking Piezo1 activation to ADAM activation remain incompletely understood, Piezo1-mediated Ca^{2+} influx provides a plausible trigger, as ADAM10 is Ca^{2+} -dependently activatable, whereas ADAM17 can be activated indirectly through Ca^{2+} using protein kinase C [49–51]. Additional evidence suggests that membrane organization and phospholipid asymmetry also regulate ADAM activity, raising the possibility that local membrane deformation and altered local lipid environment following Piezo1 activation further facilitates ADAM activation [52–56]. Previous studies have already demonstrated Piezo1-dependent ADAM-mediated shedding of substrates such as AREG, JAM-A, E-cadherin, VE-cadherin, TNFR1, and Notch1, highlighting their important role in autocrine and paracrine signaling, involving tissue remodeling and inflammatory signaling [9, 15, 16]. Additionally, studies with acute lung injury (ALI) or acute respiratory distress syndrome (ARDS) models have established ADAM17 as a key factor in the process of vascular permeability, oedema formation, cytokine release and pulmonary leukocyte recruitment proven by the use of GW or *Adam17* knockout after endotoxin challenge [17, 57, 58]. Our findings complement these observations by identifying epithelial Piezo1 activation as one upstream mechanism capable of initiating this ADAM-dependent inflammatory response by releasing a broad secretome. This secretome not only includes shed variants of established ADAM substrates on the cell surface such as HB-EGF, TGF α , EMMPRIN, CD30, ICAM1, TfR, and IL6R but also other shed membrane-associated proteins, including endoglin and uPAR [59–63], as well as additional soluble pro-inflammatory mediators like GRO α , IL-6, and ST2. While the surface molecules can be directly released through ADAM-mediated ectodomain

shedding, the secretion of other soluble mediators is more likely an indirect consequence of ADAM activity. For non-substrate mediators, indirect transcriptional regulation is a likely explanation. One possible mechanism is the induction of positive feedback loops initiated by ADAM-dependent shedding of soluble IL-6R or HB-EGF [64–66]. Subsequent activation of gp130- or EGFR-dependent signaling could further enhance transcription of secondary inflammatory mediators.

Additional evidence for the concept that Piezo1-induced inflammation is mediated through multiple signaling modules is provided by the observation that STAT3 phosphorylation was almost completely abolished following ADAM10/17 inhibition. This is consistent with the central role of IL-6/IL-6R trans-signaling in activating the JAK/STAT pathway by binding to gp130 [67, 68], since both mediators were ADAM-dependently released. In contrast, PI3K/AKT activation was only partially reduced, suggesting that this pathway integrates signals from numerous cytokine, chemokine and growth factor receptors [69], only a subset of which depends on ADAM-mediated shedding. This was also shown by the effect of ErbB family inhibition, resulting in a slight pAKT decrease. A similar principle likely applies to ERK signaling, which can likewise be activated by a broad spectrum of receptor systems [70]. The stronger sensitivity of this endothelial signaling pathway to ADAM inhibition, particularly in transfer experiments with H441-derived conditioned medium, could point towards a greater contribution of ADAM-dependent EGFR ligands such as HB-EGF or TGF α to MAPK activation [71]. However, involvement of this EGFR signaling was not confirmed by using ErbB family inhibitors, suggesting that other pathways were more critical for ADAM-dependent ERK activation. Interestingly, despite its pronounced dependence on ADAM10/17 activity, pharmacological inhibition of ERK neither altered endothelial gene expression nor affected leukocyte transmigration. These findings suggest that the functional response is not determined by individual signaling pathways activated by selected growth factors or kinases, but rather by the coordinated activity of a Piezo1-induced inflammatory signaling network in which multiple pathways cooperate to drive inflammation.

This hypothesis is further supported when comparing the transcriptional regulation with the functional transmigration process. Despite markedly reducing the expression of several inflammatory genes, PI3K inhibition had little effect on leukocyte transmigration, whereas ADAM10/17 inhibition almost completely prevented this process. These findings indicate that PI3K-dependent transcription alone is insufficient to drive endothelial activation and leukocyte transmigration. Several explanations may account for these findings. First, the remaining expression of key adhesion molecules, such as ICAM1 [72], could be sufficient to sustain endothelial activation. Alternatively, successful transmigration may depend more critically on junctional remodeling, involving ADAM10/17-dependent regulated proteins such as JAM-A, VE-cadherin or PECAM1, whose regulation was not investigated in the present study and may occur independently of PI3K signaling. Finally, whereas JAK/STAT and NF κ B signaling exerted comparatively smaller effects on transcriptional regulation, they may partially compensate for PI3K inhibition and thereby preserve the functional transmigration response. These findings indicate that transcriptional activation and functional endothelial activation are not necessarily equivalent biological endpoints.

Although PI3K has previously been described as an upstream regulator of NF κ B signaling [73], our data do not support a simple linear relationship between these pathways. Instead, the distinct effects of PI3K and NF κ B inhibition on both transcriptional responses and leukocyte transmigration suggest that these signaling cascades contribute through at least partially independent mechanisms within the complex endothelial response to epithelial Piezo1 activation.

Interestingly, despite clear quantitative differences between the two used epithelial cell models (H441 and hAELVi), the overall signaling architecture remained remarkably consistent. The stronger basal inflammatory signaling and higher endothelial gene expression resulting from H441 cells most likely reflects their phenotype as tumor-derived cell line. Epithelial tumor cells frequently exhibit constitutively elevated cytokine production and growth factor signaling, thereby generating a more pro-inflammatory secretome even under basal conditions. Importantly, the consistency in inducing the same sequence of events supports that the identified Piezo1–ADAM signaling axis represents a conserved epithelial-endothelial communication mechanism whose magnitude is influenced by the inflammatory state of the epithelial cells.

While these findings provide mechanistic insight into the intracellular signaling events underlying endothelial activation, they do not fully explain how these molecular responses translate into the functional increase in leukocyte transmigration observed in our study. Our transmigration experiments indicate that epithelial Piezo1 activation promotes leukocyte recruitment through two complementary mechanisms. On the one hand, the epithelial secretome establishes a chemotactic gradient that directs leukocytes towards the site of inflammation. On the other hand, we could show that sustained endothelial activation is also required most likely by inducing the expression and surface presentation of adhesion molecules, including ICAM1, VCAM1 and E-selectin, thereby facilitating leukocyte adhesion and generating a permissive endothelial barrier that supports efficient transmigration.

The importance of chemotactic guidance is reflected in the composition of the epithelial secretome. Besides IL-8, increased release of GRO α , MIP-3 α , MCP1, and RANTES was detected following Piezo1 activation. Among these mediators, IL-8 and GRO α are well-established leukocyte chemoattractants [74] and are therefore likely to represent the major drivers of directing the increased leukocyte migration observed in our study. However, considering the complexity of the epithelial secretome it is more likely that the combined action of several chemoattractants probably generates the robust chemotactic gradient directing leukocyte recruitment. Nevertheless, our data further demonstrate that chemotactic guidance alone is insufficient to explain the observed increase in leukocyte transmigration. Prolonged endothelial stimulation with the epithelial secretome, leading to a pro-inflammatory activation appears to be equally important for establishing a permissive endothelial phenotype. Thus, our findings indicate that ADAM-mediated epithelial signaling represents a central mechanism linking epithelial mechanotransduction to leukocyte recruitment across the alveolar-capillary barrier.

Beyond acting as a passive recipient of epithelial-derived signals, the endothelium itself may also actively contribute to amplifying the inflammatory response. Following stimulation with the epithelial

secretome, endothelial cells exhibited increased expression of *IL6*, *CXCL8*, *IL1B*, and *MCP1*, indicating that the endothelium itself may become a secondary source of inflammatory mediators. This secondary cytokine production could contribute to a positive feedback mechanism that reinforces leukocyte recruitment and sustains inflammation.

However, these signaling pathways investigated in this study may not fully explain the broad endothelial response to the epithelial secretome. Based in their established roles in endothelial activation, junctional remodeling and leukocyte transmigration, p38 MAPK, Src family kinases and RhoA/ROCK [75–78] signaling represents additional plausible candidates for future investigations. Besides the signaling pathways investigated in the present study, further downstream effectors of Piezo1 may contribute to the inflammatory epithelial response. Recent evidence demonstrated that epithelial Piezo1 promotes ventilator-induced lung injury (VILI) through activation of the calcineurin/NFATc3 pathway [79], further supporting the concept that Piezo1 orchestrates inflammatory responses through multiple parallel signaling modules, ADAM-dependent, as well as ADAM-independent. Whether crosstalk exists between calcineurin/NFAT signaling and the ADAM-dependent secretome identified in our study remains to be determined.

Despite providing detailed mechanistic insight into epithelial-endothelial communication following Piezo1 activation, several new questions remain to be addressed in further studies. First, we focused on Piezo1 by pharmacologically activating the ion channel using the selective agonist Yoda1. The pharmacological activation does not fully recapitulate the complex cellular responses induced by physiological or pathological mechanical forces which can include other mechanotransducers. Future studies employing cyclic stretch or VILI models in combination with pharmacological inhibition or genetic silencing of Piezo1 will therefore be important to validate the pathophysiological relevance of the signaling axis identified here. Second, Piezo1 activation was restricted to the epithelial compartment, whereas both epithelial and endothelial cells experience mechanical forces *in vivo*. Endothelial Piezo1 has previously been implicated in vascular barrier regulation and inflammatory signaling [8, 72, 80], suggesting that simultaneous activation of Piezo1 in both cellular compartments may further influence epithelial-endothelial communication during pulmonary injury. The present experimental design was intentionally chosen to isolate the contribution of epithelial Piezo1 signaling. However, more complex co-culture models combining mechanical stimulation of both barrier-forming cell types will be required to more closely reflect the physiological situation. Finally, we used a dual ADAM10/17 inhibitor to block most of the known shedding events. In further studies it could be of interest to identify the contribution of epithelial and endothelial ADAMs or to distinguish between the individual activity of ADAM10 and ADAM17. Cell type-specific knockdown or knockout approaches targeting ADAM10 and ADAM17 individually will therefore be essential to further dissect the cellular origin and mechanistic contribution of ADAM-dependent signaling within the epithelial-endothelial crosstalk identified in the present study. Although validation in mechanically stimulated and more physiologically complex models will be required, the mechanistic insights obtained from the present stepwise approach provide a conceptual framework for understanding how epithelial mechanotransduction may initiate inflammatory signaling across the alveolar-capillary barrier in an Piezo1- and ADAM-dependent manner.

Collectively, our study identifies a previously uncharacterized epithelial-endothelial signaling axis linking Piezo1 activation to ADAM-dependent inflammation of the alveolar-capillary barrier. By systematically dissecting the individual steps of this signaling cascade, we demonstrate that epithelial Piezo1 activation induces an ADAM-dependent inflammatory secretome that activates endothelial signaling, promotes inflammatory gene expression and ultimately enhances leukocyte transmigration. Given the central role of excessive mechanical stress in conditions such as VILI, ALI and ARDS, the identified Piezo1–ADAM signaling axis represent a promising therapeutic target. The accessibility of the lung provides an ideal basis for the local administration of specific inhibitors, thereby minimizing systemic side effects. This could help to reduce excessive endothelial activation and leukocyte recruitment, thereby preserving alveolar-capillary barrier function during inflammatory lung injury.

Declarations

Ethics approval and consent to participate

Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords of cesarean sections and polymorphonuclear neutrophils were isolated from blood of healthy volunteers, covered by the ethics approval EK241/18 from the local ethics committee.

Consent for publication

Not applicable.

Competing interest

The authors declare that they have no competing interests.

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Author Contribution

A.P., A.L. and A.B. designed the study. S.B. and L.K.R. provided methodology. A.P. and N.B. performed the experiments. A.P. and A.B. analyzed the data. A.P. performed the statistical analysis. A.P., A.L. and A.B. wrote the manuscript. All the authors have read and agreed to the entire manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information file.

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Figures

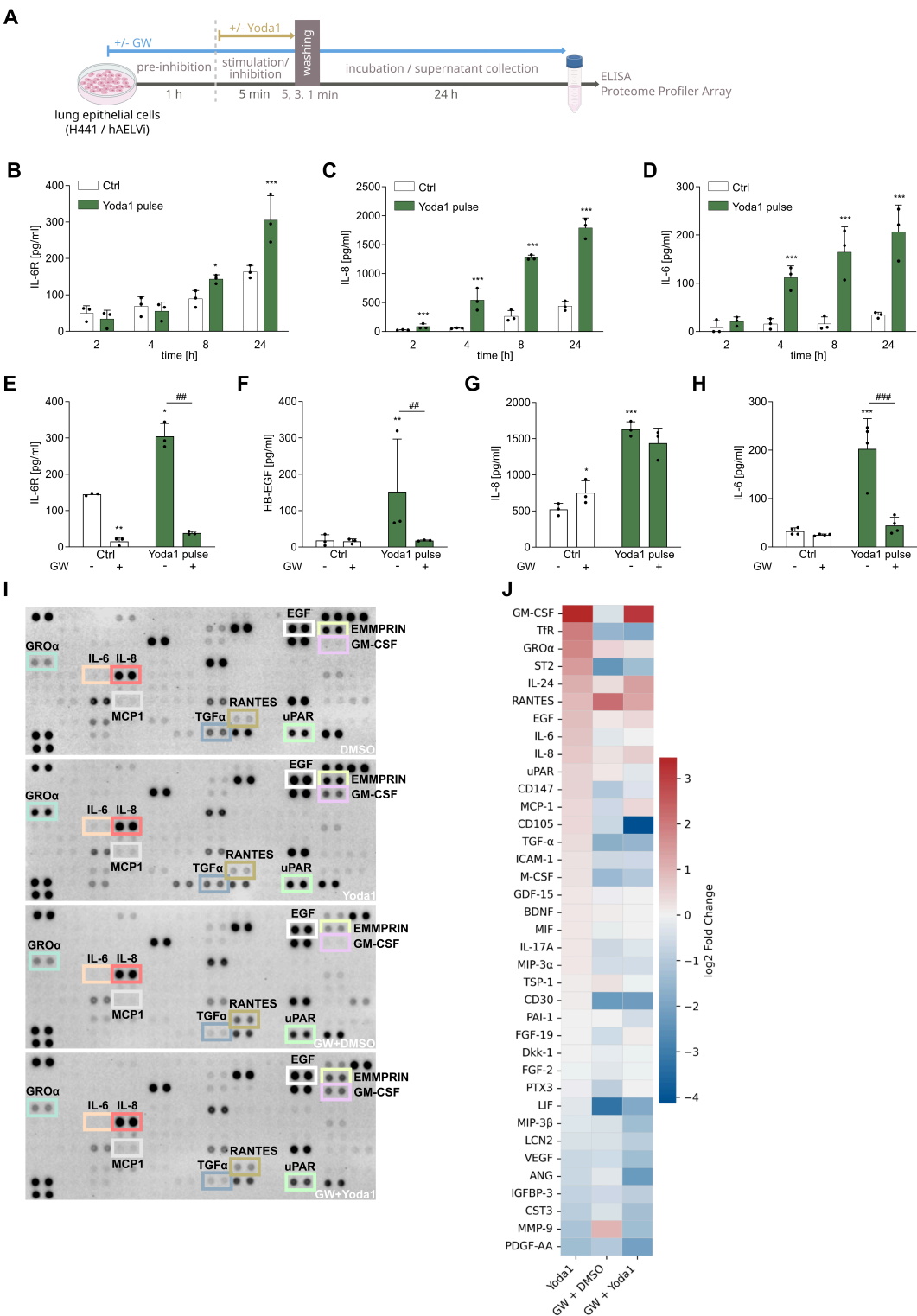


Figure 1

ADAM10/17 dependency on the Piezo1-induced release of pro-inflammatory mediators from hAELVi cells

Schematic overview of the experimental setup to stimulate epithelial cells for generating conditioned medium **(A)**. Supernatants from hAELVi cells treated with 10 μ M Yoda1 for 5 min (Yoda1 pulse) or control reagent were collected after 2, 4, 8 and 24 h and analyzed for IL-8 **(B)**, IL-6 **(C)** and sIL-6R **(D)** concentrations via ELISA. For ADAM10/17 inhibition, hAELVi cells were pre-incubated with GW280264X (GW, 10 μ M) or control reagent for 1 h, followed by a 5 min stimulation with Yoda1 or control reagent. Cells were subsequently incubated for 24 h in fresh medium containing either vehicle or GW (10 μ M), after which supernatants were collected and analyzed for IL-8 **(E)**, IL-6 **(F)**, sIL-6R **(G)** and HB-EGF **(H)** concentrations via ELISA. Supernatants as generated for **E-H** were analyzed using the human Cytokine XL Array Kit **(I)**, and $\log_2$ fold change were calculated and visualized as a heat map **(J)**. Quantitative data are shown as the means + SDs of at least three independent experiments. Statistical differences to the control are indicated by asterisks (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$), whereas differences between the treatments are indicated by hashes (# $p \leq 0.05$, ## $p \leq 0.01$, ### $p \leq 0.001$).

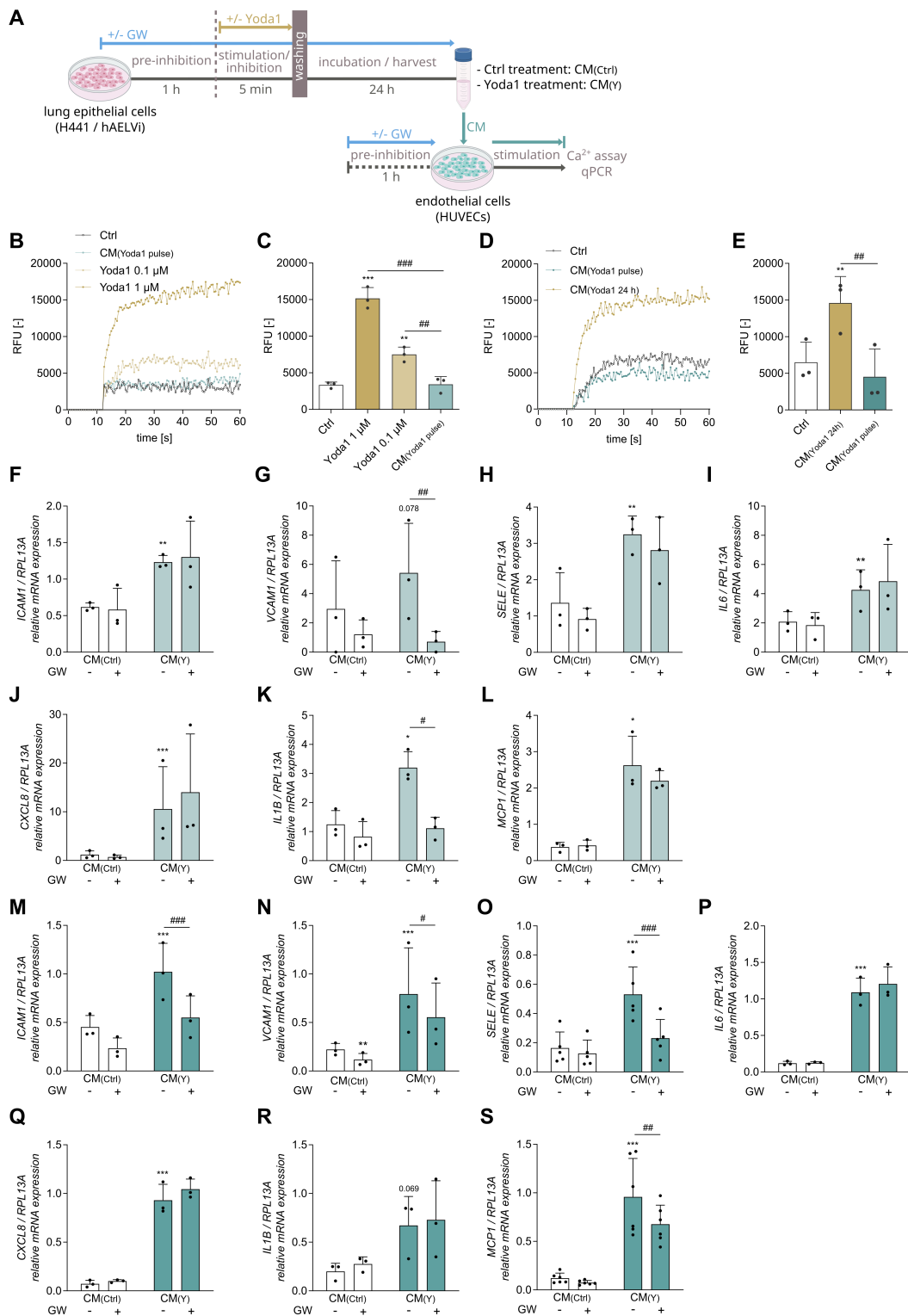


Figure 2

Upregulated gene expression of leukocyte transmigration-associated genes in endothelial cells after treatment with epithelial-derived conditioned media

Schematic overview of the experimental setup for generating conditioned medium in epithelial cells (H441/hAELVi) and its subsequent transfer to endothelial cells (HUVECs) (**A**). HUVECs loaded with

Cal590 were stimulated with control medium, 0.1 μ M or 1 μ M Yoda1, or conditioned medium obtained from Yoda1 pulse-stimulated H441 cells (CM(Yoda1 pulse)), and intracellular Ca^{2+} levels were recorded over 60 s (**B**). Ca^{2+} responses at the 60 s time point are shown separately (**C**). Ca^{2+} increase in Cal590 stained HUVECs after stimulation with control medium, CM(Yoda1 pulse) or CM containing residual Yoda1 (CM(Yoda1 24 h)) derived from hAELVi cells was monitored over 60 s (**D**). Ca^{2+} responses at the 60 s time point are shown separately (**E**). Conditioned medium from H441 (**F-L**) or hAELVi cells (**M-S**) generated after control or Yoda1 pulse stimulation in the presence or absence of the ADAM10/17 inhibitor GW (10 μ M), was applied to HUVECs. Changes in mRNA expression were analyzed by qPCR after 2 h (*IL1B*, *MCP1*, *SELE* (hAELVi)), 4 h (*IL6*, *CXCL8*, *ICAM1*, *VCAM1* (hAELVi), *SELE* (H441)), 24 h (*VCAM1* (H441)). Quantitative data are shown as the means + SDs of at least three independent experiments. Statistical differences to the control are indicated by asterisks (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$), whereas differences between the treatments are indicated by hashes (# $p \leq 0.05$, ## $p \leq 0.01$, ### $p \leq 0.001$).

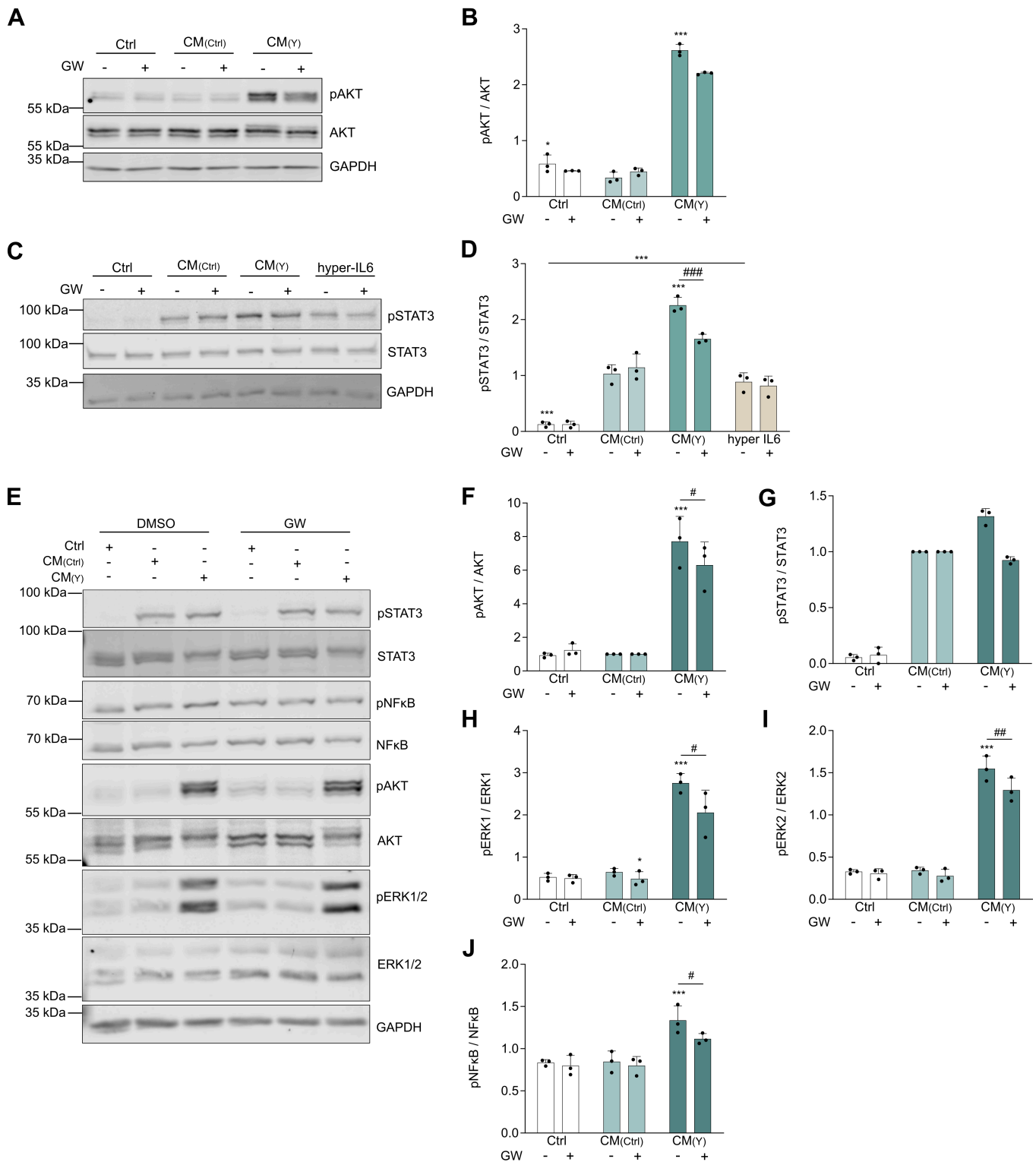


Figure 3

Endothelial signaling cascades induced by mediators released from Piezo1-activated epithelial cells

HUVECs were stimulated for 20 min with control medium or conditioned medium (Ctrl / 10 μ M Yoda1) from H441 cells in the presence or absence of ADAM10/17 inhibitor GW (10 μ M). AKT phosphorylation was subsequently analyzed by Western blot (**A**, **B**). To assess STAT3 phosphorylation, HUVECs were

additionally stimulated with hyper-IL-6 as a positive control (**C, D**). HUVECs were stimulated for 45 min with control medium or conditioned medium collected from control- or 10 μ M Yoda1-treated hAELVi cells in the presence or absence of the ADAM10/17 inhibitor GW (10 μ M). AKT (**E, F**), STAT3 (**E, G**), ERK1/2 (**E, H, I**), and NF κ B (**E, J**) phosphorylation was then analyzed. Quantitative data are shown as the means + SDs of at least three independent experiments. Statistical differences to the control are indicated by asterisks (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$), whereas differences between the treatments are indicated by hashes (# $p \leq 0.05$, ## $p \leq 0.01$, ### $p \leq 0.001$).

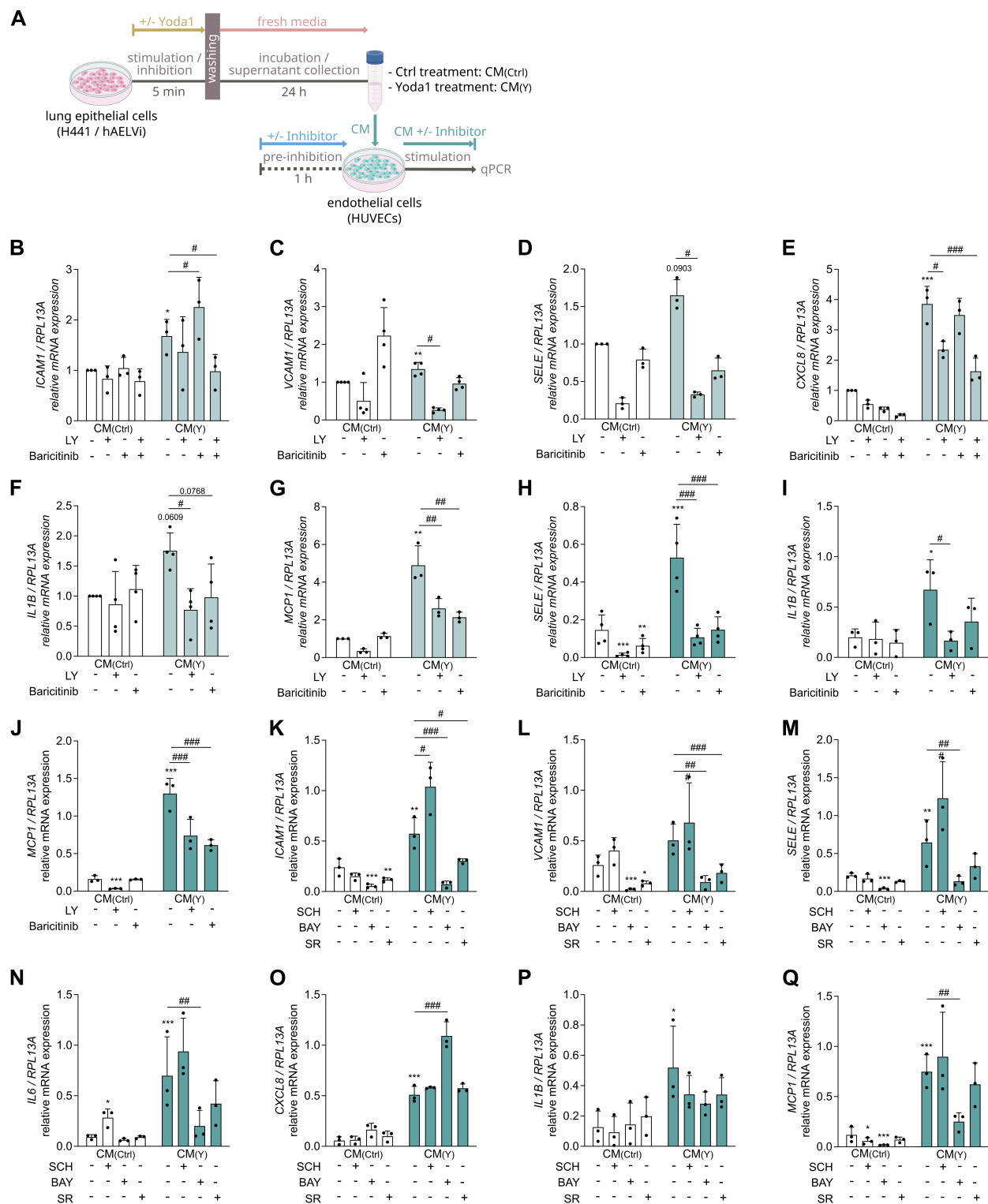


Figure 4

PI3K, JAK, and NFκB signaling mediate the endothelial transcriptional response to epithelial secretome

Conditioned medium from H441 (A-F) or hAELVi cells (G-P) generated after control or Yoda1 pulse stimulation in the presence or absence of the PI3K inhibitor LY294002 (10 μM) or the JAK1/2 Inhibitor Baricitinib (10 μM) (A-I), as well as the ERK inhibitor SCH772984 (1 μM), the NFκB inhibitor BAY11-7082 (3 μM), and the AP-1 inhibitor SR11302 (10 μM) was applied to HUVECs. Changes in gene expression were analyzed by qPCR after 2 h (*IL1B*, *MCP1*, *SELE* (hAELVi)), 4 h (*IL6*, *CXCL8*, *ICAM1*, *VCAM1* (hAELVi), *SELE*(H441)), 24 h (*VCAM1* (H441)). Quantitative data are shown as the means + SDs of at least three independent experiments. Statistical differences to the control are indicated by asterisks (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$), whereas differences between the treatments are indicated by hashes (# $p \leq 0.05$, ## $p \leq 0.01$, ### $p \leq 0.001$).

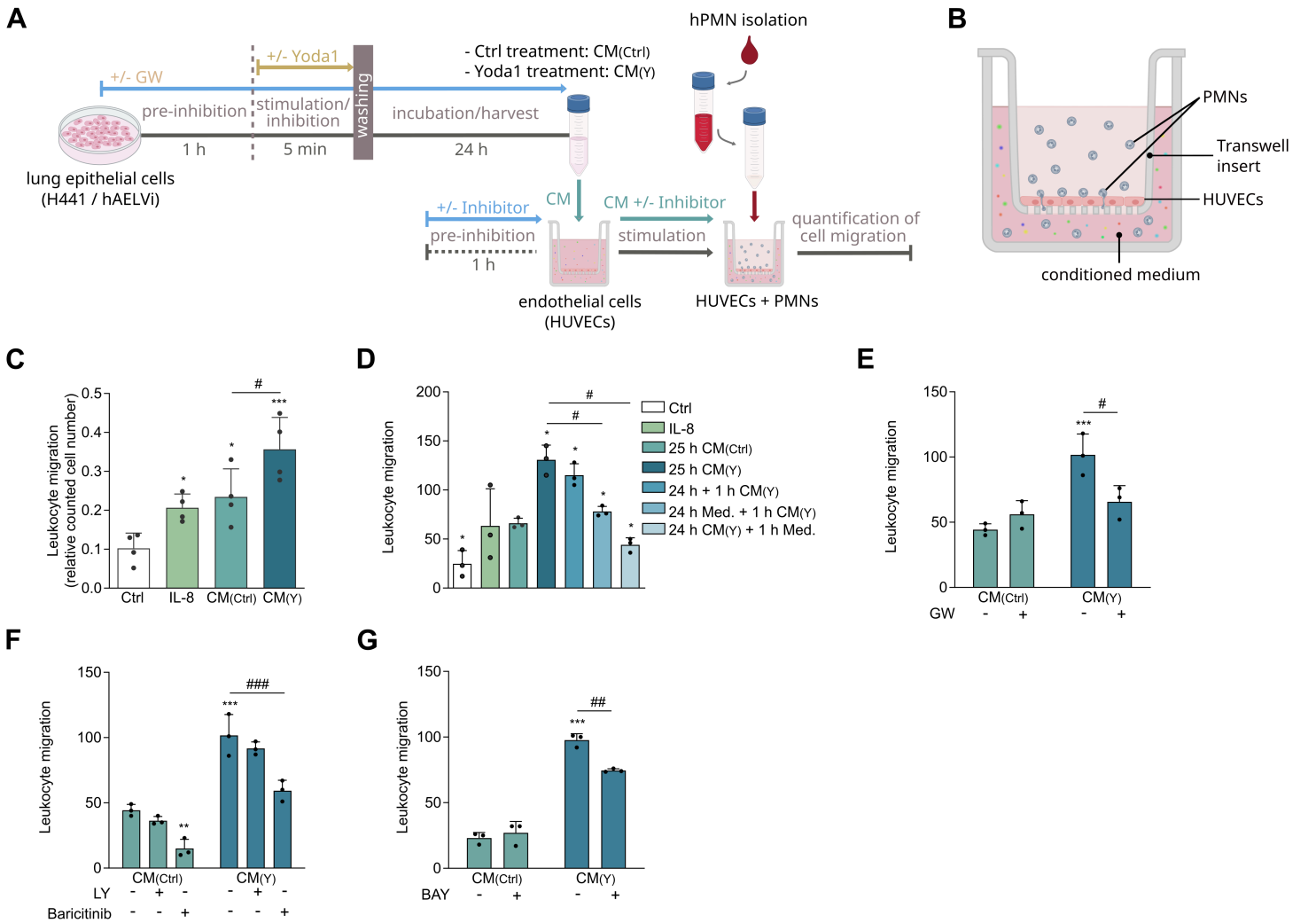


Figure 5

Piezo1 activation in epithelial cells promotes leukocyte transmigration across the endothelial barrier

Schematic overview of the leukocyte transmigration assay using a transwell system **(A)** and the transwell system in detail **(B)**. Conditioned medium from H441 **(C)** or hAELVI cells **(D)** generated after control or Yoda1 pulse stimulation was applied to HUVECs either 24 h before or during leukocyte transmigration. Vehicle control and IL-8 (10 ng/mL) served as negative and positive controls, respectively **(C, D)**. Human polymorphonuclear neutrophils (hPMN) were isolated, subsequently added to the upper transwell compartment and transmigrated cells were quantified in the lower compartment after 1 h **(C-G)**. To distinguish the contribution of epithelial-derived chemoattractants from endothelial activation, conditioned medium was added to HUVECs in four different combinations: Throughout the entire experiment (25 h CM(Y)), for 24 h followed by 1 h fresh CM (24 h + 1 h CM(Y)), for 24 h fresh medium followed by 1 h CM (24 h Med. + 1 h CM(Y)) or for 24 h CM followed by 1 h fresh medium (24 h CM(Y) + 1 h Med.) during the transmigration period **(D)**. To investigate ADAM10/17 involvement, GW280264X (GW, 10 μ M) was added to epithelial cells and remained continuously present in the conditioned media throughout the experiment **(E)**. The PI3K inhibitor LY294002 (LY, 10 μ M), the JAK1/2 inhibitor Baricitinib (10 μ M) **(F)** and the NF κ B inhibitor BAY11-7082 (BAY, 3 μ M) **(G)** were applied to HUVECs throughout the experiment. Quantitative data are shown as the means + SDs of at least three independent experiments. Statistical differences to the control are indicated by asterisks (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$), whereas differences between the treatments are indicated by hashes (# $p \leq 0.05$, ## $p \leq 0.01$, ### $p \leq 0.001$).

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