**ATRA results in irregular repair of septa and fails to inhibit proinflammatory macrophages**

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Keywords: Pulmonary alveoli, regeneration, all-trans retinol, matrix metalloproteinases, emphysema, macrophages

Word count: 4981
Abstract

**Background:** ATRA is controversially discussed in emphysema therapy. We re-evaluated ATRA in the elastase-model and hypothesized that beneficial effects should be reflected by increased alveolar surface area, elastin expression, and downregulation of inflammatory mediators and matrix metalloproteinases (MMP).

**Methods:** Emphysema was induced by porcine pancreatic elastase *versus* saline in Sprague-Dawley rats. On days 26-37, rats received daily intraperitoneal injections with ATRA (500 µg/kg b.w.) *versus* olive-oil. Lungs were removed at day 38. Rat alveolar epithelial L2 cells were incubated with/without elastase followed by ATRA- or vehicle-treatment, respectively.

**Results:** ATRA only partially ameliorated structural defects. Alveolar walls exhibited irregular architecture: increased arithmetic mean thickness, reduction in surface coverage by AEC type II. ATRA only partially restored reduced soluble elastin. It tended to increase the ratio of ED1⁺:ED2⁺ macrophages. Bronchoalveolar lavage (BAL) cells exhibited a pro-inflammatory state with high expression of IL-1β, CINC-1, TNF-α, NF-κB, MMP-2, -9, -12, TIMP-1, and -2 in emphysema with ATRA exerting only little effects. MMP-7 was highly induced by ATRA in healthy but not in emphysematous lungs. ATRA reduced both MMP-2 and TIMP-1 activity in BAL fluid of emphysematous lungs.

**Conclusions:** ATRA-therapy may bear the risk of unwanted side-effects on alveolar septal architecture in emphysematous lungs.
Introduction

Emphysema is a chronic pulmonary disease with increasing incidence and a high socio-economic burden. Destruction of the distal lung with resulting loss of gas exchanging alveoli is the defining morphological characteristic of pulmonary emphysema [1]. Therapeutic options are limited and at best allow stabilizing the current status of the disease. At present, there are no therapeutic options available that allow for the regeneration of lost alveoli in emphysematous human lungs.

All-trans retinoic acid (ATRA) was demonstrated to rescue the lungs of mice and rats from dexamethasone-induced inhibition of postnatal formation of alveolar septa [2-5]. In addition, several experimental studies in rats and mice suggested that regeneration of lost alveolar septa can be achieved by ATRA in elastase- or cigarette smoke-induced emphysema [6-8]. However, a number of studies failed to demonstrate a beneficial effect of ATRA in other emphysema models [9-14], and beneficial effects in human patients have been marginal so far [15-18]. The reason for these discrepancies is still unclear [19]. Although potential mechanisms underlying the beneficial effects of ATRA in dexamethasone-induced impairment of alveolarisation have been suggested [20-23], only little is known about the molecular mechanisms contributing to its potential regenerative effects in smoke- or elastase-induced emphysema [16, 19].

Formation of alveolar septa during postnatal lung development, repair and regeneration is characterized by complex interactions of various types of cells and extracellular matrix components involving specific transcription factors, cytokines, and proteases. Remodelling of the extracellular matrix is a key feature of postnatal alveolar septation [24] with various matrix metalloproteinases (MMPs), zinc-dependent endopeptidases, being involved [25, 26]. Gelatinase A (MMP-2) and gelatinase B (MMP-9), and macrophage metalloelastase (MMP-12) play a key role in emphysema development and progression [27-29]. However, MMPs are not
only involved in the development of pulmonary diseases, but appear to be important in repair and defence mechanisms as well. MMP-7 is involved in the control of inflammation [30], and is required for airway re-epithelialisation[31]. Both MMP-7 and MMP-9 directly modulate the activity of several growth factors, which are involved in tissue repair and remodelling [31]. Under normal physiological conditions MMPs promote remodelling of extracellular matrix, while in pathological conditions a switch in MMP expression and activity may lead to excessive lung inflammation and tissue destruction.

Because the potential of ATRA to induce alveolar regeneration in elastase-induced emphysema in rodents is controversially discussed, we tested ATRA in the same rat strain using the same experimental design as in the original study of Massaro and Massaro [7]. Quantitative stereology was used to assess alveolar tissue destruction by elastase and the potential regenerative effect of ATRA. To investigate the regenerative potential on a molecular level, we analyzed the extracellular matrix protein elastin, a key component of alveolar septa [32]. Because MMP-2, -7, -9 and -12, and their inhibitors TIMP-1 and -2 are involved in matrix remodelling and pulmonary repair, we hypothesized that the regenerative effect of ATRA, if present, should affect MMP and TIMP levels. To address this question, we studied expression and enzyme activity of MMP-2, -7, -9, -12, TIMP-1 and -2 in alveolar macrophages (AM) and whole lung tissue homogenate by means of real time RT-PCR, Western blotting, immuno histochemistry, zymography, and/or reverse zymography in control and elastase-induced emphysematous rat lungs treated with ATRA or vehicle, respectively. Furthermore, as ATRA is thought to dampen inflammatory processes, pro-inflammatory mediators TNF-α, IL-1, CINC-1, and NF-κB were investigated too. To assess the potential contribution of pulmonary epithelial cells, the effects of elastase and ATRA were studied in the rat AEC type II cell line L2.
Material and Methods

Experimental Design

All in vivo animal experiments were performed according to institutional guidelines that complied with national and international regulations, and have been approved by the regional government (Regierungspräsidium Giessen, Dezernat V 54, Giessen, Germany). Six week old male Sprague-Dawley rats with a body weight of about 150 g were randomly assigned to four different groups as follows (Figure 1):

Control-group (sham/sham = SS): at day 0, rat lungs (n=6) received a single bolus of 180 μl saline (sham) by endotracheal (ET) instillation. From day 26 to day 37, animals received an intraperitoneal injection (IP) of 300 μl/kg b.w. olive oil (sham) each day.

Emphysema-group (elastase/sham = ES): at day 0, lung emphysema was induced in rats (n=6) by ET instillation of 25 U/kg b.w. porcine pancreatic elastase (Roche Diagnostics, Mannheim, Germany). From day 26 to day 37, animals received a sham IP injection of olive oil (300 μl/kg b.w.) per day.

Emphysema-treatment-group (elastase/ATRA = EA): at day 0, lung emphysema was induced in rats (n=6) as described for group 3. From day 26 to day 37, animals received an IP injection of 500 μg/kg b.w. all-trans retinoic acid (ATRA, Sigma-Aldrich, Steinheim, Germany) dissolved in olive oil (300 μl/kg b.w.) each day.

ATRA-control-group (sham/ATRA = SA): at day 0, saline was instilled into rat lungs (n=6) by ET instillation. From day 26 to day 37, animals received an IP injection of 500 μg ATRA per kg b.w. dissolved in olive oil (300 μl/kg b.w.) each day.
All animals were sacrificed at day 38. Lungs were perfused with sterile phosphate buffered saline (PBS) to remove blood. Bronchoalveolar lavage (BAL) was performed prior to separation of the right lung for quantitative morphology and the left lung for investigation of relative mRNA expression.

**BAL and Isolation of AM**

BAL fluid was collected from both lungs by injecting each 6 ml of PBS per injection to recover a final volume of 42 ml. AM were recovered from BAL fluid by centrifugation at 1,000 rpm for 15 min, re-suspended in RLT buffer (Qiagen, Hilden, Germany), and stored at -80°C until analyzed.

**Lung Fixation**

Right lungs were fixed by airway instillation with 4% phosphate-buffered paraformaldehyde at a pressure of 20 cm water column. After overnight fixation in fresh fixative, right lung volume was determined by fluid displacement, and two fractions of lung slices were collected by systematic uniform random sampling as described elsewhere [29] for subsequent embedment into paraffin and glycolmethacrylate (GMA), respectively. In order to assess potential effects of ATRA on the inflammatory cell infiltrate, four lungs of each therapeutic group, i.e. ES and EA, were fixed without prior BAL.

**Tissue Processing**

One fraction of right lung slices was post-fixed in 1% glutardialdehyde, 1% paraformaldehyde in 0.1 M sodium cacodylate buffer followed by osmication, and *en bloc* staining with aqueous uranyl acetate prior to dehydration. These lung slices were embedded into GMA for
stereological analysis of lung parenchyma as described recently [33]. The second fraction of lung slices was directly processed for routine embedment into paraffin for subsequent immunohistochemistry.

**Immunohistochemistry**

Immunohistochemical staining for markers of inflammatory cells was performed as described recently [34]. In detail, monoclonal antibodies (mAb) ED1, ED2, and R73 were purchased from Serotec (Düsseldorf, Germany) Horseradish peroxidase conjugated rabbit anti-mouse immunoglobulins and the anti-mouse EnVision peroxidase system were supplied by DAKO (Hamburg, Germany). Polyclonal rabbit antisera against myeloperoxidase (MPO) and MMP-7 were purchased from LifeSpan Biosciences (Biozol, Eching, Germany) and Acris Antibodies (Herford, Germany), respectively. The polyclonal antiserum against surfactant protein A (SP-A) was kindly provided by Dr. Sam Hawgood (University of California, San Francisco, CA).

Slides were treated with 0.5 mg/ml Protease Type XIV (Sigma-Aldrich, Taufkirchen, Germany) in 50 mM Tris-HCl buffer, pH 7.6, containing 0.9% NaCl for 15 min at room temperature. Endogenous peroxidase activity was blocked with 1% H2O2 in PBS for 30 min. After washing in PBS, the sections were incubated for 30 min with PBS, pH 7.2, 1% BSA (Serva, Heidelberg, Germany), 0.1% NaN3 (p.a., Merck, Darmstadt, Germany) followed by overnight incubation with an appropriate dilution of primary antibody (ED1 1:500, ED2 1:200, R73 1:500) in the same solution at 4°C. On control sections, the primary antibody was omitted. Bound primary antibodies were detected using the anti-mouse EnVision peroxidase system containing 5% heat inactivated normal rat serum and 3,3’-diaminobenzidine (DAB, Sigma-Aldrich). Staining for MPO, MMP-7, and SP-D was performed by indirect immunohistochemistry using a biotinylated
goat ant-rabbit secondary antibody (Vector Laboratories, Burlingame, CA) as described earlier [35]. Sections were lightly counter-stained with hemalum.

**Quantitative Morphology (Stereology)**

From each lung slice embedded into GMA one technically excellent Richardson-stained section was used to quantify structural changes in the lung parenchyma. The following emphysema-relevant parameters were recorded as described recently [33]: mean airspace chord length (MCL) and arithmetic mean septal thickness as well as total airspace volume, total alveolar surface area, and total volume of alveolar septal wall tissue, which were normalized to body mass. In addition, paraffin sections stained for surfactant protein A by indirect immunohistochemistry were used for stereological quantification of the total alveolar surface area occupied by AEC type II as described previously [35]. Sections stained with mAbs ED1 and ED2 were used to quantify the total volume of alveolar and interstitial macrophage subpopulations, respectively, as described previously [33]. All stereological analyses were performed using a PC-based Olympus BX 51 light microscope equipped with a Cast-Grid 2.1.5 system (Olympus, Denmark) following the ATS/ERS research policy statement [36].

**Processing of Whole Lung Tissue**

Left lungs were placed on a sterile petri-dish on ice and immediately chopped up with sterile scalpel into small pieces. Chopped tissue pieces were collected in 2 ml Eppendorf tubes, dipped into liquid nitrogen for 5 min, and stored at -80 °C until analyzed.
**Total Protein Extraction and Western Blotting for Detection of Elastin.**

Total protein was extracted from 100 mg whole lung tissue (WLT) homogenate using total protein extraction kit (Biochain Institute, Hayward, USA) according to the manufacturer’s protocol. The protein amount of each sample from WLT was determined using a BCA™ protein assay kit (Pierce, Rockford, USA) according to the manufacturer’s recommendation. Equal amounts of protein (100 µg) were mixed with Tris-glycine SDS sample buffer and reduced with β-mercaptoethanol (Sigma-Aldrich, Steinheim, Germany) at 95°C for 5 min. Reduced samples along with Novex® sharp pre-stained protein standard (Invitrogen, Karlsruhe, Germany) were immediately loaded on 4-15% Tris-HCL Criterion™ pre-cast gel (Bio-Rad, Hercules, USA) and were electrophoresed at 60 V for 30 min and 120 V for 90 min. The separated proteins were transferred to Protran™ nitrocellulose membranes (Protran, Dassel, Germany), which were blocked with 5% skim milk in PBS (Biochrom, Berlin, Germany) for 1 h. Blocked membranes were incubated overnight with pre-diluted anti-elastin antibody (1:200; Biozol, Eching, Germany) followed by three times washing with PBS for 5 min and were incubated with horseradish peroxidase–linked secondary antibody (1:1000; Pierce, Rockford, USA) for 1 h. The membranes were washed three times with PBS for 5 min. The washed membranes were exposed to enhanced chemiluminescence detection (SuperSignal® West Femto, Pierce, USA) and recorded by autoradiography (CL-X Posure™ film, Pierce, USA).

Membranes were scanned and the obtained bands were quantified using computer-based densitometer and analysis software (Bio-1D V.97 software, Vilber Lourmat, France), which was carried out on negative images of the western blots. Differences in optical density of protein bands were calculated with respect to controls (100%). Since two blots were performed and each blot had three samples from each group, results were expressed as mean ± SD.
Stimulation of Epithelial Cells in vitro

Rat AEC type II (cell line L2) were seeded in 12 well plates with $1 \times 10^5$ cells per well and cultured in DMEM supplemented with 10% FBS and 1% Pen/Strep. After 24 h, L2 cells were washed with PBS and serum-free DMEM was added for 12 h in order to cause starvation of the cells. Serum-free DMEM was removed and replaced by DMEM containing 10% FBS. Accessorily 30µg/ml Elastase (Sigma Aldrich, Steinheim Germany) was added to the cells. After six hours the cells were treated with 1mM ATRA (Sigma Aldrich). L2 cell supernatants for Multiplex analysis were collected either three or five hours later. Furthermore, cells were harvested at the same time points for RT-PCR analysis.

Isolation of Total Cellular RNA and cDNA Synthesis

To investigate the relative mRNA expression in AM and WLT, total cellular RNA was extracted using RNeasy columns (Qiagen, Hilden, Germany) according to the manufacturer’s instructions as described recently [37]. Total RNA quantification and purity was determined with an Ultraspec 2100-spectrophotometer (Amersham Pharmacia Biotech, Cambridge, UK). The quantity of the isolated RNA was calculated from the absorbance at 260 nm, and the purity was determined by calculating the ratio at optical density $\text{OD}_{260\ nm}$/optical density $\text{OD}_{280\ nm}$. Total RNA integrity was assessed by analyzing 18S and 28S ribosomal RNA on 1.2% ethidium-bromide stained agarose gel electrophoresis.

First-strand cDNA was synthesized by introducing equal amounts of RNA (1.5 µg) from each sample in a total reaction volume of 20 µl using an oligo (dT)$_{12-18}$ primer (Invitrogen, Karlsruhe, Germany) and Superscript™ II Reverse Transcriptase kit (Invitrogen, Karlsruhe, Germany).
**Relative mRNA Quantification**

The real-time reverse transcription polymerase chain reaction (RT-PCR) for determining the amplification factor of the target genes (see Table 1) were performed in a 96-well format iCycler Detection System (BioRad, Hercules, USA) in 20 µl total reaction volume using 10 µl an SYBR Green PCR mix (ABgene® Thermo Fischer Scientific, UK), 1 µl each sequence-specific forward and reverse oligonucleotide primers (10 pmol), 7 µl water, and 1 µl cDNA. The thermal cycle conditions used for all reactions were as follows: Step 1: 95°C, 15 min; step 2 (40 cycles): 95°C, 50 s; step 3 (sequence-specific oligonucleotide primer’s annealing temperature, see Table 1): 40 s; step 4: 72°C, 40 s; step 5: 72°C, 5 min.

**Real-time RT-PCR: Data Analysis**

mRNA levels for target gene MMP-2, MMP-7, MMP-9, MMP-12, TIMP-1, TIMP-2, interleukin (IL)-1, CINC-1, TNF-α and NF-κB, respectively were determined relative to the endogenous control glyceraldehydes 3-phosphate dehydrogenase (GAPDH) according to the formula 2 to the power of delta cycle threshold (\(2^{\Delta Ct}\)), where \(\Delta Ct = Ct\), reference gene – Ct, test gene.

**Multiplex Analysis**

For analysis of cytokine protein levels released by L2 cells, a multiplexed bead immunoassay was performed. Samples were tested according to manufacturer’s instructions utilizing a Milliplex MAP Kit on the BioPlex 100 analyser (Bio-Rad Laboratories, USA). The kit includes five different populations of microspheres coated with specific capture antibodies, in this case for: CINC-1, TNF-α, IL-6, IL-1β, and CXCL10.
Zymography and Reverse Zymography

Gelatine zymography and reverse zymography were carried out on 200 µl of cell free lavage fluids, which were 10-fold concentrated using Centricon YM-10 columns (Millipore, Bedford, USA) with a molecular cut-off at 10 kD.

For zymography, samples were mixed with Tris-glycine SDS sample buffer (Invitrogen, Karlsruhe, Germany) and immediately loaded on Novex® 10% gelatin zymogram gels (Invitrogen, Karlsruhe, Germany). Samples were electrophoresed at 120 V for 90 min. Gels were renatured with zymogram renaturation buffer (Invitrogen, Karlsruhe, Germany), developed with zymogram developing buffer (Invitrogen, Karlsruhe, Germany), stained with Simply Blue™ Safestain (Invitrogen, Karlsruhe, Germany), and destained with double distilled water according to the manufacturer's instructions.

For reverse zymography, resolving gel and stacking gel were prepared as follows: for the resolving gel, 3.3 ml of 30% acrylamide; 2.5 ml of 1.5 M Tris-HCl, pH 8.3; 20 mg/ml gelatin (Sigma-Aldrich, Steinheim, Germany); 35 ng/ml pro-MMP-2 (Calbiochem, Darmstadt, Germany); 2.85 ml water; 100 µl of 10% SDS; 100 µl ammonium persulphate (APS); 10 µl TEMED; for the stacking gel, 1.3 ml of 30% acrylamide; 2.5 ml of 0.5 M Tris-HCl, pH 6.8; 6.1 ml water; 100 µl of 10% SDS; 100 µl APS; 10 µl TEMED. Concentrated BAL samples were mixed with Tris-glycine SDS sample buffer and immediately loaded onto the gels. Samples were electrophoresed at 120 V for 90 min. Gels were renatured, denatured, and developed as described above.
Zymography and Reverse Zymography: Data Analysis

The bands obtained were quantified by a densitometer and analysis software (Bio-1D V.97 software, Vilber Lourmat, France), which was carried out on negative images of the zymograms and reverse zymograms. Differences in optical density of bands were calculated with respect to control (100%).

Statistical Analysis

Unless stated otherwise, mean values ± s.d. are given. Student’s unpaired t-test was used to determine the level of significance of differences between sham/sham versus sham/ATRA, and elastase/sham versus elastase/ATRA, respectively. If normality and equal variance were not given at p>0.1, Mann-Whitney rank sum test was used. All analyses were performed by means of GraphPad Prism 4 software program (El Camino Real, San Diego, CA USA). Differences were considered statistically significant at p<0.05. (SigmaStat3.1, Jandel Scientific, Erkrath, Germany).
Results

Quantitative Morphology

Most of the effects seen in distal lung structures resulted from elastase-treatment whereas ATRA had no effects in healthy lungs (groups SA versus SS) and ATRA-therapy was only partially effective in emphysematous lungs (groups EA versus ES) (Table 2). Rats receiving a single endotracheal instillation of elastase followed by daily injections of vehicle for 12 days (group ES) exhibited all signs of pulmonary emphysema, i.e. heterogeneous airspace enlargement and loss of alveolar septa, whereas ATRA-therapy resulted in a partial amelioration of the effects of elastase (Figure 2). Airspace enlargement was reflected by an increase in mass-specific volume of distal airspaces (+69%) and mean chord length (+25%) in group ES compared with group SS, i.e. saline instilled, vehicle injected animals (Table 2). Loss of alveolar septa was indicated by a decrease in mass-specific alveolar septal surface area (-24%). The arithmetic mean thickness of alveolar septal walls, however, was significantly increased (+39%) in group ES compared with group SS.

Rats with elastase-induced emphysema, which received daily injections of ATRA for 12 days (group EA), were characterized by amelioration of airspace enlargement, which was indicated by a significant reduction in the mean chord length (-12%) in comparison with group ES (Table 2). Although, the loss of alveolar septal walls was slightly less pronounced in group EA, as indicated by a reduction in mass-specific alveolar septal surface area (-15%) compared with saline/ATRA-treated animals, it was not significantly different from group ES. Mean chord length and mass-specific volume of distal airspaces were still significantly higher in group EA than in group SA. Notably, the arithmetic mean thickness of alveolar septa was further increased in group EA compared with group ES (Figure 2D), as was also indicated by a significant increase in the mass-
specific volume of alveolar septal tissue (Table 2). Notably, a significant reduction in the alveolar surface area occupied by AEC type II (-38%) was observed in group EA versus group SA.

**Elastin Expression**

In whole lung tissue, elastin mRNA analyzed by RT-PCR was not significantly different between the groups under study (Figure 3A), whereas soluble elastin protein assessed by Western blotting was significantly reduced in emphysematous rat lungs (groups ES, EA) compared to controls (group SS, SA). Although, ATRA-supplementation in emphysematous lungs did not restore control levels, soluble elastin protein was significantly higher in group EA compared with ES (Figures 3B,C).

**Inflammatory Cells**

Immuno histochemical staining for major inflammatory cell types, i.e. AM (ED1⁺) (Figures 4A,D) and interstitial macrophages (ED2⁺) (Figures 4B,E), T-lymphocytes (α/β-TCR⁺) (Figures 4C,F) and neutrophilic granulocytes (not shown) demonstrated that ED1⁺ alveolar macrophages were the predominant cell type irrespective of the therapeutic treatment. The other cell types were only rarely seen. ED1⁺ alveolar macrophages appeared to be increased in response to ATRA whereas no changes were seen in ED2⁺ macrophages. As a consequence, the ratio of ED1⁺ to ED2⁺ macrophages tended to be increased (p=0.07) in the EA compared with the ES group (Figure 4 E,F).
Inflammatory Mediators

Significant effects of elastase were observed in AM for all inflammatory mediators and transcription factors under study (TNF-α, IL-1, CINC-1, NF-κB). RT-PCR revealed an increase in the mRNA levels of IL-1, CINC-1, TNF-α, and NF-κB in AM of emphysematous (group ES) in comparison with control rats (group SS). None of the inflammatory mediators under study was significantly altered by ATRA-therapy in emphysematous lungs, neither in AM (Figures 5A-D) nor in whole lung tissue (Figures 5E,F). However, mRNA levels were affected by ATRA in healthy lungs only (SA versus SS groups): CINC-1 was increased in AM whereas NF-κB and TNF-α were decreased in whole lung tissues (Figures 5E,F). Likewise, only minor effects were observed in rat AEC type II-like cells L2 treated with elastase and exposed to ATRA or control therapy, respectively (Figure 6): ATRA decreased IL-6 mRNA and protein expression in EA versus ES group (Figure 6C,D), although significance was achieved for mRNA expression only.

Matrix Metalloproteinases (MMPs) and Tissue Inhibitors of MMPs (TIMPs)

Elastase-treatment resulted in an increase in the mRNA levels of MMP-7, MMP-9, and MMP-12 as well as of TIMP-1 and TIMP-2 in AM of emphysematous rat lungs (group ES) in comparison with control group SS (Figure 7).

ATRA significantly increased mRNA expression levels of MMP-2 (Figure 7A), MMP-7 (Figure 7B), MMP-9 (Figure 7C), and TIMP-2 (Figure 7F ) in AM and MMP-7, MMP-12, and in TIMP-2 in whole lung tissues (not shown) of healthy lungs (SS versus SA). ATRA-therapy resulted in a significant reduction of MMP-12 (Figure 7D), TIMP-1 (Figure 7E), and TIMP-2 expression (Figure 7F) in AM of emphysematous lungs (group ES versus group EA), whereas no effects were seen in whole lung tissue homogenate (not shown). ATRA-therapy had no effects on MMP-7 in
emphysematous lungs, neither on mRNA expression (Figure 7B) nor on protein expression as seen by immuno histochemistry (Figure 7G,H).

**Zymography and Reverse Zymography: MMP and TIMP Activities**

In BAL fluid, enzymatic activity was detectable for MMP-2 (MW-72 kD) and TIMP-1 (MW-24 kD) by zymography and reverse zymography, respectively (Figure 8A). Densitometry based quantitative analysis of gels revealed that MMP-2 and TIMP-1 activities were significantly affected by both elastase-treatment and ATRA-therapy (Figure 8B). The activities of MMP-2 and TIMP-1 were increased by 5.1-fold and 1.8-fold in emphysematous lungs (groups ES) compared to controls (group SS), respectively. However, reduced activities of MMP-2 (0.35-fold) and TIMP-1 (0.62-fold) were observed in ATRA-treated (group EA) versus vehicle-treated (group ES) emphysematous lungs. In non-emphysematous lungs, enzyme activities did not change with ATRA-supplementation.
Discussion

Several studies performed to test the hypothesis that ATRA is able to induce regeneration of alveolar gas exchange area and to reverse emphysema reported contradictory results. Some studies in the elastase- or cigarette smoke-induced rat emphysema model reported the reversal of airspace enlargement [6-8] which, however were mainly based on mean linear intercept measurements, a highly controversially discussed parameter [36]. Focusing on elastase- and cigarette smoke-exposure models of emphysema, only one study using design-based stereology convincingly demonstrated the regeneration of lost alveolar septa after ATRA-therapy [7] whereas others did not find an effect of ATRA on emphysema-relevant histologic parameters in mice, rats, or guinea pigs [9-13]. Limited reversal of functional parameters and of an aerosol-derived measure of airspace size was seen in a papain-induced model of emphysema in dogs [38]. Likewise, clinical studies reported only limited therapeutic effects in humans [15-18]. As species- and strain-specific differences may account for these discrepancies, we used the same rat strain and therapeutic regimen as in the study performed by Massaro and Massaro [7]. Despite of this, we observed only partial reversal of airspace enlargement and no effect on alveolar gas exchange area. However, a significant increase in alveolar septal tissue mass as well as in arithmetic mean septal thickness and a significant decrease in the alveolar surface area covered by AEC type II was observed, all together being indicative of an irregular remodelling of alveolar septa. At the molecular level, the expression of elastin mRNA and soluble elastin protein, an important extracellular matrix protein, which was recently shown to be an important modifier of both lung development and response to injury in mice [32] was only partially ameliorated by ATRA-therapy. Moreover, soluble elastin does not allow drawing any conclusion about the deposition of (insoluble) elastin as a component of the extracellular scaffold of alveolar septa. Although the elastase-induced rodent model is highly specific and has clear
limitations in the investigation of the pathogenesis of the human disease, it can be used to study potential effects of therapeutics on alveolar septal maintenance and regeneration [39, 40].

Our findings are consistent with data obtained in a canine model of compensatory lung growth following right-sided pneumonectomy. In this model, four months of ATRA-supplementation resulted in a significant increase of alveolar septal volume and arithmetic mean thickness whereas no effect was seen in total alveolar surface area [41]. Quantitative electron microscopic analysis revealed that alveolar septal growth was non-uniform with a selective enhancement of capillary and endothelial growth resulting in a distorted septal architecture accompanied by a decrease in lung diffusion capacity [41]. Analysis of lung function indicated uneven distribution of ventilation consistent with distortion of alveolar geometry and/or altered small airway function induced by ATRA after right-sided pneumonectomy in the dog [42]. These data are supported by findings of Tepper and co-workers that two weeks of supplementation with ATRA had no effect on compliance, forced flows, or DL_{CO} in the rat elastase-model of emphysema, although there was a slight albeit insignificant increase in CT scan lung tissue density [43]. Notably, ATRA-supplementation had no effect on lung function, alveolar structure, or septal ultrastructure after left-sided pneumonectomy in dogs, which suggests that ATRA does not induce compensatory lung growth in the absence of existing cellular growth activities initiated by other primary signals [44]. Hence, the discrepant results of a number of studies may indicate that additional, yet unknown stimuli are necessary to induce and drive regeneration along a pathway leading to a regular architecture of regenerated alveolar septa. Because Marburg is located at approximately 380 m above sea level, hypoxia, which was suggested to be a potential factor accounting for the discrepancies observed [19], is unlikely to explain the limited effect of ATRA seen in our study.
To define the reasons why a specific intervention failed is an almost impossible task. Nevertheless, we will discuss some aspects that might be important in this respect: the role of MMPs and TIMPs as well as the role of AM. Normal tissue growth, repair, and remodelling rely on the well balanced interaction of MMP and their inhibitors, TIMPs which are produce not only by inflammatory but also by structural cells of the lung [25, 26, 31]. In AM and lung tissues of healthy rats, ATRA impressively induced transcription of MMP-7, which is involved in the control of inflammation [30] and of airway re-epithelialisation [45]. However, ATRA had no significant effect on MMP-7 mRNA expression and immune reactivity in emphysematous lungs. The failure to induce MMP-7 in emphysematous lungs, which is known to be important for repair, might partly explain the failure of ATRA-therapy to induce regular alveolar regeneration in our study. Given the plethora of MMPs and TIMPs in the lung [26], other proteases such as MMP-14 or other inhibitors such as TIMP-3, which have not been addressed in our study, may additionally contribute to the effects observed.

ATRA-therapy further enhanced MMP-2 expression in AM of emphysematous lungs, whereas increased TIMP-1 mRNA was significantly decreased. Notably, no changes were seen in whole lung tissue homogenates. The enzymatic activities of both, MMP-2 and TIMP-1, were significantly reduced in BAL fluid of emphysematous lungs supplemented with ATRA. Similarly, short term in vitro incubations of human BAL cells with ATRA demonstrated a reduction in both MMP-9 and TIMP-1 at protein and mRNA levels although at a ratio that was more favourable towards TIMP-1 [46]. Our data suggest that in rats ATRA may result in a net increase in the enzyme activity of MMP-2 over TIMP-1 in BAL fluid in comparison with control lungs thus impairing potential beneficial effects.

MMP-2 levels were correlated with the severity of airflow obstruction and disease progression in COPD patients [29, 47, 48]. Several in vitro studies suggest that ATRA affects MMP and TIMP
expression. Incubation with ATRA decreased the production and activity of MMP-2 and MMP-9, and increased TIMP-1 and TIMP-2 in capillary endothelial cells [49]. ATRA resulted in decreased MMP-9 and increased TIMP-1 in alveolar macrophages from COPD patients [46]. Likewise, ATRA reduced production and activity of MMP-9 in AM obtained from smokers with COPD in comparison to untreated cells from the same subjects, whereas no changes were seen in AM of non-smokers [16]. However, as incubation of regenerating AEC with TIMPs resulted in abnormal epithelial differentiation [50] and an inhibition of MMP-7 activity [51], a shift to a preponderance of TIMPs may give rise to an unintended negative effect on epithelial repair and promote airway fibrosis [51].

MMP expression was previously shown to be regulated at the transcriptional level by TNF-α via NF-κB signalling in several cell types including AEC [52, 53]. In this context, a further interesting finding of our study was that AM exhibited a persistently high expression of pro-inflammatory mediators that might counteract the potentially beneficial effects of ATRA. Instillation of elastase induces an early inflammatory response dominated by neutrophils and AM in rats and mice, which is almost completely resolved after seven to 20 days [54-56]. Likewise, inflammation was morphologically no longer seen in rat lungs 38 days after elastase-instillation in our study. AM were the predominant inflammatory cell type present in distal lung. However, inflammatory mediators such as TNF-α, IL-1 and CINC-1 as well as the pro-inflammatory transcription factor NF-κB were still significantly increased compared to controls, suggesting a persistent pro-inflammatory state of AM even more than five weeks after the initial injury. In line with this, we observed an increase in ED1+ AM in emphysematous rats treated with ATRA, whereas no effect was seen in macrophages binding mAb ED2, which recognizes CD163, a regulated hemoglobin scavenger receptor involved in the down-modulation of inflammatory
responses [57]. Likewise, ATRA did not affect the expression levels of cytokines in whole lung tissue of emphysematous lungs and had only limited effects in AEC in our *in vitro* experiments.

Taken together, the current study indicates that in the rat elastase-model of emphysema ATRA-therapy may lead to an irregular architecture of alveolar septa. We speculate that this might relate to the persistently high expression of pro-inflammatory cytokines of AM seen in emphysematous lungs, the failure to induce MMP-7 and an altered MMP/TIMP balance. Although we cannot present a definite conclusion about the potential cause-and-effect relationships, our findings suggest that ATRA-therapy may bear the risk of unwanted side-effects on the alveolar septal architecture.

**Acknowledgments**

The excellent technical assistance of Tanja Rausch and Larissa Greif (Marburg), Gabriele Fuchs-Moll (Giessen) as well as Franziska Ganzert and Dörte Karp (Borstel) is gratefully acknowledged. We thank Britta Wenske (Hannover) for her help in the establishment of the elastase model of emphysema. The study was supported by grant of the Deutsche Forschungsgemeinschaft (FE287/8-1; EXC 306, Cluster of Excellence “Inflammation at Interfaces”) and by the LOEWE-Centre Universities of Giessen and Marburg Lung Centre (UGMLC).
References


<table>
<thead>
<tr>
<th>Target gene</th>
<th>Sequence (FP; 5’ to 3’)</th>
<th>Annealing temperature (°C)</th>
<th>Amplicon length (bp)</th>
</tr>
</thead>
</table>
| rMMP-2      | cct gat aac cct gtc g  
gat gct tcc aac gct c | 57                         | 103                 |
| rMMP-7      | cac gga gac aac ttcc ca tt  
cag aat ggc aac aaa cag g | 52                         | 157                 |
| rMMP-9      | gcc cgcc ttt gtc ctt c  
tgc tagc cag gtc g | 57                         | 107                 |
| rMMP-12     | tcc cat ctt gat ctg c  
ttc tgtg atact ctg g | 53                         | 119                 |
| rTIMP-1     | gct ttct gtc aga cct g  
gcg tgc aat cag cat c | 58                         | 118                 |
| rTIMP-2     | gca tca ccc aga aga aag gc  
gtc cat cca gag gca ctc at | 57                         | 119                 |
| mHPRT       | agc tac tgtaat gat cag ctc acg  
aga ggt cct ttcc cac cag ca | 55                         | 198                 |
| rIL-1       | gtc acc cct gag gat ctg aa  
tgt gta cct tgg ccg tat ga | 47                         | 110                 |
| rIL-6       | gag gat acc act ccc aac aga cc  
aag tgc atc atc gtt gtt cat cca | 55                         | 117                 |
| rCINC-1     | gca ccc aaa ccg aag tca ta  
ggg gac acc ctt tagc cta | 50                         | 110                 |
| rTNF-α      | tgc ctc agc ctc ttcc gaa ctt ctc ct | 50                         | 108                 |
| rNF-κB      | acg atc tgt ttccc tca tct  
tgc ttc tct ccc cag gaa ta | 52                         | 179                 |
| rGAPDH      | cgg cca tca cgc cac aat tt  
cgc ttc ctc aca cat cga ga | 61                         | 280                 |

1 the first letter indicates the organism: r = Rattus norvegicus; m = Mus musculus
# Table 2 – Quantitative Morphological Data

<table>
<thead>
<tr>
<th>Parameter</th>
<th><strong>Group SS</strong></th>
<th><strong>Group SA</strong></th>
<th><strong>Group ES</strong></th>
<th><strong>Group EA</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Saline/Sham</strong></td>
<td><strong>Saline/ATRA</strong></td>
<td><strong>Elastase/Sham</strong></td>
<td><strong>Elastase/ATRA</strong></td>
</tr>
<tr>
<td>body weight [g]</td>
<td>359±14</td>
<td>358±25</td>
<td>343±31</td>
<td>342±22</td>
</tr>
<tr>
<td>V&lt;sub&gt;RL&lt;/sub&gt; [cm³]</td>
<td>6.0±0.6</td>
<td>6.2±0.6</td>
<td>8.5±1.4</td>
<td>7.9±0.7</td>
</tr>
<tr>
<td>mass-spec. V&lt;sub&gt;RL&lt;/sub&gt; [mm³/g]</td>
<td>16.7±1.4</td>
<td>17.4±0.9</td>
<td>24.8±3.3</td>
<td>23.8±2.4</td>
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<tr>
<td>mass-spec. V&lt;sub&gt;air&lt;/sub&gt; [mm³/g]</td>
<td>11.8±1.1</td>
<td>12.7±1.0</td>
<td>19.9±2.5</td>
<td>18.0±2.1</td>
</tr>
<tr>
<td>mass-spec. V&lt;sub&gt;ast&lt;/sub&gt; [mm³/g]</td>
<td>2.7±0.3</td>
<td>2.5±0.4</td>
<td>2.8±0.7</td>
<td>3.8±0.4*</td>
</tr>
<tr>
<td>mass-spec. S&lt;sub&gt;a&lt;/sub&gt; [cm²/g]</td>
<td>12.7±1.5</td>
<td>12.2±1.4</td>
<td>9.6±1.7</td>
<td>10.4±1.3</td>
</tr>
<tr>
<td>S&lt;sub&gt;SAEII&lt;/sub&gt; [cm²/cm²]</td>
<td>2.7±0.6</td>
<td>3.0±0.4</td>
<td>2.8±0.7</td>
<td>1.8±0.6*</td>
</tr>
<tr>
<td>τ&lt;sub&gt;ast&lt;/sub&gt; [µm]</td>
<td>2.1±0.2</td>
<td>2.1±0.2</td>
<td>2.9±0.3</td>
<td>3.7±0.1*</td>
</tr>
<tr>
<td>MCL [µm]</td>
<td>73.0±6.0</td>
<td>69.3±5.6</td>
<td>91.3±7.6</td>
<td>80.6±4.2*</td>
</tr>
</tbody>
</table>

Data are given as means ± s.d.; mass-spec. = mass-specific; V<sub>RL</sub> = volume of fixed right lung; V<sub>air</sub> = volume of distal airspaces; V<sub>ast</sub> = volume of alveolar septal tissues; S<sub>a</sub> = surface area of alveolar septa; S<sub>SAEII</sub> = fraction of alveolar surface area occupied by alveolar epithelial type II cells; τ<sub>ast</sub> = arithmetic mean thickness of alveolar septa; MCL = mean chord length of acinar airspace (alveoli and alveolar ducts); if normality and equal variance were given at p>0.1, unpaired Student’s t-test was used to test for significant differences between saline/sham versus saline/ATRA and elastase/sham versus elastase/ATRA, respectively; otherwise non-parametric Mann-Whitney U-test was performed; significances are indicated as follows: ¶p<0.05 versus group SS; *p<0.05 versus group ES
Figure legends

Figure 1

Schematic drawing of experimental setup. On day 0, rats received a single bolus of 180 µl of saline with or without 25 U of porcine pancreatic elastase per kg body weight (b.w.). From day 26 to day 37, animals received an intraperitoneal injection of 300 µl of olive oil per kg b.w. alone (sham) or with 500 µg of all-trans retinoic acid (ATRA) per kg b.w. each day. At day 38, animals were sacrificed and lungs removed for analysis by quantitative morphology and molecular biology. Group SS = saline/sham; group ES = elastase/sham; group EA = elastase/ATRA; group SA = saline/ATRA.

![Schematic drawing of experimental setup](image)

Figure 2

Histopathology of gas exchange area. Richardson-stained glycol methacrylate sections show (A) normal histology in saline/sham-treated animals (group SS), (B) slightly thickened alveolar septa in saline/ATRA-treated rats (group SA), (C) rarefaction of alveolar septa with enlarged airspaces in elastase/sham-treated rats (group ES), and (D) slightly increased airspaces with focally
considerably thickened alveolar septa in elastase/ATRA-treated animals (group EA). All micrographs were taken at identical magnification.
Figure 3 (including new data in C)

Relative mRNA and soluble protein expression for elastin in whole lung tissue (WLT) homogenate. (A) Elastin mRNA expression did not show any significant differences between groups: saline/sham versus saline/ATRA and elastase/sham versus elastase/ATRA; expression was determined by means of RT-PCR relative to the endogenous reference gene glyceraldehydes 3-phosphate dehydrogenase (GAPDH) according to the formula 2 to the power of delta cycle threshold \((2^{ΔCt})\), where \(ΔCt = Ct_{\text{reference gene}} - Ct_{\text{target gene}}\). (B) Densitometrical analysis of Western blots of soluble elastin revealed a reduction as a result of elastase-treatment. Although, ATRA-supplementation did not restore control levels, soluble elastin protein was significantly in the elastase/ATRA group higher compared with elastase/sham. Data were analyzed by means of unpaired Students’s t-test for significant differences between the groups saline/sham versus saline/ATRA and elastase/sham versus elastase/ATRA, respectively, with \(p≤0.05\) being indicated level by an asterisk.

![Graph A](image1.png)

![Graph B](image2.png)

![Image C](image3.png)
Figure 4 (new data)

Immunohistopathology of inflammatory cells. Dewaxed paraffin sections of lungs from groups ES (A, B, C) and EA (D, E, F) were stained by indirect immuno histochemistry for (A, D) ED1⁺ alveolar macrophages, (B, E) ED2⁺ interstitial macrophages, and (C, F) α/β-TCR⁺ T-lymphocytes (insert in C demonstrates presence of T-lymphocytes in bronchus-associated lymphatic tissues). ED1⁺ macrophages were the predominant cell type in the alveolar airspace in both groups whereas T-lymphocytes were rarely seen and most frequently observed with capillaries. Magnifications are indicated by scale bars. (G, H) The results of quantitative morphologic analysis of ED1⁺ and ED2⁺ macrophages suggest an increase in ED1⁺ over ED2⁺ macrophages in elastase/ATRA versus elastase/sham group. Data were analyzed by means of unpaired Students’s t-test for significant differences. alvED1⁺, ED1⁺ = alveolar ED1⁺ macrophages; alvED2⁺ = parenchymal ED2⁺ macrophages ; np ED2⁺ = non-parenchymal ED2⁺ macrophages; tot ED2⁺ = total ED2⁺ macrophages.
Relative mRNA expression of inflammatory mediators in alveolar macrophages (A-D) and whole lung tissue homogenate (E,F). (A) interleukin-1 (IL-1), (B) CINC-1, (C) nuclear factor-kappa B (NF-κB), and (D) tumour necrosis factor-alpha (TNF-α) were increased in response to elastase-treatment. However, significant effects of ATRA-supplementation were only observed with respect to CINC-1 in alveolar macrophages as well as of NF-kB and TNF-α in tissues. The mRNA levels of the target genes were determined relative to the endogenous reference gene glyceraldehydes 3-phosphate dehydrogenase (GAPDH) according to the formula $2^{\Delta Ct}$, where $\Delta Ct = C_{\text{reference gene}} - C_{\text{target gene}}$. Data were analyzed by means of unpaired Students’s t-test for significant differences between the groups saline/sham versus saline/ATRA and elastase/sham versus elastase/ATRA, respectively, with $p \leq 0.05$ being indicated level by an asterisk.
Figure 6 (new data)

Relative mRNA expression (A, C, E) and protein release (B, D, F) of inflammatory mediators rat AEC type II-like cells L2. Cells were seeded in 12 well plates with 1x10^5 cells per well and cultured in DMEM supplemented with 10% FBS and 1% Pen/Strep. After 24 h, L2 cells were washed with PBS and serum-free DMEM was added for 12 h in order to cause starvation of the cells. Serum-free DMEM was removed and replaced by DMEM containing 10% FBS. Accessorily 30µg/ml Elastase was added to the cells. After six hours the cells were treated with 1mM ATRA. Three or five hours later, cells were harvested for RT-PCR and cell supernatants for Multiplex analysis. The only significant effect observed was a decrease in mRNA expression of IL-6 after five hours in the EA group versus ES. Data were analyzed by means of unpaired Students’s t-test for significant differences between the groups saline/sham versus saline/ATRA and elastase/sham versus elastase/ATRA, respectively, with p≤0.05 being indicated level by an asterisk.
Figure 7 (including new data in G, H)

Relative mRNA expression of matrix metalloproteinases (MMP) and tissue inhibitors of MMPs (TIMP) in alveolar macrophages. Whereas (A) MMP-2, (B) MMP-7, and (C) MMP-9 showed no differences in emphysematous lungs treated with ATRA or olive oil, (D) ATRA-supplementation significantly decreased MMP-12 expression in rats treated with elastase. ATRA-supplementation significantly decreased (E) TIMP-1 and (F) TIMP-2 in rats treated with elastase, whereas TIMP-2 was increased in ATRA-treated healthy lungs. Indirect immuno histochemistry revealed no difference in cellular localization of MMP-7 and intensity in the immune reactivity between (G) elastase/sham and (H) elastase/ATRA. The mRNA levels of the target genes were determined relative to GAPDH according to the formula 2 to the power of delta cycle threshold (2^ΔCt), where ΔCt = Ct_reference gene − Ct_target gene. Data were analyzed by means of unpaired Students’s t-test for significant differences between the groups saline/sham versus saline/ATRA and elastase/sham versus elastase/ATRA, respectively, with p≤0.05 being indicated level by an asterisk.
Enzymatic activity of MMP-2 and TIMP-1 in bronchoalveolar lavage fluid. MMP-2 activity as assessed by (A, Gel 1) zymogram gel electrophoresis was highest in elastase-treated rats receiving sham injections (group ES), and was significantly reduced by ATRA-supplementation as revealed by (B) densitometry. Similarly, TIMP-1 activity as assessed by (A, Gel 2) reverse zymogram gel electrophoresis was significantly reduced by ATRA in emphysematous lungs (C). Data were analyzed by means of unpaired Students’s t-test for significant differences between the groups saline/sham versus saline/ATRA and elastase/sham versus elastase/ATRA, respectively, with p≤0.05 being indicated level by an asterisk.