473. Mechanistic studies in airway cell biology

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Acute effect of cigarette smoke on proteasome function

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Background: Chronic obstructive pulmonary disease (COPD) is associated with an abnormal inflammatory response of the lungs to cigarette smoke (CS). The products of CS oxidatively modify proteins thereby inducing severe oxidative cellular damage. The ubiquitin proteasome system serves as the major disposal system for oxidatively modified proteins and is thus essential for proper cellular

function. However, its role in CS-induced cell damage is currently unknown. We hypothesized that CS exposure impairs the function of the proteasome resulting in accumulation of oxidatively modified proteins, and exacerbation of cellular stress.
Methods and results: Treatment of human lung epithelial cells with CSE resulted in time and dose-dependent decrease of survival and increase of intracellular reactive oxygen species. The increased levels of oxidative stress correlated with accumulation of oxidatively modified proteins. CSE exposure also induced accumulation of polyubiquitinated proteins. Notably, treatment with CSE significantly impaired all three proteasomal activities at high doses. The trypsin-like activity was also inhibited at nontoxic CSE doses. Expression of the proteasome was unaffected. To confirm these observations in vivo, mice were exposed for 3 days to CS. Importantly, the trypsin-like activity of the proteasome was significantly reduced in lungs of smoked mice. We also observed increased levels of polyubiquitinated proteins in tissue extracts of smoked lungs compared controls.
Conclusion: Our data clearly indicate that acute cigarette smoke exposure impairs proteasome function in the lung. Reduced proteasomal protein degradation might thus contribute to the detrimental cellular effects of cigarette smoke exposure in COPD.

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Decreased levels of elafin in the lungs of patients with acute lung injury as a result of proteolytic cleavage by the proteasome
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Unregulated protease activity may drive dysregulated pulmonary inflammation implicated in acute lung injury (ALI). Elafin is a potent serine protease inhibitor produced locally in the lung by epithelial and inflammatory cells with anti-inflammatory properties. In this study we assessed the temporal changes in elafin concentration in patients with ALI and evaluated whether a decrease in elafin levels are due to proteolytic degradation. Patients with ALI within 48 hours of onset of ALI (n=37), day 3 (n=19) and day 7 (n=9) as well as healthy volunteers underwent bronchoalveolar lavage (BAL). Elafin was measured by ELISA. To determine whether elafin was susceptible to proteolytic cleavage, western blot analysis of recombinant elafin incubated with BAL fluid ± protease inhibitors was carried out. Elafin was significantly increased at the onset of ALI compared to healthy volunteers (39±5 ng/ml vs 0.5±0.1 ng/ml; $p<0.0004$). Elafin levels fell significantly by day 7 compared to baseline (16±4 ng/ml Vs 39±5 ng/ml; $p=0.02$). Incubation of exogenous elafin with ALI BAL fluid revealed that elafin underwent proteolytic cleavage. In contrast, proteolytic cleavage was not observed following incubation of exogenous elafin with healthy volunteer BAL fluid. Pre-incubation of ALI BALF with trypsin and chymotrypsin-like inhibitors abrogated this degradation of elafin. In addition, we demonstrated increased levels and activity of 20S proteasome in the BAL fluid of ALI patients compared to healthy volunteer BAL fluid and confirmed that 20S proteasome was responsible for cleavage of elafin in ALI BAL fluid which inactivated elafin's anti-elastase activity.

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Klotho: An important protein in the formation and development of emphysema
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Objective: Klotho is an anti-aging protein which also possesses anti-inflammatory actions and modulates the cellular responses to oxidative stress. Knock out of klotho in the mouse caused the formation of emphysema. The aim of this study is to investigate the expression of klotho in human emphysema and factors affecting its expression and activity in human bronchial epithelial cells.
Methods: Lung tissue from 5 COPD patients, 8 smokers without COPD and 13 non-smoking, non-COPD. Klotho expression was determined by quantitative real-time PCR, Western blotting and immunohistochemistry. Human bronchial epithelial cells (HBE) were treated with tumor necrosis factor (TNF)-α and hydrogen peroxide (H_2O_2), and the expression of klotho mRNA in cells and protein in cell supernatants was detected by RT-PCR and enzyme-linked immune sorbent assay (ELISA) respectively. Exogenous klotho was also added to HBE and A549 cell cultures and MTT assays were used to detect cell apoptosis.
Results: Klotho was detected in human lung tissue with a clear localization to airway epithelial cells. The level of klotho mRNA and protein in smokers with normal lung function was similar to that in non-smokers but was reduced in COPD patients The level of klotho expression was similar in COPD patients with emphysema compared with that in non-emphysematous COPD patients. In addition, we found that both TNF-α and H_2O_2 could significantly inhibit the expression and release of klotho in HBE cells. Exogenous klotho inhibited apoptosis in HBE) and A549 cells).
Conclusions: Klotho may play an important role in the formation and development of emphysema in COPD. However, further research is needed to explore the underlying mechanism.

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Immune activation in α1 antitrypsin deficiency (AATD) emphysema: Beyond the protease/antiprotease hypothesis
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The protease/antiprotease hypothesis has long set the field in the pathogenesis of AATD related emphysema, while activation of lymphocyte-driven responses has been scarcely investigated in this condition. We performed this study to evaluate the involvement of adaptive immune responses in AATD patients. By immunohistochemistry and molecular analysis we evaluated number, topographical distribution and clonality of lymphoid follicles in native lungs of AATD patients undergoing transplantation for severe emphysema (n=10). Results were compared to patients with similar disease severity, but with normal AAT levels (n=26) and to smoking (n=17) and nonsmoking (n=12) controls. Lymphoid follicles (LF) were significantly increased in the lungs of emphysematous patients, either with AATD (4.1;0.7-13 LF/cm^2) or without AATD (1.5;0-5.1) as compared to smoking (0;0-5) and nonsmoking controls (0;0-1, all $p<0.05$). Somewhat surprisingly the number of LF was even more prominent in patients with AATD than in those with normal AAT levels ($p<0.05$). Follicles in patients with AATD were predominantly located in the lower lobe, where lung destruction predominates. Molecular analysis confirmed an oligoclonal response in B cells isolated from these follicles. In conclusion, our study shows that organization of lymphocytes in follicles is a prominent feature of subjects with severe emphysema and AATD. These results challenge the current paradigm of α1antitrypsin deficiency-related emphysema from a protease/antiprotease driven process only to a more complex scenario entailing activation of adaptive immune responses.
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Impact of cigarette smoke exposure on Pseudomonas clearance in serpinB1–/– mice
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Opportunistic pathogens colonize the lungs of COPD patients and contribute to exacerbations. Cigarette smoke exposure induces pulmonary inflammation and is associated with increased incidence and morbidity of pulmonary infections but specific mechanisms linking smoke exposure to defective microbial clearance remain to be defined. Knock-out mice for serpinB1 (sB1–/–), a potent inhibitor of neutrophil proteases, have a severe defect in Pseudomonas clearance associated with increased inflammation, neutrophil death and proteolysis of antimicrobial molecules. In this study, we investigated the combined effects of cigarette smoke exposure and excess neutrophil proteases on Pseudomonas clearance. sB1–/– and wild-type mice were exposed to cigarette smoke or room air (control) for 6 weeks and infected intranasally 24h later. As shown previously, control sB1–/– mice had a severe defect in bacterial clearance compared to control wild-type mice 20h post infection. Surprisingly, clearance was dramatically improved in smoke-exposed sB1–/– mice, which had very low bacterial counts similar to smoke-exposed and control wild-type mice. At earlier time points (4 & 9h post infection), smoke-exposed wild-type mice also had lower bacterial counts than control wild-type mice. All groups of mice had similar bacterial counts 30min after infection suggesting no effect of prior smoke exposure on initial Pseudomonas survival. No increase in Pseudomonas-specific serum antibodies or neutrophil numbers was seen in smoke-exposed compared to their respective control mice. Our findings indicate that protease:inhibitor imbalance and acute cigarette smoke exposure differentially affect the kinetics of Pseudomonas clearance in the lung.

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Investigations on the role of region-specific IL-13 receptor alpha 1 expression along the airway tree in mucus production in asthma
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Increased mucus production is a critical factor impairing lung function in patients suffering from bronchial asthma. In acute and chronic mouse models of allergic asthma metaplasia of mucus producing goblet cells (GC) was found in proximal but not in distal airways although secretion of interleukin (IL)-13, the main trigger of mucus production, was significantly elevated along the entire airway tree. The current study aimed at elucidating what detains TH2 cell derived IL-13 from inducing mucus in distal airways.
BALB/c mice were sensitized with OVA/Alum and subsequently challenged with OVA for one (acute) or twelve weeks (chronic). Lungs were fixed under constant pressure with 4% PFA followed by paraffin embedding. Samples were stained with PAS to analyse mucus production. Quantitative (q)PCR was performed on microdissected airways and airway epithelial cells isolated by laser-capture microdissection (LCM).

QPCR revealed higher expression of IL-13Rα1 in proximal versus distal airways in both acutely (1.5-fold) and chronically (1.5-fold) challenged mice. Similarly, in PBS-treated control mice expression of IL-13Rα1 was higher in proximal airways (1.3-fold) and even more prominent in epithelial cells (2-fold) isolated by LCM from proximal airways.
Expression levels of transcription factors down-stream the IL-13Rα1 signalling implicated in mucus hyper-production, such as Spdef or FoxA2, were also region-specifically regulated.
These results suggest, that the low expression of IL-13Rα1 and hence, the reduced sensitivity towards IL-13, might protect distal airways from mucus plugging which would impair ventilation of the alveoli of the respective acinus.

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Role of nicotinic receptors in the regulation of cytokines production by human lung macrophages

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Background: In addition to its functions as a neurotransmitter, ACh may also serve as an autocrine/paracrine modulator of pulmonary inflammation. Our aims were to investigate the role of cholinergic receptors in the M1 (proinflammatory)/M2 (immunomodulatory) polarization of lung macrophages (LM).
Methods: LM were isolated from human resected lungs challenged for 24hrs with LPS to obtain M1 LM or with IL-13 to obtain M2 LM. Expression of α4/α7 nicotinic ACh receptors (nAChRs), M1-5 muscarinic receptors and cytokines was assessed with RT-qPCR. M1- (TNF-α, CCL3, CXCL8 and IL-6) and M2-cytokines (CCL18, CCL22) were quantified in supernatants.
Results: Expression of α7nAChR and M2 and M3 receptors was found in LM. The selective α7nAChR agonist and desensitizing agent GTS-21 (100 μM) inhibited (~65%) the production of M1 cytokines after LPS stimulation and of M2 cytokines after IL-13 stimulation. On the other hand, unstimulated LM in the presence of the α7nAChR antagonist α-bungarotoxin (10μM) showed an increased expression of M1 cytokines at both the transcriptional (5- to 157-fold) and protein level (2.5- to 46-fold), whereas M2 cytokines were not affected. Two agonists with mixed nicotinic/muscarinic activity that do not induce stable α7nAChR desensitization (acetylcholine and carbachol) and the muscarinic antagonists tiotropium and 4-DAMP were devoid of effect.
Conclusions: The blockade of α7nAChR in basal conditions favours LM polarization toward the M1 phenotype, whereas ligand-bound, but potentially non-conducting states of α7nAChR in proinflammatory conditions inhibit the production of M1 cytokines. α7nAChR may thus constitute a pharmacological target in lung inflammatory diseases.

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miRNA-17 and -144 regulate cAMP-responsive element binding protein (CREB1) signaling in murine ovalbumin-induced asthma and in human bronchial epithelial cells

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Background: MicroRNAs (miRs) are small non-coding RNAs that are essential for immune function and lung development. They are influenced by environmental exposures such as smoke or nutrition, both of which are also known to affect asthma risk development. Previously, we reported increased pulmonary expression of miR-17 and -144 in mice with OVA-induced asthma. This correlated with decreased mRNA and protein levels of CREB1, a validated target of both miRs. In addition, the cAMP-regulated transcriptional co-activators, CRTC-1 and -3, have been described to enhance CREB1-mediated gene transcription (Altarejos *et al.*, Nat Rev Mol Cell Biol., 2011, 12) and are also predicted targets of miR-17 and -144. Thus,we hypothesized that miR-17 and -144 regulate CREB1/CRTC-mediated gene transcription in experimental asthma and sought to elucidate this interdependency *in vitro*.
Methods: Human bronchial epithelial cells (16HBE) were transfected with precursor miRs or antagomiRs for miR-17 and miR-144. The expression of miR and endogenous CREB1, CRTC-1, -2, and -3 was assessed by RT-qPCR and Western blot analysis.
Results: The mRNA levels of CRTC-1 and -3, but not CRTC-2 in the lung were decreased in OVA-induced murine asthma. In 16HBE cells, the mRNA and protein levels of CREB1 and the mRNA levels of its co-activators CRTC-1,-2, and -3 were significantly decreased after transfection with precursor miR-17 and -144. Vice versa, their expression increased after inhibition of miRs by antagomiRs.
Conclusion: These findings suggest a role for microRNA-17 and -144 in the regulation of CREB1/CRTC signaling of potential relevance in asthma.