Airway epithelial innate host defence in chronic obstructive pulmonary disease

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CHAPTER 11

Summary and Discussion.

SUMMARY AND GENERAL DISCUSSION

This thesis describes a collection of studies which examined the effects of cigarette smoke (CS) and COPD disease status on airway epithelial innate host defense. In this last part, first an overview is given of the main findings of this thesis. Next, the role of alterations in airway epithelial innate host defense will be further discussed, focusing on its role in microbial colonization and infection, airway inflammation, and epithelial remodeling. Finally, a model will be proposed in which the role of airway epithelial cells in COPD will be summarized, according to the vicious circle hypothesis (1).

OVERVIEW OF THE MAIN FINDINGS IN THE THESIS

Antimicrobial proteins and peptides (AMPs) play an important role in the host defense of the lungs. In **Chapter 2** (2) it is summarized that this defense mechanism is altered in COPD and therefore may contribute to the disease. Excessive neutrophilic inflammation in COPD leads to enhanced levels of neutrophil-derived AMPs, i.e. LL-37 and alpha-defensins, which may promote inflammation and airway tissue injury and remodeling. In contrast to neutrophils, AMPs produced by the airway epithelium are suppressed by smoking or in COPD. Therefore, this may contribute to the increased susceptibility to microbial colonization and infections in patients.

In the next series of experimental studies described in this thesis, it was explored how airway epithelial expression of AMPs and other host defense mediators are affected by smoking and COPD disease status.

In **Chapter 3** (3) it is described that expression of the antimicrobial protein Ribonuclease 7 (RNase 7) was selectively induced upon microbial stimuli of undifferentiated basal cells (BCs) but not in differentiated cell cultures. Moreover, BCs present in differentiated cultures also produced RNase 7 upon transient injury caused by CS exposure. This was mediated by activation of the epidermal growth factor receptor (EGFR) and the downstream MEK/ERK1/2 signaling transduction pathway.

The microbial-induced expression of well described AMPs, i.e. human beta-defensin 2 (hBD-2), CCL20, LCN2 and S100A7, and antibacterial activity of airway epithelial cells was investigated in **Chapter 4** (4). Here we first observed an impaired antibacterial activity towards non-typeable *Haemophilus influenzae* (NTHi) in COPD airway epithelial cultures, when compared to non-COPD controls. In line with this, we furthermore detected lower NTHi-induced mRNA expression of the AMPs hBD-2 and S100A7. Next, we observed that CS exposure reduced airway epithelial expression of hBD-2, CCL20, LCN2 and S100A7 in both NTHi-stimulated COPD and non-COPD cultures. In contrast, expression of the pro-inflammatory mediators IL-8 and IL-6 persisted upon epithelial exposure to NTHi and CS. This selective impairment of AMPs expression was related to suppression of NFκB signaling, while inflammatory mediator expression was likely increased due to persistent MAPK/AP-1 signaling

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Next to microbial-induced AMPs, we further investigated in **Chapter 5** the expression of constitutively expressed AMPs and other host defense proteins in airway epithelial cells. We observed that human beta-defensin 1 (hBD-1) was highly expressed in undifferentiated airway epithelial cells, while the expression was reduced upon cell differentiation. In contrast, other AMPs and host defense proteins, i.e. SLPI, SPLUNC, LPLUNC, and PIGR, were increased in differentiated cultures and were furthermore found to be restricted in luminal epithelial cells. Using a chronic CS exposure model, we further demonstrated that impaired epithelial differentiation caused by CS was accompanied by a reduced expression of luminal cell-restricted host defense proteins. This differentiation-dependent effect could be mimicked by blocking the Notch signaling pathway

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In addition to expression of innate immune mediators, we further determined the effects of cigarette smoking on airway epithelial injury and repair, which is described in **Chapter 6** (5). Here we examined the effect of CS on the airway epithelial barrier integrity and wound repair using a calcium switch assay and mechanical injury model respectively. CS exposure caused a delay in epithelial repair in both models. In contrast, combined epithelial injury and CS exposure further enhanced RNase 7 expression. Increased number of BCs were observed at the wounded edge of CS exposed injured cultures, thereby suggesting that CS reduced BC migration. It was furthermore shown that CS-induced oxidative stress contributed to impaired wound repair. Moreover, oxidative stress increased downstream MEK ERK1/2 signaling independent of EGFR, thereby causing a further increase in RNase 7 expression.

As CS exposure of epithelial cells caused transient impairment in epithelial repair while causing low cytotoxic effects, we further examined in **Chapter 7** the effect of CS on activation of the cytoprotective integrated stress response (ISR) in airway epithelial cells. We observed that CS caused transient activation of the ISR in differentiated airway epithelial cells, which could be reproduced in undifferentiated cells exposed to CS extract. In addition we observed that CS-induced mRNA expression of the ISR target genes GADD34 and CHOP was higher in COPD cultures compared to non-COPD controls and negatively correlated with lung function. In addition, we described how CS-induced oxidative stress can induce expression of GADD34 via an ISR-independent mechanism. In the discussion, we speculated that enhanced oxidative stress-dependent expression of GADD34 is needed to promote the production of inflammatory mediators by airway epithelial cells, and therefore may contribute to airway inflammation in COPD.

Next to epithelial injury and repair, we further explore the potential influence of CS on airway tissue remodeling in **Chapter 8** (6). Here we described that the surface bound matrix metalloprotease ADAM17 contributed to increased mRNA expression and shedding of soluble IL-6 receptor (sIL-6R) and amphiregulin (AREG). Basolateral protein shedding of sIL-6R and AREG mediated by ADAM17 was significantly higher in COPD cultures. In addition to ADAM17, we furthermore observed that CS-induced EGFR signaling was involved IL-6R and AREG mRNA and protein expression, thereby further demonstrating the importance of EGFR in airway epithelial inflammation and repair/remodeling.

In **Chapter 9** we further examined the expression of the novel innate immune mediators WFDC12 in airway epithelial cultures. Similar to RNase 7, WFDC12 expression was only observed in undifferentiated cultures upon microbial stimulation. However, CS exposure did

not affect the expression in differentiated cell cultures, as was seen with RNase 7. Stimulation with the pro-inflammatory cytokines IL-1β and TNFα did however increase the expression of WFDC12 in differentiated cells, whereas TGFβ1 attenuated the expression. Constitutive expression of WFDC12 increased upon mucociliary differentiation, and was furthermore enriched in the luminal airway epithelial fraction of differentiated cell cultures. Moreover, we observed lower WFDC12 expression in COPD cultures compared to non-COPD controls, which correlated with lung function.
Finally, in **Chapter 10** (7) it is discussed how the impaired antimicrobial defense system in COPD but also other noninfectious and infectious lung diseases can be therapeutically targeted. Here it is mentioned that the COPD patients may be treated with synthetic AMPs, AMP–inspired molecules or through induction of endogenous AMPs to prevent microbial infections and colonization.

ROLE OF IMPAIRED AIRWAY EPITHELIAL ANTIBACTERIAL DEFENSE ON MICROBIAL COLONIZATION AND INFECTIONS IN COPD

The increased susceptibility of smokers and COPD patients to microbial infections raised the question whether airway epithelial innate host defense is attenuated. It has been shown that mucociliary clearance is impaired in response to CS and in COPD (8). Although this function is indeed critical for airway host defense, it has been shown in primary ciliary dyskinesia patients, which display impaired mucociliary clearance, that other host defense mechanisms allow sufficient protection against microbes (9). Studies in cystic fibrosis (CF) suggest that an additional impaired activity of antimicrobial proteins and peptides (AMPs) causes a more severe susceptibility to microbial colonization and infections (10). Therefore, we have focused on the airway epithelial defense functions mediated by AMPs, as this mechanism is largely neglected in COPD. We have observed that the expression of AMPs is differentially regulated in intact epithelial cells, upon microbial exposure, and upon injury and repair. CS and COPD disease status have different effects on the expression of these different classes of AMPs, which will be discussed further.

Constitutively produced AMPs

As shown in the seminal study by *Smith et al.*, the airway surface liquid of differentiated airway epithelial cultures from healthy individuals displayed intrinsic antibacterial activity towards respiratory pathogens (11).This defense against low number of microbes was seen without prior stimulation with pro-inflammatory mediators, suggesting that differentiated airway epithelial cells have a constitutive expression of mediators that provide baseline antibacterial defense. In line with this it is shown in **Chapter 5** that several AMPs, i.e. SLPI, SPLUNC1 and LPLUNC1, are highly expressed at a steady-state level in differentiated airway epithelial cells and allocated to the luminal airway epithelial cells. Similar to mucociliary clearance, alterations in luminal airway epithelial cell differentiation or composition may affect the expression of constitutively produced AMPs in COPD. We have shown that chronic CS exposure attenuated the expression of luminal cell-restricted AMPs by suppressing cell differentiation. In contrast to the effects of CS, we did not observe differences in the expression of constitutively produced AMPs and baseline antibacterial activity between COPD and non-COPD cultures (**Chapter 4 (4)**). This is likely caused by the low disease severity in examined

patients, whereas in more severe (GOLD IV) patients the expression might be abrogated due to persistent epithelial remodeling in culture (12, 13).

Persistent epithelial remodeling, i.e. basal cell hyperplasia and squamous metaplastic lesions, may attenuate expression of luminal cell-specific AMPs. Moreover, goblet cell metaplasia, for instance mediated by the cytokines IL-13, IL-4, IL-17 (14), may alter the expression of AMPs that are restricted to a specific luminal cell type. A previous study in our laboratory has shown that SLPI is not significantly altered in goblet cell enriched cultures (15). However, it was shown by others that SPLUNC1 and LPLUNC1 are respectively absent or restricted to goblet cells (16). In addition to the AMPs examined in this thesis, *Tanabe et al.* have also shown that the antibacterial protein $sPLA_2$ is increased upon ciliated cell differentiation and lost in goblet cell enriched cultures (17). Moreover, as shown in a secretome analysis of differentiated airway epithelial cultures (18), also other antimicrobials such as dermcidin are constitutively produced by differentiated airway epithelial cells (19). This further suggests that a broader spectrum of other AMPs are also regulated by cell differentiation and restricted to luminal cells. Further research is therefore required to understand the broad composition of constitutively-produced AMPs in differentiated airway epithelium and how epithelial remodeling may affect this. This for instance may explain the selective colonization of certain microbes in remodeled airway lesions, which might be caused by a favorable micro-environment shaped by altered expression of constitutively produced AMPs (20). In conclusion, alterations in airway epithelial differentiation due to smoking and persistent epithelial remodeling in COPD may result in a weakened constitutive host defense due to impaired expression of luminal cell-restricted AMPs. This may lead to onset of microbial outgrowth and early colonization of opportunistic respiratory pathogens in the lungs of smokers and COPD patients.

Microbial- and cytokine-induced AMPs

Next to constitutively produced AMPs, microbial infection may lead to the further induction of other types of AMPs in the airway epithelium upon activation of patter recognition receptors and downstream innate immune signaling pathways. In previous studies it was shown that CS exposure reduced the antibacterial defense against opportunistic pathogens of cultured airway epithelial cells (21). This finding corresponded with impaired expression of the AMP hBD-2 in CS-exposed cultures and with the attenuated expression of hBD-2 in tracheal washing of smokers with pneumonia. Extending these findings, we have shown in **Chapter 4** (4) that COPD airway epithelial cells displayed reduced antibacterial activity compared to non-COPD smokers, which corresponded with impaired expression of several microbial-induced AMPs including hBD-2. This finding was furthermore in agreement with observational studies in bronchial tissues from COPD patients, in which lower hBD-2 expression persisted after smoking cessation (22). We were unable to identify the molecular mechanism causing a reduced antibacterial activity of COPD airway epithelial cells, and therefore further research remains needed to determine this. However, we did gain more insight in the mechanisms underlying the impaired antibacterial activity of airway epithelial cells in response to CS. First, we observed that not only expression of hBD-2 was attenuated by CS, but also a panel of other AMPs (4). This suggests that the attenuated antibacterial activity of airway epithelial cells by smoking is the result of abrogation of several microbial-induce AMPs in addition to hBD-2. Next to impaired expression in response to microbial stimuli, we also observed that CS impaired the expression of AMPs upon stimulation with

TNFα and IL-1β (unpublished data). This suggests that attenuated expression of AMPs is not due to effects of CS at the receptor level, but due to alterations in common downstream innate immune signaling pathways. Supporting this, we found that CS impaired the NFκB signaling pathway, which in previous studies was shown to be critical in regulating epithelial expression of several AMPs (23-25). In a previous study it was shown that CS-induced oxidative stress contributed to impaired expression of hBD-2 (26). Therefore it is likely that reactive oxygen species (ROS) mediates suppression of NFκB signaling via post-translational modifications of upstream kinases (27, 28). It has previously been shown that reduced hBD-2 expression in the airways of COPD patient was localized in the upper, but not in the lower airways (22). This might be explained by differences in the density of cigarette smoke particles to which the upper and lower airways are exposed, and suggests that a certain threshold is present at which antibacterial defense is impaired. It has been shown that diesel exhaust may also impair expression of hBD-2 in differentiated airway epithelial cells (29). Therefore, attenuation of the inducible antibacterial defense system in airway epithelial cells may be a common target of air pollutants. In summary, the microbe-induced expression of AMPs is a second defense mechanism that may prevent the further outgrowth of microbes when the constitutive airway epithelial defense system is overwhelmed. Attenuation of this defense system in COPD, because of smoking or exposure to air pollutants, may increase the susceptibility to microbial infection. This mechanism may therefore contribute to microbial infections in smokers or COPD patients during disease exacerbations.

Injury and repair-induced AMPs

Although airway BCs are normally quiescent, findings in **Chapter 3** (3) and **5** suggested unique host defense responses by these cells in the context of epithelial injury and repair (30). hBD-1 has been described in previous study as a constitutively expressed AMP (21). Although this is indeed the case, we observed that hBD-1 was highly expressed in undifferentiated cultures and relatively reduced upon differentiation. This suggests that BCs display a high expression of hBD-1 upon initial phases of epithelial regeneration that may contribute to epithelial defense upon injury. Interestingly, when comparing COPD and non-COPD cell cultures, we observed that hBD-1 expression was significantly higher in COPD cultures (unpublished data). This corresponded with an observational study, which demonstrated enhanced levels of hBD-1 in sputum samples of COPD patients compared to healthy controls (31). Similar to hBD-1, we furthermore described in **Chapter 3** (3) that the antimicrobial protein RNase 7 was highly expressed by cultured BCs. Although we did not observe a significant higher expression of RNase 7 in COPD cultures (unpublished data), we observed that CS exposure increased RNase 7 expression in BCs of differentiated cells. This was related to a transient impairment in the epithelial barrier integrity, and could be further enhanced upon epithelial wounding as described in **Chapter 6** (5). This, also suggests that RNase 7 is a damage-induced AMP, which is increased in activated BCs. Indeed, stimulation of undifferentiated airway epithelial cells with NTHi, increased the expression of RNase 7 and other microbial-induced AMPs, i.e. hBD-2, CCL20, LCN2 (**Chapter 3** (3)). Therefore, BCs in damaged epithelium may display more broad antimicrobial activity that may prevent microbial colonization and infection during wound repair. In summary, increased epithelial injury in COPD and smoking is reflected by the enhanced expression of BC-produced AMPs. Moreover, the epithelial restricted expression of hBD-1 and RNase 7 in BCs of damaged epithelium, suggests that these proteins may be used as selective biomarkers that may reflect epithelial injury in smokers and COPD. The

functional contribution of hBD-1 and RNase 7 on the antibacterial defense in the injured lung requires further investigation. However, it can be speculated that the enhanced expression of these AMPs in smokers and COPD may contribute to a favorable micro-environment that leads to selective microbial outgrowth as mentioned in **2.2** (20). Another explanation, is that hBD-1 and RNase 7 do not provide sufficient protection, as the expression of other AMPs is reduced. For instance CS suppresses microbial- and cytokine-induced AMPs. Furthermore, cigarette smoke may modulate vitamin D3-induced antibacterial responses, which require further study. Interestingly, it has been shown that EGFR activation in keratinocytes leads to expression of human beta-defensin 3 and LCN2 (32). These responses are lacking in airway epithelial cells exposed to CS. This differential response between keratinocytes and airway epithelial cells might be due to tissue dependent EGFR-mediated responses. However, another explanation is that CS selectively modulates downstream EGFR signaling transduction, leading to a selective impaired expression of certain AMPs.

AIRWAY EPITHELIAL INFLAMMATORY RESPONSES

In addition to AMPs, we furthermore determined the expression of pro-inflammatory mediators as indicator of epithelial inflammatory responses. In particular the role of the chemoattractant CXCL8/IL-8 is of importance, as it reflects the contribution of airway epithelial cells in neutrophilic inflammation in COPD (33, 34). In this section the role of CS-induced airway inflammation will be discussed, highlighting the importance of epithelial injury and repair as cause of inflammation, and the contribution oxidative stress.

Cigarette smoke-induced inflammation as part of epithelial repair

In line with previous studies (35, 36), we have shown that CS exposure increases IL-8 expression in airway epithelial cells via activation of ADAM17/EGFR signaling transduction (**Chapter 3,6,8** (3, 5, 6)). In contrast to RNase 7, we observed IL-8 expression in both luminal and basal cells of differentiated cell cultures (3). It has been shown that EGFR is selectively expressed by BCs (37). Therefore, it can be speculated that luminal epithelial IL-8 expression is indirectly mediated via currently uncharacterized BC-interactions. EGFR-dependent release of secreted factors from BCs may for instance cause induction of inflammatory responses in the luminal airway epithelium. Further research is needed to understand the potential basal-luminal cross-talk, which might be targeted to dampen the inflammatory response in the epithelium. We furthermore showed that combined exposure of CS with either microbial stimuli or injury further enhanced IL-8 expression (**Chapter 4 and 6** (4, 5)). Microbe-induced activation of the innate immune system mediates pro-inflammatory responses by airway epithelial cells in large part via NFκB signaling transduction. However, also based on observations by others (38-40), it can be questioned if the NFκB system has an important role in airway epithelial inflammatory responses during cigarette smoking. As discussed in paragraph **2.3**, the NFκB-dependent innate immune responses seems to be suppressed by CS, thereby contributing to microbial colonization and infections. In contrast, sustained activation of MAPK signaling and the downstream AP-1 family of transcription factors have likely a more prominent role in the expression of inflammatory mediators in the airway epithelium.

EGFR and MAPK/AP-1 have a critical role in mediating tissue recovery after injury of

epithelial tissues. Therefore, it can be speculated that CS-induced inflammation is part of the epithelial repair mechanism, whereas immune cells such as neutrophils normally contribute to airway host defense at the site of injury. Although epithelial recovery and resolution of inflammation would normally occur, the repetitive injury caused by cigarette smoking will prevent this from happening. This may help to explain chronic neutrophilic inflammation in smokers, and its contribution to COPD development. Sustained expression of IL-8 in regenerating airway epithelium, is supported by previous studies showing elevated expression of IL-8 in a xenograft model (41). Also in **Chapter 6** (5), we observed enhanced expression of IL-8 in CS-exposed airway epithelial cells with an impaired barrier integrity. In addition, we have shown in **Chapter 5** that IL-8 expression is elevated upon chronic exposure of airway epithelial cells during cell differentiation. This furthermore suggests a relation between an injured or repairing state of the airway epithelium and expression of pro-inflammatory cytokines.

In addition to the role of epithelial repair mechanisms, inflammatory responses may also result from a loss of anti-inflammatory mediators expressed by the luminal airway epithelium, i.e. SLPI and CC10/CC16 (42, 43). These secreted factors may normally act in intact epithelial layers as inhibitory signals to maintain airway homeostasis, while loss of these mediators upon luminal cell depletion may act as a negative signal to promote inflammatory responses in BCs, but also stromal and immune cells. Similar to SLPI and CC10, WFDC12 described in **Chapter 9** may also act as such an anti-inflammatory mediator, based on its high expression in differentiated luminal cells. However, further research is needed to evaluate this.

We did not detect differences in CS- and microbial-induced inflammatory mediator expression between COPD and non-COPD airway epithelial cultures (44). Since these cultures were derived from current and former smokers, it needs to be noted that we did not compare these cultures to those of healthy never-smokers, and possibly the induction of these mediators is lower in healthy airway epithelium. Moreover, it possible that in contrast to differentiated cells, enhanced inflammatory responses may persist in undifferentiated cultures. This was shown in a previous study by *Schulz et al.* (45), which observed enhanced inflammatory mediator expression of undifferentiated airway epithelial cells from COPD patients compared to non-COPD smokers. Similar findings have also been reported in studies comparing airway epithelial cultures from CF patients and non-CF controls, whereas it was shown that enhanced inflammatory responses were only seen in undifferentiated CF airway epithelial cells (46, 47). This raises the interesting possibility that especially in injured epithelium, dominated by basal cells, differences between epithelium from patients and controls becomes apparent. Therefore, further studies are required, in which the importance of airway epithelial repair and differentiation status will be determined in the expression of inflammatory mediators in COPD cultures, as this will further reflect the importance of injured on airway epithelial inflammatory responses.

Role of CS-induced oxidative stress in inflammation

Reactive oxygen species present in smoke, and induction of oxidative stress may also contribute to airway inflammation upon smoking and in COPD. CS-induced oxidative stress causes a delay in epithelial repair as shown in **Chapter 6** (5) and therefore may lead to sustained inflammatory responses mediated by repairing signals. Moreover, oxidative stress

can increase MAPK signaling, thereby enhancing the expression of inflammatory mediators via these pathways. As speculated in **Chapter 7**, oxidative stress may furthermore control the translation of inflammatory mediators through induction of GADD34. In previous studies, it has been shown that GADD34 controls protein translation of IL-6 and interferons upon viral infection by temporary suppression of the integrated stress response (48, 49). Similar to this, CSC-induced oxidative stress may promote the translation of pro-inflammatory cytokines in airway epithelial cells via MAPK p38-dependent expression of GADD34. This mechanism may explain our recent findings in which airway inflammation is reduced in CS-exposed GADD34 deficient animals (unpublished data). However, further research is needed to confirm the contribution of GADD34 in regulating airway epithelial inflammatory responses after cigarette smoke exposure.

AIRWAY EPITHELIAL REMODELING

The airway epithelium is distinct from other mucosal tissues by its low steady-state turn-over in the lungs of healthy individuals (50). This is reflected by the quiescent state of BCs, which act as the main progenitor cells. Observations made in airway tissues from smokers, demonstrated that BCs have an enhanced turn-over (51), and it is hypothesized that the renewal capacity of airway BCs is limited (52). Smoking may therefore accelerate the aging of the airway epithelial tissues (53), as impaired regenerative capacity of BCs may underlie epithelial remodeling in smokers and COPD.

As mentioned in previous sections, EGFR has a prominent role in airway epithelial responses upon CS exposure. EGFR expression is largely restricted to BCs and plays a key role in wound repair (37, 54). Different ligands can activate EGFR, i.e. epidermal growth factor (EGF), amphiregulin (AREG), transforming growth factor-alpha (TGFα), and ligand-dependent activation of EGFR has been shown in recent studies to have a prominent role in airway epithelial remodeling. EGF and AREG are both enhanced in the airway epithelium of smokers, however the cells that express the ligands are different. EGF is mainly expressed by ciliated airway epithelium, whereas AREG is mainly expressed in BCs (37, 55). It has been shown *in vitro* that AREG and EGF are both critical in affecting airway epithelial remodeling. EGF is released by cultured epithelial cells upon exposure to CS extract, moreover exogenous stimulation of airway epithelial cells with EGF during differentiation causes a squamous cell phenotype (37). We have demonstrated in **Chapter 8** (5) that CS exposure of airway epithelial cells enhanced mRNA expression and ADAM17-dependent shedding of AREG protein, which was more prominent in COPD epithelial cultures. This enhanced release of AREG may further contribute to epithelial remodeling, as it has been shown by that AREG released by BCs, together with EGF, may affect epithelial differentiation by promoting basal cell hyperplasia and goblet cell metaplasia (55). These observations made *in vitro* were supported by a transcriptome analysis, demonstrating EGFR-dependent remodeling of the small airways of smokers and COPD patients resulting in a more proximal airway epithelial phenotype in the distal airways(56). Despite enhanced EGFR signaling, and aberrant epithelial differentiation during chronic CS exposure, we did not observe goblet cell metaplasia in our experimental model in **Chapter 5**. This might be due to a the lack of EGF expression in the luminal airway epithelial cells, which we have not addressed. Moreover, the experimental setup of the chronic

CS exposure model could have been to restricted. In our model, we observed transient and mild injury induced by CS exposure with no differences in the epithelial barrier integrity. EGF released from luminal airway epithelial cells may only affect BCs upon extensive injury, as has been shown in the case of Neuregulin (57). Therefore, it would be interesting to determine the effect of additional epithelial injury on epithelial differentiation in the chronic CS exposure model. This could for instance be achieved by incubating cells with calcium-free medium to abrogate the epithelial barrier integrity or by mechanical wounding. In addition, we did not determine the influence of chronic cigarette smoke exposure on airway epithelial cell cultures that were well-differentiated. This may lead to different alterations in epithelial differentiation compared to chronic smoke exposure in undifferentiated cells, and therefore requires further study.

Microbial products have also been shown to contribute to goblet cell metaplasia, and these were absent in our model which may help to explain the lack of goblet cell metaplasia in our CS-exposed cultures. Another explanation for the lack of goblet cell metaplasia might be the additional contribution of immune cells in airway epithelial remodeling, which are absent in our *in vitro* system. Indeed, it has been well established that cytokines such as IL-13, IL-4 and IL-17, derived from T-lymphocytes or innate lymphoid cells, may induce goblet cell differentiation (14). However, also neutrophils, the predominant cell type attracted by smoking may contribute to epithelial remodeling. As discussed in **Chapter 2** (2), neutrophils produce the AMPs LL-37 and alpha-defensins, which have been shown to modulate airway epithelial cell responses. LL-37 has been shown to mediate transactivation of EGFR by mediating matrix metalloprotease dependent shedding of EGFR-ligands (58). The ligands responsible for this are unclear, however it is possible that LL-37-mediated EGFR activation may contribute to epithelial remodeling. In addition, expression of HNPs was associated with squamous lesions in airway tissues from smokers, and it has been shown that HNPs can induce mucin expression and promote proliferation in airway epithelial cell lines (59-61). Therefore, further research examining the influence of neutrophil-derived secreted factors, together with CS exposure, may give further insight in the mechanisms underlying epithelial remodeling in smokers and in COPD.

THE VICIOUS CIRCLE HYPOTHESIS REVISITED: ROLE OF THE AIRWAY EPITHELIUM

Collectively, the studies presented in this thesis support an important role for the modulatory effect of CS on airway epithelial function in the development and progression of COPD. It is speculated that progressive impairment of airway epithelial host defense, resulting from smoke exposure, may act as an important driver in the onset of COPD in susceptible smokers. Such a suppression of constitutive expressed AMPs in luminal airway epithelial cells together with impaired mucociliary clearance may allow early microbial colonization. Microbial outgrowth and/or changes in the airway microbiota subsequently leads to activation of microbe-induced innate immune responses. Smoking may causing an imbalance in inflammation and host defense, thereby selectively decreasing microbe-induced expression of AMPs while enhancing inflammatory responses. This may be more pronounced in COPD patients, since it was found that expression of AMPs is also reduced in absence of CS exposure. Smoking and

additional damage caused by microbes or immune cells leads to further damage to the airway epithelium. This results in extensive injury, leading to loss of luminal cells and activation of BCs. In part via EGFR, activation in BCs further promotes inflammatory responses while wound repair is delayed due to smoking. Moreover, the combined effect of smoking on EGFR-mediated repair and immune cells leads to aberrant epithelial differentiation. This process will take place repeatedly until the airway epithelial tissues are persistently altered, as seen in severe COPD patients, likely due to epigenetic imprinting.

CONCLUDING REMARKS

Collectively, the studies presented in this thesis emphasize that inflammation, host defense and epithelial repair are interconnected processes that are affected by smoking and disturbed in COPD. Furthermore, the studies suggest that in particular targeting epithelial repair in COPD may have beneficial outcomes on inflammation and host defense and may be an effective approach to halt COPD progression. Since inflammation in COPD is at least part resistant to steroid treatment (62), and steroids are known to impair wound repair and lung host defense (63, 64), this furthermore stresses the potential benefit of targeting airway epithelial repair.

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