Chapter 6

General discussion

With this thesis, we aim to expand the current understanding of the importance of airway epithelial cell survival in response to environmental triggers relevant to asthma. From this aim, it can be appreciated that a central role is reserved for the airway epithelial cells throughout the thesis. As described in the introduction, the airway epithelium forms a chemical, physiological and immunological barrier between the external and internal environment [1]. Therefore, airway epithelial cells are the first in line to encounter environmental triggers such as cigarette smoke (CS), respiratory viruses and aero-allergens. Next to the fact that these three environmental triggers are associated with the inception and exacerbation of asthma, they can all affect in their own specific way airway epithelial cell survival, as described in detail in the introduction of this thesis. Until now, the role of airway epithelial cell survival in the inception and exacerbation of asthma has only moderately been explored. By taking advantage of the high expression of Pim1 kinase in airway epithelial cells and its well-known central role in cell survival [2][3], we aim to provide new insights in the role of survival of airway epithelial cells exposed to CS, respiratory viruses and aero-allergens *in vivo* and *in vitro*. However, because of its ability to phosphorylate a wide range of proteins on serine and/or threonine residues, Pim1 kinase is involved in a broader variety of cellular processes, including cell growth, cell differentiation and inflammatory responses [2][4]. Hence, in this thesis we not only explored the role of Pim1 kinase on cell survival of airway epithelial cells upon exposure to environmental triggers, but also revealed the effects of Pim1 kinase in the anti-viral inflammatory response and innate immune response upon exposure to respiratory viruses and HDM, respectively. This discussion will give a short overview of each experimental chapter and comprehensively review the effects of Pim1 kinase activity on airway epithelial cells upon exposure to the individual environmental triggers. After speculation on future perspectives of the research, an overarching conclusion will be presented.

Cigarette smoke

The role of Pim1 kinase in the response of the airway epithelium upon exposure to CS has been studied into detail *in vivo* as well as *in vitro* in **chapter 2**. First, we demonstrated that the mRNA expression of Pim1 kinase – but not the other 2 family members - in mouse lung tissue was transiently induced upon exposure to CS, highlighting an association between induction of Pim1 kinase activity and the exposure of airway epithelial cells to CS. Subsequent subchronic CS exposure of *Pim1*-deficient mice resulted in enhanced neutrophilic airway inflammation compared to *Pim1*-proficient mice. We evaluated the mechanistic basis for these remarkable results *in vitro* using the bronchial epithelial cell line BEAS-2B, stimulated with a concentration gradient of cigarette smoke extract (CSE) in the absence or presence of a pharmacological Pim1 kinase inhibitor. These experiments clearly showed that BEAS-2B cells are more susceptible towards loss of mitochondrial membrane potential and the induction of cell death upon stimulation with CSE in the absence of Pim1 kinase activity. *In vitro*, induction of cell death was accompanied by increased release of the damage-associated molecular pattern (DAMP) heat-shock protein (HSP) 70 . Although we could not detect differences in airway epithelial cell death and release of HSP70 between *Pim1*-deficient and -proficient mice *in vivo*, we observed a significant increase in release of the DAMP S100A8 in the BAL fluid of *Pim1*-deficient mice. Taken together, the data described in chapter 2 suggest that the expression of Pim1 in airway epithelial cells protects against CS-induced necrotic cell death and activation of an innate pro-inflammatory immune response.

CS is known as the main causative factor for chronic obstructive pulmonary disease (COPD), which is characterized by not fully reversible airflow limitation, neutrophilic airway inflammation and airway remodeling [5]. In addition, inflammatory infiltrates in the alveolar walls and destruction of alveolar septa might ultimately lead to the development of emphysema [5]. Since only 20% of people who smoke actually develop COPD, susceptibility to this disease is at least in part genetically

determined [6]. In susceptible smokers, exposure to CS leads to exaggerated inflammation resulting in tissue damage and structural changes of the airways [7]. To study the detrimental effects of CS, several mouse models in which mice are directly exposed to CS have been developed. Acute and sub-chronic exposure to CS for 3 days and 4 weeks, respectively, results in increased airway inflammation as determined by influx of inflammatory cells like macrophages and neutrophils into the BAL fluid [8][9]. In addition, characteristics of emphysema can be observed in models of chronic exposure to CS for 26 weeks [8]. Therefore, these mouse models are highly suitable to study the effects of CS in the airways and in particular the CS-induced neutrophilic airway inflammation.

The neutrophilic airway inflammation observed in both human subjects and mouse models upon exposure to CS, is a consequence of the release of pro-inflammatory chemokines like the neutrophilic attractant IL-8 by airway epithelial cells. Airway epithelial cells can secrete IL-8 upon various triggers, including CS-induced oxidative stress and activation of pattern recognition receptors (PRRs) expressed on these cells [7][8][9][10][11]. Although PRRs can be directly activated by CS components like lipopolysaccharide, it has been postulated that indirect activation of PRRs by DAMPs released from airway epithelial cells upon CS-induced immunogenic cell death also contributes to neutrophilic airway inflammation [7][12]. In support of this notion, CS-induced necrotic cell death, as also observed in the experiments described in this thesis, has been observed in several *in vitro* studies [13][14][15][16]. Besides immunogenic cell death, it has been shown that exposure to CS can induce autophagy [17]. Autophagy includes a homeostatic program in which cellular organelles and long-lived-proteins are integrated in double-membrane autophagosomes and lysosomally degraded [17][18]. Excessive autophagy may promote cell death, and Chen *et al* showed that increased autophagy is associated with CS-induced lung injury[17]. Moreover, a recent study by Mizumura *et al* suggested that especially mitophagy - the autophagy-dependent elimination of mitochondria upon mitochondrial dysfunction - is involved in CS-induced cell death of airway epithelial cells through

stabilization of its regulator PINK1 and leads to emphysematous changes [18]. The contribution of mitochondrial dysfunction to COPD is recently underscored by a study of Hoffmann *et al*, who showed increased expression levels of PINK1 in PBECs from COPD patients compared to healthy individuals [19]. Taken together, several cell death related mechanisms of airway epithelial cells are likely to be involved in CS-induced lung injury. While exposure to CS and the subsequent inflammatory responses are conventionally discussed in the context of COPD, these processes also bear relevance to asthma: patients with asthma who actively smoke suffer from more severe asthma symptoms, more neutrophilic airway inflammation and more airway remodeling [20]. Therefore, studying these processes in the context of asthma is certainly warranted.

Although we showed enhanced neutrophilic airway inflammation upon exposure to CS in mice deficient for Pim1 kinase activity, a causal relationship between Pim1 kinase activity and increased airway epithelial cell death *in vivo* has not formally been established. In contrast, the association between Pim1 kinase activity and airway epithelial cell death upon exposure to CS in our *in vitro* experiments is rather clear. Reduction of mitochondrial membrane potential upon increasing concentrations of CSE has been described before [15] and the stronger reduction hereof upon inhibition of Pim1 kinase activity corresponds to one of the best-studied mechanism by which Pim1 kinase exerts its pro-survival effect [21][22][23]. Initially upon exposure to CS, the intrinsic apoptotic pathway is known to be activated [14]. Permeabilization of the outer mitochondrial membrane leads to the release of pro-apoptotic mediators cytochrome C and apoptosis-inducing factor and subsequent activation of effector caspases and the execution of apoptosis [15][24]. However, as a consequence of the concentration dependent blocking effects of CS on mitochondrial respiration and ATP production, the apoptotic cell death program cannot be fully executed [14][15][25]. A switch from apoptotic into necrotic cell death will occur [14][15][25], leading to the release of DAMPs into the external environment [26].

By phosphorylating the BCL-2-associated agonist of cell death (BAD) on the mitochondrial membrane, Pim1 kinase can increase the threshold for apoptosis [23]. In contrast, absence of Pim1 kinase activity - as mimicked by the specific pharmacological Pim1 kinase inhibitor K00135 [27] - will result in predisposition towards the induction of apoptosis. With the presumption that the available amount of ATP is independent of Pim1 kinase activity, we anticipate that cells treated with the Pim1 kinase inhibitor are more susceptible to switch to necrotic cell death. In line herewith, we observed a significant increase in necrotic cell death upon stimulation with CSE in the presence of the Pim1 kinase inhibitor in chapter 2. Furthermore, we observed enhanced release of the DAMP HSP70 in the presence of the Pim1 kinase inhibitor. Unfortunately, our results obtained *in vitro* could not directly be extrapolated to our *in vivo* study, since we did not observe induction of cell death upon exposure to CS or differences in the release of HSP70 into the BAL fluid in mice exposed to CS, independent of genotype. The absence of CS-induced cell death *in vivo* could be a consequence of the relative late time point of analysis: 16 hours after the last CS exposure, which might give the airway epithelial cells in an *in vivo* system sufficient time to recover from the harmful effects of CS exposure. This lack of consistency in HSP70 release could be explained by the fact that the basal levels of HSP70 were already quite high in the FVB/Nrcl mouse strain used in this experiment, precluding further increase upon exposure to CS. Another explanation is that not HSP70 but other DAMPs are associated with CS-induced neutrophilic airway inflammation [28], which is further supported by the fact that we did observe differences in levels of another DAMP, S100A8. Indeed, neutrophilic airway inflammation in mice exposed to CS has been associated with S100A8 before [29][30], although we cannot exclude the possibility that S100A8 originates from other cells than the airway epithelial cells. A recent study by Heijink *et al* suggest that CS-induced cell death of neutrophils causes release of DAMPs, which in turn promotes the pro-inflammatory response induced in airway epithelial cells [31]. Analysis of the release of S100A8 from neutrophils was not performed in this study, but it is known from literature that together with a wide range of other cell

types, neutrophils also produce S100A8 [32]. Thus, the reduced viability of neutrophils in the absence of Pim1 kinase activity could also explain the increased levels of S100A8 observed in *Pim1*-deficient mice exposed to CS, as shown in chapter 2. It has been shown that Pim1 kinase is important for survival of eosinophils, and the viability of eosinophils is markedly reduced in the absence of Pim1 kinase activity [33]. However, whether Pim1 activity itself affects neutrophils has, as far as we know, not been established to date. Moreover, a critical role for Pim1 in neutrophil survival is not supported by the fact that we observed increased levels of neutrophils in the BAL fluid of these mice (Chapter 2).

Taken together, chapter 2 of this thesis shows that Pim1 expression can protect from neutrophilic airway inflammation induced upon exposure to CS in mice, which might be highly relevant for asthma as well as for COPD. Notwithstanding the fact that our data suggest airway epithelial cell survival to be causative in the induction of this inflammatory response, we have not unambiguously established airway epithelial cell survival as the determinant in the development of neutrophilic airway inflammation upon exposure to CS. Moreover, further studies into the role of cell survival upon exposure to CS in the inception and exacerbations of asthma are warranted.

Respiratory viruses

In **chapter 3 and 4**, the effects of a relevant respiratory virus, human Rhinovirus (HRV)-16, on airway epithelial cells were evaluated into detail in two different well-established *in vitro* culture models of human primary bronchial epithelial cells (PBECs). In **chapter 3**, we studied the effect of Pim1-dependent cell survival of PBECs from healthy individuals in monolayer cultures upon infection with HRV-16. In the presence of a pharmacological Pim1 kinase inhibitor, viral replication and release of viral particles was significantly reduced compared to PBECs infected with HRV-16 in the absence of the inhibitor. While the anti-viral inflammatory response was only marginally induced upon viral infection of these cells, we observed that the reduced viral replication observed in the virally infected PBEC cultures treated with the Pim1 kinase inhibitor was associated with enhanced induction of cell death in these cultures.

The airway epithelium fulfills a dual role in respiratory viral infections. On one hand, airway epithelial cells serve as the host cell for viruses to replicate and therefore, airway epithelial cells contribute to the severity of the viral infection. On the other hand, airway epithelial cells exert the first defense against respiratory viruses by initiating innate immune responses [34]. Upon infection, airway epithelial cells release type I and III Interferons (IFNs), which in turn activate the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway. Subsequent expression of anti-viral genes and *de novo* expression of IFNs results in prevention of viral replication and limitation of viral spread [35][36][37]. In addition, IFNs can induce apoptosis of airway epithelial cells through for instance activation of tumor suppressor gene p53 or the induction of protein kinase receptor (PKR) [38][39]. It has been postulated that this early apoptotic response is one of the key defensive mechanisms of airway epithelial cells to reduce viral load by promoting phagocytosis of infected cells and preventing viral replication and virion packaging in the infected airway epithelial cells[40]. Wark

et al showed that inhibition of apoptosis enhances the release of HRV viral particles, while induction of apoptosis by exogenous IFN-β resulted in reduced release of viral particles [40]. Especially the observed induced apoptosis upon exogenous administration of IFN-β suggests that IFN-β is the limiting factor in the early anti-viral and apoptotic response. In addition, the observed increase in viral replication in monolayer cultures of PBECs from asthmatic individuals compared to PBECs from healthy individuals was associated with resistance towards early apoptosis upon viral infection [40]. Furthermore, the association of the delayed onset of apoptosis with an impaired IFN response in these asthmatic patients underscores the importance of apoptosis as an anti-viral defense mechanism. Thus, our study described in chapter 3 is in line with these previous observations [40], but extends the current knowledge by showing that cell death can be induced not only by the administration of exogenous IFN-β, but also by interfering with endogenous survival pathways in the airway epithelial cell such as Pim1 kinase. Since the enhanced induction of cell death upon inhibition of Pim1 kinase activity was not associated with increased expression of IFNs, it is very likely that the effects of inhibition of Pim1 kinase activity are exerted through lowering of the threshold for apoptosis as described before [23]. However, with the experiments performed in our study, we could not dissect the exact mechanism by which inhibition of Pim1 kinase enhances the induction of cell death. In follow-up studies on the role of Pim1-mediated airway epithelial cell survival upon viral infection, evaluation of this exact mechanism is required.

All our studies with respiratory viruses were performed with HRV-16, a representative of the major group of the HRVs, belonging to the non-enveloped positive single stranded RNA viruses of the Picornaviridae family [34][41]. Major group HRVs enter the airway epithelial cells by binding to the intercellular adhesion molecule (ICAM)-1 and replicate inside the cells after internalization, in contrast to the minor group of the HRVs, that uses the low-density lipoprotein (LDL) receptor to

enter the cells [34]. The two groups of HRVs mainly differ in the cytopathic effects they cause in airway epithelial cells, which is more pronounced for the minor group HRVs [42]. It would therefore be interesting to study the effects of survival of airway epithelial cells on viral replication with a more aggressive minor group virus such as HRV-1B, and assess if interfering with the induction of cell death alters viral infection also for these more cytopathic strains. HRV infections are known to cause respiratory tract infections such as the common cold and lower respiratory infections, peaking in the spring and autumn, and are highly associated with the inception and exacerbations of asthma [34][41]. Next to HRVs, influenza and respiratory syncytial virus (RSV) are frequently associated with exacerbations of asthma and especially RSV infections with wheezing manifestation are a risk factor for the development of asthma [34][43]. Hence, future investigations on the effects of Pim1 kinase-dependent airway epithelial cell survival should also include infections with RSV and influenza to get a broader understanding of the role of airway epithelial cell survival in respiratory infections.

We show in Chapter 3 that viral replication is reduced upon enhanced cell survival in the presence of the Pim1 kinase inhibitor in monolayer cultures of PBECs. Notwithstanding the relevance of these observations, monolayer cultures of airway epithelial cells are a rather simplified model of the *in vivo* situation, best suited for the study of basal cell-like responses. Ideally, the effects of environmental triggers in asthma in general should be directly studied in patients actively suffering from asthma [44]. However, as a consequence of justified legal ethical and logistical restrictions, several models using animals, human tissue and human cells have been developed to allow a next-best model system for studying the airway epithelial responses to environmental triggers. While these models have resulted in an enormous increase of our current knowledge of asthma, it is important to take into account limitations of the distinct models. To start with animal, and mouse models in particular, these models offer an integrated physiological system and have provided important insights in the inflammatory and remodeling processes involved in asthma. Furthermore,

by the knock-down or overexpression of one specific gene, the function of the protein encoded by the gene could be studied in detail *in vivo* [44] [45]. Nevertheless, mouse models are not capable of reproducing all the characteristics of human asthma, which is exemplified by the fact that mice do not display spontaneous airway hyper-responsiveness (AHR) [46]. Furthermore, there are important species differences between human and mice regarding the development and structure of the airways [44] [46]. Another limitation relevant to the studies described in chapter 3 and 4 is presented by the fact that the major group of HRVs does not recognize the mouse ICAM-1 receptor and therefore, a transgenic mouse model expressing the human ICAM-1 receptor had to be generated to allow a mouse model for major group HRV infections [47]. Some of the problems with respect to the use of mouse models can be overcome by the use of lung cells derived from humans by bronchoscopy, lung resections or post-mortem procedures. With selective culture protocols, individual cell types including airway epithelial cells, central or peripheral fibroblast and airway smooth muscle cells can be outgrown from bronchial brushings, lung tissue of biopsies. Subsequent extensive culture of airway epithelial cells exposed to an air interface in the presence of retinoic acid for 21 day results in the formation of a pseudostratified epithelium containing ciliated, goblet and basal cells, which recapitulates the airway epithelial surface *in vivo* [44]. By exposing these cells to experimental conditions relevant to asthma, specific research questions can be investigated. In addition, by integrating two different cell types in one culture system, interaction between different cell types as occurring *in vivo* upon experimental conditions can be studied [44]. Nevertheless, these studies are limited by the availability of human airway material. As an alternative, airway cells can be immortalized by physical or chemical mutagenesis or the introduction of viral oncogenes, thereby creating cell lines with unlimited availability [48]. These cell lines have enormously contributed to the unraveling of the pathogenesis of asthma, though the immortalization process can have major effects on cellular differentiation, morphology and function and therefore, immortalized cell lines are

considered to be only partially representative of the *in vivo* behavior of cells [48]. Based on these considerations, we decided to extend our observations in monolayer cultures of PBECs by using the most optimal system for functional studies available to date, the air-liquid interface culture (ALI) model of PBECs.

Therefore, in **chapter 4** we explored the effects of inhibition of Pim1 kinase activity on viral infection of ALI cultures of PBECs from healthy and severe asthmatic individuals. We showed that inhibition of Pim1 kinase reduces viral replication in ALI cultures of PBECs from healthy individuals as well as from severe asthmatic individuals. Since analysis of cell death is more complicated in ALI cultures compared to monolayer cultures of PBECs and the anti-viral response in ALI cultures is much more pronounced than in monolayer cultures, we focused on the effect of Pim1 inhibition on the HRV-16-induced anti-viral inflammatory responses. We observed increased mRNA expression of IFN-β and IL-29 12 hours after HRV-16 infection of ALI cultures of PBECs from healthy individuals treated with the Pim1 kinase inhibitor, which was no longer observed 24 hours after infection. Interestingly, mRNA expression of the interferon-inducible genes IP-10 and RANTES was also significantly increased 12 hours after infection in virally infected ALI cultures upon inhibition of Pim1 kinase activity compared to the non-treated virally infected ALI cultures. While no difference between the mRNA expression of IP-10 and RANTES upon inhibition of Pim1 kinase could be observed 24 hours after infection, the protein levels of both IP-10 and RANTES were significantly increased 24 hours after infection. By analyzing the activation of the JAK-STAT pathway by the phosphorylation of STAT-1, we revealed that inhibition of Pim1 kinase activity results in an augmented interferon-induced anti-viral response upon viral infection.

As briefly described before, airway epithelial cells initiate an innate anti-viral response by the expression of the type I and III IFNs IFN-β and IL-29, respectively, upon infection with respiratory viruses. After binding of HRV-16 to the ICAM-1 receptor expressed on airway epithelial cells,

HRV-16 is endosomally internalized. Subsequent recognition of the replicative intermediate by cellular pattern recognition receptors like MDA-5, RIG-1 and TRL3 induces the expression of IFN-β and IL-29 [34][37][49]. Secreted IFN-β and IL-29 can cause dimerization of their receptor in an autocrine or paracrine manner, resulting in activation of the JAK-STAT pathway [36][50]. Translocation of activated STAT proteins from the cytoplasm to the nucleus induces the transcription of a wide range of interferon inducible genes, leading to reduction of viral replication and limitation of viral spread [50]. Impairment of this particular response has been postulated as one of the main reasons for the increased susceptibility towards viral infections observed in asthmatic patients [40][51][52]. Direct comparative studies between PBECs from healthy and asthmatic individuals by Wark *et al* and Contoli *et al* showed that viral replication was increased in monolayer PBEC cultures from asthmatic patients, which was associated with decreased expression of type I and III IFNs, respectively [40][52]. In our study, we could not detect differences in viral replication between ALI cultures of PBECs from healthy and severe asthmatic individuals. This observation is in line with current controversy in literature, since several studies in monolayer cultures as well as in ALI cultures of PBECs did not detect differences in viral replication between healthy and asthmatic individuals [53][54][55]. Since there are differences in the severity of asthma between these studies, medical treatment and subsequent asthma control might be an important determinant in the susceptibility towards viral infections and need to be considered when interpreting results. However, when evaluating our own study, the limited power of our asthmatic group should definitively also be taken into account. Notwithstanding the lack of differences in viral replication between healthy and severe asthmatic individuals, we demonstrate in chapter 4 that inhibition of Pim1 kinase can enhance STAT-1 activation and reduce viral replication in ALI cultures from asthmatic individuals, suggesting that inhibition of Pim1 kinase activity could be effective in limiting viral infection in asthma patients suffering from virally induced exacerbations.

Since Cakebread *et al* showed that the impaired IFN response observed in asthmatic patients is rather the consequence of reduced expression of IFNs than a deficient response to IFNs, exogenous administration of IFNs has been tested as novel therapy to reduce viral infections in asthmatic patients [35][56][57]. The occurrence of adverse side effects upon prolonged systemic administration of interferons tempered the enthusiasm of the promising results of these novel therapies, but recent clinical trials with local delivery of IFN-β through inhalation in virally induced exacerbations in patients with difficult-to-treat asthma have shown beneficial effects [58][57]. Therefore, new therapies improving the IFN response without actual administration of exogenous IFN might be of high interest as novel therapeutic approach. The postulated mechanisms by which inhibition of Pim1 kinase augments the IFN-induced anti-viral response is by enhanced proteosomal degradation of suppressor of cytokine signaling (SOCS) family proteins [59]. SOCS proteins represent a classical negative-feedback loop in JAK-STAT signaling, since they inhibit the cytokine signaling pathway that initially induces their expression [60]. The different SOCS proteins can directly or indirectly inhibit the activity of JAK, thereby limiting the IFN-induced anti-viral response [61]. Proteosomal degradation of SOCS proteins in an ubiquitin-mediated fashion occurs through binding to Elongin BC E3 ligases [62]. It has been shown that the binding to Elongin BC E3 ligases can be prevented by the phosphorylation of SOCS proteins by Pim1 kinase, resulting in stabilization of these SOCS proteins and enhanced suppression of JAK-STAT signaling [59]. In addition, Bedke *et al* observed that increased expression of SOCS-1 and SOCS-3 was associated with HRV infection of PBECs in a TGF-β-dependent fashion [63], suggesting that SOCS proteins contribute to the desensitization of HRV infected PBECs to Type-I interferon responses. A recent study by Gielen *et al* showed enhanced levels of SOCS-1 in bronchial epithelium of asthma patients *in situ* and these authors report that SOCS-1 suppresses the rhinovirus-induced expression of IFN [64]. Furthermore, Hashimoto and coworkers showed that SOCS proteins are involved in RSV infections as well, since they observed that transcriptional down-regulation of SOCS-1, SOCS-3 and cytokine-inducible SH2 protein (CIS) reduces viral replication of RSV in the carcinoma cell-line HEp-2 [65]. These studies all underscore the

contribution of SOCS proteins to viral replication. Unfortunately, we did not observe differences in the levels of SOCS-3 in the virally infected ALI cultures treated with the Pim1 kinase inhibitor, which might well be a consequence of high basal levels of SOCS-3 in these cultures. Nevertheless, the markedly enhanced phosphorylation of STAT-1 upon inhibition of Pim1 kinase activity as presented in chapter 4 prompted us to postulate that inhibition of Pim1 kinase leads to enhanced degradation of one of more members of the SOCS protein family, resulting in enhanced activation of JAK-STAT and subsequent augmentation of the IFN-induced anti-viral response [59].

Further research on the mechanism and the clinical relevance of our observations should reveal the potential of inhibition of Pim1 kinase activity as novel therapeutic approach in virally induced asthma exacerbations. Of interest, SOCS-3 has previously been identified as one of the key drivers of asthma gene expression levels in a large study using both GWAS data, expression profiling and immunohistochemical analyses on human lung samples in a systems biology approach [66]. By interfering with the available amount of SOCS-3, potentially therapeutic treatment with a specific Pim1 kinase inhibitor might have beneficial effects on asthma itself as well. Of note, an association between inhibition of Pim1 kinase and SOCS-3 should therefore be firmly established first.

To summarize the effects of respiratory viral infections on the airway epithelium in relation to Pim1 kinase activity, we showed that inhibition of Pim1 kinase activity augments the onset of cell death and enhanced the interferon-dependent phosphorylation of STAT-1. As a result, inhibition of Pim1 kinase activity appears to reduce viral replication through enhancement of cell death (chapter 3) and augmentation of the anti-viral response (chapter 4). These studies further establish the important role of apoptosis as protective mechanisms against respiratory viral infections and show that prolongation of the effects of IFN might also be a valid therapeutic approach for combating viral respiratory infections.

Interestingly, we showed that inhibition of Pim1 kinase could be a promising new therapeutic approach for the treatment of virally induced asthma exacerbations, since it is able to combine these two mechanisms suppressing viral replication in one single target.

Aero-allergens

In **chapter 5**, we assessed the role of Pim1 kinase in airway epithelial cells exposed to HDM. Upon pharmacological inhibition of Pim1 kinase activity, we observed a prolonged HDM-induced loss of barrier function in 16HBE cells. Interestingly, Pim1 kinase inhibition potentiated HDM-induced barrier dysfunction in PBECs. Furthermore, the HDM-induced release of the pro-inflammatory cytokine IL-1α from 16HBE cells was increased upon inhibition of Pim1 kinase activity. Consistent with our *in vitro* data, the levels of the pro-inflammatory cytokines CCL17 and CCL20 – known to be induced upon loss of airway epithelial integrity [67][68] - in lung tissue of HDM-exposed *Pim1*-deficient mice were increased compared to wild-type controls. While we observed that exposure to HDM caused increases in eosinophilic and neutrophilic airway inflammation as well as mucus metaplasia and AHR to metacholine, this increase was not affected by Pim1 kinase activity. However, levels of the Th2-cytokines IL-5 and IL-10 were significantly enhanced in the BAL fluid of *Pim1*-deficient mice, suggesting an exaggerated epithelial innate and adaptive Th2 response to HDM in the absence of Pim1 kinase activity.

As described before, modelling of asthma in mouse models is difficult since mice do not develop asthma by nature and the response displayed upon experimentally-induced asthma is different from the asthmatic response observed in humans [46][69]. The need of high concentrations of allergens to induce an asthmatic phenotype is conflicting with the low concentration of allergens normally experienced by humans [69]. Furthermore, the frequently used ovalbumin (OVA)-induced mouse model of allergic asthma requires peritoneal sensitization of OVA coupled to the adjuvant aluminum hydroxide, while allergic asthma in humans is initiated by airway epithelial cells upon inhalation of allergens [46] [70]. The development of HDM-induced mouse models of allergic asthma are therefore an enormous improvement, as sensitization to HDM is orchestrated in the lungs as presumably occurs in humans as well. Consequently, these HDM-models are thought to reflect better

the relevant pathophysiological mechanisms of asthma and are expected to offer better translation into clinical research [70]. In fact, we show that parts of the HDM-induced response in the experimental HDM-driven mouse model of allergic airway inflammation can be translated to human studies in chapter 5 of this thesis. In this chapter, we show that the enhanced innate immune response observed in HDM-challenged *Pim1*-deficient mice can also be observed in human airway epithelial cells stimulated with HDM in the presence of a specific pharmacological Pim1 kinase inhibitor, underscoring the parallels between the mouse model and the human *in vitro* studies. Differences between the OVA- and HDM-induced mouse models of allergic asthma, and the important role of airway epithelial cells herein, can be further demonstrated by comparing our study described in chapter 5 with a previous study by Shin *et al* [71]. Of note, differences in experimental set-up by using a pharmacological inhibitor of Pim1 kinase versus a germline deficient mice for *Pim1* kinase and the use of different mouse strains should certainly also be taken into account by comparing both studies. Shin and coworkers showed that pharmacological inhibition of Pim1 kinase during airway challenges in the classical OVA-induced mouse model of allergic asthma led to reduced AHR, eosinophilic airway inflammation and Th2 cytokine concentrations in the BAL fluid. In contrast, we did not observe any significant differences in the influx of eosinophilic and neutrophilic granulocytes into the airways or in AHR in HDM exposed FVB/Nrcl mice carrying a germline *Pim1*-deficiency [71]. We did, however, observe increased levels of the innate pro-inflammatory chemokines CCL17 and CCL20 in lung tissue of *Pim1*-deficient mice exposed to HDM compared to the HDM-treated wild-type controls, which was associated with enhanced Th2 activity as evidenced by increased levels of IL-5 and IL-10 in BAL fluid. Together with our *in vitro* data in which pharmacological inhibition of Pim1 kinase activity results in loss of airway epithelial integrity and subsequent release of pro-inflammatory mediators such as IL-1α, our study highlights the importance of the airway epithelial cells in the induction of allergic asthma and the important role

for Pim1 kinase in maintaining epithelial integrity. It also further underscores that the selection of the allergic asthma model is a highly important determinant for the outcome of an experimental study aimed at identifying an important pathway in asthma. The use of the HDM-driven mouse model as the preferable model to study allergic asthma *in vivo* is further supported by the recent discovery of group 2 innate lymphoid cells (ILC2s) as major producers of the traditional Th2 cytokines IL-4, IL-5 and IL-13 [72][73]. ILC2s were first identified in asthmatic humans – independent of atopy - and HDM-challenged mice that displayed eosinophilic airway inflammation together with production of Th2 cytokines in the absence of activation of the adaptive immune response [72]. ILC2s resemble Th2 cells to some extent in their cytokine production profiles, but lack antigen-specific receptors. Since ILC2 activation is dependent on the release of pro-inflammatory mediators such as IL-25, IL-33 and Thymic Stromal Lymphopoietin (TSLP) by airway epithelial cells, sensitization to allergens through the airway epithelium as achieved in the HDM-driven mouse models is essential to be able to study the involvement of ILC2s in asthma [72][74]. Although we did not specifically examine the contribution of ILC2s to the enhanced levels of IL-5 observed in the HDM-challenged *Pim1*-deficient mice, studies describing the involvement of Pim1 kinase in Th2 differentiation lend some support to the notion of the involvement IL-5-producing cells other than Th2 cells [75][76]. Jackson *et al* showed that upon inhibition of Pim1 kinase with a selective small molecule antagonist of Pim-1/3 kinase the polarization of $CD4^+$T cells towards Th1, Th2 and Th17 phenotypes was reduced, which subsequently impaired the production of IFN-γ, IL-4 and IL-21, respectively, by the polarized cells [75]. In addition, a study by Wang *et al* suggest that pharmacological inhibition of Pim1 kinase activity results in Runx3- dependent suppression of Th2 and Th17 differentiation and cytokine production in a peanut-induced allergic intestinal mouse model [76]. These studies imply that Th2 differentiation might be impaired in *Pim1*-deficient mice, thereby arguing against Th2 cells as the main source for IL-5. Hence, ILC2s could fulfill this role and increased levels of IL-5 observed in *Pim1*-deficient mice exposed to HDM might be a result of activated ILC2s.

The research described in chapter 5 suggests that Pim1 kinase protects airway epithelial cells against HDM-induced loss of airway epithelial barrier function. However, the mechanisms by which Pim1 kinase exerts this protective effect has not been studied in detail in the performed experiments. Recently, Juncadella *et al* studied the role of cell death of airway epithelial cells upon exposure to HDM [77]. They showed that upon exposure to HDM, apoptosis of airway epithelial cells was induced, followed by engulfment of apoptotic cells by viable neighboring airway epithelial cells. This process induced the expression of anti-inflammatory mediators such as IL-10, TGF-β and PGE2 in a *Rac1*-dependent fashion [77]. In mice with an airway epithelial-specific *Rac1*-deficiency, the authors observed increased numbers of eosinophils, increased AHR and enhanced levels of Th2-cytokines IL-4, IL-5 and IL-13 and the pro-inflammatory cytokine IL-33 in BAL fluid after HDM exposure [77]. These data suggest that in the absence of engulfment of apoptotic bodies, the HDM-induced inflammatory response is augmented. While we did not assess HDM-induced apoptosis and subsequent engulfment of apoptotic bodies by airway epithelial cells in our study, the increased levels of anti-inflammatory cytokine IL-10 observed in *Pim1*-deficient mice exposed to HDM could be explained by increased activity of this tolerogenic pathway due to enhanced sensitivity of the airway epithelium to HDM induced apoptosis. On the other hand, we also observed increased levels of IL-5 in BAL fluid and pro-inflammatory epithelial chemokines in lung tissue. These observations are in line with enhanced pro-inflammatory activation of the airway epithelium and enhanced activity of Th2 cells, which in the mouse can also be a source of IL-10. In addition, the lack of differences in the number of eosinophils and AHR between HDM-exposed *Pim1*-deficient mice and wild-type controls argues against an enhanced induction of tolerance in the absence of Pim1 expression. Therefore, it seems unlikely that *Pim1*-deficiency has a dominant effect on cell death of the airway epithelium upon exposure to HDM, but instead results in an augmented epithelial innate and adaptive Th2 response. Hence, a direct effect of Pim1 kinase activity on the airway epithelial barrier function,

resulting in a higher sensitivity for HDM-triggered release of pro inflammatory mediators in the absence of Pim1 likely provides a better explanation for the observed *in vivo* and *in vitro* effects of HDM exposure.

HDM exposure can disrupt the airway epithelial barrier by causing delocalization of the epithelial junction molecules E-cadherin, ZO-1 and occludin [68]. Next to the fact that the disrupted airway epithelial barrier facilitates the uptake of HDM by dendritic cells, it has been shown that loss of E-cadherin-mediated cell-cell contacts further promotes Th2-mediated airway inflammation by the release of Th2-attracting chemokines, including the chemokine CCL17 [67][68][78]. Of interest, we found CCL17 to be specifically induced upon exposure to HDM *in vivo* in absence of Pim1 kinase activity. While we did show that inhibition of Pim1 kinase activity enhances the HDM-induced loss of airway epithelial integrity and release of pro-inflammatory mediators, the mechanism by which Pim1 activity maintains airway epithelial integrity remain elusive. Epithelial-to-mesenchymal (EMT) transition of airway epithelial cells has been shown to be induced by HDM *in vitro* in the presence of TGF-β and *in vivo*, supporting a role for EMT in asthma [79][80]. One of the hallmarks of EMT is down-regulation of E-cadherin, resulting in loss of cell-cell contacts, and subsequent expression of mesenchymal makers like α-smooth muscle actin and vimentin [81]. Loss of cell-cell contacts between airway epithelial cells as a consequence of delocalization of E-cadherin, together with ZO-1 and occludin, are held at least partly responsible for defective epithelial barrier function observed in asthmatic patients [82] [83]. In addition, the role of TGF-β – one of the important EMT-inducing growth factors that is released upon damage of the airway epithelium – has been well established in airway remodeling and shown to be increased in the airways of asthmatic patients [81][84]. Notwithstanding the evidence for a role for EMT or epithelial plasticity in asthma, the precise role of this process is still a topic of discussion [81]. In contrast, the relevance of EMT is well recognized in cancer [85]. Expression of Pim1 kinase has been associated with reduced occurrence of metastases of

non-small cell lung cancer [86] and recently, expression of Pim1 in colorectal cancer was negatively associated with expression of EMT markers [87]. Since both studies imply a role for Pim1 kinase in the process of EMT in cancer, Pim1 might be involved in asthma-associated EMT as well. In support of this, we have preliminary data showing enhanced TGF-β signaling in the absence of Pim1 kinase activity in the BEAS-2B cell line. However, studies on the contribution of TGF-β signaling *in vivo in Pim1*-deficient mice as well as *in vitro* in PBECs treated with the pharmacological Pim1 kinase inhibitor are currently lacking.

To recapitulate the effects of Pim1 kinase on the airway epithelium exposed to HDM, Pim1 kinase seems to be involved in maintaining the integrity of the airway epithelial barrier and regulating the epithelial innate immune response as well as the Th2-mediated adaptive inflammatory response upon HDM exposure. However, an exact mechanism of action remains elusive and should be further investigated.

Future perspectives

In this thesis, we have studied the role of the Pim1 survival kinase in the airway epithelial response to three main environmental triggers relevant to the inception and exacerbations of asthma: CS, respiratory viruses and HDM. As described above for these three environmental triggers in detail, additional comprehensive studies on the role of airway epithelial cell survival and the facilitative role of Pim1 kinase herein, should be conducted to further reveal the importance of airway epithelial cell survival in the inception and exacerbations of asthma. While the studies described in chapter 5 do not specifically focus on the role of survival of airway epithelial cells, the HDM-driven mouse model of allergic asthma postulates a highly interesting model to investigate the effects of CS and respiratory viral infections on the inception and exacerbations of asthma in the context of aero-allergen sensitization and challenges. Given the identification of Pim1 kinase as an interesting target for intervention in virally induced asthma exacerbations, it is of interest to also further explore the theoretical implication of inhibition of Pim1 kinase activity in such combined exposure models into some detail.

With respect to the exposure to CS, several studies have investigated the effects of CS in HDM-driven mouse models of allergic asthma [88][89][90]. Although Blacquiere *et al* and Botelho *et al* showed that CS exposure mainly affects airway remodeling, Lanckacker and coworkers demonstrated the development of a more pronounced Th2-related asthmatic phenotype upon concomitant exposure to CS during allergic sensitization to HDM [88][89][90]. Exposure to CS in *Pim1*-deficient mice with previously established HDM-induced allergic asthma will provide new insights in the role of survival of airway epithelial cells in CS-induced exacerbations of asthma. Since Pim1 kinase has been shown to affect allergic asthma itself, we should also include a pharmacological approach by using a Pim1 kinase inhibitor, administered at specific times in the experiments to study the effects of cell survival on the inception or the exacerbation of asthma. In this way, we can

exclude that the observations are caused by an effect of *Pim1*-deficiency on the development of HDM-induced allergic asthma.

Similar studies should be performed with respiratory viral infections, in which *Pim1*-deficient mice are infected with respiratory viruses before or after the induction of allergic asthma upon HDM challenge. Here, to rule out the effects of Pim1 kinase on basal parameters of the allergic asthma mouse model, experiments with a pharmacological Pim1 kinase inhibitor should be taken into account as well. As described before, the major group HRV-16 is not recognized by the mouse ICAM-1 receptor and therefore, we have to use another respiratory virus like the minor HRV group virus HRV-1B of RSV. Both viruses are tested in mouse models of OVA-induced allergic asthma and have shown to especially affect the inception of allergic asthma [47][91]. Since HRV-1B was not able to exacerbate asthma in a study of Clarke *et al* [92], future experiments should focus on the establishment HDM-driven models of the inception and exacerbations of asthma, preferably with RSV. This virally induced asthma exacerbation mouse model is essential to further investigate the potential of inhibition of Pim1 kinase activity as a novel therapeutic approach in virally induced asthma exacerbations.

Pim1 kinase: a double edged sword?

Initially, we intended to use Pim1 kinase as a tool to study cell survival in airway epithelial cells exposed to environmental triggers like CS, viral infections and HDM. Pim1 kinase was originally identified as a proto-oncogene in retroviral complementation screens and was shown to be involved in cell survival by counteracting cMyc-induced apoptosis, thereby facilitating transformation of hematopoietic progenitor cells [93]. One of the best studied mechanisms by which Pim1 kinase exerts its pro-survival activity is through phosphorylation of BCL-2-associated agonist of cell death (BAD) on the mitochondrial cell membrane [22]. Nevertheless, as thoroughly reviewed in the context of cancer, Pim1 kinase is not only involved in cell survival, but also regulates cell cycle, translational, transcriptional and inflammatory processes in the cell [2][4]. In line herewith, we observed that Pim1 kinase was not merely involved in cell survival of airway epithelial cells exposed to cigarette smoke and human rhinovirus as shown in chapter 2 and 3 of this thesis, respectively, but also plays a role in the anti-viral IFN-induced inflammatory response (chapter 4) and in the HDM-induced innate immune response (chapter 5). The effects of Pim1 kinase on the different cell processes upon exposure to environmental triggers, however, are rather divergent.

As revealed by studying the effects of the environmental triggers on the airway epithelial cells in absence of Pim1 kinase activity, either by pharmacological inhibition of Pim1 kinase activity *in vitro* or by germline *Pim1*-deficiency *in vivo*, we showed that airway epithelial cells are more susceptible towards CS- and HDM-induced damage, though the mechanisms behind this increased susceptibility seem to be fairly different. CS-exposure on the one hand predisposes towards neutrophilic airway inflammation in *Pim1*-deficient mice, probably as a consequence of enhanced cell death of airway epithelial cells resulting in the release of DAMPs. Exposure to HDM in absence of Pim1 kinase activity, on the other hand, results in loss of airway epithelial barrier function, which leads to increased release of pro-inflammatory chemokines and cytokines and an augmented innate inflammatory immune response *in vivo* in

Pim1-deficient mice. However, since we did not assess HDM-induced apoptosis, in our study there is no clear link with enhanced susceptibility to HDM-induced cell death as discussed earlier. In contrast, inhibition of Pim1 kinase activity in PBECs reduces viral replication, thereby exerting a protective function. In monolayer cultures of PBECs, we showed that inhibition of Pim1 kinase activity reduces viral replication by enhancing the onset of cell death. In ALI cultures of PBECs, however, the anti-viral inflammatory response was increased upon inhibition of Pim1 kinase as a consequence of augmented IFN-induced activation of STAT-1.

Especially the observation that inhibition of Pim1 kinase activity can enhance the anti-viral inflammatory response, resulting in reduced viral replication and impairment of the subsequent spread of newly synthesized viral particles, offers opportunities for a novel therapeutic approach for virally induced asthma exacerbations. Besides the fact that interfering with proto-oncogenes might be hazardous, the divergent effects of inhibition of Pim1 kinase activity upon exposure to different environmental triggers challenge the use of Pim1 kinase activity inhibitors as novel therapeutic targets. Administration of a Pim1 kinase inhibitor to a non-smoking individual without asthma suffering from a viral respiratory infection might be beneficial in reducing the symptoms and duration of the viral infection. However, viral infections are mainly problematic for asthmatic patients, since asthmatic patients have shown to be more susceptible towards viral infections resulting in virally induced asthma exacerbations [51]. Will treatment with a Pim1 kinase inhibitor reduce the symptoms of the viral infection and have a beneficial effect on the duration and the severity of the asthma exacerbation? Or will treatment with a Pim1 kinase inhibitor increase the symptoms, duration and severity of the asthma exacerbations by its detrimental effect on the airway epithelial barrier function and Th2-mediated airway inflammation resulting in worsening of asthma control? And what would happen in an asthma patient who actively smokes and suffers from a virally induced asthma exacerbations, when treated with a Pim1 kinase activity inhibitor? Are the beneficial

effects on the reduction of viral replication transcending the detrimental effects of inhibition of Pim1 kinase activity upon exposure to CS and HDM? These questions definitively warrant more detailed studies into the mechanisms by which inhibition of Pim1 kinase protects against viral infections of airway epithelial cells. Such studies will allow the identification of downstream targets of inhibition of Pim1 kinase relevant to suppression of viral replication and allow for novel strategies in the optimization of therapeutic approaches addressing these mechanisms.

In conclusion, although inhibition of Pim1 kinase activity is a highly interesting novel therapeutic approach in virally induced asthma exacerbations, the divergent roles of inhibition of Pim1 kinase activity in airway epithelial cells exposed to environmental triggers like CS, respiratory viral infections and HDM makes it difficult to predict the effects and especially the benefits of treatment with a pharmacological inhibitor. However, the studies described in this thesis unambiguously demonstrate that further studies on the potential of inhibition of Pim1 kinase activity as novel therapeutic approach in asthma is absolutely warranted.

References

1. Nawijn MC, Hackett TL, Postma DS, van Oosterhout AJ, Heijink IH. E-cadherin: gatekeeper of airway mucosa and allergic sensitization. *Trends Immunol 2011; 32: 248-255.*

2. Nawijn MC, Alendar A, Berns A. For better or for worse: the role of Pim oncogenes in tumorigenesis. *Nat Rev Cancer 2011; 11: 23-34.*

3. de Vries M, Heijink IH, Gras R, den Boef LE, Reinders-Luinge M, Pouwels SD, Hylkema MN, van der Toorn M, Brouwer U, van Oosterhout AJ, Nawijn MC. Pim1 kinase protects airway epithelial cells from cigarette smoke- induced damage and airway inflammation. *Am J Physiol Lung Cell Mol Physiol 2014; 307: L240-51.*

4. Chen LS, Balakrishnan K, Gandhi V. Inflammation and survival pathways: chronic lymphocytic leukemia as a model system. *Biochem Pharmacol 2010; 80: 1936-1945.*

5. Cosio MG, Saetta M, Agusti A. Immunologic aspects of chronic obstructive pulmonary disease. *N Engl J Med 2009; 360: 2445-2454.*

6. Pauwels RA, Rabe KF. Burden and clinical features of chronic obstructive pulmonary disease (COPD). *Lancet 2004; 364: 613-620.*

7. Brusselle GG, Joos GF, Bracke KR. New insights into the immunology of chronic obstructive pulmonary disease. *Lancet 2011; 378: 1015-1026.*

8. Maes T, Bracke KR, Vermaelen KY, Demedts IK, Joos GF, Pauwels RA, Brusselle GG. Murine TLR4 is implicated in cigarette smoke-induced pulmonary inflammation. *Int Arch Allergy Immunol 2006; 141: 354-368.*

9. Dupont LL, Bracke KR, De Maeyer JH, Compan V, Joos GF, Lefebvre RA, Brusselle GG. Investigation of 5-HT4 receptors in bronchial hyperresponsiveness in cigarette smoke-exposed mice. *Pulm Pharmacol Ther 2014; 28: 60-67.*

10. Kirkham P, Rahman I. Oxidative stress in asthma and COPD: antioxidants as a therapeutic strategy. *Pharmacol Ther 2006; 111: 476-494.*

11. van der Vaart H, Postma DS, Timens W, Hylkema MN, Willemse BW, Boezen HM, Vonk JM, de Reus DM, Kauffman HF, ten Hacken NH. Acute effects of cigarette smoking on inflammation in healthy intermittent smokers. *Respir Res 2005; 6: 22.*

12. Pouwels SD, Heijink IH, ten Hacken NH, Vandenabeele P, Krysko DV, Nawijn MC, van Oosterhout AJ. DAMPs activating innate and adaptive immune responses in COPD. *Mucosal Immunol 2014; 7: 215-226.*

13. Groskreutz DJ, Monick MM, Babor EC, Nyunoya T, Varga SM, Look DC, Hunninghake GW. Cigarette smoke alters respiratory syncytial virus-induced apoptosis and replication. *Am J Respir Cell Mol Biol 2009; 41: 189-198.*

14. Slebos DJ, Ryter SW, van der Toorn M, Liu F, Guo F, Baty CJ, Karlsson JM, Watkins SC, Kim HP, Wang X, Lee JS, Postma DS, Kauffman HF, Choi AM. Mitochondrial localization and function of heme oxygenase-1 in cigarette smoke-induced cell death. *Am J Respir Cell Mol Biol 2007; 36: 409-417.*

15. van der Toorn M, Slebos DJ, de Bruin HG, Leuvenink HG, Bakker SJ, Gans RO, Koeter GH, van Oosterhout AJ, Kauffman HF. Cigarette smoke-induced blockade of the mitochondrial respiratory chain switches lung epithelial cell apoptosis into necrosis. *Am J Physiol Lung Cell Mol Physiol 2007; 292: L1211-8.*

16. Vayssier M, Banzet N, Francois D, Bellmann K, Polla BS. Tobacco smoke induces both apoptosis and necrosis in mammalian cells: differential effects of HSP70. *Am J Physiol 1998; 275: L771-9.*

17. Chen ZH, Kim HP, Sciurba FC, Lee SJ, Feghali-Bostwick C, Stolz DB, Dhir R, Landreneau RJ, Schuchert MJ, Yousem SA, Nakahira K, Pilewski JM, Lee JS, Zhang Y, Ryter SW, Choi AM. Egr-1 regulates autophagy in cigarette smoke-induced chronic obstructive pulmonary disease. *PLoS One 2008; 3: e3316.*

18. Mizumura K, Cloonan SM, Nakahira K, Bhashyam AR, Cervo M, Kitada T, Glass K, Owen CA, Mahmood A, Washko GR, Hashimoto S, Ryter SW, Choi AM. Mitophagy-dependent necroptosis contributes to the pathogenesis of COPD. *J Clin Invest 2014; 124: 3987-4003.*

19. Hoffmann RF, Zarrintan S, Brandenburg SM, Kol A, de Bruin HG, Jafari S, Dijk F, Kalicharan D, Kelders M, Gosker HR, Ten Hacken NH, van der Want JJ, van Oosterhout AJ, Heijink IH. Prolonged cigarette smoke exposure alters mitochondrial structure and function in airway epithelial cells. *Respir Res 2013; 14: 97-9921-14- 97.*

20. Chaudhuri R, Livingston E, McMahon AD, Lafferty J, Fraser I, Spears M, McSharry CP, Thomson NC. Effects of smoking cessation on lung function and airway inflammation in smokers with asthma. *Am J Respir Crit Care Med 2006; 174: 127-133.*

21. Aho TL, Sandholm J, Peltola KJ, Mankonen HP, Lilly M, Koskinen PJ. Pim-1 kinase promotes inactivation of the pro-apoptotic Bad protein by phosphorylating it on the Ser112 gatekeeper site. *FEBS Lett 2004; 571: 43-49.*

22. Danial NN. BAD: undertaker by night, candyman by day. *Oncogene 2008; 27 Suppl 1: S53-70.*

23. Macdonald A, Campbell DG, Toth R, McLauchlan H, Hastie CJ, Arthur JS. Pim kinases phosphorylate multiple sites on Bad and promote 14-3-3 binding and dissociation from Bcl-XL. *BMC Cell Biol 2006; 7: 1.*

24. Krysko DV, Agostinis P, Krysko O, Garg AD, Bachert C, Lambrecht BN, Vandenabeele P. Emerging role of damage-associated molecular patterns derived from mitochondria in inflammation. *Trends Immunol 2011; 32: 157-164.*

25. Wickenden JA, Clarke MC, Rossi AG, Rahman I, Faux SP, Donaldson K, MacNee W. Cigarette smoke prevents apoptosis through inhibition of caspase activation and induces necrosis. *Am J Respir Cell Mol Biol 2003; 29: 562- 570.*

26. Poon IK, Hulett MD, Parish CR. Molecular mechanisms of late apoptotic/necrotic cell clearance. *Cell Death Differ 2010; 17: 381-397.*

27. Pogacic V, Bullock AN, Fedorov O, Filippakopoulos P, Gasser C, Biondi A, Meyer-Monard S, Knapp S, Schwaller J. Structural analysis identifies imidazo[1,2-b]pyridazines as PIM kinase inhibitors with in vitro antileukemic activity. *Cancer Res 2007; 67: 6916-6924.*

28. Pouwels SD, Heijink IH, van Oosterhout AJ, Nawijn MC. A specific DAMP profile identifies susceptibility to smoke-induced airway inflammation. *Eur Respir J 2014; 43: 1183-1186.*

29. Gopal R, Monin L, Torres D, Slight S, Mehra S, McKenna KC, Fallert Junecko BA, Reinhart TA, Kolls J, Baez- Saldana R, Cruz-Lagunas A, Rodriguez-Reyna TS, Kumar NP, Tessier P, Roth J, Selman M, Becerril-Villanueva E, Baquera-Heredia J, Cumming B, Kasprowicz VO, Steyn AJ, Babu S, Kaushal D, Zuniga J, Vogl T, Rangel-Moreno J, Khader SA. S100A8/A9 proteins mediate neutrophilic inflammation and lung pathology during tuberculosis. *Am J Respir Crit Care Med 2013; 188: 1137-1146.*

30. Harada C, Kawaguchi T, Ogata-Suetsugu S, Yamada M, Hamada N, Maeyama T, Souzaki R, Tajiri T, Taguchi T, Kuwano K, Nakanishi Y. EGFR tyrosine kinase inhibition worsens acute lung injury in mice with repairing airway epithelium. A*m J Respir Crit Care Med 2011; 183: 743-751.*

31. Heijink IH, Pouwels SD, Leijendekker C, de Bruin HG, Zijlstra GJ, van der Vaart H, Ten Hacken NH, van Oosterhout AJ, Nawijn MC, van der Toorn M. Cigarette Smoke Induced DAMP Release from Necrotic Neutrophils Triggers Pro-inflammatory Mediator Release. *Am J Respir Cell Mol Biol 2014*.

32. Foell D, Wittkowski H, Vogl T, Roth J. S100 proteins expressed in phagocytes: a novel group of damage- associated molecular pattern molecules. *J Leukoc Biol 2007; 81: 28-37.*

33. Andina N, Didichenko S, Schmidt-Mende J, Dahinden CA, Simon HU. Proviral integration site for Moloney murine leukemia virus 1, but not phosphatidylinositol-3 kinase, is essential in the antiapoptotic signaling cascade initiated by IL-5 in eosinophils. *J Allergy Clin Immunol 2009; 123: 603-611.*

34. Gavala ML, Bertics PJ, Gern JE. Rhinoviruses, allergic inflammation, and asthma. *Immunol Rev 2011; 242: 69-90.*

35. Cakebread JA, Xu Y, Grainge C, Kehagia V, Howarth PH, Holgate ST, Davies DE. Exogenous IFN-beta has antiviral and anti-inflammatory properties in primary bronchial epithelial cells from asthmatic subjects exposed to rhinovirus. *J Allergy Clin Immunol 2011; 127: 1148-54.e9.*

36. Platanias LC. Mechanisms of type-I- and type-II-interferon-mediated signalling. *Nat Rev Immunol 2005; 5: 375-386.*

37. Donnelly RP, Kotenko SV. Interferon-lambda: a new addition to an old family. *J Interferon Cytokine Res 2010; 30: 555-564.*

38. Takaoka A, Hayakawa S, Yanai H, Stoiber D, Negishi H, Kikuchi H, Sasaki S, Imai K, Shibue T, Honda K, Taniguchi T. Integration of interferon-alpha/beta signalling to p53 responses in tumour suppression and antiviral defence. *Nature 2003; 424: 516-523.*

39. Balachandran S, Kim CN, Yeh WC, Mak TW, Bhalla K, Barber GN. Activation of the dsRNA-dependent protein kinase, PKR, induces apoptosis through FADD-mediated death signaling. *EMBO J 1998; 17: 6888-6902.*

40. Wark PA, Johnston SL, Bucchieri F, Powell R, Puddicombe S, Laza-Stanca V, Holgate ST, Davies DE. Asthmatic bronchial epithelial cells have a deficient innate immune response to infection with rhinovirus. *J Exp Med 2005; 201: 937-947.*

41. Jackson DJ, Sykes A, Mallia P, Johnston SL. Asthma exacerbations: origin, effect, and prevention. *J Allergy Clin Immunol 2011; 128: 1165-1174.*

42. Wark PA, Grissell T, Davies B, See H, Gibson PG. Diversity in the bronchial epithelial cell response to infection with different rhinovirus strains. *Respirology 2009; 14: 180-186.*

43. Carraro S, Scheltema N, Bont L, Baraldi E. Early-life origins of chronic respiratory diseases: understanding and promoting healthy ageing. *Eur Respir J 2014.*

44. Swindle EJ, Davies DE. Artificial airways for the study of respiratory disease. *Expert Rev Respir Med 2011; 5: 757-765.*

45. Shapiro SD. Animal models of asthma: Pro: Allergic avoidance of animal (model[s]) is not an option. *Am J Respir Crit Care Med 2006; 174: 1171-1173.*

46. Wenzel S, Holgate ST. The mouse trap: It still yields few answers in asthma. *Am J Respir Crit Care Med 2006; 174: 1173-6; discussion 1176-8.*

47. Bartlett NW, Walton RP, Edwards MR, Aniscenko J, Caramori G, Zhu J, Glanville N, Choy KJ, Jourdan P, Burnet J, Tuthill TJ, Pedrick MS, Hurle MJ, Plumpton C, Sharp NA, Bussell JN, Swallow DM, Schwarze J, Guy B, Almond JW, Jeffery PK, Lloyd CM, Papi A, Killington RA, Rowlands DJ, Blair ED, Clarke NJ, Johnston SL. Mouse models of rhinovirus-induced disease and exacerbation of allergic airway inflammation. *Nat Med 2008; 14: 199-204.*

48. Brodlie M, McKean MC, Johnson GE, Perry JD, Nicholson A, Verdon B, Gray MA, Dark JH, Pearson JP, Fisher AJ, Corris PA, Lordan J, Ward C. Primary bronchial epithelial cell culture from explanted cystic fibrosis lungs. *Exp Lung Res 2010; 36: 101-110.*

49. Takeuchi O, Akira S. MDA5/RIG-I and virus recognition. *Curr Opin Immunol 2008; 20: 17-22.*

50. Bonjardim CA, Ferreira PC, Kroon EG. Interferons: signaling, antiviral and viral evasion. *Immunol Lett 2009; 122: 1-11.*

51. Jackson DJ, Johnston SL. The role of viruses in acute exacerbations of asthma. *J Allergy Clin Immunol 2010; 125: 1178-87; quiz 1188-9.*

52. Contoli M, Message SD, Laza-Stanca V, Edwards MR, Wark PA, Bartlett NW, Kebadze T, Mallia P, Stanciu LA, Parker HL, Slater L, Lewis-Antes A, Kon OM, Holgate ST, Davies DE, Kotenko SV, Papi A, Johnston SL. Role of deficient type III interferon-lambda production in asthma exacerbations. *Nat Med 2006; 12: 1023-1026.*

53. Sykes A, Macintyre J, Edwards MR, Del Rosario A, Haas J, Gielen V, Kon OM, McHale M, Johnston SL. Rhinovirus-induced interferon production is not deficient in well controlled asthma. *Thorax 2014; 69: 240-246.*

54. Jakiela B, Gielicz A, Plutecka H, Hubalewska-Mazgaj M, Mastalerz L, Bochenek G, Soja J, Januszek R, Aab A, Musial J, Akdis M, Akdis CA, Sanak M. Th2-type cytokine-induced mucus metaplasia decreases susceptibility of human bronchial epithelium to rhinovirus infection. *Am J Respir Cell Mol Biol 2014; 51: 229-241*.

55. Lopez-Souza N, Favoreto S, Wong H, Ward T, Yagi S, Schnurr D, Finkbeiner WE, Dolganov GM, Widdicombe JH, Boushey HA, Avila PC. In vitro susceptibility to rhinovirus infection is greater for bronchial than for nasal airway epithelial cells in human subjects. *J Allergy Clin Immunol 2009; 123: 1384-90.e2.*

56. Gulraiz F, Bellinghausen C, Dentener MA, Reynaert NL, Gaajetaan GR, Beuken EV, Rohde GG, Bruggeman CA, Stassen FR. Efficacy of IFN-lambda1 to protect human airway epithelial cells against human rhinovirus 1B infection. *PLoS One 2014; 9: e95134.*

57. Djukanovic R, Harrison T, Johnston SL, Gabbay F, Wark P, Thomson NC, Niven R, Singh D, Reddel HK, Davies DE, Marsden R, Boxall C, Dudley S, Plagnol V, Holgate ST, Monk P. The Effect of Inhaled IFN-beta on Worsening of Asthma Symptoms Caused by Viral Infections. A Randomized Trial. *Am J Respir Crit Care Med 2014; 190: 145- 154.*

58. Jefferson TO, Tyrrell D. Antivirals for the common cold. *Cochrane Database Syst Rev 2001; (3): CD002743.*

59. Chen XP, Losman JA, Cowan S, Donahue E, Fay S, Vuong BQ, Nawijn MC, Capece D, Cohan VL, Rothman P. Pim serine/threonine kinases regulate the stability of Socs-1 protein. *Proc Natl Acad Sci U S A 2002; 99: 2175- 2180.*

60. Yoshimura A, Naka T, Kubo M. SOCS proteins, cytokine signalling and immune regulation. *Nat Rev Immunol 2007; 7: 454-465.*

61. Dalpke A, Heeg K, Bartz H, Baetz A. Regulation of innate immunity by suppressor of cytokine signaling (SOCS) proteins. *Immunobiology 2008; 213: 225-235.*

62. Zhang JG, Farley A, Nicholson SE, Willson TA, Zugaro LM, Simpson RJ, Moritz RL, Cary D, Richardson R, Hausmann G, Kile BJ, Kent SB, Alexander WS, Metcalf D, Hilton DJ, Nicola NA, Baca M. The conserved SOCS box motif in suppressors of cytokine signaling binds to elongins B and C and may couple bound proteins to proteasomal degradation. *Proc Natl Acad Sci U S A 1999; 96: 2071-2076.*

63. Bedke N, Sammut D, Green B, Kehagia V, Dennison P, Jenkins G, Tatler A, Howarth PH, Holgate ST, Davies DE. Transforming growth factor-beta promotes rhinovirus replication in bronchial epithelial cells by suppressing the innate immune response. *PLoS One 2012; 7: e44580.*

64. Gielen V, Sykes A, Zhu J, Chan B, Macintyre J, Regamey N, Kieninger E, Gupta A, Shoemark A, Bossley C, Davies J, Saglani S, Walker P, Nicholson SE, Dalpke AH, Kon OM, Bush A, Johnston SL, Edwards MR. Increased nuclear suppressor of cytokine signaling 1 in asthmatic bronchial epithelium suppresses rhinovirus induction of innate interferons. *J Allergy Clin Immunol 2015.*

65. Hashimoto K, Ishibashi K, Ishioka K, Zhao D, Sato M, Ohara S, Abe Y, Kawasaki Y, Sato Y, Yokota S, Fujii N, Peebles RS,Jr, Hosoya M, Suzutani T. RSV replication is attenuated by counteracting expression of the suppressor of cytokine signaling (SOCS) molecules. *Virology 2009; 391: 162-170.*

66. Hao K, Bosse Y, Nickle DC, Pare PD, Postma DS, Laviolette M, Sandford A, Hackett TL, Daley D, Hogg JC, Elliott WM, Couture C, Lamontagne M, Brandsma CA, van den Berge M, Koppelman G, Reicin AS, Nicholson DW, Malkov V, Derry JM, Suver C, Tsou JA, Kulkarni A, Zhang C, Vessey R, Opiteck GJ, Curtis SP, Timens W, Sin DD. Lung eQTLs to Help Reveal the Molecular Underpinnings of Asthma. *PLoS Genet 2012; 8: e1003029.*

67. Heijink IH, Kies PM, Kauffman HF, Postma DS, van Oosterhout AJ, Vellenga E. Down-regulation of E-cadherin in human bronchial epithelial cells leads to epidermal growth factor receptor-dependent Th2 cell-promoting activity. *J Immunol 2007; 178: 7678-7685.*

68. Post S, Nawijn MC, Hackett TL, Baranowska M, Gras R, van Oosterhout AJ, Heijink IH. The composition of house dust mite is critical for mucosal barrier dysfunction and allergic sensitisation. *Thorax 2012; 67: 488-495.*

69. Kumar RK, Foster PS. Modeling allergic asthma in mice: pitfalls and opportunities. *Am J Respir Cell Mol Biol 2002; 27: 267-272.*

70. Gregory LG, Lloyd CM. Orchestrating house dust mite-associated allergy in the lung. *Trends Immunol 2011; 32: 402-411.*

71. Shin YS, Takeda K, Shiraishi Y, Jia Y, Wang M, Jackson L, Wright AD, Carter L, Robinson J, Hicken E, Gelfand EW. Inhibition of Pim1 kinase activation attenuates allergen-induced airway hyperresponsiveness and inflammation. *Am J Respir Cell Mol Biol 2012; 46: 488-497.*

72. Lambrecht BN, Hammad H. The immunology of asthma. *Nat Immunol 2014; 16: 45-56.*

73. Klein Wolterink RG, Kleinjan A, van Nimwegen M, Bergen I, de Bruijn M, Levani Y, Hendriks RW. Pulmonary innate lymphoid cells are major producers of IL-5 and IL-13 in murine models of allergic asthma. *Eur J Immunol 2012; 42: 1106-1116.*

74. Gold MJ, Antignano F, Halim TY, Hirota JA, Blanchet MR, Zaph C, Takei F, McNagny KM. Group 2 innate lymphoid cells facilitate sensitization to local, but not systemic, TH2-inducing allergen exposures. *J Allergy Clin Immunol 2014; 133: 1142-1148.*

75. Jackson LJ, Pheneger JA, Pheneger TJ, Davis G, Wright AD, Robinson JE, Allen S, Munson MC, Carter LL. The role of PIM kinases in human and mouse CD4+ T cell activation and inflammatory bowel disease. *Cell Immunol 2012; 272: 200-213.*

76. Wang M, Okamoto M, Domenico J, Han J, Ashino S, Shin YS, Gelfand EW. Inhibition of Pim1 kinase prevents peanut allergy by enhancing Runx3 expression and suppressing T(H)2 and T(H)17 T-cell differentiation. *J Allergy Clin Immunol 2012; 130: 932-44.e12.*

77. Juncadella IJ, Kadl A, Sharma AK, Shim YM, Hochreiter-Hufford A, Borish L, Ravichandran KS. Apoptotic cell clearance by bronchial epithelial cells critically influences airway inflammation. *Nature 2013; 493: 547-551.*

78. Post S, Nawijn MC, Jonker MR, Kliphuis N, van den Berge M, van Oosterhout AJ, Heijink IH. House dust mite- induced calcium signaling instigates epithelial barrier dysfunction and CCL20 production. *Allergy 2013; 68: 1117-1125.*

79. Heijink IH, Postma DS, Noordhoek JA, Broekema M, Kapus A. House dust mite-promoted epithelial-to- mesenchymal transition in human bronchial epithelium. *Am J Respir Cell Mol Biol 2010; 42: 69-79.*

80. Johnson JR, Roos A, Berg T, Nord M, Fuxe J. Chronic respiratory aeroallergen exposure in mice induces epithelial-mesenchymal transition in the large airways. *PLoS One 2011; 6: e16175.*

81. Hackett TL. Epithelial-mesenchymal transition in the pathophysiology of airway remodelling in asthma. *Curr Opin Allergy Clin Immunol 2012; 12: 53-59.*

82. Xiao C, Puddicombe SM, Field S, Haywood J, Broughton-Head V, Puxeddu I, Haitchi HM, Vernon-Wilson E, Sammut D, Bedke N, Cremin C, Sones J, Djukanovic R, Howarth PH, Collins JE, Holgate ST, Monk P, Davies DE. Defective epithelial barrier function in asthma. *J Allergy Clin Immunol 2011; 128: 549-56.e1-12.*

83. de Boer WI, Sharma HS, Baelemans SM, Hoogsteden HC, Lambrecht BN, Braunstahl GJ. Altered expression of epithelial junctional proteins in atopic asthma: possible role in inflammation. *Can J Physiol Pharmacol 2008; 86: 105-112*.

84. Boxall C, Holgate ST, Davies DE. The contribution of transforming growth factor-beta and epidermal growth factor signalling to airway remodelling in chronic asthma. *Eur Respir J 2006; 27: 208-229.*

85. Steinestel K, Eder S, Schrader AJ, Steinestel J. Clinical significance of epithelial-mesenchymal transition. *Clin Transl Med 2014; 3: 17-1326-3-17. eCollection 2014.*

86. Warnecke-Eberz U, Bollschweiler E, Drebber U, Pohl A, Baldus SE, Hoelscher AH, Metzger R. Frequent down-regulation of pim-1 mRNA expression in non-small cell lung cancer is associated with lymph node metastases. *Oncol Rep 2008; 20: 619-624.*

87. Fromberg A, Rabe M, Aigner A. Multiple effects of the special AT-rich binding protein 1 (SATB1) in colon carcinoma. *Int J Cancer 2014; 135: 2537-2546.*

88. Blacquiere MJ, Timens W, Melgert BN, Geerlings M, Postma DS, Hylkema MN. Maternal smoking during pregnancy induces airway remodelling in mice offspring. *Eur Respir J 2009; 33: 1133-1140.*

89. Botelho FM, Llop-Guevara A, Trimble NJ, Nikota JK, Bauer CM, Lambert KN, Kianpour S, Jordana M, Stampfli MR. Cigarette smoke differentially affects eosinophilia and remodeling in a model of house dust mite asthma. *Am J Respir Cell Mol Biol 2011; 45: 753-760.*

90. Lanckacker EA, Tournoy KG, Hammad H, Holtappels G, Lambrecht BN, Joos GF, Maes T. Short cigarette smoke exposure facilitates sensitisation and asthma development in mice. *Eur Respir J 2013; 41: 1189-1199*.

91. Tourdot S, Mathie S, Hussell T, Edwards L, Wang H, Openshaw PJ, Schwarze J, Lloyd CM. Respiratory syncytial virus infection provokes airway remodelling in allergen-exposed mice in absence of prior allergen sensitization. *Clin Exp Allergy 2008; 38: 1016-1024.*

92. Clarke DL, Davis NH, Majithiya JB, Piper SC, Lewis A, Sleeman MA, Corkill DJ, May RD. Development of a mouse model mimicking key aspects of a viral asthma exacerbation. *Clin Sci (Lond) 2014; 126: 567-580*.

93. Shirogane T, Fukada T, Muller JM, Shima DT, Hibi M, Hirano T. Synergistic roles for Pim-1 and c-Myc in STAT3-mediated cell cycle progression and antiapoptosis. *Immunity 1999; 11: 709-719.*