The expression and function of RAGE and HMGB1 in airway structural cells in asthma

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by

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The expression and function of RAGE and HMGB1 in airway structural cells in asthma
Leonarda Di Candia

Abstract

Asthma is characterised by airway hyperresponsiveness, airflow obstruction, chronic inflammation and airway remodelling, with an increase in airway smooth muscle (ASM) mass and contractility. ASM also releases mediators that support inflammation and remodelling. High-mobility group box 1 (HMGB1) is a nuclear protein that is released by damaged/stressed cells and activated immune cells. HMGB1 signals through pattern recognition receptors (PRRs) including the receptor for advanced glycosylation end products (RAGE) to promote inflammation and tissue repair. HMGB1 binding and function are governed by its redox state. Evidence suggests HMGB1 elevation in asthma; however, the redox state of airway HMGB1 is unknown. Moreover, the expression and role of RAGE in regulating airway mesenchymal cells are unknown. We aimed to investigate HMGB1 and RAGE expression in bronchial tissue; the redox form of sputum HMGB1; the expression and role of HMGB1 and RAGE in airway structural cells.

HMGB1 was 3.5-fold higher in sputa of moderate-to-severe asthmatics (n=34), and the reduced form was shown to be increased in this group (n=16) for the first time. Reduced HMGB1 was chemotactic for peripheral blood leukocytes, and sputum HMGB1 correlated with sputum total cell counts. HMGB1, but not RAGE, expression was ~3-fold higher ex vivo in ASM of severe asthmatics (n=16). ASM and human bronchial epithelial cells (HBECs) expressed both HMGB1 and cell-surface RAGE in vitro. HMGB1 expression was upregulated in ASM cells stimulated with inflammatory cytokines. HMGB1 stimulation caused increased reactive oxygen species production in ASM cells from non-asthmatics, but not in asthmatics; ASM contraction and inhibition of ASM cell migration and HBEC wound healing.

These results suggest that HMGB1 promotes inflammatory cell recruitment, impairs epithelial and ASM repair, and promotes ASM contraction in asthma. Further work is required to determine whether antagonising HMGB1 or its receptors would be a viable therapeutic approach for the treatment of asthma.
Acknowledgments

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<tr>
<td>α-SMA</td>
<td>α-smooth muscle actin</td>
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<tr>
<td>AHR</td>
<td>Airway hyperresponsiveness</td>
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<tr>
<td>ASM</td>
<td>Airway smooth muscle</td>
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<tr>
<td>AUC</td>
<td>Area under the curve</td>
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<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
</tr>
<tr>
<td>BL</td>
<td>Bronchial lavage</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
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<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CXCL</td>
<td>Chemokine (C-X-C motif) ligand</td>
</tr>
<tr>
<td>DAMP</td>
<td>Damage-associated molecular pattern</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
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<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FEV₁</td>
<td>Forced expiratory volume in 1 second</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FVC</td>
<td>Forced vital capacity</td>
</tr>
<tr>
<td>GINA</td>
<td>Global Initiative for Asthma</td>
</tr>
<tr>
<td>GMA</td>
<td>Glycol methacrylate</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
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<td>Description</td>
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<tr>
<td>H$_2$DCFDA</td>
<td>2',7'-dichlorodihydrofluorescein diacetate</td>
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<tr>
<td>HMGB1</td>
<td>High mobility group box 1</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
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<td>HSP</td>
<td>Heat-shock proteins</td>
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<td>HUVECs</td>
<td>Human umbilical vein endothelial cells</td>
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<td>ICAM-1</td>
<td>Intercellular cell adhesion molecule 1</td>
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<td>ICS</td>
<td>Inhaled corticosteroids</td>
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<td>Immunohistochemistry</td>
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<td>IL</td>
<td>Interleukin</td>
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<td>ILCs</td>
<td>Innate lymphoid cells</td>
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<tr>
<td>IFNγ</td>
<td>Interferon γ</td>
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<td>IPF</td>
<td>Idiopathic pulmonary fibrosis</td>
</tr>
<tr>
<td>ITS</td>
<td>Insulin-transferrin-selenium</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<td>NF-κB</td>
<td>Nuclear factor kappa B</td>
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<tr>
<td>NOX4</td>
<td>Nicotinamide adenine dinucleotide phosphate oxidase 4</td>
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<tr>
<td>PAMPS</td>
<td>Pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
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<tr>
<td>PRR</td>
<td>Pattern recognition receptor</td>
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<td>RAGE</td>
<td>Receptor for advanced glycosylation end products</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor α</td>
</tr>
<tr>
<td>TSLP</td>
<td>Thymic stromal lymphopoietin</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule 1</td>
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List of publications and prizes arisen from this Thesis

Publications


Fellowships

Awarded an EAACI Short-term Research Fellowship 2013 (three months working at San Raffaele Scientific Institute of Milan, Italy, from September to November 2013).
Chapter 1

Introduction
1. Introduction

1.1 Asthma

Asthma is a global health burden affecting around 300 million people of all ages, ethnic backgrounds and countries. It is estimated that ~250,000 people worldwide die prematurely each year as a result of asthma [1]. The UK is one the countries with the highest asthma prevalence, with ~17 % of the population suffering from this condition [2]. Asthma is characterised by variable and recurrent symptoms including breathlessness, wheeze, chest tightness and cough; variable and reversible expiratory airflow obstruction; and/or airway hyperresponsiveness (AHR, [2], refer to Chapter 2, section 2.2 for measurements of airflow limitation). Episodes of marked worsening of the symptoms known as exacerbations can occur [3]. Inhaled corticosteroids (ICS) are commonly used as an anti-inflammatory therapy for asthma. Other treatments include short or long-acting β₂-adrenergic receptor agonists; leukotriene antagonists; and theophylline, which all act as smooth muscle relaxants; inhibitors of mast cell degranulation; oral corticosteroids; and anti-immunoglobulin (Ig) E antibodies [4]. Severity of symptoms together with measurements of lung function (such as forced expiratory volume in 1 sec (FEV₁) and FEV₁/FVC (forced vital capacity) ratio) are used to define asthma severity. Besides these parameters, the definition of asthma severity also includes the level of treatment (e.g. use and doses of inhaled corticosteroids), the responsiveness to treatment (i.e. the ease with which asthma control is achieved by therapy), the degree of symptoms control achieved through therapy, and the risk of exacerbations and chronic morbidity [1].

Approximately 5 – 10 % of asthma sufferers have severe disease, whereby symptoms and frequent exacerbations persist despite the highest level of treatment. Patients
with severe poorly controlled asthma are at a higher risk of hospitalisation and death, and often have a significantly impaired quality of life. This small population of asthmatics accounts for two-thirds of the healthcare costs attributed to asthma [3]. Early clinically-based definitions of asthma focused on two main phenotypes: extrinsic asthma, which develops in childhood and is characterised by IgE-mediated allergic disease; and intrinsic asthma, which typically develops later in life and is not associated with allergic disease [5]. However, in recent years new approaches to patient phenotyping based on statistical methods have been adopted, such as cluster analysis, which group patients based on similarities in clinical characteristics. Not only have these methods highlighted the heterogeneity of the disease, they have also informed therapy towards an increasingly tailored treatment [5]. Benton et al. (2010) identified 3 clusters of children with asthma, independently of asthma medications. Cluster 1 included predominantly males with higher airway neutrophils compared with the other two clusters; cluster 2 had predominantly females with high body mass index (BMI) and later onset asthma; cluster 3 was characterised by allergic asthma features with prevalence of airway eosinophils, worse asthma control, and high BMI [6]. In another study, asthma duration, number of asthma controller medications, and baseline lung function were found to predict cluster assignment in children with asthma [7]. This study identified 4 clusters, which could be differentiated based on lung function, atopy, symptoms, medication use, bronchial responsiveness, and comorbidity. Interestingly, all clusters included children with severe asthma, highlighting the heterogeneity of severe asthma in children [7]. Patrawalla et al. (2012) found a predominance of women (70 %) in adults with asthma, who could be clustered in patients with predominantly childhood onset atopic asthma; older subjects, with the
longest duration of asthma, increased symptoms and exacerbations; a mostly atopic group with the highest peripheral eosinophils; and older obese women with adult-onset asthma, the least atopy, and increased exacerbations [8]. Wu et al. (2014) identified 6 asthma clusters, which could be differentiated based on age of onset, symptoms, medications, and health care use. Patients with traditionally defined severe asthma were distributed across 4 clusters, and included patients with early-onset allergic asthma with low lung function and eosinophilic inflammation; patients with later-onset, mostly severe asthma with nasal polyps and eosinophilia; and patients with persistent peripheral and airway inflammation and exacerbations despite high systemic corticosteroid use [9]. Another study identified a subgroup of patients with accelerated lung function decline (FEV₁) despite appropriate asthma treatment, which predominantly comprised older men with late-onset asthma, a lower prevalence of exacerbations and a lower FEV₁ % predicted [10]. Two more clusters included (1) women with late-onset asthma and (2) subjects with early-onset asthma, atopy and long disease duration [10]. Refractory asthma (i.e. symptoms are uncontrolled despite medication) included four subgroups, which could be differentiated based on age of onset, pulmonary function, and bronchial hyperresponsiveness, in a further study [11]. Two of these clusters had a female predominance (one characterised by the most severe airway obstruction, and the other by the most enhanced bronchial hyperresponsiveness), the third group had the best-preserved airway function, and the fourth cluster included mostly male smokers [11]. Cluster analysis also revealed overlapping phenotypes among severe asthmatics and subjects with moderate-to-severe chronic obstructive pulmonary disease (COPD) based on sputum cytokine profiling [12]. This study identified 3 clusters, one with asthma predominance,
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eosinophilic, and with high T helper (Th) 2 cytokines; one cluster with asthma and COPD overlap, neutrophilic; and a third cluster with COPD predominance, mixed eosinophilic and neutrophilic [12]. Importantly, longitudinal studies have shown that phenotypic clustering is consistent over time, and may be useful to inform targeted therapeutic strategies. For example, in a large cohort (> 1,000) of children with asthma, clustering based on atopic burden, degree of airflow obstruction, and history of exacerbation was able to predict differences in pulmonary function over a period of 4 years, and also long-term asthma control, since it identified differences in responses to ICS among clusters (i.e. the two clusters with the highest rates of exacerbation and lowest lung function showed either good or no response to ICS, measured as reduction in exacerbation rates, the non-responders having a high atopic burden compared to low atopy in the responders [13]. Similarly, longitudinal cluster analysis of > 3,000 adults with asthma showed strong phenotype similarities after 10 years and > 50 % probability of remaining in the same asthma phenotype [14]. Moreover, transitions towards increased asthma symptoms were more frequently observed among non-allergic phenotypes compared with allergic phenotypes [14].

1.2 The pathophysiology of asthma

Asthma is a complex disorder of the conducting airways characterised by exaggerated contractile responses to exogenous and endogenous stimuli (airway hyperresponsiveness, AHR), changes of the airway structural elements including more fragile epithelium accompanied by increased goblet cells and mucus secretion,
thickening of the airway smooth muscle (ASM), and neovascularisation (processes collectively known as airway remodelling). Chronic inflammation is also observed in less mild forms of asthma and may contribute to airway remodelling [15].

1.2.1 Airway inflammation

Airway inflammation is a fundamental component of asthma pathogenesis and involves activation of innate and adaptive immune pathways. Early studies implicated T helper (Th) cell-mediated increased recruitment and activation of eosinophils in the pathophysiology of asthma [16,17]. Activated Th cells (CD4+ cells, co-expressing the interleukin-2 receptor (CD25+), indicative of T cell activation) were found to be increased in peripheral blood of severe asthmatics, suggesting that a T cell-mediated (adaptive) immunity is involved in the pathophysiology of severe asthma [16]. Bradley et al. (1991) found increased activated T cells (CD25+) and activated eosinophils (eosinophil cationic protein-positive, EG2+) in bronchial tissue of subjects with atopic asthma compared with non-atopic asthmatics and normal controls, with no change in neutrophils and mast cells numbers [17]. Moreover, CD25+ and EG2+ cells were increased in the bronchial mucosa of hyperresponsive subjects, and blood and tissue eosinophils correlated with AHR in asthma, suggesting that a relationship exists between eosinophilic inflammation and AHR in asthma and supporting the view that activated T cells are involved in eosinophilic inflammation [17]. To confirm this, eosinophil numbers were found to be increased in the bronchoalveolar lavage (BAL) of asthmatics [18]. In addition, BAL cells showed increased mRNA expression of Th2 type cytokines, including IL-2, IL-3, IL-4, IL-5, and granulocyte-macrophage colony-
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stimulating factor (GM-CSF), in asthmatics [18]. In agreement with previous studies, 90% of these cells were T cells (CD3+), thus providing further evidence for the activation of Th2 type inflammatory pathways in asthma [18]. This study also found increased numbers of mast cells in BAL of asthmatics [18]. IL-4, IL-5 and GM-CSF expression in BAL cells correlated with airflow obstruction and AHR in asthma [19]. T cell activation, eosinophil numbers, and IL-5 mRNA expression were also found to be higher in bronchial tissue of asthmatics [20]. Moreover, allergen challenge was able to induce T cell activation, IL-4, IL-5 and GM-CSF upregulation, and eosinophil recruitment in BAL and tissue in asthmatics [19,21]. These observations are confirmed by animal models and in vitro studies: intraperitoneal administration of IL-4 in mice caused selective accumulation of eosinophils in tissue, which could be inhibited by anti-IL-4 neutralising antibodies [22]; IL-4−/− mice and mice lacking mature CD4+ T cells developed less bronchial inflammation and eosinophil accumulation in a model of allergic asthma [23]; in vitro, IL-4 induces the adhesion of eosinophils (up to four-fold) and basophils (two-fold), but not neutrophils, to vascular endothelium through the upregulation of vascular cell adhesion molecule-1 (VCAM-1) [24]; anti-IL-5 antibodies inhibited eosinophil infiltration in the airways of allergen-challenged guinea pigs [25]. Further studies implicated Th2 inflammation in AHR and mucus production; however, IL-4 was dispensable for both processes: T cell receptor deficient transgenic mice receiving Th2 cells (producing IL-4, IL-5 and IL-13) showed airway eosinophilic inflammation, increased mucus production, and AHR following antigen challenge, whereas mice receiving Th1 cells (producing interferon γ, IFNγ) displayed neutrophilic inflammation, but no increase in mucus production or airway responsiveness [26,27]. IL-4−/− Th2 cells were not recruited to the lungs, did not cause eosinophilia in IL-4−/− mice, and no
increase in mucus production was seen; however, AHR was not diminished, and tumour necrosis factor α (TNFα) was able to restore mucus hypersecretion in the absence of IL-4, suggesting that IL-4 is crucial for cell recruitment and inflammation, but is not directly involved in mucus production or AHR [26,27].

Pre-clinical and clinical evidence points to IL-13 as an important Th2 cytokine that may be driving asthmatic features including eosinophilic and IgE-mediated inflammation, goblet cell hyperplasia, and AHR. Indeed, transgenic mice selectively overexpressing IL-13 in the lungs have been shown to develop airway eosinophilic inflammation, goblet cell hyperplasia, mucus hypersecretion, subepithelial airway fibrosis and increased airway responsiveness [28]. Moreover, administration of IL-13 and IL-4 to T cell deficient mice induced an asthma-like phenotype, with increased AHR, goblet cell hyperplasia, and increased BAL eosinophils and neutrophils, which, with the exception of airway neutrophilia, were diminished by IL-13 neutralisation [29]. In a model of allergen-induced experimental asthma, IL-13-dependent signalling was essential for IgE production [30]. Other asthmatic features such as increased mucus production, increased airway resistance, and upregulation of the pro-fibrotic cytokine transforming growth factor β (TGF-β) and the chemokine CCL11 were also critically dependent on IL-13 receptor signalling [30]. In vitro, IL-13 induces a hypersecretory phenotype in human bronchial epithelial cells, further supporting a role for IL-13 in mucus secretion [31]. In humans, IL-13 mRNA was found to be increased in bronchial tissue [32] and in sputum cells of asthmatics [33]. IL-13 protein was also increased in sputum and bronchial mucosa of asthmatics compared with controls [34,35], and sputum IL-13 correlated with AHR in asthma [36]. Whilst the majority of IL-13+ cells found in the bronchial submucosa were eosinophils (> 80 %), 8 % were mast cells [34]. Mast cells
have been found to infiltrate the ASM in asthma, suggesting a relationship between mast cells and ASM dysfunction in asthma [37]. Interestingly, increased IL-13$^+$ cells were also found in the ASM in severe asthma [35]. Another study found that all IL-13$^+$ cells infiltrating the ASM in asthma were mast cells, and approximately 50% of these also expressed IL-4, suggesting that IL-4 and IL-13 play a role in mast cell-ASM interactions [38].

IL-17 is a pro-inflammatory cytokine produced by Th17 cells and by a newly identified subset of innate lymphoid cells (ILCs), which has been implicated in more severe asthma phenotypes characterised by neutrophilic inflammation [39]. Increased numbers of IL-17-expressing cells, identifiable as T cells and eosinophils, have been found in the airways and peripheral blood of asthmatics [40]. IL-17 protein was also found to be increased in the sputum of hyperresponsive asthmatics versus controls, with levels correlating with AHR, suggesting a link between IL-17 and AHR [41]. In another study, cells isolated from the sputum of asthmatics showed increased expression of IL-17 and of the neutrophil chemoattractant IL-8, which correlated with each other and with sputum neutrophils, suggesting that IL-17 may be involved in neutrophilic inflammation [42]. IL-17 expression was also found to be increased in sputum and submucosa of COPD subjects, and correlated with lung function decline, although not with neutrophils [43]. In vivo and in vitro studies confirm the role of IL-17 in airway neutrophilia and AHR. IL-17 induces the release of IL-8 together with profibrotic cytokines in fibroblasts in vitro [40]. Th17 cells are able to attract neutrophils in vitro by producing IL-8 [44]. In their turn, neutrophils can induce Th17 cell chemotaxis in a CCL20 and CCL2-dependent manner, suggesting that a cross-talk between Th17 cells and neutrophils exist [44]. In mice, ovalbumin sensitisation of the skin caused
increased IL-17 expression in tissue, lymph nodes and peripheral blood, and ovalbumin inhalation induced neutrophil influx to the lungs and increased bronchial responsiveness, which could be inhibited by anti-IL-17 neutralising antibodies [45]. Sensitised skin DCs efficiently induced Th17 cell maturation by producing IL-23 [45]. In another model of airway allergic sensitisation, modest Th2 responses were observed whereas Th17 responses were stronger [46]. IL-17 release in the airways caused airway neutrophilia and AHR, which were abrogated in IL-17 receptor$^{-/-}$ mice and by neutrophil depletion [46]. This study also showed that Th2 and IL-17 act synergistically to promote neutrophil and eosinophil recruitment, and AHR [46]. Moreover, Th17 deficient mice showed significantly reduced airway remodelling in a model of chronic airway allergic sensitisation, independently of Th2 responses (i.e. eosinophils and IL-13 expression were not changed, whereas neutrophils were markedly reduced, [47]). ILCs are recently discovered innate lymphoid cells that do not express mature lineage hematopoietic markers; they are multipotent and competent antigen presenting cells; they expand in vivo in response to the type 2-inducing cytokines IL-25 and IL-33; and produce Th2 type cytokines [48,49]. ILCs accumulated in response to IL-25 and induced Th2 type inflammation, with IL-13, IL-5 and IL-4 production and allergen-specific Th cell maturation in mice [49]. ILCs were the main source of IL-13 during parasite infection in mice [48], and conferred protection against parasite infection in IL-25$^{-/-}$ mice [49]. Allergen challenge induced IL-33 and thymic stromal lymphopoietin (TSLP) production, eosinophilic inflammation, and mucus hyper-production in the lungs of mice, which were inhibited in mice lacking ILCs [50]. Airway inflammation and AHR caused by viral infections in mice were dependent on IL-33 signalling, together with Toll-like receptor (TLR) 7 signalling [51]. Responses to viral infection were dependent on IL-13 produced
by ILCs, and these were ultimately required for the development of AHR (i.e. depletion of ILCs abolished AHR, and injection of activated ILCs into IL-13−/− mice reconstituted AHR [51]). Similar results were found in a different experimental model of AHR: blockade or genetic deletion of IL-33 receptor inhibited AHR, and ILCs were able to restore AHR in IL-13−/− mice [52], suggesting that IL-33 release/signalling is upstream of IL-13 in the development of AHR. Importantly, IL-33-induced airway inflammation and AHR were independent of adaptive immunity, since mice defective in B and T cells were still able to develop such features, indicating that ILCs link innate and adaptive immunity in inflammatory diseases like asthma [5,51,53].

The airway epithelium is able to release cytokines such as IL-25, IL-33 and TSLP following damage or stress, and may be an important source of these cytokines. This is supported by the increased IL-33 expression observed in the bronchial epithelium of smokers [54]. Moreover, cultured epithelial cells exposed to cigarette smoke upregulate IL-33 mRNA and protein expression [54]; damaged airway epithelium releases TSLP [55]; and osmotic stress causes the release of IL-33 from murine lung explants [55]. Importantly, TSLP and IL-33 stimulation are able to activate mast cells [55]. Whilst epithelial cells express high levels of IL-33 at baseline, viral infection caused IL-33 upregulation in lung macrophages and DCs in mice, suggesting that immune cells can also produce IL-33 [51]. Lastly, in vitro experiments suggest that more than one cytokine is required to induce Th2 type inflammation, because IL-33 alone was a weak stimulator of Th2 cytokine production in ILCs in vitro, but its efficacy was greatly enhanced when combined with other cytokines including IL-2, IL-7 and TSLP [50]. It has been suggested that an altered epithelial barrier could promote Th2
differentiation eventually leading to IgE production in asthma [5]. **Figure 1.2.1** summarises the main inflammatory pathways in asthma.
Figure 1.2.1 Airway inflammation in asthma. This figure summarises the main inflammatory pathways in asthma. Details and references can be found in the text. TSLP = thymic stromal lymphopoietin; DCs = dendritic cells; ILCs = innate lymphoid cells; AHR = airway hyperresponsiveness.
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1.2.2 Airway wall remodelling

Thickening of the airway wall is a main feature of asthma that correlates with disease severity and degree of airflow obstruction. Increased ASM mass and sub-epithelial fibrosis critically participate in this process and may appear early in the pathogenesis of asthma, since evidence of airway remodelling is found in children with asthma [58]. The contribution of ASM to airway remodelling is discussed in the next section. Allergen challenge has been shown to cause sub-epithelial fibroblast accumulation, basement membrane thickening and increased procollagen expression in asthmatic patients. These changes were observed from day one following challenge and persisted at day 7, whereas inflammation had resolved by day 7. Importantly, fibrotic changes were associated with AHR, suggesting that the latter can persist after the resolution of inflammation [59]. Increased sub-epithelial fibroblast numbers and increased collagen III deposition were also associated with severe asthma in another study [60]. Fibroblast numbers and basement membrane thickness were negatively correlated with FEV$_1$ pre- and post-bronchodilator in asthmatics, suggesting that these changes are specifically associated with severe disease and fixed airflow limitation in proximal airways. Increased extracellular matrix deposition is thought to be a protective mechanism that opposes ASM shortening, which however results in progressive irreversible airflow obstruction [61].

In addition to increased deposition of extracellular matrix including collagen and proteoglycans, neovascularisation accompanied by increased vascular leakage also contributes to a greater airway wall thickness [15]. Other features of airway wall remodelling in asthma include increased neural networks, which are supported by
nerve growth factors produced by epithelial and ASM cells and inflammatory cells including eosinophils and mast cells [15].

1.2.3 The airway smooth muscle in asthma

The ASM encircles the airway completely with a transversal orientation in the trachea and a helical or geodesic pattern around the central and peripheral airways, where it occupies relatively more of the airway wall. The ASM is present since foetal development and increases in absolute volume from infancy to early adulthood but remains stable thereafter [62]. The role of ASM in healthy adult lungs is unclear; however, proposed functions include maintaining airway tone, facilitating ventilation and favouring the expulsion of inhaled particles and pathogens, although none of these functions have been conclusively demonstrated [63]. Developmental studies point to an important role of the ASM in lung development. It has been observed that the appearance of ASM closely follows the emergence of defined lung buds in the foetus. Stretching of the tissue is thought to be essential for correct lung growth and also determines the degree of ASM differentiation. Discrete populations of ASM have been identified in the proximal lung that may work as pacemakers of airway peristalsis. Thus, waves of contraction are generated causing movement of the intraluminal fluid and periodic distention of the lung buds which are crucial for correct lung growth [64]. The involvement of ASM in asthma, on the other hand, is well documented and generally recognised. Increased ASM mass together with increased sub-epithelial fibrosis are critical features of airway wall remodelling in asthma. This was shown in post-mortem studies, which found increased ASM thickness in airways of all sizes.
except for the smallest airways (< 3 mm in diameter) in cases of fatal asthma compared with deaths unrelated to respiratory causes. Moreover, the increase in ASM mass is greater in cases of fatal asthma versus non-fatal asthma [62]. Both ASM hyperplasia (increased cell number) and hypertrophy (increased cell size) may be involved in this process to different extents that may depend on the subject. In fact, early studies examining cases of fatal asthma found two types of patients: one where increased ASM mass could be observed in the central bronchi and was associated with ASM hyperplasia; and a second type where increased ASM thickness was seen along the whole airway tree and ASM hypertrophy prevailed [65]. In another study, ASM cell number was nearly twofold higher in mild-to-moderate asthmatics, whereas no change in ASM cell volume was found [66]. A recent post-mortem analysis of a larger sample of asthmatics of various severities found ASM hypertrophy in the large airways in both cases of non-fatal and fatal asthma, whereas hyperplasia was only present in fatal asthma and interested the large, medium and small airways [67]. Increased ASM area and ASM cell size, instead, were found to be associated with severe disease in another study. ASM cell size negatively correlated with FEV$_1$ pre- and post-bronchodilator in asthmatics, suggesting that ASM hypertrophy in proximal airways is associated with severe disease and fixed airflow limitation [60]. Together these studies suggest that both ASM hypertrophy and hyperplasia occur in asthma, although one process may prevail over the other in different populations of asthmatics.

*In vitro* studies suggest increased proliferation of ASM cells from asthmatics [68]; however no increase in cell proliferation markers has been found *ex vivo* [61]. Nonetheless, evidence of ASM cell proliferation has been shown in some animal models of allergic asthma, although not in others [69]. It is possible that ASM cell
proliferation occurs before or at the onset of disease or over a long period of time, which would make this process difficult to detect [61].

Recent studies suggest other sources of ASM thickening in asthma, such as mesenchymal progenitors and stem cells located within the airway tissue or derived from peripheral blood [58]. Fibrocytes are blood-derived mesenchymal progenitors that have been proposed as a source of ASM in asthma. Fibrocytes constitute 1% of peripheral blood leukocytes and express both hematopoietic and mesenchymal markers. In tissue, expression of hematopoietic markers is lost whereas mesenchymal markers including α-smooth muscle actin (α-SMA) are accentuated by TGF-β and endothelin-1, which are both increased in asthma. In experimental allergic asthma, fibrocytes are recruited to the airways after allergen exposure [58]. In patients with asthma, tissue fibrocytes are correlated with basement membrane thickness and peripheral blood fibrocytes with airflow obstruction. Importantly, increased fibrocyte numbers have been reported in the ASM of asthmatics of all severities [70]. CXCL12 and IL-33 are potential fibrocyte chemoattractants to the sub-epithelial membrane [58]. ASM-derived PDGF and CCL2 may recruit fibrocytes to the ASM [58,71]. This is supported by clinical evidence showing increased plasma levels of CCL2 in severe asthmatics and increased CCL2 in sputum of asthmatics with bronchial wall thickening [71]. Interestingly, in this study ASM cells from asthmatics were shown to release more CCL2 than controls. In addition, mast cell-derived CCL19 may recruit myofibroblasts to the ASM [72].

Pericytes may also play a role in ASM thickening. Pericytes are mesenchymal stem cells that surround blood vessels; they are thought to detach and migrate to the sub-epithelium where they differentiate in SM cells. Epithelial cells can also differentiate
into SM cells in a process called epithelial-mesenchymal transition, which is mainly mediated by TGF-β [58].

Other changes in ASM behaviour that are involved in asthma include a more contractile phenotype and the increased release of mediators. ASM seems to contract more easily in asthma: *in vitro* ASM cells from asthmatics show increased contraction compared with controls in response to histamine [73]. Although in this study there was no difference between healthy controls and asthmatics in the expression of myosin light-chain kinase, an enzyme involved in ASM contraction, there is evidence suggesting altered expression of contractile proteins in asthma. Woodruff *et al.* (2004) found 50 – 83 % higher α-SMA expression in the submucosa of mild-to-moderate asthmatics, although there was no change in the gene expression of contractile proteins [66]. In another study, increased myosin light-chain kinase expression was associated with severe asthma [60]. α-SMA expression in asthma has been shown to correlate with the degree of mast cell infiltration in the ASM; and co-culture with mast cells or β-tryptase, a serine protease released by activated mast cells, caused the upregulation of α-SMA in ASM cells *in vitro* [74]. Moreover, mast cell-derived β-tryptase was able to induce ASM contraction and both effects (α-SMA expression and ASM contraction) depended on the autocrine release of TGF-β1 in ASM cells [74].

Altered calcium homeostasis and increased oxidative stress have also been implicated in ASM hypercontractility in asthma [75]. Recent studies suggest that ASM is under an increased oxidative burden in asthma: ASM cells from asthmatics express higher levels of NOX4, a subtype of ROS-generating nicotinamide adenine dinucleotide phosphate (NADPH) oxidases [76]. Blockade or silencing of NOX4 was able to inhibit ASM contraction, thus linking NOX4 upregulation with ASM hypercontractility in asthma.
A complex relationship exists between mast cells and ASM in asthma. Mast cells have been shown to localise to the ASM bundle in asthma significantly more than in normal controls, and this is correlated with AHR [37]. ASM can respond to various stimuli including pathogens and inflammatory cytokines by producing chemokines (such as IL-8, CCL11 (eotaxin), CXCL10 and CXCL12), cytokines (including IL-6 and IL-1β) and cell adhesion molecules [77,78]. In particular, the CXCL10-CXCR3 axis has been implicated in mast cell recruitment to the ASM in asthma. CXCR3-positive mast cell localisation to the ASM was found to be higher in asthma [78]. Interestingly, ASM cells from asthmatics released more CXCL10 compared with controls, resulting in an increased mast cell migration towards asthmatic ASM cells [78]. In their turn, mast cells are able to induce ASM cell migration by releasing CCL19, suggesting a link between mast cells and airway remodelling [72]. This is relevant in vivo, because more CCL19-positive mast cells were found in the submucosa of asthmatics and CCL19 receptor CCR7 was increased in ASM/myofibroblasts in asthma [72]. ASM also supports mast cell survival and proliferation and induces allergen-independent mast cell degranulation [79]. Together, these data indicate that a crosstalk exists between ASM and mast cells which contributes to the pathophysiology of asthma.

Intrinsic differences in the ASM of healthy and asthmatic subjects also emerge from the studies discussed above (i.e. NOX4 and CXCL10 expression). Additional evidence to support this includes increased CCL2 release from ASM cells of asthmatics [71], and increased production of TGF-β-stimulated connective tissue growth factor (CTGF) in ASM cells from asthmatics, which may result in the upregulation of extracellular matrix protein expression in ASM [80].
1.3 The danger hypothesis and the “alarmins”

For many years immunologists have been trying to answer a fundamental question: what are the factors that cause the immune system to mount a response? The ability of the immune system to respond to pathogens has long been recognised. It was later observed that some foreign molecules such as toxins were also able to provoke an immune response. Based on these observations, the idea that the immune system is driven by the need to discriminate between “self” and “non-self” structures emerged. Since the immune system is tolerant to many “non-self” structures, such as the commensal bacteria in our organs, in 1989 C.A. Janeway refined this concept by proposing that the immune system responds only to potentially infectious foreign antigens [81]. Some immune responses, however, could still not be accommodated in Janeway’s model, for example the T-cell response generated in some tumours and in autoimmune diseases, where there is often no infection [82]. To explain these phenomena, in 1994 P. Matzinger formulated an alternative model, known as the “danger hypothesis”, which postulates that the immune system has evolved to respond to danger and prevent destruction rather than to protect from foreign invasion [83]. According to this model, non-physiological cell death serves as a universal danger signal, as it occurs following tissue damage as well as during infection. During necrotic cell death molecules that are normally confined to the cytoplasm, the nucleus or the cell membrane, are rapidly dispersed into the surrounding space where they may bind specific receptors on immune cells. Thus, some of the molecules released act as danger signals or “alarmins” [84], by recruiting and activating cells of the innate immune system, including antigen-presenting cells, and so also promote
adaptive immunity. Alarmins ultimately contribute to restore homeostasis, by promoting repair mechanisms with the recruitment of stem cells to the damaged tissue and the induction of their proliferation [85]. Generation of ROS at the site of injury may also play an important role in the resolution of inflammation, since some alarmins are susceptible to inactivation by oxidation [86,87].

Alarmins represent the endogenous counterpart of the exogenous pathogen-associated molecular patterns (PAMPs). These are conserved microbial structures such as bacterial cell wall components and microbial nucleic acids which are released during pathogen invasion and, similarly to alarmins, activate receptor-expressing immune cells. Thus, alarmins and PAMPs together constitute a wider group of danger signal molecules, the damage-associated molecular patterns (DAMPs) [85,82]. However, although the name DAMPs was initially used to include both alarmins and PAMPs, currently the terms alarmin and DAMP are used interchangeably to indicate endogenous danger signals [88].

The list of DAMPs is continually growing and comprises a wide variety of molecules including nuclear proteins such as high mobility group box 1 (HMGB1), IL-1α and IL-33; nucleosomes and DNA; extracellular matrix components such as hyaluronan and fibronectin; cytoplasmic proteins such as heat-shock proteins (HSPs) and S100 proteins; mitochondrial molecules such as mitochondrial DNA and N-formylated peptides; secreted proteins such as galectins and the antimicrobial proteins cathelicidins and defensins; adenosine and adenosine triphosphate (ATP); and uric acid [88,89].

As postulated by the danger theory, necrotic cells passively release DAMPs following loss of membrane integrity. Chemical modifications of DAMPs and associated cellular
structures may prevent DAMPs release or activity during apoptosis [90]. If however, apoptotic cells are not promptly cleared by phagocytic cells, they undergo secondary necrosis, which is an autolytic process resulting in cell disintegration and release of cellular components, including immunogenic DAMPs [91]. This process may have little relevance under normal physiological conditions, in which apoptotic cells are rapidly cleared by phagocytes, but it may play an important role in pathologies where scavenger capacities are impaired or insufficient, such as autoimmune disorders [91]. DAMPs are also released in another type of apoptotic process, called immunogenic cell death (ICD), which can be caused by various agents such as viruses, chemotherapeutic drugs and ionising radiations and is able to induce an immune response. The immunogenicity of ICD has been linked to the release of DAMPs, which requires endoplasmic reticulum (ER) stress coupled with increased ROS production [92]. These mechanisms are important in cancer therapy, as anti-cancer drugs that elicit ICD show higher efficacy because they activate a potent anti-cancer immune response [92].

Besides dying cells, viable cells can actively secrete DAMPs under pathophysiological states, such as innate immunity and stress. Indeed, activated monocytes, neutrophils and dendritic cells have been shown to secrete DAMPs [93,94,95], as well as structural cells under stressful conditions such as high cholesterol and oxidative stress [96,97].

**1.4 Pattern recognition receptors**

DAMPs and PAMPs signal through a heterogeneous family of receptors, collectively named pattern recognition receptors (PRRs), which include membrane-bound receptors, such as the well characterised Toll-like receptors (TLRs) and the receptor for
advanced glycosylation end products (RAGE), and cytosolic receptors, such as the nucleotide-binding (NB) domain, leucine-rich repeat (LRR)-containing receptors (NLRs). PRRs are primarily expressed on cells of the innate immune system and on epithelial cells, which together constitute the body’s first line of defence against pathogens and damage, but other cell types can express PRRs and expression can be upregulated during pathophysiological states [98].

1.5 High mobility group box 1 (HMGB1)

HMGB1 is considered the prototypical DAMP and is probably the best characterised among them. It belongs to a family of nuclear non-histone proteins that also includes HMGB2, HMGB3, and the recently described HMGB4. HMGB proteins are highly conserved; in particular, HMGB1 proteins are almost identical in all mammals (> 99 % homology). HMGB1 and HMGB2 proteins are ubiquitously expressed during embryogenesis, but HMGB2 expression declines in adult tissues where it is restricted to testis, lymphoid organs and articular cartilage [99]. In contrast, HMGB1 remains expressed in most adult tissues [100].

HMGB1 has a molecular mass of 25 KDa and is composed of two L-shaped basic domains, the DNA-binding HMG boxes A and B, followed by a short basic linker and a C-terminal 30-aminoacid long acidic tail (figure 1.5.1, [101]). HMGB1 was formerly named amphoterin due to its highly polarised structure, which is also responsible for its high electrophoretic mobility (hence the current name, [102]). HMGB1 acidic tail can interact with both boxes, thus keeping the protein in a collapsed conformation.
which screens the boxes from DNA interaction (figure 1.5.1B). HMGB1 shift from a closed to an open conformation, however, is very dynamic [101].
Figure 1.5.1 Structure of the HMGB1 protein. **A** ) HMGB1 is a 25 KDa nuclear protein of 215 amino acids. It comprises two DNA-binding domains, the HMG boxes A (red) and B (blue), and a negatively charged C-terminal tail (green) connected by short linkers (yellow). Three critical cysteines are highlighted as yellow circles in box A (C23 and C45) and box B (C106). The redox state of these cysteine residues is crucial for HMGB1 function. **B** ) Schematic representations of a hypothetical compact, “tail-bound” form of HMGB1 in which the DNA-binding faces are occluded, in equilibrium with a more open form of the protein. DNA-binding boxes are shown in red (box A) and blue (box B), the basic linkers are yellow and the acidic tail is green [101].
1.5.1 HMGB1 nuclear functions

HMGB1 is a DNA-binding architectural protein that is involved in regulating various chromatin processes such as transcription, replication, recombination, DNA repair and genomic stability. HMGB1 binds with high affinity and no sequence specificity non-canonical DNA structures such as single-stranded DNA, cruciform or bent DNA, supercoiled DNA and DNA mini-circles, hence promoting identification of damaged DNA and repair mechanisms. HMGB1 can also induce double-stranded DNA to bend, thus facilitating access for transcription factors and regulatory proteins and promoting gene transcription [102]. Targeting of HMGB1 to specific DNA sequences is usually achieved by interaction with other nuclear proteins [103]. Although structurally similar, the two DNA-binding boxes may have different functions, since box A has a higher preference than box B for distorted DNA structures, whereas box B is more effective at bending DNA [101]. HMGB1 crucial function in the nucleus is underlined by the lethal phenotype of HMGB1−/− mice which are relatively normal at birth, but die within 24 h due to the inability to catabolise glycogen [104].

1.5.2 Extracellular HMGB1

Besides nuclear functions, HMGB1 also has extracellular roles as a DAMP, promoting inflammation and tissue repair through activation of PRRs including RAGE and TLR4 [99] (figure 1.5.2). Like other DAMPs, HMGB1 is passively released by necrotic cells. In contrast, it is retained in the nucleus of apoptotic cells, due to the hypoacetylation of histone proteins which favours HMGB1 binding to chromatin [90]. Innate immune cells
can also actively secrete HMGB1 in response to various stimuli such as LPS, CpG-DNA, TNFα, IL-1β, and IFN-γ [105,106,107,108]. More than one stimulus may be required for the secretion of HMGB1, since Gardella et al. (2002) found that LPS alone is a weak stimulator of HMGB1 secretion from monocytes. In this study, LPS induced HMGB1 translocation from the nucleus to the cytoplasm into secretory lysosomes, with most of the nuclear HMGB1 being redistributed in the cytoplasm after 18 h stimulation. Addition of lysophosphatidylcholine (LPC), a bioactive lipid that appears late (i.e. after 18 h) at sites of inflammation, to LPS-stimulated monocytes induced HMGB1 extracellular release [93]. This study suggests that HMGB1 secretion from inflammatory cells is a regulated process requiring multiple signals and supports the original view that HMGB1 is a late mediator of inflammation [105]. The kinetics of HMGB1 secretion, however, may depend on other variables too, because CpG DNA causes HMGB1 release within minutes in macrophages and DCs [108].

HMGB1 acetylation and phosphorylation have been shown to regulate HMGB1 translocation from the nucleus to the cytoplasm in monocytes and macrophages, an event which is triggered by inflammatory stimuli such as LPS and TNFα [109,110]. The process does not involve new synthesis of HMGB1, but existing HMGB1 is relocated [110]. Other post-translational modifications may influence HMGB1 mobility such as methylation, causing passive diffusion of nuclear HMGB1 in the cytoplasm of neutrophils [111]. Oxidation of cysteine C106 appears to be crucial for HMGB1 redistribution in the cytoplasm [112].

Further to immune cells, a variety of structural cells can actively secrete HMGB1 under stress conditions, such as PAMPs and DAMPs or pro-inflammatory cytokine stimulation, oxidative stress, hyperglycaemia and hyperlipidaemia. For example,
increased HMGB1 expression has been demonstrated in serum and tissues of animals fed a high fat diet [113], and HMGB1 is released by human atherosclerotic plaques [96]. Interestingly, vascular smooth muscle cells as well as endothelial cells can contribute to HMGB1 elevation at the site of atherosclerosis [96]. Other structural cells have been shown to release HMGB1 including murine pituicytes and enterocytes following TNF, IL-1 and TLR agonist stimulation [114,115]; murine hepatocytes under hypoxia or hydrogen peroxide treatment [97]; human endothelial cells challenged with uric acid [116]; and LPS-stimulated primary human epithelial cells [117]. Similar to immune cells, post-translational modifications such as acetylation may regulate nuclear HMGB1 shuttling to the cytoplasm [116]. In these studies, secreted HMGB1 was measurable at variable time points following stress, but higher levels were generally detected at late time points (i.e. starting from 8 h) and were maintained for several hours after stimulation, supporting the role of HMGB1 as a late mediator of inflammation [105]. Not only can structural cells secrete HMGB1 under stress, they can also respond to it in an autocrine/paracrine manner, by proliferating, migrating and producing more HMGB1 [96].

As a DAMP, HMGB1 activates the innate immune system by promoting monocyte and neutrophil recruitment [107,118], and by stimulating the release of pro-inflammatory mediators [106,119]. Leukocyte recruitment is facilitated by the HMGB1-dependent activation of the endothelium. In these cells, HMGB1 stimulation induces the upregulation of cell adhesion molecules ICAM-1, VCAM-1 and E-selectin, thus promoting neutrophil adhesion and transendothelial migration. Moreover, HMGB1-stimulated endothelial cells secrete granulocyte colony-stimulating factor (G-CSF) and IL-8, which stimulate the bone marrow to produce granulocytes and support
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neutrophil function including differentiation and migration [120,107]. As well as activating innate immune responses, HMGB1 induces adaptive immunity by promoting DC migration, maturation and pro-inflammatory cytokine production [121,122,123]. This includes secretion of HMGB1, which acts as an autocrine/paracrine maturation signal and is also required for naïve T cell clonal expansion, survival and functional polarisation to Th1 [95,121]. HMGB1 effects on leukocyte migration, endothelial cell activation, and DC maturation are RAGE-dependent [107,118,120,122,123].

Another way in which HMGB1 promotes inflammation is by interfering with apoptotic cell clearance or efferocytosis. An early event in apoptosis is the exposure on the cell surface of “eat me” signals such as phosphatidylserine (PS), a lipid normally facing the inner leaflet of the cell membrane. Opsonins, such as milk fat globule EGF factor 8 (MFG-E8), bridge PS with receptors on phagocytes, allowing them to engulf and clear apoptotic cells. HMGB1 can bind PS thus preventing binding of opsonins and phagocytosis of apoptotic cells [124]. Moreover, HMGB1 competes with MFG-E8 for binding to the integrin αvβ3 on the surface of macrophages, thus impairing macrophage engulfing capacities [125].

Therefore, HMGB1 promotes and sustains inflammation, but it also participates in tissue regeneration. In the infarcted myocardium, HMGB1 promotes the differentiation and proliferation of resident cardiac stem cells, resulting in significant regeneration of the infarcted tissue and improved heart function [126]. Moreover, in models of dystrophic and ischemic skeletal muscle HMGB1 recruits peripheral mesodermal stem cells and endothelial progenitor cells to the site of injury and promotes their proliferation, thus also inducing neovascularisation [127,128,129]. Resident mesenchymal cells such as fibroblasts and smooth muscle cells may also
respond to HMGB1 released at the site of damage by migrating and proliferating. Once again, HMGB1 chemotactic functions are RAGE-dependent and require activation of the transcription factor nuclear factor (NF)-κB [96,130,131]. Interestingly, HMGB1 box B is able to recapitulate HMGB1 pro-inflammatory effects, whilst box A acts as an HMGB1 antagonist [132,121,133].

### 1.5.3 Other functions of HMGB1

Besides nuclear and immune functions, HMGB1 has other physiological roles, for example in neurodevelopment. It has long been known that extracellular HMGB1 stimulates neurite extension in neurons, which requires activation of RAGE signalling via the small GTPases Rac and Cdc42, but not NF-κB [134]. The role of HMGB1 in neurodevelopment has recently been elucidated further by Wang et al. (2014), who showed that HMGB1 silencing impairs neural stem cell proliferation, causes the formation of fewer and smaller neurospheres, and negatively affects mitogen-activated protein kinase (MAPK) signalling, indicating that HMGB1 is involved in the renewal, normal growth and neurulation of neural stem cells [135]. HMGB1 is also a regulator of autophagy, a programmed degradation process that eliminates and recycles damaged cellular proteins and organelles and promotes cell survival. Cellular stress generating endogenous ROS triggers HMGB1 translocation to the cytoplasm, where it is oxidised to a form containing a disulphide bond between cysteine residues 23 and 45. Disulphide-HMGB1 promotes autophagy by disrupting the interaction between the autophagy protein Beclin 1 and B cell lymphoma 2 (BCL-2), which normally suppresses autophagy [136]. Moreover, RAGE is a positive regulator of
autophagy during oxidative stress and RAGE overexpression is protective against oxidative injury, suggesting that the HMGB1-RAGE axis favours autophagy and cell survival [137].

Lastly, adenoid-derived HMGB1 shows antibacterial activity and has been proposed to contribute to antibacterial functions in the upper respiratory tract; however it is not clear whether secreted HMGB1 is responsible for this, or what the bactericidal mechanism is [138].

1.5.4 HMGB1 in complex

Various studies have shown that HMGB1 can combine with diverse ligands, including PAMPs and endogenous molecules, present them to their specific receptors and increase their efficacy. Therefore, in the presence of HMGB1 an amplification of existing inflammatory pathways is observed.

Two LPS-binding sites have been reported in the HMGB1 protein sequence, one in each DNA-binding box, with affinities for the polysaccharide and lipid A moieties of LPS, respectively [139]. HMGB1-bound LPS is transferred to CD14 on the surface of monocytes and initiates TLR4-mediated signalling leading to increased synthesis of the pro-inflammatory cytokines TNFα and IL-6 [140,141]. Similarly, HMGB1 can bind the TLR2 synthetic ligand Pam3CSK4 and induce TLR2-mediated IL-6 production [141]. In both cases, the effects of the immune complexes are synergistic compared with either ligand alone and cell activation does not require RAGE.

TLR9 is an intracellular membrane-bound PRR that localises in the ER of resting immune cells. Upon endocytosis of bacterial or viral unmethylated CpG DNA, TLR9 is
relocated to the endosomal compartment where it binds unmethylated CpG DNA and initiates an immune response. HMGB1 is able to interact with unmethylated CpG DNA and potentiates TLR9-dependent IL-6 release in macrophages [108]. Endogenous CpG DNA can also activate TLR9 signalling and this pathway is implicated in the autoimmune disease systemic lupus erythematosus (SLE). Similar to microbial CpG DNA, HMGB1 binds mammalian CpG DNA specifically and potentiates the release of TNF and IFNα from DCs [142]. This study also elucidates how endogenous CpG DNA enters the cell and activates TLR9 signalling. The co-immunoprecipitation of RAGE, HMGB1 and TLR9 upon CpG DNA stimulation suggests that RAGE is internalised following HMGB1-CpG DNA binding and interacts with TLR9. Moreover, both RAGE and TLR9 are required for HMGB1-CpG DNA-induced DC activation [142]. Together this data suggest that HMGB1-CpG DNA binds RAGE, the complex is internalised and CpG DNA is efficiently delivered to the endosome-localised TLR9.

HMGB1 modulation of TLR9 signalling may be even more complex, since another study shows that HMGB1 pre-associates with TLR9 in the ER-Golgi intermediate compartment in the absence of CpG DNA [108]. HMGB1-deficient immune cells show a delayed redistribution of TLR9 into early endosomes and an impaired cytokine response following CpG DNA stimulation, suggesting that HMGB1-TLR9 association accelerates the TLR9 redistribution in early endosomes in the presence of CpG DNA. Interestingly, TLR9-associated HMGB1 appears to originate mainly from the nucleus, although exogenous HMGB1 can rescue cytokine release in HMGB1-deficient cells, indicating that extracellular HMGB1 acts in a similar way. Together, these studies highlight a complex HMGB1-dependent modulation of CpG DNA signalling through TLR9.
Under normal physiological conditions HMGB1 is associated with chromatin and remains bound to it during apoptosis. A defect in apoptotic cell clearance, which can lead to secondary necrosis, may be implicated in autoimmune diseases such as SLE. During secondary necrosis HMGB1 remains bound to nucleosomes but can be released in complex with them. Such complexes have been found in the blood of SLE patients and are able to stimulate cytokine (TNFα, IL-10, IL-6 and IL-1β) release from macrophages [143]. The inflammatory properties of HMGB1-nucleosome complexes are dependent on HMGB1 and TLR2, whereas TLR9 and RAGE are not required, suggesting that the complex interacts with TLR2. Moreover, HMGB1-nucleosomes promote adaptive immunity, since they induce DC maturation and the development of autoantibodies against dsDNA and histones, which are hallmarks of SLE. In contrast, HMGB1-nucleosomes purified from viable cells are tolerogenic and do not stimulate cytokine release [143].

Another endogenous molecule that can associate with HMGB1 is IL-1β. HMGB1-IL-1β complexes greatly enhance the expression and release of CXCL2 and TNFα from macrophages and neutrophils compared with HMGB1 alone, an effect that can be reduced by blocking binding to IL-1 receptor [144]. HMGB1-IL-1β complexes have been detected in vivo, in the bronchoalveolar lavage (BAL) fluid of smokers and smokers with COPD [145]. In this study too, HMGB1-IL-1β complexes caused increased release of TNFα from macrophages in an IL-1 receptor-dependent manner.

In addition, HMGB1 can form complexes with the chemokine CXCL12, an interaction that may underlie HMGB1 chemotactic properties. HMGB1-induced migration was already known to be pertussis toxin (PTX)-sensitive, although the mechanism was not understood [130]. Indeed, PTX inhibits G_\text{i/o}-coupled receptors, but HMGB1 is not
known to bind G-protein coupled receptors (GPCRs). This riddle has recently been clarified. HMGB1-induced fibroblast migration was found to be completely abrogated by anti-CXCL12 antibodies and by genetic deletion or pharmacological blockade of its receptor CXCR4, a GPCR [146]. Furthermore, HMGB1 synergistically increased CXCL12-induced monocyte migration, which was inhibited by the HMGB1 antagonist glycyrrhizin, suggesting an interaction between HMGB1 and CXCL12. Using NMR, the authors showed that both HMGB1 boxes can bind CXCL12. They speculated that each molecule of HMGB1 binds two molecules of CXCL12, probably stabilising an optimal conformation for their binding to CXCR4 dimers [146]. They also found that RAGE was dispensable for cell migration; however, HMGB1-stimulated fibroblasts and monocytes released CXCL12 in a RAGE-dependent manner, suggesting that RAGE activation regulates CXCL12 expression. To confirm this, RAGE activation (NF-κB non-canonical pathway, IKKα-dependent) is essential for the release of CXCL12 [147]. On the other hand, activation of the NF-κB canonical pathway (IKKβ-dependent) leads to RAGE upregulation and is also essential for HMGB1-induced migration [131]. Together these data suggest that HMGB1 acts through RAGE to activate NF-κB canonical and non-canonical pathways, respectively leading to RAGE and CXCL12 upregulation. HMGB1 then combines with CXCL12 and optimises its delivery to CXCR4, ultimately leading to cell migration. This data is supported by in vivo evidence: injection of the HMGB1-CXCL12 complex in subcutaneous air pouches caused a significantly higher influx of leukocytes than CXCL12 or HMGB1 alone. Moreover, HMGB1-CXCL12 complexes were detected in cardiotoxin-injured muscle after 2 h, together with IL-6 and TNF. CCL2, a potent monocyte chemoattractant, and IL-10 were detectable at later time points (6 h), suggesting that the influx of leukocytes is supported by different cytokines at
different stages of inflammation. The recruitment of leukocytes to the site of injury was completely inhibited by anti-HMGB1 blocking antibodies and to a lesser extent by the HMGB1 antagonist glycyrrhizin and the CXCR4 antagonist AMD3100. However, RAGE deletion did not prevent leukocyte recruitment [146]. This confirms that in vivo HMGB1 cooperates with CXCL12 to recruit leukocytes to the damaged tissue through a CXCR4-dependent mechanism.

1.5.5 HMGB1 receptors

It is apparent from previous paragraphs that HMGB1 can interact with a variety of receptors and modulates many processes relevant to inflammation, such as cell migration and activation. In many cases, the interaction is indirect, but HMGB1 can directly bind more than one PRR, including RAGE, TLR2, TLR4 and TLR9 [108]. The evidence around HMGB1-PRR binding/signalling has been so far conflicting. In one study, HMGB1-induced cytokine release was mediated by TLR2 or TLR4 depending on the cell type, but RAGE was not involved [148]. In another study, HMGB1 was found to bind directly to RAGE, but not to TLR2 or TLR4 [142]. HMGB1 direct binding to TLR4 was confirmed in a later study, showing that cysteine 106 is essential for HMGB1-TLR4 interaction ($K_d = 1.5 \, \mu M$). Importantly, HMGB1-induced cytokine production in macrophages required TLR4, but both TLR2 and RAGE were dispensable [149].
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Figure 1.5.2 Roles of extracellular HMGB1. This figure summarises the roles of HMGB1 as a DAMP and other roles that have been identified for extracellular HMGB1. Functions attributed to disulphide-HMGB1 or all-thiol HMGB1 are highlighted in blue and green, respectively. Details and references can be found in the text.
1.5.6 HMGB1 redox state

Significant advances in understanding HMGB1-receptor interactions have been made in recent years, with the discovery that HMGB1 redox state determines its function, and that HMGB1 chemotactic and pro-inflammatory activities are mediated by distinct receptors. HMGB1 contains three conserved cysteine residues, C23, C45 and C106 (figure 1.5.1A), which are reduced by thiol groups in non-oxidative environment and terminally oxidised to sulfonic acid during oxidative stress. Moreover, a disulphide bond can form between C23 and C45 in mild oxidative environment, which stabilises the protein in a folded conformation. The latter form, containing a disulphide bond between C23 and C45 and a thiol group on C106, is responsible for the pro-inflammatory actions of HMGB1, which are mediated via TLR4 [150,133,149]; whereas fully reduced HMGB1 is the form with chemotactic properties, as demonstrated also by the fact that non-oxidisable HMGB1 (3S-HMGB1, with all three cysteines mutated to serine residues) retains this function [133]. Lastly, terminal oxidation of the cysteine residues renders the molecule inactive [133,150]. The ability of fully reduced HMGB1 to recruit leukocytes has been confirmed in vivo [133]. Therefore, in the proposed mechanism of HMGB1-induced inflammation, reduced HMGB1 is released first at the site of damage. This promotes the recruitment of inflammatory cells through two mechanisms: RAGE-mediated upregulation of CXCL12 and amplification of CXCL12 activation of CXCR4. ROS generated at the site of inflammation cause the partial oxidation of HMGB1 to disulphide-HMGB1, which activates TLR4 on inflammatory cells and causes the secretion of pro-inflammatory
mediators. More ROS is produced, which inactivates HMGB1 during the resolution phase of inflammation [87].

1.5.7 HMGB1 in pathological states

HMGB1 is implicated in several diseases. Increased levels of HMGB1 are found in human serum during sepsis, an uncontrolled systemic inflammatory response to infection, and are associated with an increased risk of death [105]. Increased serum HMGB1 concentrations were detected from 8 to 32 h in LPS-treated mice and administration of anti-HMGB1 antibodies improved mice survival, suggesting that HMGB1 is a late mediator of endotoxin lethality. However, in hepatic ischemia-reperfusion injury HMGB1 was detected as early as 1 h after injury, suggesting that the kinetics of HMGB1 release depend on the type of injury and/or tissue [151]. Importantly, HMGB1 contributed to inflammation and tissue damage in a TLR4-dependent manner.

Besides acute infection and trauma, HMGB1 is involved in chronic inflammatory disorders and autoimmune diseases. HMGB1 expression is increased in the joints and serum of patients with arthritis and contributes to inflammation and tissue destruction [152]. Expression of HMGB1 and RAGE is also increased in chronic obstructive pulmonary disease (COPD) patients [145]. Brain inflammation is an important factor in epilepsy and recently the HMGB1-TLR4 axis has been implicated in this disease. Expression of HMGB1 and TLR4 is increased in the brain of patients with epilepsy and HMGB1 was able to provoke TLR4-dependent seizures in a mouse model of epilepsy [153]. As mentioned previously, HMGB1 is also involved in the pathophysiology of SLE.
In this condition, HMGB1 leaking from necrotic cells may contribute to disease progression by impairing the clearance of damaged tissue and by promoting inflammation in complex with nucleosomes via TLR2 [154].

1.5.8 HMGB1 negative regulation

A number of endogenous molecules have been shown to inhibit HMGB1 activity. This includes endogenous neuropeptides that are involved in stress/immune responses such as vasoactive intestinal peptide (VIP, a smooth muscle relaxant and vasodilator that can be produced by leukocytes including mast cells [155]), urocortin (involved in stress responses), and pituitary adenylate cyclase-activating polypeptide (PACAP, which has different roles in various tissues. In the respiratory tract it causes bronchodilation through muscle relaxation and also stimulates mucus secretion [156]). All of these have been shown to inhibit HMGB1 nuclear translocation and release. Moreover, PACAP is able to reduce circulating HMGB1 levels and improves survival in models of endotoxemia [157]. Ghrelin (a stomach-derived hormone) and acetylcholine (a neurotransmitter) are also protective in experimental sepsis and act by stimulating bacterial elimination and by attenuating HMGB1 systemic release [158].

Anticoagulant proteins including thrombomodulin and heparin (also released by mast cells) have been reported to inhibit HMGB1 by direct interaction [158,159]. In particular, thrombomodulin reduces cytokine and HMGB1 expression and is protective against tissue damage in models of acute injury and sepsis [157]. Ethyl pyruvate is a derivative of the endogenous metabolite pyruvic acid. In models of colitis and gastric cancer, ethyl pyruvate ameliorates symptoms, inhibits HMGB1 nuclear translocation,
and reduces cytokine production, RAGE, HMGB1 and MMP-9 expression and cancer growth [157].

HMGB1 inhibitors also come from natural sources. Danshen (Salvia miltiorrhiza), a plant used in Chinese medicine, blocks the nuclear translocation and release of HMGB1 during endotoxemia and confers protection against experimental sepsis [157]. Glycyrrhizin, another natural compound found in the roots of liquorice, inhibits HMGB1 activity and protects against ischemic tissue injury. Glycyrrhizin affords its protection by attenuating HMGB1-induced apoptosis, reducing HMGB1 expression, and blocking HMGB1 secretion via suppression of nuclear HMGB1 phosphorylation (one of the signals for HMGB1 secretion). Other natural compounds including epigallocatechin-3-gallate (EGCG, the main component of green tea), quercetin (a flavonoid found in fruits, vegetables and grains), and lycopene (a carotenoid found in tomatoes and red fruits) are all able to reduce HMGB1 expression and release and are protective in endotoxemia [157].

1.6 The receptor for advanced glycosylation end products (RAGE)

RAGE is a PRR with the ability to bind several ligands, including HMGB1. RAGE was first characterised in lung tissue, where it is abundantly expressed, as a transmembrane receptor belonging to the immunoglobulin superfamily and consisting of an N-terminal signal peptide, a V-type and two C-type extracellular Ig-like domains, a single transmembrane domain, and a short C-terminal cytosolic tail [160] (figure 1.6.1A). The V-type Ig domain contains two potential N-glycosylation sites, and evidence suggests that the native receptor is a glycoprotein with a molecular weight of approximately 50
KDa. N-glycosylation of RAGE favours ligand binding and function [161,162,163]. Structural studies show that the V and C1 Ig domains form an integrated unit (VC1) which is structurally independent of the C2 domain and is likely to contain the ligand binding region [164]. RAGE VC1 structure closely resembles that of neuronal and leukocyte adhesion molecules [164]. The C-terminal cytosolic tail is highly acidic and is crucial for intracellular signalling, as deletion of this domain inhibits receptor-dependent effects [134,161].

RAGE gene transcript can be alternatively spliced to generate several isoforms. In addition to full length (FL)-RAGE, 13 splice variants have been identified in lung tissue and human primary aortic smooth muscle cells, including various soluble forms lacking the transmembrane and cytosolic domains (sRAGE), and an N-truncated form [165]. FL-RAGE was the most prevalent form in both lung and aortic smooth muscle cells, accounting for 80 % and 70 % of detected transcripts, respectively, followed by a soluble RAGE form also named endogenous secretory RAGE (esRAGE), which constituted 7 % of the total transcripts in lung and 10 % in aortic smooth muscle cells [165]. A dominant negative form (DN-RAGE) lacking the cytosolic domain has also been described in the brain [166]. It is clear from a more recent study that RAGE undergoes extensive alternative splicing in mammals [167]. This study identified a total of 22 RAGE splice variants across several human tissues including adult, foetal and tumour tissues, of which the FL-RAGE variant was the most abundant. Other forms included various C-truncated forms, potentially generating sRAGE proteins; an N-truncated form; two forms with variations in exon 4 encoding for the C1 Ig domain, which may affect ligand binding affinity; and several non-coding shorter variants [167]. Consistently with previous studies [165], FL-RAGE and a C-truncated isoform (sRAGE) were the most
abundant in foetal and adult lung, whereas other tissues showed lower levels of expression [167]. RAGE isoforms have different functions, with FL-RAGE being the signalling receptor and sRAGE and DN-RAGE acting as inhibitors of RAGE signalling. In fact, both sRAGE (secreted) and DN-RAGE (membrane-bound) have the ability to bind RAGE ligands but are unable to activate intracellular signalling; therefore decreasing RAGE activation [168]. Alternatively, sRAGE and DN-RAGE may associate with FL-RAGE at the plasma membrane, thus hindering receptor oligomerisation which is required for intracellular signalling [169]. Expression of specific RAGE isoforms appears to be cell type-dependent and may be linked to specific pathological states [165,166,170]. Besides alternative splicing, proteolytical cleavage of the membrane-bound receptor involving the metalloproteinase ADAM10 can generate sRAGE [171]. Interestingly, constitutive cleavage of membrane-bound RAGE may occur in vivo, an event that may be enhanced following exposure to RAGE ligands [171].

1.6.1 RAGE ligands

RAGE binds several structurally diverse ligands. The first RAGE ligands to be described were advanced glycosylation end-products (AGEs), a heterogeneous group of compounds deriving from non-enzymatic glycosylation and oxidation of proteins, lipids and nucleic acids. AGEs form in pro-oxidative environments where reducing sugars such as glucose react with amino groups of long lived molecules leading to the formation of irreversible adducts [173]. AGEs are increased in tissues during normal aging [174], but accumulate at an accelerated rate in pathophysiological conditions such as diabetes and associated vascular complications [175], Alzheimer’s disease
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[176], rheumatoid arthritis [177], and inflammation [174,178]. At sites of accumulation, AGEs promote leukocyte recruitment via upregulation of adhesion molecules [177], exacerbate oxidative stress [178], and cause neurototoxicity in the central nervous system [176].

RAGE is also a receptor for members of the S100 protein family, which comprises 22 EF-hand Ca\(^{2+}\) binding proteins. S100 proteins regulate a variety of intracellular activities such as protein phosphorylation, enzyme and transcription factor activity, cell growth and differentiation, cytoskeleton organisation, intracellular Ca\(^{2+}\) homeostasis, and the inflammatory response. Several S100 proteins also have extracellular functions including promotion of cell survival or apoptosis depending on their concentration, modulation of cell proliferation, leukocyte recruitment, and regulation of macrophage activation [179,180,181]. In particular, a subset of S100 proteins referred to as calgranulins have been closely related to phagocyte-driven inflammation and function as DAMPs. S100A8 (calgranulin A), S100A9 (calgranulin B) and S100A12 (calgranulin C or EN-RAGE) are prevalently found in cells of myeloid origin and together represent almost 50 % of the soluble cytosolic content of granulocytes [182]. Calgranulins are secreted by activated monocytes and granulocytes via a non-classical route requiring intact microtubules and activation of protein kinase C [183]. Elevated levels of S100A8/S100A9 heterodimer and S100A12 are found in inflamed tissue, biological fluids and serum of patients with infectious and non-infectious diseases (e.g. viral and bacterial infections, chronic arthritis, cystic fibrosis, chronic inflammatory lung and bowel diseases, autoimmune disorders, and some tumours) [184]. Increased tissue/serum calgranulin levels show a close correlation with local inflammation and might be a useful marker of phagocyte activation. The calgranulins, S100B and S100A1
have been shown to signal through RAGE [185,186,187,188]; however, it is not clear whether the pro-inflammatory effects of calgranulins are mediated exclusively via RAGE or involve other receptors such as TLR4 [189,94]. Other RAGE ligands include amyloid fibrils [190], collagen I and IV [191], the leukocyte β2 integrin Mac-1 [192,118], and HMGB1.

The ability of RAGE to bind chemically diverse ligands is puzzling, and raises questions on how RAGE recognises its ligands. Crystal structure analysis of the human VC1 domain revealed highly conserved large hydrophobic regions and positively charged patches mainly on the surfaces of the V domain, which are likely to be involved in ligand binding [193,164] (**figure 1.6.1B-C**). These studies suggest that ligand binding does not rely on the recognition of specific amino acid residues, but rather depends on hydrophobic or electrostatic interactions with the VC1 domain.
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Figure 1.6.1 Structure of the RAGE protein. A) RAGE is a 50 KDa transmembrane receptor belonging to the immunoglobulin (Ig) superfamily. It consists of an N-terminal signal peptide (black), a V-type (red) and two C-type (blue) extracellular Ig-like domains, a single transmembrane domain (orange), and a short C-terminal cytosolic tail (green). Ligands bind to the V-type domain through hydrophobic and electrostatic interactions. B-C) Crystal structures of RAGE V and C1 domains, taken from [193]. B) Hydrophobic patches in the V domain of RAGE: orange = hydrophobic residues; cyan = hydrophilic residues; white = intermediate residues. The conserved hydrophobic patch is outlined by black dotted lines. C) Electropotential surface of RAGE VC1 domain, interesting mainly the V domain and extending on the C1 domain. The conserved basic patch is outlined by a black dotted line [193].
1.6.2 RAGE activation and signalling

RAGE proteins have been shown to spontaneously form oligomers at the plasma membrane in the absence of ligands. Oligomerisation is increased in the presence of RAGE ligands, indicating that receptor clustering is important for intracellular signalling [169]. Besides increasing ligand-receptor binding, carboxylated N-glycans promote receptor clustering and signalling in the presence of S100A12, further supporting the importance of glycosylation for RAGE function [163].

RAGE cytoplasmic domain is essential for intracellular signalling, yet it lacks endogenous tyrosine kinase activity or any known motifs involved in receptor signalling, suggesting that RAGE needs a binding partner to recruit downstream effectors. Recently, diaphanous-1 (Dia-1) has been shown to interact with the intracellular domain of RAGE [194]. Dia-1 is a member of the formin protein family and is involved in cell movement. The prolin-rich FH1 domain of Dia-1 interacts with mediators of the actin cytoskeleton and signal transduction molecules to mediate reorganisation of the cytoskeleton and cell movement. In the presence of RAGE ligands such as AGEs and S100B, Dia-1 associates with RAGE cytoplasmic domain and mediates downstream activation of the small GTPases Rac-1 and Cdc42, leading to increased cell migration [194]. This confirms previous findings implicating intact RAGE/Cdc42/Rac signalling in cytoskeletal rearrangements and neurite outgrowth [134]. Dia-1 is also involved in hypoxia-induced stress responses in macrophages. Hypoxic macrophages release AGEs which activate the RAGE/Dia-1 signalling pathway leading to the activation of early growth response-1 (EGR-1) transcription factor, regulating the
expression of cytokines and genes involved in cell differentiation and mitogenesis [195].

A separate RAGE signalling pathway involves the phosphorylation of RAGE cytoplasmic domain by the protein kinase PKCζ, leading to the recruitment of the adaptor proteins TIRAP and MyD88 to RAGE. These proteins typically mediate TLRs downstream signalling and interestingly also associate with RAGE, another PRR [196]. Activation of TIRAP results in the activation of IRAK4 and that of downstream signal transducers typical of TIRAP including Akt, p38, NF-κB and caspase-8. Biological outcomes of this pathway, which may compete with that activated by Dia-1, include the upregulation of cytokines and growth factors in endothelial cells following 12 h stimulation with AGEs, and the induction of apoptosis at later time points (48 h) [196]. This study showed that TLRs and RAGE share elements of their signalling pathways generating similar biological effects; however some outcomes are specific to ligand-receptor pair, for example IFN-β expression is only induced by a TLR ligand [196].

RAGE activation also causes the generation of intracellular ROS, leading to the activation of the small GTPase Ras and MAPKs such as p38 and JNK, which are induced by stress stimuli and are involved in cell differentiation and apoptosis, and ERK1/2 which is normally activated by growth factors and mitogens [134,197-201]. MAPKs activation ultimately leads to NF-κB-mediated upregulation of genes that are typically involved in inflammatory responses such as adhesion molecules (e.g. ICAM-1, VCAM-1) and cytokines (e.g. TNFα, IL-1β, and IL-8) [202,119,203,204].

Similarly, growth factors and integrins are known to generate transient bursts of intracellular ROS, which activate mitogenic signalling or induce changes in cell shape. In this setting ROS act as second messengers which reversibly oxidise proteins such as
transcription factors, protein tyrosine phosphatases, protein tyrosine kinases, and β-actin (known as redox signalling [205]). Sources of ROS include mitochondria, auto-oxidation of glucose, and enzymatic pathways including NADPH oxidase and lipoxygenase [197,205]. It is conceivable that through the generation of ROS, RAGE also mediates the trans-activation of other receptors, for example EGF receptor, by inhibiting tyrosine phosphatase activity [206].

In addition to the Cdc42/Rac and Ras/MAPK/NF-κB pathways, there are reports of phosphatidylinositol-3 kinase (PI3-K)/Akt activation, promoting cell survival and protein synthesis, by RAGE-dependent signalling [207], although others have not found such association [169].

Specific cellular responses to RAGE ligands may depend on the cell type and on the nature and concentration of RAGE ligands. In general, activation of RAGE signalling is thought to generate a state of sustained cellular activation, which can be attributed to the overlapping distribution of RAGE and its ligands and to the prolonged presence of RAGE ligands in tissues [98,208]. Amplification mechanisms of both RAGE and ligand expressions are also involved: for example, activation of the AGEs/RAGE axis in serosal mast cells has been shown to induce ROS production, which in their turn contribute to the formation of AGEs [178]. A positive autocrine/paracrine feedback loop might also regulate HMGB1 secretion, leading to a state of sustained inflammation [96,171]. Furthermore, RAGE expression is upregulated as a result of RAGE-dependent signalling since RAGE gene promoter contains NF-κB binding sites [209,98].
1.6.3 RAGE expression and functions

RAGE is mostly studied in pathological settings; however RAGE is also involved in normal physiological processes such as development and homeostasis. RAGE is thought to play an important role during embryogenesis, especially in neural development. In fact, RAGE and HMGB1 were found to co-localise in the developing central nervous system of rats. Both the cell bodies and even more the axons were intensely positive for RAGE and HMGB1, an interaction that promoted neurite outgrowth [210]. The role of HMGB1 in inducing RAGE-mediated neurite extension was confirmed in a later study [134].

RAGE expression declines in most tissues at the completion of development; however it remains relatively high in some tissues, suggesting a role for RAGE in maintaining tissue homeostasis. Early studies conducted in bovine tissue show that RAGE expression is higher in skeletal muscle, lung, heart, and liver. At the cellular level, RAGE expression is found in the vascular endothelium and smooth muscle, mononuclear cells, cardiac myocytes, and neural tissue including peripheral nerves and cortical neurons [211].

In the lung, RAGE is upregulated during alveolar type I (ATI) epithelial cell differentiation, indicating a role in pulmonary re-epithelialisation and repair. In these cells, RAGE localises at the basolateral membrane and promotes cell adherence and spreading on collagen IV, features that may be important for ensuring effective alveolar gas exchange [191]. RAGE blocking or silencing impairs the ability of alveolar epithelial cells and pulmonary fibroblasts to adhere to the extracellular matrix and induces increased proliferation and migration in these cells [212]. An impaired
adhesion to extracellular matrix may lead to loss of cell polarisation that could result in the increased proliferation observed in these cells [213]. Together, these data suggest the involvement of RAGE in lung homeostatic functions such as alveolar gas exchange and epithelial repair. In agreement with this, over-expression of RAGE in mice causes abnormal alveolar epithelial cell differentiation culminating in severe respiratory distress and perinatal lethality [214]. Another important physiological function of RAGE is the restoration of homeostasis following tissue injury through the recruitment of phagocytes and the promotion of tissue repair. Besides ICAM-1, RAGE expressed on the vascular endothelium serves as an additional counter-receptor for the β2 integrins Mac-1 and p150,95 expressed on the surface of neutrophils and macrophages [192]. RAGE also interacts with Mac-1 on the neutrophil/macrophage surface [118]. RAGE-Mac-1 interaction is increased in the presence of RAGE ligands such as S100B and HMGB1 and this in turn enhances Mac-1 interaction with ICAM-1 on the endothelium, resulting in an increased leukocyte extravasation [192,118]. This process requires the activation of the transcription factor NF-κB [118]. In the resolution phase of inflammation, recruited macrophages clear cell debris and dead neutrophils; moreover, activated macrophages release trophic factors that support tissue repair [216]. In peripheral nerve injury, S100B-activated RAGE is essential for nerve regeneration, because it induces Schwann cells to migrate, proliferate and release cytokines and neurotrophic factors. Furthermore, HMGB1 released during skeletal and cardiac muscle injury stimulates the recruitment, proliferation and differentiation of progenitor cells in a RAGE-dependent manner [216,217].
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1.6.4 RAGE in pathological states

Sustained activation of RAGE due to the prolonged expression of RAGE ligands, however, can be deleterious and is associated with several diseases including diabetes, vascular disease, Alzheimer’s disease, and some cancers [98]. RAGE activation is also supported by a positive feedback loop that causes the upregulation of the full-length receptor itself as a consequence of RAGE signalling [209]. In addition, RAGE endocytosis and lysosomal degradation upon activation are slow processes [98]. Together these events cause a sustained activation of RAGE-expressing cells ultimately leading to chronic inflammation [98].

Functions of RAGE that are relevant in disease emerge from previous paragraphs, such as the induction of leukocyte chemotaxis combined with the upregulation of endothelial cell adhesion molecules; the stimulation of cytokine/chemokine and MMPs production; the recruitment and activation of progenitor cells. Consequently, in a pathological setting where concentrations of RAGE ligands are high, RAGE may promote several processes such as inflammation (any ligand/RAGE); oxidative stress; morphological changes in the tissue due to tissue destruction, resident and progenitor cell proliferation and differentiation; tumour growth and metastasis (HMGB1/RAGE); neurotoxicity (amyloid-β and AGEs/RAGE) [98]. The role of RAGE in disease is confirmed by the observation that blockade of RAGE signalling with blocking antibodies, sRAGE, or the overexpression of DN-RAGE are associated with beneficial effects in models of diseases where RAGE is involved [218,219]. This also points to RAGE as a potential therapeutic target in these diseases.
1.6.5 RAGE genetic variation

Several RAGE gene (AGER) polymorphisms have been described, and some of these are associated with disease [172]. For example, Hudson et al. (2001) have shown various polymorphisms in the promoter region of RAGE; particularly a –429T>C variant, a –374T>A variant, and a rare 63-bp deletion, which all result in two- to three-fold higher gene expression in vitro [220]. Importantly, the –429T>C was associated with retinopathy in a group of diabetic subjects in this study [220]. The 63-bp deletion (spanning nucleotides –407 to –345 of the AGER promoter), instead, may be associated with reduced prevalence of nephropathy in type 2 diabetes [221]. Another important genetic mutation occurs in the exon 3 region of AGER. This is a single nucleotide polymorphism (SNP) which results in a glycine-to-serine substitution at position 82 (G82S), in the ligand binding domain of RAGE that is also a potential N-glycosylation site [222,223]. This variant occurs in approximately 10% of the general population regardless of ethnicity [222]. In vitro studies showed that cells bearing the 82S RAGE variant have higher affinity for S100A12, and are activated to a higher extent in response to S100A12 stimulation (i.e. increased phosphorylation of MAPKs and doubled NF-κB activation, [224]. Moreover, mononuclear phagocytes isolated from 82G/82S heterozygous individuals showed approximately 2-fold higher MAPKs phosphorylation, cytokine (TNFα and IL-6) generation, and MMP-9 activity compared with 82G homozygous cells following stimulation with S100A12, and this was not due to differences in basal levels of cell activation [224]. The RAGE 82S allele was also more prevalent in subjects with rheumatoid arthritis compared with controls, overall suggesting that this genetic variant is associated with higher RAGE activation and
inflammatory activity, and an increased risk of developing rheumatoid arthritis [224]. A more recent study investigating the prevalence of the –429T>C, the –374T>A, and the G82S RAGE mutations in diabetic subjects found an association of the –374T>A genotype with diabetes, and of the –429T>C mutation with microvascular complications in diabetics [225]. In agreement with this, the –429T>C genotype was associated with higher circulating levels of AGEs in diabetics and even higher in diabetics with microvascular complications, indicating this allele as a risk factor for the development of cardiovascular complications in diabetes [225]. A meta-analysis of 27 studies also investigated the association of the –429T>C, the –374T>A, and the G82S RAGE mutations with cancer, and found a significant association of the G82S variant with increased risk of cancer, whilst the –374T>A variant was associated with lower risk of cancer [226]. A significant association of the G82S RAGE variant has also been shown with Alzheimer’s disease [227,228].

Genome-wide association studies investigating large cohorts of subjects with or without respiratory diseases including asthma and COPD also showed a significant association of the G82S SNP with lower FEV₁/FVC [229,230], indicating a role for this SNP in determining lung function. In agreement with this, the G82S SNP was found to be associated with lower FEV₁/FVC in children with transient early wheeze [231], and also with COPD and an increased risk of developing COPD in smokers [232]. Interestingly, soluble RAGE expression can also be affected by RAGE genetic mutations in a way that is relevant to disease progression, as shown by a study conducted in subjects with Alzheimer’s disease [228]. In this study, plasma sRAGE was decreased in subjects with Alzheimer’s disease versus controls, and the 82S heterozygous and homozygous genotypes were associated with even lower sRAGE and faster cognitive
deterioration in these patients [228]. This was also found to be true in healthy subjects: the 82G/82S genotype was associated with lower plasma sRAGE levels compared with the 82G/82G genotype, and the 82S/82S genotype had even lower sRAGE [233]. Importantly, subjects bearing the 82S/82S genotype had significantly higher circulating levels of AGEs, TNFα, and serum C-reactive protein, higher insulin resistance, and increased risk factors for cardiovascular disease such as oxidative stress, inflammation, and insulin resistance [233]. The G82S RAGE variant was also associated with lower circulating sRAGE levels in COPD patients, although there was no significant association of RAGE polymorphism with COPD itself [234].

1.7 Evidence linking RAGE and HMGB1 to respiratory diseases

RAGE is abundantly expressed in the lung where it is likely to play a homeostatic role, particularly related to alveolar development and function [214,235]; therefore alterations in RAGE expression may cause pathophysiological changes in the lung. For example, loss of RAGE expression in the lung may be central to fibrotic lung diseases such as idiopathic pulmonary fibrosis (IPF). This is indicated by the downregulation of RAGE found in the lungs of IPF patients both at the mRNA level [212,215] and at the protein level; the latter reduction affecting both FL-RAGE and sRAGE proteins [170,212,215]. The underlying mechanism linking RAGE loss and lung fibrosis is not fully understood, but RAGE silencing in pulmonary fibroblasts and alveolar epithelial cells causes reduced cell adherence to extracellular matrix and increased cell migration and proliferation, suggesting that the functional changes following RAGE downregulation are related to loss of cell polarisation [212]. Interestingly, FL-RAGE and
sRAGE downregulation can be induced by TGF-β1 and TNFα, suggesting an inflammation-driven fibrotic phenotype [212]. In agreement with this, inflammatory cytokines and TGF-β1 are able to disrupt RAGE association with cytoskeletal proteins in alveolar epithelial cells, thus leading to changes in cell morphology towards a fibrotic phenotype [236]. These findings are confirmed by animal studies, showing loss of RAGE expression in the fibrotic lungs of bleomycin-treated mice [212]. Moreover, RAGE knock-out mice spontaneously develop lung fibrosis and also develop more severe asbestos-induced lung fibrosis compared with wild-type mice [215].

Increased RAGE ligands such as S100A12 in the airways [237] and systemic HMGB1 [238] are associated with acute lung injury and acute respiratory distress syndrome (ARDS), which are characterised by epithelial damage, bacterial infections, and inflammation, which is predominantly neutrophilic [238,239]. Similarly, airway HMGB1 levels gradually increase during IPF acute exacerbations concomitantly with the chemokine CCL2, and this is associated with augmented HMGB1 expression in alveolar macrophages, alveolar epithelial cells, and with alveolar capillary lesions [240]. While increased levels of RAGE ligands are likely to reflect tissue damage and inflammation in acute lung injury [238] and IPF exacerbations [240], they may also be involved in tissue repair mechanisms. In fact, HMGB1 released during acute lung injury has been shown to promote re-epithelialisation of alveoli by stimulating the release of TGF-β1 in an IL-1β and αvβ6 integrin-dependent manner [241]. Wound healing in rat ATII cells required both TLR4 and RAGE, whereas CXCR4, which has been shown by others to be necessary for cell migration [146], was dispensable [241]. Circulating levels of sRAGE are also increased in ARDS patients, positively correlate with HMGB1 levels and are associated with patient death. This may indicate that increased systemic sRAGE in
ARDS derives from shedding of membrane-bound RAGE and therefore reflects the extent of tissue damage [238].

A role for RAGE and RAGE ligands in chronic airway inflammatory disorders is also emerging. Ferhani et al. (2010) found increased levels of HMGB1 in the bronchoalveolar lavage (BAL) of smokers with COPD which correlated with disease severity [145]. Circulating S100A12 was also found to be increased in COPD patients and correlated with disease severity [242]. Moreover, smokers with COPD showed increased expression of RAGE and HMGB1 in the submucosa, epithelium and smooth muscle of the bronchial wall and in alveolar macrophages [145]. Increased RAGE expression in the lungs of COPD subjects was confirmed by another study, also showing higher AGEs expression in the alveolar walls and bronchioles in COPD [243].

The expression of RAGE and AGEs in the lung is upregulated in other smoke-related conditions, and affects the bronchiolar epithelium, ATII cells, the endothelium, and alveolar macrophages [244]. Smoking alone is associated with increased RAGE expression and inflammation. For example, increased RAGE and HMGB1 expression is found in airway mucosal cells of healthy smokers [145], and RAGE is upregulated in the lungs of mice exposed to cigarette smoke [245]. Furthermore, tobacco smoke causes the activation of RAGE-dependent inflammatory pathways leading to cytokine secretion in alveolar epithelial cells [204]. Other studies demonstrate increased lung RAGE expression following exposure to other environmental pollutants such as particulate matter and pollutant gases, suggesting that RAGE upregulation can be activated as an early response to environmental insults [246].

Whilst RAGE and its ligands are upregulated in COPD and other smoke-related conditions, sRAGE has been found to be reduced and to correlate with more severe
disease. Systemic levels of sRAGE were reduced in COPD subjects compared with healthy controls, with the decrease correlating with a decline in lung function [247]. Moreover, disease exacerbations were associated with even lower levels of circulating sRAGE, suggesting that sRAGE may be protective for lung function [247]. Studies investigating larger cohorts of COPD patients confirmed the association of lower circulating sRAGE with COPD severity, and also found a strong correlation with severity of emphysema, indicating sRAGE as a potential biomarker of emphysema [248,234,249]. Lower systemic sRAGE was not related with smoking (i.e. no difference in sRAGE was found between healthy smokers and non-smokers [234]); however, one of these studies uncovered an association between lower circulating sRAGE and genetic polymorphisms of the AGER locus (including the G82S variant), although these were not associated with COPD clinical characteristics [234]. Together, these studies highlight a complex relationship between RAGE genetic variations and the expression levels of RAGE, RAGE ligands and sRAGE (deriving from transcriptional regulation and/or RAGE shedding) that may influence COPD development.

A small number of studies also support the elevation of HMGB1 in asthma. Sputum HMGB1 was found to be elevated in treatment-naïve asthmatics, with HMGB1 levels being correlated with disease severity and sputum neutrophils [250]. This was confirmed in a larger study showing an increase in sputum and plasma HMGB1 in asthmatic and COPD subjects compared with healthy controls [251]. HMGB1 increased with disease severity and negatively correlated with lung function; moreover, HMGB1 concentrations were higher in COPD compared with asthma [251]. In contrast with these studies, Sukkar et al. (2011) found no difference in bronchial lavage (BL) HMGB1 concentrations in asthmatics and COPD patients compared with healthy controls [252].
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However, both systemic and BL levels of sRAGE (correlating with esRAGE, a secreted splice variant of RAGE) were lower in neutrophilic asthma/COPD (neutrophils ≥ 65 % of total BL cells) compared with non-neutrophilic disease [252]. In contrast, Watanabe et al. (2011) showed a significant increase in esRAGE expression in the sputum of asthmatics [250]. Discrepancies among these studies may reflect differences in populations and sampling; however, other studies support the association of reduced sRAGE with pathological states, as discussed in this and the previous sections.

1.7.1 Lessons from mouse models

Transgenic mouse models and animal models of disease have greatly enhanced our understanding of the roles that RAGE and HMGB1 play in lung development and homeostatis, and in respiratory diseases. A total of 17 RAGE splice variants have been identified in mouse, including a full length variant, which is the most widely expressed; various potential soluble variants; and several non-coding splice variants [167]. As mentioned in the previous section, loss of AGER causes the spontaneous development of lung fibrosis in adult RAGE<sup>−/−</sup> mice, which also develop more severe fibrotic damage compared with wild-type mice in a model of pulmonary fibrosis, confirming an important role for RAGE in maintaining lung homeostatis [215]. Similarly, RAGE overexpression in mice causes significant impairment of lung development and function. These mice develop hypoplastic lungs with enlarged alveolar spaces and decreased respiratory surface, and show impaired differentiation of alveolar type I and II epithelial cells and reduced ATI cell numbers [214]. Furthermore, mice overexpressing RAGE have impaired capillary growth due to inhibition of endothelial
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expansion [254]; reduced elastin expression; diminished collagen IV deposition and production concomitantly with increased MMP-9 secretion; increased apoptosis, particularly of alveolar epithelium; and increased BALF levels of polymorphonuclear cells and inflammatory mediators [255]. Interestingly, many of these changes have also been described in COPD: firstly, the increase in RAGE expression, as outlined in the previous section; additionally endothelial dysfunction; MMP upregulation; increased apoptosis of epithelial and endothelial cells; and increased inflammation, further linking RAGE with COPD [256]. Moreover, Bodine et al. (2014) showed that transgenic mice selectively overexpressing RAGE in the airway epithelium have normal lung development and no difference in apoptotic rates, but, in the absence of stimuli, show elevated BALF total cell numbers, which are mainly accounted for by polymorphonuclear cells, and increased lung expression of the inflammatory cytokines TNFα, IL-7 and IL-14 [257]. Conversely, RAGE−/− mice show reduced release of inflammatory cytokines following tobacco smoke exposure [204]. Together, these studies further confirm that RAGE contributes to lung inflammatory processes and to smoke-related inflammation.

The involvement of HMGB1 in asthma is also supported by animal models. HMGB1 expression was found to be increased in BALF and lung tissue of ovalbumin-sensitised mice, and HMGB1 cytoplasmic expression (which is considered a hallmark of HMGB1 secretion) was observed in airway bronchial and alveolar epithelium, fibroblasts, and inflammatory cells [258]. No increase in RAGE expression was found in the lungs of these mice, whereas TLR2 and TLR4 expression was increased in bronchial and alveolar epithelial cells and infiltrating inflammatory cells [258]. Importantly, anti-HMGB1 neutralising antibodies were able to improve several aspects of the chronic allergic
asthma model, including reduced airway inflammation (neutrophils, eosinophils and lymphocytes were affected), inflammatory cell infiltration in the lungs, goblet cell hyperplasia, collagen deposition, and AHR. In particular, ovalbumin-sensitised mice showed increased numbers of CD4$^+$ cells in the lymph nodes and lungs (mainly Th17 (IL-17$^+$) cells, and also Th2 (IL-4$^+$) and Th1 (IFN$\gamma^+$) cells) which were reduced in the presence of anti-HMGB1 antibodies. In agreement with this, HMGB1 neutralisation inhibited the release of a range of cytokines including Th17, Th2, and Th1-related cytokines, TNF$\alpha$, and airway remodelling cytokines such as TGF-β1. Notably, the expression of HMGB1, TLR2 and TLR4 was also significantly attenuated by anti-HMGB1 antibodies [258]. In a separate study, the HMGB1 antagonist box A was able to inhibit the ovalbumin-induced increase in total cell counts (prevalently neutrophils) in BALF and lung tissue in mice, and also inhibited the development of methacholine-induced AHR in these mice [259]. Furthermore, this study explores in more detail the relationship between HMGB1 and Th17 polarisation in asthma. Increased numbers of Th17 cells and increased BALF levels of the Th17-associated cytokines IL-23 and IL-17 were found in these mice, and both were reduced by box A [259]. In vitro, HMGB1-stimulated bone marrow derived DCs released IL-23, which favours the expansion of Th17 cells [45], and this was dose-dependently inhibited by box A. Moreover, HMGB1-stimulated DCs co-cultured with CD4$^+$ T cells induced increased expression of IL-17 in CD4$^+$ T cells. IL-17 release was also increased and could be inhibited by box A [259]. This study suggests that HMGB1 is involved in the polarisation of Th17 cells and Th17 responses in allergic asthma.

Systemic inflammation is a consequence of hemorrhagic shock, which is associated with increased circulating levels of HMGB1, and leads to lung neutrophilic
inflammation and acute lung injury in mice [239]. Both pharmacological inhibition of HMGB1 with box A, and genetic deletion of TLR4 were shown to reduce lung inflammation and damage in this model, suggesting that TLR4 is involved in HMGB1-induced lung inflammation [239]. However, in a mouse model of allergic inflammation, both TLR4 and RAGE participated in different stages of the sensitisation and inflammatory processes [253]. TLR4 is important in the development of allergic sensitisation to house dust mite [260]. However, Ullah et al. (2014) showed that both TLR4−/− and RAGE−/− mice were similarly protected from all features of house dust mite-induced allergic airway inflammation (except for airway neutrophilia in RAGE−/− mice), suggesting that the effects of RAGE and TLR4 deletion are not synergistic. Interestingly, when cockroach extract (a different PAMP which does not signal through TLR4) was used, contrarily to RAGE−/− mice, the TLR4−/− mice were not protected across the whole spectrum of inflammatory features (i.e. total serum IgE and mucus production remained higher, and cockroach-specific IgG was produced), which may suggest that signalling pathways activated by different PAMPs converge onto RAGE. This may depend on the role that RAGE plays in DC activation [122,123]. Indeed, whereas the innate immune response was similarly compromised in both RAGE−/− and TLR4−/− mice (i.e. reduced IL-13 production), RAGE−/− mice had reduced DC recruiting capacity which could be restored by injecting RAGE+/+ DCs into these mice [253]. Two distinct waves of HMGB1 expression and release were shown to participate in this process, the first being TLR4-dependent and the second requiring RAGE. HMGB1 BALF levels increased at day 1 following house dust mite exposure, peaked at day 2 and declined at day 3. Cytoplasmic HMGB1 expression in the epithelium followed a similar pattern, with increases at day 1 and 3, indicating the airway epithelium as a source of extracellular
HMGB1. HMGB1 increase at day 1 was inhibited in TLR4−/− mice, whilst the increase at day 2 was inhibited in RAGE−/− mice. Moreover, the first wave of HMGB1 release was IL-1 receptor-dependent and was downstream of IL-1α release, whereas the type 2 innate cytokines TSLP, IL-33, and IL-25 peaked at day 3, downstream of HMGB1 release, and were diminished in both TLR4−/− and RAGE−/− mice, and by HMGB1 neutralising antibodies. Interestingly, HMGB1-IL-1α complexes were detected in BALF at day 1 and persisted until day 3 [253]. Therefore, this study presents a model in which PAMPs cause the release of endogenous DAMPs, including HMGB1, from the epithelium through the activation of specific PRRs. This is followed by the RAGE-dependent activation of DCs, which promote Th2 inflammatory responses. This study suggests that different PRRs play roles at different stages of the allergic inflammatory response and different PAMPs may converge on RAGE activation [253].

In conclusion, both clinical and pre-clinical evidence indicate that HMGB1 and its receptors are involved in respiratory diseases including chronic airway inflammatory conditions such as COPD and asthma. However, more studies are required to elucidate the role of HMGB1 and its receptors in regulating structural airway cell function in relation to airway disease.

1.8 Hypothesis

The studies outlined in the previous sections support an important role for the HMGB1-RAGE/TLR4 axes in the pathophysiology of chronic airway inflammatory conditions such as COPD and asthma. Fewer studies have investigated this in asthma,
and in particular the role of HMGB1 in regulating human structural airway cell types including ASM and bronchial epithelial cells has not been characterised.

Recent data have shown that RAGE and HMGB1 mRNAs are constitutively expressed in cultured human ASM cells [94]. Preliminary work from our laboratory has also shown that HMGB1 mRNA is constitutively expressed by ASM cells and is upregulated following activation of the viral PRR TLR3, supporting the view that HMGB1 contributes to the innate immune response of structural cells and suggesting that crosstalk between PRRs plays an important role. Human ASM cells have also been shown to express functional TLR4 (i.e. LPS stimulation causes the release of inflammatory mediators in these cells, [261]).

Whilst it is now recognised that HMGB1 can interact with several receptors, at the time when the present study was conceived and conducted RAGE was regarded as the main receptor for HMGB1; therefore, this Thesis focuses on RAGE rather than other HMGB1 receptors. The study presented in this Thesis aimed to test the following hypotheses:

1. RAGE and HMGB1 are differentially expressed in the airways of healthy and asthmatic subjects, and this is related to airway inflammation (i.e. number of inflammatory cells).

2. The alteration in airway RAGE and HMGB1 expression is also present in cultured airway mesenchymal cells and influences their function.

3. HMGB1 is upregulated and released in mesenchymal cells in the presence of stress stimuli, such as PAMP stimulation, oxidative stress and inflammatory stimuli.
4. Extracellular HMGB1 participates in disease by affecting mesenchymal cell function via PRR activation. These hypotheses were tested first in human primary ASM (HASM) cells, due to their wider availability, and preliminary experiments also investigated the expression and function of HMGB1 and RAGE in human primary bronchial epithelial cells (HBECs, figure 1.8.1).

1.9 Aims

The aims of the study were:

1. To investigate sputum HMGB1 and RAGE levels in non-asthmatic individuals and asthmatics of various severities using ELISA. The redox state of sputum HMGB1 was also investigated by Western blotting under non-reducing conditions.
2. To investigate the expression of HMGB1 and RAGE in bronchial tissue obtained from non-asthmatics and asthmatics using immunohistochemistry.
3. To characterise the expression of HMGB1 and RAGE in HASM cells from non-asthmatics and asthmatics using quantitative RT-PCR, flow cytometry, immunofluorescence and Western blotting. HMGB1 expression was also characterised following cytokine and TLR3 agonist stimulation.
4. To study the role of HMGB1 and RAGE in regulating HASM cell functions in health and asthma including cell activation, migration and contraction.
Figure 1.8.1 Hypothetical roles for HMGB1 in regulating airway cell functions in asthma. Epithelial cell damage may cause the release of HMGB1 in asthma; this is known to induce the recruitment of inflammatory cells and to promote tissue repair. We hypothesised that HMGB1 contributes to repair mechanisms in the ASM and airway epithelium, and that it induces ASM contraction. These processes are known to be involved in asthma pathophysiology. Dotted lines represent hypothetical pathways.
Chapter 2

Materials and methods
2. Materials and methods

2.1 Materials

Rabbit monoclonal anti-HMGB1 antibody was from Abcam®, clone EPR3507; mouse monoclonal anti-HMGB1 antibody was from Abnova, clone 2F6; rabbit polyclonal anti-RAGE antibody was from Millipore, catalogue number AB9714; mouse monoclonal anti-RAGE antibodies were from Santa Cruz® (clone A11) and Millipore (clone DD/A11); rabbit monoclonal anti-NF-κB p65 antibody (clone D14E12) was from New England BioLabs®; rabbit immunoglobulin fraction, mouse IgG2a and rabbit anti-mouse FITC antibody were from Dako; sheep anti-rabbit RPE-conjugated antibody was from AbD Serotec; rabbit anti-mouse and goat anti-rabbit Alexa Fluor® 488 antibodies were from Life Technologies™; goat and mouse anti-rabbit HRP-linked antibodies were from New England BioLabs®; recombinant human (rh)HMGB1, rhIL-8, rhTNFα, rhIFNγ and rhIL-1β were from R&D Systems; 3S-HMGB1 was from HMGBiotech; poly(I:C) was from InvivoGen; H₂DCFDA was from Molecular Probes®; PureCol was from CellSystems®; and bradykinin was from Sigma-Aldrich®.

2.2 Subjects

The subjects included in this study were recruited from hospital staff, the general respiratory and the ‘Difficult Asthma’ clinics at Glenfield Hospital (Leicester), local primary healthcare and by local advertising. All subjects gave written informed consent and the study was approved by the Leicestershire Ethics Committee.

Asthma was defined according to the current Global Initiative for Asthma (GINA) guidelines [2], and asthma severity was classified based on the level of treatment
required to control symptoms and exacerbations (e.g. doses of inhaled corticosteroids (ICS) and use of long-acting β₂-agonists). Therefore, GINA treatment steps 1 and 2 corresponded to mild asthma; GINA 3 was moderate asthma; and GINA 4 and 5 corresponded to severe asthma.

All subjects underwent spirometry, which was carried out using the Vitalograph® Gold Standard, model 2150. In this test, the subject breathes in fully and then breathes out through a mouthpiece as fast and as far as possible until the lungs are empty. Spirometry provides an indication of lung function and capacity. The main spirometry parameters used in this study were the forced expiratory volume in 1 sec (FEV₁) and the FEV₁/FVC (forced vital capacity) ratio. FEV₁ is the volume of air exhaled forcefully in the first second, and FVC is the total volume of air that is breathed out in one full breath; therefore FEV₁/FVC is the proportion of the lung total air that can be blown out in one second. A maximum of 8 and at least 3 acceptable readings were taken for each patient, and the best reading out of 2 within 5 % or 100 mL of each other was chosen. FEV₁ was expressed as a percentage of FEV₁ expected on the basis of age, sex, and size. Reduced predicted FEV₁ (< 80 %) and FEV₁/FVC (< 70 %) are indicative of airflow obstruction. FEV₁ and FEV₁/FVC were measured before and after the administration of bronchodilator, in order to evaluate the degree of reversibility in airflow obstruction [262]. The healthy controls included in this study had normal spirometry, whereas the asthmatic subjects had reduced lung function.
2.3 Processing of sputum samples

Sputum was induced using inhaled incremental concentrations of nebulised hypertonic saline at 3, 4 and 5 %, each administered for 5 min [263]. Sputum plugs were selected and diluted in 8 volumes (weight/volume) of phosphate buffered saline (PBS). Samples were rocked on ice for 15 min and then spun at 790 g for 10 min at 4°C. Four volumes of the PBS supernatants were collected and kept at −80°C until further use. The remaining cell and mucus pellets were resuspended in 4 volumes of 0.2 % dithiothreitol (DTT) in PBS and rocked on ice for 15 min in order to disperse the cells. The cell suspension was then filtered through a 48 µm nylon gauze and the total cell number was determined using a haemocytometer. The DTT supernatant was obtained by centrifugation as before, removed and stored at −80°C. Cells were resuspended in PBS at a density of 0.5–0.75x10^6 per mL and 75 µL of the cell suspension were adhered onto glass slides using cytospins (450 rpm for 6 min). Slides were air-dried for at least 15 min, fixed with 90 % methanol for 5 min and stained with the Rapi-Diff II Stain Kit from Atom Scientific for differential cell counts. Briefly, slides were stained with eosin y for 5 min, washed with PBS, stained with methylene blue for 10 min, washed and allowed to dry overnight. Slides were mounted with DPX mountant for histology (Sigma-Aldrich®) and coverslips and allowed to dry overnight. This staining protocol allows the differentiation of inflammatory cell types based on their morphology and coloration, since acidic structures such as cell nuclei are clearly stained in blue by methylene blue, and neutral or basic structures such as eosinophils stain purple-red with eosin y.
2.4 Human bronchial and lung tissue

Bronchial biopsy specimens were obtained by bronchoscopy from healthy volunteers and asthmatic patients attending the asthma clinics at Glenfield Hospital (Leicester). Large airway tissue was obtained from subjects undergoing surgical intervention for tumour removal. Tissue far from the tumour was isolated and processed. The procedures were approved by the Leicestershire Ethics Committee and all subjects gave written informed consent.

2.5 Sandwich enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) is a biochemical technique commonly used in immunology to detect the presence of an antigen or an antibody in biological samples. ELISAs exploit the specificity of an antigen-antibody pair interaction. There are different types of ELISAs, in which either an antigen or a specific antibody is absorbed onto a surface where samples are then incubated. Sandwich ELISAs have been used in the present study. In this type of ELISA, a primary or capture antibody is pre-coated onto a 96-well plate. After antigen binding, a secondary or detection antibody is added. This is directed against a different epitope in the antigen and binds the captured antigens so that they are “sandwiched” between the two antibodies. The detection antibody is coupled to an enzyme that reacts with a chromogenic substrate so that a measurable colour change occurs. The amount of antigen in the samples can be quantified by using standards of known concentration to construct a standard curve from which unknown sample concentrations can be derived.
2. Materials and methods

2.5.1 HMGB1 ELISA

Levels of HMGB1 in PBS sputum supernatants and cell culture supernatants were measured using a commercial sandwich ELISA kit from IBL International following the manufacturer’s instructions. The lyophilised standard and positive control (both pig HMGB1) were reconstituted in diluent buffer to give a final concentration of 320 ng/mL and 18 ± 9 ng/mL, respectively. The standard was further diluted in diluent buffer to 80 ng/mL and from this 1:2 dilutions were made (40 – 2.5 ng/mL). Diluent buffer only was the blank (0 ng/mL). Standards and samples (10 µL) were mixed with 100 µL of diluent buffer in a 96-well plate pre-coated with an anti-HMGB1 polyclonal antibody. The plate was sealed and incubated at 37°C for 24 h. The incubation solution was then discarded and wells were washed 5 times with 400 µL of wash buffer (PBS containing 0.1 % Tween® 20). A peroxidase-conjugated anti-HMGB1/2 antibody (100 µL) was added to the wells for 2 h at 25°C. This was discarded and wells were washed 5 times as before to remove any unbound antibodies. A colour-developing solution (100 µL/well), containing 25 mM hydrogen peroxide (H₂O₂) and the chromogenic substrate 3,3’,5,5’-tetramethylbenzidine (TMB), was added for 30 min at room temperature (RT). Colour development was stopped by the addition of 0.35 M sulphuric acid (100 µL/well).

2.5.2 esRAGE ELISA

Levels of endogenous secretory RAGE (esRAGE) in PBS sputum supernatants were measured using the B-Bridge International esRAGE ELISA kit following the
manufacturer’s instructions. Prior to assay the samples were concentrated 3 – 8 folds using 10 KDa cut-off Amicon® Ultra centrifugal filter units (Millipore) and the retentates were used in the ELISA. Lyophilised standard human esRAGE was reconstituted in 0.5 mL of distilled water to have a 3.2 ng/mL stock. From this, 1:2 serial dilutions were carried out in PBS-bovine serum albumin (BSA) buffer (1.6 – 0.05 ng/mL). PBS-BSA buffer only was the blank (0 ng/mL). Standards and samples (20 µL) were added to an anti-RAGE antibody-coated 96-well plate together with 100 µL of the detection antibody solution, an anti-esRAGE antibody conjugated to horseradish peroxidase (HRP), and incubated at 4°C for 24 h. The incubation solution was discarded and wells washed 4 times with wash buffer (PBS with 0.1 % Tween® 20), prior to the addition of 100 µL of substrate, containing H₂O₂ and TMB, for 30 min at RT. The reaction was stopped by the addition of 100 µL of stop solution (1 M sulphuric acid).

For both ELISAs, absorbance was measured at 450 nm using a spectrophotometer (VICTOR or EnSpire®, both from Perkin Elmer®). HMGB1 and esRAGE concentrations in the samples were derived from linear regression curve fitting of the corrected (i.e. minus blank) standards. Protein concentrations were calculated from the linear regression equation as follows:

\[ y = ax + b \]  \hspace{1cm} \text{(standard curve equation)}

\[ x = (y - b)/a \]  \hspace{1cm} \text{\(x\) is the antigen concentration in the sample; \(y\) the sample OD)}

The concentrations thus obtained were multiplied by the dilution factor of the sputum samples in PBS (x9) to derive the starting concentrations in the sputum plugs. When samples were concentrated prior to assay, these values were divided by the concentration factor (initial volume/final volume).
The detection range was 2.5 – 80 ng/mL for the HMGB1 ELISA, and 50 – 3200 pg/mL for the esRAGE ELISA. All standards and samples were assayed in duplicate. Samples for which the OD exceeded the top end of the standard curve were diluted and re-assayed.

2.6 Principles of immunohistochemistry

Immunohistochemistry (IHC) combines histological and immunological techniques to allow the visualisation of antigen localisation and distribution within a tissue section by using fluorescently or enzyme labelled-antibodies. The tissue is first rapidly preserved using chemical fixatives to prevent protein degradation and loss of architecture, then it is embedded in a wax or resin matrix that provides a rigid support for cutting. Sections (2 – 5 µm thick) are cut using a microtome and allowed to adhere to glass slides for subsequent immunological staining. Cross-linking fixatives like formaldehyde form semi-reversible methylene bridges between proteins and nucleic acids that can preclude antigen presentation and antibody binding. In this case, an “antigen retrieval” procedure is required to unmask the epitopes, such as exposure to high temperatures or enzymatic degradation. Embedding media that are not removed before staining, such as resins, can also obstruct antibody binding and may therefore require antigen retrieval. The main advantage of IHC is that the tissue architecture is preserved, so that cellular components can be studied in a more physiologically meaningful context. However, artefacts can be introduced during tissue acquisition and processing (i.e. fixation and cutting procedures), such as tearing and swelling or shrinkage of the tissue, which should be taken into account during the analysis.
2. Materials and methods

2.6.1 Processing of bronchial biopsies in glycol methacrylate (GMA)

Bronchial biopsies and resection tissue were fixed overnight at –20°C in acetone with 2 mM phenylmethylsulfonyl fluoride (a serine protease inhibitor) and 20 mM iodoacetamide (a cysteine peptidase inhibitor). The tissue was embedded into glycol methacrylate (GMA) resin blocks which were left to polymerise at 4°C for 48 h before being stored at –20°C until further use. 2 µm sections were cut using a glass knife mounted onto a semi-automated microtome (Leica RM2255) and floated onto a 0.2 % ammonia water bath. The sections were captured onto positively charged glass slides (Thermo Scientific) and left to dry overnight at RT.

2.6.2 Immunohistochemistry staining

An EnVision™ FLEX kit (Dako) was used for the IHC staining, following the manufacturer’s instructions. Unlike paraffin, GMA does not require to be removed from the tissue sections before staining. Antigen retrieval was necessary for RAGE staining and was performed by microwaving the slides in high pH citrate buffer (Tris/EDTA buffer, pH 9) at 850 W for 5 min. The slides were then immediately immersed in water at RT. Excess water was absorbed onto a tissue and sections were circled with a water-repellent pen to create an area for reagent incubation (ImmEdge™ pen, Vector Laboratories). All incubations were carried out in a humidified incubation tray covered with a lid. To block non-specific binding, endogenous peroxidase was neutralised by incubating the sections with peroxidase-blocking reagent (PBS containing hydrogen peroxide) for 10 min at RT. Slides were washed twice with wash
buffer (Tris-buffered saline solution containing Tween® 20). Specific primary antibodies or irrelevant isotype controls were applied for 1 h at RT. Unbound antibodies were removed with two washes in wash buffer. For RAGE staining an amplification step was performed with a mouse LINKER for 15 min at RT. Slides were washed twice and the HRP-EnVision™ FLEX was added for 20 min at RT. The proprietary reagent consists of a dextran polymer backbone to which multiple peroxidase molecules and goat secondary antibody molecules against rabbit and mouse immunoglobulins are attached (figure 2.6.1). This particular structure allows a greater amplification of rabbit and mouse antibodies signal and is highly sensitive. After two washes, the chromogenic substrate 3,3′-diaminobenzidine tetrahydrochloride (1 drop per mL of substrate buffer containing hydrogen peroxide) was added for 2 min. Slides were washed in ultrapure water and immersed in Mayer’s haematoxylin for an appropriate amount of time depending on the age of the dye. Haematoxylin is a basic dye that stains acidic cellular structures, such as cell nuclei, in blue or purple and is used to reveal the tissue architecture. Slides were then placed under running tap water for approximately 5 min for a process called “blueing”. Slides were left to dry overnight, mounted with DPX mountant for histology (Sigma-Aldrich®) and allowed to dry overnight.

Tissue sections were analysed under an Olympus BX50 light microscope. Areas of airway smooth muscle were identified by α-smooth muscle actin (α-SMA) staining in a sequential section, and epithelium was recognisable from the characteristic morphology and the anatomical location in the tissue. Antigen expression in muscle and epithelium was quantified by counting the positively stained cells (brown) and the negative cells. Results were expressed as number of positive cells per area of smooth
muscle/epithelium (mm$^2$) or as percentage of positive cells per total cell number. Subject phenotype was blinded during the analysis.

**2.7 Tissue culture**

Pure airway smooth muscle (ASM) bundles were isolated from bronchial biopsy specimens and large airway tissue from subjects undergoing surgical intervention. For bronchoscopy, patients were placed in a semi-recumbent position and received a mild sedation via venous access. A flexible endoscope was inserted via a nostril and the airways inspected [265]. Human primary bronchial epithelial cells (HBEC) were derived from bronchial brushings of the right lung [265]. The procedures were approved by the Leicestershire Ethics Committee and all subjects gave written informed consent. Human primary ASM cells were characterised by α-SMA expression (≥ 70 % positive cells by flow cytometry, [figure 2.7.1](#)). A human embryonic kidney (HEK) 293T cell line was donated by Dr Herbert’s laboratory in Cell Physiology and Pharmacology, University of Leicester. A human umbilical vein endothelial cell line (HUVEC) was donated by the Cardiovascular Department at the University of Leicester. HASM and HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with GlutaMAX™ supplemented with 10 % foetal bovine serum (FBS), 1 % antibiotic/antimycotic, 1 % non-essential amino acids, and 1 % sodium pyruvate (all from Gibco®). HUVECs were cultured in endothelial cell growth medium from Lonza (EGM™-2 Bulletkit™). HBECs were maintained in bronchial epithelial cell growth medium (BEGM™) from Lonza. Cells were kept at 37°C in a humidified atmosphere with 5 % CO$_2$. HASM cells were serum-starved before experiments in DMEM containing
1 % insulin-transferrin-selenium (ITS) supplement (Gibco®). Experiments were conducted in 1 % ITS DMEM or serum-free DMEM. Cells were detached using accutase™ (Sigma-Aldrich®) and counted by means of a haemocytometer. HASM cells from non-asthmatic and asthmatic donors were used at passage 2 to 5.
Figure 2.6.1 Principle of the Dako EnVision™ kit for immunohistochemical staining. Mouse or rabbit primary antibodies bind to specific antigens in the tissue. The secondary antibody consists of a dextran polymer backbone to which multiple peroxidase molecules and goat secondary antibodies against rabbit and mouse immunoglobulins are attached. This structure allows a greater amplification of rabbit and mouse antibody signal and confers higher sensitivity [264].
2. Materials and methods

Figure 2.7.1 α-SMA expression in HASM cells. **A)** Immunofluorescence: a mouse monoclonal anti-α-SMA antibody conjugated with fluorescein isothiocyanate (FITC, Sigma-Aldrich®) was used at 1:200. Cells were counterstained with DAPI (1:1000) to reveal the nuclei. **B)** Flow cytometry: the primary antibodies were a mouse monoclonal anti-human α-SMA and a mouse IgG2a (isotype control). Both antibodies were from Dako and were used at 4.2 µg/mL. The secondary antibody was a rabbit anti-mouse FITC (1:20).
2.8 Total RNA extraction

Sub-confluent HASM cells were grown in 6 cm dishes and serum-starved for 72 h in ITS-DMEM prior to RNA extraction. Total RNA was extracted using commercial kits (the Peqlab peqGOLD total RNA kit or the Qiagen RNeasy kit) following the manufacturers’ instructions. Cells were lysed using the lysis buffer provided and RNA was extracted by binding to silica-based membranes through centrifugation. Genomic DNA contamination was removed by DNA digestion using 30 Kunitz units of DNase I (from Peqlab or Qiagen) per column. Membrane-bound RNA was eluted in 30 µL nuclease-free water and RNA concentration and purity were determined by UV spectroscopy (NanoVue plus, GE Healthcare). Absorbance at 260 nm was used to quantify RNA concentration according to the Beer Lambert Law:

\[ A = \epsilon CL \]

where:

A = absorbance at the chosen wavelength

C = concentration of nucleic acid

L = path length of the spectrophotometer cuvette

\( \epsilon = \text{extinction coefficient, } 0.025 \ (\mu g/ml)^{-1} \cdot \text{cm}^{-1} \) for RNA

Absorbance was also measured at 280 nm to assess protein contamination: an \( A_{260}/A_{280} \) ratio between 1.8 and 2.1 is indicative of highly purified RNA. RNA integrity was evaluated using agarose gel electrophoresis, a ratio of 28S:18S ribosomal RNA bands of 2:1 indicating intact RNA (figure 2.8.1).
2. Materials and methods

Figure 2.8.1 Assessment of RNA integrity. Total RNA was extracted from HASM cells using commercial RNA isolation kits, and RNA integrity was assessed using 1% agarose gel electrophoresis (n = 13 donors are shown here). The sizes of 28S rRNA and 18S rRNA are 4.8 Kb and 1.8 Kb, respectively; however in a non-denaturing gel bands may migrate differently due to their complex tertiary structure. The presence of two sharp bands with a ratio of 2:1 is considered a hallmark of intact RNA.
2.8.1 Synthesis of complementary DNA

Total RNA (0.5 – 2 µg) was used as a template to synthesise complementary DNA (cDNA) using the SuperScript® VILO™ cDNA synthesis kit (Invitrogen™). The final cDNA concentration was assumed to be equal to the starting RNA concentration. The reaction mix per tube contained:

5x VILO™ reaction mix 4 µL
10x SuperScript® enzyme mix 2 µL
RNA x µL
DEPC-treated water to 20 µL

The VILO™ reagent includes random primers, MgCl₂ and deoxyribonucleotides (dNTPs) in buffer; the SuperScript® enzyme mix contains the reverse transcriptase, a ribonuclease inhibitor, and a proprietary helper protein. Tubes were incubated at 25°C for 10 min, then at 42°C for 60 min and the reaction was terminated at 85°C for 5 min. cDNA was stored at –20°C until further use.

2.9 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Polymerase chain reaction (PCR) is a technique that enables the amplification of a specific DNA sequence using thermal cycling. Template DNA is subjected to cycles of heating and cooling during which DNA is denatured, primers anneal to specific DNA sequences and a thermally stable DNA polymerase synthesises a complementary strand of DNA from the template DNA. The PCR product is then resolved using agarose
gel electrophoresis and sequenced. Reverse transcription PCR (RT-PCR) employs RNA as the starting material.

RT-PCR was performed in order to investigate the splice variants of RAGE (accession # M91211) expressed by HASM cells, using a primer pair previously employed to simultaneously detect soluble and transmembrane RAGE forms [266] (see Chapter 4.3.1). The 18S rRNA (accession # NR_003286) was also amplified as a loading control. RAGE and 18S rRNA primer sequences (Invitrogen™) are reported in **table 2.9.1**. The PCR was carried out in 0.2 mL tubes in a 50 µL volume containing 1.25 units of Taq DNA polymerase, 100 µM of each dNTP (Taq PCR master mix kit from Qiagen), 100 ng of template cDNA, and 200 nM of each primer. The details of the amplification are reported in **table 2.9.2**. Genomic contamination was assessed using 100 ng of non-transcribed RNA and contamination of the reagents using PCR master mix plus primers only.
2. Materials and methods

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Product length</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAGE</td>
<td>GATCCCCGTCCCCACCTTCTCCTGTAGC (exon 6)</td>
<td>CACGCTCCTCCTCTTCTCCTGGTTTTCTG (exon 11)</td>
<td>500–800 bp</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>GTTGGTTTTCCGGAACGAGGPosition 891</td>
<td>GCATCGTTATGGTCGGAACPosition 1090</td>
<td>200 bp</td>
</tr>
</tbody>
</table>

**Table 2.9.1** Primers used in RT-PCR for RAGE amplification. Primers used to amplify a region of *AGER* undergoing alternative splicing using RT-PCR. 18S rRNA was amplified as an internal reference; bp = base pairs.

<table>
<thead>
<tr>
<th>PCR instrument</th>
<th>Programme</th>
<th># cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mastercycler® Gradient (Eppendorf)</td>
<td>Initial denaturation (hot start)</td>
<td>94°C at 3 min</td>
</tr>
<tr>
<td></td>
<td>Denaturation</td>
<td>94°C for 30 s</td>
</tr>
<tr>
<td></td>
<td>Annealing</td>
<td>57°C for 30 s</td>
</tr>
<tr>
<td></td>
<td>Extension</td>
<td>72°C for 1 min</td>
</tr>
<tr>
<td></td>
<td>Final elongation</td>
<td>72°C for 10 min</td>
</tr>
</tbody>
</table>

**Table 2.9.2** RT-PCR programme. Steps included in the RT-PCR to amplify RAGE mRNA and 18S rRNA.
2.10 Principles of quantitative RT-PCR

Quantitative real-time PCR (qPCR) is a technique for DNA amplification in which fluorescent dyes are used to quantify the amount of PCR product after each amplification cycle. Thus, the amount of amplification product is measured in real-time, in contrast to endpoint PCR in which the analysis is carried out after a fixed number of cycles. Quantitative reverse transcription PCR (qRT-PCR) refers to the use of RNA as the starting material, from which cDNA is synthesised. In this thesis qRT-PCR was performed. The principle of quantification in qPCR is based on the observation that a quantitative relationship exists between the amount of starting material present before the PCR and the amount of PCR product formed during the amplification process. This quantitative relationship depends on the efficiency of the PCR, which is influenced by many factors including the primer design, the amplicon length and structure, the detection chemistry, the nature and concentration of some components present in the reaction mix. Therefore, the qPCR efficiency needs to be calculated and optimised for each assay and should be between 90 and 110%.

The signal detected in qPCR is fluorescence intensity, which is correlated with the amount of PCR product. Non-specific or specific detection chemistries can be used. SYBR® Green is commonly used as a non-specific fluorescent dye, as it displays low fluorescence when unbound in solution, but fluoresces brightly when intercalated with double-stranded DNA (dsDNA). Alternatively, fluorescently-tagged oligonucleotide probes are used for higher specificity. SYBR® Green was used in this Thesis.

The specificity of the reaction can be confirmed by checking the melting temperature (Tm) of the PCR product. The Tm is the temperature at which the dsDNA is denatured.
and depends on the length and composition of the PCR product. To calculate the Tm, fluorescence intensity is measured after the qPCR at increasing temperatures (e.g. 50 – 100°C) and melting curves are constructed. The negative first derivative of fluorescence intensity versus temperature (−dF/dT) is then plotted. A single peak in this graph indicates that a single product has been amplified (18S rRNA melting peaks are shown in figure 2.10.1A as an example).
Figure 2.10.1 18S rRNA amplification. **A)** Example of 18S rRNA melting peaks (dF/dT curves) derived from fluorescence intensity curves (n = 5 samples performed in triplicate) showing a melting temperature of 84°C. **B)** 18S rRNA Ct values were not different in HASM cells derived from non-asthmatics (n = 7) and asthmatics (n = 9). Mean ± SEM Ct values were 7.3 ± 0.09 and 6.98 ± 0.16, respectively (unpaired t test p = 0.084). Data are presented as mean ± SEM.
### 2.10.1 Quantitative RT-PCR

qRT-PCR was performed using the Express SYBR® GreenER™ qPCR supermix universal from Invitrogen™. Sequences of the primers used are reported in table 2.10.1. Primer pairs for the amplification of HMGB1 and RAGE cDNA were designed by a company called Primerdesign. Other primers previously used in the laboratory were from Invitrogen™. Amplification was carried out in a real-time thermal cycler, the Bio-Rad Chromo4™ or the Stratagene® Mx3000P™, in clear qPCR tubes using the steps detailed in table 2.10.2. Gene expression was normalised to 18S rRNA as an internal control, which was not differently expressed in asthmatics and non-asthmatics (figure 2.10.1B). The reaction efficiency was first tested by amplifying increasing concentrations of cDNA (0.01 – 50 ng) from HASM cells or the human mast cell line HMC-1. The reagent volumes and concentrations used in the qRT-PCR are reported in table 2.10.3. At the end of each assay, melting curves were constructed by monitoring fluorescence intensity at increasing temperatures (50 – 100°C). Samples were assayed in triplicate. Non-transcribed RNA was included as a negative control of genomic contamination of the RNA extract, and qPCR mix including primers was the control for SYBR® Green and primer mix contamination.

### 2.10.2 qRT-PCR data analysis

Low arbitrary fluorescence thresholds were chosen in the linear range of fluorescence intensity curves to derive the threshold cycle (Ct) values. The Ct value is the number of amplification cycles at which the fluorescence intensity associated with the PCR
product crosses a set fluorescence threshold. Choosing a fluorescence threshold in the linear region of the fluorescence intensity curves is fundamental for quantification, because the reaction efficiency is greater at that point.

In order to calculate the qPCR efficiency, the Ct values of increasing concentrations of cDNA (0.01 – 50 ng) were plotted against the logarithm of the cDNA concentrations and fitted using a linear regression equation. The slope of the linear regression is related to the PCR efficiency according to the formula:

\[ \text{PCR efficiency (\%)} = \left[ (10^{-1/\text{slope}}) - 1 \right] \times 100. \]

To quantify relative gene expression in different donors, an arbitrarily chosen non-asthmatic subject (calibrator) was included in each assay. Target gene expression relative to the calibrator was calculated as follows (reference gene = internal control 18S rRNA):

\[ \Delta \text{Ct sample} = \text{Ct target}_{\text{sample}} - \text{Ct reference}_{\text{sample}} \]
\[ \Delta \text{Ct calibrator} = \text{Ct target}_{\text{calibrator}} - \text{Ct reference}_{\text{calibrator}} \]
\[ \Delta \Delta \text{Ct sample} = \Delta \text{Ct sample} - \Delta \text{Ct calibrator} \]

Relative quantity = \( 2^{(-\Delta \Delta \text{Ct})} \)

To assess changes in gene transcription levels following stimulation within the same donor, Ct values were normalised to the loading control 18S rRNA and the fold change relative to unstimulated controls was calculated:

\[ \Delta \text{Ct} = \text{Ct target} - \text{Ct reference} \]
\[ \Delta \Delta \text{Ct} = \Delta \text{Ct stimulated} - \Delta \text{Ct unstimulated} \]

Fold change = \( 2^{(-\Delta \Delta \text{Ct})} \)
2. Materials and methods

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Product length</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S rRNA</td>
<td>GTTGGTTTTCGGAACTGAGG</td>
<td>GCATCGTTTATGGTGCGGAAC</td>
<td>200 bp</td>
</tr>
<tr>
<td></td>
<td>Position 891</td>
<td>Position 1090</td>
<td></td>
</tr>
<tr>
<td>HMGB1</td>
<td>GTGCAAAAGGTTGAGGTAGCTATTG</td>
<td>AATAAATAACAGCAAACATTAA CAACAC</td>
<td>88 bp</td>
</tr>
<tr>
<td></td>
<td>Position 3255</td>
<td>Position 3342</td>
<td></td>
</tr>
<tr>
<td>RAGE</td>
<td>AGGAAGGCGGACTGCTGTAGG</td>
<td>GAGTTCCAGCCCTGATCC</td>
<td>90 bp</td>
</tr>
<tr>
<td></td>
<td>Position 1032</td>
<td>Position 1121</td>
<td></td>
</tr>
<tr>
<td>IL-8</td>
<td>TGCCAAGGAGTGTGCTAAAG</td>
<td>CTCCACAACCCTCTGAC</td>
<td>197 bp</td>
</tr>
<tr>
<td></td>
<td>Position 227</td>
<td>Position 423</td>
<td></td>
</tr>
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**Table 2.10.1** Primers used in qPCR. List of primers used to amplify HMGB1, RAGE, IL-8 mRNA transcripts and the internal reference 18S rRNA by qRT-PCR.
2. Materials and methods

<table>
<thead>
<tr>
<th>Real-time PCR instrument</th>
<th>Programme</th>
<th># cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bio-Rad Chromo4™ or Stratagene® Mx3000P™</td>
<td>Initial denaturation (hot start)</td>
<td>95°C for 5 min</td>
</tr>
<tr>
<td></td>
<td>Denaturation</td>
<td>95°C for 20 s</td>
</tr>
<tr>
<td></td>
<td>Annealing</td>
<td>59°C for 30 s (HMGB1) 60°C for 30 s (RAGE)</td>
</tr>
<tr>
<td></td>
<td>Extension</td>
<td>72°C for 30 s</td>
</tr>
<tr>
<td></td>
<td>Final elongation</td>
<td>72°C for 10 min</td>
</tr>
</tbody>
</table>

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Table 2.10.2 qPCR programme. Steps used in the qRT-PCR for the amplification of HMGB1, RAGE, IL-8 mRNA and the internal reference 18S rRNA.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Template cDNA</th>
<th>Primers (F+R)</th>
<th>2x SYBR® Green</th>
<th>Nuclease-free H₂O₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMGB1/ RAGE/ IL-8</td>
<td>10 ng (2 µL)</td>
<td>200 nM final (1 µL)</td>
<td>10 µL</td>
<td>7 µL</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>10 ng (2 µL)</td>
<td>200 nM final (0.8 µL)</td>
<td>10 µL</td>
<td>7.2 µL</td>
</tr>
</tbody>
</table>

Table 2.10.3 Concentrations and volumes of reagents used per tube in the qPCR.
2.10.3 RT-PCR product purification

RT-PCR products were loaded onto a 1 – 1.7 % agarose gel stained with GelRed™ (1 µL/10 mL, Biotium) following amplification or purification. The latter was carried out using the Qiagen MinElute kit. Briefly, 5 volumes of buffer PB were mixed with 1 volume of PCR product and bound to a DNA-binding column by centrifugation (30 – 60 s). Bound DNA was washed with 750 µL buffer PE (containing ethanol) and the column was dried by centrifugation. Bound DNA was eluted with nuclease-free water in a clean tube. PCR products were allowed to migrate by electrophoresis at 80 – 90 V and then visualised under UV illumination (Gel Doc system). DNA bands were excised using a clean scalpel and extracted with the Qiagen QIAquick gel extraction kit. Briefly, 3 volumes of buffer QG were added to 1 volume of gel (volume/weight) and incubated at 50°C until the agarose had dissolved. One gel volume of isopropanol was mixed with the sample and this was added to a DNA-binding column. DNA was bound to the column by centrifugation. Two sequential washes were performed with 500 µL of buffer QG and 750 µL of buffer PE. The column was dried by centrifugation and the DNA eluted as before. Purified DNA was sequenced in-house by the Protein Nucleic Acid Chemistry Laboratory.

2.11 Transient expression of plasmid cDNA in human cells

With this molecular biology technique, a gene of interest is inserted into bacterial plasmids, which are then injected into eukaryotic cells. After a certain period of time, cells express the protein of interest and can be used for various applications. In this
Thesis, transient transfection of human cells was used to assess the specificity of anti-RAGE antibodies by Western blotting. Plasmid DNA3 (pcDNA3) containing human full length (FL)-RAGE cDNA previously used in Raucci et al. 2008 [171] was a gift from Prof Marco Bianchi (San Raffaele Institute, Milan, Italy).

2.11.1 Cloning of FL-RAGE pcDNA into bacterial cells

Sub-cloning efficiency DH5α competent bacterial cells (Invitrogen™) were thawed on ice and transformed with FL-RAGE pcDNA3 following the manufacturer’s instructions: 2 µL of pcDNA (the stock concentration was not known) or TE buffer (negative control) were added to 25 µL of cells and incubated on ice for 30 min. Cells were heat-shocked at 42°C for 35 s to allow the pcDNA to enter the cells and membrane integrity was restored by placing the cells on ice for 2 min. Cells were diluted in 300 µL of Luria-Bertani broth (table 2.11.1) and incubated at 37°C for 30 min shaking. Since pcDNA3 contains a resistance factor to ampicillin, bacterial cells were grown for 16 h at 37°C on minimum agar plates containing 100 µg/mL of ampicillin (Melford) as a selection factor. A single bacterial colony was selected and further grown shaking for 24 h at 37°C in 100 mL of Luria-Bertani broth containing 100 µg/mL of ampicillin. The pcDNA was purified from the bacterial culture using the Qiagen EndoFree plasmid purification maxi kit, which also removes endotoxin contamination. Briefly, bacterial cells were harvested by centrifugation (6,000 g for 15 min at 4°C) and the pellet resuspended in 10 mL of lysis buffer (P1). To this, 10 mL of a further lysis buffer (P2) containing sodium hydroxide and sodium dodecyl sulphate (SDS) was added and incubated at RT for 5 min. An acidic buffer (P3, 10 mL) was added and mixed to neutralise buffer P2. The cell
lysate was incubated into a QIAfilter cartridge for 10 min at RT and then filtered into a clean 50 mL tube. Buffer ER (2.5 mL) was added to this and incubated on ice for 30 min to remove endotoxin contamination. The filtered cell lysate was added to a buffer-equilibrated DNA-binding column. DNA was allowed to bind to the resin by gravity flow. The column was then washed twice with 30 mL of buffer QC and DNA was eluted with 15 mL of buffer QN. DNA was precipitated with 0.7 volumes of isopropanol and recovered by centrifugation (15,000 g for 30 min at 4°C). The DNA pellet was washed with 5 mL of endotoxin-free 70 % ethanol and spun again at 15,000 g for 10 min at 4°C. The DNA pellet was air-dried for 5 – 10 min and resuspended in endotoxin-free buffer TE. The plasmid purity was verified by agarose gel electrophoresis and it was confirmed to contain FL-RAGE cDNA by sequencing, which was carried out by the Protein Nucleic Acid Chemistry Laboratory.
### Material and Methods

#### Medium Components

<table>
<thead>
<tr>
<th>Medium</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>1x TE buffer</td>
<td>10 mL Tris HCl 1 M pH 7.5</td>
</tr>
<tr>
<td></td>
<td>2 mL EDTA 500 mM pH 8.0</td>
</tr>
<tr>
<td></td>
<td>dH₂O to make 1 L</td>
</tr>
<tr>
<td>Luria-Bertani agar</td>
<td>10 g bacto-tryptone</td>
</tr>
<tr>
<td></td>
<td>5 g bacto-yeast extract</td>
</tr>
<tr>
<td></td>
<td>10 g NaCl</td>
</tr>
<tr>
<td></td>
<td>15 g bacto-agar</td>
</tr>
<tr>
<td></td>
<td>dH₂O to make 1 L (pH 7.0)</td>
</tr>
<tr>
<td></td>
<td>(ampicillin 100 µg/mL)</td>
</tr>
<tr>
<td>Luria-Bertani broth</td>
<td>10 g/L bacto-tryptone</td>
</tr>
<tr>
<td></td>
<td>5 g/L bacto-yeast extract</td>
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<td>10 g/L NaCl</td>
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</table>

*Table 2.11.1 Recipes of buffers and media used for pcDNA cloning into bacterial cells.*
2.11.2 Transient expression of FL-RAGE pcDNA in HEK293T and HASM cells

FL-RAGE or green fluorescent protein (GFP) pcDNA (Lonza) were transfected into sub-confluent HEK293T cells by lipofection (Lipofectamine™ 2000, Invitrogen™) and into HASM cells by electroporation (Amaxa Nucleofector™, Lonza), according to the manufacturers’ instructions. Briefly, 10 µL of Lipofectamine™ 2000 were added to 4 µg of pcDNA into 500 µL of serum and antibiotic/antimycotic-free medium. This was added onto the cells for 4 – 6 h. For electroporation, 2 – 3 µg of pcDNA were transfected into 5x10^5 HASM cells. GFP expression was assessed after 24 and 48 h using a fluorescent microscope. After 48 h, cells were harvested in ice-cold 1 % triton lysis buffer and lysates centrifuged at 16,000 g for 10 min at 4°C. Supernatants were collected and total protein concentration was measured using a Bio-Rad protein assay using BSA as standard, and adjusted for use in Western blotting.
Figure 2.11.1 Plasmid maps. A) Map of pcDNA3 containing resistance factors for ampicillin and neomycin into which human FL-RAGE cDNA was subcloned [171]. B) Map of GFP pcDNA provided by Lonza.
2.12 Principles of Western blotting

Western blotting is a common biochemical technique that allows the detection of specific proteins in cell lysates or supernatants. Proteins are separated using polyacrylamide gel electrophoresis and then transferred onto a nitrocellulose or polyvinyl membrane. Proteins are detected using specific antibodies directly or indirectly conjugated with HRP, which catalyses an oxidation reaction that is accompanied by the emission of low intensity light at 428 nm. However, the light emitted can be enhanced up to 1,000 folds in the presence of certain chemicals, such as modified phenols, thus making the light easier to detect. This is the principle of enhanced chemiluminescence (ECL), which is used to reveal proteins in Western blotting. The emitted light is captured onto photosensitive films, and is used as a measure of the amounts of protein present i.e. of the number of HRP molecules that have reacted; therefore of the amount of bound antibody.

In denaturing conditions, an anionic detergent such as SDS is used to denature proteins (i.e. proteins lose their tertiary structure) and to add negative charges to the polypeptide backbone, so that migration occurs by molecular weight.

2.12.1 Western blotting

HASM and HEK293T cells were lysed in 1 % triton buffer for the detection of RAGE, as described in the previous paragraph. For the detection of HMGB1, HASM cells were lysed directly in 1x sample buffer (Laemmli buffer, table 2.12.1).
Cell lysates and sputum PBS supernatants were mixed with concentrated sample buffer (4x and 6x, respectively) and boiled at 95°C for 5 min. Samples were loaded onto 10 or 12 % SDS-polyacrylamide gels for RAGE and HMGB1 detection, respectively, and resolved by electrophoresis (90 – 180 V). Proteins were “blotted” onto polyvinylidene fluoride (PVDF) membranes (Millipore or Bio-Rad) by semi-dry transfer (15 V for 37 min). PVDF membranes were blocked in 5 % skimmed milk in TBS-Tween® 20 (TBST) for 1 h at RT with gentle agitation. Primary antibodies were added at appropriate concentrations in 5 % milk-TBST and incubated overnight at 4°C or for 1 h at RT with gentle agitation. Unbound antibodies were removed by washing the membranes three times with TBST. Appropriate secondary HRP-conjugated antibodies were added in 5 % milk-TBST for 1 h at RT with gentle agitation. β-actin (loading control) was detected using a mouse monoclonal HRP-conjugated antibody (10ng/mL, Santa Cruz, 1 h incubation at RT). Washes were carried out as before and an ECL reagent (GE Healthcare) was added onto the membranes for 1 to 2 min. Membranes were exposed to x-ray films (Kodak) in a developing cassette for appropriate amounts of time. Films were developed and fixed using an automated photographic developer (Amersham Biosciences). Band sizes were estimated against an appropriate molecular weight marker (New England BioLabs®).
## 2. Materials and methods

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 % triton lysis buffer</td>
<td>10 mM β-glycerophosphate pH 7.4, 1 mM EDTA pH 8, 1 mM EGTA, 50 mM Tris-HCl pH 7.5, 1 mM benzamidine, 1 mM sodium orthovanadate, 0.2 mM phenylmethylsulfonyl fluoride, 1 µg/mL pepstatin A, 1 µg/mL leupeptin, 0.1 % β-mercaptoethanol, 1 % triton X-100, 50 mM sodium fluoride</td>
</tr>
<tr>
<td>6x Laemmli buffer</td>
<td>375 mM Tris-HCl pH 6.8, 9 % SDS, 50 % glycerol, 0.03 % bromophenol blue, 10 % β-mercaptoethanol</td>
</tr>
<tr>
<td>10x Tris buffered saline (TBS)</td>
<td>24.2 g Tris base, 80 g sodium chloride, H$_2$O to 1 L pH 7.6</td>
</tr>
<tr>
<td>1x TBS-Tween® 20</td>
<td>Add 0.1 % Tween® 20 to 1x TBS</td>
</tr>
<tr>
<td>10x Tris-glycine buffer</td>
<td>30 g Tris base, 144 g glycine, H$_2$O to 1 L</td>
</tr>
<tr>
<td>1x running buffer</td>
<td>100 mL 10x Tris-Glycine buffer, 10 mL 10 % SDS, H$_2$O to 1 L</td>
</tr>
<tr>
<td>1x semi-dry transfer buffer</td>
<td>50 mL 10x Tris-Glycine buffer, 1 mL 10% SDS, 100 mL methanol, H$_2$O to 500 mL</td>
</tr>
</tbody>
</table>

*Table 2.12.1* Recipes of buffers used in Western blotting.
### Table 2.12.2

<table>
<thead>
<tr>
<th></th>
<th>10 % resolving gel (RAGE)</th>
<th>12 % resolving gel (HMGB1)</th>
<th>5 % stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH₂O</td>
<td>4 mL</td>
<td>3.3 mL</td>
<td>3.4 mL</td>
</tr>
<tr>
<td>30 % acrylamide mix</td>
<td>3.3 mL</td>
<td>4 mL</td>
<td>830 µL</td>
</tr>
<tr>
<td>1.5 M Tris pH 8.8</td>
<td>2.5 mL</td>
<td>2.5 mL</td>
<td></td>
</tr>
<tr>
<td>0.5 M Tris pH 6.8</td>
<td></td>
<td></td>
<td>630 µL</td>
</tr>
<tr>
<td>10 % SDS</td>
<td>0.1 mL</td>
<td>0.1 mL</td>
<td>50 µL</td>
</tr>
<tr>
<td>10 % APS</td>
<td>0.1 mL</td>
<td>0.1 mL</td>
<td>50 µL</td>
</tr>
<tr>
<td>TEMED</td>
<td>5 µL</td>
<td>5 µL</td>
<td>5 µL</td>
</tr>
</tbody>
</table>

**Table 2.12.2** Recipes for acrylamide gels. Recipes used to prepare 10 mL of resolving acrylamide gel and 5 mL of stacking gel.
2.13 Principles of flow cytometry

Flow cytometry is a technology that allows the simultaneous measurement of multiple physical characteristics in single cells as they flow in a fluid stream through a beam of light. As the laser beam hits the cells (1 – 150 µm can be analysed) light is scattered in various directions depending on the size and internal complexity of the cell. The forward light scatter gives a rough indication of the cell size, while the side scatter depends on the internal complexity and granularity of the cell. Side and forward scatters are used to identify different sub-populations within the cell suspension. Fluorophores can also be used to label cell markers. Scattered light and emitted fluorescent light are captured by a system of lenses. Light signals are then converted into electronic voltage signals, which are used for analysis.

A major advantage of flow cytometry is the capacity to measure single cell characteristics simultaneously. Thus, heterogeneous cell populations can be identified on the basis of cell morphology and marker expression. Discrete sub-populations can then be isolated and analysed separately. Another advantage of flow cytometry is the large number of cells that can be analysed in a short period of time, making this technique more quantitative compared to immunocytochemistry.

2.13.1 Flow cytometry method

HASM cells were serum-starved in ITS-containing medium for 72 h prior to experiment. One-hundred thousand cells per condition were fixed on ice for 15 min with 4 % paraformaldehyde (PFA) for cell-surface antigen detection or 4 % PFA plus 0.1 %
saponin to permeabilise the cells for intracellular antigen detection. Specific primary antibodies (table 2.13.1) or irrelevant isotype control antibodies were applied for 1 h on ice at the appropriate concentrations in PBS containing 0.5 % BSA. Saponin (0.1 %) was always added to the buffer when intracellular antigens were investigated. Excess antibodies were removed by diluting the incubation solution with the same buffer (1 mL) and cells were recovered by centrifugation (1,300 rpm for 8 min at 4°C). R-phycoerythrin (RPE) - or fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (table 2.13.1) were used at 1:10 and 1:20 dilution, respectively, in 0.5 % BSA-PBS to which 0.1 % saponin was added where needed. Secondary antibodies were applied in the dark for 1 h on ice and then washed as before. Cells were resuspended in 0.5 % BSA-PBS and fluorescence intensity was measured using a BD FACSCanto™ flow cytometer (BD Biosciences).
<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Secondary antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMGB1 Rabbit monoclonal Abcam®, clone EPR3507</td>
<td>Sheep anti-rabbit RPE AbD Serotec</td>
</tr>
<tr>
<td>RAGE Rabbit polyclonal Millipore, cat # AB9714</td>
<td>Sheep anti-rabbit RPE AbD Serotec</td>
</tr>
<tr>
<td></td>
<td>Mouse monoclonal Santa Cruz®, clone A11</td>
</tr>
</tbody>
</table>

**Table 2.13.1** Antibodies used in flow cytometry. Primary and secondary (fluorescently labelled) antibodies used for antigen staining in flow cytometry.
2. Materials and methods

2.13.2 Flow cytometry data analysis

Flow cytometry data can be visualised in density plots, in which each data point is presented as a dot that is positioned on the graph according to the forward scatter (x axis) and the side scatter (y axis). The colour gradient in the density plot reflects the density of the cell population within the plot. The population of interest is then gated (a polygonal gate is shown in figure 2.13.1B) to restrict the analysis to it. In this Thesis, flow cytometry was used to measure protein expression in discrete cell populations using fluorescently labelled antibodies. A histogram can be drawn from the selected population, showing the fluorescent signal intensity on the x axis and the cell number on the y axis. Figure 2.13.1C-D shows histogram plots for phycoerythrin (PE)-stained HMGB1. In order to obtain the percentage of cells expressing the protein of interest (% positive cells), the histogram of interest is compared with an isotype control histogram (negative control). In this Thesis, the % positive cells was calculated using the Overton cumulative histogram subtraction algorithm, which subtracts an isotype control histogram from an histogram of interest, thus generating a positive cell population [267]. (figure 2.13.1C-D). Histograms can be overlaid to better visualise differences in protein expression.
Figure 2.13.1 Flow cytometry plots. Examples of a flow cytometry density plot (A) and HASM cell population enclosed by a polygonal gate (B) accounting for 85.34 % of the total events. Negative control (C) and HMGB1-stained (D) populations are shown as histogram plots, reporting the fluorescence intensity on the x axis, and the cell number on the y axis. Histograms are used to calculate the % positive cell population with the Overton subtraction method.
2. Materials and methods

2.14 Immunofluorescence

Immunofluorescence, or immunocytochemistry, is an immunological staining method in which fluorescently labelled antibodies are used to visualise cell structures and antigens of interest. HASM cells were seeded onto 8-well glass chamber slides at 70–80 % confluence and serum-starved for 72 h in 1 % ITS supplemented medium prior to assay. Cells were fixed for 15 min at RT with 4 % PFA or fixed and permeabilised in 4 % PFA plus 0.1 % saponin. Alternatively, cells were fixed with 90 % methanol for 20 min on ice. Non-specific binding sites were blocked with PBS containing 3 % BSA for 30 min at RT. Specific primary antibodies or irrelevant isotype control antibodies were added at the appropriate concentrations in PBS plus 1 % BSA and incubated at RT for 1 h. Unbound antibodies were removed by washing 3 times with PBS containing 0.05 % Tween® 20 and appropriate secondary antibodies conjugated with FITC (1:20) or Alexa Fluor® 488 (1:200) were applied in the dark for 1 h at RT. Excess antibodies were removed as before. The nuclear fluorescent stain 4',6-diamidino-2-phenylindole (DAPI, 1:1000) was applied for 2 min and then washed as before. Slides were mounted with ProLong® Gold anti-fade reagent (Invitrogen™) and analysed under an Olympus BX50 fluorescent microscope. Images were captured using an Olympus DP72 camera and the CellF software (Olympus).

2.15 NF-κB p65 nuclear translocation

HASM cells were seeded at a density of 1x10⁴ cells per well in 8-well chamber slides and allowed to adhere overnight at 37°C. Cells were serum-starved in ITS-containing
medium for 24 h prior to assay. Increasing concentrations of rhHMGB1 (30 – 300 – 1000 ng/mL) were then added in serum-free medium for a range of times (5 – 15 – 30 – 60 and 120 min) at 37°C. Cells were stimulated with TNFα (20 ng/mL) for 30 min as a positive control of NF-kB activation (p65 nuclear translocation). NF-kB p65 nuclear translocation was visualised by immunofluorescence, according to the method described above. Briefly, HASM cells were fixed and permeabilised in 90 % ice-cold methanol for 15 min and p65 was labelled with a rabbit monoclonal anti-p65 antibody (clone D14E12 from New England BioLabs®, at 1.88 µg/mL) followed by Alexa Fluor® 488 goat anti-rabbit antibody (10 µg/mL, Invitrogen™). Rabbit immunoglobulin fraction (1.88 µg/mL, Dako) was used as a negative control. Cells were counterstained with DAPI and slides were mounted and analysed under an Olympus BX50 fluorescent microscope. Images were captured using an Olympus DP72 camera and the CellF software (Olympus).

2.16 Intracellular reactive oxygen species production (H₂DCFDA assay)

Reactive oxygen species (ROS) production was measured using the 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) assay. H₂DCFDA is a cell-permeant chemically reduced form of fluorescein that is often used as an indicator of ROS generation within the cell. H₂DCFDA is not specific and detects general ROS production, including hydroxyl, peroxyl and other reactive oxygen species. In the reduced form, H₂DCFDA is non-fluorescent, but upon internalisation H₂DCFDA acetate groups are cleaved by intracellular esterases and ROS oxidation converts it into the
highly fluorescent 2',7'-dichlorofluorescein. Green fluorescence intensity is measured using a fluorescent plate reader.

\( \text{H}_2 \text{DCFDA} \) (Molecular Probes\(^\text{®}\)) was dissolved in DMSO to make a 205 mM stock and diluted in PBS for use in the assay. The final DMSO concentration in the assay was 0.005 % which did not affect ROS signal. HASM cells were seeded in confluent monolayers (1x10\(^4\) cells per well) in black 96-well plates with optical bottom and allowed to adhere overnight at 37°C. Cells were serum-starved for 24 h in ITS-containing medium prior to assay. This was removed and cells were washed once with PBS. \( \text{H}_2 \text{DCFDA} \) (10 µM, 100 µL per well) was added to the cells and incubated in the dark for 30 min at 37°C. Excess \( \text{H}_2 \text{DCFDA} \) was then removed and cells washed once with PBS. Stimuli (rhHMGB1 or ROS generators such as hydrogen peroxide and menadione sodium bisulphite, both from Sigma-Aldrich\(^\text{®}\)) were applied in serum-free, phenol red-free high glucose DMEM at 37°C (Gibco\(^\text{®}\)). DTT/EDTA was used as a negative control for rhHMGB1. Each condition was performed in quadruplicate. Fluorescence was monitored at various time points between 0 and 2 h with the Perkin Elmer\(^\text{®}\) EnSpire\(^\text{®}\) plate reader using the following settings: 493 nm excitation and 523 nm emission (green fluorescence); bottom reading; 10 flashes.

### 2.17 Collagen gel contraction assay

1.25x10\(^5\) HASM cells were embedded into pure bovine collagen gels (PureCol, CellSystems\(^\text{®}\)) in the absence or presence of rhHMGB1 (30 – 1000 ng/mL) or corresponding concentrations of DTT as the negative control. Each gel was composed of 62 % collagen solution (3 mg/mL), 8 % Minimum Essential Medium, and 30 % cells.
Gels were allowed to set in a 24-well plate at 37°C for 90 min and then detached from the wells using a sterile spatula. Bradykinin (Sigma-Aldrich®, 1 ng/mL) was added in 10% FBS-containing medium in order to induce contraction. Images were captured at various time points (0 – 60 min) and analysed using ImageJ software. Gel contraction was assessed by measuring the gel area, which was expressed as a percentage of the well area. The area under the curve (AUC) between 0 and 60 min was calculated for each concentration and compared between rhHMGB1 and negative control using paired t tests. The reduction in AUC (DTT control minus rhHMGB1) and the % reduction in AUC (rhHMGB1 AUC was expressed as a percentage of the DTT control AUC and then subtracted from this) were calculated between 0 and 6 h and compared in non-asthmatics and asthmatics.

2.18 Wound healing assay

Primary human bronchial epithelial cells (HBECs) were seeded in confluent monolayers in 6-well plates and allowed to adhere overnight at 37°C. Wounds were created across the well by scratching the cell monolayer with a sterile 200 µL pipette tip. Cells were washed 5 times to remove cell debris and incubated in HBEC medium containing rhHMGB1 (100 – 1000 ng/mL) or corresponding DTT control for 24 h at 37°C. Images of the wounds were captured at various time points and wound closure was assessed by measuring the wound area at different time points.
2. Materials and methods

2.19 Oris™ cell migration assay

HASM cell migration in response to various stimuli was evaluated using the 96-well plate Oris™ cell migration assay from Platypus technologies. The principle of the assay is to create a cell-free area in the centre of each well where cells can migrate. This is achieved by inserting silicone stoppers in each well that constitute a physical barrier preventing cell adherence in the detection area. Cells are seeded around the stoppers and allowed to adhere. The stoppers are then removed, except for reference well stoppers, and stimuli added. The reference well stoppers are removed at the end of the incubation period and indicate whether the cell-free migration area was successfully created. Cells can then be fixed and fluorescently labelled. Cell migration is assessed by applying to the optical bottom of the plate a mask uncovering the centre of each well (i.e. detection area). Fluorescence intensity can be measured or images captured for data analysis.

HASM cells were seeded at the sub-confluent density of 5x10^3 cells per well and allowed to adhere overnight at 37°C. Stoppers were then removed and stimuli added in serum-free medium for 24 h. Stimuli included fully reduced, non-oxidisable 3S-HMGB1 (3 – 1000 ng/mL) and 10 % FBS as a positive control. Since the 3S-HMGB1 formulation contained DTT, this was used as a negative control for 3S-HMGB1-induced migration (3 – 1000 nM DTT). Each condition was performed in quadruplicate. After the incubation period, the reference well stoppers were removed, cells were washed once with PBS and fixed in 10 % neutral buffered formalin for 10 min at RT. Cells were stained with the nuclear dye Hoechst (1:5000) for 15 min at RT and washed twice with PBS. Fixed and stained cells were left in 100 µL of PBS and stored at 4°C until data
acquisition. A detection mask was applied at the bottom of the plate to uncover the detection area. Images were captured for each well at x40 magnification using a fluorescence microscope with incorporated camera (EVOS®, Life Technologies™). Images were analysed by counting the nuclei in the detection area. The number of migrated cells was expressed as a percentage of the relevant DTT control.

2.20 Peripheral blood cell chemotaxis

Chemotaxis of human blood cells towards a chemoattractant gradient was evaluated as previously described in [268] by allowing the cells to migrate through 3 µm pore Transwell® inserts into the wells of a 24-well plate over a 4 h period. Chemotactic stimuli were diluted in Hanks’s balanced salt solution (HBSS) containing 1 % FBS and 500 µL were added in duplicate at the bottom of the wells. Stimuli included 3S-HMGB1, rhHMGB1 (both at 3 µg/mL), and rhIL-8 (25 ng/mL), which was used as a positive control of chemotaxis. DTT was the negative control for 3S-HMGB1 and rhHMGB1 (3 and 9 µM respectively). 3 µm pore size Transwells® were placed on top of the wells. Whole blood was diluted 1:10 in HBSS and 200 µL of diluted blood were added onto each Transwell® insert. Cells were allowed to migrate for 4 h at 37°C. Cells that had migrated but were adherent to the bottom of the Transwells® were detached by incubating these in 300 µL of trypsin (diluted 1:10 in PBS) for 15 min at 37°C. Migrated and detached cells were then combined and counted using a haemocytometer. The number of migrated cells was expressed as fold change compared with the corresponding negative control (1 % FBS plus or minus DTT).
2.21 Statistical data analysis

The sample sizes (n numbers) used in this study were sufficiently large to find significant differences in many experiments (n = 5 or more samples were typically used). However, some analyses were carried out on smaller sample sizes (e.g. as small as n = 3). In this case, for the results that were not significant, there are two possible explanations: 1) there was truly no difference between/among groups; and 2) there was a difference, but the analysis was underpowered. These two possibilities were evaluated by visually examining the plots and by estimating the effect size (i.e. the difference in the mean values). For the plots that were not significant, the effect size was small (an example of this can be found in figure 5.2.1). For those that were significant, the standard deviations were small and there was consistency within the samples, i.e. the mean was not driven by a single sample (see example in figure 4.2.9).

Data was analysed using GraphPad Prism® 6.0. Data distribution was tested for normality, and parametric or non-parametric t tests or ANOVA were applied. A Welch’s correction was included in parametric t tests when groups with different variances were compared. Differences were considered statistically significant when p < 0.05. Results were expressed as mean ± SEM (parametric analysis) or median and interquartile range (non-parametric analysis).
Chapter 3

Ex-vivo characterisation of HMGB1 and RAGE expression in the airways in health and asthma
3. HMGB1 and RAGE ex vivo characterisation

3.1 Introduction

As a DAMP, HMGB1 and its receptors RAGE and TLR4 have been implicated in the pathophysiology of chronic inflammatory conditions, including asthma and COPD (refer to Chapter 1, section 1.7). Knowledge available at the time of conception of this study indicated the involvement of the HMGB1-RAGE axis in asthma and COPD. More recent studies also highlighted the importance of TLR4. Relevant clinical and preclinical studies are summarised in table 3.1.1. Therefore, this study focused on RAGE and HMGB1 and aimed to test the hypothesis that the HMGB1-RAGE axis is involved in asthma pathophysiology, particularly in airway smooth muscle dysfunction in asthma. The aim of this Chapter was to test a hypothesised increase in the expression of HMGB1 and RAGE in sputum and bronchial tissue in asthma compared to health. We also hypothesised that RAGE and HMGB1 expression in sputum/tissue would be related to inflammation (e.g. number of inflammatory cells) in asthma. Indeed, HMGB1 expression in sputum of asthmatics has been found to be related to disease severity and sputum neutrophil percentages [250]. Therefore, the asthmatic sample was subdivided according to GINA severity into mild (GINA 1-to-2) and moderate-to-severe (GINA 3-to-5). To this end, the expression of HMGB1 and RAGE was investigated in clinical samples obtained from healthy controls and asthmatic subjects of various severities. Levels of HMGB1 and secreted RAGE (esRAGE) were assessed in sputum samples by ELISA and Western blotting. HMGB1 and RAGE expression were also characterised in bronchial tissue using immunohistochemistry. Another aim of this Chapter was to investigate the redox state of HMGB1 in the airways, because this is an important determinant of HMGB1 activity [269], and is not
known in the airways in health or asthma. Therefore, the redox state of airway HMGB1 was investigated in sputum by Western blotting carried out under non-reducing conditions. The absolute and relative levels of expression of different HMGB1 redox forms were also quantified in sputum samples from healthy controls and asthmatics of various severities.
<table>
<thead>
<tr>
<th>Disease area</th>
<th>Main findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>COPD</td>
<td>Circulating sRAGE is associated with genetic polymorphisms of AGER, including the G82S SNP.</td>
<td>[234]</td>
</tr>
<tr>
<td>Asthma</td>
<td>The G82S SNP in AGER is associated with lower FEV$_1$/FVC in children with transient early wheeze.</td>
<td>[231]</td>
</tr>
<tr>
<td>COPD</td>
<td>The G82S SNP is associated with COPD and increased risk of developing COPD in smokers.</td>
<td>[232]</td>
</tr>
<tr>
<td>COPD</td>
<td>HMGB1 is increased in BAL of smokers with COPD, and correlates with disease severity. HMGB1 is increased in sputum and plasma in COPD subjects and correlates with neutrophil counts. RAGE and HMGB1 expression is increased in the submucosa, epithelium and smooth muscle of the bronchi of smokers with COPD and in alveolar macrophages.</td>
<td>[145,251]</td>
</tr>
<tr>
<td>COPD</td>
<td>Lung RAGE expression and AGEs expression in alveolar walls and bronchioles are increased in subjects with COPD.</td>
<td>[243]</td>
</tr>
<tr>
<td>COPD</td>
<td>Circulating sRAGE is reduced in COPD subjects.</td>
<td>[247,248]</td>
</tr>
</tbody>
</table>
and the decrease correlates with lung function decline. Exacerbations are associated with lower levels of circulating sRAGE.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Summary</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>COPD</td>
<td>Lower circulating sRAGE is correlated with severity of emphysema</td>
<td>[234,248,249]</td>
</tr>
<tr>
<td>Asthma</td>
<td>Sputum HMGB1 is elevated in treatment-naïve asthmatics, and HMGB1 levels correlate with disease severity and sputum neutrophils. Sputum esRAGE is also increased in asthmatics.</td>
<td>[250]</td>
</tr>
<tr>
<td>Asthma</td>
<td>HMGB1 is increased in sputum and plasma of asthmatics, is related to disease severity, and correlates negatively with lung function and positively with sputum neutrophils.</td>
<td>[251]</td>
</tr>
<tr>
<td>Asthma/COPD</td>
<td>Airway and systemic sRAGE is lower in neutrophilic asthma/COPD compared with non-neutrophilic disease.</td>
<td>[252]</td>
</tr>
<tr>
<td>Allergic asthma</td>
<td>HMGB1, TLR2 and TLR4 expression is increased in the airways and lung tissue of OVA-sensitised mice. Anti-HMGB1 neutralising antibodies reduce airway inflammation, goblet cell hyperplasia, collagen deposition, and AHR.</td>
<td>[258]</td>
</tr>
<tr>
<td>Allergic asthma</td>
<td>Box A inhibits OVA-induced airway inflammation and AHR in mice, and inhibits HMGB1-stimulated</td>
<td>[259]</td>
</tr>
<tr>
<td><strong>Lung injury</strong></td>
<td>Box A and TLR4 genetic deletion reduce lung inflammation and damage in a mouse model of acute lung injury.</td>
<td>[239]</td>
</tr>
<tr>
<td>----------------</td>
<td>--------------------------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td><strong>Allergic asthma</strong></td>
<td>Both TLR4−/− and RAGE−/− mice are similarly protected from all features of HDM-induced allergic airway inflammation. TLR4 is important in the first phase of HDM-induced inflammation; whereas RAGE is important for DC-driven inflammation. HMGB1 is released downstream of IL-1α, and upstream of type 2 innate cytokines TSLP, IL-33, and IL-25. Sources of HMGB1 include the airway epithelium.</td>
<td>[253]</td>
</tr>
</tbody>
</table>

**Table 3.1.1** Clinical and pre-clinical evidence linking RAGE and HMGB1 to respiratory disease. GWAS = genome-wide association studies; SNP = single nucleotide polymorphism; FEV₁ = forced expiratory volume in 1 second; FVC = forced vital capacity; BAL = bronchoalveolar lavage; OVA = ovalbumin; AHR = airway hyperresponsiveness; HDM = house dust mite; DC = dendritic cells.
3. Characterisation of HMGB1 expression in sputum samples

3.2 Characteristics of subjects included in the HMGB1 ELISA

HMGB1 concentrations were measured in sputum PBS supernatants by ELISA. Forty-two asthmatic subjects, including eight mild asthmatics (GINA 1-to-2) and thirty-four moderate-to-severe asthmatics (GINA 3-to-5), and eighteen age-matched non-asthmatic controls were included in this part of the study. Subject characteristics are summarised in table 3.2.1. The majority of the subjects were either non-smokers or ex-smokers. Only two of the forty-two asthmatics were current smokers and two were occasional smokers, whereas there were no current smokers in the control group. Lung function was assessed by spirometry, as described in the Methods, and it was worse in the GINA 3-to-5 asthmatic sample population. The FEV₁ (% of the predicted value) before the administration of bronchodilator was 82.3 ± 4.3 (mean ± SEM) in GINA 3-to-5 subjects compared with 90.7 ± 3.7 in GINA 1-to-2 subjects and 100.7 ± 6.3 % in controls (ANOVA p = 0.04). FEV₁/FVC was also lower in GINA 3-to-5 asthmatics (table 3.2.1). Sputum was induced and processed as described in Chapter 2. Sputum neutrophil counts were similar in asthmatic subjects and controls; however, sputum eosinophils were increased in GINA 3-to-5 asthmatics compared with controls. Median (interquartile range) % sputum eosinophils were 0.75 (1.38) in controls, 0.75 (6.25) in GINA 1-to-2, and 2.0 (8.56) in GINA 3-to-5 asthmatics (Kruskal-Wallis test p = 0.038).
### Table 3.2.1

Subject demographics for the HMGB1 ELISA. Results are expressed as mean ± SEM or median (interquartile range). ICS = inhaled corticosteroids; BDP = Beclometasone dipropionate; FEV\(_1\) = forced expiratory volume in 1 second; FVC = forced vital capacity; pre-BD = pre-bronchodilator. *p < 0.05 versus control

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<td>ICS - µg of BDP</td>
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<tr>
<td>equivalent</td>
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</tr>
<tr>
<td>Pre-BD FEV(_1) % predicted</td>
<td>100.7 ± 6.3</td>
<td>90.7 ± 3.7</td>
<td>*82.3 ± 4.3</td>
<td>p = 0.04</td>
</tr>
<tr>
<td>Pre-BD FEV(_1)/FVC</td>
<td>74.5 (6.0)</td>
<td>77.0 (7.25)</td>
<td>71.0 (13.0)</td>
<td>p = 0.028</td>
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<td>% sputum neutrophils</td>
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<td>61.13 (41.7)</td>
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<tr>
<td>% sputum eosinophils</td>
<td>0.75 (1.38)</td>
<td>0.75 (6.25)</td>
<td>*2.0 (8.56)</td>
<td>p = 0.038</td>
</tr>
<tr>
<td>% sputum macrophages</td>
<td>35.2 ± 5.4</td>
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<td>25.4 ± 3.4</td>
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<td>% sputum epithelial cells</td>
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3.2.2 HMGB1 ELISA

A commercial sandwich ELISA was used to measure HMGB1 concentrations in sputum supernatants. Standard curves were constructed using pig HMGB1 protein at concentrations ranging from 2.5 to 80 ng/mL. An example of the HMGB1 ELISA standard curve is shown in figure 3.2.1. In the linear regression, $r^2$ indicates the goodness of fit i.e. how well $x$ (antigen concentration) can be predicted when $y$ (sample optical density or OD) is known. An $r^2 = 1$ means that $y$ can perfectly predict $x$, therefore an $r^2$ value close to 1 indicates a good correlation. The mean $r^2$ for the HMGB1 ELISA standard curve was $0.998 \pm 0.001$ (mean ± SEM from $n = 6$ experiments).

The inter-assay repeatability of the HMGB1 ELISA was tested by running samples from the same donor in separate assays ($n = 3$). To assess protein stability three different samples were also assayed before and after a freeze-thaw cycle. In both cases, HMGB1 concentrations were comparable (paired t tests $p = 0.79$ and $p = 0.39$, respectively), indicating a good inter-assay repeatability and low HMGB1 protein degradation following a freeze-thaw cycle.
Figure 3.2.1 HMGB1 ELISA standard curve. Blank (diluent buffer) and HMGB1 standards were assayed in duplicate. The blanks mean optical density (OD) value was subtracted from those of the standards and data was fitted using a linear regression equation. This was used to calculate the concentration of HMGB1 in the samples, as described in Chapter 2. Results from \( n = 1 \) representative of \( n = 6 \) independent experiments are presented here as mean ± SD. An \( r^2 \) value of 1 indicates a perfect fit. Inter-assay variability and HMGB1 protein degradation following a freeze-thaw cycle were also tested and were not significant.
3.2.3 HMGB1 is increased in sputum of moderate-to-severe asthmatics

HMGB1 concentration was significantly elevated in sputa from moderate-to-severe asthmatics (GINA treatment steps 3-to-5) compared with healthy controls and mild asthmatics (GINA 1-to-2, figure 3.2.2A). Median (interquartile range) values were 123 (295) ng/mL in healthy controls (n = 18); 122 (259) ng/mL in GINA 1-to-2 (n = 8); and 429 (576) ng/mL in GINA 3-to-5 (n = 34, Kruskal-Wallis test p = 0.003). Parametric statistical analysis of the Log10 transformed data gave similar results (ANOVA p = 0.002). The subject smoking history did not seem to affect sputum HMGB1 levels, since non-smokers, ex-smokers, and smokers had similar sputum HMGB1 concentrations: median (interquartile range) values were 247 (783) ng/mL in non-smokers (n = 29); 342 (338) ng/mL in ex-smokers (n = 27); and 298 (327) ng/mL in current or occasional smokers (n = 4, Kruskal-Wallis test p = 0.91).

There was no correlation between sputum HMGB1 concentrations and lung function (FEV1 % predicted) in the asthmatic sample population (Spearman r = -0.05, p = 0.76), but HMGB1 levels positively correlated with sputum total cell count (TCC, Spearman r = 0.506, p = 0.0007, figure 3.2.2B). This was not restricted to asthma, as the correlation was also observed in the non-asthma group (Spearman r = 0.89, p < 0.0001, figure 3.2.2C). The relationship between sputum TCC and HMGB1 levels was better visualised by grouping the subjects on the basis of their TCC, as shown in figure 3.2.2D-E. Using a cut-off value for normal sputum TCC of 6.8 x 10^6 cells/g of sputum [270], the asthmatic and non-asthmatic sample populations were divided in two groups based on their sputum TCC. TCC values < 6.8 x 10^6 cells/g of sputum were considered low, and those ≥ 6.8 x 10^6 cells/g of sputum were considered high. HMGB1 concentration was
significantly elevated in asthmatic subjects with high sputum TCC compared with those with low sputum TCC (figure 3.2.2D). Median (interquartile range) were 772 (683) for the asthmatics with high sputum TCC (n = 13) and 227 (285) for those with low sputum TCC (n = 28, Mann-Whitney test p = 0.004). HMGB1 was also increased in non-asthmatics with high TCC versus those with low TCC (figure 3.2.2E). Median (interquartile range) were 706 (770) for the non-asthmatics with high sputum TCC (n = 4) and 98 (170) for those with low sputum TCC (n = 14, Mann-Whitney test p = 0.0007).
Figure 3.2.2 Sputum HMGB1 is increased in moderate-to-severe asthma. HMGB1 concentrations were measured by ELISA in sputum PBS supernatants. Samples were assayed in duplicate. A) Sputum HMGB1 levels were significantly increased in moderate-to-severe asthmatics (GINA 3-to-5) compared with healthy controls and mild asthmatics (GINA 1-to-2, Kruskal-Wallis test $p = 0.003$). B-C) Sputum HMGB1 concentrations positively correlated with sputum TCC in asthmatics (B; Spearman $r = 0.506, p = 0.0007$) and in non-asthmatics (C; Spearman $r = 0.89, p < 0.0001$). D) HMGB1 was elevated in asthmatics with high sputum TCC ($\geq 6.8 \times 10^6$ cells/g of sputum, $n = 13$) versus asthmatics with low TCC ($n = 28$, Mann-Whitney test $p = 0.004$). E) HMGB1 was higher in non-asthmatics with high sputum TCC ($n = 4$) compared with non-asthmatics with low sputum TCC ($n = 14$, Mann-Whitney test $p = 0.0007$). Squares represent GINA 1 subjects ($n = 5$), diamonds are GINA 2 ($n = 3$), open circles are GINA 3 ($n = 12$), triangles are GINA 4 ($n = 13$), and stars GINA 5 subjects ($n = 9$). Results are presented as median and interquartile range.
3. HMGB1 and RAGE ex vivo characterisation

[Graph A] p < 0.05

[Graph B] r = 0.506  p = 0.0007

[Graph C] r = 0.89  p < 0.0001

[Graph D] p = 0.004

[Graph E] p = 0.0007
3.2.4 Characterisation of HMGB1 redox state in sputum samples

Recent literature highlighted how the redox state of HMGB1 is crucial for determining its activity. Different research groups have shown that fully reduced HMGB1 (i.e. containing free thiol groups at cysteine residues C23, C45 and C106) is chemotactic for leukocytes; disulphide-HMGB1 (i.e. containing a disulphide bond between C23 and C45) induces inflammatory cytokine production; and terminally oxidised HMGB1 (i.e. containing sulfonic groups on all three cysteine residues) does not have any chemokine or cytokine activity, but may play a role in the resolution phase of inflammation [149,133].

HMGB1 redox state in the airways has not been investigated in health or asthma; therefore HMGB1 expression in sputum samples from asthmatics and non-asthmatic controls was assessed by Western blotting under non-reducing conditions, i.e. in the absence of reducing agents such as β-mercaptoethanol or DTT, in order to preserve HMGB1 redox state. The clinical characteristics of the subjects included in this part of the study are summarised in table 3.2.2.

First, the specificity of two anti-HMGB1 antibodies was assessed by Western blotting using HASM cell lysates. HASM cells were lysed in 1x SDS sample buffer (Laemmli buffer) and cell lysates were probed using two monoclonal antibodies, a rabbit monoclonal antibody from Abcam® (clone EPR3507) and a mouse monoclonal antibody from Abnova (clone 2F6). Both antibodies detected a single band with a molecular weight of 25 KDa, which is the predicted molecular weight/migration pattern for HMGB1 in reducing gels (n = 3, figure 3.2.3). These results confirmed the
specificity of both anti-HMGB1 antibodies. The rabbit monoclonal antibody (clone EPR3507) was chosen to assess HMGB1 expression in sputum.

Sputum HMGB1 expression was donor-dependent. The number of bands that were detected ranged from none to two with approximate molecular weights of 25 and 30 KDa. This migration pattern is consistent with that previously described for disulphide-HMGB1 and all-thiol-HMGB1, migrating respectively at 26 and 28 KDa [133]. To confirm that the bands observed were indeed oxidised and reduced forms of HMGB1, the sputum samples were pre-treated with the reducing agent DTT (5 mM) for 15 min on ice before loading onto the gel. Incubation with DTT caused a molecular weight shift of the 25 KDa band, leaving only the 30 KDa band (figure 3.2.4). This confirmed the redox nature of the proteins.

The amount of oxidised and reduced HMGB1 could be quantified in a subset of samples (nine asthmatic subjects of various severities (GINA 2-to-5) and six non-asthmatics), for which an internal control (recombinant HMGB1) was included in the blot. Each band was quantified by densitometry and data were expressed as a percentage of the rHMGB1 standard (figure 3.2.5). In these samples, both redox forms were significantly increased in asthma. Oxidised HMGB1 was 92.3 ± 27.1 % of the standard (mean ± SEM) in the asthmatics versus 8.2 ± 2.9 % in the controls (unpaired t test with Welch’s correction \( p = 0.015 \). A Welch’s correction was included to account for different variances within groups). Reduced HMGB1 was 86.6 ± 28.8 % of the standard in the asthmatics and 0 % in the controls (the mean value of the asthma group was compared with a theoretical value of zero using a one sample t test, \( p = 0.017 \)). These data support the elevation in sputum HMGB1 concentrations found in asthma by ELISA.
The relative abundance of each HMGB1 redox form could be investigated in a bigger number of sputum samples from healthy and asthmatic subjects: the relative expression of reduced and oxidised HMGB1 was calculated as a percentage of total HMGB1 in eight controls, seven mild asthmatics and sixteen moderate-to-severe asthmatics. The relative proportion of reduced HMGB1 was significantly increased in moderate-to-severe asthma, with 36.7 ± 5.6 % (mean ± SEM, n = 16) of total HMGB1 found in the reduced form compared to 11.2 ± 7.3 % in non-asthmatic controls (n = 8) and 13 ± 8.4 % in mild asthmatics (n = 7, ANOVA p = 0.015, figure 3.2.6).
### Table 3.2.2 Subject demographics for HMGB1 redox state study

Characteristics of non-asthmatic and asthmatic subjects included in the HMGB1 redox state study. Results are expressed as mean ± SEM or median (interquartile range). ICS = inhaled corticosteroids; BDP = Beclometasone dipropionate; FEV₁ = forced expiratory volume in 1 second; FVC = forced vital capacity; pre-BD = pre-bronchodilator. *p < 0.05 versus control.

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<tr>
<td>Pre-BD FEV₁ % predicted</td>
<td>92.1 ± 4.7</td>
<td>93.9 ± 6.4</td>
<td>75.2 ± 5.4</td>
<td>p = 0.052</td>
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<td>Pre-BD FEV₁/FVC</td>
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<td>67.3 ± 2.6</td>
<td>p = 0.052</td>
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<td>% sputum neutrophils</td>
<td>50.3 ± 9.0</td>
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<td>% sputum eosinophils</td>
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<td>% sputum macrophages</td>
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<td>% sputum epithelial cells</td>
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<td>1.75 (3.75)</td>
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*Table 3.2.2* Subject demographics for HMGB1 redox state study. Characteristics of non-asthmatic and asthmatic subjects included in the HMGB1 redox state study. Results are expressed as mean ± SEM or median (interquartile range). ICS = inhaled corticosteroids; BDP = Beclometasone dipropionate; FEV₁ = forced expiratory volume in 1 second; FVC = forced vital capacity; pre-BD = pre-bronchodilator. *p < 0.05 versus control.
Figure 3.2.3 Detection of HMGB1 by Western blotting. HMGB1 antibody specificity was assessed in n = 3 HASM cell lysates by Western blotting. Cells were lysed in 1x sample buffer and loaded onto 12 % SDS-polyacrylamide gels. Proteins were blotted onto PVDF membranes and probed using a rabbit monoclonal antibody (Abcam®, clone EPR3507, 0.2 μg/mL) and a mouse monoclonal antibody (Abnova, clone 2F6, 0.5 μg/mL). Both antibodies detected a single band with a molecular weight of 25 KDa. The loading control was β-actin. Lanes 1 and 3 = asthmatic donors; lane 2 = non-asthmatic donor.
Figure 3.2.4 Assessment of HMGB1 redox state in sputum. Sputum supernatants (20 μL) of two asthmatics (A) and a non-asthmatic (NA) were loaded onto a 12 % SDS-polyacrylamide gel in the presence (+) or absence (-) of 5 mM DTT as a reducing agent. Proteins were transferred onto PVDF membranes, which were probed with the rabbit monoclonal antibody (Abcam®, clone EPR3507, 0.2 μg/mL). None to two bands with molecular weights of approximately 25 and 30 KDa were visible in the absence of DTT, but only the higher molecular weight band remained when the samples were pretreated with DTT. Recombinant HMGB1 (20 ng, rHMGB1) containing a disulphide bond (partially oxidised) was used as a control and also migrated at a higher molecular weight in the presence of DTT.
Figure 3.2.5 Expression of both redox forms of HMGB1 is increased in asthma. A) Representative non-reducing Western blot showing HMGB1 expression in the sputum of a non-asthmatic subject (NA) and two asthmatic subjects (Mild is a GINA 2 subject and Severe a GINA 4 subject). The standard is rHMGB1 in the disulphide state. B) Densitometry results from \( n = 9 \) asthmatics (GINA 2-to-5) and \( n = 6 \) controls are presented as mean ± SEM percentage of the standard rHMGB1 (unpaired t tests \( p < 0.05 \)).
3. HMGB1 and RAGE ex vivo characterisation

Figure 3.2.6 Reduced HMGB1 is more represented in moderate-to-severe asthma. A) Representative non-reducing Western blot showing HMGB1 expression in the sputum of two non-asthmatic subjects (NA) and three asthmatics (lane 3 = GINA 1; lanes 4 and 5 = GINA 4). B) Relative expression of reduced HMGB1 was significantly higher in moderate-to-severe asthmatics (GINA 3-to-5, n = 16) with 36.7 ± 5.6 % of total HMGB1 found in the reduced form compared to 11.2 ± 7.3 % in healthy controls (n = 8) and 13 ± 8.4 % in mild asthmatics (n = 7, ANOVA p = 0.015). Results are presented as mean ± SEM. Squares represent GINA 1 subjects (n = 2), diamonds are GINA 2 (n = 5), open circles are GINA 3 (n = 5), triangles are GINA 4 (n = 7), and stars GINA 5 subjects (n = 4).
3. HMGB1 and RAGE ex vivo characterisation

3.2.5 HMGB1 is chemotactic for peripheral blood leukocytes

Reduced (all-thiol) HMGB1 has been shown to induce leukocyte migration [133]. The increase in the reduced form of HMGB1 in sputa of moderate-to-severe asthmatics together with the positive correlation found between sputum TCC and sputum HMGB1 levels suggested that a relationship exists between reduced HMGB1 in sputum and the number of inflammatory cells recruited to the airways. Reduced HMGB1 released by damaged or stressed airway cells may attract peripheral blood leukocytes to the airways. There, activated leukocytes may release more HMGB1, thus generating a positive feedback loop.

In order to test this hypothesis, the ability of HMGB1 to induce peripheral blood leukocyte migration was assessed using a Transwell® chemotaxis assay. Concentrations of rhHMGB1 between 1 and 5 μg/mL have been shown to promote neutrophil migration, whereas lower concentrations (100 ng/mL) can inhibit this process [268]. In sputum, the higher HMGB1 concentrations exceeded 1 μg/mL and the maximum concentration was 2.4 μg/mL; therefore rhHMGB1 was tested for chemotaxis at a concentration of 3 μg/mL. 3S-HMGB1, a mutated non-oxidisable HMGB1 protein which was characterised in Venereau et al. (JEM 2012), was also tested at the same concentration. The chemokine and neutrophil chemoattractant IL-8 (25 ng/mL) was used as a positive control. Stimuli were diluted in 500 μL of 1 % FBS-HBSS and added into a Transwell® plate. Human whole blood was diluted ten times in HBSS and 200 μL were added into 3 μm pore Transwells®. This pore size is most suitable for the migration of neutrophils; however neither the composition of the initial cell population nor that of the migrated cells was determined, as the aim was to test HMGB1 ability to
induce peripheral blood leukocytes. Cell migration was assessed following 4 h incubation by counting the cells that had migrated in the lower chambers. The number of migrated cells was related to spontaneous migration in the absence of stimuli (1 % FBS-HBSS) by expressing the results as fold change over unstimulated cells. Both rhHMGB1 (reduced protein) and 3S-HMGB1 (non-oxidisable protein) were able to induce increased peripheral blood leukocyte migration compared with baseline migration. rhHMGB1 caused a 1.82 ± 0.18-fold increase in migration (mean ± SEM, n = 6) and 3S-HMGB1 caused a 1.43 ± 0.14-fold increase (p = 0.007 for rhHMGB1 and p = 0.027 for 3S-HMGB1 using a one sample t test against a theoretical value of 1, figure 3.2.7). The difference between rhHMGB1 and 3S-HMGB1-induced migration was not significant (paired t test p = 0.094).
Figure 3.2.7 Reduced HMGB1 promotes peripheral blood leukocyte chemotaxis. Peripheral blood leukocyte migration was assessed following stimulation with reduced rhHMGB1 and non-oxidisable 3S-HMGB1 using a Transwell® chemotaxis assay. Migrated cells were counted and results expressed as fold change over baseline migration. Mean ± SEM of n = 6 donors assayed in duplicate are shown. One sample t test was performed against a theoretical value of 1 (p = 0.007 for rhHMGB1 and p = 0.027 for 3S-HMGB1).
3.3 Characterisation of soluble RAGE expression in sputum samples

3.3.1 Sputum esRAGE is not increased in asthma

Endogenous secretory RAGE (esRAGE) is a soluble, secreted form of RAGE that is thought to act as a decoy receptor for RAGE ligands, including HMGB1. Sputum esRAGE levels have been found to be upregulated in newly diagnosed, treatment-naïve asthmatics [250]. However, airway and systemic soluble RAGE (sRAGE) levels are significantly decreased in neutrophilic asthma (i.e. neutrophils ≥ 65 % of sputum total cells), suggesting a protective role for sRAGE in neutrophilic inflammation [252]. In the latter study, sRAGE concentrations correlated significantly with esRAGE concentrations both in bronchial lavage (BL) and in serum in all the subject groups investigated, suggesting that the main form of sRAGE found both in the airways and systemically is esRAGE [252].

Therefore, the levels of esRAGE were investigated in sputum supernatants of thirty-five asthmatics and fifteen non-asthmatic controls by ELISA. These subjects were a subset of those included in the HMGB1 ELISA. Nonetheless, there were some differences between the HMGB1 ELISA and the esRAGE ELISA sample populations. For example, there was a significant age difference within the esRAGE ELISA sample population, with GINA 3-to-5 subjects slightly older than GINA 1-to-2 and controls. Moreover, the % sputum eosinophils was not different among groups (table 3.3.1). Lung function was worse in GINA 3-to-5 asthmatics, as indicated by significantly lower FEV₁ % predicted and FEV₁/FEV %.

Subject clinical characteristics are summarised in table 3.3.1.
A commercial sandwich ELISA was used to measure esRAGE levels in sputum PBS supernatants. The standard (human esRAGE) concentration range was from 50 to 3200 pg/mL. An example of the standard curve is shown in figure 3.3.1A. The mean $r^2$ for the esRAGE ELISA standard curve was $0.992 \pm 0.007$ (mean ± SD of $n = 2$ assays). Samples with esRAGE concentrations below the lower end of the standard curve (50 pg/mL) but above the blank (0 pg/mL) were assigned a value of 25 pg/mL, which was then adjusted to account for sample manipulation, as described in Chapter 2.

No significant differences were found in sputum esRAGE concentrations among controls, mild asthmatics and moderate-to-severe asthmatics (figure 3.3.1B). Median (interquartile range) values were 627 (2600) pg/mL in healthy controls ($n = 15$); 573 (1307) pg/mL in GINA 1-to-2 ($n = 6$); and 460 (1417) pg/mL in GINA 3-to-5 ($n = 29$, Kruskal-Wallis test $p = 0.86$).

These results do not support the studies cited above; however this discrepancy may reflect differences in the populations and sampling. Indeed, the asthmatic patients included here were under medication, unlike the ones in the study reported by Watanabe et al. (2011) [250], and did not have what is described as neutrophilic asthma as reported in the study by Sukkar et al. (2011, defined in that study as a sputum/BL neutrophil count $\geq 65\%$ of total cells [252]).
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<td>Pre-BD FEV₁ % predicted</td>
<td>103.7 ± 7.5</td>
<td>87.8 ± 4.4</td>
<td>*81.7 ± 4.9</td>
<td>p = 0.041</td>
</tr>
<tr>
<td>Pre-BD FEV₁/FVC</td>
<td>75.0 (6.0)</td>
<td>78.0 (7.0)</td>
<td>$71.0 (14.0)</td>
<td>p = 0.006</td>
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<tr>
<td>% sputum neutrophils</td>
<td>62.3 ± 5.8</td>
<td>50.3 ± 10.0</td>
<td>61.0 ± 4.3</td>
<td>p = 0.57</td>
</tr>
<tr>
<td>% sputum eosinophils</td>
<td>0.75 (1.96)</td>
<td>3.0 (6.4)</td>
<td>1.5 (2.9)</td>
<td>p = 0.54</td>
</tr>
<tr>
<td>% sputum macrophages</td>
<td>33.4 ± 5.5</td>
<td>44.9 ± 10.9</td>
<td>25.5 ± 3.5</td>
<td>p = 0.11</td>
</tr>
<tr>
<td>% sputum epithelial cells</td>
<td>1.2 (2.13)</td>
<td>3.0 (6.38)</td>
<td>1.5 (2.86)</td>
<td>p = 0.82</td>
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**Table 3.3.1** Subject demographics for esRAGE ELISA. Characteristics of non-asthmatic and asthmatic subjects included in the esRAGE ELISA. Results are expressed as mean ± SEM or median (interquartile range). ICS = inhaled corticosteroids; BDP = Beclometasone dipropionate; FEV₁ = forced expiratory volume in 1 second; FVC = forced vital capacity; pre-BD = pre-bronchodilator. *p < 0.05 versus control; $p < 0.05 versus GINA 1-2.
Figure 3.3.1 esRAGE levels are not changed in asthma versus health. A) esRAGE ELISA standard curve (mean ± SD) representative of n = 2 independent experiments performed in duplicate. The linear regression equation was used to calculate esRAGE concentrations in the samples. B) Sputum median (interquartile range) esRAGE concentrations were similar in healthy controls (627 (2600) pg/mL, n = 15), mild asthmatics (GINA 1-to-2, 573 (1307) pg/mL, n = 6), and moderate-to-severe asthmatics (GINA 3-to-5, 460 (1417) pg/mL, n = 29, Kruskal-Wallis test p = 0.86). Squares represent GINA 1 subjects (n = 4), diamonds are GINA 2 (n = 2), open circles are GINA 3 (n = 10), triangles are GINA 4 (n = 12), and stars GINA 5 subjects (n = 7). Results are presented as median and interquartile range.
3.4 Ex vivo characterisation of HMGB1 and RAGE expression in bronchial tissue

The expression of HMGB1 and RAGE was then characterised in bronchial tissue obtained from ten non-asthmatic subjects and nineteen asthmatic subjects, whose clinical characteristics are summarised in table 3.4.1. The subjects were matched for age and were mostly non-smokers. All the asthmatic subjects were classified as severe asthmatics based on GINA classification criteria (GINA 4 and 5). Lung function was worse in the severe asthma group, with significantly lower FEV₁/FVC (mean ± SEM was 63.1 ± 3.5 % in severe asthmatics versus 84.5 ± 2.6 in non-asthmatics, unpaired t test p = 0.0009) and lower FEV₁ % predicted, although this did not reach statistical significance (mean ± SEM was 75.8 ± 6.2 % in severe asthmatics versus 95.8 ± 5.7 in non-asthmatics, unpaired t test p = 0.065). There was no difference in sputum cell counts, except for the sputum eosinophils, which were higher in the severe asthma group; mean ± SEM was 10.7 ± 2.7 % in the asthmatics versus 0.5 ± 0.2 % in the non-asthmatics (unpaired t test p = 0.002).
3. HMGB1 and RAGE ex vivo characterisation

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<th>GINA 4-5 (n = 19)</th>
<th>p value</th>
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<td>Age, years</td>
<td>46.7 ± 6.2</td>
<td>55 ± 2.8</td>
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<td>Males (%)</td>
<td>5 (50)</td>
<td>8 (42.1)</td>
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<tr>
<td>Smoking status (%)</td>
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<td>ICS - μg of BDP equivalent</td>
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<td>1600 (125)</td>
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</tr>
<tr>
<td>Pre-BD FEV₁ % predicted</td>
<td>95.8 ± 5.7</td>
<td>75.8 ± 6.2</td>
<td>p = 0.065</td>
</tr>
<tr>
<td>Pre-BD FEV₁/FVC</td>
<td>84.5 ± 2.6</td>
<td>63.1 ± 3.5</td>
<td>p = 0.0009</td>
</tr>
<tr>
<td>% sputum neutrophils</td>
<td>50.5 ± 18.7</td>
<td>52.6 ± 6.1</td>
<td>p = 0.9</td>
</tr>
<tr>
<td>% sputum eosinophils</td>
<td>0.5 ± 0.2</td>
<td>10.7 ± 2.7</td>
<td>p = 0.002</td>
</tr>
<tr>
<td>% sputum macrophages</td>
<td>38.8 ± 17.4</td>
<td>32.5 ± 5.8</td>
<td>p = 0.66</td>
</tr>
<tr>
<td>% sputum epithelial cells</td>
<td>1.0 (17.6)</td>
<td>2.75 (3.0)</td>
<td>p = 0.85</td>
</tr>
</tbody>
</table>

**Table 3.4.1** Subject demographics for HMGB1 and RAGE IHC. Characteristics of subjects included in the immunohistochemical characterisation of HMGB1 and RAGE in bronchial biopsies. Results are expressed as mean ± SEM or median (interquartile range). ICS = inhaled corticosteroids; BDP = Beclometasone dipropionate; FEV₁ = forced expiratory volume in 1 second; FVC = forced vital capacity; pre-BD = pre-bronchodilator.
3.4.1 HMGB1 expression in bronchial biopsies from severe asthmatics

Sputum HMGB1 concentrations were found to be elevated in the more severe asthma phenotype; therefore HMGB1 expression profile was investigated in bronchial tissue obtained from severe asthmatic patients (GINA 4 and 5) using immunohistochemistry. Bronchial biopsies were embedded in GMA resin and 2 µm sections were cut and probed using the rabbit monoclonal antibody (Abcam®, clone EPR3507, 20 µg/mL). Rabbit immunoglobulin fraction (Dako) was used as a negative control. No antigen retrieval was necessary for the detection of HMGB1 in GMA-embedded tissue. HMGB1 positive staining was donor-dependent and could be found on the epithelium, the smooth muscle and infiltrating inflammatory cells (figures 3.4.1 and 3.4.2). HMGB1 was predominantly nuclear, although in some cases the staining appeared cytoplasmic as well. Examples of HMGB1 cytoplasmic staining are shown in figure 3.4.1.

HMGB1 staining in the smooth muscle was quantified and expressed as the number of positive cells per smooth muscle area (mm²) and as a percentage of positive cells compared with the total cell number. Using both methods, airway smooth muscle HMGB1 expression was significantly elevated in the severe asthma group compared with controls (figure 3.4.2). The mean ± SEM number of positive cells per smooth muscle area was 546 ± 127 in the severe asthma group (n = 16) compared with 211 ± 36 in controls (n = 10, unpaired t test p = 0.021. A Welch’s correction was applied to account for different variances within groups). The median (interquartile range) percentage of HMGB1-positive cells in the smooth muscle was 32.2 (51.2) in the severe asthma group and 10.5 (19.2) in the controls (Mann-Whitney test p = 0.046). HMGB1 expression in the ASM did not correlate with parameters of lung function in the severe
asthma group. Infiltrating inflammatory cells (tryptase-positive mast cells, major basic protein-positive eosinophils, elastase-positive neutrophils, and CD3-positive lymphocytes) were counted, and correlations with HMGB1-positive ASM cells were evaluated. The % positive ASM cells correlated negatively with mast cell numbers in the lamina propria and in the smooth muscle, and also negatively with the number of eosinophils in the lamina propria (figure 3.4.3).

HMGB1 expression in bronchial epithelium was quantified similarly to expression in the ASM, by counting the positive cells per epithelium area and the percentage of positive epithelial cells. No significant differences were observed between non-asthmatics and severe asthmatics (figure 3.4.4). The number of positive cells per area of epithelium (mm$^2$) was 1882 (1528) in non-asthmatics (n = 10, median and interquartile range) and 1830 (1917) in severe asthmatics (n = 16, Mann-Whitney test p = 0.51). The median (interquartile range) percentage of HMGB1-positive epithelial cells was 68.1 (16.4) in non-asthmatics and 54.5 (31.6) in severe asthmatics (Mann-Whitney test p = 0.42).
**Figure 3.4.1** HMGB1 staining in human bronchial tissue. HMGB1 expression was assessed using a rabbit monoclonal antibody (Abcam®, clone EPR3507, 20 μg/mL). **A-B)** Isotype controls are shown at x200 and x400 magnification, respectively. **C-D)** Several inflammatory cells within the lamina propria stained positive for HMGB1 and staining appeared cytoplasmic in some cases. The asterisk indicates an inflammatory cell with HMGB1 staining in the nucleus and black arrows point to HMGB1-positive inflammatory cells with nuclear and cytoplasmic staining. A normal donor is shown (B x200 magnification; C x400 magnification). **E)** HMGB1 staining appears cytoplasmic in some bronchial smooth muscle cells (black arrow head), whilst other cells display nuclear staining (asterisk). A severe asthmatic is shown (x400 magnification). **F)** Epithelium of a normal donor with cytoplasmic HMGB1 staining (open arrow head, x400 magnification). The black arrow points to an HMGB1-positive inflammatory cell.
3. HMGB1 and RAGE ex vivo characterisation
Figure 3.4.2 HMGB1 expression is increased in the ASM of severe asthmatics. **A)** Isotype control (rabbit immunoglobulin fraction, x200 magnification). **B)** HMGB1 staining (brown, indicated by a black arrow) in the airway smooth muscle of a non-asthmatic subject (x200). **C)** HMGB1 staining was observed in the airway smooth muscle (black arrow) and occasional epithelial cells (black arrow head) of a severe asthmatic subject (x200). **D)** x400 magnification, showing HMGB1 expression in the smooth muscle and epithelium of the severe asthmatic. **E)** The number of HMGB1-positive cells per SM area was increased in the severe asthma group (unpaired t test p = 0.021). Results are presented as mean ± SEM. **F)** The percentage of HMGB1-positive SM cells was increased in severe asthma (Mann-Whitney test p = 0.046). Median and interquartile range are shown.
3. HMGB1 and RAGE ex vivo characterisation

E

F

p = 0.021

p = 0.046
Figure 3.4.3 ASM HMGB1 correlations with inflammatory cells. HMGB1 expression in ASM (% positive SM cells) in severe asthma was negatively correlated with mast cell numbers in the smooth muscle (A) and in the lamina propria (B), and also negatively correlated with eosinophil numbers in the lamina propria (C). Pearson r values and p values are reported for each correlation.
Figure 3.4.4 HMGB1 staining in human bronchial epithelium. A) Isotype control (rabbit immunoglobulin fraction) at 200x magnification. B-C) Non-asthmatic subject (B) and severe asthmatic subject (GINA 4, C). Black arrows indicate HMGB1-positive cells; x200 magnification. D-E) No significant differences in epithelial HMGB1 expression were found between non-asthmatics and severe asthmatics when measuring positive cells per area (D) or % positive cells (E). Mann-Whitney test p values were 0.51 and 0.42, respectively. Median and interquartile range are shown.
3. HMGB1 and RAGE ex vivo characterisation

A

B

C

D

E

\[ p = 0.51 \]

\[ p = 0.42 \]
3.4.2 Bronchial RAGE expression is not different in health and asthma

Next, the expression of RAGE was investigated in bronchial tissue from non-asthmatics and severe asthmatic subjects using IHC. A mouse monoclonal anti-RAGE antibody (Millipore, clone DD/A11, 10 μg/mL) was used to detect RAGE in GMA-embedded bronchial tissue. This antibody has been previously used in the study reported by Ferhani et al. (2010) to investigate RAGE expression in paraffin-embedded bronchial tissue [145]. In order to determine whether a similar pattern of staining is seen in our laboratory, the antibody was first tested on available paraffin-embedded lung resection tissue. Specific RAGE staining could be observed in the bronchial epithelium, small vessel endothelium, and circulating inflammatory cells (figure 3.4.5B-C). This staining is consistent with the RAGE expression reported previously [145]. Interestingly, RAGE expression was enhanced in areas of bronchial epithelium in which cells displayed a different morphology and seemed therefore metaplastic (figure 3.4.5B).

The antibody was then optimised for the staining of GMA-embedded tissue. Antigen retrieval was performed and a signal amplification step with mouse LINKER was also included to enhance the signal. Three antibody concentrations were tested (10, 20 and 40 μg/mL) and a concentration of 10 μg/mL was chosen because higher concentrations caused detectable non-specific staining of the isotype control. RAGE staining was nonetheless poorer in GMA sections compared with paraffin sections; however bronchial biopsies are not routinely embedded in paraffin within the laboratory, therefore GMA sections had to be used to investigate RAGE expression in asthma versus health. A similar pattern and level of staining were observed in GMA sections.
using a different mouse monoclonal antibody from Santa Cruz which detected RAGE specifically by Western blotting (Chapter 4, figure 4.3.6B).

Therefore, it was not possible to quantify RAGE-positive cells in GMA sections, but a semi-quantitative scoring system was used instead. RAGE staining in each donor was compared to the isotype control (mouse IgG2a, 10 μg/mL) and assigned a value from 0 to 3 as follows: 0 = no positive staining; 1 = little positive staining; 2 = moderate positive staining; 3 = marked positive staining (figure 3.4.6). Using this method, no difference in RAGE expression was found between non-asthmatic controls (n = 10) and severe asthmatics (n = 19). Median (interquartile range) scores were 0 (1.0) in the ASM of both controls and severe asthmatics (Mann-Whitney test p = 0.7); and in the epithelium 0.5 (1.25) in controls versus 0 (1.0) in severe asthmatics (Mann-Whitney test p = 0.6, figure 3.4.7).
Figure 3.4.5 RAGE staining in paraffin-embedded human lung resection tissue. A) Isotype control (mouse IgG2a, 10 μg/mL). B) Staining obtained with a mouse monoclonal anti-RAGE antibody (Millipore, clone DD/A11, 10 μg/mL). The arrow points to positive staining in bronchial epithelium and the arrow head indicates enhanced RAGE staining in areas of epithelium that appear metaplastic. C) RAGE staining in the endothelium of small vessel walls (open arrow head) and in inflammatory cells (asterisk). This staining pattern is consistent with what previously described [145]. One of two donors displaying similar staining is shown at 200x magnification.
3. HMGB1 and RAGE ex vivo characterisation

Isotype control  
RAGE

Score 0

Score 1

Score 2
Figure 3.4.6 Semi-quantitative scoring system for RAGE expression analysis in IHC. 200x magnification examples of isotype control (mIgG2a) and RAGE staining (clone DD/A11, indicated by black arrows) in GMA-embedded bronchial tissue are shown for each scoring value: 0 = no positive staining; 1 = little positive staining; 2 = moderate positive staining; 3 = marked positive staining. Marked staining (score 3) was not found in any of the severe asthmatics. The images shown for score 3 are of a GINA 2 subject (Epi = epithelium; SM = smooth muscle).
Figure 3.4.7 RAGE staining in GMA-embedded human bronchial tissue. A) Isotype control (mouse IgG2a, 10 μg/mL) at 200x magnification. B) A mouse monoclonal anti-RAGE antibody (Millipore, clone DD/A11, 10 μg/mL) was used to detect RAGE expression in bronchial tissue. RAGE staining (brown) was donor-dependent and could be observed in the airway smooth muscle (arrow) and the epithelium (arrow head). A severe asthmatic subject is shown at x200 magnification. RAGE staining was assessed in the ASM (C) and bronchial epithelium (D) using a semi-quantitative scoring system. There was no significant difference in RAGE expression between non-asthma (n = 10) and severe asthma (GINA 4-to-5, n = 19) in the ASM and the epithelium (Mann-Whitney tests p > 0.05). Median and interquartile range are shown.
3.5 Conclusions

This Chapter aimed to test the hypothesis that RAGE and HMGB1 expression are increased in sputum and bronchial tissue in asthma compared to health, in relation to asthma severity and inflammation. HMGB1 was found to be elevated in the sputa of moderate-to-severe asthmatics compared with controls and mild asthmatics. This partly agrees with reports that have been published concomitantly with this work [250,251], although not with others [252]. We have furthered these observations to confirm increased HMGB1 in sputa by Western blotting and in ASM in bronchial biopsies by immunohistochemistry.

HMGB1 redox state determines HMGB1 activity, with reduced (all-thiol) HMGB1 promoting RAGE-dependent cell migration and disulphide-HMGB1 causing the upregulation of pro-inflammatory cytokines via TLR4 [87]. Although other studies show HMGB1 to be elevated in the airways in asthma, the redox state of HMGB1 released in the airways has not been studied before; therefore, another aim of this Chapter was to investigate the redox state of HMGB1 released in the airways. This was investigated in sputum by Western blotting under non-reducing conditions. Despite the airways being an oxidative environment, both reduced and oxidised forms of HMGB1 were detected in a donor-dependent manner. Both forms were significantly elevated in asthma; in particular the reduced form was more represented in moderate-to-severe asthma than in health or mild asthma, which implicates a role for this form in inflammatory cell recruitment to the airways in asthma. In support of this, a positive correlation was found between airway HMGB1 levels and sputum total cell count, and reduced HMGB1 was shown to support peripheral blood leukocyte chemotaxis in vitro. Recruited
inflammatory cells may release more HMGB1 in the airways and so generate a positive feedback loop. The upregulation of oxidised HMGB1 in the sputum of asthmatics, which likely includes disulphide-HMGB1, also suggests that pro-inflammatory processes in the asthmatic airways are supported by HMGB1. Together, these data indicate HMGB1 as a potential marker of airway inflammation in moderate-to-severe asthma.

HMGB1 expression was also upregulated in bronchial ASM but not in bronchial epithelium in severe asthmatics. Interestingly, this was negatively correlated with mast cell numbers in the lamina propria and the smooth muscle, and negatively with the number of eosinophils in the lamina propria. The implications of these correlations are presently unclear.

RAGE is one of the main receptors for HMGB1; it is abundantly expressed in the lung [191], and is found in alveolar macrophages, alveolar epithelial cells, and bronchial epithelium [145,243,244]. RAGE is upregulated in chronic inflammation and in the airways of COPD subjects [98,145,243]. Here, RAGE expression was investigated in bronchial tissue from severe asthmatics and no difference was found between these and controls. However, GMA-embedded tissue did not seem to be ideal for RAGE detection, and only allowed semi-quantification of RAGE expression. Soluble RAGE forms including esRAGE are thought to act as decoy receptors for RAGE ligands and local and systemic sRAGE levels may reflect inflammatory processes. Evidence suggests that esRAGE is the main form of sRAGE present in the airways and in the circulation [252]. In the present study, levels of sputum esRAGE were measured and found to be similar in health and asthma. This is in contrast to previous reports showing significantly higher sputum esRAGE levels in asthma, regardless of severity [250].
However, in a separate study sRAGE was not different in bronchoalveolar lavage of non-neutrophilic asthmatics versus controls [252]. These discrepancies may be due to differences in patient populations and/or sampling methods.

In conclusion, HMGB1 was shown to be upregulated in the airways of moderate-to-severe asthmatics compared to healthy controls, thus providing evidence for a potential role for HMGB1 in the pathophysiology of asthma. HMGB1 may contribute to maintain a pro-inflammatory environment in asthma, by recruiting leukocytes to the airways and causing their activation. In addition, HMGB1 could potentially influence ASM behaviour in asthma, which is addressed in Chapter 5. Neither bronchial RAGE nor sputum esRAGE expression differed in asthma compared with health. These results do not support a role for differential RAGE expression in the pathophysiology of asthma; however, technical challenges in detecting RAGE in tissue were encountered and should be taken into account. Neither HMGB1 nor RAGE were shown to be differentially expressed in the epithelium in bronchial biopsies from asthmatics compared to healthy controls; however, the increased expression of HMGB1 in sputa from moderate-to-severe asthmatics compared to both mild asthmatics and healthy controls supports a potential role for HMGB1 in regulating epithelial cell behaviour in asthma; this will be addressed in Chapter 5.
Chapter 4

In vitro characterisation of HMGB1 and RAGE expression in human airway smooth muscle cells in health and asthma
4.1 Introduction

The primary aim of this Chapter was to test the hypothesis that human primary airway smooth muscle (HASM) cells express cell-surface RAGE and HMGB1, before moving on to functional studies. The expression of other RAGE forms (i.e. soluble forms) was also investigated. The second aim was to test the hypothesis that HMGB1 can be upregulated in HASM cells exposed to a pro-inflammatory environment or to PAMPs, such as viral TLR agonists. Another aim was to investigate potential differences in HMGB1 and RAGE expression between health and asthma in vitro using HASM cells isolated from non-asthmatics and asthmatics. Therefore, the expression of HMGB1 and RAGE was characterised in cultured HASM cells using RT-PCR, flow cytometry and immunofluorescence. These experiments temporally preceded the ex vivo characterisation of RAGE and HMGB1; therefore, although differential HMGB1 expression was found in a subset of asthmatics in tissue (see previous Chapter), the asthmatics were not sub-divided according to severity in the present Chapter, also due to smaller n numbers used. However, the majority of the HASM donors were moderate-to-severe asthmatics. The clinical characteristics of HASM cell donors included in the expression and functional studies (presented in Chapter 5) are summarised in Table 4.1.1. Preliminary HMGB1 and RAGE expression data in human primary bronchial epithelial cells (HBECs) are also presented at the end of this Chapter.
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<td>ICS - μg of BDP equivalent</td>
<td>0 (0)</td>
<td>800 (800)</td>
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<tr>
<td>Pre-BD FEV\textsubscript{1} % predicted</td>
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<td>77 ± 3.2</td>
<td>p = 0.015</td>
</tr>
<tr>
<td>Pre-BD FEV\textsubscript{1}/FVC</td>
<td>79.3 ± 1.6</td>
<td>67.4 ± 2.1</td>
<td>p = 0.0016</td>
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</table>

**Table 4.1.1** Subject demographics for HMGB1/RAGE *in vitro* studies. Results are expressed as mean ± SEM or median (interquartile range). ICS = inhaled corticosteroids; BDP = Beclometasone dipropionate; FEV\textsubscript{1} = forced expiratory volume in 1 second; FVC = forced vital capacity; pre-BD = pre-bronchodilator.
4.2 HMGB1 expression in HASM cells in health and asthma

4.2.1 HMGB1 mRNA expression in HASM cells

Levels of HMGB1 mRNA expression in HASM cells were investigated using quantitative RT-PCR. Primers specific for HMGB1 amplification were designed by Primerdesign at positions 3255 (forward primer) and 3342 (reverse primer) of the HMGB1 gene (accession # NM_002128), amplifying an 88-bp product. The efficiency of the qPCR was tested using increasing concentrations of cDNA isolated from HASM cells. The reaction was highly efficient, with a mean ± SEM efficiency of 97.3 ± 0.9 % (n = 4, figure 4.2.1A). The qPCR product melting profile showed that a single sequence was amplified (figure 4.2.1B) and this was confirmed by agarose gel electrophoresis, revealing a single band of the expected size (figure 4.2.2A). The purified qPCR product was also sequenced and found to be > 90 % homologous to the human HMGB1 transcript (accession # NM_002128.4, figure 4.2.2).

HMGB1 is a DAMP that may be upregulated during cellular stress. Since serum starvation may constitute a stress for the cells and functional experiments usually require a serum-free environment, the effect of serum starvation on HASM cell HMGB1 mRNA expression was tested first. Cells were grown in 10 % FBS medium or medium with 1 % ITS supplement for 72 h and RNA was extracted for qPCR analysis. The 18S rRNA was used in the qPCR as an internal reference to normalise the amount of HMGB1 transcript in each sample. A melting profile for the 18S rRNA qPCR product is shown in the Methods (Chapter 2, figure 2.10.1). Results from six donors are shown in figure 4.2.3 as ΔCt values (HMGB1 Ct – 18S rRNA Ct) and HMGB1 mRNA relative
quantity (i.e. relative to an arbitrary calibrator, which was a serum-starved normal donor). There was no significant difference in HMGB1 mRNA expression between cells grown in the presence of serum and cells that had been serum-starved in 1 % ITS medium for 72 h (paired t tests, p > 0.05, n = 6, figure 4.2.3).

Basal levels of HMGB1 expression in HASM cells isolated from seven non-asthmatic controls and nine asthmatic subjects were then quantified relative to a calibrator (non-asthmatic control) in serum-starved unstimulated cells. HMGB1 mRNA expression was relatively low: Ct values were typically around 27 for HMGB1 compared with Ct values of around 8 for 18S rRNA from 10 ng of starting cDNA. The relative HMGB1 mRNA quantity in non-asthmatic controls was 0.8 ± 0.09 (mean ± SEM, n = 7) and 0.6 ± 0.07 (n = 9) in asthmatics (GINA 1-to-5). The modest reduction in HMGB1 mRNA expression in HASM cells isolated from asthmatics did not reach statistical significance (unpaired t test p = 0.10, figure 4.2.4C).
Figure 4.2.1 Optimisation of HMGB1 qPCR. A) Efficiency curves were constructed by measuring HMGB1 mRNA levels in increasing amounts of HASM cells cDNA. The mean ± SEM Ct values are shown for $n = 4$-independent experiments performed in triplicates. The mean ± SEM efficiency from the $n = 4$ experiments is reported. B) Representative – $dF/dT$ curves, derived from melting curves of HMGB1 qPCR products, showing a single peak with a melting temperature of 77°C.
Figure 4.2.2 Purification and sequencing of the HMGB1 qPCR product. A) HMGB1 qPCR products (n = 2) were allowed to migrate by electrophoresis in a 1.7 % agarose gel. A single band of approximately 90-bp is visible after purification (lanes 2 and 5) and lower molecular weight bands are visible in the non-purified samples (lanes 1 and 4) including the negative controls (non-transcribed RNA, lanes 3 and 6). M = molecular weight marker. B-C) Nucleotide alignment of the qPCR product with the HMGB1 sequence (accession # NM_002128.4) using the forward primer (B) and the reverse primer (C).
Figure 4.2.3 Serum starvation does not affect HMGB1 mRNA expression. HMGB1 mRNA expression was measured by qPCR in HASM cells (P2 – P5) grown in 10 % FBS medium and serum-starved in 1 % ITS-containing medium for 72 h. cDNA from n = 6 donors was assayed in triplicates. A) ΔCt values, normalised to 18S rRNA, were 20.3 ± 0.1 in FBS-grown cells and 20.5 ± 0.3 in serum-starved cells (mean ± SEM, n = 6, paired t test p = 0.24). B) HMGB1 mRNA quantities relative to a calibrator were 0.8 ± 0.05 in FBS-grown cells and 0.7 ± 0.11 in serum-starved cells (mean ± SEM, n = 6, paired t test p = 0.38).
Figure 4.2.4 HMGB1 mRNA expression is not different between health and asthma. Representative fluorescence intensity curves of HMGB1 transcript amplification are shown for a non-asthmatic (A) and an asthmatic subject (B). The dashed line marks the fluorescence intensity threshold (0.005) used to derive Ct values. C) HMGB1 mRNA expression was not different between HASM cells isolated from controls (0.8 ± 0.09, n = 7) and cells isolated from asthmatics (0.6 ± 0.07, n = 9, unpaired t test p = 0.10). Data are presented as mean ± SEM.
4.2.2 HMGB1 protein expression in HASM cells

HMGB1 protein expression was characterised in HASM cells (P2 – P5) using flow cytometry and immunofluorescence. HMGB1 is a nuclear protein that can translocate to the cytoplasm in the presence of an appropriate stimulus and be secreted as a DAMP; therefore intracellular HMGB1 expression was measured in permeabilised HASM cells and compared to cell-surface expression in non-permeabilised cells.

HASM cells were serum-starved for 72 h in 1 % ITS supplemented medium, then fixed in 4 % PFA or fixed and permeabilised with 4 % PFA plus 0.1 % saponin. Cells were labelled with HMGB1-specific antibodies followed by appropriate secondary fluorescent antibodies. The specificity of the primary antibodies was first tested by western blotting (shown in Chapter 3, figure 3.2.3). The rabbit monoclonal antibody (Abcam®, clone EPR3507) was used in flow cytometry and the mouse monoclonal antibody (Abnova, clone 2F6) in immunofluorescence, because the rabbit antibody did not yield a satisfactory staining with the latter technique.

HMGB1 protein expression was investigated by flow cytometry in permeabilised and non-permeabilised HASM cells isolated from five donors. Most of the HMGB1 was intracellular, with 62.6 ± 4.8 % of permeabilised HASM cells expressing HMGB1 compared with 12.2 ± 2.9 % in non-permeabilised cells (mean ± SEM, paired t test p = 0.0008, figure 4.2.5). This small proportion of cells expressing HMGB1 at the cell surface may represent cells undergoing secondary necrosis that are leaking HMGB1.

As a DAMP, HMGB1 can be upregulated and/or released during cellular stress. Serum starvation did not change HMGB1 mRNA expression, indicating that this condition does not negatively affect HASM cells (figure 4.2.3). To confirm this, intracellular
HMGB1 protein expression was also investigated by flow cytometry. HASM cells were cultured for 72 h in medium containing 10 % FBS or 1 % ITS supplement and HMGB1 expression was measured in permeabilised cells. Serum starvation had no significant effect on intracellular HMGB1 expression, since 63.8 ± 8.06 % of serum-fed HASM cells expressed HMGB1 compared with 57.4 ± 7.1 % of serum-starved cells (mean ± SEM, n = 5, paired t test p = 0.057, figure 4.2.6).

HMGB1 is a nuclear protein; therefore the localisation of HMGB1 in unstimulated serum-starved cells was investigated next using immunofluorescence. HASM cells were seeded at a subconfluent density on glass chamber slides, fixed and permeabilised in 4 % PFA plus 0.1 % saponin. HMGB1 protein expression was probed with the mouse monoclonal antibody followed by a FITC-conjugated rabbit anti-mouse antibody (green fluorescence). Cells were counterstained with the nuclear dye DAPI (blue fluorescence). As expected, HMGB1 localised to the nuclei of most HASM cells. Figure 4.2.7 shows an example of HMGB1 expression in a non-asthmatic donor.
Figure 4.2.5 HMGB1 expression is predominantly intracellular in HASM cells. Cells were fixed with 4 % PFA in the absence or presence of 0.1 % saponin and HMGB1 expression was analysed by flow cytometry using the rabbit monoclonal antibody (clone EPR3507, 1:10) and a sheep anti-rabbit RPE antibody (1:10). The isotype control was rabbit immunoglobulin fraction (200 µg/mL). A) Representative histogram showing cell-surface HMGB1 expression in non-permeabilised HASM cells. B) Total HMGB1 expression in permeabilised HASM cells. C) The percentage of HMGB1-positive cells was calculated using the Overton subtraction method (see Chapter 2, section 2.13.2). Significantly more HASM cells expressed intracellular HMGB1 plus membrane-bound HMGB1 than cell-surface HMGB1 only (62.6 ± 4.8 % versus 12.2 ± 2.9 %, mean ± SEM, n = 5, paired t test p = 0.0008).
Figure 4.2.6 Serum starvation has no effect on HMGB1 protein expression. Serum starvation caused no significant change in intracellular HMGB1 protein expression in HASM cells. Cells were cultured for 72 h in 10 % FBS or 1 % ITS medium and HMGB1 expression was measured by flow cytometry. The percentage of HMGB1-positive cells was calculated using the Overton subtraction method (see Chapter 2, section 2.13.2) and it was 63.8 ± 8.06 in 10 % FBS and 57.4 ± 7.1 % in serum-starved cells (mean ± SEM, n = 5, paired t test p = 0.057).
Figure 4.2.7 HMGB1 localises to the nuclei of HASM cells. Left panels: HMGB1 was labelled with the mouse monoclonal antibody (Abnova, clone 2F6, 10 µg/mL) and a rabbit anti-mouse FITC-conjugated antibody (1:20, green). The panels on the right shows staining for the isotype control (mouse IgG2a, 10 µg/mL). DAPI was used to identify cell nuclei (blue). The images shown are representative of n = 11 donors.
4.2.3 HMGB1 protein expression is reduced in HASM cells from asthmatics

Levels of intracellular HMGB1 expression were then investigated in HASM cells isolated from non-asthmatics and asthmatics using flow cytometry. HASM cells were serum starved in ITS-containing medium for 72 h, then fixed and permeabilised. HMGB1 expression was probed as before using the rabbit monoclonal anti-HMGB1 antibody, clone EPR3507. The percentage of HASM cells expressing intracellular HMGB1 was significantly lower in HASM cells isolated from asthmatics compared with cells isolated from healthy controls (52.1 ± 2.0 %, n = 13 versus 62.6 ± 4.3 %, n = 10, mean ± SEM, unpaired t test p = 0.026, figure 4.2.8). This finding was confirmed using immunofluorescence. HASM cells were grown to subconfluent density on chamber slides, serum-starved in ITS-containing medium, fixed and permeabilised with 4 % PFA plus 0.1 % saponin. Cells were labelled with the mouse monoclonal anti-HMGB1 antibody (clone 2F6, 10 µg/mL) and a secondary anti-mouse Alexa Fluor® 488 antibody (1:200, green fluorescence). The isotype control was mouse IgG2a (10 µg/mL) and showed no staining at the exposure used (not shown). Cells were counterstained with DAPI to identify the nuclei (blue fluorescence). Images of at least five different fields per donor were captured using fluorescent microscopy. HMGB1-positive cells (green) were counted and expressed as a percentage of the total cell count, which was obtained from the nuclear staining (blue, figure 4.2.9). A smaller percentage of HASM cells isolated from asthmatics expressed HMGB1 (70.0 ± 5.3 %, n = 4) compared with cells isolated from non-asthmatics (92.3 ± 4.5 %, n = 3, mean ± SEM, unpaired t test p = 0.029).
Figure 4.2.8 HASM cells express less HMGB1 in asthmatics (flow cytometry). HMGB1 protein expression was reduced in HASM cells isolated from asthmatics. Cells were fixed and permeabilised and HMGB1 expression was measured by flow cytometry. Representative histograms of a non-asthmatic and an asthmatic are shown in A and B, respectively. C) HMGB1-positive cells were calculated using the Overton subtraction method (see Chapter 2, section 2.13.2) and were 62.6 ± 4.3 % in non-asthmatics (n = 10) and 52.1 ± 2.0 % in asthmatics (n = 13, unpaired t test, p = 0.026). Data are presented as mean ± SEM.
Figure 4.2.9 HASM cells express less HMGB1 in asthmatics (IF). A) Immunofluorescence staining of HMGB1 (green) in HASM cells isolated from a non-asthmatic (left) and an asthmatic donor (right). Cells were counterstained with DAPI to identify the nuclei (blue). Overlays are shown (merged). B) HMGB1 expression was lower in HASM cells isolated from asthmatics (70.0 ± 5.3 %, n = 4) compared with non-asthmatics (92.3 ± 4.5 %, n = 3, unpaired t test p = 0.029). Results are presented as mean ± SEM.
4. HMGB1 and RAGE in vitro characterisation

A

Non-asthmatic

Asthmatic

DAPI

HMGB1

Merged

B

p = 0.029

% HMGB1+ve cells

Non-asthma

Asthma
4.2.4 HMGB1 expression during inflammation and PRR activation

Next, in order to test the hypothesis that HMGB1 is upregulated in stressed HASM cells, HMGB1 expression was investigated in HASM cells that had been exposed to a pro-inflammatory environment typical of asthma. HASM cells were stimulated for 4 – 16 h with a combination of pro-inflammatory cytokines (10 ng/mL of TNFα, IL-1β and IFNγ) and HMGB1 expression was measured by qPCR and flow cytometry. There was no significant upregulation of HMGB1 mRNA after 4 – 6 h stimulation (one sample t test versus a theoretical value of 1, n = 4 to 5 donors, figure 4.2.10A). An upregulation of IL-8 mRNA (positive control, accession # NM_000584) was instead observed (figure 4.2.10B).

However, HMGB1 protein levels were increased after 16 h stimulation with 10 ng/mL of TNFα, IL-1β and IFNγ in all the donors tested, regardless of their phenotype (figure 4.2.11). HMGB1 expression was measured by flow cytometry in permeabilised HASM cells using the rabbit monoclonal antibody (clone EPR3507). The increase in % HMGB1-positive cells following cytokine stimulation was 9.7 ± 2.2 (mean ± SEM, n = 13, paired t test versus unstimulated, p = 0.0007, figure 4.2.11E). HMGB1 upregulation was similar in non-asthmatics and asthmatics (the increase in % positive cells was 6.9 ± 3.6 in non-asthmatics, n = 6, and 12.1 ± 2.5 in asthmatics, n = 7, unpaired t test p = 0.24, figure 4.2.11F).

Polyinosinic-polycytidylic acid (poly (I:C)) is a synthetic analogue of double-stranded RNA, a molecular pattern associated with viral infection, that activates TLR3 [261]. Previous work in the group has shown a ~1.8 fold upregulation of HMGB1 mRNA following poly(I:C) stimulation in ASM from both non-asthmatics and asthmatics.
(personal communication, Dr Amanda Sutcliffe); therefore, the effect of poly(I:C) stimulation on HMGB1 protein expression was investigated next. Poly(I:C) stimulation (12.5 µg/mL for 16 h) caused an upregulation of HMGB1 protein expression in most donors; however, the mean increase in % HMGB1-positive cells did not reach statistical significance (7.1 ± 3.6 increase, mean ± SEM, n = 11, paired t test versus unstimulated, p = 0.072, figure 4.2.11E).
Figure 4.2.10 Cytokine stimulation does not change HMGB1 mRNA expression. A) HMGB1 mRNA levels were not increased in HASM cells stimulated with a combination of the pro-inflammatory cytokines TNFα, IL-1β and IFNγ (10 ng/mL) for 4 – 16 h. The mean HMGB1 mRNA fold change compared with unstimulated cells was not significantly different from 1.00 between 4 and 6 h (p > 0.05). B) Conversely, IL-8 mRNA was upregulated following stimulation with TNFα, IL-1β and IFNγ (10 ng/mL).
Figure 4.2.11 HMGB1 protein is upregulated in a pro-inflammatory environment. HASM cells were stimulated with cytokines (TNFα, IL-1β and IFNγ, 10 ng/mL) or poly(I:C) (12.5 µg/mL) for 16 h and HMGB1 expression was measured by flow cytometry in permeabilised cells using the rabbit monoclonal antibody (clone EPR3507). **A-B)** Representative histograms comparing fluorescence shifts in the isotype control (A) and the HMGB1-labelled populations (B) in unstimulated and cytokine-stimulated HASM cells. **C-D)** Representative histograms comparing fluorescence shifts in the isotype control (C) and the HMGB1-labelled populations (D) in unstimulated and poly(I:C)-stimulated HASM cells. **E** Cytokine stimulation caused a significant increase in intracellular HMGB1 expression. The increase in % HMGB1-positive cells was 9.7 ± 2.2 (mean ± SEM, n = 13, paired t test versus unstimulated, p = 0.0007); whereas the change in intracellular HMGB1 expression following poly(I:C) stimulation was not significant (7.1 ± 3.6 increase, mean ± SEM, n = 11, paired t test versus unstimulated, p = 0.072). **F** There was no difference in the increase in cytokine-induced HMGB1 expression between non-asthmatics and asthmatics (the increase in % positive cells was 6.9 ± 3.6 in non-asthmatics, n = 6, and 12.1 ± 2.5 in asthmatics, n = 7, unpaired t test p = 0.24).
4. HMGB1 and RAGE in vitro characterisation

A

B

C

D

E

F

\[ p = 0.0007 \quad p = 0.072 \]

\[ p = 0.24 \]
4.3 RAGE expression in HASM cells in health and asthma

HMGB1 interacts with various PRRs, including TLR2, TLR4, TLR9 and RAGE. Ligand-receptor binding studies have found a higher HMGB1 binding affinity for RAGE ($K_d \approx 10$ nM [210]) than for TLR4 ($K_d = 1.5 \mu M$ [149]). Recently, it has been shown that HMGB1 pro-inflammatory properties and chemotactic functions are mutually exclusive, because they are initiated by different redox forms of HMGB1 and are mediated via TLR4 and RAGE, respectively. HASM cells express functional TLR4 [261], but although RAGE is abundantly expressed in the lungs [191], expression and function in some airway cell types such as ASM are less studied. Therefore RAGE expression was investigated in HASM cells using RT-PCR, flow cytometry and immunofluorescence.

4.3.1 HASM cells express various RAGE isoforms

Up to 22 different splice variants of RAGE have been described in human tissues [167], including a membrane-spanning form, which is the signalling receptor (FL-RAGE); various C-truncated forms, which result in the loss of the transmembrane and cytosolic domains and yield secreted proteins (sRAGE forms); an N-truncated form; two forms with variations in exon 4 encoding for the C1 Ig domain, which may affect ligand binding affinity; and several non-coding shorter variants [167]. FL-RAGE was the prevalent variant in all the tissues studied [165,167], and FL-RAGE and a C-truncated isoform (sRAGE) were the most abundant in foetal and adult lung [167,165]. The expression of RAGE splice variants in HASM cells is not known. Although the focus of this Thesis was to characterise FL-RAGE expression and function in airway structural
cells, the possibility that HASM cells express other RAGE isoforms was assessed first by RT-PCR. Since FL-RAGE and sRAGE variants are the main forms detected in the lung, the aim was to assess expression of these forms by amplifying the region of AGER encoding the transmembrane and cytosolic domains which undergoes alternatively splicing. This region, located between exon 6 and exon 11 of AGER (figure 4.3.1A), was therefore amplified using a previously described primer pair annealing to exon 6 (forward primer) and exon 11 (reverse primer) [266]. Using these primers, two to four bands were found in HASM cells isolated from non-asthmatic and asthmatic individuals. Two prominent bands were also detected in lung tissue, which was used as a positive control (figure 4.3.1B). The bands were excised, purified, and further characterised by sequencing: the upper band >700-bp was homologous to sRAGE3 (accession # AF537303); the band migrating at ~650-bp corresponded to sRAGE1 (accession # AF536236); the band below 600-bp was full-length RAGE (accession # M91211); and the lower band migrating above the 500-bp marker was sRAGE2 (accession # AF536237). Nucleotide sequences are reported for full-length RAGE and sRAGE3 in figures 4.3.2 and 4.3.3. All soluble RAGE isoforms contain stop codons resulting in the translation of shorter proteins that lack the cytosolic domain and are therefore secreted. The position of the bands in the gel is in agreement with the published data [266]. In contrast with this publication, however, only two forms were detected in the lung, RAGE and sRAGE2. These forms were also found in all the HASM cells analysed, whereas sRAGE1 and sRAGE3 were not observed in all the donors (figure 4.3.1B).
Figure 4.3.1 HASM cells express various RAGE splice variants. **A)** Map of the RAGE protein and transcripts encoding the full-length receptor and the C-truncated splice variants (sRAGE). The dotted box indicates the extracellular part of the RAGE protein with the Ig-like domains; the diagonal striped box indicates the transmembrane domain; the horizontal striped box is the cytoplasmic domain. The primers used were positioned in exons 6 (RAGE up) and 11 (RAGE do). Grey boxes indicate exons; black boxes alternatively spliced intronic sequences. Large boxes indicate coding parts; small boxes noncoding parts. Start and stop codons are indicated by arrows (taken from [266]). **B)** Up to four RAGE mRNA transcripts were detected in HASM cells by RT-PCR followed by 1.5 % agarose gel electrophoresis (n = 5). Cells were obtained from two non-asthmatic donors (NA) and three asthmatic donors (A). Lung tissue was used as a positive control. **C)** 18S rRNA was the loading control. M is the molecular weight marker.
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A

B

C
Figure 4.3.2 FL-RAGE RT-PCR product sequence alignment. Nucleotide sequence alignment of the RAGE RT-PCR product corresponding to the full-length receptor (accession # M91211). Sequence homology was 98% with both the forward (A) and the reverse primers (B).
Figure 4.3.3 sRAGE3 RT-PCR product sequence alignment. Nucleotide sequence alignment of the RAGE RT-PCR product corresponding to soluble RAGE variant 3 (sRAGE3, accession # AF537303). Sequence homology was 99% with the forward primer (A) and 96% with the reverse primer (B).
4.3.2 RAGE quantitative RT-PCR

Transmembrane RAGE is the signalling form of the receptor; therefore the level of membrane-bound (full-length) RAGE transcript expression was investigated next in HASM cells. At the time when these experiments were conducted (2011) ten mRNA variants of the human RAGE gene were described on the National Centre for Biotechnology Information (NCBI, accession # NC_000006.11 on chromosome 6 [271]), of which one is a non-coding variant (v10). In order to design specific primers for full-length RAGE amplification, the ten transcript variants were aligned using the NCBI Basic Alignment Search Tool (BLAST) and a region homologous to membrane-bound isoforms only (transcript variants 1, 2 and 3) was identified (circled in figure 4.3.4A). Specific primers for the amplification of FL-RAGE were designed in this region by a company, Primerdesign, to amplify a 90-bp product from 1032-bp to 1121-bp of transcript variant 1 (accession # NM_001136). In BLAST, the amplification product was 100 % homologous to transcript variants 1, 2 and 3 (all membrane-bound forms) and transcript variant 10 (non-coding RNA, figure 4.3.4B). The efficiency and specificity of the primer pair were tested by qPCR using cDNA isolated from HASM cells and the human mast cell line HMC-1. In HASM cells the PCR efficiency was very high: 147 ± 24 % (mean ± SD, n = 2). In HMC-1 cells, the PCR efficiency was lower than in HASM cells, but it still exceeded the acceptable range of 90–110 % (mean ± SD, 111.4 ± 6.4 %, n = 2). Importantly, multiple peaks were observed in the melting temperature plots in all the experiments, indicating poor primer specificity or primer dimer formation (figure 4.3.5B). To further assess the specificity of the reaction, the qPCR product was resolved onto 1 % agarose gel which revealed a single band of approximately 100-bp
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(figure 4.3.5C). The qPCR product was then purified and sequenced in-house. The resulting nucleotide sequence was longer than the expected product (169-bp versus the expected 90-bp) and shared 72 % homology with RAGE transcript variants 4, 5, 6 and 9 and 52 % homology with transcript variant 8, which are all soluble forms. Therefore, probably due to the high homology between RAGE isoforms, the primer pair did not amplify membrane-bound RAGE transcripts specifically. Considering the technical challenges involved in designing specific primers for membrane-bound RAGE using SYBR® Green, the optimisation of the RAGE qPCR was not taken further.
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Figure 4.3.4 Primer design for membrane-bound RAGE amplification in qPCR. A) BLAST alignment of the ten known RAGE transcript variants with variant 1 (NM_001136, membrane-bound RAGE) as query. The region with highest homology among membrane-bound transcripts is circled. Primers were designed in this region by a company, Primerdesign. B) The region theoretically amplified by the designed primers (from 1032-bp to 1121-bp) was 100 % homologous to mRNA variants 1, 2 and 3 (all membrane-bound) and variant 10 (non-coding) in BLAST.
**Figure 4.3.5** Optimisation of FL-RAGE qPCR. **A)** Example of RAGE qPCR efficiency curve calculated in the HMC-1 cell line (mean ± SD of n = 1 performed in triplicate). The example shown is representative of n = 4 experiments. **B)** RAGE qPCR product melting profile (-dF/dT) showing a main peak and several secondary peaks. **C)** 1 % agarose gel showing the non-purified RAGE qPCR product (NP) and the purified product (P). M is the molecular weight marker. Sequencing indicated that the product made is not membrane-bound RAGE.
4.3.3 RAGE protein expression in HASM cells

In order to assess the specificity of anti-RAGE antibodies binding to the extracellular domain of RAGE, the full-length receptor was over-expressed in HEK293T cells and HASM cells by transient transfection with a human FL-RAGE plasmid. A green-fluorescent protein (GFP) plasmid was used as a control of transfection efficiency. After 48 h the majority of HEK293T and HASM cells displayed green fluorescence (figure 4.3.6A). Cells were then lysed in 1 % triton buffer and 0.6 µg of proteins resolved using SDS-PAGE. GFP-transfected cell lysate was used as a negative control for RAGE expression. Western blotting membranes were probed with a rabbit polyclonal anti-RAGE antibody (1.2 µg/mL) and a mouse monoclonal anti-RAGE antibody (clone A11, 0.2 µg/mL). Both antibodies detected a band with a molecular weight of 50 KDa in FL-RAGE-transfected HEK293T and HASM cells, but no band was detected in the GFP-transfected cells, confirming the specificity of both antibodies (figure 4.3.6B). Endogenous RAGE expression was also detected in GFP-transfected HEK293T and HASM cells when 30 µg of proteins were loaded. Two bands with molecular weights of approximately 50 and 43 KDa were detected using the mouse monoclonal antibody (0.4 µg/mL), corresponding to full-length RAGE and probably a soluble RAGE protein (figure 4.3.6C). The level of protein expression was higher in HEK293T cells and a longer exposure was needed for RAGE detection in HASM cell lysates.

Levels of RAGE protein expression were then evaluated in HASM cells by flow cytometry using both anti-RAGE antibodies. Cell-surface RAGE expression was investigated in non-permeabilised cells, whereas cells were permeabilised with 0.1 % saponin to assess cytoplasmic RAGE expression including soluble forms. Similar results
were observed with both antibodies, showing a significantly higher RAGE expression in permeabilised cells, which may indicate that the receptor is also expressed intracellularly. Using the mouse monoclonal antibody, 37.8 ± 4.0 % of non-permeabilised HASM cells expressed RAGE versus 83.5 ± 7.9 % of permeabilised HASM cells (mean ± SEM, n = 6, paired t test p = 0.006, **figure 4.3.7**). Similar results were obtained with the rabbit polyclonal anti-RAGE antibody: 20.2 ± 3.0 % of cells expressed cell-surface RAGE versus 65.7 ± 6.7 % expressing cell-surface and intracellular RAGE (mean ± SEM, n = 6, paired t test p = 0.0007, **figure 4.3.8**). Although the mouse monoclonal antibody was more sensitive in detecting RAGE compared with the rabbit polyclonal antibody, the relative proportions of RAGE-positive cells were similar (**figure 4.3.9**). The results shown in **figure 4.3.9A** were obtained using non-permeabilised HASM cells (cell-surface RAGE, n = 9) whereas expression in permeabilised cells is shown in **figure 4.3.9B** (n = 5).

Since RAGE is involved in damage and stress related responses, the effect of serum starvation on RAGE protein expression was also tested in HASM cells. Cells were cultured for 72 h in normal growth medium containing 10 % FBS or in the absence of serum but with 1 % ITS supplement, and the expression of cell-surface RAGE was evaluated by flow cytometry; 21.3 ± 9.5 % of HASM cells cultured in 10 % FBS expressed cell-surface RAGE compared with 29.5 ± 12.8 % of cells kept in 1 % ITS (mean ± SEM, n = 4, paired t test, p = 0.094, **figure 4.3.10**).

In order to visualise RAGE expression, subconfluent HASM cells were seeded on glass chamber slides and fixed with 4 % PFA or fixed and permeabilised with 4 % PFA plus 0.1 % saponin. Methanol fixation was also tried, but better results were obtained with PFA fixation. Both the rabbit polyclonal and the mouse monoclonal anti-RAGE
antibodies were tested at various concentrations (5 – 20 µg/mL) in immunofluorescence and similar results were obtained with both antibodies (n = 5 donors for the rabbit polyclonal antibody and n = 3 for the mouse monoclonal antibody). However, a better staining was observed with the mouse monoclonal anti-RAGE antibody (10 µg/mL) followed by a rabbit anti-mouse Alexa Fluor® 488 (1:200), which emits green fluorescence. The isotype control was mouse IgG2a (10 µg/mL, figure 4.3.11).
Figure 4.3.6 Assessment of anti-RAGE antibody specificity. **A)** Bright field and green fluorescence images of GFP-transfected HASM cells after 48 h in culture, suggesting a successful transfection for FL-RAGE too. **B)** Detection of over-expressed FL-RAGE protein in HASM and HEK293T cell lysates by Western blotting: 0.6 µg of protein were resolved and probed with a rabbit polyclonal anti-RAGE antibody (Millipore, catalogue number AB9714, 1.2 µg/mL) or a mouse monoclonal anti-RAGE antibody (Santa Cruz, clone A11, 0.2 µg/mL). The loading control β-actin was probed using an HRP-conjugated mouse monoclonal antibody (Santa Cruz, 10 ng/mL). Lanes are: 1 = FL-RAGE-transfected HASM cells; 2 = GFP-transfected HASM cells; 3 = GFP-transfected HEK293T cells; 4 = FL-RAGE-transfected HEK293T cells. GFP-transfected cells were used as negative controls for RAGE expression. **C)** Endogenous RAGE protein expression was also detected in GFP-transfected HASM and HEK293T cells when loading 30 µg of protein. Membranes were probed using the mouse monoclonal anti-RAGE antibody (0.4 µg/mL) followed by an HRP-conjugated anti-mouse antibody. Lane 1 = HASM cells; lane 2 = HEK293T cells.
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A

B

50 KDa
RAGE (rabbit polyclonal)

50 KDa
RAGE (mouse monoclonal)

40 KDa
β-actin

C

50 KDa
RAGE

40 KDa
sRAGE

1 2
Shorter exposure

50 KDa
RAGE

40 KDa
β-actin

1 2
Longer exposure
Figure 4.3.7 HASM cells express intracellular RAGE (monoclonal antibody). HASM cells were fixed with 4 % PFA or fixed and permeabilised with 4 % PFA plus 0.1 % saponin and RAGE was labelled using the mouse monoclonal antibody (clone A11, 12 µg/mL) followed by a rabbit anti-mouse FITC antibody (1:20, green fluorescence). The isotype control was mouse IgG2a (12 µg/mL). A) Cell-surface RAGE expression in non-permeabilised HASM cells. B) Total RAGE expression in permeabilised HASM cells. C) The percentage of RAGE-positive cells was calculated using the Overton subtraction method (see Chapter 2, section 2.13.2). A significantly higher percentage of cells expressed cell-surface and intracellular RAGE than cell-surface RAGE only (83.5 ± 7.9 % versus 37.8 ± 4.0 %, respectively, n = 6, paired t test p = 0.006).
HASM cells express intracellular RAGE (polyclonal antibody). HASM cells were fixed with 4% PFA or fixed and permeabilised with 4% PFA plus 0.1% saponin and RAGE was labelled using the rabbit polyclonal antibody (Millipore, catalogue number AB9714, 12 µg/mL) followed by a sheep anti-rabbit RPE antibody (1:10, red fluorescence). The isotype control was rabbit immunoglobulin fraction (12 µg/mL). A) Cell-surface RAGE expression in non-permeabilised HASM cells. B) Total RAGE expression in permeabilised HASM cells. C) The percentage of RAGE-positive cells was calculated using the Overton subtraction method (see Chapter 2, section 2.13.2). Significantly more cells expressed cell-surface and intracellular RAGE than cell-surface RAGE only (65.7 ± 6.7 % versus 20.2 ± 3.0 %, n = 6, paired t test p = 0.0007).
Figure 4.3.9 Comparison of anti-RAGE monoclonal and polyclonal antibodies. RAGE detection in HASM cells by flow cytometry using the rabbit polyclonal antibody (Millipore, catalogue number AB9714) and the mouse monoclonal antibody (Santa Cruz, clone A11). The mouse monoclonal antibody showed higher sensitivity for RAGE detection; however the rabbit polyclonal antibody gave proportionally similar results. 

A) Cell-surface RAGE (n = 9); B) total RAGE (n = 5).
Figure 4.3.10 Serum starvation does not affect cell-surface RAGE expression. HASM cells were cultured for 72 h in 10 % FBS or 1 % ITS medium and cell-surface RAGE expression was measured by flow cytometry using the rabbit polyclonal antibody (Millipore, catalogue number AB9714). The percentage of RAGE-positive cells was calculated using the Overton subtraction method (see Chapter 2, section 2.13.2). No significant difference was found between serum-starved cells and cells cultured in 10 % FBS: the mean RAGE-positive cell population ± SEM was 29.5 ± 12.8 % versus 21.3 ± 9.5 %, respectively (n = 4, paired t test p = 0.094).
Figure 4.3.11 RAGE expression visualised in HASM cells by immunofluorescence. HASM cells were fixed or fixed and permeabilised in 4 % PFA minus or plus 0.1 % saponin and labelled with a mouse monoclonal anti-RAGE antibody (Santa Cruz, clone A11) or mouse IgG2a (isotype control, both at 10 µg/mL). The secondary antibody was rabbit anti-mouse Alexa Fluor® 488 (1:200, green fluorescence). Cells were counterstained with DAPI (blue) to reveal the nuclei. The upper panels show RAGE staining in non-permeabilised and permeabilised cells. The isotype control is shown in the bottom panel. The images are representative of 3 independent experiments.
4.3.4 RAGE expression levels are comparable in HASM cells and HUVECs

Primary human endothelial cells (HUVECs) are known from the literature to express functional RAGE. For example, HMGB1 stimulation has been shown to cause RAGE-dependent NF-κB activation, TNFα secretion and RAGE upregulation in HUVECs [203]. As a comparison, RAGE expression was also investigated in HUVECs and expression levels were found to be similar to those observed in HASM cells (figure 4.3.12). Cell-surface RAGE expression was tested by flow cytometry using the rabbit polyclonal (Millipore, catalogue number AB9714) and the mouse monoclonal (Santa Cruz, clone A11) antibodies, with results similar to those found in HASM cells (figure 4.3.12A). RAGE immunofluorescence staining was also comparable to HASM cells in non-permeabilised and permeabilised HUVECs (figure 4.3.12B).
Figure 4.3.12 RAGE expression in HUVECs. HUVECs were labelled with the rabbit polyclonal (Millipore, catalogue number AB9714) or the mouse monoclonal (Santa Cruz, clone A11) anti-RAGE antibodies. Rabbit immunoglobulin fraction and mouse IgG2a were the respective isotype controls. Secondary antibodies were sheep anti-rabbit RPE and rabbit anti-mouse FITC (flow cytometry) and rabbit anti-mouse Alexa Fluor® 488 (immunofluorescence). A) 21 % of HUVECs expressed cell-surface RAGE using the rabbit polyclonal antibody, whereas 57 % of HUVECs were RAGE-positive using the mouse monoclonal antibody. B) Upper panel: immunofluorescence RAGE staining using the mouse monoclonal anti-RAGE antibody (clone A11) in non-permeabilised HUVECs and in permeabilised HUVECs. Bottom panel: isotype control. The images are representative of 2 independent experiments.
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A

![Graph showing cell counts for Rabbit RAGE and Mouse RAGE](image)

- Rabbit RAGE: 21% positive cells
- Mouse RAGE: 57% positive cells

B

![Images of non-permeabilised and permeabilised cells](image)
4.3.5 Cell-surface RAGE expression in HASM cells is not changed in asthma

RAGE expression is upregulated in chronic inflammation and RAGE-dependent signalling contributes to promote and maintain a pro-inflammatory environment [98]. Although the IHC data did not show a significant upregulation of RAGE expression in the ASM bundle of severe asthmatics (see Chapter 3, figure 3.4.6C), technical challenges due to the type of tissue embedding (GMA versus paraffin) impaired the sensitivity of the technique, only allowing a semi-quantitative analysis of RAGE expression in bronchial tissue. Overall, IHC may be less sensitive than other techniques such as flow cytometry; therefore RAGE expression was investigated next in HASM cells isolated from non-asthmatics and asthmatics using flow cytometry.

Only cell-surface RAGE expression was measured, because this is the signalling form of the receptor and the focus of this Thesis. No significant difference was found between asthmatics and non-asthmatic subjects using both the polyclonal and the monoclonal anti-RAGE antibodies. Using the rabbit polyclonal antibody (Millipore, catalogue number AB9714), the percentages of RAGE-positive cells were 16.7 ± 3.6 % in non-asthmatics (n = 11) and 30.6 ± 6.8 % in asthmatics (n = 11, mean ± SEM, unpaired t test p = 0.083, figure 4.3.13A). These findings were confirmed with the mouse monoclonal anti-RAGE antibody (Santa Cruz, clone A11). Cell-surface RAGE expression was probed in a smaller number of donors using this antibody, and similar percentages of RAGE-positive HASM cells were found in healthy controls (34.1 ± 7.3 %, n = 4) and asthmatic subjects (37.9 ± 4.7 %, n = 5, mean ± SEM, unpaired t test p = 0.67, figure 4.3.13B).
4. HMGB1 and RAGE *in vitro* characterisation

**Figure 4.3.13** Cell-surface RAGE expression is not different in health and asthma. Cell-surface RAGE expression was investigated in HASM cells isolated from non-asthmatics and asthmatic donors using flow cytometry. **A)** Cells were labelled with the rabbit polyclonal anti-RAGE antibody (Millipore, catalogue number AB9714, 12 µg/mL) followed by a sheep anti-rabbit RPE antibody (1:10). Using the Overton subtraction method (Chapter 2, section 2.13.2), 16.7 ± 3.6 % of cells expressed cell-surface RAGE in non-asthmatic controls (n = 11) and 30.6 ± 6.8 % in asthmatics (n = 11, unpaired t test p = 0.083). **B)** The mouse monoclonal anti-RAGE antibody (Santa Cruz, clone A11, 12 µg/mL) was used to label HASM cells, followed by a rabbit anti-mouse FITC antibody (1:20). 34.1 ± 7.3 % of cells expressed cell-surface RAGE in healthy controls (n = 4) compared with 37.9 ± 4.7 % in asthmatics (n = 5, unpaired t test p = 0.67). Results are presented as mean ± SEM.
4. HMGB1 and RAGE expression in bronchial epithelial cells

The expression of HMGB1 and RAGE was then investigated in primary human bronchial epithelial cells (HBECs) in a small number of donors, due to the more limited availability of epithelial cells compared to ASM cells. Both HMGB1 and RAGE were constitutively expressed by HBECs and levels of expression were comparable in HASM cells and HBECs. HMGB1 mRNA expression was quantified using qPCR and normalised to 18S rRNA. Relative levels of expression were $0.5 \pm 0.08$ in HBECs (mean $\pm$ SEM, $n = 4$) and $0.75 \pm 0.3$ in HASM cells ($n = 4$, unpaired t test with Welch’s correction $p = 0.46$, figure 4.4.1A). 18S rRNA expression was not different in the two cell types (mean Ct values were $8.4 \pm 0.1$ in HBECs and $8.4 \pm 0.2$ and HASM cells). HMGB1 protein expression was also found to be similar in HBECs and HASM cells using Western blotting ($n = 3$ each, figure 4.4.1B).

RAGE expression in HBECs was evaluated by flow cytometry in two asthmatic donors using the mouse monoclonal anti-RAGE antibody (Santa Cruz, clone A11). Levels of RAGE expression were similar to those found in HASM cells, both in non-permeabilised cells (cell-surface expression) and in permeabilised cells. The percentage of cells that expressed cell-surface RAGE was 36 and 43 % for the two donors; and 89 and 87 % of permeabilised HBECs expressed RAGE (figure 4.4.1C-D).
4. HMGB1 and RAGE in vitro characterisation

Figure 4.4.1 HMGB1 and RAGE expression in HBECs. A) HMGB1 mRNA levels (normalised to 18S rRNA) were comparable in HBECs and HASM cells (mean ± SEM of n = 4 donors are shown, unpaired t test p = 0.46). B) HMGB1 protein expression was similar in HBECs and HASM cells (cells were lysed in 1x sample buffer and loaded onto 12 % SDS-polyacrylamide gels). Blots were probed with the rabbit monoclonal anti-HMGB1 antibody (Abcam®, clone EPR3507). C) HBECs were fixed in 4 % PFA and cell-surface RAGE expression was probed using the mouse monoclonal antibody (Santa Cruz, clone A11) by flow cytometry. Using the Overton subtraction method (Chapter 2, section 2.13.2), 36 % of cells were RAGE-positive. D) HBECs were fixed and permeabilised with 4 % PFA + 0.1 % saponin and RAGE expression was measured by flow cytometry. 89 % of permeabilised cells expressed RAGE.
4.5 Conclusions

This Chapter aimed to investigate levels of HMGB1 expression in HASM cells *in vitro* at baseline and under stressful conditions, which may cause HMGB1 upregulation, such as cytokine and PAMP stimulation. The data presented in this Chapter show that HASM cells express intracellular HMGB1 which localise to the nuclei, as expected from its role in chromatin organisation and regulation of transcription. Interestingly, there was a significant reduction in HMGB1 protein expression in HASM cells isolated from asthmatic subjects. This is contrasting with the elevation in HMGB1 expression found in the ASM bundle in bronchial tissue of severe asthmatics, a discrepancy that may be attributed to the separation of cultured HASM cells from their physiological environment. For example, ASM cells may express more HMGB1 in the presence of the inflammatory asthmatic environment, whereas cultured HASM cells from asthmatics may have a reduced HMGB1 synthetic capacity when isolated from their original inflammatory environment. Alternatively, it could indicate that HASM cells from asthmatics release HMGB1 constitutively *in vitro*. This is conceivable, since ASM cells from asthmatics can constitutively release more mediators than cells from non-asthmatics (for example CCL2 [71], and IL-6, unpublished data). HMGB1 could not be detected in the cell supernatants of healthy controls or asthmatics using ELISA, but this may be due to an insufficient sensitivity of the HMGB1 ELISA used. This question remains open; however, HMGB1 protein expression was upregulated when cells were exposed *in vitro* to a pro-inflammatory environment characteristic of asthma, indicating that HASM cells are able to respond to inflammatory stress by producing more HMGB1.
This Thesis focused on RAGE as one of the main receptors for HMGB1. One of the aims of this Chapter was to investigate whether HASM cells express the signalling form of RAGE (cell-surface RAGE), which may be involved in regulating HASM cell function. Cell-surface RAGE was found to be expressed in HASM cells. Moreover, permeabilised HASM cells showed increased RAGE expression compared to cell-surface RAGE expression, which could indicate that soluble forms of RAGE are also expressed. Levels of cell-surface RAGE expression were comparable in HUVECs, a primary endothelial cell type that is known to express functional RAGE [203]. Cell-surface RAGE expression was not different in HASM cells isolated from healthy subjects and asthmatic subjects, in agreement with the IHC data. These results do not support the hypothesis of RAGE playing a role in ASM dysfunction in asthma.

Preliminary data also showed HMGB1 and RAGE expression in HBECs, with baseline expression levels similar to those found in HASM cells, suggesting that HBECs may also be a source of HMGB1 and/or be capable of mounting a cellular response to it via RAGE activation.
Chapter 5

Investigating the effects of HMGB1 stimulation on HASM cell function
5.1 Introduction

In previous Chapters the expression of HMGB1 and its receptor RAGE was investigated ex vivo in human airways (i.e. in sputum and bronchial biopsies) in health and asthma and in vitro in human primary ASM cells, which are central to asthma pathophysiology \[58,75\]. Expression of HMGB1, but not RAGE was found to be increased in moderate-to-severe asthmatics in sputum and in severe asthmatics in bronchial smooth muscle. In contrast, HASM cells from asthmatics expressed less HMGB1 protein at baseline, but HMGB1 expression was upregulated following stimulation with cytokines, suggesting that a pro-inflammatory milieu typical of asthma can induce upregulation of expression and perhaps release of HMGB1 by ASM. Extracellular HMGB1 may act on ASM in a paracrine manner via PRRs such as RAGE. Cell-surface RAGE is the signalling form of RAGE and it was expressed by HASM cells, with no difference found in health compared to asthma. This prompted the question whether HMGB1 can influence ASM function in a RAGE-dependent manner, particularly in relation to ASM functions that are relevant to asthma, such as contraction and repair processes \[58,75\]. Therefore, the aim of this Chapter was to test the hypothesis that the HMGB1-RAGE axis can influence ASM functions such as contraction and migration. First, evidence of HMGB1-induced RAGE activation was sought by investigating known downstream events of RAGE signalling, such as RAGE upregulation, NF-κB activation, and ROS generation. Then the effects of HMGB1 stimulation were assessed on HASM cell contraction and migration. Since HMGB1 expression was found to be elevated in sputum of asthmatics, and HBECs expressed cell-surface RAGE, it is plausible to hypothesise that HMGB1 can also activate RAGE signalling in HBECs. Epithelial damage occurs in asthma due to
pathogen and allergen exposure [5] and HMGB1 is involved in re-epithelialisation [241]; therefore, HMGB1 could also influence bronchial epithelial repair capacities. This hypothesis was tested in HBECs in a wound healing assay.

5.2 Evidence of HMGB1-induced RAGE activation in HASM cells

5.2.1 HMGB1 stimulation does not change cell-surface RAGE expression

One of the effects of RAGE activation is the upregulation of the full-length receptor itself [98]. For example, 24 h stimulation with 50 ng/mL HMGB1 has been shown to cause the upregulation of cell-surface RAGE as well as adhesion molecules ICAM-1 and VCAM-1 in HUVECs [202]. This suggests that upregulation of cell-surface RAGE expression following exposure to RAGE ligands can be an indicator of RAGE activation. In order to test whether HMGB1 could induce the upregulation of RAGE expression in the ASM cellular model, HASM cells were stimulated with rhHMGB1 (30 and 100 ng/mL) in serum-free medium for 16 h. Cells were harvested and fixed in 4 % PFA and cell-surface RAGE expression was measured by flow cytometry using the rabbit polyclonal anti-RAGE antibody (Chapter 4, figure 4.3.6B). No difference was observed in cell-surface RAGE expression following rhHMGB1 stimulation. The percentage of RAGE-positive cells was 16.7 ± 4.4 (mean ± SEM) in unstimulated cells, 17.2 ± 4.0 and 17.2 ± 3.2 in rhHMGB1-stimulated cells (30 and 100 ng/mL, respectively, n = 5, repeated measure ANOVA p = 0.87, figure 5.2.1A). Since there was no difference between 30 and 100 ng/mL rhHMGB1, the effect of 100 ng/mL rhHMGB1 only was tested following longer incubation times (24 – 72 h). Similarly, no difference in cell-
surface RAGE expression was found at any time point analysed (figure 5.2.1B-D). The percentages of RAGE-positive cells (mean ± SEM) are summarised in table 5.2.1.
Figure 5.2.1 RAGE expression in HMGB1-stimulated HASM cells. rhHMGB1 (30 and 100 ng/mL) stimulation did not induce upregulation of cell-surface RAGE expression in HASM cells. Cells were stimulated for 16 h (A, n = 5), 24 h (B, n = 3), 48 h (C, n = 3) and 72 h (D, n = 3) in serum-free medium and cell-surface RAGE expression was measured by flow cytometry using the rabbit polyclonal antibody (Millipore, catalogue # AB9714). The percentages of RAGE-positive HASM cells shown were calculated using the Overton subtraction method (see Chapter 2, section 2.13.2). Data was analysed by repeated measure ANOVA (A) or paired t tests (B-D) for which p values are reported.
Table 5.2.1 RAGE expression in HMGB1-stimulated HASM cells. HASM cells were stimulated with rhHMGB1 (100 ng/mL) for 16 – 72 h and cell-surface RAGE expression was measured by flow cytometry using the rabbit polyclonal antibody (Millipore, catalogue # AB9714). Percentages of cell-surface RAGE-positive HASM cells were calculated using the Overton subtraction method (see Chapter 2, section 2.13.2) and are presented as mean ± SEM. Paired t test p values are reported.
5.2.2 Assessment of HMGB1-induced NF-κB activation in HASM cells

Although rhHMGB1 did not cause the upregulation of cell-surface RAGE expression in HASM cells, protein upregulation is a distant effect of RAGE signalling which may be influenced by other factors or it may not be induced in these cells. Two main flaws of the RAGE upregulation experiments were 1) there was no positive control to show that rhHMGB1 was active and 2) no positive control was used to show that cell-surface RAGE can be upregulated in HASM cells.

Therefore the activation of NF-κB was investigated next as a more general measure of RAGE activation that can lead to several downstream events. Various transcription factors are known to be induced by RAGE signalling including NF-κB and various studies have shown that HMGB1 is able to cause NF-κB activation. For example, 30 min stimulation with HMGB1 (1 μg/mL) induced nuclear translocation of the NF-κB subunit p65 in HUVECs [120]. In another study, RAGE-dependent p65 nuclear translocation was observed in HUVECs following 30 min stimulation with 60 ng/mL HMGB1 [203]. Therefore, NF-κB activation may be a more proximal and perhaps robust event following RAGE activation. Nuclear translocation of p65 was also assessed in the present study in HASM cells using immunofluorescence.

HASM cells were stimulated with TNFα (20 ng/mL) for 30 min as a positive control of p65 nuclear translocation and with rhHMGB1 (30, 300 and 1000 ng/mL) for a range of time points (0 – 120 min). Cells were then fixed and labelled with a rabbit monoclonal anti-p65 antibody (clone D14E12 from New England BioLabs®) followed by an Alexa Fluor® 488 goat anti-rabbit antibody (green fluorescence). Images of at least three fields per condition were captured and analysed using a fluorescent microscope. HASM...
cells displaying nuclear p65 staining were counted and normalised to the total cell number, corresponding to the DAPI nuclear staining. The positive control TNFα caused a clear translocation of p65, indicating NF-κB activation, in all the cells. Conversely, rhHMGB1 did not induce p65 nuclear translocation at any concentration or time point analysed (figures 5.2.2, n = at least 3).
Figure 5.2.2 HMGB1 does not induce p65 nuclear translocation in HASM cells. HASM cells were seeded onto chamber slides and stimulated with TNFα (20 ng/mL) for 30 min to induce p65 nuclear translocation or rhHMGB1 (30, 300 and 1000 ng/mL) for 5 – 120 min. Cells were fixed with methanol and labelled with a rabbit monoclonal anti-p65 antibody (clone D14E12, New England BioLabs®, at 1.88 μg/mL) or rabbit immunoglobulin fraction (isotype control, 1.88 μg/mL). p65 was visualised by immunofluorescence using an Alexa Fluor® 488 goat anti-rabbit secondary antibody (green). Cells were counterstained with DAPI (blue). A) Subcellular localisation of the NF-κB subunit p65 following 30 min stimulation with TNFα (20 ng/mL, positive control) and rhHMGB1 (30, 300 and 1000 ng/mL). The DTT control corresponds to rhHMGB1 30 ng/mL. No nuclear translocation was observed in the rhHMGB1-treated cells. The white arrow points to occasional p65 staining in the nucleus of an rhHMGB1-treated cell. Images are representative of 3 to 6 experiments. B-C) rhHMGB1 (300 and 1000 ng/mL, respectively) did not induce NF-κB p65 nuclear translocation in HASM cells over a period of 5 – 120 min. HASM cells displaying p65 nuclear staining were counted and normalised to the total cell number in each field, corresponding to the DAPI staining. Three to five fields per condition were analysed. Results are presented as mean ± SEM of n = 3 donors. Repeated measure ANOVA was performed to compare rhHMGB1-treated cells with unstimulated cells (p > 0.05).
5. HMGB1 effects of HASM cell function

A

B

C

\[
\text{% cells with nuclear pS5} \quad \text{p} = 0.38
\]

\[
\text{% cells with nuclear pS5} \quad \text{p} = 0.55
\]
5.2.3 HASM cells from asthmatics produce less ROS in response to HMGB1

Another signalling event that follows RAGE activation is ROS production [178]. HMGB1 has also been shown to cause ROS production in macrophages [272]. Therefore the production of intracellular ROS following rhHMGB1 stimulation was evaluated in HASM cells using a H$_2$DCFDA assay previously validated in our laboratories, which detects non-specific intracellular ROS. HASM cells (1x10$^4$ cells/well) were seeded in black 96-well plates with optical bottom and incubated with H$_2$DCFDA (10 µM) for 30 min. Stimuli were then applied in serum-free, phenol red-free high glucose medium and fluorescence was monitored between 0 and 120 min. Since the recombinant protein was supplied in a formulation containing DTT, appropriate concentrations of DTT were used as negative controls in the H$_2$DCFDA assay. DTT was noted to decrease ROS signal in a concentration-dependent manner: the mean % signal reductions compared with serum-free high glucose medium were 3.3 – 8.5 – 8.9 – 28.5 – 53.6 % following 120 min incubation with increasing DTT concentrations corresponding to 10 – 1000 ng/mL rhHMGB1 (n = 10). Relative fluorescence intensity (RFU) measurements following 120 min incubation with DTT are shown in figure 5.2.4A (repeated measure ANOVA p = 0.017). This finding was not surprising since DTT is a reducing agent. The effect of DTT on ROS signal was taken into account by normalising ROS production following rhHMGB1 stimulation to the respective DTT controls. rhHMGB1 (3 – 1000 ng/mL) induced different intracellular ROS production responses in non-asthmatic donors compared with asthmatics. In fact, rhHMGB1 caused a time and concentration-dependent increase in ROS production in HASM cells isolated from non-asthmatics (n = 5), but failed to induce significant ROS increase in cells isolated from asthmatics (n = 7).
Intracellular ROS production following 120 min incubation with rhHMGB1 is shown in **figure 5.2.4B** (non-asthmatics) and **5.2.4C** (asthmatics, one sample t test was performed to compare normalised values with a theoretical value of 100; p < 0.05 was considered significant). **Figure 5.2.4D** shows a comparison of intracellular ROS production in non-asthmatics and asthmatics following 120 min stimulation with rhHMGB1. The difference in rhHMGB1-induced ROS production between healthy and asthmatics was significant at 10, 30, 300 and 1000 ng/mL rhHMGB1 (unpaired t tests p < 0.05), although it did not reach significance at 100 ng/mL rhHMGB1 (**table 5.2.2**). The area under the curve (AUC) of the ROS dose-response following 120 min stimulation with rhHMGB1 was also significantly lower in asthmatics compared with non-asthmatics (matching concentrations between asthmatics and non-asthmatics were analysed i.e. between 10 and 300 ng/mL. The AUC was 292 ± 16.5, mean ± SEM, in asthmatics versus 351 ± 6.7 in non-asthmatics, unpaired t test p = 0.017, **figure 5.2.4E**). This difference was not due to impaired ROS generation capacity in the asthmatics, because baseline ROS signal was not different between groups (**figure 5.2.4F**). RFU were 551 ± 83 in healthy controls and 799 ± 236 in asthmatics (mean ± SEM, unpaired t test with Welch’s correction p = 0.35). In addition, in a previous study hydrogen peroxide has been shown to stimulate significantly higher ROS production in ASM cells from asthmatics versus non-asthmatics [76].
5. HMGB1 effects of HASM cell function

Figure 5.2.3 HMGB1 causes differential ROS production in ASM in health and asthma.

A) DTT (concentrations corresponding to rhHMGB1 10 – 1000 ng/mL) caused a concentration-dependent reduction in ROS signal. Mean ± SEM RFU after 120 min incubation are shown for n = 10 (repeated measure ANOVA p = 0.017). B) HASM cell intracellular ROS production was normalised to DTT controls; the graph shows the effect of 120 min stimulation with rhHMGB1 (3 – 1000 ng/mL) in healthy controls (n = 5, one sample t test against a theoretical value of 100, p values are reported). C) Effect of 120 min stimulation with rhHMGB1 on HASM cell ROS production in asthmatics (n = 7, one sample t test p > 0.05). D) Intracellular ROS production following 120 min stimulation with rhHMGB1 was compared in non-asthmatics and asthmatics. E) The AUC of HMGB1 (10 – 300 ng/mL)-induced ROS production at 120 min was significantly reduced in asthmatics versus non-asthmatics (unpaired t test p = 0.017). F) Baseline ROS production was not different in HASM cells from healthy and asthmatic subjects (unpaired t test p = 0.35). Results are presented as mean ± SEM.
5. HMGB1 effects of HASM cell function

A

B

C

D

E

F

\[ p = 0.017 \]

\[ p < 0.05 \]

\[ p = 0.06 \]

\[ p > 0.05 \]

\[ p = 0.017 \]

\[ p = 0.35 \]
Table 5.2.2 Comparison of ROS production in health and asthma. Mean % intracellular ROS production following 120 min stimulation with rhHMGB1 was compared in HASM cells from non-asthmatic and asthmatic donors using unpaired t tests. Differences between asthmatics and non-asthmatics were significant (p < 0.05) at all rhHMGB1 concentrations tested except for 100 ng/mL due to higher variability at this concentration. Data is presented as mean ± SEM.
5.3 Investigating HMGB1 effects on ASM contraction

5.3.1 HMGB1 is able to increase ASM contraction

A positive relationship has been found between ROS production and ASM contraction [76]. Since differential effects of rhHMGB1 stimulation were observed on ROS production in HASM cells from asthmatics versus non-asthmatics, the possibility that rhHMGB1 would also differentially affect ASM contraction was explored next. Using an established assay, HASM cells were embedded in pure bovine collagen gels in the absence or presence of rhHMGB1 (30 – 1000 ng/mL). Gels were allowed to set for 90 min and bradykinin (1 ng/mL) was added to induce contraction (this was considered time 0). Gel contraction was monitored by imaging each well at various time points between 0 and 60 min. Gel areas were measured using ImageJ software and expressed as percentages of the well area. DTT concentrations corresponding to 30 – 1000 ng/mL rhHMGB1 were used as negative controls. The AUC between 0 and 60 min was calculated for each concentration and rhHMGB1-AUC compared with DTT control-AUC (table 5.3.1 and table 5.3.2). No significant difference was observed between 30 and 300 ng/mL, but at 1000 ng/mL rhHMGB1 caused a significant increase in contraction compared with DTT control both in asthmatics and non-asthmatics (paired t tests p values are reported in table 5.3.1 and table 5.3.2). Collagen gel sizes for this concentration are shown in figure 5.3.1. Although the effect of rhHMGB1 on ASM contraction seemed greater in asthmatics (figure 5.3.1C), this difference was not significant (AUC reduction and % AUC reduction were not significantly different...
between asthmatics and non-asthmatics, unpaired t tests \( p = 0.10 \) and \( p = 0.052 \), respectively, figure 5.3.1D).
Figure 5.3.1 HMGB1 caused increased ASM collagen gel contraction. Bradykinin-mediated HASM cell contraction in collagen gels was monitored in response to rhHMGB1 (1000 ng/mL) for 0 – 60 min. Gel size was expressed as a percentage of the well area. A) Representative examples of collagen gels with no cells, HASM cells alone and in the presence of rhHMGB1 1000 ng/mL or corresponding DTT control (60 min). B) Gel contraction following incubation with rhHMGB1 (H) or DTT control (v) in HASM cells isolated from non-asthmatic donors (n = 5). C) Gel contraction was monitored in cells isolated from asthmatics (n = 4). D) No significant difference was observed between asthmatics (A) and non-asthmatics (NA) in the AUC reduction (DTT control minus HMGB1) or the % reduction in AUC over 60 min incubation with 1000 ng/mL (unpaired t tests p = 0.10 and p = 0.052, respectively).
### Table 5.3.1
ASM collagen gel contraction in non-asthmatics. HASM cells were stimulated with rhHMGB1 (30 – 1000 ng/mL) in collagen gels for 90 min before bradykinin addition (time 0) and gel contraction was monitored for 60 min following bradykinin addition. HASM cell contraction was expressed as AUC between 0 and 60 min. AUC for DTT control and rhHMGB1 were compared using paired t tests. rhHMGB1 caused a significant increase in contraction at 1000 ng/mL. Mean ± SEM AUC are reported.
### Table 5.3.2

<table>
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<th>[rhHMGB1]</th>
<th>DTT control</th>
<th>rhHMGB1</th>
<th>Paired t test p</th>
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<td>30 ng/mL</td>
<td>3739 ± 681</td>
<td>3753 ± 731</td>
<td>p = 0.9</td>
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<td>(n = 3)</td>
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<tr>
<td>100 ng/mL</td>
<td>3847 ± 809</td>
<td>3747 ± 804</td>
<td>p = 0.22</td>
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<tr>
<td>(n = 3)</td>
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<tr>
<td>300 ng/mL</td>
<td>3569 ± 701</td>
<td>3585 ± 743</td>
<td>p = 0.9</td>
</tr>
<tr>
<td>(n = 3)</td>
<td></td>
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<tr>
<td>1000 ng/mL</td>
<td>3615 ± 486</td>
<td>2940 ± 296</td>
<td>p = 0.04</td>
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<td>(n = 4)</td>
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**Table 5.3.2** ASM collagen gel contraction in asthmatics. HASM cells were stimulated with rhHMGB1 (30 – 1000 ng/mL) in collagen gels for 90 min before bradykinin addition (time 0) and gel contraction was monitored for 60 min following bradykinin addition. HASM cell contraction was expressed as AUC between 0 and 60 min. AUC for DTT control and rhHMGB1 were compared using paired t tests. rhHMGB1 caused a significant increase in contraction at 1000 ng/mL. Mean ± SEM AUC are reported.
5. HMGB1 effects of HASM cell function

5.4 HMGB1 effects on epithelial and ASM cell repair mechanisms

5.4.1 Investigating the effects of HMGB1 on epithelial wound repair

Damage of the airway epithelium, which can result in the release of DAMPs such as HMGB1, is observed in asthma [15]. The chemotactic form of HMGB1 (reduced i.e. all-thiol) is thought to be released first at the site of damage or stress [87] and promotes tissue repair by inducing cell migration and by recruiting progenitor cells in a RAGE-dependent manner [127,128]. Since this HMGB1 redox form was significantly upregulated in the sputum of moderate-to-severe asthmatics (Chapter 3, figure 3.2.6) and HBECs were found to express cell-surface RAGE (Chapter 4, figure 4.4.1), it was hypothesised that HMGB1 can induce RAGE-dependent repair mechanisms in HBECs. To test this, the effect of rhHMGB1 (reduced form containing DTT) was investigated in a HBEC wound healing assay. HBEC monolayers were wounded using a sterile pipette tip and washed to remove cell debris. Cells were incubated for 24 h in HBEC medium containing rhHMGB1 (100 – 1000 ng/mL) or corresponding DTT control and wound closure was assessed by measuring the wound area. Contrary to the hypothesis, rhHMGB1 had no effect on wound closure at 100 and 300 ng/mL (n = 5, paired t tests p = 0.09 and p = 0.6, respectively). However, at higher concentrations (1000 ng/mL) rhHMGB1 caused a significant inhibition of wound healing with 54 ± 8 % (mean ± SEM) of the wound healed in rhHMGB1-stimulated cells compared with 65 ± 9 % in DTT control-treated cells (n = 7, paired t test p = 0.015, figure 5.4.1). A slight increase in wound closure was observed with the negative control (v 1000) versus serum-free medium (59 ± 10 %, n = 7, paired t test p = 0.049).
5. HMGB1 effects of HASM cell function

Figure 5.4.1 HMGB1 inhibits bronchial epithelial wound healing. A) Representative images of HBEC wounds following 24 h treatment with rhHMGB1 (1 µg/mL) or corresponding DTT control. The light purple-shaded area represents the area of original wound at the 0 h time point. B) rhHMGB1 (H 1000 = 1 µg/mL) caused a significant inhibition of HBEC wound healing compared with DTT control (v 1000) after 24 h stimulation (n = 7, paired t test p = 0.015).
5.4.2 Evaluating the effects of HMGB1 on HASM cell migration

Increased ASM mass contributes to airway wall remodelling in asthma. This may be due to exaggerated tissue repair mechanisms in response to a chronic inflammatory environment typical of asthma [15]. Since reduced (i.e. chemotactic) HMGB1 was found to inhibit HBEC migration in a wound assay, the effects of HMGB1 on HASM cell migration were tested next.

The ASM is less likely to be wounded in a physiological setting, although it may still respond to migratory stimuli; therefore a migration assay that does not involve the creation of wounds was used (Oris™ cell migration assay). The main advantages of this assay are that cells are not damaged; there are fewer user-controlled variables like the width and position of the migration area and the mode of detection; it has a higher throughput; and data analysis is faster. Cell chemotaxis can also be measured using the Transwell® assay; however this assay works better with more motile cells such as leukocytes whereas results with HASM cells have been scarce, and previous studies within the group have been unable to obtain reproducible results studying ASM migration using this assay.

Using the Oris™ cell migration assay, cells were seeded in 96-well plates around stoppers in order to create a cell-free migration area in the centre of each well. Stoppers were then removed prior to the addition of stimuli to allow cell migration in the cell-free area. Cell migration was assessed by fluorescently labelling the cell nuclei with Hoechst and by counting the cells in the migration area.

As part of the assay validation an appropriate positive control of HASM cell migration was investigated first. HASM cells were incubated for 24 h with serum-free medium
(negative control), serum-containing medium (10 % FBS), PDGF (10 ng/mL) or TGF-β (10 ng/mL), which have all been shown to cause ASM cell migration [273]. HASM cells migrated similarly in serum-free medium and PDGF, whereas TGF-β and 10 % FBS induced the least and the highest migration, respectively (figure 5.4.2A-B, one experiment performed in quadruplicate). Therefore, 10 % FBS was chosen as a positive control of HASM cell migration to be used in subsequent experiments. Unfortunately, the positive effect of 10 % FBS on ASM migration was not consistently observed in all these experiments (figure 5.4.2C).
**Figure 5.4.2** Optimisation of the Oris™ migration assay in HASM cells. HASM cells were seeded around stoppers to create a cell-free migration area. Stoppers were removed prior to the addition of stimuli, except for reference wells, which were a time zero, no migration control. Cells were stimulated for 24 h and then fixed and stained with Hoechst. Images were captured at x40 magnification and cell nuclei in the detection area were counted. **A)** Representative images of a reference well, a well incubated with serum-free medium and one stimulated with 10 % FBS are shown. The white arrow points to non-specific staining that may be a confounding factor if automated detection methods (e.g. fluorescent plate reader) are used. **B)** Various stimuli of cell migration were tested in this experiment: 10 % FBS, PDGF (10 ng/mL) and TGF-β (10 ng/mL). Cells stimulated with 10 % FBS migrated more (n = 1 performed in quadruplicate; mean ± SD are shown). **C)** However, the effect of 10 % FBS was not consistently observed (n = 7, paired t test p = 0.31).
5. HMGB1 effects of HASM cell function

A

Reference well

Serum-free medium

10% FBS medium

B

# cells in detection area

C

# cells in detection area

p = 0.31

Rel wells
serum-free
10% FBS
PDGF 10 ng/mL
TGF β 10 ng/mL
5.4.3 HMGB1 effects on HASM cell migration are concentration-dependent

HASM cells were then stimulated with 3S-HMGB1, a mutated non-oxidisable form of HMGB1 that has been shown to promote leukocyte migration more effectively than recombinant HMGB1 [133]. Cells were incubated with 3S-HMGB1 (3 – 1000 ng/mL) or DTT control (3 – 1000 nM) for 24 h, fixed with 10 % neutral buffered formalin and stained with Hoechst. A mask was applied at the bottom of the plate and images were taken for each well at x40 magnification. Cells in the detection area were counted and 3S-HMGB1-induced cell migration was expressed as a percentage of the respective DTT control for n = 17 (3 ng/mL); n = 8 (10 ng/mL); n = 7 (30 ng/mL); n = 6 (300 ng/mL) and n = 8 (1 µg/mL) experiments. The DTT negative control (3 – 1000 nM) did not change HASM cell migration significantly compared with serum-free medium (paired t tests p > 0.2, figure 5.4.3A). Instead, trends for increased and decreased migration were observed at the lower and the higher concentrations, respectively (figures 5.4.4 and 5.4.5). These effects were significant at 3 ng/mL (one sample t test against a theoretical value of 100, p = 0.032) and at 1 µg/mL (p = 0.045, figure 5.4.3B). Statistical analysis of the raw data, however, only found a significant difference in the HMGB1-induced inhibition of migration at 1 µg/mL (paired t tests p = 0.10 and p = 0.033 respectively, figure 5.4.3C-D), indicating that the agonist effects seen at 3 ng/mL are small.
5. HMGB1 effects of HASM cell function

Figure 5.4.3 Concentration-dependent effects of 3S-HMGB1 on HASM cell migration.  

A) DTT (3 – 1000 nM, the negative control for 3S-HMGB1) did not significantly affect the migration of HASM cells (paired t test p values are reported). B) The number of migrated cells was normalised to the respective DTT controls. Small but significant effects were observed at 3 ng/mL and 1 µg/mL for increased and decreased cell migration, respectively (one sample t test p = 0.032 and 0.045). C) However, paired t test of the raw data did not find a significant difference in migration at 3 ng/mL (paired t test p = 0.10). D) Conversely, the inhibition of migration seen at 1 µg/mL was still significant when analysing the raw data (paired t test p = 0.033).
Figure 5.4.4 Effects of low concentrations of 3S-HMGB1 on HASM cell migration. HASM cells were stimulated with non-oxidisable HMGB1 (3S-HMGB1, 3 ng/mL) or 3 nM DTT (negative control) for 24 h and cell migration was assessed by counting the Hoechst-labelled cells in the detection area. Representative images are shown for a non-asthmatic control and an asthmatic subject.
Figure 5.4.5 Effects of high concentrations of 3S-HMGB1 on HASM cell migration. HASM cells were stimulated with 3S-HMGB1 (1 µg/mL) or 1 µM DTT (negative control) for 24 h and cell migration was assessed by counting the Hoechst-labelled cells in the detection area. Representative images are shown for a non-asthmatic control and an asthmatic subject.
5.5 Conclusions

This Chapter investigated potential roles of HMGB1 in the regulation of ASM functions that are relevant to asthma, such as exaggerated contraction and repair mechanisms [58,75], using in vitro models of human primary cells. The effect of HMGB1 on bronchial epithelial repair was also explored in preliminary experiments.

In Chapter 4 both HASM cells and HBECs were found to express cell-surface RAGE, one of the main receptors for HMGB1. RAGE signalling has been shown to induce downstream activation of the transcription factor NF-κB leading to the upregulation of cytokines and also of cell-surface RAGE itself [98]. Therefore, these events were investigated in HASM cells, as evidence of RAGE functionality and HMGB1 activity in these cells. Neither cell-surface RAGE upregulation nor NF-κB activation (p65 nuclear translocation) could be demonstrated following HMGB1 stimulation. It should be pointed out, however, that the rhHMGB1 used in these experiments contained DTT, a reducing agent that may affect HMGB1 ability to activate NF-κB signalling pathways. Indeed, it has been recently shown that disulphide-HMGB1 (partially oxidised) activates the NF-κB pathway via TLR4, whereas in the reduced form HMGB1 does not induce p65 nuclear translocation [133].

Activation of RAGE signalling is also known to cause increased production of intracellular ROS [178]. Interestingly, HMGB1 increased intracellular ROS production in HASM cells isolated from non-asthmatics but no significant effect was observed in cells isolated from asthmatics, suggesting disease-associated differences in ASM responses to HMGB1. Since a positive relationship has been described between ROS production and ASM contraction [76], it was intriguing to see whether HMGB1 was able to induce
ASM contraction differentially in health and asthma. HMGB1 stimulation did indeed cause an increased contraction in HASM cells, but no difference between healthy controls and asthmatics was observed, although contraction seemed greater in asthmatics. These data suggest that HMGB1 is able to induce ASM contraction, but the relationship with ROS production is not clear.

Damage of the airway epithelium occurs in asthma due to pathogen and allergen exposure [5], and impaired tissue repair responses, particularly in the airway epithelium, may be involved in asthma pathogenesis [15]. Exaggerated repair mechanisms may also contribute to ASM increase in asthma [58]. The HMGB1-RAGE axis promotes cell migration and is implicated in tissue repair [87,241]. Therefore, the ability of HMGB1 to promote HBEC and HASM cell repair processes was tested using wound healing and migration assays, respectively. HMGB1 was found to inhibit HBEC wound healing at 1 µg/mL, a concentration that is consistent with HMGB1 levels detected in the sputa of asthmatics. Similarly, in HASM cells 3S-HMGB1, a mutated non-oxidisable form of HMGB1, inhibited cell migration at 1 µg/mL; however, lower concentrations (3 ng/mL) showed some agonist effects. These results contrast with the knowledge of HMGB1 being a chemoattractant [87]; however HMGB1 chemotactic functions may be concentration and cell type-dependent.

Together these data suggest that HMGB1 promotes ASM contraction and impairs ASM and epithelial repair processes; therefore, its elevation in asthma may be relevant to asthma pathophysiology. It is possible that these functions are mediated by separate receptors i.e. RAGE or TLR4; however, the receptors involved in these functions have not been identified here and this remains an open question for future work.
Chapter 6

Discussion
6.1 Discussion

This Thesis addressed the potential role of HMGB1 and RAGE in the pathophysiology of asthma, which is suggested by a small number of studies (refer to table 3.1.1 and this section). The redox state of HMGB1 in the airways, which is crucial for its function, is not known in health or asthma, and little is known about the expression and function of HMGB1 and RAGE in airway mesenchymal cells. We hypothesised that HMGB1 levels and redox state would differ between health and asthma and that differences in airway/tissue expression of HMGB1 and RAGE would drive pathophysiological processes in asthma, in relation to inflammation and cell functions such as migration and contraction.

Asthma is a heterogeneous disease of the conducting airways characterised by variable and recurrent symptoms including breathlessness, wheeze, chest tightness and cough; variable and reversible expiratory airflow obstruction; and/or increased susceptibility of the airways to airborne irritants (airway hyperresponsiveness, AHR). Additionally, chronic inflammation and structural changes of the airway wall, named airway remodelling, are important features of asthma [5]. The airway smooth muscle (ASM) plays a central role in pathological airway wall remodelling processes and AHR, with increased mass and exaggerated reactions to contractile stimuli [61,73]. The ASM also participates in inflammation and tissue remodelling by secreting chemokines, cytokines, and matrix metalloproteinases, and by upregulating the expression of adhesion molecules in response to various stimuli including pathogens, environmental irritants, and inflammatory cytokines [77].
HMGB1 is a nuclear protein that is released as a damage-associated molecular pattern (DAMP) during cell damage and stress. HMGB1 signals through various pattern-recognition receptors (PRRs), including TLR4 and RAGE to promote inflammation and tissue repair following tissue damage and stress [87]. HMGB1 redox state critically determines binding to specific receptors [87,150]. RAGE is a multi-ligand receptor which undergoes extensive alternative splicing, yielding up to 22 splice variants in humans [165,167]. Full length, membrane-bound RAGE is the signalling receptor and is involved in pathological states [98]. Of interest are also the soluble forms of RAGE, which can derive from alternative splicing [165,167] or shedding of the protein extracellular domain [171], and are thought to act as decoy receptors for RAGE ligands [98]. HMGB1 and RAGE have been implicated in several pathologies including chronic airway disease [246]. Increased expression of HMGB1 and RAGE has been found in airway fluids and bronchial tissue of subjects with COPD [145,243,251]. HMGB1 has also been found to be increased in the peripheral blood of COPD subjects [251]. In contrast, lower levels of circulating sRAGE have been associated with worse lung function, exacerbations [98,248], and severity of emphysema [234,248,249] in COPD. HMGB1 and esRAGE (a secreted splice variant of RAGE) have been found to be increased in the sputum of asthmatics, and HMGB1 correlated with disease severity and sputum neutrophils [250]. Sputum and peripheral HMGB1 levels were also increased in another study, and correlated with disease severity, decline in lung function, and sputum neutrophils [251]. Another study found an association between lower levels of airway and circulating sRAGE and neutrophilic asthma and COPD [252]. The relevance of RAGE in respiratory disease is also supported by genetic studies, showing a relationship of RAGE polymorphism with lower lung function [229,230], and
with lower levels of circulating sRAGE [234]. Moreover, the G82S single nucleotide polymorphism of *AGER* has been found to be associated with lower FEV$_1$/FVC in children with early transient wheeze [231], and with an increased risk of developing COPD in smokers [232]. Furthermore, animal models of allergic asthma support a role for HMGB1, RAGE and TLR4 in the development of airway inflammation, remodelling and AHR in asthma [253,258,259].

Literature available at the time when this work was carried out recognised RAGE as the main receptor for HMGB1; therefore, this Thesis focused on RAGE rather than other HMGB1 receptors. We hypothesised that HMGB1 and RAGE expression would be increased in the asthmatic airways in relation to airway inflammation; that this change would be maintained in cultured airway mesenchymal cells; that HMGB1 would be upregulated and released by airway mesenchymal cells following stress; and that the HMGB1-RAGE axis would regulate airway mesenchymal cell functions, such as ASM contraction and repair mechanisms.

Since airway HMGB1 levels have been shown to be correlated with asthma severity [250,251], we investigated airway HMGB1 expression in asthmatics of various severities (defined by GINA grades) compared with non-asthmatics. HMGB1 was found to be elevated in the sputa of moderate-to-severe asthmatics (GINA 3-to-5) compared with controls and mild asthmatics (GINA 1-to-2) using ELISA. This partly agrees with reports that have been published concomitantly with this work [250,251], although not with others [252]. In the study published by Watanabe *et al.* (2011) significantly higher sputum HMGB1 expression was found in asthmatics, with HMGB1 levels correlating with disease severity [250]. It is noteworthy that the subjects included in this study were all newly diagnosed asthmatics, non-smokers and treatment-naïve [250].
Similarly, Hou et al. (2011) found increased HMGB1 levels in the sputa of asthmatics compared with healthy controls, and these also correlated with disease severity [251]. Furthermore, in both studies sputum HMGB1 correlated with the percentage of neutrophils in sputum and negatively correlated with lung function [250,251]. Consistently, in this Thesis HMGB1 sputum levels positively correlated with sputum total cell counts in asthmatics; however, no correlation with lung function could be demonstrated. In a separate study, levels of HMGB1 were similar in the bronchial lavage (BL) of non-neutrophilic asthmatics, neutrophilic asthmatics and healthy controls [252]. The discrepancies among these studies may be due to differences in HMGB1 concentrations that are detectable in sputum and BL, or differences in sample populations. In any case, the sample size investigated in Sukkar et al. (2011) was smaller than those examined in this and other studies (n = 8 non-neutrophilic asthma, n = 8 neutrophilic asthma versus n = 18 controls), which could have affected the power of their observations.

Our data are consistent with the implication of HMGB1 in asthma. This observation was extended to include the current knowledge that the redox state of HMGB1 determines HMGB1 activity, with reduced (all-thiol) HMGB1 promoting RAGE-dependent cell migration and disulphide-HMGB1 causing the upregulation of pro-inflammatory cytokines via TLR4; whereas further oxidation causes the loss of HMGB1 chemotactic and pro-inflammatory activities [87]. The redox state of HMGB1 released in the airways has not been studied before; therefore, it was important to determine HMGB1 redox state in the sputum of healthy and asthmatic subjects. This was done by Western blotting under non-reducing conditions, in order to preserve HMGB1 redox state. Despite the airways being an oxidative environment, both reduced and oxidised
forms of HMGB1 were detected in a donor-dependent manner. The molecular weights of reduced and oxidised HMGB1 forms were consistent with those previously described [133]. Moreover, pre-treatment with the reducing agent DTT caused a shift in the molecular weight of oxidised HMGB1, further confirming the redox nature of the proteins. However, it should be stated that Western blotting is an initial exploratory technique, which should be complemented by mass spectrometry analysis definitively to answer this question. The initial plan for this Thesis was indeed to analyse sputum samples by mass spectrometry in order to determine the redox state of HMGB1 in sputum; however, this could not be done in the timeframe of the PhD and is a topic for future work. Nonetheless, mass spectrometry will give a qualitative rather than quantitative assessment of HMGB1 redox forms present in sputum; whereas by using Western blotting it was possible to quantify each HMGB1 redox form in sputa of asthmatics and non-asthmatics.

Both HMGB1 redox forms were significantly elevated in the sputa of asthmatics compared with controls, confirming the results found by ELISA, which detects any redox form of HMGB1. In particular, the reduced form was more represented in moderate-to-severe asthmatics than in healthy controls or mild asthmatics, which implicates a possible role for this form in inflammatory cell recruitment to the airways in asthma. In support of this, a positive correlation was found between airway HMGB1 levels measured by ELISA and sputum total cell counts, and in vitro reduced HMGB1 was shown to support peripheral blood leukocyte chemotaxis. Recruited inflammatory cells may release more HMGB1 in the airways, thus generating a positive feedback loop. Since disulphide-HMGB1 is an intermediate redox form between reduced and fully oxidised HMGB1, the upregulation of oxidised HMGB1 in the sputum of
asthmatics is likely to include disulphide-HMGB1, thus also implicating this form, a pro-inflammatory DAMP, in the pathophysiology of asthma.

Together, these data confirm a previously proposed involvement of HMGB1 in asthma and show that reduced HMGB1 is increased in asthma and may contribute to leukocyte recruitment to the asthmatic airways.

In bronchial tissue, HMGB1 expression was found in the bronchial epithelium, smooth muscle and infiltrating inflammatory cells in the lamina propria. HMGB1 mainly localised to the cell nuclei; however, cases of HMGB1 cytoplasmic expression were observed, both in structural cells and inflammatory cells. HMGB1 cytoplasmic expression is generally regarded as an indication of HMGB1 translocation from the nucleus that precedes secretion. This suggests that HMGB1 may be released by inflammatory cells and airway structural cells under certain conditions such as allergen/pathogen stimulation and inflammation, as shown in animal models of allergic asthma [253]. When the number of cells expressing HMGB1 was quantified in the smooth muscle and the epithelium of severe asthmatics, only expression in the ASM was upregulated compared with controls. This could be due to higher baseline HMGB1 expression levels in the epithelium, or to the presence of a more complex stress factor-response network that may influence HMGB1 expression in the epithelium. The relationship between HMGB1 expression in ASM and airway inflammation was also explored: HMGB1 was found to be negatively correlated with mast cell numbers in the lamina propria and in the smooth muscle, and negatively correlated with eosinophil numbers in the lamina propria, suggesting a negative relationship between HMGB1 expressed in the ASM and tissue inflammation. The implications of these correlations are unclear, but the kinetics of HMGB1 upregulation and release may be speculated to
account for this. HMGB1 may be upregulated in the ASM and subsequently secreted as a stress response during an early phase of inflammation. HMGB1 is able to recruit inflammatory cells, and this may include mast cells and eosinophils, which have both been shown to express RAGE [274, 275]. Therefore, when inflammatory cells (perhaps including mast cells and eosinophils) have migrated to the lamina propria and/or the ASM bundle, HMGB1 in the ASM may be downregulated as a result of secretion. Whilst promoting inflammation and tissue destruction, one of the effects of mast cell and eosinophil activation may be the inactivation of HMGB1. For example, ROS released by eosinophils can oxidise HMGB1, and this is known to reduce the ability of HMGB1 to induce cytokine expression and release, and cell migration [276, 133]. Mast cell degranulation causes the release of heparin, which has been shown to bind HMGB1 and change its conformation in a way that compromises HMGB1 ability to bind RAGE and to induce cytokine production from macrophages and endothelial cells [159]. Thus, a negative feedback loop may regulate the activity of extracellular HMGB1 whereby cells that are recruited and activated by HMGB1 are also responsible for the inactivation of HMGB1 itself.

In contrast, no differences in RAGE expression were found in sputum (sRAGE) or bronchial tissue of asthmatics compared with healthy controls, although it should be noted that the GMA embedding used for immunohistochemical investigation in this study did not seem to be ideal for RAGE detection, and allowed only semi-quantification of RAGE expression.

Evidence suggests that esRAGE is the main form of sRAGE present in the airways and in the circulation [252], and esRAGE was found to be elevated in sputa of treatment-naïve asthmatics [250]. This may reflect inflammatory processes; however, no
correlation was found between sputum esRAGE and sputum neutrophils or asthma severity [250]. In other diseases including COPD and neutrophilic asthma, however, a decrease in systemic or local sRAGE has been associated with protective effects [247,248,252]. We have not investigated sRAGE levels in plasma or serum; however, we found no differences in sputum esRAGE levels between healthy controls and asthmatics of various severities, in contrast with the studies mentioned above, but in agreement with a separate study that found no differences in sRAGE levels in the bronchial lavage of non-neutrophilic asthmatics versus controls [252]. Whilst discrepancies among studies may reflect differences in patient populations and/or sampling methods, these results do not support the hypothesised increase in RAGE expression in the airways of asthmatics versus controls.

Isolated HASM cells expressed both HMGB1 and RAGE mRNA. HMGB1 mRNA expression was not different between healthy controls and asthmatics (in vitro characterisation of RAGE and HMGB1 temporally preceded the characterisation in tissue; therefore, asthmatic subjects were not grouped according to severity). Various RAGE isoforms were found to be expressed in HASM cells, including up to 3 soluble forms and the membrane-bound signalling receptor, which was the focus of this Thesis. Due to considerable homology among RAGE isoforms, it was not possible to quantify the expression of full-length RAGE mRNA using non-specific detection methods. Cell-surface RAGE protein was expressed by HASM cells, with no difference found between non-asthmatics and asthmatics. Interestingly, RAGE expression was higher in permeabilised HASM cells, indicating intracellular RAGE expression, which may represent sRAGE isoforms. The expression of a shorter RAGE protein of a molecular weight that is consistent with sRAGE was also found by Western blotting.
The significance of this finding is not clear; however, it may mean that ASM cells are able to secrete sRAGE, perhaps in the presence of RAGE ligands or other stress stimuli. Since sRAGE antagonises RAGE signalling, this could constitute a mechanism for the negative modulation of RAGE activation [98,246]. Although cell-surface RAGE expression was low in many donors, comparable levels of cell-surface RAGE expression were found in HUVECs, a primary endothelial cell type that is known to express functional RAGE (e.g. HMGB1 stimulation causes RAGE-dependent NF-κB activation, TNFα secretion and RAGE upregulation in HUVECs [203]), suggesting that this level of expression is sufficient to produce a functional response. The lack of RAGE upregulation in bronchial tissue and in isolated ASM cells from asthmatics does not support the hypothesis that increased RAGE expression participates in ASM dysfunction in asthma. However, cell-surface RAGE expression in cultured ASM cells indicates that ASM can respond to RAGE ligands such as HMGB1. HMGB1 protein expression was intracellular and localised to nuclei, as expected from its role in chromatin organisation and regulation of transcription. Unexpectedly, there was a significant reduction in HMGB1 protein expression in HASM cells isolated from asthmatic subjects. This contrasts with the elevation in HMGB1 expression found in the ASM bundle in bronchial tissue of severe asthmatics, a discrepancy that may be attributed to the separation of cultured HASM cells from their physiological environment. For example, ASM cells may express more HMGB1 in the presence of the inflammatory asthmatic environment, whereas cultured HASM cells from asthmatics may have a reduced HMGB1 synthetic capacity when isolated from their original inflammatory environment. Alternatively, it could indicate that ASM from asthmatics release HMGB1 constitutively in vitro. This is conceivable, since ASM cells from
asthmatics can constitutively release more mediators than cells from non-asthmatics (for example CCL2 [71], and IL-6, unpublished data). HMGB1 could not be detected in cell supernatants of healthy controls or asthmatics using ELISA, but this may be due to an insufficient sensitivity of the HMGB1 ELISA used. This question remains open; however, HMGB1 protein expression was upregulated when cells were exposed in vitro to a pro-inflammatory environment characteristic of asthma, showing that HASM cells were able to respond to inflammatory stress by producing more HMGB1.

Having demonstrated cell-surface RAGE expression in HASM cells, we moved on to investigate RAGE signalling and function in these cells, which play important roles in asthma. First, evidence of RAGE activation by HMGB1 was sought in HASM cells. RAGE signalling has been shown to induce downstream activation of the transcription factor NF-κB, leading to the upregulation of cytokines and also of cell-surface RAGE itself [98]. For example, 24 h stimulation with 50 ng/mL HMGB1 has been shown to cause the upregulation of cell-surface RAGE, as well as cell adhesion molecules ICAM-1 and VCAM-1 in HUVECs [202]. This suggests that upregulation of cell-surface RAGE expression following exposure to RAGE ligands can be an indicator of RAGE activation.

The expression of cell-surface RAGE was therefore investigated following stimulation with recombinant HMGB1 (30 and 100 ng/mL) at various time points between 16 and 72 h. There was no change in cell-surface RAGE expression; however, it is possible that intracellular RAGE expression (e.g. sRAGE) is affected by HMGB1 stimulation, but this was not investigated.

Various transcription factors are known to be induced by RAGE signalling, but the activation of NF-κB is more consistently described; therefore, this may be a more general measure of RAGE activation that can lead to several downstream events [98].
Various studies have also shown that HMGB1 is able to cause NF-κB activation; for example, 30 min stimulation with HMGB1 (1 μg/mL) induced nuclear translocation of the NF-κB subunit p65 in HUVECs [120]. In another study, RAGE-dependent p65 nuclear translocation was observed in HUVECs following 30 min stimulation with 60 ng/mL HMGB1 [203]. Therefore, nuclear translocation of p65 was investigated next in HASM cells using immunofluorescence. Recombinant HMGB1 (30, 300 and 1000 ng/mL) failed to induce p65 nuclear translocation across a range of time points between 0 and 120 min, whereas HASM cells stimulated with a positive control (TNFα, 20 ng/mL, for 30 min) showed clear nuclear translocation of the p65 protein. This suggests that NF-κB was not induced by rhHMGB1 under these conditions; however, it should be pointed out that the rhHMGB1 used in these experiments contained DTT, a reducing agent that may have affected the ability of HMGB1 to activate NF-κB signalling pathways. In fact, it has recently been shown that disulphide-HMGB1 (partially oxidised) activates the NF-κB pathway via TLR4, whereas in the reduced form HMGB1 does not induce p65 nuclear translocation [133]. In view of this consideration, the functionality of cell-surface RAGE in HASM cells could not be ruled out. Therefore, alternative signalling events downstream of ligand-RAGE activation were considered next.

Activation of RAGE signalling is also known to cause increased production of intracellular ROS [98,178]. Recombinant HMGB1 (10 – 1000 ng/mL) was able to induce a significant increase in intracellular ROS production in HASM cells from non-asthmatics, but interestingly no significant effect was observed in cells isolated from asthmatics, suggesting disease-associated differences in ASM responses to HMGB1. These differences were not due to impaired ROS generation capacity in the asthmatics,
because baseline ROS production was similar in healthy controls and asthmatics, and hydrogen peroxide has previously been shown to stimulate significantly higher ROS production in ASM from asthmatics versus non-asthmatics [76]. It is possible that differences in HMGB1 receptor expression levels exist in cells from asthmatics versus non-asthmatics. Since we did not find a different expression of cell-surface RAGE in healthy and asthmatics, this could indicate that a different HMGB1 receptor is involved, for example TLR4. HASM cells from non-asthmatic subjects have been shown to express functional TLR4 [261]. Whether this expression is downregulated in asthmatics is of interest, as HMGB1 has been shown to induce TLR4-dependent ROS generation in murine cardiomyocytes [277], thus downregulation of TLR4 expression in ASM from asthmatic subjects may explain the inability of HMGB1 to stimulate ROS production.

The effect of rhHMGB1 on HASM cell ROS production meant that HMGB1 can activate these cells; therefore, potential roles of HMGB1 in the regulation of ASM functions were investigated next. We focused on functions that may be dysregulated in asthma, such as exaggerated contractile responses and altered repair mechanisms [58,75], using in vitro models. Our group has previously shown that ROS production and ASM contraction are linked by a positive relationship [76]. Therefore, it was interesting to establish whether HMGB1 could induce ASM contraction and whether this was different in health and asthma. Indeed, an increase in bradykinin-induced ASM contraction was observed, but only with the highest concentration of rhHMGB1 (1 μg/mL). No significant difference was found between healthy controls and asthmatics; however, there was a trend for greater effects in asthmatics. An explanation for this could be that the HMGB1-mediated ROS production in non-asthmatic ASM results in
Discussion

HMGB1 oxidation to a greater extent than ASM from asthmatic donors. Since the redox state of HMGB1 determines its function and fully oxidised HMGB1 is inactive [87], it could be speculated that non-asthmatic ASM contract less in response to HMGB1 stimulation due to a ROS-dependent change in the oxidation state or faster inactivation of HMGB1. Additionally, the HMGB1 induction of TLR4-dependent ROS generation in murine cardiomyocytes resulted in perturbations in $Ca^{2+}$ homeostasis leading to impaired cardiomyocyte contractility, both of which were recovered by TLR4 inhibition or silencing, and by pre-treatment with antioxidants [277]. Whether differences in $Ca^{2+}$ homeostasis occur following HMGB1 treatment of ASM from non-asthmatic donors versus asthmatic donors is worthy of further investigation. In a previous study, RAGE was also implicated in reduced cardiomyocyte contractility, since both TLR4 and RAGE inhibition partly rescued HMGB1-mediated impairment of intracellular $Ca^{2+}$ availability and cardiomyocyte contraction [278]. This agrees with an experimental model of intra-abdominal sepsis in which increased serum HMGB1 and oxidative damage in the diaphragm were associated with compromised contractile function of the diaphragm. Muscle force generation and oxidative damage were improved by blockade of HMGB1 and RAGE, suggesting a role for the HMGB1-RAGE axis in ROS-derived muscle dysfunction [279].

Sputum HMGB1 levels were increased in asthma; in particular the reduced form of HMGB1 was more represented in more severe asthmatics. The bronchial epithelium lines the bronchi and is most likely to come in contact with HMGB1 present in sputum, suggesting that interactions of HMGB1 with the bronchial epithelium are possible. Although neither RAGE nor HMGB1 were found to be increased in bronchial epithelium in asthma, preliminary in vitro data showed that human bronchial epithelial cells
(HBECs) express similar levels of HMGB1 and RAGE compared to those found in HASM cells. This suggests that HMGB1 may play a role in the regulation of HBEC function and/or HBECs may be a source of extracellular HMGB1, as shown by mouse asthma models [253]. The epithelium of asthmatics appears to be more prone to being damaged, but also less able of repair. As a consequence, epithelial damage is often associated with asthma [15]. HMGB1 is involved in repair mechanisms by promoting cell migration, proliferation and differentiation [280]. Recently, the reduced form of HMGB1 has been identified as the chemotactic form of the protein [133]. We therefore explored the effect of rhHMGB1 (a reduced form, 100 – 1000 ng/mL) on bronchial epithelial repair functions using a wound healing assay. HMGB1 had no effect on epithelial wound healing at the lower concentrations, and it inhibited HBEC wound healing at the highest concentration tested (1 µg/mL). This concentration is consistent with the levels of HMGB1 detected in the sputa of asthmatics, suggesting that this observation may be of relevance in vivo. Although HMGB1 has been shown to promote wound closure in a number of other cellular models, for example in human keratinocytes [281], alveolar epithelial cells [241], mouse fibroblasts in a RAGE-dependent manner [201] and to induce vascular smooth muscle cell migration via RAGE [130], HMGB1 and RAGE have also been implicated in impaired wound healing in experimental models of disease. For example, increased tissue and serum HMGB1 has been observed in experimental gastric ulcer and HMGB1 delayed healing processes in a TLR4- and RAGE-dependent manner [282]. In diabetic mice, sRAGE was able to recover impaired wound epithelial closure, to normalise inflammation (i.e. inflammatory cell infiltration, cytokine and MMP release), and induced increased expression of PDGF and vascular endothelial growth factor (VEGF) [283]. Moreover,
the AGEs-RAGE-ROS axis has been implicated in delayed corneal epithelial wound healing \textit{ex vivo} [284]. These studies denote a complex interaction between HMGB1 and its receptors RAGE and TLR4 in tissue repair processes that may depend on ligand/receptor expression levels and the type of tissue or experimental model involved.

The ASM is less likely to be wounded \textit{in vivo}; however, exaggerated ASM repair mechanisms in response to stress stimuli may contribute to the airway remodelling observed in asthma [58]. Therefore, we tested the effect of reduced HMGB1 (3S-HMGB1, a mutated non-oxidisable form of HMGB1) in HASM cell migration. Consistently with the results found in HBECs, 3S-HMGB1 caused an inhibition in HASM cell migration at 1 µg/mL. However, lower concentrations of 3S-HMGB1 (3 ng/mL) showed weak agonist effects. These results further suggest that the effects of HMGB1 on repair processes may depend on HMGB1 concentrations and that increased levels of HMGB1 may participate in impaired repair mechanisms in asthma.

In conclusion, HMGB1 was shown to be upregulated in the airways of moderate-to-severe asthmatics, with a higher representation of the HMGB1 chemotactic form found in this group. HMGB1 may contribute to maintaining a pro-inflammatory environment in asthma, by recruiting leukocytes to the airways and causing their activation. HMGB1 was upregulated in the ASM of severe asthmatics, suggesting that these cells are a potential source of extracellular HMGB1. In agreement with this view, HASM cells were able to respond to inflammatory stress by upregulating HMGB1 expression \textit{in vitro}. Neither bronchial RAGE nor sputum esRAGE expression differed in asthma compared with health. However, both HASM cells and HBECs expressed cell-surface RAGE, and HMGB1 was able to regulate cell function by promoting ASM
contraction and inhibiting ASM and epithelial repair. Importantly, these effects were found at HMGB1 concentrations that were detected in the sputa of more severe asthmatics, suggesting that HMGB1 elevation in asthma is relevant to asthma pathophysiology. For example, HMGB1 may contribute to impaired epithelial repair processes and exaggerated ASM contractile responses in the asthmatic airways (figure 6.1.1). Whether antagonising HMGB1 would be a viable therapeutic approach for the treatment of asthma, however, is difficult to say at present. More studies are needed to further elucidate the roles of the different redox forms of HMGB1 in asthma, and specific antagonists/antibodies are needed to confirm these. Lastly, the receptors involved in these processes have not been identified here and it is possible that separate receptors, e.g. RAGE and TLR4, mediate these functions. This remains an open question for future work.
Figure 6.1.1 Proposed functions of extracellular HMGB1 in asthmatic airways. Results presented in this Thesis suggest roles for extracellular HMGB1 in regulating ASM and bronchial epithelial functions such as contraction and repair. HMGB1 was shown to induce ASM contraction and to inhibit ASM migration and epithelial healing; suggesting that increased HMGB1 in the airways of asthmatics contributes to augmented ASM contractile responses and defective tissue repair mechanisms in asthma. These may be newly identified HMGB1 functions. Dotted lines represent hypothetical processes.
6.2 Future work

The data presented in this Thesis offer convincing evidence of HMGB1 involvement in asthma and in the regulation of ASM and bronchial epithelial functions that are relevant to the pathophysiology of the disease. However, many questions remain unanswered or need further clarification.

For example, although we speculated on possible sources of HMGB1 as a DAMP, based on HMGB1 cytoplasmic expression or upregulation following cytokine stimulation, we did not identify the sources of increased HMGB1 in the airways, nor did we show that ASM can secrete HMGB1 at baseline or after stimulation. This may be partly due to insufficient sensitivity of the HMGB1 ELISA used. Both immune and structural cells are potential sources of extracellular HMGB1, in addition to necrotic cells [82]. In future experiments this question can be addressed by stimulating various airway structural and immune cell types with a variety of stress stimuli and by analysing concentrated cell supernatants using ELISA. Alternatively, fluorescently tagged HMGB1 translocation to the cytoplasm can be monitored using confocal microscopy.

The redox state of HMGB1 in sputum was investigated in an exploratory way using non-reducing Western blotting, which allowed quantification of each redox form; however, as mentioned before, mass spectrometry is needed to definitively determine the redox nature of HMGB1. This will be achieved in the future through collaborative work.

We have shown a positive effect of HMGB1 on leukocyte migration, but a negative association of HMGB1 expression with mast cell and eosinophil infiltration in bronchial tissue. Whilst the role of HMGB1 in leukocyte recruitment, including monocytes and
neutrophils, is well documented [107,268], less is known about mast cells and eosinophils chemotaxis towards HMGB1. Therefore, it would be interesting to explore this relationship further; for example, to investigate whether HMGB1 can recruit mast cells to the ASM, and also cause their degranulation. Since HMGB1 has been shown to induce the recruitment of stem cells [280], it would be interesting to investigate the effect of HMGB1 on mesenchymal progenitor cell migration, which is thought to be involved in airway remodelling [58].

Cell-surface RAGE expression was detected in HASM cells and HBECs, with no differences seen between asthmatics and non-asthmatics for ASM; however, higher levels of intracellular RAGE expression, which may represent soluble forms of RAGE, were found in both cell types. Expression of this was not compared in healthy subjects and asthmatics; moreover, it is possible that ASM and epithelial cells are able to secrete sRAGE in response to stress stimuli, which could negatively regulate RAGE activation. Although esRAGE, a secreted splice variant of RAGE, was not changed in sputa of asthmatics, we did not investigate total sRAGE in sputum or circulating sRAGE levels, which have been shown to be lower in COPD [234,247,249] and neutrophilic asthma [252]. In addition, further in vitro characterisation is needed for HMGB1 and RAGE expression in HBECs, since only a small number of donors were analysed.

A general weakness of the functional work carried out in HASM cells and HBECs is that the receptors involved in the functions regulated by HMGB1 were not identified. Since it is now clear that HMGB1 can bind additional receptors besides RAGE, such as TLR4 and TLR2, it will be important to link specific receptors to each function (i.e. contractile responses and cell migratory responses), using selective blocking antibodies, specific receptor antagonists, or by genetic manipulation of receptor expression. Additionally,
we did not show that the effects of HMGB1 are specific, for example by using HMGB1-specific antagonists or blocking antibodies. The optimisation of these reagents is currently in progress. It would also be valuable to replicate HMGB1-induced ASM contraction under more physiological conditions, e.g. using lung tissue slices and by measuring changes in AHR in animal models.

The functional effects of HMGB1 were often only observed at a high concentration: 1 μg/mL. In order to ensure that HMGB1 is not effecting cell viability or proliferation at this concentration the effects of HMGB1 on ASM and epithelial cell apoptosis and survival/proliferation will also be investigated in future experiments e.g. using annexin V/propidium iodide staining to assess apoptosis; the tetrazolium dye MTS cell viability assay; and the carboxyfluorescein succinimidyl ester (CFSE) proliferation assay.

Another interesting point for future exploration would be to investigate the mechanism behind the differential effect observed on ROS production in health versus asthma, with HASM cells from asthmatics failing to respond significantly to HMGB1 stimulation. This may be due to differences in HMGB1 receptor expression in health versus asthma that may cause variable levels of cell activation, and could relate to differences in ASM contraction. Interestingly, a trend for increased ASM contraction in asthmatics versus non-asthmatics was observed, suggesting that a disease-driven difference may exist. The power of this observation needs to be strengthened by increasing the number of donors, and/or by studying asthmatics with different severities of disease, as this effect may be related to disease severity. A difference in contraction between healthy subjects and asthmatics could be linked with differences seen in ROS production: increased HMGB1-induced ROS in non-asthmatics could mean that exogenous HMGB1 is oxidised to a greater extent in these donors. Since the redox
state of HMGB1 determines its function and fully oxidised HMGB1 is inactive [87], it could be speculated that non-asthmatic ASM contract less due to a ROS-dependent change in the oxidation state or faster inactivation of HMGB1. This hypothesis could be tested by adding antioxidants to HMGB1-stimulated non-asthmatic ASM and see whether this restores the contraction to the level observed in asthmatics; and vice versa test whether hydrogen peroxide can inhibit the HMGB1-induced contraction seen in asthmatics. Alternatively, differences in contraction could be related to differences in TLR4 expression between ASM from non-asthmatic and asthmatic subjects. Indeed, TLR4 is involved in HMGB1-mediated ROS generation and subsequent impairment of cardiomyocyte contractility [277]. Optimisation of reagents to measure TLR4 mRNA and protein expression in HASM cells from asthmatics versus non-asthmatics is currently underway.

Differences between health and asthma should also be explored in epithelial wound healing. If a differential response to HMGB1 is observed, this could help to establish whether intrinsic differences exist between non-asthmatic and asthmatic epithelium, or whether the impaired wound healing seen in asthma is likely to be a consequence of the increased amount of HMGB1 present in the sputa of asthmatics. If intrinsic differences exist in asthma versus non-asthma, it would be interesting to determine whether this is related to differential HMGB1 receptor expression levels.
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