The role of histone arginine methylation in cytokine/chemokine gene expression in airway smooth muscle cells in asthma

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Abstract

Background: Asthma is a common chronic condition, which has a negative impact on life quality of those suffering and is also a burden to the national healthcare. Airway smooth muscle (ASM) cells have a crucial role in asthma, contributing to airway remodelling, airflow obstruction, and inflammation of the airways. Epigenetic changes, particularly histone lysine acetylation/methylation, have been shown to alter ASM functions. Histone arginine modifications have been less well studied.

Hypothesis: We tested the hypothesis that histone arginine methylation is important in regulating ASM cytokine/chemokine production and that PRMTs, the enzymes which catalyse histone arginine methylation, are a therapeutic target in asthma.

Aims: To characterise the expression of PRMTs in human ASM cells, to establish whether inhibiting PRMTs could reduce the cytokine/chemokine expression and their secretion from human ASM cells, and to establish the therapeutic potential of PRMT inhibitors in asthma.

Methods: Studies were performed in cultured human ASM cells at passage six and 4-hydroxytamoxifen (OHT)-inducible PRMT1$^{FL/-}$ ER-Cre mouse embryonic fibroblasts (MEFs). The activity of PRMT1 at the cytokine/chemokine promoters, as well as the impact of inhibiting PRMT1 on the inflammation in asthmatic airways, were investigated. TNF-α stimulation was used to simulate the conditions in asthmatic airways.

Key results: The main findings of our study were that human ASM cells expressed mRNA and protein of all four PRMTs that methylate histones *in vivo*: PRMT1, PRMT4/CARM1, PRMT5 and PRMT6, but there was no significant difference in expression between ASM cells isolated from asthma patients and healthy subjects. We found that PRMT1 likely has a role in regulating the TNF-α-induced cytokine/chemokine production by ASM cells. Evidence supporting this role for PRMT1 came from studies showing that TNF-α-induced the PRMT1-catalysed histone arginine methylation mark H4R3me2a, that a pharmacological inhibitor of PRMT1 inhibited cytokine/chemokine production by ASM, and that the molecular knockout by CRISPR showed comparable results, at least for IL-6, eotaxin and CXCL8, but not for IP-10. Further evidence for a role of PRMT1 was provided by our studies in PRMT1$^{FL/-}$ ER-Cre MEFs, as the loss of PRMT1 led to a reduction in TNF-α-induced secretion of a mouse chemokine KC. We also performed experiments studying an intermediate protein CNOT7, but overall the results regarding its involvement in TNF-α-induced PRMT1 mediated cytokine/chemokine production were inconclusive. Experiments with pharmacological

inhibitors suggested that CARM1, but not PRMT5 or PRMT6, also had a regulatory role in cytokine/chemokine production in human ASM.

Conclusions: Collectively, our results show that human ASM cells express several PRMTs and that PRMT1, and possibly also CARM1, should be investigated as potential targets for development of novel asthma treatments.

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Publications

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List of abbreviations

3’-UTR	3’-untranslated region
5-LO	5-lipoxygenase
AAI	Allergic airway inflammation
Ac	Acetylation
ADMA	Asymmetrical dimethylarginine
AdoMet	S-adenosyl methionine
AHR	Airway hyperresponsiveness
AIPI	Antigen-induced pulmonary inflammation
AMI-1	Arginine methyltransferase inhibitor 1
ARRE-2	Antigen receptor response element 2
ASM	Airway smooth muscle
Aza-CdR	5-Aza-2’-deoxycytidine, Decitabine
β2M	β2-microglobulin
BALF	Bronchoalveolar lavage fluid
BCA	Bicinchoninic acid
BET	Bromodomain and extraterminal
bFGF	Basic fibroblast growth factor
BTG1	BTG Anti-Proliferation Factor 1
CARM1	Co-activator-associated arginine methyltransferase 1
CBP	cAMP response element binding protein (CREB) binding protein
CCL11	Eotaxin
CCL17	TARC, Thymus- and activation-regulated chemokine
CCL2	MCP-1, Monocyte chemoattractant protein 1
CCL5	RANTES
cDNA	Complimentary DNA
ChIP	Chromatin Immunoprecipitation
cm^2	Square centimetre
CNOT7	hCAF1, CCR4-associated factor 1, CCR4-NOT transcription complex subunit 7
COPD	Chronic obstructive pulmonary disease
COX	Cyclooxygenase
CRISPR	Clustered regularly interspaced short palindromic repeats
CTGF	Connective tissue growth factor
CXCL10	IP-10, Interferon γ–induced protein 10
CXCL8	Interleukin 8
DEP	Diesel exhaust particulate
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulphoxide
DNMT1	DNA methyltransferase 1
DOT1L	Disruptor of telomeric silencing 1-like
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme- Linked Immunosorbent Assay

ERα	Estrogen receptor α
FCS	Foetal calf serum
FLSs	Fibroblast-like synoviocytes
g	Relative centrifugal force
GAPDH	Glyceraldeyde-3-phosphate dehydrogenase
GAR	Glycine and Arginine
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GRIP-1	Glucocorticoid receptor interacting protein 1
gRNA	Guide RNA
GRO	Growth-related oncogene
H	Histone
H_2O_2	Hydrogen peroxide
HATs	Histone acetyltransferases
HDACi	HDAC inhibitors
HDACs	Histone deacetylases
HMG	High-mobility group
hnRNP A1	Heterogeneous nuclear RNP protein A1
HPRT	Hypoxanthine guanine phosphoribosyl transferase
HRP	Horseradish peroxidase
ICAM-1	Intercellular adhesion molecule 1
IFNAR1	Intracytoplasmic domain of IFNα/β
IgE	Immunoglobulin E
IGF	Insulin-like growth factor 1
IgG	Immunoglobulin G
IL	Interleukin
IL-10	Interleukin 10
IL-13	Interleukin 13
IL-17	Interleukin 17
IL-21	Interleukin 21
IL-22	Interleukin 22
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-6	Interleukin 6
IL-9	Interleukin 9
ILF3	Interleukin Enhancer Binding Factor 3
IVT	*In-vitro* transcription
JAK2	Janus kinase 2
JmjC	Jumonji C
JMJD6	Jumonji domain-containing protein 6
K	Lysine
KC	Keratinocyte-derived chemokine
lncRNA	Long non-coding RNA
LPS	Lipopolysaccharide
LTD_4	Leukotriene D4
MAPK	Mitogen-activated protein kinase
MCL	Mantle cell lymphoma
MCPs	Monocyte chemoattractant proteins
MDB	Membrane Desalting Buffer

me1	Monomethylation
me2	Dimethylation
me2a	Asymmetrical dimethylation
me2s	Symmetrical dimethylation
me3	Trimethylation
MEFs	Mouse Embryonic Fibroblasts
mg	Milligram
MIP-2	Macrophage inflammatory protein-2
miRNA	MicroRNA
MKP-1	MAP kinase phosphatase 1
µl	Microliter
ml	Millilitre
MLL-r	MLL-rearranged
µM	Micromole
MMA	Monomethylarginine
M-MLV RT	Moloney murine leukemia virus reverse transcriptase
MMP-12	Matrix metalloproteinase 12
MMP-2	Matrix metalloproteinase 2
MMP-9	Matrix metalloproteinase 9, gelatinase B
MMPs	Matrix metalloproteinases
mRNA	Messenger RNA
MT1-MMP	Membrane type 1 metalloprotease
MTT	3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide
ncRNAs	Non-coding RNAs
NF-κB	Nuclear factor kappa B
NK	Natural killer
nm	Nanometre
NM23	Nonmetastatic 23
NTP	Nucleoside triphosphate
NuRD	Nucleosome Remodeling and Deacetylase
OHT	4-Hydroxytamoxifen
oligoDT	Oligodeoxythymidylic acid
OVA	Ovalbumin
p/CAF	p300/CBP-associated factor
PAD4	Peptidyl arginine deaminase 4
PAF	Platelet activating factor
PBS	Phosphate buffered saline
PDE	Phosphodiesterase
PDE4D	Phosphodiesterase 4D
PDGF	Platelet-derived growth factor
PG	Prostaglandin
pg	Picogram
PGD_2	Prostaglandin D2
PGE_2	Prostaglandin E2
$PGF_{2\alpha}$	Prostaglandin F2α
PGH_2	Prostaglandin H2
PGI_2	Prostaglandin I2
PGM	Proline, glycine, and methionine

PIC	Protease inhibitor cocktail
piRNA	Piwi-interacting RNA
PKC	Protein kinase C
PMSF	Phenylmethylsulfonyl fluoride
PRMTs	Protein arginine N-methyltransferases
PVDF	Polyvinylidene difluoride
R	Arginine
RA	Rheumatoid arthritis
RAR	Retinoic acid receptor
rcf	Relative centrifugal force
rpm	Rotations per minute
RT	Reverse Transcription
RT-qPCR	Reverse Transcription quantitative Polymerase Chain Reaction
SAH	S-adenosylhomocysteine
SAHA	Suberoylanilide hydroxamic acid
Sam68	SRC associated in mitosis of 68 kDa
SDMA	Symmetrical dimethylarginine
siRNA	Short interfering RNA
SMN	Survival of motor neuron
snRNPs	Small nuclear ribonucleoproteins
SPF30	Splicing factor 30
SRC-1	Steroid receptor coactivator 1
SRC-3	Steroid receptor coactivator 3
ST7	Suppressor of tumorigenicity 7
STAT-1α	Signal transducer and activator of transcription 1 α
TARPP	Thymocyte cyclic-AMP regulated phosphoprotein
TBST	Tris buffered saline plus Tween 20
TDRD	Tudor domain-containing
TGF-β	Transforming growth factor β
Th	T helper
TIF1	Transcription intermediary factor 1
TIF2	Transcription intermediary factor 2
TIMP-1	Tissue inhibitor of metalloproteinase-1
TIMP-2	Tissue inhibitor of metalloproteinase-2
TNF-α	Tumour necrosis factor α
Treg	Regulatory T
TSA	Trichostatin A
TXA_2	Thromboxane A_2
V	Volt
VCAM-1	Vascular cell adhesion protein 1
VEGF	Vascular endothelial growth factor
YY1	Yin Yang 1

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Chapter 1: Introduction

1 Introduction

1.1 Asthma

Asthma is a common chronic and complex airway disorder, characterized by variable and recurring symptoms, airflow obstruction, bronchial hyperresponsiveness, and airway inflammation [1]. Despite being described in literature since antiquity (the word asthma comes from Greek, meaning *short of breath* [2]), the definition of asthma is still evolving, and there is no single parameter determining a definite diagnosis of asthma [3]. Most people suffering from asthma respond to the standard treatment with inhaled corticosteroids, inhaled bronchodilators and oral leukotriene receptor antagonists [4]. However, about 25% patients are not able to control their symptoms despite these therapies, resulting in severe chronic disease and lower quality of life [4, 5].

1.2 Asthma prevalence and impact

Asthma is one of the most common chronic diseases worldwide, estimated to affect at least 300 million people globally [3]. The prevalence in the general population has increased in the past 50 years, from about 2-4% [6, 7], to 15% -20% or more [8, 9]. This increase reflects changes in lifestyle, as the prevalence is higher in developed countries, such as the UK and Australia than in the developing countries (15-20% as opposed to 2-4%), and a Westernized lifestyle increases the risk of developing asthma [3]. The studies on non–US-born adults show a higher prevalence of asthma in relation to the number of years they had lived in the United States [10].

Although there is some evidence that asthma prevalence has declined, or at least plateaued in some countries [11-14]**,** a review from 2009 by Anadnan *et al.* concluded that there is no overall decline in asthma prevalence, and in many parts of the world, it is still increasing [15]. The decline for the need of asthma-related emergencies in some developed countries is most likely a result of improved care and management rather than decrease in prevalence [15]**.** Difficulties in stating the prevalence of asthma arise from the lack of standardized definition of the disease, variety of symptoms, changing diagnostic habits and overdiagnosis [16]. For example, Aaron *et al.* suggest that asthma is overdiagnosed in developed countries, i.e. one-third of individuals with physician-diagnosed asthma did not have asthma when objectively assessed [17]. The changes in the diagnostic habit can be illustrated by a study on Swedish children between 1985 and 2005, which showed that although the prevalence of asthma symptoms has decreased, the percentage of children with physician-diagnosed asthma has increased [18].

Asthma affects children more often than adults, however, 30-80% of children with asthma undergo remission during puberty [3]. Although the prevalence of asthma prior to puberty is higher in males than in females, the proportions are reversed in adulthood [19]. Adult women not only suffer from asthma more often than men, but also account for the majority of severe asthma cases [19].

1.2.1 Asthma in the UK

The UK has one of the highest prevalence rates of asthma in Europe [20]. 5.4 million people in the UK are currently receiving treatment for asthma, costing the NHS around £1 billion a year [20]. About 185 daily hospital admissions are due to asthma [20]. Despite the high prevalence, asthma has a relatively low mortality - 1410 people died from asthma in 2016 [20]. The UK is also amongst the countries with the highest prevalence of childhood asthma worldwide, as approximately one in 11 children in the UK has asthma [20].

1.3 Pathophysiology

Airway inflammation is one of the main characteristics of asthma, and is largely immunoglobulin E (IgE)-dependent [21]. The inflammation results in airway lumen obstruction with mucus, airway wall oedema and shedding of airway epithelium [21]. The biopsies of asthmatic airways show an increase in the numbers and activation of T-lymphocytes, eosinophils, mast cells and macrophages [21]. Basophils, dendritic cells and neutrophils are other types of inflammatory cells involved in asthma [22]. Airway hyperresponsiveness (AHR), characteristic of asthma, is defined as an exaggerated airway narrowing in response to a variety of stimuli, and its extent may also be closely linked to airway inflammation [21]. Chronic inflammation in the asthmatic airway may lead to the structural changes to the epithelium, smooth muscle, and vasculature, known as airway remodelling [21]. However, it has been shown that airway remodelling may occur very early in the onset of asthma, without underlying chronic inflammation [3, 22, 23]. Inflammatory mediators, such as histamine, prostanoids, leukotrienes, cytokines, chemokines, and endothelins, have been implicated in asthma, with airway structural cells, (including epithelial cells, endothelial cells, fibroblasts, and airway smooth muscle cells), being a major source of these mediators [21]. Epithelial cells, fibroblasts and airway smooth muscle cells have also been shown to play a role in airway remodelling [22].

1.3.1 Inflammatory cells

The inflammatory process in asthmatic airways involves a range of different inflammatory cells which, together with multiple mediators, interact with the airways, resulting in the pathophysiological features of the disease [1, 21].

1.3.1.1 T lymphocytes

A major breakthrough in understanding the development of inflammation in asthmatic airways came when CD4+ T cells were distinguished and assigned into two broad categories, T helper 1 cells and T helper 2 cells (Th1 and Th2), each having a different effect on airway function and secreting different cytokines [1, 3]. In asthma, there is a shift towards the Th2 phenotype, leading to the increased secretion of Th2 cytokines, such as interleukin 4 (IL-4), IL-5, IL-6, IL-9, and IL-13 [1, 3]. These cytokines are responsible for the eosinophilia, overproduction of IgE and hyperresponsiveness, all observed in the airways of asthmatic patients [1]. More recently, other types of T cells have been described, indicating that the dogma of asthmatic inflammation being a result of Th1/Th2 imbalance was oversimplified [22]. Regulatory T (Treg) cells are anti-inflammatory cells, which inhibit Th2 and suppress immune responses [1, 24]. It has been suggested that Treg cell numbers or function may be reduced in patients with asthma [24]. Th17 are proinflammatory cells, which produce cytokines, such as IL-17, IL-22, tumour necrosis factor-α (TNF-α), and IL-21 [22]. The major cytokine secreted by Th17 is IL-17, which is involved in development of airway neutrophilia, and induce the secretion of a variety of proinflammatory cytokines and chemokines from many different cell types [22]. Invariant natural killer (NK) T cells release large amounts of both Th1 and Th2 cytokines [22]. Although they have been suggested to be involved in the development of asthma [25], their role remains controversial and has yet to be established with certainty [22].

1.3.1.2 Eosinophils

Eosinophils may be the major effector cells in asthma [3] and most people suffering from asthma have increased numbers of eosinophils circulating in the airways [1, 3], sputum [26] and blood [27]. The elevation in eosinophils levels is also often linked to the increase in asthma severity [1, 27]. Following the onset of the inflammatory process, Th2 cells and inflammatory cells secrete various adhesion molecules (such as intercellular adhesion molecule 1, ICAM-1) and chemokines (including eotaxin), which then attract circulating eosinophils to localise into the inflamed airways [3]. Then, the activated eosinophils release toxic granule proteins, free radicals, which can cause airway tissue damage [3]. They also

generate and secrete inflammatory molecules, including prostaglandins, leukotrienes and cytokines, which elevate and prolong the inflammatory process [3]. A decrease in sputum eosinophil levels has been shown to effectively reduce asthma exacerbations [28]. Therapies targeting IL-5, a key mediator in eosinophil activation, improve asthma control and have already been approved for use in the clinic (mepolizumab, reslizumab, benralizumab) [29].

1.3.1.3 Other inflammatory cells

Other cells that are involved in the inflammatory process of asthma include mast cells, neutrophils, dendritic cells, macrophages and basophils [1, 22]. Mast cells are crucial in initiating the acute response to allergen (via high-affinity IgE receptors), and other stimuli, such as exercise [1, 21, 22]. Their activation releases bronchoconstrictor mediators, including histamine, cysteinyl-leukotrienes and prostaglandin D2 (PGD_2), as well as proinflammatory cytokines [1, 30]. The levels of neutrophils are elevated in the sputum of asthma patients during exacerbations [31, 32], but their role in the pathophysiological process in asthma is not clear [1, 21]. Increased neutrophil levels have been linked to asthma severity [33]. Dendritic cells are crucial in inducing Th2 immunity to allergens [34]. They are located at the basolateral side of epithelial cells in the lung and are one of the first immune cells that come into contact with allergens, following which they migrate to the regional lymph nodes to present the processed antigen to T cells, resulting in Th2 production from naïve T cells [34, 35]. Macrophages are the most abundant cells in the airways and may have either a pro- or anti-inflammatory role, depending on the stimulus [21, 36]. Macrophages can be grouped into M1 and M2 subsets, each having a different role and secreting different cytokines [36]. The M2 macrophage phenotype is associated with Th2 inflammatory response and is increased in individuals with asthma [37]. Basophils have a key role in the initiation of IgE-mediated chronic allergic inflammation [38] and promote the differentiation of naïve T cells to Th2, primarily through production of IL-4 [39].

1.3.2 Immunoglobin E (IgE)

IgE has an important role in allergic asthma as it mediates allergen-initiated bronchoconstriction and plays a part in the development and perpetuation of airway inflammation [1, 3]. Interleukins secreted by Th2 cells (mainly IL-4 and IL-5) induce the production of IgE antibodies, which then attach to cells via high-affinity (FcεR1) surface receptors [40, 41]. Mast cells have the largest numbers of FcεR1 receptors, although other cells, such as basophils, dendritic cells, and lymphocytes also possess high-affinity IgE receptors [1]. Following the exposure to the antigen, IgE and cells become physically cross-

linked, which induces cell activation [3, 40]. Following that, activated cells release a variety of proinflammatory mediators, such as histamine, tryptase, leukotrienes, and prostaglandins, which induce bronchoconstriction, mucus secretion, vasodilation, tissue oedema, and mucosal inflammation, among other processes [3, 40, 41]. The rapid inflammatory response initiated by binding of IgE to mast cells is known as the immediate hypersensitivity reaction [3]. In some people, this intense, but short response is followed by a slow and sustained response, called late phase reaction [3]. The prolongation of the late phase reaction is believed to account for the chronic inflammatory state in asthmatic airways, leading to bronchial hyperreactivity and airflow obstruction [3, 40].

1.3.3 Inflammatory mediators

A range of mediators, including histamine, prostanoids, leukotrienes, cytokines, chemokines, and growth factors, have been demonstrated to influence the inflammatory processes occurring in asthmatic airways [21, 42]. Histamine was the first mediator implicated in the pathophysiology of asthma [42], and, among other effects on asthmatic airways, it has been shown to induce bronchoconstriction, mucus hypersecretion, and increase vascular permeability [43, 44]. Similar effects are induced by cysteinyl leukotrienes, with the additional recruitment of inflammatory cells into the airways [43, 45]. Prostanoids can have opposing effects in the airways, for example, PGE_2 relaxes human airway smooth muscle [46], while PGD_2, $PGF_{2\alpha}$, and thromboxane A_2 (TXA_2) induce bronchoconstriction [47-49]. Cytokines are small protein mediators, which have a crucial role in orchestration of the inflammatory response in asthma [42]. Key cytokines include IL-5, which is necessary for eosinophil differentiation and survival [50], and IL-4 and IL-13, which induce IgE production from B cells [51, 52]. TNF-α, IL-6 and IL-1β are other important proinflammatory cytokines, which induce the synthesis of various mediators and amplify and perpetuate the inflammatory response [53, 54]. Chemokines are small cytokines that act as chemoattractants for leukocytes [42]. They are crucial to the inflammatory process in asthma as they recruit inflammatory cells from circulation to the airways [50]. Some important chemokines include CCL11/eotaxin, which is a potent eosinophil chemottractant [55], CXCL8/IL-8, which activates and recruits neutrophils [42], and CCL2/Monocyte chemoattractant protein 1 (MCP-1), which acts as a monocyte chemoattractant and activating factor [42]. Growth factors are cytokines, which play a part in airway remodelling, one of the cardinal features of asthma. Some of the key growth factors implicated in altering the structure of asthmatic airways include vascular endothelial growth factor (VEGF), transforming growth factor β (TGF-β), and connective tissue growth factor (CTGF) [54].

1.4 Type 2 inflammation in asthma

Although traditionally the inflammation in asthma was believed to be driven by Th2 type cytokines, it is now known that in a significant proportion of patients the disease is driven by different type of inflammation. Therefore it has been proposed that asthma can be divided into at least two distinct molecular phenotypes, "Th2-high" and "Th2-low" asthma, based on the degree of Th2 inflammation [56]. "Th2-low" asthma has been associated with poor response to steroids [56].

More recently it has been demonstrated that cytokines originally associated with Th2 cells can be secreted by other cell types, such as NK cells, type 2 innate lymphoid cells (ILC2s), and even Th1 cells under certain conditions [57]. Therefore it is common now to refer to “type 2-high” rather than “Th2-high” asthma. Patients with “type 2-high” and “type 2-low” asthma differ in their response to therapies, and although a number of approaches has been shown to reduce inflammation in “type 2” asthma, there are currently no therapies successful in treatment of patients without “type 2” inflammation [58]. Agents treating “type 2” asthma approved for use in the clinic include anti-IgE monoclonal antibody omalizumab, and anti-IL-5 monoclonal antibodies reslizumab and mepolizumab [58]. ‘‘Type 2–low” asthma is poorly defined and its biomarkers have not been described [57, 58]. The approaches investigated for treatment of ‘‘type 2–low” asthma include targeting TNF-α [59], and targeting cytokines associated with neutrophilia, such as CXCL8 [60] and IL-17 [61].

1.5 Airway smooth muscle (ASM) cells in asthma

ASM cells have a crucial role in the processes occurring in asthma. The contractile properties of ASM cells are responsible for excessive bronchoconstriction and AHR, seen in individuals with asthma [62]. Following the activation of receptors present on ASM cells, bronchoconstriction is initiated through myocyte contraction [62]. This can be a result of contractile agonists, such as histamine, secreted in response to exercise, most likely by mast cells [62, 63]. There are multiple mechanisms proposed to explain why patients suffering from asthma are hyperresponsive [62], including increased secretion of contractile mediators from mast cells [64], increased ASM mass [65], increased vagal tone [66], cytokine-induced increase in intracellular free calcium [67], and activation of the Rho kinase pathway [68]. ASM cells are also able to proliferate, undergo hypertrophy, and migrate, which contributes to airway remodelling and airflow obstruction [69]. Increased ASM mass is one of the characteristics of asthma [62], and may even be present before the first asthma symptoms appear, suggesting its role in the possible development of the disease [70].

Thickening of the ASM layer has been shown *in vivo* in lung sections isolated from patients with asthma. The thickness of the ASM layer and ASM cell size increased with disease severity [71]. The mechanisms leading to the increase ASM mass are not clear [62]. Both ASM cells hypertrophy (increase in size) and hyperplasia (increased cell number) have been suggested [72, 73]. More recently, it has been proposed that ASM cells can migrate within the airway wall [70]. This could be a result of fibroblasts [74], fibrocytes [75], or epithelial cells [76], differentiating to acquire characteristics of ASM cells; these cells are known as myofibroblasts [70, 74]. ASM cells also contribute to airway remodelling by secreting the components of extracellular matrix (ECM), which structure is altered in asthma, as well as enzymes which modify ECM [69]. ASM cells isolated from individuals with asthma have been shown to produce different amount of ECM proteins than these isolated from healthy controls; they produce more perlecan and collagen I, and less laminin α1 and collagen IV [77]. ASM cells from asthmatic patients have also been shown to hypersecrete VEGF [78], which is crucial in the regulation of angiogenesis and microvascular remodelling [79]. The importance of ASM in asthma can be demonstrated by the success of bronchial thermoplasty, a novel treatment that employs radiofrequency energy to reduce ASM, in improving symptom control and reducing exacerbations in patients [80].

1.6 Synthetic function of airway smooth muscle cells

The proinflammatory action of ASM cells results from their ability to synthesise and secrete a number of inflammatory mediators, such as cytokines, chemokines, and growth factors [81, 82]. Growth factors secreted by ASM cells, such as TGF-β, CTGF, VEGF, and matrix metalloproteinases (MMPs), also contribute to remodelling of the airways [81]. ASM cells also produce lipid mediators, such as leukotrienes, which induce bronchoconstriction, and prostanoids and isoprostanes, which can have a varied effects on ASM tone [81]. A list of inflammatory mediators secreted by ASM cell is shown in Table 1.1.

Table 1.1: Inflammatory mediators secreted by ASM cells

Chemokines	CC family	CCL5/RANTES CCL11/eotaxin CCL2/MCP-1 CCL7 CCL8 CCL17/TARC
	CXC family	CXCL10/IP-10 CXCL8/IL-8 CXCL1 CXCL2 CXCL3
	CX3C family	CX3CL1
Lipid mediators	Prostanoids	PGD_2 $PGF_{2\alpha}$ Thromboxane A_2 PGE_2 PGI_2
	Leukotrienes	LTD_4
	Isoprostanes	8-iso-PGE_1 8-iso-PGE_2 8-iso-$PGF_{2\alpha}$ 8-iso-$PGF_{3\alpha}$
Growth and Remodelling Factors	Matrix metalloproteinases (MMPs)	MMP-2 MMP-9 MMP-12
	Extracellular matrix components	Fibronectin Laminin Perlecan Chondroitin
	Other	TGF-β CTGF VEGF PDGF IGF bFGF
Other	Cytokines	IL-6 IL-5 GM-CSF Stem cell factor IL-1β IFN-γ
	Adhesion molecules	ICAM-1 VCAM-1

1.6.1 Cytokines and Chemokines

Cytokines are small, usually extracellular signalling proteins, which are involved in cell-to-cell interactions [83]. They have a crucial role in the initiation and maintaining of chronic inflammation in asthma [83]. Chemokines are low molecular weight (8–10 kDa) chemotactic cytokines, which attract inflammatory cells into tissues [81, 83]. Chemokines can be divided into several categories, based their molecular structure, with two major groups being CXC chemokines (α chemokines) and CC chemokines (β chemokines) [81, 83]. CXC and CC chemokines have different N-terminal cysteine residues, the CXC class have a single amino acid interposed between these cysteines, while in the CC class the cysteines are adjacent [84]. Following the initiation of an inflammatory process in the lung, ASM cells secrete large quantities of chemokines, which then recruit several different types in inflammatory cells into the airway milieu [81]. Chemokines then amplify the signals produced by inflammatory cells, leading to the increased inflammatory response. To some extent, chemokines are selective in their recruitment of inflammatory cells, hence eotaxin, CCL5/RANTES, and IL-5 mostly recruit eosinophils, CXCL8 recruits neutrophils and monocyte chemoattractant proteins (MCPs) attract monocytes, etc. [81]. ASM cells synthesise chemokines in response to stimulation with Th2 cytokines, e.g. IL-4 and IL-13, proinflammatory cytokines such as IL-1β and TNF-α, or mediators such as bradykinin and thrombin [81, 82].

1.6.1.1 CCL11/eotaxin

Eotaxin is a CC (β) chemokine produced by ASM cells, which recruits eosinophils from the systemic circulation to the lungs [85]. The constitutive secretion of eotaxin by ASM cells is further increased upon stimulation with proinflammatory cytokines, such as TNF-α and IL-1β [86], and Th2 cytokines, such as IL-4 and IL-13 [87]. Eotaxin expression has been shown to be increased in samples collected from patients with asthma [88, 89]. The levels of the eotaxin are markedly increased in sputum and plasma of asthmatic patients, compared to healthy controls [90]. The response to eotaxin is also enhanced in eosinophils isolated from asthmatic individuals [91]. ASM samples collected from subjects with asthma have increased eotaxin immunoreactivity compared to samples from healthy individuals, as evidenced by *in vivo* studies of airway sections [85]. Nakamura *et al.* showed that eotaxin levels are directly linked to asthma diagnosis and compromised lung function [92]. In another study, plasma eotaxin levels were also higher in patients with acute, poorly managed asthma, than in those with stable asthma [93]. Our group has demonstrated that the TNF-α-induced expression of eotaxin in ASM cells occurs via protein kinase C (PKC) βII, which recruits histone

acetyltransferase p300/CBP-associated factor (p/CAF) to increase the acetylation of histone H4 at the eotaxin promoter [94].

1.6.1.2 CXCL8/IL-8

CXCL8 is a 8.5-kDa CXC chemokine, which is crucial in the initiation and maintenance of inflammatory process in the lung [81]. This is mostly achieved by its potent chemoattractant effects on neutrophils, although CXCL8 is also acting as a mast cell chemoattractant [81, 95, 96]. CXCL8 also acts as a pro-angiogenic factor and may be important in the process of angiogenesis in asthmatic airways [97]. ASM cells have been shown to release CXCL8 in response to inflammatory mediators, such as TNF-α [98], IL-1β [99], TGF-β [100], and bradykinin [101]. Tracheal aspirates from asthmatic patients have 19 times higher levels of CXCL8 than those obtained from healthy controls [102]. CXCL8 is also upregulated in bronchial epithelium from patients with symptomatic asthma [103]. The levels of CXCL8 are significantly increased in exhaled breath condensate of asthmatic children, in comparison with healthy controls [104]. CXCL8 protein and mRNA expression was higher in ASM cells obtained from patients with asthma, than in those isolated from nonasthmatic individuals [96]. Free CXCL8 has been detected in the serum and bronchial tissue of subjects with severe atopic asthma, but it was undetectable in normal subjects and subjects with mild atopic asthma, which suggests that CXCL8 might be an indicator of asthma severity [105]. Research from our group shows that the increased secretion of CXCL8 from ASM cells in asthma results from abnormalities in histone acetylation, as ASM cells from asthmatic donors have increased histone H3 at the lysine-18 residue (H3K18) acetylation, and increased binding of histone acetyltransferase p300 compared with ASM cells from healthy subjects [106]. The functional homologue of human CXCL8 in mice is the murine keratinocyte-derived protein chemokine CXCL1/KC. Just like human CXCL8, mouse KC is associated with neutrophil recruitment and inflammation [107].

1.6.1.3 IL-6

Interleukin 6 (IL-6) is a pleotropic cytokine that acts as a proinflammatory mediator, although it has also been reported to have anti-inflammatory properties (inhibition of TNF-α and IL-1) [108]. IL-6 has been shown to promote Th2 differentiation of CD4+ T cells while suppressing Th1 differentiation [109]. Serum IL-6 levels are significantly higher in patients with asthma than in healthy controls and increase during asthmatic attacks [110]. IL-6 levels are also significantly higher in bronchoalveolar lavage fluid (BALF) obtained from patients with acute severe asthma (status asthmaticus) than in samples from patients with controlled asthma

and healthy subjects [111]. Interestingly, patients with intrinsic asthma have higher IL-6 levels in BALF, compared to patients with allergic asthma [112]. This finding is notable, as the patients with intrinsic asthma are usually more difficult to treat and require higher doses steroids; many of them are also steroid-resistant [112]. ASM cells secrete IL-6 in response to TNF-α and bradykinin [113, 114]. In ASM cells, TNF-α acts via a p38 Mitogen-activated protein kinase (MAPK)-dependent pathway to stabilize the IL-6 mRNA transcript [115]. Corticosteroids inhibit TNF-α–induced IL-6 secretion by reducing the stability of the IL-6 mRNA transcript, through upregulation of MAP kinase phosphatase 1 (MKP-1) [115].

1.6.1.4 CXCL10/IP-10

IFN-γ inducible protein of 10 kDa (IP-10) is member of the CXC chemokine subfamily and a potent chemoattractant for mast cells and T lymphocytes [116]. IP-10 has usually been associated with Th1 immune responses, as it is induced by IFN-γ and inhibited by IL-4 [117, 118], however it is not selective for Th1 type inflammation [119]. Additionally, mice overexpressing IP-10 in the lung have been shown to develop AHR and a Th2 inflammatory response, while mice deficient in IP-10 showed a reduced Th2-type allergic airway inflammation [120]. The expression of IP-10 by ASM has been shown *in vivo* in bronchial biopsies from subjects with asthma [121]. IP-10 mediates mast cell migration to ASM and has been detected in the ASM bundles in biopsies from patients with asthma, but not from healthy controls [121]. The levels of IP-10 are elevated in BALF from subjects with asthma, compared with non-smoking control subjects [122]. The induction of IP-10 release from ASM cells by cytomix (mix of cytokines IL-1β, TNF-α, and IFN-γ) is greater in cells obtained from patients with asthma, than in cells from healthy controls [123]. IFN-γ and TNF-α synergistically induce IP-10 release from human ASM cells [116]. Our group has previously demonstrated that in ASM, TNF-α activates the classical Nuclear factor kappa B (NF-κB) pathway, whereas IFN-γ activates Janus kinase 2 (JAK2)/Signal transducer and activator of transcription 1 α (STAT-1α) to induce IP-10 secretion, and that the stimulation with either of these cytokines induced histone H4 but not histone H3 acetylation at the CXCL10 promoter [116]. Moreover, our group has demonstrated that the synergy observed following IFN-γ and TNF-α stimulation results from increased recruitment of the coactivator cAMP response element binding protein (CREB) binding protein (CBP) to the IP-10 promoter along with heightened recruitment of RNA polymerase II to the promoter [116]. A different study by Hardaker *et al.* showed a synergistic effect of TNF-α and IFN-γ on IP-10 mRNA expression in human ASM cells [124]. In this study, despite IFN-γ not directly activating the NF-κB signaling pathway, the presence of both cytokines enhanced NF-κB activation.

1.6.1.5 TNF-α

TNF-α is a proinflammatory pleiotropic cytokine, which has an important role in the innate immune response, providing immediate host defence against invading organisms before the adaptive immune system is activated [125]. TNF-α is a 17kDa protein, which is cleaved from a biologically active 26kDa membrane-bound precursor protein by a TNF-α–converting enzyme [125]. The main TNF-α producing cells are macrophages, which secrete TNF-α in response to activation of membrane-bound pattern-recognition molecules, which detect common bacterial cell-surface products [125]. TNF-α is also produced by other proinflammatory, as well as structural cells. TNF-α is thought to contribute to abnormal inflammatory response in asthma and other inflammatory diseases, such as rheumatoid arthritis (RA) [53]. Patients with asthma have increased levels of TNF-α mRNA [126] and protein [127] in their airways. Furthermore, healthy individuals develop AHR and airway neutrophilia after being exposed to inhaled recombinant TNF-α [128]. The possible mechanisms for TNF-α – driven AHR include direct effect of TNF-α on ASM, or via the release of cysteinyl leukotrienes C_4 and D_4 [125]. Other mechanisms by which TNF-α affects asthmatic airways include its chemoattractant effect on neutrophils and eosinophils [129], increasing cytotoxicity of eosinophils towards endothelial cells [130], activating T cells [131], and increasing epithelial expression of adhesion molecules [132]. In severe refractory asthma, in addition to promoting AHR and inflammation, TNF-α is also thought to have a role in airway remodelling [125]. In ASM cells, TNF-α induces the production of various important cytokines and chemokines, such as eotaxin, CCL5/RANTES, CCL17/Thymus- and activation-regulated chemokine (TARC), CXCL8, Granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-6, and others [81]. TNF-α has also been shown cooperate with IFN-γ to synergistically modulate the expression of inflammatory genes in ASM. Cross talk between TNF-α and IFN-γ has been reviewed by Tliba *et al.* [133].

TNF-α – blocking agents have been subjects to numerous trials aiming to determine their potential use in asthma therapy, with varying results [59, 134-136]. Etanercept has been shown to induce a small but significant improvement in asthma control and systemic inflammation [135], but in a different study, no significant difference was seen between etanercept and placebo in subjects with moderate-to-severe persistent asthma over 12 weeks [136]. Infliximab improved asthma control in six patients who received it for at least 3 months, but two patients showed severe adverse effects (bacterial pneumonia and extension of spreading melanoma) [134]. In a study by Wenzel *et al.,* treatment with golimumab led to serious adverse events, including death and malignancies [59].

In the study described in this thesis, we have stimulated ASM cells isolated from healthy volunteers with TNF-α, to induce the secretion of cytokines from ASM cells and simulate the conditions in the airways of asthma patients. The importance of TNF-α in asthma and its potential as a target for development of asthma treatments have been review by Brightling et al. [125].

1.6.1.6 Other

Other chemokines secreted by ASM cells include CCL5/RANTES, CCL17/TARC, and CCL2/MCP-1. RANTES is a basic 8-kDa polypeptide of the CC chemokine subfamily, which acts as a potent chemoattractant for monocytes, memory T cells, and eosinophils [81, 137]. ASM cells secrete RANTES in response to stimulation with TNF-α [114], IL-1β [138] or platelet activating factor (PAF) [139]. This secretion can be further augmented by IFN-γ [137]. The levels of RANTES in plasma are significantly elevated during acute asthmatic attacks, compared to that in healthy controls [140]. In addition, plasma RANTES levels are higher during the attacks than in the asymptomatic state in the same patients [140]. CCL17/TARC is a CC chemokine, which acts as a Th2 cell chemoattractant, as it is highly specific for CCR4 receptor that is expressed on Th2 but not Th1 lymphocytes [81, 141]. TARC is upregulated in mouse model of allergic asthma, and the specific antibodies against TARC attenuate Ovalbumin (OVA)-induced airway eosinophilia and airway hyperresponsiveness [142]. TARC levels are significantly increased in the serum and sputum of subjects with asthma, compared to healthy individuals [143]. ASM cells release TARC in response to combined stimulation of TNF-α with IL-4 or IL-13, but not with any of these cytokines alone [144]. TNF-α + IL-13 or TNF-α + IL-4-induced TARC release is inhibited by β-agonist isoproterenol, cAMP analogues or forskolin, but not by dexamethasone [144]. CCL2/MCP-1 is a 13 kDa CC chemokine, which acts as a potent chemoattractant for monocytes [145]. The levels of MCP-1 in the serum of asthma patients are significantly higher compared to healthy controls, and further increase during the acute asthma attack [146]. The levels of MCP-1 are also increased in BALF and bronchial tissue of asthmatic patients, compared with healthy individuals [147, 148]. ASM cells secrete MCP-1 in response to stimulation with IL-1β, TNF-α, and endothelin-1 [138, 149-151].

1.6.2 Lipid mediators

Although lipids were traditionally considered to be solely of dietary and structural importance, it is now known that many intercellular mediators are lipid-derived [152]. Lipid mediators have now been shown to influence many processes relevant to asthma, such as

the regulation of ASM tone, chemokine production from ASM cells, and airway remodelling [152].

1.6.2.1 Prostanoids

Prostanoids are produced from membrane phospholipids, by the cyclooxygenase (COX)-mediated conversion of arachidonic acid into prostaglandin H2 (PGH_2), which is then metabolized to various prostanoids, such as PGE_2, PGD_2, and PGI_2, and TXA_2 [81, 153, 154]. There are two main isoforms of COX. COX-1 is constitutively expressed in most cells and a housekeeping gene, which is responsible for prostanoid formation under physiological conditions [81, 154-156]. COX-2 is induced in inflammatory conditions and responsible for production of prostanoids at the site of inflammation [81, 155, 156]. COX-3 is a splice variant of COX-1, which is mostly expressed in the brain and is the site of action of acetaminophen (paracetamol) [157, 158]. Inflammatory stimuli, including IL-1β and bradykinin, induce COX-2 expression in ASM cells [159, 160]. PGE_2 is the main prostanoid produced by ASM cells; other prostanoids are produced in much smaller quantities [161]. PGE_2 acts mostly as a protective, anti-inflammatory mediator in the lung, by inhibiting mast cell mediator release, eosinophil chemotaxis and survival, and IL-2 and IgE production by lymphocytes [161, 162]. Other bronchoprotective actions of PGE_2 include inhibition of ASM cell proliferation [163] and inhibition of GM-CSF production from ASM cells [164]. These protective actions suggest that the production of prostanoids may act as a negative feedback mechanism, to limit the inflammatory response [81, 162]. However, PGE_2 can also induce VEGF production from ASM cells [165], and although it acts as a bronchodilator at low concentrations, it also contracts ASM at higher concentrations [49, 162]. Additionally, ASM cells express proinflammatory prostanoids, TXB_2, $PGF_{2\alpha}$ and PGD_2 [160]. Prostanoids also have a varied effect on ASM tone; while PGD_2, $PGF_{2\alpha}$, and TXA_2, TXB_2 act as bronchoconstrictors, PGE_2 (at low concentrations) and PGI_2 have bronchodilator effects [162, 166, 167].

1.6.2.2 Leukotrienes

Leukotrienes are produced by the action of enzyme 5-lipoxygenase (5-LO) on arachidonic acid [168]. In asthma, leukotrienes have been shown to induce potent bronchoconstriction [168, 169], contribute to the inflammatory process [170], and airway remodelling [169, 171]. Leukotriene receptor antagonists have also been used to treat asthma in clinical practice [168]. However, unpublished data from our group suggest that the levels of leukotrienes secreted by ASM cells are low and not as functionally relevant as prostanoids.

1.6.2.3 Isoprostanes

Isoprostanes are prostaglandin-like compounds, which are produced by free radical and reactive oxygen species peroxidation of arachidonic acid [172]. Although isoprostanes (especially 8-iso-$PGF_{2\alpha}$) are used as markers of oxidative stress in asthma and other pulmonary diseases [172, 173], their biological effects and their contribution to asthma pathogenesis is less characterised. 8-iso-$PGF_{2\alpha}$ and 8-iso-PGE_2 contract human ASM via prostanoid TP receptors, while 8-iso-$PGF_{3\alpha}$ relaxes carbachol-precontracted ASM [174]. However, studies with canine and porcine tissues suggest that the effect of isoprostanes on ASM tone may be species-specific [175]. 8-iso-PGE_1 and 8-iso-PGE_2 enhanced the IL-1β-induced release of granulocyte-colony stimulating factor (G-CSF) from ASM cells, while inhibiting IL-1β-induced production of GM-CSF [176]. IL-17 induces the release of 8-isoprostanes from ASM cells, however, macrolides (azithromycin and erythromycin) suppress that induction [177].

1.6.3 Growth and remodelling factors

Common features of airway remodelling include increases in ASM mass, ECM deposition and vascularisation. ASM cells synthesise and secrete many growth and remodelling factors, contributing to these changes.

1.6.3.1 Matrix metalloproteinases (MMPs)

Matrix metalloproteinases (MMPs) are zinc-dependent proteolytic enzymes, which have a role in cell migration and tissue remodelling, as they are essential for the development, turnover and degradation of ECM proteins [178]. MMPs contribute to pathogenesis of asthma by several different mechanisms. MMPs-induced breakdown of ECM leads to the destruction of healthy lung parenchyma, while the degradation of ECM releases chemotactic fragments, which attract inflammatory cells into the airways [178]. Moreover, MMPs promote the extravasation of inflammatory cells through the vascular endothelium and their migration into the ECM and through the airway epithelium [178]. Human ASM cells have been shown secrete progelatinase A (precursor for MMP-2), TNF-α-induced gelatinase B (MMP-9), MMP-3 and Membrane type 1 metalloprotease (MT1-MMP) [179, 180]. They have also been shown to secrete Tissue inhibitor of metalloproteinase-1 and -2 (TIMP-1, TIMP-2), which are inhibitors of MMPs [180]. Patients with asthma have been shown to display an increased gelatinolytic activity linked to MMP-2 and MMP-9 and higher levels of TIMP-1 in their sputum [181].

1.6.3.2 Extracellular matrix components

Serum stimulation induces ASM to secrete fibronectin, laminin, perlecan, chondroitin sulfate, elastin, thrombospondin, collagen and decorin [182]. Extracellular matrix protein components are altered in asthmatic ASM cells. The secretion of perlecan and collagen I is increased in ASM cells isolated from asthma patients, while the production of laminin α1 and collagen IV is reduced, compared to cells obtained from healthy subjects [182]. Chondroitin sulfate production is detected only in ASM cells from healthy subjects [182].

1.6.3.3 Other

Other growth and remodelling factors secreted by ASM include TGF-β, CTGF, VEGF and other angiogenic factors. TGF-β has multiple roles in the remodelling process in ASM. It stimulates the synthesis of collagen, fibronectin, tenascin, vitronectin, CTGF, elastin and several proteoglycans, while downregulating synthesis of MMPs and upregulating the synthesis of TIMPs [81]. TGF-β can also act as a mitogen for ASM cells [183] and has been shown to stimulate ASM cell migration [184] and proliferation [185]. TGF-β induces CTGF production from ASM and this induction is further increased in ASM cultured from patients with asthma compared with healthy subjects [186]. Then TGF-β-induced CTGF secretion leads to the production of fibronectin and collagen I from ASM cells [187]. VEGF is a crucial angiogenic factor, which is overexpressed in asthmatic sputum and tissue [188, 189]. Increased VEGF levels are also associated with asthma severity in patients [190]. The production of VEGF from ASM cells is constitutive and increases in response to stimulation with IL-4-, IL-13 [191], IL-5, TGF-β [192], IL-1β [193] and bradykinin [194].

1.7 Epigenetics and its role in asthma

Epigenetic modifications have an important role in the development and progress of asthma [195-197]. Epigenetics is the study of heritable changes in gene expression, which are not caused by the changes in DNA sequence, but result from modifications of DNA and histone proteins, which affect chromatin structure. [195, 198]. Three main epigenetic mechanisms are DNA methylation, histone modifications, and noncoding RNAs [195]. DNA methylation and histone modifications control the state of chromatin and the availability of genomic DNA for gene transcription. Tightly condensed, “closed” chromatin state is associated with repression of transcription, while “open” chromatin state is linked to loosening of the DNA packaging and leads to active gene transcription [195]. Figure 1.1, taken from our review in CHEST [199], shows the summary of epigenetic modifications in humans.

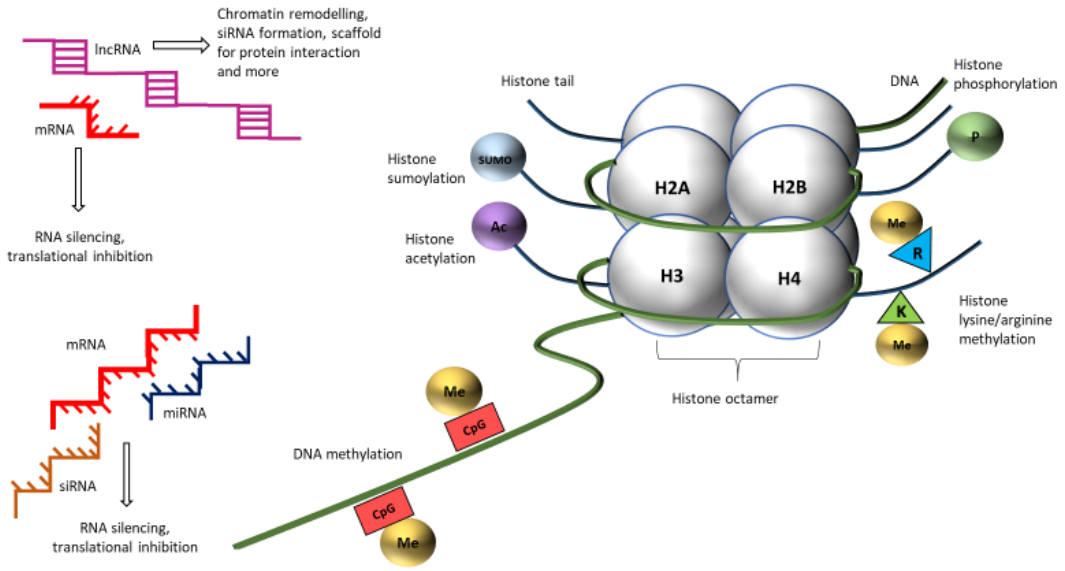

Figure 1.1: Summary of epigenetic modifications. DNA methylation, histone modifications, and noncoding RNAs, are the three main epigenetic mechanisms. Histones are organized in octamers, composed of two copies of each of the histones: H2A, H2B, H3, and H4; and wrapped by 147 base pairs of DNA. Histones have N- terminal tails, which protrude through the DNA and contain multiple residues, susceptible to posttranslational modifications, including acetylation, phosphorylation, sumoylation, ubiquination and methylation. These modifications have a role in gene regulation, leading to either gene transcription or repression, depending on the type and site of the modification. DNA methylation is the addition of the methyl group to the C5 position of cytosine residues, primarily where cytosine is followed by a guanine (CpG sites). The regions of human genome with the high content of CpG sites are called CpG islands. Hypermethylation of CpG islands leads to transcriptional repression, while their hypomethylation is linked to active transcription. Non-coding RNAs (ncRNAs) are RNA molecules, which are not translated into proteins, but can regulate the transcription, stability or translation of protein-coding genes. There are many types of ncRNAs, including long non-coding RNA (lncRNA), microRNA (miRNA), short interfering RNA (siRNA), and others, such as piwi-interacting RNA (piRNA). They act by interacting with messenger RNA (mRNA), histone modifying complexes, DNA methyltransferases, and various other proteins.

1.7.1 DNA methylation

The most described and understood mechanism to date is DNA methylation – a reversible modification of cytosine residues, primarily where cytosine is followed by a guanine (CpG sites) [198]. CpG islands are the regions of human genome with the high content of CpG sites - hypermethylation of CpG islands is associated with transcriptional repression, while hypomethylation in these regions leads to active transcription [195, 198]. Environmental factors, diet, and aging can influence DNA methylation, therefore it may provide the explanation to the impact of the environment on the development and inheritance of allergy [197]. The degree of DNA methylation differs between ASM from healthy and asthmatic individuals and varies with disease severity [200]. Comparison of healthy and non-severe asthma subjects shows differences in DNA methylation of genes involved in the regulation of cell proliferation and apoptosis. However, genes which differ in their DNA methylation

profile between samples from severe asthma and healthy subjects are associated with ASM contraction, proliferation and endocytosis [200]. Human ASM Phosphodiesterase 4D (PDE4D) is overexpressed in asthma and is associated with increased AHR. Overexpression of PDE4D in asthmatic ASM is caused by hypomethylation of PDE4D promoter [201]. The inhibition of DNA methyltransferase 1 (DNMT1) with 5-Aza-2'-deoxycytidine (Aza-CdR, or Decitabine) inhibited Platelet-derived growth factor (PDGF)-induced increase in contractility, migration and proliferation of rat ASM cells [202].

1.7.2 Non-coding RNAs

Non-coding RNAs (ncRNAs) are functional RNA molecules that are transcribed from DNA but not translated into proteins. They can however regulate the transcription, stability or translation of protein-coding genes [203]. The most extensively studied type of ncRNAs are microRNAs (miRNAs) - 19–24 nucleotide-long, single-stranded RNAs, which induce translational repression and/or messenger RNA (mRNA) degradation by sequence-specific binding to the 3'-untranslated region (3'-UTR) of target mRNA [203]. MiRNAs are known to modify many ASM functions, such as synthetic functions (miR-143-3p, miR-145, miR-708, miR-140-3p, miR-221, miR-146a, miR-155), contractility (miR-145, miR-133a, miR-708), and proliferative functions (miR-150, miR-139-5p, miR-23b, miR-143-3p, miR-145, miR-135a, miR-10a, miR-203, miR-221, miR-21, miR-138), by acting on diverse cellular targets (BCYRN1, SMAD3, NFATc1, TRPC1, PI3 kinase, c-Abl, PDK1, RhoA, JNK MAPK) [204-216].

1.7.3 Histone modifications

The basic structural units that build eukaryotic chromatin are nucleosomes, composed of two units of each of the core histones - H2A, H2B, H3, and H4 [195, 217], wrapped by 147 base pairs of DNA [217]. Nucleosome core particles are connected by the linker DNA, and a linker histone (H1 histone). The resulting structure is known as a chromatosome [218]. Histones have N- terminal tails, which protrude through the DNA and are able to undergo a series of posttranslational modifications; acetylation, phosphorylation, sumoylation, ubiquination and methylation [217].

1.7.3.1 Histone acetylation

Histone acetylation is the transfer of an acetyl group from acetyl-CoA to histone lysine (K) ε-amino groups [219]. Enzymes that catalyse the addition of acetyl groups to the histone tails are termed histone acetyltransferases (HATs), and enzymes that catalyse the removal of these groups are called histone deacetylases (HDACs) [219]. Furthermore, transcriptional coactivators, such as CBP, steroid receptor coactivator 1 (SRC-1), transcription intermediary

factor 2 (TIF2)/glucocorticoid receptor interacting protein 1 (GRIP-1), and p/CAF have been found to possess intrinsic HAT activity [220]. Following the acetylation of the core histones by HATs, chromatin remodelling occurs (carried out by the enzymes such as Brg1 [220]), co-transcriptional proteins and polymerase II are recruited, leading to the initiation of transcription [217]. Histone deacetylation by HDACs is linked to repression of transcription [217]. Acetylated lysine residues are recognized by bromodomains, including the bromodomain and extraterminal (BET) proteins, making them crucial to the regulation of gene transcription [221]. Histone acetylation has an important role in regulating chemokine secretion by ASM cells [94, 106, 116, 151].

1.7.3.2 Histone methylation

Histone methylation is the addition of a methyl group to primarily lysine (K) and arginine (R) residues on the N-terminal tail of histones. Lysine residues are mono (me1), di (me2), or tri (me3) methylated on their ε amine group, while arginine residues are mono (me1), symmetrically dimethylated (me2s), or asymmetrically dimethylated (me2a) on their guanidinyl group [222]. Histone methylation has varied effects on gene regulation, depending on its site and extent [223]. For example, histone H3 arginine (R) 17 asymmetrical dimethylation (me2a) (H3R17me2a), H3R26me2a, H3R2me2s (symmetrical dimethylation), H4R3me2a and histone H3 lysine (K) 4 trimethylation (H3K4me3) are linked to active transcription, while H3R2me2a, H4R3me2s, H3R8me2s and H3K27me3 are linked to transcriptional repression [222, 224]. H4R3me2a, an important mark associated with transcriptional activation, has been investigated in this study. The enzymes that catalyse the transfer of methyl groups from S-adenosyl methionine (AdoMet) to histones are termed histone methyltransferases and can be divided in to three families: the SET domain containing proteins and Dot1 like proteins, which catalyse lysine methylation, and protein arginine N-methyltransferases (PRMTs), which catalyse arginine methylation [222, 224-226]. Lysine demethylases remove the methyl group from lysines and can be divided into two main groups: amine oxidases and Jumonji C (JmjC) domain containing dioxygenases, [222, 227, 228]. Little is known about arginine demethylases. Peptidyl arginine deaminase 4 (PAD4) and jumonji domain-containing protein 6 (JMJD6) have been shown to be involved in arginine demethylation [229, 230]. PAD4 converts both methylated and unmethylated arginine to citrulline, rather than removing the methyl group [223]. The ability of JMJD6 to carry out arginine demethylation has also been questioned [224]. More recently, it has been shown that some lysine demethylases, such as KDM3A, KDM4E, KDM5C, can also act as arginine demethylases *in vitro* [231].

1.7.4 PRMTs

PRMTs are found in both the cytoplasm and the nucleus (with the exception of PRMT6, which is found only in the nucleus [223], PRMT3, which is exclusively cytoplasmic [232], and PRMT8, which is associated with the plasma membrane [233]) and have a role in many important cellular processes, such as DNA repair, RNA processing, regulation of transcription and signal transduction [223]. There are eleven mammalian PRMTs [223], which can be divided into three classes [234]. Following the formation of the monomethylarginine (MMA) intermediate, which is catalysed by type I, type II, and type III PRMTs, type I PRMTs (PRMT1, 2, 3, 6, 8 and PRMT4) further catalyse the intermediate to asymmetrical dimethylarginine (ADMA), while type II PRMTs (PRMT5 and PRMT9) catalyse the production of symmetrical dimethylarginine (SDMA) [223, 224, 235]. PRMT7 is the only known type III PRMT, catalysing the production of MMA only [234]. PRMTs 10 and 11 do not possess any enzymatic activity [223], while PRMT2, has only very weak type I activity [224, 236]. Figure 1.2 shows the end product of histone arginine methylation, depending on the type of PRMT catalysing the reaction. PRMTs often have a preference for methylating arginine residues that are flanked by one or more glycine residues – motifs often referred to as GAR sequences (Glycine and Arginine Rich) [224]. However, this is not the case for all of the PRMTs, for example, PRMT4/CARM1 favours arginine neighbouring a PGM-rich (proline, glycine, and methionine) motif [237], and PRMT7 targets RXR Sites (a pair of arginine residues separated by one residue) in Lysine- and Arginine-rich Regions [238]. Methylated arginine motifs are recognized and bound by the Tudor domains [239]. Tudor domain-containing proteins that interact with methylarginine include Survival of motor neuron (SMN), Splicing factor 30 (SPF30), and Tudor domain-containing (TDRD) protein 1/2/3/6/9/11 [240]. Thirty-six proteins that harbour Tudor domains have been identified in humans up to date [239, 240]. All Tudor domains have an aromatic cage, allowing for cation-pi interactions between aromatic residues of Tudor domains and the cationic carbon of methylarginine [240]. The methyl groups provide increased hydrophobicity within the Tudor domain binding pocket [240].

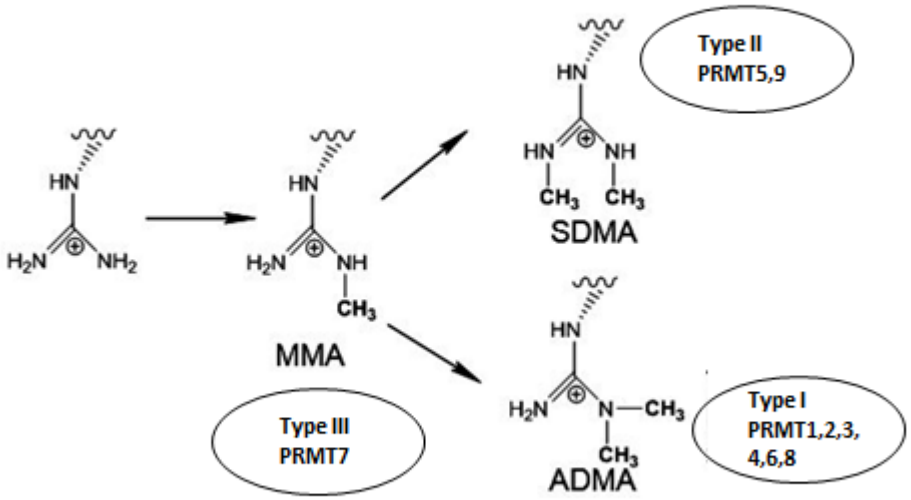

Figure 1.2: Arginine methylation mediated by three types of PRMTs. (Adapted from Yue *et al.* [241])

1.7.4.1 PRMT1

PRMT1 was the first member of the PRMT family to be identified; it is the most predominant PRMT in eukaryotic cells and carries out 85% of total protein arginine methylation [223, 224]. It is a primary type I enzyme, regulating the functions of other PRMTs [242]. It is primarily located in the cytoplasm [223]. PRMT1 -deficient mice embryos fail to develop beyond E6.5 (embryonic day 6.5) and die *in utero*, despite the embryonic stem cells remaining viable [243], highlighting the importance of PRMT1 on early development. PRMT1 is involved in the regulation of various cellular processes, such as nuclear–cytoplasm transport, signal transduction and transcription [244]. It has a role in RNA biosynthesis, processing and transport [223, 244, 245]. Lastly, PRMT1 methylates H4R3 tails, which facilitates subsequent acetylation of histone H4 by p300 [246, 247]. This event is an important step in the establishment and maintenance of an active euchromatin structure [247]. PRMT1 activity is highly dependent on two methionine residues, Met-48 and Met-155, and mutation of these residues results in a loss in activity and a change in distribution of mono- and dimethylated products [248]. The enzymatic activity of PRMT1 can be modulated by a number of proteins, including BTG1, TIS21/BTG2 [249], hCAF1/CNOT7 [250], ILF3 [251], and YY1 [252]. hCAF1, also known as CNOT7, acts as a cofactor of PRMT1, and regulates its enzymatic activity *in vitro* and in cellular systems, with different effects according to the substrate [250]. CNOT7 induces a dose-dependent inhibition of PRMT1-mediated methylation of histone H4 *in vitro* and the methylation of histone H4 is improved in MCF-7 cells transfected with CNOT7 siRNAs

[250]. ILF3 belongs to a group of proteins (including ILF1, nuclear NF45/ ILF2 and NF90) that can bind to the antigen receptor response element 2 (ARRE-2) sequence [253]. ILF3 enhances the methylation activity of PRMT1 in *in vitro* methyltransferase assays [251].

1.7.4.2 PRMT2

PRMT2 is a weak type I methyltransferase [236] and acts as a transcription co-activator by methylating histone H4 [236]. PRMT2 acts as a coactivator of both the androgen receptor and the Estrogen receptor α (ERα) [254, 255]. PRMT2 is also able to promote apoptosis, by blocking IκB-α nuclear export and inhibiting NF-κB-dependent transcription [256]. Yoshimoto *et al.* showed that PRMT2 represses the activity of E2F1 (a member of the E2F transcription factors family, which regulate cell cycle progression by controlling gene expression of key cell cycle regulators), through binding to and interacting with the retinoblastoma gene product [257]. PRMT2- knockout mice are viable and mostly normal, although their response to vascular injury is abnormal [257].

1.7.4.3 PRMT3

PRMT3 recognises its substrate via the zinc finger at its N-terminus [258]. It is found exclusively in the cytosol and considered to have no epigenetic function [224].

1.7.4.4 PRMT4/CARM1

PRMT4, also known as CARM1 (co-activator-associated arginine methyltransferase 1), was first identified as a protein binding p160 transcriptional co-activator GRIP-1 [259]. CARM1 methylates R2, R17 and R26 sites in histone H3 [223, 260]. PRMT4 is also able to methylate a number of non-histone proteins, such as CBP/p300, steroid receptor coactivator 3 (SRC-3) and RNA polymerase II [260]. CARM1-catalysed methylation of CBP/p300 modulates its co-activator transcriptional activity [261]. The methylation of SRC-3 by CARM1 promotes dissociation of the SRC-3/CARM1 coactivator complex, reducing the transcriptional response [262]. CARM1-catalysed methylation of RNA polymerase II facilitates the expression of select RNAs [263]. CARM1 functions as a transcriptional coactivator for various transcriptional factors, such as nuclear receptors, p53, E2F1 and NF-κB [259, 264-266]. PRMT4 is known to cooperate synergistically with histone acetylation. An *et al.* demonstrated that p53-dependent transcriptional activation results from p300-mediated histone acetylation, followed by H3 histone arginine methylation by CARM1 [264]. Daujat *et al.* showed that estrogen stimulation of the *pS2* promoter leads to Histone H3 K18 and K23 acetylation and subsequent CARM1 mediated methylation at R17 and transcription [267]. CARM1 mediated

H3R17me2a and H3R26me2a also work in conjunction with acetylation to inhibit the binding of corepressor Nucleosome Remodelling and Deacetylase (NuRD) and Transcription intermediary factor 1 (TIF1) family proteins to the Histone H3 tail, protecting chromatin from deacetylation [260]. CARM1-knockout mice die during late embryonic development or immediately after birth [268]. Histological examination of CARM1$^{-/-}$ embryos showed abnormalities in their lung anatomy and function [268]. CARM1-knockout mice display aberrant T cell development, probably due to the lack of thymocyte cyclic-AMP regulated phosphoprotein (TARPP) arginine methylation**.** [269]. Overexpression of CARM1 has been linked to prostate, breast and colorectal tumours [237, 270, 271]. 75% of colorectal cancer samples have been shown to overexpress CARM1 [237].

1.7.4.5 PRMT5

PRMT5 is the predominant type II PRMT [224, 272], and methylates histones H2A and H4 at R3 and histone H3 at R8, in cooperation with its cofactor MEP50 [273]. Contrary to other PRMTs, PRMT5 is generally associated with transcriptional repression [223, 224, 273, 274]. PRMT5 is present in several histone deacetylase co-repressor complexes, such as SIN3A/HDAC, MBD2/NuRD, and N-CoR/SMRT, which negatively regulate transcription [223, 272, 273]. PRMT5 also catalyses the methylation of non-histone proteins, such as Sm protein B/B, LSm4, SmD1 and SmD3 [275]. This event promotes the assembly of these proteins into small nuclear ribonucleoproteins (snRNPs), therefore PRMT5 functions in RNA processing [275]. PRMT5 also has a function in cell cycle control, DNA repair, and transcriptional elongation [275]. PRMT5-null mice die between E3.5 and E6.5 suggesting an important role in embryonic development [276]. PRMT5 is overexpressed in gastric, colorectal and lung cancer, as well as in lymphoma and leukaemia [237]. PRMT5 has oncogene-like properties, as it is able to repress a number of tumour suppressor genes, such as p53, suppressor of tumorigenicity 7 (ST7) and nonmetastatic 23 (NM23) [237, 273].

1.7.4.6 PRMT6

PRMT6 resides solely in the nucleus and undergoes automethylation [277]. It is highly expressed in human kidney and testes [277]. PRMT6 has been associated with both transcriptional repression (via methylation of H3 at R2 and H2A at R29) and transcriptional activation (when methylating H4 and H2A at R3, and H3 at R42) [278-280]. PRMT6 is the major H3R2 methyltransferase in mammalian cells [278]. Hyllus *et al.* demonstrated that the methylation of H3R2 by PRMT6 interferes with H3 K4 trimethylation, inhibiting transcription of genes which are regulated by H3 K4 trimethylation, such as Hox

genes [278]. PRMT6 also methylates a number of non-histone proteins, such as high-mobility group (HMG)1a and HMG1b, suggesting that it may have a role in RNA processing and chromatin remodelling [281-283]. PRMT6 methylates DNA polymerase β, which suggests its role in DNA base-excision repair [284]. PRMT6 is overexpressed in bladder and lung cancer cells [285].

1.7.4.7 PRMT7

There has been some controversy with regards to the product of PRMT7-catalysed methylation. Some reports claim PRMT7 is the only type III methyltransferase, with MMA being its sole product [234]. However, PRMT7 has also been reported to be a type II methyltransferase, capable of producing SDMA modifications in proteins [286], and to methylate histones H2A and H4 at R3 *in vitro* [287]. Type II methyltransferase activity of PRMT7 has later been questioned [234]. It has been suggested, that like PRMT5, PRMT7 methylates Sm proteins and mediates RNA splicing events [288]. PRMT7 functions in DNA repair, by repressing genes involved in DNA repair, such as *ALKBH5*, *APEX2*, *POLD1*, and *POLD2* [287].

1.7.4.8 PRMT8

PRMT8 is currently believed to be expressed exclusively in the brain, which suggests that it may be involved in neuronal differentiation [233]. It is also the only PRMT which is bound to the plasma membrane [233]. The abnormalities in motor behaviours in PRMT8 knockout mice indicated that PRMT8 may have a role in cerebellar-dependent behavioural systems, such as motor coordination and attention [289]. The role of PRMT8 in epigenetic regulation is poorly defined [223].

1.7.4.9 PRMT9

In comparison with other PRMTs, PRMT9 has not yet been fully characterised [224]. PRMT9 is a type II methyltransferase, capable of producing MMA and SDMA, when methylating SAP145 at arginine 508 gene [235]. It has no activity on core histones or GAR motif-containing proteins [235].

1.7.5 Histone modifications and asthma

Although traditionally the research of epigenetics in asthma focused on DNA methylation, there is an increasing volume of evidence showing a role of histone modifications in pathological processes occurring in asthma.

1.7.5.1 Histone acetylation

Bronchial biopsies show that individuals with asthma have increased HAT activity and reduced HDAC activity compared to healthy subjects [290]. It has been suggested that the abnormal histone acetylation seen in asthma contributes to the inflammation in asthmatic airways, as histone modifications regulate the transcription of genes coding for many chemokines, which are responsible for inflammatory cell recruitment in ASM. One of these chemokines is eotaxin, a potent eosinophil chemoattractant [55]. Previous research from our group shows that TNF-α-induced eotaxin expression in ASM cells is a result of increased histone H4 acetylation at the promoter, due to PKCβII and its recruitment of histone acetyltransferase p/CAF [94]. CXCL8 is a neutrophil chemoattractant, which is hypersecreted by ASM from asthmatic subjects [106]. This results from increased recruitment of the HAT p300, leading to elevated H3K18 acetylation at the CXCL8 promoter [106]. The inhibition of BET proteins, which recognise acetylated histone lysine residues, reduced CXCL8 secretion from ASM [106]. In another study, asthma related proinflammatory cytokine TNF-α increased acetylation of histone H4 at the CXCL8 promoter in human ASM cells [151]. TNF-α and IFN-γ synergistically induce IP-10/CXCL10, a mast cell and T lymphocyte chemoattractant, in ASM cells, by the acetylation of histone H4 at its promoter [116]. The synergistic effect of TNF-α and IFN-γ was regulated by the transcriptional co-activator CREB binding protein.

Besides its effect on chemokines, histone acetylation may contribute to the inflammation in asthmatic ASM cells by regulating transcription of other genes. Vascular cell adhesion protein-1 (VCAM-1) is a cell adhesion molecule responsible for recruitment and infiltration of polymorphonuclear leukocytes [291]. The acetylation of histone H4 by HAT p300 regulates TNF-α, endothelin-1 and IL-1β-induced upregulation of VCAM-1 in human ASM [291-293]. Histone deacetylase HDAC4 has also been shown to regulate TNF-α- and IL-1β-induced VCAM-1 expression via a p300-independent pathway [292, 293]. COX-2 is an enzyme responsible for prostaglandin synthesis from arachidonic acid [81]. The acetylation of histone H4 is involved in regulation of COX-2 transcription by bradykinin and IL-1β in human ASM cells [294].

Histone acetylation is also involved in the regulation of airway remodelling in asthmatic ASM. The histone deacetylase HDAC8 regulates ASM cytoskeleton dynamics [295]. Pharmacological inhibition of BET proteins reduces ASM cell proliferation induced by foetal calf serum and TGF-β. However, ASM cells obtained from subjects with asthma were partially

resistant to the effect of BET inhibitors [296]. MMP-9, which degrades ECM and basement membranes, is increased in the lungs of asthmatic patients [297]. TNF-α-induced MMP-9 upregulation in ASM is mediated by p300 and the acetylation of histone H3 [298].

Another way in which histone acetylation affects ASM in asthma is via regulating ASM contractility, associated with AHR. HDAC8 has been shown to regulate ASM contractility [295]. HDAC inhibitor Trichostatin A (TSA) abrogates methacholine-induced AHR in mice, and inhibits agonist-induced contraction in human precision-cut lung slices, possibly by disrupting Ca^{2+} release [299]. Other HDAC inhibitors: suberoylanilide hydroxamic acid (SAHA), diamide (1), and MGCD0103, significantly reduced the antigen-induced contraction of sensitised guinea pig tracheal rings as well the contraction induced by G-protein coupled receptor agonists [300]. MGCD0103 also reduced sodium fluoride-, KCl- and peroxyl radical generator-induced contraction of guinea pig tracheal rings [300].

1.7.5.2 Histone methylation

Compared to histone acetylation, there are fewer studies investigating the impact of histone methylation in asthma. Nonetheless, recent evidence suggests that histone methylation may have a role in inflammation and remodelling in asthmatic airways. Sun *et al.* showed that PRMT1 plays an important role in asthma pathogenesis - the expression of PRMT1 was significantly upregulated in the lungs of AIPI (Antigen-induced pulmonary inflammation, a rat model for asthma) rats [301]. PRMT1 knockdown, and its inhibition by arginine methyltransferase inhibitor 1 (AMI-1), reduced the IL-4- stimulated levels of eotaxin and CCR3 in the epithelial cells [301]. The same group has later demonstrated that inhibiting PRMT1 activity in primary human lung fibroblasts decreases PDGF-BB-induced cell proliferation, COX-2 production, collagen-1A1 secretion, and fibronectin production, suggesting that PRMT1 has a role in lung tissue remodelling [302]. More recently, the group showed the role of PRMT1 in remodelling in asthmatic ASM cells. PRMT1 expression was significantly increased in lung tissue sections and in ASM cells isolated from patients with severe asthma, due to reduced miR-19a expression [303]. Inhibition of PRMT1 abrogated collagen type I and fibronectin deposition, cell proliferation, and migration of ASM cells from asthmatic patients [303]. Rogerio *et al.* demonstrated that ellagic acid, an inhibitor of CARM1/PRMT4- mediated H3R17me2a [304] reduces inflammation in a murine model of ovalbumin-induced asthma [305]. CARM1 expression is significantly reduced in AIPI rat lungs [301] and the loss of CARM1 prolongs bleomycin-induced pulmonary fibrosis in mice [306]. Elevated lung levels of ADMA, a product of type I methyltransferase activity, have been found

in murine models of allergic airway inflammation (AAI) resembling asthma [307]. Elevated ADMA levels lead to an increase in AHR and collagen deposition in mice [308], and potentiate lung inflammation in a mouse model of allergic asthma [309]. ADMA and SDMA levels are also increased in lung homogenates of OVA-sensitized and -challenged (OVA/OVA) mice and in the lungs from humans in asthma [310]. The inhibition of type I PRMTs (PRMT1, 2, 3, 6, 8 and PRMT4/CARM1) could therefore reduce ADMA levels and inhibit AHR, airway remodelling and inflammation observed in asthma.

VEGF is a key angiogenic molecule, implicated in asthma severity and airflow obstruction [311]. Our group has previously shown that abnormal histone lysine methylation is responsible for increased VEGF secretion from asthmatic ASM cells [311]. The hypersecretion of VEGF is caused by the reduced association of the transcriptional repression marker histone H3K9me3 and increased association of the transcriptional activation mark H3K4me3 [311]. PRMT1 and PRMT6 could potentially regulate the secretion of VEGF, by blocking H3K9me3 and H3K4me3, respectively [311].

1.7.5.3 Histone modifications as a potential therapeutic target

The volume of evidence suggesting the role of histone modifications in inflammatory and remodelling processes in asthma makes them an emerging target for development of new treatments. Interestingly, many agents which are already used in asthma therapy have been shown to act on histones or histone modifying enzymes, although they were not originally designed to target epigenetic modifications. Glucocorticoids and β_2-agonists, which are the drugs of choice in treatment of chronic asthma, can modify histone acetylation in human ASM cells - they repress TNF-α-induced eotaxin gene transcription via the inhibition of histone H4 acetylation and NF-κB p65 binding to the eotaxin promoter [312]. Theophylline (3-methyxanthine) has been used to treat airway diseases for over 70 years, although in many industrialised countries it is only used in poorly controlled patients as an add-on therapy [313]. Although its main mechanism of action is likely to be phosphodiesterase (PDE) inhibition, theophylline has also been shown to activate HDACs [313]. Montelukast, a cysteinyl leukotriene receptor antagonist used in asthma treatment, acts by histone H3 acetylation to enhance the lipopolysaccharide (LPS)-induced expression of the anti-inflammatory cytokine IL-10 in human myeloid dendritic cells [314]. Histone modifying agents have also been used in cancer treatment for years. The HDAC inhibitors (HDACi) Vorinostat (SAHA) and Romidepsin, have been approved for cutaneous T-cell lymphoma therapy [315], and have also been investigated for treatment of many other conditions, such

as HIV infection [316]. Belinostat and Panobinostat are HDACi approved for treatment of peripheral T-cell lymphoma and multiple myeloma respectively [315]. Histone methylation as a therapeutic target has not been as extensively studied, although there are some agents currently in development. Pinometostat inhibits histone methyltransferase disruptor of telomeric silencing 1-like (DOT1L), reducing H3K79 methylation [317]. Pinometostat is being developed for treatment of relapsed or refractory MLL-rearranged (MLL-r) acute leukaemia [318]. Tazemetostat is an EZH2 inhibitor, currently in development for use in relapsed or refractory B-cell non-Hodgkin lymphoma and advanced solid tumours [319]. Histone arginine methylation is still a new target and there are not many agents targeting it in development. One of few is GSK3326595, a PRMT5 inhibitor, which is in clinical trials for treatment of solid tumours and non-Hodgkin's lymphoma [320].

1.7.5.4 Targeting PRMTs in asthma treatment

As mentioned above, there are currently no agents targeting histone arginine methylation or PRMTs, which have been approved for treatment of any conditions. It is also not known whether PRMT inhibitors (or inducers) have potential in asthma therapy, although some studies suggest they could be a new interesting target. Various studies by Sun *et al.* show PRMT1 has a role in inflammation and remodelling processes occurring in asthma [301-303]. Another promising target is CARM1, as its inhibitor ellagic acid, has been shown to reduce inflammation in a mouse asthma model [305].

Ellagic acid is a polyphenol, widely found in fruits, vegetables and wine, and known for its antioxidant and anti-inflammatory proprieties [321]. Ellagic acid was identified as a CARM1 inhibitor in 2009 [304], and is one the most promising compounds amongst PRMT inhibitors. Other compounds reported to inhibit CARM1 contain a pyrrazole core. However, studies with these compounds either did not test their cellular activity, or showed that pyrrazole – based CARM1 inhibitors lacked significant cellular activity [322, 323].

One of the biggest challenges that has to be overcome in order to introduce PRMT inhibitors as a treatment for asthma is their lack of specificity [324]. SAM analogues, including sinefungin and S-adenosylhomocysteine (SAH), inhibit PRMT1, but were also found to inhibit all PRMTs, as well as lysine methyltransferases [324]. AMI-1, one of the best studied PRMT1 inhibitors, inhibits all type I PRMTs [324]. Bonham *et al.* and Feng *et al.*, identified potential PRMT1 inhibitors, but they also lacked specificity and inhibited other PRMTs [325, 326]. Newer studies identified PRMT inhibitors with greater specificity towards PRMT1, but their potential to use in therapy is yet to be verified [324, 327, 328]. Another potential concern is

the fact that PRMTs control a broad range of cellular processes, therefore even specific inhibition may produce widespread effects beyond direct targets of PRMTs.

1.8 Summary

Asthma is a common chronic condition, which has a negative impact on life quality of those suffering and is also a burden to the national healthcare. A better understanding of the disease could lead to the development of novel therapies and help the patients, especially those who struggle with severe and poorly controlled form of asthma. ASM cells have a crucial role in asthma, contributing to airway remodelling, airflow obstruction, and inflammation of the airways. ASM cells secrete a number of inflammatory mediators, such as cytokines, chemokines, and growth factors, and cells isolated from asthmatic individuals have been shown to hypersecrete some of these mediators. Epigenetic modifications, and histone modifications in particular, have been suggested to have a role in the development and progress of asthma. Histone acetylation has been shown to be involved in regulating the chemokine secretion by ASM cells. The role of histone methylation in asthma has not been studied in as much detail yet. However, the work carried out by our group provides preliminary data suggesting that abnormal histone arginine methylation may be linked to the inflammation in asthma, particularly associated with the increased secretion of chemokines and mediators from ASM cells, and that inhibiting PRMTs may be a strategy to reduce this inflammation.

Chapter 2: Hypothesis and aims

2 Hypothesis and aims

Hypothesis: Histone arginine methylation regulates the secretion of chemokines and cytokines from ASM cells, is upregulated in asthma, and represents a novel target for the development of new therapies against asthma.

Specific aims:

- To characterise the expression of PRMTs in human ASM cells and determine how asthmatic stimuli influence their expression and function.
- To establish whether inhibiting PRMTs could reduce the cytokine/chemokine expression and their secretion from human ASM cells
- To investigate how asthmatic stimuli influences histone arginine methylation at the cytokine/chemokine promoters in ASM cells
- To establish the therapeutic potential of PRMT inhibitors in asthma

Chapter 3: Methods

3 Methods

3.1 Materials

Hyperfilm ECL™	GE Healthcare Life Sciences
Immuno-Blot® PVDF membrane for protein blotting	Bio-Rad

3.2 Reagents

0.1M DTT	Thermo Fisher Scientific
217531	EMD Millipore
3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide	Sigma-Aldrich
4-hydroxytamoxifen	Sigma-Aldrich
5X RT buffer	Thermo Fisher Scientific
Agarose	Sigma-Aldrich
Ammonium persulphate	Sigma-Aldrich
Bis/acrylamide	National Diagnostics
Blasticidin	InvivoGen
Bovine Serum Albumin (BSA)	Sigma-Aldrich
β-mercaptoethanol	Sigma-Aldrich
Clarity ™ Western ECL substrates	Bio-Rad
Deoxycholic Acid	Sigma-Aldrich
Deoxynucleoside Triphosphates (dNTPs)	Promega
DEPC treated water (RNase free)	Ambion
Dimethyl sulphoxide (DMSO)	Sigma-Aldrich
Dulbecco's modified Eagle's medium (DMEM)	Sigma-Aldrich
ECL™ detection reagents	GE Healthcare Life Sciences
Ethanol	Fisher Scientific
EPZ015666	Sigma-Aldrich
EPZ020411	Cayman Chemical
Foetal calf serum (FCS)	Fisher Scientific
GeneArt™ Platinum™ Cas9 Nuclease	Invitrogen
Glycerol	Fisher Scientific
Glycine	Sigma-Aldrich
Hydrogen peroxide 30%	VWR chemicals
Isopropanol	Fisher Scientific
Lipofectamine™ CRISPRMAX™ Cas9 Transfection Reagent	Invitrogen

KAPA SYBR FAST qPCR Master Mix	KAPA Biosystems
KAPA PROBE FAST Universal qPCR Master Mix	KAPA Biosystems
L-glutamine	Sigma-Aldrich
Lipofectamine™ 2000 Transfection Reagent	Thermo Fisher Scientific
Methanol	Sigma-Aldrich
M-MLV Reverse Transcriptase	Promega
M-MLV RT 5X Buffer	Promega
N,N,N,N-Tetramethylethylenediamine (Temed)	Sigma-Aldrich
Non-fat dry milk	Santa Cruz Biotechnology
Nuclease- free water	Qiagen
OligoDT primer	Roche
Opti-MEM™ I Medium	gibco (Life Technologies)
Penicillin/streptomycin	Sigma-Aldrich
Phenylmethanesulfonyl fluoride (PMSF)	Sigma-Aldrich
Phosphate buffered saline (PBS) tablets	Sigma-Aldrich
Protease Inhibitor Cocktail	Sigma-Aldrich
Rainbow™ molecular weight marker	GE Healthcare Life Sciences
Recombinant Human TNF-α	R&D Systems
RNasin® Ribonuclease Inhibitor	Promega
Sodium Acetate	Sigma-Aldrich
Sodium chloride (NaCl)	Fisher Scientific
Sodium dodecyl sulphate (SDS)	Sigma-Aldrich
Sodium pyruvate	gibco (Life Technologies)
SuperScript® IV Reverse Transcriptase (200U/µl)	Thermo Fisher Scientific
TC-E 5003	Tocris
Triton X 100	Sigma-Aldrich
Trizma® base (Tris base)	Sigma-Aldrich
Trypsin/EDTA	Sigma-Aldrich
Tween-20	Sigma-Aldrich

3.3 Antibodies

α-tubulin mouse monoclonal antibody	Santa Cruz Biotechnology
CARM1 rabbit polyclonal antibody	Bethyl Laboratories
CNOT7 rabbit monoclonal antibody	Abcam
GAPDH mouse monoclonal antibody	Santa Cruz Biotechnology

Goat anti- rabbit polyclonal antibody	Dako
ILF3 rabbit polyclonal antibody	Abcam
PRMT1 rabbit monoclonal antibody	Abcam
PRMT1 rabbit polyclonal antibody	Bethyl Laboratories
PRMT5 rabbit polyclonal antibody	Bethyl Laboratories
PRMT6 rabbit polyclonal antibody	Bethyl Laboratories
Rabbit anti- mouse polyclonal antibody	Dako
Polyclonal rabbit IgG	R&D Systems
Polyclonal rabbit IgG	EMD Millipore

3.4 Kits

BCA Assay kit	Thermo Fisher Scientific
ChIP-IT™ Express	Active Motif
GeneArt® Genomic Cleavage Detection Kit	Invitrogen
GeneArt™ Precision gRNA Synthesis Kit	Invitrogen
Human CCL11/Eotaxin DuoSet ELISA kit	R&D Systems
Human IL-6 DuoSet ELISA kit	R&D Systems
Human IL-8/CXCL8 DuoSet ELISA kit	R&D Systems
Human CXCL10/IP-10 DuoSet ELISA kit	R&D Systems
Mouse CXCL1/KC DuoSet ELISA	R&D Systems
NucleoSpin® RNA Extraction Kit	Macherey Nagel
RNeasy® Micro Kit	Qiagen
SimpleChIP® Plus Sonication Chromatin IP Kit	Cell Signaling Technology

3.5 Primers

CARM1 primers	Eurofins Genomics
Human IP-10, IL-6, Eotaxin, CXCL8 primers, mouse KC, PRMT1, HPRT primers	Eurofins Genomics
PRMT1, PRMT2, PRMT3, PRMT5, PRMT6, PRMT7, FBX011 primers	Sigma-Aldrich
IVT-PRMT1-gRNA primers	Invitrogen

3.6 Buffer Recipes

2X Lysis buffer

50 mM Tris HCl pH 6.8

150mM NaCl

1% v/v Triton X-100

0.1% w/v SDS

0.5% w/v Deoxycholic Acid

0.01M EDTA

Prior to use, the buffer was diluted 1:2. PMSF (1:45) and protease inhibitor cocktail (1:100) were added.

4 x Lamellae Buffer

125 mM Tris HCl pH 6.8

20% v/v Glycerol

2% v/v SDS

0.2% w/v bromophenol blue

Just before use, β-mercaptoethanol (60µl/ml) was added.

Buffer 1

1.5M Tris-HCl, 0.4% SDS, pH 8.8

Buffer 2:

0.5M Tris-HCl, 0.4% SDS, pH 6.8

10 x Running buffer

24.2g Tris base

144g glycine

10g SDS

Made up to 1L dH_2O.

10 x Transfer buffer

24.2g Tris base

144g glycine

Made up to 1L dH_2O.

Prior to use, 20% v/v methanol was added.

10 x Tris buffered saline plus Tween 20 (TBST)

24.2g Tris base

87.6g NaCl

pH 7.4 – 7.6

10mls Tween 20

Made up to 1L dH_2O.

Resolving gel (10% acrylamide)

6.66 ml 30% Bis/Acrilamide

5.2 ml Buffer 1

7.92 ml dH_2O

200 µl 10% ammonium persulphate

20 µl Temed

Stacking gel

1.3 ml 30% Bis/Acrilamide

2.5 ml Buffer 2

6.1 ml dH_2O

50 µl 10% ammonium persulphate

10 µl Temed

3.7 Cell culture

3.7.1 Human Airway Smooth Muscle Cells

Human airway smooth muscle cells (ASM) were isolated during bronchoscopy from healthy volunteers and volunteers with asthma that have been recruited to studies carried out in the Nottingham Respiratory Research Unit (NRRU). Cells were previously characterised by morphological and immunocytochemical staining techniques, as described by Pang *et al.*, to confirm that the cultured cells had the characteristics of pure ASM cells [160]. Cells were examined under the light microscope to prove that they appearance was characteristic of ASM cells in culture. The application of immunocytochemical techniques showed that over 95% of the cultured cells were positively labelled for α-actin (specific for smooth muscle cells) and desmin (specific for mature muscle cells), while staining for cytokeratin (epithelial cell marker) was negative.

Table 3.1: Details of participants who donated ASM cells used in this study. ICS = Inhaled corticosteroids; LABA = Long-acting beta agonists; Combi = Combination inhalers: Corticosteroids and long-acting beta agonists; SABA = Short-acting beta agonists; ORAL = Oral corticosteroids

Donor number	Code	Disease	Age	Gender	Ethnicity	Medication	Smoking
1	MMP-1H01	Healthy	49	M	Black	None	Never
2	AZAC11	Healthy	46	M	White	None	Never
3	AZAC03	Healthy	61	M	White	None	Never
4	AZAC05	Healthy	26	F	White	None	Never
5	AZAC07	Healthy	39	F	Asian	None	Never
6	AZAC09	Healthy	19	F	White	None	Never
7	MMP-1H10	Healthy	46	M	White	None	Never
8	AZAD01	Asthma	51	M	White	ICS+ LABA+ Combi+ SABA	Never
9	AZAD08	Asthma	57	F	White	ICS+ LABA+ Combi+ SABA	Never
10	SMACD04	Asthma	60	M	White	ICS+ LABA+ Combi+ SABA	Never
11	ALS09007	Asthma	37	M	White	ICS+ LABA+ ORAL	Not available
12	ALS09008	Asthma	50	F	White	ICS+ LABA+ ORAL	Ex
13	HASM0609	Asthma	57	M	White	ICS	Never

The cells were cultured to passage 6 in Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich) supplemented with 4mM L-glutamine (Sigma-Aldrich), 100U/ml penicillin, 100µg/ml streptomycin (Sigma-Aldrich), and 10% heat inactivated foetal calf serum (FCS, Fisher Scientific), under 5% CO_2/95% air in a humidified incubator at 37°C. In order to growth arrest

cells, they were incubated in the same conditions for 24 hours in a serum free DMEM (which contained all the supplements as the standard media, but FCS).

3.7.2 PRMT1FL/– ER-Cre Mouse Embryonic Fibroblasts (MEFs)

PRMT1$^{FL/-}$ ER-Cre Mouse Embryonic Fibroblasts (MEFs) were a kind gift from Dr Stephane Richard (Institute for Medical Research of the Jewish General Hospital in Montreal, Canada). MEFs were grown to confluence in DMEM, containing 4mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin, 20% FCS, 1mM sodium pyruvate (gibco, Life Technologies), under 5% CO_2/95% air in a humidified incubator at 37°C. Blasticidin (InvivoGen) was added to cell culture medium to maintain the ER-Cre. Once confluent the MEFs were detached with trypsin-Ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich) and seeded to 24 well plates and cultured to the density required for the experimental procedure. 4-Hydroxytamoxifen (OHT, Sigma-Aldrich) at 2µM was added to induce PRMT1 loss. MEFs without OHT treatment were used as a control. In order to growth arrest MEFs, they were incubated in the same conditions for 24 hours in a DMEM containing all the supplements as the growth media, but only 1% FCS and no sodium pyruvate

3.8 Reverse Transcription Polymerase Chain Reaction (RT-PCR) to determine the expression of PRMTs in ASM cells

3.8.1 Cell culture

Human ASM cells were cultured in 24-well plates until reaching 90-100% confluency, when they were growth arrested.

3.8.2 RNA extraction

RNA extractions were performed using NucleoSpin® RNA Extraction Kit (Macherey Nagel) as per the manufacturer's guidelines. Briefly, after removing the media, cells were washed with Phosphate buffered saline (PBS) and lysed in RA1 lysis buffer and β-mercaptoethanol (Sigma-Aldrich) at ratio 100:1. The lysates were transferred to Eppendorf tubes, vortexed and filtered through NucleoSpin® Filter by centrifuging for 1 min at 11,000 x *g*. 350µl of 70% ethanol was added into each sample and mixed by pipetting. The lysates were then transferred to NucleoSpin® RNA Column, placed in a collection tubes, and centrifuged for 30 seconds at 11,000 x *g*. The columns were desalted (350ul MDB, Membrane Desalting Buffer) and centrifuged for 1 minute at 11,000 x *g*. 95ul DNase reaction mixture (10µl reconstituted rDNase + 90µl Reaction Buffer) was added to columns, which were then incubated for 15 minutes at room temperature. 200ul Buffer RAW2 was added to columns, followed by

centrifugation for 30 seconds at 11,000 x *g*. 600µl of buffer RA3 was added to the columns and they were centrifuged for 30 seconds at 11,000 x *g.* Finally, 250ul Buffer RA3 was added into columns, which were then centrifuged for 2 min at 11,000 x *g* and placed into nuclease-free collection tubes. RNA was eluted in 40µl of RNase-free H_2O and centrifuged at 11,000 x *g* for 1 minute. RNA was immediately frozen at -80°C.

3.8.3 Reverse Transcription (RT)

3µl of 200µg/ml oligodeoxythymidylic acid (oligoDT) primer (Roche) and 5µl of 10mM Deoxynucleoside Triphosphates (dNTPs, Promega) were added to 10.7µl RNA and heated at 72°C for 5 minutes in a thermocycler. Subsequently 5µl of 5x RT buffer, 0.66µl of 200U/µl Moloney murine leukemia virus reverse transcriptase (M-MLV RT) and 0.66µl of 40U/µl RNasin® Ribonuclease Inhibitor (Promega) were added into each sample. The samples were placed in a thermocycler on the following cycle: 42°C for 90 minutes followed by 90°C for 5 minutes. The resulting complimentary DNA (cDNA) was stored at -20°C.

3.8.4 Polymerase Chain Reaction (PCR)

Predesigned primers for PRMT1, CARM1/PRMT4, PRMT5 and PRMT6 were obtained from Sigma-Aldrich (www.sigmaaldrich.com). 10µl KAPA SYBR FAST qPCR Master Mix (2X, KAPA Biosystems), 0.5µl of 10µM forward primer, 0.5µl of 10µM reverse primer and 8 µl of Diethyl pyrocarbonate (DEPC) treated water (Ambion) was added to 1µl of cDNA sample on a 96-well PCR plate. Real time PCR was performed (MX3004/5 Agilinet Technologies) using the following cycling conditions: 95°C for 30 seconds, followed by 45 cycles at 95°C for 5 seconds and 60°C for 30 seconds, finished with one cycle of 95°C for 1 minute, 60°C for 30 seconds and 95°C for 30 seconds. Negative controls consisted of no template, all the samples were run in triplicate. Primer specificity was confirmed by the analysis of the dissociation curve. To compare the levels of expression of different PRMTs, the mean Ct value of β2-microglobulin (β_2M, reference gene) was subtracted from the mean Ct value of the target gene to give the ΔCt. Fold expression was then determined by the following equation: 2^(-ΔCt).

The difference between asthmatic and non-asthmatic cells for each of the PRMT was determined by the ΔΔCt method. The mean Ct value of β_2M (reference gene) was subtracted from the mean Ct value of the target gene to give the ΔCt. The mean ΔCt from the control group (non-asthmatic cells) was then subtracted from ΔCt of each sample, to give ΔΔCt. Fold expression compared to the control was determined by the following equation: 2^(-ΔΔCt).

Table 3.2: PCR Primers used in the study (Sigma-Aldrich)

Gene	Product length (bp)	Forward Primer	Reverse Primer
PRMT1	154	5'-CAGTACAAAGACTACAAGATCC-3'	5'-AGATGTCCACCTCCTTTATG-3'
CARM1/PRMT4	243	5'-ATCGCCCTCTACAGCCATGA-3'	5'-GTACTGCACGGCAGAAGACT-3'
PRMT5	75	5'-AAATACTCTCAGTACCAGCAG-3'	5'-ATTGGTATCCTTCTCCTCTTC-3'
PRMT6	195	5'-ATGTCAGTTTTTGAAGGGTC-3'	5'-TGAGGAAGAAGTACCATAGC-3'
β_2M	110	5'-AATCCAAATGCGGCATCT-3'	5'-GAGTATGCCTGCCGTGTG-3'

3.9 Western blotting to determine protein expression of PRMTs in ASM cells

3.9.1 Preparation of cell lysates

Cells were cultured on sterile 10cm Petri dishes. Upon reaching 90-100% confluency, they were growth arrested for 24 hours. Following removal of culture medium, the cells were washed with ice- cold PBS. After removing PBS, 500µl of lysis buffer was added to each plate. The cells were then collected by scraping. The lysates were collected into Eppendorf tubes, centrifuged at 10000 rpm for 3 minutes to remove any insoluble protein, and stored at -20°C. The protein concentration in lysates was determined using bicinchoninic acid (BCA) assay (Thermo Fisher Scientific), according to manufacturer's instructions. Briefly, BSA standard solutions were prepared as per manufacturer's instructions. BCA working reagent was prepared by mixing 50 parts of Reagent A with 1 part of Reagent B. 10 µl of standards and samples was prepared in duplicates into a flat-bottomed 96 well ELISA plate. 200 µl of the prepared BCA working reagent was added into each well. The plate was covered and incubated at 37°C for 30 minutes. The absorbance was measured at 560nm on a FLUOstar Omega microplate reader (BMG Labtech). The samples were diluted 4:1 with Lamellae buffer and boiled at 95°C for 5 minutes. The samples were then stored at -20°C until further use.

3.9.2 Gel electrophoresis

The gel casting apparatus (Bio-Rad) was set up according to the manufacturer's instructions. 8µl of the Rainbow™ protein marker (GE Healthcare Life Sciences) was added to the first

lane; the subsequent lanes were loaded with 30µg of each protein sample. The gels were run on Mini-PROTEAN® Tetra Vertical Electrophoresis Cell (Bio-Rad) at 150V for 1 hour.

3.9.3 Protein transfer

Transfer to Immuno-Blot® Polyvinylidene difluoride (PVDF) membrane (Bio-Rad) was performed on Criterion™ Blotter (Bio-Rad) as per the manufacturer's instructions for 1 hour at 100V.

3.9.4 Protein detection

The membrane was washed in Tris buffered saline plus Tween 20 (TBST) and blocked at room temperature in 5% non-fat milk (in TBST) for 1 hour. The membrane was then incubated with the primary antibody overnight at 4°C. The specificity of antibodies was previously verified in published studies [329-333]. Following 3X 5 minute washes in TBST, membranes were incubated with the appropriate secondary antibody at room temperature for 1 hour.

Table 3.3: Antibodies used in western blotting

Antibody	Supplier	Dilution
PRMT1 rabbit monoclonal antibody	Abcam	1:250
CARM1 rabbit polyclonal antibody	Bethyl Laboratories	1:1000
PRMT5 rabbit polyclonal antibody	Bethyl Laboratories	1:500
PRMT6 rabbit polyclonal antibody	Bethyl Laboratories	1:1000
GAPDH mouse monoclonal antibody	Santa Cruz Biotechnology	1:5000
α-tubulin mouse monoclonal antibody	Santa Cruz Biotechnology	1:1000
Goat anti-rabbit polyclonal antibody	Dako	1:2000
Rabbit anti-mouse polyclonal antibody	Dako	1:2000

The membrane was then washed 5X5 mins in TBST, developed in the Enhanced chemiluminescence (ECL™) detection reagent (GE Healthcare Life Sciences) and then developed in the dark room, using Hyperfilm ECL™ (GE Healthcare Life Sciences).

The results were analysed by densitometry using ImageJ program. The density of each sample band and the density of each the loading control sample band were measured and the background was subtracted. The value for each sample was divided by the value of its equivalent loading control sample.

3.10 Chromatin Immunoprecipitation (ChIP) Assay for PRMT1, PRMT6, CCR4-NOT transcription complex subunit 7 (CNOT7) and Interleukin Enhancer Binding Factor 3 (ILF3)

Cells were cultured on sterile 500cm^2 plates. Upon reaching 90-100% confluency, they were growth arrested for 24 hours. Following removal of culture medium, the cells were incubated with TNF-α (1ng/ml, R&D Systems) for 30 minutes and 1 hour. ChIP assay was performed according to the manufacturer's protocol using the SimpleChIP® Plus Sonication Chromatin IP Kit (Cell Signaling Technology). Briefly, following treatment the cells were fixed in 1% formaldehyde solution in serum free media for 10 minutes at room temperature. After washing with ice cold PBS, the fixation reaction was stopped by adding a glycine solution (in PBS) for 5 minutes at room temperature. The cells were then briefly washed with ice cold PBS, removed from the flask by scraping in 2ml of scraping solution (1x PBS supplemented with 200x protease inhibitor cocktail (PIC)), collected into Eppendorf tubes and centrifuged at 720 rcf for 10 minutes at 4°C. The supernatant was removed; 1ul of 100mM phenylmethylsulfonyl fluoride (PMSF, Sigma-Aldrich) and 1µl PIC (Sigma-Aldrich) were added to each cell pellet. The cells were lysed in 1.5ml of ChIP Sonication Cell Lysis Buffer supplemented with 200x PIC, incubated on ice for 10 minutes and centrifuged at 4°C for 5 minutes at 5000 rcf. After the supernatant was removed, the pellets were resuspended in 1.5ml of ChIP Sonication Cell Lysis Buffer supplemented with 200x PIC, incubated on ice for a further 5 minutes, and centrifuged at 4°C for 5 minutes at 5000 rcf again. The supernatant was removed and the pellets were resuspended in 350µl ice cold ChIP Sonication Nuclear Lysis Buffer supplemented with 200x PIC. After incubating on ice for 10 minutes, chromatin was fragmented by using the Q800R Sonicator (Active Motif). DNA concentration was determined following DNA clean up using Nanodrop (Thermo Scientific). Chromatin samples (13µg) were incubated overnight at 4°C on a rotor in a 500µl solution containing ChIP buffer supplemented with 200x PIC and 5µg (PRMT1, PRMT6, Bethyl Laboratories) or 4µg (CNOT7, ILF3, Abcam) of antibody. The specificity of antibodies was previously verified in published studies [332, 334-336]. Protein G magnetic beads (30 µl) were then added to each tube and the reactions were then incubated for a further 2 hours at 4°C on a rotor. The chromatin bead complexes were washed and the bound chromatin was eluted from the magnetic beads by adding 150 µl of 1x ChIP Elution Buffer to each reaction, and placing in a water bath set to 65°C for 30 minutes with frequent mixing. The DNA- protein cross links were reversed and the bound proteins were removed by treatment with proteinase K. 20µl of each chromatin sample diluted in ChIP buffer supplemented with 200x PIC were used as "input control DNA",

i.e. chromatin which does not undergo immunoprecipitation procedure. DNA was purified using DNA purification spin columns provided by the manufacturer. The recovered DNA was then quantified by real-time PCR. Immunoglobulin G (IgG, EMD Millipore) was used as a negative control.

3.10.1 Real time PCR

The recovered DNA from ChIP was quantified by real-time PCR using the primers described in Table 3.4 and Dual Labeled (Taqman) Probes described in Table 3.5 (Eurofins Genomics). Primers and probes were designed by Dr Rachel Clifford, using Beacon Designer™ (PREMIER Biosoft International). The eotaxin, IL-6, IP-10, and CXCL8 primers used yielded 125, 99, 108, and 129bp products respectively, corresponding to −132 to −7, -195 to -96, -184 to -76, and -307 to -178bp relative to the transcription start site. Input control DNA and IP samples (2µl) were subjected to real-time PCR using KAPA PROBE FAST qPCR Master Mix (2X) and the MX3004/5 Agilent Technologies qPCR system. Each reaction consisted of 10µl of KAPA PROBE FAST qPCR Master Mix (2X), 0.5µl of each 10µM forward primer, 0.5µl of each 10µM reverse primer, 0.2 µl of each probe and 3.2 µl of water.

Thermal cycler conditions were as follows: 95°C for 3 minutes, followed by 40 cycles at 95°C for 3 seconds and 60°C for 20 seconds. Negative controls consisted of no template, all the samples were run in triplicate.

Expression was determined using the ΔΔCt method. The mean Ct value of the input was subtracted from the mean Ct value of the sample IP/IgG to give the ΔCt. The mean ΔCt from the basal IgG is then subtracted from ΔCt of each sample, to give ΔΔCt. Fold expression compared to the basal IgG was then determined by the following equation: 2^(-ΔΔCt).

Table 3.4: PCR Primers used in ChIP qPCR (Eurofins Genomics)

Gene	Forward Primer	Reverse Primer
IP-10	5'-CATGGTGCTAAACACAGT-3'	5'-AGCCCTTTACAGACCCTA-3'
IL-6	5'-CCAGAAAGGAAAGGAACTTC-3'	5'-CTGCATTAGGAGGTCTTTG-3'
Eotaxin	5'-CAAGGCAAGATCCAGATG-3'	5'-GTCACGATGAAGGGATTC-3'
CXCL8	5'-GCCATTAAAAGAAAATCATCCA-3'	5'-CCACAATTTGGTGAATTATCAA-3'

Table 3.5: Probes used in ChIP qPCR (Eurofins Genomics)

Gene	Sequence
IP-10	5'-CCAACTTCCAGACCTGCCGA-3'
IL-6	5'-TGACACCAGCAAAGGATAAATACAGAA-3'
Eotaxin	5'-TGCTGACCAGATTCCAGATCCTAG-3'
CXCL8	5'-TCTTGTTCTAACACCTGCCACTCTAG-3'

3.11 ChIP Assay for H4R3me2a

Cells were cultured on sterile 500cm^2 plates. Upon reaching 90-100% confluency, they were growth arrested for 24 hours. Following removal of culture medium, the cells were incubated with TNF-α (1ng/ml) for 1 and 2 hours. ChIP assay was performed according to the manufacturer's protocol using the ChIP-IT™ Express kit (Active Motif). Briefly, following treatment the cells were fixed in 1% formaldehyde solution in serum free media for 10 minutes at room temperature. After washing with ice cold PBS, the fixation reaction was stopped by adding a glycine solution (in PBS) for 5 minutes at room temperature. The cells were then briefly washed with ice cold PBS, removed from the flask by scraping in 2ml of scraping solution (1x PBS supplemented with 0.5mM PMSF), collected into Eppendorf tubes and centrifuged at 720 rcf for 10 minutes at 4°C. The supernatant was removed; 1ul of 100mM PMSF and 1µl PIC were added to each cell pellet. The cells were lysed in 1.5ml ice cold lysis buffer supplemented with 5µl PMSF and 5µl PIC, incubated on ice for 30 minutes and centrifuged at 4°C for 10 minutes at 2400 rcf. After removing the supernatant, the pellets were resuspended in 350µl shearing buffer (supplemented with 1.75 µl PIC and 1.75 µl PMSF). Chromatin was fragmented by using the Q800R Sonicator (Active Motif). DNA concentration was determined following DNA clean up using Nanodrop (Thermo Scientific). Chromatin samples (25µg) were incubated overnight at 4°C on a rotor in a 200 µl solution containing Protein G magnetic beads (25 µl), PIC (2 µl), ChIP buffer 1 (10 µl) and 1 µg of antibody. The chromatin bead complexes were washed and the bound chromatin was eluted from the magnetic beads. The DNA- protein cross links were reversed and the bound proteins were removed by treatment with proteinase K. 5µg of chromatin per sample was used as "input control DNA", i.e. chromatin which does not undergo immunoprecipitation procedure. Input DNA was phenol/chloroform extracted before being used in PCR. The recovered DNA was then quantified by real-time PCR. IgG was used as a negative control.

3.11.1 Real time PCR

Real time PCR was performed as described for "ChIP Assay for PRMT1, PRMT6, CNOT7 and ILF3" in section 3.10.

3.12 Enzyme- Linked Immunosorbent Assay (ELISA) following dosing with PRMT inhibitors

3.12.1 Preparation of inhibitor solutions

PRMT inhibitors (TC-E 5003, EPZ015666, 217531, EPZ020411) were reconstituted in Dimethyl sulphoxide (DMSO, Sigma-Aldrich) to a final concentration of 10mM. They were then aliquoted and stored at -80°C.

3.12.2 Cell culture

Human ASM cells were cultured in 48-well plates until reaching 90-100% confluency, when they were growth arrested for 24 hours. Following removal of culture medium, the cells were dosed with PRMT inhibitors diluted in serum-free media to a series of concentrations (0-10µM). The cells were then incubated at 37°C for 30 minutes, following which they were incubated with TNF-α (1ng/ml) for 24 hours. Supernatants were then collected and stored at -20°C. 6 extra wells (3 basal and 3 stimulated with TNF-α) per each inhibitor were used for cell counts.

3.12.3 Cell viability

The toxicity of PRMT inhibitors was determined by 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Following the collection of supernatants, 100 µl of serum free media containing 1 mg 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide (Sigma-Aldrich) was added into each well and incubated for 30 minutes at 37°C. This medium was removed and plates were dried overnight. 250 µl of DMSO (Sigma-Aldrich) was then added to dissolve the blue-coloured tetrazolium. The optical density was read at 550 nm in a FLUOstar Omega microplate reader (BMG Labtech). Viability was set at 100% in control cells.

3.12.4 Human IL-6 ELISA

The assay was performed according to manufacturer's instructions using the Human IL-6 DuoSet ELISA kit (R&D Systems). Briefly, a monoclonal antibody (mouse anti-human IL-6) specific for IL-6 was precoated onto a 96 well plate, overnight at room temperature. The plate was washed 3 times with wash buffer (0.05% Tween 20 in PBS, pH 7.2-7.4) and blocked for a minimum of 1 hour with reagent diluent (1% Bovine Serum Albumin, BSA, Sigma-

Aldrich, in PBS, pH 7.2-7.4, 0.2 µm filtered). Standards were diluted using 2 fold serial dilutions in reagent diluent to provide a seven point standard curve from 600 pg/ml to 0 pg/ml. Plates were washed and standard and sample supernatants (50 µl) were pipetted into the wells and incubated for 2 hours at room temperature. Any IL-6 present was bound by immobilised antibody. Plates were washed three times to remove any unbound substances and 50 µl of a polyclonal antibody (biotinylated goat anti-human IL-6) specific for IL-6 was added to the wells and incubated for an additional 2 hours at room temperature. Plates were washed again and 50 µl of Streptavidin-horseradish peroxidase (HRP) solution was added and incubated for 20 minutes. 50 µl of a substrate solution (50:50 v/v Hydrogen peroxide, H_2O_2 and tetramethylbenzidine) was then added and coloured developed in proportion to the amount of IL-6 bound in the initial step. The reaction was stopped by adding 25 µl of stop solution (2N sulfuric acid); the degree of colour generated was determined by measuring the optical density at 450 nm (reference Filter 570 nm) within 30 minutes in a FLUOstar Omega microplate reader (BMG Labtech). The IL-6 concentrations of unknown samples were calculated using the standard curve.

3.12.5 Human CXCL10/IP-10 ELISA

The assay was performed according to manufacturer's instructions (R&D Systems), similarly to that described for IL-6 in 3.6.4. A 96 well plate was precoated with a monoclonal antibody (mouse anti-human IP-10) specific for IP-10 and incubated overnight at room temperature. The plate was washed with wash buffer and blocked with reagent diluent. Standards were diluted using 2 fold serial dilutions in reagent diluent to provide a seven point standard curve from 2000 pg/ml to 0 pg/ml. Plates were washed and standard and sample supernatants were pipetted into the wells and incubated for 2 hours at room temperature. After washing the plates, 50 µl of a polyclonal antibody (biotinylated goat anti-human IP-10) specific for IP-10 was added to the wells and incubated for an additional 2 hours at room temperature. Plates were washed and after the incubation with Streptavidin-HRP solution, the reaction was stopped. The concentration of IP-10 in the unknown samples was then determined similarly to that described in 3.6.4 for IL-6.

3.12.6 Human CCL11/Eotaxin ELISA

The assay was performed according to manufacturer's instructions (R&D Systems), similarly to that described for IL-6 in 3.6.4. A 96 well plate was precoated with a monoclonal antibody (mouse anti-human eotaxin) specific for eotaxin and incubated overnight at room temperature. The plate was washed with wash buffer and blocked with reagent diluent.

Standards were diluted using 2 fold serial dilutions in reagent diluent to provide a seven point standard curve from 1000 pg/ml to 0 pg/ml. Plates were washed and standard and sample supernatants were pipetted into the wells and incubated for 2 hours at room temperature. After washing the plates, 50 µl of a polyclonal antibody (biotinylated goat anti-human eotaxin) specific for eotaxin was added to the wells and incubated for an additional 2 hours at room temperature. Plates were washed and after the incubation with Streptavidin-HRP solution, the reaction was stopped. The concentration of eotaxin in the unknown samples was then determined similarly to that described in 3.6.4 for IL-6.

3.12.7 Human IL-8/CXCL8 ELISA

The assay was performed according to manufacturer's instructions (R&D Systems), similarly to that described for IL-6 in 3.6.4. A 96 well plate was precoated with a monoclonal antibody (mouse anti-human IL-8) specific for eotaxin and incubated overnight at room temperature. The plate was washed with wash buffer and blocked with block buffer (1% BSA in PBS, pH 7.2-7.4, 0.2 µm filtered). Standards were diluted using 2 fold serial dilutions in reagent diluent (0.1% BSA, 0.05% Tween 20, Sigma-Aldrich, in PBS pH 7.2-7.4, 0.2 µm filtered), to provide a seven point standard curve from 2000 pg/ml to 0 pg/ml. Plates were washed and standard and sample supernatants were pipetted into the wells and incubated for 2 hours at room temperature. After washing the plates, 50 µl of a polyclonal antibody (biotinylated goat anti-human IL-8) specific for IL-8 was added to the wells and incubated for an additional 2 hours at room temperature. Plates were washed and after the incubation with Streptavidin-HRP solution, the reaction was stopped. The concentration of IL-8 in the unknown samples was then determined similarly to that described in 3.6.4 for IL-6.

3.13 Clustered regularly interspaced short palindromic repeats assay (CRISPR)

3.13.1 Guide RNA (gRNA) synthesis

PRMT1 primers were designed using the GeneArt™ CRISPR Search and Design tool (thermofisher.com/crisprdesign).

Table 3.6: In-vitro transcription (IVT)-PRMT1 Primers used in gRNA synthesis

Primer set	Forward Primer	Reverse Primer	CRISPR target sequence
T1	5'-TAATACGACTCACTATAGTCATAACCGGCACCTC-3'	5'-TTCTAGCTCTAAAACTGAAGAGGTGCCGGTTATG-3'	TCATAACCGGCACCTCTTCA
T2	5'-TAATACGACTCACTATAGGAGGTGCCGGTTATGA-3'	5'-TTCTAGCTCTAAAACTGTTTCATAACCGGCACCT-3'	GAGGTGCCGGTTATGAAACA
T3	5'-TAATACGACTCACTATAGGATGTCATGTCCTCAG -3'	5'-TTCTAGCTCTAAAACAACGCTGAGGACATGACAT-3'	GATGTCATGTCCTCAGCGTT
T4	5'-TAATACGACTCACTATAGCATGGAGTTGCGGTAA-3'	5'-TTCTAGCTCTAAAACTCACTTACCGCAACTCCAT-3'	CATGGAGTTGCGGTAAGTGA

The gRNA synthesis was performed according to the manufacturer's protocol using the GeneArt™ Precision gRNA Synthesis Kit (Invitrogen). Briefly, a 0.3 µM target oligonucleotide mix working solution was prepared by diluting the 10 µM target oligonucleotide mix stock solution in nuclease-free water. Each of the PCR assembly reactions were then set up by mixing 12.5 µl of Phusion™ High-Fidelity PCR Master Mix (2X), 1 µl of Tracr Fragment + T7 Primer Mix, 1 µl of 0.3 µM Target F1/R1 oligonucleotide mix, and 10.5 µl nuclease-free water. The assembly PCR was performed using the cycling parameters below:

Table 3.7: Assembly PCR cycling parameters

Cycle step	Temperature	Time	Cycles
Initial Denaturation	98°C	10 seconds	1X
Denaturation	98°C	5 seconds	32X
Annealing	55°C	15 seconds	
Final extension	72°C	1 minute	1X
Hold	4°C	Hold	1X

The *in vitro* transcription reactions were then set up by adding the reaction components in a following order: 8 µl of nucleoside triphosphate (NTP) mix (100 mM each of ATP, GTP, CTP, UTP), 6 µl of gRNA DNA template (from PCR assembly), 4 µl of 5X TranscriptAid™ Reaction Buffer, and 2 µl of TranscriptAid™ Enzyme Mix, The reactions were then incubated at 37°C for 2–3 hours. Following the addition of DNase I (1 µl into each reaction mix), the reactions were incubated at 37°C for a further 15 minutes. Nuclease-free water was added to adjust the volume of each IVT reaction to 200 µl. Binding Buffer (100 µl) was then added to each reaction and mixed thoroughly by pipetting. Similarly, 300 µl of ethanol (>96%) was then added to each reaction and mixed. The mixture was transferred to the GeneJET™ RNA Purification Micro Column and centrifuged for 30–60 seconds at 14,000 × *g*. The flow-through was discarded. 700 µl Wash Buffer 1 (diluted with 13 ml of >96% ethanol) was added to each column, following which, the columns were centrifuged for 30–60 seconds at 14,000 × *g* and the flow-through was discarded. Similarly, 700 µl of Wash Buffer 2 was added and to each column and the columns were then centrifuged. Following the removal of flow-through, this step was repeated. Empty purification columns were centrifuged for an additional 60 seconds at 14,000 × *g* to completely remove any residual Wash Buffer. The columns were then transferred to a clean 1.5-ml collection tube. 10 µl of nuclease-free water was added to the centre of each of the purification column filters. gRNA was eluted by centrifuging the columns for 60 seconds at 14,000 × *g.*

3.13.2 Cell transfection

The transfection was performed according to the manufacturer's protocol using the Lipofectamine™ CRISPRMAX™ Transfection Reagent (Invitrogen). Briefly, human ASM cells were cultured overnight in 24-well plates to reach 30-70% confluency in a standard DMEM media, as described in section 3.7.1. Cas9 nuclease/gRNA solution was prepared by adding

Opti-MEM™ I Medium (25µl/well, gibco, Life Technologies), Cas9 nuclease (500ng/well, Invitrogen), gRNA (125ng/well) and Lipofectamine™ Cas9 Plus™ Reagent (1µl/well, Invitrogen) in a single tube. Lipofectamine CRISPRMAX™ Reagent (1.5µl/well, Invitrogen) was diluted in Opti-MEM™ I Medium (25µl/well) in a different tube. Both solutions were mixed and incubated for 5 minutes at room temperature. Another tube was also prepared for control plate, containing all the reagents, but without gRNA. Cas9 nuclease/gRNA solution was then added to diluted CRISPRMAX™ Reagent, mixed, and incubated for 5-10 minutes at room temperature. Following the incubation, 50µl of this solution was added to each well and the cells were incubated for 2 days at 37°C. After 2 days, the media was changed for DMEM containing all the supplements as the standard media, but only 1% FCS and the cells were incubated for a further 4 days. Following that, the media was taken off and the cells were incubated with TNF-α (1ng/ml) in 1% FCS DMEM for 24 hours.

3.13.3 Sample collection

Cell supernatants were collected and stored at -20°C. After washing with PBS, cell viability was assessed by MTT assay, similarly to that described in section 3.12.3. RLT buffer from RNeasy® Micro Kit (Qiagen) with β-mercaptoethanol was added into wells for RNA extraction. RDL buffer with PIC was added into another wells for protein lysis. These samples were then scraped and frozen at -80°C.

3.13.4 RNA purification

RNA purification was performed using RNeasy® Micro Kit (Qiagen) as per the manufacturer's guidelines. Briefly, after removing the media, cells were washed with PBS and lysed in RLT buffer and β-mercaptoethanol at ratio 100:1. The lysates were transferred to 1.5ml Eppendorf tubes, 1 volume of 70% ethanol was added to each lysate. After mixing by pipetting, the samples were transferred to RNeasy MinElute spin columns in 2 ml collection tubes and centrifuged for 15 seconds at 8000 × *g.* Following the removal of flow-through, 350µl of RW1 buffer was added to each column and the samples were centrifuged for 15 seconds at 8000 × *g*. The flow-through was discarded. 80µl DNase reaction mixture (10µl reconstituted DNase I + 70µl Buffer RDD) was added to each of the columns, which were then incubated for 15 min at room temperature. 350µl of RW1 buffer was added to each column, followed by centrifugation for 15 seconds at 8000 × *g*. After placing the columns in new 2 ml collection tubes, 500 µl of Buffer RPE was added to each column. The samples were then centrifuged for 15 seconds at 8000 × *g*. The flow-through was discarded. 500 µl of 80% ethanol was added to each column, followed by centrifugation at 8000 × *g* for 2 minutes.

After placing the columns in new 2 ml collection tubes, the lids of the spin columns were opened, and the membranes were dried by centrifuging for 5 minutes at full speed. The columns were then transferred to a clean 1.5mL collection tube. 14 µL of RNase-free water was added to the centre of each of the spin column membranes. RNA was eluted by centrifuging the columns for 1 minute at full speed.

3.13.5 Reverse Transcription

1.25µl of 50µM oligoDT primer, 1µl of 10mM dNTPs mix and 11µl of template RNA were combined in a tube, mixed and briefly centrifuged. The RNA- primer mix was then incubated at 65°C for 5 minutes and placed on ice for 1 minute. 4µl of 5X RT buffer (Thermo Fisher Scientific), 1µl of 100mM Dithiothreitol (DTT, Thermo Fisher Scientific), 1µl of RNasin® Ribonuclease Inhibitor (Promega) and 1µl of SuperScript® IV Reverse Transcriptase (200U/µl, Thermo Fisher Scientific), were combined in a tube, mixed and briefly centrifuged. Following that, 7µl of this RT reaction mix was added to the annealed RNA. The combined reaction mixture was then incubated at 55°C for 10 minutes. Following that, the reaction was inactivated by incubating at 80°C for 10 minutes. Nuclease free water was added to a total volume of 100ul. The resulting cDNA was stored at -20°C.

3.13.6 Polymerase chain reaction

To assess PRMT1 knockout, polymerase chain reaction was carried out, similarly to that described in section 3.8.4. To assess how PRMT1 knockout affects cytokine/chemokine expression in ASM cells, polymerase chain reaction was carried out with eotaxin, IL-6, IP-10 and CXCL8 primers (Eurofins Genomics). Cycling conditions used for IL-6, IP-10 and CXCL8 were the same as described in section 3.8.4. For eotaxin, cycling conditions were as follows: 95°C for 30 seconds, followed by 45 cycles at 95°C for 5 seconds and 64°C for 30 seconds, finished with one cycle of 95°C for 1 minute, 60°C for 30 seconds and 95°C for 30 seconds. The primer concentration for eotaxin qPCR was also lowered from 0.5µl to 0.125µl of 10µM primer per well, to avoid the formation of a primer dimer.

Table 3.8: PCR primers used in CRISPR (Eurofins Genomics)

Gene	Product length (bp)	Forward Primer	Reverse Primer
IP-10	152	5'-CCACGTGTTGAGATCATTGCT-3'	5'-TGCATCGATTTTGCTCCCCT -3'
IL-6	101	5'-CAATAACCACCCCTGACCCA-3'	5'-GCGCAGAATGAGATGAGTTGTC-3'
Eotaxin	250	5'-AACCCAGAAACCACCACCTC-3'	5'-GGTCTTGAAGATCACAGCTTTCTG-3'
CXCL8	92	5'-GAAGTTTTTGAAGAGGGCTGAGA-3'	5'-TTTGCTTGAAGTTTCACTGGCA-3'
PRMT1	154	5'-CAGTACAAAGACTACAAGATCC-3'	5'-AGATGTCCACCTCCTTTATG -3'
β_2M	110	5'-AATCCAAATGCGGCATCT-3'	5'-GAGTATGCCTGCCGTGTG -3'

Expression was determined by the ΔΔCt method. The mean Ct value of β_2M (reference gene) in the control basal cells was subtracted from the mean Ct value of each sample to give the ΔCt. The mean ΔCt from the control basal cells is then subtracted from ΔCt of each sample, to give ΔΔCt. Fold expression compared to the control basal cells was then determined by the following equation: 2^(-ΔΔCt).

3.13.7 Western blotting

In order to assess PRMT1 knockout, western blotting was carried out, similarly to that described in section 3.9. After incubating membranes with PRMT1 (Abcam) and glyceraldeyde-3-phosphate dehydrogenase (GAPDH, Santa Cruz Biotechnology) antibodies, membranes were then washed in 15% H_2O_2 (VWR chemicals) for 40 minutes at room temperature, following which they were washed 2x 5 minutes in TBST and re-incubated with anti α-tubulin primary antibody (Santa Cruz Biotechnology).

3.13.8 Human IL-6, IL-8/CXCL8, CXCL10/IP-10 and CCL11/Eotaxin ELISA

The concentration of cytokines/chemokines secreted from control and PRMT1 knockout ASM cells, basally and following TNF- α stimulation, was compared. The assays were performed according to manufacturer's instructions, similarly to that described in 3.6.4.,

3.6.5, 3.6.6 and 3.6.7. Due to the technical issues, we were not able to perform cell counts, therefore the results were normalised to MTT assay.

3.14 PRMT1 knockdown in Mouse Embryonic Fibroblasts

3.14.1 Cell culture

PRMT1$^{FL/-}$ ER-Cre Mouse Embryonic Fibroblasts (MEFs) were donated from Dr Stéphane Richard (Institute for Medical Research of the Jewish General Hospital in Montreal, Canada). The cells were cultured in 24 well plates, with Blasticidin added to cell culture medium to maintain the ER-Cre. 4-Hydroxytamoxifen (OHT) at 2µM was added to to induce PRMT1 loss. MEFs without OHT treatment were used as a control. Once confluent, the MEFs were growth arrested for 24 hours, in a serum free DMEM containing all the supplements as the growth media, but FCS and sodium pyruvate. Following that, the MEFs were stimulated with TNF-α at 1ng/ml and 10 ng/ml. After 24 hours, the supernatants were collected and stored at -20°C. After washing with PBS, cell viability was assessed by MTT assay, similarly to that described in section 3.12.3. RLT buffer from RNeasy® Micro Kit (Qiagen) with β-mercaptoethanol was added into three wells from each plate for RNA extraction. RDL buffer with PIC was added into another four wells from each plate for protein lysis. These samples were then scraped and frozen at -80°C.

3.14.2 RNA purification

RNA purification was performed using RNeasy® Micro Kit (Qiagen), similarly to that described in section 3.13.4.

3.14.3 Reverse Transcription

Reverse transcription was performed similarly to that described in section 3.13.5.

3.14.4 Polymerase chain reaction

To assess PRMT1 knockdown, polymerase chain reaction was carried out, similarly to that described in section 3.8.4. The PRMT1 primer concentration was lowered from 0.5µl to 0.125µl of 10µM primer per well. Cycling conditions were as follows: 95°C for 30 seconds, followed by 45 cycles at 95°C for 5 seconds and 64°C for 30 seconds, finished with one cycle of 95°C for 1 minute, 60°C for 30 seconds and 95°C for 30 seconds. Hypoxanthine guanine phosphoribosyl transferase (HPRT) was used as a reference gene.

Table 3.9: PCR primers used in MEFs qPCR (Eurofins Genomics)

Gene (mouse)	Product length (bp)	Forward Primer	Reverse Primer
PRMT1	155	5'-GCTAATGGGATGAGCCTCCA-3'	5'-TCATCCTTCAGCATCTCCTCG-3'
HPRT	132	5'-GTTGGGCTTACCTCACTGCT-3'	5'-TCATCGCTAATCACGACGCT-3'

3.14.5 Western blotting

In order to assess PRMT1 knockdown, western blotting was carried out, similarly to that described in section 3.9. After incubating membranes with PRMT1 and GAPDH antibodies, membranes were then washed in 15% H_2O_2 for 40 minutes at room temperature, following which they were washed 2x 5 minutes in TBST and re-incubated with anti α-tubulin primary antibody.

3.14.6 Mouse keratinocyte-derived chemokine (KC) ELISA

The assay was performed according to manufacturer's instructions (R&D Systems), similarly to that described for IL-6 in 3.6.4. A 96 well plate was precoated with a monoclonal antibody (rat anti-mouse KC) specific for KC and incubated overnight at room temperature. The plate was washed with wash buffer and blocked with reagent diluent. Standards were diluted using 2 fold serial dilutions in reagent diluent to provide a seven point standard curve from 1000 pg/ml to 0 pg/ml. Plates were washed and standard and sample supernatants were pipetted into the wells and incubated for 2 hours at room temperature. After washing the plates, 50 µl of a polyclonal antibody (biotinylated goat anti-mouse KC) specific for KC was added to the wells and incubated for an additional 2 hours at room temperature. Plates were washed and after the incubation with Streptavidin-HRP solution, the reaction was stopped. The concentration of KC in the unknown samples was then determined similarly to that described in 3.6.4 for IL-6. The results were then normalised to the MTT assay and expressed as % of TNF-α stimulated control sample (10ng/ml). Then the means obtained from cells from three different experiments were combined.

3.15 SiRNA- mediated knockdown of CNOT7

3.15.1 Cell transfection

Human ASM cells were cultured overnight in 6-well plates to reach 40-50% confluency in a standard DMEM media, as described in section 3.7.1. SiRNA was kindly donated by Dr Sebastiaan Winkler (University of Nottingham, Nottingham, UK). SiRNA sequences were as follows: CNOT7 – UAACUUGCCUGAAGAAGAA, CNOT8 – CCAUAGAUCUCCUUGCUAA. Five different siRNA solutions were prepared: CNOT7, CNOT7+ CNOT8, non targetig siRNA 30nM and non targetig siRNA 60nM. SiRNA solutions were prepared by adding 60nM (or 120nM for non targetig siRNA 60nM) of siRNA to 400ul of Opti-MEM™ I Medium in a single tube. Lipofectamine™ 2000 Transfection Reagent (8ul, Thermo Fisher Scientific) was added to 400ul of Opti-MEM™ I Medium in a different tube. SiRNA solutions were then added to tubes containing diluted Lipofectamine™ 2000 Transfection Reagent and incubated for 20 minutes at room temperature. Following the incubation, 800µL of the solutions was added to each well and the cells were incubated for 3 days at 37°C. Control wells were included, containing only Opti-MEM™ I Medium, without siRNA or Lipofectamine™ 2000 Transfection Reagent. After 3 days, the media was changed for serum and antibiotic free DMEM and the cells were incubated for a further 24 hours. Following that, the media was taken off and the cells were incubated with TNF-α (1ng/ml) in serum and antibiotic free DMEM for 24 hours.

3.15.2 Sample collection

Cell supernatants were collected and stored at -20°C. After washing with PBS, RLT buffer from RNeasy® Micro Kit (Qiagen) with β-mercaptoethanol was added into wells for RNA extraction (350µl/well). These samples were then scraped and frozen at -80°C.

3.15.3 RNA purification

RNA purification was performed using RNeasy® Micro Kit (Qiagen) as per the manufacturer's guidelines, similarly to that described in section 3.13.4.

3.15.4 Reverse Transcription

Reverse transcription was performed similarly to that described in section 3.13.5.

3.15.5 Polymerase chain reaction

To assess CNOT7 knockdown, polymerase chain reaction was carried out, similarly to that described in section 3.8.4. Cycling conditions were as follows: 95°C for 5 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute, finished with one cycle of 95°C for

1 minute, 60°C for 30 seconds and 95°C for 30 seconds. CNOT7 primer sequences were: 5'-GAGCTGCAAAAATCTCAAAGG-3' and 5'-GTTGTGGTCCTATCCGTTCC -3'. The difference in expression between control and CNOT7 knockdown cells was determined by the ΔΔCt method. The mean Ct value of β2M (reference gene) in the control TNF-α - stimulated cells was subtracted from the mean Ct value of each sample to give the ΔCt. The mean ΔCt from the control TNF-α - stimulated cells is then subtracted from ΔCt of each sample, to give ΔΔCt. Fold expression compared to the control TNF-α - stimulated cells was then determined by the following equation: 2^(-ΔΔCt).

3.16 Statistical analysis

The data were subjected to statistical analysis to determine statistical significance using GraphPad Prism version 6.00 or 7.00 for Windows. $P<0.05$ was accepted as statistically significant. Mann- Whitney test was used to compare mRNA and protein PRMT expression between asthmatic and non-asthmatic cells, as the samples were not paired and not normally distributed. In ChIP experiments, ratio paired t-test was used to compare between basal and TNF-α stimulated cells, as the samples were paired and the ratios of unstimulated and TNF-α stimulated samples were consistent. In ELISA following dosing with PRMT inhibitors experiments, the means obtained from cells from three different patients were combined. Kruskal-Wallis test with Uncorrected Dunn's test was used to compare between control samples and these dosed with PRMT inhibitors at various concentrations, as the results were not normally distributed. In CRISPR experiments, ratio paired t-test was used to compare qPCR results, as the samples were paired and the ratios of TNF-α stimulated control and PRMT1 knockout samples were consistent. Paired t-test was used to compare ELISA results in CRISPR experiments, as the samples were paired and the differences between TNF-α stimulated control and PRMT1 knockout cells were consistent. In KC ELISA with MEFs, the means obtained from cells from three different experiments were combined. Kruskal-Wallis test with Uncorrected Dunn's test was used to compare between control cells and these dosed with OHT, as the results were not normally distributed.

Chapter 4: The expression of PRMTs in ASM cells

4 The expression of PRMTs in ASM cells

4.1 Introduction

PRMTs are the enzymes that catalyse histone arginine methylation, and they have a role in many important cellular processes, such as DNA repair, RNA processing, regulation of transcription and signal transduction [223]. Eleven mammalian PRMTs have been identified up to date, however, only four of them are currently known to methylate arginine residues on histones *in vivo*- PRMT1, CARM1, PRMT5 and PRMT6. PRMT1 is the most predominant PRMT in eukaryotic cells and carries out 85% of total protein arginine methylation [223, 224]. Studies by Sun *et al.* show that abnormal PRMT1 expression and function may have a crucial role in pathological processes in asthmatic airways [301-303]. CARM1 has an important role in lung development and function [268], and is required for proper differentiation of alveolar cells [337]. Inhibition of CARM1 - mediated H3R17me2a reduces inflammation in a murine model of asthma [305]. PRMT5 is the predominant type II PRMT [224, 272], and is generally associated with transcriptional repression [223, 224, 273, 274]. PRMT6 resides solely in the nucleus and has been associated with both transcriptional repression and transcription [278-280]. Not much is known about a possible role of PRMT5 and PRMT6 in asthma.

4.2 Aims

The specific aims of this chapter were:

- To determine which PRMTs are expressed in human ASM cells, at an mRNA and protein level
- To compare the levels of mRNA expression between different PRMTs in human ASM cells
- To determine whether there is are differences in PRMT expression between ASM cells isolated from subjects with asthma and healthy controls

4.3 Methods

4.3.1 Polymerase chain reaction to determine mRNA expression of PRMTs in ASM cells

ASM cells from healthy subjects and patients with asthma were cultured in 24-well plates, until reaching 90-100% confluency, when they were growth arrested for 24 hours. After removing the media, cells were washed with PBS and lysed in RA1 lysis buffer and β-mercaptoethanol at ratio 100:1. The lysates were transferred to 1.5ml microfuge tubes and the RNA extractions were performed using NucleoSpin® RNA Extraction Kit as per the

manufacturer's guidelines (see 3.2.2). RNA was immediately frozen at -80°C and stored until required. Reverse transcription was carried out, as described in section 3.8.3. PRMTs mRNA was quantified by qPCR as described in section 3.8.4. Data were normalised to β_2M which was used as a housekeeping gene. Mann- Whitney test was used to compare asthmatic and non-asthmatic cells. A value of $P<0.05$ was accepted as significant.

4.3.2 Western blotting to determine protein expression of PRMTs in ASM cells

Cells were cultured on sterile 10 cm Petri dishes, until reaching 90-100% confluency, when they were growth arrested for 24 hours. Following removal of culture medium, the cells were washed with PBS, lysed in lysis buffer and collected by scraping. After centrifugation to remove any insoluble protein, samples were stored at -20°C. The protein concentration in lysates was determined using BCA assay, according to manufacturer's instructions. The samples were diluted with Lamellae buffer, boiled and stored at -20°C. Gel electrophoresis, protein transfer and protein detection were carried out as described in sections 3.9.2, 3.9.3, and 3.9.4.

The results were analysed by densitometry using ImageJ program. Mann- Whitney test was used to compare asthmatic and non-asthmatic cells. A value of $P<0.05$ was accepted as significant

4.4 Results

4.4.1 PRMT mRNA expression in human ASM cells

The mRNA expression of PRMT1, CARM1, PRMT5 and PRMT6 in human ASM cells was investigated by qPCR. Human ASM cells expressed mRNA for all 4 PRMTs. PRMT1 was the methyltransferase with the highest mRNA expression in ASM cells, followed by CARM1 and PRMT6 (Figure 4.1). There was no difference in PRMT mRNA expression between cells isolated from patients with asthma and healthy controls (Figure 4.2).

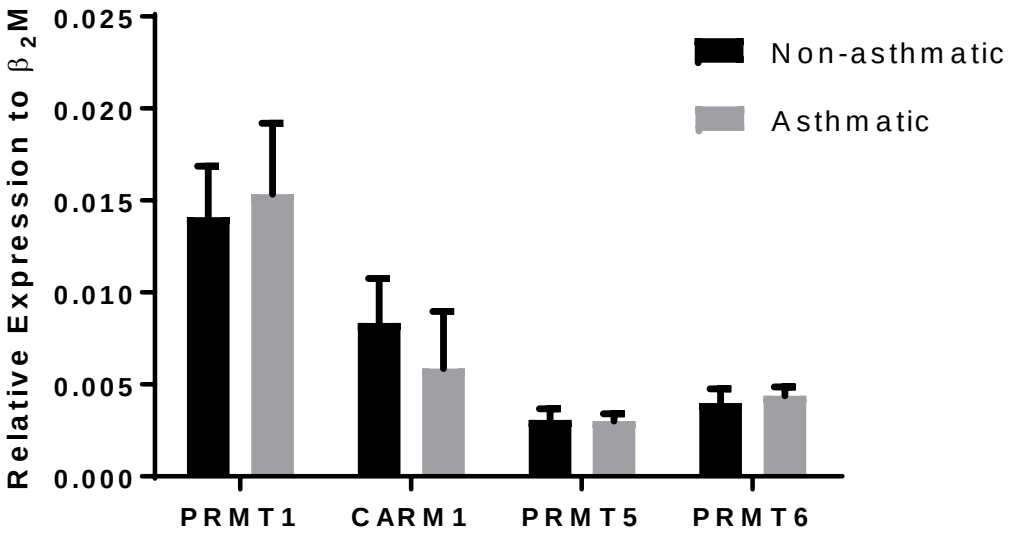

Figure 4.1: Comparison of the levels of PRMT expression in human ASM cells. The mRNA levels of different PRMTs in ASM cells isolated from patients with asthma and healthy controls were assessed by real-time RT-PCR. Data are expressed as the mean ± SEM of ASM cells collected from 5-6 asthmatic patients and 7 healthy controls.

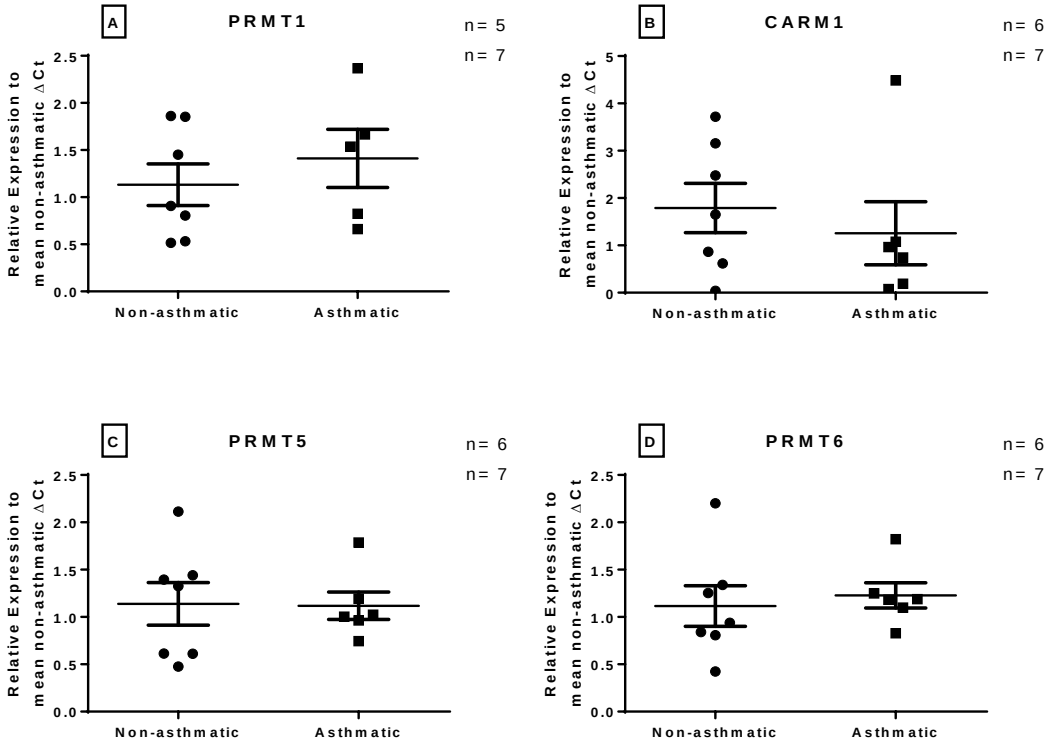

Figure 4.2: The expression of PRMTs in asthmatic and non-asthmatic ASM cells. Expression of PRMT1 (A), CARM1 (B), PRMT5 (C), and PRMT6 (D), in ASM cells isolated from asthmatic donors and healthy controls was assessed by qPCR. Data are expressed as the mean ± SEM of ASM cells collected from 5-6 asthmatic patients and 7 healthy controls, and analysed by Mann- Whitney test.

4.4.2 PRMT protein expression in human ASM cells

Western blotting was carried out to measure PRMT protein expression in human ASM cells. ASM cells expressed the proteins of all the PRMTs assessed- PRMT1, CARM1, PRMT5, and PRMT6 (Figure 4.3.) The blots were analysed by densitometry, to compare the levels of protein PRMTs expression between healthy and asthmatic cells. α- tubulin was used as a loading control for PRMT1 and PRMT6, as their molecular weight is too similar to that of GAPDH, which was the loading control for CARM1 and PRMT5. No significant difference was found between asthmatic cells and controls for any of the PRMTs assessed (Figure 4.4).

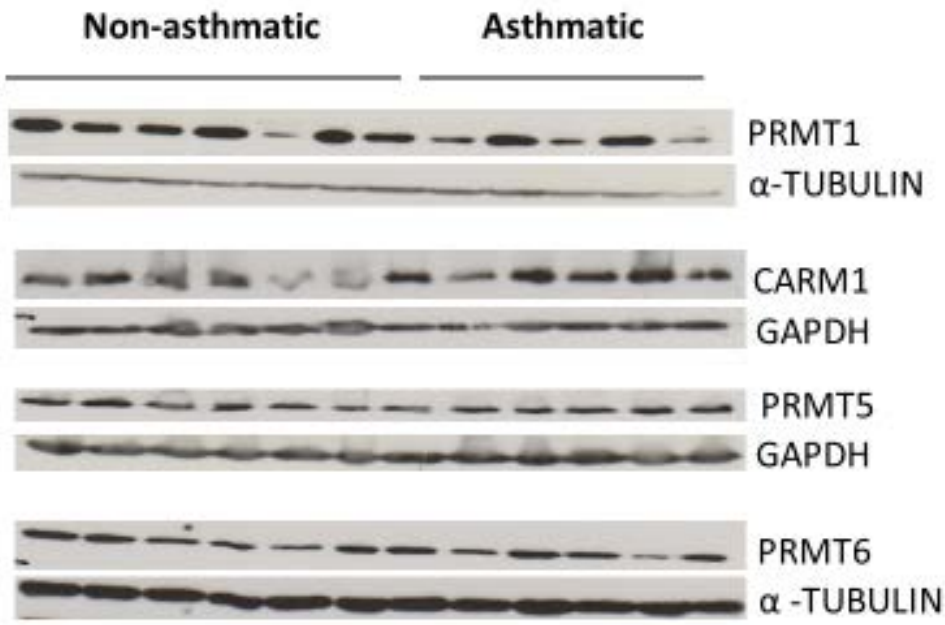

Figure 4.3: Protein expression of PRMTs in asthmatic and non-asthmatic human ASM cells. Protein expression of PRMT1, CARM1, PRMT5 and PRMT6, in cells collected from 5 asthmatic patients and 7 healthy controls, was assessed by western blotting.

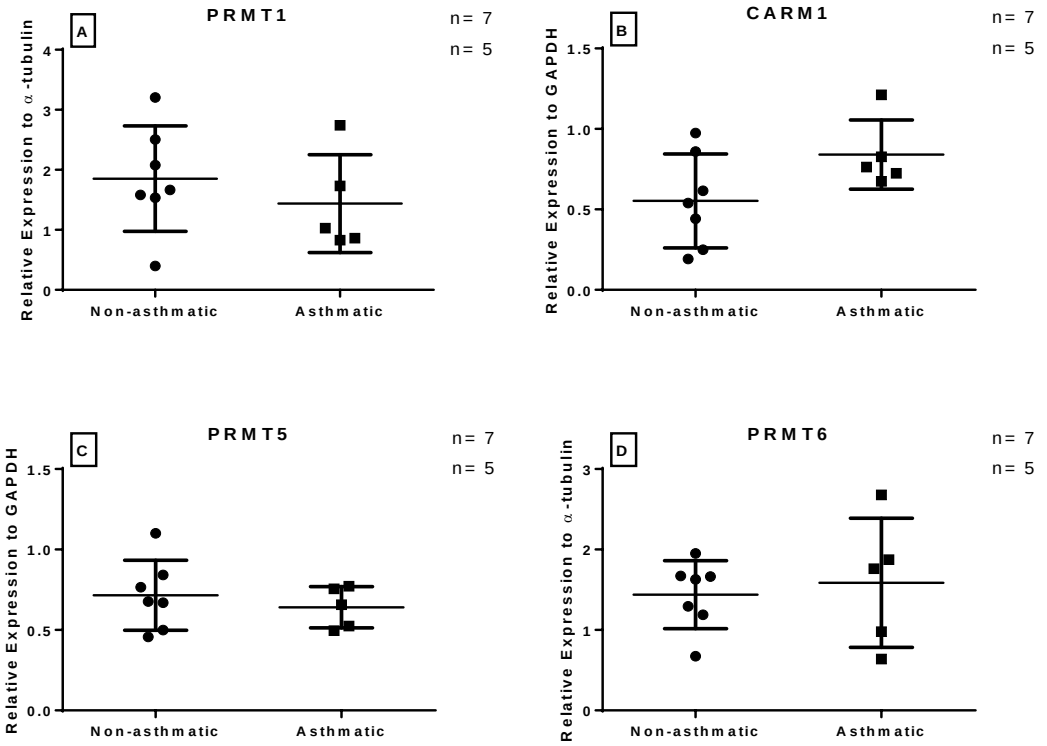

Figure 4.4: Semi-quantitative comparison of PRMT protein expression between cells isolated from asthma patients and healthy controls. The protein expression of PRMT1 (A), CARM1 (B), PRMT5 (C), and PRMT6 (D) was analysed by densitometry of western blots. Data are expressed as the mean ± SEM of ASM cells collected from 5 asthmatic patients and 7 healthy controls, and analysed by Mann-Whitney test.

4.5 Discussion

The aims of this chapter were to: confirm that human ASM cells expressed PRMTs on both mRNA and protein level, to compare the levels of mRNA expression between different PRMTs, and to determine whether human ASM cells isolated from patients with asthma differed in their PRMT expression profile from ASM cells obtained from healthy volunteers. We showed that human ASM cells expressed mRNA and protein of all four PRMTs investigated in this study, namely PRMT1, CARM1, PRMT5 and PRMT6. When this project was commenced, there were no publications showing that human ASM cells expressed any PRMT. However, during the course of the project, Sun *et al*. showed that PRMT1 was expressed in lung sections and isolated human ASM cells [303]. Our studies confirmed this, but also showed for the first time that human ASM cells express CARM1, PRMT5 and PRMT6. We also showed that PRMT1 had the highest mRNA expression in human ASM cells, followed by CARM1 and PRMT6. PRMT1 it is the most predominant PRMT in eukaryotic cells,

catalysing 85% of total protein arginine methylation [223, 224], therefore this result is perhaps not surprising.

We did not observe any difference in the expression of any of the PRMTs in ASM cells between healthy and asthmatic cells, neither in mRNA nor protein. These results contrast with a study by Sun *et al*., who found that the expression of PRMT1 was significantly higher in lung tissue sections and in isolated ASM cells of patients with severe asthma [303]. We have considered why our results contradict the findings of the Sun *et al.* study. We considered whether it might reflect the small numbers studied. Our experiments were carried out on cells isolated from only 5-6 asthmatic patients and we initially thought that repeating them with a wider range of isolated cells might show difference in PRMT expression profile between patients with asthma and healthy subjects (Sun *et al.* used samples from 8 control subjects and 9 patients with asthma). However, in further confirmation of my qPCR data, Dr Rachel Clifford within the department, as part of her studies, generated Affymetrix Human Gene 2.1 ST Arrays in a larger cohort of samples, which also showed no differential PRMT1 expression (see Appendix).

The severity of disease could be an alternative explanation for the disparity between our findings and those by Sun *et al*. This however seems unlikely, as they used ASM cells isolated from severe asthmatics and at least 5 out of 6 samples used in our study came from patients with severe asthma (based on treatment). The experiments in Sun *et al*. study were also performed in ASM cells at different passages (between passages 2 and 8), which could have introduced error, while all of our experiments were carried out with ASM cells at passage 6. Another possible reason for the contradiction in findings is that the control group in Sun *et al*. study consisted of patients who underwent lung biopsy for diagnosis of diseases other than asthma and chronic obstructive pulmonary disease (COPD), mainly for lung metastases of tumours in other organs [303], while our control group consisted of healthy volunteers. Possible lung metastases could have affected experimental results in cells obtained from these patients.

Our experiments were carried out in basal conditions, without any stimulation with proinflammatory cytokines, such as TNF-α or IL-1β, commonly used in similar studies. It is possible that even though basal expression of PRMTs was the same in ASM cells from healthy subjects and patients with asthma, it could differ in response to stimulation with a proinflammatory cytokine.

Regardless of whether the expression of PRMTs was increased in ASM cells obtained from patients with asthma, the activity of PRMTs at specific inflammatory cytokine/chemokine promoters, might be altered in asthma. Therefore even though the global expression of PRMTs was unchanged, PRMTs could still have an important role in transcription of inflammatory genes, via increased binding to the promoters, or increased activity.

The previous study by Sun *et al*. showed a significant decrease in CARM1 mRNA expression in the lungs of AIPI rats [301]. We did not observe any difference in CARM1 mRNA expression between ASM cells from patients with asthma and healthy subjects. This disparity is most likely to be attributed to the difference between rat asthma model and human ASM cells.

We analysed our results to investigate whether there was a difference in the expression of PRMTs between ASM cells isolated from males and females. However, no statistical difference was observed, neither in mRNA, nor protein (see Appendix). We also performed analysis to see whether the mRNA and protein expression of each of the PRMTs levels were correlated in ASM cells. There was a weak correlation between mRNA and protein levels for PRMT1 and PRMT6 ($r= 0.39$ for PRMT1 and $r= 0.24$ PRMT6, by Spearman's rank correlation test). However, it has been reported that the correlation between mRNA and protein levels in cells is poor and depends on many factors, such as RNA secondary structure, regulatory proteins, ribosomal density, and half-life of proteins [338].

Collectively, our findings suggested that PRMTs may have a role in ASM cell biology and their role in asthma should be further investigated. In the next chapter, we continued our study on PRMTs in ASM cells, investigating how pharmacological inhibition of each of the PRMTs affected the inflammation in ASM.

Chapter 5: The effect of pharmacological inhibition of PRMTs on TNF-α-induced cytokine/chemokine secretion from human ASM cells

5 The effect of pharmacological inhibition of PRMTs on TNF-α-induced cytokine/chemokine secretion from ASM cells

5.1 Introduction

In Chapter 4, we showed that human ASM cells expressed PRMT1, CARM1, PRMT5 and PRMT6 mRNA and protein. In this chapter, we investigated how pharmacological inhibitors of these PRMTs affected TNF-α-induced cytokine/chemokine secretion from ASM cells.

We chose to focus on eotaxin, IL-6, IP-10 and CXCL8, based on their role in recruiting different inflammatory cell types into the airways in asthma. Eotaxin has a selective chemoattractant activity for eosinophils [86], which contributes to airway eosinophilia- a prominent feature of asthma [85]. ASM is a rich source of eotaxin [81], and its release is further increased in response to proinflammatory cytokines, such as TNF-α and IL-1β [86], and Th2 cytokines, including IL-4 and IL-13 [87]. ASM samples collected from subjects with asthma have increased eotaxin immunoreactivity compared to samples from healthy individuals [85]. IL-6 is a pleiotropic cytokine that can have both proinflammatory and anti-inflammatory action in asthma [81]. Patients with symptomatic and intrinsic asthma have higher concentrations of IL-6 in BALF than healthy subjects [339]. ASM cells secrete IL-6 in response to TNF-α and bradykinin [113, 114]. IP-10 is a potent chemoattractant for mast cells and T lymphocytes, which are implicated in the pathophysiology of asthma [116]. IP-10 levels are raised in the airways of asthmatic patients, as well as in the bronchial mucosa and BALF of patients suffering from moderate to severe form of the disease, which is usually resistant to treatment with steroids [116, 122]. ASM cells do not produce IP-10 basally, but they release it in response to TNF-α and IFN-γ, and it has been shown that these cytokines synergistically enhance IP-10 release when combined [116, 124]. IP-10 is a strong inhibitor of angiogenesis, in contrast to CXCL8 [217]. CXCL8 is thought to be crucial in the initiation and maintenance of inflammatory responses, and is involved in neutrophil recruitment during exacerbations in asthma [81]. It is also an angiogenic factor, and may contribute to angiogenesis [81]. Besides its potent chemoattracting action towards neutrophils, it is also thought to be a mast cell chemoattractant [81, 95, 96].

In this chapter, we used commercially available inhibitors of PRMTs to investigate how PRMT inhibition affected the TNF-α-induced cytokine/chemokine secretion from ASM cells isolated from healthy subjects. To simplify protocols, we decided to discontinue using ASM cells isolated from asthma patients and instead chose to use ASM cells isolated from healthy volunteers, under basal and TNF-α-stimulated conditions.

5.2 Aims

The aims of this chapter were:

- To investigate how inhibitors of four different PRMTs affected TNF-α-induced secretion of eotaxin, IL-6, IP-10 and CXCL8 from human ASM cells isolated from healthy subjects

5.3 Methods

5.3.1 Preparation of inhibitor solutions

PRMT inhibitors (TC-E 5003, EPZ015666, 217531, EPZ020411) were reconstituted in DMSO to a final concentration of 10mM. They were then aliquoted and stored at -80°C.

Table 5.1: PRMT inhibitors used in the study

Inhibitor	PRMT targeted	Supplier	IC_{50}	Selectivity
TC-E 5003	PRMT1	Tocris	1.5 μM	Selective for PRMT1 over PRMT4/CARM1 and SET7/9 methyltransferases, with less than 5% inhibition at a concentration of 50 μM for SET7/9 [340].
217531	PRMT4/CARM1	EMD Millipore	7.1 μM	Inhibits PRMT3 only at much higher concentrations (by 70% and 62% with 100 μM inhibitor). Exhibits little activity against PRMT1, PRMT5, PRMT6 (IC_{50} >100 μM), or HKMTs (<20% inhibition against DOTL1, G9a, SET7, or Suv39H1 with 100 μM inhibitor) [341].
EPZ015666	PRMT5	Sigma-Aldrich	22 nM	No inhibition up to the maximum tested concentration of 50 μM against a panel of 20 other protein methyltransferases [342].
EPZ020411	PRMT6	Cayman Chemical	10 nM	Targets PRMT6 with IC_{50} of 10 nM. Targets PRMT1 and PRMT8 at higher concentrations (IC_{50} = 119 and 223 nM, respectively) [343].

5.3.2 Cell culture

Human ASM cells from three healthy volunteers were cultured in 48-well plates until reaching 90-100% confluency, when they were growth arrested for 24 hours. The cells were then dosed with PRMT inhibitors diluted in serum-free media to a series of concentrations (0-10μM) and incubated at 37°C for 30 minutes, following which they were incubated with TNF-α (1ng/ml) for 24 hours. Supernatants were then collected and stored at -20°C.

5.3.3 Cell viability

The toxicity of PRMT inhibitors was determined by MTT assay, as described in section 3.12.3.

5.3.4 Human CCL11/Eotaxin, IL-6, CXCL10/IP-10 and IL-8/CXCL8 ELISA

Elisa assays were performed as described in sections 3.12.4., 3.12.5, 3.12.6, and 3.12.7.

5.3.5 Statistical analysis

The means obtained from cells from three different patients were combined. Kruskal-Wallis test with Uncorrected Dunn's test was used to compare between control samples and these dosed with PRMT inhibitors at various concentrations. A value of $P<0.05$ was accepted as significant. The effect of the PRMT inhibitors on changes in the secretion of chemokines under basal conditions were not analysed, as secretion was low and would most likely not had any impact *in vivo*.

5.4 Results

5.4.1 The effect of PRMT inhibitors on TNF-α-induced secretion of cytokines/chemokines from human ASM cells

5.4.1.1 PRMT1 Inhibitor

TC-E 5003, a PRMT1 inhibitor, significantly inhibited TNF-α-induced secretion of eotaxin at the highest concentration tested, 10μM (Figure 5.1, p=0.04 in Kruskal-Wallis test and p= 0.027 in Uncorrected Dunn's test comparing DMSO control vs TC-E 5003 at 10μM). TC-E 5003 also inhibited the TNF-α-induced secretion of IL-6, IP-10 and CXCL8, although the result were not statistically significant (p=0.12, p=0.07, and p=0.18, respectively). Raw concentrations of the cytokines secreted by control ASM cells (i.e. with DMSO in media) are presented in Figure 10.5 (see Appendix).

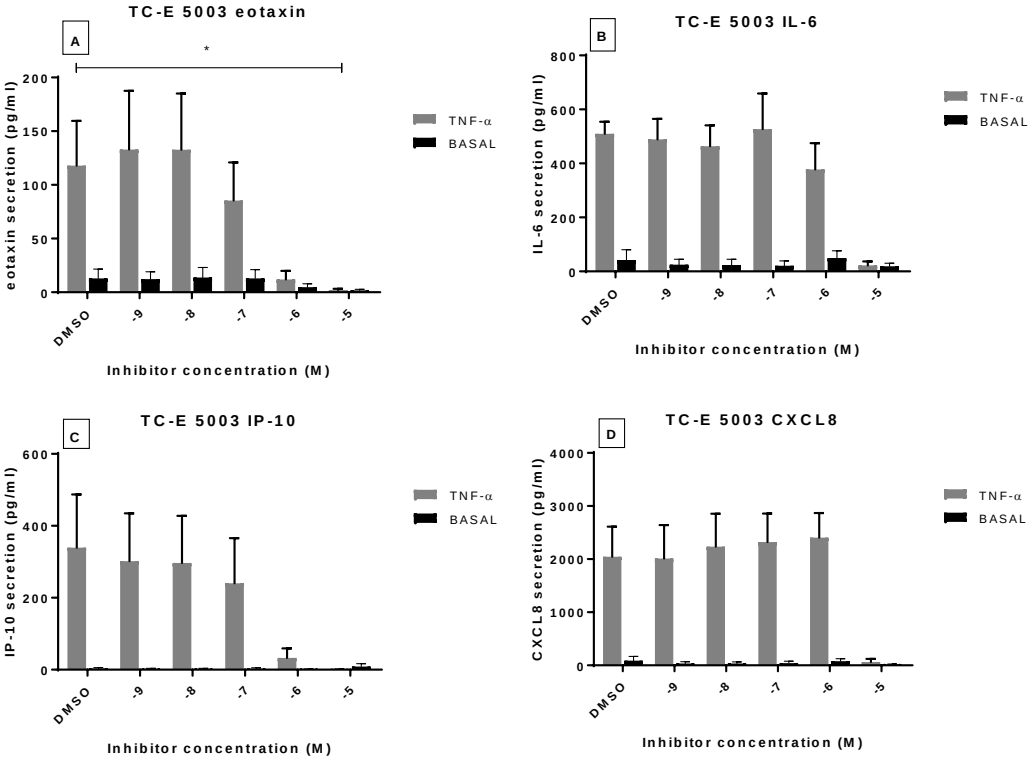

Figure 5.1: The effect of inhibition of PRMT1 with TC-E 5003 on the secretion of eotaxin (A), IL-6 (B), IP-10 (C), and CXCL8 (D) from ASM cells. The levels of eotaxin, IL-6, IP-10 and CXCL8 secreted by ASM cells following the inhibition of PRMT1 with TC-E 5003, under basal and TNF-α (1 ng/ml) stimulated conditions, were assessed by ELISA. N=3 (* $p<0.05$)

5.4.1.2 CARM1 Inhibitor

217531, a CARM1 inhibitor, inhibited TNF-α-induced secretion of eotaxin, IL-6, IP-10, and CXCL8, at the highest concentration tested, 10μM (Figure 5.2). However, the inhibition was not statistically significant (p=0.16, p=0.2, p=0.17, and p=0.17, for eotaxin, IL-6, IP-10, and CXCL8, respectively).

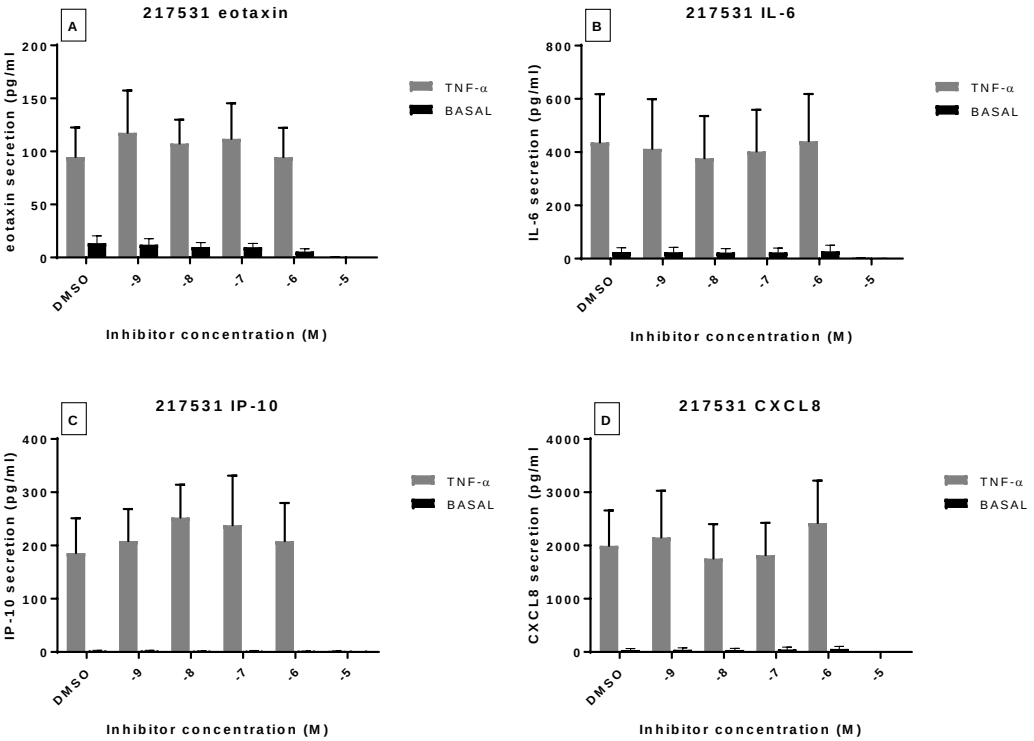

Figure 5.2: The effect of inhibition of CARM1 with 217531 on the secretion of eotaxin (A), IL-6 (B), IP-10 (C), and CXCL8 (D) from ASM cells. The levels of eotaxin, IL-6, IP-10 and CXCL8 secreted by ASM cells following the inhibition of CARM1 with 217531, under basal and TNF-α (1 ng/ml) stimulated conditions, were assessed by ELISA. N=3

5.4.1.3 PRMT5 Inhibitor

EPZ015666, a PRMT5 inhibitor, had no significant effect on the secretion of inflammatory cytokines/chemokines (Figure 5.3, p=0.33, p=0.9, p=0.88, and p=0.8, for eotaxin, IL-6, IP-10, and CXCL8, respectively).

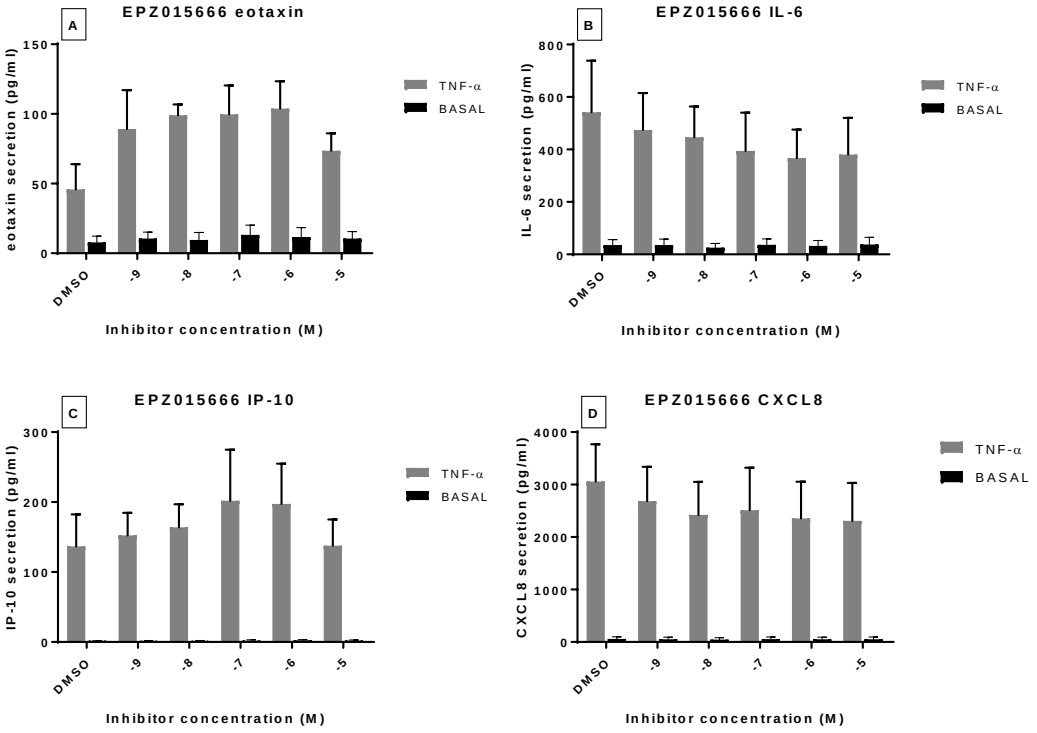

Figure 5.3: The effect of inhibition of PRMT5 with EPZ015666 on the secretion of eotaxin (A), IL-6 (B), IP-10 (C), and CXCL8 (D) from ASM cells. The levels of eotaxin, IL-6, IP-10 and CXCL8 secreted by ASM cells following the inhibition of PRMT5 with EPZ015666, under basal and TNF-α (1 ng/ml) stimulated conditions, were assessed by ELISA. N=3

5.4.1.4 PRMT6 Inhibitor

EPZ020411, a PRMT6 inhibitor, had no significant effect on the secretion of inflammatory cytokines/chemokines (Figure 5.4, p=0.75, p=0.84, p=0.87, and p=0.95, for eotaxin, IL-6, IP-10, and CXCL8, respectively).

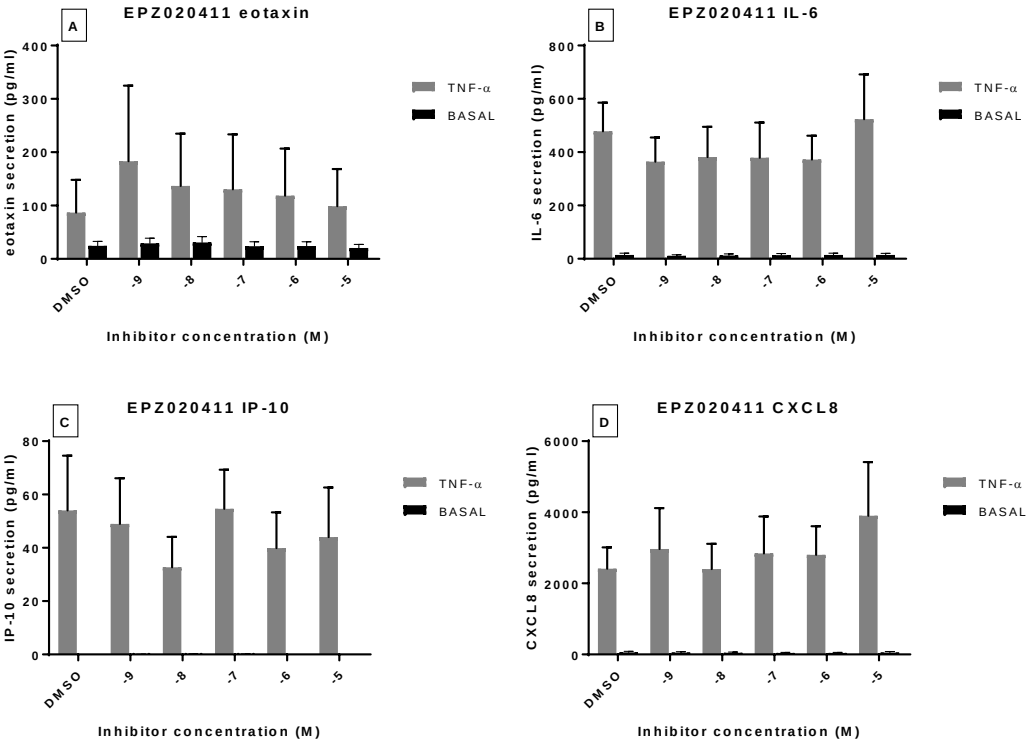

Figure 5.4: The effect of inhibition of PRMT6 with EPZ020411 on the secretion of eotaxin (A), IL-6 (B), IP-10 (C), and CXCL8 (D) from ASM cells. The levels of eotaxin, IL-6, IP-10 and CXCL8 secreted by ASM cells following the inhibition of PRMT6 with EPZ020411, under basal and TNF-α (1 ng/ml) stimulated conditions, were assessed by ELISA. N=3

5.4.2 MTT assays of cell viability

MTT assays were carried out to see whether any of the inhibitors were toxic to ASM cells and reduced cell viability. The PRMT inhibitors used in our study had no negative impact on ASM cell viability, with the exception of CARM1 inhibitor 217531, which reduced the viability of ASM cells at 10µM, to about 85% of control values under both basal and TNF-α – stimulated conditions (Figure 5.5).

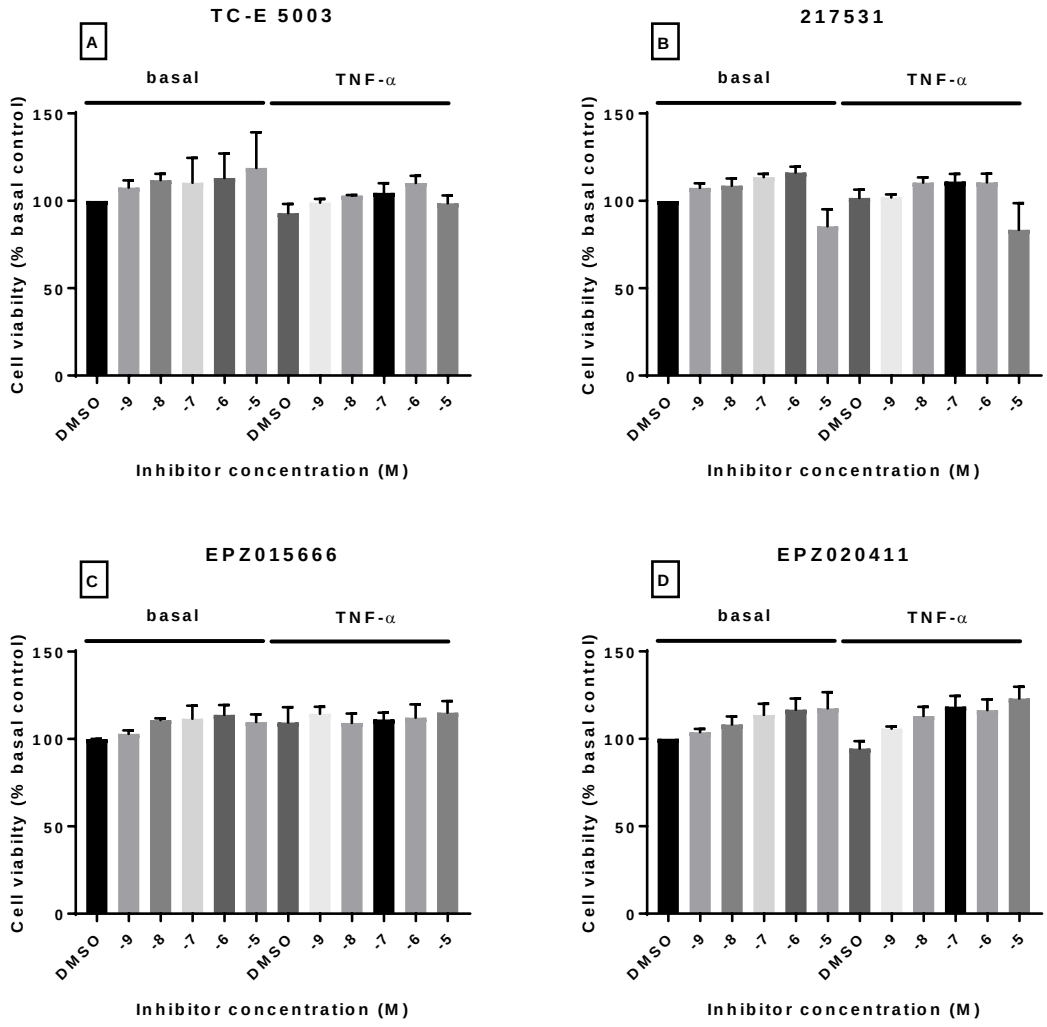

Figure 5.5: The viability of human ASM cells, following the dosing with TC-E 5003 (A), 217531 (B), EPZ015666 (C) and EPZ020411 (D). Cell viability following the dosing with PRMT inhibitors, under basal and TNF-α (1 ng/ml) stimulated conditions, was assessed by MTT assay. The results are expressed as % of DMSO controls under basal conditions. N=3

5.5 Discussion

The aim of this chapter was to investigate how inhibitors of four different PRMTs affected TNF-α-induced secretion of cytokines/chemokines in human ASM cells. The main findings were that the PRMT1 inhibitor TC-E 5003 and the CARM1 inhibitor 217531 inhibited TNF-α-induced secretion of the cytokines/chemokines, even though the inhibition failed to reach statistical significance (with the exception of TC-E 5003 inhibiting the secretion of eotaxin), whereas the PRMT5 and 6 inhibitors had no effect.

The only other studies to look at PRMT inhibitors in asthma are those of Sun *et al.* [301-303, 344]. The administration of AMI-1 (a pan-PRMT inhibitor, 50µl at a concentration of 0.1 mg/ml in PBS) in AIPI rats reduced pulmonary inflammation, humoral immune response and the production of IL-4 and COX-2. Moreover, AMI-1 reduced expression of collagen and fibronectin in ASM cells and primary lung fibroblasts. EPZ015666, a PRMT5 inhibitor used in our study, has been shown to reduce TNF-α-induced IL-6 and CXCL8 mRNA and secretions in fibroblast-like synoviocytes (FLSs) from patients with RA [25].

In our study, we used inhibitors with the greatest reported selectivity against each of the PRMTs. The main problem with the use of pharmacological inhibitors generally is lack of selectivity and off target effects. In addition, some compounds may show efficacy against a specific substrate *in vitro* but lack penetration in cell systems. We did not measure the ability of the various PRMT inhibitors to inhibit their targets in our cells as this would require an assay of specific PRMT activity in cell systems which is not currently available.

The PRMT1 inhibitor TC-E 5003 was the most promising in our studies. However we would need to use molecular tools to conclusively show a role for PRMT1 and also show that PRMT1 causes important histone arginine methylation events in our cells. We considered using AMI-1 rather than TC-E 5003, to target PRMT1. However AMI-1 also inhibits CARM1 and PRMT6 [345], while TC-E 5003 is more selective towards PRMT1 and does not inhibit CARM1 [340]. However, it has been shown that the loss of PRMT1 may lead to substrate scavenging by other PRMTs and global increases of MMA and SDMA levels, with reduction in ADMA levels [242]. Therefore it should be noted that even if the compound is selective towards PRMT1, the inhibition can alter other types of arginine methylation. TC-E 5003 inhibits PRMT1 with IC_{50} of 1.5µM in *in vitro* methylation assays [340]. However, IC_{50} values differ between *in vitro* assays and cell systems. Moreover, assay methods and conditions for IC_{50} measurements often vary in one study from another, which can result in disparities in IC_{50} reported for the

same compound between studies [346]. For example, the reported IC_{50} for AMI-1 inhibition of PRMT1 activity ranges from 1.2 μM [347] to 376 μM [326]. Therefore IC_{50} values for PRMT inhibitors reported in literature need to be interpreted with caution. In our study, TC-E 5003 significantly inhibited TNF-α-induced secretion of eotaxin, and also showed the trend towards the inhibition of other chemokines/cytokines at 10uM. Interestingly, TC-E 5003 had some non-concentration-dependent effect on CXCL8, unlike on the other cytokines/chemokines. Even though there was a trend towards the inhibition of CXCL8 at 10μM, TCE5003 actually increased TNF-α-induced secretion of CXCL8 at lower concentrations. Different effect of TC-E 5003 at lower concentrations on CXCL8, as opposed to eotaxin, IL-6, and IP-10 should be further investigated in future studies.

The CARM1 inhibitor 217531 [319] only had an effect, albeit not statistically significant, on the secretion of cytokines/chemokines at the highest concentration tested, 10μM. At this concentration, 217531 caused a slight reduction in cell viability and so results with it should be viewed with caution and additional evidence would be needed with either other inhibitors, or molecular tools, to implicate CARM1 in TNF-α- responses in human ASM cells.

EPZ015666 has a high selectivity towards PRMT5 [341, 342], and EPZ020411 inhibits PRMT6-specific H3R2me2a mark, associated with transcriptional repression [343, 348]. The fact that neither agent reduced the secretion of inflammatory mediators suggests either that PRMT5 and PRMT6 are not important in mediating TNF-α-induced responses in ASM cells, or that the inhibitors were either poorly cell permeant or are relatively ineffective at inhibiting these PRMTs. Although EPZ015666 inhibited PRMT5- catalysed symmetric arginine demethylation on multiple substrates in Mantle cell lymphoma (MCL) cell lines at 5 μM, global levels of H4R3me2s and H3R8me2s were not altered [342]. Therefore even though EPZ015666 is cell permeant and cell-potent, it is possible that it did not have any activity of histones in our experiments. EPZ020411 has been shown to inhibit PRMT6-catalysed H3R2me2a in A375, a melanoma cell line, with IC_{50} at 0.637 μM [343]. Therefore it is likely that EPZ020411 successfully inhibited PRMT6 in our experiments and our results suggest lack of a significant role of PRMT6 in TNF-α-induced inflammatory responses in ASM cells. However, it should also be noted that EPZ020411 has also been shown to inhibit PRMT1 at higher concentrations.

In summary, the results of this chapter suggested that targeting PRMT1 was the most promising way of reducing TNF-α-induced production of cytokines/chemokines. In view of this and the fact that in Chapter 4 we had shown that PRMT1 was one of the most abundantly

expressed PRMTs in human ASM, we decided to make PRMT1 our primary focus in subsequent experiments. In the next chapter, we investigated the effect of TNF-α on PRMT1-catalysed histone arginine methylation at the promoters of eotaxin, IL-6, IP-10, CXCL8.

Chapter 6: The effect of TNF-α stimulation on PRMT1- catalysed histone arginine methylation at the eotaxin, IL-6, IP-10 and CXCL8 promoters

6 The effect of TNF-α stimulation on PRMT1- catalysed histone arginine methylation at the eotaxin, IL-6, IP-10 and CXCL8 promoters

6.1 Introduction

In view of the results in Chapter 4 and 5, we decided to focus on PRMT1. In this chapter, we investigated the histone arginine methylation mark (H4R3me2a) catalysed by PRMT1 at the eotaxin, IL-6, IP-10 and CXCL8 promoters, and the effect of TNF-α on this. We also investigated if the PRMT1 inhibitor TC-E 5003 inhibited PRMT1- catalysed H4R3me2a at the promoters of these cytokines/chemokines. H4R3me2a facilities subsequent acetylation of histone H3 and H4, and is required for histone acetylation and maintenance of an active chromatin domain *in vivo* [247]. Therefore PRMT1-catalysed H4R3me2a is an important mark linked to transcriptional activation.

Previous studies from our group have shown that histone lysine acetylation and methylation are involved in the regulation of the transcription of several cytokine/chemokine, growth factor and lipid mediator genes in human ASM [106, 311, 312]. The role of histone arginine methylation in regulating cytokine/chemokine genes in human ASM has not been previously investigated.

6.2 Aims

The aims of this chapter were:

- To investigate the effect of TNF-α on the H4R3me2a at the eotaxin, IL-6, IP-10 and CXCL8 promoters
- To determine how TNF-α affects the association of PRMT1 with the cytokine/chemokine promoters
- To determine if the PRMT1 inhibitor TC-E 5003 inhibits H4R3me2a at the cytokine/chemokine promoters

6.3 Methods

6.3.1 Cell culture

Human ASM cells from healthy volunteers (n=3-4) were cultured on sterile 500cm^2 plates. Upon reaching 90-100% confluency, they were growth arrested for 24 hours. Following removal of culture medium, the cells were incubated with TNF-α (1ng/ml), at various time points, ranging from 30 minutes to 2 hours. For experiments with TC-E 5003, cells were pre-

incubated with TC-E 5003 in serum free media at 10μM for 30 minutes, following which they were incubated with TNF-α (1ng/ml) for 1 hour.

6.3.2 ChIP Assay for H4R3me2a

ChIP assay was performed using the ChIP-IT™ Express kit (Active Motif), as described in section 3.11.

6.3.3 ChIP Assay for PRMT1 and H4R3me2a with TC-E 5003

Due to technical difficulties experienced while performing experiments with ChIP-IT™ Express kit from Active Motif, ChIP assays for PRMT1 and H4R3me2a with TC-E 5003 were performed using the SimpleChIP® Plus Sonication Chromatin IP Kit (Cell Signaling Technology), as described in section 3.10.

6.3.4 Real time PCR and statistical analysis

The recovered DNA from ChIP was quantified by real-time PCR as described in section 3.10.1. The data were analysed using GraphPad Prism version 6.00 or 7.00 for Windows. The ratio paired t-test was used to compare basal and TNF-α stimulated cells. $P<0.05$ was accepted as statistically significant.

6.4 Results

6.4.1 The effect of TNF-α on H4R3me2a at the cytokine/chemokine promoters

H4R3me2a was present under basal conditions at the promoters of all four inflammatory cytokines/chemokines (Figure 6.1). Stimulation with TNF-α for 1 hour significantly increased the levels of H4R3me2a at the eotaxin ($P=0.01$), IL-6 ($P=0.01$), IP-10 ($P=0.02$) and CXCL8 promoters ($P=0.0007$, Figure 6.1). The increase was transient, and no difference in H4R3me2a was seen at 2h of TNF-α stimulation (data not shown).

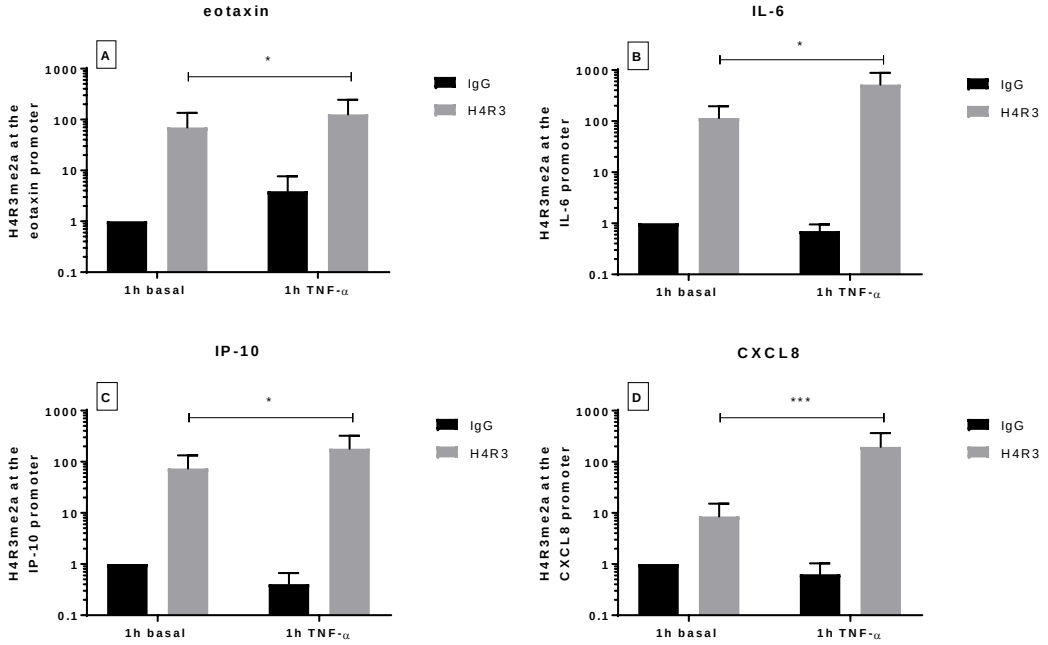

Figure 6.1: H4R3me2a at the eotaxin (A), IL-6 (B), IP-10 (C), and CXCL8 (D) promoters. H4R3me2a at the eotaxin, IL-6, IP-10 and CXCL8 promoters was assessed by ChIP under basal and TNF-α (1 ng/ml for 1 hour) stimulated conditions. Data are expressed relative to the unstimulated IgG control and presented on a log scale. N=4 (* $p<0.05$, ***$p<0.001$)

6.4.2 The effect of TNF-α on the association of PRMT1 with the cytokine/chemokine promoters

To investigate if the increase in H4R3me2a was a result of increased association of PRMT1 with the promoters, we measured PRMT1 association with the promoters by ChIP. As the increase in H4R3me2a was seen at 1h of TNF-α stimulation, we investigated PRMT1 association with the promoters at 30 minutes and 1h. 30 minutes of TNF-α stimulation caused a small increase in PRMT1 association with the cytokine/chemokine promoters, but this was not statistically significant (Figure 6.2). We repeated the experiment with 1 hour of TNF-α stimulation. Again, we saw a small increase in PRMT1 binding to the promoters of eotaxin, IL-6, IP-10 and CXCL8 but this was not statistically significant (Figure 6.3).

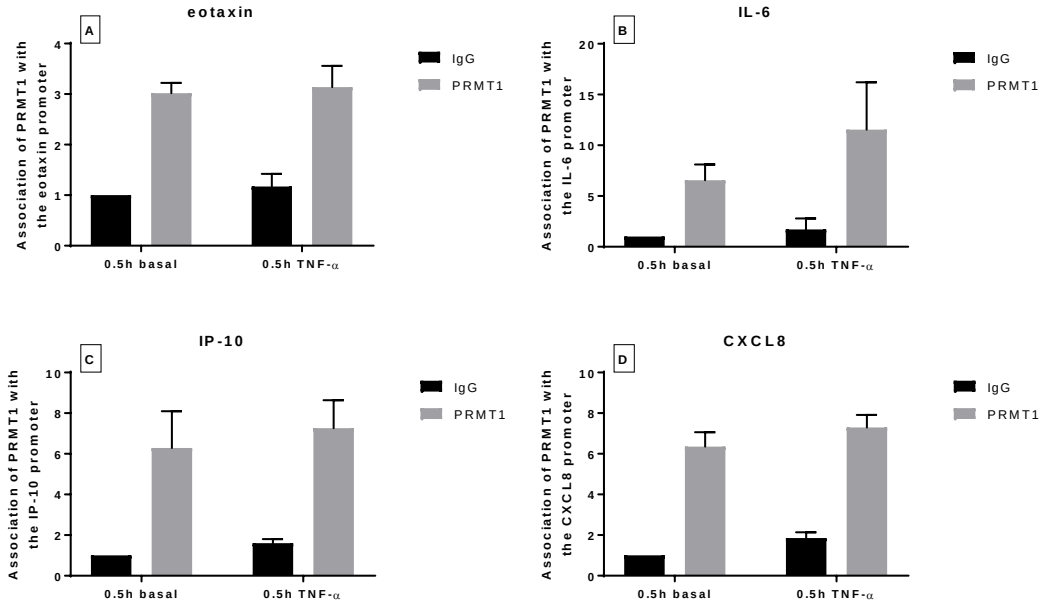

Figure 6.2: PRMT1 association with the eotaxin (A), IL-6 (B), IP-10 (C), and CXCL8 (D) promoters following stimulation with TNF-α for 30 minutes. The association of PRMT1 at the eotaxin, IL-6, IP-10 and CXCL8 promoters was assessed by ChIP under basal and TNF-α (1 ng/ml for 30 minutes) stimulated conditions. Data are expressed relative to the unstimulated IgG control. N=4

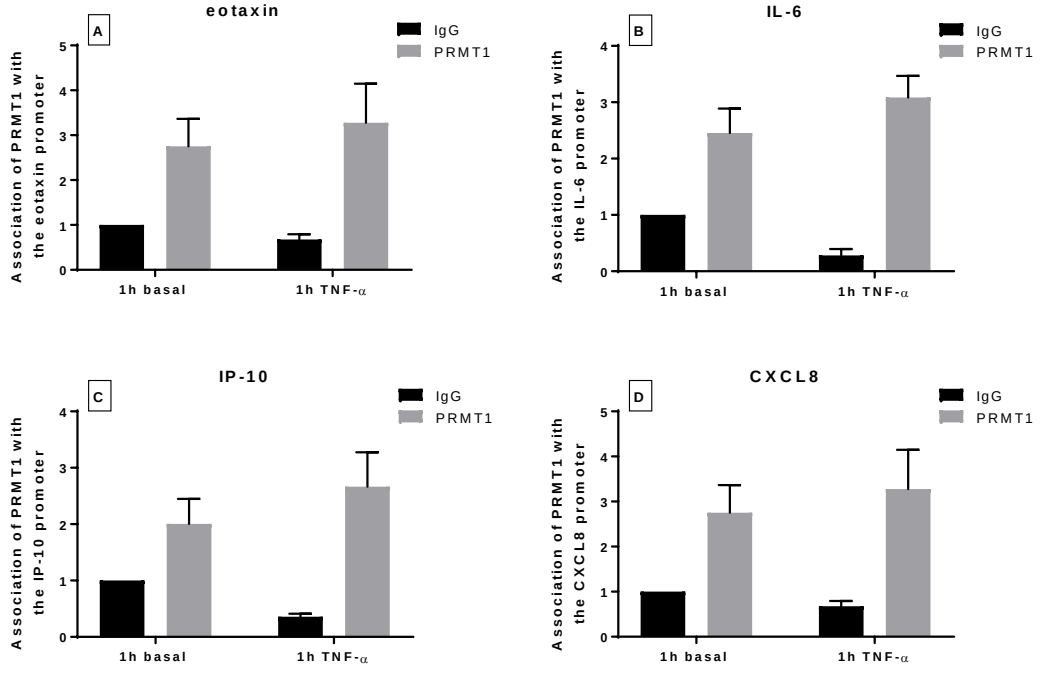

Figure 6.3: PRMT1 association with the eotaxin (A), IL-6 (B), IP-10 (C), and CXCL8 (D) promoters following stimulation with TNF-α for 1 hour. The association of PRMT1 at the eotaxin, IL-6, IP-10 and CXCL8 promoters was assessed by ChIP under basal and TNF-α (1 ng/ml for 1 hour) stimulated conditions. Data are expressed relative to the unstimulated IgG control. N=3

6.4.3 The effect of pharmacological inhibition of PRMT1 on H4R3me2a at the cytokine/chemokine promoters

The PRMT1 inhibitor TC-E 5003 significantly inhibited TNF-α-induced H4R3me2a at the CXCL8 promoter (P=0.01, Figure 6.4). However, there was no significant difference between TNF-α- stimulated cells with or without TC-E 5003, in their levels of H4R3me2a at eotaxin, IL-6, or IP-10 promoters (Figure 6.4).

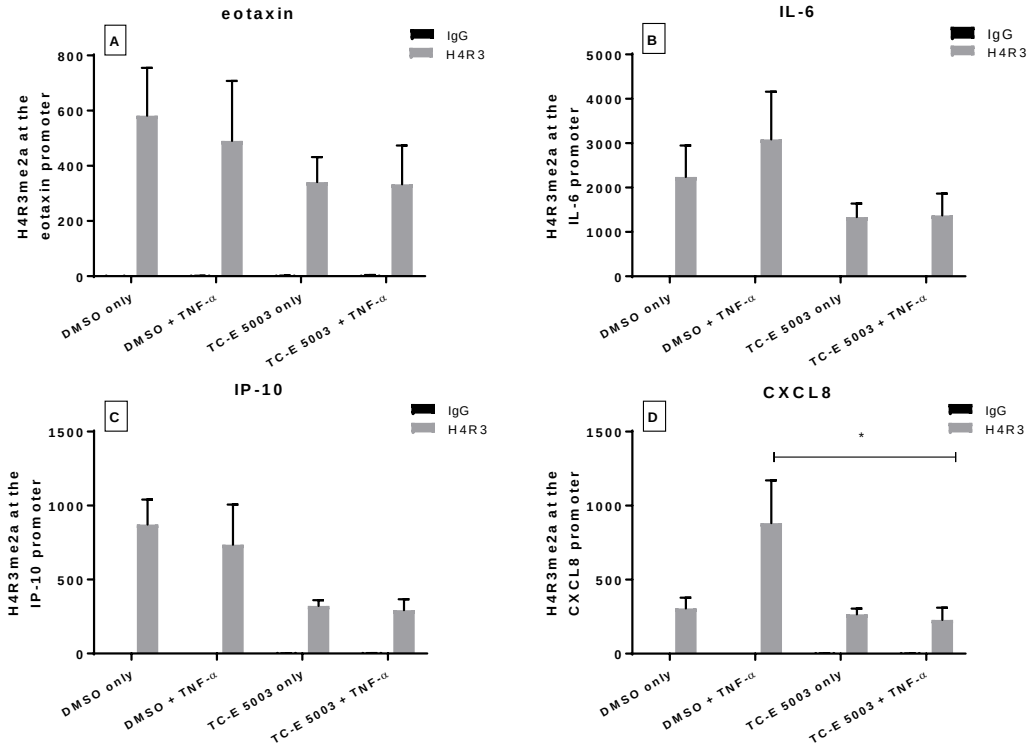

Figure 6.4: The effect of inhibiting PRMT1 with TC-E 5003 on H4R3me2a at the eotaxin (A), IL-6 (B), IP-10 (C), and CXCL8 (D) promoters. The effect of TC-E 5003 on H4R3me2a at the eotaxin, IL-6, IP-10 and CXCL8 promoters was assessed by ChIP under basal and TNF-α (1 ng/ml for 1 hour) stimulated conditions. Data are expressed relative to the unstimulated IgG control. N=3 (* $p<0.05$)

6.5 Discussion

The aims of this chapter were to investigate PRMT1- catalysed histone arginine methylation at the promoters of eotaxin, IL-6, IP-10 and CXCL8, under basal and TNF-α- stimulated conditions. We also aimed to show the association of PRMT1 with the promoter of these cytokines/chemokines, and how TNF-α affects it. Lastly, we wanted to see how pharmacological inhibition of PRMT1 affects H4R3me2a at the eotaxin, IL-6, IP-10 and CXCL8 promoters. The main finding of this chapter is that TNF-α-induced H4R3me2a at eotaxin, IL-6, IP-10 and CXCL8 promoters, but did not cause a significant increase in PRMT1 promoter association. We also demonstrated that the PRMT1 inhibitor TC-E 5003 inhibited TNF-α-induced H4R3me2a at the CXCL8 promoter but not the other promoters.

The finding that TNF-α induced H4R3me2a at the cytokine/chemokine promoters was expected from our previous results given that the PRMT1 inhibitor in Chapter 5 inhibited TNF-α-induced cytokine/chemokine production and H4R3me2a is a PRMT1 methylation mark. However, we were unable to demonstrate significant induction of PRMT1 binding to the promoters, upon TNF-α stimulation. We have considered possible explanations for this discrepancy. In our experiments, we investigated PRMT1 association with the promoters after 30 minutes and 1 hour of stimulation with TNF-α, as the levels H4R3me2a were elevated at 1 hour of TNF-α stimulation. It is still possible that TNF-α increased PRMT1 association with the promoters at a shorter time course, and the binding was extremely transient. To definitively answer this question, it would be useful to repeat the experiments with shorter lengths of TNF-α stimulation, such as 5,10, 15, or 20 minutes. We did not do it during the course of this project due to time restrictions. An alternative possibility is that the induction of H4R3me2a was a result of an increased binding of another PRMT that has been shown to catalyse H4R3me2a. PRMT6 is the only other PRMT that has been shown to catalyse H4R3me2a but the PRMT6 inhibitor was without effect on cytokine/chemokine production in Chapter 5 [278]. This possibility is further explored in next chapter. Another mechanism by which TNF-α could elevate the levels of H4R3me2a at the promoters of inflammatory mediators, is by regulating the levels of PRMT1-modulating proteins, such as BTG Anti-Proliferation Factor 1 (BTG1) and TIS21/BTG2 [249], CCR4-associated factor 1 (hCAF1)/CNOT7 [250], ILF3 [251], and Yin Yang 1 (YY1) [252]. This is also investigated in the next chapter. Lastly, TNF-α stimulation induced a small, although non-significant increase in PRMT1 binding with the promoters, in ASM cells isolated from 3-4 subjects. It is possible that the induction would be significant, if the n values were increased.

The second aim of this chapter was to investigate the effect of the PRMT1 inhibitor TC-E 5003 on TNF-α-induced H4R3me2a at the cytokine/chemokine promoters. This is the continuation of studies in Chapter 5, which showed that inhibiting PRMT1 with TC-E 5003 reduced TNF-α-induced secretion of eotaxin, IL-6, IP-10, and CXCL8 in ASM cells. Our results showed that the addition of TC-E 5003 significantly inhibited TNF-α-induced H4R3me2a at CXCL8 promoter. However, to our surprise, there was no significant inhibition of TNF-α-induced H4R3me2a at eotaxin, IL-6, or IP-10 promoters. The lack of significant inhibition seemed to be a reflection of failed TNF-α- induction, rather than a lack of effect of TC-E 5003 at these promoters. One of the possible explanations is that DMSO, used as a solvent for TC-E 5003, interfered with TNF-α induction of the methylation mark. Our group has previously had technical issues when carrying out ChIP, with the addition of DMSO in the media. TNF- α is a potent inducer of CXCL8 in ASM cells [98, 99], however, the induction is not as strong with the other cytokines/chemokines. Therefore, a possible explanation is that DMSO has reduced the response to TNF- α, and while it was still high enough to produce significant results for CXCL8, H4R3me2a at other promoters was too low. It could be interesting to repeat this experiment with other PRMT1 inhibitors that are soluble in water, such as C21 [349], to see if this alters the results. Alternatively, we could repeat the experiment using TC-E 5003 dissolved in another organic solvent, such as methanol. Nonetheless, we still demonstrated that TC-E 5003, a pharmacological PRMT1 inhibitor, inhibited TNF-α-induced H4R3me2a at CXCL8 promoter.

PRMT1 can also methylate a number of non-histone proteins, such as NF-κB [350], ERα [351], recombinant heterogeneous nuclear RNP protein A1 (hnRNP A1) [352], intracytoplasmic domain of IFNα/β (interferon-α receptors (IFNAR1) [353], SRC associated in mitosis of 68 kDa (Sam68) [354], and many others [275]. Therefore, the effects of inhibiting PRMT1 may be a result of inhibiting its methyltransferase action towards histones, or alternatively, one or more of these non-histone proteins.

We used kits from two different manufacturers to perform ChIP experiments in this chapter. We first used the kit from Active Motif to carry out ChIP with H4R3me2a antibody. However, we experienced technical difficulties with this kit later, when performing experiments with the PRMT1 antibody. A possible explanation for this difference is that the recruitment of PRMT1 to the promoters is transient, which makes it more difficult to measure, unlike the H4R3me2a mark, which is more stable and therefore easier to capture. After optimising the experimental conditions and trying different kits, we were able to produce repeatable results

for PRMT1 association using a kit from Cell Signalling. A possible difference between the two kits are the buffers used to wash magnetic beads. If too harsh, they can negatively affect the results which require more sensitivity. However, it is unlikely that it affected our results for H4R3me2a mark, as the signal was strong enough to produce results with Active Motif kit.

In summary, in this chapter we demonstrated that TNF-α-induced H4R3me2a at the eotaxin, IL-6, IP-10 and CXCL8 promoters, and that pharmacological inhibition of PRMT1 with TC-E 5003 significantly inhibited TNF-α-induced H4R3me2a at the CXCL8 promoter. However, we could not convincingly demonstrate that the increase in H4R3me2a was due to increased PRMT1 association with the cytokine/chemokine promoters. In next chapter, we investigated the possible mechanisms by which TNF-α-induced H4R3me2a at the cytokine/chemokine promoters.

Chapter 7: CNOT7 and the modulation of TNF-α-induced PRMT1 activity at the cytokine/chemokine promoters

7 CNOT7 and the modulation of TNF-α-induced PRMT1 activity at the cytokine/ chemokine promoters

7.1 Introduction

In Chapter 6, we showed that TNF-α-induced H4R3me2a at the eotaxin, IL-6, IP-10 and CXCL8 promoters, but not by inducing PRMT1 association with these promoters. In this chapter, we investigated other possible mechanisms by which TNF-α could have induced H4R3me2a at the cytokine/chemokine promoters.

One of the possible mechanisms by which TNF-α could have increased H4R3me2a at the eotaxin, IL-6, IP-10 and CXCL8 promoters, is by increasing the binding of PRMT6, another PRMT that has been associated with this histone arginine methylation mark. In 2007, Hyllus *et al*. showed that PRMT6 catalyses H4R3me2a *in vitro* [278]. However, it has not been demonstrated that PRMT6 catalyses H4R3me2a *in vivo* [278]. Nonetheless, we wanted to see whether TNF-α affects the association of PRMT6 with eotaxin, IL-6, IP-10 and CXCL8 promoters and therefore we carried out ChIP experiments to investigate PRMT6 binding with the cytokine/chemokine promoters in ASM cells, under basal and TNF-α-induced conditions.

Another possible mechanism of TNF-α-induced increase in H4R3me2a at the promoters is via the regulation of proteins which modulate PRMT1 activity. BTG1, TIS21/BTG2 [249], hCAF1/CNOT7 [250], ILF3 [251], and YY1 [252], are some of the proteins which have been demonstrated to interact with PRMT1 and regulate its activity. Therefore we chose two of the PRMT1 modulating proteins – hCAF1/CNOT7 and ILF3, and carried out ChIP experiments to investigate their levels at eotaxin, IL-6, IP-10 and CXCL8 promoters.

Our plan was to see whether there was any significant change in their levels at the promoters following TNF-α stimulation.

7.2 Aims

The aims of this chapter were:

- To investigate how TNF-α stimulation affects PRMT6 association with eotaxin, IL-6, IP-10 and CXCL8 promoters, in human ASM cells
- To determine whether TNF-α stimulation affects the association ILF3 and CNOT7 with cytokine/chemokine promoters
- To investigate the mechanisms by which TNF-α induces H4R3me2a at the cytokine/chemokine promoters in human ASM cells

7.3 Methods

7.3.1 ChIP Assay for PRMT6, ILF3 and CNOT7

Human ASM cells from healthy volunteers (n=3-5) were cultured on sterile 500cm^2 plates. Upon reaching 90-100% confluency, they were growth arrested for 24 hours. Following removal of culture medium, the cells were incubated with TNF-α (1ng/ml) for 30minutes and 1 hour (based on our results from chapter 6, in which H4R3me2a increased after 1h of TNF-α stimulation). ChIP assay was then performed as described in section 3.10. The recovered DNA from ChIP was quantified by real-time PCR as described in section 3.10.1. The data were subjected to statistical analysis using GraphPad Prism version 6.00 or 7.00 for Windows. The ratio paired t-test was used to compare between basal and TNF-α stimulated cells. $P<0.05$ was accepted as statistically significant.

7.3.2 SiRNA- mediated knockdown of CNOT7

Human ASM cells from three healthy subjects were cultured overnight in 6-well plates to reach 40-50% confluency in a standard DMEM media. Transfection with CNOT7 and CNOT8 siRNA was carried out as described in section 3.15.1. Following that, the cells were incubated with TNF-α (1ng/ml) in serum and antibiotic free DMEM for 24 hours. Samples were collected, as described in section 3.15.2. RNA purification was performed, as described in section 3.15.3. Then reverse transcription was performed as described in section 3.15.4. Then qPCR was carried out with cDNA samples, as described in section 3.15.5. The ratio paired t-test was used to compare between control and either CNOT7 or CNOT7 + CNOT8 knockdown cells. $P<0.05$ was accepted as statistically significant.

7.4 Results

7.4.1 The effect of TNF-α on PRMT6 association with the cytokine/chemokine promoters

We carried out ChIP to investigate how TNF-α stimulation affected the association of PRMT6 with the cytokine/chemokine promoters in human ASM cells. Although PRMT6 associated with eotaxin, IL-6, IP-10 and CXCL8 promoters under basal conditions, stimulation with TNF-α had no significant effect on this association (Figure 7.1).

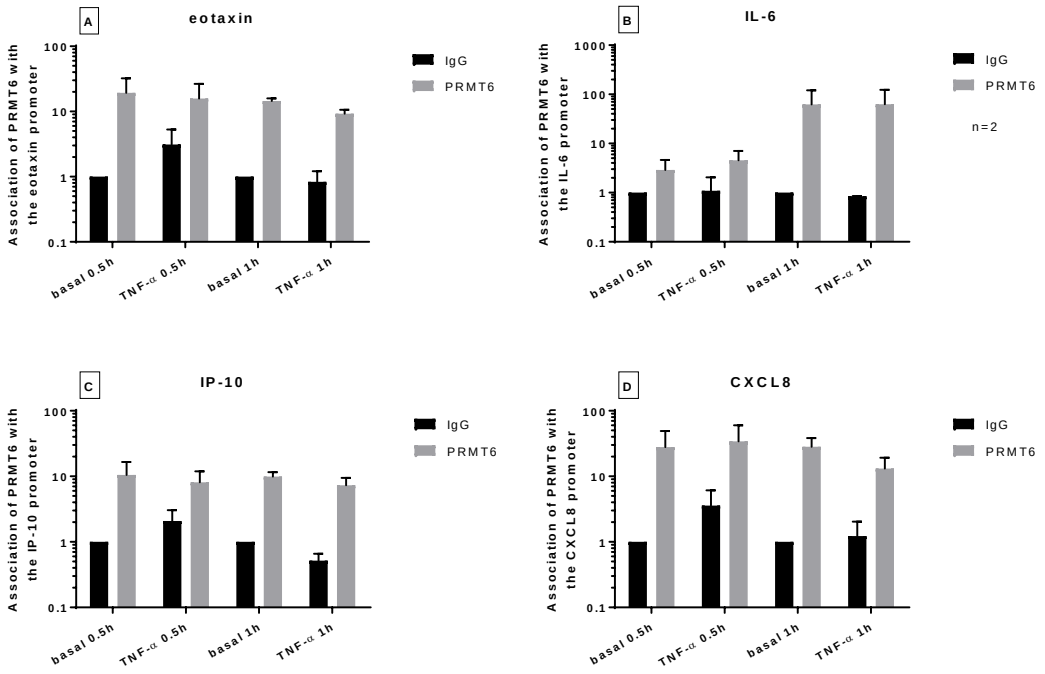

Figure 7.1: PRMT6 association with the eotaxin (A), IL-6 (B), IP-10 (C), and CXCL8 (D) promoters following stimulation with TNF-α for 30 minutes and 1 hour. The association of PRMT1 at the eotaxin, IL-6, IP-10 and CXCL8 promoters was assessed by ChIP under basal and TNF-α (1 ng/ml for 30 minutes and 1 hour) stimulated conditions. Data are expressed relative to the unstimulated IgG control and presented on a log scale. N=3 for eotaxin, IP-10 and CXCL8, N=2 for IL-6

7.4.2 The effect of TNF-α on the association of ILF3 with the cytokine/chemokine promoters

We carried out ChIP to see how TNF-α stimulation affected ILF3 association with the eotaxin, IL-6 and IP-10 promoters. 1 hour stimulation with TNF-α significantly increased ILF3 at IL-6 promoter (P=0.004), but had no significant effect on the association of ILF3 with eotaxin or IP-10 promoters (Figure 7.2).

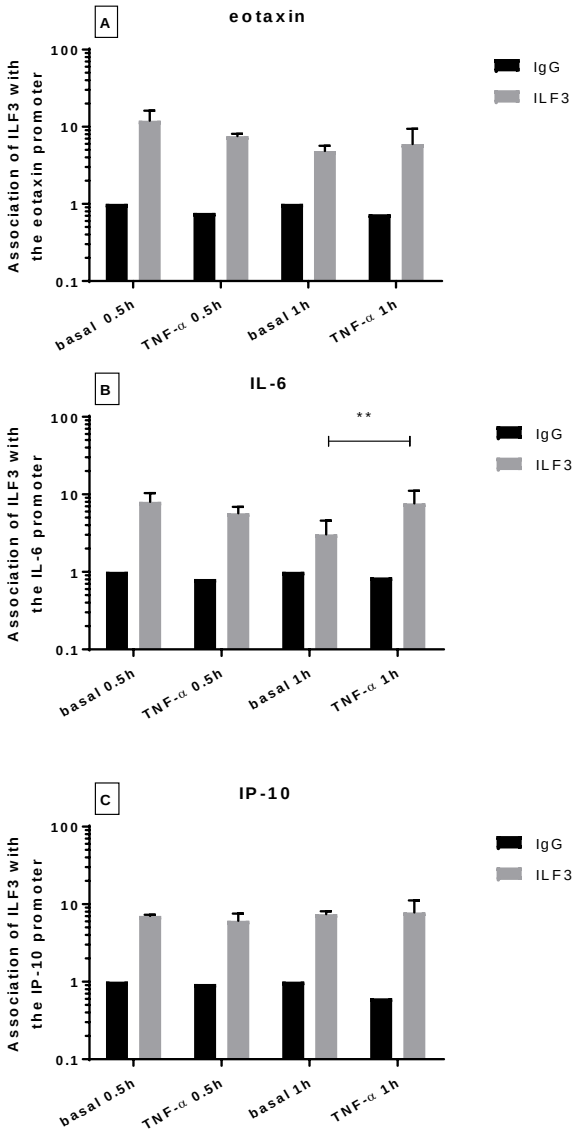

Figure 7.2: ILF3 association with the eotaxin (A), IL-6 (B), and IP-10 (C) promoters following stimulation with TNF-α for 30 minutes and 1 hour. The association of ILF3 at the eotaxin, IL-6, and IP-10 promoters was assessed by ChIP under basal and TNF-α (1 ng/ml for 30 minutes and 1 hour) stimulated conditions. Data are expressed relative to the unstimulated IgG control and presented on a log scale. N=3 (**p<0.01)

7.4.3 The effect of TNF-α on the association of CNOT7 with the cytokine/chemokine promoters

We carried out ChIP to see how TNF- α stimulation affected CNOT7 association with the eotaxin, IL-6, IP-10 and CXCL8 promoters. 30 minutes of TNF- α stimulation significantly decreased CNOT7 binding to the IL-6 (P= 0.04) and IP-10 (P=0.04) promoters (Figure 7.3). TNF- α also reduced CNOT7 association at the eotaxin and CXCL8 promoters, however, the decrease was not statistically significant (P=0.12 for both eotaxin and CXCL8).

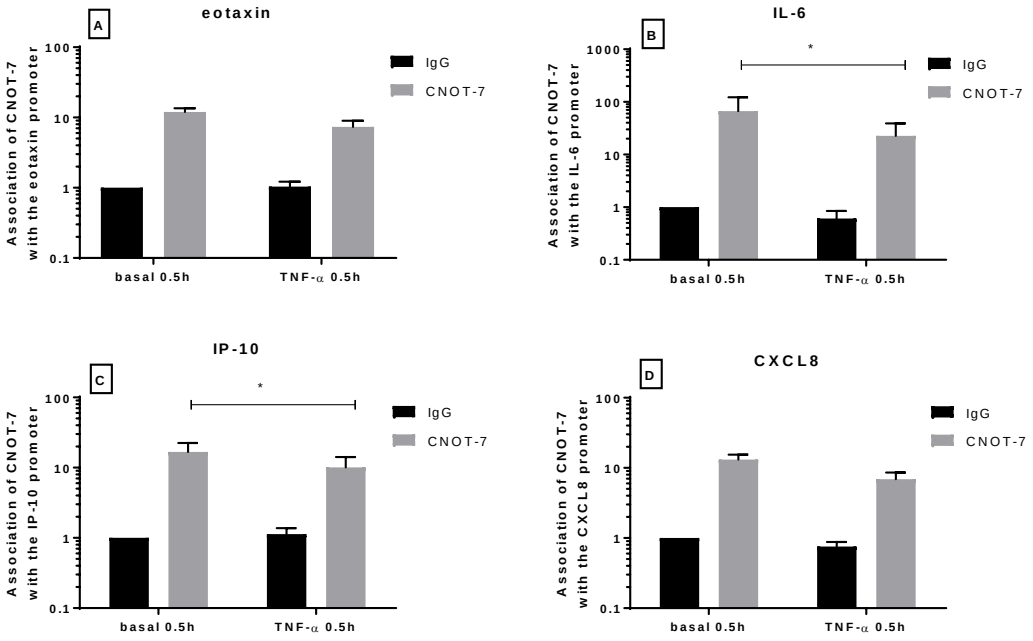

Figure 7.3: CNOT7 association with the eotaxin (A), IL-6 (B), IP-10 (C), and CXCL8 (D) promoters following stimulation with TNF-α for 30 minutes. The association of CNOT7 at the eotaxin, IL-6, IP-10 and CXCL8 promoters was assessed by ChIP under basal and TNF-α (1 ng/ml for 30 minutes) stimulated conditions. Data are expressed relative to the unstimulated IgG control and presented on a log scale. N=5 (* $p<0.05$)

7.4.4 SiRNA mediated CNOT7 knockdown and its effect on eotaxin, IL-6, IP-10 and CXCL8 mRNA expression in human ASM cells

We used siRNA to knockdown CNOT7 in human ASM cells obtained from three healthy subjects (Figure 7.4, P=0.01 for CNOT7 siRNA and P=0.004 for CNOT7 + CNOT8 siRNA). Following that, we carried out qPCRs to see whether CNOT7 knockdown changed mRNA expression of eotaxin, IL-6, IP-10 and CXCL8 in TNF-α- stimulated ASM cells. Our results were variable and there was no pattern of change in cytokine/chemokine mRNA expression following CNOT7 knockdown (Figure 7.5).

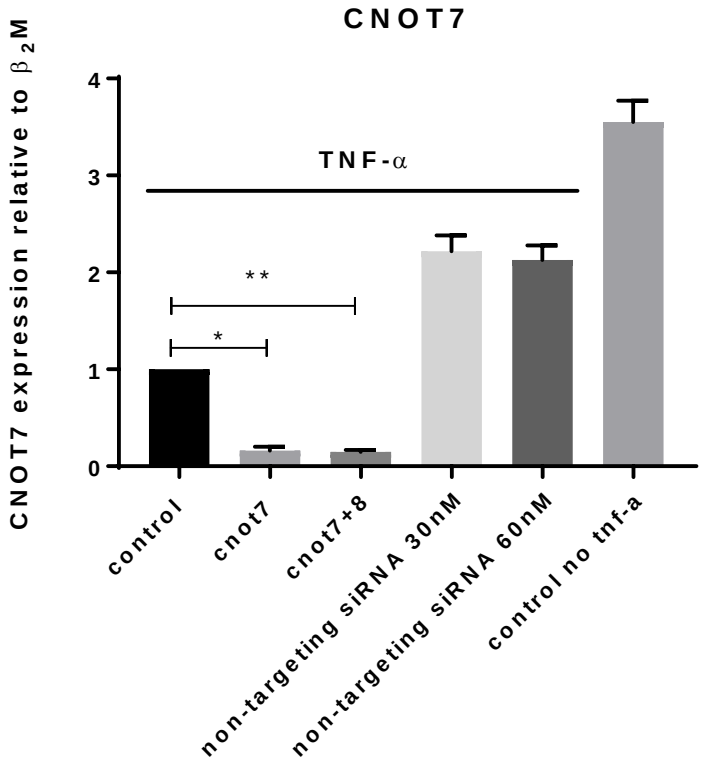

Figure 7.4: SiRNA mediated CNOT7 knockdown in human ASM cells. CNOT7 mRNA expression was measured in ASM cells from three healthy volunteers, following knockdown with CNOT7 and CNOT7+ CNOT8 siRNA at 30nM. Non-targeting siRNA at 30nM and 60nM was used a negative control. Cells were stimulated with TNF-α at 1ng/ml for 24 hours. Data are expressed relative to the TNF-α – stimulated negative control. N=3 (* $p<0.05$, ** $p<0.01$)

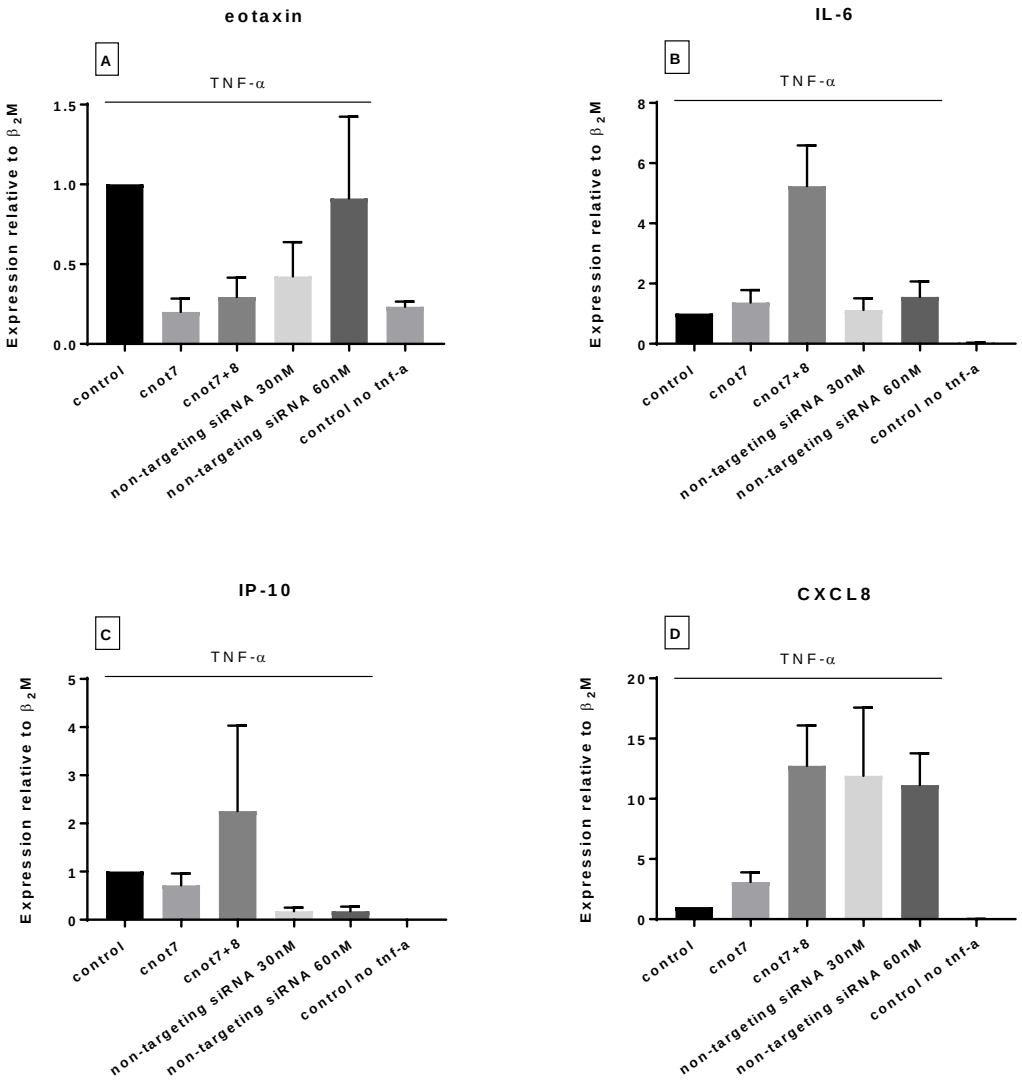

Figure 7.5: Eotaxin (A), IL-6 (B), IP-10 (C) and CXCL8 (D) mRNA expression, following siRNA mediated CNOT7 knockdown. Eotaxin, IL-6, IP-10 and CXCL8 mRNA expression was measured in ASM cells from three healthy volunteers, following knockdown with CNOT7 and CNOT7+ CNOT8 siRNA at 30nM. Non-targeting siRNA at 30nM and 60nM was used a negative control. Cells were stimulated with TNF-α at 1ng/ml for 24 hours. Data are expressed relative to the TNF-α – stimulated negative control. N=3

7.5 Discussion

The aim of this chapter was to investigate the possible mechanisms by which TNF-α-induced H4R3me2a at the eotaxin, IL-6, IP-10 and CXCL8 promoters. Our results in Chapter 6 suggested that association of PRMT1, the main PRMT associated with H4R3me2a, did not increase at the cytokine/chemokine promoters upon TNF-α stimulation. Another PRMT that has been shown to catalyse H4R3me2a is PRMT6, and we speculated that TNF-α may induce its association with the eotaxin, IL-6, IP-10 and CXCL8 promoters. However, the binding of PRMT6 with the promoters did not change following stimulation with TNF-α. We then investigated the hypothesis that TNF-α recruited additional co-activators to modulate PRMT1 activity. Therefore we carried out experiments to investigate how TNF-α affected the association of ILF3 and CNOT7 with the cytokine/chemokine promoters, as they have previously been shown to interact with PRMT1 and regulate its activity [250, 251]. The association of ILF3 was significantly increased at the IL-6 promoter upon 1 hour of stimulation with TNF-α. However, TNF-α did not affect ILF3 association at the eotaxin or IP-10 promoters, while technical difficulties prevented us from obtaining results at the CXCL8 promoter. We then turned our attention to CNOT7. CNOT7 can negatively modulate PRMT1-catalysed methylation at histone H4 [250], therefore we speculated that TNF-α would decrease CNOT7 binding with the cytokine/chemokine promoters. As expected, stimulation with TNF-α for 30 minutes resulted in significantly reduced association of CNOT7 with IL-6 and IP-10 promoters. TNF-α also reduced CNOT7 binding at the eotaxin and CXCL8 promoters, although it was not statistically significant. These results encouraged us to further investigate whether there was a link between CNOT7 and TNF-α-induced increase in H4R3me2a at the cytokine/chemokine promoters. Therefore we used siRNA-mediated gene knockdown to study how knocking down CNOT7 affected the TNF-α-induced expression of inflammatory genes in ASM cells. However, the study was inconclusive with regards to the impact of CNOT7 on the mRNA expression of inflammatory genes.

We decided to focus on CNOT7 and not ILF3, even though TNF-α significantly induced ILF3 association with the IL-6 promoter at 1 hour. This is because the CNOT7 results were more consistent, and TNF-α stimulation reduced CNOT7 association with all four promoters tested, although the decrease was not significant for eotaxin and CXCL8. The ILF3 results at eotaxin and IP-10 promoters varied, and we did not obtain a result at CXCL8 promoter due to technical difficulties, therefore we decided not to investigate ILF3 any further. Other co-activators of PRMT1 could be involved in the regulation of its activity at various promoters, however, we had to limit our research to ILF3 and CNOT7 due to time restrictions.

Although we showed a consistent reduction in the association of CNOT7 at eotaxin, IL-6, IP-10 and CXCL8 promoters, following 30 minutes of TNF-α stimulation, our knockdown study was inconclusive. We used both CNOT7 siRNA, and CNOT7 combined with CNOT8 siRNA, as the combination of CNOT7 and CNOT8 siRNA was shown to have synergistic effect in knocking down CNOT7 in MCF7 cells [355]. We also found this in ASM cells, although CNOT7 siRNA alone was enough to knockdown CNOT7. Due to time restrictions, we were only able to investigate the mRNA levels of CNOT7 in ASM cells following CNOT7 knockdown, and not protein levels. As CNOT7 is a negative regulator of PRMT1- catalysed histone H4 methylation, which is associated with gene transcription, we expected an increase in levels of mRNA cytokine/chemokine transcript, after CNOT7 knockdown. The combination of CNOT7 and CNOT8 siRNA increased the expression of IL-6 and CXCL8, but CNOT7 siRNA alone did not, even though using CNOT7 siRNA alone was enough to induce CNOT7 knockdown. Moreover, non-targeting siRNA, which was used as a negative control, also increased CXCL8 expression. Due to time restrictions, we only carried out qPCR to see how knocking down CNOT7 affects mRNA cytokine/chemokine expression in ASM cells. However, qPCR results can vary. It could be useful to carry out ELISA to see how CNOT7 knockdown affects protein secretion in ASM cells. Overall, our siRNA mediated CNOT7 knockdown studies were not conclusive enough to determine whether CNOT7 had a role in TNF-α-induced H4R3me2a at the eotaxin, IL-6, IP-10 and CXCL8 promoters in ASM cells.

Interestingly, our results showed that TNF-α stimulation for 24 hours decreased mRNA expression of CNOT7 in ASM cells (see Figure 7.4). As association of CNOT7 with the cytokine/chemokine decreased after 30 minutes of TNF-α stimulation, we hypothesised that if TNF-α stimulation for 30 minutes was enough to decrease CNOT7 expression, this could explain how TNF-α induced H4R3me2a at the cytokine/chemokine promoters. We therefore investigated the effect of TNF-α stimulation on CNOT7 expression in ASM cells at different time points (0.5, 8, and 24 hours). The decrease in mRNA expression of CNOT7 was first observed at 8 hour time point and was not seen after 30 minutes of TNF-α stimulation (see Appendix). Therefore we concluded that a decrease in CNOT7 expression was not the mechanism by which TNF-α induced H4R3me2a at the eotaxin, IL-6, IP-10 and CXCL8 promoters. We did not have sufficient time to further study the functional effects TNF-α-induced decrease in CNOT7 expression, but it may be interesting to investigate it in future.

CNOT7/hCAF1 is an evolutionarily conserved multi-subunit complex that regulates gene expression by acting on multiple cellular functions, such as initiation of transcription, controlling mRNA elongation, mRNA deadenylation and decay, and protein degradation

[356]. CNOT7 interacts with PRMT1 *in vivo* and colocalize in nuclear speckles, a sub-nuclear compartment enriched in small nuclear ribonucleoproteins and splicing factors [250]. In human cells, alternative splicing of the CNOT7 gene yields a second CNOT7 transcript leading to the formation of a shorter protein, CNOT7 variant 2 (CNOT7v2). A recent study suggested that CNOT7v2 is not involved in mRNA deadenylation and decay, but rather in the regulation of PRMT1-dependent arginine methylation [357]. Chapat *et al.* showed that PRMT1 displays a stronger affinity for CNOT7v2 than the CNOT7v1 isoform, and that the inhibition of PRMT1 activity by CNOT7v2 was stronger compared to CNOT7v1. [357]. Future studies could differentiate between these two CNOT7 isoforms and focus on CNOT7v2.

Other proteins have been shown to modulate PRMT1 activity and could have a role in TNF-α-induced increase in H4R3me2a at the cytokine/chemokine promoters. BTG1 and TIS21/BTG2 are very homologous proteins, which only differ by a small insertion in the C-terminal regions [358]. Both have been shown to associate with PRMT1 and positively regulate its activity [249]. Berthet *et al*. showed that the interaction of PRMT1 with BTG1 and BTG2 is dependent of boxC, a region of 10 amino acids found only in BTG1 and BTG2, and other members of the BTG/TOB family which do not contain boxC, do not associate with PRMT1 [359]. The interaction of PRMT1 with BTG2 has been demonstrated to have a role in retinoic acid receptor (RAR) α- mediated gene regulation [360]. YY1 (also known as δ, NF-E1, UCRBP, and CF1) is a 65 kDa member of the GLI-Krüppel family of zinc finger transcription factors, ubiquitously expressed and highly conserved among human, mouse and *Xenopus* [361]. YY1 can act both as an activator and as a repressor depending on its relative concentration, its binding partners, and on promoter context [252]. Besides PRMT1, YY1 has been shown to interact with many other histone-modifying complexes, including HATs (CBP and p300) and HDACs (HDAC1, 2 and 3) [362, 363]. Rezai-Zadeh *et al*. demonstrated that YY1 binds to PRMT1 and recruits it to YY1-activated promoters, most likely via a bridging protein DRBP76 [252]. ILF3 is a ~110kDa protein which belongs to a group of proteins that can bind to the ARRE-2 sequence, and is closely related in sequence to NF90 [251, 253]. ILF3 enhances the methylation activity of PRMT1 in *in vitro* methyltransferase assays [251]. Future studies could explore some of these interactions with PRMT1 at the eotaxin, IL-6, IP-10 and CXCL8 promoters in ASM cells.

In summary, we showed that PRMT6 was not involved in TNF-α-induced H4R3me2a at the promoters of inflammatory mediators in ASM cells. We showed that TNF-α stimulation for 1 hour induced the binding of ILF3 at the IL-6 promoter, while 30 minutes of TNF-α stimulation significantly decreased the association of CNOT7 at IL-6 and IP-10 promoters. However, our

results did not conclusively show a link between CNOT7 and TNF-α-induced increase in mRNA transcript of genes coding for cytokine/chemokine in ASM cells. In the next chapter, we investigated the effects of PRMT1 gene knockout on the cytokine/chemokine secretion from human ASM cells and mouse embryonic fibroblasts (MEFs).

Chapter 8: PRMT1 knockout and its effect on the cytokine/chemokine secretion from human ASM cells and mouse embryonic fibroblasts (MEFs)

8 PRMT1 knockout and its effect on the cytokine/chemokine secretion from human ASM cells and mouse embryonic fibroblasts (MEFs)

8.1 Introduction

In Chapter 5, we showed that the PRMT1 inhibitor TC-E 5003 reduced TNF-α-induced secretion of eotaxin, IL-6, IP-10 and CXCL8 from human ASM cells. Pharmacological inhibitors can have off-target effects, therefore we wanted to confirm these results by knocking down PRMT1 in human ASM cells using a molecular method, CRISPR, and then investigating its effect on TNF-α-induced cytokine/chemokine secretion and mRNA expression. To complement these studies, we also performed studies using PRMT1- deficient MEFs. MEF culture is a frequently used system in gene function studies, due to their easy accessibility, rapid growth rates, and the possibility of performing a large number of experiments [364]. The loss of PRMT1 is not compatible with cell viability in MEFs, so we obtained a floxed MEF cell line that can be treated with OHT to induce PRMT1 loss ($PRMT1^{FL/-}$ ER-Cre MEFs) [365]. We used these $PRMT1^{FL/-}$ ER-Cre MEFs to study how PRMT1 loss affected the TNF-α-induced secretion of a mouse chemokine KC.

Although not a true homologue, the murine keratinocyte-derived protein chemokine CXCL1/KC is the functional homologue of human CXCL8. KC belongs to the CXC mouse chemokine family and is the 8 kDa murine homologue of a human oncogene termed growth-related oncogene (GRO) [366-368]. KC binds to a unique mouse receptor homologous to human CXCR2, which mediates neutrophil chemotaxis in response to both human CXCL8 and mouse KC [107]. Therefore, just like human CXCL8, mouse KC is associated with neutrophil recruitment and inflammation. KC, along with macrophage inflammatory protein-2 (MIP-2), has been shown to orchestrate diesel exhaust particulate (DEP)-induced exacerbation of airway inflammation and AHR *in vivo* [369]. KC BALF levels are increased in the mouse model of viral asthma exacerbation [370].

8.2 Aims

The aims of this chapter were:

- To investigate how CRISPR- mediated PRMT1 knockout affects TNF-α-induced secretion and mRNA expression of eotaxin, IL-6, IP-10 and CXCL8 in human ASM cells
- To study the effects of PRMT1 loss in MEFs on TNF-α-induced secretion of a mouse chemokine KC

8.3 Methods

8.3.1 CRISPR to induce PRMT1 knockout in ASM cells

PRMT1 primers were designed using the GeneArt™ CRISPR Search and Design tool (thermofisher.com/crisprdesign). The gRNA synthesis was performed as described in section 3.13.1. Human ASM cells from four non-asthmatic subjects were cultured overnight in 24-well plates to reach 30-70% confluency in a standard DMEM media, as described in section 3.7.1. The transfection was performed as described in section 3.13.2. The cells were incubated with TNF-α (1ng/ml) in 1% FCS DMEM for 24 hours. Cell supernatants were collected and stored at -20°C. Cell viability was assessed by MTT assay, as described in section 3.12.3. RLT buffer from RNeasy® Micro Kit (Qiagen) with β-mercaptoethanol was added into wells for RNA extraction. RDL buffer with PIC was added into other wells for protein lysis. These samples were then scraped and frozen at -80°C. RNA purification was performed as described in 3.7.4. Reverse transcription was then carried out, as described in 3.7.5. To assess PRMT1 knockout, PCR was carried out, as described in section 3.13.6. In order to assess PRMT1 protein knockout, western blotting was carried out, as described in section 3.13.7. To assess cytokine/chemokine mRNA expression in ASM cells, PCR was carried out with eotaxin, IL-6, IP-10 and CXCL8 primers, as described in 3.7.6. The ratio paired t-test was used to compare cytokine/chemokine mRNA expression between TNF- α- stimulated control and PRMT1 knockout cells. $P<0.05$ was accepted as statistically significant. ELISA assays were performed as described in section 3.13.8, to compare cytokine/chemokine secretion from control and PRMT1 knockout ASM cells, under basal and TNF- α stimulated conditions. The results were then normalised to MTT assay. The data were subjected to statistical analysis using GraphPad Prism version 7.00 for Windows. Paired t-test was used to compare between control and PRMT1 knockout cells, upon TNF-α stimulation. $P<0.05$ was accepted as statistically significant.

8.3.2 PRMT1 knockdown in MEFs

$PRMT1^{FL/-}$ ER-Cre MEFs were donated from Dr Stéphane Richard (McGill University, Montreal, Canada). The generation of the PRMT1 conditional allele in mice has been described by Yu *et al.* [365]. The cells were cultured in 24 well plates, with Blasticidin added to cell culture medium to maintain the ER-Cre. OHT at 2μM was added for 24 hours to induce PRMT1 loss. MEFs without OHT treatment were used as a control. Once confluent, the MEFs were growth arrested for 24 hours. Following that, the MEFs were stimulated with TNF-α at 1ng/ml and 10 ng/ml. After 24 hours, the supernatants were collected and stored at -20°C.

Cell viability was assessed by MTT assay, as described in section 3.12.3. RLT buffer from RNeasy® Micro Kit (Qiagen) with β-mercaptoethanol was added into three wells from each plate for RNA extraction. RDL buffer with PIC was added into another four wells from each plate for protein lysis. These samples were then scraped and frozen at -80°C. RNA purification was performed as described in section 3.14.2. Reverse transcription was performed as described in section 3.14.3. To assess PRMT1 mRNA knockdown, polymerase chain reaction was carried out, as described in section 3.14.4. One way ANOVA with Tukey's multiple comparisons test was used to analyse PRMT1 mRNA knockdown, $P<0.05$ was accepted as statistically significant. Western blotting was carried out to assess PRMT1 protein knockdown, as described in section 3.14.5. The ELISA assay for mouse KC was carried out with supernatants collected from samples, as described in section 3.14.6. The results were normalised to MTT assay and expressed as % of control cells stimulated with TNF-α 10ng/ml. The means obtained from cells from three different experiments were combined. Kruskal-Wallis test with Uncorrected Dunn's test was used to compare between control cells and these dosed with OHT.

8.4 Results

8.4.1 The effect of CRISPR- mediated PRMT1 knockout in human ASM cells on TNF-α-induced secretion and mRNA expression of eotaxin, IL-6, IP-10 and CXCL8

8.4.1.1 PRMT1 is knocked out by using CRISPR

CRISPR decreased PRMT1 protein expression (Figure 8.1 (A)). PRMT1 mRNA expression was significantly decreased in CRISPR treated cells, both basally and after TNF-α- stimulation (Figure 8.1 (B), P<0.0001 for both). However, CRISPR treatment affected cell viability (Figure 8.2).

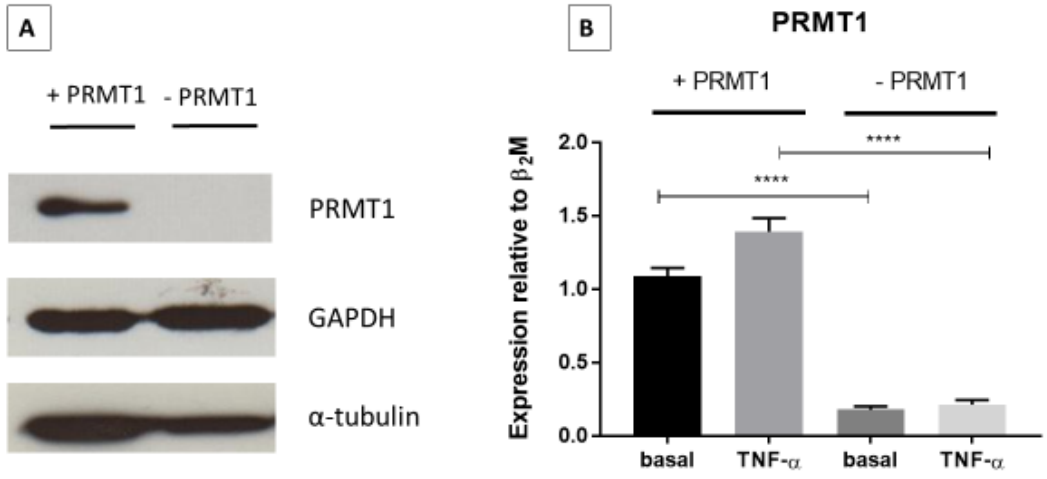

Figure 8.1: CRISPR- mediated PRMT1 knockout in ASM cells. (A) PRMT1 protein knockout in ASM cells was assessed by western blotting (representative blot of 5 experiments). (B) PRMT1 mRNA knockout in ASM cells was assessed by qPCR, under basal and TNF-α (1 ng/ml) stimulated conditions. Data are expressed relative to the basal +PRMT1 control, n=4. (**** p<0.0001)

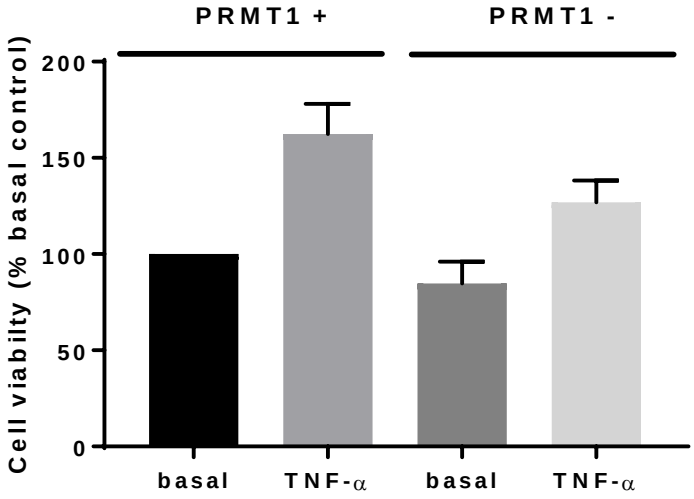

Figure 8.2: The viability of human ASM cells, following PRMT1 knockout using CRISPR. Cell viability following PRMT1 knockout, under basal and TNF-α (1 ng/ml) stimulated conditions, was assessed by MTT assay. The results are expressed as % of control cell viability under basal conditions.

8.4.1.2 Cytokine/chemokine secretion from ASM cells following PRMT1 knockout using CRISPR

Although PRMT1 knockout decreased TNF-α-induced secretion of eotaxin, IL-6 and CXCL8 from ASM cells, only the IL-6 reduction was statistically significant (P= 0.04, P=0.32, P=0.22 for IL-6, eotaxin and CXCL8, respectively). IP-10 did not follow this trend and its secretion was increased in PRMT1 knockout cells, both basally and under TNF-α- stimulated conditions, although the increase was not statistically significant (Figure 8.3).

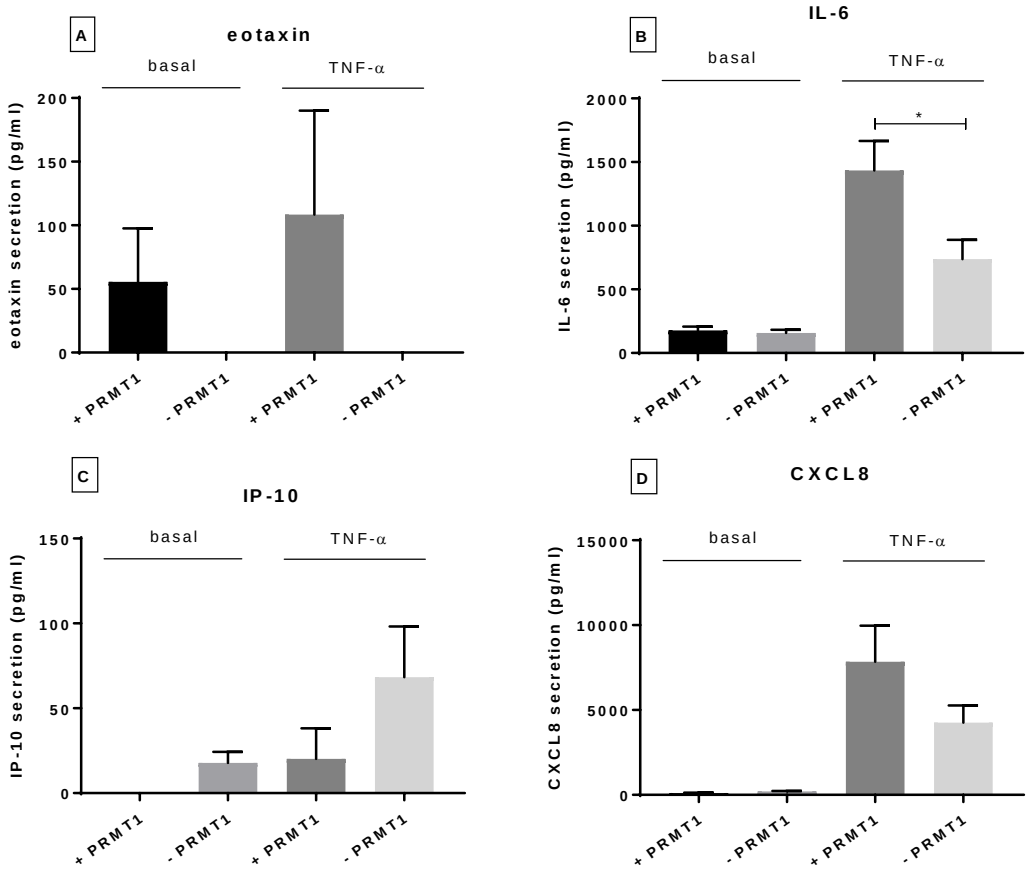

Figure 8.3: The effect of CRISPR- mediated PRMT1 knockout on the secretion of eotaxin (A), IL-6 (B), IP-10 (C), and CXCL8 (D) from ASM cells. The levels of eotaxin, IL-6, IP-10 and CXCL8 secreted by ASM cells following PRMT1 knockout under basal and TNF-α (1 ng/ml) stimulated conditions, were assessed by ELISA. The results are normalised to MTT assay. N=3-4 (* $p<0.05$)

8.4.1.3 Cytokine/chemokine mRNA expression in ASM cells following PRMT1 knockout

The changes in TNF-α-induced cytokine/chemokine mRNA expression followed the same trend as changes in their protein secretion but were all statistically significant. TNF-α-induced mRNA expression of eotaxin, IL-6 and CXCL8, was significantly decreased in PRMT1 knockout cells (Figure 8.4, P= 0.006, 0.024, and 0.0029, respectively). TNF-α-induced IP-10 mRNA expression was significantly increased in PRMT1 knockout cells (P= 0.006).

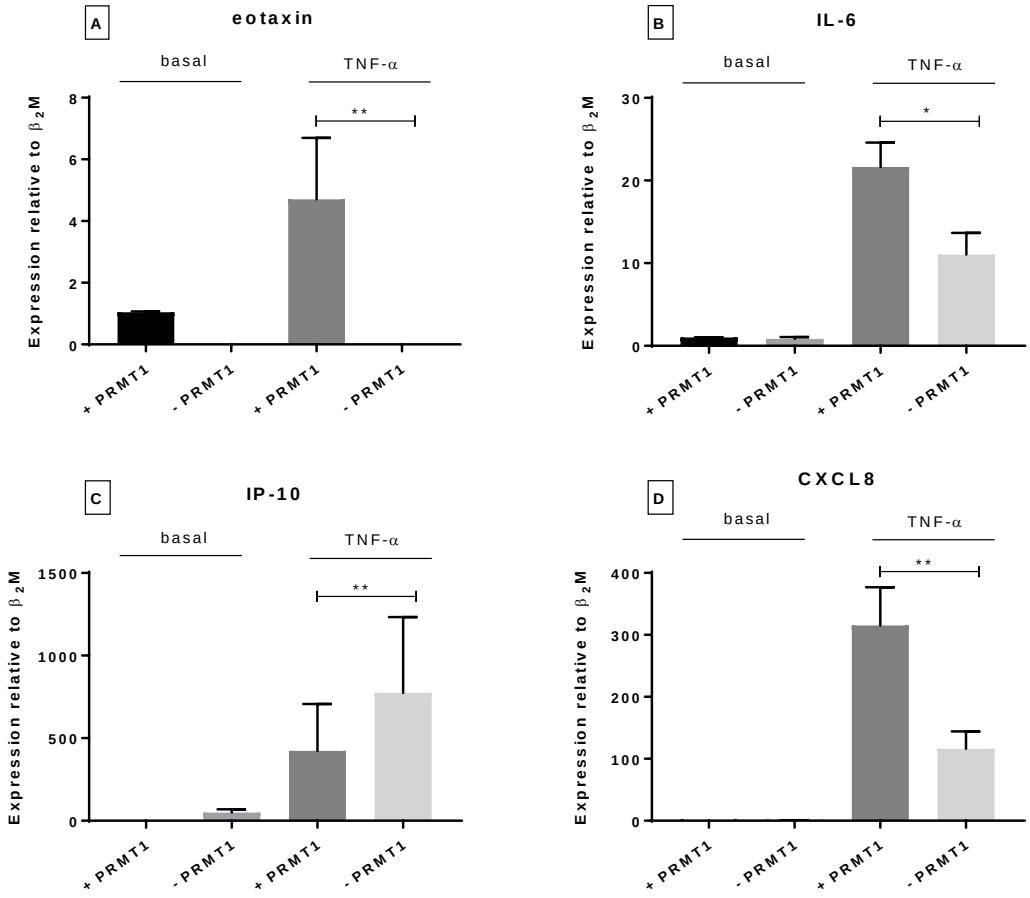

Figure 8.4: The effect of CRISPR- mediated PRMT1 knockout on the mRNA expression of eotaxin (A), IL-6 (B), IP-10 (C), and CXCL8 (D) in ASM cells. The mRNA levels of eotaxin, IL-6, IP-10 and CXCL8 expressed in ASM cells following PRMT1 knockout under basal and TNF-α (1 ng/ml) stimulated conditions, were assessed by qPCR. Data are expressed relative to the basal +PRMT1 control. N=3-4 (* p<0.05, **p<0.01)

8.4.2 The effect of PRMT1 knockdown in MEFs on TNF-α-induced secretion of KC

OHT treatment successfully decreased PRMT1 protein expression (Figure 8.5). We were not able to determine the magnitude of PRMT1 mRNA knockdown, due to the formation of a primer dimer. However, the analysis of dissociation curve and Ct values showed that PRMT1 mRNA levels were also reduced in OHT-treated cells (see Appendix).

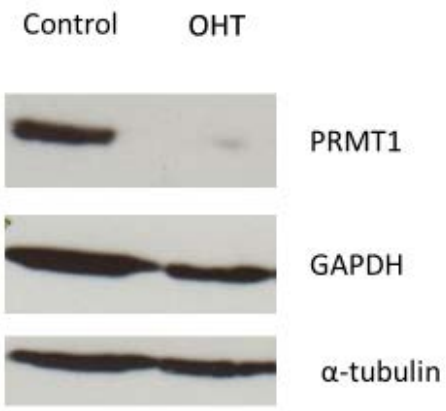

Figure 8.5: PRMT1 knockdown in MEFs. PRMT1 protein knockdown in MEFs was assessed by western blotting. Figure shows results representative of three separate experiments.

PRMT1 knockdown led to a decrease in TNF-α-induced KC secretion from MEFs (Figure 8.6 (A)), both at 1ng/ml and 10ng/ml, although the results were not statistically significant. OHT treatment had no impact on the viability of MEFs (Figure 8.6 (B)).

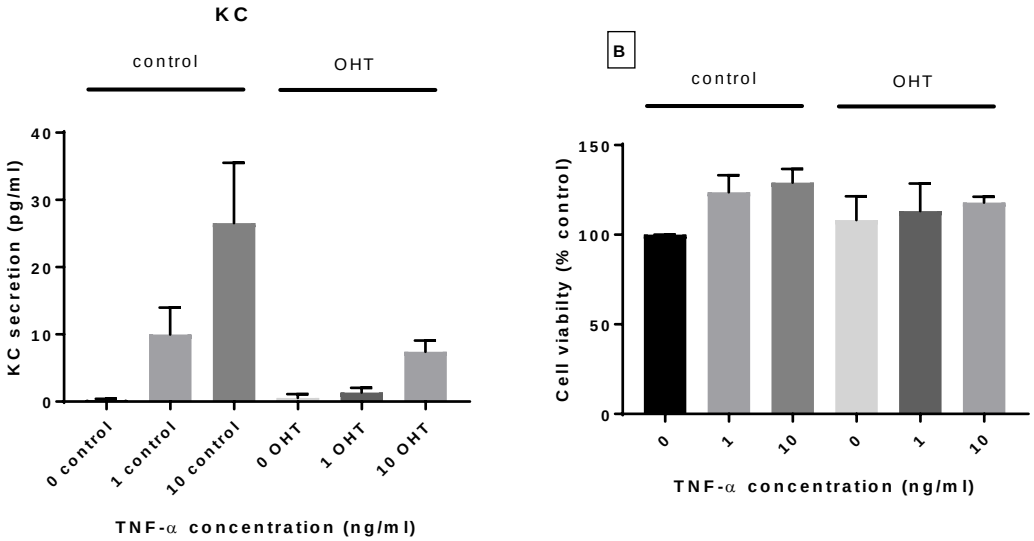

Figure 8.6: The effect of PRMT1 knockdown on KC secretion from MEFs (A), MEFs cell viability (B). (A) The levels of KC secreted by ASM cells following PRMT1 knockdown under basal and TNF-α (1 ng/ml and 10 ng/ml) stimulated conditions, were assessed by ELISA. The results are normalised to MTT assay. N=3 (B) Cell viability following PRMT knockdown, under basal and TNF-α (1 ng/ml and 10 ng/ml) stimulated conditions, was assessed by MTT assay. The results are expressed as % of control cell viability under basal conditions. N=3

8.5 Discussion

The main findings of this study were that CRISPR - mediated gene knockout of PRMT1 in human ASM cells reduced TNF-α-induced eotaxin, IL-6, and CXCL8 mRNA but had a more variable effect on protein secretion. Using OHT-inducible PRMT1 knockdown MEFs we found that downregulating PRMT1 reduced TNF-α-induced secretion of KC, a functional murine homologue of human CXCL8.

CRISPR is a relatively new genome editing technology, developed around 2014, which utilizes natural defence mechanisms of bacteria and archaea against infection. This project was the first time anyone in our group has successfully used CRISPR to knockout the expression of any gene in human ASM cells. We found that PRMT1 knockout reduced TNF-α-induced secretion of eotaxin, IL-6 and CXCL8 protein from ASM cells. However, only the inhibition of IL-6 secretion was statistically significant. PRMT1 knockout reduced cell proliferation and viability, leading to a difference in cell numbers between our samples and controls. We normalised our ELISA data to MTT assay results, to account for that difference. However, it is possible that a difference in cell numbers introduced an error to our results and the lack of statistical significance is a reflection of that. It could also reflect the fact that primary cells have variable secretion of different cytokines/chemokines and that larger numbers of donor cell lines would be required to see significant results. Another factor could be variable efficiency of the CRISPR technique between experiments.

We then investigated how loss of PRMT1 affected the mRNA expression of eotaxin, IL-6, IP-10 and CXCL8 in human ASM. PRMT1 knockout significantly reduced TNF-α-induced mRNA expression of eotaxin, IL-6, and CXCL8. This is consistent with our hypothesis that PRMT1 has a role in transcription of genes coding for proinflammatory cytokines/chemokines in ASM cells. It also adds weight to the fact that the failure to see significant effects on protein in our experiments may just reflect experimental technique or small n numbers. In contrast we found that TNF-α-induced IP-10 mRNA was significantly increased. This effect was in a similar direction to the IP-10 protein experiments and suggests a fundamental difference in the regulation of IP-10 compared to the other cytokines/chemokines. We have considered possible explanations for this difference. PRMT1 supresses TNF-α-induced activation of NF-κB, and negatively regulates transcription of NF-κB-dependent genes [350]. Moreover, PRMT5, which often antagonizes PRMT1 by carrying out symmetrical demethylation at the same sites, has been shown to enhance IP-10 induction in response to TNF-α, by methylating

NF-κB p65 [371]. It is possible that while PRMT1 positively regulated TNF-α-induced transcription of eotaxin, IL-6 and CXCL8, via its activity on histones, it suppressed TNF-α-induced transcription of IP-10, by methylating NF-κB. However, eotaxin, IL-6 and CXCL8 are also regulated by NF-κB, therefore it is unlikely that these results are NF-κB - related. Further research is needed to investigate why loss of PRMT1 increased secretion and mRNA expression of IP-10 in ASM cells. The increase in IP-10 mRNA expression and protein secretion upon PRMT1 knockout also differs from our results from Chapter 5, where pharmacological inhibition of PRMT1 decreased TNF-α-induced IP-10 secretion in ASM cells. It is possible that the results from Chapter 5 were due to off-target effects of PRMT inhibitor TC-E 5003. Another possibility is that PRMT1 could have some effect, which is not linked to its enzymatic activity. Therefore the knockout of PRMT1 could have produced different result to inhibition with TC-E 5003, which acts as a ligand by docking at the PRMT1 substrate-binding pocket [340]. Lastly, it is known now that CRISPR -mediated genome editing may also produce off targets effects [372]. Therefore further research is needed to determine which results reflect the real effect of PRMT1 inhibition on IP-10 production from ASM cells.

Another aim of this chapter was to investigate how PRMT1 loss affected the TNF-α-induced secretion of a mouse chemokine KC in MEFs. We used a floxed MEF cell line that could be treated with OHT to induce PRMT1 loss. OHT treatment for 24 hours reduced PRMT1 protein and mRNA expression, without affecting cell viability. PRMT1 loss in MEFs resulted in reduced KC secretion upon TNF-α stimulation, although the results were not statistically significant. We chose to study KC secretion, as it is thought to be the functional homologue of human CXCL8. Time restrictions prevented us from investigating how PRMT1 loss affects the secretion of murine eotaxin, IL-6, and IP-10, however, it would be interesting to investigate this in future. These results are consistent with our experiments with human ASM cells, suggesting that PRMT1 is an important regulator of cytokine/chemokine production in ASM.

To conclude, in this final chapter, we used molecular methods to show that PRMT1 knockout reduced TNF-α-induced cytokine/chemokine mRNA expression in ASM but had a more variable effect on cytokine/chemokine secretion, probably reflecting experimental variability, CRISPR efficiency and small n numbers. Furthermore, we found that downregulating PRMT1 reduced TNF-α-induced secretion of KC, a functional murine homologue of human CXCL8. Collectively these studies support our hypothesis that PRMT1 has an important role in regulating the production of inflammatory cytokines/chemokines in ASM and is a target for the development of novel asthma treatments.

Chapter 9: General discussion, conclusions and suggestions for future studies

9 General discussion, conclusions and suggestions for future studies

The main findings of our study were that human ASM cells express a number of PRMTs, and that PRMT1 likely has a role in regulating the production of cytokines/chemokines by ASM cells, in response to TNF-α. Evidence supporting this role for PRMT1 came from studies showing that TNF-α induced the PRMT1-catalysed histone arginine methylation mark H4R3me2a, that a pharmacological inhibitor of PRMT1 inhibited cytokine/chemokine production by ASM, and that the molecular knockout by CRISPR showed comparable results, at least for IL-6, eotaxin and CXCL-8, but not for IP-10. Further evidence for a role of PRMT1 was provided by our studies in $PRMT1^{FL/-}$ MEFs, as the loss of PRMT1 led to a reduction in TNF-α-induced secretion of a mouse chemokine KC.

There were however inconsistencies in some of our experiments. The expression of PRMT1 (or any other PRMT) was not upregulated in ASM cells isolated from asthma patients, unlike in study by Sun *et al.* [303]. Pharmacological inhibition of PRMT1 with TC-E 5003 had a different effect on IP-10 secretion than when PRMT1 was knocked down via CRISPR. TC-E 5003 significantly inhibited TNF-α-induced H4R3me2a at CXCL8 promoter, but not at eotaxin, IL-6 or IP-10 promoters. TNF-α significantly induced H4R3me2a at cytokine/chemokine promoters, but not the association of PRMT1 with these promoters, as expected. Some of these may have been a result of low n numbers, variable efficiency of experimental techniques, variability between primary ASM cells isolated from different subjects, cell toxicity, off target effects, or failed TNF-α induction in the presence of different drug vehicles.

Other limitations of our study include the variability between ASM cells samples isolated from asthma patients, which results from different levels of disease severity and varying anti-asthma treatments. Different medications could affect the results in samples obtained from these patients. Our results from chapter 5 should also be interpreted with caution, as the available PRMT inhibitors varied in their specificity towards selected PRMTs. We were not able to determine how these agents affected the methyltransferase activity of each of the PRMTs, as the specific assays are currently not available. Moreover, these agents inhibit the activity of PRMTs towards both histone and non-histone proteins, and we were not able to determine whether our results show strictly the effect of inhibiting histone methylation. We also did not manage to determine the pathway by which TNF-α induces H4R3me2a at cytokine/chemokine promoters, due to time restrictions.

On balance, our results provide evidence that PRMT1 is a potential target for development of novel asthma treatments and is worthy of further study. We also performed experiments

studying an intermediate protein CNOT7, but overall the results regarding its involvement in TNF-α-induced, PRMT1-mediated cytokine/chemokine production were inconclusive.

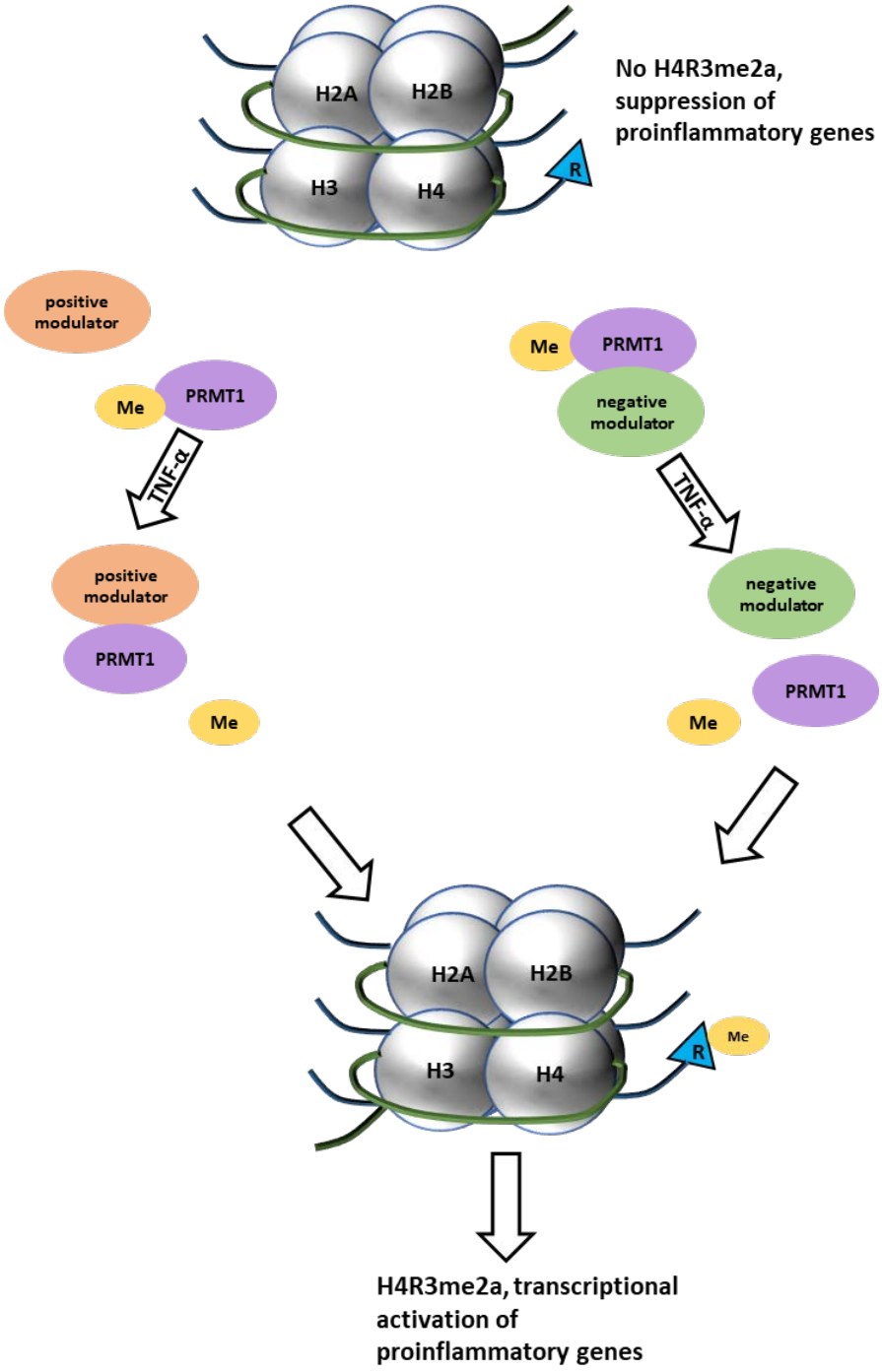

Figure 9.1: Putative mechanisms of regulation of PRMT1 activity at the cytokine/chemokine promoters in ASM cells. In non-asthmatic ASM, negative regulators of PRMT1 activity (e.g. CNOT7) inhibit PRMT1-catalysed histone arginine methylation at the cytokine/chemokine promoters, while positive regulators (e.g. ILF3, BTG1, BTG2) do not interact with PRMT1, leading to suppression of inflammatory gene transcription. Asthmatic ASM secrete TNF-α, which either induces the interaction of PRMT1 with positive modulators of its activity, or reduces the association with negative regulators. The end result is the increase in PRMT1-catalysed H4R3me2a at the cytokine/chemokine promoters, and transcriptional activation of proinflammatory genes.

Loss of PRMT1 may lead to substrate scavenging by other PRMTs and global increase in MMA and SDMA levels, while reducing ADMA levels [242]. The reduced levels of cytokines/chemokines secreted from ASM cells could therefore be a result of inhibiting PRMT1 and PRMT1-catalysed ADMA, or increased MMA and SDMA levels, catalysed by other PRMTs (or a combination of both). In future, it could be interesting to investigate how inhibiting PRMT1 with TC-E 5003 affects levels of ADMA, MMA and SDMA in ASM, and how they correlate with the levels of cytokines/chemokines secreted by ASM cells.

There were differences in the effect of PRMT1 knockout by CRISPR on TNF-α-induced IP-10, as opposed to eotaxin, IL-6 and CXCL8. The secretion and mRNA expression of IP-10 increased following PRMT1 knockout, suggesting differences in the regulation of IP-10 versus the other cytokines/chemokines. It would be interesting to investigate this further in future. The effect of CRISPR-mediated PRMT1 knockout on IP-10 differed from our results from Chapter 5, in which pharmacological inhibition of PRMT1 reduced TNF-α-induced IP-10 secretion from ASM cells. In order to establish which result was a true effect of PRMT1 inhibition, and which was an experimental artefact, we could repeat our experiments using a different PRMT1 inhibitor, such as C21, and a different method of molecular PRMT1 knockdown, such as siRNA-mediated knockdown. If they showed that TNF-α-induced IP-10 secretion and mRNA expression increased in PRMT1 knockdown cells, we could then further investigate the pathways of IP-10 regulation.

Our results showed that PRMT1 knockdown in MEFs reduced TNF-α-induced secretion of KC, a mouse chemokine, which is believed to be a functional homologue of human CXCL8. Time restrictions prevented us from investigating how PRMT1 loss affects the secretion of murine eotaxin, IL-6, and IP-10, however, it would be interesting to investigate this in future.

It would be interesting to use cells isolated from donors with asthma, alongside TNF-α-stimulated ASM cells from healthy subjects. Although there were no differences in PRMT expression between ASM from normal and asthmatic subjects, the effect of PRMT modulation might be qualitatively or quantitatively different in asthmatic cells after cytokine stimulation. Time restrictions prevented us from doing that in this project.

It would also be interesting to carry out experiments with some other proinflammatory cytokines associated with asthma, such as IL-1β, IFN-γ, bradykinin, or a mix of TNF-α, IL-1β, and IFN-γ (cytomix), to look at Th2 cytokines, such as IL-13 or IL-4, and to look at remodelling cytokines, such as TGF-β.

In our experiments, we focused on four cytokines/chemokines secreted from ASM cells-eotaxin, IL-6, IP-10 and CXCL8, based on their roles in inflammation in asthma. However, this does not mean that the regulation of inflammatory functions in ASM by PRMT1 is limited to these four cytokines/chemokines. In future, it would be interesting to broaden our research to include some other cytokines/chemokines secreted by ASM and associated with asthma, such as CCL5/RANTES, CCL2/MCP-1, IL-5, and many others (see section 1.6).

Future studies would be enhanced by the addition of animal experiments. PRMT1$^{-/-}$ mouse embryos fail to develop beyond E6.5 and die *in utero* [243], therefore mice inducible for cell type specific PRMT1 knockdown would be necessary. It would be useful to compare the expression of cytokines/chemokines in PRMT1 deficient mouse ASM cells and with the expression in cells from control mice. However, the translational value of animal studies in asthma research is limited, as no laboratory animal commonly used to study this condition, exhibits symptoms similar to humans [373].

Our studies also showed that CARM1 was expressed at high levels in ASM cells and that the CARM1 inhibitor 217531 inhibited TNF-α-induced secretion of eotaxin, IL-6, IP-10 and CXCL8, but did not study this further. In future, it would be interesting to use several pharmacological inhibitors, and molecular techniques such as CRISPR, to further evaluate the role of CARM1 in cytokine/chemokine production in ASM. Another approach is to study H3R17me2a, an important methylation mark catalysed by CARM1 and associated with transcriptional activation.

We investigated the potential role of CNOT7 as an intermediate protein in regulating histone arginine methylation and our results were inconclusive. Future studies to more definitively answer the role of CNOT7 would include ELISA, to investigate how TNF-α induced cytokine/chemokine secretion in ASM cells changes following CNOT7 knockdown, as these tend to be more reliable than mRNA studies. Another approach would be to use ChIP to study how PRMT1 association with the cytokine/chemokine promoters, and H4R3me2a at these promoters, changes in response to CNOT7 knockdown. However, this would be technically difficult, as ChIP requires a lot of cells, therefore using pharmacological inhibitors of CNOT7 could be more suitable, when they become commercially available.

It would be also interesting to investigate the regulation of H4R3me2a at the cytokine/chemokine promoters by other known PRMT1 modulators, such as BTG1, BTG2, and YY1, and whether TNF-α alters this. As the association of ILF3 with IL-6 promoter increased after TNF-α stimulation, it could be interesting to see whether it also increases at

promoters of other chemokines/cytokines secreted by ASM cells which were not included in our study (see section 1.6).

PRMT1 is known to methylate many non-histone proteins involved in transcriptional regulation, including NF-κB [350], ERα [351], IFNAR1 [353], RIP140 [374], and PGC-1α [375]. We cannot rule out the possibility that our results were affected by the activity of PRMT1 towards any of these proteins. In future, it would be necessary to investigate the effect of PRMT1 inhibition on the methylation of non-histone proteins, as opposed to the inhibition of PRMT1-catalysed histone arginine methylation, and how it affects cytokine/chemokine secretion from ASM cells.

In conclusion, our study has showed that PRMT1 may be implicated in inflammation in asthmatic airways, by catalysing histone arginine methylation mark responsible for increased production of cytokines/chemokines from ASM cells, after TNF-α stimulation. Therefore PRMT1 is a good potential target for development of novel asthma treatments. However, just like any other study, our project had its limitations, due to time restrictions, availability of experimental techniques, variability between cells and the efficiency of techniques. Future studies should concentrate on further exploring the mechanisms by which PRMT1 regulates the cytokine/chemokine secretion from ASM and investigating the effect of PRMT1 on different cytokines/chemokines. Our results suggest there is a need for development of new PRMT1 inhibitors, and investigating their efficacy and safety *in vivo*, in order to establish their potential in asthma treatment in the clinic.

Chapter 10: Appendix

10 Appendix

10.1 Additional figures

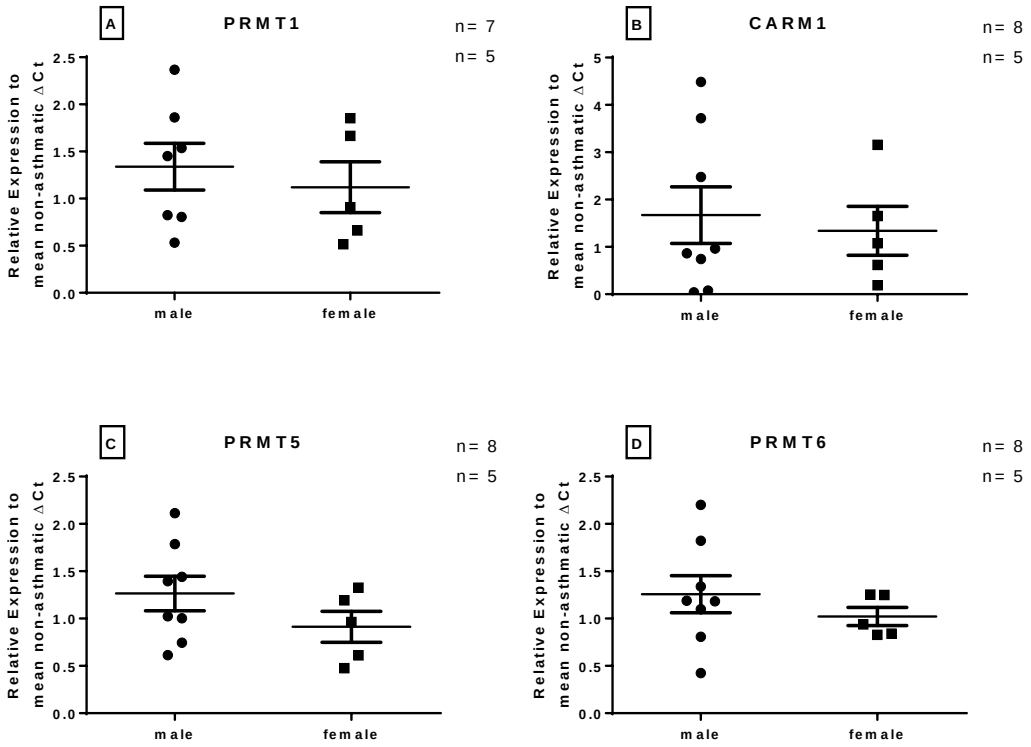

Figure 10.1 The expression of PRMTs in ASM cells isolated from males and females. Expression of PRMT1 (A), CARM1 (B), PRMT5 (C), and PRMT6 (D), in ASM cells isolated from males and females was assessed by qPCR. Data are expressed as the mean ± SEM of ASM cells collected from 7-8 males and 5 females, and analysed by Mann- Whitney test.

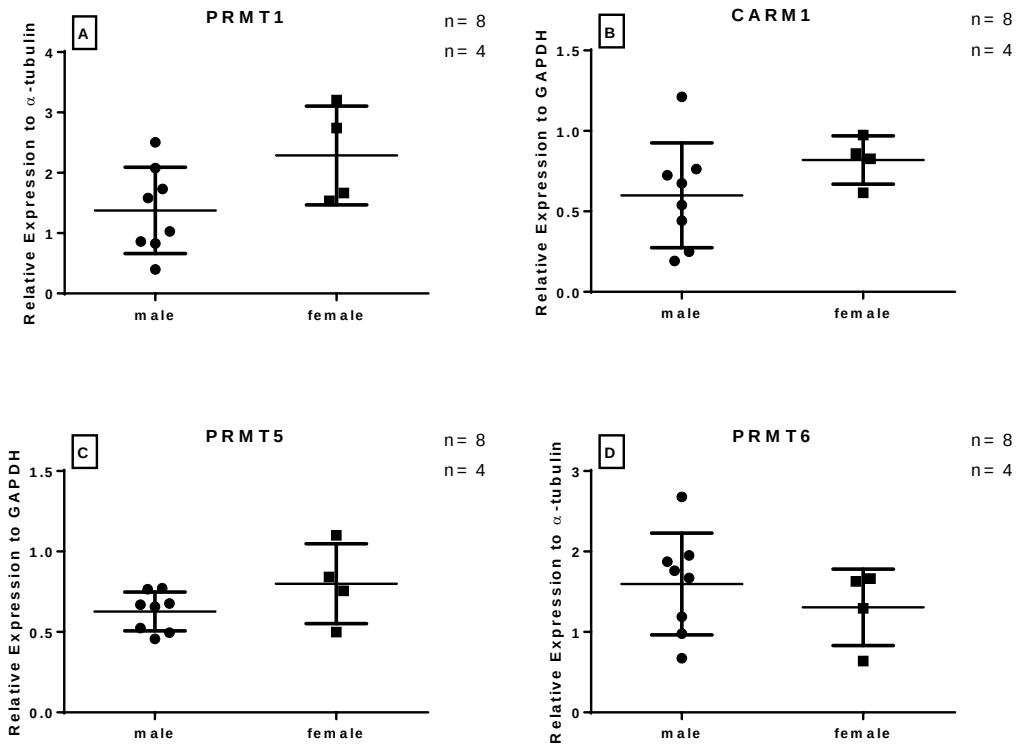

Figure 10.2 Semi-quantitative comparison of PRMT protein expression between cells isolated from males and females. The protein expression of PRMT1 (A), CARM1 (B), PRMT5 (C), and PRMT6 (D) was analysed by densitometry of western blots. Data are expressed as the mean ± SEM of ASM cells collected from 8 males and 4 females, and analysed by Mann- Whitney test.

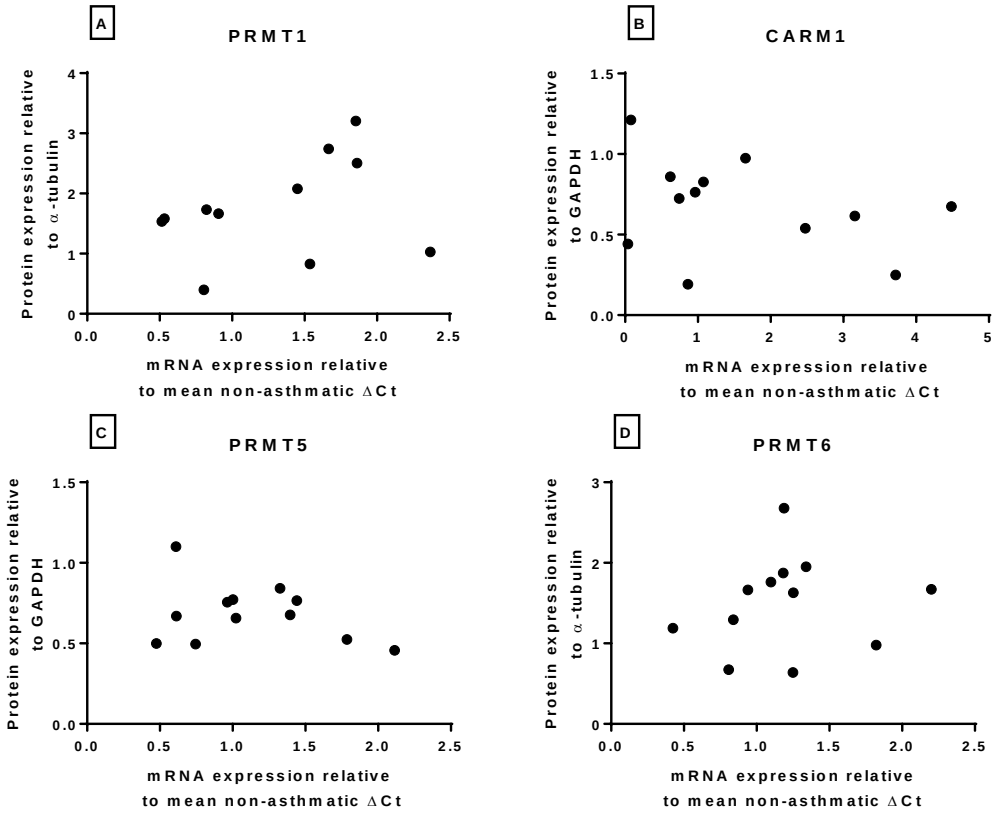

Figure 10.3: Correlation between mRNA and protein expression of PRMTs in ASM cells isolated from healthy volunteers and patients with asthma. Expression of PRMT1 (A), CARM1 (B), PRMT5 (C), and PRMT6 (D), in ASM cells isolated from asthmatic donors and healthy controls was assessed by qPCR (mRNA) and densitometry of western blots (protein). Data are expressed as the mean of ASM cells collected from 7 healthy controls and 4-5 patients with asthma and analysed by Spearman's rank correlation test.

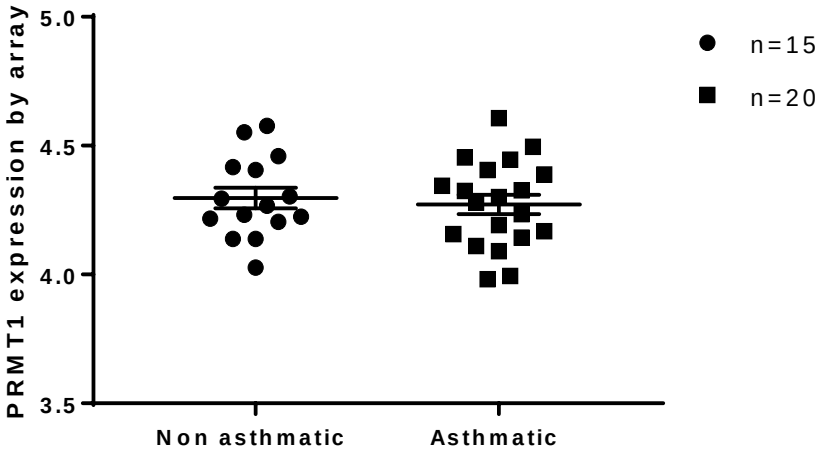

Figure 10.4: PRMT1 mRNA expression by array. Expression of PRMT1 in ASM cells isolated from asthmatic donors (n=20) and healthy controls (n=15) was assessed by Affymetrix Human Gene 2.1 ST Arrays. Data are analysed by Mann- Whitney test. A value of $P<0.05$ was accepted as significant.

Table 10.1: Details of participants who donated ASM cells used in Rachel Clifford's study. ICS = Inhaled corticosteroids; LABA = Long-acting beta agonists; Combi = Combination inhalers: Corticosteroids and long-acting beta agonists; SABA = Short-acting beta agonists; ORAL = Oral corticosteroids

Code	Disease	Age	Gender	Ethnicity	Medication	Smoking	Height (cm)	Weight (kg)
SMACD04	Asthmatic	60	M	White	ICS+ LABA+ Combi+ SABA	Never	175	Not available
MMP1-A02	Asthmatic	41	F	Asian	SABA	Never	158	55
MMP-1A08	Asthmatic	19	F	White	ICS+ LABA+ Combi+ SABA	Never	165	57
MMP-1A12	Asthmatic	22	F	White	ICS+ LABA+ Combi+ SABA	Never	171	67
AZAD03	Asthmatic	63	M	White	ICS+ LABA+ Combi+ SABA	Never	177	Not available
AZCC14	Asthmatic	36	F	White	ICS+ LABA+ Combi+ SABA	Never	158	69.1
AZCC15	Asthmatic	41	F	White	ICS+ LABA+ Combi	Never	157.4	90.8
AZCC23	Asthmatic	39	F	White	ICS+ LABA+ Combi+ SABA	Never	170	100
MMP-1A11	Asthmatic	24	M	White	ICS+ LABA+ Combi+ SABA	Never	178	71.6
MMP1-A14	Asthmatic	22	M	White	ICS+SABA	Never	182	78
MMP-1A16	Asthmatic	22	M	White	SABA	Never	175	85
AZCC01	Asthmatic	46	M	White	ICS+ LABA+ Combi+ SABA	Ex	180	Not available
ALS9007	Asthmatic	37	M	White	ICS+ LABA+ ORAL	Unknown	Not available	Not available
ALS9008	Asthmatic	50	F	White	ICS+ LABA+ ORAL	Ex	Not available	Not available
AZAD01	Asthmatic	51	M	White	ICS+ LABA+ Combi+ SABA	Never	164	Not available
MMP1-A06	Asthmatic	22	M	Asian	SABA	Never	184	82
MMP1-A03	Asthmatic	36	M	White	ICS+ LABA+ Combi+ SABA	Never	175	84
AZAC05	Non-asthmatic	26	F	White	None	Never	Not available	Not available
AZAC07	Non-asthmatic	39	M	Asian	None	Never	Not available	Not available
AZAC09	Non-asthmatic	19	F	White	None	Never	Not available	Not available
AZAC11	Non-asthmatic	46	M	White	None	Never	186	Not available
AZAC03	Non-asthmatic	61	M	White	None	Never	Not available	Not available
AZAC12	Non-asthmatic	27	M	White	None	Never	Not available	Not available
AZCC16	Non-asthmatic	54	F	Black	SABA	Ex	168.7	63.6
AZCC21	Non-asthmatic	70	M	White	None	Ex	169.5	87.2
AZCC22	Non-asthmatic	55	M	White	None	Ex	189	119.3
AZCC25	Non-asthmatic	72	M	White	None	Ex	171.2	85
AZCC26	Non-asthmatic	51	M	White	None	Never	181.3	95.5
MMP-1H10	Non-asthmatic	46	M	White	None	Never	190	86
MMP1-H02	Non-asthmatic	26	F	White	None	Never	162	70
MMP1-H01	Non-asthmatic	49	M	Black	None	Never	183	73
HASM0210	Asthmatic	46	M	Asian	Combi	Never	174	80.2
HASM0909	Asthmatic	37	M	White	Combi	Never	176	98
2803005	Asthmatic	58	F	White	Combi+SABA	Ex	166	86.5
MMP1-H04	Non-asthmatic	47	M	White	None	Never	186	59

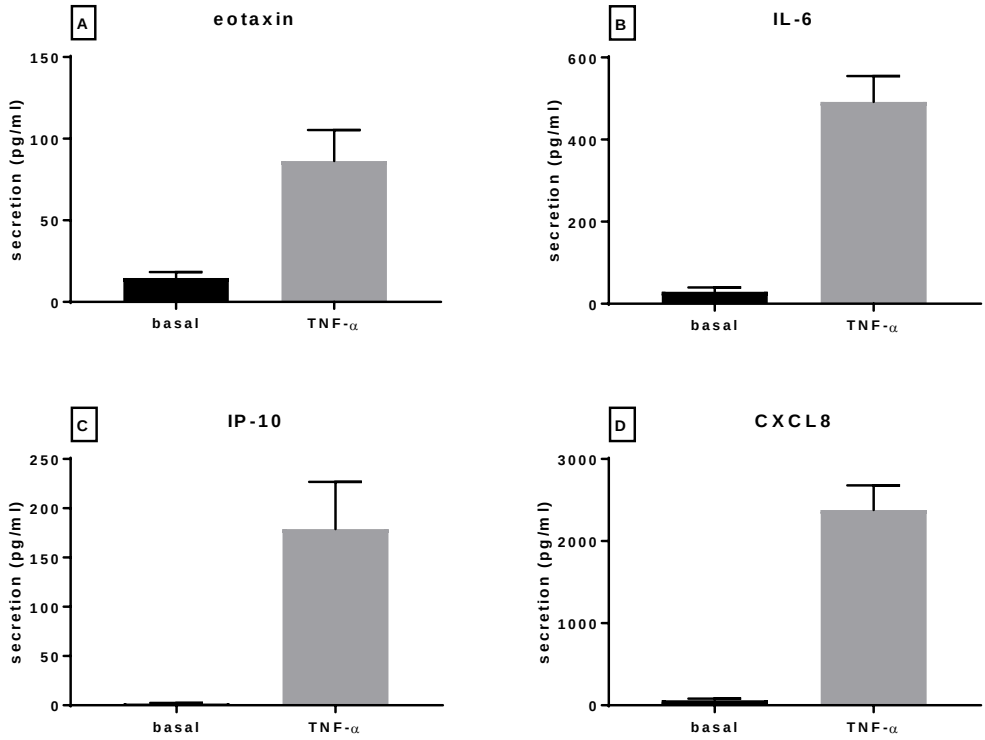

Figure 10.5: Raw concentrations of cytokines/chemokines secreted by ASM cells. The concentrations of eotaxin, IL-6, IP-10 and CXCL8, in pg/ml, secreted under basal and TNF-α stimulated conditions (1 ng/ml), by control (i.e. with DMSO) ASM cells from three healthy volunteers, were assessed by ELISA. Data show mean +/- SEM results from 12 experiments, normalised to cell counts.

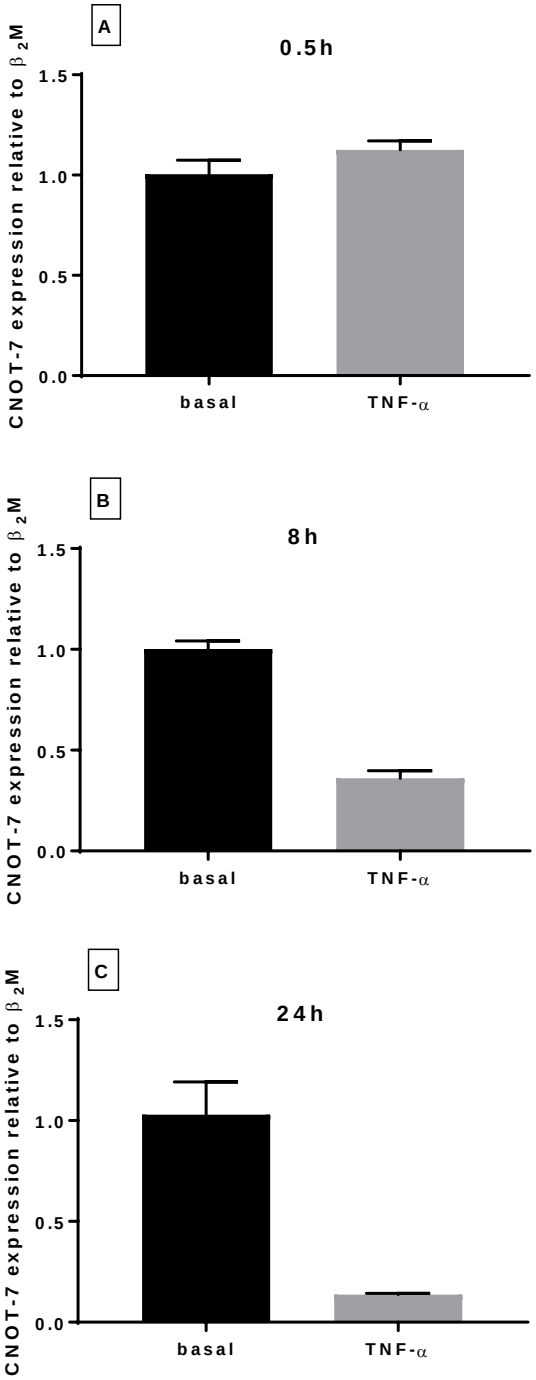

Figure 10.6: CNOT7 mRNA expression in ASM cells following TNF-α stimulation for 0.5 (A), 8 (B) and 24 (C) hours. CNOT7 mRNA expression following TNF- α stimulation at various time points in ASM cells obtained from one non-asthmatic donor was analysed by qPCR. Data are expressed relative to β_2M.

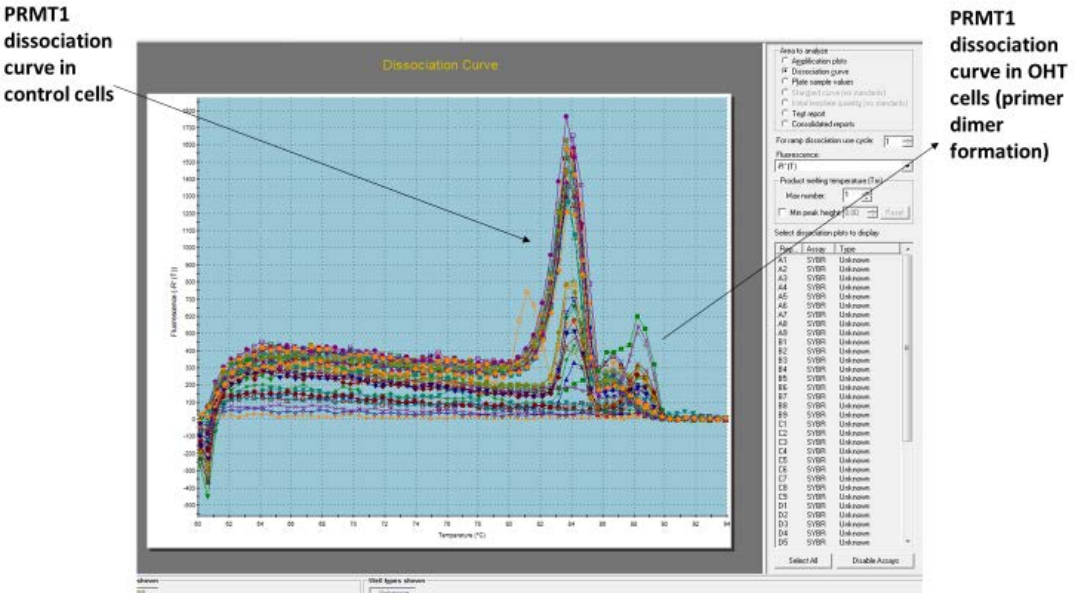

Figure 10.7: Dissociation curve from a qPCR aimed to determine PRMT1 mRNA expression in MEFs. Dissociation curve shows a formation of a primer dimer in samples from OHT-treated cells due to low PRMT1 expression in samples. This shows that PRMT1 mRNA expression is much lower in OHT-treated MEFs.

Chapter 11: Bibliography

11 Bibliography

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