UNIVERSITY OF NAPLES "FEDERICO II"

DEPARTMENT OF PHARMACY

"Role of sphingolipid and hydrogen sulphide pathways in inflammatory lung diseases"

by

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Ph.D. Thesis

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Ph.D. Programme in Pharmacology (XXVII cycle)
2012-2015

To my parents and family,
who have always supported me.

ACKNOWLEDGMENTS

First of all, I would like to thank my tutor Prof Giuseppe Cirino, who has always been an experienced guide during all my PhD studentship.

A special thanks goes to Dr Fiorentina Roviezzo, who helped me to grow (both professionally and personally) with her precious advices.

I would like to acknowledge Prof Jane A. Mitchell of the Imperial College of London and all her group, who gave me the opportunity to work in her laboratory and to spend an awesome and unforgettable year in London, full of achievements.

Thanks to Dr Rosalinda Sorrentino and Prof Aldo Pinto, who, with their deep knowledge of immune system, have revealed so helpful and made such a complex scenario so easy to me to investigate.

The last thanks go to my friends and colleagues Dr Vincenzo Brancaleone, Dr Emma Mitidieri, and Ms Ermina Donnarumma, who contributed to create such a pleasant and friendly location in the laboratory, and all the students who joined our group over these years.

INDEX OF CONTENTS

page

Index of figures vi

Index of tables vii

List of abbreviations ix

Abstract 1

Chapter 1 Introduction 2

1.1 Inflammatory lung diseases 2

1.1.1 Chronic obstructive pulmonary disease 3

1.1.2 Asthma 3

1.1.3 Pulmonary arterial hypertension 9

1.2 Relevance of sphingolipids to respiratory diseases 20

1.2.1 Metabolic and catabolic pathways 20

1.2.2 S1P and asthma 23

1.2.3 Pepducins 27

1.3 Gasotransmitters and lung pathophysiology 29

1.3.1 Hydrogen sulphide 35

Chapter 2 Materials and methods 49

2.1 Reagents 49

2.2 Animal studies (airway responsiveness assessment) 50

2.2.1 Mice 50

2.2.2 Experimental protocol 50

2.2.3 Tissue preparation 52

2.2.4 Airway responsiveness measurements 52

2.2.5 Isolated perfused mouse lung preparation and measurement 52

2.3 Animal studies (pepducin activity assessment) 53

2.3.1 Mice 53

2.3.2 Tissue preparation 54

2.3.3 Aorta responsiveness measurements 54

2.4 Animal studies (pulmonary artery contractility assessment) 55

2.4.1 Mice 55

2.4.2 Experimental protocol 55

2.4.3 Tissue preparation 56

2.4.4 Pulmonary vessel responsiveness measurements 56

2.5 Lung morphological analysis 57

2.6 Cellular studies 58
2.6.1 Flow cytometry analysis 58
2.6.2 Mixed lymphocyte reaction 59
2.6.3 RBL-2H3 cell culture and β-hexosaminidase measurement 59
2.6.4 Smooth muscle cells 60
2.6.5 Isolation of blood outgrowth endothelial cells 60
2.6.6 Fibroblast culture and proliferation assessment 60
2.6.7 Immunohystochemistry 61
2.7 ELISA measurements 62
2.8 Western blotting analysis 62
2.9 H_2S levels measurements (Methylen blue assay) 63
2.10 Statistical analysis 63

Chapter 3 Results 64
3.1 Study of cellular mechanisms of S1P-induced airway hyperreactivity and pulmonary inflammation 64
3.1.1 Rationale 64
3.1.2 Methods 65
3.1.3 Data handling and statistical analysis 66
3.1.4 Results 67
3.1.5 Summary 97
3.1.6 Conclusions 102
3.1.7 Limitations 102
3.2 Study of H_2S effects in antigen-induced airway hyperresponsiveness: role of mast cells 104
3.2.1 Rationale 104
3.2.2 Methods 105
3.2.3 Data handling and statistical analysis 105
3.2.4 Results 106
3.2.5 Summary 119
3.2.6 Conclusions 122
3.2.7 Limitations 122
3.3 Study of H_2S in models of pulmonary hypertension 123
3.3.1 Rationale 123
3.3.2 Methods 124
3.3.3 Data handling and statistical analysis 124
3.3.4 Results 125
3.3.5 Summary 139
3.3.6 Conclusions 141
3.3.7 Limitations 141

Chapter 4 Discussion 142

References 144

INDEX OF FIGURES

page

Figure 1.1. Cellular and molecular mechanisms of PAH induction 13

Figure 1.2. Catabolic and methabolic pathway of sphigolipids 21

Figure 1.3. Schematic mechanism of function of a pepducin 28

Figure 1.4. Catabolic and metabolic pathways of H_2S 38

Figure 2.1. Apparatus used for administration of aerosolised NaHS 51

Figure 3.1. Systemic S1P administration induces lung inflammation 68

Figure 3.2. S1P increases mast cell infiltration in the lung 69

Figure 3.3. Mast cells are essential for the development of S1P-induced bronchial hyperreactivity, but not for lung inflammation 71

Figure 3.4. S1P enhances pulmonary CD23 (FcεRII) expression 73

Figure 3.5. Anti-CD23 attenuates S1P-mediated effects on the lung 74

Figure 3.6. T cells play a key role in S1P-mediated effects on lung 76

Figure 3.7. Lymphocytes harvested from S1P-treated BALBC/c mice have an increased ability to proliferate 79

Figure 3.8. Adoptive transfer of CD4+ T cells from S1P-treated mice into naïve (untreated) mice mimics S1P-induced effects in BALBC/c mice 80

Figure 3.9. S1P-mediated airway inflammation is similar to that observed in OVA-treated mice 83

Figure 3.10. S1P treatment promotes the recruitment of Treg cells 85

Figure 3.11. Treatment of B cells with S1P 89

Figure 3.12. Stimulation of lung-derived B cells with S1P increases MHC I and MHC II expression at early time points (3-7 days) 90

Figure 3.13. Lung-derived B cells from S1P-treated mice increased T cell proliferation and IL-10 release 91

Figure 3.14. Stimulation of lung-derived B cells with S1P increased CD20 expression at later time points (14 days) 92

Figure 3.15. Depletion of CD20+ B cells increases S1P-induced lung airway inflammation 94

Figure 3.16. Assessment of a newly synthesised pepducin acting as a $S1P_3$ antagonist 96

Figure 3.17. Administration of hydrogen sulphide aerosol abrogates OVA-induced airway hyperreactivity 107

Figure 3.18. Hydrogen sulphide inhibits mast cells infiltration and cytokines expression in the lung 109

Figure 3.19. Hydrogen sulphide abrogates OVA-induced mast cell-dependent hyperreactivity 111

Figure 3.20. Lack of effect of hydrogen sulphide in mast cell-deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mice 113

Figure 3.21. Hydrogen sulphide inhibits pulmonary fibroblasts proliferation and differentiation 115

Figure 3.22. Hydrogen sulphide inhibits fibroblasts proliferation and differentiation in the asthma model mast cell-dependent 116

Figure 3.23. Hydrogen sulphide reduces IgE-mediated RBL-H3 cells degranulation 118

Figure 3.24. Stability of NaHS in medium 126

Figure 3.25. NaHS decreases IP10 levels released from hPASMCs and BOECs ... 128

Figure 3.26. Effect of NaHS on IL-8 release from hPASMCs and BOECs 130

Figure 3.27. NaHS does not affect proliferation rate of hPASMCs and BOECs ... 132

Figure 3.28. NaHS does not affect ET-1 production from hPASMCs and BOECs 134

Figure 3.29. Additive effect of NaHS and Sildenafil, ACT-132577, and Treprostinil sodium on IP10 release from healthy BOECs 136

Figure 3.30. Characterisation of murine experimental model of PH 138

INDEX OF TABLES

page

Table 1. WHO functional classification for patients with PAH 10

Table 2. Revised (2009) WHO classification of group 1 PAH 10

Table 3. Toxicity of H_2S 38

LIST OF ABBREVIATIONS

12-HETE	12-hydroxyeicosatetraenoic acid
15-HETE	15-hydroxyeicosatetraenoic acid
3-MST	3-mercaptopyruvate sulfurtransferase
5-HT	serotonin
α-SMA	α-smooth muscle actin
ABC transporters	ATP-binding cassette transporters
ADP	adenosine diphosphate
APCs	antigen-presenting cells
ApoM	apolipoprotein M
ASM cells	airway smooth muscle cells
ATP	adenosine triphosphate
BACE1	β-site amyloid precursor protein cleaving enzyme 1
BAL	bronchoalveolar lavage
Bcl protein	B cell lymphoma protein
bFGF	basic fibroblast growth factor
BKC	calcium-activated potassium channel (large conductance calcium-activated potassium channel)
CBS	cystathionine β-synthase
CCL5	chemokine (C-C motif) ligand 5
CcOX	cytochrome oxidase
CD	cluster of differentiation
Cdk	cyclin-dependent kinase
cGMP	cyclic guanosine monophosphate
CO	carbon monoxide
COX	cyclooxygenase
CSE	cystathionine γ-lyase
CTGF	connective tissue growth factor
CXCR	CXC (C-X-C chemokine) receptor
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EDRF	endothelium-derived relaxing factor
EGF	epidermal growth factor
ER	endoplasmic reticulum
ERK	extracellular-signal-regulated kinase
ET-1	endothelin-1
FAD	flavin adenine dinucleotide

FCεRI	“high-affinity” IgE receptor
FCεRII	“low-affinity” IgE receptor (CD23)
FGF2	fibroblast growth factor 2
FMN	flavin mononucleotide
FPR2	N-formyl peptide receptor 2
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GC	guanylyl ciclase
GCPR	G protein-coupled receptor
GM-CSF	granulocyte-macrophage colony-stimulating factor
GMP	guanosine monophosphate
GTP	guanosine-5'-triphosphate
H_2S	hydrogen sulphide
HDAC	histone deacetylase
HDL	high-density lipoprotein
HIV	human immunodeficiency virus
HLA-DR	human leukocyte-associated antigen-DR
HO	heme oxigenase
Hsp	heat shock protein
ICAM-1	intercellular adhesion molecule-1
IFN	interferon
IgA	immunoglobulin A
IgE	immunoglobulin E
IgG	immunoglobulin G
IgM	immunoglobulin M
IGF	insulin growth factor
IL	interleukin
IP10	interferon γ–inducible protein 10
IP receptor	PGI_2 receptor
K_a	acid dissociation constant
K_{ATP}	ATP-sensitive potassium channels
K_d	equilibrium dissociation constant
KLF-5	Kruppel-like factor-5
LDL	low-density lipoprotein
LPS	lipopolysaccharide
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinases
MC	mast cell
MC4R	melanocortin 4 receptor
MCP-1	monocyte chemoattractant protein-1
MCT	monocrotaline

MHC	major histocompatibility complex
MIF	macrophage migration inhibitory factor
MIP	macrophage inflammatory protein
MMP-13	matrix metalloproteinase-13
mRNA	messenger RNA (ribonucleic acid)
MYPT1	myosin phosphatase target subunit 1
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NO	nitric oxide
NOS	nitric oxide synthase
OVA	ovalbumin
P2XR	P2 (ATP) purinergic receptor type X
PAR	protease-activated receptor
PASMCs	pulmonary artery smooth muscle cells
PDE-5	phosphodiesterase type 5
PDGF	platelet-derived growth factor
PGE_2	prostaglandin E2
PGI_2	prostacyclin (prostaglandin I2)
PHB2	prohibitin 2
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase (phosphatidylinositol-3-kinases)
PKC	protein kinase C
PKD	protein kinase D
PLC	phospholipase C
ppm	parts per million
RANTES	regulated upon activation, normal T cell expressed and secreted
RNS	reactive nitrogen species
ROS	reactive oxygen species
S1P	sphingosine 1-phosphate
SEM	standard error of the mean
sGC	soluble guanylyl ciclase
SOD	superoxide dismutase
SphK	sphingosine kinase
TGF-β	transforming growth factor-β
Th2 lymphocytes	type 2 T helper lymphocytes
TIMP-1	tissue inhibitor of metalloproteinase-1
TM	transmembrane α-helices
TNF-α	tumor necrosis factor-α

TRAF2	TNF receptor-associated factor 2
Treg cells	regulatory T cells
TREK-1	TWIK-related potassium channel-1
TXA_2	thromboxane A2
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
VLA-4	very late antigen-4
VLDL	very low-density lipoprotein

ABSTRACT

The importance of sphingolipids in lung pathophysiology is becoming more and more evident over years. Despite the findings that they have such numerous activities on cells, the mechanisms by which sphingolipids elicit their effects are not fully understood. Furthermore, the role of other recently-identified mediators such as gasotransmitters in pulmonary district has started to be investigated.

My project aimed at clarifying the cellular/molecular pathways underlying the beneficial/detrimental effects of these mediators in lungs in animal experimental models by means of in vivo *and* in vitro *approaches.*

The results obtained showed that systemic administration of S1P could induce an asthma-like condition in mice and such condition involved the cooperation and activation of different cells of the immune system, i.e. *mast cells and T cells. The effects of S1P were counteracted by B cells. Conversely to the negative effects of S1P, we showed that H_2S inhalation proved to be beneficial in reducing allergen-induced airway hyperreactivity via a mast cell- and fibroblast-mediated mechanisms, without affecting lung inflammation. However, in an experimental model of pulmonary hypertension, we observed that H_2S acted as an antiinflammatory agent.*

In conclusion, a new experimental asthma-like model useful for defining the role of S1P in the mechanism of action of currently-used drugs as well as in the development of new therapeutic approaches has been characterised. In addition, the protective properties of H_2S have been further clarified, evidencing its involvement in modulating remodeling processes in inflammatory lung diseases.

CHAPTER 1
INTRODUCTION

1.1 INFLAMMATORY LUNG DISEASES

Respiratory diseases are considered ones of the major causes of mortality and morbidity worldwide: it is believed that they provoke the death of 4 million people every year[1]. The dramatic incidence of the number of cases of asthma in the last decades is very important for the public world health: currently, the patients affected by asthma are approximately 300 million and those numbers are thought to increase further. Furthermore, chronic obstructive pulmonary disease (COPD) will be the fourth principal cause of death in the next years[2]. Asthma and COPD are chronic inflammatory conditions but their aetiology and pathogenenesis sensibly differ. It is considered that asthma and COPD are caused by the exposition to environmental agents (particularly aeroallergens and cigarette smoke, respectively) in patients with a sensible background. The typical clinical evolution of these conditions is marked by times of acute exacerbations of their symptoms. Exacerbations are believed to be significant events of the progression of the disease and have huge implications for patients and physicians: exacerbations accelerate the progression of the disease, worsen the quality of life, and represent the principal cause of mortality.

Pulmonary hypertension was first identified by Ernst von Romberg in 1891 and is a haemodynamic and pathological state that can be found in multiple clinical conditions. It is defined as an increase of blood pressure in lung vasculature, leading to shortness of breath, dizziness, fainting, leg swelling and other

symptoms. Pulmonary hypertension can be a severe disease with a markedly decreased exercise tolerance and heart failure.

1.1.1 CHRONIC OBSTRUCTIVE PULMONARY DISEASE

The chronic obstructive pulmonary disease (COPD) is the most common chronic respiratory condition among adult populations. The GOLD (Global Initiative for Obstructive Lung disease), a collaboration between the World Health Organization and the National Heart Lung and Blood Institute, defines the COPD as *"a common preventable and treatable disease, characterised by persistent airflow limitation that is usually progressive, not completely reversible, and associated with an enhanced chronic inflammatory response in the airways and the lung to noxious particles or gases. Exacerbations and co-morbidities contribute to the overall severity in individual patients"*. The main etiological agent linked with BPCO is cigarette smoke. The inflammatory response consists in an activation of neutrophils, mast cells, and T lymphocytes CD8+ and differs significantly from the response observed in asthma. The lung inflammation is even more amplified by oxidative stress and an excess of proteases released by inflammatory cells recruited in lungs. Similarly in asthma, in COPD as well episodes of acute exacerbations are more and more frequent as the pathology evolves[3]. Exacerbations are the main cause of morbidity and mortality and accelerate the decline of pulmonary functionality[4] and quality of life[5].

1.1.2 ASTHMA

Asthma is the most common chronic respiratory disease and affects up to 10% of the adult population and the 30% of infants in the developed countries2. The GINA (Global Initiative for Asthma) states that asthma is defined as *"a chronic*

inflammatory disease of airways in which many cell types and cellular elements play a role. The chronic inflammation is associated with airway hyperresponsitivity that leads to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, in particular during night-time and the early hours of the day. These episodes are often associated to a diffuse but variable obstruction of airflow, which is reversible spontaneously or after pharmacological treatment". This definition responds to the key physiopathological hallmarks of the pathology: reversible obstruction of airflow and airway inflammation. The characteristic profile of the inflammation involves eosinophils, mast cells, and Th2 lymphocytes and a wide range of mediators. The exacerbations are episodes characterised by coughing, wheezing, shortness of breath, and airflow obstruction, which appears with reduction of lung functional parameters such as the peak expiratory flow. Acute exacerbations are very common and their social and economic burden is considerable, due to both direct costs for the use of the national health system and indirect costs associated with the lack of productivity. Current pharmacological therapies for asthma consist in bronchodilators and antiflammatory drugs such as β_2-agonists and corticosteroids. These are very efficient in alleviating the symptoms and reducing exacerbations down to 50%, as clinical trials reveal[6]. Even though asthma is characterised by a reversible obstruction of the airflow, the pharmacological treatment is often not effective in alleviating the airflow limitation. This lack of response is associated with remodelling of the bronchial wall, a term used to describe the structural modifications that happen through the matrix[7].

Pathophysiology

The model of the allergic asthma is the most understood and studied one. The allergic (atopic) subject has an increased production of CD4+ T cells, also known as Th2 cells, compared to healthy individuals. Such lymphocytes, morphologically identical to Th1, mostly produce IL-4, IL-5, and IL-13[8]. These cytokines help the allergic inflammation to set up. IL-4 is partly responsible of the isotypic switch of B

cells towards the production of IgE[9]. When an inhaled allergen comes in contact to IgE-bearing cells, which selectively recognise the allergen, airway resident mast cells[10,11] and macrophages[12,13] rapidly activate through the FCεRI, the "high-affinity" receptor for IgE. The activated cells rapidly release proinflammatory mediators such as histamine[14], eicosanoids[15] (in particular leukotrienes), and ROS that induce contraction of smooth muscle, mucus production, and vasodilation. Bronchial microcirculation plays an important role in this process[16]: indeed, released mediators induce capillary leakage with subsequent formation of exudate[17,18]. Plasma proteins leakage induces a thickening of bronchial walls with airways obstruction. Exudate can compromise epithelial integrity and its presence in the bronchial lumen can reduce the clearance of mucus[19]. All these effects contribute to airflow obstruction. The early inflammatory phase follows a late inflammatory phase that involves the recruitment and activation of eosinophils[20], CD4+ T cells[21], basophils[22], neutrophils[23,24], and macrophages[25]. There is a selective retention of T cells[26], expression of adhesion molecules[27,28,29], and the release of proinflammatory mediators and Th2-type cytokines involved in the recruitment and activation of inflammatory cells[30,31,32] that will be important in the chronic phase of inflammation.

The recruitment of peripheral blood cells including eosinophils, lymphocytes, and monocytes into inflamed airways is the result of adhesive interactions between circulating inflammatory and microvascular endothelial cells via the production of proinflammatory mediators, cytokines and chemokines, and the expression of cell surface adhesion molecules. Up-regulation of distinct adhesion molecules such as CD11a, CD11b, CD18, or VLA-4 on blood cells and ICAM-1 or VCAM-1 on endothelial cells is a critical step for the induction of the inflammatory response[28,33,34]. The ligand VLA-4 is not present on neutrophils[35], which in part may explain the selective recruitment of eosinophils in asthma[36]. An increase of such airway vascular adhesion molecules has been observed in asthma[37]. Recruitment of cells into the airways wall is associated with their priming and activation[38] and is also dependent on cytokines such as IL-5[39] and GM-CSF acting

to enhance eosinophil recruitment, terminal maturation[40], and expression of their adhesion molecules[35,41]. Chemokines such as RANTES[42,43] and eotaxin[44,45] also act on eosinophils and T cells[46] to markedly enhance their recruitment and possibly their activation. RANTES[47], IL-16 (a lymphocyte chemoattractant factor), and MIP-1α are found in BAL fluid of antigen-challenged asthmatics[48] and may participate in the process.

The survival of inflammatory cells in airway tissues depends on survival factors. Apoptosis, a dynamic process involved in the control of the "tissue load" of cells at inflamed sites, tends to limit inflammatory tissue injury and promote resolution rather than progression of inflammation[49]. Because apoptosis attempts to terminate the inflammatory process by reducing the number of viable inflammatory cells within the bronchial mucosa, the persistence of inflammation may be due to alterations in the regulation of cell apoptosis leading to a chronic and self-perpetuating inflammatory cell survival and accumulation. Once at the site of airway inflammation, their survival as activated cells is increased[50] as a consequence of reduced apoptosis[51,52] and possibly by increased expression of adhesion molecules on epithelial cells[53,54]. Increased eosinophil survival in asthma is associated with reduced apoptosis[51,55]. Several cytokines and chemokines may also promote cell survival, among them GM-CSF, IL-3, IL-5, and RANTES, which are overexpressed in asthmatic airways[56,57,58,59,60,61].

Inflammation in chronic asthma appears to be far more complex than a simple eosinophilic inflammation alone[62]. All cells of the airways, including T cells, eosinophils, mast cells, macrophages, epithelial cells, fibroblasts, and even bronchial smooth muscle cells, are involved in asthma and become activated. Nonetheless, eosinophils play an effector role by release of proinflammatory mediators[63,64,65,66], cytotoxic mediators[67], and cytokines[68,69,70,71,72,73], resulting in vascular leakage, hypersecretion of mucus, smooth muscle contraction, epithelial shedding, and bronchial hyperresponsiveness. These cells are also involved in the regulation of the airway inflammation and initiate the process of remodelling by the release of cytokines[68,69,70,71,72] and growth factors. For many years, bronchial

epithelial cells were considered to act mainly as a mere barrier participating in mucociliary clearance and removal of noxious agents. More recently, epithelial cells have been found to participate in inflammatory reactions by the release of eicosanoids, peptidases, matrix proteins, cytokines, and NO, as well as performing an immune function by their capacity to express HLA-DR and present antigen.

In asthma, epithelium is partly shed, ciliated cells appear swollen and vacuolised, and there is often loss of cilia[74,75]. When epithelium is reconstituted, there are greater numbers of goblet cells than normal. In fatal asthma, extensive epithelial shedding is commonly observed. Epithelial cells of asthmatics are also significantly less viable than those of normal subjects[76]. The mechanisms underlying epithelial shedding in asthma are still a matter of debate[77]. Epithelial shedding can be caused by plasma exudation[78], toxic inflammatory mediators such as eosinophil granule proteins[79,80], oxygen free radicals, TNF-α[81], mast cell proteolytic enzymes[82] or metalloproteases from epithelial cells[83], or macrophages[84]. Furthermore, the increased epithelial fragility and shedding may also be caused by a weakened attachment of superficial epithelial cells to basal cells or to their basement membrane; this probably reflecting a disturbance in cell-cell adhesion[85].

The functional consequences of epithelial shedding are still unclear. Epithelial damage may lead to heightened airway responsiveness[86,87], a failure to metabolise agonists[88], the destruction of a diffusion barrier altering permeability of the airway mucosa[89], the depletion of epithelial-derived relaxant factors[90], and loss of enzymes (neutral endoproteases) responsible for degrading proinflammatory neuropeptides including substance P[91]. The integrity of airway epithelium may influence the sensitivity of the airways to provocative stimuli by liberating a variety of bronchoactive mediators, *e.g.* lipoxygenase and cyclooxygenase products[76,92] and NO[93]. Activated epithelial cells release a wide array of mediators including 15-HETE[76], cytokines[94], eotaxin[95], growth factors[96,97], ECM proteins[76,98], and metalloproteases[99], which may induce bronchial obstruction, inflammation, and airway remodelling[100]. In asthma, epithelial cells are activated and release great amounts of 15-HETE, PGE_2, fibronectin, cytokines, growth factors, and

endothelin spontaneously or after stimulation[76,101]. There is an increased expression of membrane markers such as adhesion molecules[102,103], endothelin[104], NO synthase[93], cytokines[105,106,] or chemokines[107]. Epithelial cells can be activated by IgE-dependent mechanisms[108], viruses, pollutants[109], or proinflammatory mediators such as histamine[110]. In asthma, epithelial cells are likely to be important in repair processes. They release ECM proteins[76] including fibronectin[98,111], which appears to be of importance in cell regeneration. Bronchial epithelial cell-derived cytokines may amplify ongoing inflammatory processes via the recruitment and activation of specific subsets of inflammatory cells, as well as by prolonging their survival in the airway microenvironment[112]. Bronchial epithelial cells represent targets for paracrine acting cytokines and growth factors, which may then modulate bronchial epithelial cell functions. They may be important in the regulation of airway remodelling and fibrosis as they release fibrogenic growth factors such as IGF[96,113] and TGF-β[114], regulate fibroblast proliferation[115], and release metalloproteases[116].

Other cells play a role in the slow and complex process of remodelling. Eosinophils, through the release of growth factor[117,118], elastases[119], and metalloproteases[120], are involved. Alveolar macrophages as well are able to release cytokines and growth factor such as PDGF and bFGF or TGF-β[121]. They represent the cellular types that are mostly found in the BAL of asthmatic patients and several studies shows their extensive activation[122,123,124,125,126]. Fibroblasts show some characteristics of inflammatory cells after their activation by IL-4 and IL-13, which induce them to differentiate and proliferate[127], and they can be the precursors of various cells, including smooth muscle cells[128]; furthermore, in asthma, myofibroblasts are numerous and are concentrated in the basal reticular membrane. The high number of fibroblasts well correlates with the high levels of TGF-β found in the BAL and a slight overexpression observed in some works[129]. Finally, airway smooth muscle cells have the capacity of contraction, proliferation, and production of mediators, such as chemokines and growth factors. Chemokines and their receptors are involved in a large number of pathological processes,

which contribute to bronchial hyperresponsiveness, including recruitment of inflammatory cells and collagen deposition[130]. For example, RANTES is a potent chemiotactic substance for eosinophils, T cells, and monocytes and seems to worsen asthma[131]. One of the characteristics of these cells is their capacity to migrate, which could likely link to remodelling[132] and hyperplasia of smooth muscle, feature observed only in asthma and not in COPD.

1.1.3 PULMONARY ARTERIAL HYPERTENSION

Pulmonary hypertension (PH) is a rare but devastating disease with very low therapeutic options. It is a progressive and fatal disease if untreated.

The World Health Organization (WHO) formulated a clinical classification of the various manifestations of PH, according to similarities in pathophysiologic mechanisms, clinical presentation, and therapeutic approaches. This classification system was initially adopted in 1998 and was re-examined in 2003 and 2008. The WHO pulmonary hypertension groups consist in the following[133]:

Group 1: pulmonary arterial hypertension
Group 2: pulmonary hypertension with left heart disease
Group 3: pulmonary hypertension associated with lung disease and/or hypoxemia
Group 4: pulmonary hypertension due to chronic thrombotic and/or embolic disease
Group 5: miscellaneous (sarcoidosis, histiocytosis X, lymphangiomatosis, compression of pulmonary vessels [adenopathy, tumor, fibrosing mediastinitis])

Patients can also be classified according to the severity of their symptoms and functional abilities. The WHO functional class is determined from patient's own subjective impression of ability and symptom severity[134]:

Table 1. WHO functional classification for patients with PAH.

Class	Description
I	Patients with pulmonary hypertension but without resulting limitation of physical activity; ordinary physical activity does not cause undue dyspnea or fatigue, chest pain, or near syncope
II	Patients with pulmonary hypertension resulting in slight limitation of physical activity; they are comfortable at rest; ordinary physical activity causes undue dyspnea or fatigue, chest pain, or near syncope
III	Patients with pulmonary hypertension resulting in marked limitation of physical activity; they are comfortable at rest; less-thanordinary physical activity causes undue dyspnea or fatigue, chest pain, or near syncope
IV	Patients with pulmonary hypertension with an inability to carry out any physical activity without symptoms; these patients manifest signs of right heart failure; dyspnea and/or fatigue can even be present at rest; discomfort is increased by any physical activity

Pulmonary arterial hypertension (PAH), which is a subgroup of PH, is a group of diseases having in common vascular remodelling of the small pulmonary arteries with associated elevated pulmonary arterial pressure and right ventricular failure. Table 2 reports a classification of PAH, according to the cause.

Table 2. Revised (2009) WHO classification of group 1 PAH.

1. Pulmonary arterial hypertension (PAH)
1.1 Idiopathic (iPAH)
1.2 Familial (fPAH)
1.3 Associated with (aPAH)
1.3.1 Connective tissue disorder
1.3.2 Congenital systemic-to-pulmonary shunts
1.3.3 Portal hypertension
1.3.4 Human immunodeficiency virus infection
1.3.5 Drugs and toxins
1.3.6 Other (thyroid disorders, glycogen storage disease, Gaucher's disease, hereditary hemorrhagic telangiectasia, hemoglobinopathies, chronic myeloproliferative disorders, splenectomy)
1.4 Associated with significant venous or capillary involvement
1.4.1 Pulmonary veno-occlusive disease (PVOD)
1.4.2 Pulmonary capillary hemangiomatosis (PCH)

1.5 Persistent pulmonary hypertension of the newborn

(Data from Simonneau G. *et al.*, 2009)

The primary pathophysiology is remodelling of the small pulmonary arteries and increased pulmonary vascular resistance. Symptoms begin as shortness of breath on exertion and progress to dyspnea with normal activities and, finally, dyspnea at rest. In advanced PAH, patients are unable to perform any activity without shortness of breath or chest pain[135].

Estimates of the incidence of PAH have varied from 5 to 52 cases/million population[136,137]. The incidence of PAH associated with scleroderma is estimated at 30 to 286 cases/million population[138]. PAH occurs in both genders and in all age groups, although after puberty PAH is seen approximately twice as often in females[139]. Untreated patients with PAH face an estimated mean survival of 2.8 years, with a 1-, 3-, and 5-year survival rate of 68%, 48%, and 34% respectively[140]. Treated, the survival rates improve to 91% to 97% after 1 year and 84% to 91% after 2 years[141]. Patients with PAH associated with the scleroderma spectrum of diseases have a poorer prognosis[142].

The diagnosis of PAH consists of three hemodynamic assessments[142]: mean pulmonary artery pressure >25 mmHg; left atrial pressure or left ventricular end-diastolic pressure ≤15 mmHg; pulmonary vascular resistance >3 Wood units.

Patients suspected of PAH should undergo a thorough evaluation (history and physical examination), electrocardiogram, chest X-ray, and echocardiogram. The symptoms of PAH are non-specific and include breathlessness, fatigue, weakness, angina, syncope, and abdominal distension. Symptoms at rest are reported only in very advanced cases. The physical signs of PAH include a right parasternal lift and an accentuated pulmonary component of second heart sound. Rare findings include a pansystolic murmur of tricuspid regurgitation, a diastolic murmur of pulmonary insufficiency, and a right ventricular third sound. The electrocardiogram might suggest PAH by demonstrating a right axis deviation and right ventricular hypertrophy and strain and right atrial enlargement. Many

patients with idiopathic PAH will have abnormal chest X-ray findings with central pulmonary arterial dilation and right atrial and right ventricular enlargement in more advanced cases. Echocardiography will help to evaluate the right heart structure and function and estimate the hemodynamic alterations[143].

The diagnosis of PAH must be confirmed by right heart catheterization[142]. After the diagnosis, the patient response to therapy and disease progression is generally assessed using non-invasive tests, including transthoracic echocardiography, WHO functional class, and the 6-minute walk test. Two biomarkers, brain natriuretic peptide and N-terminal fragment of pro-brain natriuretic peptide, are used to assess disease severity, with the serum levels of brain natriuretic peptide increasing as right ventricular function fails[142]. Patients with a plasma brain natriuretic peptide <180 pg/ml, and especially patients whose brain natriuretic peptide levels decrease within 6 months of starting treatment, have a better prognosis[144].

Pathophysiology

The pathophysiology of PAH is a complex interplay of vasoconstriction, vascular wall hypertrophy, fibrosis, and thrombosis[145,146,147,148]. Pathologic findings show intimal hyperplasia and fibrosis, medial hypertrophy, and *in situ* thrombi of the small pulmonary arteries and arterioles[148]. Until recently, the underlying causes of symptoms were thought to mainly be vasoconstriction and vasodilation pathology; however, it is now believed that the processes that contribute to PAH involve abnormal proliferation of the vascular smooth muscle and endothelial cells, infiltration of the inflammatory cells, and fibrosis of vascular elements (see Figure 1.1). These changes are associated with increased ET-1 levels, decreased NO levels, and decreased PGI_2 levels. These factors influence vasodilation, vasoconstriction, and cell proliferation, among other processes.

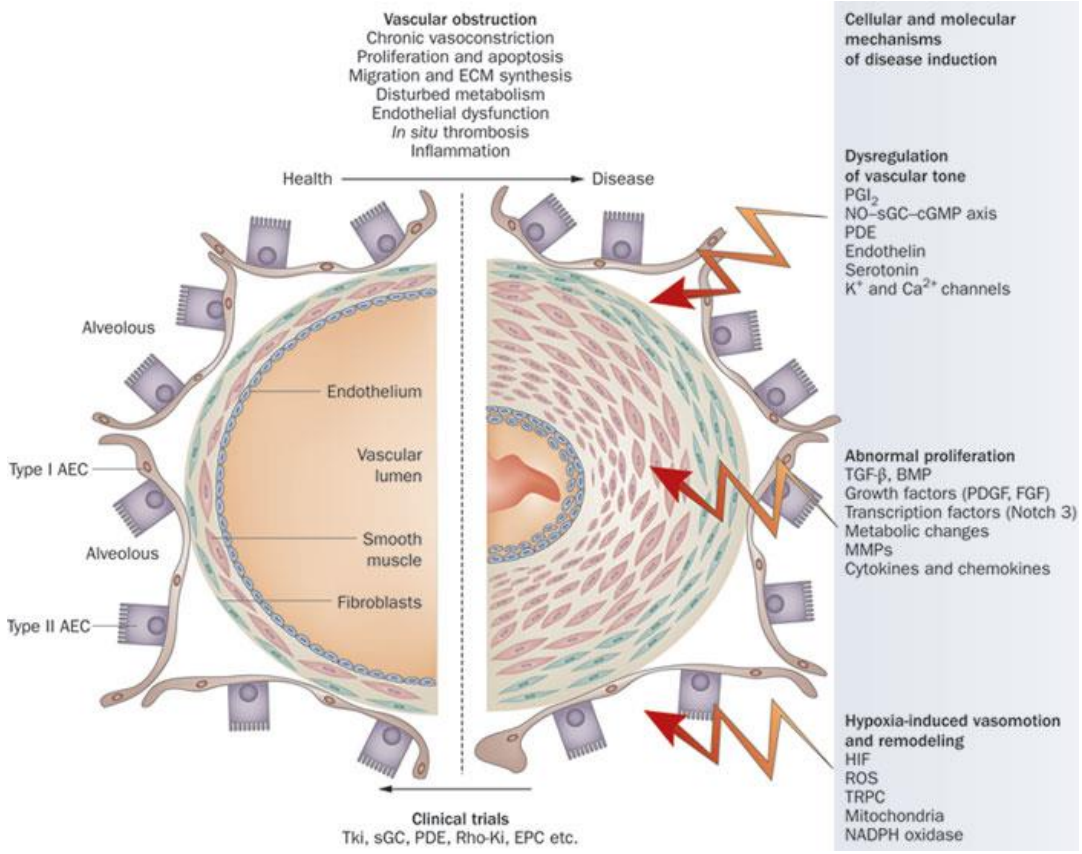


Figure 1.1. Cellular and molecular mechanisms of PAH induction (from Schermuly *et al.*, 2011).

It is well established that ET-1 is a key mediator of PAH, driving pathological changes in the lung that lead to pulmonary vascular remodelling[149,150]. The effects of ET-1 are mediated via two ET receptor subtypes, ET_A and ET_B, to which ET-1 binds with high affinity[151,152]. ET_A receptors are found in smooth muscle cells whereas ET_B receptors are located in both endothelial cells and smooth muscle cells. Endothelial cell dysfunction results in overexpression of ET-1. Released from the endothelium, it acts primarily on the underlying smooth muscle cells to cause vasoconstriction and proliferation. In addition, ET-1 acts on fibroblasts to induce contraction, proliferation, and fibrosis and on the endothelium itself to cause proliferation, vasodilation (via NO and PGI_2), and vasoconstriction (via TXA_2). All three of these ET-driven processes play an important role in lung vascular and structural remodelling[153]. A greater understanding of the ET system has elucidated a role for autocrine/paracrine signalling in the ET system and PAH pathway. The

evidence to support this hypothesis is reinforced by a large number of observations. For instance, cleavage of big ET-1 to form mature ET-1 is known to occur *in situ*[152,154,155] and circulating plasma levels of ET-1 are below the pharmacological threshold levels required to elicit vasoconstriction. This is suggestive of a local site of action[151]. Additional support for the importance of autocrine/paracrine signalling includes the polarised secretion of ET-1, whereby 80% of the ET-1 produced by the endothelial cells is released basolaterally towards the vessel wall, and hence the tissue, while only 20% is released into the bloodstream[156]. Furthermore, Davie *et al.*[157] reported an increased number and density of ET receptors in the small pulmonary arteries of patients with iPAH and PAH associated with congenital heart disease. Not only does there seem to be a high concentration of ET receptors, there also seems to be continuous occupancy of these receptors by their agonist[158,159].

PAH is associated with impaired production of the endothelium-derived vasodilator NO[160]. In healthy individuals, NO acts on smooth muscle cells to induce vasodilation and to inhibit proliferation by increasing production of the secondary messenger cGMP via the activation of soluble guanylyl cyclase (sGC)[161,162]. Treatment of pulmonary hypertension with NO-releasing agents such as nitrates has failed to produce beneficial long-term effects as, in most cases, negligible pulmonary vasodilation was counterbalanced by significant peripheral reduction in vascular resistance and reflex tachycardia[163], both of which are poorly tolerated by patients with severe PAH. An alternative therapeutic strategy targets downstream components of the NO signalling pathway by inhibiting PDE-5, the enzyme that catalyses the conversion of cGMP to GMP[164]. Sildenafil and tadalafil have been the lead substances in this group of agents, showing both acute and long-term beneficial effects in patients with PAH[165,166,167]. However, PDE-5 inhibition is not effective in all patients with PAH[168]; therefore, the full therapeutic potential of inducing the NO signalling pathway remains to be exploited.

The PGI_2 pathway has an established role in the pathogenesis of PAH[169]. This pathway is activated when PGI_2 stimulates the IP receptor, leading to increased

cyclic adenosine monophosphate and resulting in vasodilatory and antiproliferative effects[169]. PAH patients have reduced levels of endogenous PGI_2 and reduced expression of PGI_2 synthase in the lung[170,171]. Several PAH-specific therapies that target the PGI_2 pathway have been developed[169]. However, there are significant limitations associated with currently approved therapies, including inconvenient modes of administration (by continuous intravenous or subcutaneous injection or inhalation), short half-life and non-specific receptor interactions[172,173]. Currently, there are no licensed oral PGI_2 or PGI_2 analogues available in Europe or the USA. Parenteral prostanoids are available but their uptake has been poor, presumably because of difficulties with administration.

Finally, iPAH patients exhibit elevated levels of 5-HT which persist post heart-lung transplantation. This finding indicates a causal role for 5-HT in the pathogenesis of PAH[174]. 5-HT, stored primarily in the platelets, is a potent vasoconstricting agent and mitogen, which promotes smooth muscle cell proliferation. The mitogenic effects of 5-HT are mediated by the serotonin transporter, 5-HTT[175]. Indeed, chemical inhibition of 5-HTT results in a loss of 5-HT induced cell proliferation. 5-HTT is expressed at high levels on pulmonary artery smooth muscle cells (PASMCs) and increased production of 5-HTT in PASMCs is capable of triggering spontaneous PAH[176].

An interesting observation is that calcium levels in PAH smooth muscle cells have been shown to be elevated likely as a direct consequence of the observed reduction in transcript for potassium channels and low current in patients by comparison to controls. Intracellular calcium levels are regulated by voltage-gated potassium channels, which act by regulating membrane potential and controlling the release of calcium. Intracellular calcium is integral in driving contraction of PASMCs and promoting hypertrophy. The paucity of potassium channels has been posited as a potential explanation for the vasoconstriction and uncontrolled proliferation observed in patients[177].

Inflammation in PH

In recent years, great attention has been paid to PAH associated with systemic autoimmune diseases[178,179,180,181]. In these patients, important evidence of perivascular inflammation has been observed. Thus, there is a strong rationale to determine how immune-mediated vascular injury initiates and propagates alterations in metabolic function and in the phenotype of PAH vascular cells. Based on clinical and animal studies, there is now reason to suggest that advanced vascular remodelling may be reversed by approaches that address specific inflammatory and immune processes.

Pulmonary vascular lesions occurring in patients with PAH as well as in animal models of PH are characterised by varying degrees of perivascular inflammatory infiltrates, comprising T and B lymphocytes, macrophages, dendritic cells, and mast cells. Recently, correlations of the average perivascular inflammation score with intima plus media and adventitia thickness, respectively, and with mean pulmonary arterial pressure have been reported; these associations support a role for perivascular inflammation in the processes of pulmonary vascular remodelling[182].

The fact that inflammation precedes vascular remodelling in experimental PH suggests that altered immunity is a cause rather than a consequence of vascular disease[183]. Beyond increased perivascular immune cells accumulation and intravascular infiltration, circulating levels of certain cytokines and chemokines are abnormally elevated. These include IL-1β, IL-6, IL-8, MCP-1, CCL5/RANTES, and TNF-α. Some of these cytokines and chemokines correlate with a worse clinical outcome in PAH patients and may serve as biomarkers of disease progression. Some, such as IL-1β and TNF-α, have been related to an accumulation of extracellular matrix proteins such as fibronectin[184], observed in PAH lesions[185], and others, such as IL-6, have been related to the proliferation of smooth muscle cells[186]. Of particular relevance to my work and to autoimmune types of PAH is the finding that IFN is associated with the disease[187]. There are three types of IFNs, type I, type II, and type III, which operate via discreet signalling pathways and

specific receptors, all linked to STAT networks[188]. IFNs activate pulmonary vascular cells to release ET-1 and elevated levels of IFN correlated with other biomarkers and indicators of the disease, as does the ubiquitous surrogate of IFN signalling, IP10[187]. IP10 is released by all IFNs and has recently been implicated as a potential marker or mediator of PAH in its own right[187].

Recent investigations provide evidence that both pulmonary vascular cells and inflammatory cells are important local sources of chemokines and cytokines that can lead to pulmonary vascular remodelling in PAH. Indeed, the increased expression of cytokines and chemokines contribute to exaggerated contractility and proliferation of vascular cells. IL-1 can induce FGF2[189] and both FGF2 and IL-6 play an integral role in mediating the proliferative response in the smooth muscle-like cells and fibroblasts of the pulmonary vasculature[190,191,192,193,194,195] and contribute to the increased pericyte coverage in PAH. Several cytokines can directly control cell proliferation, migration, and differentiation of pulmonary vascular cells. IL-6 is prominent among these multifunctional proinflammatory cytokines and has been linked to the pathogenesis of PAH. Delivery of recombinant IL-6 protein in rodents is sufficient to cause pulmonary vascular remodelling and to exaggerate the pulmonary hypertensive response to chronic hypoxia[196,197]. Furthermore, IL-6-overexpressing mice spontaneously develop PH and pulmonary vascular remodelling and an obliterative form of remodelling in hypoxia resembling human disease, whereas IL-6-knockout mice are more resistant to the development of PH induced by chronic hypoxia[192,193]. IL-6 also induces pulmonary artery smooth muscle cell proliferation via induction of FGF2 by the transcription factor KLF-5[186]. TNF-related apoptosis-inducing ligand has recently been identified as playing a key role in apoptosis of endothelial cells and proliferation of smooth muscle cells in many PH experimental models and its inhibition is related to prevention of disease pathology[198]. In addition, osteoprotegerin, a protein regulated by serotonin and IL-1 signalling, is highly expressed in smooth muscle cells and serum of patients with PH[199] and can stimulate their migration and proliferation[200]. Recent data have demonstrated

that increased production of macrophage migration inhibitory factor plays a pivotal role in the pathogenesis of PAH[201]. Migration inhibitory factor is a critical upstream inflammatory mediator with pleiotropic actions partly explained by its binding to the extracellular domain of CD74. In endothelial cells, a migration inhibitory factor-CD74 interaction can lead to the activation of Src-family kinase, MAPK/ERK, PI3K/Akt, and NF-κB pathways, and to apoptotic resistance via repressed p53[202,203,204,205,206,207]. In addition, migration inhibitory factor can bind to CXCR2 and CXCR4 and lead to the proliferation of pulmonary artery smooth muscle cells and contribute to hypoxic PH[208,209,210,211]. In addition to elevated production of cytokines and chemokines, phenotypic alterations and functional defects in cytotoxic T and natural killer cells are linked to human PAH and experimental PH, as well as pulmonary veno-occlusive disease[212,213]. Recent data show deposition of complement C3 in idiopathic PAH patients and the protective effect of complement depletion in experimental models of PH[214], emphasizing the relevance of exploring complement-mediated vascular injury in the pathobiology of PAH.

Additional analyses of immunity in PAH support the notion that maladaptation of the immune response exists and may explain both the accumulation of perivascular inflammatory cells and the overabundance of cytokines and chemokines. Indeed, a delicate balance between immunity and tolerance exists and any disturbance may result in chronic inflammation or autoimmunity. Several types of autoantibodies directed against antinuclear antigens, endothelial cells, and fibroblasts have been found in idiopathic and systemic sclerosis-associated PAH[215,216,217,218]. These autoantibodies may play an important role in endothelial cell apoptosis and in the expression of cell adhesion molecules[219,220,221]. The role of T cells and more specifically of Treg cells in the control of self-tolerance is well-established[222,223,224] and altered Treg function has been demonstrated in patients with PAH[225]. Tregs not only control other T cells but also regulate monocytes,macrophages, dendritic cells, natural killer cells, and B cells; decreased Treg function may predispose individuals to PAH, as it does in animals. For

example, conditions associated with PAH, such as HIV, systemic sclerosis, systemic lupus erythematosus, Hashimoto thyroiditis, Sjögren syndrome, and the antiphospholipid syndrome, are characterised by abnormal CD4+ T cell number and function[226,227,228,229,230]. In animals with a congenital absence of T cells (athymic nude rats), vascular injury causes the lungs to become infiltrated with macrophages, mast cells, and B cells, similar to human PAH lesions, and disease is prevented with Treg reconstitution[183,196]. In experimental PH and clinical PAH, accumulation of immature dendritic cells in remodelled pulmonary arteries has been demonstrated, suggesting that they may contribute to PAH immunopathology[231]. Moreover, several autoimmune and infectious diseases such as systemic sclerosis, systemic lupus erythematous, HIV, herpes, and schistosomiasis infection are recognised causes of PAH. In many of these conditions, PAH is not reversible with the treatment of the causal disease[232,233,234]. The sequence of events from initiation or recurrence of inflammation to pulmonary vascular disease development remains unknown in idiopathic and even in autoimmune and infectious forms of PAH.

Recent attention has focused on the role of perivascular macrophages infiltrating pulmonary arterioles. The role of the macrophages in processes as diverse as limb or cardiac regeneration and pathogen defense indicates that it is important to comprehend the behavior of this cell in the context of the vascular milieu. CD68+ macrophages are prominent in advanced obliterative plexiform lesions observed in experimental and clinical PAH[235,236,237,238,239,240] and macrophage depletion or inactivation prevents PAH in several model systems, including experimentally induced hypoxic PH and portopulmonary hypertension[237,241]. Activation of macrophages is also closely linked to epigenetic changes that stimulate and induce proliferation of vascular fibroblasts in patients and in experimental models of PH[242]. These features involve HDAC1-mediated activation of a host of proinflammatory cytokines.

1.2 RELEVANCE OF SPHINGOLIPIDS TO RESPIRATORY DISEASES

1.2.1. METABOLIC AND CATABOLIC PATHWAYS

In origin, sphingolipids have been considered simple structural elements of the plasmatic membranes, but now it is clear that they participate in the regulation of various cellular functions[243]. Like glicerofosfolipids, sphingolipids are ubiquitous components of the mammalian cellular membranes and they can be metabolised to active molecules. One of the most important metabolites is represented by sfingosine 1-phosphate (S1P).

Ceramide, considered the scaffold of all the more complex sphyngolipids, can be either generated *de novo* at the cytosolic face of the ER or released from sphingomyelin by sphingomyelinases. Ceramide is further deacylated by ceramidase to sphingosine[244]. S1P is then generated from the phosphorilation of the sphingosine by two isoforms of the same kinase, the sphingosine kinase 1 and 2 (SphK1 and SphK2)[245] (see Figure 1.2). SphK1 is found in the cytosol of eukaryotic cells, while the SphK2 is mainly localised to the nucleus. SphK1 can be stimulated by a wide variety of growth factors, including EGF[246], VEGF[247], and other factors, such as TNF-α[248], estradiol[249], lysophosphatidic acid[250], and S1P itself[251]. These stimuli induce an activation ERK-dependent on the residues Ser225, Thr54, and Asn89 of SphK1 that leads to enhance the membrane affinity, selectively bind phosphatidylserine, and target the membrane, where its substrate is[252]. The regulation of SphK2 results in enzymatic activity and nuclear export. Recent reported evidence that EGF, the PKC activator, and phorbol ester[253] can phosphorylate SphK2 on Ser351 and Thr578[254]. PKD activates SphK2 leading to its nuclear export subsequent cellular signal[255]. SphK2 is thought to be involved in epigenetic regulation: indeed, in nucleus, SphK2 interacts with histone H3[256]. S1P regulates activity of HDAC1 and HDAC2; therefore, HDACs are direct intracellular

targets of S1P and the activity of SphK can regulate gene expression by S1P, such as cyclin-dependent kinase inhibitor p21 or the transcriptional regulator c-Fos.

The mechanisms for termination of S1P signalling are various: it can be either irreversibile metabolised by S1P lyase, leading to the formation of hexadecenal and phosphatidylethanolamine (see Figure 1.2), or dephosphorilated by one or more integral membrane lipid phosphohydrolases, including two specific S1P phosphatates (SPP1 and SPP2) and/or members of the LPP (lipid phosphate phosphohydrolases) family, back to sphingosine and recycled.

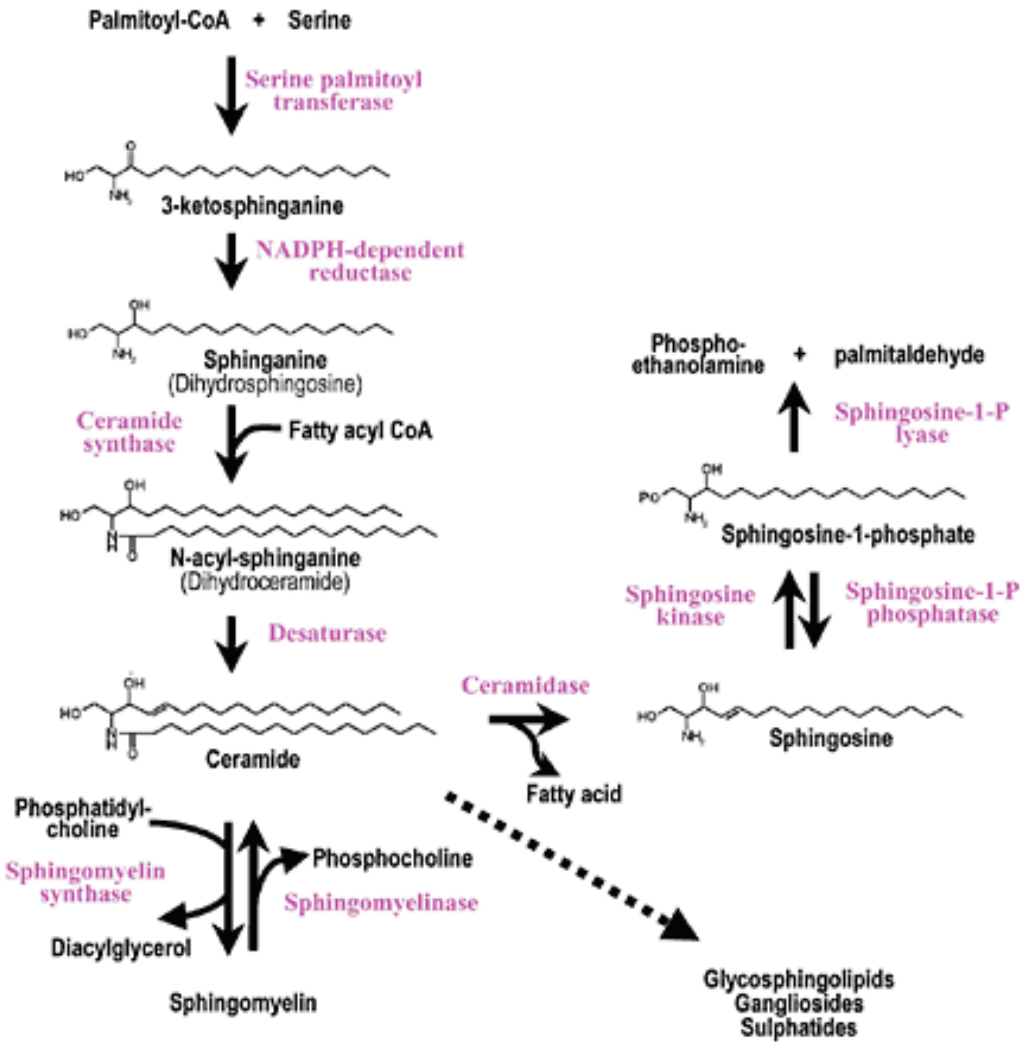


Figure 1.2. Catabolic and methabolic pathway of sphigolipids.

Because of the interconvertibility of ceramide, sphingosine, and S1P and the opposing effects on cell fate of ceramide and sphingosine (anti-growth, pro-apoptotic) compared to S1P (pro-growth, anti-apoptotic), the dynamic balance between S1P and ceramide/sphingosine has been proposed to form a ‘‘cellular rheostat’’ that determines cell survival or death[257].

S1P levels are finely regulated by the two specific kinases, by the enzymes that generate its substrate, the sphingosin, and by other enzymes, which have the role to degrade the phospholipid: thus, the levels of S1P in plasma are showed to be between 0.1 and 0.6 mM. Conversely, the amounts of S1P in tissues are very low. This concentration gradient plays an important role in driving S1P function: the S1P gradient at the tissue-vascular interface regulates the trafficking of immune cells from lymphoid organs into the circulation[258]. Since S1P is produced intracellularly, it is noting that it needs a transporter system to realise this concentration gradient. Several transporters have now been identified: these include the ABC transporters ABCA1[259], ABCC1[260], and ACG2[261], as well as the Spinster2[262]. This process is called “inside-out signalling” of S1P.
Once outside of the cell, S1P can bind to two known carriers, albumin or ApoM[263,264]. Approximately 35% of plasmatic S1P is bound to albumin and 65% to lipoproteins LDL, VLDL, and HDL. The bulk of lipoprotein-associated S1P is bound to HDL. This ApoM+HDL-bound S1P has been proposed as a primary contributor to the vasoprotective properties of HDLs[265]. How albumin or ApoM deliver S1P to specific S1PRs has yet to be characterised.

S1P acts through five different membrane receptors, belonging to the GCPRs, named $S1P_{1\text{-}5}$ (formerly known as EDG receptors), all with low nM K_d values, widely expressed in the whole organism. These receptors are associated to various subunits of heterotrimeric G proteins: G_i, G_q, $G_{12/13}$. This coupling results in the activation of small GTPases such as Rho, Rac, and Ras[266,267]. Further downstream effectors of S1P receptors include adenylate cylase, PI3K, PLC, PKC, and

intracellular calcium. By binding to its receptors, S1P triggers different actions, such as regulation of cellular growth[268] and suppression of apoptosis[269], suggesting a potential role in cancer. Through the activation of $S1P_1$, it can also regulate the trafficking of immune cells[270] and the development of blood vessels[271]; furthermore, S1P/$S1P_1$ axis is important in controlling the vascular tone and permeability, by regulating basal and inflammation-induced vascular leak[272]. $S1P_2$ and $S1P_3$ activate PLC and Rho[273]. Finally, T-bet-dependent $S1P_5$ expression is required for natural killer T cell egress from lymph organs[274], whereas $S1P_4$ plays a role in neutrophil trafficking[275].

Early studies demonstrated that S1P could induce calcium release from the ER, although no target has been conclusively identified[276]. Recently, this changed with the demonstration that S1P binds and alters the function of disparate intracellular proteins. Besides the effects of S1P on HDAC, which I have already introduced to you (see above), S1P binds the PHB2[277], modulates the activity of BACE1 in neurons[278], and is considered to be a cofactor of TRAF2[279].

1.2.2 S1P AND ASTHMA

Airway smooth muscle (ASM) cells function as the primary effector cells that modulate bronchomotor tone. However, beyond their contractile functions, ASM cells also contribute to the pathogenesis of asthma in various ways. Airway instillation of bacterial LPS into rat lungs enhances ICAM-1 expression in ASM cells[280]. Also, TNF-α induces ICAM-1 and VCAM-1 expression and may play a role in cell-cell interactions with other inflammatory cells, such as T cells[281], eosinophils[282], and mast cells[283]. Under the influence of inflammatory cytokines such as IL-6, ASM cells may be induced to undergo hyperplasia and hypertrophy and contribute ultimately to airway obstruction[284]. ASM cells can also secrete various inflammatory mediators such as GM-CSF and IL-5, which contribute to the proliferation and survival of eosinophils[285,286]. ASM cells play a prominent role in

the recruitment of inflammatory cells, as they have also been demonstrated to be a source of various chemokines such as eotaxin[287], MCP-1[288], and RANTES[289]. The initial clue that S1P plays a role in asthma came from the finding that S1P levels were elevated in the BAL fluid of asthmatics, but not healthy controls, after antigen challenge[290]. Indeed, the levels of S1P in the lavage fluid directly correlated with eosinophil numbers, another hallmark of asthma. This initial finding suggested that S1P might play a role in both acute bronchoconstriction and airway remodelling through its direct action on ASM cells[290]. Indeed muscarine-induced peripheral airway constriction was reduced by inhibition of SphK using DHS (D,L-threo-dihydrosphingosine) and DMS (N,N-dimethylsphingosine), suggesting that the SphK/S1P signalling pathway contributes to cholinergic constriction of murine peripheral airways[291]. Based on a model of human ASM cells embedded in collagen matrices, Rosenfeldt *et al.*[292] demonstrated that S1P induced the formation of stress fibers and the contraction of individual ASM cells and stimulated myosin light chain phosphorylation in a Rho-kinase-dependent manner. In another study using guinea pig tracheal smooth muscle strips, pretreatment with S1P markedly enhanced methacholine-induced contraction[293]. This effect was inhibited in the presence of a Rho-kinase inhibitor. Exogenous addition of S1P to human bronchial smooth muscle cells increased the level of active RhoA (GTP-RhoA) and phosphorylation of MYPT1. These findings demonstrate that exposure of ASM cells to S1P results in airway hyperreactivity mediated via RhoA and inactivation of myosin phosphatase[293]. In addition, Roviezzo *et al.*[294] evaluated the effects of exogenous S1P administration on isolated bronchi and whole lungs harvested from BALBC/c mice sensitised to ovalbumin (OVA) by measuring bronchial reactivity and lung resistance. They found that, in OVA-sensitised mice, S1P caused a dose-dependent contraction of isolated bronchi and increased airway resistance in the whole lung system. This was accompanied with an enhanced expression of SphK1, SphK2, $S1P_2$, and $S1P_3$[294].

Another important hallmark of chronic asthma is airway remodelling, characterised in part by increased smooth muscle mass, extracellular matrix deposition, and vasculogenesis. S1P is likely involved in all these processes. First, S1P promotes the growth of both human ASM cells and fibroblasts[295,296]. Moreover, S1P produced by SphK1 has recently been shown to be involved in fibroblast differentiation into myofibroblasts[297], cells thought to be responsible for thickening of the *lamina reticularis*. TGF-β and S1P, which are both increased in asthmatic lung, can induce α-SMA expression in lung fibroblasts, a hallmark of their differentiation into myofibroblasts[297]. Subsequently, it was shown that TGF-β stimulates SphK1, which in turn releases S1P that activates $S1P_2$ and $S1P_3$ in an autocrine manner, leading to α-SMA expression[298].

Mast cells (MCs) are tissue resident effector cells best known for their role in atopic diseases. However, these cells can alter both innate and acquired immune responses to various stimuli, by releasing a plethora of vasoactive mediators, proteases, chemokines, and cytokines that collectively enhance vascular permeability, elicit bronchoconstriction, and incite recruitment and function of leukocytes. The end effects range from local to systemic inflammation[299]. MCs develop in peripheral tissues from bone marrow-derived circulating committed progenitors, with two subpopulations that vary in the composition of their intragranular proteases: those expressing tryptase only are designated MC_T and predominate in lung, while those that also contain chymase, carboxypeptidase, and cathepsin G are termed MC_{TC} and predominate in skin. The concentration of MC_{TC} cells in ASM in asthmatics increases dramatically and correlates with bronchial hyperreactivity[283]. The presence of IgE antibodies to innocuous antigens causes dysregulation of this normally protective response, resulting in allergic inflammatory disease. Crosslinking of FcεRI on MC activates many downstream signalling molecules, including Src family tyrosine kinases, the Syk tyrosine kinase, calcium flux, PKC, and the Ras-MAPK pathway. The result is a biphasic response characterised by degranulation with release of histamine and other preformed mediators and the subsequent *de novo* synthesis and secretion of cytokines and

chemokines as well as the arachidonic acid metabolites, leukotrienes and prostaglandins. Crosslinking of the FcεRI also induces activation of SphK1, production of S1P, and subsequent activation of the $S1P_1$ and $S1P_2$ receptors[300]. Activation of $S1P_2$ is required for mast cell degranulation, while $S1P_1$ activation is important for their migration to the sites of inflammation. At least in mast cells, S1P does not appear to be released by degranulation of stored mediators, rather *de novo* formed S1P is secreted, at least in part, through ABCC1, a member of the ATP-binding cassette transporter family[260].

It has been suggested that the decisive balance between sphingosine and S1P is a determinant of the allergic responsiveness of MCs[301]. Whereas sphingosine inhibits antigen-mediated leukotriene synthesis and cytokine production by preventing activation of ERK1/2, S1P activates ERK1/2, stimulating degranulation and cytokine production[301]. Thus, SphK plays a pivotal role in MC activation by modulating the balance of these counterregulatory sphingolipid signalling molecules. Moreover, expression of MCP-1, MIP1-α, MIP1-β, MIP2 (belonging to the CC-chemokine family), and MIF, all important modulators of monocyte and eosinophil recruitment and inflammation, was significantly increased by S1P in MCs[300]. Thus, secretion of S1P by MCs can also promote inflammation by activating and recruiting other immune cells involved in allergic and inflammatory responses, including eosinophils[302] and Th2 lymphocytes[303]. Controversy surrounding the role of SphK1 and SphK2 in MCs has also been documented. Olivera *et al.* reported that SphK2 regulated mast cell activation, whereas SphK1 enhanced susceptibility to antigen challenge *in vivo* in knockout mouse models[304]. Taken together, these data suggest a key role for SphK and/or its product S1P in the responses triggered by activated mast cells.

1.2.3 PEPDUCINS

GPCRs are ones of the most important targets on which the basic pharmacological research is based: GPCRs are membrane receptors that are central in almost every signalling pathway in the human body, such as inflammation, neurotransmission, and hormone signalling. Nearly one in three commercialised compounds are designed to modulate GPCR functions. However, as researchers become more and more aware of the additional properties of this receptor superfamily, they also realise how much is not yet understood about receptor function and potential unexploited properties that could reveal new pharmacological approaches to the design of the drugs of the future.

Pepducins were developed in the late 1990s by scientists at the Tufts Medical Center: at that time, they started to look at ways to target the receptors from the inside and not from the outside surface of the cells. As pepducins can theoretically act as either agonists or antagonists, they considered interesting tools for studying receptor signalling and mechanisms in cell-based systems, as well as basis for developing new attractive drugs supporting a new mechanism of action.

Pepducins are molecules characterised by a lipid tag attached to the N-terminal of a sequence of peptides designed over the sequence of the third intracellular loop (i3) of the 7-transmembrane receptor. Although mutagenesis studies have demonstrated that the i3 loop of GPCRs largely mediates coupling between the receptor and G protein[305], further studies have showed that certain pepducins based on the i1, i2, or i4 loops could also act as agonists or antagonists of receptors, suggesting that all intracellular domains may be important for intracellular signal transduction. The four intracellular loops of GPCRs all directly interact with heterotrimeric G proteins that are composed of α, β, and γ subunits[306].

The lipid tag is essential to the proper operation of the pepducins: the lipid, usually a palmitoyl or mirystoyl residue, acts as an anchor to the outer surface of the plasmatic membrane. Biochemical and pharmacologic studies indicate that the

lipid moiety of pepducins facilitates translocation across the lipid bilayer and serves to tether the pepducin in the cytosolic face of the plasma membrane in the vicinity of the target receptor, thereby increasing the effective molarity[307]. Once flipped across the membrane, the pepducin can interact with its cognate receptor and either block or stimulate signalling to associated G proteins[308]. Figure 1.3 is a schematic example how pepducins work.

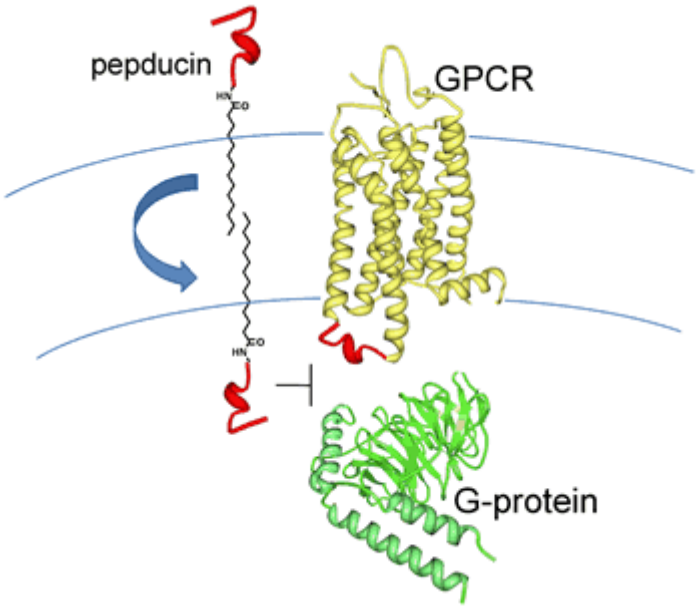


Figure 1.3. Schematic mechanism of function of a pepducin.

However, it is well recognised that GPCRs assume heterogeneous conformations from resting state to full activation and that the ionic lock between TM3 and TM6 is destabilised to selectively activate G protein. Therefore, it is highly possible that pepducins may act also by stabilizing the GPCR/G protein complex in specific on- or off-conformation, possibly by mimicking a receptor dimer[309].

Classically, GPCRs are activated by extracellular ligands. Intracellular pepducin agonists have been generated against more than a dozen receptors, including PAR1[308], PAR2[308,309], MC4R[308], CXCR4[310], $S1P_3$[311], and FPR2[312]. After the initial development of agonist pepducins, antagonist pepducins were subsequently described for the thrombin receptors PAR1 and PAR4 and most recently for the tryptase/trypsin PAR2 receptor.

1.3 GASOTRANSMITTERS AND LUNG PATHOPHYSIOLOGY

Nitric oxide, carbon monoxide, and hydrogen sulphide are the major and the most studied representatives of the class of gaseous transmitters. It is demonstrated that they have effects on lungs and airways, in both pathological and physiological conditions. A short introduction to carbon monoxide and nitric oxide follows, in order to clarify their characteristics and possible interactions with the hydrogen sulphide.

Carbon monoxide

Carbon monoxide (CO) is a colourless and odourless gas, generally produced during incomplete combustion of compounds containing carbon. It is a poisonous gas and exerts its noxious effects mostly because it can disrupt normal mitochondrial respiratory functions[313].

Synthesis of CO in living organisms is run by heme oxygenase (HO) and is linked to heme catabolism. In the initial stage, the heme is oxidised to alpha-metahydroxyheme. In the next stage, it reacts with a molecule of oxygen leading to generation of verdoheme and CO. Verdoheme is finally converted to biliverdin, which by the action of biliverdin reductase produces bilirubin (a highly effective antioxidant)[314]. There are three isoforms of HO: HO-1 (inducible isoform, also known as Hsp32), HO-2 (constitutive isoform), and HO-3 (constitutive isoform). They are the products of the expression of three different genes. HO-1 is a 32 kDa protein, one of the thermal shock proteins. HO-1 is cytoprotective against oxidative stress. The oxidative stress and a low level of glutathione lead to increased expression of the HO-1 gene. In addition, the expression of HO-1 also may be induced by a high level of heme, hypoxia or elevated body temperature, heavy metals, and ultraviolet radiation[314]. HO-2 is a constitutive enzyme with a mass of 36 kDa. The highest activity of the enzyme is detected in brain. It catabolises hemoproteins and generates CO, which functions as a signalling molecule in the nervous system, as well as in the circulatory system. Depolarization of neurons leads to increased activity of HO-2 and synthesis of

CO[314]. HO-3 (33 kDa) has a significant homology to HO-2, but its activity is low. In cells, HO-3 is a heme-binding protein and regulates production of free radicals[314,315].

CO has the ability to bind heme groups: it has about 245 times greater affinity for hemoglobin and myoglobin than oxygen. However, it should be noted that CO interference with oxygen transport in blood due to its binding to heme iron centres in hemoglobin is not a major contributor to CO toxicity, but rather the increase in CO tissue concentration leading to disruption of mitochondrial function[313]. For this capacity, one of the targets of CO is guanylyl cyclase (GC): CO can activate GC and lead to production of cGMP, even though to a lesser extent with that observed with nitric oxide. CO may be associated with other hemoproteins and inhibit their activity, *i.e.* cytochrome P450. This inhibition induces reduction of the synthesis of 12-HETE, which significantly narrows the lumen of blood vessels. CO also activates cyclooxygenase and increases PGE_2 synthesis[314]. Other targets of CO include nuclear hormone receptors[316]. The activity of various ion channels, *i.e.* BKC, TREK-1, P2XR2, or P2XR4, may be modulated by CO application, but the mechanisms by which CO regulates these channels are still unknown. Some experiments suggest that a limited range of amino acid residues confers CO sensitivity, either directly or indirectly, to particular ion channels and that cellular redox state appears to be important to the final response[317].

Similarly to other gasotransmitter, CO is a regulatory molecule, which impacts on physiological and pathological functions. CO exerts its principal effects on cardiovascular system: indeed, CO induces relaxation of smooth muscle in blood vessels, which increase their lumen. In addition, CO regulates blood pressure[318,319]. It has a positive influence in atherosclerosis, in which CO synthesis is increased. This is the effect of the increase of HO-1 expression in endothelial cells and foam cells of atherosclerotic lesion. Furthermore, an increase in the expression of HO-1 may be induced by oxidised LDL, peroxynitrite ($ONOO^-$), PDGF, TGF-β, and angiotensin II, as well as by heme from damaged cells. CO has an antiapoptotic

activity via a number of cellular mechanisms and this activity results in its protective role in atherosclerosis. CO inhibits apoptosis of blood vessel smooth muscle cells by activation of the sGC. Moreover, CO inhibits the expression of p53 that regulates transcription of many proteins, *i.e.* Bax. Activation of Bax by p53 makes the mitochondrial membrane permeable for cytochrome c, which flows from the mitochondria to the cytoplasm and activates a signalling cascade that leads to cell death[320]. Antiapoptotic activity of CO is also associated with increased activity of HO-1. HO-1 reduces the bioavailability of iron ions with pro-oxidative action engaged in the ROS production. ROS play an important role in the signal cascade leading to programmed cell death[321]. Moreover, CO maintains high levels of expression of antiapoptotic proteins such as Bcl-2 or Bcl-xL[322.] It is known that CO and HO-1 inhibit cell cycle in the G_0/G_1 phase. This inhibition is associated with a decrease in the cyclin-dependent kinase 2 activity (Cdk2). Cdk2 is responsible for transition from G_0 to G_1 and G_1 to S phase, because it is responsible for cyclin A and E phosphorylation. Ability of CO to inhibit Cdk2 activity is due to the expression of proteins, *i.e.* activators of Cdk2 and cyclin A and D1. On the other hand, CO increases the expression of Cdk2 inhibitor – a protein p21[319]. The antiinflammatory role of CO is based on the inhibition of the expression of LPS-dependent proinflammatory cytokines. These cytokines include TNF-α, IL-1β, and MIP-1β. On the other hand, CO induces expression of the antiinflammatory cytokine IL-10. In addition, CO inhibits the release of nitric oxide produced by the inducible nitric oxide synthase. Finally, CO inhibits the release of histamine from mastocytes and prevents the activation of basophils[323].

Nitric oxide

In 1980 Furchgott and Zawadzki demonstrated that, in response to muscarinic agents, the endothelium released a substance that relaxed blood vessels: the chemical nature of this substance was unknown and was named with the generically term of endothelium-derived relaxing factor (EDRF)[324]. It was only with

the independent work of Ignarro and Moncada that the EDRF could be identified as nitric oxide (NO), the first gasotransmitter discovered[161,325].

The NO is a free radical, but relatively stable compared to other radical species: in fact, it could diffuse over micron of distance before reacting with other molecules. As a small uncharged molecule, it is higly soluble in hydrophobic environment and can easily pass across biological membranes.

The chemical formule of NO is reported:

$$\cdot N{=}O$$

NO is synthesised intracellularly through the action of nitric oxide synthase (NOS) enzymes. NOS enzymes catalyze the NADPH- and O_2-dependent oxidation of L-arginine to L-citrulline and NO. Enzymatic synthesis of NO is complex and depends on the availability of prostetic groups and cofactors such as FAD, FMN, tetrahydrobiopterin, and heme[326]. Three distinct isoforms have been identified, all of which have different characteristics and generate NO at different rates, but they are all sterospecific and active as homodimers[327]. Endothelial NOS (eNOS or NOS3) generates the lowest levels of NO and was originally discovered in the vascular endothelium but it is also found in neurons, epithelial cells, and cardiac myocites[328]. Its location within the cell and activity are controlled by Ca^{2+} ions and calmodulin. Regulation of eNOS is also intimately related to the physical forces important in vascular function such as shear stress induced by blood flow[327,329]. Neuronal NOS (nNOS or NOS1) is constitutively present in neurons, skeletal muscle, and epithelial cells. It is also a Ca^{2+}/calmodulin-dependent isoform[327]. The last major NOS isoform is inducible NOS (iNOS or NOS2), which has the highest capacity to generate NO. This isoform is expressed in multiple cell types in response to inflammatory stimuli, such as those induced by endotoxin and cytokines, *e.g.* IL-1 and IL-2, TNF-α, and IFN-γ[327,330]. It has also been shown to be constitutively present in some tissues such as lung epithelium. The NOS enzymes are regulated by post-translational modifications, controlled localization within

the cell, substrate and cofactor availability, and other proteins with which they form stable complexes[327].

Once generated, NO has a number of potential biological fates. The intra- and extra-vascular half-lives of NO, which have been measured to be in the range of 2 ms to >2 s, appear to be dependent on the availability of intracellular reactants of NO[331]. The most specific and highest affinity interactions of NO with biological targets are those with metalloproteins such as sGC, CcOX, and hemoglobin.

NO reacts rapidly with other free radicals such as O^{2-} or lipid peroxyl radicals[332,333]. The best studied of these reactions is the very rapid reaction of NO with superoxide to form peroxynitrite[334]. For example, peroxynitrite (or reactive species derived from it such as nitrogen dioxide and carbonate anion radical) can mediate the oxidation and nitration of proteins, lipids, or DNA, whereas NO cannot directly mediate any of these effects. Since the realization that peroxynitrites are produced in biological systems, they have been proposed to be the major mediators of the pathological effects associated with NO, particularly inflammation[335]. Peroxynitrite reacts directly with sulfur-containing amino acids (cysteine and methionine), as well as those with aromatic structure (*e.g.* tryptophan and tyrosine). Furthermore, secondary products of peroxynitrite oxidation can also modify proteins. Although the rate constant for reaction of peroxynitrite with free cysteine is 5.9×10^{3} $m^{-1}\cdot s^{-1}$, reaction with protein thiols is much slower. This may then contribute to the selectivity in protein targets. Additionally, because NO is freely diffusible and O^{2-} is not, one of the factors controlling peroxynitrite reactivity may be the location of O^{2-} production.

Nitrites and nitrates are higher oxidation states of NO that were previously believed to be stable oxidation end-products of the redox sequence. However, Recent work suggests that these oxidised species can be recycled *in vivo* in a process that involves oral microbiota and foodstuffs. Oral bacteria can reduce dietary nitrate to nitrite, which, upon entering the acidic milieu of the stomach, undergoes non-enzymatic reductive conversion to NO. In parallel, nitrate and nitrite in blood that originates from the diet and from systemic production and

oxidation of NO can be taken up actively by the salivary glands, whereupon it enters the nitrate reductase-rich bacterial milieu of the oral cavity facilitating the cycle[336].

There are essentially two major biochemical mechanisms under current investigation through which NO can mediate signal transduction. The first is the ability of NO to bind ferrous heme. The reaction of NO with metalloproteins involves nitrosylation of ferrous iron (Fe^{2+}). However, ligand discrimination between metalloproteins differs widely because of the effects of the protein structure. For instance, sGC does not bind O_2 but does form 5-coordinate Fe^{2+}·NO complexes, which subsequently activate the enzyme and are responsible for the cGMP-dependent effects of NO. In contrast, hemoglobin and CcOX have different affinities for NO but also bind O_2[337]. The second is correlated to the capacity of NO to induce modifications on proteins. Cysteinyl thiols are targets of NO and NO from exogenous and endogenous sources has been shown to increase intracellular low molecular weight S-nitrosothiols (*e.g.* S-nitrosoglutathione), promote protein glutathiolation (protein S-glutathione), and increase thiol oxidation[338]. However, the different modifications induced by NO appear to be dependent on the RNS, the protein itself, and the subcellular localization of the protein. Recently, S-nitrosothiols have attracted interest because of i) their potential roles as intermediates in the transport, storage, and delivery of NO; ii) as post-translational protein modifications in cell signalling and inflammation; and iii) as molecular markers of RNS. Well over 100 proteins have been shown to be S-nitrosated and, as the list of S-nitrosated proteins grows, the potential relevance of this modification and other NO-induced protein modifications in redox biology and signalling is of increasing interest.

Besides the well-known activities of NO in inflammation and nervous system, Barnes[339] first suggested that NO produced by nNOS has beneficial effects in asthma, causing bronchodilation by relaxing ASM. However, NO produced by eNOS can induce vasodilation in the arterioles, with plasma extravasation and edema. High quantities of NO produced by iNOS can result in vasodilation, plasma

extravasation, increase in mucus secretion, and indirect activation by Th2 cells (mainly due to eosinophilic recruitment) to contribute to asthma physiopathology. However, the role of NO in asthma is not as simple as it has been initially described because several *in vitro* and *in vivo* studies with animal experimental models and humans have showed different results. Some authors suggested that NOS activation could exert both beneficial and detrimental effects, such as altering relevant aspects of asthma pathology and participating in inflammatory cell recruitment[340], airway responsiveness[341], and airway remodeling[342]. NO was detected in the exhaled air in normal humans, but not in animals[343]. Various researchers demonstrated that the exhaled NO (eNO) concentrations were increased in asthmatic patients[344,345] and in experimental models of chronic pulmonary inflammation[346,347]. Some authors suggested that the increase in eNO can be due to an increase in iNOS expression[348]. The eNO has been proposed as an indirect marker of inflammation to be used in clinical practice. However, the efficacy of these measurements is still a matter of controversy.

1.3.1 HYDROGEN SULPHIDE

Hydrogen sulphide (H_2S) has been considered for a long time as a mere toxic compound with the characteristic smell of rotten eggs. In the last decades, the hypothesis that H_2S could act as a transmitter within the body has risen up, because of the observation that it was endogenously produced in animal tissues. Nowaday, it is widely accepted that H_2S belongs to the class of gaseous transmitter, along with NO and CO.

Physical and chemical properties

This inorganic sulphur compound is a colourless flammable gas that is heavier than air (d=1.19). H_2S is the sulphur analogue of water, but the only similarity with it is the molecular structure. The sulphur atom is not as electronegative as oxygen so

that molecules of H_2S cannot form hydrogen-bounds among them. Because of this, its melting and boiling points are much lower than those of H_2O. Hydrogen sulphide is a very lipophylic compound that can easily pass across biological membranes, also due to its very small dimensions.

H_2S is a weak acid and in water and rapidly dissociates in HS^- and S^{2-}, according to the equilibrium:

$$H_2S <-> HS^- + H^+ <-> S^{2-} + H^+$$

The dissociation constants are respectively $K_{a1}=1.3\times10^{-7}$ M and $K_{a2}=1\times10^{-19}$ M[349] (20°C) and they are both temperature-sensitive: this can have a significant effect on the relative concentrations of H_2S and HS^- when comparing experiments performed at room temperature to those performed at body temperature (37°C). Thus, under physiological conditions (pH=7.40), only one-third of H_2S is in the undissociated form and the amounts of the undissociated form are even lower (approximately one-fifth) when experiments are run at 37°C[350].

The amount of S^{2-} is often irrelevant because it is present only at very high pH values.

H_2S is also relatively unstable in solution. H_2S is spontaneously oxidised in the presence of oxygen and metal catalysts, such as ferric iron, and the concentration of H_2S can be halved in as little as 3 hours. Addition of an iron chelator such as diethylenetriaminepentaacetic acid (DTPA) can greatly delay spontaneous oxidation; however, this can also remove ions that are vital in most biological processes.

The obnoxious smell of H_2S, which is familiar to anyone working with sulphide salts, hints at an even greater threat to proper experimental design and that is the volatility of H_2S. Even in the absence of cells, half of a dose of H_2S can be lost from open tissue culture wells in 5 minutes. This time is further reduced in bubbled

tissue baths to around 3 minutes and it is even less in the Langendorff heart apparatus that is used to study cardiac function[351].

H_2S also has reductive properties and in redox reactions with weak oxidizing agents it is oxidised to elementar sulphur, as follows:

$$H_2S(g) + 2M^{3+} \rightarrow S_0(s) + 2H^+ + 2M^{2+} + 2e^-$$

Metabolic and catabolic pathways

H_2S is endogenously produced in mammalian tissues from the sulphurated amino acid L-cysteine. The reaction is driven by two pyrydoxal 5'-phosphate-dependent enzymes: cystathionine γ-lyase (CSE or CTH) and cystathionine β-synthase (CBS)[352.] Recently, a new enzyme involved in H_2S synthesis has been discovered, the 3-mercaptopyruvate sulfurtransferase (3-MST) (see Figure 1.4). CBS and CSE are cytosolic enzymes, whereas 3-MST is predominantly localised to the mithocondrial matrix. CBS was originally considered to be the predominant enzyme for H_2S production in the brain, whereas H_2S synthesis in the heart and vasculature was attributed to CSE[353]. In some tissues, CSE and CBS are both required for H_2S synthesis, whereas in others only one of these enzymes is necessary[354]: indeed, recent studies with improved markers have provided a broader picture of enzyme distribution. Recently, CBS and CSE were identified in human plasma, where they were proposed to reduce circulating H_2S.

Hydrogen sulphide is rapidly oxidised, mainly in mitochondria, initially to thiosulfate and subsequently to sulfite and sulfate. The former oxidation is not enzymatically driven, while thiosulfate conversion to sulfate and/or sulfite is catalyzed by thiosulfate cyanide sulfurtransferase (TST). In addition, sulfite originating through this reaction is quickly oxidised to sulfate, as sulfate is the major end-product of H_2S metabolism under physiological conditions. Another catabolic pathway is represented by methylation by thiol-S-methyltransferase

(TSMT) to methanethiol and dimethyl sulphide. This reaction occurs mainly in cytosol. Methemoglobin, considered a "common sink" for endogenous gases including CO and NO, also binds H_2S as sulfhemoglobin[355] (see Figure 1.4).

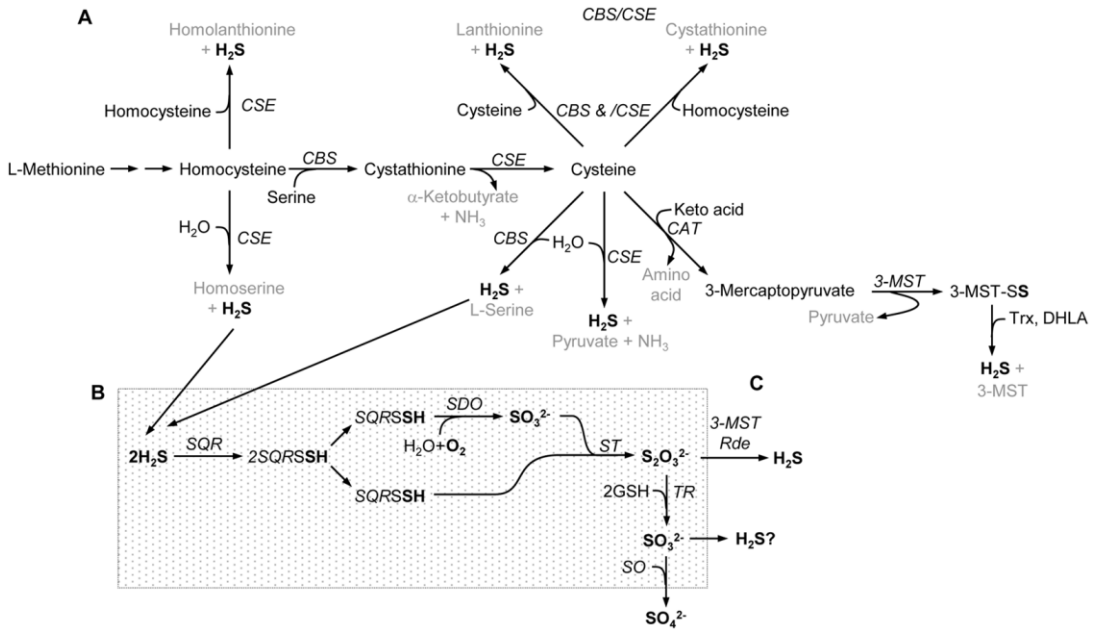


Figure 1.4. Catabolic and metabolic pathways of H_2S.

Toxicity

Like NO and CO, at higher concentrations H_2S is a toxic gas, but its toxicological properties are of low interest because intoxication by H_2S is restricted to infrequent cases of occupational exposure from industrial activities. It is thought that H_2S may target lots of organs and tissues, primarily brain and respiratory system. Table 3 reports the effects of H_2S on humans.

Table 3. Toxicity of H_2S.

Concentration of H_2S		Physiological responses
ppm	mg/m^3	
0.003-0.02	0.042-0.028	Odour threshold
3-10	4-14	Obviously unpleasant odour
20-30	28-42	Strong offensive odour
30	42	Sickening sweet odour
50	70	Conjunctival irritation

50-100	70-140	Irritation of respiratory tract
100-200	140-280	Loss of smell
150-200	210-280	Olfactory paralysis
250-500	350-700	Pulmonary oedema
500	700	Anxiety, headache, ataxia, dizziness, stimulation of respiration, amnesia, unconsciousness
500-1000	700-1400	Respiratory paralysis leading to death, immediate collapse, neural paralysis, cardiac arrhythmias, death

(adapted from Reiffeinstein *et al.*, 1992)

The toxicity of H_2S is not due to the gas itself, but to the capacity of blocking cytochrome c oxydase and forming persulphides[356].

Measurement of the concentration of thiosulfate in blood and urine is useful for determining hydrogen sulphide poisoning[357].

Study of H_2S biology

Researchers have always faced difficult challenges in the study of H_2S biology. As a toxic and volatile molecule, its use for scientific purposes has always been limited. In order to bypass those difficulties, different approaches have been used. Currently, the strategies for delivering H_2S in biological systems take advantages of molecules that release H_2S in solution. These include salts of HS^- or S^{2-} species (especially sodium salts) and synthetic or natural H_2S-releasing molecules, such as GYY4137 or diallyl disulphide (DADS) and diallyl trisulphide (DATS). The progress in the gasotransmitter research field has clarified the pathways leading to H_2S synthesis and more tools are now available. These are represented by inhibitors of the enzymes CBS, CSE, and 3-MST, such as aminooxyacetic acid (AOAA), D,L-propalgylglycine (PAG or PGG), or β-cyanoalanine (BCA), but unfortunately they are not high-specific and some of them are unstable. The creation of CBS- and CSE-knockout mice represents a more useful tool for the study of H_2S involvement in physiologic and pathologic conditions.

The last challenge is represented by the methods used for the quantification of H_2S levels in biological fluids or its production from tissue homogenates. The most common is the methylene blue assay, but it has intrinsic limitations. Apparently more precise methods include the monobromobimane method and sulphide-selective electrodes. All these methods are extensively reviewed by Nagy *et al.*[358].

H_2S signalling pathway

Unfortunately, H_2S signalling is not completely and fully understood. Unlike NO and CO, it does not seem to activate GC, even though it can bind heme groups.

Therefore, H_2S can signal in a similar way to that observed with NO: H_2S is able to form a covalent bond on cysteine residues of proteins, a mechanism called "sulfhydrilation", parallel to the "nitrosylation" process run by NO.

Sulfhydration appears to be substantially more prevalent than nitrosylation. Whereas nitrosylation typically affects only about 1-5% of most proteins, 10-25% of endogenous GAPDH, β-tubulin, and actin are basally sulfhydrated[359]. Unlike nitrosylation that provides a NO "cap" on the reactive nucleofilic group of cysteines inactivating proteins, sulfhydrilation puts an -SSH group on it, which has a lower pK_a than -HS group solely and thus make them more reactive.

Actions of H_2S

Since the discovery that H_2S could act as a gaseous transmitter, a large amount of papers regarding the H_2S involvement in several districts has been published. Here are listed the most relevant ones to our interest.

Cardiovascular system

Like NO and CO, H_2S affects cardiovascular system. Its effects are predominantly vasorelaxation, but under some conditions, such as high oxygen concentrations, it can constrict vessels as well. H_2S has the ability to relax the rat thoracic aorta, portal vein, and mesenteric artery, which suggests a more fundamental role in the regulation of contractility and blood pressure[360,361,362]. Conversely, other studies

have suggested that H_2S is a vasoconstrictor at low concentrations with a possible mechanism for suppressing NO, which, since NO is a dilator, could partially explain the contractility effects of H_2S. One of the most important pieces of evidence for the involvement of H_2S in regulation of vascular tone is the finding that CSE knockout mice develop age-dependent hypertension similar to that observed in eNOS deficient mice[363]. H_2S seems to activate K_{ATP}, at least in the cardiovascular system, by sulfhydrating the channel at Cys43 and the subsequent hyperpolarisation elicited by opening K_{ATP} channels leads to relaxation of smooth muscle cells[364]. Evidence of this hypothesis comes from the observation that glibenclamide, a potent and selective inhibitor of the K_{ATP} channel, reduces the effects of H_2S.

Before endogenous H_2S was shown to regulate blood vessel tone, exogenous H_2S had been shown to exert beneficial cardiovascular actions. Many studies have dealt with myocardial ischemia, which is substantially diminished by administration of H_2S-donors during ischemia/reperfusion of the heart[365,366]. Numerous mechanisms had been proposed for these cardioprotective actions[367,368]. Particularly promising is the evidence that H_2S inhibits apoptosis, as H_2S-donors reproducibly diminish poly(ADP-ribose) polymerase (PARP) cleavage, as well as cleavage of caspase-3[369]. H_2S also preserves mitochondrial structure and function in response to myocardial ischemia. H_2S may also be cardioprotective by decreasing the “work” of the heart, analogous to β-blockers, through diminishing contractility of cardiac myocytes, largely by inhibiting L-type calcium channels[370].

Inflammation

Literature reports very conflictive effect of H_2S on inflammation. Prominent proinflammatory effects have been reported in association with increased formation of sulphide in neutrophils as well as activation of these cells[371]. Administration of H_2S-donors seemed to accentuate inflammatory factors associated with burns, while burn injuries were reduced by treatment by the CSE inhibitor propargylglycine[372]. Lung injury elicited by bacterial sepsis can be

alleviated by treatment with propargylglycine and worsened with H_2S-donors[371]. By contrast, there are numerous reports of antiinflammatory effects for H_2S-donors as described below. A consensus has emerged in recent years that the apparently contradictory findings largely reflect variations in dose-response relationships for H_2S under different experimental conditions. At relatively low physiologic concentrations, H_2S appears to be antiinflammatory, while high concentrations elicit inflammation, a pattern reminiscent of NO, which is antiinflammatory in low concentrations and proinflammatory at high levels. CO, well known to be lethal in high doses, is also often beneficial when administered in low doses.

What physiologic mechanisms underlie influences of H_2S on inflammation? One of the best characterised involves the disposition of leukocytes, especially their adherence to vascular endothelium as well as their extravasation. H_2S-donors and sulphide salts diminish lymphocyte and neutrophil infiltration in models of inflammation, whereas inhibitors of H_2S biosynthesis increase leukocyte adherence[373]. H_2S-donors diminish edema, presumably due to inhibition of plasma exudation, while CBS and CSE inhibitors increase the formation of edema in response to inflammatory stimuli[373]. A molecular mechanism underlying antiinflammatory roles of H_2S may include its scavenging peroxynitrite, a toxic derivative of NO, as well as other oxidants[374].

H_2S has been shown to exert beneficial influences in disorders of joints, including resolving synovitis in rodents[375] and alleviating the pathology of carageenen-associated arthritis[376]. H_2S-donors also have been extensively explored in intestinal disorders, with beneficial effects in several models of colitis[377].

H_2S may participate in some actions of TNF-α. While TNF-α is regarded as proinflammatory, it does display antiapoptotic actions mediated via NF-κB. The antiapoptotic actions of NF-κB appear to be mediated by H_2S generated by CSE[378]. TNF-α treatment triples H_2S generation by stimulating the binding of the transcription factor SP1 to the CSE promoter. H_2S generated by CSE enhances the binding of NF-κB to promoters of downstream genes, whose signalling is markedly

diminished in CSE knockout mice. H_2S acts by sulfhydrating the p65 subunit of NF-κB, which promotes its binding to the co-activator ribosomal protein S3 (RPS3). The antiapoptotic influences of NF-κB are substantially reduced in CSE deleted mice[378]. The antiinflammatory influences of H_2S have led to efforts to develop therapeutic agents. Classic non-steroidal antiinflammatory drugs (NSAIDs) often cause gastric irritation by inhibiting the formation of prostaglandins, which are physiologic cytoprotectants of the gastric mucosa; H_2S, on the other hand, reduces mucosal inflammation, protects the gastrointestinal mucosa from injury, and augments tissue repair. One specific example of this is a H_2S-form of naproxen (ATB-346) developed by the company Antibe Therapeutics (www.antibethera.com). In direct comparisons of naproxen and its H_2S-linked derivative, the latter exerted comparable therapeutic efficacy with reduced gastric damage[379,380].

Asthma

Fitzgerald *et al.* have demonstrated that H_2S relaxed isolated airways smooth muscle cells and this implicated the role of the sarcolemmal K_{ATP} channel[381]. Although it seems that the effect of H_2S on smooth muscle is due to an activation of K_{ATP}, however the relaxation of animal bronchial smooth muscle is due to neither the same mechanism nor the activation of sGC, COX-1 or COX-2, or takinin receptors[382]. Nevertheless, H_2S relaxes murine bronchial airway smooth muscle preparations precontracted with carbachol very strongly, but only a small effect is seen on guinea pig bronchial rings[382].

Several studies suggest that H_2S metabolism is altered in pulmonary system-related pathologies: indeed, Wu *et al.* noted that the serum level of H_2S decreased from 75.2±13.0 μM in healthy subjects to 55.8±13.6 μM in patients with stable asthma and 31.3±2.9 μM in patients with severe acute exacerbation of asthma[383]. Serum H_2S levels positively correlated with forced expiratory volume (FEV1) and negatively with the count of sputum cells and the percentage of sputum neutrophils in patients with acute asthma. Whether the drop of H_2S level in

asthma patient serum is the cause or the consequence of asthma development has not been addressed. It is also not clear whether the lung production of H_2S was altered in these individuals. In any rate, this clinical study was mirrored by a later animal study: Chen *et al.* found that serum H_2S level in OVA-treated (an experimental model of allergic asthma) rats decreased by 81% and H_2S production from the lungs of these rats decreased by 80%[384]. The CSE expression level and CSE activity were decreased significantly in lung tissues from OVA-treated mice, which may explain the decreased endogenous levels of H_2S in lung tissues and serum in these animals. Chen *et al.* also found that the peak expiratory flow was 55.4% lower in OVA-treated rats than that of control rats and the proportions of eosinophils, lymphocytes, and neutrophils in BAL fluid were significantly increased[384]. Administration of exogenous H_2S improved peak expiratory flow and alleviated airway inflammation and airway remodelling in this model[384].

Asthma is a condition where there is an imbalance of oxidant/antioxidant influences that is at the basis of the increased oxidative stress[385]. Activated inflammatory cells generate more ROS. H_2S has reducing properties by allowing reduction of disulphide bonds and scavenging of various oxidants such as superoxide and peroxynitrite[386]. H_2S does not react readily with hydrogen peroxide but it reduces peroxynitrite-mediated toxicity and tyrosine nitration[374]. In a hypoxic pulmonary hypertension model, H_2S reduced oxidised glutathione levels and increased total antioxidant capacity in lung tissues, associated with a reduction in pulmonary artery pressures[387]. In an OVA-challenged mouse model, NaHS inhibited eosinophil and neutrophilic lung inflammation and prevented damage by increasing endogenous antioxidant with inhibition of lipid peroxidation. NaHS also increased SOD, glutathione peroxidase and glutathione reductase activity, together with a reduction in iNOS[388]. This indicates that H_2S may be useful as an antioxidant.

H_2S does act as an antioxidant also in mithocondrial milieu: mitochondrial dysfunction and oxidative stress are associated with the development and progression of asthma[389]. Antioxidants have been used to prevent and treat

mitochondrial abnormality in asthma patients[390]. Macromolecule antioxidants, such as vitamins E and C, cannot enter mitochondria to scavenge ROS. In contrast, the gasotransmitter H_2S can freely cross the plasma membrane and the mitochondrial membrane. Once inside mitochondria, H_2S acts as a reducing agent, which can help to decrease oxidative stress and enhance endogenous antioxidant defences, leading to the preservation of both mitochondrial structure and function[391]. To date, whether H_2S can attenuate airway inflammation and hyperresponsiveness in asthma by affecting the mitochondrial/oxidative stress pathway is not clear.

ASM cells contribute not only to airway narrowing in asthma, but also to bronchial inflammation through the secretion of inflammatory factors and recruitment and activation of inflammatory cells[392]. Perry *et al.* investigated the regulatory role of H_2S on the proliferation of human ASM cells. Exogenous H_2S delivered from either the fast-releasing donor NaHS or the slow-releasing donor GYY4137 suppressed airway smooth muscle cell proliferation and IL-8 release due to the inhibited phosphorylation of ERK-1/2 and p38 MAPKs. In these cells, the enzyme CBS, and not CSE, was mainly involved in the endogenous production of H_2S[393]. In other studies, administration of NaHS or the CSE blocker propargylglycine alleviated or aggravated, respectively, airway hyperresponsiveness in both the cigarette smoke exposure model and the OVA-induced asthma model in rats[394,395].

H_2S also has effects on fibrotic processes. It attenuated epithelial-mesenchymal transition of human alveolar epithelial cells induced by TGF-β1, through the inhibition of Smad2/3 phosphorylation. H_2S also suppressed human lung fibroblast proliferation induced by fetal bovine serum and PDGF-BB and TGF-β2-induced myofibroblast transdifferentiation[396].

Antiinflammatory effects of H_2S have already been discussed above. To this extent, it is worth noting that H_2S prevented particulate air pollution-induced endothelial disruption and lung vascular leakage, through scavenging ROS[397]. In terms of resolution of inflammation, H_2S could induce neutrophil apoptosis[398] and shift macrophage function to a pro-resolution phenotype[399], with enhancement of

bacterial phagocytosis and suppression of endotoxin-induced production of TNF-α. These observations indicate a potential antiinflammatory effect of exogenous H_2S in asthma and COPD.

Pulmonary hypertension

Scientific literature about the effect of H_2S on pulmonary hypertension is not very well developed so far and it deals mostly with animal experiments, so it lacks human data.

The first evidence of a possible role of H_2S in PAH was the finding that H_2S relaxed the smooth muscle of ileum, portal vein, and thoracic aorta *in vitro*, decreased the mean arterial pressure *in vivo*[400,401], and inhibited the proliferation of cultured aortic vascular smooth muscle cells of rats[402] and humans[403] via inhibition of MAPK activity. Nevertheless, Chunyu *et al.* conducted the pioneer work in this area: they found out that in an animal model of PH the gene expression and the activity of CSE enzyme in lung tissues as well as H_2S levels in plasma were reduced, and that exogenous supply of H_2S alleviated the elevation of pulmonary arterial pressure and improve vascular remodelling[404]. Furthermore, inhibition of CSE by propalglycine could further worsen the hallmarks of PH[405]. Xiaohui *et al.* confirmed the same findings of Chunyu *et al.* by inducing PH in rats with a different method[406]. It is interesting to note that H_2S levels were not affected by monocrotaline (MCT) in a rat model of PH[407], likely because the pathogenesis of MCT-induced PH is different from other animal models.

The improvement of vascular remodelling by H_2S was reported to be due to: 1) reduction of production of collagen I and III, important component of the ECM, by regulating the homeostasis of their synthesis and degradation through the up-regulation of the MMP-13 and the TIMP1 and the reduction of ET-1 and CTGF gene mRNA expression[408]; 2) apoptosis of pulmonary artery smooth muscle cells via the activation of Fas pathway and the inhibition of Bcl-2[409]; and 3) the attenuation of pulmonary smooth muscle cells proliferation[410]. In particular, the latter effect is considered to be a mixture of involvement of both NO and CO: the treatment with

exogenous H_2S attenuated the activated NO/NOS pathway and increased the production of endogenous CO (reported to inhibit vascular smooth muscle cell proliferation in hypoxic pulmonary hypertension[411]) and up-regulated the HO-1 protein expression in lung tissue in shunted rats, which might be involved in the mechanisms by which H_2S alleviates pulmonary vascular remodelling[410]. It seems that NO, CO, and H_2S might possibly interact under physiological and pathological conditions and constitute a regulatory network in the vascular system, in particular in the environment caused by PH, as other works by Yanfei *et al.*[412] and Zhang *et al.*[413] may suggest.

The hypoxic conditions used to induce the animal model of PH are widely known to generate large amounts of ROS, which can induce the progression of pulmonary vascular remodelling. The role of H_2S as an antioxidant agent in the disease was evidenced by the fact that the gasotransmitter could restore the levels of the total antioxidant capacity and lower the content of oxidised gluthatione in favour of reduced glutathione, as indicator of intracellular redox buffers; the activity of SOD enzymes was not affected by hypoxia neither before nor after administration of H_2S[387].

H_2S has been found useful to attenuate proliferation of human PASMCs treated with cobalt chloride ($CoCl_2$), via upergulation of COX-2/PGI_2 pathway[414]. $CoCl_2$ is a hypoxia-mimicking agent, which leads to a cellular model of hypoxic PAH; human PASMCs treated with this agent show a marked increase in cell proliferation, accompanied by a decrease in COX-2 expression, PGI_2 secretion, and H_2S levels. Exogenous NaHS suppressed $CoCl_2$-induced proliferation of the cells through the increase in the intracellular content of H_2S; importantly, the exposure of the cells to NaHS suppressed the $CoCl_2$-induced down-regulation of COX-2 expression and PGI_2 secretion from the PASMCs.

Finally yet importantly, H_2S has been proved to relax human pulmonary arteries precontracted with potassium chloride in a concentration-dependent manner[415] and this effect is independent of the integrity of the endothelium; however, the mechanisms by which H_2S exerts these effects have not been investigated. In

another paper, Sun *et al.* compared the vasorelaxing effect of exogenous H_2S in rat aorta and pulmonary artery: NaHS was able to induce a more pronounced vasorelaxation in aorta, compared to pulmonary artery[416]. They postulated that H_2S could more widely open K_{ATP} channels in aorta rather than pulmonary artery because of a higher presence of subunit SUR2B of K_{ATP} channels in the former; furthermore, in pulmonary artery only glibenclamide (a blocker of cellular K_{ATP} channels) affected the vasorelaxation, compared to aorta, in which both glibenclamide and 5-hydroxydecanoate (a blocker of mithocondrial K_{ATP} channels) could reduce vasorelaxation, thus further supporting their thesis.

Therefore, the molecular and cellular mechanisms by which H_2S/L-cysteine pathway affects PH are still not fully understood and needs further exploration.

CHAPTER 2
MATERIALS AND METHODS

2.1 REAGENTS

Phenilephrine (PE), N_G-nitro-L-arginine methylester (L-NAME), acetylcholine (Ach), bovine serum albumin (BSA), ovalbumin (OVA), sodium hydrosulphide (NaHS), L-cysteine, zinc acetate, N,N-dimethyl-*p*-phenylendiamine sulphate (DPD), iron(III) chloride ($FeCl_3$), collagenase Type XI, DNase I Type IV from bovine pancreas, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), FR180204, monoclonal anti-dinitrophenyl (anti-DNP) IgE, thapsigargin, ionomycin, antigen dinitrophenyl-human seralbumin (DNP-HSA), triton X-100, polyinosinic:polycytidylic acid [Poly(I:C)], lipopolysaccharide (LPS) from *E. Coli* (serotype 0111:B4), and sodium nitroprusside (SNP) were purchased from Sigma-Aldrich (Italy). Human IFN-α, mouse IFN-α, and TNF-α were purchased from R&D systems (UK). U46619 was purchased from Cayman Chemical (UK). SphK1/2 inhibitor was purchased from Tocris Bioscience (UK). S1P was purchased from Enzo Life Sciences (Italy). All salts and buffers used for Krebs solution preparation were purchased from Carlo Erba Reagenti (Italy).

KRX-725 (Compound 1) has been synthesised and kindly provided to us from group of Professors V. Santagada and G. Caliendo (Department of Pharmacy, University of Naples, Italy).

2.2 ANIMAL STUDIES (AIRWAY RESPONSIVENESS ASSESSMENT)

2.2.1 MICE

BALBC/c mice, mast cell-deficient $Kit^{W\text{-}Sh/W\text{-}sh}$ mice[417,418], and athymic nude BALBC/c mice were purchased from Charles River (Italy). They were allowed food and water *ad libitum* and housed in a controlled environment with a 12-hour light/dark cycle under specific pathogen-free conditions at the Department of Pharmacy (University of Naples, Italy). Mice were sacrificed by bleeding under isoflurane anaesthesia for flow cytometry experiments and under ketamine/xylazine (10 mg/1 $mg \cdot ml^{-1} \cdot kg^{-1}$) anaesthesia for bronchial reactivity experiments. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purposes (DM. 116/92) as well as with the EEC regulations (O. J. of E. C. L. 358/1 12/18/1986). All studies were performed in accordance with European Union regulations for the handling and use of laboratory animals and were approved by the local committee.

2.2.2 EXPERIMENTAL PROTOCOL

Mice received subcutaneous injection of 0.1 ml of S1P (10 ng/mouse) or vehicle on day 0 and 7 and sacrificed at different time points: 7, 14, and 21 days after the first subcutaneous injection of S1P. The vehicle used in the experimental procedures contained 0.001% of BSA.

In another set of experiments, an anti-CD20 mAb (rat IgG, 250 µg/mouse, i.p.)[419] was used to deplete CD20+ B cells at day 0, 3, 7, 10, and 14. The anti-CD20 mAb was injected the same day, but 30 minutes before the subcutaneous injection of S1P or vehicle. We already published that the anti-CD20 mAb depleted CD20+ B cells by around 90% compared with IgG[419]. Anti-CD20 mAb was injected every three days since the turnover of CD20+ B cells was approximately 4-5 days[419].

In another set of experiments, mice received the purified rat anti-mouse CD23 mAb (10 µg/mouse; B3B4 clone, BD Pharmingen, Italy) 30 minutes before S1P administration.

In another set of experiments, mice were injected subcutaneously with 0.4 ml of OVA (100 µg/mouse) dissolved in $Al(OH)_3$ gel (13 mg/ml in sterile saline) or vehicle (sterile saline) at day 0 and 7. Other mice were injected subcutaneously with 0.4 ml of OVA (50 µg/mouse solubilised in PBS) or vehicle (PBS only) at day 0, 2, 4, and 7. From day 7 to day 21 mice were exposed for 7 minutes on a daily basis to an H_2S-donor (NaHS) through aerosol (100 ppm), by means of the apparatus illustrated in Figure 2.1. Mice were sacrificed at day 21.

To prove the role of S1P-dependent pathway, OVA-sensitised mice were also treated with an inhibitor of SphK1/2 (10 µg/mouse), responsible of sphingosine phosphorylation.

Figure 2.1. Apparatus used for administration of aerosolised NaHS.

2.2.3 TISSUE PREPARATION

Mice were killed and bronchial tissue was rapidly dissected and cleaned from fat and connective tissue. Rings of 1 to 2 mm length were cut and placed in organ baths (2.5 ml) filled with oxygenated (95% O_2/5% CO_2) Krebs solution at 37°C, mounted to isometric force transducers (type 7006; Ugo Basile, Italy), and connected to a Powerlab 800 (ADInstruments, Italy). The composition of the Krebs solution was as follows (mol/l): NaCl 0.118, KCl 0.0047, $MgCl_2$ 0.0012, KH_2PO_4 0.0012, $CaCl_2$ 0.0025, $NaHCO_3$ 0.025, and glucose 0.01.

2.2.4 AIRWAY RESPONSIVENESS MEASUREMENTS

Rings were initially stretched until a resting tension of 0.5 g was reached and allowed to equilibrate for at least 30 minutes, during which tension was adjusted to 0.5 g when necessary and bathing solution was periodically changed. In a preliminary study, a resting tension of 0.5 g was found to develop the optimal tension to stimulation with contracting agents. In each experiment, bronchial rings were firstly challenged with Ach (10^{-6} M) until the responses were reproducible. Bronchial reactivity was assessed performing a cumulative concentration–response curve to carbachol (10^{-8}-3×10^{-5} M). Data were expressed as contraction capacity (dine/mg of tissue) compared to the concentration of carbachol.

2.2.5 ISOLATED PERFUSED MOUSE LUNG PREPARATION AND MEASUREMENTS

Lungs were perfused in a non-recirculating fashion through the pulmonary artery at a constant flow of 1 ml/minute, resulting in a pulmonary artery pressure of 2 to 3 cmH_2O. The perfusion medium used was RPMI1640 lacking phenol red (37°C). The lungs were ventilated by negative pressure (-3 and -9 cmH_2O) with 90

breaths/min and a tidal volume of approximately 200 ml. Every 5 minutes a hyperinflation (-20 cmH_2O) was performed. Artificial thorax chamber pressure was measured with a differential pressure transducer (Validyne DP 45–24; Validyne Engineering, CA) and airflow velocity with pneumotachograph tube connected to a differential pressure transducer (Validyne DP 45–15). The lungs respired humidified air. The arterial pressure was continuously monitored by means of a pressure transducer (Isotec Healthdyne Cardiovascular, PA), which was connected with the cannula ending in the pulmonary artery. All data were transmitted to a computer and analysed with the Pulmodyn software (Hugo Sachs Elektronik, Germany). The data were analysed through the following formula: $P = V \times C^{-1} + R_L \times dV \cdot dt^{-1}$, where P is chamber pressure, C pulmonary compliance, V tidal volume, R_L airway resistance. The airway resistance value registered was corrected for the resistance of the pneumotachometer and the tracheal cannula of 0.6 cmH_2O s ml^{-1}. Lungs harvested from mice of each group treated as described before were perfused and ventilated for 45 minutes without any treatment to obtain a baseline state. Subsequently, lungs were challenged with Ach. Repetitive dose-response curves of Ach (10^{-8}-10^{-5} M) were administered as 50 µl bolus, followed by intervals of 15 minutes, in which lungs were perfused with buffer only.

2.3 ANIMAL STUDIES (PEPDUCIN ACTIVITY ASSESSMENT)

2.3.1 MICE

CD1 mice were purchased from Charles River (Italy). They were allowed food and water *ad libitum* and housed in a controlled environment with a 12-hour light/dark cycle under specific pathogen-free conditions at the Department of Pharmacy (University of Naples, Italy). Mice were sacrificed by bleeding under isoflurane anaesthesia. Animal care was in compliance with Italian regulations on protection

of animals used for experimental and other scientific purposes (DM. 116/92) as well as with the EEC regulations (O. J. of E. C. L. 358/1 12/18/1986). All studies were performed in accordance with European Union regulations for the handling and use of laboratory animals and were approved by the local committee.

2.3.2 TISSUE PREPARATION

Mice were killed and thoracic aortha was rapidly removed, dissected, and cleaned from fat and connective tissue. Rings of 1 to 2 mm length were cut and placed in organ baths (2.5 ml) filled with oxygenated (95% O_2/5% CO_2) Krebs solution at 37°C, mounted to isometric force transducers (type 7006; Ugo Basile, Italy), and connected to a Powerlab 800 (ADInstruments, Italy). The composition of the Krebs solution was as follows (mol/l): NaCl 0.118, KCl 0.0047, $MgCl_2$ 0.0012, KH_2PO_4 0.0012, $CaCl_2$ 0.0025, $NaHCO_3$ 0.025, and glucose 0.01.

2.3.3 AORTA RESPONSIVENESS MEASUREMENTS

Rings were initially stretched until a resting tension of 1.0 g was reached and allowed to equilibrate for at least 30 minutes, during which tension was adjusted to 1.0 g when necessary and bathing solution was periodically changed. In a preliminary study, a resting tension of 1.0 g was found to develop the optimal tension to stimulation with contracting agents and to preserve the endothelial cell layer. In each experiment, aorthic rings were firstly challenged with PE (10^{-6} M) until the responses were reproducible. In order to verify the integrity of endothelium, Ach cumulative concentration-response curve (10^{-8}-3×10^{-5} M) was performed on the PE-precontracted rings. Vessels that relaxed less than 85% were discarded. Aortic rings were contracted with PE (10^{-6} M), and once plateau was reached, a cumulative concentration-response curve to Compound 1 (10^{-8}-3×10^{-5}

M) was performed. Relaxations were expressed as the percentage of contraction. Relaxations were determined for each concentration-response curve by nonlinear regression analysis. S1P-induced relaxation of aorta involves both $S1P_1$ and $S1P_3$ and is NO-mediated, but the $S1P_1$ and $S1P_3$ receptors are coupled to different mechanisms of signalling transduction. In particular, $S1P_3$ is coupled to a G_i-dependent ERK. In order to assess the contribution of nitric oxide or the ERK activation in the Compound 1-induced vasorelaxation, aortic rings were preincubated with the NOS inhibitor L-NAME (100 µM) or the ERK inhibitor FR180204 (10 µM). In another set of experiments, the ability of myristoyl peptides (2–20) to induce vasorelaxation when compared with Compound 1 was tested (data not shown). Upon assays, the more active compounds were discarded, and the ability of the less active compounds to inhibit the Compound 1-induced vasorelaxation was evaluated. For this purpose, aortic rings were incubated for 15 minutes with the selected compounds and a cumulative concentration-response curve to Compound 1 was performed.

2.4 ANIMAL STUDIES (PULMONARY ARTERY CONTRACTILITY ASSESSMENT)

2.4.1 MICE

Male C57Bl/6J mice (Charles River, UK) were allowed food and water *ad libitum* and housed in a controlled environment with a 12-hour light/dark cycle under specific pathogen-free conditions at the Hammersmith Hospital (Imperial College of London, UK). All studies were performed in accordance with UK Home Office Animals (Scientific Procedures) Act 1986 and institutional guidelines.

2.4.2 EXPERIMENTAL PROTOCOL

Mice (8 to 10 weeks old; ≈20 g) were housed in normal air or placed in a normobaric hypoxic chamber (FiO_2 10%) for 14 days. At day 14, animals were

weighed and anesthetised (fentanyl/fluanisone 0.25 ml/kg; midazolam 25 mg/kg i.p.), and right ventricular systolic pressure (RVSP) was measured via direct cardiac puncture using a closed-chest technique in the spontaneously breathing, anesthetised animal to verify development of pulmonary hypertension as previously described[420].

2.4.3 TISSUE PREPARATION

At day 14, mice were humanely sacrificed by bleeding under anaesthesia and lungs carefully removed from thoracic cavity. Secondary pulmonary artery was rapidly isolated and cleaned from fat and connective tissue. Rings of 2 mm length were cut and mounted in organ baths (5 ml) of an isometric Mulvany-Halpern myograph filled with oxygenated (95% O_2/5% CO_2) physiological saline solution (PSS) at 37°C. Force was recorded via a PowerLab/800 (ADInstruments Ltd., UK) and analysed using Chart 6.0 acquisition system (ADInstruments Ltd., UK). The composition of the PSS was as follows (mmol/L): NaCl 118, KCl 4.7, $CaCl_2 \cdot 6H_2O$ 2.5, $MgSO_4 \cdot 7H_2O$ 1.17, $NaHCO_3$ 25.0, $NaH_2PO_4 \cdot 2H_2O$ 1.0, Na_2EDTA 0.03, and glucose 5.5.

2.4.4 PULMONARY VESSEL RESPONSIVENESS MEASUREMENTS

Rings were mounted and allowed to equilibrate for 10 minutes. They were then stretched at a resting tension of 3.99 kPa and contracted with the thromboxane mimetic U46619 (10^{-9}-10^{-6} M). After washing, tissues were contracted with an EC_{80} concentration of U46619, followed by cumulative concentration-response curves to NaHS (10^{-6}-3×10^{-3} M) and SNP (10^{-8}-10^{-5} M). Data were expressed as force (mN) developed by the contracting agent or as percentage of U46619-induced contraction after administration of vasorelaxant compounds.

2.5 LUNG MORPHOLOGICAL ANALYSIS

Left lung lobes were fixed in OCT medium (Pella Inc., Italy) and 7 µm cryosections were cut. At least five sections were considered for each animal and the mean of the positive staining compared with the total area were plotted.

The degree of inflammation was scored by blinded observers by using hematoxylin and eosin (H&E) and Periodic Acid-Schiff (PAS) staining (Sigma-Aldrich, Italy). PAS staining was performed according to the manufacturer's instructions to detect glycoprotein. PAS+ cryosections were graded with scores 0 to 4 to describe low to severe lung inflammation as follows: 0: <5%; 1: 5–25%; 2: 25–50%; 3: 50–75%; 4: >75% positive staining/total lung area.

Mast cell activation was evaluated by toluidine blue (Sigma-Aldrich, Italy) positive staining by immunohistochemistry.

Immunohistochemical detection of CD23 was performed by using anti-CD23 or rat IgG isotype control. The diamino-benzidinic acid system was used to detect complexes. Positive staining was quantified by means of Image J software (NIH, USA) and expressed as CD23 positive staining compared with the total area of the lung section.

Immunohistochemical detection of FGF2 was performed by using anti-FGF2 or IgG isotype control (Santa Cruz Biotechnologies, USA). The diamino-benzidinic acid system was used to detect complexes. Positive staining was quantified by means of Image J software (NIH, USA) and expressed as FGF2 positive staining compared with the total area of the lung section.

2.6 CELLULAR STUDIES

2.6.1 FLOW CYTOMETRY ANALYSIS

Lungs were isolated and digested with 1 U/ml collagenase. Cell suspensions were passed through 70 µm cell strainers and red blood cells were lysed. Cell suspensions were used for flow cytometric analysis of different cell subtypes[419]. The composition of lung inflammatory cells was determined by flow cytometry (BD FacsCalibur, Italy) using the following antibodies: CD3-PeCy5.5, CD4-PE, CD8-APC, CD11c-FITC, CD11b-PeCy5.5, CD19-PeCy5.5, CD20-APC, CD25-PE, B220-PE, cKit-PeCy5.5 or -PE, FoxP3-PeCy5.5, IgE-FITC, MHC II-PE, and MHC I-FITC (Bioscience, USA).

CD4+ T cells were isolated by digesting mediastinic lymph nodes with collagenase (0.5 U/ml) and by using immunomagnetic beads for negative selection[421], according to the manufacturer's instructions (EasySep, Voden, Italy). The purity of CD4+ T cells was around 90%. CD4+ T cells were then marked for carboxyfluoresceindiacetate succinimidyl ester (CFSE; 5 µM; Molecular Probes, Invitrogen, Italy) to perform proliferation assay by using CD3/CD28 stimulation (CD3/CD28 beads, Invitrogen, Italy). CFSE flow cytometry data were analysed by means of ModFit software (BD Pharmingen).

In another set of experiments, CD4+ T cells harvested from vehicle-, S1P-, or S1P+anti-CD23-treated mice were adoptively transferred into naïve (untreated) mice. Then 1×10^6 CD4+ T cells were injected intravenously as previously described[421].

Lung B cells (CD19+ cells) were generated by digesting the lung with collagenase (5-10 U/ml), DNAse I (20 µg/ml), and antibiotics for 45 minutes. Cell suspensions were passed through 70 µm cell strainers and red blood cells were lysed. For some

experiments, CD19+ cells were isolated using a negative selection for CD19+ cells (EasySep Stem Cell, Voden, Italy). Purity was routinely around 90%, as already published[419].

2.6.2 MIXED LYMPHOCYTE REACTION

Lung CD19+ B cells were harvested, treated with PBS or S1P (0.1-1 µM), and then cultured for 24 and 72 hours with splenic CD8+ (ratio 1:10) and CD4+ T cells (ratio 1:10). Splenic CD8+ and CD4+ T cells were isolated using an EasySep Stem Cell kit (Voden, Italy). Purity was checked by flow cytometry by using anti-CD3, anti-CD19, anti-CD4, and anti-CD8 antibodies (eBioscience, USA) and was routinely around 90% (data not shown). Cell-free supernatant was tested for IFN-γ and IL-10 release. CD4+ and CD8+ T cells were tested for cell proliferation by using CFSE (1 µM) assay (eBioscience, USA).

2.6.3 RBL-2H3 CELL CULTURE AND β-HEXOSAMINIDASE MEASUREMENT

RBL-2H3 (Rat Basophilic Leukemia Mast Cell Line; Japan Health Sciences Foundation) were sensitised with the anti-DNP IgE (0.50 µg/ml). The H_2S-donor NaHS (10-1000 µM; 5 min) or the vehicle were incubated. RBL-2H3 cell degranulation was induced by i) the antigen DNP-HSA (10 ng/ml); ii) ionomycin (1 µM) and iii) thapsigargin (1 µM). Triton X-100 was added to cause exhaustive release of β-hexosaminidase. The concentration of *p*-nitrophenate release of β-hexosaminidase was measured at 405 nm.

2.6.4 SMOOTH MUSCLE CELL CULTURE

Human pulmonary artery specimens were obtained from healthy segments of lung from patients undergoing lung resection at the Royal Brompton Hospital, London, UK (Research Ethics Committee study number 02-081, sub-amendment 3). Full-informed written consent was obtained from all participants. Human pulmonary artery smooth muscle cells (hPASMCs) were isolated from those arteries, as already described[422]. Cells were serum-deprived for 24 hours and subsequently treated with increasing concentrations of NaHS (10-1000 μM) and stimulated with IFN-α (10 ng/ml) or TNF-α (10 ng/ml) for 24 hours; after this period, supernatants were collected and stored for following analysis. Cell viability was measured using the AlamarBlue® method (Life Technologies, UK) according to manufacturer's instructions.

2.6.5 ISOLATION OF BLOOD OUTGROWTH ENDOTHELIAL CELLS

Blood was collected from patients with PAH and healthy controls and blood outgrowth endothelial cells (BOECs) were isolated as per previously published protocols[423,424]. BOECs were treated with increasing concentrations of NaHS (10-1000 μM) and stimulated with IFN-α (10 ng/ml) for 24 hours; after this period, supernatants were collected and stored for following analysis. Cell viability was measured using the AlamarBlue® method (Life Technologies, UK), according to manufacturer's instructions.

2.6.6 FIBROBLASTS CULTURE AND PROLIFERATION ASSESSMENT

Murine lung fibroblasts were isolated from lungs of mice treated with OVA and/or H_2S aerosol by means of enzymatic digestion, according to Baglole *et al.*[425].

Fibroblastic phenotype was confirmed by presence of vimentin staining by immunocytochemistry (data not shown).

Cell viability was assessed using the MTT colorimetric assay, considering the ability of living cells to oxidise the yellow MTT into water-insoluble purple crystals of formazan. Briefly, cells were seeded at a density of 7,500 cells/well in a 96-well plate. After 24 hours from the seeding, the medium was discarded and replaced with 200 µl of a solution of MTT (0.25 mg/ml) solublised in RPMI1640. After 3.5 hours of incubation at 37°C, supernatants were removed and the crystals of formazan dissolved with 100 µl of DMSO. Absorbances were read at 520 nm with the Multiskan GO spectrophotometer (Thermo Scientific, Italy). The number of cells was extrapolated from absorbances against a reference curve.

2.6.7 IMMUNOCYTOCHEMISTRY

Murine lung fibroblasts were seeded on glass Lab-Tek II chamber slide™ (Thermo Scientific, USA) until confluence. They were first washed and then fixed with formalin. Fibroblasts differentiation was tested by incubating cells with mouse monoclonal antibody against α-SMA (Sigma Aldrich, Italy) or rabbit polyclonal antibody against vimentin (Santa Cruz, USA). For detection, fluorescein-labeled anti-mouse IgG (ABNOVA, Italy) or Texas-Red-labeled anti-rabbit IgG (ABNOVA, Italy) were used. Finally, glass slides were mounted with Fluoroshield™ with DAPI histology mounting medium (Sigma-Aldrich, Italy) and analysed by immunofluorescence. Myofibroblasts relative presence over fibroblasts was assessed as reported by Singh and Hall[426].

2.7 ELISA MEASUREMENTS

Total serum IgE levels were measured by means of ELISA using matched antibody pairs (BD Pharmingen, USA). The amount of serum PGD_2 was quantified using an EIA Kit (Cayman, USA).

ET-1, IP10, and IL-8 were measure on cell culture supernatans by using commercially available ELISAs (R&D System, UK; Bethyl Laboratories, UK), according to manufacturers' instructions.

TNF-α, IL-4, IL-6, IL-10, IFN-γ, Granzyme B, TGF-β (R&D Systems, UK), and IL-13 (eBioscience, USA) were measured in lung homogenates by using commercially available ELISAs and expressed as pg/mg of proteins.

2.8 WESTERN BLOTTING ANALYSIS

Lungs isolated B cells were lysed in RIPA buffer (50 mM Tris/HCl, pH 7.4, 1% Triton-X-100, 0.25%, sodium deoxycolate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 10 µg/ml aprotinin, 20 µM leupeptin, and 50 mM sodium fluoride). Lysates were centrifuged at 14,000 g in a precoooled centrifuge for 15 minutes. Lysates were separated by SDS/PAGE (10% gels) and proteins were transferred onto a PVDF membrane. Membranes were blocked by incubation in PBS containing 0.1% Tween-20 and 5% (w/v) non-fat dried skimmed milk for 1 hour, followed by overnight incubation at 4°C with anti-SphK1 (SantaCruz Biotechnologies, USA) and anti-GAPDH (Sigma-Aldrich, Italy) antibodies. The membranes were washed extensively in PBS containing 0.1% Tween-20, before incubation for 2 hours with HRP-conjugate secondary antibodies. The membranes were then washed and developed using ECL (enhanced chemiluminescence substrate) using Image Quant F40 Instrument (GE Healthcare Life Sciences, Italy) for acquisition.

2.9 H_2S LEVELS MEASUREMENT (METHYLEN BLUE ASSAY)

Serum determination of H_2S was performed as follows. Briefly, 200 µl of serum were added in Eppendorf tubes containing zinc acetate (150 µl; 1% w/v). Subsequently, DPD (100µl; 20 mM in 7.2 M HCl) and $FeCl_3$ (133 µl; 30 mM in 1.2 M HCl) were added to the reaction mixture. After 20 minutes of incubation in the dark, trichloroacetic acid (10%, 300 µl) was added to each sample. After centrifugation (10,000 r.p.m. for 10 minutes at 4°C), the optical absorbance of the newly generated methylene blue was measured at a wavelength of 667 nm. All samples were assayed in duplicate and H_2S concentration was calculated against a calibration curve of NaHS (3.9-250 µM).

2.10 STATISTICAL ANALYSIS

Results are expressed as means ± SEM. Changes observed in treated groups compared with controls were analysed using one-way analysis of variance (ANOVA), followed by Bonferroni's post-test for multiple comparisons, and/or Student's *t*-test, by using the GraphPad Prism software (USA). Two-way ANOVA was applied where required. *p*-values less than 0.05 were considered significant.

CHAPTER 3
RESULTS

3.1 STUDY OF CELLULAR MECHANISMS OF S1P-INDUCED AIRWAY HYPERREACTIVITY AND PULMONARY INFLAMMATION

3.1.1 RATIONALE

Allergic asthma is a pulmonary disease characterised by reversible bronchoconstriction, lung inflammation, and mucus hyperproduction: all these hallmarks are responsible of impaired respiration and lung functionality.

It is accepted that allergic asthma is an inflammatory condition driven by a complex mix of mediators. In line with this, S1P is found in elevated levels in the BAL of patients with asthma, after challenge with the antigen. This finding suggests that S1P could be induced in allergic asthma. Indeed, Roviezzo *et al.*[294] have demonstrated that interfering in a specific step of the sphingolipid methabolic pathway with D,L-threo-dihydrosphingosine (DTD), a SphK inhibitor, the OVA-induced hyperreactivity is abrogated. Furthermore, S1P has direct effects on SMCs. Roviezzo *et al.*[427] have already demonstrated that systemic administration of S1P in mice leads to lung inflammation, airway hyperreactivity, and goblet cell hyperplasia, associated with an increase in mast cell recruitment in lungs, but the molecular/cellular mechanisms of these effects have not been investigated. Here, we aim at delucidating the pathways responsible for the S1P-mediated effects on lungs and airways.

Thus, in this chapter, we have addressed the following hypothesis and specific aims:

Hypothesis:

"Systemic administration of S1P in mice contributes to lung inflammation, airway remodelling, and bronchoconstriction via immune system activation"

Aims:

- to investigate the effect of exogenous S1P on airway inflammation and markers of allergic asthma in mice;
- to investigate the role of mast cells in the effects of S1P on airway responses and lung inflammation *in vivo*;
- to investigate the role of T cells in the effects of S1P on airway responses and lung inflammation *in vivo*;
- to assess the role of B cells in S1P- and OVA-induced hyperresponsiveness *in vivo*;
- to assess the effects of S1P on B cells *in vitro*;
- to assess the efficacy of a new pepducin-derived $S1P_3$ antagonist.

3.1.2 METHODS

The methods relating to experiments performed in this section are described in the Chapter 2 (Materials and methods).

3.1.3 DATA HANDLING AND STATISTICAL ANALYSIS

The statistical analysis relating to experiments performed in this section are described in the Chapter 2 (Materials and methods). The details of each statistical test used are reported in the respective figure legends.

3.1.4 RESULTS

S1P induces airway smooth muscle hyperreactivity and lung inflammation

Lungs harvested from mice injected with S1P (Figure 3.1A) displayed a progressive alteration in lung morphology. The effect was maximal at 21 days after S1P challenge (Figure 3.1B) and was associated with increased mucus production as determined by PAS staining (Figure 3.1C). Plasma levels of IgE (Figure 3.1D) were significantly increased too. Flow cytometry analyses of lungs showed a significant increase in the percentage of mast cells, identified as CD11c+cKit+IgE+ cells (Figure 3.2A and Figure 3.2B). Mast cells were stained for IgE since they covalently bind to its receptor (FcεRI) on these cells[428]. Plasma levels of PGD_2 were significantly increased in S1P-treated compared with vehicle-treated mice (Figure 3.2C).

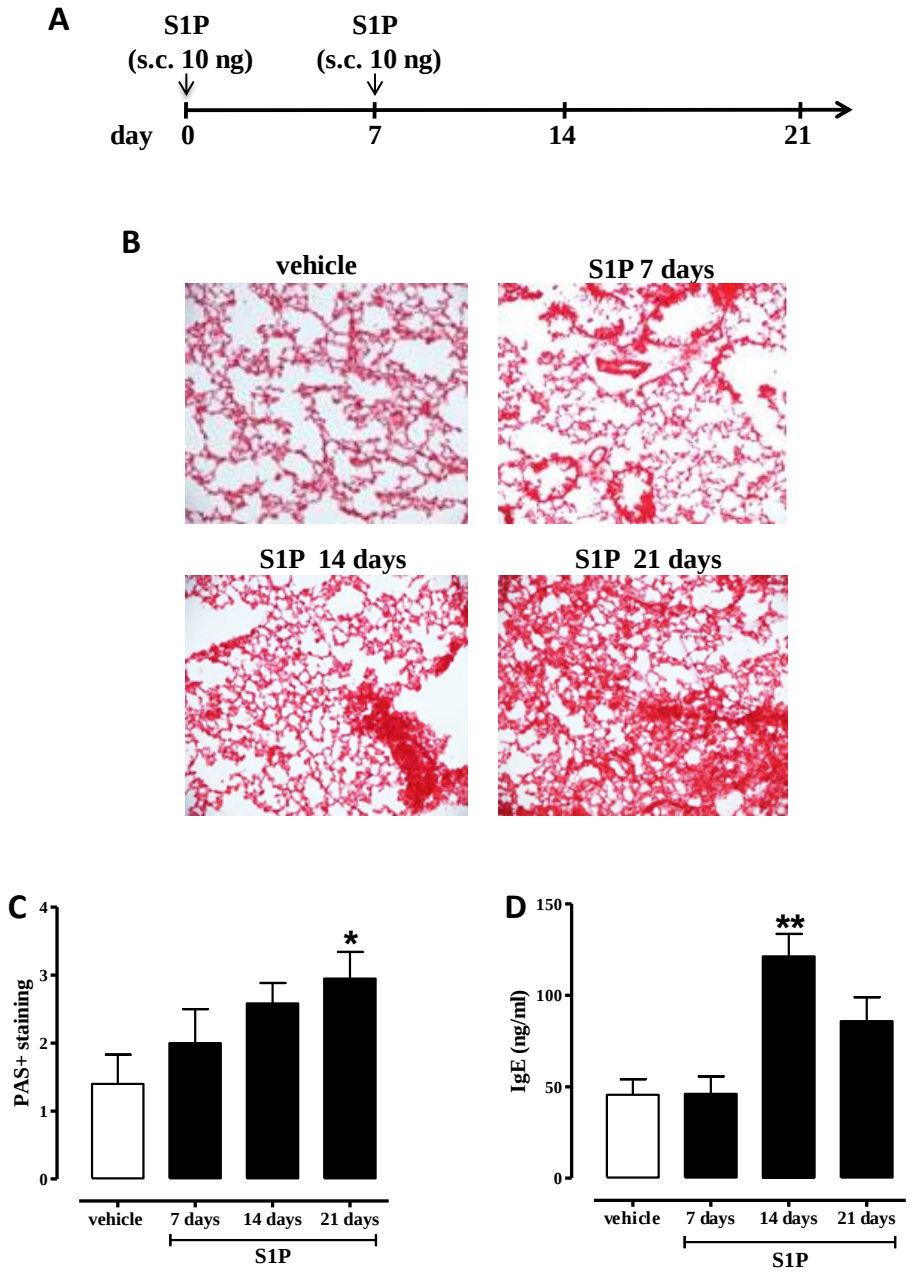


Figure 3.1. Systemic S1P administration induces lung inflammation. (A) Mice received s.c. S1P (10 ng) or vehicle (BSA 0.001%) on days 0 and 7. (B) Lung sections were fixed and stained with H&E. Lung sections were photographed under light microscopy at 10× magnification. (C) PAS was performed to detect glycoprotein (* $p<0.05$ *vs.* vehicle, one-way ANOVA with Bonferroni's post-test). (D) Sera were collected and levels of total IgE were determined by using specific ELISA (** $p<0.01$ *vs.* vehicle, one-way ANOVA with Bonferroni's post-test). Data are mean ± SEM, *n=6* mice in each group.

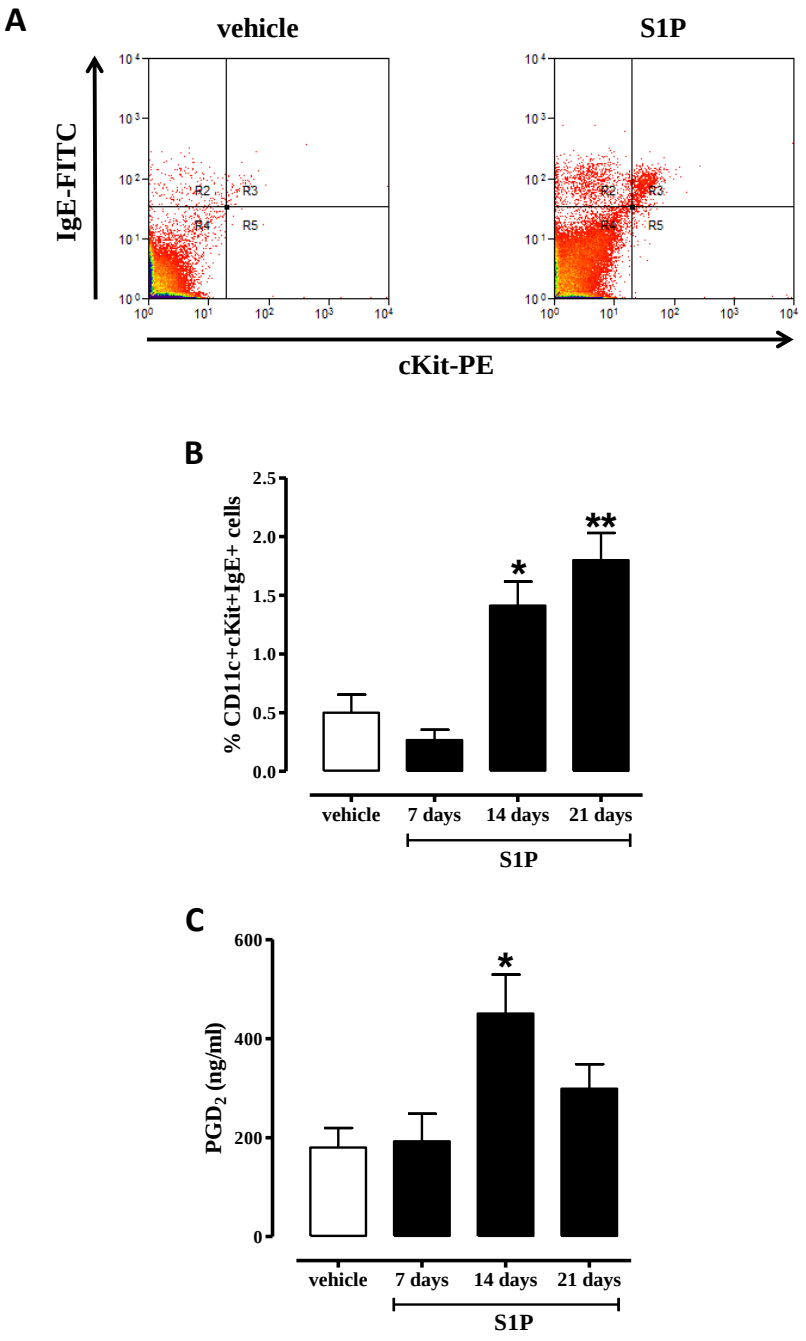


Figure 3.2. S1P increases mast cell infiltration in the lung. (A) Mast cells were identified as CD11+cKit+IgE+ cells by flow cytometry as shown in the representative dot plot. (B) Mast cell-infiltration was quantified after 7, 14, and 21 days following S1P administration (* $p<0.05$, ** $p<0.01$ *vs.* vehicle, one-way ANOVA with Bonferroni's post-test). (C) Sera were collected and PGD_2 levels were determined by using specific ELISA. (* $p<0.05$ *vs.* vehicle, one-way ANOVA with Bonferroni's post-test). Data are mean ± SEM, *n=6* mice in each group.

S1P-induced hyperreactivity, but not lung inflammation, is attenuated in mast cell-deficient Kit$^{W\text{-}sh/W\text{-}sh}$ mice

In mast cell-deficient Kit$^{W\text{-}sh/W\text{-}sh}$ mice, S1P failed to induce bronchial hyperresponsiveness (Figure 3.3A). Conversely, lungs harvested from the same animals still displayed i) altered alveolar structure (Figure 3.3B) and ii) increased mucus production (Figure 3.3C) in comparison with wild type. Basal serum IgE was significantly reduced in Kit$^{W\text{-}sh/W\text{-}sh}$ mice when compared with wild-type mice (Figure 3.3D). Nevertheless, S1P challenge significantly increased IgE levels in Kit$^{W\text{-}sh/W\text{-}sh}$ mice when compared with the basal levels of mast cell-deficient mice (Figure 3.3D).

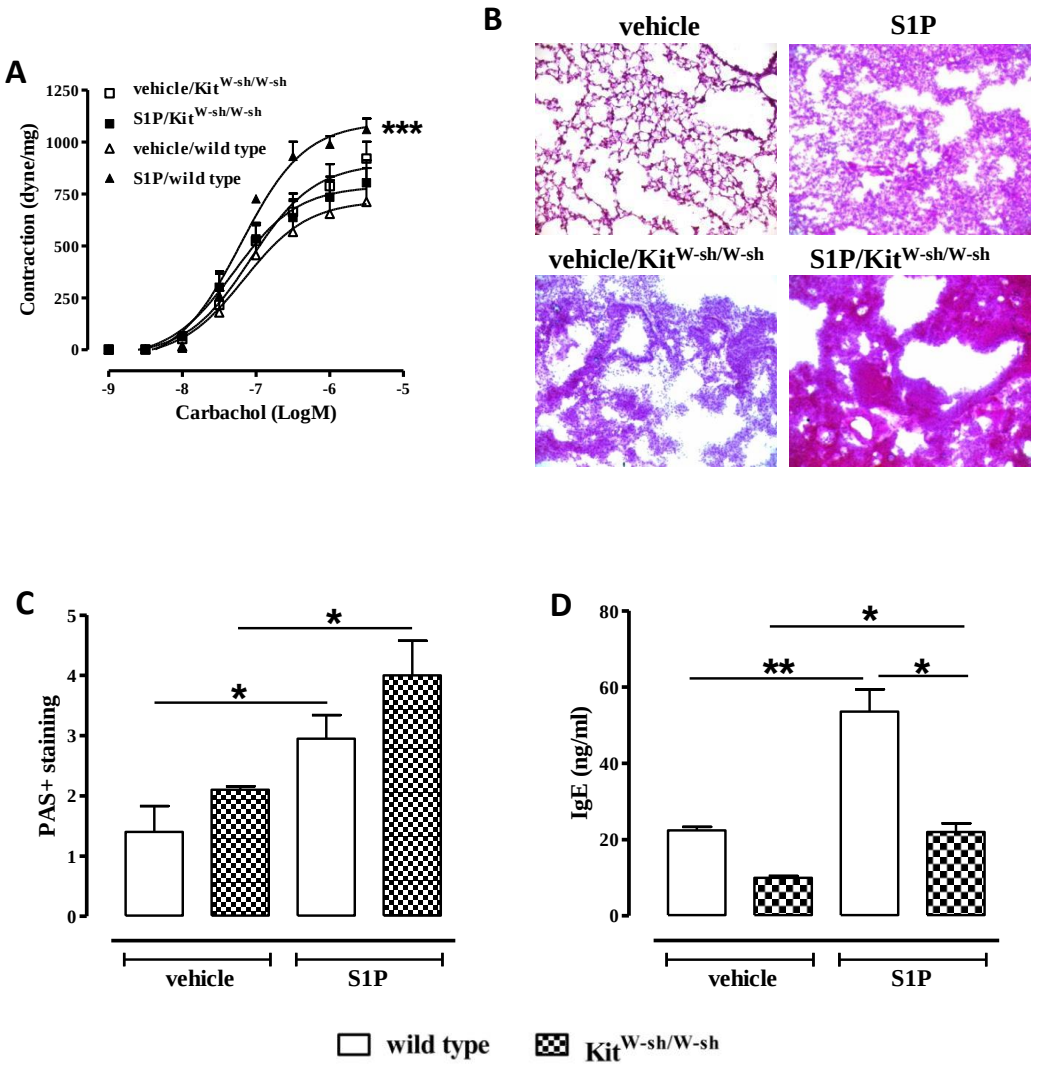


Figure 3.3. Mast cells are essential for the development of S1P-induced bronchial hyperreactivity, but not for lung inflammation. Mast cell-deficient Kit$^{W\text{-}sh/W\text{-}sh}$ or wild-type mice received s.c. S1P (10 ng) or vehicle (BSA 0.001%) on days 0 and 7. Mice were killed on day 21. (A) Assessment of bronchial reactivity to carbachol (*** p<0.001 *vs.* vehicle, two-way ANOVA with Bonferroni's post-test). (B) Lung sections were fixed and stained with PAS (* p<0.05, Student's t-test). Lung sections were photographed under light microscopy at 10× magnification. (C) PAS staining was quantified as described in Methods chapter. (D) Sera were collected and levels of IgE were determined by ELISA (* p<0.05; ** p<0.01, Student's t-test). Data are mean ± SEM, n=6 mice in each group.

S1P induces lung inflammation and airway smooth muscle hyperreactivity in an IgE-dependent manner

CD23 is an important regulatory receptor for IgE production and its interaction with IgE can amplify IgE-associated immune responses[429,430,431]. Immunohistochemical analysis (Figure 3.4A) showed that CD23 expression was significantly increased in S1P-treated mice with a maximal effect at 21 days after S1P challenge in comparison with vehicle mice (Figure 3.4B). Therefore, in order to investigate the role of IgE in S1P-induced airway dysfunction, we used an anti-CD23 mAb. Administration of anti-CD23 per se did not affect S1P-induced increase in pulmonary mast cell infiltration (Figure 3.4C). Conversely, anti-CD23 significantly reduced S1P-induced IgE increase (Figure 3.4D) thereby confirming the role of CD23 in S1P-induced IgE production. IL-4 and IL-13 are key cytokines for the initiation of Th2-mediated responses and for IgE isotype class switching[432,433]. S1P administration induced a significant increase in both IL-4 (Figure 3.4E) and IL-13 (Figure 3.4F) in the lung. Anti-CD23 inhibited S1P-induced expression of IL-13 (Figure 3.4F), but not of IL-4 (Figure 3.4E). However, the administration of anti-CD23 to S1P-treated mice prevented airway smooth muscle hyperresponsiveness (Figure 3.5A), lung damage (Figure 3.5B), and mucus production (Figure 3.5C).

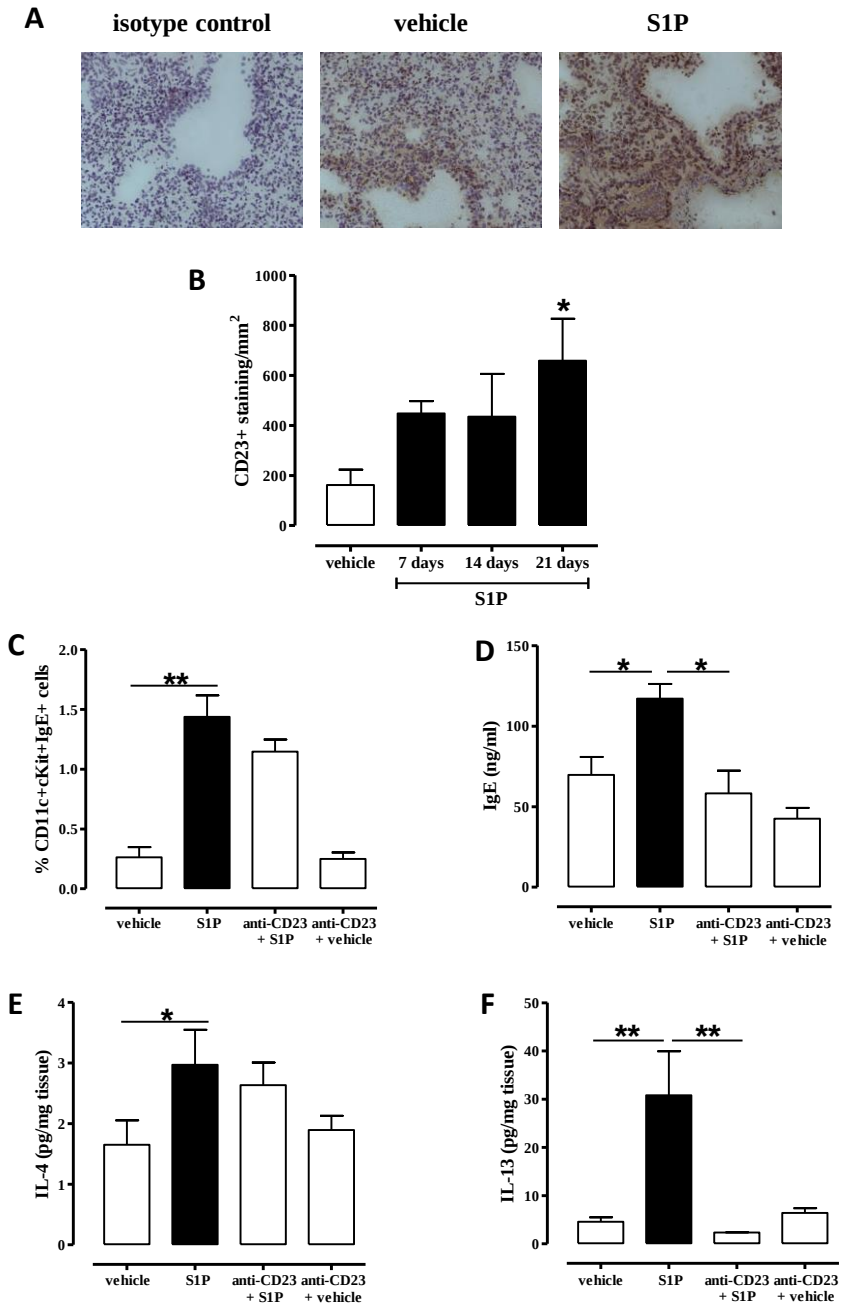


Figure 3.4. S1P enhances pulmonary CD23 (FcεRII) expression. (A) Immunohistochemical detection of CD23 was performed on lung sections harvested from mice challenged with vehicle or S1P by using anti-CD23 mAb or rat IgG isotype control as shown in the representative lung section staining. Lung sections were photographed under light microscopy at 10× magnification. (B) CD23 quantification was performed at 7, 14, and 21 days after S1P challenge (* p<0.05 *vs.* vehicle, Student's *t*-test). In another set of experiments, anti-CD23 (10 µg/mice) was administered i.p. 30 minutes before S1P or vehicle on days 0 and 7. On day 21, mice were killed. (C) Pulmonary mast cells were identified as CD11+cKit+IgE+ cells by flow cytometry (** p<0.01, Student's *t*-test). (D) Sera were collected to determine IgE levels by ELISA (** p<0.01, Student's *t*-test). (E) (F) Pulmonary expression of IL-4 and IL-13 (* p<0.05, ** p<0.01, Student's *t*-test) was determined by ELISA. Data are mean ± SEM, *n=6* mice in each group.

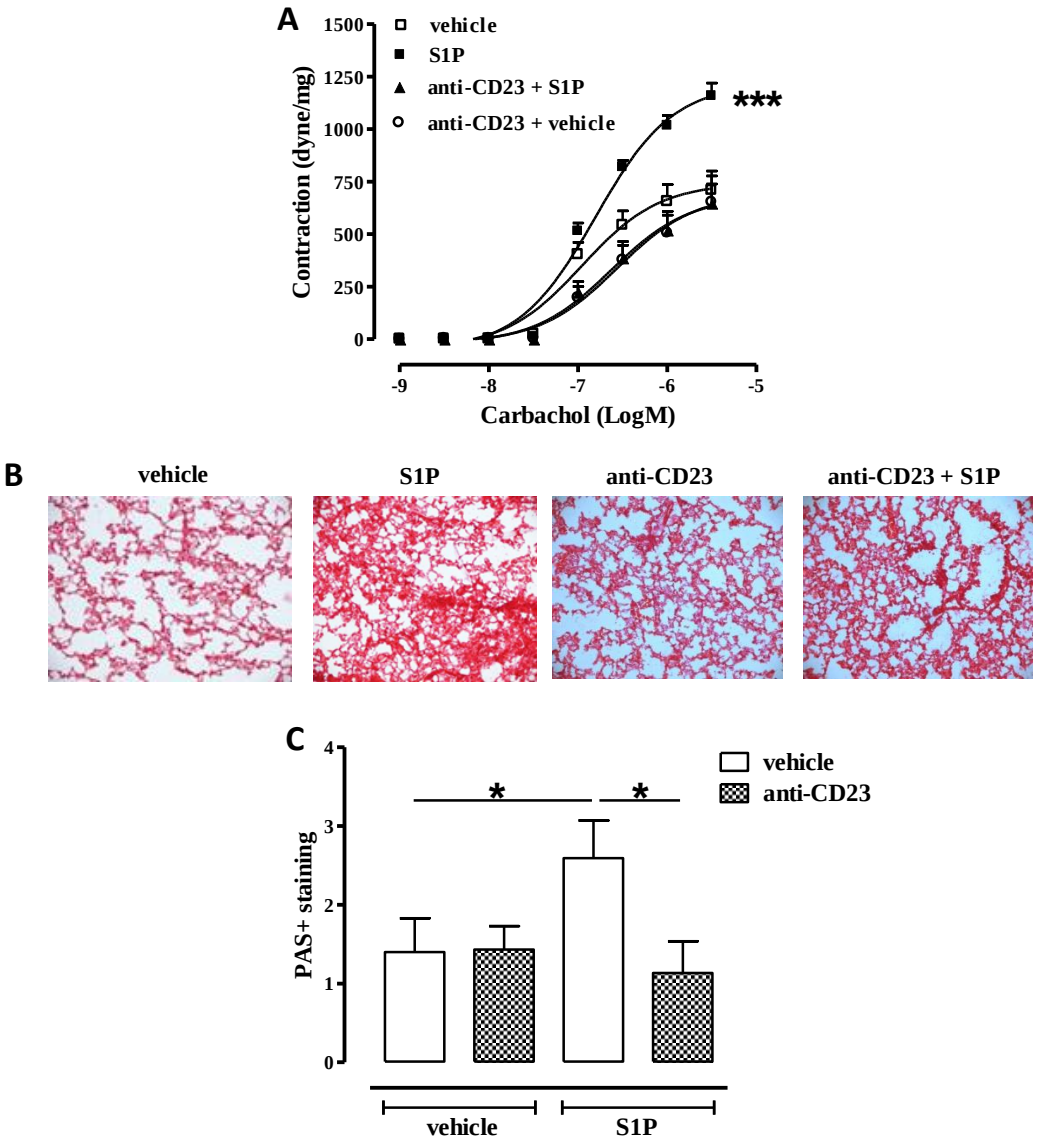


Figure 3.5. Anti-CD23 attenuates S1P-mediated effects on the lung. Anti-CD23 (10 µg/mice) was administered i.p. 30 minutes before S1P on days 0 and 7. Mice were killed on day 21. (A) Bronchial responses to carbachol were evaluated (*** $p<0.001$ *vs.* vehicle, two-way ANOVA with Bonferroni's post-test). (B) Representative staining of lung sections with H&E. (C) Quantification of PAS staining performed as described in Methods chapter (* $p<0.05$, Student's *t*-test). Lung sections were photographed under light microscopy at 10× magnification. Data are mean ± SEM, *n=6* mice in each group.

T cells are involved in S1P-induced effects on lung

The major control step in IgE synthesis is the regulation of IgE class-switch recombination, which is mostly T cell-dependent[434,435]. Our data define IgE/CD23 signalling as a key driver for the effects triggered by S1P in the airways. In order to extend our observations on the role of CD23 and to further elucidate the role of IgE-dependent mechanisms, we evaluated T cell involvement. Nude mice, which genetically lack of T cells, injected with S1P did not develop bronchial hyperresponsiveness (Figure 3.6A). In addition, alteration of lung structure (Figure 3.6B), mucus production (Figure 3.6C) and mast cell infiltration (Figure 3.6D) in S1P-treated nude mice were significantly lower than wild-type mice. Plasma levels of IgE were unaltered by S1P in nude mice compared with wild type (Figure 3.6E). Accordingly, we observed neither an increase in CD23 pulmonary expression (Figure 3.6F) nor IL-4/IL-13 overexpression in the lung (data not shown).

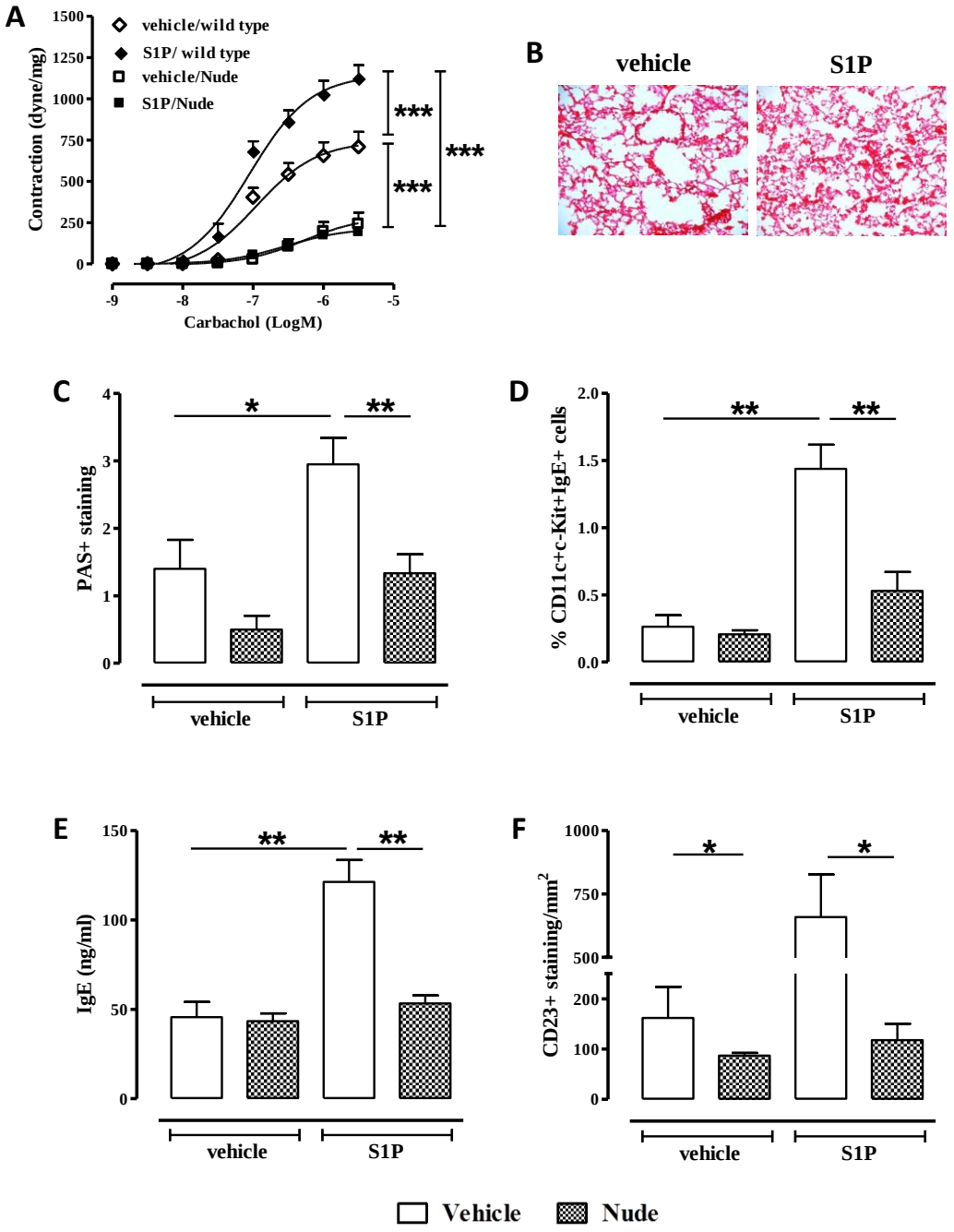


Figure 3.6. T cells play a key role in S1P-mediated effects on lung. Nude athymic mice or wild-type mice received s.c. S1P (10 ng) or vehicle (BSA 0.001%) on days 0 and 7. Mice were killed on day 21. (A) Assessment of bronchial response to carbachol (*** p<0.001, two-way ANOVA with Bonferroni's post-test). (B) Representative staining of lung sections with H&E. (C) Quantification of PAS staining performed as described in Methods chapter (* p<0.05, ** p<0.01, Student's t-test). (D) Pulmonary mast cells quantified by flow cytometry (** p<0.01, Student's t-test). (E) Sera IgE levels determined by ELISA (** p<0.01, Student's t-test). (F) Immunohistochemical detection of CD23 (* p<0.05, Student's t-test) on lung sections by using anti-CD23 mAb. Data are mean ± SEM, $n=6$ mice in each group.

Adoptive transfer of S1P-derived CD4+ T cells into naïve mice induces airway smooth muscle hyperreactivity and lung inflammation

In order to gain further insight into the cellular mechanisms, we isolated CD4+ T cells. CD4+ T cells were labelled at day 0 with CFSE (parent histogram; Figure 3.7A) and then cultured for 3 days in the presence or absence of CD3/CD28 beads. CD4+ T cells were isolated from the mediastinic lymph node of vehicle- (Figure 3.7B and 3.7C), S1P- (Figure 3.7D and 3.7E), IgG- (Figure 3.7F), anti-CD23- (Figure 3.7G and 3.7H), or S1P+anti-CD23- (Figure 3.7I and 3.7J) treated mice. CD4+ T cells harvested from S1P-treated mice (Figure 3.7E) displayed a basal increase in the proliferation rate as compared with vehicle-treated mice (Figure 3.7C), even in the absence of CD3/CD28 beads (Figure 3.7B and 3.7D). We observed that CFSE+CD4+ T cells proliferated for up to seven generations when they were obtained from S1P- (Figure 3.7D and 3.7E) but not vehicle-treated mice (Figure 3.7B and 3.7C). The proliferation rate of CD4+ T cells induced by S1P was not affected by anti-CD23 treatment (Figure 3.7I and 3.7J). IgG- (Figure 3.7F), or anti-CD23- (Figure 3.7G and 3.7H) derived CD4+ T cells did not show any significant increase in the proliferation rate. These results imply that the role of CD23 *in vivo* is to regulate negatively IgE production during S1P exposure without a direct significant effect on T cell growth or differentiation. These latter data fit well with the lack of effect of anti-CD23 in regulating IL-4 expression. To determine whether S1P could directly affect the proliferation of CD4+ T cells, we incubated CD4+ T cells obtained from vehicle-treated mice with S1P. In these experimental conditions, there was no detectable increase in the proliferation index (data not shown). Finally, adoptive transfer of CD4+ T cells, harvested from the mediastinic lymph node of S1P-treated mice, into naïve mice was performed. S1P-derived CD4+ T cell adoptively transferred into naïve mice increased mast cell infiltration (Figure 3.8A), bronchial reactivity (Figure 3.8B), lung inflammation (Figure 3.8C), IL-4 (Figure 3.8D), and IL-13 (Figure 3.8E) release. Conversely, adoptive transfer of CD4+ T cells, harvested from mice treated with anti-CD23 and S1P reversed airway smooth muscle hyperreactivity (Figure 3.8B), lung inflammation (Figure 3.8C), and

IL-13 up-regulation (Figure 3.8E). In agreement with previous data (Figure 3.4), neither mast cell infiltration (Figure 3.8A) nor IL-4 up-regulation (Figure 3.8D) were modified.

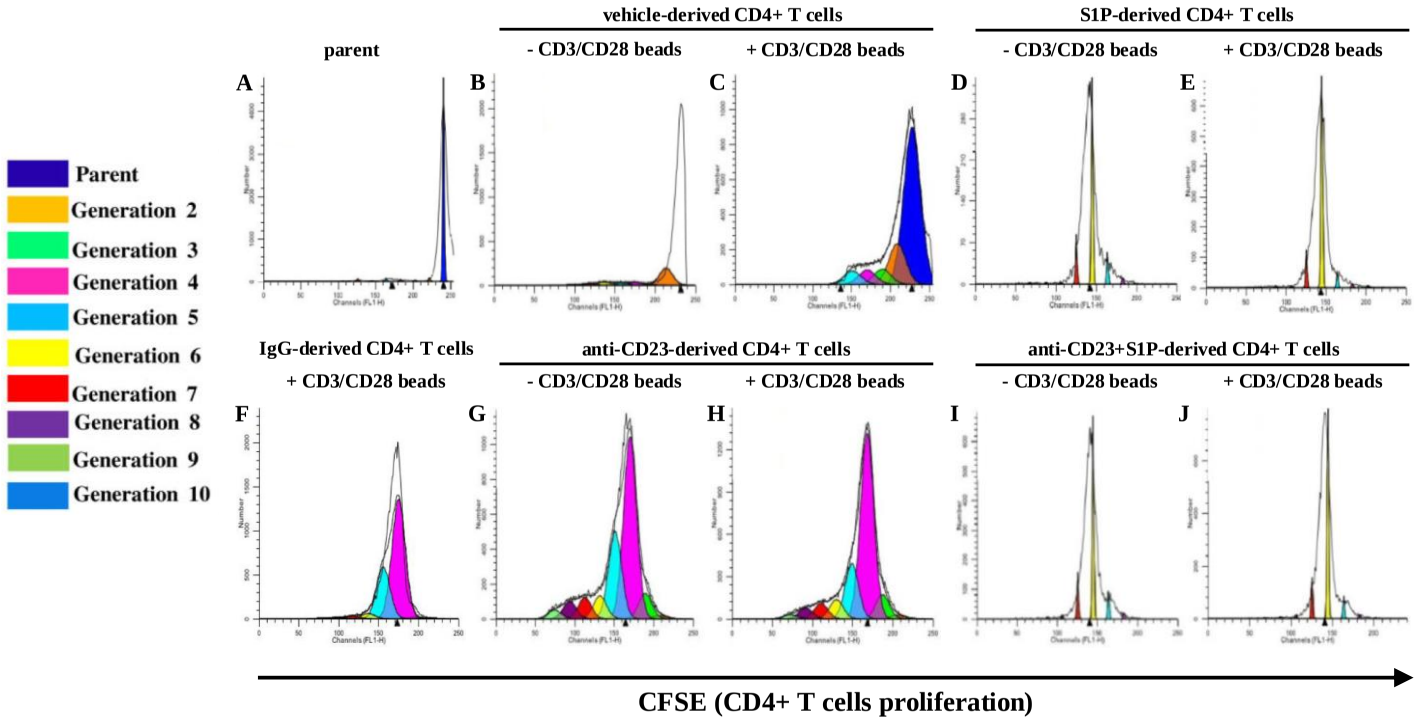


Figure 3.7. Lymphocytes harvested from S1P-treated BALB/c mice have an increased ability to proliferate. CD4+ T cells were isolated from the mediastinic lymph node of vehicle-, S1P-, IgG- or S1P+anti-CD23-treated mice. CD4+ T cells were labelled on day 0 with CFSE (parent histogram, panel A) and then cultured for 3 days in the presence (C, E, F H, J) or absence (B, D, G, I) of CD3/CD28 beads. Histograms in panels B to J represent the proliferation of CD4+ T cells isolated from the lymph node of vehicle- (B and C), S1P- (D and E), IgG- (F), anti-CD23- (G and H) and S1P+anti-CD23- (I and J) treated mice. Experiments were performed at three different experimental times. The histograms reported are representative. Data were analysed by mean of ModFlt3 program (BD FACSCalibur).

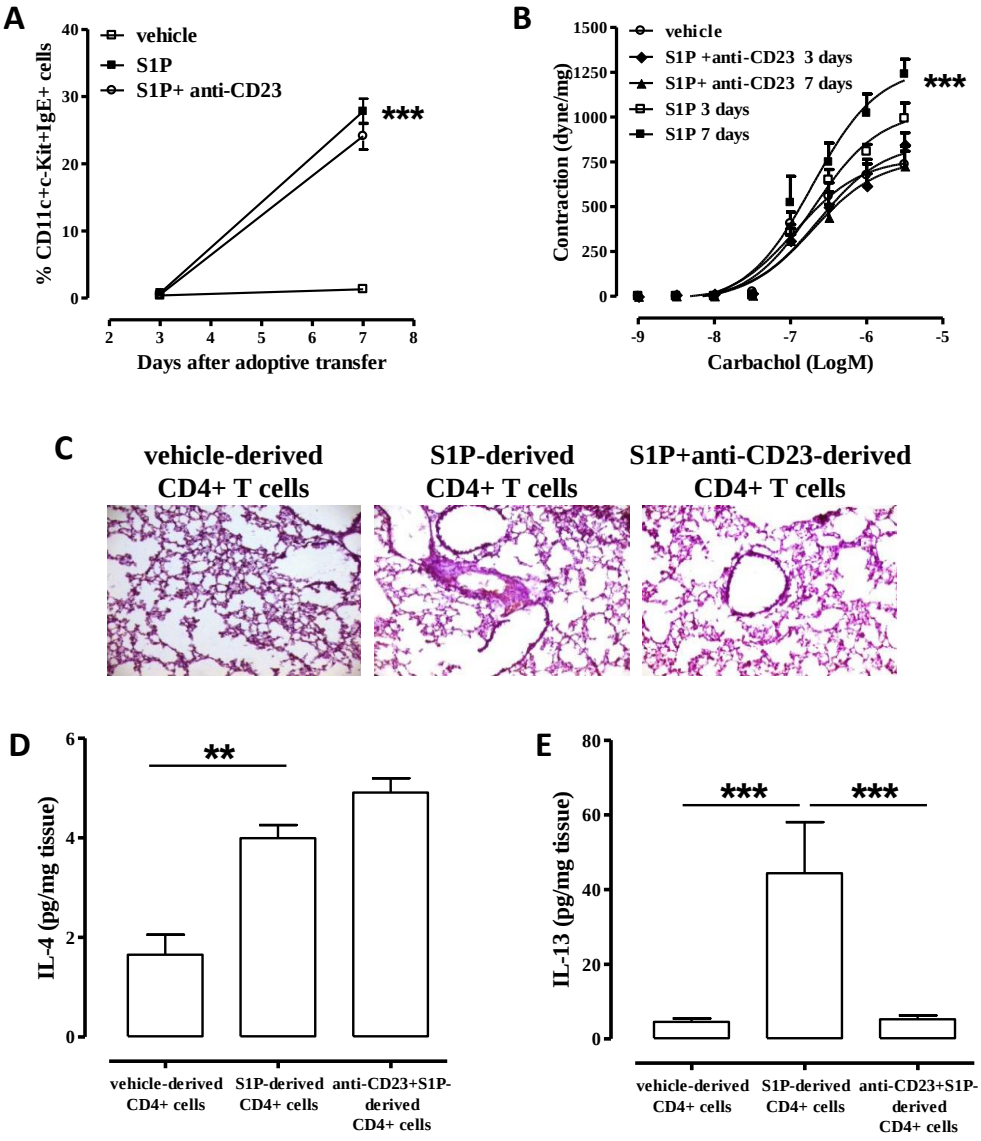


Figure 3.8. Adoptive transfer of CD4+ T cells from S1P-treated mice into naïve (untreated) mice mimics S1P-induced effects in BALB/c mice. Adoptive transfer of CD4+ T cells derived from vehicle-, S1P- or anti-CD23+S1P-treated mice into naïve BALB/c mice was performed. Mice were killed at 3 and 7 days following adoptive transfer. (A) Mast cells were identified as CD11c+cKit+IgE+ cells by flow cytometry (*** p<0.001 *vs.* vehicle, Student's *t*-test). (B) Assessment of bronchial response to carbachol (*** p<0.001 *vs.* vehicle, two-way ANOVA with Bonferroni's post-test). (C) PAS staining of lung sections harvested from mice after 7 days of adoptive transfer. (D) Pulmonary levels of IL-4 (** p<0.01, Student's *t*-test). (E) Pulmonary levels of IL-13 (*** p<0.001, one-way ANOVA with Bonferroni's post-test). Data are mean ± SEM, *n=6* mice in each group.

S1P/SphK1/2-dependent signalling is involved in B cell recruitment during OVA-induced airway hyperreactivity and inflammation

We already demonstrated that S1P/SphK1/2 signalling is involved in airway hyperresponsiveness in OVA-sensitised mice[294]. Indeed, the injection of a SphK1/2 inhibitor in OVA-treated mice significantly reduced airway reactivity to carbachol (Figure 3.9A). Because allergic airway diseases are well characterised by the high levels of immunoglobulins, we went on by evaluating the presence of B cells in the lung of OVA-sensitised mice. The percentage of B cells (identified as CD19+B220+ cells) in the lung of OVA-sensitised mice was significantly higher than control mice (Figure 3.9B) at earlier time points. Interestingly, we observed a significant reduction of B cells in the lung of OVA-sensitised mice treated with SphK1/2 inhibitor (Figure 3.9B), implying that S1P axis is responsible of B cell recruitment during allergic airway inflammation. In addition, the injection of a SphK1/2 inhibitor markedly reduced OVA-induced IL-13 (Figure 3.9C), IL-10 (Figure 3.9D), and TGF-β (Figure 3.9E). These data further confirm the role of S1P axis in airway allergic Th2-like responses.

S1P-mediated hyperresponsiveness is associated to higher presence of B cells in the lung

In order to further clarify the role of S1P signalling in B cells recruitment during sensitization, we went on exposing BALBC/c mice to S1P (10 ng/mouse). In our previous study, we already demonstrated that S1P exogenous administration, as well as OVA, increases airway responsiveness and inflammation in a time- and dose-dependent manner[427]. Similarly, in this study we observed that the treatment with S1P induced higher responsiveness to carbachol starting at day 3 (data not shown) up to 14 days (Figure 3.9F), when statistical differences were appreciated. This effect was similar to what happened in OVA-sensitised mice (Figure 3.9A). In addition, the release of IL-13, well-known airway allergic cytokine[436], was significantly increased in lung homogenates of mice treated with S1P compared to vehicle mice (Figure 3.9G). Interestingly, B cells significantly accumulated into the lung of S1P-treated mice at day 3, 7, and 14, compared to vehicle mice (Figure 3.9H), similarly to what happened to OVA-sensitised mice (Figure 3.9B). These data imply that S1P axis is involved in lung B cell recruitment during allergic airway inflammation.

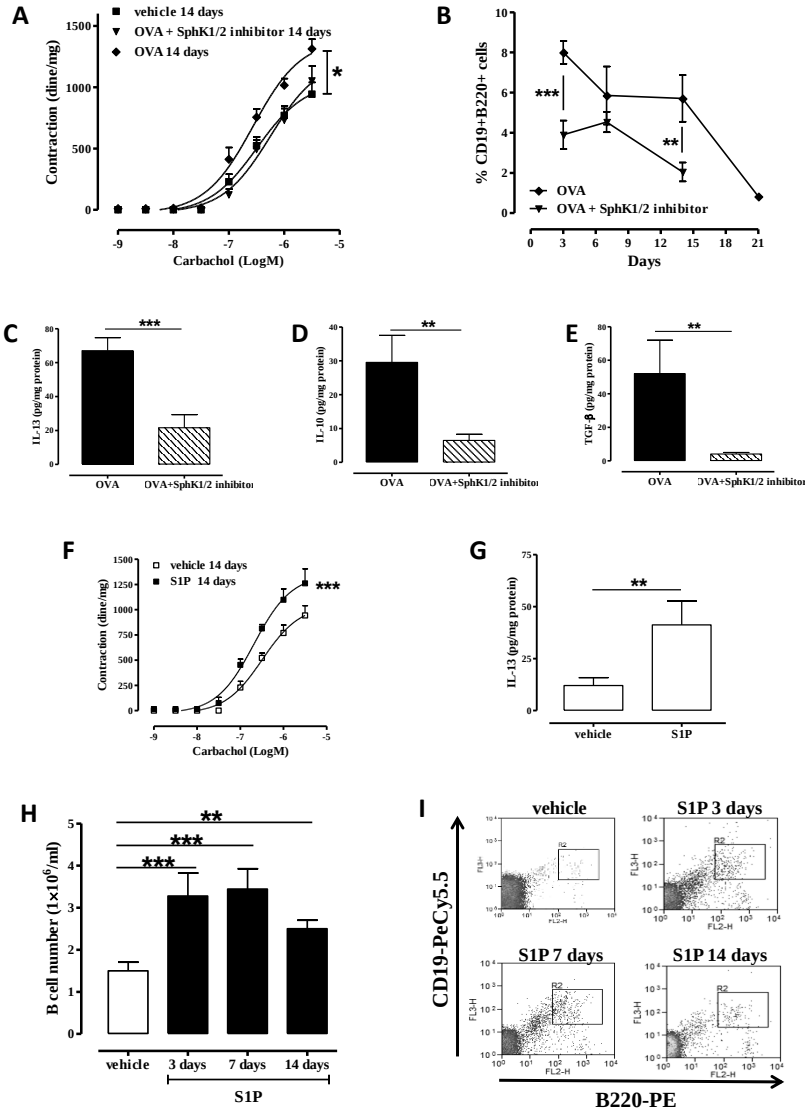


Figure 3.9. S1P-mediated airway inflammation is similar to that observed in OVA-treated mice. BALB/c mice were s.c treated with OVA (100 µg/mouse) or S1P (10 ng/mouse) at day 0 and day 7. (A) Bronchi of OVA-treated mice showed higher reactivity to cumulative administration of carbachol (10^{-9}-3×10^{-6} M), expressed as contraction ability (dine/mg), compared to vehicle (BSA 0.001%) and SphK1/2 inhibitor-treated mice (* p<0.05, two-way ANOVA with Bonferroni's post-test). (B) B cell (identified as CD19+B220+ cells) percentage was significantly increased in the lung of OVA-treated mice compared to mice treated with SphK 1/2 inhibitor (** p<0.01, *** p<0.001, Student's t-test). Lung homogenates were tested for Th2-like cytokine levels such as (C) IL-13, (D) IL-10, and (E) TGF-β (** p<0.01, *** p<0.001, Student's t-test). (F) Bronchi of S1P-treated mice showed higher reactivity to cumulative administration of carbachol (*** p<0.001 *vs.* vehicle, two-way ANOVA with Bonferroni's post-test), associated to (G) higher levels of IL-13 (** p<0.01, Student's t-test), but also to (H) higher B cell recruitment to the lung (** p<0.01, *** p<0.001, Student's t-test), as reported for OVA-treated mice. B cell recruitment is shown as B cell count (1×10^6/ml). (I) Representative dot plot of lung CD19+B220+ gated cells. Data are mean ± SEM, *n=9* mice in each group. Experiments were performed in two different experimental days.

S1P-mediated hyperresponsiveness is associated to an immune-suppressive environment

To further dissect the role of leukocytes in our mouse model, we went on and analysed the presence of immune-suppressive cells such as Treg cells. Treg were identified by means of flow cytometry analysis and gated as CD3+CD4+CD25+FoxP3+ cells. The administration of S1P significantly increased the levels of Treg into the lung only after 7 and 14 days post the first administration of S1P (Figure 3.10A) when B cell presence was reduced. This effect was also observed in OVA-sensitised mice (data not shown). In addition, the levels of IL-10 (Figure 3.10C) and TGF-β (Figure 3.10D), well-known immune-suppressive cytokines, were significantly increased in lung homogenates of S1P-treated mice sacrificed at 14 days. These cytokines were not detected at earlier time points (3-7 days) after S1P treatment. These data imply that S1P treatment can increase Treg recruitment, which has already been observed for OVA-sensitised mice[437].

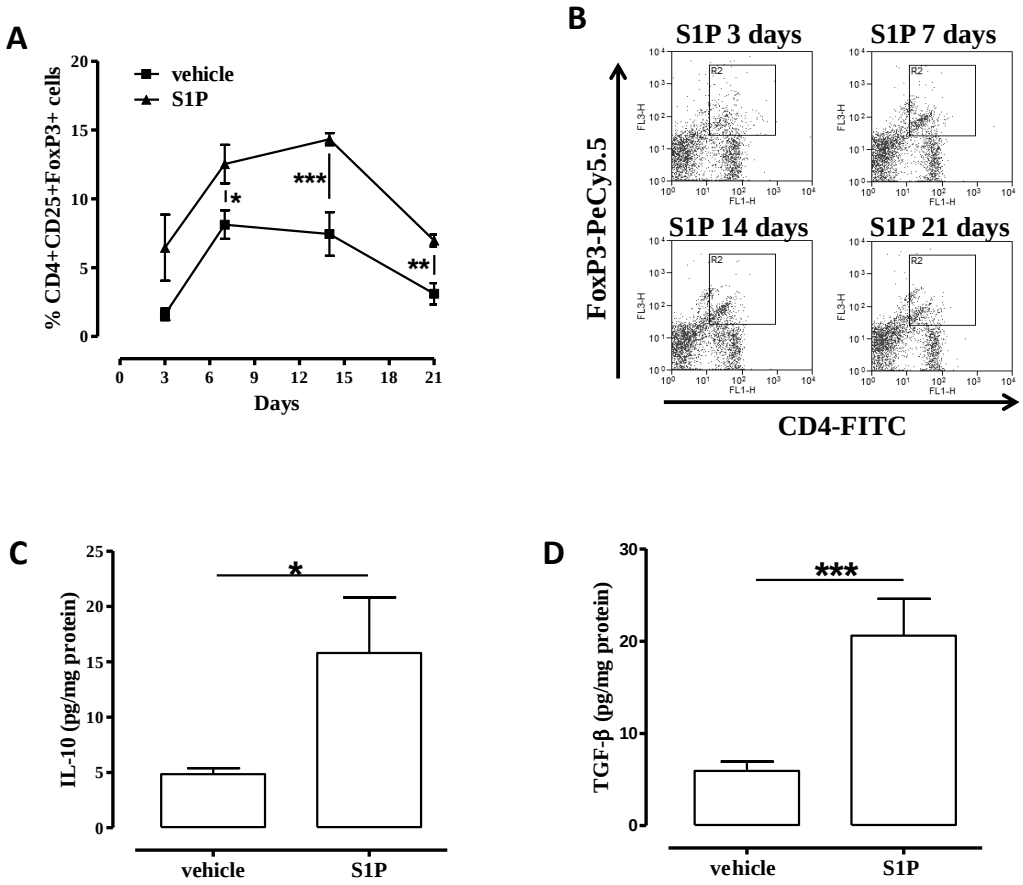


Figure 3.10. S1P treatment promotes the recruitment of Treg cells. (A) Increase of CD4-FITC, CD25-PE and FoxP3-PeCy5.5 positive Treg cells in the lung of vehicle and S1P-treated mice (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, Student's *t*-test). (B) Representative flow cytometry dot plot are reported. (C) IL-10 and (D) TGF-β levels in lung homogenates obtained from vehicle and S1P-treated mice sacrificed at 14 days (* $p<0.05$, *** $p<0.001$, Student's *t*-test). Data are mean ± SEM, *n=9* mice in each group. Experiments were performed in two different experimental days.

S1P-pulsed B cells behave as B regulatory cells

Our previous data demonstrated that S1P increased B cell influx to the lung of BALBC/c mice (Figure 3.9H). In order to understand the role of S1P axis, we isolated lung B cells after S1P or vehicle treatment. Lung-derived B cells expressed sphingosine kinase, SphK1 (Figure 3.11A), as already reported by Pi *et al.*[438]. No differences were observed in SphK1 expression in B cells obtained from the lung of both vehicle and S1P-treated mice isolated at different time points, implying that this kinase is physiologically present in these cells. In addition, the levels of the most relevant receptor for S1P, the $S1P_1$, was equally expressed on B cells derived from vehicle-, S1P-, and OVA-treated mice (Figure 3.11B).

In order to understand the phenotype of S1P-pulsed B cells, *in vitro* experiments were performed by using lung-derived B cells isolated from vehicle mice. S1P treatment (0.1-1 μM) of vehicle mice-derived lung B cells increased the levels of IL-10 (Figure 3.11C) and TNF-α (Figure 3.11D) in a significant manner. In contrast, the addition of S1P on vehicle-derived lung B cells did not increase the release of the cytotoxic protease granzyme B (Figure 3.11E) nor IL-13, a Th2-like cytokine (Figure 3.11F). However, B cells isolated from the lung of S1P-treated mice at 7 and 14 days showed significant release *in vitro* of both granzyme B (Figure 3.11D) and IL-13 (Figure 3.11E), implying that another signal was essential to induce these cells to release harmful mediators such as granzyme B and IL-13. Because of the well-described activity of B cells as antigen-presenting cells (APCs)[439], we performed flow cytometry analysis for cell surface markers. Again, B cells were isolated from vehicle- and S1P-treated mice at different time points. At 3 days post S1P or vehicle injection, the stimulation of vehicle-derived B cells with S1P *in vitro* showed higher levels of MHC I (Figure 3.12A) and MHC II (Figure 3.12B). Instead, B cells from S1P-treated mice did not show any increase in MHC I (Figure 3.12A) and MHC II (Figure 3.12B) after S1P addition. However, the basal levels of MHC I (Figure 3.12A) and MHC II (Figure 3.12B) on B cells from S1P-treated mice were already significantly higher than those from vehicle mice, implying that B cells from S1P-treated mice were already in their active phenotype.

Similarly, vehicle-derived B cells obtained at 7 days showed a significant increase of MHC I (Figure 3.12C) and MHC II (Figure 3.12D) under S1P stimulation. However, at this time point, B cells obtained from the lung of S1P-treated mice showed a significant increase in MHC I (Figure 3.12C) and MHC II (Figure 3.12D) levels after S1P treatment. However, it is to note that the stimulation *in vitro* with S1P (0.1–1 μM) of B cells obtained from mice treated *in vivo* with S1P for 3 days, had a reduced expression of MHC I (Figure 3.12A) and MHC II (Figure 3.12B), compared to the basal conditions. In addition, the different behaviour of B cells obtained from the lung of S1P-treated mice at 3 and 7 days may be due to the administration of S1P that was performed 7 days before the isolation. Interestingly, B cells isolated from the lung of S1P- or vehicle-treated at 14 days did not show any differences in both the levels of MHC I (Figure 3.12E) and MHC II (Figure 3.12F). In contrast, higher levels of CD80 were detected on S1P-derived B cells compared to vehicle at 14 days (Figure 3.12G). These data imply that, although B cells do not present antigens via MHC I-II overexpression at day 14, they can interact with T cells via CD80.

To understand the role of lung-derived B cells from mice treated with vehicle or S1P on T cells, we performed mixed lymphocyte reaction experiments *in vitro*. B cells obtained from the lung of vehicle mice were first treated with S1P or PBS and then added to CD4+ or CD8+ T cells obtained from the spleen of naïve (non-treated) mice. CFSE-marked CD4+ T cells alone did not show any increase in proliferation when S1P was added (Figure 3.13A). Similarly, CD8+ T cells did not proliferate (Figure 3.13F). In contrast, B cells obtained from mice exposed to S1P and sacrificed at 14 days induced higher proliferation rate of both CD4+ (Figure 3.13A) and CD8+ T cells (Figure 3.13F), although in the absence of an additional (exogenous) stimulus. This effect was associated to the higher release of TGF-β (Figure 3.13B) and IL-10 (Figure 3.13C) from B cells harvested from the lung of S1P-treated mice sacrificed at 14 days when added to naive CD4+ T cells. The levels of these immunosuppressive cytokines was higher than that detected in the supernatant of B cells alone (Figure 3.13B and 3.13C). In contrast, we did not

detect any increase in IFN-γ (data not shown). In addition, to understand the phenotype of CD4+ T cells after the co-culture with B cells, we went on by analysing the expression levels of FoxP3, a well-known Treg transcription factor. Indeed, FoxP3 expression was significantly higher in CD4+ T cells that were co-cultured with B cells derived from the lung of S1P-treated mice compared to vehicle mice (Figure 3.13D and 3.13E). Similar to CD4+ T cells, B cells obtained from both vehicle- and S1P-treated mice were added to CD8+ T cells derived from naïve mice. Again, we observed higher proliferation rate after the addition of CD8+ T cells to B cells from S1P-treated mice (Figure 3.13F), associated to higher release of the suppressive cytokine IL-10 (Figure 3.13G). These data suggest the ability of S1P to lead B cells toward a regulatory phenotype. Besides their APC activity, B cells behave as immunoglobulin-producing cells[419,439].

To evaluate the role of S1P on plasma cell-like B cells, we analysed the levels of the surface marker CD20, which is predominantly present on immunoglobulin-producing cells[419]. B cells isolated from the lung of mice treated with S1P and sacrificed at 3 (Figure 3.14A) and 7 days (Figure 3.14B) did not show any statistical difference in CD20 expression either in the presence or not of S1P *in vitro*. Instead, B cells isolated from the lung of S1P-treated mice sacrificed at 14 days had significantly higher basal levels of CD20 (Figure 3.14C) compared to vehicle. Conversely, the administration of S1P *in vitro* on B cells derived from vehicle- or S1P-treated mice did not alter CD20 levels (Figure 3.14C). In addition, B cells from S1P-treated mice and sacrificed at 14 days showed higher levels of IgA (Figure 3.14D), but not IgG2a (Figure 3.14E) and IgM (Figure 3.14F). Taken altogether, these data suggest that S1P can increase MHC I, MHC II, and CD20 expression on B cells, which in their regulatory phenotype are associated to the polarization of T cells toward IL-10-producing T cells and an immunosuppressive environment, as also demonstrated by the higher release of the antiinflammatory activity of IgA-producing B cells.

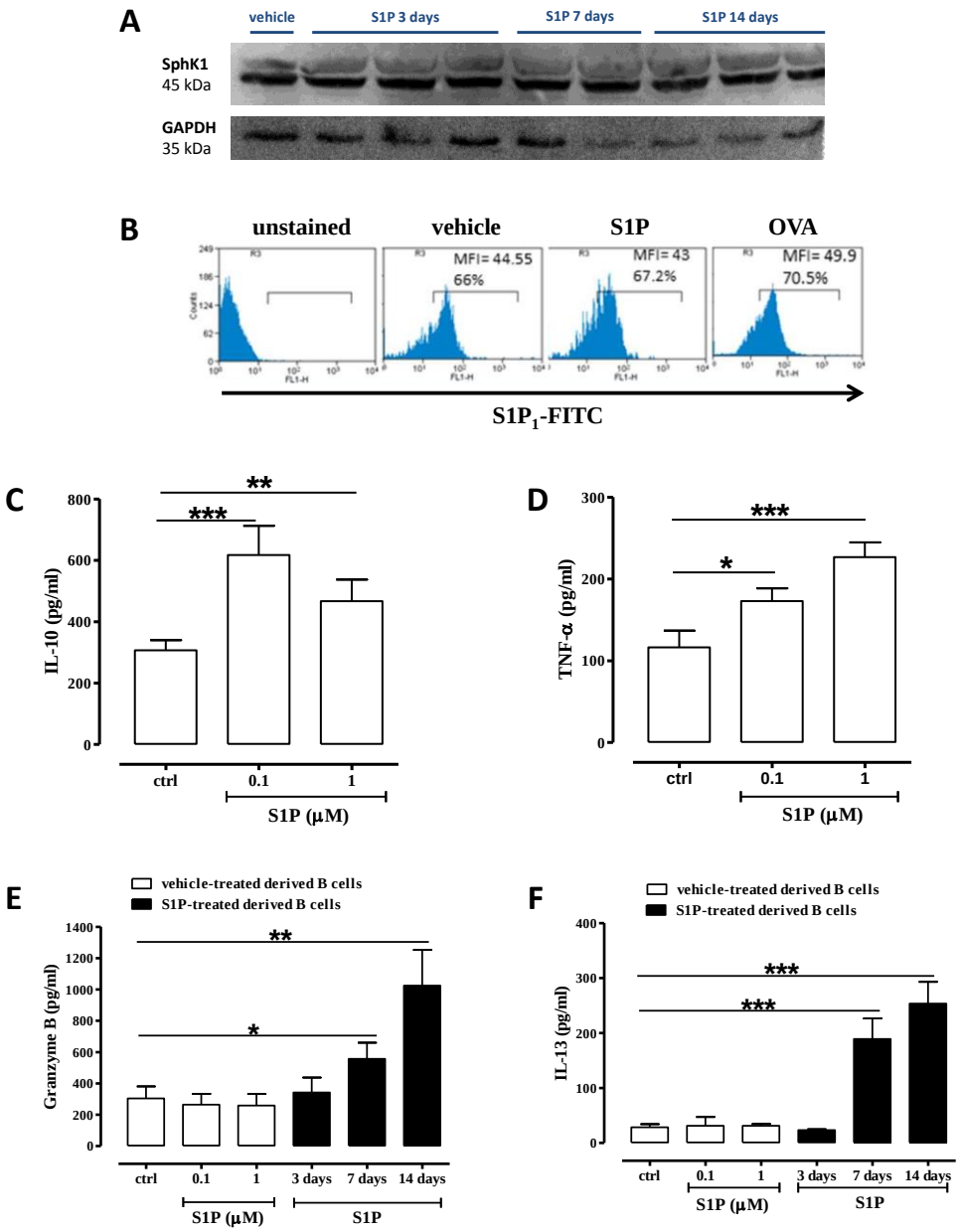


Figure 3.11. Treatment of B cells with S1P. Lung-derived B cells (0.5×10^5 cells/well) from vehicle and S1P-treated mice were isolated and then treated *in vitro* with S1P (0.1-1 µM) overnight. (A) The expression of SphK1 and (B) $S1P_1$ was equally observed on B cells isolated by both vehicle- and S1P-treated mice at different time points. S1P stimulation of vehicle-derived lung B cells promoted a significant release of (C) IL-10 and (D) TNF-α, but not of (E) granzyme B and (F) IL-13; in contrast, lung B cells obtained from S1P-treated mice, sacrificed at day 14, were able to release (E) granzyme B and (F) IL-13, even in the absence of an external stimulus (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, Student's *t*-test). Data are mean ± SEM, *n=6* mice in each group. Experiments were performed in two different experimental days.

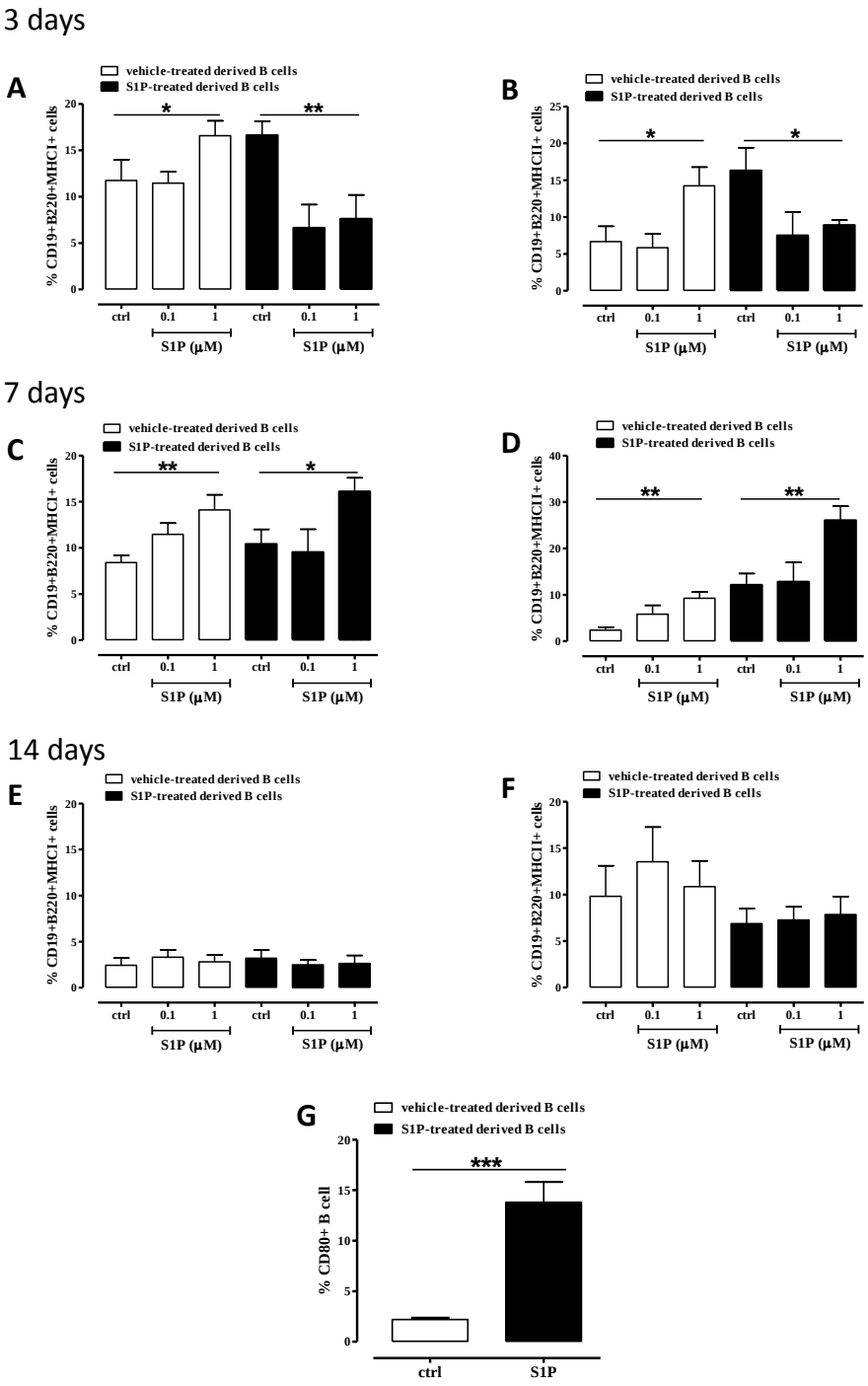


Figure 3.12. Stimulation of lung-derived B cells with S1P increases MHC I and MHC II expression at early time points (3-7 days). (A) Expression of MHC I and (B) MHC II at 3 days on lung-derived B cells (identified as CD19+B220+) obtained from vehicle- and S1P-treated mice (* $p<0.05$, ** $p<0.01$, Student's *t*-test). (C) Higher expression of MHC I and (D) MHC II at 7 days on lung-derived B cells (identified as CD19+B220+) obtained from vehicle- and S1P-treated mice (* $p<0.05$, ** $p<0.01$, Student's *t*-test). No statistical differences were observed in MHC I (E) and MHC II (F) on lung-derived B cells obtained at 14 days. (G) CD80 expression was higher on B cells obtained from S1P-treated mice at day 14 (*** $p<0.001$, Student's *t*-test). Data are mean ± SEM, *n=6* mice in each group. Experiments were performed in two different experimental days.

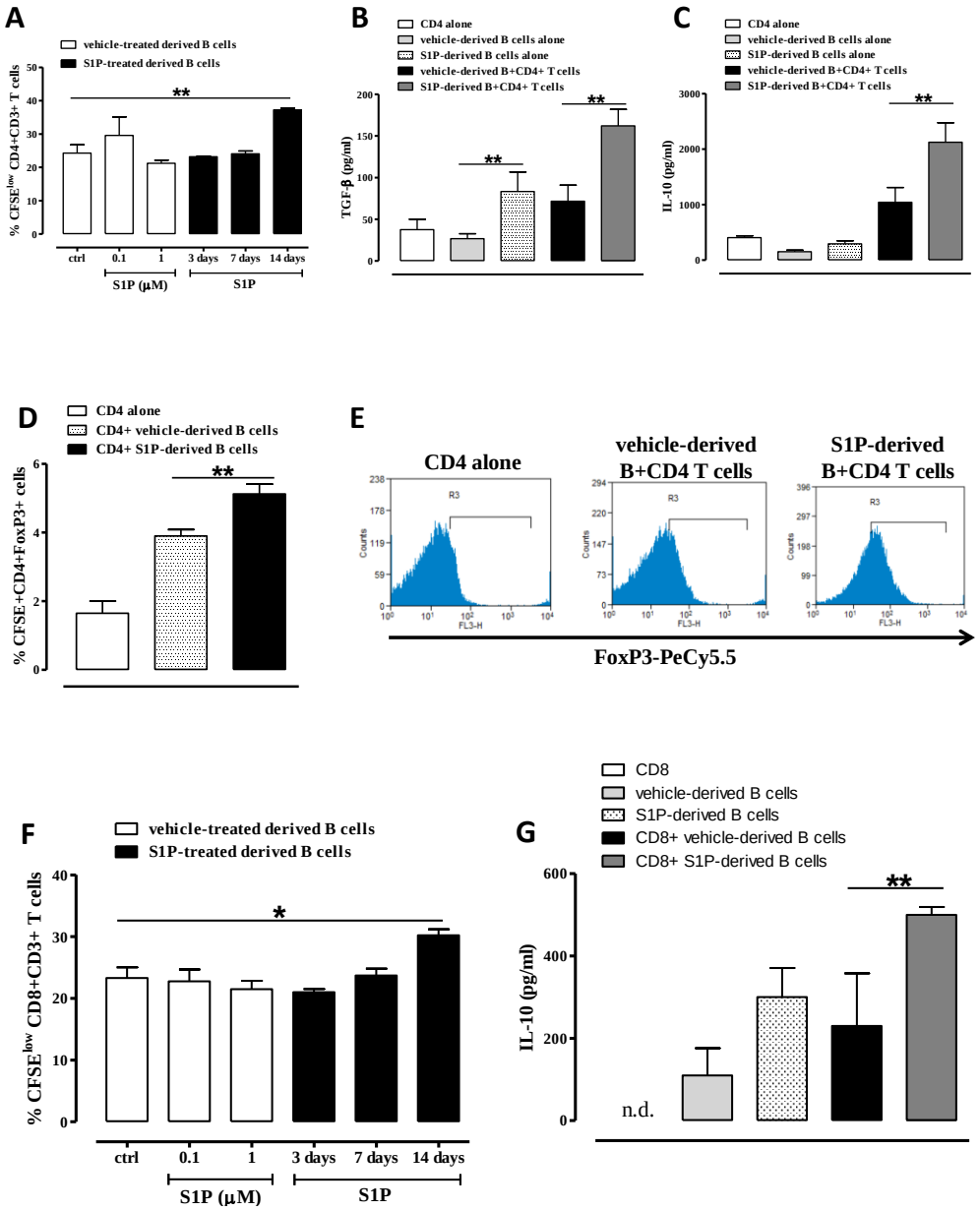


Figure 3.13. Lung-derived B cells from S1P-treated mice increased T cell proliferation and IL-10 release. (A) Lung B cells (identified as CD19+B220+) were harvested from both vehicle- and S1P-treated mice and then treated *in vitro* with PBS or S1P (0.1-1 µM) and then co-cultured for 24 and 72 hours with splenic naive CD4+ (ratio 1:10) (** $p<0.01$, Student's *t*-test). Cell-free supernatant was tested for (B) TGF-β and (C) IL-10 release by means of ELISA. T cell proliferation (72 hours) was tested by using flow cytometry analyses of CFSE (** $p<0.01$, Student's *t*-test). (D) The expression of FoxP3 was tested by means of flow cytometry after the co-culture of vehicle- or S1P-derived lung B cells with naïve (non-treated) CD4+ T cells (** $p<0.01$, Student's *t*-test). (E) Representative histograms for FoxP3 expression by means of FACS. (F) Similar to CD4+ T cells, CD8+ T cells were co-cultured with B cells obtained from vehicle and S1P-treated mice (ratio 1:10) in order to evaluate the proliferation rate and the release of suppressive cytokine, such as (G) IL-10 (* $p<0.05$, ** $p<0.01$, Student's *t*-test). Data are mean ± SEM, *n=6* mice in each group. Experiments were performed in two different experimental days.

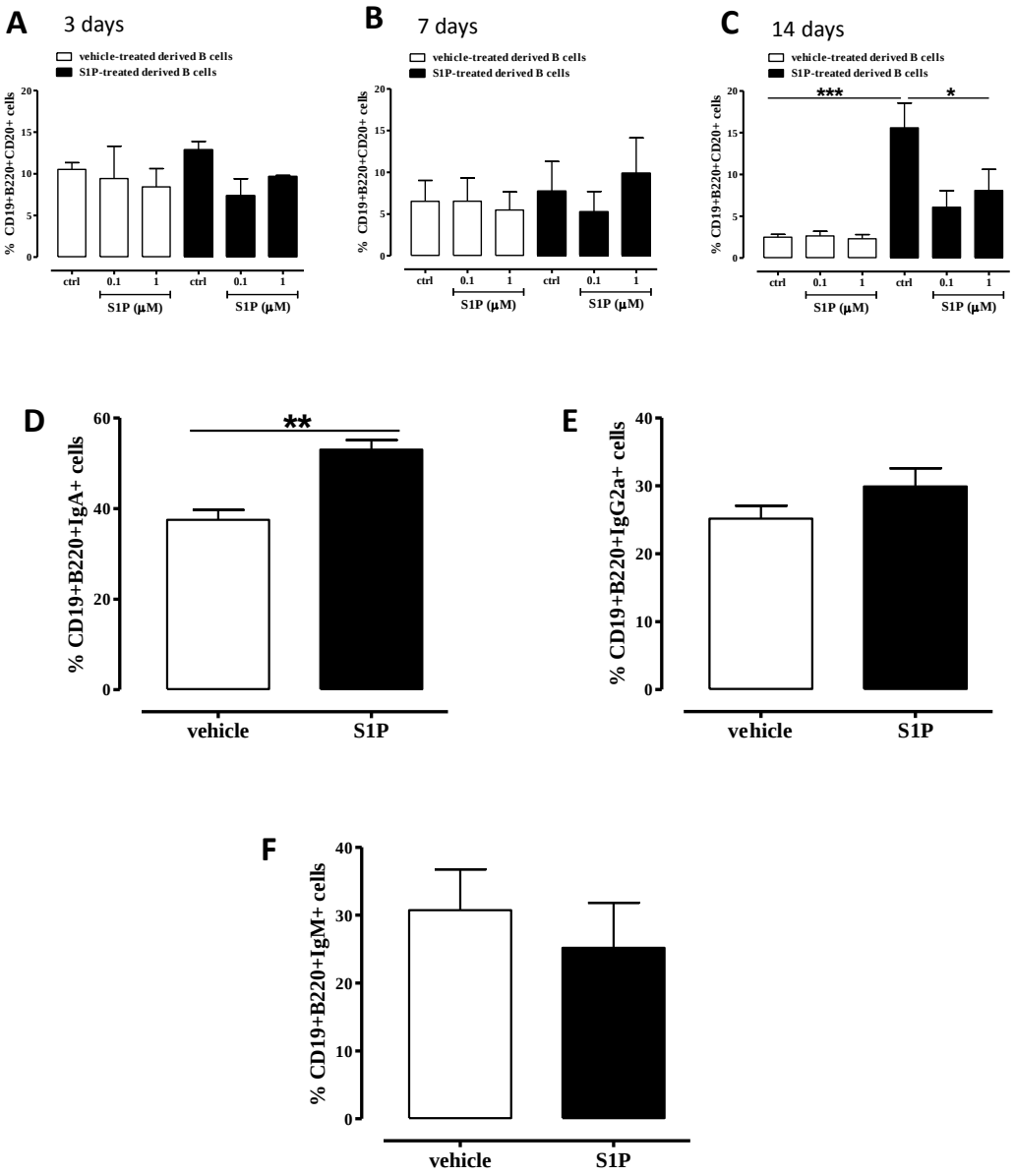


Figure 3.14. Stimulation of lung-derived B cells with S1P increased CD20 expression at later time points (14 days). Expression of CD20 after *in vitro* stimulation with S1P (0.1-1 µM) of lung-derived B cells obtained from both vehicle- and S1P-treated mice at (A) 3, (B) 7, and (C) 14 days (* $p<0.05$, *** $p<0.001$, Student's *t*-test). S1P treatment *in vivo* increased lung (D) CD19+B220+IgA+ cells, but not (E) CD19+B220+IgG2a+ and (F) CD19+B220+IgM+ cells (** $p<0.01$, Student's *t*-test). Data are mean ± SEM, *n=6* mice in each group. Experiments were performed in two different experimental days.

Depletion of CD20+ B cells increases S1P-induced airway hyperresponsiveness and inflammation

Our previous results showed that the exposure of mice to S1P increased the surface expression of CD20 on B cells at 14 days (Figure 3.14C). Since CD20 is a marker for mature B cells[440], we wanted to understand the role of these cells in our experimental protocol. Mice were treated with a specific anti-CD20 mAb prior S1P or vehicle administration. Anti-CD20 mAb was injected every three days to deplete mature B cells[419,440]. The depletion of CD20+ B cells was approximately of 90%, as already demonstrated[419]. The absence of CD20+ B cells increased S1P-induced airway responsiveness to carbachol (Figure 3.15A), compared to anti-CD20 mAb alone or vehicle-treated mice. In addition, PAS-stained lung cryosections showed that the depletion of CD20+ B cells further increased lung inflammation in S1P-treated mice compared to S1P alone and vehicle. Indeed, the number of goblet cells was significantly increased in S1P-treated mice depleted of CD20+ B cells compared to mice solely treated with S1P (Figure 3.15B). The injection of isotype control IgG or anti-CD20 mAb alone did not alter lung morphology (Figure 3.15B), as already published[419]. Importantly, the presence of Treg was significantly reduced at all time-points (from 3 up to 14 days) in the lung of anti-CD20 mAb+S1P-treated compared to S1P-treated mice (Figure 3.15C). To confirm the absence of Treg, we tested the levels of IL-10 and TGF-β. Again, the antibody-mediated depletion of CD20+ B cells reduced both IL-10 (Figure 3.15D) and TGF-β (Figure 3.15E) in the lung of S1P-treated mice.

Taken altogether, these results imply that B cells counteract S1P-mediated airway hyperresponsiveness and inflammation via the induction of an immunosuppressive environment with the attempt to affect asthma-like outcomes.

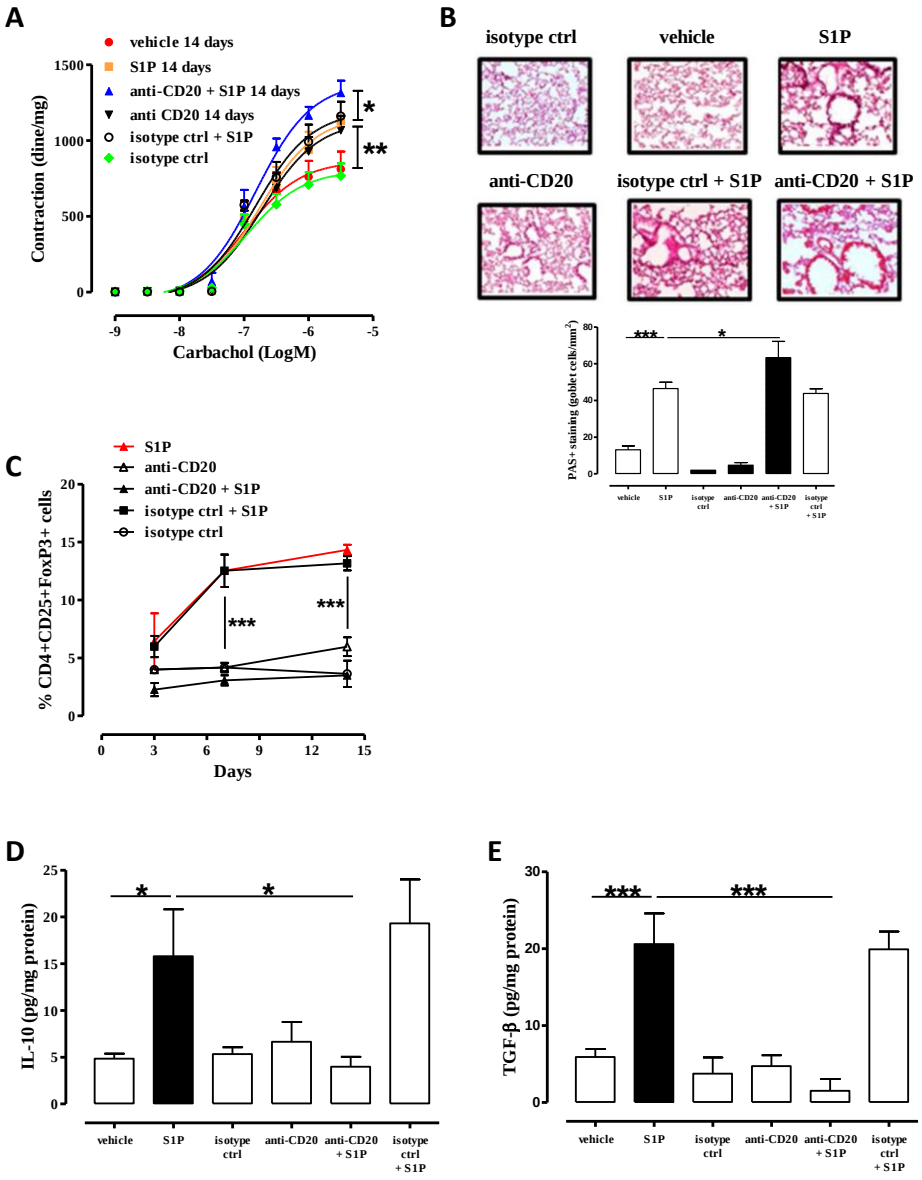


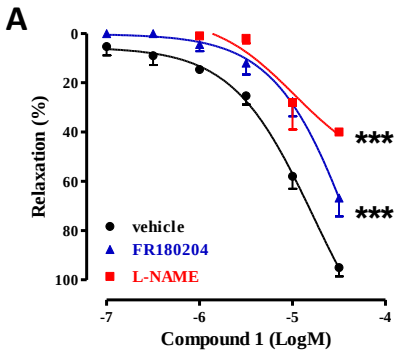
Figure 3.15. Depletion of CD20+ B cells increases S1P-induced lung airway inflammation. BALB/c mice were s.c treated with S1P (10 ng/mouse) at day 0 and day 7. Another group of mice was treated i.p. with anti-CD20 mAb (250 µg/mouse) to deplete CD20+ B cells at day 0, 3, 7, 10, and 14, or with isotype control Ab (rat IgG) or isotype control Ab+S1P. The anti-CD20 mAb was injected the same day, but 30 minutes before the s.c. injection of S1P or vehicle. (A) Bronchi of anti-CD20+S1P-treated mice showed higher reactivity to cumulative administration of carbachol (10^{-9}-3×10^{-6} M), expressed as contraction ability (dine/mg), compared to S1P-, anti-CD20-, or vehicle-treated mice (* p<0.05, ** p<0.01, two-way ANOVA with Bonferroni's post-test). (B) Upper panels: representative pictures of PAS-stained lung cryosections. Lower panel: goblet cell (PAS+ cells) quantitation per square millimeter of the bronchial basement membrane (* p<0.05, *** p<0.001, Student's t-test). (C) Lower influx of CD4+CD25+FoxP3+ T cells in the lung of anti-CD20+S1P-treated mice compared to S1P-treated mice (*** p<0.001, Student's t-test). Lung homogenates were tested for (D) IL-10 and (E) TGF-β (* p<0.05, *** p<0.001, Student's t-test). Data are mean ± SEM, n=6 mice in each group. Experiments were performed in two different experimental days.

Assessment of a new pepducin-derived $S1P_3$ antagonist in vitro

As an endogenous metabolite, S1P acts as a full agonist on all the $S1P_{1-5}$ receptors. The study of the role of these receptors is limited by the reduced availability of specific receptor agonists or antagonists. In this context our interest has been focused on $S1P_3$ becasue of its main involvement in airway diseases.

The well-described pepducin KRX-725 (Compound 1), derived from the second intracellular loop of $S1P_3$ receptor, mimics the effect of S1P, triggering specifically the $S1P_3$ receptor. In our experimental design, the effectiveness of the peptide to modulate the $S1P_3$ receptor activation was assessed by determining its ability to affect vascular tone. First, we have characterised the Compound 1-induced vasorelaxation. $S1P_3$ induces G_i-dependent ERK activation and a nitric oxide-mediated relaxation. In order to assess the ability of Compound 1 to induce a vasorelaxation with a similar mechanism of signalling transduction, we tested the ability of L-NAME (100 µM), a NOS inhibitor, or FR180204 (10 µM), an ERK inhibitor, to affect the Compound 1-induced effect on vascular tone. As shown in Figure 3.16A, both L-NAME and FR180204 significantly inhibited the Compound 1-induced vasorelaxation. These data confirmed that, also in our experimental conditions, Compound 1 activated the same signalling pathway, although triggering $S1P_3$ intracellularly.

Starting from the native pepducin, the group of Professors V. Santagada and G. Caliendo has performed an Ala-scan on KRX-725 to identify new compounds that could act as antagonists (Figure 3.16B). Unfortunately, none of these showed such activities (data not shown). Despite of these findings, a collection of deleted peptides from both the C- and N-terminus of KRX-725 (Figure 3.16C) had more promising properties. In particular, the Compound 16 showed $S1P_3$-antagonist properties: incubation of increasing concentrations of Compound 16 (1, 10, and 100 µM) on PE-precontracted aortic rings harvested from naïve mouse and relaxed with KRX-725 (10^{-7}-3×10^{-5} M) showed that Compound 16 reduced in a concentration-dependent manner the vasorelaxation provoked by the native pepducin (Figure 3.16D).



B KRX-725 (Compound 1) and its Ala-scan derivatives (2–10)

Compound	Peptide sequence
1	myristoyl-Gly-Met-Arg-Pro-Tyr-Asp-Ala-Asn-Lys-Arg-NH_2
2	myristoyl-**Ala**-Met-Arg-Pro-Tyr-Asp-Ala-Asn-Lys-Arg-NH_2
3	myristoyl-Gly-**Ala**-Arg-Pro-Tyr-Asp-Ala-Asn-Lys-Arg-NH_2
4	myristoyl-Gly-Met-**Ala**-Pro-Tyr-Asp-Ala-Asn-Lys-Arg-NH_2
5	myristoyl-Gly-Met-Arg-**Ala**-Tyr-Asp-Ala-Asn-Lys-Arg-NH_2
6	myristoyl-Gly-Met-Arg-Pro-**Ala**-Asp-Ala-Asn-Lys-Arg-NH_2
7	myristoyl-Gly-Met-Arg-Pro-Tyr-**Ala**-Ala-Asn-Lys-Arg-NH_2
8	myristoyl-Gly-Met-Arg-Pro-Tyr-Asp-**Ala**-Ala-Lys-Arg-NH_2
9	myristoyl-Gly-Met-Arg-Pro-Tyr-Asp-Ala-Asn-**Ala**-Arg-NH_2
10	myristoyl-Gly-Met-Arg-Pro-Tyr-Asp-Ala-Asn-Lys-**Ala**-NH_2

C KRX-725 (Compound 1) and its deleted derivatives (11-20)

Compound	Peptide sequence
1	myristoyl-Gly-Met-Arg-Pro-Tyr-Asp-Ala-Asn-Lys-Arg-NH_2
11	myristoyl-Gly-------Arg-Pro-Tyr-Asp-Ala-Asn-Lys-Arg-NH_2
12	myristoyl-Gly----------Pro-Tyr-Asp-Ala-Asn-Lys-Arg-NH_2
13	myristoyl-Gly----------Tyr-Asp-Ala-Asn-Lys-Arg-NH_2
14	myristoyl-Gly----------Asp-Ala-Asn-Lys-Arg-NH_2
15	myristoyl-Gly-Met-Arg-Pro-Tyr-Asp-Ala-Asn-Lys-------NH_2
16	myristoyl-Gly-------Arg-Pro-Tyr-Asp-Ala-Asn----------NH_2
17	myristoyl-Gly-------Arg-Pro-Tyr-Asp-Ala----------NH_2
18	myristoyl-Gly-------Arg-Pro-Tyr-Asp----------NH_2
19	myristoyl-Gly------Arg-Pro-Tyr----------NH_2
20	myristoyl-Gly----------Pro-Tyr-Asp-Ala-Asn----------NH_2

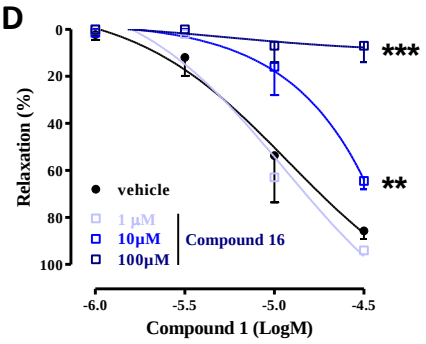


Figure 3.16. Assessment of a newly-synthesised pepducin acting as a $S1P_3$ antagonist. (A) Compound 1-induced vasorelaxation on PE-precontracted aortic rings and vasorelaxation inhibitory effects of L-NAME (100 µM) and FR180204 (10 µM) (*** p<0.001 *vs.* vehicle, two-way ANOVA with Bonferroni's post-test, *n=6* mice in each group). (B) Chemical structure of Compound 1 (KRX-725) and its Ala-scan derivatives. (C) Chemical structure of Compound 1 and its deleted derivatives. (D) Compound 16 reduces Compound 1-induced vasorelaxation in a concentration-dependent manner (** p<0.01, *** p<0.001, two-way ANOVA with Bonferroni's post-test, *n=3* mice in each group). Data are mean ± SEM.

3.1.5 SUMMARY

Roviezzo *et al.* have previously showed that *in vivo* inhibition of SphK, the ezyme responsible of phosphorylation of the sphingosine, reduced bronchial contractility in allergen-sensitised mice[294]. They went further and demonstrated that systemic administration of S1P in laboratory animals induced asthma-like features characterised by bronchial hyperreactivity, lung inflammation, and airway remodelling[427]; in this study, we have extensively cleared the cellular mechanisms responsible for S1P-mediated effects in laboratory animals. These morphological and functional alterations were associated with an increase in levels of PGD_2 and IgE as well as IL-4 and IL-13.

It is now accepted that both IgE and mast cells are key factors in establishing pathophysiological changes on airways in chronic asthma condition. Such effects include IgE-dependent regulation of mast cells, mast cell-independent IgE-mediated actions and mast cell activities that do not directly involve IgE. In order to assess the role of mast cells in our experimental conditions, we explored the effects of administration of S1P *in vivo* to mast cell-deficient $Kit^{W\text{-}sh/W\text{-}sh}$ mice. Such animals did not show bronchial hyperreactivity after treatment with the sphigolipid; however, they were still characterised by lung inflammation, high IgE levels, and mucus hyperproduction. Thus, the absence of mast cells does not affect IgE levels suggesting an alternative target for IgE in our model.

CD23 is the "low-affinity" receptor for IgE (FcεRII). It has been implicated in a number of inflammatory conditions and is considered important in the regulation of IgE production. An altered expression of CD23 has been widely associated with allergic diseases[429,430,441]. Based on these observations, we found that CD23 was highly expressed in lung of S1P-treated mice compared to vehicle. In order to define the role of CD23, we treated mice with a neutralizing antibody (B3B4 clone), extensively characterised for its high affinity for CD23 and for a reciprocal inhibitory pattern with IgE. Indeed, treatment of mice with both anti-CD23 and S1P reduced airway smooth muscle hyperresponsiveness, pulmonary hyperplasia,

and IL-13 and IgE levels, but pulmonary mast cell infiltration and IL-4 levels were not altered. Therefore, these data, taken together with previous evidence, imply that S1P/CD23 signalling is not directly involved in mast cell recruitment, but rather interferes with their activation, as suggested by the marked reduction in production of IgE. This hypothesis is also supported by the observation of reduced levels of IL-13 after anti-CD23 treatment. Similarly, we did not observe any change in IL-4 release after the treatment with anti-CD23, suggesting a predominant Th2 bias in S1P-induced asthma-like model in mice.

In order to look deeply into the cellular mechanisms, our attention focused on T cells. Effects of S1P on nude mice, which genetically lack of the adaptive immune system, were studied. Our results showed that T cells were crucial in fully explaining the effects elicited by S1P in our model, since nude mice injected with S1P did not exhibit airway hyperreactivity, mast cell infiltration, and IgE production; furthermore, IL-4, IL13, and CD23 were not induced by S1P.

The idea that all these events are T cell-dependent is further sustained by the finding that the CD4+ T lymphocytes, harvested from BALBC/c mice exposed to S1P, showed an increased ability to proliferate *in vitro*. To note, CD4+ T cells harvested from S1P-treated mice were able to proliferate even in the absence of the *in vitro* stimulation with CD3/CD28 beads. However, the proliferation rate of CD4+ T-cells, obtained from mice pretreated *in vivo* with the anti-CD23 before S1P administration, was not altered. This indicates that the major action of CD23 *in vivo* is to negatively regulate IgE production without a significant effect on T cell growth or differentiation. These data fit well with the lack of effect of anti-CD23 in regulating IL-4 release, mainly involved in a Th2 skew in the lung of S1P-treated mice, together with IL-13, IgE, and PGD_2[442]. That these mechanisms triggered by S1P require this *in vivo* cellular activation is strongly supported by the finding that CD4+ T cells, harvested from vehicle-treated mice, were not able to proliferate when exposed *in vitro* to S1P. These latter events occur in an IgE-independent manner.

The final results confirming S1P could induce a modulation of the adaptive immune system came from the adoptive transfer experiments. S1P-derived CD4+ T cells adoptively transferred into naïve mice increased mast cell infiltration, bronchial reactivity pulmonary inflammation, and IL-4 and IL-13 release. As expected, adoptive transfer of CD4+ T cells derived from S1P-treated mice receiving anti-CD23 into naïve mice did not promote any effect on the lung. In particular, we did not observe any increase in airway smooth muscle reactivity or an inflammatory response. Conversely, IL-4 overexpression was still present. These data, together with the studies performed in mast cell knockout and nude mice, confirm that CD4+ T-cells are the main cells involved in S1P-induced effects. In addition, this implies an obligatory role for CD23/IgE signalling to trigger IgE-mediated immune responses in order to observe functional changes in the lung. However, the role of innate immune cells in this context still remains to be elucidated.

We then explored the role of B cells in our experimental model. We found that B cells counteract S1P-induced allergic inflammation in the lung.

Similarly to OVA-sensitised mice, the presence of B cells was strictly correlated to the recruitment of Treg and to the higher release of IL-10 and TGF-β, well-known immuno-suppressive cytokines, in S1P-treated mice compared to the controls. In animal models of allergic airway disease, Treg can suppress established airway inflammation and airway hyperresponsiveness[443]. Existing therapies including corticosteroids and allergen immunotherapy act on Treg, in part to increase IL-10 production, while vitamin D3 and long-acting β-agonists enhance IL-10 Treg function[444].

Importantly, the inhibition of SphK1/2 reduced B cell recruitment and IL-13 release in OVA-sensitised mice, implying that the endogenous presence of S1P can participate to the inflammatory patterns observed during the allergic inflammation[427].

The involvement of S1P signalling in B cell migration was already observed for marginal zone B cells that require $S1P_1$ receptor activity to egress into the bone marrow vascular compartment and into the peripheral blood[445]. Our study, instead, is the first to our knowledge to demonstrate that B cells migrate to the inflamed lungs of S1P-treated mice. Additionally, the co-presence of Treg with B cells is at the basis of an immuno-suppressive environment characterised by IL-10 and TGF-β[446]. It is well-known that during asthmatic conditions, Th2 cells and Treg are present in an oscillatory mode in that Treg presence tries to dampen Th2-derived harmful signals[444,447,448,449]. In this scenario, B cells, important for the humoral immune responses, behave as IL-10-producing B regulatory (B reg) cells[419,446] and, more importantly, they induce CD4+ and CD8+ T cells to a suppressive phenotype associated to the release of suppressive cytokines such as TGF-β and IL-10. Upon recognition of specific antigens, B cells can present antigen(s), interact with T cells, and differentiate into plasma cells[419,440,446]. However, upon specific allergic conditions, B cells can behave as Breg and negatively regulate inflammation and immune responses through IL-10[419]. The stimulation of B cells with S1P increased the levels of IL-10 and TNF-α. The release of TNF-α *in vitro* by S1P-stimulated B cells is in line with the observation that this cytokine is overproduced in asthmatic patients and is a potent activator of SphK1/2 and subsequent production of S1P[450]. Indeed, SphK1 activation is observed in many cell types relevant to allergic disorders and occurs principally in response to inflammatory cytokines, such as TNF-α and IL-1β[450]. IL-10 is a potent immune-modulator and it can inhibit Th1 polarization and also dampen proinflammatory activities of Th2 cells[451]. IL-10-producing B cells have been shown to down-regulate inflammation in airway hyperresponsiveness. Regulatory B cells were reported to be protective against allergic airway inflammation, especially when they express high levels of CD23 and IL-10[452]. In our mouse model, lung B cells also expressed high levels of CD23 after the administration of S1P at all time-points examined (data not shown). The suppressive activity of Breg underlies the recruitment of Treg to the lungs, which mediate the suppression of lung

inflammation in an IL-10-dependent manner[452]. Indeed, we observed that the suppressive B cells obtained from the lung of S1P-treated mice were able to both induce Treg activity and to promote CD8+ T cells to release IL-10, further suggesting the role of regulatory B cells on the adaptive immunity after S1P treatment in mice. In support to our data, FTY720, a pro-S1P-mimetic, proved of successful activities against multiple sclerosis as an immunomodulator once in its phosphorylated form, mimicking S1P[453]. Moreover, we observed that B cells isolated from the lung of S1P-, but not vehicle-treated mice, were able to release high levels of granzyme B and IL-13. This effect was not a direct activity of S1P on B cells but most probably dependent on the environment these cells encountered. Granzyme B is a cytotoxic mediator. B cells were also reported to behave as cytotoxic cells and the release of granzyme B may underlie their activity to modulate T cells[419]. In addition, granzyme B was also reported to be functionally important for Treg proliferation, further supporting our data on the role of Breg/Treg in countering S1P-mediated airway allergic inflammation. The activation of B cells has also been described as a mean to induce B cell maturation in plasma cells[454]. Indeed, our *in vitro* and *in vivo* experiments showed that the administration of S1P increased the expression of CD20 on B cells. CD20 is a maturation marker for B cells, which are programmed to produce immunoglobulins as plasma cells[419]. The depletion of the mature CD20+ B cells increased airway reactivity and lung inflammation, implying the relevance of the immunomodulator activity of Ig-producing B cells. This effect was associated to the lower presence of Treg, IL-10, and TGF-β.

The sphingolipid S1P exerts its effects by activating five G protein-coupled membrane receptors, namely $S1P_{1-5}$. S1P, as an endogenous mediator, is a full agonist and unspecifically activates its receptors. In particular, activation of $S1P_3$ is associated with a pro-angiogenic[455] and pro-tumorigenic role[456,457]. It is now clear that the $S1P_3$ receptor is one of the most expressed receptor along the airways among S1P receptors. Many works have established that blocking $S1P_3$ could have

beneficial effects in animal models of lung injury in terms of controlling and pulmonary inflammation[458] and reducing airway hyperreactivity[459]. It is evident that the possibility of designing $S1P_3$-specific antagonists could be helpful in therapy, but at the moment no specific molecule exists. Furthermore, full $S1P_3$ agonists have been synthesised neither.

Our collaboration with the chemistry research group of Professors G. Caliendo and V. Santagada (Department of Pharmacy, University of Naples, Italy) cleared the way in further advancing the research in this field, pointing out the ability of pepducin-derived compound to act as a $S1P_3$ antagonist. However, further chemical studies are underway to optimise selective $S1P_3$ receptor antagonists; anyway, the unique biological properties of Compound 16 make it a useful and handle pharmacological tool for the study of S1P signalling involving the $S1P_3$ receptor.

3.1.6 CONCLUSIONS

In conclusion, systemic administration of S1P triggers a cascade of events that sequentially involves T cells, IgE, and mast cells, which leads to the asthma-like symptoms in mice. In this context, B cells have protective activities in S1P-induced allergic inflammation because they behave as Breg that highly release IL-10. The association of Breg to Treg represents a potential cellular mechanism by means the host tries to dampen S1P-mediated proinflammatory/allergic patterns.

3.1.7 LIMITATIONS

Although we have extensively studied the molecular and cellular mechanisms in the S1P model, the results are related to mice. Thus, clinical translational studies

to human beings affected by allergic asthma is recommended in order to evaluate possible affinities/differences in the activation of the immune system.

3.2 STUDY OF H_2S EFFECTS IN ANTIGEN-INDUCED AIRWAY HYPERRESPONSIVENESS: ROLE OF MAST CELLS

3.2.1 RATIONALE

The precise role and the importance of H_2S in lung pathophysiology is still a matter of debate. The controversial effects of this gaseous transmitter are far from being delucidated. Here, we have further investigated the influence of H_2S on lung function in a well-established model of allergic asthma.

In particular, the administration of H_2S *in vivo* was carried out by means of an aerosolizing apparatus, in order to exactly mirror the empiric findings that thermal waters rich in sulphurs relieve the symptoms of asthma and upper respiratory tract conditions.

Thus, in this chapter, we have addressed the following hypothesis and specific aims:

Hypothesis:

"The gaseous mediator H_2S can affect lung function"

Aims:

- to investigate the effects of H_2S inhalation in a mouse model of allergic asthma;
- to determine the role of mast cells;
- to establish an involvement of remodelling processes assessing fibroblast activity.

3.2.2 METHODS

The methods relating to experiments performed in this section are described in the Chapter 2 (Materials and methods).

3.2.3 DATA HANDLING AND STATISTICAL ANALYSIS

The statistical analysis relating to experiments performed in this section are described in the Chapter 2 (Materials and methods). The details of each statistical test used are reported in the respective figure legends.

3.2.4 RESULTS

Aerosol administration of hydrogen sulphide abolishes OVA-induced airway hyperreactivity

The effect of hydrogen sulphide aerosol administration on allergen-induced airway hyperreactivity was tested using the protocol described in Figure 3.17A.

We have assessed both bronchial reactivity (Figure 3.17B) and lung resistance (Figure 3.17C), after carbachol challenge. Bronchi harvested from OVA-treated mice showed an increased reactivity to carbachol in comparison to vehicle-treated mice (Figure 3.17B). Conversely, hyperreactivity to carbachol was reverted in bronchi harvested from mice treated *in vivo* with hydrogen sulphide aerosol (Figure 3.17B). Similarly, hydrogen sulphide brought back to control level the lung resistance in OVA-treated mice (Figure 3.17C). Hydrogen sulphide inhalation did not alter OVA-induced hyperplasia (Figure 3.17D), as evaluated by PAS staining since mucus production was still visible in pulmonary histology (Figure 3.17E). Administration of hydrogen sulphide to control mice had no effects on the parameters analysed (data not shown).

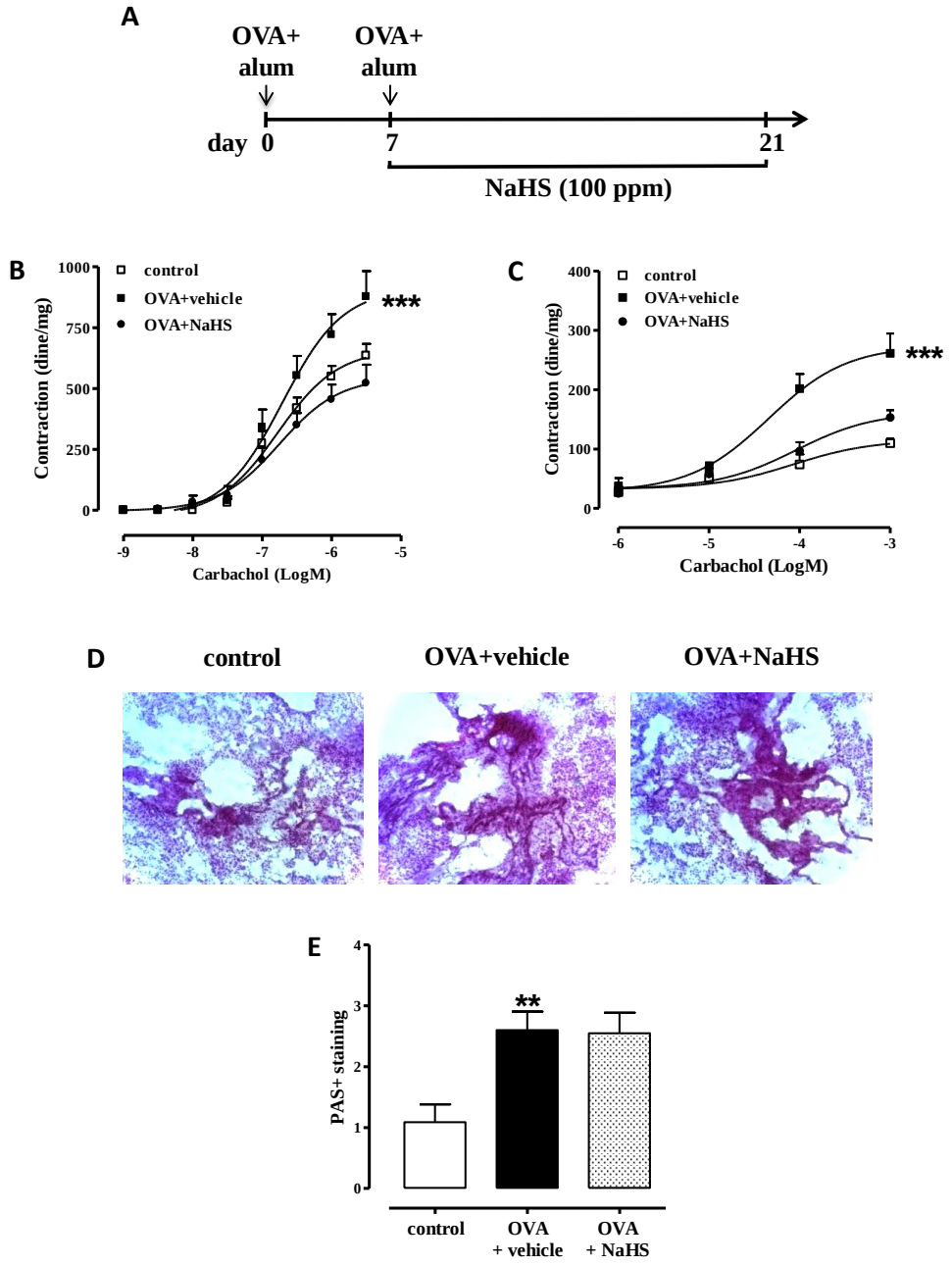


Figure 3.17. Administration of hydrogen sulphide aerosol abrogates OVA-induced airway hyperreactivity. (A) Schematic representation of the protocol used. Mice received subcutaneous administration of OVA (100 µg) adsorbed onto alum at day 0 and 7. Aerosol administration of vehicle or hydrogen sulphide (NaHS, 100 ppm) was performed daily from day 7 to day 21. Twenty-four hours after the last administration mice were sacrificed. (B) Measurement of bronchial reactivity to carbachol in organ bath (*** $p<0.001$ *vs.* control, two-way ANOVA with Bonferroni's post-test). (C) Measurement of lung resistance by using isolated perfused lung preparation (*** $p<0.001$ *vs.* control, two-way ANOVA with Bonferroni's post-test). (D) Histological analysis of pulmonary sections (H&E staining). (E) Goblet cell hyperplasia evaluation by PAS staining (** $p<0.01$ *vs.* control, one way ANOVA with Bonferroni's post-test). Lung sections have been photographed under light microscopy at 10× magnification. Data are mean ± SEM, *n=6* mice in each group.

Hydrogen sulphide reduces mast cell activation and FGF2 and IL-13 production

Flow cytometry analysis of lungs harvested from OVA-sensitised mice demonstrated a significant increase of mast cell recruitment (Figure 3.18A). Treatment of mice with aerosol administration of hydrogen sulphide did not affect OVA-induced mast cell infiltration (Figure 3.18A). Conversely, NaHS administration significantly prevented mast cell degranulation (Figure 3.18C). Sensitization caused also a significant increase in IgE plasma levels (Figure 3.18B) that was not modified by hydrogen sulphide. Finally, hydrogen sulphide reverted the OVA-induced up-regulation of two important fibrogenic cytokines in the lung such as IL-13 (Figure 3.18D) and FGF2 (Figure 3.18E). Administration of hydrogen sulphide to control mice had no effects on the parameters analysed (data not shown).

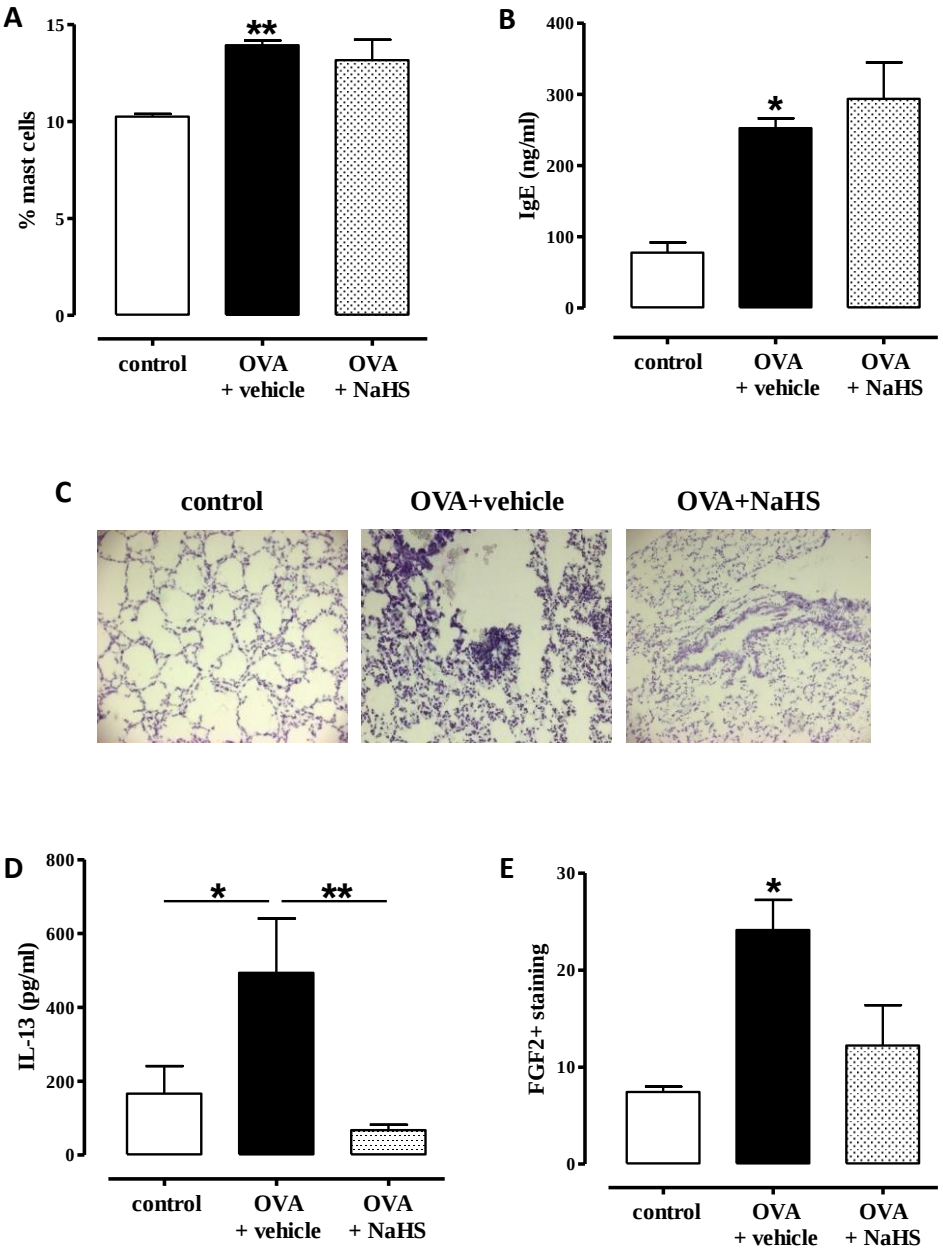


Figure 3.18. Hydrogen sulphide inhibits mast cells infiltration and cytokines expression in the lung. (A) Quantification of lung mast cells (** p<0.01 *vs.* control, one-way Bonferroni's post-test). (B) Sera IgE levels were determined by using specific ELISA (* p<0.05 *vs.* control, one-way ANOVA with Bonferroni's post-test). (C) Toluidine positive staining of lung cryosections. (D) IL-13 concentrations in whole lung homogenates have been determined by ELISA (* p<0.05, ** p<0.01, one-way ANOVA with Bonferroni's post-test). (E) Immunohistochemistry of FGF2 expression in lungs (* p<0.05 *vs.* control, one-way ANOVA with Bonferroni's post-test). Data are mean ± SEM, *n=6* mice in each group.

Hydrogen sulphide reduces the development of mast cell-dependent airway hyperreactivity

The development of airway hyperreactivity can be either mast cell-dependent or -independent in mouse models of asthma and it is widely suggested that the former is due to a sensitization without and the latter with adjuvant[460]. In order to further address the role of mast cells, we treated mice with OVA without alum using the protocol reported in Figure 3.19A. This is a chronic allergic asthma model mainly mast cell-dependent[460]. Bronchi harvested from these mice displayed bronchial hyperreactivity to carbachol (Figure 3.19B) that was abrogated by hydrogen sulphide aerosol (Figure 3.19B). PAS staining evidenced a weak increase of goblet cells, suggesting a marginal role of mast cells in the regulation of mucus production (Figure 3.19C). Finally hydrogen sulphide did not affect pulmonary metaplasia (Figure 3.19D). Administration of NaHS to vehicle-treated mice had no effects on the parameters analysed (data not shown).

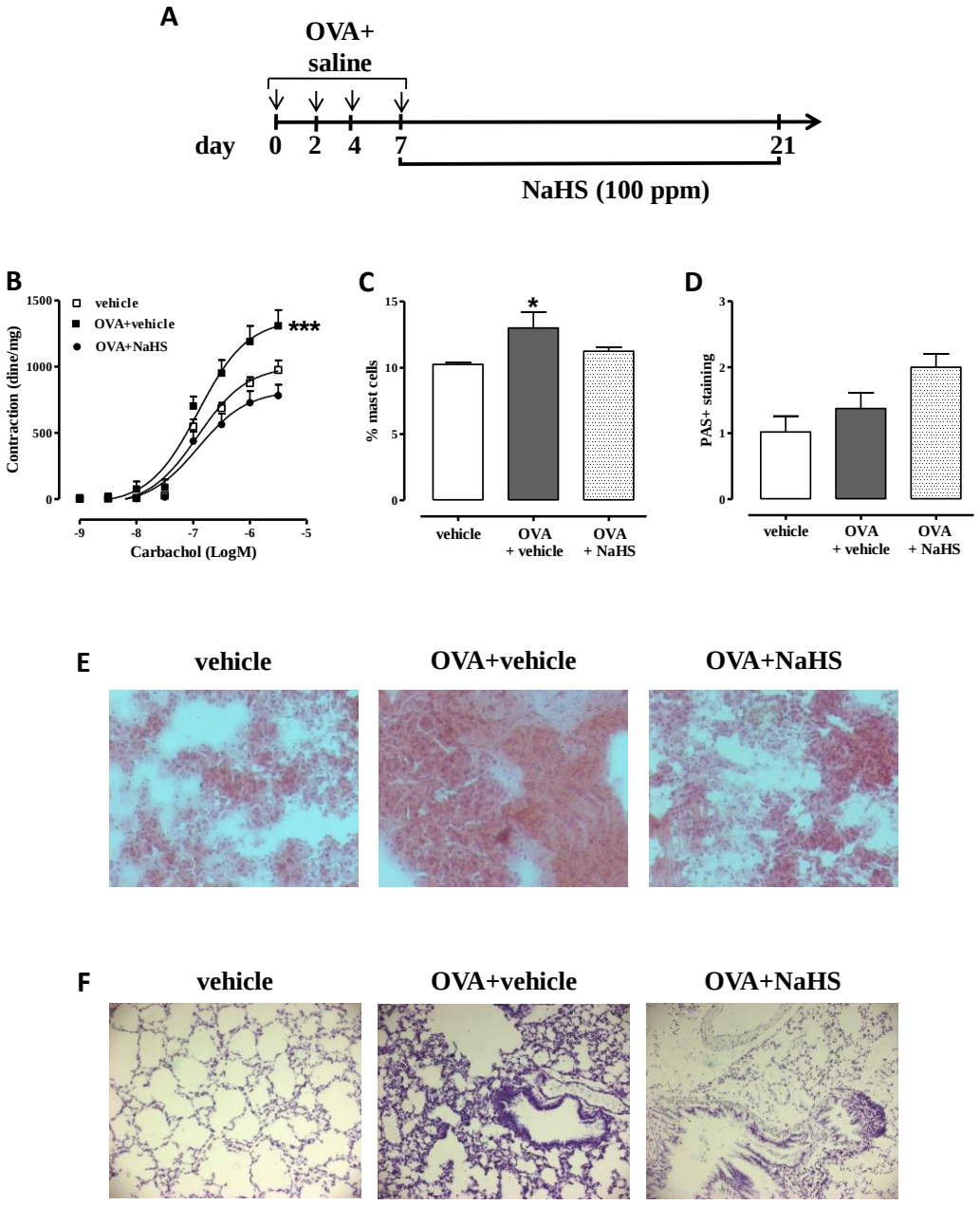


Figure 3.19. Hydrogen sulphide abrogates OVA-induced mast cell-dependent hyperreactivity. (A) Schematic representation of the protocol used. Mice received subcutaneous administration of OVA (50 µg) without alum at day 0, 2, 4, and 7. Mice were treated with either vehicle or NaHS aerosol daily from day 7 to day 21. Twenty-four hours after the last administration mice were sacrificed. (B) Measurement of bronchial reactivity to carbachol in organ bath (*** $p<0.001$ *vs.* vehicle, two-way ANOVA with Bonferroni's post-test). (C) Quantification of lung mast cells (* $p<0.05$ *vs.* vehicle, one-way ANOVA with Bonferroni's post-test). (D) Goblet cell hyperplasia evaluation by PAS staining. Lung sections have been photographed under light microscopy at 10× magnification. (E) Histological analysis of pulmonary sections (H&E staining). (F) Toluidine positive staining of lung cryosections. Data are mean ± SEM, *n=6* mice in each group.

Mast cell-deficient Kit$^{W\text{-}sh/W\text{-}sh}$ mice fail to develop airway hyperesponsiveness

The role of mast cells in the development of airway hyperreactivity has been investigated by using mast cell-deficient mice in different mouse asthma model. The lack of mast cells generally decreased the effect of carbachol both with and without allergen challenge compared to wild type. However, in mast cell-deficient Kit$^{W\text{-}sh/W\text{-}sh}$ mice bronchial hyperresponsiveness could be induced changing the protocol of antigen challenge. Under the different experimental conditions employed, it is possible to observe different effects. In particular, frequent stimulation could induce airway hyperreactivity also in these mice. To validate the data obtained by using the model described in the above paragraph, we tested if our sensitization protocol could affect airway responsiveness in mast cell-deficient Kit$^{W\text{-}sh/W\text{-}sh}$ mice. These mice exposed to OVA sensitization did not develop bronchial hyperreactivity to carbachol (Figure 3.20A). The mucus production was increased after OVA exposure (Figure 3.20B), even though sensitization failed to induce a significant increase of both IL-13 (Figure 3.20C) and FGF2 (Figure 3.20D) expression in lungs. Treatment of control mice with hydrogen sulphide did not cause any effect on all parameters considered (Figure 3.20B, 3.20C, and 3.20D).

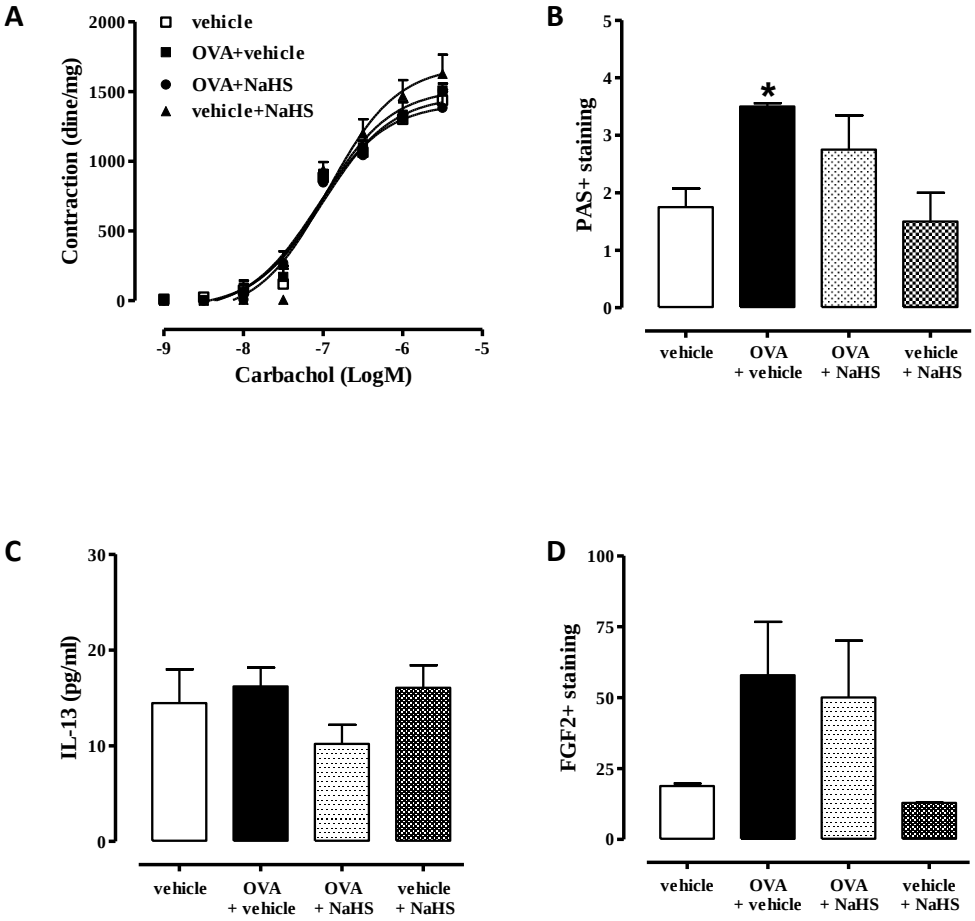


Figure 3.20. Lack of effect of hydrogen sulphide in mast cell-deficient Kit$^{W\text{-}sh/W\text{-}sh}$ mice. (A) Kit$^{W\text{-}sh/W\text{-}sh}$ mice received subcutaneous administration of OVA (100 µg) adsorbed onto alum at day 0 and 7. Mice were treated with either vehicle or hydrogen sulphide aerosol daily from day 7 to day 21. Twenty-four hours after the last administration mice were sacrificed. Bronchial reactivity to carbachol was assessed. For this protocol, see Figure 17A. (B) Goblet cell hyperplasia evaluation by PAS staining. Lung sections have been photographed under light microscopy at 10× magnification (* $p<0.05$ *vs.* vehicle, one-way ANOVA with Bonferroni's post-test). (C) Whole lung homogenates IL-13 concentrations, determined by ELISA. (D) Immunohistochemistry of FGF2 expression in lungs. Data are mean ± SEM, *n=6* mice in each group.

Hydrogen sulphide aerosol reduces fibroblast activation: ex vivo *study*

Lung mast cells are found in close proximity to fibroblasts and the number of the cells is increased in asthma. Since mast cell-fibroblast interactions contribute to increased contractile response in airways, we evaluated the effect of H_2S on fibroblasts activation *in vivo*. We isolated primary fibroblasts from mice exposed to OVA and tested their proliferation rate and differentiation. Our data show that primary fibroblasts harvested from sensitised mice displayed an increased proliferation rate (Figure 3.21A) as well as increased expression of α-SMA (Figure 3.21B and 3.21C). In fibroblasts harvested from mice treated with hydrogen sulphide aerosol both the increased proliferation rate and differentiation were reverted (Figure 3.21A, 3.21B, and 3.21C). Thus, mice exposed to hydrogen sulphide inhalation displayed a significant reduction of fibroblast activation beyond reduction of airway hyperreactivity.

In order to further demonstrate that the fibroblasts contribute to mast cell-dependent airway hyperreactivity, we evaluated fibroblast activation also in the model of mast cell-dependent hyperreactivity using the protocol described in Figure 3.19A. Our data show that primary fibroblasts harvested from these mice displayed an increased rate of proliferation (Figure 3.22A) as well as an increased expression of α-SMA (Figure 3.22B and 3.22C). When mice were treated with aerosol of hydrogen sulphide, again both the increased fibroblast proliferation and differentiation measured *ex vivo* were prevented (Figure 3.22A, 3.22B, and 3.22C), as well as the airway hyperreactivity.

Administration of hydrogen sulphide to control mice had no effects on the parameters analysed in any of the conditions tested (data not shown).

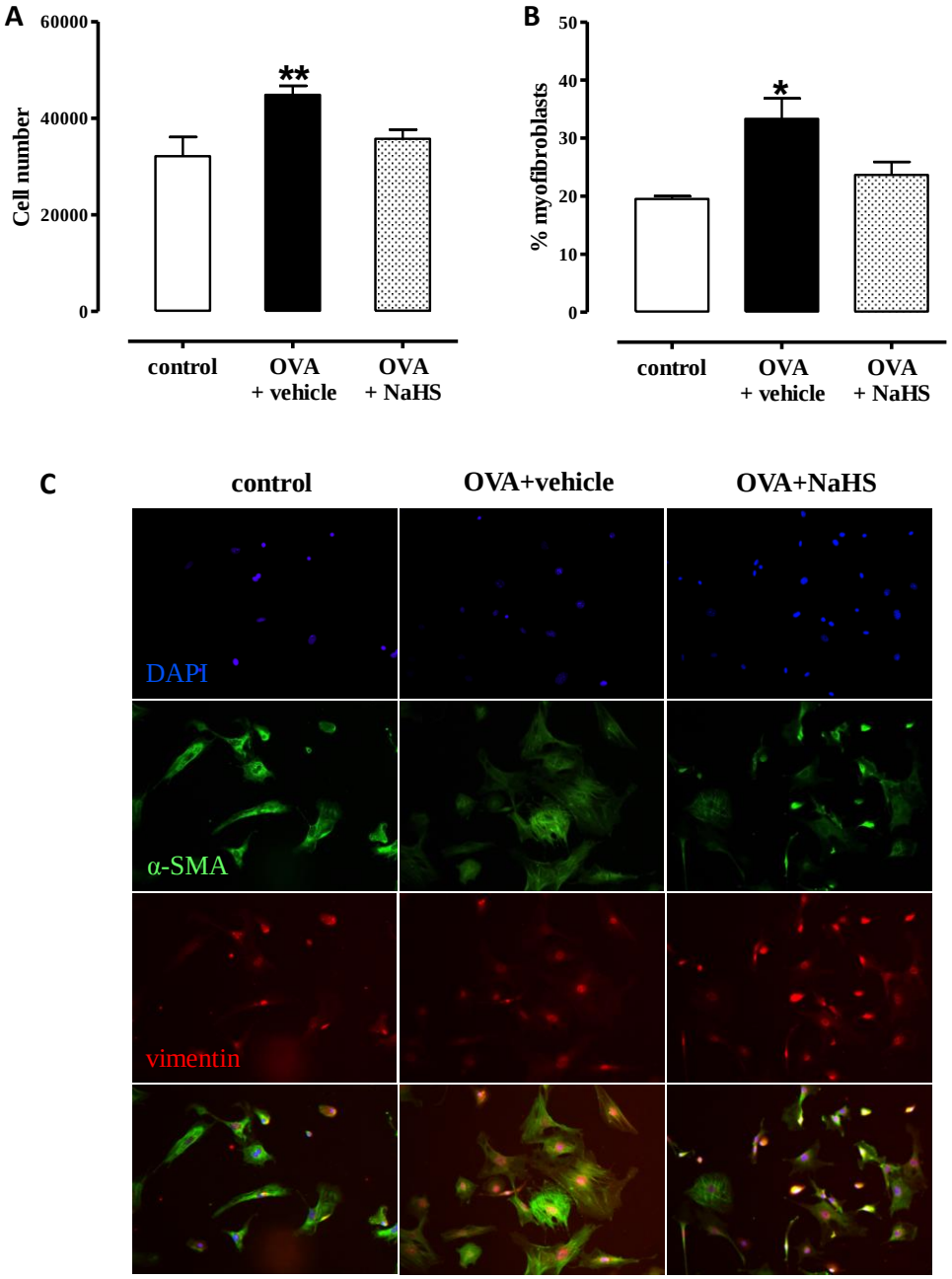


Figure 3.21. Hydrogen sulphide inhibits pulmonary fibroblasts proliferation and differentiation. (A) Lung primary fibroblasts were harvested from OVA+alum-sensitised mice exposed to vehicle or NaHS aerosol. For the *in vivo* protocol, see Figure 17A. The MTT colorimetric assay was used to assess fibroblast proliferation *in vitro* (** $p<0.01$ *vs.* control, one-way ANOVA with Bonferroni's post-test). (B) Percentage of myofibroblasts (* $p<0.05$ *vs.* control, one-way ANOVA with Bonferroni's post-test). (C) Fibroblasts were labelled with anti-vimentin antibody (red). Nuclei were stained with DAPI (blue). Myofibroblast differentiation was determined by detection of expression of α-SMA (green). Lowest panels represent the merge of upper and central panels. The percentage of myofibroblasts are easily distinguishable since they display well-developed, thick and α-SMA-positive stress fibers. Images were taken at 200× magnification. Data are mean ± SEM, *n=3* mice in each group.

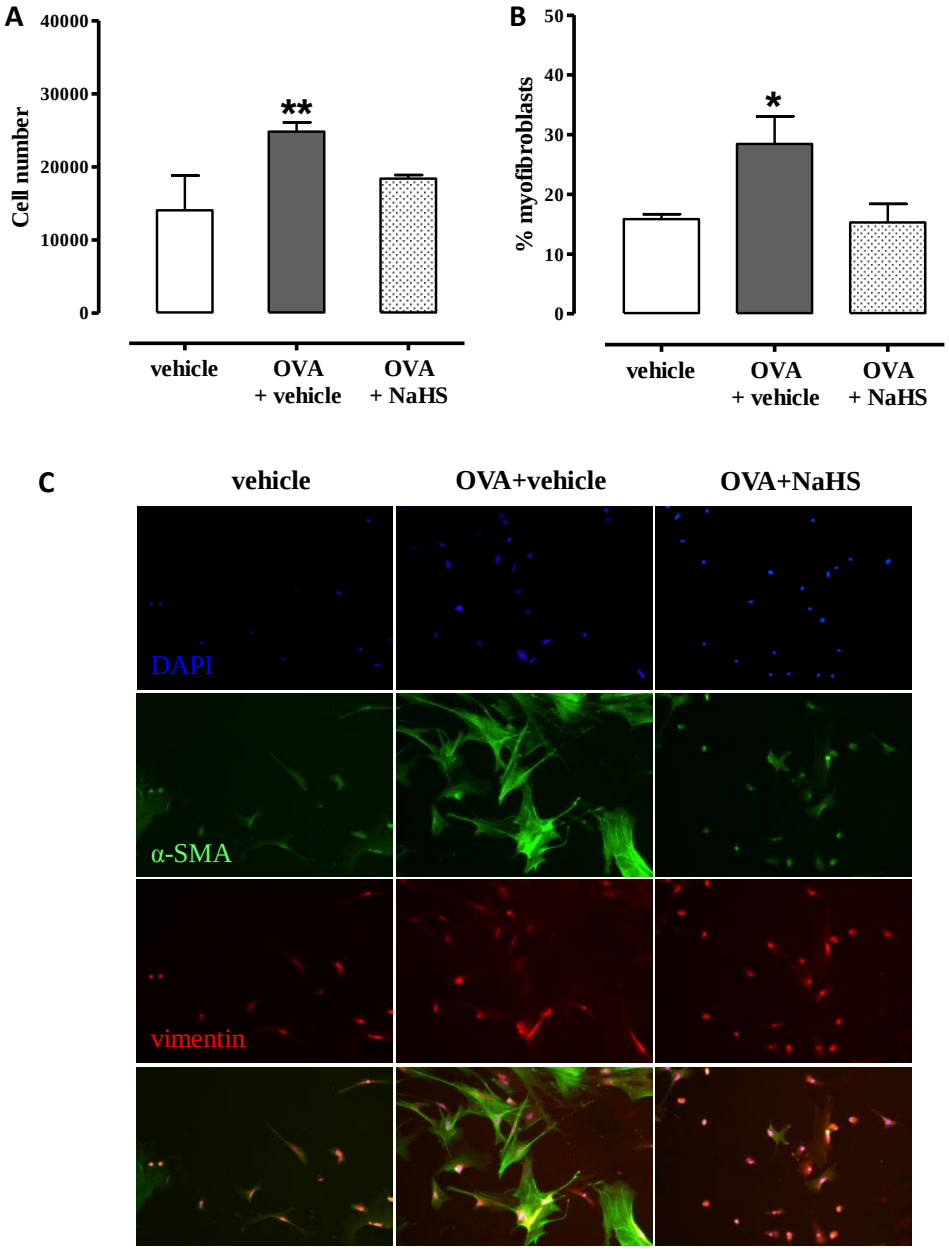


Figure 3.22. Hydrogen sulphide inhibits fibroblasts proliferation and differentiation in the asthma model mast cell-dependent. (A) Lung primary fibroblasts were harvested from mice sensitised with OVA without alum and exposed to vehicle or NaHS. For protocol, see figure 19A. Proliferation rate and differentiation of fibroblasts were evaluated. The MTT colorimetric assay was used to assess fibroblast proliferation *in vitro* (** $p<0.01$ *vs.* vehicle, one-way ANOVA with Bonferroni's post-test). (B) Percentage of myofibroblasts (* $p<0.05$ *vs.* vehicle, one-way ANOVA with Bonferroni's post-test). (C) Fibroblasts were labelled with anti-vimentin antibody (red). Nuclei were stained with DAPI (blue). Myofibroblast differentiation was determined by detection of expression of α-SMA (green). Lowest panels represent the merge of upper and central panels. The percentage of myofibroblasts are easily distinguishable since they display well-developed, thick and α-SMA-positive stress fibers. Images were taken at 200× magnification. Data are mean ± SEM, *n=3* mice in each group.

Hydrogen sulphide inhibits RBL-2H3 antigen-induced degranulation in vitro

A direct effect of hydrogen sulphide on mast cell degranulation was investigated *in vitro* by using mast cell-like RBL-2H3 cell line. The addition of the antigen DNP-HSA to presensitised RBL-2H3 cells caused an evident degranulation, indicated by a massive release of β-hexosaminidase (about 50% of the total content of the enzyme; Figure 3.23A). An almost equivalent level of degranulation was induced by the non-antigenic stimuli thapsigargin and ionomycin (Figure 3.23A). Hydrogen sulphide caused a remarkable concentration-dependent inhibition of the RBL-2H3 degranulation induced by DNP-HSA (Figure 3.23B). In contrast, hydrogen sulphide did not exhibit any significant inhibitory activity on the degranulation induced by both thapsigargin and ionomycin (Figure 3.23C).

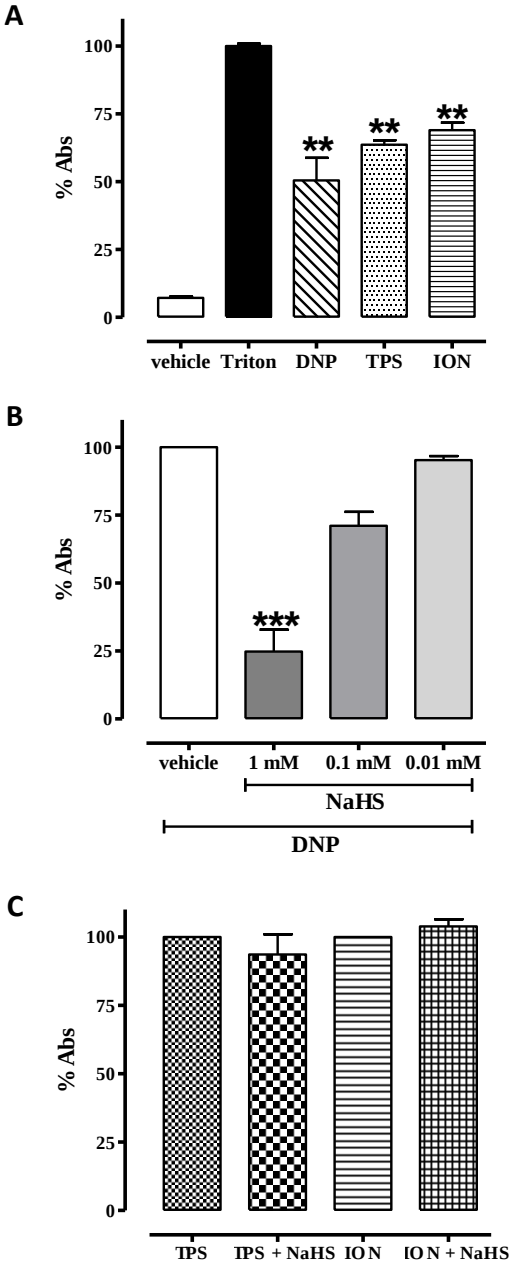


Figure 3.23. Hydrogen sulphide reduces IgE-mediated RBL-H3 cells degranulation. (A) RBL-2H3 cell degranulation was pharmacologically induced by i) the antigen DNP-HSA (DNP; 10 ng/ml); ii) ionomycin (ION; 1 µM); and iii) thapsigargin (TPS; 1 µM). In matched wells, Triton X-100 was added to elicit cell lysis and exhaustive release of β-hexosaminidase (** p<0.01 *vs.* vehicle, one-way ANOVA with Bonferroni's post-test, *n=9* in each group). (B) Cells were sensitised with DNP and then incubated for 5 minutes with either the H_2S donor NaHS (10, 100, and 1000 µM) or the vehicle. Cell degranulation was induced by DNP (*** p<0.001 *vs.* vehicle, one-way ANOVA with Bonferroni's post-test, *n=3* in each group). (C) Cells were incubated with either NaHS (1 mM) or vehicle for 5 minutes and cell degranulation induced by ION or TPS (*n=6* in each group). Data are mean ± SEM.

3.2.5 SUMMARY

Once confirmed the role of S1P and its mechanisms in inducing an asthma-like condition in mouse, we then investigated the effects of protective mediator in the pulmonary district in a well-described and recognised animal model of allergic asthma induced by OVA. Intraperitoneal treatment with H_2S have already been proved to be effective in reducing the OVA-induced effects in animals[461] and the administration of inhibitors of the enzymes deputed to its synthesis even worsened the symptoms[384]. Here, we evaluated the effect of an administration of H_2S through aerosol. H_2S, given as NaHS, reversed the OVA-induced bronchial hyperreactivity to carbachol and total lung resistance. Conversely, H_2S did not affect either cell infiltration or mucus hypersecretion as determined by histological analysis of lungs. Since goblet cell metaplasia and mucus hypersecretion are two parameters indicative of lung inflammation[462,463], these data imply that hydrogen sulphide regulatory action is not strictly dependent upon reduction of the inflammatory response in our experimental conditions.

Airway hyperresponsiveness is an important pathophysiological characteristic of asthma. It is linked to both inflammation and remodelling of the airways and is partially reversible with therapy. The mouse model used mimics the prominent features of allergic asthma associated with airway hyperreactivity such as the infiltration of mast cells into the airway smooth muscle layer and the increase in plasmatic levels of IgE[460,464]. It is now accepted that both IgE and mast cells are key factors in establishing pathophysiological changes on airways in chronic asthma condition, but the mechanisms by which H_2S affects bronchial tone are not fully understood and not deeply explored yet. Aerosol administration of hydrogen sulphide prevented mast cell activation but did not modify IgE plasma levels. These data suggest that aerosol administration of hydrogen sulphide, rather than modified systemic immunologic reactivity, had a local immunosuppressive role. Indeed, hydrogen sulphide aerosol prevented OVA-induced increase of IL-13 and FGF2 in the lung.

In order to further assess the role of mast cells, we tested the efficacy of hydrogen sulphide aerosol in a chronic allergic asthma model mainly mast cell-dependent[460]. Hydrogen sulphide abrogated airway hyperreactivity, without modulating either goblet cell metaplasia or mucus hypersecretion. To further validate these data, we performed the same protocol in mast cell-deficient mice. Bronchi harvested from these mice failed to develop an increased reactivity following OVA sensitization as well as IL-13 and FGF2 up-regulation. Aerosol administration of hydrogen sulphide did not further modify these parameters. On the other hand, in mast cell-deficient mice, OVA-induced goblet cell metaplasia and mucus hypersecretion were still present and were not affected by the hydrogen sulphide aerosol. Thus, our data indicate that mast cells are the main responsible of airway hyperreactivity.

However, we still had to define how this effect is translated into a beneficial effect on bronchial hyperreactivity. Mast cells synthesise and secrete a large number of proinflammatory cytokines, which regulate both IgE synthesis and the development of inflammation, and several pro-fibrogenic cytokines. One possible explanation was suggested by the finding that aerosol of hydrogen sulphide prevented FGF2 and IL-13 up-regulation, that are known to be fibroblast activators. Indeed, these cytokines are known to activate lung fibroblasts in asthmatic patients as well as to be up-regulated by sensitization in experimental model of asthma[465,466]. In addition, there is increasing evidence for a strong correlation between sub-epithelial fibrosis and airway hyperreactivity as it has been demonstrated in human studies[467,468]. This is suggested by the increased number of fibroblasts/myofibroblasts found in the airways of asthmatic patients[469,470]. On the basis of this clinical evidence and our findings, we have investigated the possible role played by fibroblasts in hydrogen sulphide inhibitory effect on airway hyperreactivity. In order to address this issue, we used both models of OVA-sensitised mice with or without alum. Fibroblasts harvested from mice sensitised with either OVA plus alum or OVA without alum displayed an increased proliferation rate *in vitro*. Both treatments also caused a significant increase in the percentage of fibroblasts converted in myofibroblasts. Treatment

of mice with hydrogen sulphide aerosol inhibited both the increased fibroblast proliferation rate and their differentiation into myofibroblasts. Thus, aerosolised hydrogen sulphide attenuates airway hyperreactivity and causes a selective reduction of mast cell and fibroblast activation. These data suggest that the hydrogen sulphide might modulate mast cell-mediated fibroblast activation. This hypothesis is supported by the finding that, in mast cell-deficient mice, FGF2 and IL-13 up-regulation is not induced by OVA sensitization. In clinical studies, it has been shown that IL-13+ cells, present within the airway smooth muscle of asthmatic patients, are predominantly mast cells[471]. IL-13 has been shown to elicit many of the features of human asthma as well as to regulate production of several profibrogenic cytokines including FGF2[472]. Our data, taken together with this clinical evidence, suggest that hydrogen sulphide, by inhibiting mast cell activation, modulates fibroblast activation leading to an improvement of bronchial hyperreactivity. These data also imply that mast cells are responsible of the direct or indirect production of IL-13 in our experimental model and control FGF2 up-regulation. This is of particular interest on the clinical side since recently it has been demonstrated that FGF2 levels in induced sputum significantly correlate with pulmonary function and have been proposed to be a possible biomarker of asthmatic airway remodelling[473]. In order to define if hydrogen sulphide can directly affect mast cell activation, we tested the effect of hydrogen sulphide on mast cell degranulation by using naïve or sensitised RBL-2H3 cells[474]. The data obtained showed a remarkable concentration-dependent inhibition of the RBL-2H3 degranulation by hydrogen sulphide only in sensitised cells. Indeed, hydrogen sulphide did not exhibit any effect on the degranulation induced by different non-specific stimuli such as thapsigargin and ionomycin. These results confirm that hydrogen sulphide, as it happens *in vivo*, displays an inhibitory action only once mast cells are activated.

3.2.6 CONCLUSIONS

In conclusion, the treatment of sensitised mice with the aerosol of hydrogen sulphide improves lung and bronchial hyperreactivity to carbachol. Our data indicate that these effects are due to a modulation of mast cell and myofibroblast activation. These results suggest a possible explanation of the beneficial effects clinically obtained by the inhalation of sulfurous waters. Finally, the data obtained can propose that aerosol treatment with H_2S-donors could be exploited as therapeutic complementary approach in lung diseases such asthma or COPD.

3.2.7 LIMITATIONS

Although empiric evidences already suggest that sulphurous thermal waters have beneficial properties in upper airway chronic diseases, the mechanism has not been investigated. In our model, we observed that the aerosolised H_2S ameliorates hyperreactivity in an animal model via the inhibition of activation of mast cells and fibroblasts. Future traslational studies involving patients suffering from asthma and treated with H_2S-releasing molecules is recommended in order to observe affinities/differences in the mechanism of action.

3.3 STUDY OF H_2S IN MODELS OF PULMONARY HYPERTENSION

3.3.1 RATIONALE

In the previous chapter, we demonstrated that inhaled H_2S could prevent the airway hyperreactivity associated with OVA sensitization. Since the effect of H_2S appeared to be independent of inflammation, the observations are consistent with a direct effect on airway smooth muscle. This is in line with the well-established finding that H_2S is a direct bronchodilator.

In the current chapter, we have extended our investigation into the role of H_2S biology into models of PAH. PAH is a devastating respiratory condition that could rapidly lead to death if not treated. Furthermore, the pharmacological therapy is limited and is not addressed at re-establishing the physiological conditions in the vessels, but only aims at limiting the symptoms. Indeed, current drugs used to treat PAH do not cure the disease but merely leads to a very limited delay in ultimate death.

In this part of the study, human and animal models of PH were considered. In particular, human models of PAH were established by means of *in vitro* experiments of cultured cells. As PAH affects the pulmonary vessels, only the most relevant vascular smooth muscle cell type to use is from pulmonary artery (PASMCs). Pulmonary artery endothelial cells were not used; instead, advantage was taken of a relatively new endothelial cell model where cells are grown out from progenitors in blood, the so-called “blood outgrowth endothelial cells” (BOECs). These were used because they can be easily obtained from living donors without the need for surgical specimens, allowing cells to be compared from healthy donors and from patients with PAH.

Thus, in this chapter, we have addressed the following hypothesis and specific aims:

Hypothesis:

"H_2S has protective actions in experimental models of PAH"

Aims:

- to establish the effect of H_2S on human pulmonary artery smooth muscle cell proliferation and inflammatory responses;
- to investigate the effects of H_2S on proliferation and inflammatory responses of endothelial cells grown from the blood of healthy donors and from patients with PAH;
- to determine any combined effect of H_2S and clinical drugs used to treat PAH on endothelial cell proliferation and inflammatory responses;
- to investigate the effect of PAH on tissue levels of H_2S in the hypoxic mouse model;
- to investigate the effect of PAH on the vascular effects of H_2S in pulmonary arteries from control and hypoxic mice;
- to establish the effects of PAH in human patients on plasma levels of H_2S measured using two established techniques.

3.3.2 METHODS

The methods relating to experiments performed in this section are described in the Chapter 2 (Materials and methods).

3.3.3 DATA HANDLING AND STATISTICAL ANALYSIS

The statistical analysis relating to experiments performed in this section are described in the Chapter 2 (Materials and methods). The details of each statistical test used are reported in the respective figure legends.

3.3.4 RESULTS

H_2S release from NaHS donor on in vitro *experiments*

We measured the H_2S release over time from the classical H_2S-donor NaHS to assess its relevance to *in vitro* experiments. We aimed at performing a stability experiment that could effectively mirror the conditions used for the following experiments. In order to do this, 200 µl of NaHS solution (10, 100, and 1000 µM) dissolved in complete DMEM (with 10% FCS) were put in a 96-well plate, in absence or presence of cells (hPASMCs, 8,000 cells/well), and incubated in a humidified chamber at 37°C/5% CO_2. Each condition was run in triplicate and H_2S generated by NaHS were assessed by means of methylen blue assay.

The results of our experiments proved that, in absence of cells, H_2S released by a concentration of NaHS 1000 µM dramatically dropped about 90% after 1 hour of incubation and the amount of H_2S is so low as to be irrelevant after 4 hours (Figure 3.24). In presence of cells, H_2S released instantly dropped about 15%, likely due to metabolic consequences, and after 1 hour an 88% loss was observed. As observed previously, after 4 hours the concentrations of H_2S are irrelevant. H_2S production by NaHS 100 µM, in presence and absence of cells, rapidly went down of about 86% and 92%, respectively, after 1 hour of incubation. NaHS 10 µM generated undetectable concentrations of H_2S already after 1 hour and still were so after 4 hours.

We did not try lower concentration because of the detection limit of the assay (1-2 µM).

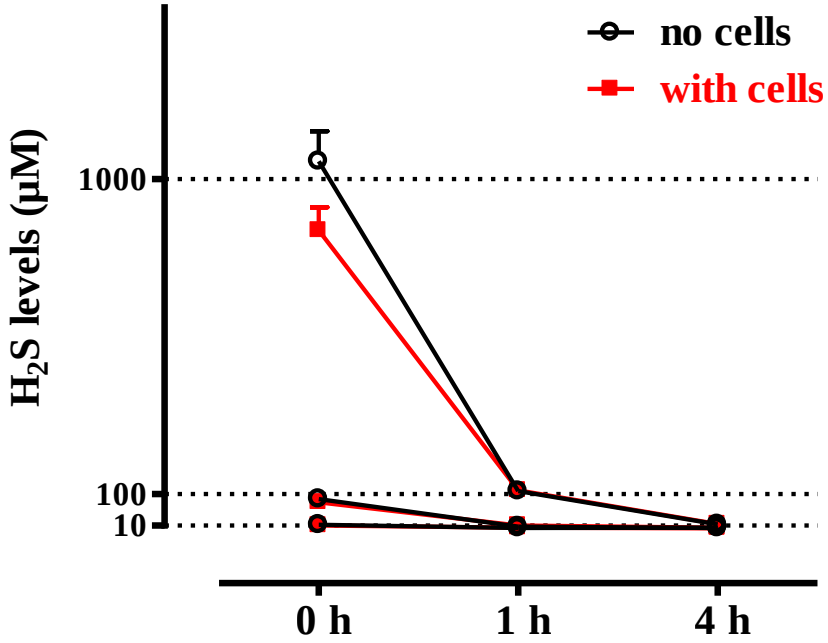


Figure 3.24. Stability of NaHS in medium. Production of H_2S from different concentrations of the H_2S-donor NaHS (10, 100, and 1000 µM) was tested, in absence and presence of cells (hPASMCs), at different time points (0, 1, and 4 h) by means of MB assay. Data are mean ± SEM, *n=4* in each group.

Effect of NaHS on IP10 release from hPASMCs and BOECs

The research group in which I completed the last part of my doctorate project had previously showed that IFN-α-primed hPASMCs released increased levels of IP10[187], as shown in Figure 3.25A. Here, we demonstrated that NaHS was able to significantly reduce IFN-α-induced IP10 release from hPASMCs in a concentration-dependent manner (Figure 3.25B). Although TNF-α co-administration accentuated IFN-induced responses, it is not an absolute prerequisite for IFN sensing in some cells. Nevertheless, NaHS showed only a slight and not significant inhibition of IP10 release after a combination of IFN-α and TNF-α, likely due to the synergistic effect of the substances[422] (data not shown).

As already demonstrated[187], BOECs cultured from blood of patients affected by PAH were more sensitive to IFN-α compared to healthy BOECs in releasing IP10 (Figure 3.25C). Similarly, NaHS was able to reduce IP10 production also in BOECs and this effect was even more marked for iPAH rather than healthy (Figure 3.25D).

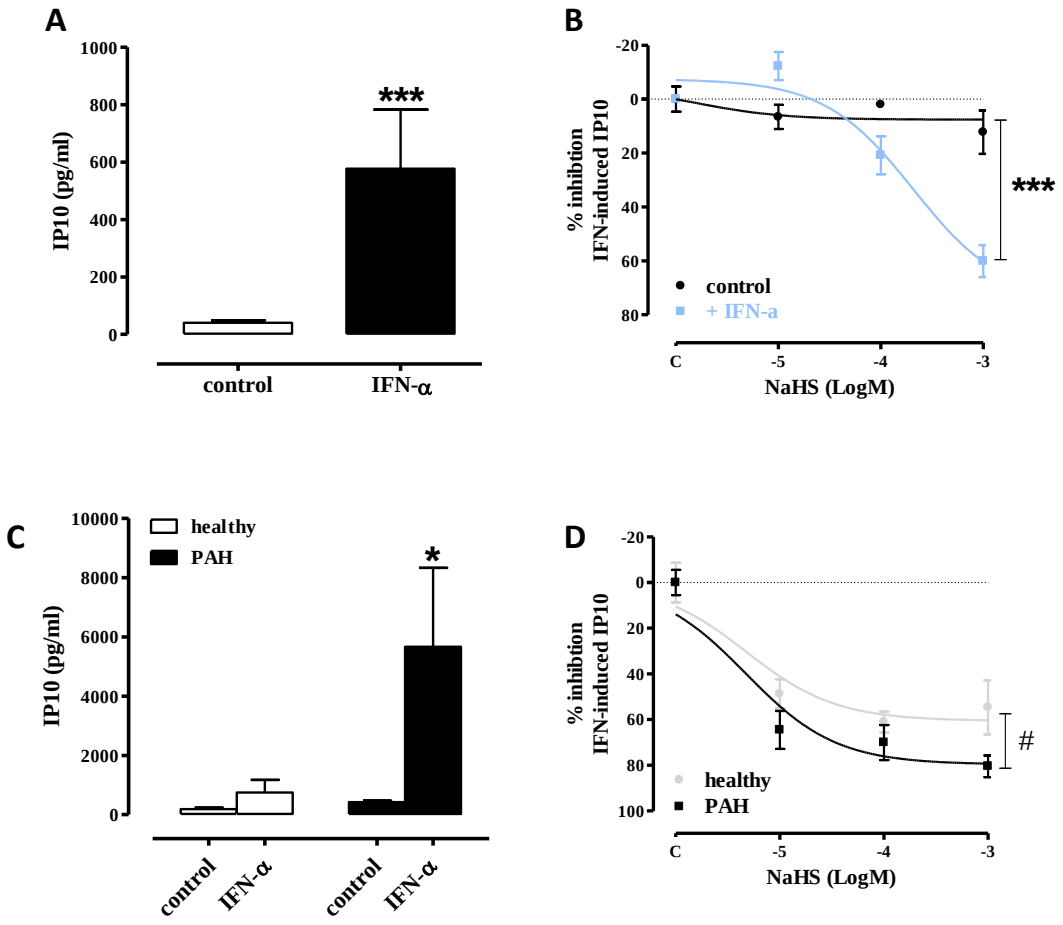


Figure 3.25. NaHS decreases IP10 levels released from hPASMCs and BOECs. (A) IP10 release from vehicle- and IFN-α-stimulated hPASMCs (*** p<0.001 vs. control, Student's t-test). (B) Decrease of IP10 release from hPASMCs stimulated with IFN-α (10 ng/ml) and treated with increasing concentrations of NaHS (10^{-5}-10^{-3} M) (*** p<0.001 *vs.* control). (C) IP10 release from vehicle- and IFN-α-stimulated BOECs (from PAH patients and healthy individuals) (* p<0.05 IFN-α *vs.* control, Student's t-test). (D) Decrease of IP10 release from BOECs (from PAH patients and healthy individuals) stimulated with IFN-α (10 ng/ml) and treated with increasing concentrations of NaHS (10^{-5}-10^{-3} M) (# p<0.05 PAH *vs.* healthy, two-way ANOVA with Bonferroni's post-test). Data are mean ± SEM, *n=4* in each group.

Effect of NaHS on IL-8 release from hPASMCs and BOECs

We then looked at the effect of NaHS on a more general inflammatory cytokine, namely IL-8. We stimulated both hPASMCs and BOECs (healthy and PH) with TNF-α (Figure 3.26A and 3.26C), as IFN-α does not promote IL-8 production. NaHS was able to reduce IL-8 release from hPASMCs (Figure 3.26B), even though this effect was less pronounced when compared to that seen on IP10; on BOECs, NaHS showed any effect on the production of the cytokine neither in healthy cells nor in PAH-affected cells (Figure 3.26D).

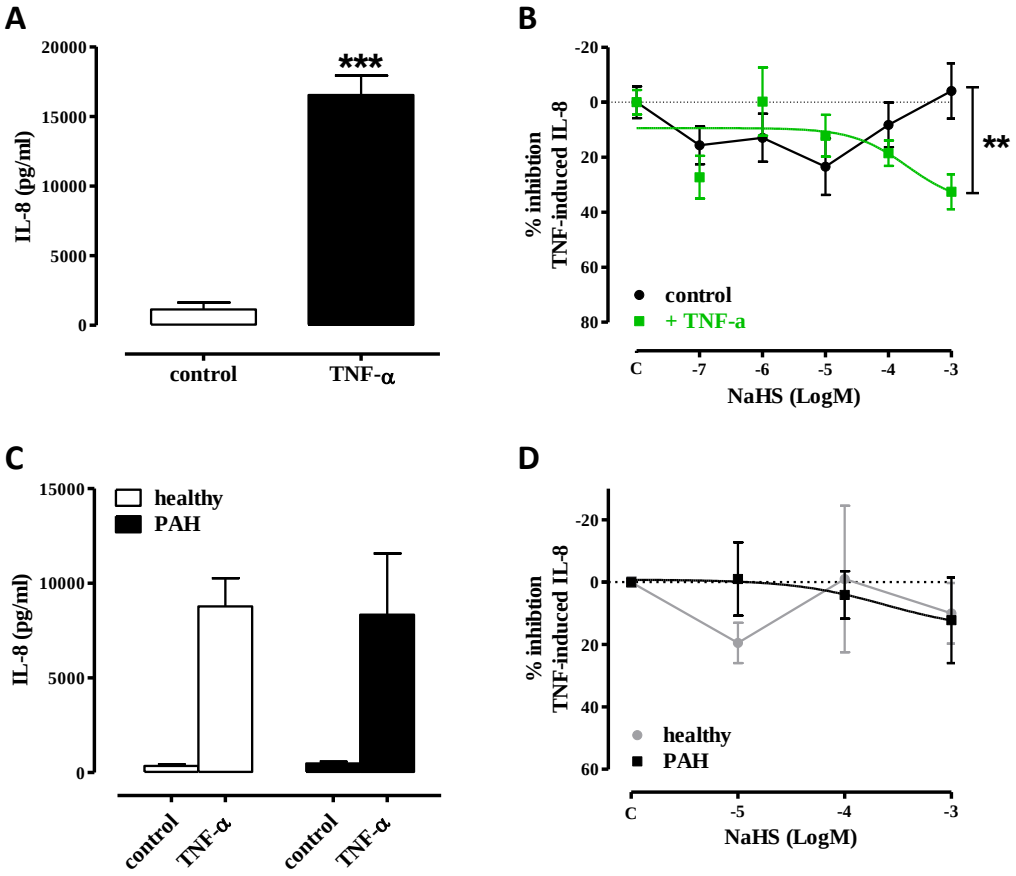


Figure 3.26. Effect of NaHS on IL-8 release from hPASMCs and BOECs. (A) IL-8 release from vehicle- and TNF-α-stimulated hPASMCs (*** $p<0.001$, Student's *t*-test, *n=5* in each group). (B) Decrease of IL-8 release from hPASMCs stimulated with TFN-α (10 ng/ml) and treated with increasing concentrations of NaHS (10^{-5}-10^{-3} M) (** $p<0.01$ *vs.* control, *n=5* in each group). (C) IL-8 release from vehicle- and TNF-α-stimulated BOECs (from PAH patients and healthy individuals) (*n=3* in each group). (D) No effect is observed on IL-8 release from BOECs (from PAH patients and healthy individuals) stimulated with TNF-α (10 ng/ml) and treated with increasing concentrations of NaHS (10^{-5}-10^{-3} M) (*n=5* in each group). Data are mean ± SEM.

Effect of NaHS on viability of hPASMCs and BOECs

Others have shown that NaHS was able to reduce proliferation rate in both human and animal smooth muscle cells both *in vivo*[409,410] and *in vitro*[414]. This is relevant to PH since the disease is characterised by hyperplasia of the inner cell layers of the vessels. However, under our experimental conditions tested, neither the stimulating agents nor the NaHS affected the viability of cells (Figure 3.27A, 3.27B, and 3.27C).

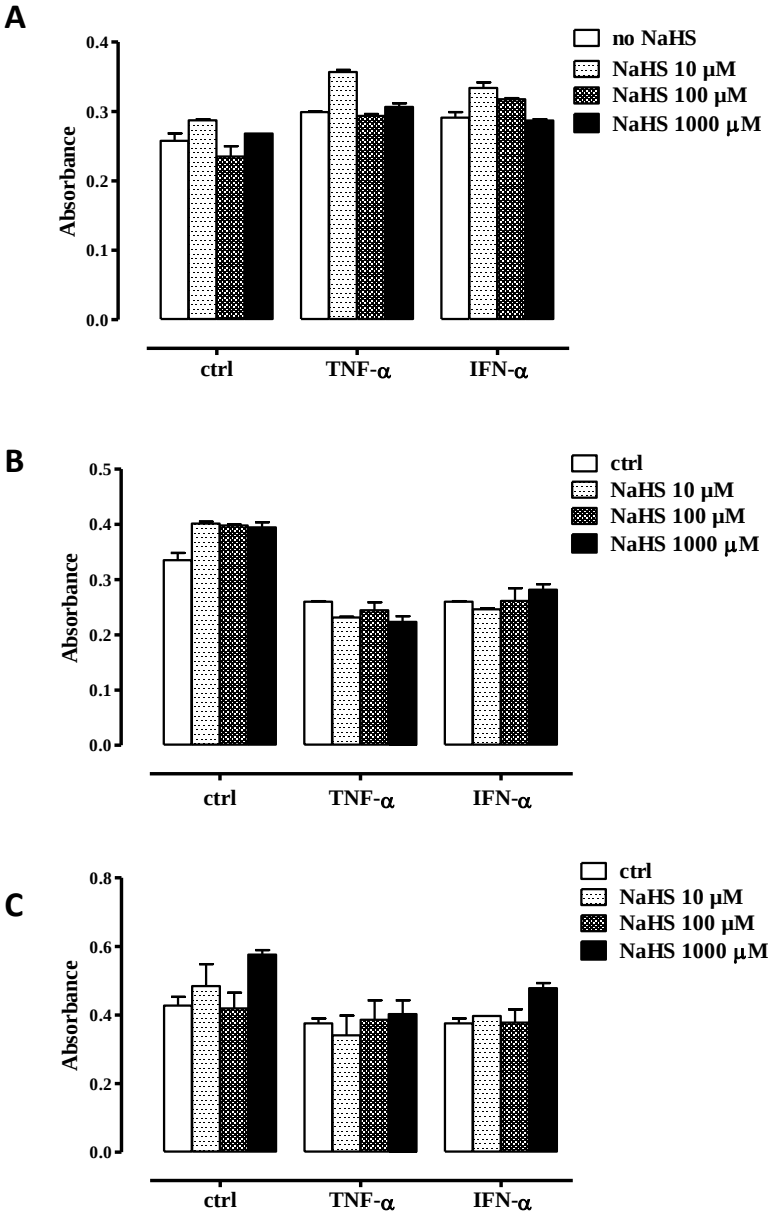


Figure 3.27. NaHS does not affect proliferation rate of hPASMCs and BOECs. Representative graphs of cell viability. (A) No effect has been observed on proliferation rate of hPASMCs not-stimulated or stimulated with IFN-α (10 ng/ml) or TNF-α (10 ng/ml) and treated with increasing concentrations of NaHS (10-1000 µM). (B) No effect has been observed on proliferation rate of BOECs (isolated from PAH-affected patients) not stimulated or stimulated with IFN-α (10 ng/ml) or TNF-α (10 ng/ml) and treated with increasing concentrations of NaHS (10-1000 µM). (C) No effect has been observed on proliferation rate of BOECs (isolated from healthy individuals) not stimulated or stimulated with IFN-α (10 ng/ml) or TNF-α (10 ng/ml) and treated with increasing concentrations of NaHS (10-1000 µM). Cell viability has been assessed by Alamar Blue® after 24 hours from treatment. Data are mean ± SEM.

Effect of NaHS on ET-1 release from hPASMCs and BOECs

As the group where I worked demonstrated that ET-1 is an IFN-regulated gene, then our attention focused on ET-1. ET-1 is a key mediator in PAH: its increased levels are responsible for hyperproliferation of SMCs, constriction state of vessels, and endothelial dysfunction. My group showed that also hPASMCs released increased levels of ET-1 when treated with an appropriate stimulus[187].

Surprisingly, unlike the observations above using IP10 as a read-out, IFN-α-induced ET-1 release was not affected in either hPASMCs (Figure 3.28A) or BOECs (healthy and PAH) (Figure 3.28B and 3.28C).

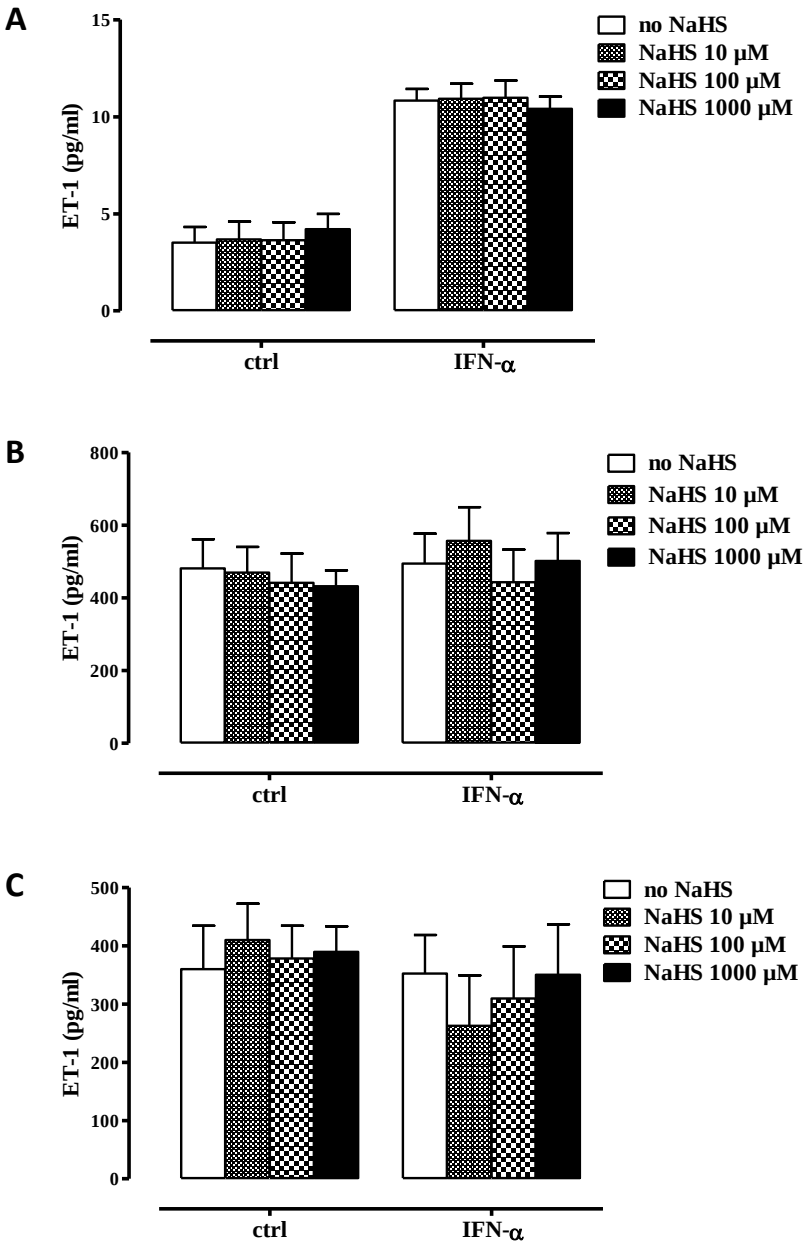


Figure 3.28. NaHS does not affect ET-1 production from hPASMCs and BOECs. (A) Increasing concentration of NaHS (10-1000 µM) showed no effect on ET-1 release from unstimulated hPASMCs or cells primed with IFN-α (10 ng/ml) or TNF-α (10 ng/ml), (*n=4* in each group). (B) Increasing concentration of NaHS (10-1000 µM) showed no effect on ET-1 release from unstimulated BOECs (from PAH patients) or cells primed with IFN-α (10 ng/ml) or TNF-α (10 ng/ml) (*n=3* in each group). (C) Increasing concentration of NaHS (10-1000 µM) showed no effect on ET-1 release from unstimulated BOECs (from healthy individuals) or cells primed with IFN-α (10 ng/ml) or TNF-α (10 ng/ml) (*n=3* in each group). Data are mean ± SEM.

Synergistic effect of NaHS and sildenafil, ACT-132577, and treprostinil on IP10 release

The current therapy for PAH is based on drugs aiming at controlling the vasoconstriction. Thus, PDE type V inhibitors, ET-1 receptor antagonists, and prostacyclin (PGI_2) analogues have been introduced in therapy. Here, we aimed at identifying a potential synergistic effect of NaHS with these substances: therefore, we pretreated IFN-α-stimulated healthy BOECs with NaHS (0, 100, and 1000 µM) and increasing concentrations of sildenafil (a PDE type V inhibitor), ACT-132577 (the active metabolite of macitentan, a new ET_A receptor antagonist), or treprostinil sodium (a synthetic analogue of PGI_2). The drugs alone had either no effect or tended to reduce the IP10 release from the cells. However, the treatment with the drugs and NaHS revealed an inhibitory effect that, where relevant, was additive. The effect of NaHS was particurarly pronounced at concentration of 1000 µM (Figure 3.29A, 3.29B, and 3.29C).

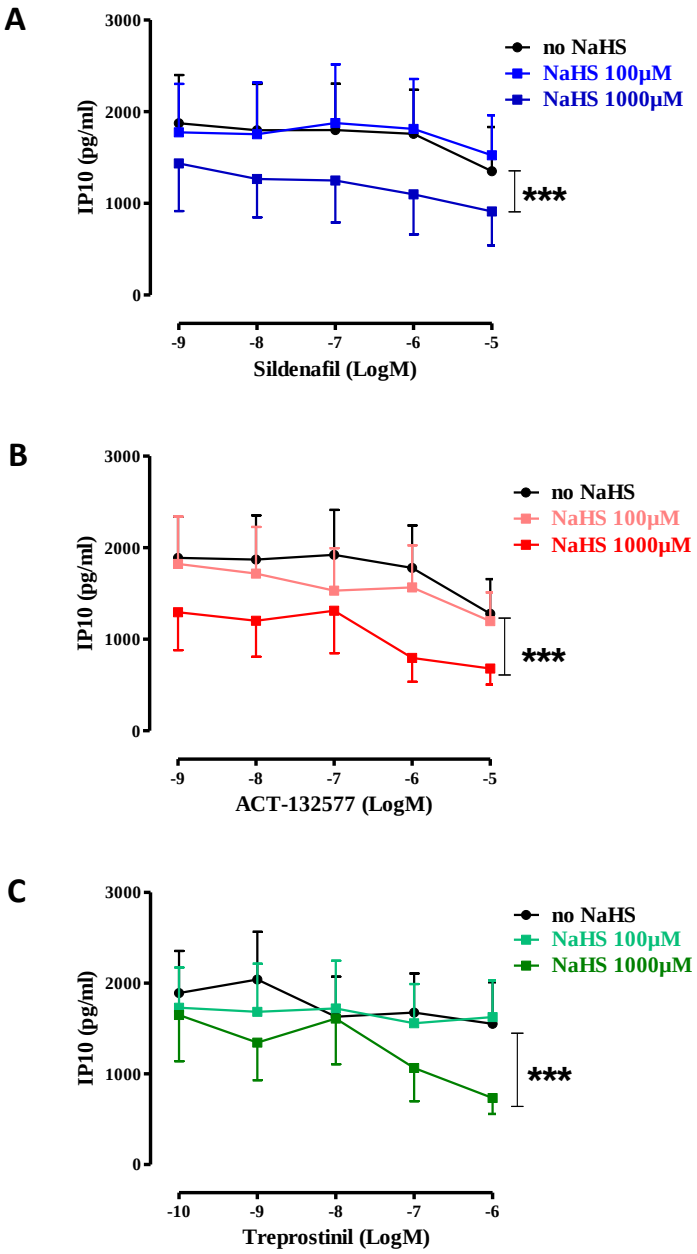


Figure 3.29. Additive effect of NaHS and Sildenafil, ACT-132577, and Treprostinil sodium on IP10 release from healthy BOECs. (A) IP10 production is decreased after co-incubation of NaHS (100 or 1000 µM) with Sildenafil (10^{-9}-10^{-5} M) (*** p<0.001 NaHS 1000 µM *vs.* no NaHS, two-way ANOVA with Bonferroni's post-test). (B) IP10 production is decreased after co-incubation of NaHS (100 or 1000 µM) with ACT-132577 (10^{-9}-10^{-5} M) (*** p<0.001 NaHS 1000 µM vs. no NaHS, two-way ANOVA with Bonferroni's post-test). (C) IP10 production is decreased after co-incubation of NaHS (100 or 1000 µM) with treprostinil sodium (10^{-10}-10^{-6} M) (*** p<0.001 NaHS 1000 µM *vs.* no NaHS, two-way ANOVA with Bonferroni's post-test). Data are mean ± SEM, *n=3* in each group.

Effect of NaHS on mice undergoing hypoxic/normoxic conditions

The following experiments aimed at looking into the effects of H_2S on an experimental model of hypoxia-induced PAH in mice using tissues *ex vivo*, in order to further understand the role of this gasotransmitter in the pathology.

First of all, we incubated parts of the lung of these mice in DMEM (with 10% FCS) and treated with Poly(I:C) (10 µg/ml), mouse IFN-α (10 ng/ml), and LPS (1 µg/ml) for 24 hours. After this period, cell-free supernatants were collected and assayed for H_2S levels by means of methylen blue assay. The data showed that untreated lungs from hypoxic mice released higher amount of H_2S in medium compared to normoxic mice and this difference was significant (Figure 3.30A). These data well correlated with the findings that serum H_2S levels from human patients affected by iPAH were significantly higher than healthy individuals (Figure 3.30B). Surprisingly, a very high increase in H_2S release was seen in IFN-α-treated tissues from normoxic mice compared to hypoxic (Figure 3.30A). No significant differences has been observed in Poly(I:C)- and LPS-treated lungs (Figure 3.30A).

Finally, pulmonary artery reactivity has been assessed as well. Pulmonary arteries precontracted with the thromboxane-mimetic U46619 were exposed to cumulative concentrations of NaHS. As already shown by others[475,476], NaHS relaxed vessels. However, the data obtained showed that there was a significant difference in relaxation as evidenced by EC_{50} and maximum effect (E_{max}) values: in arteries harvested from hypoxic mice, a more pronounced response to relaxant effects of NaHS was seen (Figure 3.30C). It seemed that this effect was selective to NaHS: the same vessels relaxed with SNP, a NO-donor, had only a slight effect attributed to hypoxia-induced PAH on EC_{50}, but the differences were not significant (Figure 3.30D). However, it is relevant to note that pulmonary arteries from hypoxic mice showed a significant increased contractile response to cumulative concentrations of U46619 (Figure 3.30E).

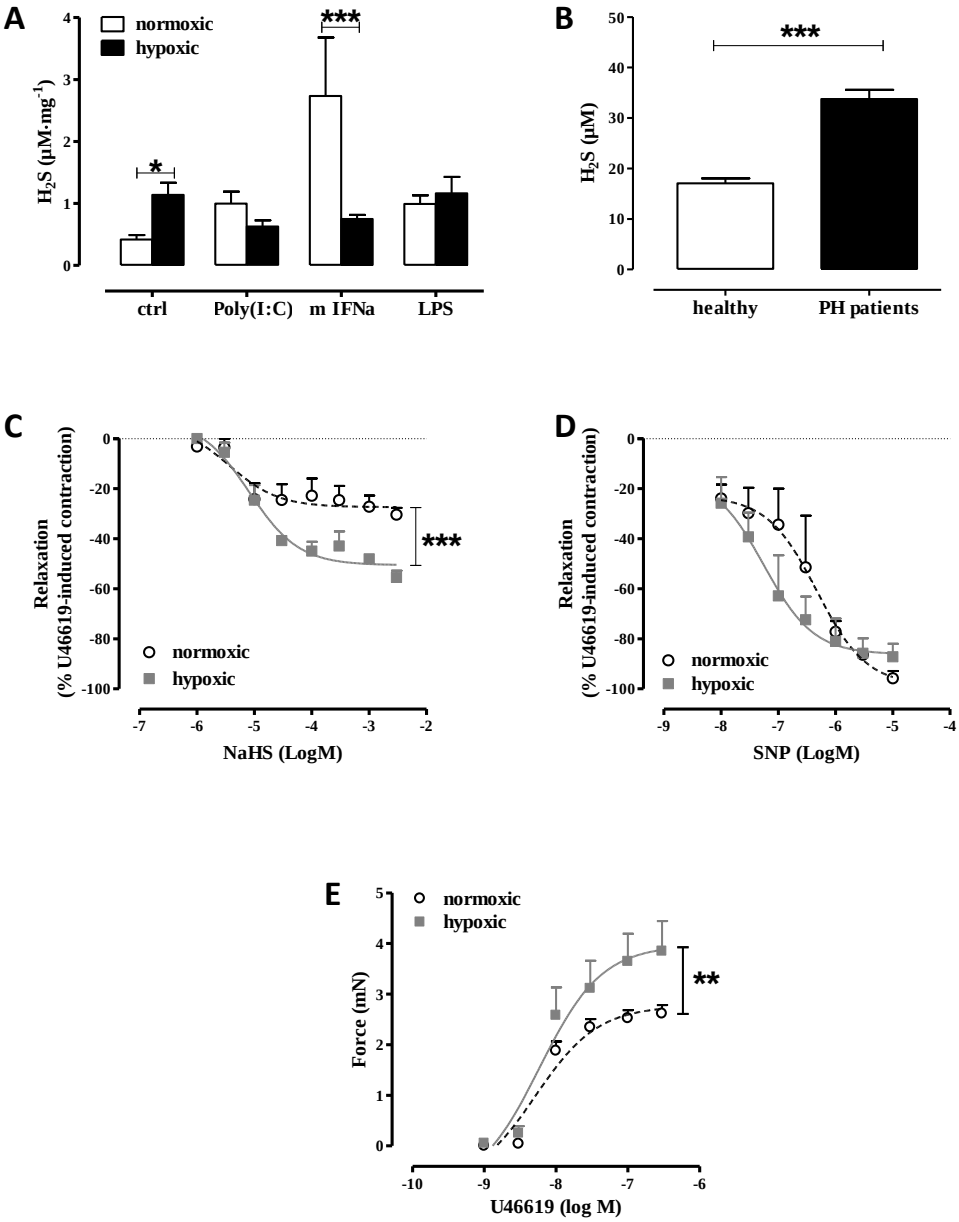


Figure 3.30. Characterization of murine experimental model of PH. (A) H_2S production in supernatants of lungs from normoxic/hypoxic mice cultured in DMEM 10% in absence of stimulus or with Poly(I:C) (10 µg/ml), mouse IFN-α (10 ng/ml), or LPS (1 µg/ml) for 24 hours in a temperature-controlled humidified incubator (* p<0.05; *** p<0.001, Student's t-test, $n=4$ in each group). (B) Serum H_2S levels from healthy and PAH-affected patients (*** p<0.001, Student's t-test, $n=9$ individuals in each group). (C) Vasorelaxant effect of NaHS (10^{-6}-3×10^{-3} M) on U46619-precontracted pulmonary artery harvested from normoxic/hypoxic mice (*** p<0.001, two-way ANOVA, $n=3$ mice in each group). (D) Vasorelaxant effect of SNP (10^{-8}-3×10^{-5} M) on U46619-precontracted pulmonary artery harvested from normoxic/hypoxic mice ($n=3$ mice in each group). (D) Contractory effect of U46619 (10^{-9}-3×10^{-7} M) on pulmonary artery harvested from normoxic/hypoxic mice (** p<0.01, two-way ANOVA, $n=3$ mice in each group). Data are mean ± SEM.

3.3.5 SUMMARY

In this part of the study, we investigated the idea that H_2S could be protective in PH. This was a relatively new idea and our work is therefore based at a preclinical level only. To investigate this, we assessed the effect of H_2S in cell and mouse tissue models relevant to PH in humans.

As a toxic gas, H_2S itself is neither easy nor recommended for use in experimental procedures. Because of this, we used the sodium hydrosulphide (NaHS) as a H_2S-donor because it is inexpensive, soluble in aqueous buffer, and has a very long history in use for scientific purposes. Moreover, as a salt, the concentrations of H_2S levels released by NaHS are very easy to control. First, we performed stability experiments in order to establish the correct concentration range of NaHS for our experimental procedures. We observed that H_2S released from NaHS dropped very rapidly (within 1 hr) to undetectable levels under the conditions tested. We can conjecture that the time-dependent H_2S loss was even shorter, as Martelli *et al.* already reported, although with a different detection method[477].

In addition to the originally accepted view that PH is a disease of remodelled and constricted vessels, it is now known that PH is also driven by inflammatory cytokines[478,479]. Previous works from my group had implicated, in particular, interferon and interferon-target genes, which include ET-1 and IP10[187]. IP10 is one of the best characterised of all the IFN-stimulated genes. IP10, which is secreted by endothelial cells (among other cell types), is the cognate ligand of CXCR3 and is an antiviral and chemoattractant that promotes the formation of lymphoid infiltrates commonly seen in viral infection and autoimmune disease[480]; it also potentiates the adhesion of T lymphocytes to the endothelium[481] and promotes the migration of CXCR3+ cells to the lung[482]. CXCR3 expression, at both the gene and protein level, has previously been demonstrated to be up-regulated in patients with PAH and IP10 has been shown to mediate endothelial dysfunction by disrupting calcium homeostasis[483]. We evaluated IP10 levels as read-out because it is increasingly recognised as a potential mediator of inflammation, including that

associated with the lung[481,482]. Furthermore, my group has shown that IP10 levels correlate with disease indicators and biomarkers in PAH[187]. Our results showed that NaHS was able to reduce IP10 levels in IFN-α-primed hPASMCS and BOECs, but not IL-8 release from TNF-α-stimulated cells, suggesting a specific role of H_2S towards lung inflammatory mediators in our experimental model. Since ET-1 is an IFN-related gene, our attention then focused on ET-1 release: surprisingly, NaHS did not affect ET-1 release from cells.

Current pharmacological approaches for PAH are not addressed at resolving the underlying causes, but only at targeting the symptoms. Here, we showed that the one of each most representative class of drugs used in therapy have a synergistic effect with H_2S in terms of reducing IP10 release and, consequently, lowering the inflammation and symptoms.

We then extendended our observations from experiments performed on human cells to *ex vivo* approaches from an animal model of PH. Even if we did not performed systemic administration of NaHS into mice, we showed that lung tissue from hypoxic mice produced higher levels of H_2S compared to normoxic ones: these findings are in line with the human data showing that iPAH-affected patients had higher levels of circulating H_2S compared to healthy individuals. However, this is in contrast with some other reports assuming that serum H_2S levels are lower in patients affected by the disease compared to control[387,404,405,406,412].

H_2S is a well-known vasorelaxant mediator of both capacitance and resistance vessels. Its vasorelaxing effects have been shown also in both human and rat pulmonary artery[475,476]. Here, for the first time to our knowledge, we compared relaxing effects of NaHS on pulmonary arteries harvested from normoxic (control) and hypoxic (with PH) mice: we demonstrated that NaHS relaxed more powerfully arteries from hypoxic mice and this effect seemed to be H_2S-specific since an NO-donor did not showed the same differences in terms of relaxation. As endothelial dysfunction of pulmonary arteries is a well-characterised hallmarks of PAH, we expected and obtained a stronger contraction from PH arteries compared to controls.

3.3.6 CONCLUSIONS

Work from this chapter shows that H_2S, via actions on inflammatory pathways associated with IFN and on pulmonary vessels from animals with established PH, could be protective in PAH. However, more efforts are necessary to fully explore the potential of these observations.

3.3.7 LIMITATIONS

The experiments we run represent only preliminary data. It is worth to evaluate the effects on inflammatory patterns from human cells by using different H_2S-donors, such as the slow-releasing molecules GYY4137 or DADS., in order to further validate the observations.

The data we collected are related only to human cellular models of pulmonary hypertension. Other suggested experiments could involve the effects of systemic/localised administration of NaHS in the animal model of pulmonary arterial hypertension in order to observe whether H_2S could have beneficial effects. Furthermore, H_2S levels should be measured also in serum from hypoxic/normoxic mice and in a larger well-defined cohort of human patients affected by PAH, by means of methylen blue assay. However, since this assay does not measure free sulphide levels but the whole pool[358], another way to measure the gasotransmitter is desirable, such as the monobromobimane method associated to HPLC.

CHAPTER 4
DISCUSSION

Inflammatory pulmonary diseases represent an authentic threat for global population since it is estimated that they will be among the first causes of death in the next years, because of their complications. Many progresses has been achieved since they were described for the fist time, but the biomedical research in this field is far from having reached an end-point. Many new drugs (including monoclonal antibodies from the area of biotechnologies and long-acting β-agonists) have been recently introduced in therapy, but they have still some limitations. In asthma, for example, an important share of patients does not respond to conventional treatments, such as corticosteroids, or suffers a loss of efficacy of β-agonists in the long term. Furthermore, in pulmonary hypertension, current pharmacological approach only aims at treating the symptoms but does not cure the causes at the basis. Here, pharmacological research in discovering new pathological pathways that could lead to designing new drugs is capital.

Our study well integrates in this perspective. Our first objective was to clarify the role of the sphingolipid S1P in lung pathophysiology. It is known that S1P affects immune system, fibroblasts, and smooth muscle cells, central effector entities in the pathophysiology of asthma. We demonstrated that T cells and mast cells are essential in mediating the effects of S1P and B cells counteract them. The model described and characterised in this study may represent a useful tool to define the role of S1P in the mechanism of action of currently used drugs, as well as in the development and characterisation of new therapeutic approaches. Furthermore, current therapies are designed to treat the symptoms of asthma trying to decrease the ongoing inflammation and reducing bronchial constriction. Further understanding of the role of IgE has led to new approaches in asthma

management that act early on in the allergic cascade of events. Therefore, we believe that the understanding of B and T lymphocyte and mast cell implication in S1P-induced asthma-like responses has considerable therapeutic implications, according to the role of the endogenous S1P in both mice and asthmatic patients.

The centrality of mast cells in this condition is also supported by our further findings. We demonstrated the beneficial properties of an aerosolised administration of H_2S in a well-described and accepted experimental model of airway hyperresponsiveness. We observed that treatment with H_2S through aerosol to OVA-sensitised mice reduced bronchial hyperresponsiveness but did not affect lung inflammation. Mast cell and fibroblast activation accounted for the blunt in airway hyperreactivity. However, the beneficial effect of H_2S in the OVA model needs to be further studied at molecular levels. In particular, the interaction of the sphingolipid methabolism with the gasotransmitter by means of our S1P-induced asthma-like model needs to be performed. Once these aspects have been defined, it will be also possible to assess the possible interaction with the other two gasotransmitters, namely CO and NO.

Finally, the role of H_2S was studied in another inflammation-based disease, the pulmonary arterial hypertension. Conversely to the previous results, in experimental cellular models of PH, H_2S acted as an antiinflammatory mediator. The difference in terms of effects of H_2S in the two experimental conditions could be attributable to the finding that in OVA-induced asthma H_2S has been administered *in vivo*, whereas in PH models it has been administered *in vitro*.

Another possible explanation is that, in different pathologies, the relative role of the H_2S pathway and its interaction with other mechanism(s) deployed leads to a different outcome. In other words, the difference in the pathophysiology between PH and the asthma-like model used may account for the different contribute of the H_2S pathway.

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