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EXPERIMENTAL STUDIES OF LIPID MEDIATOR MODULATION OF AIRWAY RESPONSIVENESS

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Experimental studies of lipid mediator modulation of airway responsiveness

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Good things in life take time and effort

POPULAR SCIENCE SUMMARY

Deep in the lungs, gas exchange is happening with oxygen taken up in the bloodstream and carbon dioxide expelled to be breathed out. To reach these deep parts of the lung, the air is guided through a series of branching tubes forming the upper (nose cavity and trachea) and lower (bronchi and bronchioles) airways. The ability of these airways to contract and relax is very important for regulating the airflow and hence the ability of the lungs to exchange gases. Asthma is a disease of the airways which results in the airways contracting too much, too often. Reason is that airway contraction is more easily triggered compared to healthy airways. The resulting excessive narrowing leads to breathing difficulties. This obstruction of the airways is also called airway hyperreactivity.

Inflammation in the airways is an important reason for airway hyperreactivity. This inflammation can be caused by for example air pollution or viruses. Another trigger is allergy. In this case the immune system gets activated by otherwise unharmful causes like dog dander or mite debris present in dust found in most houses (called house dust mites). This inflammation causes airways to contract and swell, making them narrower, just as for example when a finger gets inflamed and starts to swell.

Many cells of the immune system play a role in allergic asthma, but one cell type is especially important: the mast cell. This guardian cell can be found in all places of the body that are in contact with the outside world, for example the intestines, the skin and the lungs. They normally help the body protect itself against bacteria and parasites. However, mast cells in the lung can also cause lung disease. This happens when a person with allergic asthma is exposed to for example house dust mites, causing mast cells to become wrongly activated.

When mast cells become activated, they release different kinds of signalling molecules. Among these molecules are different kinds of lipid messengers. These are made by the body from polyunsaturated fats from food such as nuts or fish. Important lipid messengers are prostaglandins and leukotrienes. Prostaglandins are known for causing inflammation symptoms (pain, swelling, redness, heat) and fever. Drugs like ibuprofen put a brake on prostaglandin formation. That is why they are used during fever or inflammation. Leukotrienes are well known for their ability to make the airways contract, as some prostaglandins do as well. Because of this release of lipid mediators, mast cell activation can lead to inflammation and airway contraction.

However, lipid messengers are not only involved in starting inflammation, but may also contribute to stopping inflammation. One such group of lipids is called specialised pro-resolving mediators (SPMs) and the function of these SPMs is thought to be to put a brake on inflammation. This means that they block some inflammatory effects of other substances, but also activate certain parts of the immune system to clean up all the bacterial and dead cell debris after inflammation. This way the body can return to its normal, healthy state again.

In this thesis it was investigated if a selection of SPMs could reduce airway hyperreactivity. If so, future medicines based on SPMs could be developed to relieve airway hyperreactivity in asthma, and so decrease hospital visits and increase the quality of life of patients. In addition, it was assessed what mechanism lies behind the observation that drugs like ibuprofen can worsen symptoms of asthma in certain patients. And finally, it was investigated whether lung mast cells were responsible for causing airway hyperresponsiveness and contraction when exposed to house dust mite extract.

First, repeated exposure to an extract of house dust mites, but also TNFα and IL-13 were used to cause airway hyperreactivity in mice. TNFα and IL-13 are so-called cytokines and have important roles as a signalling molecule in the immune system. Second, when mice received certain SPMs together with house dust mite, TNFα or IL-13, the airway hyperreactivity was reduced. These mice were thus protected from airway hyperreactivity, in particular after exposure to the SPMs LXA_4 and three cysteinyl maresins. A possible explanation for how lipoxins mediated these protective effects could be that lipoxins directly interfere with the ability of TNFα to signal to other cells in the airways. A receptor was found that might be important in the reducing effect of cysteinyl maresins, but the exact role of this receptor remained unclear. With our experiments it was shown that the tested SPMs are promising targets for future development of drugs to treat people with difficulties breathing due to increased airway contraction, e.g., in asthma.

Additionally, it was discovered that not only SPMs had a protective function. Quite surprisingly, PGD_2, mostly regarded as to cause inflammation, also had a braking effect on activation of mast cells. This effect was found by activating mast cells in guinea pig trachea. Mast cell activation caused the guinea pig trachea to contract due to release of many substances like histamine, cysteinyl leukotrienes and PGD_2. But contrary to the other substances, the release of PGD_2 made the tracheal rings actually contract less. Also, the dampening effect of PGD_2 was lost when a specific receptor on mast cells, the DP_1 receptor, was blocked. The proposed mechanism is as follows: when airways from sensitised guinea pigs are exposed to allergens, mast cells become activated. These activated mast cells release many inflammatory and constriction-causing substances. However, they also release PGD_2 which puts a brake on the same mast cells to prevent them from releasing more substances that cause contraction. PGD_2 thus helps mast cells to not to release too much of the contractile substances and ultimately prevents too much contraction of the airways.

Finally, in mouse airways and lungs from mice made allergic to house dust mite, it was shown that the presence of mast cells was necessary to cause a contraction when exposed to house dust mite extract. Moreover, mast cells made airways more sensitive to contraction-causing substances. This resulted in increased airway contraction compared to mice not allergic to house dust mite. Also, mast cells were activated by a substance released from nerves. It could therefore be that nerves present in airways influence how mast cells and airway contract when exposed to allergens like house dust mites.

In conclusion, what was learned from these experiments is that:

- House dust mite, TNFα and IL-13 cause increased contractions of airways. This increased contraction of airways can be reduced by lipoxins and cysteinyl maresins.

- PGD_2 does not only cause inflammation but also protects airways against excessive airway constriction.

- Mast cells present in the lung trigger airway contractions and airway hyperreactivity when exposed to house dust mite, possibly through interaction with nerves present in airways.

- Development of drugs that resemble SPMs or that are directed towards mast cells could lead to potential medicines to alleviate breathlessness in inflammatory airway diseases like asthma.

POPULÊR-WITTENSKIPLIKE GEARFETTING

Djip yn de minsklike long wurde gassen útwiksele. Koalstofdiokside wurdt út it bloed wei frijjûn oan de lucht dy't útazeme wurdt. Soerstof giet krekt de oare kant op en wurdt út de ynazeme lucht wei opnommen yn it bloed. Om de djipten fan de longen te berikken, wurdt de lucht ferfierd troch in buizestelsel. Dat stelsel bestiet út de boppeste (noas en luchtpiip) en de ûnderste luchtwegen (bronkioalen en bronkiën). Om stjoere te kinnen hoefolle lucht at der yn- en útazeme wurdt, kinne de luchtwegen gearlûke en ûntspanne. Astma is in sykte wêrby't de luchtwegen just tefolle en ta gau gearlûke. Dêrtroch rekket de pasjint benaud. Dat wurdt ek wol luchtweihyperreaktiviteit (LWH) neamd.

LWH wurdt faak feroarsake troch ûntstekking fan de luchtwegen en longen. Dat soarte fan ûntstekking kin komme troch bygelyks firussen en luchtfersmoarging. In oare reden kin wêze dat it ymmúnsysteem aktivearre wurdt troch ûngefaarlike saken lykas hûnehierren en hússtofmyt. Myt is yn alle húshâldens oanwêzich en soarget gewoanwei net foar problemen. By allergyske ûntstekking lykwols, krekt as bygelyks by in ûntstutsen finger, swolle de luchtwegen op en wurde se smeller. Dat en dat se earder en krêftiger gearlûke, soarget derfoar dat it sykheljen hieltyd lestiger wurdt.

In soad sellen spylje in rol yn it ûntstekkingsproses, mar in ekstra wichtige rol is der foar mêstsellen. Dy sellen kinne fûn wurde op alle plakken mei oerflakken dy't yn kontakt steane mei de bûtenlucht, bygelyks de termen, de hûd en dus de longen. Gewoanwei helpe se om it lichem te beskermjen tsjin baktearjes en parasiten. Mar mêstsellen wurde ek aktivearre as in persoan allergysk is foar bygelyks hússtofmyt en astma hat.

At mêstsellen aktyf wurde, begjinne se in grut ferskaat oan stoffen út te stjitten, wêrûnder fet-eftige boadskipperstoffen. In wichtige groep fetten binne de prostaglandines en leukotriënen. De prostaglandines feroarsaakje ûntstekkingssymptoanen lykas pine, tining en koarts. Dêrom wurde medisinen as ibuprofen ek brûkt, want dy remje de oanmaak fan prostaglandines yn it lichem. Leukotriënen soargje derfoar dat luchtwegen harren gearlûke, in effekt dat bepaalde prostaglandines ek hawwe. Dêrtroch liedt de aktivaasje fan mêstsellen ta ûntstekking en benaudens.

Dochs binne dy feteftige stoffen fan mêstsellen net allinnich belutsen by it begjin fan ûntstekking, mar ek om dy wer del te bêdzjen. Dy groep wurdt mei in Ingelsk wurd ek wol specialised pro-resolving mediators (SPMs) neamd. Se dogge dat troch ûntstekkingseffekten fan oare stoffen te blokkearjen, mar se stimulearje ek oare parten fan it ymmúnsysteem om oerskotten fan baktearjes en deade sellen op te romjen. Sa kin it lichem wer werom nei syn normale, sûne steat.

Yn dizze dissertaasje is ûndersocht of SPMs ek LWH ferminderje koene. At dat sa wêze soe, dan soene der yn de takomst medisinen fan makke wurde kinne foar astmapasjinten, sadat se minder nei it sikehûs ta hoege en in hegere kwaliteit fan libjen hawwe. Dêrneist hawwe wy ek ûndersocht oft in bepaalde prostaglandine - prostaglandine D_2 (PGD_2) - ek in beskermjende funksje hat. Yn it lêst hawwe wy ek ûndersocht oft mêstsellen echt nedich binne foar LWH troch hússtofmiten.

Om dat te ûndersykjes waarden luchtpipen fan mûzen en bargemotten (kavia's) brûkt. De luchtpipen kinne yn libben holden wurden yn in spesjale, iiskâlde buffer besteande út sûker en sâlten. Al it fetweefsel wurdt dan fuorthelle en de luchtpiip wurdt yn ringen knipt. De ringen wurde dan ophongen yn in myograaf. Dat apparaat bestiet út lytse putsjes dêr't in waarme buffer yn sit (37 °C, lichemstemperatuer) en twa izeren pintsjes dêr't de ringen omhinne dien

wurde kinne. Sa kin mei help fan in kompjûter metten wurde hoe't se gearlûke en wer ûntspanne. Yn de SPM-stúdzjes waarden de luchtpiipringen earst fjouwer dagen yn in ynkubator holden. Yn dy fjouwer dagen waarden stoffen dy't ûntstekking en LWH feroarsaakje en SPMs tafoege. Dêrnei waard dan yn de myografen besjoen oft de SPMs ek in delbêdzjend effekt hiene op de LWH.

Wat sjoen waard, wie dat in bepaalde groep SPMs, nammentlik de lipoksinen, yndie sa'n delbêdzjend effekt hie op LWH, feroarsake troch in ekstrakt fan hûsstofmiten en TNFα. TNFα is in saneamde sytokyne en soarget foar ûntstekking en LWH. Wat ek sjoen wurde koe wie dat de LWH nei bleatstelling oan hûsstofmytekstrakt kaam troch datselde TNFα, mar dan oanmakke troch it lichem sels. Mooglik dat de lipoksinen dus direkt de wurking fan TNFα blokkearje.

It oare artikel oer SPMs wie konsintreare op in oare, nije groep SPMs. Dy binne bekend ûnder de namme systeinyl maresinen. Diskear wiene de rinkjes wer fjouwer dagen op kweek hâlden, mar no mei IL-13 as LWH-feroarsaakjende stof. IL-13 spilet in wichtige rol yn it sykteproses fan astma. Wat no wer sjoen waard, wie dat IL-13 yndie LWH feroarsake en ek dat de systeinyl maresinen dat delbêdzje koene. Eat dat it anti-astmamedisyn dexamethason net koe. Uteinliken is it net krekt dúdlik wurden hoe't dy stoffen LWH delbêdzje, mar it is no wol dúdelik dat der in soad fan ferwachte wurde kin as it giet om de behanling fan astma.

Unferwachts waard ek ûntdutsen dat PGD_2 in beskermjende funksje hie. PGD_2 wurdt makke en útstjitten troch mêstsellen fan bargemotten dy't allergysk makke binne foar ovalbumine (OVA), in aaiwyt út hinneaaien. As OVA dan tafoege wurdt oan de buffer mei de rinkjes ûnder de eksperiminten, dan stjitte de mêstsellen stoffen út lykas histamine, systeinyl leukotriënen en dus PGD_2. Lykwols yn tsjinstelling ta dy oare stoffen, soarge PGD_2 der just foar dat de rinkjes minder gearlutsen. Dat die PGD_2 troch te binen oan de mêstsellen wêr't it weikaam. Dêrtroch stjitten mêstsellen úteinlik minder stoffen út en luts de luchtpiip minder sterk gear. PGD_2 is dus in wichtige stof fan mêstsellen dy't derfoar soarget dat se net tefolle aktivearre reitsje en de luchtwegen hielendal tichtknipe.

Op it lêst waarden der noch eksperiminten dien op mêstsellen yn mûzeluchtpipen . As dy mûzen allergysk makke wiene foar hûsstofmyt en se dêroan bleatsteld waarden, dan lutsen de luchtwegen gear. Wat toand waard wie dat dêr mêstsellen foar nedich binne. At dy net yn de long oanwêzich wiene, dan knypten de luchtwegen net gear by bleatstelling oan hûsstofmyt. Dêrnjonken waard ek dúdelik dat mêstsellen aktivearre wurde kinne troch in stof dy't gewoanwei troch senuwen útstjitten wurdt. It soe dus sa wêze kinne dat senuwen in rol spylje yn hoe't en wannear't mêstsellen aktivearre wurde by bleatstelling oan allergenen lykas hússtofmyt.

De konklúzjes dy lutsen wurde kinne, binne dus:

- LWH, feroarsake troch hússtofmyt, TNFα en IL-13 kin delbêde wurde troch lipoksinen and systeinyl maresinen.

- Mêstsellen yn de longen spylje in wichtige rol yn luchtweikontraksjes feroarsake troch hússtofmyt, mooglik troch ynteraksjes mei senuwen.

- Dat makket SPM in oantreklik bestândiel yn nije medisinen tsjin benaudens. Fierder wurdt yn it ûndersyk it belang oantoand fan mêstsellen foar de ûntwikling fan nije medisinen tsjin astma.

ABSTRACT

Lipid mediators play an important role in responsiveness of the airways. The roles of prostanoids and leukotrienes in inducing airway inflammation and contraction are reasonably well established. The functions of specialised pro-resolving lipid mediators (SPMs), which are thought to mediate pro-resolving and anti-inflammatory effects, are less well-studied. The current knowledge on SPM functions, specifically in airway inflammation and contractility is limited. Furthermore, mast cells are important innate immune effectors cells found in the lung, known to release a host of pro-inflammatory and contractile cytokines and lipids in allergic airway inflammation and airway hyperreactivity. However, if the presence of mast cells in airways is necessary for antigen-induced airway contractions and induction of airway hyperreactivity remains to be clarified. In addition, though it is known that unselective cyclo-oxygenase (COX)-inhibition results in increased contractions in airways upon mast cell activation by antigen, the exact mechanism behind this is unknown. The aim of this thesis was thus to investigate if selected SPMs have anti-hyperreactive properties, how COX-inhibition results in increased airway constriction and if mast cells are necessary for antigen-induced contraction and airway hyperreactivity.

To this end, mouse and guinea pig models of airway hyperreactivity and allergic inflammation were used. Tracheae were dissected free from surrounding tissue and divided in segments of equal size. The isometric contractions of these isolated tracheal preparations were studied in myographs, either immediately after dissection from mice that received intranasal administration of HDM and SPMs beforehand, or after four days of incubation of the segments with cytokines and SPMs. Alongside this, concentrations of released mast cell mediators were determined with liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) and enzyme-linked immunosorbent assay (ELISA).

First, it was found that four-day intranasal administration of the SPMs lipoxin A_4 (LXA_4) and resolvin D1 (RvD1) could reduce house dust mite (HDM)-induced airway hyperreactivity. The reducing effect of LXA_4 was replicated when added during four days of incubation of tracheal segments with HDM and TNFα and was also present for lipoxin B_4 (LXB_4). A potential involvement of the ALX/FPR2 receptor was found, though this should be further backed up by future investigations.

Second, IL-13 induced a steroid-resistant airway hyperreactivity *in vitro* that could be reduced by cysteinyl maresins. This effect could be blocked by three different cysteinyl leukotriene (CysLT) receptor 1 antagonists. However, LTD_4 could not reproduce the anti-hyperreactive effect nor did it interfere with cysteinyl maresin signalling. The exact receptor signalling remains to be clarified.

Third, prostaglandin D_2 (PGD_2) was found to be produced by the COX-1 enzyme in mast cells present in tracheal segments from ovalbumin (OVA)-sensitised guinea pigs, when exposed to OVA *in vitro*. Further release of contractile mediators like histamine and cysteinyl leukotrienes from mast cells was inhibited by PGD_2. This was done via the DP_1 receptor, therefore PGD_2 and the DP_1 receptor likely function as an inhibitory, autocrine signalling axis for mast cells.

Fourth, the presence of mast cells in lung tissue was necessary for HDM-induced airway contractions and mast cell absence led to reduced airway hyperresponsiveness in mouse models. Repeated HDM-exposure via intranasal instillation led to airway hyperreactivity mediated by carbachol and serotonin (5-HT) in isolated tracheal segments. The data suggest that mast cell activation occurred as an interplay between nerve-endings and mast cells, as mouse mast cells expressed the M_3-receptor and activation led to release of 5-HT.

To conclude, lipid mediators and mast cells play an essential role in the modulation of airway responsiveness. They do this by either inducing contractions after antigen-exposure (mast cells), reducing cytokine and antigen-induced airway hyperreactivity (SPMs) or inhibiting release of pro-contractile mediators and ultimately airway contractions (PGD_2). This

makes SPMs and their receptors, as wells as mast cells promising future drugs or drug targets for the treatment of airway hyperreactivity as seen in for example asthma.

LIST OF SCIENTIFIC PAPERS

I. Abma W, Noreby M, Wheelock CE, Dahlén SE, Adner M, Säfholm J
Lipoxin A(4) reduces house dust mite and TNFα-induced hyperreactivity in the mouse trachea
Prostaglandins Other Lipid Mediat. 2020;149:106428

II. Mendez-Enriquez E, Alvarado-Vazquez PA, Abma W, Simonson OE, Rodin S, Feyerabend TB, Rodewald HR, Malinovschi A, Janson C, Adner M, Hallgren J
Mast cell-derived serotonin enhances methacholine-induced airway hyperresponsiveness in house dust mite-induced experimental asthma
Allergy. 2021;00:1–13

III. Säfholm J, Abma W, Liu J, Balgoma D, Fauland A, Kolmert J, Wheelock CE, Adner M and Dahlén SE
Prostaglandin D_2 inhibits mediator release and antigen induced bronchoconstriction in the guinea pig trachea by activation of DP_1 receptors
Manuscript submitted

IV. Abma W, Dahlén SE, Barret NA, Bankovac LG, Boyce JA, Wheelock CE, Adner M, Säfholm J
Cysteinyl maresins inhibit IL-13-induced hyperreactivity of mouse trachea
Manuscript in preparation

CONTENTS

LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine also know as serotonin
5-HT_{2a}	5-hydroxytryptamine 2a receptor
AA	Arachidonic acid
AC	Adenylyl cyclase
AHR	Airway hyperresponsiveness
ALX/FPR2	Lipoxin A/formyl peptide 2 receptor
COX	Cyclooxygenase, also known as prostaglandin-endoperoxide synthase
$\text{Cpa}^{\text{Cre/+}}$	Mice that express Cre-recombinase instead of mast cell carboxypeptidase A3, i.e., mast cell devoid mice
CysLT	Cysteinyl leukotriene
CysLT_1	Cysteinyl leukotriene 1 receptor
DHA	Docosahexaenoic acid
DP_1	Prostaglandin D_2 receptor 1
E_{max}	Maximal effect of a given agonist
EPA	Eicosapentaenoic acid
HDM	House dust mite
IL	Interleukin
LOX	Lipoxygenase enzyme
LPS	Lipopolysaccharide
LXA_4	Lipoxin A_4
MCTR	Maresin-conjugate in tissue repair, also cysteinyl maresin
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
OVA	Ovalbumin
pEC_{50}	Negative logarithm of EC_{50}, which is the concentration needed to elicit half-maximum response
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
$\text{PGF}_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGI_2	Prostaglandin I_2, also known as prostacyclin
RvD1	Resolvin D1
SPM	Specialised pro-resolving mediator
TNFα	Tumour necrosis factor alpha

1 INTRODUCTION

Increased contractility of the airways is a central pathophysiological component of asthma, making research into ways to dampen increased contractility highly relevant. Especially since in severe asthma, airway hyperreactivity or hyperresponsiveness (AHR) might be hard to control and leads to loss of quality of life, hospital visits and sometimes death. The central theme of this thesis is therefore hyperreactivity of airway smooth muscle under allergic inflammatory conditions and how lipid mediators can modulate this, i.e. have anti-inflammatory and anti-hyperreactive actions.

Prostanoids and leukotrienes have been studied over the decades since their discovery and generally induce inflammation or contraction of airway smooth muscle. This has also led to the successful development of cysteinyl leukotriene (CysLT) receptor antagonists such as montelukast, which are used in the management of asthma symptoms. However, the recent discovery of certain ω-3 and ω-6 polyunsaturated fatty acid products, called specialised pro-resolving lipid mediators (SPMs), has opened the door to a whole new approach. An approach in which SPMs, SPM-derivatives or other agonists for their receptors might be used to actively resolve airway inflammation and symptoms of increased airway contractility.

To aid in the development of possible future treatment strategies, mouse and guinea pig models were used to study the modulation of airway responsiveness. Cytokines and antigens were utilised to induce airway hyperreactivity or to study antigen-induced airway contraction via mast cells. These models made it possible to assess the anti-hyperreactive potential of SPMs, but also the exact role of mast cells in antigen-induced airway contractions and the mast cell-inhibiting potential of prostaglandin D_2 (PGD_2).

The studies revealed that several groups of SPMs, namely lipoxins, cysteinyl maresins and potentially resolvin D1 (RvD1), have the ability to reduce airway hyperreactivity in mouse airways. Additionally, PGD_2 was found to reduce antigen-induced mediator release and contractions in guinea pig airways. Lastly, lung mast cells were imperative for antigen-induced airway contractions in mice, possibly through nerve-mast cell interactions

2 LITERATURE REVIEW

2.1 INTRODUCTION

Asthma is an inflammatory disease of the airways. Despite its name suggesting a clear, single disease, asthma is characterised by multiple endo- and phenotypes (1). Regardless of this heterogeneity, all asthma patients show symptoms such as wheezing, coughing and shortness of breath (2). On a tissue level, predominant characteristics of the condition include bronchoconstriction, airway inflammation, AHR, airway remodelling and increased mucus secretion (2).

In airway allergic inflammation, mast cells and mast cell-derived mediators play an important role in the initiation of the inflammatory process and modulation of bronchial tone. Lipid mediators derived from ω-6 and ω-3 polyunsaturated fatty acids (PUFAs) are one group of mediators found in mast cells and other cells. They potentially play a role in the initiation, aggravation, and resolution of the asthmatic inflammatory response (3-6). Lipid mediators derived from ω-6 and ω-3 PUFAs can be divided in a more proinflammatory group: the prostaglandins, leukotrienes and thromboxanes and an anti-inflammatory and pro-resolving group: the lipoxins, resolvins, protectins and maresins, though exceptions exist (7).

The importance of these lipid mediators in inflammation and asthma is illustrated by the clinical use of leukotriene antagonists in asthma, as well as findings which suggest that decreased levels of lipoxins contribute to the pathology seen in patients with severe asthma (4, 8).

2.2 REGULATION OF AIRWAY SMOOTH MUSCLE TONE

2.2.1 Airway anatomy

The human airway starts as a single compartment in the nasal cavity. Through the pharynx and larynx, this leads to the trachea. This large conducting airway has a length of approximately 13 cm and a diameter of around 2 cm. The trachea is built up in distinct layers: an outer layer consisting of connective tissue and lymph nodes; horseshoe-shaped cartilage rings and interspersed smooth muscle tissue; a submucosa and an inner mucosal layer lined with epithelium. Further distal, the trachea bifurcates into two mainstem bronchi which further bifurcate again and enter the lung through the hila. The bronchi keep dividing and decrease in diameters <2 mm. These bronchi resemble the trachea, except for the cartilage rings which decrease in thickness and the diverse lung immune cells including resident macrophages, T-cells and mast cells (9). Further distal, the airways are called bronchioles. These bronchioles terminate in alveoli, in which the actual gas-exchange takes place (10-12).

2.2.2 The role of epithelium derived signals

The inner mucosal layer of the upper and lower airways consists of pseudostratified columnar epithelium with apical cilia, interspersed with diverse secretory cells (13, 14). Within the airways, the epithelial layer fulfils several key roles. First and foremost, it acts as a barrier against micro-organisms, in particular bacteria and fungi, but also to prevent allergens and noxious gases reaching deeper tissue layers (15, 16). Secondly, when epithelium gets activated by a trigger, it can release different cytokines such as TLSP, IL-25 and IL-33. These cytokines stimulate the immune system and initiate and regulate eventual tissue repair and regeneration (16, 17). Thirdly, the epithelium has a function in the release of mediators, for instance nitric oxide (NO), prostaglandins and SPMs. These can induce biological effects such as modulation of airway smooth muscle tone, regulation of secretion of mucus or inhibition of histamine release by lung mast cells (18-20). Finally, the epithelium contains enzymes, e.g. acetylcholinesterase or monoamine-oxidase, which degrade contractile agonists such as acetylcholine and serotonin (5-HT) respectively (21, 22).

2.2.3 Airway mast cells

One cell type prominently involved in regulation of airway contraction and responsiveness is the mast cell. This immune cell is found as a guardian cell in the whole body, often at interfaces between the interior and exterior environment such as the skin, the intestines and the lung. These tissue-resident mast cells are defined as having the high-affinity IgE receptor (FcεRI), the stem cell factor receptor KIT/CD117, and granules containing proteases, histamine and growth factors (23, 24).

A discerning feature used to distinguish different mast cell populations is the protease content of the granules. One group is formed by tryptase-positive mast cells (MC_T) and the other by the tryptase, chymase, carboxypeptidase A, and cathepsin G-like protease positive mast cells (MC_{TC}). The MC_T are mostly found at mucosal surfaces such as in the distal airways and the MC_{TC} more in connective tissue including the skin. Apart from these two distinct groups, many intermediate mast cell types exist with different phenotypes, depending on the tissue in which the mast cell resides (24).

Allergens can crosslink allergen-specific IgE-antibodies already bound to FcεRI found on the surface of mast cells. This initiates the release of pre-stored as well as newly synthesised mediators. This includes proinflammatory cytokines as for instance tumour necrosis factor α (TNFα) or IL-13, contractile agonist such as histamine, 5-HT, cysteinyl leukotrienes (CysLTs), prostaglandins and the aforementioned proteases. Not only can these compounds directly contract airway smooth muscle (histamine, CysLTs, prostaglandins), but they can also modulate airway responsiveness (TNFα, IL-13, prostaglandins) (25, 26).

2.2.4 Main signalling pathways in controlling airway smooth muscle tone

Three main G-coupled receptor pathways exist in airway smooth muscle that contribute to airway tone. Gq-signalling initiates contraction, Gs-signalling results in relaxation and Gi-signalling can result both in airway contraction with concurrent Gs-signalling modulation and alteration of Ca^{2+} sensitivity and smooth muscle growth (27).

2.2.4.1 Gq-coupled signalling

Agonists that bind Gq-coupled receptors cause binding and activation of the Gq-protein complex to the receptor. Through this receptor-binding, several down-stream second messengers get activated. The most important are phospholipase C (PLC) and subsequent formation of inositol-1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DAG). One effect of these signalling molecules is that the intracellular Ca^{2+} concentration rises, resulting in smooth muscle contraction. Another result is the activation of cytosolic phospholipase A_2 ($cPLA_2$) and subsequent liberation of fatty acids that can serve as precursors to eicosanoid formation. Some important Gq-coupled receptors mediating contraction in the human airways are the histamine H_1 receptor, the $CysLT_1$ receptor, the muscarinic M_3 receptor whilst the $5\text{-}HT_{2a}$ receptor being important in mouse airways (27).

2.2.4.2 Gs-coupled signalling

When an agonist binds a Gs-coupled receptor in airways, membrane-bound adenylyl cyclase (AC) is activated and starts converting adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP). Increased cAMP concentrations ultimately result in decreased intracellular Ca^{2+} concentrations, thus leading to relaxation of smooth muscle in the airways (27). The archetypical and clinically well utilised Gs-coupled receptor in the lungs is the β2-adrenoreceptor. Another example being the prostacyclin IP receptor (27, 28).

2.2.4.3 Gi-coupled signalling

Whereas Gs-coupled receptors activate AC, the activated Gi-coupled receptor does the opposite and thus downregulates AC-activity. It can also activate PLC and thus increase

intracellular Ca^{2+} concentrations and smooth muscle contraction. A Gi-coupled receptor found in the airway is the prostaglandin E receptor EP_3 (27, 29).

2.2.4.4 Other pathways

There are also other relevant relaxant mediators and pathways that are not G-protein coupled, one being NO. This mediator has a function in airway relaxation through guanylyl cyclase stimulation resulting in cyclic guanosine monophosphate (cGMP), and protein kinase G (PKG) activation. A diminished neuronal NO-release has been found in a guinea pig model of allergic asthma, possibly leading to increased bronchial tone (30). In addition, NO-donor molecules have been proposed as broncho-dilating therapeutics (31). Theophylline also relaxes smooth muscle, though this is done via inhibition of phosphodiesterase enzymes, leading to increased intracellular levels of cAMP and cGMP (32).

2.2.5 Airway hyperresponsiveness and hyperreactivity

Airway hyperresponsiveness is the increased sensitivity to contractile agonists such as histamine, methacholine and CysLTs, and otherwise non-harmful external stimuli as for example cold, exercise and house dust mite (33). This results in increased airway contraction at a certain concentration of an agonist (increased potency, pEC_{50}) and increased maximal contraction (increased efficacy, E_{max}), compared to healthy individuals (33, 34). Airway hyperreactivity only refers to increased maximal contraction to a contractile agonist (E_{max}), without increased sensitivity (pEC_{50}). AHR is defined as an *in vivo* phenomenon, involving airway responses to inhaled contractile agonists in intact lungs. In cultured *ex vivo* explants, the direct response of the airway smooth muscle to an agonist may be quantified, thus assessing the responsiveness only on a smooth muscle level.

AHR can be measured in individuals with asthma. This can be done either by a direct (methacholine, histamine) or indirect (mannitol, adenosine, allergen) challenge. Upon challenge, a fall in forced expiratory volume (FEV) occurs. This is often done using the protocol developed by Cockroft et al, involving two-minute tidal breathing of nebulised methacholine (35). In this protocol, the concentration that causes a 20 % fall in FEV, the PC_{20}, is used as read-out and is in asthmatics generally < 8 mg/mL, whilst in non-asthmatics being > 16 mg/mL when challenged with methacholine. Direct and indirect challenge differ in that direct challenge measures direct smooth muscle responsiveness to the agonist, whilst indirect challenge is due to activation of inflammatory cells present, releasing mediators such as histamine and CysLTs (34, 36).

AHR consists of two components: chronic or persistent AHR and more variable AHR. The persistent AHR is seen in most patients with asthma, one contributing factor being airway remodelling, leading to e.g. increased smooth muscle mass, fibrosis and increased mucus production (33, 37). Variable AHR is related to current inflammatory status in the airways, being prominent during inflammation triggered by for example respiratory infections or the activation of mast cells by a hyperosmotic environment (33, 38).

2.3 ANIMAL MODELS IN RESPIRATORY RESEARCH

Multiple reasons exist for the use of animals in research, including restricted availability of human lung samples and fewer possibilities to perform research in humans. Therefore, use of both *in vivo* and *in vitro* (tissue, cells) animal models have been of great importance in respiratory research. Model animals often used are mice, rats and guinea pigs. Mice models are preferred by many researchers due to the existence of many mouse specific molecular biological tools such as antibodies, mouse specific cytokines and in particular, the availability of genetically modified strains (33). In respiratory research though, the usage of guinea pigs as experimental models is often preferable. This is due to the guinea pig possessing an anatomy, (patho)physiology and pharmacology that better resemble healthy and diseased human lungs

and a greater ease of performing *in vivo* lung measurements (33, 39). Important differences between the human, guinea pig and mouse respiratory tract are summarised in table 1.

Table 1. Comparison of the respiratory tract in humans, guinea pigs and mice

	Human	**Guinea pig**	**Mouse**
Bronchial tree	Dichotomous	Dichotomous	Monopodial
Bronchial musculature	Densely muscled	Densely muscled	Much less smooth muscle present
Airway lining	Thick epithelial lining with mucus and basal cells	Epithelial lining with mucus and basal cells	Epithelial lining thickness only 25 % of humans. No mucus producing cells, but club cells present
Mast cell contractile mediators	Histamine, leukotrienes, prostaglandins	Histamine, leukotrienes, prostaglandins	5-HT
Mast cell activation	IgE-dependent	IgE-/ IgG1-dependent	IgE-independent
Response to allergen	Acute and chronic but can develop allergen tolerance	Acute and chronic but can develop allergen tolerance	Acute, develop allergen tolerance

References (33, 39, 40)

2.4 INFLAMMATION AND IMPORTANT MEDIATORS

Acute inflammation is a carefully programmed and protective process. It protects against infiltration of pathogenic bacteria, viruses, fungi or other stimuli recognised as potentially harmful. Classical signs of inflammation are tumor (swelling), rubor (redness), dolor (pain), calor (heat) and functio laesa (loss of function). On a cellular and molecular level, inflammation is characterised by infiltration of immune cells such as neutrophils, macrophages and later on T-cells, and a host of mediators being released. Examples of this include chemokines (e.g. CXCL8), interleukins (e.g. IL-1β and IL-13), lipid mediators (e.g., PGD_2) and leukotriene B_4 (LTB_4)), and acetylcholine and neuropeptides such as substance P, released by nerve-endings (26, 41-44). All these released substances fulfil various functions such as chemoattractant, cell activator or stimulant of smooth muscle proliferation and contraction (26, 42, 43). Although acute inflammation exerts a protective function, chronic inflammation is part of the aetiology and pathophysiology of a myriad of chronic diseases (e.g. diabetes type 2, Crohn's disease, rheumatoid arthritis (45-47).

In allergic inflammation, the inflammation is preceded by sensitisation to an allergen. During this process, exposure to the allergen leads to antigen-presentation by dendritic cells and an ensuing maturation of naïve T-cells into Th2-type cells. These cells release quintessential type 2-cytokines: IL-4, IL-5 and IL-13. IL-5 causes an influx and survival of eosinophils. IL-4 and IL-13 stimulate IgE-formation by B-cells through induction of immunoglobulin class-switch recombination (48). Consequently, released IgE activates mast cells, which results in their mediators being released.

Repetitive exposure to an allergen can change the nature of inflammation from an acute response to a more chronic state. In this chronic inflammatory state, immune cells from both the innate immune system (e.g. innate lymphoid cells (ILC), eosinophils), and the adaptive immune system (e.g. different type of T-cells including Th2-type cells, B-cells), infiltrate and stay in affected tissues. When present in tissue, these cells interact with local epithelial and structural cells including fibroblasts and smooth muscle cells. Ultimately, in the airway this can lead to AHR and airway remodelling, the latter characterised by airway thickening, increased number of goblet cells, increased mucus production and an altered extracellular matrix containing more collagen (figure 1) (23, 48).

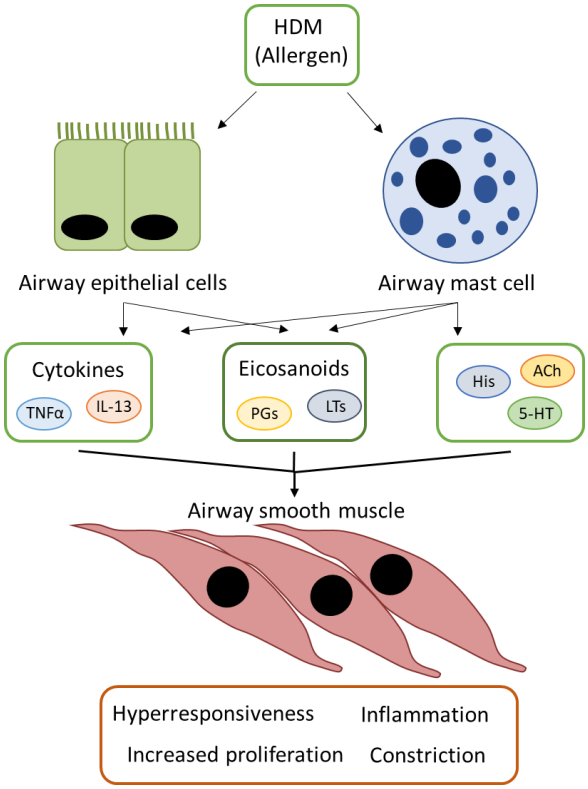

Fig. 1. HDM exposure causes airway epithelial cells and mast cells to release e.g. cytokines, eicosanoids and contractile agonists which affect smooth muscle function

2.4.1 HDM

House dust mite (HDM) is an allergen that can cause chronic inflammation in asthma. HDMs are mites mostly from the *Dermatophagoides* genus. They are commonly found in households worldwide and are an important driver of allergy and asthma (49, 50). HDM extract is a mixture of active components containing proteases, chitinases and lipopolysaccharide (LPS) (51). Compared to a commonly used antigen, ovalbumin (OVA) from chicken egg white, HDM is a more relevant allergen in asthma. This is due to the fact that HDM itself is one of the most common aeroallergens found in households, as opposed to OVA, which does not play a role in asthma but food-allergies (52, 53). In addition, OVA sensitisation and exposure leads to a less broad effect. OVA induces IgE production, mast cell activation upon challenge and lung eosinophilia when doing repeated exposure. However, on top of these effects, HDM also causes epithelial barrier damage and involvement of the innate immune system (51, 54). Another advantage of HDM in experimental research is the greater ease to sensitise by airway exposure, which is more pathophysiological relevant as compared to OVA, where intra-peritoneal injection with adjuvants is often used (54, 55).

2.4.2 TNFα

Micro-organisms, or parts thereof such as LPS found in HDM, prompt a type 1 inflammatory response. This causes release of TNFα which is a protein found in the membrane of innate immune cells that can also be found as a soluble form (56). It is a cytokine released during early inflammation, having a plethora of effects on different cells. When bound to the tumour necrosis factor 1 receptor (TNFR1), TNFα causes diverse proinflammatory effects often through NF-κB signalling. Examples of these proinflammatory functions are increased neutrophil trafficking, CXCL8 release and direct stimulation of histamine-release from mast cells (25, 56-58). Although less well known, TNFα also has immunomodulatory and tissue generative functions when signalling through the tumour necrosis factor 2 receptor (TNFR2) (56). In the respiratory tract, increased TNFα levels are found in patients suffering from COPD and asthma exacerbations and TNFα exposure causes AHR and airway hyperreactivity *in vivo* and *in vitro* in human and mouse tissue (59-62). A randomized, double-blind, placebo-controlled study with a human monoclonal TNFα antibody has been performed in asthma patients, to test whether anti-TNFα treatment could actually improve asthma symptoms. This study however did not demonstrate significant improvements in airway function, though some sub-groups benefitted from anti-TNFα treatment (63). Nevertheless, the side-effect profile discouraged further development of anti-TNFα treatment regimes in asthma.

2.4.3 IL-13

Allergens, like those also found in HDM, induce a type 2 inflammation. IL-13 is one of the typical type 2 cytokines, released by different immune cells, examples being mast cells, CD4+ T-cells and ILC-2 cells (42, 64). IL-13 can bind the IL-4R, IL-13Rα1 and IL-13Rα2 subunits of the receptor complex it signals through. Receptor activation by IL-13 causes asthma-features including AHR and increased mucus secretion (26, 64). However, through the IL-13Rα2 receptor sub chain, IL-13 also has reparative functions as it initiates airway epithelium wound repair (65). Downstream of the receptor, the signal transducer and activator of transcription (STAT6) pathway generally causes the effects of IL-13 in the allergic inflammation seen in asthma. Since the apparent critical function of IL-13 in the causation of key-features of asthma, several antibodies have been developed to treat patients with moderate-to-severe asthma, with some clinically effective (dupilumab) and others not (tralokinumab, lebrikizumab) (66-68).

2.4.4 Eicosanoids

Enzymatic liberation of the ω-6 PUFA arachidonic acid (AA) from the cell membrane and consequent actions of cyclooxygenase (COX) and lipoxygenase (LOX) enzymes results in the formation of twenty-carbon long lipid mediators collectively called eicosanoids (figure 2). Within the eicosanoid group, there are three important branches of lipids: prostaglandins, thromboxanes and leukotrienes. COX-1 and COX-2 enzymes catalyse the biosynthesis of prostaglandins and thromboxane, whereas the 5-LOX enzyme initiates the production of leukotrienes. The main prostaglandins are prostaglandin E_2 (PGE_2), PGD_2, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and prostaglandin I_2 (PGI_2). Thromboxane synthase simultaneously produces 12-HHTrE and thromboxane A_2 (TXA_2), which is highly unstable and quickly catabolised to inactive thromboxane B_2 (TXB_2) in the body (3).

All prostaglandins signal through specific G-protein coupled receptors, though almost all of them also bind the other prostaglandin receptors, but with less affinity (69). PGE_2 preferentially binds the EP_1 to EP_4 receptors, PGD_2 the DP_1 and DP_2 receptors, TXA_2 the TP receptor, $PGF_{2\alpha}$ the FP receptor and PGI_2 the IP receptor. The somewhat receptor-promiscuity of the prostaglandins results in the fact that almost all of them can contract airway smooth muscle, except for PGI_2, which has a relaxant effect in both airways and the vasculature (70, 71).

PGE_2 has multiple functions that can be characterised as both pro- and anti-inflammatory. In the airways it induces mucus secretion, leakage of plasma in the microvasculature and it

modulates the airway tone (72-74). Clear anti-inflammatory actions include the inhibition of mast cell-mediated bronchoconstriction and inhibition of inflammation caused by IL-33-ILC2 (74, 75). PGD_2 is a notable mast cell-derived lipid mediator and plays an important role in many pathological processes seen in asthma. Examples are mediating antigen-induced airway contraction, lung eosinophilia, increased airway smooth muscle mass and release of IL-4, IL-5 and IL-13 (76-78). However, recent data from a phase 3 trial of a DP_2 antagonist, fevipiprant, did not improve lung function in individuals suffering from severe asthma. This suggest that not only the DP_2 receptor, but also the DP_1 and the TP receptor play a notable role in mediating PGD_2's effects in the lung in asthma. TXA_2 and $PGF_{2\alpha}$ are potent constrictors of smooth muscle. They induce contraction in trachea, bronchi, as well as vessels, and TXA_2 also causes acetylcholine release (79, 80). As mentioned before, PGI_2 relaxes smooth muscle in the airways and vasculature. Next to this, PGI_2 signalling has also been implicated in the amelioration of allergic inflammation by reducing CD4+ T-cell activation and cytokine release (7).

Leukotriene A_4 (LTA_4) and LTB_4 make up one part of the leukotriene branch, with the LTA_4-epoxide having a short half-life and quickly converted to LTB_4 (81). The other group is the CysLTs. This group consists of leukotriene C_4 (LTC_4), leukotriene D_4 (LTD_4) and leukotriene E_4 (LTE_4). LTC_4 is glutathione conjugated to the LTA_4 backbone. LTD_4 is formed through the removal of glutamine from the glutathione group of LTC_4 by γ-glutamyl transferase and further removal of glycine by peptidases results in the formation of LTE_4.

There are two receptors for LTB_4-signalling: the BLT_1 and BLT_2 receptor. BLT_1 seems to be the main receptor as it shows high affinity for LTB_4 binding. The BLT_1 receptor is mainly found on immune cells such as neutrophils, mast cells and T-cells (81). Activation of this receptor by LTB_4 results in chemotaxis of these cells, vascular leakage and indirect vasoconstriction (82, 83). Much less is known about the low-affinity BLT_2 receptor, though in mast cell-mediated airway inflammation, BLT_2 receptor downregulation results in decreased inflammation after LPS or allergen stimulation (84). Another possible role might lie in epidermal wound healing through activation of migration of keratinocytes. This was seen when the BLT_2 receptor was activated not by LTB_4 but by 12-HHTrE formed during TXA_2 formation (85).

The CysLTs were discovered as being the main bronchoconstrictors found in what was then called slow-reacting substance of anaphylaxis (86). CysLTs are one group of important contractile agonists found in human mast cells. They induce a slow-but-sustained contraction of the airways through the $CysLT_1$ receptor. Apart from bronchoconstriction, $CysLT_1$ and $CysLT_2$ receptor activation results in permeability of the microvasculature, immune cell recruitment as well as increased airway smooth muscle proliferation and mucus hypersecretion (4). The importance of CysLTs in asthma is further illustrated by the development of the $CysLT_1$-antagonist montelukast. This drug is currently used as an add-on in treatment of asthma patients and has further potential in other diseases as for example allergic rhinitis and cardiovascular disease (4).

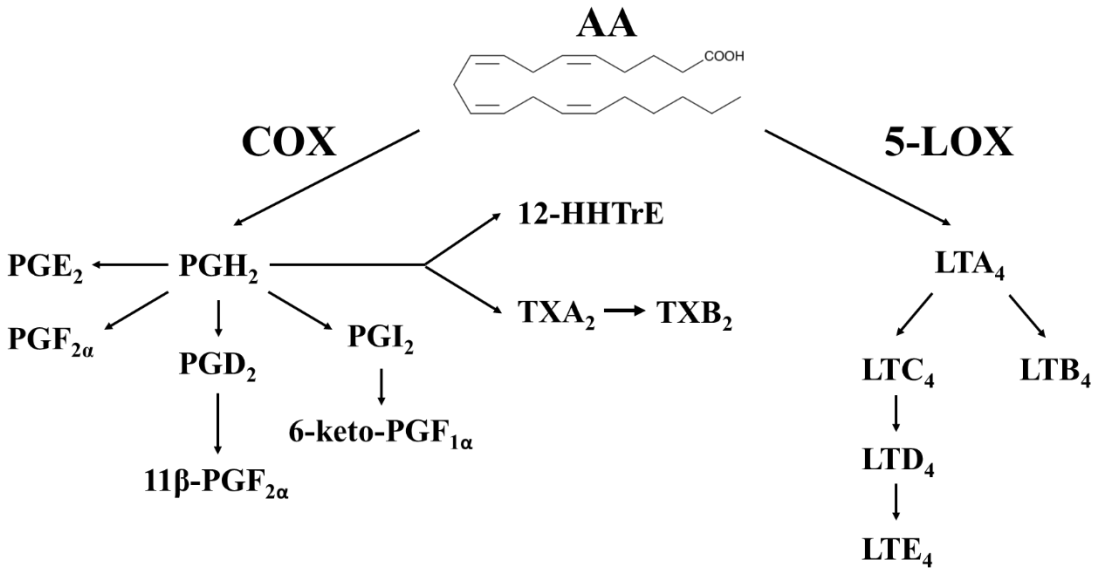

Fig. 2. Simplified scheme of eicosanoid metabolism.

2.5 SPECIALISED PRO-RESOLVING LIPID MEDIATORS

Even though inflammation is an important mechanism, unchecked inflammation is detrimental and can lead to disease (87, 88). It is therefore vital that the inflammatory process is strictly controlled and timely stopped. This process of cessation of inflammation and return to tissue homeostasis is called resolution of inflammation (89). It is an active process and at least a part of it is mediated by lipid SPMs (89, 90). Being an SPM however is not exclusive for lipid mediators, as other compounds, such as the protein Annexin a1, can also have pro-resolving functions (91).

Although SPMs all carry anti-inflammatory activities, being anti-inflammatory is not the same as being pro-resolution. Examples of anti-inflammatory effects of SPMs are the reduction of neutrophil and eosinophil recruitment to the site of inflammation, production of cytokines by immune cells and production of reactive oxygen species (5, 92, 93). On the other hand, pro-resolution functions are characterised by stimulation of macrophage phagocytosis and efferocytosis of for instance apoptotic cells, antibody production by B-cells and reduction of inflammatory pain (5, 94, 95).

SPMs are biosynthesised from both ω-6 and ω-3 PUFAs including AA, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (96). In experimental settings, most SPMs are effective in picomolar and nanomolar ranges, making them potent mediators of resolution (97-99). In disease settings, a few studies report decreased SPM production seen in various samples of patients with asthma, cystic fibrosis and Alzheimer's disease (100-103). As DHA and EPA are particularly enriched in the aquatic environment, SPM production after regular intake of seafood or supplements might explain the beneficial effects of these ω-3 PUFAs seen (104, 105). Nevertheless, since SPMs are readily oxidised, stable SPM analogues have been developed to increase bioavailability and prolong SPM exposure, thus making them a more viable treatment for inflammatory diseases (106-108). The most prominent SPM sub-families and their receptors are summarised in figure 3.

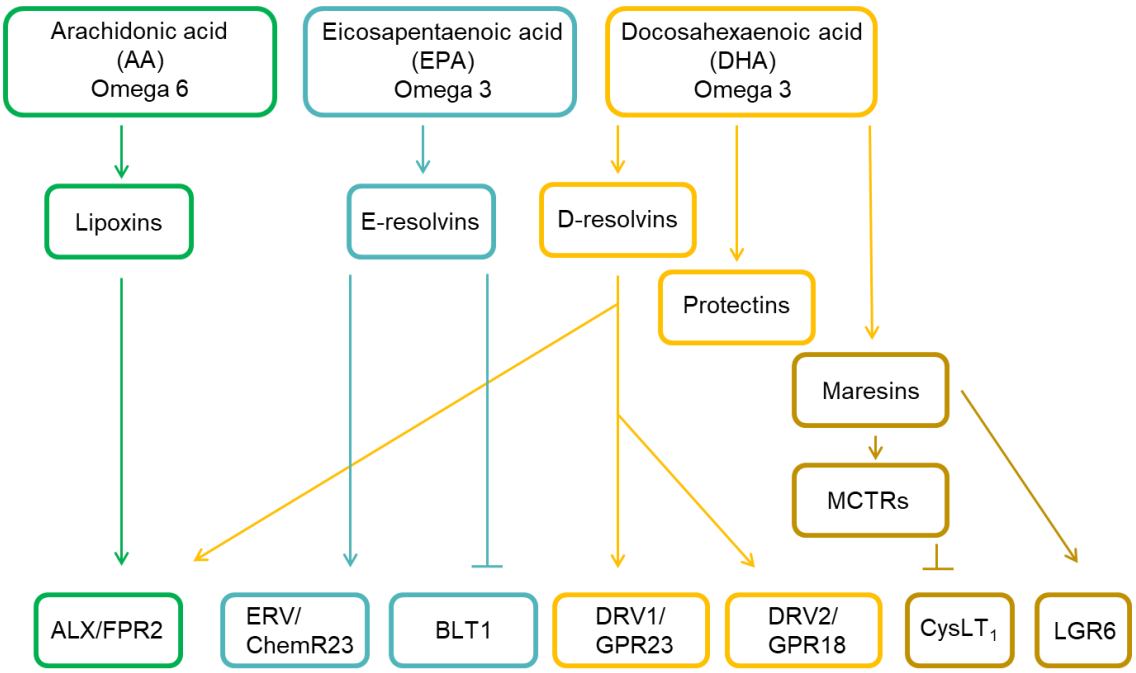

Fig. 3. Specialised pro-resolving mediators, their precursors and proposed receptors.

2.5.1 Lipoxins

The lipoxin family consists of two distinct members that are positional isomers. Lipoxin A_4 (LXA_4; 5S,6R,15S-trihydroxy-7E,9E,11Z,13E-eicosatetraenoic acid) and lipoxin B_4 (LXB_4; 5S,14R,15S-trihydroxy-6E,8Z,10E,12E-eicosatetraenoic acid). Lipoxins are generated from AA, making them the only SPMs of the main SPM groups (resolvins, maresins, protectins and lipoxins) that are generated from an ω-6 PUFA (figure 3).

The name lipoxins is derived from 'lipoxygenase interaction products', coined when they were first discovered in human leukocytes and referring to their formation by interaction of different LOX enzymes (109). Lipoxin formation is a concerted, transcellular effort of both structural as well as immune cells (110). One important pathway of lipoxin formation consists of the production and release of LTA_4 by neutrophil 5-LOX and then further metabolism by 12-LOX in adhered platelets, to LXA_4 and LXB_4 (111, 112). There is also an alternative route of biosynthesis where 15(S)-HETE (15(S)-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid) is formed by 15-LOX in epithelial cells. 15(S)-HETE is sequestered by leukocytes, where 5-LOX may convert it into LXA_4 and LXB_4 (112).

In addition to the 15(S)-enantiomers, 15(R)-lipoxins can also be produced. Normally this is done by cytochrome P450 enzymes, but the production can be boosted. One example is when acetylsalicylic acid (aspirin) is present, which irreversibly acetylates the COX-2 enzyme, causing COX-2 production of 15(R)-HETE and ultimately 15(R)-lipoxins in structural cells such as endo- and epithelial cells and immune cells such as monocytes (112). Another recent finding is that statins, for instance atorvastatin, usually used to lower cholesterol, appear to have anti-inflammatory properties (113) and S-nitrosylate the COX-2 enzyme leading to production of 15(R)-lipoxins and certain resolvin-series (110, 114, 115). In experimental settings, 15(S)- and 15(R)-lipoxins appear to exert similar functions, although differences in potency may exist (58, 116-118).

Even though now generally recognised as part of the SPM family, initial experiments in airway tissue pointed at what can be considered a more proinflammatory profile. At its discovery, it was shown that LXA_4 caused O_2^- generation and lysosomal elastase release (109). It was also shown that LXA_4 induced contraction of guinea pig lung strips through TXA_2-release and caused arteriolar dilation in the hamster cheek pouch (119, 120). However, after these findings it soon became clear that LXA_4 and LXB_4 also possess broad anti-inflammatory and pro-resolving traits.

Firstly, LXA_4 is an agonist of the ALX/FPR2 receptor. Many of the effects of LXA_4 appear to be mediated through this receptor. For example, lipopolysaccharide (LPS, a component of the cell wall of gram-negative bacteria)-induced lung injury is ameliorated by LXA_4-ALXR signalling (121) and LXA_4 can dampen TNFα-mediated pro-inflammatory effects such as CXCL8-release from airway epithelium (122). Moreover, decreased synthesis of LXA_4 as well as decreased expression of the ALX/FPR2 receptor might cause defective resolution, which in turn can be a contributing factor to chronic inflammation as seen in airway diseases including asthma (8, 123) and COPD (124, 125). Secondly, LXA_4 can inhibit plasma leakage and leukocyte adherence in the vasculature caused by LTB_4, pointing to possible antagonistic activity for the BLT receptors (82). Thirdly, LXA_4 can also displace LTD_4 bound to the $CysLT_1$-receptor, thereby antagonising leukotriene-induced effects, namely vascular leakage and airway contraction (126, 127). Finally, LXA_4 exerts anti-inflammatory effects through the aryl hydrocarbon receptor in dendritic cells, leading to modulation of pro-inflammatory cytokine production and inflammation (128).

Although discovered at the same time as LXA_4, much less is known about the possible anti-inflammatory actions and/or pro-resolving actions of LXB_4. Similarly to LXA_4, LXB_4 can antagonise the prophlogistic effect of LTB_4, albeit to a lesser degree (82). Also, LXB_4 can also inhibit TNFα-release by peripheral blood mononuclear cells (PBMCs) (116). Along with this, LXB_4 appears to have broad anti-inflammatory and pro-resolution effects in lower and upper airway allergic inflammation, causing decreased release of for example cytokines and IgE and shortening of the resolution interval (99). Interestingly, not only the innate immune system seems to be modulated by lipoxins, but also the adaptive immune system as LXB_4 boosts IgG antibody production in memory B-cells, possibly through the COX-2 enzyme (94).

2.5.2 Maresins

As with the lipoxins, the maresin family consists of two main members: maresin 1 (MaR1; 7R,14S-dihydroxy-4Z,8E,10E,12Z,16Z,19Z-docosahexaenoic acid) and maresin 2 (MaR2; 13R,14S-dihydroxy-4Z,7Z,9E,11Z,16Z,19Z-docosahexaenoic acid) formed from the ω-3 PUFA DHA. Apart from the aforementioned maresins, other maresin metabolites or modifications with biological effects have been described (e.g. 13(S),14(S)-epoxy-DHA, 14-oxo-MaR1 (129, 130)).

Maresins were named after their discovery in macrophages, with maresin being an abbreviation of ‘macrophage mediator in resolving inflammation’ (131). Transcellular synthesis also plays an important role in the biosynthesis of these SPMs. One route in maresin-formation is the formation of 13(S),14(S)-epoxy-DHA by platelet 12-LOX, this intermediate is then taken up by neutrophils to produce MaR1 (132). MaR1 can also be produced solely by macrophages or via uptake of the 13(S),14(S)-epoxy-DHA intermediate (129, 131).

Immediately at its discovery, MaR1 was tested for bioactivity, showing prototypical SPM functions such as stimulation of macrophage phagocytosis and reduction of neutrophil infiltration (131). Soon after this, a more specialised function was shown in a planaria model of tissue generation. Here MaR1 could hasten planaria head regrowth, although the same could be shown for resolvin E1 (RvE1) (133). Since then, a broad range of actions and molecular targets have been described.

On the receptor side, MaR1 has been found to signal through the human leucine-rich repeat containing G protein–coupled receptor 6 (LGR6) found on leukocytes, mediating such an archetypical SPM function as phagocytosis (134). It can also influence currents generated by the transient receptor potential V1 (TRPV1) ion channel, thereby inhibiting inflammatory pain (133).

Some actions in the lung have been described, examples being maintenance of permeability in lung epithelium (135), decrease of proinflammatory cytokines and CysLTs after harmful stimuli such as organic dust and hydrochloric acid (132, 135) and protection against bleomycin-induced lung fibrosis (136). In OVA-induced allergic airway inflammation

in mice, MaR1 levels in the lung are decreased during OVA-challenge, but increased in the resolution-phase, signifying the time-restricted production and release of MaR1 (137). The 13(S),14(S)-epoxy-DHA intermediate in MaR1 biosynthesis can also interfere with the formation of CysLTs, as it inhibits leukotriene A4 hydrolase, decreasing formation of LTB_4 (129).

Furthermore, MaR1 potentially has functions in both the innate and adaptive immune system. For example, MaR1 itself can reduce release of type 2 cytokines such as IL-5 and IL-13 from ILC-2 cells but also through upregulating the formation of T-regulatory (Treg)-cells (137, 138). In addition, it can reduce the generation of T_H1 and T_H17 T-cell populations (137, 139), possibly with microRNA-21 (miR-21) as a downstream effector (139). Two other downstream effectors of MaR1 found in bronchial epithelial cells during organic dust exposure, are the serum response element (SRE) found in DNA and PKC (140).

Not much is known about MaR2. The enzymes 12-LOX from platelets and soluble epoxide hydrolase are known to play a role in its formation. On a functional level, typical SPM effects including inhibition of neutrophil infiltration and stimulation of macrophage phagocytosis have been shown (141).

A distinct group is formed by maresin-conjugates named 'maresin-conjugates in tissue regeneration' (MCTR), with three members: MCTR1 (13-glutathionyl-14-hydroxy-docosahexaenoic acid), MCTR2 (13-cysteinylglycinyl-14-hydroxy-docosahexaenoic acid) and MCTR3 (13-cysteinyl-14-hydroxy-docosahexaenoic acid). MCTR1 is in effect MaR2 conjugated to glutathione, a three-amino-acid-long peptide consisting of glutamine, cysteine and glycine, by leukotriene C_4 synthase as well as glutathione S-transferase Mu 4 (142, 143). MCTR1 is converted in macrophages to MCTR2 through removal of glutamine by γ-glutamyl transferase (142, 144). Further removal of glycine is done by peptidases, thus forming MCTR3 (figure 4) (142).

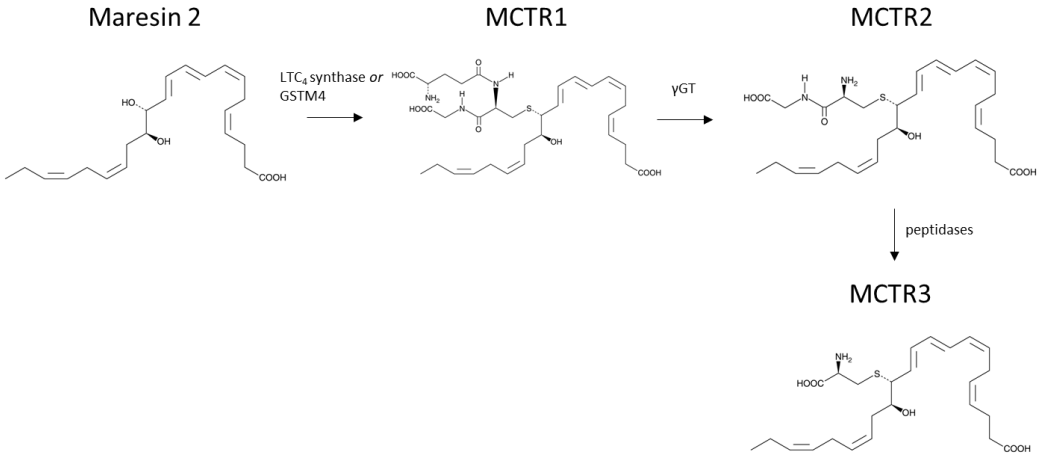

Fig. 4. Biosynthesis of maresin-conjugates

Since their cysteinyl-conjugation is analogues to the CysLTs, it is thought that MCTRs can bind the same $CysLT_1$ receptor, but in an antagonistic fashion, thus decreasing CysLT-induced pro-inflammatory and broncho-constrictive effects (145). There is indeed evidence showing MCTRs decreasing LTD_4-induced bronchoconstriction, vascular leakage and negative ionotropic effect in tunica heart (97, 145). In resting human lung tissue, MCTRs have been reported to be present at approximately three times higher levels then CysLTs, a balance that appears to be completely shifted in diseased lung tissue where the CysLT concentration is ten times higher than that of MCTRs (97). The limited data so far suggests that effects can also be distinct for each MCTR. One example is MCTR3, which seems to be more effective in ameliorating OVA-induced AHR in mice, whilst both MCTR1 and MCTR3 can dampen LPS-induced acute lung injury and AHR via different mechanisms (146, 147). Both MCTR1 and 3 dampen eosinophils infiltration after HDM stimulation as well (97). Apart from this, MCTRs also possess main SPM effects as for instance stimulation of efferocytosis by macrophages,

dampening neutrophil infiltration and the more specialised tissue-regenerative effect also seen in maresins (144, 148).

Other than the observed effects described above, much is unknown about the MCTRs and a possible specific high-affinity receptor is still at large.

2.5.3 Resolvins

The resolvin family consists of two branches. One branch is formed by the resolvin D group, produced from the ω-3 PUFA fatty acid DHA. This group consists of six members: resolvin D1 (RvD1) to resolvin D6 (RvD6). The other branch is formed by the resolvin E group, produced from the ω-3 PUFA fatty acid EPA and consisting of resolvin E1 (RvE1) to resolvin E3 (RvE3.

D-type resolvins were the first new SPMs discovered after the lipoxins. The name resolvin stands for 'resolution phase interaction product', with the D and E denoting their origin from DHA or EPA respectively (90, 149, 150). Resolvins are also formed through transcellular co-operation of different cells. D-series resolvin formation, at least for RvD1 to RvD4, is started by 15-LOX from epithelial cells. 5-LOX from neutrophils can then perform the final step to form RvD1-4 (149-151). As with the lipoxins, resolvin epimers can also be formed. In the case of the resolvin D series, aspirin acetylation of COX-2 can cause production of 17(R)-hydroxy-DHA in the vasculature. Again, 5-LOX from neutrophils then catalyses the final formation of 17(R)-resolvins (149, 151, 152). E-type resolvin formation is slightly different in that the first step of biosynthesis is performed by either acetylated COX-2 or cytochrome P450 enzymes. The final step is then again performed by 5-LOX from neutrophils, or in the case of RvE3, 12/15-LOX derived from eosinophils in mice (96, 151, 153).

Two receptors are important for the function of E-type resolvins. Firstly, Chemerin Receptor 23 (ChemR23) is important for pro-resolution effects of RvE1 in (allergic airway) inflammation (154, 155). In the case of OVA-induced airway inflammation, RvE1 causes clearance of eosinophils and antigen-specific CD4+ T-cells, in which natural killer cells (NK-cells) might play a role as an effector cell for these pro-resolving effects (155). In addition, RvE1 also exerts its effect on the smooth muscle level. It can dampen increased Ca^{2+}-sensitivity and U46619 (a TP receptor agonist)-induced smooth muscle contractions in rat and human pulmonary arteries, with or without previous exposure to TNFα and IL-6 (60, 156). Secondly, antagonism at the BLT_1 receptor has been shown in inflammatory models (154, 157). BLT_1 antagonism by RvE1 can prevent LTB_4-mediated neutrophil infiltration (154). Moreover, as shown in HDM and OVA mouse models of allergic asthma, RvE1 can reduce AHR, cell counts of eosinophils, leukocytes and macrophages, and cytokine concentrations of IL-4,IL-13 and IL-23 in BALF, possibly through decreasing cytokine release from lung macrophages (92, 157, 158).

Of the resolvin D series, 17(R)-RvD1 and RvD1 share a G-protein coupled receptor with LXA_4: the ALX/FPR2 receptor (95, 159). Moreover, they bind the receptor with the same potency as LXA_4 (160). The other G-protein coupled receptor involved in RvD1 signalling is GPR32 (161). Through these receptors, 17(R)-RvD1 and RvD1 mediate a broad pallete of anti-inflammatory and pro-resolution functions. Both can inhibit neutrophils infiltration during inflammation and stimulate macrophage phagocytosis (161, 162). Downstream mediators important herein include microRNA's (e.g. miR-208a, miR-219) and release of the anti-inflammatory cytokine IL-10 (161, 163). In OVA-induced inflammation and AHR, there could be a difference in efficacy as 17(R)-RvD1 is more efficacious that RvD1 in reducing eosinophilia and AHR (164).

Lung diseases such as cystic fibrosis or inhalation of harmful substances through smoking can lead to decreased endogenous production of RvD1 (102). Ensuing exogenous administration can reduce disease symptoms including lung destruction or development of end-stage disease such as lung emphysema (87, 165, 166). Furthermore, RvD1, but also RvD2 and LXA_4, can through their release by human airway epithelial cells, reduce histamine release by

human mast cells (18). In addition, in isolated human bronchi, RvD1 exposure has been reported to reduce Ca^{2+}-sensitivity and hyperresponsiveness induced by IL-13 (98). Further similar actions on smooth muscle function have been shown in human and rat pulmonary artery, reducing again cytokine-induced Ca^{2+}-sensitivity and contractility (156, 167).

Less is known about the other D-resolvins. It is known that RvD2 can dampen LTD_4- and TNFα-induced hypercontractility to histamine, methacholine (a more stable acetylcholine analogue) and U46619 (168). Moreover, during bacteria-induced inflammation, RvD2 reduces systemic inflammation, microbial burden and leukocyte trafficking. This is done through endothelial NO release and dampening of release of proinflammatory mediator including LTB_4, PGE_2 and several cytokines (163, 169). For RvD3 to RvD6, aside from the key SPM functions such as increased efferocytosis, protective effects have been shown in e.g. acid-induced lung injury, pathological thrombosis and neuropathic and inflammatory pain (170-175).

In summary, ω-6 and ω-3 PUFAs can give rise to lipid mediators that could be of importance in lung inflammation, resolution and modulation of airway tone. If the role of SPMs gets further substantiated by future research, this would open up the possibility to use stable SPM-analogues or SPM receptor agonists to actively shut down ongoing inflammation and decrease symptoms in inflammatory diseases like asthma.

3 RESEARCH AIMS

The general aim was to investigate if selected SPMs have anti-hyperreactive properties, how COX-inhibition results in increased airway constriction and if mast cells are necessary for antigen-induced contraction and airway hyperreactivity.

Specific aims:

- To examine whether a selection of specialised pro-resolving lipid mediators can modulate airway contractility, either by inducing contraction or relaxation or change the contractility to other contractile agonists

- To investigate if LXA_4 can reduce airway hyperreactivity induced by HDM allergen

- To examine if IL-13 induces corticosteroid-resistant airway hyperreactivity in isolated mouse trachea and whether cysteinyl maresins are able to reduce this airway hyperreactivity

- To investigate the mechanism underlying the way unselective COX-inhibition enhances OVA-induced airway constriction in guinea pig

- To assess if mast cells are necessary for HDM allergen-induced airway contraction and if they can modulate the level of airway hyperresponsiveness after chronic HDM exposure in genetically modified mice

4 MATERIALS AND METHODS

4.1 GENERAL

Two species of animals and several inflammation models were used for the studies performed in this thesis. Mice were used to study the role of selected SPMs in airway contractility and hyperreactivity (**article I, IV**) or of mast cells in allergen-induced airway contraction and hyperresponsiveness (**article II**). Because of similarities with humans in airway physiology and pharmacology, guinea pigs were used in **article III** to study increased airway contraction after unselective COX inhibition and initial studies of direct contractile or relaxant studies of SPMs (not published).

4.2 ANIMALS

All studies performed were in accordance with the ethical permits that were obtained from the regional ethical review committee for experimental animal research. Guinea pigs (Dunkin-Hartley) were obtained from Envigo and mice (BALB/c or C57BL/6) were purchased from Envigo or Charles River. All animals were housed in a 12-hour light/dark cycle with a continuous supply of food and water at their disposal. Guinea pigs were euthanised by CO_2-asphyxiation and subsequently removal of the heart. Mice were euthanised by cervical dislocation.

4.3 INTRANASAL INSTILLATION

In **article I**, a four-day instillation protocol was used. Mice received intranasal instillation of 10 ng SPM one hour before the intranasal administration of 50 µg HDM, both under isoflurane-induced anaesthesia. In **article II**, mice received intranasally 50 µg HDM every third day for a total of seven times.

4.4 TISSUE COLLECTION AND CULTURE

To ensure tissue viability, whole tracheae were immediately dissected out after animal euthanasia and kept on ice cold Krebs-Henseleit buffer until further dissection. During further dissection, guinea pig tracheae were divided in eight equal segments whilst each mouse trachea was divided in four segments. Both mouse and guinea pig segments were then immediately used in organ bath or myograph experiments (**article I-IV**) or in the case of mouse tracheal segments, incubated for a total of five days (**article I and IV**). These segments were incubated in 96 wells culture plates filled with low-glucose (1 g/L) DMEM supplemented with 1% penicillin (100 $IU \cdot mL^{-1}$) and streptomycin (100 $\mu g \cdot mL^{-1}$), which were placed in a humified incubator at 37 °C and 5% CO_2. All segments were allowed an overnight equilibration period before they were moved to new medium, and cytokines and SPMs were added for the first time. In **article I**, SPMs were added one hour before the pro-inflammatory stimulus. In **article IV** cysteinyl maresins and cytokines were added at the same time.

4.5 FUNCTIONAL STUDIES

Tracheal rings were suspended in 5 mL myographs (mouse trachea) or organ baths (guinea pig trachea) direct after dissection or after incubation. To measure the changes in force exerted by the smooth muscle in the organ baths, isometric force-displacement transducers linked to a Grass polygraph were used. After suspension, segments were left to equilibrate for 30-60 minutes. This was then followed by adjustment of the mechanical tension to 30 mN (guinea pig trachea) or 0.8 mN (mouse trachea). In studies of guinea pig trachea, the contractility of the tissue was tested with a concentration response curve of histamine (10 nM – 100 µM). In mouse trachea, this was done with 60 mM potassium chloride (KCl). Experiments in guinea pig trachea were ended with a maximal contraction induced by histamine (1 mM), acetylcholine

(1 mM) and 60 mM KCl. In mouse trachea this was done with carbachol (10 µM) and 60 mM KCl.

4.6 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Histamine was analysed by an external laboratory with an ELISA-based method (RefLab ApS, Copenhagen, Denmark). For the measurement of cysteinyl-leukotrienes (**article III**), an ELISA-kit was used. For the histamine analyses, glass fiber-coated microtiter plates were used. Samples were diluted and pipetted on the plates. After an hour of incubation at 37 °C, the plates were washed with distilled water, dried and closed off and sent for analysis. Cysteinyl leukotrienes were measured according to the manufacturers protocol (Cayman Chemical). In brief, a buffer was added to the wells of a 96-well plate pre-treated with a specific antibody. Then, standards and samples were added. Next followed the addition of a tracer of acetylcholinesterase bound to cysteinyl-leukotrienes and a cysteinyl-leukotriene monoclonal antibody. After two hours of incubation at room temperature, the plate was developed by washing and addition of Ellman's reagent, containing an acetylcholinesterase substrate, and tracer and left to develop for 90 min. The absorption of light with a wavelength between 405 and 420 nm was measured with a Biotek Instruments EL808 plate reader. The concentration of cysteinyl leukotrienes was then inversely correlated with the amount of absorbed light. Cross reactivity for N-methyl-LTC_4 was 124%, for LTC_4 and LTD_4 it was 100% and for LTE_4 65%. For other fatty acids this was <0.01%.

4.7 MASS SPECTROMETRY

Eicosanoid levels in samples were quantified via liquid chromatography coupled to triple quadrupole mass spectrometry (LC-MS/MS) (**article III**), as described in (176). In short, samples were spiked with deuterated internal standards and extracted with a pre-conditioned solid phase extraction cartridge. Second, solvent was evaporated and the samples were reconstituted in methanol. Samples were injected onto a BEH C_{18} column and quantitative mass spectral data was collected with a Xevo TQ S triple quadrupole system operating in negative scan mode.

4.8 SPECTROPHOTOMETRY

To ascertain the concentration and stability of the stocks of SPMs used, spectrophotometry was used. Depending on the concentration of the stocks, the sample taken from the stock was diluted in methanol, to achieve light absorption between 0.2 and 1.0 A.U. A blank measurement was used to correct all samples. The concentration was calculated with the help of the Lambert-Beer law: $A = \varepsilon \times l \times c$. Were A is the absorption, ε the molar absorptivity, l the length of the optical path and c the concentration of the sample. The ε values and absorption wavelengths were obtained from a public spectral book with SPM spectral data (Serhan C, Lipid Mediator Metabololipidomics LC-MS-MS Spectra Book 2016).

4.9 CALCULATIONS AND STATISTICS

All data were presented as mean ± SED. Contractions were normalised to the maximal contraction induced by KCl, histamine and acetylcholine (guinea pig trachea) or carbachol maximum (mouse trachea). GraphPad Prism 8.0 software (GraphPad Software Inc., San Diego, CA) was used for data and statistical analyses. Non-linear regression fit was used to calculate pEC_{50} and E_{max}. For bolus additions, the area under the curve (AUC) was calculated. To assess statistical significance of multiple groups, one-way ANOVA with Dunnet's post-hoc test was used, or student's T-test in the case of two groups. When possible, paired analysis was performed. The significance level was set at $p < 0.05$.

5 RESULTS AND DISCUSSION

5.1 EXPLORATION OF SPECIALISED PRO-RESOLUTION LIPID MEDIATOR EFFECTS IN GUINEA PIG AIRWAY AND VASCULAR TISSUE

5.1.1 Direct smooth muscle effect of SPMs

The SPM research field is relatively young, with most discoveries happening the last 20 years compared to for example the prostaglandins discovered already in the 1930s. Many animal studies, in particular those examining airway effects of SPMs, have been conducted in mouse airway tissue (157, 164, 177). The guinea pig offers another model with some advantages in airway research by way of similar airway anatomy and existence of similar physiology and pharmacology, i.e. similar agonists and receptor functions (33, 39). Therefore, guinea pig tissue was first used to investigate if there would be acute contractile or relaxant effects of selected SPMs. This would be in analogy to prostanoids being either contractile, e.g., $PGF_{2\alpha}$ or relaxant, e.g., PGI_2, PGE_2, or to contractile cysteinyl leukotrienes in airways.

To this end, a total of six structurally different SPMs were selected to screen for acute smooth muscle effects. The selection was made in order to have a representative SPM of each major group derived from different polyunsaturated fatty acids, i.e., AA, EPA and DHA (figure 3). These representative SPMs were added in increasing concentration to baseline or precontracted guinea pig tracheal rings. Since these experiments were used as a screening for potential effects, one or two segments were dedicated to each SPM (figure 5).

Despite the broad range of concentrations tested, picomolar up to high nanomolar, which is often referred to as the bio-active range of SPMs (90), no direct contractile (figure 5A) or relaxant effects (figure 5B) of selected SPMs were found. These data regarding SPM functions in airways outside the context of inflammation have not been reported before. However, LXA_4 has been tested in the same way in guinea pig airway tissue in previous investigations. In these investigations, it was observed that LXA_4 induced contraction in lung parenchymal strips but not tracheal strips (178, 179), suggesting a contractile effect in vessels and not airways.

Mechanistically, the early experiments pointed at activation of the $CysLT_1$ receptor and release of TXA_2 to cause the constriction observed (120, 179). In later binding studies in cells, it could be observed that LXA_4 and the epimer 15(R)-LXA_4 displaced LTD_4 from the $CysLT_1$ receptor (126), suggesting different mechanisms present in different cells or tissues. Chirality of the LXA_4 molecule, i.e. LXA_4 and 15(R)-LXA_4, can also cause differences in receptor binding and biological effects (112, 180). The current data however shows no contractile or relaxant effects of 15(R)-LXA_4 in guinea pig trachea, just as found previously for LXA_4.

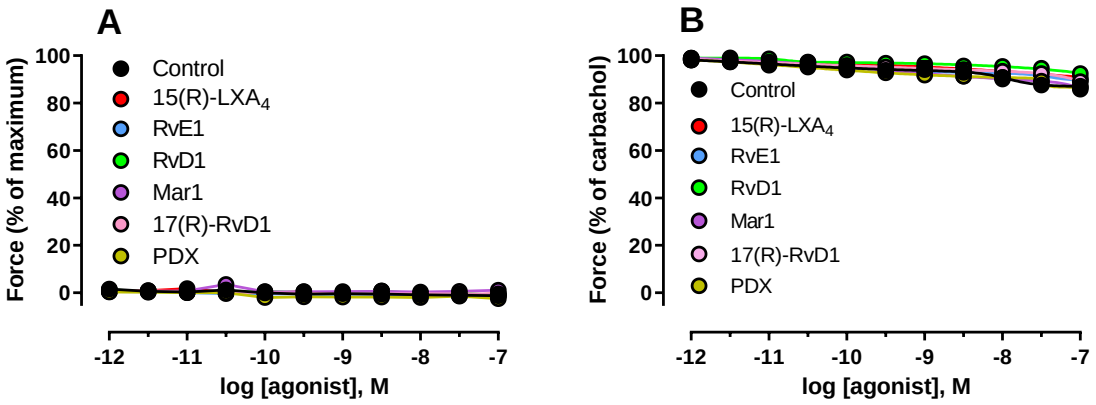

Fig. 5. Concentration-response curves of selected SPMs (1 pM – 100 nM) in presence of indomethacin (3 µM). A, addition of increasing concentrations SPMs in relaxed guinea pig tracheal rings (n = 1-2). B, addition of increasing concentrations SPMs in guinea pig tracheal segments precontracted with carbachol (10 or 100 nM) (n = 1-2). Data represent mean ± SEM. SPMs used are: lipoxins: 15(R)-lipoxin A_4 (15(R)-LXA_4); E-resolvins: resolvin E1 (RvE1); D-resolvins: resolvin D1 (RvD1) and its 17(R)-epimer 17(R)-RvD1; Protectins: protectin DX (PDX); Maresins: maresin 1 (MaR1).

5.1.2 SPM modulation of agonist-induced contractions

It was next assessed if there would be a modulatory effect of SPMs on other contractile or relaxant agonists in tracheal as well as vascular tissue, because of the apparent lack of a direct effect on smooth muscle contractile activity. It was found that maresin 1 (mar1) affected contractions induced by endothelin 1 and sarafotoxin 6b in tracheal and aorta tissue (figure 6), but not contractions induced by other contractile agonists (phenylephrine, U46619, histamine, figure 7). In the presence of mar1, the E_{max} was significantly increased (figure 6C,D) and/or leftward shifted (figure 6A-C). However, in the case of the concentration-response curves of figure 6B-D this has to be interpreted with caution since none of the curves reached the maximal contractile effect, thus there is still the possibility of them reaching the same E_{max}. Endothelin and sarafotoxin 6B are both non-selective endothelin receptor agonist, making an effect on both the ET_A and ET_B receptor possible.

In guinea pig airways, ET-induced contractions are mediated by both endothelin receptors and can be direct, e.g., in trachea, or indirect through release of COX products, possibly TXA_2, e.g. in lung parenchyma (181-183). It has to be noted that involvement of TXA_2 was restricted to lung parenchyma and in our experiments, indomethacin was used to remove a possible confounding effect of TXA_2. These results seem counter-intuitive given the nature of SPMs and how they are proposed to resolve inflammation. Especially since endothelin 1 could play a role in allergic airway inflammation (184, 185). It could however, at some point in the resolution process, be beneficial to have an increased responsiveness to endogenous released endothelin 1 to for example temporarily decrease perfusion or ventilation. Because of our focus on allergen-induced inflammation and hyperreactivity and possible ameliorating effects SPMs herein, these effects were not further investigated. Future experiments are however needed to look into the mechanism of this effects and its possible functional relevance for resolution of lung inflammation.

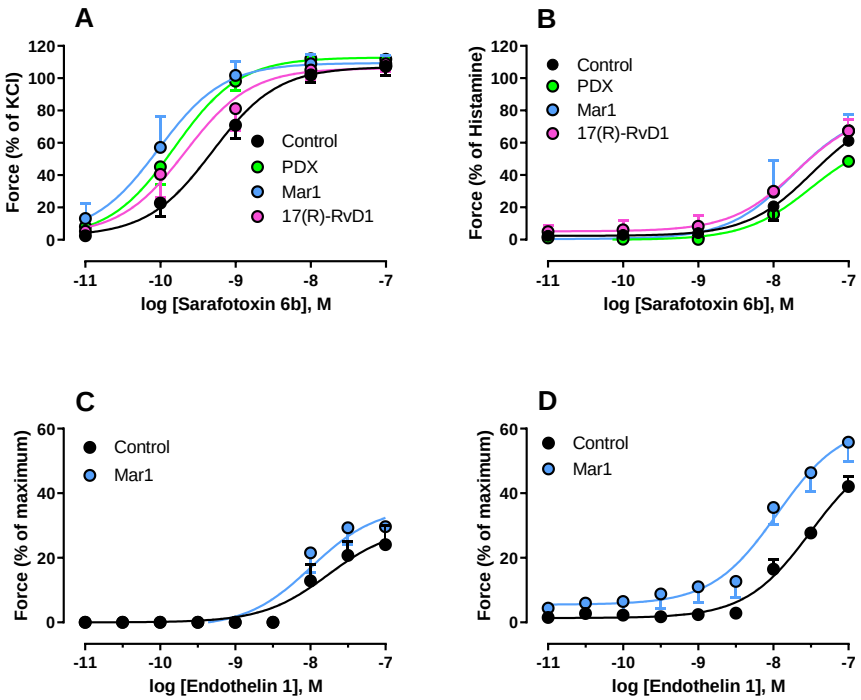

Fig. 6. Concentration-response curves of endothelin 1 or sarafotoxin 6b after 30 minutes of pre-incubation with protectin DX (PDX), maresin 1 (MaR1) or 17(R)-RvD1 (100 nM). A, in guinea pig aorta (n = 5-6). B, in guinea pig trachea (n = 2). C, mar1 in guinea pig aorta (n = 4). D, MaR1 in guinea pig trachea (n = 4). All in presence of indomethacin (3 µM). Data represent mean ± SEM.

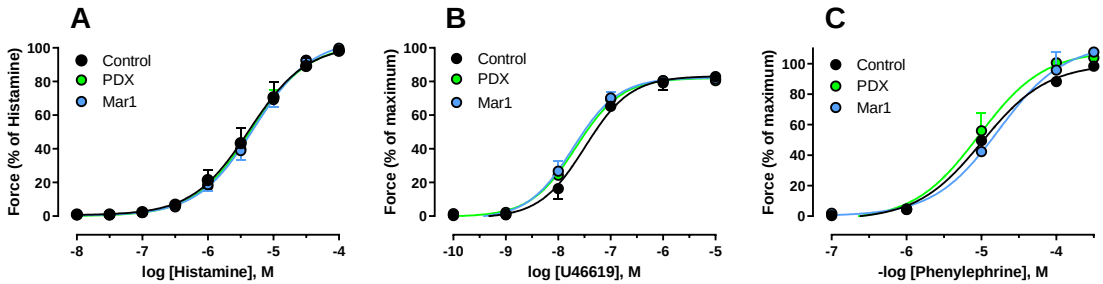

Fig. 7. Concentration-response curves after 30 minutes of pre-incubation with PDX and maresin 1 (mar1) (100 nM). A, histamine (10 nM – 100 µM) in guinea pig tracheal rings (n = 6-9). B, U46619 (100 pM – 10 µM) in guinea pig tracheal rings (n = 2). C, phenylephrine (100 nM – 300 µM) in guinea pig aorta rings (n = 1-2). All in presence of indomethacin (3 µM). Data represent mean ± SEM

5.1.3 MaR1 and PDX in OVA-induced airway contraction

In the light of the important role of mast cells as in airway inflammation (186, 187) , a possible acute effect of MaR1 and PDX on OVA-induced mast cell activation and airway contraction was investigated (figure 8). No effects of these two SPMs added 30 minutes before OVA-challenge could be seen on general OVA-induced contractions (figure 8A), nor in OVA-induced contractions mediated by histamine (figure 8B) or leukotrienes (figure 8C).

5.1.4 Summary

It was found that selected SPMs do not induced contraction nor relaxation of guinea pig tracheal rings in the organ-bath set up. However, when pre-treating aorta rings with MaR1, a significant increase in sensitivity, i.e., pEC_{50}, of sarafotoxin 6b was observed. A similar effect could be shown for endothelin 1-induced contraction in aorta and tracheal tissue as well. Nevertheless, since these are small scale exploratory studies, larger studies are warranted to confirm the results and explore the mechanisms involved.

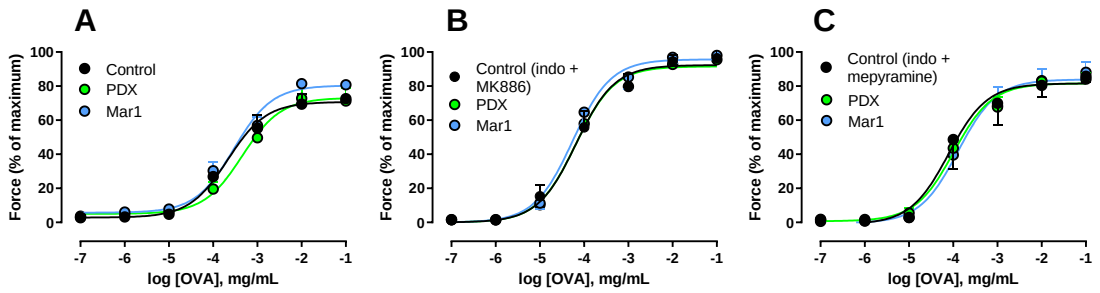

Fig. 8. Concentration-response curves of OVA after 30 minutes of pre-incubation with PDX and mar1 (100 nM) in guinea pig trachea. A, no indomethacin or antagonists present (n = 2-3). B, In presence of indomethacin (3 µM) and MK886 (10 µM) (n = 9-12). C, in presence of indomethacin (3 µM) and mepyramine (1 µM) (n = 4-6). Data represent mean ± SEM

5.2 ANTI-HYPERREACTIVE FUNCTION OF LXA_4 AND CYSTEINYL MARESINS IN AIRWAY HYPERREACTIVITY INDUCED BY TNFA AND IL-13 IN MICE (PAPER I AND IV)

It was next assessed if there were effects of selected SPMs in tracheal hyperreactivity induced by allergic inflammation. To this end, both exposure to HDM through intranasal instillation as well as to HDM or cytokines during incubation were used to induce airway hyperreactivity in mouse trachea. Mouse airway tissue was used to take advantage of a method developed in our lab to study airway hyperreactivity as well as the widespread availability of mouse specific cytokines. HDM was used to model a more complex allergic inflammation as HDM is a mixture and exposure to it causes release of both type 1 and type 2 cytokines. In addition it activates toll-like receptors (TLRs), depending on levels of LPS and biopolymers present in the HDM extract (51, 188, 189). Furthermore, by addition of TNFα or IL-13, two different inflammatory pathways which lead to airway hyperreactivity were modelled. TNFα is considered a type 1 cytokine, more involved in inflammation mediated by immune cells against intracellular micro-organisms (190, 191). IL-13 is a type 2 cytokine more involved in inflammation as part of defence against eukaryotic parasites and patho-physiologically in allergic inflammatory disease (190, 191). Both cytokines can also be released by mast cells (23).

5.2.1 Reduction of airway hyperreactivity by lipoxins and cysteinyl maresins

First, HDM exposure *in vivo* led to a pronounced upregulation of contractions induced by 5-HT (figure 9A). This HDM-induced hyperreactivity was reduced by the DHA product 17(R)-RvD1 as well as the AA product lipoxin LXA_4. These SPMs have in common that both have been shown to signal through the ALX/FPR2 receptor (6, 160). The other group of SPMs (RvE1, MaR1 and PDX) did not reduce airway hyperreactivity. One explanation for difference in activity could be involvement of the ALX/FPR2 receptor, as both LXA_4 and 17(R)-RvD1 have been shown to signal through the ALX/FPR2 receptor (6, 160), but not the SPMs found the be inactive. Another possibility could be a different potency of the ineffective SPMs, since only one dose of SPM was tested. If so, to be effective as intranasal installation, both a higher or a lower dose of these SPMs could have been necessary, as SPM dose- or concentration-response curves can be bell-shaped (122, 126-128). Nevertheless, this does not seem to be the case for LXA_4 (paper I, figure 4). Another explanation could be an increased biochemical instability of the ineffective SPMs compared to the effective ones, leading to degradation before reaching their target in the lung after installation.

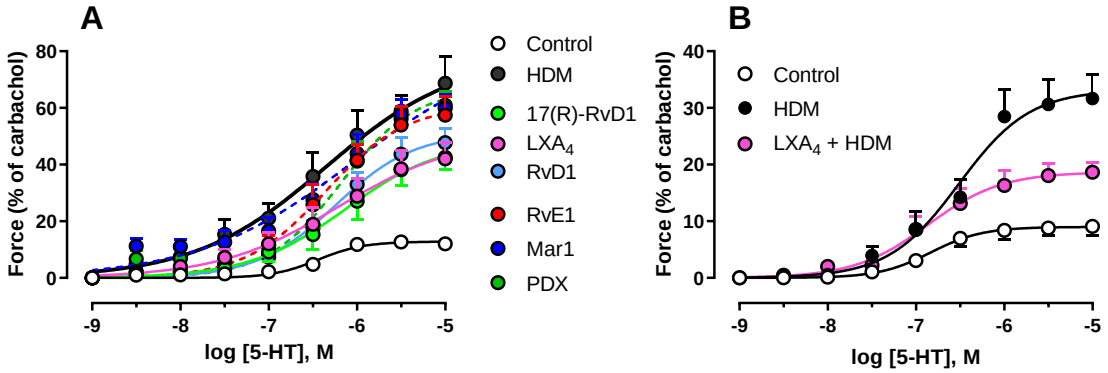

Fig. 9. Concentration-response curves of 5-HT in mouse trachea. A, after four days of intranasal instillation of selected SPMs (10 ng) one hour before challenge with HDM (50 µg) (n = 4-11). B, after four days of *in vitro* exposure to LXA_4 (100 nM) added one hour before HDM (1 µg/mL) (n = 5-8). Data represent mean ± SEM

Not only did HDM exposure via the intranasal route induce airway hyperreactivity, but the same effect was also observed with HDM exposure during four days of incubation (figure 9B). Nonetheless, the E_{max} of 5-HT was considerably lower in isolated tracheal segment after exposure during incubation than after intranasal installation in live animals. This could be due to limited availability of immune cells in the tissue explants during culturing compared to live animals, which would decrease the cytokines released and thus the hyperreactivity induced. In addition, LXA_4 reduced airway hyperreactivity both when given intranasally and during incubation. This suggests that the anti-hyperreactive effect is mediated by receptors present on cells in tracheal segments, examples being epithelial cells and smooth muscle cells and airway resident immune cells like mast cells.

As the TNFα inhibitor etanercept could partly reverse the HDM-induced airway hyperreactivity (paper I, figure 3B), it was concluded that one of the mediators released after HDM exposure was TNFα. Therefore, it was assessed if airway hyperreactivity induced by exposure to TNFα *in vitro* could also be inhibited by LXA_4 (figure 10A). TNFα did indeed induce a marked increase in 5-HT responsiveness, which corresponded with previous published research (62). In addition, LXA_4 could reduce airway hyperreactivity at three different concentrations (10, 100 and 1000 nM). This effect did not seem to be specific to LXA_4, since also the positional isomer LXB_4 reduced the E_{max} of 5-HT (figure 10B). That LXA_4 can downregulate TNFα signalling has been observed before in human pulmonary endothelial cells (121). In these cells, exposure to an LXA_4 releasing compound (oxidised 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine, OxPAPC) decreased NF-κB activation, the important downstream protein complex involved in TNFα signalling (121). Furthermore, LXB_4 has been implicated before as being an anti-inflammatory lipid mediator in upper and lower allergic inflammation, not only through decreasing TNFα release from bone marrow mononuclear cells and stabilising mast cells, but interestingly also through accelerated decrease of levels of IL-4 and IL-13 and mRNA of their receptors (99). A possible receptor mediating the effect of lipoxins is the ALX/FRP2 receptor (paper I, table I). The results of the LXA_4 studies are summarised in figure 13.

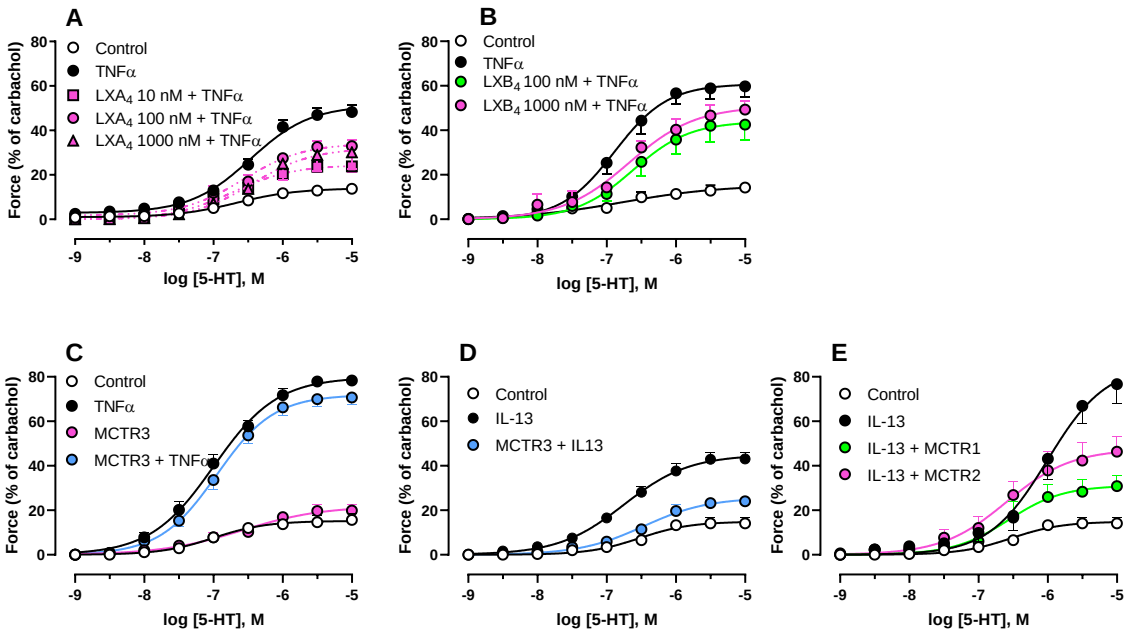

Fig. 10. Concentration-response curves of 5-HT four days of incubation with lipoxins or cysteinyl maresins and TNFα (100 ng/mL) or IL-13 (100 ng/mL). A, incubation with TNFα and presence or absence of LXA_4 (10 – 1000 nM) (n = 5-21). B, incubation with TNFα and presence or absence of LXB_4 (100 or 1000 nM) (n = 7-13). C, incubation with TNFα and presence or absence of MCTR3 (1000 nM). D-E, incubation with IL-13 and presence or absence of MCTR3 (n = 4-13) or MCTR1 or MCTR2 (1000 nM, n = 4-6). Data represent mean ± SEM

It was also assessed if a representative SPM from another SPM family branch, namely MCTR3, could reproduce the dampening effect of lipoxins. However, TNFα-induced hyperreactivity was not affected by the presence of MCTR3 (figure 10C). This suggests different mechanisms of action for these structurally different SPM subgroups, even though MCTR3 has been found to signal through the ALX/FPR2 receptor in LPS-induced acute lung injury (147).

As for the cysteinyl maresin family, though not effective in dampening TNFα-induced airway hyperreactivity, they were found to dampen IL-13-induced airway hyperreactivity (figure 10D-E). IL-13 induced a somewhat lower hyperreactivity compared to TNFα, but still markedly increased 5-HT-mediated airway contractions. This airway hyperreactivity was resistant to corticosteroids as observed in this mouse model (paper IV, figure 1C) and has been shown before in human airways (26). This thus makes this anti-hyperreactive action of cysteinyl maresins finding important, as it offers a future lead for development of treatment of corticosteroid-resistant hyperreactivity of the airways.

5.2.2 Receptors involved in lipoxin and cysteinyl maresin signalling

As stated before, one explanation for difference in effectiveness of lipoxins and cysteinyl maresins in TNFα or IL-13-induced hyperreactivity could be different receptors involved. For lipoxins, the ALX/FPR2 could play a role (paper I, table I) and for cysteinyl maresins circumstantial evidence suggests involvement of the $CysLT_1$ receptor. The anti-hyperreactive effect of MCTR3 could be blocked when pre-incubating tracheal segments with three structurally different $CysLT_1$ receptor antagonists, being montelukast, zafirlukast and pobilukast (figure 11A-B). Noteworthy is the difference in concentrations needed to antagonise the effect of MCTR3 for the different antagonists. Whilst for zafirlukast a concentration of 10 nM would suffice, for montelukast a 100-fold higher concentration was needed to obtain the same effect. This does not agree with the potencies of these antagonists blocking the CysLTs, as the potency of montelukast and zafirlukast is more or less the same for the $CysLT_1$ receptor (192). Moreover, for pobilukast the same concentration was needed as montelukast (1000 nM), even though pobilukast is 10-fold less potent than montelukast in cell models and lung tissue

(192). Though cysteinyl leukotrienes do not contract murine airways, LTD_4 was used as an agonist for the $CysLT_1$ receptor to examine the possible anti-hyperreactive function of this receptor. However, incubation with LTD_4 did neither reduce IL-13-driven airway hyperreactivity nor did it interfere with MCTR3's reducing effect (figure 11C-D).

This makes it therefore unlikely that cysteinyl maresins signal through binding at the orthosteric binding site of the $CysLT_1$ receptor. It is possible that there are one or more alternative, allosteric binding sites present on the receptor, leading to a different signalling cascade that is responsible for the effect seen. Another possibility would be that another receptor is involved, one that also binds the antagonists tested. One candidate for this was the GPR17 receptor (193-195), but stimulation of this receptor could not reproduce the anti-hyperreactive effect of cysteinyl maresins (paper IV, figure 6). Furthermore, an antagonistic effect of cysteinyl maresins on LTD_4-signalling could not be seen in mouse as well as guinea pig tissue (paper IV, figure 5), as was shown by others before (97). A way to confirm involvement of the $CysLT_1$ receptor is future experiments with $CysLT_1$ knock out ($CysLT_1^{-/-}$) mice. Until then, it remains to be proven which receptor exactly mediates the anti-hyperreactive effect of cysteinyl maresins in airways.

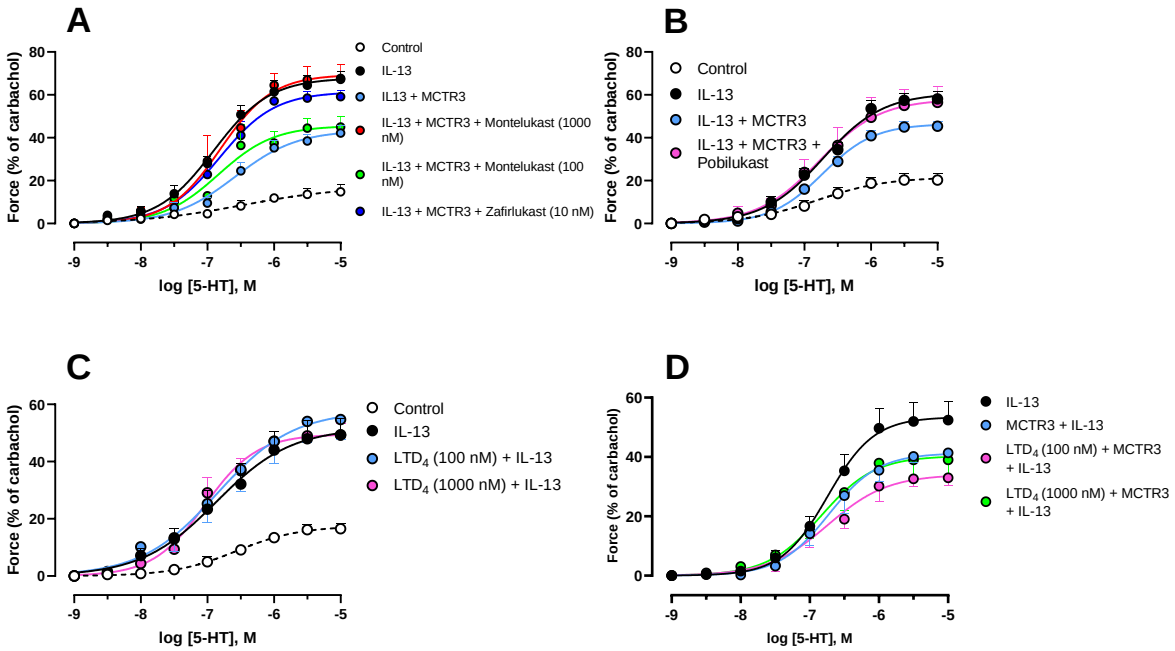

Fig. 11. Concentration-response curves of 5-HT of tracheal segments incubated with montelukast (100 and 1000 nM), zafirlukast (10 nM), pobilukast (1000 nM) or LTD_4 (100 and 1000 nM) and MCTR3 (1000 nM) and IL-13 (100 ng/mL). A-B, zafirlukast blocks MCTR3's protective effect at a lower concentration than montelukast (n = 3-12) and pobilukast (n = 7-13). C-D, LTD_4 cannot reproduce (n = 7-8) nor interfere (n = 3-5) with MCTR3's anti-hyperreactive effect. Data represent mean ± SEM

5.2.3 Other explorations of SPM modulation of airway hyperreactivity

Several other experiments were performed to further describe the possible mechanisms underlying lipoxin and cysteinyl maresin signalling in airway hyperreactivity. First, for LXA_4 it was determined if it could induce a relaxation of non-inflamed, pre-contracted tracheal rings (figure 12A). However, up to 100 nM LXA_4, which is the concentration used in all lipoxin experiments, no relaxation different from vehicle was seen. This thus excludes relaxations as a confounding factor. The same can be stated for the cysteinyl maresins, because if a general relaxant function would have been present, a decrease of the E_{max} 5-HT should have been observed not only in the IL-13-driven model but also the TNFα-driven model (figure 10C-E).

Second, the epithelium present in the trachea offers a plausible target for SPMs. Binding would then lead to a release of secondary anti-inflammatory, anti-hyperreactive mediators or a blockade of pro-inflammatory, pro-hyperreactive mediators. This would then add an extra layer

to the control of the inflammation and resolution balance in airway tissue. That these mechanisms occur has been observed for SPMs in epithelium. Both LXA_4 and MaR1 are able to inhibit migration of neutrophils over the epithelium and inhibit release of CXCL8 or TNFα (140, 196). Even so, removal of epithelium from mouse trachea did not alter the hyperreactivity induced by IL-13 nor did it change the anti-hyperreactive effect of MCTR3 (figure 12B). It makes it therefore more likely that both these substances, in this particular model, exert their effects on the smooth muscle cells. Though effects on other cells have not been excluded, such as effects on immune cells present or other structural cells.

Modulation of airway contractility in non-inflamed mouse tracheal segments was also assessed, before continuing into the inflammatory models already described. This was done in analogy to the studies in guinea pig tissue. In these studies, it was found that cysteinyl maresins, when added at 30 minutes before performing concentration-response curves of carbachol, caused a significant (MCTR2 and MCTR3) half-log shift of the pEC_{50} as well as a trend towards reduction of the E_{max} (non-significant, $p = 0.07$ for MCTR1 and 3 and $p = 0.17$ for MCTR2, figure 12C). Something which could not be seen for U46619-mediated contractions (figure 12D).

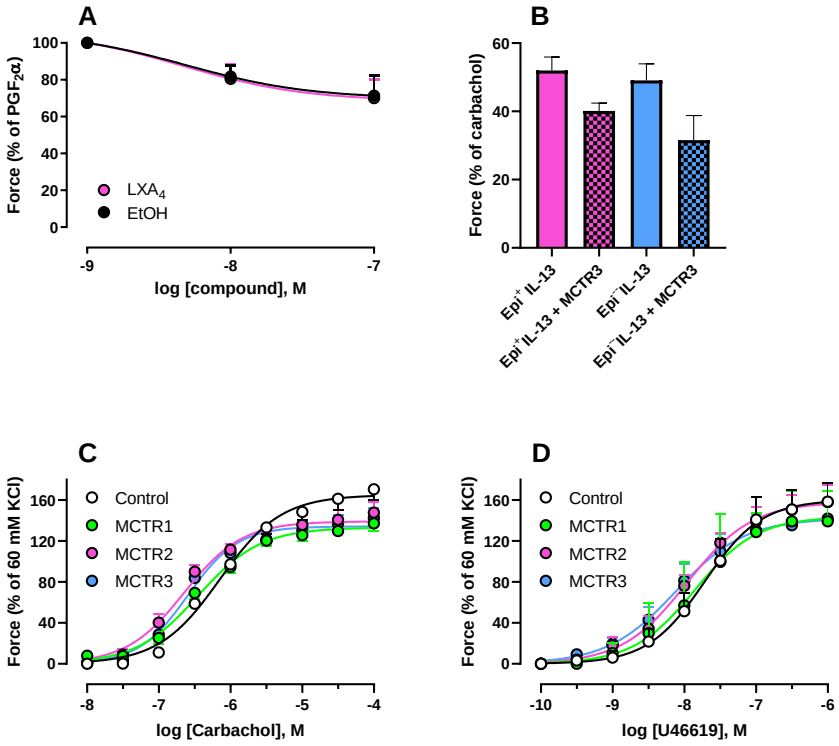

Fig. 12. Acute effects of LXA_4 and cysteinyl maresins and role of the airway epithelium segments. A, concentration-response curve for LXA_4 in $PGF_{2\alpha}$ (3 µM) pre-contracted mouse tracheal segments (n = 7-10). B, effect of four-day incubation of epithelium positive (Epi^+) and epithelium denuded (Epi^-) mouse tracheal rings with MCTR3 (1000 nM) and IL-13 (100 ng/mL, n = 6-8). C-D, concentration-response curve of carbachol and U46619 after 30 minutes of pre-incubation with cysteinyl maresins (100 nM) in mouse tracheal rings. Data represent mean ± SEM

5.2.4 Summary

In mouse trachea, HDM, TNFα and IL-13 cause a pronounced increase in airway responsiveness to 5-HT. Several types of SPM can counter this airway hyperreactivity. LXA_4 does this in HDM and TNFα-induced hyperreactivity when given via intranasal instillation, as well as during incubation of tissue explants. RvD1 and 17(R)-RvD1 can replicate this effect when given intranasally before HDM and LXB_4 during incubation together with TNFα. The ALX/FPR2 receptor might play a role in lipoxin signalling in these experiments, but not the epithelium (summarised in figure 13).

Cysteinyl maresins can reduce IL-13-induce hyperreactivity, which could be specific to this cytokine since they do not reduce hyperreactivity induced by TNFα. Cysteinyl maresin signalling is inhibited by known $CysLT_1$ antagonists, though stimulation of the receptor by LTD_4 does not modulate 5-HT mediated contractions (summarised in figure 14). This opens the possibility that cysteinyl maresins signal through a previously unknown allosteric site of the $CysLT_1$ receptor, and thus points at a possibly anti-inflammatory function of this receptor.

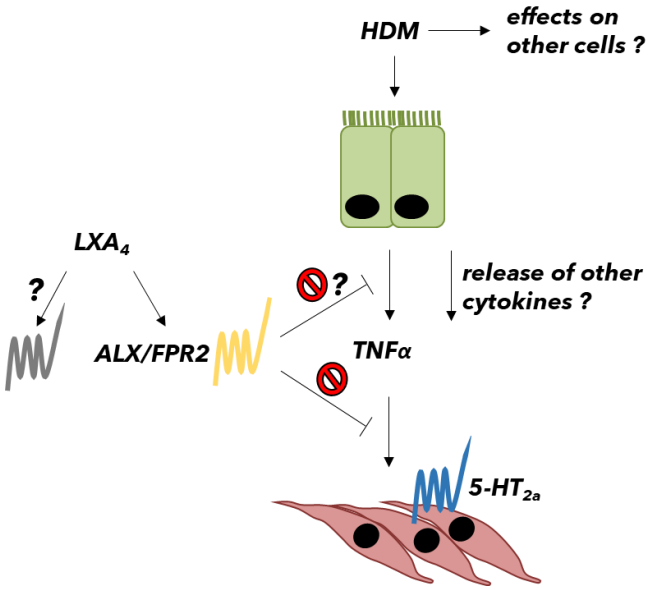

Fig. 13. Schematic summary of the main findings found in **paper I** and important remaining questions. HDM causes release of TNFα, which in turn causes airway hyperreactivity. This hyperreactivity can be reduced by LXA_4.

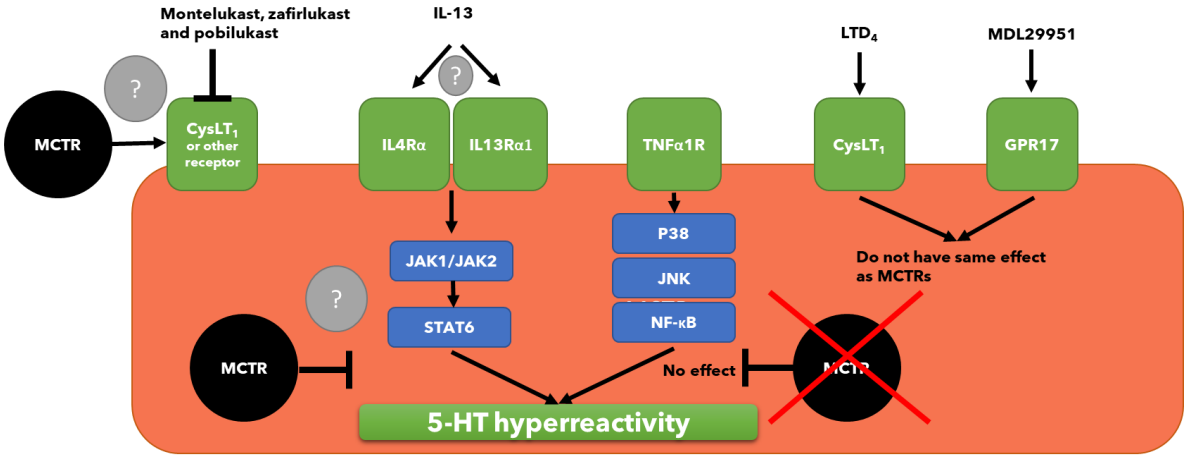

Fig. 14. Schematic summary of the main findings found in **paper IV** and important remaining questions. Cysteinyl maresins, known also as MCTRs, dampen IL-13-induced airway hyperreactivity but not TNFα-induced hyperreactivity. $CysLT_1$ antagonists block the anti-hyperreactive effect of cysteinyl maresins, but the $CysLT_1$ agonist LTD_4 does not have any dampening effect, nor does the GRP17 agonist MDL29951.

5.3 PGD_2-DP_1R AS AN AUTO-INHIBITORY AXIS IN MAST CELL MEDIATED AIRWAY CONSTRICTION (PAPER III)

One part of the effect of SPMs are their anti-inflammatory actions. But they are not the only lipid mediators having anti-inflammatory effects in airway tissue. Even though prostanoids are generally known for their pro-inflammatory functions, they also have been found to mediate anti-inflammatory effects. A well-known example is PGE_2, which can inhibit activation of mast cells in human lung tissue, thus reducing antigen-induced bronchoconstriction (74). Another example is PGI_2, which not only causes smooth muscle relaxation, but can also inhibit release of cytokines and interacts with immune cells from the innate as wells as the adaptive immune system (7, 197). This relates to the work in this thesis, which uncovers a similar mechanism for PGD_2 as for PGE_2 in OVA-induced constriction of tracheal rings from sensitised guinea pigs.

5.3.1 PGD_2 is produced by the COX-1 enzyme in mast cells and inhibits OVA-induced guinea pig airway constriction

In the initial experiments it was verified that a bolus dose of OVA induces a marked contraction tracheal segments from OVA-sensitised guinea pigs (figure 15) (198). This contraction was reduced over time, though did not return to basal tension for 90 minutes. Furthermore, when the tissue was pre-treated with the unselective COX-inhibitor indomethacin, OVA-exposure induced an increased contraction which was sustained over time. However, when the FLAP/5-LOX enzyme was blocked by MK-886, this resulted in a decreased contraction that returned to baseline tension within 45 minutes.

Three main groups of substances released from mast cells can be distinguished when analysing the concentrations of lipid mediators released after OVA exposure and blockade of COX-enzymes or the 5-LOX enzyme. First, unselective COX blockade as well as COX-2 blockade led to an almost complete inhibition of release of PGE_2, $PGF_{2\alpha}$ and the PGI_2 breakdown product 6-keto-$PGF_{1\alpha}$ (paper III, figure 2), with no effect of FLAP/5-LOX blockade. This thus showed that these lipids are formed by the COX-2 enzyme. Second, unselective COX blockade, but not COX-2-specific blockade nor FLAP/5-LOX blockade, inhibited release of PGD_2, TXB_2, and 12-HHTrE. This led to the conclusion that the latter lipids are formed by the COX-1 enzyme. Finally, the concentrations of the leukotriene LTB_4 and the contractile cysteinyl leukotriene LTE_4 were decreased by FLAP/5-LOX blockade, unchanged by COX-2-specific blockade but increased by unselective COX-inhibition. As unselective COX inhibition, but not COX-2-selective inhibition, increased the concentration of the contraction-inducing LTE_4, it was concluded that the substance mediating the inhibiting effect seen in figure 15 must come from COX-1, thus being PGD_2 or TXB_2 products.

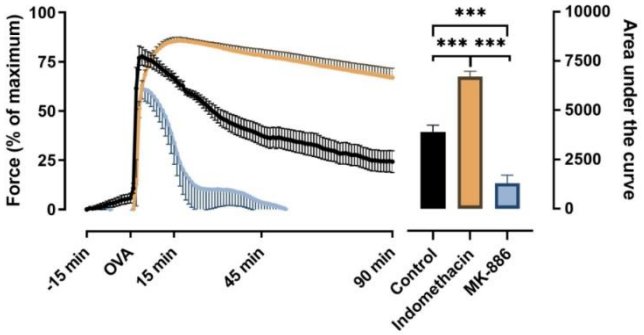

Fig. 15. Traces and area under the curve (AUC) of tracheal segments exposed to a bolus dose OVA (100 µg/mL) and presence or absence of indomethacin (3 µM) of the FLAP/5-LOX inhibitor MK-886 (10 µM). Data represent mean ± SEM with n = 5

Two inhibitors were used to specifically inhibit haematopoietic prostaglandin D_2-synthase (PPCA) or thromboxane A synthase (ozagrel) to further define which COX-1-produced lipid mediator was responsible for the dampening effect observed (figure 16). Blockade of PGD_2 formation by PPCA resulted in an increased and sustained contraction after

OVA exposure (figure 16A), an effect that was similar to that observed in figure 15. However, blockade of the TXA_2/TXB_2 pathway had no effect on OVA-induced contractions (figure 16B). Consequently, the reduction of OVA-induced contraction was attributed to PGD_2.

In the following investigations the inhibitory function was confirmed, and the receptor involved was further investigated. To start with, tracheal segments were incubated with three concentrations PGD_2, after which OVA concentration-response curves were made (figure 17A). These experiments could show that PGD_2, concentration-dependently, decreased OVA-induced contractions. Second, the dampening effect of PGD_2 could be reproduced when using another DP_1 receptor agonist (BW 245c), but not when using the DP_2 receptor agonist 15(R)-15-methyl PGD_2 (figure 17B). Finally, the involvement of the DP_1 receptor was further confirmed by usage of a DP_1 antagonist (MK-524), which concentration-dependently inhibited the DP_1 agonist (figure 17C).

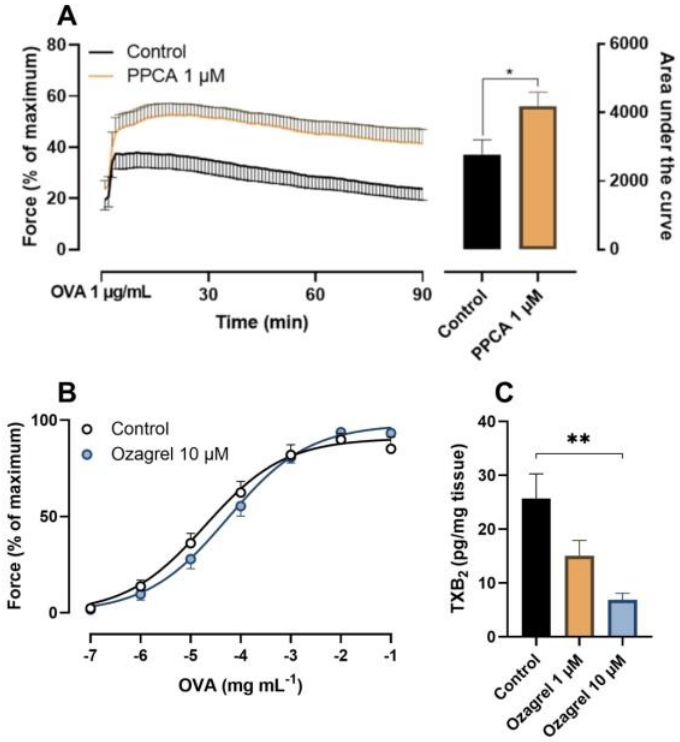

Fig. 16. A, traces and AUC of guinea pig tracheal segments exposed to a bolus dose OVA (100 µg/mL) and presence of SQ-29,548 (1 µM) and presence or absence of the haematopoietic prostaglandin D_2-synthase inhibitor PPCA (1 µM). B, concentration-response curve of OVA in presence of SQ-29,548 (1 µM) and presence or absence of the thromboxane A synthase inhibitor ozagrel (10 µM). C, Release of TXB_2 after OVA and ozagrel (1-10 µM). Data represent mean ± SEM with n = 6-9

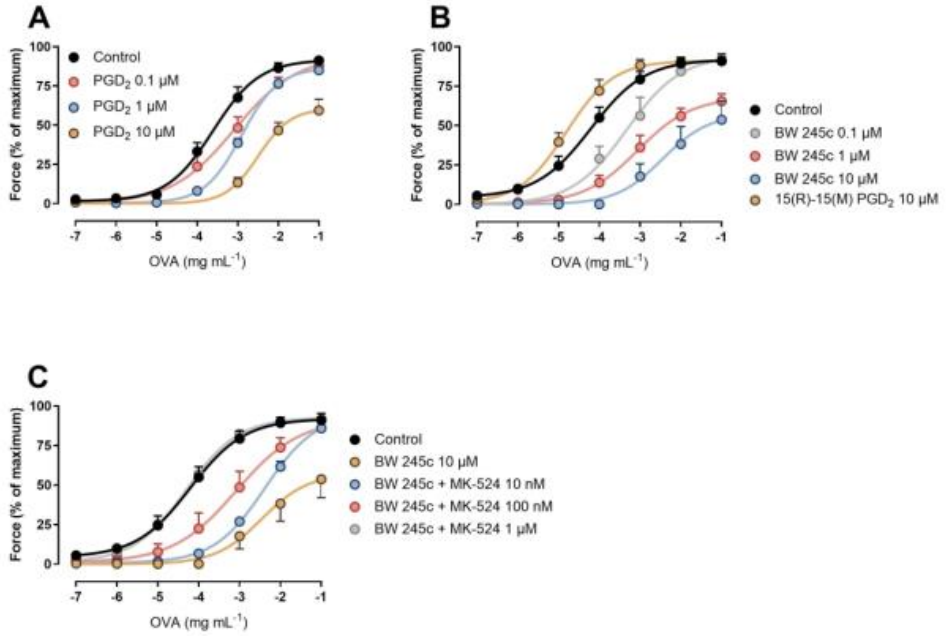

Fig. 17. OVA concentration-response curves in guinea pig trachea in presence of indomethacin (3 µM) and SQ-29-548 (1 µM). A, in presence of PGD_2 (0.1 - 10 µM). B, in presence of the DP_1 receptor agonist BW 245c (0.1 – 10 µM) or the DP_2 receptor agonist 15(R)-15-methyl PGD_2. C, in presence of BW245c (10 µM) and presence or absence of the DP_1 receptor antagonist MK-524 (10 – 1000 nM). Data represent mean ± SEM with n = 5-8

In another experimental setting it was confirmed that DP_1 receptor activation with BW 245c leads to a decreased constriction after OVA exposure. Here, increased concentrations of BW 245c led to decreased release of histamine and cysteinyl leukotrienes after addition of a bolus OVA, with a concomitant decreased E_{max} of the induced contraction (figure 18A-C). Also, the DP_1 receptor antagonist MK-524 reversed the dampening effect of BW-524 on OVA-induced contractions (figure 18A) as well as reversing the decreased release of cysteinyl leukotrienes mediated by the DP_1 agonist (figure 18C).

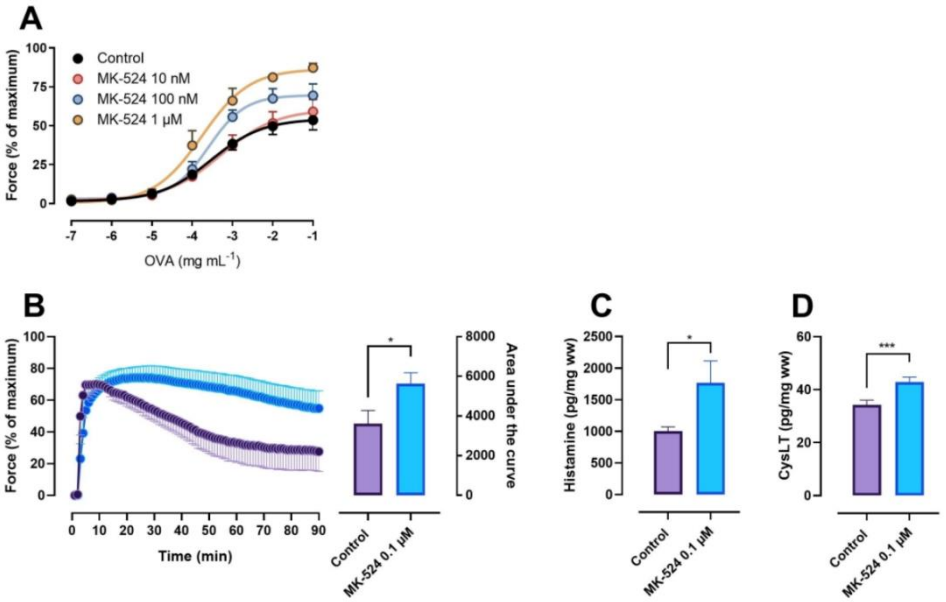

Fig. 18. Traces of OVA responses in guinea pig trachea with simultaneous release of histamine and cysteinyl leukotrienes in presence of indomethacin (3 µM), BW 245c (0.1 - 10 µM) and presence or absence of MK-524 (0.1 µM). A, OVA-induced contraction. B, histamine release after 15 min. C, cysteinyl leukotriene release after 90 min. Data represent mean ± SEM with n = 5-6

A following investigation was started to further prove that endogenously released PGD_2 mediated this inhibitory effect as well. To this end, an OVA concentration-response curve and bolus dose exposure were performed with the presence of the DP_1 receptor antagonist MK-524 present (figure 19). Here it was shown that PGD_2 is released upon OVA stimulation, and this released PGD_2 inhibits further contraction. This is done through the DP_1 receptor by inhibiting release of the contractile mediators histamine and cysteinyl leukotrienes.

As a final series of experiments, it was excluded that DP_1 receptor stimulation with PGD_2 could modulate (dampen E_{max} or right shift the pEC_{50}) histamine or LTD_4-induced contractions or that it could cause relaxation in tracheal segments (paper III, figure 7). If so, this would have been an alternative explanation for the decreased contractions seen. However, the DP_1 receptor agonist did not change histamine or LTD_4-induced contractions, nor did it induce a relaxation of pre-contracted tracheal segments.

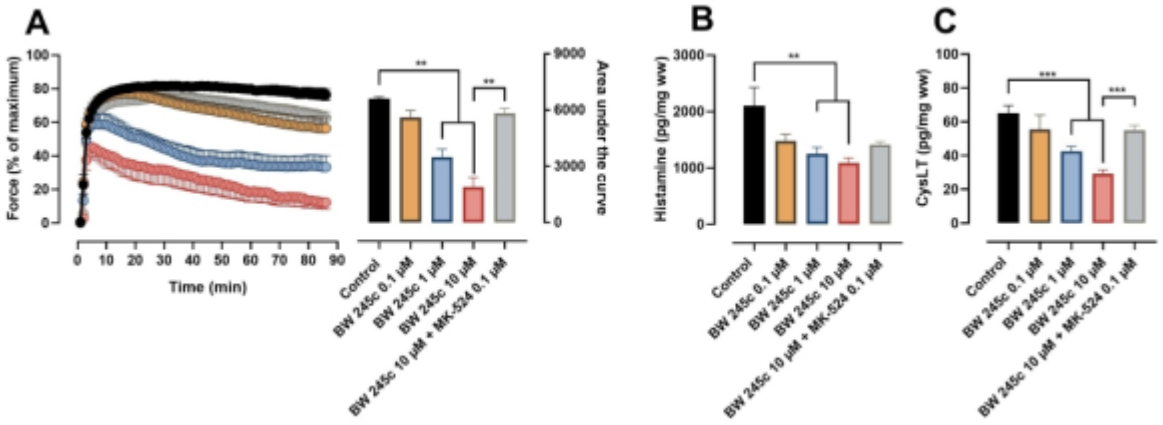

Fig. 19. Concentration-response curve and traces of OVA responses with simultaneous release of histamine and cysteinyl leukotrienes in presence of indomethacin (3 µM) and MK-524 (0.1 µM). A, OVA-induced contraction. B, OVA bolus induced contraction. C, histamine release after 15 min. D, cysteinyl leukotriene release after 90 min. Data represent mean ± SEM with n = 5-6

5.3.2 Summary

Despite the over 40-year-old knowledge that unselective COX-inhibition augments lipid mediator release and bronchoconstriction induced by antigen (199, 200) and that certain asthma patients experience increased bronchial constriction after NSAID usage (201), no mechanism underlying these observations has been proven without reasonable doubt. Though increased availability (shunting) of arachidonic acid from the COX-pathway to the 5-LOX-pathway has often been proposed, the experiments presented here now shown that this actually does not seem to be the case. PGD_2 is formed by the COX-1 enzyme in OVA-sensitised guinea pigs and released after OVA-exposure of isolated tracheal segments. This endogenously released PGD_2 acted through the DP_1 receptor, resulting in a decreased release of histamine and leukotrienes and ultimately reduced OVA-induced contractions. This effect could be replicated by the exogenous administration of PGD_2 to the organ-bath set up. PGD_2 had no effect on histamine or LTD_4 mediated contractions nor did it induce relaxation.

This thus offers evidence for a PGD_2-driven negative feedback loop present in mast cells when stimulated by antigens, similar to the inhibitory effect of PGE_2 on human mast cell-dependent bronchoconstriction, mediated by the EP_2 receptor (summarised in figure 20). Further investigation is warranted to examine if the effect of PGD_2 also is present in human mast cells. The dampening effect of PGD_2 might be somewhat surprising since PGD_2 is considered a pro-inflammatory mediator (202, 203). However, this proves that anti-inflammatory functions should not only be expected within the SPM group of lipid mediators, but within the prostanoid and even the leukotriene group as well (204). Moreover, it should be kept in mind that possible detrimental effects can occur when blocking the production of a whole group of lipid mediators, as is done with NSAIDs blocking prostanoid formation.

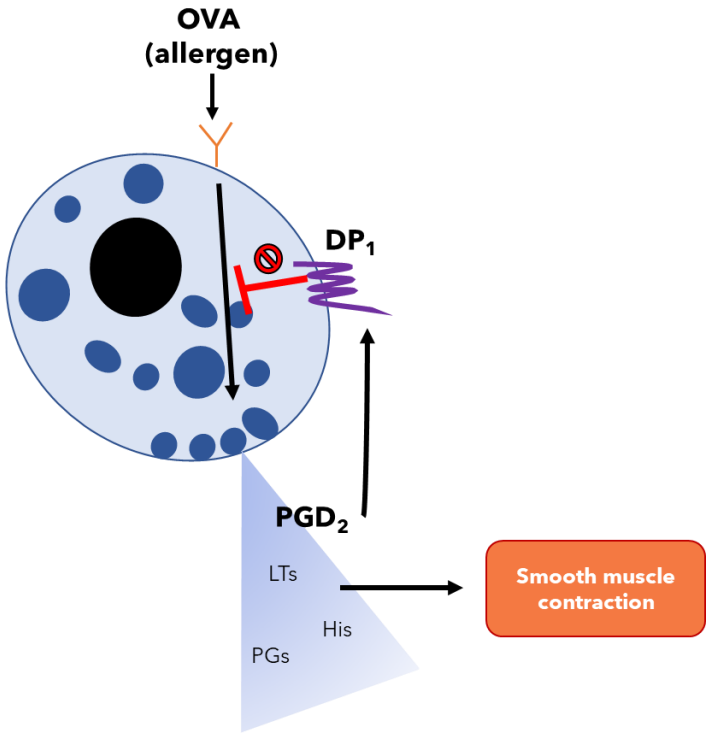

Fig. 20. Schematic summary of the main findings found in **paper III**. OVA causes mast cell activation in mast cells from OVA-sensitised guinea pigs. Mediators are released upon activation and induce contraction. However, PGD_2 also decreased further release of contractile mediators from mast cells through DP_1 agonism. This ultimately dampens OVA-induced contractions, thus making PGD_2 serve as a negative autocrine signal for guinea pig mast cells.

5.4 ROLE OF MAST CELLS AND MAST-CELL DERIVED 5-HT IN AIRWAY HYPERRESPONSIVENESS (PAPER II)

Mast cells are known effector cells for allergen-induced bronchial constriction. In **paper III** of this thesis, it has been described that lipid mediators mediate important functions in airway constriction by mast cells. However, not only lipid mediators mediate contractile and anti- or pro-inflammatory responses, but also other substances such as 5-HT have inflammatory and pro-contractile functions (23, 205-207). 5-HT causes contraction of isolated mouse tracheal segments, as also observed in paper I and IV. Histamine however does not induce contraction in mice airways, as opposed to in human or guinea pig airways (26, 198).

It is known that HDM-sensitisation and challenge causes mast cells to migrate to areas in the airway close to the epithelium and smooth muscle (208). Nevertheless, the functional implications of this migration are not clear and often investigated with usage of knock-out mice having lost *c-kit* expression, which causes mast cell depletion (208). Even so, this effect is not always mast cell specific, making it possible that the phenotypes observed are not attributable solely to the loss of mast cells (209). This investigation therefore served to dissect out mast cell mediated effects on airway hypercontractility, using another mast cell depleted mouse strain: $Cpa3^{Cre/+}$. These mice express Cre-recombinase instead of mast cell carboxypeptidase A3 (Cpa3), which more specifically initiates mast cell death.

5.4.1 Consequences of different genetical modifications of mast cells on airway hyperreactivity

HDM was administered every third day for 18 days to induce airway inflammation and hyperreactivity in mice (paper II, figure 1A). Repeated administration of HDM could induce a 10-fold increase in potency of carbachol, without effecting the E_{max} (figure 21A). In addition, there was no difference in carbachol-induced contractions between mast cell depleted ($Cpa^{Cre/+}$)

and non-depleted ($Cpa^{+/+}$) mice. Differences could be observed when looking at 5-HT-mediated contractions (figure 21B). First, as expected, HDM-exposure induced an airway hyperreactivity seen as increased airway contractions in tracheal segments from both mast cell depleted and non-depleted mice.

Second, segments from mast cell-depleted mice displayed an increased potency of 5-HT in mediating contractions, compared to those from non-depleted mice. Though at first glance this effect might be unexpected, an explanation could be that a constant release of 5-HT from mast cells causes a level of tachyphylaxis of the $5\text{-}HT_{2a}$ receptor, which is not happening in airways from mast cell depleted mice.

To demonstrate that HDM causes activation of mast cells, which in their turn release contractile substances like 5-HT and acetylcholine, tracheal segments were exposed to a bolus HDM (figure 21C). The absence of mast cells completely inhibited the HDM-induced contraction. Moreover, mice lacking functional Fc receptors due to loss of the γ-subunit ($Fcer1g^{-/-}$) were used to prove that HDM-induced contractions were due to mast cells activation by cross-linking of IgE receptors (Fc receptors). Indeed, loss of the Fc-receptor also resulted in a complete loss of HDM-induced contractions (figure 21D).

It was shown that loss of the major form of mast cell tryptase ($Mcpt6^{-/-}$ mice) does not influence the constriction induced by HDM (figure 21E). The question left then, was which mediator actually caused the contractions seen. Therefore, segments were incubated with the $5\text{-}HT_{2a}$ receptor antagonist ketanserin and the non-selective muscarinic receptor antagonist atropine (figure 21F). Both ketanserin as well as atropine almost completely inhibited HDM-induced contractions. This effect of both atropine and ketanserin has been found before (210, 211). It is still under debate which mediator, 5-HT or acetylcholine, actually activates mast cells and which one is released after antigen-exposure and caused airway constriction. It has been ascertained that mouse mast cells contain and release 5-HT (212). This released 5-HT has been hypothesised to activate airway epithelium, which would then release acetylcholine. This acetylcholine would then mediate the actual contraction (210). Nevertheless, usage of a murine OVA model also showed an epithelium-independent mechanism, in which 5-HT from mast cells caused release of acetylcholine, which again would contract the airways (211).

5.4.2 Mast cell activation by the M_3 receptor

The mechanisms of antigen-induced mast cell activation and airway contractions were dissected out in further experiments. These experiments resulted in four lines of evidence:

1. Precision cut lung slices (PLCS) showed a diminished contraction after HDM exposure in $Cpa^{Cre/+}$ mice (paper II, figure 4).
2. Mast cells synthesised 5-HT and released this upon HDM exposure, also in the lower airways (paper II, figure 4).
3. HDM-sensitised mice displayed methacholine mediated airway hyperresponsiveness, which was blocked by ketanserin and reduced after loss of mast cells (paper II, figure 5).
4. Mast cells expressed the M_3 receptor and release 5-HT upon methacholine stimulation (paper II, figure 6).

These data show that it is indeed possible that in an allergic inflammatory setting, antigen causes 5-HT-release from mast cells, which in turn initiates release of acetylcholine from parasympathetic nerve endings causing contraction of airway smooth muscle and further activation of mast cells. Still, another mechanism could also be at play. It could be that HDM-sensitisation and exposure causes a neuronal reflex leading to release of acetylcholine, which in turn stimulates 5-HT release from mast cells that then binds and activates the $5\text{-}HT_{2a}$ receptor present on the airway smooth muscle. The importance of neuronal tissue in inflammatory airway diseases and mast cell activation is known and affected by inflammation itself (213-

217). This would also be in line with data on cytokine-exposed isolated tracheal tissue from unsensitised mice, in which 5-HT was observed to directly contract smooth muscle, with only a minor component being secondary release of acetylcholine from nerve endings (62) (paper I, figure 6). Finally, other inflammatory cells could also be involved in the effects seen. Mast cells are not the only cells that can modulate airway responsiveness, as for example eosinophilic lung infiltration also increases 5-HT responsiveness, again through interactions with nerves (218).

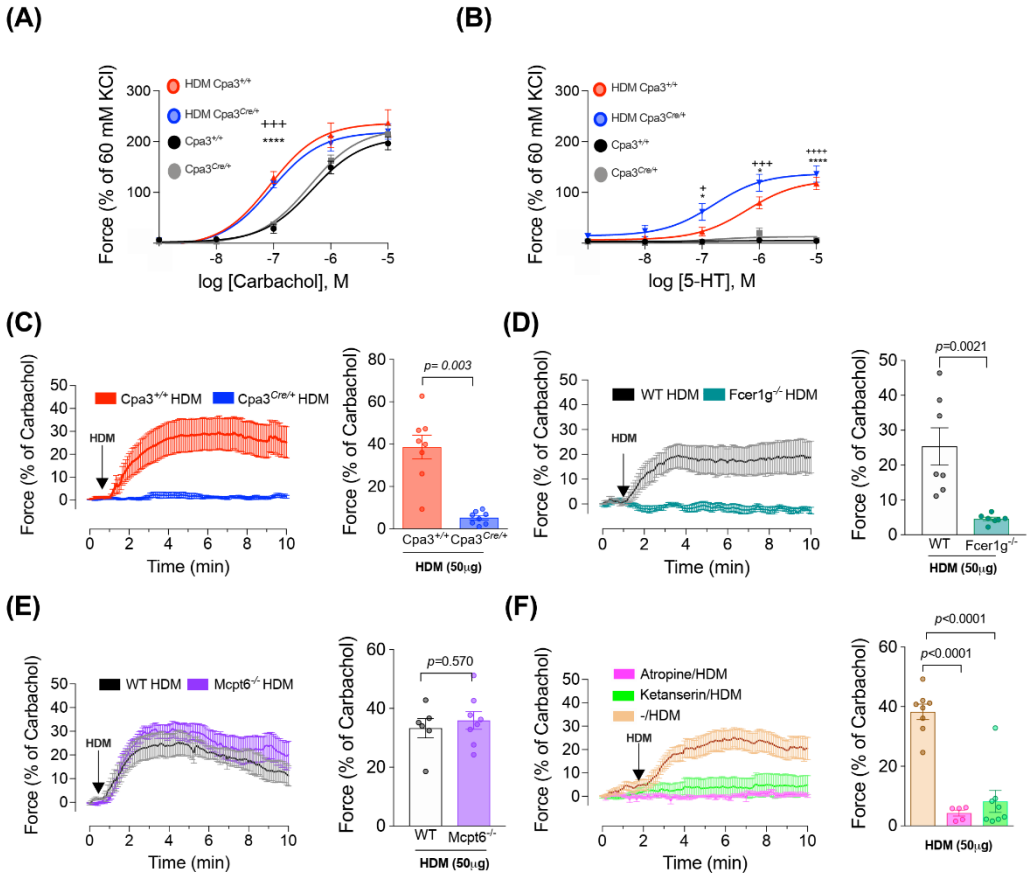

Fig. 21. Concentration-response curves, traces and E_{max} of HDM (50 µg) responses in different mouse mast cell KO models. A-B, carbachol and 5-HT in mast cell deficient ($Cpa3^{Cre/+}$) and mast cell non-deficient ($Cpa3^{+/+}$) mice. C, HDM in wildtype (WT) and $Cpa3^{Cre/+}$ mice. D, HDM in WT and Fc-receptor deficient ($Fcer1g^{-/-}$) mice. E, HDM in WT and major form of mast cell tryptase deficient ($Mcpt6^{-/-}$) mice. F, Effect of atropine and ketanserin in tracheal segments from HDM-sensitised, WT mice. Data represent mean ± SEM with n = 4-9

5.4.3 Summary

Mast cells in the lower and upper airways play an important role in antigen (HDM)-induced contractions. Their activation by HDM, which is a mixture of different enzymes, other proteins, LPS and more, results in airway smooth muscle contraction, but also enhances airway hyperresponsiveness. Frequent release of 5-HT might decrease 5-HT responsiveness of airway smooth muscle. Loss of mast cells and loss of their Fc receptors led to a complete inhibited response upon HDM exposure. Mast cells expressed the M_3 receptor and stimulation by acetylcholine and methacholine resulted in release of 5-HT and increased airway hyperresponsiveness. The released 5-HT from mast cells might release further acetylcholine from parasympathetic nerve endings but could also directly contract the airways. This is summarised in figure 22.

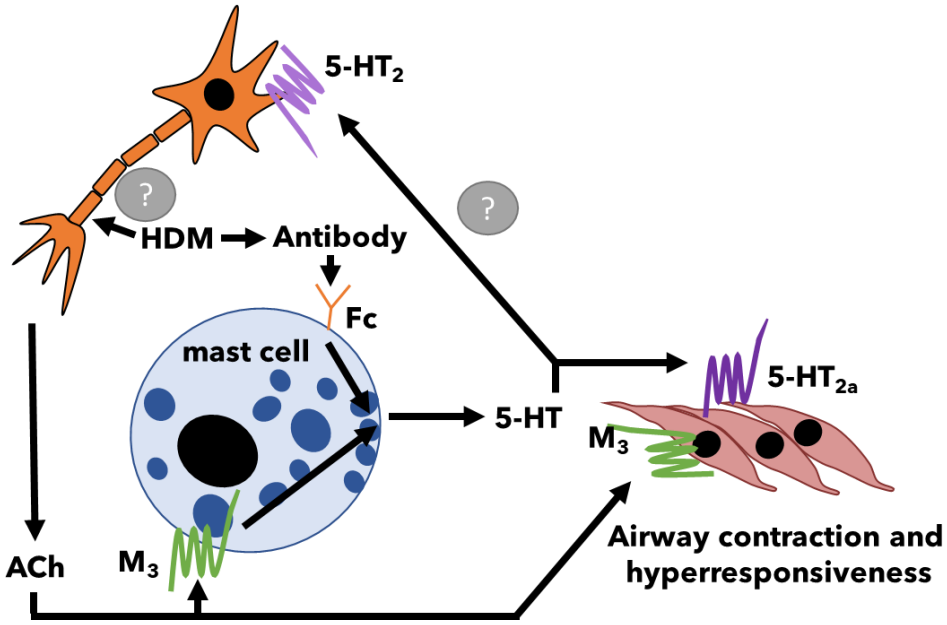

Fig. 22. Schematic summary of the main findings found in **paper II** and remaining questions. HDM causes airway contraction and hyperresponsiveness in HDM-sensitised mice. This contraction might be due to Fc receptor mediated release of 5-HT, which causes nerve-endings to release acetylcholine (Ach). This Ach might then contract the airway smooth muscle. Alternatively, HDM causes neuronal release of Ach, which activates mast cells to release 5-HT. 5-HT then contracts the airway smooth muscle.

6 GENERAL DISCUSSION

Two key pathophysiological processes that underpin many symptoms of asthma are increased contractility of the airways and airway inflammation. Though two distinct features, it is airway inflammation that can lead to the development of airway hyperreactivity (26, 36, 62, 198, 219). The beneficial effects of corticosteroids in the management of asthma symptoms also relate to their anti-inflammatory effects (220). Nevertheless, corticosteroid-resistant, airway hyperreactivity in severe asthma exists (26, 221). In addition, blockade or dampening of some pathways of inflammation, does not lead to complete resolution of airway inflammation.

Lipid mediators play important roles in mediating airway inflammation and modulation of the contractile state of the airways in asthma (222). They possess pro-inflammatory (76, 223), pro-contractile (86) as well as anti-inflammatory and pro-resolving functions (6). Therefore, these mediators constitute an attractive target for future treatment options to resolve ongoing inflammation in asthma and other chronic-inflammatory diseases. Main emphasis was put on the SPM group of lipid mediators and how they modulate airway contractility because of their anti-inflammatory and anti-hyperreactive potential. First the guinea pig was used, as it offers some advantages over mice in airway research due to e.g., similarities in receptors present in lung tissue, physiological control of airway contractility and pathophysiological mechanisms (224).

It was first investigated if SPMs would influence the contractile state of non-inflamed guinea pig airway tissue, having in mind the direct contractile effects of many lipid mediators from the AA-COX/LOX-pathways. Not many acute effects of selected SPMs were observed, except for the finding that maresin 1 potentiated sarafotoxin 6b- and endothelin 1-mediated constrictions in tracheal and aorta rings. Apart from this, the main conclusion was that either the guinea pig is not a good model for SPM and resolution biology in the airways, or SPMs are not directly involved in the contractile state of airway smooth muscle, at least not outside the inflammatory milieu. Which is not an all too surprising finding since SPMs are formed as part of the late inflammatory response (90), when a return to katabasis is needed again. Though this would not have excluded a direct relaxant effect in analogy to PGI_2, which is an anti-inflammatory lipid mediator as well (225).

However, given the essentially negative results in guinea pig and previous observations pointing at SPMs primarily being effective during inflammation, a more appropriate experimental setting was needed. Even so, without the widespread availability of guinea pig specific cytokines, investigations of SPM biology is difficult. Mouse-specific cytokines however are readily available, and several pro-inflammatory stimuli are prone to induce airway hyperreactivity (62, 212, 226, 227). HDM, TNFα and IL-13 all induced airway hyperreactivity that is observed as an increased contraction to 5-HT. Increased contractions of 5-HT are of interest as 5-HT is the mediator that is released from mouse mast cells during allergic inflammation (212). A role that is served by histamine and leukotrienes in human mast cells (23, 228).

Several SPMs were found to have an anti-hyperreactive activity in different models of airway hyperreactivity. Lipoxin activity was observed across HDM and TNFα-induced hyperreactivity, suggesting a mechanism in which lipoxins interfere with TNFα signalling (figure 13). This was further supported by the fact that part of the effect of HDM could be blocked by the TNFα inhibitor etanercept, suggesting TNFα release after HDM exposure. A mechanistic explanation would be that TNFα binding leads to upregulation of the $5\text{-}HT_{2a}$ receptor. Another would be a more general smooth muscle effect of lipoxins, such as upregulation of L-type Ca^{2+} channel upregulation. This that has been shown for CXCL8 and TNFα-induced airway hyperreactivity before (229). The anti-hyperreactive actions could be related to ALX/FPR2 signalling, as ALX-FPR2 agonists (lipoxins and RvD1) were effective in blocking HDM-induced hyperreactivity and an ALX-FPR2-antagonist partly blocked the anti-hyperreactive effect of LXA_4. A downstream mediator affected by LXA_4 signalling might

be NF-κB. This important effector protein complex of TNFα, has been found to be inhibited by LXA_4 before (174, 230, 231).

LXA_4 was not 100% effective in blocking hyperreactivity. This might have been due to the concentrations used, duration of effect of SPM vs TNFα, chemical stability or other unknown factors. Moreover, the LXA_4 function could also be specific to TNFα-NF-κB signalling in airway tissue. Furthermore, etanercept did not completely block airway-hyperreactivity after HDM exposure. HDM thus not only caused airway hyperreactivity through release of TNFα, but possibly also through release of other pro-contractile cytokines. This could be the type 2 cytokine IL-13. Airway hyperreactivity induced by IL-13 was found to actually be dampened by another group of SPMs: the cysteinyl maresins (figure 14). The cysteinyl maresin activity seemed to be restricted, in that they were not able to interfere with the pro-contractile effect of TNFα. This action of cysteinyl maresins was novel. Even more intriguing was the way the $CysLT_1$ receptor seemed to be involved, namely not through its orthostatic binding site.

Due to the shared cysteinyl moiety, research focus has been on the possible competitive antagonism of cysteinyl maresins for the $CysLT_1$ receptor (97, 145), However, our investigations showed that cysteinyl maresins could not shift the LTD_4 concentration-response curve in guinea pig tracheal tissue. Despite this, the anti-hyperreactive effect of cysteinyl maresins was inhibited by $CysLT_1$ receptor antagonists in mouse tracheal tissue. Others have found before that MCTR1 or MCTR3 could prevent LPS-induced lung or cardiac disfunction (146, 147, 232). Interestingly, one investigation pointed at cysteinyl maresin activity on another receptor: the ALX/FPR2 receptor with involvement of the airway epithelium (147). Though in our own experiments, epithelium removal had no effect on the anti-hyperreactive effect of MCTR3. A protective function of the $CysLT_1$ receptor has been found before. Here, $CysLT_1$ receptor knock-out mice showed an increased airway hyperresponsiveness in a model of irritant-induced asthma (204). Other experiments looking at $CysLT_1$ activation should further clarify if and how cysteinyl maresins bind to this receptor, possibly by biased agonism or via orthosteric modulation. It could be of interest as well to investigate if an ALX/FPR2 receptor antagonist is able to interfere with cysteinyl maresin signalling and if lipoxins can repeat the effect of cysteinyl maresins in this model.

Guinea pig tracheal tissue was used to clarify the mechanism, mediator and receptor responsible for increased OVA-induced airway constriction after unselective COX-inhibition (figure 20). Mast cell exposure to OVA-induced PGD_2 production by the COX-1 enzyme and subsequent release. PGD_2 then functioned as negative feedback signal to mast cell via the DP_1 receptor. DP_1 receptor activation of mast cells led to a decreased release of histamine and cysteinyl leukotrienes, resulting in decreased airway contraction. This mechanism was similar to the auto-inhibitory effect of PGE_2 on mast cells present in human lung tissue via the EP_2 receptor, which also resulted in decreased levels of histamine and leukotrienes and ultimately dampened airway constriction (74).

PGD_2 is well known for its pro-inflammatory role in type 2 inflammatory asthma, being associated with a decreased lung function, increased exhaled NO and lung eosinophilia (222), often through the DP_2 (CRTH2) receptor (76). An in-built anti-inflammatory function into an otherwise more pro-inflammatory mediator could serve as to prevent excessive inflammation and associated damage. This balancing act is thus seen in prostaglandin signalling, but LXA_4 could be considered in the same way. Most arachidonic acid metabolites possess predominantly pro-inflammatory traits, but simultaneous release of LXA_4 balances these, thus preventing excessive inflammation.

This also brings the attention to the usage of drugs like NSAIDs. Complete blockade of enzymes important for the production of mediators involved in many processes in the body, such as COX-enzymes, does not always go unpunished. In this case, there is a group of aspirin/NSAID intolerant asthmatics suffering from asthma worsening upon ingestion of these compounds, often referred to as aspirin-exacerbated respiratory disease (AERD) (233).

Moreover, NSAID intake leads to hospitalisations due to gastro-intestinal bleeding and respiratory symptoms (234) and other effects include increased risk of heart and renal failure (235, 236). Of course, efforts have been made to prevent this by usage of for example gastro-protective drugs like proton-pump inhibitors and the development of COX-2-specific inhibitors, but these have cardio-vascular side-effects as well (237).

Next to LOX enzymes, COX enzymes contribute to the formation of SPMs as well. In this case, COX inhibition could result in a decreased production of SPMs leading to the hampering of the resolution process (90). Result of this would be a continuation of inflammation into a more chronic form, leading to increased inflammation-associated damage. The development of stable SPM analogues or selective agonists of their receptors could therefore be a better treatment strategy, compared to NSAID-mediated blockade of pro-inflammatory pathways. SPM based drugs would then be used to induce resolution of chronic inflammation underlying inflammatory diseases. Other options are modulation of receptors. An example would be a combination of a DP_2-receptor antagonist to prevent allergen-induced airway inflammation plus a TP-receptor antagonist to prevent associated airway contractions (198). An inhaled, combined EP_2-EP_4 receptor agonist could be another method as to decrease mast cells activation and airway hyperreactivity in allergic airway disease (74).

The finding that mast cell depletion in genetically modified mice resulted in significantly reduced allergic airway inflammation and hyperreactivity, further substantiated the importance of mast cells in allergic inflammation (figure 22). However, the presence of mast cell during repeated intranasal administration of HDM was not necessary for an increased pEC_{50} of carbachol in isolated tracheal segments. Furthermore, mast cell presence resulted in a decreased 5-HT-mediated contractility in isolated segments, compared to those from mast cell depleted mice. This thus pointed at the existence of a possible counter-balancing mechanism leading to a decreased airway contraction, possibly being receptor tachyphylaxis.

Another main finding was the existence of the M_3-receptor on mast cells, as well as other immune cells like eosinophils and neutrophils. In mouse mast cells, M_3-activation would lead to release of 5-HT. This receptor was also found on human mast cells and led to Ca^{2+}-mobilisation. Though not done now, it would have been interesting to investigate if M_3 receptor stimulation would have resulted in the release of histamine and leukotrienes. If so, this would have pointed at a role of acetylcholine from nerve endings in activation of mast cells and airway contractions. It has been shown before that such an interaction between mast cells and nerves can indeed lead to inflammation and airway contraction (211, 214, 218). Nevertheless, vagal nerve ending are also implicated in the timely resolution of (airway) inflammation. Vagal nerve stimulation can activate certain cells involved in lung injury repair (238) or induce release of SPMs (239). What can be concluded is that mast cells are necessary for allergen-induced airway constriction and mast cell activation leads to aggravated airway hyperresponsiveness.

The general aim of this thesis was stated as: to investigate if selected SPMs have anti-hyperreactive properties, how COX-inhibition results in increased airway constriction and if mast cells are necessary for antigen-induced contraction and airway hyperreactivity. The investigations showed indeed that diverse lipid mediators had anti-hyperreactive activities in cytokine and antigen-induced airway hyperreactivity. Provided that these findings can be translated in humans as well, stable lipid mediator analogues or other receptor antagonists might offer future treatment strategies for inflammatory airway diseases such as asthma. In doing so, special focus should be placed on modulation of airway mast cell activity to further lessen airway inflammation and hypercontractility.

7 CONCLUSIONS

The main conclusions that can be distilled from this thesis are:

- The SPMs tested do not cause acute contraction or relaxation of guinea pig tracheal tissue. Nonetheless, maresin 1 and protectin DX might potentiate ET_a/ET_b-mediated contractions.

- LXA_4 has an anti-hyperreactive effect that dampens HDM and TNFα-induced airway hyperreactivity when given intranasally or during incubation of tracheal segments.

- Cysteinyl maresins are able to reduce steroid-resistant airway hyperreactivity induced by IL-13. This effect is possibly achieved through an allosteric binding site of the $CysLT_1$ receptor.

- Lipoxins, cysteinyl maresins and their receptors are potential new targets for the development of drugs modulating airway responsiveness.

- Prostaglandin D_2 released by guinea pig mast cells serves as an auto-inhibitory signal via the DP_1 receptor, reducing release of the contractile mediators histamine and cysteinyl leukotrienes and ultimately airway constriction.

- Lung mast cells are imperative for HDM-induced airway constriction and increase airway hyperresponsiveness possibly through mast cell-nerve interactions.

- The findings remain to be validated in humans, as animal models were used in these investigation.

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