Unravelling the Roles of $CRTH_2^+$ Cells in Asthma Pathogenesis



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Thesis submitted for the degree of

Doctor of Philosophy in Clinical Medicine,

University of Oxford, Hilary Term, 2023

Supervised by Professor Luzheng Xue, Professor Ian Pavord

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Acknowledgements

The studies in my thesis have been done under the supervision of Professor Luzheng Xue, Professor Ian Pavord, and Professor Paul Klenerman. The work of my three major projects was based on their initial scientific ideas or previous studies, and without that, I could not build up complete proposals for studies. Meanwhile, they have kindly mentored me to become an independent scientist during the DPhil project via project discussion, insight sharing and writing skill teaching. They have also supported the lab expenses, not to mention my scholarship, especially for a non-EU student with a much higher burden of living expenses. Mainly, I really appreciate Luzheng, who gave me the opportunity, the interview for this DPhil position after I wrote him an email in 2016. Since then, he has supervised me closely and firmly supported my career path. For example, when I mentioned I would like to pave the way to becoming a research scientist in the pharmaceutical industry, he provided me chances to be involved in the industrial collaboration with Novartis, which was later translated into two of my primary DPhil studies and guided me towards a real world of drug discovery and development.

I am grateful to all parties for funding my DPhil studies, including Novartis, Atopix Therapeutics and NIHR Oxford Biomedical Research Centre. In addition, Human Immune Discovery Initiative (HIDI) also kindly funded the single-cell sequencing in my eosinophil project.

Besides my supervisors, I appreciate the kind support and professional suggestions from the Transfer and Confirmation examiners of my DPhil project, Professor Graham Ogg and Professor Mona Bafadhel. I would like to especially thank my industrial collaborators in Novartis, Dr David Sandham, Dr Veit Erpenbeck, Dr Eric Shikatani and Dr Shamsah Kazani, for backing the data I generated and supports to international conferences. The delightful working experience with them further intrigued me to dedicate my expertise to developing therapeutics and curing diseases.

My current and past colleagues working in the lab offered much help and contributed to many of my published works. It was Dr Jian Luo and Dr Ryan Hoyle that accompanied me in the lab as well as most of my DPhil life in Oxford from 2016 till I left the UK. I am thankful that my students, Rowie Borst, Mohammad Mostafa Chizari, and Luisa Chocarro De Erauso, generated valuable data under my supervision and made an outstanding contribution to my thesis project. I have received other help from Simei Go, Paul Batty, Yuan Ye, Yanqiu Wu, Ni Zeng, Xuemei Liu, Shan Jiang, Jing Zhang, Qiong Huang, Timothy Powell and Jennifer Cane. I will never forget all days and nights we spent together, sitting in front of the bench, as well as those beautiful sunny afternoons when we did punting on River Cherwell.

I also owe a debt of gratitude to our clinic team: Dr Rahul Shrimanker and Dr Gareth Hynes, who coordinated clinical participants; the RMU clinical team members, Katie Borg, Clare Connolly, Christine Mwasuku, Anna Gittins, Karolina Krassowska, Jack Seymour, Laura Sanchez, Dr Rahul Shrimanker, Dr Sanjay Ramakrishnan, Dr Maisha Jabeen, Dr Simon Couillard, Dr Nayia Petousi, Dr Timothy Hinks who were involved in collecting clinical samples from participants; Samantha Thulborn, Mahdi Mahdi and Tilly Downs who process the biological samples. Indeed I am grateful for all participants who consent to our RMU cohort studies or donate blood to NHS.

I also cherish all favours from Dr Clare Hardman and Dr Li-Ying Chen, who contributed to experiment design and conduction, paper writing and proposal generation in my published papers, and all other group members in the group of Professor Graham Ogg for the discussion and suggestions. Meanwhile, I would like to thank Oxford Biomedical Data Science Training Programme, which guided me in programming, data processing and visualisation of results. I appreciate all the help from Dr Emanuele Marchi for coding and using R packages. Dr Helen Ferry and Liam Hardy supported sorting cells in the flow cytometry facility.

Beyond work, I have been fortunate to have personal support from a wide circle of friends from the UK and China. My dearest friend Jiandong always values what I have been doing during my whole study in the UK. Although we could not meet face to face often due to the long

distance between Oxford and Shanghai, he still spent countless nights chatting with me and sharing his life. Without his moral support, I could not climb out of rock bottom. Xuyang is the first person I knew in the UK to share the same interest in tea tasting, and she always reminds me to be optimistic and enjoy life while studying. Strolling around exhibitions and hunting for scrumptious food will be an unforgettable memory. I further thank all my friends, Jiahe, Yutian; Tianyu, Dian; James, Tilly, Rassin, Rowie; Qian, Zhu, Yun, Xiaopeng, David; Kedong; Beining, Ran; Zhuopei, Hanzhi; Andy, Yifei, and Xin.

Over and above all people, I would express my deep sense of gratitude to my parents, Jing and Shimeng, who always stand by me. I could only be myself today with your unconditional love and support for all my decision.

Covid-19 Impact Statement

The University of Oxford has released guidance to graduate students regarding the effects of the coronavirus pandemic on our studies. We have been advised to either apply for an extension or to submit a thesis on time containing details of how our work has been disrupted. I decided to extend the study and was supported by my supervisors.

In general, the outbreak of SARS-CoV-2 in March 2020 disrupted my work due to the closure of our laboratory and postponed patient visits between March 13th and August 7th, 2020. I have therefore had the chance to join a study of the COvid-19 Multi-omics Blood ATlas (COMBAT) Consortium. I participated in the study and contributed to the data analysis and figure generation of the cytokine profile in COVID patients using Luminex-based protein assays. The study published in Cell, Mar 2022, named A blood atlas of COVID-19 defines hallmarks of disease severity and specificity.

Regarding my thesis studies, access to the labs has been restricted until the beginning of 2021 in line with university and national guidance. This limited the lab space and blocked me from finishing the basophil study using basophils isolated from human blood in Chapter 4. Therefore, the unfinished experiment in Chapter 4 was only completed in 2021. Then, the repeating waves of the outbreak of SARS-CoV-2 raised my safety concerns, and consequently, I decided to move abroad to finish my data analysis and continue my thesis writing. Although I am a Chinese citizen, the most restricted regulation in the world and the expensive and often-cancelled fights made it difficult for me to return to my homeland. Hence, I have found a job in Germany. However, the high pressure in the company due to the lack of FTE (full-time equivalent) and COVID-infected employees instead increased my burden of the work and further delayed the thesis writing. Meanwhile, I have also been infected twice from 2021 to 2022. Therefore, although I have generated most of my data and finished the analysis before the confirmation in 2020, I could only finish the thesis writing at the end of 2022.

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Abstract

Prostaglandin D_2 (PGD_2) receptor 2 (as known as chemoattractant receptor homologous to the T helper 2 cell, $CRTH_2$) is expressed on various human immune cells including but not limited to type 2 helper T (Th2) cell, type 2 cytotoxic T (Tc2) cell, group 2 innate lymphoid cell (ILC2), eosinophil and basophil. These $CRTH_2^+$ cells are highly associated with type 2 (T2) immune response, the severity and the endotype of asthma. In this research, I demonstrated the importance of three types of $CRTH_2^+$ cells, Tc2 cells, basophils, and eosinophils, in inflammation and asthma. PGD_2 induced cell migration, adhesion and survival, evoked the production of proinflammatory cytokine and fibrotic factors, and mediated autocrine/paracrine production of PGD_2 in cultured human Tc2 cells. Using the $CRTH_2$-specific antagonist fevipiprant as a tool, I observed the inhibition of the aforementioned PGD_2-mediated activities *in vitro*, suggesting the anti-inflammatory and anti-fibrotic roles of $CRTH_2$ antagonism in asthma. In the second study, the numbers of basophils that highly express $CRTH_2$ were found to be significantly increased in blood and sputum from patients with severe eosinophilic asthma. Chemotaxis, interleukin (IL)-4/IL-13 production, granule histamine release and CD63/CD203c expression in basophils could be induced by PGD_2, which could be suppressed by $CRTH_2$ antagonism. The RNA sequencing revealed potential unique roles of the PGD_2-$CRTH_2$ pathway in basophils. In the third study, blood eosinophils from patients with eosinophilic asthma showed increased responses to the inducers of chemotaxis and degranulation *ex vivo* and *in vitro*, compared to those from non-eosinophilic asthma and healthy subjects. These enhanced responses were reversed after treatment with the anti-IL-5 monoclonal antibody mepolizumab. Mepolizumab also repressed the phosphorylation level of AKT, MERK and p38 in blood eosinophils, which was confirmed with single-cell RNA sequencing. These findings provided a better understanding of the roles of $CRTH_2^+$ cells in the pathogenic mechanisms of asthma.

Chapter 1. Introduction

1.1. Asthma

1.1.1. Summary

Asthma is a common chronic disease affecting over 300 million people worldwide (Soriano *et al.*, 2017). In the 2022 Report for asthma from the Global Initiative for Asthma (GINA), asthma is defined as the following (The Global Initiative for Asthma, 2022):

Asthma is a heterogeneous disease, usually characterised by chronic airway inflammation. It is defined by the history of respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough that vary over time and in intensity, together with variable expiratory airflow limitation. (p. 20)

1.1.2. Pathology of asthma

The asthma symptoms are usually derived from a series of alterations in the airway, including airway hyperresponsiveness (AHR), mucosal swelling, mucus plugging and airway remodelling.

Airway hyperresponsiveness is a hallmark of asthma. It is defined as "the predisposition of the airways of patients to narrow excessively in response to stimuli that would produce little or no effect in healthy subjects" (Chapman and Irvin, 2015, p. 706). The severity of asthma is, at least partially, related to the degree of AHR as measured by responses to histamine or methacholine challenges (Cockcroft *et al.*, 1977; Fowler *et al.*, 2000). Underlying mechanisms affecting AHR include inflammation, dysregulation of the neuronal system, and structural changes in the lung. Inflammation is apparently a fundamental and well-known cause of AHR as well as other signs of airflow limitation. The chronic and progressive inflammation in the airway will gradually result in mucosal swelling, increasing the secretion of mucus, and the formation of mucus plugs, further obstructing airflow (Hogg, 1982; Evans *et al.*, 2009; Fahy and Dickey, 2010). Chronic inflammation will further lead to structural changes named "airway remodelling" as the disease progresses. These include damage to the epithelium, thickening of the basement membrane, enlargement and proliferation of the smooth muscle cell, mucus metaplasia, increasing vascular structures, and fibrosis. (Beckett and Howarth, 2003). These changes make airflow

obstruction only partially or totally irreversible (Shifren *et al.*, 2012; Fehrenbach, Wagner and Wegmann, 2017). In some patients, airway remodelling is not a consequence of inflammation but a primary event, perhaps linked to an infection in children (Fehrenbach, Wagner and Wegmann, 2017).

Besides persistent airway obstruction in asthma, a sudden constriction of the muscles in the lung (an exacerbation) also leads to airway narrowing and airflow limitation. As defined by Dunican and Fahy (2015), exacerbations present as "acute or subacute episodes of progressively increasing shortness of breath, cough, wheezing or chest tightness, and decreasing airflow". In acute exacerbations, the contraction of bronchial smooth muscle is induced by a range of factors, such as allergens or irritants. Allergen-induced exacerbations are often mediated by mast cell activation. Upon recognising allergens, immunoglobulin E (IgE) crosslinks with high-affinity IgE receptors (FcεRI) on the surface of mast cells, leading to the release of various substances such as histamine, tryptase, leukotrienes, prostaglandins, and cytokines, which directly or indirectly contract airway smooth muscle cells (Barnes, 2011). Potentially, basophils may also contribute to the exacerbations following the exact mechanism, as they also highly express IgE receptor FcεRI (Ono *et al.*, 2010; Salter *et al.*, 2015, 2016). Nonetheless, exacerbations may also occur due to nonsteroidal anti-inflammatory drugs (e.g. aspirin) (Spector, Wangaard and Farr, 1979; Jenkins, Costello and Hodge, 2004), infection with respiratory viruses (Yamaya, 2012), or exercise, cold air, irritants, and stress (Anderson and Kippelen, 2008; Weiss and Rundell, 2011; Marshall, 2019).

1.1.3. Management of asthma

Asthma treatments are divided into three categories: the long-term controller that is applied to minimise the persistent symptoms, the quick reliever that is used for treating breakthrough symptoms and exacerbations, and the add-on therapy that is considered for severe asthma (The Global Initiative for Asthma, 2022).

Corticosteroids, a class of steroid hormones, function as anti-inflammatory therapeutics. They are widely used in asthma to control symptoms and prevent exacerbations (Rhen and Cidlowski, 2005; Barnes, 2010). There are two ways of administration of corticosteroids in asthma: inhaled

corticosteroids (ICS) and oral corticosteroids (OCS). ICS is the main component of long-term controllers, while OCS is used to rapidly inhibit the symptoms on short courses or for severe resistant asthma (Czock *et al.*, 2005; Barnes, 2010).

Adrenergic β_2 receptor agonists are another type of first-line drugs in asthma, which work as bronchodilators resulting in smooth muscle relaxation. There are two forms of β_2 agonists: short-acting β_2 agonists (SABAs), including albuterol, levalbuterol, and pirbuterol, providing relief from acute bronchospasm, and long-acting β_2 agonists (LABAs) including salmeterol and formoterol for long-term controllers. In addition, long-acting muscarinic antagonists (LAMAs), for example, tiotropium, are also used in asthma as an add-on therapy (Abdelrahim *et al.*, 2021; The Global Initiative for Asthma, 2022).

Other medications, including immunomodulators and macrolide antibiotics, are also considered add-on therapies depending on the phenotype of severe asthma (The Global Initiative for Asthma, 2022). The details of these treatments were discussed in Chapter 1.3. as well.

To acquire better control over the asthma process, GINA has proposed a stepwise management strategy for patients with asthma aged over 12 years old in clinical practice (The Global Initiative for Asthma, 2022). First, adjustment of treatment will be made based on assessment and review of the response, varying from step 1 low dose ICS with or without LABA to step 4 medium-to-high dose ICS/LABA, up to step 5 with add-on LAMA or other medications, with SABAs and/or OCS as the relievers (The Global Initiative for Asthma, 2022).

1.1.4. Classification of asthma

1.1.4.1. Severity

In asthma, there is no clear marker of disease severity. Inflammation can be present even in patients with mild symptoms. Despite the suppression of inflammation, symptoms and exacerbations still exist in some patients with severe asthma (Wilson *et al.*, 2016). At present, the level of treatment required to manage symptoms and exacerbations is used to determine the severity of asthma. However, such an assessment can only occur after a patient has received treatment for several months (The Global

Initiative for Asthma, 2022). It is important to note that asthma severity is not fixed and may fluctuate over time depending on the doses of treatment.

According to the GINA definition (The Global Initiative for Asthma, 2022), asthma could be classified into three groups based on severity:

- **Mild asthma** is currently defined as asthma that is well controlled with Step 1 or Step 2 treatment;
- **Moderate asthma** is asthma that is well controlled with Step 3 or Step 4 treatment (e.g. low or medium dose ICS/LABA):
- **Severe asthma** is asthma that requires a high dose of ICS/LABA to control the symptom or that even remains uncontrolled (Chung *et al.*, 2014).

1.1.4.2. Phenotypes and endotypes

Asthma is a heterogeneous syndrome. The observable characteristics (phenotypes) of asthma and their underlying mechanisms (endotypes) are complex (Wenzel, 2012). Early attempts to phenotype asthma focused on the division of allergic (extrinsic) vs nonallergic (intrinsic) asthma (Rackemann and Mallory, 1941; Corren, 2013). One notable characteristic of allergic asthma is the presence of elevated levels of allergy-specific IgE in blood and positive results of a skin prick test (Wenzel, 2012).

Current phenotyping strategies aim to link the observable phenotypes with the underlying endotypes in asthma. The endotypes are characterisations of the cellular and molecular processes of pathophysiology (Kuruvilla, Lee and Lee, 2019). This approach provides a more accurate representation of the diverse nature of asthma. In addition, the clinical administration of many target-specific treatments would rely on the endotypes or corresponding biomarkers related to the target (Shaw *et al.*, 2015; Loza *et al.*, 2016).

One of the initiations of asthma endotyping was based on the knowledge of type 2 helper T cells (Th2) and associated type 2 (T2) immunity. Previous studies (Wenzel *et al.*, 1999; Simpson *et al.*, 2006) discovered that asthma could be divided into two main inflammatory subtypes: eosinophilic and non-eosinophilic, according to the eosinophil percentage in the airway. It became increasingly evident that

eosinophilia is mainly mediated by Th2 cells (Fahy, 2015) and associated type 2 inflammation. Due to the strong association between the endotype T2 immunity and the phenotype eosinophilia, asthma was classified into: T2-high (eosinophilic) and T2-low (non-eosinophilic). It has been aware that a large group of patients with non-eosinophilic asthma do not respond to even high-dose systemic corticosteroids, whilst eosinophilic asthmatic patients are usually well responsive to corticosteroid (Hynes and Pavord, 2020). It led to the finding that T2 inflammation is suppressed by steroids (Rhen and Cidlowski, 2005). For the last two decades, researchers have been striving to investigate T2-high asthma to identify targets that could inhibit T2 inflammation. The objective is to offer comparable benefits of steroids to patients with eosinophilic asthma but without the associated adverse events related to steroid treatment (McDonough, Curtis and Saag, 2008; Fahy, 2015). This critical insight leads to the successful clinical development of biologics targeting T2 inflammation. To further understand pathogenic mechanisms underlying the T2-high endotype, I studied the immunology of T2-high (eosinophilic) asthma and dissected the functions of immune cells which participate in the T2 inflammation in severe asthma.

1.2. Type 2 inflammation in asthma

Type 2 (T2) inflammation is induced by the excess production of type 2 cytokines, including interleukin (IL)-4, -5 and -13 and mediated by Th2, type 2 cytotoxic T cells (Tc2), group 2 innate lymphoid cells (ILC2), eosinophils, basophils, mast cells and B cells (Oliphant, Barlow and Mckenzie, 2011; Wynn, 2015). Type 2 cytokines (Walker *et al.*, 1992; Motojima *et al.*, 1993; Zangrilli *et al.*, 1995) and type 2 cells, including Th2 (Palikhe *et al.*, 2016), Tc2 (Hilvering *et al.*, 2018), ILC2 (Bartemes *et al.*, 2014; Nagakumar *et al.*, 2016), eosinophils (Fahy, 2015) and basophils (Brooks *et al.*, 2017) are enriched in bronchoalveolar lavage (BAL), sputum or blood from asthma.

IL-4 is crucial for the differentiation of naïve T (T0) cells to T2 cells, which switches antibody isotypes to IgE in B cells. IL-4 and IL-13 also activate epithelial cells from the skin, lung, or gut (Zhu *et al.*, 1999). Activated epithelial cells produce alarmin cytokines, including IL-25, IL-33, and thymic stromal lymphopoietin (TSLP). Afterwards, those cytokines further provoke Th2, Tc2 and ILC2s to produce type 2 cytokines (Xue *et al.*, 2005; Salimi *et al.*, 2017; Hilvering *et al.*, 2018).

IL-5, instead, is responsible for the maturation, differentiation, recruitment, degranulation and activation of eosinophils (Stein *et al.*, 2008; Kouro and Takatsu, 2009; Dougan, Dranoff and Dougan, 2019). Degranulation of eosinophils results in the rapid release of four principal cytotoxic proteins, namely major basic protein (MBP), eosinophil peroxidase (EPX), eosinophil cationic protein (ECP), and eosinophil-derived neurotoxin (EDN), from the cytoplasmic granules of eosinophils. This eosinophil biological process constitutes the core of type 2 inflammation (Kay, Phipps and Robinson, 2004; Amin, Janson and Bystrom, 2016; George and Brightling, 2016; Pavord *et al.*, 2018). In addition, eosinophil-produced cytokines, lipid mediators and oxygen radicals contribute to type 2 inflammation as well. These factors derived from eosinophils lead to the recruitment of type 2 T and dendritic cells (DCs), damage to epithelium and endothelium, and the induction of fibrosis. These effects ultimately result in airway hyperactivity and airway remodelling. (Tanaka *et al.*, 2004; Nissim Ben Efraim and Levi-Schaffer, 2008; Brusselle, Maes and Bracke, 2013; Yasukawa *et al.*, 2013; Doran *et al.*, 2017). All of the effects mentioned above significantly impact both chronic asthma symptoms and acute exacerbations. Although basophils, another type of granulocyte, share some functions with eosinophils, their roles in type 2 inflammation have been less well studied.

1.2.1. Adaptive type 2 immunity in asthma

Type 2 adaptive immunity is traditionally initiated by basophils, mast cells and Th2 cells (Kubo, 2017). In allergic patients, antigen-presenting cells (APCs) like DCs recognise allergens and present antigens to T cell receptors (TCR) on T cells in lymph nodes. Naïve Th cells differentiate into Th2 cells, migrate into the inflammatory site and release type 2 cytokines when activated (Oliphant, Barlow and Mckenzie, 2011; Kubo, 2017). T cells further interact with B cells eventually leading to release antigen-specific IgE. The IgE crosslinks with FcεRI on mast cells and basophils, which causes cell activations and release of mediators (Ballesteros-Tato *et al.*, 2016). Mediators, including type 2 cytokines, histamine, serglycin, proteoglycans, proteases, and lipid mediators (platelet-activating factor, sphingolipids, prostaglandins, and leukotrienes) drive the recruitment and activation of eosinophils, basophils and T cells in the lung and amplify the inflammatory responses. Similar to eosinophil activation, basophil and mast cell activation after allergen exposure also evokes exacerbation via the construction of smooth

muscle (Schleimer *et al.*, 1985; Marone, Triggiani and De Paulis, 2005; Stone, Prussin and Metcalfe, 2010; Sawaguchi *et al.*, 2012).

It has been commonly accepted for a long time that type 2 cytokines are mainly produced by Th2 cells in type 2 immunity, but more recently, the research I have participated in also suggested that Tc2 cell is another important source of type 2 cytokines (Hilvering *et al.*, 2018). However, the functions of Tc2 cells in asthma have not been well studied compared to Th2, and it will be important to understand how Tc2 cells contribute to the pathogenesis of asthma, especially eosinophilic (T2-high) asthma.

1.2.2. Innate type 2 immunity in asthma

Because of the strong association of eosinophil numbers with type 2 inflammation, eosinophil counts in the blood or airway are used as a biomarker to reflect the feature of T2-high asthma (Pavord *et al.*, 2018). Eosinophil depletion has been confirmed as a valuable therapy for controlling T2-high asthma (Patterson, Borish and Kennedy, 2015). An interesting finding from the DREAM trial (Pavord *et al.*, 2012), which investigated eosinophil depletion therapy, was that half of the patients with severe eosinophilic asthma were not atopic. Therefore, adaptive immune response might not be the only endotype in the development of severe asthma. Especially for individuals with non-allergic asthma, further understanding of the contribution of innate immunity is required.

ILC2s are present at a low frequency in the blood and are enriched in the blood and sputum of patients with severe eosinophilic asthma (Bartemes *et al.*, 2014; Nagakumar *et al.*, 2016; Kim *et al.*, 2019). ILCs are key nonspecific innate immune effectors. Despite the lack of TCR, ILCs could mirror the functions of T cells bypassing APC interaction. Upon stimulation with the epithelial cytokines IL-25, IL-33 and TSLP, ILC2s produce high levels of type 2 cytokines such as IL-4, IL-5 and IL-13 (Spits *et al.*, 2013), which suggests ILCs as a potential source of type 2 cytokines, especially in non-allergic asthma.

Furthermore, PGD_2, a principal product of activated mast cells after IgE crosslinking (Lewis *et al.*, 1982), is detected in both eosinophilic and non-eosinophilic asthma (Hilvering *et al.*, 2018), suggesting an IgE-independent PGD_2 production. All type 2 inflammation related immune cells, including eosinophils (Hirai *et al.*, 2001), basophils (Hirai *et al.*, 2001), Th2 (Hirai *et al.*, 2001), Tc2 (Hilvering

et al., 2018) and ILC2s (Xue *et al.*, 2014) highly express the PGD_2 receptor 2 ($CRTH_2$ or DP2). Through $CRTH_2$, PGD_2 elicits many pro-inflammatory activities in type 2 cells, including type 2 cytokine production (Gervais *et al.*, 2001; Hirai *et al.*, 2001; Xue *et al.*, 2005, 2014; Hilvering *et al.*, 2018). Therefore, the PGD_2/$CRTH_2$ axis could play an important role in type 2 immunity in eosinophilic asthma. This thesis will further explore and discuss the functions of the PGD2/CRTH2 axis in Tc2 and basophils in severe eosinophilic asthma.

1.3. Targeted therapies for asthma

Based on the better understanding of the pathogenesis and immunology of asthma in the recent two decades, many new clinical approaches have been developed or are under development to control severe asthma. The growing understanding of type 2 inflammation has expedited the development of several therapeutics for T2-high asthma, targeting one of the specific components which contribute to type 2 inflammation, including cytokines and their receptors, lipids and their receptors and other proteins. Other attempts have been made to develop drugs for non-eosinophilic asthma.

1.3.1. IgE

Omalizumab (Roche and Novartis), a monoclonal antibody against human IgE, has been used in the treatment of asthma for two decades. Omalizumab neutralises IgE and inhibits DC, mast cell and basophil activation (Djukanović *et al.*, 2004; Massanari *et al.*, 2010). It effectively improved lung functions (Boulet *et al.*, 1997; Fahy *et al.*, 1997; Busse *et al.*, 2001), decreased sputum eosinophil counts (Milgrom *et al.*, 1999; Djukanović *et al.*, 2004) and reduced airway inflammation and exacerbation rates in allergic asthma (Solèr *et al.*, 2001; Djukanović *et al.*, 2004). However, due to the nature of IgE biology, patients with non-allergic asthma could barely benefit from this antibody.

1.3.2. IL-4Rα

IL-4 receptor α chain (IL-4Rα) is a subunit for both IL-4 and IL-13 receptors. Chapter 1.2 mentions that IL4 and IL-13 are vital elements in type 2 inflammation. Therefore, their common binding site, IL-4Rα, is an attractive drug target in T2-high asthma (Wenzel *et al.*, 2013; Castro *et al.*, 2018). Dupilumab

(Sanofi) is humanised monoclonal antibody against human IL-4Rα. The administration of dupilumab in patients with eosinophilic asthma has reduced asthma symptoms and exacerbation rates and improved lung functions (Wenzel *et al.*, 2013). The patients with high fractional exhaled nitric oxide (FeNO), a biomarker reflecting IL-4/13 -mediated epithelium activation, showed better responses (Barranco *et al.*, 2017).

1.3.3. IL-5

IL-5 is secreted by multiple cells, including Th2, Tc2, ILC2, and mast cells (Lorentz *et al.*, 2000; Xue *et al.*, 2005; Salimi *et al.*, 2017; Hilvering *et al.*, 2018). As we mentioned before, IL-5 is actively involved in multiple biological processes of eosinophils (Kouro and Takatsu, 2009) via binding to the IL-5 receptor, a heterodimer consisting of an IL-5-specific chain (IL-5Rα, CD125) and a common β chain (CD131, existing in receptors of IL-3, IL-5, and GM-CSF) (Broughton *et al.*, 2015). The level of IL-5 in the airway is highly associated with the eosinophil number and eosinophilic inflammation in asthma (Sur *et al.*, 1995). Because IL-5 effectively and specifically targets eosinophils and basophils, it is considered one of the key drivers in the pathogenesis of eosinophilic asthma.

Three antibodies targeting IL-5 or the IL-5 receptor have been approved for treating severe asthma. Mepolizumab (GlaxoSmithKline) and reslizumab (Teva Pharmaceutical Industries) are humanised monoclonal antibodies against human IL-5. Benralizumab (AstraZeneca) is a humanised antibody that binds to IL-5Rα directly. They all reduced the exacerbation rate and almost abolished blood eosinophil counts (Ortega *et al.*, 2016; FitzGerald *et al.*, 2018; Menzella *et al.*, 2019). In addition, FeNO and the level of serum IgE were not affected by these IL-5 therapies (Yancey *et al.*, 2017; Chipps *et al.*, 2018).

1.3.4. PGD_2 receptor – $CRTH_2$

PGD_2 is a metabolite through the arachidonic acid cascade, mainly produced by activated mast cells during an allergic response. There are two G protein-coupled PGD_2 receptors with distinct binding affinity and function: D prostanoid receptor (DP1) and $CRTH_2$. $CRTH_2$ is highly expressed in type 2 immune cells with a higher binding affinity to PGD_2 (Xue *et al.*, 2015; Salimi *et al.*, 2017; Hilvering *et al.*, 2018). By binding to $CRTH_2$, PGD_2 induces cell migration, degranulation, cytokine production, and

cell survival (Gervais *et al.*, 2001; Hirai *et al.*, 2001; Xue *et al.*, 2005, 2014; Hilvering *et al.*, 2018). Since $CRTH_2$ activation induced by PGD_2 is one of the causes of IL-4/5/13 secretion, it has been considered a potential therapeutic target to block excessive type 2 immune responses in T2-high asthma. A few $CRTH_2$ antagonists were under development when the thesis study started in 2017, including fevipiprant (Novartis), GB001 (Gossamer Bio) and timapiprant (Atopix Therapeutics). Although fevipiprant (Novartis) has been terminated due to unsatisfactory outcomes in their phase III clinical trials, in phase II trials in severe eosinophilic asthma, airway eosinophils level dropped (Barnes *et al.*, 2012; Shrimanker *et al.*, 2019; Asano *et al.*, 2020), exacerbation rates reduced (Asano *et al.*, 2020) and lung function improved (Barnes *et al.*, 2012; Berair *et al.*, 2015; Shrimanker *et al.*, 2019) in eosinophilic asthmatic patients treated with fevipiprant.

1.3.5. Epithelial alarmins

TSLP, IL-25 and IL-33 are cytokines predominantly produced by airway epithelial cells after stimulation and upregulated in the airways of patients with severe asthma (Ying *et al.*, 2008; Préfontaine *et al.*, 2009, 2010; Cheng *et al.*, 2014). They could also activate ILC2s, eosinophils, basophils and Th2 cells to secrete IL-4/5/13, leading to the excess of type 2 inflammation (Pecaric-Petkovic *et al.*, 2009; Murakami-Satsutani *et al.*, 2014; Salter *et al.*, 2015; Camelo *et al.*, 2017). Because the excess production of these alarmins leads to direct airway reactivities and upstream type 2 inflammation, blocking these alarmins may be more effective than inhibiting type 2 cytokines to control type 2 immunity. Results from phase II and III clinical trials showed promising effects of an anti-TSLP antibody (tezepelumab from AstraZeneca and Amgen) on the significant reduction in acute exacerbations, blood eosinophils and FeNO regardless of the phenotypes (Corren *et al.*, 2017; Menzies-Gow *et al.*, 2021). Anti-IL-33 antibodies, including REGN3500 (Sanofi) and etokimab (AnaptysBio), and anti-ST2 (receptor of IL-33) antibodies GSK3772847 (GlaxoSmithKline) and AMG282 (Roche) improved the loss of asthma control and the lung function, and decreased blood eosinophil counts and exacerbations (Porsbjerg *et al.*, 2020; Saikumar Jayalatha *et al.*, 2021). Although IL-25 appears to play a crucial role in asthma, no clinical studies of anti-IL-25 antibodies are in progress.

1.3.6. Other targets

Despite a high interest in developing antagonists of chemokine receptors to treat inflammatory airway diseases, neither eosinophil-targeted CCR3 nor neutrophil-targeted CXCR2 has demonstrated clinical success (Donnelly and Barnes, 2006). While drug discovery and development research for asthma has traditionally concentrated on targeting eosinophilic inflammation and type 2 immunity, other targets involve type 1 and type 17 inflammatory responses in non-eosinophilic asthma. A clinical trial has shown that infliximab (Janssen Biotech), an anti-TNF antibody, has the potential to reduce exacerbations in patients with moderate asthma. However, the study needed more power to observe a significant difference (Erin *et al.*, 2006). Brodalumab (AstraZeneca and Valeant) is an antibody against IL17Rα, a receptor of IL-17, and presented no clinical benefits in a study in patients with severe asthma (Busse *et al.*, 2013). CJM112 (Novartis), an anti-IL-17A monoclonal antibody, did not improve lung functions but significantly improved the life quality of patients with uncontrolled severe asthma (ClinicalTrials.gov identifier: NCT03299686).

1.3.7. Use of new therapies in asthma research

Above pharmacological and clinical practices suggest that many components in the type 2 immunity cascade could be used as drug targets to manage the immune response and airway inflammation in T2-high asthma. These drugs or potential drugs could also be used as valuable tools in the research to deconvolute the immune mechanisms underlying the disease.

Anti-IL-5 biologics result in a disease model close to an "eosinophil knockout" in humans. We could use this model to dissect the roles of eosinophils under physiological and pathological conditions by treating patients with those eosinophil-depleting medications. More remarkably, mepolizumab depleted around 80-90 per cent, but not all eosinophils in the blood, based on previous results in trials (Leckie *et al.*, 2000; Flood-Page *et al.*, 2007), so comparing the remaining eosinophils after treatment with the eosinophils before treatment would validate the functions of IL-5 on eosinophils *in vivo*. In this study, mepolizumab clinical treatment will be used to address my scientific questions.

The $CRTH_2$ antagonism has demonstrated some clinical benefits in eosinophilic asthma, suggesting that the $PGD_2/CRTH_2$ axis, at least, contributes to the pathogenesis of the disease. Although roles of the $PGD_2/CRTH_2$ axis in eosinophils (Carstensen *et al.*, 2019), Th2 cells (Sykes *et al.*, 2016) and ILC2s (Hardman *et al.*, 2019) have been reported, their roles in some other $CRTH_2^+$ immune cells such as Tc2 cells and basophils are still not fully understood. In this thesis, I will use fevipiprant as a tool compound to investigate the effects of the $CRTH_2$ pathway in basophils and Tc2 cells *in vitro* in severe eosinophilic asthma.

1.4. Aims

Severe eosinophilic asthma patients have the highest healthcare utilisation and mortality in asthma. Therefore, there is an unmet need for the effective and safe treatment of patients with severe symptoms despite optimised standard treatment. IgE, IL-4 and IL-5 targeted therapeutics may address this unmet need, but the high cost of biologics is still an enormous burden for the healthcare system. Therefore, further studies of the mechanism of severe eosinophilic asthma are crucial for developing alternative or even better drugs.

As mentioned above, the mechanism of T2-high eosinophilic asthma, especially atopic asthma, has been broadly studied. However, the innate immune response could play an important role in non-atopic asthma, which is common in severe eosinophilic asthma. There is increasing evidence of the impact of $CRTH_2^+$ Th2 and ILC2s on innate type 2 inflammation in severe asthma. However, the functions of other $CRTH_2^+$ type-2-immunity-associated cells, such as Tc2, basophils and eosinophils in severe eosinophilic asthma, are still poorly understood.

In this thesis, I will address the roles of Tc2, basophils and eosinophils in severe eosinophilic asthma. Firstly, properties of the PGD_2-$CRTH_2$ pathway in Tc2 cells and basophils will be studied in order to improve our understanding of the immunological functions of $CRTH_2$ in asthma, which will help us gain a comprehensive picture of the mechanism of action (MoA) of $CRTH_2$ antagonism and biology of Tc2 cells or basophils. In addition, the contribution of eosinophils in the pathogenesis of severe eosinophilic asthma will also be examined. Finally, using an anti-IL-5 antibody as a tool, I will further

study the importance of IL-5 in eosinophil biology and explore how anti-IL-5 therapy provides a clinical impact. In summary, the thesis aims to achieve a more comprehensive understanding of innate immunity in severe eosinophilic asthma by revealing novel functions of $CRTH_2^+$ immune cells and aims to contribute to further drug discovery.

Chapter 2. General Methodology

2.1. Study design and participants

Patients and healthy control subjects were recruited from John Radcliffe Hospital, Oxford. The definition of severity (mild-to-moderate or severe asthma) of asthmatic patients met the American Thoracic Society/European Respiratory Society definition of severity of asthma (Chung *et al.*, 2014). Patients with eosinophilia (eosinophilic asthma) were identified as those whose blood eosinophil counts were greater than > 3% in total leukocytes. Patients with OCS were defined as patients who had any doses of OCS in the past 7 days when recruited. The studies were approved by South Central-Oxford B Research Ethics Committee, UK (18/SC/0361). The peripheral blood samples were collected from patients and healthy controls, directly used in assays mentioned in Chapter 4.2.2 and Chapter 5.2.3 - 5.2.4, and further processed to isolate eosinophils for assays in Chapter 5.2.1 - 5.2.2 and 5.2.5.

All primary T cells used in Chapter 3 and primary basophils used in Chapter 4 were isolated from human CD Leucocyte Cones from National Blood Service, Oxford, UK. The T cells were isolated following the protocol in Chapter 3.2.1 and used for assays mentioned in Chapter 3.2.2 - 3.2.9. The basophils were isolated following the protocol in Chapter 4.2.3 and used for assays mentioned in Chapter 4.2.4 - 4.2.8.

Due to no access to animal models with lung inflammation in the group, I only conducted experiments using human samples. However, fresh human blood was widely used as a model *ex vivo* to project the functions of immune cells under physiological conditions. Purified or cultured primary cells were applied for the assays that could not dissect cell-specific signals from a complex multiple-cell-type environment or require a large number of pure cells.

2.2. Cellular assays

2.2.1. Cell activation

As mentioned in 2.1, cell activation was conducted *in vitro* or *ex vivo*. In this thesis, cellular morphological features combined with biochemical properties using the following biochemical assays

could evaluate the response of immune cells to various stimuli or conditions to understand cellular functions, signalling pathways, and potential therapeutic targets, including $CRTH_2$ and IL-5. I will discuss in detail the two following assay types.

2.2.2. Cell apoptosis

There are several different approaches to detecting cell apoptosis, such as caspase activity, signal pathway or nuclear fragmentation. Considering the limitation of cell numbers of human primary immune cells, in this thesis, I will use Annexin V staining in cells using flow cytometry to evaluate the extent of cellular apoptosis. As Annexin V binds to apoptosis-induced membrane-exposed phosphatidylserine (Reutelingsperger and Van Heerde, 1997), Annexin V could detect apoptotic cells.

2.2.3. Cell migration

Cell migration could be measured in different ways. They have different advantages and disadvantages. The most common and direct technique to measure cell migration is the chemotaxis assay, which requires a certain number of purified cells under a less physiological condition (e.g. *in vitro*). It is also incompatible with adherent cells, and a scratching assay would be an alternative. The cell shape change is a biomarker of cell migration and can be used with a small amount of fresh blood without cell isolation. To make the measurement close to a physiological condition, I conducted a cell shape change assay *ex vivo* in fresh whole blood first whenever possible, and then confirmed the results using a chemotaxis assay *in vitro*. I will further explain these three aforementioned approaches separately.

2.2.3.1. Shape change assay

The shape change assay is a flow-cytometry-based cell migration assay. Changes in forward scatter are correlated with observed shape change under the microscope (Cole *et al.*, 1995). Microscopic shape change has been shown to associate with granulocyte movement and chemotaxis (Yuli and Snyderman, 1984; Haston and Shields, 1985). Therefore, shape change assay could indicate the cellular responses to chemoattractants. In the thesis, I applied less than 0.1 ml whole blood instead of pure eosinophils per condition and ran hundreds of specimens with a high throughput adapter set in an LSRII flow cytometer.

Hence, the shape change assay is the primary assay determining the migratory function of eosinophils from patients with a limited amount of blood samples in the large cohort.

2.2.3.2. Chemotaxis

The chemotaxis assay was performed in all chapters to demonstrate the potency and efficacy of chemoattractants and antagonists blocking the chemoattractant receptors on suspension cells. Compared to flow-cytometry-based shape change assay with higher throughput, it returns a more accurate readout but requires a much higher number of purified cells. Therefore, chemotaxis was used to 1) confirm the migratory activities of cells from shape change assay using whole blood; 2) profile the chemoattractants and corresponding antagonists.

2.2.3.3. Scratching assay

Vertical movement in the well is rather difficult for adherent cells such as fibroblasts and epithelial cells. Although adherent cells could be made to suspension by trypsinisation, their responses to chemoattractants could be changed after immediate protease treatment. Therefore, chemotaxis and shape change are not applicable to coherent cells. Scratching assay could exhibit the horizontal migration of adherent cells towards the empty space by manually creating the scratch in the well.

2.3. Genomic analysis

2.3.1. Real-time quantitative polymerase chain reaction (qPCR)

qPCR is a cheap and convenient way to quantify the relative levels of gene transcripts, which was performed in all projects to detect the expression of intracellular mRNA. Primarily, qPCR in this thesis facilitates 1) alignment with the protein data; 2) detecting the signal of analytes with no protein assay available or well-validated; and 3) confirming the mRNA data achieved from RNA microarray or sequencing.

Methods: The total RNA of human cells are extracted using an RNeasy Mini kit (Qiagen). cDNA of all samples is prepared from the same amount of RNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qPCR is conducted using Master Mix and Probe (for IL4, IL5 and IL13,

Roche) or Fast SYBR Green Master Mix (For all other genes, Thermo Fischer) in a LightCycler 480 Real-Time PCR System. GAPDH is used as the reference gene. The mRNA level changes compared with untreated samples are calculated according to the manufacturer's instructions. Primers and probes used are listed in Appendix Table 2.1.

2.3.2. RNA sequencing (RNA-seq)

RNA-seq was performed in Chapter 4 and 5. It could broadly screen the whole transcriptome in a single assay and allow me to detect both known and novel functional genes regulated in a pathway. The capabilities of this high-throughput sequencing method provide insight into the underlying mechanism of cell biology formulated by differentially expressed genes, pathways, gene association and so on. In this thesis, RNA-seq was used to identify the functions of PGD_2 on human basophil biology and the mechanism of action of IL-5 blocking on human eosinophils. In the eosinophil project, due to the low number of eosinophils in the blood, particularly after treatment, single-cell RNA-seq (scRNA-seq), which requires a much lower cell number, was performed. Meanwhile, I aim to disclose the subgroups of eosinophils that might contribute to the pathogenesis of severe asthma.

The details of the method were presented in the corresponding chapters.

2.4. Protein analysis

Different protein assays will be performed based on the purpose of the experiments and the features of the samples.

2.4.1. Enzyme-linked immunosorbent assay (ELISA)

ELISA was applied to measure the released proteins. In particular, this is one of the most well-established assays for selected proteins (e.g. cytokines and chemokines) detection in either cell culture or biological fluid. The details of the method were presented in the corresponding chapters.

2.4.2. Luminex and Meso Scale Discovery (MSD)

Luminex and MSD are the two widely used multiplex platforms, which can measure a panel of proteins in a small volume of fluid samples in a single assay.. Luminex (R&D Systems) is more robust in multiplexing (up to 50 analytes per plate in Luminex versus 10 in MSD) but is less sensitive than the MSD V-plex assays (Meso Scale) on some proteins. Therefore, Luminex, in the thesis, was conducted to screen the biological components which might illustrate the trend of regulation in the cellular assays or fluid from patients. Since the MSD platform provides higher sensitivity and a broader range than Luminex and ELISA, it was applied to confirm those minor alterations of levels of components that Luminex could not capture. The details of the method were presented in the corresponding chapters.

2.4.3. Flow cytometry

Flow cytometry is a potent tool to measure proteins on the cell surface or in the cytoplasm of suspension cells, which was used in all subjects in the thesis (eosinophils, basophils and Tc2 cells). Notably, it is robust in measuring proteins at the single-cell level. Therefore, I conducted flow cytometry in order to 1) dissect certain desired types of cells via pan markers in tissues such as blood and sputum; 2) monitor the number of those desired cells in tissues from different groups (e.g. treatment, severity, etc.) of patients; 3) study the vital functional activities of cells via detection of pan markers, activation markers, receptors for chemokines or cytokines, intracellular cytokine, etc. *ex vivo* and *in vitro*; 4) detect the signalling of kinase phosphorylation; and 5) check the purity of isolated and cultured human cells. The details of the method were presented in the corresponding chapters.

2.4.4. Western blotting

Western blotting is a tool to identify a protein of interest in a complex mixture *in vitro*, which, particularly, is well-validated for phosphorylated protein measurement. In this thesis, western blotting was performed to capture the signal of protein phosphorylation in eosinophils. The details of the method were presented in Chapter 5.

2.5. Statistical analysis

436 Data are presented as means with SEM. Data were analysed using either the T test or one-way ANOVA,
437 followed by Tukey's test. Values of $p < 0.05$ were considered statistically significant.

Chapter 3. Novel Functions of $PGD_2/CRTH_2$ Axis in Type 2 Cytotoxic T Cells*

3.1. Introduction

Type 2 cytokine producing cells, such as Th2 cells and ILC2s, are well-known vital players in T2 high eosinophilic asthma (Dunican and Fahy, 2015; Fahy, 2015). However, compared to Th2 cells and ILC2s, the potential roles of Tc2 cells in the pathogenesis of eosinophilic asthma are less researched.

We have recently found that Tc2 cells are significantly enriched in the peripheral blood and airways of patients with severe eosinophilic asthma (Hilvering *et al.*, 2018). Our previous study has also revealed the corresponding increase of PGD_2 in the airways of patients in the same group. Similar to Th2 cells and ILC2s, Tc2 cells express $CRTH_2$ and respond to PGD_2. Furthermore, activation of $CRTH_2$ elicits pro-inflammatory reactions in Tc2 cells, including cell migration, type 2 cytokine production, and suppression of cell apoptosis *in vitro*. Considering all evidence above, Tc2 cells might play a key role in T2-high asthma, especially with severe disease. However, many potential functions of Tc2 cells in the pathogenesis of T2-high asthma remain unclear.

To confirm the contribution of Tc2 cells in T2 inflammation, I further investigated comprehensively the roles of $PGD_2/CRTH_2$ in Tc2 cell activations. I have used $CRTH_2$ antagonists, fevipiprant and TM30089, to dissect PGD_2-mediated Tc2 reactions. Fevipiprant is a selective and potent $CRTH_2$ antagonist that has clinical benefits for patients with severe eosinophilic asthma in phase 2 clinical trials (Gonem *et al.*, 2016), suggesting the inflammatory functions of $PGD_2/CRTH_2$ axis in the disease. Although two recent phase 3 fevipiprant clinical studies had not reached a consistent statistically significant reduction in asthma exacerbations, there were suggestive trends. In the same phase 2 clinical trial mentioned above, fevipiprant also reversed airway remodelling by improving epithelial integrity and reducing airway smooth muscle mass (Gonem *et al.*, 2016). As mentioned in Chapter 1, inflammation partially mediates airway remodelling (Shifren et al., 2012; Fehrenbach, Wagner and

* Some passages in sections, Chapter-specific materials and methods, Results and Discussion, have been quoted verbatim or paraphrased from my own written publication (Chen *et al.*, 2021)

Wegmann, 2017), suggesting that $CRTH_2$ activation contributes not only to T2 inflammation but also potentially to airway remodelling. However, the cellular and molecular mechanism underlying this is still unclear, and the roles of Tc2 cells in airway remodelling haven't been explored.

As mentioned in Chapter 1, the level of PGD_2 is not always correlated with the level of IgE in asthma patients, though allergen-induced mast cell activation after IgE crosslinking is considered the primary source of PGD_2. Multiple $CRTH_2$ antagonists consistently showed benefits in severe eosinophilic asthma, but about half of such patients are non-atopic with low IgE levels. It raised a question about where PGD_2 is generated in these IgE-low patients. Increasing evidence suggests that PGD_2 can also be produced by some FcεRI -low or -negative cells, such as DCs, macrophages, eosinophils, Th2 cells, and ILC2s, in an IgE-independent manner. It is unclear whether Tc2 cells also possess such capacity to produce PGD_2 and contribute the lipid mediator to the disease.

In this study, I have tried to confirm the pro-T2 inflammatory activities of Tc2 cells and explored previously unknown functions linked to airway remodelling and PGD_2 autocrine production induced by $CRTH_2$ activation using fevipiprant as an inhibitor. My findings support the importance of Tc2 cells in severe eosinophilic asthma and emphasise the roles of $CRTH_2$ in both features of asthma - inflammation and airway remodelling.

3.2. Chapter-specific materials and methods

3.2.1. Human $CD8^+$ $CRTH_2^+$ Tc2 cell preparation and treatment

Human Tc2 cells were isolated from fresh clinical blood samples collected in heparin-coated tubes or CD Leucocyte Cones (National Blood Service, Oxford, UK). First, peripheral blood mononuclear cells (PBMCs) were prepared by gradient with Lymphoprep (Stemcell Technology), and then $CD3^+CD8^+CD4^-CRTH_2^+$ cells were sorted into 96-well plates using a BD FACSAriaTM III sorter. Next, cells were amplified in culture for about a month with RPMI 1640 medium supplemented with 10% human serum, 250 IU/ml IL-2, 50ng/ml anti-CD3 antibody, and irradiated feeder PBMCs. The purity ($CD3^+CD8^+CD4^-CRTH_2^+$ cells > 90%) of the cells after expansion and their response to PGD_2 was confirmed with flow cytometry and chemotaxis before use.

For cytokine or other protein production assays, Tc2 cells were incubated with 100-200 nM PGD_2 in the presence or absence of various concentrations of fevipiprant (Novartis Pharma AG), BW245C, BW868C or TM30089 (Cayman) as indicated in the results for 4 hours at 37°C. The cell supernatants were harvested for ELISA or Luminex assays, and the cell pellets were stored at -80°C for quantitative real-time PCR (qRT-PCR or qPCR) and microarray.

For PGD_2 and LTE_4 assays, Tc2 cells were treated with 400 nM DK-PGD_2 or 5 µg/ml anti-CD3/CD28 antibodies for various lengths of time in the absence or presence of 1 µM fevipiprant, or after 1-hour pre-incubation with 10 µM diclofenac (SIGMA), 10 µM flurbiprofen (Abcam), or 1 µM hematopoietic prostaglandin D synthase (hPGDS) inhibitor I (Cayman) or 20-minute pre-incubation with 30 µM arachidonyl trifluoromethyl ketone (ATK, Cayman) or 10 µM U-73122 (Cayman). In addition, the cell supernatants were harvested for enzyme immunoassay assays, and the cell pellets were stored at -80°C for qPCR.

3.2.2. Fibroblast and Tc2 co-culture

Human fibroblast cell line MRC-5 cells (ATCC) were seeded in a 96-well plate at $1x10^3$ cell/well in a MEM medium (Gibco) supplemented with 10% FCS and cultured overnight. After a gentle wash with PBS, $1x10^4$ Tc2 cells were loaded on the top of fibroblasts in a final volume of 200 µl MEM containing

2% FCS in the presence or absence of 200 nM PGD_2 or/and 1 µM fevipiprant. As a negative control, 200 µl of the same media were added into fibroblast cultures without Tc2 cells. In some experiments, Tc2 cells were added into a 0.5 µm filter insert on the top of the wells containing fibroblasts. Otherwise, Tc2 conditioned media after treatment with 200 nM PGD_2 in the presence or absence of 1µM fevipiprant or media containing various concentrations of IL-4/13 were loaded instead of Tc2 cells. The IncuCyte ZOOM (Essen Bioscience) captured images of the cell cultures at different time points. The confluence of fibroblasts was quantified as the area of fibroblasts from images using the Trainable Weka Segmentation, a plugin in Fiji of ImageJ (Arganda-Carreras *et al.*, 2017). The supernatants of the MRC-5 cultures were harvested for ELISA tests and the cell pellets were collected for qPCR analysis.

3.2.3. Epithelial cell and Tc2 co-culture

Human epithelial cell lines A549 and BEAS-2B (ATCC) were seeded in a 96-well plate at $4x10^4$ cell/well in an F-12K medium (Gibco) supplemented with 10% FCS and cultured overnight. After a gentle wash with PBS, $1x10^4$ Tc2 cells were loaded on the top of epithelial cells in a final volume of 200 µl F-12K containing 2% FCS in the presence or absence of 200 nM PGD_2 or/and 1 µM fevipiprant. As a negative control, 200 µl of the same media were added into F-12K cultures without Tc2 cells.

For the wound healing assay (scratching assay), A549 and BEAS-2B cells (ATCC) were seeded in a 96-well plate at $1x10^5$ cell/well in an F-12K medium (Gibco) supplemented with 10% FCS and cultured until they reached 90% of confluence. After a gentle wash with PBS, a scratch was made using the WoundMaker tool (Essen Bioscience), followed by two more washes with the medium. $1x10^4$ Tc2 cells were loaded into wells in a final volume of 200 µl F-12K containing 2% FCS in the presence or absence of 200 nM PGD_2 or/and 1 µM fevipiprant.

Images of the cell cultures were captured at different time points by using the IncuCyte ZOOM (Essen Bioscience). The confluence of epithelial cells was quantified as the area of epithelial cells from images using the Trainable Weka Segmentation.

3.2.4. Chemotaxis

Tc2 cells were resuspended in RPMI 1640 media containing 10% human serum and then incubated with various concentrations of fevipiprant, BW245C, BW868C, TM830089 or medium for 1 hour at 37°C in the upper chamber (5μm pore size) of HTS Transwell 96 well permeable supports (Corning). The upper chamber was then placed into the lower chamber of the supports containing pre-warmed media supplemented with 100 nM PGD_2, 1 μM BW245C or medium for further 1-hour incubation. The cell migration to the lower chamber was quantified using an IncuCyte ZOOM.

3.2.5. Immunoassay

The levels of IL-4, IL-5, IL-13, TGF-β1 (Invitrogen) or collagen I α1 (Bio-Techne) in the supernatants after the cell treatments were assayed with ELISA kits according to the method mentioned in Chapter 2. The concentrations of PGD_2 and LTE_4 in the supernatants were measured with a PGD_2–MOX enzyme immunoassay kit and LTE_4 enzyme immunoassay kit (Cayman Chemicals) respectively according to the manufacturer's instructions. The results were measured in an EnVision Multilabel Plate Reader (PerkinElmer). The levels of IL-3/8, CCL4, FasL, Galectin-3, GM-CSF, M-CSF and TNFα in the supernatants after the cell treatments were measured with Human Magnetic Luminex Assay kits (Bio-Techne) following the manufacturer's instructions. The results were obtained with a Bio-Plex 200 System (Bio-Rad Laboratories).

3.2.6. Microarray

RNAs from cultured Tc2 cells treated with or without PGD_2 for 4 hours were extracted with an RNeasy Mini kit. Microarrays were conducted using an Illumina HumanHT-12v4 Expression Beadchip at the Transcriptomics Core Facility, The Jenner Institute, University of Oxford. Pre-processing data analysis was performed using R Bioconductor packages. Genes involved in tissue remodelling functions in Gene Ontology (GO term: 0060429, 0030198, 0048771, 0042060) which reached absolute log2 fold change > 1 and significance at a $p < 0.05$ were selected using Limma package (Ritchie *et al.*, 2015). Heatmap was generated using the pheatmap package (https://CRAN.R-project.org/package=pheatmap).

3.2.7. Apoptosis

Tc2 cells were incubated in RPMI 1640 medium in the presence or absence of 1 µM PGD_2 or/and 1 µM fevipiprant for 12 hours at 37°C. Cells treated with a standard culture medium containing human serum were used as a control. The cells were harvested and stained with Annexin V (BioLegend) in an Annexin V binding buffer (BioLegend) following the BioLegend instructions. Results were acquired with an LSRII flow cytometer (BD Biosciences).

3.2.8. Cell aggregation analysis

Cell aggregation was photographed using a Nikon Eclipse TS100 microscope. Images were analysed with the Trainable Weka Segmentation in ImageJ. Briefly, cell clumps were identified using the Otsu method of thresholding. The intensity and size of identified objects were measured. Results are reported as integrated intensity.

3.2.9. Flow cytometry

For the *ex vivo* cytokine production assays, PBMCs isolated from the fresh blood of the patients with severe eosinophilic asthma were incubated with 1 µM PGD_2, 1 µM fevipiprant or in combination for 6 hours at 37°C. Brefeldin A (5 µg/ml, BioLegend) was added 30 minutes after starting the stimulation. Then the cells were stained with an antibody panel against CD3, CD4 and CD8 with Zombie Aqua dye (BioLegend) followed by fixation with IC fixation buffer (Invitrogen) overnight at 4°C. The cells were treated with permeabilization buffer (Invitrogen) followed by intracellular staining with anti-human IL-13 and IL-5 (BioLegend) antibodies. $CD3^+CD4^-CD8^+IL\text{-}5/13^+$ cells were detected with the flow cytometer LSRII.

For hPGDS staining, fresh blood was labelled with the antibody panel against CD3, CD4 and CD8 with Zombie Aqua dye followed by red blood cell lysis with a FACS Lysing Solution (BD Biosciences) and cell permeabilisation with permeabilisation buffer and then intracellularly stained with antibody to hPGDS (kind gift from Advanced Technology and Development, BML, Inc., Japan).

For cyclooxygenase (COX) staining in cultured Tc2 cells, the cells were treated with 400 nM DK-PGD_2 or 5 µg/ml anti-CD3/CD28 antibodies in the absence or presence of 1 µM fevipiprant for 4 hours and

then fixed and permeabilised using a FOXP3 Fix/Perm Buffer (BioLegend) followed by intracellular staining with anti-human COX-1 and COX-2 antibodies (BD Biosciences).

For $CRTH_2$ endocytosis analysis, cultured Tc2 cells with or without pre-incubation with 10 μM flurbiprofen or 1 μM hPGDS inhibitor 1 for 1 hour were stimulated with 200 nM PGD_2 or 5 μg/ml anti-CD3/CD28 antibodies in the absence or presence of 1 μM fevipiprant for 4 hours and then stained with anti-$CRTH_2$ antibody together with Zombie Aqua.

3.3. Results

3.3.1. Inflammatory functions of Tc2 cells mediated by PGD_2/$CRTH_2$ axis

Since an elevated number of Tc2 cells are observed in the lung of severe eosinophilic asthma (Hilvering *et al.*, 2018), I first evaluated how PGD_2-$CRTH_2$ is involved in Tc2 enrichment in the airway by examining cell migration, adhesion, and survival.

In a chemotaxis assay, the strong Tc2 cell migration was induced by PGD_2 (100 nM) but not the DP1 agonist BW245C, which was inhibited by only $CRTH_2$ antagonists TM30089 and fevipiprant but not the DP1 antagonist BW868C (Fig 3.1A). In addition, dose-dependent inhibition of PGD_2-induced Tc2 migration was detected in fevipiprant-treated cells (IC50=3.5±3.6 nM).

I then investigated the effect of $CRTH_2$ on cell survival. Serum privation in cell culture increased apoptosis of the Tc2 cells. Apoptosis was rescued by PGD_2 (1 μM), which was reversed by 1 μM fevipiprant (Fig 3.1B).

The aggregation of cells is a feature of cell adhesion during *in vitro* culture. Tc2 cell aggregation appeared within an hour and lasted 2-4 hours after PGD_2 (200 nM) treatment (Fig 3.1C). It was suppressed by fevipiprant in a dose-dependent way (Fig 3.1C,D). ICAM and PECAM, two adhesion molecules, were upregulated by PGD_2 stimulation and repressed by fevipiprant (Fig 3.1E). Neutralisation of ICAM and PECAM with specific antibodies significantly reduced the cell aggregation induced by PGD_2 (Fig 3.1F,G), suggesting aggregation was profoundly facilitated by ICAM and PECAM.

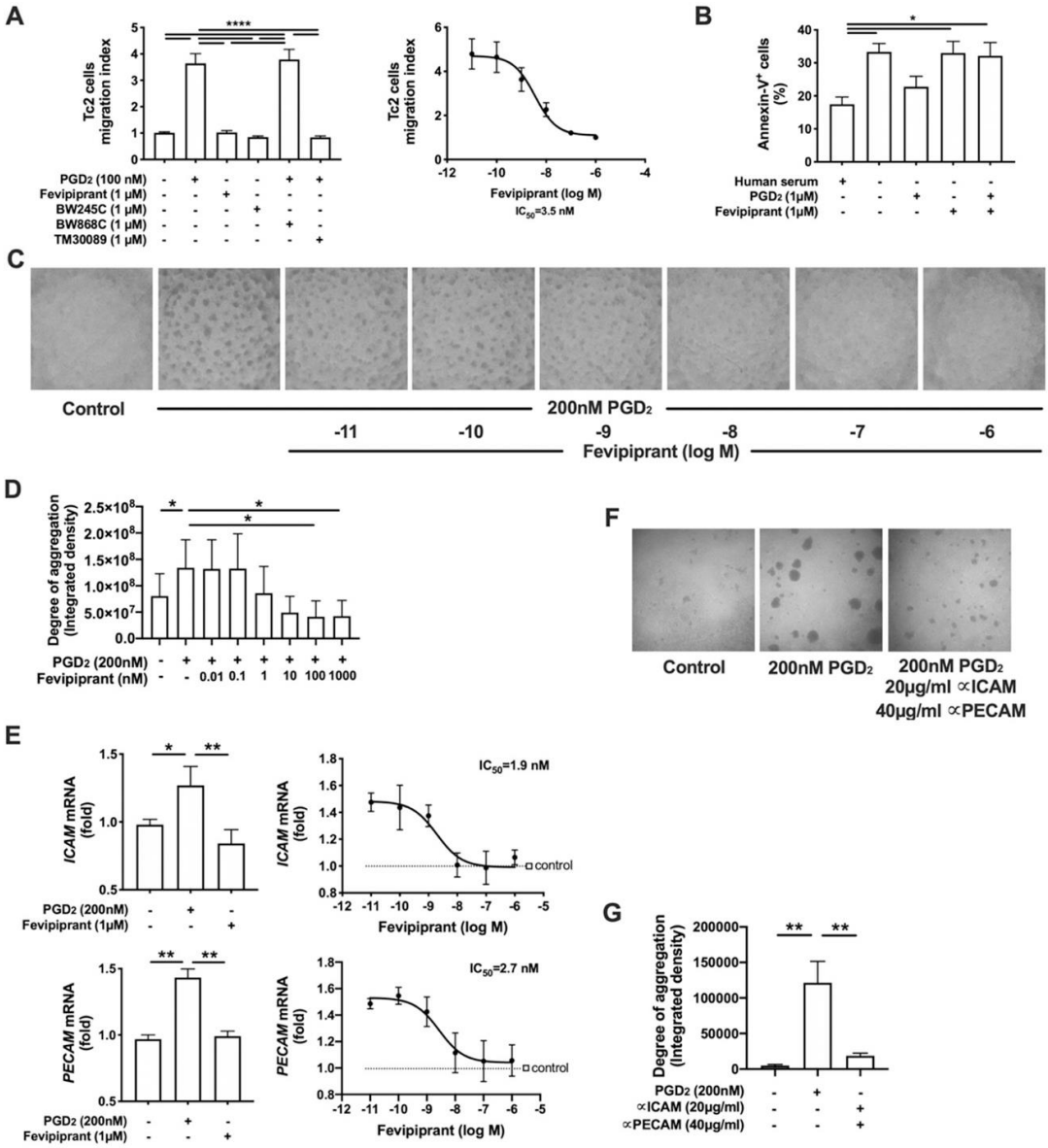


Fig 3.1 PGD_2-induced cell migration, survival, and aggregation in cultured Tc2 cells were inhibited by fevipiprant.
A. Tc2 cell migration to 100 nM PGD_2 in the presence or absence of 1 μM BW245C (DP1 agonist), 1 μM BW868C (DP1 antagonist), 1 μM TM30089 ($CRTH_2$ antagonist) or various concentrations of fevipiprant determined by chemotaxis assay. The data are presented as the migration index of luminescence intensity divided by the untreated samples. B. Annexin V^+ apoptotic Tc2 cells induced by human serum deprivation in the presence or absence of 1 μM PGD_2 and/or 1 μM fevipiprant detected with flow cytometry. C-D. Tc2 cell aggregation after incubation with 200 nM PGD_2 in the absence or presence of various concentrations of fevipiprant for 2 hours recorded under microscopy (C) and then quantified with ImageJ (D). E. mRNA levels of *ICAM* and *PECAM* after PGD_2 stimulation in the absence or presence of various concentrations of fevipiprant examined with qPCR. The data are presented as the fold change $([2^{-(Cq_{target\ gene} - Cq_{GAPDH})}]_{treated} \div [2^{-(Cq_{target\ gene} - Cq_{GAPDH})}]_{untreated})$ **. F-G. Cell aggregation after incubation with 200 nM PGD_2 in the absence or presence of neutralisation antibodies to ICAM (20 μg/ml) and PECAM (40 μg/ml) recorded under microscopy (F) and then quantified with ImageJ (G). * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$; n=10 for A, n=5 for B, n=7 for D, n=3 for E, and n=4 for G.**

IL-4, IL-5 and IL-13 production by Tc2 cells increased following exposure to PGD_2 but not the DP1 agonist, which was suppressed by TM30089 and fevipiprant but not BW868C (Fig 3.2A,B). Fevipiprant

inhibited the cytokine production dose-dependently at both mRNA (IC_{50} = 1.17 nM for *IL4*, 0.5 nM for *IL5* and 6.3 nM for *IL13*) and protein (IC_{50} = 9.1 nM for IL-4, 0.7 nM for IL-5 and 5.9 nM for IL-13) levels.

To compare the difference between health and severe eosinophilic asthma on the cell capacities to produce type 2 cytokines and the potency of the $CRTH_2$ antagonist, Tc2 cells were isolated from both groups and expanded. The cells from severe asthma patients produced more IL-13 in response to PGD_2 than those from healthy donors. The maximum responses by the cells from asthma were about 1.4-fold higher than those from healthy. Still, there was no significant difference in the potencies (IC_{50}) of fevipiprant on the inhibition of IL-13 production (Fig 3.2C).

Besides the *in vitro* culture, I conducted flow cytometry to measure cytokine production in Tc2 in the blood *ex vivo* in order to verify the type 2 cytokine production under a more physiological condition. IL-5 and IL-13 were stained intracellularly in Tc2 cells in the fresh peripheral blood of the patients with severe eosinophilic asthma (Fig 3.2D). The ratio of $CD3^+CD4^-CD8^+$ T cells with $IL\text{-}5^+$ or $IL\text{-}13^+$ in the blood was increased following the treatment of PGD_2 (1μM), and fevipiprant (1μM) blocked the increase. No such increase could be detected in the blood derived from healthy donors (data not shown).

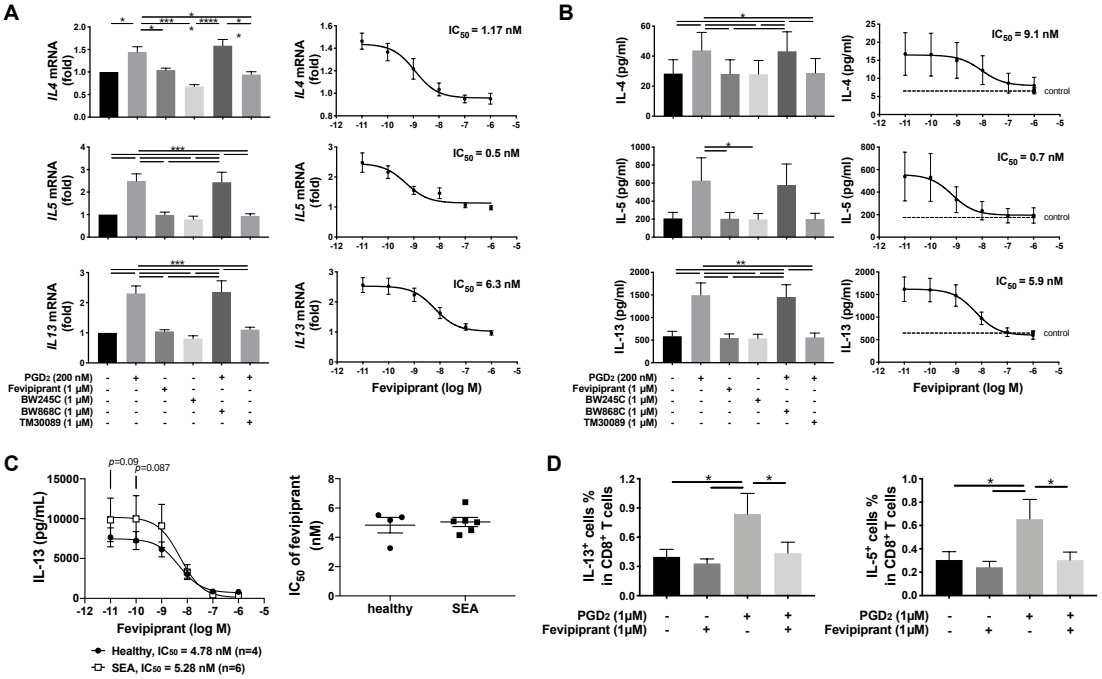


Fig 3.2 $CRTH_2$-mediated type 2 cytokine production in Tc2 cells was blocked by fevipiprant *in vitro* and *ex vivo*.

A-B. mRNA (A) or protein (B) levels for type 2 cytokines in cultured Tc2 cells after treatments with 200 nM PGD_2 in the presence or absence of BW245C, BW868C, TM30089 or various concentrations of fevipiprant, measured with qPCR or ELISA, respectively. The mRNA data are presented as the fold change $([2^{-(Cq_{target\ gene} - Cq_{GAPDH})}]_{treated} \div [2^{-(Cq_{target\ gene} - Cq_{GAPDH})}]_{untreated})$**. C. Effect of fevipiprant on IL-13 production induced by PGD_2 (200 nM) in cultured Tc2 cells derived from healthy controls or severe eosinophilic asthmatics (SEA). IC_{50} values of fevipiprant were compared between the donor groups. D. IL-5 and IL-13 producing Tc2 cells induced by PGD_2 and/or fevipiprant in fresh blood from severe asthmatics detected by using intracellular staining for flow cytometry. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; n=5 for A and C, n=9-19 for B, n=4-6 for D.**

In addition to type 2 cytokines, the production of other pro-inflammatory cytokines, including IL-3, GM-CSF, M-CSF, TNFα, IL-8, CCL4, FasL and galectin-3, was also upregulated by PGD_2 in Tc2 cells, both at the mRNA and protein levels (Fig 3.3). Fevipiprant impaired all cytokine production in a dose-dependent manner with potent IC50s at the magnitude of nanomolar (Fig 3.3).

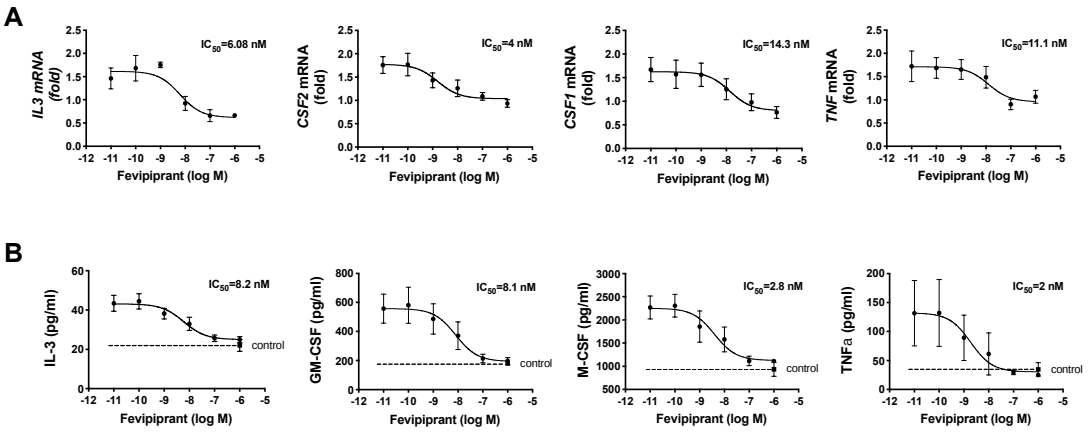


Fig 3.3 PGD_2-induced pro-inflammatory cytokine productions in cultured Tc2 cells were inhibited by fevipiprant.
A-B. mRNA or protein levels for IL-3, GM-CSF, M-CSF and TNFα from Tc2 cells after treatments with 200 nM PGD_2 in the presence of various concentrations of fevipiprant measured with qPCR (A) or Luminex (B) respectively. The mRNA data are presented as the fold change $([2^{-(Cq_{target\ gene} - Cq_{GAPDH})}]_{treated} \div [2^{-(Cq_{target\ gene} - Cq_{GAPDH})}]_{untreated})$**. n=6**

3.3.2. Fibrotic effects of Tc2 cells promoted by PGD_2-$CRTH_2$ pathway

Considering the clinical benefits of fevipiprant on both inhibiting inflammation and airway remodelling (Singh, Ravi and Southworth, 2017), I further explored the potential involvement of Tc2 cells in airway remodelling in the disease. Tissue remodelling associated genes in response to PGD_2 (100 nM) in Tc2 cells were extracted from our RNA microarray data set (Fig 3.4A).

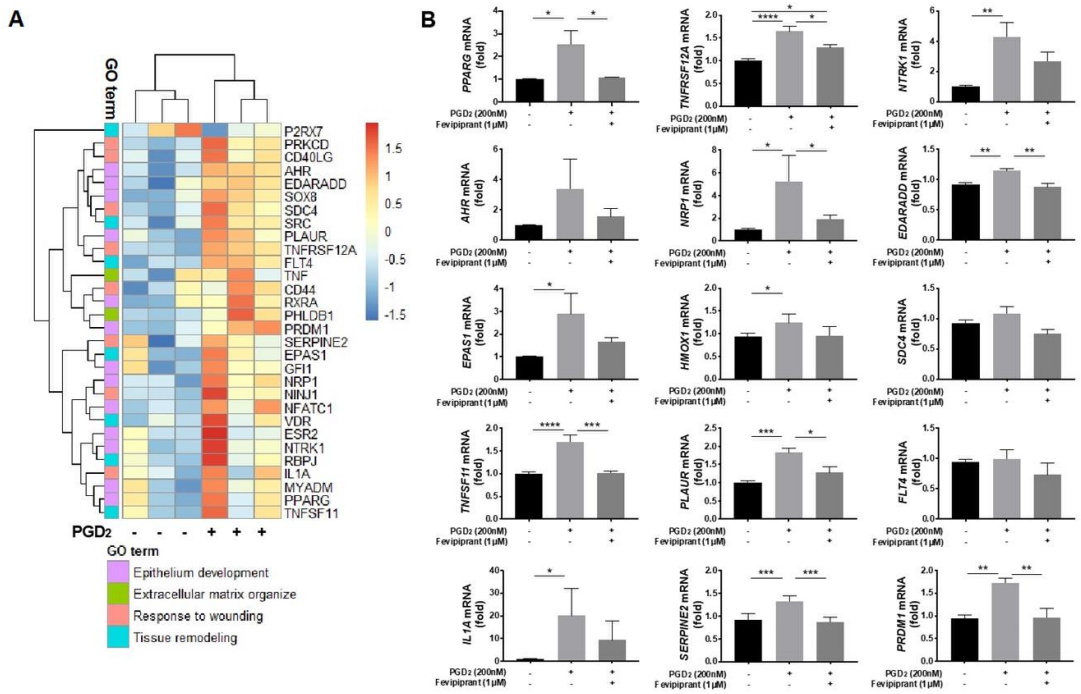


Fig 3.4 Upregulation of tissue remodelling genes in cultured Tc2 cells from Leucocyte Cones by PGD_2 was inhibited by fevipiprant.
A. Heatmap showing tissue remodelling genes significantly ($p < 0.05$) upregulated by PGD_2 (100 nM) in cultured Tc2 cells detected by microarray. B. The upregulation of tissue remodelling genes by PGD_2 was inhibited by fevipiprant. The mRNA levels of tissue remodelling genes in cells after treatment with PGD_2 (200 nM) in the presence or absence of fevipiprant (1 μM) measured with qPCR. The data are presented as the fold change ($[2^{-(Cq_{target\ gene} - Cq_{GAPDH})}]_{treated} \div [2^{-(Cq_{target\ gene} - Cq_{GAPDH})}]_{untreated}$). *$p < 0.05$; **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. n=3 for A or 6-7 for B.

PGD_2 upregulated a significant number of pro-tissue-remodelling genes expressed in Tc2 cells. Among all, the upregulation of *PPARG, AHR, EPAS1, TNFSF11, IL1A, TNFRSF12A, NRP1, HMOX1, PLAUR, SERPINE2, NTRK1, EDARADD, SDC4, FLT4, PRDM1* (Fig 3.4B), and *TNF* (TNFα) were confirmed by qPCR (Fig 3.3A). Treatment with fevipiprant (1 μM) obstructed the upregulation of those genes induced by PGD_2 significantly (*PPARG, TNFSF11, TNFRSF12A, NRP1, PLAUR, SERPINE2, EDARADD, TNF*) or partially at least (Fig 3.4B, Table 3.1). Therefore, Tc2 might promote tissue remodelling via these regulated proteins contributing to epithelium development, fibroblast proliferation and extracellular matrix (ECM) organisation (gene functions as indicated in GO in Fig 3.4A) initiated by $CRTH_2$ activation.

Table 3.1 Comparison of tissue remodelling gene among different conditions in Tc2 cells by qPCR

Gene symbol	Full name	Short name	Significance[$]	
			C vs P	P vs PF
PPARG	Peroxisome proliferator-activated receptor gamma	PPAR-γ	*	*
AHR	Aryl hydrocarbon receptor	AhR	ns	ns
EPAS1	Endothelial PAS domain-containing protein 1	EPAS-1	*	ns
TNFSF11	Receptor activator of nuclear factor kappa-B ligand	RANKL	****	***
IL1A	Interleukin 1 alpha	IL-1a	*	ns
TNFRSF12A	TWEAK receptor	TWEAKR	****	*
NRP1	Neuropilin-1	NRP1	*	*
HMOX1	Heme oxygenase 1	HMOX1	*	ns
PLAUR	Urokinase receptor	uPAR	***	*
SERPINE2	Glia-derived nexin	GDN	***	***
NTRK1	Tropomyosin receptor kinase A	TrkA	**	ns
EDARADD	Ectodysplasin-A receptor-associated adapter protein	EDARADD	**	**
SDC4	Syndecan-4	Syndecan-4	ns	ns
FLT4	FMS-related tyrosine kinase 4	FLT4	ns	ns
PRDM1	PR domain zinc finger protein 1	BLIMP-1	**	**

[$] C stands for control (unstimulated samples), P stands for PGD_2 treated samples, and PF stands for PGD_2 and fevipiprant co-treated samples.
***p* < 0.05; **p* < 0.01, ***p* < 0.001, ****p* < 0.0001; ns *p*≥0.05**

As Tc2 cells may orchestrate tissue repair directly and/or indirectly by targeting fibroblasts and epithelial cells, to confirm the pro-tissue remodelling roles of Tc2 cells, I conducted co-culture assays to examine the effects of activated Tc2 cells on fibroblasts.

First, a fibroblast cell line, MRC-5, was co-cultured with PGD_2-activated Tc2 cells. Tc2 cells were loaded into MRC-5 cultures with or without PGD_2 (200nM) in the presence or absence of fevipiprant (1 μM). The confluence of MRC-5 cells was recorded using photographs at various time points (Fig 3.5A) to determine the speed of fibroblast proliferation. Tc2 cells with PGD_2 significantly increased the MRC-5 confluence after 24 hours of culture compared to the culture without PGD_2. The enhancement became more obvious after 48 hours but disappeared when fevipiprant was added together with PGD_2

in the MRC-5-Tc2 co-culture (Fig 3.5B). This enhancement was not due to direct interaction between PGD_2 and MRC-5 cells because no such enhancement was observed in the same culture without Tc2 (Fig 3.5C).

To confirm whether physical cell-cell contact is required for this enhanced fibroblast expansion, two systems of co-culture were applied. Tc2 cells were mixed and cultured with MRC-5 cells or separated with a 0.5 μm filter insert during the culture. PGD_2-treated Tc2 enhanced the growth of fibroblasts in both systems, whilst slightly more response was observed in physically contacted co-culture (without insert), which indicated that both soluble tissue-remodelling factors released by Tc2 cells and cell-cell cross-talk contribute to the fibroblast proliferation (Fig 3.5D). Interestingly, culturing MRC-5 with the conditioned medium harvested from pre-activated Tc2 cells was less effective compared to two co-culture methods on the MRC-5 growth (Fig 3.5E), suggesting that promotion of MRC-5 growth requires long interaction with activated Tc2.

Furthermore, type 2 cytokines IL-4 and IL-13 also promoted MRC-5 proliferation, as IL-4 and IL-13 increased MRC-5 confluence in a dose-dependent manner (Fig 3.5F, left). However, considering the rather low concentration of cytokines measured in the supernatant of co-culture (IL-13: 0.1-1 ng/ml; IL-4: not detectable), it had a minor impact on the MRC-5 growth (Fig 3.5F, right).

In addition to fibroblast proliferation, functions relating to ECM organisation were also investigated. Collagens are a class of key components of ECM(Frantz, Stewart and Weaver, 2010). Upregulation of collagen I α1 (COL1A1) was detected at both protein (Fig 3.5G, left) and mRNA (Fig 3.5H, left) levels in the MRC-5 cells after co-culture with Tc2 and PGD_2, which were blocked in the presence of fevipiprant. Other markers indicating activation of fibroblasts were also increased at transcriptional [*ACTA2* (actin α2) *and VIM* (vimentin)] (Fig 3.5H, mid and right) or translational levels [TGF-β1 (transforming growth factor beta 1)] (Fig 3.5G, right). All these upregulations were seen neither in Tc2 microarray data nor in the incubation of fibroblasts with PGD_2 (Fig 3.5G,H). Therefore, these regulations were not induced by PGD_2 directly in fibroblasts or Tc2 cells.

Epithelial cell lines A549 and BEAS-2B were also used to confirm the functions of Tc2 on epithelium development. However, although genes detected in the microarray should potentially be associated with epithelium development, no enhancement of epithelial cell proliferation was observed in the Tc2-epithelial cell co-culture. In addition, no enhanced epithelial cell migration was detected using a cell scratching assay. These findings indicated that PGD_2-induced Tc2 activation would likely only impact fibroblast but not epithelial cells in the modulation of airway remodelling.

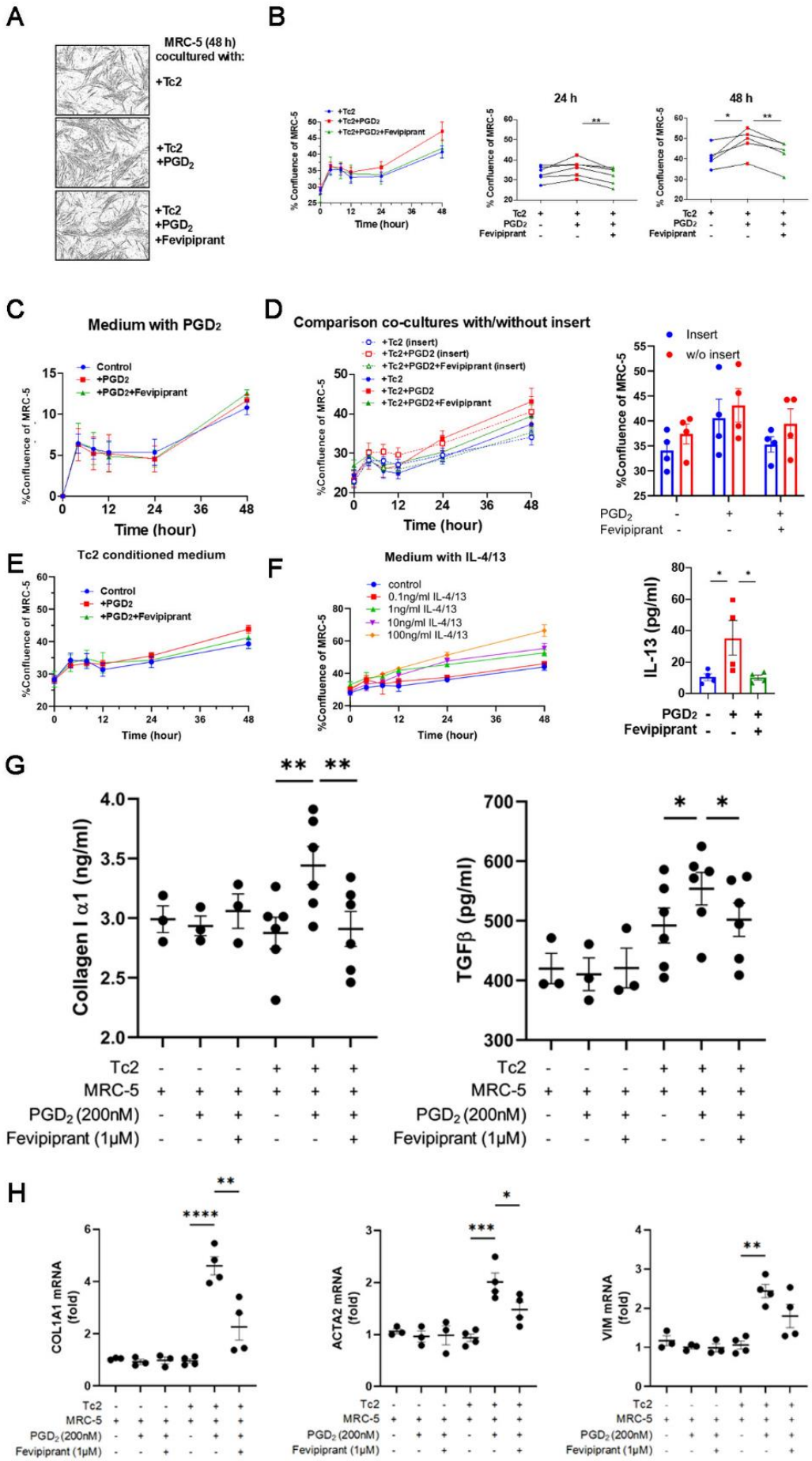

A
MRC-5 (48 h) cocultured with:
+Tc2
+Tc2 +PGD2
+Tc2 +PGD2 +Fevipiprant
B
+Tc2
+Tc2+PGD2
+Tc2+PGD2+Fevipiprant
% Confluence of MRC-5
Time (hour)
24 h
48 h
Tc2
PGD2
Fevipiprant
C
Medium with PGD2
Control
+PGD2
+PGD2+Fevipiprant
%Confluence of MRC-5
Time (hour)
D
Comparison co-cultures with/without insert
+Tc2 (insert)
+Tc2+PGD2 (insert)
+Tc2+PGD2+Fevipiprant (insert)
+Tc2
+Tc2+PGD2
+Tc2+PGD2+Fevipiprant
Insert
w/o insert
E
Tc2 conditioned medium
F
Medium with IL-4/13
control
0.1ng/ml IL-4/13
1ng/ml IL-4/13
10ng/ml IL-4/13
100ng/ml IL-4/13
IL-13 (pg/ml)
G
Collagen I α1 (ng/ml)
TGFβ (pg/ml)
Tc2
MRC-5
PGD2 (200nM)
Fevipiprant (1μM)
H
COL1A1 mRNA (fold)
ACTA2 mRNA (fold)
VIM mRNA (fold)

Fig 3.5 The tissue remodelling effect of Tc2 cells cultured from Leucocyte Cones induced by PGD_2 was abolished by fevipiprant.
A-B. Promoting effect of Tc2 cells on MRC-5 cell growth induced by PGD_2 was abolished by fevipiprant. The confluence of MRC-5 cells in the co-culture with Tc2 cells in a control medium, a medium containing 200 nM PGD_2 or a medium containing 200 nM PGD_2 and 1 μM fevipiprant for various time lengths were recorded with IncuCyte (A) and quantified with Fiji (B). C. The confluence of MRC5 cells cultured with medium alone or medium containing 200 nM PGD_2 in the presence or absence of 1 μM fevipiprant. D. Comparison of MRC-5 cell confluence in the MRC-5-Tc2 co-cultures with or without using insert over 48 hours. E-F. The confluence of MRC-5 cells after cultured with the supernatants from Tc2 cells stimulated with PGD_2 in the presence or absence of fevipiprant (E), or with MEM medium containing different concentrations of IL-4 and IL-13 (F). G. The concentration of collagen I α1 and TGFβ detected with ELISA in the supernatants of MRC-5 cells with or without Tc2 cell co-culture. H. mRNA levels of *COL1A1*, *ACTA2* and *VIM* measured with qPCR in MRC-5 cells with or without Tc2 cell co-culture. The data are presented as the fold change ($[2^{-(Cq_{target\ gene} - Cq_{GAPDH})}]_{treated} \div [2^{-(Cq_{target\ gene} - Cq_{GAPDH})}]_{untreated\ MRC-5}$)**. *$p$ < 0.05; **p < 0.01, ***p < 0.001, ****p < 0.0001. n=5-6 for B, 4 for C,D,F, 3 for E, 3-6 for G and 3-4 for H.**

3.3.3. PGD_2 production in Tc2 cells

Increased levels of PGD_2 were detected in the airways of asthma (Hilvering *et al.*, 2018). To determine whether Tc2 cells could contribute to PGD_2 upregulation in the disease, I examined PGD_2 production in activated Tc2 cells *in vitro* under different stimulation conditions. To enable differentiation of stimulator and product PGD_2 in the PGD_2 ELISA, DK-PGD_2, a particular $CRTH_2$ agonist that has no cross-reactivity with the ELISA, was used to stimulate $CRTH_2$. Anti-CD3/CD28 antibodies were also administrated as an alternative stimulation. The PGD_2 production, which peaked 4 hours after the stimulations, was driven by both DK-PGD_2 and anti-CD3/CD28 (Fig 3.6A). The PGD_2 production triggered by DK-PGD_2 was significantly inhibited by fevipiprant (Fig 3.6B), suggesting an autocrine or paracrine PGD_2 production in Tc2 cells via $CRTH_2$ activation. Remarkably, the production of PGD_2 induced by TCR also decreased significantly by fevipiprant.

To further confirm the synthesis pathway for PGD_2 in Tc2 cells, I examined the expression of enzymes that are involved in the typical arachidonic acid metabolism, including hematopoietic prostaglandin D synthase (hPGDS), cyclooxygenase (COX) -1, COX-2, phospholipase (PL) A2 and PLC.

HPGDS is the enzyme catalysing the conversion of the precursor PGH_2 into PGD_2. It was detected in Tc2 cells in blood with flow cytometry, and the frequency of $hPGDS^+$ Tc2 cells was correlated with blood eosinophil counts in asthma (Fig 3.6C,D). The expression of hPGDS was elevated by DK-PGD_2 but not by anti-CD3/CD28, and fevipiprant reversed this elevation (Fig 3.6E).

COX-1 and COX-2 are enzymes that function upstream of the hPGDS, which converts the arachidonic acid to PGH_2. The expression of COX-2 in Tc2 cells was slightly upregulated by DK-PGD_2 at both mRNA (*PTGS2* for COX-2) and protein levels, which was significantly reduced by fevipiprant. On the other hand, mRNA of COX-1 (*PTGS1*) was significantly increased with PGD_2 treatment in Tc2, but the change of protein level of COX-1 was difficult to determine due to all cells showing COX-1 positive staining. Furthermore, TCR activation specifically upregulated COX-2, which was not influenced by fevipiprant.

hPGDS inhibition using hPGDS Inhibitor 1 in Tc2 cells blocked PGD_2 production completely induced by DK-PGD_2 but only partially blocked the production mediated by TCR (Fig 3.6H). Inhibiting COXs with diclofenac or flurbiprofen led to the entire blocking of PGD_2 release in Tc2 mediated by both TCR and $CRTH_2$.

I also examined the enzymes upstream of COX, cytosolic PLA2 (cPLA2) and PLC, required for PGD_2 synthesis. In Tc2 cells, both enzymes, but mainly cPLA2, were expressed (Fig 3.6I). However, they were not significantly regulated by $CRTH_2$ or TCR activation (Fig 3.6J). ATK, an inhibitor of cPLA2, repressed the production of PGD_2 in Tc2 cells, which was much stronger compared to the PLC inhibitor U-73122. Although U-73122 only partially reduced PGD_2 production, the combination of ATK and U-73122 additionally suppressed the PGD_2 synthesis (Fig 3.6K).

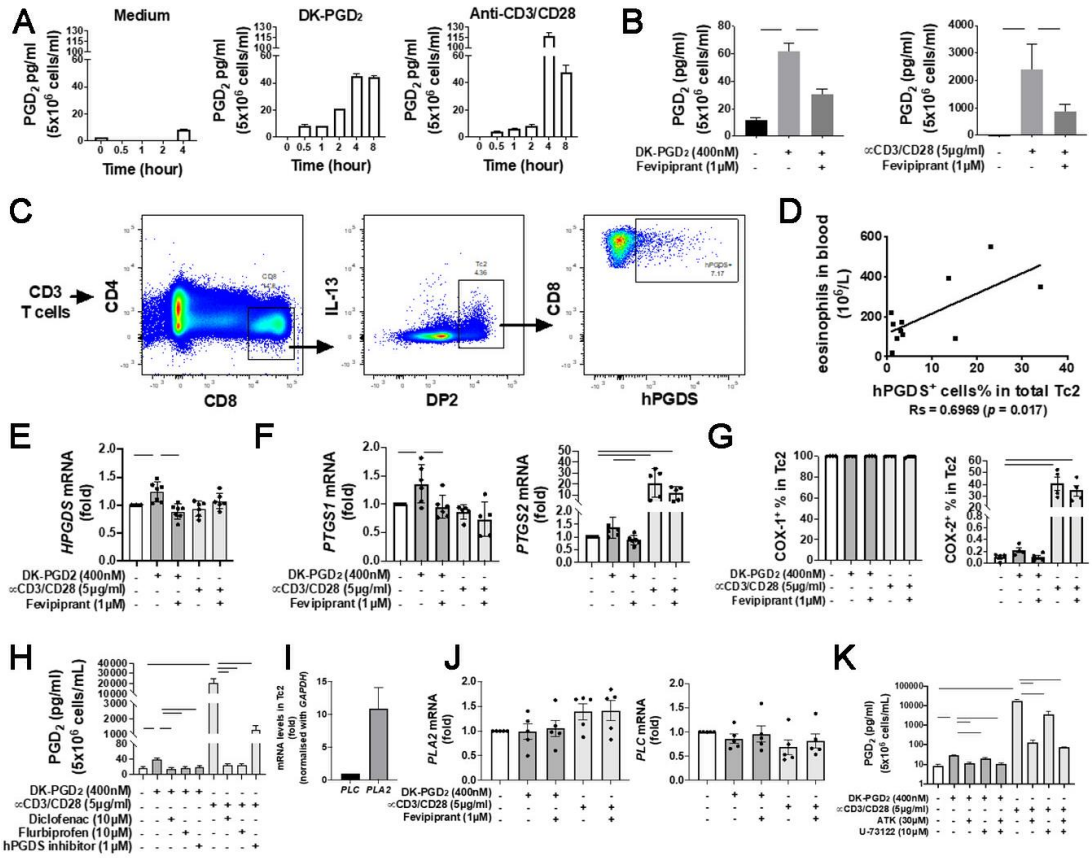


Fig 3.6 Autocrine PGD_2 production in cultured Tc2 cells from Leucocyte Cones was attenuated by inhibition of DP2, hPGDS and COXs.

A. PGD_2 production from cultured Tc2 cells after stimulation with DK-PGD_2 (400 nM) or anti-CD3/CD28 (5μg/ml) at different time points measured with MOX enzyme immunoassay. B. Levels of PGD_2 production after stimulation with DK-PGD_2 or anti-CD3/CD28 in the presence or absence of fevipiprant for 4 hours. C. Gating strategy to detect $hPGDS^+$ Tc2 cells in fresh blood with flow cytometry. D. Correlation of the level of $hPGDS^+$ Tc2 cells and blood eosinophil counts in asthmatic patients. E-F. The mRNA levels of *HPGDS* (E), *PTGS1* or *PTGS2* (F) in cultured Tc2 cells after treatments with DK-PGD_2 or anti-CD3/CD28 antibodies in the absence or presence of fevipiprant for 4 hours determined with qPCR. G. Expression of COX-1 and COX-2 in cultured Tc2 cells after treatments with DK-PGD_2 or anti-CD3/CD28 antibodies in the absence or presence of fevipiprant measured with flow cytometry. H. PGD_2 production in cultured Tc2 cells after pre-incubation with diclofenac, flurbiprofen or hPGDS inhibitor I for 1 hour, followed by stimulation with DK-PGD_2 or anti-CD3/CD28 for 4 hours. I. Comparison of mRNA levels between *PLA2* and *PLC* in Tc2 cells. J. Transcription levels of *PLA2* and *PLC* after indicated treatments. K. PGD_2 production after DK-PGD_2 or anti-CD3/CD28 in the absence or presence of ATK or U-73122. The mRNA data in E, F, I and J are presented as the fold change $([2^{-(Cq_{target\ gene} - Cq_{GAPDH})}]_{treated} \div [2^{-(Cq_{target\ gene} - Cq_{GAPDH})}]_{untreated})$. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. n=3 for A, 6 for B, 5 for E, 4 for F, 3-4 for G, 10 for H, 5 for I,J and 7 for K.

3.4. Discussion and conclusions

Our previous study has discovered that Tc2 cells but not ILC2s, are enriched in both peripheral blood and airways in severe eosinophilic asthma (Hilvering *et al.*, 2018). We have also confirmed that the production of proinflammatory cytokines, including IL-4/5/13 and GM-CSF induced by PGD_2, could be a potential role of Tc2 in airway eosinophilia and inflammation. In this study, two unknown functions of Tc2 cells, including tissue remodelling and IgE-independent PGD_2 autocrine production, were further explored. The PGD_2-$CRTH_2$ pathway was observed to be heavily involved in all functions related to inflammation, tissue remodelling and PGD_2 production in Tc2 cells. My results showed that antagonism of $CRTH_2$ not only totally blocked cell migration, adhesion, survival and pro-inflammatory cytokine production in Tc2, which leads to airway inflammation, but also reduced the profibrotic effects and the autocrine production of PGD_2 in Tc2, which would contribute to the airway remodelling and severe symptoms in non-atopic patients, respectively. These results will give us a better understanding of the possible role of Tc2 cells in the pathogenesis of severe eosinophilic asthma.

Airway remodelling is associated with a decline in lung function because it results in irreversible or partially reversible airflow obstruction (Homer and Elias, 2005). Thus, airway remodelling is an important feature of asthma which has been explained in the aforementioned Chapter 1.1.2. The mechanisms regulating airway remodelling are rather complex, and limited knowledge has been obtained, especially in the relationship between Tc2 and airway remodelling.

In this study, I demonstrated that $CRTH_2$ activation in Tc2 cells regulated the expression of many genes that could modulate fibroblast proliferation, extracellular matrix organisation and tissue repair. IL-1α, TNFα, syndecan-4, TrkA, NRP1 and TWEAK/Fn14 are capable of stimulating fibroblast and smooth muscle cell proliferation (Schmidt *et al.*, 1982; Porreca *et al.*, 1993; Hoefer *et al.*, 2002; Aravamudan *et al.*, 2012; Jang *et al.*, 2012; Lin *et al.*, 2015; Zhu *et al.*, 2018). IL-1α, TNFα and NRP1 can promote fibrosis (Piguet *et al.*, 1993; Distler *et al.*, 2008; Cao *et al.*, 2010). TWEAK/Fn14, Glia-derived nexin and TrkA can induce extracellular matrix generation and deposition (Mustonen, Ruskoaho and Rysä, 2012; Li *et al.*, 2016; J. Liu *et al.*, 2019; Z. Liu *et al.*, 2019). The results of my co-culture assay provided direct evidence that Tc2 have functional activities promoting fibrosis *in vitro*. Tc2 facilitated fibroblast

proliferation and production of collagen I α1, actin α2, vimentin and TGFβ via $CRTH_2$ activation on Tc2, which was inhibited by fevipiprant.

Collagen I α1 is a member of the type 1 collagens, which are the most abundant components of the extracellular matrix. Pathological deposition of collagens causes basement membrane thickening and airway remodelling in asthma (Shifren *et al.*, 2012). Actin α2 is a biomarker of myofibroblasts and smooth muscle differentiation (Rockey, Weymouth and Shi, 2013). Myofibroblasts are differentiated from fibroblasts that have a greatly upregulated production of ECM components and are found enriched in fibrosis (Kendall and Feghali-Bostwick, 2014). Therefore, the expression of actin α2 is considered a biomarker of lung fibrosis (Zhang *et al.*, 1996). Vimentin plays an important role in fibroblast proliferation during wound healing and is a pan marker of fibroblast activation (Cheng *et al.*, 2016; Sliogeryte and Gavara, 2019). TGFβ is a key factor in fibrosis and mediates the expression of type 1 collagen and actin α2 in fibroblasts (Verrecchia and Mauviel, 2004; Van Bruaene *et al.*, 2009; Kumawat *et al.*, 2016). It was suggested in previous studies that fevipiprant reduced airway smooth muscle mass and improved lung functions in asthma patients (Gonem *et al.*, 2016; Bateman *et al.*, 2017), suggesting a tissue remodelling role of the $CRTH_2$ pathway in asthma. My results support their observation and provide a novel mechanism that, besides eosinophils, PGD_2-activated Tc2 cells also contribute to airway remodelling by activating fibroblast functions, leading to the potential use of $CRTH_2$ antagonists in clinical applications for severe eosinophilic asthma with airway remodelling.

Traditionally, PGD_2 was considered a lipid mediator mainly released from mast cells following an IgE crosslinking to its high-affinity receptor, FcεR1 (Lewis *et al.*, 1982; Schleimer *et al.*, 1985; Marone, Triggiani and De Paulis, 2005; Stone, Prussin and Metcalfe, 2010). However, PGD_2 is detected in both eosinophilic and non-eosinophilic asthma (Hilvering *et al.*, 2018), suggesting an IgE-independent PGD_2 production. Recent reports showed that macrophages (Jandl *et al.*, 2016), eosinophils (Luna-Gomes *et al.*, 2011), Th2 cells (Tanaka *et al.*, 2000) and ILC2s (Maric *et al.*, 2019) are alternative sources of PGD_2. In this study, I revealed that Tc2 cells also have the capacity to produce PGD_2 in an IgE-independent manner. The capacity of PGD_2 production in Tc2 cells is similar to that in Th2 cells and about half of that in mast cells. Both exogenous $CRTH_2$ agonists and TCR activation positively

enhanced the production of endogenous PGD_2 in Tc2 cells. This production is impaired by blockage of $CRTH_2$ with fevipiprant, even in the TCR-mediated activation, suggesting that TCR-mediated PGD_2 production at least partially relies on the secondary autocrine or paracrine production of PGD_2. That is, the endogenous PGD_2 initiated from TCR-activated Tc2 cells binds to $CRTH_2$ on the same cells and subsequently initiates the autocrine or paracrine pathway.

The molecular machinery for PGD_2 synthesis occurs through the phospholipid - arachidonic acid - prostaglandin cascade. In a classic PGD_2 metabolic pathway, arachidonic acid derived from phospholipids via cPLA/PLC/PLD catalysis is converted into PGH_2, a precursor for prostaglandins, by COXs, which is then isomerised into PGD_2 by hPGDS (Kanaoka and Urade, 2003; Hanna and Hafez, 2018; Mitchell and Kirkby, 2019). Therefore, along with our *in vitro* finding that inhibition of hPGDS, COXs and cPLA2/PLC blocked $CRTH_2$-induced PGD_2 production in Tc2 cells, Tc2 cells use the routine PGD_2 conversion pathway and machinery in their PGD_2 autocrine.

Stimulation of TCR strongly promotes PGD_2 production in Tc2 cells, which is similar to what has been seen in Th2 cells (Tanaka *et al.*, 2000). This suggests that the TCR-dependent mechanism in T cells could evoke the production of PGD_2 bypassing mast cell and IgE cross-linking. TCR activation, unlike $CRTH_2$ activation, might not follow the regular known metabolic pathway of PGD_2. Firstly, inhibition of hPGDS and cPLA2/PLC could not completely block the PGD_2 production induced by anti-CD3/28 stimulation. As PLD is also involved in arachidonic acid generation (Hanna and Hafez, 2018), the roles of PLD in TCR-activated PGD_2 production ought to be investigated. As for hPGDS, although prostaglandin D_2 synthase (PTGDS) is another enzyme with similar roles, it is preferentially expressed in the brain and could not be detected in Tc2 cells (https://www.proteinatlas.org/ENSG00000107317-PTGDS/) (Uhlén *et al.*, 2015), which indicates some unknown enzymes function in parallel with hPGDS in the TCR activation pathway. In addition, TCR activation did not upregulate COX-1 and hPGDS, and COX-2 upregulation by TCR was not reduced by fevipiprant. These results suggested that further study is required to dissect the pathways related to TCR activation to control PGD_2 production in Tc2.

I have observed that Tc2 cells displayed a low but spontaneous baseline PGD_2 secretion even without stimulation. Although stimulation with anti-CD3/28 or PGD_2 elicited increasing in the enzyme expression, all Tc2 cells constitutively and highly express COX-1. Therefore, the rate of PGD_2 synthesis might be related to the amount of hPGDS that could convert PGH_2 to PGD_2. I discovered that the level of hPGDS was elevated in Tc2 cells activated by PGD_2, and hPGDS-positive Tc2 cells increased in eosinophilic asthma. If further investigation could confirm the correlation between PGD_2 level and $hPGDS^+$ type 2 cell number both in the airways or blood, hPGDS level could be a biomarker of PGD_2 high patients and a predictor of $CRTH_2$ antagonist responders. Meanwhile, considering blood eosinophil counts have a positive correlation with hPGDS-positive Tc2 cells in the blood but not serum IgE in asthma, as showed in Chapter 5, Tc2 cells could potentially amplify the inflammation and airway remodelling through PGD_2 autocrine signalling independently of IgE mediation in non-atopic severe eosinophilic asthma which accounts for 20% of severe asthma (Haughney *et al.*, 2018).

In summary, I have, for the first time to my knowledge, revealed that $CRTH_2$-specific activation not only promotes migration, survival, adhesion, and cytokine production that drives type 2 inflammation in severe eosinophilic asthma but also contributes to the airway remodelling in asthma by regulating fibroblast proliferation, differentiation and activation. Furthermore, all activities can be echoed by endogenous PGD_2 production via autocrine/paracrine pathways. Overall, these results will improve our understanding of Tc2 biology and its role in the pathogenesis of asthma, especially severe eosinophilic asthma. Moreover, my findings of the inhibitory effects of $CRTH_2$ antagonism in Tc2 cells support the further development of two $CRTH_2$ antagonist candidates in ongoing clinical trials.

Chapter 4. Potential Functions of PGD_2 and $CRTH_2$ in Basophils Associated with Severe Eosinophilic Asthma

4.1. Introduction

Basophils are one type of leukocyte in a small population (0.5 - 1%) in peripheral blood. The $CD34^+$ myeloid hematopoietic progenitors maturate and differentiate into basophils in the bone marrow. Then these bone-marrow-originated cells enter the circulating blood in a mature phenotype with approximately 5 days lifespan in humans (Stone, Prussin and Metcalfe, 2010). Basophils are functionally distinct from other leukocytes with the expression of FcεR1 and a capacity of activation by IgE-mediated FcεR1 crosslinking. Based on the similarity to mast cells, basophils are often considered as possibly "redundant and negligible" circulating mast cells. Activation of basophils evokes fast release of preformed histamine, newly synthesised leukotriene C_4 (LTC_4) and type 2 cytokines IL-4 and IL-13 (Marone, Triggiani and De Paulis, 2005). Both histamine and LTC_4 are potent bronchoconstrictors (Dale and Laidlaw, 1919; Dahlén *et al.*, 1980; Thangam *et al.*, 2018), which causes acute asthma attack by smooth muscle constriction. IL-4 and IL-13 are important cytokines mediating T2 cell differentiation and epithelium activation, resulting in type 2 inflammation in asthma (Wynn, 2015). However, in contrast to other inflammatory effector cells, the understanding of the roles of basophils in the pathogenesis of asthma is very limited.

In circulating blood, human basophils are 100% $CRTH_2^+$, and the expression level of $CRTH_2$ in basophils is one of the highest (Hirai *et al.*, 2001) among all $CRTH_2^+$ cell types, including Th2, Tc2, ILC2, basophils and eosinophils. Although the PGD_2-$CRTH_2$ pathway in Th2 (Xue *et al.*, 2005), Tc2 (Hilvering *et al.*, 2018) and ILC2 (Xue *et al.*, 2014) showed vital effects on type 2 inflammation, only little is known about the biology of the PGD_2/$CRTH_2$ axis in basophils. Previously, it has been reported that basophil migration was promoted by PGD_2 via $CRTH_2$ *in vitro* (Hirai *et al.*, 2001). CD203c (an activation marker of basophils) and CD11b (an adhesion molecule on basophils) were upregulated by PGD_2 treatment (Monneret *et al.*, 2005). However, according to the reports, PGD_2 could not trigger histamine release or CD63 expression by basophils. Therefore, it is still unclear whether PGD_2 is an

inducer of cytokine production and degranulation in basophils. Increasing interest in the topic of IgE-independent basophil activation rationalises the study of the PGD_2-$CRTH_2$ pathway in basophils. To date, high-content RNA sequencing techniques have not been applied to the study of human basophils, particularly to discover their new functions and roles in asthma or inflammation. The limitation of the studies on human basophils is partially due to their small number in the blood, the short lifespan of the cells, and the technical difficulties of cell purification and culture.

In this chapter, I have characterised the basophils from peripheral blood in a cohort of different asthma phenotypes and healthy controls. I compared the distribution levels of blood basophils in different patient groups. The effects of PGD_2 and its blockade by using $CRTH_2$ antagonist fevipiprant on the *In vitro* and *ex vivo* activation of basophils were then investigated. I revealed that PGD_2 is a potent stimulator to induce $CRTH_2$-dependent IL-4 and IL-13 production, cell migration and adhesion, and degranulation in basophils. To broadly understand the biology of basophils, RNA sequencing was conducted on purified blood basophils treated with different stimuli. The results suggested that, compared with type 2 T cells, basophils exhibited a milder immune response to PGD_2 and presented a type 2 focused response compared to the response to IgE. PGD_2-activated basophils might also potentially contribute to the interaction with neurons and other cells via Sdk2 adhesion. Collectively, these findings reveal the critical roles of PGD_2 on basophils and open new clinical strategies for basophil-based therapeutics in severe eosinophilic asthma.

4.2. Chapter-specific materials and methods

4.2.1. Flow cytometry

For the *ex vivo* basophil profile study, fresh blood of the patients and healthy subjects were stained with an antibody panel against CD3, CD45, CD193, $CRTH_2$, CD123, HLA-DR, ST2 and TSLPR (anti-ST2 from mdbio products, all others from BioLegend) for 30 minutes at 4°C followed by lyse/fixation with lysis buffer (BD) for 20 minutes in the dark at room temperature. Samples were ready to be measured after two times of washing with PBS.

For the shape change assay, fresh human blood was incubated in the presence or absence of 1µM PGD_2 in RPMI-1640 with 10% FCS for 50 minutes at 37 °C with 5% CO_2. All samples were stained with additional anti-CD123 and anti-HLA-DR antibodies for further 10 minutes. The samples were fixed with a Cytofix Fixation Buffer (BD Biosciences) followed by red blood cell lysis with RBC Lysis Solution (Qiagen). Flow cytometry was conducted to measure basophil shape changes. Basophils were gated as side-scatter^{low} (SSC), CD123^{+} and HLA-DR^{-}. The changes in shapes were defined by the increasing shift of forward scatter.

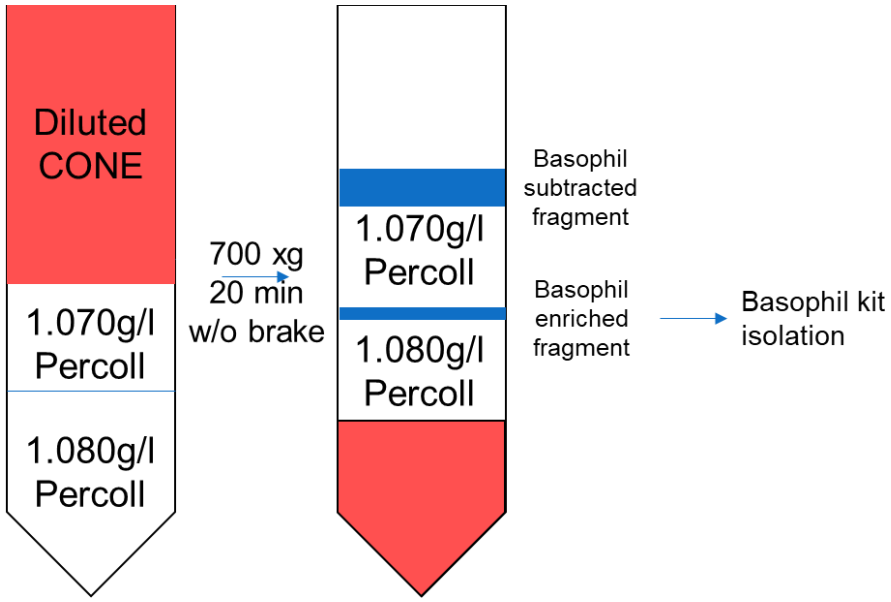
For the measurement of CD11b, CD63 and CD203c, fresh blood from the patients and healthy controls were treated with 100 nM PGD_2 and/or 1 µM fevipiprant for 20 minutes at 37°C under 5% CO_2. The reaction was stopped by adding cold PBS plus 1 mM EDTA into the blood. Cells were then stained with an antibody panel against CD123, HLA-DR, $CRTH_2$, CD63, CD203c and CD11b (BioLegend), followed by the standard method as mentioned above.

Results of the above staining were acquired with BD LSRFortessa or LSRII flow cytometers.

4.2.2. Human basophils isolation

One part of 10x PBS was diluted with nine parts of Percoll (GE Healthcare), which was further diluted with 1x HBSS to have two Percoll solutions with final densities of 1.070 g/ml and 1.080 g/ml separately. Each human leukocyte cone was diluted with HBSS into a final volume of 50 ml. As illustrated in the figure below, 12.5 ml 1.080 g/ml Percoll was layered first in the 50 ml falcon tube, followed by 12.5

ml 1.070 g/ml Percoll and 25 ml diluted cone blood on the top. After centrifugation without braking at 700xg for 20 minutes, the thinnest fragment was harvested for MACS basophil isolation, as illustrated by Meltenyi Biotec (https://www.miltenyibiotec.com/GB-en/products/basophil-isolation-kit-ii-human.html). In brief, cell pellets were resuspended in MACS buffer (Ca^{2+}/Mg^{2+} free PBS plus 10% FCS and 0.5 mM EDTA) and then incubated with FcR Blocking Reagent and Basophil Biotin-Antibody Cocktail for 10 minutes under 4°C, followed by incubation with Anti-Biotin MicroBeads for further 15 minutes. After magnetic separation by LS Columns, basophils were collected in the flow-through medium. For validation of the cell quality, cells were counted and stained with an antibody panel against CD3, CD45, CD193, $CRTH_2$, CD123, HLA-DR and CD63 (BioLegend).



4.2.3. Chemotaxis

Basophils were resuspended in RPMI 1640 media containing 10% human serum and then pre-incubated with various concentrations of fevipiprant, 1 µM BW868C, 1 µM TM830089 or medium as indicated in the results for 1 hour at 37°C in the upper chamber (5 µm pore size) of HTS Transwell 96 well permeable supports (Corning). The upper chamber was then placed into the lower chamber of the supports containing pre-warmed 100 nM PGD_2, 1 µM BW245C or medium for further 1-hour incubation. The cell migration to the lower chamber was quantified using an IncuCyte ZOOM.

4.2.4. Basophil activation *in vitro*

Isolated basophils were rested with RPMI 1640 medium supplemented with 10% human serum and 10 IU/ml IL-3 (Miltenyi).

For cytokine production assays, basophils were incubated with 0.01-10 µM PGD_2 or 0.5 µg/ml anti-IgE in the presence or absence of various concentrations of fevipiprant or other compounds (1 µM BW245C, 1 µM BW868C or 1 µM TM30089) as indicated in the results for 4 hours at 37°C (except the mRNA detection of IL4 and IL13 in the presence of a series of concentrations of PGD_2). For cell aggregation assay, basophils were checked under the microscope at 0-, 0.5-, 1-, and 2-hour time points and the results were recorded with photos. The cell supernatants were harvested for ELISA assays, and the cell pellets were stored at -80°C for qPCR and RNA-seq.

For histamine assays, basophils were treated with 1µM PGD_2 in the presence or absence of 1 µM fevipiprant for 1 hour at 37°C. The cell supernatants were harvested for ELISA assays.

4.2.5. ELISA

The levels of IL-4, IL-5 and IL-13 in the supernatants after the cell treatments were measured with ELISA kits (Invitrogen) according to the manufacturer's instructions. The concentrations of histamine in the supernatants were measured with a competitive ELISA kit (Abcam) according to the manufacturer's instructions. The results were detected in an EnVision Multilabel Reader (PerkinElmer).

4.2.6. RNA sequencing

Total RNA from the basophil pellets after the treatments, as indicated in the results, was extracted by using RNeasy Plus Mini Kit (Qiagen). RNA samples were sent to Novogene company and sequenced by NovaSeq platforms with paired-end 150 bp reads aiming at an average depth of 10 million reads/sample. Data quantification and normalisation were performed by the company as well. A file of mapped gene counts was returned. Differential expression (DE) was performed using the DESeq2 (Love, Huber and Anders, 2014). Genes were considered differentially expressed if they had an absolute log2 fold change (FC) more than 2 and the *p* value lower than 0.01. Pathway enrichment analysis was

conducted using the clusterProfiler and ReactomePA (Yu and He, 2016) to validate the enriched pathways with differentially expressed genes.

For cross-cell type analysis, differential expression analysis of eosinophil microarray data were generated by GEO2R on NCBI (GEO accession: GSE75142 (Sippel *et al.*, 2018)). Differential expression analysis of Tc2 and Th2 microarrays was previously published (Xue *et al.*, 2015; Hilvering *et al.*, 2018). Differential expression analysis of Tc2 RNA-seq was kindly provided by Ryan Hoyle from his project.

For heatmap analysis, the value of normalised counts was converted into Z-score. The Z-score was calculated by subtracting the overall average gene counts in the sample from the raw expression and dividing that result by the standard deviation (SD) of all of the measured counts across all conditions in the model.

4.3. Results

4.3.1. Elevation of basophil counts in severe eosinophilic asthma

To investigate the potential role of basophils in asthma, I compared the basophil profiles in the fresh blood from different asthma phenotypes in this Oxford cohort of 109 participants (Table 4.1) with flow cytometry.

Firstly, blood basophils were identified as SSC^{low} $CD193^{+}$ cells (Fig 4.1A, upper left). On those cells, high levels of conventional markers CD193, FcεRI, $CRTH_2$ and CD123, moderate levels of CD45 and low levels of HLA-DR were detected regardless of the patient's phenotypes (Fig 4.1A). The level of $CRTH_2$ expression on basophils was about 4-fold higher than that on Tc2 or Th2 ($p < 0.01$ for both comparisons) whilst slightly less compared to that on eosinophils (no significance, $p > 0.9$, Fig 4.1B,C). In addition, no expression of alarmin receptors ST2 or TSLPR was found in the basophils (Fig 4.1D,E). These data suggested that $CRTH_2$ but not ST2 or TSLPR, may play important roles in basophil activation.

Table 4.1 General characteristics of the study subjects

Characteristic	Healthy control	Mild non-eosinophilic asthma	Mild eosinophilic asthma	Severe non-eosinophilic asthma	Severe eosinophilic asthma	Severe non-eosinophilic asthma with OCS	Severe eosinophilic asthma with OCS
Number	19	14	14	17	31	4	10
Sex (%female)	52.63%	35.71%	57.14%	75.00%	58.82%	50.00%	77.42%
BMI	33.2±9.0	27.7±4.2	27.1±5.5	30.2±5.6	29.8±6.1	28.5±4.1	31.9±8.7
Age	34.7±14.5	49.8±15.2	46.5±16.5	50.1±16.7	56.9±13.8	56.4±11.8	50.2±15.9
FeNO (ppb)	13.8±4.6	35.9±25.0	80.2±55.0	28.7±23.4	55.1±52.8	33.6±42.8	70.7±65.7
Pre FEV1/FVC ratio	0.75±0.04	0.72±0.09	0.74±0.12	0.67±0.14	0.65±0.13	0.69±0.12	0.63±0.12
Blood eosinophils (per µL)	99±72	179±74	495±169	144±77	765±414	134±80	275±208

BMI (body mass index) = Weight (kg)/Height^2 (m).
FeNO, a fractional concentration of exhaled nitric oxide.
FEV1, forced expiratory volume in one second
FVC, forced vital capacity

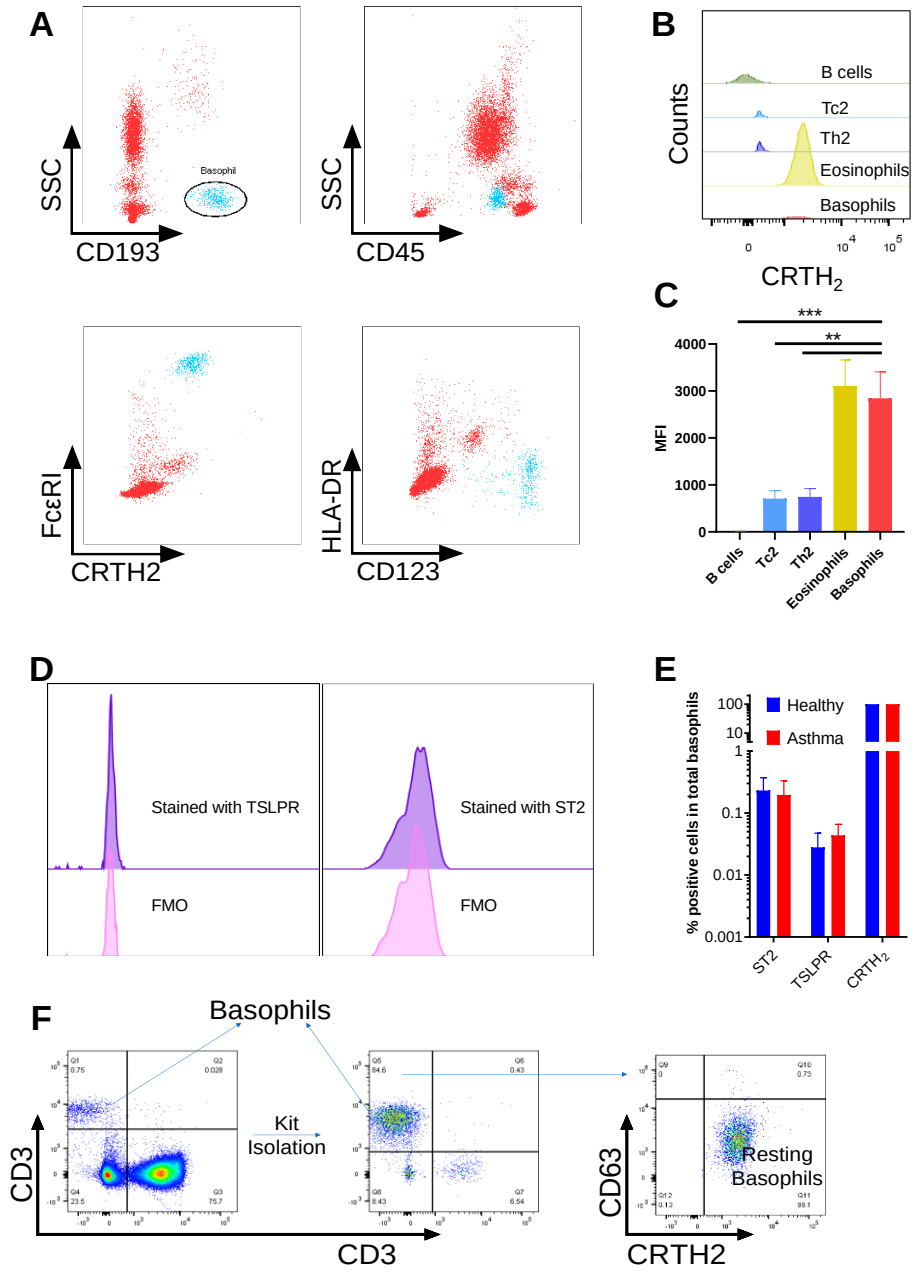


Fig 4.1 Pan markers of basophils in human blood.

A. Expression levels of CD193, FcεRI, CRTH$_2$, CD123, CD45, and HLA-DR on blood basophils. Basophils were coloured blue, and other blood cells red. B. Comparison of mean fluorescence intensity (MFI) among B cells, Tc2, Th2, eosinophils and basophils. C. Expression levels of CRTH$_2$ among different type 2 cells presented by MFI in blood from asthma. D. Comparison of MFI between stained (in purple) against TSLPR or ST2 and unstained (in pink) basophils in blood. E. Expression levels of ST2, TSLPR and CRTH$_2$ presented by a percentage of positively expressed basophils in total basophils from healthy and asthma. F. Purity of basophils in the procedure of basophil isolation (left: basophil-enriched fragment after gradient isolation, mid: cells after MACS beads kit isolation, right: level of CD63 in purified basophils). n=5 for C. n=24 for E.

I then used the expression profile of basophils to define basophils as $SSC^{low}CD193^{+}CD123^{+}HLA\text{-}DR^{-}CRTH_2^{+}$ cells. The blood result from the cohort study illustrated those basophils, presented by absolute cell number (nB) or relative cell percentage in total leucocytes (%B), were significantly elevated in patients with severe eosinophilic asthma compared with healthy controls ($p < 0.001$ in nB and $p < 0.05$ in %B) or severe non-eosinophilic asthma ($p < 0.01$ in nB and $p < 0.001$ in %B) (Fig 4.2A). Despite no significance ($p = 0.16$), the absolute cell number in patients with severe eosinophilic asthma also relatively increased compared to that in patients with mild-to-moderate asthma. So did the absolute cell number in patients with mild-to-moderate compared to healthy donors ($p = 0.29$). The percentage of basophils was also significantly higher in severe eosinophilic asthma compared to mild-to-moderate eosinophilic asthma ($p < 0.05$). Oral administration of corticosteroids relatively abolished (on the border of the significance in %B, $p = 0.06$) the enrichment of basophils in the blood of patients with severe eosinophilic asthma.

The increase of the peripheral blood basophils showed a trend of association with the severity of asthma and eosinophilia (Fig 4.2B). The amount (nB and %B) of basophils was well correlated with eosinophil number in blood but interestingly not with the blood IgE (Fig 4.2C), which indicated that basophils might participate in the pathogenesis of asthma in an IgE-independent manner.

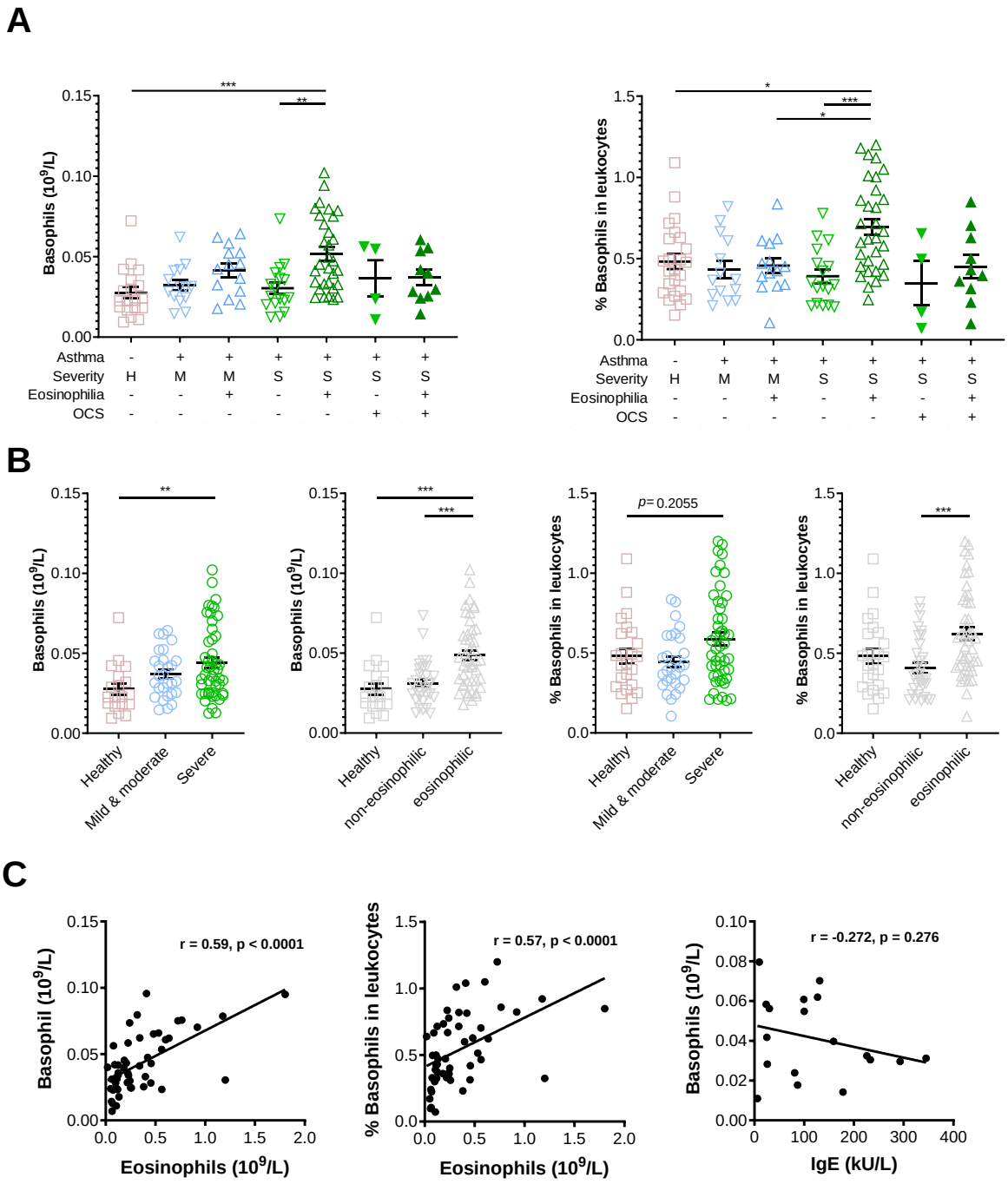


Fig 4.2 Comparison of basophil frequencies in different phenotypes of asthma.
A. Basophil counts (left) or percentage in total leucocytes (right) in healthy donors and patients from different asthma groups with or without OCS treatment determined with flow cytometry. B. Basophil counts or percentages in total leucocytes in healthy donors or patients with different severity or various levels of blood eosinophils. C. Correlation of levels of basophils in blood and blood eosinophil counts or blood IgE concentrations. *$p < 0.05$, **$p < 0.001$, and ***$p < 0.0001$.

4.3.2. Migration of basophils regulated by PGD_2

To perform *in vitro* assays with purified basophils, I established and optimised the methods of basophil isolation. $CD193^{+}FcεRI^{+}CRTH_2^{+}CD123^{+}HLA\text{-}DR^{-}$ basophils were isolated from human leukocyte cone using Percoll gradient centrifugation and MACS beads purification. The purity was over 85%, and the yield reached at least one basophil purified from 1000 leukocytes in general. 99% of isolated basophils expressed low levels of CD63 (Fig 4.1D), suggesting that they were resting basophils and not activated during the isolation procedure.

Since basophils are enriched in the sputum of eosinophilic asthma (Brooks *et al.*, 2017), I first examined the functions of PGD_2 on the cell migration and adhesion that could potentially contribute to basophil recruitment to the lung. Basophil migration induced by PGD_2 was mediated explicitly by $CRTH_2$, as chemotaxis in response to PGD_2 (100 nM) was robust and inhibited by $CRTH_2$ antagonist TM30089. Neither DP1 agonist BW245C nor DP1 antagonist BW868C showed effects on the cell migration (Fig 4.3A, upper panel). Fevipiprant inhibited basophil migration induced by PGD_2 (100 nM) in a dose-dependent manner with IC_{50}=1.5±2.2 nM (Fig 4.3A, bottom panel). In addition to chemotaxis, I also measured cell aggregation in culture *in vitro*. Basophils treated with high doses of PGD_2 (0.1-10 μM) quickly formed aggregates within 30 minutes and lasted for 2 hours, which was inhibited by fevipiprant (Fig 4.3B).

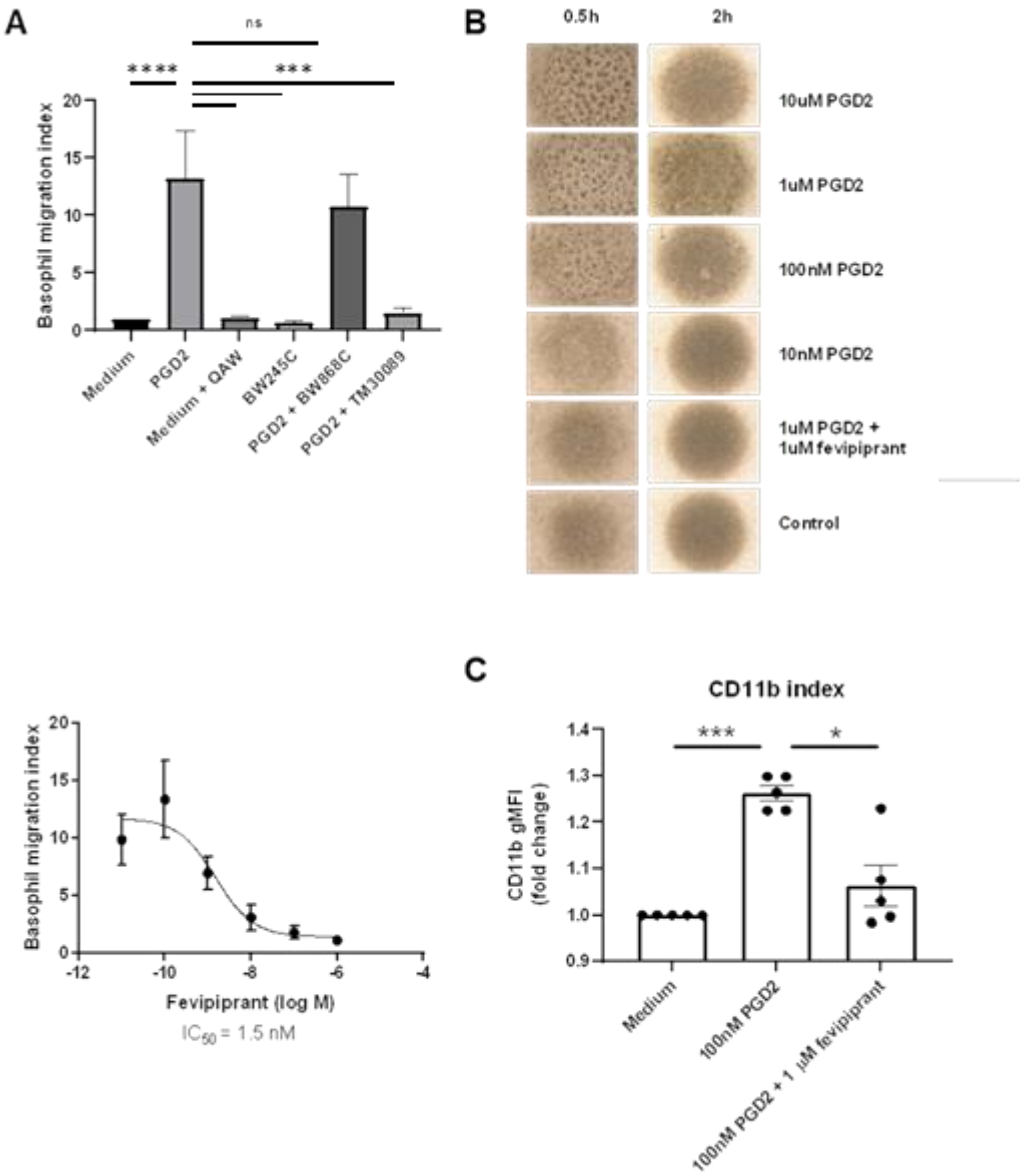


Fig 4.3 PGD_2-mediated cell migration, aggregation and adhesion in purified basophils were inhibited by $CRTH_2$ antagonism.

A. Basophil cell migration to 100 nM PGD_2 in the presence or absence of 1 μM BW245C (DP1 agonist), 1 μM BW868C (DP1 antagonist), 1 μM TM30089 ($CRTH_2$ antagonist) (upper) or various concentrations of fevipiprant (lower) determined by chemotaxis assay. The data are presented as the migration index of luminescence intensity divided by the untreated samples. B. Basophill aggregation after incubation with various concentrations of PGD_2 in the absence or presence of 1 μM fevipiprant at 0.5 or 2 hours recorded under microscopy. C. Level of CD11b in basophils after treatment with medium (unstimulated) or PGD_2 in the absence or presence of fevipiprant examined with flow cytometry. The data are presented as the fold change of mean fluorescence intensity to the untreated (medium) samples. * $p < 0.05$, *** $p < 0.001$; n=6 for A, n=5 for C.

I next examined the effect of PGD_2 on basophil migration *ex vivo* by measuring the adhesion molecule CD11b in whole blood. Basophils in the blood expressed increasing levels of CD11b after administration of PGD_2 (100nM) in whole blood, whilst this upregulation was suppressed by the addition of fevipiprant (1 μM, Fig 4.3C). Attempts have been made to measure the shape change of basophils by flow cytometry, but no apparent differences could be observed between control and PGD_2-stimulated basophils in human blood (Appendix Fig 4.1).

4.3.3. Cytokine production and degranulation in basophils induced by PGD_2

PGD_2 has been proven to be a potent inducer of type 2 cytokine production in various $CRTH_2^+$ type 2 immune cells, including Th2, Tc2 and ILC2 cells. However, it is still unclear whether PGD_2 has such effects on cytokine production in basophils, where the expression level of $CRTH_2$ is much higher than the above type 2 cytokine producing cells. Since basophils have the capacity to produce IL-4 and IL-13 in an IgE-dependent pathway (Sawaguchi *et al.*, 2012), I examined the impact of PGD_2 on the IL-4/13 production in blood basophils.

Basophils isolated from human blood were stimulated with a series of concentrations of PGD_2 for 2 hours for mRNA analysis or for 4 hours for protein analysis. PGD_2 increased IL-4 and IL-13 expression at both the levels of mRNA (EC_{50}= 181 nM for *IL4* and 321 nM for *IL13*) and protein (EC_{50}= 169 nM for IL-4 and 378 nM for IL-13) in a dose-dependent manner (Fig 4.4A). The production of IL-5 was undetectable.

This PGD_2-induced IL-4/13 production in basophils was explicitly mediated by $CRTH_2$ as the reaction was repressed entirely with 1 μM TM30089 (Fig 4.4B). Similar to the responses in

migration assays, the DP1 pathway did not show an effect on the IL-4 and IL-13 production in basophils. Neither did fevipiprant alone (1μM) impact the production (Fig 4.4B). This IgE-independent PGD_2-induced activation of basophils led to similar levels of IL-4 and IL-13 production induced by PGD_2 (1μM) to those induced by IgE/anti-IgE (0.5 μg/ml) stimulation (Fig 4.4B). On average, every million basophils produce 5.0 pg IL-4 and 12.1 pg IL-13 with PGD_2 (1μM) stimulation and 5.2 pg IL-4 and 14.8 pg IL-13 with IgE/anti-IgE (0.5 μg/ml) stimulation.

Fevipiprant blocked the cytokine production induced by PGD_2 (1 μM) in a dose-dependent manner at both mRNA (IC_{50} = 2.37 nM for *IL4* and 0.97 nM for *IL13*) and protein (IC_{50} = 0.40 nM for IL-4, and 7.18 nM for IL-13) levels (Fig 4.4C). Meanwhile, fevipiprant *per se* did not promote the IL-4 or IL-13 production as applying 1μM fevipiprant to the basophils showed no increase of IL-4 or IL-13 at both mRNA and protein levels compared to control (Fig 4.4B).

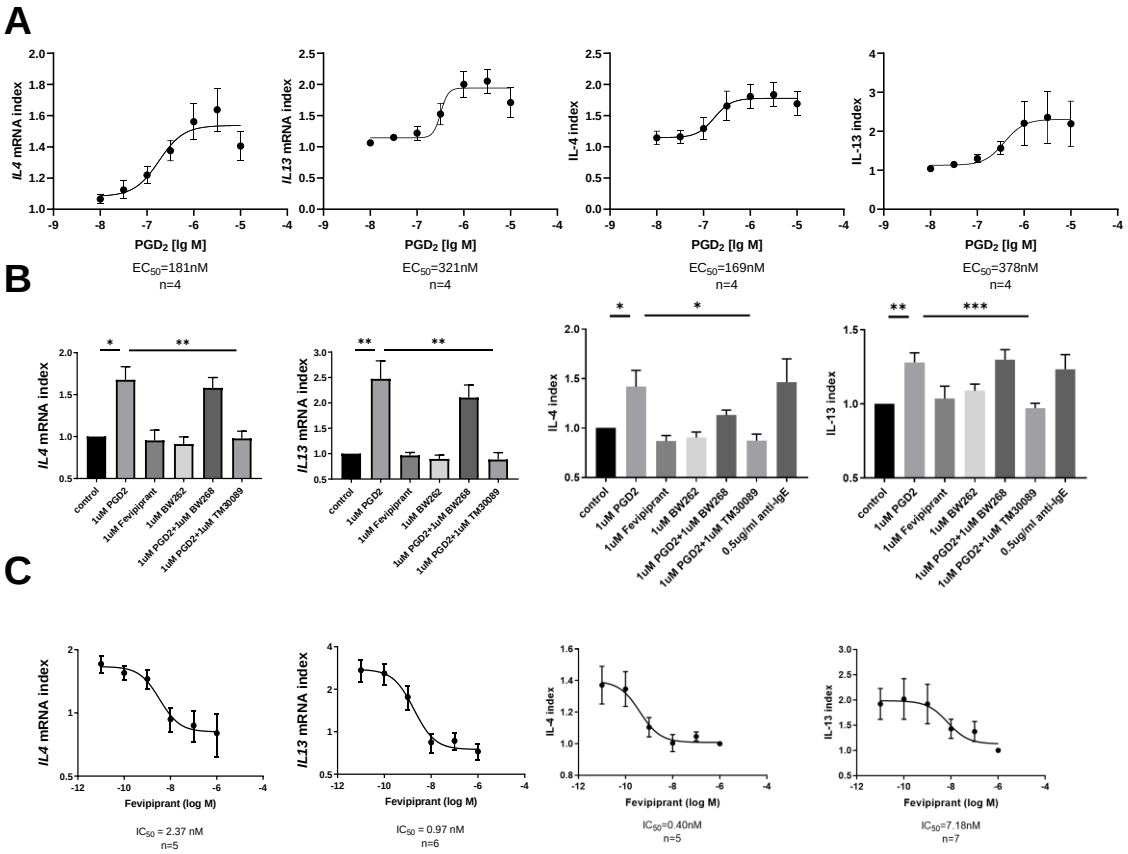


Fig 4.4 PGD_2-induced type 2 cytokine production in basophils was mediated by $CRTH_2$.
A-C. mRNA or protein levels of IL-4 and IL-13 in basophils after treatments with various concentrations of PGD_2 (A) or with 1 μM PGD_2 in the presence or absence of BW245C, BW868C, TM30089 (B) or various concentrations of fevipiprant (C) measured with qPCR or ELISA respectively. The mRNA index data are presented as the fold change $\left([2^{-(Cq_{target\ gene}-Cq_{GAPDH})}]_{treated} \div [2^{-(Cq_{target\ gene}-Cq_{GAPDH})}]_{control}\right)$. The protein index data are presented as the fold change of concentration $(C_{treated} \div C_{control})$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; n=4 for A-C.

As mentioned in the introduction, one of the well-known biological functions of basophils in allergy and asthma is to release pro-inflammatory mediators by degranulation in response to IgE-induced FcεR1 crosslinking (Stone, Prussin and Metcalfe, 2010). It has been reported that eosinophil degranulation could be induced by PGD_2 (Gervais et al., 2001). In my study, I also examined the effect of PGD_2 on basophil degranulation.

Fresh human blood was incubated with PGD_2 (100 nM) in the absence or presence of fevipiprant (1 µM) for 20 minutes, and the degranulation markers of basophils, CD63 (anaphylactic degranulation) and CD203c (piecemeal degranulation) (Eberlein *et al.*, 2015; Hemmings *et al.*, 2018) were measured by flow cytometry. Both levels of CD63 and CD203c on basophils were significantly upregulated by PGD_2, which was significantly inhibited by fevipiprant (Fig 4.5A).

Histamine release is a pan marker of basophil degranulation (Stone, Prussin and Metcalfe, 2010). Increasing amounts of histamine were detected following 1 µM PGD_2 treatment, which was significantly suppressed by the addition of 1 µM fevipiprant (Fig 4.5B). It suggested that PGD_2/$CRTH_2$ axis could not only activate basophils to produce pro-inflammatory cytokines but also cause basophil degranulation to fast-release cytotoxic components from their granules.

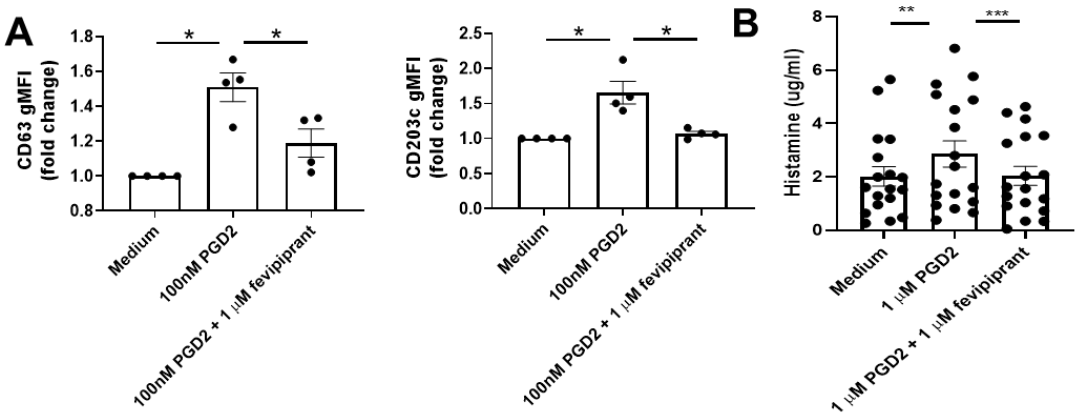


Fig 4.5 PGD_2-induced basophil degranulation was mediated by $CRTH_2$.
A. Levels (mean fluorescence intensity) of CD63 and CD203c in basophils after PGD_2 (100 nM) stimulation in the absence or presence of fevipiprant (1 µM) examined with flow cytometry. The data are presented as the fold change of mean fluorescence intensity to the untreated (medium) samples. B. Levels of histamine in the supernatants of basophils after treatments with PGD_2 (1 µM) in the absence or presence of fevipiprant (1 µM) measured with ELISA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; n=4 for A, n=18 for B.

4.3.4. Basophil transcriptome and PGD_2 regulation

To undertake a more comprehensive investigation of the role of the PGD_2/$CRTH_2$ axis in basophils, I performed basophil RNA-seq with 4 conditions (6 samples for each): 1) control; 2) treated with 1 µM PGD_2 (PGD_2); 3) treated with 1 µM PGD_2 and 1 µM fevipiprant (Fevi); and 4) treated with 0.5 µg/ml anti-IgE (IgE) for 4 hours. Basophils were isolated from the leukocyte cone, and RNAs were purified from the cells after the above treatments for RNA sequencing. Because all cone samples were anonymous from the National Blood Service, and no clinical information was available, not even gender, height, weight or age. Therefore, the sample number was increased to 6 instead of 3, and all samples were collected within two days to minimise the variations.

First, differential expression analysis was used to decipher the crucial differences between PGD_2-stimulated and unstimulated samples. PGD_2 group significantly and differentially ($|log2FC| > 2$) expressed 18 genes compared with unstimulated control (16 genes). (Fig 4.6, upper). The top 10 genes enriched in each condition were listed below (Table 4.2, left). The low expressed genes (baseMean no more than one) were filtered out as too few transcripts might not represent meaningful biological functions. Although previous data showed that PGD_2 induced IL-4 and IL-13 expression in basophils, the changes recorded in RNA-seq were significant but relatively small (FC = 1.1 for *IL4* and 1.3 for *IL13*) between the control and PGD_2 groups. Interestingly, PTGS2 was upregulated in PGD_2-stimulated basophils compared to unstimulated basophils.

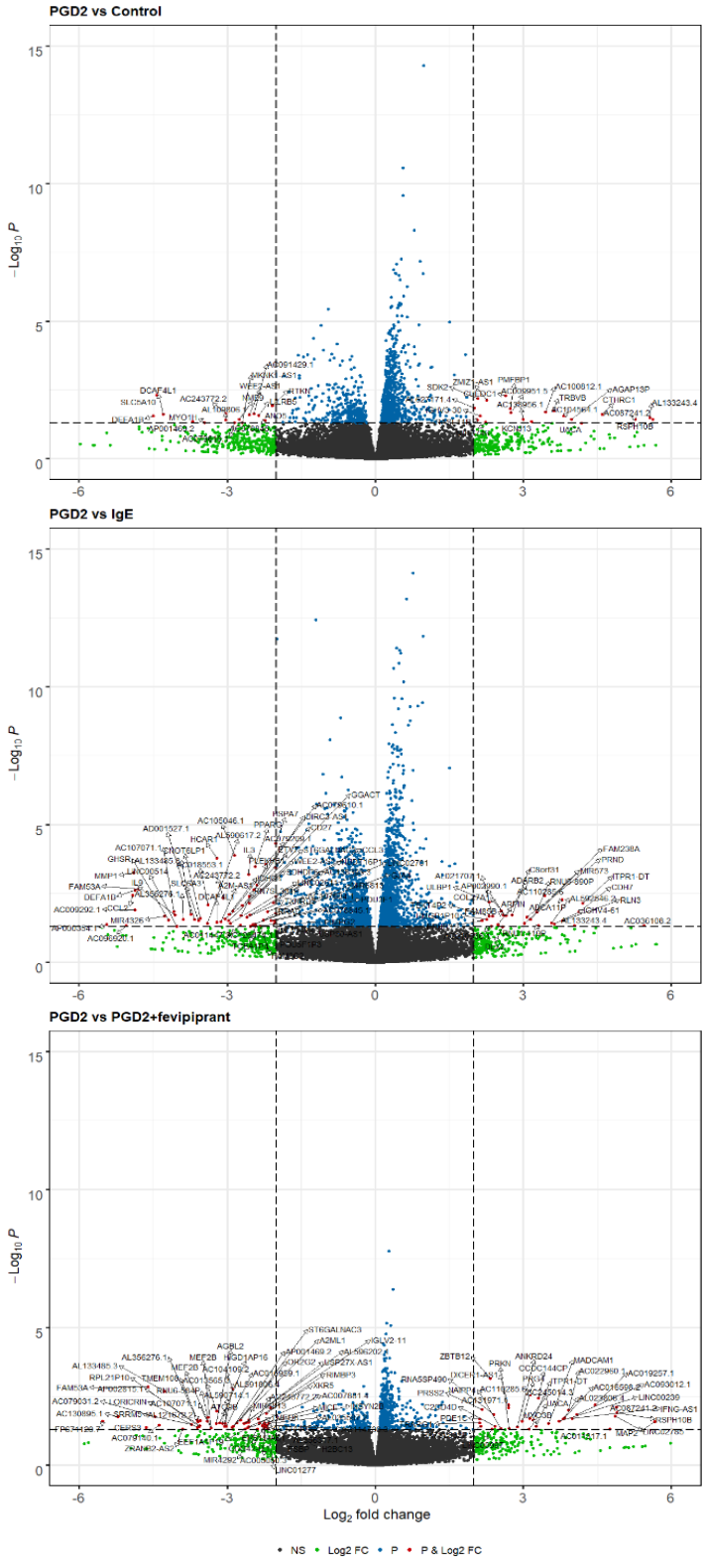


Fig 4.6 Differential expression analysis of RNA-seq from isolated basophils after treatment with 1 µM PGD_2 in the presence or absence of 1µM fevipiprant or 0.5 µg/ml anti-IgE.
Genes were highlighted in blue ($p < 0.01$), green ($|\log_2FC| > 2$) or red (matched both conditions) in the comparison between PGD_2 and control (upper), IgE and PGD_2 (middle), and PGD_2 and PGD_2 with fevipiprant (lower).

Table 4.2 Top genes from differential expression analysis

Control vs PGD_2				PGD_2 vs IgE			
Control	log2FC	PGD_2	log2FC	IgE	log2FC	PGD_2	log2FC
DEFA1B	-4.50	UACA	4.19	MMP1	-3.60	CCDC80	3.08
SLC5A10	-4.43	KCNJ13	3.17	CCL2	-3.39	AC093512.2	3.08
MYO1H	-3.47	PMFBP1	2.74	GPR22	-3.26	SLC5A10	2.82
NME9	-2.76	CUEDC1	2.64	KCNJ13	-2.70	GAS2	2.53
LILRB5	-2.36	SDK2	2.08	HCAR1	-2.64	ZNF221	2.40
ANO5	-2.26	RASL11B	2.07	COL15A1	-2.34	CTXN1	2.25
RTKN	-2.09	PTGS2	2.01	IL3	-2.23	SLC6A12	2.10
				PPARG	-2.09	SLC25A23	2.08
				CCL3L1	-2.08	PTGS2	2.00

Genes were filtered by $p < 0.05$, $|log2FC| > 2$ and baseMean > 1, and ordered by $|log2FC|$.

I did pathway enrichment analysis to further characterise the clustered pathways of differentially expressed genes. Genes that were upregulated in the PGD_2 group compared with the control mainly play roles in type 2 related pathways, including “IL−4 and IL−13 signalling”, “MAPK pathway”, “p75NTR recruits signalling complexes”, “RAF−independent MAPK1/3 activation” and “signalling by interleukins” according to the Reactome database. On the other hand, unstimulated basophils presented type 1 favoured pathways: “interferon gamma

signalling" and "interferon signalling" (Fig 4.7). This suggested that PGD_2-derived basophil activation might be highly relevant to type 2 immune responses.

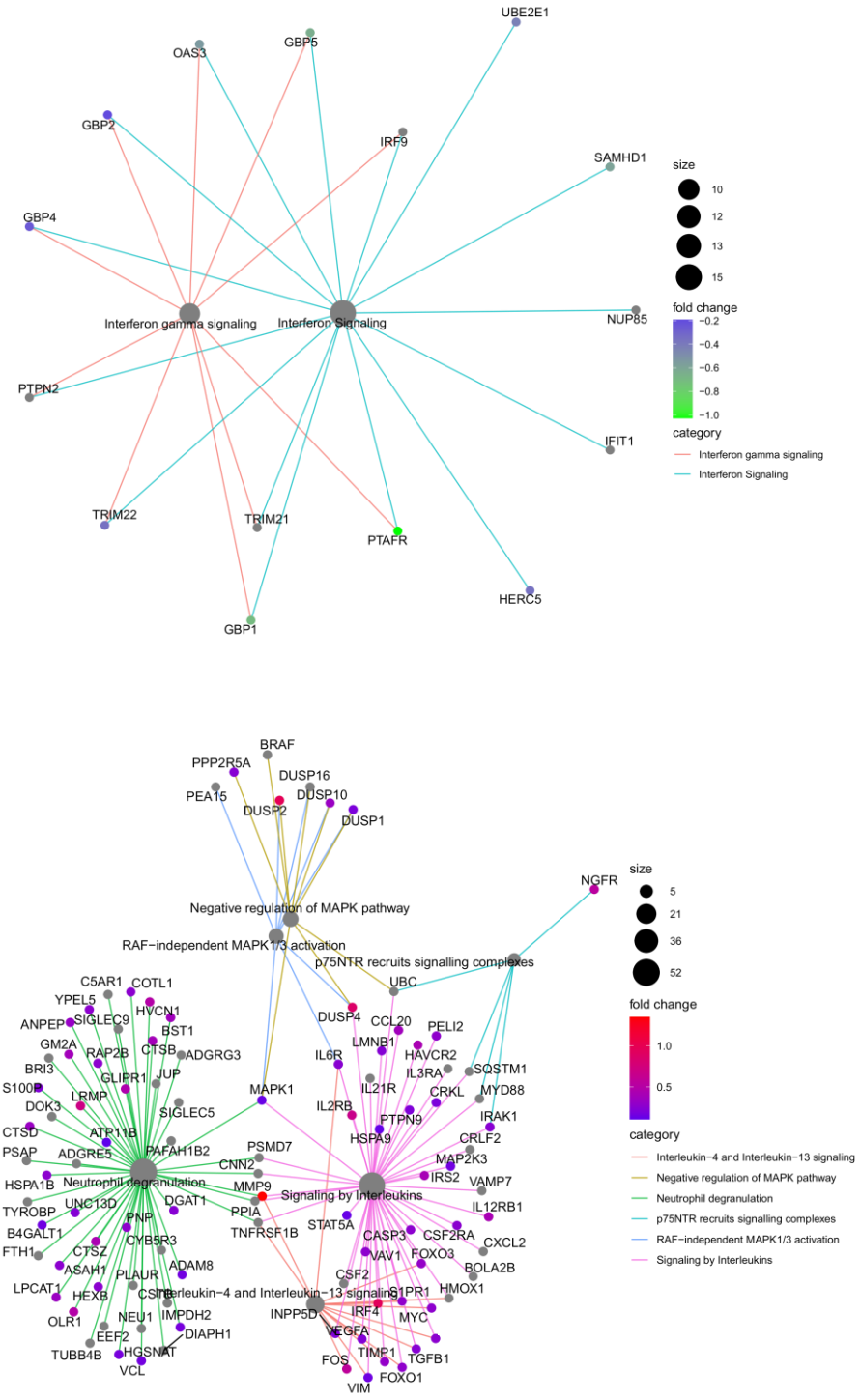
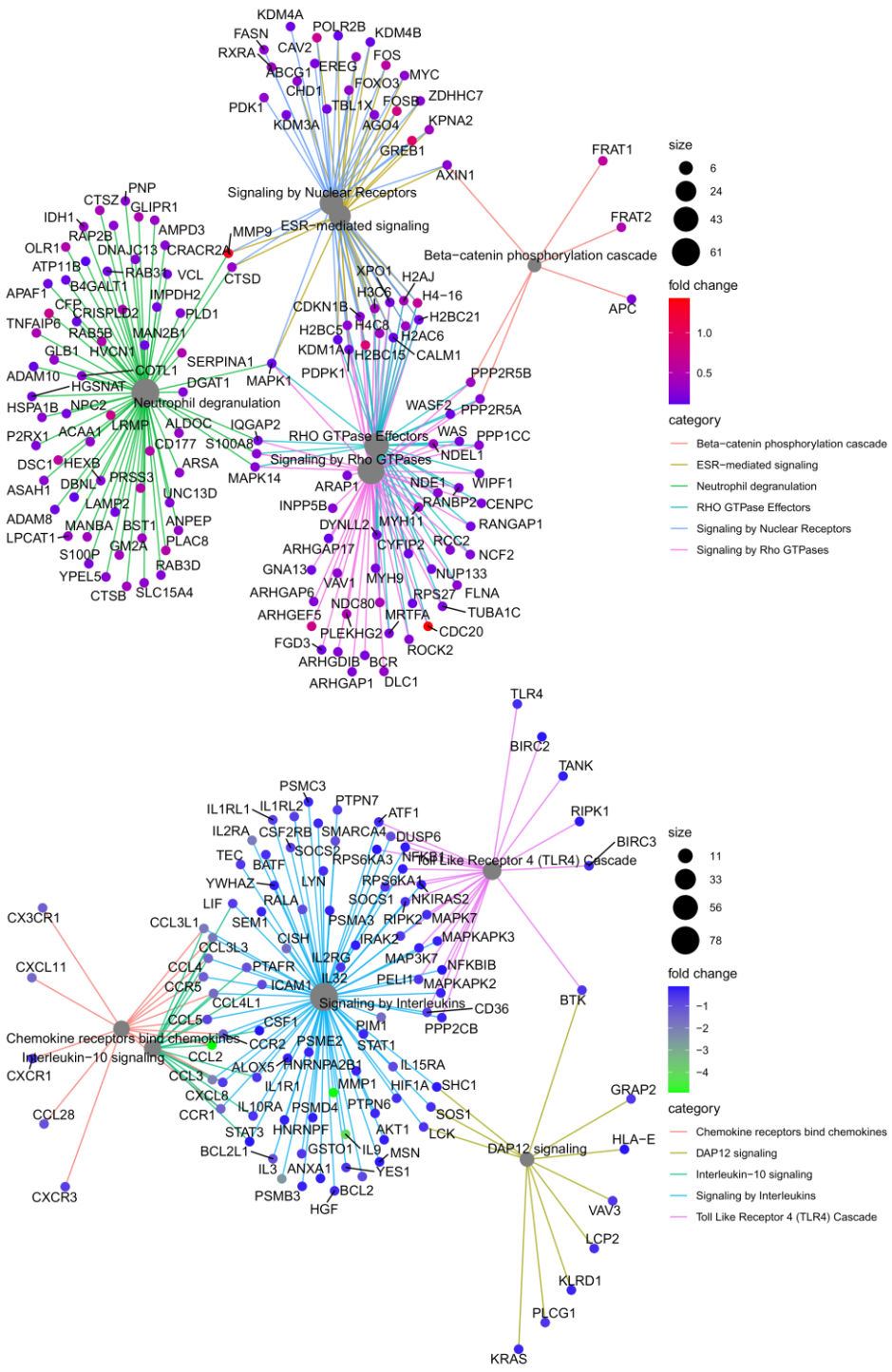


Fig 4.7 Correlations between enriched pathways in the comparison of control (upper) and PGD_2 (lower) and related genes.
Every big and grey node represents a pathway sized by the number of differentially expressed genes in the pathway. Each small node represents a gene coloured by the value of log2FC. Every link represents a gene related to the pathway coloured by the name of the pathway.

Since the typical allergic reaction mediated by IgE-dependent basophil activation is also associated with type 2 immunity, further to dissect the differences between IgE and PGD2-induced basophil activation, I performed differential expression analysis comparing anti-IgE- and PGD_2- treated samples (Fig 4.6, middle). 24 genes were enriched in the PGD_2 group compared with the IgE group (21 genes). Like the previous comparison, the top 10 genes enriched in each condition were listed below (Table 4.2, right). The low-expressed genes (baseMean no more than one) were filtered out. PTGS2 was also upregulated in PGD_2-stimulated basophils compared to anti-IgE-stimulated basophils.

I compared the signal pathways composed of differentially expressed genes in IgE and PGD_2 groups. As expected, IL-4 and IL-13 signalling genes were upregulated in both stimulation groups and resulted in no significant differences in the comparison. However, a few type 2 related genes, including CSF1, CSF2RB and IL9, were only upregulated in the IgE group (Fig 4.8). Interestingly, different signalling pathways were enriched in these two groups. Basophils activated by IgE expressed higher levels of many non-type 2 cytokines and chemokines as well as receptors (**Fig 4.8** low panel) compared to those activated by PGD_2 (**Fig 4.8** upper panel). These IgE-regulated cytokines (such as IL3) and cytokine receptors (such as IL1R1, IL2RA, IL15RA, etc.) are involved in the pathways of “signalling by interleukins” and “interleukin−10 signalling” (Fig 4.8 lower panel); the chemokines (such as CCL2, CCL3, CCL5, etc.) and

chemokine receptors (such as CX3CR1, CCR5, CCR1, etc.) were associated with the pathway of "Chemokine receptors bind chemokines" (Fig 4.8 lower panel), which suggested that, compared to PGD_2-induced activation, IgE might cause broader and more general immune responses in basophils, but PGD_2 might only contribute to type 2 responses in basophils. PGD_2 stimulation led to the upregulation of genes involved in the pathways of "signalling by Rho GTPases", "ESR (estrogens) and nuclear receptors", and "beta−catenin phosphorylation cascade" (Fig 4.8 upper panel), which might be related to metabolism. The pathway of "neutrophil degranulation" (Reactome DOI: 10.3180/R-HSA-6798695.1) is the only degranulation pathway among all granulocytes in the Reactome database. Although the contents of granules may be distinct (Uciechowski and Rink, 2018), neutrophils and basophils have similar procedures of the exocytosis of granule lumen or granule membrane proteins (Lacy, 2006; Cromheecke, Nguyen and Huston, 2013), mechanistically. This pathway may link to basophil-related degranulation activities (Fig 4.8 upper panel).



1240

1241 **Fig 4.8 Correlations between enriched pathways involved in the genes regulated by PGD_2 (upper**
1242 **panel) and IgE (lower panel) in basophils detected with RNA-seq.**
1243 **Every big and grey node represents a pathway sized by the number of differentially expressed**
1244 **genes in the pathway. Each small node represents a gene coloured by the value of log2FC. Every**
1245 **link represents a gene related to the pathway coloured by the name of the pathway.**

To further investigate the consistency and uniqueness of the effect of PGD_2 in basophils compared to that in other type 2 cells, I compared the differential gene expression by PGD_2 vs control among different cell types, including basophils, eosinophils, Th2 and Tc2 cells. I used previously published eosinophil (Sippel *et al.*, 2018), Tc2 (Hilvering *et al.*, 2018), and Th2 (Xue *et al.*, 2015) microarray data as well as unpublished Tc2 RNA-seq data generated in our group. All lists of PGD_2-upregulated genes in the condition of PGD_2 vs control were extracted. According to the Venn diagram (Fig 4.9A), basophils shared 5 genes with Tc2 (RNA-seq: LAYN, XCL1, MMP9, DUSP4, UACA), 2 genes with Th2 (XCL1, DUSP4) and 1 gene with Tc2 (microarray: DUSP4). Among basophil, Tc2 (RNA-seq) and Th2, there were two common genes, XCL1 and DUSP4, upregulated by PGD_2, suggesting these genes might contribute to a general pathway of PGD_2-induced cell activation. However, no gene was shared between eosinophils and basophils. On the other hand, there were still 32 genes that only appeared in basophils, suggesting that these genes might be associated with the unique activities of basophils. To better understand the function of these genes, I performed pathway enrichment analysis (Fig 4.9B, Fig 4.10) using the unique genes that appeared only in one cell type (Fig 4.9A, 93 in Th2, 425 in Tc2 RNA-seq, 32 in basophil and 157 in eosinophil dataset). The most significant pathways obtained from the basophil dataset were “IL12 signalling” (BOLA2, BOLA2B), “factors involved in megakaryocyte development and platelet production” (DOCK6, GATA6, KIF2C), and “Cell-Cell communication” (PARD6B, SDK2). Among these genes, the upregulation of SDK2 was the most obvious (log2FC=2.07). There were four other genes, KCNJ13, PMFBP1, CUEDC1, and RASL11B, that did not fall into any pathways but were still highly upregulated (log2FC > 2) by PGD_2 in basophils. Further study on the functions of these

genes in the future might provide a better understanding of the uniqueness of PGD_2-induced activation on basophils. No prominent type 2 pathways, such as "signalling by interleukins and IL-4 and IL-13 signalling", were observed in the pathway comparison with Th2 microarray or Tc2 RNA-seq data sets, indicating that the upregulation of type 2 related genes by PGD_2 in basophils was not as strong as those in Th2 and Tc2 cells (Fig 4.10). Eosinophils also presented somewhat different profiles of pathways related to the metabolism of carbohydrates.

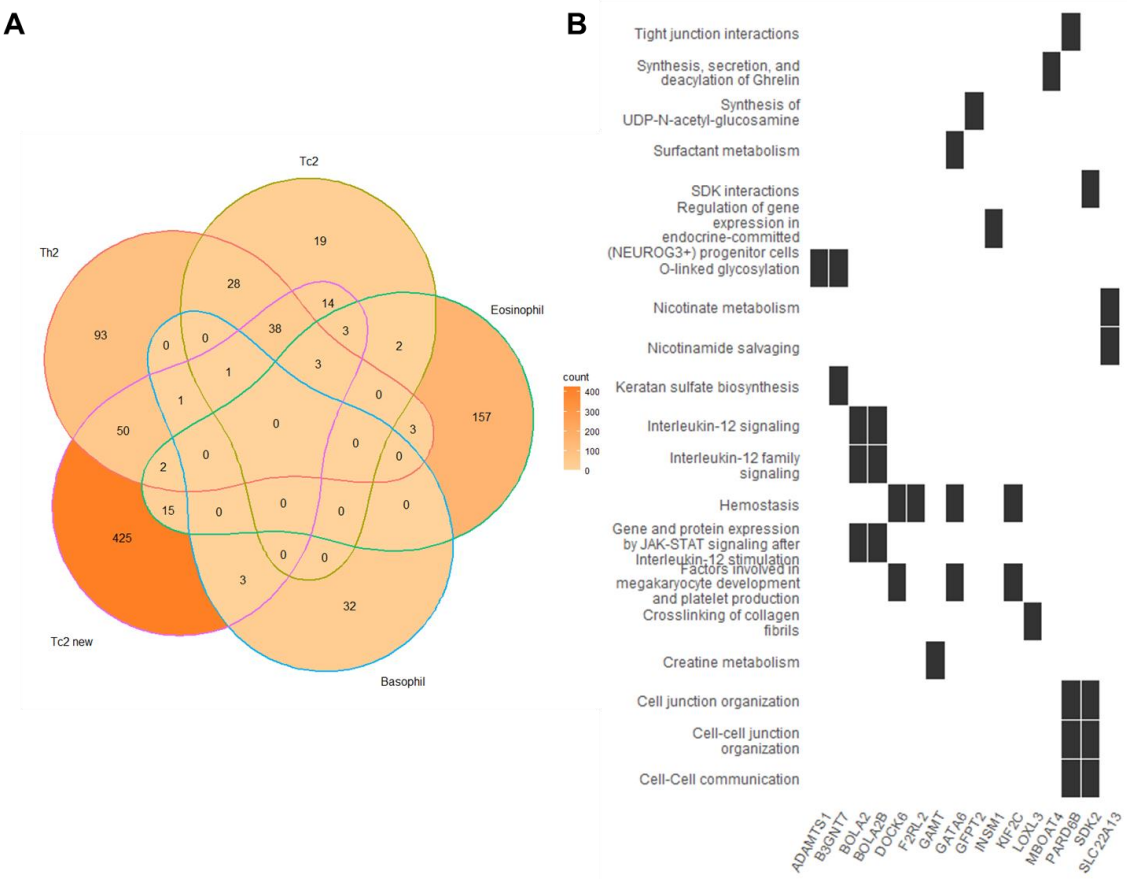


Fig 4.9 Comparison of PGD_2-upregulated genes among different type 2 cell types.
A. Venn diagram of the common or unique genes regulated by PGD_2 among RNA-seq data sets from basophils and Tc2 (new) generated in-house, and microarray data from Th2, Tc2 and eosinophils (published). The grape was shaded in the number of genes. B. Heatmap of top pathways analysed from 32 PGD_2-upregulated genes uniquely detected in basophils.

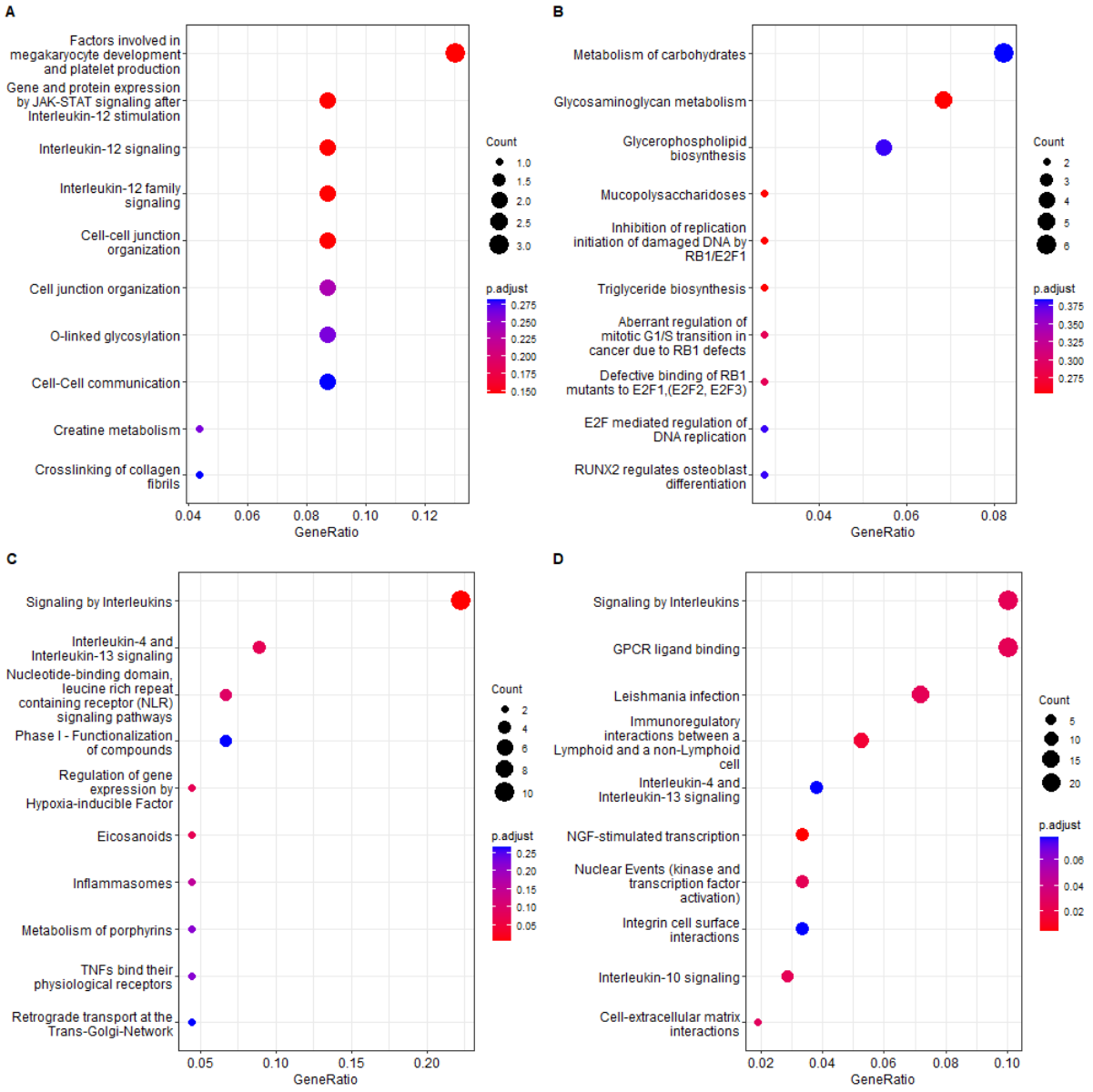


Fig 4.10 Enriched pathways analysis of PGD_2-upregulated genes.
A-D. The genes were uniquely detected in basophils (A), eosinophils (B), Th2 (C) or Tc2 (D) cells during the cell type comparison in Figure 4.9. The pathways were ordered by the counts of differentially expressed genes in the pathway and coloured by the p value using the Reactome database.

To further confirm that the effect of PGD_2-induced gene regulation is mediated via $CRTH_2$, I analysed the condition Fevi in the RNA-seq (Fig 4.6, lower). Expression of type 2 signature genes *IL4* (log2FC=0.1, $p < 0.05$) and *IL13* (log2FC=0.5, $p < 0.05$) was downregulated by Fevi

compared with those in the condition of PGD_2, which confirmed the pattern of CRTH2-specific PGD_2-modulated type 2 cytokine production on basophils.

Interestingly, all genes upregulated by PGD_2 (Table 4.2) were suppressed by fevipiprant, at least partially (Fig 4.11). Among them, the expression of *PTGS2* and *UACA* were almost completely blocked (log2FC=2.0 and 3.3, respectively, $p < 0.00001$) in Fevi, suggesting that the regulation of *PTGS2* and *UACA* by PGD_2 are entirely mediated by $CRTH_2$.

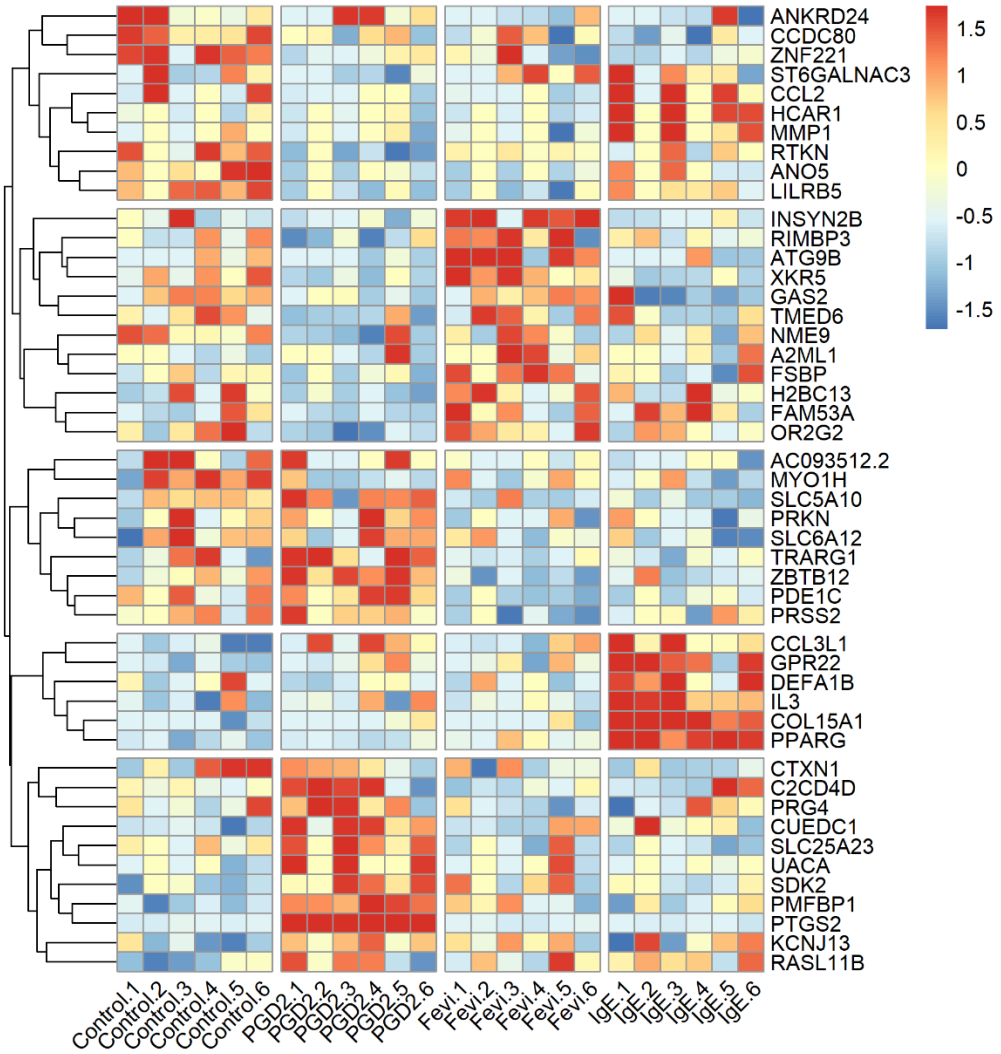


Fig 4.11 Heatmap of top genes in each condition.
Genes that had $p < 0.05$, $|log2FC| > 2$ and baseMean > 1 in any of 3 comparisons (1. Control vs PGD_2, 2. PGD_2 vs IgE, 3. PGD_2 vs Fevi) were selected. Each cell is coloured by the Z-score.

4.4. Discussion and conclusions

Basophils are essential in allergic diseases, including allergic asthma, via IgE-dependent activation (Macfarlane *et al.*, 2000; Marone, Triggiani and De Paulis, 2005). However, whether basophils influence the pathogenesis of asthma in an IgE-independent pathway or not is not well-studied. In this chapter, I observed enriched $CRTH_2^{high}$ blood basophils in severe eosinophilic asthma and revealed the functions of the $PGD_2/CRTH_2$ axis in the basophil activations, including cell migration, adhesion, cytokine production and degranulation. I also surmised the gene profile contributing to PGD_2-induced basophil activation. These findings indicate the potential roles of PGD_2 in the pathogenesis of asthma via basophil activation.

Although it was suggested that sputum basophil counts could be used as a biomarker for eosinophilic asthma due to the increase of airway basophils (Suzuki *et al.*, 2017), the profile of basophils in blood from asthma is unclear. Basophils are a small population in human blood among the main types of leucocytes, so the clinical blood counts of basophils usually turn out to be near zero. Therefore, we could not correctly interpret the impact of basophils in asthma using the basophil counts from whole blood tests. I managed to apply flow cytometry and observed the elevation of blood basophils in severe eosinophilic asthma, and correlated with the disease severity and eosinophil counts, suggesting that blood basophils could also be a biomarker to determine severe eosinophilic asthma. However, further investigation is required to quantify whether “basophilia” is also valuable for the clinical setting and to determine the threshold of basophilia in asthma.

Nevertheless, the fact that the enrichment of blood basophils in severe eosinophilic asthma was not correlated with blood IgE level suggested that basophils could play roles in type 2 inflammation in an IgE-independent manner. Alarmins IL-33 and TSLP are considered drivers of IgE-independent basophil activation, which contributes to type 2 inflammation (Pecaric-Petkovic *et al.*, 2009; Siracusa *et al.*, 2011). However, in this study, I have found that blood basophils consistently expressed $CRTH_2$ but not ST2, the receptor for IL-33, or TSLPR, the receptor for TSLP. Without expression of the receptors, it is hard to imagine how these alarmins function in human blood basophils, even after migration to the airways, and whether these receptors could develop in these short-lifespan basophils or not. Instead, $CRTH_2$ is known to bind PGD_2 and regulate the activation of Th2 (Xue, Barrow and Pettipher, 2009), Tc2 (Hilvering *et al.*, 2018) and ILC2 (Xue *et al.*, 2014), in consequence of IL-4, IL-5 and IL-13 release. To date, little is known about the role of the PGD_2/$CRTH_2$ axis in basophils except that PGD_2 induces basophil chemotaxis (Hirai *et al.*, 2001). That is the rationale of my study to investigate the functions of PGD_2 and $CRTH_2$ in basophils. I not only confirmed the migratory effect of PGD_2 but also revealed the functions of PGD_2 in mediating IL-4 and IL-13 production and promoting cell degranulation in human basophils. Those two phenomena are mainly considered as consequences of allergen challenge and IgE crosslinking on basophils. Hence, those elevated levels of blood basophils might migrate into the airways after chemoattraction by PGD_2 and then are activated in a PGD_2-induced IgE-independent manner in the lung, leading to inflammation and tissue remodelling (Crivellato, Travan and Ribatti, 2010).

To better mimic the type 2 cytokine release from basophils under physiological conditions, *ex vivo* simulation using whole blood and cytokine intracellular staining approach has been used as what had been done in Chapter 3 for Tc2 cells. However, due to the pandemic of COVID-19, we had difficulty collecting blood samples, and the experiment had to be postponed in this study until we resumed our capacity to recruit patients.

Histamine is a well-known pro-inflammatory mediator released from basophil and mast cell degranulation (Thangam *et al.*, 2018) induced by IgE crosslinking or other factors like IL-3, which evoke bronchoconstriction in asthma (Dale and Laidlaw, 1919). My data demonstrated that PGD_2 could promote histamine release from basophils suggesting that PGD_2 might directly influence the smooth muscle cells and contribute to the bronchoconstriction associated with acute asthma exacerbations. Although *in vitro* treatment of fevipiprant inhibited the histamine release induced by PGD_2 from basophils, $CRTH_2$ antagonism might only prevent part of the overall histamine release, i.e. that mediated by $PGD_2/CRTH_2$ but not total histamine release in patients, which is evidenced by the primary outcome (annual exacerbation rate versus placebo) of a phase 3 clinical trial of fevipiprant which only reached borderline significance in eosinophilic asthmatic patients (p = 0.06). Compared with IgE crosslinking or IL-3, PGD_2 might not be a robust stimulator for basophil degranulation. In general, each basophil contains around 3 pg histamine (Yamaga *et al.*, 2020). In my experiments, 3 µg/ml histamine on average (1-7 µg/ml) could be detected in a volume of 50 µl medium with half a million basophils, which is equal to 0.3 pg histamine (10% of total) per cell released. However, both IgE crosslinking (Smiljkovic *et al.*, 2017) and IL-3 (Tschopp *et al.*, 2006) could cause a 100% release of

histamine from basophils. Also, compared with Tc2 data in Chapter 3, basophils produce similar levels of IL-4 (around 5 pg/million cell) but only 2.5% of IL-13 compared to IL-13 from cultured Tc2 cells (300 pg/million Tc2 cells *vs* 12 pg/million basophils) in response to PGD_2. The comparison among different transcriptome datasets also illustrated that Th2 and Tc2 cells expressed higher levels of type 2 signature genes in response to PGD_2 than basophils. In summary, PGD_2 widely regulated basophil activities but with a moderate degree of type 2 responses.

Although PGD_2 upregulated genes in the pathway of "IL-4/13 signalling" in basophils based on RNA-seq results, none of the signature genes in this pathway, such as IL-4, IL-13, GATA3 or STAT6, was significantly and highly expressed in basophils. IgE did not enormously change the expression of *IL4* and *IL13* as well compared to PGD_2, suggesting that basophils might not be the predominant source of type 2 cytokines. Nevertheless, basophils are already mature in the blood and have the capacity to produce type 2 cytokines. They might still play crucial roles in initiating the inflammation in the airways by releasing IL-4 and IL-13 to help the differentiation of Th2 and Tc2 from T0 cells (Fahy, 2015; Lambrecht and Hammad, 2015) and to stimulate airway epithelial cells to produce eosinophil chemokines eotaxins (Lilly *et al.*, 1997; Hilvering *et al.*, 2018). Meanwhile, as the *PTGS2* (gene for COX2) was mainly upregulated by PGD_2 but not by IgE crosslinking, basophils could potentially be a robust source of PGD_2 production like mast cells, or like Tc2 and ILC2s in an autocrine manner. It has been confirmed that basophils have the machinery for PGD_2 synthesis (Ugajin *et al.*, 2011), so further

investigation is needed to verify whether there is any autocrine production of PGD_2 from the basophil via the PGD_2/$CRTH_2$ axis.

Besides *PTGS2*, PGD_2 also specifically upregulated some other genes, such as *SLC5A10*, *SLC6A12*, *SLC25A23* and *CCDC80* (Table 4.2), which has never been recognised in previous basophil studies. *SLC5A10*, *SLC6A12* and *SLC25A23* are genes belonging to a family of solute carriers (Schumann *et al.*, 2020). SLC5A10 is a sodium/glucose transporter with a high affinity to monosaccharides, such as mannose fructose, glucose and galactose (Grempler *et al.*, 2012; Fukuzawa *et al.*, 2013). Mannose receptors and mannose were reported to facilitate type 2 inflammation (Lee *et al.*, 2002; Zhou *et al.*, 2018). Therefore, SLC5A10 in basophils induced by PGD_2 might impair the homeostasis of sugar transportation and further enhance type 2 inflammation. SLC6A12 transports betaine and γ-aminobutyric acid (GABA) (Zhou *et al.*, 2012). In the central nervous system, coregulation of GABA and PGD_2 was observed (Zhao, Ohinata and Yoshikawa, 2009; Hao *et al.*, 2021), suggesting a potential interaction between the nervous system and basophils via PGD_2. However, whether basophils contribute to GABA release needs to be confirmed. SLC25A23 is an important solute carrier mediating the ATP-magnesium transport. It is known that calcium and magnesium ions are required by PGD_2 synthesis (Inoue *et al.*, 2003), so it again suggested a potential role of PGD_2 in the autocrine PGD_2 synthesis in basophils by promoting the uptake of Mg via upregulation of SLC25A23. CCDC80 has been shown to be implicated in cell adhesiveness with ECM (Manabe *et al.*, 2008), confirming the mechanism of how PGD_2 enhances basophil migration and adhesion to the lung tissue by upregulation of CCDC80. In general, those upregulated genes by PGD_2 but not by

anti-IgE indicated specific functions of PGD_2 in basophils using an IgE-independent mechanism, which could contribute to airway inflammation.

SDK2 was significantly upregulated in PGD_2-activated basophils but not in other type 2 immune cells, including Tc2, Th2 or eosinophils. *SDK2* encodes an adhesion protein molecule Sdk2, which is required by specific synaptic connectivity in the nervous system (Yamagata and Sanes, 2012). Its expression or regulation has never been observed in basophils. My RNA-seq data indicated potential crosstalk between basophils and neurons via PGD_2-induced Sdk2 binding. Moreover, since Sdk2 could form homophilic dimers and tight conjunctions for cell connection (Tang *et al.*, 2018), basophils could utilise this molecule apart from the basophil-presenting adhesion molecules, integrins, selectins, ICAMs and PECAM for cell adhesion (Bochner *et al.*, 1994). By targeting $CRTH_2$, we might be able to block the Sdk2-mediated interaction between basophils and other cells, which could result in a reduction of basophil activation and type 2 inflammation in the disease.

The effects of PGD_2 in basophils are dominantly mediated by $CRTH_2$, as DP1 agonist and antagonist showed no effect in basophils, and $CRTH_2$ antagonist could completely remove the effects of PGD_2 in the cells. I also demonstrated that fevipiprant is a potent and specific inhibitor of the $CRTH_2$ pathway in basophils, as it did in Tc2 cells. It not only inhibited established basophil migration but also suppressed newly discovered cell adhesion, cytokine production and degranulation in basophils. This study might provide further rational design for $CRTH_2$ antagonist trials by considering not only eosinophil but also basophil numbers. In summary, this study provides a better understanding of the effects of the PGD_2/$CRTH_2$ axis in basophils

and their potential roles in the pathogenesis of severe eosinophilic asthma, which might help to identify therapeutic strategies for the disease.

Chapter 5. Increased Activation of Eosinophils in Eosinophilic Asthma and Reversal by IL-5 Inhibition

5.1. Introduction

Eosinophils are not only a biomarker indicating the severity and endotype of asthma (Yancey *et al.*, 2017) but also, as introduced in Chapter 1.2., contribute to the development of type 2 inflammation cascade, airway remodelling and exacerbation in asthma (Nakagome and Nagata, 2018). Due to the importance of eosinophils in asthma development, increasing research studies are focusing on the molecular and cellular level of eosinophil biology. There are many biological elements responsible for the fate of eosinophils, but IL-5 is the most important and versatile player. IL-5 is considered to modulate progenitor proliferation and maturation in bone marrow (Menzies-Gow *et al.*, 2003). The IL-5/IL-5R axis is also characterised by eosinophil migration. IL-5 induces eosinophil chemotaxis and boosts eosinophil adherence (Walsh *et al.*, 1991; Sehmi *et al.*, 1992; Czech *et al.*, 1993; Wong, Ip and Lam, 2003). IL-5 could further promote degranulation and the secretion of cytokines and eosinophilic extracellular traps from eosinophils (Lopez *et al.*, 1988; Fujisawa *et al.*, 1990; Dvorak *et al.*, 1992; Hall *et al.*, 1998; Choi *et al.*, 2018). The clinical success of anti-IL-5 and anti-IL-5R antibodies also confirms the critical roles of IL-5 in asthma. In this study, I will investigate the effects of IL-5 on sensitising responses of human blood eosinophils to chemotaxis, adhesion, degranulation and kinase activation.

Although IL-5 is a robust regulator of eosinophil activities, the incomplete depletion of eosinophils by mepolizumab (Pavord *et al.*, 2012) also indicates other influencers on eosinophil

biology. For example, benralizumab diminishes eosinophils since it binds to eosinophils and causes antibody-dependent cellular cytotoxicity. In fact, heterogeneity of eosinophils has been hypothesised (Fukuda and Gleich, 1989; Mesnil *et al.*, 2016). In mice, Siglec-8^{+}CD62L^{+}IL-$3R^{low}$ resident eosinophils were found, which functionally differ from Siglec-8^{+}CD62L^{low}IL-$3R^{high}$ eosinophils. This distinct eosinophil subset was described as "IL-5–independent parenchymal cells with a ring-shaped nucleus". IL-5 receptor level did not alter in lung resident eosinophils from mice challenged with house dust mites (Mesnil *et al.*, 2016). However, no evident phenotypic changes were found in human samples. Therefore, more studies are required to confirm and understand these subpopulations in humans. Here, I have applied a cutting-edge technique, single-cell RNA sequencing, to focus on the profile of human blood eosinophils. Therefore, not only the heterogeneity of eosinophils was investigated in humans, but also I explored the impact of IL-5 on these subsets by the administration of mepolizumab to remove IL-5 in patients with severe eosinophilic asthma.

In this chapter, I demonstrated that eosinophils from eosinophilic asthma showed enhanced responses to chemokines, including PGD_2 and IL-5 *ex vivo*, compared to those from non-eosinophilic asthma and healthy control. An elevated level of blood IL-5 in eosinophilic asthma indicated a priming role of IL-5 on eosinophil activation. It was also confirmed with the observation that these enhanced responses were reversed or even further suppressed by clinical treatment with mepolizumab. Sequencing of eosinophil transcriptome revealed that IL-5 blocking reduced kinase activities, including MAPK (mitogen-activated protein kinase) and PI3K (phosphoinositide 3-kinase), and inhibited enhanced activities of eosinophil in asthma,

related to inflammation, tissue damage and airway remodelling. Together, these findings indicate the critical roles of IL-5 on eosinophils and basophils and severe eosinophilic asthma.

This project focuses on the transcriptomics of eosinophils in different phenotypes of asthma under different treatments. We will compare samples between 6 groups of patients: healthy donors, non-eosinophilic asthma, eosinophilic asthma and eosinophilic asthma with OCS, mepolizumab or dupilumab (anti-IL-4/13 as negative control) treatment, with 5 persons in each group (see the figure below). At least 500,000 eosinophils from their blood will be sorted at FACS lab in NDM Experimental Medicine. Cell pellets will be sent to the MRC WIMM Single Cell Facility for bulk RNA-seq. The data will be analysed by R and other software.

By using this technique, we will broadly understand the gene expression and signal pathway activities in eosinophils from different phenotypes of asthma, particularly eosinophilic asthma, and the effects of clinical drugs in eosinophils. These understanding will help us to discover the key drivers of eosinophilia.

5.2. Chapter-specific materials and methods

5.2.1. Eosinophil isolation

Human eosinophils were isolated from fresh peripheral blood samples collected in heparin-coated tubes. PBMCs were removed by gradient centrifugation with Lymphoprep (Stemcell Technology). The remaining deposited red blood cell layer was washed with PBS, and the layer, after centrifugation, was added to the same volume of 3% dextran solution. After a 30-minute incubation, the supernatant upper layer was collected and centrifuged. The granulocyte-rich cell pellet was lysed with ice-cold water to remove red blood cells and collected for either further purification or cryopreservation. For *in vitro* functional assays, the granulocyte-rich cell pellet was further labelled with CD16 MicroBeads (Miltenyi) to remove $CD16^+$ neutrophils by MACS separation. The purity (> 90%) of the eosinophils after isolation was confirmed with flow cytometry as $CD193^+CD16^-$ cells before use. For single-cell RNA sequencing, cells were cryopreserved in heat-inactivated FCS containing 10% DMSO at −80°C for one week, and then cryovials were transferred to liquid nitrogen tanks.

5.2.2. Eosinophil chemotaxis *in vitro*

For chemotaxis assays, purified human eosinophils were resuspended in RPMI 1640 medium. 25 µl of eosinophil suspension were applied to the upper chambers, and 29 µl test compounds, as indicated in the results, were added into lower chambers in a 5-mm pore-sized 96-well ChemoTx plate (Neuro Probe). After one-hour incubation at 37 °C, cell suspension in the lower

chambers was collected and mixed with a Cell Titer-Glo Luminescent Cell Viability Assay kit (Promega). The luminescence was measured by an EnVision Multilabel Reader.

5.2.3. Eosinophil treatment *ex vivo*

For shape change assays, fresh human blood was incubated with a series concentration of IL-5, PGD_2 or medium for 1 hour at 37 °C with 5% CO_2. The samples were fixed with a Cytofix Fixation Buffer (BD Biosciences) followed by red blood cell lysis with RBC Lysis Solution (Qiagen). Flow cytometry was conducted to measure eosinophil shape changes. Eosinophils were gated from granulocytes according to their high autofluorescence and high side scatter. The changes in eosinophil shapes were defined by the increasing shift of percentage from a baseline in forward scatter. The baseline of shape change is obtained by a fixed gate of forward-scatter^{high} eosinophils (Appendix Fig 5.1). For fold change of shape change calculation, the corresponding value from healthy that was run in each experiment in parallel with other patient samples was set as denominators (i.e. Fold change = $\frac{\%\text{ shape change (patient)}}{\%\text{ shape change (paired healthy)}}$).

For expression of activation markers or eosinophil peroxidase (EPX), fresh blood was incubated with a series concentration of IL-5, PGD_2 or medium for 40 minutes at 37 °C with 5% CO_2. For activation marker detection, blood was labelled with anti- CD16-PerCP, CD69-BV421, CD11b-PE and CD63-APC antibody (Biolegend) followed by red blood cell lysis with a BD FACS Lysing Solution (BD Biosciences). For intracellular EPX staining, after surface marker labelling with anti-CD16-PerCP, cells were fixed with IC Fixation Buffer (Invitrogen) at 4 °C overnight and then treated with the Permeabilization Buffer (Invitrogen) followed by incubation with anti-EPX-APC antibody (Novus Biologicals).

5.2.4. PhosFlow cytometry

Fresh peripheral blood samples were fixed with Lysis/Fixation Buffer (BD Biosciences) at 37 °C for 12 minutes. Cell pellets were harvested after centrifugation and washed with PBS. The cells were then permeabilised by using Perm Buffer II (BD Biosciences) at 0 °C for 30 minutes, washed with Stain Buffer (BD Biosciences), followed by incubation with anti-pAKT, pERK1/2, or pp38 antibodies conjugated with Alexa Flour 647 for one hour. After the antibody was washed off with Stain Buffer, the stained eosinophils were ready for flow cytometry analysis.

5.2.5. scRNA-seq

5.2.5.1. Eosinophil flow cytometry sorting

Granulocyte-rich cells were prepared as described in 5.2.1. from two healthy donors and three paired samples of patients before and after mepolizumab treatment (Appendix Table 5.1) and stored in liquid nitrogen tanks. Before sorting, the cryovials with cryopreserved granulocyte-rich cells were thawed in the water bath and then rapidly transferred to warmed RPMI 1640 medium with 10% FCS. The cell suspensions were stained with Annexin V-Alexa Fluor 647 (Biolegend), SYTOX Green (Invitrogen) and DAPI (Sigma-Aldrich). Single eosinophil was gated as Annexin V^-/SYTOX $Green^-$/$DAPI^-$/SSC^{high}/$autoflorescence^{high}$ and sorted in 2 µl of Lysis Buffer [containing 0.4% v/v Triton X-100 and 2U/µl RNase inhibitor (Promega), ERCC spike in, 2.5mM dNTPs (Thermo-Fisher) and 2.5µM Oligo-dT30VN] in 96-well plates, and then centrifuged and immediately frozen at −80°C.

5.2.5.2. Library construction and sequencing

Reverse transcription and PCR were performed as described in Smart-seq 2 protocol (Picelli *et al.*, 2014). In summary, SMARTScribe Reverse Transcriptase (Clontech) was applied for first-strand synthesis, and cDNA was synthesised by SCA Chemistry SeqAmp DNA Polymerase (Clontech). The cDNA libraries for sequencing were prepared using Nextera XT DNA Sample Preparation Kit and Nextera XT 96-Index Kit (Illumina). Cell cDNA libraries from the same sample were pooled and cleaned up by AMPure XP beads (Beckman Coulter). Pooled samples were sent to Novogene company and sequenced by NovaSeq platforms with paired-end 150 bp reads aiming at an average depth of 1 million reads/cell.

5.2.5.3. Transcriptome quantification and quality control

Quality and adapter trimming was performed by Trim Galore! (https://github.com/FelixKrueger/TrimGalore) and FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). cDNA sequences from GRCh38 and ERCC were used for alignment by STAR (Dobin *et al.*, 2013) and mapping by Subread (Liao, Smyth and Shi, 2019). Standard scRNA-seq quality control was performed using scatter (McCarthy *et al.*, 2016). Cells were filtered based on different quality parameters (mitochondrial reads < 25%, ERCC reads < 50%, unmapped reads < 50%, number of detected genes > 1000, and number of mapped reads > 100000). 484 cells passed quality control, whilst one paired patient sample was excluded completely.

5.2.5.4. Dimension reduction

Principal Component Analysis (PCA), tSNE and Uniform Manifold Approximation and Projection (UMAP) were used for normalised log counts. PCA and tSNE were calculated by the RunPCA and RunTSNE functions correspondingly. 30 Principal Components (PCs) and 30 for perplexity were used for tSNE calculation. UMAP was generated using uwot. A subset of the matrix (100 patients X 5000 features) was used for supervised training.

5.2.5.5. Downstream analysis

Differential expression (DE) was performed using the DESeq2 (Love, Huber and Anders, 2014) with the LRT test. Genes were considered differentially expressed if they had an absolute log2 fold change (FC) of more than 2 (1 for the healthy control group vs pre-mepolizumab group) and the p value lower than 0.01. Pathway enrichment analysis was conducted using the clusterProfiler and ReactomePA (Yu and He, 2016) to validate the significantly enriched ($p < 0.05$) pathways with differentially expressed genes.

5.3. Results

5.3.1. Enhanced eosinophil migration and activation in eosinophilic asthma and reverse effect of mepolizumab

In contrast to healthy control individuals and/or non-eosinophilic asthmatic patients, eosinophilic asthmatic patients exhibited significantly higher levels of sputum and blood eosinophil counts (Fig 5.1A). Four-week administration of mepolizumab significantly diminished elevated levels of eosinophils in both the blood and sputum of patients with eosinophilic asthma (Fig 5.1B).

Eosinophil shape change assay is a well-established method indicating eosinophil migration (Choi *et al.*, 2003). To investigate whether mepolizumab also impacts eosinophil migration, I examined eosinophil shape change in response to chemoattractant IL-5 or PGD_2 from different groups of individuals using a shape change assay.

Exogenous IL-5 and PGD_2 induced eosinophil shape changes in fresh blood in a dose-dependent manner regardless of the phenotypes of patients (Fig 5.1C). To compare the difference accurately, I paired patient and healthy in parallel for each experiment and converted the percentage of shape change into fold change index (Fig 5.1D). Interestingly, eosinophils from eosinophilic asthma had substantially elevated responses to almost all concentrations of chemoattractant, whilst those from non-eosinophilic asthma presented similar profiles compared to healthy donors. After treatment with one course of oral corticosteroids or mepolizumab, the enhanced shape change effect of the eosinophils from patients with

eosinophilic asthma was strongly inhibited. Those eosinophils even developed a weaker response to IL-5 (across all concentrations of IL-5 for OCS-treated eosinophils and at low concentrations of IL-5 for mepolizumab-treated eosinophils) compared to those in healthy.

Next, I evaluated the baseline of eosinophil shape change, which was the background of the assay without stimulation (Fig 5.1E,F). The baseline value for the eosinophil shape change was highly correlated with blood eosinophil counts (Fig 5.1E, r=0.6345, $p < 0.01$). Compared with those in healthy donors and non-eosinophilic asthma, the baseline in eosinophilic asthma was much higher ($p < 0.01$) (Fig 5.1F). OCS suppressed the climbing baseline level of shape change in eosinophilic asthma to that in healthy. The mepolizumab treatment acted similarly to steroid, which attenuated the baseline dramatically in eosinophilic asthma ($p < 0.001$), and eosinophils from some patients even presented lower forward scatter in flow cytometer compared to healthy blood. Therefore, when comparing the shape change from healthy, pre-mepolizumab and post-mepolizumab groups, I shifted the baseline gate to a lower forward scatter in order to obtain all positive baselines for further analysis.

I further investigated how mepolizumab altered the potency of chemokines on the eosinophil shape change using EC_{50} of IL-5 and PGD_2 in shape change assays (Fig 5.1G). Mepolizumab treatment remarkably reduced the potency of IL-5 or PGD_2 to the eosinophils from eosinophilic asthma as both EC_{50} significantly increased ($p < 0.05$) after mepolizumab treatment. Besides the baseline shape change, EC_{50} was also correlated with blood eosinophil counts, which suggests that both background and response of eosinophils to chemokines could contribute to blood eosinophil counts (Fig 5.1H).

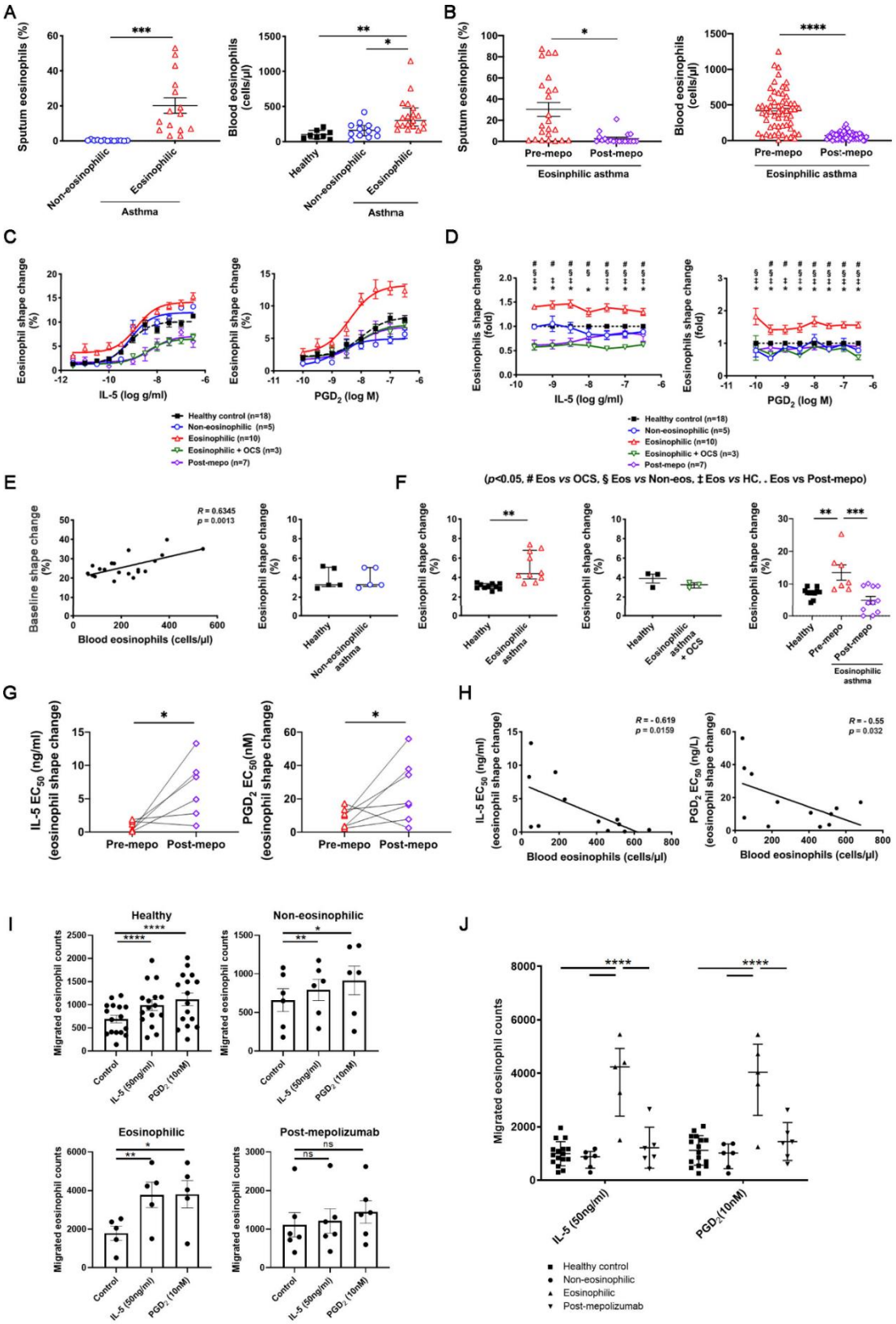


Fig 5.1 Enhanced eosinophil migration in eosinophilic asthma was reversed by mepolizumab.

A-B. Eosinophil levels in sputum and blood among different asthma groups (A) or before and after mepolizumab treatment for 4 weeks (B). C-D. Blood eosinophil shape change responding to a series of concentrations of IL-5 or PGD_2 from different individuals with or without OCS or mepolizumab treatment (C) and their comparison to healthy control (D). The data (D) are presented as the fold change of blood eosinophil shape change to the healthy control samples. E-F. Correlation (E) or comparison (F) of background eosinophil shape change and blood eosinophil counts. G-H. Potencies of IL-5 and PGD_2 in the induction of blood eosinophil shape change before and after mepolizumab treatment in eosinophilic asthma (G) and their correlation with blood eosinophil counts (H). I-J. Eosinophil chemotaxis induced by IL-5 and PGD_2 in isolated eosinophils from different asthma conditions and mepolizumab treatment (I) and their comparison (J). *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$ and ****$p < 0.00001$.

To confirm the migratory activities of eosinophils, an additional chemotaxis assay was performed to measure the migration of purified blood eosinophils *in vitro*. IL-5 or PGD_2 promoted eosinophil chemotaxis and induced migration of eosinophils wherever isolated from healthy donors, non-eosinophilic or eosinophilic asthma. However, this reaction was not observed in the blood eosinophils of patients with mepolizumab treatment (Fig 5.1I). Unlike the shape change assay, where a weak but typical reaction was illustrated in post-mepolizumab samples, they only showed a trend of migration in the chemotaxis assay. More robust migration was induced in eosinophils from eosinophilic asthma compared with others, and this marked response was suppressed by the biological therapeutics (Fig 5.1J), which also supported the finding in the shape change assays.

Apart from enrichment in the lung, eosinophils also strongly contribute to asthma pathogenesis by general activation, including degranulation. Therefore, I examined the eosinophil activation among different groups of patients and the influence of mepolizumab on cell activation. Changes of several surface proteins on eosinophils, including CD11b (Wong, Ip and Lam, 2003; Johansson *et al.*, 2013), CD63 (Carmo *et al.*, 2016) and CD69 (Hartnell *et al.*, 1993; Mawhorter

et al., 1996) have been reported to demonstrate cell activation and even act as potential biomarkers in asthma (Hartnell *et al.*, 1993; Pignatti *et al.*, 2002). CD69 was nearly absent with a low gMFI (518 on average) on resting blood eosinophils, and no significant difference was detected among the three groups (Fig 5.2A, top panel). CD69 levels in eosinophils were elevated *ex vivo* by IL-5 or PGD_2 in all groups. The expression level of CD63 was increased up to near 6-fold in eosinophilic asthma, which was significantly higher compared to levels in healthy individuals as well as post-mepolizumab groups. This upregulation of expression was also detected in other activation markers (Fig 5.2A, middle and bottom panel). Compared to the eosinophils from healthy and post-mepolizumab groups, the eosinophils from eosinophilic asthma were dramatically activated by IL-5 or PGD_2 and significantly enhanced the expression of CD11b and CD63. Thus, eosinophils from eosinophilic asthma also presented enhanced activation, which was reversed by mepolizumab. Unstimulated eosinophils from eosinophilic asthma also intrinsically contain higher levels of CD11b and CD63 than those from healthy individuals, and mepolizumab could reverse the elevated levels of these activation markers.

Because CD63 is tightly associated with degranulation in eosinophils (Carmo *et al.*, 2016), the degranulation activities in eosinophils were also determined by measuring granule proteins EDN and ECP. In eosinophilic asthma, eosinophils contain slightly more total EDN (Fig 5.2B, left) and much more total ECP (Fig 5.2C, left) in the cells than those in healthy donors or non-eosinophilic asthma. Under PGD_2 stimulation, blood eosinophils showed a higher capacity to secrete EDN in eosinophilic asthma than that in healthy and non-eosinophilic samples (up to 20-fold more than healthy control, Fig 5.2B, right), but the higher secretory capacity was not

observed on ECP (Fig 5.2C, right). This finding confirmed the previous result that the upregulation of CD63 in activated eosinophils was much greater in eosinophilic asthma and indicated that eosinophil degranulation was related to enhanced eosinophil activation.

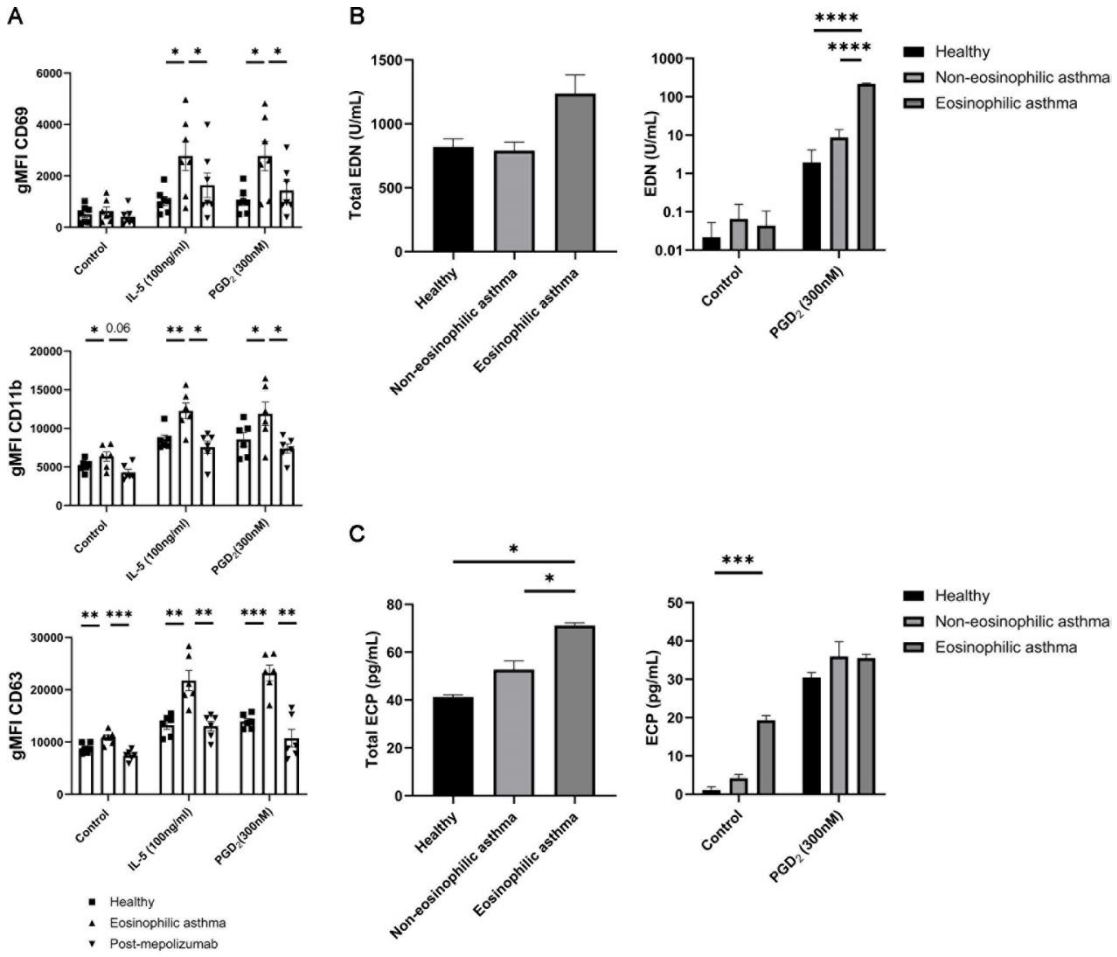
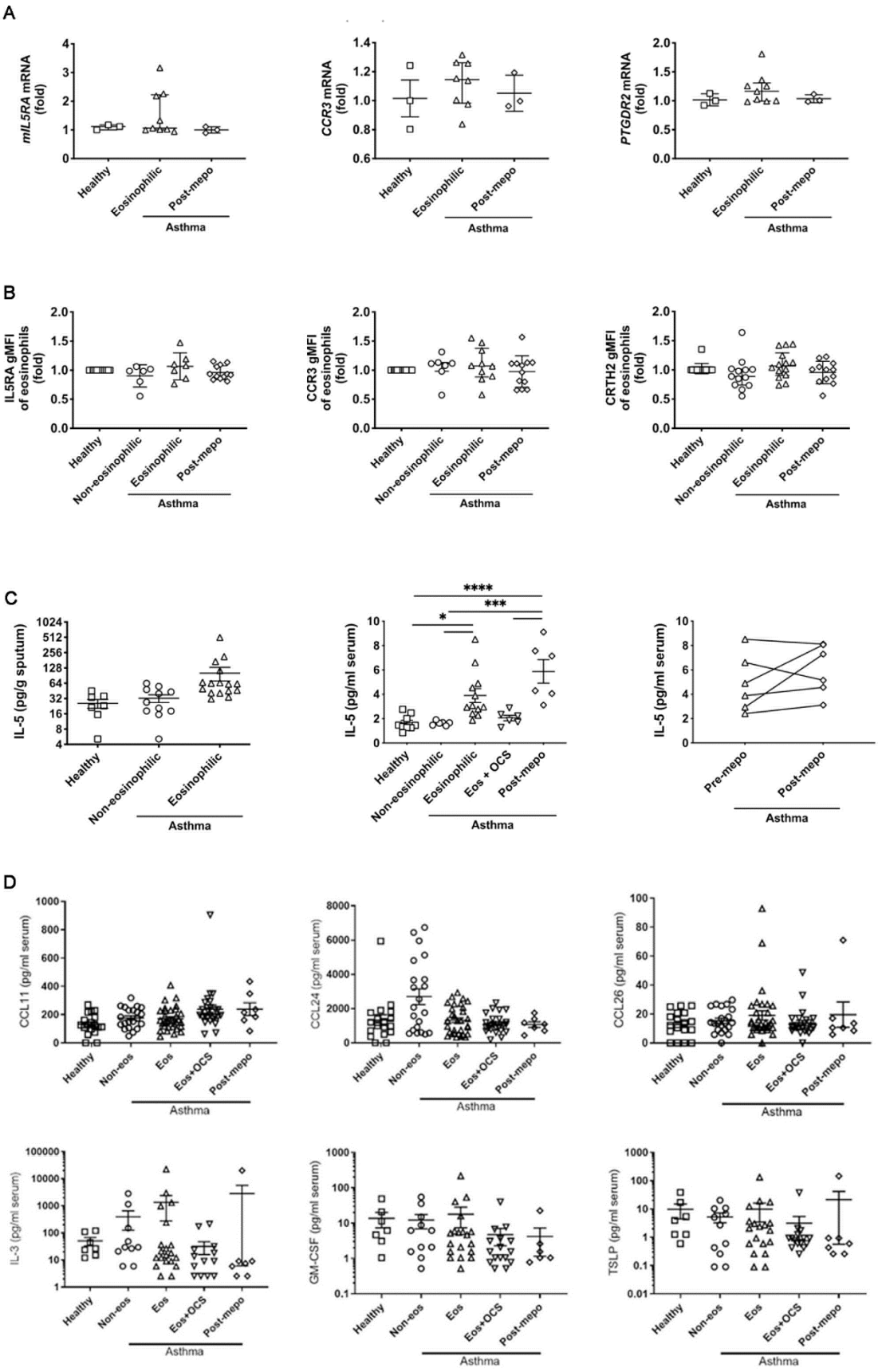


Fig 5.2 Eosinophil activation was enhanced in eosinophilic asthma.
A. Expression of CD69 (top), CD11b (middle) and CD63 (bottom) in blood eosinophils in healthy and eosinophilic asthma before and after mepolizumab treatment with or without IL-5 or PGD_2 stimulation detected with flow cytometry. B. *In vitro* detection of total EDN in granules (left panel) or released EDN after degranulation (right panel) in eosinophils isolated from blood in healthy or different groups of asthma. C. *In vitro* detection of total ECP in granules (left panel) or released ECP after degranulation (right panel) in eosinophils isolated from blood in healthy or different groups of asthma. *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$ and ****$p < 0.00001$. n=6-16 for A, n=2 for B and C.

5.3.2. Priming factor in enhancing eosinophil responses in eosinophilic asthma

Since the enhanced activities of eosinophils, including migration and activation distinguished in eosinophilic asthma, were initiated by eosinophil-related chemokines, to understand whether the enhancement was caused by the increased expression level of chemokine receptors, the levels of receptors for IL-5 (determined by the subunit IL-5Ra, *IL5RA* for the gene), PGD_2 ($CRTH_2$, *PTGDR2* for gene) and eotaxins (CCR3, *CCR3* for gene) in eosinophils from different sample groups were measured. Neither transcriptional levels (Fig 5.3A) nor protein levels (Fig 5.3B) of these receptors were significantly up- or down- regulated. I could not detect any significant change after mepolizumab treatment as well.

Previous reports had suggested that eosinophils could be primed by cytokines such as IL-3, IL5, and GM-CSF *in vitro* and exhibit enhanced chemotaxis activities to stimuli (Sehmi *et al.*, 1992; Warringa *et al.*, 1993). Therefore, in order to determine the potential priming factors in eosinophilic asthma, I further measured and compared the levels of these cytokines in the blood sera from different patient groups. The IL-5 level increased in both sputum and serum from eosinophilic patients compared to that from healthy control and non-eosinophilic asthma. Interestingly, serum IL-5 levels were only reduced by OCS but not altered by mepolizumab treatment (Fig 5.3C). However, no significant alteration was detected in other cytokines, including eotaxins (CCL11, CCL24, and CCL26), IL-3, GM-CSF and TSLP in the blood samples from eosinophilic asthma (Fig 5.3D). Although PGD_2 could be a potential candidate for eosinophil priming, the level of this lipid mediator in blood was shallow or even undetectable (Data not shown). Therefore, IL-5 is likely the priming factor of eosinophils in the blood from eosinophilic asthma.


A
mIL5RA mRNA (fold)
CCR3 mRNA (fold)
PTGDR2 mRNA (fold)
Healthy
Eosinophilic
Post-mepo
Asthma
B
IL5RA gMFI of eosinophils (fold)
CCR3 gMFI of eosinophils (fold)
CRTH2 gMFI of eosinophils (fold)
Healthy
Non-eosinophilic
Eosinophilic
Post-mepo
Asthma
C
IL-5 (pg/g sputum)
Healthy
Non-eosinophilic
Eosinophilic
Asthma
IL-5 (pg/ml serum)
****
***
*
Healthy
Non-eosinophilic
Eosinophilic
Eos + OCS
Post-mepo
Asthma
IL-5 (pg/ml serum)
Pre-mepo
Post-mepo
Asthma
D
CCL11 (pg/ml serum)
CCL24 (pg/ml serum)
CCL26 (pg/ml serum)
IL-3 (pg/ml serum)
GM-CSF (pg/ml serum)
TSLP (pg/ml serum)
Healthy
Non-eos
Eos
Eos+OCS
Post-mepo
Asthma

1696

Fig 5.3 Expression of chemoattractant receptors on eosinophils and cytokine levels in different groups of asthma.
A-B. Expression of chemoattractant receptors on purified eosinophils at mRNA level with qPCR (A) and at the protein level with flow cytometry (B) in healthy, non-eosinophilic asthma or eosinophilic asthma before and after mepolizumab treatment. The mRNA data are presented as the fold change $\left([2^{-(Cq_{target\ gene} - Cq_{GAPDH})}]_{samples} \div mean\ of\ [2^{-(Cq_{target\ gene} - Cq_{GAPDH})}]_{healthy}\right)$**. The flow cytometry data are presented as the fold change of mean fluorescence intensity to the healthy samples. C. IL-5 level in sputum or serum among different asthma groups and medical treatments. D. Levels of CCL11, CCL24, CCL26, IL-3, GM-CSF, and TSLP in serum among different asthma groups and medical treatments. *$p < 0.05$, ***$p < 0.0001$ and ****$p < 0.00001$.**

5.3.3. Transcript profile of blood eosinophils from severe eosinophilic asthma before and after mepolizumab intervention

Since IL-5 is a priming factor of eosinophils, the raised level of IL-5 in blood potentially leads to enhanced eosinophil activation. Blocking IL-5 by mepolizumab inhibits the boosted activities of eosinophils. To further broadly investigate the alteration of eosinophils in eosinophilic asthma and the role of the eosinophil priming effect of IL-5 in the disease, I explored the transcriptome of blood eosinophils from healthy individuals, eosinophilic asthma before and after mepolizumab treatment using scRNA-seq. Single-cell instead of bulk techniques were performed because of the low number of blood eosinophils that could be obtained after mepolizumab treatment. In addition, due to the low yield of RNA extracted from granulocytes, I chose plate-based but not droplet-based scRNA-seq protocols in order to increase the depth of reads.

Blood was collected from female individuals with similar BMI and age. Live and non-apoptotic eosinophils were sorted, and cells from different individuals were mixed in each plate to further prevent batch effects. To avoid changes in the transcriptome in eosinophils by stress during

sorting, activation markers of eosinophils (CD63 and CD11b) were checked before and after the cell sorting. Little difference (< 5% changes in gMFI) was found in all samples.

To quantitate scRNA-seq data and perform quality control, I referred to the Smart-seq2 protocol and workflow from Human Cell Atlas (https://data.humancellatlas.org/pipelines/smart-seq2-workflow). Normalised data were then clustered by semi-supervised UMAP. The overall pattern of clustering differed among groups (Fig 5.4A). Whilst a proportion of healthy and eosinophilic asthma fell into each other's cluster, eosinophils from post-mepolizumab groups stood out and were well separated from the other two groups. **No heterogeneity of eosinophils was observed in this dataset (i. e. no clear and unique cluster was noticed representing a subtype of eosinophils).**

To understand the vast differences among the three groups, a gene differential analysis was conducted. A total of 10 genes were significantly ($p < 0.01$) and differentially expressed ($|\log_2 FC| > 1$) between eosinophils from healthy and eosinophilic asthma, and 3849 genes were significantly ($p < 0.01$) and differentially expressed ($|\log_2 FC| > 2$) between eosinophils from eosinophilic asthma before and after mepolizumab treatment (Fig 5.4B).

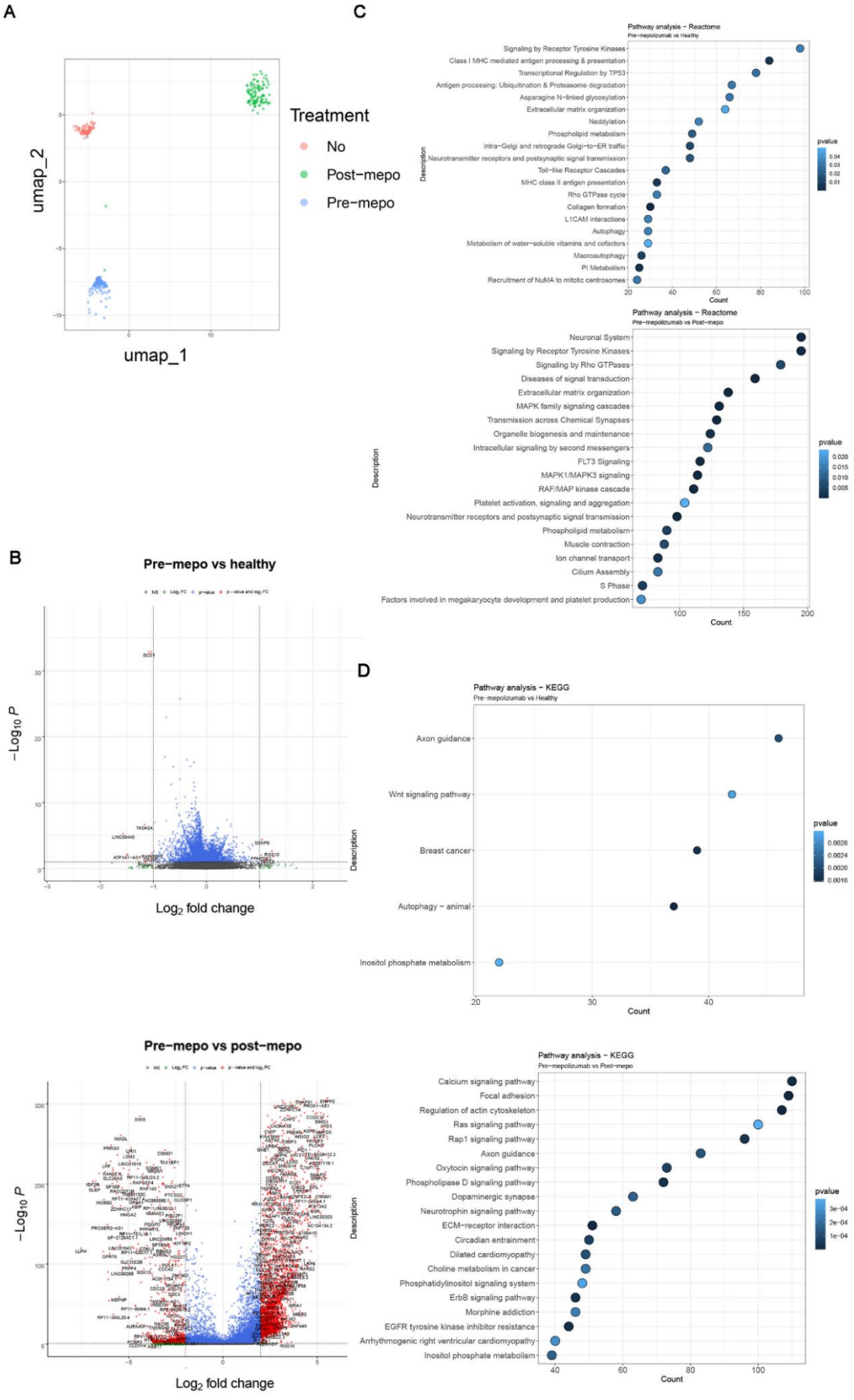

A
umap_2
umap_1
Treatment
No
Post−mepo
Pre−mepo
B
Pre−mepo vs healthy
−Log10 P
Log2 fold change
Description
Pre−mepo vs post−mepo
−Log10 P
Log2 fold change
Description
C
Pathway analysis − Reactome
Pre−mepolizumab vs Healthy
Signaling by Receptor Tyrosine Kinases
Class I MHC mediated antigen processing & presentation
Transcriptional Regulation by TP53
Antigen processing: Ubiquitination & Proteasome degradation
Asparagine N−linked glycosylation
Extracellular matrix organization
Neddylation
Phospholipid metabolism
Intra−Golgi and retrograde Golgi−to−ER traffic
Neurotransmitter receptors and postsynaptic signal transmission
Toll−like Receptor Cascades
MHC class II antigen presentation
Rho GTPase cycle
Collagen formation
L1CAM interactions
Autophagy
Metabolism of water−soluble vitamins and cofactors
Macroautophagy
PI Metabolism
Recruitment of NuMA to mitotic centrosomes
Description
Count
pvalue
Pathway analysis − Reactome
Pre−mepolizumab vs Post−mepo
Neuronal System
Signaling by Receptor Tyrosine Kinases
Signaling by Rho GTPases
Diseases of signal transduction
Extracellular matrix organization
MAPK family signaling cascades
Transmission across Chemical Synapses
Organelle biogenesis and maintenance
Intracellular signaling by second messengers
FLT3 Signaling
MAPK1/MAPK3 signaling
RAF/MAP kinase cascade
Platelet activation, signaling and aggregation
Neurotransmitter receptors and postsynaptic signal transmission
Phospholipid metabolism
Muscle contraction
Ion channel transport
Cilium Assembly
S Phase
Factors involved in megakaryocyte development and platelet production
Description
Count
pvalue
D
Pathway analysis − KEGG
Pre−mepolizumab vs Healthy
Axon guidance
Wnt signaling pathway
Breast cancer
Autophagy − animal
Inositol phosphate metabolism
Count
pvalue
Pathway analysis − KEGG
Pre−mepolizumab vs Post−mepo
Calcium signaling pathway
Focal adhesion
Regulation of actin cytoskeleton
Ras signaling pathway
Rap1 signaling pathway
Axon guidance
Oxytocin signaling pathway
Phospholipase D signaling pathway
Dopaminergic synapse
Neurotrophin signaling pathway
ECM−receptor interaction
Circadian entrainment
Dilated cardiomyopathy
Choline metabolism in cancer
Phosphatidylinositol signaling system
ErbB signaling pathway
Morphine addiction
EGFR tyrosine kinase inhibitor resistance
Arrhythmogenic right ventricular cardiomyopathy
Inositol phosphate metabolism
Count
pvalue

Fig 5.4 Differential expression analysis of scRNA-seq of isolated eosinophils from healthy and eosinophilic asthma before and after mepolizumab treatment.
A. Semi-supervised UMAP projection of the scRNA-seq consisting of eosinophils (196 eosinophils from two healthy, 196 eosinophils from two pre-mepolizumab eosinophilic asthma and 196 eosinophils from paired post-mepolizumab eosinophilic asthma coloured by groups of patients. B. differential gene expression analysis in pre-mepolizumab eosinophilic asthma compared to healthy (upper) or post-mepolizumab (lower) groups where genes were highlighted in blue ($p < 0.01$), green [$|\log_2 FC| > 1$ (upper), or > 2 (lower)] or red (matched both conditions). C-D. Enriched pathways analysis in the comparison of pre-mepolizumab eosinophilic asthma and healthy (upper) or post-mepolizumab (lower) ordered by the counts of differentially expressed genes in the pathway and coloured by the *p* value ($p < 0.05$) using Reactome (C) or KEGG (D) database.

To further identify the functions of differentially expressed genes, I conducted pathway enrichment analysis (Fig 5.4C,D, Fig 5.5). There are a comprehensive set of databases providing information on biological pathways, including Reactome and KEGG. Because these databases differ in pathway number, names, hierarchy, details of pathways and protein number per pathway, I analysed the data by employing multiple databases to capture the most information. To pinpoint confident pathways that were shared by diverse sources, I extracted pathway networks where pathways sharing the same genes were connected as a cluster and shared pathway information from Reactome, KEGG and GO (Fig 5.6). Due to the small number of pathways enriched in the comparison of healthy control and pre-mepolizumab groups by KEGG analysis, only networks of pathways from the comparison between pre- and post-mepolizumab were illustrated in Fig 5.6C.

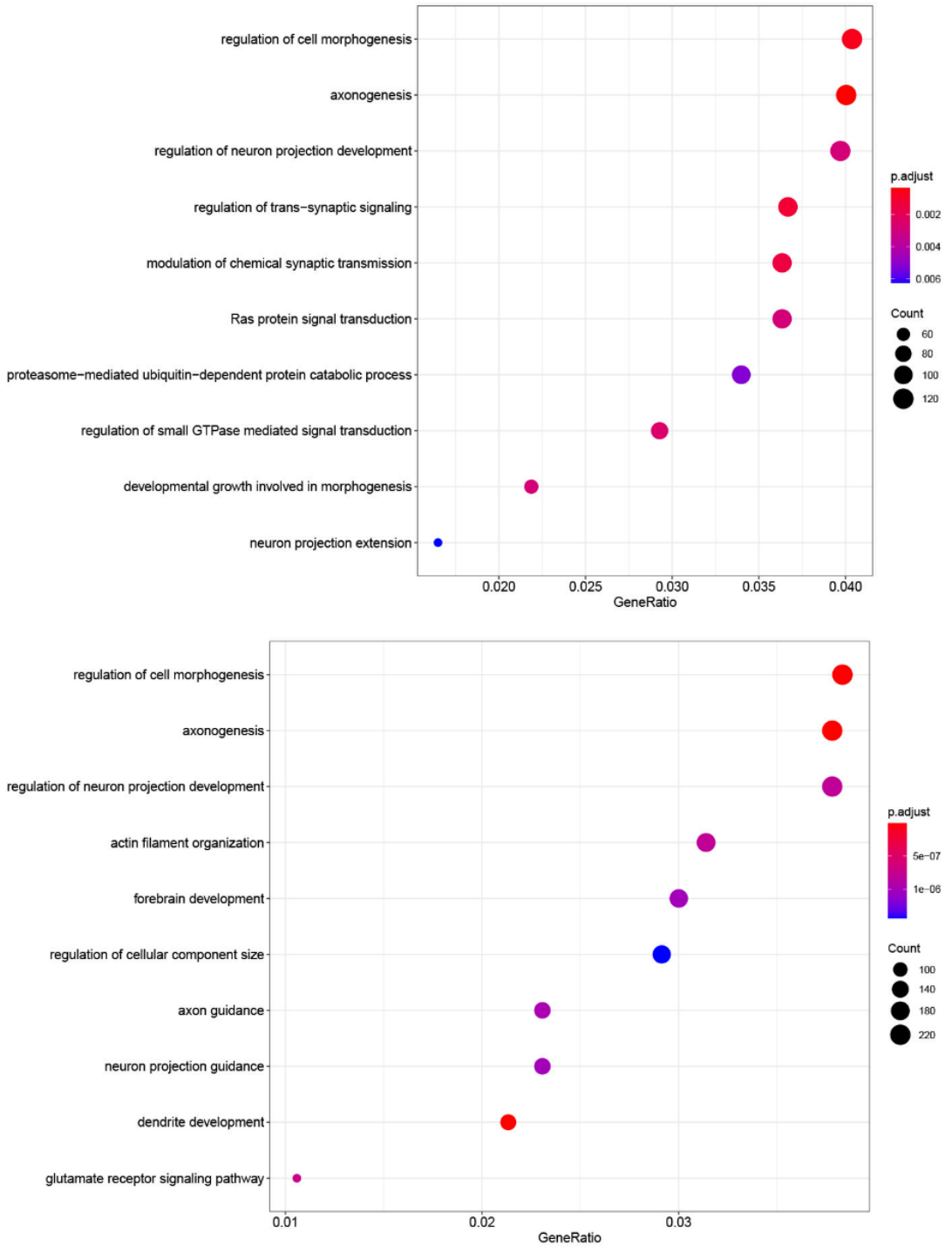


Fig 5.5 Enriched pathways analysis in the comparison of pre-mepolizumab eosinophilic asthma and healthy or post-mepolizumab

Pre-mepolizumab eosinophilic asthma vs healthy (upper) or pre-mepolizumab eosinophilic asthma vs post-mepolizumab (lower) were ordered by the ratio of counts of differentially expressed genes in the total gene set involved in the pathway and coloured by the *p* value ($p < 0.05$) using GO database.

In all three types of analysis, the biggest cluster of the pathway network shared functions about cell growth, proliferation, and especially ECM organisation in both comparisons of pre-mepolizumab with healthy and with a post-mepolizumab group (Fig 5.6A). Eosinophils from eosinophilic asthma highly expressed genes (FC > 4) including collagens (COL11A2, COL1A1, COL15A1, COL11A1, COL18A1, COL4A6, COL28A1, COL23A1, COL13A1, COL9A1, COL19A1, COL22A1, COL4A4, COL4A1, COL9A2, COL27A1), laminins (LAMA1, LAMA5, LAMC3), and their receptors integrins (ITGA2B, ITGB8, ITGA10, ITGA2, ITGB2, ITGA11, ITGB5, ITGA9, ITGA7) (Fig 5.8A). This suggested a potentially enhanced activity related to tissue remodelling in eosinophils from eosinophilic asthma besides migration and degranulation. Mepolizumab treatment would therefore reduce the function of tissue remodelling in eosinophils.

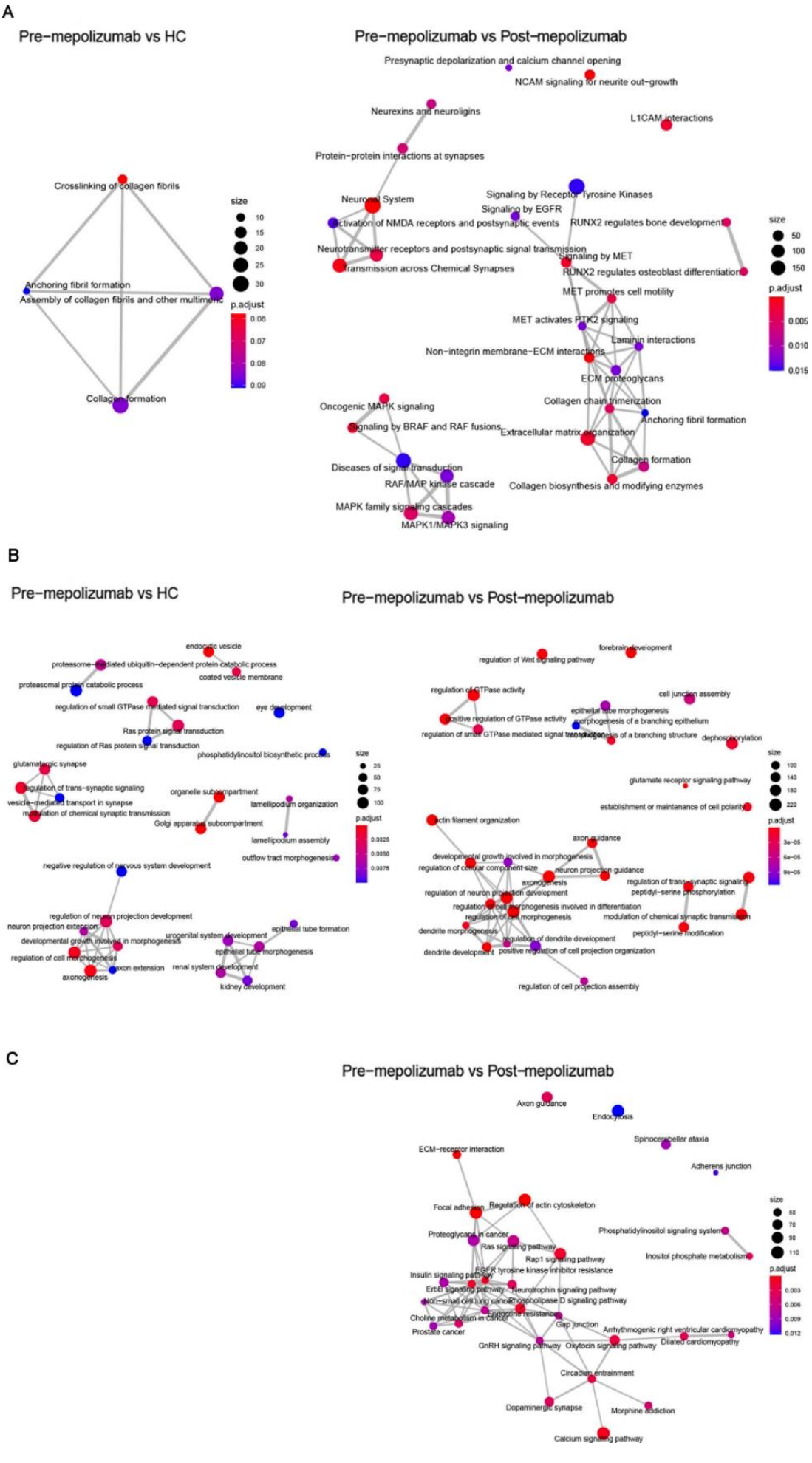

A
Pre−mepolizumab vs HC
Crosslinking of collagen fibrils
Anchoring fibril formation
Assembly of collagen fibrils and other multimeric
Collagen formation
size
10
15
20
25
30
p.adjust
0.06
0.07
0.08
0.09
Pre−mepolizumab vs Post−mepolizumab
Presynaptic depolarization and calcium channel opening
NCAM signaling for neurite out−growth
Neurexins and neuroligins
L1CAM interactions
Protein−protein interactions at synapses
Neuronal System
Signaling by Receptor Tyrosine Kinases
Signaling by EGFR
Activation of NMDA receptors and postsynaptic events
RUNX2 regulates bone development
Neurotransmitter receptors and postsynaptic signal transmission
Signaling by MET
Transmission across Chemical Synapses
RUNX2 regulates osteoblast differentiation
MET promotes cell motility
MET activates PTK2 signaling
Laminin interactions
Non−integrin membrane−ECM interactions
ECM proteoglycans
Oncogenic MAPK signaling
Collagen chain trimerization
Anchoring fibril formation
Signaling by BRAF and RAF fusions
Extracellular matrix organization
Collagen formation
Diseases of signal transduction
RAF/MAP kinase cascade
Collagen biosynthesis and modifying enzymes
MAPK family signaling cascades
MAPK1/MAPK3 signaling
size
50
100
150
p.adjust
0.005
0.010
0.015
B
Pre−mepolizumab vs HC
endocytic vesicle
proteasome−mediated ubiquitin−dependent protein catabolic process
coated vesicle membrane
proteasomal protein catabolic process
regulation of small GTPase mediated signal transduction
eye development
Ras protein signal transduction
regulation of Ras protein signal transduction
phosphatidylinositol biosynthetic process
glutamatergic synapse
regulation of trans−synaptic signaling
organelle subcompartment
vesicle−mediated transport in synapse
lamellipodium organization
modulation of chemical synaptic transmission
Golgi apparatus subcompartment
lamellipodium assembly
outflow tract morphogenesis
negative regulation of nervous system development
regulation of neuron projection development
neuron projection extension
epithelial tube formation
urogenital system development
developmental growth involved in morphogenesis
epithelial tube morphogenesis
regulation of cell morphogenesis
axon extension
renal system development
axonogenesis
kidney development
size
25
50
75
100
p.adjust
0.0025
0.0050
0.0075
Pre−mepolizumab vs Post−mepolizumab
forebrain development
regulation of Wnt signaling pathway
regulation of GTPase activity
cell junction assembly
epithelial tube morphogenesis
positive regulation of GTPase activity
morphogenesis of a branching epithelium
regulation of small GTPase mediated signal transduction
morphogenesis of a branching structure
dephosphorylation
glutamate receptor signaling pathway
establishment or maintenance of cell polarity
actin filament organization
axon guidance
developmental growth involved in morphogenesis
regulation of cellular component size
neuron projection guidance
axonogenesis
regulation of trans−synaptic signaling
regulation of neuron projection development
peptidyl−serine phosphorylation
regulation of cell morphogenesis involved in differentiation
regulation of cell morphogenesis
modulation of chemical synaptic transmission
dendrite morphogenesis
peptidyl−serine modification
regulation of dendrite development
dendrite development
positive regulation of cell projection organization
regulation of cell projection assembly
size
100
140
180
220
p.adjust
3e−05
6e−05
9e−05
C
Pre−mepolizumab vs Post−mepolizumab
Axon guidance
Endocytosis
Spinocerebellar ataxia
ECM−receptor interaction
Adherens junction
Focal adhesion
Regulation of actin cytoskeleton
Proteoglycans in cancer
Phosphatidylinositol signaling system
Ras signaling pathway
Inositol phosphate metabolism
Rap1 signaling pathway
EGFR tyrosine kinase inhibitor resistance
Insulin signaling pathway
ErbB signaling pathway
Neurotrophin signaling pathway
Non−small cell lung cancer
Phospholipase D signaling pathway
Endocrine resistance
Choline metabolism in cancer
Gap junction
Prostate cancer
Arrhythmogenic right ventricular cardiomyopathy
GnRH signaling pathway
Oxytocin signaling pathway
Dilated cardiomyopathy
Circadian entrainment
Dopaminergic synapse
Morphine addiction
Calcium signaling pathway
size
50
70
90
110
p.adjust
0.003
0.006
0.009
0.012

Fig 5.6 Correlations between enriched pathways in the comparison of pre-mepolizumab eosinophilic asthma and healthy (HC, left) or post-mepolizumab (right).
A-C. Different databases, including Reactome (A), GO (B), and KEGG (C) were used. Every node represents a pathway coloured by the p value ($p < 0.05$) and is sized by the number of differentially expressed genes in the pathway. Every link represents shared genes between two pathways. No linked pathways were found in the comparison of pre-mepolizumab and healthy using KEGG.

In addition, this tissue remodelling function derived from ECM pathways was connected to signalling pathways by receptor tyrosine kinases in the network analysis. The main classes of receptor tyrosine kinases involved were epidermal growth factor (EGF), Platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), insulin and ephrin receptor families. In Reactome pathway analysis, eosinophils from eosinophilic asthma showed higher gene expression related to *Signalling by Receptor Tyrosine Kinases*, compared to both the healthy and the treated group (Fig 5.4C). *Signalling by MET* (receptor of HGF) and *signalling by EGFR* (receptor of EGF) were the most significant ones among all entries (p = 0.001 and p = 0.01, respectively) when I evaluated the impact of mepolizumab on enriched pathways (Fig 5.6A). GO analysis, on the other hand, did not indicate any pathway associated with receptor tyrosine kinases. For instance, *epithelial tube formation* jointed the hub of tissue development, but no significant pathways related to kinases were linked to this node (Fig 5.6B). As for KEGG, although no conclusion could be drawn when I compared healthy and eosinophilic asthma, mepolizumab did alter the expression of genes belonging to the receptor tyrosine kinases family in KEGG pathway analysis. Two pathways, *EGFR tyrosine kinase inhibitor resistance* and *ErbB signalling pathway* (p = 0.001 for both), which could be equivalent to the concept of *signalling by EGFR* in Reactome, were in the centre of the network (Fig 5.6C). Furthermore, mepolizumab also seemed to facilitate the modulation

of the insulin signalling pathway since *Insulin signalling pathway* was another top hit in the KEGG network (p = 0.008).

Eosinophils are considered to promote tissue remodelling via producing TGF-β (Minshall *et al.*, 1997), but mRNA expression of the TGF-β family (TGFB1, TGFB2, TGFB3) did not change between healthy and eosinophilic asthma in RNA-seq. Remarkably, the receptor submits for TGF-β, TGFBR1, and TGFBR3 are significantly highly expressed in eosinophils from the patients with eosinophilic asthma compared to healthy or patients treated with mepolizumab (Fig 5.7A). The KEGG pathway analysis revealed that some Smad genes (Smad2/3) downstream of the TGFR were also upregulated in eosinophilic asthma compared to healthy and post-mepolizumab groups (Fig 5.7A,B).

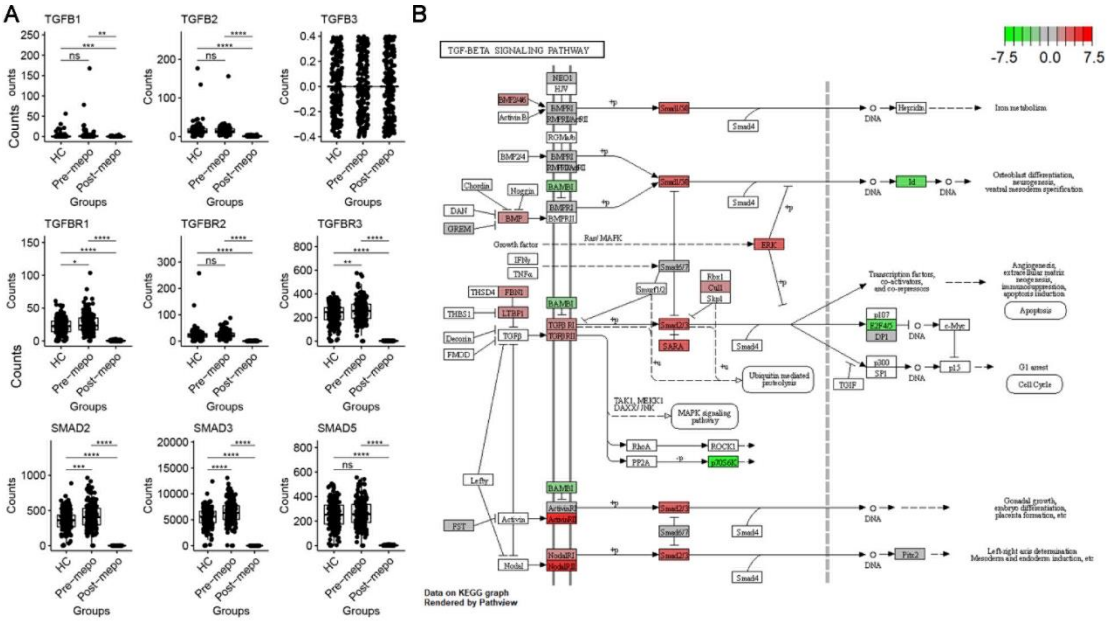


Fig 5.7 TGF-β associated gene expression in scRNA-seq.
A. comparison of gene counts among healthy (HC) and eosinophilic asthma before (pre-mepo) and after (post-mepo) mepolizumab treatment. B. Visualisation of differential expression of TGF-β associated genes between pre-mepo (red) and post-mepo (green) in a pathway map of TGF-beta Signalling Pathway integrated and drawn by KEGG (ID: hsa04350). The details of map notation can be found at https://www.genome.jp/kegg/document/help_pathway.html. *p < 0.05, **p < 0.001, ***p < 0.0001 and ****p < 0.00001.

Mepolizumab treatment also suppressed the mitogen-activated protein kinase (MAPK) signalling and reduced kinase expression (PKC, MAPK1, JNK) compared to those before the treatment (Fig 5.8B). *MAPK family signalling cascades* (Reactome) or *MAPK signalling pathway* (KEGG) was significantly enriched in the comparison before and after treatment (p = 0.005 or 0.02, respectively). Since endogenous IL-5 was nearly neutralised by mepolizumab, I also checked whether the IL-5-mediated JAK-STAT signalling pathway was changed or not. However, a significant difference was seen in neither KEGG (p = 1) nor Reactome analysis (not listed).

Remarkably, all analyses directed to pathways about neuron development (Fig 5.6) where differentially expressed genes from eosinophilic asthma (GABRA2, ADCY1) or post-mepolizumab (NRXN1) were highly enriched.

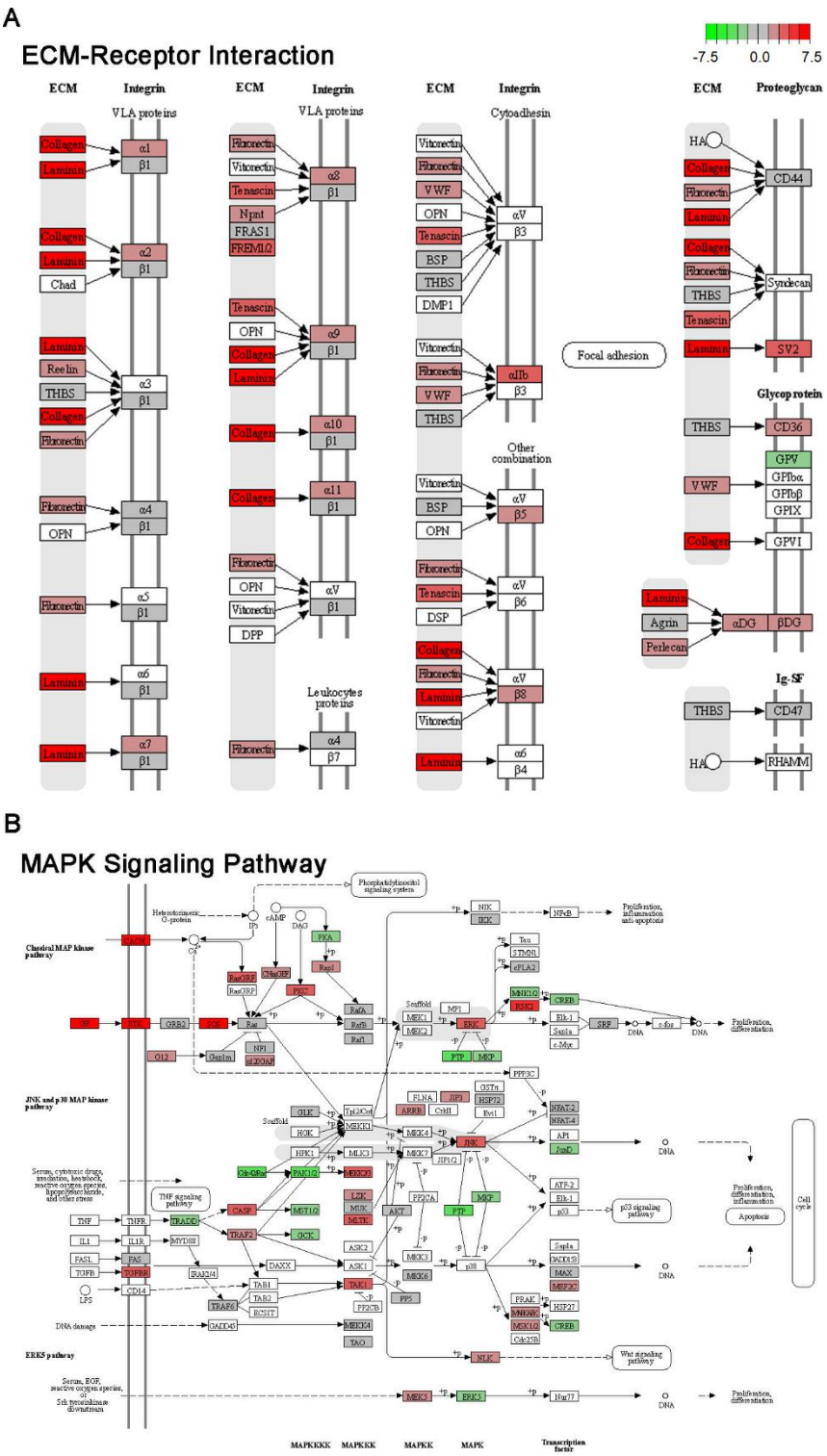


Fig 5.8 Visualisation of differential expression of pathway-associated genes in pathway maps. A-B. Comparison between pre-mepolizumab (red) and post-mepolizumab (green) groups in a pathway map of ECM-Receptor Interaction (A) or MAPK Signalling Pathway (B) integrated and drawn by KEGG (ID: hsa04512 or hsa04010, respectively). The details of map notation can be found at https://www.genome.jp/kegg/document/help_pathway.html.

5.3.4. MAPK and PI3K-Akt kinase signalling in eosinophils regulated by IL-5 inhibition

MAPK kinases have been known to contribute to cell migration (Bates, Green and Bertics, 2000), so the enhanced migration activities in eosinophils from eosinophilic asthma may be contributed by the enriched expression of kinases as found in scRNA-seq. To confirm the gene differential expression in MAPK kinase signalling, eosinophils from healthy individuals and eosinophilic asthma before and after the mepolizumab treatment were isolated, and then the levels of total kinase protein, as well as phosphorylated proteins in the cells were examined. Extracellular signal-regulated kinases 1/2 (Erk1/2) and p38 mitogen-activated protein kinases (p38) for MAPK signalling pathways were selected to determine the signals of the MAPK pathway. Besides MAPK signalling cascades, the PI3K-Akt signalling pathway is also involved in eosinophil migration (Zhu and Bertics, 2011). Therefore, Akt for the PI3K-Akt pathway was also included in the assays.

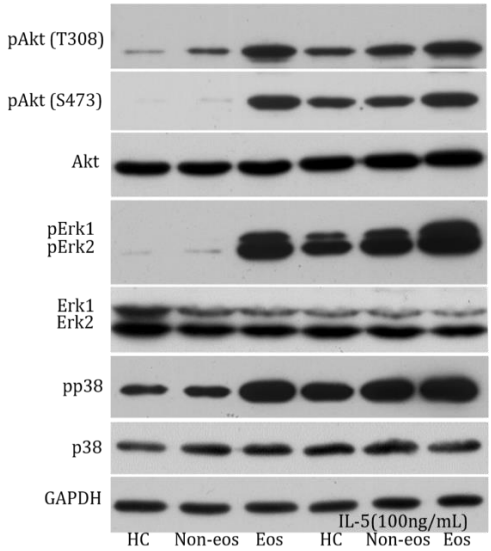


Fig 5.9 Levels of constitutive or phosphorylated ERK, p38 and AKT in purified blood eosinophils with or without IL-5 treatment among different groups of patients were detected by Western blotting.
GAPDH was used as a reference protein.

Phosphorylated kinases and total kinases were dissected by western blotting (Fig 5.9, Fig 5.10). Although eosinophils among various groups contained a similar amount of total Akt, Erk1/2 and p38, there were much more phosphorylated kinases existed in eosinophils from eosinophilic asthma than those from healthy control and non-eosinophilic asthma (Fig 5.9, Fig 5.10A). For MAPK pathways, signals of pErk1/2 and pp38 were significantly amplified in eosinophils from eosinophilic asthma (~20 fold higher for pErk1/2 and ~1.5 fold higher for pp38 compared to HC/non-eosinophilic) (Fig 5.10A, top left). When eosinophils were primed with IL-5 (100ng/ml) *in vitro*, the levels of pErk and pp38 were upregulated regardless of patient phenotypes. However, the ratio of increased signals was much lower in eosinophilic asthma, and primed eosinophils almost reached similar peak levels of pp38 and pErk among all groups (Fig 5.9, Fig 5.10A, bottom). Comparable results were exhibited in the PI3K-Akt pathway. The rising of pAkt level was apparent on both phosphorylation sites T308 and S473 (~2 fold more) (Fig 5.10A, top left), and primed eosinophils displayed a high but close level of pAkt among groups (Fig 5.10A, middle). Because only the MAPK but not the PI3K-Akt pathway was disclosed in the scRNA-seq analysis, PhosFlow assays were completed to confirm the changes in PI3K-Akt signalling. Consistent with the results from Western blotting, more $pAkt^+$ eosinophils were detected by flow cytometry in eosinophilic asthma than in healthy controls. To evaluate the impact of mepolizumab on the signalling pathways, I performed PhosFlow assays as no sufficient cells for Western blotting could be isolated from blood after the treatment. One course of mepolizumab significantly reduced the number of $pAkt^+$ or $pErk^+$ eosinophils, and the number of $pp38^+$ eosinophils declined slightly as well ($p = 0.08$). Blocking

IL-5 in the blood, therefore, as illustrated by RNA-seq results, strongly impaired the enhanced kinase activation in eosinophils from eosinophilic asthma.

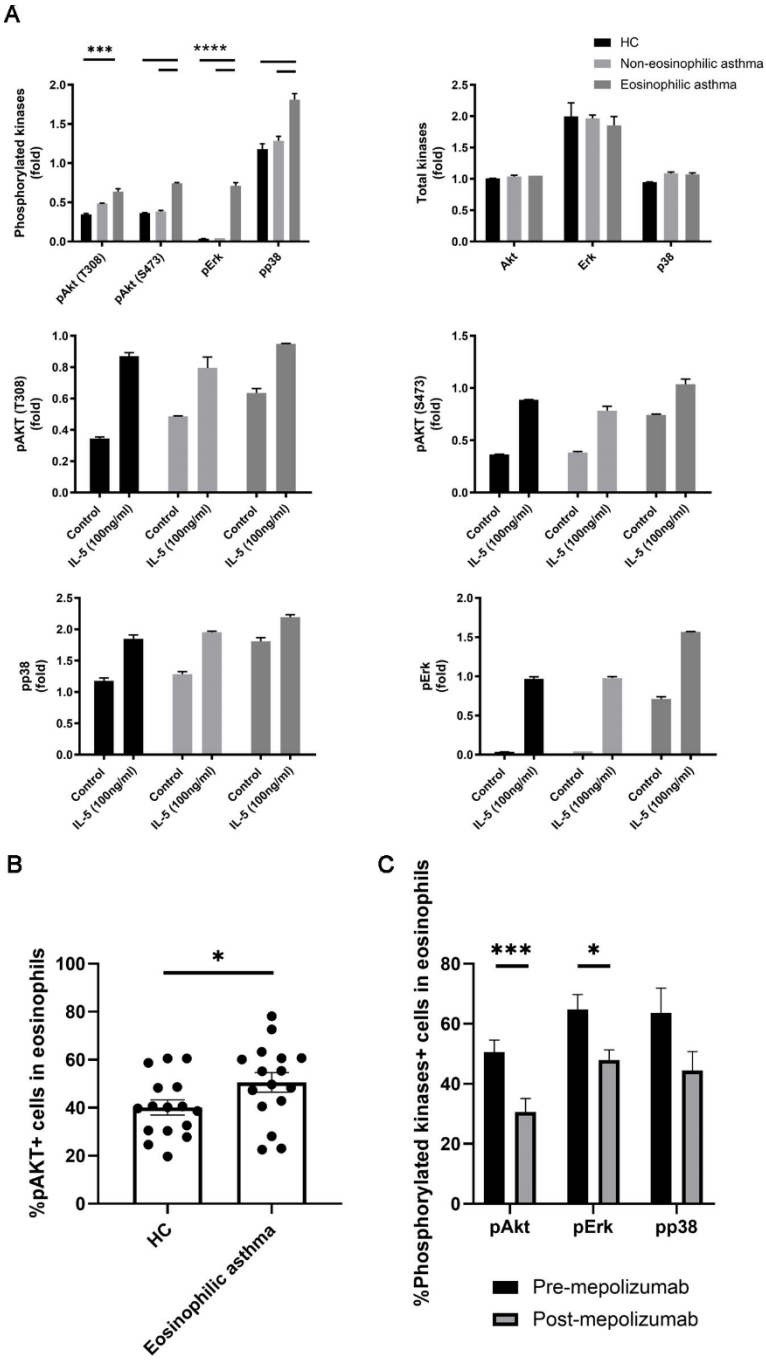


Fig 5.10 Activation of PI3K or MAPK pathways were enhanced in eosinophils from eosinophilic asthma.

A. Expression of total or phosphorylated kinases (Akt, Erk and p38) in purified blood eosinophils among asthma groups (top), and comparison of phosphorylation levels in the absence or presence of IL-5 stimulation (middle and bottom) by Western blotting. The data are presented as the fold change of protein band intensities to intensities of GAPDH. B. Expression of pAkt in eosinophils in blood from healthy (HC) or eosinophilic asthma determined by PhosFlow. C. Effect of mepolizumab treatment on phosphorylation of kinases in blood eosinophils from eosinophilic asthma measured by PhosFlow. *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$ and ****$p < 0.00001$. n=3 for A, n=3-6 for C.

5.4. Discussion and conclusions

Eosinophils are an established biomarker and drug target for severe eosinophilic asthma. They play crucial roles in asthma pathogenesis. In this study, I demonstrated that eosinophils from eosinophilic asthma contained substantially greater capacity for migration, degranulation, more active signal transduction and potentially enhanced tissue remodelling function when compared with eosinophils from healthy individuals or non-eosinophilic asthmatic patients. I also revealed that the blood IL-5 level was elevated in eosinophilic asthma, which potentially initiated enhanced eosinophil activities. Using mepolizumab to neutralise serum IL-5 in patients, not only dramatically reduced eosinophil number, but also diminished the high inflammatory capacity of eosinophils. These findings improved the understanding of eosinophils in the pathogenesis of asthma.

Previous studies characterised the higher responsiveness of eosinophils to chemoattractants in asthma. Migration of eosinophils isolated from allergic asthma was significantly greater than those from healthy donors (Warringa *et al.*, 1992, 1993). Interestingly, those responses in eosinophils from healthy donors could be boosted after the incubation of eosinophils with a low dose of IL-3, IL-5, or GM-CSF (down to ~10 pg/ml) and was comparable with the level of migration in asthma. However, eosinophils from allergic asthma could not gain further enhancement of migratory effect by IL-5 treatment. Therefore, researchers named this phenomenon a priming effect and hypothesised that elevated levels of growth factors of eosinophils *in vivo* primed cells migrate differently. As they had discovered before, I also confirmed that eosinophils in eosinophilic asthma presented an enhanced migration activity in

terms of shape changing, chemotaxis and adhesion (i. e. the higher expression of adhesion molecule CD11b after IL-5 or PGD_2 stimulation). CD63 (degranulation marker) was spontaneously increased in blood eosinophils, suggesting the eosinophils in eosinophilic asthma were more active even at the resting stage, paralleled with the priming definition. The more incredible release of EDN as well as higher expression of surface CD63 in eosinophils from eosinophilic asthma, evidenced the enhanced degranulation. In addition, the consistent trend of a more considerable amount of total ECP and EDN pointed out that these eosinophils possibly maintained more granule contents which would further amplify the secretion from degranulation. The increased activated status, including surface activation marker expression, the response of degranulation, as well as kinase phosphorylation, suggested that the priming is a universal effect on eosinophil biology instead of simply targeting migration.

Although *in vitro* assays and animal studies in previous reports have confirmed that several growth factors would prime the eosinophils, due to the lack of proper measurement of these growth factors in human samples and ethical methods to modulate the levels of these factors in the human body, it is still unclear 1) if the priming is true in humans with asthma; 2) if it is true in humans, where growth factor dominates the priming effect in asthma; and 3) if the primed eosinophils in patients could recover when growth factors are erased. My study implicated that IL-5 but no other cytokines or their receptors were significantly increased in blood from eosinophilic asthma, so IL-5 is the best candidate for priming of eosinophils. Moreover, I, for the first time, demonstrated that all enhanced activities in eosinophils were diminished after patients were treated with mepolizumab (i. e. removing IL-5 specifically in humans). Evidence

from the proper “IL-5 depletion” model in humans and the IL-5 level in blood unveiled that an increasing level of IL-5 in the blood from eosinophilic asthma primed the eosinophils. Whilst blocking IL-5 could reverse the hyperreactivity of these eosinophils. This finding may reflect that Il-5 not only participated in the fate of eosinophils (differentiation, maturation, migration, and activation) but also played a vital role in eosinophil priming, which sensitises the eosinophils and amplifies the inflammatory responses in asthma.

Although I attempted to discover the subclass of eosinophils in humans via scRNA-seq, there was no apparent heterogeneity of mRNA in human eosinophils. There are many studies illustrating the map of cell groups in the human lung or blood from patients with asthma (Tang *et al.*, 2022), but no study has managed to identify the proper group of eosinophils. Single-cell analysis of granulocytes generally remains a significant challenge due to the intrinsic low RNA content and relatively high levels of RNases. Especially, the commercially available RNase inhibitors, including the one used in the study* are not effective against RNase 2 (EDN) and RNase 3 (ECP) which are the major contents in eosinophil granules. Therefore, this is still a pioneering study in this field focusing on eosinophil single-cell sequencing. The data could be used as guidance for any following larger scale of sequencing.

As discussed in Chapter 3.4, airway remodelling is a crucial feature of asthma. Eosinophils are believed to provoke airway remodelling with a dual mechanism. Granule proteins, enzymes and reactive oxygen species (ROS) released will damage the tissue, and cytokines and lipid mediators synthesised by eosinophils will arouse abnormal tissue repair (Kariyawasam and Robinson, 2006).

An essential aspect of airway remodelling is epithelium damage mediated by eosinophil degranulation. The granule proteins released by eosinophils, MBP, EPX and ECP are considered cytotoxic to epithelial cells. MBP, the major content of eosinophil granules, was reported to damage the respiratory epithelial cells *in vitro* (Gleich *et al.*, 1979) and in mammal models (Frigas, Loegering and Gleich, 1980). *In vitro* study showed that damage, exfoliation, bleb formation and ciliostasis of guinea pig tracheal epithelial cells were caused by ECP or EPX in a dose-dependent manner (Motojima *et al.*, 1989). Eosinophils also produce leukotrienes (LTC_4 and LTD_4), ROS and substance P which could increase the vascular permeability and bronchoconstriction (Dahlén *et al.*, 1980; Chanez *et al.*, 1990; Garland *et al.*, 1997). In this study, I have demonstrated that eosinophils from eosinophilic asthma expressed a higher level of CD63, a degranulation marker of eosinophils, at the resting stage as well as upon stimulation. Once blood eosinophils infiltrate into tissue and degranulate, they would release more cytotoxic proteins and worsen the wounding because of the increasing number of cells and the enhanced activity. However, although more ECP proteins were measured in the granules in eosinophilic asthma, the concentration of released proteins seemed to be similar among groups. Given the small number of samples (n=2) in the assay, further investigation with a larger sample size and a more comprehensive panel, including EPX and MBP, should be conducted to confirm the result. Due to the limited number of eosinophils isolated from the blood in the post-mepo group, it was also rather challenging to value the impact of mepolizumab on degranulation. A more sensitive method requiring fewer cells like flow cytometry would be ideal for comparison between pre- and post- mepo groups. Unfortunately,

there are no high-quality and validated antibodies against human granule proteins available in the application of flow cytometry.

In addition to tissue damage, eosinophils also contribute to abnormal tissue repair, which is associated with basement membrane thickening. The airway basement membrane is an extracellular structure connecting the epithelium with the ECM (Fehrenbach, Wagner and Wegmann, 2017). The ECM contains two types of molecules: proteoglycans and fibrous proteins. Proteoglycans include small leucine-rich proteoglycans, modular proteoglycans and cell-surface proteoglycans, and the fibrous proteins are collagens, elastins, fibronectins and laminins (Frantz, Stewart and Weaver, 2010). Pathological deposition of collagens, elastin, fibronectin, tenascin, laminin, and proteoglycans causes basement membrane thickening and airway remodelling in asthma (Shifren *et al.*, 2012). Some of these protein genes have been detected in the scRNA-seq of this study including collagens (COL11A2, COL1A1, COL15A1, COL11A1, COL18A1, COL4A6, COL28A1, COL23A1, COL13A1, COL9A1, COL19A1, COL22A1, COL4A4, COL4A1, COL9A2, COL27A1), laminins (LAMA1, LAMA5, LAMC3), and their receptors integrins (ITGA2B, ITGB8, ITGA10, ITGA2, ITGB2, ITGA11, ITGB5, ITGA9, ITGA7).

Whilst eosinophils promote airway inflammation which indirectly causes tissue repair, eosinophils are also one of the predominant sources of TGF-β, which is a key cytokine in tissue remodelling and found increased in asthma (Minshall *et al.*, 1997). TGF-β triggered smooth muscle hyperplasia and hypertrophy, and controlled the differentiation, activation, and proliferation of fibroblasts. Application of corticosteroids reduced TGF-β expression in the

lung, collagen deposition, and peribronchial fibrosis in mice as a consequence of a reduction of lung eosinophil and macrophage number (Miller *et al.*, 2006). Accordingly, in a trial study, ECM deposition in the reticular basement membrane in asthma was repressed by an anti-IL-5 antibody as a result of eosinophil attenuation (Flood-Page *et al.*, 2003). In parallel, Conversely, IL-5 overexpression in mice resulted in an increase of eosinophils, collagens and TGF- β (Tanaka *et al.*, 2004; Nakagome *et al.*, 2007). In this study, the TGF-β family did not change among healthy and eosinophilic asthma before and after Mepolizumab treatment in RNA-seq, although eosinophils might generate TGF-β only when being activated. TGF-β receptors can be homo- or heterodimeric, assembled by TGFBR1 and TGFBR2, and transduce the TGF-β signal into the cytoplasm. Thus, the upregulation of TGFBR1 I found in the RNA-seq might result in a more vigorous response in eosinophils induced by eosinophil-derived TGF-β.

The key function of the TGF-β signal in airway remodelling is to initiate the production of proteins controlling ECM deposition and degradation in fibroblasts. Despite the fact that no change of ECM genes was observed in eosinophils in the presence and absence of TGF-β in a previous report (Miller *et al.*, 2006), I found that eosinophils differentially expressed many ECM genes. Upregulation of collagens, laminins, and their receptors integrins, and downregulation of MMP7 was founded in eosinophilic asthma. Collagens are the core structural protein in ECM. Aberrant accumulation of collagens will lead to fibre deposition and epithelial fibrosis (Fehrenbach, Wagner and Wegmann, 2017). Besides scaffolding functions, Laminin α1 and α2 chains are required for lung branching and differentiation of airway smooth muscle cells (Schuger *et al.*, 1997; Relan *et al.*, 1999). Increasing the expression of laminins would

thicken smooth muscle layers (Dekkers *et al.*, 2010). MMP7 is a member of matrix metalloproteinases, mediating ECM degradation induced in the lung and the gut during inflammation (Greenlee, Werb and Kheradmand, 2007). Downregulation of MMP7 in eosinophilic asthma might dampen the degradation of ECM and cause fibrosis. On the basis of dysregulation of tissue-remodelling-related genes, eosinophils may contribute to airway remodelling not only via producing TGF-β but also via a succeeding activation of TGF-β signalling in eosinophils and generation of ECM components as occurring in fibroblasts. Eosinophils in eosinophilic asthma possibly enhanced the capacity of producing ECM components by IL-5 priming, which would further regulate airway remodelling and contribute to asthma pathogenesis. Consequently, using mepolizumab to improve lung function might be not only associated with the reduction of eosinophil number, but also the impaired functions of tissue remodelling in the remaining eosinophils.

To confirm that remaining eosinophils after treatment with mepolizumab have diminished potential to drive airway remodelling, I studied TGF-β receptor signalling to bridge between the initial signal from the TGF-β receptor and matrix protein synthesis. Mechanisms of TGF-β receptor signalling are rather complex and many intracellular molecules are dimerised and phosphorylated to properly transduce the signal into nuclear (Van Bruaene *et al.*, 2009). One important complex involved is Smad 2/3, formed by two Smad proteins. Smad will regulate gene expression of collagens, MMPs and their inhibitors, and tissue inhibitors of metalloproteinases (TIMPs) in fibroblasts to promote the ECM deposition (Khalil *et al.*, 2017; Lin *et al.*, 2017). In this study, increased levels of *TGFBR1/3*, *SMAD2/3* and ECM genes

2036 occurred in eosinophils from eosinophilic asthma, signifying the possibility that eosinophils,
2037 like fibroblasts, have the machinery to produce ECM genes through typical Smad-dependent
2038 TGF-β signalling, and eosinophils from eosinophilic asthma contribute to airway remodelling
2039 by IL-5-induced TGF-β production and TGF-βR-mediated matrix preservation.

2040 Recent studies pointed out that besides the integrins and proteoglycans, ECM molecules also
2041 bind to receptor tyrosine kinases directly with a low affinity, which implicated the aberrant
2042 tissue repair (Shrivastava *et al.*, 1997; Vogel *et al.*, 1997; Iozzo *et al.*, 1999; Scott Swindle *et*
2043 *al.*, 2001; Schenk *et al.*, 2003). My data suggested that receptors for EGF, HGF and insulin
2044 were enriched in the eosinophils from eosinophilic asthma, and particularly, EGFR was
2045 significantly increased in eosinophilic asthma compared to healthy and post-mepolizumab
2046 groups. EGFR happens to be the receptor that researchers confirmed to interact with ECM
2047 components such as laminins and one of the proteoglycans, decorin (Tran, Griffith and Wells,
2048 2004). Laminin-5, which was also found to increase in eosinophils from eosinophilic asthma
2049 by RNA-seq, could promote migratory signals in breast epithelial cells (Giannelli *et al.*, 1997).
2050 Actually, synergy effects between EGF-EGFR and ECM-integrin signalling were revealed.
2051 The proliferation of glomerular epithelial cells mediated via EGFR activation was facilitated
2052 by contact with ECM (Cybulsky, McTavish and Cyr, 1994). Besides ECM, integrins could also
2053 activate EGFR signalling and induce EGF-specific proliferation and migration (Doerr and
2054 Jones, 1996; Jones, Crack and Rabinovitch, 1997). In response to cell-ECM adhesion, integrin
2055 forms a complex with EFGR, p130Cas, Crk, and c-Src, and the EGF receptor is phosphorylated
2056 with no exogenous EGF in cell culture (Moro *et al.*, 2002). Therefore, the synergy of receptor

tyrosine kinases and integrin pathways appears to regulate multiple processes in eosinophils, including receptor tyrosine kinases-mediated eosinophil proliferation, survival and ECM-induced eosinophil migration.

Eosinophil transcriptome exhibited that levels of growth factors such as transforming growth factor-alpha (TGF-α), EGF, HGF, PDGF and IGF, apart from their receptors, also altered in eosinophils from eosinophilic asthma. Both TGF-α and EGF bind to EGFR and promote the repairing of epithelium wounds (Todd *et al.*, 1991). While TGF-α was induced by IL-5 activation on eosinophils, blocking the EGFR on epithelial cells significantly reduced mucin production mediated by eosinophil-derived TGF-α (Burgel *et al.*, 2001). Previously, EGF was not found in activated eosinophils either at mRNA or protein levels (Todd et al., 1991; Davoine and Lacy, 2014). Surprisingly, both TGF-α and EGF were detected in resting eosinophils by scRNA-seq. Their expression levels significantly declined after anti-IL-5 therapeutics. HGF, which shared a similar trend with the two mentioned above, inhibited migration and cytokine production in activated human eosinophils (Ito *et al.*, 2007; Yamauchi *et al.*, 2016). Eotaxin- or PGD_2-induced chemotaxis was suppressed by HGF with attenuated Erk and p38 signalling (Ito *et al.*, 2007). HGF also impaired the secretion of TGF-β and VEGF induced by IL-5 (Yamauchi *et al.*, 2016). PDGF, However, was downregulated in eosinophilic asthma in my data. Like EGF and TGF-α, PDGF is known to be released by eosinophils and promote collagen production from fibroblasts (Kardas *et al.*, 2020). Due to the mixed signals, the effects of eosinophil-derivative growth factors on tissue remodelling need to be further investigated.

MAPK pathways and PI3K pathways are two classes of signal transduction pathways that are important in regulating cell proliferation, differentiation, migration, metabolism, apoptosis and protein production (Wei and Liu, 2002; Fruman *et al.*, 2017). Both have been reported to be activated by IL-5 priming in eosinophils *in vitro* (Bates, Green and Bertics, 2000; Zhu and Bertics, 2011). The authors claimed that signalling pathway activation is a consequence of IL-5 priming and results in the augmented migratory reaction in eosinophils. However, levels of pERK and pAkt only peaked at 10-15 minutes with low doses of IL-5 incubation, whilst this signal decayed after long-time incubation (more than 30 minutes). It raised a question about how such a transient phosphorylation signalling could affect eosinophil function in chronic inflammation in humans. Therefore, *in vivo* data are crucial to determine the real-time signalling in human eosinophils after priming. Due to the elevated level of blood IL-5 and enhanced activities of eosinophils in eosinophilic asthma, I assumed that blood eosinophils were primed by IL-5 in eosinophilic asthma under physiological conditions. According to the protein assays, eosinophils from eosinophilic asthma exhibited higher levels of pERK, pp38 and pAkt *in vivo*, and the MAPK pathway was highly enriched at mRNA levels. Those findings indicated that activation of MAPK pathways and PI3K pathway by IL-5 priming is consistent and maybe dynamic in the blood but not as transient as it was illustrated *in vitro.*

Interestingly, I also further revealed some potential genes that might also play an important role in enhanced migration. Shape change, which precedes the movement and migration of cells, follows chemokine receptor stimulation and cytoskeleton remodelling (Willetts *et al.*, 2014). In the pathway analysis of scRNA-seq, many hits were upregulated in eosinophilic asthma

including WAVE2, Arp2/3, LIMK and Myosins, which is controlled by chemokine, growth factor or ECM-induced MAPK/PI3K pathways activation (Fig 5.11). Unlike healthy cells, cancer cells have a substantially modified actin cytoskeleton, which is associated with enhanced cellular motility and impacts many processes in cancer, including invasion and metastasis (Efremov *et al.*, 2015). Many cytoskeleton regulators have been implicated in cancer cell motility previously, including all revealed by scRNA-seq (Olson and Sahai, 2009). It suggested that the enhanced IL-5-primed migration of eosinophils might be a result of permanent cytoskeleton remodelling induced by activation of MAPK/PI3K pathways and upregulation of WAVE2, Arp2/3, LIMK and Myosins.

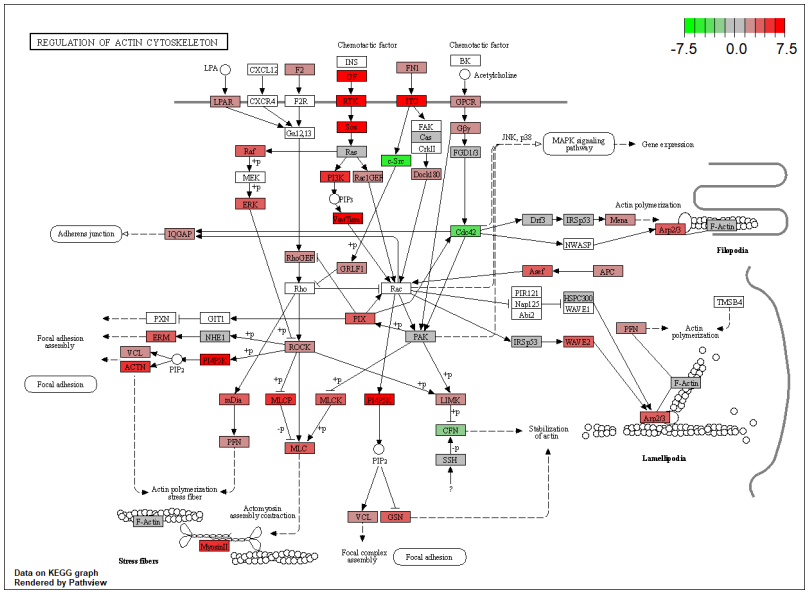


Fig 5.11 Visualisation of differential expression of cytoskeleton-related genes. Comparison between pre-mepolizumab (red) and post-mepolizumab (green) groups in a pathway map of Regulation of Actin Cytoskeleton integrated and drawn by KEGG (ID: hsa04810). The details of map notation can be found at https://www.genome.jp/kegg/document/help_pathway.html.

The distinct impact of IL-5 priming in eosinophils provides new information about the roles of eosinophils in asthma and the pharmacology of IL-5 blockage. Eosinophils with enhanced

activities contribute to inflammation, tissue damage and airway remodelling in asthma, and mepolizumab reverses all activities initiated by IL-5 priming. Future experiments to confirm the protein production of ECM and growth factors, signalling pathway of RTK and ECM should clarify the molecular mechanisms of priming and functions of eosinophils in eosinophilic asthma.

Chapter 6. General Discussion

6.1. Summary of studies

I have illustrated that $CRTH_2$ plays a crucial role in the biology of Tc2 and basophils. Inhibition of $CRTH_2$ on Tc2 cells confirmed the PGD_2-induced migration, aggregation, survival and proinflammatory cytokine production were mediated specifically by $CRTH_2$. Meanwhile, for the first time, I have shown the profibrotic effects of the $PGD_2/CRTH_2$ axis from Tc2 cells. Tc2 cells not only act as the effector cells of PGD_2 but also were found in this study as a source of PGD_2 *de novo* synthesis. All these cellular activities could be potentially induced and enhanced by the endogenous production of PGD_2 via autocrine signalling.

Barring allergic responses, the functions of basophils in asthma are often undervalued. In this thesis, I observed that blood basophils were enriched in severe eosinophilic asthma and their number was associated with eosinophil counts only but not serum IgE, suggesting a robust role of basophils in the severe eosinophilic asthma regardless of atopy. Interestingly, PGD_2 functions in basophils are similar to those in Tc2 cells. Further studies of $CRTH_2$ on basophils revealed that PGD_2 elicited basophil migration, cytokine production and degranulation via the $CRTH_2$ receptor, which implies that PGD_2 might also contribute to the pathogenic roles of basophils in severe eosinophilic asthma.

Reducing eosinophils in the lung has been used as a clinical strategy to suppress type 2 inflammation in severe asthma. Following this strategy, anti-IL-5 therapeutics have been developed and achieved clinical success. In this thesis, I revealed that, apart from acting as a

critical chemoattractant, IL-5 primed eosinophils, enhanced the capacity of eosinophil migration, activation and degranulation, and altered the transcriptome of eosinophils compared to healthy subjects. The administration of mepolizumab in severe eosinophilic asthma obstructed IL-5 function and significantly reversed the excess eosinophil activities. The transcriptomic study also indicated that IL-5 in humans contributes to the potential roles of eosinophils in tissue damage and remodelling in asthma.

6.2. Discussion of $CRTH_2$ antagonism *in vitro* and *in vivo*

The discovery of $CRTH_2$ in both mouse (Abe *et al.*, 1999) and human (Nagata *et al.*, 1999) T cells initiated the study of the biology of this PGD_2-binding G protein coupled receptor. Soon in the following years, it was reported to be the most reliable pan marker identifying type 2-cytokine-producing T cells in human peripheral blood (Cosmi *et al.*, 2000). With the accumulation of knowledge, it is now known that $CRTH_2$ is also expressed in basophils, eosinophils and ILC2s (Hirai *et al.*, 2001; Mjösberg *et al.*, 2011). Pro-inflammatory actions of PGD_2 were described almost 50 years ago (Flower, Harvey and Kingston, 1976), but it was only in 2001 that PGD_2 was shown to act on eosinophils via $CRTH_2$ (Gervais *et al.*, 2001; Hirai *et al.*, 2001; Monneret *et al.*, 2001). Scientists have then discovered the functions of $CRTH_2$ on many type 2 cells, which implies that $CRTH_2$ is not simply a biomarker of type 2 cells but a functionally important receptor. Binding with the ligand PGD_2, $CRTH_2$ contributes to inflammation by provoking cell migration and type 2 cytokine production in most type 2 cells (Hirai *et al.*, 2001; Xue *et al.*, 2005, 2014; Hilvering *et al.*, 2018). In light of these results, $CRTH_2$ has emerged as one of the most promising pharmacological targets for the treatment of

asthma. In the following decade, many antagonists including TM30089 (Uller *et al.*, 2007), OC000459 (Pettipher *et al.*, 2012), and MK-7246 (Gil *et al.*, 2014), with high potency and selectivity for human $CRTH_2$ were designed and exhibited *in vitro* and *in vivo* biological functions against inflammation. Fevipiprant (QAW039) might not be the earliest player in the $CRTH_2$ space, although this 7-Azaindole-3-acetic acid derivative showed the most advanced progression in clinical studies when the project started in 2016 (Sandham *et al.*, 2009; Luu *et al.*, 2015; Erpenbeck, Popov, *et al.*, 2016; Erpenbeck, Vets, *et al.*, 2016). Therefore, fevipiprant was applied in both Tc2 and basophil studies as a potent tool compound to deconvolute the $CRTH_2$-mediated functions in those cell types. However, these robust results *in vitro* did not coincide with the projected clinical outcome. Fevipiprant failed in phase III trials, although a borderline significant reduction in exacerbation was seen, suggesting a limited functional role. Neither did the two remaining candidates, OC000459 (ClinicalTrials.gov identifier: NCT04049175) and GB001 (ClinicalTrials.gov identifier: NCT03683576), achieve clinical success. The approved drugs, CysLTR antagonists like montelukast, which is another derivative of eicosanoids functioning similarly *in vitro* (mentioned in Chapter 1), showed weak benefits in improving lung function (biomarker: FEV1) and reducing IL-13-driven inflammation (biomarker: FeNO) (Sandrini *et al.*, 2003; Menzies *et al.*, 2007; Ye *et al.*, 2015). However, trials comparing the efficacy between montelukast and ICS/LABA also revealed that montelukast had no better effects on FEV1 and asthma exacerbations (Kanniess *et al.*, 2002; Bjermer *et al.*, 2003). Since montelukast has been on the market since 1998, the standard of clinical endpoints is much more stringent today. Especially the significant effects of biologics such as anti- IL-5, IL-5R, IL-4R and TSLP make $CRTH_2$ antagonists difficult to be

differentiated from the standard of care, even if these CRTH2 antagonists did illustrate mild therapeutic effects on protection against asthma worsening and exacerbation rate (Brightling *et al.*, 2021). The table below (Table 6.1) briefly illustrates the comparison of some efficacy outcomes among phase III trials of fevipiprant (Brightling *et al.*, 2021), montelukast (Bjermer *et al.*, 2003; Sandrini *et al.*, 2003; Paggiaro and Bacci, 2011; Ye *et al.*, 2015), mepolizumab (Pavord *et al.*, 2012; Ortega *et al.*, 2014), dupilumab (Castro *et al.*, 2018) and tezepelumab (Menzies-Gow *et al.*, 2021). Unlike the mechanism of action of dupilumab (blocking IL-4/13 activation) or mepolizumab (blocking IL-5 activation), clinical benefits of $CRTH_2$ antagonism, on the other hand, are highly associated with the reduction of sputum eosinophil counts and inflammation (Gonem *et al.*, 2016; Brightling *et al.*, 2021), suggesting the mechanism of action of $CRTH_2$ antagonists is predominately related to PGD_2-mediated chemotaxis but not type 2 cytokine production or cytokine-driven cellular activities downstream. This contradicts the finding *in vitro,* suggesting $CRTH_2$ negative cells might produce type 2 cytokines or $CRTH_2$ positive cells could still produce type 2 cytokines bypassing $PGD_2/CRTH_2$ axis. In the future plan below, I will discuss the possible work to demonstrate the effectiveness of $CRTH_2$ antagonism in asthma.

6.3. Future plan

6.3.1. RNA sequencing analysis and follow up confirmation

For the basophil and eosinophil projects, further work should be done to confirm whether the levels of differentially expressed genes found in RNA-seq are also changed in human cells. For

the eosinophil project, cells should be isolated from healthy and eosinophilic asthma patients, and PCR should be done with unstimulated cells. For the basophil project, cells should be isolated from leukocyte cones, and treated with the same stimuli, and PCR/protein studies should be done to check the PGD_2- or IgE- mediated gene profile changes.

6.3.2. Why could the robust bioactivities of $CRTH_2$ antagonism *in vitro* not be echoed in clinical trials of $CRTH_2$ antagonists?

6.3.2.1. $CRTH_2$/CysLTR combination

Since the combination of PGD_2 and leukotrienes enhanced type 2 cell activities (Xue *et al.*, 2015; Salimi *et al.*, 2017), blocking $CRTH_2$ or CysLTR alone might lead to the compensation of increasing activities of the other pathway. The combination of $CRTH_2$ antagonists and CysLTR antagonists administration might result in a significant improvement in disease symptoms. Therefore, a reanalysis of the trials of $CRTH_2$ antagonists might reveal whether patients taking montelukast in the treated group showed better efficacy outcomes compared to those not taking montelukast in the standard of care protocol.

6.3.2.2. Alternative source of type 2 cytokines

Since the fevipiprant phase III trials (Table 6.1) demonstrated that fevipiprant could not block IL-5 (biomarker: blood eosinophil counts) and IL-4/13 signalling (biomarker: FeNO), another possibility is that $CRTH_2$- cells might have critical roles in type 2 inflammation, especially when the signalling is suppressed in $CRTH_2$-antagonised $CRTH_2^+$ cells. From our in-house studies, we discovered that $CRTH_2^-$ $CD4^+$ or $CD8^+$ cells could express moderate amounts of

IL-4, IL-5 and IL-13 (data not shown). When $CRTH_2$ is blocked by fevipiprant in humans, $CRTH_2$- T cells might be activated, and type 2 cytokines would be released via the salvage pathway. Additionally, TCR-mediated T cell activation would also facilitate cytokine production. Hence, these $CRTH_2^-$ T cells might contribute to IL-4/5/13-mediated lung inflammation via the interaction of antigen-presenting cells in the lung. Considering the anti-TSLP antibody tezepelumab significantly reduced the FeNO and blood eosinophil counts, TSLP-induced cytokine production from ILC2, basophils, and epithelial cells could also bypass the $PGD_2/CRTH_2$ axis *in vivo.* In the following studies, so one should confirm the capacity of cytokine production from $CRTH_2^-$ cells via TCR or TSLP-mediated activation. One could also explore if patients with lower levels of alarmins, including TSLP or excess PGD_2 levels in the lung, would be better responders to $CRTH_2$ antagonists.

Table 6.1 Summary of efficacy outcomes among phase III trials of fevipiprant, montelukast, mepolizumab, dupilumab and tezepelumab.

Variables	Fevipiprant	Montelukast	Mepolizumab	Dupilumab	Tezepelumab
ΔPrebronchodilator FEV1 (Litre)	0.04-0.07, $p > 0.1$	-0.08, p unknown	0.1, $p < 0.05$	0.14, $P < 0.001$	0.13, $p < 0.001$
Lower Ratio of Annualised Rate of Asthma Exacerbations (treated vs placebo)	0.69-1.04, $p > 0.05$	No change, $p > 0.1$	0.39-0.52 $p < 0{\cdot}001$	0.477, $p < 0.001$	0.44, $p < 0.001$
Δ FeNO level (ppb)	No change, p unknown	Unknown	> -10, $p > 0.1$	(-14,-10), $p < 0.01$ §	-13.8, $p < 0.0001$ #
Δ Blood eosinophil count (cells/μ)	No change, p unknown	-30, p unknown	(-220, -120), $p < 0{\cdot}0001$	(23, 41) $p > 0.1$ §	-130 $p < 0.0001$ #

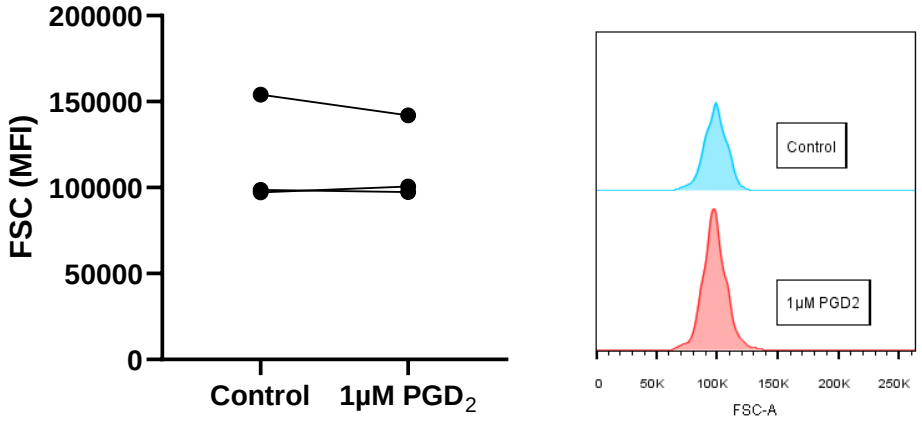
The data were retrieved from the released results of trials (Bjermer *et al.*, 2003; Sandrini *et al.*, 2003; Paggiaro and Bacci, 2011; Pavord *et al.*, 2012; Ortega *et al.*, 2014; Ye *et al.*, 2015; Castro *et al.*, 2018; Brightling *et al.*, 2021; Menzies-Gow *et al.*, 2021)

Δ = data from treatment groups – data from placebo groups

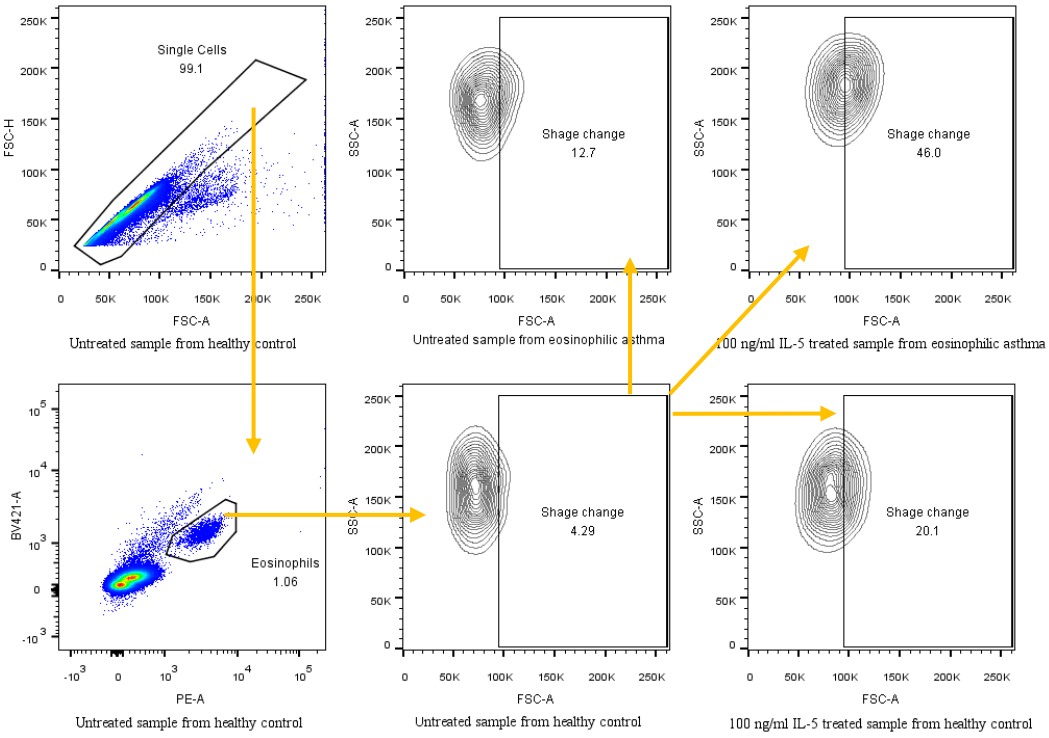
§ Unpaired t test was performed using the data reported (Rabe *et al.*, 2018). No p value was presented in the phase III trial

 # Unpaired t test was performed using the data reported (Menzies-Gow *et al.*, 2021). No p value was presented in the phase III trial

Appendix I – Figures and Tables



Appendix Fig 4.1 Detection of PGD_2-mediated basophil migration using shape change assay *ex vivo* in whole blood.
Paired-wise comparison of MFI of FSC between basophils in the presence or absence of 1μM PGD_2 (left) and the illustration of flow cytometry data on the histogram (right).



Appendix Fig 5.1 Gating strategy of shape change assay.
The PE (x axis) and BV421 (y axis) in the lower left figure represent the channel used for the corresponding dye reflecting the autoflorescence, whilst cells were not actually stained.

11 **Appendix Table 2.1 Primer list for qPCR**

Name	Sequence (F)	Sequence (R)
CCL11	ATGAAGGTCTCCGCAGCA	GGTGGTTGGGACAGAAGC
CCL24	AATTCCTGAGAACCGAGTGG	GCCCTTCTTGGTGGTGAAG
CCL26	CTGGGTGCGAAGCTATGAAT	TCTTGCCTCTTTTGGTAGTGAA
CD69	CAAGTTCCTGTCCTGTGTGC	CAATTGTATTGGCCCACTGA
$CRTH_2$	CCTGTGCTCCCTCTGTGC	TCTGGAGACGGCTCATCTG
CSF1 (V1)	GCAAGAACTGCAACAACAGC	ATCAGGCTTGGTCACCACAT
GAPDH	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC
GM-CSF	TCTCAGAAATGTTTGACCTCCA	GCCCTTGAGCTTGGTGAG
ICAM1	CCTTCCTCACCGTGTACTGG	AGCGTAGGGTAAGGTTCTTGC
IL13	AGCCCTCAGGGAGCTCAT	CTCCATACCATGCTGCCATT
IL3	TTGCCTTTGCTGGACTTCA	CTGTTGAATGCCTCCAGGTT
IL4	CACCGAGTTGACCGTAACAG	GCCCTGCAGAAGGTTTCC
IL5	GGTTTGTTGCAGCCAAAGAT	TCTTGGCCCTCATTCTCACT
PECAM1	GCAACACAGTCCAGATAGTCGT	GACCTCAAACTGGGCATCAT
TGFB1	CGACTACTACGCCAAGGAGGT	TGCTTGAACTTGTCATAGATTTCG
TNF	CAGCCTCTTCTCCTTCCTGAT	GCCAGAGGGCTGATTAGAGA
CRLF2/TSLPR	AGCGACTGGTCAGAGGTGA	AATTTGGACAGCTTTGGTTTG
MMP9	GAACCAATCTCACCGACAGG	GCCACCCGAGTGTAACCATA
TIMP1	CTGTTGTTGCTGTGGCTGAT	AACTTGGCCCTGATGACG
B2M	TTCTGGCCTGGAGGCTATC	TCAGGAAATTTGACTTTCCATTC
TUBB2A	TGCAGCTGGAGAGAATCAAC	GGATGGCCCGAGGTACATA
RACK1	TTCAGCCCTAACCGCTACTG	GATCTTTCCCTCTAAATCCCAGA
CASC3	ACCACCGCCTCATCTGTATC	TGGGCGGGGTTATAGTAGGT
RPLP0	TCTACAACCCTGAAGTGCTTGAT	CAATCTGCAGACAGACACTGG

UBC	GGAAGGCATTCCTCCTGAT	CCCACCTCTGAGACGGAGTA
UBE2D2	AATGGCAGCATTTGTCTTGA	GGATTGGGATCACACAACAGA
YWHAZ	CGAGATCCAGGGACAGAGTC	AGCTCATTTTTATCCATGACTG G
SDHA	CAGACCATCTACGGAGCAGAG	GATGGGCTTGGAGTAATCGT
HMBS	AGCTATGAAGGATGGGCAAC	TTGTATGCTATCTGAGCCGTCT A
PPIA	ATGCTGGACCCAACACAAAT	TCTTTCACTTTGCCAAACACC
TNFRSF12A	GACCGCACAGCGACTTCT	CACGAAGGTCAGGCTCAGA
PPARG	GACAGGAAAGACAACAGACAAATC	GGGGTGATGTGTTTGAACTTG
NTRK1	CAATGCCTCGGTGGATGT	GCAGACCCCCAGATTTCA
AHR	CAACATCACCTACGCCAGTC	GCTTGGAAGGATTTGACTTGA
NRP1	GAGATTATCCTGGAATTTGAAAGC	CACAGTAACGCCCAATGTGA
EDARADD	ACTGCCCACGAAATTCAGATA	TGGAAGAGGATCTCCAGTGC
EPAS1	GACATGAAGTTCACCTACTGTGATG	CGGAGTCTAGCGCATGGTA
HMOX1	GGCAGAGGGTGATAGAAGAGG	AGCTCCTGCAACTCCTCAAA
SDC4	GGCAGGAATCTGATGACTTTG	GGCCGATCATGGAGTCTTC
TNFSF11	TGATTCATGTAGGAGAATTAAACA GG	GATGTGCTGTGATCCAACGA
FLT4	GTGACGTGTGGTCCTTTGG	CTGGCAGAACTCCTCATTGAT
PRDM1	GTGGTGGGTTAATCGGTTTG	GAAGCTCCCCTCTGGAATAGA
PLAUR	AGCCTTACCGAGGTTGTGTG	CTTCGGGAATAGGTGACAGC
IL1A	GGTTGAGTTTAAGCCAATCCA	TGCTGACCTAGGCTTGATGA
SRC	CCATGTTCACTCCGGTTTTAC	CTCAGCGTCCTCATCTGGTT
CD44	GACACCATGGACAAGTTTTGG	CGGCAGGTTATATTCAAATCG
SERPINE2	TCTGCCTGTGATTCCATCAA	GGTGAGCACACCATCAATAAG AT
PRKCD	ATTATCCCCGCTGGATCAC	CTTGGTTGGTTCCCTTTCAA
NFATC1	CCAAGGTCATTTTCGTGGAG	GGTCAGTTTTCGCTTCCATC

IL1RL1	TTGTCCTACCATTGACCTCTACAA	GATCCTTGAAGAGCCTGACAA
ADRB2	CCATGTCCAGAACCTTAGCC	GATCTGCGGAGTCCATGC
TNFSF4	GTATCCTCGAATTCAAAGTATCAAAGT	CTGAGTTGTTCTGCACCTTCA
TNFRSF4	CCTGCACGTGGTGTAACCT	AGCGGCAGACTGTGTCCT
PLA2	CCTCCCCACCCTGAAAAA	TGCCAAAATTTTAAATGGAACAC
PLC	CAGTGTTAATGAGAACCACTCCA	TTGCTGACTCTCTTCTCTCTTAACC
COL19A1	GGGGAGAAAGGCGATGAG	TAAGCCAGGGGGTCCAGT
ITGB2	GTGGGGGAGCTGTCTGAG	GGAAGACCCTGGAGGAGAGT

12

Appendix Table 5.1 General characteristics of the study subjects at baseline and end of the trial (eosinophil RNA-seq)

Characteristic	Healthy control		Severe eosinophilic asthma					
			Pre-mepolizumab	Post-mepolizumab	Pre-mepolizumab	Post-mepolizumab	Pre-mepolizumab	Post-mepolizumab
ID	1	2	3		4 #		5	
Sex (%female)	F	F	F		F		F	
BMI	28.9	35.7	43.1		20.3		37.3	
Age	45	36	57		52		39	
FeNO (ppb)	22	8	34	49	22	32	10	10
Pre FEV1 (L)	2.74	3.01	1.95	2.39	4.09	4.33	2.33	2.06
Blood eosinophils (per μL)	530	200	320	110	160	60	550	230

BMI (body mass index) = Weight (kg)/Height2 (m).
FeNO, fractional concentration of exhaled nitric oxide.
FEV1, forced expiratory volume in one second
 # patient NO. 4 was excluded in the RNA-seq analysis
This is a one-month follow-up study

Appendix II - Journal Articles and Conference Abstracts During the DPhil Studies

DPhil thesis study:

Publications:

1. **Chen, W.**, Luo, J., Ye, Y., Hoyle, R., Liu, W., Borst, R., Kazani, S., Shikatani, E. A., Erpenbeck, V. J., Pavord, I. D., Klenerman, P., Sandham, D. A., & Xue, L. (2021). The Roles of Type 2 Cytotoxic T Cells in Inflammation, Tissue Remodelling, and Prostaglandin (PG) D2 Production Are Attenuated by PGD_2 Receptor 2 Antagonism. *The Journal of Immunology*, *206*(11), 2714–2724. https://doi.org/10.4049/JIMMUNOL.2001245

Conferences:

American Thoracic Society International Conference 2018, 2019, 2020

European Respiratory Society International Congress 2018, 2019

Other contributions to DPhil study:

Publications:

1. Ye, Y., Luo, J., Zeng, N., Jiang, S., Chen, W., Hoyle, R. D., Klenerman, P., Pavord, I. D., & Xue, L. (2022). Neuromedin U promotes human type 2 immune responses. *Mucosal Immunology*, *15*(5), 990–999.
2. COvid-19 Multi-omics Blood ATlas (COMBAT) Consortium (2022). A blood atlas of COVID-19 defines hallmarks of disease severity and specificity. *Cell*, *185*(5), 916-938.e58.
3. Luo, J., Chen, Y.-L., Chen, W., Duncan, D. A., Mentzer, A., Knight, J. C., Ogg, G., Klenerman, P., Pavord, I. D., & Xue, L. (2021). Pre-existing asthma as a comorbidity

does not modify cytokine responses and severity of COVID-19. *Allergy, Asthma & Clinical Immunology*, *17*(1), 1–12.

4. Youngs, J., Provine, N. M., Lim, N., Sharpe, H. R., Amini, A., Chen, Y.-L., Luo, J., Edmans, M. D., Zacharopoulou, P., Chen, W., & others. (2021). Identification of immune correlates of fatal outcomes in critically ill COVID-19 patients. *PLoS Pathogens*, *17*(9), e1009804.

5. Hardman, C., Chen, W., Luo, J., Batty, P., Chen, Y.-L. L., Nahler, J., Wu, Y., Pavord, I. D., Erpenbeck, V. J., Sandham, D. A., Xue, L., Ogg, G., & others. (2019). Fevipiprant, a selective prostaglandin D2 receptor 2 antagonist, inhibits human group 2 innate lymphoid cell aggregation and function. *Journal of Allergy and Clinical Immunology*, *143*(6), 2329–2333.

6. Hilvering, B., Hinks, T. S. C. C., Stöger, L., Marchi, E., Salimi, M., Shrimanker, R., Liu, W., Chen, W., Luo, J., Go, S., Powell, T., Cane, J., Thulborn, S., Kurioka, A., Leng, T., Matthews, J., Connolly, C., Borg, C., Bafadhel, M., … others. (2019). Synergistic activation of pro-inflammatory type 2 CD8+ T lymphocytes by lipid mediators in severe eosinophilic asthma. *Mucosal Immunology*, *12*(2), 581.

60 Abbreviations

Abbreviation	Long format
ACQ	Asthma Control Questionnaire
ANOVA	analysis of variance
APC	Antigen-presenting cells or allophycocyanin, context-dependent
ATS	American Thoracic Society
BAL	bronchoalveolar lavage
BMI	body mass index
BV	Brilliant Violet™
CCL	C-C chemokine ligand (e.g., CCL17, CCL18 etc.)
CCR	C-C chemokine receptor (e.g., CCR3, CCR7 etc.)
CD	cluster of differentiation
cDNA	complementary DNA
COPD	chronic obstructive pulmonary disease
$CRTH_2$	chemoattractant receptor-homologous molecule expressed on Th2 cells
CSF	colony-stimulating factor
DC	dendritic cell
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DP1	prostaglandin D2 receptor 1
DP2	prostaglandin D2 receptor 2 ($CRTH_2$)
ECM	extracellular matrix
ECP	eosinophil cationic protein
EDN	eosinophil-derived neurotoxin
EGF	epidermal growth factor

EPX	eosinophil peroxidase
Erk	extracellular signal-regulated kinase
ERS	European Respiratory Society
FACS	fluorescence-activated cell sorting
FC	fold change
FCS	foetal calf serum
FeNO	fractional exhaled nitric oxide
FEV1	forced expiratory volume in 1 second
FVC	forced vital capacity
GABA	γ-aminobutyric acid
GINA	Global Initiative for Asthma
GM-CSF	granulocyte-macrophage colony-stimulating factor
GO	Gene Ontology
GPCR	G protein-coupled receptor
HC	healthy control
HLA	human leukocyte antigen
HPGDS	haematopoietic prostaglandin D synthase
ICS	inhaled corticosteroids
Ig	immunoglobulin
IL	interleukin
ILC	innate lymphoid cell
ILC2	group 2 innate lymphoid cell
ILR	interleukin receptor (e.g., IL6R, IL4R, etc.)
KEGG	Kyoto Encyclopaedia of Genes and Genomes
LT	leukotriene
MAPK	mitogen-activated protein kinase
MBP	major basic protein

M-CSF	macrophage colony-stimulating factor
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
mRNA	messenger RNA
OCS	oral corticosteroids
padj	adjusted p-value
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PE	phycoerythrin
PerCP	peridinin chlorophyll protein
PGD_2	prostaglandin D2
PI3K	phosphoinositide 3-kinase
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
qPCR	Real-time quantitative polymerase chain reaction
RNA	ribonucleic acid
RNA-seq	RNA sequencing
scRNA-seq	Single-cell RNA sequencing
T0	naïve T
T2	type 2
Tc	cytotoxic T
Tc2	type 2 cytotoxic T
TCR	T-cell receptor
TGF	transforming growth factor
Th	T helper
Th2	type 2 T helper

TNF	tumour necrosis factor
TP	thromboxane receptor
TSLP	thymic stromal lymphopoietin
US	unstimulated

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BIBLIOGRAPHY

Abdelrahim, M.E.A. *et al.* (2021) 'The Type of Patients Requiring Aerosol Therapy (to Treat What and Where?)', *Essentials of Aerosol Therapy in Critically ill Patients*, pp. 1–25. Available at: https://doi.org/10.1007/978-3-030-85026-5_1.

Abe, H. *et al.* (1999) 'Molecular cloning, chromosome mapping and characterization of the mouse CRTH2 gene, a putative member of the leukocyte chemoattractant receptor family', *Gene*, 227(1), pp. 71–77. Available at: https://doi.org/10.1016/S0378-1119(98)00599-X.

Amin, K., Janson, C. and Bystrom, J. (2016) 'Role of Eosinophil Granulocytes in Allergic Airway Inflammation Endotypes', *Scandinavian Journal of Immunology*, 84(2), pp. 75–85. Available at: https://doi.org/10.1111/sji.12448.

Anderson, S.D. and Kippelen, P. (2008) 'Airway injury as a mechanism for exercise-induced bronchoconstriction in elite athletes', *Journal of Allergy and Clinical Immunology*, 122(2), pp. 225–235. Available at: https://doi.org/10.1016/j.jaci.2008.05.001.

Aravamudan, B. *et al.* (2012) 'Brain-derived neurotrophic factor induces proliferation of human airway smooth muscle cells', *Journal of Cellular and Molecular Medicine*, 16(4), pp. 812–823. Available at: https://doi.org/10.1111/j.1582-4934.2011.01356.x.

Arganda-Carreras, I. *et al.* (2017) 'Trainable Weka Segmentation: A machine learning tool for microscopy pixel classification', *Bioinformatics*, 33(15), pp. 2424–2426. Available at: https://doi.org/10.1093/bioinformatics/btx180.

Asano, K. *et al.* (2020) 'A Phase 2a Study of DP2 Antagonist GB001 for Asthma', *Journal of Allergy and Clinical Immunology: In Practice*, 8(4), pp. 1275-1283.e1. Available at: https://doi.org/10.1016/j.jaip.2019.11.016.

Ballesteros-Tato, A. *et al.* (2016) 'T Follicular Helper Cell Plasticity Shapes Pathogenic T Helper 2 Cell-Mediated Immunity to Inhaled House Dust Mite', *Immunity*, 44(2), pp. 259–273. Available at: https://doi.org/10.1016/j.immuni.2015.11.017.

Barnes, N. *et al.* (2012) 'A randomized, double-blind, placebo-controlled study of the CRTH2 antagonist OC000459 in moderate persistent asthma', *Clinical and Experimental Allergy*, 42(1), pp. 38–48. Available at: https://doi.org/10.1111/j.1365-2222.2011.03813.x.

Barnes, P.J. (2010) 'Inhaled Corticosteroids', *Pharmaceuticals*, 3(3), pp. 514–540. Available at: https://doi.org/10.3390/ph3030514.

Barnes, P.J. (2011) 'Pathophysiology of allergic inflammation', *Immunological Reviews*, 242(1), pp. 31–50. Available at: https://doi.org/10.1111/j.1600-065X.2011.01020.x.

Barranco, P. *et al.* (2017) 'Dupilumab in the management of moderate-to-severe asthma: the data so far', *Therapeutics and Clinical Risk Management*, Volume 13, pp. 1139–1149. Available at: https://doi.org/10.2147/TCRM.S125964.

Bartemes, K.R. *et al.* (2014) 'Enhanced innate type 2 immune response in peripheral blood from patients with asthma', *Journal of Allergy and Clinical Immunology*, 134(3), pp. 671-678.e4. Available at: https://doi.org/10.1016/j.jaci.2014.06.024.

Bateman, E.D. *et al.* (2017) 'Fevipiprant, an oral prostaglandin DP2 receptor (CRTh2) antagonist, in allergic asthma uncontrolled on low-dose inhaled corticosteroids', *The European respiratory journal*, 50(2), p. 1700670. Available at: https://doi.org/10.1183/13993003.00670-2017.

Bates, M.E., Green, V.L. and Bertics, P.J. (2000) 'ERK1 and ERK2 activation by chemotactic factors in human eosinophils is interleukin 5-dependent and contributes to leukotriene C4 biosynthesis', *Journal of Biological Chemistry*, 275(15), pp. 10968–10975. Available at: https://doi.org/10.1074/jbc.275.15.10968.

Beckett, P.A. and Howarth, P.H. (2003) 'Pharmacotherapy and airway remodelling in asthma?', *Thorax*, 58(2), pp. 163–74. Available at: https://doi.org/10.1136/thorax.58.2.163.

Berair, R. *et al.* (2015) 'LATE-BREAKING ABSTRACT: Effect of QAW039, an oral prostaglandin D2 receptor (DP2/CRTh2) antagonist, upon bronchial epithelial integrity in treatment-resistant asthma in a randomized, placebo controlled study', in *5.1 Airway*

Pharmacology and Treatment. European Respiratory Society, p. OA290. Available at: https://doi.org/10.1183/13993003.congress-2015.OA290.

Bjermer, L. *et al.* (2003) 'Montelukast and fluticasone compared with salmeterol and fluticasone in protecting against asthma exacerbation in adults: One year, double blind, randomised, comparative trial', *British Medical Journal*, 327(7420), pp. 891–895. Available at: https://doi.org/10.1136/bmj.327.7420.891.

Bochner, B.S. *et al.* (1994) 'Function and expression of adhesion molecules on human basophils', *Journal of Allergy and Clinical Immunology*, 94(6), pp. 1157–1162. Available at: https://doi.org/10.1016/0091-6749(94)90326-3.

Boulet, L.P. *et al.* (1997) 'Inhibitory effects of an anti-IgE antibody E25 on allergen-induced early asthmatic response', *American Journal of Respiratory and Critical Care Medicine*, 155(6), pp. 1835–1840. Available at: https://doi.org/10.1164/ajrccm.155.6.9196083.

Brightling, C.E. *et al.* (2021) 'Effectiveness of fevipiprant in reducing exacerbations in patients with severe asthma (LUSTER-1 and LUSTER-2): two phase 3 randomised controlled trials', *The Lancet Respiratory Medicine*, 9(1), pp. 43–56. Available at: https://doi.org/10.1016/S2213-2600(20)30412-4.

Brooks, C.R. *et al.* (2017) 'Sputum basophils are increased in eosinophilic asthma compared with non-eosinophilic asthma phenotypes', *Allergy: European Journal of Allergy and Clinical Immunology*, 72(10), pp. 1583–1586. Available at: https://doi.org/10.1111/all.13185.

Broughton, S.E. *et al.* (2015) 'The βc receptor family - Structural insights and their functional implications', *Cytokine*, 74(2), pp. 247–258. Available at: https://doi.org/10.1016/j.cyto.2015.02.005.

Van Bruaene, N. *et al.* (2009) 'TGF-β signaling and collagen deposition in chronic rhinosinusitis', *Journal of Allergy and Clinical Immunology*, 124(2), pp. 253–9, 259.e1–2. Available at: https://doi.org/10.1016/j.jaci.2009.04.013.

Brusselle, G.G., Maes, T. and Bracke, K.R. (2013) 'Eosinophils in the Spotlight: Eosinophilic airway inflammation in nonallergic asthma', *Nature Medicine*, 19(8), pp. 977–979. Available at: https://doi.org/10.1038/nm.3300.

Burgel, P.-R. *et al.* (2001) 'Human Eosinophils Induce Mucin Production in Airway Epithelial Cells Via Epidermal Growth Factor Receptor Activation', *The Journal of Immunology*, 167(10), pp. 5948–5954. Available at: https://doi.org/10.4049/jimmunol.167.10.5948.

Busse, W. *et al.* (2001) 'Omalizumab, anti-IgE recombinant humanized monoclonal antibody, for the treatment of severe allergic asthma', *Journal of Allergy and Clinical Immunology*, 108(2), pp. 184–190. Available at: https://doi.org/10.1067/mai.2001.117880.

Busse, W.W. *et al.* (2013) 'Randomized, Double-Blind, Placebo-controlled Study of Brodalumab, a Human Anti–IL-17 Receptor Monoclonal Antibody, in Moderate to Severe Asthma', *American Journal of Respiratory and Critical Care Medicine*, 188(11), pp. 1294–1302. Available at: https://doi.org/10.1164/rccm.201212-2318OC.

Camelo, A. *et al.* (2017) 'IL-33, IL-25, and TSLP induce a distinct phenotypic and activation profile in human type 2 innate lymphoid cells', *Blood Advances*, 1(10), pp. 577–589. Available at: https://doi.org/10.1182/bloodadvances.2016002352.

Cao, S. *et al.* (2010) 'Neuropilin-1 promotes cirrhosis of the rodent and human liver by enhancing PDGF/TGF-β signaling in hepatic stellate cells', *Journal of Clinical Investigation*, 120(7), pp. 2379–2394. Available at: https://doi.org/10.1172/JCI41203.

Carmo, L.A.S. *et al.* (2016) 'CD63 is tightly associated with intracellular, secretory events chaperoning piecemeal degranulation and compound exocytosis in human eosinophils', *Journal of Leukocyte Biology*, 100(2), pp. 391–401. Available at: https://doi.org/10.1189/jlb.3a1015-480r.

Carstensen, S. *et al.* (2019) 'Fevipiprant inhibits eosinophil activation induced by multiple metabolites of prostaglandin D2', in *Allergy and immunology*. European Respiratory Society, p. PA4391. Available at: https://doi.org/10.1183/13993003.congress-2019.PA4391.

Castro, M. *et al.* (2018) 'Dupilumab Efficacy and Safety in Moderate-to-Severe Uncontrolled Asthma', *New England Journal of Medicine*, 378(26), pp. 2486–2496. Available at: https://doi.org/10.1056/nejmoa1804092.

Chanez, P. *et al.* (1990) 'Generation of oxygen free radicals from blood eosinophils from asthma patients after stimulation with PAF or phorbol ester', *European Respiratory Journal*, 3(9), pp. 1002–1007. Available at: https://doi.org/10.1183/09031936.93.03091002.

Chapman, D.G. and Irvin, C.G. (2015) 'Mechanisms of airway hyper-responsiveness in asthma: the past, present and yet to come', *Clinical & Experimental Allergy*, 45(4), pp. 706–719. Available at: https://doi.org/10.1111/cea.12506.

Chen, W. *et al.* (2021) 'Prostaglandin D2 drives tissue remodelling functions and prostaglandin D2 autocrine production in type 2 cytotoxic T cells', *The journal of immunology*, 206, pp. 2714–2724.

Cheng, D. *et al.* (2014) 'Epithelial interleukin-25 is a key mediator in Th2-high, corticosteroid-responsive asthma', *American Journal of Respiratory and Critical Care Medicine*, 190(6), pp. 639–648. Available at: https://doi.org/10.1164/rccm.201403-0505OC.

Cheng, F. *et al.* (2016) 'Vimentin coordinates fibroblast proliferation and keratinocyte differentiation in wound healing via TGF-β-Slug signaling', *Proceedings of the National Academy of Sciences of the United States of America*, 113(30), pp. E4320–E4327. Available at: https://doi.org/10.1073/pnas.1519197113.

Chipps, B.E. *et al.* (2018) 'Benralizumab efficacy by atopy status and serum immunoglobulin E for patients with severe, uncontrolled asthma', *Annals of Allergy, Asthma and Immunology*, 120(5), pp. 504-511.e4. Available at: https://doi.org/10.1016/j.anai.2018.01.030.

Choi, E.N. *et al.* (2003) 'A parallel signal-transduction pathway for eotaxin- and interleukin-5-induced eosinophil shape change', *Immunology*, 108(2), pp. 245–256. Available at: https://doi.org/10.1046/j.1365-2567.2003.01565.x.

Choi, Y. *et al.* (2018) 'Biological function of eosinophil extracellular traps in patients with severe eosinophilic asthma', *Experimental and Molecular Medicine*, 50(8), p. 104. Available at: https://doi.org/10.1038/s12276-018-0136-8.

Chung, K.F. *et al.* (2014) 'International ERS/ATS guidelines on definition, evaluation and treatment of severe asthma', *European Respiratory Journal*, 43(2), pp. 343–373. Available at: https://doi.org/10.1183/09031936.00202013.

Cockcroft, D.W. *et al.* (1977) 'Bronchial reactivity to inhaled histamine: a method and clinical survey.', *Clinical Allergy*, 7(3), pp. 235–43. Available at: https://doi.org/10.1111/j.1365-2222.1977.tb01448.x.

Cole, A.T. *et al.* (1995) 'A Flow Cytometric Method to Measure Shape Change of Human Neutrophils', *Clinical Science*, 89(5), pp. 549–554. Available at: https://doi.org/10.1042/cs0890549.

Corren, J. (2013) 'Asthma phenotypes and endotypes: An evolving paradigm for classification', *Discovery Medicine*, 15(83), pp. 243–249. Available at: https://pubmed.ncbi.nlm.nih.gov/23636141/ (Accessed: 17 May 2021).

Corren, J. *et al.* (2017) 'Tezepelumab in Adults with Uncontrolled Asthma', *New England Journal of Medicine*, 377(10), pp. 936–946. Available at: https://doi.org/10.1056/nejmoa1704064.

Cosmi, L. *et al.* (2000) 'CRTH2 is the most reliable marker for the detection of circulating human type 2 Th and type 2 T cytotoxic cells in health and disease.', *European Journal of Immunology*, 30(10), pp. 2972–9. Available at: https://doi.org/10.1002/1521-4141(200010)30:10<2972::AID-IMMU2972>3.0.CO;2-%23.

Crivellato, E., Travan, L. and Ribatti, D. (2010) 'Mast Cells and Basophils: A Potential Link in Promoting Angiogenesis during Allergic Inflammation', *International Archives of Allergy and Immunology*, 151(2), pp. 89–97. Available at: https://doi.org/10.1159/000235998.

214 Cromheecke, J.L., Nguyen, K.T. and Huston, D.P. (2013) 'Emerging Role of Human Basophil
215 Biology in Health and Disease', *Current Allergy and Asthma Reports 2013 14:1*, 14(1), pp. 1–
216 9. Available at: https://doi.org/10.1007/S11882-013-0408-2.

217 Cybulsky, A. V., McTavish, A.J. and Cyr, M.D. (1994) 'Extracellular matrix modulates
218 epidermal growth factor receptor activation in rat glomerular epithelial cells', *Journal of*
219 *Clinical Investigation*, 94(1), pp. 68–78. Available at: https://doi.org/10.1172/JCI117350.

220 Czech, W. *et al.* (1993) 'Induction of intercellular adhesion molecule 1 (ICAM-1) expression
221 in normal human eosinophils by inflammatory cytokines', *Journal of Investigative*
222 *Dermatology*, 100(4), pp. 417–423. Available at: https://doi.org/10.1111/1523-
223 1747.ep12472082.

224 Czock, D. *et al.* (2005) 'Pharmacokinetics and pharmacodynamics of systemically
225 administered glucocorticoids', *Clinical Pharmacokinetics*. Springer, pp. 61–98. Available at:
226 https://doi.org/10.2165/00003088-200544010-00003.

227 Dahlén, S.-E. *et al.* (1980) 'Leukotrienes are potent constrictors of human bronchi', *Nature*,
228 288(5790), pp. 484–486. Available at: https://doi.org/10.1038/288484a0.

229 Dale, H.H. and Laidlaw, P.P. (1919) 'Histamine shock', *The Journal of Physiology*, 52(5), pp.
230 355–390. Available at: https://doi.org/10.1113/jphysiol.1919.sp001837.

231 Davoine, F. and Lacy, P. (2014) 'Eosinophil cytokines, chemokines, and growth factors:
232 Emerging roles in immunity', *Frontiers in Immunology*. Frontiers Media S.A. Available at:
233 https://doi.org/10.3389/fimmu.2014.00570.

234 Dekkers, B.G.J. *et al.* (2010) 'The laminin β1-competing peptide YIGSR induces a
235 hypercontractile, hypoproliferative airway smooth muscle phenotype in an animal model of
236 allergic asthma', *Respiratory Research*, 11(1), p. 170. Available at:
237 https://doi.org/10.1186/1465-9921-11-170.

Distler, J.H.W. *et al.* (2008) 'The controversial role of tumor necrosis factor α in fibrotic diseases', *Arthritis and Rheumatism*, 58(8), pp. 2228–2235. Available at: https://doi.org/10.1002/art.23645.

Djukanović, R. *et al.* (2004) 'Effects of treatment with anti-immunoglobulin E antibody omalizumab on airway inflammation in allergic asthma', *American Journal of Respiratory and Critical Care Medicine*, 170(6), pp. 583–893. Available at: https://doi.org/10.1164/rccm.200312-1651OC.

Dobin, A. *et al.* (2013) 'STAR: Ultrafast universal RNA-seq aligner', *Bioinformatics*, 29(1), pp. 15–21. Available at: https://doi.org/10.1093/bioinformatics/bts635.

Doerr, M.E. and Jones, J.I. (1996) 'The roles of integrins and extracellular matrix proteins in the insulin-like growth factor I-stimulated chemotaxis of human breast cancer cells', *Journal of Biological Chemistry*, 271(5), pp. 2443–2447. Available at: https://doi.org/10.1074/jbc.271.5.2443.

Donnelly, L.E. and Barnes, P.J. (2006) 'Chemokine receptors as therapeutic targets in chronic obstructive pulmonary disease', *Trends in Pharmacological Sciences*, 27(10), pp. 546–553. Available at: https://doi.org/10.1016/j.tips.2006.08.001.

Doran, E. *et al.* (2017) 'Interleukin-13 in Asthma and Other Eosinophilic Disorders', *Frontiers in Medicine*, 4(SEP), p. 139. Available at: https://doi.org/10.3389/fmed.2017.00139.

Dougan, M., Dranoff, G. and Dougan, S.K. (2019) 'GM-CSF, IL-3, and IL-5 Family of Cytokines: Regulators of Inflammation', *Immunity*, 50(4), pp. 796–811. Available at: https://doi.org/10.1016/j.immuni.2019.03.022.

Dunican, E.M. and Fahy, J. V. (2015) 'The role of type 2 inflammation in the pathogenesis of asthma exacerbations', *Annals of the American Thoracic Society*, 12(Suppl 2), pp. S144–S149. Available at: https://doi.org/10.1513/AnnalsATS.201506-377AW.

Dvorak, A.M. *et al.* (1992) 'Mature eosinophils stimulated to develop in human-cord blood mononuclear cell cultures supplemented with recombinant human interleukin-5. II. Vesicular

transport of specific granule matrix peroxidase, a mechanism for effecting piecemeal degranulation.', *The American Journal of Pathology*, 140(4), p. 795. Available at: /pmc/articles/PMC1886371/?report=abstract (Accessed: 16 December 2022).

Eberlein, B. *et al.* (2015) 'Optimizing of the basophil activation test: Comparison of different basophil identification markers', *Cytometry Part B - Clinical Cytometry*, 88(3), pp. 183–189. Available at: https://doi.org/10.1002/cyto.b.21203.

Efremov, Y.M. *et al.* (2015) 'Distinct impact of targeted actin cytoskeleton reorganization on mechanical properties of normal and malignant cells', *Biochimica et Biophysica Acta - Molecular Cell Research*, 1853(11), pp. 3117–3125. Available at: https://doi.org/10.1016/j.bbamcr.2015.05.008.

Erin, E.M. *et al.* (2006) 'The effects of a monoclonal antibody directed against tumor necrosis factor-α in asthma (Retraction in: American Journal of Respiratory and Critical Care Medicine (2011) 183:3 (418))', *American Journal of Respiratory and Critical Care Medicine*, 174(7), pp. 753–762. Available at: https://doi.org/10.1164/rccm.200601-072OC.

Erpenbeck, V.J., Vets, E., *et al.* (2016) 'Pharmacokinetics, Safety, and Tolerability of Fevipiprant (QAW039), a Novel CRTh2 Receptor Antagonist: Results From 2 Randomized, Phase 1, Placebo-Controlled Studies in Healthy Volunteers', *Clinical pharmacology in drug development*, 5(4), pp. 306–313. Available at: https://doi.org/10.1002/CPDD.244.

Erpenbeck, V.J., Popov, T.A., *et al.* (2016) 'The oral CRTh2 antagonist QAW039 (fevipiprant): A phase II study in uncontrolled allergic asthma', *Pulmonary Pharmacology and Therapeutics*, 39, pp. 54–63. Available at: https://doi.org/10.1016/j.pupt.2016.06.005.

Evans, C.M. *et al.* (2009) 'Mucus hypersecretion in asthma: Causes and effects', *Current Opinion in Pulmonary Medicine*. NIH Public Access, pp. 4–11. Available at: https://doi.org/10.1097/MCP.0b013e32831da8d3.

Fahy, J. V. (2015) 'Type 2 inflammation in asthma-present in most, absent in many', *Nature Reviews Immunology*, 15(1), pp. 57–65. Available at: https://doi.org/10.1038/nri3786.

Fahy, J. V. and Dickey, B.F. (2010) ‘Airway Mucus Function and Dysfunction’, *New England Journal of Medicine*, 363(23), pp. 2233–2247. Available at: https://doi.org/10.1056/nejmra0910061.

Fahy, J.V. *et al.* (1997) ‘The effect of an anti-IgE monoclonal antibody on the early- and late-phase responses to allergen inhalation in asthmatic subjects’, *American Journal of Respiratory and Critical Care Medicine*, 155(6), pp. 1828–1834. Available at: https://doi.org/10.1164/ajrccm.155.6.9196082.

Fehrenbach, H., Wagner, C. and Wegmann, M. (2017) ‘Airway remodeling in asthma: what really matters’, *Cell and Tissue Research*, 367(3), pp. 551–569. Available at: https://doi.org/10.1007/s00441-016-2566-8.

FitzGerald, J.M. *et al.* (2018) ‘Predictors of enhanced response with benralizumab for patients with severe asthma: pooled analysis of the SIROCCO and CALIMA studies’, *The Lancet Respiratory Medicine*, 6(1), pp. 51–64. Available at: https://doi.org/10.1016/S2213-2600(17)30344-2.

Flood-Page, P. *et al.* (2003) ‘Anti-IL-5 treatment reduces deposition of ECM proteins in the bronchial subepithelial basement membrane of mild atopic asthmatics’, *Journal of Clinical Investigation*, 112(7), pp. 1029–1036. Available at: https://doi.org/10.1172/JCI17974.

Flood-Page, P. *et al.* (2007) ‘A study to evaluate safety and efficacy of mepolizumab in patients with moderate persistent asthma’, *American Journal of Respiratory and Critical Care Medicine*, 176(11), pp. 1062–1071. Available at: https://doi.org/10.1164/rccm.200701-085OC.

Flower, R.J., Harvey, E.A. and Kingston, W.P. (1976) ‘Inflammatory effects of prostaglandin D2 in rat and human skin.’, *British Journal of Pharmacology*, 56(2), pp. 229–33. Available at: https://doi.org/10.1111/j.1476-5381.1976.tb07446.x.

Fowler, S.J. *et al.* (2000) ‘Screening for bronchial hyperresponsiveness using methacholine and adenosine monophosphate: Relationship to asthma severity and β2-receptor genotype’,

American Journal of Respiratory and Critical Care Medicine, 162(4 I), pp. 1318–1322. Available at: https://doi.org/10.1164/ajrccm.162.4.9912103.

Frantz, C., Stewart, K.M. and Weaver, V.M. (2010) 'The extracellular matrix at a glance', *Journal of Cell Science*, 123(24), pp. 4195–4200. Available at: https://doi.org/10.1242/jcs.023820.

Frigas, E., Loegering, D.A. and Gleich, G.J. (1980) 'Cytotoxic effects of the guinea pig eosinophil major basic protein on tracheal epithelium', *Laboratory Investigation*, 42(1), pp. 35–43. Available at: https://pascal-francis.inist.fr/vibad/index.php?action=getRecordDetail&idt=PASCAL8050240028 (Accessed: 5 May 2021).

Fruman, D.A. *et al.* (2017) 'The PI3K Pathway in Human Disease', *Cell*, 170(4), pp. 605–635. Available at: https://doi.org/10.1016/j.cell.2017.07.029.

Fujisawa, T. *et al.* (1990) 'Regulatory effect of cytokines on eosinophil degranulation.', *The Journal of Immunology*, 144(2), pp. 642–646. Available at: https://doi.org/10.4049/JIMMUNOL.144.2.642.

Fukuda, T. and Gleich, G.J. (1989) 'Heterogeneity of human eosinophils', *The Journal of allergy and clinical immunology*, 83(2 Pt 1), pp. 369–373. Available at: https://doi.org/10.1016/0091-6749(89)90120-6.

Fukuzawa, T. *et al.* (2013) 'SGLT5 Reabsorbs Fructose in the Kidney but Its Deficiency Paradoxically Exacerbates Hepatic Steatosis Induced by Fructose', *PLOS ONE*, 8(2), p. e56681. Available at: https://doi.org/10.1371/JOURNAL.PONE.0056681.

Garland, A. *et al.* (1997) 'Activated eosinophils elicit substance P release from cultured dorsal root ganglion neurons', *American Journal of Physiology - Lung Cellular and Molecular Physiology*, 273(5 17-5). Available at: https://doi.org/10.1152/ajplung.1997.273.5.l1096.

George, L. and Brightling, C.E. (2016) ‘Eosinophilic airway inflammation: Role in asthma and chronic obstructive pulmonary disease’, *Therapeutic Advances in Chronic Disease*, pp. 34–51. Available at: https://doi.org/10.1177/2040622315609251.

Gervais, F.G. *et al.* (2001) ‘Selective modulation of chemokinesis, degranulation, and apoptosis in eosinophils through the PGD2 receptors CRTH2 and DP’, *Journal of Allergy and Clinical Immunology*, 108(6), pp. 982–988. Available at: https://doi.org/10.1067/mai.2001.119919.

Giannelli, G. *et al.* (1997) ‘Induction of cell migration by matrix metalloprotease-2 cleavage of laminin-5’, *Science*, 277(5323), pp. 225–228. Available at: https://doi.org/10.1126/science.277.5323.225.

Gil, M.A. *et al.* (2014) ‘Anti-inflammatory actions of Chemoattractant Receptor-homologous molecule expressed on Th2 by the antagonist MK-7246 in a novel rat model of Alternaria alternata elicited pulmonary inflammation’, *European journal of pharmacology*, 743, pp. 106–116. Available at: https://doi.org/10.1016/J.EJPHAR.2014.09.021.

Gleich, G.J. *et al.* (1979) ‘Cytotoxic Properties of the Eosinophil Major Basic Protein’, *The Journal of Immunology*, 123(6), pp. 2925–2927. Available at: https://doi.org/10.4049/jimmunol.123.6.2925.

Gonem, S. *et al.* (2016) ‘Fevipiprant, a prostaglandin D2 receptor 2 antagonist, in patients with persistent eosinophilic asthma: a single-centre, randomised, double-blind, parallel-group, placebo-controlled trial’, *The Lancet Respiratory Medicine*, 4(9), pp. 699–707. Available at: https://doi.org/10.1016/S2213-2600(16)30179-5.

Greenlee, K.J., Werb, Z. and Kheradmand, F. (2007) ‘Matrix metalloproteinases in lung: Multiple, multifarious, and multifaceted’, *Physiological Reviews*, 87(1), pp. 69–98. Available at: https://doi.org/10.1152/physrev.00022.2006.

Grempler, R. *et al.* (2012) 'Functional characterisation of human SGLT-5 as a novel kidney-specific sodium-dependent sugar transporter', *FEBS Letters*, 586(3), pp. 248–253. Available at: https://doi.org/10.1016/J.FEBSLET.2011.12.027.

Hall, L.R. *et al.* (1998) 'An Essential Role for Interleukin-5 and Eosinophils in Helminth-Induced Airway Hyperresponsiveness', *Infection and Immunity*, 66(9), pp. 4425–4430. Available at: https://doi.org/10.1128/IAI.66.9.4425-4430.1998.

Hanna, V.S. and Hafez, E.A.A. (2018) 'Synopsis of arachidonic acid metabolism: A review', *Journal of Advanced Research*, 11, pp. 23–32. Available at: https://doi.org/10.1016/j.jare.2018.03.005.

Hao, Y.F. *et al.* (2021) 'Targets and underlying mechanisms related to the sedative and hypnotic activities of saponins from: Rhodiola rosea L. (crassulaceae)', *Food and Function*, 12(21), pp. 10589–10601. Available at: https://doi.org/10.1039/d1fo01178b.

Hardman, C. *et al.* (2019) 'Fevipiprant, a selective prostaglandin D2 receptor 2 antagonist, inhibits human group 2 innate lymphoid cell aggregation and function', *Journal of Allergy and Clinical Immunology*, 143(6), pp. 2329–2333. Available at: https://doi.org/10.1016/j.jaci.2019.02.015.

Hartnell, A. *et al.* (1993) 'CD69 is expressed by human eosinophils activated in vivo in asthma and in vitro by cytokines.', *Immunology*, 80(2), pp. 281–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8262555%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC1422202.

Haston, W.S. and Shields, J.M. (1985) 'Neutrophil leucocyte chemotaxis: a simplified assay for measuring polarising responses to chemotactic factors', *Journal of Immunological Methods*, 81(2), pp. 229–237. Available at: https://doi.org/10.1016/0022-1759(85)90208-X.

Haughney, J. *et al.* (2018) 'A retrospective cohort study in severe asthma describing commonly measured biomarkers: Eosinophil count and IgE levels', *Respiratory Medicine*, 134, pp. 117–123. Available at: https://doi.org/10.1016/j.rmed.2017.12.001.

Hemmings, O. *et al.* (2018) 'Basophil Activation Test: Old and New Applications in Allergy', *Current Allergy and Asthma Reports*, 18(12), p. 77. Available at: https://doi.org/10.1007/s11882-018-0831-5.

Hilvering, B. *et al.* (2018) 'Synergistic activation of pro-inflammatory type-2 CD8+ T lymphocytes by lipid mediators in severe eosinophilic asthma', *Mucosal Immunology*, 11(5), pp. 1408–1419. Available at: https://doi.org/10.1038/s41385-018-0049-9.

Hirai, H. *et al.* (2001) 'Prostaglandin D2 selectively induces chemotaxis in T helper type 2 cells, eosinophils, and basophils via seven-transmembrane receptor CRTH2', *Journal of Experimental Medicine*, 193(2), pp. 255–261. Available at: https://doi.org/10.1084/jem.193.2.255.

Hoefer, I.E. *et al.* (2002) 'Direct evidence for tumor necrosis factor-α signaling in arteriogenesis', *Circulation*, 105(14), pp. 1639–1641. Available at: https://doi.org/10.1161/01.CIR.0000014987.32865.8E.

Hogg, J.C. (1982) 'The pathophysiology of asthma', *Chest*, 82(1 Suppl.), pp. 8s–12s. Available at: https://doi.org/10.1378/chest.82.1.8S.

Homer, R.J. and Elias, J.A. (2005) 'Airway remodeling in asthma: Therapeutic implications of mechanisms', *Physiology*. American Physiological Society, pp. 28–35. Available at: https://doi.org/10.1152/physiol.00035.2004.

Hynes, G. and Pavord, I.D. (2020) 'Targeted biologic therapy for asthma', *British Medical Bulletin*, 133(1), pp. 16–35. Available at: https://doi.org/10.1093/BMB/LDAA004.

Inoue, T. *et al.* (2003) 'Mechanism of metal activation of human hematopoietic prostaglandin D synthase', *Nature Structural Biology*, 10(4), pp. 291–296. Available at: https://doi.org/10.1038/nsb907.

Iozzo, R.V. *et al.* (1999) 'Decorin is a biological ligand for the epidermal growth factor receptor', *Journal of Biological Chemistry*, 274(8), pp. 4489–4492. Available at: https://doi.org/10.1074/jbc.274.8.4489.

Ito, W. *et al.* (2007) 'Hepatocyte growth factor attenuates eotaxin and PGD2-induced chemotaxis of human eosinophils', *Allergy: European Journal of Allergy and Clinical Immunology*, 62(4), pp. 415–422. Available at: https://doi.org/10.1111/j.1398-9995.2006.01299.x.

Jandl, K. *et al.* (2016) 'Activated prostaglandin D2 receptors on macrophages enhance neutrophil recruitment into the lung', *Journal of Allergy and Clinical Immunology*, 137(3), pp. 833–843. Available at: https://doi.org/10.1016/j.jaci.2015.11.012.

Jang, E. *et al.* (2012) 'Syndecan-4 proteoliposomes enhance fibroblast growth factor-2 (FGF-2)-induced proliferation, migration, and neovascularization of ischemic muscle', *Proceedings of the National Academy of Sciences of the United States of America*, 109(5), pp. 1679–1684. Available at: https://doi.org/10.1073/pnas.1117885109.

Jenkins, C., Costello, J. and Hodge, L. (2004) 'Systematic review of prevalence of aspirin induced asthma and its implications for clinical practice', *British Medical Journal*. BMJ Publishing Group, pp. 434–437. Available at: https://doi.org/10.1136/bmj.328.7437.434.

Johansson, M.W. *et al.* (2013) 'Anti-IL-5 attenuates activation and surface density of β2-integrins on circulating eosinophils after segmental antigen challenge', *Clinical and Experimental Allergy*, 43(3), pp. 292–303. Available at: https://doi.org/10.1111/j.1365-2222.2012.04065.x.

Jones, P.L., Crack, J. and Rabinovitch, M. (1997) 'Regulation of tenascin-C, a vascular smooth muscle cell survival factor that interacts with the α(v)β3 integrin to promote epidermal growth factor receptor phosphorylation and growth', *Journal of Cell Biology*, 139(1), pp. 279–293. Available at: https://doi.org/10.1083/jcb.139.1.279.

Kanaoka, Y. and Urade, Y. (2003) 'Hematopoietic prostaglandin D synthase', *Prostaglandins Leukotrienes and Essential Fatty Acids*, 69(2–3), pp. 163–167. Available at: https://doi.org/10.1016/S0952-3278(03)00077-2.

Kanniess, F. *et al.* (2002) 'Montelukast versus fluticasone: effects on lung function, airway responsiveness and inflammation in moderate asthma', *The European respiratory journal*, 20(4), pp. 853–858. Available at: https://doi.org/10.1183/09031936.02.00244602.

Kardas, G. *et al.* (2020) 'Role of Platelet-Derived Growth Factor (PDGF) in Asthma as an Immunoregulatory Factor Mediating Airway Remodeling and Possible Pharmacological Target', *Frontiers in Pharmacology*. Frontiers Media S.A. Available at: https://doi.org/10.3389/fphar.2020.00047.

Kariyawasam, H.H. and Robinson, D.S. (2006) 'The eosinophil: The cell and its weapons, the cytokines, its locations', *Seminars in Respiratory and Critical Care Medicine*. Semin Respir Crit Care Med, pp. 117–127. Available at: https://doi.org/10.1055/s-2006-939514.

Kay, A.B., Phipps, S. and Robinson, D.S. (2004) 'A role for eosinophils in airway remodelling in asthma', *Trends in Immunology*, 25(9), pp. 477–482. Available at: https://doi.org/10.1016/j.it.2004.07.006.

Kendall, R.T. and Feghali-Bostwick, C.A. (2014) 'Fibroblasts in fibrosis: Novel roles and mediators', *Frontiers in Pharmacology*, 5 MAY, p. 123. Available at: https://doi.org/10.3389/fphar.2014.00123.

Khalil, H. *et al.* (2017) 'Fibroblast-specific TGF-β-Smad2/3 signaling underlies cardiac fibrosis', in *Journal of Clinical Investigation*. American Society for Clinical Investigation, pp. 3770–3783. Available at: https://doi.org/10.1172/JCI94753.

Kim, J. *et al.* (2019) 'Innate immune crosstalk in asthmatic airways: Innate lymphoid cells coordinate polarization of lung macrophages', *Journal of Allergy and Clinical Immunology*, 143(5), pp. 1769-1782.e11. Available at: https://doi.org/10.1016/j.jaci.2018.10.040.

Kouro, T. and Takatsu, K. (2009) 'IL-5- and eosinophil-mediated inflammation: From discovery to therapy', *International Immunology*, 21(12), pp. 1303–1309. Available at: https://doi.org/10.1093/intimm/dxp102.

Kubo, M. (2017) ‘Innate and adaptive type 2 immunity in lung allergic inflammation’, *Immunological Reviews*, 278(1), pp. 162–172. Available at: https://doi.org/10.1111/imr.12557.

Kumawat, K. *et al.* (2016) ‘Cooperative signaling by TGF-β1 and WNT-11 drives sm-α-actin expression in smooth muscle via Rho kinase-actin-MRTF-A signaling’, *American Journal of Physiology - Lung Cellular and Molecular Physiology*, 311(3), pp. L529–L537. Available at: https://doi.org/10.1152/ajplung.00387.2015.

Kuruvilla, M.E., Lee, F.E.H. and Lee, G.B. (2019) ‘Understanding Asthma Phenotypes, Endotypes, and Mechanisms of Disease’, *Clinical Reviews in Allergy and Immunology*, 56(2), pp. 219–233. Available at: https://doi.org/10.1007/S12016-018-8712-1/METRICS.

Lacy, P. (2006) ‘Mechanisms of Degranulation in Neutrophils’, *Allergy, Asthma & Clinical Immunology*, 2(3), p. 98. Available at: https://doi.org/10.1186/1710-1492-2-3-98.

Lambrecht, B.N. and Hammad, H. (2015) ‘The immunology of asthma’, *Nature Immunology*, 16(1), pp. 45–56. Available at: https://doi.org/10.1038/ni.3049.

Leckie, M.J. *et al.* (2000) ‘Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response’, *Lancet*, 356(9248), pp. 2144–2148. Available at: https://doi.org/10.1016/S0140-6736(00)03496-6.

Lee, S.J. *et al.* (2002) ‘Mannose Receptor-Mediated Regulation of Serum Glycoprotein Homeostasis’, *Science*, 295(5561), pp. 1898–1901. Available at: https://doi.org/10.1126/SCIENCE.1069540.

Lewis, R.A. *et al.* (1982) ‘Prostaglandin D2 generation after activation of rat and human mast cells with anti-IgE.’, *The Journal of Immunology*, 129(4), pp. 1627–1631. Available at: https://doi.org/10.4049/jimmunol.129.4.1627.

Li, X. *et al.* (2016) ‘Overexpression of SerpinE2/protease nexin-1 Contribute to Pathological Cardiac Fibrosis via increasing Collagen Deposition’, *Scientific Reports*, 6(1), pp. 1–12. Available at: https://doi.org/10.1038/srep37635.

Liao, Y., Smyth, G.K. and Shi, W. (2019) 'The R package Rsubread is easier, faster, cheaper and better for alignment and quantification of RNA sequencing reads', *Nucleic Acids Research*, 47(8). Available at: https://doi.org/10.1093/nar/gkz114.

Lilly, C.M. *et al.* (1997) 'Expression of eotaxin by human lung epithelial cells. Induction by cytokines and inhibition by glucocorticoids', *Journal of Clinical Investigation*, 99(7), pp. 1767–1773. Available at: https://doi.org/10.1172/JCI119341.

Lin, P.S. *et al.* (2017) 'Transforming growth factor beta 1 increases collagen content, and stimulates procollagen I and tissue inhibitor of metalloproteinase-1 production of dental pulp cells: Role of MEK/ERK and activin receptor-like kinase-5/Smad signaling', *Journal of the Formosan Medical Association*, 116(5), pp. 351–358. Available at: https://doi.org/10.1016/j.jfma.2016.07.014.

Lin, Y. Te *et al.* (2015) 'Galectin-1 accelerates wound healing by regulating the neuropilin-1/Smad3/NOX4 pathway and ROS production in myofibroblasts', *Journal of Investigative Dermatology*, 135(1), pp. 258–268. Available at: https://doi.org/10.1038/jid.2014.288.

Liu, J. *et al.* (2019) 'TWEAK/Fn14 Signals Mediate Burn Wound Repair', *Journal of Investigative Dermatology*, 139(1), pp. 224–234. Available at: https://doi.org/10.1016/j.jid.2018.05.036.

Liu, Z. *et al.* (2019) 'p75 neurotrophin receptor regulates NGF-induced myofibroblast differentiation and collagen synthesis through MRTF-A', *Experimental Cell Research*, 383(1), p. 111504. Available at: https://doi.org/10.1016/j.yexcr.2019.111504.

Lopez, A.F. *et al.* (1988) 'Recombinant human interleukin 5 is a selective activator of human eosinophil function', *The Journal of experimental medicine*, 167(1), pp. 219–224. Available at: https://doi.org/10.1084/JEM.167.1.219.

Lorentz, A. *et al.* (2000) 'Human Intestinal Mast Cells Are Capable of Producing Different Cytokine Profiles: Role of IgE Receptor Cross-Linking and IL-4', *The Journal of Immunology*, 164(1), pp. 43–48. Available at: https://doi.org/10.4049/jimmunol.164.1.43.

Love, M.I., Huber, W. and Anders, S. (2014) 'Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2', *Genome Biology*, 15(12). Available at: https://doi.org/10.1186/s13059-014-0550-8.

Loza, M.J. *et al.* (2016) 'Validated and longitudinally stable asthma phenotypes based on cluster analysis of the ADEPT study', *Respiratory Research*, 17(1). Available at: https://doi.org/10.1186/s12931-016-0482-9.

Luna-Gomes, T. *et al.* (2011) 'Eosinophils as a Novel Cell Source of Prostaglandin D2: Autocrine Role in Allergic Inflammation', *The Journal of Immunology*, 187(12), pp. 6518–6526. Available at: https://doi.org/10.4049/jimmunol.1101806.

Luu, V.T. *et al.* (2015) 'Synthesis of a high specific activity methyl sulfone tritium isotopologue of fevipiprant (NVP-QAW039)', *Journal of labelled compounds & radiopharmaceuticals*, 58(5), pp. 188–195. Available at: https://doi.org/10.1002/JLCR.3281.

Macfarlane, A.J. *et al.* (2000) 'Basophils, eosinophils, and mast cells in atopic and nonatopic asthma and in late-phase allergic reactions in the lung and skin', *Journal of Allergy and Clinical Immunology*, 105(1 I), pp. 99–107. Available at: https://doi.org/10.1016/S0091-6749(00)90184-2.

Manabe, R. *et al.* (2008) 'Transcriptome-based systematic identification of extracellular matrix proteins', *Proceedings of the National Academy of Sciences of the United States of America*, 105(35), p. 12849. Available at: https://doi.org/10.1073/PNAS.0803640105.

Maric, J. *et al.* (2019) 'Cytokine-induced endogenous production of prostaglandin D2 is essential for human group 2 innate lymphoid cell activation', *Journal of Allergy and Clinical Immunology*, 143(6), pp. 2202-2214.e5. Available at: https://doi.org/10.1016/j.jaci.2018.10.069.

Marone, G., Triggiani, M. and De Paulis, A. (2005) 'Mast cells and basophils: Friends as well as foes in bronchial asthma?', *Trends in Immunology*, 26(1), pp. 25–31. Available at: https://doi.org/10.1016/j.it.2004.10.010.

Marshall, G.D. (2019) ‘Psychological stress, immunity, and asthma: developing a paradigm for effective therapy and prevention’, *Current Opinion in Behavioral Sciences*. Elsevier Ltd, pp. 14–19. Available at: https://doi.org/10.1016/j.cobeha.2019.01.006.

Massanari, M. *et al.* (2010) ‘Effect of omalizumab on peripheral blood eosinophilia in allergic asthma’, *Respiratory Medicine*, 104(2), pp. 188–196. Available at: https://doi.org/10.1016/j.rmed.2009.09.011.

Mawhorter, S.D. *et al.* (1996) ‘Identification of surface molecules associated with physiologic activation of eosinophils: Application of whole-blood flow cytometry to eosinophils’, *The Journal of Immunology*, 156(12), pp. 4851–4858.

McCarthy, D. *et al.* (2016) ‘scater: pre-processing, quality control, normalisation and visualisation of single-cell RNA-seq data in R’, *Scater: Pre-processing, quality control, normalization and visualization of single-cell RNA-seq data in R*, p. 069633. Available at: https://doi.org/10.1101/069633.

McDonough, A.K., Curtis, J.R. and Saag, K.G. (2008) ‘The epidemiology of glucocorticoid-associated adverse events’, *Current Opinion in Rheumatology*, 20(2), pp. 131–137. Available at: https://doi.org/10.1097/BOR.0B013E3282F51031.

Menzella, F. *et al.* (2019) ‘Efficacy and steroid-sparing effect of benralizumab: Has it an advantage over its competitors?’, *Drugs in Context*. Bioexcel Publishing LTD, p. 212580. Available at: https://doi.org/10.7573/dic.212580.

Menzies, D. *et al.* (2007) ‘Differential anti-inflammatory effects of large and small particle size inhaled corticosteroids in asthma’, *Allergy*, 62(6), pp. 661–667. Available at: https://doi.org/10.1111/J.1398-9995.2007.01376.X.

Menzies-Gow, A. *et al.* (2003) ‘Anti-IL-5 (mepolizumab) therapy induces bone marrow eosinophil maturational arrest and decreases eosinophil progenitors in the bronchial mucosa of atopic asthmatics’, *Journal of Allergy and Clinical Immunology*, 111(4), pp. 714–719. Available at: https://doi.org/10.1067/mai.2003.1382.

568 Menzies-Gow, A. *et al.* (2021) 'Tezepelumab in Adults and Adolescents with Severe,
569 Uncontrolled Asthma', *New England Journal of Medicine*, 384(19), pp. 1800–1809. Available
570 at: https://doi.org/10.1056/nejmoa2034975.

571 Mesnil, C. *et al.* (2016) 'Lung-resident eosinophils represent a distinct regulatory eosinophil
572 subset', *The Journal of clinical investigation*, 126(9), pp. 3279–3295. Available at:
573 https://doi.org/10.1172/JCI85664.

574 Milgrom, H. *et al.* (1999) 'Treatment of Allergic Asthma with Monoclonal Anti-IgE Antibody',
575 *New England Journal of Medicine*, 341(26), pp. 1966–1973. Available at:
576 https://doi.org/10.1056/nejm199912233412603.

577 Miller, M. *et al.* (2006) 'Corticosteroids prevent myofibroblast accumulation and airway
578 remodeling in mice', *American Journal of Physiology - Lung Cellular and Molecular*
579 *Physiology*, 290(1). Available at: https://doi.org/10.1152/ajplung.00252.2005.

580 Minshall, E.M. *et al.* (1997) 'Eosinophil-associated TGF-β1 mRNA Expression and Airways
581 Fibrosis in Bronchial Asthma', *American Journal of Respiratory Cell and Molecular Biology*,
582 17(3), pp. 326–333. Available at: https://doi.org/10.1165/ajrcmb.17.3.2733.

583 Mitchell, J.A. and Kirkby, N.S. (2019) 'Eicosanoids, prostacyclin and cyclooxygenase in the
584 cardiovascular system', *British Journal of Pharmacology*, 176(8), pp. 1038–1050. Available
585 at: https://doi.org/10.1111/bph.14167.

586 Mjösberg, J.M. *et al.* (2011) 'Human IL-25- and IL-33-responsive type 2 innate lymphoid cells
587 are defined by expression of CRTH2 and CD161', *Nature immunology*, 12(11), pp. 1055–1062.
588 Available at: https://doi.org/10.1038/NI.2104.

589 Monneret, G. *et al.* (2001) 'Prostaglandin D2 is a potent chemoattractant for human eosinophils
590 that acts via a novel DP receptor', *Blood*, 98(6), pp. 1942–1948. Available at:
591 https://doi.org/10.1182/BLOOD.V98.6.1942.

592 Monneret, G. *et al.* (2005) 'Effects of prostaglandin D2 and 5-lipoxygenase products on the
593 expression of CD203c and CD11b by basophils', *Journal of Pharmacology and Experimental*
594 *Therapeutics*, 312(2), pp. 627–634. Available at: https://doi.org/10.1124/jpet.104.074823.

595 Moro, L. *et al.* (2002) 'Integrin-induced epidermal growth factor (EGF) receptor activation
596 requires c-Src and p130Cas and leads to phosphorylation of specific EGF receptor tyrosines',
597 *Journal of Biological Chemistry*, 277(11), pp. 9405–9414. Available at:
598 https://doi.org/10.1074/jbc.M109101200.

599 Motojima, S. *et al.* (1989) 'Toxicity of eosinophil cationic proteins for guinea pig tracheal
600 epithelium in vitro', *American Review of Respiratory Disease*, 139(3), pp. 801–805. Available
601 at: https://doi.org/10.1164/ajrccm/139.3.801.

602 Motojima, S. *et al.* (1993) 'Clinical significance of measuring levels of sputum and serum ECP
603 and serum IL-5 in bronchial asthma', *Allergy*, 48(s17), pp. 98–106. Available at:
604 https://doi.org/10.1111/j.1398-9995.1993.tb04709.x.

605 Murakami-Satsutani, N. *et al.* (2014) 'IL-33 promotes the induction and maintenance of Th2
606 immune responses by Enhancing the function of OX40 ligand', *Allergology International*,
607 63(3), pp. 443–455. Available at: https://doi.org/10.2332/allergolint.13-OA-0672.

608 Mustonen, E., Ruskoaho, H. and Rysä, J. (2012) 'Thrombospondin-4, tumour necrosis factor-
609 like weak inducer of apoptosis (TWEAK) and its receptor Fn14: Novel extracellular matrix
610 modulating factors in cardiac remodelling', *Annals of Medicine*, 44(8), pp. 793–804. Available
611 at: https://doi.org/10.3109/07853890.2011.614635.

612 Nagakumar, P. *et al.* (2016) 'Type 2 innate lymphoid cells in induced sputum from children
613 with severe asthma', *Journal of Allergy and Clinical Immunology*, 137(2), pp. 624-626.e6.
614 Available at: https://doi.org/10.1016/j.jaci.2015.06.038.

615 Nagata, K. *et al.* (1999) 'Selective Expression of a Novel Surface Molecule by Human Th2
616 Cells In Vivo', *The Journal of Immunology*, 162(3), pp. 1278–1286. Available at:
617 https://doi.org/10.4049/jimmunol.162.3.1278.

Nakagome, K. *et al.* (2007) 'IL-5-Induced Hypereosinophilia Suppresses the Antigen-Induced Immune Response via a TGF-β-Dependent Mechanism', *The Journal of Immunology*, 179(1), pp. 284–294. Available at: https://doi.org/10.4049/jimmunol.179.1.284.

Nakagome, K. and Nagata, M. (2018) 'Involvement and Possible Role of Eosinophils in Asthma Exacerbation', *Frontiers in Immunology*, 9, p. 2220. Available at: https://doi.org/10.3389/FIMMU.2018.02220/BIBTEX.

Nissim Ben Efraim, A.H. and Levi-Schaffer, F. (2008) 'Review: Tissue remodeling and angiogenesis in asthma: The role of the eosinophil', *Therapeutic Advances in Respiratory Disease*, 2(3), pp. 163–171. Available at: https://doi.org/10.1177/1753465808092281.

Oliphant, C.J., Barlow, J.L. and Mckenzie, A.N.J. (2011) 'Insights into the initiation of type 2 immune responses', *Immunology*, 134(4), pp. 378–385. Available at: https://doi.org/10.1111/j.1365-2567.2011.03499.x.

Olson, M.F. and Sahai, E. (2009) 'The actin cytoskeleton in cancer cell motility', *Clinical and Experimental Metastasis*, 26(4), pp. 273–287. Available at: https://doi.org/10.1007/s10585-008-9174-2.

Ono, E. *et al.* (2010) 'CD203c expression on human basophils is associated with asthma exacerbation', *Journal of Allergy and Clinical Immunology*, 125(2). Available at: https://doi.org/10.1016/j.jaci.2009.10.074.

Ortega, H.G. *et al.* (2014) 'Mepolizumab Treatment in Patients with Severe Eosinophilic Asthma', *New England Journal of Medicine*, 371(13), pp. 1198–1207. Available at: https://doi.org/10.1056/nejmoa1403290.

Ortega, H.G. *et al.* (2016) 'Severe eosinophilic asthma treated with mepolizumab stratified by baseline eosinophil thresholds: a secondary analysis of the DREAM and MENSA studies', *The Lancet Respiratory Medicine*, 4(7), pp. 549–556. Available at: https://doi.org/10.1016/S2213-2600(16)30031-5.

643 Paggiaro, P. and Bacci, E. (2011) ‘Montelukast in asthma: A review of its efficacy and place
644 in therapy’, *Therapeutic Advances in Chronic Disease*. SAGE Publications, pp. 47–58.
645 Available at: https://doi.org/10.1177/2040622310383343.

646 Palikhe, N.S. *et al.* (2016) ‘Elevated levels of circulating CD4+CRTh2+ T cells characterize
647 severe asthma’, *Clinical and Experimental Allergy*, 46(6), pp. 825–836. Available at:
648 https://doi.org/10.1111/cea.12741.

649 Patterson, M.F., Borish, L. and Kennedy, J.L. (2015) ‘The past, present, and future of
650 monoclonal antibodies to IL-5 and eosinophilic asthma: A review’, *Journal of Asthma and*
651 *Allergy*, 8, pp. 125–134. Available at: https://doi.org/10.2147/JAA.S74178.

652 Pavord, I.D. *et al.* (2012) ‘Mepolizumab for severe eosinophilic asthma (DREAM): A
653 multicentre, double-blind, placebo-controlled trial’, *The Lancet*, 380(9842), pp. 651–659.
654 Available at: https://doi.org/10.1016/S0140-6736(12)60988-X.

655 Pavord, I.D. *et al.* (2018) ‘After asthma: redefining airways diseases’, *The Lancet*. Lancet
656 Publishing Group, pp. 350–400. Available at: https://doi.org/10.1016/S0140-6736(17)30879-
657 6.

658 Pecaric-Petkovic, T. *et al.* (2009) ‘Human basophils and eosinophils are the direct target
659 leukocytes of the novel IL-1 family member IL-33’, *Blood*, 113(7), pp. 1526–1534. Available
660 at: https://doi.org/10.1182/blood-2008-05-157818.

661 Pettipher, R. *et al.* (2012) ‘Pharmacologic profile of OC000459, a potent, selective, and orally
662 active D prostanoid receptor 2 antagonist that inhibits mast cell-dependent activation of T
663 helper 2 lymphocytes and eosinophils’, *The Journal of pharmacology and experimental*
664 *therapeutics*, 340(2), pp. 473–482. Available at: https://doi.org/10.1124/JPET.111.187203.

665 Picelli, S. *et al.* (2014) ‘Full-length RNA-seq from single cells using Smart-seq2’, *Nature*
666 *Protocols*, 9(1), pp. 171–181. Available at: https://doi.org/10.1038/nprot.2014.006.

667 Pignatti, P. *et al.* (2002) 'Increased CD69 expression on peripheral blood eosinophils after
668 specific inhalation challenge', *Allergy: European Journal of Allergy and Clinical Immunology*,
669 57(5), pp. 411–416. Available at: https://doi.org/10.1034/j.1398-9995.2002.23454.x.

670 Piguet, P.F. *et al.* (1993) 'Interleukin 1 receptor antagonist (IL-1ra) prevents or cures
671 pulmonary fibrosis elicited in mice by bleomycin or silica', *Cytokine*, 5(1), pp. 57–61.
672 Available at: https://doi.org/10.1016/1043-4666(93)90024-Y.

673 Porreca, E. *et al.* (1993) 'Effect of interleukin-1 receptor antagonist on vascular smooth muscle
674 cell proliferation', *Atherosclerosis*, 99(1), pp. 71–78. Available at:
675 https://doi.org/10.1016/0021-9150(93)90052-V.

676 Porsbjerg, C.M. *et al.* (2020) 'Anti-alarmins in asthma: Targeting the airway epithelium with
677 next-generation biologics', *European Respiratory Journal*. European Respiratory Society.
678 Available at: https://doi.org/10.1183/13993003.00260-2020.

679 Préfontaine, D. *et al.* (2009) 'Increased Expression of IL-33 in Severe Asthma: Evidence of
680 Expression by Airway Smooth Muscle Cells', *The Journal of Immunology*, 183(8), pp. 5094–
681 5103. Available at: https://doi.org/10.4049/jimmunol.0802387.

682 Préfontaine, D. *et al.* (2010) 'Increased IL-33 expression by epithelial cells in bronchial
683 asthma', *Journal of Allergy and Clinical Immunology*. Mosby Inc., pp. 752–754. Available at:
684 https://doi.org/10.1016/j.jaci.2009.12.935.

685 Rabe, K.F. *et al.* (2018) 'Efficacy and Safety of Dupilumab in Glucocorticoid-Dependent
686 Severe Asthma', *New England Journal of Medicine*, 378(26), pp. 2475–2485. Available at:
687 https://doi.org/10.1056/nejmoa1804093.

688 Rackemann, F.M. and Mallory, T.B. (1941) 'Intrinsic Asthma.', *Transactions of the American*
689 *Clinical and Climatological Association*, 57, pp. 60–73. Available at:
690 http://www.ncbi.nlm.nih.gov/pubmed/21407628 (Accessed: 17 May 2021).

691 Relan, N.K. *et al.* (1999) ‘Cell elongation induces laminin α2 chain expression in mouse
692 embryonic mesenchymal cells: Role in visceral myogenesis’, *Journal of Cell Biology*, 147(6),
693 pp. 1341–1350. Available at: https://doi.org/10.1083/jcb.147.6.1341.

694 Reutelingsperger, C.P.M. and Van Heerde, W.L. (1997) ‘Annexin V, the regulator of
695 phosphatidylserine-catalyzed inflammation and coagulation during apoptosis’, *Cellular and*
696 *Molecular Life Sciences*, 53(6), pp. 527–532. Available at:
697 https://doi.org/10.1007/S000180050067/METRICS.

698 Rhen, T. and Cidlowski, J.A. (2005) ‘Antiinflammatory Action of Glucocorticoids — New
699 Mechanisms for Old Drugs’, *New England Journal of Medicine*, 353(16), pp. 1711–1723.
700 Available at: https://doi.org/10.1056/nejmra050541.

701 Ritchie, M.E. *et al.* (2015) ‘Limma powers differential expression analyses for RNA-
702 sequencing and microarray studies’, *Nucleic Acids Research*, 43(7), p. e47. Available at:
703 https://doi.org/10.1093/nar/gkv007.

704 Rockey, D.C., Weymouth, N. and Shi, Z. (2013) ‘Smooth muscle α actin (Acta2) and
705 myofibroblast function during hepatic wound healing.’, *PloS one*. Edited by M.A. Avila, 8(10),
706 p. e77166. Available at: https://doi.org/10.1371/journal.pone.0077166.

707 Saikumar Jayalatha, A.K. *et al.* (2021) ‘The central role of IL-33/IL-1RL1 pathway in asthma:
708 From pathogenesis to intervention’, *Pharmacology & Therapeutics*, 225, p. 107847. Available
709 at: https://doi.org/10.1016/j.pharmthera.2021.107847.

710 Salimi, M. *et al.* (2017) ‘Cysteinyl leukotriene E4 activates human group 2 innate lymphoid
711 cells and enhances the effect of prostaglandin D2 and epithelial cytokines’, *Journal of Allergy*
712 *and Clinical Immunology*, 140(4), pp. 1090-1100.e11. Available at:
713 https://doi.org/10.1016/j.jaci.2016.12.958.

714 Salter, B.M. *et al.* (2015) ‘Thymic stromal lymphopoietin activation of basophils in patients
715 with allergic asthma is IL-3 dependent’, *Journal of Allergy and Clinical Immunology*, 136(6),
716 pp. 1636–1644. Available at: https://doi.org/10.1016/j.jaci.2015.03.039.

Salter, B.M. *et al.* (2016) 'Expression of activation markers in circulating basophils and the relationship to allergen-induced bronchoconstriction in subjects with mild allergic asthma', *Journal of Allergy and Clinical Immunology*, 137(3), pp. 936-938.e7. Available at: https://doi.org/10.1016/j.jaci.2015.08.024.

Sandham, D.A. *et al.* (2009) '7-Azaindole-3-acetic acid derivatives: Potent and selective CRTh2 receptor antagonists', *Bioorganic & Medicinal Chemistry Letters*, 19(16), pp. 4794–4798. Available at: https://doi.org/10.1016/J.BMCL.2009.06.042.

Sandrini, A. *et al.* (2003) 'Effect of montelukast on exhaled nitric oxide and nonvolatile markers of inflammation in mild asthma', *Chest*, 124(4), pp. 1334–1340. Available at: https://doi.org/10.1378/CHEST.124.4.1334.

Sawaguchi, M. *et al.* (2012) 'Role of Mast Cells and Basophils in IgE Responses and in Allergic Airway Hyperresponsiveness', *The Journal of Immunology*, 188(4), pp. 1809–1818. Available at: https://doi.org/10.4049/jimmunol.1101746.

Schenk, S. *et al.* (2003) 'Binding to EGF receptor of a laminin-5 EGF-like fragment liberated during MMP-dependent mammary gland involution', *Journal of Cell Biology*, 161(1), pp. 197–209. Available at: https://doi.org/10.1083/jcb.200208145.

Schleimer, R.P. *et al.* (1985) 'Role of human basophils and mast cells in the pathogenesis of allergic diseases', *The Journal of Allergy and Clinical Immunology*, 76(2 PART 2), pp. 369–374. Available at: https://doi.org/10.1016/0091-6749(85)90656-6.

Schmidt, J.A. *et al.* (1982) 'Interleukin 1, a potential regulator of fibroblast proliferation', *The Journal of immunology (1950)*, 128(5), pp. 2177–2182.

Schuger, L. *et al.* (1997) 'Laminin α1 chain synthesis in the mouse developing lung: Requirement for epithelial-mesenchymal contact and possible role in bronchial smooth muscle development', *Journal of Cell Biology*, 139(2), pp. 553–562. Available at: https://doi.org/10.1083/jcb.139.2.553.

Schumann, T. *et al.* (2020) 'Solute Carrier Transporters as Potential Targets for the Treatment of Metabolic Disease', *Pharmacological Reviews*, 72(1), pp. 343–379. Available at: https://doi.org/10.1124/PR.118.015735.

Scott Swindle, C. *et al.* (2001) 'Epidermal growth factor (EGF)-like repeats of human tenascin-C as ligands for EGF receptor', *Journal of Cell Biology*, 154(2), pp. 459–468. Available at: https://doi.org/10.1083/jcb.200103103.

Sehmi, R. *et al.* (1992) 'Interleukin-5 selectively enhances the chemotactic response of eosinophils obtained from normal but not eosinophilic subjects', *Blood*, 79(11), pp. 2952–2959. Available at: https://doi.org/10.1182/blood.v79.11.2952.2952.

Shaw, D.E. *et al.* (2015) 'Clinical and inflammatory characteristics of the European U-BIOPRED adult severe asthma cohort', *Eur Respir J*, 46, pp. 1308–1321. Available at: https://doi.org/10.1183/13993003.01414-2015.

Shifren, A. *et al.* (2012) 'Mechanisms of Remodeling in Asthmatic Airways', *Journal of Allergy*, 2012, pp. 1–12. Available at: https://doi.org/10.1155/2012/316049.

Shrimanker, R. *et al.* (2019) 'Late Breaking Abstract - Effect of timapiprant, a DP2 antagonist, on airway inflammation in severe eosinophilic asthma', *European Respiratory Journal*, 54(suppl 63), p. RCT3784. Available at: https://doi.org/10.1183/13993003.congress-2019.rct3784.

Shrivastava, A. *et al.* (1997) 'An orphan receptor tyrosine kinase family whose members serve as nonintegrin collagen receptors', *Molecular Cell*, 1(1), pp. 25–34. Available at: https://doi.org/10.1016/S1097-2765(00)80004-0.

Simpson, J.L. *et al.* (2006) 'Inflammatory subtypes in asthma: Assessment and identification using induced sputum', *Respirology*, 11(1), pp. 54–61. Available at: https://doi.org/10.1111/j.1440-1843.2006.00784.x.

Singh, D., Ravi, A. and Southworth, T. (2017) 'CRTH2 antagonists in asthma: Current perspectives', *Clinical Pharmacology: Advances and Applications*, 9, pp. 165–173. Available at: https://doi.org/10.2147/CPAA.S119295.

Sippel, V. *et al.* (2018) 'Activation of IL5R and CRTH2 on human eosinophils elicit a similar molecular response and reveal a synergistic effect', *European Journal of Molecular and Clinical Medicine*, 5(1), pp. 1–11. Available at: https://doi.org/10.5334/ejmcm.1.

Siracusa, M.C. *et al.* (2011) 'TSLP promotes interleukin-3-independent basophil haematopoiesis and type 2 inflammation', *Nature*, 477(7363), pp. 229–233. Available at: https://doi.org/10.1038/nature10329.

Sliogeryte, K. and Gavara, N. (2019) 'Vimentin Plays a Crucial Role in Fibroblast Ageing by Regulating Biophysical Properties and Cell Migration', *Cells*, 8(10). Available at: https://doi.org/10.3390/cells8101164.

Smiljkovic, D. *et al.* (2017) 'BTK inhibition is a potent approach to block IgE-mediated histamine release in human basophils', *Allergy*, 72(11), pp. 1666–1676. Available at: https://doi.org/10.1111/ALL.13166.

Solèr, M. *et al.* (2001) 'The anti-IgE antibody omalizumab reduces exacerbations and steroid requirement in allergic asthmatics', *European Respiratory Journal*, 18(2), pp. 254–261. Available at: https://doi.org/10.1183/09031936.01.00092101.

Soriano, J.B. *et al.* (2017) 'Global, regional, and national deaths, prevalence, disability-adjusted life years, and years lived with disability for chronic obstructive pulmonary disease and asthma, 1990–2015: a systematic analysis for the Global Burden of Disease Study 2015', *The Lancet Respiratory Medicine*, 5(9), pp. 691–706. Available at: https://doi.org/10.1016/S2213-2600(17)30293-X.

Spector, S.L., Wangaard, C.H. and Farr, R.S. (1979) 'Aspirin and concomitant idiosyncrasies in adult asthmatic patients', *The Journal of Allergy and Clinical Immunology*, 64(6 PART 1), pp. 500–506. Available at: https://doi.org/10.1016/0091-6749(79)90059-9.

Spits, H. *et al.* (2013) 'Innate lymphoid cells-a proposal for uniform nomenclature', *Nature Reviews Immunology*, 13(2), pp. 145–149. Available at: https://doi.org/10.1038/nri3365.

Stein, M.L. *et al.* (2008) 'Anti-IL-5 (mepolizumab) therapy reduces eosinophil activation ex vivo and increases IL-5 and IL-5 receptor levels', *Journal of Allergy and Clinical Immunology*, 121(6), pp. 1473-1483.e4. Available at: https://doi.org/10.1016/j.jaci.2008.02.033.

Stone, K.D., Prussin, C. and Metcalfe, D.D. (2010) 'IgE, mast cells, basophils, and eosinophils', *Journal of Allergy and Clinical Immunology*, 125(2 SUPPL. 2), pp. S73–S80. Available at: https://doi.org/10.1016/j.jaci.2009.11.017.

Sur, S. *et al.* (1995) 'Eosinophilic inflammation is associated with elevation of interleukin-5 in the airways of patients with spontaneous symptomatic asthma', *The Journal of Allergy and Clinical Immunology*, 96(5), pp. 661–668. Available at: https://doi.org/10.1016/S0091-6749(95)70265-2.

Suzuki, Y. *et al.* (2017) 'Airway basophils are increased and activated in eosinophilic asthma', *Allergy: European Journal of Allergy and Clinical Immunology*, 72(10), pp. 1532–1539. Available at: https://doi.org/10.1111/all.13197.

Sykes, D.A. *et al.* (2016) 'Fevipiprant (QAW039), a slowly dissociating CRTh2 antagonist with the potential for improved clinical efficacy', *Molecular Pharmacology*, 89(5), pp. 593–605. Available at: https://doi.org/10.1124/mol.115.101832.

Tanaka, H. *et al.* (2004) 'Role of interleukin-5 and eosinophils in allergen-induced airway remodeling in mice', *American Journal of Respiratory Cell and Molecular Biology*, 31(1), pp. 62–68. Available at: https://doi.org/10.1165/rcmb.2003-0305OC.

Tanaka, K. *et al.* (2000) ' Cutting Edge: Differential Production of Prostaglandin D 2 by Human Helper T Cell Subsets ', *The Journal of Immunology*, 164(5), pp. 2277–2280. Available at: https://doi.org/10.4049/jimmunol.164.5.2277.

Tang, H. *et al.* (2018) 'Architecture of cell–cell adhesion mediated by sidekicks', *Proceedings of the National Academy of Sciences*, 115(37), pp. 9246–9251. Available at: https://doi.org/10.1073/PNAS.1801810115.

Tang, W. *et al.* (2022) 'Single-cell RNA-sequencing in asthma research', *Frontiers in Immunology*, 13, p. 7271. Available at: https://doi.org/10.3389/FIMMU.2022.988573.

Thangam, E.B. *et al.* (2018) 'The role of histamine and histamine receptors in mast cell-mediated allergy and inflammation: The hunt for new therapeutic targets', *Frontiers in Immunology*, 9(AUG). Available at: https://doi.org/10.3389/fimmu.2018.01873.

The Global Initiative for Asthma (2022) *Global Strategy for Asthma Management and Prevention*.

Todd, R. *et al.* (1991) 'The eosinophil as a cellular source of transforming growth factor alpha in healing cutaneous wounds', *American Journal of Pathology*, 138(6), pp. 1307–1313. Available at: /pmc/articles/PMC1886395/?report=abstract (Accessed: 7 May 2021).

Tran, K.T., Griffith, L. and Wells, A. (2004) 'Extracellular matrix signaling through growth factor receptors during wound healing', *Wound Repair and Regeneration*. John Wiley & Sons, Ltd, pp. 262–268. Available at: https://doi.org/10.1111/j.1067-1927.2004.012302.x.

Tschopp, C.M. *et al.* (2006) 'Granzyme B, a novel mediator of allergic inflammation: Its induction and release in blood basophils and human asthma', *Blood*, 108(7), pp. 2290–2299. Available at: https://doi.org/10.1182/blood-2006-03-010348.

Uciechowski, P. and Rink, L. (2018) 'Neutrophil, Basophil, and Eosinophil Granulocyte Functions in the Elderly', *Handbook of Immunosenescence*, pp. 1–27. Available at: https://doi.org/10.1007/978-3-319-64597-1_22-1.

Ugajin, T. *et al.* (2011) 'FcεRI, but Not FcγR, Signals Induce Prostaglandin D2 and E2 Production from Basophils', *The American Journal of Pathology*, 179(2), pp. 775–782. Available at: https://doi.org/10.1016/J.AJPATH.2011.04.023.

Uhlén, M. *et al.* (2015) 'Tissue-based map of the human proteome', *Science*, 347(6220). Available at: https://doi.org/10.1126/science.1260419.

Uller, L. *et al.* (2007) 'Antagonism of the prostaglandin D2 receptor CRTH2 attenuates asthma pathology in mouse eosinophilic airway inflammation', *Respiratory Research*, 8(1), p. 16. Available at: https://doi.org/10.1186/1465-9921-8-16.

Verrecchia, F. and Mauviel, A. (2004) 'TGF-β and TNF-α: Antagonistic cytokines controlling type I collagen gene expression', *Cellular Signalling*, 16(8), pp. 873–880. Available at: https://doi.org/10.1016/j.cellsig.2004.02.007.

Vogel, W. *et al.* (1997) 'The discoidin domain receptor tyrosine kinases are activated by collagen', *Molecular Cell*, 1(1), pp. 13–23. Available at: https://doi.org/10.1016/S1097-2765(00)80003-9.

Walker, C. *et al.* (1992) 'Allergic and Nonallergic Asthmatics Have Distinct Patterns of T-Cell Activation and Cytokine Production in Peripheral Blood and Bronchoalveolar Lavage', *American Review of Respiratory Disease*, 146(1), pp. 109–115. Available at: https://doi.org/10.1164/ajrccm/146.1.109.

Walsh, G.M. *et al.* (1991) 'Interleukin-5 enhances the in vitro adhesion of human eosinophils, but not neutrophils, in a leucocyte integrin (CD11/18)-dependent manner', *International Archives of Allergy and Applied Immunology*, 94(1–4), pp. 174–178. Available at: https://doi.org/10.1159/000235355.

Warringa, R.A.J. *et al.* (1992) 'In vivo priming of platelet-activating factor-induced eosinophil chemotaxis in allergic asthmatic individuals', *Blood*, 79(7), pp. 1836–1841. Available at: https://doi.org/10.1182/blood.v79.7.1836.1836.

Warringa, R.A.J. *et al.* (1993) 'Upregulation of formyl-peptide and interleukin-8-induced eosinophil chemotaxis in patients with allergic asthma', *The Journal of Allergy and Clinical Immunology*, 91(6), pp. 1198–1205. Available at: https://doi.org/10.1016/0091-6749(93)90323-8.

867 Wei, Z. and Liu, H.T. (2002) ‘MAPK signal pathways in the regulation of cell proliferation in
868 mammalian cells’, *Cell Research*. Science Press, pp. 9–18. Available at:
869 https://doi.org/10.1038/sj.cr.7290105.

870 Weiss, P. and Rundell, K.W. (2011) ‘Exercise-induced lung disease: Too much of a good
871 thing?’, *Pediatric, Allergy, Immunology, and Pulmonology*, 24(3), pp. 149–157. Available at:
872 https://doi.org/10.1089/ped.2011.0066.

873 Wenzel, S. *et al.* (2013) ‘Dupilumab in Persistent Asthma with Elevated Eosinophil Levels’,
874 *New England Journal of Medicine*, 368(26), pp. 2455–2466. Available at:
875 https://doi.org/10.1056/nejmoa1304048.

876 Wenzel, S.E. *et al.* (1999) ‘Evidence that severe asthma can be divided pathologically into two
877 inflammatory subtypes with distinct physiologic and clinical characteristics’, *American
878 Journal of Respiratory and Critical Care Medicine*, 160(3), pp. 1001–1008. Available at:
879 https://doi.org/10.1164/ajrccm.160.3.9812110.

880 Wenzel, S.E. (2012) ‘Asthma phenotypes: The evolution from clinical to molecular
881 approaches’, *Nature Medicine*, 18(5), pp. 716–725. Available at:
882 https://doi.org/10.1038/nm.2678.

883 Willetts, L. *et al.* (2014) ‘Eosinophil shape change and secretion’, *Methods in Molecular
884 Biology*, 1178, pp. 111–128. Available at: https://doi.org/10.1007/978-1-4939-1016-8_11.

885 Wilson, S.J. *et al.* (2016) ‘Severe asthma exists despite suppressed tissue inflammation:
886 findings of the U-BIOPRED study on behalf of the U-BIOPRED Study Group 16’, *Eur Respir
887 J*, 48, pp. 1307–1319. Available at: https://doi.org/10.1183/13993003.01129-2016.

888 Wong, C.K., Ip, W.K. and Lam, C.W.K. (2003) ‘Interleukin-3, -5, and granulocyte macrophage
889 colony-stimulating factor-induced adhesion molecule expression on eosinophils by p38
890 mitogen-activated protein kinase and nuclear factor-κB’, *American Journal of Respiratory Cell
891 and Molecular Biology*, 29(1), pp. 133–147. Available at: https://doi.org/10.1165/rcmb.2002-
892 0289OC.

Wynn, T.A. (2015) ‘Type 2 cytokines: Mechanisms and therapeutic strategies’, *Nature Reviews Immunology*, 15(5), pp. 271–282. Available at: https://doi.org/10.1038/nri3831.

Xue, L. *et al.* (2005) ‘ Prostaglandin D 2 Causes Preferential Induction of Proinflammatory Th2 Cytokine Production through an Action on Chemoattractant Receptor-Like Molecule Expressed on Th2 Cells ’, *The Journal of Immunology*, 175(10), pp. 6531–6536. Available at: https://doi.org/10.4049/jimmunol.175.10.6531.

Xue, L. *et al.* (2014) ‘Prostaglandin D2 activates group 2 innate lymphoid cells through chemoattractant receptor-homologous molecule expressed on TH2 cells’, *Journal of Allergy and Clinical Immunology*, 133(4), pp. 1184-1194.e7. Available at: https://doi.org/10.1016/j.jaci.2013.10.056.

Xue, L. *et al.* (2015) ‘Prostaglandin D2 and leukotriene E4 synergize to stimulate diverse TH2 functions and TH2 cell/neutrophil crosstalk’, *Journal of Allergy and Clinical Immunology*, 135(5), pp. 1358-1366.e11. Available at: https://doi.org/10.1016/j.jaci.2014.09.006.

Xue, L., Barrow, A. and Pettipher, R. (2009) ‘Novel Function of CRTH2 in Preventing Apoptosis of Human Th2 Cells through Activation of the Phosphatidylinositol 3-Kinase Pathway’, *The Journal of Immunology*, 182(12), pp. 7580–7586. Available at: https://doi.org/10.4049/jimmunol.0804090.

Yamaga, S. *et al.* (2020) ‘Decreased intracellular histamine concentration and basophil activation in anaphylaxis’, *Allergology International*, 69(1), pp. 78–83. Available at: https://doi.org/10.1016/J.ALIT.2019.05.009.

Yamagata, M. and Sanes, J.R. (2012) ‘Expanding the Ig Superfamily Code for Laminar Specificity in Retina: Expression and Role of Contactins’, *Journal of Neuroscience*, 32(41), pp. 14402–14414. Available at: https://doi.org/10.1523/JNEUROSCI.3193-12.2012.

Yamauchi, Y. *et al.* (2016) ‘The effect of hepatocyte growth factor on secretory functions in human eosinophils’, *Cytokine*, 88, pp. 45–50. Available at: https://doi.org/10.1016/j.cyto.2016.08.013.

Yamaya, M. (2012) 'Virus infection-induced bronchial asthma exacerbation', *Pulmonary Medicine* [Preprint]. Available at: https://doi.org/10.1155/2012/834826.

Yancey, S.W. *et al.* (2017) 'Biomarkers for severe eosinophilic asthma', *Journal of Allergy and Clinical Immunology*. Mosby Inc., pp. 1509–1518. Available at: https://doi.org/10.1016/j.jaci.2017.10.005.

Yasukawa, A. *et al.* (2013) 'Eosinophils Promote Epithelial to Mesenchymal Transition of Bronchial Epithelial Cells', *PLoS ONE*, 8(5). Available at: https://doi.org/10.1371/journal.pone.0064281.

Ye, Y.M. *et al.* (2015) 'Addition of Montelukast to Low-Dose Inhaled Corticosteroid Leads to Fewer Exacerbations in Older Patients Than Medium-Dose Inhaled Corticosteroid Monotherapy', *Allergy, asthma & immunology research*, 7(5), pp. 440–448. Available at: https://doi.org/10.4168/AAIR.2015.7.5.440.

Ying, S. *et al.* (2008) 'Expression and Cellular Provenance of Thymic Stromal Lymphopoietin and Chemokines in Patients with Severe Asthma and Chronic Obstructive Pulmonary Disease', *The Journal of Immunology*, 181(4), pp. 2790–2798. Available at: https://doi.org/10.4049/jimmunol.181.4.2790.

Yu, G. and He, Q.Y. (2016) 'ReactomePA: An R/Bioconductor package for reactome pathway analysis and visualization', *Molecular BioSystems*, 12(2), pp. 477–479. Available at: https://doi.org/10.1039/c5mb00663e.

Yuli, I. and Snyderman, R. (1984) 'Rapid changes in light scattering from human polymorphonuclear leukocytes exposed to chemoattractants. Discrete responses correlated with chemotactic and secretory functions.', *The Journal of clinical investigation*, 73(5), pp. 1408–1417. Available at: https://doi.org/10.1172/JCI111345.

Zangrilli, J.G. *et al.* (1995) 'sVCAM-1 levels after segmental antigen challenge correlate with eosinophil influx, IL-4 and IL-5 production, and the late phase response.', *American Journal*

944 *of Respiratory and Critical Care Medicine*, 151(5), pp. 1346–1353. Available at:
945 https://doi.org/10.1164/ajrccm.151.5.7537586.

946 Zhang, H.Y. *et al.* (1996) 'Lung fibroblast α-smooth muscle actin expression and contractile
947 phenotype in bleomycin-induced pulmonary fibrosis', *American Journal of Pathology*, 148(2),
948 pp. 527–537. Available at: https://pubmed.ncbi.nlm.nih.gov/8579115/ (Accessed: 25 May
949 2021).

950 Zhao, H., Ohinata, K. and Yoshikawa, M. (2009) 'Central prostaglandin D2 exhibits anxiolytic-
951 like activity via the DP1 receptor in mice', *Prostaglandins and Other Lipid Mediators*, 88(3–
952 4), pp. 68–72. Available at: https://doi.org/10.1016/j.prostaglandins.2008.10.001.

953 Zhou, Y. *et al.* (2012) 'The betaine-GABA transporter (BGT1, slc6a12) is predominantly
954 expressed in the liver and at lower levels in the kidneys and at the brain surface', *American*
955 *Journal of Physiology - Renal Physiology*, 302(3). Available at:
956 https://doi.org/10.1152/ajprenal.00464.2011.

957 Zhou, Y. *et al.* (2018) 'Mannose receptor modulates macrophage polarization and allergic
958 inflammation through miR-511-3p', *Journal of Allergy and Clinical Immunology*, 141(1), pp.
959 350-364.e8. Available at: https://doi.org/10.1016/j.jaci.2017.04.049.

960 Zhu, C. *et al.* (2018) 'TWEAK/Fn14 interaction induces proliferation and migration in human
961 airway smooth muscle cells via activating the NF-κB pathway', *Journal of Cellular*
962 *Biochemistry*, 119(4), pp. 3528–3536. Available at: https://doi.org/10.1002/jcb.26525.

963 Zhu, Y. and Bertics, P.J. (2011) ' Chemoattractant-Induced Signaling via the Ras–ERK and
964 PI3K–Akt Networks, along with Leukotriene C 4 Release, Is Dependent on the Tyrosine
965 Kinase Lyn in IL-5– and IL-3–Primed Human Blood Eosinophils ', *The Journal of Immunology*,
966 186(1), pp. 516–526. Available at: https://doi.org/10.4049/jimmunol.1000955.

967 Zhu, Z. *et al.* (1999) 'Pulmonary expression of interleukin-13 causes inflammation, mucus
968 hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production',

969 *Journal of Clinical Investigation*, 103(6), pp. 779–788. Available at:
970 https://doi.org/10.1172/JCI5909.