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NEW INSIGHTS ON TYPE 2 IMMUNITY KEY DRIVERS: MAST CELLS AND GROUP 2 INNATE LYMPHOID CELLS

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New insights on type 2 immunity key drivers: Mast cells and group 2 innate lymphoid cells.

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There exists this stupendous beauty called compassion
And therefore, the world exists.

-Poet Thiruvalluvar

ABSTRACT

Type 2 immune responses are characterized by the production of cytokines IL-4, IL-5 and IL-13. They are known to have crucial physiological and pathophysiological functions in the body. Data from recent studies indicate their importance in the containment and clearance of helminth infections. They are induced to generate rapid response against helminth infections even before the elicitation of specific adaptive responses. On the other hand, Th2 responses are also involved in the promotion of inflammatory conditions such as allergy and asthma. Studies from the past have probed the mounting of type 2 immune responses and Th2 cytokine production by innate immune cells. In this thesis, we set out to study two innate cells, which are considered to be one of the key drivers of allergic conditions – mast cells and group 2 innate lymphoid cells.

In **paper I**, an optimized protocol to efficiently isolate mast cells from human lung tissue with high yield and cell viability is described. Mast cells are heterogeneous, tissue-resident inflammatory cells characterized by the expression of high-affinity IgE receptor, FcεRI, and CD117/KIT, the receptor for stem cell factor (SCF). The isolation of mast cells was performed by a sequential combination of washing, enzymatic digestion, mechanical disruption and Percol centrifugation (WEMP). The yield from WEMP protocol was significantly high when compared to the conventional enzyme-based method. The isolated cells were further used for flow cytometry-based characterization studies and single cell RNA sequencing.

Paper II is a study of human lung mast cells at the single cell level using RNA sequencing. The classic marker expression by mast cells revealed the integrity of the sort and the sequencing reactions. Following this, analyses of sequencing data was performed to depict highly variable genes, highly abundant genes and the presence of subpopulation. Preliminary data displayed homogeneity within the sorted population of the isolated lung mast cells with no clear subpopulations.

In **paper III**, the fundamental aim was to determine the effects of prostaglandin D_2 on ILC2. ILC2 play critical roles in the initiation and promotion of type 2 immune responses. The results indicated the constitutive expression of prostaglandin D_2 synthase by ILC2 and the endogenous PGD_2 production was involved in the activation of ILC2 through CRTH2 receptors in a para/autocrine fashion.

In **paper IV**, the study was designed to elucidate the effect of PGE_2 on ILC2. ILC2 were isolated, cultured and expanded under different treatment conditions to study the effect. PGE_2 was found to have suppressive effect on GATA3 and IL-2 receptor (CD25) expression and

cytokine production by ILC2. The suppressive effects were mediated by EP2 and EP4 receptors. The use of EP2 and EP4 agonists can therefore serve as potential therapeutic target in controlling ILC2 mediated inflammation.

In conclusion, the results from our studies add to the already known information about initiation, maintenance and regulation of type 2 immune responses. They serve as basis for future mast cell-ILC2 interaction studies that could provide potential insights into their contribution in allergic and other TH2-driven conditions.

LIST OF SCIENTIFIC PAPERS

I. **Ravindran A**, Rönnberg E, Dahlin JS, Mazzurana L, Säfholm J, Orre AC, Al-Ameri M, Peachell P, Adner M, Dahlén SE, Mjösberg J and Nilsson G
An Optimized Protocol for the Isolation and Functional Analysis of Human Lung Mast Cells.
Front Immunol. 2019 Oct

II. **Ravindran A**, Mazzurana L, Rönnberg E, Dahlin J, Säfholm J, Ann-Charlotte Orre[4], Al-Ameri M, Adner M, Dahlén SE, Mjösberg J* and Nilsson G*
Deciphering the heterogeneity of human lung mast cells by single cell RNA sequencing.
In manuscript

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III. Maric J, **Ravindran A**, Mazzurana L, Van Acker A, Rao A, Ekoff M, Thomas D, Fauland A, Nilsson G, Wheelock C, Dahlén SE, Ferreiros Bouzas N, Geisslinger G, Friberg D, Heinemann A, Konya V and Mjösberg J
Cytokine-induced endogenous production of PGD2 is essential for ILC2 activation.
J Allergy Clin Immunol. 2018 Dec -Article In Press

IV. Maric J, **Ravindran A**, Mazzurana L, Björklund ÅK, Van Acker A, Rao A, Friberg D, Dahlén SE, Heinemann A, Konya V and Mjösberg J
Prostaglandin E2 suppresses human group 2 innate lymphoid cell function.
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Induction of human regulatory innate lymphoid cells from group 2 innate lymphoid cells by retinoic acid.
J Allergy Clin Immunol. 2019 Jan

CONTENTS

LIST OF ABBREVIATIONS

AA – Arachidonic acid

CCL – Chemokine ligands

COPD – Chronic Obstructive pulmonary disease

COX – Cyclooxygenase

CRTH2 – Chemoattractant receptor-homologous molecule (CRTH2),

CysLT – Cysteinyl leukotrienes

EP receptors – E-prostanoid receptors

FLAP – 5-Lipooxygenase-activating protein

IBD – Inflammatory bowel disease

IgE – Immunoglobulin E

IgG – Immunoglobulin G

IL – Interleukin

ILC – Innate lymohoid cells

ILC2 – Group 2 Innate Lymphoid cells

KLRG1 – Killer cell lectin like receptor G1

LPS – Lipopolysaccharide

LT – Leukotrienes

MAPK – Mitogen-activated protein kinase

MHC-II – Major histocompatibility complex class II

NKT – Natural killer T cells

PAR – Protease-activating reeptors

PG – Prostaglandin

PI3K – Phosphoinositide-3-kinase

PTGDR – Prostaglandin D_2 receptor 1

PUFA – Poly unsaturated fatty acid

RAG2 – Recombination-activating gene 2

RORα – Retinoic acid-related orphan receptor alpha

SCF – Stem cell factor

TH2 – Type 2 helper

TLR – Toll-like receptors

TNF – Tumor necrosis factor

TSLP – Thymic stromal lymphopoietin

VEGF – Vascular endothelial growth factor

1 INTRODUCTION

Our immune system is an extensively evolved phenomenal defence system made up of a humongous array of cells primarily dedicated to protect our body from invading pathogens. They can not only distinguish foreign from its own but also can recognize the subtle differences between the foreign molecules and therefore can display remarkable specificity. Traditionally, immune responses to pathogens are broadly divided into two main categories - innate and adaptive. The very first line of defence upon pathogen encounter occurs in a non-specific, yet effective manner by the recruitment of a number of innate cell types. This type of response is important for the rapid initial action to pathogen entry and exists from the birth. In adaptive immune responses, the initial recognition of the pathogen is relatively delayed yet is followed by the mounting of an appropriate effector response specific to the pathogen in order to eliminate or neutralize them. Adaptive arm of the immune system mainly constitutes B and T cells, which can respond in a highly specific way. The subsequent exposure to the same antigen induces a more accelerated memory response by B and T cells. The innate arm, on the other hand, is comprised of rapidly responding cells such as macrophages, neutrophils, dendritic cells that are readily present at the infection site and actively participate in the control and clearance of infection.

1.1 TYPE 2 IMMUNE RESPONSES

Type 2 innate immune responses are characterized by the production of type 2 cytokines IL-4, IL-5, IL-9 and IL-13 by the recruitment and activation of TH2 cells, eosinophils, mast cells, ILC2 and basophils. Protective aspect of type 2 immunity lies majorly in orchestrating resistance against parasitic infections at the cutaneous and mucosal surfaces [1, 2]. Recent evidences suggest their protective roles in autoimmune diseases by suppressing type 1 driven tissue damage, inflammation and fibrosis [3-6]. Contrastingly, they play critical roles in the promotion of allergic inflammatory conditions like asthma, atopic dermatitis and anaphylaxis [7, 8].

In response to allergens and helminths, epithelial cells produce TSLP, IL-25 and IL-33, which serve as the major inducers of type 2 immune response [9-11]. TSLP is identified as a potent Th2 activating cytokine that causes CD4+ T cell differentiation by the induction of dendritic cells. These TSLP-activated dendritic cells in turn produce Th2 attracting chemokines CCL17, CCL22, CCL24 [12, 13]

In mice, IL-25 promotes TH2 response by bringing about eosinophilia and IgE production leading to type 2 cytokine release especially in the lung. This type of IL-25 induced Th2

response advocates inflammatory conditions in the lung [14, 15]. IL-33-activated basophils build up type 2 responses by generating IL-4, IL-5, IL-6 and IL-13. IL-33 administration in mice caused the IgE release, hyperplasia of goblet cells and eosinophilia [16-18].

The cytokine mediated effects of these responses can be both specific and non-specific. Recruitment and activation of innate cells by IL-25 and IL-33 caused the production of IL-5 and IL-13. This effect was shown to occur entirely independent of B and T cells [14, 19, 20]. As mentioned previously, mast cells and basophils produce TH2 cytokines upon activation. In addition to this, NKT cells and ILC2 have IL-25 receptor (IL25R) and therefore can be activated by IL-25 that ultimately causes IL-13 release [21, 22]. In addition to suppression of TH1 driven inflammation as discussed above, type 2 cytokines such as IL-4 and IL-13 have found to be driving macrophages to promote tissue repair and regeneration following inflammation [23]. Increase in Th2 cytokine levels in response to chronic helminth infection often result in fibrotic lesions. Initial fibrosis resulting from worm egg storage in liver, lung and gut is highly altered by IL-4, IL-5 and IL-13 in addition to TH2 activated eosinophils and macrophages causing type 2 fibrotic condition [24, 25]. Such contrasting roles of type 2 immunity in health and diseases explain the persistent efforts made in the medical field to carefully target their pro inflammatory roles without sacrificing their effective functions in health.

1.1.1 Type 2 immunity in allergic inflammation

Repetitive exposure to allergen leads to chronic inflammatory condition. Long term persistence of inflammation leads to asthma characterized by airflow obstruction, hyper responsiveness, mucus accumulation and airway remodelling. Asthma is recognized as a type 2 inflammatory disorder characterized by type 2 cytokine-driven inflammation [10]. IL-4 induces the basophil activation and IgE-dependent mast cell degranulation leading to the release of their mediators. IL-5 and IL-9 autonomously induce mast cell hyperplasia and eosinophilia. IL-13, on the other hand, promotes the hyperplasia of goblet cells during mucus production and airway hyper responsiveness in asthma. Elementary mouse models of asthma revealed the crucial roles of TH2 cells, mast cells and eosinophils in driving the disease [26, 27].

IL-33 is a potent activator of IL-5 and IL-13-producing ILC2, which are important during initial inflammatory phases. They also create in interface between IL-33 and eosinophils to facilitate eosinophilic inflammation as observed in ILC-deficient mice [28-30]. TSLP-deficient asthma mice models demonstrated resistance to airway hyper responsiveness indicating their allergy promotion roles [31]. The detailed pro inflammatory roles of mast

cells and ILC2 during the latent and chronic phase of allergic inflammation has been discussed in the following sections of this thesis.

1.2 MAST CELLS

Mast cells have remained inexplicable for over a century since they were first described as 'mastzellen' by Paul Enrich in the year 1878. Persistent research carried out in the field has helped unravel their characteristics and functions over the years.

1.2.1 Mast cells

The progenitors of mast cells originate from the pluripotent hematopoietic cells of the bone marrow and circulate in blood at low frequencies. The progenitors migrate to tissues where they mature under the influence of epithelial and mesenchymal-derived growth factors such as SCF and become exclusively tissue resident [32, 33]. These non-proliferative mature tissue resident cells are well-characterized by their surface expression of classic markers such as the high affinity immunoglobulin E (IgE) receptor, FcεRI, stem cell factor receptor CD117 [34]. The secretion of various mediators and cytokines by mast cells has been shown to play significant roles in inflammatory and allergic responses. Mast cells are found throughout the body, particularly in connective tissues. They contribute to body's first line of defence by their presence particularly in tissues in close contact with the environment; i.e., skin, lung and the gastrointestinal tract [35, 36].

1.2.2 Heterogeneity and activation

The heterogeneous nature of mast cells is due to the variation displayed by them in terms of granularity, protease content, responsiveness to agents and mediator release. Based on the above factors, mast cells are compartmentalized in different parts of the same tissue such as the lung. Mast cells are classically divided into two main subsets based on their protease content – MC_T and MC_{TC}. Tryptase positive cells, often found in lung parenchyma and mucosal tissues, are classified as MC_T. Mast cells that express chymase, carboxypeptidase and cathepsin G in addition to tryptase are called MC_{TC}, predominantly found in connective tissues such as the skin [37]. A recent study described the presence of a new mast cell subset in airway epithelium of asthmatic patients expressing tryptase and carboxypeptidase A3, but not chymase[38]. Detailed description and characterization of such mast cell subsets are crucial in order to understand their role in homeostatic and disease conditions. Researching mast cells in such depth is made possible by recent advancements in the field that help us study them at single cell resolution.

As described above, mast cells are the storehouses of numerous granules such as histamine, serine proteases, chymase, heparin, and cytokines [39]. The classic and well-defined mast cell activation occurs through cross-linking of two IgE-molecules bound to high-affinity IgE receptors cross-linking, an activation that is more relevant in allergic responses. Initial exposure to the allergen causes the binding of IgE to the FcεRI receptor on the mast cells. Subsequent allergen exposure results in the cross linkage of FcεRI resulting in the initiation of signalling cascade. The proceeding series of events ultimately lead to mast cell degranulation – release of their cellular contents. Upon their activation, mast cells also de novo synthesize and release inflammatory cytokines and lipid mediators that mediates various effects. [40].

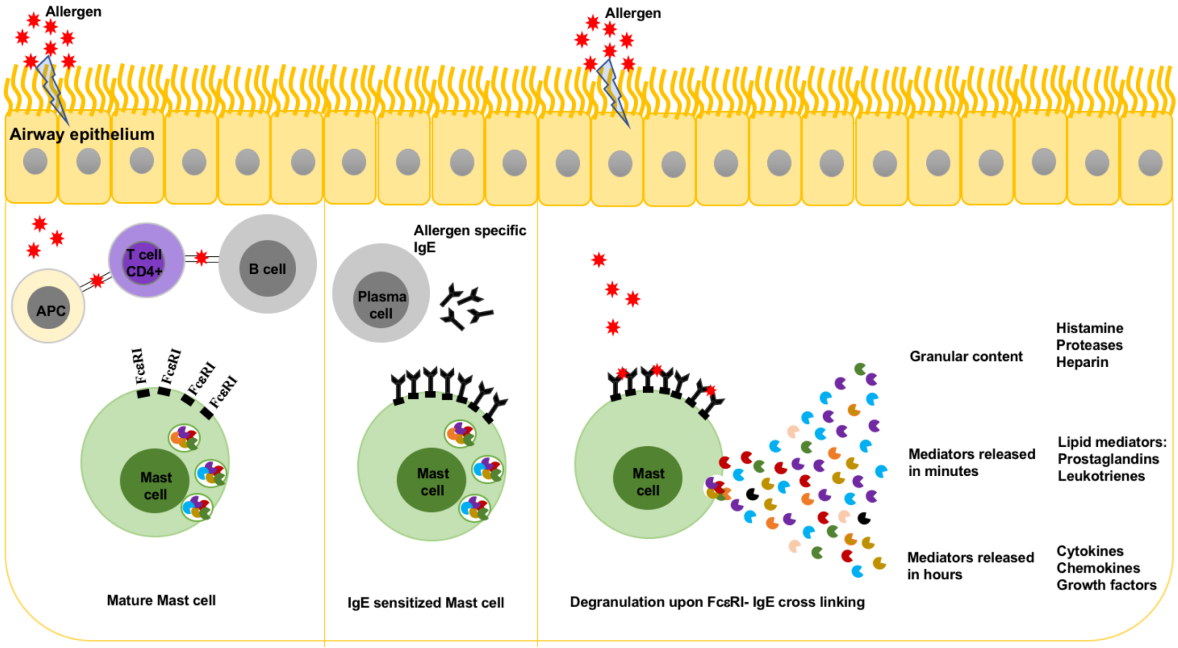

Figure 1: IgE dependant mast cell activation.

Mast cell activation occurs also through a number of IgE independent pathways. Their location proximal to the external environment makes their encounter of pathogen likely and therefore their activation through pattern recognition receptors dependent [35]. Products of inflammation such as complements, IgG, cytokines, chemokines, adenosine etc. can also trigger mast cell activation. Mast cells located at the sensory nerve endings can be activated by neurotrophins and neuropeptides suggesting their contribution to neuro-inflammatory disorders [41]. Recent studies identified the activation of MC_{TC} by Mas-related G protein-coupled receptor X2 (MRGPRX2) leading to phospholipase C mediated release of cytoplasmic calcium release. [42].

1.2.3 Mast cells in type 2 immune response

In parasitic infections, antigen specific IgE-dependent mast cell degranulation results in release of growth factors like IL-3, IL-4, SCF, IL-9, which aids in regulating gastrointestinal permeability and recruitment of immune cell to the infection site. Mast cells play crucial role in the regulation of type 2 immune responses to helminth infections. Th2 responses have been shown to be inhibited when mice lacking mast cells (Kit^{W}/ $Kit^{W\text{-}v}$ and $^{KitW\text{-}Sh}$) were infected with parasitic helminth *Heligmosomoides polygyrus bakeri*. Apart from antigen specific IgE dependent degranulation, mast cells can also be activated in an IgE independent manner in response to parasites. During intestinal helminth infections, the secretory products serve as ample stimulatory molecules that can be sensed by mast cells through their surface TLRs, protease-activating receptors (PARs) and cause their degranulation [43]. The tissue derived cytokines IL-33 and TSLP released in response to pathogens and allergens can act on mast cells affecting their functions [44-46].

1.2.4 Physiological role of mast cells

The widespread distribution particularly at the interface between host and external environment put mast cells in a privileged position to take part in biological actions and be one of the first responders to external stimuli [47]. Mast cells contribute to the body's innate immune response by recognizing and interacting with pathogens, antigens and toxins.

Pattern recognition receptors like TLR expressed by mast cells facilitate the direct binding of pathogens to them ultimately causing mast cell degranulation [48]. The release of inflammatory mediators thereby aids the control and clearance of pathogens. Release of such mast cell mediators upon TLR binding depends on the ligand and the receptor [49]. Classic examples of such reactions include the recognition and binding of TLR2 by bacterial peptidoglycan, TLR4 by LPS [50, 51].

Mast cells also react to viral infection by producing interleukins IL-1, IL-6 and chemokine ligands like CCL3, CCL4, CCL8 [52, 53].

Number of mediators released during mast cell activation induces various physiological and immunomodulatory functions in endothelial, nervous and epithelial tissues. Mast cells maintain the tissue function and homeostasis through mediators such as histamine, tryptase and VEGF [54]. Mast cell tryptase has been shown to activate protease-activating receptor 2, which inhibits differentiation of osteoclasts [55]. On the other hand, mast cell mediators such as platelet activating factor (PAF), leukotrienes, t-plasminogen (tPA), heparin, tryptase are released by them in response to tissue injury that regulate fibrinolytic mechanisms thereby promoting tissue repair and remodelling [56, 57].

1.2.5 Pathological role of mast cells

In contrast to their multifunctionality in homeostasis, mast cells have typically been associated with pathological conditions such as allergy, asthma, mastocytosis and so on. Improper regulation of mast cells causes them to respond to harmless antigens in an imprudent manner. Under such circumstances, antigen-specific IgE that are produced by B cells bind to the FcεRI receptor on mast cells causing sensitization. Subsequent antigenic exposure ultimately results in mast cell degranulation and release of pre-formed and newly synthesized mast cell mediators and cytokines. The released pro-inflammatory mast cell contents such as histamine, prostaglandins and leukotrienes trigger allergic reactions causing symptoms like mucus production, edema and itch [48]. The release of cytokine leads to the recruitment and activation of other immune cells like T cells, eosinophils, basophils causing persistent chronic inflammation, tissue remodelling and fibrosis that can be observed in disorders like asthma [58, 59]. Crohn's disease is characterized by the chronic inflammation of the gastrointestinal tract. Mast cells have shown to contribute to the disease pathology by releasing IL-16. The mast cell produced IL-16 causes the recruitment of circulating T lymphocytes to the inflammatory site [60]. In mastocytosis, a primary mast cell-driver disease, the clonal accumulation of mast cells is caused by a c-KIT activating mutations that causes mast cell accumulation in bone marrow, GI tract, skin and other organs [61].

Phenotypic modifications of mast cells under the influence of micro environmental changes in the tissue, e.g., during inflammation, determine their roles in health and pathophysiological conditions. Such multifunctionality brings about a need to study these cells in depth. In this thesis, we have described two such studies – a protocol for the effective isolation of human lung mast cells and their detailed characterization using single cell RNA sequencing platform.

1.3 INNATE LYMPHOID CELLS

Innate lymphoid cells are a novel heterogeneous family of cells consisting of 3 main subsets - group 1 (ILC1), group 2 (ILC2), and group 3 (ILC3) [62, 63]. They play roles in various bacterial, fungal, viral and parasitic infections [64]. Their improper regulation causes the worsening of chronic inflammatory conditions such as allergy, asthma, COPD, IBD etc [65, 66]. ILCs also have roles in driving certain cancers and autoimmune disorders [67]. The homeostatic and inflammatory roles of ILCs are being extensively studied for the better understanding of their roles.

1.3.1 Group 2 innate lymphoid cells

Group 2 innate lymphoid cells (ILC2) originate from the common lymphoid progenitor of bone marrow and become committed to ILC2 lineage. The maturation, maintenance and survival of ILC2 mainly rely on the transcription factor GATA3 and RORα expression. Genetic knockout studies in mice proved GATA3 to be a crucial transcription factor in the ILC2 development and regulation. Mature ILC2s are characterized by their predominant expression of GATA3 and production of type 2 cytokines IL-4, IL-5, IL-9 and IL-13 [68-70]. These cells lack antigenic receptor expression on their surface unlike B and T cells. Owing to this reason, ILC2 confer immune responses in a non-specific manner making them a strong part of the innate immune family [71]. ILC2 are highly responsive to and activated by IL-33, IL-25 and TSLP [72]. CRTH2 a receptor for prostaglandin D2 (PGD_2) is expressed in human ILC2 and can activate them upon CRTH2-PGD_2 binding [73].

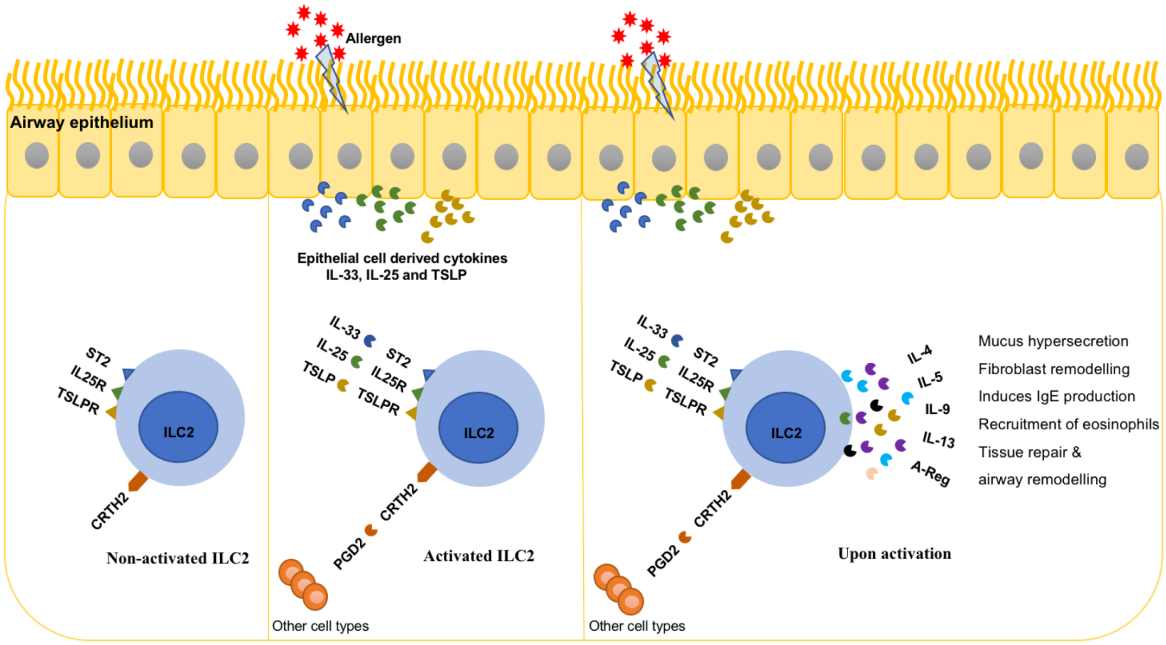

Figure 2: Activation of ILC2

The two major classes of ILC2 are natural ILC2 (nILC2) and inflammatory ILC2 (iILC2). Natural ILC2, as the name suggests, mostly possess functions related to homeostasis [74] and respond to IL-33. Recent experiments on the synovial samples of arthritic patients suggest a possible IL-9 mediated anti-inflammatory role of ILC2 by restriction of inflammation and cell death. Their close proximity to regulatory T cells at the site of inflammation and their abundant IL-9 production were observed in these samples [75]. On the contrary, iILC2 that respond to IL-25, has pro-inflammatory roles that promote certain chronic inflammatory conditions through their surface expression of KLRG1 and IL25R. These pro inflammatory

ILC2 are highly responsive to chemotactic signals and migrate between mucosal sites promoting cytokine mediated type 2 inflammation [76].

Recent advancement in the field has enabled the high throughput, in-depth analyses of ILC2 using RNA-seq, CHIP-seq platforms in order to better understand their transcriptional and regulatory network [77, 78]. Heterogeneity in the ILC2 exits because of their interaction with the different environmental situations. The gene expression profile and phenotypic variations exist from counterbalancing their anti and pro inflammatory roles [79]. The above described natural and inflammatory ILC2s are a result of the establishment of such an equilibrium. ILC2 subclasses have been identified based on their gene expression - ILC1/2 also expressing *Gzma, Hopx Epas1* and ILC2/3 expressing *Cxcl2, Cxcl3, Arg1* [80].

1.3.2 ILC2 plasticity

ILCs are known for their plastic nature, which was first observed in the ILC3 present in gut, where they modify their gene expression to transform into ILC1 [81]. ILC2 are able to show plasticity by upregulating T-bet in the presence of IL-33 and IL-1β and produce interferon γ (IFNγ) to become ILC1 [66, 82, 83]. Administration of IL-25 in mice resulted in the generation of IL-17 producing inflammatory ILC2, a typical ILC3 cytokine, which is in addition to their well-defined type 2 cytokine profile [74]. In another notch ligand mediated condition, ILC2 upregulate Rorγt and produce IL-17 and IL-13 causing ILC2 to ILC3 transformation [84]. In addition to the above heterogeneous nature of ILC, recent mice studies identified a group of ILC that has regulatory roles through IL10 production [85].

1.3.3 Role of ILC2 in type 2 immune responses

ILC2, as discussed above, performs various pro- and anti-inflammatory roles in the body through their predominant production of type 2 cytokines, i.e., IL-4, IL-5, IL-9 and IL-13 upon activation through IL-25, IL-33 and TSLP. Due to the absence of an antigenic receptor, therefore a lack of specificity, they stand unique from the CD4+ Th2 helper cells. ILC2 play a role in orchestrating type 2 immune responses such as eosinophilic induction, class switching of B cells, expansion of CD4+ T cells, initiation of adaptive immune response.

ILC2 from the spleen and lymph nodes of mice expressed MHC-II and co-stimulatory molecules such as CD80 and CD86. Combined expression of these molecules allowed the CD4+ T cell interaction, activation and expansion, which is critical in the clearance of murine helminth infection [86]. In humans, ILC2 are found in tonsils, skin, spleen, lymph nodes, liver, blood and lungs [87-91]. ILC2 population were assessed in helminth infected children in Zimbabwe. Children aged 6-13 had lower ILC2 counts when compared to uninfected

cases. While 14-18-year-old groups has higher ILC2 counted in comparison with uninfected individuals. These findings suggest an early role of ILC2 in parasitic infection and also in the initiation of Th2 immune response [92]. ILC2 are found to be located in proximity to the enteric neurons. These neurons are capable of activation and cytokine production of ILC2 by interacting through mediators. The vasoactive intestinal peptide (VIP) receptor engagement causes the enhancement of type 2 cytokine production by ILC2 [93]. Further ILC2 helps in the pathogen clearance and tissue repair in the lung through post viral infection through the release of epidermal growth factor amphiregulin, which is predominantly expressed by Th2 cells [94].

Apart from the protective roles, ILC2 critically contribute to the promotion of chronic inflammatory condition by means of cytokine production. They are said to be the key drivers of type-2 mediated inflammatory diseases like asthma. Intranasal delivery of the ILC2 activating epithelial cytokines such as IL-25 and IL-33 resulted in the induction of an allergic response even in the absence of B and T cells implicating the involvement of ILC2 in the promotion of such response [14, 29]. In the absence of T cells, papain induced lung inflammation by means of ILC2 suggesting the ability of these cells to stand alone in promoting Th2 inflammation [28]. Furthermore, ILC2 are found to be enriched in chronic rhinosinusitis and atopic dermatitis patients suggesting their pro-inflammatory roles in other sites as well. Skin-residing ILC2, however, are mainly activated by TSLP and remain resistant to the action of IL-25 and IL-33 [95]. This observation indicated the variability of ILC2 characteristics based on where they reside [96]. Much remains to be studied about biology and functions of these fairly new cells. The possibility of these cells to be a potential therapeutic target poses the need to study their key anti-inflammatory functions. In this thesis, we have probe into the factors involved in the activation and inhibition of ILC2 functions that provide new insights in understanding them better.

1.4 LIPID MEDIATORS

1.4.1 Arachidonic acid

Lipid mediators have many essential physiological and pathological functions. They often engage in cell proliferation, migration and apoptosis. Arachidonic acid (AA) is a well-studied PUFA composed of 20 carbon atoms. Egg, fish, seafood and poultry are some of its abundant sources [97, 98]. They are generally found to be fused with the phospholipids in the cell cytoplasm. One fourth of phospholipid fatty acids in cells such as mononuclear cells, neutrophils, platelets and tissues such as brain, muscle, liver is in the form of AA [99, 100]. They help in maintaining selective permeability and integrity of the cell membrane serving

as a basic constituent of the cell [101, 102]. They are necessary for general growth and development, especially in newborns where they are an important nutrient supplement [103, 104].

AA is present adjacent to the endoplasmic reticulum and nuclear membrane of the cell [99]. Upon cellular activation, AA is freed from phospholipids by the action of enzyme phospholipase A_2 (PLA) [105]. The metabolism of the released AA can lead to two main pathways – either by the enzyme cyclooxygenase (COX) or by lipoxygenase (LOX). The downstream products of these pathways are collectively known as eicosanoids and consist of prostaglandins, leukotrienes, thromboxanes and lipoxins. Eicosanoids have various biological roles in cell growth, vascular permeabilization, inflammation, blood pressure maintenance, platelet aggregation etc. [106]. The generation of prostaglandin G_2 (PGG_2) from the intracellular AA is catalysed by the COX enzymes [107]. Further activation of synthases leads to the conversion of PGG_2 to PGH_2. The downstream metabolites of AA encompass inflammatory roles such as immune cell activation, bronchoconstriction and vasodilation [108, 109].

1.4.2 Prostaglandins & Leukotrienes

Prostaglandins are found almost throughout the body and have both pro- and anti-inflammatory effects. Consequent to activation, mast cells release AA metabolites such as PGD_2 and leukotriene C_4 (LTC_4) that have significant roles in health and diseases. PGD_2 is a biologically active lipid that is enzymatically derived from PGH_2 by the action of PGD_2 synthase [107]. PGD_2 is particularly expressed by mast cells and is a classic mast cell marker. CRTH2 and PTGDR are the well-defined receptor for PGD_2 [110, 111]. They participate in the recruitment of immune cells such as Th2 cells, eosinophils and basophils. They play critical role in the development of allergic conditions such as asthma owing to their roles in bronchial constriction, vasodilation and mucus production [112].

The potential effects of PGD_2 on ILC2 have been studied extensively in both human samples and mouse models [113]. One of their major interaction pathways is via CRTH2, a well-known surface receptor expressed on human ILC2. Studies have demonstrated that PGD_2 can induce ILC2 to produce IL-13 through CRTH2 receptor activation [114]. It has also been shown that PGD_2 induced the production of IL-5 and IL-13 by human skin ILC2 [73]. They help in the chemotaxis of human blood ILC2 [115]. Upon treatment of CRTH2 antagonist, IgE-activated mast cells were found to have lesser stimulatory effects[73]. Helminth mouse models were found to have increased ILC2 accumulation upon PGD_2 action [116]. Adding

to these, PGD_2 is found in considerable amounts in brain and its signalling blockade in krabbe mouse models lowered neuro-inflammatory effects [117].

Unlike pro-inflammatory effects, the role of PGD_2 and its distal products in the resolution of inflammation is not well studied. LPS induced inflammation in human models causes flu-like symptoms combined with pyrexia. The PGJ_2, a downstream product of PGD_2 metabolism has been shown to have anti-pyretic effects in these models [118]. Undermining of inflammation by PGD_2 is observed in models of pleuritis, a lung inflammatory condition [119].

PGE_2 is another lipid mediator derived from arachidonic acid. It acts through the binding of four different E-type prostanoid receptors such as EP1, EP2, EP3 and EP4. The sensitivity and activation pathways of these EP receptors diverge, which explains the pro and anti-inflammatory roles of PGE_2. The protective roles of PGE_2 in allergy and asthma is supported by recent studies in mouse models [120, 121]. In human lung, PGE_2 was observed to inhibit bronchoconstriction induced by allergen [122]. Furthermore, immune cells such as macrophages, mast cells, eosinophils, neutrophils and Th2 cells are inhibited by PGE_2 [123-127]. In addition to this, PGE_2 has been found to support the production of suppressive factors such as IL-10 [128] and trombospondin [129] by dendritic cells. They also enhance the ability of dendritic cells to cause T cell expansion [130-132]. EP2 and EP4 mediated PGE_2 signalling promotes the production of Th17 cells [133-136].

Leukotrienes (LT) are also a class of bioactive lipids derived from intracellular AA through LOX enzymes. The nuclear membrane AA initially binds to 5-lipooxygenase-activating protein (FLAP) which transfers AA to LOX enzyme producing an unstable LTA_4 [137]. Further conversion of LTA_4 is controlled based on the cell type, like in neutrophils, LTA_4 hydration by LTA_4 hydrolase (LTA_4H) converted it to LTB_4 [138]. The action of LTC_4 synthase causes the conversion of LTA_4 to LTC_4 in mast cells [139]. The extracellular cleavage of LTC_4 produces LTD_4 and LTE_4. LTD_4 has a notable contribution in mediating bronchoconstriction during allergic conditions such as asthma [140].

1.5 INTERACTION BETWEEN MAST CELLS AND ILC2

Extensive pro- and anti-inflammatory roles of mast cells and ILC2 in health and diseases suggests possible interactions between them. The elucidation of their interactions and therefore their ultimate effect could potentially facilitate the understanding of various inflammatory conditions better. Efforts have been made in the field to figure out the means of communication between them and this part of the thesis will review important finding from such studies.

As discussed in the previous section, ILC2 mediate immune responses via type 2 cytokine production playing roles in inflammation, allergy and tissue repair. Murine lung ILC2 isolated from wildtype, $RAG2^{-/-}$ and $STAT6^{-/-}$ animals when treated with cysteinyl leukotrienes (cysLTs), interact with their receptors on ILC2. This leukotriene-mediated activation of ILC2 leads to their production of type 2 cytokines [141]. ILC2 express leukotriene receptors $cysLT_1$ and especially found to be increased in atopic subjects. A recent study investigated the role of cysteinyl leukotrienes on ILC2. The results from this study showed that LTE_4 facilitated the migration and cytokine production of human ILC2 *in vitro*. Further they enhanced the action of known ILC2 activators – IL-25, IL-33, TSLP and PGD_2. These effects were hindered by cysLT1 antagonist. Further, LTE_4, in presence of IL-2, upregulated the expression of IL-25 and IL-33 receptors [142]. Mast cells have long known for their production of lipid mediators such as prostaglandins and leukotrienes following FcεRI activation. This classic characteristic of mast cells suggests a possibility of their interaction with ILC2 via cysLTs. Further, prostaglandin-mediated mast cell-ILC2 interaction through CRTH2 has been demonstrated. It was observed that ILC2 were activated upon treatment with PGD_2 from human skin tissues [73]. Further follow-up study in mice lung tissues showed the critical role of CRTH2-PGD_2 interaction in ILC2 activation and cytokine release. This kind of ILC2 activation via CRTH2 was found commonly in lung tissues in comparison with skin ILC2. Hence the production of the potent activator PGD_2 by mast cell, resulted in the ILC2 activation and promotion of allergic reaction in lung [116].

Recent studies showed that serine proteases generated by activated mast cells produced mature forms of IL-33 that had potent effects on ILC2. The effects of tryptase-mediated cleavage of IL-33 3-fold increase in action when compared to the full-length IL-33. The cleaved IL-33 therefore caused strong increase in ILC2 and eosinophil expansion *in vivo* and lead to the production of Th2 cytokines such as IL-5 and IL-13 by ILC2 [143].

Th9 cells promote lung inflammation mainly by the production of IL-9. Studies conducted in cystic fibrosis patient samples lead to the proposal of a positive feedback mechanism wherein the released IL-9 induced the IL-2 production by mast cells. The action of IL-2 lead to the activation of CD25+ ILC2 ultimately causing the increase in IL-9 production. In addition to IL-9, the cytokines released from the ILC2 activation during this loop contributed to the inflammatory reactions [144].

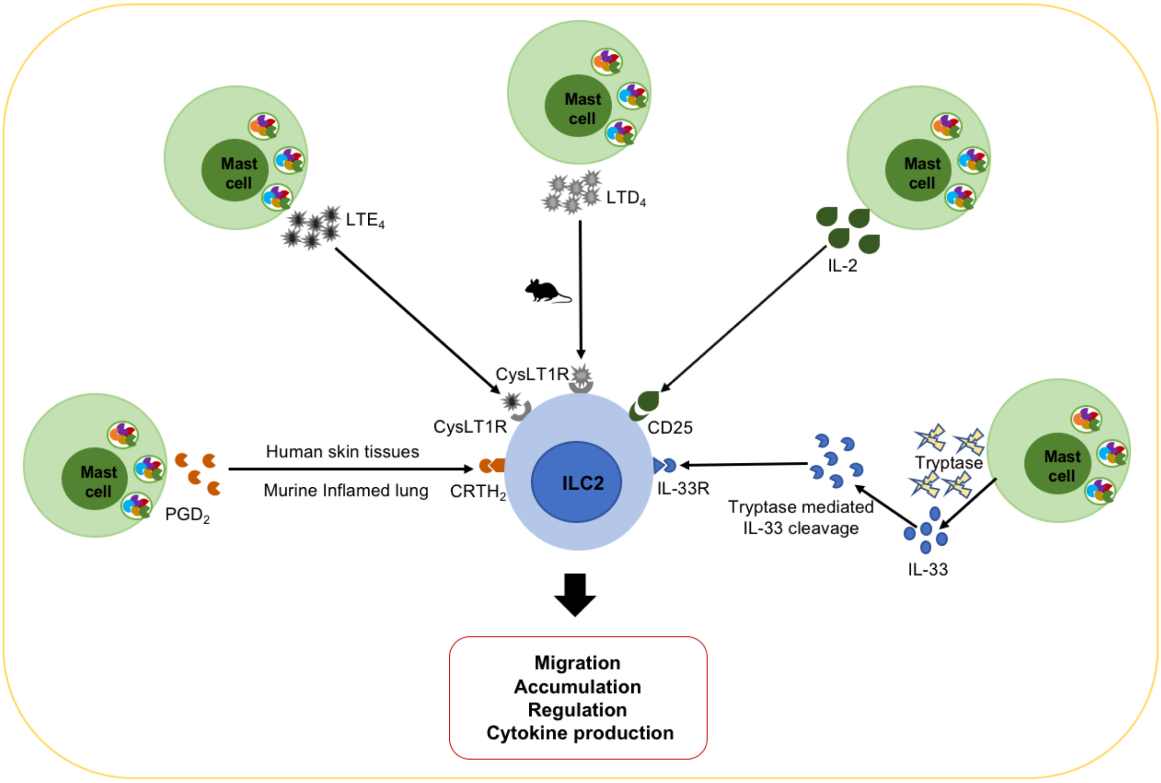

Figure 3: Interaction between mast cells and ILC2

Yet another mast cell-ILC2 interaction pathway has been recently proposed in helminth infected mice models. Intestinal epithelial cells undergo apoptosis during helminth infection. The released ATP molecule induce mast cell degranulation and IL-33 release, which causes the activation of ILC2 that results in IL-13 production. This ILC2 activation pathway ultimately leads to goblet cell hyperplasia and the clearance of helminth infection [145]. Continuous research carried out in the field could result in more interesting insights into the possible mast cell-ILC2 interaction mechanisms. These data consistently help in unravelling the role of these cells in health and diseases.

2 METHODS

Patient sample collection

All the studies included in this thesis were done on freshly obtained and processed human samples from surgeries. We value these samples a lot and preciously protect the interests of our donors.

Lung

Lung tissue samples used in papers I and II were obtained from the lung resection surgery of tumor patients at Karolinska University Hospital, Solna, Sweden. A portion of the lung tissue was cut out from the region far from tumor site. The obtained tissue was stored in Krebs-henseleit buffer on ice until the beginning of tissue processing. It was taken into account that the patients did not receive chemotherapy or radiotherapy. The donors were provided with informed written consent and the study was approved by regional review board in Stockholm.

Tonsil and Buffy coats

Tonsil and blood samples were the main sources of immune cells used in papers III and IV. The tonsil tissues were obtained from the routine tonsillectomy operations conducted on patients with obstructive sleep and apnea syndrome at ear nose throat clinic, Karolinska University Hospital, Huddinge, Sweden. Blood samples were provided by the blood bank at Karolinska University Hospital, Huddinge. The use of such samples were used with proper consent from patients and the studies were approved by the regional ethical board at Karolinska University Hospital.

Flow cytometric analyses and sorting

Flow cytometric analyses and sorting is one of the main methods used in all the four studies included in this thesis. The technique enables the identification and distinguishing of various cell populations at once based on the different surface molecules they express. The isolated cells from the human tissue were incubated with antibodies that can bind to the surface molecules on the cells of our interest. Each of these antibodies are tagged with different flourochromes. The focus of papers I and II involved the efficient isolation and single cell sequencing of mast cells. The papers III and IV involved the sorting of ILC2, expanding them and analyzing these cells using flow cytometer. In order to perform this, mast cells, NK cells and ILCs were stained for their surface markers by incubating the cells under question in a

pool of fluorescently labelled antibodies that bound to the surface molecules on our desired cells. Thereafter, the cells were stained for live/dead markers to rule out the dead cells from analyses. Intracellular tryptase staining of mast cells was performed by permeabilization of cells followed by blocking step to rule out non-specific binding using blocking buffer consisting of 5% dry milk and 2% FCS in PBS. Cells were thereafter stained with Tryptase antibody conjugated with a flourochrome and then subjected to flowcytometric analyses. Similarly, intracellular cytokine staining of ILCs were performed by permeabilizing and then staining them with appropriate antibodies. The data obtained from the flow experiments were analyzed using the Flowjo software.

Single cell RNA sequencing

In paper II, single cell RNA sequencing was performed using Smart-seq2 that facilitated full-length characterization of the entire transcriptome. The sequencing process was carried out by our collaborators from Science for life laboratory, Solna. A brief description of the entire process is presented in this section. The mast cells isolated from fresh lung tissue were sorted as single cells into 384 well plates using a flow cytometer. The lysis buffer contained free dNTPs and oligo-Dt primers necessary for cDNA synthesis. Once sorted, the single mast cells were lysed and the released RNA were converted to cDNA by reverse transcriptase enzyme. This enzyme acts by the addition of 2-5 nucleotides on the 5' end of mRNA thus creating overhangs mostly composed of cytosine. A template switch oligo with the same anchor sequence as the oligo-dT at its 5' facilitates the CDNA extension with template switch oligo as template. The template switch oligo contains special locked nucleic acid that increases the cytosine binding strength and thermal stability. The cDNA thus carries same known anchor sequencing facilitating the subsequent PCR reaction. The cDNA is randomly amplified with Tn5 transposase containing adaptor sequence. Finally, an enrichment PCR introduced unique adaptor sequences to be able to pool them and run on the same lane of Illumina sequencing instrument.

Culture, expansion and treatments of mast cells and ILC2

Freshly sorted ILC2 were cultured in IMDM media containing Yssel's supplement, 1% serum, penicillin and streptomycin. The cells were expanded and maintained in culture for two weeks with irradiated PBMC and JY cells as feeders. The culture was done under the presence of IL-2, IL-4 and phytohemagglutinin. The cells were maintained in U bottom 96 well plates for one week in 200ul media and then to a 24 well plate, where they were maintained as 1ml cultures. Media was changed once in every two days. In order to treat

them with different culture conditions, the maintained cells were seeded as $5x10^4$ cells/well. Seeded cells were thereafter subjected treatment conditions depending on the aim of the experiment. For LC-MS, cells were seeded at a concentration of $2.5x10^5$ and incubated for 24 hours. Supernatants were collected following each experiment to assess the effects using ELISA.

ELISA

The effect of various treatment conditions was deciphered by measuring the type and theamount of cytokines released by the cells into the supernatant. This is done by ELISA, a sandwich immunoassay that detects the specific cytokine levels. IL-5 and IL-13 were detected by the commercially available Duo set kit from R&D systems and human IL-13 kit from Sanquin.

RT-qPCR

Total RNA was extracted from the tonsil cultures using RNeasy kit from Qiagen. cDNA synthesis was performed by using commercially available iScript cDNA synthesis kit. For PCR reactions, gene specific primers and SsoAdvancedTM Universal SYBR® Green supermix (Bio-Rad) were used. The reactions were performed on CFX Connect Real-Time PCR Detection System (Bio-Rad).

3 AIMS

In this thesis, we set out to study mast cells and ILC2 and their role in type-2 immune responses in detail.

Paper I – To optimize a protocol to efficiently isolate mast cells from human lung tissues.

Paper II – To study human lung mast cells on a single cell level using RNA sequencing platform.

Paper III – To determine the production of PGD_2 by human ILC2 and to investigate their endogenous effects on ILC2.

Paper IV – To elucidate the role of PGE_2 in human ILC2 regulation.

4 RESULTS AND DISCUSSION

4.1 PAPER I: AN OPTIMIZED PROTOCOL FOR THE ISOLATION AND FUNCTION ANALYSIS OF HUMAN LUNG MAST CELL

The aim of this study was to optimize a protocol to isolate mast cells from human lung tissue by increasing yield and not sacrificing cell viability. Modern day techniques such as flow cytometry, RNA sequencing at single cell level can be used to study cells, including mast cells, on a highly detailed level. This will enable us to understand the biology of these cell types and unravel their physiological and pathophysiological roles. However, mast cell being a highly granular tissue-resident, it is necessary to optimize a protocol to facilitate their efficient isolation from complex tissues such as human lung. In this paper, we developed a protocol named WEMP – Washing, Enzymatic digestion, Mechanical disruption and Percoll purification, where each and every step is optimized to reduce cell death and significant increase the mast cell yield. During wash, red blood cells and blood pockets in the tissue are gently removed in order not to lose the immune cells **(Paper I, Figure 1B-C, 1J-O)** and to avoid treating final cell suspension with repeated ACK lysis buffer to lyse RBC, which can affect cell viability. Increasing enzymatic digestion time can increase total number of cells isolated. Enzymatic digestion with collagenase can affect cell surface marker, which is vital for fluorescence-based cell analyses. In this protocol, we however did not change enzymatic digestion time from conventional protocol (45 minutes). We instead included an additional step called mechanical disruption, where we collect enzymatically digested tissue pieces and use a 50ml syringe to mechanically disrupt it 10 times (repeated thrice) to squeeze out tissue-resident cells such as mast cells **(Paper I, Figure 1 S-U)**. This step will not only squeeze mast cell in particular but also other tissue-bound cells such as macrophages. Also, a conventional Percoll based mast cell purification by using 70%-30% Percoll interface might not be efficient to isolate all mast cells. Therefore, we re-suspended cells from mechanical disruption in 30% Percoll, centrifuged it and isolated all immune cells along with remaining RBCs. Using this Percoll gradient, larger alveolar macrophages will be collected in the supernatant along with cell debris **(Paper I, Figure 1W).** Cells are then washed and treated with ACK lysis buffer to get rid of RBC **(Paper I, Figure 1X)**. Yield of human lung mast cell from WEMP protocol was significantly increased compared to conventional protocol (enzymatic digestion) **(Paper I, Figure 3)**. We also isolated ILC2 and compared it with conventional protocol. Percentage of ILC2 of CD45 was similar between WEMP and conventional protocols. However, increase in total number of cells isolated using WEMP

protocol means that increase in total number of ILC2. This is important as ILC2 are known to be rare in number and in *in vitro* experiments using freshly isolated ILC2 and single cell sorting, such increase will be effective. Another application of WEMP protocol is isolation of all immune cells using 30% Percoll, which enables us to isolate all immune cells including lymphocytes and granulocytes. This is particularly helpful in studying different cell types at once, as we managed to do the flowcytometric analysis and sorting on lymphocytes and granulocytes using same FACS panel. Also, more cell numbers mean that cells from such a tissue can be used for different studies and scientific collaborations at same time.

4.2 PAPER II: DECIPHERING THE HETEROGENEITY OF HUMAN LUNG MAST CELLS BY SINGLE CELL RNA SEQUENCING

The aim of this study was to characterize human lung mast cells on a more detailed level using single cell RNA sequencing platform. Human lung samples received post-surgery were processed using WEMP protocol as described in paper I. Using fluorescent-activated single cell sorting, mast cells, ILC and NK cells were sorted in 96 well plates containing lysis buffer **(Paper II, Figure 1).** Following the quality control analyses, sequencing of the sorted cell populations was carried out by SciLifeLab Eukaryotic Single-cell Genomics Facility, Stockholm, and delivered to 183 Uppmax c2016002. Principle component analyses of the sequencing data showed three different sorted populations – mast cells, ILC and NK cells. Further results of the analyses are summarized with a particular focus on mast cells as this is the aim of our study. Primarily, the expression of key mast cell markers such as CD117/KIT, IgE receptor FcεRI, IL-33 receptor IL1RL1, tryptase, chymase, carboxypeptidase, histidine carboxylase, prostaglandin synthase and arachidonate-5-lipoxygenase i.e., 5-lipoxygenase, were confirmed from the sequencing data, which as validates the single cell sort **(Paper II, Figure 2A-2D).** Key observation here was that not all mast cells expressed chymase. This data is cohesive with the fact that chymase is generally not expressed by mucosal mast cells [146].

Following this, top 50 highly expressed genes were found that made up to 24% of totally gene expressed. Well studied mast cell markers observed among the highly expressed genes were tyrosine kinase receptor KIT, tryptase genes TPSAB1 and TPSAB2, IL-33 receptor and GATA2 transcription factor.

We then set out to decipher the highly variable genes (HVG) within the sorted cell population as shown in the violin plots **(paper II, Figure 3A).** Elucidation of HVG expression remains as a basic aspect of this kind of studies as it helps us identify subpopulations within a cell type and therefore unravel their biological functions. Recognizing subsets within a cell

population and understanding their roles *in vivo* can serve as a basis for being able to specifically target them in various treatment strategies. Among the identified HVG from the sequencing data, cathepsin G and prostaglandin endoperoxide synthase 2 (PTGS2) were well-known mast cell markers [147, 148]. Cathepsin G has been shown to be generally expressed in the mast cell subset MC_{TC}. PTGS2 is a main enzyme that catalyses prostaglandin biosynthesis. Dual specificity phosphatases (DUSP6) is a MAP kinase phosphatase that has main role in mast cell stimulation during PI3K pathway [149]. Among the other identified HVG were TSC22D1, ZNF331, CD83, MAOB, SGK1, SLC43A3.

Hierarchical clustering of batch-corrected log- HVG expression were used to cluster them into putative sub-populations. This analysis did not indicate the presence of subpopulation. The results from t-SNE plots were also consistent with this.

Top 50 abundant genes from the sequencing data was then looked at. Among them were RSG1 and RSG2 – regulators of mast cell signalling in inflammation, FOS – regulators of mast cell degranulation, SRGN – hematopoietic core glycoprotein critical in production and storage of mast cell proteases, HDC – histidine decarboxylase in histamine formation and thereby mediating allergic diseases. Preliminary pathway analyses based on the above observations was carried out using enricher. Gene expression profile noted from these results followed by pathway analysis confirmed the involvement of mast cells in TNF, PI3K and MAPK pathways.

Such thorough in-depth studies form the basis for better understanding of mast cell functions and building a targeted approach for the development of new therapeutic strategies.

4.3 PAPER III: CYTOKINE-INDUCED ENDOGENOUS PRODUCTION OF PGD2 IS ESSENTIAL FOR HUMAN ILC2 ACTIVATION

ILC2 have long been known to be activated by epithelial derived cytokines IL-33, IL-25 and TSLP to produced their signature type 2 cytokines IL-5 and IL-13[150]. Although ILC2 can be activated by each one of these epithelial cytokines individually, their synergistic effect leads to a very potent ILC2 activation [151]. This highly potent type 2 cytokine producing effect of ILC2 is not only significant in the induction of type 2 immune responses, but also in maintaining them during inflammatory conditions such as allergy and asthma[152]. In addition to the above mentioned epithelial derived cytokines, PGD_2 was also shown to be important for ILC2 activation, chemotaxis and proliferation [73, 115, 116]. Therefore, in this study our aim was to determine the possibility of PGD production by human ILC2 and its role in activation, proliferation and regulation of ILC2 functions.

Cells suspensions isolated from tonsil tissue were used to sort ILC2 by FACS. Sorted ILC2s were expanded in Yssel's medium in the presence of IL-2 and IL4. After 2 weeks of expansion, ILC2 phenotype was confirmed by flow cytometry analysis.

Our first goal was to investigate the effect of COX1/2 inhibition in ILC2 activation. For this, ILC2 were pre-treated for 40 minutes with flurbiprofen, a COX1/2 inhibitor and incubated with IL-2, IL-33, IL-25 and TSLP. Type 2 cytokines, IL-5 and IL-13, production was observed in the presence of IL-33, IL-25 and TSLP (along with IL-2). No cytokine production was observed in non-stimulated condition (only IL-2). However, IL-5 and IL-13 production by ILC2 were shown to be inhibited following flurbiprofen pre-treatment. Both IL-5 and IL-13 release and production per cell analysed through ELISA and intracellular flow cytometry respectively showed that COX1/2 is required for ILC2 activation. Also, transcription factor GATA3, previously shown to be important for ILC2 activation, was upregulated following IL-33, IL-25 and TSLP stimulation. However, COX1/2 inhibition reduced GATA3 upregulation **(Paper III Figure 1)**. Above data suggest COX1/2 blockade inhibited the prostaglandin production, which might be critical for ILC2 activation.

To confirm and investigate further, supernatant from non-stimulated ILC2 cultures, cytokine stimulated ILC2 cultures in presence or absence of flurbiprofen pre-treatment were analysed by high sensitivity mass spectrometry. Results from this analysis revealed presence of PGD_2 and its metabolites PGJ_2, delta-12-PGJ_2 (Δ^{12}-PGJ_2) and 13, 14 dihydro-15-keto PGD_2 in supernatants of cytokine stimulated ILC2. However, supernatant from non-stimulated ILC2 did not contain lipid mediator and the flurbiprofen pre-treatment inhibited release of lipid mediators by cytokine stimulation **(Paper III, Figure 2).**

Upon confirming endogenous production of PGD_2 and its metabolites by ILC2, our next aim was to look into effect of endogenous PGD_2 on ILC2 activation. To investigate this, we aimed at inhibiting PGD_2 release by blocking HPGDS inhibitor (KMN698) and look at its effect in ILC2 activation. As expected, blocking HPGDS inhibited IL-5 and IL-13 release and also reduced the cytokine-induced CD25 expression **(Paper III, Figure 4)**. In the next experiment, we used a selective CRTH2 antagonist (CAY10471) to block CRTH2 receptor. Expanded ILC2 were pre-treated with the CRTH2 antagonist for 20 minutes and stimulated with IL-33, IL-25 and TSLP. CRTH2 antagonist pre-treatment inhibited IL-5 and IL-13 production by ILC2 **(Paper III, Figure 5)**. Inhibition of IL-5 and IL-13 production from activated ILC2 by blocking COX2, HPGDS and CRTH2 suggests that endogenously produced PGD_2 by ILC2 upon activation is required for GATA3 upregulation and ILC2 activation.

It was previously shown that PGD_2 produced by mast cell is important for ILC2 activation. In this study we showed that ILC2 can endogenously produce PGD_2 and its metabolites upon activation. However, it is noteworthy that the amount of PGD_2 produced from ILC2 is much less when compared to mast cells. This finding is crucial as CRTH2 blocking can be a potent therapeutic target for ILC2 inhibition.

4.4 PAPER IV: PROSTAGLANDIN E2 SUPPRESSES IL-5 AND IL-13 PRODUCTION IN HUMAN TONSIL ILC2

In Paper IV, we investigate the effect of another prostaglandin, PGE_2, in human ILC2 function. Taken into account the abundance of PGE_2 in lung tissue and the roles of ILC2 in asthma conditions, it is worthwhile to investigate the PGE_2 effects on ILC2 [120, 121]. This hypothesis is also supported by the fact that PGE_2 has suppressive effects on Th2 cells, macrophages, mast cells and neutrophils [123-127].

Similar to paper III, ILC2 were sorted from human tonsil tissue using flow cytometry-based sorting. These sorted ILC2 were expanded for 2 weeks in Yssel's medium with IL-2, IL-4 and irradiated feeder cells for 2 weeks. Phenotype of expanded ILC2s were confirmed by flow cytometric analysis.

Expanded tonsil ILC2, in the presence of IL-2, were stimulated with IL-33, IL-25 and TSLP in presence or absence of PGE_2. IL-33, IL-25 and TSLP combination resulted in IL15 and IL-13 release, which was analysed by ELISA. PGE_2 (30nM) was shown to inhibit IL-5 and IL-13 release. However, PGE_2 had no effect on ILC2 cell viability and cell proliferation after 24 hours. Further intracellular IL-5 and IL-13 production in ILC2 were analysed by flowcytometry **(Paper IV, Figure 1)**. These results confirmed PGE_2 inhibitory effect of IL-5 and IL-13 on per cell basis.

As production of IL-5 and IL-13 was inhibited, it was interesting to measure the effect on transcription factor GATA3, which is required for ILC2s cytokine production. As already shown in other studies, high intracellular GATA3 protein expression was high in non-stimulated ILC2 and this was increased by cytokine stimulation[69]. Intracellular GATA3 expression was significantly lowered in presence of PGE_2. CD25, a receptor for IL-2, is also important for ILC2 co-stimulation. CD25 expression was upregulated following 24-hour cytokine stimulation and presence of PGE_2 was shown to significantly lower CD25 expression **(Paper IV, Figure 2A-C)**.

From the above results, it is understood that PGE_2 effect on GATA3 and CD25 expression are part of a mechanism supporting IL-5 and IL-13 production in ILC2.

Our next aim was to investigate long lasting effect of PGE_2 on ILC2. To study this effect, we incubated the expanded ILC2 in the above-mentioned treatment conditions for 72 hours. Compared to 24-hour time point ILC2 produced higher amount of IL-5 and IL-13 after 72-hour incubation period. This IL-5 and IL-13 production was significantly lowered in the presence of PGE_2 after 72-hour time point **(Paper IV, Figure 3A-B)**. Also, GATA3 expression and CD25 expression were low in the presence of PGE_2 at 72-hour time point. PGE_2 effect on CD25 expression was much stronger at the 72-hour time point when compared to 24-hour time **(Paper IV, Figure 2D-F).** This raised the question on the long lasting PGE_2 effect on ILC2 proliferation as IL-2-CD25 interaction is important for proliferation. To answer this question, we used cell proliferation assay – cell trace violet dye at 72-hour time point.

Cells were loaded with dye before treatment condition, as cells divide fluorescence of dye in cells diluted depending on number of cell division undergone. Cytokine stimulation of ILC2 induced cell proliferation, which also could be attributed to increase in IL-5 and IL-13 production compared to 24-hour time point. However, we observed the inhibitory effect on ILC2 proliferation in presence of PGE_2 and most of the cells failed to undergo second cycle cell division **(Paper IV, Figure 2G-I)**. This further confirms that PGE_2 effect on per cell basis in reducing GATA3 and CD25 expression in ILC2.

Importantly, similar inhibitory effect of PGE_2 was observed in freshly sorted ILC2 from tonsil and buffy coat. Microscopic image of 5-day treatment condition of freshly sorted Tonsil and buffy coat ILC2 showed lower number of cells in presence of PGE_2. In addition to this, reduced CD25 expression of ILC2 in presence of PGE_2 confirmed PGE_2 effect in ILC2 proliferation **(Paper IV, Figure 4)**.

Next, we investigated the mode of action of PGE_2. PGE_2 is known to act through 4 of its E-prostanoid (EP) receptors, EP1, EP2, EP3 and EP4. The EP2 and EP4 mRNA expression was observed in tonsil ILC2. These expression levels were significantly higher when compared to EP1 and EP3 mRNA expression. This result was consistent with the data obtained from single cell study[153] **(Paper IV, Figure 5).**

To further investigate role of EP2 and EP4 receptors in ILC2, we pre-treated expanded cells with EP2 and EP4 antagonist for 20 mins. Following this, the cells were incubated with IL-2, IL-33, IL-25 and TSLP. IL-5 and IL-13 levels in supernatant were analysed with ELISA at 24-hour time point. Partial suppression of IL-5 and IL-13 release was observed in presence of one of the EP receptor antagonists. This indeed proved that both EP receptor antagonist was necessary to completely reverse PGE_2 suppressive effect, confirming the EP2 and EP4 receptor mediated PGE_2 effect on ILC2. Similar involvement of EP2 and EP4 receptor effect

was observed in intracellular IL-5 and IL-13 production, CD25, GATA3 expression and ILC2 proliferation **(Paper IV, Figure 6).**

Comprehensively, this study showed the negative regulation of ILC2 by PGE_2 through EP2 and EP4 receptors. Adding to this effect of PGE_2 in IL-5 and IL-13 production, we also showed their inhibitory action on ILC2 proliferation. Hence, PGE_2-EP2/EP4 interaction can be a potential therapeutic target in allergy and asthma.

Future perspectives

The functions of type 2 immune responses in health and diseases are still left to be fully explored. By studying the critical roles played by mast cells and ILC2 can deepen our current knowledge about such responses. The data obtained from such studies can serve as a basis for future research.

In order to probe the mast cell biology and functions, we employed a up-to-date single cell RNA sequencing technique. The extensive information that could be obtained from such studies can potentially put us one step ahead in fully understanding mast cells. To be able to perform an effective sequencing reaction that could yield good quality data, it was crucial for us to isolate intact mast cells from complex tissues such as the lung. Application of the WEMP protocol in human lung tissue has facilitated this and thereafter, WEMP protocol can be further optimized for the isolating and studying mast cells from other relevant tissues. It would be of great value to extend the studies by having the mast cell sequencing data as the basis. The gene expression profile of mast cells from lung can be compared with that of the other tissues such as skin to understand their tissue-specific roles.

ILC2 stand in need for various signals for their activation and regulation. ILC2 play significant roles in initiating and maintaining type 2 immune responses. Improved knowledge about the regulation of these cells is necessary for the development of novel therapeutics. Several studies are being conducted to study the lipid mediators and their effects on ILC2. The elucidation of the activating and suppressive action of two such lipid mediators, PGD_2 and PGE_2, on ILC2 from human tissues will serve as a basis for further studies. Following the observations from these studies, it will be worthwhile to investigate the effects of various other lipid mediators on ILC2 and also to determine their long-term effect. The prostaglandins studied here can further be exploited as a therapeutic targets by CRTH2 antagonist or EP2/4 agonist in treating inflammatory conditions due to their activating and suppressing effects.

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