Mapping intracellular cytokine pools in granulocytes: Dynamic storage and trafficking sites of interleukin-9 and interleukin-13 in human eosinophils.

by

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Abstract

Eosinophils are immune cells with constantly evolving roles in both innate and adaptive immunity and have long been associated with the predominant pathology of the human airways, asthma. Eosinophils are specialized in their granulocytic nature, possessing several membrane-bound secretory organelles that store potent immunoregulatory mediators that can be rapidly and differentially released in the presence of an inflammatory stimulus. Among this arsenal of immunomodulatory factors are T helper 2 (Th2) cytokines that include interleukins (IL-) 4, 5, 9, and 13 which are pivotal proteins involved in immune cell crosstalk and pro-inflammation associated with asthma pathogenesis. While the pathobiological actions of eosinophils and their derived Th2 cytokines in asthma are increasingly appreciated and have translated to becoming approved therapeutic targets of asthma, the modes in which these cytokines, particularly IL-9 and IL-13, are delivered to the extracellular environment is not well known. In this study, I aimed to identify whether the secretory organelles crystalloid granules or recycling endosomes (REs) found in human blood eosinophils were associated with trafficking IL-9 and IL-13 in the presence of the inflammatory stimulus platelet activating factor (PAF). I hypothesize that REs act as a novel trafficking site for IL-9 and IL-13 upon stimulation in human eosinophils as REs have been implicated in exocytic transport of newly synthesized cytokines in different immune cells. Furthermore, crystalloid granules would mainly act as a storage site for these cytokines that could later be subsequently released. Human eosinophils were double-stained for IL-9 or IL-13 and transferrin receptor (TfnRc) as a marker of endosomal compartments or CD63 as a marker of crystalloid granules. Cells were imaged using super-resolution microscopy and the degree of colocalization between cytokines and secretory organelles of interest was measured with Pearson's

correlation coefficient and an object-based colocalization protocol. Intracellular cytokine levels were measured as mean immunofluorescence intensity values. Increases or decreases in the degree of colocalization between cytokines and secretory organelles of interest is suggestive of cytokine trafficking into or out of these membrane-bound compartments, respectively. Meanwhile elevated or reduced levels of intracellular cytokine following 60 min of PAF stimulation would be suggestive of *de novo* protein synthesis or cytokine secretion, respectively. In this study, IL-9 immunofluorescence intensity significantly increased upon PAF stimulation. Similarly, IL-9 colocalization with TfnRc significantly increased upon PAF stimulation while colocalization between IL-9 and CD63 transiently increased at 5 and 15 min of PAF stimulation prior to returning to baseline levels. Furthermore, 3.5 times the amount of immunolabelled IL-9 was found in $TfnRc^+$ compartments compared to $CD63^+$ granules in resting eosinophils. This data suggests for the first time that $TfnRc^+$ endosomes are important trafficking sites of IL-9 in human eosinophils. It is also suggested that PAF induces *de novo* IL-9 synthesis and this newly made IL-9 is shuttled into $TfnRc^+$ endosomes for release at the cell surface. Some newly made IL-9 may also be shuttled into $CD63^+$ granules for storage and release. In contrast, both IL-13 colocalization with CD63 and IL-13 immunofluorescence intensity decreased upon PAF stimulation while IL-13 colocalization with TfnRc transiently increased at 5 min of PAF stimulation. This data suggests IL-13 is being shuttled out of $CD63^+$ crystalloid granules for cytokine secretion and is predominantly preformed in these granules. Small amounts of IL-13 may also be shuttled into $TfnRc^+$ compartments upon PAF stimulation as a static pool of intracellular IL-13 poised for release. Overall, we identified $TfnRc^+$ endosomes in human eosinophils as a novel site for IL-9 and IL-13 trafficking. These novel trafficking sites provide potential therapeutic targets for asthma that aim to dampen the pro-inflammatory effects of eosinophils and Th2 cytokines in this disease.

Preface

The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name "Molecular Mechanisms Regulating Leukocyte Activation", Pro00000942, 2020.

Literature review and figures presented in Chapter 1 are my original work, as well as data analysis presented with figures found in Chapter 4.

Portions of research presented in Chapters 2 and 3 have been published as a collaborative work by S Almas, N Fayad, O Srivastava, M Siddique, N Touret, and P Lacy in the brief conclusive report "Cytokine trafficking of interleukin-9 and interleukin-13 through TfnRc^{+} vesicles in activated human eosinophils" in the Journal of Leukocyte Biology (2020). I contributed towards original and revised manuscript composition as well as data collection and analysis. I performed tasks involving blood donor recruitment, blood collection, cell isolation, cell imaging, image processing and statistical analysis. I was responsible for collecting the data of 6 blood donors presented in Figure 3.6 and performed statistical analyses for the whole donor dataset. S Almas was responsible for collecting the data of 8 blood donors presented in both Figures 3.5 and 3.6. I also prepared Figures 3.1, 3.2, 3.3, and 3.4 from raw cell images collected by S Almas. I developed the object-based colocalization method together with Dr. X Sun presented in Chapter 2 and Figure 2.2. I collected and analyzed data from this method for Figures 3.7 and 3.8 on raw cell images collected by S Almas. N Touret and S Almas contributed to designing the experimental method in Figure 2.1. O Srivastava contributed to revising the manuscript text. M Siddique aided in panel size formatting for Figures 3.1-3.4. P Lacy was the supervisory author and was involved with concept formation and manuscript composition.

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Abbreviations

ASM	Airway smooth muscle
c/EBP	CCAT-enhancer binding protein
CBN	Cholesterol butyrate nanoparticles
CCL	C-C motif chemokine ligand
CCR3	C-C motif chemokine receptor 3
ECP	Eosinophil cationic protein
EDN	Eosinophil-derived neurotoxin
EoSV	Eosinophil sombrero vesicle
EPX	Eosinophil peroxidase
ER	Endoplasmic reticulum
FDA	Food and Drug Administration
GATA	GATA-binding protein
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte-macrophage colony stimulating factor
GTPase	Guanosine triphosphatase
ICS	Inhaled corticosteroid
IFN-γ	Interferon-γ
IL	Interleukin
IL-13Rα1/2	Interleukin-13 receptor α1/2 subunit
IL-4Rα	Interleukin-4 receptor α subunit
JAK	Janus family of protein kinases

LABA Long-acting β_2-agonist

LPS Lipopolysaccharide

mAb Monoclonal antibody

MBP Major basic protein

NK cell Natural killer cell

PAF Platelet activating factor

PFA Paraformaldehyde

Q-/R-SNARE Glutamine-/Arginine-SNARE

RANTES Regulated upon activation, normal T cell expressed and secreted

RE Recycling endosome

SNARE Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor

STAT Signal transducer and activator of transcription

Tfn Transferrin

TfnRc Transferrin receptor

TGF-β Transforming growth factor-β

Th2 T helper 2

TNF Tumor necrosis factor

v-/t-SNARE Vesicle-/target-SNARE

VAMP Vesicle-associated membrane protein

Vti1b Vesicle transport through interaction with t-SNAREs homolog 1B

γc Common γ chain receptor subunit

Chapter 1:

Introduction

1.1.1 Eosinophils in inflammation and immunity

Since being first described by Paul Ehrlich in 1879, the role eosinophils play in the immune response has been a consistently evolving field of research. Eponymously, eosinophils are cells stained by the acidophilic dye, eosin, and belong to a subset of granule-containing immune cells, termed granulocytes, along with neutrophils and basophils. Early findings of elevated circulating eosinophil levels in individuals with helminthic infections, allergic skin reactions, and asthma implicated their role in antiparasitic defense and inflammation.[1] It is now appreciated, with their high degree of intracellular compartmentalization, eosinophils synthesize and store a plethora of mediators involved in antimicrobial defense (not limited to helminths) as well as immunoregulation.[2,3] Specifically, released cationic proteins from granules have cytotoxic effects on bacteria, viruses, and parasites, while released cytokines and chemokines allow for immune cell crosstalk leading to cell survival, activation, and recruitment.[4] Aside from producing and rapidly releasing these effector molecules, eosinophils express pattern recognition receptors (PRRs) for the surveillance of pathogenic microbes and can phagocytose foreign agents.[5,6] Furthermore, eosinophils present antigens to T lymphocytes on major histocompatibility complex (MHC) class II, with the ability to polarize adaptive immune responses towards a T helper 2 (Th2) response.[7,8]

1.1.2 Eosinophil development, differentiation, and displacement

Through an interplay of transcription factors and cytokine signaling, pluripotent haematopoietic stem cells in the bone marrow are instructed to follow the myeloid cell lineage and become a differentiated eosinophil. Transcription factors PU.1, CCAT-enhancer binding protein (c/EBP) members (c/EBPα, β, ε), and, most notably, GATA-1 have been cited as critical in the development of mature eosinophils.[9–11] Meanwhile, cytokines including granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-3, and IL-5 are implicated in the differentiation and maturation of eosinophils.[12] Once fully differentiated, eosinophils exit the bone marrow and shortly enter circulation, making up less then 5% of circulating leukocytes. Under non-inflammatory conditions, eosinophils reside in the thymus, gastrointestinal tract, uterus, and mammary gland.[13–16] Commonly, upon airway inflammation, eosinophils migrate to the lungs and are highly responsive to chemoattractants such as eotaxin-1, -2, and -3 (also known as C-C motif chemokine ligand (CCL)11, CCL24, and CCL26, respectively), macrophage inflammatory protein (MIP)-1α, and regulated upon activation, normal T cell expressed and secreted (RANTES or

CCL5).[17] Here in inflamed tissues, the cytokine and chemokine environment prolong eosinophil survival while promoting cell activation, where cells live for 2-5 day compared to its 18 hour half-life in circulation.[18]

1.1.3 Intracellular complexities of an eosinophil: crystalloid granules and their contents

Human eosinophils exhibit a unique cellular morphology. Possessing bilobed nuclei and multiple secretory organelles, including primary granules, secretory vesicles, eosinophil sombrero vesicles (EoSVs), and crystalloid granules (also termed secondary or specific granules), crystalloid granules are arguably the noteworthiest intracellular trait of eosinophils **(Figure 1.1)**.[19] Given their name from observations with transmission electron microscopy (TEM), these large granules (greater than 0.5 micrometers in diameter) contain a crystalline core consisting of major basic protein 1 (MBP-1) surrounded by a lesser stained matrix.[19–21] In addition to MBP-1, eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN) and eosinophil peroxidase (EPX) are housed in crystalloid granules, with these cytotoxic basic proteins attributing to the cell's acidophilic staining with eosin.[22] Preformed pools of cytokines, including tumor necrosis factor (TNF), interferon-γ (IFN-γ), IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 and IL-13, are also found in crystalloid granules for rapid release upon cell stimulation.[23–29] To note, IL-13 is the most abundant preformed cytokine found within eosinophils.[23] Preformed chemokines CCL5/RANTES and CCL11/eotaxin are also housed within crystalloid granules.[30,31] Moreover, crystalloid granule membranes are decorated with transmembrane proteins including cytokine and chemokine receptors and CD63, a tetraspanin associated with selective mediator release upon eosinophil stimulation which moves from the granular membrane to the cell periphery and cell surface.[32–34] To add an additional level of complexity, vesiculotubular membranous structures, which eventually give rise to cytoplasmic EoSVs, exist within crystalloid granules of activated human eosinophils to further compartmentalize cargo for eventual release.[35–38]

1.1.4 Eosinophil degranulation: modes of stored mediator release

Given the arsenal of secretory organelles they house, several modes for the rapid release of stored mediators exist **(Figure 1.2)**.

1.1.4.1 Classical and compound exocytosis

In classical exocytosis, the entirety of granular content is released by fusion of the granule membrane with the cell membrane; scenarios in which granule-granule fusions occur

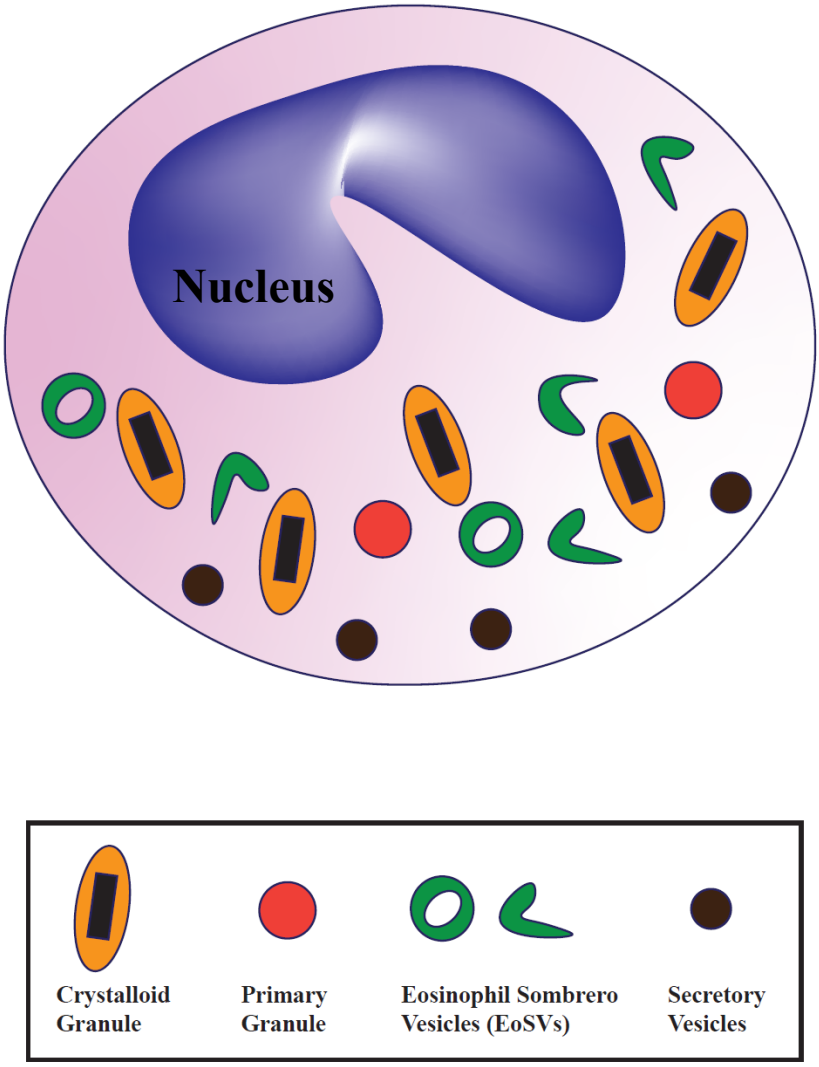


Figure 1.1. Eosinophil secretory organelles. As a granule-containing immune cell, eosinophils possess several membrane-bound compartments capable of storing and secreting preformed immunoregulatory mediators. Under TEM, eosinophil crystalloid granules are most easily identified based on their electron-dense crystalline core. Primary granules lack this electron-dense labelling. Additionally, eosinophils possess highly mobilizable secretory vesicles and circular or C-shaped eosinophil sombrero vesicles (EoSVs) that bud off eosinophil crystalloid granules for selective mediator release.

prior to release of all granular cargo is termed compound exocytosis.[19,22,39] While relatively uncommon as a mode of mediator release in this cell type, eosinophils undergo classical and compound exocytosis upon interactions with helminth parasites that have been coated with immunoglobulin (Ig) A or IgG through Fc receptor activation in vitro.[40,41]

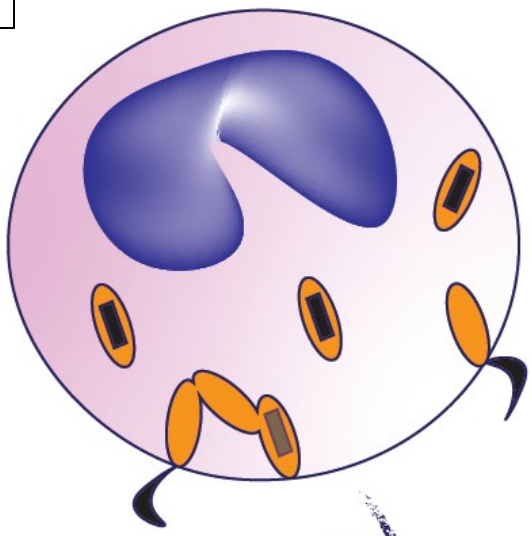
1.1.4.2 Cytolysis

Human eosinophils can also release intact granules, that are then competent of selective secretion of preformed mediators, through a process called cytolysis.[42,43] During cytolysis, nuclear DNA is de-condensed, the permeability of nuclear and cell membranes are compromised, and both nuclear DNA and intact granules are extruded from the cell.[44,45] Released DNA and granule contents may go onto form eosinophil extracellular traps (EETs) that assist in the entrapment and killing of microbes.[46] Meanwhile, the presence of membrane-bound free extracellular eosinophils granules (FEGs) has been documented in vivo in many cases of respiratory diseases, such as asthma and allergic rhinitis.[44,45,47–53] By bearing chemokine receptors like C-C motif chemokine receptor 3 (CCR3) and cytokine receptors like IFN-γ receptor α-chain (IFNGR1), these FEGs are responsive to ligands CCL5 and CCL11 or IFN- γ, respectively, and selectively release cargo based on the stimulant.[42] Specifically, in response to IFN-γ, FEGs have been reported to release IL-4 and IL-6 as opposed to preformed IL-10, IL-12, and IL-13 found within them.[42]

1.1.4.3 Piecemeal degranulation

Another frequent mode of degranulation in human eosinophils relies on the formation of secretory vesicles and EoSVs within crystalloid granules and subsequent movement of these vesicles to the cytoplasm and towards the cell membrane.[35,36,38] This process in which secretory vesicles mobilize portions of granular contents for release whilst preserving the integrity of crystalloid granules is termed piecemeal degranulation.[54,55] Recruitment of cytokine and chemokine receptors to crystalloid granule membranes has been a suggested mechanism for the sorting of soluble mediators to be released.[56,57] A well described example of piecemeal delivery and receptor-sorting involves release of IL-4 in human eosinophils stimulated with CCL11.[57] Upon stimulation, IL-4 receptor subunit α (IL-4Rα) is enriched within eosinophil granule membranes to bind IL-4 and subsequently extrudes this cytokine from granules, emerging as IL-4Rα and IL-4 bearing cytoplasmic secretory vesicles.[57] Intragranular MBP is also released by piecemeal degranulation upon CCL11 stimulation.[35] In addition to CCL11, within 1 h, piecemeal degranulation can be

A



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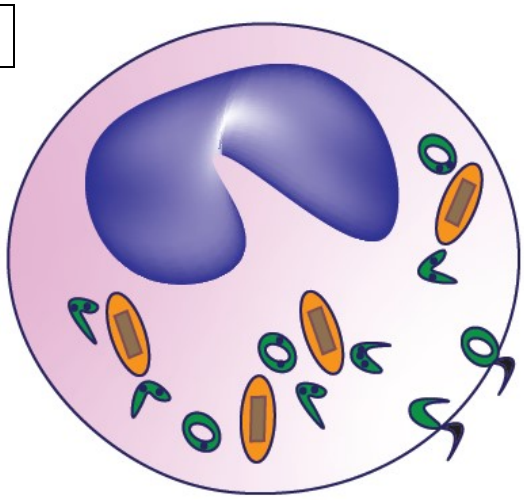


Figure 1.2. Modes of eosinophil mediator release. Eosinophil secretory organelles store a multitude of immunomodulatory mediators and release these mediators through several modes, often based on the nature and recurrence of stimuli. The entirety of a granule's contents or the contents of multiple granules which fused together can be released by membrane fusion with the plasma membrane in classical exocytosis and compound exocytosis, respectively (granules are orange with contents in black, A). Cells with compromised nuclear and plasma membranes can extrude chromatin (purple streaks) and intact granules, which are later competent in releasing granular contents, during cytolysis (B). Lastly, select portions of granular content can be released via eosinophil sombrero vesicles (green) during piecemeal degranulation (C).

observed in human eosinophils responding to CCL5 and the lipid mediator platelet activating factor (PAF).[36]

1.1.5 Eosinophils in asthma

As mentioned prior, eosinophils are present at relatively low levels in circulation and typically reside in the thymus, gut tissue, uterus, and mammary glands.[13–16] Exceptionally, eosinophil counts in peripheral blood and lung tissue are elevated in asthmatics and are linked to disease severity.[58–67] Differential white blood cell counts of greater than 3% eosinophils in sputum and peripheral blood have been reported as cut-off values for eosinophilia in asthma.[68] In the simplest sense, asthma is defined as airway inflammation that clinically presents with decreased lung function (i.e. limited expiratory airflow with reversible bronchoconstriction), airway remodeling and hyperresponsiveness, and enhanced mucus production.[69,70] Recently, it is appreciated that asthma is a heterogenous respiratory disease consisting of multiple phenotypes that are increasingly being defined by a common thread of pathobiology accompanied with biomarkers and foreseeable responses to therapeutics.[71] Eosinophilic asthma is arguably the most clinically important asthma phenotype, making up approximately half of severe asthma cases defined by symptoms being uncontrolled by mainstay treatments for at least a year.[69,70,72] Eosinophil crystalloid granule proteins MBP, ECP, and EPX are toxic to airway epithelium leading to overall remodeling of damaged tissue in which increasing numbers of collagen-depositing fibroblasts, airway smooth muscle (ASM) cells, and mucus-producing goblet cells effectively narrow the airways.[73–76] Eosinophil-derived transforming growth factor β (TGF-β) and lipid mediators such as cysteinyl leukotrienes further promote proliferation of fibroblasts and ASM cells, respectively.[77–82] As will be noted, eosinophil-derived PAF and a plethora of eosinophil-derived cytokines orchestrate multiple aspects of asthma pathology.

1.1.5.1 PAF in eosinophilic asthma

PAF (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a pro-inflammatory lipid mediator produced by several immune cells, including eosinophils, that advances asthma pathology.[83,84] PAF inhalation studies in humans have associated PAF with bronchoconstriction and pulmonary gas exchange impairment.[83] Mechanistically, PAF is appreciated as a potent chemoattractant of eosinophils in the airways.[85] Indeed, human eosinophils subject to PAF treatment morphologically change from round to stretched, crawling cells.[86] This shape change is due to cytoskeletal

remodeling in which globular (G-) actin reversibly transforms to filamentous (F-) actin which is enriched at edges of the cell and forms contractile stress fibers for cell motility.[86] As a link for the regulatory role PAF has on eosinophils in asthma, PAF receptor expression is elevated in peripheral blood eosinophils in atopic asthmatics compared to normal individuals.[87] Additionally, PAF acts as a secretagogue and induces the release of EPX, IL-9, IL-13, RANTES/CCL5, and eotaxin/CCL11 in human eosinophils.[88] Notably, PAF-mediated EPX and CCL5 release from human eosinophils occurs independent of interactions with PAF receptor and suggests novel receptors and signaling pathways remain to be elucidated.[88]

1.1.5.2 Th2 cytokines in eosinophilic asthma

A type 2 inflammatory response in which Th2 cells produce cytokines IL-4, IL-5, IL-9, and IL-13 is implicated in eosinophilic asthma **(Figure 1.3)**.[69,70,89] Critically, eosinophils are additional producers of these Th2 cytokines.[23,24,27,28,38,88] Despite the prevalence of Th2 cytokines and eosinophilia in atopic or allergic asthma, pollutants and pathogens (i.e. non-allergens) can stimulate a similar inflammatory response with group 2 innate lymphoid cells (ILC2s).[90]

1.1.5.2.1 Roles of IL-4 and IL-13 in eosinophilic asthma

Both IL-4 and IL-13 elicit pro-inflammatory roles in asthma and do so with a shared cytokine receptor system. IL-4 can either signal through (i) a Type I receptor consisting of the ubiquitously-expressed IL-4Rα which binds IL-4 with high-affinity and a common γ chain (γc) subunit typically expressed on lymphocytes and myeloid cells or (ii) a Type II receptor consisting of IL-4Rα and IL-13 receptor α1 subunit (IL-13Rα1) typically expressed in myeloid cells and non-haematopoietic cells like airway epithelial cells and ASM cells.[91–94] Meanwhile, IL-13 can signal through the same Type II receptor complex as IL-4 by binding to IL-13Rα1.[91–94] To note, IL-13 can also bind IL-13Rα2, however, this receptor mainly acts as a decoy with limited signaling capabilities and generally sequesters IL-13 from signaling through its Type II receptor.[95] IL-4 or IL-13 bound receptor complexes initiate a JAK-STAT signaling pathway through IL-4Rα in which Janus family of protein kinases (Jak) phosphorylate the Signal transducer and activator of transcription 6 (Stat6) transcription factor which promotes expression of Th2-related genes.[92,94,96,97] Jak proteins and downstream transcription factors can vary between receptor complexes to convey different signals to cells.[92,94,98–100] Additionally, as mentioned, the expression profiles of γc or IL-13Rα1 subunits vary in that lymphocytes are less responsive to IL-13 compared to IL-4 as they express fewer of

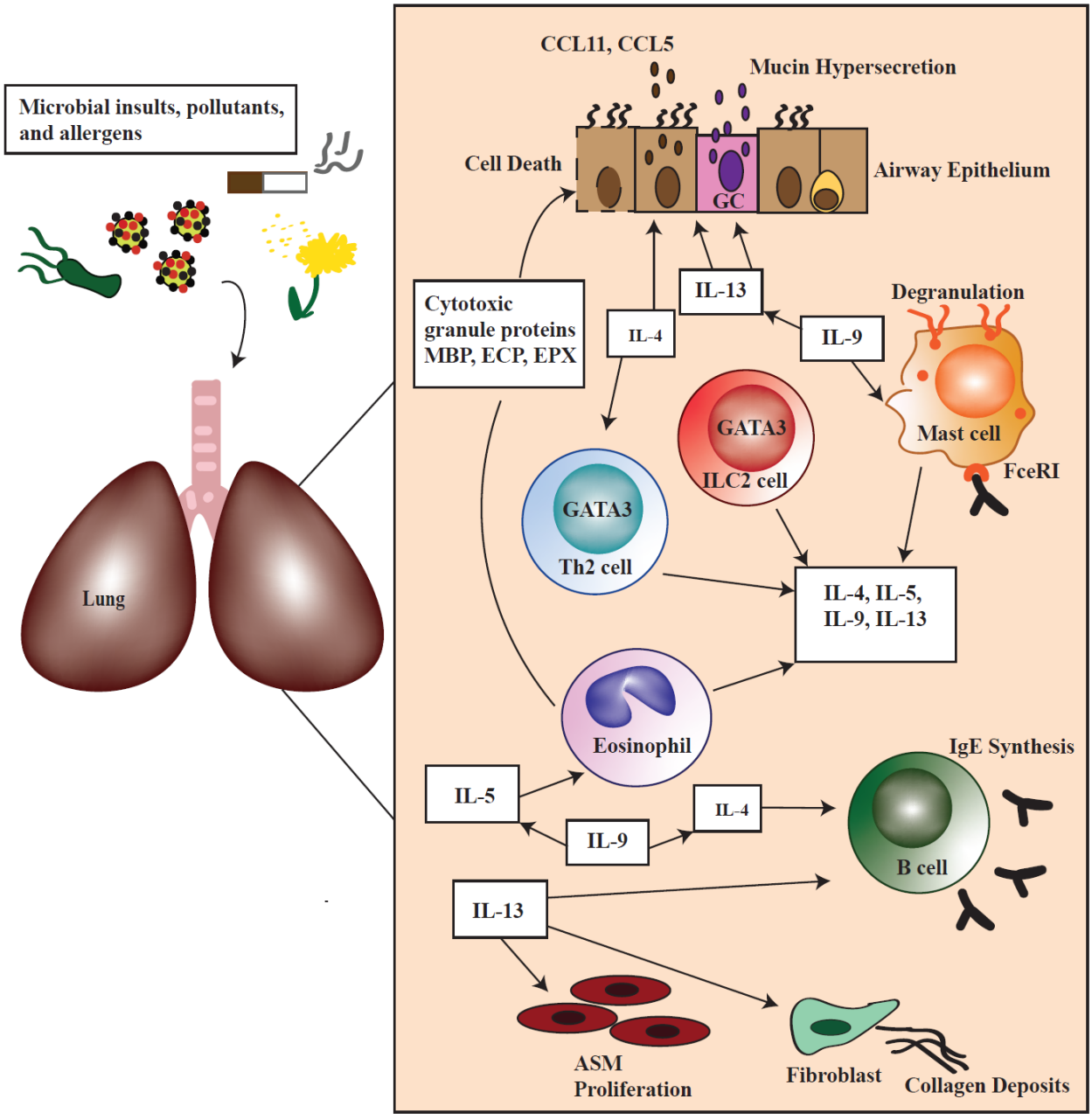

Microbial insults, pollutants, and allergens
Lung
CCL11, CCL5
Mucin Hypersecretion
Cell Death
GC
Airway Epithelium
Cytotoxic granule proteins MBP, ECP, EPX
IL-4
IL-13
IL-9
Degranulation
Mast cell
FceRI
GATA3
ILC2 cell
GATA3
Th2 cell
IL-4, IL-5, IL-9, IL-13
Eosinophil
IgE Synthesis
IL-5
IL-4
IL-9
B cell
IL-13
ASM Proliferation
Fibroblast
Collagen Deposits

Figure 1.3. Th2 cytokines in eosinophilic asthma. Microbial insults, pollutants, and allergens are all triggers of Th2 inflammation in eosinophilic asthma. Th2 and ILC2 cells share the GATA3 transcription factor which allows for expression of the pro-inflammatory cytokines IL-4, IL-5, IL-9, and IL-13. Eosinophils and mast cells additionally produce these Th2 cytokines. Both IL-4 and IL-13 enhance IgE synthesis by B cells and induce the expression of chemokines CCL11 and CCL5 by airway epithelial cells. IL-4 is also important in supporting Th2 cell differentiation. IL-13 promotes ASM proliferation, increased subepithelial collagen deposition by fibroblasts, and mucus hypersecretion by goblet cells (GCs) in the airway epithelium. IL-5 is pivotal in eosinophil differentiation, recruitment, and activation. Activated eosinophils release cytotoxic granule proteins that lead to tissue damage at the airway epithelium. IL-9 potentiates the effects of IL-4 on B cells, IL-5 effects on eosinophils, and IL-13 effects on GCs. Furthermore, IL-9 promotes mast cell proliferation and degranulation and upregulates their expression of Th2 cytokines.

IL-13Rα1 and more of γc; the opposite is true for non-haematopoietic cells.[91–94] For these reasons, IL-4 is often associated with orchestrating lymphocyte functions whereas IL-13 mainly affects airway epithelial cells, goblet cells, and ASM cells in asthma.[100–107] For example, IL-4 signaling in $CD4^+$ helper T cells leads to Stat6-mediated upregulation of the transcription factor GATA-binding protein 3 (GATA-3) which is specific to the Th2 lineage and allows for expression of IL-4, IL-5, IL-9, and IL-13.[100,101] Interestingly, chronic IL-4 signaling in combination with TGF-β induces a switch in Th2 cells in which GATA-3 expression is dampened and IL-9 expression is promoted leading to differentiation into a Th9 subset.[108] Meanwhile, Stat6-independent IL-13 signaling is implicated in airway hyperresponsiveness in asthma.[99] IL-13 further acts in all aspects of airway remodeling by promoting ASM cell proliferation, goblet cell proliferation and transformation to an increased mucus-producing state, and conversion of fibroblasts to an increased collagen-depositing state.[109–112] Certainly, there are many instances in which IL-4 and IL-13 mediate shared roles as in the promotion of IgE production by B cells which is a hallmark of an allergic response.[113–117] Increased IgE synthesis enhances expression of FcεRI receptors which bind IgE with high affinity and are often found on the surface of mast cells, an immune cell attributed to initial allergic asthma symptoms.[118,119] Once IgE/FcεRI complexes bind allergens and become crosslinked for activation, mast cells release preformed mediators that induce bronchoconstriction, vasodilation, and mucus production.[120] Lastly, both IL-4 and IL-13 are involved in recruitment of eosinophils to the lungs by upregulating the expression of chemokines CCL11 and CCL5 in airway epithelial cells and airway fibroblasts and inducing expression of vascular cell adhesion molecule-1 (VCAM-1) in endothelial cells to which integrin α4β1 on the surface of eosinophils binds to for initiating the movement of these cells from circulation and into tissue.[112,121–131]

1.1.5.2.2 IL-5 in eosinophilic asthma

IL-5 is exquisitely involved in promoting all effector functions of an eosinophil, consequently connecting this cytokine to the pathological burden of eosinophils in asthma.[132] Notably, IL-5 levels are elevated in both sputum and serum samples of individuals with severe asthma, implicating elevated IL-5 in disease severity.[133–135] These heightened concentrations of IL-5 increasingly instruct pluripotent haematopoietic stem cells in the bone marrow to differentiate into eosinophils, effectively resulting in eosinophilia.[133,136,137] IL-5 additionally contributes to eosinophil adhesion to the endothelium via activation of eosinophil cell surface integrins which

allow binding to intracellular adhesion molecule 1 (ICAM-1) and eventually lead to the cells exit from blood and into airways.[138–140] Once these cells have infiltrated the airways, IL-5 signaling also induces transcriptional changes that promote eosinophil proliferation, prolong survival through inhibition of programmed cell death, and induce activation with the onset of degranulation.[141–144] In order to convey these transcriptional changes to eosinophils, IL-5 binds to its receptor complex through an IL-5Rα subunit highly expressed on the surface of eosinophils that then joins with a βc subunit to initiate JAK-STAT and alternative signaling pathways.[145]

1.1.5.2.3 IL-9 in eosinophilic asthma

Despite being lesser studied, IL-9 has been linked to allergic inflammation that often accompanies eosinophilic asthma.[146,147] In fact, asthmatic airways have elevated IL-9 expression as well as heightened expression of its receptor, IL-9Rα, when compared to the lungs of healthy individuals.[148–150] In ASM cells, IL-9 signals through IL-9Rα to initiate a Jak signaling cascade that activates STAT3 and leads to upregulation of CCL11.[151] This elevated CCL11 by ASM cells enhances the recruitment of eosinophils to the lungs. [151] IL-9 also promotes the expansion of mast cells and upregulates their expression of mast cell proteases that mediate tissue injury in early phases of allergic inflammation in atopic asthma.[152–154] IL-9 further upregulates expression of IL-4, IL-5, IL-13, and IL-9 itself in these mast cells.[152,154] With regards to the airway epithelium, IL-9 directly upregulates expression of mucin genes leading to overproduction of mucus that further narrows the inflamed airways of asthmatic individuals.[155–158] IL-9 also indirectly influences airway remodeling by upregulating the production of IL-13 in hematopoietic cells.[159] Similarly, studies have outlined IL-9 as an enhancer of IL-4 and IL-5 effects.[160–162] In synergy, IL-9 potentiates IL-4-mediated IgE synthesis from human B cells.[160,161] Moreover, IL-9 enhances expression of IL-5Rα on early precursor cells of eosinophils such that they readily differentiate into mature eosinophils in response to elevated IL-5 levels observed in asthma.[162]

1.1.5.3 Asthma therapeutics

The first line of treatment for asthma has long included inhaled corticosteroids (ICS) combined with long-acting β_2-agonists (LABA).[163,164] ICS have broad anti-inflammatory effects in asthmatic airways through inhibition of inflammatory transcription factors such as NF-κB, c-Jun, and Fos.[165–167] These inflammation-related transcription factors are involved in upregulation of all Th2 cytokines and their receptors, chemokines such as CCL11 and CCL5, and cell adhesion molecules

like ICAM-1 and VCAM-1.[165–167] LABA are added onto ICS treatments when patients' symptoms are inadequately controlled with ICS doses alone, accompanied with proper adherence and inhaler technique.[70,164,168] LABA primarily act as bronchodilators by countering ASM contraction through activation of their β_2 adrenoreceptor (β_2AR).[168] If the ICS and LABA mainstay treatments do not control symptoms, coexisting conditions must be considered that can range from psychosocial factors like depression to environmental factors like exposure to tobacco smoke.[70,163] Several add-on therapies exist to further aid in resolving asthmatic symptoms and include the bronchodilator tiotropium, a long-acting muscarinic antagonist (LAMA) that also antagonizes ASM contractions though their muscarinic M3 receptors, and even antimicrobials such as macrolides.[70,163,169–171] Continued poor response to these nonbiologic add-on therapies, necessitates personalized treatment of the asthma phenotype with biologics that target airway-infiltrating cell types and their associated immunomodulatory mediators that contribute to disease.[70,163,172] Biologics here refer to treatments which are composed of products made from living organisms; the use of antibodies will specifically be discussed.

1.1.5.3.1 Biologics for severe asthma: targeting Th2 inflammation

The first Food and Drug Administration (FDA) approved biologic for severe allergic asthma was the humanized monoclonal antibody (mAb), omalizumab.[70,172] A mAb is generated from a single clone of an antibody-producing plasma B cell and, because of this singular origin, detects a single 6-8 amino acid component of a protein termed an epitope.[173] If multiple clones of plasma B cells were involved which produce antibodies to multiple epitopes of a protein, the antibodies would be termed polyclonal antibodies (pAb). [173] Humanized mouse mAbs contain a mostly human antibody amino acid sequence but only differ in the complementarity determining regions (CDR) within the variable region of an antibody, which originate from mice that were immunized with the target protein of interest.[174] Therefore, these humanized mouse mAbs are identified as self in humans while containing a part of the molecule from mice that allows it to specifically bind a drug target.[174] Omalizumab is a humanized mouse mAb that specifically targets released IgE in severe allergic asthma patients with elevated total IgE serum levels and positive allergen testing. [175–177] Binding of soluble IgE blocks its interaction with FcεRI on the surface of mast cells and basophils and leads to downregulation of this receptor which effectively prevents the release of inflammatory mediators from these cells in allergic disease.[70,175] Omalizumab has been reported to reduce asthma

exacerbation and hospitalization rates and reduce patient dependency on ICS and β_2 agonist use.[175–177]

After 12 years of omalizumab being approved in asthma therapeutics, biologics developed to specifically treat severe eosinophilic asthma were approved by the FDA and included mepolizumab, reslizumab and benralizumab (approved 2015, 2016, and 2017, respectively).[178] Mepolizumab and reslizumab are mAbs that target and sequester IL-5, which reduces blood and sputum eosinophil levels in asthmatic patients.[179–185] Similarly, benralizumab acts to reduce blood and sputum eosinophil levels by targeting IL-5Rα and in this way prevents released IL-5 from delivering pro-survival/activation signaling through its receptor on eosinophils.[186–189] Benralizumab also promotes antibody-dependent cell-mediated cytotoxicity (ADCC) of eosinophils. During this process, benralizumab bound to IL-5Rα on the surface of eosinophils has an exposed Fc portion which can be recognized by FcγIIIa on natural killer (NK) cells.[189] This recognition leads to the death of target eosinophils through the release of proapoptotic proteins by NK cells.[189] Through treatment with mepolizumab, reslizumab, and benralizumab asthma exacerbation rates are reduced and in the case of mepolizumab and benralizumab patient dependency on oral steroid use is reduced.[179,180,182–184,186,187,190,191]

The latest biologic, approved in 2018, for eosinophilic asthma accompanied with atopy is the fully human mAb dupilumab.[172] Dupilumab binds to IL-4Rα which is shared with the Th2 cytokines IL-4 and IL-13 and blocks their respective intracellular signaling cascades that are critical to the development of asthma.[192,193] Contrary to anti-IL-5 and anti-IL5 Rα biologics, dupilumab temporarily increases blood eosinophil levels.[192,193] Despite this observation, dupilumab lowers IgE levels in eosinophilic atopic asthma, reduces exacerbation rates, increases lung function as measured with increased forced expiratory volume (FEV), and lowers patient dependency on oral steroids.[192,193]

1.1.5.3.2 Furthering personalized therapeutics in severe asthma

To reiterate, eosinophilic asthma comprises approximately half of severe asthma cases and is associated with Th2 inflammation which may or may not be accompanied with atopy.[69,70,72] As the importance of personalizing therapeutics was profoundly appreciated in the last decade, asthma therapeutics became available to target this asthma phenotype.[194] Despite these developments, all FDA approved biologics have yet to be compared to one another in terms of clinical effectiveness

in randomized clinical trials.[195] Directly switching biologic treatments has been shown to improve asthma control and patient health without adverse effects in the case of a switch from omalizumab to mepolizumab.[196] However, further complicating physicians' decisions in personalizing asthma therapeutics, a standardized time at which changing treatments should be considered in non-responding patients has yet to be set.[195] Meanwhile, the steep prices of these biologics render them not cost-effective, in which the Institute for Clinical and Economic Review (ICER) recommended a 60-80% drop in the price of all biologics to allow for cost-effectiveness as all biologics exhibited a comparable halving effect on asthma exacerbation rates.[197]

In juxtaposition, the other half of severe asthma cases are not responsive to mainstay treatments and anti-Th2 biologics and are often associated with elevated sputum neutrophils (~40-60% of white blood cells in sputum) and a Th17 response in what is termed neutrophilic asthma.[198–201] This phenotype will be discussed to appreciate an area of therapeutics left to be developed in the context of asthma.

1.1.5.3.2.1 Neutrophilic inflammation in asthma

Yet another granulocyte implicated in asthma pathogenesis, neutrophils canonically work as first-responding leukocytes that defend against microbial insults through the quick release of granular antimicrobial proteins, cytokines and chemokines, and reactive oxygen species (ROS).[202] Neutrophils are highly migratory blood cells that follow chemokine gradients, specifically those involving IL-8 (CXCL-8), to inflammatory sites in tissue.[203] Within inflamed tissue, the cytokine environment, precisely granulocyte colony-stimulating factor (G-CSF), GM-CSF, IL-8, and TNF, largely extends the lifespan of neutrophils.[18] As well as cytokines, bacterial endotoxins such as lipopolysaccharides (LPS) prolong the survival and activation of neutrophils such that they may continue their bacterial killing in the case of an infection.[18] While important in innate immunity, an extended homing and activation of neutrophils in the lungs leads to the development of asthma. In fact, excessive release of bacteria-killing neutrophil extracellular traps (NETs) consisting of chromatin and cytotoxic granular proteins potentiate tissue damage in asthma.[204,205] Neutrophil-derived proteases such as matrix metalloprotease-9 (MMP-9) further inflict damage to airway tissue leading to remodeling.[206,207] Akin to eosinophils, neutrophils also produce pro-fibrotic TGF-β leading to elevated deposition of collagen beneath the airway epithelium.[208] Smoking, obesity, and microbial infections are all linked to neutrophil infiltration in asthmatic airways.[209–212] This

neutrophilic asthma is associated with elevated levels of IL-8, GM-CSF, IL-17A, and TNF in bronchial alveolar lavage (BAL) fluid and plasma, all of which can be produced by neutrophils themselves as positive feedback for cell survival and recruitment.[213–217] Th17 inflammation also greatly contributes to neutrophilia in asthma.[218] Th17 cells are a subset of $CD4^+$ helper T cells that largely produce IL-17A.[219] IL-17A in turn induces expression of IL-8 by airway epithelial cells to recruit neutrophils to the lungs and induces G-CSF expression to promote neutrophil production in the bone marrow.[220–223]

1.1.5.3.2.2 Targeting neutrophilic asthma

The use of a class of antimicrobials, macrolides which include azithromycin and clarithromycin, have been studied for treating neutrophilic asthma, as they possess both antimicrobial and immunomodulatory activity.[224] Azithromycin reduced asthma exacerbation rates and improved quality of life in severe asthma patients.[225] Meanwhile, clarithromycin significantly reduced IL-8, MMP-9, and neutrophil elastase concentrations and neutrophil counts in the airways leading to improved quality of life in severe asthmatics.[226] A clinical trial testing a mAb against GM-CSF presented no evidence of significant improvements in asthma control or exacerbation rates.[227] Likewise, an antagonist of the CXCR2 receptor, which is stimulated by IL-8/CXCL-8, has been well-tolerated with patients but did not reduce exacerbation rates in individuals with uncontrolled neutrophilic asthma.[228,229] Notably, mAbs developed against IL-17A receptor and TNF, as well as inhibitors of TNF such as etanercept, have not displayed treatment effects in severe asthma patients; however, these studies have not yet assessed treatments based on subpopulations of severe asthma patients (i.e. asthmatics with neutrophilia versus eosinophilia or Th2 inflammation versus Th17 inflammation).[230–232]

1.2 Cytokine trafficking pathways

Evident from discussions thus far, cytokines act as soluble messenger proteins synthesized from one immune cell which have potent biological effects on other target cells.[233] Specifically, Th2 cytokines to a large degree orchestrate the development of severe eosinophilic asthma through cell-cell crosstalk.[69,70,89] To execute these cell-cell communications, cytokines first must navigate through trafficking pathways within cells for their eventual release. While the plethora of biological effects induced by Th2 cytokines is well-studied, their trafficking pathways in eosinophils remain to be elucidated.

1.2.1 The classical secretory pathway

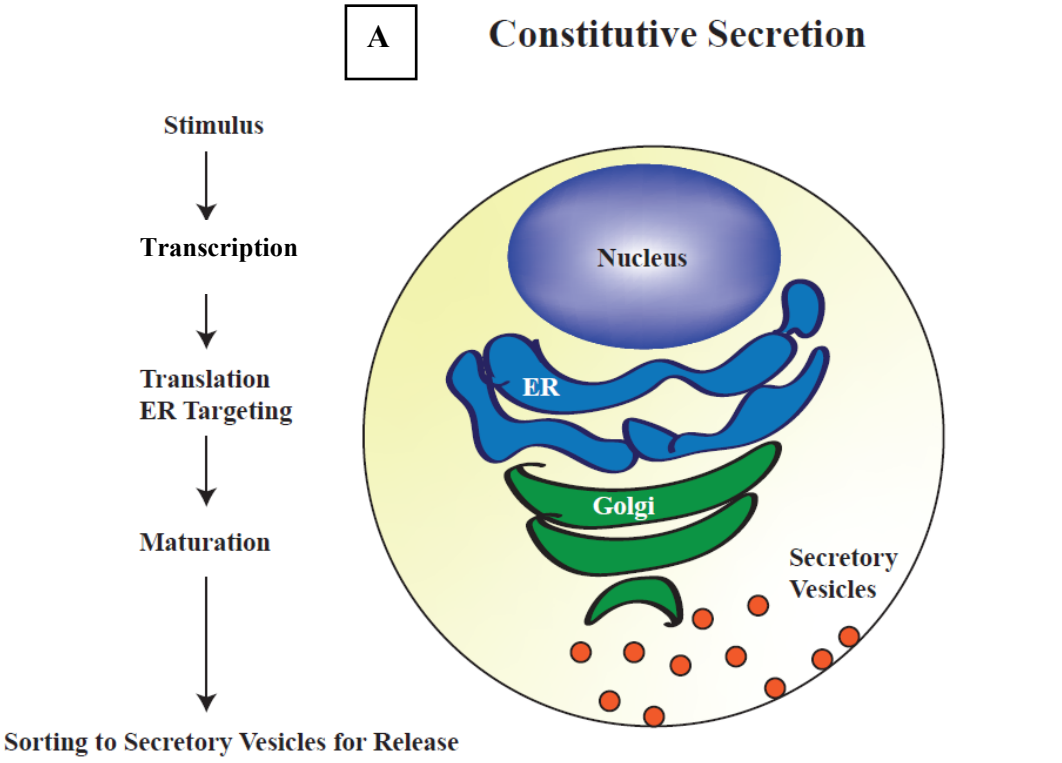
Most cytokines possess a signal sequence at their amino (N)- terminus which acts to direct cytosolic ribosomes translating nascent proteins to the rough endoplasmic reticulum (ER).[234–236] For instance, the Th2 cytokines IL-4, IL-5, IL-9, and IL-13 discussed prior all possess this signal sequence (UniProtKB: P05112, P05113, P15248, and P35225, respectively). This effectively targets these cytokines to the classical secretory pathway **(Figure 1.4)**.[234–236] In this pathway, newly made proteins in the ER lumen are sorted into transport vesicles that can fuse with other transport vesicles to form the *cis*-Golgi reticulum.[236] Coat protein complex II (COPII) are a series of cytosolic proteins that mediate both the budding of these transport vesicles and sorting of ER lumen proteins to be included for transport to the *cis*-Golgi.[237] Once cargo is delivered to the *cis*-Golgi stack, it will migrate further away from the ER to a *trans* position in which post-translational modifications of luminal proteins occur throughout this progression.[236] From the *trans*-Golgi reticulum, *de novo* proteins may be immediately released in secretory vesicles that fuse with the plasma membrane in a process called constitutive or continuous secretion **(Figure 1.4)**.[236] Constitutive secretion occurs in all cell types.[236] Meanwhile, specialized cells can sort proteins from the *trans*-Golgi network into secretory vesicles or granules for storage that will eventually be released upon an appropriate stimulus which is termed regulated secretion **(Figure 1.4)**.[236] As detailed in section 1.1.4, eosinophils are examples of these specialized cells that can house preformed cytokines in crystalloid granules prior to their regulated release in classical or compound exocytosis or piecemeal degranulation.

1.2.2 The non-classical secretory pathway

In contrast, a limited set of cytokines lack an ER signal sequence and are synthesized completely in the cell cytoplasm and include IL-1β and macrophage migration inhibitory factor (MIF).[238,239] These cytokines follow lesser-studied non-classical pathways for secretion which do not rely on the ER and Golgi network.[238–240] Involvement of protein transporters on the cell membrane, multivesicular bodies fusing with the cell surface for subsequent cytokine-containing exosome release, and cell lysis are all proposed models of non-classical secretion.[238–240]

1.3 Cytokine trafficking machinery

Cytokine release via the classical secretory pathway relies on trafficking machinery, a variety of proteins that are often found on cell membranes and mediate the sorting and loading of cargo into


A
Constitutive Secretion
Stimulus
Transcription
Translation
ER Targeting
Maturation
Sorting to Secretory Vesicles for Release
Nucleus
ER
Golgi
Secretory
Vesicles

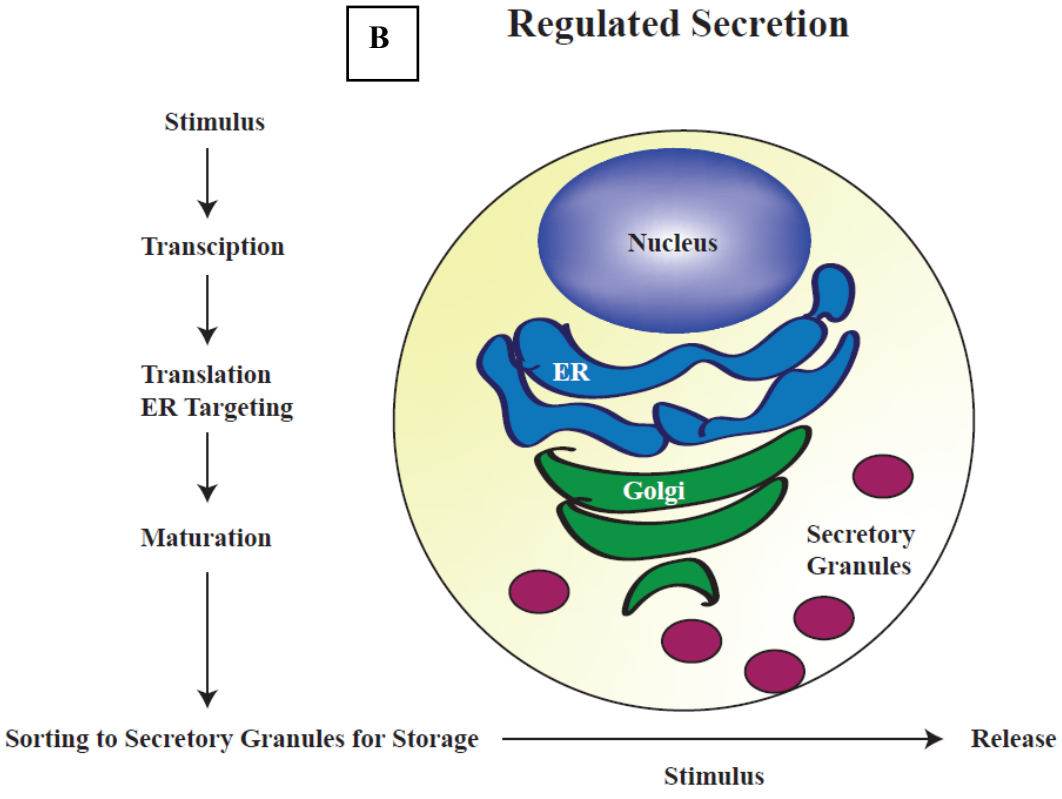

B
Regulated Secretion
Stimulus
Transciption
Translation
ER Targeting
Maturation
Sorting to Secretory Granules for Storage
Release
Stimulus
Nucleus
ER
Golgi
Secretory
Granules

Figure 1.4. Classical secretory pathways. In the presence of a stimulus, nuclear DNA is transcribed to messenger RNA and exported to the cytosol where it can interact with ribosomes for protein translation. Protein being translated by ribosomes are then targeted to the ER based on the presence of a signal sequence and enter the classical secretory pathway. Once in the ER lumen, proteins are properly folded and can bud off into vesicles that will fuse with the Golgi. Once the protein progress through the Golgi network, migrating further away from the ER, it can once again bud off into secretory vesicles that will fuse with the plasma membrane in a process termed constitutive secretion (A). This secretion occurs across all cell types. In granule-containing cells, such as eosinophils, proteins from the Golgi can instead be sorted for storage in secretory granules. In the presence of a later stimulus, stored proteins in secretory granules can be delivered to the plasma membrane for regulated secretion (B).

vesicles, movement of cargo-filled vesicles within the cell, and docking of secretory vesicles to a target membrane for fusion and release of its contents. This trafficking machinery largely involves soluble *N*-ethylmaleimide-sensitive factor (NSF) attachment protein receptors (SNAREs) and the Rab family of guanosine triphosphatases (GTPases).

1.3.1 SNAREs: mediating membrane fusion events

The final step in classical secretion involves a membrane-bound organelle carrying cargo molecules fusing with the plasma membrane such that its contents are released to the extracellular environment.[241] SNARE proteins drive this membrane fusion event.[242–245] With 36 members in humans, SNARE proteins are a family of proteins which share a SNARE motif that is commonly followed by a transmembrane domain at the carboxyl (C)-terminus.[244–246] These SNARE motifs allow for the formation of a stable four-helix bundle between SNARE protein complexes associated with opposing vesicle (v-SNARE) and target (t-SNARE) membranes.[244,245] A release in free energy results from this conformational change and mediates the fusing of opposing membranes.[244,245] Each helix in the four-helix bundle is composed of three Q-SNARE motifs (Qa, Qb, and Qc) and one R-SNARE motif, in which the Q and R denote glutamine and arginine residues, respectively, that are found in the centre of this conformation.[244,245] Interestingly, 3 out of the 36 human SNARE proteins contain two Q-SNARE motifs (Qb and Qc) at their N- and C-terminus as opposed to one SNARE motif and include synaptosome-associated protein of 25 kDa (SNAP-25), SNAP-23, and SNAP-29.[244] SNARE proteins with a SNARE motif homologous to the N-terminal motif of SNAP25 make up a S25N Qb subfamily, whereas those with a motif homologous to the C-terminal motif make up a S25C Qc subfamily.[244] Furthermore, the syntaxin subfamily consist of proteins with Qa-SNARE motifs and the vesicle-associated membrane protein (VAMP) or synaptobrevin subfamily consist of proteins with R-SNARE motifs.[244] NSF mediates the dissociation of this four-helix bundle such that SNARE motifs are readily available for other membrane fusion events.[244,245,247]

1.3.1.2 SNAREs associated with eosinophil secretory organelles

Most SNARE proteins are localized to a specific membrane-bound organelle.[244,245] This SNARE sorting poises the secretory organelle for a membrane fusion event by ensuring the correct combination of SNARE proteins (i.e. presence of Qa-c SNAREs and a R-SNARE) exist at vesicle and target membranes.[244,245] A greater number of v-SNAREs found on a secretory organelle is

associated with a greater propensity for vesicle secretion as more combinations of SNARE complexes can be formed to fuse with the plasma membrane.[248] For instance, the highly mobilizable secretory vesicles associated with piecemeal degranulation in human eosinophils bear the v-SNAREs VAMP-2, VAMP-7, and VAMP-8.[56,249,250] Specifically, VAMP-2^+ secretory vesicles colocalize with the chemokine CCL5/RANTES, known to be stored in crystalloid granules prior to release, and mobilize to the plasma membrane in as rapid as 5 min post-stimulation with IFN-γ **(Figure 1.5)**.[249] VAMP-2-bearing secretory vesicles are also implicated in the regulated secretion of ECP in human eosinophils.[251] Meanwhile, only VAMP-7 and VAMP-8 are localized to crystalloid granules of human eosinophils, in which VAMP-7 is predominantly implicated in classical exocytosis events.[250] Inhibition of VAMP-7 *in vitro* using an anti-VAMP-7 mAb hindered release of crystalloid-granule-derived EPX and EDN in human eosinophils.[250] Likewise, in mice with eosinophil-specific deletions of VAMP-7, which was found on $CD63^+$ crystalloid granules, EPX and MBP release with PAF stimulation was reduced compared to controls.[252] Interestingly, VAMP-7 deletion in these mice selectively lowered levels of IL-9 release by eosinophils in response to PAF and ionomycin stimulation and was associated with decreased airway hyperresponsiveness in ovalbumin (OVA)-challenged mice, a well-established model of allergic asthma.[252] VAMP-7 deletion in mice did not affect release of other Th2 cytokines IL-4, IL-5, and IL-13 suggesting a selective role for VAMP-7 in cytokine and granule protein release.[252] Human eosinophils also express the t-SNAREs syntaxin-4 (Qa-SNARE) and SNAP-23 (Qbc-SNARE) which are predominantly found at the plasma membrane and likely form complexes with VAMP-2^+ (R-SNARE) secretory vesicles during piecemeal degranulation **(Figure 1.5)** and VAMP-7^+ crystalloid granules during exocytosis.[250,253] The Qa-SNARE syntaxin-17 is also found on crystalloid granules and granule-derived EoSVs of human eosinophils and could contribute to piecemeal degranulation by EoSVs complexing with SNAP-23 at the plasma membrane along with an unknown R-SNARE.[254]

1.3.2 Rab GTPases: recruiters and regulators of trafficking machinery

Rab GTPases act as global regulators of trafficking machinery.[255] With more than 60 members in humans, Rab GTPases are carefully localized to specific membrane-bound compartments throughout the cell in which they encounter critical trafficking machinery.[255,256] Here these proteins interact with coat protein complexes, motor proteins, and SNARE protein regulators to coordinate vesicle formation and selection of luminal contents, vesicle movements that utilize cell

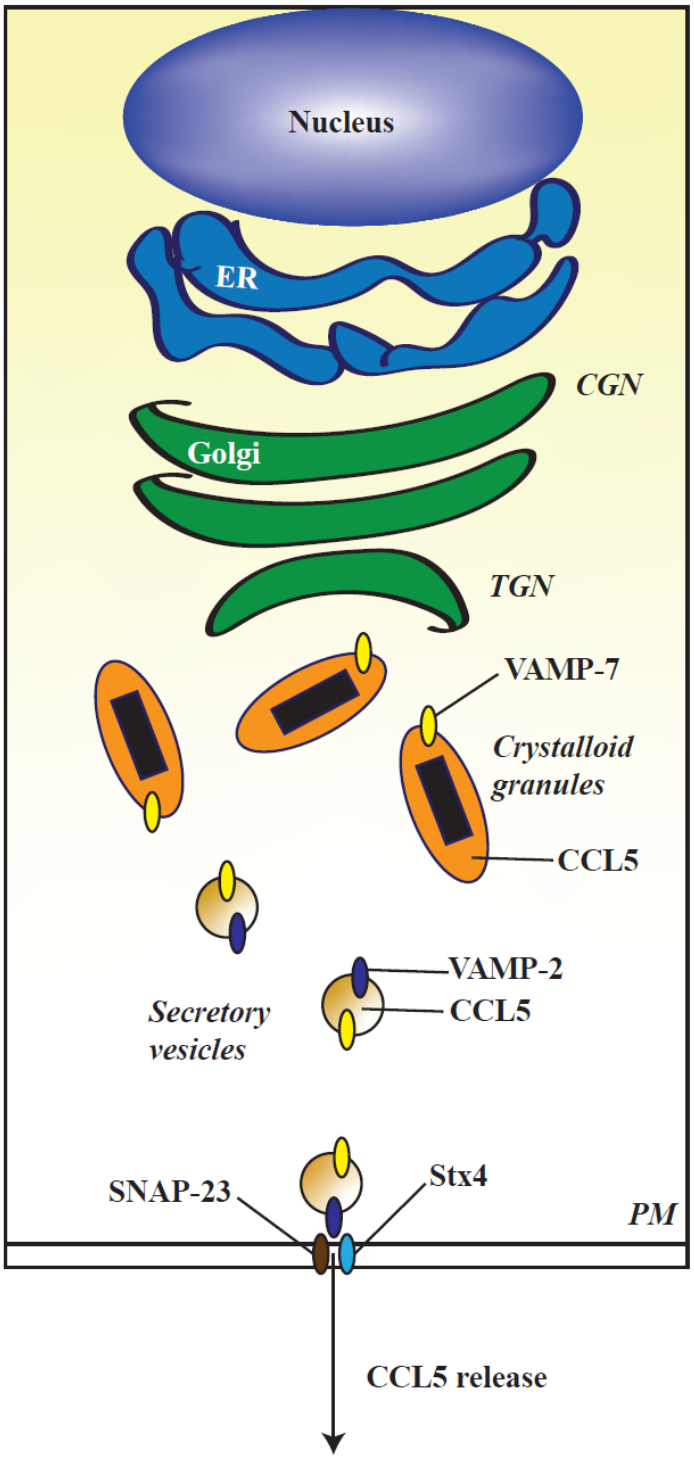

Nucleus
ER
CGN
Golgi
TGN
VAMP-7
Crystalloid granules
CCL5
VAMP-2
CCL5
Secretory vesicles
SNAP-23
Stx4
PM
CCL5 release

Figure 1.5. SNAREs associated with regulated CCL5 secretion in eosinophils. Newly made CCL5 classically traffics through the ER to *cis*-Golgi network (CGN) to *trans*-Golgi network (TGN) where it buds off for granular storage. Preformed CCL5 is found within eosinophil crystalloid granules which bear the v-SNARE VAMP-7. Upon certain stimuli, CCL5 is selectively sorted out of crystalloid granules and into secretory vesicles with the v-SNARE VAMP-2. VAMP-2 interacts with SNAP-23 and syntaxin-4 (Stx4) at the plasma membrane to mediate membrane fusion and release of CCL5.

cytoskeletal components, and vesicle membrane fusion for content release, respectively.[255,257,258] Many of these protein-protein interactions are dependent on an active conformational state of Rab GTPases.[255] Classically, Rab GTPases are active when bound to GTP and inactive when bound to GDP.[255] Several proteins are involved in converting Rab GTPases to active or inactive states. Active GTP-bound Rab proteins hydrolyse GTP to GDP intrinsically and this hydrolysis is accelerated by GTPase-activating proteins (GAPs).[255,259] This process converts Rab proteins to an inactive GDP-bound form. Subsequently, guanine nucleotide exchange factors (GEFs) trigger the release of GDP from these Rab proteins and cytosolic GTP can readily bind to complete conversion to its active state once again.[255,260] Rab GDP dissociation inhibitors (GDIs), given its name, counter this process by inhibiting the release of GDP.[261] GDIs also chaperone cytosolic Rab GTPases towards their associated intracellular membrane where membrane bound GDI displacement factors (GDFs) trigger GDI dissociation and allow Rab proteins to associate with their cognate membranes.[261–265] In this way GDIs and GDFs effectively sort Rab proteins back to their membrane of origin following vesicle trafficking events that carry Rabs away and towards a target membrane.[255,265]

1.3.2.1 Rab27a in eosinophil degranulation

Rab27a is well-studied with regards to its role in regulated secretion of secretory vesicles in immune cells and secretory cells, namely melanocytes.[255] Specifically, deficiencies in this Rab protein hinder regulated exocytosis in killer T cells, NK cells, basophils, neutrophils, and melanocytes.[266–271] Consequently, humans with mutations in the *RAB27A* gene develop a rare disorder characterized by partial loss of pigment in skin, hair, and eyes and immunodeficiency termed Griscelli syndrome type 2.[272] Rab27a is also associated with eosinophils in asthma as human eosinophils express this protein at elevated levels in asthmatics compared to a normal control group and this Rab plays an important role in eosinophil exocytosis.[271,273] In resting human eosinophils Rab27a is partially localized to $CD63^+$ crystalloid granules within the cell cytoplasm and these structures migrate towards the cell periphery following PAF stimulation, which is known to induce EPX release.[273] Furthermore, Rab27a-deficient *Ashen* mice exhibit hindered release of EPX following PAF stimulation.[273] While several effectors that bind to Rab27a to elicit regulated secretion have been identified in secretory cells, the mechanisms underlying Rab27a-mediated secretion in eosinophils remains unknown. For example, Rab27a found on membranes of melanosomes, which store and release the pigment melanin in melanocytes, recruit the protein

melanophilin.[274] Melanophilin then bridges melanosomes to the actin motor myosin Va that transports these vesicles to the plasma membrane for release.[274]

1.4 Endocytosis and membrane recycling

Accompanying the stream of cytokines being secreted from eosinophils through diverse pathways is the uptake of molecules from the cell's extracellular environment through endocytosis. During this endocytic process select lipids and protein receptors on the plasma membrane invaginate within the cell cytosol to form a membrane-bound compartment that is often associated with a clathrin coat.[275,276] Endocytosed cargo is first delivered to early endosomes which have a mildly acidic pH to trigger release of proteins coupled to transmembrane receptors.[275] Here, early endosomes play an important role in regulating cell signaling through cell surface receptors; endocytosed receptors may be downregulated by sorting these proteins into the late endosome/lysosome pathway for degradation or recycled back to the plasma membrane for continued cell signaling directly from early endosomes (fast recycling) or by tubulovesicular recycling endosomes (REs) (slow recycling).[275–277] Well-studied receptors that are sorted for recycling in these endosomes are transferrin receptors (TfnRc) responsible for iron uptake through transferrin (Tfn), integrin proteins involved in cell adhesion, and tyrosine kinase and G protein-coupled receptors (RTK and GPCRs) involved in cytokine and chemokine signaling, respectively.[278–280]

1.4.1 Recycling endosomes in cytokine secretion

Notably, REs possess a noncanonical role in cytokine secretion in immune cells that will be discussed here.[234,235,277] In particular, these endosomes and their associated trafficking machinery, VAMP-3 and Rab-11, are critical in exocytosis of *de novo* synthesized TNF in macrophages **(Figure 1.6)**.[281] Interestingly, LPS and IFN-γ, which are associated with bacterial and viral infections, respectively, upregulate expression of both VAMP-3 and Rab-11 which then aids in pro-inflammatory TNF release.[281] At the *trans*-Golgi network and Golgi-derived vesicles, the Q-SNAREs syntaxin-6/syntaxin-7/vesicle transport through interaction with t-SNAREs homolog 1B (Vti1b) allow for the exchange of newly produced TNF to REs through the R-SNARE VAMP-3 **(Figure 1.6)**.[281,282] TNF-bearing REs can then fuse with the Q-SNARE complex syntaxin-4/SNAP-23 at the plasma membrane for cytokine delivery **(Figure 1.6)**.[281,283] TNF remains associated to the plasma membrane until a TNF alpha converting enzyme (TACE) proteolytically

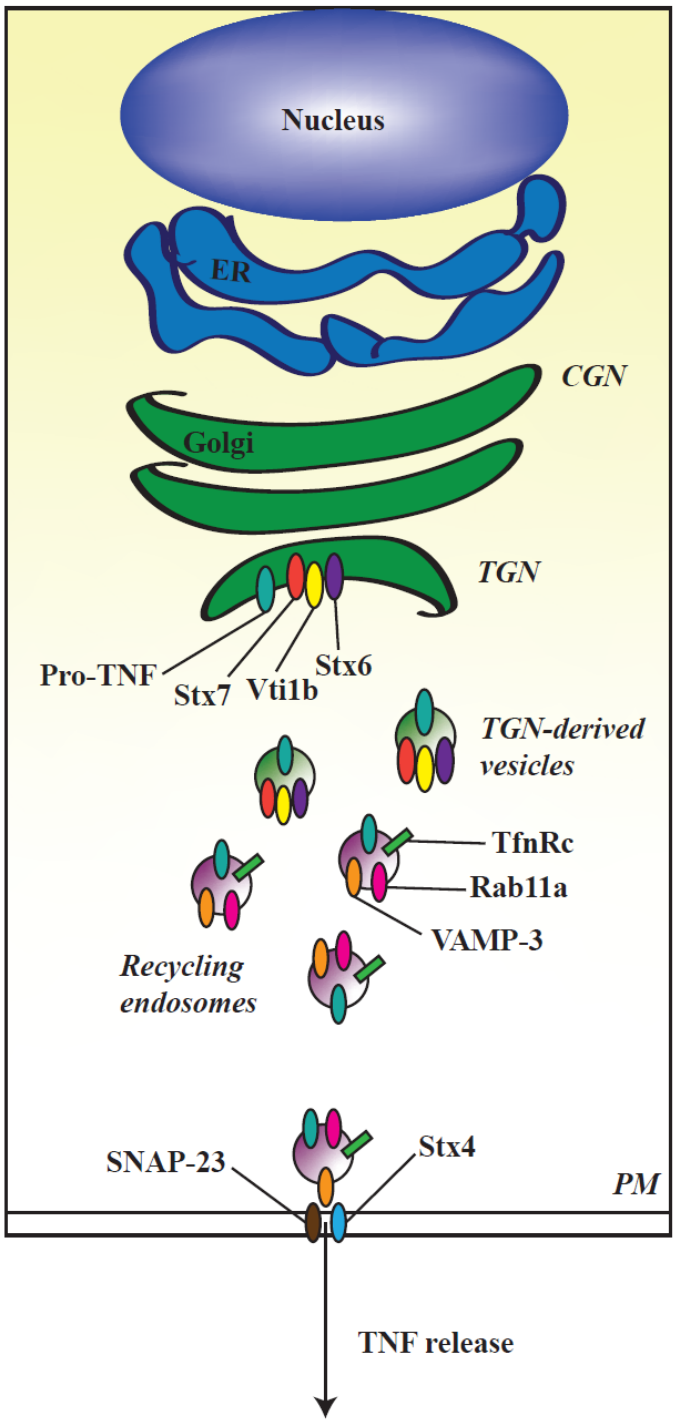

Nucleus
ER
CGN
Golgi
TGN
Pro-TNF
Stx7
Vti1b
Stx6
TGN-derived vesicles
TfnRc
Rab11a
VAMP-3
Recycling endosomes
SNAP-23
Stx4
PM
TNF release

Figure 1.6. Constitutive TNF release in macrophages via recycling endosome trafficking. Newly synthesized TNF exists in a pro-form that is membrane-associated and migrates classically from the ER to *cis*-Golgi network (CGN) to *trans*-Golgi network (TGN). At the TGN and vesicles which bud from the TGN, pro-TNF is found on the membrane along with the SNARE complex composed of Stx6, Vti1b, and Stx7. This pro-TNF is then transferred to recycling endosomes bearing TfnRc, the v-SNARE VAMP-3, and Rab11a. VAMP-3 can then interact with SNAP-23 and Stx4 at the plasma membrane to mediate membrane fusion. Proteolytic cleavage of pro-TNF at the cell surface by TNF alpha converting enzyme allows for release of soluble TNF.

cleaves it into a soluble form.[284] In addition to exporting newly made TNF, REs increase macrophage surface area for phagocytosis, the process in which immune cells engulf large particles such as pathogens for subsequent antigen processing and presentation or apoptotic somatic cells in membrane-bound phagosomes.[281,285] Indeed, TNF and VAMP-3 from REs are enriched in membrane contributing to the initial forming of a phagosome, the phagocytic cup, in macrophages.[281] Similar to TNF, newly synthesized IL-6 in macrophages is observed budding off into trans-Golgi-derived vesicles and fusing with VAMP-3 and TfnRc positive REs.[286] The importance of syntaxin-6 and Vti1b associated with the *trans*-Golgi and VAMP-3 on REs are shared for IL-6 and TNF secretion in macrophages.[286] Within IL-6 containing REs, cargo such as TNF and Tfn are also found and are differentially secreted. TNF and VAMP-3 are released into phagocytic cups while IL-6 and TfnRc do not exhibit this polarized release.[286] This differential release from cytokines sharing a common RE as their carrier illustrates a compartmentalizing of cargo in these endosomal compartments.[286] To note, *de novo* synthesized TNF and IFN- γ in NK cells also rely on TfnRc-bearing REs and associated Rab11 and VAMP-3 for cell surface delivery and release.[287] Interestingly, REs provide a means of re-routing these pro-inflammatory cytokines for non-polarized release as opposed to the directional release of preformed cytolytic granules in NK cells that directly aim to kill target cells in an immunological synapse.[287]

1.5 A need for eosinophil-like cell models

Eosinophils and other granulocytes characteristically have quick cell turnover rates in circulation.[18,288] Under noninflammatory conditions and infrequent encounters with pro-survival cytokines, eosinophils survival is measured to be approximately 3 days.[18,288] This short lifespan alone makes experimental manipulations of primary eosinophils extremely time-sensitive and limited in terms of prolonged cell immunolabelling experiments. Compounding these limitations to experimentation, primary immune cells have proven to be challenging to work with in gene manipulation studies. Transfections are commonly used for these studies in which exogenous nucleic acids are introduced to a cell to enhance or silence the expression of a certain gene.[289] In primary immune cells, transfection rates are often low due to induction of cell death during protocols.[289] To circumvent these issues, human leukemia cell lines have been explored as a proxy for primary immune cells.[290,291]

1.5.1 HL-60 and HL-60 Clone 15 cell lines

The HL-60 human leukemia cell line consists of multipotent promyelocytes which can be induced to follow certain granulocyte lineages of differentiation. Treatment of these cells with chemical inducers such as retinoic acid and dimethyl sulfoxide (DMSO) trigger neutrophilic differentiation.[292,293] While primary human neutrophils classically contain $CD63^+$ primary granules, $CD66b^+$ secondary granules, and $CD11b^+$ tertiary granules, neutrophil-like HL-60 cells only contain primary granules and lack certain granule components which result in impaired abilities in producing extracellular traps or reactive oxygen species for studies assaying microbial killing.[293–295] HL-60 cells that were previously cultured in slightly alkaline pH (7.6-7.8) for 2 months, known as HL-60 clone 15 cells, and then treated with 0.5 mM sodium butyrate or butyric acid for 5-7 days have a high propensity for eosinophil-like differentiation.[292,296,297] Cell growth arrest, change in nuclear shape from round to lobular, change from blue (basophilic) to pink (acidophilic) cytoplasmic staining with Giemsa-like stains, and increased expression of eosinophil-associated genes (i.e. MBP, IL-5Rα, and CCR3) are associated with this eosinophil-like differentiation.[291,298,299] Butyrate inhibits histone deacetylation in these cells, leading to a prevalence of acetylated, transcriptionally accessible regions of DNA.[298] Here c/EBPα, c/EBPβ, and c/EBPε transcription factors bind to inhibit cell proliferation and induce eosinophil-specific gene expression.[298] The granulocyte-like cells are then commonly used for chemotaxis assays.[299,300] Additionally, these cells are susceptible to electroporation-based introduction of nucleic acid to cells, which can be used for gene knockdown studies or introducing the expression of fluorescently tagged proteins for live cell imaging.[301–304] Recently the development and use of lipid nanoparticles has advanced nucleic acid delivery in difficult-to-transfect cell lines, enhancing both uptake of nucleic acid and cell viability when compared to traditional transfection methods.[305] Furthermore, lipid nanoparticles can be loaded with chemical mediators. For instance, cholesterol butyrate nanoparticles (CBNs) passively diffuse through the plasma membrane of HL-60 cells, surpassing the need for cell surface expression of butyrate transport proteins for classical butyrate uptake, and induce cell cycle arrest and apoptotic death.[306] This induction of apoptosis in HL-60 cells with CBNs provides experimental results in favor of butyrate as a candidate anti-cancer drug.[306]

1.6 Rationale

Eosinophils are an undeniable facet of asthma pathogenesis. Affecting close to 2.6 million Canadians over the age of 12 in 2018, severe asthma inflicts a large economic burden on our healthcare system and overall lowers quality of life (Statistics Canada Table 13-10-0096-08 Asthma, by age group). As a highly compartmentalized granulocyte, eosinophils are uniquely poised to rapidly release preformed cytotoxic granule proteins and Th2 cytokines for microbial defence. In a tip of the balance that is required in homeostasis and immunity, continuous recruitment of these cells to tissue enriched in pro-survival mediators leads to prolongment of their typically short lifespan and chronic cell activation leading to pathology. Eosinophils and the Th2 cytokines they release have been widely studied for their roles in asthma pathology, evident in them being widely targeted by current FDA-approved asthma therapeutics. Meanwhile, the trafficking pathways in which these Th2 cytokines, namely IL-9 and IL-13, follow for cell surface delivery and release by eosinophils are largely unknown and have not been fully localized to intracellular membranes. Given eosinophils house over 30 cytokines and chemokines and only a third have been localized to crystalloid granules, the RE known to traffic cytokines in other immune cells is newly suggested here to act as an additional cytokine trafficking site in eosinophils. Knowledge of these Th2 cytokine trafficking sites and secretion pathways contribute novel therapeutic targets in severe asthma that aim to dampen their release and subsequent systemic proinflammatory effects. In Chapter 3, I present results regarding the storage and trafficking sites of IL-9 and IL-13 in peripheral blood eosinophils from atopic donors at baseline and in the presence of the stimulant PAF.

1.7 Hypothesis

I hypothesize that REs act as a novel trafficking site for IL-9 and IL-13 upon PAF stimulation in human eosinophils as REs have been implicated in exocytic transport of newly synthesized cytokines in different immune cells. Furthermore, crystalloid granules would mainly act as a storage site for these cytokines that could later be subsequently released.

1.8 Objectives

1. To assess the presence and distribution of recycling endosomes in both resting and PAF-stimulated eosinophils through immunolabelling of $TfnRc^{+}$ vesicles followed by super

resolution microscopy. Throughout this study, I assume $TfnRc^+$ vesicles represent endosomal compartments.

2. To determine the subcellular localization of IL-9 and IL-13 at baseline and throughout a PAF-stimulation time course in eosinophils; specifically, whether these cytokines localize to $TfnRc^+$ vesicles and/or $CD63^+$ crystalloid granules. Eosinophils are immunolabelled for IL-9 or IL-13 and TfnRc or CD63 and imaged by super resolution microscopy. Colocalization or association between a cytokine of interest and organelle of interest are measured with Pearson's Correlation Coefficient (PCC). Changes between associations of the cytokine of interest and organelle of interest at resting state compared to a PAF-stimulated state are indicative of cytokine trafficking within the cell (i.e. shuttling into or out of membrane-bound compartments).
3. To determine differences in the detection of intracellular IL-9 or IL-13 protein at baseline compared to following PAF stimulation. Mean fluorescence intensity values obtained from immunolabelling IL-9 or IL-13 are used as a measure of protein levels within the cell.
4. To determine the proportion of total intracellular IL-9 found within $TfnRc^+$ vesicles or $CD63^+$ crystalloid granules. Eosinophils are immunolabelled for IL-9 and TfnRc or CD63 and imaged by super resolution microscopy. An object-based colocalization method I developed defines immunolabeled IL-9 and organelles as 3-D spots on a per cell basis. These cytokine spots and organelle spots can then be colocalized and quantified.

Chapter 2:
Materials & Methods

2.1 Donor recruitment.

Volunteers with self-reported allergies and/or asthma who were not taking any form of medication for at least two weeks prior to their donation appointment were recruited for the study in Chapter 3. Donors with over 3% circulating eosinophils were selected for this study. Both non-allergic and atopic individuals, meeting the same no medication criteria mentioned prior, were recruited for human neutrophil data presented in Appendix A. All participants were 18 years or older and provided informed consent prior to blood collection. These protocols received research ethics approval from the University of Alberta Research Ethics Board, Project Name "Molecular Mechanisms Regulating Leukocyte Activation", Pro00000942, 2020.

2.2 Phlebotomy.

Human peripheral venous blood was collected in 10 ml EDTA (K2) Vacutainer blood collection tubes (cat. # 366643) and drawn with Safety-Lok butterfly needle sets (cat. #367281) from BD Biosciences (Franklin Lakes, NJ, USA). A total of 30 ml of whole blood was collected from eosinophil donors, while 10 ml of whole blood was collected from neutrophil donors.

2.3 White blood cell differential count.

Differential counts were performed to assess the percentage of circulating eosinophils or neutrophils found in whole blood collected from donors prior to conducting isolations for these cell types. Whole blood was first stained using freshly made Kimura stain, prepared as previously described in reference 306 and filter-sterilized with a 0.22 μm Millex-GS syringe filter unit (cat. # SLGS033SS; Millipore Sigma, Burlington, MA, USA).[307] The cytoplasm of human eosinophils uniquely stain green with Kimura and thus are easily identifiable among other white blood cells. Human neutrophils do not stain green but have characteristic multilobed nuclei. Whole blood was diluted 1:10 with Kimura stain prior to loading 10 μl of this solution into a 4-Chip disposable hemocytometer (cat. # DHC-N404; Bulldog Bio, Portsmouth, NH, USA). White blood cell differential counts were then performed under bright field microscopy using a Fisherbrand manual differential counter (cat. # 13-684-140).

2.4 Human eosinophil isolation from whole blood.

Whole blood samples were transferred from blood collection tubes to 50 ml conical centrifuge tubes (cat. # 430290) from Corning (Corning, NY, USA). Eosinophils were isolated from 30ml whole blood samples by negative immunomagnetic selection following manufacturer's protocols

using the MACSxpress human eosinophil isolation kit (cat. # 130-104-446) and MACSxpress separator (cat. # 130-098-308) from MACS Miltenyi Biotec (Bergisch Gladbach, Germany). Eosinophils were carefully collected from the supernatant in the MACSxpress separator, as to avoid collecting cells adhered to the walls of the tube, using 10 ml serological pipettes (cat. # 4488) from Corning and placed into a new 50 ml centrifuge tube. From this point on, cells were kept on ice. RPMI 1640 cell media (cat. # 11875093) from Gibco (Waltham, MA, USA) was then added to the supernatant to reach a total volume of 45 ml. Eosinophils were then gently pelleted by centrifugation at 300 x g for 7 min at 4°C. Following this first centrifugation step, the supernatant was discarded, and the cell pellet was gently resuspended in RPMI 1640 using a transfer pipette (cat. # 13-7119-AM; Fisher Scientific, Waltham, MA, USA). The volume of this cell suspension was topped up with RPMI-1640 to 45 ml and centrifuged at 300 x g for 7 min at 4°C once again as a second wash step. Isolated eosinophils were then gently resuspended in 1 ml of cell media as prior and a small sample was taken for Kimura staining as outlined in section 2.3 for cell counts. On average, eosinophil isolation kits resulted in $\geq$ 97% cell purity. Isolated eosinophils were finally diluted in RPMI 1640 to obtain a final concentration of $1x10^6$ eosinophils/ml.

2.5 Human neutrophil isolation from whole blood.

Whole blood samples were transferred from blood collection tubes to 15 ml conical centrifuge tubes (cat. # 430052) from Corning. Neutrophils were isolated in a close manner to eosinophils by following manufacturer's protocols MACSxpress human neutrophil isolation kit (cat. # 130-104-434) and use of the same MACSxpress separator. A 5ml serological pipette (cat. # 4487, Corning) was used to collect neutrophils from the supernatant formed after incubation on the magnetic MACSxpress separator and to transfer the solution to a new 15 ml conical tube. Neutrophils were kept on ice and washed twice with 10 ml of RPMI 1640 as described in section 2.4. Similarly, a small sample of isolated cells was counted with Kimura stain as outlined in section 2.3 and revealed an average purity of $\geq$ 98%. Isolated neutrophils were also diluted in cell media to a final concentration of $1x10^6$ neutrophils/ml.

2.6 Short-term culture of human eosinophils.

Isolated eosinophils were cultured over circular glass coverslips (cat. # 12-545-80) in 6-well plates (cat. # 12-556-004) from Fisher Scientific. Coverslips were previously cleaned with 95% ethanol and left to air dry prior to being placed at the bottom of wells with curved, fine point metal forceps.

Each well contained 5 coverslips and was seeded with $1x10^6$ eosinophils in RPMI 1640. Cells were then incubated at 37°C with 5% CO2 for 30 min to allow for adherence to glass coverslips. Following this incubation, wells were looked at under an inverted light microscope to ensure coverslips were homogeneously coated with adherent cells. To simulate cells, C18-PAF (cat. # P6537; Millipore Sigma) was added to a final concentration of 5 μM per well and incubated for 0, 5, 15, or 60 min at 37°C with 5% CO2. Following stimulation, media was aspirated from the corresponding well and coverslips were washed once with 1X PBS (diluted from 10X, cat. # SH3025801) from HyClone (Logan, UT, USA). Cells were then fixed onto coverslips by incubation with 4% paraformaldehyde (PFA) (diluted from 16%, cat. # 15710; Electron Microscopy Sciences, Hatfield, PA, USA) for 30 min at room temperature. PFA was later aspirated from wells and cells were washed thrice with 1X PBS. Coverslips were stored in wells in 1X PBS at 4°C for up to one week until use for immunofluorescence staining.

2.7 Short-term culture of human neutrophils.

The same culture plates and coverslips, inclusive of coverslip cleaning and placement in wells, described in section 2.6 were used for culturing of isolated neutrophils. Each well was also seeded with $1x10^6$ neutrophils and were left to adhere to glass coverslips for 30 min at 37°C with 5% CO2. Neutrophils were then stimulated with 10 ng/ml of lipopolysaccharide (LPS from *Escherichia coli* O111:B4, cat. # L3024; Millipore Sigma) for either 0, 30, and 60 min or 0, 2, 4, and 6 h at 37°C with 5% CO2. Washing steps with 1X PBS and fixation with 4% PFA were followed as in section 2.6.

2.8 Immunofluorescence staining of human eosinophils.

Human eosinophils fixed onto coverslips were first permeabilized and blocked with 0.1% saponin (cat. # S4521, Millipore Sigma), 2% bovine serum albumin (BSA, cat. # 126579, Millipore Sigma), and 10% heat-inactivated human serum in 1X PBS for 30 minutes at room temperature in a humidified chamber. This solution was used in subsequent washes and as a diluent for all staining solutions to ensure saponin was kept on cells throughout the staining protocol.

Eosinophils were then immunolabeled with antibodies specific to IL-9 (Alexa Fluor 647 Mouse α-Human IL-9, cat. # 560815; BD Biosciences) or IL-13 (Alexa Fluor 647 Mouse α-Human IL-13, cat. # FAB2131R; R&D Systems, Minneapolis, MN, USA) at 7 μg/ml. At the same time, cells were stained with Alexa Fluor 488 Rabbit α-Human TfnRc (cat. # bs-0988R-A488; Bioss,

Woburn, MA, USA) to label endosomes or Alexa Fluor 488 Mouse α-human CD63 (cat. # MA5-18149; Invitrogen, Waltham, MA, USA) to label crystalloid granules at 10 μg/ml. Another set of coverslips were also set aside for staining with the following isotype control antibodies: Alexa Fluor 647 Mouse IgG1 κ (cat. # 557783, BD Biosciences) at 7 μg/ml and Alexa Fluor 488 Rabbit IgG (cat. # bs-0295P-A488, Bioss) or Alexa Fluor 488 Mouse IgG1 (cat. # IC002G, R&D Systems) at 10 μg/ml. In addition to these antibodies, eosinophils were stained with Rhodamine Phalloidin to label F-actin (cat. # R415; Thermo Fisher Scientific, Waltham, MA, USA) diluted 1:200 and 4, 6-Diamidino-2-Phenylindole (DAPI, cat. # D1306; Thermo Fisher Scientific) diluted 1:15,000 for nuclear staining. Cells were incubated with conjugated antibodies, phalloidin, and DAPI for 45 min at room temperature in a humidified chamber.

Subsequently, coverslips were washed once with 1X PBS quickly and then washed thrice with 1X PBS after leaving the solution on for 4 min between washes. Lastly, three quick washes with Milli-Q water were performed. Once fully washed, coverslips were mounted onto glass slides (cat. # 22-037-246, Fisher Scientific) using ProLong Glass Antifade Mountant (cat. # P36980, Invitrogen) with a curing time of overnight to 24 hours prior to imaging slides.

2.9 Immunofluorescence staining of human neutrophils.

In pilot experiments, fixed neutrophils were permeabilized and blocked with 0.1% saponin, 2% BSA and 10% human serum as described in section 2.8. For troubleshooting experiments, 0.1% or 0.2% Triton X-100 (cat. # T9284, Millipore Sigma) in 1X PBS was used instead to permeabilize neutrophils for 3 min at room temperature. After the Triton X-100 incubation, cells on coverslips were washed thrice with 1X PBS and then blocked with 2% BSA and 10% human serum in 1X PBS for 30 min at room temperature.

Once cells were permeabilized and blocked, the following primary antibodies were used for IF staining of TNF in human neutrophils: Mouse α-human TNF (cat. # MAB610; Novus Biologicals, Littleton, CO, USA), Rabbit α-human TNF (cat. # 654250, Millipore Sigma), Alexa Fluor 488 Mouse α-human TNF (cat. # 557722, BD Biosciences), Alexa Fluor 594 Rabbit α-human TNF (cat. # bs-2081R-A549, Bioss), Goat α-human/mouse TNF (cat. # AF-410-NA, R&D Systems). Two primary antibodies were also sampled for immunolabelling of IL-8 in neutrophils: Mouse α-human IL-8 (cat. # AHC0982, Thermo Fisher Scientific) and Mouse α-human IL-8 (cat. # MAB208, R&D Systems). In addition to staining for cytokines, Rab GTPases, SNARE proteins,

CD66b, and TfnRc were immunolabelled in IF experiments using the following antibodies: Rabbit α-human Rab27a (cat. # sc-22756; Santa Cruz Biotechnology, Dallas, TX, USA), Rabbit α-human Rab27a (cat. # R4655, Millipore Sigma), Rabbit α-human SNAP23 (cat. # 111202; Synaptic Systems, Göttingen, Germany), FITC Mouse α-human CD66b (cat. # 60086FI.1; STEMCELL Technologies, Vancouver, BC, Canada), Mouse α-human Syntaxin-4 (cat. # sc-101301, Santa Cruz Biotechnology), Alexa 488 Rabbit α-human TfnRc (cat. # bs-0988R-A488, Bioss), Rabbit α-human Rab11a (cat. # 715300, Invitrogen), Mouse α-human VAMP-3 (cat. # sc-136162, Santa Cruz Biotechnology), Rabbit α-human VAMP-3 (cat. # NB300-510, Novus Biologicals), and Rabbit α-human VAMP-3 (cat. # OSS00046G, Thermo Fisher Scientific). All primary antibodies were used at a concentration of 10 μg/ml and incubated for 45 min at room temperature.

For unconjugated primary antibodies, the following secondary antibodies were then used: Alexa Fluor 647 AffiniPure Donkey α-mouse IgG (cat. # 715-605-151; Jackson ImmunoResearch Laboratories, West Grove, PA, USA), Cy3 AffiniPure Donkey α-rabbit IgG (cat. # 711-165-152, Jackson ImmunoResearch Laboratories), Cy3 AffiniPure α-mouse IgG (cat. # 715-165-150, Jackson ImmunoResearch Laboratories), Alexa Fluor 488 AffiniPure Donkey α-mouse IgG (cat. # 715-545-151, Jackson ImmunoResearch Laboratories), and Alexa 488 Donkey α-goat IgG (cat. # A11055, Invitrogen). Prior to adding secondary antibodies, coverslips were quickly washed twice with 1X PBS and washed thrice where 1X PBS was left on for 4 min between washes. All secondary antibodies were diluted 1:200 and incubated for 30 min in the dark at room temperature.

Alongside all IF staining, isotype control antibodies were included at the same concentrations mentioned on a separate set of coverslips. These antibodies included: ChromePure Rabbit IgG (cat. # 011-000-003, Jackson ImmunoResearch Laboratories), Mouse IgG1 isotype control (cat. # MA5-14453, Life Technologies), Mouse IgG1 isotype control (cat. # M5284, Millipore Sigma), Purified Mouse IgG2b κ isotype control (cat. # 401201; BioLegend, San Diego, CA, USA), Alexa Fluor 488 Rabbit IgG isotype control (cat. # bs-0295P-A488, Bioss), FITC Mouse IgM isotype control (cat. # 401607, BioLegend), Alexa Fluor 488 Mouse IgG1 isotype control (cat. # 557702, BD Biosciences), Alexa Fluor 594 Rabbit IgG isotype control (cat. # bs-0295P-A594, Bioss), and Normal Goat IgG isotype control (cat. # AB-108-C, R&D Systems).

DAPI was used alongside the last antibody incubation as described in section 2.8. Two phalloidin stains were also used, such as Alexa Fluor 647 Phalloidin (cat. # A22287, Invitrogen) and Alexa

Fluor 405 (cat. # A30104, Thermo Fisher Scientific) at 1:40 or 1:100 dilution. Coverslips were finally washed and mounted as described in section 2.8.

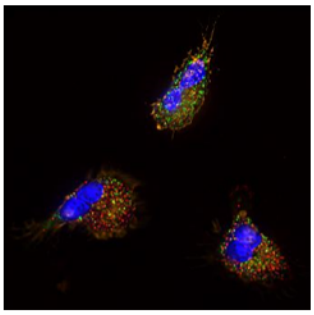
2.10 Image collection and processing with Softworx and Volocity software.

Microscope slides were imaged on a DeltaVision OMX super-resolution microscope system (Applied Precision Inc, Issaquah, WA, USA) at the Faculty of Medicine and Dentistry's Cell Imaging Centre (KATZ, University of Alberta). A 60X/1.42 oil immersion objective lens was used to collect all images. Z-stacks were captured for 50 cells per cell stimulation time point. For z-stacks, the bottom and top of the cells were defined and 0.25 μm sections were imaged throughout the cell volume. Softworx Suite 2.0 software (Applied Precision Inc) was used to align and deconvolve z-stacks.

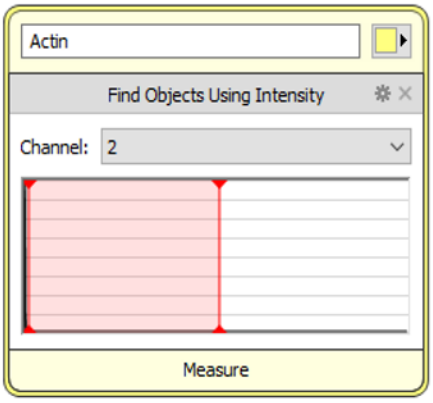
Next, colocalization between immunolabelled cytokines and organelles of interest was measured using Pearson's correlation coefficient (PCC), as practiced in the field of cell biology.[308,309] An automated protocol was developed to obtain PCC values on a per cell basis from the images collected using Volocity Analysis software (Version 6.5.1; Quorum Technologies Inc, Puslinch, ON, Canada) **(Figure 2.1)**. This protocol was developed to expedite measurements and remove potential selection bias. Mean fluorescence intensity (MFI) values for immunolabelled cytokines were also measured on a single cell basis using this protocol.

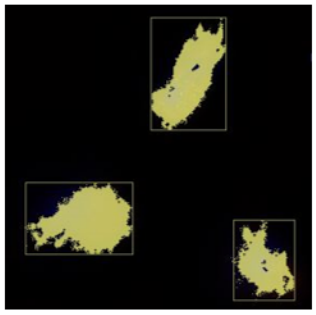
2.11 Object-based colocalization protocol in Imaris software.

Colocalization between immunolabelled IL-9 and granules marked by CD63 or endosomes marked by TfnRc in human eosinophils was additionally measured using a novel object-based colocalization protocol in Imaris software (Version 9.5; Bitplane, Zürich, Switzerland) **(Figure 2.2)**. This software was made available by the Cell Imaging Facility in the Department of Oncology (Cross Cancer Institute, University of Alberta). A 3-D masking feature of Imaris software was used to design "spots" that best represented immunolabelled intracellular IL-9, $CD63^+$ granules, and $TfnRc^+$ endosomes in imaged cell volumes (**Figure 2.2**). All "spots" were defined based on IF staining of cells imaged at baseline (0 min of PAF stimulation), with these same settings applied to the remaining cells imaged post-PAF-stimulation. Two criteria were considered during "spots" creation, (i) the average diameter of immunolabelled cytokine pools or organelles and (ii) being above a threshold of MFI values which represented background signal. The diameters of a minimum of 10 immunolabelled IL-9 pools or $CD63^+$ granules or $TfnRc^+$ endosomes were

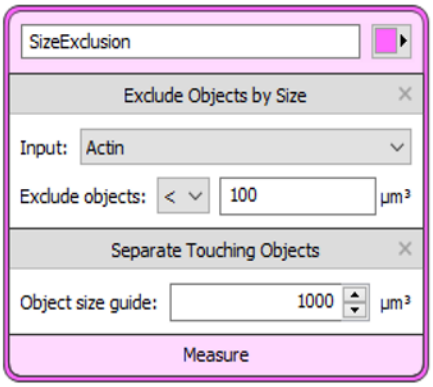


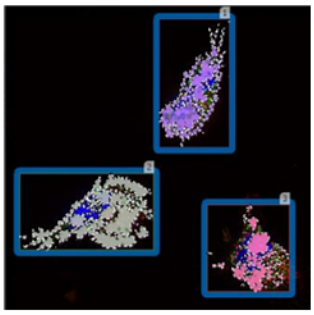
Original Image



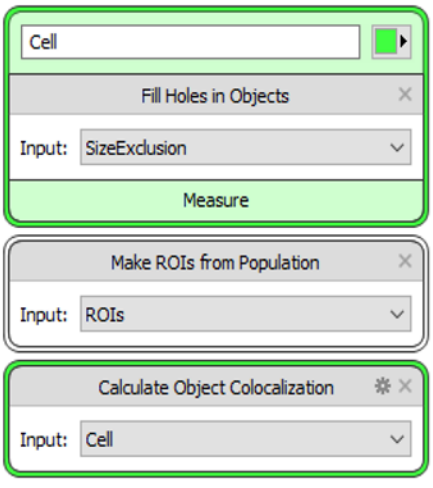


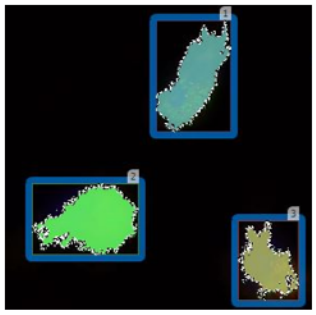
Defining ROI
Cell defined by staining intensity of F-actin using rhodamine phalloidin





Size Exclusion + Separation of Touching ROIs
ROIs < 100 µm^3 excluded from selection and touching other ROIs were separated





Filling of ROIs + Measurement of PCC
Filling of ROIs to ensure full cell cytoplasm coverage for measurements of PCC

Figure 2.1. Automated measurements of colocalization using Pearson's correlation coefficient (PCC) on a single-cell basis using Volocity software (version 6.3). Cells were selected based on having a mean fluorescence intensity (MFI) for actin cytoskeleton staining by phalloidin above a set threshold for background signals (yellow boxes). Selected cells determined by phalloidin staining were defined as regions of interest (ROIs). ROIs were refined through exclusion of less than 100 μm^3 and by separation of touching objects producing single cell ROIs (magenta boxes). Lastly, ROIs were filled such that the entire cell volume was selected, to overcome potential heterogeneity in phalloidin staining. Within these single cell ROIs, PCC was then measured between immunolabeled cytokines of interest and intracellular compartments of interest for each ROI (green boxes). Cell images shown in black boxes (center) were collected by S Almas, the remainder of the figure is my original work.

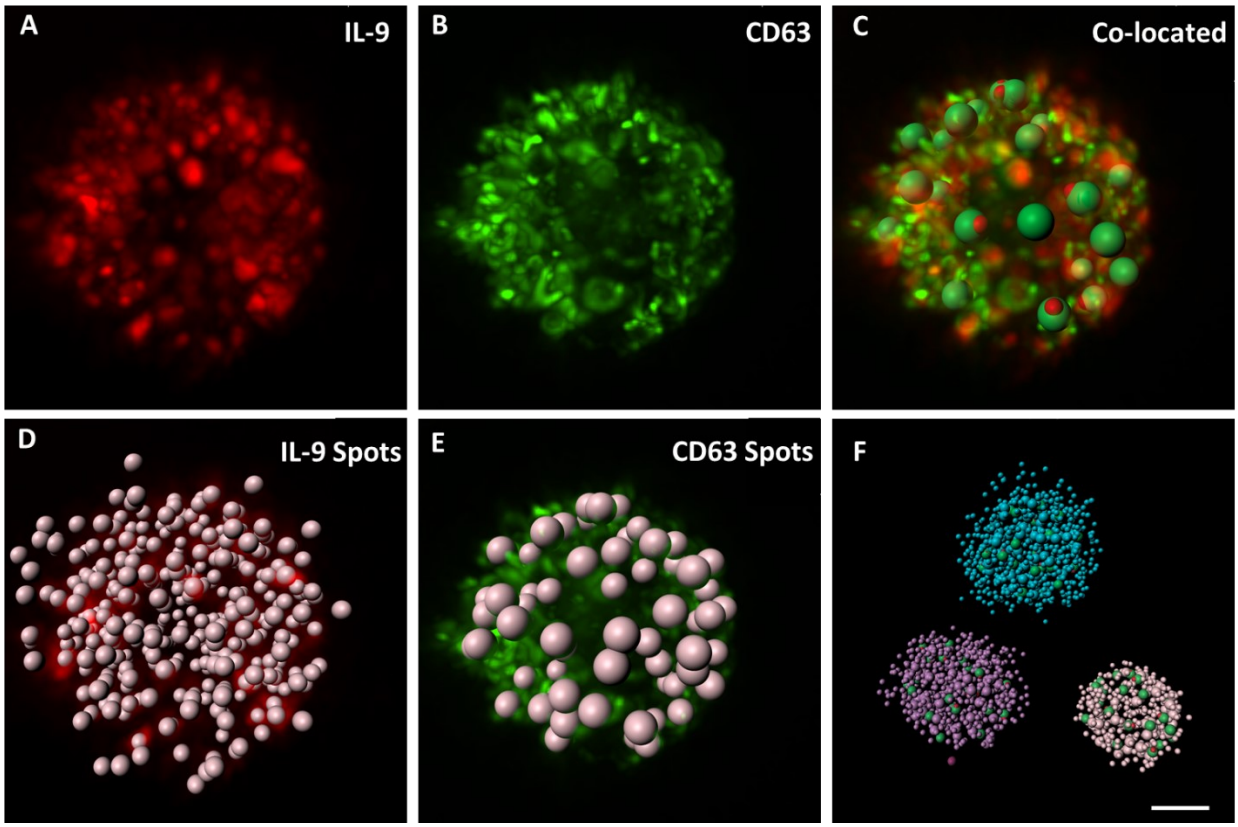


Figure 2.2. Object-based colocalization of IL-9 and CD63$^+$ granules in human eosinophils with Imaris software. Within cell volumes, intracellular IL-9 (A) and CD63+ granules (B) were defined as "spots", a 3D masking feature of Imaris software (Version 9.5). Both IL-9$^+$ spots (D) and CD63$^+$ spots (E) were defined based on average diameter and a mean immunofluorescence intensity threshold set on baseline conditions. IL-9$^+$ spots found within CD63+ granules were identified by the software as co-located spots (red and green spots seen in C) through the input of a specified nearest distance. Non-co-located IL-9 and CD63 spots of three distinct cells are shown (cyan, purple, and ivory spots to discriminate between cells in a single view) as well as co-located IL-9 and CD63 spots (red and green spots) (F).

measured in the software to compute their corresponding average "spot" diameter (in this case, the diameters were as follows: 0.45 μm, 0.90 μm, and 0.80 μm, respectively).

Once "spots" were defined for immunolabelled probes of interest, a function in Imaris software termed "Co-locate spots" was used. This plug-in also relies on MATLAB software (Version 9.7; Natick, MA, USA). A distance within which two defined "spots" must be found relative to one another was inputted to quantify colocalized or "co-located spots". In this protocol, this distance was calculated as follows: average diameter of organelle of interest minus the average diameter of IL-9 pools. As such, IL-9$^+$ "spots" co-located with CD63$^+$ or TfnRc$^+$ "spots" were identified **(Figure 2.2C)**. Given multiple cells were present in a single image, the function "Split spots" was used to ensure all defined "spots" were assigned to an individual cell **(Figure 2.2F)**. Finally, these preparations allowed for enumeration of total IL-9$^+$, CD63$^+$, and TfnRc$^+$ "spots", as well as quantification of IL-9$^+$ spots which co-located with CD63$^+$ or TfnRc$^+$ "spots", on a per cell basis.

2.12 HL-60 clone 15 cell culture and eosinophil-like differentiation.

HL-60 clone 15 cells (ATCC, CRL-1964) were kindly provided by Dr. M Kulka. HL-60 clone 15 cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM, cat. # 12440061, Thermo Fisher Scientific) supplemented with 50,000 U of Penicillin/Streptomycin (cat. # SV30010, Thermo Fisher Scientific) and 10% Fetal Bovine Serum (FBS Canadian origin, cat. # F1051, Millipore Sigma). Supplemented IMDM was filter-sterilized using Stericup vacuum filtration units (cat. # S2GPU05RE, Millipore Sigma) and 50 ml aliquots were prepared in 50 ml conical tubes and stored at 4°C. Media was refreshed every 2-3 days and cultures were maintained at 37°C with 5% CO2. When changing media, cells were spun down at 500 x g for 5 min at room temperature and old media was then aspirated and replaced with fresh complete media. Total cell counts and cell viability were measured with a Countess Automated Cell Counter (cat. # AMQAX1000, Thermo Fisher Scientific) in a 1:1 ratio of cells with 0.4% Trypan Blue stain (cat. # 15250-061, Gibco). Stained cells were loaded into Countess cell counting chamber slides (cat. # C10228, Thermo Fisher Scientific).

To differentiate HL-60 clone 15 cells into an eosinophil-like lineage, cells at 5 x 10^5 cells/ml were added to Falcon T25 tissue culture treated flasks (cat. # 08-772-1F, Fisher Scientific). Cells were grown in the presence of sodium butyrate (NaBu, cat. # B5887, Millipore Sigma) at 0.1, 0.5 or 1 mM for 5 days. Additionally, cholesterol butyrate nanoparticles (CBNs) provided and produced

by Dr. M Kulka were used at the same concentrations as sodium butyrate to treat cells for 5 days. Untreated cells were included as a control throughout the time course.

2.13 Flow cytometry analysis of HL-60 clone 15 cells.

Following 5-day treatment with NaBu, HL-60 clone 15 cells were analyzed for expression of eosinophil-associated proteins CCR3 and Siglec-8 by flow cytometry analysis on the BD Fortessa X-20 in the Flow Cytometry Facility of the Faculty of Medicine and Dentistry (KATZ, University of Alberta). Untreated cells were also analyzed as a negative control. Per sample, 0.5-1.0 x 10^6 cells were collected into 1.5 ml microfuge tubes (cat. # 05-408-129, Fisher Scientific), pelleted at 500 x g for 5 min at 4°C, and washed with cold 1X PBS. After cells were centrifuged at the same settings and supernatant was aspirated, cells were fixed in 2% PFA for 10 min at room temperature or overnight at 4°C.

Subsequently, cells were washed with 5% BSA in 1X PBS, pelleted, supernatant was aspirated off, and then permeabilized with 0.2% Triton X-100 for 3 minutes at room temperature. This permeabilization was omitted for analysis of surface protein expression as opposed to intracellular proteins. After a wash with cold 1X PBS, cells were transferred to 5ml polypropylene tubes (cat. # 14-959-1A) and blocked in 5% BSA for 30 min. Cells were then incubated with the following antibodies at the recommended test volumes for flow analysis: FITC Mouse α-human CD193/CCR3 (cat. # 310719, BioLegend) and PE/Dazzle Mouse 594 α-human Siglec-8 (cat. # C10228, BioLegend). The following isotype control antibodies from BioLegend were included to establish gates for fluorescence readings that were considered background: FITC Mouse IgG2b κ isotype control (cat. # 310719) and PE/Dazzle 594 Mouse IgG1 κ isotype control (cat. # 400175). Anti-Mouse Ig, κ/Negative Control Compensation Particles Set (cat. # 552843, BD Biosciences) were kindly provided by N Shrestha and Dr. H Vliagoftis. These compensation beads together with one of the two antibodies used were used as fluorescence minus one (FMO) controls for compensation done by accompanying BD FACSDiva software. All antibodies were incubated for 30 min. Cells were washed with cold 1XPBS and then resuspended in 5% BSA prior to reading cells at the flow cytometer.

Chapter 3: Results

3.1 Endosomal compartments bearing TfnRc are found in human peripheral blood eosinophils and increasingly colocalize with intracellular IL-9 upon PAF stimulation.

While human eosinophils are known to express and release IL-9 in response to PAF, intracellular sites of storage and mechanisms of secretion are unknown.[88] I hypothesize that recycling endosomes are involved in storing and trafficking IL-9 to the cell surface in the presence of the stimulus PAF. To test my hypothesis, resting and PAF-stimulated human blood eosinophils were double-stained for intracellular IL-9 and TfnRc, a marker of recycling endosomes as well as early and late endosomal compartments, by immunofluorescence microscopy. Colocalization of immunolabeled IL-9 and $TfnRc^+$ endosomes would suggest these endosomes are a novel site of storage of intracellular IL-9. Furthermore, changes in the association and subcellular distribution of cytokine and endosomes would shed light on pathways of IL-9 secretion.

Immunolabeling of $TfnRc^+$ endosomes appear as densely granular shapes often localized near cell nuclei and near cell peripheries at resting conditions (0 min) and throughout PAF stimulation **(Figure 3.1, TfnRc panels)**. Intracellular IL-9 appears as faint, punctate, and diffuse staining at baseline **(Figure 3.1, 0 min)**. Meanwhile, upon PAF stimulation, bright and enriched granular staining of IL-9 at perinuclear areas of the cell and near the cell surface arise **(Figure 3.1, 5 and 15 and 60 min)**. In terms of cell morphology, resting eosinophils appear as compact round cells, approximately 17 micrometers in diameter, based on staining of actin filaments in the cell cytoskeleton with phalloidin **(Figure 3.1, 0 min)**. At 15 and 60 min of PAF stimulation, eosinophils exhibit a stretched morphology with filopodia seen at the poles of the cell **(Figure 3.1, Phalloidin panels)**. The compact bilobed nuclei of eosinophils stained with DAPI of resting cells similarly become elongated with PAF stimulation **(Figure 3.1, Merged)**. When channels are merged, magnified inserts show IL-9 and TfnRc colocalize in perinuclear regions of the cell at baseline, with cytokine pools found within endosomal compartments **(Figure 3.1, 0 min)**. At 5 min, 15min, and 60 min of PAF stimulation, IL-9 and TfnRc colocalization appears to be present at a larger extent in perinuclear regions and at the cell periphery **(Figure 3.1, Merged)**. Intracellular IL-9 is found almost completely overlapping with endosomal compartments at these timepoints when compared to baseline **(Figure 3.1, 5 and 15 and 60 min Merged)**.

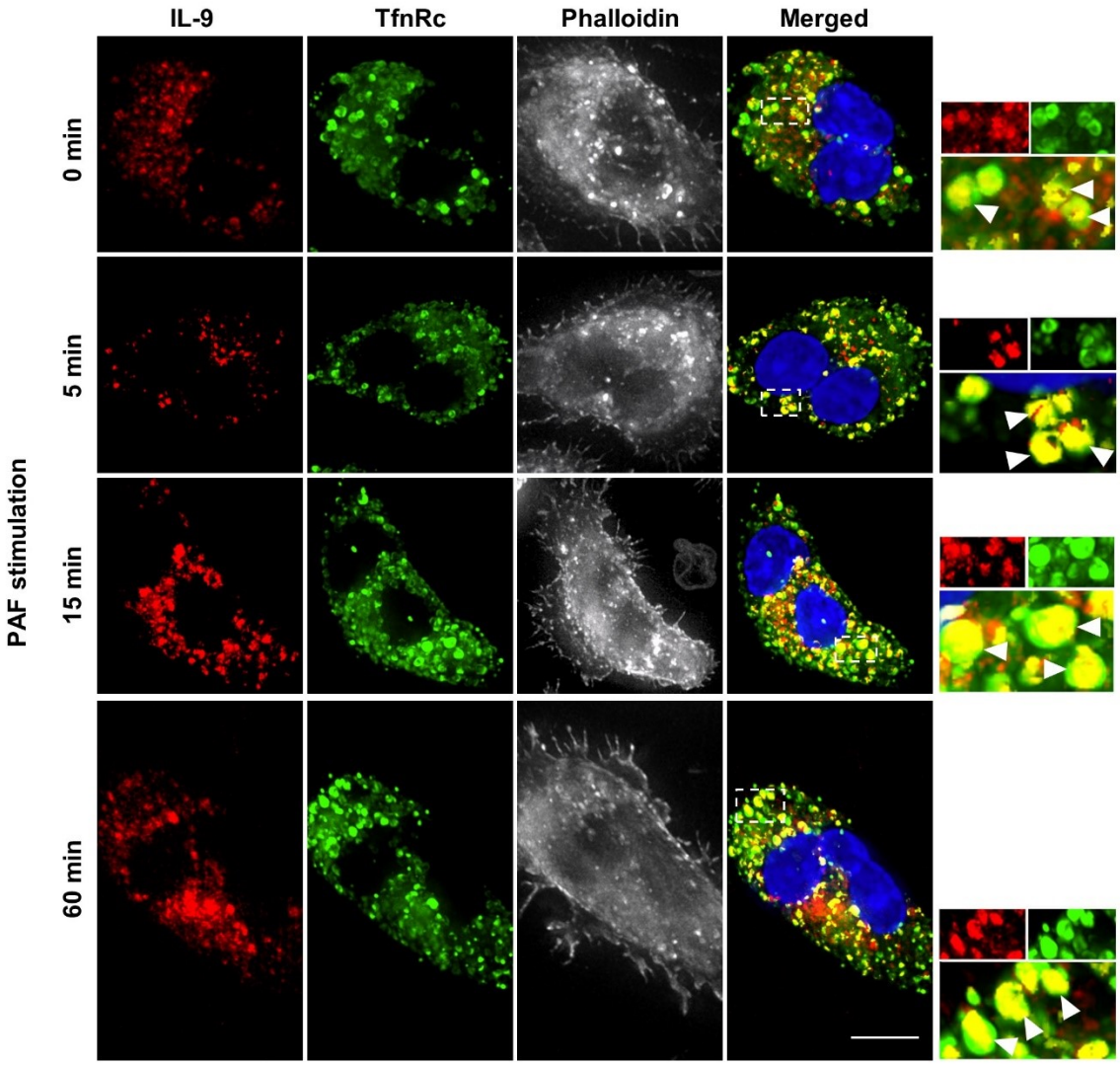

IL-9
TfnRc
Phalloidin
Merged
PAF stimulation
0 min
5 min
15 min
60 min

Figure 3.1. Increased IL-9 colocalization with TfnRc$^+$ endosomes in PAF-stimulated eosinophils. Human eosinophils adhered to glass coverslips were stimulated with PAF for 0, 5, 15, and 60 min. Following fixation and permeabilization, cells were labeled with anti-IL-9 (red) and anti-TfnRc (green) as a marker for recycling endosomes. Cells were then imaged with a DeltaVision OMX super-resolution microscope. Actin cytoskeleton was stained with rhodamine-phalloidin (grey). Nuclei were stained with DAPI (blue). Merged images with high magnification insets are shown at right; white arrowheads indicate yellow regions in which colocalization between intracellular cytokine and endosomes exist. Cells shown were prepared and imaged by S Almas. Image processing in Imaris software involving creation of yellow masks in merged panels to note colocalization between cytokine and organelles and creation of high magnification inserts were my original work. Maximum intensity projections (MIPs) from Imaris software are shown here. Scale bar represents 5 μm.

3.2 Intracellular IL-9 colocalizes with CD63-positive crystalloid granules in human eosinophils at baseline and upon PAF stimulation.

As eosinophil crystalloid granules house a plethora of cytokines, I hypothesized IL-9 could also be stored here. IF staining was employed once again to double-label for intracellular IL-9 and CD63, a marker of crystalloid granule membranes, in resting and PAF-stimulated human peripheral blood eosinophils. By immunostaining at all these conditions, I tested (a) whether IL-9 was stored in crystalloid granules at resting or stimulated conditions and (b) if IL-9 is stored in crystalloid granules, whether IL-9 storage in this compartment changes upon stimulation, which would be suggestive of cytokine trafficking.

CD63 labeled granules largely appear as ring-shaped structures and densely populate the cell cytosol at baseline and after 5 min of PAF stimulation **(Figure 3.2, CD63 panels)**. At 15 and 60 min of PAF stimulation, CD63 more crisply decorates the crystalloid granule membrane with less staining observed within the centre of granules **(Figure 3.2, CD63 panels)**. Once again, intracellular IL-9 staining was enriched in perinuclear regions at resting state and increasingly distributed towards the cell edges by later time points of PAF stimulation **(Figure 3.2, IL-9 panels)**. IL-9 and $CD63^+$ granules mostly colocalize in perinuclear regions in resting condition **(Figure 3.2, 0 min Merged)**. At 5 min of PAF stimulation, a greater degree of colocalization between IL-9 and granules is seen near the cell periphery before returning to colocalization at perinuclear and cytosolic regions at 15 and 60 min of PAF stimulation **(Figure 3.2, Merged)**. Notably, magnified inserts display the pattern of colocalization at resting conditions and 5 min of PAF stimulation to be that of CD63 completely encapsulating IL-9 within the granules, whereas at 15 and 60 min of PAF stimulation only portions of CD63 colocalize with granular IL-9 **(Figure 3.2, Merged)**.

3.3 Intracellular IL-13 stably colocalizes with endosomal compartments bearing TfnRc in eosinophils at baseline and during PAF stimulation.

While delineating storage and trafficking sites of IL-9, I also hypothesized that IL-13 could similarly be found stored in recycling endosomes in eosinophils. Immunolabeling for intracellular IL-13 pools in both resting and PAF-stimulated eosinophils consistently appeared as evenly distributed granular shapes within the cell cytoplasm **(Figure 3.3., IL-13 panels)**. To note, IL-13 puncta appeared brightest in resting cells and most faint at 60 min of PAF stimulation **(Figure 3.3,**

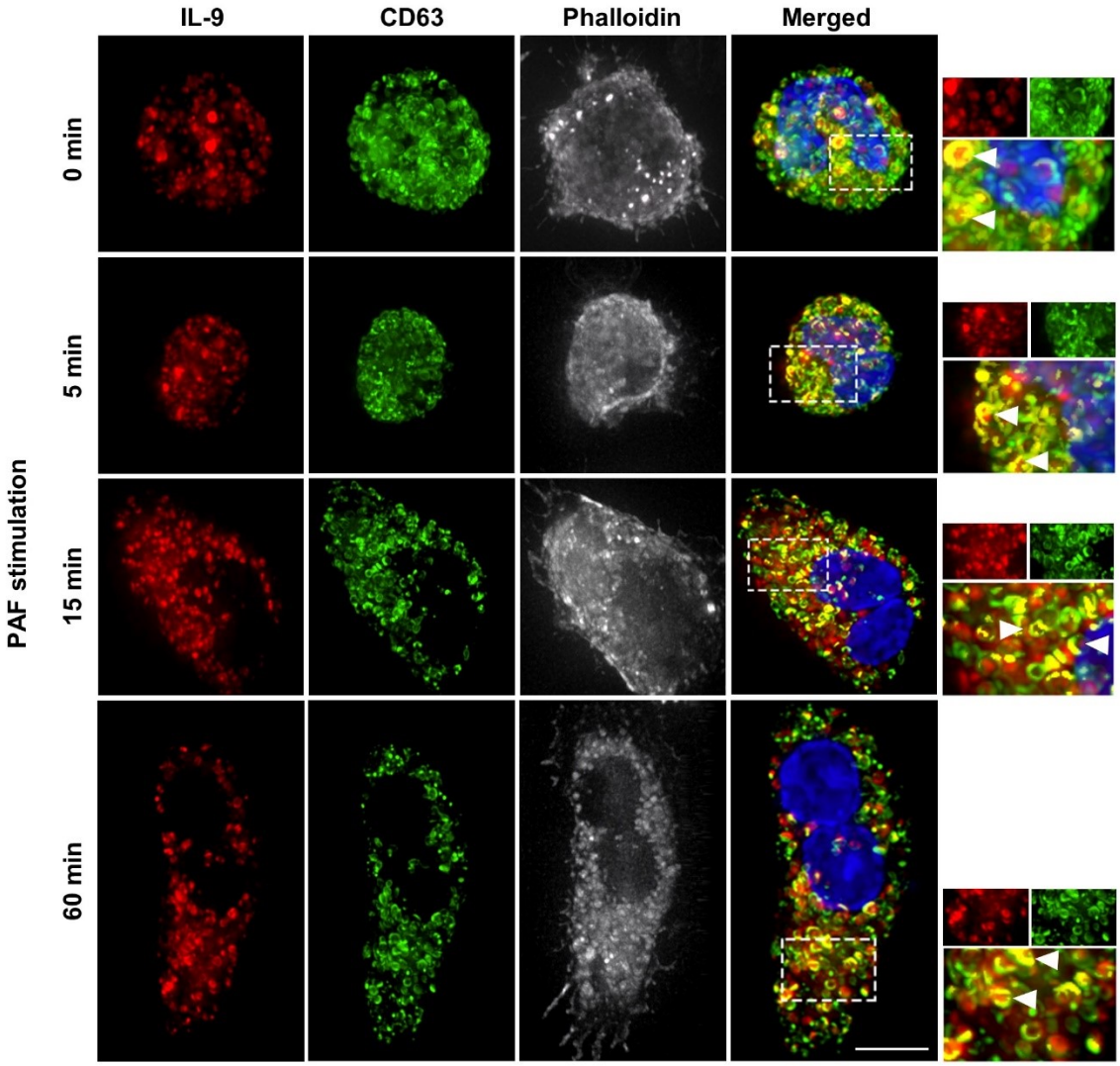

IL-9
CD63
Phalloidin
Merged
PAF stimulation
0 min
5 min
15 min
60 min

Figure 3.2. IL-9 colocalizes with CD63$^+$ crystalloid granules in resting and PAF-stimulated eosinophils. Human eosinophils were prepared as described in Figure 3.1. Here, cells were labeled with anti-IL-9 (red) and anti-CD63 (green) as a marker for crystalloid granules. White arrowheads in magnified inserts at right indicate yellow regions in which colocalization between intracellular cytokine and crystalloid granules exist. Cells shown were prepared and imaged by S Almas. Image processing in Imaris software involving creation of yellow masks in merged panels to note colocalization between cytokine and organelles and creation of high magnification inserts were my original work. Maximum intensity projections (MIPs) from Imaris software are shown here. Scale bar represents 5 μm.

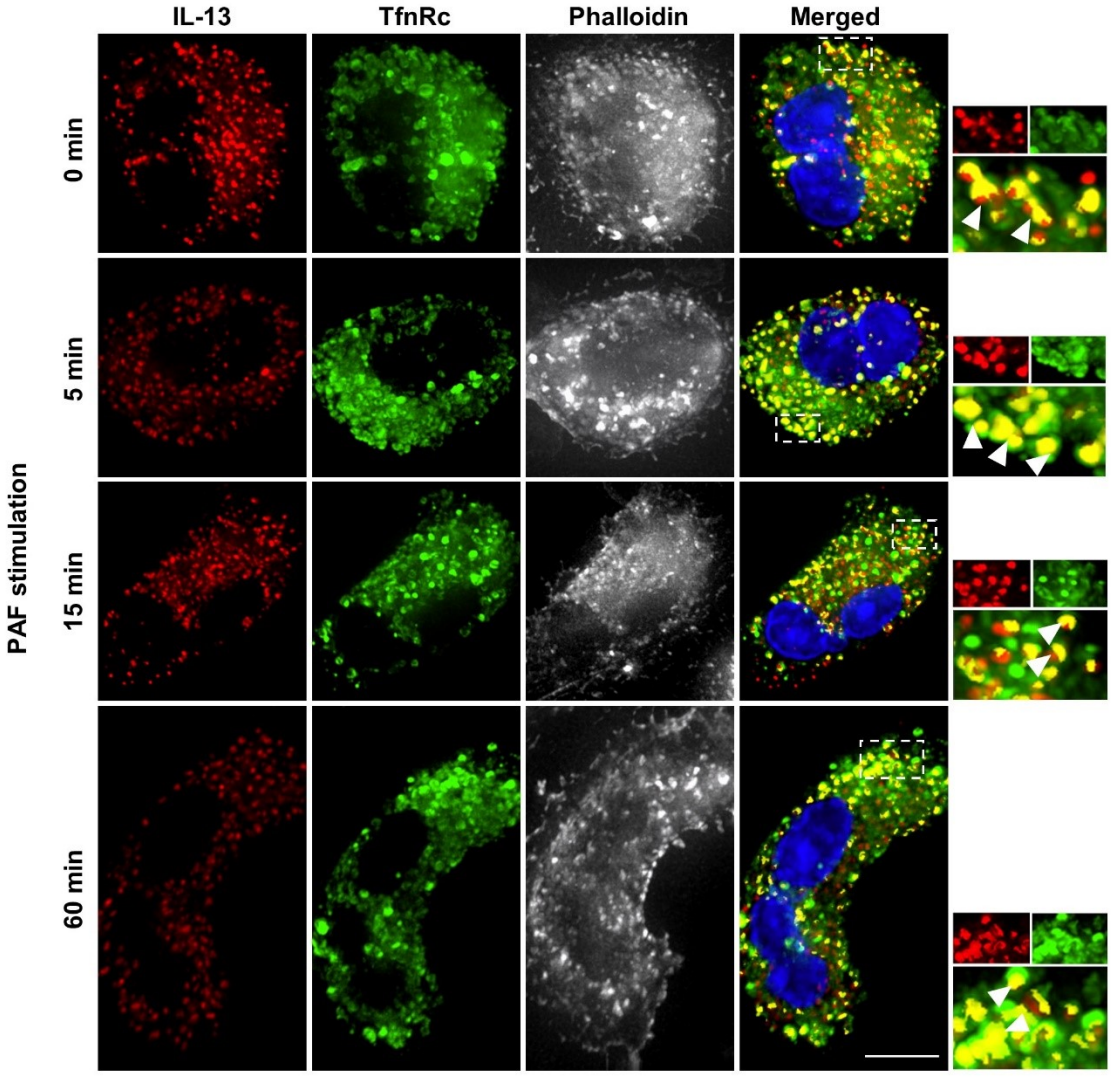

IL-13
TfnRc
Phalloidin
Merged
PAF stimulation
0 min
5 min
15 min
60 min

Figure 3.3. IL-13 colocalizes with TfnRc^{+} endosomes in resting and PAF-stimulated eosinophils. Human eosinophils were prepared as described in Figure 3.1. Here, cells were labeled with anti-IL-13 (red) and anti-TfnRc (green) as a marker for recycling endosomes. White arrowheads in magnified inserts at right indicate yellow regions in which colocalization between intracellular cytokine and endosomes exist. Cells shown were prepared and imaged by S Almas. Image processing in Imaris software involving creation of yellow masks in merged panels to note colocalization between cytokine and organelles and creation of high magnification inserts were my original work. Maximum intensity projections (MIPs) from Imaris software are shown here. Scale bar represents 5 μm.

IL-13 panels). Merging of IL-13 and TfnRc$^+$ endosomes immunolabelling revealed colocalization throughout the cytosol and at the cell surface of resting human blood eosinophils **(Figure 3.3, 0 min Merged)**. Colocalization between IL-13 and TfnRc upon PAF stimulation was similarly dispersed throughout the cell cytosol and at the cell periphery; however, there were many observations of IL-13 completely filling TfnRc$^+$ endosomes at 5 min of PAF stimulation **(Figure 3.3., 5 and 15 and 60 min Merged)**.

3.4 Intracellular IL-13 decreasingly colocalizes with CD63-positive crystalloid granules upon PAF stimulation in human eosinophils.

Immunolabeling for IL-13 and CD63$^+$ crystalloid granules revealed degrees of colocalization throughout the cell cytosol in resting eosinophils **(Figure 3.4, 0 min Merged)**. At 5, 15, and 60 min of PAF stimulation, there is a gradual decrease in IL-13 and CD63 colocalization in the cell cytosol **(Figure 3.4, Merged)**. While IL-13 appears to occupy the majority of the CD63$^+$ granule compartment in resting cells, cytosolic IL-13 is increasingly observed outside of granules at 15 and 60 min of PAF stimulation **(Figure 3.4, white arrowheads in Merged)**.

3.5 Dynamic changes of IL-9 and IL-13 colocalization with crystalloid granules and endosomal compartments upon PAF stimulation through measurements with PCC.

While colocalization was visually represented in merged channels of immunolabelled cytokines and organelles of interest, PCC was also used as a measure for colocalization between labelled probes on a per cell basis as described in Section 2.10 of Materials and Methods. MFI values of cytokine staining were additionally collected on a per cell basis as a measure of intracellular protein levels. PCC and MFI measurements were extracted from 50 eosinophils at baseline (0min) and at 5, 15, and 60 min of PAF stimulation for every donor **(Figure 3.5)**. These measurements from each donor were also averaged and analyzed together as a grouped dataset **(Figure 3.6)**.

In an individual donor, IL-9 MFI values significantly increased upon PAF stimulation in eosinophils **(Figure 3.5A)**. This donor is representative of the trend seen when IL-9 MFI measurements of five donors were grouped, in which IL-9 MFI significantly increases at 5, 15, and 60 min of PAF stimulation ($p < 0.01$) **(Figure 3.6A, D1)**. Contrastingly, in an individual donor, IL-13 MFI values significantly decreased at 5 and 60 min of PAF stimulation **(Figure 3.5D)**. This donor, along with two others, is representative of the significant decrease in IL-13 MFI at 60 min of PAF stimulation observed in the grouped dataset ($p < 0.01$) **(Figure 3.6D, D1-3)**.

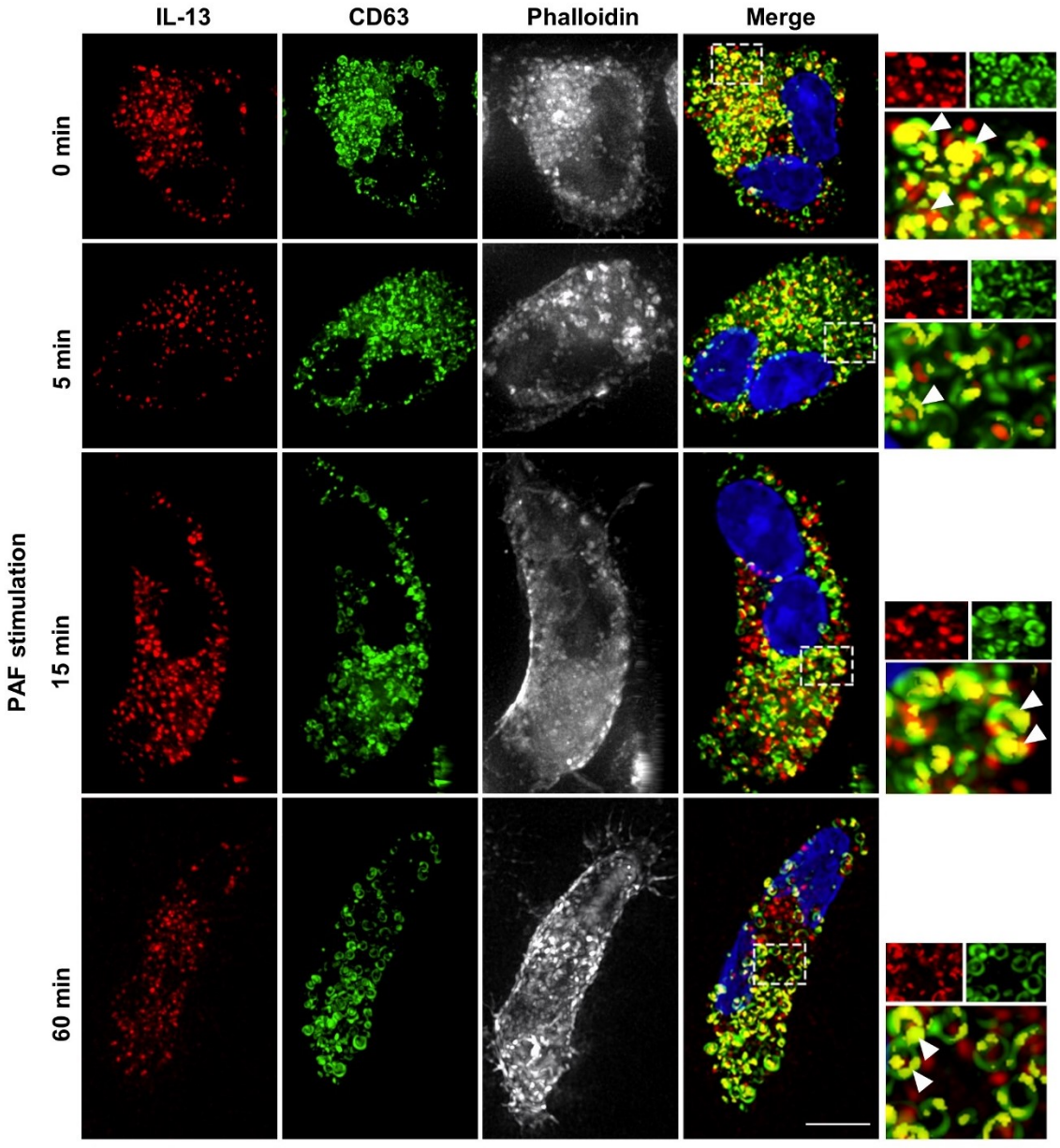

IL-13
CD63
Phalloidin
Merge
PAF stimulation
0 min
5 min
15 min
60 min

Figure 3.4. IL-13 decreasingly colocalizes with CD63^{+} crystalloid granules upon PAF stimulation in human eosinophils. Human eosinophils were prepared as described in Figure 3.1. Here, cells were labeled with anti-IL-13 (red) and anti-CD63 (green) as a marker for crystalloid granules. White arrowheads in magnified inserts at right indicate yellow regions in which colocalization between intracellular cytokine and crystalloid granules exist. Cells shown were prepared and imaged by S Almas. Image processing in Imaris software involving creation of yellow masks in merged panels to note colocalization between cytokine and organelles and creation of high magnification inserts were my original work. Maximum intensity projections (MIPs) from Imaris software are shown here. Scale bar represents 5 μm.

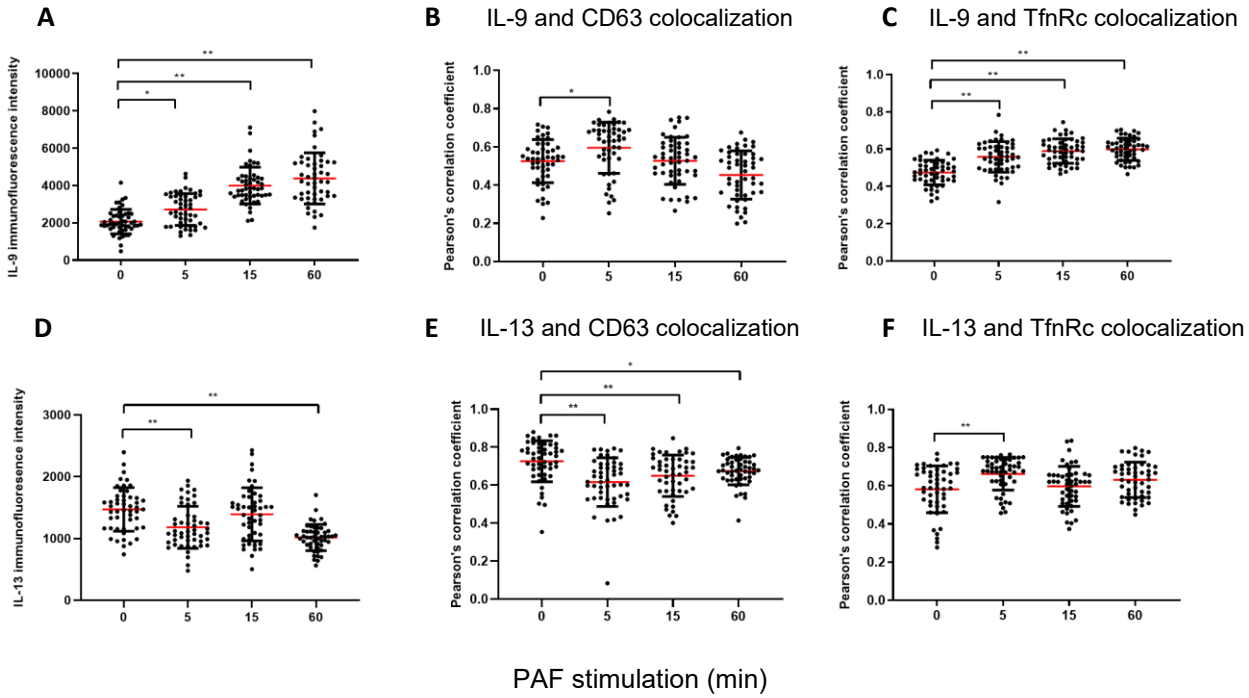


Figure 3.5. IL-9 and IL-13 intensity and colocalization with CD63$^+$ crystalloid granules and TfnRc$^+$ vesicles change dynamically in response to PAF stimulation. Immunofluorescence intensity values were measured for intracellular IL-9 (A) and IL-13 (D) in a time course analysis of PAF stimulation using Volocity analysis software. Pearson's correlation coefficients were measured for intracellular IL-9 and CD63 (B) or TfnRc (C), as well as IL-13 and CD63 (E) or TfnRc (F). In all plots a minimum of 50 eosinophils were analyzed within an individual donor. Donor data presented here was collected by S Almas. Means are shown by red lines. Error bars indicate SD. Kruskal-Wallis test followed by Dunn's multiple comparisons test were performed in GraphPad Prism. * $p < 0.05$ and ** $p < 0.01$.

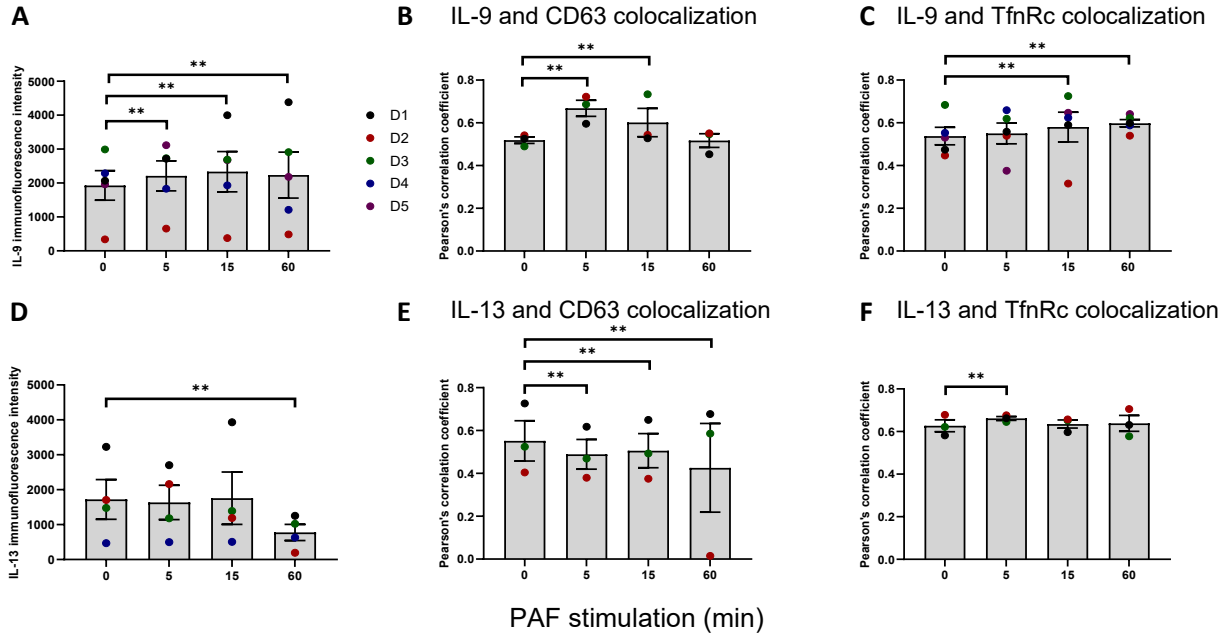


Figure 3.6. Intensity and colocalization of IL-9 and IL-13 with CD63$^+$ granules and TfnRc$^+$ vesicles changes with PAF stimulation in eosinophils grouped from all donors recruited. Mean immunofluorescence intensity (A, D) and Pearson's correlation coefficient (B, C, E and F) were measured in a time course analysis of PAF stimulation using Volocity analysis software. Each plot displays a minimum of 3 donors, from which 50 eosinophils were analyzed with their measurements averaged for each PAF stimulation timepoint and shown as a coloured dot. Donor numbers represent replicates for each data set and are not assigned to an individual. S Almas collected data for 8 donors presented here. I collected data for 6 donors presented and performed data analysis for the whole grouped donor dataset. Mean values of 150-250 measurements from all donors at each time point are shown by a bar. Error bars indicate SEM. A two-way ANOVA followed by Dunnett's multiple comparisons test were performed in GraphPad Prism. *$p < 0.05$, and **$p < 0.01$.

In terms of IL-9 and CD63 colocalization, eosinophils from all three donors recruited for this dataset displayed a significant increase in colocalization at 5 min of PAF stimulation (0.67 ± 0.04) compared to resting conditions (0.52 ± 0.02) ($p < 0.01$) **(Figure 3.5B and Figure 3.6B)**. IL-9 and CD63 colocalization also significantly increased at 15 min of PAF stimulation compared to baseline for one donor **(Figure 3.6B, D3)**. IL-9 and TfnRc colocalization significantly increased at 5, 15, and 60 min of PAF stimulation relative to resting conditions in one donor (**Figure 3.5C)**. When data from four donors was grouped, IL-9 and TfnRc colocalization also significantly increased at 15 min of PAF stimulation (0.58 ± 0.07) and 60 min of PAF stimulation (0.60 ± 0.02) compared to baseline (0.54 ± 0.04) ($p < 0.01$) **(Figure 3.6C)**.

Next, IL-13 and CD63 colocalization significantly decreased at 5 (0.50 ± 0.07), 15 (0.51 ± 0.08), and 60 min (0.43 ± 0.21) of PAF stimulation compared to resting control (0.55 ± 0.09) ($p < 0.01$) in a grouped data set of three donors **(Figure 3.5E and Figure 3.6E)**. Meanwhile, IL-13 and TfnRc colocalization increased at 5 min of PAF stimulation (0.66 ± 0.01) prior to returning to measurements close to baseline at 15 (0.64 ± 0.02) and 60 min (0.64 ± 0.04) of PAF stimulation **(Figure 3.5F and Figure 3.6F)**.

3.6 Intracellular IL-9 is predominantly found in TfnRc-positive endosomal compartments relative to CD63-positive granules in resting human eosinophils based on a novel object-based colocalization method.

Prior data measuring IL-9 colocalization with $CD63^+$ granules or $TfnRc^+$ endosomes with PCC values revealed significant changes in granule- and endosome-associated cytokine pools upon PAF stimulation. To explore other methods of measuring colocalization between IL-9 and organelles of interest, I developed an object-based colocalization protocol in Imaris as described in section 2.11. This method also tests for the proportion of intracellular IL-9 found within organelles of interest in resting and PAF-stimulated eosinophils on a per cell basis. Inversely, the proportion of organelles of interest containing IL-9 was also calculated per cell under the same conditions.

At baseline, 6.4% of intracellular IL-9 was found within $CD63^+$ granules and significantly decreased after 15 (0.9% ± 0.72) and 60 min (3.3% ± 1.81) of PAF stimulation (**Figure 3.7A**). Meanwhile, 21% of IL-9 was found within $TfnRc^+$ endosomes at baseline and significantly increased following 5 min (29% ± 11.00) of PAF stimulation (**Figure 3.7C**).

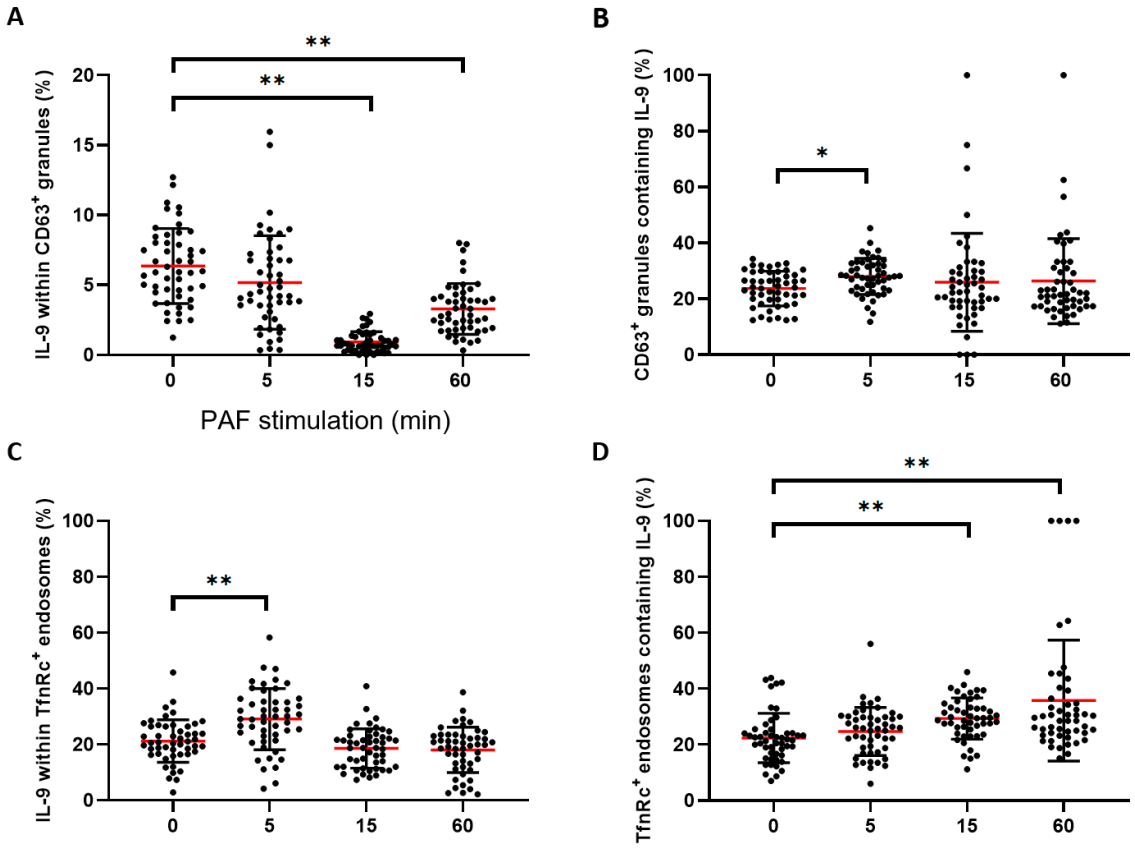


Figure 3.7. Greater proportions of intracellular IL-9 are found in TfnRc$^+$ endosomes when compared to proportions of IL-9 within CD63$^+$ crystalloid granules. Human eosinophils were prepared and imaged as described in Figure 3.1 by S Almas. Intracellular IL-9, CD63$^+$ granules, and TfnRc$^+$ endosomes were then defined on a per cell basis in Imaris software as described in the protocol I developed in Section 2.11. Total counts of these 'spots' were also extracted per cell. IL-9 defined 'spots' co-located within CD63$^+$ granules or TfnRc$^+$ endosomes were then quantified on a per cell basis. Percentages of total IL-9 found within CD63$^+$ granules (A) or TfnRc$^+$ endosomes (C) could then be measured per cell, as well as percentages of total CD63$^+$ granules or TfnRc$^+$ endosomes containing IL-9 (B and D). At each timepoint, 50 eosinophils were analyzed from an individual donor. Object-based colocalization data collection and analysis shown here is my original work. Means are represented as red lines and error bars indicate SD. Kruskal-Wallis test followed by Dunn's multiple comparisons test were performed in GraphPad Prism (version 8). *$p < 0.05$, ** $p < 0.01$.

In resting eosinophils, 24% of $CD63^+$ granules contained IL-9 and this proportion significantly increased after 5 min (28% ± 6.45) of PAF stimulation **(Figure 3.7B)**. In addition, the proportion of $TfnRc^+$ endosomes containing IL-9 significantly increased at 15 (29% ± 7.35) and 60 min (36% ± 22) of PAF stimulation compared to resting cells (22% ± 8.84) **(Figure 3.7D)**.

Prior to stimulation, 257 IL-9^+ spots were defined in eosinophils and significantly increased at 5 (383 ± 152), 15 (802 ± 228), and 60 min (337 ± 102) of PAF stimulation **(Figure 3.8A)**. Eosinophils at baseline contained 69 $CD63^+$ spots with the number of spots decreasing at 15 and 60 min of PAF stimulation (31 ± 25 and 47 ± 23 $CD63^+$ spots, respectively) **(Figure 3.8B)**. On average, resting eosinophils contained 89 $TfnRc^+$ spots which significantly decreased to 74 ± 35 and 62 ± 37 at 5 and 60 min of PAF stimulation, respectively **(Figure 3.8C)**.

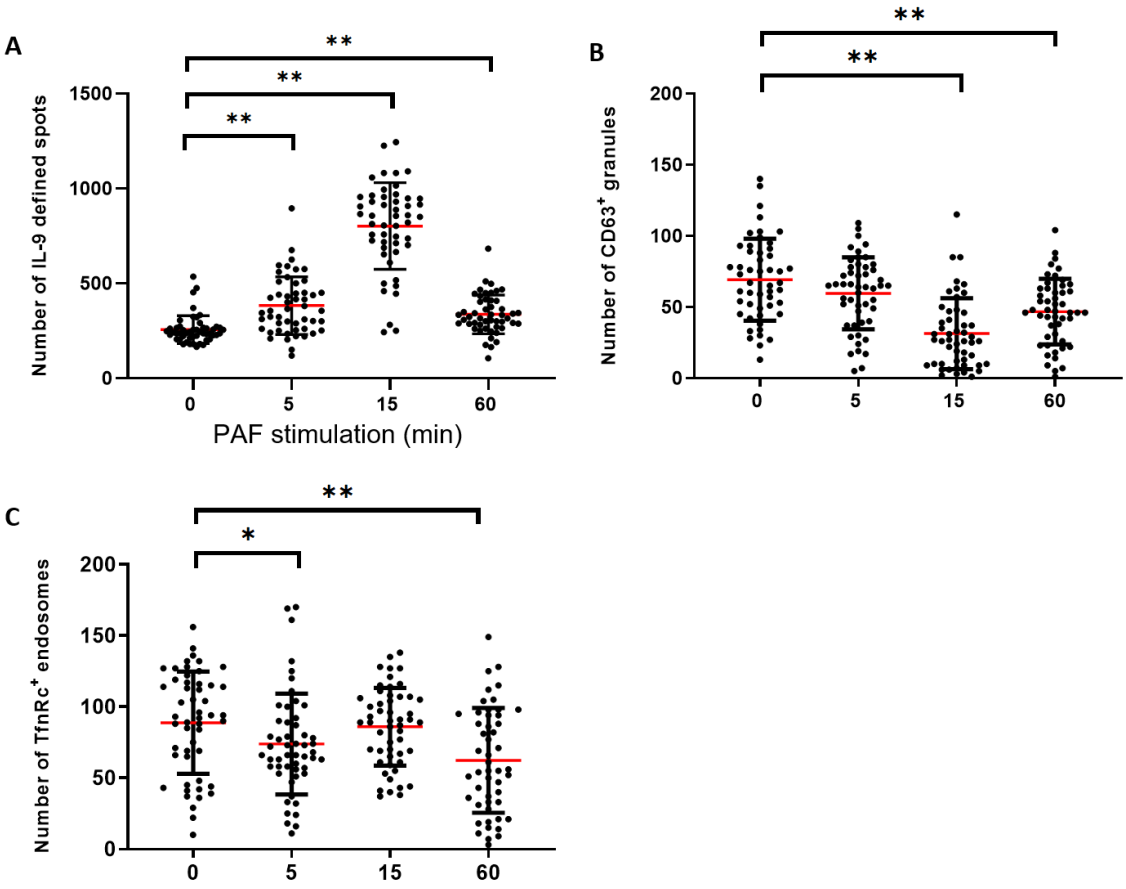


Figure 3.8. PAF stimulation drastically alters the proportion of intracellular IL-9, CD63$^+$ granules, and TfnRc$^+$ endosomes found in eosinophils. Human eosinophils were prepared and imaged as described in Figure 3.1 by S Almas. Then, eosinophils were analyzed in the protocol I developed as described in Section 2.11 and Figure 3.7. Briefly, intracellular IL-9 (A), CD63$^+$ granule (B), and TfnRc$^+$ endosome (C) 'spots' were quantified on a per cell basis. At each timepoint, 50 eosinophils were analyzed from an individual donor. Object-based colocalization data collection and analysis shown here is my original work. Means are represented as red lines and error bars indicate SD. Kruskal-Wallis test followed by Dunn's multiple comparisons test were performed in GraphPad Prism (version 8). *$p < 0.05$, ** $p < 0.01$.

Chapter 4: Discussion

As will be discussed in this chapter, this study reveals for the first time an association between $TfnRc^+$ endosomes and cytokine trafficking in eosinophils. Furthermore, by investigating associations of intracellular IL-9 and IL-13 with both $TfnRc^+$ endosomes and $CD63^+$ granules in a PAF stimulation time course, these two cytokines are found to have differing models of cytokine trafficking in eosinophils.

4.1 Upon PAF stimulation in human eosinophils, detection of intracellular IL-9 increases and $TfnRc^+$ endosomes become novel traffickers of intracellular IL-9 while smaller pools of IL-9 are localized to $CD63^+$ granules.

In this study, PAF was used as a stimulant of human eosinophils to investigate intracellular IL-9 and IL-13 trafficking. PAF has been reported to induce the release of both cytokines from human eosinophils within 60 min.[88] PAF also acts as an eosinophil chemoattractant and characteristically induces a change in cell shape, indicative of actin cytoskeletal remodeling.[86] Poles of the cells become enriched with filamentous actin that then mediate cell motility. Morphologically, within 15 min of PAF stimulation, eosinophil shape changes from round compact cells to stretched cells seen with phalloidin staining **(Figures 3.1, 3.2, 3.3, and 3.4)**. Stretching of the bilobed nucleus stained with DAPI is also observed in PAF-stimulated eosinophils **(Figures 3.1, 3.2, 3.3, and 3.4)**. These morphological changes provide cues that PAF is indeed stimulating these cells and should be accompanied with trafficking and secretion of IL-9 and IL-13.

Through immunofluorescence microscopy images, we observed that IL-9 immunostaining is increasingly detected in perinuclear regions and throughout the cell cytosol after 60 min of PAF stimulation **(Figures 3.1 and 3.2)**. This staining distribution could suggest IL-9 is increasingly being translated in the presence of PAF; perinuclear IL-9 staining may represent nascent protein found in the ER or Golgi apparatus and cytoplasmic granular staining suggests IL-9 is trafficked to membrane-bound compartments in the cell for secretion or storage. While IL-9 mRNA and protein have previously been detected in human blood eosinophils, IL-9 storage and trafficking sites have not been fully elucidated.[310] As recycling endosomes bearing markers such as TfnRc, VAMP-3, and Rab11 have been described in constitutive release of newly synthesized TNF and IL-6 in macrophages **(Figure 1.6)**, I hypothesized endosomal compartments bearing TfnRc could also be involved in trafficking newly made IL-9.[286] Interestingly, perinuclear IL-9 at 5 and 15 min of PAF stimulation increasingly colocalize with $TfnRc^+$ endosomes **(Figure 3.1, white**

arrowheads). This suggests preformed IL-9, synthesized prior to PAF stimulation given the brief timeframe, is leaving the trans-Golgi network and is being transported into $TfnRc^+$ endosomes akin to *de novo* IL-6 and TNF in macrophages.[286] At 60 min of PAF stimulation, IL-9 again colocalizes with $TfnRc^+$ endosomes in perinuclear regions and near the cell periphery **(Figure 3.1, white arrowheads)**. IL-9 and TfnRc colocalization at perinuclear regions after 60 min of PAF stimulation further suggests PAF induces IL-9 *de novo* synthesis. IL-9 and TfnRc colocalization at the cell periphery suggests these endosomes are involved in trafficking newly made IL-9 to the plasma membrane for release.

Eosinophil crystalloid granules are also well-recognized sites of storage for cytokines and I hypothesize IL-9 can be stored within these compartments. By immunolabelling IL-9 and $CD63^+$ crystalloid granules, IL-9 is found to colocalize with $CD63^+$ granules in the absence of PAF **(Figure 3.2, 0 min white arrowheads)**. This suggests IL-9 can be found stored in eosinophil crystalloid granules at rest in human eosinophils. At 5 min of PAF stimulation increasing amounts of IL-9 colocalized with $CD63^+$ granules near the cell surface **(Figure 3.2, white arrowheads)**. In comparison, at 15 and 60 min of PAF stimulation, more IL-9 is seen outside of granules in the cell cytosol and near the nucleus **(Figure 3.2, merged)**. These observations implicate that while crystalloid granules may store IL-9 at low basal levels, the increasing amounts of IL-9 that may be due to *de novo* synthesis are not observed to shuttle into $CD63^+$ granules throughout PAF stimulation.

To substantiate the previous observations in immunofluorescence microscopy images, measures of PCC and MFI were collected on a per cell basis to discern changes in colocalization between cytokines and organelles and to measure intracellular protein levels, respectively. Additionally, an object-based colocalization method was employed to quantify the levels of intracellular IL-9 found within organelles of interest and proportions of organelles containing IL-9 throughout the PAF stimulation time course. Upon PAF stimulation, IL-9 immunofluorescence intensity significantly increased compared to resting cells which is suggestive of increased intracellular IL-9 protein levels **(Figures 3.5A, 3.6A)**. In the object-based colocalization method, $IL\text{-}9^+$ spots also significantly increased relative to cells at baseline **(Figure 3.8A)**. This increased detection of IL-9 further suggests that *de novo* synthesis of IL-9 is induced by PAF.

Given these eosinophils were briefly cultured during PAF stimulation time courses, it is also possible that IL-9 is being endocytosed from the extracellular environment leading to elevated intracellular IL-9 levels and the increased immunofluorescence intensity or IL-9$^+$ spots observed by IF. Recently, it has been suggested that the uptake of extracellular cytokines by immune cells functions as negative feedback regulation on the biosynthesis of cytokines.[311] Both newly synthesized and endocytosed cytokines can be found within recycling endosomes and signal through their respective receptors found within the endosomal membrane to downregulate cytokine expression.[311] Notably, in our studies, increased IL-9 staining was paired with an increased colocalization with TfnRc$^+$ endosomes upon PAF stimulation measured with two distinct colocalization methods **(Figures 3.5C, 3.6C)**. Furthermore at 15 and 60 min of PAF stimulation, there are significant increases in TfnRc$^+$ endosomes containing IL-9 compared to resting cells **(Figure 3.7D)**. As previously discussed, newly synthesized IL-9 from the trans-Golgi network may be shuttling into these TfnRc$^+$ endosomes and account for these increases in endosomal-associated-IL-9. Now, I also pose that IL-9 found within TfnRc$^+$ endosomes may be taken up from the extracellular milieu and potentially regulate subsequent transcription or translation of IL-9 through IL-9Rα.

While intracellular IL-9 was increasingly associated with TfnRc$^+$ endosomes throughout PAF-stimulation, IL-9 was also increasingly associated with CD63$^+$ granules at 5 and 15 min of PAF stimulation **(3.5B, 3.6B, and 3.7B)**. These results suggest that IL-9 is additionally being trafficked into CD63$^+$ crystalloid granules as well as TfnRc$^+$ endosomes. Notably, object-based colocalization revealed that 3.5 times the amount of intracellular IL-9 is found in TfnRc$^+$ endosomes as opposed to CD63$^+$ crystalloid granules in resting cells **(Figure 3.7A and C)**. This result emphasizes that TfnRc$^+$ endosomes are major traffickers of IL-9 in human eosinophils at rest and to a greater extent upon PAF stimulation which is paired with elevated IL-9 levels. These findings are consistent with the interpretation that IL-9 is newly synthesized upon PAF stimulation, follows the classical secretory pathway and shuttles into TfnRc$^+$ endosomes for release, as reported for *de novo* synthesized TNF in macrophages.[281] Results from IL-9 and CD63 colocalization suggest lesser amounts of newly made IL-9, relative to TfnRc$^+$ endosomes, are shuttled into crystalloid granules for cytokine storage upon PAF stimulation. Crystalloid granules bearing CD63 and VAMP-7 have previously been implicated in IL-9 release in mouse eosinophils.[252] In this study, IL-9 release from PAF-stimulated eosinophils was not measured but a similar association

between $CD63^+$ crystalloid granules and IL-9 was found in human eosinophils. While not a direct measure of cytokine release, the number of $CD63^+$ spots did significantly decrease at 15 and 60 min of PAF stimulation suggesting these granules are undergoing membrane fusions and could be involved in releasing stored IL-9 **(Figure 3.8B)**. $TfnRc^+$ spots also decreased at 5 and 60 min of PAF stimulation suggesting endosomes were engaging in membrane fusion events **(Figure 3.8C)**. This suggests for the first time that intracellular IL-9 is increasingly trafficked into $TfnRc^+$ recycling endosomes, as well as other compartments of the endosomal network, potentially routed to the cell surface for membrane fusion and release.

4.2 Upon PAF stimulation in human eosinophils, detection of intracellular IL-13 decreases, and IL-13 is decreasingly associated with $CD63^+$ granules while $TfnRc^+$ endosome-associated IL-13 remains relatively static.

IL-13 has previously been identified as the most abundant preformed cytokine in human eosinophils and has been localized to $CD63^+$ crystalloid granules in subcellular fractionation experiments.[23] Indeed, in this study, immunofluorescence labeling revealed colocalization between IL-13 and $CD63^+$ granules throughout the cytoplasm of resting human eosinophils **(Figure 3.4, 0 min white arrowheads)**. Upon PAF stimulation, there appears to be a decrease in IL-13 and CD63 colocalization, in which more IL-13 immunolabelling is observed outside of $CD63^+$ granules at 15 and 60 min of PAF stimulation **(Figure 3.4, merged)**. Given these results, it is suggested that IL-13 is being shuttled out of storage in eosinophil crystalloid granules upon PAF stimulation and routed for extracellular release. Notably, the granular, diffuse staining of IL-13 throughout the cell cytosol also appeared faintest in immunofluorescence images of eosinophils stimulated with PAF for 60 min compared to resting cells **(Figure 3.3 and 3.4)**. This lowered detection of IL-13 suggests this cytokine was indeed secreted from PAF-stimulated eosinophils. Using flow cytometry-based multiplex cytokine detection assays or ELISAs on cell supernatants, PAF has been reported to induce IL-13 release in human eosinophils at a concentration of about 7 pg/ml in 60 min.[88]

As a novel finding, IL-13 was also observed to colocalize with $TfnRc^+$ endosomes in immunofluorescence imaging of resting and PAF-stimulated eosinophils **(Figure 3.3, merged)**. At 5 min of PAF stimulation, a greater degree of IL-13 almost completely colocalized with $TfnRc^+$ endosomes, often seen in perinuclear regions and at the cell surface **(Figure 3.3, white**

arrowheads). At 5 min PAF stimulation timepoint, a small burst of *de novo* IL-13 leaving the Golgi may have shuttled into $TfnRc^+$ endosomes for constitutive release at the cell surface. As eosinophil crystalloid granules should be an abundant source of preformed IL-13 and IL-13 was observed shuttling out of $CD63^+$ granules upon PAF stimulation, it is possible that small amounts of this cytokine trafficked into endosomal compartments as well. IL-13 often colocalized with $TfnRc^+$ endosomes at the cell peripheries at 15 and 60 min of PAF stimulation potentially poising the cells for IL-13 release at the plasma membrane **(Figure 3.4, merged)**.

Upon PAF stimulation and in contrast to IL-9, IL-13 immunofluorescence intensity decreases which suggests intracellular IL-13 protein levels are decreased **(Figure 3.5D and 3.6D)**. This supports the notion that IL-13 is being secreted by PAF-stimulated eosinophils. Along with results of a decrease in IL-13 detection in eosinophils stimulated with PAF, IL-13 colocalization with $CD63^+$ granules measured with PCC significantly decreased at each time point of PAF stimulation **(Figure 3.5E and 3.6E)**. This decreased colocalization between IL-13 and CD63 once again suggests IL-13 is shuttling out of $CD63^+$ granules potentially routed for release at the cell surface.

Interestingly, IL-13 was also found to increasingly colocalize with $TfnRc^+$ endosomes at 5 min of PAF stimulation in PCC measurements before returning to baseline **(Figure 3.5F and 3.6F)**. As opposed to IL-9 that was increasingly associated with $TfnRc^+$ endosomes throughout PAF stimulation, endosome-associated IL-13 was relatively static in that there was not a constant influx or efflux of cytokine to this compartment that would be reflected in changes in PCC in the PAF time course. This static pool of IL-13 in $TfnRc^+$ endosomes despite IL-13 detection within cells decreasing suggests that these endosomes are not delivering IL-13 to the cell surface for release. Given recycling endosomes are associated with exocytic transport of *de novo* synthesized cytokines, it appears that relatively little IL-13 is being newly made upon PAF stimulation and being shuttled into $TfnRc^+$ endosomes. Instead, IL-13 appears to predominantly exist as a preformed cytokine in $CD63^+$ crystalloid granules. This preformed IL-13 may be differentially released from crystalloid granules in secretory vesicles bearing VAMP-2 as has been described for CCL5.[249] It is also possible that $TfnRc^+$ endosomes labelled here can act as an intermediate vesicle for IL-13 release from crystalloid granules.

4.3 Concluding remarks

This project aimed to elucidate trafficking sites of IL-9 and IL13 in human eosinophils with hopes to identify therapeutic targets for asthma that would dampen this cytokine release and alleviate airway pathology. Findings with super resolution microscopy and colocalization analyses suggest varying trafficking pathways for these two cytokines upon PAF stimulation in human eosinophils, described in models as follows **(Figure 4.1 and 4.2)**:

- PAF stimulation may induce *de novo* IL-9 synthesis since low basal levels of the cytokine are found in resting cells. Resting eosinophils have been found to contain functional IL-4 and IL13 transcripts that are readily translated when stimulated.[312] Thus, resting eosinophils may also contain functional IL-9 mRNA that can be rapidly translated upon PAF stimulation and mature within the ER and Golgi. $TfnRc^{+}$ endosomes then act as novel traffickers of newly made IL-9 that leave the trans-Golgi, delivering this cytokine to the plasma membrane. If these $TfnRc^{+}$ endosomes also bear VAMP-3, a SNARE complex involving VAMP-3/Syntaxin-4/SNAP-23 at the cell membrane will mediate IL-9 release. Meanwhile at PAF stimulation time points, $TfnRc^{+}$ endosomes may also house endocytosed IL-9 where, in the presence of IL-9Rα, cytokine signaling can regulate cellular IL-9 production rates. Lesser amount of newly made IL-9 are shuttled into $CD63^{+}$ crystalloid granules for storage. Overall, this model suggests that IL-9 is mainly constitutively secreted from human eosinophils in the presence of PAF.
- PAF stimulation may induce regulated secretion of abundant, preformed IL-13 found in $CD63^{+}$ crystalloid granules. Secretory vesicles bearing VAMP-2 may be differentially releasing IL-13 to the cell membrane bearing Syntaxin-4 and SNAP-23. IL-13 can also be found in $TfnRc^{+}$ endosomes, however, trafficking into and out of this compartment is relatively static. Endosomal-associated IL-13 may be newly made or preformed IL-13 originating from granules. Overall, this model suggests that IL-13 mainly exists as a preformed cytokine that is most likely released via piecemeal degranulation from granules upon PAF stimulation.

Attempts to address further personalizing therapeutics for neutrophilic asthma by investigating TNF and IL-8 trafficking sites, with similar methods and aims to this study, can be found in

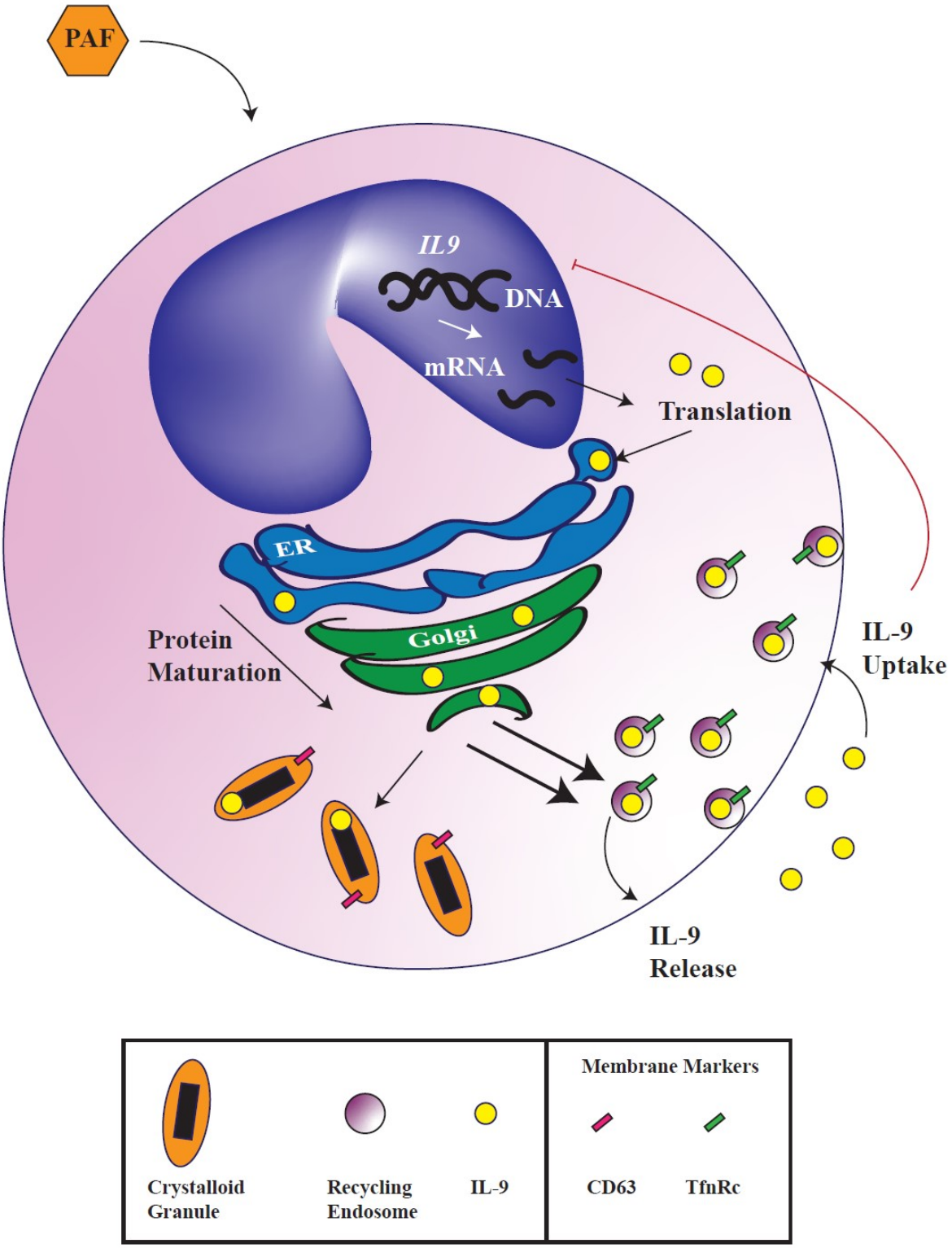

PAF
IL9
DNA
mRNA
Translation
ER
Golgi
Protein
Maturation
IL-9
Uptake
IL-9
Release
Crystalloid
Granule
Recycling
Endosome
IL-9
Membrane Markers
CD63
TfnRc

Figure 4.1. Proposed IL-9 trafficking pathway in human eosinophils stimulated with PAF involves constitutive secretion via TfnRc$^+$ endosomes. PAF induces translation of IL-9 from newly made or previously abundant IL-9 mRNA. Newly made IL-9 follows the classical secretory pathway, targeted to the ER and Golgi for protein maturation. At the *trans*-Golgi, newly made IL-9 is shuttled into TfnRc$^+$ endosomes for delivery to the plasma membrane for release. A lesser amount of this newly made IL-9 is shuttled into CD63$^+$ granules for storage and can be released upon further stimulation. Extracellular IL-9 that has been endocytosed can also be found in TfnRc$^+$ endosomes and has the potential to downregulate IL-9 biosynthesis through IL-9Rα on endosomal membranes.

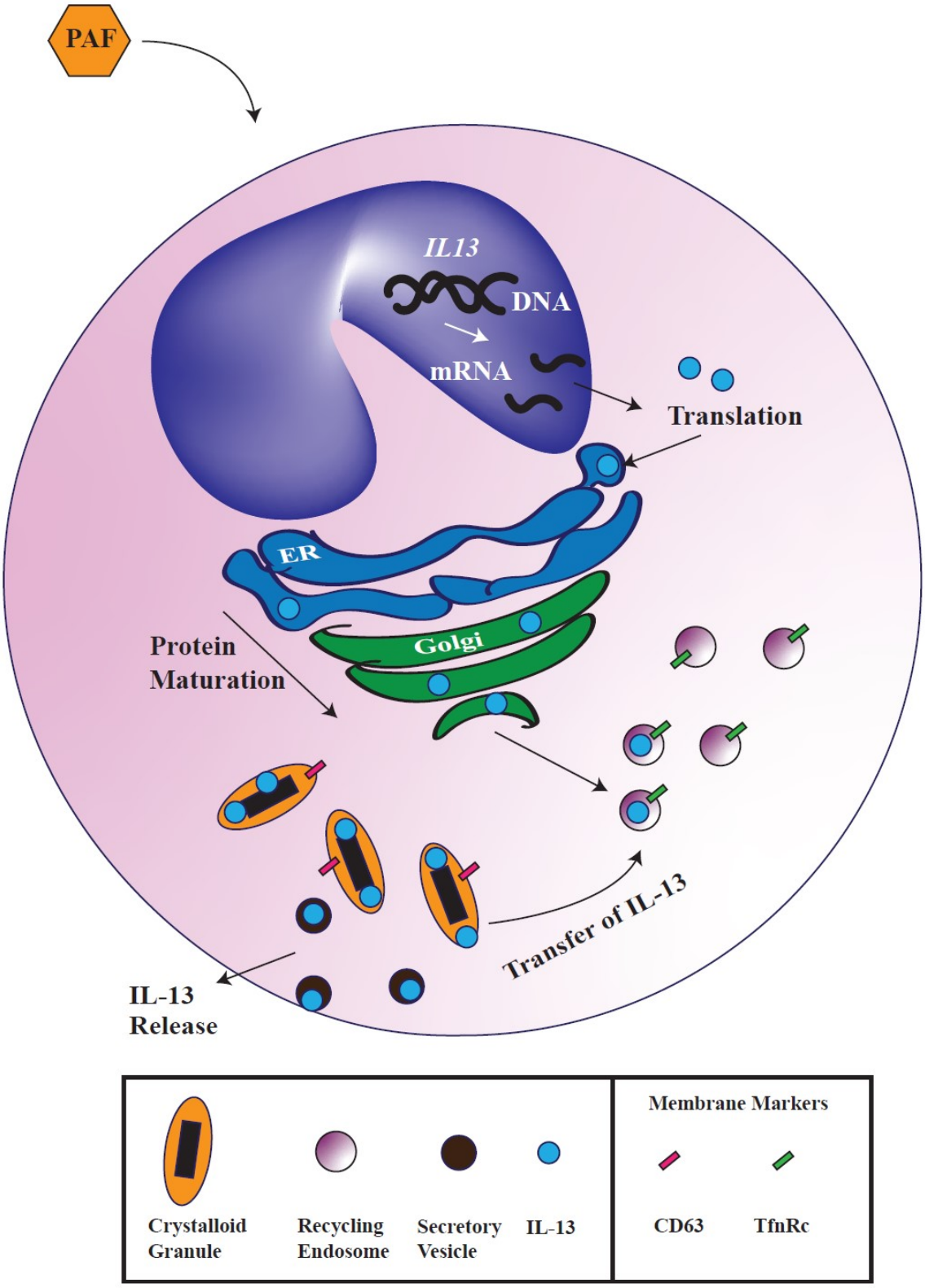

PAF
IL13
DNA
mRNA
Translation
ER
Golgi
Protein
Maturation
Transfer of IL-13
IL-13
Release
Membrane Markers
Crystalloid
Granule
Recycling
Endosome
Secretory
Vesicle
IL-13
CD63
TfnRc

Figure 4.2. Proposed IL-13 trafficking pathway in human eosinophils stimulated with PAF involves regulated secretion of preformed IL-13 in CD63$^+$ crystalloid granules. PAF induces secretion of preformed IL-13 stored in CD63$^+$ crystalloid granules. Secretory vesicles budding from crystalloid granules are likely involved in piecemeal degranulation of stored IL-13. PAF may induce a small burst of IL-13 translation that follows the classical secretory pathway. At the *trans*-Golgi, newly made IL-13 is shuttled into TfnRc$^+$ endosomes as a static pool that may be delivered to the cell membrane at later stimulation timepoints. Preformed IL-13 from crystalloid granules can also be transferred to TfnRc$^+$ endosomes.

Appendix A. Furthermore, preliminary data for optimization of priming the HL-60 clone 15 cell line for eosinophil-like differentiation can be found in Appendix B.

4.3 Future directions

1. In this study, cytokines that were detected could not be differentiated as newly made or preformed. To delineate whether PAF induces *de novo* synthesis of IL-9 in human eosinophils, isolated cells could be treated with the protein translation inhibitor cycloheximide or transcription inhibitor actinomycin D prior to PAF stimulation and immunofluorescence staining. If a significant increase in IL-9 immunofluorescence intensity is observed after stimulation and use of actinomycin D, this suggests functional IL-9 transcripts are already present in resting eosinophils and are readily translated. If a significant increase in IL-9 immunofluorescence intensity is not observed after stimulation and the use of cycloheximide, the hypothesis that PAF induces *de novo* IL-9 synthesis is supported. If IL-9 continues to be detected at elevated levels in stimulated cells treated with cycloheximide, PAF stimulation may be increasingly unmasking the epitope of IL-9 that is recognized by IF antibodies. Previous attempts at treating human eosinophils with cycloheximide in our laboratory resulted in decreased cell attachment to glass coverslips, preventing efficient imaging of cells by immunofluorescence microscopy. Other assays listed in the next point to measure IL-9 levels can be used instead if cycloheximide proves incompatible for use in immunofluorescence microscopy assays.
2. More sensitive assays such as Meso Scale Discovery assays, western blots, and flow cytometry-based cytokine measuring assays could also be used to measure changes in intracellular and secreted IL-9 or IL-13 levels upon PAF stimulation. In our laboratory, enzyme-linked immunosorbent assays (ELISAs) aimed to measure IL-9 and IL-13 release throughout the PAF stimulation time course were unable to detect secreted cytokines, thus the aforementioned assays should be opted for in future studies. Reverse transcription quantitative polymerase chain reactions (RT-qPCRs) could also be used to measure changes in cytokine mRNA expression in PAF-stimulated eosinophils relative to cells at baseline.
3. To specifically label recycling endosomes in these experiments, additional markers should be utilized in future IF experiments and include VAMP-3 and Rab11a. Previous attempts at labelling VAMP-3 and Rab11a in human eosinophils by immunofluorescence

microscopy resulted in no detection of these markers. If this assay continues to produce negative results, VAMP-3 and Rab11a can be labelled instead in subcellular fractions containing recycling endosomes as described in the next point.

4. Subcellular fractionation experiments may be developed to isolate recycling endosomes and protein levels of IL-9 and IL-13 could be measured in these fractions to supplement colocalization done by immunofluorescence microscopy here.
5. Since constitutively secreted cytokines such as IL-6 have been reported to mainly signal through receptors in recycling endosomes and this signaling reduces IL-6 *de novo* synthesis, it would also be interesting to immunolabel for IL-9Rα and measure its colocalization with recycling endosomes to test whether IL-9 signaling can indeed take place within recycling endosomes and affect IL-9 biosynthesis.[311]
6. Given VAMP-7 has been implicated in IL-9 exocytosis in mice, immunolabelling for VAMP-7 and measuring colocalization with CD63 and IL-9 in human eosinophils would draw parallels to these reports.[252] As immunolabelling for VAMP-7 in human eosinophils has yet to be detected by immunofluorescence microscopy in our laboratory, subcellular fractions containing crystalloid granules may be needed to label for VAMP-7, CD63, and IL-9.
7. Rab27a has been reported to localize to crystalloid granules in eosinophils and cited important trafficking machinery in eosinophil degranulation.[273] Immunolabelling for Rab27a and CD63 and cytokines would implicate this GTPase in regulating their release. Rab27a has also proven undetectable in human eosinophils by immunofluorescence microscopy done previously in our laboratory. As in the previous point, crystalloid granules could be isolated in subcellular fractionation experiments to allow for labeling of Rab27a and CD63. To note, mouse eosinophils allow for immunolabeling of both VAMP-7 and Rab27a and may be used as a model for investigating cytokine trafficking pathways in eosinophils by immunofluorescence microscopy.
8. VAMP-2 has been reported to localize to secretory vesicles shuttling CCL5 from crystalloid granules to the cell surface.[249] Colocalization between VAMP-2 and TfnRc would be interesting to outline whether these markers delineate distinct compartments.
9. Knockdown studies of trafficking machinery associated with recycling endosomes (VAMP-3, Rab11a), crystalloid granules (VAMP-7, Rab27a), and secretory vesicles

(VAMP-2) and later measuring IL-9 and IL-13 release in human eosinophils would provide greater detail on the mechanisms of their secretion. However, given human eosinophils limited lifespan, *in vitro* gene manipulation studies such as this are extremely challenging to perform. Optimization of eosinophil-like differentiation in the HL-60 clone 15 cell line may allow for performance of these knockdown studies in this model.

Bibliography

1. Gleich GJ. Historical overview and perspective on the role of the eosinophil in health and disease. In: *Eosinophils in Health and Disease*. Elsevier Inc.; 2013:1-11. doi:10.1016/B978-0-12-394385-9.00001-8

2. Ravin KA, Loy M. The eosinophil in infection. *Clinical Reviews in Allergy and Immunology*. 2015;50(2):214-227. doi:10.1007/s12016-015-8525-4

3. Rosenberg HF, Dyer KD, Foster PS. Eosinophils: Changing perspectives in health and disease. *Nature Reviews Immunology*. 2013;13(1):9-22. doi:10.1038/nri3341

4. Shamri R, Xenakis JJ, Spencer LA. Eosinophils in innate immunity: An evolving story. *Cell and Tissue Research*. 2011;343(1):57-83. doi:10.1007/s00441-010-1049-6

5. Kvarnhammar AM, Cardell LO. Pattern-recognition receptors in human eosinophils. *Immunology*. 2012;136(1):11-20. doi:10.1111/j.1365-2567.2012.03556.x

6. Shamri R, Xenakis JJ, Spencer LA. Eosinophils in innate immunity: an evolving story. *Cell and Tissue Research*. 2011;343(1):57-83. doi:10.1007/s00441-010-1049-6

7. Akuthota P, Wang HB, Spencer LA, Weller PF. Immunoregulatory roles of eosinophils: A new look at a familiar cell. *Clinical and Experimental Allergy*. 2008;38(8):1254-1263. doi:10.1111/j.1365-2222.2008.03037.x

8. Spencer LA, Weller PF. Eosinophils and Th2 immunity: Contemporary insights. *Immunology and Cell Biology*. 2010;88(3):250-256. doi:10.1038/icb.2009.115

9. Nerlov C, Graf T. PU.1 induces myeloid lineage commitment in multipotent hematopoietic progenitors. *Genes & Development*. 1998;12(15):2403-2412. www.genesdev.org

10. Nerlov C, Mcnagny KM, Dö Derlein G, Kowenz-Leutz E, Graf T. Distinct C/EBP functions are required for eosinophil lineage commitment and maturation. *Genes & Development*. 1998;12(15):2413-2423. www.genesdev.org

11. Hirasawa R, Shimizu R, Takahashi S, et al. Essential and instructive roles of GATA factors in eosinophil development. *Journal of Experimental Medicine*. 2002;195(11):1379-1386. doi:10.1084/jem.20020170

12. Esnault S, Kelly EA. Essential mechanisms of differential activation of eosinophils by IL-3 compared to GM-CSF and IL-5. *Critical Reviews in Immunology*. 2016;36(5):429-444. doi:10.1615/CritRevImmunol.2017020172

13. Throsby M, Herbelin A, Pléau J-M, Dardenne M. CD11c + Eosinophils in the murine thymus: Developmental regulation and recruitment upon MHC Class I-

restricted thymocyte deletion. *The Journal of Immunology*. 2000;165(4):1965-1975. doi:10.4049/jimmunol.165.4.1965

14. Yantiss RK. Eosinophils in the GI tract: How many is too many and what do they mean? *Modern Pathology*. 2015;28:S7-S21. doi:10.1038/modpathol.2014.132

15. Perez MC, Furth EE, Matzumura PD, Lyttle CR. Role of Eosinophils in Uterine Responses to Estrogen'. *Biology of reproduction*. 1996;54:249-254. https://academic.oup.com/biolreprod/article-abstract/54/1/249/2761942

16. Gouon-Evans V, Rothenberg ME, Pollard JW. Postnatal mammary gland development requires macrophages and eosinophils. *Development*. 2000;127:2269-2282.

17. Hogan SP, Rosenberg HF, Moqbel R, et al. Eosinophils: Biological properties and role in health and disease. *Clinical and Experimental Allergy*. 2008;38(5):709-750. doi:10.1111/j.1365-2222.2008.02958.x

18. Geering B, Stoeckle C, Conus S, Simon HU. Living and dying for inflammation: Neutrophils, eosinophils, basophils. *Trends in Immunology*. 2013;34(8):398-409. doi:10.1016/j.it.2013.04.002

19. Melo RCN, Weller PF. Contemporary understanding of the secretory granules in human eosinophils. *Journal of Leukocyte Biology*. 2018;104(1):85-93. doi:10.1002/JLB.3MR1217-476R

20. Miller F, de Harven E, Palade GE. The structure of eosinophil leukocyte granules in rodents and in man. *Journal of Cell Biology*. 1966;31(2):349-362. https://rupress.org/jcb/article-pdf/31/2/349/444417/349.pdf

21. Gleich GJ, Loegering DA, Mann KG, Maldonado JE. Comparative properties of the Charcot-Leyden crystal protein and the Major Basic Protein from human eosinophils. *Journal of Clinical Investigation*. 1976;57(3):633-640.

22. Weller PF, Spencer LA. Functions of tissue-resident eosinophils. *Nature Reviews Immunology*. 2017;17(12):746-760. doi:10.1038/nri.2017.95

23. Spencer LA, Szela CT, Perez SAC, et al. Human eosinophils constitutively express multiple Th1, Th2, and immunoregulatory cytokines that are secreted rapidly and differentially. *Journal of Leukocyte Biology*. 2008;85(1):117-123. doi:10.1189/jlb.0108058

24. Moqbel R, Ying S, Barkans J, et al. Identification of messenger RNA for IL-4 in human eosinophils with granule localization and release of the translated product. *Journal of Immunology*. 1995;155(10):4939-4947. http://www.jimmunol.org/content/155/10/4939

25. Beil WJ, Weller PE, Tzizik DM, Galli SJ, Dvorak AM. Rapid Communication I Ultrastructural immunogold localization of tumor necrosis factor-a to the matrix

compartment of eosinophil secondary granules in patients with idiopathic hypereosinophilic syndrome. *The Journal of Histochemistry and Cytochemistry*. 1993;41(11):1611-1611.

26. Levi-Schaffer F, Barkans J, Newman TM, et al. Identification of interleukin-2 in human peripheral blood eosinophils. *Immunology*. 1996;87(1):155-161.

27. Moller GM, de Jong TAW, Overbeek SE, van der Kwast H, Postma DS, Hoogsteden HC. Brief Report ultrastructural immunogold localization of interleukin 5 to the crystalloid core compartment of eosinophil secondary granules in patients with atopic asthma. *The Journal of Histochemistry and Cytochemistry*. 1996;44(1):67-69.

28. Dubucquoi S, Desreumaux P, Janin SA, et al. Interleukin 5 synthesis by eosinophils: association with granules and immunoglobulin-dependent secretion. *Journal of Experimental Medicine*. 1994;179(2):703-708.

29. Woerly G, Lacy P, Younes AB, et al. Human eosinophils express and release IL-13 following CD28-dependent activation. *Journal of Leukocyte Biology*. 2002;72(4):769-779.

30. Lacy P, Mahmudi-Azer S, Bablitz B, et al. Rapid Mobilization of Intracellularly Stored RANTES in Response to Interferon-in Human Eosinophils. *Blood*. 1999;94(1):23-32. https://ashpublications.org/blood/article-pdf/94/1/23/1655498/23.pdf

31. Nakajima T, Yamada H, Iikura M, et al. Intracellular localization and release of eotaxin from normal eosinophils. *Federation of European Biochemical Societies Letter*. 1998;434(3):226-230.

32. Muniz VS, Weller PF, Neves JS. Eosinophil crystalloid granules: structure, function, and beyond. *Journal of Leukocyte Biology*. 2012;92(2):281-288. doi:10.1189/jlb.0212067

33. Carmo LAS, Bonjour K, Ueki S, et al. CD63 is tightly associated with intracellular, secretory events chaperoning piecemeal degranulation and compound exocytosis in human eosinophils. *Journal of Leukocyte Biology*. 2016;100(2):391-401. doi:10.1189/jlb.3a1015-480r

34. Mahmudi-Azer S, Downey GP, Moqbel R. Translocation of the tetraspanin CD63 in association with human eosinophil mediator release. *Blood*. 2002;99(11):4039-4047. https://ashpublications.org/blood/article-pdf/99/11/4039/1685817/h81102004039.pdf

35. Melo RCN, Spencer LA, Perez SAC, et al. Vesicle-mediated secretion of human eosinophil granule-derived major basic protein. *Laboratory Investigation*. 2009;89(7):769-781. doi:10.1038/labinvest.2009.40

36. Melo RCN, Perez SAC, Spencer LA, Dvorak AM, Weller PF. Intragranular vesiculotubular compartments are involved in piecemeal degranulation by activated human eosinophils. *Traffic*. 2005;6(10):866-879. doi:10.1111/j.1600-0854.2005.00322.x

37. Melo RCN, Spencer LA, Dvorak AM, Weller PF. Mechanisms of eosinophil secretion: large vesiculotubular carriers mediate transport and release of granule-derived cytokines and other proteins. *Journal of Leukocyte Biology*. 2008;83(2):229-236. doi:10.1189/jlb.0707503

38. Melo RCN, Spencer LA, Perez SAC, Ghiran I, Dvorak AM, Weller PF. Human eosinophils secrete preformed, granule-stored interleukin-4 through distinct vesicular compartments. *Traffic*. 2005;6(11):1047-1057. doi:10.1111/j.1600-0854.2005.00344.x

39. Spencer LA, Bonjour K, Melo RCN, Weller PF. Eosinophil secretion of granule-derived cytokines. *Frontiers in Immunology*. 2014;5(OCT):496-undefined. doi:10.3389/fimmu.2014.00496

40. Scepek S, Moqbel R, Lindau M. Compound exocytosis and cumulative degranulation by eosinophils and their Role in parasite killing. *Parasitology Today*. 1994;10(7):276-278.

41. Mclaren DJ, Mackenzie CD, Ramalho-Pinto FJ. Ultrastructural observations on the in vitro interaction between rat eosinophils and some parasitic helminths (Schistosoma mansoni, Trichinella spiralis and Nippostrongylus brasiliensis). *Clinical & Experimental Immunology*. 1977;30(1):105-118.

42. Neves JS, Perez SA, Spencer LA, et al. Eosinophil granules function extracellularly as receptor-mediated secretory organelles. *Proceedings of the National Academy of Sciences*. 2008;105(47):18478-18483. doi:https://doi.org/10.1073/pnas.0804547105

43. Neves JS, Perez SAC, Spencer LA, Melo RCN, Weller PF. Subcellular fractionation of human eosinophils: Isolation of functional specific granules on isoosmotic density gradients. *Journal of Immunological Methods*. 2009;344(1):64-72. doi:10.1016/j.jim.2009.03.006

44. Ueki S, Konno Y, Takeda M, et al. Eosinophil extracellular trap cell death-derived DNA traps: Their presence in secretions and functional attributes. *Journal of Allergy and Clinical Immunology*. 2016;137(1):258-267. doi:10.1016/j.jaci.2015.04.041

45. Ueki S, Melo RCN, Ghiran I, Spencer LA, Dvorak AM, Weller PF. Eosinophil extracellular DNA trap cell death mediates lytic release of free secretion-competent eosinophil granules in humans. *Blood*. 2013;121(11):2074-2083. doi:10.1182/blood-2012-05

46. Mukherjee M, Lacy P, Ueki S. Eosinophil Extracellular Traps and Inflammatory Pathologies—Untangling the Web! *Frontiers in Immunology*. 2018;9. doi:10.3389/fimmu.2018.02763

47. Persson CGA, Erjefält JS. "Ultimate activation" of eosinophils in vivo: lysis and release of clusters of free eosinophil granules (Cfegs). *Thorax*. 1997;52(6):569-574.

48. Persson CGA, Erjefält JS. Eosinophil lysis and free granules: an in vivo paradigm for cell activation and drug development. *Trends in Pharmacological Sciences*. 1997;18(4):117-123. doi:https://doi.org/10.1016/S0165-6147(97)01042-0

49. Persson CGA. Centennial notions of asthma as an eosinophilic, desquamative, exudative, and steroid-sensitive disease. *The Lancet*. 1997;350(9083):1021-1024. doi:https://doi-org.login.ezproxy.library.ualberta.ca/10.1016/S0140-6736(96)02335-5

50. Erjefält JS, Persson CGA. New aspects of degranulation and fates of airway mucosal eosinophils. *Pulmonary Perspective American Journal of Respiratory and Critical Care Medicine*. 2000;161:2074-2085. www.atsjournals.org

51. Erjefält JS, Greiff L, Andersson M, et al. Allergen-induced eosinophil cytolysis is a primary mechanism for granule protein release in human upper airways. *American Journal of Respiratory and Critical Care Medicine.* Vol 160.; 1999. www.atsjournals.org

52. Watanabe K, Misu T, Edamatsu H. Cytolysis of eosinophils in nasal secretions. *Annals of Otology, Rhinology, and Laryngology*. 2003;112(2):169-173.

53. Greiff L, Erjefält JS, Andersson M, Svensson C, Persson CGA. Generation of clusters of free eosinophil granules (Cfegs) in seasonal allergic rhinitis. *Allergy*. 1998;53:200-203.

54. Dvorak AM, Furitsu T, Letourneau L, et al. Mature Eosinophils Stimulated to Develop in Human Cord Blood Mononuclear Cell Cultures Supplemented with Recombinant Human Interleukin-5 Part I. Piecemeal Degranulation of Specific Granules and Distribution of Charcot-Leyden Crystal Protein and the Charles. *American Journal of Pathology*. 1991;138(1).

55. Karawajczyk M, Sevéus L, Garcia R, et al. Piecemeal Degranulation of Peripheral Blood Eosinophils A Study of Allergic Subjects during and out of the Pollen Season. *American Journal of Respiratory Cell and Molecular Biology*. 2000;23:521-529. www.atsjournals.org

56. Moqbel R, Coughlin JJ. Differential secretion of cytokines. Maqbel. *Science's Signal Transduction Knowledge Environment*. 2006;2006(338):pe26-undefined.

57. Spencer LA, N Melo RC, C Perez SA, Bafford SP, Dvorak AM, Weller PF. Cytokine receptor-mediated trafficking of preformed IL-4 in eosinophils identifies

an innate immune mechanism of cytokine secretion. *Proceedings of the National Academy of Science*. 2006;103(9). www.pnas.orgcgidoi10.1073pnas.0508946103

58. Bousquet J, Chanez P, Lacoste JY, et al. Eosinophilic Inflammation in Asthma. *New England Journal of Medicine*. 1990;323(15):1033-1039. doi:10.1056/NEJM199010113231505

59. Price DB, Rigazio A, Campbell JD, et al. Blood eosinophil count and prospective annual asthma disease burden: a UK cohort study. *The Lancet Respiratory Medicine*. 2015;3(11):849-858. doi:10.1016/S2213-2600(15)00367-7

60. Horn BR, Robin ED, Theodore J, van Kessel A. Total Eosinophil Counts in the Management of Bronchial Asthma. *New England Journal of Medicine*. 1975;292(22):1152-1155. doi:10.1056/NEJM197505292922204

61. Taylor KJ, Luksza AR. Peripheral blood eosinophil counts and bronchial responsiveness. *Thorax*. 1987;42(6):452-456. doi:10.1136/thx.42.6.452

62. Griffin E, Håkansson L, Formgren H, Jörgensen K, Venge P. Increased chemokinetic and chemotactic responses of eosinophils in asthmatic patients. *Allergy*. 1991;46(4):255-265. doi:10.1111/j.1398-9995.1991.tb00583.x

63. ULRIK CS. Peripheral eosinophil counts as a marker of disease activity in intrinsic and extrinsic asthma. *Clinical Experimental Allergy*. 1995;25(9):820-827. doi:10.1111/j.1365-2222.1995.tb00024.x

64. Marouf R, Khadadah ME, Onadeko BO, et al. The Effect of Corticosteroid Therapy on Blood Eosinophils and Eosinophilic Cationic Protein in Patients with Acute and Chronic Asthma. *Journal of Asthma*. 1999;36(7):555-564. doi:10.3109/02770909909087292

65. Lewis SA, Pavord ID, Stringer JR, Knox AJ, Weiss ST, Britton JR. The Relation Between Peripheral Blood Leukocyte Counts and Respiratory Symptoms, Atopy, Lung Function, and Airway Responsiveness in Adults. *Chest*. 2001;119(1):105-114. doi:10.1378/chest.119.1.105

66. Tran TN, Khatry DB, Ke X, Ward CK, Gossage D. High blood eosinophil count is associated with more frequent asthma attacks in asthma patients. *Annals of Allergy, Asthma & Immunology*. 2014;113(1):19-24. doi:10.1016/j.anai.2014.04.011

67. Zhang X-Y, Simpson JL, Powell H, et al. Full blood count parameters for the detection of asthma inflammatory phenotypes. *Clinical & Experimental Allergy*. 2014;44(9):1137-1145. doi:10.1111/cea.12345

68. Kostikas K, Brindicci C, Patalano F. Blood Eosinophils as Biomarkers to Drive Treatment Choices in Asthma and COPD. *Current Drug Targets*. 2018;19(16):1882-1896. doi:10.2174/1389450119666180212120012

69. Wenzel SE. Asthma phenotypes: the evolution from clinical to molecular approaches. *Nature Medicine*. 2012;18(5):716-725. doi:10.1038/nm.2678

70. Israel E, Reddel HK. Severe and difficult-to-treat asthma in adults. *New England Journal of Medicine*. 2017;377(10):965-976. doi:10.1056/NEJMra1608969

71. Lötvall J, Akdis CA, Bacharier LB, et al. Asthma endotypes: A new approach to classification of disease entities within the asthma syndrome. *Journal of Allergy and Clinical Immunology*. 2011;127(2):355-360. doi:10.1016/j.jaci.2010.11.037

72. de Groot JC, Brinke A ten, Bel EHD. Management of the patient with eosinophilic asthma: A new era begins. *European Respiratory Journal Open Research*. 2015;1(1). doi:10.1183/23120541.00024-2015

73. FRIGAS E, GLEICH G. The eosinophil and the pathophysiology of asthma. *Journal of Allergy and Clinical Immunology*. 1986;77(4):527-537. doi:10.1016/0091-6749(86)90341-6

74. Navarro S, Aleu J, Jiménez M, Boix E, Cuchillo CM, Nogués M v. The cytotoxicity of eosinophil cationic protein/ribonuclease 3 on eukaryotic cell lines takes place through its aggregation on the cell membrane. *Cellular and Molecular Life Sciences*. 2008;65(2):324-337. doi:10.1007/s00018-007-7499-7

75. van DALEN CJ, KETTLE AJ. Substrates and products of eosinophil peroxidase. *Biochemical Journal*. 2001;358(1):233. doi:10.1042/0264-6021:3580233

76. Bergeron C, Tulic MK, Hamid Q. Airway remodelling in asthma: From benchside to clinical practice. *Canadian Respiratory Journal*. 2010;17(4):e85-e93. doi:10.1155/2010/318029

77. Ohno I, Nitta Y, Yamauchi K, et al. Transforming growth factor beta 1 (TGF beta 1) gene expression by eosinophils in asthmatic airway inflammation. *American Journal of Respiratory Cell and Molecular Biology*. 1996;15(3):404-409. doi:10.1165/ajrcmb.15.3.8810646

78. Birkland TP, Cheavens MD, Pincus SH. Human eosinophils stimulate DNA synthesis and matrix production in dermal fibroblasts. *Archives of Dermatological Research*. 1994;286(6):312-318. doi:10.1007/BF00402221

79. Makinde T, Murphy RF, Agrawal DK. The regulatory role of TGF-β in airway remodeling in asthma. *Immunology & Cell Biology*. 2007;85(5):348-356. doi:10.1038/sj.icb.7100044

80. Levi-Schaffer F, Garbuzenko E, Rubin A, et al. Human eosinophils regulate human lung- and skin-derived fibroblast properties in vitro: A role for transforming growth factor beta (TGF-beta). *Proceedings of the National Academy of Sciences*. 1999;96(17):9660-9665. doi:10.1073/pnas.96.17.9660

81. Henderson WR, Chiang GKS, Tien Y, Chi EY. Reversal of allergen-induced airway remodeling by CysLT $_1$ receptor blockade. *American Journal of Respiratory and Critical Care Medicine*. 2006;173(7):718-728. doi:10.1164/rccm.200501-088OC

82. Halwani R, Vazquez-Tello A, Sumi Y, et al. Eosinophils induce airway smooth muscle cell proliferation. *Journal of Clinical Immunology*. 2013;33(3):595-604. doi:10.1007/s10875-012-9836-3

83. Kasperska-Zajac A, Brzoza Z, Rogala B. Platelet activating factor as a mediator and therapeutic approach in bronchial asthma. *Inflammation*. 2008;31(2):112-120. doi:10.1007/s10753-007-9056-9

84. Lee T-C, Lenihan DJ, Malone B, Roddy LL, Wassermanii SI. Increased biosynthesis of platelet-activating factor in activated human eosinophils. *The Journal of Biological Chemistry*. 1984;259(9):5526-5530. http://www.jbc.org/

85. Wardlaw AJ, Moqbel R, Cromwell O, Kay AB. Platelet-activating factor. A potent chemotactic and chemokinetic factor for human eosinophils. *Journal of Clinical Investigation*. 1986;78(6):1701-1706. doi:10.1172/JCI112765

86. Lacy P, Willetts L, Kim JD, et al. Agonist activation of F-actin-mediated eosinophil shape change and mediator release is dependent on Rac2. *International Archives of Allergy and Immunology*. 2011;156(2):137-147. doi:10.1159/000322597

87. Kishimoto S, Shimadzu W, Izumi T, et al. Comparison of platelet-activating factor receptor mRNA levels in peripheral blood eosinophils from normal subjects and atopic asthmatic patients. *International Archives of Allergy and Immunology*. 1997;114(1):60-63. doi:10.1159/000237720

88. Dyer KD, Percopo CM, Xie Z, et al. Mouse and human eosinophils degranulate in response to platelet-activating factor (PAF) and LysoPAF via a PAF-receptor–independent mechanism: Evidence for a novel receptor. *The Journal of Immunology*. 2010;184(11):6327-6334. doi:10.4049/jimmunol.0904043

89. Renauld J-C. New insights into the role of cytokines in asthma. *Journal of Clinical Pathology*. 2001;54(8):577-589. doi:10.1136/jcp.54.8.577

90. Lambrecht BN, Hammad H. The immunology of asthma. *Nature Immunology*. 2015;16(1):45-56. doi:10.1038/ni.3049

91. LaPorte SL, Juo ZS, Vaclavikova J, et al. Molecular and structural basis of cytokine receptor pleiotropy in the interleukin-4/13 system. *Cell*. 2008;132(2):259-272. doi:10.1016/j.cell.2007.12.030

92. Junttila IS. Tuning the cytokine responses: An update on interleukin (IL)-4 and IL-13 receptor complexes. *Frontiers in Immunology*. 2018;9. doi:10.3389/fimmu.2018.00888

93. Maes T, Joos GF, Brusselle GG. Targeting interleukin-4 in asthma: Lost in translation? *American Journal of Respiratory Cell and Molecular Biology*. 2012;47(3):261-270. doi:10.1165/rcmb.2012-0080TR

94. Bao K, Reinhardt RL. The differential expression of IL-4 and IL-13 and its impact on type-2 immunity. *Cytokine*. 2015;75(1):25-37. doi:10.1016/j.cyto.2015.05.008

95. Chandriani S, DePianto DJ, N'Diaye EN, et al. Endogenously expressed IL-13Rα2 attenuates IL-13–mediated responses but does not activate signaling in human lung fibroblasts. *The Journal of Immunology*. 2014;193(1):111-119. doi:10.4049/jimmunol.1301761

96. Kuperman D, Schleimer R. Interleukin-4, interleukin-13, signal transducer and activator of transcription factor 6, and allergic asthma. *Current Molecular Medicine*. 2008;8(5):384-392. doi:10.2174/156652408785161032

97. Oh CK, Geba GP, Molfino N. Investigational therapeutics targeting the IL-4/IL-13/STAT-6 pathway for the treatment of asthma. *European Respiratory Review*. 2010;19(115):46-54. doi:10.1183/09059180.00007609

98. Heller NM, Qi X, Junttila IS, et al. Type I IL-4Rs selectively activate IRS-2 to induce target gene expression in macrophages. *Science Signaling*. 2008;1(51):ra17-ra17. doi:10.1126/scisignal.1164795

99. Blease K, Schuh JM, Jakubzick C, et al. Stat6-deficient mice develop airway hyperresponsiveness and peribronchial fibrosis during chronic fungal asthma. *The American Journal of Pathology*. 2002;160(2):481-490. doi:10.1016/S0002-9440(10)64867-5

100. Chatila TA. Interleukin-4 receptor signaling pathways in asthma pathogenesis. *Trends in Molecular Medicine*. 2004;10(10):493-499. doi:10.1016/j.molmed.2004.08.004

101. Chatila TA, Li N, Garcia-Lloret M, Kim H-J, Nel AE. T-cell effector pathways in allergic diseases: Transcriptional mechanisms and therapeutic targets. *Journal of Allergy and Clinical Immunology*. 2008;121(4):812-823. doi:10.1016/j.jaci.2008.02.025

102. Wills-Karp M, Luyimbazi J, Xu X, et al. Interleukin-13: Central mediator of allergic asthma. *Science*. 1998;282(5397):2258-2261. doi:10.1126/science.282.5397.2258

103. Grunig G, Warnock M, Wakil AE, et al. Requirement for IL-13 Independently of IL-4 in Experimental Asthma. *Science*. 1998;282(5397):2261-2263. doi:10.1126/science.282.5397.2261

104. Lewis CC, Aronow B, Hutton J, et al. Unique and overlapping gene expression patterns driven by IL-4 and IL-13 in the mouse lung. *Journal of Allergy and Clinical Immunology*. 2009;123(4):795-804.e8. doi:10.1016/j.jaci.2009.01.003

105. Perkins C, Wills-Karp M, Finkelman FD. IL-4 induces IL-13–independent allergic airway inflammation. *Journal of Allergy and Clinical Immunology*. 2006;118(2):410-419. doi:10.1016/j.jaci.2006.06.004

106. Finkelman FD, Hogan SP, Hershey GKK, Rothenberg ME, Wills-Karp M. Importance of Cytokines in Murine Allergic Airway Disease and Human Asthma. *The Journal of Immunology*. 2010;184(4):1663-1674. doi:10.4049/jimmunol.0902185

107. Corry DB, Folkesson HG, Warnock ML, et al. Interleukin 4, but not interleukin 5 or eosinophils, is required in a murine model of acute airway hyperreactivity. *The Journal of Experimental Medicine*. 1996;183(1):109-117. doi:10.1084/jem.183.1.109

108. Veldhoen M, Uyttenhove C, van Snick J, et al. Transforming growth factor-β "reprograms" the differentiation of T helper 2 cells and promotes an interleukin 9–producing subset. *Nature Immunology*. 2008;9(12):1341-1346. doi:10.1038/ni.1659

109. Bossé Y, Thompson C, Audette K, Stankova J, Rola-Pleszczynski M. Interleukin-4 and interleukin-13 enhance human bronchial smooth muscle cell proliferation. *International Archives of Allergy and Immunology*. 2008;146(2):138-148. doi:10.1159/000113517

110. Richter A, Puddicombe SM, Lordan JL, et al. The Contribution of Interleukin (IL)-4 and IL-13 to the epithelial–mesenchymal trophic unit in asthma. *American Journal of Respiratory Cell and Molecular Biology*. 2001;25(3):385-391. doi:10.1165/ajrcmb.25.3.4437

111. Kondo M, Tamaoki J, Takeyama K, et al. Elimination of IL-13 reverses established goblet cell metaplasia into ciliated epithelia in airway epithelial cell culture. *Allergology International*. 2006;55(3):329-336. doi:10.2332/allergolint.55.329

112. Zhu Z, Homer RJ, Wang Z, et al. Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. *Journal of Clinical Investigation*. 1999;103(6):779-788. doi:10.1172/JCI5909

113. PUNNONEN J, YSSEL H, DEVRIES J. The relative contribution of IL-4 and IL-13 to human IgE synthesis induced by activated CD4 or CD8 T cells. *Journal of*

Allergy and Clinical Immunology. 1997;100(6):792-801. doi:10.1016/S0091-6749(97)70276-8

114. van der Pouw Kraan, van der Zee, Boeije, de Groot, Stapel, Aarden. The role of IL-13 in IgE synthesis by allergic asthma patients. *Clinical and Experimental Immunology*. 1998;111(1):129-135. doi:10.1046/j.1365-2249.1998.00471.x

115. Punnonen J, Aversa G, Cocks BG, et al. Interleukin 13 induces interleukin 4-independent IgG4 and IgE synthesis and CD23 expression by human B cells. *Proceedings of the National Academy of Sciences*. 1993;90(8):3730-3734. doi:10.1073/pnas.90.8.3730

116. Cocks BG, de Waal Malefyt R, Galizzi J-P, de Vries JE, Aversa G. IL-13 induces proliferation and differentiation of human B cells activated by the CD40 ligand. *International Immunology*. 1993;5(6):657-663. doi:10.1093/intimm/5.6.657

117. Defrance T, Carayon P, Billian G, et al. Interleukin 13 is a B cell stimulating factor. *The Journal of Experimental Medicine*. 1994;179(1):135-143. doi:10.1084/jem.179.1.135

118. Yamaguchi M, Lantz CS, Oettgen HC, et al. IgE Enhances Mouse Mast Cell FcεRI Expression In Vitro and In Vivo: Evidence for a Novel Amplification Mechanism in IgE-dependent Reactions. *Journal of Experimental Medicine*. 1997;185(4):663-672. doi:10.1084/jem.185.4.663

119. Saini SS, MacGlashan DW, Sterbinsky SA, et al. Down-Regulation of Human Basophil IgE and FCεRIα Surface Densities and Mediator Release by Anti-IgE-Infusions Is Reversible In Vitro and In Vivo. *Journal of Immunology*. 1999;162(9):5624-5630. http://www.jimmunol.org/content/162/9/5624

120. Oettgen HC, Geha RS. IgE regulation and roles in asthma pathogenesis. *Journal of Allergy and Clinical Immunology*. 2001;107(3):429-441. doi:10.1067/mai.2001.113759

121. Pope SM, Brandt EB, Mishra A, et al. IL-13 induces eosinophil recruitment into the lung by an IL-5– and eotaxin-dependent mechanism. *Journal of Allergy and Clinical Immunology*. 2001;108(4):594-601. doi:10.1067/mai.2001.118600

122. Li L, Xia Y, Nguyen A, et al. Effects of Th2 cytokines on chemokine expression in the lung: IL-13 potently induces eotaxin expression by airway epithelial cells. *Journal of Immunology*. 1999;162(5):2477-2487. http://www.jimmunol.org/content/162/5/http://www.jimmunol.org/content/162/5/2477.full#ref-list-1

123. Terada N, Hamano N, Nomura T, et al. Interleukin-13 and tumour necrosis factor-alpha synergistically induce eotaxin production in human nasal fibroblasts. *Clinical Experimental Allergy*. 2000;30(3):348-355. doi:10.1046/j.1365-2222.2000.00750.x

124. Rothenberg ME, Luster AD, Leder P. Murine eotaxin: an eosinophil chemoattractant inducible in endothelial cells and in interleukin 4-induced tumor suppression. *Proceedings of the National Academy of Sciences*. 1995;92(19):8960-8964. doi:10.1073/pnas.92.19.8960

125. Garcia-Zepeda EA, Rothenberg ME, Ownbey RT, Celestin J, Leder P, Luster AD. Human eotaxin is a specific chemoattractant for eosinophil cells and provides a new mechanism to explain tissue eosinophilia. *Nature Medicine*. 1996;2(4):449-456. doi:10.1038/nm0496-449

126. Stellato C, Matsukura S, Fal A, et al. Differential regulation of epithelial-derived C-C chemokine expression by IL-4 and the glucocorticoid budesonide. *Journal of Immunology*. 1999;163(10):5624-5632. http://www.jimmunol.org/content/163/10/http://www.jimmunol.org/content/163/10/5624.full#ref-list-1

127. Mochizuki M, Schröder J-M, Christophers E, Yamamoto S. IL-4 induces eotaxin in human dermal fibroblasts. *International Archives of Allergy and Immunology*. 1999;120(1):19-23. doi:10.1159/000053587

128. Matsukura S, Stellato C, Plitt JR, et al. Activation of eotaxin gene transcription by NF-κB and STAT6 in human airway epithelial cells. *Journal of Immunology*. 1999;163(12):6876-6883. http://www.jimmunol.org/content/163/12/6876

129. Hoeck J, Woisetschläger M. STAT6 mediates eotaxin-1 expression in IL-4 or TNF-α-induced fibroblasts. *Journal of Immunology*. 2001;166(7):4507-4515. doi:10.4049/jimmunol.166.7.4507

130. Bochner BS, Klunk DA, Sterbinsky SA, Coffman RL, Schleimer RP. IL-13 selectively induces vascular cell adhesion molecule-1 expression in human endothelial cells. *Journal of Immunology*. 1995;154(2):799-803. http://www.jimmunol.org/content/154/2/799

131. Schleimer RP, Sterbinsky SA, Kaiser J, et al. IL-4 induces adherence of human eosinophils and basophils but not neutrophils to endothelium. Association with expression of VCAM-1. *Journal of Immunology*. 1992;148(4):1086-1092. http://www.jimmunol.org/content/148/4/

132. Pelaia C, Paoletti G, Puggioni F, et al. Interleukin-5 in the pathophysiology of severe asthma. *Frontiers in Physiology*. 2019;10. doi:10.3389/fphys.2019.01514

133. Dorman SC, Efthimiadis A, Babirad I, et al. Sputum CD34+IL-5Rα+ cells increase after allergen: evidence for in situ eosinophilopoiesis. *American Journal of Respiratory and Critical Care Medicine*. 2004;169(5):573-577. doi:10.1164/rccm.200307-1004OC

134. Greenfeder S, Umland SP, Cuss FM, Chapman RW, Egan RW. Th2 Cytokines and asthma: The role of interleukin-5 in allergic eosinophilic disease. *Respiratory Research*. 2001;2(2):71-79. doi:10.1186/rr41

135. Park SW, Kim DJ, Chang HS, et al. Association of interleukin-5 and eotaxin with acute exacerbation of asthma. *International Archives of Allergy and Immunology*. 2003;131(4):283-290. doi:10.1159/000072140

136. Wood LJ, Sehmi R, Dorman S, et al. Allergen-induced increases in bone marrow T lymphocytes and interleukin-5 expression in subjects with asthma. *American Journal of Respiratory and Critical Care Medicine*. 2002;166(6):883-889. doi:10.1164/rccm.2108015

137. Bhalla A, Mukherjee M, Nair P. Airway Eosinophilopoietic and autoimmune mechanisms of eosinophilia in severe Asthma. *Immunology and Allergy Clinics of North America*. 2018;38(4):639-654. doi:10.1016/j.iac.2018.06.003

138. Sano M, Leff AR, Myou S, et al. Regulation of interleukin-5–induced β2-Integrin adhesion of human eosinophils by phosphoinositide 3-kinase. *American Journal of Respiratory Cell and Molecular Biology*. 2005;33(1):65-70. doi:10.1165/rcmb.2005-0076OC

139. Barthel SR, Jarjour NN, Mosher DF, Johansson MW. Dissection of the hyperadhesive phenotype of airway eosinophils in asthma. *American Journal of Respiratory Cell and Molecular Biology*. 2006;35(3):378-386. doi:10.1165/rcmb.2006-0027OC

140. Johansson MW, Annis DS, Mosher DF. αMβ2 integrin–mediated adhesion and motility of IL-5–stimulated eosinophils on periostin. *American Journal of Respiratory Cell and Molecular Biology*. 2013;48(4):503-510. doi:10.1165/rcmb.2012-0150OC

141. Pazdrak K, Stafford S, Alam R. The activation of the Jak-STAT 1 signaling pathway by IL-5 in eosinophils. *Journal of Immunology*. 1995;155(1):397-402. http://www.jimmunol.org/content/155/1/397

142. Stout BA, Bates ME, Liu LY, Farrington NN, Bertics PJ. IL-5 and granulocyte-macrophage colony-stimulating factor activate STAT3 and STAT5 and promote Pim-1 and Cyclin D3 protein expression in human eosinophils. *Journal of Immunology*. 2004;173(10):6409-6417. doi:10.4049/jimmunol.173.10.6409

143. Pazdrak K, Olszewska-Pazdrak B, Stafford S, Garofalo RP, Alam R. Lyn, Jak2, and Raf-1 kinases are critical for the antiapoptotic effect of interleukin 5, whereas only Raf-1 kinase is essential for eosinophil activation and degranulation. *Journal of Experimental Medicine*. 1998;188(3):421-429. doi:10.1084/jem.188.3.421

144. Schwartz C, Willebrand R, Huber S, et al. Eosinophil-specific deletion of IκBα in mice reveals a critical role of NF-κB–induced Bcl-xL for inhibition of apoptosis. *Blood*. 2015;125(25):3896-3904. doi:10.1182/blood-2014-10-607788

145. Molfino NA, Gossage D, Kolbeck R, Parker JM, Geba GP. Molecular and clinical rationale for therapeutic targeting of interleukin-5 and its receptor. *Clinical & Experimental Allergy*. 2012;42(5):712-737. doi:10.1111/j.1365-2222.2011.03854.x

146. Goswami R, Kaplan MH. A brief history of IL-9. *The Journal of Immunology*. 2011;186(6):3283-3288. doi:10.4049/jimmunol.1003049

147. Zhou Y, McLane M, Levitt RC. Th2 cytokines and asthma- interleukin-9 as a therapeutic target for asthma. *Respiratory Research*. 2001;2(2):80-84.

148. Erpenbeck VJ, Hohlfeld JM, Discher M, et al. Increased expression of interleukin-9 messenger RNA after segmental allergen challenge in allergic asthmatics. *Chest*. 2003;123(3):370S. doi:10.1378/chest.123.3_suppl.370S-a

149. Shimbara A, Christodoulopoulos P, Soussi-Gounni A, et al. IL-9 and its receptor in allergic and nonallergic lung disease: Increased expression in asthma. *Journal of Allergy and Clinical Immunology*. 2000;105(1):108-115. doi:10.1016/S0091-6749(00)90185-4

150. Bhathena PR, Comhair SAA, Holroyd KJ, Erzurum SC. Interleukin-9 receptor expression in asthmatic airways in vivo. *Lung*. 2000;178(3):149-160. doi:10.1007/s004080000018

151. Yamasaki A, Saleh A, Koussih L, Muro S, Halayko AJ, Gounni AS. IL-9 induces CCL11 Expression via STAT3 signalling in human airway smooth muscle cells. *PLoS ONE*. 2010;5(2):e9178. doi:10.1371/journal.pone.0009178

152. Lora JM, Al-Garawi A, Pickard MD, et al. FcεRI-dependent gene expression in human mast cells is differentially controlled by T helper type 2 cytokines. *Journal of Allergy and Clinical Immunology*. 2003;112(6):1119-1126. doi:10.1016/j.jaci.2003.08.042

153. Matsuzawa S, Sakashita K, Kinoshita T, Ito S, Yamashita T, Koike K. IL-9 enhances the growth of human mast cell progenitors under stimulation with stem cell factor. *Journal of Immunology*. 2003;170(7):3461-3467. doi:10.4049/jimmunol.170.7.3461

154. Wiener Z, Falus A, Toth S. IL-9 increases the expression of several cytokines in activated mast cells, while the IL-9-induced IL-9 production is inhibited in mast cells of histamine-free transgenic mice. *Cytokine*. 2004;26(3):122-130. doi:10.1016/j.cyto.2004.01.006

155. Longphre M, Li D, Gallup M, et al. Allergen-induced IL-9 directly stimulates mucin transcription in respiratory epithelial cells. *Journal of Clinical Investigation*. 1999;104(10):1375-1382. doi:10.1172/JCI6097

156. Louahed J, Toda M, Jen J, et al. Interleukin-9 upregulates mucus expression in the airways. *American Journal of Respiratory Cell and Molecular Biology*. 2000;22(6):649-656. doi:10.1165/ajrcmb.22.6.3927

157. Reader JR, Hyde DM, Schelegle ES, et al. Interleukin-9 induces mucous cell metaplasia independent of inflammation. *American Journal of Respiratory Cell and Molecular Biology*. 2003;28(6):664-672. doi:10.1165/rcmb.2002-0207OC

158. Vermeer PD, Harson R, Einwalter LA, Moninger T, Zabner J. Interleukin-9 induces goblet cell hyperplasia during repair of human airway epithelia. *American Journal of Respiratory Cell and Molecular Biology*. 2003;28(3):286-295. doi:10.1165/rcmb.4887

159. Steenwinckel V, Louahed J, Orabona C, et al. IL-13 mediates in vivo IL-9 activities on lung epithelial cells but not on hematopoietic cells. *The Journal of Immunology*. 2007;178(5):3244-3251. doi:10.4049/jimmunol.178.5.3244

160. Dugas B, Renauld JC, Pène J, et al. Interleukin-9 potentiates the interleukin-4-induced immunoglobulin (IgG, IgM and IgE) production by normal human B lymphocytes. *European Journal of Immunology*. 1993;23(7):1687-1692. doi:10.1002/eji.1830230743

161. Petit-frere C, Dugas B, Braquet P, Mencia-Huerta JM. Interleukin-9 potentiates the interleukin-4-induced IgE and IgG1 release from murine B lymphocytes. *Immunology*. 1993;79:146-151.

162. Gounni AS, Gregory B, Nutku E, et al. Interleukin-9 enhances interleukin-5 receptor expression, differentiation, and survival of human eosinophils. *Blood*. 2000;96(6):2163-2171. doi:10.1182/blood.V96.6.2163

163. *Global strategy for asthma management and prevention. Updated 2020*.; 2020. www.ginasthma.org

164. Barnes PJ. Scientific rationale for inhaled combination therapy with long-acting β2-agonists and corticosteroids. *European Respiratory Journal*. 2002;19(1):182-191. doi:10.1183/09031936.02.00283202

165. Barnes PJ. Inhaled corticosteroids. *Pharmaceuticals*. 2010;3(3):514-540. doi:10.3390/ph3030514

166. Barnes PJ. Glucocorticosteroids: current and future directions. *British Journal of Pharmacology*. 2011;163(1):29-43. doi:10.1111/j.1476-5381.2010.01199.x

167. Rhen T, Cidlowski JA. Anti-inflammatory action of glucocorticoids — New mechanisms for old drugs. *New England Journal of Medicine*. 2005;353(16):1711-1723. doi:10.1056/NEJMra050541

168. Billington CK, Penn RB, Hall IP. β2 Agonists. *Handbook of Experimental Pharmacology*. 2017;237:23-40. doi:10.1007/164_2016_64

169. Dreher M, Müller T. Add-on therapy for symptomatic asthma despite long-acting beta-agonists/inhaled corticosteroid. *Tuberculosis and Respiratory Diseases*. 2018;81(1):1-5. doi:10.4046/trd.2017.0102

170. Pelaia G, Vatrella A, Busceti MT, et al. Pharmacologic rationale underlying the therapeutic effects of tiotropium/olodaterol in COPD. *Therapeutics and Clinical Risk Management*. 2015;11:1563-1572. doi:10.2147/TCRM.S84151

171. Wong EHC, Porter JD, Edwards MR, Johnston SL. The role of macrolides in asthma: current evidence and future directions. *The Lancet Respiratory Medicine*. 2014;2(8):657-670. doi:10.1016/S2213-2600(14)70107-9

172. Eger KA, Bel EH. The emergence of new biologics for severe asthma. *Current Opinion in Pharmacology*. 2019;46:108-115. doi:10.1016/j.coph.2019.05.005

173. Nelson PN, Reynolds GM, Waldron EE, Ward E, Giannopoulos K, Murray PG. Demystified ...: Monoclonal antibodies. *Molecular Pathology*. 2000;53(3):111-117. doi:10.1136/mp.53.3.111

174. Mallbris L, Davies J, Glasebrook A, Tang Y, Wolfgang G, Nickoloff BJ. Molecular insights into fully human and humanized monoclonal antibodies-What are the differences and should dermatologists care? *The Journal of Clinical Aesthetic Dermatology*. 2016;9(7):13-15. http://www.who.int/medicines/services/inn/BioRev2014.pdf.

175. Busse W, Corren J, Lanier BQ, et al. Omalizumab, anti-IgE recombinant humanized monoclonal antibody, for the treatment of severe allergic asthma. *Journal of Allergy and Clinical Immunology*. 2001;108(2):184-190. doi:10.1067/mai.2001.117880

176. Normansell R, Walker S, Milan SJ, Walters EH, Nair P. Omalizumab for asthma in adults and children. *Cochrane Database of Systematic Reviews*. Published online January 13, 2014. doi:10.1002/14651858.CD003559.pub4

177. Hanania NA, Wenzel S, Rosén K, et al. Exploring the effects of omalizumab in allergic asthma. *American Journal of Respiratory and Critical Care Medicine*. 2013;187(8):804-811. doi:10.1164/rccm.201208-1414OC

178. Busse WW. Biological treatments for severe asthma. *Current Opinion in Allergy and Clinical Immunology*. 2018;18(6):509-518. doi:10.1097/ACI.0000000000000487

179. Pavord ID, Korn S, Howarth P, et al. Mepolizumab for severe eosinophilic asthma (DREAM): a multicentre, double-blind, placebo-controlled trial. *The Lancet*. 2012;380(9842):651-659. doi:10.1016/S0140-6736(12)60988-X

180. Ortega HG, Liu MC, Pavord ID, et al. Mepolizumab treatment in patients with severe eosinophilic asthma. *New England Journal of Medicine*. 2014;371(13):1198-1207. doi:10.1056/NEJMoa1403290

181. Haldar P, Brightling CE, Hargadon B, et al. Mepolizumab and exacerbations of refractory eosinophilic asthma. *New England Journal of Medicine*. 2009;360(10):973-984. doi:10.1056/NEJMoa0808991

182. Castro M, Zangrilli J, Wechsler ME, et al. Reslizumab for inadequately controlled asthma with elevated blood eosinophil counts: results from two multicentre, parallel, double-blind, randomised, placebo-controlled, phase 3 trials. *The Lancet Respiratory Medicine*. 2015;3(5):355-366. doi:10.1016/S2213-2600(15)00042-9

183. Corren J, Weinstein S, Janka L, Zangrilli J, Garin M. Phase 3 study of reslizumab in patients with poorly controlled asthma. *Chest*. 2016;150(4):799-810. doi:10.1016/j.chest.2016.03.018

184. Bjermer L, Lemiere C, Maspero J, Weiss S, Zangrilli J, Germinaro M. Reslizumab for inadequately controlled asthma with elevated blood eosinophil levels. *Chest*. 2016;150(4):789-798. doi:10.1016/j.chest.2016.03.032

185. Mukherjee M, Aleman Paramo F, Kjarsgaard M, et al. Weight-adjusted Intravenous reslizumab in severe asthma with inadequate response to fixed-dose subcutaneous mepolizumab. *American Journal of Respiratory and Critical Care Medicine*. 2018;197(1):38-46. doi:10.1164/rccm.201707-1323OC

186. Bleecker ER, FitzGerald JM, Chanez P, et al. Efficacy and safety of benralizumab for patients with severe asthma uncontrolled with high-dosage inhaled corticosteroids and long-acting β2-agonists (SIROCCO): a randomised, multicentre, placebo-controlled phase 3 trial. *The Lancet*. 2016;388(10056):2115-2127. doi:10.1016/S0140-6736(16)31324-1

187. FitzGerald JM, Bleecker ER, Nair P, et al. Benralizumab, an anti-interleukin-5 receptor α monoclonal antibody, as add-on treatment for patients with severe, uncontrolled, eosinophilic asthma (CALIMA): a randomised, double-blind, placebo-controlled phase 3 trial. *The Lancet*. 2016;388(10056):2128-2141. doi:10.1016/S0140-6736(16)31322-8

188. Sehmi R, Lim HF, Mukherjee M, et al. Benralizumab attenuates airway eosinophilia in prednisone-dependent asthma. *Journal of Allergy and Clinical Immunology*. 2018;141(4):1529-1532.e8. doi:10.1016/j.jaci.2018.01.008

189. Pelaia C, Calabrese C, Vatrella A, et al. Benralizumab: From the basic mechanism of action to the potential use in the biological therapy of severe eosinophilic asthma. *BioMed Research International*. 2018;2018:1-9. doi:10.1155/2018/4839230

190. Bel EH, Wenzel SE, Thompson PJ, et al. Oral glucocorticoid-sparing effect of mepolizumab in eosinophilic asthma. *New England Journal of Medicine*. 2014;371(13):1189-1197. doi:10.1056/NEJMoa1403291

191. Nair P, Wenzel S, Rabe KF, et al. Oral glucocorticoid–sparing effect of benralizumab in severe asthma. *New England Journal of Medicine*. 2017;376(25):2448-2458. doi:10.1056/NEJMoa1703501

192. Castro M, Corren J, Pavord ID, et al. Dupilumab efficacy and safety in moderate-to-severe uncontrolled asthma. *New England Journal of Medicine*. 2018;378(26):2486-2496. doi:10.1056/NEJMoa1804092

193. Rabe KF, Nair P, Brusselle G, et al. Efficacy and safety of dupilumab in glucocorticoid-dependent severe asthma. *New England Journal of Medicine*. 2018;378(26):2475-2485. doi:10.1056/NEJMoa1804093

194. McGregor MC, Krings JG, Nair P, Castro M. Role of biologics in asthma. *American Journal of Respiratory and Critical Care Medicine*. 2019;199(4):433-445. doi:10.1164/rccm.201810-1944CI

195. Anderson WC, Szefler SJ. Cost-effectiveness and comparative effectiveness of biologic therapy for asthma. *Annals of Allergy, Asthma & Immunology*. 2019;122(4):367-372. doi:10.1016/j.anai.2019.01.018

196. Chapman KR, Albers FC, Chipps B, et al. The clinical benefit of mepolizumab replacing omalizumab in uncontrolled severe eosinophilic asthma. *Allergy*. 2019;74(9):1716-1726. doi:10.1111/all.13850

197. Institute for Clinical and Economic Review. *Biologic Therapies for Treatment of Asthma Associated with Type 2 Inflammation: Effectiveness, Value, and Value-Based Price Benchmarks: Final Evidence Report. Boston, MA.*; 2018. Accessed June 28, 2020. https://icer-review.org/material/asthma-final-evidence-report/

198. Haldar P, Pavord ID. Noneosinophilic asthma: A distinct clinical and pathologic phenotype. *Journal of Allergy and Clinical Immunology*. 2007;119(5):1043-1052. doi:10.1016/j.jaci.2007.02.042

199. Loza MJ, Djukanovic R, Chung KF, et al. Validated and longitudinally stable asthma phenotypes based on cluster analysis of the ADEPT study. *Respiratory Research*. 2016;17(1):165. doi:10.1186/s12931-016-0482-9

200. Moore WC, Hastie AT, Li X, et al. Sputum neutrophil counts are associated with more severe asthma phenotypes using cluster analysis. *Journal of Allergy and Clinical Immunology*. 2014;133(6):1557-1563.e5. doi:10.1016/j.jaci.2013.10.011

201. McGrath KW, Icitovic N, Boushey HA, et al. A large subgroup of mild-to-moderate asthma is persistently non-eosinophilic. *American Journal of Respiratory and Critical Care Medicine*. 2012;185(6):612-619. doi:10.1164/rccm.201109-1640OC

202. Nicolás-Ávila JÁ, Adrover JM, Hidalgo A. Neutrophils in homeostasis, immunity, and cancer. *Immunity*. 2017;46(1):15-28. doi:10.1016/j.immuni.2016.12.012

203. Kunkel SL, Standiford T, Kasahara K, Strieter RM. Interleukin-8 (IL-8): The major neutrophil chemotactic factor in the lung. *Experimental Lung Research*. 1991;17(1):17-23. doi:10.3109/01902149109063278

204. Dworski R, Simon H-U, Hoskins A, Yousefi S. Eosinophil and neutrophil extracellular DNA traps in human allergic asthmatic airways. *Journal of Allergy and Clinical Immunology*. 2011;127(5):1260-1266. doi:10.1016/j.jaci.2010.12.1103

205. Lachowicz-Scroggins ME, Dunican EM, Charbit AR, et al. Extracellular DNA, neutrophil extracellular traps, and inflammasome activation in severe asthma. *American Journal of Respiratory and Critical Care Medicine*. 2019;199(9):1076-1085. doi:10.1164/rccm.201810-1869OC

206. Cundall M, Sun Y, Miranda C, Trudeau JB, Barnes S, Wenzel SE. Neutrophil-derived matrix metalloproteinase-9 is increased in severe asthma and poorly inhibited by glucocorticoids. *Journal of Allergy and Clinical Immunology*. 2003;112(6):1064-1071. doi:10.1016/j.jaci.2003.08.013

207. Mautino G, Capony F, Bousquet J, Vignola AM. Balance in asthma between matrix metalloproteinases and their inhibitors. *Journal of Allergy and Clinical Immunology*. 1999;104(3):530-533. doi:10.1016/S0091-6749(99)70319-2

208. Chu HW, Trudeau JB, Balzar S, Wenzel SE. Peripheral blood and airway tissue expression of transforming growth factor β by neutrophils in asthmatic subjects and normal control subjects. *Journal of Allergy and Clinical Immunology*. 2000;106(6):1115-1123. doi:10.1067/mai.2000.110556

209. Scott HA, Gibson PG, Garg ML, Wood LG. Airway inflammation is augmented by obesity and fatty acids in asthma. *European Respiratory Journal*. 2011;38(3):594-602. doi:10.1183/09031936.00139810

210. Polosa R, Thomson NC. Smoking and asthma: dangerous liaisons. *European Respiratory Journal*. 2013;41(3):716-726. doi:10.1183/09031936.00073312

211. Green BJ, Wiriyachaiporn S, Grainge C, et al. Potentially pathogenic airway bacteria and neutrophilic inflammation in treatment resistant severe asthma. *PLoS ONE*. 2014;9(6):e100645. doi:10.1371/journal.pone.0100645

212. Busse WW, Lemanske RF, Gern JE. Role of viral respiratory infections in asthma and asthma exacerbations. *The Lancet*. 2010;376(9743):826-834. doi:10.1016/S0140-6736(10)61380-3

213. Teran LM, Campos MG, Begishvilli BT, et al. Identification of neutrophil chemotactic factors in bronchoalveolar lavage fluid of asthmatic patients. *Clinical & Experimental Allergy*. 2006;27(4):396-405. doi:10.1111/j.1365-2222.1997.tb00724.x

214. Jatakanon A, Uasuf C, Maziak W, Lim S, Chung KF, Barnes PJ. Neutrophilic inflammation in severe persistent asthma. *American Journal of Respiratory and Critical Care Medicine*. 1999;160(5):1532-1539. doi:10.1164/ajrccm.160.5.9806170

215. Simpson JL, Grissell T v, Douwes J, Scott RJ, Boyle MJ, Gibson PG. Innate immune activation in neutrophilic asthma and bronchiectasis. *Thorax*. 2007;62(3):211-218. doi:10.1136/thx.2006.061358

216. Uddin M, Lau LC, Seumois G, et al. EGF-induced bronchial epithelial cells drive neutrophil chemotactic and anti-apoptotic activity in asthma. *PLoS ONE*. 2013;8(9):e72502. doi:10.1371/journal.pone.0072502

217. Chakir J, Shannon J, Molet S, et al. Airway remodeling-associated mediators in moderate to severe asthma: Effect of steroids on TGF-β, IL-11, IL-17, and type I and type III collagen expression. *Journal of Allergy and Clinical Immunology*. 2003;111(6):1293-1298. doi:10.1067/mai.2003.1557

218. Newcomb DC, Peebles RS. Th17-mediated inflammation in asthma. *Current Opinion in Immunology*. 2013;25(6):755-760. doi:10.1016/j.coi.2013.08.002

219. Bettelli E, Korn T, Oukka M, Kuchroo VK. Induction and effector functions of TH17 cells. *Nature*. 2008;453(7198):1051-1057. doi:10.1038/nature07036

220. Laan M, Cui Z-H, Hoshino H, et al. Neutrophil recruitment by human IL-17 via C-X-C chemokine release in the airways. *Journal of Immunology*. 1999;162(4):2347-2352. http://www.jimmunol.org/content/162/4/http://www.jimmunol.org/content/162/4/2347.full#ref-list-1

221. Ye P, Rodriguez FH, Kanaly S, et al. Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense. *Journal of Experimental Medicine*. 2001;194(4):519-528. doi:10.1084/jem.194.4.519

222. Schwarzenberger P, Huang W, Ye P, et al. Requirement of endogenous stem cell factor and granulocyte-colony-stimulating factor for IL-17-mediated granulopoiesis. *Journal of Immunology*. 2000;164(9):4783-4789. doi:10.4049/jimmunol.164.9.4783

223. Stark MA, Huo Y, Burcin TL, Morris MA, Olson TS, Ley K. Phagocytosis of apoptotic neutrophils regulates granulopoiesis via IL-23 and IL-17. *Immunity*. 2005;22(3):285-294. doi:10.1016/j.immuni.2005.01.011

224. Altenburg J, de Graaff CS, van der Werf TS, Boersma WG. Immunomodulatory effects of macrolide antibiotics – Part 2: Advantages and disadvantages of long-term, low-dose macrolide therapy. *Respiration*. 2011;81(1):75-87. doi:10.1159/000320320

225. Gibson PG, Yang IA, Upham JW, et al. Effect of azithromycin on asthma exacerbations and quality of life in adults with persistent uncontrolled asthma (AMAZES): a randomised, double-blind, placebo-controlled trial. *The Lancet*. 2017;390(10095):659-668. doi:10.1016/S0140-6736(17)31281-3

226. Simpson JL, Powell H, Boyle MJ, Scott RJ, Gibson PG. Clarithromycin targets neutrophilic airway inflammation in refractory asthma. *American Journal of Respiratory and Critical Care Medicine*. 2008;177(2):148-155. doi:10.1164/rccm.200707-1134OC

227. Molfino NA, Kuna P, Leff JA, et al. Phase 2, randomised placebo-controlled trial to evaluate the efficacy and safety of an anti-GM-CSF antibody (KB003) in patients with inadequately controlled asthma. *British Medical Journal Open*. 2016;6(1):e007709. doi:10.1136/bmjopen-2015-007709

228. Nair P, Gaga M, Zervas E, et al. Safety and efficacy of a CXCR2 antagonist in patients with severe asthma and sputum neutrophils: a randomized, placebo-controlled clinical trial. *Clinical & Experimental Allergy*. 2012;42(7):1097-1103. doi:10.1111/j.1365-2222.2012.04014.x

229. O'Byrne PM, Metev H, Puu M, et al. Efficacy and safety of a CXCR2 antagonist, AZD5069, in patients with uncontrolled persistent asthma: a randomised, double-blind, placebo-controlled trial. *The Lancet Respiratory Medicine*. 2016;4(10):797-806. doi:10.1016/S2213-2600(16)30227-2

230. Busse WW, Holgate S, Kerwin E, et al. 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*. 2013;188(11):1294-1302. doi:10.1164/rccm.201212-2318OC

231. Wenzel SE, Barnes PJ, Bleecker ER, et al. A randomized, double-blind, placebo-controlled study of tumor necrosis factor-α blockade in severe persistent asthma.

American Journal of Respiratory and Critical Care Medicine. 2009;179(7):549-558. doi:10.1164/rccm.200809-1512OC

232. Holgate ST, Noonan M, Chanez P, et al. Efficacy and safety of etanercept in moderate-to-severe asthma: a randomised, controlled trial. *European Respiratory Journal*. 2011;37(6):1352-1359. doi:10.1183/09031936.00063510

233. Dinarello CA. Historical insights into cytokines. *European Journal of Immunology*. 2007;37(S1):S34-S45. doi:10.1002/eji.200737772

234. Lacy P, Stow JL. Cytokine release from innate immune cells: association with diverse membrane trafficking pathways. *Blood*. 2011;118(1):9-18. doi:10.1182/blood-2010-08-265892

235. Stanley AC, Lacy P. Pathways for cytokine secretion. *Physiology*. 2010;25(4):218-229. doi:10.1152/physiol.00017.2010

236. Lodish H, Berk A, Zipursky S, Matsudaira P, Baltimore D, Darnell J. *Molecular Cell Biology*. 4th edition. W. H. Freeman; 2000. Accessed July 1, 2020. https://www.ncbi.nlm.nih.gov/books/NBK21471/

237. Barlowe C. COPII: A membrane coat formed by Sec proteins that drive vesicle budding from the endoplasmic reticulum. *Cell*. 1994;77(6):895-907. doi:10.1016/0092-8674(94)90138-4

238. Rubartelli A, Cozzolino F, Talio M, Sitia R. A novel secretory pathway for interleukin-1 beta, a protein lacking a signal sequence. *The European Molecular Biology Organization Journal*. 1990;9(5):1503-1510. doi:10.1002/j.1460-2075.1990.tb08268.x

239. Flieger O, Engling A, Bucala R, Lue H, Nickel W, Bernhagen J. Regulated secretion of macrophage migration inhibitory factor is mediated by a non-classical pathway involving an ABC transporter. *Federation of European Biochemical Societies Letters*. 2003;551(1-3):78-86. doi:10.1016/S0014-5793(03)00900-1

240. Eder C. Mechanisms of interleukin-1β release. *Immunobiology*. 2009;214(7):543-553. doi:10.1016/j.imbio.2008.11.007

241. Bonifacino JS, Glick BS. The Mechanisms of Vesicle Budding and Fusion. *Cell*. 2004;116(2):153-166. doi:10.1016/S0092-8674(03)01079-1

242. Söllner T, Whiteheart SW, Brunner M, et al. SNAP receptors implicated in vesicle targeting and fusion. *Nature*. 1993;362(6418):318-324. doi:10.1038/362318a0

243. McNew JA, Parlati F, Fukuda R, et al. Compartmental specificity of cellular membrane fusion encoded in SNARE proteins. *Nature*. 2000;407(6801):153-159. doi:10.1038/35025000

244. Hong W. SNAREs and traffic. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*. 2005;1744(2):120-144. doi:10.1016/j.bbamcr.2005.03.014

245. Jahn R, Scheller RH. SNAREs - Engines for membrane fusion. *Nature Reviews Molecular Cell Biology*. 2006;7(9):631-643. doi:10.1038/nrm2002

246. Bock JB, Matern HT, Peden AA, Scheller RH. A genomic perspective on membrane compartment organization. *Nature*. 2001;409(6822):839-841. doi:10.1038/35057024

247. Vivona S, Cipriano DJ, O'Leary S, Li YH, Fenn TD, Brunger AT. Disassembly of all SNARE complexes by N-Ethylmaleimide-sensitive factor (NSF) is initiated by a conserved 1:1 interaction between α-soluble NSF attachment protein (SNAP) and SNARE Complex. *Journal of Biological Chemistry*. 2013;288(34):24984-24991. doi:10.1074/jbc.M113.489807

248. Mollinedo F, Calafat J, Janssen H, et al. Combinatorial SNARE complexes modulate the secretion of cytoplasmic granules in human neutrophils. *The Journal of Immunology*. 2006;177(5):2831-2841. doi:10.4049/jimmunol.177.5.2831

249. Lacy P, Logan MR, Bablitz B, Moqbel R. Fusion protein vesicle-associated membrane protein 2 is implicated in IFN-γ–induced piecemeal degranulation in human eosinophils from atopic individuals. *Journal of Allergy and Clinical Immunology*. 2001;107(4):671-678. doi:10.1067/mai.2001.113562

250. Logan MR, Lacy P, Odemuyiwa SO, et al. A critical role for vesicle-associated membrane protein-7 in exocytosis from human eosinophils and neutrophils. *Allergy*. 2006;61(6):777-784. doi:10.1111/j.1398-9995.2006.01089.x

251. Hoffmann HJ, Bjerke T, Karawajczyk M, Dahl R, Knepper MA, Nielsen S. SNARE proteins are critical for regulated exocytosis of ECP from human eosinophils. *Biochemical and Biophysical Research Communications*. 2001;282(1):194-199. doi:10.1006/bbrc.2001.4499

252. Willetts L, Felix LC, Jacobsen EA, et al. Vesicle-associated membrane protein 7-mediated eosinophil degranulation promotes allergic airway inflammation in mice. *Communications Biology*. 2018;1(1):83. doi:10.1038/s42003-018-0081-z

253. Logan MR, Lacy P, Bablitz B, Moqbel R. Expression of eosinophil target SNAREs as potential cognate receptors for vesicle-associated membrane protein-2 in exocytosis. *Journal of Allergy and Clinical Immunology*. 2002;109(2):299-306. doi:10.1067/mai.2002.121453

254. Carmo LAS, Dias FF, Malta KK, et al. Expression and subcellular localization of the Qa-SNARE syntaxin17 in human eosinophils. *Experimental Cell Research*. 2015;337(2):129-135. doi:10.1016/j.yexcr.2015.07.003

255. Stenmark H. Rab GTPases as coordinators of vesicle traffic. *Nature Reviews Molecular Cell Biology*. 2009;10(8):513-525. doi:10.1038/nrm2728

256. Pereira-Leal JB, Seabra MC. Evolution of the rab family of small GTP-binding proteins. *Journal of Molecular Biology*. 2001;313(4):889-901. doi:10.1006/jmbi.2001.5072

257. Schwartz SL, Cao C, Pylypenko O, Rak A, Wandinger-Ness A. Rab GTPases at a glance. *Journal of Cell Science*. 2007;120(22):3905-3910. doi:10.1242/jcs.015909

258. Zerial M, McBride H. Rab proteins as membrane organizers. *Nature Reviews Molecular Cell Biology*. 2001;2(2):107-117. doi:10.1038/35052055

259. Pan X, Eathiraj S, Munson M, Lambright DG. TBC-domain GAPs for Rab GTPases accelerate GTP hydrolysis by a dual-finger mechanism. *Nature*. 2006;442(7100):303-306. doi:10.1038/nature04847

260. Delprato A, Merithew E, Lambright DG. Structure, exchange determinants, and family-wide Rab specificity of the tandem helical bundle and Vps9 domains of Rabex-5. *Cell*. 2004;118(5):607-617. doi:10.1016/j.cell.2004.08.009

261. Matsui Y, Kikuchi A, Araki S, et al. Molecular cloning and characterization of a novel type of regulatory protein (GDI) for smg p25A, a ras p21-like GTP-binding protein. *Molecular and Cellular Biology*. 1990;10(8):4116-4122. doi:10.1128/MCB.10.8.4116

262. Ullrich O, Horiuchi H, Bucci C, Zerial M. Membrane association of Rab5 mediated by GDP-dissociation inhibitor and accompanied by GDP/GTP exchange. *Nature*. 1994;368(6467):157-160. doi:10.1038/368157a0

263. Ullrichss O, Stenmarksy H, Alexandrovs K, et al. Rab GDP dissociation inhibitor as a general regulator for the membrane association of Rab proteins. *Journal of Biological Chemistry*. 1993;268(24):18143-18150.

264. Soldati T, Shapiro AD, Dirac Svejstrup AB, Pfefffer SR. Membrane targeting of the small GTPase Rab9 is accompanied by nucleotide exchange. *Nature*. 1994;369(6475):76-78. doi:10.1038/369076a0

265. Sivars U, Aivazian D, Pfeffer SR. Yip3 catalyses the dissociation of endosomal Rab–GDI complexes. *Nature*. 2003;425(6960):856-859. doi:10.1038/nature02057

266. Stinchcombe JC, Barral DC, Mules EH, et al. Rab27a is required for regulated secretion in cytotoxic T lymphocytes. *Journal of Cell Biology*. 2001;152(4):825-834. doi:10.1083/jcb.152.4.825

267. Gazit R, Aker M, Elboim M, et al. NK cytotoxicity mediated by CD16 but not by NKp30 is functional in Griscelli syndrome. *Blood*. 2007;109(10):4306-4312. doi:10.1182/blood-2006-09-047159

268. Goishi K, Mizuno K, Nakanishi H, Sasaki T. Involvement of Rab27 in antigen-induced histamine release from rat basophilic leukemia 2H3 cells. *Biochemical and Biophysical Research Communications*. 2004;324(1):294-301. doi:10.1016/j.bbrc.2004.09.050

269. Munafó DB, Johnson JL, Ellis BA, Rutschmann S, Beutler B, Catz SD. Rab27a is a key component of the secretory machinery of azurophilic granules in granulocytes. *Biochemical Journal*. 2007;402(2):229-239. doi:10.1042/BJ20060950

270. Hume AN, Collinson LM, Rapak A, Gomes AQ, Hopkins CR, Seabra MC. Rab27a regulates the peripheral distribution of melanosomes in melanocytes. *Journal of Cell Biology*. 2001;152(4):795-808. doi:10.1083/jcb.152.4.795

271. Coughlin JJ, Odemuyiwa SO, Davidson CE, Moqbel R. Differential expression and activation of Rab27A in human eosinophils: Relationship to blood eosinophilia. *Biochemical and Biophysical Research Communications*. 2008;373(3):382-386. doi:10.1016/j.bbrc.2008.06.033

272. van Gele M, Dynoodt P, Lambert J. Griscelli syndrome: a model system to study vesicular trafficking. *Pigment Cell & Melanoma Research*. 2009;22(3):268-282. doi:10.1111/j.1755-148X.2009.00558.x

273. Kim JD, Willetts L, Ochkur S, et al. An essential role for Rab27a GTPase in eosinophil exocytosis. *Journal of Leukocyte Biology*. 2013;94(6):1265-1274. doi:10.1189/jlb.0812431

274. Wu XS, Rao K, Zhang H, et al. Identification of an organelle receptor for myosin-Va. *Nature Cell Biology*. 2002;4(4):271-278. doi:10.1038/ncb760

275. Gruenberg J. The endocytic pathway: a mosaic of domains. *Nature Reviews Molecular Cell Biology*. 2001;2(10):721-730. doi:10.1038/35096054

276. Cullen PJ, Steinberg F. To degrade or not to degrade: mechanisms and significance of endocytic recycling. *Nature Reviews Molecular Cell Biology*. 2018;19(11):679-696. doi:10.1038/s41580-018-0053-7

277. Taguchi T. Emerging roles of recycling endosomes. *Journal of Biochemistry*. 2013;153(6):505-510. doi:10.1093/jb/mvt034

278. Mayle KM, Le AM, Kamei DT. The intracellular trafficking pathway of transferrin. *Biochimica et Biophysica Acta (BBA) - General Subjects*. 2012;1820(3):264-281. doi:10.1016/j.bbagen.2011.09.009

279. Bridgewater RE, Norman JC, Caswell PT. Integrin trafficking at a glance. *Journal of Cell Science*. 2012;125(16):3695-3701. doi:10.1242/jcs.095810

280. Cendrowski J, Mamińska A, Miaczynska M. Endocytic regulation of cytokine receptor signaling. *Cytokine & Growth Factor Reviews*. 2016;32:63-73. doi:10.1016/j.cytogfr.2016.07.002

281. Murray RZ. A role for the phagosome in cytokine secretion. *Science*. 2005;310(5753):1492-1495. doi:10.1126/science.1120225

282. Murray RZ, Wylie FG, Khromykh T, Hume DA, Stow JL. Syntaxin 6 and Vti1b form a novel SNARE complex, which is up-regulated in activated macrophages to facilitate exocytosis of tumor necrosis factor-α. *Journal of Biological Chemistry*. 2005;280(11):10478-10483. doi:10.1074/jbc.M414420200

283. Pagan JK, Wylie FG, Joseph S, et al. The t-SNARE Syntaxin 4 is regulated during macrophage activation to function in membrane traffic and cytokine secretion. *Current Biology*. 2003;13(2):156-160. doi:10.1016/S0960-9822(03)00006-X

284. Black RA, Rauch CT, Kozlosky CJ, et al. A metalloproteinase disintegrin that releases tumour-necrosis factor-α from cells. *Nature*. 1997;385(6618):729-733. doi:10.1038/385729a0

285. Arango Duque G, Descoteaux A. Macrophage cytokines: involvement in immunity and infectious Diseases. *Frontiers in Immunology*. 2014;5. doi:10.3389/fimmu.2014.00491

286. Manderson AP, Kay JG, Hammond LA, Brown DL, Stow JL. Subcompartments of the macrophage recycling endosome direct the differential secretion of IL-6 and TNFα. *Journal of Cell Biology*. 2007;178(1):57-69. doi:10.1083/jcb.200612131

287. Reefman E, Kay JG, Wood SM, et al. Cytokine secretion is distinct from secretion of cytotoxic granules in NK Cells. *Journal of Immunology*. 2010;184(9):4852-4862. doi:10.4049/jimmunol.0803954

288. Shen Z-J, Malter JS. Determinants of eosinophil survival and apoptotic cell death. *Apoptosis*. 2015;20(2):224-234. doi:10.1007/s10495-014-1072-2

289. Przybylski S, Gasch M, Marschner A, et al. Influence of nanoparticle-mediated transfection on proliferation of primary immune cells in vitro and in vivo. *PLOS ONE*. 2017;12(5):e0176517. doi:10.1371/journal.pone.0176517

290. Koeffler HP, Golde DW. Human myeloid leukemia cell lines: A review. *Blood*. 1980;56(3). www.bloodjournal.org

291. Ishihara K. Eosinophil Cell Lines. In: Walsh GM, ed. *Eosinophils: Methods and Protocols*. Springer New York; 2014:45-51. doi:10.1007/978-1-4939-1016-8_5

292. Fischkoff SA, Condon ME. Switch in differentiative response to maturation inducers of human promyelocytic leukemia cells by prior exposure to alkaline conditions. *Cancer Research*. 1985;45:2065-2069.

293. Mollinedo F, Santos-Beneit AM, Gajate C. The human leukemia cell line HL-60 as a cell culture model to study neutrophil functions and inflammatory cell

responses. In: Clynes M, ed. *Animal Cell Culture Techniques*. Springer Berlin Heidelberg; 1998:264-297. doi:10.1007/978-3-642-80412-0_16

294. Yaseen R, Blodkamp S, Lüthje P, et al. Antimicrobial activity of HL-60 cells compared to primary blood-derived neutrophils against Staphylococcus aureus. *Journal of Negative Results in BioMedicine*. 2017;16(1):2. doi:10.1186/s12952-017-0067-2

295. Faurschou M, Borregaard N. Neutrophil granules and secretory vesicles in inflammation. *Microbes and Infection*. 2003;5(14):1317-1327. doi:10.1016/j.micinf.2003.09.008

296. Fischkoff SA. Graded increase in probability of eosinophilic differentiation of HL-60 promyelocytic leukemia cells induced by culture under alkaline conditions. *Leukemia Research*. 1988;12(8):679-686. doi:10.1016/0145-2126(88)90103-8

297. Fischkoff SA, Pollak A, Gleich GJ, Testa JR, Misawa S, Reber TJ. Eosinophilic differentiation of the human promyelocytic leukemia cell line, HL-60. *The Journal of Experimental Medicine*. 1984;160(1):179-196. doi:10.1084/jem.160.1.179

298. Ishihara K, Hong J, Zee O, Ohuchi K. Possible mechanism of action of the histone deacetylase inhibitors for the induction of differentiation of HL-60 clone 15 cells into eosinophils. *British Journal of Pharmacology*. 2004;142(6):1020-1030. doi:10.1038/sj.bjp.0705869

299. Fu C-H, Tsai W-C, Lee T-J, Huang C-C, Chang P-H, Su Pang J-H. Simvastatin inhibits IL-5-induced chemotaxis and CCR3 expression of HL-60-derived and human primary eosinophils. *PLOS ONE*. 2016;11(6):e0157186. doi:10.1371/journal.pone.0157186

300. Millius A, Weiner OD. Chemotaxis in neutrophil-like HL-60 Cells. In: Jin Tian and Hereld D, ed. *Chemotaxis: Methods and Protocols*. Humana Press; 2009:167-177. doi:10.1007/978-1-60761-198-1_11

301. de la Fuente J, Manzano-Roman R, Blouin EF, Naranjo V, Kocan KM. Sp110 transcription is induced and required by Anaplasma phagocytophilumfor infection of human promyelocytic cells. *BMC Infectious Diseases*. 2007;7(1):110. doi:10.1186/1471-2334-7-110

302. Nakata Y, Tomkowicz B, Gewirtz AM, Ptasznik A. Integrin inhibition through Lyn-dependent cross talk from CXCR4 chemokine receptors in normal human CD34+ marrow cells. *Blood*. 2006;107(11):4234-4239. doi:10.1182/blood-2005-08-3343

303. Carter BZ, Milella M, Tsao T, et al. Regulation and targeting of antiapoptotic XIAP in acute myeloid leukemia. *Leukemia*. 2003;17(11):2081-2089. doi:10.1038/sj.leu.2403113

304. Johnson JL, Ellis BA, Munafo DB, Brzezinska AA, Catz SD. Gene transfer and expression in human neutrophils. The phox homology domain of p47phox translocates to the plasma membrane but not to the membrane of mature phagosomes. *BMC Immunology*. 2006;7(1):28. doi:10.1186/1471-2172-7-28

305. Duguay BA, Huang KW-C, Kulka M. Lipofection of plasmid DNA into human mast cell lines using lipid nanoparticles generated by microfluidic mixing. *Journal of Leukocyte Biology*. 2018;104(3):587-596. doi:10.1002/JLB.3TA0517-192R

306. Foglietta F, Serpe L, Canaparo R, et al. Modulation of butyrate anticancer activity by solid lipid nanoparticle delivery: An in vitro investigation on human breast cancer and leukemia cell lines. *Journal of Pharmacy & Pharmaceutical Sciences*. 2014;17(2):231. doi:10.18433/J3XP4R

307. Kimura I, Moritani Y, Tanizaki Y. Basophils in bronchial asthma with reference to reagin-type allergy. *Clinical Experimental Allergy*. 1973;3(2):195-202. doi:10.1111/j.1365-2222.1973.tb01321.x

308. Dunn KW, Kamocka MM, McDonald JH. A practical guide to evaluating colocalization in biological microscopy. *American Journal of Physiology-Cell Physiology*. 2011;300(4):C723-C742. doi:10.1152/ajpcell.00462.2010

309. Adler J, Parmryd I. Quantifying colocalization by correlation: The Pearson correlation coefficient is superior to the Mander's overlap coefficient. *Cytometry Part A*. 2010;77A(8):733-742. doi:10.1002/cyto.a.20896

310. Gounni AS, Nutku E, Koussih L, et al. IL-9 expression by human eosinophils: Regulation by IL-1β and TNF-α. *Journal of Allergy and Clinical Immunology*. 2000;106(3):460-466. doi:10.1067/mai.2000.109172

311. Revelo NH, ter Beest M, van den Bogaart G. Membrane trafficking as an active regulator of constitutively secreted cytokines. *Journal of Cell Science*. 2020;133(5):jcs234781. doi:10.1242/jcs.234781

312. Gessner A, Mohrs K, Mohrs M. Mast Cells, basophils, and eosinophils acquire constitutive IL-4 and IL-13 transcripts during lineage differentiation that are sufficient for rapid cytokine production. *Journal of Immunology*. 2005;174(2):1063-1072. doi:10.4049/jimmunol.174.2.1063

Appendix A

Table A1. Troubleshooting of immunofluorescence experiments involving LPS-stimulated primary human neutrophils. Primary human neutrophils were isolated, stimulated and immunolabelled as described in Sections 2.5, 2.7, and 2.9 of Materials and Methods. [#] A successful or unsuccessful stain is denoted by a 'Y' for yes and 'N' for no. Stains were defined as successful if the fluorescent signal appeared to be above background and isotype antibody staining intensities. * LPS stimulation of neutrophils was conducted for 2 h, 4 h, and 6 h. **LPS stimulation of neutrophils was conducted for 2h and 4h.

Experiment number	Permeabilization agent	Targets	Antibody	Successful Stain (Y/N)[#]
1	Saponin (0.1%)	TNF	Mouse α-human TNF [cat. # MAB610, Novus Biologicals]	N
		Rab27a	Rabbit α-human Rab27a [cat. # sc-22756, Santa Cruz Biotechnology]	N
		SNAP23	Rabbit α-human SNAP23 [cat. # 111202 Synaptic Systems]	N
		CD66b	FITC Mouse α-human CD66b [cat. # 60086FI.1, STEMCELL Technologies]	N
2	Saponin (0.1%)	TNF	Mouse α-human TNF [cat. # MAB610, Novus Biologicals]	N
		Syntaxin-4	Mouse α-human Syntaxin-4 [cat. # sc-101301, Santa Cruz Biotechnology]	N
		TfnRc	Alexa 488 Rabbit α-human TfnRc [cat. # bs-0988R-A488, Bioss]	Y
		Rab11a	Rabbit α-human Rab11a [cat. # 715300, Invitrogen]	Y
3	Saponin (0.1%)	TNF	Mouse α-human TNF [cat. # MAB610, Novus Biologicals]	N
		TfnRc	Alexa 488 Rabbit α-human TfnRc [cat. # bs-0988R-A488, Bioss]	N

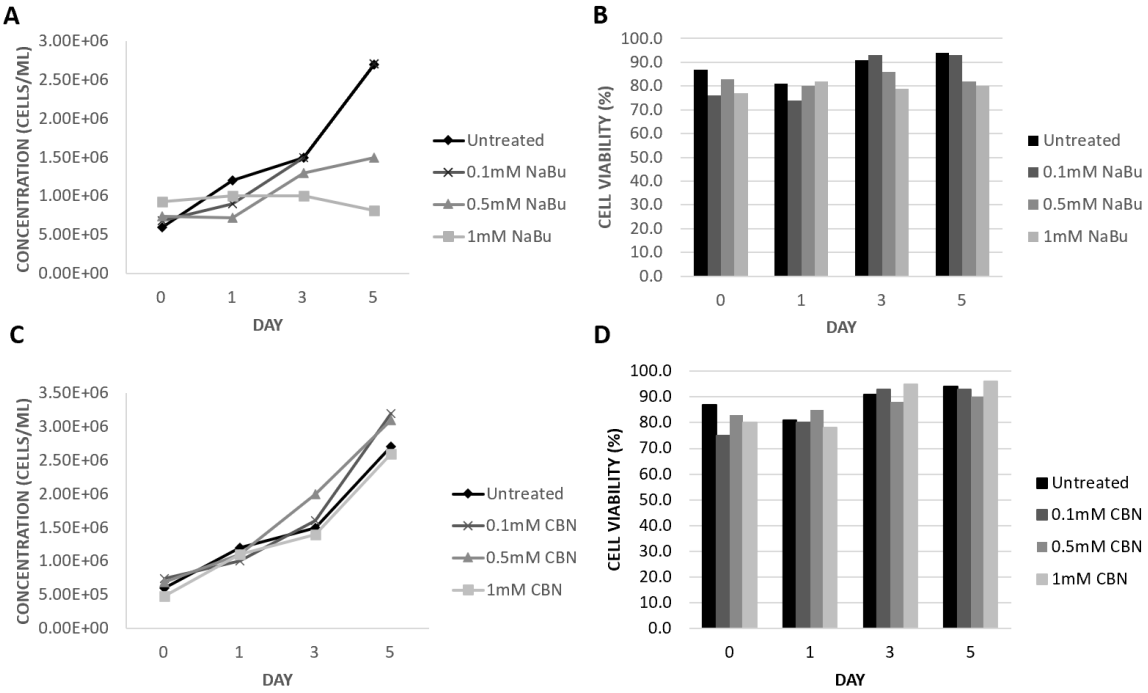
4	Triton X-100 (0.2%)	TNF	Mouse α-human TNF [cat. # MAB610, Novus Biologicals]	N
		VAMP-3	Rabbit α-human VAMP-3 [cat. # NB300-510, Novus Biologicals]	N
		TfnRc	Alexa 488 Rabbit α-human TfnRc [cat. # bs-0988R-A488, Bioss]	Y
5	Triton X-100 (0.1%)	Rab27a	Rabbit α-human Rab27a [cat. # R4655, Millipore Sigma]	N
		CD66b	FITC Mouse α-human CD66b [cat. # 60086FI.1, STEMCELL Technologies]	Y
6	Triton X-100 (0.1%)	TNF	Rabbit α-human TNF [cat. # 654250, Millipore Sigma]	N
		CD66b	FITC Mouse α-human CD66b [cat. # 60086FI.1, STEMCELL Technologies]	Y
7	Triton X-100 (0.1%)	TNF	Rabbit α-human TNF [cat. # 654250, Millipore Sigma]	N
		CD66b	FITC Mouse α-human CD66b [cat. # 60086FI.1, STEMCELL Technologies]	Y
8	Triton X-100 (0.2%)	TNF	Mouse α-human TNF [cat. # MAB610, Novus Biologicals]	N
		VAMP-3	Rabbit α-human VAMP-3 [cat. # OSS00046G, Thermo Fisher Scientific]	Y
9	Triton X-100 (0.2%)	TNF	Alexa Fluor 488 Mouse α-human TNF [cat. # 557722, BD Biosciences]	N
10	Triton X-100 (0.1%)	IL-8	Mouse α-human IL-8 [cat. # AHC0982, Thermo Fisher Scientific]	N
		VAMP-3	Rabbit α-human VAMP-3 [cat. # OSS00046G,	Y

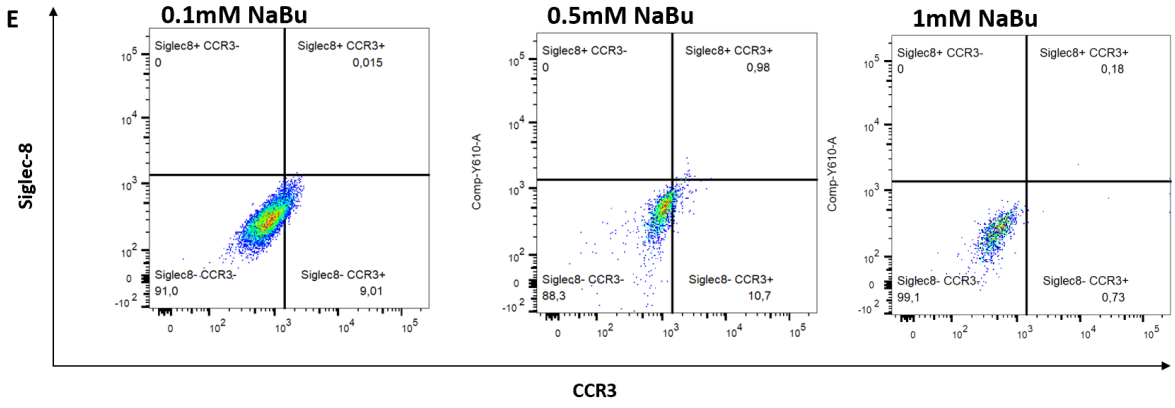
			Thermo Fisher Scientific]	
11*	Triton X-100 (0.1%)	TNF	Rabbit α-human TNF [cat. # 654250, Millipore Sigma]	N
		CD66b	FITC Mouse α-human CD66b [cat. # 60086FI.1, STEMCELL Technologies]	Y
12*	Triton X-100 (0.1%)	IL-8	Mouse α-human IL-8 [cat. # AHC0982, Thermo Fisher Scientific]	N
		VAMP-3	Rabbit α-human VAMP-3 [cat. # OSS00046G, Thermo Fisher Scientific]	Y
13*	Triton X-100 (0.1%)	TNF	Alexa Fluor 594 Rabbit α-human TNF [cat. # bs-2081R-A549, Bioss]	N
		CD66b	FITC Mouse α-human CD66b [cat. # 60086FI.1, STEMCELL Technologies]	N
14**	Triton X-100 (0.1%)	TNF	Goat α-human/mouse TNF [cat. # AF-410-NA, R&D Systems]	N
		VAMP-3	Rabbit α-human VAMP-3 [cat. # OSS00046G, Thermo Fisher Scientific]	N

A total of five primary antibodies raised against TNF could not successfully detect the cytokine in immunolabelling experiments of human neutrophils **(Table A1)**. I considered that use of saponin as a permeabilization agent may be too mild for primary neutrophils and began to use the harsher detergent Triton X-100 in hopes of granting antibodies greater access to bind intracellular proteins **(Table A1, experiment numbers 4-14)**. While implementation of Triton X-100 allowed for positive staining of TfnRc, CD66b, and VAMP-3 in human neutrophils, TNF remained undetected **(Table A1, experiment numbers 4-8)**. Similarly, IL-8 could not be detected in human neutrophils through immunolabelling with two primary antibodies raised against IL-8 **(Table A1)**. Longer incubations of LPS with human neutrophils were implemented with hopes of enhancing intracellular TNF of IL-8 levels and allowing for detection of these cytokines by immunofluorescence experiments **(Table A1, experiment numbers 11-14)**. This elongation of

LPS stimulation resulted in the same negative staining for TNF and IL-8. Overall, the lack of detection of IL-8 and TNF in resting and LPS stimulated human neutrophils suggests cytokine protein levels are below the detection limit of the immunofluorescence assay. Subcellular fractionation of human neutrophils followed by western blotting or mass spectrometry of fractions containing granules and endosomal compartments for detection of TNF and IL-8 should be employed next.

Appendix B


A
CONCENTRATION (CELLS/ML)
3.00E+06
2.50E+06
2.00E+06
1.50E+06
1.00E+06
5.00E+05
0.00E+00
0
1
3
5
DAY
Untreated
0.1mM NaBu
0.5mM NaBu
1mM NaBu
B
CELL VIABILITY (%)
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0
1
3
5
DAY
Untreated
0.1mM NaBu
0.5mM NaBu
1mM NaBu
C
CONCENTRATION (CELLS/ML)
3.50E+06
3.00E+06
2.50E+06
2.00E+06
1.50E+06
1.00E+06
5.00E+05
0.00E+00
0
1
3
5
DAY
Untreated
0.1mM CBN
0.5mM CBN
1mM CBN
D
CELL VIABILITY (%)
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0
1
3
5
DAY
Untreated
0.1mM CBN
0.5mM CBN
1mM CBN


E
Siglec-8
CCR3
0.1mM NaBu
Siglec8+ CCR3-
0
Siglec8+ CCR3+
0,015
Siglec8- CCR3-
91,0
Siglec8- CCR3+
9,01
0.5mM NaBu
Siglec8+ CCR3-
0
Siglec8+ CCR3+
0,98
Comp-Y610-A
Siglec8- CCR3-
88,3
Siglec8- CCR3+
10,7
1mM NaBu
Siglec8+ CCR3-
0
Siglec8+ CCR3+
0,18
Comp-Y610-A
Siglec8- CCR3-
99,1
Siglec8- CCR3+
0,73

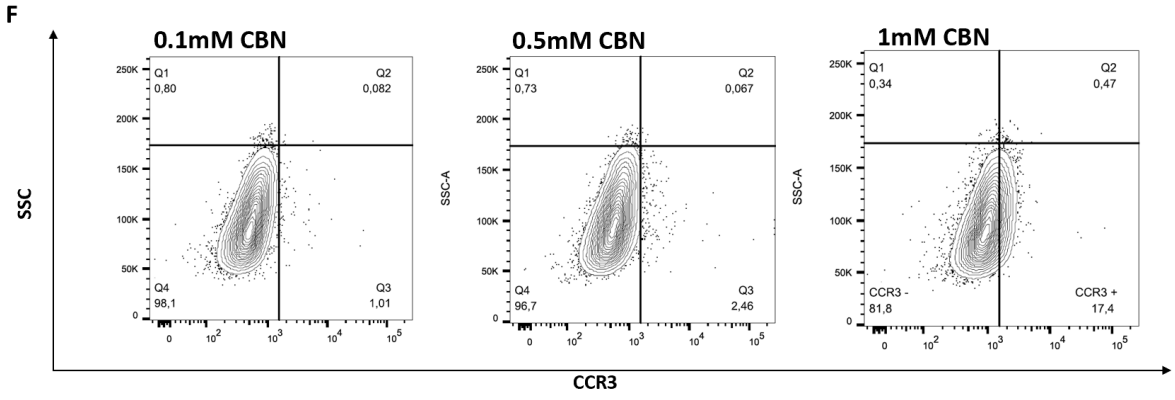

F
SSC
CCR3
0.1mM CBN
Q1
0,80
Q2
0,082
Q4
98,1
Q3
1,01
0.5mM CBN
SSC-A
Q1
0,73
Q2
0,067
Q4
96,7
Q3
2,46
1mM CBN
SSC-A
Q1
0,34
Q2
0,47
CCR3 -
81,8
CCR3 +
17,4

Figure B1. HL-60 clone 15 cell growth and viability is unaltered by cholesterol butyrate nanoparticle (CBN) treatment and induces CCR3 expression at high doses, as opposed to sodium butyrate (NaBu) treatment. HL-60 clone 15 cells were treated with 0.1 mM, 0.5 mM, and 1 mM of NaBu or CBN for five days. At days 0, 1, 3, and 5, cells were collected and loaded into the Invitrogen Countess^{TM} Automated Cell Counter to meausre cell concentration and viability (A-D). Cell viability was assessed by trypan blue exclusion. Untreated cells were also included as a normal control. Expression of eosinophil-associated proteins, Siglec-8 and CCR3, assessed by flow cytometry is shown for NaBu-treated HL-60 clone 15 cells at day 5 (E). Expression of CCR3 assessed by flow cytometry is shown for CBN-treated HL-60 clone 15 cells at day 5 (F).

A proxy for primary eosinophils which can withstand prolonged immunolabelling in time-lapse microscopy or gene manipulation experiments would be a valuable tool to further investigate IL-9 andIL-13 trafficking sites. While the use of NaBu as an inducer of eosinophil-like differentiation in HL-60 clone 15 cells has been described as a substitute for use of primary eosinophils, the protocol remains to be optimized. Here NaBu at 0.5 mM and 1mM is seen to hinder HL-60 clone 15 cell growth and lower cell viability during a 5-day treatment relative to untreated cells **(Figure B1 A, B)**. Meanwhile, use of CBN was hypothesized to be more easily taken up by HL-60 clone 15 cells compared to NaBu and with this increased uptake enhance the expression of eosinophil associated genes to a greater level then NaBu. When CBN was used to treat cells for 5 days, cell growth and viability appeared similar to that of untreated cells **(Figure B1 C, D)**. When expression of eosinophil-associated proteins such as CCR3 and Siglec-8 were assessed by flow cytometry, pilot studies revealed slightly elevated CCR3 expression in HL-60 clone 15 cells treated with 1 mM CBN **(Figure B1 F)**. Flow cytometry results for NaBu-treated cells were inconclusive **(Figure B1 E)**. Studies comparing NaBu and CBN treatment effects on cell growth, viability, and eosinophil-like differentiation in HL-60 clone 15 cells need to be repeated. HL-60 clone 15 cells treated with nanoparticles alone (i.e. not containing butyrate) should also be included as a vector control group in these studies. Furthermore, a positive control is needed for future flow cytometry experiments and should include primary human eosinophils which will express CCR3 and Siglec-8. More protein markers associated with eosinophils such as EPX and IL-5Rα should also be assessed by flow cytometry. Lastly, nanoparticles containing GFP-expressing plasmids could be used to assess the degree of nanoparticle uptake by HL-60 clone 15 cells by measuring GFP-positive cells by flow cytometry.