INVESTIGATING FLUID SHEAR STRESS IN EOSINOPHIL MIGRATION

INVESTIGATING THE EFFECT OF FLUID SHEAR STRESS-INDUCED CALCIUM RELEASE ON MIGRATION-ASSOCIATED MORPHOLOGICAL CHANGES IN HUMAN PERIPHERAL EOSINOPHILS

By

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TITLE: Investigating the effect of fluid shear stress-induced calcium release on migration-associated morphological changes in human peripheral eosinophils

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ABSTRACT

Elevated eosinophil counts in the circulation and/or tissues is considered a clinical feature and biomarker of several chronic airway diseases including asthma. As such, many therapeutic biologics for asthma developed within the past decade target eosinophil recruitment to and accumulation in the airways to mixed success. Although the nature of adhesive interactions and directional migration of eosinophils has been well studied, there remains a lack of comprehensive understanding regarding the components which modulate eosinophil movement from the blood into respiratory tissues that impacts the efficacy of these clinical studies; therefore, continued research in this area may reveal novel therapeutic targets and ultimately improve clinical outcomes of patients with eosinophilia-mediated diseases.

The Janssen lab serendipitously discovered that the mere perfusion of standard media without pharmacological additives over human eosinophils *in vitro* induced the release of intracellular calcium (Ca^{2+}) reminiscent of chemokine-induced Ca^{2+} release well documented in the literature. The central focus of my doctoral research was to characterize this novel phenomenon of the perfusion-induced calcium response (PICR), and to determine its physiological role in the eosinophil extravasation process to inflamed tissue sites.

In our first research objective, we optimized a protocol of eosinophil isolation directly from whole blood with emphases on maximizing population purity and yield efficiency while minimizing cell activation that could potentially interfere with secondary functional assays. For our latter two studies, we utilized real-time fluorescent confocal

microscopy and immunofluorescence staining to investigate the PICR. We observed that the latency to the PICR post-perfusion was significantly shorter in eosinophils subjected to physiological rates of shear stress, suggesting a temporal-regulatory function of eosinophil mechanosensitivity. Furthermore, the disruption of the PICR via pharmacological inhibitors significantly reduced eosinophil motility by increasing the latency to cytoskeletal rearrangements (flattening onto substrate-coated surfaces, formation of membrane protrusions that explore the environment) necessary for cell migration out of the vasculature. Detailing the role of eosinophil sensitivity to the mechanical trigger of fluid shear stress expands upon the current paradigm of eosinophil recruitment and will contribute to the development of clinical strategies.

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PREFACE

This is a *sandwich*-formatted thesis as outlined in the guide for the *Preparation of Master's and Doctoral Theses* available through the School of Graduate Studies, McMaster University, Hamilton, Ontario. Chapter 1 of this thesis focuses on introductory concepts relevant to eosinophil biology, calcium signaling pathways, and actin cytoskeleton regulation. Chapter 2 provides a brief methodology overview that supplements the information provided in the body. Chapters 3-5 compose the body of the thesis, each one an independent study that is either published or in the process of being submitted to a peer-reviewed scientific journal. The nature of contributions made by each of the authors are outlined for all scientific articles. The final chapter of this thesis (Chapter 6) is comprised of a discussion covering topics such as the clinical relevance, limitations, and future directions of these studies.

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LIST OF ABBREVIATIONS

AHR	Airway Hyperresponsiveness
AM	Acetoxymethyl
Arp2/3	Actin-Related Protein 2/3
BALF	Bronchoalveolar Lavage Fluid
BM	Bone Marrow
CICR	Calcium-Induced Calcium Release
CCE	Capacitative Calcium Entry
CCL	C-C Chemokine Ligands
CCR3	C-C Chemokine Receptor 3
Cdc42	Cell Division Cycle 42
COPD	Chronic Obstructive Pulmonary Disorder
CPA	Cyclopiazonic Acid
DAG	Diacyl-Glycerol
EC	Endothelial Cell
ECM	Extracellular Matrix
ECP	Eosinophil Cationic Protein
EDN	Eosinophil-Derived Neurotoxin
EGPA	Eosinophilic Granulomatosis with Polyangiitis
EoP	Eosinophil Progenitors
ER	Endoplasmic Reticulum
EPX	Eosinophil Peroxidase
F-Actin	Filamentous Actin
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HES	Hypereosinophilic Syndrome
HPC	Hematopoietic Progenitor Cell
ICAM-1	Intercellular Adhesion Molecule 1
IL	Interleukin
ILC2	Type 2 Innate Lymphoid Cell

IP_3	Inositol-1,4,5-trisphosphate
LAD	Leukocyte Adhesion Deficiency
mAb	Monoclonal Antibody
Mac-1	Macrophage-1 Antigen
MBP	Major Basic Protein
MIP-1	Macrophage Inflammatory Protein 1
MS	Multiple Sclerosis
NCX	Sodium-Calcium Exchanger
PI3K	Phosphoinositide-3 Kinase
PICR	Perfusion-Induced Calcium Response
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PLC	Phospholipase C
PMCA	Plasma Membrane Calcium ATPase
Rac1	Ras-related C3 Botulinum Toxin Substrate 1
RANTES	Regulated on Activation, Normal T Cell Expressed and Secreted
RhoA	Ras Homolog Family Member A
ROC	Receptor-Operated Channel
ROCK	Rho-Associated Protein Kinase
ROI	Region of Interest
RyR	Ryanodine Receptor
SEA	Severe Eosinophilic Asthma
SERCA	Sarcoplasmic-Endoplasmic Reticulum Calcium ATPase
SOC	Store-Operated Channel
TEM	Transendothelial Migration
T_H2	T-Helper Type-2
VCAM-1	Vascular Cell Adhesion Molecule 1
VLA-4	Very Late Antigen-4
VOC	Voltage-Operated Channel
XBP1	X-box-binding Protein 1

Chapter 1: Introduction

1.1 The Eosinophil: Friend or Foe?

Paul Ehrlich is credited for first staining the eosinophil leukocyte in 1879 that later led to its identification, and named them for their capacity to be stained by the acidophilic dye, eosin. Eosinophils are granulocytes, characterized by their bi-lobed, segmented nucleus and the presence of cytoplasmic granules which contain cell-specific cytotoxic molecules which are secreted to combat various pathogens including helminth parasites (Giembycz & Lindsay, 1999). The eosinophil has been discovered in virtually all vertebrates, strongly suggesting an evolutionary lineage of millions of years (Lee et al., 2010; Lee et al., 2012). Although the conservation of this cell type and its role in immunity highlights the benefits eosinophils must provide to their host, clinicians have persistently discovered an increased level of eosinophils, termed eosinophilia, in both circulation and in tissues of patients with allergic diseases such as asthma (Bochner, 2018). This invariable association found across scientific literature has ultimately led to therapeutic strategies that target eosinophilia.

1.1.1 Eosinophilopoiesis

Similar to other leukocytes, eosinophils develop from hematopoietic progenitor cells (HPC) that can be identified by the surface expression of CD34 (Krause et al., 1996). This monomeric transmembrane O-sialylated glycoprotein is highly expressed in early precursor stages, and gradually decreases in expression through the maturation process and lost on terminally differentiated cells (Krause et al., 1996). In steady state conditions, HPC will commit to eosinophil lineage in the bone marrow (BM) with the stimulation of the

eosinophilopoietin interleukin-5 (IL-5) in combination with granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-3 (Clutterbuck, Hirst, & Sanderson, 1989). The IL-5 receptor (IL-5R) has both a unique binding α-chain and a common β-chain shared with both GM-CSF and IL-3 (Tavernier et al., 1991). The upregulation of IL-5Rα on $CD34^{+}$ progenitors is commonly accepted as the initial, necessary step for eosinophil lineage commitment, thereby suggesting that CD34+IL-5Rα+ cells represent the earliest phenotype of eosinophil progenitor cells (EoPs) (Southam et al., 2005). The upregulation of IL-5Rα on HPC is preceded by signaling from several fate-determining transcription factors such as GATA-binding factor (GATA)-1 and GATA-2 (McNagny & Graf, 2002). Additionally, the X-box-binding protein 1 (XBP1) was found to be critical for eosinophil development; in fact, XBP1-deficient mice were unable to produce fully mature granule proteins necessary for eosinophil survival (Bettigole et al., 2015). Studies have shown that eosinophilopoiesis is tightly regulated with respect to granule protein development, in which significant deviance from the norm would result in dysfunctional differentiation (Doyle et al., 2013).

1.1.2 In Situ *Eosinophilopoiesis*

Although the majority of eosinophils will mature in the bone marrow (BM), there has been increasing data that indicate a migration of EoPs from the BM to sites of allergic inflammation post-allergen exposure for in situ differentiation to provide a consistent source of inflammatory effector cells (Sehmi et al., 2003). In subjects with mild allergic asthma, the number of BM-derived EoP were increased in the mucosa of lower and upper

airways, the peripheral blood, and airway secretions in positive correlation with disease severity (Kim et al., 1999; Robinson et al., 1999; Sergejeva et al., 2005; Sehmi et al., 1996). Moreover, a 10-fold increase in sputum EoP were found in corticosteroid-dependent severe asthmatics compared to mild asthmatics, even though EoP numbers were comparable in the peripheral blood of both groups, further cementing the idea that tissue and blood eosinophilia do not necessarily correlate (Sehmi et al., 2016). The increase in progenitor cells throughout various compartments in response to stress, tissue injury, and/or inflammation has given rise to the theory that HPC can differentiate appropriately outside the BM depending on the cytokines present in the local microenvironment (Salter & Sehmi, 2017). Based on these findings, there is a possibility that both the mobilization of EoP from the BM and their ensuing proliferation and maturation *in situ* in highly eosinophilopoietic environments contribute to eosinophilic inflammation in the airways; therefore controlling for both these factors may be key in diminishing inflammatory symptoms of asthma (Southam et al., 2005).

1.2 Eosinophilia

Eosinophils are generally considered to be a rare cell, with circulating counts in healthy populations often found between 30-350 cells/μL. Eosinophilia is a condition in which the absolute blood eosinophil count is observed to be significantly higher than normal, with the threshold commonly set as greater than 500 cells/μL (Kovalszki & Weller, 2016). Although eosinophilia can be caused by primary or idiopathic factors, the majority of cases are a result of secondary, or reactive, means which include asthma, allergic rhinitis,

eosinophilic esophagitis, and other allergy-associated disorders (Kovalszki & Weller, 2016; Butt et al., 2017). The degree of eosinophilia often determines the prognosis and/or treatment of these various diseases; in fact, there is a direct correlation between eosinophil counts and the clinical severity of asthma (Klion, 2015; Kay, 2005). However, it is critical to note the distinction between blood and tissue eosinophilia. Clinicians have conventionally relied on measurements of peripheral blood as a reflection of eosinophil accumulation in the tissue. This is due to the enumeration of tissue eosinophil counts being contingent on the analyses of biopsies (sputum and bronchoalveolar lavage) which are comparably more invasive and difficult to obtain in non-specialist centres (Wedzicha, 2016). Yet, current studies have shown that the assumption that blood eosinophilia is an authentic representation of tissue eosinophilia can no longer be conclusively accepted (Wedzicha, 2016; Turato et al., 2017).

1.2.1 General Asthma Overview

Asthma is a chronic respiratory disease that affects nearly 300 million people worldwide, in which the predominant pathophysiological defect is the manifestation of airway narrowing resulting from both acute bronchoconstriction and thickening of the airway wall (Murphy & O'Byrne, 2010; O'Byrne & Inman, 2003; Braman, 2006). The reversible constriction of the upper airways precipitates common symptoms such as wheezing and coughing (Kirshnan et al., 2012). Airway inflammation, mucus hypersecretion, and airway hyperresponsiveness (AHR) are additional core histological features (Broide et al., McBrien & Menzies-Gow, 2017) (Figure 1.1).

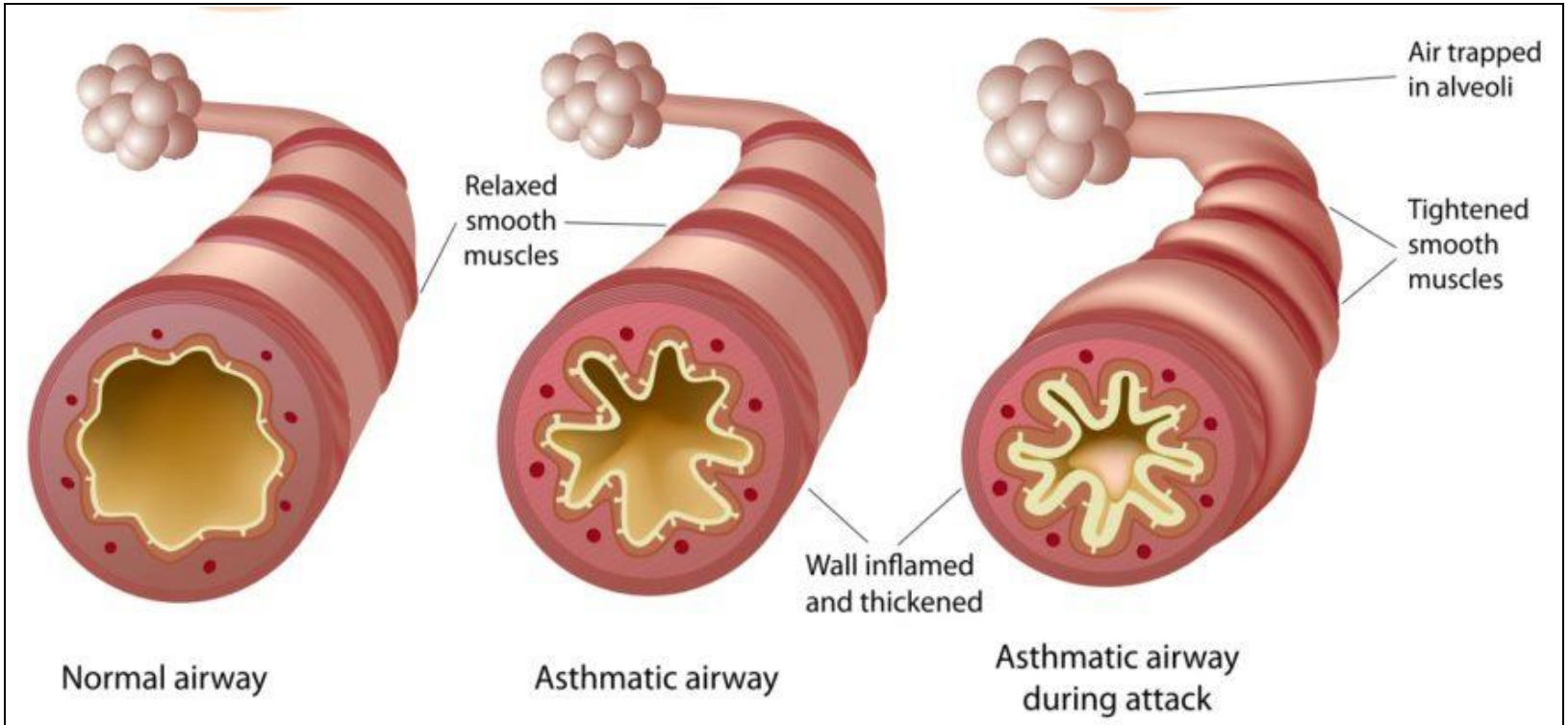

Figure 1.1 The asthmatic airway exhibits considerable narrowing as a result of several histological and functional changes. Inflammation and mucus production lead to a significant reduction in cross-sectional area which dramatically increases the resistance of the vessel to airflow and gives rise to symptoms of respiratory distress. (Image adapted from Canadiem, 2017)

Although no cure for asthma has been discovered, current medications attempt to manage and control the symptoms by targeting the inflammation. Unfortunately, asthma remains poorly controlled in more than half of diagnosed patients resulting in an overall decrease in their quality of life marked by worsening of symptoms, increased hospital visits, and days lost at school or work. As a result, asthma continues to exert a staggering global socioeconomic burden for both patients and healthcare systems worldwide (Bahadori et al., 2009).

Corticosteroids remain the most effective gold standard therapy for treating asthma, a small subset of individuals with severe asthma remain unresponsive and present with uncontrolled eosinophilia (Bateman et al., 2004). In these patients, new treatments directly targeting airway eosinophilia via IL-5 have been developed and shown to be effective in decreasing exacerbations and mature blood eosinophil counts (Nair et al., 2009; Mukherjee, Sehmi, Nair, 2014). In concurrence with seeming success, these drugs have additionally highlighted novel information regarding the origins of the airway eosinophils (Sehmi et al., 2016). The accumulation of tissue eosinophils is a result of the recruitment of (1) mature eosinophils from the vasculature and/or (2) in situ differentiation of EoPs in response to local cytokines such as IL-5 (Cameron et al., 2000). A study by Sehmi et al., (2016) showed that the treatment of severe asthmatics with mepolizumab which functions by inactivating IL-5 resulted in significant attenuation of blood eosinophils with an increase in blood EoP, but no apparent effect in suppressing local eosinophilopoeisis as indicated by unaffected sputum eosinophil counts. Adequate targeting of local eosinophilopoetic events using a drug with improved tissue availability such as benralizumab (a fucosylated anti-IL-5R

antagonist) was effective at depleting both EoP and sputum eosinophils underscoring the importance of tissue mediated generation of eosinophils in asthma (Sehmi et al., 2018).

1.2.2 The Role of Eosinophils in Asthma

Eosinophils are frequently studied as terminally differentiated effector cells that play major roles in driving the pathophysiology of allergic inflammatory processes and tissue remodeling in asthma (Doherty & Broide, 2007; Munitz, Bachelet, Levi-Schaffer, 2006; Kariyawasam & Robinson, 2007). For example, silencing GATA gene expression in mouse models have shown to mitigate eosinophilia and tissue remodeling in addition to improving airflow (Uhm, Kim, Chung, 2012).

Eosinophil activation can induce the secretion of various cytokines including IL-13, which promotes both AHR and mucus hypersecretion by stimulating goblet cell (Grünig et al., 1998). Indeed, the absence of AHR and mucus hypersecretion has been observed in eosinophil-deficient mouse models (Lee et al., 2004). However, separate animal studies have shown that eosinophils may not be the sole contributor to AHR-associated pathophysiology as IL-13 is also produced by other T-helper type-2 (T_H2) cells and even by type 2 innate lymphoid cells (ILC2s) (Humbles et al., 2004; Kay, 2005; McBrien & Menzies-Gow, 2017). In fact, while therapeutic monoclonal antibodies (mAb) to IL-5 in human clinical trials have resulted in significantly reduction of blood eosinophilia, the AHR feature remained (Leckie et al., 2000).

However, eosinophil effector functions causing significant lung tissue damage has been well documented in the literature. The activation of tissue eosinophils leads to the

degranulation and secretion of various molecules including four eosinophil-specific cytotoxic proteins: major basic protein (MBP), eosinophil peroxidase (EPX), eosinophil cationic protein (ECP), and eosinophil-derived neurotoxin (EDN) (Acharya & Ackerman, 2014). These granule proteins play an evolutionarily anti-pathogenic role, as they generate highly reactive oxygen species that non-specifically degrade invading pathogens such as helminth parasites (Giembycz & Lindsay, 1999). However, their release of activated eosinophils in airway tissues results in major damage to the airway epithelial lining, thereby accelerating antigen-penetration and amplifying the inflammatory response (Kay, 2005).

Given the highly destructive potential of eosinophils, the past decade has witnessed the development of several anti-IL-5 biologic therapies targeting eosinophilia, a prominent feature in asthma exacerbations. The reduction of exacerbations has been seen with the anti-IL-5 mAbs mepolizumab and reslizumab (Haldar et al., 2009; Pavord et al., 2012; Ortega et al., 2014; Castro et al., 2015). Furthermore, treatment of benralizumab, a mAb against the IL-5Rα has demonstrated effective depletion of both blood and tissue eosinophilia (Laviolette et al., 2013). However, other studies have shown a mixed success rate, as a proportion of steroid-insensitive severe eosinophilic asthmatics (SEAs) continued to exhibit persistent inflammation even with adjunct mAb treatment, suggesting alternative mechanisms contributing to eosinophil accumulation in respiratory tissues (Mukherjee, Sehmi, Nair, 2014).

1.3 Eosinophil Extravasation

1.3.1 The Classical Leukocyte Extravasation Model

The migration of circulating leukocytes to sites of injury, inflammation, and/or infection is a major component of innate immunity and occurs through a series of complex interactions between the adhesion molecules expressed on the cell surface and the endothelium (Figure 1.2). The leukocytes will travel passively through the circulation, carried by the laminar flow of blood. However, once they reach postcapillary venules, the reduced vessel radius and changes in hemodynamics increases the likelihood of cell contact with the endothelial cells (ECs) lining the vessel. The presence of inflammatory mediators at these sites stimulate the surface expression of P-selectins on the luminal surface of ECs which interact with selectin ligands expressed on leukocytes (Ulfman et al., 2001). The interaction of selectin-ligand binding has rapid on and off rates which allows quickly moving leukocytes to form tentative, transient bonds with the ECs through processes known as *tethering* and *rolling* (Grailer et al., 2009; Calvey & Toldeo-Pereyra, 2007; Muller, 2013). Furthermore, proinflammatory cytokines can induce ECs to express additional selectins such as E-selectin to promote *slow rolling* (Kunkel & Ley, 1996; Ley et al., 2007). Rolling as the first step in the extravasation process brings the leukocytes into close proximity to the endothelium to provide opportunities for further activation by chemokines and other proinflammatory molecules (Metcalf et al., 2008; Gonlugur & Efeoglu, 2004; Lowell & Berton, 1999).

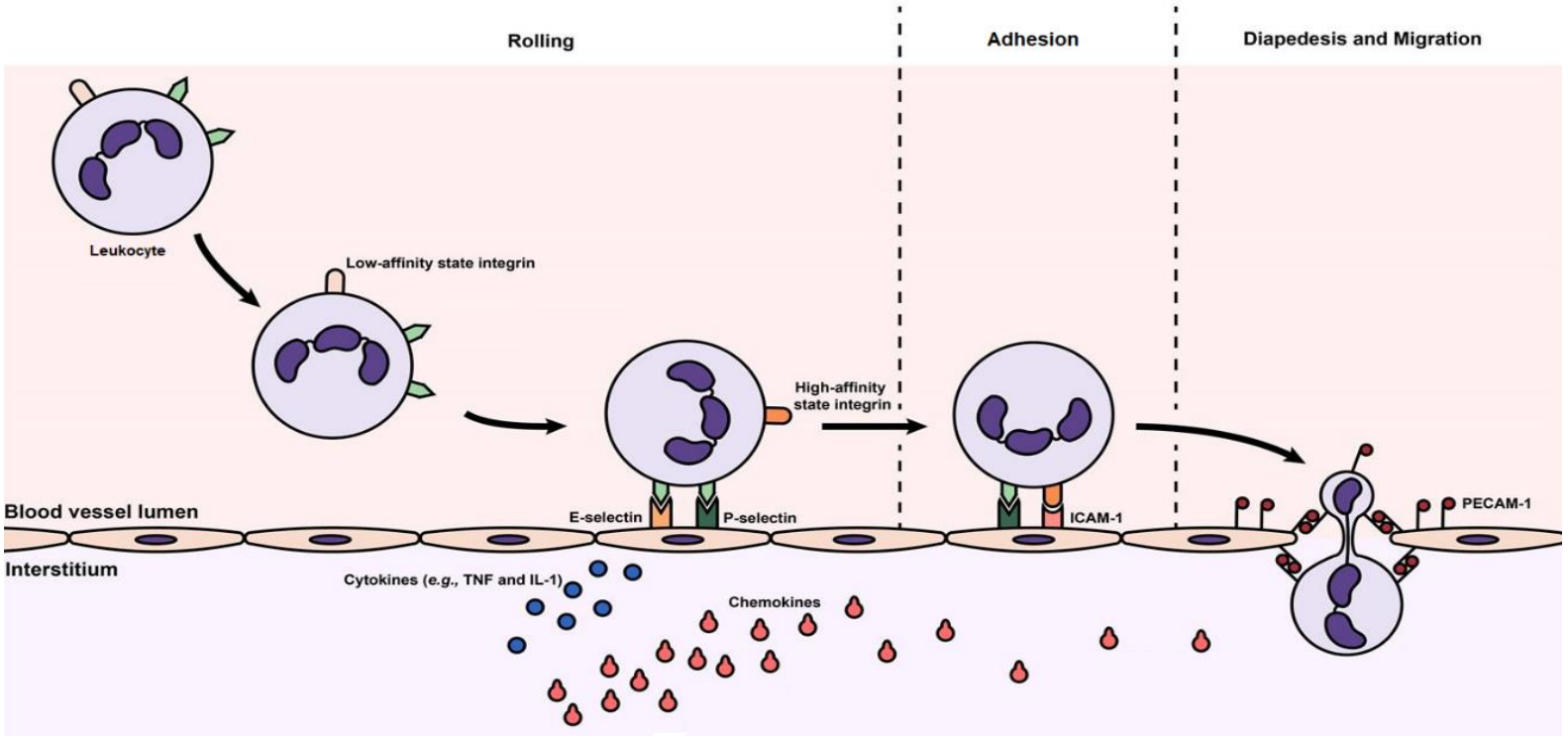

Figure 1.2. Eosinophil extravasation into tissues is controlled by a sequence of molecular interactions mediated by adhesion molecules, chemokines (eotaxin), and cytokines (IL-5, IL-13). The leukocytes will roll onto the endothelial wall, transiently attaching via selectin binding. Firm adhesion is mediated by integrin receptors binding to VCAM-1 or ICAM-1 on the endothelium to ensure subsequent diapedesis and migration through the vessel wall into the surrounding tissue. (Image adapted from MedBullets, 2018)

The binding of chemokines, which are produced by either the ECs or interstitial inflammatory cells, to transmembrane chemokine receptors on the leukocyte surface transduces signals leading to integrin activation. Integrins are heterodimeric, transmembrane proteins composed of an α- and β-subunit, thereby displaying immense structural and functional complexity in contrast to selectin adhesion receptors (Mould et al., 2003; Lowell & Mayadas, 2012). With 18 different α-subunits and 8 β-subunits, there are numerous dimerization possibilities with the capacity for distinct binding and signaling (Humphries, 2000). Integrin receptors exist in one of two conformational states that differ by their binding affinity for their counter-ligand (Johansson & Mosher, 2013). The lower-affinity state is widely expressed in unstimulated cells and is distinguished by its retracted extracellular ligand-binding domain that sterically obstructs high-affinity binding to prevent unwarranted adhesion and extravasation of eosinophils (Humphries, 2000; Johansson et al., 2006). Chemokine binding provides the intracellular signal required for the conformational transition of the integrin into its high-affinity state (Barthel et al., 2008). This *inside out* integrin activation allows for *firm adhesion*, the second step in the extravasation process.

The final two steps are crawling and transendothelial migration (TEM), also known as locomotion and diapedesis respectively (Gonlugur & Efeoglu, 2004). The crawling of adhered leukocytes towards junctions between ECs is mediated by integrin interaction with EC adhesion molecules ICAM-1 (intercellular adhesion molecule 1) and VCAM-1 (vascular cell adhesion molecule 1) which are upregulated during inflammation (Sans et al., 1999; Barthel et al., 2008). Subsequently, leukocytes can squeeze through EC borders in

an ameboid-like manner into the interstitial tissue for effector functions (Muller 2013). The TEM is often thought of as the point of no return whereas the previous three steps of extravasation are all considered reversible (Muller, 2007).

1.3.2 Eotaxin, an Eosinophil-Selective Chemoattractant

There have been several chemokines characterized as eosinophil chemoattractants including RANTES (regulated on activation, normal T cell expressed and secreted) and MIP-1 (macrophage inflammatory protein 1α), neither of which are specific for eosinophils. However, the eotaxins, members of the CC chemokine family identified by their Cys-Cys residues near the N-terminus, were observed to be uniquely eosinophil-selective (Rankin, Conroy, Williams, 2000; Rosenberg, Phipps, Foster, 2007). There are three isoforms of eotaxin: eotaxin-1 (CCL11), eotaxin-2 (CCL24), and eotaxin-3 (CCL26), each with different binding affinities to the chemokine receptor 3 (CCR3) due to variation in their amino acid sequence homologies (Rankin, Conroy, Williams, 2000). Despite being expressed on basophils and CD4+ T_H2 lymphocytes, CCR3 is present in higher densities on mature eosinophils; therefore, they are commonly used are eosinophil cell markers (Giembycz & Lindsay, 1999).

A study by Jose et al. (1994) was the first to describe eotaxin, found as a novel element of bronchoalveolar lavage fluid (BALF) of allergen sensitized guinea pigs. Eotaxin-1 is produced and secreted by epithelial cells through the T_H2 immune response and is thought to play a supporting role to IL-5 in eosinophil recruitment to airway tissues in asthmatics (Pease, 2006; Simson & Foster, 2000; Pope et al., 2001). Although eotaxin-2

shares only approximately 30% sequence homology with eotaxin-1, it also functions as a chemoattractant through CCR3 binding (Rankin, Conroy, Williams, 2000; Forssmann et al., 1997). The expression of eotaxin-1 was detected at earlier timepoints (6 hours) post allergen challenge in mouse models relative to eotaxin-2 (24 hours) (Rosenberg, Phipps, Foster, 2007). However, the role of eotaxin-1 in human severe asthma eosinophil recruitment has been questioned, as CCR3 antagonist and inhibitor clinical trials were shown to be ineffective (Neighbour et al., 2014; Gauvreau et al., 2018). Studies have indicated that eotaxin-2 and IL-13 cooperate in synergistic fashion to promote eosinophil recruitment in response to allergen challenge (Pope et al., 2005). Eotaxin-3 is also involved in mediating eosinophilia and has been identified as a biomarker for the eosinophilic esophagitis (EoE) disorder (Blanchard et al., 2006).

1.3.3 Eosinophil Integrins

Eosinophil extravasation from the postcapillary venules of bronchial circulation to the airway wall is regulated by selectin and integrin adhesion receptors. Bronchial circulation is a separate system from the pulmonary system, in that it is systemic and supplies oxygen and nutrients to the cells that constitute the lung whereas pulmonary circulation carries deoxygenated blood to the lung to bring back oxygenated blood for circulation throughout the body. Eosinophils express seven distinct heterodimeric integrin receptors (Figure 1.3), each interacting uniquely with their own corresponding ligands deposited throughout the extracellular matrix or on other cells (Barthel et al., 2008).

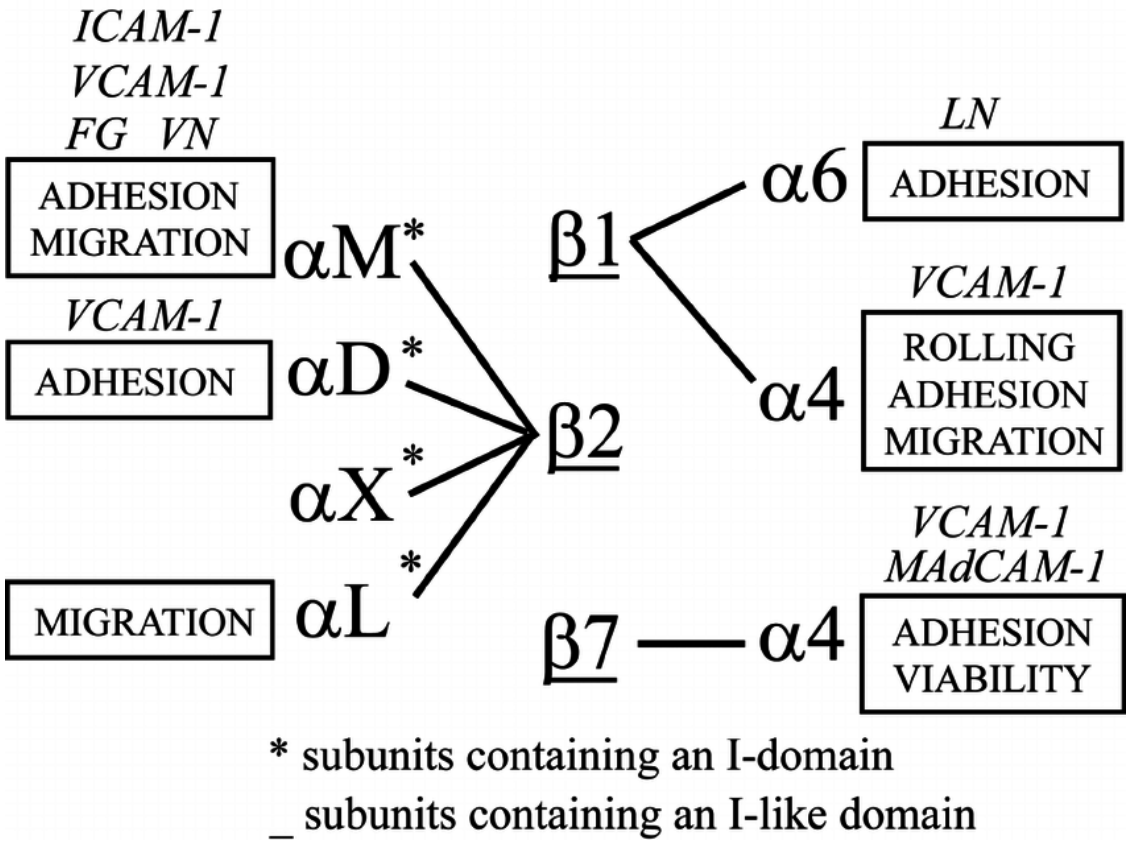

Figure 1.3. Schematic of the seven heterodimeric eosinophilic integrin receptors with their corresponding ligands and functions. (Image adapted from Barthel et al., 2008)

The very late antigen-4 (VLA-4, α4β1) integrin is the most extensively studied eosinophil integrin due to its central role in mediating eosinophil rolling and adhesion to VCAM-1 on the endothelium (Sriramarao et al., 1994; Sriramarao et al., 2000; Matsumoto et al., 1997). VLA-4 expression on neutrophils is negligible (Bochner et al., 1991; Taooka et al., 1999) or even absent (Weller et al., 1991; Kirveskari et al., 2000), implying that eosinophil recognition of VCAM-1 allows for selective infiltration of eosinophils in asthma. Indeed, a study using anti-α4 blocking antibodies showed inhibition of eosinophil migration across endothelial cell layers in response to eotaxin (Jia et al., 1999). Furthermore, the upregulated expression of VCAM-1 is induced by T_H2 mediators, thereby suggesting its critical function in VLA-4-mediated recruitment to the asthmatic airway (Masinovsky, Urdal, Gallatin, 1990; Weller et al., 1991).

The macrophage-1 antigen (Mac-1, αMβ2) integrin remains in a constitutively less active conformational state relative to the α4β1 integrins (Barthel et al., 2008). In contrast to VLA-4, which ligates VCAM-1 even in the absence of cytokine stimulation, Mac-1 activation is necessary for counter-receptor recognition (Barthel et al., 2008). Studies have shown that IL-5 enhanced Mac-1 adhesion to ICAM-1 and modules 1 and 4 of VCAM-1 under static conditions, and this interaction was mediated through phosphoinositide-3 kinase (PI3K) (Zhu et al., 2000; Sano et al., 2005). Interestingly, a preferential engagement of eosinophil adhesion to VCAM-1 through the β2 subunit was found when PI3K inhibitors (wortmannin and LY294002) completely abolished adhesion despite this interaction being mediated by both VLA-4 and Mac-1 integrins (Zhu et al., 2000; Sano et al., 2005). Furthermore, a study by Barthel et al. (2006) paradoxically showed that IL-5 incubation

actually decreased adhesion to VCAM-1 under flow conditions that was reversed by an anti-αM antibody.

In summary, VLA-4 integrins mediate rolling on the endothelium in unstimulated blood eosinophils and initiates firm adhesion through binding VCAM-1 expressed on ECs. The presence of eotaxins or IL-5 in inflammatory states can induce high-affinity conformational states in Mac-1 integrins, which allows competition with VLA-4 for VCAM-1 binding sites (Shattil, Kim, Ginsberg, 2010; Tachimoto et al., 2002). Eventually, a priority shift will occur in favor of Mac-1 as the dominant migratory integrin. However prolonged IL-5 activation may result in the release of Mac-1 from VCAM-1 within minutes, suggesting a narrow time window for eosinophil diapedesis towards airway tissues. The remaining five eosinophil integrins likely play supportive roles in extravasation; unfortunately, further studies must be completed to illuminate the exact roles they play in this complex interaction. Notably, isolated tissue and BALF eosinophils from patients with asthma patients expressed elevated levels of high-affinity surface integrins in comparison to their healthy counterparts, implicating a role for abnormal integrin activation in the pathogenic phenotype (Barthel et al., 2006).

1.3.4 Extracellular Matrix

The extracellular matrix (ECM) refers to a collection of secreted extracellular molecules that provide structural and biochemical support to the environments between the vascular endothelium and the tissue epithelium (Michel et al., 2010). It is comprised of: (1) proteins such as collagen and fibronectin that function to form macromolecular structures

and regulate cell adhesion; (2) matricellular proteins responsible for modulating cell-matrix interactions and functions; and (3) proteoglycans which provide tissue turgidity and facilitate molecular transport (Hubmacher & Apte, 2013). Although the ECM is fundamentally made up of similar proteins, each tissue has its own unique ECM composition due to dynamic cellular communication during tissue development (Frantz et al., 2010). The variability in ECM stiffness and elasticity has direct implications in cellular migration as cells have the ability to detect ECM rigidity and can undergo preferential migration via durotaxis (Engler et al., 2006). For example, the VLA-4 integrin that binds to VCAM-1 on ECs will also bind to its fibronectin ligand in the ECM (Barthel et al., 2008). The general role of VLA-4 and other integrins to mechano-activation or induce mechanotransduction in leukocytes is further reviewed in section 1.5.2.

1.4 Fluid Shear Stress

Two categories of superficial stress develop within blood vessels in response to haemodynamic conditions: [1] circumferential stress due to pulse variation and [2] shear stress due to blood flow (Papaioannou & Stefanadis, 2005). According to the simplified Hagen-Poiseuille equation ($\Delta P = (8\mu LQ)/(\pi R^4)$; ΔP, pressure difference; μ, dynamic viscosity; L, length of vessel; Q, volumetric flow rate; R, radius of vessel), shear stress is directly proportional to flow rate and inversely proportional to vessel diameter. With respect to blood flow, fluid viscosity, which is a measure of combined adhesion and cohesion effects, offers resistance to flow (Papaioannou & Stefanadis, 2005). Blood, which is a non-Newtonian fluid, has a viscosity that is dependent on shear rate which in turn is

determined by the presence of platelets, erythrocytes, and other molecules in the bloodstream (Papaioannou & Stefanadis, 2005). Therefore, the fluctuating viscosity of blood throughout the circulatory system leads to varying shear stresses against the vascular endothelium as well. However, the exact values of fluid shear stress on circulating and adherent leukocytes *in vivo* remains a major discussion point that has yet to be clearly answered.

A major point of contention lies in the fact that *in vitro* experiments suggest that comparably low degrees of fluid shear stress can induce biological responses; yet physiologically, notable differences between fluid normal and shear stress exist in circulation (Makino et al., 2007). Leukocytes in the centre of the vessel (moving with the blood and undergoing no deceleration whatsoever) are subjected to minimal shear stresses, whereas leukocytes near the endothelial wall would experience shear stresses relatively comparable to what the endothelial cells would be subject to. However, in single file cells in capillaries, the fluid shear stress is determined by the lubrication layer between their membrane and the endothelium, and has been suggested to be between 10 to 50 dyn/cm^2 (Sugihara-Seki & Schmid-Schönbein, 2003). However, in post-capillary venules, such as ones where eosinophils would extravasate from *in vivo*, the answer is not quite as simple. When the eosinophil makes first contact with the endothelium in venules, it undergoes rolling and tethering steps of extravasation. When the integrins become activated and the eosinophil is firmly adhered, they are subject to significantly increased levels of fluid shear stress with a non-uniform distribution over its membrane (Makino et al., 2007). In fact, the shear stresses for circulating and adherent leukocytes in microvessels were found to be 10

dyn/cm^2 and 100 dyn/cm^2 respectively, indicating that shear stresses experienced by leukocytes may be several orders of magnitude larger than the endothelial cells at identical sites in the absence of adhered leukocytes (Makino et al., 2007). Another interesting factor to consider is the aforementioned non-uniform distribution of shear stress over the leukocyte plasma membrane. The membrane ruffles on their cell surface lead to local non-uniformities, resulting in above average shear stresses experienced at critical points in the cell membrane.

The discussion revolving around relevant fluid shear stress levels remains in contention. For example, shear stress against venules have been reported to be as low as ~15 dyn/cm^2, yet as high as ~45 and ~55 dyn/cm^2 in capillaries and arterioles respectively (Lipowsky et al., 1980; Lipowsky et al., 1978). Other studies done as early as the 1980s in arterial systems have indicated shear stresses up to 28 dyn/cm^2 (Ku et al., 1985). A more recent review by Cheng et al., (2007) showed a comparison in arterial shear stress levels across different animals. They observed an approximate average of 10 dyn/cm^2 in humans, with much higher shear stresses in small organisms with reduced vessel diameters (Cheng et al., 2007). However, as the fluid shear stress is inversely proportional to vessel diameter, we can extrapolate from these data that the magnitude of shear stress in post-capillary venules may exceed those observed in the arterial systems by several fold. Indeed, some studies have shown wall shear stresses ranging between 2.8 to 95.5 dyn/cm^2 (Koutsiaris et al, 2007). The high variance in reported values for *in vivo* shear stress levels reflects the complexity in considering the many factors in determine an appropriate range for our *in vitro* experiments. The predominant concern for using high fluid shear stresses lies in the

potential for viscoelastic deformation of the cells. Yet, with respect to migrating leukocytes such as eosinophils, the increased interactions with the endothelium during the rolling and adhesion stages of extravasation will allow the mechanosensors expressed on the surface to detect an elevated magnitude of fluid shear stress caused by the surrounding blood flow.

1.5 Actin Cytoskeleton

Leukocyte migration is mechanistically different from the processes of mesenchymal cells such as fibroblasts or ECs with respect to their flexibility in response to environmental cues. These signals such as cytokines, chemokines, lipids, ECM fragments affect leukocyte activation by inducing adhesiveness and contributing to changes in cell shape and motility. The morphological changes associated involve cell flattening, multidirectional development of membrane protrusions, and the establishment of cell polarity (Lammermann & Germain, 2014).

The development of cell polarity with an actin-rich front and a contracting cell rear is critical for cell movement and is often seen in leukocyte chemoattractant-mediated chemotaxis. The relevant morphological changes are regulated by several members of the Rho family of small GTPases: Rac1, Cdc42, and RhoA. Rac1 and Cdc42 are localized to the leading edge where they mediate F-actin polymerization and leading edge persistence, respectively (Germena & Hirsch, 2013; Tybylewicz & Henderson, 2009). On the other hand, RhoA is localized to the cell rear to modulate the contractile activity in cell migration. Actin polymerization provides the central protrusive force at the membrane at the cell front as filament traverse backwards into the cell in a process known as retrograde actin flow

(Renkawitz & Sixt, 2010). The retrograde activity is made possible through integrin adhesion, which essentially couples the actin cytoskeleton to the underlying substrate (Hogg, Patzak, & Willenbrock, 2011; Abram & Lowell, 2009).

The most prominent factor in nucleating actin filaments for polymerization is the actin-related protein (Arp) 2/3 complex. This protein aggregate in conjunction with two actin monomers provides the *pre-existing* physical foundation upon which daughter actin filaments can bind to and create a branched actin network (Rotty, Wu, Bear, 2013). The continuous branching of the Arp2/3 complex may overcome plasma membrane tension to enable forward movement and membrane protrusions through thin sheet-like architectural features including lamellipodia and ruffles (Pollard & Borisy, 2003; Small et al., 2002). The activation of the Arp2/3 complex was found to be required for lamellipodia and ruffle formation as deletion and knockout models showed defects in the formation of these elements (Suraneni et al., 2012; Rotty, Wu, Bear, 2013).

1.5.1 Changes in Eosinophil Morphology in Response to Activation Factors

Eosinophils will respond to cytokine and chemokine presence by undergoing a specific subset of morphological changes, and these functions are heightened in disease and inflammatory states. Indeed, the stimulation of eosinophils *ex vivo* through eotaxin-2 and PAF resulted in rapid actin polymerization detected by confocal microscopy using fluorescent F-actin probes (Lacy et al., 2011). Furthermore, greater proportions of activated eosinophils were observed in children with severe asthma relative to their healthy counterparts (Muniz-Junquiera, Barbosa-Marques, Junquiera, 2013). These activated

eosinophils showed a greater propensity for morphological changes that include adhesion, flattening for crawling, membrane protrusions, migration, and degranulation (Muniz-Junquiera, Barbosa-Marques, Junquiera, 2013). Each of these events involve distinct changes in the cytoskeleton that occur in a coordinated, temporally-regulated sequence comprising of a myriad of regulatory stimuli and signaling events. In order to investigate the underlying mechanisms of morphological change upon cellular activation, an understanding of the regulation of the actin cytoskeleton is fundamentally necessary.

1.5.2 Mechanotransduction Signaling Pathways

Cells are constantly exposed to mechanical stimulation from various sources ranging from blood flow to the stiffness of the extracellular matrix. The detection of changes in the physical environment often induces outside-in signal transduction to elicit meaningful biological responses, in a process known as mechanotransduction (Martino et al., 2018). The predominant site of force transmission in cells lies within the cellular membrane, where contact is formed with any extracellular space. For circulating leukocytes, this may be the endothelial cell wall, whereas for structural cells, this may be the extracellular matrix. These points of contact are often described as focal adhesions or focal points, dependent on the transience of the formation, and take the form of discrete multiprotein complexes with intra- and extracellular components. Integrins, as mentioned before, are transmembrane proteins with receptors present on the cell surface that form the core of these focal points. Indeed, specific integrin heterodimer combinations were observed to maintain different functions in mechanosensing and force generation

(Seetharaman & Etienne-Manneville, 2018). For example, the deletion of β3 integrin subunits resulted in an increase in traction force whereas β1 deletion resulted in a decrease of contraction (Martino et al., 2018). VLA-4 is a β1 integrin whose mechanosensitive properties has been extensively studied. Leukocytes adhered through VLA-4 integrins were found to adapt to external fluid forces by forming upstream anchors to stabilize adhesion, a process observed to be dependent on Rac as well as PI3K (Rullo et al., 2012). The capacity of integrin-mediated adhesion is often dependent on their cytoplasmic associations. Indeed, the inhibition of the interaction between the α4 subunit of VLA-4 and the cytoplasmic adaptor protein paxillin was shown to impair adhesion strengthening in Jurkat T cells to VCAM-1 under shear stress conditions (Alon et al., 2005).

The development of focal points requires a coordinated sequence of protein recruitment, of which focal adhesion kinase (FAK) is critical. Its activation through autophosphorylation is reinforced in a positive feedback loop by downstream signals such as cytoskeletal contraction and cell spreading (Lachowski et al., 2018). The relationship between FAK and the contracting cytoskeleton is tightly regulated in order to maintain tension at key contact points and control intracellular signaling strength. In fact, it has been suggested that the mechanosensing capabilities of FAK are homeostatic and self-adjusting to balance sensitivity with extracellular substrate (Bell & Terentjev, 2017). In addition to FAK, talin and vinculin are studied as key adaptor proteins heavily involved in the development of focal points. The N-terminal globular head of talin binds to the cytoplasmic domain of the β-integrin subunit whereas its FERM domain binds to filamentous actin (F-actin) (Ciobanasu et al., 2018). Its intermediate rod domain features additional binding sites

for both integrins and vinculin (Gingras et al., 2005). Without a mechanical force stimulus, the vinculin binding sites (VBS) on the talin rod domain remain inhibited; however, as the applied force increases, VBS are gradually exposed thereby activating vinculin binding (Haining et al., 2016). Indeed, talin-vinculin mechanosensitivity is considered a necessary step for talin-F-actin stabilization and outside-in signaling cascades (Humphries et al., 2007). Cells without vinculin exhibited decreased levels of contractility, a phenomenon that was rescued by restoring vinculin tail domains (Mierke et al., 2008). The actual contractility of the cytoskeleton is induced by F-actin interaction with the motor protein myosin II, held together by various crosslinking proteins such as α-actinin and filamin.

There are several actin-binding proteins that regulate the actin cytoskeletal reorganization in response to extracellular triggers such as nucleation factors Arp2/3 and depolymerization factors (cofilin). The actin cytoskeleton is stabilized by inhibiting cofilin activity, which severs F-actin fibers to expose actin filaments that can be depolymerized to reduce cell tension. However, mechanical triggers lead to the activation of the LIMK kinase in the RhoA/ROCK pathway, which continuously phosphorylates cofilin rendering it inactive (Fukata et al., 2001; Hayakawa et al., 2011). RhoA-ROCK activation can also induce myosin II activation via phosphorylation of myosin light chain (MLC) and/or the inhibitor of MLC phosphatase (MLCP) (Amano et al., 1996). The inhibition of Rho or its downstream molecules significantly attenuates its capacity to respond to the extracellular environment. A reduction in myosin II leads to contractile defects, reduced focal points and inhibition of their maturation (Burridge & Chrzanowska-Wodnicka, 1996).

1.6 Calcium Signaling

1.6.1 Decoding Calcium Signaling

The calcium ion is a prevalent secondary messenger found throughout cell signaling and is known to impact numerous cellular functions include motility, contractility, apoptosis, and transcription (Clapham, 2007). There are several major types of calcium signaling that differ on factors such as cell type, activation trigger, and downstream cell function. For example, in neuronal calcium signaling, fluorescent imaging in acute mouse brain slices has shown that increasing the tonic firing rate generated elevated calcium signals multi-fold greater than predicted (Hage & Khaliq, 2015). Other studies have verified the nature of *frequency-dependent signaling* in dendritic cells, with observations that increased frequency of stimulation allowed for elevated calcium entry (Msghina et al., 1999). Furthermore, calcium spikes detected in dendritic cells were found to have corresponding amplitudes to the degree of synaptic input, suggesting a role of *amplitude-dependent signaling* (Suzuki & Larkum, 2017). The nature of calcium spikes in neuronal cells have been detected to rapidly rise within < 2 milliseconds while decaying over 70 ms (Helmchen, Imoto, Sakmann, 1996). However, the inhibition of hyperpolarization through endogenous autacoids such as histamine and serotonin have shown possibilities of a kinetically slower, singular calcium spike occurring between 0.5 to 15 seconds (Cordoba-Rodriguez et al., 1999).

In another example, cytoplasmic calcium regulates contractility in smooth muscle cells and tissues (Sanders, 2001). The absolute increase in calcium throughout the cytoplasm required for contraction may be triggered through calcium entry via

depolarization or through a release from intracellular stores (Sanders, 2001). Both extracellular calcium $[Ca^{2+}]_e$ entry and intracellular calcium $[Ca^{2+}]_i$ release are mediated by specialized calcium transporters and ion channels with distinct compartments capable of facilitating calcium movement along electrochemical gradients (Sanders, 2001). In addition, the depletion of $[Ca^{2+}]_i$ stores was found to be coupled to activation of calcium entry in a phenomenon known as capacitative calcium entry (CCE) that contributes to calcium homeostasis (Putney & Ribeiro, 2000). The activity of specialized sarco/endoplasmic reticulum Ca^{2+}-ATPases is vital to the reuptake of cytosolic calcium into internal stores, and maintains a 10,000-fold gradient between the reticulum lumen and cytoplasm (Sanders, 2001). The complexity of regulating calcium homeostasis in part to numerous specialized proteins speaks to the vital role this secondary messenger plays in essential cell signaling pathways.

1.6.2 Eotaxin and CCR3 Signal Transduction

The binding of the eosinophil-selective chemoattractant, eotaxin, to its CCR3 receptor elicits calcium-dependent changes in cellular function including migration, hormone secretion, cell survival and death, and gene transcription (Schwab et al., 2012). The binding interaction activates the $G_{q/11}$ pathway (Figure 1.4), which in turn activates membrane-bound phospholipase C (PLC) (Amerio et al., 2003). PLC acts as a catalyst for the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) into inositol-1,4,5-trisphosphate (IP_3) and diacyl-glycerol (DAG). While DAG remains associated with the cell membrane due to its hydrophobic properties, IP_3 diffuses into the cytosol where it binds

to IP_3-gated calcium channels (IP_3R) on the endoplasmic reticulum surface (Berridge et al., 2003). IP_3R activation releases calcium from intracellular stores, which can promote additional release of Ca^{2+} ions from ER-bound ryanodine receptor (RyR) via a process known as calcium-induced calcium release (CICR) (Sanders, 2001).

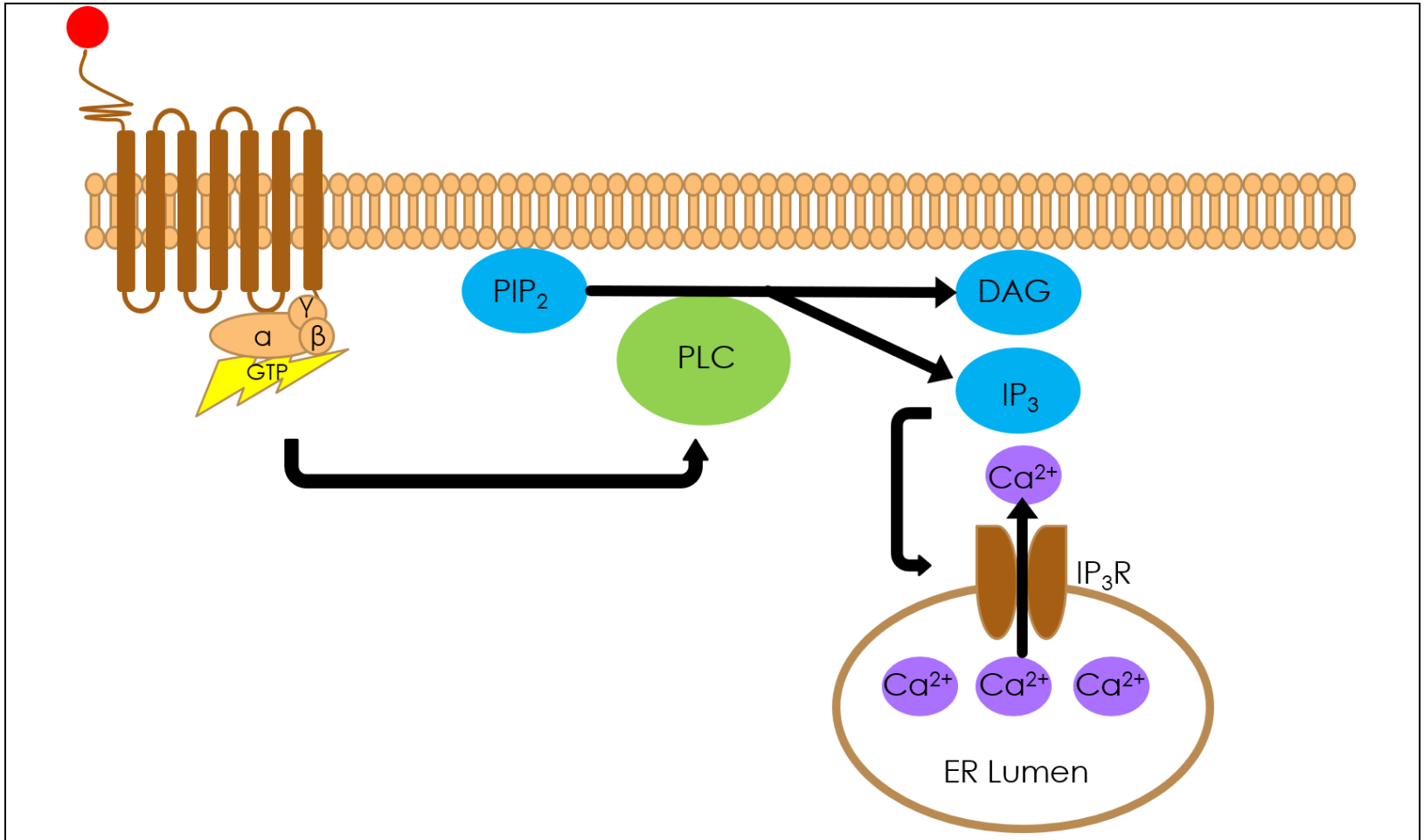

Figure 1.4 The binding of the chemoattractant, eotaxin (red circle) to the CCR3 transmembrane receptor elicits IP_3 mediated intracellular calcium release

Due to calcium playing critical roles across multiple cellular functions, intracellular calcium concentrations ($[Ca^{2+}]_i$) are tightly regulated. Mammalian cells use membrane proteins and ion pumps to keep the $[Ca^{2+}]_i$ within a physiologically tolerable range (Berridge et al., 2003). Ca^{2+} pumps and exchangers such as the sodium-calcium exchanger (NCX), plasma membrane calcium ATPase pump (PMCA) on the plasmalemma, and the sarcoplasmic-endoplasmic reticulum calcium ATPase pump (SERCA) on the ER work to expel Ca^{2+} ions from the cytosol subsequent to agonist-induced increases in $[Ca^{2+}]_i$ (Berridge et al., 2003). Each of these pumps and exchangers have specific inhibitors: ryanodine for the ryanodine receptor, U-71322 inhibits the PLC, cyclopiazonic acid (CPA) inhibits the SERCA pump, and Xestospongin inhibits IP_3R. The influx and efflux of Ca^{2+} ion homeostasis is coordinated by multiple mechanisms that can be influenced by the presence of eotaxin-1 in disease states to trigger cell migration in a calcium-dependent manner (Giembycz & Lindsay, 1999).

1.6.3 Calcium Signaling in Eosinophil Migration

The regulatory role of calcium in adhesion and migratory functions has been well documented in the literature for multiple cell types (Minton, 2014; Wei et al., 2012). For example, a study by Fay et al., (1993) showed transient fluctuations in calcium concentration have been shown to be critical in newt eosinophil migration (Brundage et al., 1993). The newt eosinophils exhibited a heterogenous distribution of calcium throughout the cytoplasm, with markedly increased levels in cells changing direction as opposed to those persistently migrating in a singular direction (Brundage et al., 1993). Fay's study was

the first to investigate calcium-mediated chemotactic activity in the eosinophil. However, the considerable size of newt eosinophils at 100 μm in comparison to human eosinophils (10-15 μm) allowed for the easier visualisation of intracellular calcium localization and cellular polarization. Unfortunately, further research on the exact role of calcium in human eosinophil migration has been minimal, and therefore, the molecular mechanisms remain poorly understood.

1.6.4 Calcium Signaling and Mechanosensitivity

Leukocyte interaction with the endothelium or ECM proteins is dependent on the formation of nascent selectin- and integrin-mediated interactions with their counter-ligands. The binding of integrins, in particular, induces regulatory intracellular signaling events which results in the formation of physical links between integrin subunits and the actin cytoskeleton through adaptor proteins such as talin and vinculin (Wehrle-Haller, 2012). Stabilizing high-affinity integrin receptors to the cytoskeleton allows the leukocytes to explore the surrounding environment through membrane protrusions such as lamellipodia and ruffles (Wehrle-Haller, 2012).

Clusters of bound integrin subunits in focal dots are responsible for relaying changes of both intra- and extracellular tension into signaling pathways that modify their own behavior, directly influencing the migratory and contractile state of the cell (Wehrle-Haller, 2012). An emerging concept in recent years suggests that these integrin clusters can act as mechanical transducing devices equipped with mechanosensing functions (Wehrle-Haller, 2012; Vincente-Manzanares et al., 2009). Indeed, studies have shown that both the

spacing/concentration and rigidity of ECM substrates have determined the density and rate of turnover of integrins in focal dots, which support the idea of inherent mechanosensors to probe the surrounding microenvironment of the cell (Wehrle-Haller, 2012).

The lateral pull of integrins proteins triggered through RhoA activation exposes stress-sensitive binding sites for adaptor proteins which ultimately results in high-density clusters that require constant drag to maintain signaling levels (Wehrle-Haller, 2012). It has been suggested that calcium influx may stimulate RhoA and the subsequent activation of RhoA-kinase required for rear detachment of cells, which is crucial for migrating cells as the rapid release of focal adhesions at the cell rear allow for quicker migration (Wehrle-Haller, 2012).

1.6.5 Calcium Modulation of Cytoskeleton Dynamics in Cell Migration

The involvement of calcium in various signaling pathways necessitates the appropriate detection of diverse initial stimuli through factors of spatiotemporal control of calcium increase, localization of the cytosolic spike, the rate of increase, and kinetics of its decay to basal levels (Berridge, 2014). Various calcium transporters are responsible for the tight, temporal control of signaling, to allow for the range of calcium spikes that last from microseconds in neuronal exocytosis to hours seen in oocyte fertilization (Martin-Romero et al., 2016). The most prevalent transporters include: (1) voltage-operated channels (VOCs) which are regulated by the net electric charge across the membrane during depolarization, (2) receptor-operated channels (ROCs) regulated by agonist binding (e.g. ATP, acetylcholine) (Frings, 1997), and (3) store-operated channels (SOCs) regulated by the state

of intracellular calcium stores, particularly those within the endoplasmic reticulum (ER). Of these, store-operated calcium entry (SOCE) has been considered of particular importance, as it maintains permanence of calcium-dependent signaling. STIM1 is a transmembrane protein containing a calcium-sensitive domain that triggers plasma membrane SOCs when internal stores are depleted (Martin-Romero et al., 2016). A study by Smyth et al. (2007) observed a colocalization of fluorescence-tagged STIM1 and endogenous tubulin, suggesting calcium modulation of the cytoskeleton. Indeed, studies have shown that activation of calcium entry and reuptake into internal stores allowed for increased association between STIM1 and microtubules (Pozo-Guisado et al., 2013; Pozo-Guisado & Martin-Romero, 2013).

To further support the role of calcium in cellular adhesion and migration, PIP_2, which is an intermediate in the aforementioned IP_3/DAG pathway, is responsible for the recruitment of functionally active adaptor proteins in focal adhesions. For example, the adaptor protein talin is auto-inhibited by the interactions between its head and tail domain. The opening of talin is linked to PIP_2-dependent recruitment to the plasma membrane, where once bound to PIP_2, talin subdomains interact with intracellular motifs of integrin β-subunits (Wehrle-Haller, 2012). The binding of talin to the F-actin scaffold is necessary to maintain the open configuration (Wehrle-Haller, 2012). Similarly, vinculin is yet another adaptor protein that is constitutively expressed in an inactive, closed conformation in the cytoplasm (Wehrle-Haller, 2012). The unclasping of vinculin is induced by PIP_2 binding to a regulatory site in its tail-domain (Wehrle-Haller, 2012). The commonality of the presence of PIP_2 in the intracellular calcium signaling pathway and the necessary role it

plays in recruiting adaptor proteins shows a potential link that has yet to be carefully explored.

1.7 Central Hypothesis and Research Objectives

1.7.1 Project Rationale

The relative infancy and imperfect success of clinical trials targeting eosinophil migration and accumulation, in conjunction with gaps of knowledge regarding migration-associated molecular mechanisms provided the axis along which my doctoral studies were conducted. A couple of years prior to my involvement with the Janssen lab, a graduate student was researching the effects of eotaxin-induced calcium release in human peripheral eosinophils on its adhesion and migration functions. They serendipitously discovered that the perfusion of media without any chemical agents as a negative control over human peripheral eosinophils adhered on both uncoated and extracellular matrix (ECM) protein-coated coverslips elicited the release of calcium, not dissimilar to the calcium flux induced via eotaxin (Ahmadzai et al, 2015). This observation, the perfusion-induced calcium response (PICR), provided a unique opportunity to investigate a novel phenomenon in my graduate studies.

1.7.2 Central Hypothesis

Despite the importance of $[Ca^{2+}]_i$ mobilization in cellular migration being well documented in several cell types, few have attempted to illuminate the underlying pathways in eosinophils. Therefore, studying the novel pathway of eosinophil perfusion-induced

calcium response (PICR), in addition to characterizing the accompanying cellular morphological changes within the first 5-10 minutes post-perfusion, is critical for understanding eosinophil biology in inflammatory disease and provide tools to modulate these steps which in turn could be used to understand the role eosinophils play in the pathogenesis of asthma and/or other eosinophil-mediated diseases. **We hypothesize that the mechanical stimulus of fluid shear stress is a critical initial step in the sequence of events that modulate eosinophil migrational response.** Furthermore, we hypothesize that both the magnitude of shear stress and application of pharmacological agents disrupting relevant signaling pathways will affect characteristics of the PICR, namely the strength of the calcium spike. The additional investigation of the early morphological changes of non-adherent spherical eosinophils into flattened, amoeboid morphology will supplement our study of the initial events of eosinophil commitment to transendothelial migration.

1.7.3 Research Objectives

The isolation of eosinophils from human peripheral whole blood is commonly the first step for any downstream *in vitro* assays. A limitation of the initial study was the lack of purity of the isolated eosinophil population. In **Chapter 3**, we perform a comparative analysis between eosinophil isolation techniques: (1) the standard, negative selection of eosinophils from density-centrifugation separated granulocyte populations through anti-CD16 antibody conjugated magnetic microbeads, and (2) recently developed isolation kits that utilize cocktails of magnetically-conjugated antibodies targeting non-eosinophilic surface markers directly from whole blood. Head-to-head comparisons allowed us to

determine that the newer kits that bypass density-centrifugation and allow immunonegative selection from peripheral blood yielded eosinophil populations with higher purity and recovery with a lower baseline activation of cells.

In **Chapter 4**, we characterized the PICR by subjecting purified populations of blood-extracted eosinophils to varying degrees of fluid shear stress and measuring their responses with respect to time to calcium spike post-perfusion, strength of calcium spike, and increase in cell area. We demonstrated that there was no significant difference in the latter two outcome variables at various flow rates, suggesting an all-or-nothing nature of the cellular response. However, at the most physiologically-relevant shear stresses of ~3.75 dynes/cm^2, we observed a significantly shorter latency to calcium release in response to perfusion. Therefore, we proposed that these physiological levels of shear stress *in vivo* provides an additional signal in conjunction to chemoattractants to reinforce the extravasation process. In addition, we utilized immunofluorescence staining to visualize the localization of activated β1 integrin subunit and the adaptor proteins talin and vinculin over various timepoints post-perfusion.

In **Chapter 5**, we incubated purified eosinophils extracted from blood of non-atopic human volunteers with pharmacological inhibitors to calcium signaling and actin regulatory pathways prior to fluid shear stress stimulation. We observed that cytoskeletal rearrangement reminiscent of migration succeeded the calcium spike within two minutes, indicating that calcium signaling modulates changes in cell morphology. A temporal linkage between these two pathways was demonstrated, as the inhibition of either pathway resulted in a significant loss of eosinophil ability to traverse across the underlying substrate

in comparison to untreated eosinophils. We theorized that targeting PICR disruption might be a novel therapeutic strategy to reduce eosinophil recruitment from the circulation resulting in development of tissue eosinophilia which precede eosinophil activation and associated pathophysiological features of specific disease pathology.

Chapter 2: Experimental and Analytic Methodology

2.1 Eosinophil Isolation

The optimization of our eosinophil isolation protocol is thoroughly outlined in Chapter 3 as our first research objective.

2.2 Parallel-Plate Flow Chamber

The low-profile parallel-plate fluid flow chamber was utilized as our *in vitro* model to simulate fluid shear stress against eosinophils adhered onto fibronectin-coated glass coverslips. The chamber could be disassembled into top and bottom halves and screwed back together whilst sandwiching a silicon gasket with a vertical height of 125 μm to represent the side walls. Eosinophils were transferred onto the coverslip secured on the bottom half of the chamber prior to assembly. The perfusate was introduced through a peristaltic pump with an arbitrary rate setting of 0.0-10.0 (PS-200; Living Systems Instruments, VT, USA). We were able to validate the physiological relevance of the shear stress levels that the adhered eosinophils would experience by plugging in the necessary elements into Poiseuille's law equation: $\tau = 3Q\mu / 2WH^2$ (τ = shear stress; Q = flow rate; μ = dynamic viscosity; W = width of the flow chamber; H = one-half height of the flow chamber). By measuring the volume output of various pump settings, we calculated that each increment was equal to a flow rate increase of 0.0667 mL/min. With a chamber width of 2.5 mm, height of 125 μm, and dynamic viscosity of 0.733 cP (Poon, 2020), we were able to determine that each increment was equivalent to **1.25 dyn/cm^2**. Therefore, we were able to manipulate the peristaltic pump settings to achieve desired shear stresses.

2.3 Confocal Fluorescence Microscopy and Calcium Imaging

A brief overview of confocal microscopy is delineated in the methodology sections of Chapters 4 and 5, as well as Figure 5, seen below. In detail, approximately 3 x 10^5 eosinophils were loaded with fluo-3 acetoxymethyl (AM) ester and incubated in the dark at room temperature for 10 minutes prior to transfer into the parallel-plate flow chamber. Although fluo-3 is unable to penetrate the cell membrane on its own, loading can be achieved through the AM ester derivative. The non-specific esterases within the cell will cleave the AM group, essentially trapping the fluo-3 inside and allowing it to bind to calcium ions, upon which it will sharply fluoresce with emission wavelengths measured at approximately 510 nm.

The eosinophils were visualized via inverted Nikon Eclipse TE2000-4 microscope (ON, Canada), and recordings were obtained through a custom-built apparatus. Oscillating mirrors at 8 kHz and 30 kHz allowed the respective scanning of cells at 488 nm in the X- and Y- planes using a 20mW photodiode laser (Coherent Technologies, CA, USA). Fluorescent emissions were measured by a photomultiplier and digitized into .tiff images at a 480x400 pixel resolution. The *Video Savant* v4.0 software (IO Industries, ON, Canada) was utilized to create sequential .tiff images by averaging 10 (Chapter 4) to 15 (Chapter 5) images taken in quick succession every 2 (Chapter 4) or 1.5 (Chapter 5) seconds at a 30 Hz frame rate.

In transitioning from Chapter 4 to Chapter 5, we also manipulated the scanning width of the mirrors to “zoom in” onto a smaller field of view (FOV), while maintaining the same 480x400 pixel resolution. In essence, this greatly enhances the resolution, which

can be defined as the capacity to resolve two closely-spaced objects within a FOV. The resolution is also dependent on several factors include: (i) wavelength of light utilized (488 nm); (ii) whether the lens requires water or oil-immersion as opposed to air; (iii) and the numerical aperture (NA) of the objective lens, which reflects the magnitude of light the objective collects. For our experiments, we utilized a 20x objective with 0.4 NA to capture images. The improved resolution in Chapter 5 allowed us to capture minute fluctuations in calcium concentrations and/or changes in cell morphology.

2.4 Signal Processing and Data Analysis

The eosinophils loaded with fluo-3 displayed minimal fluorescence at baseline/resting state prior to fluid perfusion. However, the release of intracellular calcium into the cytoplasm resulted in a very obvious increase in fluorescence. The fluorescence recordings were encoded in 8-bit; in other words, the pixels of the fluorescent image were given an intensity value between 0 to 255, representing the range of no calcium to extremely high calcium concentrations, respectively.

The .tiff image files were exported and analyzed using the QuimP plug-in (University of Warwick, UK) for the open platform software *ImageJ* (National Institutes of Health, MD, USA). In order to quantify fluorometric tracings, an analysis module of QuimP was utilized to threshold each greyscale pixel into black or white to differentiate between background signals and regions of interest (ROIs). The ROIs were then tracked over the duration of the experiment to produce outcome measurements for variables of interest including but not limited to fluorescence, cell area, and cell displacement. Additional information regarding data analysis can be found in Chapters 4 and 5.

Chapter 3: Publication in the

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Improved recovery of functionally active eosinophils and neutrophils using novel immunomagnetic technology

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Abstract

Clinically relevant and reliable reports derived from in vitro research are dependent on the choice of cell isolation protocols adopted between different laboratories. Peripheral blood eosinophils are conventionally isolated using density-gradient centrifugation followed by immunomagnetic selection (positive/negative) while neutrophils follow a more simplified dextran-sedimentation methodology. With the increasing sophistication of molecular techniques, methods are now available that promise protocols with reduced user-manipulations, improved efficiency, and better yield without compromising the purity of enriched cell populations. These recent techniques utilize immunomagnetic particles with multiple specificities against differential cell surface markers to negatively select non-target cells from whole blood, greatly reducing the cost/time taken to isolate granulocytes. Herein, we compare the yield efficiencies, purity and baseline activation states of eosinophils/neutrophils isolated using one of these newer protocols that use immunomagnetic beads (MACSxpress isolation) vs. the standard isolation procedures. The study shows that the MACSxpress method consistently allowed higher yields per mL of peripheral blood compared to conventional methods ($P < 0.001$, $n = 8$, Wilcoxon paired test), with high isolation purities for both eosinophils ($95.0 \pm 1.7\%$) and neutrophils ($94.2 \pm 10.1\%$) assessed by two methods: Wright's staining and flow cytometry. In addition, enumeration of CD63+ (marker for eosinophil activation) and CD66b+ (marker for neutrophil activation) cells within freshly isolated granulocytes, respectively, confirmed that conventional protocols using density-gradient centrifugation caused cellular activation of the granulocytes at baseline compared to the MACSxpress method. In conclusion, MACSxpress isolation kits were found

to be superior to conventional techniques for consistent purifications of eosinophils and neutrophils that were suitable for activation assays involving degranulation markers.

Key words: eosinophils, neutrophils, isolation, yield efficiency, purity

Abbreviations: 7-AAD – 7-aminoactinomycin; ANOVA – analysis of variance; APC – allophycocyanin; CD – cluster of differentiation; COPD - chronic obstructive pulmonary disease; DNA – deoxyribonucleic acid ; EDTA – ethylenediaminetetraacetic acid; EPX – eosinophil peroxidase; FACS – fluorescence-activated cell sorting; fMLP – N-formyl-methionyl-leucyl-phenylalanine; FITC – fluorescein isothiocyanate; HBSS – Hanks' Balanced Salt Solution; ICAM-1 – intercellular adhesion molecule 1; Ig – immunoglobulin; LTC4 – leukotriene C4; NH4Cl – ammonium chloride; PAF – platelet-activating factor; PB – peripheral blood; PBS – phosphate-buffered saline; PMD – piecemeal degranulation; PE – phycoerythrin; SEM – standard error of mean

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1. Introduction

Eosinophils and neutrophils belong to the granulocyte family of leukocytes which are characterized by the presence of granules in the cytoplasm (Geering et al., 2013). The granules contain a diverse array of cytotoxic molecules that are secreted under specific conditions to combat invading extraneous agents like pathogens and allergens. In disease conditions, these molecules also collaterally damage host cell tissues and often contribute to the inflammatory state (Jacobsen et al., 2007; Mantovani et al., 2011). Indeed, both eosinophils and neutrophils are important effector cells with established pathogenicity in chronic airway diseases, including asthma, chronic obstructive pulmonary disease (COPD), and bronchitis (D'silva et al., 2011). Intact sputum eosinophil counts, and in particular measures of airway eosinophil activity (free granules and eosinophil peroxidase levels in sputum), are associated with disease severity in asthma (Nair, Ochkur et al. 2013). Again, a significantly higher percentage of neutrophils were found in the sputum of patients diagnosed with moderate to severe COPD relative to mild airflow obstruction (Kay, 2005; D'silva et al., 2011).

Both clinical studies and in vitro/ex vivo experimentation suggest several distinct mechanisms of degranulation in eosinophils including piecemeal degranulation (PMD) and cytolytic release of intact granules (ECL) (Lee et al., 2012). PMD and ECL degranulation in human eosinophils have been well studied and documented, degranulation mechanisms in mouse models of human disease, in particular to the airways, have been subjected to limited reproducibility and subjective interpretation (Stelts et al., 1998; Lacy and Moqbel, 2001; Ochkur et al., 2007; Ochkur et al., 2012). The inconsistencies of mouse eosinophils in

replicating human eosinophil functions essentially highlight the importance of continued in vitro research using human peripheral blood eosinophils to investigate underlying mechanisms of recruitment, survival, migration, degranulation and other effector functions that contribute to disease progression and steroid insensitivity.

The accurate assessment and interpretation of cellular functions inferred from in vitro studies rely heavily on the isolation protocol used, as well as the population of target cells studied. In early studies, the standard isolation technique for individual granulocyte populations commonly involved sedimentation in high molecular weight polysaccharide solutions, such as dextran, followed by discontinuous density gradients which were greatly limited by low granulocyte recovery and purity, particularly for eosinophils. In fact, isolations using this technique generated extremely poor yields for eosinophils from healthy donors, and made comparative studies between healthy and diseased subjects a logistical impossibility.

In the 1990s, target-cell selection using antibody-conjugated microbeads post density-gradient centrifugation of whole blood was introduced. Negative immunomagnetic selection of eosinophils was made possible by using anti-CD16 antibody-coated magnetic beads, since neutrophils express high levels of this cell surface marker compared to eosinophils in granulocyte preparations (Pillay et al., 2013). This method was deemed to be preferable over discontinuous density (Percoll) gradient methods (Sedgwick et al., 1996) and, to this date, remains the most commonly used protocol. In some cases, isolation techniques were expanded to improve negative selection of eosinophils using biotinylated antibodies of multiple specificities (for example, Eosinophil Isolation Kit, Miltenyi Biotec, 130-092-010,

uses anti-CD2, CD14, CD16, CD19, CD56, CD123, CD235a).

Conflicting data remains regarding the purity and activation states of eosinophils isolated by these methods (Schefzyk et al., 2009; Percopo et al., 2010). Nevertheless, the use of immunomagnetic beads of multiple specificities that exploit differential expression of surface cellular markers between target and non-target cells are commercially available, including the newly introduced MACSxpress kit (Miltenyi Biotec Inc., Bergisch Gladbach, Germany), along with the EasySep kit (STEMCELL Technologies Inc., BC, Canada), which are gaining popularity. However, eosinophils and neutrophils isolated using various approaches show variability in their activation profiles, with a tendency towards cells becoming refractory to stimulation if dextran, Lymphoprep, or Histopaque sedimentation of whole blood is carried out for too long a period (>30 minutes), if red blood cell lysis is damaging to cells, or if cells are subjected to excess mechanical handling because of repeated centrifugation steps (personal communication). In spite of this, neutrophil isolation involving dextran sedimentation of whole blood followed by Ficoll separation remains the method of choice (Oh et al., 2008), although similar immunomagnetic bead-based kits are available.

Here we report a comparative analysis of granulocyte isolation from healthy individuals using immunomagnetic microbead cocktail-based methods and standard protocols with respect to granulocyte recovery (yield), purity and baseline activation states of the freshly isolated cells.

2. Materials and Methods

2.1 Subjects and Blood Sampling

Healthy donors (n = 8) with no known medical conditions were recruited with written consent (Hospital Research Ethics Board, St. Joseph's Healthcare, Hamilton, ON, Canada). The complete blood count was assessed clinically for determining the circulating absolute values of eosinophils and neutrophils (St. Joseph's Healthcare Core Lab). The neutrophil counts ranged from 3.3 – 4.6 x 109 cells / mL whereas the eosinophil counts were 0.2 x 109 cells / mL for all volunteers. A total of 60 mL of venous blood was drawn for eosinophil isolations and 40 mL for neutrophil isolations on separate days. The comparative isolation protocols were initiated in parallel, immediately after sample draw. A schematic of the work flow is summarized in Figure 1.

2.2 Isolation Protocols

Column-Based CD16 Negative Selection Isolation

A volume of 25 mL of peripheral blood collected into heparin anticoagulant blood collection tubes (BD Biosciences, ON, Canada) from venipuncture was mixed at a 1:1 ratio with RPMI media (Invitrogen, CA, USA) and layered on top of Lymphoprep (STEMCELL Technologies Inc.) at a 2:1 volume ratio for density-gradient sedimentation. Subsequent to centrifugation (20 min, 1200 g, 20°C, no brakes), the bottom-most granulocyte layer was harvested. Erythrocytes were lysed with an ice-cold ammonium chloride solution (NH4Cl,

155 mM; KHCO3, 10 mM; EDTA, 0.1 mM; pH 7.4) and further centrifuged (10 min, 300 g, 4°C) for removal from the granulocyte layer. The remaining granulocyte population was treated with immunomagnetic anti-CD16 microbeads (#130-45-701, Miltenyi Biotec Inc.) to negatively select for eosinophils. The remainder of the protocol was carried out as per manufacturer's protocol, and an aliquot of the final population was removed and stained with trypan blue for cell counts via hemocytometer.

2.2.1 Easy Sep (STEMCELL Technologies)

Briefly, peripheral blood samples were diluted with PBS and layered on top of Ficoll (GE Healthcare, Little Chalfont, UK) and centrifuged (30 min, 300 g, room temperature, no brakes) for density separation according to the manufacturer's protocol. The granulocyte layer was removed and residual erythrocytes were lysed with 1 mL of endotoxin-free water. Cells were divided into aliquots of 107 cells / mL, and were then treated with a human eosinophil enrichment cocktail (catalogue#: 19256, STEMCELL Technologies Inc.) containing magnetic nanoparticles as per manufacturer's instruction. An aliquot of the final population was removed for cell counting as mentioned previously.

2.2.2. MACSxpress Isolation Kit (Miltenyi Biotec Inc.)

Samples of 30 mL of venous blood were collected into EDTA anticoagulant blood collection tubes for eosinophil isolation, while only 8 mL was procured for neutrophil

isolation. The isolation procedure was performed according to the manufacturer's protocol (catalogue #: 130-104-446, for eosinophils; 130-104-434, for neutrophils). In brief, peripheral blood was mixed with cocktail containing magnetically-labelled antibodies targeting cell surface markers of cells of non-interest. Enriched cells remained in supernatants while other cell populations were segregated magnetically using the MACSxpress Separator (#130-098-308). Residual erythrocytes were lysed with 9 mL of cold sterile endotoxin-free water for 20 sec (cold water/hypotonic lysis), then 1 mL of 10X HBSS media without calcium and magnesium (Invitrogen) was added. An aliquot of the final population was removed for cell counting as mentioned previously.

2.2.3 Dextran Sedimentation and Percoll Gradient Purification

A 60-mL syringe (BD Biosciences) containing 4.4 mL of 3.8% sodium citrate anticoagulant was used to draw 30 mL of venous blood for dextran sedimentation. This protocol utilizes discontinuous plasma-Percoll gradients as described previously (Haslett et al., 1985), with the difference of the leukocyte layer being under-layered with 60% and 70% Percoll instead. A cold water lysis was performed to eradicate any erythrocyte contamination. Subsequently, an aliquot of the final population was removed for cell counting as mentioned previously.

2.3. Cell counts, Cytospin, and Viability Determination

Cytospin slides were prepared with Shandon Cytospin 3 centrifuge (6 min, 30g, room temperature) (GMI Inc, MN, USA). Prepared slides were stained with Wright's method and cell differentials (as a percent of total cell count) were reported for each isolation in duplicate. Cell viability was determined by trypan blue (Thermo Fischer, MA, USA) exclusion, as well as flow cytometry analysis using 7-aminoactinomycin (7-AAD) (BD Biosciences) which is a DNA intercalator that undergoes a spectral shift in dead cells.

2.4. Flow Cytometry

2.4.1 Antibodies for Flow Cytometry

Flow cytometry data was acquired on a BD FACS Canto analyzer (BD Biosciences) and analyzed using FlowJo version 9.0 (FlowJo LLC, OR, USA). Surface staining was performed using the following antibodies conjugated to the indicated fluorescent dyes (Miltenyi Biotec Inc.): CD15-FITC (clone VIMC6), CD16-APC-Vio770 (clone VEP13), CD63-PE (clone H5C6), CD66b-PE (clone REA306), and Siglec-8-APC (clone 7C9). Dead cell exclusion was performed using 7-AAD (BD Biosciences). Isotype controls (IgM-FITC, IgG1-APC, IgG1-APC-Vio770, IgG1-PE) were used to verify a lack of non-specific antibody mediated fluorescence (Miltenyi Biotec Inc.).

The rationale for using the above markers for assessing purity of isolated eosinophils/neutrophils, in addition to their activated states using flow cytometry is as

follows: (i) CD15 is an adhesion molecule found on the surface of both eosinophils and neutrophils (Lampinen et al., 2005), (ii) CD16 is a low affinity Fc receptor found on the surface of neutrophils, but at low levels on eosinophils (Pillay et al., 2013), (iii) Siglec8 is a specific marker for eosinophils that is expressed late in differentiation (Kikly et al., 2000), (iv) CD63 is a transmembrane-4 glycoprotein and is considered a surface marker for activation and degranulation of eosinophils as a result of crystalloid granule exocytosis (Carmo et al., 2016), (v) CD66b is stored in neutrophil granules and mobilized to the surface from lactoferrin-containing secondary granules upon activation (Lampinen et al., 2005).

2.4.2 Stimulation of Cells Prior to Flow Cytometry

Following isolation, a portion of cells from the final eosinophil isolation population were treated with 10 µM platelet-activating factor (PAF) for 30 min at 37°C to induce cellular activation, indicated by upregulation of CD63 on cell surfaces. Neutrophils, on the other hand, were stimulated with 1 µM of N-formyl-methionyl-leucyl-phenylalanine (fMLP) for 15 min at 37°C to stimulate activation leading to upregulation of CD66b expression. A dose-response curve was performed for both eosinophil and neutrophil stimulation (PAF: 0 µM, 10 µM, 20 µM; fMLP: 0 µM, 0.1 µM, 1 µM, 10 µM). Stimulated cells were used for confirming the gating strategy for flow cytometry analysis of the respective activation markers, otherwise absent on unstimulated cells.

2.5 Statistical Analysis

GraphPad Prism 7 (La Jolla, CA, USA) was used for statistical analysis. Wilcoxon paired signed-rank test was used to evaluate end-points between the isolation methods. Kruskal-Wallis was used for comparing > 2 groups. All data are reported as mean ± SD unless indicated otherwise.

3. Results

3.1 Retrospective Analysis of Commonly Used Eosinophil Isolation Methods

A qualitative comparison (Table 1) between the commonly used techniques showed that the newer methods, EasySep and MACSxpress using antibody cocktail of multiple specificities were more cost-effective and gave better results compared to the conventional single-antibody targeted depletion method (anti-CD16). An unpaired non-parametric analysis using retrospectively collected data from consecutive eosinophil isolations (MACSxpress vs. EasySep) conducted in three independent laboratories is presented in Figure 2. Both the EasySep method (n = 18) and MACSxpress (n = 20) consistently yielded eosinophils with purity > 90% (based on cytology staining). However, the yield of eosinophils per mL of peripheral blood was significantly greater for the former method ($P < 0.0001$, Mann-Whitney, Fig. 2). To address whether immunomagnetic cocktail (antibodies with multiple specificities) based techniques are superior to conventional single antibody CD16-bead based negative selection, a parallel head-to-head study was conducted. Though ideal, logistically it was improbable to perform all three isolations (MACSxpress vs. EasySep vs. CD16 depletion) in parallel. However, considering that the yield efficiency for MACSxpress (7.3 ± 16.3%; range: 50.9 – 101.8%, Fig. 2B) was significantly greater than EasySep method (43.1 ± 24.8%; range: 5.0 – 94.0% Fig. 2B) along with less user-manipulations (Fig. 1) and time taken per isolation (Table 1), the former was selected for comparison. Another issue with the EasySep method was the selection of CD45+CD16-CD66b+ eosinophils (as per the manufacturer's data sheet), when CD66b is considered an activation marker for eosinophils (Tak et al., 2016). To avoid confounding experimental work with the use of apparently activated CD66b+ eosinophils,

the use of MACSxpress is recommended. Furthermore, the EasySep employs an initial common step of employing post density-gradient centrifugation of whole blood similar to CD16 depletion method, which is likely to cause procedural activation of the isolated cells.

3.2 Determination of Purity and Absolute Yield in Enriched Populations

Two methods were utilized in assessing the purity of eosinophils and neutrophils derived from the different isolation techniques. Immune cells were identified based on their morphology and differential staining property with Wright's stain (representative micrographs of cytospin slides Fig. 3A, 3C). The cell differential count showed consistently higher purities isolated from the MACSxpress kit for eosinophils (96.6 ± 2.8%) and neutrophils (95.7% ± 8.4%) relative to their respective conventional counterparts: column-based (77.1 ± 22.5%) and dextran-sedimentation methods (81.6 ± 7.8%) (eosinophils, Fig. 3B, $P = 0.005$, Two-way ANOVA, Sidak Correction; neutrophils, Fig. 3D, $P < 0.0001$, Two-way ANOVA, Sidak Correction). For flow cytometry, the gating strategy for assessing viable eosinophil- (7AAD- CD16- CD15+ Siglec8+) and neutrophil- (7AAD- CD15+ CD16+) enriched populations is outlined (Fig. 3E, 3F). Absolute values obtained simultaneously from cytospin-based and flow analysis strongly correlated (eosinophils, Fig. 4A, $r = 0.97$, $P < 0.0001$; neutrophils, Fig. 4B, $r = 0.99$, $P < 0.0001$) and were computed to be in agreement (eosinophils, Fig. 4C, Bias = -1.216, SD = 21.01, Bland-Altman plot; neutrophils, Fig. 4D, Bias = 0.022, SD = 0.15). Indeed, the absolute yield (per mL of blood) for both eosinophils and neutrophils were comparable when calculated using the isolation purities from either

method (Fig. E1, $P > 0.05$). The mean values from both applications were hereafter used to calculate yield efficiencies reported throughout study.

3.3 Assessing Eosinophil Purity and Absolute Yields

In a head-to-head comparison, the MACSxpress kit produced higher absolute eosinophil values (calculated as eosinophil purity % multiplied by total number of cells/mL) relative to numbers isolated from the column-based protocol per mL of peripheral blood (Fig. 5A, $P = 0.01$, Wilcoxon test). In order to justify the broad overlap in range of values, we provide an illustration of absolute eosinophils number isolated by both methods for every individual donor in Figure 5B. MACSxpress isolation kit constantly yielded higher eosinophil counts ($P < 0.0001$, Two-way ANOVA, Holm-Sidak Correction). Next, the eosinophil yield efficiency was calculated as percentage of absolute eosinophils isolated by the respective method per mL of peripheral blood compared to the absolute eosinophil values/mL, reported on the complete blood count done clinically (SJHH core lab) for each donor. Again, the yield efficiency was greater with the MACSxpress protocol (73.9 ± 15.9%; range: 51.2 – 100.5%) versus the column-based isolation (49.2 ± 11.4%; range: 33.3 – 61.8%) (Fig. 5C, $P = 0.01$, Wilcoxon test). With respect to purity, the MACSxpress isolation method consistently yielded purer populations (95.0% ± 1.7%; range: 92.8 - 97.5%) compared to column-based method (73.1% ± 26.2%; range: 24.0 - 93.6%) (Fig. 5D, $P = 0.01$, Wilcoxon test).

3.4 Assessing Neutrophil Purity and Absolute Yields

We found higher absolute neutrophil yield/mL of blood derived from the MACSxpress kit compared to the dextran-sedimentation protocol in our head-to-head comparison (Fig. 6A, $P = 0.007$, Wilcoxon test). In contrast to the eosinophil analysis, there was no overlap in the range of values between the two isolation methods. This was further reflected when values for individual donors were plotted and MACSxpress method was documented to consistently yield higher neutrophil counts (Fig. 6B, $P < 0.0001$, Two-way ANOVA, Holm-Sidak Correction). The MACSxpress protocol had significantly greater yield efficiency (78.7 ± 14.1%; range: 63.5 – 101.4%) compared to the dextran-sedimentation method (22.6 ± 11.3%; range: 10.1 – 46.4%) (Fig. 6C, $P = 0.007$, Wilcoxon test). However, there was no significant difference in the purities of neutrophil populations isolated from the MACSxpress isolation kit (94.2 ± 10.1%; range: 69.3 – 98.1%) relative to the dextran-sedimentation method (82.5 ± 6.6%; range: 69.8 – 89.9%) (Fig. 6D, $P = 0.07$, Wilcoxon test).

3.5 Baseline Activation States of Isolated Target Cells

CD63 surface expression as a function of eosinophil activation was compared between the two methods. CD63 surface expression was consistently higher in eosinophils freshly isolated using the column-based method (68.2 ± 22.7%; range: 41.5 – 98.7%) compared to the MACSxpress kit (33.5 ± 18.1%; range: 17.9 – 55.3) (Fig. 7E, $P = 0.014$, Wilcoxon test). As a method for determining eosinophil activation measured as CD63 expression, increasing concentrations of PAF were used (Kroegel and Matthys, 1993), and

the gating strategy was elucidated in Figures 7A and 7B. A dose-response curve was generated by stimulating eosinophils with 0 µM, 10 µM, and 20 µM PAF, in which CD63 expression directly increased with an increase in stimulation (Fig. 7C).

Similarly, CD66b surface expression was utilized as a representation of activation of neutrophils isolated from the two different methods. Neutrophils isolated from the dextran-sedimentation method consistently showed higher CD66b surface expression (87.1 ± 7.9%; range: 74.6 – 96.9%) compared to cells isolated using the MACSxpress kit (33.9 ± 13.3%; range: 19.9 – 55.7%) (Fig. 8C, P = 0.0003, Wilcoxon test). fMLP was used as to induce CD66b surface expression (Fig. 8A).

4. Discussion

In this study, we carried out a comparative analysis of commonly available isolation techniques for both eosinophils and neutrophils to identify optimal methods for recovery of target cells suitable for downstream in vitro research. Our study confirmed that conventional methods using density-gradient sedimentation (Ficoll/Dextran) showed significantly compromised yields and higher rates of contaminated immune cells compared with immunomagnetic bead methods. Moreover, density-gradient methods caused target cells to become activated in the course of exposure to density gradient materials as well as extensive handling and centrifugation, evident by the higher frequency of CD63+ eosinophils and CD66b+ neutrophils.

Irrespective of research conducted in a clinical setting or basic science laboratory, specific cell isolation techniques are commonly used for a range of downstream applications. In particular, eosinophil research has been hampered by earlier isolation techniques, since these granulocytes constitute a minute fraction of the total leukocyte pool. Consequently, early researchers used patients with hypereosinophilic syndrome as donors so that sufficient numbers of eosinophils could be obtained for in vitro analysis (Roberts and Gallin, 1985). A disadvantage of using eosinophils from hypereosinophilic patients is that these cells exhibit unique or unusual features which are not typically observed in healthy or asthmatic subjects. Hence, we need improved techniques with more efficient recoveries and the procurement of intact, responsive cells, which would allow adequate eosinophil numbers from healthy and diseased donors for downstream functional assays.

Indeed, every decade has seen introduction of newer techniques for isolating eosinophils. Currently a number of methods are commercially available and routinely used in different laboratories globally, viz., (i) CD16-bead based depletion (ii) eosinophil isolation kit (antibody cocktail with multiple specificities, Miltenyi Biotec), and (iii) EasySep (StemCell Technologies) that requires density-gradient centrifugation and is labour-intensive. The most recent immunomagnetic bead based technique that allows eosinophil isolation without the requirement of density-gradient centrifugation is MACSxpress (Miltenyi Biotec). In addition to our head-to-head comparison of the more conventionally used CD16 depletion method vs. MACSxpress (Fig. 5), and retrospective analysis with another common method i.e. EasySep (Fig. 2) we show evidence (in our online supplementary, Fig. E2) that MACSxpress remains superior to its immediate previous generation of isolation protocol that uses a multiple antibody cocktail for depletion of non-target cells (Eosinophil Isolation Kit, 132-092-010, Miltenyi Biotec). As evident in Figure E2-A (refer to online supplementary), the yield efficiency of eosinophils from whole blood by MACSxpress is improved by approximately 75% compared to older generations ($P = 0.002$). In context to baseline procedural activation, MACSxpress shows a trend towards lower CD63 surface expression (Fig. E2-B, $P = 0.08$). The viability of the cells purified for the performed isolation techniques was comparable between the methods: CD16 depletion ($96.2 \pm 4.9\%$), multiple antibody depletion (Miltenyi) ($94.6 \pm 4.5\%$), and MACSxpress ($95.3 \pm 4.4\%$). However, it is important to note that in our observation study (both retrospective and prospective) only MACSxpress and EasySep methods have consistently yielded an enriched population of eosinophils > 90% purity (Fig. 2B). Though CD16 depletion is routinely used in many labs, we show that it is

inconsistent and can yield a population with compromised purity sporadically, as observed from our outliers plotted in Figure 5D. Though a head-to-head comparison between all these four methods would be more convincing, nevertheless we believe it is the initial density-gradient centrifugation steps and increased user manipulation (Fig. 1) that compromises the yield and leads to the observed cellular activation.

When activated, neutrophils can utilize effector responses such as degranulation and oxidative burst for host defense against foreign antigen infection (Chen and Junger, 2012). In eosinophils, the various activation states of circulating and extravasated cells may be associated with asthmatic activity and pulmonary function (Johansson, 2014). Therefore, studies investigating or comparing cellular function should consider an isolation method that minimizes unintended activation of the cells as a consequence of the protocol itself, in order to reduce confounding factors in data analysis.

Indeed, several reports comparing different isolation techniques have shown inadvertent activation of target cells via these methods. With respect to eosinophils, different research groups have reported that for various in vitro assays, cells isolated through anti-CD16 magnetic beads were unable to functionally respond to lipid chemoattractants or interleukin-8 (Rozell et al., 1996; Casale et al., 1999). The column-based CD16 isolation method was also shown to induce significantly increased expression of CD18 and CD54 (ICAM-1) which are surface markers that play roles in cellular adhesion and transmigration, both of which are hallmarks of eosinophil activation (Sedgwick et al., 1996). Additionally, Sedgwick et al. (1996) found spontaneous superoxide and leukotriene (LTC4) generation in eosinophils isolated using anti-CD16 isolation. These earlier reports, coupled with our data

on CD63 surface expression (Fig. 7E, Fig. E3B), supports the notion that target cells are being activated or primed during the process of purification.

With regards to neutrophils, obtaining high yields has not been problematic, since these cells comprise 50-70% of circulating leukocytes (Sagiv et al., 2015). However, previous research has shown that using dextran in neutrophil isolation methods led to priming or activation of unstimulated cells (Watson et al., 1992). The same study also showed an altered capacity for neutrophils to generate oxidative species and increased expression of CD11b and CD16, suggestive of procedural priming and/or activation of isolated cells. We used CD66b as a marker for neutrophil activation since it is known to be upregulated and mobilized to the cell surface upon activation by clinically relevant extraneous agents such as cigarette smoke and allergens (Stocks et al., 1995; Lampinen et al., 2005; Friedrichs et al., 2014; Pelikan, 2014). Therefore, when comparing physiological levels of such markers in ex vivo conditions to determine health and disease, it is concerning if the isolation method itself increases surface expression of target markers, as seen for CD66b in our study (Fig. 8C). We acknowledge that our current study design does not let us directly evaluate the baseline activation state of neutrophils/eosinophils isolated via MACSxpress compared to the physiological circulating levels ex vivo. Since we have used healthy non-atopic donors with normal leukocyte counts, it is unlikely that there would be circulating levels of activated granulocytes.

Contaminating/non-target cells in a supposedly “enriched” population raise questions regarding the validity of functional assays/experimental end-points. In the present study, we document that eosinophil populations isolated using the column-based anti-CD16 method

had detectable numbers of neutrophils and lymphocytes as depicted in Figure 3B (lower purity) compared to MACSxpress method. Again, we could identify eosinophils (12.5 ± 2.8%) within the enriched neutrophil population isolated with dextran-sedimentation (Fig. 3C). Therefore, in addition to our current observation, the anti-CD16 column method was also documented in previous studies to have suboptimal yield efficiencies and contaminating immune cells identified using cytological stains (Sedgwick et al., 1996; Percopo et al., 2010).

We acknowledge that extensive comparative functional assays for our present study were not conducted. However, MACSxpress protocols have been used in downstream functional assays/read-outs showing a lack of procedural cellular activation. For instance, EPX levels in supernatants collected from MACSxpress-isolated eosinophils incubated at 37˚C for 3 h, were comparable to control wells (media only, no cells), suggesting minimal baseline activation (Fig. E3, A). Again, while investigating IgG-induced eosinophil degranulation, measurement of the release of lactose dehydrogenase, as a marker of membrane integrity, showed no detectable differences between unstimulated eosinophils (isolated using MACSxpress) vs. control media (Fig. E3, B). Other readouts could be determined in future studies, including lipid mediator release and respiratory burst responses.

Finally, the use of ammonium chloride in erythrocyte eradication during an eosinophil isolation using anti-CD16 depletion method was shown to be responsible for procedural cellular activation (Ide et al., 1994). While two different erythrocyte lysis methods in this head-to-head comparison study were used (NH4Cl for CD16 depletion vs. cold water lysis for MACSxpress), the main objective was to maintain routinely practiced lysis methods used

in many labs with respective isolation protocols. Nevertheless, in a direct comparison (n = 5) between NH4Cl vs. cold water lysis for the CD16 depletion protocol, we found that although purity was improved with cold water lysis (75.7 ± 29.3% vs. 98.0 ± 1.2%, $P < 0.01$, Fig. E4A), there was no difference in yields (48.6 ±13.1 vs. 42.7 ± 37.1, $P = 0.41$, Fig. E4B), or in numbers of activated CD63+ eosinophils (62.1 ± 19.2 vs. 44.8 ± 28.8, $P = 0.22$, Fig. E4C). Therefore, we do not believe that using the cold water lysis method for CD16 depletion protocol would have significantly changed our findings regarding the superiority of the MACSxpress method in 'consistently' yielding high numbers of minimally activated eosinophils.

Research laboratories within similar disciplines have often found discrepancies in inferred observations or error in reproducibility of data. We surmise that the lack of standardized isolation protocols contributes to this. In an attempt to choose a method most suitable for in vitro translational research, we summarized our current knowledge regarding the commonly used techniques in Table 1. It is important to note that with respect to both length of processing and cost of eosinophil isolation, the MACSxpress kit is cheaper, uses less blood per sample, and is substantially more time-efficient compared to standard column-based methods. Despite the MACSxpress isolation kit for neutrophils being twice as expensive as the dextran-sedimentation method, overall, neutrophil isolation kits are significantly cheaper than eosinophil kits. Additionally, the shorter processing time allows for more same-day downstream functional assays to be run if necessary.

5. Conclusions

In conclusion, comparisons between granulocyte functions from different research laboratories must take into consideration the particular use of different isolation protocols. We hereby report that negative selection of granulocytes using immunomagnetic beads of multiple specificities targeted against different surface markers expressed on non-target cells (bypassing density-gradient centrifugation steps), yields an enriched population with minimal procedural activation and is thus suitable for in vitro functional studies. Importantly, this method has a greater yield efficiency/recovery from minimum volume of blood draw, which allows more freedom with ethical approval, experimental design and sample size.

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Table Legends:

Table 1: A qualitative comparison of the isolation techniques

Figure legends:

Figure 1. Schematic overview of various granulocyte isolation protocols used.

Figure 2: Retrospective analysis of consecutive eosinophil isolations: (A) Analysis of yield efficiencies and (B) purity of eosinophils using the EasySep Kit (STEMCELL Technologies Inc.) (n = 18) and MACSxpress kit (Miltenyi Biotec Inc.) (n = 20). Unpaired analysis with Mann Whitney Test.

Figure 3: Determination of purity in enriched populations: (A,C) Cell differential method using Wright staining on cytospin slides of isolated eosinophils/neutrophils using column-based CD16 negative selection/dextran-sedimentation methods and MACSxpress method (contamination indicated by black arrows). (B,D) Cell differentials as a percentage of total number of cells counted in final isolated populations for each method (n = 8), data presented as mean ± SD, P **** indicates $P < 0.0001$, Two-way ANOVA, Sidak correction. (E,F) Flow cytometry method of identifying live eosinophils/neutrophils using gating strategy of 7AAD- (PerCP) CD16- (APC-Cy7) CD15+ (FITC) Siglec8+ (APC) & 7AAD- (PerCP) CD16+ (APC-Cy7) CD15+ (FITC) respectively.

Figure 4: Assessing techniques for determining isolation purity: (A,C) Correlation and (B,D) Bland-Altman plots comparing the absolute (A,B) eosinophil and (C,D) neutrophil (cells / mL of blood) yield using the two isolation purity assessment strategies. N.B. Absolute yield calculated as eosinophil purity% multiplied by total number of cells/mL.

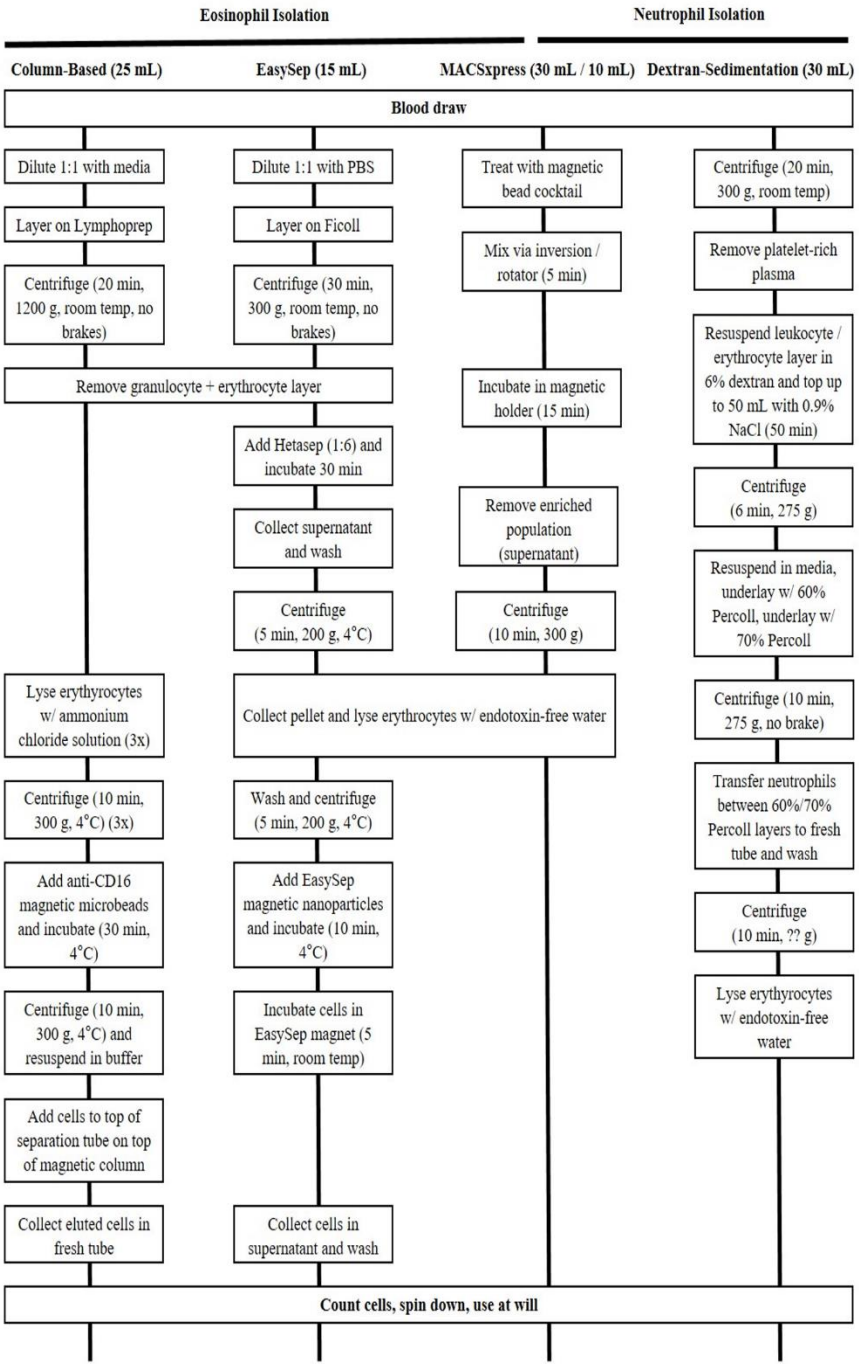
Figure 5: Assessment of eosinophil purity and yield: Paired comparison of (A) absolute eosinophil yield per mL of peripheral blood (PB) isolated in parallel using Miltenyi MACSxpress vs. Column based CD16 negative selection method, calculated as eosinophil purity % *multiplied by total number of cells/mL; (B) Absolute eosinophil yield between paired isolations for n = 7 healthy volunteers. P >0.0001, significant difference between absolute eosinophils obtained between the isolation methods, Two-way ANOVA, Holm-Sidak correction; (C) % eosinophil yield per mL of PB, or yield efficiency calculated as absolute eosinophil yield/ mL divided by the absolute eosinophil values /mL, reported on the complete blood count done clinically (SJHH core lab) for each healthy volunteer; (D) Variability in eosinophil purity between the methods for all 7 paired isolations performed. The dotted line represents 90% threshold; N.B. The eosinophil purity is taken as the average of cytospin and flow methods. For paired test analysis of P values reported Wilcoxon matched-pairs signed rank test was performed.

Figure 6: Assessment of neutrophil purity and yield: Paired comparison of (A) Absolute neutrophil yield per mL of peripheral blood (PB) isolated in parallel using MACSxpress vs. dextran sedimentation method, calculated as neutrophil purity% *multiplied by total number of cells/mL; (B) Absolute neutrophil yield between paired isolations for n=8 healthy volunteers. P >0.0001, significant difference between absolute neutrophil obtained between the isolation methods, Two-way ANOVA, Holm-Sidak correction; (C) % neutrophil yield per mL of PB, calculated as absolute neutrophil yield/ mL divided by the absolute neutrophil values /mL, reported on the complete blood count done clinically (SJHH core lab) for each healthy volunteer; (D) Variability in isolation purity between the methods for all 8 paired isolations performed. The dotted line represents 90% threshold; N.B. The neutrophil purity is taken as the average of cytospin and flow methods. For paired test analysis of P values reported Wilcoxon matched-pairs signed rank test was performed.

Figure 7: Assessing baseline activation state of eosinophils: Gating strategy for assessing CD63 expression in eosinophils (A) unstimulated vs. (B) 10 μM PAF stimulated eosinophils from healthy PB isolated via MACSxpress. (C) CD63 expression in (naïve) unstimulated vs. increasing platelet-activating factor (PAF) stimulated eosinophils isolated from PB; (D) Representative plot showing baseline expression of CD63 in freshly isolated unstimulated eosinophils using MACSxpress (purple) and column-based CD16 negative selection (green) method, and (E, F) CD63+ eosinophils (percent of total, MFI values) are plotted for n = 7 paired isolations, *indicates significant difference in CD63 expression, paired t test.

Figure 8: Assessing baseline activation state of neutrophils: (A) CD66b expression in (naïve) unstimulated vs. 1 µM fMLP stimulated neutrophils isolated from PB (B) Representative plot showing baseline expression of CD66 in freshly isolated unstimulated neutrophils using MACSxpress (purple) and dextran sedimentation (green) method, and (C,D) CD66b+ neutrophils (percent of total, MFI values) are plotted for n =7 paired isolations, showing a trend towards increased CD66 expression in neutrophils isolated via dextran-sedimentation, *indicates significant difference in CD66b expression, Paired t test.

Figure 1. Son et al., 2017



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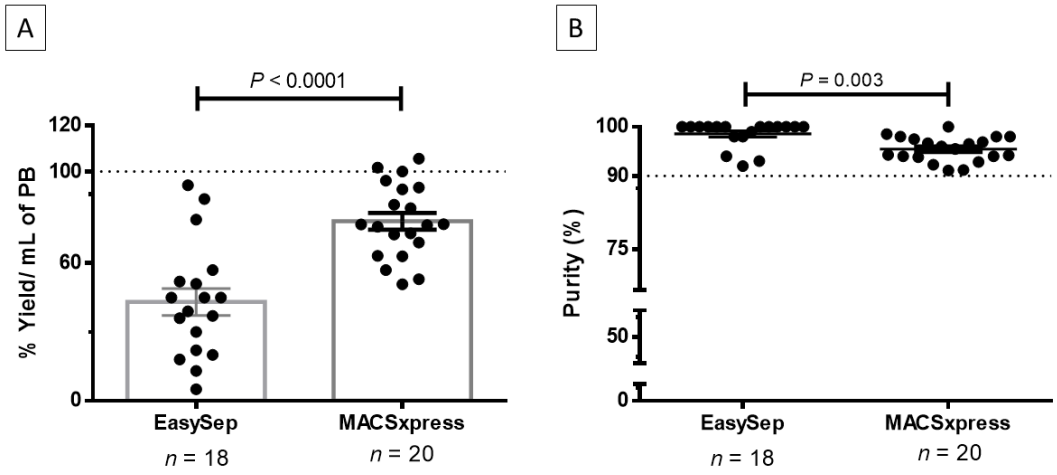

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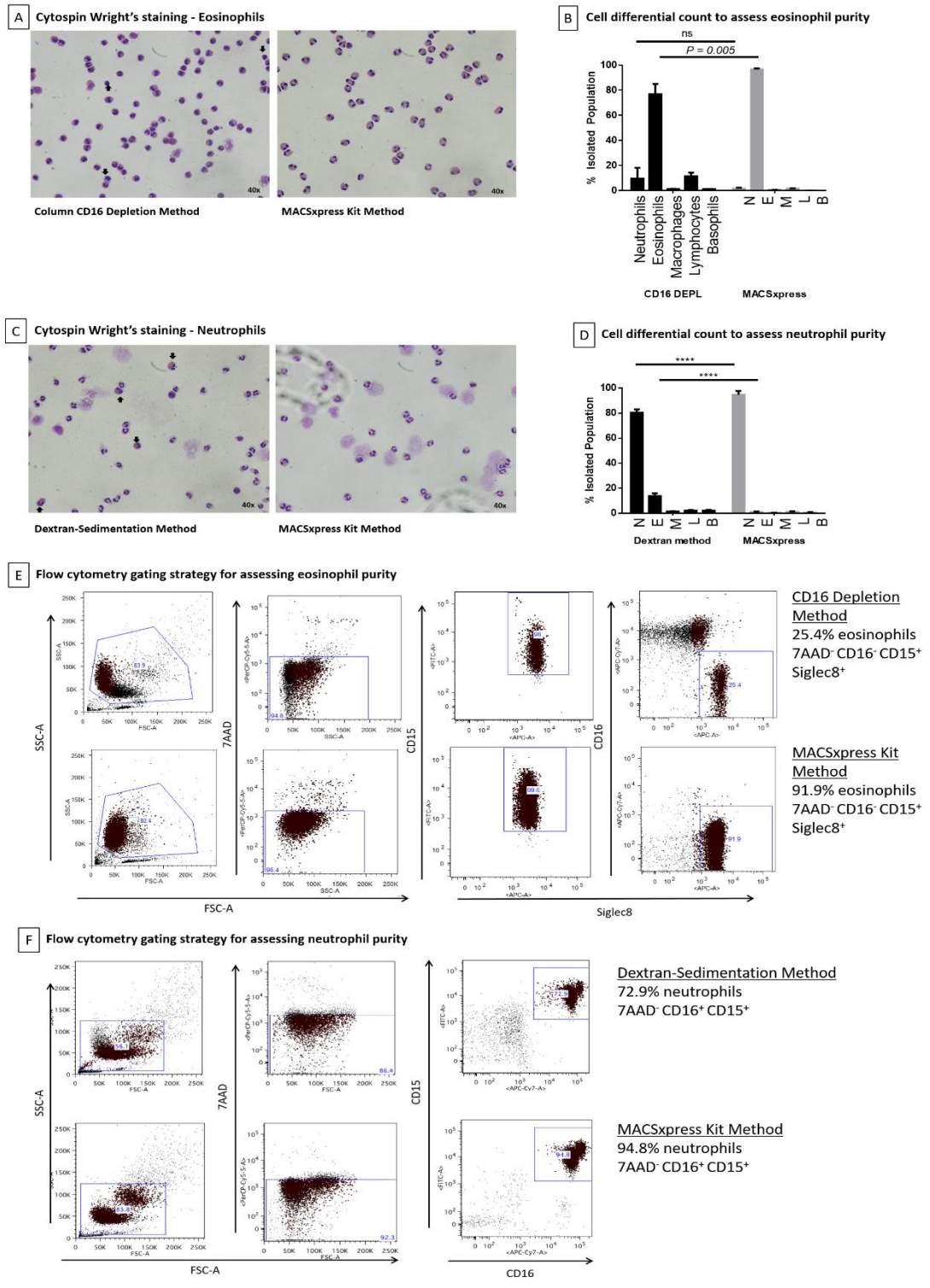

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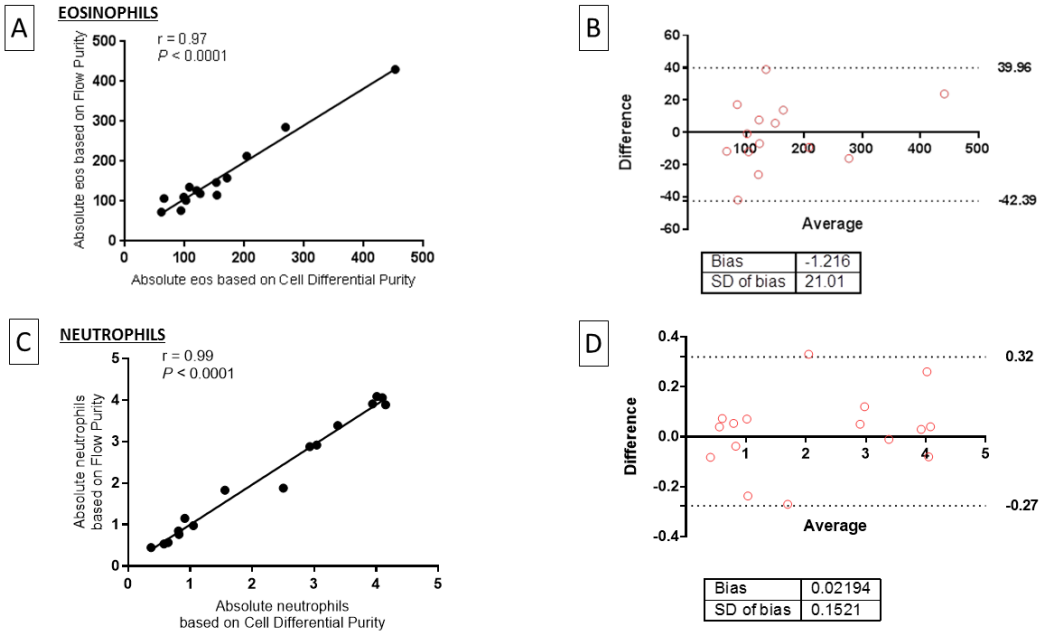

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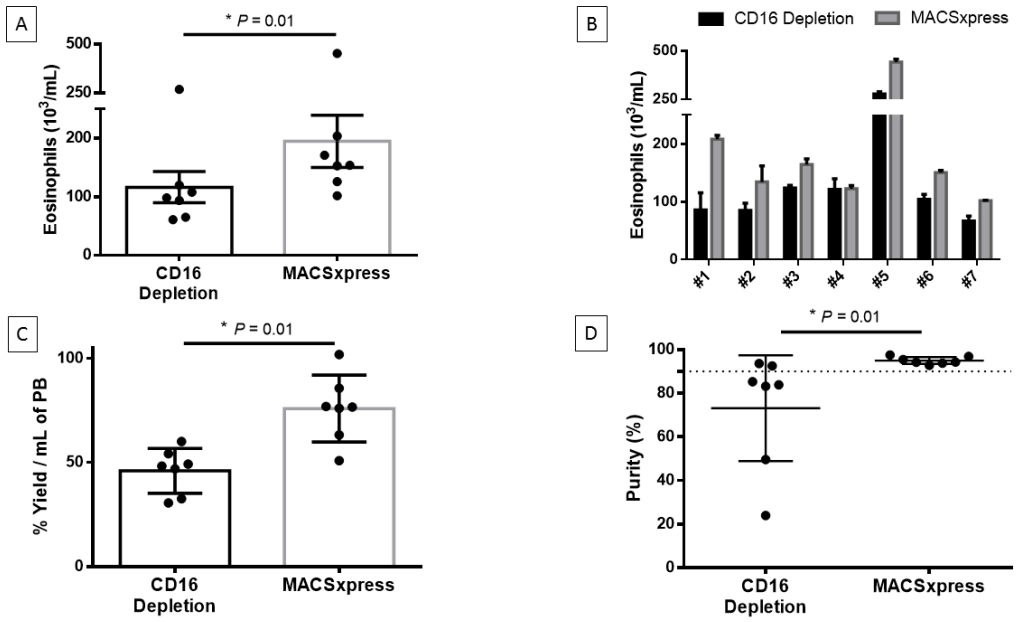

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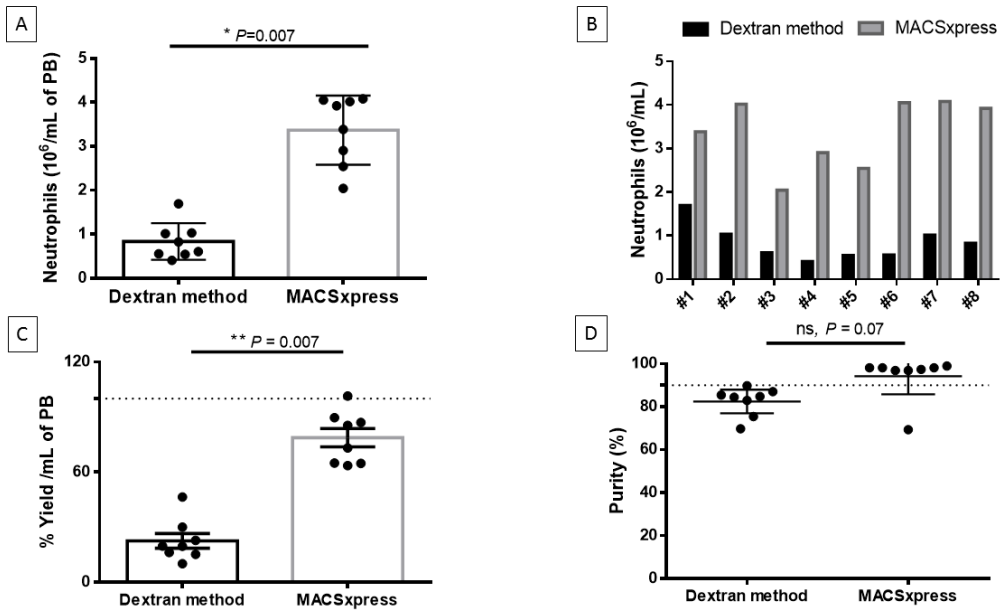

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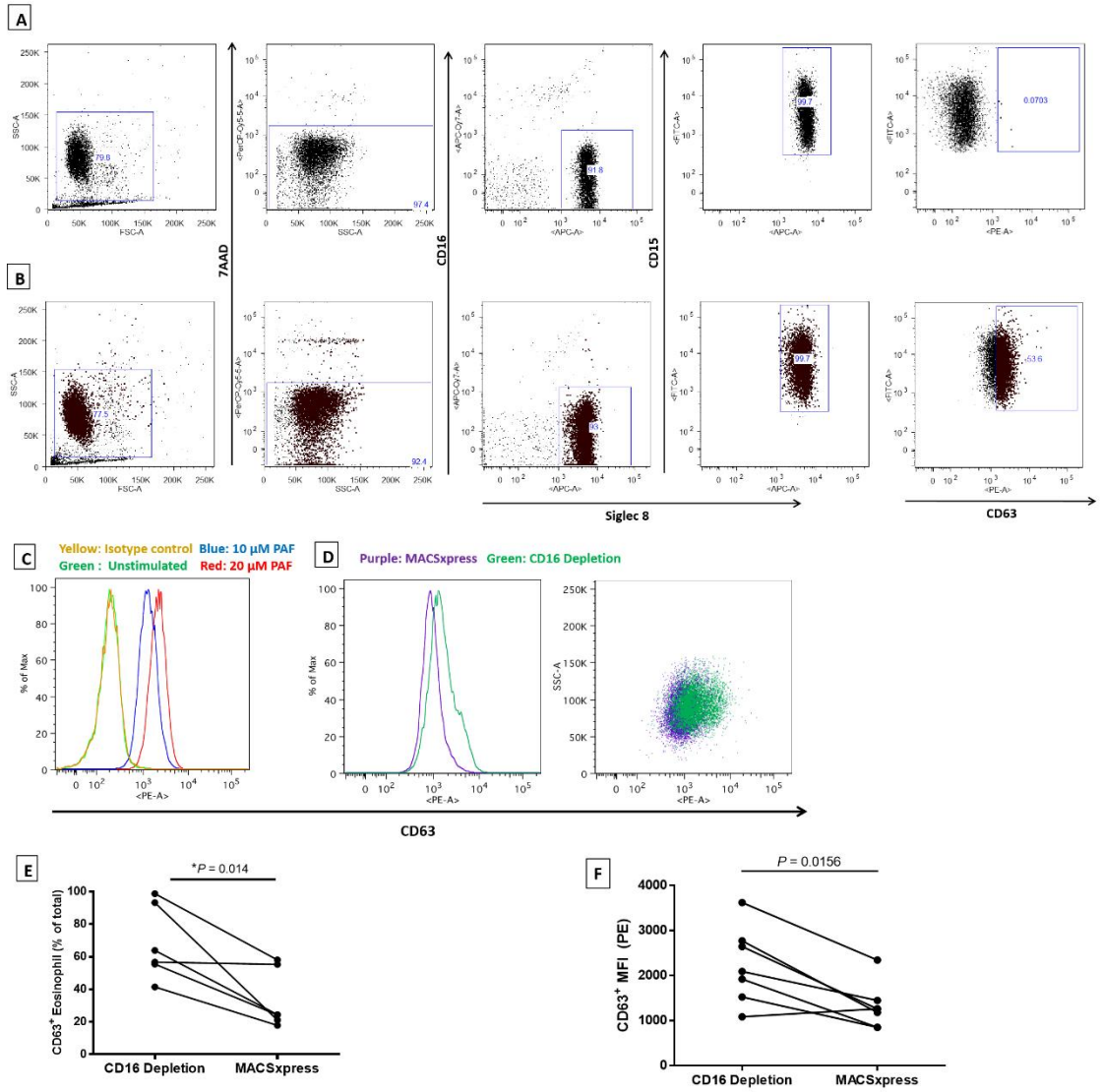

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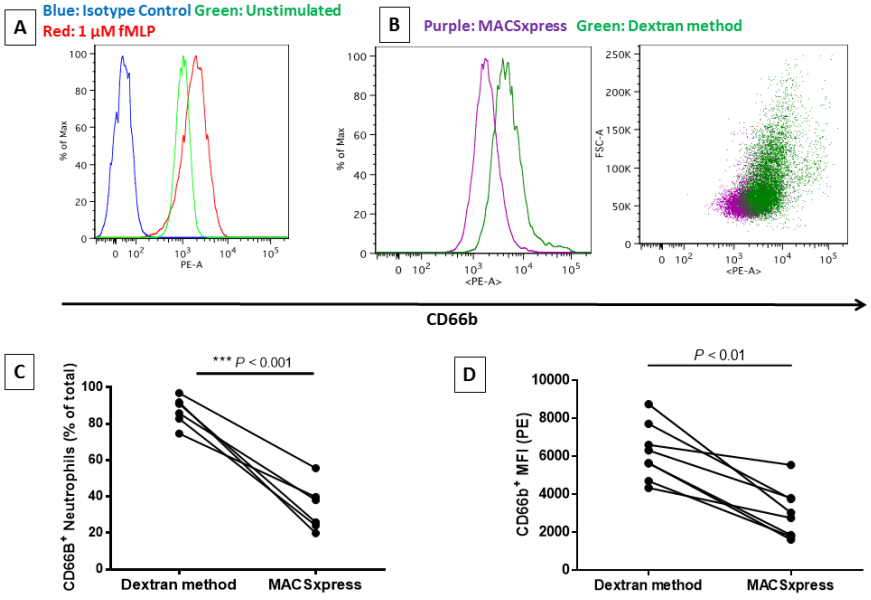


Table 1. Son et al., 2017

Parameters	Eosinophils			Neutrophils	
Isolation Method	Column-Based	EasySep	MACSxpress	Dextran-Sedimentation	MACSxpress
Purity	++	+++	+++	+++	+++
Yield	+	++	+++	+	+++
Activation	+++	n/a	+	+++	+
Hours	4-5 hrs	4-5 hrs	1-2 hrs	3-4 hrs	1-2 hrs
Cost (approx.)	$250	$150	$150	$23	$60

Supplementary Information

Title: Improved recovery of functionally active eosinophils and neutrophils using novel immunomagnetic technology

Figure Legends

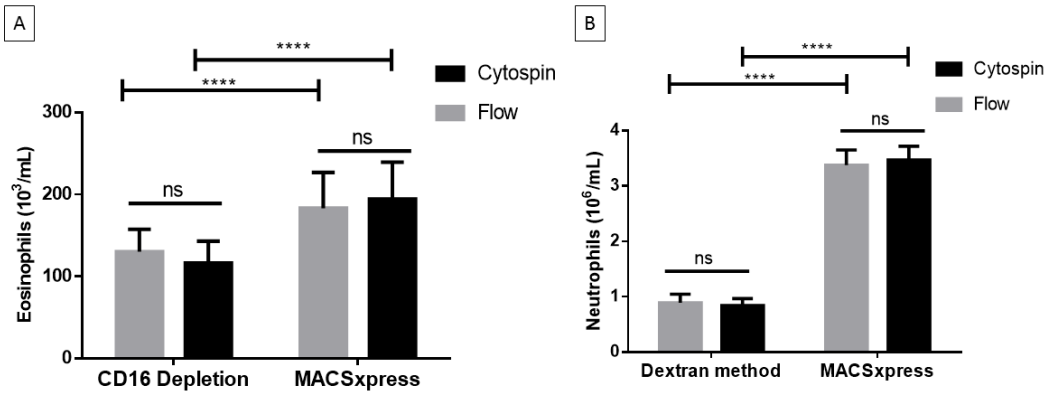
Supplementary Figure E1: Absolute number of isolated (A) eosinophils and (B) neutrophils for both isolation methods calculated based on isolation purity using cytospin vs. flow cytometry methods, **** indicates P<0.0001, Two-way ANOVA, Sidak correction

Supplementary Figure E2: Comparing eosinophil methods: Paired comparisons using CD16 depletion, multiple antibody depletion (Miltenyi), and MACSxpress methods (choice of RBC lysis in all three techniques: endotoxin-free cold water lysis) with respect to (A) eosinophil yield per mL of whole blood ($P < 0.01$) and (B) enumeration of the $CD63^+$ surface marker in freshly isolated eosinophils ($P = 0.08$). Kruskal-Wallis comparison with Dunn's multiple test, n = 5. (C) Representative histogram of CD63 expression in (naïve) unstimulated eosinophils isolated using three different methods, and isotype control.

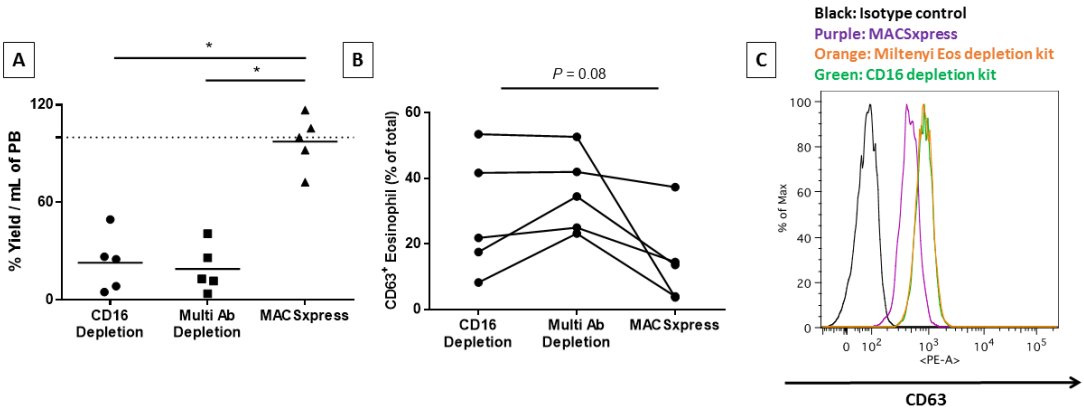
Supplementary Figure E3: (A) EPX release measured by ELISA (Nair et al., Allergy 2013) in untreated and 0.1% Triton treated eosinophils. Data presented as mean ± SEM, n = 4 healthy donors. * indicates significant difference from control wells (no cells) and untreated eosinophils, ns indicates no significant difference in detectable EPX between control wells and unstimulated cells. Kruskal-Wallis, Dunn's multiple test; (B) Lactose dehydrogenase activity (LDH assay, Abcam) as a function of NADH measured in media supernatants collected over given time-points from control wells (media), untreated (eosinophils only) and IgG-stimulated eosinophils (5ug/mL of ChromPure IgG, Jackson ImmunoResearch Laboratories Inc., 009-000-003). *p <0.05, Two-way ANOVA , n = 4. Eosinophils used for both data-set were isolated using MACSxpress method.

Supplementary Figure E4: Comparison of eosinophil isolation using different RBC lysis method: (A) Purity (B) yield efficiency and (C) $CD63^{+}$ surface expression of freshly isolated eosinophils using CD16 depletion method using two RBC lysis methods, NH_4Cl or endotoxin-free sterile cold water. Wilcoxon paired rank test, ns: not significant

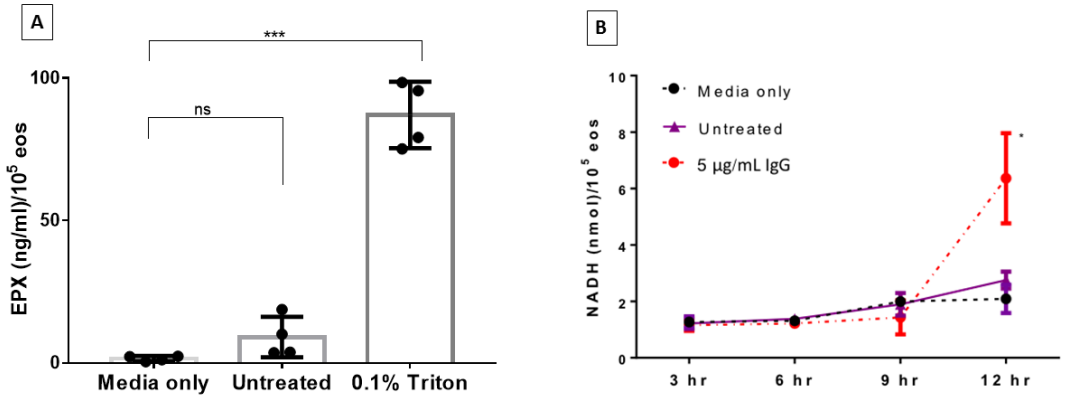
Supplementary Figure E1. Son et al., 2017



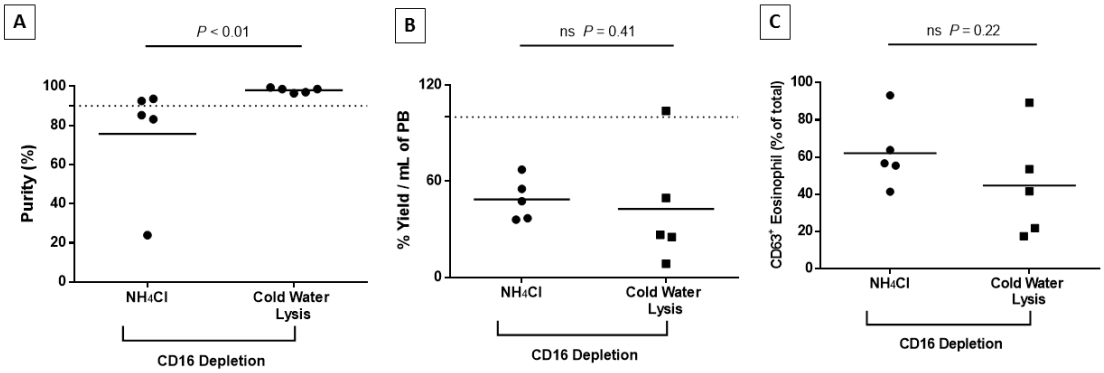
Supplementary Figure E2. Son et al., 2017



Supplementary Figure E3. Son et al., 2017



Supplementary Figure E4. Son et al., 2017



Chapter 4: Publication to the

Journal of Leukocyte Biology, 2020

Title: The eosinophil actin cytoskeleton undergoes rapid rearrangement in response to fluid shear stress.

Summary Sentence: The changes in human eosinophil cytoskeleton architecture induced by fluid perfusion at physiologically-relevant flow rates are augmented by and temporally-linked to calcium release.

Running Title: Eosinophils respond to mechanical stress stimulus

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Key Words: integrin, calcium, migration, mechanosensing, adhesion, podosomes

Abbreviations Page

AM	Acetoxymethyl
Arp	Actin-related protein
BSA	Bovine serum albumin
CCR3	C-C chemokine receptor type 3
DABCO	1,4-diazabicyclo[2.2.2]octane
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
EGTA	Egtazic acid
IL-5	Interleukin 5
IP_3	Inositol 1,4,5-trisphosphate
KCl	Potassium chloride
Mac-1	Macrophage-1 antigen
MAdCAM-1	Mucosal addressin cell adhesion molecule-1
$MgCl_2$	Magnesium chloride
MES	2-(N-morpholino)ethanesulfonic acid
NaN_3	Sodium azide
PBS	Potassium-buffered saline
PICR	Perfusion-induced calcium response
RhoA	Ras homolog gene family, member A
ROCK	Rho-associated protein kinase
VCAM-1	Vascular cell adhesion protein 1

VLA-4 Very Late Antigen-4

Abstract

The regulatory processes involved in eosinophil trafficking into tissues are poorly understood; therefore, it is crucial to elucidate these mechanisms to advance the quality of clinical care for patients with eosinophil-mediated diseases. The complex interactions between eosinophil integrin receptors and their corresponding ligands on the post-capillary venules of the bronchial endothelium result in distinct modifications to the cytoskeletal architecture that occur in coordinated, temporally-regulated sequences. The current study utilizes real-time confocal microscopy and time-based immunofluorescence staining to further characterize the effects of physiologically-relevant fluid shear stress on this novel phenomenon of perfusion-induced calcium response.

We found that the mere perfusion of fluid over adhered human eosinophils induced a release of intracellular calcium observed in conjunction with changes in cell morphology (flattening onto the coverslip surface, an increase in surface area, and a loss of circularity), suggesting a previously unknown mechanosensing aspect of eosinophil migration out of the vasculature. Although changes in morphology and degree of calcium release remained consistent across varying perfusion rates, the latency of the response was highly dependent on the degree of shear stresses.

Eosinophils were fixed post-perfusion at specific timepoints for immunofluorescence staining to track proteins of interest over time. The distribution of proteins was diffuse throughout the cell prior to perfusion; however, they quickly localized to the periphery of the cell within 5 minutes. The actin cytoskeleton became markedly built

up at the cell edges rapidly after stimulation, forming punctate dots by 4 minutes, suggesting a pivotal role in directed cell motility.

Introduction

Eosinophils are inflammatory effector cells that belong to the granulocyte family of leukocytes[1]. The magnitude of eosinophil accumulation in respiratory tissues often correlates to disease severity and is seen as a driving force in the pathogenesis of inflammatory processes[2]; therefore, it is critical to decipher the regulatory mechanisms involving eosinophil trafficking into tissues to advance the treatment of clinical patients with eosinophilia-related diseases[3]. Of these processes, the involvement of chemokines (e.g. IL-5, eotaxin) in eosinophil recruitment has been well documented[4]. The binding of eotaxin to chemokine receptor 3 (CCR3) activates an outside-in transduction cascade via the $G_{q/11}$ pathway which ultimately leads to the IP_3-mediated release of calcium ions from the endoplasmic reticulum[4,5]. Chemokine-stimulated eosinophils that develop an intracellular calcium ($[Ca^{2+}]_i$) gradient have shown a greater propensity to undergo morphological changes such as adhesion, flattening, lamellipodium formation, and migration[6–10]. These events involve distinct modifications to the cytoskeletal architecture that occur in a coordinated, temporally-regulated sequence comprising of a myriad of stimuli and signaling events.

The extravasation of eosinophils from the postcapillary venules of bronchial circulation to the airway wall involves complex interactions between integrin receptors in various activation states and their analogous ligands on the bronchial vascular endothelium[11]. Of the seven distinct heterodimeric integrin adhesion molecules expressed on the eosinophil cell surface, the very late antigen-4 (VLA-4, $\alpha_4\beta_1$) and macrophage-1 antigen (Mac-1, $\alpha_M\beta_2$) integrins have been extensively studied due to their predominant

role in mediating rolling and adhesion on the vascular cell adhesion molecule-1 (VCAM-1) expressed by endothelial cell surface, an integrin counter-ligand that is upregulated in the asthmatic lung[11–14]. Eosinophil integrin binding to their counter ligands on both the endothelium and extracellular matrix (ECM) triggers a series of signaling events that recruit adaptor proteins such as talin, vinculin, and the Arp2/3 complex for the rearrangement of the actin cytoskeleton to form physical links between the cell and its environment[15,16]. The stability of these connections determine the duration of sustained polarization of actin polymers and subsequent induction of directed transmigration of cells[15]. However, the precise relationship and directionality between $[Ca^{2+}]_i$ fluctuations via chemokine binding versus actin cytoskeleton rearrangement through integrin activation in eosinophils has yet to be realized.

We have previously shown that the mere perfusion of media without eotaxin over adhered human peripheral eosinophils induced a release of $[Ca^{2+}]_i$[17]. This perfusion-induced (or fluid shear stress-induced) calcium response (PICR) was observed in conjunction with hallmark migrational morphological changes (flattening, pseudopodium extension), suggesting a hitherto unknown mechanosensing aspect of eosinophil extravasation out of the vasculature[17]. The present study utilizes real-time confocal microscopy and time-based immunofluorescence staining to further characterize the effects of physiologically-relevant levels of fluid shear stress on the temporality of both $[Ca^{2+}]_i$ mobilization and actin cytoskeleton regulating signaling cascades in eosinophils. Detailing the role of cell surface molecular mechanosensors in eosinophil migration may provide novel therapeutic targets to control and/or modify disease pathology in conditions

characterized by tissue eosinophilia including eosinophilic asthma.

Materials and Methods

Ethics Statement

Ethical approval was granted by the institutional ethics committee (Hamilton Integrated Research Ethics Board; HIREB) to obtain blood samples from healthy adult volunteers. The inclusion criteria merely asked for donors without a history of asthma, currently off any type of medication, and without having been recently ill prior to donation. Participants provided written informed consent to participate in this study.

Eosinophil Enrichment

Eosinophils were isolated from whole blood draws using the MACSxpress Eosinophil Isolation Kit (catalogue #: 130-104-446; Miltenyi Biotec Inc., Bergisch Gladbach, Germany) as previously outlined in detail per Son et al.[18]. Eosinophil populations (purity: 95.0 ± 1.7%; viability: 95.3 ± 4.4%)[18] were resuspended in RPMI1640 and used within 12 hours of isolation to prevent poor cell function due to loss of viability over time.

Confocal Fluorescence Microscopy

A calcium binding dye, Fluo-3 AM (4 µl at 3.5 µM in DMSO w/ 0.01% pluronic acid) was loaded into 500 µL of 10^6 cells/mL and incubated in the dark for 10 min. The cells were transferred onto a fibronectin-coated (2 µg/mL; Sigma Aldrich, ON, Canada) glass coverslip (40 mm; Warner Instruments, CT, USA) which was then placed within a low-profile parallel plate flow chamber (RC-31; Harvard Apparatus, MA, USA) for further

incubation in the dark for 50 min. RPMI1640 (0.42 mM calcium) was perfused into the chamber using a PS-200 peristaltic pump (Living Systems Instruments, VT, USA) which allowed for controlled perfusion rates. Eosinophil recordings were obtained and digitized as previously described per Ahmadzai et al.[17]. All experiments were conducted at room temperature that typically lasted 3-10 minutes.

Microscopy Signal Processing and Statistical Analysis

The exported .tiff image files were analyzed using the QuimP plug-in (University of Warwick, UK) for the open platform software *ImageJ* (National Institutes of Health, MD, USA). An analysis module of QuimP first transformed images into binary form by thresholding each greyscale pixel into white or black to differentiate between background signals versus regions of interest (ROIs). Individual cells represented by their corresponding ROIs were tracked over the duration of the experiment to quantify parameters of interest in cell morphology (adhesion, cell area) and fluorescence intensity (latency and intensity of calcium flux). A representation of raw data prior to normalization is given in Figure 1.

In the current study, multiple cells (5-15) were analyzed from purified eosinophil populations isolated from a given subject on a particular day, then averaged to generate mean values (± SD) for each individual. These intra-subject mean values were pooled in order to generate population means for data analysis; reported sample size refers to the number of unique subjects for whom each experimental condition was replicated. GraphPad Prism 6 (La Jolla, CA, USA) was used for statistical analysis, which involved

Kruskal-Wallis test to compare means of two or more groups, followed by Dunn's multiple comparison *post hoc* test. In all cases, $p < 0.05$ was considered to be significant.

Immunofluorescence Staining

Mature eosinophils are considered to be terminally differentiated by the time they egress from haematogenous regions of the bone marrow into the blood[19]. *Ex-vivo*, the viability of eosinophils drops dramatically in as little as 24 hrs post-isolation[20]. Consequently, there lies an inherent difficulty in maintaining viable mature eosinophils *ex-vivo* without affecting cellular function. In addition, the reduced lifespan of these cells does not allow for viral transduction or other similar methods to visualize the real-time localization of fluorescence-tagged proteins of interest. In order to compensate for these shortcomings, we perfused 4% formaldehyde (Sigma Aldrich) in cytoskeletal buffer (CBS; 10 mM MES pH 6.1, 3 mM $MgCl_2$, 2 mM EGTA, 138 mM KCl) for 10 min through the peristaltic pump at specific time points (0 sec - 5 min) after the initial perfusion of solution to fix the cells in the process of morphological change. Coverslips with fixed, adherent eosinophils were removed from the flow chamber, washed three times with PBS, and permeabilized with 0.25% Triton X-100 in PBS for 5 min. We did not observe any morphological changes in eosinophils due to the fixative or permeabilizing agents. After three more washes with PBS, coverslips were incubated with blocking buffer (2% BSA, 0.1% Triton X-100, 0.02% NaN_3 in PBS) overnight at 4°C.

The eosinophils were incubated with primary antibodies the next day for 2 hrs at room temperature and washed three times with PBS before adding the secondary antibodies

for 45 min at room temperature in the dark. The coverslips were rinsed three to five times with PBS before incubating with phalloidin for 30 min at room temperature in the dark. All fluorescent probes and antibodies (primary and secondary) used for immunofluorescence staining and their respective dilutions in blocking buffer are listed in Table 1. After three final rinses with PBS, the coverslips were mounted in Mowiol mounting medium (0.2 M Tris-HCl pH 8.5, 25% glycerol, 10% Mowiol 4-88 (Sigma Aldrich), 2% DABCO (1,4-Diazabicyclo(2.2.2)octane, Sigma Aldrich)) and allowed to harden overnight. An Olympus IX81 Nipkow scanning disc confocal microscope was utilized to picture protein localization, and images were analyzed using Metamorph image analysis software (Molecular Devices, CA, USA).

Results

Calibration of the Peristaltic Pump and Shear Stress Calculation

In order to consider the physiological relevance of the applied flow stimuli in our experiments (1-10 dynes/cm^2 in post-capillary venules), we ascertained the shear stresses produced within the parallel plate flow chamber. Each increment in peristaltic pump flow rate setting increased flow output by 0.0667 mL/min. Given the dimensions of our parallel plate flow chamber and using the Poiseuille's law equation: $\tau = 3Q\mu / 2WH^2$ (τ = shear stress; Q = flow rate; μ = dynamic viscosity; W & H = width and height of the flow chamber), we calculated that these flow rate increments equate to a fluid shear stress increase of 1.25 dynes/cm^2.

The Effect of Fluid Shear Stress on Individual Eosinophils

Prior to perfusion, the cells were observed to be spherical with a smooth periphery, with very little fluctuation in fluorescence signal (Fig. 2). A baseline recording of eosinophils at rest was taken for at least 60 seconds before application of perfusion. Approximately 60-200 seconds after the onset of perfusion, the eosinophils experienced a sharp increase in $[Ca^{2+}]_i$ as indicated by the spike in emission, followed by a less rapid return of the fluorescence back toward baseline (Fig. 1). The sharp increase in fluorescence always coincided with an increase in cell area which precedes the loss of sphericity due to the flattening onto the coverslip surface and extending pseudopod formations (Fig. 2).

The Effect of Fluid Shear Stress on the Perfusion-Induced Calcium Response

First, the level of adherence of the eosinophil population to fibronectin was tested by perfusing media without pharmacological agents in increasing amounts. This parameter was determined by cataloguing the number of cells in the FOV pre-perfusion, and determining whether they remained visible by the end of the experiment. The majority of eosinophils remained adhered to the coverslip at low (93.8 ± 10.8%) and medium (91.6 ± 12.0%) flow rates; however, approximately 23% of eosinophils were washed away at higher (77.6% ± 20.1%) applied shear stresses (Fig. 3).

We investigated the effect of varying degrees of perfusion on PICR with regards to changes in area expansion (Fig. 4). The area ratio was calculated by dividing the average area of individual eosinophils over the last 30 seconds of the recording by the average area of the cells at rest (see Fig. 1). We noticed that the flattening and subsequent increase in surface area of eosinophils in response to perfusion occurred at all flow rates (low: 1.51 ± 0.13, medium: 1.51 ± 0.14, high: 1.58 ± 0.20), with no statistically significant difference in the peak magnitude of area ratio across our range of shear stresses (Fig. 4A). However, we observed two distinct time-courses for this area ratio change. Although a small proportion of eosinophils increased in surface area gradually over the course of the experiment (Fig. 4B), the majority of eosinophils across our flow rates (low: 73%, medium: 82%, high: 85%) (Fig. 4D) exhibited a sudden increase in area that peaked within 90 seconds and then remained constant for the remainder of the experiment (Fig. 4C). When we took five individual area tracings of eosinophils from the latter subpopulation and superimposed them by their respective peak fluorescent values,

we noted that the calcium flash occurred just prior to the initial increase in cell surface area (Fig. 4E).

Finally, we compared the latency to peak calcium-response and the maximal intensity of the calcium flux at the three ranges of shear stress. For the fluorescence ratio, the peak emission signal was divided by the average fluorescence of baseline eosinophils (see Fig. 1). Similar to area ratio, different flow rates (low: 2.38 ± 0.62, medium: 2.75 ± 0.16, high: 2.60 ± 0.27) had no statistically significant effect on the absolute increase in fluorescence intensity (Fig. 5A). The latency was measured as the time interval between the onset of flow and the maximum fluorescence value of the calcium spike (see Fig. 1). We found that eosinophils subjected to intermediate shear stresses had a significantly shorter mean latency (110 ± 35 sec; $p < 0.01$) relative to cells experiencing lower (202 ± 50 sec) and higher (225 ± 47 sec; $p < 0.001$) flow rates (Fig. 5B). Although we found the range of latencies to be widely varied, the paired data of experiments from a single donor across flow rates consistently show cells with the shortest latency at the medium flow rate (Fig. 5C). Representative individual fluorimetric cell tracings of eosinophils subjected to low (Fig. 5D), medium (Fig. 5E), and high (Fig. 5F) flow rates are shown to demonstrate these significant differences.

Timepoint Localization of Adaptor Proteins Involved in Actin Cytoskeleton Rearrangement

Eosinophils subjected to shear stress of 3.75 dynes/cm^2 were fixed at various time points (30, 60, >120 seconds) following perfusion and double-stained with phalloidin to

detect F-actin and either talin (Fig. 6A), activated β1-integrin subunit (Fig. 6B), or vinculin (Fig. 6C). Individual and merged fluorescent channels are shown to visualize the amount of co-localization of proteins. All images obtained prior to fluid shear stress stimulation show diffuse staining throughout the cell with the exception of F-actin, which showed stronger presence in the periphery of the eosinophil. This marginal localization of F-actin becomes more pronounced after fluid shear stress is applied to the cells, with a clustering in distinct focal dots up to 4 minutes post-perfusion (Fig. 6A, 6B). Similarly to F-actin, the other three proteins of interest also re-distributed towards the outer edges of the eosinophil. There was minimal co-localization between F-actin and the other proteins of interest: the latter migrated to regions of the periphery distinct from those which became enriched with F-actin (this lack of co-localization is emphasized in merged images).

The Effect of Disrupting Calcium and Cytoskeletal Pathway Regulators on the PICR

Preliminary studies were performed to investigate the relationship between the calcium flash and the accompanying PICR-induced cytoskeletal rearrangement by examining the effects of various pharmacological interventions upon the response to a fluid shear stress of 3.75 dynes/cm^2 (Fig. 7).

The eosinophils were both incubated in and perfused with media containing specific drugs: ryanodine (10μM) directly inhibits the release of intracellular stores, U-73122 (10μM) inhibits phospholipase C activity, Y-27632 (10μM) functions as a ROCK inhibitor to prevent actin stress-fibre formation, and CK-666 inhibits the formation of the

Arp2/3 complex responsible for filopodia and lamellipodia formation. Relative to the aforementioned PICR-characterizing experiments, we noticed high variance of eosinophil responses within a single experiment; therefore, rather than taking the average response as we had done previously, individual cellular responses were analyzed to represent this discrepancy. With respect to area ratio, only CK-666 was found to markedly abrogate changes in morphology (Fig. 7A; $p < 0.0067$), specifically flattening and corresponding increase in surface area. However, ryanodine, CK-666, and U-73122 were both found to significantly supress intracellular calcium release, indicated by the lower fluorescence ratio compared to the control group (Fig. 7B; $p < 0.05$).

Discussion

The specific mechanisms of eosinophil extravasation out of the vasculature into inflamed lung tissue have yet to be fully elucidated. A strong positive correlation exists between the degree of eosinophilia and the severity of respiratory disease including asthma; however, treatments targeting eosinophil accumulation in disease models haven't shown promising levels of success, which may owe to a poor understanding of cellular processes at the molecular level[6]. To our knowledge, no study has attempted to link mechanical stimuli to the mobilization of human eosinophils and their cytosolic calcium fluctuations. In this study, we examined the temporality of changes in eosinophil morphology and the directionality of this relationship to changes in $[Ca^{2+}]_i$ upon the induction of fluid shear stress via perfusion.

We have enhanced our experimental set up since first describing the flow-induced calcium response[17]: a modified recording chamber allowed us to more carefully control and assess perfusion rates, and in turn, magnitudes of fluid shear stress. According to the Hagen-Poiseuille equation, fluid shear stress is directly proportional to flow rate and inversely proportional to the vessel diameter. There are various estimations of biologically pertinent shear stresses at various points in the circulation, with the many fluid shear stress studies examining values between 1-10 dynes/cm^2.

Although we had initially sought to coat our coverslips with endothelial cells for physiological relevance, we were concerned with confounding variables: (1) the endothelial cells may uptake residual calcium dye that would ultimately interfere with our fluorescence signal as they also respond to shear stress[22] and (2) stimulating the

endothelial cells with cytokines to express VCAM-1 and mucosal addressin cell adhesion molecule-1 (MAdCAM-1) for binding may have unintended effects on the eosinophils. We eventually utilized fibronectin as a suitable substitute, as the $\alpha_4\beta_1$ integrin on the eosinophil surface responsible for binding to VCAM-1 and MAdCAM-1 also binds to this ECM protein[23].

Cells moving within the blood may experience, upon transient adherence to the vessel wall via selectin binding, an increase in fluid shear stress. We propose that changes in shear stress provide an additional signal to the cell to extravasate, reinforcing or synergizing with other signals that precede extravasation such as chemokine presence. Although we observed that eosinophils will eventually respond in an all-or-none fashion with respect with surface area increase (Fig. 3A) and degree of $[Ca^{2+}]_i$ mobilization (Fig. 5A) upon persistent stimulation *in vitro*, we found statistical difference in the time to flash at relevant post-capillary shear stress intensities compared to lower and higher stress levels (Fig. 5B). We surmise that *in vivo*, this quicker response time allows the activation of signaling pathways that align with the cell's biological imperative to ensure appropriate spatial and temporal conditions for extravasation (e.g. shear stress magnitude, engagement of integrin subunits to appropriate counter ligands, mobilization of internal proteins to necessary locations), whereas cells experiencing the 'wrong' levels of shear stress would dissociate from their temporary bonds to the endothelial cell wall. This is of particular importance from a health/disease standpoint, as it would be undesirable for eosinophils to extravasate out of the circulation without proper regulatory triggers,

potentially resulting in unwarranted degranulation-mediated inflammation of peripheral tissues.

The general concept of cellular migration could be depicted as a cyclic process, in which a cell responds to a migration-promoting signal by extending protrusions to explore their nearby environment[24]. These extensions are conventionally lamellipodia and/or filopodia, both of which are driven by actin polymerization and are stabilized by the adhesion of integrin receptors to the endothelium or ECM[24]²⁴. The transmembrane integrin receptors are linked to the actin cytoskeleton via intracellular adaptor proteins such as talin and vinculin[25,26]. Although the nature of the adhesion points will vary from cell to cell, in invasive cells such as eosinophils, podosomes are thought to be the primary type of contact point formed[27,28] as opposed to the extensively studied focal adhesion complexes[28]. The nascent formation of podosomes requires rapid turnover of actin filaments to palpate their environment[29,30] and mature into contact points that serve as a motor for cell motility[31], whereas focal adhesions require a longer period of time to form[32]. Unfortunately, the inherent difficulty in tracking protein movements in eosinophils results in uncertainty in confirmation of structural form[27]. However, our immunofluorescent images clearly indicate a rapid mobilization of F-actin and relevant proteins (talin, vinculin, activated β1-integrin subunit) to the cell periphery within 4 minutes of shear stress stimulation (Fig. 6), thus supporting the idea that the primary actin structure responsible for migration in eosinophils are podosomes.

The adhesion contact points are responsible for relaying intra- and extracellular tension into signaling pathways that modify their own behavior, thereby directly

influencing the migratory and contractile state of the cell[15]. For example, they also serve as traction sites, allowing for the migrating cell to move over them while simultaneously being disassembled at the rear of the cell[24]. This phenomenon may explain why our adaptor proteins and integrin subunits seemingly prefer the cell periphery opposite to our phalloidin stains (Fig. 6). We propose that the phalloidin is binding to the copious amounts of actin being polymerized at the leading edge of the cell where lamellipodia and filopodia protrusions occur, whereas our antibodies are binding to their respective adaptor proteins (Table 1) as the protein complexes are being taken apart at the tail-end of the eosinophil.

Altogether, our previous study showed that peak adherence (which is dependent, in part, upon activation of integrins) coincided with the calcium spike[17]. Adherence would produce a sudden increase in shear stress experienced by the cell, and has also been shown to lead to rolling of the cell along the vascular wall for some short distance before flattening and extravasation occur. The current study further corroborates the relationship by illuminating the presence of a temporal link between the calcium spike and flattening of the cell (Fig. 4E). We wanted to explore the directionality of the relationship between these two cellular responses by observing changes in the PICR in eosinophils treated with pathway-disrupting drugs. We noted that disruptors of the calcium pathway such as ryanodine and U-71322 had no effect on the cells' ability to flatten on the fibronectin-surface, whereas the ROCK inhibitor Y-27632 did not seem to significantly affect either the calcium or the actin regulation pathways. However, the chemical CK-666 which prevents Arp2/3 complex formation necessary in filopodia and lamellipodia formation

was shown to significantly decrease the area ratio in treated eosinophils while dramatically decreasing the degree to which calcium is released from intracellular stores.

The CK-666 data suggests that despite the actin cytoskeleton polymerizing via a multitude of distinct pathways, the Cdc42-WASP-Arp2/3 pathway may be dependent on the presence of cytoplasmic calcium whereas the RhoA-ROCK-MLC pathway may be activated independently of the calcium pathway[25]. In fact, the dynamic nature of granulocytes relies on rapid turnover (<5 min) of actin filaments in filopodia and lamellipodia formation to explore their immediate vicinity prior to drastic morphological changes preceding migration from the vasculature. Therefore, we surmise that the transiency of the calcium flash serves as a checkpoint for Arp2/3 formation; indeed, protein crystallography has shown an association of calcium ions to the Arp2/3 complex[33], and some studies have indicated a reliance of Arp2/3 subunits to calcium-bound calmodulin for strengthened function[34].

Despite the limited published literature on the role of calcium in eosinophil migration, the importance of calcium as a regulator of migration in other cell types such as metastatic cancer cells and fibroblasts has been well documented[35]. For example, migrating cells exhibit a gradient of calcium that is thought to be critical for calpain-mediated cleavage of adhesion protein complexes at the rear of the cell[35,36]. The recognised relationship between calcium release and migration[37,38] provides confidence regarding the pivotal role calcium plays in regulating eosinophil migration as well. However, further studies should be performed to confirm the nature of the relationships

between fluid shear stress, intracellular calcium release, and podosome formation in eosinophils.

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Author Contributions: KS, RS, and LJ designed the study. KS and MS conducted experiments. KS prepared the first draft of the manuscript. RS and LJ supervised the data interpretation and contributed to manuscript development. KS takes overall guarantee for the paper.

Conflict-of-Interest Disclosure: None of the authors have any conflict of interest pertaining to this work.

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Table Legends:

Table 1. List of antibodies and dyes. All reagents used for immunofluorescence staining and their respective sources and reactivities are outlined above.

Figure Legends:

Figure 1. Representative primary fluorimetric and area tracings of an individual eosinophil before and during onset of perfusion at a shear-stress of 3.75 dynes/cm^2. The fluorimetric tracing (green) shows an elevation of $[Ca^{2+}]_i$ in response to the onset of perfusion (blue line), peaking in fluorescence emission (purple circle) almost simultaneously with a ~1.5x fold increase in cell surface area (red).

Figure 2. Microscopy images before and during onset of perfusion at a shear-stress of 3.75 dynes/cm^2. The brightfield (top) and fluorescence (bottom) microscopy images of live (not fixed) eosinophils at baseline (left), at approximate peak fluorescence post-perfusion (middle), and clear pseudopod formation (right).

Figure 3. Percentage of eosinophils that remain adhered in the field of view (FOV) post-perfusion of media at increasing magnitudes of fluid shear stress. The number of observed cells were counted pre- and post-application of media at low (n = 11), medium (n = 14), and high (n = 11) flow rates. The percentage was calculated by determining the proportion of cells that remain visible for the duration of the experiment. One-way ANOVA data presented as mean ± SD, Kruskal-Wallis test.

Figure 4. Characterizing changes in cell area in response to fluid shear stress. The extent of increase in surface area of eosinophils from a top-down microscopy view does not vary across different flow rates (low: $n = 7$, medium: $n = 7$, high: $n = 4$) (A). Some cells (n = 5 shown) present with a nonphasic, gradual increase in area post-perfusion (B, 3.75 dynes/cm^2 shear stress), while others ($n = 5$ shown) exhibit a biphasic nature, with a dramatic increase in cell surface area (C, 3.75 dynes/cm^2 shear stress) that corresponds with the calcium spike denoted by the asterisks. The blue line at 60 seconds represents the onset of perfusion. (D) The proportion of eosinophils at various flow rates (low: $n = 26$, medium: $n = 45$, high: $n = 20$) that exhibit a gradual increase in area (orange) versus a biphasic, dramatic increase (blue) (E) Area tracings of seven individual eosinophils were synchronized by their calcium flash denoted by the dotted line. Each of the cells exhibited dramatic biphasic increase in area ratio coinciding within 90 seconds of the peak emission signal. One-way ANOVA data presented as mean ± SD, Kruskal-Wallis test.

Figure 5. Characterizing changes in $[Ca^{2+}]_i$ release in response to fluid shear stress. Although the magnitude of the calcium flash remains similar for all shear stresses (low: $n = 8$, medium: $n = 6$, high: $n = 6$, $P = 0.1849$) (A), the latency to peak emission differs greatly for different perfusion rates (low: $n = 8$, medium: $n = 12$, high: $n = 9$, $P < 0.0002$) (B). Although the range of activation latency is seemingly large, paired analysis of the various flow rates for eosinophils isolated from a single donor still shows a shorter latency for the medium flow rate (C). Fluorimetric tracings of five individual cells for

each perfusion rate (low: D, medium: E, high: F) are shown for comparison. The blue line at 60 seconds represents the onset of perfusion. One-way ANOVA data presented as mean ± SD, Kruskal-Wallis test.

Figure 6. Localization of F-actin and various adhesion proteins at specific timepoints post-perfusion at 3.75 dynes/cm^2. (A) Talin (B) β1-Integrin Subunit (C) Vinculin

Figure 7. Characterizing changes in area and $[Ca^{2+}]_i$ release in response to pharmacological agents. Eosinophils were subjected to the perfusion of various drugs targeting either the mobilization of the actin cytoskeleton (A) (Untreated: 1.53 ± 0.14 vs. CK-666: 1.10 ± 0.06, $P < 0.05$) or the calcium signaling pathway (B) (Untreated: 2.75 ± 0.16 vs Ryanodine: 1.90 ± 1.21, U-73122: 0.80 ± 0.12, CK-666: 0.86 ± 0.24, $P < 0.05$) at fluid shear stresses of 3.75 dynes/cm^2. One-way ANOVA data presented as mean ± SD, Kruskal-Wallis test.

Table 1

Primary Antibody	**Species**	**Source**	**Dilution**
Activated β1 Integrin	Mouse	Millipore (MAB2079Z)	1:100
Talin 1 & 2	Mouse	Abcam (ab11188)	1:100
Vinculin	Rabbit	Abcam (ab129002)	1:100
Secondary Antibody	**Wavelength**	**Source**	**Dilution**
Donkey anti-mouse	488	Abcam (ab150105)	1:500
Goat anti-rabbit	488	Abcam (ab150077)	1:500
Probe	**Conjugation**	**Source**	**Dilution**
Phalloidin (F-actin)	Texas Red®-X	Thermo Fischer (T7471)	As Per Manufacturer

Figure 1. Son et al., 2020

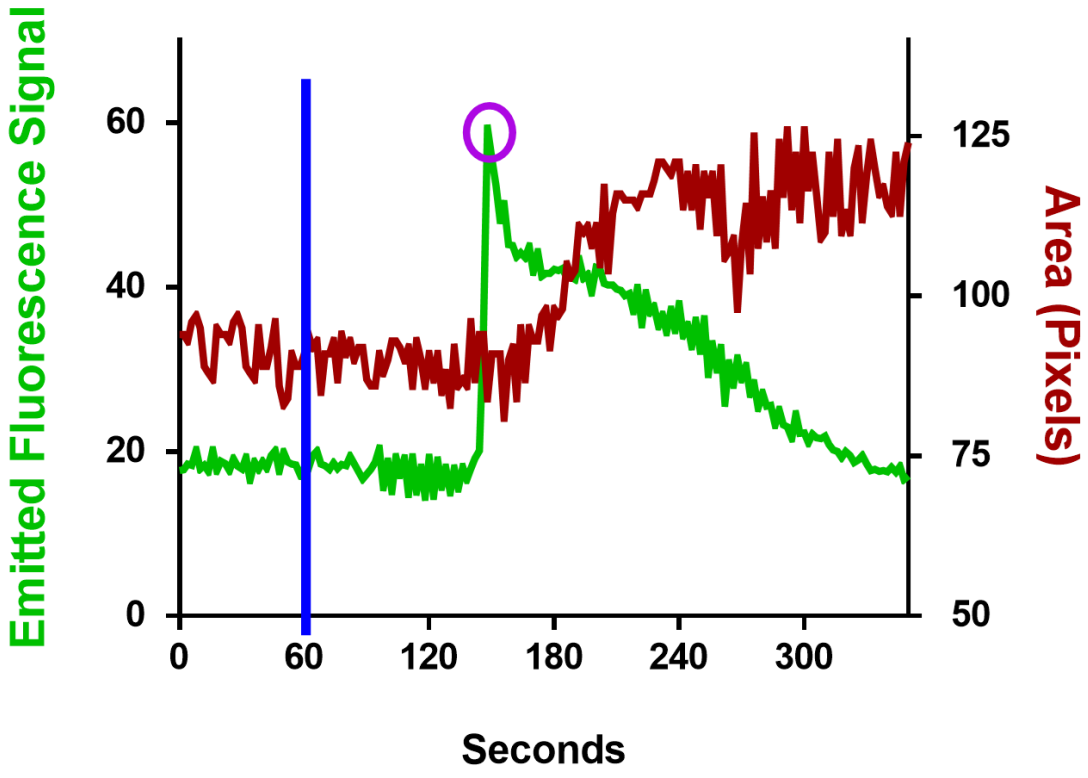


Figure 2. Son et al., 2020

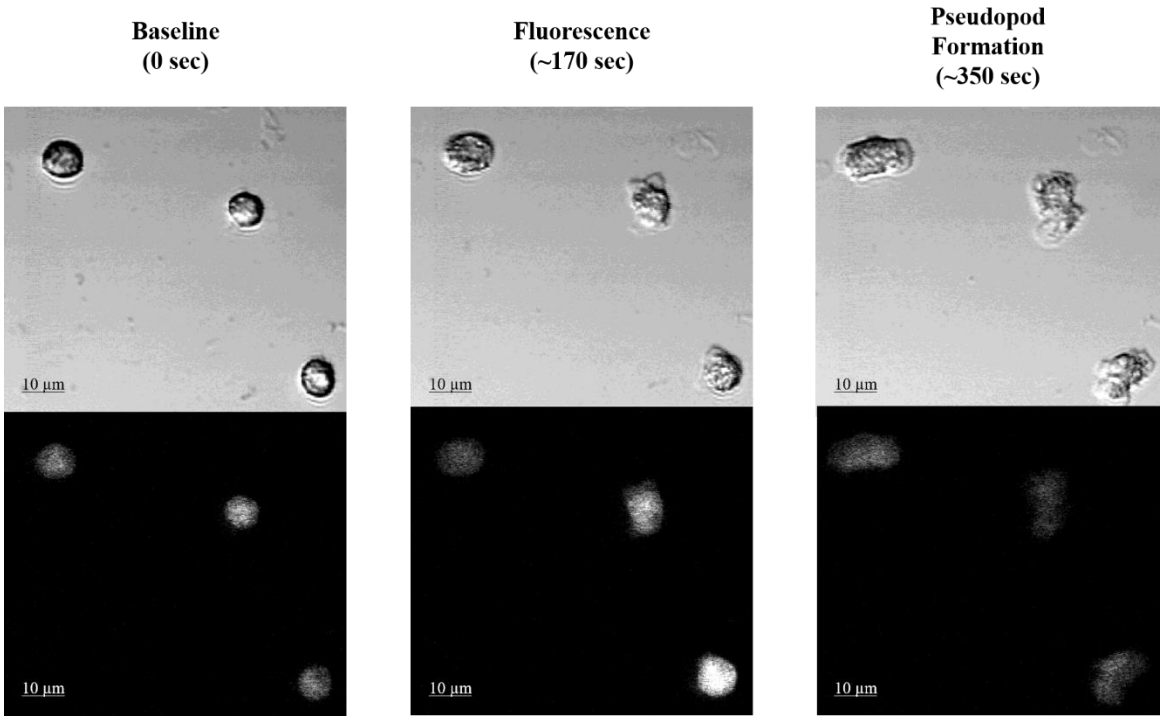


Figure 3. Son et al., 2020

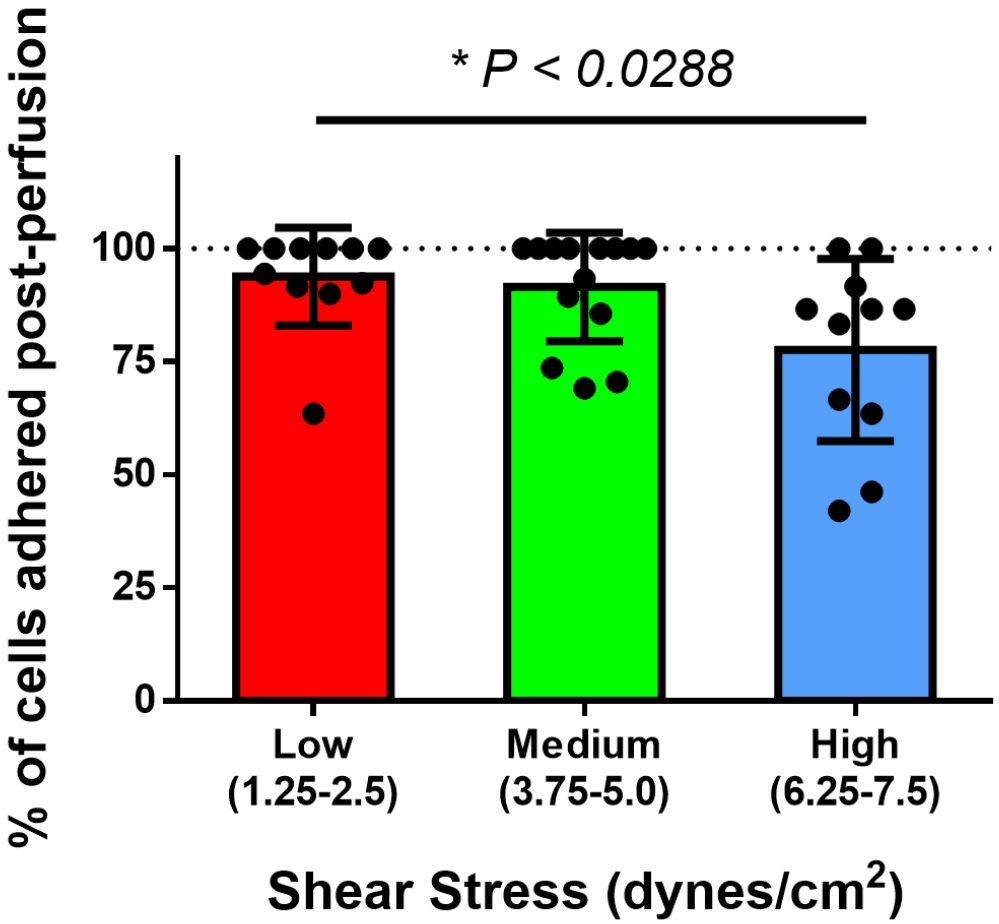


Figure 4. Son et al., 2020

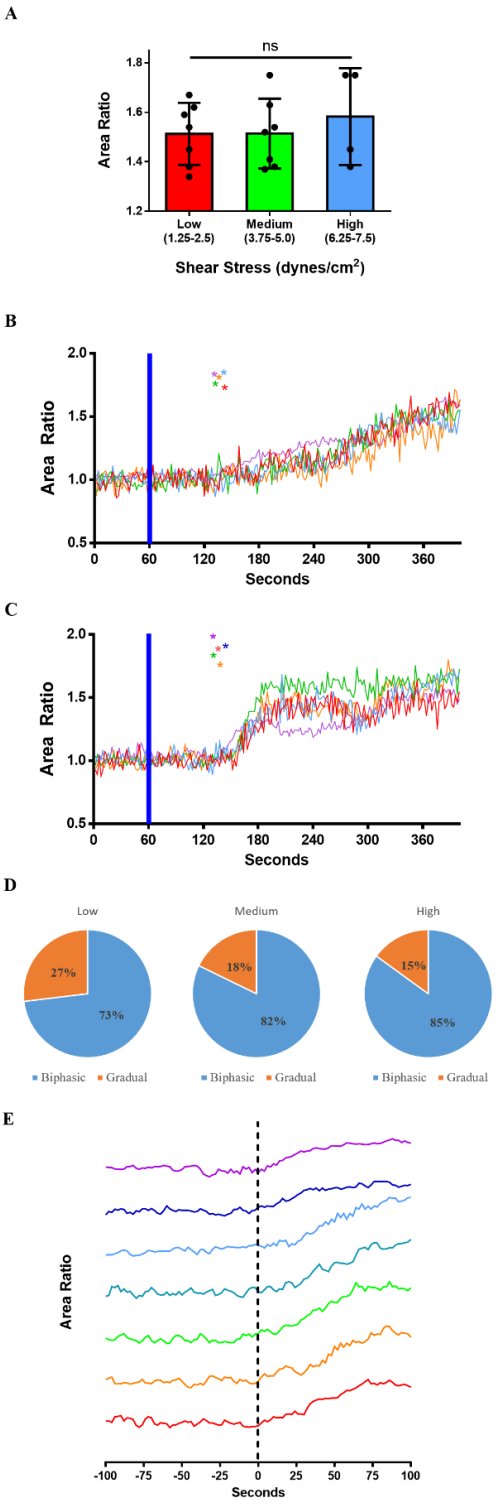


Figure 5. Son et al., 2020

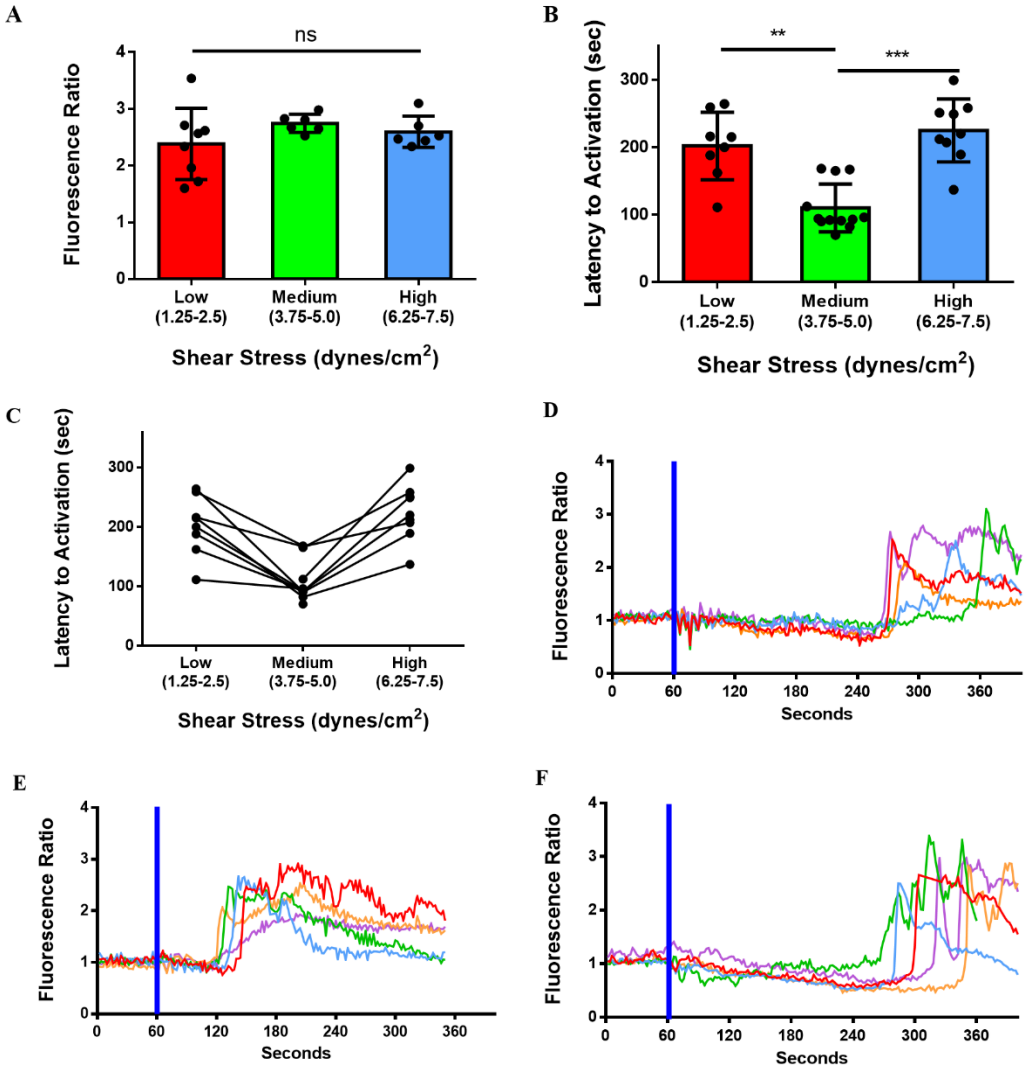


Figure 6. Son et al., 2020

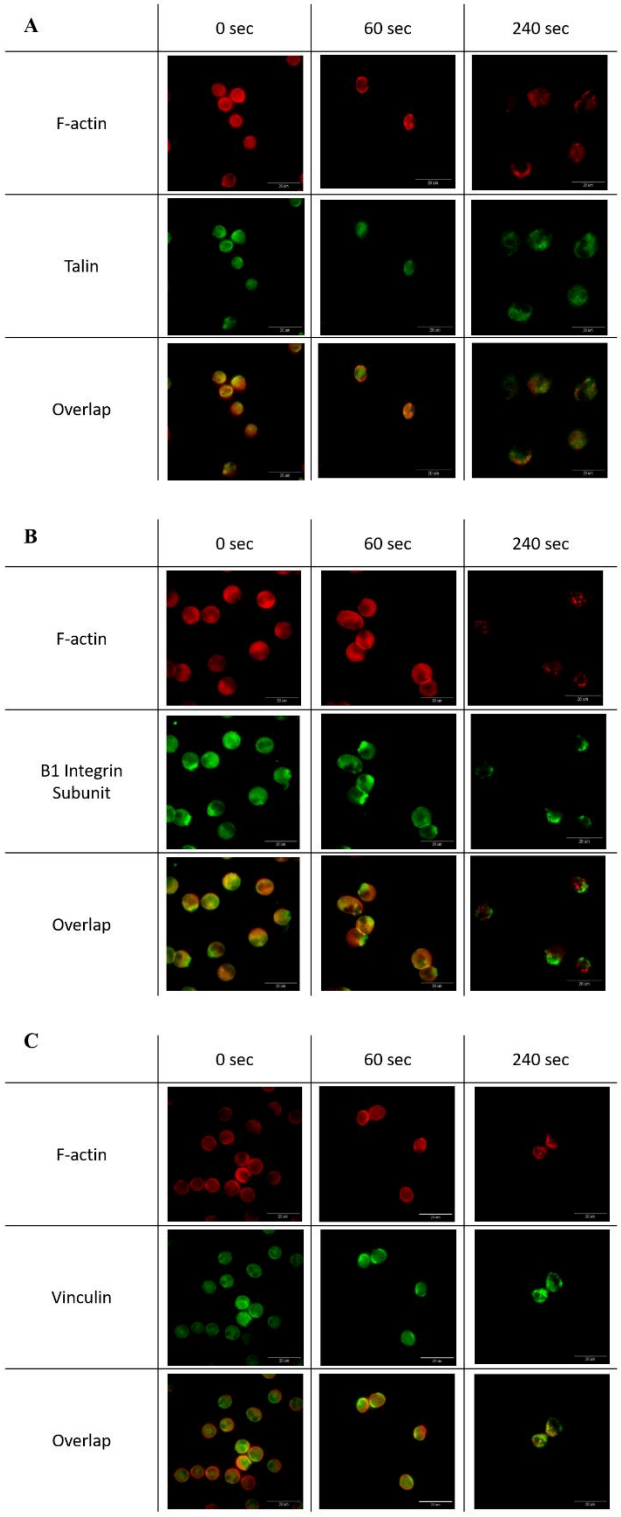
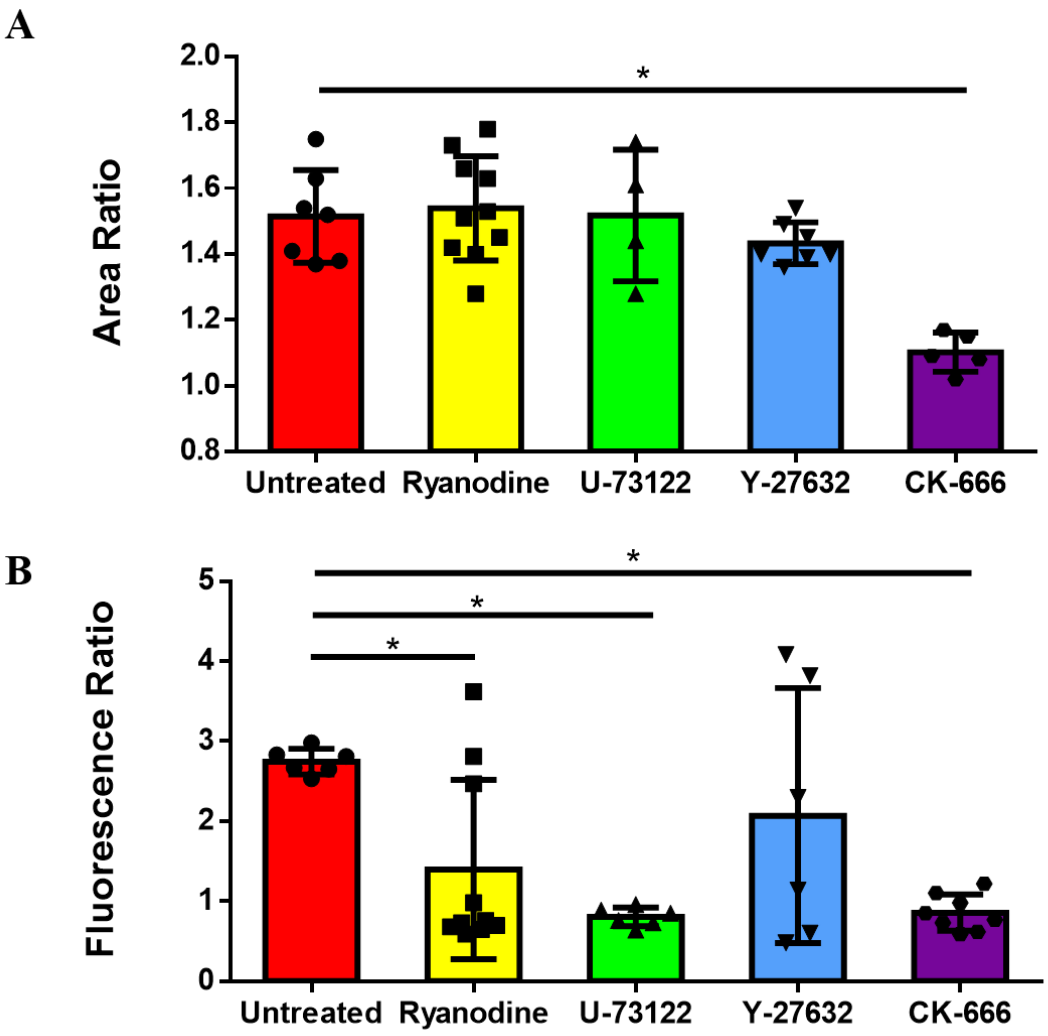
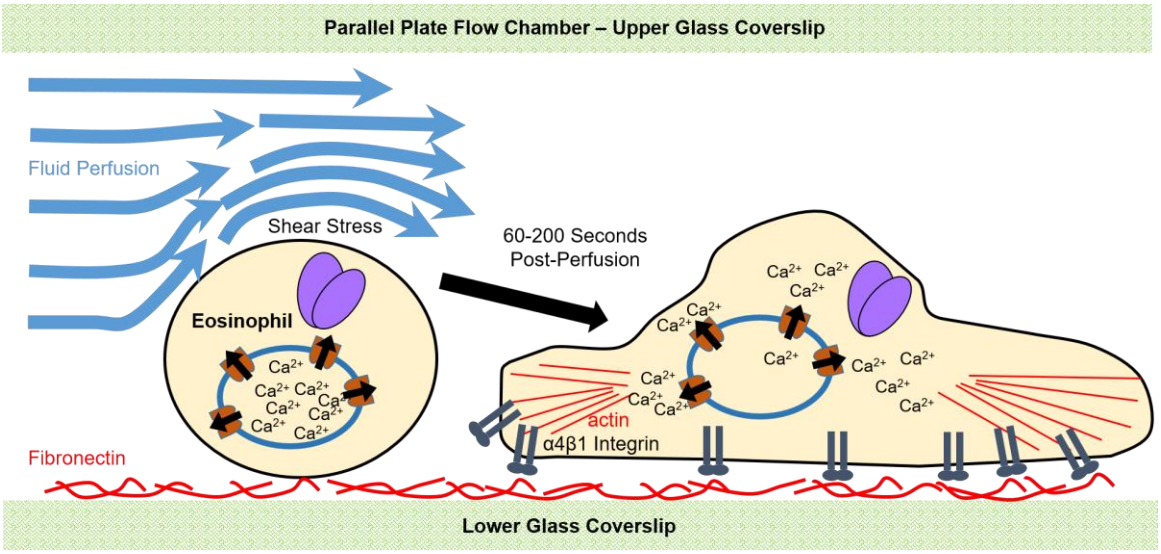


Figure 7. Son et al., 2020



Graphical Abstract. Son et al., 2020



Appendix A

The determination in Figure 4D of whether the individual eosinophil exhibited a gradual vs biphasic area increase was performed by calculating the first integral of the cell area tracing. A linear slope through the duration of the experiment was indicative of a gradual increase, whereas observing a sharp peak within the first integral graph suggested a biphasic area increase.

Chapter 5: Publication to *Cells: Special Issue* – Eosinophils Beyond IL-5, 2021

The cycling of intracellular calcium released in response to fluid shear stress is critical for migration-associated actin reorganization in eosinophils

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Abstract:

The magnitude of eosinophil mobilization into respiratory tissues drives the severity of inflammation in several airway diseases. In classical models of leukocyte extravasation, surface integrins undergo conformational switches to high-affinity states via chemokine binding activation. Recently, we learned that eosinophil integrins possess mechanosensitive properties that detect fluid shear stress, which alone was sufficient to induce activation. This mechanical stimulus triggered intracellular calcium release and hallmark migration-associated cytoskeletal reorganization including flattening for increased cell-substratum contact area and pseudopodia formation. The present study utilized confocal fluorescence microscopy to investigate the effects of pharmacological inhibitors to calcium signaling and actin polymerization pathways on shear stress-induced migration in vitro. Morphological changes (cell elongation, membrane protrusions) succeeded the calcium flux in untreated eosinophils within 2 minutes, suggesting that calcium signaling was upstream of actin cytoskeleton rearrangement. The inhibition of ryanodine receptors and endomembrane Ca^{2+}-ATPases corroborated this idea, indicated by a significant increase in time between the calcium spike and actin polymerization. The impact of the temporal link is evident as the capacity of treated eosinophils to move across fibronectin-coated surfaces was significantly hampered relative to untreated eosinophils. Furthermore, we determined that the nature of cellular motility in response to fluid shear stress was nondirectional.

Keywords: actin, calcium signaling, confocal microscopy, integrin, mechanosensing, membrane ruffles, pseudopodia, shear stress

1. Introduction

Eosinophils are a subset of granulocytes that play a vital role in the inflammatory processes associated with a number of airway diseases including asthma [1], eosinophilic granulomatosis with polyangiitis (EGPA) [2], and hypereosinophilic syndrome (HES) [3]. As the positive correlation between eosinophil accumulation in pulmonary tissues and disease severity has been ascertained [4], the past decade has seen the development of several biologics that target interleukin-5 (IL-5), a TH2 cytokine largely responsible for the mobilization, recruitment, and survival of eosinophils at sites of inflammation [5]. Although some of these anti-IL-5 clinical trials have reported effective reduction and/or depletion of eosinophils from the blood and airways of severe asthmatic patients [5,6], it has become a point of interest to investigate other factors such as epithelial cell-derived cytokines (IL-25, TSLP) and IL-13-secreting type 2 innate lymphoid cells (ILC2) that may also contribute significantly to eosinophil migration [5,7-8]. It is vital to untangle the series of signaling events that lead to specific actin cytoskeleton reorganization which allow for (1) cellular adhesion to the endothelium and (2) transmigration through the extracellular matrix into the surrounding tissues by utilizing membrane protrusions such as pseudopodia.

In the current model of eosinophil extravasation, inactive integrins expressed on the surface of circulating eosinophils will undergo a conformational change to a highly active state upon exposure to chemokines (e.g. eotaxin) near the bronchial vascular endothelium [9,10]. In particular, the very late antigen-4 (VLA-4, $\alpha 4\beta 1$) integrin on the eosinophilic cell surface will bind to the vascular cell adhesion molecule-1 (VCAM-1) counter ligand that is upregulated in the asthmatic lung [10–12]. Furthermore, *in vivo* pre-activation and/or

priming of the cells mediated by increased IL-5 levels in asthmatics result in intermediate-activated integrin conformations displayed on the surface [9], resulting in augmented integrin activation, extravasation from circulation, and an overaccumulation of eosinophil populations in respiratory tissues oftentimes leading to subsequent airway inflammation and obstruction.

Our previous study identified a novel phenomenon in eosinophils which we coined the perfusion-induced calcium response (PICR), in which the perfusion of media without any pharmacological agents over adhered eosinophils from peripheral blood elicited an integrin-mediated release of intracellular calcium $[Ca^{2+}]_i$ from internal stores. The calcium flux was observed concurrently with changes in eosinophil morphology (flattening, membrane protrusions) [11] suggesting that: (1) the signaling pathways involved in actin skeleton restructuring may be regulated by $[Ca^{2+}]_i$ mobilization, and (2) mechanical stimuli of fluid shear stress detected by integrin mechanosensors on the eosinophil surface may play a pivotal role in eosinophil migration from the vasculature. Indeed, eosinophils in vivo would experience varying magnitudes of shear stress corresponding to their strength of adhesion to the vascular endothelium, in turn triggering the PICR under optimal environmental conditions. In a subsequent study, we observed in response to physiologically-relevant fluid shear stress levels, a temporal link between $[Ca^{2+}]_i$ release and increased cell surface contact area with the fibronectin-coated substratum via cell flattening [14]. Unfortunately at the time, we were neither able to clearly elucidate the correlated/causative nature nor the directionality of this relationship in eosinophils. The present study utilizes improved real-time confocal microscopy techniques and refined

analytic methods to investigate the effects of pharmacological inhibitors to calcium and actin cytoskeleton signaling pathways on the PICR in eosinophils. Improving upon the limited knowledge regarding eosinophil trafficking processes may assist in the future development of novel treatments and therapies of eosinophilia-driven pathologies such as asthma.

2. Materials and Methods

Ethics Statement

Healthy adult volunteers (n = 8) were recruited with written consent to participate in this study. The institutional ethics committee (Hamilton Integrated Research Ethics Board; HIREB) granted ethical approval to obtain blood samples from participants (McMaster University REB Project #12-3764). The inclusion criteria for donors were as follows: no history of asthma, not on any type of medication, and no illness prior to blood donation.

Eosinophil Isolation

The MACSxpress Eosinophil Isolation Kit (catalogue #: 130-104-446; Miltenyi Biotec Inc., Bergisch Gladbach, Germany) was utilized to isolate eosinophils by negative selection from whole blood draws as previously outlined in detail per Son et al. [15]. Briefly, the kit contains a cocktail of magnetically labelled antibodies conjugated to various surface markers of non-eosinophilic cells. This solution is mixed with 30 mL of peripheral blood samples collected into EDTA anticoagulant vacutainers (BD Bioscience, MD, USA), and incubated for 15 minutes within a specialized magnet to allow for segregation. Residual erythrocytes in the enriched population were lysed with 9 mL of sterile endotoxin-free water for 20 sec (hypotonic lysis) and topped off with 1 mL of 10X HBSS media without calcium and magnesium (Invitrogen, CA, USA). The isolated eosinophil populations (purity: 95.0 ± 1.7%; viability: 95.3 ± 4.4%) [15] were resuspended in RPMI1640 (0.42 mM calcium) at 10^6 cells/mL and kept at 4°C for a minimum of 2 hours to allow pre-

activated or primed eosinophils to return to an unstimulated, baseline state. Experiments were run within 12 hours of isolation to avoid defective cell function due to viability loss over time.

Confocal Fluorescence Microscopy

Glass coverslips (40 mm diameter, 1.5 mm thick; Warner Instruments, CT, USA) were cleaned in 55°C 1M HCl overnight then washed twice in distilled water and twice more in double distilled water for 10 minutes each. Afterwards, the coverslips were rinsed with 100% ethanol and allowed to dry before storing them in a clean container for future use. The fibronectin solution (Sigma Aldrich, ON, Canada) was prepared at 2 µg/mL in PBS, of which 1 mL was pipetted onto an aforementioned acid-washed coverslip resulting in an approximate 4 µg/cm^2 coating when dried overnight.

A low-profile parallel plate flow chamber (RC-31; Harvard Apparatus, MA, USA) was utilized as the experimental apparatus for confocal imaging. The bath chamber could be disassembled into top and bottom halves and screwed back together whilst sandwiching a silicon gasket with a vertical height of 125 µm to represent the side walls. Eosinophils transferred onto the fibronectin-coated coverslip would be placed securely on top of the bottom half of the chamber prior to assembly. The perfusate was introduced through a peristaltic pump (PS-200; Living Systems Instruments, VT, USA) at a perfusion rate inducing fluid shear stress at 3.75 dynes/cm^2, which was within the physiologically relevant range of shear stress experienced by eosinophils migrating from post-capillary venules.

300 µL of the resuspended eosinophil population was loaded with 3 µl of the calcium binding dye, Fluo-3 AM (3.5 µM in DMSO w/ 0.01% pluronic acid), and incubated in an opaque 1.5 mL tube for 20 minutes prior to transferring onto the fibronectin-coated coverslips for an additional 40 minute incubation in the dark. Where applicable, the eosinophils were incubated for 10 minutes with various pharmacological agents before loading with Fluo-3 AM. To interfere with the calcium signaling pathways, we treated eosinophils with either ryanodine (10 µM) or cyclopiazonic acid (CPA) (10 µM). The inhibition of actin cytoskeletal reorganization was analyzed with compounds CK-666 (10 µM) and Y-27632 (10 µM). All inhibitor concentrations were previously optimized, and the experiments were conducted at room temperature. The experiment recordings generally took 10 minutes to complete and were digitized as outlined per Ahmadzai et al. [13].

Signal Processing and Statistical Analysis

The untreated and treated eosinophil recordings were processed as outlined per Son et al.[14]. Briefly, the QuimP plug-in (University of Warwick, UK) for the open platform software ImageJ (National Institute of Health, MD, USA) was utilized for .tiff file analysis. The QuimP module allowed images to be transformed into binary black-or-white images by thresholding greyscale pixels to differentiate regions of interest (ROIs) from baseline background noise. The ROIs could be tracked over the course of the experiment to quantify parameters of interest for both morphology (area ratio, adhesion) and fluorescence (intensity and latency of calcium release).

A couple noteworthy improvements have been implemented for the current study. Firstly, a higher magnification was employed for the field of view (FOV), effectively observing fewer cells but obtaining more precise and accurate measurements of our parameters of interest. As a direct consequence, we were able to expand our outcome variables to include cell elongation, displacement, membrane ruffling, and pseudopodia formation. Furthermore, the higher magnification allowed for the detection for multiple calcium spikes in some eosinophils, a markedly absent feature in our earlier studies. Secondly, we increased the number of images averaged per frame from 10 to 15 to reduce background noise and reduced the frame rate from 2 to 1.5 seconds per frame to better resolve fleeting changes in cell morphology and fluorescence.

In our previous study [14], area and fluorescence ratios were obtained by averaging values from multiple cells isolated from a given subject on a particular day. However, we noted significant day-to-day variance in certain outcome variables for our untreated control group: not just between different blood donors, but also in eosinophils from a given donor on different days. Therefore, to minimize this variance, outcome measurements for all treatment groups were normalized to the average value of the untreated control group of the corresponding donor and day. For example, in quantifying cell flattening in response to shear stress (calculated by dividing the average area of individual cells over the final 60 seconds of the recording by the average area of the cell at baseline), the control cells on a given day might exhibit a 53% increase in area, while cells of a given treatment group might exhibit a 38% increase in area: we therefore recorded a normalized area change in that treatment group on that day of 38/53. This was done for all replicates of treatment

groups studied on various days and report here the mean normalized area change (+/- SD). The same was done in quantifying the fluorometric response (calculated by dividing the peak of the transient spike-elevation by the average of the baseline fluorescence before onset of perfusion). However, we were unable to run all four of our different treatment conditions every single experiment day, due to the low eosinophil population count isolated from healthy donors (~1.5 million cells per 25-30 mL venous blood). As a result, we ran the untreated, control eosinophil group more often ($n = 6$) than the treatment groups ($n = 4$ for each), with approximately 2-5 cells analyzed per experiment.

Graphpad Prism 6 (La Jolla, CA, USA) was used to conduct all statistical analyses and generate figures. Statistical analyses involved Kruskal-Wallis tests to compare means of two or more groups, followed by Dunn's multiple comparisons post hoc test. Fischer's exact test was used to compare event frequencies (e.g. membrane ruffling, pseudopodia) of treated cells against the untreated control group. In all cases, $p < 0.05$ was considered to be significant.

3. Results

3.1. Pharmacological Agents

A total of four pharmacological inhibitors were utilized to disrupt the calcium signaling and actin regulatory pathways. Cyclopiazonic acid (CPA) is a specific inhibitor of sarco(endo)plasmic reticulum Ca^{2+}-ATPases, which are responsible for transporting cytoplasmic calcium ions back into the ER. Ryanodine binds to ryanodine receptors (RyRs), a family of intracellular calcium release channel proteins, keeping them in closed conformations at micromolar concentrations. With respect to actin cytoskeleton rearrangement, the small molecule CK-666 functions as an allosteric antagonist to the Arp2/3 protein complex, which is well known player in actin nucleation. Lastly, the compound Y-27632 binds to the catalytic site of ROCK kinases, which are critical for the actin stabilization and the formation of stress fibres.

3.2. Normalizing Treatment Groups to the Control for Area Ratio

As previously mentioned, the area ratio was calculated by dividing the average area for each individual cell over the final 60 seconds of the recorded experiment by the average cell area prior to perfusion. Using the refinements to our imaging and data analysis described in Methods (above), we first confirmed the inhibitory effect of CK-666 upon cytoskeletal reorganization that we noted previously, contrasted against the lack of effect of disrupting Ca^{2+}-handling using CPA or ryanodine (Figure 1). The Rho-kinase inhibitor Y-27632 also showed a trend toward decreasing the cell flattening, although this did not reach statistical significance.

3.3. Characterizing the Effect of Pharmacological Agents on Shear Stress-Induced Calcium Release

We first investigated whether the inhibitor treatments impacted the occurrence of an intracellular calcium response to shear stress, which is regularly observed in untreated eosinophils. All eosinophils in the control group exhibited the PICR as expected, evident by a peak fluorescence emission occurring approximately 2 minutes post-perfusion, whereas a few eosinophils per treatment group did not; the difference was only statistically significant in the case of Y-27632 (Figure 2A).

In those cells which did show a fluorescent response, the magnitude still represented a dramatic elevation over baseline: previously, we have shown in control cells that this comprises a 2- to 3-fold increase over baseline over the course of a few seconds, followed by a return to baseline over the course of one to two minutes [13,14]. Nonetheless, there was a small reduction in the magnitude of this still very sharp calcium signal, as reflected in mean values of control-normalized fluorescence responses of less than unity (Figure 2B); the reduction was only statistically significant in the case of the two inhibitors of calcium signaling (CPA and ryanodine). More importantly, however, we observed that the time taken for the eosinophil to display this calcium flash was nearly doubled in the ryanodine-treated cells ($p < 0.05$ compared to control; Figure 2C). The time to calcium flash was measured by determining the number of frames post-perfusion taken to reach peak fluorescence emission, and converting the value into seconds (1 frame = 1.5 seconds). Latency was not statistically changed in the other treatment groups.

3.4. Investigating the Effect of Fluid Shear Stress on Loss of Cell Circularity

We have previously shown how the eosinophils, which are normally spherical in shape at rest, respond to a variety of excitatory stimuli including eotaxin and shear stress by rapidly flattening and extending cell membrane protrusions in various directions [13,14]; this morphological change takes place over the course of two or three minutes, thus representing the earliest stage of migration and chemotaxis which have been studied in detail elsewhere [17,18]. We used the QuimP analysis module of ImageJ to quantify this morphological change: this module superimposes a best-fitting ellipse on top of the cellular ROI to track changes in the ellipse's major and minor axes. In order to ascertain whether the application of pharmacological agents influenced this response, we measured cell elongation by dividing the length of the major axis of the cell by the length of the minor axis, which were both tracked over the duration of the experiment. The highest value in this ratio (which represents peak elongation) was not statistically significant different across treatment groups (Figure 3A).

In contrast, certain of the inhibitors tested here markedly changed the time-course of this morphological response to shear stress, as reflected in an increased latency between peak calcium-response and peak elongation (Figure 3B). This increase was highly statistically significant in the case of CK-666 and CPA, but not for Y-27632 or ryanodine (although there was a trend toward an increase in the latter group).

3.5. Pseudopodia Formation and Membrane Ruffling

We observed two distinct morphological features of untreated eosinophils subjected to perfusion-induced shear stress: (1) pseudopodia, in which the membrane morphs non-circularly and extends, often preceding preliminary cell movement in the matching direction (Figure 4A), and (2) membrane ruffling, during which the cell membrane remains circular and intact, with small actin protrusions observed just beyond the membrane (Figure 4B). We noted that the application of our inhibitors impacted the formation of these morphological features. To examine the affect of our agents, we counted whether pseudopodia (Figure 4C) and membrane ruffling (Figure 4D) were formed for individual cells in each treatment group and compared the frequency distribution against the control group. The treatment of Y-27632 ($p < 0.01$) impeded pseudopodia formation (Figure 4C), whereas CK-666 ($p < 0.05$) and ryanodine ($p < 0.01$) impacted the formation of membrane ruffling (Figure 4D).

3.6. Directionality of Eosinophil Motility

Although the duration of these experiments is too brief to document bone fide migration, we observed that some cells made initial movements across the surface of the fibronectin-coated coverslip throughout the course of the 10 minutes post-perfusion. To investigate the effect of our inhibitors on eosinophil motility, we measured the movement of the centroid location for each cell over the course of the experiment to assess the distance traveled. We found that the distances travelled by cells treated with cytoskeleton inhibitors (CK-666; Y-27632) or with the calcium reuptake inhibitor CPA were highly statistically

significantly less than control cells (Figure 5A). The ryanodine-treated group also showed lower distance traveled, but this effect was not statistically significant (Figure 5A).

In addition, we assessed the latency between the calcium flash and its succeeding directional shift. We found that, similarly to elongation, that latency was significantly longer in cells treated with calcium inhibitors (Figure 5B). Although the actin signaling inhibitors also had longer time periods between events, the latter were not statistically significantly different (Figure 5B).

Furthermore, we were interested in whether the direction the eosinophil membrane was protruding (and presumably crawling) towards relative to their centroid position was affected by the direction that the perfusion was applied. Although the media was perfused from left to right in our microscopy field of view for the video recordings, the cells did not show a directional preference of actin cytoskeletal protrusion and/or movement (Figure 5C).

4. Discussion

The modifications to the cytoskeletal architecture due to stimuli ranging from mechanical fluid shear stress to ligand binding chemokines occurs in a systematic fashion to ensure proper spatial and temporal conditions are met for migration-related behavior. Yet, the molecular processes of eosinophil extravasation detailing the activation of its integrin receptors and the subsequent outside-in signaling transduction cascades have not been fully unraveled. Although many groups are investigating the migrational responses triggered and guided by molecular stimuli (chemotactic and chemokinetic ligands), we are pioneering a role for physical stimuli (shear stress) in triggering the extravasation of activated eosinophils from the circulation and into the vessel wall. That is, shear stress elicits a complex and carefully choreographed sequence of morphological changes which all take place within 10 minutes, and sets the stage for the migrational response which occurs over the next many hours. Furthermore, we are finding that the immediate triggering event is connected to intracellular calcium fluxes. In the present study, we disrupt both actin cytoskeleton and calcium pathways with pharmacological agents to probe the eosinophil response to physiologically relevant shear stresses experienced by the cell once it adheres to the vessel wall.

To link the two crucial steps in classically studied leukocyte extravasation of adhesion and membrane protrusion, we utilized fibronectin as our substrate coating. It is well documented that firm cell adhesion to the endothelium and the extracellular matrix is primarily mediated through integrin engagement. The VLA-4 integrin on the eosinophil surface not only binds to VCAM-1 upregulated on the endothelium in disease models [19],

but also readily adheres to fibronectin [20], allowing our experimental model to better simulate in vivo endothelium environments. Integrin activation has been shown to activate Rac1 which in turn signals to the Arp2/3 complex [21], a seven-subunit protein aggregate necessary for actin nucleation and actin filament polymerization for numerous essential cell functions including vesicle trafficking, migration, and membrane protrusions (lamellipodia and pseudopodia) [21,22]. Rac1 promotes the linkage between the Arp2/3 complex with vinculin, an adaptor protein that physically links the transmembrane integrin receptor to the actin cytoskeleton [24]. This connection may be further stimulated by PIP_2, which induces a conformational change in vinculin to expose additional binding sites for the Arp2/3 complex [25]. PIP_2 itself is also involved in the activated Gq/11 pathway which results in IP_3-mediated calcium release. As such, we were motivated to determine if the inhibition of the integrin/Rac1/Arp2/3 pathway, central components to both cellular adhesion and motility, with the compound CK-666 which would interfere with the eosinophil PICR. We also utilized the compound Y-27632 as a cell-permeable Rho-associated protein kinase (ROCK)-selective inhibitor to reduce actin filament stabilization [26]. The GTPase Rho emerged in the late 20th century as a major facilitator of actin skeleton rearrangement necessary for actin stress fiber formation and cell migration through focal adhesions [26]. ROCK was found to be a key downstream Rho effector molecule specifically responsible for stress fiber formation, and itself inhibits actin filament depolymerization [26].

In our study, both CK-666 and Y-27632-treated eosinophils displayed stunted capabilities with regards to environment exploration and cell motility. The inability of the Arp2/3 complex to transiently bind to vinculin and integrin receptor aggregates because of

CK-666 inhibition was markedly reflected in its smaller area ratio (Figure 1B) and lack of membrane ruffling (Figure 4D). However, the CK-666-treated eosinophils boasted a robust and rapid calcium spike (Figure 2B-C), suggesting that intracellular calcium release signaling remained upstream or independent from actin rearrangement. It is interesting to note however, the perfusion of Y-27632 did not significantly affect the eosinophils' response to fluid shear stress outside of pseudopodia formation (Figure 4C) and distance traveled (Figure 5A). In fact, eosinophils treated with Y-27632 exhibited all of the hallmark features of the fluid shear stress response indicated by the lack of significant differences in area ratio, fluorescence ratio, and high positive correlations between calcium flux, elongation, and directional shift timepoints. These results indicate that pseudopodia formation requires long term stability of actin polymerization relative to membrane ruffling, and is a necessary checkpoint for substantial eosinophil motility.

However, calcium signaling inhibitor data supports our theory that eosinophil PICR is responsible for triggering actin cytoskeleton rearrangement. The compound ryanodine functions as a full antagonist to ryanodine receptors (RyRs) at micromolar concentrations [27]. Although stimulated RyRs are predominantly known to trigger $[Ca^{2+}]_i$ release from the sarcoplasmic reticulum in skeletal muscle cells to drive contraction [28], it was recently discovered that RyRs are also expressed on the endoplasmic reticulum of other cell types including immune cells [29]. Subsequent to $[Ca^{2+}]_i$ release in cells, the Ca^{2+}-ATPases on the plasma membrane and endomembrane are responsible for restoring calcium homeostasis by pumping Ca^{2+} into the extracellular space and back into intracellular stores,

respectively [30]. Therefore, treating cells with the ER Ca^{2+}-ATPase inhibitor, CPA, would deplete calcium stores over time.

Although the CPA-treated eosinophils displayed fluorescence ratios of smaller magnitude, it did not influence the latency of the calcium flux post-perfusion (Figure 2C). The CPA data strongly suggest that cycling intracellular calcium ions between the cytosol and ER storage is integral to migration-related actin rearrangement. In essence, as a consequence of calcium ion reuptake inhibition, the smaller PICR in this treatment group resulted in weak temporal association with both cellular elongation and directional changes in movement. On the other hand, we surmised that the reason ryanodine-treated eosinophils did not abolish the PICR (Figure 2A) despite taking a significantly longer time achieving it (Figure 2C) was due to the involvement of alternative calcium signaling pathways such as the PLC-IP_3 pathway.

The acquisition of spatial asymmetry in cells is critical for the onset of cellular migration. In essence, polarized cell morphology with distinguished front and back ends allows the cell to continue the formation of actin polymerization at the leading region with filaments released from adhesions at the rear [31,32]. In order for sustained migration to occur, the membrane protrusions must be firmly adhered to the substrate. If the pseudopodia fail to stabilize adhesions, they will retract towards the cell body [33]. On the other hand, although membrane ruffles lack adhesion sites and cannot promote displacement [34], it has been suggested the ruffles play an indirect role in promoting migration. Membrane ruffling is associated with macropinocytosis, a transient endocytic process that internalizes extracellular components in addition to parts of the cell membrane

and their surface receptors [34]. Specifically, the ruffles may be responsible for redistributing the β1 integrins towards newly formed adhesion sites at the leading edge and the establishment of the front-rear-axis of the cell. The necessity for both pseudopodia and membrane ruffling formation in cell ruffling is evident in the ryanodine treatment group. Although these eosinophils had the most comparable degree of distance traveled relative to the control group due to their higher ratio of pseudopodia formation, the complete lack of membrane ruffling appears to be limiting their potential.

The calcium-spike is a very rapid, all-or-none, pan-cellular event: these are all good and useful features of a finely tuned trigger mechanism. Our data suggest that the morphological changes (flattening; ruffling; pseudopod-formation) can still occur after disruption of this trigger, but their latency is greatly increased. This might offer up a new avenue for treatment strategy for eosinophilic inflammation. That is, in the physiological setting, circulating eosinophils become primed by agents (such as IL-5), then roll along the vessel wall until they are stimulated by a chemoattractant signal (such as eotaxin) to extravasate. The markedly increased shear stress produced during the rolling phase triggers the PICR, which in turn commits the cells to adhere strongly and accelerate extravasation. If that PICR could be disrupted, the primed cells might otherwise continue to roll and eventually be swept away back into the systemic circulation, thus reducing inflammation at that site. In conclusion, we theorize that adequate strength of the PICR 'jump-starts' the extravasation process in vivo and plays a significant role in the temporal aspect of migration.

The study of mechanosensors on circulating leukocytes is fairly novel, and the majority of shear stress research remains dominantly limited to non-migratory cells such as

the endothelium. However, it is crucial that we begin to differentiate between the role of mechanical stimuli for the various cell types. For example, the β1 integrins highly expressed on endothelial cells (ECs) were recently found to sense unidirectional flow and promote cellular alignment for vascular homeostasis, suggesting a role distinct from the β1 integrins on eosinophils [35]. Furthermore, some studies have shown that mechanical stress on ECs triggered the influx of calcium from extracellular sources [36], whereas our study indicates the increase in cytosolic calcium are from internal stores. In conclusion, investigating the distinct role of integrin mechanosensors in eosinophils and their role in mobilization may have future therapeutic implications for asthma and other eosinophilia-driven conditions.

Author Contributions: Conceptualization, K.S., R.S., and L.J; methodology, K.S., R.S. and L.J.; validation, K.S. and A.H.; formal analysis, K.S.; investigation, K.S. and A.H.; resources, K.S., R.S. and L.J.; data curation, K.S.; writing—original draft preparation, K.S.; writing—review and editing, K.S., R.S. and L.J..; visualization, K.S.; supervision, R.S. and L.J.; project administration, L.J.; funding acquisition, L.J. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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Figure 1: Characterizing changes in cell-substrate contact area in response to pharmacological inhibitors. Control-normalized cell flattening (see Methods) was significantly reduced in CK-666-treated cells compared to control cells (control: $n = 20$, CK-666: $n = 12$, CPA: $n = 12$, ryanodine: $n = 10$, Y-27632: $n = 11$; $p < 0.05$, Kruskal Wallis test).

Figure 2: Characterizing changes in PICR in response to pharmacological inhibitors. (A) Proportion of cells exhibiting (blue bars) or not exhibiting (red bars) a PICR (control: n = 20 cells with Ca2+ flux vs 0 cells without; CK-666: n = 10 vs 3; CPA: n = 10 vs 2; ryanodine: n = 8 vs 2; Y-27632: n = 8 vs 3, p = 0.037, Fischer's exact test). (B) The calcium signaling pathway inhibitors CPA (0.80 ± 0.23) and ryanodine (0.80 ± 0.19) significantly reduced the magnitude of the PICR compared to untreated eosinophils (1.00 ± 0.16, p = 0.022, Kruskal Wallis test). (C) Ryanodine markedly increased the latency to calcium flux (322 ± 118 sec) in comparison to the control (162 ± 94 sec, p = 0.002, Kruskal Wallis test).

Figure 3: Analyzing the relationship between loss of circularity and the PICR in eosinophils. (A) The peak elongation ratio was similar for all pharmacological treatments (control: $n = 20$; CK-666: $n = 12$; CPA: $n = 12$; ryanodine: $n = 10$; Y-27632: $n = 11$; $p = 0.385$) **(B)** The time it took for the eosinophil to begin losing circularity after the calcium spike increased significantly in CK-666 (291 ± 132 sec) and CPA (309 ± 151 sec)

treatments with respect to the untreated controls (104 ± 59 sec, $p < 0.001$). One-way ANOVA data presented as mean ± SD, Kruskal-Wallis test.

Figure 4: Assessing morphological changes in response to fluid shear stress. Eosinophils may exhibit distinct actin cytoskeletal protrusions in response to fluid shear stress. **(A)** Pseudopodia extension generally precedes cellular movement in the matching direction whereas **(B)** membrane ruffles maintain membrane circularity and do not associate with cell motility. The cell tracker image (right) traces the cell membrane over the course of the experiment with a green-to-yellow color transition indicator. **(C)** Eosinophils subjected to Y-27632 had significantly reduced capacity to form pseudopodia (control: $n = 16$ with pseudopodia vs 5 without; Y-27632: $n = 2$ vs 9, $p = 0.003$, Fischer's exact test). **(D)** CK-666-treated eosinophils had diminished membrane ruffling formation whereas ryanodine treatment abolished it completely (control: $n = 15$ with ruffles vs 5 without; CK-666: $n = 4$ vs 9, $p = 0.029$; Y-27632: $n = 0$ vs 10, $p = 0.0002$, Fischer's exact test).

Figure 5: Evaluating eosinophil motility response and its relationship with the PICR. (A) The centroid of analyzed eosinophils was tracked over the course of the experiment to determine the total distance traveled (μm) (control: 6.44 ± 2.26 μM; CPA: 2.79 ± 1.43 μM; ryanodine: 4.01 ± 2.17 μM; CK-666: 3.05 ± 1.12 μM; Y-27632: 2.89 ± 1.44 μM; $p < 0.0001$, Kruskal Wallis test). (B) A number of eosinophils (control: n = 18; CPA: n = 10; ryanodine: n = 7; CK-666: n = 12; Y-27632: n = 6) changed their direction of movement subsequent

to the calcium flash. The time in between the events was significantly shorter in the control eosinophils compared to eosinophils treated with calcium signaling inhibitors (control: 93 ± 43 sec; CPA: 230 ± 98 sec; ryanodine: 232 ± 135 sec; $p = 0.008$, Kruskal Wallis test). (C) Percentage of cells in each group which altered their migration in one of four different directions (perfusion moves from left to right in the FOV).

Figure 1. Son et al., 2021

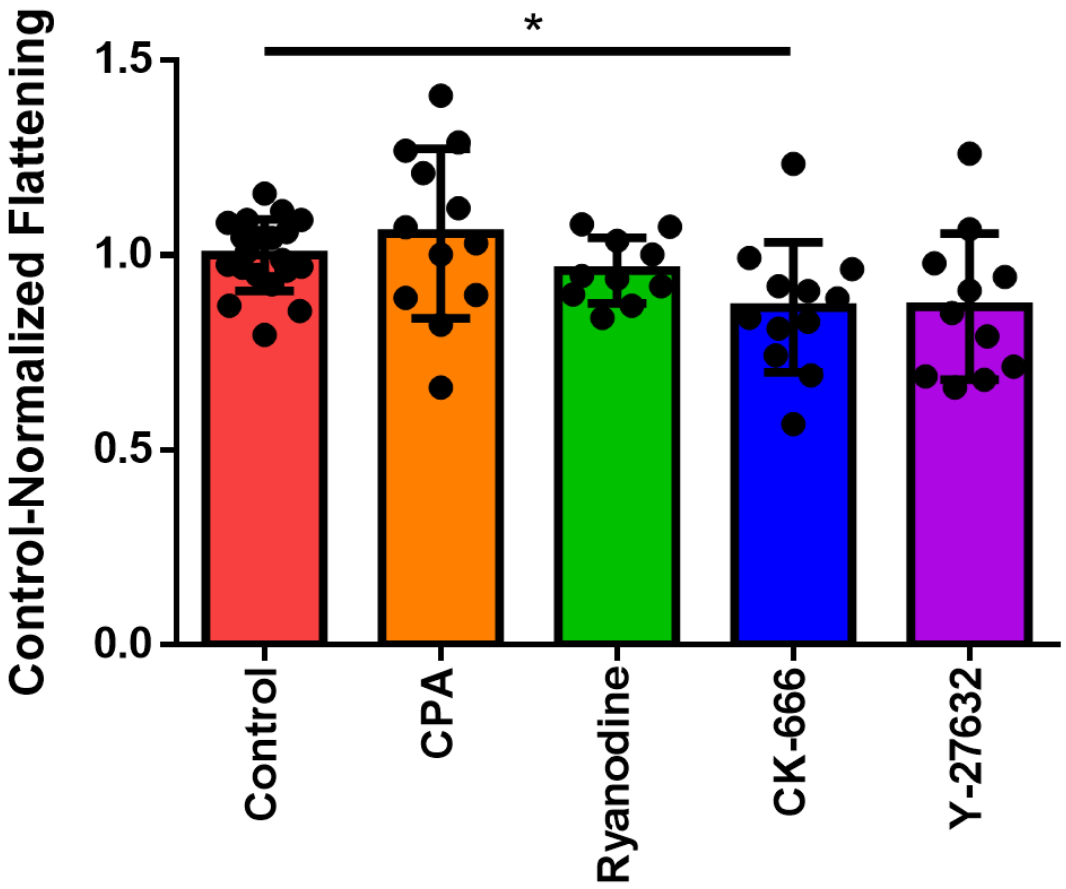


Figure 2. Son et al., 2021

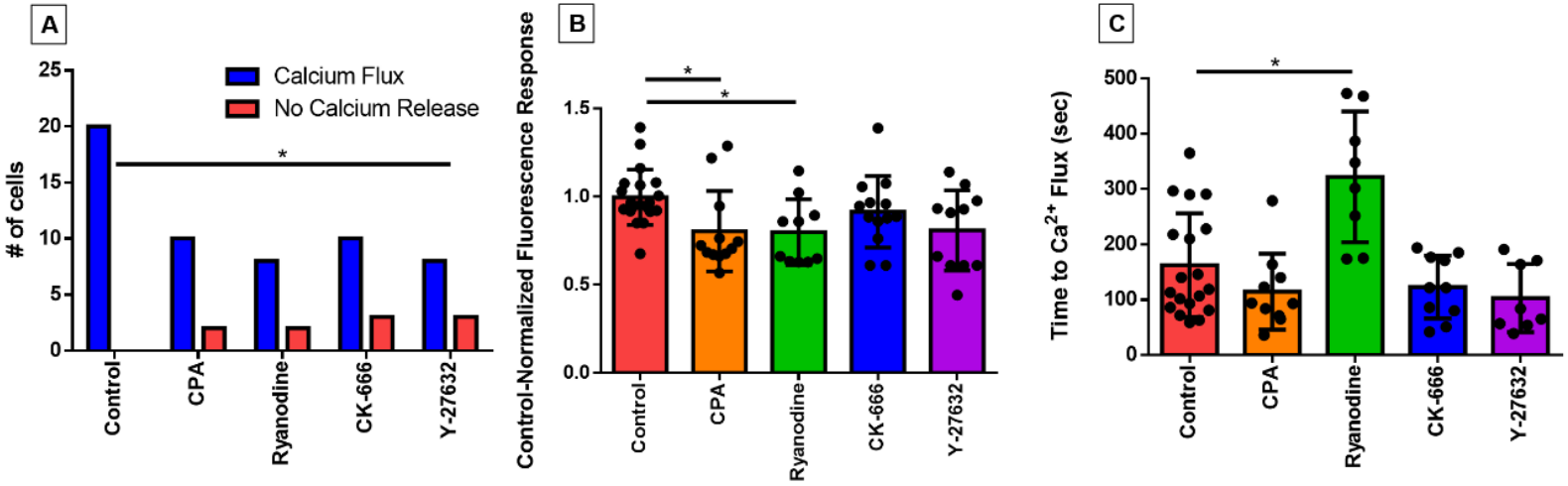


Figure 3. Son et al., 2021

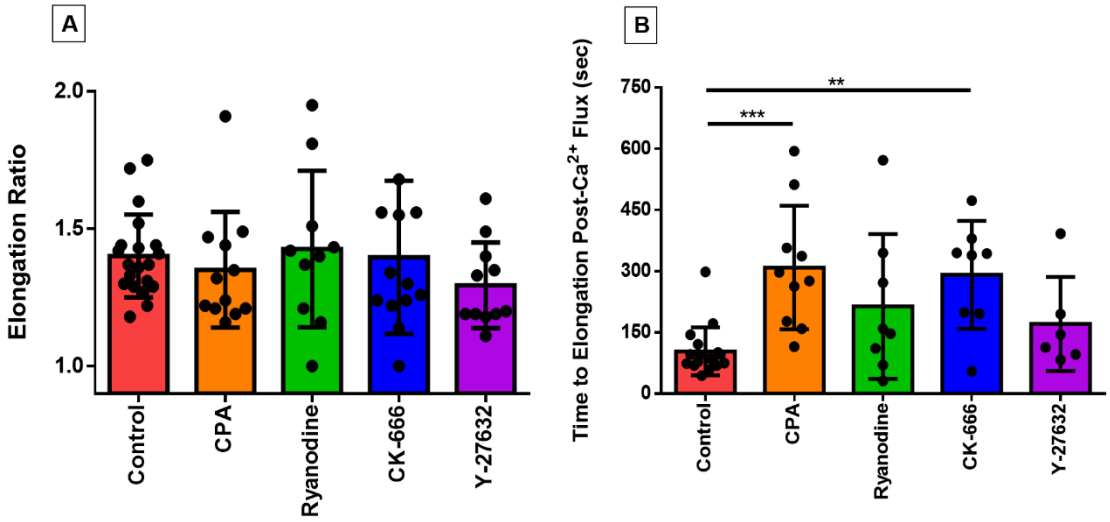


Figure 4. Son et al., 2021

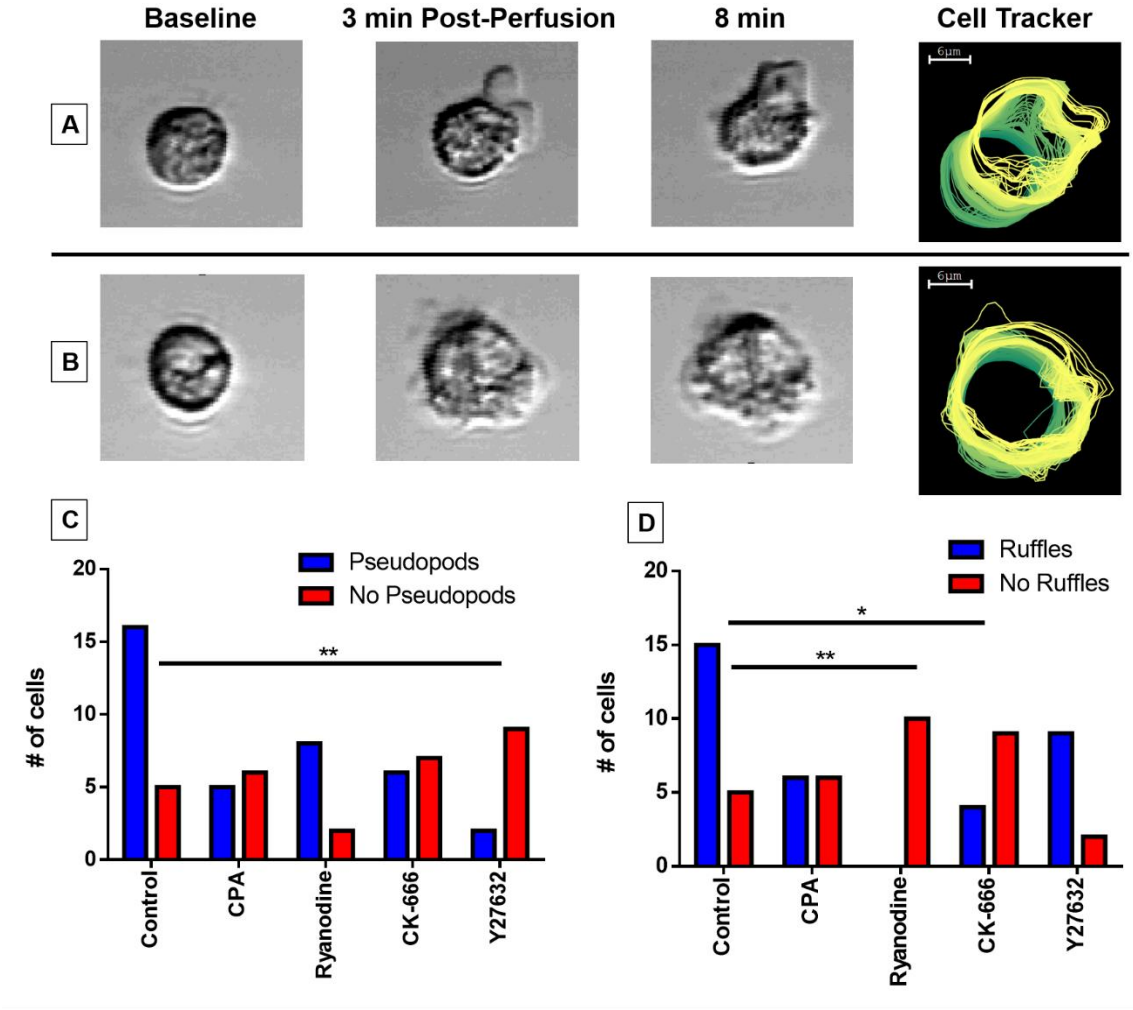
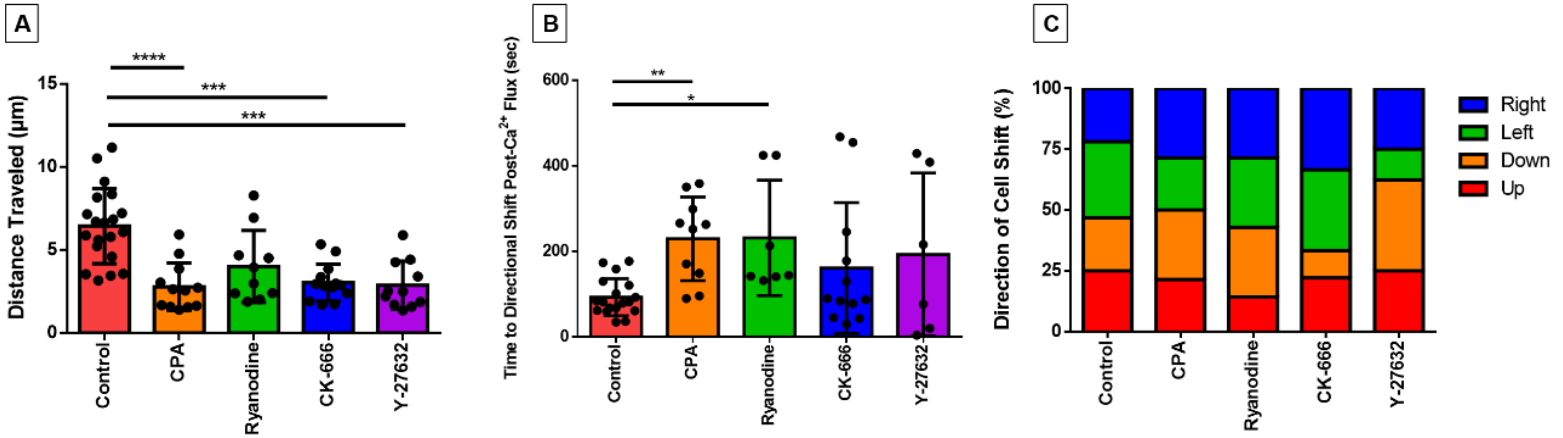


Figure 5. Son et al., 2021



Chapter 6: Overall Discussion and Synthesis

Eosinophils play a major role in the pathophysiology of multiple respiratory disorders that present with inflammatory pathology including asthma (Nair, 2013), chronic obstructive pulmonary disorder (COPD) (Hargreave & Leigh, 1999), eosinophilic granulomatosis with polyangiitis (EGPA) (Vaglio, Buzio, Zwerina, 2013), and hypereosinophilic syndrome (HES) (Busse et al., 2010). Indeed, it has been well-documented that eosinophil counts in the airway tissues will directly correlate with the severity of inflammatory symptoms in asthmatics, as increased eosinophil activation results in higher levels of tissue damage (Kay, 2005). Therefore, several therapeutic interventions have been developed in the past decade that directly attempt to attenuate eosinophil accumulation in respiratory tissues by targeting IL-5, a key player in the maturation, mobilization, activation, and survival of eosinophils at sites of inflammation (Rothenberg & Hogan, 2006; Lee et al., 2010; Garcia et al., 2013; Gleich, 2000). However, the outcomes from clinical trials of various anti-IL-5 biologics have highlighted the complexity of eosinophil biology pertaining to eosinophil recruitment and activation. Benralizumab, a monoclonal antibody that targets the alpha subunit of the IL-5 receptor, showed an increased efficacy of eosinophil depletion compared to its contemporary biologics, reslizumab and mepolizumab, and the initial trial documented reduced exacerbations and improved lung functions (Ghazi & Trika, 2012; Busse et al., 2010). However, despite multiple studies indicating a significant depletion in circulating eosinophil numbers with the adjunct mAb treatment, some subsets of asthmatics continued to present with airway eosinophilia and detectable levels of granule proteins including ECP (Busse et al., 2010;

Laviolette et al., 2013). This phenomenon suggests the existence of alternative mechanisms to IL-5-mediated eosinophil recruitment and survival in tissues.

Leukocyte migration typically occurs in response to chemotactic cytokines or chemokines, often secreted by the endothelial cells (ECs) creating a chemical gradient along which the cells migrate along. Of the various chemokines known to stimulate eosinophil chemotaxis, only eotaxin has shown to selectively mediate eosinophil trafficking to the asthmatic lung (Griffiths-Johnson et al., 1993; Conroy & Williams, 2001). Indeed, eotaxin levels in animal models have shown an elevated expression that directly corresponds to eosinophil counts in the bronchoalveolar lavage fluid (BALF) (Griffiths-Johnson et al., 1993; Jose et al., 1994). Furthermore, increased eotaxin mRNA expression was observed in bronchial biopsies of asthmatic patients compared to the healthy population (Ying et al., 1997). In fact, prior to the development of anti-IL-5 biologics, the drug product TPI ASM8 was developed which contains two modified antisense oligonucleotides designed to suppress the expression of both the surface molecule receptor C-C chemokine receptor type 3 (CCR3) which binds eotaxin, and the β chain, a shared receptor for IL-5, IL-3, granulocyte macrophage-colony stimulating factor (GM-CSF) (Gauvreau et al., 2008). The study yielded a reduction of reduced sputum/airway eosinophil presence by approximately half (Gauvreau et al., 2008). Unfortunately, a separate study investigating the role of the molecular antagonist to the CCR3 receptor, GW766994, was unable to demonstrate decreases in neither eosinophil nor their progenitor populations (Neighbour et al., 2014), further underscoring the complex biology revolving around eosinophil extravasation from the vasculature.

The binding of eotaxin to CCR3 in both mouse models and *in vitro* experiments with human eosinophils have shown to induce the release of intracellular calcium (Rothenberg, 1996; Tenscher et al., 1996). In addition, the regulatory role of calcium in adhesion and migratory functions has been well documented in the literature for multiple cell types, including newt eosinophils (Minton, 2014; Wei et al., 2012; Brundage et al., 1993). Therefore, when we first observed that perfusion-induced fluid shear stress elicited a calcium response in eosinophils *in vitro* (Ahmadzai et al., 2015), we were keen on determining whether this mechanical stimulus played a contributing role in eosinophil migration.

To my knowledge, we are the first research group to examine and publish on the role of fluid shear stress on eosinophil migration. The neoteric nature of the investigation merited meticulous protocol validation and experimental optimization in the initial stages of the project. Indeed, I was unable to produce reproducible data in my first year of graduate research as I observed substantial variances in multiple areas of our time-lapse confocal microscopy videos including levels of cell viability, adhesion, and intracellular calcium release. I determined via Wright-Giemsa stain, that my isolated 'eosinophil' populations were contaminated with a significant proportion of neutrophils, which in turn may have influenced any data obtained from downstream migration assays we had conducted. Thus, we developed our first research objective, to comparatively assess two eosinophil isolation techniques with respect to population purity, yield efficiency from whole blood, and potential activation of eosinophils as a result of the isolation protocol itself. The first eosinophil isolation protocol utilized density-gradient centrifugation to separate out the

granulocyte population, from which eosinophils were negatively selected for via treatment of antibody-coated magnetic microbeads targeting CD16, a highly expressed receptor on the neutrophil surface but absent on unstimulated eosinophils (Son et al., 2017). The second procedure was the recently developed MACSxpress isolation kit (Miltenyi Biotec Inc., Germany), in which an immunomagnetic antibody cocktail conjugated to multiple non-eosinophilic surface markers is employed to bypass the centrifugation process and negatively select for eosinophils directly from whole blood (Son et al., 2017).

Reproducibility and Replicability in Science

Over recent decades, scientific research has witnessed an exponential growth of emerging and intersecting disciplines resulted in over 2.3 million science and engineering research articles published worldwide in 2016 alone (National Science Foundation, 2018). Furthermore, the increased availability of data and access to computational resources have transformed numerous fields and has allowed for the adoption of large-scale machine learning and artificial intelligence assisted analytical methodology that was not possible in previous eras. However, amidst these dynamic revolutions, reproducibility and replicability has and should remain at the forefront of the development of science. For example, researchers have developed an emphasis on randomized experimentation with blinding as well as proper sizing and powering of experiments to counteract questions of validity.

Unfortunately, more than half of scientists today believe the community is facing a *replication crisis* (Baker, 2016), which collectively entails the following: (1) the absence of replication studies in published literature (Makel, Plucker, Hegarty, 2012), (2) inability

to reproduce results of published studies in replication projects (Begley & Ellis, 2012), (3) publication bias (Fanelli, 2010), (4) increasing prevalence of false positives in the literature (Simmons, Nelson, & Simonsohn, 2011), and (5) lack of transparency in reporting methodology and data analysis (Nuijten, 2016). Not surprisingly, even the extremely specialized field of eosinophil research has fallen prey to limited reproducibility. For example, although degranulation has been well documented in human eosinophils *in vitro* and *ex vivo* models, the mechanism for degranulation in mouse models has yet to be reliably replicated (Lee et al., 2012; Lacy and Moqbel, 2001; Ochkur et al., 2007). As such, the inconsistency of mouse models to replicate key human eosinophil functions underscore the critical role of *in vitro* research that utilize human peripheral blood eosinophils to study effector functions contributing to disease progression. Regardless of whether the nature of the conducted research is translational or basic science, cell isolation techniques are the first step for secondary experimentation of cell function. With respect to eosinophils, a major disadvantage lies within the relatively low circulating levels in healthy subjects, resulting in notably increased blood draw volumes to obtain comparable population counts to other leukocytes. This drawback is further compounded by the fact that studies investigating basic molecular mechanisms may not have access to blood from eosinophilic patients to make comparative analyses as cells from these donors may exhibit features different from non-eosinophilic populations. A necessary second step in our future research would be to compare differences between these populations to better understand whether diseases modify shear stress-induced cellular responses. Therefore, it is vital to use

isolation techniques that efficiently yield highly pure and undamaged eosinophil populations for secondary functional assays.

The assessment of our first research objective led to the observation that the MACSxpress kit consistently produced a larger and highly enriched eosinophil population (>90% purity) whereas the CD16 depletion method occasionally yield populations with compromised purity. In addition, the reduced processing time of approximately 2 hours associated with the MACSxpress isolation kit allows for researchers to isolate cells and run functional assays on the same day. On the other hand, we showed that the increased mechanical handling of the cells in the CD16 depletion protocol led to higher proportion of activated eosinophils immediately following the isolation. This poses a serious logistical issue, as primed and/or activated eosinophils would have to be allotted the necessary incubation time of several hours to return to their baseline states. As the procedure itself requires 5-8 hours to complete, eosinophils isolated from blood drawn in the morning would not be ready for function evaluation until the evening. This is critical as blood eosinophils are viable for up to 24 hours in circulation (Farahi et al., 2012), although survival factors such as IL-5 present in the lungs may prolong the lifespan up to 72 hours in tissues (Carlens et al., 2009). Furthermore, a recent study showed that blood eosinophils from healthy subjects incubated in standard conditions (5% CO_2, 37°C, serum-free growth medium) only yielded a population viability of 65% at 24 hours (Januskevicius et al., 2020). However, as the overarching focus of my doctoral research was on eosinophil migration, we did not utilize survival factors and were consequently keen on running our assays of

interest within a tighter temporal window. Therefore, we naturally gravitated towards the simplicity of the MACSxpress and the logistical advantage it provided.

Even within the CD16 depletion method, which has been considered the gold standard for eosinophil isolation thus far, global variability in the step-by-step procedure remains including type of anticoagulant for blood draws, reagents used for density-gradient centrifugation and erythrocyte contamination (Sedgwick et al., 1996; Ide et al., 1994), and incubation time periods. The significance of the accumulation of these seemingly minute differences is evident in the persistent discrepancies in observations and limited reproducibility of results in eosinophil labs worldwide. The potential standardization of highly efficient eosinophil isolation techniques with decreased handling to minimize cell activation will allow for more meaningful comparisons of eosinophil data.

Physiological Relevance of Eosinophil Mechanosensitivity

Once we optimized an isolation protocol that reliably provided pure eosinophil populations from healthy subjects, we returned our focus towards characterizing the perfusion-induced calcium response observed in our *in vitro* confocal microscopy experiments. We first attempted to validate a physiological significance of the perfusion-induced calcium response triggered by fluid shear stress on eosinophil migration.

Indeed, several studies have already outlined the induction of calcium release from mechanical stimuli in non-migratory cell types including endothelial cells (Naruse, Yamada, & Sokabe, 1998), neurons (Shibasaki et al., 2010), and myocytes (McCain & Parker, 2011). Interestingly, the actin polymerization pathway in human neutrophils

required for adhesion and migration was found to be independent of intracellular calcium mobilization (Elsner et al., 1996). Furthermore, neutrophils have been shown to de-activate and retract pseudopods in response to shear stress (Komai & Schmid-Schonbein, 2005). Both of these observations **made in human neutrophils** conflict with what we have routinely observed in human eosinophils. In essence, the combined investigation of the roles of fluid shear stress, calcium release, and cell migration is novel and has yet to be investigated in migrating granulocytes. Furthermore, a previous study by the Janssen lab showed the pharmacologically inhibiting integrin receptors significantly reduced the PICR in a dose-dependent fashion, suggesting that the PICR is mediated through integrin receptors as opposed to being a non-specific by-product *in vitro* (Ahmadzai et al., 2015).

Eosinophils suspended in circulation will experience little or no shear stress. However, they will experience increased contact with the endothelium as they travel through blood vessels of narrower proportions, potentially forming transient adhesions through binding of selectins expressed on endothelial cells. Through these nascent adhesions, the eosinophils will begin to experience markedly increased levels of shear stress proportional to the degree of *slow rolling* or drag caused by increasing binding affinity to the endothelium. We proposed that, if fluid shear stress had a physiologically relevant effect on eosinophil migration, manipulating the perfusion flow rate may modify the PICR.

Therefore, for our second research objective, we directly upgraded several aspects to the experimental set up utilized to study eosinophil migration in earlier Janssen lab publications (Ahmadzai et al., 2015). Previously, the perfusion chamber in our previous

study had to be crudely assembled using silicon and glass slides and the perfusion of media was applied using a gravity-controlled fluid reservoir: as such, the magnitude of the shear stress was unknown and uncontrollable. For the present study, we used a commercial parallel-plate flow chamber and peristaltic pump to precisely control shear stress at three distinct magnitudes to observe meaningful eosinophil response: low shear stress (1.25 dynes/cm^2), medium (3.75 dynes/cm^2), and high (6.25 dynes/cm^2). Furthermore, we opted to exclusively utilize fibronectin as the substrate-coating on the coverslip for our experiments as the α_4 subunit of the VLA-4 integrin on the eosinophil surface responsible for binding to VCAM-1 and MAdCAM-1 on the endothelium also binds to this ECM protein (Johannson et al., 2012).

We discovered that the PICR was induced in an all-or-nothing manner across all flow rates, with negligible differences with respect to fluorescence and area ratios. The all-or-nothing nature of these two cellular functions suggests that the PICR represents the eosinophil commitment to the migratory process. From a cellular perspective, regulatory checkpoints would prevent any unnecessary extravasation of eosinophils or other migratory cell types to preserve the considerable energy and resources required to undertake these processes. Eosinophil migration is tightly regulated in the healthy population to minimize accidental trafficking to tissues and to reduce the risk of inadvertent activation-mediated inflammation. Indeed, the first three stages of extravasation (rolling, adhesion, locomotion) are all considered to be reversible processes, whereas initiating diapedesis is accepted as the point of no return.

In stark contrast to the lack of a discernible relationship between those two aspects of the PICR, we found that the latency of the PICR was powerfully dependent on perfusion rate (i.e., shear stress): PICR latency was shortened by half during the medium rate of perfusion (shear stress ~4 dynes/cm^2) compared to lower or higher rates of perfusion. This observation might suggest that eosinophil extravasation is suppressed or delayed in the conduit arteries and smaller arterioles but promoted or accelerated in the postcapillary venules.

This critical observation confirmed that the PICR was a *bona fide* physiological mechanism with potential pathological significance. In the classical eosinophil migration in disease paradigm, the cells sense various chemical signals such as increased IL-5 and eotaxin levels near the inflamed tissues prior to migration. We proposed that appropriate levels of fluid shear stress provide information regarding the cells' location within circulation, and act as a reinforcing spatial trigger for extravasation from the postcapillary venules whereas unsuitable magnitudes of shear stress would promote the disassociation from the endothelium of the larger upstream (conduit arteries and arterioles) and downstream (larger venules) vessels. The quicker response time to activated calcium signaling pathways in the medium shear stress treatment group further indicates the presence of a temporal trigger, as the induced calcium release may potentially act upstream of other contributing pathways such as the polymerization of the actin cytoskeleton.

In addition to that all-or-none Ca^{2+}-response ~1 minute after that optimal shear stress stimulus, our immunofluorescent staining allowed us to visualize the rapid mobilization of F-actin and adaptor proteins to the cell periphery within 5 minutes of shear

stress stimulation. The membrane protrusions extended by adhered eosinophils are necessary to explore their environment for the final TEM step of extravasation. The nascent formation of the exploratory actin architecture requires a rapid turnover of filaments with the potential of developing into a high-density integrin cluster to serve as a stabilizing point for the eosinophil. Interestingly, we observed a distinct spatial asymmetry as our adaptor proteins and F-actin stained at opposite ends of the cell. We hypothesized that the F-actin was being stained at the leading edge of the cell where an abundance of actin filaments would be polymerized, whereas the talin and vinculin proteins responsible for linking integrin subunits to the actin cytoskeleton were stained at the cell rear as the protein complexes were being disassembled. Indeed, the asymmetry we detected is consistent with observed calcium polarization that modulated migration in newt eosinophils (Brundage et al., 1993). The swift localization of F-actin, talin, and vinculin to the cell periphery within 5 minutes of perfusion is indicative of how quickly eosinophil extravasation is initialized.

Altogether, then, the loose adhesion of the eosinophils to an activated endothelium would result in increased shear stress as the circulating blood rushes around the slowed eosinophil, and trigger an all-or-none Ca^{2+}-response (that which we have labelled the PICR) after a latency which is modulated by the magnitude of that shear stress. This all-or-none Ca^{2+}- response is concurrent with a slowly-developing breakdown of the actin cytoskeleton (which we witnessed as all-or-none cell flattening over the course of a couple minutes). The latter is not dependent upon elevated cytosolic calcium concentrations, since it persisted when the calcium spike was abolished, but did seem to be accelerated by $[Ca^{2+}]$ elevation. The Ca^{2+}-response and cell flattening were also concurrent with an even more

slowly-developing cell polarization (Chapter 4, Figure 6) and lamellipodia formation and ruffling within 8 minutes after the shear stress stimulus (Chapter 5, Figure 4). Migration per se (a completely different process) then follows even later yet (not measured in these experiments) once the cell is in the interstitial space and being guided by the concentration gradient of various chemotactic stimuli.

Investigating the Relationship between Calcium and Actin Signaling Pathways

In the previous study, we were unable to clearly uncover correlated or causative nature of the relationship between the calcium spike and changes in morphology. Therefore, for our third and final research objective, we sought to elucidate the nature of this relationship by applying pharmacological inhibitors to the calcium signaling pathway and regulators of actin cytoskeleton polymerization and monitoring its effects on the PICR. We further upgraded our confocal microscopy technique and analytical methods for improved accuracy in fluorometric and morphological readings.

We consistently observed a distinct lack of change in both cytosolic calcium concentrations and eosinophil morphology for up to two minutes immediately following the onset of perfusion. Afterwards, we detected a calcium spike over the rapid course of mere seconds followed by the flattening of the eosinophil onto the underlying fibronectin-coated coverslip over the next couple of minutes, which was the first indicator of the possibility that the delayed decoding of a single signal may be responsible for both events. A major observation was that the release of intracellular calcium always preceded changes in cell morphology (flattening, membrane protrusions), often within a 2-minute time

window. Furthermore, we saw evidence of multiple calcium flashes within a single eosinophil, a phenomenon we were unable to detect in prior studies. However, the CPA-treated eosinophils were unable to exhibit an oscillation of cytosolic calcium concentration due to their reduced capacity to uptake and cycle calcium ions through their endoplasmic stores. Consequently, this treatment group displayed the weakest temporal association between the PICR and initial changes in cell elongation and directional movement, verifying a vital role for calcium cycling in eosinophil migration.

The versatility of the calcium ion as a secondary messenger is well documented in the literature. Cytosolic free calcium has been shown to regulate leukocyte chemotaxis (Pettit & Fay, 1998; Ridley et al., 2003) as well as mediate tumour metastasis (Prevarskaya, Skryma, Shuba, 2011). In fact, the gradient of the calcium concentration exists at a 10,000-fold degree across the plasma membrane as well as the ER membrane and the cytosol (Wei, Wang, Cheng, 2012). This allows for the rapid mobilization of ions down their electrochemical gradient across these membranes to trigger a myriad of signaling transduction cascades. In addition, calcium will act as the physiological ligand to IP_3 and ryanodine receptors, which in turn releases intracellularly stored calcium. The precise study of the spatiotemporal architecture of calcium dynamics is relatively novel, and researchers are currently utilizing single-molecule tracking and super-resolution imaging (see section 2.3). to decipher the exact role of calcium in the many variations of cellular migration (amoeboid vs. endothelial vs. neurite pathfinding vs. mesenchymal). To my knowledge, we are the first to demonstrate fluid shear stress-induced calcium release in human non-endothelial cells.

In another observation, the treatment of CK-666, an inhibitor of the Arp2/3 complex significantly attenuated its ability to flatten onto the underlying substrate and develop membrane ruffles. In chemoattractant-stimulated leukocytes, the ruffling of the plasma membrane occurs rapidly within seconds of chemoattractant exposure, and often precedes the development of more stabilized actin polymerization events such as pseudopodia (Harvath, 1998). As well, the mitigation of pseudopodia formation in our Y-27632-treated eosinophils further supported the idea that membrane ruffling required less stability than pseudopods. Indeed, a study found that high membrane ruffling rates were observed in epithelial cells with inefficient migration and low pseudopodia persistence (Borm et al., 2005). The neutralization of the Arp2/3 complex and ROCK via CK-666 and Y-27632 respectively was evident in their reduced capacity for movement. In addition, despite inhibitor mechanisms of calcium inhibitors ryanodine and CPA not directly impacting actin polymerization, we observed a lack of membrane ruffling in ryanodine-treated eosinophils, and a decreased capacity for movement across the coverslip in CPA-treated cells, further indicating the key role calcium plays in cell migration. However, we also found that a small minority of eosinophils in treated groups had flattened without a prior calcium spike, suggesting the calcium release was not absolutely necessary for cytoskeletal rearrangement. Furthermore, in Chapter 4, we observed that the majority of eosinophils underwent a biphasic increase in area ratio as opposed to a gradual increase in response to shear stress. Therefore, we surmise that a singular, trigger event bifurcates and transduces calcium signaling and actin cytoskeleton rearrangement through separate pathways, and that calcium release may act as an accelerant on contractile and/or cytoskeletal rearrangement

pathways. However, the cross-talk between these pathways cannot be ignored, as the inhibition of one indirectly impacts the strength of response of the other.

Conclusion

Studies investigating the effect of fluid shear stress on cells have been conducted predominantly on endothelial cells thus far (Ando et al., 1990; Yamamato et al., 2000; Cuvelier et al., 2005). Their mechanosensors may possess greater sensitivity to changes in fluid shear stress in comparison to rolling leukocytes with selectin-mediated bonding as the protrusion of adhered leukocytes against circulating blood flow prior to cellular flattening results in a greater subjected shear stress. Understanding the role of fluid shear stress on leukocyte function and development and/or updating corresponding models is an emerging field in health science research. Recent studies have shown fluid shear stress to mediate integrin activation in adhered neutrophils in the presence of platelet-activating factor (Mitchell, 2014), but promote the de-activation of neutrophils in suspension (Komai & Schmid-Schonbein, 2005). Yet another study found that high levels of shear stress induced apoptosis of adherent neutrophils (Shive, Salloum, Anderson, 2000). The ‘infancy’ of fluid shear stress research in leukocytes contributes to an overall lack of agreement in its role in cell adhesion and migration. The stark contrast between the neutrophil fluid shear stress studies and our own indicate that shear stress as a trigger does not induce identical pathways in various cell types. Furthermore, as we are currently the sole group researching the effects of physical stimuli on eosinophil adhesion and migration, we strive to build a reliable foundation other research groups can build upon in the future.

In our latest proposed extravasation model, eosinophils in the post-capillary venules of bronchial circulation will form temporary bonds with selectins expressed on the endothelial wall. The presence of eosinophil-selective molecules such as eotaxin or IL-5 in the microenvironment will strengthen the adhesion through activating integrins. Other T_H2 cytokines including IL-4 and IL-13 that stimulate the endothelium to upregulate adhesion molecules such as VCAM-1 may also impact the perceived shear stress experienced by the eosinophils. Despite no changes in blood flow rate, the eosinophils will experience increased levels of shear stress due to this binding interaction.

Fluid shear stress within an appropriate range will serve as an additional, regulatory checkpoint, and can induce the release of intracellular calcium, supplementing chemokine-induced calcium release. The fluctuating levels of calcium release modulates cytoskeletal rearrangement, particularly actin polymerization at the leading edge during the locomotion stage of extravasation. The interaction between fluid shear stress, calcium signaling, and actin reorganization is responsible for eosinophil commitment to diapedesis during inflammatory states of eosinophilia-mediated diseases.

Clinical Implications, Limitations, & Future Directions

Based on our observations, we propose that targeting the PICR may be a novel therapeutic intervention in attenuating airway eosinophilia. In our latest study, the disruption of the PICR significantly increased the latency to morphological changes post-calcium spike, indicating a strong coupling between these cellular events. Through this interference, the eosinophil *in vivo* may disassociate from the endothelium and be swept

back into the circulation. Whereas eosinophils in circulation are unlikely to directly contribute to inflammatory states in eosinophilia-mediated diseases, the activation of tissue eosinophils has high potential for significant damage to the airway epithelium. Therefore, targeting PICR interference compliments the current therapeutic focus to reduce eosinophil accumulation in tissues and the associated inflammatory pathology.

Integrins are the predominant mechanosensors on the eosinophil surface. The inhibition of VLA-4 and Mac-1, the two eosinophil integrins responsible for adhesion and migration, may be of clinical interest as a viable drug target for respiratory diseases. In fact, the blockade of VLA-4 on circulating leukocytes through anti-CD49 mAb was shown to prevent airway eosinophilia in mouse models (Henderson et al., 1997). The development of small molecule VLA-4 inhibitors such as BIO-1211 allowed researchers to demonstrate inhibition of eosinophil chemotaxis *in vitro* (Zhao et al., 2003) and reduced inflammation in experimental asthma models in sheep (Abraham et al., 1999). Unfortunately, these types of antagonists have restrictions based on the route of administration and dosage as peptidomimetics, and their studies have been restricted to *in vitro* and animal models thus far. In one case, a multiple sclerosis (MS) patient receiving natalizumab treatments, an inhibitor to the α4 integrin subunit of VLA-4 commonly used to treat MS patients, counterintuitively developed pulmonary eosinophilia which was attenuated after cessation of treatment (Curto et al., 2016). In addition, the defects in the β2 subunit present in Mac-1 integrins amongst others, causes leukocyte adhesion deficiency (LAD) in patients which subsequently leads to immune deficiency (Kishimoto et al., 1987). Therefore, the global therapeutic inhibition of Mac-1 and VLA-4 may result in unfavorable consequences.

Lastly, we hypothesize that the latent PICR in the low and high shear stress treatment groups is an experimental artefact as a by-product of allowing the eosinophils to firmly adhere onto the fibronectin-coated coverslip via integrin binding prior to microscopy recordings. In essence, the longer time to activation for eosinophils experiencing low and high shear stresses in our *in vitro* model symbolizes the missed window of opportunity to commit to migration, and we surmise that the eosinophils would dissociate from their transient bonds via selectins *in vivo* at non-physiological flow rates. However, although eosinophils displayed migration-associated morphological behavior in our model, relevant levels of shear stress *in vivo* are not sufficient for cellular trafficking: the latter also requires chemical triggers. In order to verify this study limitation, further experiments investigating a range of incubation periods as well as utilizing different substrate-coating may be necessary. Although a smaller proportion of eosinophils would firmly adhere with a shorter incubation period, this experimental condition may be more consistent with *in vivo* events.

A better understanding of the PICR is necessary for our work to be eventually useful in identifying novel biomarkers and/or therapeutic targets for eosinophilia. For example, characterizing the PICR from eosinophils isolated from asthmatics, or priming healthy eosinophils with pro-inflammatory chemokines and cytokines produced in the asthmatic airways should provide additional insights into the role of eosinophil mechanosensitivity in migration. Furthermore, as the nature of leukocyte migration largely depends on its immediate environment, utilizing a 3-dimensional migration model consisting of ECs and ECM proteins may serve as a better representation of *in vivo* conditions. The examination of potential differences in eotaxin- or IL-5-induced versus shear stress-induced calcium

signaling by applying additional inhibitors such as IP_3R blocker xestospongin may also contribute to filling in the current gaps of knowledge. The novel and exciting research on eosinophil mechanosensitivity should continue to advance our understanding as well as the applicability of these concepts in years to come.

Appendix A: Book Chapter Publication in *Eosinophils.*

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Title: Functionally Active Eosinophil Purification from Peripheral Blood

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Key Words

Eosinophils, Isolation, Population purity, Negative selection, Flow cytometry, Degranulation, Cytospin, Immunomagnetic

Abstract

The choice of isolation technique for human peripheral blood eosinophils contributes to the nature of clinically relevant data derived from *in vitro* research. Since the 1990s, eosinophils have been conventionally isolated via density-gradient centrifugation followed by negative immunomagnetic selection using anti-CD16 antibody-coated magnetic beads. Due to recent advancements in molecular techniques, 'newer' methods have been made commercially available that drastically reduce user-handling and processing time while maintaining high population purity. Here, we describe the isolation procedure using one of these methods, the MACSxpress® Whole Blood Isolation Kit – human, as well as outlining protocols for differential staining and flow cytometry analysis to evaluate the purity and activation state of the isolated population. In addition, we highlight an *in vitro* degranulation assay that may be used to verify the intact functionally of the isolated eosinophils.

Key Words

Eosinophils, Isolation, Population purity, Negative selection, Flow cytometry, Degranulation, Cytospin, Immunomagnetic

1. Introduction

Eosinophils are innate immune cells with well-established roles in host defense against parasites, and pathogenicity in chronic airway diseases such as asthma [1-2]. In general, these granulocytes will fully differentiate into their mature, non-proliferative state before exiting the bone marrow into circulation, where it has a short half-life of 8 to 18 hours prior to migrating into tissue compartments [3]. For several decades, clinical and basic science researchers have isolated peripheral blood eosinophils to assess their cellular functions *in vitro* through various assays. The importance of *in vitro* research was further underscored by the unfortunate consequence of mouse eosinophil models being unable to reliably replicate human eosinophil functions [4]. Even so, the differences in isolation protocol used across eosinophil labs worldwide contribute to the questionable reproducibility of the data and the disparity in its interpretation.

The earliest techniques included sedimentation in dextran followed by discontinuous density gradients; unfortunately, these methods resulted in both poor recovery and population purity in subjects with normal or slightly elevated eosinophil counts [5]. The standard protocol since then has involved a density-gradient centrifugation to select for the granulocyte population, followed by the negative immunomagnetic selection of eosinophils using anti-CD16 antibody-coated magnetic beads [6]. This conventional isolation method required numerous centrifugation steps and took approximately 6 hours from blood draw to complete. The extensive handling of the cells may potentially be responsible for the procedural activation of the eosinophils prior

to even the evaluation of their cellular functions through secondary assays, inadvertently skewing the data.

Recently, more sophisticated isolation kits have been developed that utilize immunomagnetic antibody cocktails with multiple specificities against differential cell surface markers, which allows for the negative selection of eosinophils directly from whole blood. These techniques considerably reduce the processing time down to approximately 1-1.5 hours while simultaneously preserving population purity and improving the yield efficiency [6]. In this chapter, we have chosen to outline the isolation protocol for one of these newer cocktail kits, the MACSxpress® Whole Blood Isolation Kit – human, commercially available through Miltenyi Biotec Inc., based on the routine use of this product in our laboratories. However, there are comparable kits available through other biotechnology manufacturers including STEMCELL Technologies Inc.

There are several methods to verify the population purity of the eosinophils post-isolation. We utilize the Diff-Quik stain, a commercially available modified Wright-Giemsa stain that rapidly stains and differentiates samples. It contains an eosinophilic solution that allows for the quick visualization of our target cells against contaminants such as neutrophils or lymphocytes. Fluorescence-activated cell sorting (FACS) is used to further validate the population purity and assesses the activation states of the cells.

2. Materials

All solutions should be prepared (unless indicated otherwise) by using cell culture-grade reagents and ultrapure water that is created by purifying deionized, endotoxin-free water to obtain a sensitivity of 18 MΩ-cm at 25°C. If not specified, the preparation, storage, and usage of reagents should be done at room temperature. Ensure that appropriate waste disposal protocols are utilized for each reagent.

2.1 Eosinophil Isolation

1. Human blood donor
2. 21-gauge butterfly needles with luer-lock compatibility
3. 10 mL EDTA anticoagulant blood collection tubes
4. 15 mL and 50 mL conical polypropylene centrifuge tubes
5. MACSxpress® Whole Blood Eosinophil Isolation Kit, human (Miltenyi Biotec Inc., Bergisch Gladbach, Germany; catalogue #: 130-104-446). Stored at 4°C. Components include:
    a. 3 cocktail vials of immunomagnetic microbeads conjugated to monoclonal antibodies targeting cell surface markers of non-eosinophilic cells (*see* **Note 1**).
    b. 25 mL of Buffer A (undisclosed, proprietary information)
    c. 25 mL of Buffer B (undisclosed, proprietary information)
6. MACSxpress Separator (catalogue #: 130-098-308): This apparatus can hold either a 5 mL polystyrene round-bottom test tube, 15 mL tube, or 50 mL tube. It induces a

strong magnetic field around the tube, thereby allowing for magnetically labelled cells to adhere to the tube walls while aggregated erythrocytes will deposit to the bottom.

7. MACSmix™ Tube Rotator (catalogue #: 130-090-753): This apparatus has a pre-determined setting of 12 rpm that is suggested for optimal results as per manufacturer's instructions. If unavailable, any tube rotator / rocker comparable mixing capabilities can be used.
8. Hanks' balanced salt solution (HBSS) with calcium and magnesium, and without sodium bicarbonate and phenol red, 10x stock solution.
9. Sterile endotoxin-free water. Store at 4°C and remove from storage for immediate use.

2.2 Assessing Population Purity via Cytospin Staining

1. 5% fetal bovine serum (FBS) in phenol red-free RPMI 1640. The media does not have to contain HEPES, bicarbonate, or glutamine as the cells will not be incubated for a long period of time
2. Cytocentrifuge (e.g. Shandon CytoSpin 3, GMI Inc., MN, USA)
3. Cytospin consumables: glass microscope slides, cytospin funnel, cytoclip, and filter paper
4. Phosphate-buffered saline (PBS), 1x stock solution (pH 7.4)
5. Diff-Quik staining kit which should include:
    a. Fixative agent (methanol, blue-green)
    b. Solution I (eosinophilic, orange)
    c. Solution II (basophilic, dark blue)

6. Sterile endotoxin-free water

2.3 Evaluating Population Purity and Cell Activation State via Flow Cytometry

1. Flow Cytometry Buffer (FCB): 1x PBS with 2 mM EDTA and 0.5% BSA (pH 7.4)
2. Fluorochrome-conjugated antibodies. Ensure that the antibodies for the following cell surface markers are conjugated to their own unique fluorochrome: CD15, CD16, Siglec 8, CD63, and 7-AAD. For our studies, we used CD15-FITC (clone VIMC6), CD16-APC-Vio770 (clone VEP13), Siglec-8-APC (clone 7C9), CD63-PE (clone H5C6), and 7-AAD-PerCP.
    a. CD15 is constitutively expressed on granulocytes and monocytes, and plays a role in cell tethering and rolling during extravasation [7]
    b. CD16 is a low affinity Fc receptor that is highly expressed on neutrophils, but is lacking or minimal on eosinophil surfaces of nonallergic subjects [8]
    c. Siglec 8 is a late differentiation antigen expressed on eosinophils [9]
    d. CD63 is a transmembrane-4 glycoprotein and is a surface marker for eosinophil degranulation [10]
    e. 7-AAD is used to identify non-viable cells by intercalating their DNA at G-C rich regions
3. 10 µM platelet activating factor (PAF)

3. Methods

3.1 Eosinophil Isolation

1. Utilizing appropriate venipuncture protocols and techniques, draw four tubes (*see* **Note 2**) of peripheral blood into 10 mL EDTA anticoagulant (*see* **Note 3**) blood collection tubes.
2. Remove the MACSxpress® Whole Blood Eosinophil Isolation Kit from 4°C storage and allow reagents to warm to room temperature prior to use. The following procedure should be performed within a biosafety cabinet to reduce likelihood of sample contamination.
3. Reconstitute the lyophilized pellet in the cocktail vial with 7.5 mL of Buffer A. Gently pipette up and down several times to ensure homogenous suspension.
4. In a fresh 15 mL tube, prepare the isolation mix by pipetting the 7.5 mL of the reconstituted pellet in *Step 3* with 7.5 mL of Buffer B (*see* **Note 4**).
5. Gently pour 30 mL of peripheral blood into a fresh 50 mL tube. Add the isolation mix, close the tube, and mix gently through inversion several times.
6. Add the 50 mL tube to the MACSmix™ Tube Rotator, and let the mixture incubate for 5 minutes at a run speed of 12 rpm.
7. Remove the sample from the MACSmix™ Tube Rotator, unscrew the tube cap, and place the open tube into the MACSxpress Separator for 15 minutes (*see* **Note 5**). During this time the magnetically labelled cells (non-eosinophilic) will magnetically adhere to the side walls of the tube, whereas the erythrocytes will deposit at the

bottom of the tube. The eosinophil population will remain in the supernatant (*see* **Figure 1**).

8. Leaving the tube as is, carefully collect the supernatant in a new 50 mL tube. Use a 5 mL pipette for the first 20 mL of supernatant. The remaining ~10 mL of supernatant should be collected using a P1000 micropipette (*see* **Note 6**).
9. Centrifuge the supernatant (10 min, 300 *g*, room temperature). There will be a small percentage of erythrocyte contamination, denoted by the "red-ness" of the pellet. Aspirate the supernatant and resuspend the pellet with 9 mL of cold (4°C), endotoxin-free water for 20 seconds (cold water / hypotonic lysis) (*see* **Note 7**). Immediately add 1 mL of 10x HBSS media without calcium and magnesium to make a total volume of 10 mL.
10. Remove an aliquot of the final population for cell counting and trypan blue exclusion test of cell viability.

3.2 Identification of Pure Populations via Cytospin Differential

1. Following eosinophil isolation (*see* **Section 3.1**), resuspend 1.0 x 10^5 cells in 100 μL in 5% FBS-supplemented RPMI media.
2. Assemble the cytospin complex in order from top to bottom: funnel, filter paper (rough side up), glass microscope slide (labelled side up), and cytoclip
3. Once assembled, place the cytospin into the Shandon CytoSpin 3 Cytocentrifuge, and balance appropriately with a blank or duplicate on the opposite end.

4. Spin 1: Pre-wet the glass slide by pipetting 85 µL PBS into the funnel. Spin for 3 minutes at 300 rpm.
5. Spin 2: Load the 100 µL of eosinophils into the funnel. Spin for 5 minutes at 500 rpm.
6. Allow the slide to air dry following the second spin cycle.
7. Open all the solution containers for Diff-Quik staining (*see* **Note 8**).
8. Gently dip the slide into the fixative for one second, then remove. Repeat five times and remove excess fluid by gently dabbing onto a paper towel.
9. Repeat *Step 8*, but dip the slide into Solution I.
10. Repeat *Step 8*, but dip the slide into Solution II.
11. Rinse the slide using sterile, endotoxin-free water until the slide is pink and the water runs clear. Allow the slide to airdry vertically (*see* **Note 9**).
12. Microscopically analyze the Diff-Quik stain for population purity. Eosinophils should present with a blue nucleus, blue cytoplasm, and red granules (*see* **Note 10, Figure 2**).

3.3 Evaluating Population Purity and Cell Activation State via Flow Cytometry

1. Resuspend 2.0 x 10^5 eosinophils in 200 µL of 5% FBS-supplemented RPMI containing 10 µM PAF and incubate for 30 minutes at 37°C. This will serve as a positive control for eosinophil activation.
2. From the remaining eosinophils (*see* **Section 3.1**, *Step 10*), resuspend 1.4 x 10^6 cells in 700 µL flow cytometry buffer (FCB). Divide these cells evenly into seven separate flow cytometry-compatible tubes.

3. Label eight tubes as follows:
    a. Unstimulated, unstained control (no antibodies)
    b. Single stain: CD15
    c. Single stain: CD16
    d. Single stain: CD63
    e. Single stain: Siglec 8
    f. Single stain: 7-AAD
    g. Unstimulated control, all stains (CD15, CD16, CD63, Siglec8, 7-AAD)
    h. 10 µM PAF stimulated, all stains (CD15, CD16, CD63, Siglec8, 7-AAD). Add the cells from *Step 1* into this tube following incubation.
4. Add 10 µL of the corresponding antibodies into their respective tubes. Mix well and incubate for 10 minutes in the dark at 4°C.
5. Wash the eosinophils by adding another 1 mL of FCB, followed by centrifugation (10 minutes, 300 *g*, room temperature). Aspirate the supernatant completely.
6. Resuspend the pellet in 500 µL of FCB and add 1 µL of 7-AAD for viability discrimination.
7. The gating strategy for our eosinophil population is: SSC^{hi} $CD16^{-}$ $CD15^{+}$ $Siglec8^{+}$ (*see* **Note 11, Figure 3**).
8. Utilize 7-AAD positive gating to check for dead cells, and CD63 positive cells as a reflection of eosinophil activation (*see* **Note 12, Figure 3**).

4. Notes

1. Each cocktail vial can process a maximum of 30 mL of whole blood. Use >1 vial for blood draw volumes exceeding 30 mL. Any leftover cocktail solution can be stored at 4°C and must be utilized within one week of reconstitution for optimal results.
2. The outlined protocol for eosinophil isolation has been written for a blood draw of 30 mL. Using four 10 mL blood collection tubes ensures blood volume of >30 mL, as certain collection tubes may have lost internal vacuum over time. Discard excess blood carefully using appropriate disposal techniques.
3. There are other anticoagulant-containing blood collection tubes commercially available, e.g., heparin or sodium citrate. However, using EDTA is strongly suggested, as alternative anticoagulants will lower the yield of eosinophils per mL of peripheral blood drawn.
4. If the volume of the blood draw is not 30 mL, different volumes of reconstituted cocktail and Buffer B should be used correspondingly. The ratio of whole blood, to reconstituted pellet in Buffer A, to Buffer B is 4:1:1 (*see* Table 1).
5. mix by mixing appropriate volumes of the reconstituted pellet from step 1 and Buffer B (Table 1) in tube of appropriate size. Use the isolation mix immediately after preparation.
6. The volume of supernatant collected should approximately equal the original volume of blood drawn. For optimal eosinophil yields, the supernatant should be collected by placing the tip of the micropipette against the front wall of the tube (*see* Figure 1).

7. There are other known methods of erythrocyte lysis, such as using ammonium chloride solutions. However, the use of ammonium chloride to remove red blood cells has shown to activate the eosinophils during the isolation process [11].
8. Prior to usage, the solutions from the Diff-Quik staining kit should be stored in airtight containers large enough to dip a glass microscope slide and immerse the cytospin smear. Be careful to avoid contact with skin and/or clothing, as Diff-Quik stains are difficult to remove.
9. Do not wipe or blot out the top of the slide with tissues, otherwise the cells may be removed or destroyed.
10. Commonly contaminating cells may include neutrophils (blue nucleus, pink cytoplasm, violet granules), monocytes (violet nucleus, light blue cytoplasm), and basophils (dark blue/purple nucleus, violet granules). Eosinophils can be further characterized by their bi-lobed nuclei, distinct in both eosinophils and neutrophils (*see* Figure 2).
11. Neutrophils will be positive for CD16, whereas basophils are CD15 negative. Siglec 8 is a cell surface marker expressed on mature human eosinophils, and to a lesser extent, basophils.
12. There will always be, to an extent, a proportion of activated eosinophils (*see* Figure 3). The nature of this activation is primarily due to mechanical handling of the cells throughout the isolation and/or flow cytometry experimental processes. However, a comparative study between commonly used isolation protocols has shown that

eosinophils obtained via the MACSxpress method had a significantly lower percentage of activated cells [6].

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Table Legends

Table 1: Different volumes of reconstituted cocktail and Buffer B must be used, dependent on the volume of blood drawn.

Figure Legends

Figure 1: Schematic of the isolation apparatus: (A) Front and side view of the whole blood-antibody cocktail mixture in a 50 mL Falcon tube within the MACSxpress Separator.

Figure 2: Assessment of purity in isolated populations: Isolated eosinophils imaged at 40x magnification, post staining. (A) Staining of cytospin slides can be used on enriched populations for differential cell counts. (B) Differential cell counts for final populations isolated from five healthy donors.

Figure 3: Gating strategy for assessing eosinophil population: A. A schematic representing the gating strategy for assessing eosinophil purity within the cell suspension post isolation protocol using surface expression as $CD16^-$ $CD15^+$ $Siglec8^+$ eosinophils. To assess baseline activation state of the isolated eosinophils, CD63 surface expression is used. Activation with platelet activating factor (PAF) is used as positive control for activated CD63+ eosinophils. Generated flow cytometry plots for $CD63^+$ (PE) expression in isolated eosinophils (B) unstimulated (C) stimulated with 10µM platelet-activating factor (PAF) for 30 min, 37˚C. (D) Individual plots for n = 4 paired isolations, showing

baseline expression (i.e. unstimulated) of CD63 in freshly isolated eosinophils using MACSxpress® (purple) compared to another column negative selection (green) method (method commonly used, not described here).

Table 1. Son et al., 2020

Blood Volume	Cocktail in Buffer A	Buffer B	Total Volume	Appropriate Tube
4 mL	1 mL	1 mL	6 mL	15 mL
8 mL	2 mL	2 mL	12 mL	15 mL
20 mL	5 mL	5 mL	30 mL	50 mL
30 mL	7.5 mL	7.5 mL	45 mL	50 mL
40 mL	10 mL	10 mL	60 mL	2 x 50 mL

Figure 1. Son et al., 2020

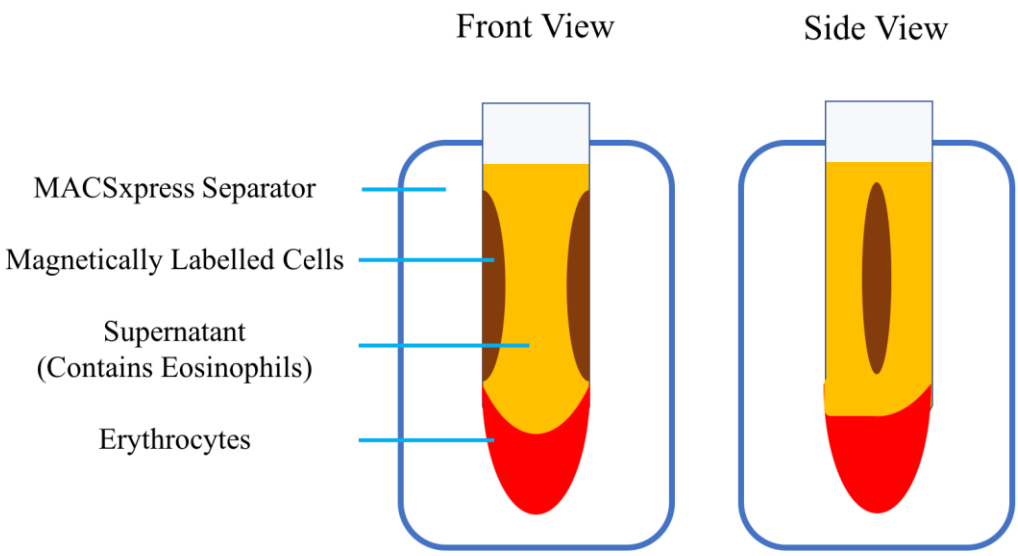
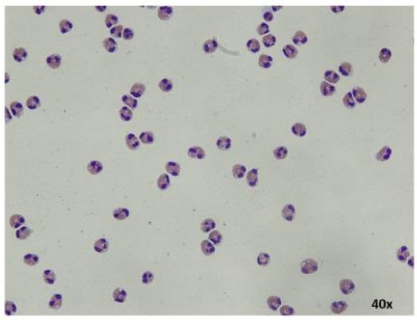


Figure 2. Son et al., 2020

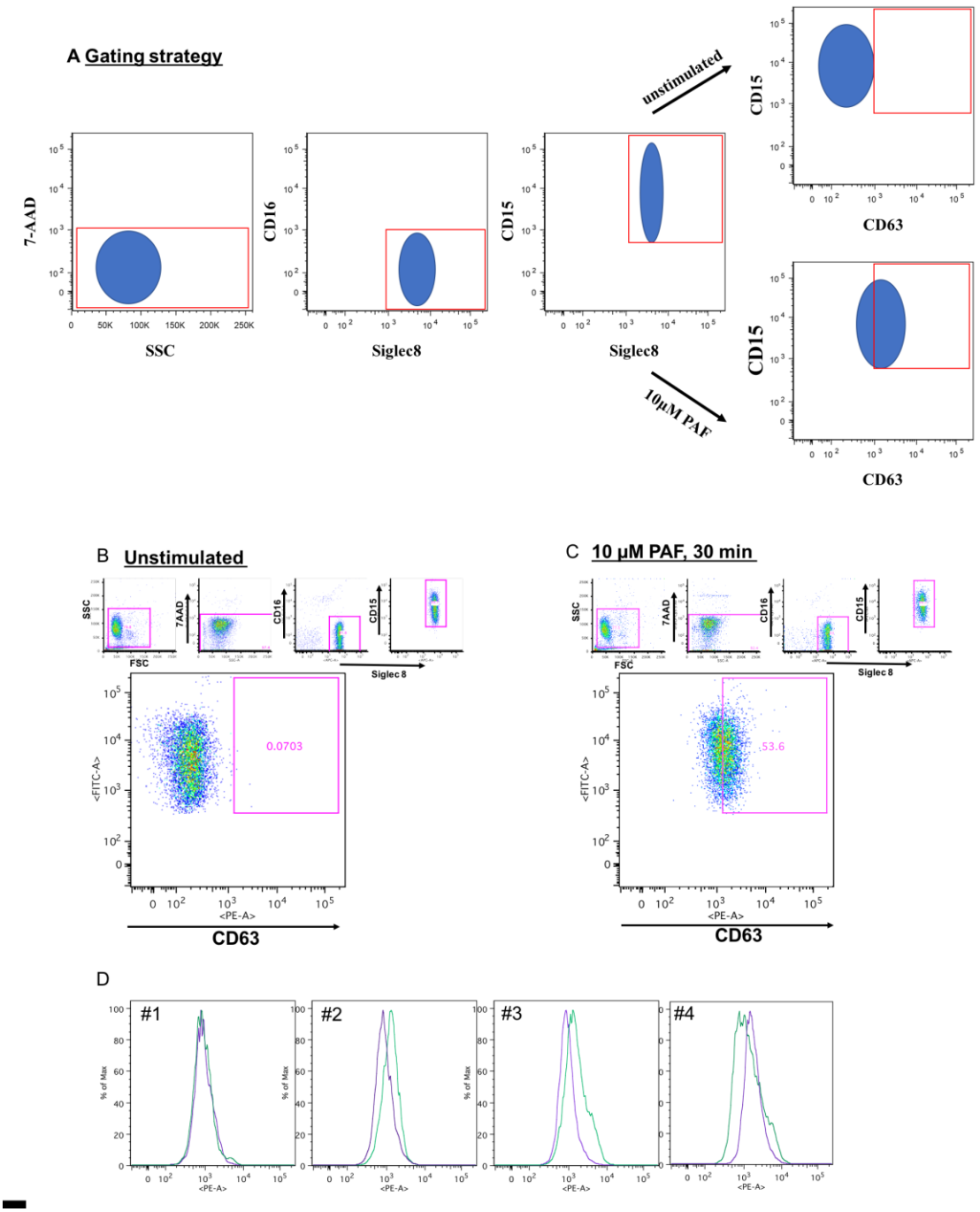
A



B

Subject	Eosinophils	Neutrophils	Macrophages	Lymphocytes	Basophils
1	95.0%	1.3%	1.5%	2.3%	0.0%
2	99.3%	0.0%	0.3%	0.5%	0.0%
3	99.5%	0.0%	0.3%	0.3%	0.0%
4	97.3%	1.0%	0.0%	1.8%	0.0%
5	99.5%	0.3%	0.0%	0.0%	0.0%
Average	**98.1%**	**0.5%**	**0.4%**	**1.0%**	**0.0%**

Figure 3. Son et al., 2020



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