INTEGRINS ARE MECHANOSENSORS THAT MODULATE HUMAN EOSINOPHIL ACTIVATION

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ABSTRACT

Eosinophils are end-point effectors of inflammation that contribute to the clinical severity of asthma. Eosinophil homing to the asthmatic lung is primarily guided by eotaxin-1, which is an eosinophil-selective chemokine. The mechanism by which eotaxin-1 augments intracellular calcium during cell migration is incompletely understood but is integral to the extravasation of eosinophils at sites of inflammation. We consequently report here that fluid shear stress, like eotaxin-1, unexpectedly activates human eosinophils in a calcium-dependent manner.

We used confocal fluorescence microscopy to study calcium-handling in purified human eosinophils. Application of eotaxin-1 augmented the $[Ca^{2+}]_i$ in a concentration-dependent manner. Pre-treatment of cells with ryanodine (10 μM) completely abolished the eotaxin-mediated calcium response, indicating that this phenomenon is dependent on Ca^{2+}-release from the ER. Several SOCC blockers (2-APB, 100 μM; Gd^{3+}, 10 μM; SKF-96365, 100 μM) attenuated SOCE, suggesting that these channels may directly contribute towards the eotaxin-1 calcium response in human eosinophils.

In the presence of fluid-perfusion, eosinophils displayed a robust perfusion-induced calcium response (PICR) demonstrating that eosinophils are mechanically sensitive. The PICR rapidly induced adhesion and non-directional migration in eosinophils, suggesting that some hitherto unknown molecular mechanosensor permits these cells to detect and respond to changes in shear-stress.

Pre-treatment of eosinophils with the non-selective tripeptide integrin receptor blocker, Arg-Gly-Asp (RGD), abrogated the PICR. The highly selective, dual $\alpha_4\beta_7/\alpha_4\beta_1$ integrin receptor blocker, CDP-323, was used to ascertain whether these highly expressed integrin subtypes

mediate the PICR in eosinophils. Pre-treatment of cells with CDP-323 completely abolished the PICR, in addition to the eotaxin-mediated calcium response in a shear-dependent manner.

Taken together, our results support a novel role for the $\alpha_4\beta_7/\alpha_4\beta_1$ integrin receptors as mechanosensors that directly modulate $[Ca^{2+}]_i$, adhesion and migration in human eosinophils. On-going experiments will seek to quantify the shear-response thresholds at which eosinophils activate and the time-course of the associated calcium response. This study suggests that the recruitment and activation of eosinophils are regulated by chemical and mechanical stimuli via overlapping, calcium-dependent signal transduction cascades. Given that the PICR is mediated by the eosinophil-specific $\alpha_4\beta_7/\alpha_4\beta_1$ integrin receptors, we conclude that integrin receptors are molecular mechanosensors that may facilitate eosinophil activation, adhesion and non-directional migration independently of, or in conjunction with, chemokine signaling.

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LIST OF ABBREVIATIONS

2-APB	2-Aminoethoxydiphenyl Borate
ANOVA	Analysis of Variance
AR	Allergic Rhinitis
ASM	Airway Smooth Muscle
ASMC	Airway Smooth Muscle Cell
AUC	Area Under the Curve
BALF	Bronchoalveolar Lavage Fluid
BSA	Bovine Serum Albumin
Ca^{2+}	Calcium Ion
$[Ca^{2+}]_i$	Intracellular Calcium Ion Concentration
CAM	Cell Adhesion Molecule
CCR3	C-C Chemokine Receptor 3
CICR	Calcium-Induced Calcium Release
CPA	Cyclopiazonic Acid
DAG	Diacyl-glycerol
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's Phosphate Buffered Saline
EC	Endothelial Cell
ECM	Extracellular Matrix
ECP	Eosinophil Cationic Protein
EDN	Eosinophil-derived Neurotoxin
EDTA	Ethylenediaminetetraacetic Acid
EGTA	Ethylene Glycol Tetraacetic Acid
EPO	Eosinophil peroxidase
ER	Endoplasmic Reticulum
FBS	Fetal Bovine Serum
FcεR	High Affinity IgE Receptor
FN	Fibrinogen
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HBSS	Hank's Buffered Saline Solution
HSPG	Heparan-sulphate Proteoglycan
ICAM-1	Intercellular Adhesion Molecule 1
IgE	Immunoglobulin E
IL-5	Interleukin-5

IP_3	Inositol-1,4,5-trisphosphate
K_d	Equilibrium dissociation constant
LN	Laminin
LPAM-1	Lymphocyte Peyer's Patch-Associated Molecule 1
mAb	Monoclonal Antibody
MAdCAM-1	Mucosal Addressin Cell Adhesion Molecule 1
MBP	Major Basic Protein
MCP	Monocyte Chemoattractant Protein
MLCK	Myosin Light Chain Kinase
NCX	Sodium-Calcium Exchanger
NLF	Nasal Lavage Fluid
PICR	Perfusion-Induced Calcium Response
PKC	Protein Kinase C
PIP_2	Phosphatidylinositol-4,5-bisphosphate
PLC	Phospholipase C
PMCA	Plasma Membrane Calcium ATPase
RANTES	Regulated on Activation, Normal T-Cell Expressed and Secreted
ROI	Region of Interest
RyR	Ryanodine Receptor Channel
SEM	Standard Error of the Mean
SERCA	Sarcoplasmic-Endoplasmic Reticulum Calcium ATPase
SOCC	Store-Operated Calcium Channel
SOCE	Store-Operated Calcium Entry
STIM	Stromal-Interacting Molecule
T_H2	Type 2 T-Helper
7-TMR	7-Transmembrane Receptor
TNF-α	Tumour Necrosis Factor Alpha
TRP	Transient Receptor Potential
VCAM-1	Vascular Cell Adhesion Molecule 1
VLA-4	Very Late Antigen 4
VN	Vitronectin

Chapter 1: Introduction

1.1 Epidemiology and Clinical Features of Asthma

Asthma is a chronic respiratory disease in which reversible constriction of the upper airways precipitates intermittent episodes of airflow obstruction and symptoms of wheezy breathlessness, coughing and/or chest tightness (Murphy & O'Byrne, 2010). Airway narrowing is the main pathophysiological defect of asthma that results from a combination of acute bronchoconstriction and chronic thickening of the airway wall (Figure 1; Murphy & O'Byrne, 2010; O'Byrne & Inman, 2003). Asthma afflicts 3-5% of the Canadian population and nearly 300 million people worldwide (Peters et al., 2006; Urbano, 2006). The clinical presentation of asthma is remarkably heterogeneous and the severity of the condition varies from *mild intermittent* to *severe persistent*, depending on the extent to which symptoms affect quality of life and the extent to which they can be controlled (Shahidi & Fitzgerald, 2010).

Asthma-related mortality has declined over the last decade but the incidence of the disease has doubled in developed nations and similarly increased in nations that have undergone Western industrialization (Braman, 2006; Koenig, 1999). Globally, asthma remains a social burden that continues to incur tremendous economic and personal costs (*e.g.*, direct medical expenses, restriction of lifestyle opportunities; Braman, 2006). Ultimately, various genetic and environmental factors contribute to the onset of asthma via complex immunological, developmental and neuro-hormonal interactions (Kay, 2001; Murphy, 2012). The development of any therapeutic intervention is therefore contingent upon elucidating the mechanistic basis by which these interactions cause disease.

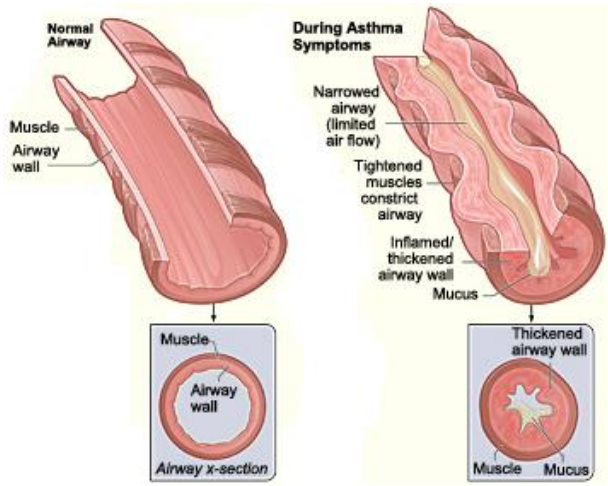


Figure 1. Pathophysiology of asthma. The flow of air to and from the alveoli is determined by the cross-sectional area of the conducting vessels (Tulic et al., 2001). Unlike in healthy individuals (left panel), the asthmatic airway exhibits considerable narrowing as a result of several histological and functional changes. An asthmatic exacerbation consequently results from the aberrant contraction of airway smooth muscle cells (ASMCs) that circumscribe the airway lumen, leading to an exponential reduction in the cross-sectional area of that airway (Weinberger, 2008). This reduction in cross-sectional area dramatically increases the resistance of the vessel to airflow and gives rise to symptoms of respiratory distress. (Image adapted from NLBH, 2012)

1.2 Role of Inflammation in the Pathogenesis of Asthma

Airway inflammation is a salient histological feature of asthma that contributes to alterations in the structural composition and the functional properties of the airway wall (Broide, 2008). These long-term changes are irreversible and collectively include: hyperresponsiveness and hypertrophy of airway smooth muscle cells (ASMCs), collagen deposition, sustained angiogenesis and goblet cell hyperplasia (Broide, 2008).

Normally, inflammation constitutes a homeostatic response mounted by the immune system that serves to mitigate the spread of infectious pathogens (Geering et al., 2013; Thomas & Schroder, 2013; Lacy, 2006). During this process, inflammatory effector cells migrate out of the bone marrow and infiltrate distant tissue sites where they elaborate a cascade of potent, anti-microbial agents that directly destroy pathogens and/or infected host cells (Remijsen et al., 2011).

In asthma, dysregulation of the inflammatory response heralds rampant destruction of host airway tissues following the aberrant accumulation and activation of immune effector cells in these regions (Persson, 2013; Gauvreau et al., 2009; Denburg & Keith, 2008). The unwarranted destruction of bystander cells sets off a run-away cycle of inflammation that results in tissue destruction/remodeling (Broide, 2008).

Several extrinsic factors trigger inflammation in the asthmatic airway, including allergens, infections and exercise (Figure 2; Kay, 2001). The exact manner in which inflammation manifests is consequently governed by the nature of the insult (sterile *vs.* non-sterile), the health status of the individual (immunocompetent *vs.* immunocompromised) and/or their immunological history (unexposed *vs.* sensitized). Ultimately, allergens, infections and exercise act through distinct pathways to induce the same pathophysiological endpoint (*i.e.*, inflammation; Kay, 2001; Weinberger, 2012). Inflammation *per se* therefore comprises a critical event in the onset of asthma and is viewed by some as a global underlying factor in the pathogenesis of this disease (Kay, 2001; Kay 1991; Broide, 2008; Weinberger 2012).

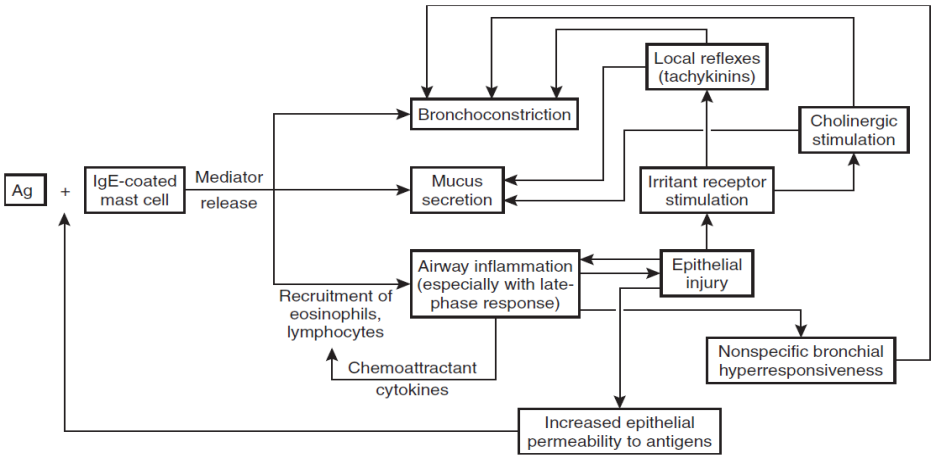


Figure 2. Factors that contribute to the onset of asthma. Allergens, exercise and/or pulmonary infection contribute to the onset of asthma (Weinberger, 2008). Allergens induce airway inflammation via binding with immunoglobulin E (IgE) antibodies, which act on mast cells to induce bronchoconstriction and mucous secretion (Murphy, 2012). Allergic inflammation is protracted and self-enforcing. Exercise triggers bronchoconstriction and/or mucous secretion by stimulating irritant receptors and/or local neural reflexes that control bronchial contractility (Weinberger, 2008). Infection induces the proliferation of T- and B-lymphocytes and thereby facilitates immunological sensitization against allergens. Altogether, any of these triggers may give rise to a twitchy, hyperresponsive ASM phenotype. (Image from Weinberger, 2008).

Disentangling the events involved in the inflammatory response is central to better understanding the pathogenesis of asthma and inflammatory diseases. Allergic (or atopic) asthma constitutes the most common and the best-studied phenotype of the disease (Busse & Lemanske, 2001). The time-course of the asthmatic response has been well-characterized in atopic humans and in animal models of allergic asthma, and continues to inform our understanding of how airway inflammation contributes to the pathogenesis of asthma. Briefly, allergic asthma features

a marked proliferation in CD4+ Type 2 T-helper (T_H2) lymphocytes, which drive the production and secretion of allergen-specific antibodies (Busse & Lemanske, 2001; Kay, 2001). T_H2 cells function as distress beacons that guide, amplify and fine-tune the recruitment of inflammatory effector cells to the airways. Ultimately, these effector cells constitute end-point perpetrators of tissue damage, and so many groups have sought to target effector cell functions in the development of asthma therapies (Kay, 2001).

Indeed, anti-inflammatory drug therapies that blunt the inflammatory response are currently used in conjunction with bronchodilator agents that aim to reverse airflow obstruction by inducing relaxation of airway smooth muscle (ASM) (Weinberger, 2008). Inflammation-based therapies include the use of inhaled corticosteroids, lipooxygenase inhibitors and leukotriene receptor antagonists, which collectively counteract the effects of endogenous inflammatory mediators (Weinberger, 2008; Kay, 2005).

1.3 Eosinophils in Allergic and Non-Allergic Airway Inflammation

Of the various inflammatory effector cells recruited to the asthmatic airway, the myeloid-derived, eosinophil leukocytes most directly contribute to the persistent cycle of inflammation and tissue destruction/remodeling seen in asthma (Bousquet et al., 1990; Busse & Sedgwick, 1992; Conroy & Williams, 2001; Humbles et al., 2004; Kay, 2005; Makino & Fukuda, 1995; Rankin et al., 2000). Indeed, Castro et al. (2008) recently showed that blocking mAbs directed against the interleukin (IL-) 5 receptor decreases sputum eosinophil counts in patients with poorly controlled asthma. This decrease in sputum eosinophil was accompanied by marked improvements in airway function relative to the placebo (Castro et al., 2008). These findings were largely consistent with several previous landmark studies, which similarly highlighted the

pathological role of eosinophils in the onset of asthma and allergic disease (Busse & Sedgwick, 1992; Oddera et al., 1996).

1.3.1 Properties of Eosinophils

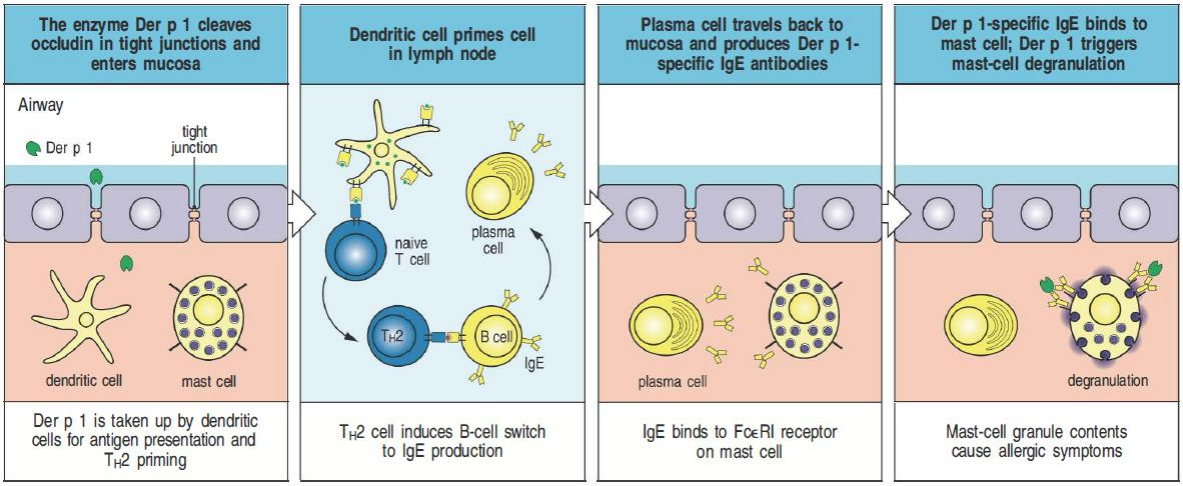
Eosinophils are inflammatory effector cells that belong to the granulocyte family of leukocytes, which also includes mast cells and basophils (Kolaczkowska & Kubes, 2013). In humans, mature eosinophils are approximately 10-15 μm in diameter and are characterized by the presence of bi-lobed nuclei and diffuse, cytoplasmic granules that contain toxins like major basic protein (MBP), eosinophil-derived neurotoxin (EDN), eosinophil peroxidase (EPO) and eosinophil cationic protein (ECP) (Giembycz & Lindsay, 1999). These cytotoxins are released into the extracellular milieu through a process known as degranulation following activation of the respiratory burst pathway. Once secreted, these compounds generate highly reactive oxygen species that non-specifically degrade invading pathogens (Giembycz & Lindsay, 1999).

Given their immense destructive potential, eosinophils normally reside in the gastrointestinal epithelial lining, where they facilitate host immunity against helminthic parasites (Giembycz & Lindsay, 1999; Amerio et al., 2003). As an added safeguard, the circulating fraction of eosinophils is tightly regulated under normal conditions such that eosinophils constitute only 1-2% of all leukocytes (Giembycz & Lindsay, 1999). In the presence of helminthic infection, however, this number may increase to 30-50% as eosinophils are rapidly mobilized from the bone marrow (Giembycz & Lindsay, 1999).

Allergic diseases like asthma, rhinitis and atopic dermatitis are characterized by marked increases in the numbers of circulating and tissue eosinophils (Teixeira et al., 1995; Rankin et al., 2000). Interestingly, the increased incidence of eosinophilic disease in the developed world has paralleled the advent of water sanitation policies that have reduced helminthic parasite infections

within the population overall. This has prompted some to speculate that the role of eosinophils in the pathogenesis of allergic disease may relate to under-stimulation of the host immune system by helminthic organisms (Griffiths, 2011).

In asthma, the mechanisms by which eosinophils contribute to airway inflammation extend beyond their ability to directly destroy bystander cells. Eosinophil-mediated release of EPO, EDN, MBP and/or ECP damages the airway epithelial lining, which normally precludes allergens from entering the tissues (Giembycz & Lindsay, 1999). Destruction of the epithelium following eosinophil activation accelerates antigen-penetration into the airways and thereby intensifies the inflammatory response as whole (Figure 3; Kay, 2005). Eosinophils can also exacerbate the initial allergic response, however, by functioning as antigen-presenting cells that stimulate the production of allergen-specific immunoglobulin E (IgE) molecules (Murphy, 2012; Rankin et al., 2000). IgE molecules bind to the high affinity IgE receptor (FcεR) expressed on the surface of mast cells (Murphy, 2012; Kay, 2001). Following subsequent allergen-exposure, IgE-bound mast cells degranulate and release bronchoconstricting agents that induce airway narrowing (Murphy, 2012).



<u>Figure 3.</u> Destruction of the airway epithelium exacerbates airway inflammation. Airway thickening results from hypertrophy and/or hyperproliferation of airway smooth muscle cells, hyperproliferation of mucous-producing goblet cells, deposition of extracellular matrix proteins and angiogenesis. Much of the pathological dysfunction seen in asthma is attributable to the activity of T-helper type 2 (T_H2) cells, which secrete cytokines that stimulate the recruitment of inflammatory cells like neutrophils, basophils, and eosinophils to the airways (Makino & Fukuda, 1995; Broide, 2008). The mechanism by which allergen sensitization precipitates asthma in these atopic individuals is unclear, but likely involves some combination of genetic and/or environmental interactions that predispose these individuals towards immunological hypersensitivity against the allergen (Murphy, 2012). In allergic asthmatics, airway inflammation is mediated by antibodies secreted as part of a misdirected humoral immune response. These allergen-specific antibodies are produced subsequent to B- and T-lymphocyte activation at sub-mucosal sites, where allergens enter the body by penetrating the epithelial barrier (Kay, 2001). In the absence of infection, however, the immune system suppresses lymphocyte activation and thereby mitigates the risk of mounting an immune response, be this against a pathogen or an otherwise innocuous allergen. For this reason, allergen-exposure requires the presence of an infection so that immunosuppressive signals preventing the proliferation of lymphocytes are blocked (Image from Murphy, 2012).

1.4 Eosinophil Homing to the Asthmatic Lung

Eosinophil counts in the blood and in the airway tissues directly relate to the clinical severity of asthma such that greater eosinophil activation in the tissues heralds more extensive tissue damage (Kay, 1991; Kay, 2005). Although not all subtypes of asthma are characterized by tissue eosinophilia, the role of eosinophils in the pathogenesis of airway disease is generally well-accepted and several therapeutic interventions have sought to attenuate eosinophil accumulation in the asthmatic airway (Soth, 2004; Bel, 2004; Kay, 2005). These attempts have met with varying levels of success, however, as the exact mechanism by which eosinophils are recruited to the airways remains incompletely understood. For this reason, the molecular and sub-cellular events coordinating the sustained recruitment of eosinophils into the asthmatic airway merit greater consideration.

1.4.1 Selective Eosinophil Recruitment by Eotaxin

Most cells migrate at some point throughout their life cycles (Schwab et al., 2012). Typically, migration occurs in response to chemotactic cytokines (or chemokines) secreted by other cells. Cells migrate along gradients of these chemoattractant molecules, during which they undergo a series of cytoskeletal re-arrangements whereby the leading edge of the cell membrane protrudes outward and adheres to the surrounding substrate, while the trailing edge is retracted inward (Schwab et al., 2007; Schwab et al., 2012). Functional studies demonstrate that several chemokines mobilize eosinophils from the bone marrow and promote their homing to the lungs. These include: interleukin (IL)-5, regulated on activation, normal T cell expressed and secreted (RANTES), granulocyte-macrophage colony stimulating factor (GM-CSF) and eotaxin (Rankin et al., 2000; Amerio et al., 2003).

Eotaxin is the only chemokine that selectively and potently mediates eosinophil recruitment to the asthmatic lung (Griffiths-Johnson et al., 1993; Conroy & Williams, 2001; Pease & Williams, 2001). Studies using guinea-pig models of airway disease have shown that the level of eotaxin expression in the airway tissue directly relates to the numbers of eosinophils isolated from the bronchoalveolar lavage fluid (BALF) (Griffiths-Johnson et al., 1993; Jose et al., 1994). Similarly, human studies have shown that the incidence of asthma is accompanied by an increase in the mRNA expression level of eotaxin in bronchial biopsies (Ying et al., 1997). Lastly, the pronounced migratory effect of eotaxin on eosinophils motivated the development of the humanized anti-CCL11 antibody, bertilimumab, which recently entered phase II clinical trials for the treatment of inflammatory bowel disorder (Ding et al., 2004).

1.4.2 Eotaxin Signaling and Calcium Homeostasis

Eotaxin belongs to the CC family of chemokines, which are characterized by the presence of Cys-Cys residues found near the N-terminus of the protein (Amerio et al., 2003; Rankin et al., 2000). Three isoforms of eotaxin exist: eotaxin-1 (CCL11), eotaxin-2 (CCL24) and eotaxin-3 (CCL26), which vary in their amino acid sequence homologies and their binding affinities for their cognate receptor, chemokine receptor 3 (CCR3; Rankin et al., 2000). CCR3 is a 7-transmembrane receptor (7-TMR) that is highly expressed on mature eosinophils, but is also found on basophils and on CD4+ T_H2 lymphocytes. The surface expression of CCR3 is highest in eosinophils that have fully matured (Rankin et al., 2000). CCR3 thus constitutes a surface marker for eosinophils (Giembycz & Lindsay, 1999; Ying et al., 1997). In these mature cells, CCR3 activation evokes robust changes in cell function in a calcium-dependent manner (*see below*). While RANTES and monocyte chemoattractant protein (MCP) also induce eosinophil migration, they do not bind to CCR3 selectively, whereas all eotaxin isoforms almost exclusively

bind to CCR3 (Giembycz & Lindsay, 1999). Given that ≥95% of all eosinophil-chemoattractant interactions are mediated by the CCR3 receptor, eotaxin is thought to sub-serve an especially critical role in eosinophil migration to the lungs.

Mechanistically, eotaxin-1 binding to CCR3 has been shown to activate the $G_{q/11}$ pathway, which culminates in the activation of membrane-bound phospholipase C (PLC) (Amerio et al., 2003; Kampen et al., 2000). PLC catalyzes the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) into inositol-1,4,5-trisphosphate (IP_3) and diacyl-glycerol (DAG). By virtue of its hydrophobic properties, DAG remains associated with the cell membrane while hydrophilic IP_3 molecules freely diffuse into the cytosol, where they bind to IP_3-gated calcium channels (IP_3R) expressed on the endoplasmic reticulum (ER) (Berridge et al., 2003). IP_3R activation leads to calcium-release from intracellular calcium stores, which may further stimulate Ca^{2+} ion release via nearby, ER-bound Ryanodine receptor channels (RyRs) through a process known as calcium-induced calcium release (CICR) (Sanders, 2001).

Alongside its role in migration, Ca^{2+} regulates several cell functions, including excitation-contraction, hormone secretion, cell survival/death and gene transcription (Schwab et al., 2012; Ramsey et al., 2006; Janssen et al., 2009). Consequently, mammalian cells utilize membrane-bound channel proteins and ion pumps in order to maintain the prevailing intracellular calcium ion concentration ($[Ca^{2+}]_i$) within a physiologically tolerable, sub-nanomolar range (Berridge et al., 2003). Following agonist-induced increases in $[Ca^{2+}]_i$, several families of Ca^{2+} pumps and exchangers extrude Ca^{2+} ions from the cytosol. These include the sodium-calcium exchanger (NCX) and the plasma membrane calcium ATPase (PMCA) pump expressed on the plasmalemma, and the sarcoplasmic-endoplasmic reticulum calcium ATPase (SERCA) pump expressed on the ER (Berridge et al., 2003). Collectively, these mechanisms of

Ca^{2+} homeostasis coordinate the flux of Ca^{2+} into and out of the cell and therefore transduce the migratory effects of eotaxin.

1.4.3 Directional Eosinophil Migration

In human and non-human eosinophils, eotaxin binding to CCR3 raises the $[Ca^{2+}]_i$ and triggers cell migration and/or degranulation in a strictly calcium-dependent manner (Rothenberg et al., 1996; Giembycz & Lindsay, 1999). The fact that cell migration depends on the coordinated adhesion of the front of the cell with detachment of the rear of the cell, however, implies that Ca^{2+} ions and other resources are somehow spatially divided within migrating eosinophils (Schwab et al., 2012; Elsner et al., 1996). Indeed, Newt eosinophil migration has been shown to depend on an intracellular calcium gradient that spatio-temporally modulates actin (de)polymerization and myosin II contraction (Walker et al., 1998). Given that Newt CCR3 receptors couple to the PLC pathway, agonist-binding generates IP_3 and DAG molecules in cell regions that are closest to the chemotactic source (Brundage et al., 1993; Brundage et al., 1991; Fay et al., 1995). Hydrophobic DAG remains bound to the cell membrane and acts in concert with PKC to attenuate calcium-influx at the chemokine-facing region of the cell. In this manner, $[Ca^{2+}]_i$ is low at the anterior of the cell but high at the posterior of the cell (Brundage et al., 1993; Brundage et al., 1991).

In certain species, the $[Ca^{2+}]_i$ is similarly high in the posterior of migrating neutrophils where region-specific activation of the calcium-sensitive protease, calpain, is thought to occur (Lokuta et al., 2003). Activated calpain at the rear of the cell serves to cleave interactions between cytoskeletal actin and actin-binding proteins at the plasmalemma (Bhadriraju et al., 2007). Actin depolymerisation leads to retraction of the posterior cell membrane towards the cell body (Potter et al., 1998; Perrin et al., 2006). In *Dictostellium* amoebae and in Newt eosinophils,

this inward recoil of the posterior cell membrane is further reinforced by contraction of myosin fibres subsequent to myosin phosphorylation by calcium-dependent myosin light chain kinase (MLCK; Perrin et al., 2006).

This general description of directional cell migration primarily describes how eosinophils navigate within the interstitium towards their final destination within the tissues (Friedl & Gilmour, 2009; Borchers et al., 2002). While the exact mechanism of human eosinophil migration has yet to be confirmed definitively, it is evident that the prevailing model of chemotaxis can only apply to eosinophils that have extravasated into the tissues and are poised to unleash their cytotoxins. By extension, the pathological accumulation of eosinophils in the asthmatic airway is contingent upon the initial entry of eosinophils into the pulmonary interstitium from the vascular compartment. Strictly speaking, eosinophil departure from the vascular circulation necessarily precedes its migration and degranulation, and thus constitutes an altogether different aspect of the eosinophil recruitment program that merits greater scrutiny.

1.5 Multi-step Paradigm of Leukocyte Extravasation

Eosinophils capture at the post-capillary endothelium comprises an early, critical event in the development of eosinophilic inflammation and disease (Ulfman et al., 2001; Williams et al., 2011; Weis, 1994). Eosinophil recruitment to the airway tissues is a dynamic, multi-layered event that involves bi-directional communication between eosinophils and endothelial cells (ECs), which line the luminal surface of the blood vascular capillary. ECs thus constitute a physical barrier that eosinophils must traverse in order to enter the airway interstitium (Wang et al., 2007; Ulfman et al., 2001). Given its indispensible role in eosinophilic inflammation, eotaxin must guide eosinophils to the lungs by imparting the instructive signals needed for them to

surpass the endothelium. This unfolds as part of a 4-step sequence of events, which comprise: rolling, adhesion, crawling and transmigration (Gonlugur & Efeoglu, 2004). This process, broadly referred to as extravasation, is governed by the serial and cooperative engagement of a series of adhesion receptor molecules variously expressed on the surface of the eosinophil and the EC (Middleton et al., 2002; Gonlugur & Efeoglu, 2004). While extravasation categorically results in the transmigration of cells into the interstitium, it is important to note that one or more of the preceding stages may not always occur as eosinophils extravasate into the tissues.

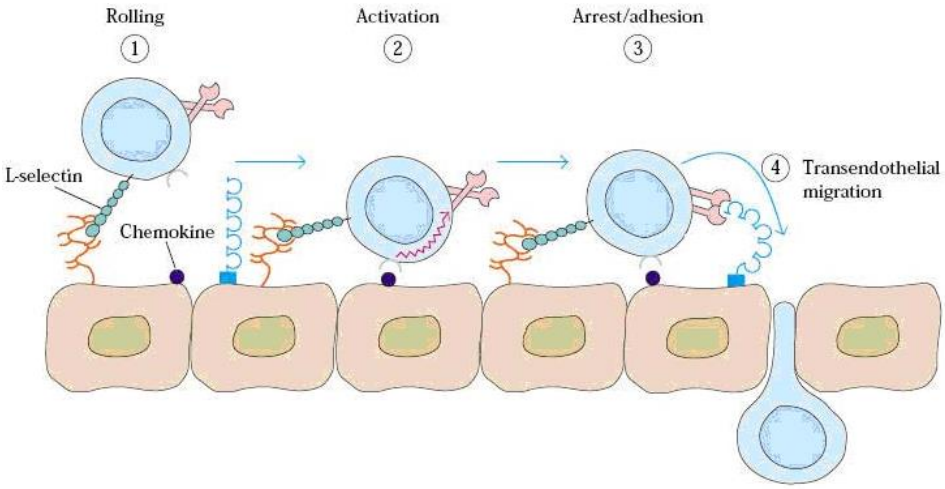


Figure 4. Eosinophil extravasation occurs at the vascular endothelium. Eosinophil recruitment entails four functionally distinct steps that collectively anchor the leukocyte to the endothelium in order to facilitate its migration into the interstitium. Eosinophil rolling (1) occurs as the eosinophil briefly and weakly interacts with the endothelium. The L-selectin receptors expressed on the eosinophil engages glycoproteins expressed on the activated endothelium, which slows the cell down long enough for it to become activated (2) subsequent to chemokine binding (Spoelstra et al., 1998). Chemokine-binding triggers an intracellular signal transduction cascade that leads to integrin activation. Once activated, integrins bind to their ligands expressed on the surface of ECs, causing the cell to arrest (3) on the endothelium. Following its arrest/adhesion, the cell crawls (not shown) towards transendothelial junctions, between which it migrates (4). At the outset of transmigration, the eosinophil extends cytoskeletal projections, known as podosomes, towards the basement membrane and into the interstitial space. From this point onwards, the cell's journey is dictated by the gradient of chemokine molecules produced within the interstitium by inflammatory cells (Image from Huttenlocher & Horwitz, 2011).

Eosinophil extravasation begins as the cell rolls on the vascular endothelium and forms weak, transient bonds between the selectin family of adhesion receptors and their counter-ligands

(Grailer et al., 2009). Selectins are preferentially expressed on the vascular endothelium during inflammation and on inactive, circulating leukocytes (Calvey & Toledo-Pereyra, 2007). Structurally, selectins are monomeric transmembrane glycoproteins that bind to carbohydrate residues via conserved lectin-binding domains (Appendix 1; Panes & Granger, 1998). Selectin-binding assists eosinophils in adhering firmly to the endothelium by bringing the endothelial and eosinophil membranes in close proximity to one another (Von Andrian et al., 1992; Metcalf et al., 2008; Cheung et al., 2011). In the lungs, eosinophil adhesion can altogether bypass any selectin-mediated tethering of the cell (Gonlugur & Efeoglu, 2004). In this case, firm adhesion occurs directly and is initiated by a second, distinct class of cell adhesion receptors, known as integrins (Berton & Lowel, 1999).

1.6 Integrin Adhesion Receptors

Integrins are heterodimeric, transmembrane proteins composed of an α- and a β-subunit (Mould et al., 2003). Integrin receptors, in contrast to the selectins, display tremendous structural and functional complexity (Baldwin et al., 1998). Integrins are expressed on eosinophils and bind to cell adhesion molecules (CAMs) on the endothelial surface or to extracellular matrix (ECM) proteins (Barthel et al., 2006a). In mammals, there are 18 different α- and 8 different β-subunits, any two of which may dimerize in order to form a unique integrin receptor with distinct ligand-binding and signaling capabilities (Humphries, 2000). By virtue of this diversity, many different integrin receptor subtypes exist and participate in leukocyte recruitment.

Firm adhesion of eosinophils primarily involves two α_4- integrins: lymphocyte Peyer patch associated molecule (LPAM, or $\alpha_4\beta_7$) and very late antigen-4 (VLA-4, or $\alpha_4\beta_1$) (Appendix 2, Table describing expression of integrins in eosinophils; Gonlugur & Efeoglu, 2004;

Tachimoto et al., 2000; Spoelstra et al., 1998). Integrins can bind to a wide range of ligands with considerable overlap (Figure 5; Barthel et al., 2008). In the gastrointestinal tract, LPAM preferentially binds to the highly expressed mucosal addressin cell adhesion molecule-1 (MAdCAM-1). In the lungs, however, LPAM primarily interacts with vascular cell adhesion molecule 1 (VCAM-1), the expression of which is greatly up-regulated on the inflamed capillary endothelium in response to the production of pro-inflammatory cytokines (*e.g.*, tumor necrosis factor (TNF)-α; Gonlugur & Efeoglu, 2004). VCAM-1 is also capable of binding to VLA-4, although these interactions are thought to be mediated by different binding domains within the VCAM-1 molecule itself (Figure 5; Gonlugur & Efeoglu, 2004). In spite of its apparent promiscuity, VCAM-1 interactions are uniquely critical to the eosinophil recruitment cascade since eosinophils express higher levels of the cognate α_4- integrins than do neutrophils, the exception being a small sub-population of immature neutrophil precursor cells (Gonlugur & Efeoglu, 2004; Diamond et al., 1990).

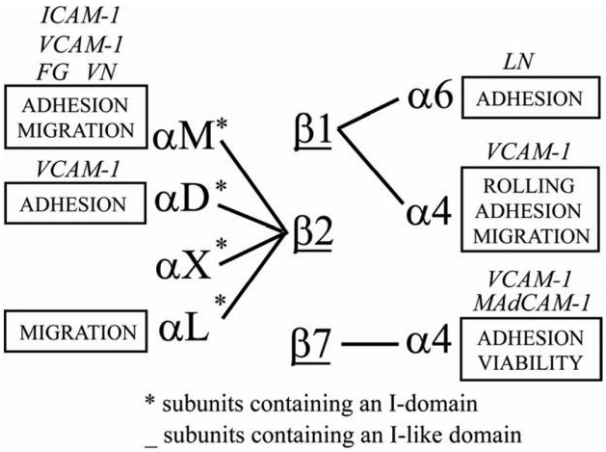
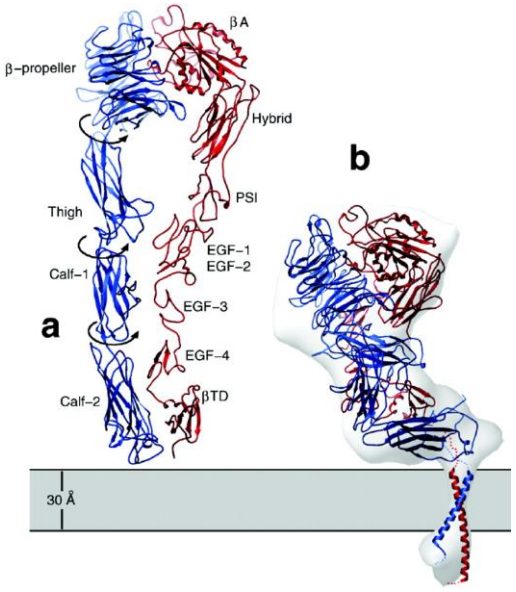


Figure 5. Integrins interact with a diverse range of ligands expressed in the ECM and on ECs. Integrins vary remarkably in their ligand-binding specificities, which are determined the by the receptors' constituent α- and β-subunits (Staunton et al., 1988). Eosinophil recruitment in the lungs is predominantly mediated by VCAM-1, which binds to the $\alpha_4\beta_1$ and to the $\alpha_4\beta_7$ integrin receptors. Eosinophils also express the $\alpha_M\beta_2$ (CD11b, Mac-1 receptor) integrin, which orchestrates a wide range of binding activities (Lim & Hotchin, 2012). Incidentally, $\alpha_M\beta_2$ also facilitates eosinophil activation consequent to complement binding (Image from Barthel et. al., 2008).

1.7 Conformational Regulation of Integrins by Chemokines

Like all other integrin receptor subtypes, LPAM and VLA-4 exist in two distinct conformational states that exhibit varying degrees of affinity for their counter-ligands (Johansson & Mosher, 2013). The lowest-affinity state predominates in unstimulated eosinophils and is characterized by an inwardly retracted, extracellular ligand-binding domain (Figure 6; Humphries, 2000). This folded conformation sterically hinders ligands from efficiently engaging the binding-site on the integrin receptor, which precludes the unnecessary adhesion and extravasation of eosinophils to otherwise healthy tissues (Johansson et al., 2006).



<u>Figure 6.</u> Structural conformations of integrin receptors. The 3-D structure of $\alpha_2\beta_3$ has been determined from x-ray crystallography studies. (a) The left hand structure exemplifies the high-affinity state of the integrin receptor, in which the ligand-binding pocket is extended outward and available to interact with its ligand(s). (b) The same integrin receptor is shown on the right in a low-affinity conformational state. In this state, the binding-pocket located in between the β-propeller and βA domains is sterically hindered. The integrin binds its ligand with low affinity in this state, indicating that while interactions may still occur, these are generally considered to be weak and of little biological significance. (Image from Adair & Yeager, 2002).

The fact that integrins primarily occupy a low-affinity state on inactive leukocytes constitutes a homeostatic mechanism by which unwarranted eosinophil extravasation and activation are controlled (Humphries, 2000). Before eosinophil adhesion can occur, an intracellular signal must therefore prime the inactive integrin to adopt its high-affinity, adhesive conformation (Barthel et al., 2008). The putative model of leukocyte extravasation suggests that

integrins undergo this low-to-high affinity conformational transition subsequent to chemokine-binding (in't Veen et al., 1998). Chemokine-binding therefore constitutes an upstream event to integrin-mediated eosinophil adhesion and transmigration (Gane & Stockley, 2012). In the context of eosinophil recruitment, eotaxin-binding to CCR3 must generate an 'inside-out' signal that activates integrin receptors (Shattil et al., 2010). In eosinophils, many studies suggest that this signal is relayed by a network of secondary messengers and downstream effectors mobilized following activation of the PLC pathway by agonist-binding to CCR3 (Amerio et al., 2003). In this regard, eotaxin permits eosinophil integrins to bind to their counter-ligands on the endothelial surface and/or in the ECM during adhesion and transmigration (Figure 7; Cheresh & Mecham, 1994; Tachimoto et al., 2002).

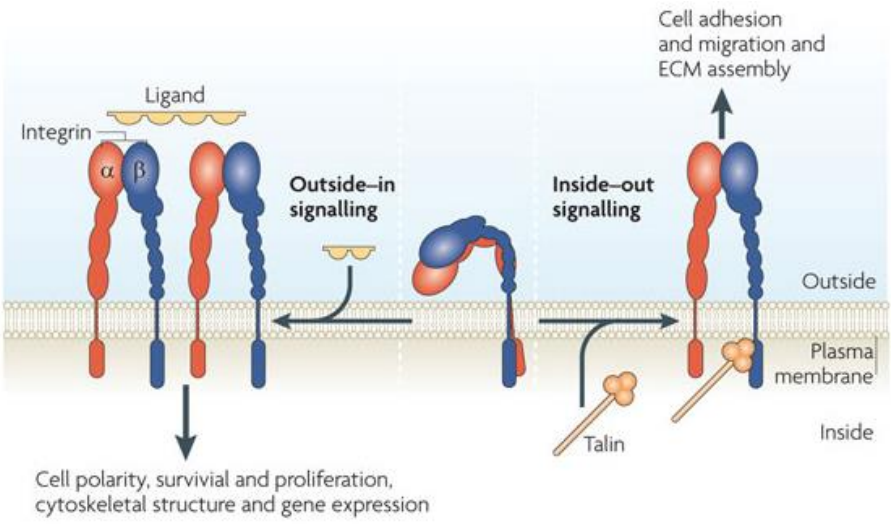


Figure 7. Integrins undertake bidirectional signaling. Integrins reside in one of two conformational states. The inactive, inwardly-bent conformation (centre) prevails on the surface of leukocytes during homeostatic conditions. During extravasation, chemokine-binding initiates an inside-out signal transduction cascade that modifies the cytoplasmic tail of the integrin. This induces a conformational change in the receptor and permits the ligand-binding domain to swing outward (right). In this state, the integrin may bind to its counter-ligand in the ECM or on ECs during adhesion or migration. Binding of the integrin to its counter-ligand initiates an outside-in signal that precipitates further changes in cell function and structure (left). In this regard, integrins serve as prolific signaling hubs that act on the cell-surroundings and that, in turn, permit the surroundings to act on the cell (Image from Barthel et. al., 2007).

Activated integrins bind to their ligands on the extracellular surface. Ligand-binding in turn triggers a reciprocal, inwardly-directed 'outside-in' signal that evokes changes in cell morphology (Johansson & Mosher, 2013). In some cases, this outside-in signal recruits ubiquitously expressed cytoskeletal proteins, like talin, which engage the N-terminal clasp on the

cytosolic surface of the integrin and regulate the activity of actin binding proteins at the plasmalemma (Johansson & Mosher, 2013). Outside-in signaling thereby modulates cell adhesion, polarization and crawling (Massena et al., 2011; Barthel et a., 2006b; Brundage et al., 1991; Fay et al., 1995). Interestingly, tissue and bronchoalveolar lavage fluid (BALF) eosinophils isolated from asthmatic subjects display a higher proportion of high-affinity integrins on their surface relative to healthy controls, suggesting that aberrant integrin activation may precipitate their pathogenic, hyper-adhesive phenotype (Barthel et al., 2006b).

Despite the widely-accepted view that eotaxin recruits eosinophils to the asthmatic lung, the mechanism by which eotaxin activates integrins *in vivo* is not yet fully understood (Witt & Lander, 1994). From a physiological perspective, eotaxin is produced and retained in the airway interstitium during inflammation and the ever-present flow of blood in the vascular compartment precludes the possibility of continuous, chemical gradients forming between the vessel lumen and the inflamed interstitium (Fay et al., 1995; Witt & Lander, 1994). From this perspective, it is generally unclear how leukocytes sense and respond to chemokine signals, raising a key, fundamental question: how does eotaxin-1 modulate the activity of integrins and facilitate eosinophil adhesion if cells do not encounter the chemokine while they transit through the inflamed vasculature (Rot, 1994; Kreuger et al., 2006).

The current paradigm of leukocyte extravasation attempts to resolve this question by suggesting that chemokines generated within the pulmonary interstitial spaces are transported from the basolateral to the apical endothelial membranes via transcytosis (Middleton et al., 1997). Once transcytosed, chemokine molecules are retained at the endothelial surface via non-covalent interactions with heparan-sulfate proteoglycans (HSPGs) expressed in the glycocalyx

(Kuschert et al., 1999; Pablos et al., 2003). HSPGs are primarily negatively-charged whereas most chemokine molecules are positively-charged (Sadir et al., 2004). The resultant interaction between HSPGs and most pro-inflammatory chemokines is electrostatically favourable and may serve to immobilize eotaxin at the inflamed capillary bed (Proudfoot et al., 2003). Early histological studies of neutrophilic inflammation provide convincing evidence in support of this view. Middleton et al. (1997) showed that the level of IL-8 binding to the vascular endothelium directly corresponds to the number of neutrophils that infiltrate those tissue regions. In this manner, chemokines presumably decorate the blood vascular endothelial surface and demarcate the boundaries at which eosinophil extravasation occurs.

HSPGs exhibit tremendous promiscuity in their interactions with chemokines, however, and their roles in chemokine-presentation have primarily been studied in the context of non-sterile neutrophilic inflammation (Forsberg & Kjellen, 2001; Kreuger et al., 2006; Lortat-Jacob et al, 2002). As a result, much of our current understanding of eosinophil extravasation has been inferred from these early studies, despite the fact that HSPG expression is remarkably heterogeneous with respect to tissue type, species and/or genetic factors (Lortat-Jacob et al., 2002; Massena et al., 2010; Wu et al., 1996; Holub et al., 2003). The few studies that have nevertheless focused on eotaxin-HSPG interactions failed to adequately discriminate between the monomeric and the multimeric forms of eotaxin generated following HSPG-binding, which differ in their affinities for CCR3 (Sadir et al., 2004; Goger et al., 2002; Handel et al., 2005).

With regard to monomeric eotaxin bound to HSPG, binding studies show that the chemokine's interaction with HSPG is more favourable than its interaction with its cognate receptor, CCR3 (K_d<5 nM *vs.* K_d=0.4 µM, respectively; Ellyard et al., 2007;). Ultimately, the association of eotaxin with HSPGs might therefore serve to diminish, rather than to promote,

integrin activation as HSPGs prevent eotaxin from binding to CCR3. Most importantly, *in vivo* evidence has yet to be uncovered corroborating the functional role of the HSPG-chemokine association in the recruitment of inflammatory effector cells (Ellyard et al., 2007). Whether eotaxin oligomers presented on the endothelium are even capable of binding and activating CCR3 is therefore unclear.

1.8 Hypothesis

Various studies showcase the central role of Ca^{2+} in eotaxin-mediated eosinophil migration, but far fewer have sought to explore how the eotaxin-Ca^{2+} cascade modulates integrin activity, especially considering that: integrins directly modulate changes in cell shape; that many of the proteins that regulate cytoskeletal architecture are Ca^{2+}-dependent; and that sufficient evidence highlights that HSPG-bound eotaxin might fail to bind to its cognate receptor on eosinophils transiting through the microvasculature (Lortat-Jacob et al., 2002; Forsberg & Kjellen, 2001; Ellyard et al., 2007).

The finding by Liu et al. (1999) that eotaxin-1 fails to induce eosinophil migration when Ca^{2+} ions are completely chelated out of the preparation categorically emphasizes the calcium-dependence of eosinophil homing to the tissues. The structural and functional properties of eosinophils vary considerably across species, however, motivating the need to better identify the ion channels and the calcium pools involved in human eosinophils *per se* (Giembycz & Lindsay, 1999; Mestas & Hughes, 2004). Ultimately, a deeper understanding of the Ca^{2+}-handling mechanisms may provide substantial insight into how eotaxin augments $[Ca^{2+}]_i$ and how Ca^{2+}, in turn, modulates integrin receptor activity.

Over the last decade, improvements in genetic, molecular and cellular biology techniques have contributed towards our understanding of inflammation in the context of chronic illness. Although it is unclear whether airway inflammation is a cause or a consequence of asthma, the association between the two is consistent (Kay, 1991; Kay, 2005). Whether evoked by allergens (house dust mite, cat hair) or by irritants (heavy metals, nebulized microparticles), airway inflammation precipitates the influx of inflammatory effector cells into the tissues. Nevertheless, the question of how eotaxin mediates integrin activation (and therefore eosinophil adhesion)

poses a logistical dilemma that must be reconciled with our understanding that eosinophil recruitment is simultaneously regulated by eotaxin and integrin receptor signaling. To these ends, the advent of confocal microscopy has proven to be an effective technique for studying calcium handling events in live-time (McCormack & Cobbold, 1991). Furthermore, advances in optical and laser technology over the past decade have improved the resolution with which intracellular phenomena can be observed (Paredes et al., 2008). We consequently set out to characterize the calcium-handling mechanisms that mediate the eotaxin response in human eosinophils using confocal fluorescence microscopy.

While previous studies used monolayers of millions of cells, our set-up allows us to study fluctuations in cytosolic calcium in individual eosinophils alongside changes in cell function and morphology. By imaging real-time fluctuations in $[Ca^{2+}]_i$, we seek to elucidate the calcium-handling pathways involved in eotaxin signaling (McCormack & Cobbold, 1991). *We hypothesize that the migratory response of eosinophils evoked by eotaxin is carefully orchestrated by a combination of the internal and external calcium pools, and that this dynamic process depends critically on the concerted function of several ion channel families expressed on the plasmalemma and the endoplasmic reticulum. Further to this, we hypothesize that eosinophil activation is a phenomenon that depends on an additional stimulus that works in concert with eotaxin to facilitate eosinophil recruitment.*

Chapter 2: Materials and Methods

2.1 Granulocyte Isolation from Peripheral Human Blood

Ethical approval was granted by the institutional ethics committee for all experiments involving human subjects. Blood was obtained from these subjects and the granulocyte layer was isolated from each blood sample as per the methods described by Sedgwick et al. (1996). Briefly, approximately 30 mL of peripheral blood was collected from consenting adult male or female subjects into heparin-containing vacutainers (BD Bioscience, MD, United States). The whole blood was then diluted in equal parts by volume into McCoy's 5A Medium and mixed thoroughly. Approximately 25 mL of the diluted blood was gently layered onto 15 mL Percoll density gradients and centrifuged (Heraeus Instruments, Osterode, Germany) at 800xg for 20 minutes with no brake. The supernatant was removed and the bottom-most layer comprising the granulocyte fraction of cells was transferred into fresh tubes. From this point onward, all samples were kept on ice.

In order to lyse the erythrocytes, chilled ammonium chloride (NH_4Cl) was added to the mixture for a final volume of 50 mL. The mixture was inverted several times and subsequently incubated on ice for approximately 15 minutes, or until it appeared blackish-red. The mixture was then centrifuged at 300xg for 10 minutes with brake. The supernatant was removed and the whitish pellet was gently re-suspended in NH_4Cl solution and incubated on ice for approximately 15 minutes, as described previously. The cell suspension was centrifuged and re-suspended in NH_4Cl solution at least twice more in this manner in order to thoroughly lyse any remaining erythrocytes. After the final lysis, the bright pink granulocyte pellet was washed and re-suspended in 5 mL of MACS buffer solution.

2.2 Eosinophil Enrichment

The granulocyte mixture was centrifuged at 300xg for 10 minutes with no brake and the supernatant was removed. Eosinophils were purified by negative selection from the granulocyte fraction using MACS magnetically-labelled CD16-selective MicroBeads (Miltenyi Biotec, MA, United States). As per the manufacturer's instructions, cells were co-incubated with 50 µL magnetic microbeads (per $50x10^6$ cells) and 50 µL MACS buffer solution for approximately 60 minutes in the dark and on ice (Sedgwick et al., 1996; Roberts & Gallin, 1985). Cells were subsequently washed with 5 mL MACS buffer before being centrifuged at 300xg for 10 minutes. The supernatant was removed and the pellet was re-suspended in 500 µL of MACS buffer.

The magnetically-labelled cell suspension was pre-filtered using MACS pre-separation filters (Miltenyi Biotec, MA, United States) in order to remove large chunks of debris. The filtered suspension was subsequently added drop-wise to an MS separatory column (Miltenyi Biotec, MA, United States), which was placed in a MACS magnetic cell separator (Miltenyi Biotec, MA, United States). Neutrophils express high levels of the CD16 molecules on their surface. As a result, neutrophils are readily extracted from the mixture as the suspension elutes through the separatory column in the presence of a magnetic field. The eluate is highly enriched in eosinophils and is collected and subsequently re-suspended in RPMI 1640 Media for use in calcium studies. While this procedure also tends to increase basophil counts, the extent to which this occurs has been found to be negligible. Blood samples were not differentiated with regards to the donor's disease status. For most subjects, the final eosinophil count was found to be between ~$1\text{-}5x10^6$ cells. Cell viability was assessed using a Trypan blue stain, which revealed >90% cell viability using this isolation protocol. Using this method of isolation yields a purified

population of eosinophils, where ~90-95% of cells are eosinophils and the remaining contaminating cells are neutrophils and/or basophils.

2.3 Confocal Fluorescence Microscopy

2.3.1 Experimental Set-up and Recordings

Approximately $1x10^6$ cells were loaded with the calcium-binding dye, Fluo-3 AM (3.5 μM in DMSO with 0.01% pluronic acid), and incubated in the dark for 60 minutes at room temperature. Cells were subsequently placed in between two glass slides that were separated by a thin layer of silicone, as demonstrated by Figure 8 below. Initially, we sought to characterize how eotaxin-1 affects calcium handling in eosinophils by applying various combinations of pharmacological agents to cells using a gravity-controlled fluid reservoir. The heights of these reservoirs could be easily adjusted and permitted us to control the rate at which drugs/fluids were applied. Although basophils are also known to express CCR3, these cells are known to constitute a small proportion of the total granulocytes isolated by this method. While neutrophils also contribute to contamination of the cell population, they do not express the CCR3 receptor and therefore do not respond to eotaxin-1.

As our research question evolved, we sought to determine how fluid shear stress might also modulate $[Ca^{2+}]_i$ in eosinophils. We consequently used this drug-delivery system to simulate fluid shear stress by perfusing cells with RPMI 1640 (unless otherwise noted). Our 'sandwich preparation' provided a convenient set-up for conducting flow experiments as it resembles the parallel-plate flow chambers used by other groups to study the physiological effects of fluid shear stress on other cell populations.

During flow experiments, cells were perfused with a continuous stream of physiological solution. The rate of fluid perfusion was maintained at a constant level for all flow experiments by assuring that the height of the reservoir and the column of the fluid was consistent between all experiments. In some cases, cells were layered on glass slides that were coated with either rat tail collagen (25 µg/cm^2) or with bovine fibronectin (2-4 µg/cm^2). Experiments were conducted at room temperature and typically lasted an average of approximately 45-60 minutes. All recordings were collected using a personal computer running *Windows XP* operating system (Microsoft Corporation, Redmond, WA, United States).

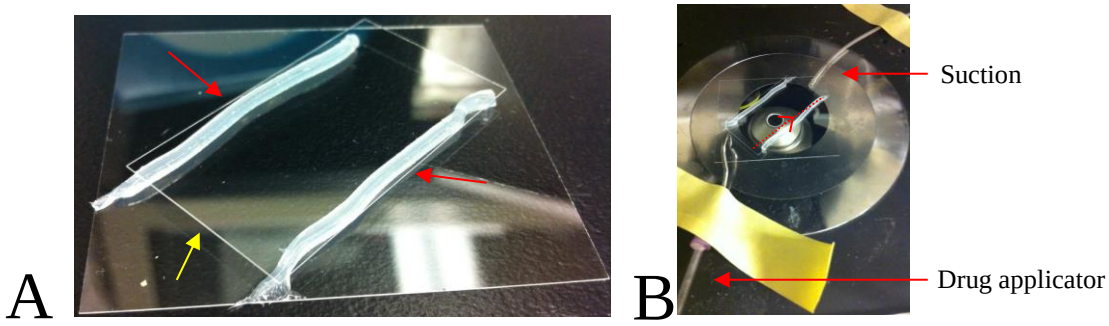


Figure 8. Overview of experimental set-up. **(A)** Cells were placed on a 5 x 4.5 cm glass cover slip. Two thin strips of silicone (red arrows) were then applied diagonally, onto which a second 2 x 4 cm glass slide was gently placed. The "sandwich" preparation provided a decisive gap through which drug-containing, physiological salt solution could be drawn towards the cells by capillary action (yellow arrow). **B**: Drugs and fluids were applied at one corner and aspirated via suction at the opposite corner. The rate at which drugs/fluids perfused through the preparation was controlled by adjusting the heights of the reservoirs relative to the ground (not shown). Fluid that was drawn through the chamber was siphoned away by a vacuum suction that was placed at the opposite corner. In this manner, a variety of drugs and solutions could be rapidly added to cell preparation.

2.3.2 Calcium Imaging Experiments

Cells were viewed using an inverted Nikon Eclipse TE2000-4 microscope (Mississauga, ON, Canada) with a 20x objective. Intracellular calcium recordings were obtained using a custom-built apparatus. During recordings, eosinophils were scanned with a 20 mW photodiode laser (Coherent Technologies, CA, United States) at 488 nm in the X- and Y- plane using two mirrors oscillating at 8 kHz and 30 kHz, respectively. The fluorescence light emitted at 510 nm was measured by a photomultiplier, which digitized the signal and produced .tiff images at a resolution of 480x400 pixels. These .tiff images were created by averaging 8 images recorded in rapid succession every 2 s at a frame rate of 30 Hz. *Video Savant* v4.0 software (IO Industries, London, ON, Canada) was used to generate videos by stacking these .tiff images in chronological order. Averaging images proved especially effective in reducing the background "noise" during recordings. Unless otherwise noted, all recordings were consequently obtained at this frame rate.

2.4 Data Analysis

2.4.1 Fluorescence measurements and cell tracking

Eosinophils are highly mobile cells that rapidly change their size and position. This posed a particular challenge for precisely quantifying fluorescence in eosinophils that moved around in the field of view (FOV). To address this issue, we used the *Particle Analysis* plug-in of the open platform software, *ImageJ* v1.6r (National Institutes of Health, Bethesda, MD, United States), in order to simultaneously track the spatial location and the fluorescence intensity of a given cell at each point in time (Figure 9).

Eosinophils that are loaded with Fluo-3 dye only exhibit fluorescence when there is an increase in $[Ca^{2+}]_i$. Since all fluorescence recordings are encoded in 8-bit, the constituent pixels of a fluorescence image are assigned an intensity value that ranges from 0 (darker, lower calcium) to 255 (brighter, higher calcium). As with most eukaryotic cells, the intracellular calcium ion concentration tends to vary widely within the eosinophil cytosol and between sub-cellular compartments. At any given point in time, the $[Ca^{2+}]_i$ of an eosinophil is therefore given by the average value of all pixels that combine to form the fluorescence image of that cell (Figure 9).

We determined average cell fluorescence using a multistep analysis approach. Briefly, confocal images were first subjected to a process referred to as "thresholding", whereby the grayscale fluorescence image was transformed into a black-and-white binary image. During this process, pixel values <24 were re-assigned a value of 0 and pixel values >25 were re-assigned a value of 255 (Figure 9C). In this manner, low levels of calcium at the periphery of a cell could be easily detected and readily observed.

The built-in particle analysis module of *ImageJ* then generated regions of interest (ROIs) from the binary image "map" of each cell. Using these ROIs, we then measured the mean fluorescence in a given cell at each frame of the recording. Thus, our analysis technique allowed us to measure a number of qualitative parameters, including the area, position, speed of movement and shape of the cell. In some cases, however, cells could not be automatically tracked using the *ImageJ* plug-in, and were instead manually tracked using the open source *Manual Tracking* plug-in available at http://rsb.info.nih.gov/ij/plugins/track/track.html. (Institut Curie, Orsay, France). In these instances, the cell's centroid position was manually determined for each cell at frame of a recording.

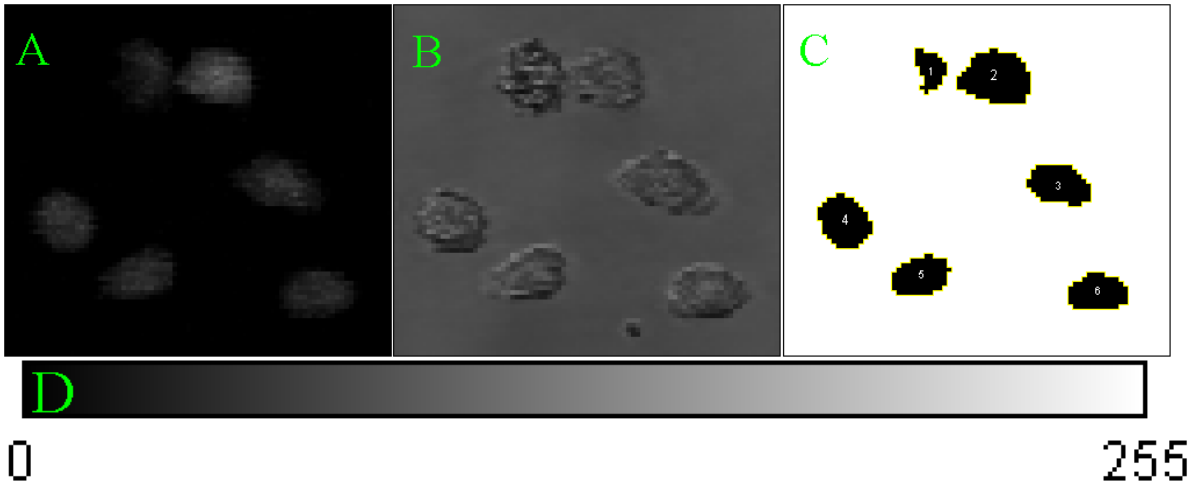


Figure 9. Processing Fluorescence Images. **(A)** *Confocal fluorescence image.* The grayscale confocal image depicts the heterogeneous distribution of calcium within these cells. Regions with higher $[Ca^{2+}]_i$ are brighter and regions with lower $[Ca^{2+}]_i$ are darker. **(B)** *Light image.* The corresponding light image of these same cells demonstrates that fluorescence remarkably corresponds well with the position of a given cell. The centroid of a cell's fluorescent image can therefore be used to describe the spatial position of that cell. **(C)** *Binary image.* The thresholded, binary image derived from the confocal image is used to calculate regions of interest (ROI) corresponding to each cell. These ROIs (enumerated 1 through 6) can be used to measure fluorescence over the entirety of each cell in the corresponding confocal image (in panel *A*). **(D)** *Fluorescence intensity scale.* As $[Ca^{2+}]_i$ increases at a given point in the cell, the pixel value also increases up to a maximum possible value of 255. These shades of gray are eliminated during the thresholding process as pixel values are re-assigned a value of 0 or 255 depending on where they fall along this gray-scale spectrum.

2.4.2 Signal Processing and Quantification of Calcium Response

Where applicable, tracings serve to display average fluorescence in a single cell as a function of time. Figure 10 consequently depicts a typical fluorescence *vs.* time tracing obtained from a recording. Upwards deflections indicate a rise $[Ca^{2+}]_i$ and downwards deflections denote a decrease in $[Ca^{2+}]_i$. The drastic nature in which $[Ca^{2+}]_i$, fluctuates even under baseline conditions creates ambiguity in interpreting the effects of a pharmacological compound. In some cases, responses were processed using a 7-car box filter, which averaged the cell's fluorescence intensity in any given image with the three adjacent frames that immediately precede and follow the frame in question.

Broadly speaking, $[Ca^{2+}]_i$ fluctuations in other cell types (neurons, myocytes, fibroblasts) occur as countable spikes that are otherwise absent in eosinophils. Nevertheless, the total measurable fluorescence of a given cell should change as $[Ca^{2+}]_i$ increases or decreases. For these reasons, we quantified calcium responses in eosinophils in terms of the total, cumulative fluorescence measured in a cell over the time period prior to and immediately after application of a treatment. As demonstrated by Equation (1) and Figure 10, changes in $[Ca^{2+}]_i$ can be described by the ratio of the area under the curve (AUC) in the presence of a drug (AUC_D), to the AUC in the absence of that drug (AUC_{ND}):

$$AUC_f\ Ratio = \frac{AUC_D}{AUC_{ND}} \qquad (1)$$

In order to calculate a meaningful fluorescence AUC ratio (AUC_f Ratio), the time intervals over which the AUC_D and the AUC_{ND} are measured must be of equal length and should encompass segments of the curve that lie immediately adjacent to the frame at which a treatment was applied (Figure 10). The AUC_f Ratio is a dimensionless parameter that effectively describes the extent to which a treatment increased or decreased $[Ca^{2+}]_i$. As a result, the AUC ratio

provides a succinct description of whether a response increased (AUC>1), decreased (AUC<1) or did not affect (AUC=1) $[Ca^{2+}]_i$.

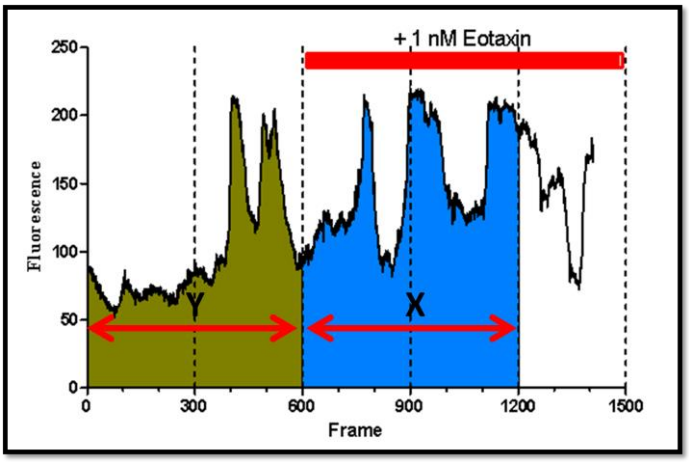


Figure 10. Quantifying changes in $[Ca^{2+}]_i$. In this representative tracing, the rise in cellular calcium cannot be easily discerned by counting the peaks of the oscillations in fluorescence. Consequently, dividing AUC_D measured following application of agonist (X, in blue) by the AUC_{ND} measured prior to application of agonist (Y, in gold) yields an AUC ratio that is greater than 1. Note that the segment of the curve beyond frame 1200 is not included in the calculation.

2.5 Statistical Analysis

GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, United States) and SigmaPlot 12.5 (Systat Software Inc., San Jose, CA, United States) were used to conduct all statistical analyses and to generate all figures. Where applicable, data are expressed as mean values ± SEM. Multiple cells (approximately 5-10) were analyzed from a given subject, and the responses from many different subjects were pooled in order to generate this mean value. For our purposes, sample sizes refer to the number of unique subjects in which each experimental condition was replicated.

Statistical analysis involved one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test for comparing the difference of means. In all cases, $p<0.05$ was considered to be significant. Reported sample sizes describe the number of donors in which a given response was replicated. The average response was measured in multiple (≥ 5) cells from a given donor. This value was used to compute an overall, pooled mean that describes the average response in *n*-number of unique subjects. Sample sizes consequently refer to the total number of unique participants in which a given response was replicated.

2.6 Chemicals and Reagents

Percoll was obtained from VWR (Mississauga, ON, Canada) and density gradients were prepared in Dulbecco's Phosphate Buffered Saline (DPBS) and HBSS at a concentration of 65% (v/v). Recombinant human eotaxin-1 (CCL11) was obtained from Reprokine (Tel Aviv, Israel). Ryanodine, gadolinium chloride ($GdCl_3$) 2-aminoethoxydiphenyl borate (2-APB), cyclopiazonic acid (CPA) and SKF 96365 were all obtained from Tocris (Burlington, ON, Canada). Ammonium chloride lysis buffer used during granulocyte isolation contained (in mM): 155 NH_4Cl, 10 KCO_3 and 0.1 ethylenediaminetetraacetic acid (EDTA), pH 7.2. All reagents were dissolved in RPMI 1640 Media, which sometimes contained 10% FBS and P/S (Fisher Scientific, Walkersville, MD, United States), or in (Ca^{2+}-free) HBSS (Gibco, Grand Island, NY, United States), pH 7.2-7.4. DPBS, McCoy's 5A Medium and fluo-3 AM were obtained from Invitrogen (Burlington, ON, Canada). CDP-323 (Zauritegraste) was obtained from Adooq Bioscience (Irvine, CA, United States). Recombinant Arg-Gly-Asp (RGD) tripeptide, rat tail collagen (Type I) and bovine fibronectin were obtained from Sigma Aldrich (Oakville, ON,

Canada). MACS buffer used during eosinophil enrichment was prepared in 1x DPBS and contained the following (in mM): 2 EDTA and 0.5% BSA (w/v).

Chapter 3: Results

3.1 Concentration-effect Relationship of Eotaxin-1

Eotaxin-1 is a CCR3 chemokine that orchestrates eosinophil migration through a calcium-dependent pathway. We used confocal fluorescence microscopy to study the effects of eotaxin-1 on the intracellular calcium concentration ($[Ca^{2+}]_i$) in purified human eosinophils. In order to meaningfully interpret any agonist-induced biological responses, however, it was pertinent to examine how $[Ca^{2+}]_i$ changes in eosinophils under resting conditions and in the absence of any stimulation. In preliminary recordings, eosinophils exhibited slight, random fluctuations in $[Ca^{2+}]_i$ that occurred as cells changed position or shape in the presence of drug-free media. Such recordings were invaluable for contextualizing how eotaxin-1 elevates $[Ca^{2+}]_i$ and modulates eosinophil function. Consequently, application of a physiological range of eotaxin-1 to eosinophils significantly increased $[Ca^{2+}]_i$ in a concentration-dependent manner relative to the control (Figures 11 and 12). The calcium response was greatest following eosinophil treatment with 100 nM eotaxin-1, which augmented $[Ca^{2+}]_i$ by 71.2 ± 12.9 %. In contrast, 1 nM eotaxin-1 increased $[Ca^{2+}]_i$ by 19.5 ± 5.91 %, while 10 nM and 20 nM eotaxin-1 increased $[Ca^{2+}]_i$ by 45.8 ± 4.82 % and 62.1 ± 7.75 %, respectively. In almost all cases, the agonist-induced calcium response was accompanied by demonstrable changes in cell motility and shape (Figure 11). Following application of eotaxin-1 and the onset of the calcium flux, cells moved around rapidly throughout the FOV. Eosinophil motility was non-directional and most dramatic in the presence of intermediate concentrations of eotaxin-1 (10-20 nM), although the calcium response was greatest following application of 100 nM eotaxin-1.

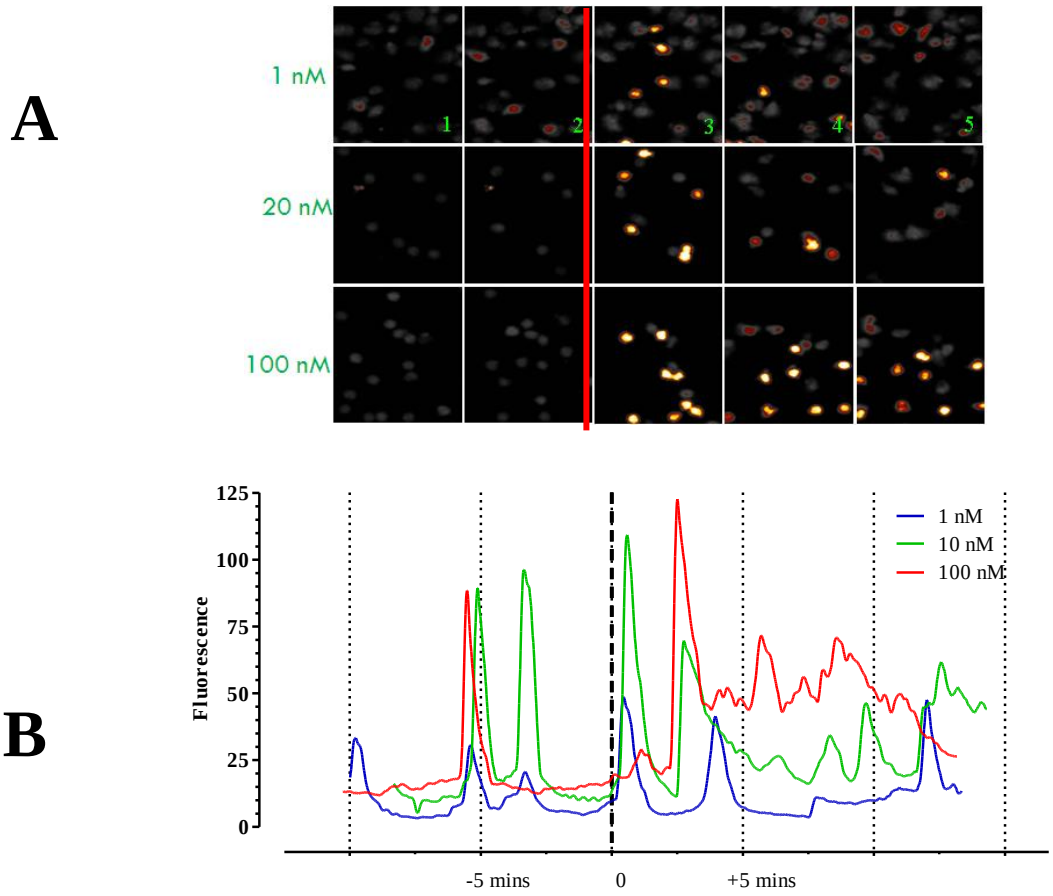


Figure 11. **Eotaxin-1 increases $[Ca^{2+}]_i$ in human eosinophils**. (**A**) *Representative confocal fluorescence image showing baseline and agonist-evoked changes in* $[Ca^{2+}]_i$. Before eotaxin-1 was applied, eosinophils were quiescent and $[Ca^{2+}]_i$ was persistently low as cells remained localized to one particular region of the FOV (panels 1-2). When eotaxin-1 was added (red line, panels 3-5), eosinophils exhibited a rapid increase in fluorescence and motility. Some cells were washed away upon drug perfusion. Enumarated panels are time-lapse images taken at approximately 300 s intervals. (**B**) *Representative time-course of eotaxin-1-mediated calcium response*. Three separate cells demonstrated sporadic fluctuations in baseline $[Ca^{2+}]_i$, which rapidly increased following application of eotaxin-1 (t=0 min). The onset of the calcium response was remarkably synchronous, with the initial calcium spike occurring within the first 15-30 seconds for all eotaxin-1 concentrations. The calcium response peaked within 1-3 minutes and was sustained throughout the course of the recording. Curves represent responses seen in many cells taken from at least 3 different preparations. X-axis: time in minutes. Y-axis: fluorescence

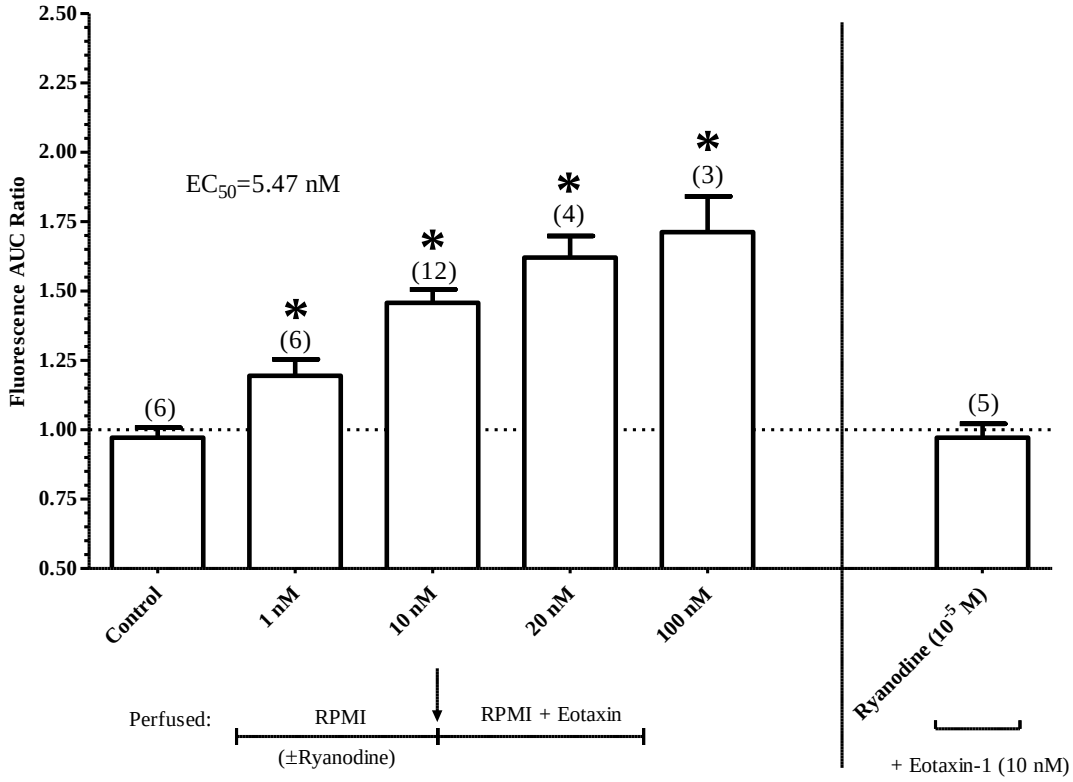


Figure 12. Store- and concentration-dependence of eotaxin-1 signaling. As indicated by the inset, cells were initially perfused with a chemokine-free solution of RPMI for a given time period, after which a solution of RPMI containing eotaxin-1 was subsequently added to the cells. In all cases, the magnitude of the fluorescence AUC ratio was significantly greater than the control, indicating that eotaxin-1 evoked a wholesale increase in eosinophil $[Ca^{2+}]_i$. Some eotaxin-1 concentrations elevated eosinophil $[Ca^{2+}]_i$ more robustly than other concentrations of the drug, indicating that the eotaxin-1-evoked calcium response is concentration-dependent. Each bar shows the aggregate effect of a given eotaxin-1 concentration on $[Ca^{2+}]_i$ measured in many cells (~7-15) from various different subjects (enumerated in brackets). In separate sets of experiments, cells were pretreated with ryanodine (10 μM) prior to the application of 10 nM eotaxin-1. Eosinophil pretreatment with ryanodine abolished the calcium response normally evoked by eotaxin-1 at that concentration. Values represent mean ± SEM. Sample size enumerated in brackets. Symbols indicate responses that significantly differ from unity ($p<0.05$).

Typically, the molecular mechanisms regulating intracellular Ca^{2+} levels are specialized to support the biological roles in which a cell participates. The increase in $[Ca^{2+}]_i$ induced by eotaxin-1 can be due to calcium-entry through extracellular pathways or to calcium-release via intracellular pathways (see Introduction: *Calcium homeostasis*). Calcium-release is in part mediated by the ryanodine receptor (RyR) family of calcium channels expressed on the endoplasmic reticulum (ER), which can be selectively blocked by the plant alkaloid, ryanodine, at micromolar concentrations. In order to further characterize the calcium-dependence of eotaxin-1 signaling, we consequently treated eosinophils with ryanodine (10 μM) before applying stimulating concentrations of eotaxin-1. Whereas 10 nM eotaxin-1 evoked a 45.8 ± 4.82 % increase in $[Ca^{2+}]_i$, ryanodine pretreatment completely blocked the eotaxin-1 calcium response, suggesting that eotaxin-1 increases $[Ca^{2+}]_i$ by mobilizing intracellular calcium stores (Figure 12).

3.2 Store-operated Calcium Entry in Eosinophils

Calcium-release from the ER can eventually deplete intracellular calcium stores, which must be replenished in order for agonist-signaling and normal cell-functions to continue. One means by which this occurs is via plasmalemmal store-operated calcium channels (SOCCs). SOCCs respond to intracellular Ca^{2+} depletion by conducting Ca^{2+} into the lumen of the ER. The eotaxin-1 calcium response can be abolished with ryanodine, which lead us to suspect that SOCCs might play a role in the eotaxin-1 signaling cascade.

Store-operated calcium entry (SOCE) occurs following depletion of the intracellular calcium pool, which we induced by treating eosinophils with the cell-permeable ER calcium ATPase inhibitor, cyclopiazonic acid (CPA; 10^{-5} M). In agonist-stimulated cells, application of CPA in the absence of extracellular calcium evoked a calcium spike that precipitously decayed to levels below baseline $[Ca^{2+}]_i$ (Figure 13a). After the intracellular calcium pool was depleted, re-

introduction of calcium to the extracellular medium elicited a 37.0 ± 6.71 % increase in $[Ca^{2+}]_i$ in a manner consistent with SOCE ($p<0.05$, Figure 13b). This increase in fluorescence was attenuated by three SOCC blockers to varying degrees. In cells that were pretreated with Gd^{3+} or with SKF-96365, there was no change in fluorescence after calcium was re-introduced to the bathing medium. Unexpectedly, eosinophil pretreatment with 2-aminoethoxydiphenyl borate (2-APB) caused a 35.8 ± 7.49 % decrease in $[Ca^{2+}]_i$ after calcium was reintroduced to the extracellular medium ($p<0.05$, Figure 13b).

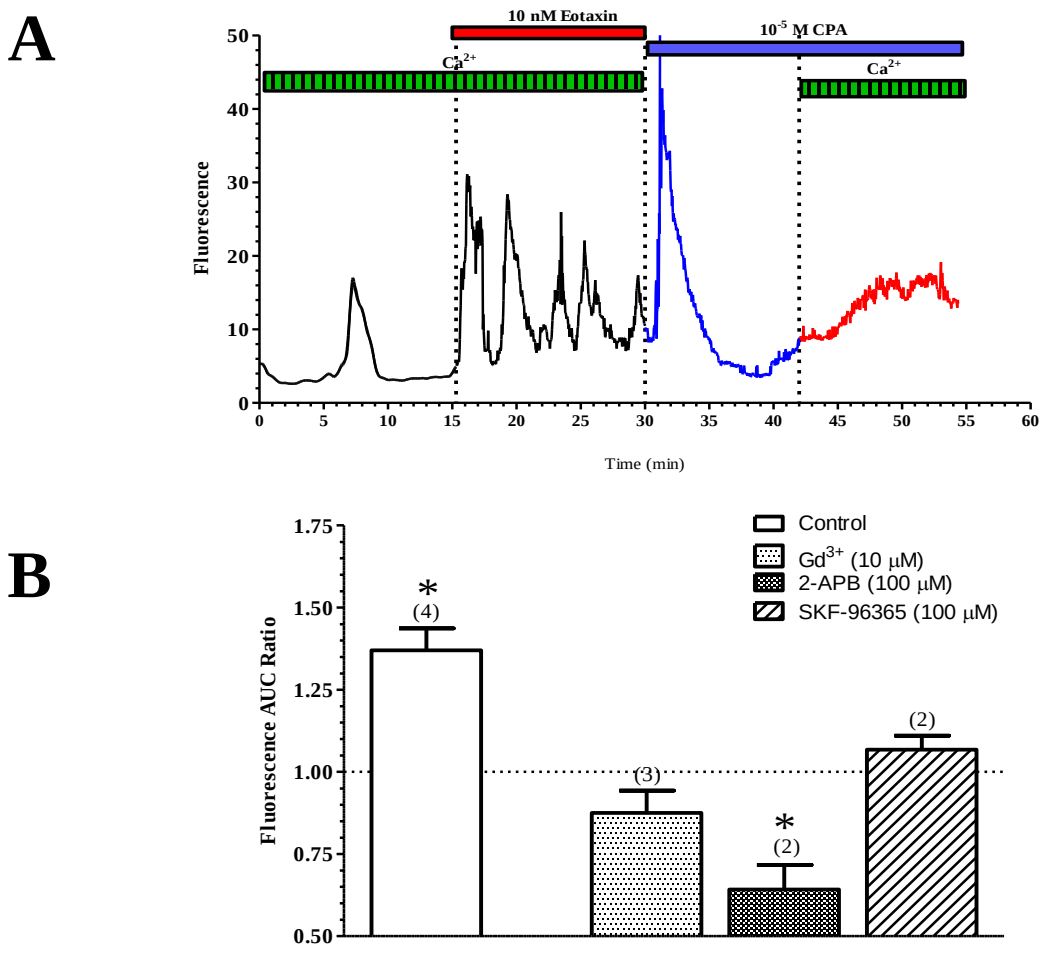


Figure 13. SOCC-dependence of the eotaxin-1 calcium response. (**A**) *Representative time-course of SOCE in human eosinophils.* Application of 10 nM eotaxin-1 evoked fluctuations in $[Ca^{2+}]_i$ (red bar), which rapidly decreased following addition of CPA in the absence of extracellular calcium (blue segment of tracing). Following CPA-treatment, reintroduction of extracellular calcium into the bathing medium stimulated a rapid and sustained increase in fluorescence (red segment of tracing). (**B**) *Gd^{3+}, 2-APB or SKF-96396 block SOCE in human eosinophils*. Pretreatment of store-depleted eosinophils with various SOCC antagonists mitigated the increase in cellular calcium evoked by re-introduction of Ca^{2+} to store-depleted eosinophils. Eosinophils that were pretreated with either gadolinium ion (Gd^{3+}, 10 μM) or SKF-96365 (100 μM), showed no discernible change in the fluorescence whereas cells that were pretreated with 2-APB (100 μM), displayed a decrease in fluorescence. Values represent mean ± SEM. Sample size enumerated in brackets. Symbols indicate responses that significantly differ from unity ($p<0.05$).

3.3 Perfusion-induced Calcium Response (PICR) in Human Eosinophils

During our initial characterization of the eotaxin-1 calcium response, we noted that eotaxin-1 frequently evoked two distinct and prominent spikes (Figure 11). In some experiments, the initial calcium spike occurred well before cells could have been fully exposed to activating concentrations of eotaxin-1. In addition, re-perfusing cells with the same eotaxin-1 concentration stimulated a calcium response even though there was no net change in the eotaxin-1 bath concentration. This prompted us to speculate that some aspect of the drug application process *per se* also contributes to the eosinophil calcium response on some level.

In follow-up experiments, we found that superfusion of drug-free RPMI or HBSS (as diluents controls) evoked calcium responses in almost all eosinophils (Figure 14; Appendix 3). This perfusion-induced calcium response (PICR) occurred in the absence of any pharmacological stimuli. Although we did not further study the time-course of the PICR, higher rates of fluid perfusion appeared to increase $[Ca^{2+}]_i$ more dramatically than lower rates of fluid perfusion.

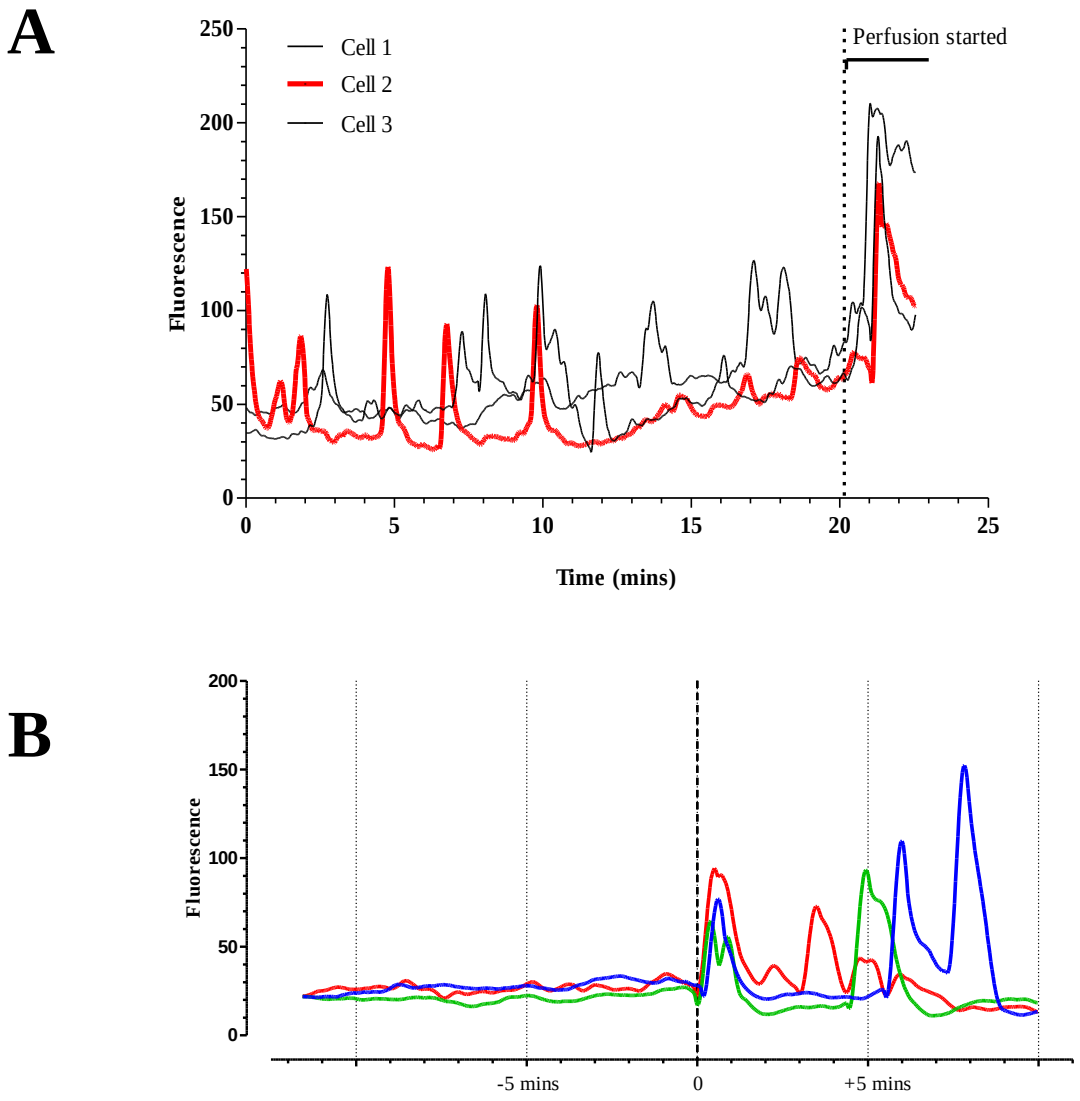
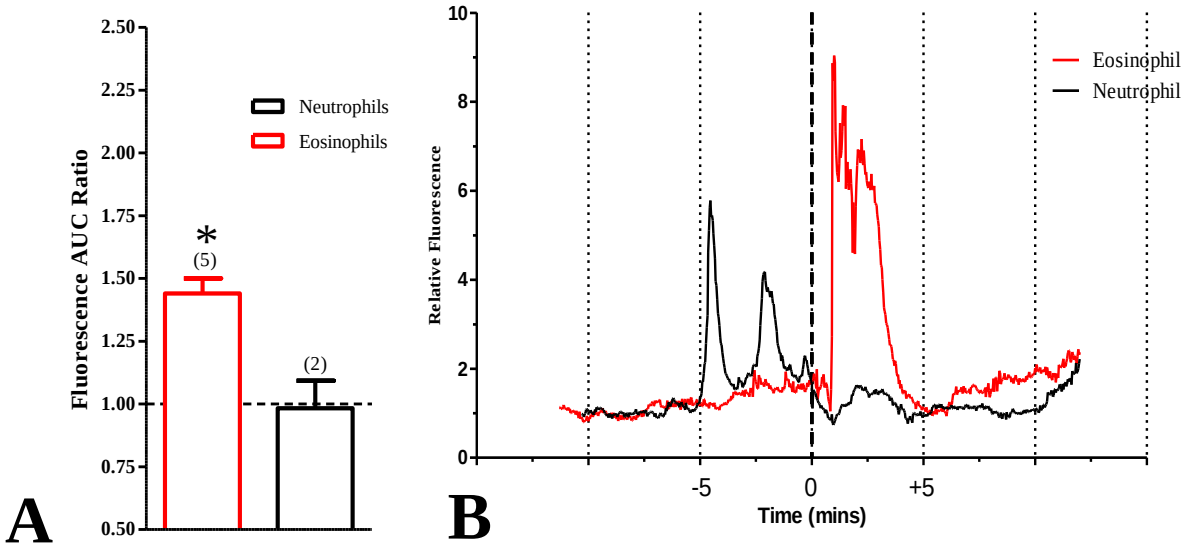


Figure 14. Effect of fluid perfusion on human eosinophils. **(A)** *Primary tracing of the PICR.* In at least three cells taken from the same preparation, perfusion of RPMI evoked a dramatic increase in intracellular calcium (dotted line). This rapid increase in fluorescence was unexpected given that the perfusing medium was free of any chemical stimulus. X-axis: time in minutes. Y-axis: fluorescence. **(B)** *Representative time-course of the PICR in three cells*. In follow-up experiments, we replicated the PICR in eosinophils taken from a different subject. For at least the first 10 minutes of the recording, the $[Ca^{2+}]_i$ is relatively low and stable. At t=0 min, RPMI perfusion begins and is accompanied by a rapid increase in fluorescence within ~1 min. X-axis: time in minutes. Y-axis: fluorescence.

Before further characterizing this PICR, we sought to determine whether this phenomenon is non-specific or unique to eosinophils. We consequently ascertained that perfusion increases $[Ca^{2+}]_i$ in eosinophils but not in neutrophils, suggesting that this mechanism uniquely modulates eosinophil function. In eosinophils, fluid perfusion evoked a 44.1 ± 5.98 % increase in $[Ca^{2+}]_i$ in eosinophils but did not affect $[Ca^{2+}]_i$ in neutrophils (Figure 15). Henceforth, the fluid level and the height of the reservoir were maintained at consistent levels in order to ensure that the rate of fluid perfusion was constant for all experiments.



<u>Figure 15</u>. Effect of fluid perfusion on $[Ca^{2+}]_i$ in human neutrophils and eosinophils. (A) *Fluid perfusion uniquely augments $[Ca^{2+}]_i$ in eosinophils*. In five separate preparations, superfusion of drug-free RPMI evoked a sustained increase in $[Ca^{2+}]_i$ in eosinophils, with an average fluorescence increase of approximately 44.8 ± 5.98 %. The same stimulus, however, did not affect the $[Ca^{2+}]_i$ in neutrophils. Values represent mean ± SEM. Symbols indicate responses that significantly differ from unity ($p<0.05$). Sample size enumerated in brackets. **(B)** *Representative time-course of the PICR in neutrophils and eosinophils*. The neutrophil fraction (isolated as a by-product of the eosinophil enrichment process) was exposed to fluid perfusion at t=0 min. While fluid perfusion rapidly increased $[Ca^{2+}]_i$ in eosinophils, the same response was absent in neutrophils.

3.4 Effects of the PICR on Eosinophil Adhesion and Migration

Most remarkably, the PICR was associated with changes in eosinophil function. Firstly, un-adhered eosinophils rapidly attached to the substratum subsequent to fluid perfusion and the incipient PICR (Figure 16a). In addition, newly- and formerly-adhered eosinophils exhibited increased non-directional migration and shape changes consistent with chemokinesis (Figure 16b). To these ends, we further characterized the perfusion-evoked eosinophil displacement by comparing the initial and final cell positions following exposure to fluid perfusion (Figure 17). While the vast majority of eosinophils exhibited a PICR and migrated to varying degrees, there was no clear association between the direction of the cells' motion and the direction of the fluid perfusion.

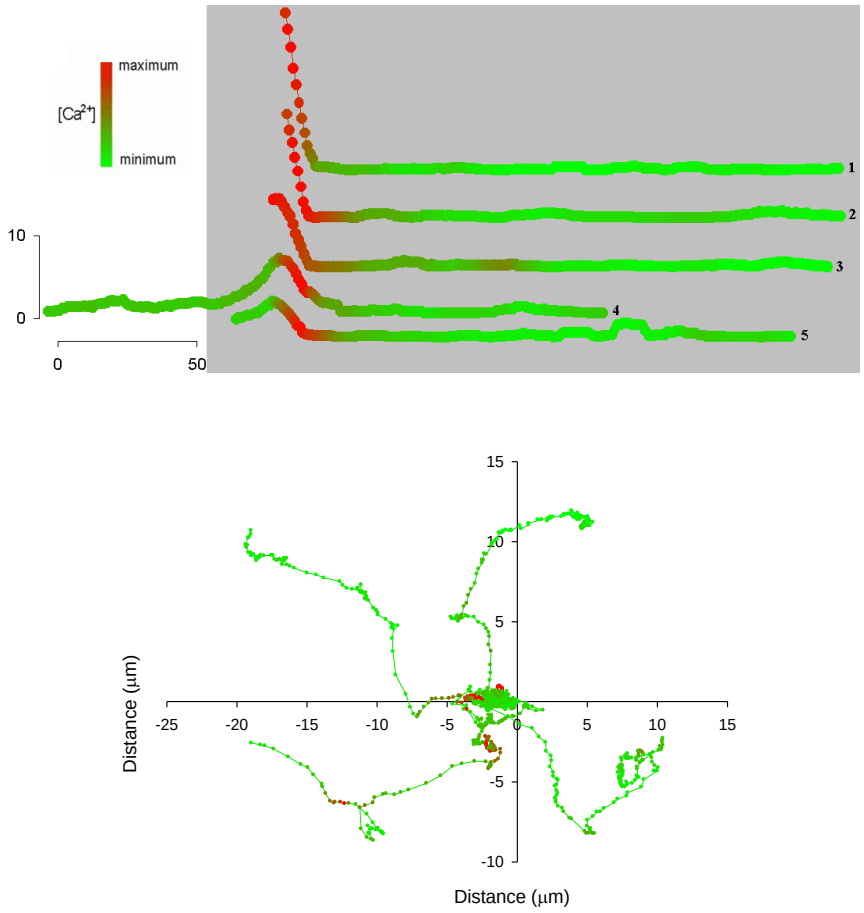


Figure 16. The PICR modulates eosinophil function. (A) *Fluid shear stress promotes eosinophil adhesion***.** In the presence of fluid perfusion, these five non-adherent eosinophils (taken from three separate subjects) displayed a prominent increase in $[Ca^{2+}]_i$ that precipitated cell adhesion. Before perfusion begins, only cell 4 is located within the FOV. Cells 1-3 in addition to cell 5 are located elsewhere in the preparation, upstream to the FOV. The onset of perfusion dislodges cell 4 and evokes a PICR (red portion), which in turn triggers the deceleration and adhesion of the cell to the substrate. In parallel, cells 1-3 and cell 5 are carried into the FOV by the perfusing fluid, whereupon they undergo a similar sequence of PICR and adhesion. Horizontal bar represents time in seconds. Vertical bar represents distance in micrometers. **(B)** *Fluid shear stress promotes eosinophil migration*. The onset of fluid perfusion rapidly augments $[Ca^{2+}]_i$ in eosinophils, and promotes their migration. The direction in which cells move, however, does not appear to correspond to the direction of the flow of fluid (arrow). Points represent cell position measured 8 s apart. Colour changes denote fluctuations in $[Ca^{2+}]_i$.

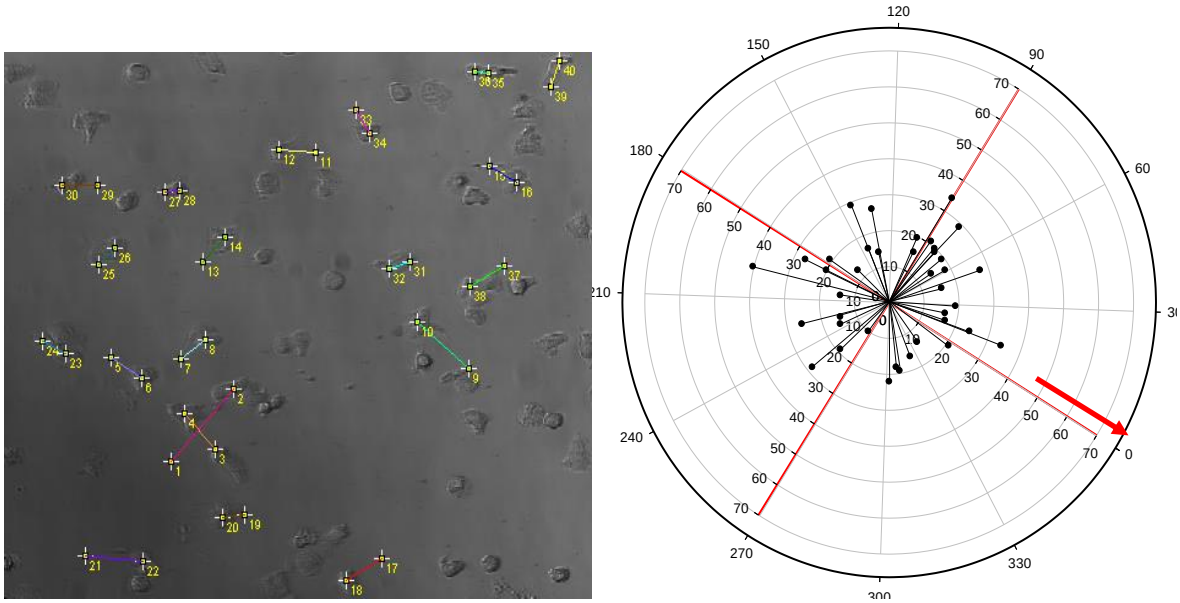


Figure 17. **Directionality of perfusion-induced eosinophil migration. (A)** *Post-perfusion eosinophil displacement*. Following exposure to fluid perfusion, the net eosinophil displacement was assessed using the initial and final cell positions. The pre- and post-perfusion time-frames were approximately 10 min in length. The cell's initial position taken at the beginning of the recording is indicated by the lower number and the final position, taken at the end of the recording, is indicated by the higher number. Almost all viable and responsive cells migrated to some extent. (**B**) *Spatial location of eosinophils following perfusion-induced eosinophil migration*. The direction in which eosinophils migrated was independent of the direction of the fluid perfusion (red arrow). The starting point of each cell was superimposed on the origin and the final location of each cell was plotted as a point on a polar grid. Circular axis: degrees. X- and Y-axes: distance in micrometers.

The wholesale increase in cell motility was nevertheless significant and ultimately provided a decisive metric for quantifying how fluid perfusion affects eosinophil function (Figure 18). When layered on collagen, eosinophils migrated a cumulative distance of 58.7 ± 2.59 μm and 46.3 ± 2.59 μm in the presence and absence of fluid perfusion, respectively ($p<0.05$). Similarly, eosinophils migrated a cumulative distance of 68.4 ± 3.33 μm in the presence of fluid perfusion on fibronectin, which was greater than the cumulative distance travelled in the absence of fluid perfusion (46.3 ± 2.59 μm, $p<0.05$; Figure 18b). Interestingly, cumulative eosinophil displacement in response to fluid perfusion was greater when cells were layered on fibronectin as opposed to collagen ($p<0.05$).

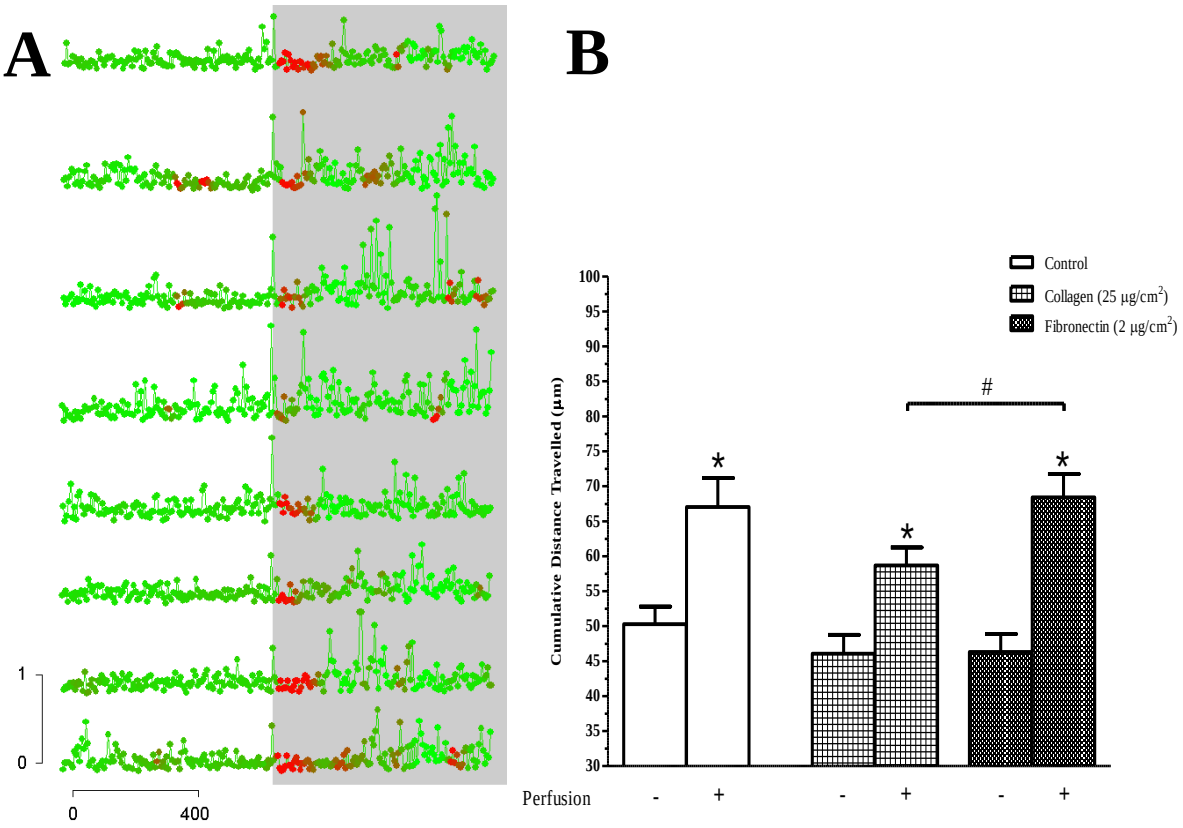


Figure 18. **Fluid perfusion increases eosinophil motility**. **(A)** *The PICR increases eosinophil motility*. Flow-induced eosinophil migration is a non-directional phenomenon associated with increased overall cell movement. Under control conditions, eosinophils are relatively immobile (clear background). In almost all eosinophils, the onset of RPMI perfusion (grey background) evokes a drastic increase in $[Ca^{2+}]_i$ that is proceeded by a marked increase in cell motility. Horizontal bar represents time in seconds. Vertical bar represents distance travelled micrometers. Points represent concatenated measurements taken at 8 s intervals and colour reflects fluctuations in $[Ca^{2+}]_i$. Cells were taken from the same preparation but are representative of responses seen in all perfusion experiments. **(B)** *Fluid perfusion categorically increases the cumulative distance travelled by eosinophils*. In the absence of fluid perfusion, eosinophils exhibited a low baseline level of non-directional migration. In the presence of fluid perfusion applied over a comparable time period (~10 min), the cumulative distance travelled increased nearly two-fold for all cells. *$p<0.05$ relative to perfusion control; $^{\#}p<0.05$ between-group comparisons.

Our preliminary experiments investigating the functional consequences of fluid perfusion prompted us to further characterize the underlying mechanisms of the PICR. Consequently, we determined the calcium pools involved in the PICR by pre-treating eosinophils with ryanodine (10 μM) for approximately 10 minutes before stimulation with fluid perfusion. Interestingly, ryanodine significantly decreased the magnitude of the PICR but failed to completely eliminate the calcium response (Figure 19). Depletion of the ER with CPA (10^{-5} M), however, abolishes the PICR. Taken together, our findings indicate that the PICR can be pharmacologically manipulated using blockers of the intracellular calcium release pathway.

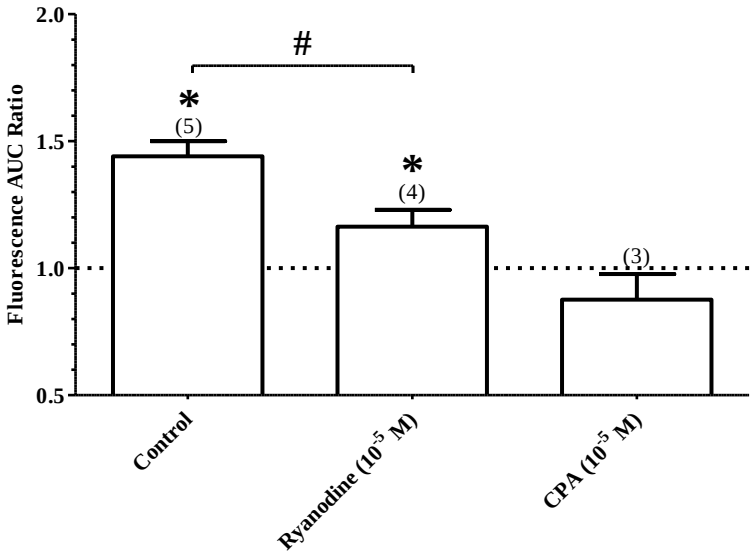
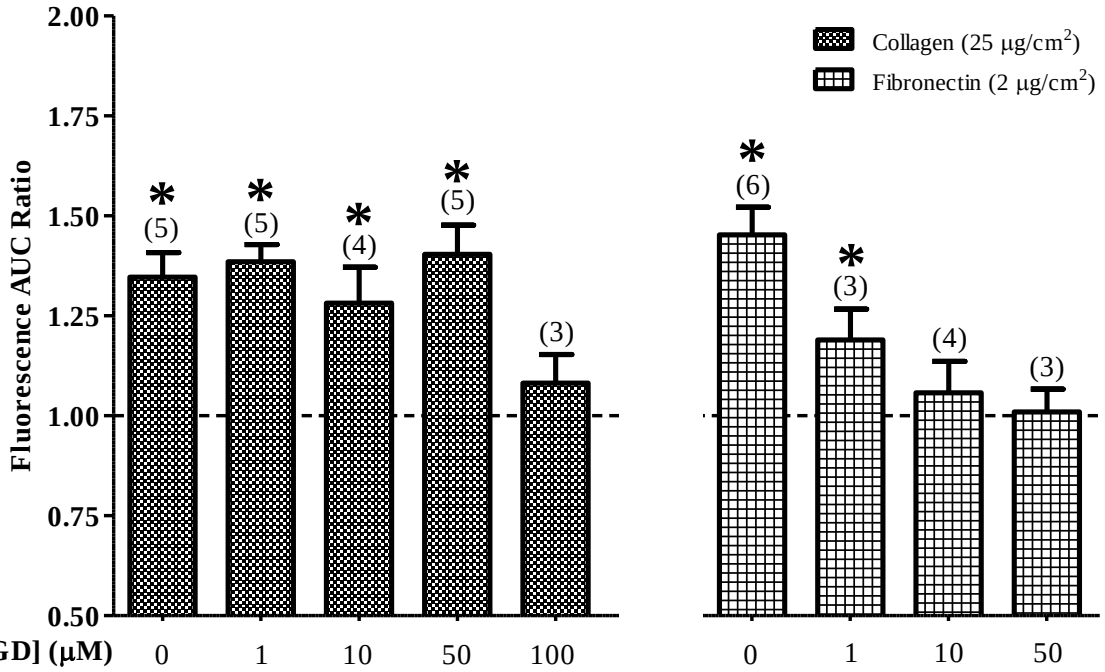


Figure 19. Store-dependence of PICR. Fluid perfusion elicits a 44.1 ± 5.98 % increase in $[Ca^{2+}]_i$ in eosinophils. When cells are pretreated with ryanodine (10 μM), fluid perfusion promotes a 16.4 ± 6.60 % increase in $[Ca^{2+}]_i$, which is significantly lower than the control ($p<0.05$). In contrast, CPA (10^{-5} M) blocks the PICR. Values represent mean AUC ratio ± SEM and brackets indicate sample size. *$p<0.05$ relative to unity; $^{\#}p<0.05$ between-group comparisons.

3.5 Effect of Arg-Gly-Asp (RGD) and CDP-323 on the PICR

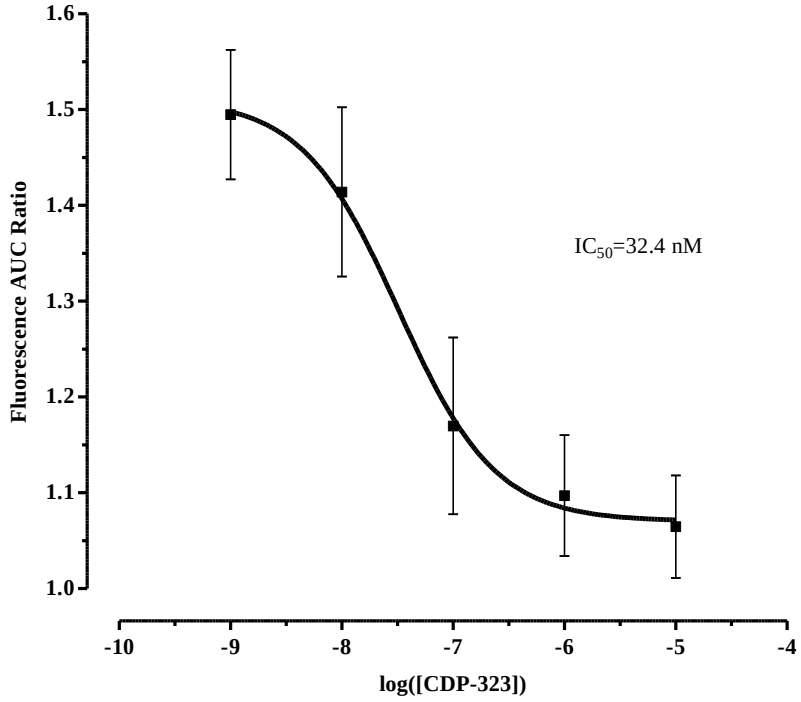
We subsequently sought to identify the molecular cognate of the PICR in eosinophils using a series of increasingly selective pharmacological agents. Previous studies have implicated the integrin receptor family of adhesion molecules as force transducers in various cell types, including vascular endothelial cells and T-lymphocytes. Eosinophils primarily express the $\alpha_4\beta_7$ and $\alpha_4\beta_1$ integrin receptor subtypes, which overlap in their affinities for ligands presented on the surface of vascular endothelial cells and within the ECM. The Arg-Gly-Asp (RGD) motif is an amino acid sequence ubiquitously expressed in the binding domains of many integrin receptor ligands (Ruoslahti, 1996; D'Souza et al., 1991). We consequently blocked eosinophil integrin receptor activity by incubating cells with increasing concentrations of recombinant RGD before stimulating them with fluid shear stress (Figure 20).

As shown in Figure 20, pre-treating eosinophils with RGD attenuated the PICR in a concentration-dependent manner when cells were layered on fibronectin (Figure 19). In stark contrast, RGD failed to block the PICR in eosinophils that were layered on collagen. A concentration of 100 μM RGD was required in order to effectively block the PICR in eosinophils layered on collagen, whereas 10 μM RGD was sufficient to block the PICR in eosinophils layered on fibronectin.



<u>Figure 20</u>. Eosinophil Pre-treatment with RGD attenuates the PICR. The RGD-mediated suppression of the PICR is concentration-dependent in eosinophils that are layered on fibronectin. In contrast, only the highest RGD concentration (100 μM) of RGD blocked the PICR in eosinophils layered on collagen. Values represent means ± SEM. Symbols indicate responses that significantly differ from unity ($p<0.05$). Sample sizes are enumerated in brackets.

Our experiments using RGD suggest that the PICR is an integrin-mediated phenomenon. We consequently proceeded to test the effects of a highly selective $\alpha_4\beta_7/\alpha_4\beta_1$ dual integrin receptor antagonist, known as CDP-323 (or Zaurategrast), on activation of the PICR in eosinophils. Similar to previous experiments with RGD, eosinophils were pre-treated with CDP-323 for ~20-25 minutes and stimulated with fluid perfusion. Consistent with our previous findings, eosinophil pretreatment with CDP-323 blocked the PICR in a concentration-dependent manner (Figure 21; IC_{50}=32.4 nM).

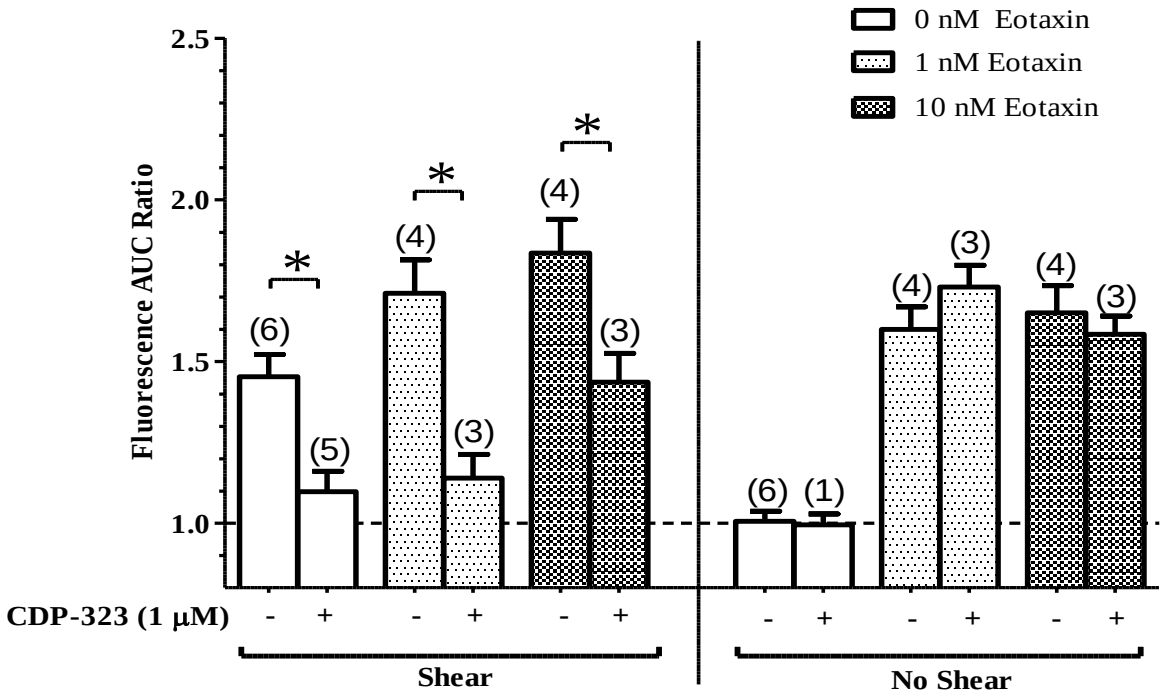


<u>Figure 21</u>. Effect of CDP-323 on eosinophil PICR. Eosinophil pretreatment with the $\alpha_4\beta_7/\alpha_4\beta_1$ dual-integrin antagonist, CDP-323, blocked the PICR in a concentration-dependent manner at all concentrations greater than 10^{-7} M. Values represent means ± SEM.

3.6 Effect of the PICR on Eotaxin-1-mediated Calcium Signaling

The finding that fluid perfusion augments $[Ca^{2+}]_i$ by mobilizing intracellular calcium stores is functionally interesting in light of the central role of Ca^{2+} as a secondary messenger in the eotaxin-1 signal transduction cascade. Given the intimate involvement of chemokine and integrin receptors in mediating eosinophil adhesion and migration, our next step was to investigate whether or not these integrin receptors participate in cross-talk with the eotaxin-1 signaling cascade and/or whether integrin function can affect eotaxin-1-mediated eosinophil activation. To these ends, we concurrently stimulated eosinophils with both eotaxin-1 and fluid perfusion after pretreating cells with blocking concentrations of CDP-323 (1 μM).

Contrary to our expectations, the PICR observed following concerted application of eotaxin-1 and fluid perfusion did not surpass the calcium response evoked by eotaxin-1 alone. Although eotaxin-1 and fluid perfusion did not act synergistically to increase $[Ca^{2+}]_i$, we were nevertheless surprised to find that CDP-323 completely obliterated the eotaxin-1-mediated calcium response when the chemokine was applied via perfusion. Of particular interest was the fact that CDP-323 failed to block the eotaxin-1-induced calcium response when the chemokine was applied in the absence of fluid perfusion (Figure 22).



<u>Figure 22</u>. Effect of CDP-323 on the eotaxin- and perfusion-induced calcium response. *Left panel*: Fluid perfusion and eotaxin-1 increase $[Ca^{2+}]_i$ in eosinophils. The concerted, stimulatory effects of perfusion and chemokine application were nearly completely abolished by pretreating cells with blocking concentrations of the selective integrin antagonist, CDP-323 (1 μM). The calcium response evoked by the application of 10 nM eotaxin-1 by perfusion was attenuated by CDP-323, but the magnitude of this inhibition was smaller than that seen in cells treated with 0 (control) or 1 nM eotaxin-1. *Right panel*: Application of eotaxin-1 without perfusion augments $[Ca^{2+}]_i$ in CDP-323-treated cells and in the treatment controls. In the absence of fluid shear stress, CDP-323 did not block the eotaxin-1-mediated calcium response. Fluid shear stress appears to facilitate the integrin-mediated blockage of eotaxin-1 signaling. Values represent means ± SEM. Sample size enumerated in brackets. Symbols indicate significant differences ($p<0.05$).

Chapter 4: Discussion

Ionic calcium (Ca^{2+}) is an intracellular second messenger that regulates myriad cellular functions, including the migration and degranulation of eosinophils (see: Chapter 1, *Calcium homeostasis;* (Berridge et al., 2003). Eotaxin-1 promotes eosinophil homing to the asthmatic lung by augmenting the intracellular calcium ion concentration ($[Ca^{2+}]_i$) consequent to CCR3 activation (Brundage et al., 1993; Conroy & Williams, 2001). The signal transduction cascade of eotaxin-1 has been previously studied *in vivo* and *in vitro* in humans, mice, Newts and various cell lines, but the downstream molecular events triggered by CCR3-activation vary according to the cell type and the species under investigation (Brundage et al., 1993). While characterizing the ionic regulatory mechanisms of the eotaxin-1 calcium-response, we found that eosinophils activate in response to shear-stress. This shear-sensitivity triggers a calcium response that accompanies marked changes in cell shape and function consistent with adhesion and/or migration. To the best of our knowledge, our study provides the first pharmacological description of a molecular mechanosensor in human eosinophils capable of directly regulating eosinophil activation and engaging in cross-talk with the eotaxin-1 signal transduction cascade.

4.1 Baseline Fluctuations in $[Ca^{2+}]_i$ in Human Eosinophils

Before we could study the effects of eotaxin-1 on $[Ca^{2+}]_i$ in human eosinophils, it was pertinent to understand how $[Ca^{2+}]_i$ changes in these cells under baseline conditions. In the absence of any external excitatory stimulus, $[Ca^{2+}]_i$ sporadically increased in quiescent eosinophils. These transient fluctuations in $[Ca^{2+}]_i$ occurred without any discernible pattern and varied in magnitude within and between subjects. This phenomenon occurred in relatively few of the many dozens of cells in the field of view (FOV), but was persistent enough that it could

prevent us from discerning agonist-induced responses from background activity. Baseline calcium fluxes most often accompanied slight modifications in cell shape and position, suggesting that they may be associated with the cell's motile nature and its related role as an effector cell of the innate immune response (Fay et al., 1995; Geering et al., 2013).

In a physiological setting, eosinophils respond to various signals pertinent to host defence (Geering et al., 2013). In order to accomplish this, eosinophils continuously survey their environment for pathogen/inflammation-related signals by extending cytoplasmic projections into their surroundings (Brundage et al., 1993). The ensuing changes in cell shape result from (de)polymerization of actin filaments and are largely Ca^{2+}-dependent. The random Ca^{2+}-fluxes that we observed in unstimulated eosinophils might therefore underlie how these cells are able to detect and respond to local, inflammation-specific signals generated during host infection (Geering et al., 2013; Brundage et al., 1993).

It is conceivable that the baseline oscillations in $[Ca^{2+}]_i$ that we observed during our early experiments, however, may have been due to the localized, *ex vivo* release of stimulatory factors by other eosinophils and/or contaminating cells (e.g., neutrophils) found in the preparation (Giembycz & Lindsay, 1999; Geering et al., 2013). Even under ideal conditions, the use of magnetic beads does not yield a 100% pure population of eosinophils (Chihara et al., 1995). Human eosinophils are known to synthesize, store and secrete chemokines like regulated on activation, normal T-cell expressed and secreted (RANTES), which also augments $[Ca^{2+}]_i$ in eosinophils (Alam et al., 1993). The fact that several groups have shown that eosinophils constitutively express RANTES mRNA and secrete biologically active concentrations of the protein supports the possibility that, in our hands, $[Ca^{2+}]_i$ in eosinophils may have been affected by the activity of other cells present in our preparation (Velazquez et al., 2000).

Ultimately, the fact that $[Ca^{2+}]_i$ could dramatically vary within individual cells emphasizes the merit in standardizing responses using an internal control. Quantifying the relative amount by which the total fluorescence increased or decreased in each cell following application of a given treatment therefore permitted us to disentangle *bone fide* calcium responses from the random, background oscillations in Ca^{2+} that may occur in these cells.

4.2 Effect of Eotaxin-1 on $[Ca^{2+}]_i$ and Eosinophil Adhesion

We applied a range of eotaxin-1 concentrations to primary human eosinophils via perfusion with a gravity-controlled apparatus that delivered the chemokine at a near-constant flow rate. In nearly all of the eosinophils that we studied, eotaxin-1 evoked a robust increase in $[Ca^{2+}]_i$. Higher concentrations of eotaxin-1 elicited a proportionally greater increase in $[Ca^{2+}]_i$, demonstrating that the eotaxin-mediated calcium response is concentration-dependent.

In stark contrast to this finding, previous functional studies of eosinophil migration variously suggest that the chemotactic effect of eotaxin-1 is biphasic. Using Boyden chamber assays, Smith et al. (2012) have shown that low and intermediate concentrations of eotaxin-1 induce greater eosinophil chemotaxis than higher ($\geq$ 100 nM) eotaxin-1 concentrations, which preferentially elicit eosinophil degranulation. Elsewhere, Zimmerman et al. (2000) used a murine model of allergic disease to similarly show that subcutaneous administration of an intermediate eotaxin-1 dose rapidly and preferentially induces eosinophil accumulation at the site of injection.

The biphasic effect of eotaxin-1 on eosinophil migration reflects the cell's ability to cater its functional response according to the physiological compartment in which it encounters the chemokine (Smith et al., 2012). In principle, circulating eosinophils located in the blood vasculature encounter and bind relatively few molecules of eotaxin-1. This low-level chemokine-

binding, however, must be proficient at alerting the cell to the presence of an infectious agent elsewhere in the body. Although we did not directly assess the relationship between the magnitude of calcium response and the extent of eosinophil migration, we suspect that these low(er) eotaxin concentrations should readily promote eosinophil migration rather than eosinophil degranulation.

In the interstitium, by contrast, eosinophils encounter relatively high concentrations of eotaxin-1 that, according to *in vitro* functional studies, induce eosinophil degranulation (Smith et al., 2012). Ca^{2+} participates in both migration- and degranulation-related events in eosinophils, however, since the fusion of cytoplasmic vesicles to the cell membrane is calcium-dependent event (Barclay et al., 2005). In our experiments, the fact that high eotaxin-1 concentrations induced the greatest calcium responses in human eosinophils might therefore be consistent with the role of Ca^{2+} in eosinophil migration and degranulation (Berridge et al., 2003; Barclay et al., 2005).

In the context of previous functional studies, our results suggest that migration and degranulation are mechanistically related events that lie on opposite ends of a continuous functional spectrum. We therefore suspect that low $[Ca^{2+}]_i$ promotes migration while high $[Ca^{2+}]_i$ promotes degranulation and further postulate that some threshold $[Ca^{2+}]_i$ exists in eosinophils that, once surpassed, preferentially induces eosinophil degranulation.

The fact that eotaxin-1 promotes a widespread increase in eosinophil adhesion is consistent with its *in vivo* role as an eosinophil-selective chemoattractant (see: Chapter 1; (Conroy & Williams, 2001; Griffiths-Johnson et al., 1993). Qualitatively, eosinophil adhesion occurred when cells flattened against the surface of the glass (Curtis, 1964). In addition,

adhesion was characterized by a marked change in shape whereby cells that were initially circular and balled-up rapidly adopted an amorphous and globular appearance. This functional response was almost ubiquitously preceded by an increase in $[Ca^{2+}]_i$ that occurred shortly after chemokine application (see above). Although adhesion-related shape changes proved difficult to quantify using this technique, the persistent association between adhesion and Ca^{2+}-flux is unlikely to be circumstantial given that a vast array of calcium-dependent proteins (*e.g.*, RhoA) directly modulate cytoskeletal architecture (see: Chapter 1; Alblas et al., 2001).

It is important to note that our experimental setup prevents us from making *in vivo-in vitro* inferences regarding cell function, however, since the manner in which eotaxin-1 was applied does not adequately recapitulate the physiological environment. Classically, eosinophil homing to the asthmatic airway is thought to be guided by gradients of chemokine molecules generated in the airway interstitium during inflammation (Conroy & Williams, 2001; Pease & Williams, 2001). While our experimental approach assessed the calcium response evoked by eotaxin-1 at a given chemokine concentration, it failed to simulate the kind of chemotactic gradient that cells would encounter *in vivo*. The pulmonary vascular capillary bed exists in a 3-dimensional network that is spatially distinct from the setup we used during out experiments. It was consequently difficult to ascertain the effects of eotaxin-1 on the directional migration of eosinophils and to draw comparisons between how this functional response differs from *in vivo* studies.

In eosinophils that responded to eotaxin-1, the onset of the initial calcium transient occurred 15-30 seconds following the start of chemokine perfusion. In our setup, the calcium flux that we measured in eosinophils constitutes one of many downstream events triggered by CCR3 activation. The apparent delay in the eotaxin-mediated calcium response might therefore

reflect the time needed to generate intracellular second messengers, which in turn must activate various calcium channels expressed by the cell (Berridge et al., 2003). While previous studies report that eotaxin-1 evokes a rapid, near-instantaneous rise in $[Ca^{2+}]_i$ in human and murine eosinophils, it is important to note that these groups measured the average fluorescence using monolayers of millions of eosinophils, the corollary being that the exact time-course of the eotaxin-mediated calcium-response reported by these experiments is difficult to discern (Kernen et al., 1991; Brundage et al., 1991; Brundage et al., 1993). Based on electrophysiological characterizations of calcium channel activation in other cell types, the 15-30 s lapse associated with the eotaxin calcium-response is consistent with the time-course involved in the activation of ER-bound calcium channels, which are generally known to orchestrate migration-related events in other immune cells, including neutrophils (Luciani et al., 2009; Jaconi et al., 1997; Kawa, 1989).

4.3 Store-dependence of the Eotaxin-1 Calcium-Response

The increase in $[Ca^{2+}]_i$ evoked by eotaxin-1 is mediated by activation of ion channels that conduct the influx of Ca^{2+} from the extracellular milieu and/or the release of Ca^{2+} from the ER (see Chapter 1: *Calcium homeostasis;* (Berridge et al., 2003; Schwab et al., 2012). The calcium response elicited by eotaxin-1 was completely blocked by pre-treating cells with ryanodine (10 μM), which is a plant alkaloid that selectively binds to ryanodine receptor channels (RyRs) expressed on the ER (Fill & Copello, 2002). Under physiological conditions, RyRs are activated by Ca^{2+} ion binding at the channel's cytosolic surface (Brini et al., 2013). RyR activation can therefore conduct the release of Ca^{2+} ions into the cytosol and potentiate any rise in $[Ca^{2+}]_i$ induced by an agonist. This phenomenon, known as calcium-induced (CICR), is critical in

mediating a number of calcium-dependent cellular responses including contraction of ASM, hormone secretion and gene transcription (Dulhunty, 2006).

Studies have previously suggested that CCR3 couples to the PLC pathway and thereby promotes the IP_3-dependent release of Ca^{2+} ions from IP_3Rs following agonist-binding (Walker et al., 1998). The fact that ryanodine blocks the eotaxin-1-mediated calcium response provides strong evidence in support of a Ca^{2+}-release-dependent mechanism, possibly downstream of PLC activation. Since we did not directly or indirectly assess IP_3 production, however, we cannot definitively conclude that the eotaxin-1 calcium-response is mediated by PLC activation. In non-human eosinophils, however, others report that CCR3 activation is pertussis-sensitive, indicating that the effects of eotaxin may be transduced through an altogether different signal transduction cascade (Kampen et al., 2000; Pease & Williams, 2001).

4.4 Store-operated Calcium Entry in Human Eosinophils

The prevailing Ca^{2+} level within the ER lumen can further affect $[Ca^{2+}]_i$ in the cytosol via an additional, distinct mechanism involving plasmalemmal calcium channels that specifically activate following ER calcium-depletion (Varnai et al., 2009). This so-called store-operated calcium entry (SOCE) is mediated by: the stromal-interacting molecule (STIM) family of ER-bound proteins, which sense changes in ER $[Ca^{2+}]$; and the Orai family of plasmalemmal calcium channels, which are recruited by STIM following store depletion through some currently unknown mechanism (Varnai et al., 2009; Soboloff et al., 2012). While several isoforms of Orai and STIM are expressed based on alternative splicing, the STIM1/2 and Orai1 isoforms interact preferably in order to mediate SOCE in neutrophils and T-cells during chemotaxis (Varnai et al., 2009; Schaff et al., 2010).

Our investigation of SOCE in eosinophils required us to deplete the ER calcium stores while removing extracellular calcium ions from the bathing medium (Ashmole et al., 2012). The depletion of the ER would activate SOCCs, but SOCE could not occur until extracellular Ca^{2+} ions are reintroduced (Soboloff et al., 2012). Consequently, the removal of extracellular Ca^{2+} ions was critical, as any subsequent fluorescence increase noted following the controlled re-introduction of Ca^{2+} would strongly suggest the presence of SOCE (Ashmole et al., 2012; Soboloff et al., 2012).

We depleted stores using cyclopiazonic acid (CPA) and we removed extracellular calcium by supplementing the bathing medium with the calcium chelator, EGTA (1 mM). Importantly, CPA-containing and Ca^{2+}-free media were introduced only once cells had been stimulated with activating concentrations of eotaxin-1. CPA inhibits the SERCA pump, which normally facilitates the active re-uptake of cytosolic Ca^{2+} into the ER (Liang & Sze, 1998). The CPA-induced build-up of Ca^{2+} ions corresponds to the spike in fluorescence that is proceed by a precipitous decay (Figure 12a, blue portion of tracing), which occurs as Ca^{2+} ions are extruded out of the cytosol by the plasmalemmal NCX and/or PMCA (see Chapter 1: *Calcium Homeostasis*; (Brini et al., 2013).

The clear and persistent rise in $[Ca^{2+}]_i$ that was evoked by re-introduction of extracellular Ca^{2+} provides strong evidence in support of SOCE involvement in the eotaxin-1 calcium-response (Parekh & Putney, 2005). This finding is consistent with the purported involvement of STIM/Orai channels with eosinophil migration in mouse eosinophils. In their study, Wang et al. (2012) used a murine model of allergic rhinitis (AR) to show that calcium signaling through store-operated calcium channels may contribute to the severity of AR. Using lenti-virus gene

silencing, the group abrogated the expression of this ion channel, which alleviated symptoms of AR and reduced the number of eosinophils in the nasal lavage fluid (NLF; Wang et al., 2012).

To further determine whether STIM-Orai channels facilitate SOCE in eotaxin-1-treated cells, we used SOCC blockers to inhibit SOCE by pre-treating cells with Gd^{3+}, SKF96365 or 2-APB following ER depletion and extracellular Ca^{2+} chelation (Bourne & Trifaro, 1982;). Under these conditions, the finding that Gd^{3+} and 2-APB categorically reduced the rise in fluorescence that was previously associated with SOCE lends further support for the expression of SOCC in human eosinophils (Bootman et al., 2002; DeHaven et al., 2008). It is important to note, however, that the lack of selectivity of these blockers simply confirm that SOCE indeed occurs in human eosinophils and cannot be used to decisively characterize the exact calcium channel subtype that mediates this phenomenon (Singh et al., 2010). Both Gd^{3+} and 2-APB, for instance, have been shown to also block activation of transient receptor potential (TRP) channels and calcium-dependent potassium channels in various species (Singh et al., 2008; Hagenston et al., 2009; Song & Yuan, 2010). Similarly, the Gd^{3+} ion also targets IP_3-gated calcium channels in astrocytes, fibroblasts and epithelial cells, which further complicates any interpretation as to the molecular constituents of SOCE in these cells (Ostrow & Sachs, 2005).

Over the course of our investigation of SOCE in eosinophils, cells were only briefly exposed to eotaxin-1 at the outset of each experiment for a number of reasons. Firstly, one study of primary human eosinophils reported that surface expression of CCR3 rapidly decreases to ~60% of control following treatment with 10 nM eotaxin-1 for ~15 minutes (Zimmerman et al., 1999). In addition, Teixeira et al (1997) reported that treating eosinophils with eotaxin for 10 minutes results in CCR3 downregulation and gives rise to eosinophil unresponsiveness towards eotaxin following *in vivo* transfer up to 8 hours following initial chemokine stimulation. This

suggests that the cellular events triggered by brief exposure of eosinophils to eotaxin persist long after the chemokine has been withdrawn from the bathing medium. In addition, the fact that eosinophils remained adherent and somewhat migratory after removal of the chemokine but not of extracellular Ca^{2+} supports the assumption that the calcium channels activated by CCR3 continue to participate in calcium flux events in the absence of the chemokine. Our collective findings regarding the ER- and SOCC-dependence of eotaxin-1 are thus largely consistent with various studies on calcium-handling in eosinophils.

4.5 Perfusion-induced Calcium Response (PICR)

We conducted diluent-control experiments during which we simply sought to corroborate that the excitatory response evoked by eotaxin-1 in prior experiments was indeed attributable to activation of CCR3. During these experiments, we applied either RPMI or HBSS to cells shortly after they were layered onto the glass preparation but before they had been subjected to any excitatory stimulus, and found that $[Ca^{2+}]_i$ drastically increased in eosinophils. After initially dismissing this response as an artefact, subsequent experiments showed that the same stimulus (*i.e.*, perfusion) could repeatedly induce a calcium-response in eosinophils isolated from other subjects.

The fact that perfusion evokes a robust and dramatic calcium-response in nearly all cells prompted us to further investigate whether this phenomenon is unique to eosinophils or whether it occurs in any cell exposed to fluid perfusion as a consequence of non-specific distortion of the cell membrane, which has been found elsewhere to facilitate Ca^{2+}-flux in cardiac myocytes and dorsal root ganglion neurons (Bongrand, 1995; McCain & Parker, 2011; Howe et al., 1977). We consequently tested whether this PICR also occurs in neutrophils using the polymorphonuclear

cell fraction that was normally discarded during the magnetic separation of eosinophils (see: Chapter 2: *Eosinophil Purification;* Sedgwick et al., 1996). Using the exact same experimental protocol described previously, application of fluid perfusion to neutrophils failed to elicit a PICR. This observation is consistent with the study conducted by Shin et al. (2008), who demonstrated that fluid shear-stress induces pseudopod retraction in human neutrophils.

Overall, the fact that perfusion only augmented $[Ca^{2+}]_i$ in eosinophils suggests that the PICR sub-serves a unique biological role in human eosinophils (Schaff et al., 2008). In light of the limited sample size (n=2), however, these findings must be further replicated before any decisive conclusions can be drawn as to whether or not the PICR occurs exclusively in eosinophils. It is also conceivable that the use of anti-CD16 beads during the extraction/isolation process may have interfered with neutrophil responsiveness, although the manufacturer's instructions specifically indicate that this protocol can be reliably implemented in order to study neutrophil migration, adhesion and signaling events (Sedgwick et al., 1996).

4.6 Effects of the PICR on Eosinophil Adhesion and Migration

The consistency with which the PICR could be replicated in eosinophils taken from multiple subjects was remarkable but did not rule out the possibility that the Ca^{2+}-flux evoked by perfusion might still constitute a biologically insignificant artefact. The finding that the PICR evokes global, unambiguous changes in cell function, however, provides the most compelling evidence in support of a physiological role for the PICR. Our results demonstrate that adhesion and migration are two key functional changes in cell behaviour that can be readily observed following the onset of perfusion and the PICR. Adhesion induced by the PICR qualitatively resembled the shape changes that were evoked by eotaxin-1 in earlier experiments: cells layered

on glass in stagnant RPMI (or HBSS) adopted a flat appearance once they were exposed to perfusion. In some cases, anterior/posterior regions could be readily discerned as cells underwent polarization and began migrating about the surface of the glass.

Widespread eosinophil adhesion occurred subsequent to the PICR but never prior to the application of fluid perfusion. Indeed, extended recordings of eosinophils layered on glass in stagnant RPMI or HBSS (≥ 40 minutes) reveal that in the absence of fluid perfusion, there is no increase in $[Ca^{2+}]_i$ and/or eosinophil adhesion comparable to that seen during the PICR (Curtis, 1964). The cells in which perfusion-induced adhesion occurred were presumably quiescent and non-adherent elsewhere in the preparation, and were washed into the FOV once perfusion was started. The fact that the PICR occurred concomitantly with perfusion is consistent with the role of Ca^{2+} in eosinophil adhesion and migration and suggests that the PICR induces the rapid deceleration and arrest of eosinophils onto the surface of the glass (Curtis, 1964).

The widespread migratory response triggered by the PICR (Figure 18) did not appear to follow any definitive pattern (Figure 17). In contrast to this finding, intravital microscopy studies have shown that neutrophils crawl on the luminal surface of the microvasculature perpendicularly to the direction of the fluid flow (Megens et al., 2011; Phillipson et al., 2009; Dixit et al., 2011). Contrary to our expectations, no such correlation could be observed between the direction of eosinophil migration and the direction of fluid flow. While random cell motion evoked by a chemical stimulus is referred to as chemokinesis, we propose that the random cell motion evoked by perfusion and initiated by the PICR constitutes a form of ‘mechanokinesis’, since the excitatory stimulus is mechanical in nature.

4.7 Role of Shear-Stress in the PICR

Of the many eosinophils situated in the FOV at the outset of a given recording, however, a sizeable proportion would be washed away by the fluid perfusion. In light of this, it might be tempting to conclude that the PICR is an anomalous event observed in only a subset of eosinophils. Two key observations suggest otherwise, however, and should be taken into consideration: namely, re-application of perfusion to cells that have already adhered elicits additional PICRs; and widespread eosinophil adhesion occurs at downstream regions of the flow chamber, showing that the cells washes away from the FOV may have in fact adhered at a different part of the glass surface. This interpretation serves to highlight that although some cells are indeed washed out of the FOV by perfusion, they may have simply adhered elsewhere in the preparation and are fundamentally difficult to detect since they lie outside the scope/magnification of the FOV.

Although the PICR may have been a consequence of receptor activation induced by the underlying substrate, it is important to note that cells were usually exposed to the underlying (*e.g.*, glass, collagen, fibronectin) substrate for at least 10-15 minutes before perfusion was applied (Curtis, 1964). As a result, $[Ca^{2+}]_i$ was measured in non-perfusing physiological solution over a time period that permitted receptor-mediated events to unfold and to affect $[Ca^{2+}]_i$. The fact that the $[Ca^{2+}]_i$ increased only following the onset of perfusion, however, confirms that the calcium response seen in eosinophils is caused by fluid flow rather than a non-specific interaction with the surrounding substrate.

From a physiological perspective, our experimental set-up can be seen to replicate the physical perturbation that an eosinophil experiences as it flows through the circulation and

collides with the walls of the vasculature and/or with other cells in the blood. The resultant perturbation is described as fluid shear stress, and is a function of the velocity of the fluid and its intrinsic viscosity (Papaioannou & Stefanadis, 2005; Traub & Berk, 1998).

In our set-up, fluid shear-stress includes the deformative forces of the fluid that act on eosinophils in a direction parallel to the surface of the underlying glass (Sundd et al., 2011; Papaioannou & Stefanadis, 2005). As the perfusing RPMI washes over the cell and imparts acceleration, cells begin to flow between the two glass slides with some non-zero velocity. In the presence of laminar fluid flow, the fluid's flow rate is zero at the glass boundary and greatest in the middle of the two plates (Papaioannou & Stefanadis, 2005). Eosinophils transitioning/spanning from the middle to the top/bottom regions of the preparation are therefore subjected to a differential gradient of fluid flow (Curtis, 1964; Papaioannou & Stefanadis, 2005). Different spatial regions of the cell (de)accelerate with respect to one another according to where the cell is situated within this gradient: cells regions that are closer to the edges of the vessel decelerate more rapidly than those regions that are closer to the centre, subjecting the cell to shear stress (Papaioannou & Stefanadis, 2005). In addition to this fluid-derived shear stress, any collision of these now-flowing cells with the underlying, stationary glass ultimately causes that portion of the cell membrane to decelerate relative to the opposite surface. The resultant mechanical (as opposed to fluid) stress can also contribute to shearing of the eosinophil.

The shear stress imparted upon eosinophils in our preparation fundamentally results from the frictional forces generated by the cells' collision with the glass and/or the rate at which the perfusion is applied. In the body, shear-stress might arise as the eosinophil collides with the wall of the blood vasculature (Traub & Berk, 1998). Physiologically, the shear experienced by eosinophils *in vivo* might be influenced by several critical factors. Firstly, blood constitutes a

suspension of proteins and cells that, for a number of reasons, might exhibit turbulent (rather than laminar) flow (Papaioannou & Stefanadis, 2005). Secondly, regional differences in blood pressure result in eosinophils experiencing drastic variations in blood flow as they travel from the heart towards the peripheral microvasculature (Papaioannou & Stefanadis, 2005). Lastly, adhesion receptor molecules expressed on eosinophils could bind to the vascular endothelium and provide a tensional force that opposes the direction of the cell's flow and thereby reinforces the shear stress that the cell experiences at the blood-endothelial interface (Sundd et al., 2011).

4.8 Identifying the Molecular Cognate of the PICR

The experimental findings described thus far suggest that the PICR constitutes a *bone fide* physiological phenomenon that merits closer investigation. We subsequently sought to determine the hitherto unidentified mechanosensor that serves as the molecular cognate of the PICR in human eosinophils. The most logical candidates that might sub-serve this role are the integrin receptors that have been previously described for their central role in eosinophil adhesion (see: Chapter 1; Sigal et al., 2000; Ogneva, 2013; Chase et al., 2012). Since integrins can bind to ECs and/or to the ECM, their unique capacity to engage in bidirectional signaling enables them to both sense and to respond to physical perturbations via intracellular signal transduction cascades, particularly via the influx/release of Ca^{2+} ions (Johansson et al., 2013; Gottlieb et al., 2004).

Physiologically, the binding of integrins to their counter-ligands on the vascular endothelium during eosinophil recruitment could physically transmit the tensile force required to decelerate the eosinophil as it adheres to the vascular endothelium (Lessey et al., 2012). As a result, integrin-binding could feasibly impart a shearing stress upon the cell and thereby trigger

PICR. Most importantly, however, the adhesive contact between the integrin and its counter-ligand comprises a molecular hub through which signals are transmitted from the ECM to the actin cytoskeletal network, which subsequently relays a signal back towards the integrin that either reinforces or severs the adhesive contact (Alon & Dustin, 2007; Gopalan et al., 1997). Any possible involvement of integrin adhesion receptors in the PICR would therefore likely depend on their unique structural and functional properties.

4.8.1 RGD-binding Integrins Mediate the PICR

Our subsequent experiment therefore sought to explore whether or not integrins mediate the PICR in human eosinophils. Although integrins exhibit remarkable diversity in structure and function, it is unsurprising that at least half of the 20 known integrin receptors have been implicated in some form of mechanotransduction (Alon & Dustin, 2007; Alon & Ley, 2008). Most likely, this is due to the fact that many of these overlap in their abilities to recognize and bind to the Arg-Gly-Asp (RGD) peptide sequence expressed in the ECM proteins or within their counter-ligands (*e.g.*, VCAM-1; Ruoslahti, 1996; Ruoslahti & Pierschbacher, 1987; D'Souza et al., 1991). Consequently, the use of RGD tripeptides has long been critical to the study of integrin receptors: insoluble RGD tripeptides serve to activate integrins, whereas soluble peptides inactivate integrins (Ruoslahti, 1996; Takagi, 2004). In our experiments, pretreating cells with soluble RGD abolished the PICR in a concentration-dependent manner, confirming that at least one of the RGD-binding integrins expressed on eosinophils is mechanosensitive and directly participates in the PICR and downstream adhesion/migration in these cells.

The role of integrins as mechanosensors of shear stress has been well-acknowledged in the literature (Katsumi et al., 2004; Shyy & Chien, 2002; Egger et al., 2002; Cortijo et al., 2006;

Vanderslice et al., 2004). In fact, various groups have used monoclonal antibodies (mAbs) directed against the active/inactive conformations of integrin receptors to show that, in the presence of physiological levels of shear stress, the active integrin conformation dominates (Woodside & Vanderslice, 2008). Remarkably, this phenomenon can be blocked by blocking mAbs or peptidomimetics that occupy the ligand-binding domain of the receptor, suggesting that this specific region of the receptor is directly involved in the mechanosensitive response (Frick et al., 2005).

The finding that RGD can block the PICR is consistent with previous studies that have variously described mechanosensor functions for RGD-binding integrins receptors that are expressed on other cell types (Gehlsen et al., 1988). In particular, Komai & Schmid-Schonbein (2005) have shown that neutrophils undergo de-activation in the presence of fluid shear-stress in an erythrocyte-dependent manner. Similarly, Phillipson et al. (2009) used intravital microscopy to show that the $\alpha_M\beta_2$integrin (CD11b/Mac-1) and/or the $\alpha_L\beta_2$ integrin (LFA-1) receptor mediates intraluminal crawling of murine neutrophils on the vascular endothelium in a manner perpendicular to the flow of blood. In addition, Traub & Berk (1998) have described that endothelial cells respond to hypertension-induced increases in fluid-shear stress by reorienting themselves in a direction parallel to the direction of blood flow, which in turn minimizes the fluid shear-stress on these cells and confers an athero-protective effect (Davies et al., 1986; Traub & Berk, 1998; Inoue et al., 2009).

To the best of our knowledge, whether or not integrins are mechanosensors that modulate $[Ca^{2+}]_i$ dynamics has yet to be shown in human eosinophils. Tachimoto et al. (2002) have certainly suggested that the functional responses of eosinophils depend on the presence of external fluid flow. Elsewhere, it has similarly been shown elsewhere that the perfusion of

untreated eosinophils induces their adhesion in an $\alpha_4\beta_1$ integrin-dependent manner (Tachimoto et al., 2000). The scope of these studies, however, have been largely limited to the functional effects (*i.e.*, adhesion) of shear stress and few, if any, have described how shear stress modulates $[Ca^{2+}]_i$ in eosinophils.

4.8.2 Selective Antagonism of the $\alpha_4\beta_1/\alpha_4\beta_7$ Integrins Attenuates the PICR

The $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrin receptor subtypes comprise the most highly expressed integrins in eosinophils and are thought to mediate the migration of these cells to the airways and to the gut in both health and disease (Giembycz & Lindsay, 1999). Early studies characterizing the so-called 'hyper-adhesive' phenotype of eosinophils motivated the development of small molecular integrin receptor antagonists that, unlike their humanized mAb counterparts, exhibit vastly superior bioavailability and pharmacokinetic parameters (Barthel et al., 2006b; Walker et al., 1993). We consequently used a second generation, dual $\alpha_4\beta_1/\alpha_4\beta_7$ integrin antagonist, known as CDP-323 (Zaurategrast^{TM}), to test our suspicion the PICR is mediated by these two RGD-binding integrins in human eosinophils. CDP-323 is a highly potent and selective analogue of the precursor, TR-14035, and was originally developed for use as a pharmacological intervention for multiple sclerosis (Prat & Stuve, 2012; Cortijo et al., 2006; Egger et al., 2002). Pre-treating eosinophils with CDP-323 resulted in a drastic decrease in the magnitude of the PICR in a manner consistent with the effects of RGD tripeptides.

The antagonistic effects of CDP-323 and RGD were evident only when eosinophils were layered onto fibronectin-coated glass. Fibronectin is an ECM protein that possesses the RGD binding sequence in the cell-binding domain, located in module III (Clements et al., 1994; Takagi, 2004). The attenuation in the PICR induced by both CDP-323 and RGD strongly

support the involvement of $\alpha_4\beta_1$ and/or $\alpha_4\beta_7$ integrin receptors in mediating mechanosensitivity in eosinophils. The initial study that motivated the development of CDP-323 demonstrated that blocking $\alpha_4\beta_1$ and/or $\alpha_4\beta_7$ integrins leads to decreased eosinophil accumulation and airway hyperresponsiveness in an allergic model of airway disease in Brown Norway rats (Cortijo et al., 2006). The improvements in symptoms reported in their study occurred despite persistently high levels of IL-5 production. Given that IL-5 primes eosinophils towards eotaxin-1 responsiveness, these findings indirectly suggest that the eotaxin-mediated migration of eosinophils was effectively abolished by the dual $\alpha_4\beta_1/\alpha_4\beta_7$ integrin antagonist (Cortijo et al., 2006; Egger et al., 2002). Our final sets of experiments therefore sought to elucidate whether or not these integrins engage in cross-talk with the eotaxin-1 signal transduction cascade.

4.9 Blocking the Mechanosensitive $\alpha_4\beta_1/\alpha_4\beta_7$ Integrins Abolishes CCR3 Signaling

We stimulated cells with fluid perfusion and/or increasing concentrations of eotaxin-1. Accordingly, the calcium-response evoked by perfusion in eotaxin-free mediate corresponds to the PICR described thus far (Figures 22 and 14). The application of fluid perfusion alongside 1 or 10 nM eotaxin-1 results in seemingly greater calcium-responses, but the magnitudes of the calcium-responses evoked under either of these conditions are not statistically different from those elicited in the absence of perfusion. The magnitude of the calcium-response induced by application of 1 nM (or 10 nM) eotaxin-1 is as great as the magnitude of the response evoked by 1 nM (or 10 nM) eotaxin-1 + perfusion. Contrary to our expectations, the calcium-responses induced by the combination of eotaxin-1 and fluid perfusion are therefore not summative.

It is important to note, however, that the PICR and the calcium-response triggered by eotaxin-1 are both mediated by calcium-release from the ER. The stores of Ca^{2+} ions within the

ER are finite and utilized by both stimuli. For this reason, either stimulus could foreseeably consume the very same pool of Ca^{2+} ions needed to transduce the signal activated by the other stimulus. The fact that both perfusion and eotaxin-1 mobilize ER Ca^{2+} as part of their respective signaling programs ultimately highlights the central role of Ca^{2+} ions in both integrin and chemokine receptor signaling.

In the presence of fluid perfusion, CDP-323 can dramatically abolish the Ca^{2+}-response normally elicited by eotaxin. In contrast, CDP-323 fails to affect the eotaxin-mediated calcium response in the absence of fluid shear stress. The perfusion-dependence of this observation suggests that the signaling program of eotaxin-1 is uniquely influenced by the presence of fluid shear stress, which is likely sensed by the mechanosensitive $\alpha_4\beta_1$ and/or $\alpha_4\beta_7$ integrins. Given that CDP-323 fails to attenuate the calcium-response elicited by eotaxin-1 in the absence of fluid shear stress, we postulate that the mechanosensitive signals transduced by the $\alpha_4\beta_1/\alpha_4\beta_7$ integrins override and pre-dominate the Ca^{2+}-handling mechanisms that determine the global $[Ca^{2+}]_i$ in human eosinophils.

It is tempting to extrapolate our findings to the physiological context, in which eosinophils are exposed to both eotaxin and shear stress in the vascular lumen. In the blood vasculature, it would be particularly advantageous for the behavior and function of cells to be primarily dictated by the activation state of shear-sensitive integrin receptors. As the eosinophil travels through the blood vasculature towards the site of inflammation, a mechanism that permits the cell to respond to shear stress imparted by increased blood flow (e.g., through a dilated capillary) could facilitate the cell's adhesion and extravasation at the inflamed tissue, even in the absence of chemokine signaling. In their comprehensive report, Papaioannou & Stefanadis (2005) indicate that arterioles and capillaries experience the highest shear stress and shear rates

compared to any other regions of the blood vascular circuit. The shear stress and shear rate experienced in the arteriole is approximately ~50 dyn/cm^2 and >1500 s^{-1}, respectively. By contrast, the ascending aorta experiences shear stress and shear rates on the order of ~10 dyn/cm^2 and <250 s^{-1}. Most intriguing is the finding that the arteriolar and the capillary segments of the vasculature exhibit the greatest and most dramatic increases in both shear-stress. Taken together, these findings support the possibility that the PICR constitutes a biological mechanism by which eosinophils activate as they transition into inflamed microcirculation (Papaioannou & Stefanadis, 2005).

Once they have entered the tissues, however, eosinophils have no need or use for a mechanosensitive mechanism since the surrounding extracellular fluid is stagnant. In this case, it follows that chemokine signaling would be highly prioritized, permitting the cell to migrate towards the chemotactic source. Ultimately, the excitatory effects of fluid perfusion and eotaxin-1 on eosinophils may provide a more complete and holistic explanation for how eosinophils exit the vasculature and enter the tissues during states of health and disease.

4.10 Conclusions and Future Directions

The phenomenon of mechanotransduction has been described in a variety of cellular systems, including neurons, myocytes and endothelial cells. While other groups have remarked on the flow-dependence of eosinophil adhesion, there is a dearth of evidence regarding the molecular mechanisms and the signal transduction cascades involved in this response. Most importantly, the functional effects of fluid perfusion have yet to be described in relation to the ionic Ca^{2+} handling mechanisms in human eosinophils. Our study elegantly demonstrates that $\alpha_4\beta_1$ and/or $\alpha_4\beta_7$ integrins function as molecular mechanosensors that directly regulate the $[Ca^{2+}]_i$

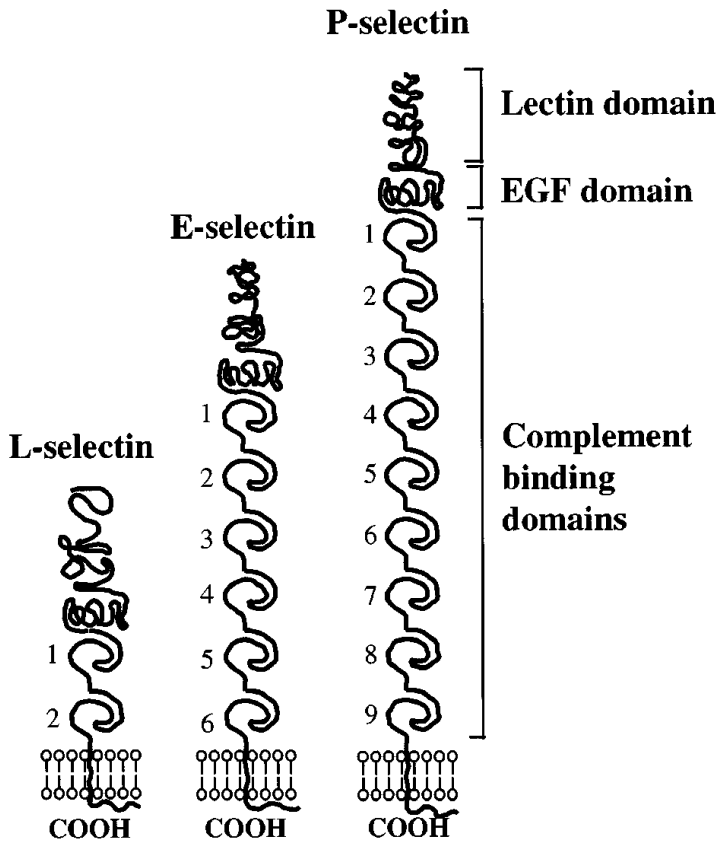
and the adhesion/migration of human eosinophils. *In vivo*, the putative mechanosensitive properties of eosinophils suggest that these cells might respond to acute variations in blood flow that accompany states of health and disease. The potential for $\alpha_4\beta_1/\alpha_4\beta_7$ integrins to fine-tune eosinophil sensitivity and responsiveness to inflammatory stimuli, particularly eotaxin-1, is noteworthy.

Local inflammation activates the kinin-kallikrein system by mobilizing a series of blood proteins/enzymes, which elicit hemodynamic changes that include vasodilation of pre-capillary arterioles and vasoconstriction at post-capillary venules (Guyton & Hall, 2006). This augments the net leakage of fluids into the tissues and promotes the formation of edema, which in turn facilitates the increased entry of inflammatory cells into the inflamed capillary bed. In addition to the influx of inflammatory cells, however, alterations in the diameter of the (pre/post)capillary endothelium affect the rate at which eosinophils flow through these vessels and determine the shear-stress that these cells experience (Papaioannou & Stefanadis, 2005). Under inflammatory conditions, we propose that the mechanosensitive $\alpha_4\beta_1$ and/or $\alpha_4\beta_7$ integrins permit eosinophils to respond towards altered blood flow rates, promoting their adhesion and extravasation to various tissue sites.

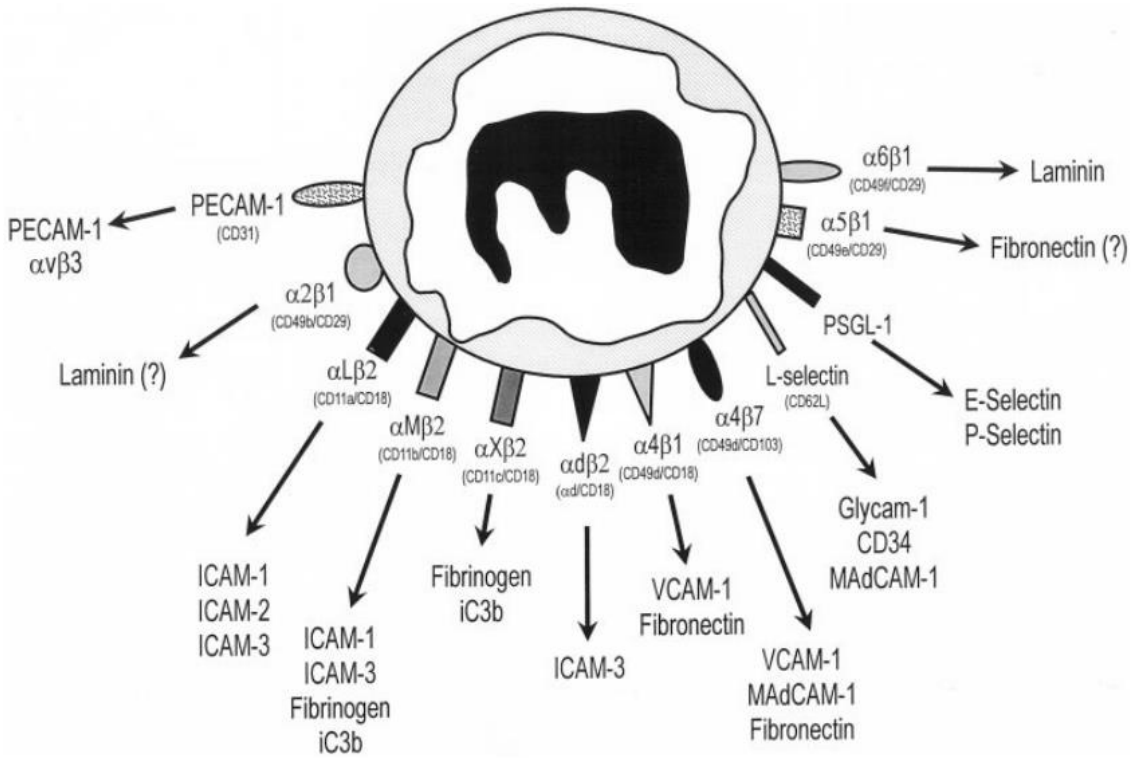
In large part, the purported inability of eotaxin-1 molecules to dissociate from HSPGs on the vascular endothelium and to thereby activate CCR3 on the surface of eosinophils transiting through the vasculature must be reconciled with our established understanding that airway eosinophilia is preeminently mediated by eotaxin-1 (Peter et al., 2009; Conroy & William, 2001; Rankin et al., 2000). The failure of anti-IL5 (and other) therapies to block eosinophil accumulation in severe allergic asthmatics favours the possibility that some hitherto unrecognized and non-chemokine-dependent mechanism might also facilitate the infiltration of

eosinophils into the tissues (Garcia et al., 2013). Our model thus expands upon the established paradigm of leukocyte extravasation and provides an added layer of complexity whereby the activation of integrin receptors might in fact govern the chemokine receptor activation and signaling.

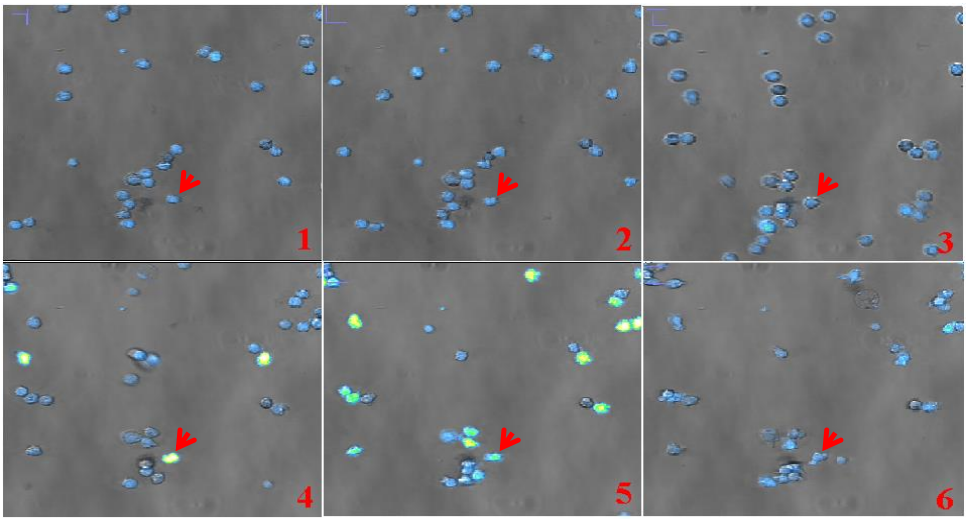
Our lab is currently seeking to rule out the contribution of other, non-$\alpha_4\beta_1/\alpha_4\beta_7$ integrins that might be involved in the PICR. Further molecular experiments involving the use of blocking mAbs would be ideal in addressing this question, as it would allow us to examine how perfusion modulates the activation states of related integrins expressed on eosinophils. In order to appropriately contextualize the physiological significance of the PICR, future experiments should also quantify the magnitude of the shear-stress to which our cells were exposed in relation to previously reported levels of physiological shear-stress in the blood (Papaioannou & Stefanadis, 2005). Lastly, although we strived to ensure that perfusion was applied at a near constant and reasonable rate during all experiments, a direct measurement of the physical stress imposed upon cells would be vital to drawing any decisive conclusions about how fluid shear-stress affects the $[Ca^{2+}]_i$ and the function of human eosinophils.



Appendix 1. Structure of L-, E- and P-selectins. All three selectin receptors possess an extracellular lectin binding domain. The lectin binding domain mediates ligand-receptor interactions and is a highly conserved region in all three receptor subtypes. In addition, the selectins possess complement binding domains that may engage with complement proteins that are involved in the removal of foreign extracellular pathogens. The lengths of these domains vary, however, with P-selectin possessing the largest complement binding domain out of all three selectin subtypes. (Image from Panes & Granger, 1998).



<u>Appendix 2.</u> Eosinophil integrins and their counter-ligands. Eosinophils express a multitude of integrin receptors, which exhibit distinct ligand-binding properties. Integrin-ligand interactions are primarily determined by the constituent α- and β-subunits of the receptor, with most integrins recognizing common sets of amino acid binding sequences (e.g., RGD). Although several different integrin receptors might be capable of binding to the same ligand (e.g., fibronectin), this association might occur through distinct ligand-binding sites expressed on the receptor, which in turn may or may not recognize different motifs present in the ligand. (Image from Giembycz & Lindsay, 1999).



<u>Appendix 3.</u> Morphological changes in human eosinophils consequent to the PICR. In the first three panels, the physiological bathing medium is stagnant, cells are primarily balled-up and average calcium levels are low. Once perfusion is applied (panel 4-6), most cells exhibit a PICR (green flashes) that precede a rapid change in shape and position. Cells that were round/circular become polarized and oblong, and subsequently begin moving about the surface of the glass. The change in cell position can be seen to occur as a result of cell activation rather than fluid perfusion-induced displacement, since some cells appear to move opposite to the direction of the fluid flow (black arrow). Panels represent time-lapse images taken 40 s apart, where green represents maximal $[Ca^{2+}]_i$ and blue shading represents minimal $[Ca^{2+}]_i$.

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