

THE EFFECTS OF PPAR AGONISTS ON EOSINOPHIL FUNCTION

THE EFFECTS OF PPAR AGONISTS ON EOSINOPHIL FUNCTION

By STEVEN GREGORY SMITH

A Thesis Submitted to the School of Graduate Studies in Fulfilment of the
Requirements for the Degree, Doctor of Philosophy

McMaster University © Copyright Steven Gregory Smith, October 2012

DOCTOR OF PHILOSOPHY (2012)

Medical Science

McMaster University

Hamilton, Ontario

The Effects of PPAR Agonists on Eosinophil Function

Steven Gregory Smith, Master of Science

Dr. G.M. Gauvreau

xv, 191

Abstract

PPAR agonists have been suggested as novel therapeutics for the treatment of inflammatory lung disease, such as allergic asthma. Treatment with PPAR agonists have been shown to inhibit peripheral eosinophilia in murine models of allergic asthma, which can occur through several mechanisms including decreased cytokine/chemoattractant (IL-5/eotaxin) release, decreased eosinophil migration and/or decreased eosinophil differentiation. This is the first study to show that PPAR γ is expressed at the protein level in human airway eosinophils sampled from induced sputum, and confirms PPAR γ protein expression in human peripheral blood eosinophils. We demonstrated the novel observation that peripheral blood eosinophil PPAR γ protein expression, as measured by flow cytometry, is not different in eosinophils purified from asthmatic subjects compared to healthy controls and these observations suggest that the level of PPAR γ expressed in human eosinophils is not related to asthmatic status. Our study also confirms, by real time PCR, the detection of mRNA for PPAR γ in airway-derived leukocytes, collected from bronchial washings, increases 24hrs after whole lung allergen challenge. This increase is regulated by Symbicort® and Pulmicort® treatment in most subjects. This is the first study to show increased chemokinesis (random stimulated movement) of eosinophils *in vitro* at low concentrations of a PPAR γ agonist. We have generated data to suggest this is through an effect on calcium signalling. We also observed that higher concentrations of PPAR agonists directly inhibit eotaxin-stimulated eosinophil directional migration. Finally, using methycellulose cultures of non-adherent mononuclear cells and CD34+ progenitor cells, we demonstrate that PPAR activation can inhibit the differentiation of eosinophils *in vitro*. Collectively, these data demonstrate that PPAR γ is expressed

constitutively on eosinophils in peripheral blood and airways, and suggest signaling through this receptor with nanomolar concentrations of agonist regulates eosinophilia through inhibition of both eosinophil migration and eosinophil differentiation.

Acknowledgments

I would like to thank Dr. Gauvreau for believing in my potential to learn, perform and produce quality research. As my mentor, she has taught me how to improve my experimental design, how to write in a concise scientific manner and how to work effectively in the research community. These lessons have formed the basis for my future research experiences and I hope to make her proud. I would also like to thank Dr. Sehmi for her involvement in my PhD project. Her encouragement has helped me to develop and achieve my project goals. I appreciate her ongoing support and for making me feel like my research mattered. Another great mentor I had during my PhD was Dr. Killian. He always challenged my way of thinking and he taught me several lessons on being a good scientist during my McMaster University career. His lessons have influenced my research and I will always be grateful. I'd also like to thank Dr. O'Byrne for his support over the last 6 years. I have enjoyed every minute of the lab and I will miss it immensely. The cardiorespiratory research unit has been a great place to learn and grow, and I appreciate this wonderful opportunity. I would also like to thank Tara Strinich. Tara has been an entertaining co-worker and she could always brighten my day. Last, but not least, I would like to thank Jamie Corbett, my wife, for all her encouragement and endless support. She believed in me from the start and has had the patience of a saint. Thank you and I love you.

Table of Contents

	Page		Page
Abstract	iii	Chapter 3	62
Acknowledgments	v	3.1 Introduction	63
List of Tables	vii	3.2 Materials and Methods	65
List of Figures	viii	3.3 Results and Discussion	80
List of Abbreviations	xi	3.4 Conclusion	84
		3.5 Appendix	86
Chapter 1	1	3.6 Tables and Figures	90
1.1 Introduction	2	3.7 References	101
1.2 References	14		
		Chapter 4	112
Chapter 2	27	4.1 Introduction	113
2.1 Introduction	28	4.2 Materials and Methods	114
2.2 Materials and Methods	29	4.3 Results and Discussion	121
2.3 Results and Discussion	47	4.4 Conclusion	123
2.4 Conclusion	49	4.5 Appendix	125
2.5 Tables and Figures	51	4.6 Tables and Figures	130
2.6 References	56	4.7 References	141
		Chapter 5	149
		5.1 Introduction	150
		5.2 References	172

List of Tables

	Page
Table 2.1. PPAR γ expression in peripheral blood eosinophils isolated from allergic asthmatic and healthy control subjects.	50
Table 3.1 Bradford assay results for eosinophil protein (mg/mL) from 4 subjects after treatment with the PPAR γ agonist rosiglitazone.	89
Table 3.2 Eosinophil expression of CCR3 and phosphorylation of ERK1/2 and p38 after treatment with the PPAR γ agonist rosiglitazone.	90

List of Figures

	Page
Figure 2.1. Gating strategy used to isolate peripheral blood eosinophils from the whole blood population.	51
Figure 2.2. A representative western blot of PPAR γ expression in isolated eosinophils.	52
Figure 2.3. A fluorescent image of PPAR γ expression in the sputum of an allergic asthmatic subject.	53
Figure 2.4. The effect of Pulmicort® and Symbicort® treatment on the expression of PPAR γ mRNA in BW cells from allergic asthmatics 24hrs after allergen challenge.	54
Figure 3.1. Gating strategy used to isolate peripheral blood eosinophils from the isolated population.	91
Figure 3.2. ELISA standard curves for phosphorylated ERK1/2 and p38.	92
Figure 3.3. The effect on eotaxin-induced eosinophil migration of agonists to PPAR γ (a; rosiglitazone), PPAR α (b; GW9578), and PPAR β/δ (c; GW50516) at low (0.1 – 100 nM; 9 subjects) and high concentrations (1000 – 100,000 nM; 6 subjects).	93
Figure 3.4. The effect on eosinophil chemokinesis of agonists to PPAR γ (a; rosiglitazone), PPAR α (b; GW9578), and PPAR β/δ (c; GW50516) at low (0.1 – 100 nM; 9 subjects) and for PPAR γ (a; rosiglitazone) at high concentrations (1000 – 100,000 nM; 6 subjects).	94

Figure 3.5. The effect of rosiglitazone (PPAR γ agonist) on SDF-1 α -induced migration of IL-5R α + CD34+ cells isolated from cord blood (6 subjects).	95
Figure 3.6. The effect of 100 nM rosiglitazone treatment on the eotaxin-induced increase in [Ca ²⁺] _i , measured by the frequency of calcium oscillations and maximum fluorescence intensity (representative tracing from 3 experiments).	96
Figure 3.7. Eotaxin-induced eosinophil chemotaxis using MTS dye assay.	97
Figure 3.8. Light microscope image of a polycarbonate filter after the eotaxin-induced chemotaxis assay that has been stained with Diff-Quik®.	98
Figure 3.9. Eotaxin-induced eosinophil chemotaxis using Cell Glo chemluminescence.	99
Figure 4.1. Optimization of IL-5-induced eosinophil differentiation.	129
Figure 4.2. An Eo/B-CFU after 2-week incubation with IL-5 in Methocult®.	130
Figure 4.3. The effect of rosiglitazone (PPAR γ agonist) on IL-5-induced eosinophil differentiation of non-adherent mononuclear cells isolated from the peripheral blood of allergic subjects.	131
Figure 4.4. The effect of troglitazone (PPAR γ agonist) on IL-5-induced eosinophil differentiation of non-adherent mononuclear cells isolated from the peripheral blood of allergic subjects.	132

Figure 4.5. The effect of rosiglitazone (PPAR γ agonist; upper panel), GW9578 (PPAR α agonist; middle panel), and GW501516 (PPAR β/δ agonist; lower panel) on IL-5-induced eosinophil differentiation of CD34 cells isolated from isolated from the peripheral blood of allergic subjects.	133
Figure 4.6. The effect of rosiglitazone (PPAR γ agonist) on IL-5/IL-3- induced eosinophil differentiation of CD34+ cells isolated from peripheral blood of allergic subjects.	134
Figure 4.7. The effect of rosiglitazone (PPAR γ agonist) on IL-5- induced eosinophil differentiation of non-adherent mononuclear cells isolated from the peripheral blood of COPD subjects.	135
Figure 4.8. The effect of rosiglitazone (PPAR γ agonist) on IL-5- induced eosinophil differentiation of non-adherent mononuclear cells isolated from the bone marrow of allergic asthmatic subjects.	136
Figure 4.9. FlowJo® plots of the phosphorylation time course for ERK1/2, p38 and STAT5.	137
Figure 4.10. FlowJo® plots of CD34+ cell gating strategy.	138
Figure 4.11. The effect of rosiglitazone (PPAR γ agonist) on IL-5- induced ERK1/2 phosphorylation.	139

List of all Abbreviations and Symbols

15d-PGJ ₂	15-Deoxy- Δ 12,14-Prostaglandin J2
2-ME	2-Mercaptoethanol
AHR	Airway hyperresponsiveness
AM	Acetoxymethyl
ANOVA	Analysis of variance
APC	Allophycocyanin
ATP	Adenosine triphosphate
BAL	Bronchoalveolar lavage
BME	Basal medium Eagle
BSA	Bovine serum albumin
BW	Bronchial wash
CB	Cord blood
CCR3	C-C chemokine receptor type 3
CD45	Cluster differentiation 45
cDNA	Complementary DNA
CO ₂	Carbon dioxide
COX-2	Cyclooxygenase-2
CXCR4	Chemokine receptor type 4
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide

DPBS	Dulbecco PBS
DTT	Dithiothreitol
EC50	half maximal effective concentration
ECP	Eosinophil cationic protein
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
Eo/B-CFU	eosinophil/basophil colony-forming units
EPC	Endothelial progenitor cells
EPX	Eosinophil peroxidase
ERK1/2	Extracellular signal-regulation kinase-1
FACS	Fluorescence-activated cell sorting
FBS	Fetal Bovine Serum
Fc	Fragment, crystallisable
FEV ₁	Forced expired volume in 1 second
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GINA	Global Initiative for Asthma
GMCSF	Granulocyte/macrophage colony-stimulating factor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish peroxidase
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IL-3	Interleukin-3

IL-5R α	Interleukin-5 receptor- α
IMDM	Iscove's modified Dulbecco's medium
JAK	Janus Kinase
LAR	Late asthmatic response
LC	Langerhan cells
LPS	Lipopolysaccharide
MACS	Magnetic-activated cell sorting
MAPK	Mitogen-activated protein kinase
MCh PC ₂₀	Methacholine provocative concentration that causes a 20% fall in FEV ₁
MEK	MAPK kinase
MFI	Median Fluorescence intensity
mRNA	Messenger ribonucleic acid
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NAMNC	Non-adherent mononuclear cells
NFAT	Nuclear factor of activated T-cells
NF κ B	Nuclear factor κ B
NH ₄ Cl	Ammonium chloride
NSAID	Nonsteroidal anti-inflammatory drugs
NTC	No template control
OVA	Ovalbumin
PBS	Phosphate buffered saline
PE	Phycoerythrin

Penstrep	Penicillin/Streptomycin
PFA	Paraformaldehyde
PMT	Photomultiplier tube
PPAR	Peroxisome Proliferator-activated Receptor
PPRE	PPAR response elements
RBC	Red blood cell
RIPA	Radioimmunoprecipitation assay
RPMI	Roswell Park Memorial Institute
RT-PCR	Real time polymerase chain reaction
RXR	Retinoid X receptor
SDF-1 α	Stromal cell-derived factor-1 α
STAR	Signal transduction assay
STAT	Signal transducers and activators of transcription
Th2	T helper 2
TMB	3,3',5,5'-Tetramethylbenzidine
TNF α	Tumor necrosis factor α
β c	Common β chain

Declaration of Academic Achievement

I have been the main driving force behind the work in this thesis. However, I would like to acknowledge those who helped along the way. First, I'd like to thank Dr. Sehmi for introducing me to progenitor cell migration. Dr. Sehmi and her students, Neha Punia and Anam Irshad showed me the techniques I needed for the study. I'd also like to thank Dr. Janssen for introducing me to confocal microscopy. Without your help calcium measurements would have not been possible. With the help of a summer student, Mike Hill, the eosinophil differentiation experiments in COPD subjects were possible. Finally, I'd like to thank Karen Howie and Adrian Baatjes for introducing me to the differentiation assay and flow cytometry, respectively. These techniques were an important part of my work. Thank you.

Chapter 1: Peroxisome Proliferator-Activated Receptors and Allergic Asthma

1.1 Introduction

Eosinophils and Allergic Asthma

Asthma is an inflammatory disease, characterized by airflow obstruction, airway hyperresponsiveness and airway inflammation, which affects 3 million Canadians (Statistics Canada, 2010). There are an estimated 300 million asthmatics worldwide (Masoli, *et al.*, 2004). The prevalence of asthma in the 84 different countries studied ranged from 1 to 18% of the population (Masoli *et al.*, 2004). The World Health Organization estimates that asthma represents 1% of the global disease burden (Masoli *et al.*, 2004). Annually there are 250,000 deaths from asthma worldwide (Masoli *et al.*, 2004). Although asthma treatment is a substantial economic burden, the costs associated with uncontrolled asthma are even more substantial (Chaudhuri *et al.*, 2006). Asthmatics symptoms can be triggered by exercise, viral infections, exposure to allergens, and/or inhaled chemical irritants (GINA Science Committee, 2011). Allergen exposure is extensively studied, is a significant cause of asthma development (Sporik *et al.*, 1990; Nelson, 2000), and is linked to asthma severity (Zock, *et al.*, 1994; Nelson, 2000). Avoidance of allergens results in an improvement in asthmatic symptoms (Nelson, 2000; Piacentini *et al.*, 1996) and sensitivity to house dust mite, cat, dog and mold spores is associated with an increased risk of asthma (Nelson, 2000; Sears *et al.*, 1989). Thus the immunological response to allergens is a significant focus of asthma research.

Corticosteroids, β -agonists and leukotriene inhibitors (Zdanowicz, 2007) are the best therapies to date for treatment of asthma, but they are associated with side effects. Corticosteroids are associated with osteoporosis, growth suppression, adrenal

suppression, oral candidiasis (Zdanowicz, 2007), and in some cases patients do not respond to treatment (Strek, 2006; Leung *et al.*, 2002). Leukotriene inhibitors have fewer side effects than corticosteroids (Zdanowicz, 2007). However, they are less effective than inhaled corticosteroids alone (Busse *et al.*, 2001) and less effective as a combination therapy than long acting β -agonists (Nelson *et al.*, 2000). Long acting β -agonists maybe associated with tachycardia (Zdanowicz, 2007) and with desensitization of the β -adrenergic receptor (Lipworth, 1997). Thus novel approaches targeting the inflammatory pathways of asthma are currently being examined in hopes of developing improved asthma therapies.

Allergens must overcome protective barriers, such as the cough reflex and the mucocillary apparatus, to enter the airway and elicit an exacerbation (Lambrecht *et al.*, 2003). Once past these barriers, allergens can interact with IgE antibodies and dendritic cell appendages that protrude into the epithelial cell layer (Hammad & Lambrecht, 2006). Acute bronchoconstriction can be observed within 10 minutes of allergen inhalation (O'Byrne, 1988) as a result of allergen activation of mast cells and basophils through crosslinking of IgE bound to their high affinity IgE receptors. The crosslinking leads to degranulation and acute bronchoconstriction induced by the release of mediators, such as histamine and cysteinyl leukotrienes (Holgate, 2008). This is known as the early asthmatic response (O'Byrne, 1988). Approximately 50% of adult subjects develop a second bronchoconstriction approximately 3 hours later (O'Byrne, 1988), which is known as the late asthmatic response (O'Byrne, 1988). The late asthmatic response is driven by the uptake of allergens by dendritic cells causing their activation and migration to the local lymph nodes where they stimulate proliferation of T and B cells (Hammad & Lambrecht, 2006). Antigen specific T cells

differentiate into T helper cells (Lambrecht *et al.*, 2003) and migrate to the affected area where they recruit macrophages, basophils and eosinophils during the late asthmatic response, through release of Th2 cytokines such as IL-3, IL-4, IL-5, IL-9, IL-13 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Holgate, 2008).

Links have been made between the physiological responses after allergen challenge and the cellular mechanisms that are potentially responsible. A correlation of inflammatory cell infiltration into the lung with physiological responses (FEV₁ and methacholine PC₂₀) has been observed in asthmatics post-allergen challenge (Gauvreau *et al.*, 1999). Increased amounts of circulating IgE and airway hyperresponsiveness predicts the presence of the early asthmatic response (Cockcroft *et al.*, 2007), whereas the influx of eosinophils into the airway correlates with the late asthmatic response and airway hyperresponsiveness (Cockcroft *et al.*, 2007). While early studies have formed the framework for examining the cellular mechanisms involved in the pathogenesis of allergic asthma, the cellular targets have evolved over the years.

Asthma therapy is focused on reducing the associated symptoms and frequency of exacerbation by attempting to modify the mechanisms of lung inflammation and bronchoconstriction. Eosinophils represent a major effector cell in asthma (Hogan *et al.*, 2008) thus is a major target for drug development. Recently, studies using anti-IL-5 therapy have shown that although eosinophils are a marker of the late asthmatic response and AHR, they may not be the major underlying cause (Phipps *et al.*, 2004; Leckie *et al.*, 2000). These negative results have initiated debate about the importance of eosinophils in asthma. However, subsequent studies have

since re-established the eosinophil as an important effector cell in asthma. Using similar treatment methods as the previous study, mepolizumab, an anti-IL-5 therapy, was shown to reduced eosinophils in brochial biopsies by 50% (Flood-Page *et al.*, 2003a) and this reduction was associated with a significant decrease in extracellular matrix protein deposition (Flood-Page *et al.*, 2003b), which has been linked with increased asthma severity (Chetta *et al.*, 1997). Also Nair and colleagues have shown that treatment with mepolizumab improved asthma control up to 8 weeks after treatment and caused a significant decreased in the prednisone dose required by asthmatic patients with persistent eosinophilia (Nair *et al.*, 2009). Another study showed that patients with refractory eosinophilic asthma that were on mepolizumab treatment had significantly fewer severe exacerbations than placebo over a 50 week period (Haldar *et al.*, 2009). These findings suggest that tissue eosinophils may be important in airway remodeling associated with the pathogenesis of asthma and that eosinophils play a dominate role in a subset of asthmatics.

Peroxisome Proliferator-Activated Receptors

The peroxisome proliferator-activated receptors (PPARs) have been evaluated for their effects on asthma outcomes. PPARs are metabolite-activated transcription factors that regulate metabolic and inflammatory responses (Bensinger & Tontonoz, 2008). There are three identified subtypes of PPARs (Desvergne & Wahli, 1999). PPAR α was the first PPAR cloned and was subsequently recognized as a member of the steroid hormone receptor superfamily (Issemann & Green, 1990a). PPAR α was found to be activated by rodent hepatocarcinogens and upon activation it caused proliferation of hepatic peroxisomes, thus it was named the peroxisome proliferator-

activated receptor (Issemann & Green, 1990b). PPAR β and PPAR γ were cloned based on sequence homology and due to their regulation of adipose gene expression in response to known PPAR α ligands (Kliwer *et al.*, 1994). PPAR β was originally cloned from *Xenopus* oocyte (Dreyer *et al.*, 1992). Two years later a similar sequence was cloned from mouse, but due to low sequence homology it was considered the fourth PPAR and named PPAR δ (Kliwer *et al.*, 1994). After characterizing and comparing PPARs in chicks, mice, *Xenopus* and mammals, it was decided that there were only 3 PPARs and that PPAR β and PPAR δ would be designated as PPAR β/δ (Michalik *et al.*, 2006).

PPAR α is mainly expressed in organs that perform fatty acids catabolism and fatty acid oxidation, such as brown adipose tissue, liver, heart, kidney and intestine (Mandard *et al.*, 2004). PPAR α knockout mice suffer from a variety of metabolic defects during fasting, including hypoketonemia, hypothermia, elevated plasma free fatty acid levels and hypoglycemia (Kersten *et al.*, 1999; Leone, Weinheimer & Kelly, 1999). PPAR β/δ , on the other hand, is widely expressed in tissues, including skin, gut, placenta, skeletal muscle, adipose tissue and brain (Barak *et al.*, 2002; Bastie *et al.*, 1999; Braissant *et al.*, 1996; Michalik *et al.*, 2006; Michalik *et al.*, 2001; Peters *et al.*, 2000). PPAR β/δ , just like its expression, effects numerous biological processes, such as cholesterol transport (Oliver *et al.*, 2001), tumorigenesis in the colon (He, *et al.*, 1999), placental development (Barak *et al.*, 2002), wound healing (Michalik *et al.*, 2001) and atherosclerosis (Lee *et al.*, 2003). PPAR γ has two structural isoforms, PPAR γ 1 and PPAR γ 2, which differ at the N-terminus, PPAR γ 2 having an extra 30 amino acids (Zieleniak *et al.*, 2008). These isoforms are found in human heart, fat, liver and muscle tissue (Vidal-Puig *et al.*, 1997). PPAR γ is pivotal for adipose tissue

differentiation, for maintaining adipocyte function, glucose metabolism (Zieleniak *et al.*, 2008), and just like PPAR α , PPAR γ regulates inflammation (Ricote *et al.*, 1998). PPAR γ gene knockout is embryonically lethal due to a deficiency in placental vascularization, severe myocardial thinning and lipodystrophy (Barak *et al.*, 1999). In tissues that express both isoforms, PPAR γ 1 receptor density is greater than PPAR γ 2 (Fajas *et al.*, 1997), however PPAR γ 2 shows higher activity in response to insulin (Auwerx, 1999).

Activated PPARs can form heterodimers with retinoid X receptors (RXRs) and subsequently bind to PPAR response elements (PPREs) in the promoter region of target genes, which results in an inhibition or activation of gene expression (Tan *et al.*, 2005). Efforts have been made to identify functional PPREs in the promoter regions of human genes (Heinaniemi *et al.*, 2007). However, this is not the only mechanism that PPARs can utilize to influence gene expression, there are also effects independent of the RXR and PPREs (Tan *et al.*, 2005). An example of this is the effect of PPAR α on co-activator activity (Tan *et al.*, 2005). PPAR α can interact with the p65 subunit of NF- κ B, which sequesters this co-activator away from NF- κ B thus inhibiting it (Delerive *et al.*, 2002). Interaction of RXR, PPAR isotypes, ligands and co-activator availability, makes it difficult to predict the influence of PPAR activation.

Since the discovery of PPARs, substances such as unsaturated fatty acids, eicosanoids, low-density lipoproteins, very-low-density-lipoproteins and linoleic acid have been identified as endogenous ligands of PPARs (Bensinger & Tontonoz, 2008). PPAR activation occurs when changes in metabolism, such as physiological (feeding) or pathophysiological (inflammation), increase substrate levels for specific enzymes, such as 5-lipoxygenase and cyclooxygenase, that produce PPAR ligands, such as

leukotriene B4 and 15-deoxy- Δ -12,14-prostaglandin J2 (15d-PGJ₂) (Michalik *et al.*, 2006). However, most of these ligands are not unique to PPAR receptors therefore they have PPAR-independent effects (Michalik *et al.*, 2006). This is why synthetic PPAR ligands have been valuable in determining PPAR function. Rosiglitazone and troglitazone are the frequently used PPAR γ agonists. However, rosiglitazone is more commonly used because it has a higher binding affinity for PPAR γ than troglitazone (Young *et al.*, 1998). These agonists belong to a class of drugs known as thiazolidinediones, which are used as an antidiabetic medication (Bensinger & Tontonoz, 2008). They activate PPAR γ and subsequently cause insulin sensitization (Bensinger & Tontonoz, 2008). The mechanisms underlying these effects have yet to be determined and it remains an area of intense research (Bensinger & Tontonoz, 2008).

Recent reviews have suggested that the PPARs are novel anti-inflammatory targets (Belvisi *et al.*, 2006; Spears *et al.*, 2006). PPAR α and PPAR γ have been suggested to play a role in chronic inflammatory disease (Kota *et al.*, 2005). However, PPAR γ has been the focus of most lung disease research due to its preferential expression on human airway smooth muscle (Patel *et al.*, 2003), its expression in inflammatory cells (Spears *et al.*, 2006), and due to its increased expression during inflammation (Benayoun *et al.*, 2001).

PPAR γ and Asthma

Studies using murine models of allergic asthma have described a potential role for PPARs in the pathogenesis. PPAR γ expression increases in ovalbumin (OVA) sensitized mice after airway challenge (Kim *et al.*, 2005; Lee *et al.*, 2005; Lee *et al.*,

2006). Expression of PPAR γ can be further enhanced by administration of a PPAR γ agonist (Kim *et al.*, 2005; Lee *et al.*, 2005). Cytokines, IL-4, IL-5, IL-10, and eosinophilic cationic protein increase after airway challenge (Kim *et al.*, 2005; Lee *et al.*, 2005). The post-allergen levels of these mediators can be decreased by treatment with a PPAR γ agonist (Kim *et al.*, 2005; Lee *et al.*, 2005). PPAR γ agonists also reduce reactive oxygen species, Th2 cytokines, adhesion molecules, chemokines, and vascular endothelial growth factor (Lee *et al.*, 2006). PPAR agonists can also inhibit inflammatory cell infiltration into the airway. Trifilieff and colleagues observed an inhibition of eosinophil and lymphocyte influx after treating OVA sensitized mice with a PPAR γ agonist (Trifilieff *et al.*, 2003). Using OVA-pulsed dendritic cells (DCs) treated with a PPAR γ agonist, a reduced proliferation of Ag-specific T cells in mediastinal lymph nodes, an increased production of IL-10 by T cells, and a decreased recruitment of eosinophils in bronchoalveolar lavage was observed (Hammad *et al.*, 2004). Murine models have allowed researchers to explore not only the extensive inhibition of inflammatory cell infiltration into the lung, but also the effect of PPARs on airway remodelling.

Honda *et al* showed that PPAR γ is expressed in airway epithelium of mice after airway challenge and treatment with a PPAR γ agonist decreased airway hyperresponsiveness to methacholine, and indices of remodeling such as basement membrane thickness, mucus production, collagen deposition and TGF- β synthesis (Honda *et al.*, 2004). PPAR γ agonists also inhibit pro-inflammatory cytokine (eotaxin and monocyte chemoattractant protein-1) production in cultured human airway smooth muscle cells treated with TNF α (Nie *et al.*, 2005). Several studies have concluded that the inhibitory effects of PPAR γ ligands on remodeling and AHR in OVA sensitized

mice indicate PPAR γ agonists as possible therapeutics for asthma (Honda *et al.*, 2004; Ward *et al.*, 2006).

Other investigators have compared PPAR agonist to current therapeutics. PPAR γ activation inhibits the release of granulocyte-macrophage colony-stimulating factor, just like dexamethasone steroid treatment (Patel *et al.*, 2003). However, PPAR γ agonists, unlike dexamethasone, inhibit the release of granulocyte colony-stimulating factor, which may regulate the eosinophil differentiation and lineage expansion (Patel *et al.*, 2003). Activation of PPAR γ was also more effective at inhibiting serum-induced human airway smooth muscle cell growth in culture than dexamethasone (Patel *et al.*, 2003). PPAR γ and glucocorticoid receptor protein-protein interactions were observed during TNF α stimulation of cultured human airway smooth muscle cells (Nie *et al.*, 2005). These interactions were enhanced by β 2 agonist treatment (Nie *et al.*, 2005). Interactions between PPARs and current therapeutic mechanisms may have important consequences in the treatment of asthma.

The anti-inflammatory capability of PPAR γ agonists have been observed both in cell culture studies and in allergic animal models (Spears *et al.*, 2006). Several clinical studies have also examined the potential of PPAR γ agonists. Benayoun and colleagues, using immunohistochemistry, show that PPAR γ is overexpressed in the bronchial mucosa, airway epithelium, and airway smooth muscle of asthmatics (Benayoun *et al.*, 2001). They also showed that PPAR γ expression correlates with subepithelial membrane thickening, collagen deposition, proliferation, and was negatively related to FEV₁ values (Benayoun *et al.*, 2001). Thus PPAR γ may be a marker of asthma severity. Kobayashi *et al* showed that there was a trend towards a downregulation of PPAR γ mRNA in BAL cells collected from asthmatics compared

to healthy controls (Kobayashi *et al.*, 2005a). This downregulation appeared to be present 24-hours after segmental challenge. Furthermore two single nucleotide polymorphisms have been associated with the development of asthma (Oh *et al.*, 2009). However, no study has looked at protein expression or the effect of allergen challenge on PPAR γ expression in a specific cell type, such as eosinophils.

Currently two studies have suggested a role for PPAR γ in eosinophil chemotaxis. In one such study, Ueki *et al* showed troglitazone, a PPAR γ agonist, inhibited the chemotaxis of cultured human eosinophils stimulated with eotaxin (Ueki *et al.*, 2003). Similar results were found by Woerly *et al*, who also showed a decrease in eotaxin and IL-5-induced eosinophil chemotaxis after treatment with rosiglitazone or ciglitazone (PPAR γ agonists) (Woerly *et al.*, 2003). The selectivity of the PPAR γ induced inhibition of chemotaxis was confirmed by the lack of inhibition after treatment with GW9662, a PPAR γ antagonist (Woerly *et al.*, 2003). The effect of PPAR γ on eosinophil chemotaxis appears to be dose and ligand-dependent. A recent paper described an increase in eosinophil chemotaxis at low concentrations of 15d-PGJ₂ and a decrease at high concentrations (10⁻⁴M) (Forman *et al.*, 1995). Other PPAR γ studies have confirmed this observation (Fukunaga *et al.*, 2001). The increased migration after 15d-PGJ₂ treatment (low concentration) was observed to be independent of MAPK and NF κ B (Kobayashi *et al.*, 2005b). Thus the mechanism behind this has yet to be clarified, but should be taken into consideration when examining whether PPAR γ may modulate eosinophil migration in asthma.

IL-5 is the principal regulatory cytokine for eosinophils (Chihara *et al.*, 1990). During an allergic response, it stimulates the release of eosinophils from bone marrow cells resulting in blood eosinophilia (Collins, *et al.*, 1995) and can enhance blood

eosinophil recruitment into tissue by eotaxin, a chemoattractant for eosinophils that signals through CCR3 (Okada *et al.*, 1997; Jose *et al.*, 1994). Once in the tissue, IL-5 prolongs the survival of eosinophils by delaying their apoptosis (Simon, 2001). Along with the important role for IL-5 in recruitment and survival, it is also important in eosinophil differentiation. Eosinophils develop in the bone marrow from multipotent hematopoietic stem cells upon exposure to IL-5 (Sanderson, 1992). PPAR γ agonists have been observed to have an inhibitory effect on IL-5 induced eosinophil chemotaxis and survival (Ueki *et al.*, 2003; Woerly *et al.*, 2003). Also PPAR γ has an inhibitory effect on differentiation of several cell types, including erythroid cells (Bernardo *et al.*, 2009; Nagasawa *et al.*, 2005; Rosen & Spiegelman, 2001) Therefore PPAR γ may antagonize other functions of IL-5 such as eosinophil differentiation, which has not been examined.

Rationale

To date there are several effective anti-eosinophilic therapies for allergic asthma that have been studied in clinical trials. However, novel therapeutics are currently in development to improve a patients' quality of life through the optimization of symptom treatment and therapeutic cost. For example, antibody therapies are associated with substantial cost or have the potential for severe adverse events. The gold standard therapy of asthma, corticosteroids, are not effective in subsets of asthmatics. PPAR agonists represent a novel therapy of allergic asthma. It is associated with a lower cost compared to antibody therapies and has diverse anti-inflammatory effects similar to corticosteroids. Along with the anti-inflammatory effects, PPAR agonists can also cause bronchodilation. Eosinophils are an important effector cells in allergic asthma and currently the effects of PPAR agonists on eosinophil function are unknown. This study will determine the effect of PPAR agonists on two pathophysiological mechanisms that lead to peripheral eosinophilia, migration and differentiation.

Hypothesis: PPAR γ plays a role in the pathogenesis of allergic asthma through its inhibitory effects on eosinophil function.

Chapter 2

Specific Objective 1: To examine PPAR γ expression in eosinophils from asthmatics and healthy controls.

Specific Objective 2: To determine the effects of interventions such as inhaled allergen challenge, and treatment with Pulmicort® and Symbicort® on the expression of PPAR γ in cells derived from human airways.

Chapter 3

Specific Objective 1: To determine the effect of PPAR γ agonists on eosinophil migration and chemokinesis *in vitro*.

Specific Objective 2: To determine the effect of PPAR γ agonists on eosinophil migrational signalling pathways, such as expression of CCR3, the phosphorylation of ERK1/2 and p38, and calcium signalling.

Specific Objective 3: To determine the effect of PPAR γ agonists on migration of immature eosinophils.

Chapter 4

Specific Objective 1: To determine the effect of PPAR γ agonists on eosinophil differentiation *in vitro*.

1.2 References

- Auwerx, J. (1999). PPARgamma, the ultimate thrifty gene. *Diabetologia*, 42, 1033-1049.
- Barak, Y., Liao, D., He, W., Ong, E. S., Nelson, M. C., Olefsky, J. M. *et al.* (2002). Effects of peroxisome proliferator-activated receptor delta on placentation, adiposity, and colorectal cancer. *Proc.Natl.Acad.Sci.U.S.A*, 99, 303-308.
- Barak, Y., Nelson, M. C., Ong, E. S., Jones, Y. Z., Ruiz-Lozano, P., Chien, K. R. *et al.* (1999). PPAR gamma is required for placental, cardiac, and adipose tissue development. *Mol.Cell*, 4, 585-595.
- Bastie, C., Holst, D., Gaillard, D., Jehl-Pietri, C., & Grimaldi, P. A. (1999). Expression of peroxisome proliferator-activated receptor PPARdelta promotes induction of PPARgamma and adipocyte differentiation in 3T3C2 fibroblasts. *J.Biol.Chem.*, 274, 21920-21925.
- Belvisi, M. G., Hele, D. J., & Birrell, M. A. (2006). Peroxisome proliferator-activated receptor gamma agonists as therapy for chronic airway inflammation. *Eur.J.Pharmacol.*, 533, 101-109.
- Benayoun, L., Letuve, S., Druilhe, A., Boczkowski, J., Dombret, M. C., Mechighel, P. *et al.* (2001). Regulation of peroxisome proliferator-activated receptor gamma expression in human asthmatic airways: relationship with proliferation, apoptosis, and airway remodeling. *Am.J.Respir.Crit Care Med.*, 164, 1487-1494.

- Bensinger, S. J. & Tontonoz, P. (2008). Integration of metabolism and inflammation by lipid-activated nuclear receptors. *Nature*, 454, 470-477.
- Bernardo, A., Bianchi, D., Magnaghi, V., & Minghetti, L. (2009). Peroxisome Proliferator-Activated Receptor-gamma Agonists Promote Differentiation and Antioxidant Defenses of Oligodendrocyte Progenitor Cells. *J.Neuropathol.Exp.Neurol.*
- Braissant, O., Foufelle, F., Scotto, C., Dauca, M., & Wahli, W. (1996). Differential expression of peroxisome proliferator-activated receptors (PPARs): tissue distribution of PPAR-alpha, -beta, and -gamma in the adult rat. *Endocrinology*, 137, 354-366.
- Busse, W., Raphael, G. D., Galant, S., Kalberg, C., Goode-Sellers, S., Srebro, S. *et al.* (2001). Low-dose fluticasone propionate compared with montelukast for first-line treatment of persistent asthma: a randomized clinical trial. *J.Allergy Clin.Immunol.*, 107, 461-468.
- Chaudhuri, R., Livingston, E., McMahon, A. D., Lafferty, J., Fraser, I., Spears, M. *et al.* (2006). Effects of smoking cessation on lung function and airway inflammation in smokers with asthma. *Am.J.Respir.Crit Care Med*, 174, 127-133.
- Chetta, A., Foresi, A., Del, D. M., Bertorelli, G., Pesci, A., & Olivieri, D. (1997). Airways remodeling is a distinctive feature of asthma and is related to severity of disease. *Chest*, 111, 852-857.
- Chihara, J., Plumas, J., Gruart, V., Tavernier, J., Prin, L., Capron, A. *et al.* (1990). Characterization of a receptor for interleukin 5 on human eosinophils: variable

- expression and induction by granulocyte/macrophage colony-stimulating factor. *J.Exp.Med.*, 172, 1347-1351.
- Cockcroft, D. W., Hargreave, F. E., O'Byrne, P. M., & Boulet, L. P. (2007). Understanding allergic asthma from allergen inhalation tests. *Can.Respir.J.*, 14, 414-418.
- Collins, P. D., Marleau, S., Griffiths-Johnson, D. A., Jose, P. J., & Williams, T. J. (1995). Cooperation between interleukin-5 and the chemokine eotaxin to induce eosinophil accumulation in vivo. *J.Exp.Med.*, 182, 1169-1174.
- Delerive, P., De, B. K., Vanden, B. W., Fruchart, J. C., Haegeman, G., & Staels, B. (2002). DNA binding-independent induction of IkappaBalpha gene transcription by PPARalpha. *Mol.Endocrinol.*, 16, 1029-1039.
- Desvergne, B. & Wahli, W. (1999). Peroxisome proliferator-activated receptors: nuclear control of metabolism. *Endocr.Rev.*, 20, 649-688.
- Dreyer, C., Krey, G., Keller, H., Givel, F., Helftenbein, G., & Wahli, W. (1992). Control of the peroxisomal beta-oxidation pathway by a novel family of nuclear hormone receptors. *Cell*, 68, 879-887.
- Fajas, L., Auboeuf, D., Raspe, E., Schoonjans, K., Lefebvre, A. M., Saladin, R. *et al.* (1997). The organization, promoter analysis, and expression of the human PPARgamma gene. *J.Biol.Chem.*, 272, 18779-18789.
- Flood-Page, P. T., Menzies-Gow, A. N., Kay, A. B., & Robinson, D. S. (2003a). Eosinophil's role remains uncertain as anti-interleukin-5 only partially depletes numbers in asthmatic airway. *Am.J.Respir.Crit Care Med.*, 167, 199-204.
- Flood-Page, P., Menzies-Gow, A., Phipps, S., Ying, S., Wangoo, A., Ludwig, M. S. *et al.* (2003b). Anti-IL-5 treatment reduces deposition of ECM proteins in the

- bronchial subepithelial basement membrane of mild atopic asthmatics. *J.Clin.Invest*, 112, 1029-1036.
- Forman, B. M., Tontonoz, P., Chen, J., Brun, R. P., Spiegelman, B. M., & Evans, R. M. (1995). 15-Deoxy-delta 12, 14-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR gamma. *Cell*, 83, 803-812.
- Fukunaga, Y., Itoh, H., Doi, K., Tanaka, T., Yamashita, J., Chun, T. H. *et al.* (2001). Thiazolidinediones, peroxisome proliferator-activated receptor gamma agonists, regulate endothelial cell growth and secretion of vasoactive peptides. *Atherosclerosis*, 158, 113-119.
- Gauvreau, G. M., Watson, R. M., & O'Byrne, P. M. (1999). Kinetics of allergen-induced airway eosinophilic cytokine production and airway inflammation. *Am.J.Respir.Crit Care Med.*, 160, 640-647.
- GINA Science Committee (2011). Global strategy for asthma management and prevention.
- Haldar, P., Brightling, C. E., Hargadon, B., Gupta, S., Monteiro, W., Sousa, A. *et al.* (2009). Mepolizumab and exacerbations of refractory eosinophilic asthma. *N.Engl.J Med*, 360, 973-984.
- Hammad, H., de Heer, H. J., Soullie, T., Angeli, V., Trottein, F., Hoogsteden, H. C. *et al.* (2004). Activation of peroxisome proliferator-activated receptor-gamma in dendritic cells inhibits the development of eosinophilic airway inflammation in a mouse model of asthma. *Am.J.Pathol.*, 164, 263-271.
- Hammad, H. & Lambrecht, B. N. (2006). Recent progress in the biology of airway dendritic cells and implications for understanding the regulation of asthmatic inflammation. *J.Allergy Clin.Immunol.*, 118, 331-336.

- He, T. C., Chan, T. A., Vogelstein, B., & Kinzler, K. W. (1999). PPARdelta is an APC-regulated target of nonsteroidal anti-inflammatory drugs. *Cell*, 99, 335-345.
- Heinaniemi, M., Uski, J. O., Degenhardt, T., & Carlberg, C. (2007). Meta-analysis of primary target genes of peroxisome proliferator-activated receptors. *Genome Biol.*, 8, R147.
- Hogan, S. P., Rosenberg, H. F., Moqbel, R., Phipps, S., Foster, P. S., Lacy, P. *et al.* (2008). Eosinophils: biological properties and role in health and disease. *Clin.Exp.Allergy*, 38, 709-750.
- Holgate, S. T. (2008). Pathogenesis of asthma. *Clin.Exp.Allergy*, 38, 872-897.
- Honda, K., Marquillies, P., Capron, M., & Dombrowicz, D. (2004). Peroxisome proliferator-activated receptor gamma is expressed in airways and inhibits features of airway remodeling in a mouse asthma model. *J.Allergy Clin.Immunol.*, 113, 882-888.
- Issemann, I. & Green, S. (1990a). Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. *Nature*, 347, 645-650.
- Issemann, I. & Green, S. (1990b). Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. *Nature*, 347, 645-650.
- Jose, P. J., Griffiths-Johnson, D. A., Collins, P. D., Walsh, D. T., Moqbel, R., Totty, N. F. *et al.* (1994). Eotaxin: a potent eosinophil chemoattractant cytokine detected in a guinea pig model of allergic airways inflammation. *J.Exp.Med.*, 179, 881-887.

- Kersten, S., Seydoux, J., Peters, J. M., Gonzalez, F. J., Desvergne, B., & Wahli, W. (1999). Peroxisome proliferator-activated receptor alpha mediates the adaptive response to fasting. *J.Clin.Invest*, 103, 1489-1498.
- Kim, S. R., Lee, K. S., Park, H. S., Park, S. J., Min, K. H., Jin, S. M. *et al.* (2005). Involvement of IL-10 in peroxisome proliferator-activated receptor gamma-mediated anti-inflammatory response in asthma. *Mol.Pharmacol.*, 68, 1568-1575.
- Kliwer, S. A., Forman, B. M., Blumberg, B., Ong, E. S., Borgmeyer, U., Mangelsdorf, D. J. *et al.* (1994). Differential expression and activation of a family of murine peroxisome proliferator-activated receptors. *Proc.Natl.Acad.Sci.U.S.A*, 91, 7355-7359.
- Kobayashi, M., Thomassen, M. J., Rambasek, T., Bonfield, T. L., Raychaudhuri, B., Malur, A. *et al.* (2005a). An inverse relationship between peroxisome proliferator-activated receptor gamma and allergic airway inflammation in an allergen challenge model. *Ann.Allergy Asthma Immunol.*, 95, 468-473.
- Kobayashi, Y., Ueki, S., Mahemuti, G., Chiba, T., Oyamada, H., Saito, N. *et al.* (2005b). Physiological levels of 15-deoxy-Delta12,14-prostaglandin J2 prime eotaxin-induced chemotaxis on human eosinophils through peroxisome proliferator-activated receptor-gamma ligation. *J.Immunol.*, 175, 5744-5750.
- Kota, B. P., Huang, T. H., & Roufogalis, B. D. (2005). An overview on biological mechanisms of PPARs. *Pharmacol.Res.*, 51, 85-94.
- Lambrecht, B. N., Hoogsteden, H. C., & Diamant, Z. (2003). *The Immunological Basis of Asthma*. (vols. 174) New York: Marcel Dekker Inc.

- Leckie, M. J., ten, B. A., Khan, J., Diamant, Z., O'Connor, B. J., Walls, C. M. *et al.* (2000). Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response. *Lancet*, 356, 2144-2148.
- Lee, C. H., Chawla, A., Urbiztondo, N., Liao, D., Boisvert, W. A., Evans, R. M. *et al.* (2003). Transcriptional repression of atherogenic inflammation: modulation by PPARdelta. *Science*, 302, 453-457.
- Lee, K. S., Kim, S. R., Park, S. J., Park, H. S., Min, K. H., Jin, S. M. *et al.* (2006). Peroxisome proliferator activated receptor-gamma modulates reactive oxygen species generation and activation of nuclear factor-kappaB and hypoxia-inducible factor 1alpha in allergic airway disease of mice. *J.Allergy Clin.Immunol.*, 118, 120-127.
- Lee, K. S., Park, S. J., Hwang, P. H., Yi, H. K., Song, C. H., Chai, O. H. *et al.* (2005). PPAR-gamma modulates allergic inflammation through up-regulation of PTEN. *FASEB J.*, 19, 1033-1035.
- Leone, T. C., Weinheimer, C. J., & Kelly, D. P. (1999). A critical role for the peroxisome proliferator-activated receptor alpha (PPARalpha) in the cellular fasting response: the PPARalpha-null mouse as a model of fatty acid oxidation disorders. *Proc.Natl.Acad.Sci.U.S.A*, 96, 7473-7478.
- Leung, D. Y., Spahn, J. D., & Szefler, S. J. (2002). Steroid-unresponsive asthma. *Semin.Respir.Crit Care Med.*, 23, 387-398.
- Lipworth, B. J. (1997). Airway subsensitivity with long-acting beta 2-agonists. Is there cause for concern? *Drug Saf*, 16, 295-308.

- Mandard, S., Muller, M., & Kersten, S. (2004). Peroxisome proliferator-activated receptor alpha target genes. *Cell Mol.Life Sci.*, 61, 393-416.
- Masoli, M., Fabian, D., Holt, S., & Beasley, R. (2004). The global burden of asthma: executive summary of the GINA Dissemination Committee report. *Allergy*, 59, 469-478.
- Michalik, L., Auwerx, J., Berger, J. P., Chatterjee, V. K., Glass, C. K., Gonzalez, F. J. *et al.* (2006). International Union of Pharmacology. LXI. Peroxisome proliferator-activated receptors. *Pharmacol.Rev.*, 58, 726-741.
- Michalik, L., Desvergne, B., Tan, N. S., Basu-Modak, S., Escher, P., Rieusset, J. *et al.* (2001). Impaired skin wound healing in peroxisome proliferator-activated receptor (PPAR)alpha and PPARbeta mutant mice. *J.Cell Biol.*, 154, 799-814.
- Nagasawa, E., Abe, Y., Nishimura, J., Yanase, T., Nawata, H., & Muta, K. (2005). Pivotal role of peroxisome proliferator-activated receptor gamma (PPARgamma) in regulation of erythroid progenitor cell proliferation and differentiation. *Exp.Hematol.*, 33, 857-864.
- Nair, P., Pizzichini, M. M., Kjarsgaard, M., Inman, M. D., Efthimiadis, A., Pizzichini, E. *et al.* (2009). Mepolizumab for prednisone-dependent asthma with sputum eosinophilia. *N.Engl.J.Med.*, 360, 985-993.
- Nelson, H. S. (2000). The importance of allergens in the development of asthma and the persistence of symptoms. *J.Allergy Clin.Immunol.*, 105, S628-S632.
- Nelson, H. S., Busse, W. W., Kerwin, E., Church, N., Emmett, A., Rickard, K. *et al.* (2000). Fluticasone propionate/salmeterol combination provides more effective asthma control than low-dose inhaled corticosteroid plus montelukast. *J.Allergy Clin.Immunol.*, 106, 1088-1095.

- Nie, M., Corbett, L., Knox, A. J., & Pang, L. (2005). Differential regulation of chemokine expression by peroxisome proliferator-activated receptor gamma agonists: interactions with glucocorticoids and beta2-agonists. *J.Biol.Chem.*, 280, 2550-2561.
- O'Byrne, P. M. (1988). Allergen-induced airway hyperresponsiveness. *J.Allergy Clin.Immunol.*, 81, 119-127.
- Oh, S. H., Park, S. M., Lee, Y. H., Cha, J. Y., Lee, J. Y., Shin, E. K. *et al.* (2009). Association of peroxisome proliferator-activated receptor-gamma gene polymorphisms with the development of asthma. *Respir.Med.*, 103, 1020-1024.
- Okada, S., Kita, H., George, T. J., Gleich, G. J., & Leiferman, K. M. (1997). Transmigration of eosinophils through basement membrane components *in vitro*: synergistic effects of platelet-activating factor and eosinophil-active cytokines. *Am.J.Respir.Cell Mol.Biol.*, 16, 455-463.
- Oliver, W. R., Jr., Shenk, J. L., Snaith, M. R., Russell, C. S., Plunket, K. D., Bodkin, N. L. *et al.* (2001). A selective peroxisome proliferator-activated receptor delta agonist promotes reverse cholesterol transport. *Proc.Natl.Acad.Sci.U.S.A*, 98, 5306-5311.
- Patel, H. J., Belvisi, M. G., Bishop-Bailey, D., Yacoub, M. H., & Mitchell, J. A. (2003). Activation of peroxisome proliferator-activated receptors in human airway smooth muscle cells has a superior anti-inflammatory profile to corticosteroids: relevance for chronic obstructive pulmonary disease therapy. *J.Immunol.*, 170, 2663-2669.

- Peters, J. M., Lee, S. S., Li, W., Ward, J. M., Gavrilova, O., Everett, C. *et al.* (2000). Growth, adipose, brain, and skin alterations resulting from targeted disruption of the mouse peroxisome proliferator-activated receptor beta(delta). *Mol.Cell Biol.*, 20, 5119-5128.
- Phipps, S., Flood-Page, P., Menzies-Gow, A., Ong, Y. E., & Kay, A. B. (2004). Intravenous anti-IL-5 monoclonal antibody reduces eosinophils and tenascin deposition in allergen-challenged human atopic skin. *J.Invest Dermatol.*, 122, 1406-1412.
- Piacentini, G. L., Martinati, L., Mingoni, S., & Boner, A. L. (1996). Influence of allergen avoidance on the eosinophil phase of airway inflammation in children with allergic asthma. *J.Allergy Clin.Immunol.*, 97, 1079-1084.
- Ricote, M., Li, A. C., Willson, T. M., Kelly, C. J., & Glass, C. K. (1998). The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation. *Nature*, 391, 79-82.
- Rosen, E. D. & Spiegelman, B. M. (2001). PPARgamma : a nuclear regulator of metabolism, differentiation, and cell growth. *J.Biol.Chem.*, 276, 37731-37734.
- Sanderson, C. J. (1992). Interleukin-5, eosinophils, and disease. *Blood*, 79, 3101-3109.
- Sears, M. R., Herbison, G. P., Holdaway, M. D., Hewitt, C. J., Flannery, E. M., & Silva, P. A. (1989). The relative risks of sensitivity to grass pollen, house dust mite and cat dander in the development of childhood asthma. *Clin.Exp.Allergy*, 19, 419-424.
- Simon, H. U. (2001). Regulation of eosinophil and neutrophil apoptosis--similarities and differences. *Immunol.Rev.*, 179, 156-162.

- Spears, M., McSharry, C., & Thomson, N. C. (2006). Peroxisome proliferator-activated receptor-gamma agonists as potential anti-inflammatory agents in asthma and chronic obstructive pulmonary disease. *Clin.Exp.Allergy*, 36, 1494-1504.
- Sporik, R., Holgate, S. T., Platts-Mills, T. A., & Cogswell, J. J. (1990). Exposure to house-dust mite allergen (Der p I) and the development of asthma in childhood. A prospective study. *N.Engl.J.Med.*, 323, 502-507.
- Statistics Canada (2010). Asthma. <http://www.statcan.gc.ca/pub/82-625-x/2011001/article/11458-eng.htm> [Electronic version].
- Strek, M. E. (2006). Difficult asthma. *Proc.Am.Thorac.Soc.*, 3, 116-123.
- Tan, N. S., Michalik, L., Desvergne, B., & Wahli, W. (2005). Multiple expression control mechanisms of peroxisome proliferator-activated receptors and their target genes. *J.Steroid Biochem.Mol.Biol.*, 93, 99-105.
- Trifilieff, A., Bench, A., Hanley, M., Bayley, D., Campbell, E., & Whittaker, P. (2003). PPAR-alpha and -gamma but not -delta agonists inhibit airway inflammation in a murine model of asthma: *in vitro* evidence for an NF-kappaB-independent effect. *Br.J.Pharmacol.*, 139, 163-171.
- Ueki, S., Adachi, T., Bourdeaux, J., Oyamada, H., Yamada, Y., Hamada, K. *et al.* (2003). Expression of PPARgamma in eosinophils and its functional role in survival and chemotaxis. *Immunol.Lett.*, 86, 183-189.
- Vidal-Puig, A. J., Considine, R. V., Jimenez-Linan, M., Werman, A., Pories, W. J., Caro, J. F. *et al.* (1997). Peroxisome proliferator-activated receptor gene expression in human tissues. Effects of obesity, weight loss, and regulation by insulin and glucocorticoids. *J.Clin.Invest*, 99, 2416-2422.

- Ward, J. E., Fernandes, D. J., Taylor, C. C., Bonacci, J. V., Quan, L., & Stewart, A. G. (2006). The PPAR γ ligand, rosiglitazone, reduces airways hyperresponsiveness in a murine model of allergen-induced inflammation. *Pulm.Pharmacol.Ther.*, 19, 39-46.
- Woerly, G., Honda, K., Loyens, M., Papin, J. P., Auwerx, J., Staels, B. *et al.* (2003). Peroxisome proliferator-activated receptors alpha and gamma down-regulate allergic inflammation and eosinophil activation. *J.Exp.Med.*, 198, 411-421.
- Young, P. W., Buckle, D. R., Cantello, B. C., Chapman, H., Clapham, J. C., Coyle, P. J. *et al.* (1998). Identification of high-affinity binding sites for the insulin sensitizer rosiglitazone (BRL-49653) in rodent and human adipocytes using a radioiodinated ligand for peroxisomal proliferator-activated receptor gamma. *J.Pharmacol.Exp.Ther.*, 284, 751-759.
- Zdanowicz, M. M. (2007). Pharmacotherapy of asthma. *Am.J.Pharm.Educ.*, 71, 98.
- Zieleniak, A., Wojcik, M., & Wozniak, L. A. (2008). Structure and physiological functions of the human peroxisome proliferator-activated receptor gamma. *Arch.Immunol.Ther.Exp.(Warsz.)*, 56, 331-345.
- Zock, J. P., Brunekreef, B., Hazebroek-Kampschreur, A. A., & Roosjen, C. W. (1994). House dust mite allergen in bedroom floor dust and respiratory health of children with asthmatic symptoms. *Eur.Respir.J.*, 7, 1254-1259.

Chapter 2: PPAR γ Expression in Eosinophils

2.1 Introduction

The anti-inflammatory effects of PPAR γ agonists have been observed in several allergic models (Spears *et al.*, 2006). Several studies have also examined the expression of PPAR γ and its correlation to disease presence and severity. PPAR γ expression is increased in the bronchial mucosa, airway epithelium, and airway smooth muscle of asthmatics (Benayoun *et al.*, 2001). This expression correlates with increased airway remodelling (subepithelial membrane thickening and collagen deposition) and decreased clinical outcomes (FEV₁) (Benayoun *et al.*, 2001). Thus the expression of PPAR γ may be related to the severity of asthma. PPAR γ expression is constitutively expressed in BAL cells collected from asthmatics compared to healthy controls (Kobayashi *et al.*, 2005). In asthmatic subjects a decrease in expression was observed in BAL cells before and 24-hours after segmental allergen challenge (Kobayashi *et al.*, 2005). Currently, no study has examined the expression of PPAR γ protein in eosinophils isolated from asthmatic subjects compared to healthy controls. Our study will confirm PPAR γ expression in eosinophils in the peripheral blood of mild atopic asthmatics compared to healthy controls and will determine the expression of PPAR γ in the sputum of allergic asthmatic subjects. These findings will help verify the role of PPAR in the pathogenesis of allergic asthma. Furthermore, PPAR shares similar anti-inflammatory mechanisms to other asthma therapies, such as the glucocorticoids (Ogawa *et al.*, 2005). Furthermore, glucocorticoids have been observed to stimulate PPAR mRNA synthesis (Lemberger *et al.*, 1994). However, the effect of inhaled allergen challenge and asthma therapy on eosinophil PPAR γ expression has not been determined. This study will also determine the effect of

Pulmicort® and Symbicort® treatment on the expression of PPAR γ 24 hours after allergen challenge in the BAL of mild asthmatics.

2.2 Materials and Methods

All challenges and treatments were conducted after approval from the McMaster University Research Ethics Board.

2.2.1 Expression of PPAR γ in Bronchial Washings

Patients

Six mild asthmatic subjects and 4 healthy controls of either sex, aged between 18-65 years participated in the study. Subjects were recruited through advertisements approved by the research ethics board at McMaster University. The Pulmicort® and Symbicort® study used a subset of subjects from a previously published study (Kelly *et al.*, 2010).

Sample Size

A conservative approximation of the estimated sample size was used to study the expression of PPAR γ expression. Sample size was based on a previous study of PPAR γ expression in the BAL of asthmatics (Kobayashi *et al.*, 2005). This approximation is meant to minimize the number of subjects exposed to allergen, but to ensure appropriate power for analysis. The sample size was re-assessed after data from 6 subjects had been collected.

Inclusion and Exclusion Criteria

Study subjects were non-smoking, mild-to-moderate atopic asthmatics that were well controlled on β 2-agonists and had a baseline FEV₁ more than 70% of predicted normal. Subjects were excluded from the study if they have had an airway infection or an exacerbation in the last 4 weeks because this indicates instability of the disease. Other exclusion criteria include the use of drugs that inhibit the development of allergen-induced responses, such as inhaled and/or oral steroids during the preceding 4 weeks and/or antihistamines during the previous 48 hours. Inhaled β 2-agonists were allowed as a bronchodilator during the study provided they were withheld 8 hours before the study visits.

The inclusion and exclusion criteria were designed to decrease the possibility of confounding variables thereby increasing the accuracy of inferences made from the results. Any conditions that induce or inhibit inflammation (e.g. medication, infection, or smoking) were not allowed because they would have an effect on airway responsiveness to allergen. The use of intermittent β 2-agonists therapy was approved for this study because it will have no effect on the late asthmatic response (O'Byrne *et al.*, 1987). Mild atopic asthmatics that are 18-65 years old represent a convenient sample population because they can easily tolerate the allergen challenge. The majority of them don't have co-morbidities and are not prescribed steroids for asthma control. Mild-to-moderate asthmatics are classified by the GINA as those patients with a FEV₁ of >80% predicted normal (GINA Science Committee, 2008) and moderate asthmatics are those with a FEV₁ of 60% to 80% predicted normal. According to our inclusion criteria, a baseline FEV₁ more than 70% of predicted normal, our patients were mild to moderate GINA classification. Our classifications allowed us to recruit a

more diverse asthmatic population while ensuring that the confounding variables (e.g. number of asthma medications) were well controlled.

Study Design

Subjects participated in a whole lung allergen challenge study. A screening period assessed inclusion and exclusion criteria. Subjects underwent procedures including complete history, physical examination and spirometry. In addition, methacholine inhalation challenge and skin-prick testing was performed to assess airway hyperresponsiveness and determine atopic status respectively. Allergen challenges were carried out to document the development of early and late asthmatic responses. If the criteria were met, subjects were enrolled into the study. On the first visit, spirometry and airway hyperresponsiveness were measured. Also at this time baseline blood and sputum samples were collected. The next day, baseline spirometry was measured. If the spirometry had changed less than 10% the subject underwent a whole lung allergen challenge. Subjects returned 24 hours following allergen inhalation challenge for spirometry, bronchoscopy and bronchial wash collection and measurement of airway hyperresponsiveness.

Methacholine Challenge

Methacholine inhalation was performed as described by Cockcroft *et al* (Cockcroft *et al.*, 1977). Subjects were instructed to wear nose-clips and to breathe normally from the mouthpiece during a 2-minute inhalation period. Normal saline, then doubling concentrations of methacholine (0.03 to 16.0 mg/mL) were inhaled from a Wright nebulizer and each inhalation period is separated by a 5 minute wait

period. The subject's FEV₁ was measured at 30, 90, 180 and 300 seconds after each inhalation. Spirometry was measured with a Collins water sealed spirometer and kymograph. The test was terminated when a fall in FEV₁ of 20% of the baseline value occurred, and the methacholine provocative concentration 20% (PC₂₀) was then calculated.

The inflammatory cell infiltration into the lung that causes the late asthmatic response after inhaled allergen is also related to the development of airway hyperresponsiveness (O'Byrne, 1988). Hyperresponsiveness can last up to 24 hours after allergen exposure, which coincides with the time of maximum lung eosinophilia (O'Byrne, 1988; Gauvreau *et al.*, 1999). While airway hyperresponsiveness can be measured with several bronchoconstrictor stimuli (O'Byrne, 1988), methacholine, a muscarinic agonist, is the standard agent used clinically to test bronchial hyperresponsiveness. An adequate washout period (1 day) is required before starting the allergen challenge after a methacholine PC₂₀ test. Measuring baseline FEV₁ before allergen challenge ensured that the bronchoconstriction induced by methacholine has resolved.

Allergen Challenge

Allergen inhalation challenge was performed as previously described (Inman *et al.*, 1995). A Wright nebulizer operated by oxygen at 50 psi and at a flow rate of 8 L/min producing an output of 0.13 mL/min was used to deliver allergen solutions. Subjects were instructed to wear nose-clips and to breathe normally from the mouthpiece during a 2-minute inhalation period. All subjects inhaled allergen to which they were sensitized, and allergen was selected using results from a skin prick

test. The starting concentration of allergen was also determined from the skin prick test and from the methacholine PC₂₀ according to the formula¹ published by Cockcroft *et al* (Cockcroft *et al.*, 2005). The skin prick test was performed using extracts of several allergens. Duplicate skin-prick tests were performed with doubling dilutions of allergen from 1:4 to 1:4096. The wheal diameter was measured in two perpendicular directions at 10 minutes and the mean wheal diameter is determined for each dilution. A positive response was defined as the threshold concentration that produces a 2-mm wheal over the diluent response. The extract that produces a 2-mm wheal in the skin prick test was the same extract used for the allergen inhalation. The starting concentration for the allergen challenge was two doubling concentrations lower than that predicted to cause a 20% fall in FEV₁ (allergen PC₂₀).

Each concentration of allergen was inhaled for 2 minutes and the FEV₁ was measured 10 minutes after the inhalation. If the FEV₁ fell by 10% or more, the measurement was repeated at 10-minute intervals until no further fall was observed. The dose of inhaled allergen was increased in a doubling manner until the FEV₁ had decreased by at least 20%. After inhalation of the highest allergen concentration, the FEV₁ was measured at 10, 20, 30, 45, 60, 90, and 120 minutes, and then at hourly intervals until 7 hours after the inhalation. Subjects returned the next day for their 24 hour FEV₁ measurement. Subjects were included in the study if a decrease in FEV₁ greater than 20% was observed during the early asthmatic response (0 to 2 hours) of the first challenge and if a decrease in FEV₁ greater than 15% was observed during the late asthmatic response (3 to 7 hours) of either challenge (O'Byrne *et al.*, 1987).

¹ $\log(\text{allergen PC}_{20}) = 0.31 \log(\text{MCh PC}_{20}) + 0.50 \log(\text{skin prick endpoint} - 0.42)$

The time course of an early and late phase response after allergen challenge has been well described in the literature (O'Byrne *et al.*, 1987; O'Byrne, 1988). The observed early phase bronchoconstriction occurs within 10 minutes of allergen exposure and reaches a maximum after 30 minutes. The early response usually resolves within 1 to 3 hours. The late response occurs after 3 to 4 hours and reaches a maximum around 6 to 10 hours. The late response can last for 24 hours or more. Subjects who developed at least a 15% fall in FEV₁ within 7 hours of challenge were included, providing an adequate subject population for our study.

Whole lung allergen challenge as opposed to segmental allergen challenge was used in this study. Although segmental allergen challenge is commonly employed because it elicits a larger albeit only local inflammatory response, whole lung allergen challenge permits the measurement of physiological outcomes such as the late asthmatic response (Calhoun *et al.*, 1993). Whole lung allergen challenge also does not distribute the allergen homogeneously in the lung (Kavuru *et al.*, 1999). This might cause dilution of inflammatory response, perhaps explaining why the immune response is larger in segmental than in whole lung allergen challenge. However, inhalation of allergen is a more physiologically relevant challenge.

As mentioned previously, pretreatment with a β_2 -agonist (salbutamol) should inhibit the early asthmatic response without affecting the late asthmatic response (O'Byrne *et al.*, 1987). Our protocol required the early asthmatic response as a marker of when to discontinue allergen delivery. In other words, since our subjects are bronchodilated, a larger allergen dose will be required to induce a 15% fall in FEV₁ (allergen challenge cut off). To ensure a subject is not receiving too much allergen, the current allergen concentration delivered was compared to previous allergen

challenges. Our study required a screening allergen challenge to determine if the subject was a dual responder (has both an early and late), or if the subject was an isolated early responder (O'Byrne *et al.*, 1987). This screening challenge served as a guide to the treatment challenges.

Inhaled Pulmicort® and Symbicort® Allergen Challenge Study

This study was a crossover study in which bronchial washes were performed on asthmatic subjects 24hrs after diluent challenge and after allergen challenge. Study subjects were randomly allocated to treatment with placebo, budesonide (Pulmicort® 200µg, 2 inhalations twice daily); or the combination of budesonide and formoterol (Symbicort® 200, 200µg budesonide and 6µg formoterol, 2 inhalations twice a daily) for 8 days. Bronchial washes were performed 24 hours after allergen challenge, a time point known to have a significant increase in eosinophils (Metzger *et al.*, 1986). The subjects were recruited using the same criteria as above. The study examined the expression of PPAR γ mRNA in bronchial wash cells 24hrs following allergen challenge after the treatment with placebo, budesonide alone, and the combination of budesonide and formoterol. After a 3-week washout period, subjects were allocated to the next treatment period. The study consisted of 4 periods, composed of a screening period with allergen and diluent challenge, and 3 treatment periods, with allergen challenge, which was performed in the same manner as Inman and colleagues (Inman *et al.*, 1995). Fiberoptic bronchoscopy and bronchial washes were performed according to the recommendations of the National Institutes of Health (National Institutes of Health workshop summary, 1985) as a day-case procedure in the endoscopy unit by the same endoscopist each time. Bronchial wash specimens were

taken from segmental and subsegmental carinae of the lung. The lung to be sampled first was randomly assigned, and subsequently the opposite lung was sampled at the next procedure.

To obtain bronchial wash (BW) samples, fiberoptic bronchoscopy was performed using a bronchoscope in accordance with international guidelines. Fasting subjects withheld anti-asthma medications for 24 h and had spirometry measured before and after salbutamol (400 µg) inhalation. They received premedication with atropine (0.6 mg i.m.) and topical anaesthesia of the upper airways, which was achieved with lidocaine gargle (2 mL of 4% lidocaine solution for 60 s) and four sprays of 1% lidocaine (10 mg/spray). The nasal passages were lubricated with 2% lidocaine gel. The bronchoscope was passed through the nose to just above the glottis and 4% lidocaine in 2 mL aliquots were applied to the vocal cords and later to the trachea. The bronchoscope was then wedged in the right middle lobe or lingula and 20 mL of sterile normal saline at room temperature was instilled and aspirated back through the bronchoscope with gentle suction. Samples were kept on ice and processed within 30 min.

The volume of BW fluid was recorded and centrifuged at -4°C at 1,000 rpm for 10 min. The supernatant was removed and stored at -70°C. The cell pellet was washed with D-PBS, centrifuged for 10 min at 1,000 rpm and the supernatant discarded. The cell pellet was resuspended in 10 mL of D-PBS. Total cell counts and viability, using a hemocytometer and Trypan blue staining respectively, and differential cell counts, using Diff-Quick staining, were performed. The remaining cells in suspension were analyzed by RT-PCR.

Whole lung allergen challenge as opposed to segmental allergen challenge was used in this study. Although segmental allergen challenge is commonly employed when BW is sampled because it elicits a larger, albeit only local inflammatory response, whole lung allergen challenge permits the measurement of physiological outcomes such as the late asthmatic response (Calhoun *et al.*, 1993). Although inflammatory cell phenotype is an outcome in this experiment, the primary outcome is the evaluation of asthma treatment on the expression of PPAR γ . Whole lung allergen challenge also does not distribute the allergen homogenously in the lung (Kavuru *et al.*, 1999) which might cause dilution of inflammatory response. However, examination of inflammatory cells in BW after whole lung allergen challenge has previously been performed therefore it is not a limitation (Grootendorst *et al.*, 1997).

Examining PPAR γ mRNA expression in the BW will select for the less adherent cells. Cells that are bound to the airway epithelium are less active than the non-adherent cells (Lambrecht, 2006) therefore their phenotype maybe different. However, more cells, such as alveolar macrophages, are collected by BW than biopsy or sputum (Grootendorst *et al.*, 1997), which makes it a more effective experimental approach. Even though sputum induction is non-invasive and an easier technique to perform than bronchoscopy, the limited numbers of cells collected from sputum would potentially limit the mRNA analysis. By only examining the PPAR γ mRNA in the BW, we are not attempting to elucidate the source. However, we can determine if asthma treatment has an effect on PPAR γ mRNA expression in mild atopic asthmatics after allergen challenge. This crossover allergen challenge provides evidence that correlates asthma treatment with changes on PPAR γ expression.

RT-PCR

The BW cells, collected from the bronchoscopy, were disrupted by adding the RLT buffer. The lysate was homogenized using a blunt 20-gauge needle. The lysate was then transferred to an Allprep DNA spin column (Qiagen, Toronto, Ontario) and centrifuged for 30s at 8000xg. The flow through was used for subsequent RNA purification. Ethanol (100%) was added to the sample and the sample was then transferred to an RNeasy spin column and was centrifuged for 15s at 8000xg. RW1 Buffer was added to the RNeasy spin column and centrifuged for 15s at 800xg. RPE buffer was added to the RNeasy spin column and the centrifuge was repeated. RNase-free water was added to the spin column membrane and the column was centrifuged at 8000xg for 1min. Genomic DNA was eliminated using a mixture containing gDNA wipeout buffer. The reverse-transcription master mix (Quantiscript Reverse Transcriptase, Quantiscript RT buffer and RT primer mix) was prepared on ice. Template RNA was added to the reverse-transcription master mix and incubated for 15min at 42°C. After the 15mins, the reaction solution was incubated for 3min at 95°C to inactivate Quantiscript Reverse Transcriptase (Qiagen, Toronto, Ontario).

The next step is the real time polymerase chain reaction (RT-PCR) analysis of PPAR γ expression. A PCR reaction mix was made according to the manufactures instructions (QuantiTect SYBR Green PCR, QuantiTect Primer Assay and RNase-free water). The template cDNA was added to individual PCR wells containing the reaction mix. The real-time cycler was programmed according to manufactures instructions (15min initial activation step at 95°C and 40 cycles of: 15s denaturing at 94°C, 30s annealing at 55°C and 30s extension at 72°C). The individual Ct values were calculated using the MyiQ (Biorad, Mississauga, Ontario) and the melting curves

were analyzed to determine purity of the DNA. All results were compared to the housekeeping gene, β actin. A no template control (NTC) and a no reverse transcriptase control were also used.

Statistical Analysis

All data are expressed as the mean \pm 95% confidence intervals unless otherwise stated. Statistical analyses were performed using Prism version 5 (GraphPad Software, La Jolla, CA). Repeated measures ANOVA was used to compare PPAR agonist treatments versus diluent at the various doses, with post-hoc Tukey tests for pre-specified comparisons. For data that was not normally distributed, the statistical analyses were performed on the log-transformed data. Statistically significant differences were accepted at $P < 0.05$.

2.2.2 Expression of PPAR γ in Blood Eosinophils and Sputum

Subjects

Asthmatic subjects recruited for the flow cytometry study were 29 ± 16 years old with 2 male and 4 female volunteers. All asthmatic subjects had a positive skin prick test and the geometric mean MCh PC₂₀ was 4mg/mL (range 0.6 to 11.6mg/mL). The healthy control subjects recruited for the flow cytometry study were 39 ± 7 years old with 1 male volunteer and 3 female volunteers. All healthy subjects had a negative skin prick test and a MCh PC₂₀ that was greater than 32mg/mL.

Flow Cytometry

Whole blood samples were stained for PPAR γ surface expression using mouse anti-human APC/Cy7-CD45, PE-CD16 (Becton-Dickinson Biosciences, Mississauga, ON, Canada) and intracellular Alexa 488-PPAR γ (H-100; Cayman Chemicals, Ann Arbor, Michigan, USA), as well as the isotype control antibodies for PPAR γ . Cells were permeabilized with BD Cytoperm™ buffer to internalize the PPAR γ antibody (BD Biosciences, Mississauga, ON, Canada). A negative control, without BD Cytoperm™, was used to determine the selectivity of the antibody. To separate eosinophils from contaminating cells a CD45⁺CD16⁻ gating strategy was utilized (Figure 2.1). CD45 is a protein tyrosine phosphatase receptor type C expressed on leukocytes (Omary *et al.*, 1980) and CD16, as mentioned previously, is expressed on the surface of neutrophils, natural killer cells, T cells and macrophages (LoBuglio *et al.*, 1967; Messner & Jelinek, 1970; Henson, 1969; Perlmann *et al.*, 1976). We used a CD16⁻, high granularity gating strategy to isolate eosinophils (Terstappen *et al.*, 1990). PPAR γ positive staining was determined from an isotype control, which represents the specific antibody without the epitopes. Using standard FACS methods, the isotype control, which represents non-specific binding, was set at 2% positive. The positive specific binding was determined from this set gate. A minimum of 3000 CD45⁺CD16⁻ cells were collected for analysis. Two parameters were determined from this gating strategy, the percent stained and the median fluorescence intensity (MFI). The percent stained is the percentage of the parent population, in this case CD45⁺CD16⁻ cells that express the PPAR γ . The MFI is the median fluorescent value for the specific antibody fluorochrome on the parent population. This is an index of the number of receptors per cell. Cells were acquired with an LSR II flow cytometer

(Becton Dickinson Instrument Systems; Becton-Dickinson, Mississauga, ON, Canada) using the FACSDiva software program (Becton-Dickinson Biosciences). Flow cytometers were first developed in 1965 by Fulwyler, although the Coulter principle was first issued in a 1953 U.S. Patent (Fulwyler, 1965; Coulter, 1953). The Coulter principle states that particles moving through an electric field cause measurable disturbances in that field. This principle was eventually applied to cells which lead to the development of flow cytometry. Fluorometric compensation was set to minimize autofluorescence, a known issue surrounding eosinophils and flow cytometry (Thureau *et al.*, 1996).

Western Blot

One hundred millilitres of peripheral blood was collected from each subject into sodium heparin vacutainers. Sodium heparin is an anticoagulant that binds to antithrombin III, which inactivates thrombin (Porter *et al.*, 1967). Peripheral blood was diluted with an equal volume of McCoy's 5A (Invitrogen Canada Inc, Burlington, ON, Canada) and eosinophils were purified using an accuprep™ density gradient (Accurate Chemical & Scientific Corporation, Westbury, NY, USA) followed by a MACS (magnetic-activated cell sorting) column CD16+ neutrophil depletion (Miltenyi Biotec, Auburn, CA, USA). McCoy's 5A is a general purpose media, which contains various amino acids, vitamins and buffers, such as bicarbonate that keeps the cells at ideal conditions during the isolation process. Accuprep™ is a mixture of sodium diatrizoate and polysaccharide that forms a 1.077 g/mL density liquid. Layering the sample on the media causes the mononuclear cells, which have a density lower than 1.077 g/mL, to form as a layer at the sample/media interface (Boyum,

1968). It also results in a layer at the bottom of the 1.077 g/mL media consisting of red blood cells, eosinophils and neutrophils.

After removing the eosinophil layer, red blood cells were lysed with ammonium chloride. Ammonium chloride (NH_4Cl) is a buffer optimized for the gentle lysis of erythrocytes with a minimal effect on leukocytes (Shortman *et al.*, 1972). Red blood cell lysis occurs due to an unbalanced osmotic pressure because of their permeability to NH_4Cl (Jacobs & Stewart, 1942). The method of NH_4Cl lysis has several effects on eosinophils after 4 days in culture, including decreased survival and degranulation (Ide *et al.*, 1994). This is not a concern for this *in vitro* assay due to the acute nature of the protein measurements and the fact that degranulation is not an outcome.

MACS column was used to purify the eosinophils from the granulocyte population after red blood cell (RBC) lysis. The MACS separation system uses antibodies bound to microbeads that are retained in the column by magnetism, which allows unlabeled cells to pass through. To separate eosinophils from the granulocyte population a CD16 antibody was utilized. CD16 is a low affinity Fc receptor for IgG antibodies. It is expressed on the surface of neutrophils, natural killer cells, T cells and macrophages (LoBuglio *et al.*, 1967; Messner & Jelinek, 1970; Henson, 1969; Perlmann *et al.*, 1976). Granulocytes are incubated with the CD16 antibody diluted in phosphate buffered saline (PBS) with 0.5% BSA and 6 mM EDTA for 45 minutes then added to a LD column that is placed in a magnetic field. The PBS, BSA and EDTA provide the cells with a solution that buffers against changes in pH, decreases non-specific binding and can prevent cell coagulation. The LD column is designed to retain even weakly labeled cells in the column. The column is composed of

ferromagnetic spheres that, when placed in the magnetic field, amplifies the magnetism by 10,000 fold, which ensures neutrophils stay in the column and eosinophils flow through for subsequent analysis.

The purity of each eosinophil sample was determined with a cytoprep stained for a cell differential count (Diff-Quik®; Siemens Healthcare Diagnostics, Deerfield, IL, USA). After the cells are diluted to a concentration of 1×10^6 cell/mL, 50 μ L of cell suspension is placed into plastic cuvettes that have been pre-wet with DPBS (doesn't contain calcium and magnesium). The cuvettes are centrifuged at 500g for 5min forming a cell pellet on the surface of the glass slide. After the pellet has dried the slide is stained using Diff-Quik®. This consists of a fixative (Triarylmethane dye), an acidic dye (Xanthene dye) and a basic dye (Thiazine dye). This method was developed by Paul Ehrlich in 1878. In his dissertation “Contributions to the Theory and Practice of Histological Staining” at Leipzig University, Ehrlich first described eosinophils as acidophils based on the preference to stain with acidic dyes (Ehrlich, 1878). It was modified in 1891 by Ernst Malachowski and Dmitry Romanowsky to make the dyes more stable and water soluble (Romanowsky, 1891). Current Diff-Quik® staining methods use variations of the Romanowsky protocol.

Light microscopy demonstrated that the eosinophil preparations were >90% pure and the majority of contaminating cells were neutrophils. The purity was determined from the average of 2 slides after a minimum of 400 cells were counted on each, to minimize measurement variability. The vast majority contaminating cells were neutrophils, because the layering process isolates the granulocyte population. However, during the granulocyte layer retrieval it is possible to get contaminating lymphocytes (<1%).

Purified eosinophils were boiled in western buffer (Novex buffer, 2-ME) and distilled water) and stored at -70°C. Protein lysates from eosinophils were separated by gel electrophoresis. Two protein standards, See Blue Plus2 and HRP-linked protein standards ladder, were used to visualize protein size. Also a protein loading standard, 125ng human PPAR γ protein, will be used to ensure equal loading. The gel was run at 100 volts for 2.5 hours and then transferred onto a nitrocellulose membrane at 30 volts for 90 minutes. The membrane was blocked overnight at 4°C with 3% milk blocking buffer (0.6g milk in 20mL TBS-T). The next morning the membrane was washed and incubated at room temperature for 90 min with PPAR γ IgG antibody (corresponding to the N-terminus of PPAR γ 1 and PPAR γ 2) that was diluted in blocking buffer. Following that, the membrane was washed and incubated at room temperature for 60 min with Polyclonal Goat Anti-Rabbit HRP-linked Immunoglobulins diluted in blocking buffer. Finally the membrane was washed and incubated with Chemiglow for 8 mins and development on a Fluorochem.

Sputum Induction

Sputum was induced with an aerosol of inhaled hypertonic saline using a modification of the method of Pin *et al* (Pin *et al.*, 1992) after pretreatment with inhaled salbutamol 200 μ g (Pizzichini *et al.*, 1996). The modification consisted of inhaling the hypertonic saline in concentrations of 3, 4 and 5% each for 7 min. Subjects were instructed to rinse the mouth with water and blow their nose after each inhalation to avoid contamination with saliva and postnasal drip. The sample was collected in a sterile container and was examined as soon as possible, within 2 h. The whole sample of sputum and saliva was transferred to a Petri dish. Sputum plugs were

selected and transferred into a new 15 mL conical tube. The weight of the sputum plugs was recorded. Fresh DTT in a balanced salt solution (Sputolysin 10%; Calbiochem Corp., San Diego, CA, USA) diluted to 0.1% by the addition of distilled water was prepared. The sample was treated with a volume of 0.1% DTT of four times the weight. The portions were agitated in a vortex mixer for 15 sec, and gently aspirated in and out of a pipette to ensure mixing. The polystyrene tubes were then placed in a bench rocker (Dade Tube Rocker; Baxter Diagnostics Corp., Miami, USA) and rocked for 15 min. A further four volumes of DPBS were added to the sample, giving a final concentration of 0.05% DTT in the solution, and was rocked for a further 5 min. The suspensions were then filtered through 48 µm nylon gauze (B & SH Thompson, Scarborough, Ontario, Canada); which does not result in selected cell loss (Efthimiadis *et al.*, 1994) to remove cell debris and mucus. The resulting suspension was spun at 790×g for 10 min and the supernatant was aspirated and stored in Eppendorf tubes at -70°C for later analysis. The pellet was resuspended in a volume of 1000 µL of DPBS to obtain a total cell count in a Neubauer haemocytometer. The viability of nonsquamous cells in the sample was evaluated by the trypan blue exclusion method (the blue cells being considered dead). The total number of cells per millilitre of processed sputum was calculated for both portions, as well as the absolute number of nonsquamous and live cells in each portion (absolute total cell count per portion minus the squamous and dead cell counts). The cell suspension was then adjusted to 1.0×10^6 cells/mL and 50µL of cell suspension was placed into the cuvettes of a Shandon III cytocentrifuge (Shandon Southern Instruments, Sewickley, PA, USA). Two cytospins were prepared at 500 g for 5 min. The slides were air-dried and stained using DiffQuik stain. Four hundred nonsquamous cells were counted.

Immunofluorescence Staining

PPAR γ expression was examined by immunofluorescence in baseline sputum samples from allergic asthmatic subjects. Sputum was processed using standard procedures (Pizzichini *et al.*, 2002), which includes the digestion of mucus using DTT (Efthimiadis *et al.*, 1997). Several studies have shown that DTT does not affect cell morphology, differential counts or cytokine supernatant levels (Efthimiadis *et al.*, 1997). Four cytopspins were prepared on APTEX coated slides and fixed for 10min in 4% PFA. After the 10min incubation the slides were treated for 10min in 15% sucrose in PBS. The slides were then stored at -70°C until fluorescence staining. Before staining, the slides were permeabilized with 1% triton X-100 in PBS for 10 min. The cells were then treated with 1% BSA in PBS-T to block the Fc receptor sites, followed by incubation with selective PPAR γ and eosinophil peroxidase (EPX) antibodies. EPX is a specific marker for sputum eosinophils (Metso *et al.*, 2002). The primary antibodies used were rabbit anti-human PPAR γ IgG (H-100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse anti-human EPX IgG (Diagnostic Development, Uppsala, Sweden). The secondary antibodies used were goat anti-rabbit ALEXA 488 IgG and donkey anti-mouse ALEXA 555 IgG (Molecular Probes, Burlington, ON). The primary antibodies were incubated at a 1/100 dilution with 1% BSA in PBS-T for 1hr. A negative slide was also prepared without the primary antibody. Slides were washed and the secondary antibody at a 1/100 dilution was incubated for 1hr. After incubation, slides were washed and a coverslip was mounted using aqueous mounting medium with DAPI. Slides were stored in the dark at 4°C until image acquisition. Fluorescence images were visualized with a Leica DMI 600 B

widefield deconvolution microscopy (Concord, Ontario, Canada) in the biophotonics facility at McMaster University. The microscope is equipped with an EXFO metal halide lamp (Mississauga, Ontario, Canada) which was set to excite cells at 488nm and images were acquired with a Hamamatsu Orca ER-AG camera (Bridgewater, NJ, USA) at 400x magnification using Volocity 4 acquisition software (Waltham, Massachusetts, USA). First eosinophils were located using the ALEXA 555 filter, Semrock TRITC-A-000 (Rochester, New York, USA). After the location of an eosinophil an image using all filters were acquired. Filters included in this study were ALEXA 555 (Semrock TRITC-A-000), ALEXA 488 (Semrock 3035B) and DAPI (Semrock 5060B). The Volocity 4 files were converted to jpg images and the combined image was created with ImageJ (developed at the National Institutes of Health).

2.3 Results and Discussion

Western Blot of PPAR γ in Isolated Peripheral Blood Eosinophils

Western blots were used to examine PPAR γ protein expression in eosinophils isolated from the peripheral blood of asthmatics. PPAR γ protein was observed when using protein extracted from 1×10^6 eosinophils (Figure 2.2). Purified PPAR γ protein was used as a positive control. This verifies results from previous reports (Woerly *et al.*, 2003) demonstrating that circulating eosinophils express PPAR γ protein.

Immunofluorescence Staining of PPAR γ in Sputum Eosinophils

Sputum eosinophils, identified using EPX, were examined at baseline in an asthmatic subject. A negative control that contained no primary antibody was used to

determine the selectivity of the EPX antibody and the PPAR γ antibody. In both cases no staining was observed. DAPI was used to locate cells by staining their nucleus. PPAR γ is expressed in eosinophils in the sputum of an allergic asthmatic subject (Figure 2.3). PPAR γ expression is predominately in the cytoplasm of eosinophils, in agreement with previous BAL results (Kobayashi *et al*, 2005). Cells that were positive for DAPI but negative for EPX and PPAR γ were also observed (Figure 2.3). By developing novel methods to examine PPAR γ expression in sputum eosinophils, we will be able to correlate physiological changes observed after allergen challenge with changes in eosinophil number and PPAR γ expression.

PPAR γ Expression in Allergic Asthmatics Compared to Healthy Controls

Peripheral blood was collected from allergic asthmatics and healthy control subjects. Both the percent (%) stained and MFI was determined for eosinophils (CD45+C16-) and neutrophils (CD45+CD16+) (Table 2.1). No significant differences were observed between asthmatics and healthy control subjects. This confirms the baseline mRNA observations made in the BAL (Kobayashi *et al.*, 2005). The gating strategy used allowed us to examine neutrophil expression in addition to eosinophil expression. Our study and previous reports (Reddy *et al.*, 2008) have shown that PPAR γ is also expressed in neutrophils. However, no significant difference in intracellular expression of PPAR γ was observed between asthmatics and healthy control subjects.

PPAR γ Expression in Bronchial Washings of Allergic Asthmatics; the Effect of Allergen Challenge and Inhaled Pulmicort® and Symbicort® on PPAR γ Expression

We examined the effect of allergen inhalation on PPAR γ mRNA from bronchial washings. PPAR γ mRNA increased with 24h post-allergen challenge with placebo treatment compared to diluent in 3 out of 6 subjects. We examined the effect of Pulmicort® and Symbicort® treatment on the expression of PPAR γ mRNA in BW cells from allergic asthmatics 24hrs after allergen challenge. Treatment with Symbicort® and Pulmicort® showed no difference from the placebo treatment (Figure 2.4). Kobayashi *et al* (2005) showed a decrease in PPAR γ mRNA expression in the BAL of asthmatics 24hrs after allergen challenge. However, in their study PPAR γ mRNA was normalizing to internal GAPDH (glyceraldehyde 3-phosphate dehydrogenase) expression levels. It has been suggested that GAPDH and β -actin are not good housekeeping genes for asthmatic subjects (Glare *et al.*, 2002). However, this effect may only be observed in large numbers (Glare *et al* studied 92 asthmatics to observe the effect). Furthermore, Kobayashi and colleagues examined BAL compared to our study that examined BW cells. BW will favour the collection of non-adherent cells whereas BAL, with the extra washing, would yield a higher proportion of adherent cells. Another difference between the studies is the method used to induce the allergic response. Kobayashi *et al* used segmental allergen challenge compared to our study, which used whole lung allergen challenge. As described previously, segmental allergen challenge induces a robust, albeit only local, inflammatory response (Calhoun *et al.*, 1993). This large induction of inflammation may result in changes to the PPAR γ expression, which may explain the differences observed.

2.4 Conclusion

This study is the first to demonstrate PPAR γ expression in sputum eosinophils from allergic asthmatics. We also confirmed the expression of PPAR γ in peripheral blood eosinophils and neutrophils. This study also confirms previous reports that PPAR γ expression is not different at baseline in asthmatics compared to healthy controls (Kobayashi *et al.*, 2005). Furthermore, we have described the novel effects of Symbicort® and Pulmicort® treatment on PPAR γ mRNA expression 24hrs after whole lung allergen challenge in BW cells. Although PPAR γ mRNA expression increased in some subjects after allergen challenge, no statistically significant increase was observed. Our study confirms the expression of PPAR γ protein in eosinophils, and suggests there are no differences in the level of expression between subjects with asthma and healthy controls. As such, constitutive expression of PPAR γ at baseline does not appear to be responsible for the pathogenesis of asthma. We and others have observed that allergen challenges elevate the expression of PPAR γ . However there is no significant regulation by Symbicort® or Pulmicort® treatment on the elevated level of PPAR γ expression. Therefore a targeted approach such as PPAR γ agonists may be beneficial for treatment of asthma exacerbation.

2.6 Tables and Figures

Table 2.1. PPAR γ expression in peripheral blood eosinophils isolated from allergic asthmatic and healthy control subjects. Data represent arithmetic mean \pm standard deviation.

	% Stained		MFI	
	Healthy Controls	Asthmatics	Healthy Controls	Asthmatics
Neutrophils	53 \pm 11	67 \pm 20	167 \pm 37	444 \pm 404
Eosinophils	39 \pm 17	72 \pm 28	858 \pm 288	1076 \pm 784

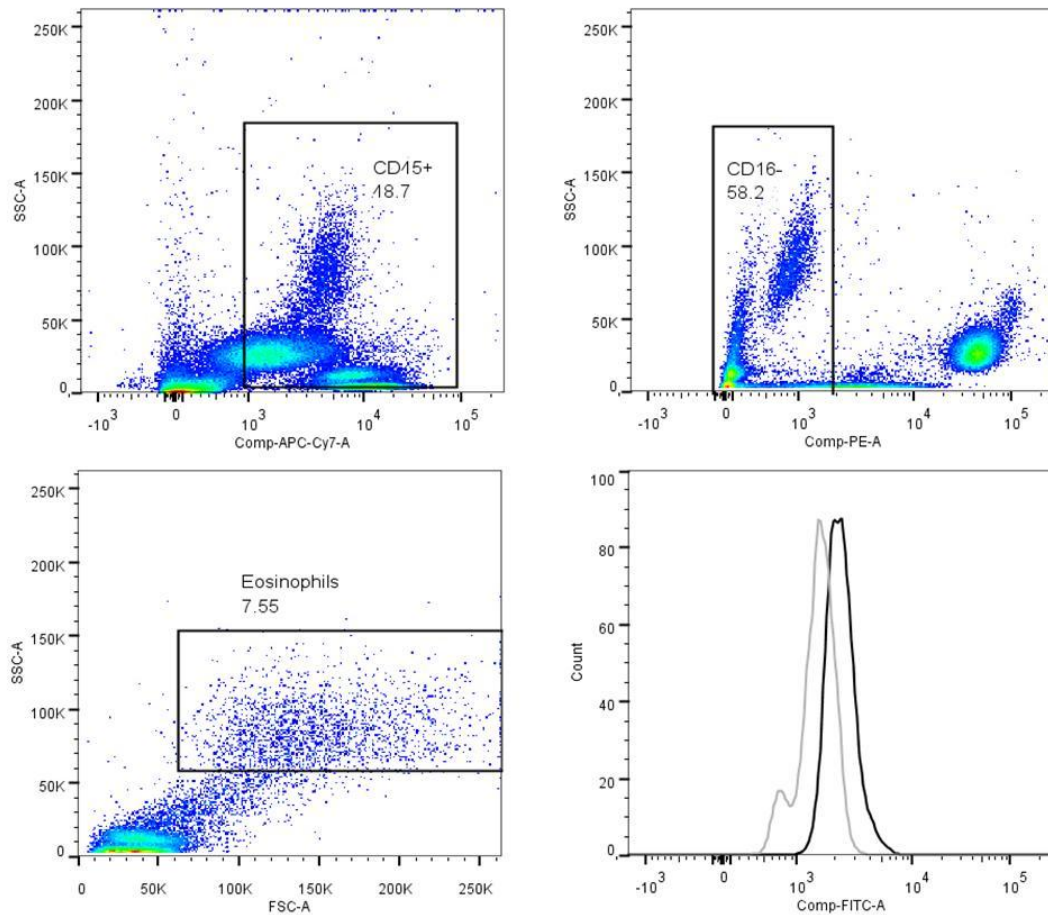


Figure 2.1. Gating strategy used to isolate peripheral blood eosinophils from the whole blood population. PPAR γ expression on eosinophils was examined using CD45⁺CD16⁻ with high granularity (top left, top right and bottom left graph respectively). The MFI for the isotype (grey line) and PPAR γ antibody (black line) is shown in the bottom right graph.

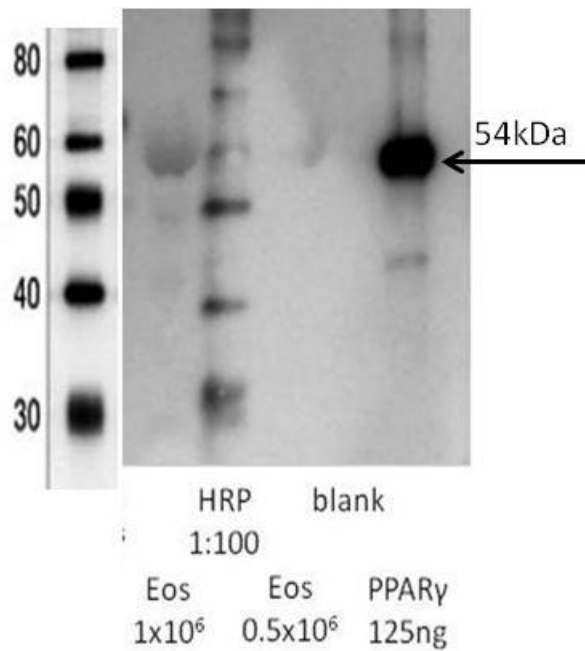


Figure 2.2. A representative western blot of PPAR γ expression in isolated eosinophils. Lysates from 1×10^6 eosinophils in 25uL western buffer was determined to be the optimal cell number. PPAR γ protein appears at 54 kDa. The first column shows the product insert HRP linked molecular weight ladder, second column shows protein from 1×10^6 eosinophils, third shows the HRP linked protein ladder that was run on the gel, fourth shows protein from 0.5×10^6 eosinophils, fifth column is blank and the last column is 125ng of PPAR γ protein.

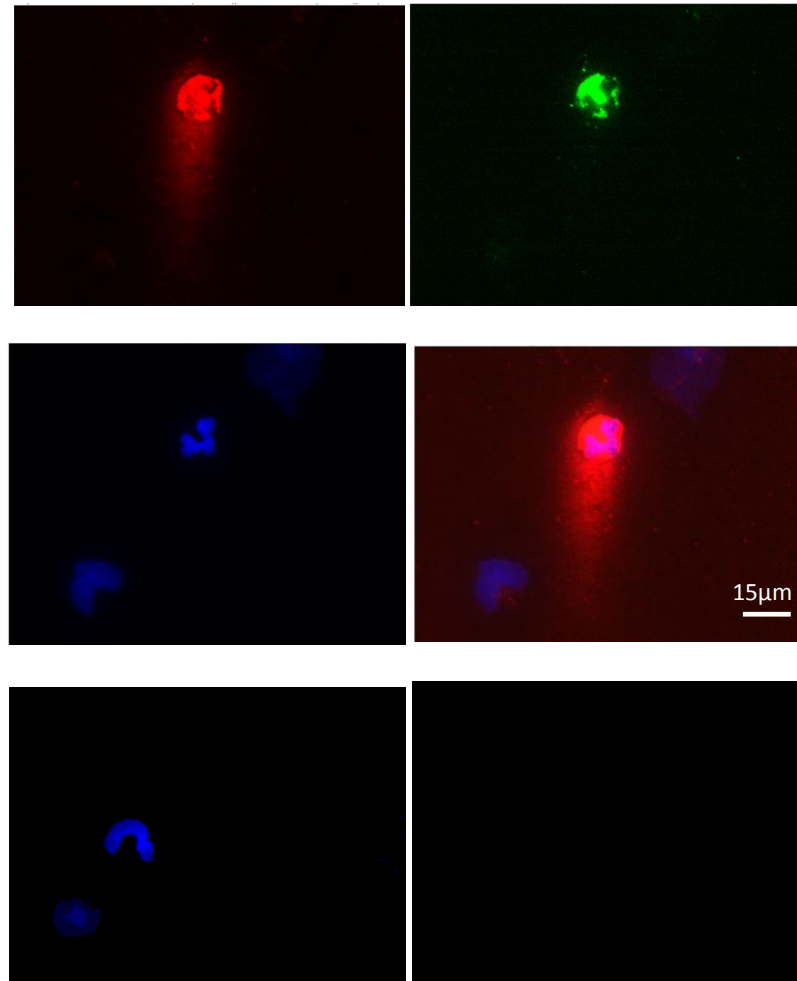


Figure 2.3. A fluorescent image of PPAR γ expression in the sputum of an allergic asthmatic subject. Images show eosinophil peroxidase (EPX), PPAR γ , and DAPI expression (top left, top right and middle left image respectively). The middle right image shows the combined image, which localizes the PPAR γ and EPX expression. The bottom image shows the DAPI (bottom left) and PPAR γ for the isotype control (bottom right).

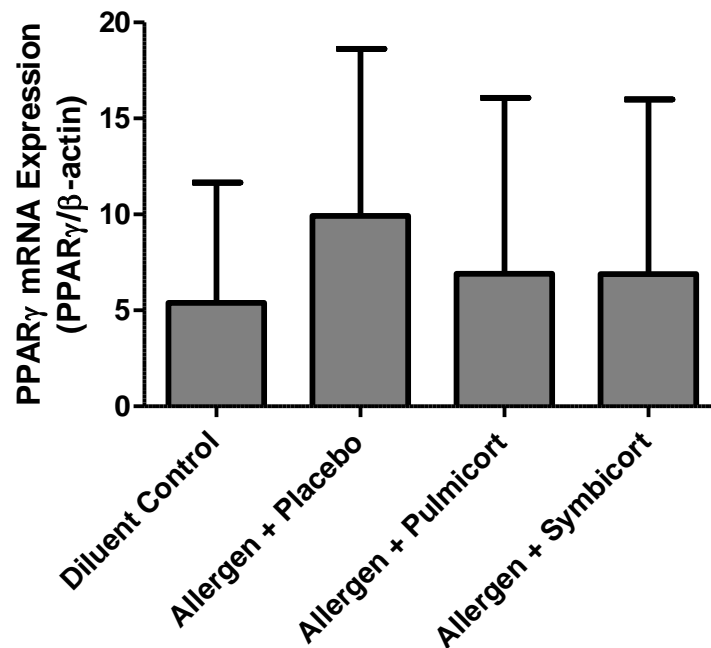


Figure 2.4. The effect of Pulmicort® and Symbicort® treatment on the expression of PPAR γ mRNA in BW cells from allergic asthmatics 24hrs after allergen challenge. PPAR γ mRNA showed a trend towards an increased in half of the subjects on placebo after allergen challenge compared to diluent challenge. Symbicort® and Pulmicort® showed no difference from the diluent response. Data are arithmetic mean \pm 95% confidence intervals.

2.7 References

- Benayoun, L., Letuve, S., Druilhe, A., Boczkowski, J., Dombret, M. C., Mechighel, P. *et al.* (2001). Regulation of peroxisome proliferator-activated receptor gamma expression in human asthmatic airways: relationship with proliferation, apoptosis, and airway remodeling. *Am.J.Respir.Crit Care Med.*, 164, 1487-1494.
- Boyum, A. (1968). Separation of leukocytes from blood and bone marrow. Introduction. *Scand.J.Clin.Lab Invest Suppl*, 97, 7.
- Calhoun, W. J., Jarjour, N. N., Gleich, G. J., Stevens, C. A., & Busse, W. W. (1993). Increased airway inflammation with segmental versus aerosol antigen challenge. *Am.Rev Respir.Dis.*, 147, 1465-1471.
- Cockcroft, D. W., Davis, B. E., Boulet, L. P., Deschesnes, F., Gauvreau, G. M., O'Byrne, P. M. *et al.* (2005). The links between allergen skin test sensitivity, airway responsiveness and airway response to allergen. *Allergy*, 60, 56-59.
- Cockcroft, D. W., Ruffin, R. E., Dolovich, J., & Hargreave, F. E. (1977). Allergen-induced increase in non-allergic bronchial reactivity. *Clin.Allergy*, 7, 503-513.
- Coulter, W. H. (1953). [Patent No. 2,656,508]. U.S. Patent and Trademark Office. Ref Type: Patent
- Efthimiadis, A., Pizzichini, M. M., Pizzichini, E., Dolovich, J., & Hargreave, F. E. (1997). Induced sputum cell and fluid-phase indices of inflammation: comparison of treatment with dithiothreitol vs phosphate-buffered saline. *Eur.Respir.J.*, 10, 1336-1340.

- Efthimiadis, A., Popov, T., Kolendowicz, Dolovich, J., & Hargreave, F. E. (1994). Increasing the yield of sputum cells for examination. *Am J Respir Crit Care Med*, 149, A949.
- Ehrlich, P. (1878). *Beiträge zur Theorie und Praxis der histologischen Färbung.* (Doctoral dissertation) Universität Leipzig, Leipzig, Saxony, Germany.
- Fulwyler, M. J. (1965). Electronic separation of biological cells by volume. *Science*, 150, 910-911.
- Gauvreau, G. M., Watson, R. M., & O'Byrne, P. M. (1999). Kinetics of allergen-induced airway eosinophilic cytokine production and airway inflammation. *Am.J.Respir.Crit Care Med.*, 160, 640-647.
- Glare, E. M., Divjak, M., Bailey, M. J., & Walters, E. H. (2002). beta-Actin and GAPDH housekeeping gene expression in asthmatic airways is variable and not suitable for normalising mRNA levels. *Thorax*, 57, 765-770.
- GINA Science Committee (2008). *Global strategy for asthma management and prevention.*
- Grootendorst, D. C., Sont, J. K., Willems, L. N., Kluin-Nelemans, J. C., Van Krieken, J. H., Veselic-Charvat, M. et al. (1997). Comparison of inflammatory cell counts in asthma: induced sputum vs bronchoalveolar lavage and bronchial biopsies. *Clin.Exp.Allergy*, 27, 769-779.
- Henson, P. M. (1969). The adherence of leucocytes and platelets induced by fixed IgG antibody or complement. *Immunology*, 16, 107-121.
- Ide, M., Weiler, D., Kita, H., & Gleich, G. J. (1994). Ammonium chloride exposure inhibits cytokine-mediated eosinophil survival. *J.Immunol.Methods*, 168, 187-196.

- Inman, M. D., Watson, R., Cockcroft, D. W., Wong, B. J., Hargreave, F. E., & O'Byrne, P. M. (1995). Reproducibility of allergen-induced early and late asthmatic responses. *J.Allergy Clin.Immunol.*, 95, 1191-1195.
- Jacobs, M. H. & Stewart, D. R. (1942). THE ROLE OF CARBONIC ANHYDRASE IN CERTAIN IONIC EXCHANGES INVOLVING THE ERYTHROCYTE. *J.Gen.Physiol*, 25, 539-552.
- Kavuru, M. S., Dweik, R. A., & Thomassen, M. J. (1999). Role of bronchoscopy in asthma research. *Clin.Chest Med.*, 20, 153-189.
- Kelly, M. M., O'Connor, T. M., Leigh, R., Otis, J., Gwozd, C., Gauvreau, G. M. *et al.* (2010). Effects of budesonide and formoterol on allergen-induced airway responses, inflammation, and airway remodeling in asthma. *J.Allergy Clin.Immunol.*, 125, 349-356.
- Kobayashi, M., Thomassen, M. J., Rambasek, T., Bonfield, T. L., Raychaudhuri, B., Malur, A. *et al.* (2005). An inverse relationship between peroxisome proliferator-activated receptor gamma and allergic airway inflammation in an allergen challenge model. *Ann.Allergy Asthma Immunol.*, 95, 468-473.
- Lambrecht, B. N. (2006). Alveolar macrophage in the driver's seat. *Immunity*, 24, 366-368.
- Lemberger, T., Staels, B., Saladin, R., Desvergne, B., Auwerx, J., Wahli, W. (1994). Regulation of the peroxisome proliferator-activated receptor alpha by glucocorticoids. *J Biol Chem*, 269, 24527-30.
- LoBuglio, A. F., Cotran, R. S., & Jandl, J. H. (1967). Red cells coated with immunoglobulin G: binding and sphering by mononuclear cells in man. *Science*, 158, 1582-1585.

- Messner, R. P. & Jelinek, J. (1970). Receptors for human gamma G globulin on human neutrophils. *J.Clin.Invest*, 49, 2165-2171.
- Metso, T., Venge, P., Haahtela, T., Peterson, C. G., & Seveus, L. (2002). Cell specific markers for eosinophils and neutrophils in sputum and bronchoalveolar lavage fluid of patients with respiratory conditions and healthy subjects. *Thorax*, 57, 449-451.
- Metzger, W. J., Richerson, H. B., Worden, K., Monick, M., & Hunninghake, G. W. (1986). Bronchoalveolar lavage of allergic asthmatic patients following allergen bronchoprovocation. *Chest*, 89, 477-483.
- National Institutes of Health workshop summary. Summary and recommendations of a workshop on the investigative use of fiberoptic bronchoscopy and bronchoalveolar lavage in individuals with asthma (1985). *J Allergy Clin.Immunol.*, 76, 145-147.
- O'Byrne, P. M. (1988). Allergen-induced airway hyperresponsiveness. *J.Allergy Clin.Immunol.*, 81, 119-127.
- O'Byrne, P. M., Dolovich, J., & Hargreave, F. E. (1987). Late asthmatic responses. *Am.Rev.Respir.Dis.*, 136, 740-751.
- Ogawa, S., Lozach, J., Benner, C., Pascual, G., Tangirala, R. K., Westin, S. et al. (2005). Molecular determinants of crosstalk between nuclear receptors and toll-like receptors. *Cell*, 122, 707-721.
- Omary, M. B., Trowbridge, I. S., & Battifora, H. A. (1980). Human homologue of murine T200 glycoprotein. *J.Exp.Med*, 152, 842-852.

- Perlmann, H., Perlmann, P., Pape, G. R., & Hallden, G. (1976). Purification, fractionation and assay of antibody-dependent lymphocytic effector cells (K cells) in human blood. *Scand.J.Immunol., Suppl 5*, 57-68.
- Pin, I., Gibson, P. G., Kolendowicz, R., Girgis-Gabardo, A., Denburg, J. A., Hargreave, F. E. *et al.* (1992). Use of induced sputum cell counts to investigate airway inflammation in asthma. *Thorax*, 47, 25-29.
- Pizzichini, E., Pizzichini, M. M., Efthimiadis, A., Hargreave, F. E., & Dolovich, J. (1996). Measurement of inflammatory indices in induced sputum: effects of selection of sputum to minimize salivary contamination. *Eur.Respir.J.*, 9, 1174-1180.
- Pizzichini, E., Pizzichini, M. M., Leigh, R., Djukanovic, R., & Sterk, P. J. (2002). Safety of sputum induction. *Eur.Respir.J.Suppl*, 37, 9s-18s.
- Porter, P., Porter, M. C., & Shanberge, J. N. (1967). Heparin cofactor and plasma antithrombin in relation to the mechanism of inactivation of thrombin by heparin. *Clin.Chim.Acta*, 17, 189-200.
- Reddy, R. C., Narala, V. R., Keshamouni, V. G., Milam, J. E., Newstead, M. W., & Standiford, T. J. (2008). Sepsis-induced inhibition of neutrophil chemotaxis is mediated by activation of peroxisome proliferator-activated receptor-gamma. *Blood*, 112, 4250-4258.
- Romanowsky, D. L. (1891). Zur Frage der Parasitologie und Therapie der Malaria. *St Petersburg Med Wochenschr*, 16, 297-302.
- Shortman, K., Williams, N., & Adams, P. (1972). The separation of different cell classes from lymphoid organs. V. Simple procedures for the removal of cell

debris. Damaged cells and erythroid cells from lymphoid cell suspensions. *J.Immunol.Methods*, 1, 273-287.

Spears, M., McSharry, C., & Thomson, N. C. (2006). Peroxisome proliferator-activated receptor-gamma agonists as potential anti-inflammatory agents in asthma and chronic obstructive pulmonary disease. *Clin.Exp.Allergy*, 36, 1494-1504.

Terstappen, L. W., Hollander, Z., Meiners, H., & Loken, M. R. (1990). Quantitative comparison of myeloid antigens on five lineages of mature peripheral blood cells. *J Leukoc.Biol.*, 48, 138-148.

Thurau, A. M., Schylz, U., Wolf, V., Krug, N., & Schauer, U. (1996). Identification of eosinophils by flow cytometry. *Cytometry*, 23, 150-158.

Woerly, G., Honda, K., Loyens, M., Papin, J. P., Auwerx, J., Staels, B. *et al.* (2003). Peroxisome proliferator-activated receptors alpha and gamma down-regulate allergic inflammation and eosinophil activation. *J.Exp.Med.*, 198, 411-421.

Chapter 3: Effects of PPAR Agonists on Eosinophil Migration

3.1 Introduction

Eosinophils are effector cells which contribute to the pathology of allergic diseases (Hogan *et al.*, 2008). They are recruited from the blood into the inflamed tissue by local release of chemokines (Okada, *et al.*, 1997; Jose *et al.*, 1994). The first observations of leukocyte chemotaxis occurred in 1888, when Leber described the movement of leukocytes into the avascular cornea of a rabbit following irritation with various microorganisms (Leber, 1888). The study of migration was at a standstill until Boyden developed an efficient *in vitro* technique for measuring chemotaxis (Boyden, 1962). Using this newly developed technique, researchers showed that the response to a chemoattractant is a combination of random and directionally stimulated movement (Keller & Sorkin, 1966; Keller & Sorkin, 1967) and that it can be a cell specific process (Keller & Sorkin, 1969; Kay, 1970). Eotaxin-1, which is one of the most potent eosinophil chemokines, signals through CCR3. Novel approaches toward inhibiting migration of effector cells, such as eosinophils, are being investigated for treatment of allergic asthma.

PPARs are metabolite-activated transcription factors that have been shown to regulate metabolic and inflammatory responses (Bensinger & Tontonoz, 2008). They have attracted interest as therapeutic targets in lung disease due to their preferential expression on human airway smooth muscle (Patel *et al.*, 2003), their expression on inflammatory cells (Spears *et al.*, 2006), and increased expression during inflammatory events (Benayoun *et al.*, 2001; Kobayashi *et al.*, 2005a). In murine models of allergic asthma, PPAR α and PPAR γ agonists (rosiglitazone and GW9578, respectively) inhibit eosinophil influx to the lung following airway antigen challenge

(Kim *et al.*, 2005; Trifilieff *et al.*, 2003). The PPAR γ agonist was more effective than the PPAR α agonist, while the PPAR β/δ agonist had no effect (Trifilieff *et al.*, 2003).

At low concentrations, however, PPAR agonists have been shown to enhance eosinophil migration *in vitro* (Kobayashi *et al.*, 2005b). The observed enhancement in eosinophil migration could be due to an increase in chemotaxis or chemokinesis (Zigmond & Hirsch, 1973). The current study will compare the effects of low concentrations of the PPAR agonists GW9578, GW501516 and rosiglitazone on eosinophil migration and chemokinesis.

Eotaxin binding to CCR3 receptor on the surface of eosinophils leads to the initiation of migration through a transient rise in Ca²⁺ and phosphorylation of tyrosine on downstream signaling proteins. MAPKs, p44 and p42, also known as extracellular signal-regulated kinase-1 (ERK-1) and ERK-2 are involved in eotaxin induced rolling and migration through the regulation of cytoskeletal rearrangements (Boehme *et al.*, 1999). In the current study, the effect of PPAR agonist treatment on eotaxin-induced phosphorylation of ERK1/2 and p38 was investigated. The intracellular calcium oscillations were also examined.

Cell migration is a multistep process, which involves both chemotaxis and chemokinesis in response to a chemoattractant (Friedl & Weigelin, 2008). Chemotaxis is defined as stimulated directional migration towards a chemoattractant, whereas chemokinesis is defined as stimulated non-directional or random migration. Chemokinesis, alone, is not sufficient for cell accumulation, but may contribute considerably by priming a cell to respond more vigorously to subsequent chemotactic stimuli (Dunn, 1983). In the current study, the effects of PPAR agonists on eotaxin-

induced eosinophil directional and PPAR-induced random migration were investigated.

3.2 Materials and Methods

Subjects

Blood was obtained from 10 male and 13 female, non-smoking, allergic donors aged 19-60 years old. Subjects were recruited using research ethics board approved advertisement posted at the McMaster University campus. Smoking status, which can influence the migration of peripheral blood leukocytes (Pandit *et al.*, 2006), was confirmed as non-smoking during the initial interview. A subject's allergic status was determined by skin prick testing. Only subjects with a positive skin prick test were selected. Selecting subjects with allergies increased the chance of collecting sufficient numbers of eosinophils for *in vitro* analysis because patients with allergic disease can have a higher circulating eosinophil counts (Felarca & Lowell, 1967). Subjects were not currently using steroidal or non-steroidal anti-inflammatory medications or antihistamines. All of these medications would have affected the total number and function of the isolated eosinophils.

Samples of cord blood, from 6 individuals, were obtained from the hospital delivery room. The samples were collected in the delivery room at St. Joseph's Hospital after informed consent was received from the patient for the analysis of progenitor cell migration. An interview was not performed during the procedure so allergic status of the mother and current medication were not determined. The newborn allergic status was also not determined because no follow up visits were scheduled. Cord blood is a convenient sample to isolate progenitor cells due to the

larger number cells compared with peripheral blood (Prindull *et al.*, 1978) and the ease of collection compared to bone marrow. The studies were approved by the FHS/HHS Research Ethics Board and St. Joseph's Healthcare, and all subjects gave informed consent to participate.

Eosinophil Purification

One hundred millilitres of peripheral blood was collected from each subject into sodium heparin vacutainers for *in vitro* experiments. Peripheral blood was diluted with an equal volume of McCoy's 5A (Invitrogen Canada Inc, Burlington, ON, Canada) and eosinophils were purified using an accuprep™ density gradient (Accurate Chemical & Scientific Corporation, Westbury, NY, USA) followed by a MACS column CD16+ neutrophil depletion (Miltenyi Biotec, Auburn, CA, USA). Along with a lymphocyte layer, Accuprep™ also forms a layer at the bottom of the 1.077 g/mL media consisting of red blood cells, eosinophils and neutrophils.

After collecting the eosinophil layer, red blood cells were lysed with ammonium chloride. The method of NH₄Cl lysis has several effects on eosinophils after 4 days in culture, including decreased survival and degranulation (Ide *et al.*, 1994). This is not a concern for this *in vitro* assay due to the acute nature of the migration measurements and the fact that degranulation is not an outcome.

MACS column was used to purify the eosinophils from the granulocyte population after RBC lysis. To separate eosinophils from the granulocyte population a CD16 antibody was utilized. Granulocytes were incubated with the CD16 antibody diluted in PBS with 0.5% BSA and 6 mM EDTA for 45 minutes. The granulocytes were then added to a LD column that is placed in a magnetic field.

The purity of each eosinophil sample was determined with a cytoprep stained for a cell differential count (Diff-Quik®; Siemens Healthcare Diagnostics, Deerfield, IL, USA). After the cells are diluted to a concentration of 1×10^6 cell/mL, 50 μ L of cell suspension is placed into plastic cuvettes that have been pre-wet with DPBS (doesn't contain calcium and magnesium). The cuvettes are centrifuged at 500g for 5min forming a cell pellet on the surface of the glass slide. After the pellet has dried the slide is stained using Diff-Quik®.

Light microscopy demonstrated that the eosinophil preparations were >90% pure and the majority of contaminating cells were neutrophils. The purity was determined from the average of 2 slides after a minimum of 400 cells were counted on each, to minimize measurement variability. The vast majority contaminating cells were neutrophils, because the layering process isolates the granulocyte population. However, during the granulocyte layer retrieval it is possible to get contaminating lymphocytes (<1%).

Purified eosinophils were resuspended in RPMI complete (10% Fetal Bovine Serum (FBS), 1M HEPES in RPMI 1640) and incubated for 20 minutes with 0.1 nM–100 μ M of either a PPAR α agonist (GW9578; Cayman Chemical, Ann Arbor, MI, USA), a PPAR β/δ agonist (GW501516; Axxora LLC, San Diego, CA, USA), or a PPAR γ agonist (rosiglitazone; Cayman Chemical, Ann Arbor, MI, USA), all in a final concentration of 0.1% DMSO, or with diluent (RPMI complete in 0.1% DMSO). Roswell Park Memorial Institute (RPMI) media 1640 was developed by Moore *et al* for culturing human leukocytes (Moore, *et al.*, 1967). RPMI 1640 media utilizes a bicarbonate buffering system with varying amounts of amino acids and vitamins to ensure cell viability and growth. HEPES increases the buffer strength of the medium

and raises the maximum buffering range and FBS provides nutrients and growth factors for the cells. Prior to use in cell growth medium, FBS was heat inactivated (heated to 56 °C in a water bath) to destroy heat-labile complement proteins. The 20 min incubation with PPAR agonist prior to the migration assay was performed so methods in this study would mimic methods observed in previous studies (Kobayashi *et al.*, 2005b). Although 20 min may not be a sufficient time for protein translation (depending on the size of the protein) to occur, PPARs have non-genomic effects and can influence signaling through transrepression (Ricote & Glass, 2007). DMSO is an organosulfur that can dissolve both polar and nonpolar compounds. DMSO is used to reconstitute the PPAR agonists into solution, so the same concentration of DMSO was used in the negative condition to control for DMSO induced effects, such as increased erythroid differentiation (Friend, *et al.*, 1971).

Treatment with PPAR agonists had no effect on eosinophil viability as determined by trypan blue exclusion, being > 98% viable before and after incubation. It was important to determine the viability of the cells due to the assumption of normal cellular behaviour made in the migration assay. Dye exclusion is the method utilized by trypan blue, which is a derivative of toluidine. It determines viable cells that do not absorb the dye versus dead cells that readily absorb the dye. This selective staining method was first developed by Paul Ehrlich in 1904 to selectively kill trypanosomes (Ehrlich & Shiga, 1904).

Eotaxin-Induced Eosinophil Migration

In 1881, Dr. Engelmann was first to describe the method to measure the movement (aerotaxis) of cells (bacteria) towards oxygen producing algae (Engelmann,

1881). However, methods for measuring migration were further developed by Dr. Boyden (Boyden, 1962). The migration assay used in this study is a modified 48-well Boyden chamber assay, following protocols from previous studies on eosinophil migration (Sehmi *et al.*, 1992). The Boyden chamber utilizes two chambers that are separated by a semi-permeable filter that allows the chemoattractant to readily pass through. The cells, however, cannot pass through without the induction of migration (shape change). The PPAR agonists were left in the cell suspension, and the upper wells of the Boyden chamber were filled with 50 μ L of eosinophils at a concentration of 3×10^6 cells/mL. The PPAR agonist was balanced in the upper and lower wells so the only concentration gradient would be towards eotaxin. Therefore, we could determine the specific effect of PPAR agonists on the migration response to eotaxin. This mimics the concentrations observed in previous work (Kobayashi *et al.*, 2005b). Eotaxin at a concentration of 10 nM (R&D Systems, Minneapolis, MN, USA) in the presence of a PPAR agonist (0.1 nM -100 μ M) were placed in the lower wells of the microchemotaxis assembly (Neuro Probe Inc., Gaithersburg, MD, USA). In initial studies, we determined the suboptimal concentration of eotaxin (10nM), which was comparable to other work (Kobayashi *et al.*, 2005b). Kobayahsi *et al* (2005b) used troglitazone, a strong PPAR γ and weak PPAR α agonist, as a control response to examine the migration of 15d-PGJ₂. Troglitazone was observed to enhance and inhibit migration in a dose dependent manner. However, the EC₅₀ of troglitazone is 10x lower than rosiglitazone (0.55 μ M versus 0.043 μ M respectively) (Willson *et al.*, 2000) and rosiglitazone, unlike troglitazone, is full agonist for PPAR γ (Walker *et al.*, 1998; Camp *et al.*, 2000). In our study the concentration range selected for the PPAR

agonists was used to examine the full spectrum of responses, both the inhibition and enhancement of migration (observed in previous work) (Kobayashi *et al.*, 2005b).

The upper and lower wells were separated by a nitrocellulose filter with an 8 μ m pore and the chamber was incubated in high humidity for 90 minutes at 37 $^{\circ}$ C. The migration results obtained from nitrocellulose filters do not differ significantly from polycarbonate filters (Jungi, 1978). However, nitrocellulose filters allow further examination of chemotaxis and chemokinesis in one assay (Zigmond & Hirsch, 1973). Polycarbonate filters, which were used in the Kobayashi *et al* (2005b) study, have defined pores punched into the 10 μ m thick surface that allow for chemoattractant diffusion and cell migration (Wilkinson, 1998). Since gradients only form towards these defined pores, chemotaxis can only occur across the surface of the filter, therefore, adhesion to the filter is critical (Wilkinson, 1998). On the other hand, nitrocellulose filters form a 150 μ m thick 3-D matrix for the cells to move towards the chemoattractant (Wilkinson, 1998). This extra depth allows the cells to develop both a random and directionally stimulated migrational response thus, allowing separation of chemokinesis from chemotaxis in the migration process (Zigmond & Hirsch, 1973), as described in detail below. A filter with an 8 μ m pore was used in the eosinophil migration assays because eosinophils are 12-15 μ m in diameter, which requires them to undergo shape change to pass through the filter.

The chamber was then disassembled and the nitrocellulose filter was fixed with mercuric chloride overnight and stained with haematoxylin and chromotrope 2R. Mercuric chloride fixes the filter while haematoxylin and chromotrope 2R stain the iron and alum in the cytoplasm/nucleus and the basic proteins in the granules respectively. The filter was dehydrated through graded alcohols followed by a 30

minute xylene treatment, then mounted on a glass slide and coverslipped with Permount™ (Fisher Scientific, Pittsburgh, PA, USA). Dehydration with alcohol replaces any water left in the filter with alcohol, which is miscible with xylene. It is important to remove the water because it is not miscible with xylene, so the clearing agent would not work efficiently. Xylene is used as a clearing agent to remove excess stain, which helps to decrease background staining. The number of eosinophils was quantified from 10 random fields at the leading edge, which are counted from the underside of the filter using a light microscope at 400x magnification. To deal with the unpredictable way the cells can drop off the bottom of the filter (Keller *et al.*, 1972), Zigmond and Hirsch introduced a new technique, known as leading front (Zigmond & Hirsch, 1973). Cells aren't allowed to migrate through the whole depth of the filter and only those cells that migrated the furthest were counted. This technique was utilized in this study. Random fields were counted to control for any variations across the filter in each well. These methods were adapted from previous work (Sehmi *et al.*, 1992).

PPAR Agonist-Induced Eosinophil Chemokinesis

To determine the effects of PPARs on eosinophil chemokinetic responses, eosinophils were incubated with PPAR agonists (0.1 nM-100 µM) for 20 minutes then loaded into the upper chamber with similar concentrations of the PPAR agonist in the lower chambers. Cell migration is a multistep process, which involves chemotaxis and chemokinesis in response to a chemokine (Friedl & Weigelin, 2008). Chemotaxis is defined as directed migration towards a chemokine, whereas chemokinesis is defined as non-directional velocity induced migration. Dissecting chemotaxis from

chemokinesis is critical since chemokinesis alone is not a good mechanism for cell accumulation, but may contribute considerably by priming a cell to respond more vigorously to chemotactic stimuli (Dunn, 1983). Nitrocellulose filters form a 3-D matrix for the cells to move through, towards the chemoattractant (Wilkinson, 1998). This extra space allows the cells to develop both a chemotactic and chemokinetic response thus you can separate chemotaxis from chemokinesis in the migration process (Zigmond & Hirsch, 1973). This could not have been done in previous studies that observed increase migration after troglitazone treatment (Kobayashi *et al.*, 2005b). Adding the same concentration of the PPAR agonist in the upper and lower well eliminates a concentration gradient, which allows us to determine the chemokinetic component of the response.

Eosinophil CCR3 Expression

Purified eosinophils were incubated with rosiglitazone at 0.1 nM, 10 nM or diluent for 110 minutes. An effective dose and a low dose that had minimal effect were selected to examine the possible mechanism behind the enhanced migration. The incubation time examined was the total exposure time used in the migration assay. The cells were then stained for CCR3 surface expression using mouse anti-human Pacific Blue-CD45 (eBioscience, San Diego, CA, USA), FITC-CD16 (Becton-Dickinson Biosciences, Mississauga, ON, Canada) and PE-CCR3 (Medical & Biological Laboratories, Naka-ku, Nagoya, Japan), as well as the isotype control antibodies for CCR3. To separate eosinophils from contaminating cells a CD45⁺CD16⁻ gating strategy was utilized (Figure 3.1). CD45 is a protein tyrosine phosphatase, receptor type, C expressed on leukocytes (Omary *et al.*, 1980) and

CD16, as mentioned previously, is expressed on the surface of neutrophils, natural killer cells, T cells and macrophages (LoBuglio *et al.*, 1967; Messner & Jelinek, 1970; Henson, 1969; Perlmann *et al.*, 1976). Although we used a purified eosinophil population, these markers verified the cells of interest. CCR3 positive staining was determined from an isotype control, which represents the specific antibody without the epitopes. Using standard FACS methods, the isotype control, which represents non-specific binding, was set at 2% positive. The positive specific binding was then determined from this set gate. A minimum of 3000 CD45⁺CD16⁻ cells were collected for analysis. Two parameters were determined from this gating strategy, the % stained and the MFI. Cells were acquired with an LSR II flow cytometer (Becton Dickinson Instrument Systems; Becton-Dickinson, Mississauga, ON, Canada) using the FACSDiva software program (Becton-Dickinson Biosciences).

Eosinophil Migration – Signal Transduction Pathways

Purified eosinophils were treated with rosiglitazone at concentrations which were previously reported to induce cell migration (0.1 nM, 10 nM) (Kobayashi *et al.*, 2005b) or with diluent for 110 minutes in the absence or presence of 10 nM eotaxin. The doses and the incubation time were selected with the same strategy as the CCR3 expression assay. Eotaxin was added after 20 min of PPAR agonist pretreatment to initiate phosphorylation of ERK1/2 and p38. A negative control, in the absence of eotaxin, was examined to determine the baseline effects of the PPAR agonist. After rosiglitazone treatment, eosinophils were lysed and the protein concentration was standardized using a Bradford assay (Table 3.1). Eosinophils were lysed with RIPA buffer containing protease and phosphatase inhibitors. RIPA buffer contains

denaturing chemicals, such as sodium dodecyl sulfate, which disrupts non-covalent bonds in the proteins causing them to lose their conformation. Protease and phosphatase inhibitors are included in the buffer to ensure degradation of the protein or the phosphorylation state will not occur. A Bradford assay was required to minimize the protein loading variability between subjects. Bradford assay was developed by Dr. Bradford in 1976. It is a colorimetric assay that measures the protein concentration based on an absorbance shift of a dye. The Coomassie® brilliant blue G-250 dye that is used in this assay shifts from 465 to 595nm when protein binding occurs (Bradford, 1976). Elevated concentrations of detergent, such as SDS, can interfere with protein binding, however if it is kept at less than 0.1%, as in the current study, no deleterious effects are observed (Bradford, 1976).

Phosphorylation of ERK1/2 and p38 were analyzed using signal transduction assay reaction (STAR) ELISA kits (Millipore, Temecula, CA) and were quantified by measuring the absorbance at 450nm using an EL800 plate reader (BioTek Instruments, Winooski, VT). ELISA was first developed in 1971 (Van Weemen & Schuurs, 1971; Engvall & Perlmann, 1971), however the development was made possible by the ability to link antibodies to polysaccharides (Wide & Porath, 1966) and enzymes (Avrameas & Uriel, 1966). ELISA involves immobilizing a protein of interest using a specific antibody bound to a polystyrene microplate. A detection antibody, which is also specific for the protein of interest, is then added to the well. This is followed by the addition of an enzyme linked antibody that binds to the Fc portion of the specific antibody. Finally, a dye is added to the wells, which, when converted by the enzyme allows the specific detection of the protein of interest. A standard curve is preformed

in parallel to allow the quantification of the protein of interest. Samples are run in triplicate to reduce the variability observed between wells.

MAPKs, p44 and p42, also known as extracellular signal-regulated kinase-1 (ERK-1) and ERK-2 are involved in eotaxin induced rolling and migration through the regulation of cytoskeletal rearrangements (Boehme *et al.*, 1999). MAPK p38 was also observed to be an important mechanism in eotaxin-induced chemotaxis (Kampen *et al.*, 2000). In the current study we used rabbit anti-human antibodies specific for the phosphorylated form of the tyrosine/threonine on ERK-1/2 and on p38. The secondary antibody was mouse anti-rabbit directed to the Fc portion of the specific antibody and was HRP linked. After the incubation period TMB dye was added and HRP enzymatically converts TMB into a diimine, which appears as a blue colour. When the stop solution is added, usually sulfuric acid, TMB turns yellow and can be read at 450nm on absorbance reader (Josephy, Eling, & Mason, 1982). Protein standards were supplied by the manufacturer and used in triplicate to form the standard curve (Figure 3.2). Samples were also tested in triplicate and a negative control (TMB only) was used to manage background absorbance.

Eosinophil Progenitor Cell Transwell Migration

Immediately after delivery, umbilical cord blood (CB) was collected into a 60ml syringe (Becton Dickinson, Mississauga, ON) containing heparin (1000 units/ml; Sigma Aldrich, Oakville, ON). CB was depleted of red blood cells by gravity sedimentation in 1% (vol/vol) dextran (Sigma Aldrich, Oakville, ON) at a 1:5 dilution at 37°C, 5% CO₂ for 45 min in 50ml conical tubes (Becton Dickinson, Mississauga, ON). Cord blood was diluted with an equal volume of McCoy's 5A (Invitrogen

Canada Inc) and mononuclear cells were purified using an accuprep density gradient (Accurate Chemical & Scientific Corporation, Westbury, NY, USA). Access to cord blood was provided by Dr. Sehmi at St. Joseph's Hospital. Umbilical cord blood is a convenient sample that provides a high number of progenitor cells for subsequent analysis (Kirshner & Goldberg, 1980). Mononuclear cells were then resuspended in McCoy's 3+ (McCoy's 5A with 10% fetal bovine serum, 1% penicillin/streptomycin (pen/strep) and 1% 2-ME and incubated for 2-hours in 5% CO₂ at 37°C and high humidity to remove monocytes. McCoy's 3+ is a general purpose media, providing various amino acids, vitamins and buffers, plus heat inactivated FBS for extra nutrients, penstrep for antibiotic protection and 2-ME for preventing protein oxidation. Using glassware, Dr. Levis and Robbins observed that adherent mononuclear cells, that do not undergo blastogenesis, can be removed from the lymphocyte population (Levis & Robbins, 1972). This provides a purer cell population for subsequent CD34 isolation. CD34+ progenitor cells were isolated using a MACS column CD34+ positive selection (Miltenyi Biotec, Auburn, CA, USA). The CD34+ progenitor cell preparations were >90% viable after isolation. Cluster of differentiation molecule 34 (CD34) is a glycoprotein expressed on the surface of hematopoietic progenitor cells (Civin *et al.*, 1984) and may function as an adhesion molecule (Fina *et al.*, 1990). The antibody, known as My-10, that recognized human hematopoietic progenitor cells was first developed in 1984 in a KG-1a human leukemic cell line (Civin *et al.*, 1984). It was not until later in 1984 that the cluster differentiation (CD) nomenclature was created at the 1st International Workshop and Conference on Human Leukocyte Differentiation Antigens (Bernard & Boumsell, 1984), and My-10 was renamed

CD34. In this study CD34 was used as the marker for the positive selection of progenitor cells.

The non-adherent mononuclear cells were incubated with Fc receptor block and CD34 antibody, which was diluted in PBS with 0.5% BSA and 6 mM EDTA. Cells were incubated for 30 minutes before being added to a MS column that is placed in a magnetic field. The Fc receptor block decreases non-specific binding of the CD34 antibody, which increases the purity of the isolated population. The PBS, BSA and EDTA provide the cells with a solution that buffers against changes in pH, decreases non-specific binding, and can prevent cell coagulation. The MS column is designed for selection of strongly labeled cells in the column.

Purified CD34⁺ progenitor cells were resuspend at 1×10^6 cells/mL in RPMI complete (10% fetal bovine serum, 1M HEPES in RPMI 1640) with 100–1000 nM of PPAR α agonist (GW9578), PPAR β/δ agonist (GW501516), or PPAR γ agonist (rosiglitazone) agonist, all in a final concentration of 0.1% DMSO, or diluent (RPMI complete in 0.1% DMSO). The PPAR agonist concentration was based on previously reported effective doses in migration assays (Kobayashi *et al.*, 2005b; Ueki *et al.*, 2003; Woerly *et al.*, 2003). Stromal derived factor-1 α (SDF-1 α , R&D Systems) at a concentration of 100 ng/mL and in the presence of equivalent concentrations of PPAR agonists (100–1000 nM), was placed in the lower wells of the transwell assembly. SDF-1 α was first cloned in 1993 from a bone marrow stromal cell line (Tashiro *et al.*, 1993). SDF-1 α is a highly potent mononuclear cell chemoattractant (Bleul *et al.*, 1996). In previous studies, an increase in SDF-1 α -induced endothelial progenitor cell migration was observed in patients with coronary artery disease on a once daily dose of pioglitazone, a PPAR γ agonist (Werner *et al.*, 2007). The concentration of SDF-1 α

utilized was based on previously published reports on progenitor cell migration to SDF-1 α (Dorman *et al.*, 2005; Imaoka *et al.*, 2011). The chamber was incubated in high humidity for 4 hours at 37⁰C. This incubation period was previously optimized by Dorman *et al* (Dorman *et al.*, 2005).

The chamber was then disassembled and the cells from the lower well were stained for CD34 and IL-5 receptor- α (IL-5R α) surface expression using mouse anti-human CD34-APC, CD45-FITC and PE-IL-5R α (Becton-Dickinson Biosciences), as well as the isotype control antibodies. CD34 is expressed on hematopoietic progenitor cells, hemangioblasts, and endothelial progenitor cells (Civin *et al.*, 1984; Loges *et al.*, 2004; Peichev *et al.*, 2000). CD45+CD34+IL-5R α + cells represent a unique population of progenitor cells that are eosinophil lineage committed progenitors (Sehmi *et al.*, 1997). IL-5 signaling, which leads to eosinophilopoiesis, requires the activation of the IL-5 receptor α subunit along with the common β chain (Tavernier *et al.*, 1991). For these reasons, the current study utilized the IL-5R α as a marker of eosinophil progenitors. Cells were acquired with an LSR II flow cytometer (Becton Dickinson Instrument Systems; Becton-Dickinson) using the FACSDiva software program (Becton-Dickinson Biosciences). Migration was expressed as percent (%) of total CD45+CD34+IL-5R α + cells plated. Using a % of total expression allowed us to reduce the variability observed between samples.

Image Acquisition and Measurement of [Ca²⁺]_i

Intracellular changes in calcium were measured using confocal microscopy, as previously described (Janssen *et al.*, 2009). The principle of confocal microscopy was first patented in 1957 by Dr. Minsky (Minsky, 1957). The confocal microscope used

in the current study was constructed by Dr. Janssen, and uses two mirrors vibrating at two different frequencies to reflect a laser across the sample. The laser light (488 nm) excites a calcium-sensitive dye, which in turn emits fluorescent light (>510 nm) (described in more detail below). A photomultiplier tube (PMT) transforms the fluorescent light into an electrical signal that is recorded by the computer.

Isolated eosinophils (1×10^6 cells/mL) were loaded for 1 hr on ice with $3.5 \mu\text{M}$ of a Ca^{2+} -sensitive fluorescent probe, fluo-3 AM (Invitrogen Canada) dissolved in dimethyl sulfoxide with 0.01% pluronic F-127. Fluo-3 fluoresces at wavelengths greater than 510 nm, when excited by 488 nm light. Fluorescence is greatly increased upon binding of calcium to the polyanionic molecule Fluo-3 (Minta, Kao, & Tsien, 1989). Being such a highly charged molecule, Fluo-3 itself does not penetrate the cell membrane easily. However, neutralization of those charges by addition of AM ester groups allows Fluo-3-AM to be easily absorbed. Once inside the cell, the ester groups are cleaved by endogenous esterases, which trap fluo-3 in the cytosol. Pluronic F-127 is a hydrophilic non-ionic surfactant that helps dissolve the water-insoluble fluo-3 dye (Cohen *et al.*, 1974; Schmolka, 1973). The eosinophils were then loaded onto a culture dish and placed on the stage of a custom-built confocal microscope equipped with a 20x objective. The bathing solution for all experiments was RPMI, maintained at 37°C , which was exchanged constantly using superfusion throughout the experiment.

Eosinophils were then illuminated using 488 nm light from a 20 mW photodiode laser (Coherent Technologies; CA). During the recordings eosinophils were exposed to stimuli (10nM eotaxin or diluent control) and treatment with 100 nM rosiglitazone or diluent control. Images (480×640 pixels) were generated continuously

at 30 Hz with the imaging software ‘Video Savant’ (IO Industries; London, ON, Canada). However, only 9 consecutive images were saved. These images were averaged to produce a single videoframe, at 1.5 second intervals, giving a final image frame rate of 0.67 Hz. The image analysis software, ‘Scion’ (Scion Corporation, Frederick, MD), was used to determine the pixel intensity of a user-defined region of interest in 10 individual cells for measurement of $[Ca^{2+}]_i$. Fluorescence intensities of these regions of interest were saved and plotted against time. An increase in the average fluorescence intensity was interpreted as an increase in $[Ca^{2+}]_i$ and an increase in the average frequency of $[Ca^{2+}]_i$ spikes was interpreted as an increase in $[Ca^{2+}]_i$ oscillations.

Statistical Analysis

All data are expressed as the mean \pm 95% confidence intervals unless otherwise stated. Statistical analyses were performed using Prism version 5 (GraphPad Software, La Jolla, CA). Repeated measures ANOVA was used to compare PPAR agonist treatments versus diluent at each concentration, with post-hoc Tukey tests for pre-specified comparisons. For data that was not normally distributed the statistical analyses were performed on the log-transformed data. Statistically significant differences were accepted at $P < 0.05$.

3.3 Results and Discussion

Subjects

Blood donors that were recruited for the study were 31 ± 12 years old with mild blood eosinophilia ($3.9 \pm 2\%$).

Effects of PPAR Agonists on Eotaxin-Induced Eosinophil Migration

The chemokine eotaxin (10 nM) induced significant eosinophil migration compared to diluent control (242.9 ± 148.6 versus 46.1 ± 68.1 cells/10 HPF, $P < 0.0001$). PPAR agonists were tested at low concentrations ($n=9$, Figure 3.3) and high concentrations ($n=6$, Figure 3.3). Pre-incubation with PPAR γ agonist rosiglitazone (100 μ M) significantly inhibited eotaxin-induced eosinophil migration (Figure 3.3; $P=0.0042$). By contrast, there was no effect of PPAR α agonist, GW9578 ($P=0.9$) or PPAR β/δ agonist, GW501516 ($P=0.3$) on eotaxin-induced migration (Figure 3.3).

Rosiglitazone (PPAR γ agonist) treatment alone at a concentration of 100 nM both above and below the nitrocellulose filter in the micro-Boyden chamber assay significantly increased the eosinophil chemokinesis compared to diluent control ($P=0.0038$; Figure 3.4). In contrast, no chemokinetic responses were observed when eosinophils were incubated with equivalent concentrations of GW9578 (PPAR α agonist, $P=0.9$) or GW501516 (PPAR β/δ agonist, $P=0.9$) above and below the filter (Figure 3.4), or with a high concentration of rosiglitazone (Figure 3.4, $P=0.05$).

Previously it was not known if the increase in eotaxin-induced migration by PPAR γ agonists were due to chemotaxis, chemokinesis, or both. Although PPAR agonists in this *in vitro* study are used at concentrations similar to the reported EC₅₀s for rosiglitazone, GW9578 and GW501516 (43 nM, 50 nM and 1.1 nM, respectively) (Lehmann *et al.*, 1995; Brown *et al.*, 1999; Oliver, Jr. *et al.*, 2001), this study has determined that the enhanced migration by rosiglitazone (PPAR γ agonist) is likely due to a chemokinetic effect. Such “priming” of cells may enhance their response to other

stimuli, including chemokines such as eotaxin that are found in the microenvironment of allergic tissue, thus leading to eosinophilia.

Effect of Rosiglitazone on Eosinophil CCR3 Surface Expression and Signaling

Incubation with rosiglitazone at 0.1 and 10 nM had no effect on eosinophil CCR3 surface expression (Table 3.2). Furthermore, eosinophil incubation with rosiglitazone in the presence or absence of eotaxin had no effect on the level of phosphorylation of ERK1/2 or p38 MAPK (Table 3.2).

To improve our understanding of how PPAR agonists regulate eotaxin-induced eosinophil migration, we investigated the effects of rosiglitazone on the surface expression and down-stream signalling of the eotaxin receptor, CCR3. Consistent with studies of other PPAR γ agonists, 15d-PGJ₂ and troglitazone (Kobayashi *et al.*, 2005b), which were studied at similar concentrations, we observed no effect of rosiglitazone on the cell surface expression of CCR3 or on phosphorylation of the down-stream signaling molecules ERK1/2 and p38.

Effects of PPAR Agonist on SDF-1 α -Induced Eosinophil Progenitor Cell Migration

Compared to diluent control, SDF-1 α (100 ng/mL) induced migration of CD34+IL-5R α + cells isolated from cord blood (8.2 \pm 6.2 vs 33.2 \pm 6.1% of total cells migrated, P=0.0479). Low concentrations of rosiglitazone increased the migrational response of cord blood-derived CD34+IL-5R α + cells to SDF-1 α compared to diluent control. However, this change did not reach the level of statistical significance (Figure 3.5; P=0.054). No changes in migration of CD34+IL-5R α + cells were observed with

equivalent concentrations of GW9578 (PPAR α agonist, P=0.8) or GW501516 (PPAR β/δ agonist, P=0.6).

Examination of the effect of PPAR agonists on the immature eosinophil population of CD34+IL-5R α + cells purified from cord blood samples demonstrated that migration in response to the potent chemoattractant SDF-1 α is likewise enhanced by pre-treatment of low concentrations of rosiglitazone (PPAR γ agonist). This finding is novel, and mirrors our and Kobayashi's (Kobayashi *et al.*, 2005b) observations in mature eosinophils, which shows enhanced migration by low concentrations of PPAR γ agonist, with no effect of PPAR α and β/δ agonists.

Image acquisition and measurement of $[Ca^{2+}]_i$

Calcium flux was examined in 3 separate eosinophil preparations. Addition of eotaxin to eosinophil preparations caused an increase in the maximum fluorescence intensity (Figure 3.6; diluent 16.6 \pm 6.2 vs eotaxin 66.7 \pm 27.4 at 510nm) and the number of calcium oscillations (diluent 0.007 \pm 0.004Hz vs eotaxin 0.038 \pm 0.016Hz). Treatment with 100 nM rosiglitazone consistently reduced the frequency of calcium oscillations observed after the addition of 10 nM eotaxin (control 0.038 \pm 0.016Hz vs treatment 0.016 \pm 0.007Hz). However, no consistent effect of rosiglitazone was observed on the maximum fluorescence intensity in the 3 preparations studied (control 66.7 \pm 27.4 vs treatment 58.1 \pm 25.2).

Previous studies have shown that pre-treatment with a MEK inhibitor or a p38 MAPK inhibitor had no effect on enhanced migration, nor did inhibition of the NF- κ B pathway or inhibition of genomic transcription with actinomycin D (Kobayashi *et al.*, 2005b). We and others have shown that PPAR agonists have a modulatory effect on

eotaxin-induced calcium mobilization (Kobayashi *et al.*, 2005b), which would suggest that PPAR agonists have down-stream targets that have not been identified yet.

3.4 Conclusion

This is the first study to show increased chemokinesis of eosinophils *in vitro* at low concentrations of rosiglitazone, which has a 100-fold higher binding affinity for PPAR γ than troglitazone (Young *et al.*, 1998). The results of this study also confirm previous observations showing that high concentrations of a PPAR γ agonist inhibit eosinophil migration (Kobayashi *et al.*, 2005b).

We demonstrated that the PPAR γ agonist rosiglitazone, at a concentration of 100 nM, significantly increased eosinophil chemokinesis *in vitro*. This finding supports the data demonstrating increased eosinophil migration reported by Kobayashi (Kobayashi *et al.*, 2005b). We also demonstrated that rosiglitazone treatment had no effect on the level of cell surface expression of the eotaxin receptor, CCR3, on eosinophils, or on the downstream signalling events following eotaxin/CCR3 binding, such as phosphorylation of ERK1/2 and p38 MAPK. This suggests that the observed effects of rosiglitazone are not related to alterations in signalling through the eotaxin receptor. At these same concentrations there was no effect of the PPAR α agonist, GW9578 or the PPAR β/δ agonist, GW501516.

In summary, at low concentrations the PPAR γ agonist rosiglitazone enhanced chemokinesis of eosinophils isolated from the peripheral blood of atopic subjects, and also enhanced the migration of eosinophil progenitors. This chemokinetic effect may partially explain the enhancement of eosinophil migration seen at these concentrations. The enhanced chemokinesis was specific for the PPAR γ agonist, as

there was no effect of PPAR α or PPAR β/δ agonists. In light of our findings, that a selective PPAR γ agonist can enhance eosinophil chemokinesis at a concentration of 100 nM and decrease eosinophil migration at a concentration of 100 μ M, the therapeutic window for PPAR γ agonists as an anti-inflammatory therapy is narrow and dosage must be titrated carefully.

3.5 Appendix

Eosinophil Migration Assay – CellTiter 96

In the initial optimization several techniques were utilized to determine the effect of PPAR agonists on eosinophil migration. Previously in our lab, Rabyah Murji optimized a migration assay for neutrophils. This technique used a 96 well modified Boyden chamber with a polycarbonate filter and measured the number of cells that migrated by measuring the absorbance on an ELISA plate reader after incubation with CellTiter 96® Aqueous One Solution. CellTiter 96 contains MTS, which is a redox indicator, and phenazine ethosulfate, which is an electron coupling reagent that stabilizes the solution.

The current study modified the existing neutrophil protocol. Briefly, a modified Boyden Chamber (Neuro Probe Inc., Gaithersburg, MD) with a 96 well clear microplate was used to assess eotaxin-induced eosinophil migration. Eotaxin was used at concentrations of 0.1-10 nM. The bottom wells of the microplate were loaded with 400ul of eotaxin producing a slight positive meniscus. A polycarbonate filter with a 5µm pore (Neuro Probe Inc., Gaithersburg, MD) was placed between the upper and lower wells. The top chamber was secured fastened and 50µL of eosinophils at 3×10^6 cells/mL were loaded into the lower compartment of the upper wells followed by 175µl of RPMI buffer into the upper compartment. The cells were incubated in the Boyden chamber for 1 hour in a 5% CO₂ incubator at 37°C. RPMI alone was used as a negative control to determine migration from baseline movement.

After the incubation, the chemotaxis chamber was disassembled and the fluid from the upper wells was aspirated. The fluid in the lower wells was mixed

thoroughly and 200 μ L was transferred into a new transparent 96-well plate. The number of cells that migrated through the 5 μ m-pore filter was quantified by using CellTiter 96 AQueousOne Solution Reagent (Promega Corporation, Madison, WI, USA). MTS-based assays detect the number of viable cells by measuring redox reactions. Viable cells transfer electrons from their metabolic by-products such as NADH or NADPH to MTS, which is consequently reduced to a soluble detectable dye (formazan). MTS (40 μ L) was added into each 200 μ L sample. The plate was incubated for 2 hours at 37°C in humidified air with 5% CO₂. The absorbance at 490 nm was measured after 2 hours using an EL800 plate reader (BioTek Instruments, Winooski, VT).

Unfortunately this technique was not successful for eosinophil migration. No dose response to eotaxin could be observed (Figure 3.7). Several reasons could explain these results, including eosinophil autofluorescence, and the MTS assay susceptible to colormetric interference. Eosinophils emission spectrum include wavelengths from 490-600nm when excitation spectrum of 450nm is used (Weil & Chused, 1981). Since the assay measures absorbance at 490nm it is possible that excitation and emissions from the eosinophils themselves could produce a significant background signal thus hampering the CellTiter measurement. During this assay eosinophils were suspended in RPMI, which contains phenol red, a pH indicator. Again the phenol red could interfere with background absorbance measurements thus making it more difficult to measure the absorbance produced by the CellTiter dye. In an attempt to quantify the number of cells that had migrated, the filter was stained using Diff-Quik® (Siemens Healthcare Diagnostics, Deerfield, IL, USA). Regrettably, because the filter was so thin, it was difficult to determine the location, which side of

the filter the eosinophils were located (Figure 3.8). This technique was abandoned and CellTiter Glo® was attempted.

Eosinophil Migration Assay – CellTiter Glo®

This technique also used a 96 well modified Boyden chamber with a polycarbonate filter and measured the number of cells that migrated by measuring the luminescence on the Alpha Innotech FluorChem® camera (Quansys Biosciences, Logan, Utah, USA) after incubation with CellTiter Glo® (Promega Corporation, Madison, WI, USA), which is an ATP indicator and a luciferase reagent. CellTiter Glo® generates a luminescent signal that is proportional to the amount of ATP present which is an indicator of the number of cells present (Crouch, *et al.*, 1993).

The migration protocol used was the same as the protocol used for the CellTiter 96 assay with minor changes. One hundred microlitres aliquots of the sample from the lower wells of the modified Boyden chamber were placed in an opaque 96 well plate. A negative control was prepared to account for background luminescence. Equal volume of CellTiter Glo® was added to the sample and was mixed on an orbital shaker for 2 min to induce lysis. The plate was then incubated at room temperature for 10min. Images of the plate were then acquired using the Alpha Innotech FluorChem® camera and densitometry was performed with the provided software. Unfortunately this technique was also not successful for eosinophil migration. No dose response to eotaxin could be observed (Figure 3.9). Several reasons could explain these results, including eosinophil autofluorescence, and cellular ATP content. As mentioned previously, it is possible that eosinophils themselves could produce a significant background signal thus hampering the

luminescence measurement. Another possible explanation is that different cells vary considerably in their ATP content (Crouch *et al.*, 1993). The induction of migration itself may also have an effect on the linear relationship between cell number and luminescence through metabolism of ATP. This technique was abandoned and the nitrocellulose microscopy assay was adopted.

3.6 Tables and Figures

Table 3.1. Bradford assay results for eosinophil protein (mg/mL) from 4 subjects after treatment with the PPAR γ agonist rosiglitazone. Concentrations were adjusted to 0.3mg/mL after the assay.

Subject	Diluent	0.1 nM	10 nM
100	0.4	0.3	0.8
101	0.6	0.5	0.4
102	0.5	1.3	0.5
103	1.1	1.2	1.2

Table 3.2. Eosinophil expression of CCR3 and phosphorylation of ERK1/2 and p38 after treatment with the PPAR γ agonist rosiglitazone.

		Diluent	0.1 nM	10 nM
CCR3 (%)		29.3 \pm 20	23.5 \pm 15	19.8 \pm 12
CCR3 (MFI)		8.3 \pm 12	6.8 \pm 9	5.4 \pm 3
Phospho-ERK1/2 (units/mL)	Diluent	3.5 \pm 2.4	3.3 \pm 1.9	3.3 \pm 1.6
	Eotaxin	3.7 \pm 0.6	3.9 \pm 1.1	4.0 \pm 0.9
Phospho-p38 (units/mL)	Diluent	3.7 \pm 3.2	4.7 \pm 4.3	4.5 \pm 6.0
	Eotaxin	6.7 \pm 4.6	6.2 \pm 3.6	7.0 \pm 3.6

Data represent mean \pm SD.

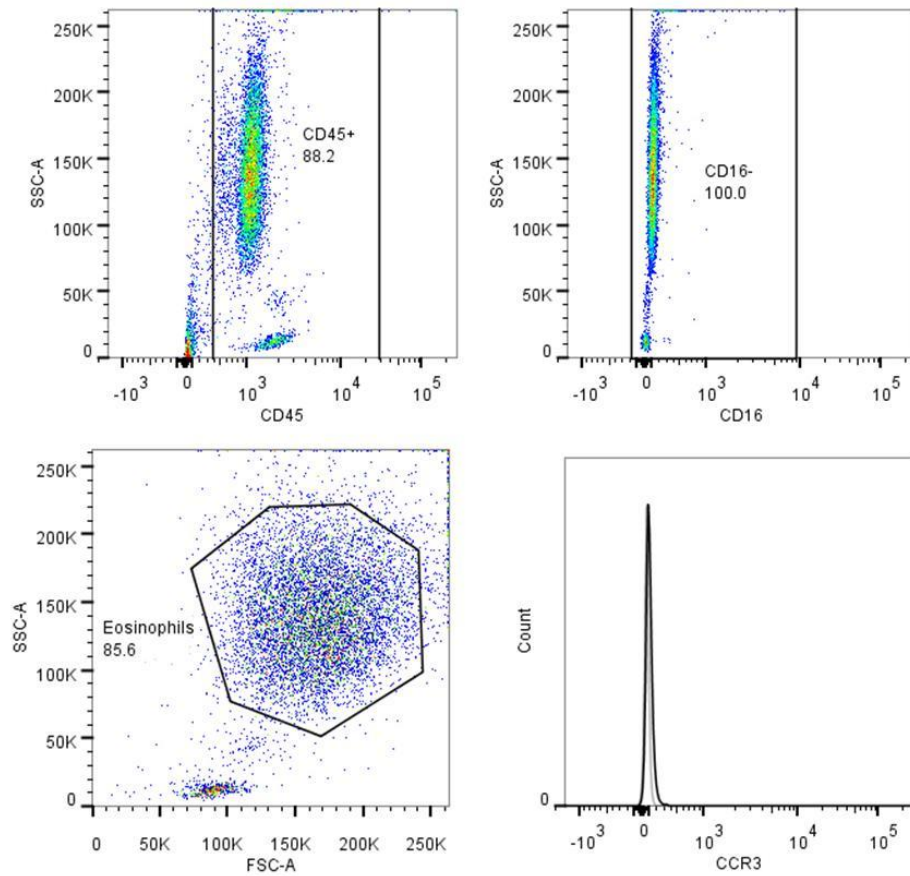


Figure 3.1. Gating strategy used to isolate peripheral blood eosinophils from the isolated population. CCR3 expression on eosinophils was examined using CD45⁺CD16⁻ with high granularity (top left, top right and bottom left graph respectively). The MFI for the isotype (grey line) and CCR3 antibody (black line) are shown in the bottom right graph.

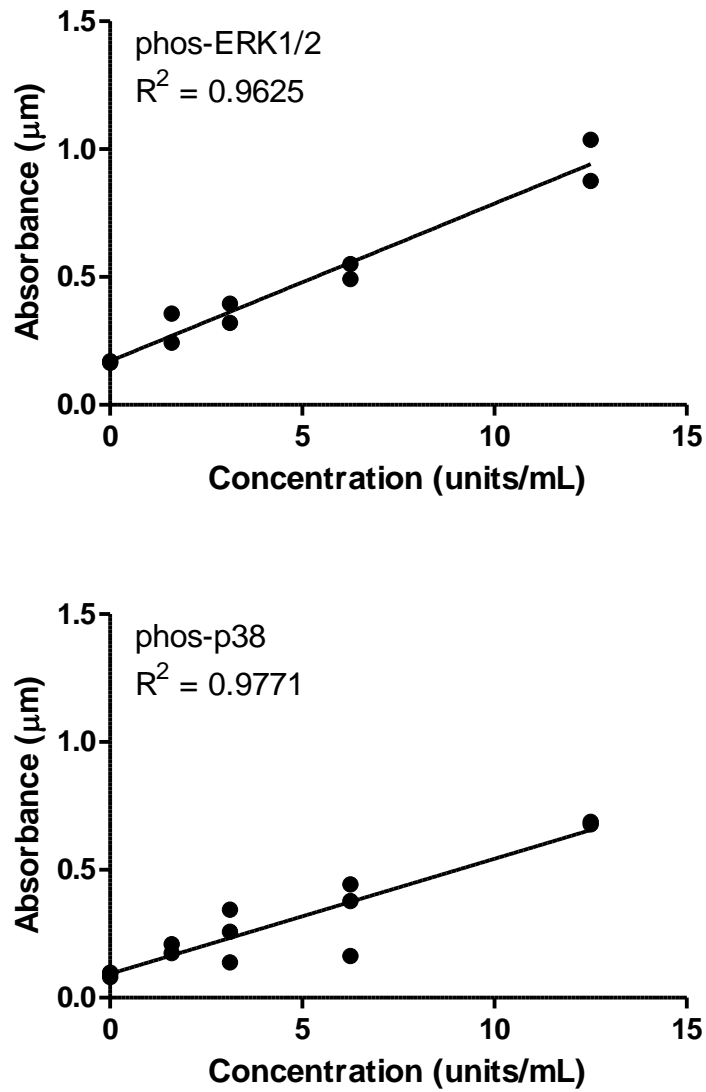


Figure 3.2. ELISA standard curves for phosphorylated ERK1/2 and p38.

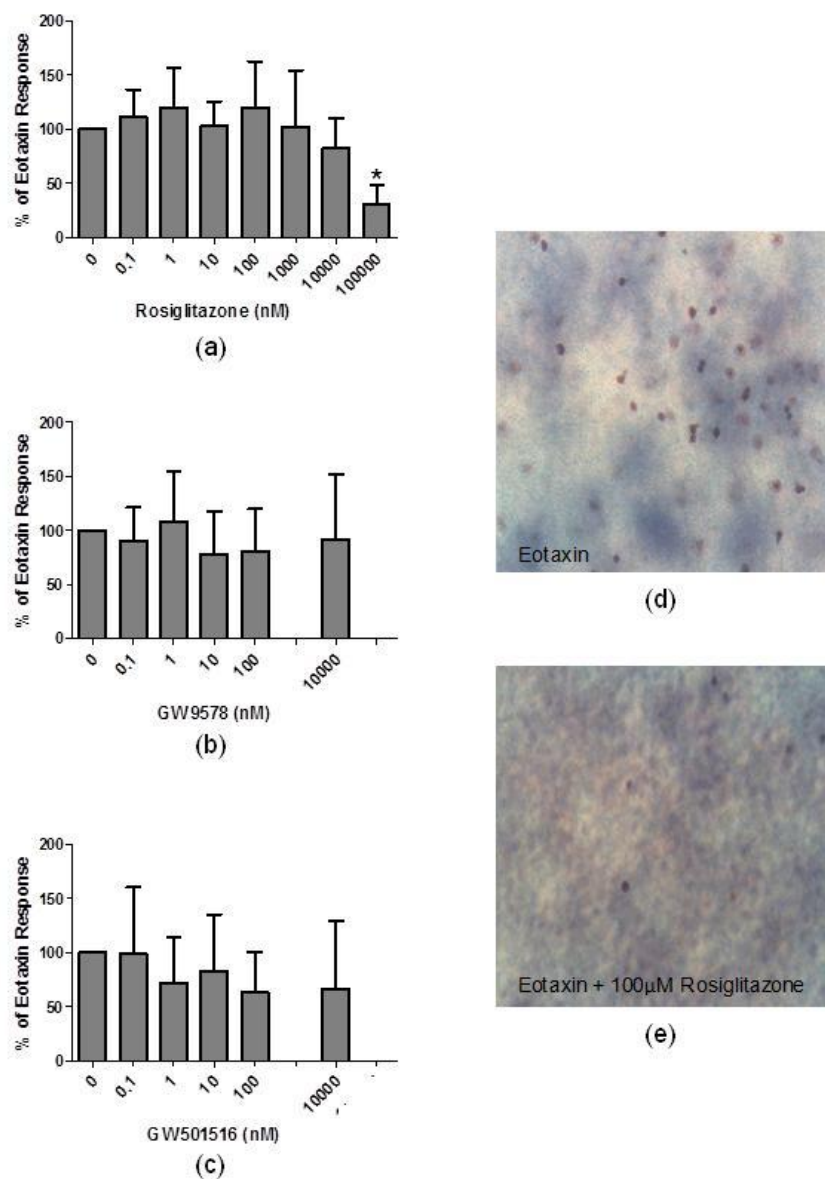


Figure 3.3. The effect on eotaxin-induced eosinophil migration of agonists to PPAR γ (a; rosiglitazone), PPAR α (b; GW9578), and PPAR β/δ (c; GW501516) at low (0.1 – 100 nM; 9 subjects) and high concentrations (1000 – 100,000 nM; 6 subjects). Representative pictures at 200x magnification of the leading edge of the nitrocellulose filter after incubation with eotaxin (d) and eotaxin with 100 μ M rosiglitazone (e). Data are mean \pm 95% confidence intervals and are expressed as % of the response to eotaxin.

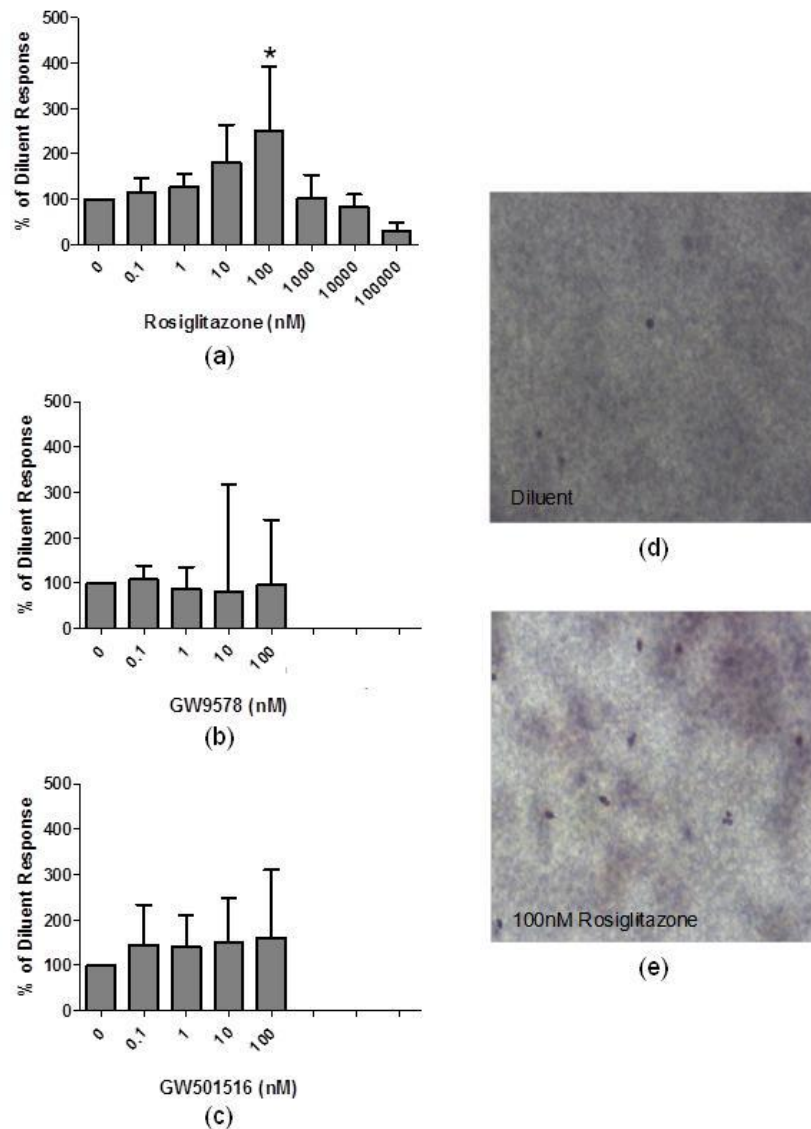


Figure 3.4. The effect on eosinophil chemokinesis of agonists to PPAR γ (a; rosiglitazone), PPAR α (b; GW9578), and PPAR β/δ (c; GW501516) at low (0.1 – 100 nM; 9 subjects) and for PPAR γ (a; rosiglitazone) at high concentrations (1000 – 100,000 nM; 6 subjects). Representative pictures at 200x magnification of the leading edge of the nitrocellulose filter after incubation with diluent (d) and 100 nM rosiglitazone (e). Data are mean \pm 95% confidence intervals and are expressed as % of the response to diluent.

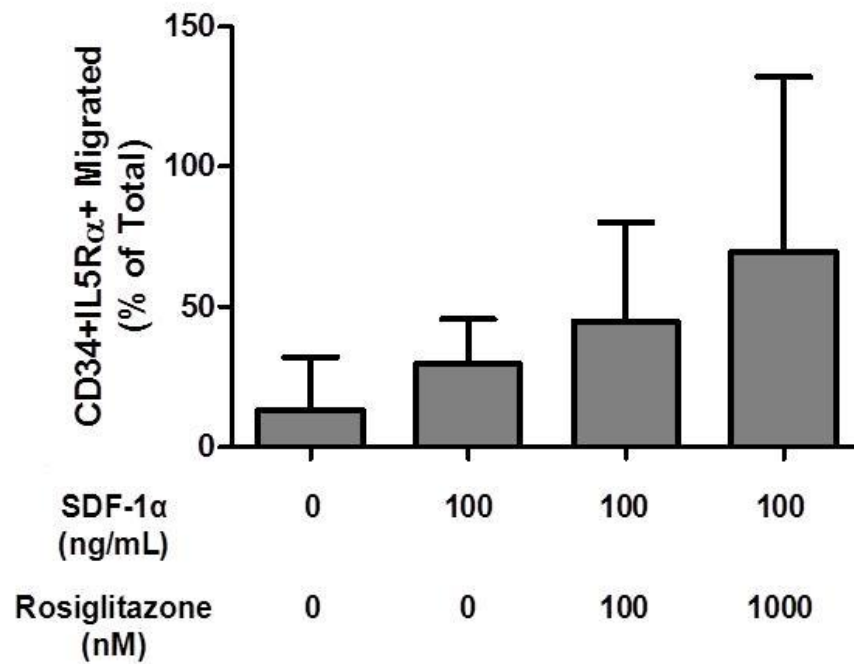


Figure 3.5. The effect of the PPAR γ agonist rosiglitazone on SDF-1 α -induced migration of CD34+ IL-5R α + cells isolated from cord blood (6 subjects). Data are mean \pm 95% confidence intervals and are expressed as a % of the total CD34+IL-5R α + cells.

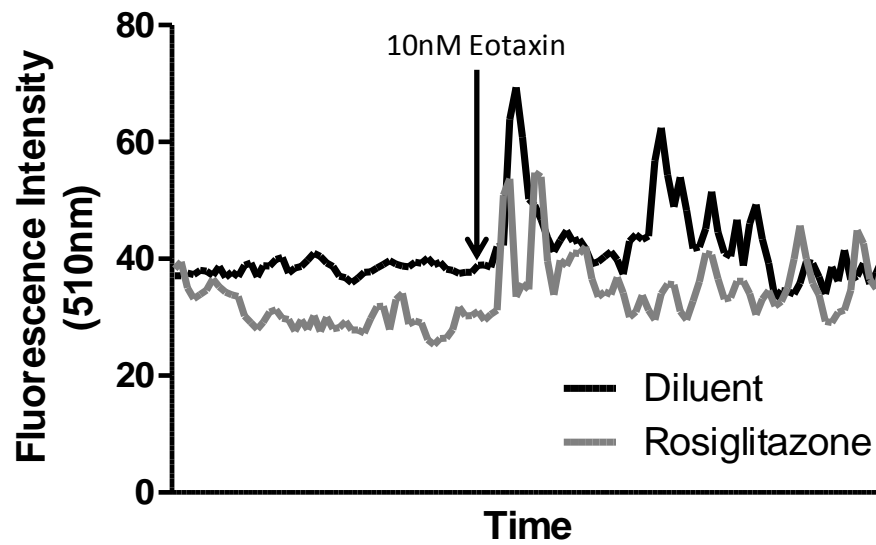


Figure 3.6. The effect of 100 nM rosiglitazone treatment on the eotaxin-induced increase in $[Ca^{2+}]_i$, measured by the frequency of calcium oscillations and maximum fluorescence intensity (representative tracing from 3 experiments). Arrow indicates the addition of 10nM eotaxin.

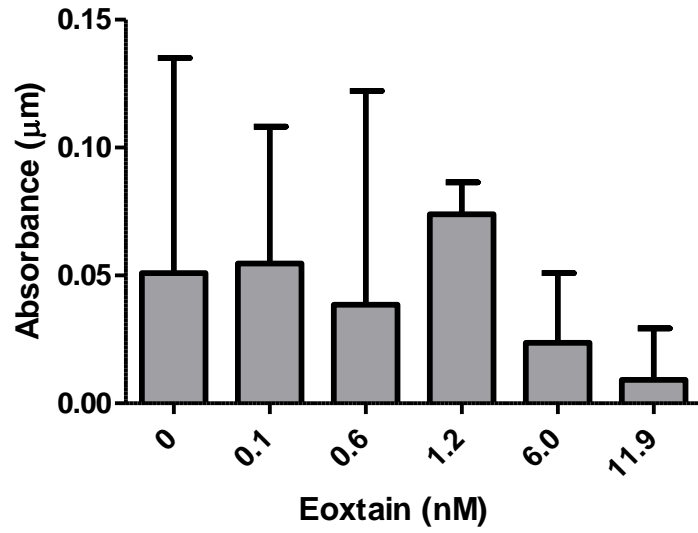


Figure 3.7: Eotaxin-induced eosinophil chemotaxis using MTS dye assay. No changes in eosinophil migration were observed. Data are mean \pm 95% confidence intervals.

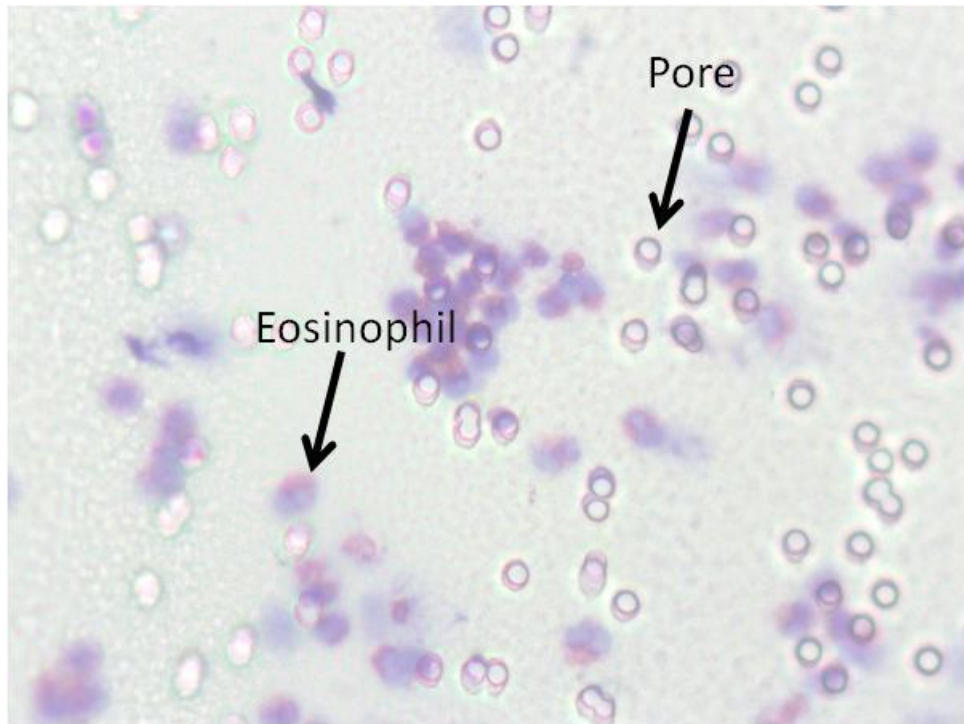


Figure 3.8: Light microscope image of a polycarbonate filter after the eotaxin-induced chemotaxis assay that has been stained with Diff-Quik®. Image is at 400x magnification.

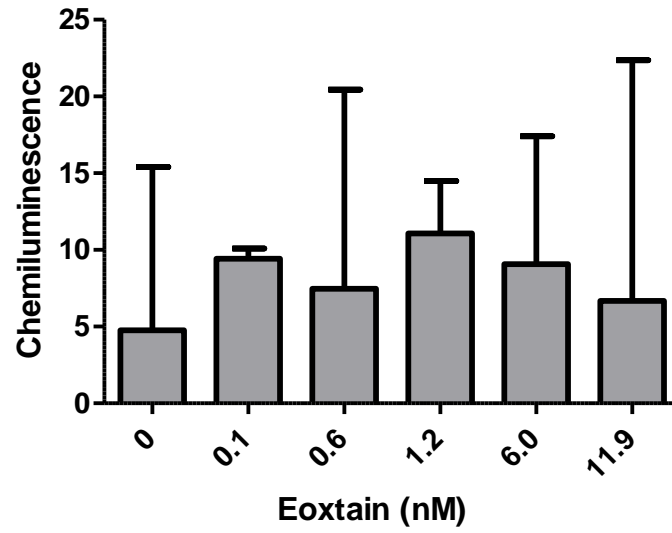


Figure 3.9: Eotaxin-induced eosinophil chemotaxis using Cell Glo chemluminescence. No changes in eosinophil migration were observed. Data are mean \pm 95% confidence intervals.

3.7 References

- Avrameas, S. & Uriel, J. (1966). [Method of antigen and antibody labelling with enzymes and its immunodiffusion application]. *C.R.Acad.Sci.Hebd.Seances Acad.Sci.D.*, 262, 2543-2545.
- Benayoun, L., Letuve, S., Druilhe, A., Boczkowski, J., Dombret, M. C., Mechighel, P. *et al.* (2001). Regulation of peroxisome proliferator-activated receptor gamma expression in human asthmatic airways: relationship with proliferation, apoptosis, and airway remodeling. *Am.J.Respir.Crit Care Med.*, 164, 1487-1494.
- Bensinger, S. J. & Tontonoz, P. (2008). Integration of metabolism and inflammation by lipid-activated nuclear receptors. *Nature*, 454, 470-477.
- Bernard, A. & Boumsell, L. (1984). [Human leukocyte differentiation antigens]. *Presse Med*, 13, 2311-2316.
- Bleul, C. C., Fuhlbrigge, R. C., Casasnovas, J. M., Aiuti, A., & Springer, T. A. (1996). A highly efficacious lymphocyte chemoattractant, stromal cell-derived factor 1 (SDF-1). *J.Exp.Med*, 184, 1101-1109.
- Boehme, S. A., Sullivan, S. K., Crowe, P. D., Santos, M., Conlon, P. J., Sriramarao, P. *et al.* (1999). Activation of mitogen-activated protein kinase regulates eotaxin-induced eosinophil migration. *J.Immunol.*, 163, 1611-1618.
- Boyden, S. (1962). The chemotactic effect of mixtures of antibody and antigen on polymorphonuclear leucocytes. *J.Exp.Med.*, 115, 453-466.

- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal.Biochem.*, 72, 248-254.
- Brown, P. J., Winegar, D. A., Plunket, K. D., Moore, L. B., Lewis, M. C., Wilson, J. G. *et al.* (1999). A ureido-thioisobutyric acid (GW9578) is a subtype-selective PPARalpha agonist with potent lipid-lowering activity. *J.Med.Chem.*, 42, 3785-3788.
- Camp, H. S., Li, O., Wise, S. C., Hong, Y. H., Frankowski, C. L., Shen, X. *et al.* (2000). Differential activation of peroxisome proliferator-activated receptor-gamma by troglitazone and rosiglitazone. *Diabetes*, 49, 539-547.
- Civin, C. I., Strauss, L. C., Brovall, C., Fackler, M. J., Schwartz, J. F., & Shaper, J. H. (1984). Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells. *J.Immunol.*, 133, 157-165.
- Cohen, L. B., Salzberg, B. M., Davila, H. V., Ross, W. N., Landowne, D., Waggoner, A. S. *et al.* (1974). Changes in axon fluorescence during activity: molecular probes of membrane potential. *J.Membr.Biol.*, 19, 1-36.
- Crouch, S. P., Kozlowski, R., Slater, K. J., & Fletcher, J. (1993). The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity. *J.Immunol.Methods*, 160, 81-88.
- Dorman, S. C., Babirad, I., Post, J., Watson, R. M., Foley, R., Jones, G. L. *et al.* (2005). Progenitor egress from the bone marrow after allergen challenge: role of stromal cell-derived factor 1alpha and eotaxin. *J.Allergy Clin.Immunol.*, 115, 501-507.

- Dunn, G. A. (1983). Characterising a kinesis response: time averaged measures of cell speed and directional persistence. *Agents Actions Suppl*, 12, 14-33.
- Ehrlich, P. & Shiga, K. (1904). Farbtherapeutische Versuche bei Trypanosomenerkrankung. *Berliner klinische Wochenschrift*, 41, 329-332.
- Engelmann, T. (1881). Neue Methoden zur Untersuchung der Sauerstoffausscheidung pflanzlicher und tierischer Organismen. *Pfluger's Archiv Gesamte Physiol*, 25, 285-292.
- Engvall, E. & Perlmann, P. (1971). Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. *Immunochemistry.*, 8, 871-874.
- Felarca, A. B. & Lowell, F. C. (1967). The total eosinophil count in a nonatopic population. *J.Allergy*, 40, 16-20.
- Fina, L., Molgaard, H. V., Robertson, D., Bradley, N. J., Monaghan, P., Delia, D. *et al.* (1990). Expression of the CD34 gene in vascular endothelial cells. *Blood*, 75, 2417-2426.
- Friedl, P. & Weigelin, B. (2008). Interstitial leukocyte migration and immune function. *Nat.Immunol.*, 9, 960-969.
- Friend, C., Scher, W., Holland, J. G., & Sato, T. (1971). Hemoglobin synthesis in murine virus-induced leukemic cells *in vitro*: stimulation of erythroid differentiation by dimethyl sulfoxide. *Proc.Natl.Acad.Sci.U.S.A*, 68, 378-382.
- Henson, P. M. (1969). The adherence of leucocytes and platelets induced by fixed IgG antibody or complement. *Immunology*, 16, 107-121.
- Hogan, S. P., Rosenberg, H. F., Moqbel, R., Phipps, S., Foster, P. S., Lacy, P. *et al.* (2008). Eosinophils: biological properties and role in health and disease. *Clin.Exp.Allergy*, 38, 709-750.

- Ide, M., Weiler, D., Kita, H., & Gleich, G. J. (1994). Ammonium chloride exposure inhibits cytokine-mediated eosinophil survival. *J.Immunol.Methods*, 168, 187-196.
- Imaoka, H., Punia, N., Irshad, A., Ying, S., Corrigan, C. J., Howie, K. *et al.* (2011). Lung homing of endothelial progenitor cells in humans with asthma after allergen challenge. *Am.J.Respir.Crit Care Med*, 184, 771-778.
- Issemann, I. & Green, S. (1990). Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. *Nature*, 347, 645-650.
- Janssen, L. J., Farkas, L., Rahman, T., & Kolb, M. R. (2009). ATP stimulates Ca(2+)-waves and gene expression in cultured human pulmonary fibroblasts. *Int.J.Biochem.Cell Biol.*, 41, 2477-2484.
- Jose, P. J., Griffiths-Johnson, D. A., Collins, P. D., Walsh, D. T., Moqbel, R., Totty, N. F. *et al.* (1994). Eotaxin: a potent eosinophil chemoattractant cytokine detected in a guinea pig model of allergic airways inflammation. *J.Exp.Med.*, 179, 881-887.
- Josephy, P. D., Eling, T., & Mason, R. P. (1982). The horseradish peroxidase-catalyzed oxidation of 3,5,3',5'-tetramethylbenzidine. Free radical and charge-transfer complex intermediates. *J.Biol.Chem.*, 257, 3669-3675.
- Jungi, T. W. (1978). Evaluation of various filter membranes for Boyden-type leukocyte migration studies. *J.Immunol.Methods*, 21, 373-382.
- Kay, A. B. (1970). Studies on eosinophil leucocyte migration. II. Factors specifically chemotactic for eosinophils and neutrophils generated from guinea-pig serum by antigen-antibody complexes. *Clin.Exp.Immunol.*, 7, 723-737.

- Kampen, G. T., Stafford, S., Adachi, T., Jinqun, T., Quan, S., Grant, J. A. *et al.* (2000). Eotaxin induces degranulation and chemotaxis of eosinophils through the activation of ERK2 and p38 mitogen-activated protein kinases. *Blood*, 95, 1911-1917.
- Keller, H. U., Borel, J. F., Wilkinson, P. C., Hess, M. W., & Cottier, H. (1972). Re-assessment of Boyden's technique for measuring chemotaxis. *J.Immunol.Methods*, 1, 165-168.
- Keller, H. U. & Sorkin, E. (1969). Studies on Chemotaxis. XIII. Differences in the Chemotactic Response of Neutrophil and Eosinophil Polymorphonuclear Leucocytes. *Int.Arch.Allergy*, 35, 279-287.
- Kim, S. R., Lee, K. S., Park, H. S., Park, S. J., Min, K. H., Jin, S. M. *et al.* (2005). Involvement of IL-10 in peroxisome proliferator-activated receptor gamma-mediated anti-inflammatory response in asthma. *Mol.Pharmacol.*, 68, 1568-1575.
- Kirshner, J. J. & Goldberg, J. (1980). Types of colonies formed by normal human bone marrow, peripheral blood and umbilical cord blood CFUc. *Exp.Hematol.*, 8, 1202-1207.
- Kliwer, S. A., Forman, B. M., Blumberg, B., Ong, E. S., Borgmeyer, U., Mangelsdorf, D. J. *et al.* (1994). Differential expression and activation of a family of murine peroxisome proliferator-activated receptors. *Proc.Natl.Acad.Sci.U.S.A*, 91, 7355-7359.
- Kobayashi, M., Thomassen, M. J., Rambasek, T., Bonfield, T. L., Raychaudhuri, B., Malur, A. *et al.* (2005a). An inverse relationship between peroxisome

- proliferator-activated receptor gamma and allergic airway inflammation in an allergen challenge model. *Ann.Allergy Asthma Immunol.*, 95, 468-473.
- Kobayashi, Y., Ueki, S., Mahemuti, G., Chiba, T., Oyamada, H., Saito, N. *et al.* (2005b). Physiological levels of 15-deoxy-Delta12,14-prostaglandin J2 prime eotaxin-induced chemotaxis on human eosinophils through peroxisome proliferator-activated receptor-gamma ligation. *J.Immunol.*, 175, 5744-5750.
- Leber, T. (1888). Uber die Entstehung der Entzundung und die Wirkung der entzundungserregenden Schadlichkeiten. *Fortschritte der Medizin*, 6, 460-464.
- Lehmann, J. M., Moore, L. B., Smith-Oliver, T. A., Wilkison, W. O., Willson, T. M., & Kliewer, S. A. (1995). An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor gamma (PPAR gamma). *J.Biol.Chem.*, 270, 12953-12956.
- Levis, W. R. & Robbins, J. H. (1972). Methods for obtaining purified lymphocytes, glass-adherent mononuclear cells, and a population containing both cell types from human peripheral blood. *Blood*, 40, 77-89.
- LoBuglio, A. F., Cotran, R. S., & Jandl, J. H. (1967). Red cells coated with immunoglobulin G: binding and sphering by mononuclear cells in man. *Science*, 158, 1582-1585.
- Loges, S., Fehse, B., Brockmann, M. A., Lamszus, K., Butzal, M., Guckenbiehl, M. *et al.* (2004). Identification of the adult human hemangioblast. *Stem Cells Dev.*, 13, 229-242.
- Messner, R. P. & Jelinek, J. (1970). Receptors for human gamma G globulin on human neutrophils. *J.Clin.Invest*, 49, 2165-2171.

Michalik, L., Auwerx, J., Berger, J. P., Chatterjee, V. K., Glass, C. K., Gonzalez, F. J. *et al.* (2006). International Union of Pharmacology. LXI. Peroxisome proliferator-activated receptors. *Pharmacol.Rev.*, 58, 726-741.

Minsky, M. (1957). [Patent No. US3013467]. U.S. Patent and Trademark Office.

Ref Type: Patent

Minta, A., Kao, J. P., & Tsien, R. Y. (1989). Fluorescent indicators for cytosolic calcium based on rhodamine and fluorescein chromophores. *J.Biol.Chem.*, 264, 8171-8178.

Moore, G. E., Gerner, R. E., & Franklin, H. A. (1967). Culture of normal human leukocytes. *JAMA*, 199, 519-524.

Okada, S., Kita, H., George, T. J., Gleich, G. J., & Leiferman, K. M. (1997). Transmigration of eosinophils through basement membrane components *in vitro*: synergistic effects of platelet-activating factor and eosinophil-active cytokines. *Am.J.Respir.Cell Mol.Biol.*, 16, 455-463.

Oliver, W. R., Jr., Shenk, J. L., Snaith, M. R., Russell, C. S., Plunket, K. D., Bodkin, N. L. *et al.* (2001). A selective peroxisome proliferator-activated receptor delta agonist promotes reverse cholesterol transport. *Proc.Natl.Acad.Sci.U.S.A*, 98, 5306-5311.

Omary, M. B., Trowbridge, I. S., & Battifora, H. A. (1980). Human homologue of murine T200 glycoprotein. *J.Exp.Med*, 152, 842-852.

Pandit, T. S., Sikora, L., Muralidhar, G., Rao, S. P., & Sriramarao, P. (2006). Sustained exposure to nicotine leads to extramedullary hematopoiesis in the spleen. *Stem Cells*, 24, 2373-2381.

- Patel, H. J., Belvisi, M. G., Bishop-Bailey, D., Yacoub, M. H., & Mitchell, J. A. (2003). Activation of peroxisome proliferator-activated receptors in human airway smooth muscle cells has a superior anti-inflammatory profile to corticosteroids: relevance for chronic obstructive pulmonary disease therapy. *J.Immunol.*, 170, 2663-2669.
- Peichev, M., Naiyer, A. J., Pereira, D., Zhu, Z., Lane, W. J., Williams, M. *et al.* (2000). Expression of VEGFR-2 and AC133 by circulating human CD34(+) cells identifies a population of functional endothelial precursors. *Blood*, 95, 952-958.
- Perlmann, H., Perlmann, P., Pape, G. R., & Hallden, G. (1976). Purification, fractionation and assay of antibody-dependent lymphocytic effector cells (K cells) in human blood. *Scand.J.Immunol.*, Suppl 5, 57-68.
- Porter, P., Porter, M. C., & Shanberge, J. N. (1967). Heparin cofactor and plasma antithrombin in relation to the mechanism of inactivation of thrombin by heparin. *Clin.Chim.Acta*, 17, 189-200.
- Prindull, G., Prindull, B., & Meulen, N. (1978). Haematopoietic stem cells (CFUc) in human cord blood. *Acta Paediatr.Scand.*, 67, 413-416.
- Ricote, M. & Glass, C. K. (2007). PPARs and molecular mechanisms of transrepression. *Biochim.Biophys.Acta*, 1771, 926-935.
- Schmolka, I. R. (1973). [Patent No. US3740421]. U.S. Patent and Trademark Office.
Ref Type: Patent
- Sehmi, R., Wardlaw, A. J., Cromwell, O., Kurihara, K., Waltmann, P., & Kay, A. B. (1992). Interleukin-5 selectively enhances the chemotactic response of

eosinophils obtained from normal but not eosinophilic subjects. *Blood*, 79, 2952-2959.

Sehmi, R., Wood, L. J., Watson, R., Foley, R., Hamid, Q., O'Byrne, P. M. *et al.* (1997). Allergen-induced increases in IL-5 receptor alpha-subunit expression on bone marrow-derived CD34+ cells from asthmatic subjects. A novel marker of progenitor cell commitment towards eosinophilic differentiation. *J.Clin.Invest*, 100, 2466-2475.

Spears, M., McSharry, C., & Thomson, N. C. (2006). Peroxisome proliferator-activated receptor-gamma agonists as potential anti-inflammatory agents in asthma and chronic obstructive pulmonary disease. *Clin.Exp.Allergy*, 36, 1494-1504.

Tashiro, K., Tada, H., Heilker, R., Shirozu, M., Nakano, T., & Honjo, T. (1993). Signal sequence trap: a cloning strategy for secreted proteins and type I membrane proteins. *Science*, 261, 600-603.

Tavernier, J., Devos, R., Cornelis, S., Tuypens, T., Van der, H. J., Fiers, W. *et al.* (1991). A human high affinity interleukin-5 receptor (IL5R) is composed of an IL5-specific alpha chain and a beta chain shared with the receptor for GM-CSF. *Cell*, 66, 1175-1184.

Trifilieff, A., Bench, A., Hanley, M., Bayley, D., Campbell, E., & Whittaker, P. (2003). PPAR-alpha and -gamma but not -delta agonists inhibit airway inflammation in a murine model of asthma: *in vitro* evidence for an NF-kappaB-independent effect. *Br.J.Pharmacol.*, 139, 163-171.

- Ueki, S., Adachi, T., Bourdeaux, J., Oyamada, H., Yamada, Y., Hamada, K. *et al.* (2003). Expression of PPARgamma in eosinophils and its functional role in survival and chemotaxis. *Immunol.Lett.*, 86, 183-189.
- Van Weemen, B. K. & Schuurs, A. H. (1971). Immunoassay using antigen-enzyme conjugates. *FEBS Lett.*, 15, 232-236.
- Walker, A. B., Naderali, E. K., Chattington, P. D., Buckingham, R. E., & Williams, G. (1998). Differential vasoactive effects of the insulin sensitizers rosiglitazone (BRL 49653) and troglitazone on human small arteries *in vitro*. *Diabetes*, 47, 810-814.
- Weil, G. J. & Chused, T. M. (1981). Eosinophil autofluorescence and its use in isolation and analysis of human eosinophils using flow microfluorometry. *Blood*, 57, 1099-1104.
- Werner, C., Kamani, C. H., Gensch, C., Bohm, M., & Laufs, U. (2007). The peroxisome proliferator-activated receptor-gamma agonist pioglitazone increases number and function of endothelial progenitor cells in patients with coronary artery disease and normal glucose tolerance. *Diabetes*, 56, 2609-2615.
- Wide, L. & Porath, J. (1966). Radioimmunoassay of proteins with the use of Sephadex-coupled antibodies. *Biochim.Biophys.Acta*, 130, 257-260.
- Wilkinson, P. C. (1998). Assays of leukocyte locomotion and chemotaxis. *J.Immunol.Methods*, 216, 139-153.
- Willson, T. M., Brown, P. J., Sternbach, D. D., & Henke, B. R. (2000). The PPARs: from orphan receptors to drug discovery. *J.Med.Chem.*, 43, 527-550.

Woerly, G., Honda, K., Loyens, M., Papin, J. P., Auwerx, J., Staels, B. *et al.* (2003).

Peroxisome proliferator-activated receptors alpha and gamma down-regulate allergic inflammation and eosinophil activation. *J.Exp.Med.*, 198, 411-421.

Young, P. W., Buckle, D. R., Cantello, B. C., Chapman, H., Clapham, J. C., Coyle, P.

J. et al. (1998). Identification of high-affinity binding sites for the insulin sensitizer rosiglitazone (BRL-49653) in rodent and human adipocytes using a radioiodinated ligand for peroxisomal proliferator-activated receptor gamma. *J.Pharmacol.Exp.Ther.*, 284, 751-759.

Zigmond, S. H. & Hirsch, J. G. (1973). Leukocyte locomotion and chemotaxis. New

methods for evaluation, and demonstration of a cell-derived chemotactic factor. *J.Exp.Med.*, 137, 387-410.

Chapter 4: Effects of PPAR Agonists on Eosinophil Differentiation

4.1 Introduction

Asthma therapy is focused on reducing the associated symptoms and frequency of exacerbation through modification of the mechanisms of lung inflammation, or bronchoconstriction, or both. In 1985, Denburg and colleagues discovered an association between atopy and greater numbers of circulating eosinophil/basophil colony-forming units (Eo/B-CFU) which could be grown in culture (Denburg *et al.*, 1985). They also observed increased numbers of circulating CD34+ progenitor cells (Sehmi *et al.*, 1996). Along with this is discovery in atopic subjects, further associations were found in allergic asthmatics. An increase in Eo/B-CFU was observed in the peripheral blood of asthmatics during an exacerbation and the numbers decreased upon recovery from symptoms (Gibson *et al.*, 1990). In the allergen challenge model, a significant increase in circulating Eo/B-CFUs and CD34+ cells can be observed 24-hours after whole lung allergen challenge in atopic asthmatic subjects who develop early and late phase bronchoconstriction (Gibson *et al.*, 1991; Sehmi *et al.*, 1997). Examination of this progenitor cell population allowed Denburg and colleagues to determine that allergen exposure upregulates the expression of IL-5 receptors on CD34+ cells in the bone marrow of atopic asthmatics with an early and late phase bronchoconstriction (Denburg *et al.*, 1999). Since IL-5 is the principal regulatory cytokine for the differentiation of eosinophils (Chihara *et al.*, 1990) and eosinophils represent a major effector cell in asthma (Hogan *et al.*, 2008), progenitor cells may be an important target for drug development.

Recent studies have shown that treatment with mepolizumab, anti-IL-5 therapy, was ineffective at completely attenuating eosinophil numbers in bronchial

biopsies from asthmatic subjects, which is the site of disease pathology (Flood-Page *et al.*, 2003). However, treatment improved asthma control and significantly reduced the prednisone dose required by asthmatic patients with persistent eosinophilia (Nair *et al.*, 2009). Researchers continue to search for a more effective strategy to completely ablate eosinophils at the site of disease, including investigational drugs that target eosinophil progenitor cells (Kolbeck *et al.*, 2008; Imaoka *et al.*, 2010). This may lead to a more effective therapy for controlling asthma.

Recent reviews have suggested that PPARs are novel anti-inflammatory targets (Belvisi *et al.*, 2006; Spears *et al.*, 2006) and PPAR α as well as PPAR γ have been suggested to play a role in chronic inflammatory disease (Kota *et al.*, 2005). The anti-inflammatory capability of PPAR γ agonists have been observed both *in vitro* studies and in allergic animal models (Spears *et al.*, 2006), including inhibitory effects on differentiation of several cell types, including erythroid cells (Bernardo *et al.*, 2009; Nagasawa *et al.*, 2005; Rosen & Spiegelman, 2001b). Currently no studies have addressed the effects of PPARs on IL-5-induced eosinophil/basophil cell differentiation.

4.2 Materials and Methods

Subjects

Forty atopic donors participating in the study were male and female, non-smoking, between 18-65 years old. Subjects were recruited using research ethics board approved advertisement posted on the McMaster University campus. Smoking status, which can influence the migration of peripheral blood leukocytes (Pandit *et al.*, 2006), was confirmed as non-smoking during the initial interview. A subject's allergic

status was determined by skin prick testing. Only subjects with a positive skin prick test were selected. Patients with progenitor cells that range from 20 to 1040 cells per million peripheral blood non-adherent mononuclear cells are considered in the normal range (Sehmi *et al.*, 1996). Selecting subjects with allergies increased the chance of collecting efficient numbers of progenitor cells for *in vitro* analysis because patients with allergic disease can have a higher progenitor cell count (Sehmi *et al.*, 1996). Prior to the laboratory visit, subjects could not use steroidal or non-steroidal anti-inflammatory medications and antihistamines were withheld for 48 hours prior to the blood draw. All of these medications would have affected the total number and function of the isolated progenitor cells. Peripheral blood was used because it was frequently available compared to cord blood samples and easier to collect compared to bone marrow. The study was approved by the FHS/HHS Research Ethics Board, and subjects gave informed consent to participate.

Progenitor Cell Purification

One hundred millilitres of peripheral blood was collected from each subject into sodium heparin vacutainers for *in vitro* experiments. Peripheral blood was diluted with an equal volume of McCoy's 5A (Invitrogen Canada Inc, Burlington, ON, Canada) and mononuclear cells were purified using an accuprep™ density gradient (Accurate Chemical & Scientific Corporation, Westbury, NY, USA) followed by a MACS column CD34+ progenitor cells positive selection (Miltenyi Biotec, Auburn, CA, USA).

Mononuclear cells were then resuspended in McCoy's 3+ (McCoy's 5A with 10% fetal bovine serum, 1% penicillin/streptomycin (pen/strep) and 1% 2-ME and

incubated for 2-hours in 5% CO₂ at 37°C and high humidity to remove monocytes. CD34⁺ progenitor cells were isolated using a MACS column CD34⁺ positive selection (Miltenyi Biotec, Auburn, CA, USA). The NAMNC and CD34⁺ progenitor cell preparations were >90% viable after isolation. In this study CD34 was used as the marker for the positive selection of progenitor cells.

The non-adherent mononuclear cells that were used for culture were incubated with the Fc receptor block and CD34 antibody diluted in PBS with 0.5% BSA and 6 mM EDTA for 30 minutes. The cells were then added to a MS column that is placed in a magnetic field.

Progenitor Cell Cultures

CD34-containing NAMNCs or purified CD34⁺ progenitor cells were resuspended in Iscove's 2+ (Iscove's modified Dulbecco's medium with 1% penicillin/streptomycin and 1% 2-ME) and placed in Methocult® cultures (Stemcell technologies, Vancouver, BC, Canada) in the presence of 16% fetal bovine serum and 10ng/mL interleukin-5 (IL-5) or 10ng/mL IL-5 and 25ng/mL IL-3. IMDM 2+ is a media that is ideal for rapid cell proliferation and high density culture. It provides various amino acids, vitamins and buffers, plus penicillin/streptomycin were added for their antibiotic properties, and 2-ME was added for preventing protein oxidation. IMDM is medium that based on DMEM with modifications. It contains selenium, additional amino acids and vitamins, sodium pyruvate, HEPES buffer, and potassium nitrate instead of ferric nitrate (Iscove & Melchers, 1978). DMEM was developed from BME, which was originally developed by Dr. Eagle to maintain HeLa cells in culture (Eagle, 1955a; Eagle, 1955b). DMEM contains 4 times more vitamins and

amino acids compared to BME (Vogt & Dulbecco, 1960). Iscove *et al* demonstrated that IMDM supports erythroid, granulocytic and macrophage colony forming cultures (Iscove *et al.*, 1980; Schreier & Iscove, 1980).

Methylcellulose is a synthetic compound produced by heating cellulose with a caustic solution, such as sodium hydroxide, in the presence of methyl chloride. The earliest mention of methylcellulose cultures, that I could determine, was by Pijper *et al*. In 1947, Pijper *et al* used methylcellulose, known as "Methocel" from the Dow Chemical Company to examine bacterial motility (Pijper, 1947). However, it wasn't until 1951 that Sanford *et al* used methylcellulose to measure the proliferation of tissue cultures (Sanford *et al.*, 1951). The advantages of methylcellulose over solid medium, such as agar, include its non-fluorescence properties, it can be completely removed after culturing is complete and it is non-toxic to the cells (Hotchin, 1955). Since its development, methylcellulose cultures have been the standard method to examine stem cell differentiation, which included characterization of adult human marrow hematopoietic progenitors after CD34 enrichment (Lu *et al.*, 1987). Several companies have developed methylcellulose based products, however, we have used Methocult® H4236 from Stemcell technologies because it contains no erythropoietin, no additional cytokines, and is serum free, which gives us more control in the experimental design to determine the effects of PPAR agonists on IL-5- or IL-5 plus IL-3-induced differentiation. Eosinophils can arise from bone marrow or peripheral blood CD34+ cells (Shalit *et al.*, 1995; Bot *et al.*, 1988), which express the receptors for IL-5 and IL-3 (Sehmi *et al.*, 1997; Kuo *et al.*, 2001; Sato *et al.*, 1993). The IL-5 and IL-3 concentrations utilized in our cultures (Figure 4.1) were based on those previously observed in differentiation of CD34+ cells isolated from the peripheral blood

of adult healthy donors (Shalit *et al.*, 1995). The colony numbers seen in our study are comparable to those observed previously (Shalit *et al.*, 1995).

The NAMNC cells were cultured at a concentration of 0.25×10^6 cells/ml and the CD34 cells were cultured at a concentration of 0.016×10^6 cells/ml. The cells were co-cultured, with diluent (Iscove's 2+ with 0.04% dimethyl sulfoxide), or with 1-1000nM of a PPAR α agonist (GW9578; Cayman Chemical, Ann Arbor, MI, USA), PPAR β/δ agonist (GW501516; Axxora LLC, San Diego, CA, USA) or PPAR γ agonist (rosiglitazone; Cayman Chemical, Ann Arbor, MI, USA), for 2-weeks at 5% CO₂, with high humidity at 37°C in 24 well plates. DMSO is used to reconstitute the PPAR agonists therefore the same concentration of DMSO was used in the negative control condition. Previous studies have shown DMSO concentrations higher than 2% increase erythroid differentiation (Friend *et al.*, 1971), so the dilutions were designed for a minimal DMSO concentration (0.04%). The concentrations of PPAR agonists that were studied correspond to those surrounding the reported EC₅₀s for rosiglitazone, GW9578 and GW501516 (43 nM, 50 nM and 1.1 nM, respectively) (Lehmann *et al.*, 1995; Brown *et al.*, 1999; Oliver, Jr. *et al.*, 2001). Co-incubating the PPAR agonists with the cytokines for two weeks maximizes the CD34+ cells exposure to the treatment, allowing the potential genomic or non-genomic effects on the cells. A two-week incubation period was based on previous studies on eosinophil differentiation (Denburg *et al.*, 1983). The number of Eo/B-CFU was quantified using an inverted light microscope at 40x magnification (Figure 4.2). Quantification methods were based on those previously published (Sehmi *et al.*, 1996). Eosinophils/basophil colonies have a distinct morphology. They are dense, circular colonies, which have cells that are granular in appearance (Shalit *et al.*, 1995). A

colony was defined as a cluster of eosinophils/basophils with a minimum density of 40 cells. The entire well was examined for colony numbers in duplicate plates, and the average colony forming units were determined.

Statistical Analysis

All data are expressed as mean \pm 95% confidence intervals unless otherwise stated. Arithmetic mean is only suitable to present data that have a normal distribution. Arithmetic mean is the sum of the samples divided by the number collected, meaning, outliers and skewed data have a large effect on the result. It is important to represent the human data with 95% confidence intervals because this allows the reader to determine the response range of 95% of the population studied. Unlike mice, which are clones and have standard controls, patients have varying backgrounds and environmental exposures that result in a wide variation in their responses. Again, using 95% confidence intervals allow the reader to visualize this variation.

Statistical analyses were performed using Prism version 5 (GraphPad Software, La Jolla, CA). ANOVA with repeated measures was used to compare PPAR agonist treatments versus diluent at the various doses, with post-hoc Tukey tests for pre-specified comparisons. Repeated measures ANOVA compares the means of three or more matched groups where treatments are repeated. The null hypothesis states that the means are equal for each group. If the overall P value is small, then it is unlikely that the differences observed are due to random sampling and we can reject the null hypothesis. A repeated-measures ANOVA is more powerful than an ordinary

ANOVA because it separates between-subject variability from within-subject variability.

There are three sources of experimental variability, between treatments, between individuals, and random or residual. The total sum-of-squares (SST) is made up of those three components. The SST measures the sum of the difference between each measurement and the overall mean for that group. It then is adjusted based on the number of groups and number of subjects, which is the degrees of freedom, to compute two F ratios. The two F ratios test the null hypothesis that the treatment means are identical and/or the individual means are identical. The latter being a test of the effectiveness of the repeated measures pairing. The repeated measures ANOVA is only valid if the samples have a normal distribution, samples have equal variances and the samples are independent. All assumptions were verified before testing.

Tukey's test is a single-step multiple comparison statistical test that is commonly used in conjunction with an ANOVA to find the means that are significantly different from one another. It is essentially a t-test that corrects for an experiment-wise error rate, meaning that with more comparisons there will, more likely, be a type I error (false rejection of the null hypothesis). The formula uses the difference between the two means divided by the pooled standard error and compares this value, known as q_s , to the studentized range distribution (q). Studentized range distribution is the range of the first sample divided by the standard error of the second sample. If q_s is larger than q , the two means are significantly different. Tukey's test assumes that the observations are independent and that the variation is equal across observations, both of which are true in our data sets. For data not normally distributed the statistics were performed on the log-transformed data. Statistically significant

differences were accepted at $p < 0.05$. The alpha was set to 5%, which means that the chance of making a Type I errors is 5%.

4.3 Results and Discussion

Subjects

Subjects recruited for the study were 32 ± 12 years old with 16 male and 24 female volunteers. All subjects had a positive skin prick test.

The Effects of Rosiglitazone on NAMNC Cultures

NAMNCs grew significantly higher numbers of Eo/B-CFU in response to IL-5 compared to diluent control (2.5 ± 1.5 versus 11.6 ± 5.9 Eo/B-CFU, $p = 0.0001$). Incubation with 10-1000nM rosiglitazone significantly inhibited IL-5 induced Eo/B-CFU formation ($P = 0.0011$; Figure 4.3). The maximum inhibition of Eo/B-CFU was 42.8% at the 10nM dose. Troglitazone, another PPAR γ agonist, was also examined to determine the robustness of the inhibitory response. Troglitazone treatment significantly decreased ($P = 0.0004$) Eo/B-CFU at 2265nM dose (Figure 4.4).

NAMNC cultures contain progenitor cells and lymphocytes (Levis & Robbins, 1972). PPAR γ agonists are known to modulate T cell function, including inhibition of activation and cytokine release (Mueller *et al.*, 2003; Yang *et al.*, 2000). It is important to consider this when interpreting the results of the NAMNC cultures. The PPAR agonist induced inhibition of Eo/B-CFU could be due to a secondary effect on lymphocytes rather than a direct effect on the progenitor cells. To determine how consistent the observations are, the experiment was repeated with isolated CD34+ progenitor cells.

The Effects of PPAR Agonists on CD34+ Progenitor Cultures

CD34+ progenitor cells cultures had a significantly higher number of Eo/B-CFU in response to IL-5 compared to diluent control (0.05 ± 0.15 versus 4.6 ± 6.1 Eo/B-CFU, $p < 0.0001$). Incubation with 100-1000nM rosiglitazone significantly inhibited IL-5 induced Eo/B-CFU formation ($P = 0.0078$; Figure 4.5). The maximum inhibition was 45.9% at the 100nM dose. Conversely, there was no effect of GW9578 ($P = 0.3$) or GW501516 ($P = 0.7$) on IL-5-induced differentiation (Figure 4.5). CD34+ progenitor cell cultures also demonstrated a significant increase in Eo/B-CFU in response to co-stimulation with IL-5 plus IL-3 compared to diluent control (0.05 ± 0.16 versus 12.5 ± 12.5 Eo/B-CFU, $p = 0.0114$). Incubation with 1 and 1000nM rosiglitazone significantly inhibited IL-5/IL-3 induced Eo/B-CFU formation ($P = 0.0144$; Figure 4.6). The maximum inhibition was 50.8% at the 1000nM dose.

In previous reports, PPAR agonists have been shown to have inhibitory effects on differentiation of several cell types, including erythroid cells (Bernardo *et al.*, 2009; Nagasawa *et al.*, 2005; Rosen & Spiegelman, 2001). PPAR agonists also inhibit IL-5-stimulated, but not spontaneous, eosinophil survival in a concentration-dependent manner (Ueki *et al.*, 2003). IL-5 is a cytokine that modulates eosinophil function (Chihara *et al.*, 1990) and prolongs the survival of eosinophils by delaying apoptosis (Simon, 2001). However, no study has examined the effect of PPAR agonists on IL-5-induced eosinophil differentiation.

Eosinophil differentiation is an important mechanism in the allergic response. An increase in Eo/B-CFU was observed in asthmatics during an exacerbation and the

numbers decreased upon recovery from symptoms (Gibson *et al.*, 1990). In the allergen challenge model of allergic asthma, a significant increase in Eo/B-CFUs and CD34+ cells can be observed 24-hours after whole lung allergen challenge in atopic asthmatic subjects who develop early and late phase bronchoconstriction (Gibson *et al.*, 1991; Sehmi *et al.*, 1997). Previous studies have observed that, in asthmatic subjects, sputum CD34+ cells increased at 7 hours after allergen inhalation. This increase is sustained for 24 hours only in subjects that get a late asthmatic response (LAR), which also correlates with increased sputum CD34+IL-5R α + cells, eosinophils, and IL-5. The authors concluded that eosinophil progenitors can migrate to the airways and may differentiate toward an eosinophilic phenotype (Dorman *et al.*, 2004). Therefore progenitor cells may be an important target for drug development.

4.4 Conclusion

This is the first study to show inhibition of eosinophil progenitor cell differentiation *in vitro* in both cultures of non-adherent mononuclear cells as well as isolated CD34+ progenitor cells from the peripheral blood of allergic subjects. The results of this study also provide further evidence that PPAR γ agonist have the potential to inhibit peripheral eosinophilia, which can be observed in asthmatic subjects after insults to the airways, such as allergen challenge (Kim *et al.*, 2005; Trifilieff *et al.*, 2003).

We demonstrated that the PPAR γ agonist, rosiglitazone, at a concentration of 10 nM, significantly inhibited IL-5-induced eosinophil differentiation *in vitro*. This finding supports the data demonstrating decreased eosinophil migration reported after allergen challenge (Kim *et al.*, 2005; Trifilieff *et al.*, 2003). We also demonstrated that

rosiglitazone treatment was effective on NAMNC, isolated CD34+ progenitor cell and was also effective in the presence of other cytokines such as IL-3. This suggests that the inhibitory effects of rosiglitazone are robust.

In summary, the PPAR γ agonist, rosiglitazone, inhibited eosinophil differentiation of NAMNCs isolated from the peripheral blood of allergic subjects. It also inhibited eosinophil differentiation of isolated CD34+ progenitor cells. The inhibition of eosinophil differentiation was specific for the PPAR γ agonist, as there was no effect of PPAR α or PPAR β/δ agonists. In light of our findings that a selective PPAR γ agonist can inhibit eosinophil differentiation at a concentration of 10-1000nM and inhibit eosinophil migration at similar doses, PPAR γ -agonists have a promising therapeutic potential as an anti-inflammatory therapy.

4.5 Appendix

Allergic Asthma, Healthy Controls and Chronic Obstructive Pulmonary Disease (COPD)

Eosinophil differentiation in healthy control subjects, allergic asthmatics and in subjects with chronic obstructive pulmonary disease was examined using the same procedure as the allergic group. This allowed us to examine other inflammatory disease phenotypes and to examine a control group with no active disease. All subjects were recruited through advertisements posted on the McMaster University campus. Healthy controls were confirmed by a complete medical history, negative skin prick test and a MCh PC₂₀ > 32mg/mL. Allergic asthmatics were determined by a complete medical history, positive skin prick test and a MCh PC₂₀ < 16mg/mL. COPD subjects were identified by smoking history and by spirometry. All COPD subjects were 37 years of age or older, with a cigarette smoking history of >10 pack years (Pack Years = [Number of cigarettes/day X years of smoking]/20). All COPD subjects had been diagnosed by a physician. All subjects conformed to the following spirometric criteria: FEV₁ < 80% predicted normal and FEV₁/VC < 70% predicted normal. Subjects with significant diseases other than COPD, or who had a myocardial infarction, cardiac arrhythmia, or respiratory infection in the 6 months prior to enrollment were excluded from this study. Any subjects who required daily oxygen therapy, had active tuberculosis, or who were enrolled in a pulmonary rehabilitation program within 4 weeks of enrollment were excluded. One hundred milliliters of peripheral blood was sampled by venous puncture and eosinophil differentiation was examined using the methycellulose culture procedures as described above. Non-

adherent mononuclear cells were isolated from the peripheral blood of COPD subjects. A significant decrease ($P=0.0117$) in the number of IL-5-stimulated Eo/B-CFU was observed at 279 and 2798nM rosiglitazone (Figure 4.7). No significant effect of rosiglitazone was observed in allergic asthma or healthy subjects, however all 3 subjects studied in each group demonstrated a decrease in the number of Eo/B-CFU at 27nM and 2798nM rosiglitazone respectively. It was important to examine other inflammatory diseases to determine if the effects of PPAR agonists on eosinophil colony formation were consistent.

Bone Marrow Progenitors

Eight milliliters of bone marrow was collected from allergic subjects into a 10mL syringe with 1mL of heparin (1000U/mL). The bone marrow was then passed through two gauges of needle (16 and 18 gauge) to eliminate clumps. The sample was then diluted in 40mL McCoy's 5A and the same procedure as the peripheral blood from the allergic group was followed. The only modification to the procedure was 1ng/mL IL-5 was used to induce IL-5 eosinophil differentiation. This concentration was previously optimized in our lab. Non-adherent mononuclear cells were isolated from the bone marrow of allergic asthmatic subjects and cultured for 2-weeks with the rosiglitazone. No significant effect was observed in the 5 subjects studied (Figure 4.8). The discrepancy between peripheral blood and bone marrow PPAR induced effects may indicate a different role for PPAR in the bone marrow, possibly centered on migration instead of differentiation.

Phosphorylation of ERK, p38 and STAT5

To examine the pathways involved in PPAR agonist inhibition of IL-5-induced eosinophil differentiation ERK1/2, p38 and STAT5 phosphorylation was measured. Progenitor cell isolation methods were the same as the eosinophil differentiation assay. Briefly, 100mL of peripheral blood was collected from each subject into sodium heparin vacutainers for *in vitro* experiments. Peripheral blood was diluted with an equal volume of McCoy's 5A (Invitrogen Canada Inc, Burlington, ON, Canada) and mononuclear cells were purified using an accuprep™ density gradient (Accurate Chemical & Scientific Corporation, Westbury, NY, USA) followed by a MACS column CD34+ progenitor cells positive selection (Miltenyi Biotec, Auburn, CA, USA). The CD34+ progenitor cell preparations were >90% viable after isolation.

Purified CD34+ progenitor cells were resuspended in RPMI complete in the presence of 10ng/mL IL-5. The cells were co-cultured with IL-5 in the presence of diluent (RPMI complete with 0.04% DMSO), or 1000nM of the PPAR γ agonist (rosiglitazone; Cayman Chemical, Ann Arbor, MI, USA) for 15 min in 5% CO₂ with high humidity and at 37°C. Timing was optimized using 10ng/mL IL-5 and varying incubation times (0, 2, 5, 15, 30 and 45min; Figure 4.9). The IL-5 concentration was kept at 10ng/mL because it was the concentration used in the eosinophil differentiation experiments.

After the incubation period, cells were fixed immediately to maintain the phosphorylation state with BD Phosflow fix buffer (Becton-Dickinson Biosciences) that had been warmed to 37°C. Cells were then incubated in a water bath at 37°C for 15min. BD Phosflow Perm buffer III (Becton-Dickinson Biosciences) that had been cooled to -20°C was slowly added to permeabilize the cells, which allows the

intracellular antibodies to enter. Cells were then incubated for 30min on ice. They were then resuspended in mouse block buffer (5% mouse serum, 5% human serum in FACS buffer) and incubated in the fridge for 15min. After the block period, antibodies were added at optimal concentrations. Cells were stained for CD45, CD34 and phosphorylated ERK, p38 and STAT5 using mouse anti-human CD34-Pacific Blue (eBioscience), CD45-FITC and PE-phos-ERK, PE-phos-STAT5 and PE-phos-p38 (Becton-Dickinson Biosciences), as well as the isotype control antibodies. The gating strategy used (Figure 4.10) to determine the CD34 progenitor population was developed by Dr. Sehmi (Sehmi *et al.*, 1997). Three thousand CD45⁺CD34⁺ cells were acquired with an LSR II flow cytometer (Becton Dickinson Instrument Systems; Becton-Dickinson) using the FACSDiva software program (Becton-Dickinson Biosciences).

A significant decrease ($P=0.0369$) in ERK1/2 phosphorylation was observed at 1000nM rosiglitazone (Figure 4.11). There are several mechanisms through which IL-5-induced eosinophil differentiation occurs. The IL-5 receptor is composed of a common β chain (β c), which is shared with IL-3 and granulocyte/macrophage colony-stimulating factor (GM-CSF), and a specific IL-5R α (Guthridge *et al.*, 1998). The α chain binds the ligand itself and forms a high-affinity signal competent receptor only when combined with the β c. IL-5 signaling uses tyrosine phosphorylation of cellular substrates after IL-5 stimulation to transmit the signal to the appropriate target genes. The JAK/STAT pathway is one of the pathways involved in IL-5 signaling (Caldenhoven *et al.*, 1998). JAK are a family of cytokine receptor associated tyrosine kinases that upon ligand binding to the IL-5 receptor subsequently phosphorylate the cytokine receptor (Darnell, Jr., 1997). This leads to activation of STAT, which

dimerise, migrate to the nucleus and regulate gene transcription. IL-5 can also activate alternative tyrosine phosphorylation pathways such as ERK1/2 and p38.

STAT5, ERK1/2 and p38 pathways have all been implicated in eosinophil differentiation (Stafford *et al.*, 2002; Pazdrak *et al.*, 1998; Adachi *et al.*, 2000; Caldenhoven *et al.*, 1998). In the current study the antibodies used to measure phosphorylation of STAT5, p38 and ERK1/2 were directed to phosphorylated tyrosine and threonine (Y694, T180/Y182, and T202/Y204 respectively). When stimulating eosinophil differentiation with IL-5 and IL-3, p38 pathway plays a greater role than ERK1/2 (Adachi *et al.*, 2000). Rosiglitazone was still able to inhibit eosinophil differentiation, suggesting it has a robust effect on differentiation mechanisms. A proper time point to examine STAT5 and p38 was not determined so no conclusion can be made about the effects of rosiglitazone on these signaling mechanisms.

4.6 Tables and Figures

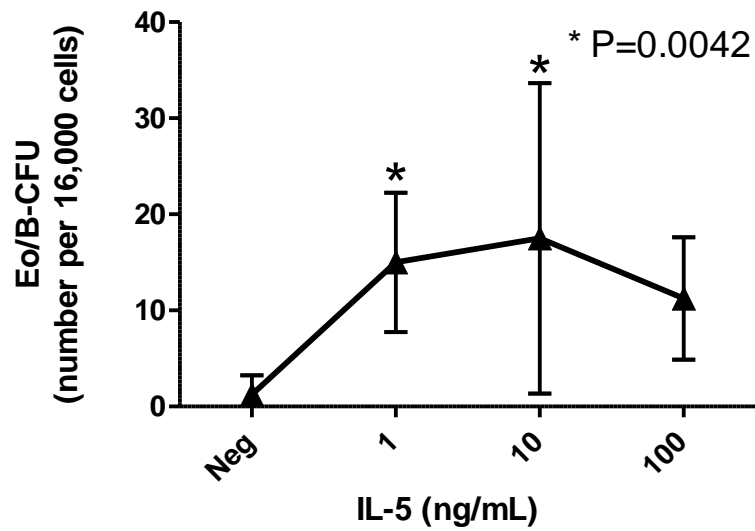


Figure 4.1. Optimization of IL-5-induced eosinophil differentiation of CD34+ cells isolated from peripheral blood of allergic subjects. A significant increase ($P=0.0042$) in Eo/B-CFU number per 0.016×10^6 cells plated was observed at 1 and 10ng/mL IL-5. Data are arithmetic mean \pm 95% confidence intervals.

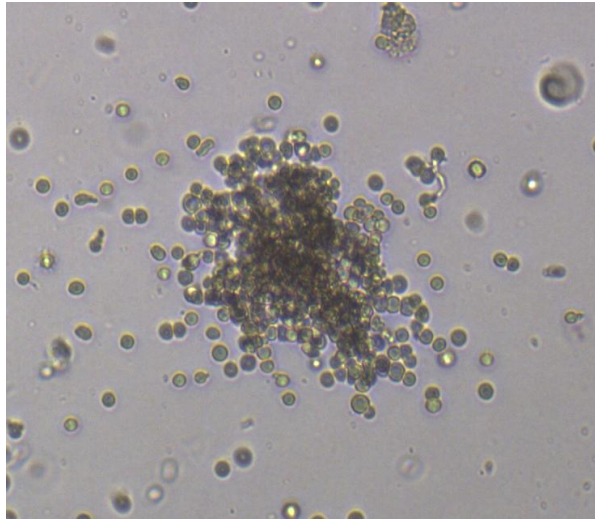


Figure 4.2. An Eo/B-CFU after 2-week incubation with IL-5 in Methocult®. The light microscope images were acquired at 200x magnification.

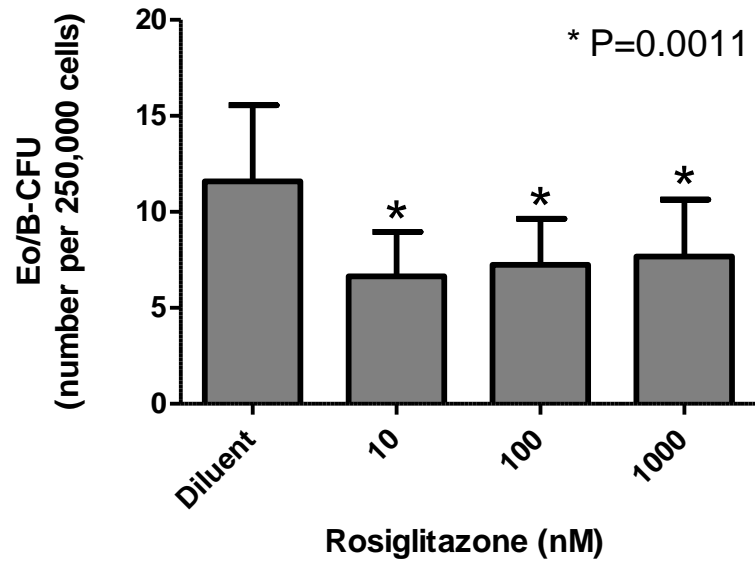


Figure 4.3. The effect of rosiglitazone (PPAR γ agonist) on IL-5-induced eosinophil differentiation of non-adherent mononuclear cells isolated from the peripheral blood of allergic subjects. A significant decrease ($P=0.0011$) in Eo/B-CFU was observed at 10, 100 and 1000nM rosiglitazone. Data are arithmetic mean \pm 95% confidence intervals.

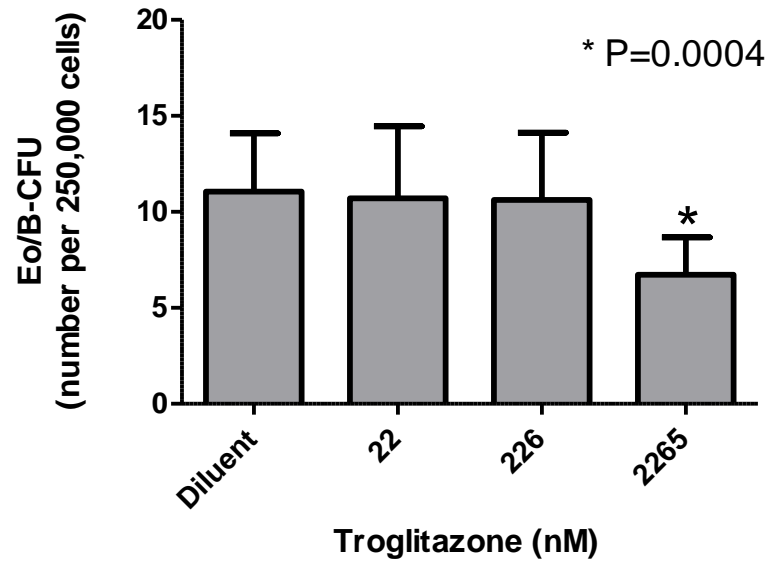


Figure 4.4. The effect of troglitazone (PPAR γ agonist) on IL-5-induced eosinophil differentiation of non-adherent mononuclear cells isolated from the peripheral blood of allergic subjects. A significant decrease ($P=0.0004$) in Eo/B-CFU was observed at 2265nM troglitazone. Data are arithmetic mean \pm 95% confidence intervals.

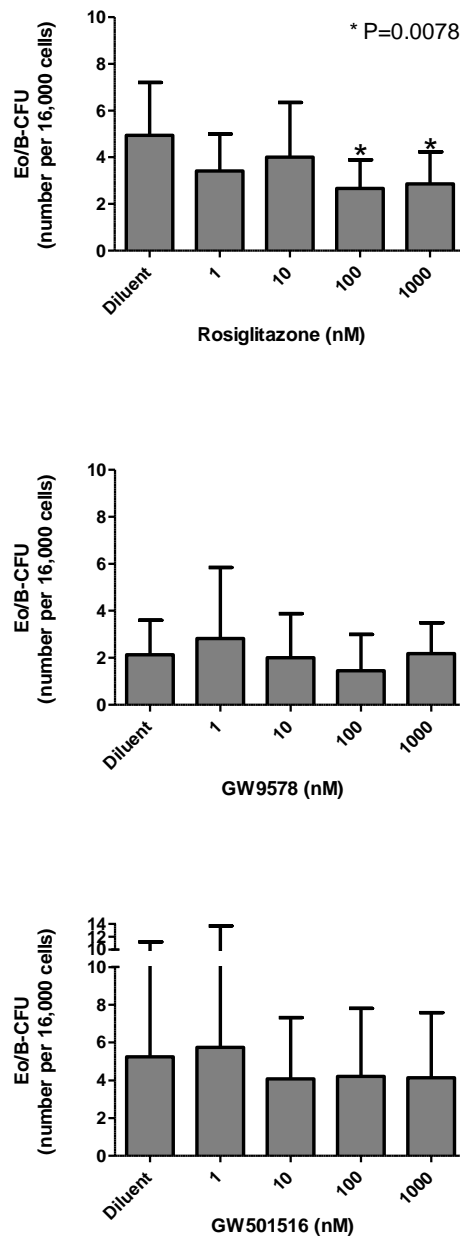


Figure 4.5. The effect of rosiglitazone (PPAR γ agonist; upper panel), GW9578 (PPAR α agonist; middle panel), and GW501516 (PPAR β/δ agonist; lower panel) on IL-5-induced eosinophil differentiation. CD34 $^+$ cells were isolated from peripheral blood of allergic subjects. A significant decrease ($P=0.0114$) in Eo/B-CFU was observed at 1 and 100nM rosiglitazone. Data are arithmetic mean \pm 95% confidence intervals.

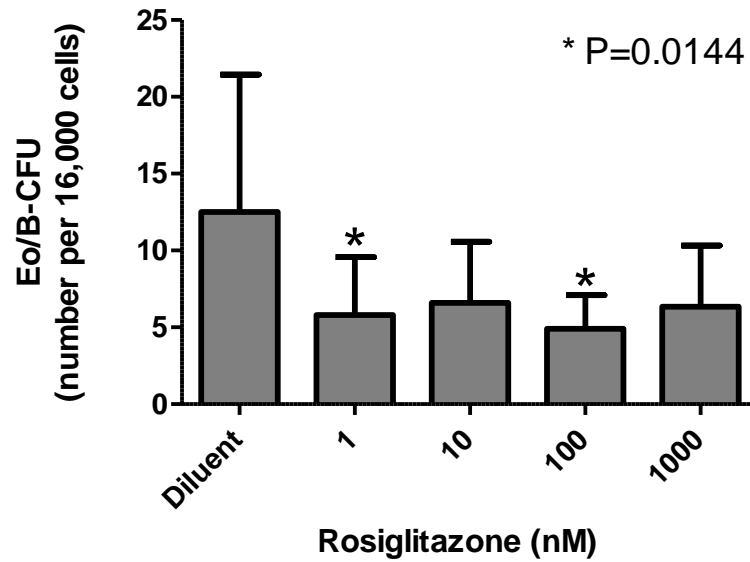


Figure 4.6. The effect of rosiglitazone (PPAR γ agonist) on IL-5/IL-3-induced eosinophil differentiation. CD34⁺ cells were isolated from peripheral blood of allergic subjects. A significant decrease ($P=0.0114$) in Eo/B-CFU was observed at 1 and 100nM rosiglitazone. Data are arithmetic mean \pm 95% confidence intervals.

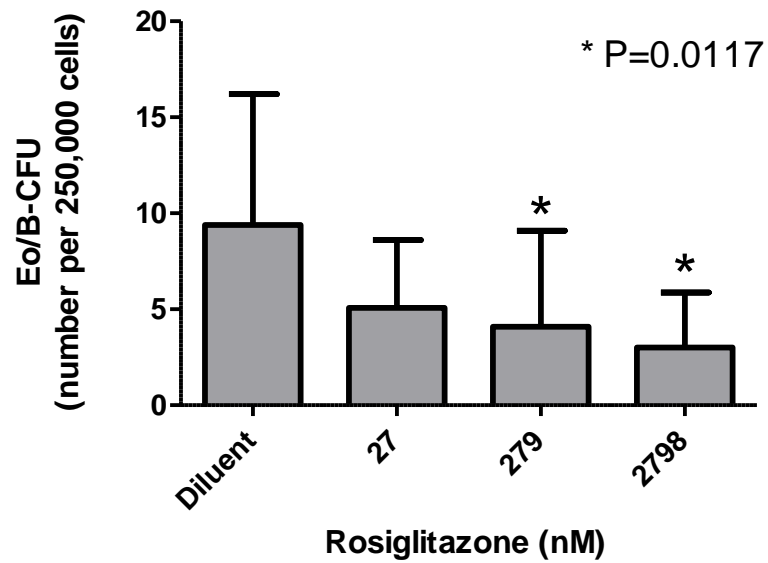


Figure 4.7. The effect of rosiglitazone (PPAR γ agonist) on IL-5-induced eosinophil differentiation. Non-adherent mononuclear cells were isolated from the peripheral blood of COPD subjects. A significant decrease ($P=0.0004$) in Eo/B-CFU was observed at 279 and 2798nM rosiglitazone. Data are arithmetic mean \pm 95% confidence intervals.

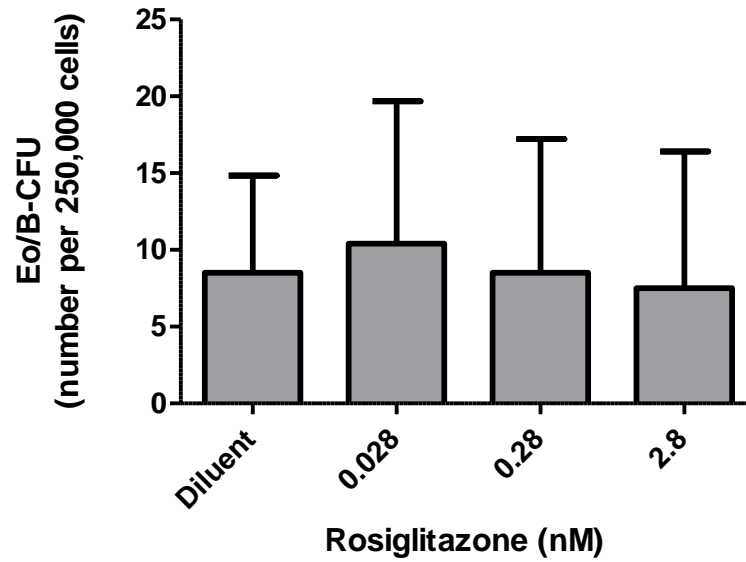


Figure 4.8. The effect of rosiglitazone (PPAR γ agonist) on IL-5-induced eosinophil differentiation of non-adherent mononuclear cells isolated from the bone marrow of allergic asthmatic subjects. No significant effect was observed in the 5 subjects studied. Data are mean \pm 95% confidence intervals.

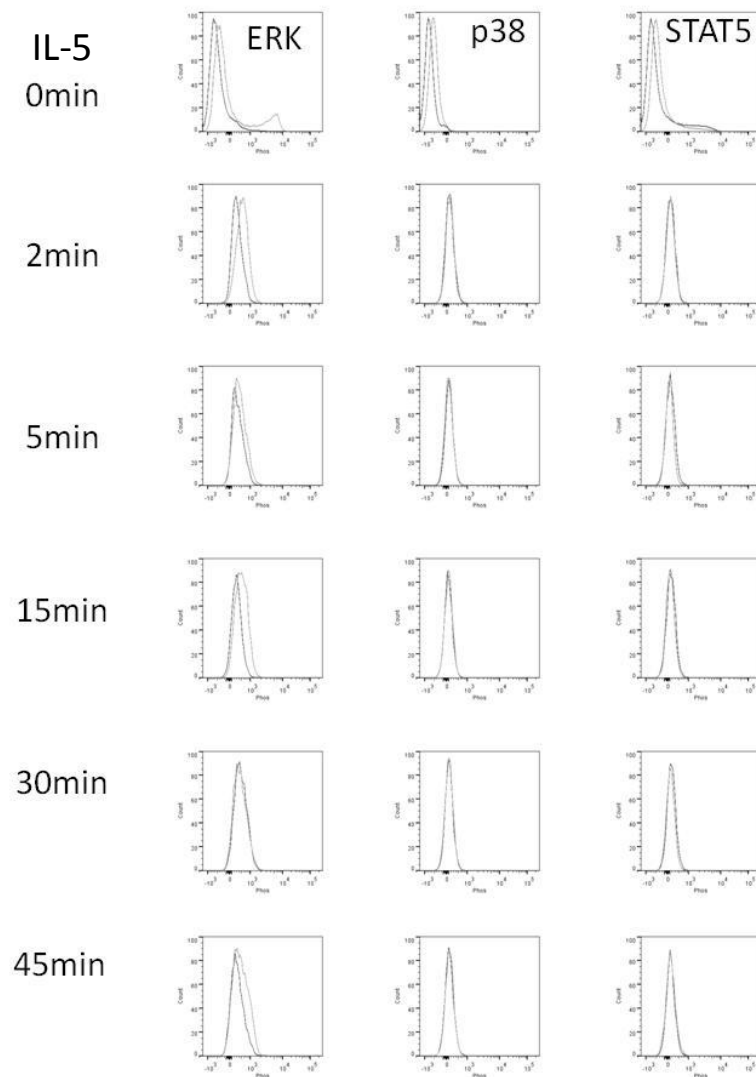


Figure 4.9. FlowJo® plots of the phosphorylation time course for ERK1/2, p38 and STAT5. Histograms were then used to determine the MFI of IL-5-induced phosphorylated ERK1/2, p38 and STAT5 at 0, 2, 5, 15, 30 and 45min. The negative condition was compared to the IL-5 treated cells and an increase in ERK1/2 phosphorylation was observed after 15min incubation. No responses were observed for STAT5 or p38. The plots shown are representative sample from 1 subject. The black lines represent the isotype control and the grey lines represent the phosphorylated antibody.

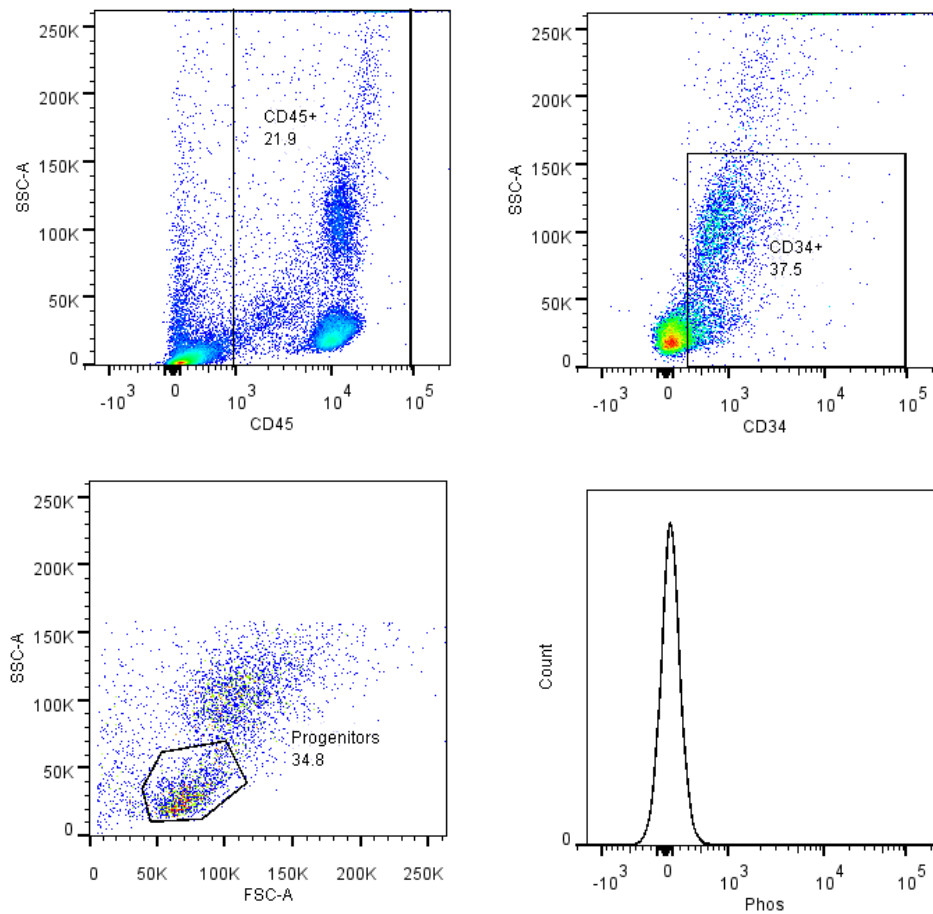


Figure 4.10. FlowJo® plots of CD34⁺ cell gating strategy. Cells were stained for CD45, CD34 and phosphorylated ERK, p38 and STAT5 using mouse anti-human CD34-Pacific Blue (eBioscience), CD45-FITC and PE-phos-ERK, PE-phos-STAT5 and PE-phos-p38 (Becton-Dickinson Biosciences), as well as the isotype control antibodies. Cells were isolated based on CD45⁺ (upper left panel), CD34⁺ (upper right panel), low granularity and small size (lower left panel). Histograms were then used to determine the MFI of the phosphorylated ERK1/2, p38 and STAT5 (lower right panel). The plots shown are a representative sample from 1 subject.

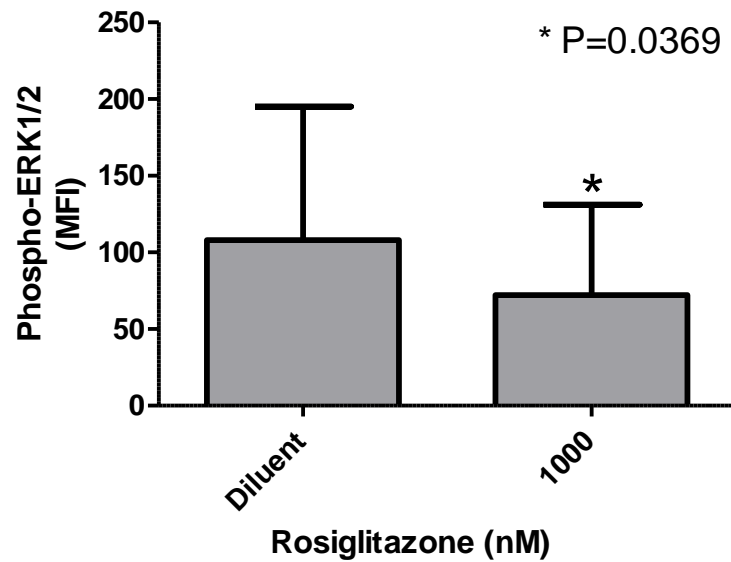


Figure 4.11. The effect of rosiglitazone (PPAR γ agonist) on IL-5-induced ERK1/2 phosphorylation. CD34 $^{+}$ cells were isolated from the peripheral blood of allergic subjects. A significant decrease ($P=0.0369$) in ERK1/2 phosphorylation was observed at 1000nM rosiglitazone. Data are arithmetic mean \pm 95% confidence intervals.

4.7 References

- Adachi, T., Choudhury, B. K., Stafford, S., Sur, S., & Alam, R. (2000). The differential role of extracellular signal-regulated kinases and p38 mitogen-activated protein kinase in eosinophil functions. *J.Immunol.*, 165, 2198-2204.
- Belvisi, M. G., Hele, D. J., & Birrell, M. A. (2006). Peroxisome proliferator-activated receptor gamma agonists as therapy for chronic airway inflammation. *Eur.J.Pharmacol.*, 533, 101-109.
- Bernardo, A., Bianchi, D., Magnaghi, V., & Minghetti, L. (2009a). Peroxisome Proliferator-Activated Receptor-gamma Agonists Promote Differentiation and Antioxidant Defenses of Oligodendrocyte Progenitor Cells. *J.Neuropathol.Exp.Neurol.*
- Bot, F. J., Dorssers, L., Wagemaker, G., & Lowenberg, B. (1988). Stimulating spectrum of human recombinant multi-CSF (IL-3) on human marrow precursors: importance of accessory cells. *Blood*, 71, 1609-1614.
- Brown, P. J., Winegar, D. A., Plunket, K. D., Moore, L. B., Lewis, M. C., Wilson, J. G. *et al.* (1999). A ureido-thioisobutyric acid (GW9578) is a subtype-selective PPARalpha agonist with potent lipid-lowering activity. *J.Med.Chem.*, 42, 3785-3788.
- Caldenhoven, E., van Dijk, T. B., Tijmensen, A., Raaijmakers, J. A., Lammers, J. W., Koenderman, L. *et al.* (1998). Differential activation of functionally distinct STAT5 proteins by IL-5 and GM-CSF during eosinophil and neutrophil

differentiation from human CD34+ hematopoietic stem cells. *Stem Cells*, 16, 397-403.

Chihara, J., Plumas, J., Gruart, V., Tavernier, J., Prin, L., Capron, A. *et al.* (1990). Characterization of a receptor for interleukin 5 on human eosinophils: variable expression and induction by granulocyte/macrophage colony-stimulating factor. *J.Exp.Med.*, 172, 1347-1351.

Darnell, J. E., Jr. (1997). STATs and gene regulation. *Science*, 277, 1630-1635.

Denburg, J. A., Richardson, M., Telizyn, S., & Bienenstock, J. (1983). Basophil/mast cell precursors in human peripheral blood. *Blood*, 61, 775-780.

Denburg, J. A., Sehmi, R., Upham, J., Wood, L., Gauvreau, G., & O'Byrne, P. (1999). Regulation of IL-5 and IL-5 receptor expression in the bone marrow of allergic asthmatics. *Int.Arch.Allergy Immunol.*, 118, 101-103.

Denburg, J. A., Telizyn, S., Belda, A., Dolovich, J., & Bienenstock, J. (1985). Increased numbers of circulating basophil progenitors in atopic patients. *J.Allergy Clin.Immunol.*, 76, 466-472.

Dorman, S. C., Efthimiadis, A., Babirad, I., Watson, R. M., Denburg, J. A., Hargreave, F. E. *et al.* (2004). Sputum CD34+IL-5Ralpha+ cells increase after allergen: evidence for in situ eosinophilopoiesis. *Am.J.Respir.Crit Care Med*, 169, 573-577.

Eagle, H. (1955a). The minimum vitamin requirements of the L and HeLa cells in tissue culture, the production of specific vitamin deficiencies, and their cure. *J.Exp.Med*, 102, 595-600.

Eagle, H. (1955b). The specific amino acid requirements of a human carcinoma cell (Stain HeLa) in tissue culture. *J.Exp.Med*, 102, 37-48.

- Flood-Page, P. T., Menzies-Gow, A. N., Kay, A. B., & Robinson, D. S. (2003). Eosinophil's role remains uncertain as anti-interleukin-5 only partially depletes numbers in asthmatic airway. *Am.J.Respir.Crit Care Med.*, 167, 199-204.
- Friend, C., Scher, W., Holland, J. G., & Sato, T. (1971). Hemoglobin synthesis in murine virus-induced leukemic cells *in vitro*: stimulation of erythroid differentiation by dimethyl sulfoxide. *Proc.Natl.Acad.Sci.U.S.A*, 68, 378-382.
- Gibson, P. G., Dolovich, J., Girgis-Gabardo, A., Morris, M. M., Anderson, M., Hargreave, F. E. *et al.* (1990). The inflammatory response in asthma exacerbation: changes in circulating eosinophils, basophils and their progenitors. *Clin.Exp.Allergy*, 20, 661-668.
- Gibson, P. G., Manning, P. J., O'Byrne, P. M., Girgis-Gabardo, A., Dolovich, J., Denburg, J. A. *et al.* (1991). Allergen-induced asthmatic responses. Relationship between increases in airway responsiveness and increases in circulating eosinophils, basophils, and their progenitors. *Am.Rev.Respir.Dis.*, 143, 331-335.
- Guthridge, M. A., Stomski, F. C., Thomas, D., Woodcock, J. M., Bagley, C. J., Berndt, M. C. *et al.* (1998). Mechanism of activation of the GM-CSF, IL-3, and IL-5 family of receptors. *Stem Cells*, 16, 301-313.
- Hogan, S. P., Rosenberg, H. F., Moqbel, R., Phipps, S., Foster, P. S., Lacy, P. *et al.* (2008). Eosinophils: biological properties and role in health and disease. *Clin.Exp.Allergy*, 38, 709-750.
- Hotchin, J. E. (1955). Use of methyl cellulose gel as a substitute for agar in tissue-culture overlays. *Nature*, 175, 352.

- Imaoka, H., Babirad, I., Kwon, Y., Killian, K., O'Byrne, P., Gauvreau, G. *et al.* (2010). Increase in sputum levels of vascular endothelial progenitor cells in asthmatic subjects following allergen challenge [abstract]. *Eur.Respir.J.*, 36, 86s.
- Iscove, N. N., Guilbert, L. J., & Weyman, C. (1980). Complete replacement of serum in primary cultures of erythropoietin-dependent red cell precursors (CFU-E) by albumin, transferrin, iron, unsaturated fatty acid, lecithin and cholesterol. *Exp.Cell Res.*, 126, 121-126.
- Iscove, N. N. & Melchers, F. (1978). Complete replacement of serum by albumin, transferrin, and soybean lipid in cultures of lipopolysaccharide-reactive B lymphocytes. *J.Exp.Med*, 147, 923-933.
- Kim, S. R., Lee, K. S., Park, H. S., Park, S. J., Min, K. H., Jin, S. M. *et al.* (2005). Involvement of IL-10 in peroxisome proliferator-activated receptor gamma-mediated anti-inflammatory response in asthma. *Mol.Pharmacol.*, 68, 1568-1575.
- Kolbeck, R., Kozhich, A., Humbles, A., Erjefalt, J., Koike, M., Peng, L. *et al.* (2008). MEDI-563, a humanized anti-IL-5Ra antibody with enhanced effector function, induces reversible blood eosinopenia in mild asthmatics [abstract]. *J.Allergy Clin.Immunol.*, S47.
- Kota, B. P., Huang, T. H., & Roufogalis, B. D. (2005). An overview on biological mechanisms of PPARs. *Pharmacol.Res.*, 51, 85-94.
- Kuo, H. P., Wang, C. H., Lin, H. C., Hwang, K. S., Liu, S. L., & Chung, K. F. (2001). Interleukin-5 in growth and differentiation of blood eosinophil progenitors in asthma: effect of glucocorticoids. *Br.J.Pharmacol.*, 134, 1539-1547.

- Lehmann, J. M., Moore, L. B., Smith-Oliver, T. A., Wilkison, W. O., Willson, T. M., & Kliewer, S. A. (1995). An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor gamma (PPAR gamma). *J.Biol.Chem.*, 270, 12953-12956.
- Levis, W. R. & Robbins, J. H. (1972). Methods for obtaining purified lymphocytes, glass-adherent mononuclear cells, and a population containing both cell types from human peripheral blood. *Blood*, 40, 77-89.
- Lu, L., Walker, D., Broxmeyer, H. E., Hoffman, R., Hu, W., & Walker, E. (1987). Characterization of adult human marrow hematopoietic progenitors highly enriched by two-color cell sorting with My10 and major histocompatibility class II monoclonal antibodies. *J.Immunol.*, 139, 1823-1829.
- Mueller, C., Weaver, V., Vanden Heuvel, J. P., August, A., & Cantorna, M. T. (2003). Peroxisome proliferator-activated receptor gamma ligands attenuate immunological symptoms of experimental allergic asthma. *Arch.Biochem.Biophys.*, 418, 186-196.
- Nagasawa, E., Abe, Y., Nishimura, J., Yanase, T., Nawata, H., & Muta, K. (2005). Pivotal role of peroxisome proliferator-activated receptor gamma (PPARgamma) in regulation of erythroid progenitor cell proliferation and differentiation. *Exp.Hematol.*, 33, 857-864.
- Nair, P., Pizzichini, M. M., Kjarsgaard, M., Inman, M. D., Efthimiadis, A., Pizzichini, E. *et al.* (2009). Mepolizumab for prednisone-dependent asthma with sputum eosinophilia. *N.Engl.J.Med.*, 360, 985-993.
- Oliver, W. R., Jr., Shenk, J. L., Snaith, M. R., Russell, C. S., Plunket, K. D., Bodkin, N. L. *et al.* (2001). A selective peroxisome proliferator-activated receptor delta

agonist promotes reverse cholesterol transport. *Proc.Natl.Acad.Sci.U.S.A*, 98, 5306-5311.

Pandit, T. S., Sikora, L., Muralidhar, G., Rao, S. P., & Sriramarao, P. (2006). Sustained exposure to nicotine leads to extramedullary hematopoiesis in the spleen. *Stem Cells*, 24, 2373-2381.

Pazdrak, K., Olszewska-Pazdrak, B., Stafford, S., Garofalo, R. P., & Alam, R. (1998). Lyn, Jak2, and Raf-1 kinases are critical for the antiapoptotic effect of interleukin 5, whereas only Raf-1 kinase is essential for eosinophil activation and degranulation. *J.Exp.Med*, 188, 421-429.

Pijper, A. (1947). Methylcellulose and Bacterial Motility. *J.Bacteriol.*, 53, 257-269.

Rosen, E. D. & Spiegelman, B. M. (2001). PPARgamma : a nuclear regulator of metabolism, differentiation, and cell growth. *J.Biol.Chem.*, 276, 37731-37734.

Sanford, K. K., Earle, W. R., Evans, V. J., Waltz, H. K., & Shannon, J. E. (1951). The measurement of proliferation in tissue cultures by enumeration of cell nuclei. *J.Natl.Cancer Inst.*, 11, 773-795.

Sato, N., Caux, C., Kitamura, T., Watanabe, Y., Arai, K., Banchereau, J. *et al.* (1993). Expression and factor-dependent modulation of the interleukin-3 receptor subunits on human hematopoietic cells. *Blood*, 82, 752-761.

Schreier, M. H. & Iscove, N. N. (1980). Haematopoietic growth factors are released in cultures of H-2-restricted helper T cells, accessory cells and specific antigen. *Nature*, 287, 228-230.

Sehmi, R., Howie, K., Sutherland, D. R., Schragge, W., O'Byrne, P. M., & Denburg, J. A. (1996). Increased levels of CD34+ hemopoietic progenitor cells in atopic subjects. *Am.J.Respir.Cell Mol.Biol.*, 15, 645-655.

- Sehmi, R., Wood, L. J., Watson, R., Foley, R., Hamid, Q., O'Byrne, P. M. *et al.* (1997). Allergen-induced increases in IL-5 receptor alpha-subunit expression on bone marrow-derived CD34+ cells from asthmatic subjects. A novel marker of progenitor cell commitment towards eosinophilic differentiation. *J.Clin.Invest*, 100, 2466-2475.
- Shalit, M., Sekhsaria, S., & Malech, H. L. (1995). Modulation of growth and differentiation of eosinophils from human peripheral blood CD34+ cells by IL5 and other growth factors. *Cell Immunol.*, 160, 50-57.
- Spears, M., McSharry, C., & Thomson, N. C. (2006). Peroxisome proliferator-activated receptor-gamma agonists as potential anti-inflammatory agents in asthma and chronic obstructive pulmonary disease. *Clin.Exp.Allergy*, 36, 1494-1504.
- Stafford, S., Lowell, C., Sur, S., & Alam, R. (2002). Lyn tyrosine kinase is important for IL-5-stimulated eosinophil differentiation. *J.Immunol.*, 168, 1978-1983.
- Trifilieff, A., Bench, A., Hanley, M., Bayley, D., Campbell, E., & Whittaker, P. (2003). PPAR-alpha and -gamma but not -delta agonists inhibit airway inflammation in a murine model of asthma: *in vitro* evidence for an NF-kappaB-independent effect. *Br.J.Pharmacol.*, 139, 163-171.
- Ueki, S., Adachi, T., Bourdeaux, J., Oyamada, H., Yamada, Y., Hamada, K. *et al.* (2003). Expression of PPARgamma in eosinophils and its functional role in survival and chemotaxis. *Immunol.Lett.*, 86, 183-189.
- Vogt, M. & Dulbecco, R. (1960). VIRUS-CELL INTERACTION WITH A TUMOR-PRODUCING VIRUS. *Proc.Natl.Acad.Sci.U.S.A*, 46, 365-370.

Yang, X. Y., Wang, L. H., Chen, T., Hodge, D. R., Resau, J. H., DaSilva, L. *et al.* (2000). Activation of human T lymphocytes is inhibited by peroxisome proliferator-activated receptor gamma (PPARgamma) agonists. PPARgamma co-association with transcription factor NFAT. *J.Biol.Chem.*, 275, 4541-4544.

Chapter 5: Conclusion

5.1 Conclusion

PPAR and Eosinophil Migration

We demonstrated that the PPAR γ agonist rosiglitazone, at a concentration of 100 nM, significantly increased eosinophil chemokinesis *in vitro*. This finding supports the data demonstrating increased eosinophil migration reported by Kobayashi (Kobayashi *et al.*, 2005). At these same concentrations there was no effect of the PPAR α agonist, GW9578 or the PPAR β/δ agonist, GW501516. Cell migration is a multistep process, which involves both chemotaxis and chemokinesis in response to a chemoattractant (Friedl & Weigelin, 2008). Chemotaxis is defined as directed migration towards a chemoattractant, whereas chemokinesis is defined as non-directional stimulated migration.

Chemokinesis, non-directional migration, is not sufficient for cell accumulation, but may contribute considerably by priming a cell to respond more vigorously to chemotactic stimuli (Dunn, 1983). Previously it was unknown whether the increase in eotaxin-induced migration by PPAR γ agonists was due to chemotaxis or chemokinesis, or both. Although PPAR agonists in this *in vitro* study are used at concentrations similar to the reported EC50s for rosiglitazone, GW9578 and GW501516 (Lehmann *et al.*, 1995; Brown *et al.*, 1999; Oliver, Jr. *et al.*, 2001), this study has determined that the enhanced migration by rosiglitazone is likely due to a chemokinetic effect. Such “priming” of cells may enhance their response to other stimuli including chemokines like eotaxin, which are found in the microenvironment of allergic tissue, such as asthmatic airways, which may lead to eosinophilia.

Eosinophil migration is important mechanism that contributes to overall inflammation during an allergic asthmatic response. Eosinophilia has been observed in the airways of asthmatic individuals with no clinical symptoms compared with healthy controls (Kirby *et al.*, 1987; Pin *et al.*, 1992). Furthermore, airway eosinophilia increases in atopic asthmatic subjects following events such as allergen challenge (Aalbers *et al.*, 1993; de Monchy *et al.*, 1985; Fahy *et al.*, 1994). The increase in airway eosinophilia is associated with the development of the late asthmatic response and AHR to methacholine (Gauvreau *et al.*, 1996; Gauvreau *et al.*, 1997; O'Byrne *et al.*, 1987). In a diluent-controlled crossover allergen challenge study, the increase in the number of sputum eosinophils also coincided with an increase in eosinophils immunopositive for IL-5, eotaxin, and RANTES after allergen inhalation (Gauvreau *et al.*, 1999).

In response to an allergen challenge cells are recruited from the bone marrow and blood into the airways (Wood *et al.*, 1998). Many asthma therapies are focused on reducing the number or activity of pro-inflammatory cells that enter the airway and affect the bronchial reactivity of the patients. Targeting the migration of eosinophils has been previously tested using a CCR3 antagonist. Eotaxin is the primary chemokine that recruits eosinophils to the airways of asthmatic patients (Ying *et al.*, 1997; Brown *et al.*, 1998). CCR3 has been identified as the eotaxin receptor (Combadiere *et al.*, 1995; Ponath *et al.*, 1996; Daugherty *et al.*, 1996) and is the dominant chemokine receptor on eosinophils (Heath *et al.*, 1997). Several therapies directed against eotaxin are in clinical development for treatment of allergic asthma. So far two small molecule CCR3 antagonists (GW-766994, DPC-168) were studied in clinical trials and another compound (BMS-639623) was suggested as a candidate for

a clinical trial. GW766994, developed by GlaxoSmithKline, is a selective, non-toxic compound that exhibits anti-inflammatory effects in the rat and guinea-pig. AHR and sputum eosinophilia was observed in a placebo-controlled parallel group study of repeated (10 day; twice daily) GW766994 dose treatment in mild to moderate asthmatics (GlaxoSmithKline, 2011). However, the effectiveness is undetermined because no statistical information was reported in the GSK clinical trial report. The Phase I development of DPC-168 has been terminated due to toxicity (De Lucca *et al.*, 2005). BMS639623 had equal potency to DPC168 but was less toxic in various species (mouse, rat, and dog) (Santella *et al.*, 2008). However, the Bristol-Myers Squibb Company halted development of BMS-639623 (no publications on this antagonist since 2008).

Another approach for inhibition of eosinophil migration has been through antisense oligonucleotides, such as TPI ASM8, which targets both CCR3 and the common β -chain of the IL-3/IL-5/GM-CSF receptor (Gauvreau *et al.*, 2011; Gauvreau *et al.*, 2008). Sputum eosinophils and clinical outcomes were studied in mild asthmatic subjects that underwent allergen challenge. TPI ASM8 inhibited sputum eosinophilia, eosinophil cationic protein (ECP), and decreased the total cell influx after allergen challenge (Gauvreau *et al.*, 2008). TPI ASM8 also significantly inhibited the early and late airway responses (Gauvreau *et al.*, 2011). It also inhibited the increase in ECP clinical outcomes including the early and late airway responses and airway responsiveness to methacholine (Gauvreau *et al.*, 2011). TPI ASM8 also reduced the number of CCR3 positive progenitor cells and IL-5R α positive progenitor cells in the sputum (Imaoka *et al.*, 2011). Using RNA as a therapy has some inherent drawbacks, including instability, the potential for an immunogenic response, and it

typically requires a delivery vehicle for efficient transport to the targeted cells (Burnett & Rossi, 2012). This may be associated with increased production costs compared to molecular antagonist synthesis.

In our study, rosiglitazone enhanced or inhibited eosinophil migration in a dose dependant manner. The effect of rosiglitazone could be through modulation of IL-5 signalling which changes CCR3 expression. Previous studies have shown that co-culture with IL-5 increases expression of CCR3 on mature eosinophils (Stirling *et al.*, 2001). In addition, PPAR γ agonists increase IL-5 induced survival of mature eosinophils (Ueki *et al.*, 2003), showing that PPAR γ agonists can modulate IL-5 induced mechanisms. Thus, we investigated whether the observed PPAR γ agonist-induced modulation of migration could be through the regulation of CCR3 expression on eosinophils. We demonstrated that rosiglitazone treatment had no effect on the level of CCR3 cell surface expression on eosinophils, and these observations reinforce those reported previously showed no changes in eosinophil CCR3 receptor expression in response to troglitazone treatment (Kobayashi *et al.*, 2005).

We tested another possibility, whether the PPAR γ agonist induced modulation of migration could be through changes in downstream signalling events. Rosiglitazone induces ERK1/2 and p38 MAPK activation in brown adipose tissue (Teruel *et al.*, 2003). MAPKs, p44 and p42, also known as extracellular signal-regulated kinase-1 (ERK-1) and ERK-2, are involved in eotaxin induced rolling and migration through the regulation of cytoskeletal rearrangements (Boehme *et al.*, 1999). MAPK p38 was also observed to be an important mechanism in eotaxin-induced chemotaxis (Kampen *et al.*, 2000). Kampen *et al* showed eotaxin induces a dose-dependent phosphorylation of ERK2 and p38 and that the phosphorylation occurred after 30 seconds and reached

a plateau at 1 minute (Kampen *et al.*, 2000). In our study a 10-fold lower dose of eotaxin was used, a dose optimized for migration in our experimental setup, and the 110 min incubation period was used to mimic the migration assay. We observed no effect on downstream signaling, phosphorylation of ERK1/2 or p38 MAPK, suggesting the observed effects of rosiglitazone on eosinophil migration are not related to alterations in signalling through the eotaxin receptor. These observations reinforce data reported from a study that showed no effect of a MEK inhibitor or a p38 MAPK inhibitor on the enhanced eosinophil migration observed after PPAR activation (Kobayashi *et al.*, 2005).

We report that the eotaxin receptor, CCR3, expression and phosphorylation are not affected by rosiglitazone. However, PPAR agonists could be affecting other important pathways that regulate migration, such as Ca^{2+} signalling. Calcium signalling in immune cells is known to be important for growth, death, differentiation and proper function (Crabtree & Clipstone, 1994; Gold & DeFranco, 1994; Fanger *et al.*, 1995). Calcium signalling occurs in two distinct patterns, a repetitive transient release (oscillation) or a sustained release (plateaux) (Clapham, 1995; Berridge, 1993). NF- κ B and NFAT are Ca^{2+} -sensitive transcriptional factors that promote the expression of haematopoietic growth factors and inflammatory cytokines (Crabtree & Clipstone, 1994; Fanger *et al.*, 1995; Baeuerle & Henkel, 1994; Frantz *et al.*, 1994; Timmerman *et al.*, 1996; Rao, 1994). Dolmetsch *et al* showed NF- κ B is activated by a large transient intracellular Ca^{2+} rise, whereas NFAT is activated by a low sustained Ca^{2+} plateau (Dolmetsch *et al.*, 1997). The authors concluded that Ca^{2+} can achieve signalling specificity using the Ca^{2+} sensitivity and kinetic behaviour of different transcription factors (Dolmetsch *et al.*, 1997). Previous studies have shown that the

enhanced migration was not due to changes in receptor signalling, ERK1/2 or p38, changes in the NF- κ B pathway or due to genomic transcription (Kobayashi *et al.*, 2005). However, the enhanced migration that we and others have shown, describes a PPAR agonist induced modulatory effect on eotaxin-induced calcium mobilization (Kobayashi *et al.*, 2005). This suggests that PPAR agonists have down-stream targets which have not been identified yet. In our study rosiglitazone reduced the frequency of eotaxin-induced calcium oscillations, which represents the mobilization of Ca^{2+} back into the cell (Clapham, 1995; Berridge, 1993). However, no consistent effect of rosiglitazone was observed on the maximum fluorescence intensity which represents the release of Ca^{2+} from intracellular stores (Clapham, 1995; Berridge, 1993). This suggests that the rosiglitazone is having an effect through modulation of Ca^{2+} channels, which should be examined in the future studies.

PPAR and Migration of Eosinophil Progenitors

In 1985 Denburg and colleagues discovered an association between atopy and greater numbers of circulating Eo/B-CFU which could be grown in culture (Denburg *et al.*, 1985), as well as increased numbers of circulating CD34+ progenitor cells (Sehmi *et al.*, 1996). Along with this is discovery in atopic subjects, similar associations were found in allergic asthmatics. An increase in peripheral blood-derived Eo/B-CFU was observed in allergic asthmatics during an exacerbation and numbers decreased upon recovery from symptoms (Gibson *et al.*, 1990). In the allergen challenge model, a significant increase in Eo/B-CFUs and CD34+ cells can be observed 24-hours after allergen inhalation challenge in atopic asthmatic subjects who develop early and late phase bronchoconstriction (Gibson *et al.*, 1991; Sehmi *et*

et al., 1997). Examination of this progenitor cell population showed that allergen exposure upregulates the expression of IL-5 receptors on CD34⁺ cells in the bone marrow of atopic asthmatics with an early and late phase bronchoconstriction (Denburg *et al.*, 1999). Since IL-5 is the principal regulatory cytokine for the differentiation of eosinophils (Chihara *et al.*, 1990) and eosinophils represent a major effector cell in asthma (Hogan *et al.*, 2008), progenitor cells could be an important target for drug development.

Recent studies have shown that treatment with mepolizumab, anti-IL-5 therapy, was ineffective at completely attenuating eosinophil numbers in bronchial biopsies from asthmatic subjects, which is the site of disease pathology (Flood-Page, Menzies-Gow, Kay, & Robinson, 2003). However, mepolizumab treatment improved asthma control and significantly reduced the prednisone dose required by asthmatic patients with persistent eosinophilia (Nair *et al.*, 2009). Researchers continue to search for a more effective strategy to completely ablate eosinophils at the site of disease, including investigational drugs that target the lung-homing and localized tissue-differentiation of eosinophil progenitor cells (Kolbeck *et al.*, 2008; Imaoka *et al.*, 2010). This may lead to a more effective therapy for controlling asthma.

SDF-1 α was first discovered as a pre-B-cell stimulating factor in a stromal cell line, PA6 (Nagasawa, Kikutani, & Kishimoto, 1994). After generating mutant mice with a targeted disruption in the SDF-1 α gene, it was found to play an important role in bone-marrow myelopoiesis (Nagasawa *et al.*, 1996). It is produced in the bone marrow by stromal cells and causes retention of progenitor cells in the bone marrow through the CXCR4 (Aiuti *et al.*, 1997; Ma *et al.*, 1998; Sweeney & Papayannopoulou, 2001). Migration of stem cells from the bone marrow into the

blood is caused by the decreased CXCR4 responsiveness to SDF-1 α and a reduction of SDF-1 α levels in the bone marrow (Levesque *et al.*, 2003; Petit *et al.*, 2002). Therefore the SDF-1 α /CXCR4 pathway has been suggested as a possible therapeutic target to prevent progenitor cell migration from the bone marrow into the blood (Petit *et al.*, 2002). This axis has also been shown to play an important role in allergic asthma. It has been reported that CXCR4 expression is decreased on CD34⁺ cells isolated from the bone marrow of atopic asthmatics (Dorman *et al.*, 2005). Similarly, CD34⁺ cells from atopic asthmatics showed decreased SDF-1 α -induced migration compared to normal individuals (Dorman *et al.*, 2005). In atopic asthmatic subjects with early and late phase bronchoconstriction following inhaled allergen challenge, there was a further decrease in CXCR4 expression on CD34⁺ cells and decreased SDF-1 α levels in the bone marrow post-challenge. Furthermore, the migrational response of these CD34⁺ cells to SDF-1 α was attenuated indicating reduced responsiveness to holding forces within the BM-niche, which promotes the egress of progenitors to the peripheral circulation (Dorman *et al.*, 2005). On the other hand, localized increases in SDF-1 α within the lung, promotes progenitor cell homing in allergic inflammatory responses (Southam *et al.*, 2005; Doyle *et al.*, 2011) Thus targeting the SDF-1 α /CXCR4 axis may be beneficial to modulating the traffic and lung-homing of progenitor cells in allergic asthma.

Our study observed a trend towards an increase in SDF-1 α -induced progenitor cell migration after rosiglitazone treatment. This was similar to the effects observed on eosinophil migration at nM doses (Smith *et al.*, 2012). This trend was also observed in a clinical trial examining pioglitazone (a PPAR γ agonist) treatment in patients with coronary artery disease and normal glucose tolerance. In response to

pioglitazone treatment, endothelial progenitor cells (EPC) increased in the peripheral blood, there was increased migration towards SDF-1 α and there was an increase in the number of EPC CFUs (Werner *et al.*, 2007). These results suggest that EPC mobilization from the bone marrow is increased by PPAR γ agonists. Therefore in addition to enhanced migration of mature eosinophils, PPAR γ agonists at nanomolar levels have effects on the SDF-1 α /CXCR4 axis, which may lead to enhanced airway eosinophilia in allergic asthma.

PPAR and Migration of Mature Eosinophils

Synthetic PPAR ligands have been valuable in determining PPAR function. Rosiglitazone and troglitazone are the most frequently studied PPAR γ agonists. However, rosiglitazone is preferred because it has a higher binding affinity for PPAR γ than troglitazone (Young *et al.*, 1998). Both of these agonists belong to a class of drugs known as thiazolidinediones, which are used as anti-diabetic medications (Bensinger & Tontonoz, 2008). They activate PPAR γ and subsequently cause insulin sensitization (Bensinger & Tontonoz, 2008). In our study of eosinophil migration, only the PPAR γ agonists, but not the PPAR α or PPAR β/δ agonists, had an effect. In contrast, others have reported an inhibitory effect of PPAR α agonists. Woerly *et al* depicted a 50% reduction in migration in response to eotaxin after treatment with WY14653 (Woerly *et al.*, 2003), which is primarily a PPAR α activator, but has been demonstrated to activate PPAR γ as well (Lehmann *et al.*, 1997; Devchand *et al.*, 1996; Hsu *et al.*, 1995; Staels *et al.*, 1998). Furthermore, Woerly *et al* observed an inhibition of IL-5-induced eosinophil migration after treatment with rosiglitazone or WY-14653 (Woerly *et al.*, 2003). Conversely, Sehmi *et al* demonstrated IL-5 produces

only a very weak migrational response in eosinophils (Sehmi *et al.*, 1992), however the main function of IL-5 is to prime eosinophils to migrate towards other chemokines (Sehmi *et al.*, 1992). Differences in migration assays utilized and chemotactic stimuli could contribute to the conflicting results for the effects of PPAR α on eosinophil migration.

In summary, at low concentrations the PPAR γ agonist rosiglitazone significantly enhanced chemokinesis of eosinophils isolated from the peripheral blood of atopic subjects, and appears to enhance the migration of eosinophil progenitors, although this was not found to be statistically significant. This chemokinetic effect may at least partially explain the enhanced eosinophil migration observed by Kobayashi (Kobayashi *et al.*, 2005). The enhanced chemokinesis was specific for the PPAR γ agonist, as there was no effect of PPAR α or PPAR β/δ agonists. Finally, the effect of the PPAR γ agonist appears to be through the modulation of calcium signalling. In light of our findings that a selective PPAR γ agonist can enhance eosinophil chemokinesis at a concentration of 100 nM and decrease eosinophil migration at a concentration of 100 μ M, the therapeutic window for PPAR γ agonists as an anti-inflammatory therapy must be titrated carefully.

PPAR and Eosinophil Progenitor Cell Differentiation

This is the first study to show a PPAR γ agonist inhibits eosinophil differentiation *in vitro* when added to cultures of non-adherent cells and isolated CD34⁺ progenitor cells collected from the peripheral blood of allergic subjects. The results of this study also provide further evidence that PPAR γ agonists work through a mechanism leading to inhibition of eosinophilia after allergen challenge (Kim *et al.*,

2005; Trifilieff *et al.*, 2003). We demonstrated that rosiglitazone treatment was effective to reduce the number of Eo/B-CFU in cultures of NAMNC grown with IL-5 and also with cytokines such as IL-3, suggesting the inhibitory effects of rosiglitazone are robust. We have described attenuation of ERK1/2 phosphorylation after rosiglitazone treatment in CD34 progenitor cells. STAT5, ERK1/2 and p38 pathways have all been implicated in eosinophil differentiation (Stafford *et al.*, 2002; Pazdrak *et al.*, 1998; Adachi *et al.*, 2000; Caldenhoven *et al.*, 1998). During IL-5 and IL-3 co-stimulated eosinophil differentiation, p38 pathway plays a greater role than ERK1/2 (Adachi *et al.*, 2000). However, rosiglitazone was still able to inhibit eosinophil differentiation, suggesting it has a robust inhibitory effect.

Differentiation of progenitor cells into eosinophils is an important mechanism for expansion of the eosinophil population in inflammatory lung disease. Denburg *et al.* has demonstrated that bone marrow progenitor commitment to the Eo/B lineage is regulated by IL-3, IL-5 and GM-CSF (Denburg *et al.*, 1983; Denburg *et al.*, 1994). IL-5 induces eosinophil differentiation from the Eo/B committed progenitor *in vivo* and co-cultured with IL-3 has an additive effect to the number of colonies (Lamkhioued *et al.*, 2003; Lu *et al.*, 1990). There are increased numbers of CD34 cells in the bone marrow of atopic compared with non-atopic subjects and this correlates with increased Eo/B-CFU grown from the blood and bone marrow (Sehmi *et al.*, 1996). Atopic asthmatics that underwent allergen challenge had increased Eo/B-CFU from cells isolated from the blood and bone marrow (Gauvreau *et al.*, 1998; Wood *et al.*, 1998). Asthmatic subjects that developed airway eosinophilia expressed higher levels of the IL-5R α than subjects without airway eosinophilia (Sehmi *et al.*, 1997; Chou *et al.*, 1999). Intravenous IL-5 increases the number of eosinophil progenitor cells in the

peripheral blood and increase the expression of CCR3 on mature eosinophils (Stirling *et al.*, 2001). Thus IL-5 increases the opportunity for peripheral eosinophilia to occur (Stirling *et al.*, 2001). In the bronchial mucosa, IL-5 caused a decrease in CD34+IL-5R α + cells, which suggests that IL-5 is important for eosinophil differentiation in tissue as well as peripheral blood (Menzies-Gow *et al.*, 2007). Mepolizumab, an anti-IL-5 therapy, caused a decrease in CD34+IL-5R α + cells in the bronchial mucosa (Menzies-Gow *et al.*, 2003), again, suggesting a role for IL-5 in peripheral tissue eosinophil differentiation and its importance in the development of peripheral eosinophilia.

CD34+ cells have been observed in different airway compartments in humans, including mucosa of the upper and lower airway (Robinson *et al.*, 1999; Cameron *et al.*, 2000). Increased progenitor cells have also been observed in nasal polyps from asthmatic subjects versus healthy controls and in sputum samples (Kim *et al.*, 1999; Dorman *et al.*, 2004). Furthermore, the number of progenitor cells increased after allergen stimulation (Dorman *et al.*, 2004). When compared to normal controls, the number of progenitor cells also increased in the airways of asthmatics (Robinson *et al.*, 1999). In *ex vivo* cultures of nasal mucosa, IL-5 or allergen decreased the number of CD34+ cells and this coincided with an increase in major basic protein-positive cells, which could be a subset of eosinophils (Cameron *et al.*, 2000). Inhaled budesonide treatment has been observed to inhibit the turnover of a subpopulation of bone marrow-derived progenitors in allergic asthmatics (Wood *et al.*, 1999). Budesonide treatment reduced the number of eosinophils in the blood and the number of Eo/B-CFU grown in culture (Gauvreau *et al.*, 2000). However, *in vitro* studies have described a stimulatory effect of steroids on colony formation (Rosenthal *et al.*, 1987).

This phenomenon is observed in specific *in vivo* models, such as an acute stress model (Elsas *et al.*, 2004). Cyr *et al* have shown that in the presence of budesonide, there was increased production of Eo/B-CFU in response to IL-5 (Cyr *et al.*, 2008). This budesonide-induced increase was a result of an increase in GATA-1 transcription factor expression. This suggests a subset of patients with eosinophilia maybe be less responsive to steroid therapy due to ineffective blockade of eosinophil differentiation. PPAR agonists might represent an alternative therapy in these subsets. The effect of PPAR agonist on GATA-1 expression was not investigated in this study but should be considered in the future.

In summary, the PPAR γ agonist, rosiglitazone inhibited eosinophil differentiation of NAMNCs isolated from the peripheral blood of allergic subjects. It also inhibited eosinophil differentiation of isolated CD34+ progenitor cells. This inhibitory effect may at least be partially explained by an inhibition of ERK1/2 phosphorylation. The inhibition of eosinophil differentiation was specific for the PPAR γ agonist, as there was no effect of PPAR α or PPAR β/δ agonists. In light of our findings that a selective PPAR γ agonist can inhibit eosinophil differentiation at a concentration of 10-1000nM and inhibit eosinophil migration at similar doses, PPAR γ -agonists have a promising therapeutic potential as an anti-inflammatory therapy, when used within the specified dosage.

PPAR as therapeutics and side effects

Allergic asthma is a complex disease with several phenotypes, neutrophilic, eosinophilic and paucigranular (Wenzel, 2012). Therapeutic success has been observed with mepolizumab, the anti-IL-5 therapy, in asthma and eosinophilic

eosinophilic esophagitis (Stein *et al.*, 2006; Nair *et al.*, 2009). The more successful asthma therapies have been those that target multiple cell types and pathways, such as glucocorticoids. Glucocorticoids and PPAR agonists use similar mechanisms to inhibit the inflammatory response in allergic asthma. The anti-inflammatory effects of PPAR agonists affect a broad range of inflammatory mechanisms, not just eosinophils. Trifilieff and colleagues observed that in OVA sensitized mice treated with a PPAR α or PPAR γ agonist (but not PPAR β/δ), inhibited the OVA-induced influx of eosinophils and lymphocytes into the lung (Trifilieff *et al.*, 2003). Birrell *et al.* showed treatment with either rosiglitazone, a PPAR γ agonist, or SB219994, a dual PPAR α and PPAR γ agonist, reduced the number of neutrophils in the murine lung after airway challenge with lipopolysaccharide (LPS) (Birrell *et al.*, 2004). In a study using OVA-pulsed DCs treated with a PPAR γ agonist, a reduced proliferation of Ag-specific T cells in mediastinal lymph nodes, an increased production of IL-10 by T cells and a decreased recruitment of eosinophils in bronchoalveolar lavage was observed (Hammad *et al.*, 2004). In a murine model, PPAR γ was found to be expressed in epidermal Langerhans cells (LCs) and LC migration induced by TNF- α was inhibited by PPAR γ activation (Angeli *et al.*, 2003). In a model of contact allergen-induced LC migration, PPAR γ activation impeded LC migration and prevented contact hypersensitivity responses after challenge (Angeli *et al.*, 2003). Furthermore after intratracheal sensitization, PPAR γ activation decreased the migration of DCs from the airway mucosa to the thoracic lymph nodes and decreased the priming of T lymphocytes (Angeli *et al.*, 2003). The murine model allowed researchers to explore not only the extensive inhibition of inflammatory cell infiltration into the lung, but also the effect of PPARs on airway remodelling.

Honda *et al* showed that PPAR γ is expressed in airway epithelium of mice after airway challenge. Treatment with a PPAR γ agonist decreased airway hyperresponsiveness to methacholine, and indices of remodelling such as basement membrane thickness, mucus production, collagen deposition and TGF- β synthesis (Honda *et al.*, 2004). In cultured human airway smooth muscle cells, PPAR γ agonists reduced smooth muscle proliferation compared to non-treated cells (Ward *et al.*, 2004). PPAR γ agonists also inhibit pro-inflammatory cytokine, eotaxin and monocyte chemoattractant protein-1, production in cultured human airway smooth muscle cells treated with TNF α (Nie *et al.*, 2005). Several studies have concluded that the inhibitory effects of PPAR γ ligands on remodelling and AHR in OVA sensitized mice indicate PPAR γ agonists as possible therapeutics for asthma (Honda *et al.*, 2004; Ward *et al.*, 2006).

Development of PPAR mutant models has further shown the importance of PPARs in the allergen-induced inflammatory response in the lung. After intranasal exposure to LPS, PPAR α -/- mice show a greater increase in neutrophils and macrophages in bronchoalveolar lavage, increased levels of TNF α , keratinocyte derived-chemokine, macrophage inflammatory protein-2 and monocyte chemoattractant protein-1, and increased activity of matrix metalloproteinase-9 compared to PPAR α +/+ mice (ayre-Orthez *et al.*, 2005). These differences were further increased by administration of a PPAR α agonist, which inhibited these responses in the PPAR α +/+ mice (ayre-Orthez *et al.*, 2005). PPAR α -/- mice sensitized to OVA showed an increase in lung eosinophils, AHR, and antigen-specific IgE concentrations after airway challenge (Woerly *et al.*, 2003). Both mouse models point

towards the PPAR α receptor as major molecular mechanism leading to the pathogenesis of an asthmatic response.

Other investigators have shown that PPARs are also important in mediating the response to current therapeutics. PPAR γ activation inhibits the release of granulocyte-macrophage colony-stimulating factor, similar to treatment with the glucocorticoid, dexamethasone (Patel *et al.*, 2003). However, PPAR γ agonists, unlike dexamethasone, inhibit the release of granulocyte colony-stimulating factor (Patel *et al.*, 2003). Activation of PPAR γ was more effective at inhibiting serum-induced human airway smooth muscle cell growth in culture than dexamethasone (Patel *et al.*, 2003). Pang and colleagues observed that PPAR γ activators, like NSAIDs, induced COX-2 expression in cultured human airway smooth muscle cells (Pang *et al.*, 2003). Along with COX-2 expression NSAIDs induced activity of a COX-2 reporter construct that contains a PPRE (Pang *et al.*, 2003). Increased transcriptional activity of COX-2 reporter construct also enhanced IL-1 β effects (Pang *et al.*, 2003). The presence of PPRE was found to be required to cause NSAID-induced COX-2 expression (Pang *et al.*, 2003). PPAR γ and glucocorticoid receptor protein-protein interactions were also observed during TNF α stimulation of cultured human airway smooth muscle cells. These interactions were enhanced by β 2 agonist treatment (Nie *et al.*, 2005). Interactions between PPARs and current therapeutic mechanisms are likely to have important consequences in the treatment of asthma.

A previous study that examined LPS-inducible genes in primary mouse macrophages in the presence of dexamethasone, a glucocorticoid, or rosiglitazone, a PPAR agonist, showed 126 overlapping genes (Ogawa *et al.*, 2005). LPS infection also had 53 unique genes that were affected by PPAR treatment (Ogawa *et al.*, 2005).

Corticosteroids, β -agonists and leukotriene inhibitors (Zdanowicz, 2007) are the most effective therapies to date for asthma, but they are associated with side effects. Corticosteroids are associated with osteoporosis, growth suppression, adrenal suppression, oral candidiasis (Zdanowicz, 2007), and in some cases patients do not respond to treatment (Strek, 2006; Leung *et al.*, 2002). Leukotriene inhibitors have fewer side effects than corticosteroids (Zdanowicz, 2007). However, they are less effective than inhaled corticosteroids alone (Busse *et al.*, 2001) and less effective as a combination therapy than long acting β -agonists (Nelson *et al.*, 2000). Long acting β -agonists maybe associated with tachycardia (Zdanowicz, 2007) and with desensitization of the β -adrenergic receptor (Lipworth, 1997). Thus novel approaches targeting the inflammatory pathways of asthma are currently being examined in hopes of developing improved asthma therapies.

That NF- κ B was discovered in mature B cells bound to the kappa immunoglobulin light-chain enhancer (hence its name, nuclear factor κ B) (Sen & Baltimore, 1986). NF- κ B is controlled by a cytoplasmic inhibitory protein called I κ B (Baeuerle & Baltimore, 1988). NF- κ B increases expression of the adhesion molecules E-selectin, VCAM-1, and ICAM-1, thus inhibition of NF- κ B will reduce leukocyte adhesion and transmigration (Chen *et al.*, 1995). The increased cytokine profile and increased adhesion molecule expression observed in bronchial biopsies from asthmatic patients' correlate with the level of NF- κ B activation (Hart *et al.*, 1998). Glucocorticoids, a current asthma therapy, induce expression of I κ B α which causes enhanced retention of NF- κ B in the cytosol (Auphan *et al.*, 1995; Scheinman *et al.*, 1995). Specifically, dexamethasone increases I κ B α , which increase the cytoplasmic retention of p65 (Scheinman *et al.*, 1995). PPARs inhibit inflammatory gene

expression by several mechanisms, including ligand-dependent transactivation, ligand-dependent transrepression and ligand-independent repression (Ricote & Glass, 2007). PPAR can specifically interact with p60 and p50 subunit of NF- κ B, which creates competition for a limited pool of co-activators (Lee *et al.*, 2003; Delerive *et al.*, 1999). Again, this describes the parallels between the current therapies and PPAR agonists, suggesting it may be valuable in the treatment of asthma.

Future Directions

Eosinophil migration is a multistep process, which involves rolling, activation, firm adhesion, transendothelial migration, and subendothelial migration (Gonlugur & Efreoglu, 2004). In our study, a modified Boyden chamber was used to determine the effects of PPAR agonists on eotaxin-induced eosinophil migration. This method simplifies the migration process into the one component, chemotaxis. This allowed us to make conclusions about the effects of PPAR agonists on chemotaxis alone. On the other hand, it ignores the effects of PPAR agonists on the other components, such as eosinophil adhesion. However, a previous study has shown that troglitazone inhibits eosinophil adhesion to intercellular adhesion molecule-1, an important in integrin regulated firm adhesion (Hirasawa *et al.*, 2008). Other components of migration could be examined, such as adhesion molecules could be examined by flow cytometry or eosinophil migration by examining shape change. However, the most effective way to determine the overall effect on migration is to study eosinophils *in vivo*. Using mouse models, we could instill eotaxin into the lungs of mice treated with a PPAR agonist. We would then compare the eosinophilia in PPAR agonist treated and untreated

animals. This would allow us to make an all encompassing conclusion about the effects of PPAR agonists on the *in vivo* migration.

The effects of PPAR agonists on the intracellular signalling mechanisms are complex. We were able to describe an inhibitory effect on eotaxin-induced calcium signalling in response to rosiglitazone. However, no responses were observed on the phosphorylation of ERK1/2 or p38. Kampen *et al* showed eotaxin induces a dose-dependent phosphorylation of ERK2 and p38 and that the phosphorylation occurred after 30 seconds and reached a plateau at 1 minute (Kampen *et al.*, 2000). In our study, we incubated for 110 min to mimic the chemotaxis assay conditions. In future experiments we could examine if rosiglitazone has a short term effect on the ERK1/2 and p38 signalling. However, I anticipate there to be no effect on signalling because in a previous study 15d-PGJ₂, an endogenous PPAR γ agonist, did not affect the eotaxin-induced phosphorylation of ERK1/2 and p38 after 1 min and 3 min respectively (Kobayashi *et al*, 2005). We also tried to determine the effect of rosiglitazone on IL-5-induced ERK1/2, p38 and STAT5 signalling. Using an IL-5-induced time course we tried to determine the optimal time to examine these effects. Unfortunately, an optimal time was not determined and no mention of an optimal time for IL-5-induced phosphorylation of CD34 cells could be found in the literature. It is possible that, similarly to eotaxin, IL-5 induces phosphorylation of p38 at 1 min. Further studies should examine these earlier time points.

In our study we examined the effects of synthetic PPARs on eosinophil function. In future experiments we could examine the role of synthetic ligands on these same outcomes. However, I would anticipate similar migration results based on previous work (Kobayashi *et al*, 2005). Kobayashi using an endogenous ligand, 15d-

PGJ₂, observed an inhibition of eosinophil migration at high doses. The effect was not due to phosphorylation of ERK1/2 or p38, a change in calcium signalling appear to parallel the migration response. No studies have examined the endogenous ligands on eosinophil differentiation.

The second major outcome in our study was the effect of PPAR agonists on peripheral blood progenitor cells. The peripheral blood is not the major source of progenitor cells. However, the peripheral blood is an important compartment in allergic asthma. In previous work, an increase in Eo/B-CFU was observed in the peripheral blood of asthmatics during an exacerbation and the numbers decreased upon recovery from symptoms (Gibson *et al.*, 1990). In the allergen challenge model of allergic asthma, a significant increase in Eo/B-CFUs can be observed 24-hours after whole lung allergen challenge in atopic asthmatic subjects who develop early and late phase bronchoconstriction (Gibson *et al.*, 1991). In our study we used IL-5 and a co-culture of IL-5 and IL-3 to induce eosinophil differentiation. This is an effective way to determine the direct effect of PPAR agonist treatment on these specific differentiation pathways. However, *in vivo* the cells are exposed to other growth factors, such as GM-CSF and stem cell factor, in addition to the growth factors that were studied. In future work, a more generalized conclusion could be made about eosinophil differentiation by studying the effects of PPAR agonist treatment on eosinophil differentiation induced by the serum from allergic asthmatics. The serum would be an all inclusive growth factor sample that could represent the *in vivo* conditions. Although the peripheral blood can produce eosinophils from hematopoietic progenitor cells, the bone marrow is still the major site of haematopoiesis. Our preliminary results showed that PPAR agonists have no effect on

progenitor cells from the bone marrow. However, the doses studied in these experiments were below the reported EC50 of rosiglitazone (Willson *et al.*, 2000), concentrations of rosiglitazone that are much lower than what was shown to be effective in NAMC from peripheral blood. Furthermore, only 5 subjects were studied. Future studies should examine the effects of PPAR agonists on eosinophil differentiation in the bone marrow from 15 (number studied in the peripheral blood experiments) subjects, and at concentrations to match the experiments conducted using peripheral blood.

PPAR agonists have been suggested as novel anti-inflammatory therapeutics for the treatment of chronic lung disease (Belvisi *et al.*, 2006). The experiments completed in this thesis have described the potent anti-eosinophilic effects of PPAR agonists *in vitro*. To date one clinical trial has examined the effect of rosiglitazone (PPAR γ agonist) treatment on allergen-induced responses in allergic asthmatics. Rosiglitazone inhibited the allergen-induced late asthmatic response by a modest 15% (Richards *et al.*, 2010). This inhibition of the late asthmatic response by rosiglitazone was a modest 15% reduction. There was also a modest inhibition on airway hyperresponsiveness (Richards *et al.*, 2010). Unfortunately no direct measure of allergen-induced airway inflammation was reported. Rosiglitazone, as a potential anti-inflammatory agent to treat asthma does not appear to be a viable option, due to the well documented side effects (Home *et al.*, 2009). This however does not exclude the possibility that new drugs with higher specificity for PPAR γ will have a better safety and efficacy profile when targeting PPAR γ . Drugs currently under investigation include GW570 (Yang *et al.*, 2010) and KR62980 (Won *et al.*, 2010). Another therapeutic approach being developed is dual agonists that activate both the PPAR α

and PPAR γ receptors, which appear to have a better safety profile (Jeong *et al.*, 2011). During the clinical development, it is important that we continue to study the impact that these PPAR agonists have on various mechanisms which promote the allergic asthmatic response. Presuming Health Canada approval, a future study would involve the examination of rosiglitazone treatment on the late asthmatic response in eosinophilic asthmatics undergoing a double blind placebo controlled crossover allergen challenge. The study would recruit allergic asthmatics with more than 3% sputum eosinophils, similar to previous studies (Nair *et al.*, 2009). The primary outcomes would include the late response, MCh PC₂₀, sputum eosinophils and Eo/B colony formation from bone marrow derived progenitor cells. This would allow us to determine if the *in vitro* effects are seen *in vivo* and if rosiglitazone has an effect on the symptom outcomes of allergic asthma.

5.2 References

- Aalbers, R., Kauffman, H. F., Vrugt, B., Koeter, G. H., & de Monchy, J. G. (1993). Allergen-induced recruitment of inflammatory cells in lavage 3 and 24 h after challenge in allergic asthmatic lungs. *Chest*, 103, 1178-1184.
- Adachi, T., Choudhury, B. K., Stafford, S., Sur, S., & Alam, R. (2000). The differential role of extracellular signal-regulated kinases and p38 mitogen-activated protein kinase in eosinophil functions. *J.Immunol.*, 165, 2198-2204.
- Aiuti, A., Webb, I. J., Bleul, C., Springer, T., & Gutierrez-Ramos, J. C. (1997). The chemokine SDF-1 is a chemoattractant for human CD34+ hematopoietic progenitor cells and provides a new mechanism to explain the mobilization of CD34+ progenitors to peripheral blood. *J.Exp.Med.*, 185, 111-120.
- Angeli, V., Hammad, H., Staels, B., Capron, M., Lambrecht, B. N., & Trottein, F. (2003). Peroxisome proliferator-activated receptor gamma inhibits the migration of dendritic cells: consequences for the immune response. *J.Immunol.*, 170, 5295-5301.
- Auphan, N., DiDonato, J. A., Rosette, C., Helmberg, A., & Karin, M. (1995). Immunosuppression by glucocorticoids: inhibition of NF-kappa B activity through induction of I kappa B synthesis. *Science*, 270, 286-290.
- ayre-Orthez, C., Becker, J., Guenon, I., Lagente, V., Auwerx, J., Frossard, N. *et al.* (2005). PPARalpha downregulates airway inflammation induced by lipopolysaccharide in the mouse. *Respir.Res.*, 6, 91.

- Baeuerle, P. A. & Baltimore, D. (1988). I kappa B: a specific inhibitor of the NF-kappa B transcription factor. *Science*, 242, 540-546.
- Baeuerle, P. A. & Henkel, T. (1994). Function and activation of NF-kappa B in the immune system. *Annu.Rev.Immunol.*, 12, 141-179.
- Barr, R. D., Volaric, Z., & Koekebakker, M. (1987). Stimulation of human eosinophilopoiesis by hydrocortisone *in vitro*. *Acta Haematol.*, 77, 20-24.
- Belvisi, M. G., Hele, D. J., & Birrell, M. A. (2006). Peroxisome proliferator-activated receptor gamma agonists as therapy for chronic airway inflammation. *Eur.J.Pharmacol.*, 533, 101-109.
- Bensinger, S. J. & Tontonoz, P. (2008). Integration of metabolism and inflammation by lipid-activated nuclear receptors. *Nature*, 454, 470-477.
- Berridge, M. J. (1993). Inositol trisphosphate and calcium signalling. *Nature*, 361, 315-325.
- Birrell, M. A., Patel, H. J., McCluskie, K., Wong, S., Leonard, T., Yacoub, M. H. *et al.* (2004). PPAR-gamma agonists as therapy for diseases involving airway neutrophilia. *Eur.Respir.J.*, 24, 18-23.
- Boehme, S. A., Sullivan, S. K., Crowe, P. D., Santos, M., Conlon, P. J., Sriramarao, P. *et al.* (1999). Activation of mitogen-activated protein kinase regulates eotaxin-induced eosinophil migration. *J.Immunol.*, 163, 1611-1618.
- Brown, J. R., Kleimberg, J., Marini, M., Sun, G., Bellini, A., & Mattoli, S. (1998). Kinetics of eotaxin expression and its relationship to eosinophil accumulation and activation in bronchial biopsies and BAL of asthmatic patients after allergen inhalation. *Clin.Exp.Immunol.*, 114, 137-146.

- Brown, P. J., Winegar, D. A., Plunket, K. D., Moore, L. B., Lewis, M. C., Wilson, J. G. *et al.* (1999). A ureido-thioisobutyric acid (GW9578) is a subtype-selective PPARalpha agonist with potent lipid-lowering activity. *J.Med.Chem.*, 42, 3785-3788.
- Burnett, J. C. & Rossi, J. J. (2012). RNA-based therapeutics: current progress and future prospects. *Chem.Biol.*, 19, 60-71.
- Busse, W., Raphael, G. D., Galant, S., Kalberg, C., Goode-Sellers, S., Srebro, S. *et al.* (2001). Low-dose fluticasone propionate compared with montelukast for first-line treatment of persistent asthma: a randomized clinical trial. *J.Allergy Clin.Immunol.*, 107, 461-468.
- Caldenhoven, E., van Dijk, T. B., Tijmensen, A., Raaijmakers, J. A., Lammers, J. W., Koenderman, L. *et al.* (1998). Differential activation of functionally distinct STAT5 proteins by IL-5 and GM-CSF during eosinophil and neutrophil differentiation from human CD34+ hematopoietic stem cells. *Stem Cells*, 16, 397-403.
- Cameron, L., Christodoulopoulos, P., Lavigne, F., Nakamura, Y., Eidelman, D., McEuen, A. *et al.* (2000). Evidence for local eosinophil differentiation within allergic nasal mucosa: inhibition with soluble IL-5 receptor. *J.Immunol.*, 164, 1538-1545.
- Chen, C. C., Rosenbloom, C. L., Anderson, D. C., & Manning, A. M. (1995). Selective inhibition of E-selectin, vascular cell adhesion molecule-1, and intercellular adhesion molecule-1 expression by inhibitors of I kappa B-alpha phosphorylation. *J.Immunol.*, 155, 3538-3545.

- Chihara, J., Plumas, J., Gruart, V., Tavernier, J., Prin, L., Capron, A. *et al.* (1990). Characterization of a receptor for interleukin 5 on human eosinophils: variable expression and induction by granulocyte/macrophage colony-stimulating factor. *J.Exp.Med.*, 172, 1347-1351.
- Chou, C. L., Wang, C. H., & Kuo, H. P. (1999). Upregulation of IL-5 receptor expression on bone marrow-derived CD34+ cells from patients with asthma. *Changeng.Yi.Xue.Za Zhi.*, 22, 416-422.
- Clapham, D. E. (1995). Calcium signaling. *Cell*, 80, 259-268.
- Combadiere, C., Ahuja, S. K., & Murphy, P. M. (1995). Cloning and functional expression of a human eosinophil CC chemokine receptor. *J.Biol.Chem.*, 270, 16491-16494.
- Crabtree, G. R. & Clipstone, N. A. (1994). Signal transmission between the plasma membrane and nucleus of T lymphocytes. *Annu.Rev.Biochem.*, 63, 1045-1083.
- Cyr, M. M., Baatjes, A. J., Dorman, S. C., Crawford, L., Sehmi, R., Foley, R. *et al.* (2008). In vitro effects of budesonide on eosinophil-basophil lineage commitment. *Open.Respir Med J*, 2, 60-66.
- Daugherty, B. L., Siciliano, S. J., DeMartino, J. A., Malkowitz, L., Sirotina, A., & Springer, M. S. (1996). Cloning, expression, and characterization of the human eosinophil eotaxin receptor. *J.Exp.Med*, 183, 2349-2354.
- De Lucca, G. V., Kim, U. T., Vargo, B. J., Duncia, J. V., Santella, J. B., III, Gardner, D. S. *et al.* (2005). Discovery of CC chemokine receptor-3 (CCR3) antagonists with picomolar potency. *J.Med Chem.*, 48, 2194-2211.

- de Monchy, J. G., Kauffman, H. F., Venge, P., Koeter, G. H., Jansen, H. M., Sluiter, H. J. *et al.* (1985). Bronchoalveolar eosinophilia during allergen-induced late asthmatic reactions. *Am.Rev.Respir.Dis.*, 131, 373-376.
- Delerive, P., De, B. K., Besnard, S., Vanden, B. W., Peters, J. M., Gonzalez, F. J. *et al.* (1999). Peroxisome proliferator-activated receptor alpha negatively regulates the vascular inflammatory gene response by negative cross-talk with transcription factors NF-kappaB and AP-1. *J.Biol.Chem.*, 274, 32048-32054.
- Denburg, J. A., Richardson, M., Telizyn, S., & Bienenstock, J. (1983). Basophil/mast cell precursors in human peripheral blood. *Blood*, 61, 775-780.
- Denburg, J. A., Sehmi, R., Upham, J., Wood, L., Gauvreau, G., & O'Byrne, P. (1999). Regulation of IL-5 and IL-5 receptor expression in the bone marrow of allergic asthmatics. *Int.Arch.Allergy Immunol.*, 118, 101-103.
- Denburg, J. A., Telizyn, S., Belda, A., Dolovich, J., & Bienenstock, J. (1985). Increased numbers of circulating basophil progenitors in atopic patients. *J.Allergy Clin.Immunol.*, 76, 466-472.
- Denburg, J. A., Woolley, M., Leber, B., Linden, M., & O'Byrne, P. (1994). Basophil and eosinophil differentiation in allergic reactions. *J.Allergy Clin.Immunol.*, 94, 1135-1141.
- Devchand, P. R., Keller, H., Peters, J. M., Vazquez, M., Gonzalez, F. J., & Wahli, W. (1996). The PPARalpha-leukotriene B4 pathway to inflammation control. *Nature*, 384, 39-43.
- Dolmetsch, R. E., Lewis, R. S., Goodnow, C. C., & Healy, J. I. (1997). Differential activation of transcription factors induced by Ca²⁺ response amplitude and duration. *Nature*, 386, 855-858.

- Dorman, S. C., Babirad, I., Post, J., Watson, R. M., Foley, R., Jones, G. L. *et al.* (2005). Progenitor egress from the bone marrow after allergen challenge: role of stromal cell-derived factor 1alpha and eotaxin. *J.Allergy Clin.Immunol.*, 115, 501-507.
- Dorman, S. C., Efthimiadis, A., Babirad, I., Watson, R. M., Denburg, J. A., Hargreave, F. E. *et al.* (2004). Sputum CD34+IL-5Ralpha+ cells increase after allergen: evidence for in situ eosinophilopoiesis. *Am.J.Respir.Crit Care Med*, 169, 573-577.
- Doyle, T. M., Ellis, R., Park, H. J., Inman, M. D., & Sehmi, R. (2011). Modulating progenitor accumulation attenuates lung angiogenesis in a mouse model of asthma. *Eur.Respir J*, 38, 679-687.
- Dunn, G. A. (1983). Characterising a kinesis response: time averaged measures of cell speed and directional persistence. *Agents Actions Suppl*, 12, 14-33.
- Elsas, P. X., Neto, H. A., Cheraim, A. B., Magalhaes, E. S., Accioly, M. T., Carvalho, V. F. *et al.* (2004). Induction of bone-marrow eosinophilia in mice submitted to surgery is dependent on stress-induced secretion of glucocorticoids. *Br.J.Pharmacol.*, 143, 541-548.
- Fahy, J. V., Liu, J., Wong, H., & Boushey, H. A. (1994). Analysis of cellular and biochemical constituents of induced sputum after allergen challenge: a method for studying allergic airway inflammation. *J.Allergy Clin.Immunol.*, 93, 1031-1039.
- Fanger, C. M., Hoth, M., Crabtree, G. R., & Lewis, R. S. (1995). Characterization of T cell mutants with defects in capacitative calcium entry: genetic evidence for the physiological roles of CRAC channels. *J.Cell Biol.*, 131, 655-667.

- Flood-Page, P. T., Menzies-Gow, A. N., Kay, A. B., & Robinson, D. S. (2003). Eosinophil's role remains uncertain as anti-interleukin-5 only partially depletes numbers in asthmatic airway. *Am.J.Respir.Crit Care Med.*, 167, 199-204.
- Frantz, B., Nordby, E. C., Bren, G., Steffan, N., Paya, C. V., Kincaid, R. L. *et al.* (1994). Calcineurin acts in synergy with PMA to inactivate I kappa B/MAD3, an inhibitor of NF-kappa B. *EMBO J.*, 13, 861-870.
- Friedl, P. & Weigelin, B. (2008). Interstitial leukocyte migration and immune function. *Nat.Immunol.*, 9, 960-969.
- Gauvreau, G. M., Boulet, L. P., Cockcroft, D. W., Baatjes, A., Cote, J., Deschesnes, F. *et al.* (2008). Antisense therapy against CCR3 and the common beta chain attenuates allergen-induced eosinophilic responses. *Am.J.Respir.Crit Care Med.*, 177, 952-958.
- Gauvreau, G. M., Doctor, J., Watson, R. M., Jordana, M., & O'Byrne, P. M. (1996). Effects of inhaled budesonide on allergen-induced airway responses and airway inflammation. *Am.J.Respir.Crit Care Med*, 154, 1267-1271.
- Gauvreau, G. M., Jordana, M., Watson, R. M., Cockcroft, D. W., & O'Byrne, P. M. (1997). Effect of regular inhaled albuterol on allergen-induced late responses and sputum eosinophils in asthmatic subjects. *Am.J.Respir.Crit Care Med*, 156, 1738-1745.
- Gauvreau, G. M., O'Byrne, P. M., Moqbel, R., Velazquez, J., Watson, R. M., Howie, K. J. *et al.* (1998). Enhanced expression of GM-CSF in differentiating eosinophils of atopic and atopic asthmatic subjects. *Am.J.Respir.Cell Mol.Biol.*, 19, 55-62.

- Gauvreau, G. M., Pageau, R., Seguin, R., Carballo, D., Gauthier, J., D'Anjou, H. *et al.* (2011). Dose-response effects of TPI ASM8 in asthmatics after allergen. *Allergy*, 66, 1242-1248.
- Gauvreau, G. M., Watson, R. M., & O'Byrne, P. M. (1999). Kinetics of allergen-induced airway eosinophilic cytokine production and airway inflammation. *Am.J.Respir.Crit Care Med.*, 160, 640-647.
- Gauvreau, G. M., Wood, L. J., Sehmi, R., Watson, R. M., Dorman, S. C., Schleimer, R. P. *et al.* (2000). The effects of inhaled budesonide on circulating eosinophil progenitors and their expression of cytokines after allergen challenge in subjects with atopic asthma. *Am.J.Respir.Crit Care Med*, 162, 2139-2144.
- Gibson, P. G., Dolovich, J., Girgis-Gabardo, A., Morris, M. M., Anderson, M., Hargreave, F. E. *et al.* (1990). The inflammatory response in asthma exacerbation: changes in circulating eosinophils, basophils and their progenitors. *Clin.Exp.Allergy*, 20, 661-668.
- Gibson, P. G., Manning, P. J., O'Byrne, P. M., Girgis-Gabardo, A., Dolovich, J., Denburg, J. A. *et al.* (1991). Allergen-induced asthmatic responses. Relationship between increases in airway responsiveness and increases in circulating eosinophils, basophils, and their progenitors. *Am.Rev.Respir.Dis.*, 143, 331-335.
- GlaxoSmithKline (2011). A randomized, double-blind, placebo-controlled, parallel group study to compare GW766994 (an oral CCR3 receptor antagonist) versus placebo in patients with asthma and sputum eosinophilia. GSK Clinical Trial Register [<http://download.gsk-clinicalstudyregister.com/files/eccc7537-3f6b-4866-9211-4c11748f5e79>].

- Gold, M. R. & DeFranco, A. L. (1994). Biochemistry of B lymphocyte activation. *Adv.Immunol.*, 55, 221-295.
- Gonlugur, U. & Efeoglu, T. (2004). Vascular adhesion and transendothelial of eosinophil leukocytes. *Cell Tissue Res.*, 318(3), 473-82.
- Hammad, H., de Heer, H. J., Soullie, T., Angeli, V., Trottein, F., Hoogsteden, H. C. *et al.* (2004). Activation of peroxisome proliferator-activated receptor-gamma in dendritic cells inhibits the development of eosinophilic airway inflammation in a mouse model of asthma. *Am.J.Pathol.*, 164, 263-271.
- Hart, L. A., Krishnan, V. L., Adcock, I. M., Barnes, P. J., & Chung, K. F. (1998). Activation and localization of transcription factor, nuclear factor-kappaB, in asthma. *Am.J.Respir.Crit Care Med*, 158, 1585-1592.
- Heath, H., Qin, S., Rao, P., Wu, L., LaRosa, G., Kassam, N. *et al.* (1997). Chemokine receptor usage by human eosinophils. The importance of CCR3 demonstrated using an antagonistic monoclonal antibody. *J.Clin.Invest*, 99, 178-184.
- Hirasawa, H., Chiba, T., Ueki, S., Kamada, Y., Ito, W., Takeda, M. *et al.* (2008). The synthetic PPARgamma agonist troglitazone inhibits eotaxin-enhanced eosinophil adhesion to ICAM-1-coated plates. *Int.Arch.Allergy Immunol.*, 146 Suppl 1, 11-15.
- Hogan, S. P., Rosenberg, H. F., Moqbel, R., Phipps, S., Foster, P. S., Lacy, P. *et al.* (2008). Eosinophils: biological properties and role in health and disease. *Clin.Exp.Allergy*, 38, 709-750.
- Home, P. D., Pocock, S. J., Beck-Nielsen, H., Curtis, P. S., Gomis, R., Hanefeld, M. *et al.* (2009). Rosiglitazone evaluated for cardiovascular outcomes in oral agent

combination therapy for type 2 diabetes (RECORD): a multicentre, randomised, open-label trial. *Lancet*, 373, 2125-2135.

Honda, K., Marquillies, P., Capron, M., & Dombrowicz, D. (2004). Peroxisome proliferator-activated receptor gamma is expressed in airways and inhibits features of airway remodeling in a mouse asthma model. *J.Allergy Clin.Immunol.*, 113, 882-888.

Hsu, M. H., Palmer, C. N., Griffin, K. J., & Johnson, E. F. (1995). A single amino acid change in the mouse peroxisome proliferator-activated receptor alpha alters transcriptional responses to peroxisome proliferators. *Mol.Pharmacol.*, 48, 559-567.

Imaoka, H., Babirad, I., Kwon, Y., Killian, K., O'Byrne, P., Gauvreau, G. *et al.* (2010). Increase in sputum levels of vascular endothelial progenitor cells in asthmatic subjects following allergen challenge [abstract]. *Eur.Respir.J.*, 36, 86s.

Imaoka, H., Campbell, H., Babirad, I., Watson, R. M., Mistry, M., Sehmi, R. *et al.* (2011). TPI ASM8 reduces eosinophil progenitors in sputum after allergen challenge. *Clin.Exp.Allergy*, 41, 1740-1746.

Jeong, H. W., Lee, J. W., Kim, W. S., Choe, S. S., Kim, K. H., Park, H. S. *et al.* (2011). A newly identified CG301269 improves lipid and glucose metabolism without body weight gain through activation of peroxisome proliferator-activated receptor alpha and gamma. *Diabetes*, 60, 496-506.

Kampen, G. T., Stafford, S., Adachi, T., Jinquan, T., Quan, S., Grant, J. A. *et al.* (2000). Eotaxin induces degranulation and chemotaxis of eosinophils through

the activation of ERK2 and p38 mitogen-activated protein kinases. *Blood*, 95, 1911-1917.

Kim, S. R., Lee, K. S., Park, H. S., Park, S. J., Min, K. H., Jin, S. M. *et al.* (2005).

Involvement of IL-10 in peroxisome proliferator-activated receptor gamma-mediated anti-inflammatory response in asthma. *Mol.Pharmacol.*, 68, 1568-1575.

Kim, Y. K., Uno, M., Hamilos, D. L., Beck, L., Bochner, B., Schleimer, R. *et al.*

(1999). Immunolocalization of CD34 in nasal polyposis. Effect of topical corticosteroids. *Am.J.Respir.Cell Mol.Biol.*, 20, 388-397.

Kirby, J. G., Hargreave, F. E., Gleich, G. J., & O'Byrne, P. M. (1987).

Bronchoalveolar cell profiles of asthmatic and nonasthmatic subjects. *Am.Rev.Respir.Dis.*, 136, 379-383.

Kobayashi, Y., Ueki, S., Mahemuti, G., Chiba, T., Oyamada, H., Saito, N. *et al.*

(2005). Physiological levels of 15-deoxy-Delta12,14-prostaglandin J2 prime eotaxin-induced chemotaxis on human eosinophils through peroxisome proliferator-activated receptor-gamma ligation. *J.Immunol.*, 175, 5744-5750.

Kolbeck, R., Kozhich, A., Humbles, A., Erjefalt, J., Koike, M., Peng, L. *et al.* (2008).

MEDI-563, a humanized anti-IL-5Ra antibody with enhanced effector function, induces reversible blood eosinopenia in mild asthmatics [abstract]. *J.Allergy Clin.Immunol.*, S47.

Lamkhioued, B., Abdelilah, S. G., Hamid, Q., Mansour, N., Delespesse, G., & Renzi,

P. M. (2003). The CCR3 receptor is involved in eosinophil differentiation and is up-regulated by Th2 cytokines in CD34+ progenitor cells. *J.Immunol.*, 170, 537-547.

- Lee, C. H., Chawla, A., Urbiztondo, N., Liao, D., Boisvert, W. A., Evans, R. M. *et al.* (2003). Transcriptional repression of atherogenic inflammation: modulation by PPARdelta. *Science*, 302, 453-457.
- Lehmann, J. M., Lenhard, J. M., Oliver, B. B., Ringold, G. M., & Kliewer, S. A. (1997). Peroxisome proliferator-activated receptors alpha and gamma are activated by indomethacin and other non-steroidal anti-inflammatory drugs. *J.Biol.Chem.*, 272, 3406-3410.
- Lehmann, J. M., Moore, L. B., Smith-Oliver, T. A., Wilkison, W. O., Willson, T. M., & Kliewer, S. A. (1995). An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor gamma (PPAR gamma). *J.Biol.Chem.*, 270, 12953-12956.
- Leung, D. Y., Spahn, J. D., & Szefer, S. J. (2002). Steroid-unresponsive asthma. *Semin.Respir.Crit Care Med.*, 23, 387-398.
- Levesque, J. P., Hendy, J., Takamatsu, Y., Simmons, P. J., & Bendall, L. J. (2003). Disruption of the CXCR4/CXCL12 chemotactic interaction during hematopoietic stem cell mobilization induced by GCSF or cyclophosphamide. *J.Clin.Invest*, 111, 187-196.
- Lipworth, B. J. (1997). Airway subsensitivity with long-acting beta 2-agonists. Is there cause for concern? *Drug Saf*, 16, 295-308.
- Lu, L., Lin, Z. H., Shen, R. N., Warren, D. J., Leemhuis, T., & Broxmeyer, H. E. (1990). Influence of interleukins 3,5, and 6 on the growth of eosinophil progenitors in highly enriched human bone marrow in the absence of serum. *Exp.Hematol.*, 18, 1180-1186.

- Ma, Q., Jones, D., Borghesani, P. R., Segal, R. A., Nagasawa, T., Kishimoto, T. *et al.* (1998). Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in C. *Proc.Natl.Acad.Sci.U.S.A*, 95, 9448-9453.
- Menzies-Gow, A., Flood-Page, P., Sehmi, R., Burman, J., Hamid, Q., Robinson, D. S. *et al.* (2003). Anti-IL-5 (mepolizumab) therapy induces bone marrow eosinophil maturational arrest and decreases eosinophil progenitors in the bronchial mucosa of atopic asthmatics. *J.Allergy Clin.Immunol.*, 111, 714-719.
- Menzies-Gow, A. N., Flood-Page, P. T., Robinson, D. S., & Kay, A. B. (2007). Effect of inhaled interleukin-5 on eosinophil progenitors in the bronchi and bone marrow of asthmatic and non-asthmatic volunteers. *Clin.Exp.Allergy*, 37, 1023-1032.
- Nagasawa, T., Hirota, S., Tachibana, K., Takakura, N., Nishikawa, S., Kitamura, Y. *et al.* (1996). Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature*, 382, 635-638.
- Nagasawa, T., Kikutani, H., & Kishimoto, T. (1994). Molecular cloning and structure of a pre-B-cell growth-stimulating factor. *Proc.Natl.Acad.Sci.U.S.A*, 91, 2305-2309.
- Nair, P., Pizzichini, M. M., Kjarsgaard, M., Inman, M. D., Efthimiadis, A., Pizzichini, E. *et al.* (2009). Mepolizumab for prednisone-dependent asthma with sputum eosinophilia. *N.Engl.J.Med.*, 360, 985-993.
- Nelson, H. S., Busse, W. W., Kerwin, E., Church, N., Emmett, A., Rickard, K. *et al.* (2000). Fluticasone propionate/salmeterol combination provides more effective asthma control than low-dose inhaled corticosteroid plus montelukast. *J.Allergy Clin.Immunol.*, 106, 1088-1095.

- Nie, M., Corbett, L., Knox, A. J., & Pang, L. (2005). Differential regulation of chemokine expression by peroxisome proliferator-activated receptor gamma agonists: interactions with glucocorticoids and beta2-agonists. *J.Biol.Chem.*, 280, 2550-2561.
- O'Byrne, P. M., Dolovich, J., & Hargreave, F. E. (1987). Late asthmatic responses. *Am.Rev.Respir.Dis.*, 136, 740-751.
- Ogawa, S., Lozach, J., Benner, C., Pascual, G., Tangirala, R. K., Westin, S. *et al.* (2005). Molecular determinants of crosstalk between nuclear receptors and toll-like receptors. *Cell*, 122, 707-721.
- Oliver, W. R., Jr., Shenk, J. L., Snaith, M. R., Russell, C. S., Plunket, K. D., Bodkin, N. L. *et al.* (2001). A selective peroxisome proliferator-activated receptor delta agonist promotes reverse cholesterol transport. *Proc.Natl.Acad.Sci.U.S.A*, 98, 5306-5311.
- Pang, L., Nie, M., Corbett, L., & Knox, A. J. (2003). Cyclooxygenase-2 expression by nonsteroidal anti-inflammatory drugs in human airway smooth muscle cells: role of peroxisome proliferator-activated receptors. *J.Immunol.*, 170, 1043-1051.
- Patel, H. J., Belvisi, M. G., Bishop-Bailey, D., Yacoub, M. H., & Mitchell, J. A. (2003). Activation of peroxisome proliferator-activated receptors in human airway smooth muscle cells has a superior anti-inflammatory profile to corticosteroids: relevance for chronic obstructive pulmonary disease therapy. *J.Immunol.*, 170, 2663-2669.
- Pazdrak, K., Olszewska-Pazdrak, B., Stafford, S., Garofalo, R. P., & Alam, R. (1998). Lyn, Jak2, and Raf-1 kinases are critical for the antiapoptotic effect of

interleukin 5, whereas only Raf-1 kinase is essential for eosinophil activation and degranulation. *J.Exp.Med*, 188, 421-429.

Petit, I., Szyper-Kravitz, M., Nagler, A., Lahav, M., Peled, A., Habler, L. *et al.* (2002). G-CSF induces stem cell mobilization by decreasing bone marrow SDF-1 and up-regulating CXCR4. *Nat.Immunol.*, 3, 687-694.

Pin, I., Gibson, P. G., Kolendowicz, R., Girgis-Gabardo, A., Denburg, J. A., Hargreave, F. E. *et al.* (1992). Use of induced sputum cell counts to investigate airway inflammation in asthma. *Thorax*, 47, 25-29.

Ponath, P. D., Qin, S., Post, T. W., Wang, J., Wu, L., Gerard, N. P. *et al.* (1996). Molecular cloning and characterization of a human eotaxin receptor expressed selectively on eosinophils. *J.Exp.Med*, 183, 2437-2448.

Rao, A. (1994). NF-ATp: a transcription factor required for the co-ordinate induction of several cytokine genes. *Immunol.Today*, 15, 274-281.

Richards, D. B., Bareille, P., Lindo, E. L., Quinn, D., & Farrow, S. N. (2010). Treatment with a peroxisomal proliferator activated receptor gamma agonist has a modest effect in the allergen challenge model in asthma: a randomised controlled trial. *Respir.Med.*, 104, 668-674.

Ricote, M. & Glass, C. K. (2007). PPARs and molecular mechanisms of transrepression. *Biochim.Biophys.Acta*, 1771, 926-935.

Robinson, D. S., Damia, R., Zeibecoglou, K., Molet, S., North, J., Yamada, T. *et al.* (1999). CD34(+)/interleukin-5⁺alpha messenger RNA⁺ cells in the bronchial mucosa in asthma: potential airway eosinophil progenitors. *Am.J.Respir.Cell Mol.Biol.*, 20, 9-13.

- Rosenthal, R. L., Wald, N., Yager, A., & Litwins, J. (1950). Effects of cortisone and ACTH therapy on eosinophils of the bone marrow and blood. *Proc.Soc.Exp.Biol.Med*, 75, 740-741.
- Santella, J. B., III, Gardner, D. S., Yao, W., Shi, C., Reddy, P., Tebben, A. J. *et al.* (2008). From rigid cyclic templates to conformationally stabilized acyclic scaffolds. Part I: the discovery of CCR3 antagonist development candidate BMS-639623 with picomolar inhibition potency against eosinophil chemotaxis. *Bioorg.Med Chem.Lett.*, 18, 576-585.
- Scheinman, R. I., Cogswell, P. C., Lofquist, A. K., & Baldwin, A. S., Jr. (1995). Role of transcriptional activation of I kappa B alpha in mediation of immunosuppression by glucocorticoids. *Science*, 270, 283-286.
- Sehmi, R., Howie, K., Sutherland, D. R., Schragge, W., O'Byrne, P. M., & Denburg, J. A. (1996). Increased levels of CD34+ hemopoietic progenitor cells in atopic subjects. *Am.J.Respir.Cell Mol.Biol.*, 15, 645-655.
- Sehmi, R., Wardlaw, A. J., Cromwell, O., Kurihara, K., Waltmann, P., & Kay, A. B. (1992). Interleukin-5 selectively enhances the chemotactic response of eosinophils obtained from normal but not eosinophilic subjects. *Blood*, 79, 2952-2959.
- Sehmi, R., Wood, L. J., Watson, R., Foley, R., Hamid, Q., O'Byrne, P. M. *et al.* (1997). Allergen-induced increases in IL-5 receptor alpha-subunit expression on bone marrow-derived CD34+ cells from asthmatic subjects. A novel marker of progenitor cell commitment towards eosinophilic differentiation. *J.Clin.Invest*, 100, 2466-2475.

- Sen, R. & Baltimore, D. (1986). Multiple nuclear factors interact with the immunoglobulin enhancer sequences. *Cell*, 46, 705-716.
- Smith, S. G., Imaoka, H., Punia, N., Irshad, A., Janssen, L. L., Sehmi, R. *et al.* (2012). The Effect of PPAR Agonists on the Migration of Mature and Immature Eosinophils. *PPAR Research*, Vol. 2012, 1-8.
- Southam, D. S., Widmer, N., Ellis, R., Hirota, J. A., Inman, M. D., & Sehmi, R. (2005). Increased eosinophil-lineage committed progenitors in the lung of allergen-challenged mice. *J.Allergy Clin.Immunol.*, 115, 95-102.
- Staels, B., Koenig, W., Habib, A., Merval, R., Lebret, M., Torra, I. P. *et al.* (1998). Activation of human aortic smooth-muscle cells is inhibited by PPARalpha but not by PPARgamma activators. *Nature*, 393, 790-793.
- Stafford, S., Lowell, C., Sur, S., & Alam, R. (2002). Lyn tyrosine kinase is important for IL-5-stimulated eosinophil differentiation. *J.Immunol.*, 168, 1978-1983.
- Stein, M. L., Collins, M. H., Villanueva, J. M., Kushner, J. P., Putnam, P. E., Buckmeier, B. K. *et al.* (2006). Anti-IL-5 (mepolizumab) therapy for eosinophilic esophagitis. *J.Allergy Clin.Immunol.*, 118, 1312-1319.
- Stirling, R. G., van Rensen, E. L., Barnes, P. J., & Chung, K. F. (2001). Interleukin-5 induces CD34(+) eosinophil progenitor mobilization and eosinophil CCR3 expression in asthma. *Am.J.Respir.Crit Care Med*, 164, 1403-1409.
- Strek, M. E. (2006). Difficult asthma. *Proc.Am.Thorac.Soc.*, 3, 116-123.
- Sweeney, E. A. & Papayannopoulou, T. (2001). Increase in circulating SDF-1 after treatment with sulfated glycans. The role of SDF-1 in mobilization. *Ann.N.Y.Acad.Sci.*, 938, 48-52.

- Teruel, T., Hernandez, R., Benito, M., & Lorenzo, M. (2003). Rosiglitazone and retinoic acid induce uncoupling protein-1 (UCP-1) in a p38 mitogen-activated protein kinase-dependent manner in fetal primary brown adipocytes. *J.Biol.Chem.*, 278, 263-269.
- Timmerman, L. A., Clipstone, N. A., Ho, S. N., Northrop, J. P., & Crabtree, G. R. (1996). Rapid shuttling of NF-AT in discrimination of Ca²⁺ signals and immunosuppression. *Nature*, 383, 837-840.
- Trifilieff, A., Bench, A., Hanley, M., Bayley, D., Campbell, E., & Whittaker, P. (2003). PPAR-alpha and -gamma but not -delta agonists inhibit airway inflammation in a murine model of asthma: *in vitro* evidence for an NF-kappaB-independent effect. *Br.J.Pharmacol.*, 139, 163-171.
- Ueki, S., Adachi, T., Bourdeaux, J., Oyamada, H., Yamada, Y., Hamada, K. *et al.* (2003). Expression of PPARgamma in eosinophils and its functional role in survival and chemotaxis. *Immunol.Lett.*, 86, 183-189.
- Ward, J. E., Fernandes, D. J., Taylor, C. C., Bonacci, J. V., Quan, L., & Stewart, A. G. (2006). The PPARgamma ligand, rosiglitazone, reduces airways hyperresponsiveness in a murine model of allergen-induced inflammation. *Pulm.Pharmacol.Ther.*, 19, 39-46.
- Ward, J. E., Gould, H., Harris, T., Bonacci, J. V., & Stewart, A. G. (2004). PPAR gamma ligands, 15-deoxy-delta12,14-prostaglandin J2 and rosiglitazone regulate human cultured airway smooth muscle proliferation through different mechanisms. *Br.J.Pharmacol.*, 141, 517-525.
- Wenzel, S. E. (2012). Asthma phenotypes: the evolution from clinical to molecular approaches. *Nat.Med.*, 18, 716-725.

- Werner, C., Kamani, C. H., Gensch, C., Bohm, M., & Laufs, U. (2007). The peroxisome proliferator-activated receptor-gamma agonist pioglitazone increases number and function of endothelial progenitor cells in patients with coronary artery disease and normal glucose tolerance. *Diabetes*, 56, 2609-2615.
- Willson, T. M., Brown, P. J., Sternbach, D. D., & Henke, B. R. (2000). The PPARs: from orphan receptors to drug discovery. *J.Med.Chem.*, 43, 527-550.
- Woerly, G., Honda, K., Loyens, M., Papin, J. P., Auwerx, J., Staels, B. *et al.* (2003). Peroxisome proliferator-activated receptors alpha and gamma down-regulate allergic inflammation and eosinophil activation. *J.Exp.Med.*, 198, 411-421.
- Won, H. Y., Min, H. J., Ahn, J. H., Yoo, S. E., Bae, M. A., Hong, J. H. *et al.* (2010). Anti-allergic function and regulatory mechanisms of KR62980 in allergen-induced airway inflammation. *Biochem.Pharmacol.*, 79, 888-896.
- Wood, L. J., Inman, M. D., Watson, R. M., Foley, R., Denburg, J. A., & O'Byrne, P. M. (1998). Changes in bone marrow inflammatory cell progenitors after inhaled allergen in asthmatic subjects. *Am.J.Respir.Crit Care Med*, 157, 99-105.
- Wood, L. J., Sehmi, R., Gauvreau, G. M., Watson, R. M., Foley, R., Denburg, J. A. *et al.* (1999). An inhaled corticosteroid, budesonide, reduces baseline but not allergen-induced increases in bone marrow inflammatory cell progenitors in asthmatic subjects. *Am.J.Respir.Crit Care Med*, 159, 1457-1463.
- Yang, L., Stimpson, S. A., Chen, L., Wallace, H. W., & Rockey, D. C. (2010). Effectiveness of the PPARgamma agonist, GW570, in liver fibrosis. *Inflamm.Res.*, 59, 1061-1071.

- Ying, S., Robinson, D. S., Meng, Q., Rottman, J., Kennedy, R., Ringler, D. J. *et al.* (1997). Enhanced expression of eotaxin and CCR3 mRNA and protein in atopic asthma. Association with airway hyperresponsiveness and predominant co-localization of eotaxin mRNA to bronchial epithelial and endothelial cells. *Eur.J.Immunol.*, 27, 3507-3516.
- Young, P. W., Buckle, D. R., Cantello, B. C., Chapman, H., Clapham, J. C., Coyle, P. J. *et al.* (1998). Identification of high-affinity binding sites for the insulin sensitizer rosiglitazone (BRL-49653) in rodent and human adipocytes using a radioiodinated ligand for peroxisomal proliferator-activated receptor gamma. *J.Pharmacol.Exp.Ther.*, 284, 751-759.
- Zdanowicz, M. M. (2007). Pharmacotherapy of asthma. *Am.J.Pharm.Educ.*, 71, 98.